

Role of cysteinyl leukotrienes in airway inflammation and responsiveness following RSV infection in BALB/c mice

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Cysteinyl leukotrienes (CysLTs) contribute to the development of airway obstruction and inflammation in asthma; however little information is available on the role of these molecules in the pathophysiology of respiratory syncytial virus (RSV) bronchiolitis. This study was designed to evaluate the effects of RSV infection on CysLTs production in a well-established mouse infection model. Furthermore, we assessed the effect of anti-inflammatory agents (a leukotriene receptor antagonist, MK-571, and dexamethasone) on the functional and immune changes induced by RSV infection. Six to 8-wk-old BALB/c mice were infected with human RSV (strain A2). Measurements of airway function were performed using whole body plethysmography. Lung inflammation was assessed by cell counts, measurement of cytokines and CysLTs in bronchoalveolar lavage fluid (BALF) in the absence and presence of treatment with MK-571 or dexamethasone. RSV infection produced a marked increase in CysLTs in the BALF and lung tissue, recruitment of neutrophils and lymphocytes into the airways, increased IFN- γ levels and airway hyperresponsiveness (AHR). Treatment with MK-571 decreased RSV-induced AHR without affecting the cellular and inflammatory responses to RSV. Dexamethasone decreased AHR and markedly reduced the recruitment of inflammatory cells and production of IFN- γ . Our findings suggest CysLTs play an important role in the pathogenesis of RSV-induced airway dysfunction. Treatment with MK-571 decreases RSV-induced AHR but does not appear to alter the lung inflammatory responses to RSV. In contrast, dexamethasone decreases RSV-induced AHR but interferes with recruitment of inflammatory cells, resulting in decreased Th₁ cytokines (a potentially Th₂-prone environment) in this model. These studies support recent reports on the beneficial effects of CysLT receptor antagonist in human trials and provide a model for investigating the role of CysLTs in RSV bronchiolitis.

**Jason J. Fullmer¹, Amir M. Khan²,
Okan Elidemir¹, Connie Chiappetta²,
James M. Stark² and
Giuseppe N. Colasurdo²**

¹Department of Pediatrics, Texas Children's Hospital and Baylor College of Medicine, Houston, TX, USA,

²Department of Pediatrics, University of Texas-Houston Medical School, Houston, TX, USA

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Giuseppe N. Colasurdo, MD, 6431 Fannin Street, MSB 3.228, Houston, TX 77030
Tel.: +1 713 500 5650
Fax: +1 713 500 0653
E-mail: giuseppe.n.colasurdo@uth.tmc.edu

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Respiratory syncytial virus (RSV) is the single most important respiratory pathogen in infants and young children, and is a major health burden worldwide (1, 2). Because of the frequency of its epidemics during the winter months, it's ubiquitous and highly contagious nature, and an incomplete immune response, this agent infects virtually all children in their first 2 yr of life. The clinical features produced

by RSV in children include upper or lower respiratory tract infection (LRTI). LRTI may be manifest as pneumonia or viral-induced wheezing (bronchiolitis). Between 1% and 2% of previously healthy infants will require hospitalization (3). Several genetic and environmental factors may determine the outcome of RSV infection on airway function, and an association between RSV in early life and long-term

pulmonary abnormalities has been repeatedly suggested (4). The pathogenic mechanisms underlying RSV-induced airway dysfunction are poorly understood. As a result, therapeutic options are limited and rather ineffective in preventing hospitalization, reducing hospital stays, or decreasing recurrent wheezing later in childhood (5–7). The development of new therapeutic or prophylactic agents remains a major challenge and yet a major clinical need.

The role of cysteinyl leukotrienes (CysLTs) in the pathogenesis of airway obstruction and inflammation has been recognized and targeted in the treatment of asthma. CysLTs are capable of affecting several cellular functions within the lung leading to the clinical features seen in response to RSV infection: bronchoconstriction (8), mucus production (9), airway infiltration by inflammatory cells, and increased vascular permeability (10, 11). CysLT have been demonstrated in nasopharyngeal secretions of human infants with RSV infection, and found to be significantly elevated in children with RSV-induced bronchiolitis compared with secretions from children with RSV upper respiratory infection alone (12).

The therapeutic effects of Montelukast, a CysLT receptor antagonist, have recently been investigated in the treatment of infants with RSV bronchiolitis. In these early studies, Montelukast significantly decreased exacerbations of reactive airway disease after RSV bronchiolitis in infants (13). In a murine model, elevated leukotriene concentrations were correlated with increasing respiratory rates and the degree of prolongation of expiratory time. Administration of Zileuton, an inhibitor of leukotriene synthesis, reduced airway constriction and the number of inflammatory cells in the lung (14). There is also emerging data that corticosteroids, which have broader anti-inflammatory action, initiated early and at high doses may be beneficial in RSV LRTI (15, 16). Although there is mounting evidence that CysLTs may play an important role in the regulation of the inflammatory and functional responses to respiratory pathogens in the lung, a paucity of data is available on the role played by these molecules in the pathogenesis of RSV infection in humans and mammalian models. This study was designed to evaluate the production of CysLTs in a well-established murine model of RSV infection, and to assess the effects of currently available therapeutic agents, a leukotriene receptor antagonist (MK-571) and dexamethasone, on the functional and cellular changes produced by RSV infection.

Methods

Experimental animals

Female BALB/c mice, obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN, USA were used in all the studies. Animals were exposed to the various treatments beginning at 6–8 wk of age. All the procedures employed in this study were approved by the Animal Care and Use Committee of the University of Texas Health Science Center and conformed to NIH guidelines.

RSV infection and treatments

After sedation with ketamine and xylazine, animals were infected intranasally with human, sucrose purified RSV, strain A2 (5×10^5 pfu of virus in 100 μ l of saline/mouse). This experimental protocol has been previously shown to produce viral replication within the respiratory tract of mice and other mammalian models (17). Control animals received uninfected culture medium treated in a similar manner. After preliminary time- and dose-response studies, *in vivo* experiments of lung mechanics and analysis of BAL fluid were performed at 5 days post-infection in control and RSV-infected mice. In separate experiments, we studied animals infected with RSV and treated with MK-571, a selective LTD₄ receptor antagonist (50 mg/kg i.p. daily for 4 days) or dexamethasone (0.5 mg/kg i.p. daily for 4 days). Both agents were diluted in normal saline.

Measurement of airway responsiveness

Airway responsiveness (AR) was measured by barometric plethysmography in unrestrained animals using whole body plethysmography (Buxco, Troy, NY, USA) as previously described (18–21). AR was expressed as an enhanced pause (Penh), a calculated value that reflects the pulmonary resistance measured by a conventional, two-chamber plethysmograph in ventilated animals. Mice were exposed to nebulized physiologic saline for 3 min and then to increasing concentrations of nebulized methacholine (3–50 mg/ml) using an ultrasonic nebulizer. Recordings were taken for 3 min after each nebulization. The Penh values measured during this period were averaged and expressed as a percentage of baseline Penh values following exposure to saline (mean $\pm$ SE). A full dose-response to methacholine was performed for each animal studied. As a result, Penh values were obtained at baseline and after exposure to increasing concentrations

of methacholine: 3, 6, 12, 25 and 50 mg/ml. These studies were performed in seven control animals, seven RSV-infected mice, seven RSV-infected mice treated with dexamethasone and seven RSV-infected mice treated with MK-571.

Bronchoalveolar lavage fluid studies

Following studies of airway mechanics, animals underwent lung lavage with three aliquots of 0.7 ml of PBS. This method consistently yields > 90% recovery of the instilled volume. Total cell count was determined using a hemocytometer (mean $\pm$ SE). Cell differentials were calculated from slides prepared with modified Wright's stain and expressed as total BALF cell counts. The observer was blinded to the source of the cells counted.

Cysteinyl leukotriene concentrations in the BALF and lung tissue were measured in duplicate by ELISA (Amersham Pharmacia Biotech, Buckinghamshire, England, UK). Individual BAL samples were not pooled but simply processed in two different wells for ELISA. The values obtained from these 'duplicate' measurements were averaged and then analyzed as described in Statistical Analysis. Samples were processed, and assays completed according to the manufacturer's instructions. These kits contain a detector antibody labeled against leukotriene C₄, providing a rapid method of determination of leukotriene C₄/D₄/E₄ in the range of 0.75 to 48 pg/well. Results were expressed as pg/ml (mean $\pm$ SE). Cytokine concentrations (IFN- γ and IL-4) in BALF were measured in duplicate by ELISA (e-Bioscience, San Diego, Ca) according to manufacturer's instructions. The limits of detection for the cytokines studied were 4 pg/ml and 8 pg/ml, for IL-4 and IFN- γ , respectively. Results were expressed in terms of pg/ml BALF (mean $\pm$ SE).

Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM). Student's paired and unpaired *t* tests and two-way analysis of variance were used to determine the level of differences, where appropriate. A value of $p < 0.05$ was considered significant. The 'n' provided refers to the total number of animals studied. The results were obtained from at least two to three sets of separate experiments.

Results

Based on previous time and dose response experiments in our laboratory, and others, we

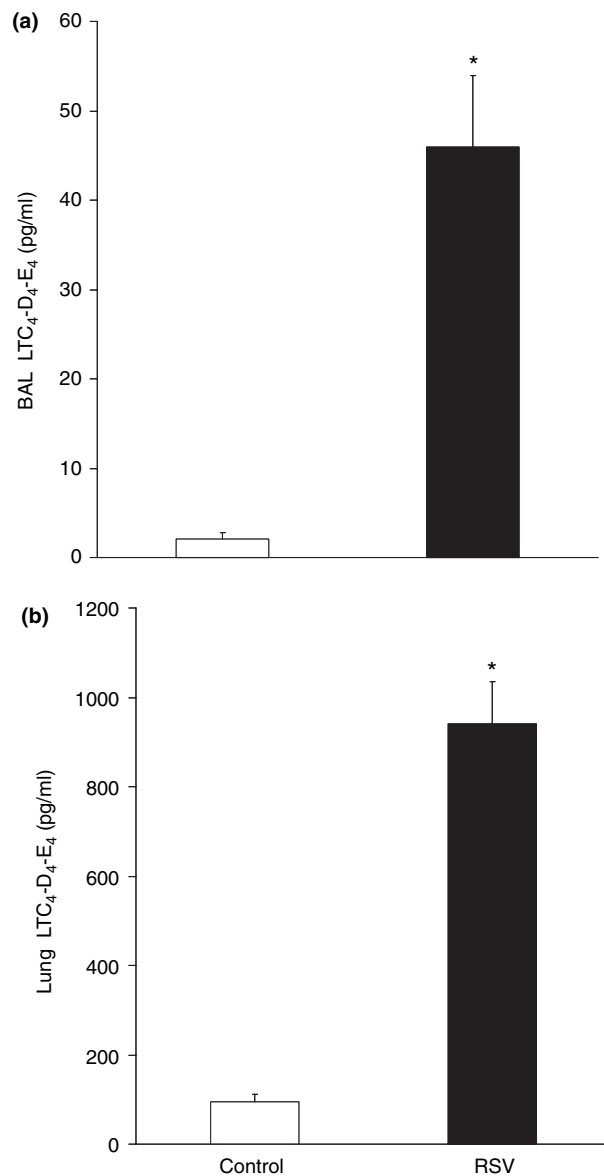


Fig. 1. Lung cysteinyl leukotrienes (CysLTs) following respiratory syncytial virus (RSV) infection. BALB/c mice were infected with RSV and studies performed on day 5 following RSV exposure. CysLTs were measured in Bronchoalveolar lavage fluid (a), (control $n = 8$, RSV $n = 7$) and lung tissue homogenates (b), (control: $n = 11$; RSV: $n = 6$). Error bars indicate standard error of the mean. * $p < 0.001$ vs. control.

determined that the RSV titers peak at day 5 post-infection. RSV infection produced a marked increase in CysLT levels in BALF (Fig. 1a) and lung tissue (Fig. 1b) at day 5 when compared with control animals: 46.5 ± 7.0 ($n = 8$) vs. 1.13 ± 0.3 pg/ml ($n = 7$, $p = 0.001$) and 940.0 ± 86.4 ($n = 11$) vs. 93.6 ± 17.1 pg/ml ($n = 6$, $p = 0.0001$), respectively.

RSV infection induced airway hyperresponsiveness, as demonstrated by enhanced Penh

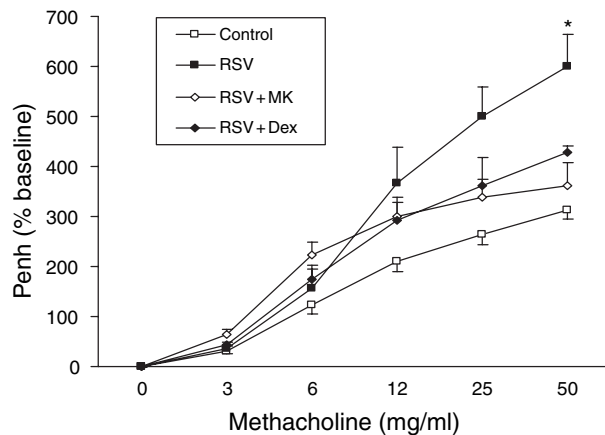


Fig. 2. Airway responsiveness following respiratory syncytial virus (RSV) infection. Airway hyperresponsiveness was assessed in BALB/c mice 5 days following RSV exposure. Response to methacholine was measured by barometric plethysmography in unrestrained animals. Treatment with MK-571 (RSV + MK) and dexamethasone (RSV + DEX) reduced RSV-induced airways hyperresponsiveness to methacholine. (Control: $n = 7$; RSV: $n = 7$; RSV + MK: $n = 7$; RSV + DEX: $n = 7$). Error bars indicate standard error of the mean. * $p < 0.05$ for RSV compared with RSV + MK, RSV + DEX, and control.

responses to Mch when compared with control animals (Fig. 2). There was a leftward shift of the dose-response curve, and greater Penh values at 50 mg/ml of Methacholine (Max Penh) when compared with control mice: $654.2 \pm 79\%$ ($n = 7$) and $313 \pm 18.7\%$ ($n = 7$), respectively. Both treatments with MK-571, (RSV + MK), and dexamethasone (RSV + Dex) reduced RSV-induced airways responsiveness to Mch (Fig. 2, $p < 0.05$).

Treatment with MK-571 did not alter RSV-induced production of CysLTs in the BALF: 46.5 ± 7.0 and 45.4 ± 8.1 pg/ml for RSV and RSV + MK-571, respectively. Treatment with dexamethasone produced a decrease in CysLT levels that did not reach statistical significance when compared with RSV and RSV + MK-571 groups (Fig. 3, $p = 0.06$).

The analysis of BALF cells revealed a significant increase in the total number of inflammatory cells at day 5 following RSV infection compared with control values (Fig. 4a: $22.3 \pm 2.2 \times 10^4$ and $3.6 \pm 0.6 \times 10^4$ respectively, $p < 0.05$). There was also an associated increase in total number of lymphocytes and neutrophils into the BALF following RSV infection (Fig. 4b). Treatment with MK-571 did not affect the recruitment of inflammatory cells ($22.3 \pm 2.2 \times 10^4$ and $21.3 \pm 4.0 \times 10^4$ for RSV and RSV + MK-571 respectively), nor did it affect the differential cell count (Fig. 4). In contrast, dexamethasone markedly decreased the total number of macrophages,

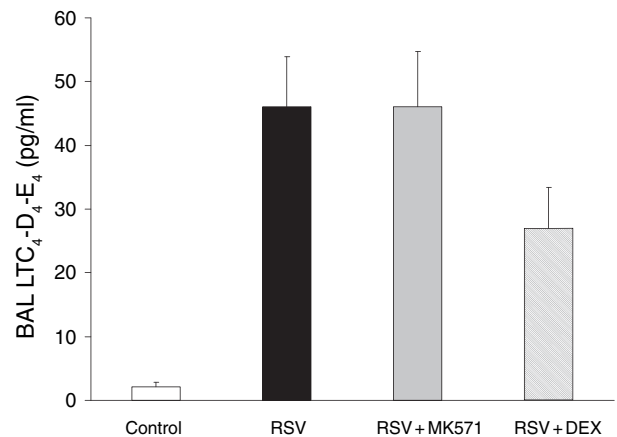


Fig. 3. Effect of MK-571 and dexamethasone on cysteinyl leukotriene production following respiratory syncytial virus (RSV) infection. BALB/c mice were infected with RSV in the absence/presence of MK-571 (RSV + MK) or dexamethasone (RSV + DEX) and BAL was performed on day 5. Cysteinyl leukotrienes (CysLTs) (combined LTC₄, LTD₄, and LTE₄) were measured in the BALF by enzyme immunoassay. Control: $n = 8$; RSV: $n = 7$; RSV + MK: $n = 7$; RSV + DEX: $n = 7$). Error bars indicate standard error of the mean.

neutrophils and lymphocytes in the BAL fluid (Fig. 4, $p < 0.05$ when compared with RSV and RSV + MK-571).

The Th₁/Th₂ cytokine balance was investigated by measuring IFN- γ and IL-4 in the BALF. The levels of IFN- γ in the BALF were markedly increased by RSV infection when compared with control values: 3505.9 ± 221.0 pg/ml ($n = 14$) and 313.4 ± 54.8 pg/ml ($n = 10$), respectively (Fig. 5a, $p = 0.0001$). Treatment with dexamethasone ($n = 14$) significantly decreased RSV-induced production of IFN- γ to 1296.0 ± 390 pg/ml (Fig. 5a, $p = 0.001$). The level of IFN- γ in RSV-infected animals was not altered by treatment with MK-571 (Fig. 5a).

The levels of IL-4 in the BALF were unchanged by RSV infection when compared with control values: 66.0 ± 6.6 pg/ml ($n = 10$) and 50.4 ± 2.6 pg/ml ($n = 14$), respectively (Fig. 5b). After treatment with dexamethasone and MK-571, IL-4 levels were similar to control values: 42.6 ± 9.6 pg/ml ($n = 14$) and 56.9 ± 5.0 pg/ml ($n = 11$), respectively, (Fig. 5b).

RSV infection produced a significant increase in IFN- γ /IL-4 compared with control animals, suggesting a predominant Th₁ immune response (Fig. 6, $p < 0.05$). Treatment with MK-571 did not significantly alter the IFN- γ /IL-4 ratio. In contrast, treatment with dexamethasone altered the Th₁ immune response induced by RSV by decreasing the IFN- γ /IL-4 (Fig. 6, $p < 0.05$).

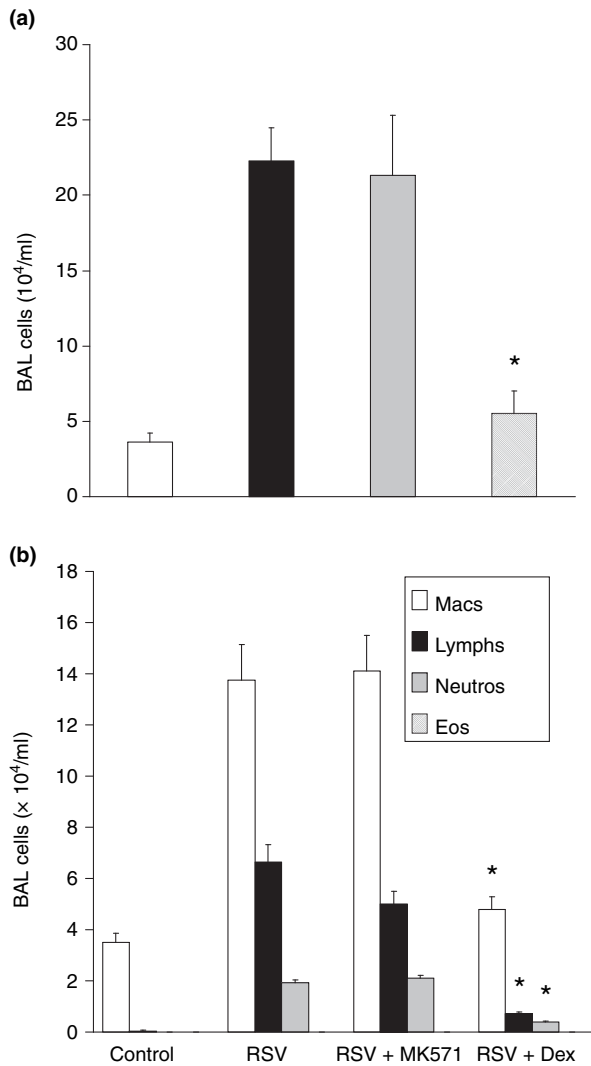


Fig. 4. Effect of MK-571 and dexamethasone on respiratory syncytial virus (RSV)-induced airway inflammation. BALB/c mice were infected with RSV in the absence/presence of MK-571 (RSV + MK) or dexamethasone (RSV + DEX) and BAL was performed on day 5. Total BALF cells (a) and differential cell count (b). Treatment with dexamethasone markedly decreased the total BALF cell count, and the number of total macrophages, neutrophils and lymphocytes. Error bars indicate standard error of the mean. * $p < 0.05$ when compared with RSV and RSV + MK-571.

Discussion

This study demonstrates that RSV infection in a murine model causes a marked increase in CysLT production in the murine lung and AHR. This increased AHR could be reduced by treatment with either MK-571 (a receptor antagonist) or dexamethasone. The effect of MK-571 on AHR occurred without an apparent effect on airway inflammation or cytokine (IFN- γ or IL-4) production, suggesting a direct relationship between CysLT receptor activation and the

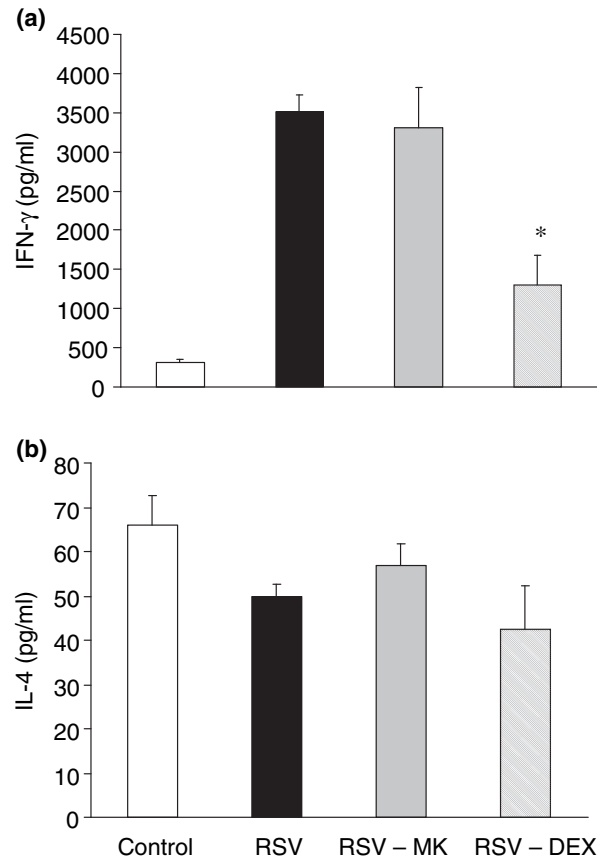


Fig. 5. Effect of MK-571 and dexamethasone on IFN- γ and IL-4 production following respiratory syncytial virus (RSV) infection. BALB/c mice were infected with RSV in the absence/presence of MK-571 (RSV + MK) or dexamethasone (RSV + DEX) and BAL was performed on day 5. IFN- γ and IL-4 was measured by enzyme immunoassay (a and b, respectively). Treatment with dexamethasone decreased RSV-induced production of IFN- γ , but had no effect on BAL IL-4 levels. Error bars indicate standard error of the mean. * $p < 0.001$.

development of airway hyperresponsiveness. RSV infection was associated with recruitment of inflammatory cells (measured in the BALF), which could be inhibited by dexamethasone but was unaffected by MK-571. Moreover, MK-571 had no effect on the overall production of Th₁ and Th₂ cytokines (IFN- γ and IL-4, respectively). These studies are in agreement with previous studies in a mouse RSV infection model, that showed a correlation between increased BALF leukotriene levels and alteration in lung function (respiratory rate and prolongation of the expiratory phase) and lung inflammation (number of macrophages and lymphocytes in BALF) (14). Moreover, leukotriene levels were shown to be significantly elevated in human subjects with altered lung function (bronchiolitis or recurrent wheezing) when compared with subjects with

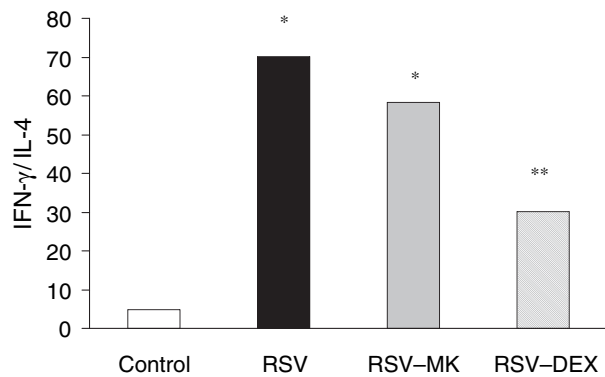


Fig. 6. Effect of MK-571 and dexamethasone on IFN- γ /IL-4 following respiratory syncytial virus (RSV) infection. BALB/c mice were infected with RSV in the absence/presence of MK-571 (RSV + MK) or dexamethasone (RSV + DEX) and BAL was performed on day 5. Ratio of IFN- γ /IL-4 measured in the BALF (Fig. 5). RSV infection produced a significant increase in the IFN- γ /IL-4 which was unaltered by treatment with MK-571. Treatment with Dexamethasone decreased the IFN- γ /IL-4. * $p < 0.05$ compared with control; ** $p < 0.05$ compared with RSV and RSV + MK.

upper respiratory infection alone (12). Taken together, these studies suggest that the CysLT have a direct effect on airway hyperresponsiveness following RSV infection.

CysLT are potent bronchoconstrictors as well as proinflammatory mediators, causing increased mucosal blood flow, edema, and increased vascular permeability. These properties make CysLT reasonable therapeutic targets for RSV bronchiolitis and its sequelae. Recent studies have reported the effects of CysLT inhibitors and/or receptor antagonist as therapeutic agents in RSV-induced lung disease. In a murine model, treatment with Zileuton, a 5-lipoxygenase inhibitor, 1 day prior to, and daily for 5 days after RSV infection markedly prevented airway constriction and the recruitment of inflammatory cells into the mouse lung (14). In a human study, Montelukast, a CysLT receptor antagonist, given for 28 days, starting within 7 days of clinical symptoms significantly decreased post RSV-induced pulmonary exacerbations in infants 3–36 months of age (13). In our study, treatment with MK-571, a selective leukotriene D₄ receptor antagonist decreased RSV-induced AHR, measured by airway plethysmography, without affecting the inflammatory changes induced by viral replication (measured by BALF cell count and IL-4 and IFN- γ levels). Our results differ from those reported by Welliver *et al.* (14), in that our study did not demonstrate a change in the inflammatory cell infiltrate with MK-571 treatment. This could be related to the fact that we used a selective LTD₄ receptor

antagonist, whereas Welliver's study utilized Zileuton, a 5-lipoxygenase (LO) inhibitor, which would alter production of both the Cys LT and other 5-LO products, including LTB₄, a potent pro-inflammatory mediator and chemoattractant (14).

The use of whole body plethysmography as a measure of airway resistance in this study is potentially an area of controversy. Penh is a calculated value based on changes in the breathing pattern of unrestrained mice and has been correlated with changes in lower airway resistance (21). Although Penh values may be affected by other non-specific changes in breathing pattern, this methodological approach has been widely used and accepted for this type of investigations (18–21). Further, measurement of airway responsiveness to inhaled methacholine by barometric whole-body plethysmography in mouse models has been found to have a good correlation with pulmonary resistance when measured with more invasive techniques (21). Finally, recent studies in this laboratory using invasive measures of lung function have demonstrated similar airway responses following RSV infection when measured by non-invasive (Buxco) and invasive techniques (Flexivent, data not shown).

In the present study, RSV infection produced a significant increase in IFN- γ with no change in IL-4 levels in the BALF, thus generating a preferential Th₁ immune response, and potentially providing a protective milieu against the development of a Th₂ environment (22–25). However, reports on RSV infection, Th₁/Th₂ phenotype and allergic sensitization in mouse models indicate that this relationship is not straightforward. Several laboratories have reported that RSV infection followed by allergic sensitization is associated with a Th₂ phenotype and enhanced allergic sensitization, increased eosinophilia, and airway hyperresponsiveness (AHR). Leibovitz reported increased levels of serum IgE in mice if RSV infection occurred prior to ragweed sensitization (26). Likewise, Schwarze reported that RSV infection prior to ovalbumin sensitization and challenge resulted in allergic sensitization, increased eosinophilia and increased AHR that could be blocked by treating with antibody to IL-5 (24, 27). Lukacs *et al.* found that RSV given 21 days prior to sensitization to cockroach antigen caused increased AHR, increased eosinophilia, and increased IL-13, and that these phenotypes could be prevented by treatment with antibody to IL-13 following the acute RSV infection (28). A prospective study of children recruited antenatally (with one asthmatic, atopic parent) demonstrated

decreased IFN- γ and increased IL-4 (increased Th₂/Th₁ ratio) in nasal washes obtained 5–7 days following acute onset of bronchiolitis (29). In contrast, Hussell *et al.* reported that primary infection of BALB/c mice with RSV resulted in production of IFN- γ and low levels of IL-4 and IL-5 (23). Similarly, Peebles *et al.* reported that primary infection of BALB/c mice resulted in a primarily Th₁ phenotype: increased IFN- γ , and almost undetectable IL-5 or IL-13 (30). Our experimental protocol differed from these previous reports in that it was not designed to evaluate the relationship of RSV to allergen sensitization. Focusing on the effects of primary RSV infection, our findings are in accordance with those of Hussell *et al.* (23) and Peebles *et al.* (30), demonstrating an increase in IFN- γ , and no significant airway eosinophilia after primary RSV infection in BALB/c mice.

Treatment with MK-571 did not alter the IFN- γ /IL-4, suggesting that CysLT do not contribute to the early inflammatory/lymphocyte responses to RSV. In contrast, treatment with Dexamethasone on day 1 of RSV infection decreased RSV-induced AHR, but also affected the recruitment of inflammatory cells and lowered the levels of IFN- γ in murine lungs. Together, these findings suggest that the use of dexamethasone may induce a Th₂-prone environment, as measured by IFN- γ /IL-4 ratio. The clinical significance of this finding is uncertain, as it was generated in the mouse model of RSV infection. However, this model has provided important new insights on host immune response to RSV and other respiratory pathogens. For instance, much of our current understanding of the mechanisms of the enhanced pulmonary pathology following early RSV vaccines has resulted from studies in the mouse model (23, 25). Further investigations will be necessary to define the acute and long-term effect of therapeutic agents commonly used to treat viral bronchiolitis.

Clinical studies have provided conflicting data on the therapeutic efficacy of corticosteroids during acute RSV lower respiratory infection. Early studies showed no benefit from the use of corticosteroids in acute bronchiolitis (31–35). In this respect, the largest randomized-controlled double-blind study did not reveal any beneficial effects of dexamethasone when compared with placebo (32). However, recent studies have suggested that early use of relatively high doses of corticosteroids in RSV infection may be of some benefit by reducing the length of hospitalization (15), and

decreasing the need for hospital admission (16). Additional controlled studies with larger patient numbers are needed to support the use of corticosteroids during acute RSV bronchiolitis. However, one of the major variables, the time of initiation of corticosteroid therapy relative to time of infection, could be responsible for the differences in outcome observed and cannot be controlled. To this end, it has been postulated that early treatment, when there is substantial reversible inflammation, may be beneficial whereas treatment occurring later in the clinical course, when the characteristic dense plugs of mucus, alveolar debris and fibrin have already formed, would not be likely to decrease clinical illness (34). While the use of corticosteroids is not common practice in bronchiolitis, this modality is typically used in patients with recurrent virus-induced wheezing. It is currently unknown, but intriguing to speculate, whether treatment of respiratory virus-induced illness with corticosteroids will modify the potentially protective effect on T-cell memory induced by these pathogens, as suggested by our data on lymphocyte recruitment and IFN- γ production. Studies in other disease states, such as organ transplantation have shown corticosteroids to preferentially block Th₁ cytokine expression, guiding the differentiation of helper T cells toward a Th₂ phenotype (35). Therefore, the use of selective anti-inflammatory agents, such as CysLT receptor antagonists, capable of modulating virus-induced AHR without affecting the normal host inflammatory and memory responses, would be an attractive strategy. Further studies are needed to determine the long-term effects of anti-inflammatory agents such as CysLT receptor antagonists and corticosteroids on T-cell memory, adaptive immune responses, and allergic sensitization following RSV infection.

In summary, this study showed that RSV infection in a murine model induced a marked increase in CysLT production in mouse lungs. This was associated with AHR, recruitment of inflammatory cells into the lung and an increased IFN- γ production. Treatment with MK-571 or dexamethasone both decreased virus-induced airway dysfunction. Treatment with MK-571 did not significantly alter the inflammatory cell infiltrate or the Th₁/Th₂ cytokine balance suggesting that the activation of the CysLT receptor is an important step in the development of AHR but not inflammatory cell recruitment following RSV lower respiratory infection.

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