

ORIGINAL ARTICLE Basic Mechanisms

CXCL1 is a negative regulator of mast cell chemotaxis to airway smooth muscle cell products *in vitro*

H. Alkhouri¹, L. M. Moir^{2,3}, C. L. Armour^{1,*} and J. M. Hughes^{1,3}

Respiratory Research Group, ¹Faculty of Pharmacy and ²Discipline of Pharmacology, The University of Sydney, and ³Woolcock Institute of Medical Research, Glebe; New South Wales, Australia

Clinical & Experimental Allergy

Summary

Background Activated mast cells (MC) numbers on airway smooth muscle (ASM) are increased in eosinophilic asthma. *In vitro*, asthmatic cytokine-stimulated ASM cell-conditioned medium (CM) induces more MC chemotaxis than CM from nonasthmatic ASM cells. Intriguingly the nonasthmatic ASM CM inhibits MC chemotaxis to the asthmatic ASM CM. However, the inhibitory factor(s) in the nonasthmatic ASM CM is still to be identified.

Objective To identify the factor(s) released by nonasthmatic ASM cells that inhibits MC chemotaxis.

Methods Confluent, serum-starved ASM cells from donors with and without asthma were stimulated with IL-1 β and T-helper (Th)1 (TNF α and IFN γ) or Th2 (IL-4, IL-13) cytokines, or left unstimulated. CM samples were collected after 24 h, and a potential inhibitory factor identified using cytokine protein arrays. Its production was assessed using ELISA and RT-PCR and inhibitory role investigated in MC chemotaxis and Ca²⁺ mobilization assays.

Results Only CXCL1 was produced in greater amounts by nonasthmatic than asthmatic ASM cells following Th1 and Th2 cytokine stimulation. CXCL1 mRNA expression was also increased. Exogenous rh-CXCL1 significantly inhibited MC intracellular Ca²⁺ mobilization and chemotaxis to either CXCL10, CXCL8 or CM collected from asthmatic ASM cells following Th1 or Th2 cytokine stimulation. Neutralizing CXCL1 in nonasthmatic ASM CM or blocking its receptor significantly promoted MC chemotaxis.

Conclusions CXCL1 was a major factor regulating MC chemotaxis *in vitro*. Its differential release by ASM cells may explain the differences observed in MC localization to the ASM of people with and without asthma.

Clinical Relevance CXCL1 inhibition of MC recruitment to the ASM may lead to new targets to limit asthma pathophysiology.

Keywords airway smooth muscle, asthma, chemotaxis, cytokines, mast cells

Submitted 16 May 2013; revised 7 October 2013; accepted 17 October 2013

Correspondence:

Hatem Alkhouri, Faculty of Pharmacy A15, University of Sydney, Glebe, NSW, 2006 Australia.

E-mail: hatem.alkhouri@sydney.edu.au

Cite this as: H. Alkhouri, L. M. Moir, C. L. Armour and J. M. Hughes, *Clinical & Experimental Allergy*, 2014 (44) 381–392.

*Current address: Discipline of Pharmacology, The University of Sydney and Woolcock Institute of Medical Research, Glebe; New South Wales, Australia

Introduction

Asthma is characterized by variable airflow obstruction, airway hyperresponsiveness (AHR) and inflammation. The latter is a multicellular process, but several independent groups [1–4] have found activated mast cells (MC) numbers are increased within the airway smooth muscle (ASM) in asthma and correlate with AHR or severity. Thus, it was hypothesized that MC–ASM cell interactions are central to the development of the disordered physiology in asthma [3]. Recently, we and others have revealed the complexity of MC–ASM cell interactions and possible functional consequences [5–7].

Mast cells localization within tissues may be controlled by many chemokines because MC express a range of chemokine receptors [8]. The chemokine receptors CCR1, CCR3, CCR4, CCR5, CCR7 and CXCR1, CXCR2, CXCR3, CXCR4 and CXCR6 have been identified on human MC [8, 9]. Chemokine receptor expression differs on MC in different tissues and is influenced by cytokines in the vicinity of the MC. Additionally, a chemokine receptor might only be expressed on a subset of cells in a tissue [10, 11]. In particular, CCR3, CXCR1, CXCR3 and CXCR4 are commonly expressed by human lung mast cells (HLMC), with CXCR3 the most abundantly expressed chemokine receptor on MC within the

ASM layer in asthma [11]. Therefore, identifying the chemokines that modulate tissue-specific MC migration is a current research priority, especially in allergic eosinophilic asthma.

Airway smooth muscle cells release a broad range of cytokines, chemokines and growth factors [12–15] and express cell adhesion molecules, co-stimulatory molecules and secrete ECM proteins that may promote inflammatory cell recruitment and adhesion to ASM cells [6, 16, 17]. Some ASM-derived chemokines important in MC recruitment under different inflammatory conditions include CXCL8 (IL-8) binding to CXCR1 [18], CXCL10 (IP-10) binding to CXCR3 [12], CXCL12 (SDF-1) binding to CXCR4 [19], CCL5 (RANTES) binding to CCR1, CCR3 and CCR5 [20] and CCL11 (eotaxin) binding to CCR3 [18]. These chemokines, in conjunction with other ASM cytokines such as SCF and TGF- β , are likely to be involved in the localization of MC to the ASM [21].

Previously we have shown that the human mast cell line (HMC-1) and HLMC migrate in higher numbers to asthmatic compared with nonasthmatic ASM cell-conditioned medium (CM) collected following ASM cell stimulation with IL-1 β and either T-helper cells 1 (Th1) or Th2 cytokines [12, 18]. We used chemokine neutralizing and chemokine receptor blocking antibodies to demonstrate that under Th1 inflammatory conditions, MC chemotaxis is driven predominantly by ASM-derived CXCL10 activating CXCR3 on the MC [12]. Whereas under Th2 inflammatory conditions, it is driven partially by ASM-derived CXCL8 and CCL11 activating CXCR1 and CCR3, respectively, on the HMC-1 and HLMC [18]. The identity of other MC chemoattractants involved under Th2 inflammatory conditions is not currently known.

Whilst it is not clear why MC chemotactic responses towards CM from asthmatic and nonasthmatic ASM cells exposed to IL-1 β and Th1 cytokines are different, asthmatic ASM cells generally produce higher levels of CXCL10 than nonasthmatic ASM cells, which might partly explain the difference. However, asthmatic and nonasthmatic ASM cells produce similar levels of the MC chemoattractants CXCL8 and CCL11 when exposed to IL-1 β and Th2 cytokines [18]. Most importantly, titration of asthmatic ASM CM with nonasthmatic ASM CM inhibited MC chemotaxis to a larger extent than titration with medium only [18], providing evidence that the nonasthmatic ASM cells produce factor(s) that can inhibit MC chemotaxis. However, the nature of the inhibitory factor(s) in the nonasthmatic ASM cell CM remains unknown.

In this study, we hypothesized that nonasthmatic ASM cells produce significantly greater quantities of a factor(s) that inhibits MC chemotaxis to ASM cells. Therefore, the aims of this study were to identify

cytokines produced in larger amounts by nonasthmatic ASM cells and when identified, to investigate the effects of these proteins of interest on MC chemotaxis induced by asthmatic ASM CM.

Materials and methods

Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (Sydney, Australia). Recombinant Human IFN γ (BD Biosciences, Sydney, Australia), IL-1 β and TNF α (R&D systems, Minneapolis, MN, USA) were reconstituted and stored as recommended by the manufacturers. Anti-human CXCL1 affinity-purified goat IgG antibody, normal goat IgG isotype control, anti-human CXCR2-purified mouse monoclonal IgG2A antibody, purified mouse monoclonal IgG2A isotype control and recombinant human CXCL1 were also purchased from R&D Systems. Anti-human CXCR2 and anti-human α -tubulin antibodies suitable for Western blotting were purchased from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively. All antibodies and their isotype controls were supplied in a lyophilized form and were solubilized using sterile PBS to an appropriate stock solution made up into multiple aliquots and stored at -20°C .

ASM cell culture

Airway smooth muscle was obtained from bronchial biopsies from donors with mild to moderate asthma (classified according to the GINA guidelines) [22] and healthy controls or resected lung tissue from donors undergoing surgery for lung cancer or lung transplantation. Donors with asthma ($n = 16$, mean age 33.4 years, range 20–69 years) had a positive bronchial challenge to mannitol [23] or methacholine [17] and symptoms in the last 12 month, whereas other donors ($n = 16$, mean age 61.1 years, range 27–80 years) 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 [6, 24].

Mast cells

The human mast cell line HMC-1 was kindly provided by Dr J. Butterfield, from the Mayo clinic (Rochester, USA). The HLMC used in this study were isolated within 24 h from resected macroscopically normal lung tissue obtained from three donors aged 49, 56 and 73 years as described previously [6] and used in experiments immediately after cell isolation.

Generation of asthmatic and nonasthmatic ASM cell CM

To generate CM or detect cytokine/chemokine release in the CM, ASM cells from donors with and without asthma were cultured in six-well plates for 7 days and then growth-arrested in serum-free RPMI with 0.1% BSA. After 48 h, the cells were left unstimulated or stimulated with IL-1 β and Th1 cytokines (TNF- α and IFN- γ) [12] or Th2 cytokines (IL-4 and IL-13) [18], or with the cytokines individually, at 10 ng/mL each. After 24 h, CM were collected and stored at -20°C, and the number of cells in each well was counted.

Cytokine screening and quantification

To identify potential MC chemotaxis inhibitory factors, CM from stimulated or unstimulated ASM cells was screened for 120 cytokines using RayBio® human cytokine antibody array G series 7 (RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. Levels of the cytokine of interest (CXCL1) were confirmed and quantified using CXCL1 Duo-set ELISA kits (R&D Systems).

Cytokine-induced CXCL1 mRNA production

Serum-deprived ASM cells from people with and without asthma were stimulated with cytokines as above. CXCL1 mRNA levels were measured following 0, 3, 6, 12 and 24 h of cytokine stimulation. Total RNA was extracted using Isolate RNA Mini kit (Bioline, Sydney, Australia). RNA was reverse transcribed using Revert-Aid First Strand cDNA Synthesis kit (Fermentas Life Sciences, Hanover, MD, USA) and performed on a PTC-200 DNA Engine (MJ Research, MA). The resulting cDNA (3 μ L) was amplified by PCR using VIC-labelled human CXCL1 and FAM-labelled 18S rRNA or FAM-labelled GAPDH TaqMan probes on an ABI PRISM 7500 (Applied Biosystems, Melbourne, Australia) 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 s, 60°C for 1 min. Chemokine mRNA levels were normalized against the two reference genes; 18S rRNA and GAPDH to analyse RT-PCR data accurately.

Mast cell chemotaxis

HMC-1 or HLMC chemotactic activity in response to ASM cell CM was assessed using sterile transwell inserts with an 8- μ m pore-size membrane (BD Falcon, Melbourne, Australia) in 24-well plates as previously described [12, 18] but with minor modifications. Briefly,

prior to the chemotaxis assay, both sides of the membranes were coated overnight at 4°C with sterile fibronectin (40 μ g/mL in PBS). The fibronectin was removed, and the inserts washed with PBS and dried. ASM cell CM or medium controls were placed into the wells of a 24-well plate (300 μ L/well) followed by the prepared inserts. MC (2×10^5 cells) in 200 μ L RPMI-1640 supplemented with 2% v/v FBS (Bovogen, Melbourne, Australia), 100 U/mL penicillin G, 0.25 μ g/mL amphotericin B, 100 μ g/mL streptomycin sulphate, 2 mM L-Glutamine and 20 mM HEPES were added to each insert. The cells were then incubated for 3 h at 37°C in a 5% CO₂ in air, humidified atmosphere. After this, any solution inside the inserts and wells was aspirated and the top side of each insert membrane was carefully washed with PBS and wiped with cotton buds to remove any remaining MC. MC that had migrated through to the underside of the insert membranes were immediately fixed, stained and dried *in situ* using DiffQuick reagents and protocol (Lab Aids, Sydney, Australia). Once the inserts were completely dried, the membranes were cut out and mounted on glass slides. The cells were counted using a light microscope in 14 consecutive fields of view ($\times 200$ magnification) across the two diameters of each filter by an observer blinded to the treatments.

Determining the effects of CXCL1 in MC chemotaxis

To determine the effects of CXCL1 on MC chemotaxis, chemotaxis assays towards ASM cell CM were conducted using freshly isolated HLMC or HMC-1 as described before [12, 18]. MC chemotaxis was also investigated after (1) adding CXCL1 (25–100 ng/mL) to the asthmatic ASM CM, CXCL10 or CXCL8 in the bottom of the transwells or (2) by neutralizing it in nonasthmatic ASM CM using anti-human CXCL1 antibody or its isotype control (1 μ g/mL).

CXCR2 expression and involvement in MC chemotaxis

To assess MC CXCR2 expression levels, MC lysates were prepared from HMC-1 and HLMC treated with IgE/anti-IgE or left untreated. Samples were subjected to SDS-PAGE and Western blotting. CXCR2 (molecular mass ~ 41 kDa) was visualized using mouse anti-human CXCR2 monoclonal antibody (Abcam) diluted 1 : 500 and normalized to α -tubulin bands visualized using mouse anti-human α -tubulin monoclonal antibody (Santa Cruz Biotechnology) diluted 1 : 5000.

CXCR2 involvement in the effects of nonasthmatic ASM CM on MC chemotaxis was also assessed. HMC-1 cells were incubated with an anti-human CXCR2 blocking antibody or its isotype control (both at 5 μ g/mL) for 1 h prior to adding them to the inserts and conducting the chemotaxis assays as described above.

Measurement of changes in HMC-1 intracellular calcium concentration

HMC-1 resuspended in Fluo-4 Direct™ Ca²⁺ assay buffer (Invitrogen, Carlsbad, CA, USA) at 2.5×10^6 cells/mL were plated in black-walled 96-well plates (50 µL/well) and incubated for 1 h at 37°C. The cells were then treated for 30 min with Fluo-4 direct supplemented with 2.5 mM probenecid (Invitrogen). Fluorescence intensity at 485 nm before and after injecting 100 µL of CM from asthmatic ASM cells stimulated with Th1 or Th2 cytokines unmodified, or containing exogenous rh-CXCL1 (100 ng/mL), was quantified using a Polarstar plate reader (BMG, Australia).

Data analysis

For cytokine profiling, the densitometry values were expressed as fold change over the array internal positive control and the values were averaged and corrected for cell number. An arbitrary cut-off value of 50 units/ 10^5 cells was used. To identify potential MC chemotaxis inhibitors produced by nonasthmatic ASM cells, a cytokine level ratio (nonasthmatic/asthmatic) was calculated. For ELISA, chemokine protein levels from duplicate treatments were averaged for each experiment and corrected for cell number (pg/ 10^5 cells). For RT-PCR studies, CXCL1 mRNA levels from the average of duplicate treatments were expressed as fold change over unstimulated. For chemotaxis assays, the number of HLMC or HMC-1 on the bottom side of the filter for each treatment in each experiment was expressed as a fold change over those for nonasthmatic CM or as percentage of cytokine-stimulated ASM CM control. To measure the changes in MC intracellular calcium concentration, the mean change in fluorescence intensity over time was calculated after subtracting the background intensity for each experiment. Statistical analyses for all studies were performed using PRISM 5.01 (GraphPad, San Diego, CA, USA). All results were expressed as the mean $\pm$ SEM, and the significance of results ($P < 0.05$) was determined by either one-way or two-way ANOVA with Bonferroni *post hoc* tests.

Results

Identification of potential MC inhibitory factors produced by nonasthmatic ASM cells

Many high and low abundance cytokines were detected in CM from unstimulated or stimulated ASM cells from donors with and without asthma. Representative cytokine antibody arrays (G series 7) are presented in Fig. 1. In all ASM CM tested, CXCL2, CXCL 3 and CXCL 8; CCL2 and CCL 5; TIMP1 and TIMP 2; osteoprotegerin;

angiogenin; IL-6; and HGF were produced constitutively at ≥ 50 arbitrary units (AU) / 10^5 cells and at similar levels by the asthmatic and nonasthmatic ASM cells (Figs 1a and d). Following stimulation with IL-1 β and Th1 or Th2 cytokines, CXCL1, CXCL 6 and CXCL 11; CCL3, CCL 4, CCL 7, CCL 8 and CCL 16; IGF-BP-6; FGF-4; IL-1 α and 5; IL-2 R α ; ICAM-1; GM-CSF and GTR; or CXCL1; CCL2, CCL 7, CCL 8 and CCL 11; IL-1 α , -5 and -10; GM-CSF; IL-6 receptor; MIF; IGF-BP-6 and GTR, respectively, were released. Only CXCL1 (Gro- α) was released in significantly greater amounts by nonasthmatic than asthmatic Th1 cytokine-stimulated ASM cells (protein spot L2, Figs 1b and e; Table 1). Although some cytokines, including CXCL1, were slightly higher in nonasthmatic than asthmatic Th2-stimulated ASM cell CM, the differences were not statistically significant (Table 1).

CXCL1 levels were also significantly greater in nonasthmatic than asthmatic ASM CM when detected using ELISA. Nonasthmatic Th1- and Th2-stimulated ASM cells released 53.2 ± 13.7 and 50.9 ± 7.4 ng/ 10^5 cells, whereas asthmatic cells released around threefold less (12.8 ± 5.1 and 17.6 ± 7.4 ng/ 10^5 cells, respectively; Fig. 1g). CXCL1 release by both unstimulated cell types was < 0.7 ng/ 10^5 cells (Fig. 1g). Individually, only IL-1 β and IL-4 induced significant levels of CXCL1. Notably, levels were higher in CM from nonasthmatic than asthmatic ASM cells following IL-1 β stimulation (Fig. 1h).

Cytokine-induced CXCL1 mRNA production

The pattern of cytokine-induced CXCL1 mRNA production by ASM cells from people with and without asthma differed. In both cell types, the combination of IL-1 β with Th1 or Th2 cytokines induced significant levels of CXCL1 mRNA levels by 3 h (Figs 2a and b). However, in the nonasthmatic ASM cells, CXCL1 mRNA levels increased sharply by 3 h and by 24 h reached 69 986-fold and 62 711-fold over basal levels with both cytokine stimulations. In contrast, the asthmatic ASM CXCL1 mRNA levels peaked at 3–6 h (677-fold and 515-fold over basal levels) and by 24 h were reduced to 311-fold and 341-fold over basal levels following Th1 and Th2 cytokine stimulation, respectively (Figs 2a and b).

Determining the effects of CXCL1 on MC chemotaxis

To determine the effects of CXCL1 on MC chemotaxis, it was tested against individual chemokines and asthmatic ASM cell CM and it was also inhibited in nonasthmatic and asthmatic ASM cell CM.

Both CXCL10 and CXCL8 at 100 ng/mL, but not 50 ng/mL, induced significant HMC-1 chemotaxis

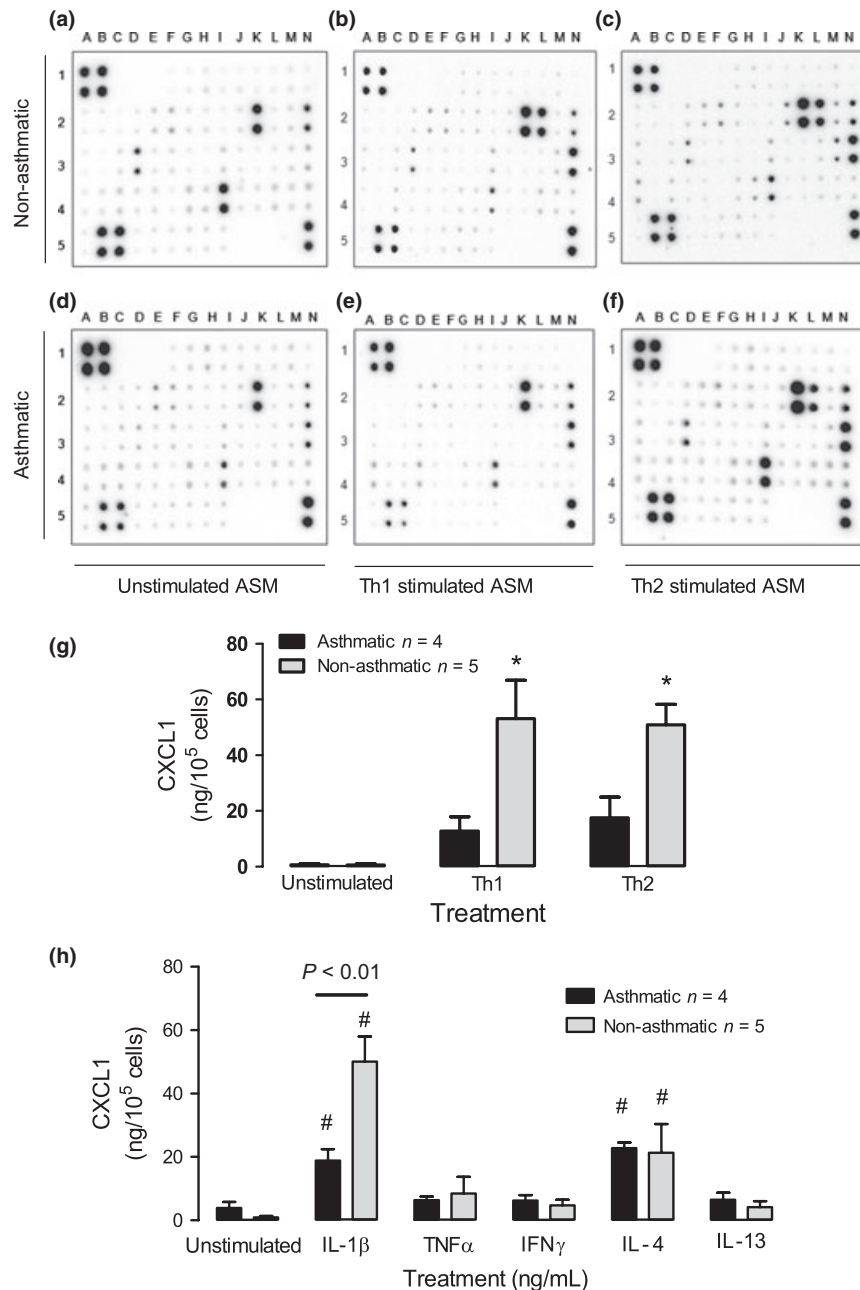


Fig. 1. Human airway smooth muscle (ASM) cell cytokine release. Cytokine arrays were exposed to conditioned medium (CM) collected from asthmatic and nonasthmatic ASM cells after 24-h stimulation with IL-1 β combined with Th1 (b and e) or Th2 (c and f) cytokines or no stimulation (a and d). CXCL1 in the CM was quantified using ELISA (g and h). A1, B1 and N5, positive control spots; L2, CXCL1. Bars, mean $\pm$ SEM; * P < 0.05; two-way ANOVA or # P < 0.05 vs. unstimulated cells, one-way ANOVA.

(9.9- and 5.8-fold over control, respectively; Figs 3a and b). Added rh-CXCL1 at 100 ng/mL, but not 50 ng/mL, significantly inhibited the HMC-1 chemotaxis induced by CXCL10 and CXCL8 (0.4- and 1.4-fold of control, respectively; Figs 3a and b).

As shown previously [12, 18], CM from asthmatic ASM cells induced more MC chemotaxis than CM from nonasthmatic cells. CM from asthmatic ASM cells stimulated with Th1 or Th2 cytokines induced 3.1-fold and

2.9-fold more HMC-1 chemotaxis, respectively, than those from nonasthmatic ASM cells (Fig. 4a). Similarly, HLMC chemotaxis was 3.1-fold and 2.7-fold higher, respectively, than towards CM from nonasthmatic cells (Fig. 4b).

Exogenous CXCL1 added to CM from asthmatic ASM cells inhibited MC chemotaxis. Notably, CXCL1 50 and 100 ng/mL inhibited HMC-1 chemotaxis when added to the CM from cells stimulated with Th1 (56 $\pm$ 18% and

Table 1. Cytokines detected at > 50 arbitrary units (AU)/ 10^5 cells and released in larger amounts in conditioned medium (CM) of nonasthmatic ($n = 4$) than asthmatic ($n = 4$) airway smooth muscle (ASM) cells stimulated with Th1 or Th2 cytokines or left unstimulated

Cytokine = 50 AU/ 10^5 cells	Nonasthmatic (NA) mean $\pm$ SEM	Asthmatic (A) mean $\pm$ SEM	Ratio NA : A
No stimulation			
None detected			
Th1 stimulation			
IL-6	880.9 $\pm$ 157.8	769.1 $\pm$ 118.7	1.15
CXCL1, 2 and 3	691.4 $\pm$ 98.7	533.6 $\pm$ 71.0	1.29
CXCL1 (GRO- α)	561.8 $\pm$ 57.6	343.1 $\pm$ 39.1	1.64*
HGF	207.2 $\pm$ 45.3	192.1 $\pm$ 63.4	1.08
Angiogenin	163.4 $\pm$ 74.1	151.3 $\pm$ 33.3	1.08
IGFBP-4	140.6 $\pm$ 43.9	130.7 $\pm$ 29.6	1.08
GITR	104.3 $\pm$ 34.3	88.9 $\pm$ 14.9	1.17
CCL16 (HCC-4)	85.3 $\pm$ 26.1	71.6 $\pm$ 12.7	1.19
CXCL6 (GCP-2)	64.4 $\pm$ 27.6	56.3 $\pm$ 24.7	1.14
Th2 stimulation			
IL-6	729.0 $\pm$ 139.4	612.3 $\pm$ 77.9	1.19
CCL2 (MCP-1)	611.0 $\pm$ 82.5	607.4 $\pm$ 68.2	1.01
CXCL1	490.9 $\pm$ 32.0	375.9 $\pm$ 42.8	1.31
CXCL1, 2 and 3	475.7 $\pm$ 25.8	428.4 $\pm$ 47.4	1.11
Angiogenin	229.4 $\pm$ 77.6	228.3 $\pm$ 68.1	1.01
CXCL6	152.8 $\pm$ 50.8	131.7 $\pm$ 49.1	1.17
IGFBP-4	147.3 $\pm$ 45.9	117.1 $\pm$ 42.6	1.26
CCL11 (Eotaxin)	123.7 $\pm$ 29.32	109.7 $\pm$ 34.4	1.13
IL-6 Receptor	100.4 $\pm$ 41.27	95.1 $\pm$ 18.4	1.06
MIF	74.5 $\pm$ 39.49	73.2 $\pm$ 14.6	1.02

* $P < 0.05$, two-way ANOVA.

$82 \pm 4\%$ of CM control) and Th2 ($67 \pm 4\%$ and $86 \pm 5\%$) cytokines, respectively (Figs 4c and e). Further, 25 and 50 ng/mL CXCL1 also significantly inhibited HLMC chemotaxis to the CM from cells stimulated with Th1 ($44 \pm 18\%$ and $56 \pm 17\%$) and Th2 ($27 \pm 5\%$ and $60 \pm 10\%$) cytokines, respectively (Figs 4d and f). By itself, rh-CXCL1 did not induce HMC-1 or HLMC chemotaxis (data not shown).

Inhibiting CXCL1 in the CM collected from nonasthmatic ASM cells increased MC chemotaxis. When a specific CXCL1 neutralizing antibody was added to the CM collected from nonasthmatic ASM cells stimulated with Th1 or Th2 cytokines, HMC-1 ($160 \pm 20\%$ and $240 \pm 47\%$) and HLMC ($329 \pm 53\%$ and $211 \pm 49\%$) chemotaxis were significantly increased compared to control CM, respectively (Figs 5a–d). In contrast, neutralizing CXCL1 in the CM from asthmatic ASM cells did not significantly affect MC chemotaxis (data not shown). MC chemotaxis was not affected by the isotype control.

CXCR2 expression and involvement in MC chemotaxis

As CXCL1 is a ligand for CXCR2, MC expression of CXCR2 was investigated. CXCR2 protein was detected

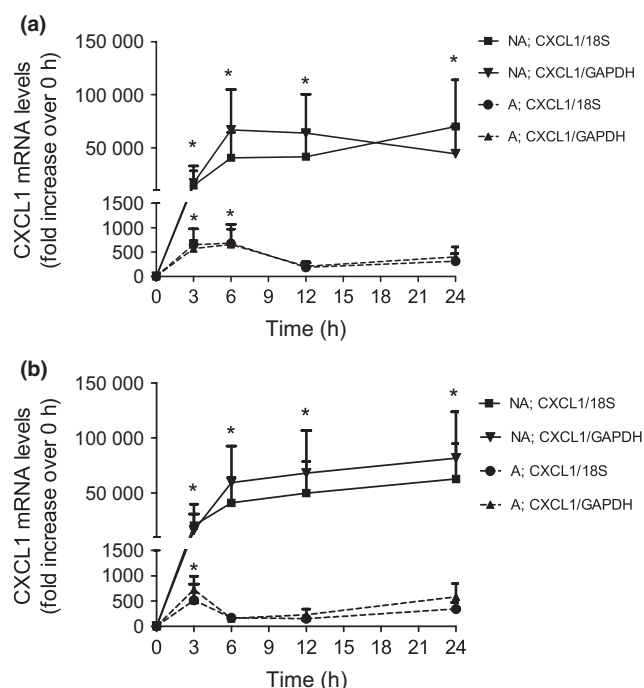


Fig. 2. Airway smooth muscle (ASM) CXCL1 production. CXCL1 mRNA production by asthmatic ($n = 3$) and nonasthmatic ($n = 4$) ASM cells after stimulation with IL-1 β with Th1 (a) or Th2 (b) cytokines for 24 h. CXCL1 mRNA levels were quantified using RT-PCR, referenced against 18S rRNA or GAPDH mRNA and expressed as fold change over 0 h. Bars, mean $\pm$ SEM; * $P < 0.05$ vs. control (0 h); one-way ANOVA.

in cell lysates from both HMC-1 cells (2.9 ± 0.4 densitometry units, $n = 3$) and HLMC (2.5 ± 0.2 densitometry units, $n = 3$) using Western blotting. The expression levels were not affected by IgE/anti-IgE treatment of the cells (Fig. 5e).

Blocking CXCR2 on MC also increased chemotaxis towards nonasthmatic ASM cell CM. In HMC-1 pretreated with a CXCR2 receptor blocking antibody, chemotaxis towards CM from nonasthmatic ASM cells stimulated with Th1 or Th2 cytokines was increased to $242 \pm 36\%$ and $277 \pm 66\%$ of control, respectively (Figs 5f and g). HMC-1 chemotaxis was not affected by the isotype control. Blocking CXCR2 did not alter HMC-1 chemotaxis towards CM collected from asthmatic ASM cells stimulated with the cytokines (data not shown).

CXCL1 effects on MC intracellular calcium induced by ASM cell CM

CXCL1 inhibited intracellular Ca^{2+} mobilization in HMC-1 induced by CM from asthmatic ASM cells. Intracellular Ca^{2+} levels peaked at about 5 s after addition of CM from asthmatic ASM cells stimulated with Th1 and Th2 cytokines (peak $15\,618 \pm 5107$ and $10\,443.5 \pm 1759$, respectively) and was sustained for at

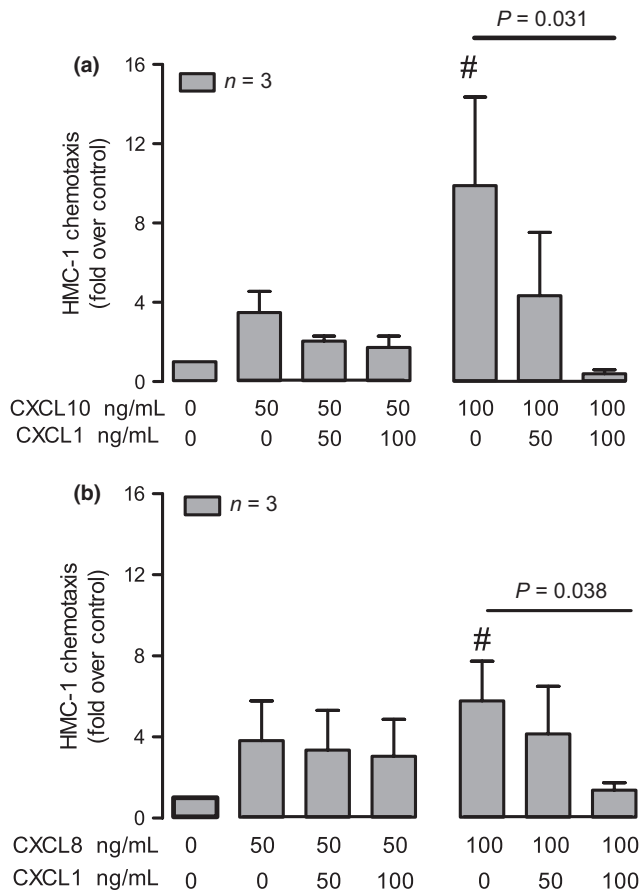


Fig. 3. CXCL1 effects on HMC-1 chemotaxis to CXCL10 and CXCL8. HMC-1 chemotaxis towards serum-free RPMI supplemented with 0.1% BSA and 50 or 100 ng/mL CXCL1 and CXCL10 (50 or 100 ng/mL) (a) or CXCL8 (50 or 100 ng/mL) (b). HMC-1 chemotaxis is expressed as % untreated medium control. Bars, mean $\pm$ SEM; # P < 0.05 vs. control, one-way ANOVA.

least 1 min (Figs 6a and b). The addition of rh-CXCL1 100 ng/mL to asthmatic ASM cell CM from ASM cells stimulated with Th1 and Th2 cytokines reduced HMC-1 intracellular Ca^{2+} mobilization to 79% and 31% of CM control, respectively (Figs 6a and b).

Discussion

Chemokines are critical in regulating airway inflammation in asthma, and their role in regulating MC localization to the ASM bundles is important in its pathophysiology. We aimed to identify factors in non-asthmatic ASM CM that might inhibit MC recruitment to the ASM. This is the first study to compare the release of a wide range of cytokines/chemokines by asthmatic and nonasthmatic ASM cells under Th1 and Th2 inflammatory conditions. Although differential release of chemokines was detected, only CXCL1 was produced in larger amounts by nonasthmatic than asthmatic ASM cells following Th1 and Th2 cytokine

stimulation. It inhibited MC chemotaxis to CXCL10 and CXCL8 and had demonstrable inhibitory effects on MC chemotaxis induced by CM from stimulated ASM cells, acting via its receptor CXCR2 on MC and reducing their intracellular Ca^{2+} mobilization. Thus, this study has provided the first evidence of how nonasthmatic ASM cells can down-regulate MC localization in the ASM.

Neutralizing CXCL1 in nonasthmatic ASM cell CM was sufficient to make these CM attract MC up to levels approaching those of the asthmatic ASM cell CM *in vitro*. In view of previous findings, it was not surprising the amount of chemotaxis was not always as much as to CM from asthmatic ASM cells. When Th1 cytokines are used to stimulate the ASM cells, the levels of the major chemokine involved, CXCL10, are generally less in CM from nonasthmatic ASM cells [12]. Although CXCL8 and CCL11 are involved in MC chemotaxis to the CM from Th2-stimulated ASM cells, they are released from asthmatic and nonasthmatic cells at similar levels, and the other chemokines involved in inducing MC chemotaxis have not been identified as yet [18].

CXCL1 has been detected in the airway epithelium of healthy controls and is increased with diesel exposure [25]. CXCL1 up to 1 ng/mL has also been detected in BAL fluid from healthy volunteers [26] and in higher levels in COPD [27]. However, we are not aware of any studies comparing CXCL1 expression in people with and without asthma, or that have examined CXCL1 expression in the ASM particularly.

In this study, the nonasthmatic ASM cells released nearly threefold greater amounts of CXCL1 than asthmatic ASM cells. Although in the experiments where we have quantified CXCL1 using ELISA, the mean age of people with asthma was 40 years and for people without asthma 58 years, and the numbers in each group are small, we do not believe age is confounding the results. ASM cells from individuals with asthma generally released less CXCL1 than cells from individuals without asthma. Release from nonasthmatic cells of all other cytokines detected using cytokine arrays was not increased. Further immune responses generally decline with age, and B-cell CXCL1 production is decreased in old compared with young mice [28].

CXCL1 production following stimulation with IL-1 β and either Th1 or Th2 cytokines resulted from a very rapid increase in CXCL1 gene transcription. Production was driven by IL-1 β in agreement with Issa and colleagues [29], who first reported that CXCL1 is released by nonasthmatic ASM cells stimulated with IL-1 β and to a lesser extent by TNF- α . However, they found no effect of the Th2 cytokines IL-4 or IL-10 on CXCL1, whereas we found IL-4 induced significant, but lower

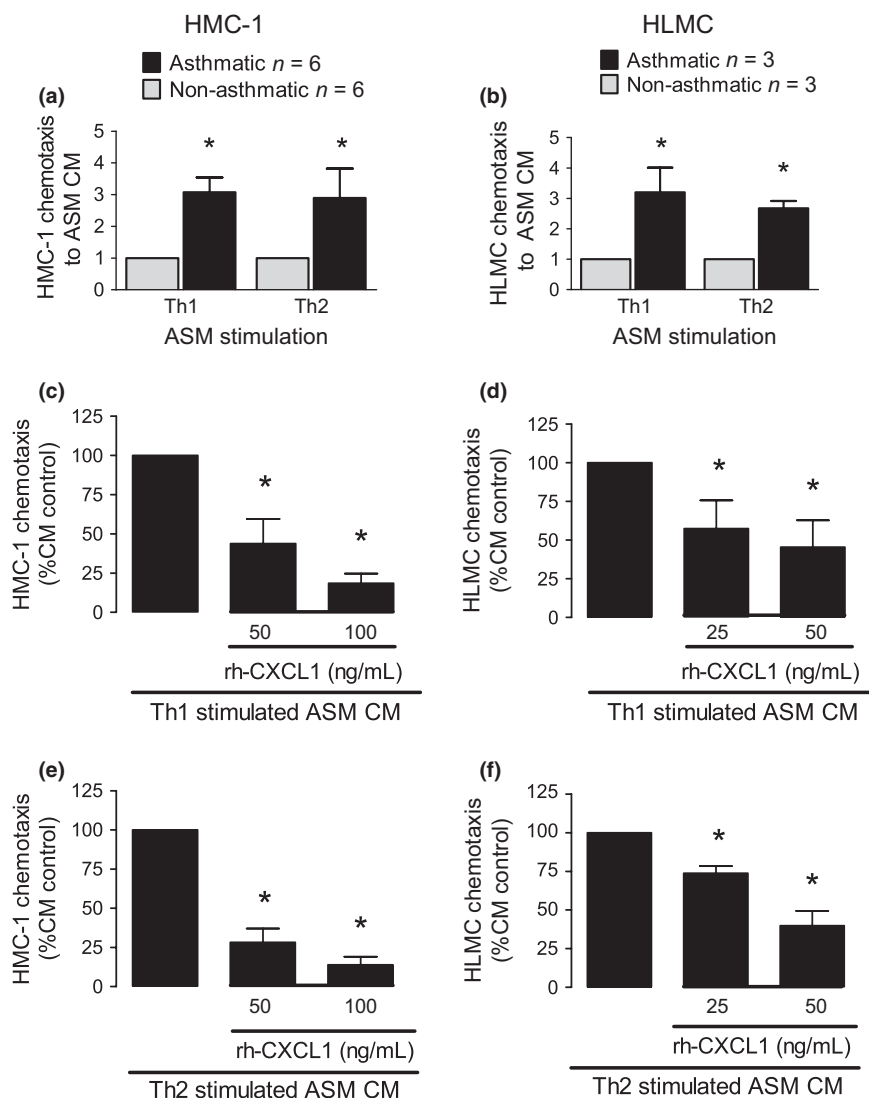


Fig. 4. CXCL1 effects on mast cells (MC) chemotaxis to airway smooth muscle (ASM) cell-conditioned medium (CM). HMC-1 (a, c and e) and human lung mast cells (HLMC) (b, d and f) chemotaxis to CM from asthmatic ASM cells stimulated with IL-1 β and Th1 or Th2 cytokines in the absence (a and b) and presence of rh-CXCL1 (c–f). MC chemotaxis is expressed as fold change over nonasthmatic ASM CM (a and b: * P < 0.05, unpaired t -test) or % CM control (* P < 0.05, one-way ANOVA). Bars, mean $\pm$ SEM.

levels of release than IL-1 β , which was similar from asthmatic and nonasthmatic cells. This may be due to slight differences in the experimental protocols used in the two studies. The confluence and/or quiescence of the ASM cells at the time of cytokine stimulation may have differed, and in our study, CXCL1 release was adjusted for cell number.

The differential release of CXCL1 between asthmatic and nonasthmatic ASM cells could be due to inherent differences in signalling pathways between asthmatic and nonasthmatic ASM cells. CXCL1 production is mediated through the activation of both ERK and JNK MAPKs [29]. It is unlikely that ERK phosphorylation is involved in the increased release of CXCL1 by nonasthmatic ASM cells, as ERK phosphorylation is greater in

asthmatic than nonasthmatic cells in the absence of strong mitogenic stimuli [30]. However, JNK phosphorylation might be involved as we have recently reported that JNK activation is greater in nonasthmatic than asthmatic ASM cells following stimulation with IL-1 β and Th1 cytokines as used in this study [31]. This differential activation of JNK is one of several abnormalities present in asthmatic ASM cells that contribute to their altered synthetic, proliferative and/or contractile responses.

The rationale for using the cytokine arrays in this study was to determine the release profiles for as many cytokines as possible for asthmatic and nonasthmatic ASM cells at baseline and when ASM cells were exposed to different inflammatory conditions. Although different

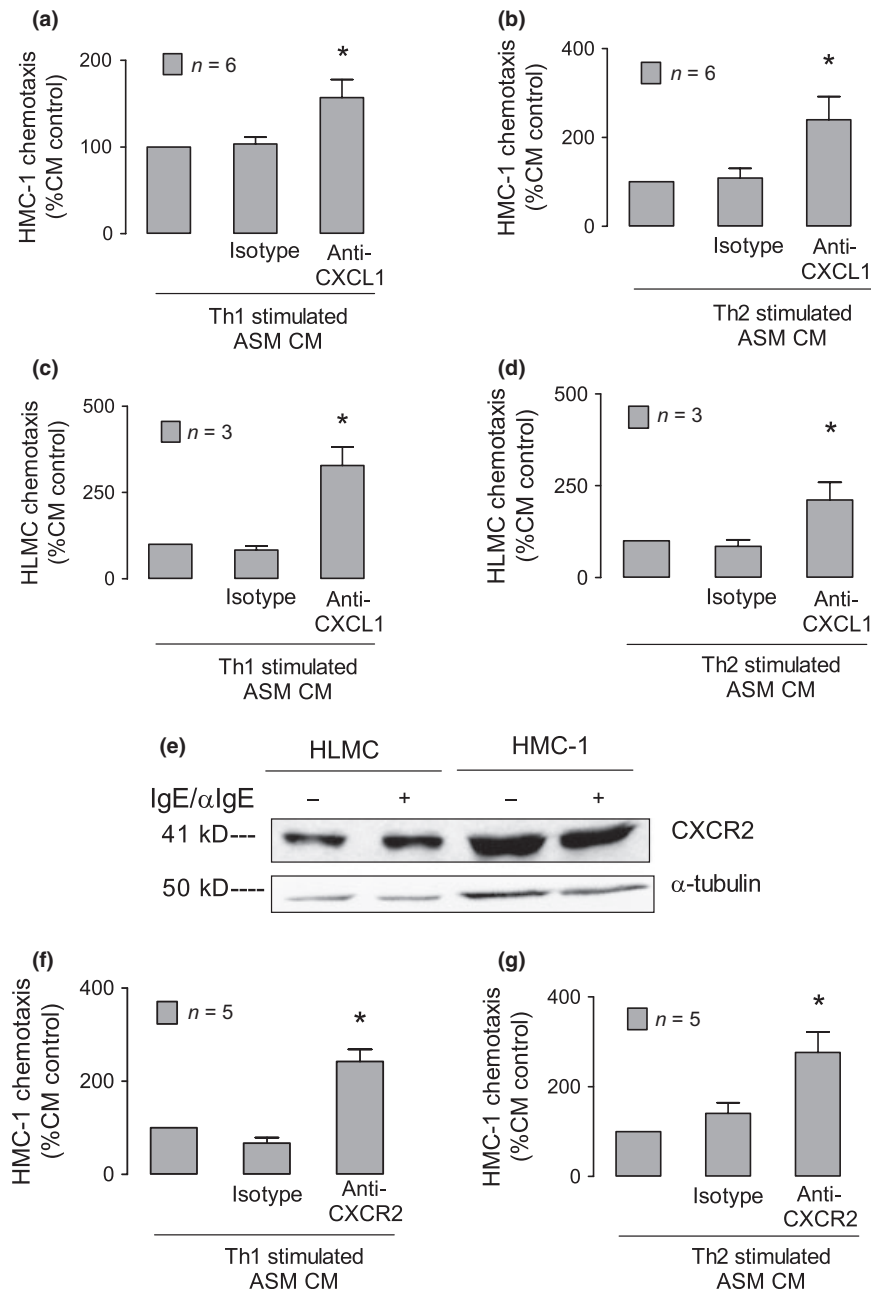


Fig. 5. Role of CXCL1 and its receptor CXCR2 in mast cells (MC) chemotaxis. HMC-1 and human lung mast cells (HLMC) chemotaxis towards conditioned medium (CM) collected from nonasthmatic airway smooth muscle (ASM) cells after stimulation with IL-1 β and Th1 (a, c and f) or Th2 (b, d and g) cytokines. The CM was treated with neutralizing anti-CXCL1 or isotype control antibodies (1 μ g/mL each) (a–d), or the HMC-1 cells were pretreated with anti-CXCR2 or isotype control antibodies (f and g). (e) A representative blot of CXCR2 expression levels in HMC-1 (*n* = 3) and HLMC (*n* = 3) treated or untreated with IgE/anti-IgE (2.5 and 1 μ g/mL, respectively). MC chemotaxis is expressed as % untreated CM control. Bars, mean $\pm$ SEM; **P* < 0.05 vs. isotype, one-way ANOVA.

groups have reported the release of some of the cytokines detected in the arrays (30–32), only nonasthmatic ASM cells were used in those studies, so no comparison between patterns of cytokine release by asthmatic and nonasthmatic ASM cells were made previously. However, it was only possible to do this for ASM cells from a small number of donors. Further studies are needed to confirm

these findings in ASM cells from a larger number of people using a rigorous method for quantifying the levels of all the different cytokines.

To investigate the role of CXCL1 in MC chemotaxis, it was tested against individual chemokines and ASM cell CM in chemotaxis assays mainly using the cell line HMC-1 and key findings were confirmed in HLMC.

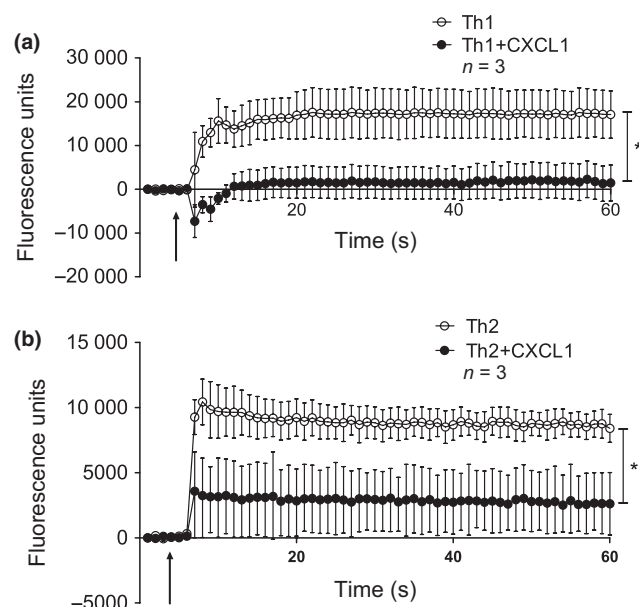


Fig. 6. CXCL1 effects on asthmatic airway smooth muscle (ASM) cell-conditioned medium (CM)-induced HMC-1 intracellular calcium flux. Fluorescence intensity of Fluo-4 loaded HMC-1 treated, in the presence or absence of exogenous rh-CXCL1 (100 ng/mL), with CM collected from asthmatic ASM cells stimulated with the combination of IL-1 β and (a) Th1 and (b) Th2 cytokines. Points, mean $\pm$ SEM; * P < 0.05, two-way ANOVA.

It was not possible to use HLMC for all the experiments as the supply of large enough pieces of human lung for successful MC isolation was limited during the course of this study; thus, we opted to test initial hypotheses using the cell line HMC-1. However, we have previously established that HMC-1 and HLMC chemotaxis in response to CM from asthmatic ASM cells stimulated in the same way to this study are comparable and involve similar chemokines and receptors [18].

CXCL1 plays a major role in inflammation by attracting neutrophils [32]. It binds to and acts exclusively through CXCR2. To the best of our knowledge, CXCL1 is not a natural antagonist of another chemokine receptor, as CXCL10 is for CCR3 [33]. This study provides novel evidence that CXCL1 acts as an endogenous inhibitor of a range of chemokines to prevent MC chemotaxis. However, CXCL1 binding to CXCR2 does inhibit structural cell migration [34]. Just how CXCL1 inhibits the activities of several chemokines that engage different receptors in this current study is likely to be complex and requires further investigation. However, Wang et al. have demonstrated that CXCL1–CXCR2 binding is dependent on the cell surface heparan sulphate and that it can compete with CXCL10 by binding to its specific heparan-sulphate-binding site [35]. This particularly relevant here because, together with our

collaborators, we have shown that HMC-1 and HLMC produce the heparan sulphate proteoglycan perlecan [36] and HLMC chemotaxis induced by asthmatic Th1-stimulated ASM cells is predominantly driven by CXCL10 and CXCR3 and provided evidence of their involvement in MC localization in the ASM in people with asthma [12]. Therefore, further studies are needed to investigate the effects of perlecan on chemokine receptor engagement on MC and their chemotactic responses.

CXCL1's inhibitory effect on HMC-1 chemotaxis was prevented by blocking CXCR2 using a specific receptor blocking antibody in this study. We detected CXCR2 protein in both HMC-1 cells and HLMC using Western blotting, but currently, there is conflicting evidence about MC CXCR2 expression. Several groups have demonstrated functional CXCR2 receptors on cultured MC or MC precursors [37–40], but CXCR2 was not detected in ASM resident MC from bronchial mucosal biopsies using immunohistochemistry [12] or on tissue resident HLMC using flow cytometry [11]. However, there is also evidence that CXCR2 is located intracellularly on specific granules [38] and thus may not have been easily detectable using those methods. In tissues, MC surface expression of certain chemokine receptors might be influenced by their degranulation/activation status and/or nearby cytokines [8].

Nilsson and co-authors reported that CXCL1 binds to MC CXCR2 and induces HMC-1 chemotaxis [39]. These results are in contrast to the findings reported here; CXCL1 at equivalent concentrations to those used in their study (50 and 100 ng/mL) did not induce HLMC or HMC-1 chemotaxis, but did significantly inhibit HLMC and HMC-1 chemotaxis. Nevertheless, both studies are in agreement that CXCL1 reduces HMC-1 calcium mobilization induced by chemokines.

Irrespective of these seeming inconsistencies in the literature, in this study, exogenous rh-CXCL1, in similar amounts to those in the nonasthmatic ASM cell CM, inhibited MC chemotaxis towards individual chemokines and CM from asthmatic ASM cells stimulated with Th1 or Th2 cytokines. Neutralizing CXCL1 in CM, or blocking its receptor on MC, promoted MC migration to nonasthmatic ASM CM up to levels approaching those induced by asthmatic ASM CM. Thus, CXCL1 may be acting indirectly via cell surface perlecan and CXCR2, or directly through CXCR2, to disrupt early MC signalling events and block MC chemotaxis towards nonasthmatic stimulated ASM cell CM.

Although the molecular mechanisms of chemokine- or antigen-induced MC migration are not completely understood, it is well established that MC chemotaxis in response to chemoattractants or IgE activation is highly dependent on Ca²⁺ mobilization [41]. This, together

with the reduced HMC-1 intracellular Ca^{2+} mobilization after rh-CXCL1 addition to CM from asthmatic ASM cells, leads us to suggest that CXCL1 inhibition of MC chemotaxis involves inhibiting chemokine-induced changes in intracellular Ca^{2+} levels.

In summary, we have shown for the first time that there is a factor produced by ASM cells which inhibits MC chemotaxis. Production of this factor, CXCL1, is reduced in asthmatic ASM cells, and this would appear to be a mechanism for increased localization of activated MC on the ASM in asthma. This increased knowledge of how MC localization to the ASM is regulated may lead to alternative strategies to reduce chronic inflammation in asthma.

Acknowledgements

We acknowledge the collaborative effort of the cardio-pulmonary transplant team at St Vincent's Hospital, SSWAHS theatre and pathology staff, Dr M Baraket and Assoc Prof. G King (Woolcock Institute of Medical Research, Sydney), Prof. I Young (Royal Prince Alfred Hospital, Sydney) and our RRG colleagues who helped with lung sample/biopsy collection. This study was funded by project grants from the NHMRC of Australia.

Conflicts of interest

The authors have no conflicts of interest to declare.

References

- Ammit AJ, Bekir SS, Johnson PR, Hughes JM, Armour CL, Black JL. Mast cell numbers are increased in the smooth muscle of human sensitized isolated bronchi. *Am J Respir Crit Care Med* 1997; 155:1123–9.
- Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-de-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma. *Thorax* 2007; 62:8–15.
- Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002; 346:1699–705.
- Carroll NG, Mutavdzic S, James AL. Distribution and degranulation of airway mast cells in normal and asthmatic subjects. *Eur Respir J* 2002; 19:879–85.
- Hollins F, Kaur D, Yang W *et al.* Human airway smooth muscle promotes human lung mast cell survival, proliferation, and constitutive activation: cooperative roles for CADM1, stem cell factor, and IL-6. *J Immunol* 2008; 181:2772–80.
- Alkhouri H, Hollins F, Moir LM, Brightling CE, Armour CL, Hughes JM. Human lung mast cells modulate the functions of airway smooth muscle cells in asthma. *Allergy* 2011; 66:1231–41.
- Kaur D, Saunders R, Berger P *et al.* Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. *Am J Respir Crit Care Med* 2006; 174:1179–88.
- Juremalm M, Nilsson G. Chemokine receptor expression by mast cells. *Chem Immunol Allergy* 2005; 87:130–44.
- Scott K, Bradding P. Human mast cell chemokines receptors: implications for mast cell tissue localization in asthma. *Clin Exp Allergy* 2005; 35:693–7.
- Romagnani P, De Paulis A, Beltrame C *et al.* Tryptase-chymase double-positive human mast cells express the eotaxin receptor CCR3 and are attracted by CCR3-binding chemokines. *Am J Pathol* 1999; 155:1195–204.
- Brightling CE, Kaur D, Berger P, Morgan AJ, Wardlaw AJ, Bradding P. Differential expression of CCR3 and CXCR3 by human lung and bone marrow-derived mast cells: implications for tissue mast cell migration. *J Leukoc Biol* 2005; 77:759–66.
- Brightling CE, Ammit AJ, Kaur D *et al.* The CXCL10/CXCR3 Axis Mediates Human Lung Mast Cell Migration to Asthmatic Airway Smooth Muscle. *Am J Respir Crit Care Med* 2005; 171:1103–8.
- Lazaar AL, Albelda SM, Pilewski JM, Brennan B, Pure E, Panettieri RA Jr. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J Exp Med* 1994; 180:807–16.
- Panettieri RA Jr, Lazaar AL, Pure E, Albelda SM. Activation of cAMP-dependent pathways in human airway smooth muscle cells inhibits TNF- α -induced ICAM-1 and VCAM-1 expression and T lymphocyte adhesion. *J Immunol* 1995; 154:2358–65.
- Pang L, Knox AJ. PGE2 release by bradykinin in human airway smooth muscle cells: involvement of cyclooxygenase-2 induction. *Am J Physiol* 1997; 273(6 Pt 1):L1132–40.
- Johnson PRA, Burgess JK, Underwood PA *et al.* Extracellular matrix proteins modulate asthmatic airway smooth muscle cell proliferation via an autocrine mechanism. *J Allergy Clin Immunol* 2004; 113:690–6.
- Ko FW, Diba C, Roth M *et al.* A comparison of airway and serum matrix metalloproteinase-9 activity among normal subjects, asthmatic patients, and patients with asthmatic mucus hypersecretion. *Chest* 2005; 127:1919–27.
- Sutcliffe A, Kaur D, Page S *et al.* Mast cell migration to Th2 stimulated airway smooth muscle from asthmatics. *Thorax* 2006; 61:657–62.
- Juremalm M, Hjertson M, Olsson N, Harvima I, Nilsson K, Nilsson G. The chemokine receptor CXCR4 is expressed within the mast cell lineage and its ligand stromal cell-derived factor-1 α acts as a mast cell chemotaxin. *Eur J Immunol* 2000; 30:3614–22.
- Juremalm M, Olsson N, Nilsson G. Selective CCL5/RANTES-induced mast cell migration through interactions with chemokine receptors CCR1 and CCR4. *Biochem Biophys Res Commun* 2002; 297:480–5.
- Berger P, Girodet PO, Begueret H *et al.* Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. *FASEB J* 2003; 17:2139–41.
- Bateman ED, Hurd SS, Barnes PJ *et al.* Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* 2008; 31:143–78.
- Baraket M, Oliver BG, Burgess JK, Lim S, King GG, Black JL. Is low dose

- inhaled corticosteroid therapy as effective for inflammation and remodeling in asthma? A randomized, parallel group study. *Respir Res* 2012; 13:11.
- 24 Johnson PR, Roth M, Tamm M *et al.* Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001; 164:474–7.
 - 25 Salvi SS, Nordenhall C, Blomberg A *et al.* Acute exposure to diesel exhaust increases IL-8 and GRO-alpha production in healthy human airways. *Am J Respir Crit Care Med* 2000; 161(2 Pt 1):550–7.
 - 26 Belperio JA, Keane MP, Burdick MD *et al.* CXCR2/CXCR2 ligand biology during lung transplant ischemia-reperfusion injury. *J Immunol* 2005; 175:6931–9.
 - 27 Traves SL, Culpitt SV, Russell RE, Barnes PJ, Donnelly LE. Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax* 2002; 57:590–5.
 - 28 Hu L, Dixit VD, de Mello-Coelho V, Taub DD. Age-associated alterations in CXCL1 chemokine expression by murine B cells. *BMC Immunol* 2004; 5:15.
 - 29 Issa R, Xie S, Lee KY *et al.* GRO-alpha regulation in airway smooth muscle by IL-1beta and TNF-alpha: role of NF-kappaB and MAP kinases. *Am J Physiol Lung Cell Mol Physiol* 2006; 291:L66–74.
 - 30 Burgess JK, Lee JH, Ge Q *et al.* Dual ERK and phosphatidylinositol 3-kinase pathways control airway smooth muscle proliferation: differences in asthma. *J Cell Physiol* 2008; 216:673–9.
 - 31 Alrashdan YA, Alkhouri H, Chen E *et al.* Asthmatic airway smooth muscle CXCL10 production: mitogen-activated protein kinase JNK involvement. *Am J Physiol Lung Cell Mol Physiol* 2012; 302:L1118–27.
 - 32 Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 2008; 118:3546–56.
 - 33 Palmqvist C, Wardlaw AJ, Bradding P. Chemokines and their receptors as potential targets for the treatment of asthma. *Br J Pharmacol* 2007; 151: 725–36.
 - 34 Tsai HH, Frost E, To V *et al.* The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell* 2002; 110:373–83.
 - 35 Wang D, Sai J, Richmond A. Cell surface heparan sulfate participates in CXCL1-induced signaling. *Biochemistry* 2003; 42:1071–7.
 - 36 Jung M, Lord MS, Cheng B *et al.* Mast cells produce novel shorter forms of perlecan that contain functional endorepellin: a role in angiogenesis and wound healing. *J Biol Chem* 2013; 288:3289–304.
 - 37 Hallgren J, Jones TG, Abonia JP *et al.* Pulmonary CXCR2 regulates VCAM-1 and antigen-induced recruitment of mast cell progenitors. *Proc Natl Acad Sci USA* 2007; 104:20478–83.
 - 38 Lippert U, Artuc M, Grutzkau A *et al.* Expression and functional activity of the IL-8 receptor type CXCR1 and CXCR2 on human mast cells. *J Immunol* 1998; 161:2600–8.
 - 39 Nilsson G, Mikovits JA, Metcalfe DD, Taub DD. Mast cell migratory response to interleukin-8 is mediated through interaction with chemokine receptor CXCR2/Interleukin-8RB. *Blood* 1999; 93:2791–7.
 - 40 Ochi H, Hirani WM, Yuan Q, Friend DS, Austen KF, Boyce JA. T helper cell type 2 cytokine-mediated comitogenic responses and CCR3 expression during differentiation of human mast cells in vitro. *J Exp Med* 1999; 190:267–80.
 - 41 Holowka D, Calloway N, Cohen R *et al.* Roles for Ca(2+) mobilization and its regulation in mast cell functions. *Front Immunol* 2012; 3:104.