Airway smooth muscle CXCR3 ligand production: regulation by JAK-STAT1 and intracellular calcium.

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Abstract

In asthma, airway smooth muscle (ASM) CXCR3 ligand production may attract mast cells or T-lymphocytes to the ASM where they can modulate ASM functions. In ASM cells (ASMC) from people with or without asthma, we aimed to investigate JAK-STAT1, JNK and calcium involvement in CXCL10 and CXCL11 production stimulated by interferon-γ, interleukin-1β and tumour necrosis factor-α combined (cytomix). Confluent, growth-arrested ASMC were treated with inhibitors for pan-JAK (pyridone-6), JAK-2 (AG490), JNK (SP600125) or sarco/endoplasmic reticulum calcium ATPase (SERCA) pump (thapsigargin), the calcium chelator (BAPTA-AM) or vehicle, prior to and during cytomix stimulation for up to 24 hr. Signalling protein activation, CXCL10/11 mRNA and protein production were examined using immunoblotting, real time PCR and ELISA respectively. Cytomix-induced STAT-1 activation was lower and CXCR3 ligand mRNA production more sensitive to P6 and AG490 in asthmatic than nonasthmatic ASMC, but CXCL10/11 release was inhibited by the same proportion. Neither agent caused additional inhibition of release when used in combination with the JNK inhibitor SP600125. Conversely P65 NFκB activation was higher in asthmatic than nonasthmatic ASMC. BAPTA-AM abolished early CXCL10/11 mRNA production, while thapsigargin reduced it in asthmatic cells and inhibited CXCL10/11 release by both ASMC types. Despite these inhibitory effects, neither calcium agent affected early activation of STAT1, JNK or P65 NFκB. In conclusion, intracellular calcium regulated CXCL10/11 production, but not early activation of the signalling molecules involved. In asthma, reduced ASM STAT1-JNK activation, increased NFκB activation and altered calcium handling, may contribute to rapid CXCR3 ligand production and enhanced inflammatory cell recruitment.

Keywords: CXCL11; asthma; CXCL10; STAT1; calcium signalling

Abbreviations: AG490, α-Cyano-(3,4-dihydroxy)-N-benzylcinnamide; ASM, airway smooth muscle; ASMC, airway smooth muscle cell; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester); FEV1, forced expiratory volume in one second; FVC, forced vital capacity; IFNγ, interferon-γ; IL-1β, interleukin-1β; JAK, Janus-activated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated kinase; NFκB, nuclear factor-κB; P6, pyridone-6; SERCA, sarcoplasmic/endoplasmic recticulum calcium ATPase; STAT, signal transducers and activators and transcription; TNFα, tumour necrosis factor-α; $[Ca^{2+}]_i$, intracellular calcium concentration.

Introduction

In asthma, airway hyperresponsiveness may be the result of inflammation (16, 56) and aberrant airway smooth muscle (ASM) function (28, 29, 52). In addition to its contractile properties, the ASM can directly contribute to the proinflammatory cytokine milieu via de novo synthesis of cytokines and chemokines (12, 23, 57). This function of ASM may serve to activate and direct the recruitment of immune cells into the airways to establish and maintain the hyperresponsive state. In asthmatic airways, there is an increase in the microlocalisation of immune cells within the ASM bundles (10, 12, 13). Key among these immune cells are mast cells (12, 13) and T lymphocytes (44).

The increase in ASM mast cell numbers in asthmatic lungs may be due to phenotypic changes occurring in both mast cells and ASM cells (ASMC). Compared to mast cells found in other parts of the lung tissue, ASM-associated mast cells have high expression of the CXCR3 chemokine receptor (12). Not surprisingly, CXCR3 expression regulated the chemotaxis of lung mast cells as blockade of this receptor significantly inhibited mast cell chemotaxis (12). In addition, CXCR3 is also expressed on CD4+and CD8+ T lymphocytes (44). These findings indicate that CXCR3 ligands are likely to be important regulators of mast cell and T lymphocyte migration into the ASM.

We have previously used immunohistochemistry on biopsies to show that ASM from people with asthma is positive for the CXCR3 ligand CXCL10 (interferon-γ inducible protein 10 kDa, (IP-10)) more than ASM from controls (12). Thus sufficient levels of CXCL10-inducing cytokines must be present in the ASM layer in asthma to induce CXCL10 production by the ASMC. These findings complement other reports of elevated levels of CXCL10 protein in bronchoalveolar lavage fluid (BALF) and bronchial biopsies (37) from people with asthma.

In vitro we have also shown asthmatic ASMC secrete more CXCL10 than nonasthmatic ASMC in response to stimulation with the Th1 cytokines interferon-γ (IFNγ), tumour necrosis factor-α (TNFα) and the proinflammatory cytokine interleukin-1β (IL-1β) (12). In contrast, the Th2 cytokines IL-4 and IL-13 did not induce ASMC CXCL10 or CXCL11 production (50). However in a mouse model of allergic airway disease CXCL10 production was increased following allergen challenge. Although it is normally regarded as a Th1 cytokine, in mice over-expressing CXCL10 there were raised levels of the Th2 cytokine IL-4, eosinophilia and airway hyperresponsiveness (36). Even though secretion of another CXCR3 ligand CXCL11 (Interferon-inducible T-cell alpha chemoattractant (ITAC) or Interferon-gamma-inducible protein 9 (IP-9)) was not significantly higher in Th1 cytokine-treated ASMC from adults with asthma, there was a clear trend (12) and CXCL11 BALF levels are elevated in children with asthma in comparison with BALF from healthy children (24).

Recently we reported that the ability of IL-1β combined with the Th1 cytokines TNFα and IFNγ to stimulate CXCL10 release by ASMC from people with or without asthma was dependent on the c-Jun N-terminal kinase (JNK) mitogen activated protein kinase (MAPK), as well as the nuclear factor-κB (NFκB) pathways (2). We also found JNK activation did not contribute to p65 NFκB activation and was markedly reduced in ASMC from people with asthma. However, IFNγ also signals through the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathways (21) and regulates CXCL10 production in human ASMC (15). JAK-STAT activation by Th1 and proinflammatory cytokines and its role in regulating cytokine-induced production of CXCR3 ligands in ASMC from people with asthma has not been investigated.

Maintenance of airway bronchomotor tone is regulated by oscillatory changes in intracellular calcium concentration ($[Ca^{2+}]_i$) as well as calcium sensitivity mechanisms in ASMC (47, 48).

However, in other cell types $[Ca^{2+}]_i$ signalling is known to regulate a diverse range of cellular function including inflammation. IL-1β and IL-8 production in human bronchial epithelial cells in response to ambient particulate matter is regulated by Ca^{2+} signalling (46). The importance of Ca^{2+} signalling in regulating inflammation has also being confirmed in monocytes whereby inhibition with the intracellular Ca^{2+} chelator BAPTA-AM not only down regulated TNFα production, but also inhibited NFκB activation (25). There is evidence that in ASMC, $[Ca^{2+}]_i$ homeostasis may also regulate cytokine production (26), as well as signalling pathways that regulate inflammation (31, 38, 39). In nonasthmatic ASMC, TNFα induced IL-6 secretion and ICAM-1 expression is regulated by Ca^{2+}-dependent activation of NFκB (4, 31).

The ASMC from people with asthma have intrinsic differences, including faster proliferation (29, 50) and altered $[Ca^{2+}]_i$ handling (34, 52). Trian and colleagues demonstrated that the asthmatic cells proliferated faster because of increased mitochondrial biogenesis which is Ca^{2+}-dependent. More recently Mahn and colleagues reported that asthmatic ASMC have reduced function and expression of the sarco/endoplasmic reticulum calcium ATPase (SERCA) pump (34, 52). In view of these findings we hypothesised that abnormal $[Ca^{2+}]_i$ handling leads to dysregulated signalling and synthesis of inflammatory mediators such as the CXCR3 ligands in ASMC from people with asthma.

The aims of this study were in ASMC from people with and without asthma to investigate and compare 1) JAK-STAT involvement in cytomix stimulated production of the CXCR3 ligands CXCL10 and CXCL11 and 2) the effects of modulating $[Ca^{2+}]_i$ on the production of these two CXCR3 ligands, as well as the early signalling events that regulate their production.

Materials and Methods

Reagents

Recombinant Human IFNγ (BD Biosciences, Australia), IL-1β and TNFα (R&D Systems, Minneapolis, MN) were reconstituted and stored as recommended by the manufacturers. The SERCA inhibitor thapsigargin, the cell membrane permeant Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), the pan JAK inhibitor pyridone-6 (2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one, the selective JAK2 inhibitor AG490 (α-Cyano-(3,4-dihydroxy)-N-benzylcinnamide) and the CaMK II inhibitor KN93, were all supplied by Calbiochem (San Diego, CA), reconstituted in dimethylsulfoxide (DMSO) (Sigma Aldrich, Castlehill, Australia). The JNK inhibitor SP600125 was supplied by A.G. Scientific (San Diego, CA) and was also reconstituted with DMSO. All agents were stored at –20°C, except for IFNγ which was stored at -80°C.

Airway smooth muscle cell culture and treatment

Human ASMC were obtained from bronchial biopsies from nonasthmatic donors, donors with mild to moderate asthma, resected lung tissue from donors undergoing surgery for thoracic malignancies or from patients undergoing lung transplantation (refer to supplementary table 1 for patient details). 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.

ASMC were maintained in culture as previously described (29) in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich) supplemented with 10% v v^{-1} foetal bovine serum (FBS), 100 units ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin sulphate, 25 μg ml^{-1} amphotericin B, 4 mM L-glutamine, 20 mM HEPES, (pH 7.4) and grown in humidified 5%

CO_2/air at 37°C. ASMC expressed α-smooth muscle actin and h-calponin upon immunostaining (35).

ASMC (passages 4-7) were put into plates at 1×10^4 cells cm^{-2} in growth medium, cultured for 7 days and then serum deprived for 48 hr in FBS-free DMEM supplemented with 0.1% v v^{-1} bovine serum albumin (BSA). ASMC were then treated with or without vehicle (0.1% v v^{-1} DMSO in the FBS-free medium), thapsigargin (1 μM), BAPTA-AM (50 μM), SP600125 (22.7 μM) or KN93 (5 μM) for 45 min prior to and during stimulation with cytomix which is a combined mixture of 10 ng ml^{-1} each of IL-1β, TNFα and IFNγ for 0-24 hr.

The viability of the cells was routinely checked using phase contrast microscopy prior to treatment and prior to collection of culture medium samples for measurement of chemokine release or preparation of cell lysates for RNA extraction or protein detection using Western blotting. The effects of these treatments on mitochondrial activity was also tested in nonasthmatic ASMC from 4 different donors using standard MTS assays.

CXCL10 and CXCL11 mRNA production

ASMC plated on 6 well plates were treated with 0.1% v v^{-1} DMSO vehicle, 50 μM BAPTA-AM or 1 μM thapsigargin for 45 min before and during stimulation with cytomix for 3 hr. Total RNA was extracted using the guanidine thiocyanate/phenol chloroform method (14). RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD) and performed on a PTC-200 DNA Engine (MJ Research, MA). The resulting cDNA (2.5 μl) was amplified by PCR using FAM-labeled human CXCL10 and CXCL11 and VIC labeled-18srRNA TaqMan probes on an ABI Prism 7500 (Applied

Biosystems) according to the following cycle parameters: 50°C for 2 min, 1 cycle; 95°C for 10 min and 40 cycles of 95°C for 15 sec, 60°C for 1 min. Chemokine mRNA levels were normalized against 18srRNA levels.

CXCL10 and CXCL11 protein release

ASMC in 24 well plates were treated with the agents or vehicle as described above and stimulated with cytomix for 24 hr. Supernatants were harvested and CXCL10 and CXCL11 protein levels were detected using the Duoset enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN) according to the manufacturer's protocol.

Western blotting for total and phosphorylated STAT1, JNK, IκBα and p65 NFκB protein

ASMC in 6 well plates were treated with the agents or vehicle as described above and stimulated with cytomix for 2.5, 5, 10 & 30 min. Whole cell lysates were prepared as previously described (30). Each sample (25 μL) was subjected to SDS-PAGE and Western blotting. Total and phospho-STAT1 (MW approximately 84 & 91 kDa), p54 and p46 JNK (MW approximately 54 and 46 kDa respectively), IκBα (MW approximately 40 kDa) and p65 NFκB (MW approximately 65 kDa) bands were detected by incubating membranes with rabbit anti-human monoclonal antibodies against the target proteins (Cell Signalling Technology, Danvers, MA) diluted 1:1000 in 5% v v^{-1} milk tris-buffered saline solution containing 0.1% v v^{-1} Tween. Band intensities were quantified by densitometric analysis using ImageJ version 1.43u software (National Institute of Health, Bethesda, MD). Phospho-protein band densities were expressed relative to their respective total protein band density.

Measurement of changes in intracellular calcium concentration

At the time of the assay, ASMC plated on black wall with clear bottom 96 well plates were incubated for 1 hr at 37ºC with culture media containing 2.5 mM probenecid (Invitrogen) and Fluo-4 AM Direct calcium detection dye (Invitrogen) prepared according to the manufacturer's protocol provided in the Fluo-4 Direct Calcium Assay kit (Invitrogen). During this incubation period, the cells were pretreated with 0.1% v v^{-1} DMSO, 50 μM BAPTA-AM or 1 μM thapsigargin for 45 min before and during stimulation with 500 nM histamine to stimulate $[Ca^{2+}]_i$ signalling. Cells were excited with 485 nm wavelength, and emission of 520 nm wavelength measured using a Polarstar plate reader (BMG, Adelaide, Australia).

Data analysis

Statistical analyses for all studies were performed using Prism 5.03 (GraphPad, La Jolla, CA). All results were expressed as the mean ± standard error of the mean (SEM). The tests used to determine the level of significance of results ($p<0.05$) are indicated in the figure legends. For ELISA studies, CXCL10 and CXCL11 protein levels from the average of duplicate treatments were expressed as a percentage of the vehicle control. For real time PCR studies, relative CXCL10 and CXCL11 mRNA levels from the average of duplicate treatments were expressed as fold change over unstimulated. For Western blot studies, densitometric measurements for phosphorylated proteins were normalized to the respective total protein, and expressed as the fold change from unstimulated levels.

Results

STAT1 signalling in cytomix-induced CXCR3 ligand mRNA and protein production.

As we have previously reported, cytomix significantly increased CXCL10 and CXCL11 release by ASMC from people with and without asthma. Basal release of CXCL10 protein into culture supernatants occurred (nonasthmatic: 590±310 pg ml^{-1}, n=4 and asthmatic 2400±2200 pg ml^{-1}, n=4). Cytomix stimulation for 24 hr markedly increased release to 38,000±6,000 pg ml^{-1} (n=4, $p<0.05$, cf basal) and 29,000±10,000 pg ml^{-1} (n=4, $p<0.05$, cf basal) in nonasthmatic and asthmatic ASMC respectively. Similarly, CXCL11 protein was detected in ASMC culture supernatants under basal conditions, albeit at lower levels. Stimulation with cytomix for 24 hr induced an increase in CXCL11 protein levels from 19.6±12.4 pg ml^{-1} to 1100±400 pg ml^{-1} (n=4, $p<0.05$) and from 42±23 pg ml^{-1} to 2300±1500 pg ml^{-1} (n=4, $p<0.05$) in nonasthmatic and asthmatic ASMC respectively.

CXCL10 and CXCL11 mRNA in ASMC from people with and without asthma could only be detected reliably following stimulation with cytomix for at least 3 hr (data not shown). The pan-JAK inhibitor pyridone-6 (P6) had differential effects on this early CXCR3 ligand mRNA production by asthmatic and nonasthmatic ASMC. At 0.3 and 1 μM it partially inhibited CXCL10 mRNA expression after 3 hr in asthmatic ASMC only (figure 1A), whereas it partially inhibited both asthmatic and nonasthmatic CXCL11 mRNA at 0.3 and 1 μM (figure 1A). Despite the lack of effect on early gene transcription in nonasthmatic ASMC, pyridone-6 partially inhibited cytomix-induced CXCL10 and CXCL11 protein secretion from both nonasthmatic and asthmatic ASMC (figure 1B). Pyridone-6 at 3 μM reduced CXCL10 release to 42±24% (n=4, $p<0.05$, cf cytomix) and 57±19% (n=4, $p<0.05$, cf cytomix) (figure 1A) and CXCL11 release to 32±13% (n=4, $p<0.05$, cf cytomix) and 22±9% (n=4, $p<0.05$, cf cytomix) (figure 1B) of the cytomix response in nonasthmatic and asthmatic ASMC respectively. The increase in CXCL10 and CXCL11 mRNA and protein secretion stimulated by cytomix was preceded by a marked increase in STAT1 phosphorylation at tyrosine 701

which was completely abolished in the presence of pyridone-6 (figure 1C). Pyridone-6 had no effect on the viability of the ASMC with any of these treatments.

As pyridone-6 inhibited CXCR3 ligand release and STAT-1 activation, we then investigated whether the JAK2 inhibitor AG490 also inhibited CXCR3 ligand production. AG490 only significantly inhibited CXCL10 and CXCL11 mRNA expression in the ASMC from people with asthma (figure 2A). Unlike its effects on mRNA expression, AG490 partially inhibited cytomix-induced CXCL10 and CXCL11 protein release by both asthmatic and nonasthmatic ASMC. However this effect was only significant at 100 μM where AG490 reduced CXCL10 release to 73±7% ($n=6$, $p<0.05$, cf cytomix) and 51±15% ($n=4$, $p<0.05$, cf cytomix) and CXCL11 release to 64±14% ($n=6$, $p<0.05$, cf cytomix) and 52±19% ($n=5$, $p<0.05$, cf cytomix) of the cytomix response in nonasthmatic and asthmatic ASMC respectively (figure 2B). AG490 had no effect on the viability of the ASMC with any of these treatments.

Previously we reported that cytomix-induced MAPK JNK activation was important in CXCL10 production, but did not contribute to the NFκB pathway and was reduced in asthmatic ASMC. Further JNK activation leading to activation at S727 on STAT3, which has homology with STAT1, has been reported. Therefore we investigated whether JAK-STAT and JNK were part of the same or different pathways leading to CXCR3 ligand production by treating nonasthmatic and asthmatic ASMC with the JNK inhibitor SP600125 (22.7 μM), combined with pyridone-6 or AG490. SP600125 alone only caused a slight reduction in CXCL10 release. Even so, this level of inhibition was not significantly enhanced by the addition of increasing concentrations of either pyridine-6 up to 3 μM (data not shown) or AG490 up to 100 μM (figure 2B). SP600125 reduced cytomix-stimulated CXCL11 production to a greater extent than CXCL10, but again concomitant pan-JAK inhibition with pyridone-6 (data not shown), or JAK-2 inhibition with AG490 (figure 2B), did not cause

significantly more inhibition of CXCL11 release than SP600125 alone. In addition, the cytomix-induced rapid phosphorylation of STAT1 at positions tyrosine 701 (data not shown) and serine 727 (figure 2C) were not affected by pretreatment of asthmatic and nonasthmatic ASMC with the JNK inhibitor. SP600125 had no effect on the viability of the ASMC. These findings are consistent with JAK-2 contributing to the same pathway as JNK.

STAT1 activation in asthmatic ASMC and intracellular calcium involvement

As CXCR3 ligand mRNA expression in asthmatic ASMC was more sensitive to JAK-STAT inhibition than nonasthmatic ASMC and JNK activation is reduced in asthmatic ASMC, we compared STAT1 activation in ASMC from people with and without asthma. Cytomix-induced phosphorylation of STAT-1 at tyrosine 701 was markedly lower in asthmatic compared to nonasthmatic ASMC (figure 3A). In nonasthmatic ASMC, cytomix stimulation for 30 min caused a 33±10 ($n=5$, $p<0.01$, cf unstimulated) fold increase in phosphorylated STAT1 levels from basal levels. In contrast, in asthmatic ASMC cytomix treatment for the same period only induced a 15±5 ($n=7$, $p<0.01$, cf unstimulated) fold increase in phosphorylated STAT1 levels from basal levels (figure 3A). This difference in STAT1 phosphorylation between the two cell types was significant ($p<0.05$, 2-way ANOVA).

In view of this finding, we investigated whether $[Ca^{2+}]_i$ regulates STAT1 activation as calcium handling is altered in asthmatic ASMC. ASMC treatment for 45 min prior to and during cytomix stimulation with either 50 μM BAPTA-AM (intracellular Ca^{2+} chelator), or 1 μM thapsigargin (SERCA pump inhibitor), did not affect the rapid cytomix-induced phosphorylation of STAT1 at tyrosine 701 in either asthmatic or nonasthmatic ASMC (figure 3B). Both these agents did inhibit the rapid histamine-induced increase in $[Ca^{2+}]_i$ in nonasthmatic ASMC (figure 3C), however cytomix stimulation did not induce any rapid

changes in $[Ca^{2+}]_i$ in ASMC (figure 3C). Similarly, cytomix induced rapid phosphorylation of P46 and P54 JNK was not modulated by concomitant treatment with either BAPTA-AM or thapsigargin (figure 4A and 4B).

NFκB activation in asthmatic ASMC and intracellular calcium involvement

In contrast to the differences observed in STAT1 activation, cytomix-induced activation of the transcription factor P65 NFκB was significantly higher in asthmatic compared with nonasthmatic ASMC (figure 5A). Cytomix stimulation for 30 min mediated a 7.4±2 fold increase from unstimulated phosphorylated levels of the P65 subunit of NFκB in asthmatic ASMC (n=5). In comparison, nonasthmatic ASMC (n=4) only achieved a 2.4±0.5 fold increase in phosphorylated P65 NFκB at the same time point (figure 5A). The higher levels of NFκB activation by cytomix stimulation in asthmatic ASMC in comparison to nonasthmatic ASMC was statistically significant (p=0.0159, t-test).

This effect of cytomix was not altered in any way in the presence of either BAPTA-AM or thapsigargin (figure 5B). Similarly, the degradation of IκBα, which precedes the phosphorylation of p65 NFκB was also unaffected by BAPTA-AM and thapsigargin (figure 5C).

Intracellular calcium and calcium calmodulin kinase II involvement in CXCR3 ligand production

As early signalling events leading to CXCR3 ligand gene expression were not affected by BAPTA-AM and thapsigargin, we investigated the effects of these agents on mRNA and

protein levels. BAPTA-AM treatment prior to and during 3 hr cytomix stimulation completely abolished the induction of CXCL10 and CXCL11 mRNA expression in both nonasthmatic and asthmatic ASMC (figure 6A). The effects of BAPTA on CXCL10 and CXCL11 protein secretion during 24 hr cytomix stimulation were not assessed as BAPTA caused all the ASMC to detach from the well bottom by 16 hr. Cell attachment and viability were however not affected during 3 hr treatment with BAPTA (data not shown).

In contrast, thapsigargin treatment partially inhibited cytomix-induced CXCL10 and CXCL11 mRNA expression only in ASMC from people with asthma. However, CXCL10 and CXCL11 release after 24 hr cytomix stimulation were significantly inhibited by thapsigargin down to 29±5% (n=3, $p<0.05$ cf with cytomix) and 9.6±1.3% (n=3, $p<0.05$ cf with cytomix) in nonasthmatic ASMC, and 26.2±7% (n=3, $p<0.05$ cf with cytomix) and 8.2±3% (n=3, $p<0.05$ cf with cytomix) in asthmatic ASMC, respectively (figure 6B). These treatments with thapsigargin did not affect the viability of the ASMC.

The pharmacological inhibitor KN93 was used to inhibit the calcium dependent calmodulin kinase II protein which regulates STAT1 activation and CXCL10 production by other cell types. In both ASMC from people with or without asthma, treatment with 5 μM KN93 prior to and during 24 hr cytomix stimulation did not significantly inhibit CXCL10 and CXCL11 release (figure 6C).

Discussion

The role of mast cells in the airways of people with eosinophilic and severe asthma remains controversial (8, 20). Mast cell derived products such as histamine, prostaglandin D_2 (PGD_2) (9) and leukotriene D_4 (LTD_4) (45), induce ASM contraction, while transforming growth factor-β (TGF-β) can stimulate airway remodelling (11). In contrast, some mast cell products have apparently beneficial effects in vitro. For example, mast cell tryptase reduces levels and activity of the eosinophil chemoattractant proteins CCL11 (eotaxin) and CCL5 (RANTES) (41). Further, newly-synthesised mast cell products released by IgE-anti-IgE activation reduce serum-induced proliferation of ASMC from people with asthma (1). Thus mast cells may have diverse effects on ASM functions in vivo. Understanding the mechanisms leading to ASMC production of mast cell chemoattractants may help us better understand how to intervene and thus determine the significance of mast cell-ASM interactions in asthma. In the present study, we found that asthmatic ASMC CXCL10 and CXCL11 production were more sensitive to the inhibitors of JAK-STAT and $[Ca^{2+}]_i$ signalling. In addition, compared to nonasthmatic ASMC, cytomix-induced STAT1 activation was significantly diminished, while p65 NFκB activation was enhanced in ASMC from people with asthma. How these alterations in signalling contribute to elevated ASM CXCR3 ligand production in people with asthma requires further extensive investigation.

In this study nonasthmatic ASMC were used as a comparison group. When available, the ASMC used were isolated from airways within macroscopically normal lung tissue but the possibility remains that the ASMC may have been affected by the lung environment prior to resection. Previously, we and others have established that ASMC from people with asthma do have proliferative and stimulus-specific synthetic abnormalities (29). In past studies in which responses have been compared between ASMC cultures established from either donor lungs

unsuitable for transplantation, or bronchial biopsies from healthy volunteers, and the 'nonasthmatic' ASMC from people with other lung diseases, the responses were similar (29).

The differences in CXCL10 and CXCL11 levels released by nonasthmatic and asthmatic ASMC either basally or following stimulation were not statistically significant. Previously, when we examined chemokine production by ASMC from a larger number of donors with and without asthma and adjusted production for cell number, there was a significant increase in cytomix-induced CXCL10 release by the asthmatic compared with nonasthmatic cells (12), perhaps as a result of more rapid CXCL10 gene transcription (2). A similar trend was observed for CXCL11 but the difference was not statistically significant.

Previously we showed that cytomix induced CXCL10 production was regulated by NFκB and JNK MAPK (2). However, since JNK and NFκB appeared to be acting on different pathways to regulate CXCL10 production, we hypothesised that JNK may be regulating another transcription factor STAT1, which is involved in ASMC CXCL10 production following activation by IFNγ, (15). The early activation of STAT1 induced by IFNγ requires phosphorylation at Y701 by JAK1 or JAK2 (21). In the present study, STAT1 signalling was blocked by inhibiting multiple JAKs or JAK2 specifically. CXCL10 mRNA production was only inhibited by the JAK inhibitors in the asthmatic cells, whereas CXCL11 mRNA was inhibited in both cell types but only with pyridone-6 at the highest concentration tested. This is the first evidence that CXCL10 gene expression in asthmatic compared with nonasthmatic ASMC is much more sensitive to disruption of the JAK-STAT1 signalling pathway, probably because of the reduced activation of STAT1 in asthmatic cells following cytomix stimulation.

In contrast to the mRNA effects, both CXCL10 and CXCL11 protein production were inhibited to a similar extent by JAK inhibition in nonasthmatic and asthmatic cells. Although this study is confirmation that STAT1 is an important regulator of ASMC CXCR3 ligand

production, the reduction in CXCR3 ligands was greater when multiple JAKs were inhibited, which may indicate that multiple JAK or STAT proteins regulate it.

The activation of STAT1 is dependent on tyrosine phosphorylation at Y701 (21), but we found JNK does not regulate this. Although not required, STAT1 activation can be further enhanced by serine phosphorylation at S727 (17, 58). STAT3, which has homology with STAT1, can be phosphorylated at S727 by JNK in COS-1 cells (32). However, we found no evidence that JNK was able to modulate phosphorylation of STAT1 at either Y701 or S727 in ASMC. The fact that neither pyridone-6 nor AG490 was able to further enhance the inhibitory effects of the JNK inhibitor SP600125 on CXCL10 and CXCL11 production is evidence that JNK and JAK-2 at least are regulating CXCR3 ligand production via the same pathway. If this is indeed the case, then JNK is not participating in the early events in STAT1 activation. Alternatively, cytomix-induced JAK-2 activation may mediate JNK activation in the ASMC, as occurs in macrophages in response to a range of stimuli (53, 55).

Contractile agonists such as acetylcholine and histamine mediate $[Ca^{2+}]_i$ signalling in ASMC (45). Asthmatic ASMC may be hyperresponsive to these agonists due to altered $[Ca^{2+}]_i$ signalling as a result of diminished SERCA activity (34) and increased Ca^{2+}-dependent mitochondrial biogenesis (50). In addition, $[Ca^{2+}]_i$ signalling has the ability to regulate transcription factors involved in inflammation such as NFκB and nuclear factor of activated T-cells (NFAT) (19, 42). Based on these insights, we explored the role of $[Ca^{2+}]_i$ signalling in CXCL10 and CXCL11 production. Thapsigargin leads to the depletion of calcium from the sarcoplasmic reticulum store by inhibiting SERCA but not plasma membrane Ca^{2+}-ATPases (33, 51). This effect was clearly demonstrated here by the absence of a rise in $[Ca^{2+}]_i$ immediately following histamine stimulation of the ASMC in the presence of thapsigargin. In agreement with previous reports of TNFα effects (3, 5), cytomix did not directly mediate any

transient increase in $[Ca^{2+}]_i$.Despite this, we found thapsigargin treated ASMC had diminished CXCR3 ligand release following cytomix stimulation but only asthmatic ASMC also had reduced mRNA production. This is the first evidence that store released Ca^{2+} is important in CXCR3 ligand production by ASMC from donors with asthma. The regulatory role of Ca^{2+} on CXCR3 ligand production was confirmed by rapidly sequestering $[Ca^{2+}]_i$ using BAPTA-AM (54) which completely abolished cytomix-induced CXCL10 and CXCL11 mRNA production in both nonasthmatic and asthmatic ASMC. The effects of thapsigargin and BAPTA-AM in this study are unequivocal evidence that CXCR3 ligand production is somehow regulated by cytosolic and/or store released Ca^{2+}.

$[Ca^{2+}]_i$ signalling requires Ca^{2+} sensitive binding proteins that are able to transduce changes in $[Ca^{2+}]_i$ into signalling events. In certain cell types, IFNγ-induced activation of STAT1 requires the calcium-dependent calmodulin kinase II (CaMKII) (38). In the present study, cytomix-induced CXCR3 ligand production was not regulated by CaMKII. However, ASMC are known to express different types of calmodulin kinases such as CaMKIV (52), which is activated to a higher extent in asthmatic ASMC (52). Hence it is possible that other calmodulin kinases may be involved in converting $[Ca^{2+}]_i$ signalling into CXCR3 ligand production.

Further experiments were performed to elucidate whether $[Ca^{2+}]_i$ regulated the signalling pathways and transcription factors that we have shown in this and a previous study (2) to be involved in CXCR3 ligand production. Phosphorylation of JNK, STAT1 and NFκB and degradation of IκBα following cytomix addition were completely unaffected by either thapsigargin or BAPTA-AM. These results are evidence that the very early signalling events induced by cytomix are not likely to be regulated by $[Ca^{2+}]_i$ signalling. For $[Ca^{2+}]_i$ to participate, it would most likely be regulating later events such as nuclear translocation of

STAT1 and/or NFκB and/or transcriptional activation. This is certainly a possibility as TNFα mediated NFκB transcriptional activation, but not nuclear translocation, can be abolished by thapsigargin (4).

Several studies have found that TNFα enhances $[Ca^{2+}]_i$ signalling by contractile agonists such as bradykinin, carbachol (5, 6) and histamine (18). In addition, TNFα can also enhance ASM contraction (49) and the sensitivity of the cellular contractile machinery to Ca^{2+} (27, 43). Although the exact mechanisms of these effects by TNFα have not been fully elucidated, there is some evidence that TNFα can augment store operated Ca^{2+} entry (SOCE) which is induced in response to Ca^{2+} emptying from the sarcoplasmic reticulum by contractile agonists (7). Even though TNFα was unable to immediately generate $[Ca^{2+}]_i$ signals in this study, treatment of ASMC in culture with TNFα for several hours has been shown to elevate basal $[Ca^{2+}]_i$ levels which was dependent on Ca^{2+} entry through transient receptor potential C3 (TRPC3) channels on the plasma membrane (59). This enhancement of $[Ca^{2+}]_i$ required increased expression of TRPC3. If this effect was occurring in our studies in response to prolonged cytomix stimulation, no rise in $[Ca^{2+}]_i$ would have been detected during the shorter time periods (60 seconds) we investigated. Thus we speculate that the potential increase in basal $[Ca^{2+}]_i$ in response to prolonged cytomix stimulation may be a possible mechanism contributing to CXCR3 ligand production.

ASMC in culture are able to retain many of their in vivo characteristics (40). Numerous studies from the Sydney Respiratory Research Group and others have previously uncovered phenotypic differences between ASMC derived from nonasthmatic and asthmatic people (22). Previously, we demonstrated that cytomix-induced JNK activation was reduced in asthmatic ASMC (2). Similarly in this study, we found evidence that STAT1 activation by cytomix was also significantly reduced in asthmatic ASMC. These findings may also help explain why in

asthmatic ASMC, CXCL10 and CXCL11 mRNA production was more sensitive to JAK inhibitors than in nonasthmatic cells. Further studies are required to ascertain how reduced JNK and STAT1 activation could lead to higher production of CXCL10 in ASMC from people with asthma. In asthma, the ASMC nuclear transcriptional machinery regulating CXCR3 ligand expression may be more sensitive to the signalling from JNK and STAT1 or they may be more reliant on other signalling pathways to direct CXCR3 ligand production. The latter point has some merit as cytomix-stimulated p65 NFκB activation was higher in asthmatic ASMC, indicating that the NFκB proinflammatory pathway may be abnormally enhanced in the ASM of people with asthma.

In conclusion, in this study we have demonstrated that multiple signalling pathways are involved in CXCR3 ligand production. In particular we have shown that while JAK-STAT does regulate CXCR3 ligand production in ASMC from people with or without asthma, JAK-STAT activation is reduced in asthmatic cells, whereas NFκB activation is increased. Based on this and a previous study (2), JNK is an important regulator of CXCR3 ligand production and contributes to the same pathway as JAK-STAT, even though its activation is also reduced in asthmatic cells. In addition, cytokine-stimulated $[Ca^{2+}]_i$ signalling events also regulate CXCR3 ligand production. Thus enhanced NFκB activation and altered $[Ca^{2+}]_i$ homeostasis in the ASM may contribute to more rapid/enhanced CXCR3 ligand production and facilitate mast/T cell recruitment to the ASM in asthma.

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Figure captions

Figure 1: Effects of the pan JAK inhibitor pyridone-6 (P6) on cytomix-induced CXCL10 (A) and CXCL11 (A) mRNA and (B) protein synthesis. ASMC were stimulated with cytomix and CXCL10/11 mRNA and protein were measured after 3 and 24 hr using real time PCR and ELISA respectively. (C) Representative blot from 3 experiments and grouped densitometric data of cytomix-induced STAT1 phosphorylation at Y701 detected after 5-30 minutes using Western blotting. Results are expressed as means ± SEM of the relative fold change from cytomix levels for mRNA, % of cytomix+vehicle (0.1% v v^{-1} DMSO) levels for CXCL10/11 protein and phospho-STAT1 levels normalized to total-STAT1 levels expressed as change from t=0 for Western blot studies. Significant effects were determined by one-way ANOVA with Bonferroni post hoc test (*$p < 0.05$, ** $p<0.01$, *** $p<0.001$ cf cytomix for (A) and (B) or t=0 for (C)) or with student t-test (# $p<0.05$ nonasthmatic vs asthmatic).

Figure 2: Effects of JAK-2 and/or JNK inhibition on cytomix-induced CXCL10 (left) and CXCL11 (right) production and STAT1 phosphorylation. ASMC were stimulated with cytomix and the effects of the JAK-2 inhibitor AG490 on nonasthmatic and asthmatic ASMC (A) CXCL10 and CXCL11 mRNA measured using real time PCR production after 3hr and (B) protein release after 24 hr, in the presence or absence of the JNK inhibitor SP600125 (22.7 μM), measured using ELISA. (C) Representative Western blots from 3 experiments investigating the effects of SP600125 on phospho(S727)-STAT1 levels determined over 60 min following cytomix addition. Results are expressed as mean ± SEM of the relative fold change from cytomix levels for mRNA, % of cytomix+vehicle (0.1% v v^{-1} DMSO) levels for CXCL10/11 protein release and phospho-STAT1 levels normalized to total-STAT1 levels expressed as change from t=0 for Western blot studies. Significant effects were determined by one-way ANOVA with Bonferroni post hoc test (*$p < 0.05$, ** $p<0.01$, *** $p<0.001$ cf cytomix for (A) and (B)

Figure 3: STAT1 activation in asthmatic and nonasthmatic ASMC and the effects of intracellular calcium. ASMC were stimulated with cytomix for 30 min (A) in the absence or (B) presence of either the intracellular calcium chelator BAPTA-AM (50 μM), SERCA

pump inhibitor thapsigargin (1 μM), or vehicle (0.1% v v^{-1} DMSO) (A) and phospho(Y701)-STAT1 levels determined using Western blotting. (C) ASMC were loaded with Fluo-4 AM and stimulated for 45 seconds with cytomix, alone or with histamine (500 nM), in the presence of either BAPTA-AM, thapsigargin or vehicle (0.1% v v^{-1} DMSO). Cytosolic calcium was detected using plate-based fluorescence imaging. Each trace (representative of 3 experiments) shows the change in calcium fluorescence over time (F_t) normalised to the initial baseline fluorescence at t=0 s (F_0). Results are expressed as mean ± SEM of the phospho-STAT1 levels normalized to total-STAT1 levels and expressed as change from t=0. Significant differences were determined by one-way or two-way ANOVA with Bonferroni post-hoc test. *p<0.05, compared with cytomix.

Figure 4: Effects of intracellular calcium on JNK phosphorylation. Serum-deprived ASMC were stimulated with cytomix in the presence or absence of BAPTA-AM (50 μM), thapsigargin (5 μM) or vehicle (0.1% v v^{-1} DMSO) for 0-30 minutes. The phospho-proteins of interest were measured using Western blot. Effects of BAPTA-AM and thapsigargin on cytomix-stimulated p54 and p46 JNK phosphorylation in nonasthmatic (left) and asthmatic (right) ASMC. Representative blots are shown in (A) above each graph (B). Each graph shows the grouped densitometric data normalised to total JNK and expressed as the mean ± SEM fold change from unstimulated levels at t=0. Significant effects were determined by one-way ANOVA with Bonferroni post-hoc test. *p<0.05, **p<0.01, compared with unstimulated.

Figure 5: NFκB activation in asthmatic and nonasthmatic ASMC and the effects of intracellular calcium. Serum-deprived ASMC from nonasthmatic and asthmatic donors were stimulated with cytomix for 30 min in the (A) absence or (B-C) presence of BAPTA-AM (50 μM), thapsigargin (1 μM) or vehicle (0.1% v v^{-1} DMSO). Phosphorylation of P65 NFκB and IκBα degradation were measured using Western blot. Effects of BAPTA-AM and thapsigargin on cytomix-stimulated (B) p65 NFκB phosphorylation and (C) IκBα degradation are shown. Each graph is the grouped densitometric data from asthmatic ASMC normalised to total (B) p65 NFκB or (C) α-tubulin and expressed as the mean ± SEM fold change from t=0. Representative blots for nonasthmatic and asthmatic ASMC

are shown on the right of each graph. Significant effects were determined by one-way or two-way ANOVA with Bonferroni post-hoc test. *$p<0.05$, **$p<0.01$, compared with t=0.

Figure 6: Effects of intracellular calcium on CXCR3 ligand production by asthmatic and nonasthmatic ASMC. ASMC were stimulated with cytomix in the absence or presence of either BAPTA-AM (50 μM), thapsigargin (1 μM), the CaMK II inhibitor KN93 (5 μM) or vehicle (0.1% v v^{-1} DMSO). CXCL10/11 (A) mRNA and (B-C) protein levels were measured after 3 and 24 hr using real time PCR and ELISA respectively. Each bar represents the mean ± SEM CXCL10/11 levels expressed as (A) fold change from cytomix stimulated levels or (B-C) percentage of cytomix+vehicle control. Significant effects were determined by one-way ANOVA with Bonferroni post-hoc test. *$p<0.05$, compared with cytomix.