Rapid publication

IL-11 expression is increased in severe asthma: Association with epithelial cells and eosinophils

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Background: IL-11 is a pleiotropic cytokine produced by a variety of stromal cells. Targeted overexpression of this cytokine in mice results in a remodeling of the airways and the development of airway hyperresponsiveness and airway obstruction.
Objectives: Because these alterations mimic important pathologic and physiologic changes in the airways of some asthmatic patients, we investigated the expression of IL-11 messenger RNA (mRNA) within the airways of patients with mild to severe asthma and nonasthmatic control subjects.
Methods: Fiberoptic bronchoscopy to obtain bronchial biopsy specimens was performed on patients with mild (n = 13), moderate (n = 10), and severe (n = 9) asthma and on nonasthmatic control subjects (n = 9).
Results: These patients differed in their extent of airway fibrosis with types I and III collagens being noted in greater quantities in the biopsy specimens from the severe and moderate asthmatics than in those from controls ($P < .05$). IL-11 mRNA expression was observed in the epithelial and subepithelial layers of asthmatic and nonasthmatic control subjects. The number of cells within the epithelium and subepithelium expressing IL-11 mRNA was greater in those with moderate and severe asthma compared with mild asthma and nonasthmatic subjects ($P < .001$). There were also greater numbers of IL-11 mRNA-positive cells within the subepithelium in severe compared with moderate asthma ($P < .001$). Immunostaining for IL-11 within the airway tissues confirmed translation of the mRNA into IL-11–immunoreactive protein in airway epithelial cells. Colocalization of IL-11 mRNA and immunoreactivity with resident inflammatory cells demonstrated that this cytokine was also expressed by major basic protein–positive eosinophils.
Conclusion: These results suggest that IL-11 is involved in the chronic remodeling seen in asthmatic airways and is associated with increasing severity of the disease. (J Allergy Clin Immunol 2000;105:232-8.)

Key words: *Severe asthma, IL-11, airway remodeling, airways inflammation, collagen deposition*

The pathologic features of asthma are associated with airway remodeling as a consequence of hypertrophy/hyperplasia of the airway smooth muscles and of subepithelial fibrosis.[1,2] The latter response is most prominent in the lamina reticularis and results from interstitial collagen, fibronectin, and proteoglycan deposition.[3,4] Although initially described in postmortem studies, these structural alterations can be observed even in mild and newly diagnosed cases of asthma.[3,5] Because any thickening of the airway wall will profoundly increase the maximal degree of airway narrowing caused by airway smooth muscle contraction, it has been proposed that architectural changes similar to those observed in asthmatic patients contribute to the development of chronic airway hyperresponsiveness and the progressive deterioration in lung function over time.[6] To date, many studies have focused on the mechanisms underlying the acute presentations of bronchial asthma. In contrast, the factors responsible for the more chronic structural changes within the airway are poorly defined. Although myofibroblasts are recognized as the cell type responsible for subepithelial collagen deposition in these individuals,[7] the mechanisms contributing to the onset of airway fibrosis in asthma and the role of inflammatory cells, in particular eosinophils and epithelial cells, remains to be established.

IL-11 was originally described as a soluble factor derived from stromal cells, which was capable of stimulating plasmacytoma cell proliferation.[8] It is a member of the IL-6 family of cytokines that share a common receptor β-subunit gp130 molecule.[9] To date, IL-11 has been ascribed a variety of functions including the ability to regulate hematopoiesis, bone metabolism, and epithelial proliferation.[10] Consistent with this pleiotropic nature, IL-11 is produced by various cell types such as stromal cells, fibroblasts, osteoblasts, endothelial cells, and epithelial cells.[8,11-14] Studies from our own laboratories suggest that IL-11 may contribute to the structural airway remodeling and alteration in immune functioning evident

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Abbreviations used
ICC: Immunocytochemistry
ISH: In situ hybridization
MBP: Major basic protein
mRNA: Messenger RNA
TGF-β: Transforming growth factor-β

in asthmatic subjects. Thus we have demonstrated that IL-11 is produced by human lung fibroblasts and alveolar and airway epithelial cell lines in response to cytokines,[11,14,15] histamine,[16] eosinophil-derived major basic protein (MBP),[17] and respiratory viruses.[15] We have also reported that overexpression of IL-11 within the lungs results in subepithelial fibrosis[18,19] and promotes the accumulation of myocytes and myofibroblasts.[19] Moreover, the functional sequelae of chronic IL-11 expression recapitulate aspects of the abnormal pulmonary physiologic features observed in severe asthma, including the development of airway hyperresponsiveness and baseline airway obstruction.[15,19]

Given these actions of IL-11, we hypothesized that the expression of IL-11 was increased within the airways of asthmatics and that it would be particularly associated with the most severely remodeled individuals. Our aim was therefore to investigate the expression of IL-11 in a range of asthmatic patients with mild to severe disease with use of in situ hybridization (ISH) and to colocalize IL-11 to inflammatory cell types within the airways. These results showed a significantly increased expression of IL-11 messenger RNA (mRNA) within the airways of subjects with severe asthma compared with those with mild asthma and with nonasthmatic control subjects. The IL-11 mRNA expression within the lungs of asthmatic individuals was observed primarily within airway epithelial cells and MBP-positive eosinophils.

METHODS

Subjects studied

To study the expression of IL-11 mRNA in a range of asthmatic subjects, we recruited 32 individuals with mild to severe asthma from the asthma clinic at the Laval Hospital (Sainte Foy) and 9 control subjects from the asthma clinic at the Montreal Chest Hospital (Montreal). Asthmatic severity was defined on the basis of prebronchodilator-measured FEV_1 values, with mildly asthmatic subjects (n = 13) having an FEV_1 value greater than 80% predicted, moderately asthmatic subjects (n = 10) having FEV_1 values between 60% and 80% predicted, and severely asthmatic subjects (n = 9) having FEV_1 values <60% predicted. FEV_1 represented the accepted level of control with therapy. In moderate asthma the mean FEV_1 improved >30% after β_2-agonist administration and further in some subjects with oral prednisone, whereas in severe asthma FEV_1 was less than the historic maximum in some subjects. All patients fulfilled the American Thoracic Society criteria for asthma,[20] had typical clinical symptoms, documented airways reversibility (>15% improvement in FEV_1), and increased airway responsiveness to methacholine (<8 mg/mL) performed only if FEV_1 >70%. On enrollment, the medical history of each patient was taken and a physical examination was performed. None of the subjects had a history of respiratory tract infection within the previous 6 weeks or immunotherapy within the previous 12 months. All subjects were atopic on the basis of positive skin wheals (>3 mm) to one or more of 13 common allergens and were currently nonsmokers (2 and 4 ex-smokers in the moderate and severe asthma groups, respectively).

Seven subjects with severe asthma required the regular use of oral corticosteroids (mean 42 ± 7.7 mg) to maintain acceptable control of symptoms and 2 used inhaled steroids only. In addition, those with severe asthma used inhaled β_2-agonists and theophyline as necessary. Those with moderate asthma had their symptoms controlled by regular use of β_2-agonists and inhaled corticosteroids (mean 1177 ± 225 μg beclomethasone dipropionate equivalent). Three patients also required the regular use of oral corticosteroids. Those with mild asthma used inhaled β_2-agonists only. Nonsmoker nonatopic control subjects volunteered to participate in the study and none had taken corticosteroids in the year preceding the study. Informed consent, approved by the Montreal Chest Research Institute and Laval Hospital Ethics Review Committees, was obtained from all patients before entry into this study.

Fiberoptic bronchoscopy and tissue processing

The technique of fiberoptic bronchoscopy and the methods for processing of bronchial biopsy specimens have been described elsewhere in detail.[21]

Probe preparation

A digoxigenin-labeled complementary RNA probe coding for IL-11 mRNA was prepared from complementary DNA as described previously.[22] In brief, complementary DNA was inserted into PGEM vectors, linearized, and transcribed in vitro in the presence of digoxigenin-11-uridine triphosphate and either SP6 or T7 polymerases. Antisense (complementary to mRNA) and sense probes (identical to mRNA) were prepared.

ISH

Sections of lung tissue were processed for ISH for IL-11 mRNA according to Ying et al.[22] Briefly, after permeabilization with Triton X-100, the tissue sections were then briefly washed in PBS and immersed in a proteinase K solution for 20 minutes at 37°C. The samples were subsequently fixed in 4% paraformaldehyde, washed, and air dried. Hybridization was carried out with use of the hybridization mixture containing the appropriate sense or antisense. Each section was then covered and incubated overnight at 40°C in a humid chamber. Posthybridization, involving a series of high stringency washes of the samples in decreasing concentrations of saline–sodium citrate buffer at 42°C, was then performed. To remove any unbound RNA probes, the samples were washed with ribonuclease solution for 20 minutes at 42°C. The hybridization signal was visualized by incubating the sections for 4 hours with sheep polyclonal antidigoxigenin antibodies (1:1000) conjugated with alkaline phosphatase. Color development was achieved by adding the freshly prepared substrate (X-phosphate-5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium). Once the reaction was completed, the tissue sections were counterstained with hematoxylin, mounted with a coverslip, and examined under a graduated microscope for positive signals.

Immunocytochemistry

Immunostaining for IL-11 immunoreactivity within the tissues to confirm translation of the IL-11 mRNA was performed with use of the avidin-biotin peroxidase complex method as previously described.[23] Tissue sections (5 μm) from the asthmatic and nonasthmatic subjects were incubated overnight at 4°C with the primary goat antihuman IL-11 antibody (AB-218-NA, R&D Systems, Minneapolis, Minn) or with the primary rabbit antihuman type I or type III collagen antibodies

(Biodesign, Pasadena, Calif). According to the manufacturer's specifications, the IL-11 antibody exhibits no cross-reactivity with other cytokines tested, including IL-6 and leukemia inhibitory factor in direct ELISA. As a control, sections were processed in the absence of the primary antibody with an isotype-matched IgG.

Combined immunocytochemistry and ISH

To ascertain the expression of IL-11 mRNA by eosinophils, we simultaneously applied radiolabeled ISH for IL-11 mRNA with MBP immunoreactivity as previously described in detail elsewhere.[24]

Sequential immunostaining

Double sequential immunostaining for IL-11 immunoreactivity protein and MBP immunoreactivity was performed to localize the protein products of the IL-11 mRNA to eosinophils. The method for sequential immunostaining has been previously published.[25]

Quantification

Slides were coded and positive cells were counted blindly with use of ×100 magnification. Cells with positive signal in the subepithelium were counted and the results were expressed as the mean number of positive cells per square millimeter of submucosa. IL-11 mRNA-positive cells within the epithelium were counted by optical analysis and expressed as a semiquantitative score on the basis of the percentage of the epithelium demonstrating positive signal/total epithelium (0: no staining; 1: less than 12.5%; 2: 12.5%-25%; 3: 25%-37.5%; 4: 37.5%-50%; 5: 50%-62.5%; 6: 62.5%-75%; 7: 75%-87.5%; 8: 87.5%-100%). The within-observer coefficient of variation for repeated measures was less than 5%. The extent of collagen staining in the subepithelium was measured with an image analysis system. The thickness of the collagen layer was taken below the basement membrane and these data are expressed as the mean of 3 measurements.

Statistical analysis

Normality and variance assumptions were tested. The numbers of cells expressing IL-11 mRNA in normal and asthmatic airways were compared with the nonparametric Kruskal-Wallis test. Statistically significant differences between groups were subsequently analyzed with a Mann-Whitney *U* test (Systat version 7.0, SPSS, Chicago, Ill). Correlation coefficients were calculated from Pearson's moment coefficient and were corrected for multiple comparisons by the use of Bonferroni's correction factor. Results were considered statistically significant for P values <.05.

RESULTS

IL-11 expression within the airways

IL-11 mRNA expression was visualized as dark purple staining of individual cells and was seen in the airway submucosa and epithelial cell layer of asthmatic patients and nonasthmatic controls (Fig 1, *A* and *B*, respectively). No positive signals for IL-11 mRNA were observed when the sense probe was used or when the tissue sections were treated with ribonuclease before hybridization of the antisense probe. To confirm transcription of the mRNA for IL-11, the presence of specific immunoreactivity for this cytokine within the tissue sections was confirmed with use of polyclonal antihuman IL-11 antibody. The presence of specific brown staining after the immunocytochemistry (ICC) within the airways of asthmatic patients and nonasthmatic control subjects was indicative of IL-11 immunoreactive protein (Fig 1, *C*). This staining was not observed when the isotype-matched control antibody was used (Fig 1, *D*).

Expression of IL-11 mRNA in mild, moderate, and severe asthma

To further define the potential role of IL-11 in the pathophysiologic mechanisms of asthma, we compared the expression of IL-11 mRNA in nonasthmatic and asthmatic subjects with varying degrees of airway obstruction and fibrosis (Fig 2, *A* and *B*). Within the subepithelial cell layer, the numbers of cells expressing IL-11 mRNA were significantly greater in moderate (10.8 ± 1.5 cells per mm^2 of submucosal tissue) and severe asthma (21.1 ± 2.0 cells per mm^2 of submucosal tissue) compared with mild asthma (2.9 ± 0.7 cells per mm^2 of submucosal tissue) and nonasthmatic control subjects (1.3 ± 0.5 cells per mm^2 of submucosal tissue, $P < .001$). Within the subepithelium, there were also significantly greater numbers of IL-11 mRNA-positive cells in severe asthma compared with moderate asthma ($P < .001$).

In the epithelial cell layer there was an increase in the epithelial score for IL-11 mRNA expression for those with moderate (2.2 ± 0.3) and severe (2.8 ± 0.2) asthma compared with those with mild asthma (0.5 ± 0.2) and the nonasthmatic control subjects (0.2 ± 0.1, $P < .001$). There were no significant differences between the moderate and severe asthmatics in their expression of IL-11 mRNA within the epithelium.

Colocalization of IL-11 mRNA expression

The studies noted above clearly demonstrate that IL-11 is expressed at the mRNA level by cells having eosinophil-like morphologic features within the airway subepithelium. To confirm the identity of these cells, both combined ISH and ICC and double-sequential ICC were used (Fig 1, *E*). In the subepithelium of patients with moderate and severe asthma IL-11 mRNA was mostly colocalized to MBP-positive eosinophils (mean ± SEM, 61% ± 9%, n = 6).

Correlation of IL-11 mRNA expression with an index of lung physiology features

To gain insight into the potential effector functions of IL-11, we compared index values of IL-11 production and airway physiologic features (FEV_1). There were significant inverse correlations between the numbers of cells expressing IL-11 mRNA within the epithelium (Fig 3, *B*, $r^2 = 0.55$) and subepithelium (Fig 3, *A*,, $r^2 = 0.75$) and the FEV_1 values for the group of asthmatic subjects ($P < .05$).

Characterization of extent of remodeling

As noted above, the severity of asthma was initially assessed on the basis of pulmonary function parameters. To see whether this correlated with the extent of airway remodeling or airway fibrosis, the expression of types I and III collagens was assessed in the biopsy specimens from the healthy and asthmatic subjects. The presence of specific collagen immunoreactivity was visualized as brown staining beneath the lamina reticularis after the ICC reaction (Fig 1, *F*). Fig 4 demonstrates the relationship between asthma severity and

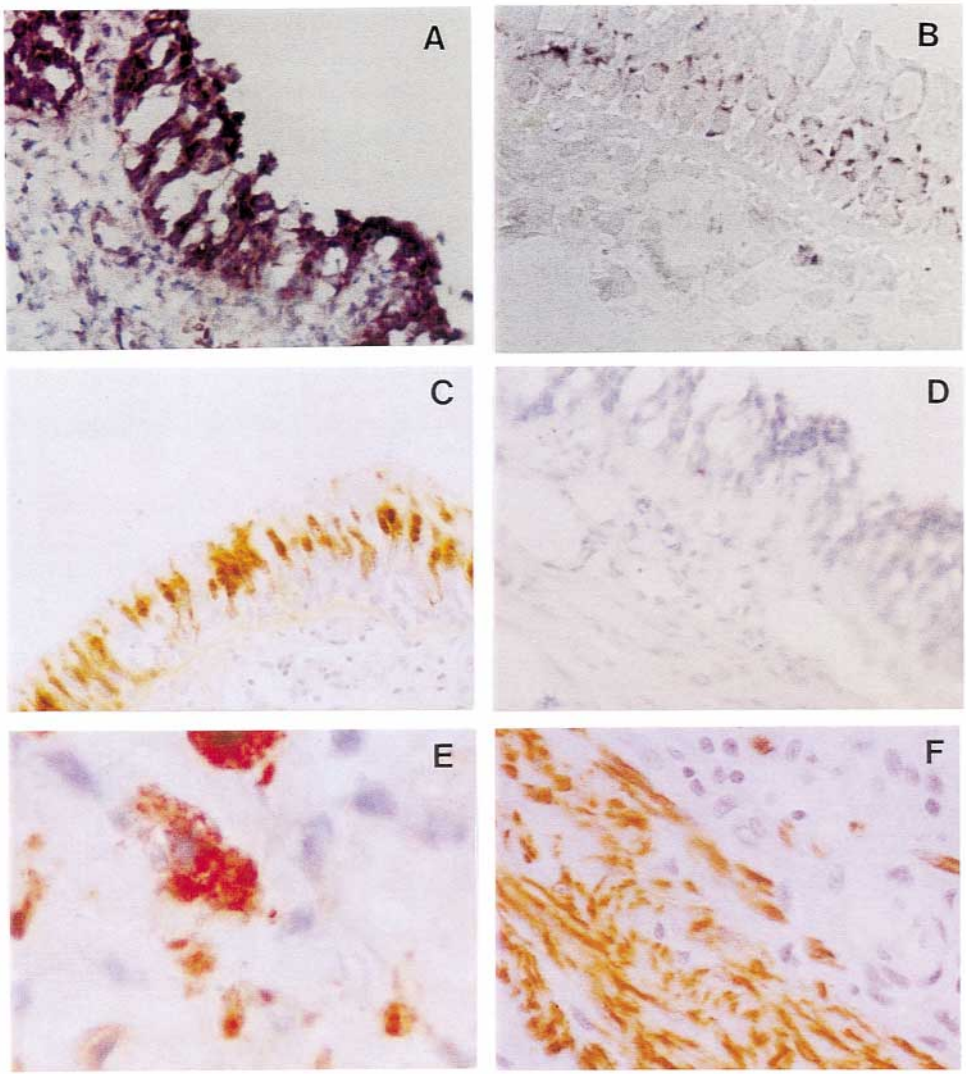

FIG 1. ISH with digoxigenin for IL-11 mRNA in bronchial biopsy specimens from **(A)** patient with severe asthma and **(B)** nonasthmatic control subject. Note presence of dark brown/purple staining indicative of IL-11 mRNA within airways epithelium and associated with individual cells in subepithelium. **C,** Immunostaining for IL-11 in individual with severe asthma. **D,** Primary antibody isotype control for IL-11 immunoreactivity. **E,** Colocalization of IL-11 immunoreactivity and eosinophil specific marker (MBP) in individual with severe asthma. IL-11 immunoreactivity is shown as brown coloration that localizes to MBP-positive cells *(red staining).* **F**, Collagen (type III) immunoreactivity, as determined by avidin-biotin peroxidase method, in individual with severe asthma is shown as brown staining.

collagen immunoreactivity. It shows that for both type I and type III collagen there is increasing expression with the severity of the disorder. There was a significantly greater staining for collagen type III in severe and moderate asthma compared to mild asthma and healthy subjects ($P < .05$). The extent of type I collagen immunoreactivity was also greater in severe asthma compared with the mildly asthmatic and healthy subjects ($P < .05$) and in moderate asthma compared with healthy control subjects ($P < .05$).

Relationships between IL-11 expression and the extent of collagen staining

Correlational analyses were performed between the expression of IL-11 in epithelium, in subepithelium, and the expression of type I and III collagens for all the subjects (nonasthmatics, mild, moderate, and severe asthmatics) included in the study. No significant correlation was found between any of these parameters.

DISCUSSION

Previous studies in mice have demonstrated the capacity of IL-11 to recapitulate many of the features observed in chronic obstructive airway diseases such as severe asthma. To investigate the contribution of this cytokine to the structural alterations evident in a range of asthmatic individuals, we examined the expression of IL-11 mRNA and immunoreactivity and its association with

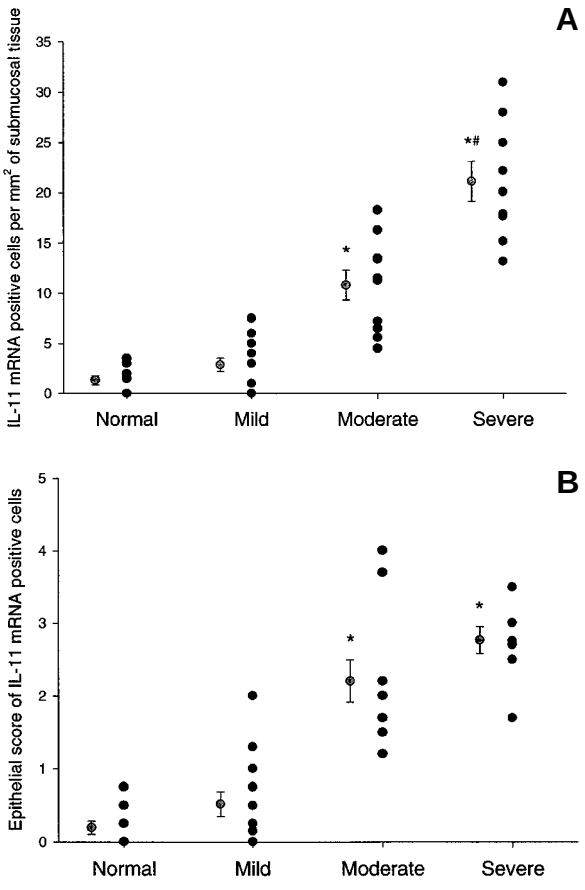

FIG 2. IL-11 mRNA expression within **(A)** subepithelium and **(B)** epithelial cell layer of subjects with mild, moderate, and severe asthma and of nonasthmatic control subjects. There was significant increase in numbers of cells expressing IL-11 mRNA in moderate (n = 10) and severe (n = 9) asthma compared with mild asthma (n = 13) and nonasthmatic control subjects (n = 9, *asterisk, P* < .001 compared with controls and mild asthmatics). Within submucosa, patients with severe asthma had significantly increased numbers of IL-11 mRNA-positive cells compared with those with moderate asthma (*pound sign, P* < .05).

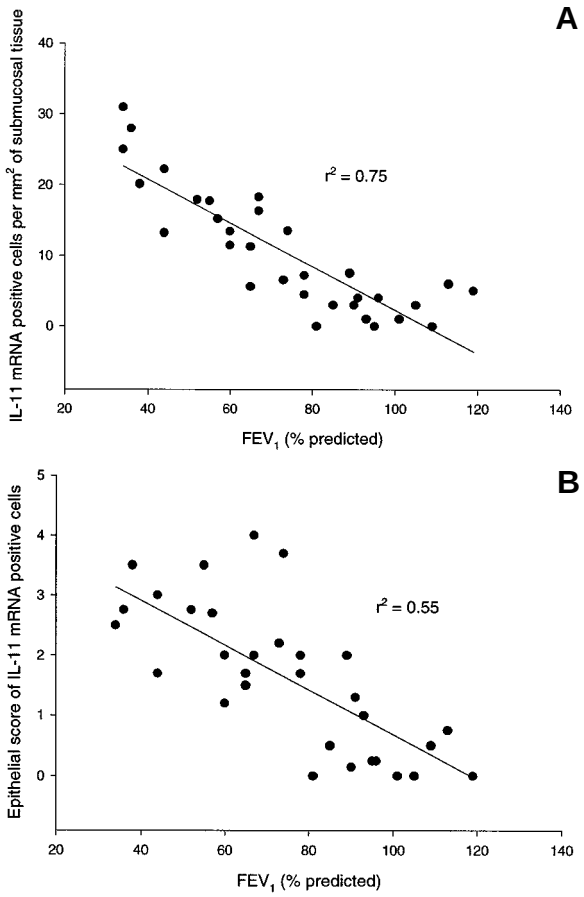

FIG 3. Correlational relationships between percent predicted FEV_1 values and numbers of cells expressing IL-11 mRNA within **(A)** subepithelium and **(B)** epithelial cell layer. There were significant correlations between numbers of IL-11 mRNA-positive cells in both airway epithelium and subepithelial regions and this index of pulmonary function ($P < .05$).

inflammatory cells with use of a combination of ISH and ICC techniques. In addition, we investigated the expression of collagen types I and III within the airways of our asthmatic and healthy individuals. Our results documented the increased numbers of cells expressing IL-11 within the airways of moderate and severe asthmatics and localized the majority of this expression to epithelial cells and to MBP-positive eosinophils. IL-11 expression was directly associated with disease severity but inversely correlated with FEV_1 measurements, suggesting that IL-11 expression is associated with abnormal lung physiologic features. Immunoreactivity for collagen types I and III expression were increased within the airways of our asthmatic subjects, and this was related to the severity of the disorder, as based on FEV_1 values.

IL-11 is a cytokine with potent immunomodulatory properties and the ability to induce substantial remodeling of the airways; however, there was little evidence concerning the importance of IL-11 in human disorders. Therefore the increased numbers of cells expressing IL-11 in moderate and severe asthma is an original finding that suggests that this cytokine may contribute to the sequelae of inflammatory events and structural modifications characterizing these asthmatic individuals. Although prior reports have shown IL-11 immunoreactivity in nasal aspirates from children with viral upper respiratory tract infections, particularly those who exhibited wheezing,[15] our study is the first demonstration that inflammatory and structural cells express striking amounts of IL-11 in vivo in a human disease. It is also the first report showing the expression of IL-11 in normal human epithelial cells in vivo because previous studies have used alveolar and bronchial epithelial cell lines.[14,15]

The mechanisms whereby IL-11 exerts its activity in the airways are complex. IL-11 has previously been shown to have fibrogenic potential in an animal model in eliciting subepithelial fibrosis and the local accumulation of fibroblasts, myofibroblasts, and smooth muscle cells.[18,19] Interestingly, IL-11 enhances the accumulation of collagen type III (and to a lesser extent type I), which

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