ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
October 2017; 16(5): 386-395.

The Changes of Th17/Treg and Related Cytokines: IL-17, IL-23, IL-10, and TGF-β in Respiratory Syncytial Virus Bronchiolitis Rat Model

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Received: 7 July 2016; Received in revised form: 3 June 2017 ; Accepted: 28 June 2017

ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of bronchiolitis and hospitalization that lead to high morbidity and mortality among young infants. T helper 17 (Th17) cells and regulatory T cells (Tregs) play essential roles in the pathogenesis of autoimmune, cancer, and inflammatory diseases. However, whether changes in T-cell subsets are related to the systemic immune responses in RSV-caused bronchiolitis merit further investigation.

Three-week-old Sprague Dawley (SD) rats were randomly divided into the normal control (NC) and RSV bronchiolitis (RSV-B) groups. An RSV-B model was successfully established using nasal drip containing RSV. Furthermore, pathological changes in the lung tissues were observed using hematoxylin and eosin staining. Flow cytometry determined the levels of Th17 and Treg subsets. The related cytokines were measured using enzyme-linked immunosorbent assay (ELISA). The expression levels of related transcription factors, such as RORγt and FOXP3, were examined using real-time quantitative PCR and western blot analysis.

The RSV-B group exhibited pulmonary interstitial hyperemia and edema, inflammatory cell infiltration, wide alveolar septa, and bronchial collapse and deformation. The percentage of Th17 cells in RSV-B group was about 2.3 fold higher than that of NC group, and the concentration of IL-17, IL-23 and RORγt was higher than in NC group. In contrast, the percentage of Treg cells in the RSV-B group was approximately 0.7 fold lower than that in the NC group, and the levels of IL-10, TGF-β, and FOXP3 in the RSV-B group were lower than those in the NC group.

The above results were statistically significant. The changes of Th17/Treg, and their associated cytokines, specific transcription factors, are present in RSV bronchiolitis model rats, which may play an important role in the pathogenesis of RSV bronchiolitis.

Keywords: Bronchiolitis; Th17 cells regulatory; T cells; Respiratory syncytial virus

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INTRODUCTION

Bronchiolitis is the most common lower respiratory infectious disease among infants and young children

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

worldwide. Infants younger than 2 years are often infected by the respiratory syncytial virus (RSV) which is the main pathogen of bronchiolitis, but one year old and kids below one year are highly prone to infections.[1, 2] After being infected by the virus, the body generates an immune response. However, the reinfection rate is considerably high because RSV cannot induce an effective immunological memory.[3] Children that are repeatedly infected with RSV have a high risk of suffering from recurrent wheezing and asthma.[4] RSV bronchiolitis evidently brings a heavy burden on both the patients and society at large.[5] The complicated pathogenesis of bronchiolitis remains to be elucidated; this disease has recently been considered a T cell-mediated immune disorder.

Studies have confirmed that RSV bronchiolitis is associated with the severe imbalance of T helper (Th)1/Th2 cytokines, with deficient Th1 and excess Th2,[6-8] and the current development of RSV accine is ongoing, but safe and effective for now since clinical vaccine is still not available.[9-11] Recent studies have shown that Th17/ Regulatory T cells (Tregs) also play a role in RSV bronchiolitis.[12] Th17 cells and Tregs are another T cell subsets except for Th1 and Th2. Th17 cells are reportedly involved in the pathogenesis of airway inflammation which is induced by microbial pathogens.[13] Previous studies suggested that interleukin (IL)-17, which is secreted by Th17 cells, recruits and activates neutrophils and eosinophils in lung tissue and airways, hence stimulate them to secrete inflammatory mediators and mobilize innate immunity[14, 15]. Tregs are the other subtype of a cluster of differentiation 4 (CD4)+T cells; Treg cells suppress aberrant immune responses that are harmful to the host to maintain immune homeostasis.[16, 17] Transferring Treg cells into allergen-induced mice alleviates the manifestations of asthma and inflamed lungs.[18] Treg cells limit inflammation, whereas Th17 cells enhance this process. Therefore, both Treg and Th17 cells play vital roles in maintaining immune homeostasis. Clinical studies have shown that Th17/Treg plays a vital role in the pathogenesis of allergic airway inflammation[19], Several studies have confirmed that, Th17/Treg is essential in the pathogensis of RSV infection, and plays an opposite role.[12, 20] This study aims to investigate the role of Th17 and Treg in the pathogenesis of RSV bronchiolitis by comparing the ratio of Th17 and Treg in PBMC and the related cytokines, transcription factor mRNA and protein level of RSV bronchiolitis model rats.

MATERIALS AND METHODS

RSV Suspensions Preparation

Approximately 10 μL of RSV (Jinan, China) was added into 2 mL of RPMI-1640 medium (HyClone, USA) supplemented with 2% fetal bovine serum (FBS; HyClone, USA) and then introduced into HeLa cells (Department of Immunology, Binzhou Medical University, China) with good growth. The cytopathic effect was observed after 2–3 days. When the cytopathic effect reached 90%–100%, the 25 cm^2 plastic flask was vigorously shaken, and freezing and thawing were repeated thrice. After that, the cell suspensions were collected by centrifugation (1000 r/min, 5 min) to obtain RSV suspensions. The 50% tissue culture infective dose ($TCID_{50}$) was then adjusted to 5×10^4 $TCID_{50}$/0.1 mL. Finally, the HeLa cell culture supernatants without RSV infection were collected and used as the negative control.

Animal Model Preparation

Thirty 3-week-old Sprague–Dawley (SD) rats, comprising 30 females, were obtained from LuYe Pharma Company (Yantai, China). The rats were randomly divided into the normal control (NC) and RSV-caused bronchiolitis (RSV-B) groups (n=15 each). In brief, the rats were anesthetized with ether (Fuchen Reagent Company, Tianjin, China). For the NC group, RSV-free medium (0.4 μL/g) was dropped on the nostrils of each rats. For the RSV-B group, 0.4 μL/g RSV suspension was dropped into the nostrils of the rats. After virus inoculation, all rats were isolated and were deprived of food; the rat cages were also disinfected thrice a week. The process mentioned above was repeated once a week for 8 weeks, after which all rats were sacrificed. All experiments were performed in accordance with the protocols of the National Institute of Health and Regulations of Laboratory Animals in China (No. 伦研批第2017-54).

Blood Sample Preparation

Blood from the abdominal aorta of rats was anticoagulated with heparin and diluted 1:2 with phosphate-buffered saline (PBS). Thereafter, blood was layered on the surface of a lymphoprep (Hao Yang Biological Manufacture Co., Ltd., Tianjin, China) and centrifuged at 400 g for 20 min at room temperature. After washing twice using PBS, peripheral blood mononuclear cells (PBMCs) were collected.

Simultaneously, plasma samples were collected and preserved at −80 °C to detect cell cytokines by using enzyme-linked immunosorbent assay (ELISA).

Flow Cytometric Staining and Analysis

For Th17 subset analysis, PBMCs were suspended at 2 × 10^6 cells/mL and were cultured (6 h, 37 °C, 5% CO_2) in RPMI-1640 medium with 10% FBS, 150 U/mL penicillin, and 150 U/mL streptomycin. PBMCs were activated for 5 h with 50 ng/mL phorbol myristate acetate (PMA; Sigma, US) and 1 μg/mL ionomycin (Sigma, USA). Consequently, the PBMCs were stimulated for an additional 2 h in the presence of 10 μg/mL Brefeldin A (Sigma, USA). The cells were then moved into a polystyrene round bottom test tube (BD, Biosciences, USA) for centrifugation at 300 g for 5 min. The cells were surface labeled using anti-CD4-FITC antibody (eBioscience, USA). After incubation for 15 min at room temperature, the cells were treated with a fixation reagent (eBioscience, USA), washed, permeated (eBioscience, USA), and then stained with anti-IL-17A-PE (eBioscience, USA) for 20 min. For Treg subset analysis, PBMCs were suspended at 4 × 106 cells/mL in RPMI-1640 with 10% FBS by using a polystyrene round bottom test tube for centrifugation at 300 g for 5 min. The cells were surface labeled using FITC antibody and CD25–phycoerythrin–cyanin5 antibody (B&D, USA), and then incubated in the dark for 30 min at 4°C. After washing, suspension, and permeation, the cells were stained using CD127–phycoerythrin antibody (B&D, USA). Then the cells were detected using a flow cytometer (Beckman, USA). The results were presented as a percentage of positive cells.

Measurement of Related Cytokines

The plasma levels of IL-17, IL-23, IL-10, and transforming growth factor beta (TGF-β) were measured using ELISA in accordance with the manufacturer's instructions (IL-17, IL-23, IL-10, and TGF-β ELISA kits, R&D, US).

Lung Histological Analysis

Lung tissue was obtained, fixed in 4% paraformaldehyde for a week, and then embedded in paraffin after washing with running water for 24 h. Lung sections were cut into 4 μm sections and stained using hematoxylin–eosin (H&E). The pathological and inflammatory changes in the lungs were observed under a light microscope (Olympus, Japan).

qRT-PCR Analysis

Total RNA was extracted from the lungs and PBMCs by using TRIzol (TaKaRa, Japan). Thereafter, complementary DNA (cDNA) was reversed using a PrimeScript RT reagent Kit (TaKaRa, Japan) in accordance with the manufacturer's instructions. The mRNA expression levels of ROR and FOXP3 were detected using a SYB'R Premix Ex Taq (TaKaRa, Japan) in a 20 μL reaction volume containing 2 μL of cDNA and 0.2 μM primers. The reaction condition was initiated with a 30 s denaturation at 95°C, followed by 50 cycles of 95°C for 5 s, 60°C for 20 s, and 4°C for 1 min. All of the tests were performed twice. The final mRNA expression levels of ROR and FOXP3 were estimated relative to that of the housekeeping gene GAPDH. At the end of the reaction, the gene expression of the NC group was set to 1. Hence, the gene expression of the RSV-B group was presented as fold change compared with that of the NC group. Finally, the following primer pairs (Sangon Biotech, China) were used as shown as below.

GAPDH	5′-ACAGCAACAGGGTGGTGGAC-3′ (F)
	5′-TTTGAGGGTGCAGCGAACTT-3′ (R)
RORγt	5′-CCTCCTGCCACCTTGAGTAT-3′ (F)
	5′-TCTGAGCCCTGTTCTGGTTC-3′ (R)
FOXP3	5′-TTCACCTATGCCACCCTCAT-3′ (F)
	5′-CCCTTCTCACTCTCCACTCG-3′ (R)

Western Blot Analysis

After washing twice with ice-cold PBS, the lung tissues were homogenized and lysed in radioimmunoprecipitation assay buffer with protease inhibitor cocktail (Sigma, USA) to obtain the extracts of lung tissue proteins. Protein content was measured using the bicinchoninic acid (BCA) assay (Thermo Scientific, USA). Equivalent protein samples were loaded to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels and then it was electrotransferred to a polyvinylidene difluoride membrane. The membrane was incubated with anti-ROR gamma antibody and anti-FOXP3 antibody (Abcam, USA), and blocked with 5% nonfat dry milk in the presence of Tris Buffered Saline, with Tween-20 (TBST). The blots were incubated with a horseradish peroxidase-conjugated secondary antibody and were washed three times with TBST. The membranes were

stripped and were visualized with enhanced chemiluminescence(ECL) reagents (Thermo, USA). All membranes were analyzed using an image analysis program (ImageJ Version 1.42, USA) to quantify protein expression.

Statistical Analysis

All data are expressed as means ± standard deviation (SD). Statistical analysis was performed using SPSS16.0(IBM, USA). Differences between the NC and RSV-B groups were analyzed using independent samples T-test. Statistical significance was considered at $p<0.05$.

RESULTS

Observation of Specimens and Pulmonary Histological Changes

After being inoculated with RSV, the rats from the RSV-B group exhibited several abnormal behaviors, such as reductive activity, shaggy hair, rapid breathing, and reluctance to eat. In contrast, the rats from the NC group did not exhibit abnormalities. The lung tissue from the NC group exhibited an average pink color, good elasticity, smooth surface, and no bleeding points (Figure 1A). In contrast, the lung tissue from the RSV-B group presented diffused congestion, edema, and loose texture (Figure 1B). The NC group presented normal pulmonary histological changes (Figure 1C), whereas the RSV-B group exhibited pulmonary interstitial hyperemia and edema, considerable inflammatory cell infiltration, and significantly wide alveolar septa (Figure 1D). The lung tissue from the RSV-B group obviously showed highly serious pulmonary interstitial inflammation. These results suggested that a rat bronchiolitis model was successfully established by using nasal drip with RSV.

Detection of Percentages of Treg and Th17 Cells in PBMCs via Flow Cytometry

As shown in Figure 2A, the percentage of Treg cells showed a decrease of 0.7 fold in the RSV-B group when compared tothe NC group (Table 1, NC vs. RSV-B: (13.39±1.01)% vs. (10.22 ± 0.66)%, $*p< 0.01$ vs. NC group). However, the percentage of Th17 cells (Figure 2B) showed 2.3 fold increase in the RSV-B group compared to the NC group (Table 1, NC vs. RSV-B: (1.65±0.41) % vs. (3.73±0.45)%, $**p<0.01$ vs. NC group).

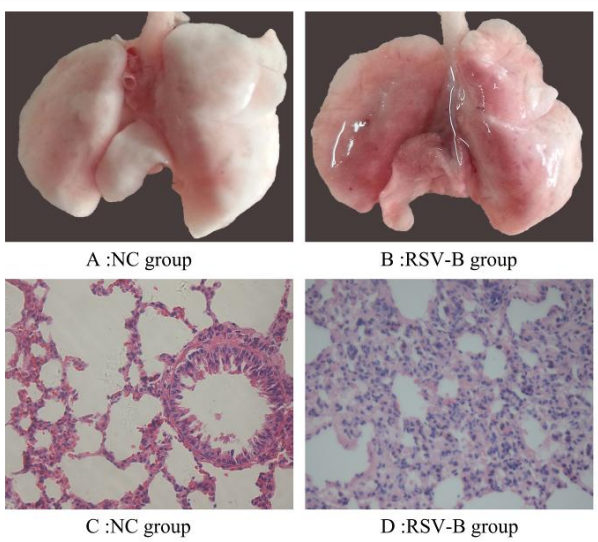

Figure 1. Specimens and histopathological changes in lung tissue. (A) Normal control group: rat lung tissue surface is smooth, good elasticity, the surface is uniform powdery white, no blood point and congestive area, under the light microscope observation, the alveolar tissue structure is complete, the uninflammatory cell infiltrates. (B) Respiratory syncytial virusB group: rat lung tissue is widely spread in the bleeder, with diffuse hyperemia swelling, have more inflammatory secretions, were observed under light, pulmonary interstitial inflammation, wide alveolar septa, alveolar space reduction, interval surrounded by a large number of lymphocyte, eosinophil infiltration, at the same time with epithelial cells degeneration necrosis caused by tracheal collapse deformation. (C) Normal control group: histopathological changes in lung tissue in the normal control group (HE,×400). (D) Respiratory syncytial virus - B group: histopathological changes in lung tissue in rats inoculated with RSV-caused bronchiolitis (HE,×400).

The alteration of both Treg and Th17 cells significantly reduced the ratio of the former to the latter in the RSV-B group (Table. 1, NC vs. RSV-B: (8.65±2.25) % vs. (2.78 ±0.32) %, $***p<0.01$ vs. NC group). These results indicate that the imbalance between Treg and Th17 cells is involved in the development of RSV bronchiolitis at the cellular level.

mRNA Expression of FOXP3 and RORγt in the Lung Tissue and PBMCs

As shown in Figure 3A, the mRNA level of FOXP3

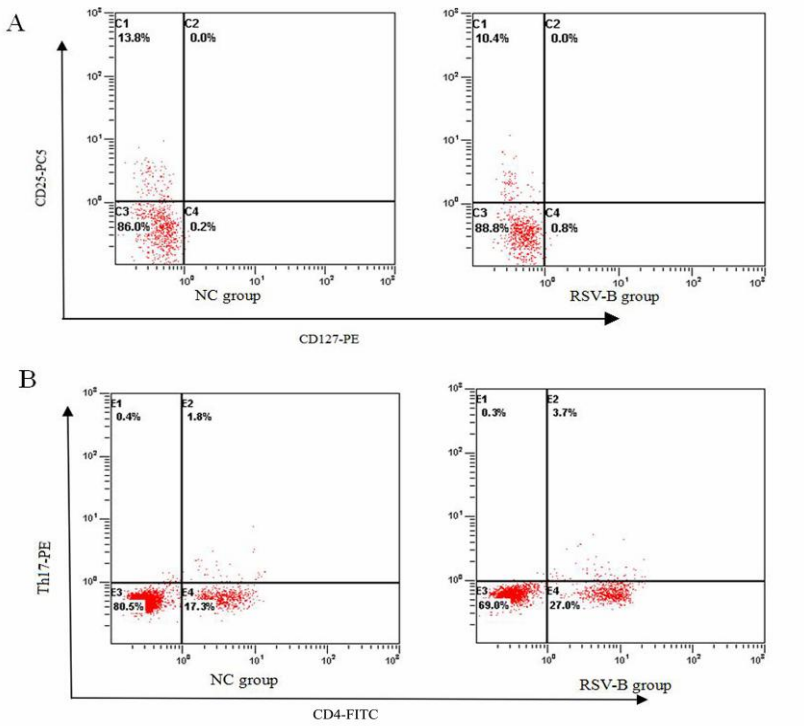

Figure 2. Percentages of $CD4^+CD25^+CD127^+$ T cells (Treg) and $CD4^+IL\text{-}17^+$ T cells (Th17) in each group. The percentage of Treg in the NC group is higher than that in the RSV-B group, on the contrary, the percentage of Th17 cells is higher in RSV-B group. (A) Representative FACS analysis of Treg cells gated from $CD4^+$ cell subsets in the normal control and respiratory syncytial virus -B groups, comparing the levels of $CD4^+CD25^+CD127^+$ and $CD4^+$ cells. (B) Representative FACS analysis of Th17 cells in the normal control and respiratory syncytial virus -B groups, comparing the levels of $CD4^+IL\text{-}17^+$ and $CD4^+$ cells.

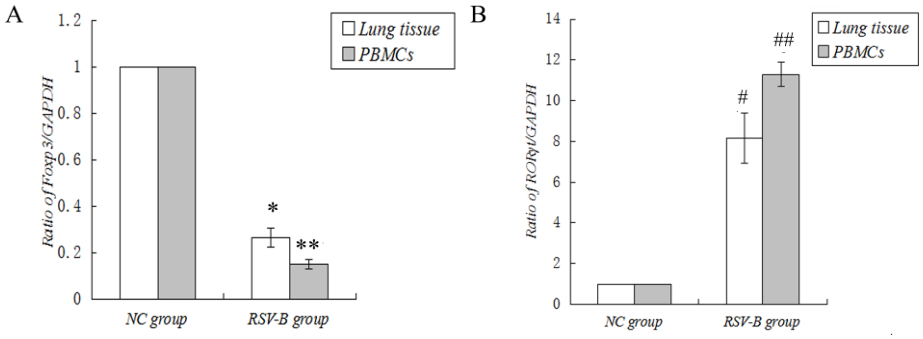

Figure 3. Expression levels of Foxp3 mRNA and RORγt mRNA in lung tissue and PBMCs from the respiratory syncytial virus -B and normal control groups by performing qRT–PCR analysis. The levels of mRNA in lung tissue showed the same trend with PBMCs, however, the trend of FoxP3 is in the opposite direction with RORγt.(A) The ratio of FOXP3/GAPDH mRNA was compared between the respiratory syncytial virus -B and normal control groups in lung tissue and PBMCs. (B) The ratio of RORγt/GAPDH mRNA was compared between the respiratory syncytial virus -B and normal control groups in lung tissue and PBMCs. All values are presented as mean±SD, n=15; $^*p<0.01$ vs. normal control group; $^{**}p<0.01$ vs. normal control group; $^{\#}p<0.05$ vs. normal control group; $^{\#\#}p<0.01$ vs. normal control group.

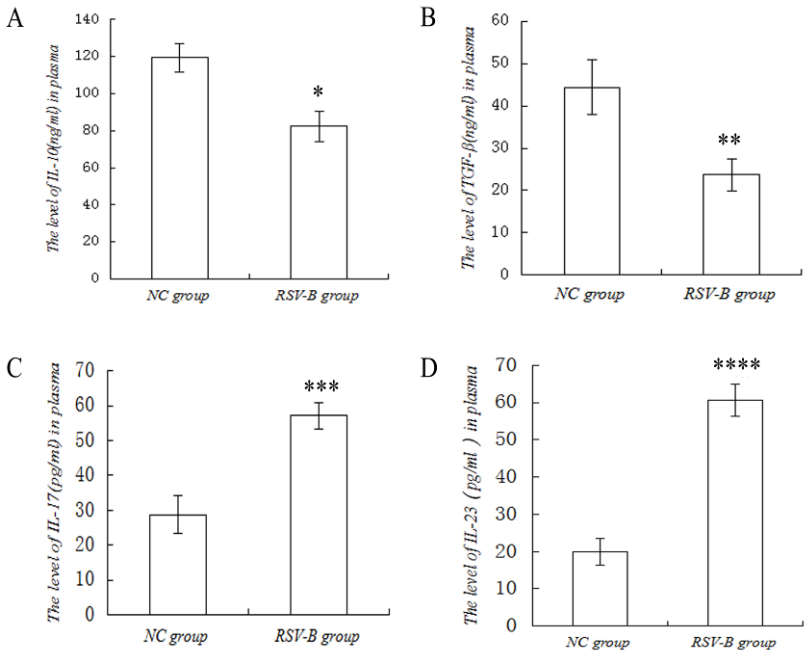

Figure 4. Concentrations of Treg- and Th17-related cytokines in plasma. (A) IL-10 concentration was compared between respiratory syncytial virus -B and normal control groups in plasma. (B) TGF-β plasma concentration was compared between respiratory syncytial virus -B and normal control groups. (C) IL-17 plasma concentration was compared between the respiratory syncytial virus -B and NC groups. (D) IL-23 plasma concentration was compared between respiratory syncytial virus -B and normal control groups. All the values were presented as mean±SD, n = 15; $^{*}p< 0.01$ vs. normal control group; $^{**}p<0.01$ vs. normal control group; $^{***}p< 0.01$ vs. normal control group; $^{****}p< 0.01$ vs. normal control group.

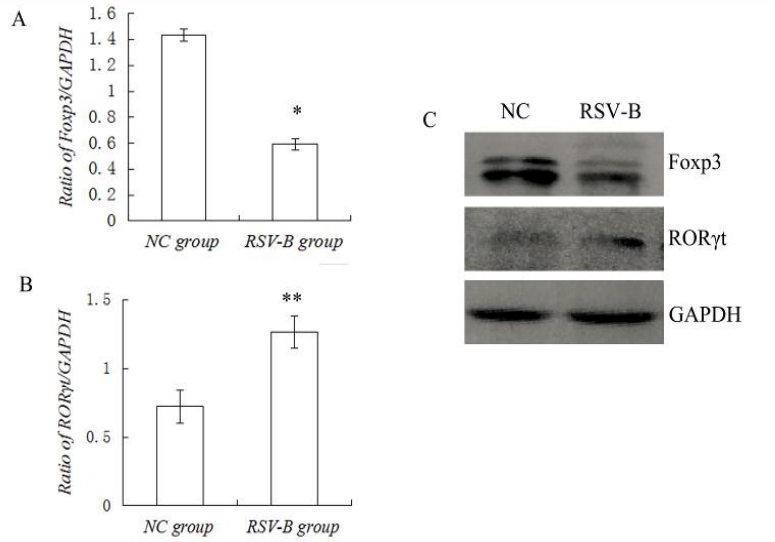

Figure 5. Western blot analysis was performed to detect the protein expression levels of FOXP3 and RORγt in lung tissue. (A) The ratio of FOXP3/GAPDH mRNA was compared between lung tissues of respiratory syncytial virus-B and normal control groups. (B) The ratio of RORγt/GAPDH mRNA was compared between lung tissues of respiratory syncytial virus -B and normal control groups. All the values were presented as mean±SD; $^{*}p<0.01$ vs. normal control group; $^{**}p<0.05$ vs. normal control group.

Table 1. Detection of percentages of both Treg and Th17 cells in PBMCs. Data are expressed as mean±SD, n=15; $^{*}p< 0.01$ vs. NC group; $^{**}p<0.01$ vs. NC group; $^{***}p<0.01$ vs. NC group.

Group	n	Treg (%)	Th17 (%)	Treg/Th17 ratio
NC group	15	13.39 ± 1.01	1.65 ± 0.41	8.65 ± 2.25
RSV–B group	15	10.22 ± 0.66*	3.73 ± 0.45**	2.78 ± 0.32***

$^{*}p<0.01$ vs. NC group; $^{**}p<0.01$ vs. NC group; $^{***}p<0.01$ vs. NC group

was significantly lower in the lung tissue and PBMCs of the RSV-B group than in those of the NC group ($^{*}p<0.01$ vs. NC group, $^{**}p<0.01$ vs. NC group); this result was more prominent in the PBMCs than in the lung tissue. As illustrated in Fig. 3B, the mRNA level of RORγt was higher in the lung tissue and PBMCs of The RSV–B group than in those of the NC group ($^{\#}p<0.05$ vs. NC group, $^{\#\#}p<0.01$ vs. NC group); this change was more prominent in the PBMCs than in the lung tissue. These results suggest that a decrease in FOXP3 mRNA and an increase in RORγt mRNA are associated with inflammation development in the RSV-B group.

Plasma Concentrations of Treg- and Th17-Related Cytokines

The plasma concentrations of Treg-related (IL-10 and TGF-β) and Th17-related cytokines (IL-17 and IL-23) were analyzed using ELISA. As shown in Figure 4, IL10 and TGF-β concentrations were consistently lesser in the RSV-B group than in the NC group (Figure 4A, $^{*}p<0.01$ vs. NC group; Figure 4B, $^{**}p<0.01$ vs. NC group). However, the amounts of IL-17 and IL-23 were significantly higher in the RSV-B group than in the NC group (Figure 4C, $^{***}p<0.01$ vs. NC group; Figure 4D, $^{****}p<0.$ 01 vs. NC group). These data indicate that changes in the expression of Treg- and Th17-related cytokines promote the occurrence of inflammation in bronchiolitis.

Protein Expression of Treg and Th17 Transcription Factors

The protein expression levels of FOXP3 and RORγt were determined in the lung tissues of rats from each group. The gene expression of FOXP3 was significantly reduced in the RSV-B group when compared to the NC group (Figure 5A, $^{*}p< 0.01$ vs. NC group). By contrast, the protein expression level of RORγt was higher in the RSV-B group than in the NC group (Figure 5B, $^{**}p<0.05$ vs. NC group). These results suggest that both Treg and Th17 cells are involved in bronchiolitis pathogenesis at the protein level.

DISCUSSION

RSV infection is the primary cause of bronchiolitis and hospitalization among children who are below two years old. RSV bronchiolitis can lead to severe tissue hypoxia, respiratory failure, and even death. However, the mortality rate of RSV bronchiolitis remains unclear to date.[21, 22] The current treatment for RSV bronchiolitis is limited to symptomatic treatments instead of specific treatment.[23] Therefore, studying the pathogenesis of bronchiolitis and determining new treatments are significant.

Th17 cell is a novel cell lineage of CD4+T that is characterized by the secretion of distinct IL-17 cytokines. Previous studies reported that the Th17 cells and its IL-17 promote inflammation in allergic asthma.[24] IL-17 can stimulate lung fibroblasts, airway epithelial cells, and other inflammatory cells to produce pro-inflammatory cytokines and chemokines, which can lead to severe tissue inflammation by recruiting neutrophils and macrophages. Moreover, asthmatic mice injected with the IL-17 monoclonal antibody do not exhibit wheezing, shortness of breath, and airway hyperresponsiveness[25]. Meanwhile, Wakeland et al[26] determined that reducing IL-17 concentration in asthmatic mice alleviates inflammation in the airway. Consistent with these previous findings, the result of the current study shows that the percentage of Th17 cells in PBMCs and the level of IL-17 in plasma were significantly higher in the RSV-B group (Figs. 2B and 4C) than in the NC group. These results imply that the increase in Th17 and IL-17 significantly contribute to the occurrence of bronchiolitis inflammation and that Th17 immune response is enhanced in RSV bronchiolitis. However, the explicit mechanisms by which Th17 immune response is enhanced in RSV bronchiolitis warrants further analysis.

IL-23 is another important pro-inflammatory cytokine that regulates the proliferation, differentiation, and maintenance of Th17 cells.[27] A study has suggested

that IL-23, produced by antigen-presenting cells, can induce CD4+T cells to differentiate into Th17 cells, which promote inflammation by secreting IL-17[28]. Similarly, the IL-23 level was significantly higher in the RSV-B group than in the NC group in the present study (Figure 4D). The increase in IL-23 may promote the proliferation and differentiation of Th17 cells, thereby making the latter significantly stable. The stable Th17 cells could considerably cause sustainable inflammation by secreting IL-17. These results suggest that IL-23 plays an integral role in the Th17 immune response of RSV bronchiolitis.

RORγt, is a key transcription factor that consistently regulatesg the differentiation of Th17 cellswhich is upregulated in mature Th17 cells.[16, 29] Transferring the retrovirus of the encoding RORγt gene into native T cells induces the differentiation of these cells into mature Th17 cells[30]. Ivanov et al [31] also determined that the lack of RORγt can reduce the number of Th17 cells. The studies as mentioned above indicate that RORγt is crucial in regulating the development and function of Th17 cells. In the present study, the RSV-B group had higher mRNA (Figure 3B) and protein (Figure 5B) expression levels of RORγt than the NC group. These results indicate that high RORγt levels are involved in Th17 immune response at the transcriptional and translational levels. Nonetheless, the mechanisms of the preternatural RORγt in RSV bronchiolitis remain unclear.

Treg cellsis another new subtype of CD4+T cells, it secretes IL-10 and other inhibitory cytokines to suppress the immune response harmful to a host.[16, 32] Kearley et al[33] also determined that transplanting exogenous Treg cells into asthmatic mice alleviates airway inflammation and hyperresponsiveness. IL-10 can inhibit the activation of eosinophils and mast cells, as well as suppress the aggregation of inflammatory cells and cytokines in the airways.[34] These processes demonstrate that Treg cells and its IL-10 secretion reduce the inflammatory response of the host. The current study determined that the percentage of Treg cells and the concentration of IL-10 were overtly lower in the PBMCs of the RSV-B group than in those of the NC group (Figures 2A and 4A). This result indicates that the number of Treg cells is reduced to suppress the inhibition of inflammatory factors in RSV bronchiolitis. However, the mechanism underlying the decrease in Treg cells in RSV bronchiolitis warrants further investigation.

The role of TGF-β in Treg immune response remains controversial. Previous studies reported that the lack of TGF-β upregulates FOXP3 expression[35] and that TGF-β regulates FOXP3 expression.[36] Thus, the role of TGF-β in Treg immune response may rely on the immunization environment. In the present study, TGF-β level was lower in the RSV-B group than in the NC group (Figure 4B). RegardingBasing from these results, we inferred that the decrease in TGF-β weakens the ability of Treg cells to suppress the inflammatory response.

FOXP3 is a specific transcription factor of the Treg cells that plays an important role in the development, morphology, and function of Treg cells[16]. The lack of FOXP3 contributes to the occurrence of allergic diseases. Chaudhry et al [37, 38] determined that the positive expression of FOXP3 in Tregs cells inhibits inflammation and suppresses effector T cells to transdifferentiate into another proinflammatory Th17 cell. In the present study, the RSV-B group had lower mRNA (Fig. 3A) and protein (Figure 5A) expression levels of FOXP3 than the NC group. Therefore, FOXP3 is possibly important for Treg immune response. The low expression of FOXP3 may be associated with the low capacity of Treg cells to suppress inflammation.

However, animal experiments have limitations. In this study we investigated the changes in the levels of tissue and cells. We can further study the therapeutic effect of protecting cytokines in this experiment by using gene transfection mode, and provide some new methods for clinical treatment. In conclusion, the Treg/Th17 ratio evidently decreased (Table 1, ***$p<$ 0.01).The increase in Th17 cells and a decrease in Treg cells were deemed important features of RSV bronchiolitis. The upregulated expression of IL-23, IL-17, and RORγt and decreased expression of IL-10, TGF-β, and FOXP3 may contribute to the abnormal Th17 and Treg immunity in RSV bronchiolitis. However, the causal role of Th17 and Treg immunity in the pathogenesis of RSV bronchiolitis requires further analysis. In the future, a new treatment associated with the regulation of Th17 and Treg immune responses for RSV bronchiolitis will be performed.

ACKNOWLEDGEMENTS

This study is supported by a grant from the Medical and Health Development Project Foundation of

Shandong Province (NO. 2013WS0312) and grants from the Shandong Province Natural Science Foundation (ZR2014HL001).

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