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Asthmatic airway smooth muscle CXCL10 production: mitogen-activated protein kinase JNK involvement

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Respiratory Research Group, ¹Faculty of Pharmacy and ²Discipline of Pharmacology, The University of Sydney, Sydney; ³Woolcock Institute of Medical Research, Glebe, New South Wales, Australia; ⁴Institute for Lung Health, Department of Infection, Inflammation and Immunity, University of Leicester, Leicester, United Kingdom

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Alrashdan YA, Alkhouri H, Chen E, Lalor DJ, Poniris M, Henness S, Brightling CE, Burgess JK, Armour CL, Ammit AJ, Hughes JM. Asthmatic airway smooth muscle CXCL10 production: mitogen-activated protein kinase JNK involvement. *Am J Physiol Lung Cell Mol Physiol* 302: L1118–L1127, 2012. First published March 2, 2012; doi:10.1152/ajplung.00232.2011.—CXCL10 (IP10) is involved in mast cell migration to airway smooth muscle (ASM) bundles in asthma. We aimed to investigate the role of cytokine-induced MAPK activation in CXCL10 production by ASM cells from people with and without asthma. Confluent growth-arrested ASM cells were treated with inhibitors of the MAPKs ERK, p38, and JNK and transcription factor NF- κ B, or vehicle, and stimulated with IL-1 β , TNF- α , or IFN- γ , alone or combined (cytomix). CXCL10 mRNA and protein, JNK, NF- κ B p65 phosphorylation, and I κ -B α protein degradation were assessed using real-time PCR, ELISA, and immunoblotting, respectively. Cytomix, IL-1 β , and TNF- α induced CXCL10 mRNA expression more rapidly in asthmatic than nonasthmatic ASM cells. IL-1 β and/or TNF- α combined with IFN- γ synergistically increased asthmatic ASM cell CXCL10 release. Inhibitor effects were similar in asthmatic and nonasthmatic cells, but cytomix-induced release was least affected, with only JNK and NF- κ B inhibitors halving it. Notably, JNK phosphorylation was markedly less in asthmatic compared with nonasthmatic cells. However, in both, the JNK inhibitor SP600125 reduced JNK phosphorylation and CXCL10 mRNA levels but did not affect CXCL10 mRNA stability or I κ -B α degradation. Together, the JNK and NF- κ B inhibitors completely inhibited their CXCL10 release. We concluded that, in asthmatic compared with nonasthmatic ASM cells, JNK activation was reduced and CXCL10 gene expression was more rapid following cytomix stimulation. However, in both, JNK activation did not regulate early events leading to NF- κ B activation. Thus JNK and NF- κ B provide independent therapeutic targets for limiting CXCL10 production and mast cell migration to the ASM in asthma.

IFN-inducible protein 10; mRNA levels and stability; NF- κ B; p38; ERK

IN ASTHMA, ACTIVATED MAST CELL densities are greatest in the airway smooth muscle (ASM) and mucosal glands, and their degranulation is highest in fatal asthma (7). In addition, in mild to moderate asthma, mast cell numbers in the ASM are inversely correlated with lung function (6) and are higher in people with atopic than nonatopic asthma (1). Degranulated mast cells are in direct contact with the ASM cells, which are hypertrophic (3). As individual mast cell mediators affect

ASM, their localization to the ASM in higher numbers in asthma may contribute to the pathogenesis (6).

Previously we identified the CXCL10 (IP10)-CXCR3 axis as mediating mast cell microlocalization to the ASM in asthma (5). CXCL10 is elevated in the bronchoalveolar lavage (BAL) and airway mucosa of people with stable asthma compared with healthy controls (21), and BAL levels are also increased following allergen challenge (4, 22). Although CXCL10 is regarded as a marker of Th1 activity, CXCL10 transgenic mice develop airway hyperresponsiveness and a Th2 inflammatory response (20). Furthermore, CXCR3 is expressed on all mast cells in the ASM layer, and CXCL10 has been detected in the ASM bundles in biopsies from people with asthma but not from healthy controls (5).

We previously reported that CXCL10 release is greater by ASM cells from people with asthma than without asthma when induced by the cytokines IL-1 β , TNF- α , and IFN- γ combined (cytomix) (5), although the mechanisms underlying this difference are unknown. In microglial cells signaling via the Janus tyrosine kinases (Jak)-signal transducer and activator of transcription (STAT)-1 pathway following IFN- γ stimulation (26) and MAPK, JNK and NF- κ B following stimulation with IFN- γ and TNF- α (10) are involved in CXCL10 production. As well, in ASM cells from donors with chronic obstructive pulmonary disease and healthy controls, NF- κ B (9, 13), CREB-binding protein, and STAT-1 (9) are involved in CXCL10 production following IFN- γ and TNF- α stimulation.

Activation of NF- κ B and the MAPKs, following stimulation by IL-1 β , TNF- α , and IFN- γ , alone and in combination, is involved in human ASM production of other chemokines (14, 28, 32), but their involvement in CXCL10 production by ASM cells from people with asthma has not been established. We hypothesized that differences in signaling via MAPKs precede NF- κ B activation and enhance CXCL10 production by asthmatic ASM cells following cytokine stimulation. Thus the aims of this study were to investigate the role of cytokine-induced MAPK activation in CXCL10 production by ASM cells from people with and without asthma.

MATERIALS AND METHODS

Reagents. Recombinant human IFN- γ (BD Biosciences, San Jose, CA), IL-1 β , and TNF- α (R&D Systems, Minneapolis, MN) were reconstituted and stored as recommended by the manufacturers. They were used at 10 ng/ml except where indicated. Dimethylfumarate (DMF; Sigma-Aldrich, Sydney, Australia), SP600125 (A.G. Scientific, San Diego, CA), PD98059, SB203580, and SB202474 (Calbio-

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chem, San Diego, CA) were reconstituted in DMSO and stored at -20°C . The CXCL10 primer sets (Hs00171042_m1) and the 18S rRNA endogenous control probe were prepared by Assays on Demand (Applied Biosystems, Melbourne, Australia). Phosphorylated and nonphosphorylated controls and antibodies to SAPK/JNK (cat. no. 9258), phospho-SAPK/JNK (Thr183/Tyr185; 9251), phospho-NF- κ B p65 (Ser276; 3037) (all from Cell Signaling, Danvers, MA), I κ -B α (cat. no. sc-371) and α -tubulin (cat. no. sc-32293) (both from Santa Cruz Biotechnology, Santa Cruz, CA) were used with prestained standards (Gibco, Grand Island, NY).

ASM cell culture. ASM was obtained from bronchial biopsies from donors with mild to moderate asthma (classified according to the GINA guidelines) (12) and healthy controls or resected lung tissue from donors undergoing surgery for lung cancer or lung transplantation. Donors with asthma ($n = 29$, mean age 32.9, range 15–66 yr) had a positive bronchial challenge to mannitol (2) or methacholine (18) and symptoms in the last 12 mo, whereas other donors ($n = 31$, 54.4, 22–76 yr) had no doctor diagnosis of asthma. Samples were obtained with the donor's informed consent and approval from Sydney South West Area Health Service, Australian Red Cross and The University of Sydney Human Ethics Committee.

ASM cells were established in culture as described previously (17) in DMEM with 10% FBS, 100 U/ml penicillin G, 100 $\mu\text{g/ml}$ streptomycin sulphate, 25 $\mu\text{g/ml}$ amphotericin B, 4 mM L-glutamine, 20 mM HEPES (pH 7.4), in humidified 5% CO_2 -air at 37°C . The cells established and maintained in this way continued to exhibit a hill and valley pattern of growth typical of ASM cells and express α -smooth muscle actin and calponin. Harvested cells (passages 5–8) were put into well plates at 1×10^4 viable cells/ cm^2 in medium and cultured for 7 days before experiments. Cell viability following harvest was assessed using light microscopy and Trypan blue dye exclusion. Following any treatments, before the collection of culture supernatants or cell lysates, the viability of the cells in the wells was routinely checked using phase-contrast microscopy.

Cytokine-induced CXCL10 mRNA production. Well cultures were washed twice with PBS, growth arrested for 48 h in DMEM supplemented as above, except with 0.1% vol/vol BSA, not FBS. Then the ASM cells were stimulated with IL-1 β , TNF- α , or IFN- γ or all three combined (cytomix), each at 10 ng/ml. CXCL10 mRNA levels were determined following 0-, 1.5-, 3.0-, 6.0-, and 24.0-h stimulation. Total RNA was extracted using a NucleoSpin RNA 11 kit and protocol (Macherey-Nagel, Duren Germany). Reverse transcription of 1 μg of extracted RNA was performed using the RevertAid First Strand cDNA Synthesis Kit and protocol for the use of random hexamer primers (Fermentas Life Sciences, Hanover, MD). The resulting cDNA (2 μl) was amplified using the CXCL10 primer set (Assays on Demand). Samples were multiplexed with a eukaryotic 18S rRNA endogenous control probe, and the following cycle parameters used: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s, 60°C for 1 min 40 cycles. Results for CXCL10 were normalized against those for 18S.

Cytokine-induced CXCL10 release. CXCL10 release by growth-arrested ASM cells stimulated with IL-1 β and/or TNF- α , IFN- γ alone, with IL-1 β or TNF- α , or cytomix (at 0.1, 1.0 or 10.0 ng/ml) for 1.5, 3.0, 6.0, and/or 24.0 h was also determined. Well-culture supernatants were collected and stored at -20°C until CXCL10 levels were measured using Duo-set ELISA kits and protocol (R&D Systems).

NF- κ B inhibition and CXCL10 production. The role of NF- κ B in CXCL10 release was confirmed using DMF, a known inhibitor of NF- κ B activation (31), which we have previously shown inhibits p65 activity in ASM cells (23, 25). Growth-arrested ASM cells were treated with DMF at final concentrations of 10 and 100 μM (23, 31) or vehicle (0.01 and 0.1% vol/vol DMSO) for 1 h before and during cytokine stimulation for 24 h.

The effect of 100 μM DMF on CXCL10 mRNA production was also determined. ASM cells were treated as above and then stimulated with cytomix for 3 h. Total RNA was extracted using the guanidine

thiocyanate/phenol chloroform method (8), and mRNA expression was quantified by real-time PCR as described above.

The effects of DMF on I κ -B α degradation and p65 phosphorylation in the first 30 min following cytomix stimulation were also confirmed. Total cell lysates were prepared (19) and subjected to SDS-PAGE gel electrophoresis and Western blotting. The I κ -B α and phosphorylated p65 bands were detected using Phospho-NF- κ B p65 (Ser276) and I κ -B α antibodies diluted 1/1,000 and 1/500, respectively. Bands were detected by enhanced chemiluminescence (Perkin Elmer, Amsterdam, The Netherlands) and following exposure to X-ray film (Fujifilm, Brookvale, Australia) were quantified using densitometry and ImageJ version 1.43u software (Bethesda, MD), and the levels were normalized against α -tubulin.

Role of MAPKs. The roles of the MAPKs in CXCL10 release were determined using the inhibitors PD98059 (ERK or p42/p44), SB203580 (p38), and SP600125 (JNK). Growth-arrested ASM cells were treated with PD98059 (30 μM), SB203580 (10 μM), SP600125 (10 μM), or the negative control SB202474 (10 μM), as previously reported (19, 29). The cells were treated with the inhibitors or their vehicle (0.1% vol/vol DMSO) 45 min before and during stimulation with the cytokines for 24 h.

JNK activation and inhibition. The time course of JNK p46 and p54 phosphorylation in asthmatic and nonasthmatic ASM cells and the effects of SP600125 on that were examined. Growth-arrested ASM cells were treated with SP600125 (10 μM) or vehicle (0.1% vol/vol DMSO) 45 min before and during cytomix stimulation for 0, 5, 10, 30, and 60 min. Total cell lysates were prepared (19, 29) and, together with prestained standards and JNK controls, subjected to SDS-PAGE gel electrophoresis and Western blotting. Total JNK and phosphorylated JNK bands were visualized using SAPK/JNK and phospho-SAPK/JNK (Thr183/Tyr185) antibodies (diluted 1/1,000), respectively. Phosphorylated JNK levels were normalized against total JNK.

JNK inhibition and CXCL10 mRNA expression and stability. The effects of SP600125 on CXCL10 mRNA production and its stability were determined. Growth-arrested cells were treated with SP600125 (22.7 μM) or vehicle (0.1% vol/vol DMSO) 45 min before and during cytomix stimulation for 3 h. Total RNA was then extracted using the guanidine thiocyanate/phenol chloroform method (8). For mRNA stability studies, after 3 h cytomix stimulation in the presence of SP60125 or its vehicle, the cells were washed, and fresh serum-starving medium containing actinomycin D (5 $\mu\text{g/ml}$) was added to stop new mRNA synthesis (0 h). Total RNA was harvested after 2-, 4-, 6-, or 24-h incubation and extracted as above. CXCL10 mRNA expression was quantified by real-time PCR.

JNK inhibition, I κ -B α degradation, and p65 phosphorylation. To determine the effects of SP600125 on early events preceding NF- κ B nuclear translocation, I κ -B α degradation and p65 phosphorylation were examined. Cell lysates were prepared from cytomix-stimulated cells, and I κ -B α and phosphorylated p65 bands were detected and quantified as described above.

Simultaneous inhibition of JNK and NF- κ B. The effect of simultaneous inhibition of JNK and NF- κ B activation on CXCL10 release was also assessed. As described above, growth-arrested ASM cells were treated with DMF at 3–100 μM in the presence and absence of 22.7 μM SP600125 or their vehicle (0.2% vol/vol DMSO), and cytomix-induced CXCL10 release was quantified at 24 h.

Data analysis. CXCL10 concentrations (ng/ml) from replicate treatments were averaged and for inhibitor studies expressed as percentage of cytokine control. For CXCL10 mRNA production values for replicates were averaged and expressed as fold change over 0 h (time course) or cytokine control (inhibitor studies). The mean $\pm$ SE was then calculated for the asthmatic and nonasthmatic cell lines. Statistical analyses were performed using Statview, and significance ($P < 0.05$) was determined by Student's t -test followed by one or two-way ANOVA and Fisher's post hoc test. For the mRNA stability studies, the amount of mRNA remaining was expressed as a percentage of the mRNA present as the actinomycin D was added (0 h). Prism

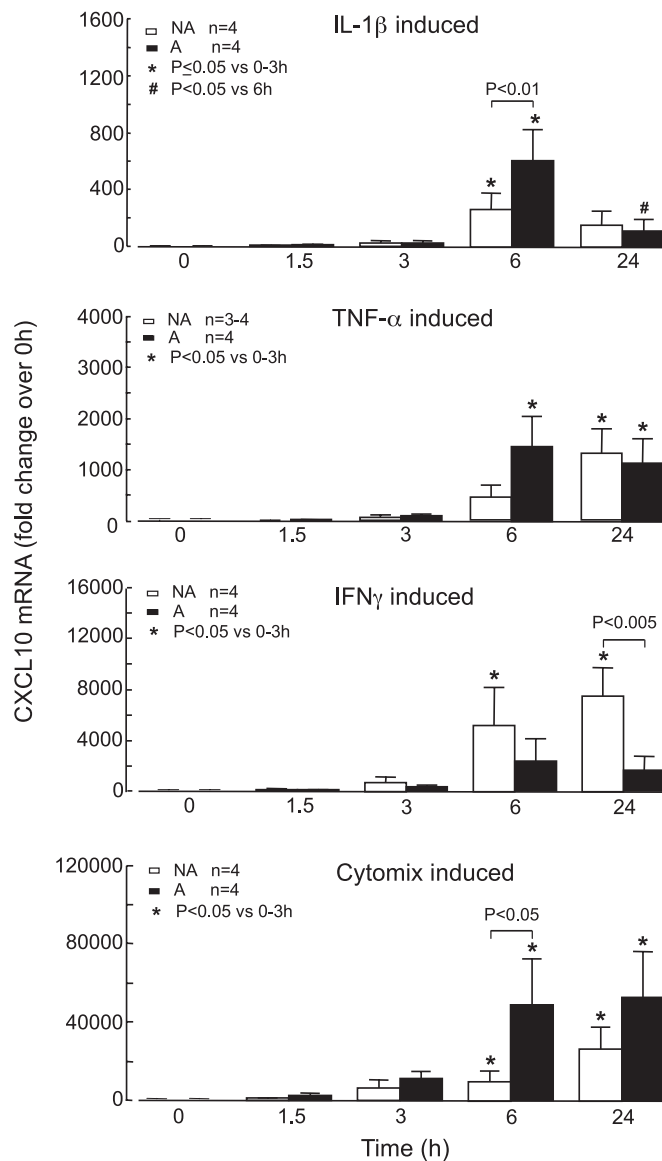


Fig. 1. Cytokine-induced CXCL10 mRNA production by asthmatic (A) and nonasthmatic (NA) airway smooth muscle (ASM) cells. Confluent, serum-deprived ASM cells were stimulated with 10 ng/ml of IL-1 β , TNF- α , IFN- γ , or cytomix for up to 24 h, and CXCL10 mRNA levels were quantified using real-time PCR and expressed as fold change over 0 h. Bars, mean $\pm$ SE.

4 for Windows (version 4.00) and Student's *t*-tests were used to calculate the decay constants (*K*) $\pm$ SE and to determine the significance of any differences in *K* (*P* < 0.05).

RESULTS

Cytokine-induced CXCL10 mRNA production. The pattern of cytokine-induced CXCL10 mRNA production by ASM cells from asthmatics and nonasthmatics differed. In both cell types, the individual cytokines induced high CXCL10 mRNA levels rapidly, with IL-1 β < TNF- α < IFN- γ (Fig. 1). Maximum CXCL10 mRNA levels induced were higher in asthmatic than nonasthmatic ASM cells following stimulation with IL-1 β and TNF- α at 6 h but lower with IFN- γ at 24 h. Cytomix induced a synergistic upregulation of CXCL10 mRNA levels, and,

similar to IL-1 β and TNF- α , these were higher (*P* < 0.05) in asthmatic ASM cells ($>48,000 \times 0$ h) than in nonasthmatic cells ($<11,000 \times 0$ h) at 6 h (Fig. 1).

Cytokine-induced CXCL10 release. CXCL10 release from the above asthmatic and nonasthmatic ASM cells also varied with the cytokine stimulus and was greatest in asthmatic cells stimulated with cytomix (Fig. 2A). Individually IL-1 β , TNF- α , and IFN- γ induced low CXCL10 release, which was only detectable at 24 h. In contrast cytomix induced significant release by 6 h, which by 24 h was synergistically increased over the release induced by the individual cytokines (Fig. 2A). Cytomix-induced release was concentration dependent, and synergism was only observed when IL-1 β and/or TNF- α were combined with IFN- γ in both asthmatic (Fig. 2B) and nonasthmatic cells (data not shown).

NF- κ B inhibition and CXCL10 production. DMF, which inhibits ASM cell NF- κ B p65 activity, significantly reduced CXCL10 release to a similar extent in asthmatic and nonasthmatic ASM cells, but the magnitude of the effects varied with the cytokine stimulus used (Fig. 3A). Release induced by IL-1 β or TNF- α was more sensitive to DMF than IFN- γ -

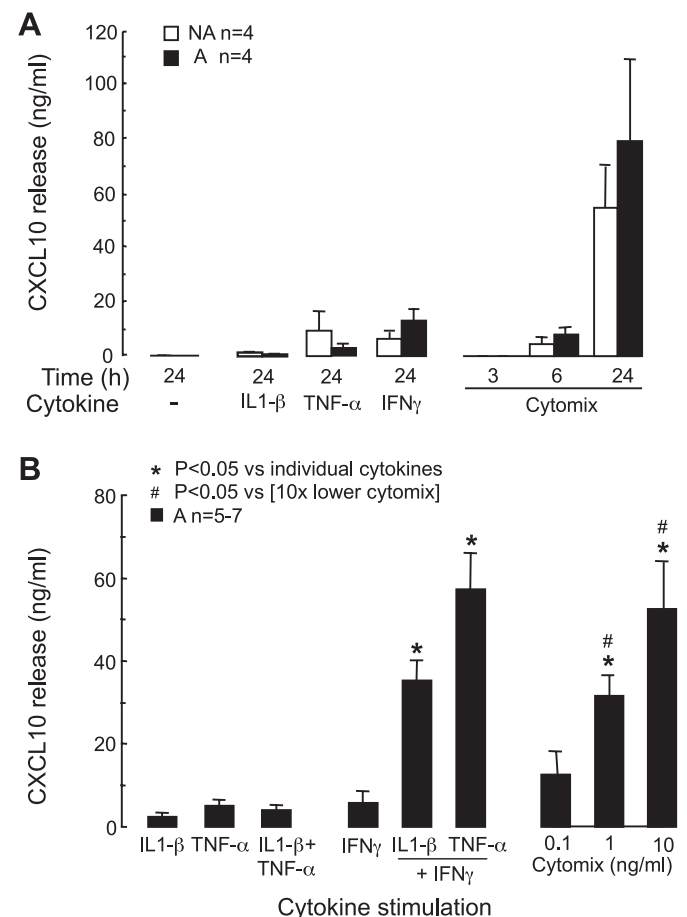


Fig. 2. Cytokine-induced CXCL10 release by asthmatic (solid bars) and nonasthmatic (open bars). Confluent, serum-deprived ASM cells were stimulated with 10 ng/ml of IL-1 β , TNF- α , IFN- γ , or cytomix for up to 24 h to investigate when CXCL10 release was first detectable (A), or for 24 h only (B) when the effects of IFN- γ alone and together with IL-1 β and/or TNF- α , including cytomix at 0.1, 1.0 and 10.0 ng/ml, on CXCL10 release were investigated. CXCL10 protein levels in the culture medium (supernatants) collected at the times indicated were quantified using ELISA. Bars, mean $\pm$ SE.

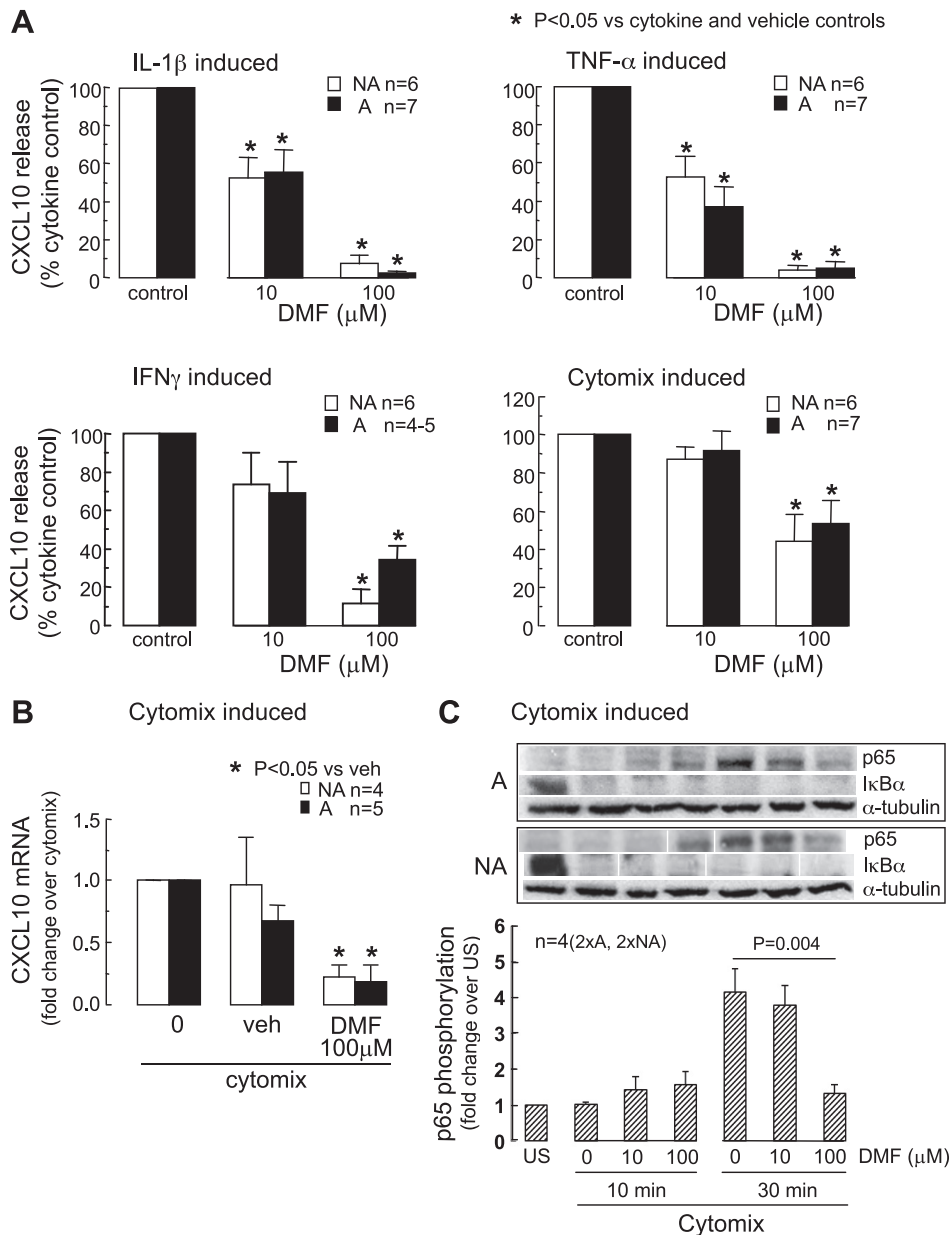


Fig. 3. The effects of dimethylfumarate (DMF) on CXCL10 production by asthmatic and non-asthmatic ASM cells. Confluent, serum-deprived ASM cells were treated with DMF (10 and 100 μ M) or their vehicles (0.01 and 0.1% vol/vol DMSO, respectively) for 1 h before and during stimulation with IL-1 β , TNF- α , IFN- γ , or cytomix. CXCL10 release after 24-h stimulation was quantified using ELISA and expressed as a percentage of the cytokine-inducing release (A). CXCL10 mRNA production after 3-h stimulation was quantified using real-time PCR and expressed as fold change over cytomix-induced expression (B). Phosphorylation of NF- κ B p65 in cytomix-stimulated and unstimulated (US) ASM cell lysates prepared 10 and 30 min after cytomix addition was detected using immunoblotting, quantified using densitometry, and expressed as fold change over US (C). White lines indicate where immunoblot images have been spliced. Bars, mean $\pm$ SE.

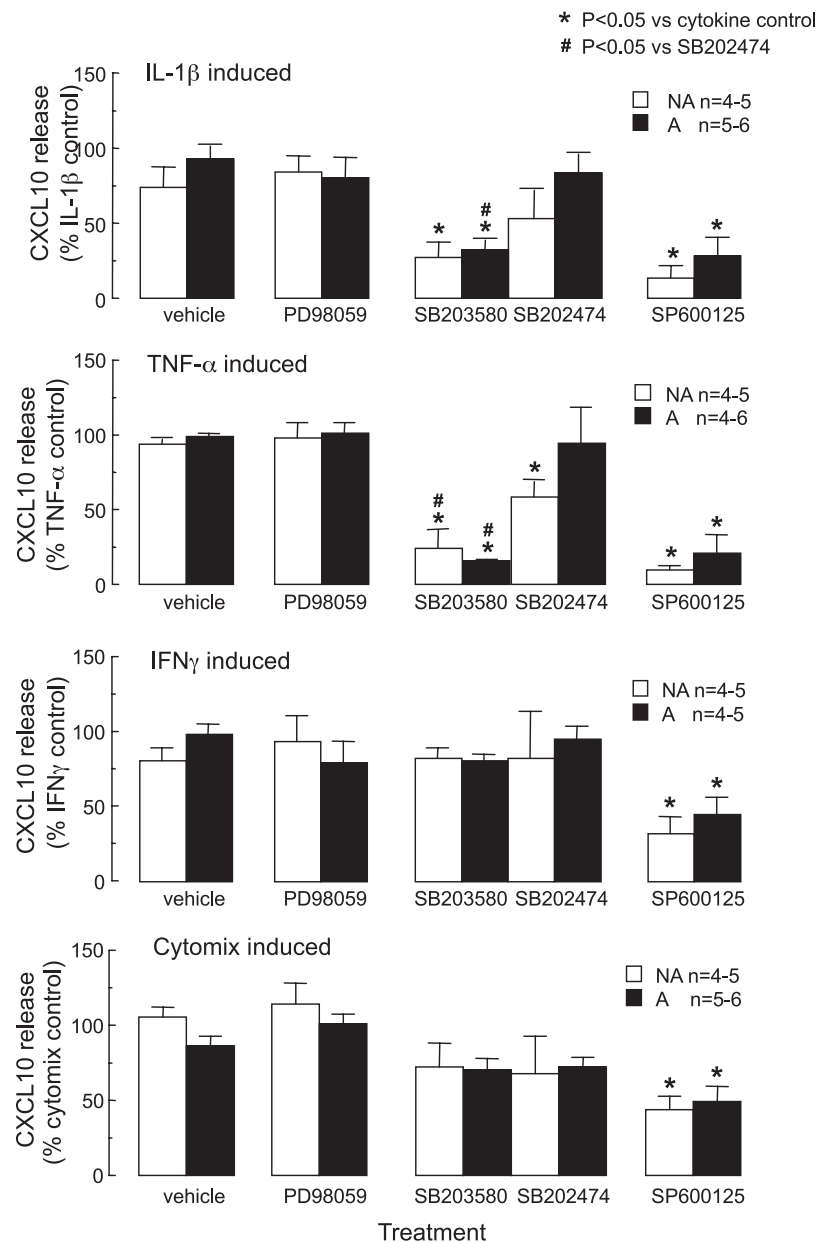
or cytomix-induced release because both 10 and 100 μ M DMF significantly reduced it. Only IL-1 β - and TNF- α -induced CXCL10 release were affected by the vehicle for DMF, and then only at the higher concentration of 0.1% vol/vol DMSO (asthmatic: $68.9 \pm 12.0\%$ and $82.1 \pm 6.3\%$ cytokine control; nonasthmatic: $66.7 \pm 12.9\%$ and $66.0 \pm 12.0\%$ cytokine control). Predictably the higher DMF concentration markedly reduced cytomix-induced CXCL10 mRNA levels (Fig. 3B) and p65 phosphorylation but not I κ -B α degradation (Fig. 3C). DMF used at these concentrations did not affect ASM cell metabolic activity or viability (data not shown).

Role of MAPKs. There were no differences between asthmatic and nonasthmatic ASM cells in the proportional effects of the MAPK inhibitors on CXCL10 release, but the effects of the inhibitors varied (Fig. 4). The JNK inhibitor SP600125 always reduced cytokine-induced release, whereas the ERK

inhibitor PD98059 always had no effect. However, the p38 inhibitor effects were dependent on the cytokine stimulus because SB203580 only reduced IL-1 β - or TNF- α -induced release significantly compared with its negative control SB202474 (Fig. 4). All three inhibitors markedly reduced cytomix-induced phosphorylation of their target MAPK, and there was no effect of SP600125 on phosphorylation of either ERK or p38 (data not shown).

JNK activation and inhibition. There was a significant difference between asthmatic and nonasthmatic cells in JNK phosphorylation (Fig. 5A). Both JNK isoforms P-54 and P-46 were rapidly phosphorylated, reaching a peak over 10–30 min following cytomix stimulation (Fig. 5, A–C). However, P46 and P54 phosphorylation were lower in asthmatic compared with nonasthmatic cells (Fig. 5A). The JNK inhibitor SP600125 reduced the early P-54 phosphorylation more than P-46 phosphorylation in the asthmatic cell lysates (Fig. 5B). In

Fig. 4. The effects of the MAPK inhibitors on CXCL10 release by asthmatic and nonasthmatic ASM cells. Confluent, serum-deprived ASM cells were treated with the ERK, p38, and JNK inhibitors PD98059 (30 μ M), SB203580 (10 μ M) (its negative congener SB202474 10 μ M), and SP600125 (10 μ M), respectively, or vehicle, for 45 min before and during stimulation with IL-1 β , TNF- α , IFN- γ , or cytomix for 24 h, and CXCL10 release was quantified using ELISA. Bars, mean $\pm$ SE; vehicle, 0.1% vol/vol DMSO.



nonasthmatic cells SP600125 reduced P-54 and P-46 phosphorylation in a similar way (Fig. 5C).

JNK inhibition and CXCL10 mRNA. Although there were no differences in the effects of JNK inhibition on CXCL10 mRNA, there may be a trend for CXCL10 mRNA to be less stable in nonasthmatic than asthmatic ASM cells (Fig. 6). Predictably the JNK inhibitor SP600125 significantly reduced the CXCL10 mRNA levels reached after 3 h cytomix stimulation (Fig. 6A). Interestingly the CXCL10 mRNA produced was very stable, with $t_{1/2} = 31$ h (decay constant $k = -0.02 \pm 0.01$) for asthmatic and $t_{1/2} = 19$ h ($k = -0.04 \pm 0.03$) for nonasthmatic cells (Fig. 6B). However, in both, SP600125 did not affect CXCL10 mRNA stability.

JNK inhibition and NF- κ B activation. Surprisingly, JNK inhibition also did not affect I κ -B α degradation or NF- κ B p65 phosphorylation, which precedes its entry into the nucleus and DNA binding. Neither the rapid decrease in

I κ -B α levels in the first 10 min following cytomix stimulation, nor its later reappearance at 60 min, was affected by SP600125 in the asthmatic or nonasthmatic ASM cells (Fig. 7A). Similarly, the increased phosphorylation of p65 following 30 min of cytomix stimulation was also not affected by JNK inhibition (Fig. 7B).

Simultaneous inhibition of JNK and NF- κ B. As well, similar effects on CXCL10 release were observed in asthmatic and nonasthmatic ASM cells when activation of both JNK and NF- κ B were inhibited simultaneously (Fig. 8). The addition of the JNK inhibitor SP600125 to DMF caused further inhibition, with release completely inhibited at the highest DMF concentration in both asthmatic and nonasthmatic cells (Fig. 8, A and B, respectively). ASM cell metabolic activity and viability were not affected by DMF and SP600125 used at these concentrations alone or in combination (data not shown).

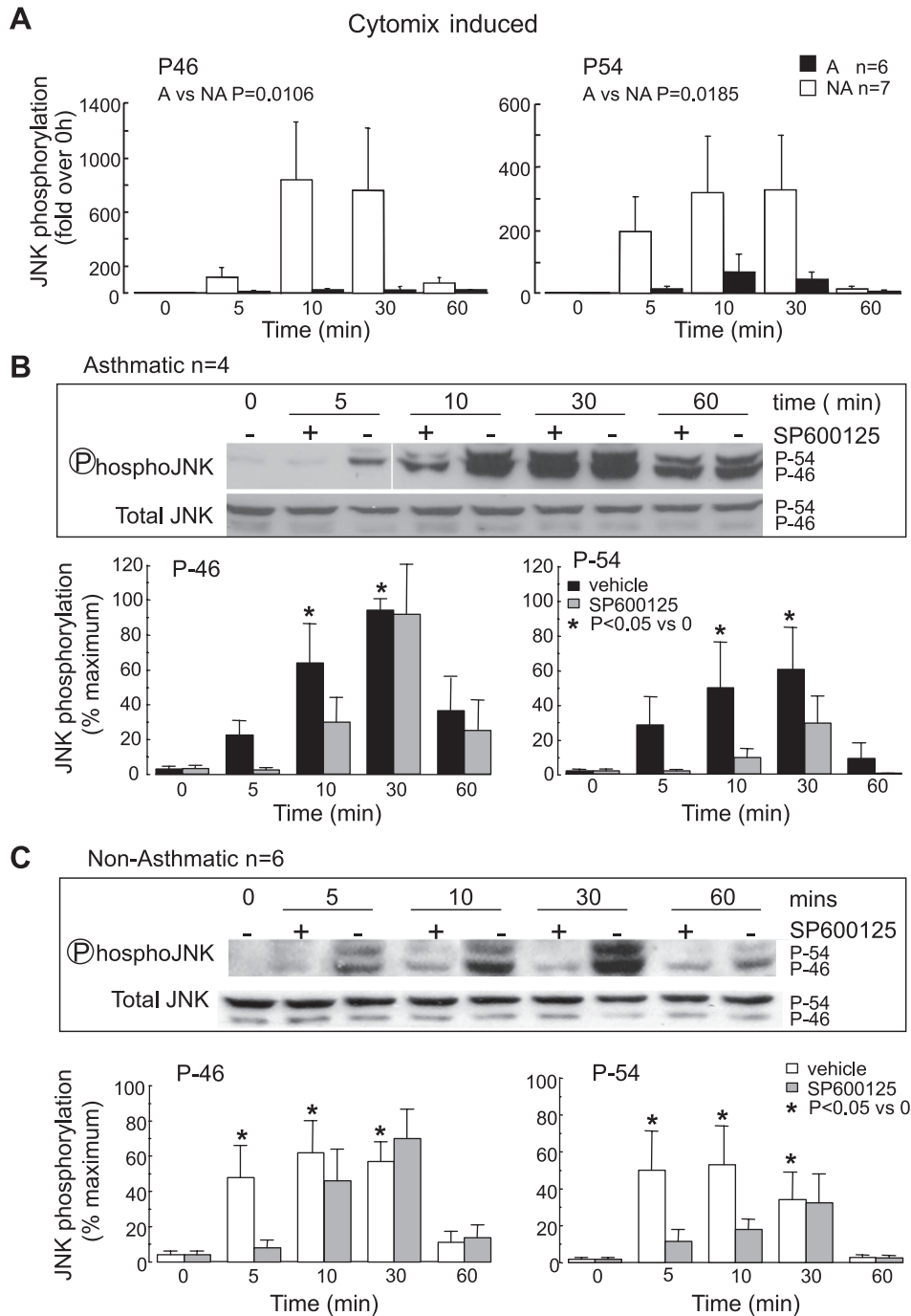


Fig. 5. JNK activation and the effects of its inhibitor SP600125 in ASM cells. Confluent serum-deprived asthmatic and nonasthmatic ASM cells were stimulated with cytomix for up to 1 h. Levels of phosphorylated P-46 and P-54 JNK in the cell lysates were detected using immunoblotting and quantified using densitometry. Differences in phosphorylated P-46 and P-54 levels (expressed as fold change over 0 h) between A ($n = 6$) and (NA) ($n = 7$) cells (A). Representative immunoblots and densitometry of changes with time in cytomix-induced P-46 and P-54 JNK phosphorylation (expressed as percentage of maximum phosphorylation) and the effects of SP600125 on it for A ($n = 4$, B) and NA ($n = 6$, C) ASM cells. The cells were treated with SP600125 or vehicle for 45 min before and during the cytomix stimulation. White lines indicate where immunoblot images have been spliced. Bars, mean $\pm$ SE; vehicle 0.1% vol/vol DMSO.

DISCUSSION

This is the first study to investigate the kinetics and mechanisms underlying CXCL10 production by ASM cells from people with asthma. The pattern of CXCL10 production induced by IL-1 β , TNF- α , and IFN- γ and MAPK signaling molecule involvement were determined. We demonstrated that JNK phosphorylation was markedly reduced, whereas the induction of CXCL10 mRNA expression was more rapid in asthmatic compared with nonasthmatic ASM cells following stimulation with the combined cytokines. In addition, we have provided evidence that JNK activation is a key component of a separate signaling pathway(s) to the

NF- κ B pathway in ASM cell CXCL10 production under those conditions, even in asthmatic cells. Previously, we reported that CXCL10 protein production by the ASM cells from people with asthma is increased and provided evidence linking it to mast cell migration to the ASM in asthma (5). Now we have extended those findings and identified that both JNK and NF- κ B are key signaling molecules involved in CXCL10 production and propose that they provide alternative independent therapeutic targets for limiting ASM cell CXCL10 release and thus mast cell myositis in asthma.

Both ASM cell CXCL10 production and the effects of the signaling inhibitors on it were cytokine stimulus dependent.

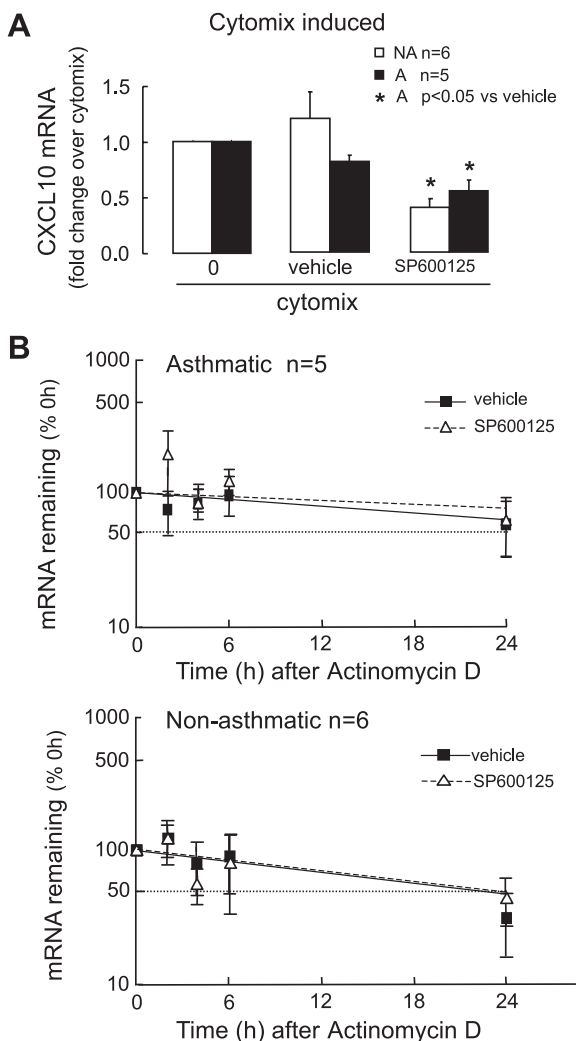


Fig. 6. Effects of the JNK inhibitor SP600125 on CXCL10 mRNA levels and its stability in asthmatic and nonasthmatic ASM cells. Confluent serum-deprived ASM cells were treated with SP600125, or its vehicle, for 45 min before and during stimulation with cytomix for 3 h. CXCL10 mRNA levels were quantified using real-time PCR and expressed as fold change over cytomix-induced expression (A). The stability of the mRNA generated in the presence of SP600125 or its vehicle was also assessed. At the end of the cytomix stimulation, the cells were immediately washed and Actinomycin D, or its vehicle, were added (0 h) to prevent new mRNA synthesis. The mRNA remaining was quantified as above at intervals over the next 24 h and expressed as a percentage of the mRNA levels present at 0 h (B). Bars/points, mean $\pm$ SE; vehicle 0.1% vol/vol DMSO.

CXCL10 release was greatest when the cells were stimulated with IFN- γ in combination with IL-1 β and/or TNF- α . Although NF- κ B is involved in CXCL10 production, DMF, which inhibited cytokine-induced ASM cell NF- κ B p65 activity as previously reported (23), was less effective when the cells were stimulated with IFN- γ alone or in combination. Also, the magnitude of its effects were similar in asthmatic and nonasthmatic cells. As well, we determined that the MAPK JNK always mediated CXCL10 production, whereas ERK was never involved and p38 only mediated CXCL10 release induced by IL-1 β or TNF- α . However, the JNK inhibitor SP600125 was also less effective when the stimulus involved IFN- γ . Surprisingly perhaps, the relative sensitivity of cytomix-induced JNK phosphorylation and

CXCL10 release to SP600125 was similar in asthmatic and nonasthmatic ASM cells. JNK inhibition reduced CXCL10 mRNA production but did not affect its stability or I κ -B α degradation and subsequent NF- κ B p65 activation, which precede NF- κ B nuclear translocation. As well, CXCL10 release was further reduced when both the JNK inhibitor and DMF were used. These latter findings are consistent with signaling through JNK contributing to activation of a different transcription factor pathway to the NF- κ B pathway, following stimulation with the combined cytokines. Further studies are needed to determine the role of JNK in the signaling preceding CXCL10 gene transcription, especially in asthmatic ASM cells, where its activation appears to be reduced and yet still contributes significantly.

In this study we used real-time PCR to establish that CXCL10 gene transcription was more rapid in asthmatic than nonasthmatic ASM cells following stimulation with IL-1 β and TNF- α , alone and in combination with IFN- γ . No statistically significant differences in mRNA stability were observed in asthmatic and nonasthmatic cells following the latter stimulation. Thus more rapid induction of CXCL10 mRNA expression, not altered mRNA stability, may underlie our previous observations of increased CXCL10 release over 24 h by the ASM cells from asthmatic compared with nonasthmatic donors (5). However, this apparent difference may not be sustained over longer periods, as the mRNA levels in nonasthmatic cells continued to increase throughout the 24-h period and thus may lead to increased CXCL10 release at later time points.

Synergistic upregulation of ASM signaling molecules and proinflammatory mediators by IFN- γ in combination with TNF- α has been observed previously. ASM production of the chemokines CCL5 (16) and CX3CL1 (28), as well as CD38, an enzyme involved in cyclic ADP ribose formation (30), are all synergistically increased when these cytokines are used in combination. Hardaker et al. (13) and Clarke et al. (9) reported synergistic increases in CXCL10 release by ASM cells from nonasthmatic donors following IFN- γ and TNF- α stimulation, which was due to recruitment of CREB binding protein and increased RNA polymerase II (9). This study of CXCL10 production by ASM cells from asthmatic, as well as nonasthmatic, donors extended those findings and established that IFN- γ , combined with IL-1 β as well as TNF- α , caused a synergistic increase in CXCL10 release.

Intriguingly, reduced activation of the MAPK JNK may contribute to the more rapid onset of CXCL10 gene transcription in ASM cells from people with asthma. MAPKs are involved in ASM production of various chemokines, but which MAPK is involved depends on the stimulus and the chemokine being produced. In this study, pharmacological inhibitors were used to determine which MAPKs were involved in ASM CXCL10 production. We found it varied: p38, but not ERK, had a role when the cells were stimulated with IL-1 β or TNF- α , but not IFN- γ , whereas JNK signaling was always involved irrespective of the cytokine stimulus. This is in contrast to CX3CL1 production, which is synergistically induced by TNF- α and IFN- γ following p38 and JNK activation (28). In asthmatic and nonasthmatic ASM cells, although the proportional effects of JNK inhibition were similar following cytomix stimulation, their JNK activation was very different. Phosphorylation of P-46 and

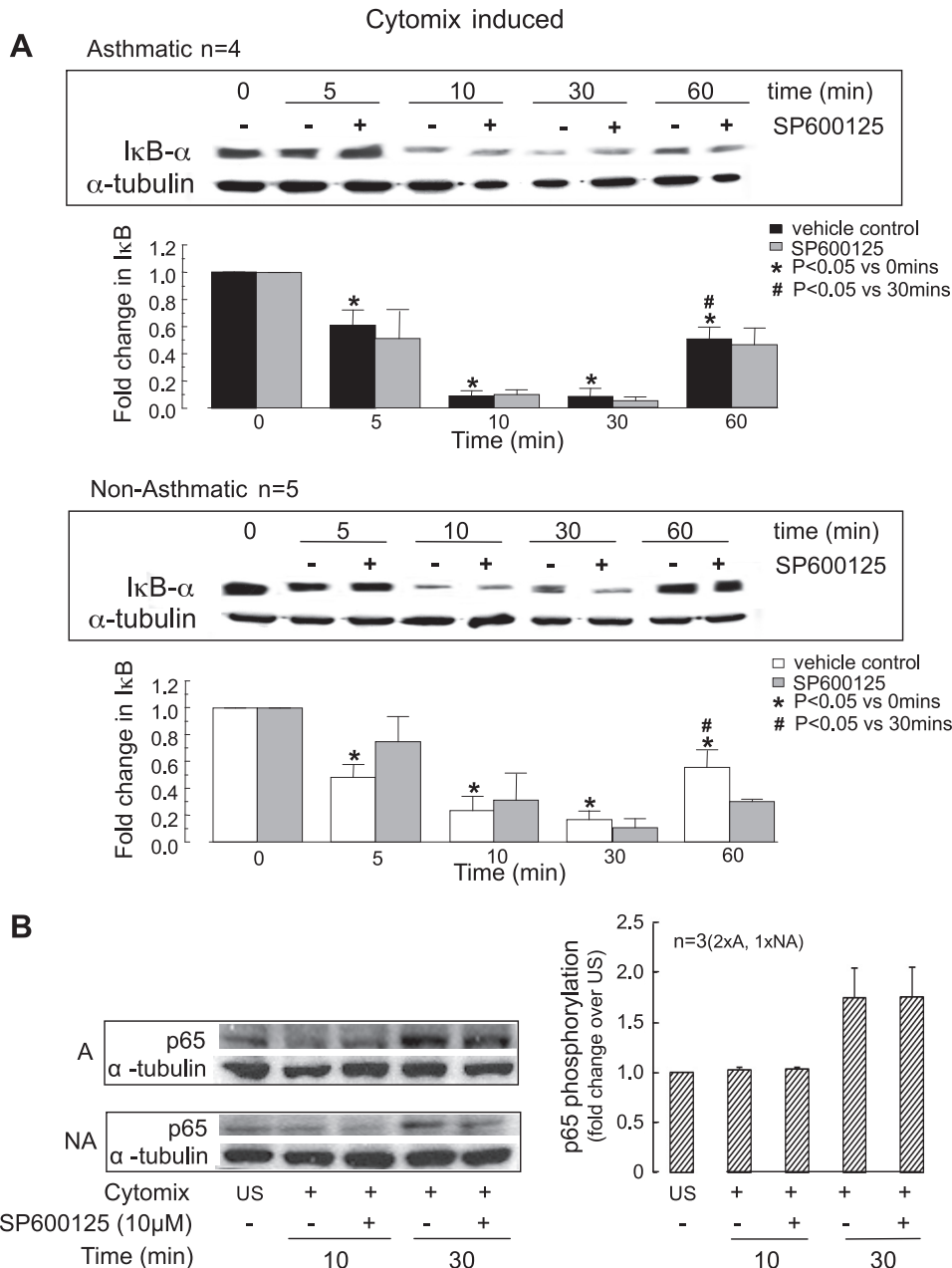


Fig. 7. The effects of the JNK inhibitor SP600125 on early signaling events leading to NF- κ B nuclear translocation. ASM cells were treated with SP600125 or its vehicle for 45 min before and during stimulation with cytomix. I κ B- α levels over 60 min (A) and p65 phosphorylation at 10 and 30 min (B) were detected in whole cell lysates using immunoblotting and quantified using densitometry and expressed as fold change over 0 h and US levels, respectively. Bars, mean $\pm$ SE; vehicle 0.1% vol/vol DMSO.

P-54 was markedly reduced in asthmatic cells but followed the same time course as in nonasthmatic cells. Thus decreased JNK activation may contribute, either directly or indirectly, to earlier CXCL10 gene transcription in the asthmatic cells.

It is unlikely that signaling events before NF- κ B translocation account for the faster CXCL10 gene expression in asthmatic ASM cells. DMF reduced CXCL10 production induced by each of the cytokines, consistent with previous findings of NF- κ B involvement in CXCL10 production (9, 13) and extending them to IL-1 β -stimulated cells and to ASM cells from donors with asthma. However, in both asthmatic and nonasthmatic cytomix-stimulated cells, I κ B- α degradation followed a similar time course, and DMF inhibited CXCL10 mRNA and protein production to a similar extent. Altered NF- κ B activation is also not involved in the increased CXCL8 (IL8) pro-

duction by asthmatic compared with nonasthmatic ASM cells (15). Rather increased binding of p65 and other factors to the CXCL8 promoter underlie its increased production by asthmatic cells (15).

Importantly, this study has also provided evidence that signaling through JNK may not lead to NF- κ B activation, even though both are involved in ASM CXCL10 production. Although SP600125 reduced the rapid P-54 JNK phosphorylation in asthmatic and nonasthmatic ASM cells following cytomix stimulation, I κ B- α degradation and NF- κ B p65 phosphorylation over the same period were not affected, consistent with signaling through JNK not being upstream of NF- κ B activation. Additional support for this hypothesis was provided when the JNK inhibitor and DMF were used together and significantly greater inhibition of CXCL10 release was observed compared with either agent alone. Thus it is likely that JNK

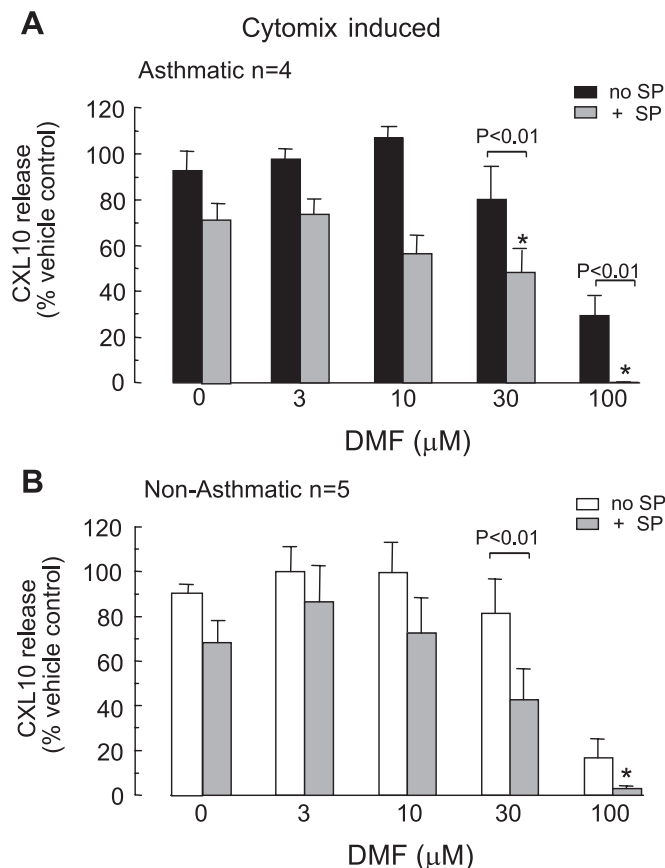


Fig. 8. Effects of the JNK inhibitor SP600125, alone and in combination with DMF, on CXCL10 release by asthmatic (A) and nonasthmatic (B) ASM cells. ASM cells were treated with SP600125 (SP) alone and in combination with DMF for 1 h before and during stimulation with cytomix for a further 24 h. CXCL10 release in culture supernatants was quantified using ELISA. Bars, mean $\pm$ SE; vehicle 0.1% vol/vol DMSO.

signaling contributes to activation of other pathways, not NF- κ B, in CXCL10 production following cytomix stimulation.

Binding motifs for other transcription factors in addition to NF- κ B are present in the CXCL10 promoter. Although there is an activator protein (AP)-1 binding motif, Clarke and colleagues (9) have already established that AP-1 is not involved in CXCL10 production following IFN- γ stimulation of ASM cells. Thus, although DMF has been reported to inhibit AP-1 activity in fibroblasts (24), it is unlikely that inhibition of AP-1 activity underlies the effects of DMF or that JNK signaling to cJUN in the AP-1 complex is directly involved in cytomix-induced CXCL10 production reported here. However, the transcription factor STAT-1 is involved in CXCL10 production when ASM cells are stimulated with IFN- γ and TNF- α (9). In this study, perhaps JNK activation following cytomix stimulation directly or indirectly modulated STAT-1 activity.

Several groups have detected mast cells located on the ASM and demonstrated that their numbers and/or activation correlate with decline in lung function and/or severity in asthma (1, 3, 6, 7). Brightling et al. (6) hypothesized that this mast cell micro-localization plays a central role in the pathogenesis of asthma. There is clear evidence of remodeling in ASM, where mast cells are in close contact (3). Thus the regulation of mast cell

migration to the ASM is an area of considerable interest. Previously we reported findings consistent with CXCL10 being a key regulator of this process in asthma (5). Asthmatic ASM produces CXCL10 in vivo, and mast cells on the ASM all express CXCR3, the receptor for CXCL10. In addition, human lung mast cell migration to supernatants from cytokine-stimulated ASM cells from asthmatics was blocked by neutralizing CXCL10 in the supernatants or blocking CXCR3 on the mast cells (5). Now we have investigated key molecules involved in CXCL10 production by the ASM cells from asthmatics and identified ways to reduce their activation and CXCL10 production. The latter may have clinical relevance because in vivo CXCL10 production is increased by rhinovirus (27) and oral corticosteroids (11).

In summary, production of the mast cell chemoattractant CXCL10 by ASM cells from asthmatic and nonasthmatic donors was synergistically induced by IL-1 β and/or TNF- α in combination with IFN- γ . JNK activation was always involved but was markedly less in asthmatic cells, and their CXCL10 mRNA levels rose more rapidly and were higher in the first 24 h compared with nonasthmatic ASM cells. In addition, signaling through JNK did not precede NF- κ B activation. Thus these intracellular molecules may provide alternative independent therapeutic targets for limiting CXCL10 production and mast cell migration to the ASM in asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: Y.A.A., H.A., D.J.L., S.H., C.E.B., J.K.B., C.L.A., A.J.A., and J.M.H. conception and design of research; Y.A.A., H.A., E.C., D.J.L., M.P., S.H., A.J.A., and J.M.H. performed experiments; Y.A.A., H.A., E.C., D.J.L., S.H., A.J.A., and J.M.H. analyzed data; Y.A.A., H.A., E.C., D.J.L., M.P., S.H., C.E.B., J.K.B., C.L.A., A.J.A., and J.M.H. interpreted results of experiments; Y.A.A., H.A., C.E.B., J.K.B., C.L.A., A.J.A., and J.M.H. edited and revised manuscript; Y.A.A., H.A., E.C., D.J.L., M.P., S.H., C.E.B., J.K.B., C.L.A., A.J.A., and J.M.H. approved final version of manuscript; H.A. and J.M.H. prepared figures; J.M.H. drafted manuscript.

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