# Montelukast during Primary Infection Prevents Airway Hyperresponsiveness and Inflammation after Reinfection with Respiratory Syncytial Virus

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*Rationale*: **Respiratory syncytial virus (RSV) bronchiolitis in infants may be followed by the development of asthma-like symptoms. Age at first infection dictates consequences upon reinfection. Reinfection of mice initially exposed as neonates to RSV enhanced development of airway hyperresponsiveness (AHR), eosinophilic inflammation, and mucus hyperproduction. RSV lower respiratory tract disease is associated with activation of the leukotriene pathway.**

*Objectives*: **To determine the effects of montelukast (MK), a cysteinyl leukotriene (cysLT) receptor antagonist, in primary and secondary RSV-infected newborn and adult mice.**

*Methods*: **BALB/c mice were infected with RSV at 1 week (neonate) or 6 to 8 weeks (adult) of age and reinfected 5 weeks later. MK was administered 1 day before the initial infection and through Day 6 after infection. Seven days after primary or secondary infection, airway function was assessed by lung resistance to increasing doses of inhaled methacholine; lung inflammation, goblet cell metaplasia, and cytokine levels in bronchoalveolar lavage fluid were monitored.**

*Measurements and Main Results*: **RSV infection induced cysLT release in bronchoalveolar lavage fluid. MK decreased RSV-induced AHR, airway inflammation, and increased IFN-γ production in primary infected adult and neonatal mice. MK, administered during initial infection of neonates but not during secondary infection, prevented subsequent enhancement of AHR, airway eosinophilia, and mucus hyperproduction upon reinfection.**

*Conclusions*: **MK attenuated the initial responses to primary RSV infection in both age groups and altered the consequences of RSV reinfection in mice initially infected as neonates. These data support an important role for cysLT in RSV-induced AHR and inflammation.**

**Keywords:** airway; inflammation; RSV; cysteinyl leukotrienes

Respiratory syncytial virus (RSV) is the leading cause of viral lower respiratory tract infections during infancy and early childhood worldwide. Approximately 65% of children are infected with RSV within the first year of life, and almost all children are infected at least once by 2 years of age (1, 2). Repeated infection is common at all ages; however, therapeutic options are limited and rather ineffective. The development of new therapeutic or prophylactic agents remains a major challenge and a clinical priority (3).

Several studies have indicated an association between RSV infection in early life and the subsequent development of persistent wheezing and asthma in childhood (4–7). However, the underlying mechanisms are not fully defined. In an earlier study, we demonstrated that the age at primary RSV infection dictated the subsequent consequences upon reinfection (8). Initial infection of mice at weaning elicited a protective airway response upon reinfection characterized by an increased airway inflammatory response but without the development of airway hyperresponsiveness (AHR) or eosinophilia and decreased IL-13 levels. In contrast, neonatal infection failed to protect the airways and resulted in enhanced AHR after reinfection. This secondary response was associated with the development of airway eosinophilia, increased IL-13 levels, and mucus hyperproduction. IFN-γ (9), RSV-specific IgE (10), and T cells (11) have been implicated in this altered response of neonates to RSV reinfection.

Cysteinyl leukotrienes (cysLT) are lipid mediators derived from arachidonic acid. CysLT exert their functions through two receptors: cysLT1R and cysLT2R. CysLT1R is the most studied and is the target of the drug montelukast (MK), which is a potent and specific cysLT1R antagonist (12–14). The role of cysLT in the pathogenesis of airway obstruction and inflammation has been recognized and targeted in the treatment of asthma (15, 16). CysLT have multiple effects that contribute to airway obstruction and inflammation in asthma: bronchoconstriction, mucus production, recruitment of inflammatory cells to the airway, and increased vascular permeability. These features render cysLT as possible mediators in RSV-induced AHR and inflammation, especially in reinfected mice that were initially infected as neonates.

CysLT levels in children with RSV-induced bronchiolitis were significantly higher than in control subjects (17–19). Similarly, significantly elevated levels of LTC4 were seen in the nasopharyngeal secretions of infants with RSV-induced bronchiolitis compared with secretions from children with RSV upper respiratory infection alone (20, 21). There was a significant correlation of eosinophil activation and cysLT production in infants with RSV bronchiolitis (22). Based on these observations, we hypothesized that cysLT may play a critical

### AT A GLANCE COMMENTARY

#### Scientific Knowledge on the Subject

Although cysteinyl leukotriene levels are elevated in response to respiratory syncytial virus infection, their role in eliciting altered airway function and inflammation is not well defined.

#### What This Study Adds to the Field

This study demonstrates the important role of cysteinyl leukotrienes during primary infection of neonates in dictating the airway response to reinfection with respiratory syncytial virus.

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role during primary RSV infection, particularly in determining the airway response to subsequent RSV reinfection. To test this hypothesis, BALB/c mice were infected with RSV at 1 week (neonate) or at 6 to 8 weeks (adult) of age and reinfected 5 weeks later. The results demonstrated that MK administered during initial infection of neonates prevented the subsequent enhancement of AHR and the development of airway eosinophilia and mucus hyperproduction upon reinfection. Some of the results of these studies have been previously reported in abstract form (23).

## METHODS

### Animals

BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were maintained under pathogen-free conditions at the Biological Resource Center, National Jewish Health. Mice were used under an experimental protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

### Virus Preparation

Stocks of purified human RSV (strain A2) were produced as previously described (8). Briefly, RSV A2 Strain (VR-1302) and HEp-2 cells (CCL-23) were obtained from ATCC (Rockville, MD). The virus was propagated in HEp-2 cells. At maximum cytopathic effect, cells were harvested and disrupted by sonication. The suspension was clarified by centrifugation at 10,000 × *g* for 15 minutes at 10°C, and the resulting supernatant was layered on top of a sucrose gradient (30% in 50 mM Tris-buffered saline solution containing 1 mM EDTA [pH 7.5]) and centrifuged at 100,000 × *g* for 2 hours at 4°C. The pellet containing purified virus was resuspended in 10 mM phosphate-buffered saline (PBS) (pH 7.4) containing 15% sucrose and stored in aliquots at −70°C. Viral titers were determined by a standard plaque assay combined with immunostaining for RSV using a biotinylated goat anti-human RSV Ab (Accurate Chemical and Scientific, Westbury, NY) and an avidin-biotin peroxidase detection system (Dako, Glostrup Denmark). Ultraviolet (UV)-inactivated RSV was generated by exposing the virus to 302 nm of ultraviolet radiation (Chromato-Vue Lamp UVM-57; UVP, Uplands, CA) for 16 hours at 4°C. Plaque assay confirmed the absence of viable virus.

### RSV Infection and Treatments

Mice were lightly anesthetized with inhaled isofluorane before intranasal inoculation with $10^6$ plaque-forming units of purified RSV (in endotoxin-free PBS) at the indicated age. MK (30 mg/kg) was orally administered daily from 1 day before primary or secondary RSV infection through Day 6 after infection. Age-matched control mice were inoculated with PBS. Secondary RSV infection was performed 5 weeks after primary infection. Airway function and inflammation were assessed on Day 7 after primary or secondary RSV infection.

### Assessment of Airway Function

Airway function was assessed in anesthetized, tracheostomized, mechanically ventilated animals by measuring changes in lung resistance ($R_L$) in response to increasing doses of inhaled methacholine (MCh) (Sigma-Aldrich, St. Louis, MO) as described (24). Ventilation was achieved at 160 breaths per minute at a tidal volume of 0.16 ml with a positive end-expiratory pressure of 2 to 4 cm $H_2O$ with a ventilator (SN-480-7; Shinano Seisakusho, Tokyo, Japan). $R_L$ was continuously computed (Labview; National Instruments, Austin, TX) by fitting flow, volume, and pressure to an equation of motion using a recessive least-squares algorithm. Aerosolized MCh was administered through bypass tubing via an ultrasonic nebulizer (model 5500D; DeVilbiss, Glendale Heights, IL) placed between the expiratory port of the ventilator and the four-way connector. Aerosolized MCh was administered for 8 seconds with a tidal volume of 0.45 ml and frequency of 60 breaths per minute using a second ventilator. The data of RL were continuously collected for up to 3 minutes, and maximum values were taken. Data are expressed as the percent change from baseline $R_L$ obtained after inhalation of saline.

### Airway Inflammation and Lung Histopathology

Immediately after measurement of AHR, lungs were lavaged through the trachea with 1 ml of Hanks' balanced salt solution in adult mice or twice with 0.5 ml of Hanks' balanced salt solution in the youngest mice. Airway inflammation was assessed by total and differential counting of cells recovered in bronchoalveolar lavage fluid (BALF). After the BALF was obtained, the lungs were fixed in 10% formalin and embedded in paraffin. Lung tissue sections (5 μm thickness) were cut from the paraffin blocks and stained with hematoxylin and eosin. Mucus-producing goblet cells were detected by staining of tissue sections using periodic acid-Schiff (PAS). For quantitative analyses, the data were normalized to the perimeter of the basement membrane (BM) of the airway epithelium as previously described (8). All measurements (the groups were blinded to the observer) were performed on at least three serial tissue sections cut from the paraffin blocks every 50 μm. The measured values were averaged for each animal, and the mean values were determined for each group. The data are expressed as the mean ± SEM of $PAS^+$ cells per millimeter of BM.

### Lung Viral Titers

In separate experiments, the amounts of replicating virus were examined after inoculation from the lungs of different groups at different time points. The lungs were homogenized and used for determination of lung titers by culture plaque assay combined with confirmatory immunostaining of syncytia for RSV, as described previously.

### Measurement of Cytokine Levels

Levels of IFN-γ, IL-4, IL-5, and IL-6 were measured in BALF using commercial ELISA kits according to the manufacturer's instructions (eBioscience, San Diego, CA), as was IL-13 (R&D Systems, Minneapolis, MN).

### Measurement of cysLT Levels

cysLT concentrations in BALF were measured by ELISA according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

### *In Vitro* Cytokine Production by Peribronchial Lymph Node Cells after Restimulation with RSV

Seven days after secondary RSV infection, the peribronchial lymph nodes (PBLNs) were isolated from each mouse and minced using tissue forceps and scissors. The cell suspensions were filtered through a 70-μm cell strainer, centrifuged at 300 × *g* for 5 minutes at room temperature, and resuspended in RPMI 1640 medium containing 10% fetal calf serum. Cells ($2 \times 10^5$) were cultured in 96-well plates in the presence of $10^6$ plaque-forming units of UV-inactivated RSV for 96 hours. The concentrations of IL-4, IL-5, IL-6, IL-13, and IFN-γ in the supernatants were measured by ELISA.

### Statistical Analysis

All results were expressed as mean ± SEM. Data were analyzed by analysis of variance using the StatView 4.5 statistical analysis software package (Abacus Concepts, Piscataway, NJ). Student's *t* tests and one-way analysis of variance were used to determine the level of differences, where appropriate. Nonparametric analysis using the Mann-Whitney U test was used to confirm that the statistical differences remained significant even if the underlying distribution was uncertain. The *P* values for significance were set to 0.05 for all tests.

## RESULTS

### RSV Infection Increases cysLT Levels in BALF

We initially determined the temporal relationship of cysLT release and AHR after RSV infection. Mice (6–8 wk of age) were inoculated with RSV on Day 0, and airway responsiveness to inhaled MCh was measured on Days 1, 3, 5, 7, 10, and 14. On each day, BAL was obtained for cysLT determinations. Airway responsiveness to inhaled MCh increased beginning on Day 5 and reached a maximum on Day 7 before declining on Days 10

and 14 (*see* Figure E1A in the online supplement). Figure E1B shows the levels of cysLT in the BALF. The concentrations of cysLT were elevated above baseline 3 days after infection and peaked 5 to 7 days after infection. Significantly greater quantities of cysLT were noted from Day 3 to Day 10 compared with Day 0. Thus, the time pattern of RSV-induced cysLT release paralleled and likely preceded RSV-induced AHR.

### Effect of MK Treatment on Primary RSV Infection in Adult Mice

The association of cysLT release and RSV-induced AHR suggested that cysLT may play a role in the development of airway dysfunction after RSV infection. To examine the effect of MK in primary RSV infection, mice were infected at 6 to 8 weeks of age; MK was administered orally daily from 1 day before RSV infection through Day 6 after infection. Airway function and inflammation were assessed on Day 7 after infection. We first determined the dose-dependent response to MK. Based on previous studies (25–30), MK was given at 1, 5, 10, 30, and 50 mg/kg. In the group treated with 1 mg/kg of MK, there were no significant differences in AHR when compared with nontreated RSV control mice (Figure E2A). In the groups treated with 5 and 10 mg/kg of MK, a decrease in AHR was observed. However, mice treated with 30 and 50 mg/kg developed significantly decreased AHR when compared with positive control mice. Based on these results, a dose of 30 mg/kg was chosen for the study (Figure 1A).

After primary RSV infection of these mice, the number of total cells, lymphocytes, and neutrophils recovered in the BALF were significantly increased compared with the age-matched negative control group. Treatment with MK significantly reduced the number of lymphocytes in the BALF (Figure 1B). There were no significant differences in the numbers of macrophages or neutrophils. Few eosinophils were detected in primary RSV-infected adult mice in the presence or absence of MK treatment, as previously shown (8). The MK-treated group had significantly higher levels of IFN-γ in BALF (Figure 1C), but levels of IL-4, IL-5, IL-6, and IL-13 were not different in the MK-treated and control RSV groups (below the level of detection; data not shown).

Treatment with MK did not alter RSV-induced levels of cysLT in the BALF of the untreated group (38.3 ± 3.0 pg/ml) compared with the MK-treated group (40.5 ± 2.5 pg/ml).

Virus replication and clearance were examined in both groups by measuring the amounts of replicating virus recovered from lung at different time points after inoculation. RSV titers peaked at Day 4 postinfection (Figure 1D). No significant differences were detected in the MK-treated and control groups.

### Effect of MK on Primary RSV Infection in Neonatal Mice

We determined whether MK had similar effects on RSV-infected neonatal mice. Mice were infected shortly after birth (<1 wk of age), and MK (30 mg/kg) was administered by gavage daily from 1 day before RSV infection through Day 6 after infection. Airway function and inflammation were assessed on Day 7 after infection. As previously shown (8), RSV infection of neonates resulted in the development of AHR to inhaled MCh as demonstrated in adult mice, although baseline resistance was significantly higher in the younger group (2.104 ± 0.138 cm $H_2O$/ml · $s^{-1}$ for neonates and 0.903 ± 0.101 cm $H_2O$/ml · $s^{-1}$ for adult mice). Treatment with MK significantly reduced AHR without altering baseline resistance (1.92 ± 0.042 cm $H_2O$/ml · $s^{-1}$ for neonates and 0.831 ± 0.075 cm $H_2O$/ml · $s^{-1}$ for adult mice) (Figure 2A).

The number of lymphocytes recovered in BALF was significantly increased compared with age-matched, noninfected mice. However, the extent of the lymphocytosis was lower in the neonatal mice than in adult mice. Treatment with MK

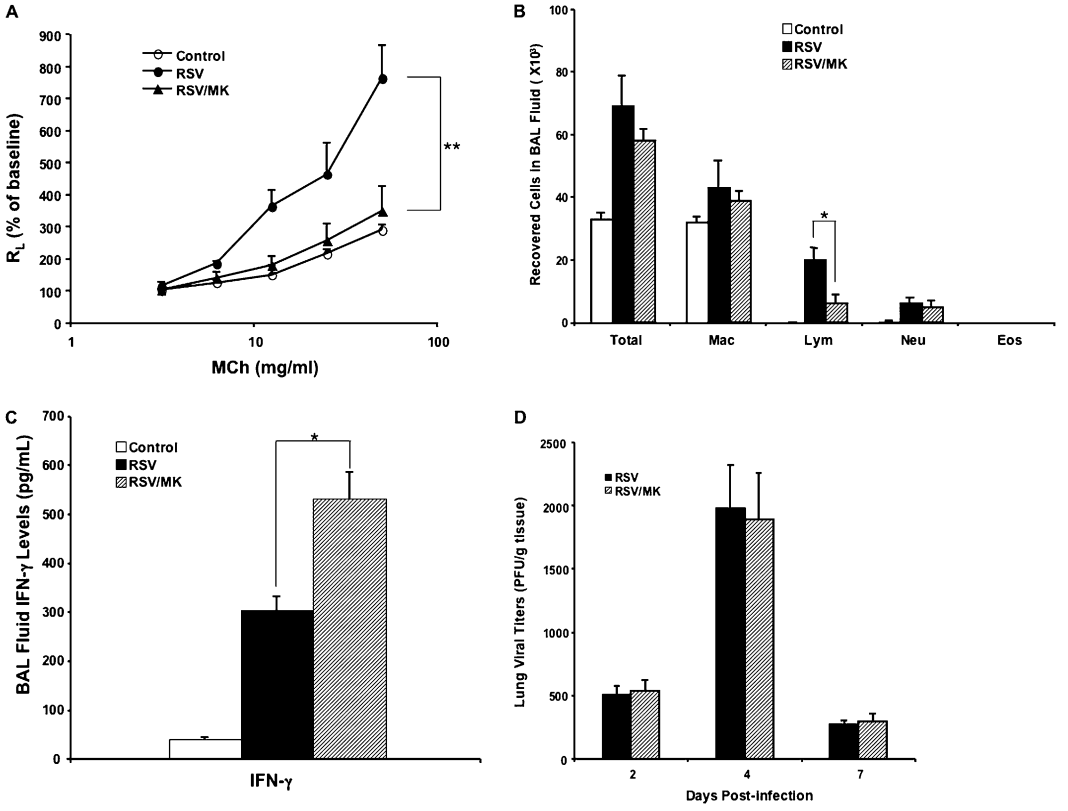


*Figure 1.* Effect of montelukast (MK) treatment on airway responsiveness to primary respiratory syncytial virus (RSV)-infected adult mice. BALB/c mice (6–8 wk old) were inoculated with RSV (n = 6 per group). MK at 30 mg/kg was administered 1 day before RSV infection and through 6 days after infection. Airway responsiveness to (*A*) inhaled methacholine, (*B*) bronchoalveolar lavage cellularity, and (*C*) bronchoalveolar lavage fluid IFN-γ levels were assessed on Day 7 after infection. (*D*) Lung viral titers were examined on Days 2, 4, and 7 after RSV infection. The data are expressed as mean ± SEM. **P* < 0.05; ***P* < 0.01 compared with control or RSV-MK to RSV. Eos = eosinophil; Lym = lymphocyte; Mac = macrophage; MCh = methacholine; Neu = neutrophil; PFU = plaque-forming units; $R_L$ = lung resistance.

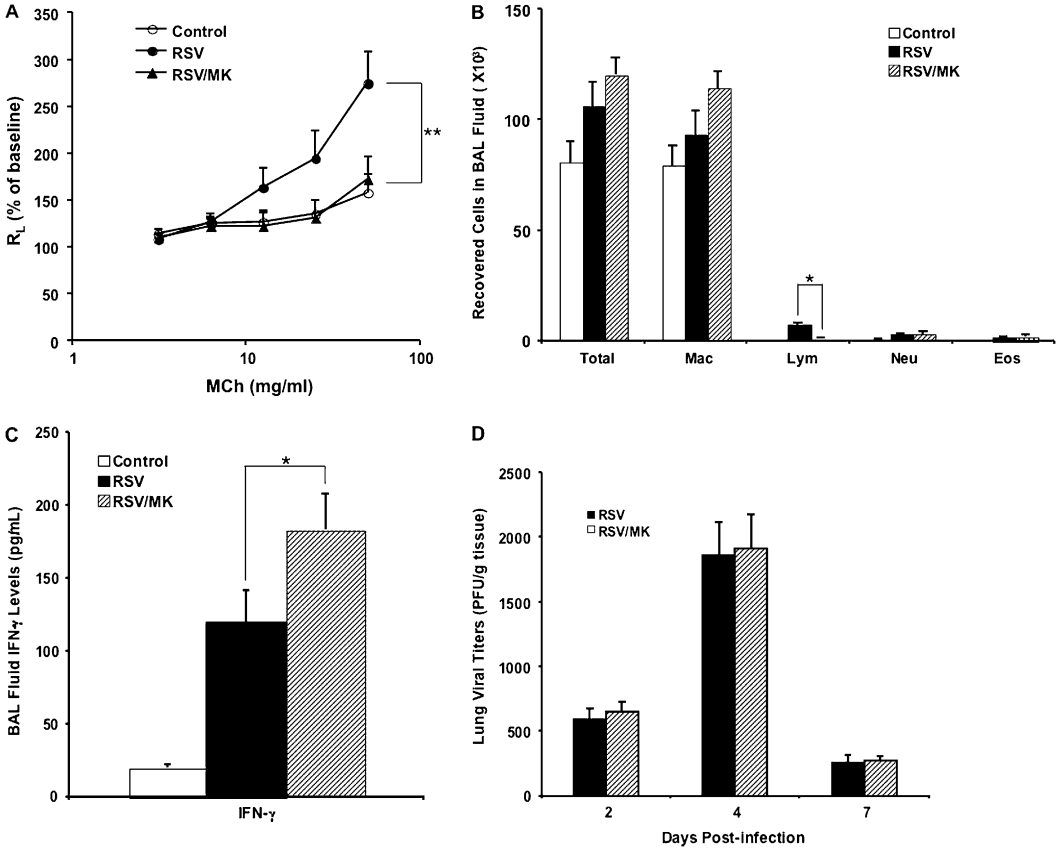


*Figure 2.* Effect of montelukast (MK) treatment on airway responsiveness and inflammation in primary respiratory syncytial virus (RSV)-infected neonatal mice. BALB/c mice were inoculated as newborns (<1 wk of age) with RSV (n = 6 per group). MK (30 mg/kg) was administered 1 day before RSV infection and through 6 days after infection. Airway responsiveness (*A*) to inhaled methacholine, (*B*) bronchoalveolar lavage cellularity, and (*C*) bronchoalveolar lavage fluid (BAL) IFN-γ levels were assessed on Day 7 after infection. (*D*) Lung viral titers were examined on Days 2, 4, and 7 after RSV infection. The data are expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$ compared with control or RSV-MK to RSV. MCh = methacholine; PFU = plaque-forming units; $R_L$ = lung resistance.

significantly reduced the number of lymphocytes in the BALF of neonatal mice (Figure 2B).

Compared with adult mice, neonatal mice showed lower levels of IFN-γ in BALF after RSV infection, and MK treatment significantly increased the amount of IFN-γ (Figure 2C). The levels of IL-4, IL-5, IL-6, and IL-13 were not different in the MK-treated control RSV groups (data not shown).

The rates of virus replication and clearance in neonatal mice were similar to those in adult mice. There were no significant differences in the MK-treated and control groups (Figure 2D).

### Effect of MK during Primary Infection on Secondary RSV Infection in Neonatal Mice

To determine whether MK administered during primary infection at an early age would affect the consequences of reinfection, mice were infected with RSV shortly after birth (<1 wk of age) and then reinfected 5 weeks later, a time when no residual AHR or airway inflammation could be detected after recovery from the primary RSV infection. Airway function and inflammation were assessed on Day 7 after secondary infection. Mice initially infected as neonates developed enhanced AHR upon reinfection with RSV (Figure 3A). Administration of MK during primary neonatal infection prevented the enhancement of AHR on reinfection, although these mice developed levels of AHR similar to mice initially infected at 6 weeks.

Total cell numbers recovered in the BALF were significantly increased after secondary RSV infection compared with the numbers recovered after primary infection. Secondary infection induced significant airway eosinophilia in the BALF. Administration of MK significantly reduced the number of total cells and eosinophils in the BALF, and there were no differences in the numbers of macrophages, lymphocytes, or neutrophils in the treated and untreated groups (Figure 3B).

RSV reinfection induced a marked increase in the numbers of PAS+ cells, and administration of MK during primary infection reduced this goblet cell metaplasia (from 130.5 ± 17.9 to 34.9 ± 5.8 PAS+ cells/mm BM; $P < 0.01$) (Figure 3C). In the BALF, IFN-γ and Th2 (IL-5 and IL-13) cytokine levels were increased after RSV reinfection compared with levels after primary infection. Treatment with MK during primary infection decreased IL-5 and IL-13 levels; IFN-γ levels were higher but did not reach statistical significance (Figure 3D).

To further assess the T-cell cytokine response after RSV reinfection, PBLN cells were isolated 7 days after RSV reinfection, stimulated with UV-inactivated RSV *in vitro*, and concentrations of IL-4, IL-5, IL-6, IL-13, and IFN-γ in the supernatants were measured. Lower levels of IL-5 and IL-13 were detected when PBLNs were obtained from MK-treated mice (Figure 3E). There were no differences in IL-4, IL-6, or IFN-γ levels in these two groups.

cysLT levels in the BALF were measured 7 days after secondary infection. Secondary infection induced much higher levels of cysLT when compared with levels after primary RSV infection (Figure 3F). The levels of cysLT were decreased in MK-treated mice in concert with the reduced number of total cells in the BALF (Figure 3F).

### Effect of MK during Secondary Infection on Secondary RSV Infection

To determine if MK affected the response during reinfection, mice received primary RSV infection as neonates and were reinfected 5 weeks later. MK was administered just before and after reinfection (Figure 4A). Administration of MK in this way did not affect the development of enhanced AHR (Figure 4B), the increases in total BAL cell counts, or lymphocyte and eosinophil numbers (Figure 4C). Similarly, MK did not affect

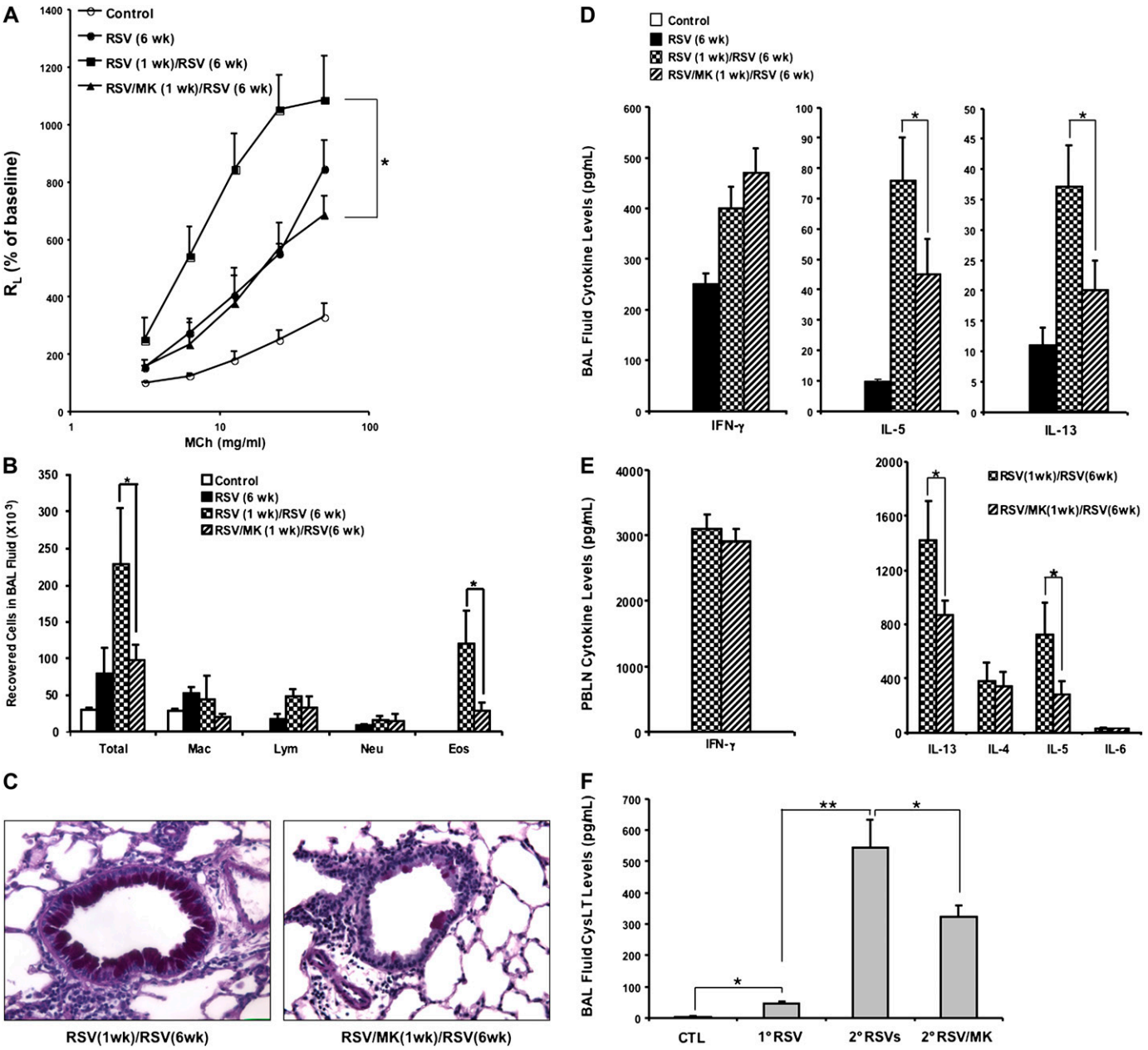


***Figure 3.*** Effect of montelukast (MK) treatment on airway responsiveness and inflammation on reinfection of newborn mice. Newborn BALB/c mice were treated with MK administered during primary neonatal respiratory syncytial virus (RSV) infection. Airway responsiveness to (*A*) inhaled methacholine, (*B*) bronchoalveolar lavage (BAL) cellularity, (*C*) lung histopathology, (*D*) BAL fluid cytokine levels, (*E*) *in vitro* cytokine production by peribronchial lymph node mononuclear cells after RSV stimulation, and (*F*) BAL fluid cysLT levels on Day 7 after secondary RSV infection. The data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$. MCh = methacholine; PBLN = peribronchial lymph node; $R_L$ = lung resistance; 1°RSV = primary RSV infection; 2°RSV = secondary RSV infection.

BAL cysLT levels (Figure 4D). Thus, unlike the effects of MK on reinfection responses when administered during primary infection, MK was without effect on the reinfection responses when administered only during secondary infection.

## DISCUSSION

In the current study, the effects of a cysLT1R antagonist, MK, on RSV-induced AHR and airway inflammation were investigated, focusing on the development of altered airway function after reinfection with RSV. The results demonstrated that RSV infection induced increased levels of cysLT release in the airway. The time pattern of cysLT release paralleled and even preceded RSV-induced AHR. MK treatment, which did not affect the levels of cysLT in BALF, decreased RSV-induced AHR and airway inflammation in primary infected adult and neonatal mice. MK administered during initial infection but not during secondary infection of neonates prevented the subsequent enhancement of AHR and the development of airway eosinophilia and mucus hyperproduction upon reinfection.

Age at primary infection is critical to the outcome of secondary RSV infection. Neonatal RSV infection was previously shown to predispose mice to develop more severe airway disease on reinfection (8, 31). This amplified and altered response

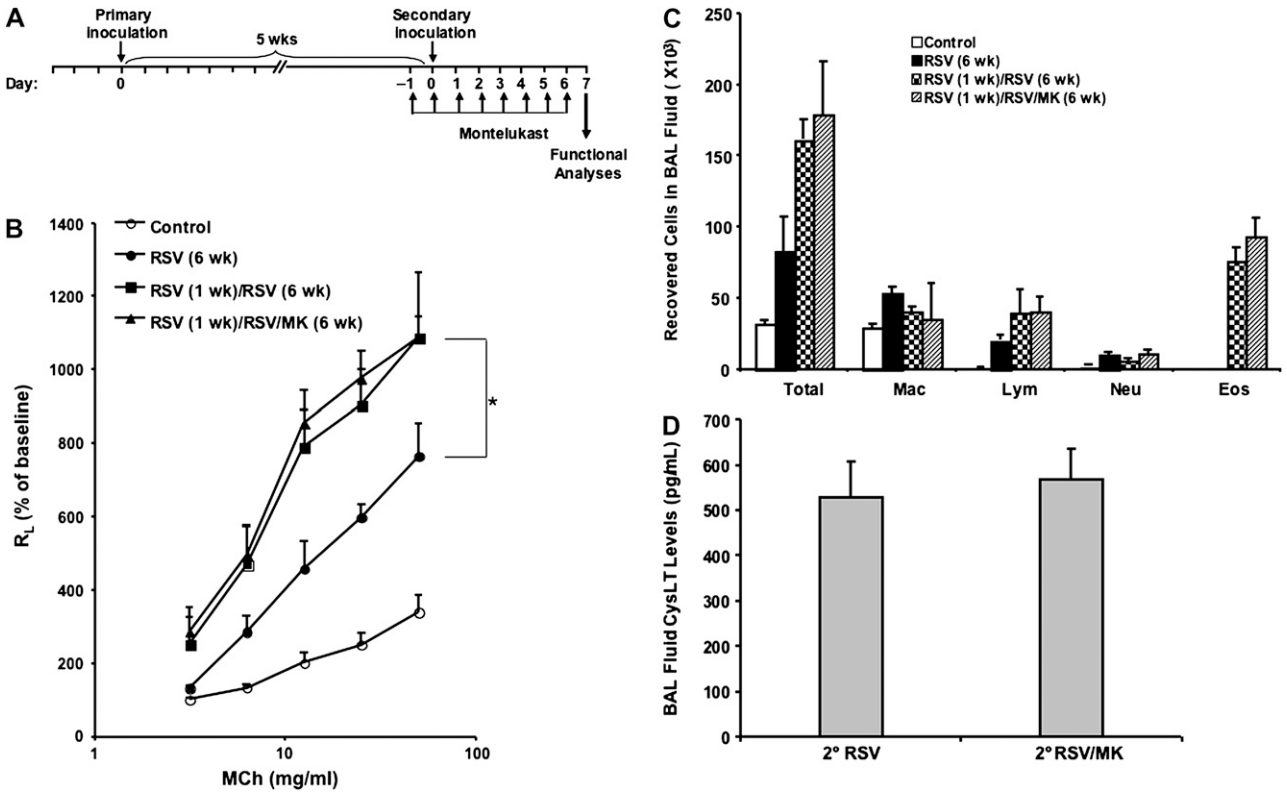


*Figure 4.* Effect of montelukast (MK) treatment during secondary infection on airway responsiveness and inflammation after reinfection of newborn mice. Newborn BALB/c mice were infected with respiratory syncytial virus (RSV) and reinfected with RSV 5 weeks later. MK was administered during secondary RSV infection. (*A*) Protocol. (*B*) Airway responsiveness to inhaled methacholine. (*C*) Bronchoalveolar lavage cellularity. (*D*) Bronchoalveolar lavage fluid cysLT levels on Day 7 after secondary RSV infection. The data are expressed as mean ± SEM. *$P < 0.05$. Mch = methacholine; $R_L$ = lung resistance.

to reinfection was characterized by the development of significant AHR associated with marked airway eosinophilia and mucus hyperproduction. IFN-γ, RSV-specific IgE, and T cells were shown to be involved in this altered response to RSV reinfection.

Central to the protective effects of MK administered during initial infection of neonates on development of AHR and lung histopathology on reinfection may be the increases seen in levels of IFN-γ. Compared with weanling mice, neonates demonstrated a lower IFN-γ response to initial RSV infection. This role of IFN-γ was confirmed in IFN-γ–deficient mice reconstituted with recombinant IFN-γ. The results showed that IFN-γ was required during initial RSV infection for the expression of protective responses against development of AHR and lung histopathology on reinfection (9). The effects of MK treatment on IFN-γ levels have been demonstrated in other systems. In patients with asthma and in some healthy volunteers, levels of IFN-γ were increased from mononuclear cells after cysLT1R antagonism with MK (32). In patients with allergic rhinitis, treatment with MK increased IFN-γ levels in nasal secretions (33). Here, MK treatment during primary RSV infection increased the levels of IFN-γ after primary and secondary infection, potentially preventing the increases in Th2 responses (IL-5 and IL-13) associated with development of AHR, airway eosinophilia, and goblet cell metaplasia. These decreases in levels of Th2 cytokines after MK treatment were confirmed in *in vitro* cultures of PBLN cells stimulated with RSV.

The mechanisms whereby MK treatment resulted in increased IFN-γ levels are not entirely clear. Several lines of evidence indicate that dendritic cells (DCs) and activated T cells, which express cysLT1R, are targets of cysLT. cysLT has been shown to modulate cytokine production from allergen-stimulated DCs (34). Murine bone marrow DCs pulsed with mite antigen plus LTD4 produced higher amounts of IL-10 compared with antigen-pulsed DCs alone, whereas DCs pulsed with antigen in the presence of a cysLT1R antagonist secreted increased levels of IL-12 and decreased levels of IL-10. When these cysLT1R antagonist-treated DCs were administered intranasally into BALB/c recipients subsequently challenged with antigen, the concentrations of IFN-γ in BALF were increased. This study indicates that cysLT1 may direct DC function in a Th2-dominant manner. CysLT1R signaling may affect the initiation of immune responses by modulating DC migration. In patients with allergic asthma, pranlukast treatment attenuated the decrease in circulating myeloid DCs seen 3 hours after allergen challenge (35). Another study in mice demonstrated that cysLT enhanced DC migration to draining lymph nodes (36). cysLT1 may also have effects on T cells. MK treatment after TCR activation increased IFN-γ production (37). Sensitized and challenged mice lacking leukotriene C4 synthase, the terminal enzyme for cysLT generation, exhibited reduced AHR, eosinophil infiltration, Th2 cytokine levels, and goblet cell metaplasia compared with wild-type control; Th1 cell–dependent responses remained intact (38). Together, these findings indicate that the major activity of cysLT1 in allergen-induced inflammatory responses is to enhance Th2-type responses. T-cell responses in neonatal mice are, in general, biased toward a Th2-like phenotype (39, 40). In the present study, neonatal mice infected with RSV mounted

Th2-dominant responses on reinfection with RSV. It appears that the root of this skewed response is an impairment of IFN-γ production at the time of initial infection (8, 9) and is contrasted by the response of older mice to initial infection and reinfection. Treatment with MK at the time of initial RSV infection clearly attenuated the skewing toward Th2 differentiation and was associated with increased levels of IFN-γ.

The exact mechanisms resulting in AHR are not clear, and there is no single common pathway that leads to this alteration. cysLT are potent bronchoconstrictors (41) as well as proinflammatory mediators (42), both of which might be involved in RSV-induced AHR. In our study, it is difficult to elucidate which one is more important in primary RSV infection in both age groups because treatment with MK decreased AHR and airway inflammation simultaneously. However, after secondary infection, when MK was administered only during primary infection, the decreased AHR in MK-treated mice after secondary infection, weeks after MK administration, cannot be attributed to decreased bronchoconstriction with MK. This conclusion is further supported by the findings that, when administered during secondary infection, MK was without effect on the development of enhanced AHR.

The dose of MK chosen in this study was based on initial dose–response experiments for preventing the development of AHR. Different doses of MK have been shown to have different effects in the mouse after exposure to allergen, including continuous subcutaneous delivery (27). In the study by Ihaku and colleagues, only a reduction in mononuclear cells was found when lower doses (3 mg/kg) of MK were used (28), whereas Blain and Sirois showed that the maximum inhibition of airway eosinophilia was achieved with 100 mg/kg of MK-517 (29). Wu and colleagues demonstrated that the dose required for antiinflammatory effects was higher (25 mg/kg) than the dose required for inhibiting bronchial smooth muscle constriction (30). One reason for the high doses apparently required in mice is that plasma clearance of MK is considerably higher in mice than in humans (30). It is not possible to rule out the potential for off-target effects of MK at the doses used that may be independent of cysLT1R blockade. In support of the role for leukotrienes mediating the RSV-induced changes in lung function and airway inflammation, other leukotriene synthesis inhibitors have shown efficacy in a primary RSV infection model (26).

The cellular sources of cysLT in mice triggered by RSV infection are unclear. Similar to the asthmatic response, a variety of inflammatory cells have the capacity to produce cysLT. These cells are of myeloid origin and are resident in the lung (e.g., mast cells, macrophages) or are recruited to the lung after RSV infection (e.g., eosinophils, neutrophils, lymphocytes) (43). In this study, we found that the levels of cysLT were significantly increased in BALF after primary RSV infection in both age groups. The increase in cysLT levels was even more pronounced after secondary RSV infection. This may have been the result of the increased accumulation of inflammatory cells and in particular eosinophils after secondary RSV infection. In association with alterations in many other parameters, including a significant reduction in airway eosinophilia, the levels of cysLT in the BALF of secondary infected mice were also reduced by MK treatment in the neonatal period. Airway eosinophilia may not be the sole or primary source for the increased cysLT levels in infected mice. Primary airway epithelial cells can release appreciable amounts of LTC4 when activated (44). Because epithelial cells are activated by RSV but do not express the cysLT1R after infection with RSV (45), the continued production of some cysLT may occur in the absence of inflammation. Reinfection with RSV may have induced airway epithelial cells to release more cysLT when compared with primary RSV infection.

The data indicate that cysLT release and the consequences are to some extent linked, but it is possible that MK acts in different ways during primary and secondary infection. Based on the data accumulated in this model, the early exposure of neonatal mice to RSV resulted in a Th2 skewing of the response to reinfection (8, 31). In the absence of MK treatment during primary infection, the presumption is that cysLT acted on a number of cell types expressing a cysLT receptor, leading to their recruitment and activation. It is likely that this response conditioned the neonatal mice in a way that resulted in the Th2 skewing on reinfection. MK treatment blocked these responses (e.g., airway eosinophilia) at the receptor level, whereas cysLT levels remained unchanged. After secondary infection, it is unlikely that the cysLT receptors remained blocked by the MK treatment administered 5 weeks earlier. More likely are the findings that MK treatment during primary infection modified the neonatal response to RSV, preventing Th2 skewing by enhancing IFN-γ release. This prevention of Th2 skewing and increases in IFN-γ were sustained on reinfection.

The failure of MK treatment during secondary infection to alter the development of enhanced AHR and airway eosinophilia suggests that these altered responses to secondary infection may be governed by pathways that are (now) independent of cysLT/cysLT1R interactions, unlike the responses to primary infection in neonatal and adult mice. These findings may have important clinical relevance, emphasizing the importance of preventing the responses during initial encounters with RSV.

These findings confirm that the early encounter with RSV sets the stage for the responses to reinfection and that MK treatment of young mice during primary infection significantly alters their responses to reinfection. MK has been studied in the prophylaxis against asthma exacerbations with effects shown in the fall and winter compared with placebo (46–48). Although the associations between RSV infection and triggering of asthma are not clear, it appears that severe RSV infection requiring hospitalization may show such an association (49–51). In these cohorts, there is no comment on whether the infants had been reinfected.

In summary, the results of this study support the notion that cysLT production at the time of initial RSV infection may be one of the critical factors that determine the outcomes of subsequent reinfection with RSV. Under the conditions described, MK may act beyond cysLT1R blockade; nonetheless, treatment with MK attenuated the initial responses to primary RSV infection and altered the consequences of RSV reinfection in mice initially infected as neonates. These findings identify an important role for cysLT in RSV-induced airway responses and provide novel insights into prophylactic approaches for the prevention of RSV-mediated long-term sequelae in infants with severe RSV bronchiolitis.

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