

Letter to the Editor

Steroids and extracellular signal-regulated kinase 1/2 activity suppress activating transcription factor 3 expression in patients with severe asthma

To the Editor:

Severe or refractory asthma, which affects 5% to 15% of all asthmatic patients, is a state of heightened morbidity that is refractory to normal pharmacologic treatment options and responsible for more than half of the health burden associated with the disease. Although glucocorticoids are the mainstay of

treatment in asthmatic patients, they are unable to fully control the disease in patients with severe asthma. Glucocorticoid resistance can stem from genetic irresponsiveness, although this is a rare occurrence. Moreover, glucocorticoid withdrawal leads to worsening of symptoms in patients with severe asthma, supporting the notion that glucocorticoids are still playing a role but are inefficient at controlling the severity of the disease.

Interestingly, a feature of severe asthma is an inflammatory process that often combines the presence of neutrophils with more typical features of allergic inflammation. Stratification of asthmatic patients based on mixed granulocytes present in sputum

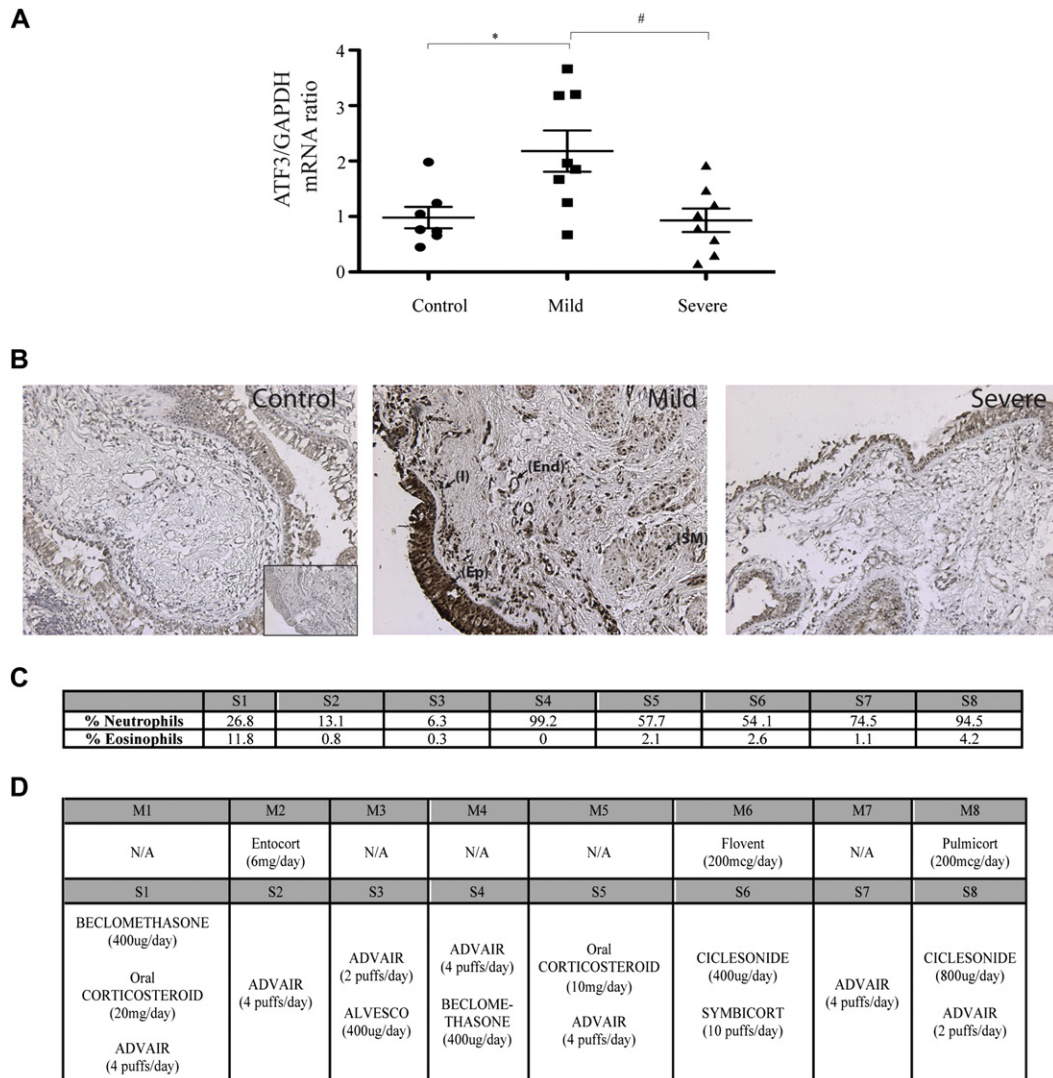


FIG 1. *ATF3* expression is lost in patients with severe asthma compared to mild asthmatics. **A**, *ATF3* mRNA abundance (PCR primers: *ATF3* reverse, 5'- TTT CTT TCT CGT CGC CTC TTT T -3'; *ATF3* forward, 5'- AAG TGA GTG CTT CTG CCA TC -3'). **P* < .05, patients with mild asthma compared with control subjects. #*P* < .05, patients with severe asthma compared with patients with mild asthma (ANOVA followed by Bonferroni multiple comparison test). *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase. **B**, *ATF3* localization. *End*, Endothelial cells; *Ep*, epithelial cells; *I*, inflammatory cells; *SM*, smooth muscle cells. Panels are $\times 200$ magnifications, and the *insert* is the isotype control. **C**, Percentages of neutrophils and eosinophils in WBCs of sputum from patients with severe asthma determined as published in Hastie et al.¹ **D**, Use and dosage of oral and inhaled glucocorticoids in patients with mild (*M*) and severe (*S*) asthma. *N/A*, Not applicable.

showed that a high percentage of eosinophils ($\geq 2\%$) combined with a high percentage of neutrophils ($\geq 40\%$) was associated with worst lung function and worst asthma control.¹ Accordingly, neutrophil-rich inflammation is generally found to be poorly responsive to glucocorticoids.²

Recently, activating transcription factor 3 (ATF3), a member of the basic-region leucine zipper transcription factor family, was shown to be a negative regulator of allergic inflammation in mice challenged with ovalbumin.³ Mice lacking ATF3 had increased levels of IL-4, IL-5, and IL-13, as well as enhanced pulmonary eosinophilia and airway hyperresponsiveness.³ Moreover, ATF3-deficient mice have an increased susceptibility to endotoxic shock and increased serum levels of IL-6 and IL-12.⁴ IL-6 is an essential signal for the differentiation of T_H17 lymphocytes, whereas IL-12 plays a role in promoting the proliferation of T_H1 cells. These results identify ATF3 not only as a negative regulator of allergic inflammation (linked to T_H2 immunity) but also of T_H1 - and T_H17 -driven immunity. Because the inflammation seen in patients with severe asthma has features of T_H2 and T_H17 immunity,⁵ we investigated the expression levels of ATF3 in nonasthmatic subjects, as well as in patients with mild and severe asthma.

Adult endobronchial tissue specimens were obtained from the Fonds de Recherche en Santé du Québec Respiratory Health Network Tissue Bank. Written consent was obtained from all subjects, and the study protocol was approved by a hospital research ethics board. A clinical diagnosis of asthma was made by a respiratory physician for all subjects based on the American Thoracic Society Workshop for Refractory Asthma. Tissue RNA was extracted, and mRNA levels of *ATF3* were analyzed by means of quantitative real-time PCR, as described previously.⁶

A significant increase in *ATF3* mRNA was observed in patients with mild asthma compared with that seen in nonasthmatic subjects, but this difference was no longer present in the patients with severe asthma (Fig 1, A). Immunohistochemical staining of ATF3 on bronchoscopic biopsy specimens with an ATF3 antibody (catalog no. 467-MO1; Abnova, Taipei City, Taiwan) resulted in similar results to the mRNA profile: high expression in patients with mild asthma that was lost in patients with severe asthma (Fig 1, B). ATF3 expression was most apparent in airway epithelial cells, endothelial cells, airway smooth muscle cells, and infiltrating inflammatory cells (Fig 1, B). Interestingly, when the patients with severe asthma were stratified based on sputum white blood cell (WBC) percentage of eosinophils and neutrophils,¹ 5 of 8 biopsy specimens showed high neutrophil counts in the matching sputum, with 3 of those also showing high eosinophil counts (Fig 1, C). No WBC count was available for sputum from patients with mild asthma. This clearly supports the presence of neutrophilic and mixed granulocytic inflammation in some patients with severe asthma. Therefore in patients with severe asthma, the expression of a negative regulator of inflammation is lost, correlating well with the increase in inflammation.

We then investigated the signals that might be responsible for ATF3 repression in patients with severe asthma. TNF- α has been linked with the severe but not mild or moderate forms of asthma.⁷ TNF- α (10 ng/mL) increased mRNA levels of *ATF3* in primary human airway smooth muscle cells, airway epithelial cells, and microvascular endothelial cells (Fig 2). This increased expression was fully reversed by pretreating cells for 1 hour with the glucocorticoid dexamethasone (100 nmol/L). Moreover, blocking the activation of the extracellular signal-regulated kinase (ERK) 1/ERK2 mitogen-activated protein kinases with the

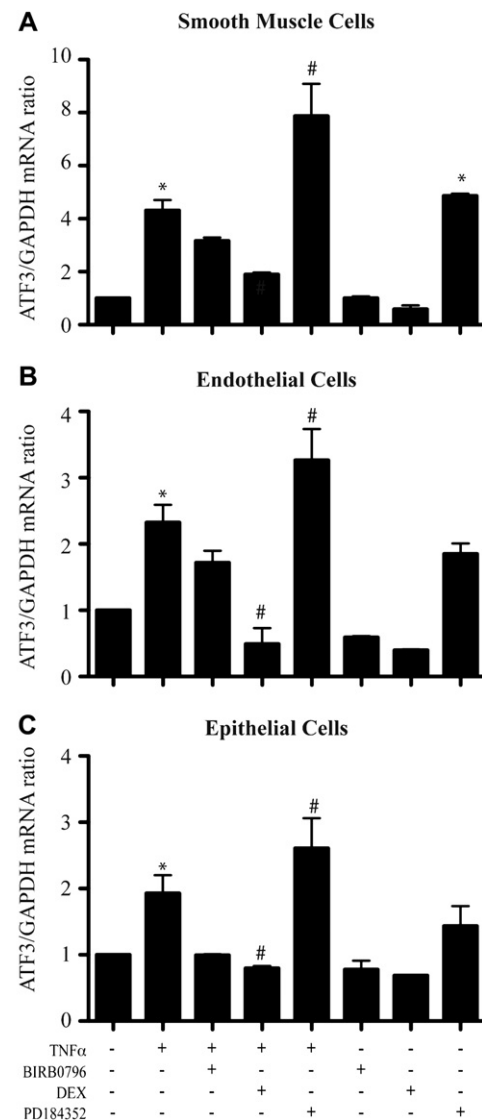


FIG 2. Steroids and ERK1/ERK2 activity suppress TNF- α -driven ATF3 expression. Airway smooth muscle cells (A), human microvascular endothelial cells (HMEC-1; B), and airway epithelial cells (Beas-2B; C) were left untreated or pretreated with 100 nmol/L dexamethasone (DEX), 2 μ mol/L PD184352, or 0.1 μ mol/L BIRB0796 for 1 hour before exposure to 10 ng/mL TNF- α for 8 hours. ATF3 mRNA levels were measured as in Fig 1. * $P < .05$, TNF- α treated compared with unstimulated. # $P < .05$, dexamethasone or PD184352 treated compared with TNF- α treated. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

pharmacological inhibitor PD18435 (2 μ mol/L) increased ATF3 expression, whereas blocking p38 mitogen-activated protein kinase activity with BIRB0796 (0.1 μ mol/L) had no effect (Fig 2, A). Taken together, these results demonstrate that glucocorticoids and ERK1/ERK2 suppress ATF3 expression. Interestingly, patients with severe asthma by definition take high doses of glucocorticoids (see the table in Fig 1, D) and were previously shown to have higher ERK1/ERK2 activity,⁸ 2 events decreasing ATF3 expression, which might explain the increase in inflammation. However, currently, it is not possible to distinguish between the intrinsic role played by increased ERK1/ERK2 activity and the extrinsic contribution of glucocorticoid exposure to ATF3 repression in patients with severe asthma. Therefore glucocorticoids might have

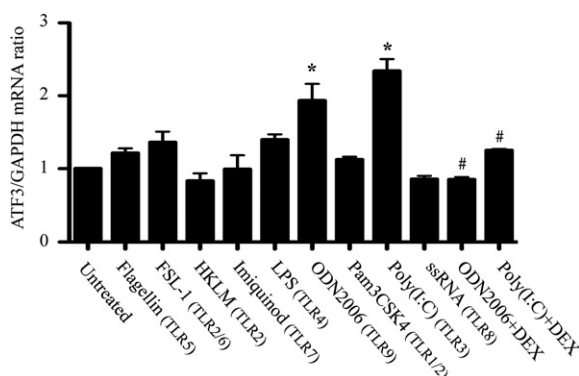


FIG 3. TLR3 and TLR9 agonists are the most potent inducers of ATF3 expression in epithelial cells. Beas-2B cells were left untreated or pretreated with 100 nmol/L dexamethasone and incubated 24 hours in the absence or presence of Pam3CSK4 (1 μ g/mL), heat-killed *Listeria monocytogenes* (HKLM; 10^8 cells/mL), Poly I:C (10 μ g/mL), *Escherichia coli* K12 LPS (100 ng/mL), *Salmonella typhimurium* flagellin (100 ng/mL), FSL-1 (1 μ g/mL, a synthetic diacylated lipoprotein), imiquimod (1 μ g/mL), ssRNA40 (1 μ g/mL), or ODN2006 (2 μ mol/L). The TLR activated is indicated in brackets. ATF3 mRNA levels were measured as in Fig 1. * $P < .05$, TLR agonist treated compared with unstimulated. # $P < .05$, dexamethasone compared with respective TLR agonist. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

an unwanted effect by preventing the expression of a negative regulator of inflammation. The corollary is that therapies aimed at increasing ATF3 levels might be beneficial in patients with severe asthma. This could be achieved by suppressing ERK1/ERK2 activity or by driving ATF3 expression.

We found that a Toll-like receptor (TLR) 3 agonist (Poly I:C) and a TLR9 agonist (CpG oligodeoxynucleotide [ODN]) were the most potent of the TLR activators in inducing ATF3 expression in epithelial cells (Fig 3). However, because activation of TLR3 would occur during viral infections linked with asthma exacerbation, ATF3 expression downstream of TLR3 appears not to be sufficient to counterbalance the innate immune activation mediated by this receptor, such as the synthesis of type I interferons. Interestingly, TLR9 activation has already shown promise in treating allergic diseases.⁹ Our data further support a protective role of CpG ODN by increasing ATF3 expression. It is worth noting that TLR3- and TLR9-driven ATF3 expression is suppressed by dexamethasone before treatment (Fig 3).

For the first time, we have provided evidence of decreased ATF3 expression in the airways of patients with severe asthma compared with that seen in patients with mild asthma. Both the presence of glucocorticoids and the increased activity of ERK1/ERK2 are involved in repressing ATF3 expression, thus preventing the action of this transcription factor in negatively regulating inflammation. However, we cannot exclude the possibility that the

disease process between mild and severe asthma differs and that only the former would involve a change in ATF3 expression. We propose that increasing ATF3 expression by using CpG ODN or by inhibiting ERK1/ERK2 activity might lead to significant improvements in patients with severe asthma.

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