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Immunomodulation with IL-4R α Antisense Oligonucleotide Prevents Respiratory Syncytial Virus-Mediated Pulmonary Disease

Michael J. Ripple,¹ Dahui You,^{1,2} Srinivasa Honnagowda, Joseph D. Giaimo, Andrew B. Sewell, David M. Becnel, and Stephanie A. Cormier

Respiratory syncytial virus (RSV) causes significant morbidity and mortality in infants worldwide. Severe RSV infections in infants cause bronchiolitis, wheeze, and/or cough and significantly increase the risk for developing asthma. RSV pathogenesis is thought to be due to a Th2-type immune response initiated in response to RSV infection, specifically in the infant. Using a neonatal mouse system as an appropriate model for human infants, we sought to determine whether local inhibition of IL-4R α expression during primary RSV infection in the neonate would prevent Th2-skewed responses to secondary RSV infection and improve long-term pulmonary function. To reduce IL-4R α expression, antisense oligonucleotides (ASOs) specific for IL-4R α were administered intranasally to neonatal mice at the time of primary infection. Mice were initially infected with RSV at 1 wk of age and were reinfected at 6 wk of age. Administration of IL-4R α ASOs during primary RSV infection in neonatal mice abolished the pulmonary dysfunction normally observed following reinfection in the adult. This ablation of pulmonary dysfunction correlated with a persistent rebalancing of the Th cell compartment with decreased Th2 responses (i.e., reduced goblet cell hyperplasia, Th2 cells, and cytokine secretion) and increased Th1 responses (i.e., elevated Th1 cell numbers and type I Abs and cytokines). Our data support our hypothesis that a reduction in the Th2 immune response during primary infection in neonates prevents Th2-mediated pulmonary pathology initially and upon reinfection and further suggest that vaccine strategies incorporating IL-4R α ASOs may be of significant benefit to infants. *The Journal of Immunology*, 2010, 185: 4804–4811.

Respiratory syncytial virus (RSV) is an important cause of acute respiratory tract infections in infants (and the elderly), causing significant morbidity and mortality. The World Health Organization estimates the global burden of RSV

disease at 64 million cases and 160,000 deaths annually. In the United States, RSV is responsible for 85,000–144,000 infant hospitalizations annually (1). Health care costs are estimated at \$365–585 million per year (2), and the economic impact, in relation to days lost from work, is greater than that of influenza (3). Primary RSV infection causes severe bronchiolitis requiring hospitalization in 30–40% of infants, particularly in infants 2–5 mo of age (4). Interestingly, infants who are younger than 3 mo of age and who develop RSV bronchiolitis show a persistent increase in IL-4 production following infection (5) and are at an increased risk for developing recurrent wheeze/asthma (4, 6–15). Despite an urgent need, no safe and effective vaccine for RSV exists.

In preclinical mouse models of infantile RSV infection, age at initial infection determines whether RSV predisposes to long-term lung dysfunction and dictates the type of immune response (Th1 versus Th2) observed following secondary infection with RSV (16–19). When primary infection with RSV occurs in the first week of life, mice develop airway hyperresponsiveness (AHR) that lasts into adulthood (19). Furthermore, a subsequent RSV infection elicits enhanced immunopathology, with even greater increases in AHR (17). In contrast, when primary infection with RSV occurs in the third week of life (weanling), AHR is not induced in response to secondary infection, although significant airway inflammation exists (17).

As the age at primary infection increases, the Th2 response decreases, and the Th1 response increases. This switch from a Th2 bias to more of a Th1 bias in response to RSV infection occurs at ~1 wk of age in mice (16, 20), and epidemiological data suggest that it occurs at ~4 mo of age in humans (4). Prior to this time point, there is a window of immunological immaturity that results in an aberrant response to the virus and primes the host to respond with an adverse Th2 response upon reinfection later in life (16). Indeed, the failure of the RSV vaccine of the 1960s is believed to be due,

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Abbreviations used in this paper: AHR, airway hyperresponsiveness; AR, mice receiving antisense oligonucleotide and infected with respiratory syncytial virus; ARR, mice receiving antisense oligonucleotide and reinfected with respiratory syncytial virus; ASO, antisense oligonucleotide; BALF, bronchoalveolar lavage fluid; CK, BALF cytokines; DC, dendritic cell; dpi, days postinfection; Eos, eosinophils; Epi, epithelial cells; i.n., intranasally; Lym, lymphocytes; Mac, macrophages; mDC, myeloid DC; MeCh, methacholine; MFI, mean fluorescence intensity; MM, mismatch oligonucleotide; MR, mice receiving mismatch oligonucleotide and infected with respiratory syncytial virus; MRR, mice receiving mismatch oligonucleotide and reinfected with respiratory syncytial virus; Neu, neutrophils; NP, nucleoprotein; PAS, periodic acid-Schiff; PFT, pulmonary function testing; RSV, respiratory syncytial virus; SR, mice receiving saline and infected with RSV; SRR, mice receiving saline and reinfected with RSV; TCID₅₀, median tissue culture infective dose.

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in part, to the exacerbated Th2 response to community-acquired RSV following inoculation with formalin-inactivated virus (21). Understanding this age-related difference in pathophysiological response to RSV infection is critical to appreciate the problems associated with the development of an effective pediatric vaccine for RSV.

IL-4 and IL-13 are classical signaling mediators of the Th2 response. Both of these cytokines bind their respective receptors containing the IL-4R α subunit. IL-4 has two receptors: type I and II. The type I receptor is composed of IL-4R α and the common γ -chain; it binds IL-4 exclusively and initiates Th2 cell differentiation. The type II IL-4R is composed of the IL-4R α and IL-13R α 1 subunits. It binds IL-4 or IL-13 and is thought to cause the adverse effects observed following neonatal RSV infection, including AHR, lung remodeling, and mucus hyperproduction (22). Signaling through both of these receptors occurs via a JAK/STAT pathway (23) and is important in the neonatal response to RSV. Inhibition or depletion of IL-4 and/or IL-13 helps to reduce the adverse effects seen in neonatal infections in mice (17, 24), most notably decreased AHR and mucus hyperproduction. The recent association of IL-4/IL-13 haplotypes and IL-4R α gain-of-function polymorphisms with RSV hospitalizations and disease severity (25–28) suggests that IL-4R α may play a key role in RSV-mediated pulmonary pathologies in human infants.

Despite nearly half a century of research, no vaccine has been clinically approved for RSV, and few viable treatments exist. In this study, we sought to determine whether reduction of IL-4R α in the pulmonary compartment is sufficient to prevent the adverse pulmonary events observed following neonatal RSV infection. To accomplish this, we used an antisense oligonucleotide (ASO) specific for IL-4R α to reduce receptor expression. Our results indicate that suppression of pulmonary IL-4R α protects from subsequent RSV-mediated pulmonary inflammation and lung dysfunction. Our data further suggest that a pediatric-vaccine strategy employing inhaled IL-4R α ASO may be effective to prevent complications of RSV-induced wheeze.

Materials and Methods

Mice

BALB/c mice were purchased as breeders from Harlan Laboratories (Indianapolis, IN). All mice were housed in the vivarium at Louisiana State University Health Sciences Center and were maintained in ventilated microisolator cages housed in a specific pathogen-free animal facility. Sentinel mice within this animal colony were negative for Abs to specific viral and other known mouse pathogens. Breeders were time mated, and 2-day-old pups were used for experiments. All animal protocols were prepared in accordance with the Guide for the Care and Use of Laboratory Animals (29) and approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center.

Oligonucleotides: preparation and dosage

IL-4R α ASOs and mismatch oligonucleotides (MMs) were kindly provided by Isis Pharmaceuticals (Carlsbad, CA). The oligonucleotides were synthesized and purified as previously described (30). Both oligonucleotides were designed to avoid any murine immune-stimulatory motifs and were 20 bases in length, with 2'-O-methoxyethylribose modification on bases 1–5 and 16–20 (underlined). Their estimated half-life following single-dose inhalation studies is 4 d in the mouse lung (30). The sequences of the ASO and MM are shown below; the mismatched bases are shown in lower case: ASO: 5'-CCGCTGTTCTCAGGTGACAT-3' MM: 5'-CCaCtCaTcaTcGcTGaCtT-3'.

The oligonucleotides were suspended in sterile saline and administered intranasally (i.n.) to mouse pups at a dose of 100 or 500 μ g/kg body weight. Data for both doses were similar for all experiments. Therefore, we chose to present all inflammatory data at the highest dose (500 μ g/kg body weight), to demonstrate that administration of the oligonucleotides does not elicit inflammatory responses greater than that induced by RSV; the

pulmonary function data at the lowest dose (100 μ g/kg body weight), to demonstrate lower dose efficacy; and the cytokine data at both doses (100 and 500 μ g/kg body weight), to demonstrate that at higher doses the MM oligonucleotide has effects that are not due to downregulation of IL-4R α . Control pups received sterile saline.

Experimental design

The experimental design is outlined in Fig. 1. IL-4R α ASO, MM, or saline was administered i.n. to mice on protocol days –5, –3, –1, and 1. On protocol day 0 (7 d of age), mice were infected with RSV or were sham infected. Mice receiving ASO, MM, or saline and infected with RSV are referred to as AR, MR, and SR, respectively. Those mice receiving saline and sham-infected are referred to as SHAM. For all secondary infections, mice were reinfected with RSV (ARR, MRR, SRR) or vehicle (SHAM) on protocol day 35. Various end points were measured, including IL-4R α levels, T cell populations in the lung, pulmonary function, bronchoalveolar lavage cellularity and cytokine profile, pulmonary viral copy number, lung histology, and RSV-specific Ab levels in serum, at the indicated time points.

RSV infection and pulmonary viral load determination

Human RSV strain A-2 was purchased as a sucrose-gradient purified virus from Advanced Biotechnologies (Columbia, MD). The virus preparation was determined to be free of bacteria, yeast, and fungi. Seven-day-old mice (protocol day 0; Fig. 1) were anesthetized with 5% isoflurane and infected i.n. with 2×10^5 median tissue culture infective dose (TCID₅₀) per gram body weight of RSV in 10 μ l serum-free media (Invitrogen) or media alone (18, 19). Adult mice (protocol day 39) were similarly infected with RSV (2×10^5 TCID₅₀/gram body weight) in 50 μ l the same media.

To determine lung viral load, we used the TCID₅₀ method of Spearman-Kärber, as previously published (19), using whole-lung homogenates isolated from mice at 6 d postinfection (dpi). Vero cells were seeded on a 96-well plate and then inoculated with 10-fold serial dilutions of whole-lung homogenate. Cells were incubated at 37°C and 5% CO₂ for 4 d; wells showing syncytia were counted, and TCID₅₀ values were calculated.

Viral load was also quantified using real-time PCR. Lungs were isolated at 6 dpi, quick-frozen in liquid nitrogen, and stored at –80°C until processing. RNA was extracted from the lungs with TRIzol reagent (Invitrogen, Carlsbad, CA), purified with an RNeasy Mini Kit (Qiagen, Valencia, CA), and treated with DNase (Ambion, Austin, TX). The genomic RNA was then reverse transcribed into cDNA with an RSV-nucleoprotein (NP)-specific primer (5'-GCGATGTCTAGGTTAGGAAGAA-3') or oligonucleotide (dT) for GAPDH using the Superscript III-RT kit (Invitrogen) and the following conditions: 65°C for 5 min, 4°C for 1 min, 42°C for 50 min, and 85°C for 5 min. The samples were incubated at 4°C for 1 min, RNase H was added, and the samples were incubated at 37°C for 20 min. Real-time PCR was performed using LUX (Invitrogen) primers specific for RSV-NP: 5'-TTGGGTAGTAAGCCTTTGTAA[FAM]G-3' (forward primer) and 5'-CTGCTTACAGCCGTGATTAGGA-3' (reverse primer), which amplified the region between nucleotides 544–623 to give an amplicon of 79 bp. After a 2-min denaturation at 95°C, PCR cycling conditions were 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. This was followed by a melt-curve analysis: 1 min denaturation at 95°C, 1 min anneal at 55°C, and a 55–90°C melt-curve (+0.5°C/cycle; 30 s) in a Bio-Rad iQ5 Machine (Bio-Rad, Hercules, CA). Selected PCR products were cloned into a TA cloning vector (pGEM-T; Promega, Madison, WI), and the sequence was determined to confirm the identity of the virus detected by the PCR reaction. GAPDH internal control (Invitrogen Mouse/Rat GAPDH-Certified JOE-labeled LUX Primer Set) was used to confirm equal quantities of input cDNA. RSV-NP copy number was determined from standard curves of a plasmid vector containing a fragment of the RSV-NP gene.

Isotype determination and quantification of RSV-specific Ab

Serum was isolated from the left ventricle of euthanized mice following primary infection (7 and 12 dpi) using serum separator tubes (BD Biosciences, Bedford, MA) and was stored at –80°C until use. Microtiter plates (Nunc-Immuno Maxisorp, Nalge Nunc International, Rochester, NY) were coated with 50 μ l RSV (5×10^4 PFU/ml) overnight at 4°C in PBS. The plates were blocked with 1 \times Blocker BSA (Pierce, Rockford, IL) for 5 min, and 25 μ l serum was added to each well and allowed to incubate for 2 h at room temperature. Bound RSV-specific Ab was then isotyped and quantified using peroxidase-conjugated goat Abs specific for mouse IgA, IgG1, IgG2a, and IgE (Southern Biotech, Birmingham, AL) and 3,3',5,5'-tetramethylbenzidine (Pierce, Rockford, IL) as substrate. Color development was stopped with 2 M H₂SO₄, and the absorbance was read at 450 nm. RSV-specific Ab levels were determined by subtracting the

absorbance value of the blank wells (media only) from the absorbance of each serum sample.

Determination of IL-4R α surface expression and assessment of pulmonary T and dendritic cell populations

A single-cell suspension of lung cells was prepared using a standardized protocol (31). Lungs were perfused, excised, cut into small pieces, and incubated at 37°C for 1 h in RPMI 1640 media (HyClone, Logan, UT) supplemented by 5% heat-inactivated FBS (HyClone), 100 U/ml penicillin, 100 mg/ml streptomycin (HyClone), 1 mg/ml collagenase I (Invitrogen), and 150 μ g/ml DNase I (Sigma-Aldrich, St. Louis, MO). After incubation, single cells were obtained by mashing the lung pieces through a 40- μ m cell strainer (BD Biosciences, San Jose, CA). RBCs were lysed using RBC lysis buffer (eBioscience), and cells were stained with combinations of the following Abs purchased from BD Biosciences and eBioscience (San Diego, CA): Pacific Blue-CD3e (17A2), PerCP-CD4 (RM4-5), FITC-CD8a (53-6.7), Biotin-CD124 (mIL4R-M1), allophycocyanin-CD11b (MI/70), PE Cy7-CD11c (N418), and FITC-E-cadherin (36/E-cadherin).

For determining T cell subsets, lung cells (single-cell suspensions prepared as above and after RBC lysis) were stimulated for 5 h with 5 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of a protein-transport inhibitor (1 μ l/10⁶ cells; GolgiPlug, BD Biosciences). After stimulation, cells were harvested, stained for surface markers (i.e., CD3, CD4, and CD8), fixed, permeabilized (Fixation and Permeabilization Buffer; eBioscience), and stained with PE-IFN- γ (XMG1.2) to identify Th1/Tc1 cells and PE-Cy7-IL-4 (BVD4-24G2) to identify Th2/Tc2 cells. Cell staining was determined with a FACSCanto II (BD Biosciences) flow cytometer after gating on specific live cell populations, as determined by forward and side scatter properties, and on CD3⁺ cells for T cell population analyses. A total of 100,000 events were analyzed per mouse lung.

For determining myeloid dendritic cell (mDC) subsets, lung cells (single-cell suspensions prepared as above and after RBC lysis) were stained for surface expression of CD11b and CD11c. After selecting the live non-lymphocyte, nonmonocyte cells using forward and side scatter properties, expression of CD11c^{hi} and CD11b⁺ was used to identify mDCs. A total of 300,000 events were analyzed per mouse lung. Isotype control Abs were used in all flow-cytometry experiments. Flow data were analyzed and plotted using FlowJo software (version 7.2.2 for Windows; Tree Star, Ashland, OR).

Assessment of pulmonary function

Six days after secondary infection, lung resistance and compliance to increasing doses of methacholine (MeCh; 0, 12.5, 25, and 50 mg/ml in isotonic saline; Sigma-Aldrich) were assessed using the forced-oscillation technique. Animals were anesthetized with ketamine/xylazine (180/10 mg/kg) and mechanically ventilated at a tidal volume of 10 ml/kg and a frequency of 2.5 Hz using a computer-controlled piston ventilator (FlexiVent Ver. 5.2R02, SCIREQ, Montreal, Quebec, Canada). Resistance and compliance data were calculated using the single-compartment model. For comparison among the groups, all data were normalized to their individual baseline resistance values ($[\text{value}-\text{baseline}]/\text{baseline}$) and plotted as normalized resistance. Baseline values were not statistically different among the groups.

Determination of bronchoalveolar lavage fluid cellularity and cytokine measurement

Bronchoalveolar lavage fluid (BALF) was isolated in 0.9 ml PBS containing 2% BSA. Total bronchoalveolar lavage cellularity was determined with a hemocytometer. Cells (20,000) were centrifuged onto slides and were fixed and stained using the Hema-3 staining kit (Fisher Scientific, Pittsburgh, PA). Two unbiased observers counted 200–300 cells per slide using standard morphological criteria to classify individual leukocyte populations. Cytokine levels were measured from 50 μ l cell-free BALF using a high-throughput multiplex cytokine assay system (x-Plex Mouse Assay; Bio-Rad), according to the manufacturer's instructions. Three to six BALF samples per group were analyzed in duplicate on the Bio-Plex 200 system (Bio-Rad). Standards ranging from 0.2 to 6296 pg/ml (depending on the analyte) were used to quantitate a dynamic range of cytokine concentrations. The concentrations of analytes in the samples were quantified using a standard curve, and a five-parameter logistic regression was performed to derive an equation that was then used to predict the concentration of the unknown samples. The following cytokines were assayed: IL-4, IL-5, IL-12(p40), IL-13, and IFN- γ . The data presented exclude any number outside the range of sensitivity for the particular analyte.

Pulmonary histopathology

Lungs were perfused with PBS containing 20 U/ml heparin, inflated gently to total lung capacity, and fixed in HistoChoice Tissue Fixative (AMRESCO, Solon, OH) for 24 h at 4°C. These tissues were then embedded in paraffin, cut in 4- μ m frontal sections, and stained with H&E or periodic acid-Schiff (PAS) to show inflammation and mucus hyperproduction in airway goblet cells, respectively. To evaluate the level of inflammation associated with secondary RSV infection, two independent observers quantified the total number of airways in each lung section and then scored each of these airways for inflammation (0, no inflammation; 1, inflammation at least three cells thick). These data are reported as the percentage of inflamed airways/total number of airways per lung section.

Statistical analyses

Power analysis was used to determine the appropriate number of subjects for each experiment. All data were plotted as means \pm SEM and analyzed using GraphPad Prism software (version 5.02; GraphPad, San Diego, CA). Two-way ANOVA and Bonferroni post hoc tests were used to test for differences in the pulmonary function tests between the groups. One-way ANOVA and Bonferroni post hoc tests were used to test for differences in BALF cellularity, BALF cytokine levels, Ab levels, lung inflammation, and T cell populations. The Student *t* test was used for surface expression of IL-4R α . Differences were considered statistically significant at *p* < 0.05.

Results

Surface expression of IL-4R α is reduced on pulmonary mDCs and epithelial cells following IL-4R α ASO inhalation

IL-4R α ASOs, which were shown to downregulate IL-4R α protein in an adult mouse model of allergic asthma (30), were used to reduce the expression of IL-4R α on pulmonary immune and structural cells in the neonatal mouse lung. We administered 500 μ g/kg of IL-4R α ASO or MM i.n. to neonatal mice on days -5, -3, and -1 (Fig. 1). On protocol day 0 (i.e., 7 d of age), lung cells were recovered after collagenase digestion of the tissue and analyzed for surface expression of IL-4R α . Downregulation of IL-4R α protein on the surface of specific lung cell populations was determined using flow cytometry (Fig. 2). The mean fluorescence intensity (MFI) of IL-4R α was significantly reduced on mDC populations by $21 \pm 1.6\%$ and on E-cadherin⁺ epithelial cells by $12.5 \pm 3.0\%$ (mean \pm SEM) compared with saline (vehicle) controls. The decreased MFI data for epithelial cells and mDCs paralleled the reduction in the percentage of IL-4R α -expressing cells. No difference in IL-4R α expression levels by MFI was observed on CD4⁺ or CD8⁺ T cells. There was a slight, but significant, reduction in the percentage of CD4⁺ cells expressing IL-4R α compared with saline, as well as a decrease in the percentage of epithelial cells expressing IL-4R α (Fig. 2B). Similar to previous studies, administration of MM had no effect on IL-4R α expression levels, as assessed by MFI of IL-4R α or by the percentage of IL-4R α -expressing cells. Because absorption of inhaled ASO into the systemic circulation is <1% of the deposited lung dose (30), IL-4R α expression in other tissues was not assessed. These observations indicate that inhaled ASO effectively targets mDCs in the neonatal lung.

Downregulation of IL-4R α during primary RSV infection increases Th1 cellular and Ab response

Because signaling through IL-4R α is required for Th2 differentiation, downregulation of IL-4R α should significantly impair the development and differentiation of Th2 cells. To test this theory, we treated mice with IL-4R α ASO, infected them with RSV, and measured T cell populations at 6 dpi (Fig. 1). RSV infection elicited an increase in pulmonary Th2 (CD4⁺IL4⁺) cell populations compared with control mice (SR, $0.347 \pm 0.033\%$ versus SHAM, $0.033 \pm 0.003\%$ or, Fig. 3). Treatment with IL-4R α ASO significantly reduced the percentage of Th2 cells in the lungs of

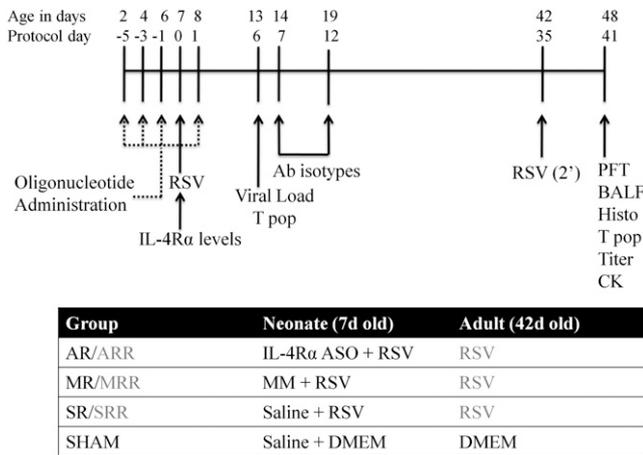


FIGURE 1. Study timeline and group summary. Mice were treated with IL-4Rα ASO (AR/ARR), MM (MR/MRR), or saline (SR/SRR and SHAM) on protocol days -5, -3, -1, and 1 and infected with RSV (AR/ARR, MR/MRR, and SR/SRR) or vehicle (SHAM) on protocol day 0. For secondary studies, mice were reinfected with RSV (ARR, MRR, and SRR) or vehicle (SHAM) on protocol day 35. Various end points were measured: IL-4Rα levels on pulmonary cells (IL-4Rα levels), RSV-specific Ab levels in serum (Ab isotypes), viral load (RSV copy number in the lung), T cell populations in the lung (T pop), pulmonary function testing (PFT), cell differential counts in BALF (BALF), lung histology (Histo); and BALF cytokines (CK).

RSV-infected mice (AR, 0.127 ± 0.032%) compared with the SR group. Th1 (CD4⁺IFN-γ⁺) cell populations were increased in RSV-infected mice compared with controls (0.280 ± 0.025% versus 0.040 ± 0.006% for SR and SHAM, respectively), and ASO treatment further increased this Th1 population (AR, 0.767 ± 0.048%). These data indicate that treatment with IL-4Rα ASO significantly shifts the initial immune response to RSV infection toward a Th1 profile.

Tc2 (CD8⁺IL-4⁺) cells, another important source of IL-4, were increased in the SR group compared with controls (0.217 ± 0.058% and 0.043 ± 0.003%, respectively) and were reduced in the MR and AR groups compared with controls (0.110 ± 0.012% and 0.087 ± 0.018%, respectively), although neither achieved statistical significance. Tc1 (CD8⁺IFN-γ⁺) cells, which are important in viral clearance, were significantly increased in the SR group compared with controls (2.27 ± 0.113% and 0.033 ± 0.003%,

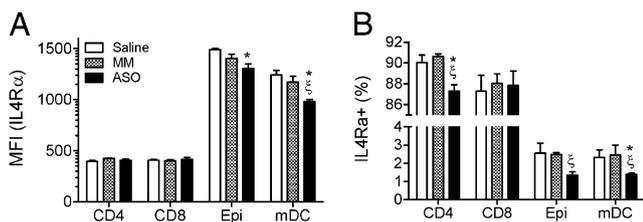


FIGURE 2. Cell surface expression of IL-4Rα and percentage of IL-4Rα⁺ cells after inhalation of ASO in neonatal mice. The ASO group was treated with 500 μg/kg IL-4Rα ASO on protocol days -5, -3, and -1. The MM group was treated with 500 μg/kg MM. Saline mice were treated with saline. On protocol day 0 (age 7 d), lung cells were isolated, labeled with differentiation markers, and analyzed by flow cytometry. **A**, MFI of IL-4Rα on pulmonary cell subsets. **B**, Percentage of IL-4Rα⁺ cells of each cellular subset. CD4, CD4⁺ T cells (CD3⁺, CD4⁺, IL-4Rα⁺); CD8, CD8⁺ T cells (CD3⁺, CD8⁺, IL-4Rα⁺); Epi (E cadherin⁺, IL-4Rα⁺); mDC (CD11b^{hi}, CD11c⁺, IL-4Rα⁺). Data are representative of three independent experiments and are expressed as means ± SEM (*n* = 4/group). **p* < 0.05, compared with saline; †*p* < 0.05, compared with MM. Epi, epithelial cells.

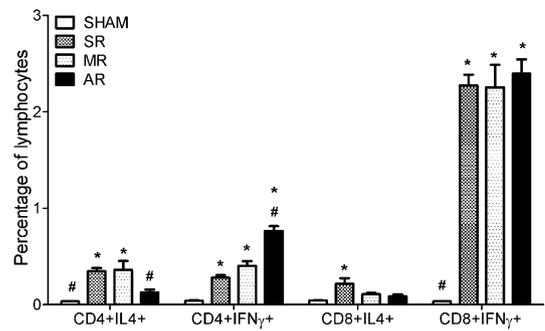


FIGURE 3. T cell subpopulations after primary infection with RSV. The AR group was treated with 500 μg/kg IL-4Rα ASO or MM on protocol days -5, -3, -1, and 1 and infected with RSV on protocol day 0. The SR group was treated with saline and infected with RSV. Control (SHAM) mice were treated with saline and sham infected with vehicle. Six dpi, lung cells were isolated and stained with differentiation markers and analyzed by flow cytometry. Th1 cells (CD4⁺IFN-γ⁺), Th2 cells (CD4⁺IL4⁺), Tc1 cells (CD8⁺IFN-γ⁺), and Tc2 cells (CD8⁺IL4⁺) in the lung were analyzed. Data are representative of three independent experiments and are expressed as means ± SEM (*n* = 3/group). **p* < 0.05, compared with SHAM; #*p* < 0.05, compared with SR.

respectively) and seemed to be unaffected by ASO administration (2.397 ± 0.148%). There were no differences in the percentage of T cell subsets (i.e., Th2, Th1, Tc2, Tc1) in the SR and MR groups. Similar trends were also observed when the total numbers of each of these T cell subsets were calculated (Supplemental Fig. 1).

Because the isotype of Abs produced in response to a pathogen is another indicator of the type of immune response initiated (i.e., Th1 versus Th2), we analyzed RSV-specific Ab isotypes by indirect ELISA in sera of mice infected with RSV as neonates with or without IL-4Rα ASO treatment. At 7 dpi, neonatal RSV infection (SR) resulted in detectable levels of IgG1, IgG2a, IgE, and IgA (Fig. 4A). However, treatment with IL-4Rα ASO during neonatal RSV infection significantly boosted IgG2a levels compared with controls (AR: 2.31 ± 0.58-fold greater than SR and 2.09 ± 0.54-fold greater than MR). By 12 dpi (Fig. 4B), IgG2a remained elevated in the sera of IL-4Rα ASO-treated mice, suggesting that suppression of IL-4Rα expression results in elevated Th1-like IgG2a responses to neonatal RSV infection. Ab isotypes produced in response to RSV infection were similar between the SR and MR groups at 7 and 12 dpi.

IL-4Rα ASO treatment has no effect on viral load during RSV infection

Pulmonary viral load may be associated with increased disease severity in some human cases (32). To address the possible effects

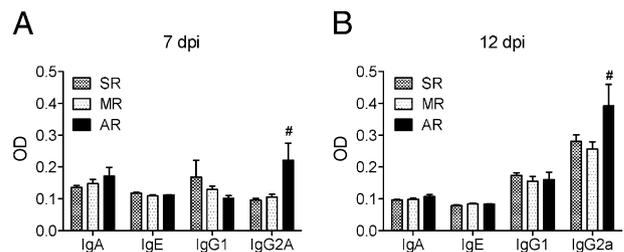


FIGURE 4. Serum Ab isotypes after primary RSV infection at 7 dpi (**A**) and 12 dpi (**B**). RSV-specific Abs in serum were determined by indirect ELISA. Absorbance values (OD) are plotted. Data are representative of two independent experiments. Dose of ASO or MM was 500 μg/kg (*n* = 4-6/group). #*p* < 0.05, compared with SR.

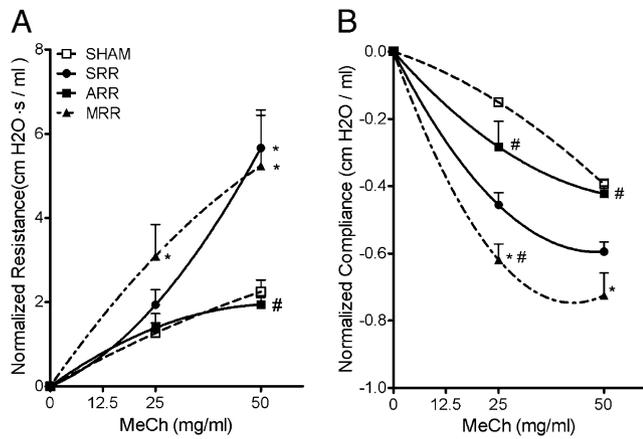
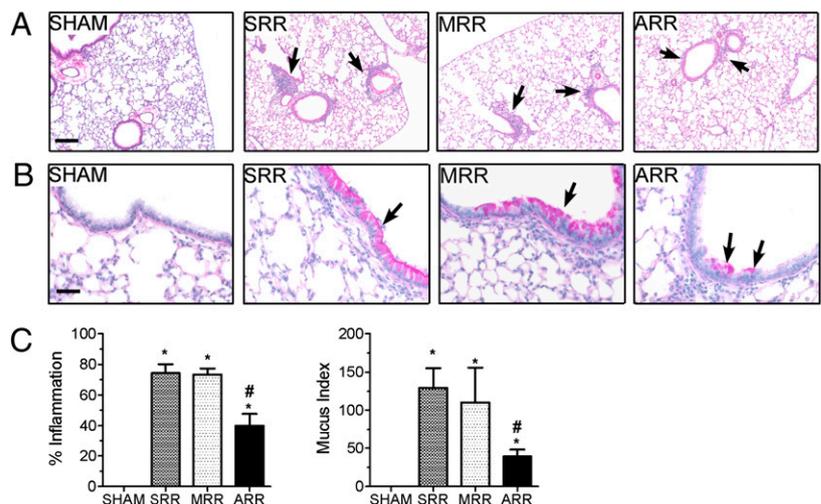


FIGURE 5. Pulmonary function after secondary RSV infection. Mice were treated with ASO, MM, or saline, infected with RSV or sham infected as neonates, and reinfected with RSV at 6 wk of age (ARR, MRR, SRR, and SHAM, respectively). Six days later, pulmonary function was measured in all four groups. Lung resistance (A) and compliance (B) were measured in response to increasing doses of MeCh. Resistance values were normalized to individual baseline resistance at 0 mg/ml MeCh. Dose of ASO or MM was 100 μ g/kg to demonstrate efficacy even at lower dose. Data are representative of three independent experiments and are expressed as means \pm SEM ($n = 6$ /group). * $p < 0.05$, compared with SHAM; # $p < 0.05$, compared with SRR.

of ASO treatment on RSV replication in the lung, we assessed viral load using traditional TCID₅₀ methodology and real-time PCR to determine pulmonary RSV-*NP* copy numbers. Viral loads were measured at 6 dpi. Viral load in the lungs of ASO-treated, RSV-infected mice (AR group) was similar to control mice infected with RSV but not receiving ASO (SR group) or mice receiving MM (MR group). The TCID₅₀ for RSV was 781 ± 219 in the lungs of the AR group and 765 ± 72 in the lungs of the SR group. The cycle threshold values for RSV-*NP* were 30.2 ± 0.662 , 30.3 ± 0.213 , and 30.8 ± 0.426 for the SR, AR, and MR groups, respectively. Cycle threshold values for the reference gene, GAPDH, were also similar (20.0 ± 0.710 , 19.5 ± 0.663 , and 18.6 ± 0.685 for the SR, AR, and MR groups, respectively), indicating that equivalent amounts of experimental sample were analyzed. No viral particles were detected in the lungs of any mice after 8 dpi during primary or secondary infection, suggesting that IL-4R α ASO treatment does not hinder viral clearance during primary or secondary infection.

FIGURE 6. Histology after secondary RSV infection. Lung tissue was obtained at 6 d after secondary infection. A, H&E staining shows inflammatory cells in the lung (arrows). Original magnification $\times 100$; scale bar, 50 μ m. B, Staining with PAS shows mucus production in airway epithelial cells. Original magnification $\times 400$; scale bar, 200 μ m. C, Morphometric analysis of lung inflammation and mucus. Data are expressed as mean percentage of inflamed airways to total airways or as mean mucus index as quantified from frontal sections of the lungs from each mouse. The mucus index was determined as follows: [(area of PAS staining/total area of the airway epithelium) \times number of airways counted per lung]. Dose of ASO or MM was 100 μ g/kg to demonstrate efficacy at a lower dose. Micrographs are representative of three independent experiments and are expressed as means \pm SEM ($n = 4$ mice/group). * $p < 0.05$, compared with SHAM; # $p < 0.05$, compared with SRR.



IL-4R α ASO treatment provides long-term pulmonary protection even after rechallenge with RSV

Data from the failed RSV vaccine trials of the 1960s indicated that vaccinated children suffered from enhanced bronchiolitis and respiratory disease with community-acquired RSV compared with unvaccinated children. To determine whether treatment with IL-4R α ASO was capable of preventing enhanced respiratory disease following reinfection with RSV, we treated neonatal mice with IL-4R α ASO during primary RSV infection. Five weeks later, these same mice were reinfected with RSV. Six days after secondary infection (protocol day 42), airway function and inflammation were assessed in these animals.

Pulmonary function. The SRR group showed significant increases in airway resistance compared with SHAM mice at the 500 and 100 μ g/kg doses (data not shown and Fig. 5A, respectively). IL-4R α ASO treatment during the initial infection provided protection from AHR in response to secondary RSV infection. In fact, lung resistance at 50 mg/ml of MeCh in the SRR group was ~ 2.5 -fold higher than in the SHAM or ARR group at the 100 μ g/kg dose of ASO. Lung compliance followed the same trend (Fig. 5B). Compliance in the ARR group was similar to that in the SHAM mice, whereas the SRR group showed substantially lower compliance at 50 mg/ml of MeCh (1.5-fold) compared with the SHAM or ARR groups. Treatment with MM during initial RSV infection (MRR group) failed to alter pulmonary resistance or compliance following secondary RSV infection compared with infection alone (SRR group).

Pulmonary histopathology. Histological evaluation was performed on lung sections obtained 6 d after secondary infection. Significant inflammation was visible in the peribronchiolar and perivascular areas in the lungs of mice infected with RSV (SRR group) or treated with MM and subsequently infected with RSV (MRR group). Pulmonary inflammation was significantly diminished by ASO administration (ARR group; Fig. 6A, 6C, Supplemental Fig. 2). Mice reinfected with RSV exhibited markedly enhanced mucus production; this effect was mostly abolished with IL-4R α ASO treatment at the time of primary infection (Fig. 6B, 6C). These data correlate with the increased airway resistance observed after secondary infection in the SRR group and the reduction of airway resistance to baseline levels in the ARR group (Fig. 5).

BALF cellularity. The total number of leukocytes recovered in the BALF of all RSV-infected mice was significantly elevated after

reinfection at 5 wk (Fig. 7). This increase seemed to be due primarily to increases in macrophage/monocyte and lymphocyte populations. IL-4R α ASO treatment during primary infection significantly reduced the total number of leukocytes present in the BALF following reinfection with RSV (ARR group), and these decreases were mainly due to a reduction in macrophage and lymphocyte numbers. Although not statistically different, BALF eosinophil numbers were slightly reduced from mice receiving IL-4R α ASO treatment compared with those not receiving treatment (2.1×10^3 versus 5.0×10^3 for ARR and SRR, respectively; $p = 0.14$).

Th1 cell subsets and cytokine responses are maintained in the adult mouse upon rechallenge with RSV

Unlike in adult mouse models in which primary infection with RSV induces predominant Th1 responses, primary infection with RSV in neonates induces Th2 responses upon reinfection and is associated with enhanced disease (i.e., Th2 cell expansion, IL-4 production, and eosinophilia) (16, 33, 34). To address the possible mechanisms by which IL-4R α ASO treatment alleviated pulmonary disease upon RSV reinfection, T cell responses (i.e., Th1/Th2 and Tc1/Tc2 responses) were investigated 6 d after reinfection (Fig. 8). Th1, Th2, Tc1, and Tc2 cells migrated to lungs, as evidenced by significantly greater numbers of all four cell types in the lungs of RSV-reinfected mice (SRR and ARR groups) than in the SHAM mice. The lungs from mice infected with RSV as neonates and reinfected as adults (SRR group) contained significantly greater numbers of Th2 cells ($1.88 \pm 0.148\%$) compared with control mice (SHAM, $0.220 \pm 0.061\%$). Administration of IL-4R α ASO at the time of primary infection significantly reduced the Th2 population in the lungs upon secondary infection (ARR, $1.00 \pm 0.188\%$) compared with the SRR group. Tc1 and Tc2 populations were significantly increased in response to secondary RSV infection compared with uninfected controls. Administration of IL-4R α ASO also increased Tc1 levels (ARR, $26.4 \pm 3.10\%$) compared with the SRR ($18.4 \pm 1.76\%$) and SHAM groups. Similar data were observed in the total numbers of T cell subsets (Supplemental Fig. 1)

BALF cytokine levels were also measured 6 d following secondary infection (Fig. 9). RSV reinfection resulted in secretion of a variety of cytokines, including IL-4, -5, -12(p40), and -13 and IFN- γ . IL-4R α ASO treatment (ARR group) during primary infection in neonatal mice led to a reduction in Th2 cytokines, including IL-5 and IL-13, following secondary infection. The decreased Th2 cytokine production in the BALF was consistent with the reduced AHR and mucus observed in the ARR group. No

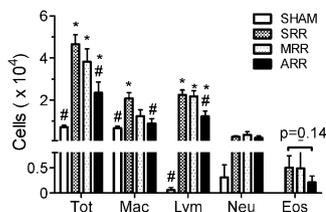


FIGURE 7. BALF cellularity after secondary RSV infection. Mice were treated with ASO, MM, or saline; infected with RSV or sham infected as neonates; and reinfected at 6 wk of age (ARR, MRR, SRR, and SHAM, respectively). Six days later, BALF was collected, and WBC differentials were counted. Dose of ASO or MM was 500 $\mu\text{g}/\text{kg}$. Data are representative of three independent experiments and are expressed as means \pm SEM ($n = 6\text{--}10/\text{group}$). Two independent observers counted 200–300 cells per mouse. * $p < 0.05$, compared with SHAM; # $p < 0.05$, compared with SRR. Eos, eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils.

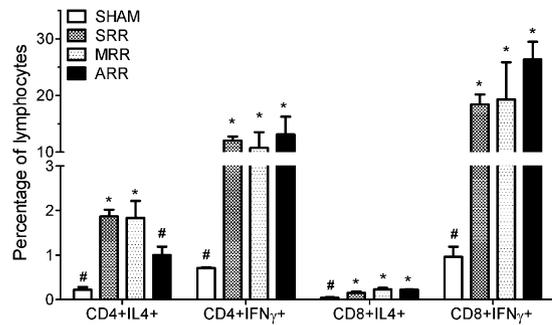


FIGURE 8. T cell populations after secondary RSV infection. Mice were treated with ASO, MM, or saline on protocol days $-5, -3, -1,$ and 1 ; infected with RSV on protocol day 0 ; and reinfected with RSV on protocol day 35 . Six days after secondary infection (protocol day 41), lung cells were isolated, stimulated in vitro with ionomycin and PMA, stained with differentiation markers, and analyzed by flow cytometry. Th1 cells (CD4⁺IFN- γ ⁺), Th2 cells (CD4⁺IL4⁺), Tc1 cells (CD8⁺IFN- γ ⁺), and Tc2 cells (CD8⁺IL4⁺) in the lung were measured. Dose of ASO or MM was 500 $\mu\text{g}/\text{kg}$. Data are representative of three independent experiments and are expressed as means \pm SEM ($n = 4/\text{group}$). * $p < 0.05$, compared with SHAM; # $p < 0.05$, compared with SRR.

differences were observed between the ARR group and nontreated mice (SRR group) for other cytokines, including the prominent Th1 cytokines IL-12 and IFN- γ .

Discussion

The age of primary infection with RSV is important in dictating the initial and subsequent immunological responses to infection. Neonatal RSV infection results in the development of persistent pulmonary dysfunction in humans (4) and mice (16–19), as the result of an immature immune system in the neonates. Therefore, manipulation of the immune system during this window of immunological immaturity may provide significant long-term respiratory benefit to neonates infected with RSV and yield an opportunity for effective vaccination. Data from the present study support our hypothesis that the administration of IL-4R α ASO during neonatal RSV infection rebalances the Th cell compartment, decreasing Th2 responses (i.e., reduced goblet cell hyperplasia and Th2 cytokine secretion) and increasing Th1 responses (i.e., elevated Th1 cell numbers and type I Abs and cytokines). Administration of IL-4R α ASO reduced multiple pathophysiological parameters associated with neonatal RSV infection, including pulmonary inflammation and mucus hyperproduction.

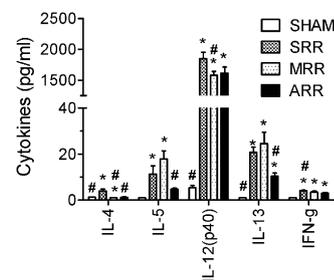


FIGURE 9. BALF cytokine levels after secondary RSV infection. Following secondary RSV infection, BALF was isolated at 6 dpi, and the cell-free supernatant was analyzed for the following cytokines: IL-4, IL-5, IL-12(p40), IL-13, and IFN- γ . Dose of ASO or MM was 500 $\mu\text{g}/\text{kg}$. Data are representative of three independent experiments and are expressed as means \pm SEM ($n = 4/\text{group}$). * $p < 0.05$, compared with SHAM; # $p < 0.05$, compared with SRR.

Furthermore, it completely abolished the development of AHR following adult reinfection with RSV and altered the initial and subsequent T cell responses to RSV infection, demonstrating its potential as part of a vaccine strategy.

Administration of IL-4R α ASO significantly downregulated IL-4R α on neonatal pulmonary cells, including mDCs, suggesting that IL-4R α mediates T cell rebalancing via modulation of pulmonary mDC development and function. Sriram et al. (35) demonstrated that IL-4 suppresses the maturation of DCs (e.g., expression of costimulatory molecules, antiviral genes) by rendering the DC less responsive to type I IFNs. They further demonstrated that the lack of responsiveness of the DCs to type I IFNs attenuated the autocrine positive feedback response required to generate high levels of type I IFNs via suppression of STAT1 transcription. Although it was beyond the scope of this study to explore this mechanism in greater detail, we observed substantially reduced levels of type I IFN responses in neonatal RSV infection compared with responses in adult RSV infection (data not shown). Because type I IFN signaling is required for DCs to help initiate and maintain the Th1 response, these data suggest that DCs developing in the presence of IL-4/IL-13 are helping to initiate or maintain a Th2 immune response. More importantly, our data suggest that the use of IL-4R α ASO helps to rebalance the Th cell compartment in response to neonatal RSV infection. In addition, administration of IL-4R α ASO resulted in reduction of IL-4R α expression on lung epithelial cells and might account, in part, for the reduced production of goblet cells and mucus (36).

Overall, our studies reveal a role for IL-4R α in the pathogenesis of neonatal RSV infection, which agrees with previous studies demonstrating that IL-4 (24) and IL-13 (17, 19, 24) are central mediators in RSV-mediated airways disease in mouse models. More importantly, our findings support recent data from human studies indicating that gain-of-function variants of IL-4R α (26) play a major role in increasing the severity of RSV disease that occurs as a result of neonatal RSV infection. The concentration of IL-13 in the BALF isolated after primary (data not shown) and secondary RSV infection was significantly reduced in IL-4R α ASO-treated mice compared with untreated controls. This decrease in IL-13 correlated with decreased Th2 cells in the lung, significantly reduced mucus production in pulmonary epithelial cells, and normal pulmonary function in ASO-treated mice following secondary RSV infection. These data are consistent with a series of recently published studies demonstrating the importance of IL-13 in RSV-mediated pathophysiology (17, 24). The mechanism(s) via which IL-4R α ASO treatment during primary RSV infection decrease IL-13 concentrations during reinfection was not explored in this study, but it may simply be due to the reduced numbers of Th2 cells present after primary and secondary RSV infection in the ASO-treated groups.

Our study clearly indicates that reduction of IL-4R α in the lung at the time of initial infection inhibits the development of Th2 cell subsets following RSV infection. In light of recent data from Zaghouani's laboratory demonstrating the IL-4/IL-13-dependent specific deletion of Th1 cells in neonates (20, 37), our data further suggest that downregulating a component of the type II IL-4R complex allows for the survival of Th1 cells in the presence of Th2 cytokines, such as IL-4 and IL-13. In support of this theory, we observed increased numbers of Th1 cells following primary RSV infection in ASO-treated mice (Fig. 3).

IL-4R α ASO treatment changed the primary T cell responses to RSV in infected neonates. Analysis of T cell populations and Ab isotypes showed that ASO treatment increased the Th1 response and decreased the Th2 response to RSV. This change was not an

on-off effect; however, it was sufficient to properly alter the Th1/Th2 balance and to ensure that the immune responses to RSV reinfection were beneficial, as evidenced by the fact that pulmonary function, inflammation, and mucus production in ASO-treated mice were more similar to noninfected controls. The importance of the fine balance between Th1 and Th2 responses to a viral infection is well recognized in many studies, and it was eloquently demonstrated in a study in which rRSV expressing IFN- γ was used to infect mice with the hope of preventing pulmonary disease following RSV reinfection (38). Unfortunately, overexpression of IFN- γ , the canonical Th1/Tc1 cytokine, during primary infection in adults led to detrimental effects, including enhanced weight loss and more severe pulmonary inflammation upon reinfection. A follow-up study using rRSV expressing IL-4 or IFN- γ in neonates showed that neonatal mice infected with RSV expressing IFN- γ have increased maturation of immune cells responsible for the innate response (39). This reduces neutrophil and NK cell recruitment upon rechallenge with RSV, which is mediated by macrophages. Priming the neonate with RSV in an environment rich in IFN- γ may help to promote the maturation of macrophages, thereby preventing neutrophil- and NK cell-mediated pathology upon rechallenge as an adult. In contrast, administration of IL-4R α ASO moderately increased Th1 responses while significantly reducing the pulmonary pathology (i.e., AHR and mucus hyperproduction) characteristic of neonatal RSV reinfection.

Recent data using an adult model of RSV infection demonstrated that inhibiting IL-13 expression or function may promote Th17 inflammation (40). Although we observed decreased levels of IL-13 in the BALF of RSV-infected pups treated with IL-4R α ASO, IL-17 levels were not statistically different from saline-treated, SHAM-infected controls (data not shown). This suggests that, at least in our neonatal model of RSV infection, the use of IL-4R α ASO does not have the unintended consequence of upregulating Th17 cytokine production.

Despite almost half a century of intense research, there is no vaccine available for RSV. One of the major reasons is that the usual vaccination strategy uses an attenuated virus to inoculate infants with the hope of eliciting protective memory responses in a rather immature or weakened immune system. As an alternative, we used a wild-type virus to infect neonatal mice along with immunomodulation at the time of infection. The results, so far, are promising. We demonstrated that treating neonates with ASO specific for IL-4R α during primary RSV infection completely protects against the pulmonary dysfunction usually observed following RSV reinfection. This protection was achieved by inducing a sufficiently strong immune response with a wild-type virus instead of an attenuated one, as well as by delicately balancing Th1 and Th2 responses with IL-4R α ASO treatment during infection. We believe that our IL-4R α ASO treatment offers a good opportunity for future vaccine-development strategies and suggest its potential use as a therapeutic for pediatric RSV infection.

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Disclosures

A patent application has been filed by Louisiana State University Health Sciences Center as a result of this research, with S.A.C. as the inventor. The other authors have no financial conflicts of interest.

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