Respiratory Virus-Induced Regulation of Asthma-Like Responses in Mice Depends upon CD8 T Cells and Interferon- γ Production

Joost J. Smit,* Louis Boon,[†] and
Nicholas W. Lukacs*

From the Department of Pathology,* University of Michigan
Medical School, Ann Arbor, Michigan; and Bioceros,[†] Utrecht,
The Netherlands

Respiratory virus infections can significantly influence the development of airway disease by both predisposing and exacerbating the developing lung immune environment. In contrast, the initiation of a more desirable anti-viral response may better prepare the local environment and protect it from developing an adverse long-term disease phenotype. BALB/c or C57BL/6 mice exposed to respiratory syncytial virus (RSV) infection at the same time as allergen sensitization were assessed for airway function, cytokine responses, and inflammatory parameters. Depending on the genetic strain of mouse used, BALB/c versus C57BL/6, RSV could differentially protect against the development of airway allergen responses. Although RSV was able to block allergen sensitization and induction of airway hyperresponsiveness and eosinophilic inflammation in C57BL/6 mice, the infection did not reduce the allergic responses in BALB/c mice. The alteration of airway responsiveness did not depend on the timing of RSV infection in C57BL/6 mice in conjunction to the allergen sensitization protocol. Neutralization experiments demonstrated that interferon- γ contributed significantly to the RSV-induced airway attenuation of the allergic responses, whereas transfer of CD8 T cells from RSV-infected animals suggested that they were partially responsible for the altered environment. These data suggest that a respiratory viral infection impacts on the local lung environment and may reflect specific aspects of the hygiene hypothesis. However, the outcome of this interaction depends on the immunological response of the host. (*Am J Pathol* 2007, 171:1944–1951; DOI: 10.2353/ajpath.2007.070578)

Respiratory syncytial virus (RSV) is one of the most pervasive viruses, causing the majority of infant hospitalizations for bronchiolitis throughout the world.^{1–3} RSV, like other respiratory viruses, is able to induce respiratory illness by itself but also can exacerbate established asthma or chronic obstructive pulmonary disease in susceptible groups especially in children and the elderly.⁴ Although the hygiene hypothesis suggests that bacterial and viral infections during early childhood may protect from development of allergic diseases, RSV has been suggested to increase the risk of long-term pulmonary disease and asthma.^{5–10} However, this has been a matter of much debate and the focus of several studies.¹¹ It has been shown that severe RSV bronchiolitis in early infancy was a strong risk factor for the occurrence of allergic sensitization and asthma in childhood and early adolescence.¹² On the other hand, the Tucson Children's Respiratory Study¹³ and others¹⁴ found an association between RSV infection and wheezing later in life, but this was not accompanied by an increased risk of allergic sensitization.

Interactions between the responses induced by viruses and those by chronic respiratory illnesses are complex and may involve multiple aspects of the immune system. Therefore, a mouse model, with its obvious limitations, has the unique potential to yield specific information to help sharpen the focus of the human studies. Models for RSV-mediated lung disease in the mouse have already proven to be essential to decipher RSV-mediated immune responses. Interestingly, different effects of RSV infection on allergic responses have also been observed in murine studies. In the mouse, RSV exacerbated established asthmatic responses when administered shortly before or during allergen provocation challenge.^{15,16} In contrast, RSV infection before allergic sensitization either exacerbated^{17–19} or low-

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Current address of J.J.S.: Institute for Risk Assessment (IRAS), Utrecht University, Utrecht, The Netherlands. E-mail: j.j.smit@uu.nl.

Address reprint requests to Nicholas W. Lukacs, Dept. of Pathology, University of Michigan Medical School, 4059 BSRB, 109 Zina Pitcher Pl., Ann Arbor, MI 48109-2200. E-mail: nlukacs@umich.edu.

ered airway responses.¹⁶ The key factor for these latter observations may be the timing of viral and allergen exposure, the type of specific viral isolate used,²⁰ or the genetic background of the mouse strain used. C57BL/6 mice are less permissive for RSV infection than BALB/c mice²¹ because RSV induced strong specific CD8 T-cell responses in C57BL/6 comparable to the responses described for BALB/c mice.^{22,23}

The extent to which animal models reflect human immunopathology is not entirely clear. Studies comparing different RSV isolates have demonstrated that there are even differences in viruses that can be exploited to better understand disease phenotypes and immunological responses in models of disease.^{20,24} Although complex, the responses established in different mouse strains are very useful for further deciphering the impact of the immune responses to chronic disease. In this study, the effect of RSV infection at different time points on development of allergic disease was studied in mice using a model of airway-only sensitization and challenge to more appropriately examine the mucosal immune responses.

Materials and Methods

Animals

Female BALB/c/J or C57BL/6J mice, 6 to 8 weeks of age, were obtained from the Jackson Laboratory, Bar Harbor, ME. All mice were housed under specific pathogen-free conditions within the animal care facility at the University of Michigan. The University of Michigan Committee on the Use and Care of Animals approved all experiments. Groups consisted of five mice and are indicated in the figure legends.

RSV Propagation

RSV virus (line 19, A strain) was derived from a clinical isolate at the University of Michigan. Hep2 or VERO cells were infected with RSV until syncytia were formed. Hereafter, cells were frozen at -80°C overnight, and the supernatant was harvested, clarified, and aliquoted. To determine viral titers in culture supernatants, an immunoplaque assay was performed as previously described.²⁵

Experimental Design

Mice were intranasally sensitized for 7 consecutive days (days 0 to 7) with 1 μg of cockroach allergen (CRA; Hollister Stier Laboratories, Spokane, WA), followed by one intranasal (1 μg) and two intratracheal (4 μg) CRA challenges on days 35, 40, and 42, respectively. In addition, on days -21 , 0, or 21, mice were intratracheally infected with $\sim 1 \times 10^5$ plaque-forming units of RSV. An intraperitoneal injection of monoclonal anti-mouse interferon (IFN)- γ (clone XMG 1.2) on days -1 , 0, 1, 4, and 7 was used *in vivo* to neutralize IFN- γ during RSV infection. For transfer of CD4 or CD8 T cells from RSV-infected mice, 6 days after RSV infection, single-cell suspensions of lung-draining lymph nodes were prepared by isolating

mediastinal lymph nodes, pushing cells through a nylon mesh using a syringe and lysing red blood cells. Hereafter, CD4 or CD8 T cells were isolated by positive selection on a magnetic cell-sorting column using CD4 or CD8 beads (Miltenyi Biotec, Auburn, CA). Cells were injected intratracheally on day 34, 1 day before the start of the CRA challenge protocol.

Measurement of Allergic Responses and Collection of Bronchoalveolar Lavage

One day after final CRA challenge, airway reactivity in anesthetized mice was measured as previously described.²⁶ Briefly, mice were anesthetized with sodium pentobarbital, and the trachea was cannulated and ventilated using a pump ventilator. After baseline measurements, mice were injected intravenously with 2.5 μg of methacholine (Sigma, St. Louis, MO), and the peak airway resistance was recorded. After measurement of airway reactivity, bronchoalveolar lavage was collected by injection of 1 ml of phosphate-buffered saline (PBS) in the trachea. Cells were resuspended in 100 μl of PBS, counted, and cytopsin-fixed and stained with Diff Quick (Dade Behring Ag, Deerfield, IL).

Lymph Node Restimulation

Single-cell suspensions of lung-draining lymph nodes were prepared as described above. Cells were cultured in RPMI medium (RPMI 1640 with 10% fetal calf serum, L-glutamine, and Pen/Strep; Life Technologies, Inc., Grand Island, NY) at a concentration of 5.10^6 cells/ml in the presence of either sterile CRA (2 $\mu\text{g}/\text{ml}$) or medium alone and incubated at 37°C . After 3 to 4 days the supernatants were isolated and stored at -80°C until further analysis.

A standardized sandwich enzyme-linked immunosorbent assay was performed to measure cytokine levels in culture supernatants. All antibody pairs were obtained from R&D Systems, Minneapolis, MN, and used according to company recommendations. In addition, in some experiments cytokines were measured using the Bioplex system (Bio-Rad, Hercules, CA) according to the instructions by the manufacturer.

Histology

Lungs were inflated and maintained in formalin overnight before being processed into paraffin using standard histological techniques. Lung tissue sections were stained with hematoxylin and eosin (H&E) for analysis of inflammatory cell accumulation.

Statistical Analysis

Differences between groups were analyzed by using one-way analysis of variance with a Bonferroni posthoc test. Differences with a *P* value of <0.05 were considered significant.

Figure 1. RSV infection and allergen sensitization and challenge in C57BL/6 and BALB/c mice. C57BL/6 and BALB/c mice were RSV infected (RSV), allergen sensitized (CRA), or RSV infected and allergen sensitized (CRA + RSV). **A:** Airway responses were measured after one dose of methacholine and compared to basal measurements. **B:** Lung lavage was performed, and eosinophils were counted in stained cytopsin slides. **C:** Lung lymph node cells were restimulated with allergen, and cytokines were measured in culture supernatants 24 hours after challenge. Data are represented as mean airway resistance in cmH₂O/ml/second, number of cells, or ng/ml $\pm$ SEM, respectively. * P < 0.01 compared to RSV-infected control mice, # P < 0.05 compared to CRA-sensitized mice, n = 5 mice.

Results

RSV Infection Regulates Allergen-Induced Airway Responses and Th2 Cytokine Production

To examine the effect of RSV on allergic sensitization, we infected mice with RSV at the first day of airway CRA sensitization, followed by CRA exposure 2 weeks later. Sensitization and challenge of both C57BL/6 and BALB/c mice with allergen alone elicited a significant increase in airway resistance compared to controls (Figure 1A). RSV infection of C57BL/6 mice blocked the allergen-induced airway hyperreactivity whereas no significant change in airway hyperreactivity was observed in BALB/c mice. CRA sensitization and challenge induced an influx of eosinophils in the bronchoalveolar space of C57BL/6 and BALB/c mice (Figure 1B). However, RSV infection of C57BL/6 mice significantly decreased the number of eo-

sinophils but did not affect the number of eosinophils in BALB/c mice. Thus, airway responses elicited in the C57BL/6 and BALB/c mice were relatively comparable with allergen alone, but only the C57BL/6 strain of mice were significantly altered by the presence of RSV.

To investigate the effect of RSV infection on the underlying T-cell responses during CRA sensitization and challenge, lymph nodes were isolated and cells dispersed and stimulated *in vitro* with CRA. Restimulation of lymph node cells after CRA challenge alone of C57BL/6 mice demonstrated a significant increase in interleukin (IL)-5 and IL-13 (Figure 1C). Interestingly, production of these cytokines was significantly reduced in RSV-infected C57BL/6 mice, suggesting that in the presence of RSV responses allergen sensitization is modified in these mice. In contrast, when lymph nodes from BALB/c mice were restimulated *in vitro*, the Th2 cytokines, including IL-4, were further increased in the RSV-treated animals compared to allergen-alone-challenged mice. Thus, the

Figure 2. Timing of RSV infection and CRA sensitization and challenge. **A:** C57BL/6 mice were RSV infected (RSV), allergen sensitized (CRA), or RSV infected and allergen sensitized (CRA + RSV) at different time intervals. **B:** Airway responses were measured after one dose of methacholine and compared to basal measurements. Data are represented as mean airway resistance in cmH₂O/ml/second, number of cells, or ng/ml $\pm$ SEM, respectively. * $P < 0.01$ compared to RSV-infected control mice, # $P < 0.05$ compared to CRA-sensitized mice, $n = 5$ mice.

responses elicited in the two strains of mice were differentially altered by the presence of RSV at the time of allergen sensitization. IFN- γ was undetectable in the T-cell culture supernatants of both strains upon allergen restimulation (data not shown).

The Effect of RSV Infection on Allergic Responses in C57BL/6 Mice Is Not Dependent on Timing of Infection

To extend our understanding of what conditions provided protection to allergen challenge during RSV infection, we focused our studies on the responses in C57BL/6 mice. To study whether the effect of RSV infection in C57BL/6 mice is dependent on simultaneous exposure of mice to both RSV and CRA allergen, a study was established to examine different timing of RSV infection. Instead of exposing mice to RSV simultaneously with allergen, mice were infected with RSV 3 weeks before or after the first day of CRA sensitization as indicated in Figure 2A. Mice were then given their final allergen challenge and assessed for changes in airway hyperreactivity. Time points were determined by the kinetics of RSV infection and CRA sensitization. Acute manifestations of RSV infection had mostly disappeared by 14 to 21 days, and RSV G protein RNA was not detectable in lungs from C57BL/6 and BALB/c mice from day 14 on (data not shown). This indicates that in our model, RSV does not seem to be persistent. RSV infection, when given before or after sen-

sitization, was able to inhibit allergen-induced airway hyperresponsiveness (Figure 2B), in a similar manner as with the simultaneous exposure of allergen. Thus, no matter the timing, RSV is able to inhibit allergic responses in C57BL/6 mice.

IFN- γ Neutralization Partially Restores Allergic Responses after RSV Infection in C57BL/6 Mice

Next, studies determined whether the observed effects of RSV infection on the allergic responses in C57BL/6 mice were mediated via IFN- γ . IFN- γ was neutralized by a specific antibody during RSV infection and CRA sensitization. This antibody did not affect CRA sensitization (Figure 3). As shown before, RSV infection blocked the development of airway hyperresponsiveness, eosinophilia, and production of Th2 cytokines (Figure 3A). However, the airway response after IFN- γ neutralization in RSV and CRA-treated mice was significantly elevated compared with RSV-infected CRA-treated control mice. In addition, the number of eosinophils in the bronchoalveolar lavage (BAL) was significantly elevated in animals treated with anti-IFN- γ compared with RSV-infected CRA-treated mice (Figure 3B). Furthermore, blockade of IFN- γ also significantly elevated IL-4, IL-5, and IL-13 levels after RSV infection and CRA challenge compared to control antibody-treated mice. Finally, a limited analysis of histology of lung sections showed a reduction of inflammation in the lung after RSV infection and CRA sensitization and challenge (Figure 4A). In CRA animals this inflammation consisted of mononuclear cells and eosinophils (Figure 4B). Again, neutralization of IFN- γ restored the inflammation in the RSV-infected and CRA-challenged mice. Clearly, IFN- γ participated in the RSV-mediated protection on CRA sensitization and challenge, whereas neutralization of IFN- γ partially restored the allergic response in RSV-treated mice.

Transfer of CD4 and CD8 T Cells of RSV-Infected Mice Decreases Allergic Responses

To investigate further the underlying immunological mechanism of the above described effects, C57BL/6 mice were infected with RSV, and CD4 or CD8 T cells from these mice were transferred into CRA-sensitized mice shortly before final challenge with CRA (Figure 5A). Transfer of CD8 T cells but not CD4 T cells significantly ameliorated the airway hyperreactivity after CRA challenge (Figure 5B). In addition, transfer of CD8 T cells from RSV-infected mice inhibited CRA-induced IL-5 and IL-13 production by lung lymph node cells (Figure 5C). Transfer of CD4 T cells partially inhibited only IL-5 production. Correspondingly, the transfer of either CD4 or CD8 T cells from RSV-infected mice significantly increased the production of IFN- γ , with CD8-cell transfer having the greatest effect.

Figure 3. Influence of IFN- γ on RSV-mediated changes on allergen sensitization and challenge. C57BL/6 mice were RSV infected (RSV); allergen sensitized (CRA); allergen sensitized and anti-IFN- γ treated (CRA + anti-IFN- γ); RSV infected and allergen sensitized (CRA + RSV); or RSV infected, allergen sensitized, and anti-IFN- γ treated (CRA + RSV + anti-IFN- γ). **A:** Airway responses were measured after one dose of methacholine and compared to basal measurements. **B:** Lung lavage was performed, and eosinophils were counted in stained cytopsin slides. **C:** Lung lymph node cells were restimulated with allergen, and cytokines were measured in culture supernatants after 24 hours. Data are represented as mean airway resistance in cmH₂O/ml/second, number of cells, or ng/ml $\pm$ SEM, respectively. * P < 0.01 compared to RSV-infected control mice, # P < 0.05 compared to CRA-sensitized mice, α P < 0.05 compared to CRA + RSV-sensitized mice, n = 5 mice.

Discussion

Epidemiological studies that examine the influence of RSV infection on the establishment of allergy and asthma have been inconclusive and in some cases have demonstrated opposing results.^{1,12,27–30} In the present study, an allergic asthma model was used in which both the allergen sensitization and challenge consisted of airway-only administration, without the use of adjuvant. This study showed that RSV infection in two different mouse strains with their distinct immunological progression leads to opposite effects of viral infection on allergic, asthma-like responses. The different responses in these two mouse strains has previously been identified with allergic responses.³¹ However, because RSV initially infects epithelial cells, the different responses we observe in C57BL/6 and BALB/c mice might be a difference in epithelial cell activation. These cells can secrete a number of inflammatory cytokines and chemokines, which influence the subsequent immune response.³² Differential chemokine expression after respiratory virus infection reflects Th1- or Th2-biased immunopathology and may result in the recruitment of specific T-cell subsets. The outcome of this response after allergen exposure or RSV infection, is dependent on the balance between the direct effects of RSV infection on antigen-presenting cells and the indirect effects of RSV infection through the production of inflammatory cytokines and chemokines from RSV-infected airway resident cells, such as epithelial cells and macrophages.³³ The effects observed in the present

study, however, are clearly defined by the type of T-cell-derived cytokines that are produced regardless of the chemokine environment that is generated.

Some suggest that C57BL/6 mice appear to be less susceptible to RSV infection.²¹ In contrast, others reported no difference in RSV-induced airway responses between BALB/c and C57BL/6 mice.³⁴ In our own studies, we have published distinct differences in the nature and level of immune responses elicited by RSV in BALB/c versus C57BL/6 mice. Although the BALB/c mice respond with an IL-13-associated response,^{17,20,35} the C57BL/6 mice appear to mount an efficient anti-viral immunity that is reliant at least on IL-12.³⁶ Additional studies have demonstrated that the heightened allergen responsiveness found in the BALB/c mice on RSV infection is associated with increases in IL-13 production.¹⁷ Thus, the balance of Th1- versus Th2-type cytokines appears to be critical in shaping the immune environment of the lung. These concepts help our understanding of why and under what circumstances these events occur. Furthermore, these studies demonstrated that RSV infection significantly inhibited allergic responses no matter what time point the virus was given in C57BL/6 mice (before, after, or during allergen sensitization). However, because both allergen exposure and RSV infection likely occur simultaneously in patients, a model that utilizes infection during allergen sensitization may best reflect the clinical situation.

The concept of whether early infectious processes in infants are protective has been consolidated into a still

A

B

Figure 4. Influence of IFN- γ on RSV-mediated changes on allergen-induced airway inflammation. C57BL/6 mice were RSV infected (RSV); CRA sensitized (CRA); CRA sensitized and anti-IFN- γ treated (CRA + anti-IFN- γ); RSV infected and CRA sensitized (CRA + RSV); or RSV infected, CRA sensitized, and anti-IFN- γ treated (CRA + RSV + anti-IFN- γ). Lungs were isolated and processed, and sections were stained with H&E. Shown are representative lung sections. **Arrows** indicate eosinophils. Original magnifications: $\times 200$ (**A**); $\times 400$ (**B**).

developing hygiene hypothesis.^{9,37} Although no set of studies has clearly indicated that there is a link between early childhood exposure to specific infectious agents and asthma or atopy, strong correlations have been identified between specific immune environments and disease severity. It is more likely that an interplay between genetic predisposition and environmental factors determine whether an individual progresses toward an asthmatic phenotype.³⁸ Factors that may be protective in

individuals that are low risk, ie, from families with no history of atopy or asthma, are likely not protective factors in individuals that have a higher genetic predisposition. Thus, it may be difficult to have a consolidated model that can be generally applied to an entire population.

An abundance of clinical data has been generated examining whether a specific virus is linked to development of atopy and/or asthma.^{7,11,29,39–43} Although this may be epidemiologically interesting, perhaps it limits our

Figure 5. CD4 and CD8 T cells from RSV-infected mice mediate changes on allergen sensitization and challenge. C57BL/6 mice were RSV infected, and CD4 or CD8 T cells were isolated from lung lymph nodes. **A:** CRA-sensitized mice were injected by tail vein intravenously with vehicle alone (CRA), CD4 T cells (CRA + CD4⁺), or CD8 T cells (CRA + CD8⁺). **B:** Airway responses were measured after one dose of methacholine and compared to basal measurements. **C:** Lung lymph node cells were restimulated with CRA, and cytokines were measured in culture supernatants after 24 hours. Data are represented as mean airway resistance in cmH₂O/ml/second or ng/ml $\pm$ SEM, respectively. **P* < 0.01 compared to RSV-infected control mice, #*P* < 0.05 compared to CRA-sensitized mice, *n* = 5 mice.

interpretation of the responses and the type of respiratory infection may not matter as much as the initiation of the local immune environment. In the present set of studies, like other similar investigations, the least pathogenic response to RSV infection appears to be linked to increased Th1 responses and IFN- γ production leading to a protective immune environment. This appears to be controlled partially by the activation of CD8 T cells. The CD8 T-cell responses have multiple roles including clearance of the viral infection and establishment of a type 1 immune environment.⁴⁴ In line with our results are data generated from a C57BL/6 mouse model that demonstrated that influenza-specific effector memory CD8 T cells inhibited allergic responses by antigen-independent production of IFN- γ .⁴⁵ In contrast, transfer of CD8 T cells from RSV-infected BALB/c mice enhanced allergic airway sensitization.⁴⁶ Interestingly, the percentage of CD4 and CD8 T cells expressing IFN- γ was lower in BALB/c than in C57BL/6 mice after RSV infection.²³ These studies together with the present data suggest a model in which induction of CD8 T cells in C57BL/6 mice not only leads to protective immunity to RSV but also to diminished development of allergic responses. The IFN- γ -mediated responses in C57BL/6 mice might alter the infiltration, maturation, and differentiation of Th2-type cells having a direct impact on the airway itself. In addition, because timing of the viral infection was not limiting, the IFN- γ -associated Th1 environment might also alter the established Th2 immune responses by affecting eosinophilic cell accumulation, survival, and/or activation.^{47,48} The combination of all of these effects could

significantly impact on the outcome of the developing responses.

These studies clearly demonstrate that whereas RSV can alter and predispose some mice (BALB/c) to a more severe allergic response, it also has the ability to protect other genetically different animals (C57BL/6) from a similar allergic response. Although mouse models have their limitations, the different responses observed in this study may be valuable for modeling and studying human disease phenotypes.

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