The Failure of Interleukin-10–deficient Mice to Develop Airway Hyperresponsiveness Is Overcome by Respiratory Syncytial Virus Infection in Allergen–sensitized/challenged Mice

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Although respiratory syncytial virus (RSV) bronchiolitis in early life is associated with later episodes of wheezing during childhood (1), the existing data are controversial concerning the role of RSV as a true risk factor in asthma development and allergic sensitization. The study by Sigurs and coworkers (2) found bronchiolitis during the first year of life to be a risk factor both for the development of asthma and sensitization to common allergens during the subsequent 2 yr. In the Tucson cohort study, however, the association of frequent wheezing and early RSV bronchiolitis was not associated with an increased risk of allergic sensitization (1). In another cohort study, positive serology for RSV infection correlated with aeroallergen sensitization during the first year of life but not at later times (3). It seems likely, therefore, that predisposing factors such as altered airway function and/or immunogenetic factors determine which children ultimately develop asthma.

RSV infection has been shown to induce expression of interleukin-10 (IL-10) in human macrophages (4) and in mouse pulmonary T cells (5). Interleukin-10 is an important regulatory cytokine that can mediate a number of biological activities (6). Data derived from both in vivo and in vitro studies suggest that the biological effects of IL-10 can vary depending on the surrounding cytokine and cellular milieu and timing of expression during the immune response. For example, in a murine allergic sensitization model, IL-10 suppressed or delayed development of pulmonary eosinophilia when administered at the time of antigen challenge: given 1 h after the challenge, the cytokine had no effect (7). In vitro, preincubation of resting T cell clones with IL-10 enhanced their capacity to produce cytokines after subsequent activation (8). However, when IL-10 was added during the activation step, inhibition of IL-2 synthesis was observed.

The role of IL-10 in asthma remains controversial. Some studies found IL-10 expression to be higher in subjects with asthma than in control subjects (9–11) whereas others found lower IL-10 levels (12–15). Diminished IL-10 production could result in Th2 cytokine skewing in allergic mice, as demonstrated in a model of bronchopulmonary aspergillosis (16). In a mouse model of allergic sensitization, we have recently shown that IL-10 may play an important role in the development of airway hyperresponsiveness (AHR). IL-10–deficient mice sensitized and challenged with ovalbumin (OVA) developed a robust pulmonary inflammatory response but not AHR (17). After reconstitution with IL-10 through adenovirus–mediated gene transfer, the mice developed AHR. van Scott and coworkers observed an increase in AHR despite a decrease in pulmonary inflammation when allergen-sensitized and challenged wild-type mice were administered recombinant IL-10 protein (18). In the present study, we further evaluated the role of IL-10 in allergic lung inflammation, analyzing the effects of RSV infection on airway function and inflammation in sensitized and challenged IL-10–/– mice.

METHODS

Animals

Homozygous IL-10–deficient mice (IL-10–/–) on a C57BL/6 background (C57BL/6-IL-10(tm1Cgn) (19) were originally obtained from Dr. Werner Müller, Cologne, Germany. These mice and littermate control mice were bred and housed in specific pathogen-free conditions and maintained on an OVA-free diet in the Biological Resources Center at National Jewish Medical and Research Center. Both female and male mice, 6–10 wk of age were used in the experiments. Control mice were matched with the deficient mice with regard to both age and sex in each experimental group.

Virus

Human respiratory syncytial virus (Long strain, type A) was obtained from American Type Culture Collection (ATCC; Manassas,
Monoclonal Antibody Treatments

Anti-mouse IL-5 monoclonal antibody (mAb), TRFK-5 (IgG2b), was used in this study. One hundred microliters of the stock mAb was diluted with PBS in a total volume of 100 μl, which was then given to sensitized mice as a single intravenous injection, 2 h before the first airway challenge. As a control, purified rat IgG2b at the same dose and volume was administered.
and sham-infection (light microscopy).

Differentiated cells were counted under a microscope and stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA) and differentiated in a blinded fashion by counting at least 200 cells under light microscopy. PAS-stained histological sections of murine lungs. Normal airways and vessels after sensitization with OVA and exposure to nebulized PBS in IL-10−/− mice (A). Representative sections from an IL-10−/− mouse after RSV infection (B), after OVA sensitization/challenge and sham-infection (C), and OVA sensitization/challenge and RSV infection (D). Note the staining of single goblet cells within the respiratory epithelium in the RSV-infected OVA-sensitized/challenged mouse.

Bronchoalveolar Lavage

After assessment of RL, lungs were lavaged (17). Cytospin slides were stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA) and differentiated in a blinded fashion by counting at least 200 cells under light microscopy.

Measurement of Serum Immunoglobulins

Serum levels of total IgE, OVA-specific IgE, and IgG1 were measured by ELISA as previously described (23).

Measurement of Cytokines in Bronchoalveolar Lavage Fluid

Interferon (IFN)-γ, IL-4, and IL-5 in the bronchoalveolar lavage fluid (BALF) supernatants were detected by enzyme immunoassay (EIA) as previously described (24). For IL-10, the OptEIA set was used according to the manufacturer’s directions (PharMingen). For IL-13, a commercial kit was used (R&D Systems, Minneapolis, MN). Cytokine levels were determined by comparisons with the known standards. The limits of detection were 30 pg/ml for IL-10 and 10 pg/ml for the other cytokines.

Histologic and Immunohistochemistry Studies

After obtaining the BALF, lungs were inflated through the tracheal tube with 2 ml air and fixed in 10% formalin and blocks of lung tissue were prepared (17). Tissue sections, 5 μm thick, were affixed to microscope slides and deparaffinized. The slides were stained with hematoxylin and eosin (H&E), and periodic acid-Schiff (PAS) for identification of mucus-containing cells, and examined under light microscopy. For quantitating mucus staining, PAS-positive goblet cells in the airways were counted and the length of the basement membrane (BM) in each studied section was measured using NIH Image software (version 1.62). The results are given as mean number of PAS-positive goblet cells per millimeter of BM after evaluating several airways of three to five mice per group in a blinded fashion.

Cells containing major basic protein (MBP) in lung sections were identified by immunohistochemical staining as described using rabbit anti-mouse MBP (provided by Dr. J. Lee, Mayo Clinic Scottsdale, Arizona, AZ) (25). The slides were examined in a blinded fashion with a Nikon microscope equipped with a fluorescin filter system. Numbers of eosinophils in the perivascular, peribronchial, and peripheral tissues were evaluated using the IPLab2 software (Signal Analytics, Vienna, VA) for the Macintosh computer counting five sections per animal (three mice per group).

Statistical Analysis

The data were analyzed with the JMP statistical software package (SAS Institute Inc., Cary, NC). Analysis of variance was used to determine the levels of difference between all groups in measurements of RL. Comparisons for all pairs were performed by Tukey–Kramer honest significant difference (HSD) test. Significance levels were set at a p value of 0.05. Values for all measurements are expressed as mean ± SEM. Differences in cytokine levels between groups were analyzed by nonparametric ANOVA, the Kruskal–Wallis test. When significant differences between groups were observed, comparison for pairs was made by the Wilcoxon test with Bonferroni correction. Significance levels were set at a p value of 0.05.

RESULTS

Airway Hyperresponsiveness in Allergen-sensitized and Challenged Mice Infected with RSV

As shown in our previous study and confirmed here, IL-10-deficient mice that were sensitized and challenged with OVA (OS groups) (and in this case exposed to inactivated RSV, sham infection) did not develop significant changes in RL (Figure 1A) when compared with wild-type (WT) mice (Figure 1B). The airway response to inhaled MCh was also low in the IL-10−/− mice infected with RSV (R) groups compared with the WT mice. Further, even following sensitization, RSV infection of sensitized (but not challenged) IL-10−/−mice had only marginal effects (Figure 1A, ipR) without allergic sensitization. However, when allergen-sensitized/challenged IL-10−/−mice were infected with RSV, airway responsiveness was significantly enhanced (Figure 1A) (OR groups). An enhancement of AHR following RSV infection of sensitized and challenged mice was also seen (Figure 1B). Inactivated RSV induced only a marginal increase in AHR in the allergen sensitized and challenged mice, a response that was significantly lower than following live virus infection, indicating the importance for live virus infection in this response.
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BAL Analysis of Cells

There were no significant differences in the cellular profile in BALF between naive IL-10−/− and WT mice. After allergen sensitization and challenge, the percentage of eosinophils was significantly lower and neutrophil and macrophage percentages were higher in the IL-10−/− mice than in the WT mice (Figure 2). RSV infection significantly increased the number of eosinophils in the IL-10−/− mice, to the levels in WT mice. There were no detectable eosinophils in the BALF of nonsensitized and challenged mice.

Histopathology

The allergen sensitization and challenge protocol induced significant mononuclear and eosinophilic cell infiltration perivascularly and peribronchially in both the IL-10−/− and WT mice, as we have shown earlier (17). In the H&E-stained sections, no obvious differences could be detected between sham- and RSV-infected animals after allergen challenge of sensitized mice (not shown). However, PAS staining of the sections clearly showed an increase in mucus production, that is, airway goblet cell hyperplasia, both in the IL-10−/− and the WT mice after RSV infection (Figures 3 and 4).

Eosinophils in the pulmonary tissue were identified by immunofluorescence using an MBP-specific antibody (Figure 5). Eosinophil counts were significantly lower in the IL-10−/− (Figure 6A) compared with the WT mice (Figure 6B). These decreases were seen when eosinophil numbers were quantitated in the perivascular, peribronchial, and peripheral airways. However, after RSV infection the numbers increased to levels observed in WT mice. These results paralleled those in the BALF (Figure 2).

Cytokines and Immunoglobulins

Naive IL-10−/− and WT mice had undetectable or very low levels of IFN-γ, IL-4, IL-5, and IL-13 in the BAL fluid. These cytokines were analyzed at two different time points after allergen sensitization and challenge, 12 h and 48 h after the last airway challenge. There was a significant increase at both time points in all of these cytokines except IFN-γ. The most prominent difference between the groups was that IL-10−/− mice had significantly lower levels of IL-5 than WT mice after allergen sensitization/challenge. However, in OVA-sensitized/challenged IL-10−/− mice that were infected with RSV, the IL-5 levels were comparable to those in WT mice (Figure 7). A similar trend was seen with IL-13, but did not reach significance. RSV infection did not have a significant effect on cytokine levels in the WT mice.

Antibody levels, total IgE concentrations and OVA-specific IgG1 and IgG2a levels in serum were significantly higher in the IL-10−/− sensitized/challenged mice than in the WT mice, as demonstrated in our earlier study (17) (data not shown). Although there was a tendency for RSV infection to reduce all of these antibody levels, none of the differences between the IL-10−/− and WT mice reached significance.

Treatment with Anti-IL5

To define the role of IL-5 in the increases in eosinophil numbers and AHR, IL-10−/− mice were treated with anti–IL-5, 2 h before the first airway challenge. This resulted in a dramatic decrease in both the number of eosinophils in the BALF and also AHR (Figure 8).

Effect of RSV Strain Lacking the G and SH Genes

Mice immunized with formalin-inactivated RSV vaccine develop pulmonary eosinophilia after challenge with live virus. The G glycoprotein appeared to be important in directing this type of host response (26). To define whether the G protein was involved in the enhancement of AHR in IL-10−/− mice, infection with a mutant RSV strain (CP52), which lacks both G (glycoprotein) and SH (small hydrophobic protein) genes, was carried out; as a control, the parental B1 strain was used. As can be seen in Figure 9, both the B1 and the CP52 strains induced similar increases in AHR in sensitized/challenged mice.

Figure 5. Staining of eosinophils with an MBP antibody. A representative figure from IL-10−/− mice with OVA challenge only (A), RSV infection (B), OVA sensitization/challenge (C), and OVA sensitization/challenge and RSV infection (D).

Figure 9.
BAL fluid and in the lung tissue. Goblet cell hyperplasia and was seen as increases in numbers of eosinophils both in the infected, allergic mice when compared with allergic mice. This inflammatory response was enhanced in inflammatory response, cytokine levels, and increases in goblet IL-10 deficiency. Perhaps contributing were changes in the viral infection may override the consequences of how this viral infection may override the consequences of IL-10–deficient mice as treatment with anti–IL-5 resulted in both a decrease in eosinophil numbers and normalized the airway responsiveness to inhaled Mch. How eosinophils contribute to altered airway function is not clear (34). Moreover, the presence of eosinophils per se does not indicate their state of activation. Indeed, mouse eosinophils may not degranulate, at least not in response to triggers that result in human eosinophil degranulation (35). Further, there are currently no markers of eosinophil activation.

Eosinophils have been shown to alter muscarinic control of airway function (36). Guinea pigs sensitized/challenged with OVA and infected with parainfluenza virus demonstrated muscarinic (M2) receptor dysfunction that was abolished by pretreatment with anti–IL-5. The authors interpreted these findings to mean that sensitization to a nonviral antigen altered the inflammatory response to viral infection, so that M2R dysfunction and hyperreactivity were ultimately eosinophil dependent. Similar data have been obtained in allergic human subjects.

DISCUSSION

Previous data from this laboratory demonstrated that IL-10−/− mice do not develop AHR after allergen sensitization and challenge despite a significant pulmonary inflammatory response including increased numbers of tissue and BAL fluid eosinophils (17). When reconstituted with the IL-10 gene, these mice became responsive to inhaled MCh, indicating an important role for IL-10 in the development of altered airway function. Here, we demonstrate that allergen-sensitized and -challenged IL-10−/− mice infected with RSV are capable of developing altered airway responsiveness to inhaled Mch despite the absence of IL-10. Neither infection alone nor sensitization/challenge alone was capable of doing so. The response to RSV in allergic mice was examined in an attempt to explain how this viral infection may override the consequences of IL-10 deficiency. Perhaps contributing were changes in the inflammatory response, cytokine levels, and increases in goblet cell numbers. The inflammatory response was enhanced in infected, allergic mice when compared with allergic mice. This was seen as increases in numbers of eosinophils both in the BAL fluid and in the lung tissue. Goblet cell hyperplasia and increased IL-5 levels were also observed in these mice. Moreover, these effects of infection of allergic mice were more pronounced in the IL-10−/− than in the WT animals. The effects of RSV infection followed by allergic sensitization up to 3 wk after virus inoculation have been shown to result in enhanced AHR and inflammation (27) and this enhancing effect could be adoptively transferred by T lymphocytes, especially CD8 positive cells (28). This combination of RSV infection and allergen sensitization and challenge resulted in changes in airway function similar to those seen following IL-10 gene reconstitution of allergic mice (17).

AHR is a complex phenomenon involving both immunological and neural mechanisms. Eosinophilic inflammation is important for expression of AHR in many animal models (24, 25, 29, 30), but it is neither sufficient, as seen in our earlier work with the IL-10−/− mice (17), nor perhaps necessary for development of AHR in some models (31–33). Indeed, eosinophil responses considerably lower than observed in the BAL fluid and pulmonary tissue of IL-10−/− mice have been associated with AHR in some studies. Therefore, it is unlikely that the increase in eosinophils induced by RSV alone was sufficient to reconstitute AHR in the IL-10–deficient mice. That notwithstanding, the eosinophilic component appeared necessary for the response in infected and sensitized/challenged IL-10−/− mice as treatment with anti–IL-5 resulted in both a decrease in eosinophil numbers and normalized the airway responsiveness to inhaled Mch. How eosinophils contribute to altered airway function is not clear (34). Moreover, the presence of eosinophils per se does not indicate their state of activation. Indeed, mouse eosinophils may not degranulate, at least not in response to triggers that result in human eosinophil degranulation (35). Further, there are currently no markers of eosinophil activation.

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**Figure 6.** Tissue eosinophilia is significantly increased in allergen-sensitized/challenged IL-10−/− mice infected with RSV. Immunohistochemical localization of lung tissue eosinophils was performed using an MBP antibody as described in Methods. Shown are results from IL-10−/− (A) and WT (B) mice after OVA challenge alone (O), RSV infection alone (R), OVA-sensitized/challenged mice infected with RSV (OR), and sham-infected, OVA-sensitized/challenged mice (OS). Eosinophil numbers (per mm²) in the perivascular (PV), peribronchial (PB), and peripheral regions (PAR) were quantified. Results of each group are expressed as mean ± SEM. *Significant difference (p < 0.05) between RSV- and sham-infected allergic IL-10−/− mice.

**Figure 7.** RSV infection increases IL-5 levels in IL-10−/− mice. RSV-infected IL-10−/− mice have levels comparable to the WT mice. OVA sensitized (two intraperitoneal injections 14 d apart) mice were infected with RSV (OR) or sham preparation (OS) on Day 26 and challenged via the airways by OVA on Days 28, 29, and 30. Twelve hours after the last airway challenge mice were killed and BAL was collected. IL-5 and other cytokines were measured by ELISA. Level of detection 10 pg/ml. *Significant difference between groups (p < 0.05).
Th2 cytokine production, and goblet cell hyperplasia were all parameters including eosinophilic inflammation, IL-10 could be useful in controlling allergic inflammation. Of interest, immunotherapy was associated with enhanced levels in the BAL fluid were reduced. In mice, sensitization and subjects with asthma, both IL-10 mRNA transcripts and IL-10 shown to be major cause of exacerbations of asthma both in adults and in children (39, 40).

The role of IL-10 in allergic inflammation is complex (41). On the one hand, IL-10 has been shown to inhibit IL-5 production, eosinophilic inflammation, and chemotaxis (42–44), but on the other, may interfere with Th1 cytokine synthesis (41). In subjects with asthma, both IL-10 mRNA transcripts and IL-10 levels in the BAL fluid were reduced. In mice, sensitization and challenge resulted in significantly lower BAL IL-10 levels (45). Of interest, immunotherapy was associated with enhanced IL-10 production (46). Cumulatively, the evidence suggests that IL-10 could be useful in controlling allergic inflammation.

Thus, it was somewhat surprising that in IL-10−/− mice many of the parameters including eosinophilic inflammation, Th2 cytokine production, and goblet cell hyperplasia were all lower than in IL-10 sufficient mice. In addition, the deficient mice failed to increase AHR to inhaled Mch in vivo when lung resistance was measured. All of these responses were altered when sensitized and challenged mice were infected with live RSV. The cytokine data suggest that whereas the response of IL-10−/− mice to allergic sensitization and challenge is less skewed to a Th2 phenotype than the WT mice, a more pronounced Th2 response is promoted by RSV infection. Firstly, IL-5 levels in the BAL fluid of IL-10−/− sham-infected allergic mice were significantly lower than in the WT mice but following RSV infection, the IL-5 levels were increased. This is in agreement with a recent study in which IL-10−/− mice sensitized to allergen showed significantly reduced IL-5 production (47). In addition, we observed increases in IFN-γ mRNA in the lung (data not shown); naive IL-10−/− deficient mice demonstrated several fold higher levels of IFN-γ mRNA than WT mice. The combination of allergen sensitization and RSV infection resulted in lower levels of expression of IFN-γ mRNA. IFN-γ has been shown to modify AHR in allergen-sensitized and -challenged mice (48, 49). Most adult mice deficient in IL-10 develop a CD4 T cell-dependent and IFN-γ-mediated enterocolitis (50). In a recent study, spleen cells isolated from IL-10−/− mice infected with Chlamydia trachomatis also exhibited increased IFN-γ production (51). Addition of neutralizing antibody to IL-10 in vitro cultures of peripheral blood mononuclear cells of normal human subjects also increased production of IFN-γ (12).

In allergen-sensitized/challenged IL-10−/− mice, RSV infection resulted in increases in airway goblet cell hyperplasia and mucus staining, as it did in WT mice. Discharge of goblet cell mucin content induced by RSV infection superimposed on allergic sensitization has been demonstrated (52). Because mucin production seems to be under the control of Th2 cytokines such as IL-4, IL-9, and IL-13 (53–55), this provides additional support that RSV infection can enhance a Th2 phenotype in the allergic IL-10−/− mice.

Peebles and coworkers (56) recently described a model using BALB/c mice where RSV infection was given to OVA-sensitized mice during the allergen challenge period. Consistent with our results (57), they found increased AHR in the OVA/RSV group on Day 15, at a time when AHR decreased.
to basal levels in mice that were only sensitized to allergen. They also found increased inflammation in the lungs of OVA/RSV mice compared with sham-infected allergic mice. Surprisingly, no AHR was found in the WT mice infected with RSV without allergic sensitization. This is in contrast to the findings described here and previously (27, 28) as well as the findings of van Schaik and coworkers (58). Similarly, in guinea pigs, RSV infection has been shown to cause AHR and to potentiate the effects of allergic sensitization on both AHR and inflammation (59–61).

Formalin-inactivated RSV vaccine, used for a limited time in the 1960s, induced an atypical pulmonary inflammatory response in children who later encountered RSV. This immunopathological enhancement of disease has been studied extensively in mice (reviewed in 62). In mouse models, one of the important components of the virus that induces the eosinophilic response is the major glycoprotein (G protein) (26). G protein has been shown to modulate cytokine production in human PBMC in vitro, such as inducing pronounced increases in IL-10 production (63). We questioned, therefore, whether G protein could be of importance in the enhancement of AHR in the IL-10−/− mice. The data, however, showed that the enhancing effect on AHR was independent of the expression of both G and SH genes.

To summarize, the present study illustrates that RSV infection can contribute in an essential way to the development of AHR in allergen-sensitized/challenged IL-10−/− mice. The mechanism whereby active RSV infection together with allergen sensitization can overcome the failure in development of AHR in the IL-10−/− mice is not clear but may relate to the combination of effects on lung inflammatory responses, changes in cytokine levels, and goblet cell hyperplasia. These findings may bear on the relationship between viral infection and human asthma. Asthmatic/allergic patients may demonstrate a relative IL-10 deficiency compared with healthy control subjects (45). It is interesting therefore to note that following RSV infection, changes in airway function and cytokine responses can be induced and enhanced in IL-10−/− mice.

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References


