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IL-4Rα on CD4⁺ T cells plays a pathogenic role in respiratory syncytial virus reinfection in mice infected initially as neonates

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ABSTRACT
RSV is the major cause of severe bronchiolitis in infants, and severe bronchiolitis as a result of RSV is associated with subsequent asthma development. A biased Th2 immune response is thought to be responsible for neonatal RSV pathogenesis; however, molecular mechanisms remain elusive. Our data demonstrate, for the first time, that IL-4Rα is up-regulated in vitro on human CD4⁺ T cells from cord blood following RSV stimulation and in vivo on mouse pulmonary CD4⁺ T cells upon reinfection of mice, initially infected as neonates. Th cell-specific deletion of Il4ra attenuated Th2 responses and abolished the immunopathophysiology upon reinfection, including airway hyper-reactivity, eosinophilia, and mucus hyperproduction in mice infected initially as neonates. These findings support a pathogenic role for IL-4Rα on Th cells following RSV reinfection of mice initially infected as neonates; more importantly, our data from human cells suggest that the same mechanism occurs in humans. J. Leukoc. Biol. 93: 933-942; 2013.

Introduction
RSV is the leading cause of acute respiratory tract infections in infants younger than 1 year of age [1, 2]. Typically children are infected with RSV in their first RSV season, and 50% of them are reinfected by 2 years of age [3, 4]. Long-term resistance to RSV infection does not develop, and reinfection is common, particularly in those with chronic lung or heart diseases, where severity of the illness is much greater [5]. Although there is considerable worldwide, clinical impact from RSV infection, we have neither vaccines nor specific therapeutics against RSV infection. Development of vaccines or therapeutics has been slow as a result of limited understanding of the virus, the host immune system at the time of initial infection, and the interaction between the virus and the host immune system.

Studies have demonstrated the importance of age at the time of initial RSV infection in determining the pathophysiological responses [6–11]. In humans, severe RSV infection in infants leads to respiratory dysfunction that can persist into early adulthood [12–15]. In mice, we have demonstrated that age of initial infection with RSV determines respiratory function in later life [8, 9]. Mice infected with RSV as neonates (<7 days of age) develop long-term airway dysfunction [8], whereas mice infected initially as weanlings fail to do so [9]. Studies from other laboratories have demonstrated further that mice infected as neonates respond to RSV reinfection with a skewed Th2 immune response [10, 11]. Increased susceptibility to RSV and disease severity in infants are thought to be, in part, a result of the developmental state of their immune system [16, 17], although other factors, such as airway size and maturation status of the lung, may also contribute to the pathology observed.

In humans, Th2 cytokines IL-4 and IL-13 and their receptors have been associated with severe RSV disease [18–23]. In mouse models, IL-4/13 and their receptors have been shown to play a key role in the multiple pathophysiologies associated with infant RSV infection, including airway hyper-reactivity, eosinophilic inflammation, and mucus hyperproduction [18–23]. Interestingly, IL-4 and IL-13 share a common receptor, the type II IL-4R, which is composed of IL-4Rα and IL-13Rα1 and is thought to be re-
sponsible for the pathological changes observed in asthma and RSV-associated disease [20, 24, 25]. IL-4 also binds to a second receptor, the type 1 receptor, which is composed of IL-4Rα and a γ chain and is believed to initiate Th2 differentiation. All of these data point to a potential role of IL-4Rα in RSV pathogenesis in infants.

Here, we confirm that upon reinfection, mice infected initially with RSV as neonates responded with a skewed Th2 immune response associated with airway hyper-reactivity, eosinophilia, and mucus hyperproduction. This is not the case when the initial infection occurs in adults. The immunophysiological changes following reinfection of neonatally infected mice correlated with increased expression of IL-4Rα on pulmonary CD4+ Th cells and elevated proliferation of these cells, especially the Th2 subset. Specific deletion of Il4ra from Th cells attenuated pulmonary Th2 responses and abolished the immunopathophysiology associated with RSV reinfection. Validating these data in the human system, we observed elevated IL-4Rα expression on CD4+ Th cells from cord blood following stimulation with RSV in vitro.

Collectively, our data reveal a mechanism, whereby IL-4Rα signaling on Th cells in neonates results in an exaggerated Th2 immune response upon reinfection with RSV and adds significantly to our understanding of RSV pathogenesis, which may be applicable to humans.

MATERIALS AND METHODS

Mice

BALB/c breeder pairs and female adult mice (6–8 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN, USA) and were maintained under a specific, pathogen-free condition in the Division of Animal Care at LSUHSC (New Orleans, LA, USA). Breeders were time-mated, and pups born on the same date were used for experiments. The CD4+ T cell-specific, IL-4Rα-deficient mice (Lck−/−Il4ra−/-Lox) were generated by crossing IL4RαLox/Lox BALB/c mice and Lck−/−Il4ra−/-BALB/c mice (gifts from Drs. Debroski Herbert and Frank Brombacher, University of California, San Francisco, USA, and University of Cape Town, South Africa). In these mice, Cre recombinase is under the control of the T cell-specific promoter Lck; however, Lck−/−Il4ra−/-Lox mice showed effective deletion of Il4ra in CD4+ T cells but incomplete and variable deletion in other T cells, including CD8+ T cells, NKT cells, and γδT cells [26].

All animal protocols were prepared in accordance with the Guide for the Care and Use of Laboratory Animals [27] and were approved by the Institutional Animal Care and Use Committee at LSUHSC.

Human cord or peripheral blood samples

Cord and peripheral blood samples were obtained with informed, written consent from term neonatal subjects (between 38 and 41 weeks of gestation), delivered at the Children’s & Women’s Health Centre of British Columbia (Vancouver, BC, Canada), and from healthy adult volunteers at the Child & Family Research Institute (Vancouver, BC, Canada), respectively. Consent was obtained for children by their parent or legal guardian. Protocols were approved by the Clinical Research Ethics Board (H07-02081).

RSV infection

The original human RSV strain A2 was purchased from Advanced Biotechnologies (Columbia, MD, USA). For all mouse studies, the virus was propagated in Vero cells (American Type Culture Collection, Manassas, VA, USA) using serum-free medium (SFM/MegaVir; HyClone, Logan, UT, USA) and stored at −80°C. Five-day-old pups or adults were anesthetized with 5% isoﬂurane and infected intranasally with 2 × 105 50% tissue culture-infective dose/g body weight of RSV in 10 μl (for pups) or 50 μl (for adults) serum-free medium. In some experiments, mice were sham-infected with serum-free medium. For stimulation of human PBMCs and CBMCs, the virus was propagated in HEp-2 cells (Hybrid Diagnostics, Athens, OH, USA) using minimal essential medium with Earle’s salts, containing 2% FBS and 1 mM sodium pyruvate (HyClone), and purified further by ultracentrifugation on a discontinuous sucrose gradient [28].

Pulmonary function test

Airway resistance upon MeCh (Sigma-Aldrich, St. Louis, MO, USA) challenge was measured using the flexiVent system (Scireq, Montreal, QC, Canada). Mice were anesthetized with ketamine/xylazine (180/10 mg/kg body weight), tracheotomized, and ventilated with a computer-controlled piston pump. The pressure and volume of the airway were recorded and fitted into a single compartment model to calculate airway resistance. Raw data were normalized by subtracting individual baseline values (resistance at 0 mg/ml MeCh) and plotted as normalized resistance. Baseline values were not statistically different among the groups.

Lung histopathology

Mice were killed and retrograde perfusion performed to remove blood from the lungs, which were then gently inflated and fixed with zinc formalin (Thermo Fisher Scientiﬁc, Waltham, MA, USA). After fixation, lungs were embedded in paraffin, sectioned, and mounted on slides; then, the slides were stained with H&E or PAS to identify cellular infiltrates or mucus production in the airway epithelial cells, respectively. Lung sections were stained by H&E and visualized under high magnification (60X). Eosinophils were quantified in the peribronchial tissues of lung specimens in a blinded manner and expressed as number of eosinophils/surface area of the lung parenchyma. Mucus expression in the airway epithelial cells was detected by PAS staining and images acquired. Images were analyzed with ImageJ [29]. Mucus expression was quantified and expressed as area of mucus-positive cells/surface area of airway epithelial cells.

T cell activation assay

To measure the proliferative ability of lung Th cells in vitro, T cell activation assay was performed [30]. Briefly, pulmonary CD11c+ cells were isolated from naive, adult mice with a mouse CD11c-positive selection kit from Stemcell Technologies (Vancouver, BC, Canada) and infected with RSV (MOI=50) for 18 h. Lung CD4+ T cells were selected with a mouse CD3-positive selection kit (Stemcell Technologies) and stained with 0.5 μM CFSE (Life Technologies, Carlsbad, CA, USA). CD4+ cells (5×104) were then cocultured with 5 × 105 CD11c+ cells in the presence of 1 μg/ml CD3 antibody (clone #17A2; e Bioscience, San Diego, CA, USA) for 72 h. The cells were stimulated, stained, and subjected to flow cytometry to determine the Th populations and CFSE signals in each population. The feasibility of this T cell activation assay was tested using increasing infectious doses of RSV, with or without CD3 antibody (Supplemental Fig. 1).

BALF cellularity

BALFs were isolated by flushing lungs with 1 ml PBS containing 2% heat-inactivated FBS. Total cell numbers were counted with a hemocytometer. BALF cells were centrifuged onto slides and stained with a Hema-3 staining kit (Thermo Fisher Scientiﬁc). Differential cell counts were determined by two unbiased observers using standard morphological criteria.

Cytokine assay

Cytokine levels in the BALF were measured using a Milliplex mouse cytokine/chemokine assay kit (Millipore, Billerica, MA, USA) on the Bioplex system (Bio-Rad Laboratories, Hercules, CA, USA), as per the manufacturer’s instruction. Each sample was assayed in duplicate for type I cytokines, IFN-γ and IL-12 (p40), and type II cytokines, IL-4 and IL-13. Data presented herein excluded any number outside of the sensitivity level of each cytokine.
Cell staining and flow cytometry

To determine the Th cell population in the lung, lung single-cell suspension was isolated using the gentleMACS Octo Dissociator and lung dissociation kit (Miltenyi Biotec, Auburn, CA, USA). The cells were then stimulated for 5 h at 37°C in stimulation media containing RPMI 1640 (HyClone), 10% heat-inactivated FBS (Life Technologies), 100 U/ml penicillin (HyClone), 100 mg/ml streptomycin (HyClone), 5 ng/ml PMA (Sigma-Aldrich), and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of a protein transport inhibitor (1 µl/100 cells; GolgiPlug; BD Biosciences, Franklin Lakes, NJ, USA). After stimulation, the cells were stained with fixable viability dye (eBioscience); fixed and permeabilized with intracellular fixation and permeabilization buffer (eBioscience); and stained with antibodies to CD3 (clone #SP34-2; eBioscience), T-bet (clone #4B10; eBioscience), and IL-4R (clone #hIL4R-M57; BD Biosciences). The staining profile was acquired on an LSR II flow cytometer (BD Biosciences), and the flow data were analyzed using FlowJo software (version 7.6.5 for Windows; Tree Star, Ashland, OR, USA).

To determine the proliferation of Th cell populations in the T cell activation assay, the cells collected from the CD11c Cre+ and CD4+ cell coculture were stimulated and stained as above and subjected to flow cytometry to measure the CFSE signal in each Th population.

To measure IL-4Ra expression level on pulmonary Th cells, lungs cells were isolated as above. The single-cell suspension was then stained with fixable viability dye and antibodies to CD3 (clone #17A2; eBioscience), CD4 (clone #RM4-4; BioLegend, San Diego, CA, USA), IFN-γ (clone #XMG1; eBioscience), and IL-4 (clone #BVD6-24G2; eBioscience). The flow data were acquired with a Canto II flow cytometer (BD Biosciences), analyzed, and plotted with FlowJo software (version 7.6.5 for Windows; Tree Star, Ashland, OR, USA).

RESULTS

Neonatal RSV infection induced airway hyper-reactivity and lung histopathology upon reinfection

To model the unique pathophysiological effects that RSV infection has on infants, we infected 5-day-old pups or adult mice (6–8 weeks) with RSV and then reinfected these mice at 4 weeks postprimary infection. At 6 days postreinfection, we assessed pulmonary function and evaluated pulmonary pathology. Pulmonary viral load data from other groups have shown that RSV infects and replicates in the lung during secondary infection [11]. As shown in Fig. 1A, NRR mice exhibited significantly greater airway resistance following MeCh challenge than ARR mice (6.16 ± 0.99 vs. 1.88 ± 0.26 cm H2O · s · ml at 50 mg/ml MeCh), indicating that neonatal primary infection but not adult primary infection results in airways hyper-reactivity upon reinfection. Histological analysis of lung sections from NRR and ARR mice revealed similar levels of perivascular and peribronchiolar inflammation between NRR and ARR mice (Fig. 1B). However, significantly more eosinophils were observed in the lungs of NRR versus ARR mice (932 ± 151 vs. 46 ± 15 cells/mm2 surface area of epithelium). Additionally, we observed a substantial amount of mucus cell hyperplasia and mucus hyperproduction in mice infected as neonates, whereas ARR mice exhibited little-to-moderate levels of mucus-positive staining cells in the airways (Fig. 1C). In fact, the area of mucus-positive cells in the airways of NRR mice was significantly greater than in ARR mice (0.47 ± 0.04 vs. 0.08 ± 0.02 µm2/µm2 epithelium). Occasionally, we observed sloughing epithelial cells in the airway of NRR mice (data not shown), and this was not observed in any of the lung sections from ARR mice.

Based on our previous studies in neonates and weanlings [9], we thought it was highly unlikely that age at reinfection (i.e., 5 or 10–12 weeks) had a substantial effect on Th cell responses following reinfection. However, to address this, we monitored pulmonary viral loads at 1, 2, and 4 dpi and Th cell responses at 6 days postprimary infection from mice infected at 5 or 12 weeks old. As expected, there was no difference in pulmonary viral load or primary T cell response (Supplemental Fig. 2) between these two groups. These data sustain that the differences we are observing are associated with the age difference at primary but not secondary infection.

Neonatal RSV infection induced an exaggerated Th2 response upon reinfection

We further examined the immune responses to RSV reinfection in the lungs of mice infected as neonates (NRR) or adults (ARR). Six days postreinfection, lung cells were isolated from NRR and ARR mice and Th subsets analyzed by flow cytometry (Fig. 2A and B). RSV reinfection induced substantial amounts of Th1 (IFN-γ-expressing CD4+ T) cells in both NRR and ARR mice (21.03 ± 2.65 and 16.19 ± 2.21%), respectively. However, NRR mice recruited sixfold more Th2 (IL-4-expressing CD4+ T) cells to the lung compared with ARR mice (3.8 ± 0.98 vs. 0.46 ± 0.12%). More interestingly, NRR mice had a subset of “multifunctional” Th cells that coexpressed the prototypical Th1 cytokine IFN-γ and Th2 cytokine IL-4. These data demonstrate that mice infected as neonates elicited a Th2-biased immune response in the lung upon secondary RSV infection.

Statistics

All data were plotted as means ± SEM using Prism5 (GraphPad Software, La Jolla, CA, USA). The statistics were analyzed with Prism5 or InStat 3 (GraphPad Software). Two-way ANOVA and Bonferroni post hoc tests were used to compare between the groups for the pulmonary function tests. Student’s t-test was used for comparisons between ARR versus NRR mice for Th populations, BALF cellularity, cytokines, and IL-4Ra expression. Mann-Whitney test was used to compare between the groups for the pulmonary function tests. Student’s t-test was used to compare the expression level of IL-4Ra on Th cells from CBMCs and adult PBMCs, with or without RSV stimulation in vitro. For this purpose, PBMCs from seven healthy adult volunteers and CBMCs from seven term neonatal subjects were isolated by Ficoll extraction. PBMCs or CBMCs were infected with RSV or media at a MOI of 2 in RPMI 1640 (HyClone), containing 10% heat-inactivated FBS (HyClone) and 1 mM sodium pyruvate. Cells were harvested after 48 h, resuspended in FACS lysing solution (BD Biosciences), and stored at −80°C until staining. For staining, cells were thawed; permeabilized in FACS permeabilization buffer (eBioscience); and stained by antibodies to CD3 (clone #SP34-2 from BD Biosciences or clone #OKT3 from eBioscience), CD4 (clone #RPA-T4; eBioscience), T-bet (clone #RB10; eBioscience), and IL-4Ra (clone #HIILR-M57; BD Biosciences). The staining profile was acquired on an LSR II flow cytometer (BD Biosciences). Given the heterogeneity in human immune responses, we calculated the iMFI of IL-4Ra-positive cells by multiplying the relative frequency (percent positive) of cells expressing IL-4Ra with the MFI of that population. iMFI had been shown to correlate better with vaccine-induced protection from Leishmania major challenge than either the percentage or the MFI of the cytokine-positive population [31].

Online Supplemental material

Supplemental Fig. 1 demonstrated the feasibility of using CD5 antibody in the in vitro Th activation assay. Supplemental Fig. 2 showed that pulmonary viral load and Th responses during primary infection were similar, between 5- and 12-week-old mice.
There was no significant difference in the total number of leukocytes recovered from the BALF between NRR and ARR mice (Fig. 2C). Significantly more macrophages were observed in the BALF of NRR compared with ARR mice (79,987 ± 12,516 vs. 38,456 ± 5367). In concordance with the exaggerated Th2 response, NRR mice also possessed a substantially greater number of eosinophils in the BALF compared with ARR mice (33,217 ± 14,988 vs. 0; Fig. 2C). Finally, Th2 cytokines, such as IL-4 and IL-13, were readily detectable in the BALF of NRR mice and were secreted at significantly elevated levels compared with ARR mice (9.40 ± 2.09 pg/ml vs. nondetectable and 68.42 ± 10.56 vs. 23.46 ± 1.85 pg/ml, respectively; Fig. 2D). Consistent with increased Th2 cytokine secretion, NRR mice secreted significantly less IL-12(p40) (15.81 ± 1.6 vs. 47.51 ± 4.7 pg/ml) compared with ARR mice. No significant difference was observed in IFN-γ levels between NRR and ARR mice.

It is possible that the exaggerated Th2 response observed in mice infected initially as neonates was a result of memory responses resulting from a skewed Th2 primary response. To test this, we measured the primary CD4⁺ T cell response in the lungs of neonatal or adult mice at 6 dpi. As shown in Fig. 2E, there were no difference in IL-4⁺ or IL-4⁺IFN-γ⁺ expressing CD4⁺ T cells between NR and AR. Interestingly, RSV infection of neonates resulted in significantly lower levels of pulmonary Th1 (IFN-γ-expressing CD4⁺) cells compared with adults (2.99 ± 0.35 vs. 0.60 ± 0.08%), suggesting that the exaggerated Th2 response upon reinfecition in neonatally infected mice was not a result of a biased Th2 primary response.

IL-4Rα was up-regulated on Th cells upon reinfecction of mice infected initially as neonates

As IL-4 signaling is a determinant for Th cell development, we measured the expression of the common component of the IL-4R and IL-13R—IL-4Rα—on pulmonary Th cells of RSV-infected mice. Five-day-old pups or adults were infected with RSV and reinfected 4 weeks postprimary infection. At 2 days postreinfection, lung cells were isolated and the expression of IL-4Rα analyzed by flow cytometry. IL-4Rα expression was higher on Th cells from NRR versus ARR mice (MFI: 735.60 ± 29.58 vs. 810.60 ± 20.33, respectively; Fig. 3A and B). Furthermore, Th1 and Th2 cells from NRR mice expressed more IL-4Rα than those cells from the lungs of ARR mice (MFI: 891.00 ± 15.55 vs. 1208.60 ± 44.07 vs. 767.80 ± 20.33, respectively; Fig. 3C). Interestingly, the expression of IL-4Rα on Th2 cells from NRR mice was significantly higher than its expression on Th1 cells from the same mice.

It is possible that IL-4Rα expression actually increased on neonatal Th cells during primary infection. To test this, we measured IL-4Rα expression on neonatal and adult CD4⁺ T cells at 2 days after primary infection. Surprisingly, IL-4Rα expression was decreased on naïve neonatal versus adult CD4⁺ T cells. However, with RSV infection, we observed a decrease in IL-4Rα expression on adult but not neonatal CD4⁺ T cells, and as a result, IL-4Rα expression was similar on CD4⁺ T cells between RSV-infected neonates and adults. More importantly and relevantly, neonatal Th1 and Th2 cells expressed higher
levels of IL-4Ra compared with their adult counterparts at primary infection. Without RSV infection, neonatal Th2 cells showed significantly greater expression of IL-4Ra compared with adult Th2 cells.

**IL-4Ra expression was up-regulated on Th cells purified from cord blood and stimulated with RSV in vitro**

To begin to explore the translational potential of our findings, we compared the expression level of IL-4Ra on CD4+ T cells purified from cord blood or adult peripheral blood. As shown in Fig. 3D, Th cells from CBMCs or PBMCs expressed similar levels of IL-4Ra without stimulation, as indicated by the comparable iMFI values (3462±1132 vs. 4766±1186). However, when stimulated with RSV in vitro, IL-4Ra levels on Th cells from CBMCs increased substantially and significantly compared with IL-4Ra levels on Th cells from PBMCs (iMFI: 13,931±3095 vs. 3462±1132). These data demonstrate that IL-4Ra expression on infant CD4+ T cells was up-regulated, responding to RSV infection in humans.

**Elevated IL-4Ra on CD4+ T cells upon reinfection was associated with hyperproliferation in vitro**

To determine the mechanism by which Th2 bias occurs to RSV reinfection in neonatally infected mice, we examined the proliferative ability of the Th cells from mice infected as neonates (NRR) or adults (ARR) in vitro. Five-day-old pups or adults were infected with RSV and reinfected at 4 weeks postprimary infection. At 6 days after reinfection, lung CD4+ T cells were isolated and cocultured with RSV-infected lung CD11c+ cells as APCs. Three days after coculture, CD4+ T cell subsets and their proliferation were measured. As shown in Fig. 4A and B, CD4+ T cells from NRR mice, when stimulated in vitro with RSV, possessed the ability to differentiate into Th1 (20.68±0.31%) and Th2 (7.57±0.48%) cells. In contrast, CD4+ T cells from ARR mice developed predominantly into Th1 cells (50.19±0.85%), with only 0.09% developing into Th2 cells. With the use of CFSE dilution, we analyzed the proliferative capacity of Th cells from NRR and ARR mice. We observed that 86.9±0.44% of the Th2 cells from NRR mice had undergone cell division, whereas only 39.04±7.59% of the Th2 cells from ARR mice divided into further generations (Fig. 4C and D). Th1 cells also proliferated with 65.9±0.76% from NRR mice and 17.17±3.33% from ARR mice undergoing cell division. In agreement with the IL-4Ra expression data, these data indicated that CD4+ T cells from NRR mice were more proliferative, that there was increased proliferation of Th2 cells from NRR mice, and that Th2 cells predominated in NRR mice.

**Th cell-specific deletion of Il4ra abolished airway hyper-reactivity and lung histopathology upon reinfection in mice infected initially as neonates**

As greater levels of IL-4Ra expression on pulmonary CD4+ T cells were associated with neonatal RSV infection-induced physiolog-ical consequences, we hypothesized that IL-4Ra signaling was responsible for the observed phenotype. To investigate the role of IL-4Ra on CD4+ T cells, we infected mice specifically lacking IL-4Ra on CD4+ T cells (LckcreIL4Rα−/lox−) and their littermate controls (IL-4Ra+/lox−) at 5 days of age and subsequently reinfeeted those mice with RSV, 4 weeks later (LckcreIL4Rα−/lox−-RR and IL4Rα−/lox−-RR, respectively). Control mice, including IL-4Ra-deficient and -competent mice, were mock-infected with viral production media (LckcreIL4Rα−/lox−-Sham and IL4Rα−/lox−-Sham, respectively). Pulmonary function and lung histopathology were determined at 6 days after reinfection.
Figure 3. IL-4Ra was up-regulated upon reinfection on Th cells from mice infected originally as neonates. Neonates (5 days old; NRR) or adults (6–8 weeks old; ARR) were infected and reinfected with RSV at 4 weeks postprimary infection. The expression of IL-4Ra on Th cells and Th subsets was determined by flow cytometry at 2 days postsecondary infection. Lung cells were isolated from NRR and ARR mice, stimulated in vitro, and stained with dead cell, excluding dye and antibodies to CD3, CD4, IFN-γ, IL-4, and IL-4Ra. (A) Relative expression level of IL-4Ra on CD4⁺ Th cells indicated by MFI of IL-4Ra; n = 5. (B) Representative histogram showing the staining profile of IL-4Ra on Th cells. IL-4Ra fluorescence minus one (FMO) serves as a negative control for IL-4Ra staining. (C) Relative expression level of IL-4Ra on CD4⁺ Th1 or Th2 cells, indicated by MFI of IL-4Ra; n = 5. (D) Relative expression level of IL-4Ra on circulating Th cells from cord blood of healthy term infants or from peripheral blood of healthy adults at baseline or upon in vitro stimulation with RSV. MFI was calculated by multiplying the relative frequency (percent positive) of cells expressing IL-4Ra with MFI of that population; n = 7. (E) IL-4Ra expression on neonatal or adult CD4⁺ T cells during primary RSV infection in mice. Neonates (5 days) or adults (6–8 weeks) were infected with RSV (NR and AR, respectively) or media (NS and AS, respectively). IL-4Ra expression on CD4⁺ T cells was measured at 2 dpi; n = 5. NS, pups infected with media; AS, adults infected with media. (F) IL-4Ra expression on neonatal or adult Th1 cells during primary RSV infection. Mice were treated and IL-4Ra measured as in E; n = 5. (G) IL-4Ra expression on neonatal or adult Th1 cells during primary RSV infection. Mice were treated and IL-4Ra measured as in E; n = 5; *p < 0.05.

As shown in Fig. 5A, no statistical difference in airways resistance was observed between the two sham-infected mouse genotypes (i.e., LckcreIL4Rα−/−Lox-Sham and IL4Rα−/−Lox-Sham), indicating that specific deletion of Il4ra in CD4⁺ T cells did not induce a baseline change in pulmonary function. As expected, RSV reinfection of the IL-4Ra-competent mice (IL4Rα−/−Lox-RR) induced airway hyper-reactivity, as evidenced by the increase in airway resistance compared with the sham-infected control mice (IL4Rα−/−Lox-Sham) in response to MeCh challenge. While in the absence of CD4⁺ T cell-specific IL-4Ra, RSV reinfection failed to induce airway hyper-reactivity (LckcreIL4Rα−/−Lox-RR vs. IL4Rα−/−Lox-RR; 2.17 ± 0.44 vs. 3.01 ± 0.34 cm H₂O/s/ml). Furthermore, there was no difference between CD4⁺ T cell-specific, IL-4Ra-deficient mice that were RSV- or SHAM-reinfected (LckcreIL4Rα−/−Lox-RR vs. LckcreIL4Rα−/−Lox-Sham; 2.17 ± 0.44 vs. 2.43 ± 0.18 cm H₂O/s/ml), indicating a complete abrogation of airway hyper-reactivity in the CD4⁺ T cell-specific, IL-4Ra-deficient mice.

Supporting the pulmonary function data, we observed a substantial decrease in perivascular and peribronchiolar inflammation in CD4⁺ T cell-specific, IL-4Ra-deficient mice (LckcreIL4Rα−/−Lox-RR) compared with the IL-4Ra-competent mice (IL4Rα−/−Lox-RR; Fig. 5B). In particular, substantially less eosinophils were observed in LckcreIL4Rα−/−Lox-RR mice compared with IL4Rα−/−Lox-RR mice (10 ± 6 vs. 559 ± 128 cells/mm² surface area). Additionally, little-to-no mucus was observed in the airways of the CD4⁺ T cell-specific, IL-4Ra-deficient mice, whereas we observed a significant amount of mucus production and mucus cell hyperplasia in the IL-4Ra-competent mice (Fig. 5C; 0.006 ± 0.005 vs. 0.25 ± 0.04 μm² area of mucus+cells/μm² area of epithelium). No pulmonary inflammation or airway mucus was observed in IL-4Ra-deficient or -competent, sham-infected mice (data not shown). These data demonstrated a requirement for CD4⁺ T cell-specific IL-4Ra in pulmonary dysfunction and histopathology associated with RSV reinfection in neonatally infected mice.

Th-specific deletion of Il4ra attenuated Th2 responses to reinfection in mice infected initially as neonates

To understand how the absence of IL-4Ra on CD4⁺ T cells affected the pulmonary immune response to RSV reinfection, we analyzed the pulmonary immune response in the above mice.
Pulmonary immunophenotyping, including CD4+ T cell profile and BALF cellularity and cytokine levels, were measured 6 days after reinfection. As shown in Fig. 6A and B, sham-infected, IL-4Rα-deficient and -competent mice failed to induce effector CD4+ T cells (i.e., few CD4+ T cells expressed effector cytokines: IFN-γ or IL-4). Reinfection with RSV induced substantial and similar amounts of the IFN-γ-expressing Th1 cells in IL-4Rα-deficient and -competent mice (21.80±1.41 vs. 20.48±3.52%) but failed to induce significant amounts of IL-4-expressing Th2 cells or multifunctional Th cells coexpressing IFN-γ and IL-4 (1.94±0.46 vs. 4.48±0.81% and 1.86±0.33 vs. 5.3±1.07%, respectively).

Consistent with the reduction in pulmonary Th2 cells, we observed a significant reduction in eosinophils in the BALF of RSV-reinfected, IL-4Rα-deficient versus -competent mice (1527±541 vs. 19,290±12,089; Fig. 6C). No differences in total BALF cell numbers, alveolar macrophages, lymphocytes, or neutrophils were observed between the two groups of mice. In addition, we observed a significant decrease in Th2 cytokine IL-13 and an increase in Th1 cytokine IL-12 (p<0.01) in the BALF of IL-4Rα-deficient compared with -competent mice (59.43±11.35 vs. 32.34±5.36 pg/ml and 21.58±2.47 vs. 12.18±2.28 pg/ml, respectively; Fig. 6D). There was no difference in IFN-γ between IL-4Rα-deficient and -competent mice upon RSV reinfection. These data demonstrated that CD4+ T cell-specific ablation of Il4ra attenuated the pathogenic, Th2-biased immune responses associated with RSV reinfection in the neonatally infected mice.

DISCUSSION

It has been proposed that a biased Th2 immune response is responsible for RSV-induced pulmonary pathophysiology in infants. In this study, we first confirmed that an exaggerated Th2 response was induced by RSV reinfection in mice originally infected as neonates but not as adults, and this Th2 immune response correlated with airway hyper-reactivity, mucus hyperproduction, and eosinophilia. We further observed increased expression of IL-4Rα on CD4+ T cells from mice infected initially with RSV as neonates and reinfeared with RSV as adults. Increased expression of IL-4Rα on Th cells was associated with an increased ability of these cells to proliferate and develop into Th2 cells. Specific deletion of Il4ra on Th cells abolished airway hyper-reactivity and mucus hyperproduction and attenuated airway eosinophilia and pulmonary Th2 responses. We demonstrated further that IL-4Rα expression was increased on human CD4+ T cells from cord blood but not peripheral blood from adults upon RSV stimulation in vitro, suggesting that the mechanisms that we have identified in mice are translatable to humans.

Whereas human epidemiological studies suggest a gain-of-function association of IL4RA polymorphisms with severe RSV disease in infants [22], few studies have investigated the function of IL-4Ra in a neonatal model. We clearly showed that expression of IL-4Rα on CD4+ T cells, including Th1 and Th2 cells, was increased upon reinfection when the mice were infected initially as neonates. This observation is in line with the study of Li et al. [32], which showed increased expression of IL-13Rα1, the other component of the type II IL-4R complex, on neonatal Th1 cells upon antigen recall. Whereas Li and coworkers’ data demonstrated increased expression of IL-13Rα1, correlated with enhanced Th1 apoptosis, and suggested this as a mechanism skewing the neonatal immune response toward a Th2-dominant response, we were unable to observe any changes in Th1 apoptosis between RSV-infected and noninfected mice after secondary RSV infection (data not shown). What we did observe is that Th1 and Th2 cells from mice infected initially as neonates...
possess better proliferative ability than cells from mice infected as adults. Moreover, Th2 cells from mice infected initially as neonates showed the greatest ability to proliferate. This is consistent with the observation that RSV reinfection resulted in increased numbers of pulmonary Th2 cells without an increase in the numbers of Th1 cells in mice infected initially as neonates compared with mice infected initially as adults. Cumulatively, these data suggest exaggerated Th2 cell proliferation rather than Th1 apoptosis as a mechanism inducing biased Th2 responses during reinfection in neonatally infected mice.

It was unexpected that in mice, primary RSV infection did not increase the expression of IL-4Rα on neonatal CD4+ T cells but rather decreased IL-4Rα expression on adult CD4+ T cells. This is in contrast to the ex vivo human CD4+ T cell data, which demonstrated an increase in IL-4Rα expression on CD4+ T cells from CBMCs versus adult PBMCs exposed to RSV. Although not significant but similar to the mouse in vivo data, IL-4Rα expression appeared to decrease on PBMC CD4+ T cells. This discrepancy between mouse and human data could be a result of a variety of factors. First, humans are reinected by RSV throughout life [4], and therefore, a portion of each genotype were treated with serum-free medium (LckcreIL4Rα/Lox-RR) and -competent mice (IL4Rα+/Lox-RR) were infected with RSV at 5 days old and reinfected at 4 weeks postprimary infection. Control mice of each genotype were treated with serum-free medium (LckcreIL4Rα+/Lox-RR, and IL4Rα+/Lox-RR, respectively). Pulmonary function and lung histopathology were determined at 6 days postreinfection. (A) Airway resistance was measured at baseline (0 mg/ml MeCh) and increasing doses of intratracheally delivered MeCh to quantify airway hyper-reactivity. At baseline, airway resistance was not different between the groups; therefore, individual responses were normalized to their prospective baselines and normalized resistance plotted; n = 5–7; *P < 0.05. (B) H&E staining, demonstrating pulmonary inflammation. The arrow points to cellular infiltrates. Original scale bar = 50 μm. (C) PAS staining showing mucus. The arrow points to mucus staining. The right panels are enlargements of the rectangles in the left panels. Original scale bar = 200 μm.

Figure 5. CD4+ T cell-specific deletion of IL4ra abolished neonatal RSV infection-induced immunopathophysiology upon reinfection. Th-specific, IL-4Rα-deficient (LckcreIL4Rα/Lox-RR) and -competent mice (IL4Rα+/Lox-RR) were infected with RSV at 5 days old and reinfected at 4 weeks postprimary infection. Control mice of each genotype were treated with serum-free medium (LckcreIL4Rα+/Lox-RR, and IL4Rα+/Lox-RR, respectively). Pulmonary function and lung histopathology were determined at 6 days postreinfection. (A) Airway resistance was measured at baseline (0 mg/ml MeCh) and increasing doses of intratracheally delivered MeCh to quantify airway hyper-reactivity. At baseline, airway resistance was not different between the groups; therefore, individual responses were normalized to their prospective baselines and normalized resistance plotted; n = 5–7; *P < 0.05. (B) H&E staining, demonstrating pulmonary inflammation. The arrow points to cellular infiltrates. Original scale bar = 50 μm. (C) PAS staining showing mucus. The arrow points to mucus staining. The right panels are enlargements of the rectangles in the left panels. Original scale bar = 200 μm.

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observed following RSV reinfection indicate an IL-4Rα-independent Th2 commitment/maintenance mechanism, as has been suggested previously in the context of different pathogens or allergens [34–38]. In addition, the abrogation of the Th2-associated pathophysiology, despite the presence of residual Th2 cells in the lungs, suggests that either Th2 effector functions require a minimal number of Th2 cells (or a minimal release of Th2 effector cytokines) to induce functional responses or current measurements of Th2-induced pathophysiology are not sensitive enough to detect these responses. It is also possible that undefined mediators released by IL-4Rα-expressing CD4+ T cells are responsible for RSV-induced pathophysiology and that upon ablation of these cells, those mediators are lost, resulting in a reduction in pathophysiology.

As mentioned above, reinfection of neonatally infected mice induced not only Th2 cells expressing IL-4 but also a multifunctional Th subset expressing the prototypical Th2 and Th1 cytokines, IL-4, and IFN-γ. Intriguingly, this multifunctional subset was only observed in neonatally infected mice, where IL-4Rα was up-regulated upon reinfection and not in mice infected as adults. Furthermore, the number of multifunctional Th cells was reduced drastically in Th-specific, IL-4Rα-deficient mice. These observations indicated that IL-4Rα is partially required for the development of these cells. The exact role of these multifunctional cells is uncertain, and a few studies suggest that they are simply an immature cell type (i.e., Th0 cells) in the process of committing to a Th1 or Th2 cell [25, 39, 40]. From our data, it is unclear if and how these cells might function in the pathogenesis of neonatal RSV infection.

In summary, we confirmed that skewed Th2 immune responses with airway hyper-reactivity, mucus hyperproduction, and eosinophilia are induced upon RSV reinfection, specifically in neonatally infected mice. This skewed Th2 response correlated with increased IL-4Rα expression on CD4+ T cells. Furthermore, deletion of Il4ra from Th cells attenuated Th2 responses and abolished Th2-associated immunopathophysiology. The increase in IL-4Rα expression was also observed in CD4+ T cells from CBMCs, suggesting that IL-4Rα may also have a role in RSV pathogenesis in human infants. These findings elucidate a pathogenic role for Th-specific IL-4Rα during RSV reinfection in mice infected as neonates and provide important insight for infant RSV vaccine development.

AUTHORSHIP

S.A.C. was the principal investigator in this study, designed the research, analyzed the data, and wrote the manuscript. D.Y. designed and performed most of the experiments, analyzed the data, and wrote the manuscript draft. B.S. and G.L. assisted in some of these experiments. N.M. and S.E.T. collected and analyzed human Th cell data and assisted in preparing the manuscript. J.S.S. studied the Th profile of the Th-specific, IL-4Rα-deficient mice. F.B. and D.H. provided the Th-specific, IL-4Rα-deficient mice.
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DISCLOSURES

A U.S. patent application has been filed by LSUHSC as a result of this research, with S.A.C. as the inventor. The other authors have no financial conflicts of interest. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the U.S. National Institutes of Health.

REFERENCES


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viral infection - infants - T helper cells