



Natural helper cells are associated with the exacerbated airway inflammation seen during RSV reinfection of neonatally primed mice

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ABSTRACT

Infection with respiratory syncytial virus (RSV) in neonatal mice causes more aggressive airway disease when the mice are reinfected with the same virus as adults. However, the underlying mechanisms responsible for this phenomenon are not entirely defined. Natural helper (NH) cells are considered a key factor for virus-induced or exacerbated airway inflammation and airway hyper-responsiveness by producing type 2 cytokines. To confirm whether NH cells are involved in the aggravated lung pathology seen during reinfection, BALB/c mice were initially infected as neonates and reinfected in adulthood. We observed that neonatal RSV infection resulted in an enhanced infiltration of eosinophils and neutrophils in the lungs, in parallel with a significant increase in the levels of IL-5 and IL-13 in bronchoalveolar lavage fluids on day 2 after reinfection. It seems likely that pulmonary NH cells may play a role in the occurrence, since mice first infected at 1 wk of age developed an additional increase in the number of NH cells as well as IL-5- and IL-13-producing NH cells in the lungs than those first infected as young adults. In fact, an elevated expression of mRNAs for IL-5 and IL-13 in pulmonary NH cells was detected in mice first infected as neonates. Furthermore, adoptive transfer of NH cells into neonatal mice was able to boost eosinophilic infiltration as well as the production of type 2 cytokines in the lungs after reinfection at adulthood. In contrast, the expression of mRNA for the type 1 cytokine IFN- γ was down-regulated markedly by adoptive transfer of NH cells. Thus, these results suggest that Th2-type NH cells may play a role in the exacerbated airway inflammation seen during RSV reinfection of neonatally primed mice.

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1. Introduction

Respiratory syncytial virus (RSV) is the most common cause of respiratory-tract infections in infants and young children [1]. RSV infection of infants can lead to serious respiratory disease, sometimes requiring hospitalization. Severe infantile RSV infections are associated with recurrent wheezing and an asthma diagnosis in childhood and adulthood [2,3]. It is clear that RSV infection during early infancy preferentially promotes a Th2-like response in the respiratory tract with local production of type 2 cytokines, such as IL-4 and IL-5 [4]. RSV infection can occur throughout life. However, compared with a delay in the initial infection of RSV, neonatal RSV infection produces more severe weight loss and increases inflammatory cell recruitment during reinfection in adulthood,

suggesting that the timing of the primary RSV infection has a profound effect on the outcome of RSV reinfection [5,6]. Indeed, the level of IL-13 as well as eosinophilic infiltration in the lungs of mice first infected with RSV as neonates can be significantly enhanced after a secondary adult infection [7]. However, the underlying mechanisms responsible for these significant age differences are not entirely defined.

Although T cells may play an important role in the development of RSV-induced airway hyper-reactivity and airway inflammation [8–10], T cell depletion during secondary infection does not completely abrogate the RSV-induced airway inflammation or weight loss, which can be observed as early as 2 days after the infection [11], suggesting that other cell types, especially innate immune cells, may also contribute to RSV-related respiratory tract diseases. The depletion of NK cells has been reported to be able to attenuate weight loss and airway inflammation, particularly eosinophilic infiltration, during RSV reinfection [12].

Recently, a novel population of non-B/non-T innate immune cells, in this paper termed natural helper (NH) cells, was identified in lung parenchyma [13–15]. NH cells do not express antigen receptors and lineage markers, but do express Sca-1, CD25 and the IL-33 receptor ST2

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[13,14,16]. NH cells have the capacity to produce large amounts of type 2 cytokines, particularly IL-5 and IL-13 after exposure to the proinflammatory cytokines IL-25 and IL-33, two cytokines linked to the type 2 response [13,17–19]. In mice, NH cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity [20,21]. Infection with respiratory syncytial virus has been reported to be able to induce an increase in the number of pulmonary NH cells, and these NH cells produce abundant IL-13, which is essential for the accumulation of eosinophils into infected lungs [21–23]. However, up until now, little has been known about the role of NH cells in the exacerbated airway inflammation during secondary reinfection of mice first infected as neonates.

Thus, in this paper, by using BALB/c mice that were infected with RSV as neonates or young adults and reinfected in adulthood, the role of pulmonary NH cells in airway inflammation on day 2 after reinfection of neonatally infected mice was investigated.

2. Materials and methods

2.1. Mice and virus stocks

BALB/c mice were purchased from Shanghai Laboratory Animal Center and maintained in individual filter cages at the Laboratory Animal Center, China Medical University. Breeders were time-mated, and pups born on the same date were used for experiments. The human RSV type A2 (RSV A2) strain was grown in HEp-2 cells (ATCC) and assayed for infectivity as described previously [24]. The viral titer was expressed as a 50% tissue culture infectious dose (TCID₅₀), calculated using the method of Reed and Muench [25].

2.2. Experimental design

Neonatal mice (1-week-old) were infected intranasally with RSV in 10 μ l of sterile phosphate-buffered saline (PBS) containing 1×10^5 TCID₅₀ per mouse, and reinfected at 8 wk of age at a dose of 2×10^5 TCID₅₀ per mouse (expressed as Neonatal 2[○]). Young adult mice (4-week-old) were infected intranasally with RSV at an inoculum dose of 2×10^5 TCID₅₀ per mouse, and reinfected with 10 μ l of PBS containing 2×10^5 TCID₅₀ per mouse at 8 wk of age (expressed as Adult 2[○]). The mice used in the normal control group were inoculated intranasally with sterile PBS. Samples were collected on day 2 after reinfection. This work was approved by the Institutional Animal Care and Use Committee of China Medical University, China.

2.3. Histopathological examination for airway inflammation

Airway inflammation was assessed by total and differential counting of cells recovered in bronchoalveolar lavage (BAL) fluids and by histopathological examination of lung tissue sections stained with hematoxylin and eosin (H&E). The inflammation index was quantified by manually counting the number of peribronchiolar/perivascular inflammatory cells divided by the peribronchiolar/perivascular areas (mm²).

2.4. Preparation of single-cell suspensions from lungs

Mice were anesthetized and the lung was flushed *in situ* with 20 ml of PBS via cannulation of the heart to remove the intravascular blood pool. Minced lung tissue samples were incubated at 37 °C for 1 h on a rocker with 200 μ g/ml collagenase D and 40 μ g/ml DNase I (Roche Molecular Biochemicals). Single-cell suspensions from the digested lung were collected through density-gradient centrifugation with lymphocyte separation solution and resuspended in RPMI 1640 medium.

2.5. Flow cytometry

For identification of pulmonary NH cells, single-cell suspensions from lung parenchyma were incubated with anti-mouse CD16/32

mAb to block the Fc receptor and stained with FITC-conjugated lineage marker mAbs (CD3, CD4, CD5, CD8, CD11b, Gr-1, CD19, B220, DX5 or NK1.1 and TCR α), APC-conjugated anti-CD45 mAbs and PerCP-conjugated anti-ST2 mAbs (eBioscience). The CD45⁺ Lin[−] ST2⁺ cells were classified as NH cells [13,21]. For intracellular cytokine staining, approximately 2×10^6 lung cells were stimulated for 4 h with 2 μ l/ml Cell Activation Cocktail (containing 10 μ g/ml brefeldin A, 100 μ g/ml PMA and 10 μ g/ml ionomycin, Biolegend) in the presence of a protein-transport inhibitor (GolgiPlug, BD Biosciences). Cells (the percentage of live cells was >86%) were surface stained for 20 min on ice, followed by fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences). Intracellular cytokine staining for IL-5, IL-13 and IFN- γ (PE-conjugated anti-cytokine antibodies, BD Biosciences) was assayed according to manufacturer's instructions.

2.6. Adoptive transfer of pulmonary NH cells

NH cells were FACS-sorted from pooled lungs of normal adult mice (8 wk of age) using a FACS cell sorter (BD Biosciences). The purified cells (purity was >90%) were pooled and resuspended to 1×10^6 cells/ml of PBS. Then, 1×10^4 cells in 10 μ l of PBS were inoculated intranasally into neonatal mice (1 wk of age) 2 h before the primary RSV infection.

2.7. Measurement of cytokine gene expression

Total RNA was isolated from lung homogenates or FACS-sorted NH cells frozen in TRIzol (Life Technologies) according to the manufacturer's instructions. First-strand cDNA was synthesized from the RNA using SuperScript III Reverse Transcriptase (Life Technologies). Real-time PCR of cDNA was performed in a StepOnePlus PCR system using SYBR Green Master Mix (Life Technologies). Primer sequences used were as follows:

IL-5-F, 5'-GGCTTCCTGCTCCCTACTCAT-3',
IL-5-R, 5'-TCCTCGCCACACTTCTCTTT-3';
IL-13-F, 5'-AGCATGGTATGGAGTGTGGA-3',
IL-13-R, 5'-TTGCAATTGGAGATGTTGGT-3';
IFN- γ -F, 5'-TATCTGGAGAACTGCCAAA-3',
IFN- γ -R, 5'-GGTGTGATTCAATGACGCTT-3';
 β -actin-F, 5'-CAACGAGCGGTTCCGATG-3',
 β -actin-R, 5'-GCCACAGGATTCCATACCCA-3'.

Real-time RT-PCR was run in a LightCycler® 480 system (Roche Molecular Biochemicals) under identical amplification condition. The results are normalized to β -actin expression and presented as fold change in mRNA expression (fold change = $2^{-\Delta\Delta CT}$).

2.8. Cytokine assessment in BAL fluids

BAL fluids were obtained by cannulating the trachea and flushing the lungs with 1 ml of PBS. The levels of IL-5, IL-13 and IFN- γ in BAL fluids were quantified by ELISA kit (eBioscience).

2.9. Statistical analysis

Data are presented as the mean $\pm$ SEM of six to ten mice in each group. Statistical analyses were performed using Prism 5 (GraphPad Software). One-way ANOVA with a Tukey post-test was used to compare differences between groups. *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Neonatal RSV infection enhances airway inflammation after reinfection

To define the extent of airway responsiveness to a secondary RSV infection in mice, lung histopathology and bronchoalveolar lavage cellularity were assessed on day 2 after reinfection. The total cell numbers

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