

T_H2 cytokine-associated transcription factors in atopic and nonatopic asthma: Evidence for differential signal transducer and activator of transcription 6 expression

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Background: The expression of IL-4 and IL-5 is increased in patients with atopic asthma compared with control subjects and correlates with indices of pulmonary function. In nonatopic asthma the expression of IL-4, unlike IL-5, fails to correlate with pulmonary function, and compared with their atopic counterparts, these patients have fewer cells expressing IL-4 receptor (IL-4R). As such, a deficiency in the IL-4 signaling pathway may be implicated in nonatopic asthma. The transcription factors GATA-3 and cMAF mediate IL-4 and IL-5 synthesis, whereas signal transducer and activator of transcription 6 (STAT-6) is critical for IL-4R signaling.

Objective: This study examines the expression profile of these transcription factors in asthma, according to atopic status.

Methods: With immunocytochemistry, the expression of GATA-3, cMAF, and STAT-6 protein was determined in sections of bronchial biopsy specimens from patients with atopic asthma (n = 7), patients with nonatopic asthma (n = 8), and control subjects (n = 8).

Results: Higher numbers of cells expressing GATA-3 and cMAF were observed in patients with atopic and those with nonatopic asthma than in control subjects and patients with tuberculosis ($P < .001$). There were also more STAT-6-immunoreactive cells in patients with atopic and those with nonatopic asthma than in control subjects ($P < .0001$, $P < .05$). Notably, however, fewer cells expressing STAT-6 protein were observed in nonatopic versus atopic asthma ($P < .0001$).

Conclusions: These results demonstrate the upregulation of GATA-3 and cMAF in both variants of asthma and indicate that reduced IL-4R signaling, because of lower STAT-6 expression, may be a feature of nonatopic asthma.

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Key words: Atopic asthma, nonatopic asthma, transcription factors, cMAF, GATA-3, STAT-6, IL-4

Asthma can be divided clinically into 2 variants, according to atopic status.¹ Most patients with asthma have atopy, determined by skin test positivity to common aeroallergens and elevated levels of serum IgE, but some patients with asthma do not have atopy and have normal or only slightly elevated serum IgE levels.² Both asthma phenotypes exhibit impaired pulmonary function and an abundance of inflammatory cells within the bronchial mucosa, predominantly T cells and eosinophils.² Although it is well accepted that the T_H2 cytokines IL-4 and IL-5 are increased in atopic asthma,³ there is conflicting evidence as to whether nonatopic asthma is similarly mediated. Walker et al⁴ reported that IL-5, but not IL-4, is increased in BAL fluid from patients with nonatopic asthma compared with control subjects, whereas Humbert et al⁵ demonstrated higher numbers of cells expressing IL-5 and IL-4 mRNA in patients with nonatopic asthma versus control subjects. Furthermore, this study⁵ also showed that unlike IL-5, IL-4 did not correlate with the concentration of methacholine that induces a 20% fall in FEV₁ (PC₂₀) or asthma-severity scores.

IL-4 drives naive T cells toward the T_H2 phenotype and also induces B cells to isotype switch to IgE.⁶⁻⁸ IL-5 is essential for eosinophil differentiation,⁹ accumulation at sites of inflammation,^{3,10} and survival within the tissue.¹¹ Both IL-4 and IL-5 function through specific α chain receptors,^{12,13} and therefore the presence of receptors, rather than expression per se, is considered a more accurate reflection of cytokine activity. We previously demonstrated increased numbers of cells expressing α IL-4 receptor (α IL-4R) and α IL-5R in the bronchial mucosa of patients with asthma than in the mucosa of control subjects.^{14,15} However, there were fewer IL-4R-expressing cells in patients with nonatopic asthma,¹⁵ suggesting that the IL-4R pathway may represent a fundamental point of variance between these asthma phenotypes.

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TABLE I. Patient characteristics

	Control	Nonatopic asthma	Atopic asthma
n	8	7	8
Sex (F/M)	6/2	5/2	4/4
Age (y)*	28.8 ± 7.24	42.9 ± 13.4	29.4 ± 13.2
FEV ₁ (L)*	3.34 ± 0.84	2.96 ± 0.83	3.16 ± 1.03
FEV ₁ (% predicted)*	97.7 ± 9.35	85.3 ± 19.3	87.9 ± 21.06
PC ₂₀ range (μg/mL)	>16	0.11–4.0	0.52–4.0
IgE (IU/mL)*	29.1 ± 12.3	33.8 ± 24.7	643.75 ± 425.1
Inhaled steroid BDP (μg)*	0	1100 ± 547.7	1125 ± 353.6

BDP, Beclomethasone dipropionate.

*Data expressed as mean ± SD.

Abbreviations used

IL-4R: IL-4 receptor

PC₂₀: Concentration of methacholine that induces a 20% fall in FEV₁

STAT-6: Signal transducer and activator of transcription 6

Production of IL-4 and IL-5 is mediated by the transcription factors GATA-3 and cMAF. These factors are found at low levels in naive T cells and are upregulated during T_H2 phenotype acquisition.^{16,17} GATA-3 appears particularly important for development of the T_H2 cytokine milieu. A GATA-3 transgene was associated with increased T_H2 cytokine expression in T_H1 T cells, whereas inhibition of GATA-3 synthesis in T_H2 cells decreased expression of IL-4, IL-5, IL-6, IL-10, and IL-13.^{18,19} On the other hand, cMAF has been reported to be more selective because it activates the promoter for IL-4, but not those for IL-5 and IL-13.^{16,20} Signal transducer and activator of transcription 6 (STAT-6) is a component of the αIL-4R signaling pathway²¹ and plays an integral role in immune responses by mediating IL-4-induced T_H2-cell differentiation^{22,23} and B-cell production of IgE.^{24–26} There are STAT-6 response elements in the promoters for a number of IL-4-inducible genes, including IgE,²⁶ IL-4,²⁷ αIL-4R,²⁸ and the low-affinity IgE receptor CD23.²⁹ Furthermore, STAT-6 knockout experiments have been associated with reduced T_H2-cytokine expression and only low levels of serum IgE after antigen challenge.^{24,25}

In an attempt to characterize transcription factor expression in asthma and to determine whether there are differences according to atopy status, we examined GATA-3, cMAF, and STAT-6 protein in sections of bronchial biopsy tissue from patients with atopic and those with nonatopic asthma as well as healthy control subjects. Our results demonstrate an upregulation of all 3 transcription factors in patients with asthma compared with control subjects and that nonatopic asthma is associated with fewer STAT-6-expressing cells than atopic asthma.

METHODS

Patients

Twenty-three subjects were recruited for this study from 3 centers composing the Québec Respiratory Health Network, including the Montreal Chest Institute, Sacré Cœur Hospital, and Laval University,

Quebec, Canada. Approval was obtained from the ethical committees of each center, and all subjects gave written informed consent. According to the American Thoracic Society guidelines,³⁰ atopic asthma was defined on the basis of clinical history, positive skin prick test results to 1 or more common aeroallergens, and total serum IgE concentration greater than 100 IU/mL. Patients with nonatopic asthma had a clinical history of perennial symptoms of asthma with no clear allergic trigger, negative skin prick test result with positive histamine reaction, and total serum IgE concentrations less than 100 IU/mL. Control subjects were asymptomatic and nonatopic with normal spirometry. All subjects were nonsmokers or ex-smokers with a smoking history of less than 6 pack-years and had ceased smoking for at least 3 years. All patients with asthma were treated with short-acting β₂-agonist as needed and inhaled corticosteroids at a beclomethasone dipropionate daily dose equivalent ranging from 200 to 4000 mg for a minimum of 1 month before the study. None of the patients had received oral leukotriene antagonists, methylxanthines, antihistamines, or oral corticosteroids for at least 3 months before the study. Exclusion criteria included age less than 18 or greater than 55 years, FEV₁ less than 60% predicted, evidence of upper respiratory tract infection within the previous 6 weeks, pregnancy, breast-feeding, or any chronic medical illness other than asthma.

Bronchoscopy and tissue processing

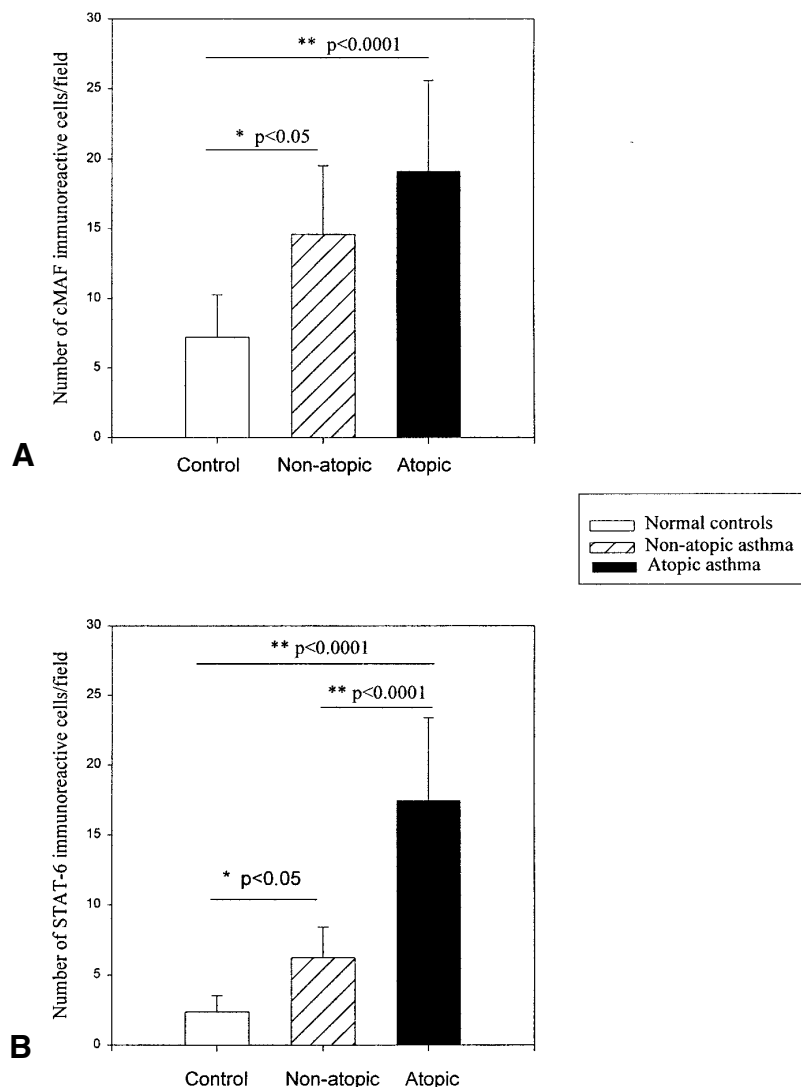
Bronchial biopsy specimens were obtained by fiberoptic bronchoscopy according to American Thoracic Society guidelines.³¹ Six to 8 specimens were obtained from the subsegmental airways of the right lung with alligator forceps (Olympus Corp, Tokyo, Japan). Nebulized salbutamol was administered to all subjects before bronchoscopy to prevent bronchoconstriction. The biopsy specimens were fixed in 4% paraformaldehyde, washed in 15% sucrose/PBS, and blocked in OCT embedding medium (RA Lamb Ltd, London, UK). Cryostat sections were prepared and stored at –80°C. Specimens were shipped on dry ice to the Meakins-Christie Laboratories, Montreal, Canada, for processing.

Antibodies

All antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, Calif). The GATA-3 (HG3-31) antibody is mouse monoclonal IgG₁ raised against human recombinant GATA-3 protein; STAT-6 (C-9) is mouse monoclonal IgG₁ generated with a recombinant STAT-6 protein, and it does not cross-react with STAT-1, STAT-2, STAT-3, STAT-4, or STAT-5; cMAF is an affinity-purified rabbit polyclonal antibody that recognizes the carboxy terminus of vMAF (C-20) and reacts with vMAF and cMAF proteins.

Immunocytochemistry

Alkaline phosphatase antialkaline phosphatase immunocytochemistry was performed³² with antibodies against GATA-3, cMAF, and STAT-6 on cryostat sections of bronchial biopsy tissue. The bridging

**FIG 1.**

Transcription factor expression in control subjects (*open bars*), patients with nonatopic asthma (*hatched bars*), and patients with atopic asthma (*filled bars*). **A**, There were significantly more cells expressing cMAF immunoreactivity in both asthmatic groups compared with control subjects. **B**, The number of STAT-6-immunoreactive cells was also increased in patients with asthma compared with control subjects; however, note that there were statistically more STAT-6 cells in patients with atopic versus nonatopic asthma.

secondary layer for the detection of GATA-3 and STAT-6 was a rabbit antimouse IgG₁ (Dako Diagnostics, Oakville, Ontario, Canada), and the tertiary layer was alkaline phosphatase conjugated to a rat antirabbit IgG₁ (Dako Diagnostics). For detection of cMAF, the secondary Ab used was a swine antirabbit Ig (Dako Diagnostics), and the tertiary layer was an alkaline phosphatase-conjugated Streptavidin complex (Dako Diagnostics). Fast red TR (Sigma Chemical Co, St Louis, Mo) was dissolved in alkaline phosphatase substrate, and once it was applied to the sections, cells expressing the protein in question could be visualized by the precipitation of this chromogen, resulting in positive cells staining red.

Quantification and statistics

The positive signal was quantified with an Olympus light microscope (Carson Group Inc, Calif) at 20× magnification and a grid attachment of 0.25 mm². Data were acquired by counting the number of immunoreactive cells, those with the cell-associated Fast Red

precipitate. These counts were reported as the mean ± SD of 6 to 8 grids. Statistical significance was determined with the unpaired Student *t* test (Systat version 5.0; Systat Inc, Evanston, Ill).

RESULTS

Patient demographics are represented in Table I. All patients with asthma had mild-to-moderate disease severity. Characteristically, subjects in the nonatopic group were older, had lower IgE levels, and tended to have better FEV₁ values than those in the atopic group.

GATA-3 and cMAF expression in atopic and nonatopic asthma

The number of cells expressing immunoreactivity for GATA-3 and cMAF (Fig 1, A) was higher in biopsy spec-

imens obtained from patients with atopic (15.6 ± 5.2 , 19.11 ± 6.5) as well as those with nonatopic (12.0 ± 4.6 , 14.6 ± 4.9) asthma, compared with healthy subjects (3.6 ± 1.6 , 7.1 ± 3.0) and patients with tuberculosis (1.8 ± 0.5 , 2.3 ± 0.8) ($P < .001$). The density of cells expressing either GATA-3 or cMAF (Fig 1, A) was not different between the 2 asthma phenotypes ($P > .05$) (Fig 2).

STAT-6 expression in atopic and nonatopic asthma

There were also higher numbers of cells expressing STAT-6 (Fig 1, B) in patients with atopic (17.5 ± 6.0 , $P < .0001$) and nonatopic (6.2 ± 2.2 , $P < .05$) asthma compared with healthy control subjects (2.4 ± 1.2) and patients with tuberculosis (2.9 ± 1.3). However, unlike GATA-3 and cMAF, fewer cells expressing STAT-6 were observed in patients with nonatopic than in those with atopic asthma (Fig 1, B; $P < .0001$). There were no correlations between GATA-3, cMAF, or STAT-6, and FEV₁, PC₂₀, or IgE levels.

DISCUSSION

The underlying pathologic differences between atopic and nonatopic asthma remain elusive. Histologically, biopsy material from these 2 groups appears highly similar, and immunocytochemical analyses reveal that the inflammatory cell infiltrate does not seem to differ.² Although there are varying reports on cytokine expression patterns, at least 1 group has observed increased levels of IL-4 in nonatopic asthma and that this expression fails to correlate with pulmonary function.⁵ Furthermore, significantly fewer IL-4R⁺ cells in nonatopic compared with atopic asthma has recently been observed.¹⁵ Together, these findings suggest that the IL-4 signaling pathway may be impaired in nonatopic asthma. As such, in this study we examined expression of transcription factors implicated in T_H2 cytokine expression and/or function—GATA-3, cMAF, and STAT-6. We observed that all 3 are constitutively expressed in healthy subjects and are upregulated in atopic as well as nonatopic asthma. Interestingly, we also demonstrated that the density of cells expressing STAT-6, contrary to that of cells expressing GATA-3 and cMAF, is significantly lower in nonatopic than in atopic asthma.

We previously reported that, compared with healthy control subjects, patients with asthma had increased GATA-3 expression within their bronchial mucosae.³³ The present work indicates that regulation of this transcription factor is not dependent on atopy because it is similarly increased in nonatopic asthma. This study is the first to investigate cMAF in human beings. We demonstrate comparable expression within the bronchial mucosa of both patients with atopic and those with nonatopic asthma, at levels significantly higher than those of control subjects. GATA-3 and cMAF mediate activity of both the IL-4 and IL-5 promoters.^{16,18} We have not investigated IL-4 or IL-5 levels in these patients; however, the fact that GATA-3 and cMAF expression is in nonatopic and atopic

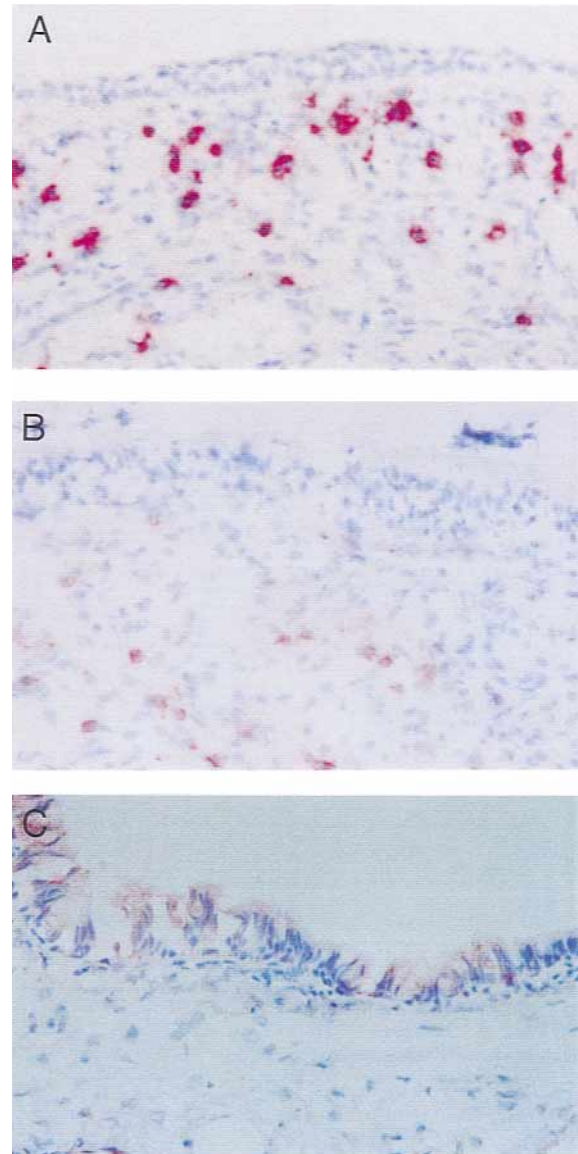


FIG 2. Representative example of STAT-6 immunoreactivity in bronchial biopsy specimens from a patient with atopic asthma (A), a patient with nonatopic asthma (B), and a healthy control subject (C).

asthma may suggest that they are elevated. However, a recent study indicates that GATA-3–induced cytokine expression may be due to interaction with other transcription factors, like NFAT.³⁴ If this is the case, documentation of the presence of a particular transcription factor may not be sufficient evidence to assume function.

Our observation of the absence of significant levels of GATA-3, cMAF, and STAT-6 in tissue from patients with tuberculosis (T_H1-mediated disease) supports the concept that these transcription factors are mainly involved in the regulation of T_H2-type cytokines. In addition, this suggests that the increased expression of these transcription factors in asthmatic airways is not the consequence of air-

way inflammation per se, but rather and more specifically, the T_H2 immune response associated with asthma.

We observed constitutive and elevated expression of STAT-6 in the lungs of healthy control subjects and patients with asthma, respectively, which agrees with our previous report of STAT-6 expression in the nasal mucosa of control subjects and patients with allergic rhinitis.³⁵ STAT-6 mediates signaling of the IL-4R,²¹ and disruption of the STAT-6 gene results in a loss of IgE production.^{24,25} Indeed, the fact that there were significantly fewer cells expressing STAT-6 in nonatopic asthma corresponds with the low level of serum IgE observed in these patients. Although the numbers of cells expressing STAT-6 (Fig 1, B) and α IL-4R¹⁵ were higher in atopic than in nonatopic asthma, there were significantly more of both mediators in the latter group than in control subjects. This difference could be ascribed merely to a "more active" response by patients with atopic asthma; alternatively, real genetic differences may be involved. Mitsuyasu et al³⁶ showed that amino acid substitution of Ile for Val in the IL-4R augments STAT-6 activation and transcriptional activity of the *I ϵ* promoter by IL-4. Rosa-Rosa et al³⁷ demonstrated that homozygosity for an IL-4R variant was significantly increased in individuals with atopic asthma and suggested it as an indicator of atopy susceptibility. From these studies, it could be postulated that patients with atopic asthma may express a more highly active variant of the IL-4R, possibly resulting in a super increase in STAT-6 expression and subsequently in IL-4/STAT-6-responsive genes such as IgE²⁶ and the α IL-4R.²⁸

The ability of IL-4 to inhibit production of IFN- γ and subsequent T_H1 cell differentiation has been shown to involve STAT-6. Goenka et al³⁸ demonstrated that STAT-6 binds and inhibits the IFN- γ regulatory factor 1 site in the IFN- γ promoter, whereas Kubo et al³⁹ observed that in T_H2 but not T_H1 cells STAT-6 inactivates a silencer of IL-4 gene activity. This would suggest that in patients with nonatopic asthma with lower STAT-6 levels, the expression of IFN- γ may be elevated. Indeed, one report indicates that IFN- γ levels are higher in nonatopic than in atopic asthma.⁴ IFN- γ is a potent activator of eosinophils⁴⁰ and induces their release of mediators such as RANTES.⁴¹ Furthermore, antibodies against this cytokine are reported to completely abolish airway hyperresponsiveness in allergen-challenged mice.⁴² As such, it is conceivable that IFN- γ may also be involved in the development of asthma symptoms in patients with nonatopic asthma with lowered STAT-6 levels.

This study is the first to characterize and compare the expression of the T_H2 cytokine-associated transcription factors in atopic and nonatopic asthma. GATA-3 and cMAF are upregulated in asthma, regardless of atopic status. Although STAT-6 is also increased in both asthma subtypes, it was substantially lower in nonatopic than in atopic asthma. This work supports the previously ventured notion that the IL-5 pathway, mediated by GATA-3, coincides with asthma, whereas IL-4 and STAT-6 are more closely associated with atopy. Furthermore, these findings indicate that a fundamental difference between

these 2 phenotypes of asthma may be the degree of IL-4R signaling, attributed to varying expression of STAT-6.

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Correction

In the article "IgE regulation and roles in asthma pathogenesis" in the March 2001 issue of the Journal (Oettgen HC, Geha RS. 2001;107:429-40), the following items were omitted from the list of references:

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