ORIGINAL ARTICLE AIRWAY DISEASES

# Human lung mast cells modulate the functions of airway smooth muscle cells in asthma

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airway smooth muscle; chemokines; extracellular matrix; mast cells; proliferation.

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## Abstract

**Background:** Activated mast cell densities are increased on the airway smooth muscle in asthma where they may modulate muscle functions and thus contribute to airway inflammation, remodelling and airflow obstruction.
**Objectives:** To determine the effects of human lung mast cells on the secretory and proliferative functions of airway smooth muscle cells from donors with and without asthma.
**Methods:** Freshly isolated human lung mast cells were stimulated with IgE/anti-IgE. Culture supernatants were collected after 2 and 24 h and the mast cells lysed. The supernatants/lysates were added to serum-deprived, subconfluent airway smooth muscle cells for up to 48 h. Released chemokines and extracellular matrix were measured by ELISA, proliferation was quantified by [$^3$H]-thymidine incorporation and cell counting, and intracellular signalling by phospho-arrays.
**Results:** Mast cell 2-h supernatants reduced CCL11 and increased CXCL8 and fibronectin production from both asthmatic and nonasthmatic muscle cells. Leupeptin reversed these effects. Mast cell 24-h supernatants and lysates reduced CCL11 release from both muscle cell types but increased CXCL8 release by nonasthmatic cells. The 24-h supernatants also reduced asthmatic, but not nonasthmatic, muscle cell DNA synthesis and asthmatic cell numbers over 5 days through inhibiting extracellular signal-regulated kinase (ERK) and phosphatidylinositol (PI3)-kinase pathways. However, prostaglandins, thromboxanes, IL-4 and IL-13 were not involved in reducing the proliferation.
**Conclusions:** Mast cell proteases and newly synthesized products differentially modulated the secretory and proliferative functions of airway smooth muscle cells from donors with and without asthma. Thus, mast cells may modulate their own recruitment and airway smooth muscle functions locally in asthma.

Asthma is characterized by airway inflammation and remodelling which contribute to airway hyperresponsiveness and episodic airflow obstruction. The airway walls of patients with asthma are thickened with increases in muscle mass, extracellular matrix (ECM) and blood vessel area, which may facilitate inflammatory cell recruitment. These changes lead to markedly reduced airway calibre and more severe bronchoconstriction (1–3). Whether these changes, which are not easily reversed by current asthma therapies (4), result from inflammatory-mesenchymal cell interactions or whether in asthma there are abnormalities in mesenchymal cells such as the airway smooth muscle (ASM) is not clear (5).

There are fundamental abnormalities in ASM cells from people with asthma, when compared with ASM cells from controls, which may contribute to the airway wall remodelling. Cultured asthmatic ASM cells proliferate faster because of an altered pattern of matrix protein deposition (6, 7), increased mitochondrial biogenesis (8) and lack the

**Abbreviations**
AP-1, activator protein 1; ASM, airway smooth muscle; C/EBP, CCAAT/enhancer binding protein; CPM, counts per minute; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; MC, mast cells; NF-$_\kappa$B, nuclear factor- kappa B; PI3-K, phosphatidylinositol 3-kinase; $PGE_2$, prostaglandin $E_2$; SN, supernatant(s).

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full-length antiproliferative transcription factor C/EBPα (9). With a strong proliferative stimulus, they depend on PI 3-kinase rather than extracellular signal-regulated kinase (ERK) signalling, whereas both pathways are activated in nonasthmatic ASM cells (10). In addition, the ASM layer is thicker (11), with increased deposition of collagens I, III and V and fibronectin and decreased collagen IV and elastin (7, 12, 13). Furthermore, asthmatic cells secrete increased amounts of the mast cell (MC) chemoattractant CXCL10 (14) and release less $PGE_2$ (15).

Mast cells are present in different compartments of the airways in people with asthma (16). They are the predominant inflammatory cells present in ASM bundles in asthma, but not eosinophilic bronchitis or in healthy controls, which makes MC localization on the ASM cell a specific feature of the asthmatic phenotype (16). In addition, MC degranulation in the ASM correlates with hyperresponsiveness (17). Degranulated MC numbers are further increased in allergic compared with nonallergic asthma (17, 18) and correlate with severity, being highest in the ASM layer in fatal asthma (18).

Mast cells are a potent source of preformed mediators, as well as many cytokines, chemokines and other newly synthesized lipid mediators. They differ from other inflammatory cells in the lung by releasing large amounts of the proteases tryptase and, depending on location, chymase (16, 19). It is well known that numerous MC-derived mediators, when used individually, directly affect ASM function [reviewed in (20, 21)]. However, some MC mediators, when studied in isolation on ASM function, have opposing effects to other mediators that are released at the same time. For example, β-tryptase induces ASM cell proliferation (22), while chymase dramatically reduces it (23). However, proteases degrade ASM pericellular matrix, resulting in increased release of fibronectin (23) which enhances ASM proliferation (7, 24).

The relative balance of MC products in the vicinity of ASM cells will determine their overall effect on the ASM cells. Thus, the aims of this study were to determine the effects of granule-derived and newly synthesized mediators released by human lung MC on the proliferative and secretory functions of ASM cells from people with and without asthma.

## Materials and methods

Approval for the use of human lung was provided by the Human Ethics Committees of Leicestershire (UK), Sydney South West Area Health Service and the University of Sydney (Australia). All donors provided written informed consent.

Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (Sydney, Australia).

### ASM cell culture

Airway smooth muscle was obtained from donors with ($n$ = 22, mean age [range] 39 [18–72] years) and without ($n$ = 25, 56 [22–80] years) asthma recruited in Sydney. ASM was dissected and cultured from bronchi as described previously (6, 25). For all experiments, cells from passages 4–7 were plated into either 6- or 96-well plates at a density of $1 \times 10^4$cells/cm$^2$ in growth medium containing 10% heat-inactivated FBS for 24 h and then serum-deprived for 72 h prior to the addition of MC supernatants (SN) or lysates. Table 1 outlines relevant information of all participants who donated lung samples or bronchial biopsies from which ASM cells were studied.

### Human lung MC isolation and activation

Human lung MC were isolated from resected macroscopically healthy lung tissue ($n$ = 10 from Midlands Lung Tissue Consortium, UK and $n$ = 12 from Sydney, Australia) as previously described (26, 27). The lung tissue was chopped, washed and then digested with collagenase and hyaluronidase for 90 min at 37°C. MC present in the digest were positively selected using anti-CD117 (Bioscientific, Sydney, Australia)-coated immunomagnetic Dynabeads (Dynal, Oslo, Norway). The selected MC were counted and finally resuspended in DMEM + 10% heat-inactivated FBS (HyClone, Logan, UT, USA), 100 units/ml penicillin G, 100 μg/ml streptomycin sulphate, 25 μg/ml amphotericin B (Gibco, Sydney, Australia), 4 mM L-glutamine and buffered with 20 mM HEPES (pH 7.4) at a density of $1 \times 10^6$cells/ml. They were immediately stimulated with IgE (2.5 μg/ml) and anti-IgE (1 μg/ml). Culture medium supernatant (SN) was collected after 2 h of stimulation, and the cells were resuspended in fresh culture medium which was then collected after 24 h of stimulation. Cells were then lysed using sonication and the lysates and SN stored at −80°C prior to simultaneously testing their effects on ASM cells from an asthmatic and a nonasthmatic.

### ECM and chemokine production

Quadruplicate well cultures of serum-deprived ASM cells from asthmatic and nonasthmatic donors were treated with MC SN or lysates at 0, 2.5, 5, 10, 20, 40% v/v in DMEM + 10% FBS. After 48 h exposure, ASM SN were collected and stored at −20°C for later measurement of chemokine release. ECM free of cells was prepared by treating the cell layer in each well with sterile $NH_4OH$ (0.016 N) for 30 min as previously described (28).

To determine whether MC proteases were involved in modulating chemokines or ECM production by the ASM cells, MC SN and lysates were pretreated with leupeptin (50 μM), or two protease inhibitor cocktails (Set III, Cat. No. 539134; EMD, Darmstadt, Germany; Complete, Cat. No. 11 836 170 001; Roche, Mannheim, Germany), or heat inactivated at 56°C for 30 min and then added to the ASM cells in culture.

CXCL10 (IP-10), CXCL8 (IL-8) and CCL11 (eotaxin) released by the ASM and MC SN were quantified using Duo-set ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The detection limits of these assays were 30, 30 and 15 pg/ml, respectively. Deposited fibronectin and collagen IV were detected using *in situ* ELISAs and biotinylated antibodies to the matrix proteins as described previously (29).

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**Table 1** Airway smooth muscle cell donor's characteristics

| Donor no. | Diagnosis | Surgical procedure | Sex | Age (y) |
|---|---|---|---|---|
| 1 | Asthma | Bronchoscopy | M | 33 |
| 2 | Asthma | Bronchoscopy | F | 18 |
| 3 | Asthma | Bronchoscopy | M | 22 |
| 4 | Asthma | Bronchoscopy | M | 20 |
| 5 | Asthma | Bronchoscopy | M | 30 |
| 6 | Asthma | Bronchoscopy | F | 50 |
| 7 | Asthma | Bronchoscopy | F | 56 |
| 8 | Asthma | Bronchoscopy | M | 60 |
| 9 | Asthma | Bronchoscopy | M | 27 |
| 10 | Asthma | Bronchoscopy | M | 21 |
| 11 | Asthma | Bronchoscopy | M | 38 |
| 12 | Asthma | Bronchoscopy | M | 50 |
| 13 | Asthma | Bronchoscopy | M | 42 |
| 14 | Asthma | Bronchoscopy | M | 30 |
| 15 | Asthma | Bronchoscopy | M | 72 |
| 16 | Asthma | Bronchoscopy | F | 31 |
| 17 | Asthma | Bronchoscopy | F | 54 |
| 18 | Asthma + Pulmonary hypertension | Transplantation | M | 33 |
| 19 | Asthma | Bronchoscopy | M | 62 |
| 20 | Asthma | Bronchoscopy | F | 21 |
| 21 | Asthma | Bronchoscopy | M | 55 |
| 22 | Asthma | Bronchoscopy | F | 40 |
| 21 | Carcinoma | Resection | F | 73 |
| 22 | CCHD | Transplantation | M | 48 |
| 23 | COPD | Transplantation | M | 56 |
| 24 | Carcinoma | Resection | F | 80 |
| 25 | Healthy | Bronchoscopy | M | 43 |
| 26 | Pulmonary hypertension | Transplantation | F | 22 |
| 27 | Carcinoma | Resection | M | 74 |
| 28 | Emphysema | Transplantation | M | 59 |
| 29 | Carcinoma | Resection | F | 74 |
| 30 | Carcinoma | Resection | M | 55 |
| 31 | Carcinoma | Resection | M | 69 |
| 32 | Pulmonary fibrosis | Transplantation | F | 49 |
| 33 | Pulmonary fibrosis | Transplantation | M | 61 |
| 34 | Pulmonary fibrosis | Transplantation | F | 47 |
| 35 | Hypersensitivity pneumonitis | Transplantation | M | 59 |
| 36 | Carcinoma | Resection | M | 53 |
| 37 | Emphysema | Transplantation | F | 56 |
| 38 | Pulmonary fibrosis | Transplantation | M | 56 |
| 39 | Pulmonary hypertension | Transplantation | F | 36 |
| 40 | Sarcoidosis | Transplantation | M | 54 |
| 41 | Pulmonary fibrosis | Transplantation | F | 55 |
| 42 | Carcinoma | Resection | F | 46 |
| 43 | Carcinoma | Resection | F | 56 |
| 44 | Emphysema | Transplantation | F | 64 |
| 45 | Emphysema | Transplantation | F | 58 |

M, Male; F, Female; CCHD, complex congenital heart disease; COPD, chronic obstructive pulmonary disease.

### ASM proliferation

*DNA synthesis and cell number*

Serum-deprived ASM cells from asthmatic and nonasthmatic donors were left untreated or treated with IL-4 (15 ng/ml), IL-4 receptor (IL-4 R) antibody (1 μg/ml) or $IgG_{2A}$ isotype control (1 μg/ml) (all from R&D Systems), the $EP_{1,2,3}$ and $DP_1$ receptor antagonist AH6809 (10 μM) (30, 31) or the $EP_4$ and $TP_1$ AH23848 (30 μM; Cayman, San Diego, CA, USA) (31) in DMEM supplemented with 10% FBS in triplicate or quadruplicate well cultures for 30 min before the addition of MC SN or lysates as described above. After 24 h, DNA synthesis was quantified by [$^3$H]-thymidine (1 μ Ci/well; PerkinElmer, Wellesley, MA, USA) incorporation over the following 5 h. Asthmatic ASM cells were harvested after 3 and 5 days exposure to MC 24-h SN and viable cells (trypan blue negative) counted on a haemocytometer using a light microscope.

*Intracellular signalling leading to proliferation*

To analyse the activation states of mitogen activated protein kinase (MAPKs) and other serine/threonine kinases involved in proliferation, serum-deprived ASM cells from asthmatic donors were treated with 40% v/v MC 24-h SN in DMEM + 10%FBS or left untreated. Total cell lysates were collected after 15 and 120 min and stored at −20°C for later analysis by protein arrays using the human phospho-MAPK array kits (Proteome Profiler™; R&D Systems) according to the manufacturer's instructions. Levels of proteins were visualized by chemiluminescence and within a linear range of exposure quantified by Quantity One® software (Bio-Rad, Hercules, CA, USA). Protein levels on each array were standardized against an internal positive control.

### MC prostaglandin $E_2$ ($PGE_2$) production

In the MC SN and lysates used, $PGE_2$ was quantified using an enzyme immuno-assay (Cayman), according to the manufacturer's instructions. The limit of detection of the assay was 15 pg/ml.

### Data analysis

Data from replicate treatments for each experiment were averaged and then expressed as a percentage of the control. The mean ± SEM was then calculated for the asthmatic and nonasthmatic cell lines. Statistical analyses were performed on all data using Statview®, and significance ($P \leq 0.05$) was determined by 1-way or 2-way analysis of variance (ANOVA) followed by Fishers *post hoc* test.

## Results

### Effects of MC on ASM cell chemokine production

The effects of MC SN and lysates on chemokine production by ASM cells from donors with and without asthma varied. ASM cell CXCL10 production was not induced by 10%FBS

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alone or when combined with MC SN or lysates (data not shown). In contrast, CCL11 release was induced from asthmatic (81 ± 25 pg/ml) and nonasthmatic (356 ± 200 pg/ml) ASM cells. Culture of ASM cells with 40% MC 2-h SN significantly reduced detectable CCL11 release by asthmatic and nonasthmatic ASM cells to 60 ± 13% and 40 ± 18% of control respectively (Fig. 1A). The 24-h SN at 20 and 40% markedly reduced detectable CCL11 released from asthmatic cells to 37 ± 15% and 52 ± 17% of control and nonasthmatic cells to 46 ± 27% and 36 ± 25% of control, respectively (Fig. 1B). In addition, 40% lysates caused a similar reduction to 53 ± 17% (asthmatic) and 32 ± 13% (nonasthmatic) of control, respectively (Fig. 1C). ASM CCL11 release was not affected by IgE/anti-IgE at the level present in the MC SN/lysates, and no CCL11 was detected in the SN/lysates used (data not shown).

The effects of the MC SN/lysates on CXCL8 release by asthmatic and nonasthmatic ASM differed markedly. Asthmatic cell CXCL8 release following 10% FBS stimulation was greater (750 ± 414 pg/ml) than nonasthmatic cell CXCL8 release (292 ± 11 pg/ml). The 40% MC 2-h SN significantly increased CXCL8 release by both asthmatic and nonasthmatic cells up to 170 ± 49% and 178 ± 36% respectively (Fig. 2A). CXCL8 production by nonasthmatic cells

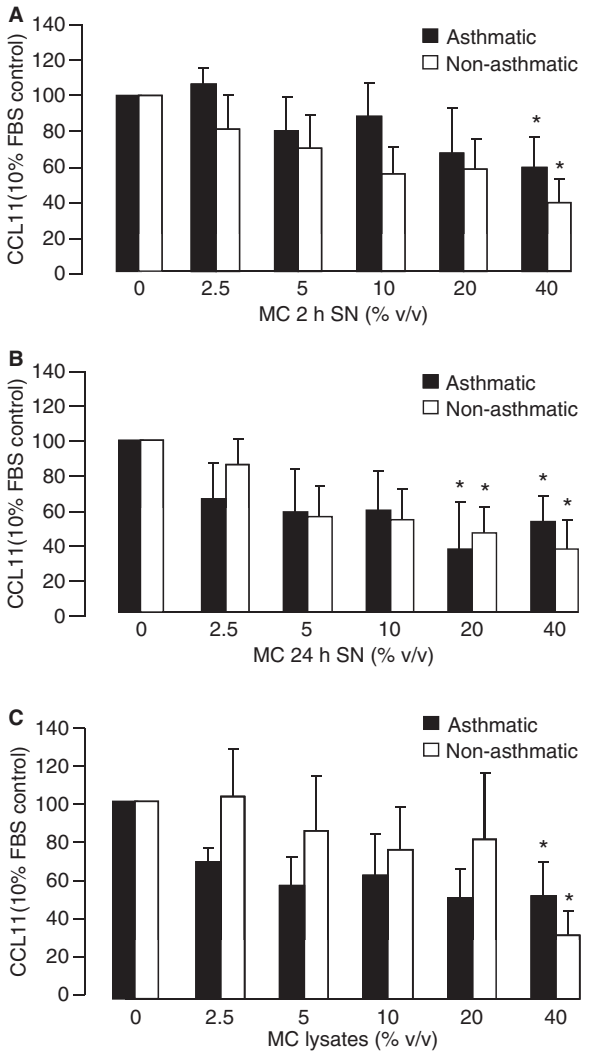


**Figure 1** The effects of activated human lung MC SN and lysates on CCL11 release. CCL11 release by serum-deprived asthmatic ($n = 6$) and nonasthmatic ($n = 6$) Airway smooth muscle cells following 48-h incubation with different percentages of the MC 2-h SN (A), MC 24-h SN (B) or MC lysates (C) in DMEM+10%FBS. Bars, means ± SEM; $^{*}P < 0.05$ *vs* relevant 10% FBS control (0% SN/lysates), 2-way ANOVA.

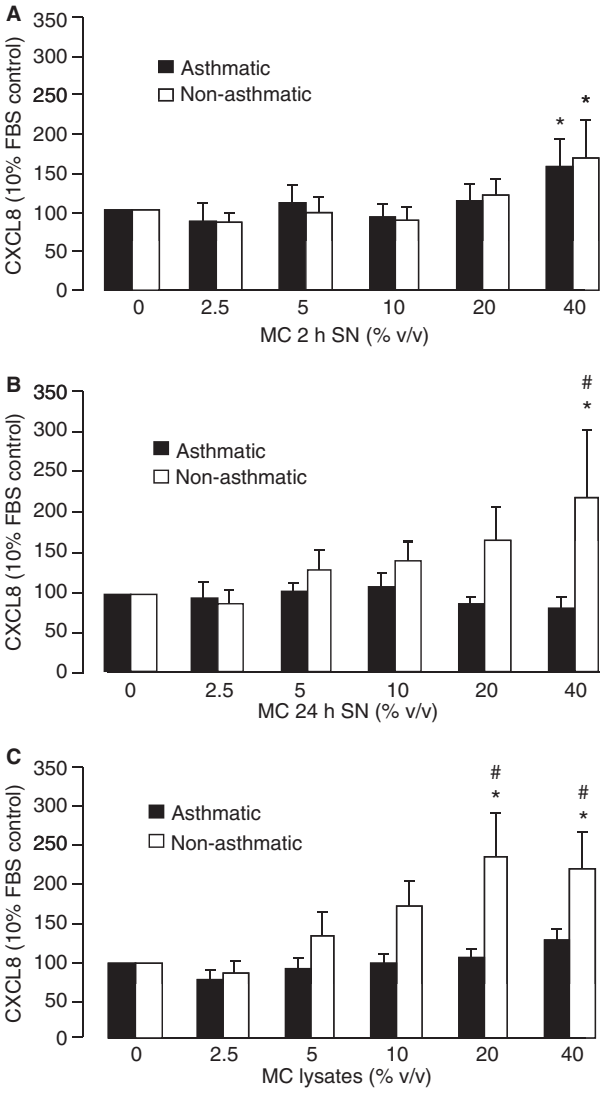


**Figure 2** The effects of activated human lung MC SN and lysates on CXCL8 release. CXCL8 release by serum-deprived asthmatic ($n = 6$) and nonasthmatic ($n = 6$) airway smooth muscle (ASM) cells following 48-h incubation with different percentages of the MC 2-h SN (A), MC 24-h SN (B) or MC lysates (C) in DMEM+10%FBS. Bars, means ± SEM; $^{*}P < 0.05$ *vs* relevant 10% FBS control (0%SN/lysates), 2-way ANOVA; $^{\#}P < 0.05$ asthmatic versus nonasthmatic ASM cells, 2-way ANOVA.

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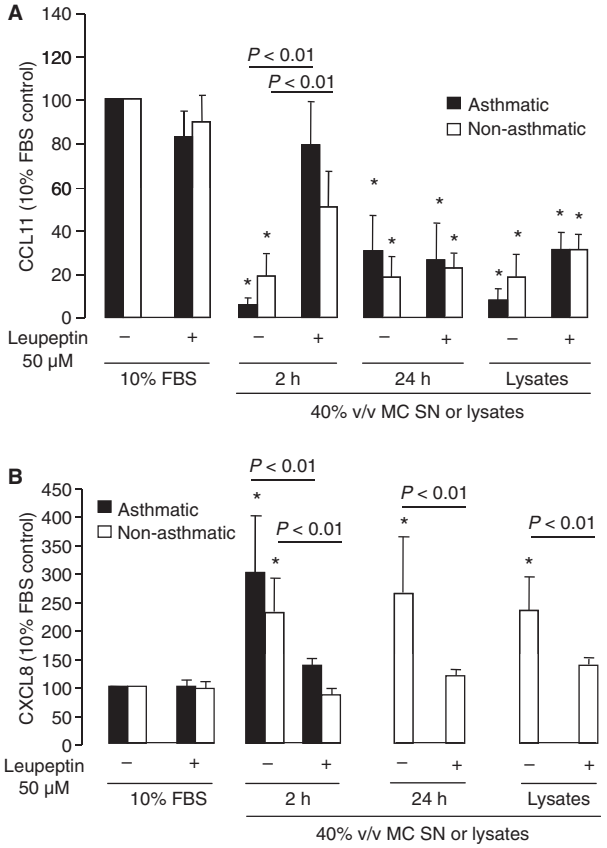


**Figure 3** The effects of the protease inhibitor leupeptin on human lung MC induced changes in airway smooth muscle (ASM) cell chemokine levels. (A) CCL11 and (B) CXCL8 release by serum-deprived asthmatic ($n = 3$) and nonasthmatic ($n = 3$–4) ASM cells following 48-h treatment with 40% v/v of MC SN/lysates untreated or pretreated with leupeptin (50 μM). Bars, mean ± SEM; $^*P < 0.05$ *vs* relevant 10% FBS control (0% SN/lysates), 1-way ANOVA.

only was also markedly increased in the presence of MC 24-h SN and lysates (Fig. 2B, C). CXCL8 release was not affected by IgE/anti-IgE at the level present in the MC SN/lysates, and no CXCL8 was detected in the SN/lysates used (data not shown).

Where the changes in asthmatic or nonasthmatic ASM cell CCL11 or CXCL8 release had been detected, whether or not those changes were as a result of MC proteolytic activity was then determined and found to differ. MC SN and lysates were pretreated with the serine protease inhibitor leupeptin. Interestingly, leupeptin pretreatment prevented the reduction in detectable CCL11 levels caused by the MC 2-h SN, but not the reduction caused by the 24-h SN or lysates in both asthmatic and nonasthmatic cells (Fig. 3A). Furthermore, pretreatment of the 24-h SN and lysates with either of two broad spectrum protease inhibitor cocktails, or heat inactivation at 56°C, did not prevent their inhibition of CCL11 release by either asthmatic ($n = 1$) or nonasthmatic ($n = 1$) ASM cells. In contrast, leupeptin pretreatment not only reversed the increases MC 2-h SN caused in CXCL8 release by both ASM cell types, but also reversed the increases caused by 24-h SN and lysates in CXCL8 release by nonasthmatic ASM cells (Fig. 3B).

### Effects of MC products on ASM cell ECM deposition

The effects of the MC SN and lysates on fibronectin and collagen IV deposition by asthmatic and nonasthmatic ASM cells differed. There were no significant effects of activated MC SN or lysates on asthmatic and nonasthmatic ASM cell collagen IV deposition (data not shown).

Fibronectin deposition, however, was affected. Deposition by asthmatic ASM cells was increased significantly to 143 ± 16% control by the MC 2-h SN. Although the nonasthmatic ASM cell fibronectin deposition appeared to follow a similar pattern, no statistically significant increase was observed (Fig. 4A, B). The MC 24-h SN or lysates had no effect on fibronectin deposition by either cell type (data not shown). Leupeptin pretreatment of the MC 2-h SN prevented the increase in fibronectin deposition by the asthmatic ASM cells (Fig. 4B). Fibronectin deposition was not affected by IgE/anti-IgE at the level present in the MC SN, and no fibronectin was detected in the SN used (data not shown).

### Effects of MC products on ASM cell proliferation

The effects of activated MC products on asthmatic and nonasthmatic ASM cell proliferation were different. DNA synthesis following 10% FBS stimulation was 4609.7 ± 1209 counts per minute (CPM) by asthmatic and 3104.3 ± 523.2 CPM by nonasthmatic ASM cells. MC 2-h SN or lysates had no significant effects on DNA synthesis by either asthmatic or nonasthmatic ASM cells (data not shown). Surprisingly, the MC 24-h SN inhibited asthmatic, but not nonasthmatic, cell DNA synthesis in a concentration-related manner. It was significantly reduced to 62 ± 13% of control by the 24-h SN at 40% v/v (Fig. 5A). DNA synthesis was not affected by the IgE/anti-IgE at the level present in the MC SN/lysates (data not shown). Asthmatic ASM cell numbers were also significantly reduced after 3 and 5 days of treatment with MC 24-h SN (Fig. 5B).

As $PGE_2$ inhibits ASM proliferation, $PGE_2$ levels were measured in MC SN and lysates. In MC SN, $PGE_2$ was below detection levels, while in MC lysates it was 97 ± 18 pg/ml (data not shown). As $PGE_2$ was unlikely to be involved, but other prostanoids or thromboxanes might be, asthmatic ASM cells were pretreated with the $EP_{1,2,3}$ and $DP_1$ receptor antagonist AH6809 (10 μM) or the $EP_4$ and $TP_1$ receptor antagonist AH23848 (30 μM). However, these antagonists did not prevent the MC 24-h SN inhibiting DNA synthesis in the asthmatic ASM cells (Fig. 5C).

IL-4 also inhibits ASM cell proliferation, so the effect of an IL-4 Rα chain blocking antibody on MC 24-h SN inhibition of asthmatic ASM cell DNA synthesis was investigated. Asthmatic ASM cell treatment with MC 24-h SN, IL-4 or IL-13 significantly reduced DNA synthesis to 48 ± 8%, 63 ± 10% and 68 ± 8% of control, respectively. Blocking

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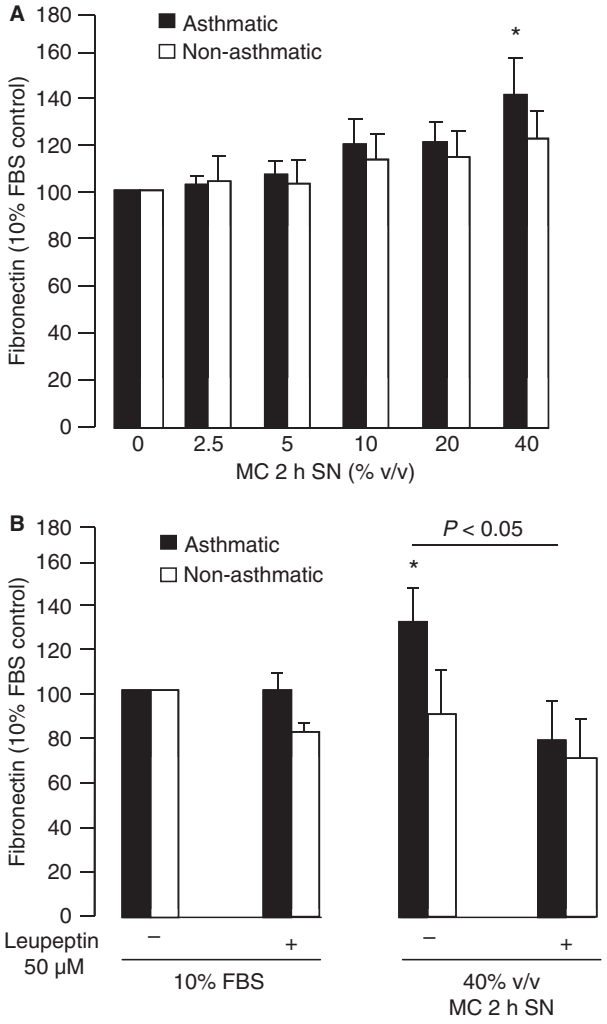


**Figure 4** The effects of activated human lung MC SN on fibronectin deposition. Fibronectin deposition by serum-deprived asthmatic ($n$ = 6) and nonasthmatic ($n$ = 6) airway smooth muscle (ASM) cells following 48-h incubation with different percentages of the MC 2-h SN (A) or asthmatic ($n$ = 3) and nonasthmatic ($n$ = 3) ASM cells incubated with 40% v/v of MC 2-h SN which had been pretreated with leupeptin (50 μM) or left untreated (B). Bars, mean ± SEM; *$P$ < 0.05 *vs* relevant 10% FBS control (0% SN), 1-way ANOVA.

IL-4 Rα did not block the inhibitory effect of the MC SN, but did block the inhibitory effects of IL-4 and IL-13 (Fig. 5D).

### Effects of MC products on asthmatic ASM cell signalling

As the MC 24-h SN reduced asthmatic ASM cell proliferation, the two signalling pathways which lead to ASM cell proliferation were investigated. The effects of MC 24-h SN on the ERK and PI3-kinase pathways were assessed using phospho-arrays. Several MAPK proteins were activated after 15 and 120 min in asthmatic ASM cells treated with 40% v/v MC 24-h SN or the 10% FBS control (Fig. 6A). Although the MC SN did not affect the early MAPK phosphorylation at 15 min (Fig. 6B), they did significantly reduce the sustained phosphorylation of ERK1, ERK2, Akt1-3 and Akt pan after 120 min (Fig. 6C).

## Discussion

This is the first study to examine the overall effects of MC products released at different times after activation on asthmatic and nonasthmatic ASM cell function. MC products and lysates reduced CCL11 release by asthmatic and nonasthmatic ASM cells, but only the decrease caused by MC products released early was because of serine protease activity. In contrast, early MC protease secretion increased CXCL8 release and fibronectin deposition by asthmatic ASM cells and later protease activity increased nonasthmatic ASM CXCL8 release. Unexpectedly, we found that asthmatic, but not nonasthmatic, ASM cell DNA synthesis and proliferation was reduced by exposure to newly synthesized MC products. The MC products reduced proliferation by preventing sustained ERK and PI3-kinase activation in the asthmatic ASM cells. However, prostaglandins, thromboxanes, IL-4 or IL-13 were not involved in these antiproliferative effects.

In this study, we examined the effects of MC mediators released together over different time periods after IgE receptor activation on ASM function in the absence of direct cell–cell contact. Different percentages of the MC SN/lysates were used to reflect what occurs in asthma, where MC infiltrate the ASM layer in different numbers and are activated (16, 18). They have partially or largely empty secretory cytoplasmic granules and their size is reduced compared to submucosal MC and compared to MC infiltrating the ASM in healthy controls (11). In addition, in asthma ASM cells and MC are in close proximity, but not all ASM cells are directly in contact with a MC (11, 16). However, it is apparent from our study that MC products can still affect ASM function in the absence of cell–cell contact.

Products released from MC within 2 or 24 h of IgE/anti-IgE activation were tested for their effects on ASM chemokine release, ECM deposition and proliferation in this study. In case mediators with different effects remained in the MC 24 h postactivation, cell lysates were also prepared and tested. Not unexpectedly, the effects of the 2- and 24-h SN on ASM function often differed from each other, whereas the lysates generally had similar effects to the 24-h SN. Thus, it is unlikely that further MC mediator release after that period would have lead to substantially different effects on the ASM cell secretory and proliferative functions examined.

Firstly, in this study the overall effect of MC products on the ASM chemokine production was examined. No ASM cell CXCL10 release was detected in the presence or absence of 10% FBS, or the MC SN and lysates. This was not surprising, as CXCL10 production is not constitutively produced but rather, is induced by IFNγ and other Th1 cytokines (14, 32). In contrast, CCL11 and CXCL8 were detected, but the levels of their release were found to be quite variable. This is

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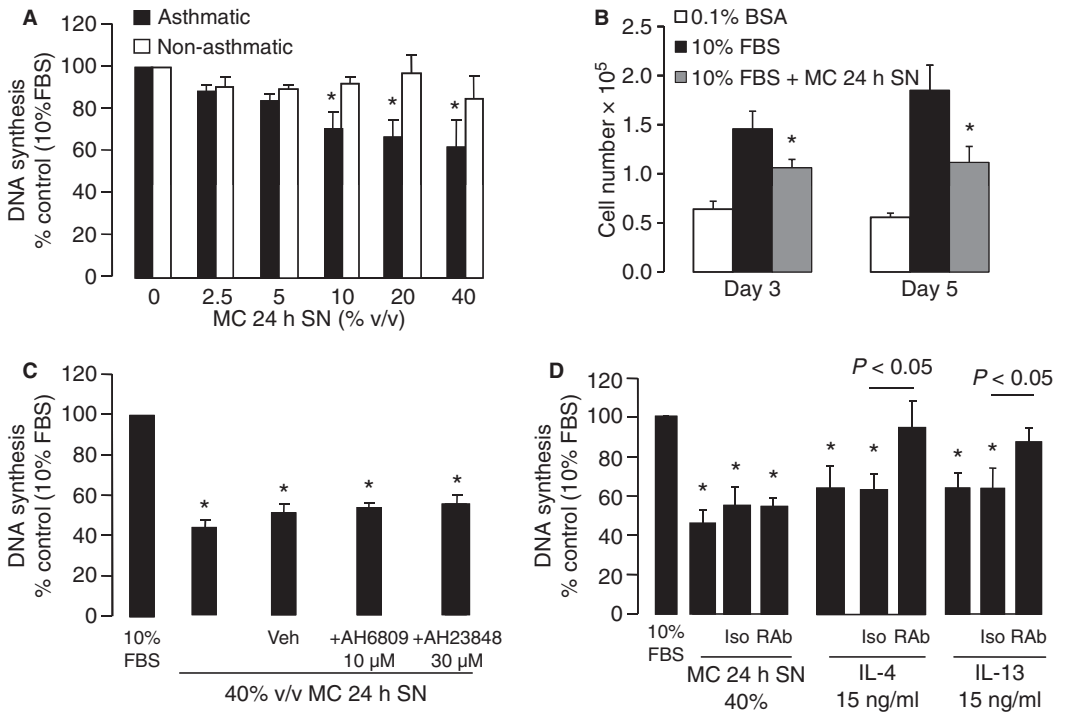


**Figure 5** The effects of activated human lung MC SN on airway smooth muscle (ASM) DNA synthesis. (A) DNA synthesis over a 5-h period in serum-deprived asthmatic ($n = 8$) and nonasthmatic ($n = 8$) ASM cells following 24-h treatment with different percentages of the MC 24-h SN in DMEM+10%FBS. (B) Asthmatic ASM cell numbers at day 3 and day 5 following treatment with 40% v/v of MC 24-h SN. Asthmatic ASM cell DNA synthesis in the presence of (C) 40% v/v MC 24-h SN following ASM cell pretreatment with AH6809 (10 μM) or AH23849 (30 μM) ($n = 6$) or (D) 40% v/v MC 24-h SN, IL-4 or IL-13 following ASM cell incubation with the anti-IL-4 Rα antibody (R Ab) or its isotype control (Iso) ($n = 5$). Bars represent mean ± SEM; $^*P \leq 0.05$ *vs* relevant 10% FBS control (0% SN), 1-way ANOVA.

perhaps not surprising as we used cells derived from lung samples from different donors, not immortalized ASM cell or MC lines. CCL11 and CXCL8 not only induce MC chemotaxis (27), but also induce the migration of other inflammatory cells such as eosinophils and neutrophils that form part of local airway inflammatory responses. Exogenous MC proteases β-tryptase and chymase each selectively cleave ASM-derived CCL11 and thus reduce its chemotactic activity for eosinophils (33). Furthermore, in ASM cell-MC co-culture β-tryptase degrades ASM-derived CCL11, but leaves CCL11 mRNA expression unaffected (34). Our findings extend these previous studies. In addition to confirming that ASM cell CCL11 levels are reduced by the early release of serine protease(s) from activated MC, we have provided the first evidence that newly synthesized MC products may inhibit ASM cell synthesis of CCL11. As neither a cocktail that inhibited aminopeptidases, as well as serine, cysteine and aspartic proteases nor heat inactivation prevented the MC 24-h SN or lysates from reducing ASM cell CCL11 levels, it is unlikely that the inhibition is because of MC enzymatic activity. Our findings are consistent with the MC having prolonged inhibitory effects on local CCL11 levels in the ASM layer. Decreased levels of active CCL11 may in part underlie the relative lack of eosinophils in the ASM layer in asthma.

The increased release of CXCL8 by both asthmatic and nonasthmatic ASM cells following exposure to MC protease activity could be important for the recruitment of inflammatory cells to the ASM. Mullan et al.(35) previously demonstrated that β-tryptase induces CXCL8 release by ASM cells via a PAR-2-independent pathway which is mediated by the transcription factors C/EBP, AP-1 and NF-$_K$B. However, asthmatic ASM cells lack the expression of full-length C/EBPα transcription factor (9) and CXCL8 release was increased by MC protease activity in asthmatic ASM cells. Thus, it would be interesting in future studies to determine whether MC protease activity induces CXCL8 production by them via NF- $_K$B and/or AP-1 and if the increase specifically in nonasthmatic ASM cell CXCL8 release induced by MC newly synthesized proteases is mediated through the activation of C/EBPα.

Fibronectin is abundant within the ASM and has previously been shown to be increased in the airway wall in fatal asthma (12). It enhances ASM proliferation (36), migration (37) and cell survival (38). *In vitro*, asthmatic ASM cells produce more fibronectin (28) and MC chymase increases its release from ASM cells (23). Our findings are consistent with the overall activity of human lung MC granule-derived proteases increasing ASM cell fibronectin deposition, but the

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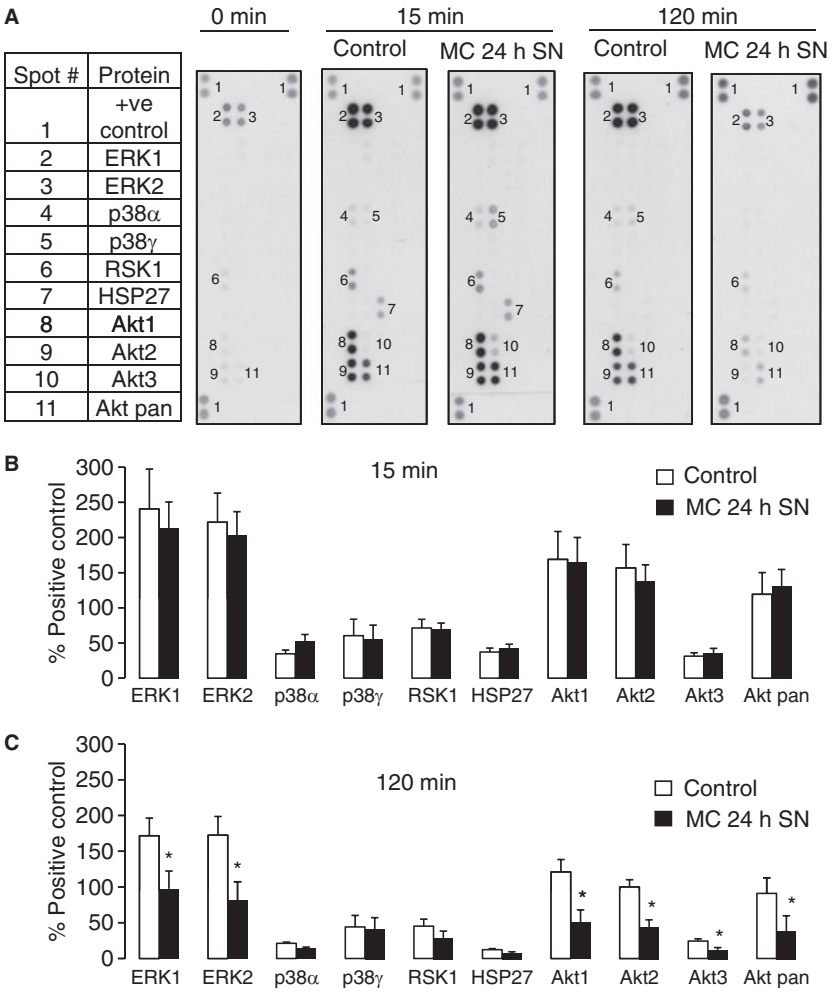


**Figure 6** The effects of activated human lung MC 24-h SN on asthmatic airway smooth muscle (ASM) cell signalling pathways leading to proliferation. Phosphorylation of signalling proteins in serum-deprived asthmatic ASM cells at 15 and 120 min after the addition of MC 24-h SN or 10% FBS (control) is shown in representative protein arrays (A). After densitometric analyses of the arrays, the data were expressed as % array internal positive control and are shown for 15 min (B) and 120 min (C). Bars represent mean ± SEM from ($n$ = 4) asthmatic ASM cells; *$P < 0.05$ *vs* relevant 10% FBS control, 1-way ANOVA.

mechanisms underlying this are still to be determined. This fibronectin-rich matrix might facilitate MC adhesion, migration and survival in the ASM as it has been shown to do *in vitro* (39–41). ASM from subjects with asthma secrete significantly more collagen I and less collagen IV than control cells *in vitro* (28). However, in this study there was no effect of MC products or lysates on asthmatic collagen I and IV deposition, which matches *in vivo* observations that collagen content within the ASM is similar in fatal asthma, nonfatal asthma and controls (12).

The effect of ASM resident MC on ASM proliferation is an area of active investigation. We have demonstrated that human lung MC had no effect on asthmatic and nonasthmatic ASM cell number when direct contact between the two cell types was possible in a 2D culture set up (42). In contrast, Ceresa et al. (43) has reported nonasthmatic ASM cell proliferation, measured using the proliferation marker Ki67, is reduced in 3D collagen gels compared with 2D culture but the addition of HMC-1 (human MC-1 cell line) to the gels partially restores proliferation, whereas HMC-1-conditioned medium does not. Our study has extended these findings by exploring the overall effects of granule-derived and newly synthesized products from human lung MC on asthmatic and nonasthmatic ASM cell proliferation. We measured DNA synthesis in ASM cells using [$^3$H]-thymidine incorporation, a well-established marker of proliferation (6, 44) and confirmed the key finding with cell counts. Interestingly, while the MC granule-derived products had no effect, the newly synthesized products inhibited the proliferation of asthmatic ASM cells, but not nonasthmatic cells.

Furthermore, the MC newly synthesized products did this by reducing the sustained upregulation of the principal sig-

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nalling pathways involved in ASM cell proliferation, ERK and PI3-kinase. However, the inhibition was not because of the antiproliferative actions of $PGE_2$ (25), IL-4 (44) or IL-13, so further investigations are needed to identify the MC mediator(s) involved. Importantly, our findings are consistent with *in vivo* observations. Whether the increase in ASM mass in the asthmatic airway wall is attributed to hyperproliferative ASM cells has been investigated by assessing Ki67 or proliferating cell nuclear antigen expression (45). Currently there is little evidence of actively proliferating ASM cells *in vivo* (46) and a number of other mechanisms have been proposed to explain the increased ASM mass. These include the following: ASM migration (47, 48), myofibroblast (49) or fibrocyte migration and differentiation into ASM cells (50) and increased deposition of ECM proteins (11, 12). Whether or not in asthma MC attract and promote the differentiation of myofibroblasts/fibrocytes into ASM cells, rather than promote ASM proliferation themselves, is not known.

An important limitation of this study is that our observations of altered ASM cell function in the presence of MC products were made *in vitro* using primary ASM cells in culture and MC isolated from human lung. It is widely accepted that the phenotype of ASM cells is altered by culturing, but we have previously demonstrated that ASM cell synthetic and proliferative activities similar to those measured in this study occur in overlapping cell populations (51), and ASM cells from people with asthma retain intrinsic differences in culture [reviewed in (52)]. Furthermore, to minimize any culture artefact, we were very particular and used MC SN and lysates from the same donor simultaneously on asthmatic and nonasthmatic ASM cells which were at the same passage in the initial experiments to determine whether there were differences in their responses to the MC. However, MC are heterogeneous and their contents vary between tissue sites in asthma (16). For this study, MC were isolated from human lung parenchyma, not bronchi, and none of the parenchyma came from people with asthma, so the effects of asthmatic bronchial MC might well be different. Nonetheless, it is notable that our observations in asthmatic ASM cells of decreased proliferation and CCL11 release alongside increased fibronectin deposition are consistent with asthma airway pathology. Although there is increased smooth muscle bulk in the airways, as discussed above, there is little evidence of active proliferation (45, 53, 54) unless they were in contact with T lymphocytes in severe asthma (55). However, ECM deposition, particularly fibronectin, is increased within the muscle bundles (28). In addition, eosinophils are rarely found in the muscle layer (33), while the numbers of activated MC there are increased in asthma (16, 17).

In conclusion, the airways in people with asthma have evidence of chronic inflammation and remodelling and activated MC numbers are increased in the ASM. MC mediators released from granules soon after activation, or newly synthesized and then released later, differentially modulated proliferation, chemokine and ECM secretion by ASM cells from donors with and without asthma. Significantly, the secretory and proliferative responses of the asthmatic and nonasthmatic ASM cells to these MC mediators were not always the same, providing further evidence that intrinsic changes in the asthmatic ASM cells determine their functional responses. We have also shown that granule-derived mediators and newly synthesized products from MC differentially modulate CXCL8 and CCL11 production consistent with MC being key regulators of local inflammation in the airways. In addition, MC granule-derived products increased asthmatic ASM cell deposition of the ECM protein fibronectin, which is increased in the ASM in asthma. Thus, our findings strongly support the hypothesis that MC modulate their own and other inflammatory cell recruitment to the smooth muscle and ASM cell functions locally. Inhibiting their proteolytic activity particularly might offer a novel approach for asthma therapy.

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## Conflict of interest

The authors have no conflict of interest to declare.

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