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Role of Interleukin-12 and Stat-4 in the Regulation of Airway Inflammation and Hyperreactivity in Respiratory Syncytial Virus Infection

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Respiratory syncytial virus (RSV) is a respiratory pathogen that can cause significant morbidity in infants and young children. Interestingly, the majority of children who acquire a RSV infection do not exhibit severe symptoms. Development of a Th1 response has been associated with resolution of symptoms in viral infections and may explain mild RSV illness. The current study investigated the cytokine response observed in mild disease in C57BL/6 mice that had low airway resistance and mucus production with little pulmonary inflammation. RSV infection in these mice was accompanied by a fourfold increase in interleukin-12 (IL-12). Treatment of RSV-infected mice with anti-IL-12 resulted in an increase in airway hyperreactivity, mucus production, and airway inflammation (eosinophilia). Since IL-12 activation is dependent on Stat-4-mediated intracellular signal transduction, similar experiments were performed in Stat-4 deficient mice and demonstrated similar results to those obtained from anti-IL-12 treated mice. Again, there was an increase in airway hyperreactivity and mucus production, and goblet cell hypertrophy. These studies support the importance of IL-12 in the immune response to RSV infection resulting in resolution of disease and protection from inappropriate inflammatory responses. *(Am J Pathol 2001, 159:631–638)*

RSV is an important viral pathogen that causes annual epidemics worldwide.[1] It is responsible for a large proportion of hospitalizations of infants in the winter months in the United States and consumption of millions of health care dollars. There can be significant morbidity associated with RSV bronchiolitis, both acutely and chronically, as a link between RSV bronchiolitis and later development of asthma has been proposed.[1–3] Mortality rates up to 5% have been reported with the highest rates in infants and young children with congenital heart disease and chronic lung disease. In severely immunocompromised children and adults, mortality rates can climb to near 90%.[4] Studies have identified specific cytokines involved in the immune response to RSV, but their importance related to physiological responses observed has not been fully elucidated. Further complicating the situation, and likely resulting in varied reports of "important" cytokines involved in the immune response to RSV, is the observation that not all humans respond the same to RSV infection. There is a wide spectrum of clinical illness and symptoms in infants and children infected with RSV. Symptoms can range from a mild cold-like illness to severe respiratory distress and death even in previously healthy children.[5–9] A spectrum of physiological symptoms also occurs in genetically different mouse strains[10–12] with BALB/c and DBA/2J strains having pronounced airway pathology in response to the virus and C57BL6 mice having a much milder response.

The present studies examined the role of IL-12 in the immune response to RSV infection in a mouse model using C57BL/6 mice. IL-12 is important in the initial phase of bacterial, parasitic, and viral infections, and for the development of the T helper type 1 (Th1) response.[13–17] Production of IL-12 is thought to favor differentiation and function of (Th1) T cells while inhibiting the differentiation of Th2 cells. In many viral infections, IL-12 promotes viral clearance and host recovery from infection.[18–21] The role of IL-12 in RSV infection in relation to airway hyperreactivity and mucus production has not been specifically addressed, but other studies have speculated on its importance in acute bronchiolitis in humans.[22] This study focused on the physiological and immune response to RSV infection in the C57BL/6 mouse strain, a strain that appears to have a favorable response to RSV.

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Materials and Methods

Animals

Specific pathogen-free C57BL/6 mice (H-2^b) and DBA/J mice (H-2^d) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in University of Michigan animal facilities under pathogen-free conditions. Stat-4 deficient mice were grown and maintained by Dr. Mark Kaplan at Indiana University.

Virus

RSV A2 was grown and harvested in Dr. Maassab's lab at the University of Michigan School of Public Health. The virus had been through 48 passages in Hep-2 cells (human epidermoid carcinoma cells from the larynx). It was subsequently passed multiple times in Vero cells (green monkey kidney cells), MRC-5 cells (human lung cells), and Hep-2 cells for growth and amplification of the virus.

Infection

Pathogen-free mice were infected intratracheally with 3×105 plaque- forming units (PFU) in 30 μl media. Control mice received 30 μl of vehicle intratracheally. Mice were anesthetized with sodium pentobarbital (50 mg/kg) and ketamine (40 mg/kg) injected intraperitoneally. A tracheostomy was performed and RSV was injected directly into the trachea with a Hamilton syringe (Reno, NV). Following infection, the incision was closed with surgical staples and mice were allowed to recover. No mice died from this level of viral infection or from anesthesia.

Measurement of Airway Hyperreactivity

Airway hyperreactivity was measured using a Buxco mouse plethysmograph (Buxco, Troy, NY) which is specifically designed for low tidal volumes as previously described.[23,24] Briefly, the mouse to be tested was anesthetized as described above, and intubated via cannulation of the trachea with an 18-gauge metal tube. The mouse was placed on a Harvard pump ventilator (Harvard, Holliston, MA) (tidal volume 0.4 ml, frequency 120 breaths/min, positive end-expiratory pressure 2.5–3.0 cm H_2O) and was ventilated for 5 minutes before the methacholine challenge. The plethysmograph was sealed and readings monitored by computer. Since the box is a closed system, a change in lung volume was represented by a change in box pressure (P_{box}) that was measured by a differential transducer. The system was calibrated with a syringe that delivered a known volume of 2 ml. A second transducer was used to measure the pressure swings at the opening of the trachea tube (P_{aw}), referenced to the body box (ie, pleural pressure), and to provide a measure of transpulmonary pressure (P_{tp}) ($P_{tp} = P_{aw} - P_{box}$). The tracheal transducer was calibrated at a constant pressure of 20 cm H_2O. Resistance is calculated by the Buxco software by dividing the change in pressure (P_{tp}) by the change in flow (F) ($\delta P_{tp}/\delta F$; units = $cmH_2O/ml/s$) at two time points from the volume curve based on a percentage of the inspiratory volume. Once baseline levels were stabilized and initial readings were taken, a methacholine challenge was given intravenously via cannulation of one of the tail veins with a 27-gauge needle. A dose-response curve (0.01 to 0.5 mg/kg) was performed and an optimal dose of 0.1 mg/kg of methacholine was obtained. This dose was used throughout the rest of the experiments in this study. After the methacholine challenge, the response was monitored and the peak airway resistance was recorded as a measure of airway hyperreactivity.

Lung Homogenates

The right upper lobe from each mouse was flash-frozen in liquid nitrogen and kept frozen at −80°C. Just before running the ELISA assays, the samples were weighed and homogenized in 1 ml of homogenization buffer containing 1 Complete tablet (Boehringer Mannheim, Germany) and 0.1% Triton-X in 50 ml of phosphate-buffered saline (PBS).

ELISAs

Cytokines were quantitated from homogenized PBS lung aqueous extracts using a double ligand ELISA system. The murine ELISAs have been developed in our laboratories using a previously described method.[25] ELISAs were conducted as follows: flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F, Lincolnshire, IL) were coated with capture antibody diluted to 3.2 μg/ml in coating buffer (borate-buffered saline, pH 8.6) and incubated overnight at 4°C. Nonspecific binding sites were blocked with 2% bovine serum albumin (BSA) in PBS and incubated for 1 hour at 37°C. Plates were washed and specimens added in triplicate followed by incubation at 37°C and washing. Biotinylated detection antibody was added and the plates incubated at 37°C for 1 hour. Plates were washed and conjugated streptavidin-peroxidase was added, followed by washing and the addition of chromogen substrate (OPD). Finally plates were incubated at room temperature, the reaction terminated with 3 mol/L H_2SO_4 and read at 490 nm in an ELISA reader. The individual polypeptides were standardized to total protein (ng/μg total protein). Our ELISAs routinely detect protein at concentrations above 50 pg/ml. These ELISAs are specific and do not cross-react to any other chemokine or cytokine.

Production of Anti-IL-12 Antibodies

Rabbit anti-murine IL-12 antibodies were prepared by multiple-site immunization of New Zealand White rabbits with recombinant murine IL-12 (R&D, Rochester, MN) in Complete Freund's Adjuvant. Polyclonal antibodies were titered by direct ELISA and specifically verified by the failure to cross-react to mIL-3, mIL-1α, mTNF, mIL-4, hIL-12, mIL-10, mMIP-1α, IL-6, mJE, mMIP-1β, hMCP-1, hIL-8, hRANTES, hMIP-1α, hTNF, and hMIP-1α. The IgG

portion of the serum was purified over a protein A column and used in a sandwich ELISA. Whole serum (0.5 ml) was used *in vivo* to block IL-12 during the RSV infection.

In Vivo *Neutralization of IL-12*

Neutralization of IL-12 was carried out using a polyclonal rabbit anti-murine IL-12 antibody developed in our laboratory as above. The anti-IL-12 or control antibody was administered intraperitoneally 1 hour before infection and every 2 days postinfection until day 14. The *in vivo* half-life of the antibody was ~30 hours.

BAL Microscopy

Bronchoaveolar lavage samples (BAL) samples were collected from each mouse just after airway hyperreactivity data were collected and after cervical dislocation. 1 ml of sterile 0.9 N saline was instilled intratracheally and was suctioned out after a few seconds. Samples were placed in Eppendorf tubes and centrifuged for 1500 rpm for 5 minutes. Supernatant was removed and cells were resuspended in PBS and cytospin-fixed and stained with Diff-Quick (Dade Behring Ag., Dudingen, Switzerland).

Morphometric Analysis of Peribronchial Eosinophil Accumulation

Mouse lungs from each time point were preserved in 1 ml of 4% paraformaldehyde. The fixed lungs were embedded in paraffin, and multiple 50-μm sections were differentially stained with hematoxylin-eosin for the identification of eosinophils. Eosinophils were quantified by counting 100 hpf/lung using multiple step sections of the lung. The eosinophils counted were only in the peribronchial region to assure enumeration of only those eosinophils within or immediately adjacent to the airway.

Goblet Cell Hypertrophy

Periodic acid-Schiff (PAS)-stained lung sections (prepared as above) were used to assess goblet cells in the small-to-moderate sized airways. Goblet cell hypertrophy was assessed qualitatively and quantitatively (counting numbers per airway).

Statistics

Numerical results were expressed as means ± SEM. Analysis of variance was used to determine the level of difference between groups. Pairs of groups were compared by unpaired two-tailed Student's *t*-test. Analysis of variance was used to compare changes between different strains with same treatment and significance was determined with P values <0.05.

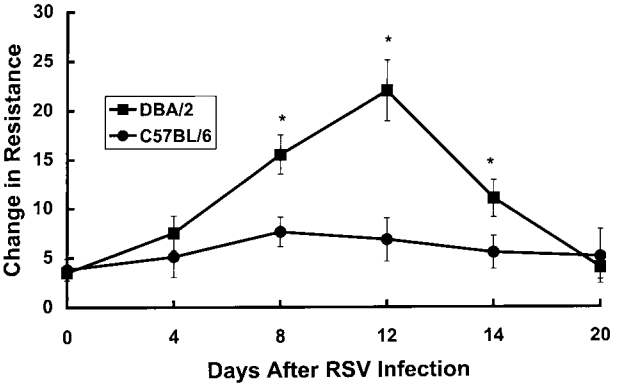

Figure 1. Airway hyperreactivity in C57BL6 (H-2b) and BALB/c (H-2d) strains of mice after RSV infection. Change in airway resistance between baseline values and after challenge with 0.4 mg/kg methacholine intravenously measured by plethysmography, $n = 10$ per strain for each time point (Resistance units = cm H_2O/ml/sec). Values represent means ± SEM; experiment was repeated three times with similar results obtained for each. * Significant changes ($P < 0.05$) in airway hyperreactivity.

Results

Airway Hyperreactivity after RSV Infection in Genetically Different Mouse Strains

The hypothesis that host genetic difference affects response to RSV was tested by comparing airway hyperreactivity (AHR) responses and BALs in C57BL/6 (H-2^b) mice to those in DBA/2 mice (H-2^d) during primary RSV infection. Pathophysiological response to RSV was determined by measuring change in airway resistance after a methacholine challenge. Comparison of the strains showed that the BALB/c and DBA/2 mice had significantly higher airway hyperreactivity over almost the entire time course after RSV exposure when compared to the C57BL/6 mice (Figure 1). Increased hyperreactivity was apparent by day 8 in DBA/2 mice, continued to increase to day 12, and then declined to baseline by later time points postinfection. The C57BL/6 mice had minimal change in hyperreactivity over the same postinfection time course. Interestingly, C57BL/6 and DBA-2 mice had similar levels of virus on day 4 of infection with no evidence of productive virus at the time of peak airway hyperreactivity at day 12 (data not shown).

Examination of BALs from RSV-infected mice showed minimal epithelial sloughing and little mucus production in the C57BL/6 mice with a prominent increase in DBA/2 mice (data not shown). Epithelial cell sloughing was most prominent early postinfection and increasing mucus production correlated with increasing hyperreactivity. Lung histology revealed similar findings with more inflammation observed in the DBA/2 mice (data not shown). Thus, the C57BL/6 mice appeared to respond more appropriately to the RSV infection with lower pathophysiologic abnormalities.

Immune Cytokine Profile in Mice with Mild Response to Primary RSV Infection

The pulmonary physiological response observed in the different mouse strains may be due to the cytokine envi-

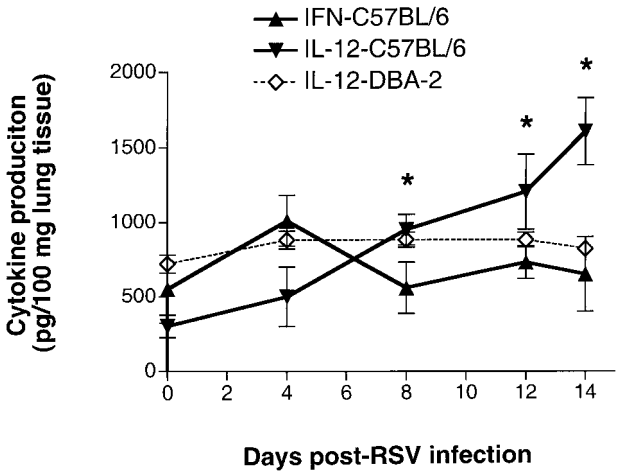

Figure 2. Th-1 cytokines produced in C57BL/6 mouse lungs after RSV infection. Cytokines were measured by ELISA assay from lung homogenates. Each time point represents mean ± SEM from 10 mice. * Significant differences ($P < 0.05$) from baseline values.

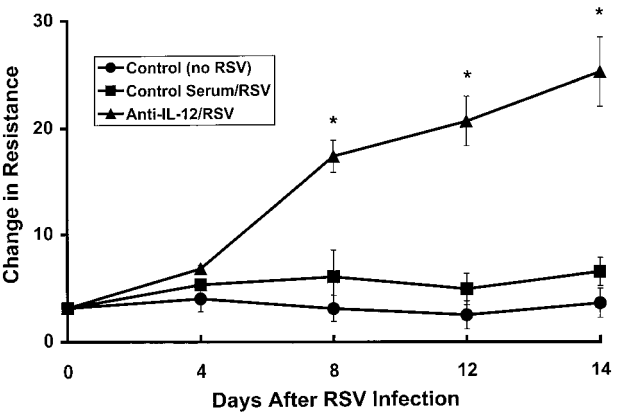

Figure 3. Airway hyperreactivity in control C57BL/6 mice versus anti-IL-12 treated mice after RSV infection. Graph shows control (intratracheal vehicle only) versus RSV control (normal serum injected IP) versus RSV (anti-IL-12 IP). Each time point represents mean ± SEM for 8 mice with experiment repeated three times with similar results obtained for each. * Significant differences ($P < 0.05$) between normal serum control/RSV mice and anti-IL-12 treated mice.

ronment established at the site of infection. In these studies, we were interested in examining the basis for the mild pathophysiological response to RSV seen in the C57Bl/6 mouse strain. Cytokine profiles were determined by running ELISA assays on whole lung homogenates. Cytokines examined included interferon-γ, IL-4, IL-12, and IL-13. The production of IFN-γ showed an increase early in the response while IL-12 production was up-regulated at later time points; specifically, at day 8 through day 14 of infection (Figure 2). IL-13 and IL-4 showed slight decreases over the same time period (data not shown). Increased IL-12 levels appeared to correlate with mild response to RSV infection suggesting that it may be an important cytokine involved in the immune response to RSV in the C57BL/6 mice. Accordingly, IL-12 production in lungs of infected DBA/2 mice did not increase over the same period of infection over background levels, ranging from 0.65 to 0.85 ng/lung on the various days (Figure 2).

Neutralization of IL-12 Causes Increased Airway Hyperreactivity, Increased IL-13 Production, and Mucus Production

IL-12 is known to be an important immunoregulatory cytokine favoring differentiation and function of Th1 cells while inhibiting differentiation of Th2 cells.[14,26] Although there is not a great deal of information available on the importance of IL-12 in RSV infection, several investigators have suggested that it may have an immunomodulatory role.[10,22,27] To confirm that IL-12 was in fact the cytokine responsible for the mild response to RSV, antibodies specific for IL-12 were used to neutralize the cytokine *in vivo.* Neutralizing anti-IL-12 or control antibodies were given intraperitoneally 1 hour before intratracheal RSV (3×10^5 PFU) and every other day thereafter until day 12 of infection. The mice were then examined for changes in airway hyperreactivity at specific time points after infection (Figure 3). The mice treated with neutralizing antibodies against IL-12 had a significant increase in airway hyperreactivity, whereas untreated mice and mice treated with control antibodies had minimal airway hyperreactivity, comparable to previous experiments. IL-12 levels were reduced to baseline values in neutralized animals (data not shown) while IL-13 levels showed a slow increase with peak levels observed on day 12 (Figure 4). In control, uninfected mice, anti-IL-12 had no effect on airway hyperreactivity (data not shown). Microscopic examination of bronchoalveolar lavage fluid demonstrated minimal mucus production in control serum-treated mice and untreated mice, with a large amount of mucus production seen in the anti-IL-12-treated animals at days 8 and 12 (Figure 5). Histologically, the anti-IL-12-treated mice had increased inflammation (Figure 6) with a significantly greater number of pulmonary eosinophils (Figure 6). PAS staining revealed increased numbers of mucus-producing goblet cells in anti-IL-12 treated mice compared to RSV/control mice (Figure 7). Thus, production of IL-12 during RSV infection appears to be an important

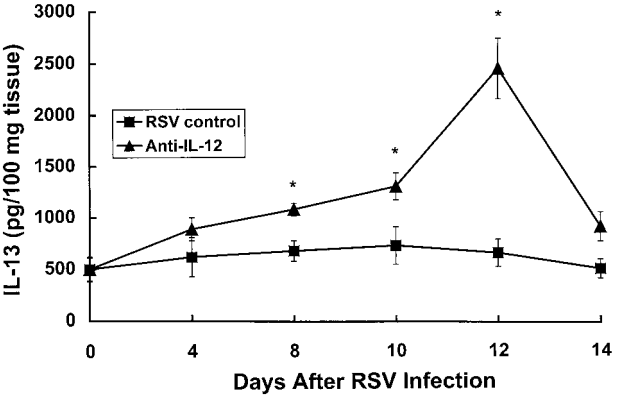

Figure 4. IL-13 production in control C57BL/6 mice versus anti-IL-12 treated mice. RSV control mice were injected intratracheally with RSV and intraperitoneally with control serum. Anti-IL-12 treated mice were injected IT with RSV and IP with anti-IL-12 antibody. Each time point represents mean ± SEM for 8 mice with experiment repeated three times with similar results obtained for each. * Significant differences ($P < 0.05$) between normal serum control/RSV mice and anti-IL-12 treated mice.

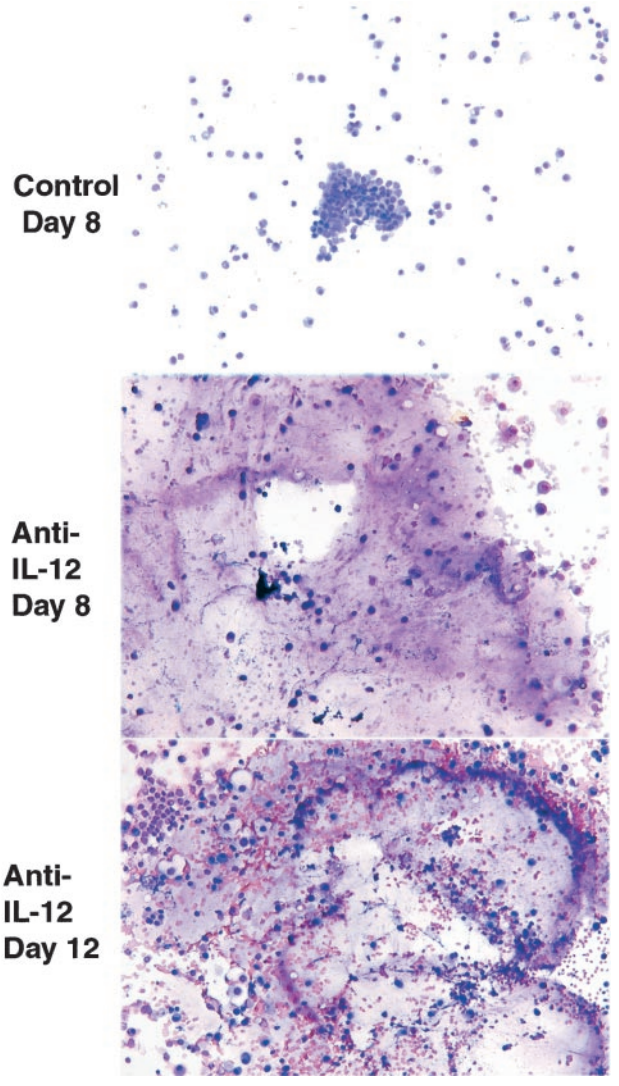

Figure 5. Light microscopy of BAL fluid from control C57BL/6 mice and anti-IL-12 treated mice after RSV infection. BALs were collected and processed as previously described using Diff-Quick stain. Magnification, ×400.

component in protecting the host from detrimental pathophysiologic responses.

Stat-4 Deficient Mice Have Pronounced Airway Hyperreactivity after RSV Infection

Th1 and Th2 lymphocytes are thought to be activated by specific cytokines resulting in a differentiated immune response. Binding of the cytokine to its receptor results in activation of its associated signal transduction pathways resulting in a unique response. The signal transducer and activator of transcription (Stat) family of proteins are involved in one of these intracellular signal transduction pathways. IL-12 activates T helper cells via a Stat-4-induced signaling pathway. To confirm the importance of IL-12 in abrogating severe airway hyperreactivity responses in RSV infection, we performed similar RSV infection experiments in Stat-4-deficient mice. Figure 8 shows that Stat-4−/− mice on a C57BL/6 background had significantly higher airway hyperreactivity responses compared to Stat 4+/+ mice. This pronounced effect was present as soon as day 4 after infection and persisted at day 12 which is similar to the effects observed in anti-IL-12-treated mice. Histologically, Stat 4 −/− mice had increased numbers of eosinophils and goblet cell hypertrophy when compared to RSV controls but not to the extent observed in anti-IL-12-treated C57BL/6 mice (data not shown).

Discussion

RSV causes airway epithelial cell damage, peribronchial inflammation, hyperreactivity, and increased mucus production leading to respiratory distress, hospitalization, and even death in those infants and small children most severely affected. Yet, most children who are infected develop only mild symptoms. Others have described cytokine mediators involved in the severe response,[28–30] but there is little information on mediators involved in an asymptomatic or mildly symptomatic infection. The current study was designed to study the cytokine(s) that were important in mild disease. Because IL-12 was the cytokine produced in the largest amount in these mildly "symptomatic" C57Bl/6 mice after RSV infection, this study focused on its importance in modifying the pathophysiology. Others have shown IL-12 to be related to the development of the Th1 immune response and cell-mediated immunity.[17,31,32] It also has been associated with viral clearance[18,33,34] in some cases independent of IFN-γ.[31,34,35] IL-12 has been shown to be inversely related to length of respiratory failure in children with RSV,[36] and decreased levels of IL-12 have been shown in humans with asthma and RSV bronchiolitis further supporting its role in preventing severe symptoms in airway disease.[22,37] Additional studies have demonstrated that exogenous IL-12 can attenuate allergen-induced airway hyperreactivity and decreases pulmonary eosinphilia in murine models.[38,39] Altogether, IL-12 appears to be a protective factor against the development of airway hyperreactivity and eosinophilia.

The intensity and nature of the response to RSV may be determined by the local cellular and cytokine response. The specific cytokines produced at the site of infection may then direct the immune response with the recruitment and activation of specific immune cells.[40] An alternative hypothesis might suggest that primary RSV infection drives an immature immune system toward a specific immune response that persists and acts in subsequent viral infections and on development of allergen sensitivity.[41–43] These responses can be broadly categorized into Th1 or Th2-like immune responses. RSV infection is thought to induce a Th2-like response in infected individuals who exhibit severe forms of the disease.[28,30] This immune response has also been observed in individuals with asthma and development of this Th2 type response may be the link between early RSV bronchiolitis and development of severe asthma. Prominent cytokines involved in Th2 responses are IL-4, -5, and -13. Mouse models of RSV infection in BALB/c (H-2d) mice after

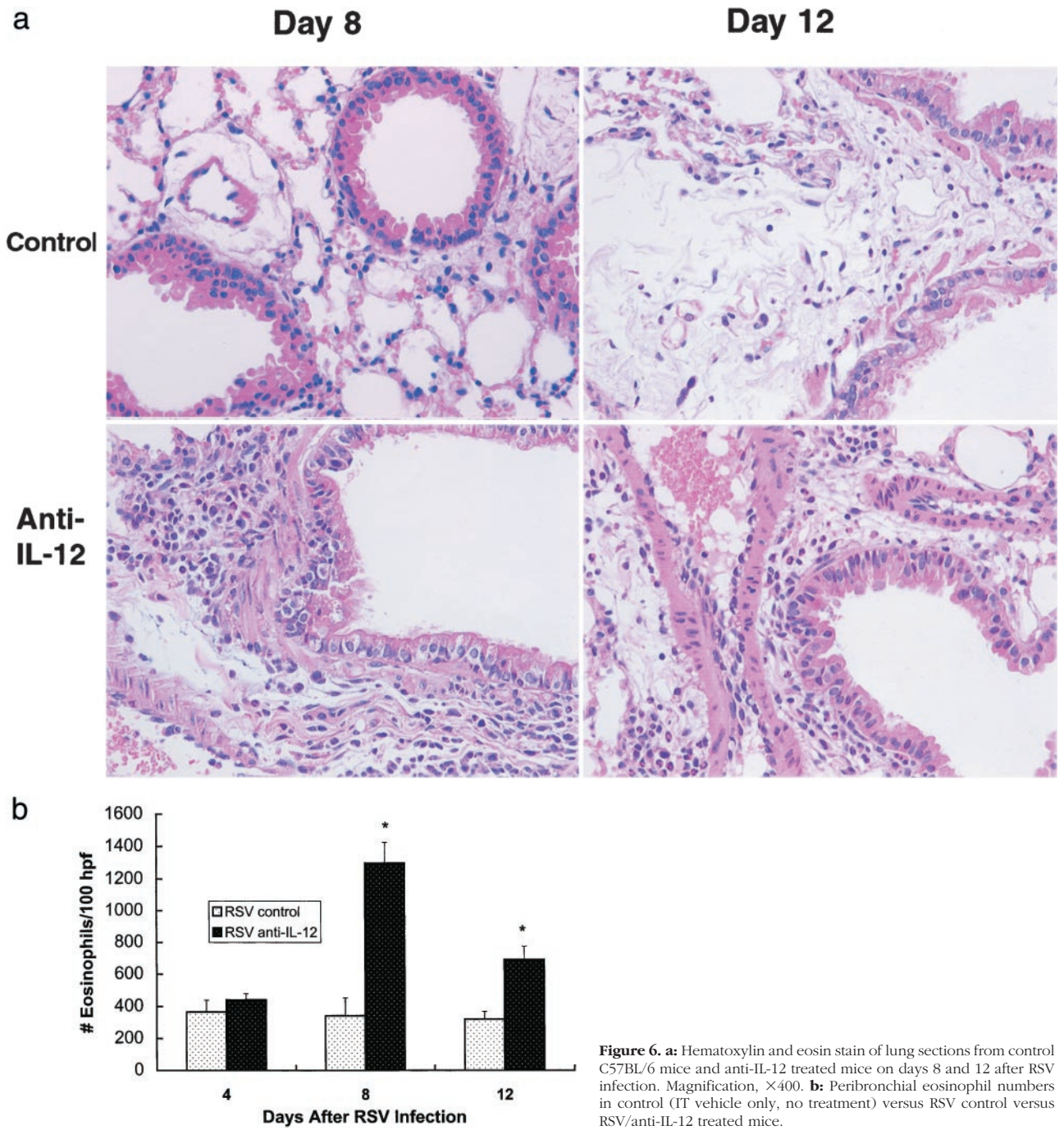

Figure 6. a: Hematoxylin and eosin stain of lung sections from control C57BL/6 mice and anti-IL-12 treated mice on days 8 and 12 after RSV infection. Magnification, ×400. **b:** Peribronchial eosinophil numbers in control (IT vehicle only, no treatment) versus RSV control versus RSV/anti-IL-12 treated mice.

sensitization with viral antigens, have demonstrated increased mucus production and increased airway hyperreactivity with a preponderance of Th2 cytokines.[44–46] IL-13 is a key cytokine involved in asthmatic responses and has been associated with increased mucus production and hyperreactivity.[47,48] In contrast, the Th-1 cytokine IL-12 observed in the infected mice in these studies is associated with pathophysiologically milder response to the infection. The current study has suggested that IL-13 production is inversely related to IL-12 production, possibly indicating an IL-12/IL-13 balance during RSV responses. We are presently addressing these issues with additional *in vitro* and *in vivo* studies. In support of this concept, recent studies in our laboratory have shown that IL-13 is elevated in DBA/2 and BALB/c (H-2d) mice during RSV infection.[49] These latter strains of mice have pronounced airway hyperreactivity and mucus production after RSV infection and low IL-12 levels. Neutralization of IL-13 in the DBA/J and BALB/c mice resulted in abrogation of airway hyperreactivity, increase in pulmonary IL-12 levels, and decreased evidence of mucus production suggesting that IL-13 is a key mediator of pulmonary pathology leading to morbidity in RSV infection. This correlates well with the rise in IL-13 before and

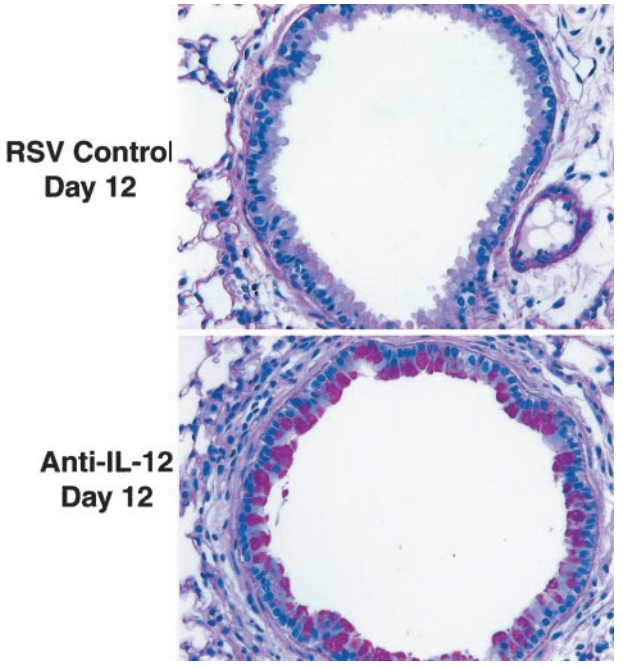

Figure 7. PAS stain of lung sections from control C57BL/6 mice and anti-IL-12 treated mice on day 12 after RSV infection.

during the most severe pathophysiologic responses, suggesting that IL-13 is inducing other mediators, such as mucus and eosinophil products, for the pulmonary dysfunction. The relationship between IL-13 and mucus production in models of RSV infection and asthma has been strongly established.[48,49] The reduction in IL-13 levels before the reduction in airway physiology may indicate the prolonged detrimental effect of the IL-13-induced responses similar to those found in the previous study in RSV-infected DBA-2 mice.[49]

The mechanism of IL-12 function in diminishing severe pathology in RSV and other viral diseases may be contentious. IL-12 binds to its receptor and through a Stat-4 signal transduction pathway, acts to increase IFN-γ.[50–52] The over-expression of IFN in the airway greatly diminishes the adverse affects observed in severe RSV infections.[53] Other studies have demonstrated that monocyte-derived IL-12 production is inversely related to duration of respiratory failure[54] and IL-12-induced NK cells can reduce lung eosinophilia and do not enhance disease severity.[55] Interestingly, administration of IL-12 during RSV antigen vaccination reduced subsequent Th2 responses, but did not improve vaccine-enhanced RSV lung illness.[56] There are a number of pathways in addition to IFNγ production that IL-12 and Stat-4 may be affecting during the pulmonary responses. IL-12 may be acting directly on recruited leukocytes to enhance viral clearance by activating effector cell populations.[31,34,35,41] This could be occurring via the recruitment and activation of NK cells and/or CD8+ cytotoxic T cells. Another pathway may function via the regulation of other cytokines by IL-12, such as IL-13, and maintaining an effective anti-viral response in the lungs of infected animals. Whatever the specific mechanism, IL-12 appears to modulate adverse responses, including mucus production, eosinophil accumulation and airway hyperreactivity. Thus, administration of exogenous IL-12 may have therapeutic applications, especially in those individuals who have low endogenous levels.

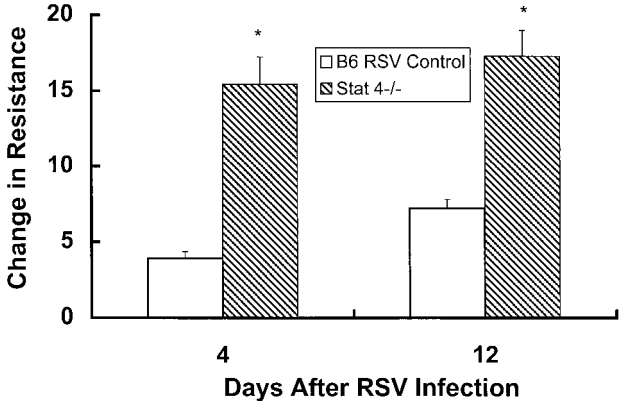

Figure 8. Airway hyperreactivity in Stat4 +/+ mice versus Stat 4 −/− mice after RSV infection. Change in airway resistance measured as described in Materials and Methods section and Figure 1. Each time point represents mean ± SEM from 10 mice. * Significant differences ($P < 0.05$) from baseline values.

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