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Human Airway Smooth Muscle Cells from Asthmatic Individuals Have CXCL8 Hypersecretion Due to Increased NF-κB p65, C/EBPβ, and RNA Polymerase II Binding to the CXCL8 Promoter

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Abstract

CXCL8 is a neutrophil and mast cell chemoattractant that is involved in regulating inflammatory cell influx in asthma. Here, we investigated the transcriptional mechanism involved in CXCL8 induction by TNF-α in cultured human airway smooth muscle (HASM) cells and compared these in cells from nonasthmatic and asthmatic individuals. Transfection studies with mutated CXCL8 promoter constructs identified NF-κB, activating protein-1, and CAAT/enhancer binding protein (C/EBP)β as key transcription factors, and binding of these three transcription factors to the CXCL8 promoter after TNF-α stimulation was confirmed by chromatin immunoprecipitation analysis. Cells derived from asthmatic individuals produced significantly higher levels of CXCL8 than nonasthmatic cells both basally and following 24 h of stimulation with TNF-α ($p < 0.001$). Furthermore, chromatin immunoprecipitation studies detected increased binding of NF-κB p65 and RNA polymerase II to the CXCL8 promoter of asthmatic HASM cells both in the presence and absence of TNF-α stimulation. This was not due to either an increased activation or phosphorylation of NF-κB per se or to an increase in its translocation to the nucleus. Increased binding of C/EBPβ to the CXCL8 promoter of unstimulated cells was also detected in the asthmatic HASM cells. Collectively these studies show that HASM cells from asthmatic individuals have increased CXCL8 production due to the presence of a transcription complex on the CXCL8 promoter, which contains NF-κB, C/EBPβ, and RNA polymerase II. This is the first description of an abnormality in transcription factor binding altering chemokine expression in airway structural cells in asthma.

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Human airway smooth muscle (HASM) cells play an integral role in the dysfunctional repair mechanisms that cause the airway remodeling and poorly reversible airflow obstruction in asthma due to their ability to proliferate, undergo hypertrophy, and migrate (1). Additionally, HASM cells synthesize and release a diverse repertoire of biologically active inflammatory mediators, including cytokines, chemokines, and growth factors, which contribute both to the inflammatory process and to airway remodeling within the bronchial wall (2, 3).

We, along with other authors, have in recent years advanced the paradigm that HASM cells have important synthetic functions in asthma. In particular, these cells are a rich source of chemokines that are capable of regulating numerous inflammatory responses in the airways via effects on both leukocytes and structural cells. Chemokines including CCL11/eotaxin (4, 5), CCL5/RANTES (6, 7), CXCL8/IL-8 (8, 9), and CXCL10/IP-10 (10, 11) are synthesized by HASM cells in response to stimulation with inflammatory mediators, including TNF-α, IL-1β (8, 12), TGFβ (13), or bradykinin (9). These chemokines have also been detected in the airways of asthmatic patients (14). Although asthma was for many years considered to be an “eosinophilic disease”, more recent studies have identified increased neutrophil numbers in asthmatic airways. In particular, elevated neutrophils numbers have been associated with asthma exacerbations (15, 16), sudden onset fatal asthma (17), status asthmaticus (18), and severe persistent asthma (19-21).

The increase in neutrophilic inflammation in patients with severe asthma appears to correlate with an increase in production of CXCL8 within the lungs (16, 20). CXCL8 is an 8.5-kDa ELR-containing CXC chemokine that plays a major role in the initiation and maintenance of inflammatory responses in the lung via its potent chemoattractant effects on neutrophils (22). Additionally, CXCL8 found in bronchoalveolar lavage samples (23) and in the supernatants of cytokine-stimulated HASM cells (10, 24) has been shown to mediate migration of mast cells via interactions with CXCR1 receptors (25). The chemokine also functions as an proangiogenic factor, thereby contributing to the regulation of angiogenesis, a prominent feature of the histopathology of inflammatory diseases such as asthma, rheumatoid disease, and inflammatory bowel disease (26).

In asthma, eosinophils and neutrophils have been shown to co-localize within the airways, although evidence suggests that patients with increasing asthma severity exhibit elevated neutrophil numbers and lower eosinophil numbers than do individuals with milder forms of the disease (21, 27). Patients with severe persistent asthma often exhibit symptoms that are difficult to control due to their failure to respond adequately to inhaled corticosteroids (27, 28). Interestingly, these patients often exhibit elevated CXCL8 levels in the airways even in the presence of high-dose corticosteroids despite in vitro evidence suggesting that corticosteroids can successfully inhibit CXCL8 production in major structural cell types from nondiseased airways including epithelial (29) and smooth muscle cells (8).

The core CXCL8 promoter contains binding sites for NF-κB, activating protein (AP)-1, and CAAT/enhancer binding protein (C/EBP) (30). Studies of CXCL8 induction in normal human cells suggest that while NF-κB activation is essential for gene transcription in

multiple cell types, AP-1 and C/EBPβ binding are dispensable for transcriptional activation in some cells but contribute to transcription in others (30, 31).

In recent years, a number of studies have compared the functional responses of normal and asthmatic HASM cells and determined that cells from asthmatic patients exhibit an inherent abnormality leading to increased contractile (32) and proliferative (33) responses. HASM cells from asthmatic subjects also appear to be hypersecretory compared with nonasthmatic HASM cells exhibiting increased levels of CCL11 basally (34) and elevated CCL11 and CXCL10 concentrations following stimulation with inflammatory cytokines (10, 34).

Here, we characterize the transcriptional mechanism regulating basal and TNF-α-induced CXCL8 production in nonasthmatic HASM cells and compare these with cells from asthmatic individuals.

Materials and Methods

Abs and reagents

Monoclonal and polyclonal Abs used for Western blot analysis, immunofluorescence, or chromatin precipitation experiments were obtained from Santa Cruz Biotechnology or Cell Signaling Technologies. FITC-labeled secondary Abs and TPCA-1 were obtained from Sigma-Aldrich.

Cell cultures

Primary cultures of HASM cells from asthmatic and nonasthmatic individuals were isolated from bronchial biopsies and large airway tissue from subjects undergoing surgery at Glenfield Hospital, Leicester, and City Hospital Nottingham as previously described (8, 35, 36). All studies directly comparing chemokine production or transcription factor levels by Western blot or chromatin immunoprecipitation (ChIP) were performed in cells obtained from the Glenfield Hospital to minimize any variation in biopsies or isolation techniques utilized at the two hospital sites. Asthmatic subjects recruited at Glenfield Hospital were carefully characterized and presented with an appropriate history and objective evidence of variable airflow obstruction and/or airway hyperresponsiveness as described previously (35). For 6 wk before bronchoscopy the subjects were free from exacerbations requiring systemic corticosteroids and/or antibiotics and were reasonably well controlled on stable medication. Disease severity was defined using the Global Initiative for Asthma (GINA) guidelines. All patients gave their written, informed consent, and the studies were approved by the Leicestershire Ethics Committees and the Nottingham City Hospital Research Ethics Committee. Additional human bronchial smooth muscle cells were purchased from Clonetics (Lonza) and were used exclusively for analysis of mutant CXCL8 promoter activity at passages 5–7. In all other experiments using cells obtained from bronchial biopsies performed in the Glenfield Hospital or at Nottingham City Hospital, experiments were performed with cells at passage 6.

Cells were cultured in DMEM containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml), and L-glutamine (4 mM) in humidified 5% CO_2, 95% air at 37°C. HASM cells were cultured in 6-well cell culture plates for determination of

CXCL8 protein production by ELISA and for real-time analysis of mRNA expression. For chromatin precipitation experiments cells were grown to confluence in T75-cm^2 or T150-cm^2 cell culture flasks. HASM cells used for luciferase reporter assays were seeded at 1.25 × 10^4 cells/well and cultured to 50–60% confluence in 24-well cell culture plates. All cells were growth-arrested in serum-free DMEM for 24 h before experiments.

RNA isolation and RT-PCR

Time- and concentration-dependent effects of TNF-α on CXCL8 mRNA expression were analyzed by real-time PCR. HASM cells were stimulated with TNF-α (10 ng/ml) for 0, 2, 4, 8, 12, and 24 h. Total RNA was isolated by using the RNeasy Plus mini kit (Qiagen) following the manufacturer's protocol. One to 2 μg of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase. mRNA levels were subsequently analyzed by real-time PCR amplification using the Stratagene MX3000P real-time PCR system and Excite 2× Master Mix with SYBR Green I. Primers sequences for analysis of CXCL8 mRNA were as follows: sensem 5′-ATG ACT TCC AAG CTG GCC GTG GCT-3′, antisense, 5′-TCT CAG CCC TCT TCA AAA ACT TCT C-3′. Equality of DNA loading was determined by the amplification of β_2-microglobulin using the following primers: sense, 5′-AATCCAAATGCGGCATCT-3′ and antisense, 5′-GAGTATGCCTGCCGTGTG-3′ as previously described (4).

Real-time PCR reactions were initially denatured for 10 min at 95°C followed by PCR amplification for up to 40 cycles using the following cycling parameters: denaturation at 95°C for 30 s, primer annealing at 59°C for 1 min, primer extension at 72°C for 30 s. A SYBR Green dissociation step of 1 min at 95°C, 55°C for 30 s, and 95°C for 30 s was also included at the end of the PCR amplification. mRNA expression levels were calculated using MxPro software.

For studies of CXCL8 mRNA stability, normal and asthmatic HASM cells were stimulated for 2 h with TNF-α, washed in serum-free media, and then incubated with actinomycin D (ActD) at a final concentration of 5 μg/ml. Cells were harvested for RNA isolation at 0, 2, 4, 7, and 10 h after ActD exposure. CXCL8 and β_2-microglobulin mRNA levels were determined by real-time RT-PCR and the time course of mRNA degradation was calculated.

ELISA analysis

CXCL8 concentrations in the culture medium were determined by ELISA (R&D Systems) according to the manufacturer's instructions and data were expressed as picograms per milliliter of CXCL8 per 10^5 cells. NF-κB p65 levels were detected in cytoplasmic and nuclear extracts using a TransAm NF-κB p65 Chemi kit (Active Motif) according to the manufacturer's protocol.

Vectors and transfections

The vectors encoding either the wild-type CXCL8 promoter (−162/+44), luciferase reporter, or site mutations of one of the binding sites (AP-1, C/EBPβ, or NF-κB) in the CXCL8 promoter region were provided by A. R. Braiser (Department of Medicine, Sealy Center for Molecular Science, Galveston, TX) (22, 23). HASM cells were cotransfected using

Lipofectamine 2000 (Invitrogen) with one of these vectors and control vector (pRL-TK, Promega) for 4 h, then cultured in fresh serum for a further 16 h before the TNF-α treatment for 60 min. The cells were then collected and lysed. Firefly and *Renilla* luciferase activities were measured by luminometer using the dual-luciferase reporter assay system (Promega). The normalized luciferase activities were calculated by dividing firefly activities by *Renilla* activities.

Western blotting

Nuclear and cytoplasmic extracts from TNF-α-stimulated or untreated control cells were prepared by Nu-Clear extraction kit (Sigma-Aldrich) according to the manufacturer's instructions. The protein concentration of cell extracts was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories) and 20–30 μg of protein was loaded onto SDS-poly-acrylamide gel (100 V, 1 h). Following transfer to polyvinylidene difluoride membrane, blots were blocked for 1 h at room temperature in blocking reagent (5% fat-free dried milk powder in PBS (pH 7.4) with 0.3% Tween 20 (PBST)), incubated with Abs against NF-κB p65, NF-κB p65 phospho Ser^{276}, NF-κB p65 phospho Ser^{536}, GAPDH, or anti-human lamin A/C for either 1 h at room temperature or overnight at 4°C. Membranes were washed with PBST and incubated with species-appropriate secondary Abs coupled to HRP for 1 h at room temperature. ECL detection was performed by washing the membrane with PBST, incubating it with the SuperSignal CL-HRP substrate system (Pierce) for 1 min, and finally exposing it to Hyperfilm ECL (Amersham Life Science).

Immunofluorescence

HASM cells were seeded and cultured on coverslips or 8-well chamber slides. After the HASM cells were treated by TNF-α, the cells were fixed in ice-cold methanol for 10 min and washed three times in PBS. All primary and FITC-labeled secondary Ab incubations were performed at room temperature for 30 min in 0.1% BSA. Cells were mounted in Vectashield mounting medium (Vector Laboratories) and photographed on an Olympus BX40 microscope.

ChIP analysis

ChIP analysis was performed using the ChIP-IT Express kit (Active Motif) according to the manufacturer's instructions. Briefly, smooth muscle cells were grown to confluence in T150- or T75-cm^2 flasks, and following TNF-α stimulation the protein-DNA interactions were cross-linked by formaldehyde fixation. Following cellular and nuclear lysis, isolated chromatin was sheared by sonication and subsequently incubated overnight at 4°C with protein G magnetic beads and 5 μg of polyclonal Ab against NF-κB p65, c-jun, C/EBPβ, or RNA polymerase II (Pol II). Negative control samples were also included. In these samples chromatin from cells stimulated for 1 h with TNF-α were incubated either with magnetic beads in the absence of Ab (no Ab control) or were incubated with magnetic beads mixed with IgG. Chromatin was isolated by magnetic bead separation, washed according to the manufacturer's instructions, and analyzed by real-time PCR with SYBR Premix Ex *Taq* (Lonza) and CXCL8 promoter (−121/+61)-specific primers with the following sequences: −121 forward, 5′-GGG CCA TCA GTT GCA AAT C and +61 reverse, 5′-TTC CTT CCG

GTG GTT TCT TC. For C/EBPβ ChIP experiments, CXCL8 promoter (−191/−20)-specific primers were used: −190 forward, 5′-AAGAAAACTTTCGTCATACGTCCG and −20 reverse, 5′-TGGCTTTTTATATCATCACCCTAC. For analysis of RNA Pol II binding within the CXCL8 coding sequence, primers were designed to amplify the coding sequence regions in exon 1 and exon 2 of the gene sequence. Exon 1 primers were: +3769 forward, 5′-GGACAAGAGCCAGGAAGAAA and +3918 reverse, 5′-GGAAAACGCTGTAGGTCAGAA. Exon 2 primers were: +4686 forward, 5′-ATCACTTTTTCCCCCAACAG and +4838 reverse, 5′-AATTTCTGTGTTGGCGCAGT. Chromatin samples were initially denatured for 30 s at 95°C followed by PCR amplification for up to 40 cycles using the following cycling parameters: denaturation at 95°C for 5 s, primer annealing at 60°C for 30 s, primer extension at 72°C for 15 s. A SYBR Green dissociation step of 1 min at 95°C, 55°C for 30 s, and 95°C for 30 s was also included at the end of the PCR amplification. Results were calculated using MxPro software (Stratagene).

Statistical analysis

Data were expressed as the means ± SE of *n* observations. Statistical significance between the means was compared with a Mann-Whitney *U* test or in experiments where multiple comparisons were made the analysis was performed using the Kruskal-Wallis test followed by a Dunn's post-test. Linear regression analysis was performed using Graphpad Prism 4.00. For all statistical analysis a value of $p < 0.05$ was accepted as significant.

Results

TNF-α induces CXCL8 mRNA and protein production

We performed TNF-α concentration response and time course experiments to determine the optimal concentration of cytokine for use in our experiments and assessed CXCL8 production at both the mRNA and protein levels in HASM cells from nonasthmatic patients. In concentration-response experiments in which cells were stimulated for 8 h with increasing concentrations of TNF-α, CXCL8 concentrations ranged from 751 ± 164 pg/ml/10^5 cells in unstimulated HASM cells to a maximum of 4165 ± 587 pg/ml/10^5 cells in response to 100 ng/ml TNF-α (Fig. 1*A*). Analysis of the time course of CXCL8 production following stimulation with 10 ng/ml TNF-α showed CXCL8 concentrations ranging from 498 ± 149 pg/ml/10^5 cells at 2 h poststimulation to 13,855 ± 3,979 pg/ml/10^5 cells after 24 h (Fig. 1*B*). A time-dependent increase in CXCL8 release was also detected in unstimulated HASM cells with cells secreting 2711 ± 104 pg/ml/10^5 cells after 24 h. Real time RT-PCR analysis also showed concentration- and time-dependent increases in CXCL8 mRNA expression following TNF-α stimulation (Fig. 1, *C* and *D*, respectively). Peak CXCL8 mRNA expression was detected at 4 h poststimulation with TNF-α (10 ng/ml), although mRNA levels were significantly elevated compared with control up to 12 h poststimulation. Pretreatment of cells with the transcription inhibitor ActD (5 μg/ml) before TNF-α stimulation completely abrogated TNF-α-induced CXCL8 mRNA expression (Fig. 1*E*) and subsequent CXCL8 secretion (Fig. 1*F*) from HASM cells, confirming that induction of CXCL8 by TNF-α is transcriptionally regulated.

Mutations in the NF-κB, AP-1, and C/EBPβ binding sites in the CXCL8 promoter reduce TNF-α-induced luciferase activity

To identify the transcription elements involved in CXCL8 induction, vectors containing either the wild-type CXCL8 promoter or mutated binding sites for AP-1, C/EBPβ, or NF-κB within the CXCL8 promoter were cotransfected into HASM cells with the control vector pRL-TK. Cells transfected with the wild-type (wt)CXCL8 promoter and stimulated with TNF-α showed an ~3-fold increase in luciferase activity compared with untreated controls (Fig. 2). The fold increase in TNF-α-induced luciferase activity recorded in cells transfected with each of the transcription factor mutant plasmids was lower than that seen following transfection with wtCXCL8. The most notable difference in activity was detected following transfection of the NF-κB mutant plasmid. TNF-α stimulation of these cells resulted in only a small increase in luciferase activity and effectively shows that TNF-α-induced reporter activity expression is significantly inhibited by 84% when NF-κB is unable to bind to the CXCL8 promoter. In the absence of AP-1 or C/EBPβ binding, CXCL8 reporter activity was reduced, but not as extensively as that seen with the NF-κB mutant. It appears that all three transcription factors play a role in maximal TNF-α-induced CXCL8 transcription in airway smooth muscle cells but that only NF-κB is essential in the process.

Inhibition of IκB kinase 2 (IKK2) by 2-[(aminocarbonyl)amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide (TPCA-1) reduces CXCL8 production in HASM cells

The importance of NF-κB activation in the synthesis and release of CXCL8 from unstimulated and TNF-α-stimulated HASM cells was confirmed by pretreatment of the cells with TPCA-1 and inhibitor of IKK2 (Fig. 2*B*). In the absence of IKK2-mediated phosphorylation of IκBα, IκBα is not degraded and NF-κB is unable to translocate to the CXCL8 promoter in the nucleus and regulate CXCL8 transcription. TPCA-1-mediated inhibition of IKK2 activity was found to significantly inhibit CXCL8 release in both TNF-α-stimulated and unstimulated HASM cells at both the 1 and 10 μM concentrations.

TNF-α increases NF-κB, AP-1, and C/EBPβ binding to the CXCL8 promoter

We next assessed the effect of TNF-α stimulation on transcription factor binding to the CXCL8 promoter using ChIP, a technique that allows us to detect binding of native transcription factors to the CXCL8 promoter with HASM cells. Following ChIP analysis, real-time PCR using CXCL8 promoter-specific primers determined that in unstimulated cells there was a low level of NF-κB p65, c-jun (part of the AP-1 complex), and C/EBPβ binding to the CXCL8 promoter (Fig. 3). For each transcription factor, we detected an increase in transcription factor binding to the CXCL8 promoter following TNF-α stimulation. A small increase in NF-κB p65 binding compared with the unstimulated control (0 h) was detected 30 min after stimulation, but peak binding to the CXCL8 promoter was seen 1 h poststimulation (Fig. 3*A*). A similar pattern of binding was seen for c-jun (Fig. 3*B*), although C/EBPβ binding appeared to peak 30 min after TNF-α stimulation (Fig. 3*C*). In each case transcription factor binding was returning, or had already returned, to the baseline level detected in unstimulated cells by 2.5 h poststimulation. The no Ab control (NAC) bar represents a negative control sample of TNF-α-stimulated chromatin (1 h incubation). In these samples the ChIP reaction was performed on chromatin incubated with magnetic beads

in the absence of Ab. In all cases the level of CXCL8 promoter DNA detected in the negative control samples was either undetectable or lower than that detected in the untreated control samples incubated with transcription factor Ab.

Nuclear translocation of NF-κB p65 following TNF-α stimulation

We further confirmed the involvement of NF-κB in chemokine production following TNF-α stimulation of airway smooth muscle cells by Western blotting. Cytoplasmic and nuclear extracts were isolated from TNF-α-stimulated cells, separated by SDS-PAGE gel, and the Western blot membranes were probed with anti-NF-κB p65 Ab (Fig 4*A*). Within the cytoplasmic extracts, NF-κB p65 was at its highest level in unstimulated cells and was reduced over time following TNF-α stimulation. In contrast, NF-κB was expressed at a low level in nuclear extracts from unstimulated cells, and the level increased following TNF-α stimulation, peaking at 60 min. These data were also confirmed by immunofluorescence staining, which demonstrated that TNF-α stimulation of HASM cells causes translocation of NF-κB p65 from the cytoplasm to the nucleus (Fig. 4*B*). In parallel experiments we also confirmed that IκBα, which is complexed to NF-κB p65 and inhibits its nuclear translocation, was degraded in the cytoplasm following stimulation with TNF-α (data not shown). AP-1 and C/EBPβ were located in the nuclei under resting conditions by immunofluorescence staining and we were unable to detect further translocation after stimulation (data not shown). This is consistent with other studies showing that unlike NF-κB, C/EBPβ and AP-1 are activated by phosphorylation within the nucleus and are not required to translocate to the nucleus before activation (37-39).

CXCL8 hypersecretion in asthmatic cells

We next compared CXCL8 production in airway smooth muscle cells isolated from the asthmatic (n = 7) and nonasthmatic (n = 4) patients detailed in Table I. CXCL8 expression was analyzed at the protein and mRNA levels. The tissue culture media from asthmatic HASM cells serum starved for 24 h and then maintained in culture for a further 8 h were found to contain significantly higher levels of CXCL8 (5340 ± 2031 pg/ml/10^5 cells) than were supernatants collected from nonasthmatic cells (752 ± 165 pg/ml/10^5 cells) (Fig. 5*A*). Real-time RT-PCR analysis of mRNA expression, where all data are represented as fold change compared with the unstimulated nonasthmatic cells, demonstrated that basal CXCL8 mRNA levels in unstimulated cells were significantly increased in HASM cells cultured from asthmatic lungs (Fig. 5*B*). In time course experiments, significantly higher CXCL8 protein production was detected in HASM cells from asthmatic individuals at the 8, 12, and 24 h time points after TNF-α stimulation (Fig. 5*C*). Peak CXCL8 levels were detected after 24 h of TNF-α stimulation at 42,998 ± 4,889 pg/ml/10^5 cells compared with 13,855 ± 3,979 pg/ml/10^5 cells in nonasthmatic cells.

We detected a rapid induction of CXCL8 mRNA expression following TNF-α stimulation in time course experiments, with the highest level of expression being detected in nonasthmatic cells at 4 h (Fig. 5*D*). Although the maximum level of CXCL8 mRNA induction following TNF-α stimulation was generally seen in cells from nonasthmatic patients, basal CXCL8 mRNA levels in unstimulated cells were significantly higher in the asthmatic cells (Fig. 5*B*). CXCL8 mRNA levels in both cell types were returning to baseline

levels by 24 h poststimulation. Concentration-response experiments also confirmed that asthmatic HASM cells secreted higher levels of CXCL8 than did cells from nonasthmatic individuals both in response to TNF-α stimulation at each concentration (0.1–100 ng/ml) and also basally (Fig. 5*E*). To determine whether the increased secretion of CXCL8 from asthmatic HASM cells could be attributed to different levels of mRNA stability in cells from asthmatic and nonasthmatic individuals, the HASM cells were stimulated for 2 h with TNF-α (10 ng/ml) and treated with ActD. Following inhibition of transcription by ActD, we detected a rapid decrease in mRNA levels that was similar in both nonasthmatic and asthmatic cells, resulting in only 10% of CXCL8 mRNA remaining 2 h after ActD treatment (Fig. 5*F*).

To assess whether the level of CXCL8 release by asthmatic HASM cells was correlated with the severity of asthma, we performed linear regression analysis to compare TNF-α-induced CXCL8 levels at 24 h with the bronchodilator (BD) response and inhaled corticosteroid (ICS) dosage recorded from each of the patients (Table I). We detected significant correlation with the CXCL8 levels found to be highest in patients showing the greatest BD response and receiving the highest concentrations of ICS (Fig. 6).

Total and phosphorylated NF-κB p65 levels in nonasthmatic and asthmatic HASM cells

Having identified NF-κB p65 binding to the promoter as a key factor in regulating CXCL8 transcription in nonasthmatic HASM cells, we compared p65 levels in nuclear and cytoplasmic extracts from nonasthmatic and asthmatic HASM cells by NF-κB p65 ELISA (Fig. 7*A*). The NF-κB p65 ELISA showed that the global amount of p65 and time course of nuclear translocation were similar in asthmatic and nonasthmatic HASM cells. Following TNF-α treatment, a low level of NF-κB p65 was detected in the nucleus of both cells types under basal conditions, and peak levels were seen at 15 min after TNF-α stimulation. These findings were also confirmed by Western blot analysis (Fig. 7*B*) and by immunofluorescence staining (Fig. 7*C*) for NF-κB p65 in which peak nuclear p65 levels were seen after 20 min of TNF-α stimulation.

We also compared the levels of phosphorylated NF-κB p65 in nuclear extracts of nonasthmatic and asthmatic cells by Western blot. We detected phosphorylation of NF-κB p65 at Ser^{276} in both nonasthmatic and asthmatic cells, with the maximal level detected at 30 min after TNF-α stimulation (Fig. 7*D*). However, we did not detect any increased basal NF-κB p65 Ser^{276} phosphorylation in unstimulated cells of either cell type, and NF-κB p65 Ser^{276} phosphorylation levels were not higher in the asthmatic cells. The pattern of phosphorylation of NF-κB p65 at Ser^{536} was also found to be similar in nuclear extracts of HASM cells from both nonasthmatic and asthmatic individuals (Fig. 7*D*). In both cell types, only a low level of phosphorylation was detected basally. However, TNF-α induced rapid phosphorylation at Ser^{536}, which was maximal at 5 min and maintained up to 15 min after cytokine stimulation before returning to baseline levels at 30–60 min in both diseased and nondiseased cells. We were unable to detect TNF-α-induced phosphorylation of NF-κB p65 at Ser^{468} in either nonasthmatic or asthmatic cells over a 60-min time course (data not shown).

Increased basal and TNF-α-induced transcription factor binding to CXCL8 promoter in asthmatic HASM cells

Studies of translocation and analysis of total nuclear and cytoplasmic p65 would not detect selective association of NF-κB p65 with a specific inflammatory gene promoter within asthmatic and nonasthmatic cells. We used ChIP analysis to examine the level of binding specifically at the CXCL8 promoter both basally and up to 1 h poststimulation with TNF-α. NF-κB p65 binding to the promoter had similar kinetics in both cell types where the level of binding compared with unstimulated cells was slightly increased at 30 min poststimulation and was maximal at 1 h (Fig 8*A*). However, the asthmatic cells showed between 5- and 10-fold higher p65 binding to the CXCL8 promoter than did the nonasthmatic cells. This significant increase in p65 binding to the CXCL8 promoter was detected both following TNF-α stimulation and in the absence of cytokine stimulation.

In order for the transcription factor NF-κB to mediate its effects on mRNA expression, it must be joined in a multiprotein-DNA transcription complex by other accessory factors, transcriptional activators and coactivators, along with RNA Pol II enzyme, which is ultimately responsible for transcribing mRNA. We therefore also examined the pattern of binding of RNA Pol II to the CXCL8 promoter in nonasthmatic and asthmatic HASM cells (Fig. 8, *B* and *C*). In the nonasthmatic HASM cells, stimulation with TNF-α resulted in an increased level of RNA Pol II binding to the CXCL8 promoter, peaking at 30 min poststimulation. However, as was also seen with the binding of NF-κB p65, RNA Pol II binding to the CXCL8 promoter was significantly higher in the asthmatic cells both basally and following TNF-α stimulation. As RNA Pol II binding within the coding region of a gene provides further evidence for active transcription, we also compared the levels of RNA Pol II binding to the coding sequence of the CXCL8 gene spanning exon 1 and exon 2. In unstimulated cells, RNA Pol II binding was significantly increased in asthmatic cells in the promoter and both regions of the coding sequence, suggesting that active transcription is present in the asthmatic cells basally.

Finally, we also assessed the level of C/EBPβ binding to the CXCL8 promoter in unstimulated cells from nonasthmatic and asthmatic individuals (Fig. 8*D*). The level of basal C/EBPβ binding to the CXCL8 promoter in nonasthmatic HASM cells was not significantly different from the IgG-negative control, suggesting that binding of this transcription factor is either absent or occurs at very low levels in unstimulated nondiseased cells. In contrast, we detected a significant ~3-fold increase in the level of C/EBPβ binding to the CXCL8 promoter in the cells isolated from the lungs of asthmatic individuals. This altered binding of C/EBPβ to the promoter occurred despite the detection of similar levels of nuclear C/EBPβ in cells from nonasthmatic and asthmatic individuals (data not shown).

Discussion

In the present study we examined the transcriptional mechanisms regulating CXCL8 production in HASM cells both basally and following exposure to TNF-α and extended our studies to compare CXCL8 production in HASM cells obtained from nonasthmatic and asthmatic individuals. Our studies are the first to perform a detailed analysis of the regulatory elements of the CXCL8 promoter in HASM cells, and through ChIP and

transfection studies we have determined that while NF-κB binding to the CXCL8 promoter is essential for CXCL8 transcription to occur, maximal promoter activation also requires binding of AP-1 and C/EBPβ. These findings are in agreement with a number of previous studies that have determined that while NF-κB binding is a central feature of CXCL8 gene expression, the precise involvement of AP-1 and C/EBPβ varies according to the stimulus and cell type being studied (31, 40-42).

Following our identification of the major transcriptional control of CXCL8 in HASM cells isolated from nonasthmatic individuals, we then directly compared CXCL8 production in cells cultured from nonasthmatic and asthmatic patient bronchial biopsies. We found that asthmatic HASM cells not only had higher CXCL8 levels following TNF-α stimulation, but that basal levels were also increased, and we went on to examine the mechanism underlying this increase. Two previous reports had suggested that asthmatic HASM cells were hypersecretory for the chemokines CCL11 (34) and CXCL10 (10). Collectively, these and ours are among a number of studies that show that smooth muscle cells have an abnormal phenotype in asthma. Our study is the first to show increased CXCL8 production in asthmatic HASM cells, and our mechanistic studies suggested that this was due to increased transcription, with asthmatic cells exhibiting basally higher CXCL8 mRNA levels. However, the stability of CXCL8 mRNA in the asthmatic and nonasthmatic cells was similar.

One previous report from Brightling et al. compared CXCL8 production in asthmatic and nonasthmatic cells, but only following stimulation with a cocktail of inflammatory cytokines containing IFN-γ, TNF-α, and IL-1β (10). The authors detected no difference in the level of CXCL8 produced by the two types of cells following stimulation with the cytokine cocktail. However, this study did not report the basal level of CXCL8 released by the normal and asthmatic cells or the effect of the individual cytokines on CXCL8 release. Keslacy et al. recently showed that IFN-γ is a potent inhibitor of a number of TNF-α-inducible inflammatory genes, including CXCL8, and that IFN-γ mediates these inhibitory effects by acting on NF-κB transactivation via the modulation of histone deacetylase function (43). It is possible that the increased TNF-α-induced CXCL8 production detected in the asthmatic cells within our study may have been masked in the study of Brightling et al. by the presence of IFN-γ in the cell culture medium and its inhibitory effects on NF-κB signaling.

As our studies suggested an abnormality in the transcriptional control of CXCL8 and that NF-κB was the most important transcription factor in this process, we next focused on whether NF-κB activation in the asthmatic cells was abnormal. Previous reports of constitutive expression of CXCL8 in vitro have been limited to cancer cells that have been shown to express high levels of a number of different chemokines even in the absence of growth factors or inflammatory stimuli. In many cases the dysregulated expression of chemokine genes in these cancer cells results from abnormal signaling in the NF-κB pathway within the cells, namely an increase in constitutive activation of IKKs and a subsequent increase in NF-κB activity (44).

We used a number of techniques to assess the NF-κB pathway, including ELISA analysis, Western blotting, and immunofluorescence staining, but we were unable to detect any

differences in the total level of NF-κB p65 in the nucleus of unstimulated asthmatic and nonasthmatic cells. These findings suggested that the increased CXCL8 production we detected in the asthmatic cells did not result from a global increase in nuclear NF-κB levels or increased NF-κB translocation. Having excluded a global increase or altered translocation of NF-κB as a cause of the increased CXCL8 production from the asthmatic HASM cells, we next compared the phosphorylation status of NF-κB in asthmatic and nonasthmatic cells. In recent years NF-κB activity has been shown to be regulated by posttranslational modification of the DNA-binding subunits by phosphorylation, acetylation, and ubiquitination (45, 46). Phosphorylation of NF-κB p65 at multiple sites, including at Ser^{276} by PKAc (47) and MSK-1 (48), at Ser^{486} by GSK3 (49) or IKKβ (50), and at Ser^{536} by IKKβ (51), has been shown to regulate its transcriptional activity potential. Western blot analysis of nuclear extracts determined that the elevated CXL8 release from the asthmatic cells was not associated with an increase in the total level of phosphorylation of NF-κB p65 at Ser^{276} or at Ser^{536} either basally or following TNF-α stimulation.

We next determined if there was a local alteration in binding of NF-κB to the CXCL8 promoter in the asthmatic and nonasthmatic cells. We found an increased level of NF-κB p65 protein binding to the CXCL8 promoter of asthmatic cells both after TNF-α stimulation and under basal conditions, consistent with our mRNA and ELISA studies. These findings suggest that although there appears to be no global increase in NF-κB activity within asthmatic HASM cells, there was a selective increase in NF-κB binding to the CXCL8 promoter.

To further examine the hypothesis that the CXCL8 promoter in asthmatic cells is transcriptionally activated compared with the nonasthmatic cells, we expanded our ChIP analysis to also examine the binding of the transcription factor C/EBPβ and the enzyme RNA Pol II and found increased binding of both to the promoter in asthmatic cells. C/EBPβ is a member of a family of basic region leucine zipper transcription factors and is known to form functional and physical associations with members of the NF-κB family, including NF-κB p65 (52). The NF-κB and C/EBP binding sites are found in close proximity in the CXCL8 promoter, and simultaneous binding of these transcription factors has been shown to have both synergistic and co-operative effects on CXCL8 transcription following induction by inflammatory stimuli (53, 54). It is still not clear whether these effects are mediated via alterations in DNA binding or transactivation potential of the transcription factors when both are associated with the CXCL8 promoter. The increased level of NF-κB p65, C/EBPβ, and RNA Pol II binding to the CXCL8 promoter in the unstimulated asthmatic cells suggests the presence of an active transcription complex in association with the promoter DNA. The detection of increased RNA Pol II binding within the coding sequence of the CXCL8 gene in unstimulated asthmatic cells provided further evidence of active transcription taking place even in the absence of cytokine stimulation. Although present basally and following TNF-α stimulation in the asthmatic cells, the components of this transcription complex are only detected in nonasthmatic cells following stimulation with TNF-α. This active transcription complex in the asthmatic cells appears to be responsible for an increase in mRNA synthesis and the elevated release of CXCL8.

In some cancer cells, constitutive chemokine production appears to result from constitutive activation of IκB kinase, resulting in prolonged activation of NF-κB. Once the level of chemokine secretion has been elevated, expression may be maintained by an autocrine feedback loop in which the secreted chemokine induces further activation of the NF-κB signaling pathway (44). The mechanism of the increased CXCL8 seen in our studies is distinct from this, as we found no evidence of abnormal activation of NF-κB per se but rather increased recruitment to the promoter.

In conclusion, our study clearly shows that CXCL8 levels are elevated in airway smooth muscle cells from asthmatic individuals both basally and following TNF-α stimulation and that this hypersecretion is related to increased binding of NF-κB, C/EBPβ, and RNA Pol II to the CXCL8 promoter.

Acknowledgments

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Abbreviations used in this paper

HASM cell	human airway smooth muscle cell
ActD	actinomycin D
AP-1	activating protein-1
BD	bronchodilator
C/EBP	CAAT/enhancer binding protein
ChIP	chromatin immunoprecipitation
ICS	inhaled corticosteroid
IKK2	IκB kinase 2
Pol II	polymerase II
TPCA-1	2-[(aminocarbonyl)amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide
wt	wild-type

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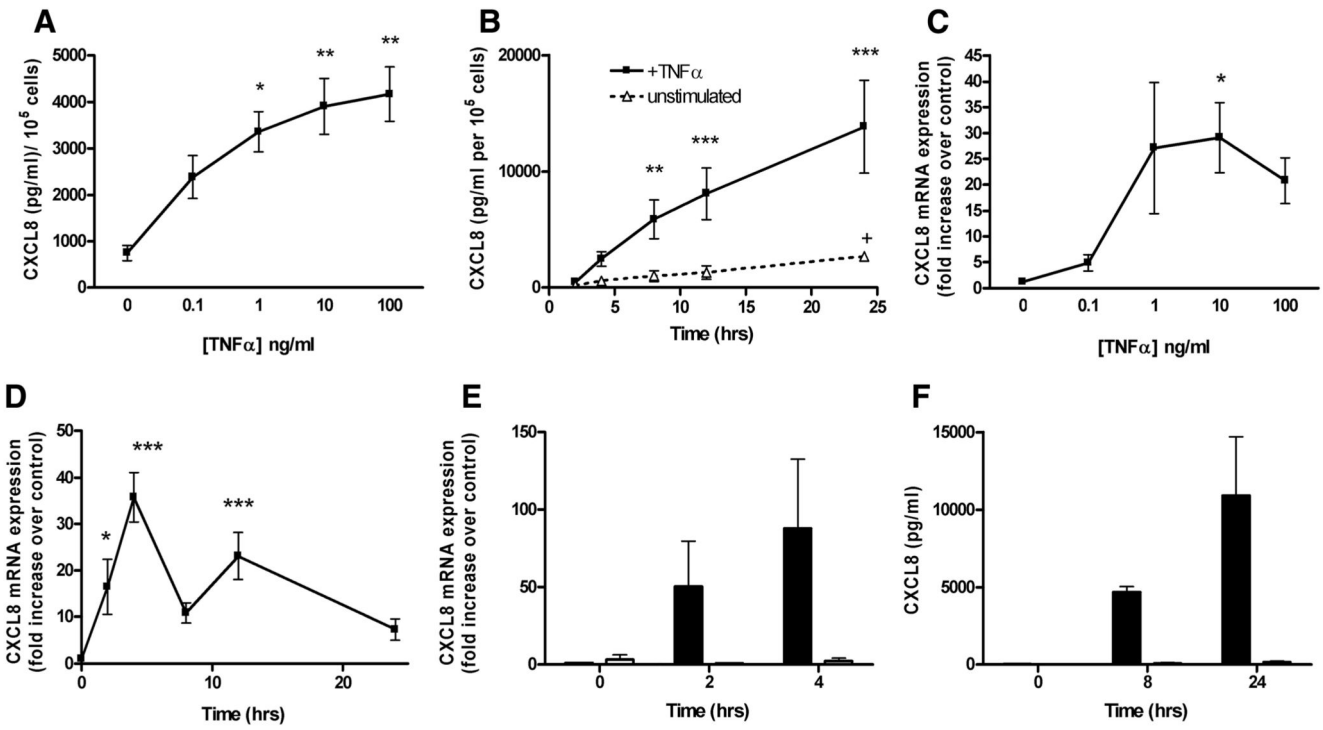

FIGURE 1. Concentration and time-dependent increases in CXCL8 production from airway smooth muscle cells in response to TNF-α stimulation.

Serum-deprived confluent cells were incubated with increasing concentrations of TNF-α up to a maximum of 100 ng/ml for 8 h and CXCL8 protein (*A*) and mRNA (*C*) levels were assessed by real-time RT-PCR and ELISA, respectively. For time course experiments, cells were serum deprived for 24 h and stimulated for up to 24 h with 10 ng/ml TNF-α before analysis of CXCL8 protein (*B*) and mRNA levels (*D*). Serum-starved cells were pretreated for 30 min with ActD (5 μg/ml; open bars) or vehicle control (closed bars) before TNF-α (10 ng/ml) for up to 4 h for mRNA (*E*) and 24 h for protein (*F*). Data are the means ± SE of nonasthmatic HASM cells collected from three different individuals. A p value of <0.05 was deemed significant and denoted as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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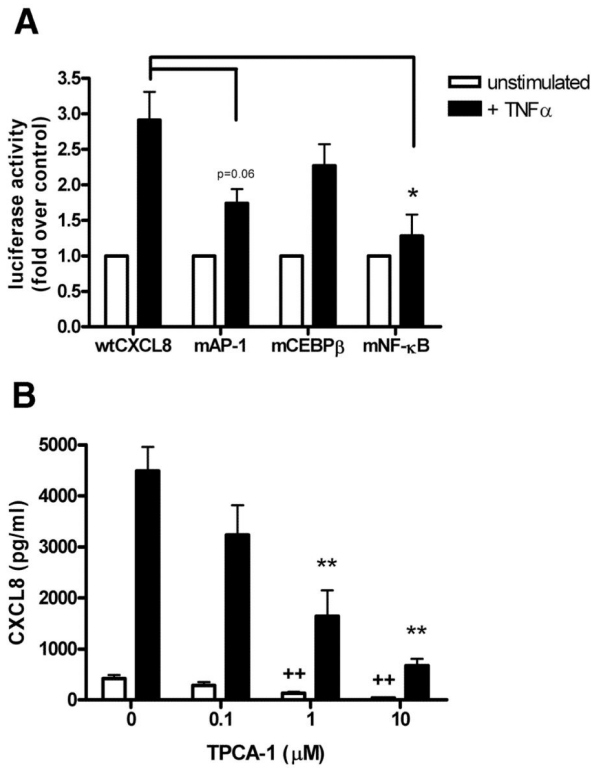

FIGURE 2. Identification of binding sites essential for transcription of CXCL8.

A, Wild-type (wtCXCL8) and mutant vectors (mAP-1, mC/EBPβ, and mNF-κB) were cotransfected with the control vector pRL-TK into HASM cells for 4 h, cultured for 16 h, and incubated for 1 h in the presence or absence of TNF-α (10 ng/ml). Normalized luciferase activity was calculated by dividing firefly by *Renilla* luciferase readings, and data are represented as fold increase in luciferase activity in cells treated with TNF-α (closed bars) compared with unstimulated cells (open bars). Each datum point represents the mean ± SE of nonasthmatic HASM cells collected from three individuals and statistical analysis comparing luciferease activity in wild-type and mutant transfected cells was deemed significant a p value of $p < 0.05$ (*). *B*, Serum-deprived confluent HASM cells were pretreated for 30 min with increasing concentrations of TPCA-1 (0–10 μM) before incubation of the cells for 24 h in the presence or absence of TNF-α (10 ng/ml). The effect of TPCA-1 on CXCL8 production was analyzed in cell supernatants by ELISA. Each datum point represents the mean ± SE of HASM cells collected from three individuals. Statistical analysis of the effects of TPCA-1 on CXCL8 release was deemed significant at a p value of <0.05 and is denoted as *, $p < 0.05$; **, $p < 0.01$ in TNF-α-stimulated cells and as ++, $p < 0.01$ in unstimulated cells.

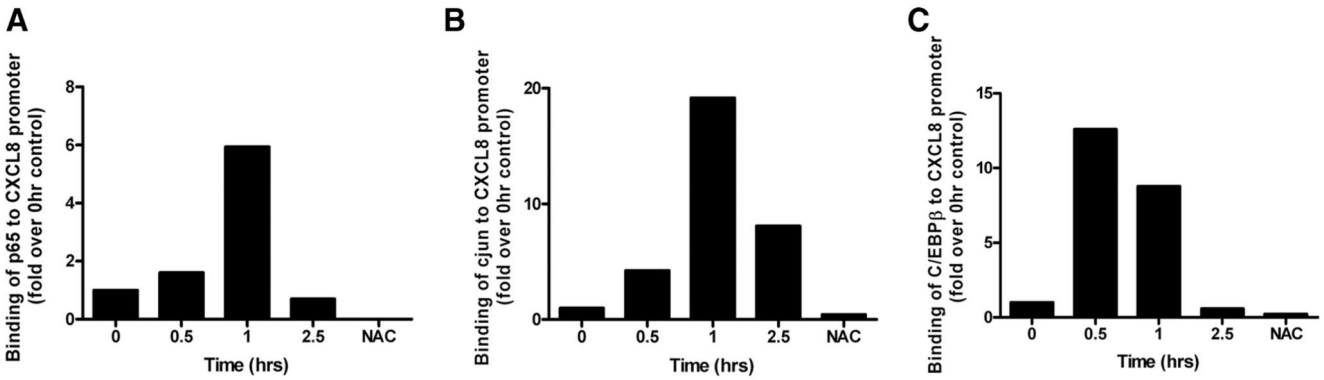

FIGURE 3. TNF-α-induced transcription factor binding to CXCL8 promoter.

Binding of NF-κB p65 (*A*), c-jun (*B*), and C/EBPβ (*C*) to the CXCL8 promoter were assessed by ChIP following stimulation with TNF-α (10 ng/ml). Transcription factor binding was analyzed at 0, 0.5, 1, and 2.5 h after cytokine stimulation by real-time PCR analysis using CXCL8 promoter-specific primers. Negative control samples incubated in the absence of transcription factor binding Ab are included (NAC) and represent the background level of CXCL8 promoter DNA in the samples. Data are expressed as fold change compared with unstimulated (0 h) samples and are representative data from HASM cells obtained from one of three nonasthmatic individuals.

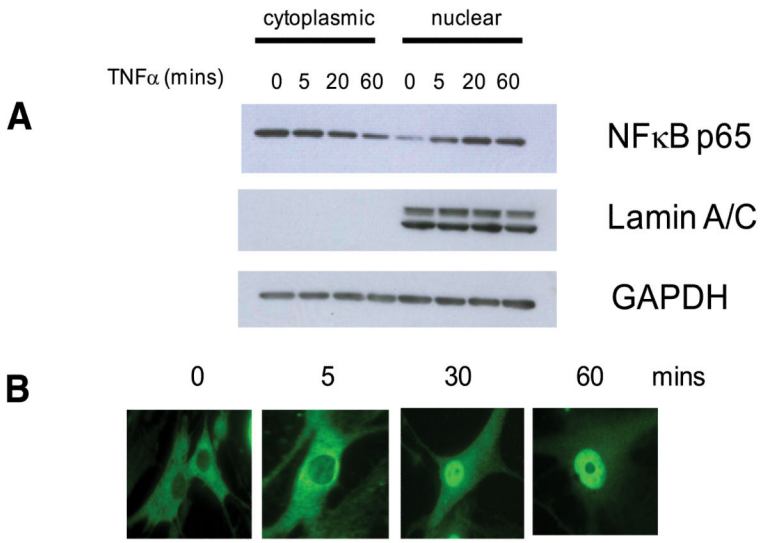

FIGURE 4. Nuclear translocation of NF-κB p65 following TNF-α stimulation.

The level of NF-κB p65 in cytoplasmic and nuclear extracts from HASM cells following TNF-α stimulation (10 ng/ml) for up to 60 min was assessed by Western blot analysis (*A*). Twenty micrograms of protein was loaded to each lane, and equality of nuclear and cytoplasmic sample loading was confirmed with control lamin A/C and GAPDH Abs, respectively. Immunofluorescence staining (*B*) was also performed in serum-deprived HASM cells to localize NF-κB p65 within the cells following stimulation with TNF-α (10 ng/ml). Data are samples derived from HASM cells of a single individual but are representative of findings in cells from three separate nonasthmatic individuals.

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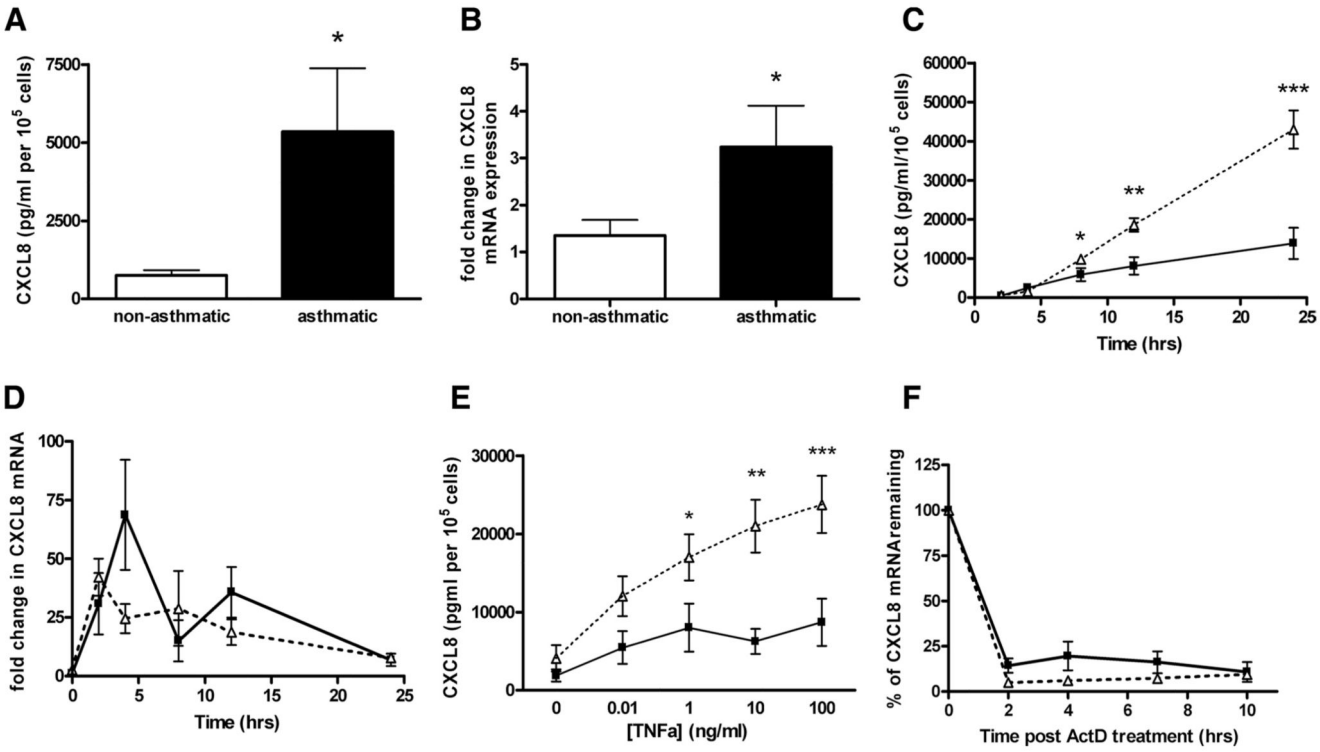

FIGURE 5. Comparison of CXCL8 production in asthmatic and nonasthmatic cells.

HASM cells isolated from nonasthmatic and asthmatic individuals were grown to confluence and serum starved for 24 h before analysis. Basal CXCL8 protein (*A*) production in unstimulated HASM cells was assessed in supernatants collected at 8 h. Basal mRNA levels were compared in unstimulated nonasthmatic and asthmatic HASM cells by real-time RT-PCR (*B*). The time course of CXCL8 protein (*C*) and mRNA (*D*) expression in nonasthmatic (closed symbol; solid line) and asthmatic (open symbol; dotted line) HASM cells following treatment with TNF-α (10 ng/ml) for 2, 4, 8, 12, and 24 h were assessed by ELISA and real-time RT-PCR, respectively. CXCL8 protein (*E*) levels were also compared following stimulation with increasing concentrations of TNF-α (0.1-100 ng/ml) for 8 h. mRNA stability (*F*) was compared in nonasthmatic and asthmatic HASM cells stimulated with TNF-α (10 ng/ml) for 2 h and treated with ActD (5 μg/ml). RNA samples were collected at various time points up to 10 h. CXCL8 mRNA levels were determined by real-time RT-PCR with the level detected 0 h after ActD treatment represented as 100% in both the nonasthmatic and asthmatic cells. Data points are means ± SE of cells from three to four nonasthmatic and three to four asthmatic individuals. Statistical analysis comparing expression in nonasthmatic and asthmatic data was deemed significant at a p value of <0.05 and denoted as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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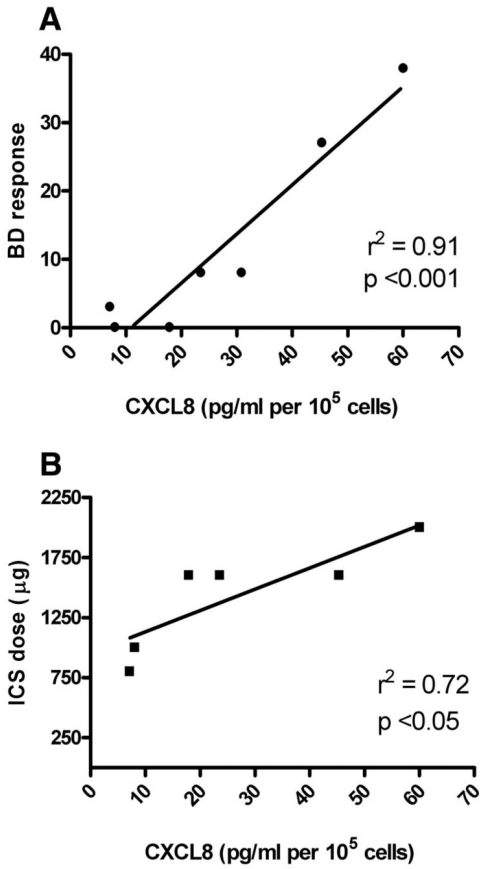

FIGURE 6. Correlation of CXCL8 production in asthmatic HASM cells with patient BD responses and ICS dosage.

HASM cells from asthmatic individuals (n = 7) were grown to confluence and deprived of serum for 24 h before stimulation with TNF-α (10 ng/ml). Supernatants were collected after 24 h and CXCL8 levels were assessed by ELISA analysis. Linear regression was performed to correlate the CXCL8 production from TNF-α-stimulated HASM cells in vitro to the BD response and ICS concentrations recorded in the asthmatic patients.

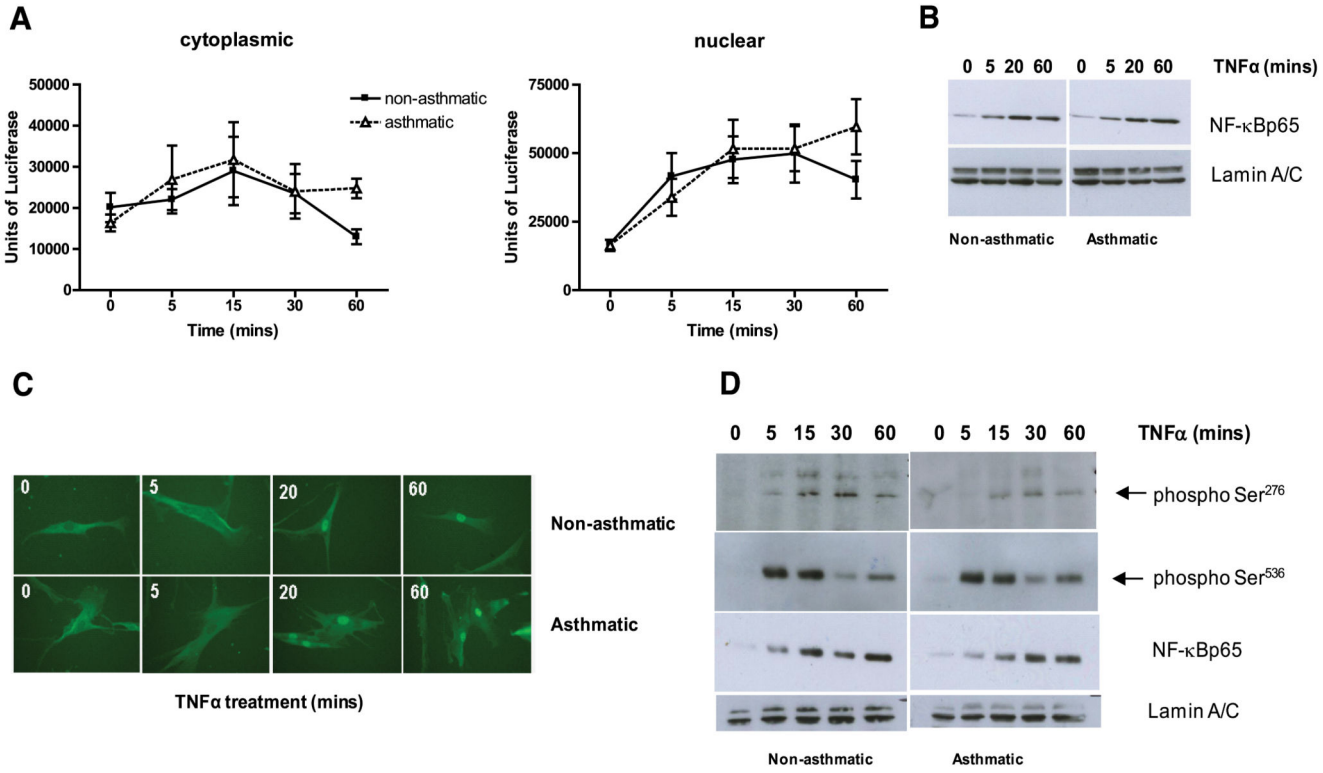

FIGURE 7. TNF-α-stimulated nuclear translocation and phosphorylation of NF-κB p65 in asthmatic and nonasthmatic airway smooth muscle cells.

Cytoplasmic and nuclear levels of NFκB p65 were assessed by ELISA in TNF-α-stimulated HASM cells from nonasthmatic (closed symbols; solid lines) and asthmatic (open symbols; dotted lines) individuals (*A*). Data points are means ± SE of cells from three nonasthmatic and three asthmatic individuals. Western blot analysis was performed with nuclear extracts (20 μg protein/well) from serum-deprived nonasthmatic and asthmatic HASM cells stimulated for up to 60 min with TNF-α (10 ng/ml) and were incubated with Abs against NF-κB p65 (*B*), NF-κB p65 phospho-Ser^{276}, and NF-κB p65 phospho-Ser^{536} (*D*). Equality of nuclear protein loading was assessed with control Abs against lamin A/C. The pattern of NF-κB p65 nuclear translocation was also compared in the two cells types by immunofluorescence staining (*C*). Western blot and immunofluorescence data are representative of findings in HASM cells from three asthmatic and nonasthmatic individuals.

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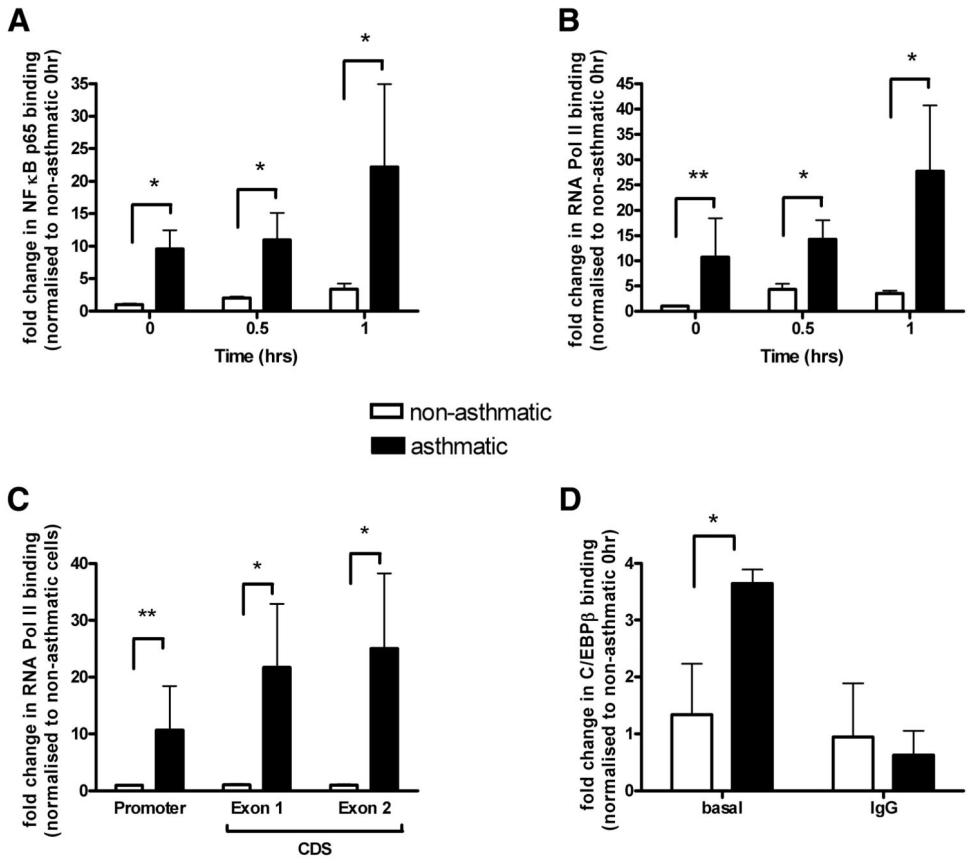

FIGURE 8. NF-κB p65 and RNA Pol II binding to the CXCL8 promoter in asthmatic and nonasthmatic airway smooth muscle cells.

The level of NF-κB p65 and RNA Pol II binding to the CXCL8 promoter were compared in nonasthmatic (open bars) and asthmatic (closed bars) HASM cells at 0, 0.5, or 1 h after stimulation with TNF-α (10 ng/ml). C/EBPβ binding was compared in basal (unstimulated) HASM cells from nonasthmatic and asthmatic individuals. Chromatin was incubated with IgG in place of transcription factor Ab as a negative control. Levels of binding of NF-κB p65 (*A*), RNA Pol II (*B* and *C*), and C/EBPβ (*D*) to the CXCL8 promoter DNA were analyzed by real-time PCR using promoter-specific primers. RNA Pol II binding was also assessed using primers spanning exon 1 or exon 2 of the CXCL8 gene (*C*). All data are normalized to the 0 h time point/basal expression in the nonasthmatic cells, and data points represent fold increase in binding compared with the 0 h/basal nonasthmatic cells. Data are means ± SE fold change in HASM cells from three nonasthmatic and three asthmatic individuals. Statistical analysis comparing the level of binding in nonasthmatic and asthmatic cells at each time point was deemed significant at a p value of <0.05 and denoted as *, $p < 0.05$; **, $p < 0.01$.

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Table I

Clinical characteristics of nonasthmatic and asthmatic airway smooth muscle cell donors[a]

	Control (n = 4)	Asthma (n = 7) (GINA I = 1, II = 0, III = 1, IV = 2, and V = 3)
Age (yr)	61 (10)	45 (5)
Sex (M:F)	1:3	4:3
Atopy (n)	0	4
Smoking status never/ex/current	2/1/1	2/5/0
Pack years	10(8)	4(1)
BDP equivalent (jg)	None	1475 (136)
BD response (%)	None	11.99 (5.15)
Oral prednisolone (mg/24 h)	None	2.8 (1.5)
ICS (μg)	None	1229(256)
LABA (n)	None	6
FEV_1 % predicted	90 (11)	80 (11)
FEV_1/FVC	75 (6)	68 (4)
Peripheral blood eosinophils × 10^9/L	0.2 (0.06)	0.5 (0.20)

[a]Data are represented as means (±SE). BDP indicates beclomethasone dipropionate; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; LABA, long-acting β_2-agonist.