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Corticosteroid insensitivity of chemokine expression in airway smooth muscle of patients with severe asthma

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Abstract

Background—Patients with severe asthma are less responsive to the beneficial effects of corticosteroid therapy.

Objective—We investigated whether corticosteroid insensitivity was present in airway smooth muscle cells (ASMCs) of patients with severe asthma.

Methods—ASMCs cultured from bronchial biopsy specimens of nonasthmatic control subjects (n = 12) and patients with nonsevere (n = 10) or severe (n = 10) asthma were compared for the effect of dexamethasone on suppression of TNF- α - and IFN- γ -induced CCL11 (eotaxin), CXCL8 (IL-8), and CX3CL1 (fractalkine) expression. The mechanisms of corticosteroid insensitivity are also determined.

Results—CCL11 release was higher in ASMCs of patients with nonsevere but not severe asthma and nonasthmatic control subjects; CXCL8 and CX3CL1 release were similar in all groups. In patients with severe asthma, dexamethasone caused less suppression of CCL11 and CXCL8 release induced by TNF- α . Dexamethasone potentiated TNF- α - and IFN- γ -induced CX3CL1 release equally in all 3 groups. TNF- α -induced phosphorylated p38 mitogen-activated protein kinase levels were increased in ASMCs from patients with severe asthma compared with those from patients with nonsevere asthma and nonasthmatic subjects, whereas TNF- α -induced phosphorylated c-Jun N-terminal kinase and phosphorylated extracellular signal-related kinase levels were increased in all asthmatic groups. A p38 inhibitor increased the inhibitory effect of dexamethasone.

Conclusions—ASMCs of patients with severe asthma are corticosteroid insensitive; this might be secondary to heightened p38 mitogen-activated protein kinase levels.

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Keywords

Airway smooth muscle; asthma; corticosteroid insensitivity; CX3CL1; CCL11; CXCL8

Asthma is a chronic disease of the airways characterized by persistent airway inflammation, reversible airway obstruction, and airway remodeling.¹ Smooth muscle hyperplasia and hypertrophy are recognized features of airway wall remodeling,² and an increase in airway smooth muscle mass has been reported in asthmatic patients, particularly in those with severe asthma, in whom the mass is greater than in those with nonsevere asthma.^{3,4} In addition to their contractile properties, airway smooth muscle cells (ASMCs) have the ability to synthesize and release biologically active inflammatory mediators, including cytokines, chemokines, and growth factors, which might contribute to the inflammatory process and to airway wall remodeling.⁵ Chemokines, such as CCL11 (eotaxin),⁶ CCL5 (RANTES),⁷ CXCL8 (IL-8),⁸ and CX3CL1 (fractalkine),⁹ are synthesized by ASMCs in response to stimulation by inflammatory cytokines, such as TNF- α and IL-1 β . ASMCs from asthmatic patients have now been reported to be hyperproliferative and to produce and express more chemokines, such as CCL11 and CXCL8, than ASMCs from nonasthmatic patients.¹⁰⁻¹³ In addition, the proliferative response of ASMCs in asthmatic patients has been reported to be less sensitive to the suppressive effects of corticosteroids.¹⁴

A proportion of asthmatic patients continue to experience uncontrolled asthma despite receiving maximal asthma therapies, including corticosteroids, which is often referred to as having severe or refractory asthma.¹⁵⁻¹⁷ These patients can present with chronic airflow obstruction and recurrent exacerbations of asthma and are less responsive to the beneficial effects of corticosteroid therapy, which usually works most effectively in those with non-severe asthma.¹⁸⁻²⁰ Thus patients with severe asthma are distinct from other asthma groups whose asthma symptoms remain controlled with existing asthma therapies. We reasoned that in patients with severe asthma, ASMCs could exhibit corticosteroid insensitivity. We therefore studied ASMCs obtained from bronchial biopsy specimens to probe some of the potential mechanisms underlying the corticosteroid insensitivity observed in ASMCs from patients with severe asthma.

METHODS

More details on the methods used in this study are provided in the Methods section in this article's Online Repository at www.jacionline.org.

Subjects' characteristics

All nonasthmatic subjects were healthy volunteers without any disease with negative PC₂₀ values to methacholine and normal spirometric results (Table I). Patients with severe asthma needed either continuous or near-continuous oral corticosteroids, high-dose inhaled corticosteroids, or both to achieve a level of mild-to-moderate persistent asthma, and by 2 or more minor criteria.¹⁶ Patients with nonsevere asthma used inhaled beclomethasone (0-1000 μ g/d or equivalent) with perfect control of their asthma symptoms. Current smokers and exsmokers of greater than 5 pack years were excluded. All patients provided informed consent to participate in this study, which was approved by the local ethics committee. They underwent fiberoptic bronchoscopy, during which bronchial biopsy specimens were obtained.²¹ All the subjects were free from upper respiratory tract infections and acute exacerbations within 3 months before bronchoscopy.

ASMC isolation and culture

Bronchial biopsy specimens were cut into small pieces ($<1 \text{ mm}^2$) and transferred to 6-well culture plates. At confluence, cells were harvested and split into larger flasks at each passage. ASMCs were identified by the characteristic “hill and valley” morphology and by their expression of calponin, smooth muscle α -actin, and myosin heavy chain in more than 95% of the cells.²² Only cells at passages 4 and 5 were used.

ASMC activation and chemokine assay

Cells were plated in 6-well culture plates at 80,000 cells per well. At 90% confluency, cells were serum starved for 24 hours and stimulated with cytokines (see below). Supernatant levels of CXCL8, CCL11, and CX3CL1 were measured by using an ELISA. Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed with random primers and Avian Myeloblastosis Virus Reverse Transcriptase (Promega, Madison, Wis). cDNA was quantified by using quantitative real-time PCR (qRT-PCR; Rotor Gene 3000; Corbett Research, Mortlake, Australia). Specific primers for *CCL11*, *CXCL8*, *CX3CL1*, and *18S* (see Table E1 in this article's Online Repository at www.jacionline.org) were designed by using the GenScript online primer design software (GenScript, Piscataway, NJ).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed with the ChIP assay kit (Millipore, Temecula, Calif). Cells were fixed in 1% formaldehyde for 10 minutes and DNA fragmented by means of sonication (5×15 -second pulses). After adding ChIP dilution buffer, 4 μg of antibody was added to precleared chromatin solution overnight. Antibody/DNA complexes were captured, washed, eluted, and reverse cross-linked. Both the DNA and input fractions were purified by means of phenol/chloroform washing and ethanol precipitation. The precipitated DNA was resuspended, and quantitative PCR was performed. Sample DNA was normalized to input DNA.

Western blotting

The protein membrane was incubated with rabbit antibody for anti-phosphorylated p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), or extracellular signal-regulated kinase (ERK), followed by anti-rabbit-horseradish peroxidase antibody. Antibody-bound proteins were visualized with ECL or ECL plus (Amersham Biosciences, Piscataway, NJ). The membranes were then reprobbed with rabbit anti-total p38, JNK, or ERK (Cell Signaling, Danvers, Mass) or with mouse anti- β -actin mAb (Santa Cruz Biotechnology, Santa Cruz, Calif) to control for protein loading. Relevant band intensities were quantified by using scanning densitometric analysis.

Statistical analysis

The Wilcoxon matched pairs test was used for intragroup analysis before and after cytokine treatment. One-way ANOVA with the Dunnett multiple comparison was used to compare the effect of dexamethasone and MAPK inhibitor compared with cytokine stimulation alone. The Kruskal-Wallis test with the Dunn multiple comparison was used to compare results between the 3 groups. *P* values of less than .05 were taken as significant.

RESULTS

Regulation of CCL11, CXCL8, and CX3CL1 in ASMCs

In preliminary studies we confirmed a dose-dependent release of CCL11 and CXCL8 by TNF- α in ASMCs from nonasthmatic subjects and the synergistic effect of TNF- α and IFN-

γ leading to a concentration-dependent increase in CX3CL1 release (see Fig E1 in this article's Online Repository at www.jacionline.org). ASMCs were treated with either TNF- α (10 ng/mL, for CCL11 and CXCL8) or a combination of TNF- α and IFN- γ (10 ng/mL each, for CX3CL1) for 24 hours. Baseline and induced CCL11 release were significantly higher in patients with nonsevere asthma compared with values seen in either nonasthmatic subjects or patients with severe asthma (Fig 1, A). qRT-PCR also showed increased mRNA expression induced by TNF- α in patients with nonsevere asthma (Fig 1, B). In contrast, there was no difference in CXCL8 and CX3CL1 release or expression between nonasthmatic subjects and patients with nonsevere asthma and those with severe asthma (Fig 1, C-F).

Promoter recruitment of p65 component of nuclear factor κ B

To determine whether differences in the recruitment of p65, a major component of nuclear factor κ B (NF- κ B), to the promoter of the NF- κ B-dependent *CCL11* gene could explain the increase in CCL11 expression in ASMCs from patients with nonsevere asthma, we used ChIP assays. We first determined that p65 was recruited to the promoters of *CCL11*, *CXCL8*, and *CX3CL1*, with maximal promoter occupancy occurring at 60 minutes after stimulation with TNF- α (10 ng/mL) or in combination with IFN- γ (see Fig E2 in this article's Online Repository at www.jacionline.org). We compared the recruitment of p65 with the *CCL11*, *CXCL8*, and *CX3CL1* gene promoters in ASMCs of nonasthmatic subjects and patients with nonsevere and severe asthma and found no differences in the degree of recruitment (Fig 2).

Corticosteroid suppression of CCL11 and CXCL8 expression

ASMCs were pretreated with dexamethasone (10^{-10} to 10^{-6} mol/L) for 2 hours and stimulated with 10 ng/mL TNF- α for 24 hours. Dexamethasone suppressed CCL11 release in a concentration-dependent manner, with a significantly reduced suppression in ASMCs of patients with severe asthma compared with that observed in both nonasthmatic subjects ($P < .05$) and patients with nonsevere asthma ($P < .05$; Fig 3, A). Similarly, dexamethasone (10^{-7} mol/L) suppressed TNF- α -induced CCL11 mRNA expression by 45.9% and 61.38% in ASMCs of nonasthmatic subjects and patients with nonsevere asthma, respectively, but without any suppression in patients with severe asthma (Fig 3, B-D). Similar results were seen with CXCL8 release (Fig 4).

Corticosteroid potentiation of CX3CL1 expression

ASMCs were preincubated with dexamethasone (10^{-10} to 10^{-6} mol/L) for 2 hours and then treated with TNF- α and IFN- γ , 10 ng/mL each, for 24 hours. The degree of CX3CL1 potentiation by dexamethasone was similar in ASMCs of the 3 groups (Fig 5, A). qRT-PCR also showed that dexamethasone potentiated the induced CX3CL1 mRNA expression to a similar level in all groups (Fig 5, B-D).

Role of TNF- α -induced MAPK activation in corticosteroid insensitivity

Phosphorylated and total p38, JNK, and ERK MAPK expression in response to TNF- α , with maximal activation between 15 and 30 minutes, was measured by means of Western blotting (see Fig E3 in this article's Online Repository at www.jacionline.org). We then compared the induction of MAPK phosphorylation at 15 minutes after stimulation in ASMCs of nonasthmatic subjects and patients with nonsevere and severe asthma. Induced p38 phosphorylation in patients with severe asthma was significantly higher than in both nonasthmatic subjects ($P < .01$) and patients with nonsevere asthma ($P < .05$; Fig 6, A). Induced JNK phosphorylation was higher in both patients with nonsevere and those with severe asthma compared with that seen in nonasthmatic subjects ($P < .05$ and $P < .01$,

respectively), whereas there was no significant difference between the former 2 groups (Fig 6, *B*). Similarly, although ASMCs from asthmatic patients expressed significantly higher ERK phosphorylation than ASMCs from nonasthmatic subjects ($P < .05$), there was no significant difference between patients with nonsevere and those with severe asthma (Fig 6, *C*).

We next studied the effect of the selective p38 α inhibitor GW-856553 or the selective JNK inhibitor SP600125 on TNF- α -induced release of CCL11 and CXCL8. GW-856553 suppressed TNF- α -induced CXCL8 release in a concentration-dependent manner, with maximal suppression of 31.8% ($P < .05$) at 10^{-6} mol/L (Fig 7, *B*), whereas it had minimal effect on CCL11 release (Fig 7, *A*). To determine whether there was additivity or synergy of effects between dexamethasone and the p38 MAPK inhibitor, we pretreated ASMCs of patients with severe asthma with GW-856553 (10^{-8} or 10^{-6} mol/L), dexamethasone (10^{-9} to 10^{-6} mol/L), or both for 2 hours, followed by stimulation with 10 ng/mL TNF- α for 24 hours. In the absence of GW-856553, dexamethasone (10^{-6} mol/L) suppressed induced CCL11 and CXCL8 release by 21.88% and 32.79%, respectively. With GW-856553 (10^{-6} mol/L), dexamethasone (10^{-6} mol/L) suppressed induced CCL11 and CXCL8 release by 55.02% ($P < .05$) and 63.45% ($P < .05$), respectively (Fig 7, *C* and *D*). In contrast, inhibition of JNK with SP600125 did not improve the suppressive effects of dexamethasone (Fig 7, *E* and *F*).

DISCUSSION

We have shown that TNF- α -induced release of both CCL11 and CXCL8 were less suppressible by dexamethasone in ASMCs from patients with severe asthma compared with those from patients with nonsevere asthma or nonasthmatic subjects. This is despite the greater release of CCL11 and mRNA expression induced by TNF- α in ASMCs of patients with nonsevere asthma. p38 activation was heightened in ASMCs of patients with severe asthma compared with that seen in patients with nonsevere asthma, and a selective inhibitor of p38 MAPK activation improved the suppression of TNF- α -induced CCL11 and CXCL8 release by dexamethasone, indicating a potential role for p38 activation in corticosteroid insensitivity. This is the first demonstration of impaired corticosteroid inhibition of CCL11 and CXCL8 from ASMCs of patients with severe asthma, extending the previously reported¹⁴ impaired corticosteroid suppression of serum-induced ASMC proliferation of ASMCs from asthmatic patients. We now extend the presence of corticosteroid insensitivity in alveolar macrophages and in blood mononuclear cells from patients with severe asthma^{23,24} to ASMCs. Furthermore, as previously shown in alveolar macrophages, p38 MAPK activation was greatest in ASMCs from patients with severe asthma, and a p38 MAPK inhibitor led to an improved suppressive effect of corticosteroids.

We have previously shown the synergistic effect of TNF- α and IFN- γ on CX3CL1 release and mRNA expression in ASMCs and the potentiating effect of dexamethasone on CX3CL1 release and expression.^{9,25} An increase in the expression of CX3CL1 in airway tissues of patients with asthma has been reported,²⁶ and CX3CL1 levels in bronchoalveolar lavage fluid are increased after segmental allergen challenge.²⁷ The potentiating effect of TNF- α - and IFN- γ -induced CX3CL1 expression by dexamethasone is likely through a transactivation with the binding of activated glucocorticoid receptor (GR) to its positive glucocorticoid-responsive element site on the CX3CL1 promoter²⁸ rather than a transrepressive mechanism underlying the suppressive effect of dexamethasone on CCL11 and CXCL8. The fact that the potentiating effect of dexamethasone on CX3CL1 expression and release was similar in the 3 groups of subjects indicates that there are no differential effects of dexamethasone on glucocorticoid-responsive element binding.

We also showed that CCL11, but not CXCL8, release and expression induced by TNF- α was increased in patients with nonsevere asthma compared with values seen in those with severe asthma and nonasthmatic subjects. Similarly, increased release of CCL11 is induced by IL-13 and IL-1 β in ASMCs of asthmatic subjects.¹⁰ However, there was no increase in CXCL8 release after TNF- α stimulation of ASMCs from patients with nonsevere asthma, as previously reported.¹² We found no increased recruitment of p65 to the promoter of *CCL11* and *CXCL8* using ChIP assays in any of the patient groups and could not confirm increased p65 binding to the *CXCL8* promoter, as previously reported.¹² The mechanism underlying the differential expression of *CCL11* in ASMCs from patients with nonsevere asthma requires further investigation. Posttranslational modification of transcription factors can affect catalytic activity, stability, trafficking, and protein-protein interactions, such as cofactor recruitment.²⁹ For example, serine 276 on p65 is a substrate for p38 α ,³⁰ and phosphorylation of serine 276, although not interfering with translocation or DNA-binding affinity, might be essential for transcriptional activity.³¹

MAPKs regulate various cellular activities, such as gene expression, cell proliferation, and cell survival/apoptosis.^{32,33} Enhanced MAPK activity in the airways of patients with severe asthma has been reported,³⁴ and increased activation of p38, JNK, and ERK might have a potential role in steroid-insensitive asthma.^{24,35-38} Activation of these MAPKs was heightened in asthmatic patients compared with that seen in nonasthmatic subjects, but only p38 activation was significantly higher in patients with severe asthma compared with patients with nonsevere asthma. IL-2 and IL-4 exposure of blood monocytes reduces the corticosteroid ligand-binding affinity caused by phosphorylation of the GR, which can be reversed by p38 MAPK inhibition,³⁵ or through an indirect effect on the ligand-binding domain of GR.³⁹ p38 MAPK activation can also be involved in the stabilization⁴⁰ and increased translation of proinflammatory cytokine mRNA, such as *CXCL8*,⁴¹ which was less suppressible by dexamethasone in ASMCs of patients with severe asthma.

One of the limitations of this study is that the cultured human ASMCs at passages 4 to 5 might not reflect the situation *in vivo*. However, these *in vivo* data are concordant with the presence of chronic airflow obstruction and increase in airway smooth muscle mass, despite treatment with corticosteroid therapy. The chronic airflow obstruction in patients with severe asthma can also be contributed to by airway wall inflammation and edema and by intraluminal mucus. The lack of effect of corticosteroids in suppressing CCL11 and CXCL8 release from ASMCs in patients with severe asthma can contribute further to the severity of asthma.

Severe asthma is a condition that does not respond fully to the beneficial effects of corticosteroid therapy. The airway smooth muscle is a site of corticosteroid insensitivity, which might contribute to the severity of asthma, and reversibility of corticosteroid insensitivity in airway smooth muscle could lead to better control of severe asthma.

METHODS

ASMC isolation and culture

ASMCs from nonasthmatic subjects were either from healthy transplant donors or from endobronchial biopsy specimens, whereas all ASMCs from asthmatic patients were obtained from bronchial biopsy specimens of the right lower bronchus taken at bronchoscopy. Biopsy specimens were cut with an aseptic needle into small pieces sized less than 1 mm². Each piece of tissue was transferred to a 6-well culture plate for culture. When confluent, cells were harvested and split into 25-cm² and then 75-cm² flasks at the next passages. Subsequently, they were split into 150-cm² flasks in preparation for experiments or stored at -80°C in liquid nitrogen.

Cells were cultured in Dulbecco modified Eagle medium supplemented with 10% FCS, 4 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B. The presence of ASMCs was confirmed by identifying the characteristic “hill and valley” morphology by means of light microscopy. Cell stocks were kept in 150-cm² flasks at 37°C and 5% CO₂ in a humidified atmosphere. Cells between passages 4 and 5 were used for experiments. At 90% confluency, the cells were serum deprived for 24 hours in Dulbecco modified Eagle medium supplemented with 4 mL L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin B, 1% nonessential amino acids, and 0.1% BSA.

Chemokine production

ASMCs were seeded in 6-well culture plates at a density of 80,000 cells per well. At 90% confluency, cells were serum starved for 24 hours and stimulated as indicated. At the end of the experiment, the supernatant was collected. The concentrations of CXCL8, CCL11, and CX3CL1 in the supernatant were measured by using an ELISA-based method (R&D Systems, Abington, United Kingdom), according to the manufacturer's instructions.

RNA extraction, cDNA preparation, and qRT-PCR

ASMCs were seeded in 6-well culture plates at a density of 80,000 cells per well. At 90% confluency, the cells were serum starved for 24 hours and then stimulated as indicated. Total RNA was isolated from ASMCs by using the RNeasy Mini Kit (Qiagen) and quantified by using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Single-stranded cDNA was then synthesized from 0.5 µg of total RNA by using random hexamers (50 ng/µL) and Avian Myeloblastosis Virus Reverse Transcriptase (1 U/µL, Promega).

cDNA was quantified by means of qRT-PCR (Rotor Gene 3000, Corbett Research); amplification was performed with a 20-µL reaction containing 10 µL of Universal SYBR Green PCR Master Mix (Qiagen). Specific primers for CXCL8, CCL11, CX3CL1, and 18S (Table I) were designed according to their published sequences by using the GenScript online primer design software and synthesized by Sigma-Genosys (The Woodlands, Tex). Primer specificity was assessed with BLAST software. The cycling conditions were 15 minutes at 95°C (enzyme activation), followed by 35 to 60 cycles of 20 seconds at 94°C (denaturing step), 20 seconds at 60°C (annealing step), and 20 seconds at 72°C (elongation step). Melting curve analysis and agarose gel electrophoresis were carried out to ensure the presence of a specific PCR product. Data were analyzed through Rotor-Gene6 software (Corbett Research) by using a standard curve. Relative quantification of gene expression was normalized to 18S expression.

ChIP assays

ChIP assays were performed by using the ChIP assay Kit supplied by Millipore and the protocol therein. Briefly, ASMCs were seeded in a 75-cm² flask. At 90% confluency, cells were serum deprived for 24 hours. After treatment, the cells were fixed in 1% formaldehyde for 10 minutes. After neutralization with 0.125 mol/L glycine, cells were scraped down, pelleted, and lysed in 1% SDS lysis buffer. The DNA was fragmented by 5 pulses of sonication for 15 seconds. After addition of ChIP dilution buffer, 100 µL of sample was saved as input. Four micrograms of antibody was added to a 900-µL precleared chromatin solution, and the sample was incubated overnight. Antibody/DNA complexes were captured, washed, eluted, and reverse cross-linked, as described in the protocols. Both the DNA and input fractions were purified by using phenol/chloroform washing and ethanol precipitation. The precipitated DNA was resuspended in 50 µL of nuclease-free water, and quantitative PCR was performed on 5 µL of sample, as described above. Sample DNA was normalized to input DNA. The primers sequences are listed in Table E1.

Western blotting

Protein concentration was determined by using the bicinchoninic acid protein assay (Thermo Scientific). Protein extract (20 µg per lane) was fractionated by means of SDS-PAGE on a 4% to 12% bis-Tris precast gel (Invitrogen, Carlsbad, Calif) and then transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was incubated overnight at 4°C with rabbit antibody for anti-phosphorylated p38, JNK, or ERK (Cell Signaling), followed by anti-rabbit-horseradish peroxidase antibody (Dako, Cambridgeshire, United Kingdom) for 45 minutes at room temperature. Antibody-bound proteins were visualized with ECL or ECL plus (Amersham Biosciences). The membranes were stripped and then reprobed with rabbit anti-total p38, JNK, or ERK (Cell Signaling) or total GR (Abcam, Cambridge, United Kingdom) antibody or with rabbit anti-β-actin mAb (Abcam) to control for protein loading. Relevant band intensities were quantified by means of scanning densitometric analysis with software from Ultra-Violet Products (Cambridge, United Kingdom). Densitometric data were normalized to β-actin.

Data analysis

The Wilcoxon matched pairs test was used for intragroup analysis before and after cytokine treatment. One-way ANOVA with the Dunnett multiple comparison was used to compare the effect of dexamethasone or MAPK inhibitor compared with cytokine stimulation alone. The Kruskal-Wallis test followed by the Dunn multiple comparison was used to compare results between the 3 groups. *P* values of less than .05 were taken as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

ASMC	Airway smooth muscle cell
ChIP	Chromatin immunoprecipitation
ERK	Extracellular signal-regulated kinase
GR	Glucocorticoid receptor
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor κB
qRT-PCR	Quantitative real-time PCR

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Key messages

- Cultured ASMCs of patients with severe asthma display relative insensitivity to the effects of corticosteroids in terms of CCL11 and CXCL8 release when compared with cells from patients with nonsevere asthma.
- p38 MAPK inhibition partly restores corticosteroid sensitivity in ASMCs of patients with severe asthma, indicating a therapeutic potential for p38 MAPK inhibitors in the treatment of severe asthma.

FIG 1.

Cytokine-induced protein release and mRNA expression of inflammatory chemokines in ASMCs of nonasthmatic subjects and patients with nonsevere and severe asthma. After exposure to cytokines for 24 hours, CCL11 (A and B), CXCL8 (C and D), and CX3CL1 (E and F) expression was assessed by using ELISA and qRT-PCR. Horizontal lines represent medians. US, Unstimulated. # $P < .05$, ## $P < .01$, and ### $P < .001$ versus unstimulated. * $P < .05$, ** $P < .01$, and *** $P < .001$.

FIG 2.

Comparison of cytokine-induced recruitment of p65 to promoters of inflammatory genes in ASMCs of nonasthmatic subjects and patients with nonsevere and severe asthma. ASMCs were stimulated with TNF- α or with a combination of TNF- α and IFN- γ for 60 minutes. p65 recruitment to promoters of CCL11 (A), CXCL8 (B), and CX3CL1 (C) was assessed by using ChIP assays. Horizontal lines represent medians.

FIG 3.

Impaired suppression of induced CCL11 by dexamethasone in patients with severe asthma. ASMCs were pretreated with dexamethasone (10^{-10} to 10^{-6} mol/L, 2 hours) and stimulated with TNF- α (10 ng/mL, 24 hours). CCL11 release (A) and mRNA expression (B-D) were assessed by means of ELISA and qRT-PCR, respectively. *Points* and *bars* represent means $\pm$ SEMs. *US*, Unstimulated. * $P < .05$ versus nonasthmatic subjects. # $P < .05$ versus patients with nonsevere asthma. \$\$\$ $P < .001$.

FIG 4.

Impaired suppression of induced CXCL8 release and mRNA expression by dexamethasone in ASMCs of patients with severe asthma. ASMCs were treated in an identical way as described in Fig 3. CXCL8 release (**A**) and mRNA expression (**B-D**) were assessed by means of ELISA and qRT-PCR, respectively. *Points* and *bars* represent means $\pm$ SEMs. *US*, Unstimulated. * $P < .05$ and ** $P < .01$ versus nonasthmatic subjects. \$ $P < .05$ and \$\$\$ $P < .001$.

FIG 5.

Effect of dexamethasone on cytokine-induced CX3CL1 in ASMCs of nonasthmatic subjects and patients with nonsevere and severe asthma. ASMCs were pretreated with dexamethasone (10^{-10} to 10^{-6} mol/L, 2 hours) and stimulated with TNF- α and IFN- γ (10 ng/mL each, 24 hours). CX3CL1 release (**A**) and mRNA expression (**B-D**) were assessed by means of ELISA and RT-PCR, respectively. Points and bars represent means $\pm$ SEMs. US, Unstimulated. $$$P < .01$ and $$$$P < .001$.

FIG 6.

Comparison of induced MAPKs in ASMCs of nonasthmatic subjects and patients with nonsevere and severe asthma. ASMCs were stimulated with TNF- α (10 ng/mL) for 15 minutes. Phosphorylated and total p38 (**A**), JNK (**B**), and ERK (**C**) levels were assessed by means of Western blotting. Horizontal lines represent medians. * $P < .05$ and ** $P < .01$.

FIG 7.

Effect of p38 α and JNK inhibitors on the suppressive effect of dexamethasone. ASMCs were pretreated with either GW-856553 or SP600125, dexamethasone, or both for 2 hours and stimulated with TNF- α for 24 hours. CCL11 (A, C, and E) and CXCL8 (B, D, and F) release were assessed by means of ELISA. Bars and points represent means $\pm$ SEMs in 4 to 6 ASMCs of patients with severe asthma. * $P < .05$, ** $P < .01$, and *** $P < .001$.

TABLE I

Subjects' characteristics

	Nonasthmatic subjects	Patients with nonsevere asthma	Patients with severe asthma
No.	12	10	10
Age (y)	46.1 ± 12.2	42.3 ± 17.2	46.2 ± 14.4
Sex (female/male)	7/5	4/6	5/5
Duration of asthma (y)	NA	23.9 ± 12.3	35.6 ± 12.0 [*]
Inhaled corticosteroid dose (μg BDP equivalent)	0	740 ± 353	1563 ± 272 [†]
Atopy (no.) [§]	3	7	5
Receiving oral corticosteroids (no.)	0	0	5 [*]
FEV ₁ (L)	3.35 ± 0.81	2.88 ± 0.41	2.24 ± 0.36 [†]
FEV ₁ (% predicted)	96.8.0 ± 12.4	88.6 ± 11.1	74.9 ± 12.4 [*]
FEV ₁ /FVC ratio (%)	80.3 ± 12.0	77.9 ± 4.6	67.7 ± 10.0 [†]
β-agonist reversibility (%)	NA	15.7 ± 5.7	22.8 ± 10.6
PC ₂₀ (mg/mL)	>16	2.91 ± 2.88	0.91 ± 0.72 [*]

Data are shown as means ± SEMs.

BDP, Beclomethasone dipropionate; *FVC*, forced vital capacity; *NA*, not available.

^{*} $P < .05$,

[†] $P < .01$,

[‡] $P < .001$ versus patients with nonsevere asthma.

[§] Defined as positive skin prick test responses to 1 or more common aeroallergens.

^{||} Measured as the percentage increase in FEV₁ after 400 μg of salbutamol.