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Immunomodulation with IL-4 Receptor-α Antisense Oligonucleotide Prevents RSV-Mediated Pulmonary Disease[1]

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Abstract

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 of 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 if local inhibition of IL-4Rα 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-4Rα expression, antisense oligonucleotides (ASO) specific for IL-4Rα were administered intranasally to neonatal mice at the time of primary infection. Mice were initially infected with RSV at one week of age and reinfected at six weeks of age. Administration of IL-4Rα ASO 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 and Th2 cells and cytokine secretion) and increased Th1 responses (i.e. elevated Th1 cell numbers and type I antibodies 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-4Rα ASO may be of significant benefit to infants.

Introduction

Respiratory syncytial virus (RSV) is an important cause of acute respiratory tract infections in infants (and the elderly) causing significant morbidity and mortality. The WHO estimates the global burden of RSV disease at 64 million cases and 160,000 deaths annually. Yearly in the U.S., RSV is responsible for 85,000 to 144,000 infant hospitalizations (1). Health care

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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 months of age(4). Interestingly, infants who are younger than three months of age and who develop RSV bronchiolitis show persistent increase in IL-4 production following infection (5); and are at an increased risk to develop recurrent wheeze/asthma (4,6–15). Despite dire need, no safe and effective vaccine for RSV currently 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 vs 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 age (weanling), AHR is not induced in response to secondary infection even though 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 about one week of age in the mouse (16,20), and epidemiological data suggests that it occurs at approximately four months of age in the human (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 (21). Indeed, the failure of the RSV vaccine of the 1960s is believed to be due, in part, to the exacerbated Th2 response to community-acquired RSV following inoculation with formalin-inactivated virus (22). Understanding this age-related difference in pathophysiological response to RSV infection is critical to understand 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-4 receptor alpha (IL-4Rα) subunit. IL-4 has two receptors, the Type I and Type II receptor. The Type I receptor is composed of IL-4Rα and the common gamma chain (γ_c) and binds IL-4 exclusively and initiates Th2 cell differentiation. The Type II IL-4 receptor is composed of the IL-4Rα and IL-13 receptor α1 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 (23). Signaling through both of these receptors occurs via a JAK/STAT pathway (24) 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 (17,25), most notably decreased AHR and mucus hyperproduction. The recent association of IL-4/IL-13 haplotypes and IL-4Rα gain-of-function polymorphisms with RSV hospitalizations and disease severity (26–29) suggests that IL-4Rα may play a key role in RSV-mediated pulmonary pathologies in human infants.

Despite nearly a half-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-4Rα in the pulmonary compartment is sufficient to prevent the adverse pulmonary events observed following neonatal RSV infection. To accomplish this, we utilized an antisense oligonucleotide (ASO) specific for IL-4Rα to reduce receptor expression. Our results indicate that suppression of pulmonary IL-4Rα protects from subsequent RSV-mediated pulmonary inflammation and lung dysfunction. The observed protection from RSV-driven immunopathology further suggests its use in a novel pediatric vaccine strategy and suggests

that inhaled IL-4Rα ASO may be effective as a preventive therapy for complications of RSV-induced wheeze.

Methods

Mice

BALB/c mice were purchased as breeders from Harlan Laboratories. All mice were housed in the vivarium at Louisiana State University Health Sciences Center (New Orleans, LA) and were maintained in ventilated micro-isolator cages housed in a specific pathogen-free animal facility. Sentinel mice within this animal colony were negative for antibodies to specific viral and other known mouse pathogens. Breeders were time-mated and two 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 (30) and approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center.

Oligonucleotides – Preparation and Dosage

IL-4Rα antisense oligonucleotides (ASO) and mismatch oligonucleotides (MM) were kindly provided by Isis Pharmaceuticals (Carlsbad, CA). The oligonucleotides were synthesized and purified as previously described (31). 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 to 5 and 16 to 20 (underlined). Their estimated half-life following single dose inhalation studies is 4 d in the mouse lung (31). The sequences of the ASO and MM are shown below; the mismatched bases are presented in lower case.

ASO: 5′-CCGCTGTTCTCAGGTGACAT-3′

MM: 5′-CCaCTcaTCaCtGcTGACtT-3′.

The oligonucleotides were suspended in sterile saline and administered intranasally (i.n.) to mouse pups at a dose of 100 or 500 μg/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 μg/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 μg/kg body weight) to demonstrate lower dose efficacy, and the cytokine data at both doses (100 and 500 μg/kg body weight) to demonstrate that at higher doses the MM oligonucleotide has effects that are not due to downregulation of IL4Rα. Control pups received sterile saline.

Experimental Design

The experimental design is outlined in Figure 1. IL-4Rα ASO, MM, or saline was administered intranasally (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 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 endpoints were measured including IL-4Rα levels, T cell populations in the lung, pulmonary function, bronchoalveolar lavage cellularity and cytokine profile, pulmonary viral copy number, lung histology, and RSV-specific antibody levels in serum at the indicated time points.

Respiratory Syncytial Virus Infection and Pulmonary Viral Load Determination

Human RSV strain A-2 was purchased as a sucrose-gradient purified virus from Advanced Biotechnologies, Inc. The virus preparation was determined to be free of bacteria, yeast, and fungi. Seven day old mice (protocol day 0; Figure 1) were anesthetized with 5% isofluorane and infected intranasally (i.n.) with 2×10^5 $TCID_{50}$ per gram body weight of RSV in 10 μl serum-free media (VP-SFM; Invitrogen) or media alone (18,19). Adult mice (protocol day 39) were similarly infected with RSV (2×10^5 $TCID_{50}$/gram body weight) in 50 μl of the same media.

To determine lung viral load, we employed the $TCID_{50}$ method of Spearman-Kärber as previously published (19) using whole lung homogenates isolated from mice at 6 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 days; wells showing syncytia were counted and $TCID_{50}$ values were calculated.

Viral load was also quantified using real-time PCR. Lungs were isolated at 6 days post-infection (dpi), quick-frozen in liquid nitrogen, and stored at −80°C until processing. RNA was extracted from the lungs with TRIzol Reagent (Invitrogen) and purified with RNeasy Mini Kit (Qiagen) and DNase treated (Ambion). The genomic RNA was then reverse transcribed into cDNA with an RSV-NP specific primer (5′-GCGATGTCTAGGTTAGGAAGAA-3′) or oligo 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 placed at 4°C for 1 min, RNase H was added, and the samples were incubated 37°C for 20 min. Real-time PCR was performed using LUX™ (Invitrogen) primers specific for RSV NP: forward primer (5′-cgttacTTGGGTAGTAAGCCTTTGTAa[FAM]G-3′) and reverse primer (5′-CTGGTCTTACAGCCGTGATTAGGA-3′) 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 sec, 60°C for 30 sec, and 72 °C for 30 sec. 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 sec) in a BioRad iQ5 Machine. Selected PCR products were cloned into a TA cloning vector (pGEM-T, Promega), 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 LUX™ Primer Set [JOE]) 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 Antibody

Serum was isolated from the left ventricle of euthanized mice following primary infection (7 and 12 dpi) using serum separator tubes (BD) and stored at −80°C until use. Microtiter plates (Nunc-Immuno Maxisorp) were coated with 50 μl of RSV (5×10^4 pfu/ml) overnight at 4°C in PBS. The plates were blocked with 1x Blocker BSA (Pierce) for 5 min, 25 μl of serum was added to each well and allowed to incubate for 2 h at room temperature. Bound RSV-specific antibody was then isotyped and quantified using peroxidase-conjugated goat antibodies specific for mouse IgA, IgG1, IgG2a, and IgE (Southern Biotech) and 3,3′,5,5′-tetramethylbenzidine (TMB) (Pierce) as substrate. Color development was stopped with 2M H_2SO_4 and the optical density was read at 450 nm. RSV-specific antibody levels were determined by subtracting the absorbance value of the blank wells (media only) from the absorbance of each serum sample.

Determination of IL-4Rα Surface Expression and Assessment of Pulmonary T and Dendritic Cell Populations

A single-cell suspension of lung cells was prepared using a standardized protocol (32). Lungs were perfused, excised, cut into small pieces, and incubated at 37°C for 1 h in RPMI 1640 media (HyClone) supplemented by 5% heat-inactivated FBS (HyClone),100 U/ml penicillin, 100 mg/ml streptomycin (HyClone), 1 mg/ml collagenase I (Invitrogen), and 150 μg/ml DNase I (Sigma-Aldrich). After incubation, single cells were obtained by mashing the lung pieces through a 40-μm cell strainer (BD Biosciences). Red blood cells were lysed using RBC lysis buffer (eBioscience) and cells were stained with combinations of the following antibodies purchased from BD and eBioscience Pacific Blue-CD3e (17A2), PerCP-CD4 (RM4–5), FITC-CD8a (53-6.7), Biotin-CD124 (mIL4R-M1), APC-CD11b (M1/70), PE Cy7-CD11c (N418), and 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 phorbol-12-myristate-13-acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of a protein transport inhibitor (1 μl/10^6 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, non-monocyte cells using forward and side scatter properties; expression of $CD11c^{hi}$ and CD11b+ were used to identify mDCs. A total of 300,000 events were analyzed per mouse lung. Isotype control antibodies were utilized in all flow cytometry experiments. Flow data were analyzed and plotted using FlowJo software (version 7.2.2 for Windows, Tree Star).

Assessment of Pulmonary Function

Six days after secondary infection, lung resistance and compliance to increasing doses of methacholine (MeCh, Sigma; 0, 12.5, 25, and 50 mg/ml in isotonic saline) 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). 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 ((value-baseline)/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 of PBS containing 2% BSA. Total BAL cellularity was determined with the use of a hemocytometer. Cells (20,000) were centrifuged onto slides and were fixed and stained using the Hema-3 staining kit (Fisher Scientific). Two unbiased observers counted 200–300 cells per slide using standard morphological criteria to classify individual leukocyte populations. Cytokine levels were measured from 50 μl of 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 6,296 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 herein excluded 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, Inc) for 24 hours at 4°C. These tissues were then embedded in paraffin, cut in 4 μm frontal sections and stained with hematoxylin and eosin (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 or 1: inflammation consisting of ≥3 cells thick). These data are reported as percentage of inflamed airways/total number of airways per lung section.

Statistical Analyses

Power analysis was used to determine appropriate number of subjects for each experiment. All data were plotted as means ± SEM and analyzed using GraphPad Prism software (version 5.02). Two-way ANOVA and Bonferroni post-hoc tests were used to test for differences between the groups for the pulmonary function tests. One-way ANOVA and Bonferroni post-hoc tests were used to test for differences in BALF cellularity, BALF cytokine levels, antibody levels, lung inflammation, and T cell populations. Student's t test was used for surface expression of IL-4Rα. Differences were considered statistically significant if $p < 0.05$; *$p<0.05$ compared to vehicle controls (Saline or SHAM) and $^{\#}p<0.05$ compared to untreated but infected mice (SR orSRR).

Results

Surface expression of IL-4Rα is reduced on pulmonary myeloid dendritic cells and epithelial cells following IL-4Rα ASO inhalation

IL-4Rα antisense oligonucleotides (ASO), which have been previously shown to down-regulate IL-4Rα protein in an adult mouse model of allergic asthma (31), were used to reduce the expression of IL-4Rα on pulmonary immune and structural cells in the neonatal mouse lung. We administered 500 μg/kg of IL-4Rα ASO or MM i.n. to neonatal mice on days -5, -3, and -1. On protocol day 0 (i.e. seven days of age), lung cells were recovered after collagenase digestion of the tissue and analyzed by for surface expression of IL-4Rα. Down-regulation of IL-4Rα protein on the surface of specific lung cell populations was determined using flow cytometry (Figure 2A,B). The mean fluorescence intensity (MFI) of IL-4Rα was significantly reduced on myeloid dendritic cell (mDC) populations by 21 ± 1.6% and on E-cadherin positive epithelial cells by 12.5 ± 3.0% (mean ± SEM) compared to Saline (vehicle controls). The decreased MFI data for epithelial cells and mDCs paralleled the reduction in the percent of IL-4Rα expressing cells. No difference in IL-4Rα expression levels by MFI were observed on CD4+ or CD8+ T cells. There was a slight but significant reduction in the percentage of CD4+ cells expressing IL-4Rα compared to Saline as well as a decrease in the percentage of epithelial cells expressing IL-4Rα (Figure 2B). Similar to previous studies, administration of a controlled mismatch oligonucleotide (MM) had no

effect on IL-4Rα expression levels as assessed by MFI of IL-4Rα or by percent of IL-4Rα expressing cells. Since absorption of inhaled ASO into the systemic circulation is less than 1% of the deposited lung dose (31), IL-4Rα expression in other tissues was not assessed. These observations indicate that inhaled ASO effectively targets mDCs in the neonatal lung.

Downregulation of IL-4Rα during primary RSV infection increases Th1 cellular and antibody response

Since signaling through IL-4Rα is required for Th2 differentiation, down-regulation of IL-4Rα should significantly impair the development and differentiation of Th2 cells. To test this theory, we treated mice with IL-4Rα ASO, infected them with RSV, and measured T cell populations six days after infection (Figure 1). RSV infection elicited an increase in pulmonary Th2 (CD4+IL4+) cell populations compared to control mice (SR, 0.347 ± 0.033% vs. SHAM, 0.033 ± 0.003% or, Figure 3). Treatment with IL-4Rα ASO significantly reduced the percentage of Th2 cells in the lungs of RSV infected mice (AR; 0.127 ± 0.032%) compared to SR mice. Th1 (CD4+IFNγ+) cell populations were increased in RSV infected mice compared to controls (SR, 0.280 ± 0.025% vs. SHAM, 0.040 ± 0.006%), 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 towards a Th1 profile.

Tc2 (CD8+IL-4+) cells, another important source of IL-4, were increased in SR mice compared to controls (0.217 ± 0.058 vs. SHAM, 0.043 ± 0.003%) and were reduced in both MR and AR mice compared to controls (0.110 ± 0.012% and 0.087 ± 0.018%, respectively) though neither achieved statistical significance. Tc1 (CD8+IFNγ+) cells, which are important in viral clearance, were significantly increased in SR mice compared to controls (2.27 ± 0.113% vs. SHAM, 0.033 ± 0.003%) and appeared 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) from SR and MR mice. Similar trends were also observed when the total numbers of each of these T cell subsets were calculated (Supplemental Figure 1).

Since the isotype of antibodies produced in response to a pathogen is another indicator of the type of immune responses initiated (i.e, Th1 vs. Th2), we analyzed RSV-specific antibody 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, and IgE (Figure 4A). Treatment with IL-4Rα ASO during neonatal RSV infection, however, significantly boosted IgG2a levels compared to controls (AR:2.31 ± 0.58 fold greater than SR and AR:2.09 ± 0.54 greater than MR). By 12 dpi (Figure 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. Antibody isotypes produced in response to RSV infection were similar between SR and MR mice at both 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 (Fodha et al., 2007). To address the possible effects of ASO treatment on RSV replication in the lung, we assessed viral load using traditional $TCID_{50}$ 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) was similar to control mice infected with RSV but not receiving ASO (SR) or receiving MM (MR). The $TCID_{50}$ for RSV was 781 ± 219 in the lungs of AR mice and 765 ± 72 in the lungs of SR mice. The cycle threshold values for RSV-NP were 30.2 ± 0.662, 30.3 ± 0.213, and 30.8 ±

0.426 (SR, AR, and MR; 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; SR, AR, and MR) indicating that equivalent amounts of experimental sample were analyzed. No viral particles were detected in the lungs of any mice after 8 dpi during either primary or secondary infection suggesting that IL-4Rα ASO treatment does not hinder viral clearance during primary or secondary infection.

IL-4Rα 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 to unvaccinated children. To determine whether treatment with IL-4Rα ASO was capable of preventing enhanced respiratory disease following reinfection with RSV, we treated neonatal mice with IL-4Rα 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—Saline treated, RSV infected (SRR) mice showed significant increases in airway resistance compared to sham-infected (SHAM) mice at both the 500 and 100 μg/kg doses (data not shown and Figure 5A, respectively). IL-4Rα 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 mice was about 2.5-fold higher than in the SHAM or ARR mice at the 100 μg/kg dose of ASO. Lung compliance followed the same trend (Figure 5B). Compliance in the ARR mice was similar to that in the SHAM mice, while SRR mice showed substantially lower compliance at 50 mg/ml of MeCh (1.5-fold) compared to the SHAM or ARR group. Treatment with MM during initial RSV infection (MRR) failed to alter pulmonary resistance or compliance following secondary RSV infection as compared to infection alone (SRR).

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

Bronchoalveolar lavage fluid cellularity—The total number of leukocytes recovered in the BALF of all RSV infected mice was significantly elevated after reinfection at 5 wk (Figure 7). This increase appeared to be due primarily to increases in macrophage/monocyte and lymphocyte populations. IL-4Rα ASO treatment during primary infection significantly reduced the total number of leukocytes present in the BALF following reinfection with RSV (ARR) and these decreases were mainly due to reduction in macrophage and lymphocyte numbers. Although not statistically different, BALF eosinophil numbers were slightly reduced from mice receiving IL-4Rα ASO treatment compared to those not receiving treatment (ARR: 2.1×10^3 vs SRR:5.0×10^3, p=0.14).

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

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

BALF cytokine levels were also measured six days following secondary infection (Figure 9). RSV reinfection resulted in secretion of a variety of cytokines, including IL-4, -5, -12(p40), -13, and IFN-γ. IL-4Rα ASO treatment (ARR) during primary infection in neonatal mice led to a reduction of 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 mice. No differences were observed between ARR mice and non-treated mice (SRR) in 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 response to infection. Neonatal RSV infection results in the development of persistent pulmonary dysfunction in humans (4) and mice (16–19) due to 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. Our data presented here support our hypothesis that the administration of IL-4Rα 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 antibodies and cytokines). Administration of IL-4Rα ASO reduced multiple pathophysiological parameters associated with neonatal RSV infection including pulmonary inflammation, and mucus hyperproduction. Furthermore, it completely abolished the development of AHR following adult reinfection with RSV and altered both the initial and subsequent T cell responses to RSV infection demonstrating its potential as part of a vaccine strategy.

Administration of IL-4Rα ASO significantly downregulated IL-4Rα on neonatal pulmonary cells including mDCs suggesting that IL-4Rα mediates T cell rebalancing via modulation of pulmonary mDC development and function. Previously, Sriram (35) and colleagues demonstrated that IL-4 suppresses the maturation of DCs (i.e. expression of costimulatory molecules, antiviral genes, etc.) 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 beyond the scope of this study to explore this mechanism in greater detail, we have observed substantially reduced levels of type I IFN responses in neonatal RSV infection compared to responses in adult RSV infection (data not shown). Since 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-4/13 are helping to either initiate or maintain a Th2 immune response. More importantly, our data suggest that the use of IL-4Rα ASO helps to rebalance the Th cell compartment in response to neonatal RSV infection. In addition, administration of IL-4Rα ASO resulted in reduction of IL-4Rα expression on lung epithelial cells and may account in part for the reduced production of goblet cells and mucus (36).

Overall, our studies reveal a role for IL-4Rα in the pathogenesis of neonatal RSV infection, which agrees with that of previous studies demonstrating that IL-4 (25) and IL-13 (17,19,25) 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-4Rα (27) 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 both primary (data not shown) and secondary RSV infection was significantly reduced in IL-4Rα ASO treated mice compared to 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 (17,25). The mechanism(s) via which IL-4Rα ASO treatment during primary RSV infection decreases IL-13 concentrations during reinfection was not explored in this study, but may simply be due to 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-4Rα 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 lab 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-4 receptor 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 (Figure 3).

IL-4Rα ASO treatment changed the primary T cell responses to RSV in infected neonates. Analysis of both T cell populations and antibody isotypes showed that ASO treatment increased Th1 and decreased 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 non-infected controls. The importance of the fine balance between Th1 and Th2 responses to a viral infection is well recognized in many studies, and is eloquently demonstrated in a study in which recombinant RSV expressing IFN-γ (rRSV/IFN-γ) was used to infect mice with the hope of preventing pulmonary disease following RSV reinfection (39). 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 recombinant RSV expressing IL-4 or IFN-γ in neonates shows that neonatal mice infected with RSV expressing IFN-γ have increased maturation of immune cells responsible for the innate response(40). 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 promote the maturation of macrophages, thereby preventing neutrophil- and NK cell-mediated pathology upon rechallenge as an adult. Administration of IL-4Rα ASO, in contrast, 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 demonstrates that inhibiting IL-13 expression or function may promote Th17 inflammation (38). Although we observed decreased levels of IL-13 in the BALF of RSV infected pups treated with IL-4Rα ASO, IL-17 levels were not statistically different from saline treated, SHAM infected controls (data not shown). This suggests, that as least in our neonatal model of RSV infection, the use of IL-4Rα ASO does not have the unintended consequence of up-regulating Th17 cytokine production.

Despite almost half-a-century of intense research, there is still 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-4Rα during primary RSV infection completely protects against the pulmonary dysfunction usually observed following RSV reinfection. This protection was achieved by inducing a strong enough immune response with a wild-type virus instead of an attenuated one and by delicately balancing Th1 and Th2 responses with IL-4Rα ASO treatment during infection. We believe that our IL-4Rα ASO treatment offers a good opportunity for future vaccine development strategies and suggests its potential use as a therapeutic for pediatric RSV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used in this manuscirpt

RSV	respiratory syncytial virus
DC	dendritic cell
IL-4Rα	IL-4 receptor alpha
ASO	antisense oligonucleotides
MM	mismatch oligonucleotides
BALF	bronchoalveolar lavage fluid
AHR	airway hyperresponsiveness

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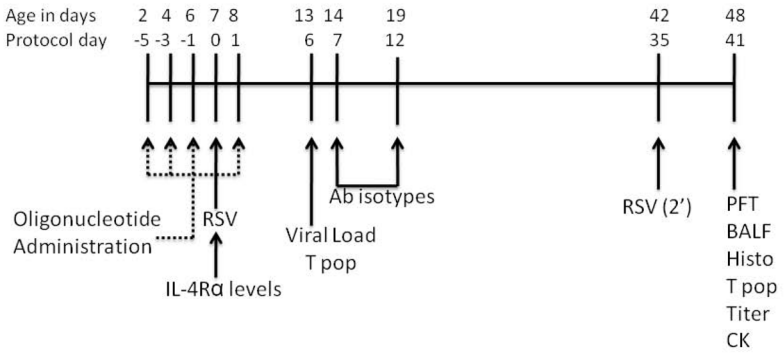

Group	Neonate (7d old)	Adult (42d old)
AR/ARR	IL-4Rα ASO + RSV	RSV
MR/MRR	MM + RSV	RSV
SR/SRR	Saline + RSV	RSV
SHAM	Saline + DMEM	DMEM

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 endpoints were measured as indicated. IL-4Rα levels: IL-4Rα levels on pulmonary cells; Viral load: RSV copy number in the lung; T pop: T cell populations in the lung; PFT: pulmonary function testing; BALF: cell differential counts in bronchoalveolar lavage fluid; Histo: lung histology; Ab isotypes: RSV-specific antibody levels in serum; and CK: BALF cytokines.

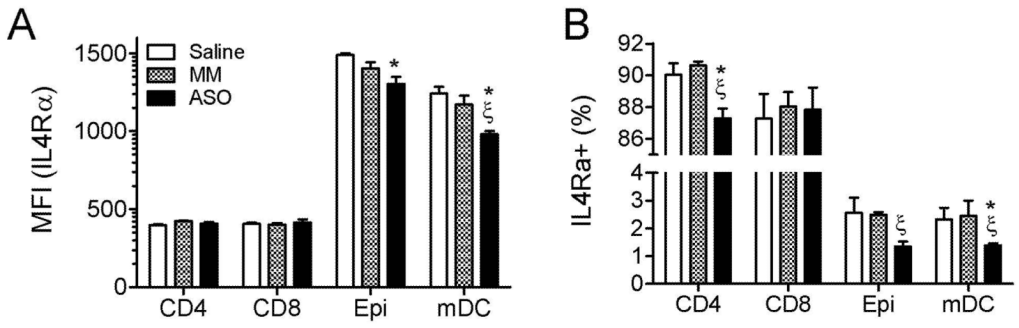

Figure 2. Cell surface expression of the IL-4 receptor α and percentage of IL-4 receptor α positive cells after inhalation of ASO in neonatal mice

Three groups were included in this study: ASO, MM, and Saline. ASO mice were treated with 500 μg/kg IL-4Rα ASO on protocol days -5, -3, and -1. MM mice were treated with 500 μg/kg mismatch oligonucleotide. Saline mice were treated with saline. On protocol day 0 (age 7d), lung cells were isolated, labeled with differentiation markers, and analyzed by flow cytometry. **A.** Mean fluorescent intensity of IL-4Rα on pulmonary cell subsets. **B.** Percent IL-4Rα+ cells of each cellular subset. CD4 indicates CD4+ T cells (CD3+, CD4+. IL-4Rα+); CD8, CD8+ T cells (CD3+, CD8+, IL-4Rα+); Epi, epithelial cells (E cadherin+, IL-4Rα+); mDC, myeloid dendritic cells ($CD11b^{hi}$, CD11c+, IL-4Rα+). Data are representative of three independent experiments and are expressed as means ± SEM. n=4/group. *$p<0.05$ compared to saline. $^{\xi}p<0.05$ compared to MM.

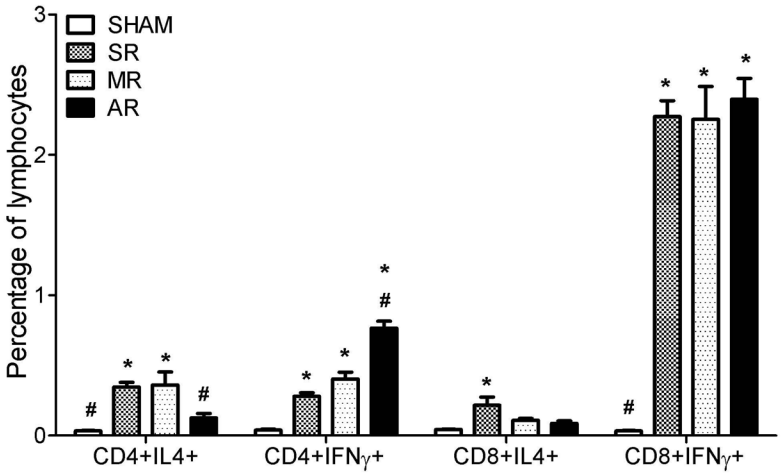

Figure 3. T cell subpopulations after primary infection with RSV

Four groups were included in this study: AR, SR, MR, and SHAM. AR mice were 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. SR mice were treated with saline and infected with RSV. Control (SHAM) mice were treated with saline and sham infected with vehicle. Six days after infection, lung cells were isolated and stained with differentiation markers and analyzed by flow cytometry. Th1 cells (CD4+IFNγ+), Th2 cells (CD4+IL4+), Tc1 cells (CD8+IFNγ+) 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 to SHAM, $^{\#}p<0.05$ compared to SR.

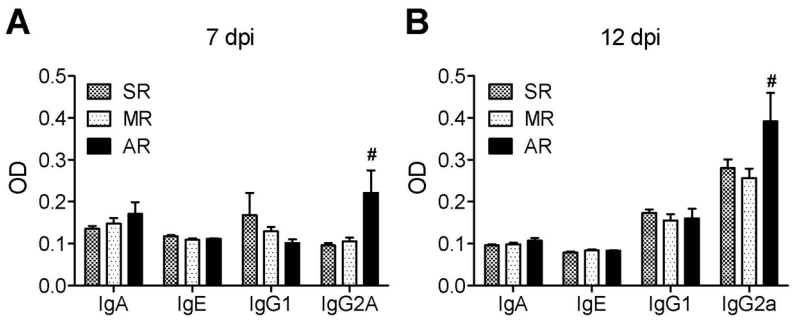

Figure 4. Serum antibody isotypes after primary RSV infection at 7dpi (A) and 12dpi (B)
RSV-specific antibodies in serum were determined by indirect ELISA. Absorbance values (OD) are plotted. Data are representative of two independent experiments. Dose of ASO or MM was 500 μg/kg. n=4–6/group. $^{\#}p<0.05$ compared to SR.

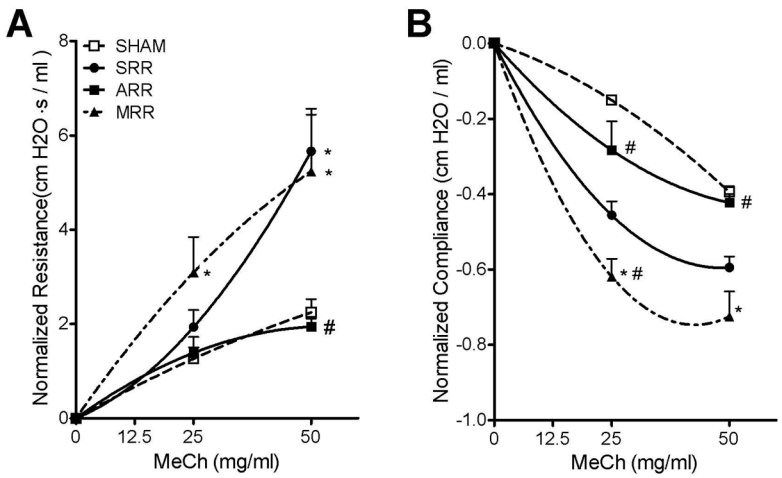

Figure 5. Pulmonary function after secondary RSV infection

Mice were treated with ASO, MM, or saline; then infected with RSV or sham infected as neonates; and reinfected with RSV at 6 weeks of age (ARR, MRR, SRR, and SHAM, respectively). Six days later, pulmonary function was measured in all three groups. Lung resistance was measured in response to increasing doses of methacholine (MeCh). Resistance values were normalized to individual baseline resistance at 0 mg/ml methacholine. Dose of ASO or MM was 100 μg/kg to demonstrate efficacy even at lower dose. Data are representative of three independent experiments and are expressed as means ± SEM. n=6/group. *$p<0.05$ compared to SHAM, $^{\#}p<0.05$ compared to SRR.

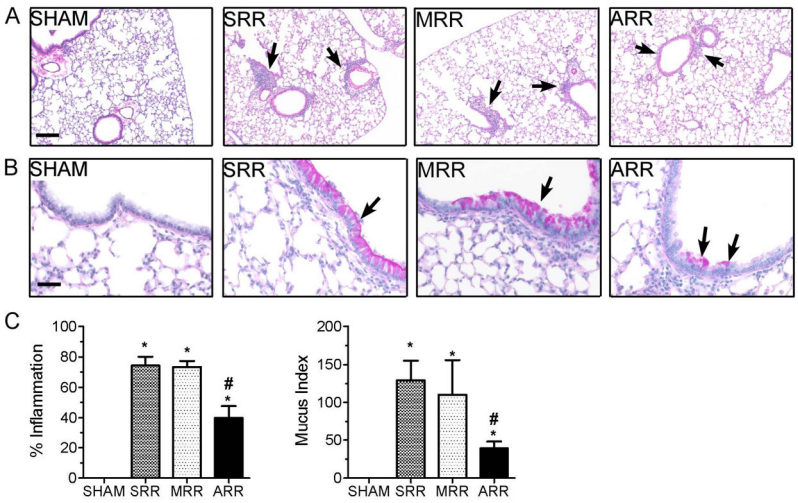

Figure 6. Histology after secondary RSV infection

Lung tissue was obtained at six days after secondary infection. **A.** H&E staining shows inflammatory cells in the lung (arrows). Scale bar = 50μm. **B.** Staining with PAS shows mucus production in airway epithelial cells. Scale bar = 200μm. **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) × number of airways counted per lung). Dose of ASO or MM was 100 μg/kg to demonstrate efficacy even at lower dose. Micrographs are representative of three independent experiments and are expressed as means ± SEM. n=4 mice/group. *$p<0.05$ compared to SHAM, $^{\#}p<0.05$ compared to SRR.

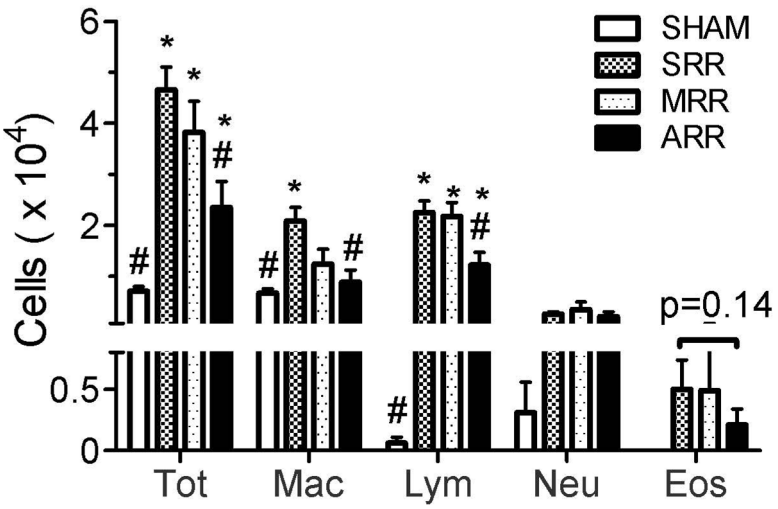

Figure 7. BALF cellularity after secondary RSV infection

Mice were treated with ASO, MM, or saline; infected with RSV or sham infected as neonates; and reinfected at 6 weeks of age (ARR, MRR, SRR, and SHAM, respectively). Six days later, bronchoalveolar lavage fluid was taken and white blood cell differentials were counted. Mac indicates macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils. Dose of ASO or MM was 500 μg/kg. Data are representative of three independent experiments are expressed as means ± SEM. n=6–10/group. Two independent observers counted 200–300 cells per mouse. *$p<0.05$ compared to SHAM, $^{\#}p<0.05$ compared to SRR.

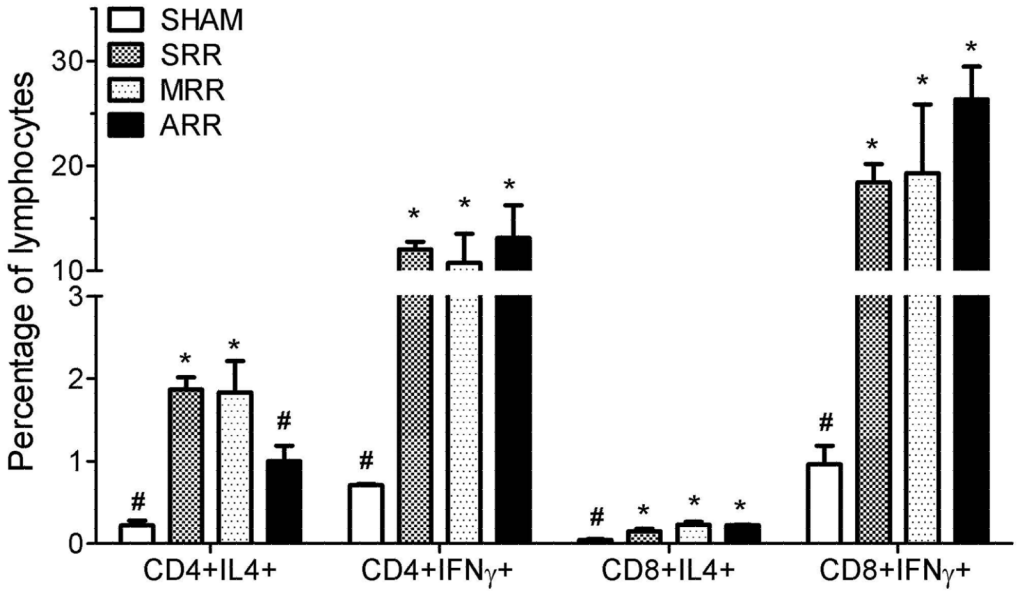

Figure 8. T cell populations after secondary RSV infection

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+IFNγ+), Th2 cells (CD4+IL4+), Tc1 cells (CD8+IFNγ+) and Tc2 cells (CD8+IL4+) in the lung were measured. Dose of ASO or MM was 500 μg/kg. Data are representative of three independent experiments are expressed as means ± SEM. n=4/group. *$p<0.05$ compared to SHAM, $^{\#}p<0.05$ compared to SRR.

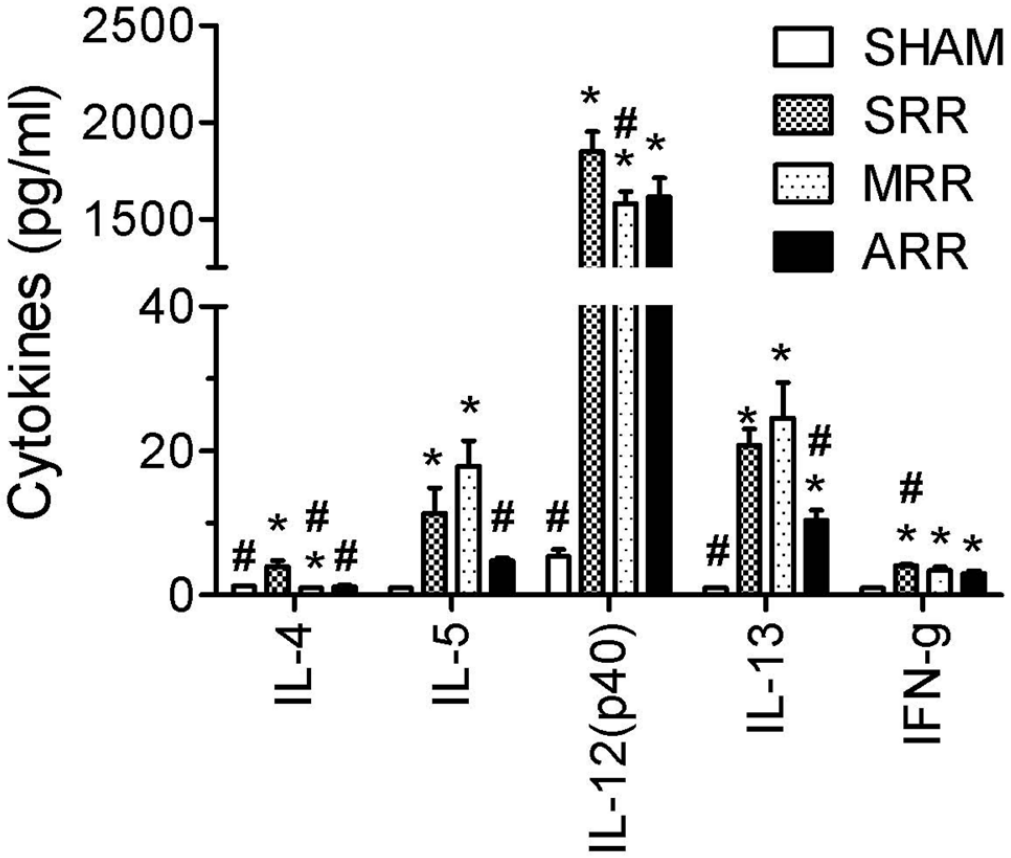

Figure 9. BALF cytokine levels after secondary RSV infection

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 (A) 100 μg/kg or (B) 500 μg/kg. Data are representative of three independent experiments and are expressed as means ± SEM. n=4/group. *$p<0.05$ compared to SHAM, $^{\#}p<0.05$ compared to SRR.