IL-13-Induced Airway Hyperreactivity During Respiratory Syncytial Virus Infection Is STAT6 Dependent

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IL-13-Induced Airway Hyperreactivity During Respiratory Syncytial Virus Infection Is STAT6 Dependent

Kim K. Tekkanat,* Hunein F. Maassab,‡ David S. Cho,‡ Joyce J. Lai,‡ Alison John,‡ Aaron Berlin,† Mark H. Kaplan,§ and Nicholas W. Lukacs*†

Airway damage and hyperreactivity induced during respiratory syncytial virus (RSV) infection can have a prolonged effect in infants and young children. These infections can alter the long-term function of the lung and may lead to severe asthma-like responses. In these studies, the role of IL-13 in inducing and maintaining a prolonged airway hyperreactivity response was examined using a mouse model of primary RSV infection. Using this model, there was evidence of significant airway epithelial cell damage and sloughing, along with mucus production. The airway hyperreactivity response was significantly increased by 8 days postinfection, peaked during days 10–12, and began to resolve by day 14. When the local production of Th1- and Th2-associated cytokines was examined, there was a significant increase, primarily in IL-13, as the viral response progressed. Treatment of RSV-infected mice with anti-IL-13 substantially inhibited airway hyperreactivity. Anti-IL-4 treatment had no effect on the RSV-induced responses. Interestingly, when IL-13 was neutralized, an early increase in IL-12 production was observed within the lungs, as well as a significantly lower level of viral Ags, suggesting that IL-13 may be regulating an important antiviral pathway. The examination of RSV-induced airway hyperreactivity in STAT6−/− mice demonstrated a significant attenuation of the response, similar to the anti-IL-13 treatment. In addition, STAT6−/− mice had a significant alteration of mucus-producing cells in the airway. Altogether, these studies suggest that a primary factor leading to chronic RSV-induced airway dysfunction may be the inappropriate production of IL-13. The Journal of Immunology, 2001, 166: 3542–3548.

Asthma is one of the fastest-growing diseases in developed countries, especially in young children (1, 2). The explosion of asthma cases in hospital emergency rooms, especially in poor urban areas, is having a devastating effect on the health of children and is putting a strain on health system resources. Asthma-like responses can be induced and/or exacerbated by viral infections that impact lung function through a combination of viral- and inflammation-induced damage (3–9). Recent evidence suggests that respiratory viral infections are one of the most common causes of asthma exacerbations. Respiratory syncytial virus (RSV)3 is known to cause asthma exacerbations, and in many young children less than 2 years of age. RSV infections can significantly alter airway function, leading to long-term airway hyperreactivity. The mechanisms that promote these long-term pulmonary problems are not clear; however, it appears that there may be a genetic predisposition toward the development of a specific immunologic response. The clinical evidence of varied responses to RSV infection supports this contention; i.e., not all children respond in a detrimental way, and most appear to clear the virus appropriately. This suggests that a particular disease phenotype may be associated with RSV responses in the lung. Recent evidence has indicated that cytokines produced during an allergic or infectious response dictate how the pulmonary response will proceed (4, 10). If the individual responds to a viral infection with a battery of Th1-type cytokines, including IL-12 and IFN, a cellular immune response is promoted, and the virus is quickly disposed of (11–15). In contrast, if a Th2-type response, including IL-4 and IL-13, is initiated, the antiviral defense may not be sufficient, and the appropriate cellular immune responses may be attenuated. These preferential cytokine profiles during viral infections can also be observed using in-bred mouse strains. In fact, a recent study has demonstrated that mice respond to RSV in a MHC-dependent manner (16). Thus, much information may be gained using animal models of infection to decipher immune responses to RSV.

It is the latter response for which researchers have begun to identify pharmaceutical targets that alter detrimental cytokine responses in the lung. This may help alleviate the long-term lung damage that initiates and/or maintains chronic lung problems. Recent evidence has identified several cytokines that may participate in setting up the appropriate inflammatory milieu that allows the progression of lung damage after RSV infection, leading to long-term pulmonary problems. In particular, IL-13, produced during allergic asthma, has been associated with increased mucus production, goblet cell hypertrophy, and airway hyperreactivity in animal models (17, 18). This cytokine may also be an essential molecule that contributes to the prolonged pulmonary responses leading to lung damage and airway hyperreactivity during RSV infection, thus setting the stage for long-term lung hyperresponsiveness. In these studies, the experiments were designed to examine the role of IL-13 as a pivotal cytokine for altering the anti-viral immune response, as well as in promoting a pulmonary environment that proceeds with lung dysfunction. A number of important features of...
the RSV infection model were examined after neutralization of IL-13, including airway hyperreactivity and mucus production.

Materials and Methods

Animals

Specific pathogen-free DBA/2J mice (H-2b) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in University of Michigan animal facilities under pathogen-free conditions. STAT6−/− BALB/c mice (H-2b) were grown and maintained by Dr. Mark Kaplan at Indiana University (Indianapolis, IN).

Virus and infection

DBA/2J mice were infected intratracheally with 30 μl (3 × 106 PFU) of human (h) RSV A2 strain wild type that was propagated in Vero cells. The virus was mycoplasma free. The level of cytokine and chemokines in the stock solution, including IL-13, was below detectable levels in Vero cells. The virus was mycoplasma free. The level of cytokine and chemokines in the stock solution, including IL-13, was below detectable levels in Vero cells. The virus was mycoplasma free. The level of cytokine and chemokines in the stock solution, including IL-13, was below detectable levels in Vero cells. The virus was mycoplasma free. The level of cytokine and chemokines in the stock solution, including IL-13, was below detectable levels in Vero cells.

Measurement of airway hyperreactivity

Airway hyperreactivity was measured using a Buxco (Troy, NY) mouse airway plethysmograph. The plethysmograph was sealed, and readings were recorded as a measure of airway hyperreactivity. 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.

Determination of RSV Ags in lungs of infected mice

Total RSV Ag levels in lung homogenates were measured by a specific ELISA as previously described (19, 20). Briefly, the lung samples (50 μl) were incubated in goat anti-RSV Ab (Ab1128; Chemicon, Temecula, CA)-coated 96-well plates for 60 min. After a three-step wash, the plates were incubated with a secondary Ab, a mouse anti-RSV polyclonal Ab (NCL-RSV3; Vector Laboratories, Burlingame, CA). After a final incubation with a peroxidase-labeled goat anti-mouse IgG Ab (Boehringer Mannheim, Indianapolis, IN), positive wells were assessed by tetramethylbenzidine development as the substrate. OD readings were determined at 490 nm. A standard line was constructed with samples of known viral titers, and the OD readings were used for comparison on the standard line, giving results that represent viral Ag load. It appears that the viral Ag peaks after known infectious particles in the lung and correlates to the peak in the immune response and damage. The accuracy of the ELISA in lung homogenates was verified by spiking lung samples with known quantities of virus and demonstrating recovery of the spiked sample.

Results

Neutralization of IL-13 was conducted using a polyclonal rabbit anti-mIL-13 Ab developed in our laboratory as described above. The anti-IL-13 or control Ab was administered i.p. 1 h before infection and every 2 days postinfection until day 12. The in vivo half-life of the Ab was ~30 h.

Plaque-forming assay

Plaque assays were performed in a manner similar to that previously described (24). Lungs were removed and snap frozen. Tissues were homogenized on ice, and the debris was removed by centrifugation. Supernatants were added onto subconfluent Hep G2 cell monolayers in 12-well plates (Costar, Corning, Corning, NY). After 1 h of gentle agitation, plates were incubated at 37°C and washing. Biotinylated detection Ab was added, and the plates were incubated at room temperature, and the reaction was terminated with 3 M H2SO4 and read at 490 nm in an ELISA reader. The individual polypeptides were standardized to total protein (nanograms per microgram 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-13 Abs

Rabbit anti-murine IL-13 Abs were prepared by multiple-site immunization of New Zealand White rabbits with murine (m) rIL-13 (R&D Systems, Rochester, MN) in CFA. Polyclonal Abs were titered by direct ELISA and were specifically verified by the failure to cross-react to mIL-3, mIL-1α, mTNF, mIL-4, hIL-13, mIL-10, mIL-12, murine macrophage inflammatory protein (MIP)-1α, IL-6, murine monocyte chemotactant protein-1, mMIP-1β, human monocyte chemoattractant protein-1, hMIP-1α, hMIP-1β, hMIP-1β, and hMIP-1β. 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-13 during the RSV infection.

In vivo neutralization of IL-13

Animals

Specific pathogen-free DBA/2J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in University of Michigan animal facilities under pathogen-free conditions. STAT6−/− BALB/c mice (H-2b) were grown and maintained by Dr. Mark Kaplan at Indiana University (Indianapolis, IN).
mice had significant changes that began by day 8, continued to increase at day 10, peaked by days 12–14, and was back near baseline by day 20 postinfection. BALB/c/J (H-2d) mice demonstrated airway hyperreactivity changes similar to those of the DBA/J (H-2d) mice. The instillation of heat-killed virus induced none of the changes observed above (data not shown). Other strains of mice including C57BL6/J (H-2b) and CBA/J (H-2k) demonstrated relatively mild RSV-induced responses (data not shown). Altogether, these data indicate significant airway damage and altered airway physiology after RSV infection in mice with an H-2d haplotype. These data correlate well with previous data suggesting that RSV-induced responses are MHC restricted (16). Thus, we continued to examine these latter strains of mice to determine the mechanism of how they respond to RSV.

IL-13 expression during RSV infection

Previous investigations have determined that the severity of RSV-induced damage may center on the cytokine environment established during RSV infection. To address this issue, the lungs from RSV-infected mice were homogenized and examined for levels of specific cytokines. A number of Th1- and Th2-associated cytokines were analyzed including IL-4, IL-13, and IFN-\(\gamma\). There were significant increases observed in IFN-\(\gamma\) at earlier time points after virus challenge (Fig. 3). When Th2-type cytokines were examined, IL-4 levels showed little change; however, there was a significant increase in IL-13 levels observed by day 8 post-RSV infection (Fig. 3). This increase in pulmonary IL-13 levels paralleled changes observed in airway hyperreactivity. From these data, it appeared that the changes in IL-13 represented a significant alteration in the immune response that led to the pathological changes observed during RSV infection. These changes include mucus production and airway hyperreactivity.

Neutralization of IL-13 abrogates airway hyperreactivity and increases IL-12 production

Recent data have established an essential role for IL-13 in airway hyperreactivity responses and mucus production (17, 18). Because many of the pathophysiological changes in RSV infections may be related to similar mechanisms, Abs specific for IL-13 were used to neutralize IL-13 in vivo. Neutralizing anti-IL-13 or control Abs were given i.p. 1 h before intratracheal RSV (3 \(\times\) 10^5 PFU) and...
then every other day thereafter until day 12 of infection. The mice were examined for changes in airway hyperreactivity at specific time points after infection (Fig. 4). The mice treated with neutralizing Abs to IL-13 showed no increase in airway hyperreactivity, whereas those animals treated with control serum demonstrated significant changes as described above. In addition, examination of the bronchoalveolar lavage (BAL) fluid showed a significant difference in the amount of mucus produced; i.e., the anti-IL-13-treated animals had almost no mucus in the BAL fluid compared with the RSV controls (Fig. 5). Interestingly, the anti-IL-13-treated mice also showed an early and significant rise in IL-12 (Fig. 6), suggesting that IL-13 produced during the response observed in these experiments may have a role in down-regulating the Th1-type response. Treatment of mice with anti-IL-4 had no effect on the development of airway hyperreactivity during RSV infection (data not shown), corresponding to the observation of no increase in IL-4 production. Infectious virus titers peak in lungs of DBA and BALB/c mice at day 4, with no detectable infectious virus by day 8. We found no difference between the control and anti-IL-13-treated animals by plaque analysis at day 4 (data not shown). However, we also examined the RSV-specific Ag in the lungs by ELISA as an indication of the viral load (Fig. 7). Significant levels of viral Ag (as represented by ELISA OD readings) was easily detectable at day 4 and increased by day 8, the time of peak IL-13 production. The anti-IL-13-treated animals demonstrated significantly lower levels of viral Ag in their lungs at day 8, suggesting that the virus-specific protein was cleared more efficiently. Thus, the overproduction of IL-13 during RSV infection may regulate specific responses that are associated with detrimental pathophysiologic reactions observed within the infected lungs.

**Abrogation of RSV-induced airway responses in STAT6**

**Abrogation of RSV-induced airway responses in STAT6**

The activation of specific Th responses, Th1 or Th2, appears to be regulated by intracellular signal pathways that include STAT protein activation (25, 26). Specific cytokine ligand binding to cytokine receptors can activate certain STAT protein pathways. For example, it has been demonstrated that IL-12 specifically activates cells via STAT4, whereas IL-4 and IL-13 activate cells via STAT6 (27). Thus, we investigated whether mice genetically deleted of their STAT6 would respond similarly to mice with Ab depletions of IL-13. Using mice with a STAT6 deletion, we observed a significant reduction in peak RSV-induced airway hyperreactivity at days 8 and 12 postinfection, compared with control mice with an intact STAT6 pathway (Fig. 8). Interestingly, IL-13 levels were similar in the STAT6**+/-** and STAT6**−/−** mice (data not shown), indicating that STAT6 had a downstream effect on the airway reactivity. These findings are similar to those obtained in the IL-13 neutralization experiments with Ab and may provide a basis for describing mechanisms of RSV-induced airway hyperreactivity.

In addition to the alteration of airway hyperreactivity, we also observed decreases in mucus in the BAL fluid from the STAT6**−/−** similar to those in the anti-IL-13-treated animals (data not shown). When histological sections were stained for mucus using PAS staining methodology, we found a complete abrogation of the presence of PAS-positive cells in the airways, as represented in Fig. 9. This correlates directly with the abrogation of airway hyperreactivity and the apparent decrease in mucus in the airway. In addition, these data strongly support the concept that STAT6-mediated pathways induce production of mucus during inappropriate airway responses.
siveness. This contention is supported by several studies that epithelial cell barrier over long periods, resulting in hyperresponsiveness. The airway may cause continued immune responses and damage to the epithelium.

Because IL-12 has been linked to anti-viral responses (35–37) with its ability to neutralize IL-13 in the current study, supports the work of others.

Discussion

Pulmonary dysfunction in RSV infection may result from a number of interacting factors including the specific host inflammatory response, tissue damage, and possibly an airway-remodeling response during the resolution phase (28). The direction of the immune/inflammatory response within the lung during a RSV infection is likely dictated by locally expressed cytokines that direct the recruitment and activation of appropriate immune cells (29, 30). The present study examined the role of the Th2-type cytokine IL-13 in RSV infection and showed that it was related to airway hyperresponsiveness and mucus production. Interestingly, during this primary RSV infection, there was very little IL-4, a cytokine historically associated with severe Th-2 responses, produced in the lung. Recent studies using animal models that showed that IL-13 exacerbated asthma-like airway hyperreactivity and appeared to be responsible for mucus production and goblet cell hyperplasia within the airway agree with the present studies (17, 18). IL-13-induced overproduction of mucus in itself can be a pathologically devastating event that can lead to airway congestion and lung dysfunction (31, 32). Studies using IL-13-transgenic mice with targeted IL-13 overproduction in the lung resulted in an overabundance of mucus production, severe airway hyperreactivity, and end-stage fibrosis around the airway (33). Thus, over-production of IL-13 leads to a number of devastating events locally within the lung. The abrogation of severe pathophysiological findings with the neutralization of IL-13 in the current study supports the work of others. These data further demonstrate that IL-13 may be not only a contributing factor, but also the primary cytokine that initiates RSV-induced lung dysfunction. As in the current study, others have shown that the physiological changes seen in RSV infection can be independent of IL-4 (34), thus further supporting a primary role for IL-13 in this response.

In addition to its role in causing a severe immune response to RSV, IL-13 may also have an immunomodulatory role. Neutralization of IL-13 allowed the increased production of IL-12, suggesting that IL-13 exerts a regulatory effect on IL-12 production. Because IL-12 has been linked to anti-viral responses (35–37) with RSV as well as with numerous other viruses, one potential effect of the overproduction of IL-13 is altered viral clearance related to altered IL-12 production. The persistence of viral Ags within the airway may cause continued immune responses and damage to the epithelial cell barrier over long periods, resulting in hyperresponsiveness. This contention is supported by several studies that showed that prolonged airway hyperreactivity in RSV-infected animals related directly to the persistence of virus and its Ags (38–40). This may be especially interesting considering that peak viral titers from the lungs occur around day 4 of infection, whereas the pathophysiological responses occur at a later time point. Altered immune response related to IL-13 regulation of IL-12 correlates with recent studies demonstrating a decrease in IL-12 in those children who developed RSV-induced bronchiolitis (41). In these studies, we found increases in IFN-γ levels in lung homogenates at earlier time points that preceded peak airway hyperreactivity. Production of IFN-γ and airway hyperreactivity may not be linked in these mice because we found no alteration in IFN-γ when we neutralized IL-13 and demonstrated reduced airway hyperreactivity. Previously, investigators have identified IFN-γ production primarily by intracellular cytokine staining of T lymphocytes or by using higher virus levels for the challenge (16, 42, 43). It appears that there is a paucity of IFN-γ in this system because the addition of exogenous IFN-γ significantly protects mice against airway damage and physiologic alterations (44, 45).

Further complicating the understanding of the precise nature of the immune response to RSV is recent data that has demonstrated that genetic predisposition may play a significant role in the responses to RSV (16, 46, 47). In fact, when examining responses to vaccines directed to RSV, the genetic MHC background of mice directly determined the type of response that was elicited upon rechallenge with virus (16). In these studies, we used mice with a MHC background, H-2d, that responds to RSV in a detrimental way. The results in this study concur directly with those previous findings and suggest that the production of specific cytokines (IL-13 in particular) likely dictates the severity of the RSV response in the lung. In ongoing studies in our laboratory, other genetic strains of mice (H-2b MHC background, C57BL6 mice) have an effective response to RSV and relatively low airway hyperreactivity that centers on high IL-12 and reduced IL-13 (unpublished data). These ongoing studies correlate with original studies concerning IL-12-induced cellular cytotoxicity and viral clearance (48–50). Thus, the IL-12/IL-13 axis may be central in determining the regulation of the response to RSV and, furthermore, may dictate the severity of the lung dysfunction, which includes airway hyperreactivity, goblet cell hyperplasia, and mucus production. Previous studies that coupled RSV infection with allergic responses indicated that the exacerbation of allergen-induced airway hyperreactivity is associated with RSV-induced IL-5 (4). We found little increase in IL-5 in our studies and few, if any,
cosinophils during primary RSV infection. These findings are consistent with previous observations examining primary RSV infections (51, 52). However, because the response was induced via a STAT6 pathway, an overall Th2-type environment may be initiated during the RSV response (24, 26). STAT6 pathways are involved in allergen-induced airway hyperreactivity and mucus production (53, 54). It is likely that multiple factors contribute to the pathophysiology during airway disease, including the genetic background and the cytokine profiles that are generated locally.

Clinically, the attenuation of IL-13 early in disease may have benefits for children who may otherwise go on to have long-term airway reactivity/asthma problems. Several clinical studies have indicated a correlation of decreased IL-12 with severe cases of RSV-infected airway disease (46, 55, 56). These findings, along with a number of animal studies in allergy/asthma, suggest that this cytokine axis may play a significant role in determining the overall direction of the pulmonary response. These results have altogether demonstrated that during a primary RSV infection the regulation of cytokine responses can dictate the airway reactions to RSV. The ability to control the level of IL-13 within the lung may have a notably beneficial effect, not only for the RSV infection, but also for subsequent asthmatic responses within the airway. These and other studies will help to define beneficial and detrimental cytokine responses during RSV infection.

References


CORRECTIONS


In Figure 1, panel C was omitted. The corrected figure is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.

In the author line, the sequence of the first two authors is reversed. The corrected author line is shown below.

Krishnamurthy Malathi, Xiaogui Li, Olga Krizanova, Karol Ondrias, Kirk Sperber, Vitaly Ablamunits, and Thottala Jayaraman


The fourth author’s name, Cindy Banh, was omitted. The correct list of authors and affiliations is shown below.

Valerie Pasquetto,* Huynh-Hoa Bui,* Rielie Giannino,* Cindy Banh,* Fareed Mirza,† John Sidney,* Carla Oseroff,* David C. Tscharke,‡ Kari Irvine,* Jack R. Bennink,* Bjoern Peters,* Scott Southwood,* Vincenzo Cerundolo,§ Howard Grey,* Jonathan W. Yewdell,* and Alessandro Sette**

*La Jolla Institute for Allergy and Immunology, San Diego, CA 92109; †Tumor Immunology Unit, Weatherall Institute of Molecular Medicine, Oxford University, Oxford, United Kingdom; ‡Epimmune Incorporated, San Diego, CA 92121; §Laboratory of Viral Diseases, National Institutes of Health, Bethesda, MD 20892; and **Division of Immunology and Infectious Diseases, Queensland Institute of Medical Research, Herston, Queensland, Australia


In **Materials and Methods**, in the first sentence under the heading Intranasal administration of recombinant adenovirus-containing HO-1 cDNA, the source for adenoviral HO-1 cDNA was incorrectly attributed. The source is stated in the corrected sentence below.

Mice were anesthetized with methoxyflurane, and then 5 × 10^8 PFU of adenoviral HO-1 (Ad-HO-1) (a gift from K. Kolls, University of Pittsburgh Medical Center, Pittsburgh, PA, and J. Alam, Alton Ochsner Medical Foundation, New Orleans, LA) (29) or adenoviral β-galactosidase (Ad-LacZ) (BD Biosciences) were administered intranasally to each mouse in a volume of 50 μl as described previously (12).

The authors also wish to add the reference shown below.


In Figure 1, a sentence regarding the solid and broken lines was omitted from the legend. The corrected legend is shown below.

**FIGURE 1.** Specificity of the CM4 mAb. A, YB2 or RNK cells transfected with Ly49 constructs were stained with medium or first layer Abs followed by AF488 goat anti-mouse Ig. Solid lines: staining by CM4. Left broken line: medium control. Right broken line: staining by positive control Abs Ly49A = A1, Ly49B = 1A1, Ly49C = 4D12, Ly49D = 4E5, Ly49E = 4D12, Ly49F = HBF, Ly49G = 4G11, Ly49H = 3D10, Ly49I = YBI. B, Cross-competition between Abs. YB2 cells transfected with Ly49E (YB2-E) and RNK cells transfected with Ly49F (RNK-F) were incubated with medium or saturating quantities of the unlabeled Ly49 Abs shown on the y-axis. After 20 min, AF488-labeled CM4, 4D12, or HBF Ab was added, and incubation was continued for an additional 20 min. Median fluorescence values were determined by flow cytometry, and the percentage inhibition caused by pretreatment with each unlabeled Ab is plotted on the y-axis. The likelihood that the inhibition observed was due to chance variation was determined by Student’s *t* test (*, *p* < 0.05, **, *p* < 0.01, ***, *p* < 0.001). The experiments shown are representative of three similar experiments of each type that were performed.

In Figure 9A, the gel image labeled Ly49A is inverted. The corrected figure is shown below.

Figure 10, demonstrating intracellular trafficking of HLA-DR after the introduction of HIV proteins, is incorrect. The corrected figure is shown below.

![Corrected Figure](image)


In Materials and Methods, in the first sentence under the heading RSV infection, the designation of the virus type should be human RSV A strain, not A2 strain.


In Materials and Methods, in the first sentence under the heading Virus and infection, the designation of the virus type should be human RSV A strain, not A2 strain.

Figure 3B, demonstrating the apoptotic effect of gp120 on CD4 and CD8 cells; Figure 4B, depicting the apoptotic effect of Fas-FasL interactions in CD4 and CD8 T cells cocultured with 43\textsubscript{HIV} cells; and Figure 6B, showing the apoptotic activity of fractionated supernatant from the 43\textsubscript{HIV} cell line, are inaccurate. The corrected figures are shown below.

In Figure 5, demonstrating the inability of HIV-1-infected 43 cells to present antigen to HLA-DR2 and DR4 T cells, panels A and B are the same. The corrected figure is shown below.