

Original article

H₄ histamine receptor mediates optimal migration of mast cell precursors to CXCL12

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Background: CXCL12, a constitutive chemokine (ligand of CXCR4 and CXCR7), is expressed in the skin and airway epithelium and plays a significant role in allergic airway diseases. The pleiotropic effects of CXCL12 are enhanced by cofactors specific to the target cell.

Objective: We hypothesized that histamine, a major mediator of allergic reactions, could interact with CXCL12 to promote human mast cell (MC) migration.

Methods: The chemotactic effects of CXCL12 alone or in combination with histamine were evaluated on human precursor and mature MCs by using *in vitro* migration assays.

Results: CXCL12 exerts a chemotactic activity on both precursor and fully mature MCs. Histamine and supernatants from IgE-activated MCs enhanced CXCL12 chemotactic activity on the precursor MC population. The synergy between histamine and CXCL12 was not observed with mature MCs, CD4⁺ T cells, and monocytes. Inhibition of histamine receptors pharmacologically or with specific small interfering RNA (siRNA) indicated that synergy between histamine and CXCL12 required the H₄ receptor.

Conclusion: Histamine released by allergen-activated mature MCs might promote MC-rich allergic inflammation by enhancing recruitment of their precursors in tissues constitutively expressing CXCL12, including skin and airways.

Clinical implications: This work highlights a novel role of the H₄ receptor in the perpetuation of allergic responses and provides evidence for the utility of H₄ receptor antagonists in the treatment of allergic diseases. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Mast cell precursors, CXCL12, histamine, H₄ receptor, cell migration

Mast cells (MCs) are the only hematopoietic cells that leave the bone marrow as committed immature precursors. After a short blood stage, precursors of mast cells (PrMCs) reach vascularized tissues or serosal cavities, where they undergo the terminal stage of their differentiation, maturation, or both under the influence of microenvironmental factors.¹ Once mature, these long-living cells defend the host against intestinal helminthic parasites and gram-negative bacterial infections, and they play a pivotal role in allergic reactions, during which sensitized MCs are rapidly activated after allergen exposure through high-affinity IgE receptors (FcεRI).²

During host responses to infections or in many other settings associated with persistent inflammation, the expansion of MC populations can be achieved, as well as local changes in their tissue distribution. For instance, MCs can accumulate in the airway smooth muscle of asthmatic patients.³ The increased number of MCs can be due to either their ability to re-enter the cell cycle to proliferate or to an increase in PrMC recruitment.¹ Migration of PrMCs from blood to tissues and the migration of mature MCs from one tissue compartment to another are under the control of chemokines and adhesion molecules.⁴ Human PrMCs express functional CXCR4, CXCR2, CCR3, and CCR5,⁵ and leukotriene B₄ (LTB₄) is a potent chemoattractant for PrMCs derived *in vitro* from human cord blood progenitors.⁶ Chemotactic factors for human mature MCs have been more extensively studied. CXCL12 (stromal cell-derived factor 1), CXCL8 (IL-8), CCL11 (eotaxin), CCL5 (RANTES), and CX3CL1 (fractalkine) control mature MC migration.^{7,8}

CXCL12 is constitutively expressed in a variety of tissues, such as the skin⁹ and airway epithelium,¹⁰ and it is a powerful chemoattractant for leukocytes.¹¹ Baseline expression of CXCL12 is detectable in the absence of stimuli provided by pathogens or immune disorders, suggesting a role for this chemokine in homeostasis. However, CXCL12 and its receptor CXCR4 also play a significant role in allergic airway diseases.^{12,13} Indeed, neutralization of CXCL12 or CXCR4 reduced lung eosinophilia and airways hyperresponsiveness in mouse models of allergic airway disease.^{12,13} The pleiotropic effects of CXCL12, such as cell migration and survival, are enhanced by

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Abbreviations used

APC:	Allophycocyanin
FITC:	Fluorescein isothiocyanate
FSC:	Forward side scatter
LTB ₄ :	Leukotriene B ₄
MC:	Mast cell
PC5:	Peridinin chlorophyll protein-cyanin 5.5
PE:	Phycoerythrin
PrMC:	Mast cell precursor
SCF:	Stem cell factor
VEGFR:	Vascular endothelial growth factor

additional cofactors, depending on the cell type. For instance, stem cell factor (SCF) enhances the chemotactic activity of CXCL12 on progenitor CD34⁺ cells,¹⁴ whereas thrombopoietin and IL-10 are the cofactors involved for megakaryocyte precursors and B lymphocytes, respectively.^{15,16} Based on these findings, we attempted to analyze novel mechanisms potentially involved in MC accumulation in allergic reactions. In the present study we report the chemotactic activity of CXCL12 on both human PrMCs and mature MCs. We identify histamine as a potent cofactor of CXCL12 for this chemotactic process, highlighting its possible relevance in allergy.

METHODS

Reagents and antibodies

Recombinant human CXCL12 was a generous gift from Dr F. Baleux (Pasteur Institute, Paris, France). Histamine agonists and antagonists were obtained from Sigma-Aldrich (St Quentin-Fallavier, France). JNJ 777120 (JNJ) was kindly supplied by Johnson & Johnson Pharmaceutical Research and Development (San Diego, Calif). The following mAbs were used in this study: fluorescein isothiocyanate (FITC)-anti-CXCR4 (clone 12G5; BD Biosciences, San Diego, Calif), phycoerythrin (PE)-anti-FcεRI (Clinisciences, Montrouge, France), FITC-anti-CD14, FITC-anti-CD16, FITC-anti-CD34, PE-anti-IL-3Rα, PE-anti-CD33, PE-anti-CD123, peridinin chlorophyll protein-cyanin 5.5 (PC5)-anti-c-kit, PC5-anti-CD4, allophycocyanin (APC)-anti-CD13, APC-anti-CD14, APC-anti-CD3 (BD Biosciences), APC-anti-vascular endothelial growth factor (VEGF) receptor 2, and APC-anti-VEGF receptor 3 (R&D Systems, Lille, France). Polyclonal rabbit antibody anti-H₁ receptor, polyclonal goat antibodies anti-H₂ receptor and anti-H₄ receptor, and horseradish peroxidase-conjugated anti-rabbit or anti-goat secondary antibodies were purchased from Tebu-Bio (Le Perray-en-Yvelines, France). Mouse anti-chymase mAb was obtained from Millipore (Molsheim, France), and anti-tryptase was obtained from Chemicon International, Inc (Temecula, Calif).

MCs

The 5C6 clone of the human leukemic MC line HMC-1 (HMC1) was grown in RPMI 1640 Glutamax (Invitrogen, Cergy Pontoise, France) supplemented with 10% FCS, 100 IU/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). Highly purified cord blood-derived MCs were obtained by means of long-term culture of cord blood progenitor cells, as previously described.¹⁷ Expression of chymase and tryptase by MCs was analyzed by using immunocytochemistry, as previously described.⁵

Flow cytometry

Cells (1×10^6) were washed in PBS 1× and resuspended in 100 μL of RPMI 1640 with 10% human AB serum to block Fc receptors. After a 30-minute incubation at 4°C, specific mAbs or their corresponding isotype controls were added at saturating concentrations and incubated for a further 30 minutes in the dark. Cells were then washed in PBS 1× and fixed with PBS 1×/paraformaldehyde 2% until FACS analysis. Intracellular staining was performed with a Cytotfix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions. FACS analysis was performed on a flow cytometer (FACScan, Becton Dickinson).

Chemotaxis assays

Chemotactic responses of MCs were examined with 24-well Transwell plates (Costar, Fisher Bioblock, Illkirch, France) with 8-μm-pore-size polycarbonate filters. Two hundred microliters of cells was added to the top wells at 1.5×10^6 cells/mL in RPMI 1640 with 20 mmol/L HEPES and 10% FCS (migration buffer), and 600 μL of migration buffer containing or not containing chemoattractant factors was added to the lower wells. CXCL12 was used at 100 ng/mL, SCF at 80 ng/mL, LTB₄ at 50 ng/mL, and histamine at 10^{-6} mol/L. Absence of histaminases was verified in FCS to avoid histamine degradation in functional assays. After 3 hours at 37°C in 5% CO₂, migrated cells were harvested from lower chambers, fixed in PBS 1×/paraformaldehyde 2%, and counted by means of flow cytometry on the basis of side-angle light scatter (SSC) and forward side scatter (FSC) parameters for HMC1 cells and mature MCs. Results are expressed as the number of migrated cells after correction for basal migration. Chemotaxis of PrMCs was evaluated by using cells from 4-week progenitor MC cultures. Migrated cells were double stained with PC5-anti-c-kit and APC-anti-CD13 mAbs and counted. Results are expressed as the percentage of migrated c-kit⁺CD13⁺ PrMCs among c-kit⁺CD13⁺ cells added to the wells.

Chemotaxis of freshly isolated PBMCs was measured in a similar way to PrMCs, except that 5-μm-pore-size polycarbonate filters were used, and migrated cells were stained with APC-anti-CD3 and PC5-anti-CD4 for CD4⁺ T cells and FITC-anti-CD14 for monocytes.

Actin polymerization assay

Briefly, cells were stained, as previously described,¹⁶ before and after activation by chemoattractant factors, with an intracellular fluorescent probe (FITC phalloidin) that specifically recognizes polymerized actin. All experiments were done with CXCL12 at 100 ng/mL and histamine at 10^{-6} mol/L. Mean fluorescence was measured with a flow cytometer (FACScan, Becton Dickinson).

MC activation

Cord blood MCs (1×10^6 cells/mL) were incubated for 4 days at 37°C with 2.5 μg/mL human myeloma IgE and IL-4 (20 ng/mL). Then cells were harvested, washed in complete Iscove's Modified Dulbecco's medium, and incubated with 1 μg/mL goat anti-human IgE (Vector Laboratories, Biovalley, Marne la Vallée, France) for 30 minutes. Some cells were treated with human myeloma IgE alone to control basal secretion of mediators. MCs incubated with IgG (2.5 μg/mL) and goat anti-human IgG (1 μg/mL) served as controls for the cross-linking. The supernatants were collected by means of centrifugation and frozen at -80°C until use. Histamine levels were determined by using a specific immunoassay kit (Immunotech, Lumigny, France).

siRNA transfection

RNA interference was used to inhibit the expression of H₁, H₂, and H₄ receptors. HMC1 cells were nucleofected with small interfering RNA (siRNA) specific for H₁ receptor (400 nmol/L, Tebu-Bio), H₂

receptor (200 nmol/L, Tebu-Bio), and H₄ receptor (200 nmol/L, Tebu-Bio) or nonspecific control (random) siRNA (400 nmol/L, Tebu-Bio) by using Amaxa technology (Amaxa, Cologne, Germany), according to the manufacturer's instructions. Cells were used 48 hours after transfection in functional assays. Inhibition of histamine receptor expression was verified by means of Western blotting. The average inhibition monitored by using densitometric analysis of immunoblots was 40%.

RT-PCR

Total RNA from purified cells was extracted by using Trizol reagent (Invitrogen) and treated with DNase (Roche Diagnostics, Meylan, France) to remove any genomic DNA contamination. cDNA was prepared with Superscript III reverse transcriptase (Invitrogen) by using oligo(dT) as the primer. RT products were amplified by using the real-time PCR Lightcycler method for histamine receptors and CXCR4. PCR conditions were as follows. All samples were preheated for 15 minutes at 95°C and then subjected to denaturing conditions at 95°C for 5 seconds. After annealing, genes were amplified for 15 seconds per cycle. CXCR7 cDNA amplification was realized, as previously described.¹⁸ The primer sequences and amplification conditions are summarized in Table E1 (available in the Online Repository at www.jacionline.org). The PCR products were run on a 1.2% agarose gel with ethidium bromide, and DNA was visualized with UV light.

Immunoblotting

Cell lysates were boiled for 5 minutes in sample buffer and loaded onto 7.5% SDS-PAGE gels at volumes corresponding to equivalent amounts of proteins. After electrophoresis, resolved proteins were transferred onto nitrocellulose membranes and probed with the appropriate primary antibodies, according to the manufacturer's recommendations. After multiple washing in TBS (10 mmol/L Tris [pH 7.5], 100 mmol/L NaCl)/0.1% Tween, membranes were probed with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour. Membranes were developed by using the ECL system. The intensity of bands was quantified by using film direct scans of immunoblots.

Influence of histamine on CXCR4 expression

Cells were incubated for 30, 120, or 240 minutes at 37°C with histamine (10^{-6} mol/L) to evaluate CXCR4 distribution between cytoplasmic and surface pools. After incubation, cells were stained with anti-CXCR4 antibody (mAb 12G5), as described above.

Cells were incubated for 10 and 90 minutes at 37°C with 1 μ g/mL CXCL12 alone or with histamine (10^{-6} mol/L) to evaluate CXCR4 recycling. After incubation, cells were immediately put on ice to prevent receptor turnover and stained with anti-CXCR4 antibody, as described above.

Statistical analysis

Throughout the text, data are expressed as means $\pm$ SE. Statistical significance (*P* value) was determined by using the Wilcoxon test for grouped variables (with the Bonferroni correction for comparison of concentration-response curve data). Analysis was performed with GraphPad Prism 3.0 (GraphPad Software, San Diego, Calif). *P* values of less than .05 were considered significant.

RESULTS

Chemotactic effect of CXCL12 on PrMCs and mature MCs

MCs were cultured from human umbilical cord blood by using an established method.¹⁷

At 4 weeks, a population of c-kit⁺ cells ($43\% \pm 6\%$, $n = 5$) was observed that exhibited the characteristics of PrMCs (SSC^{low}c-kit⁺CD13⁺IL-3R α ^{low}Fc ϵ RI⁻; see Fig E1, A, in this article's Online Repository at www.jacionline.org). A population of FSC^{low}c-kit⁺CD13⁺ cells was also present ($20\% \pm 3\%$). Resting cells were SSC^{low}c-kit⁺CD13⁻. Specific markers for other cell types, including CD14 (monocytes), CD16 (neutrophils), CD133, VEGF receptor 2, and VEGF receptor 3 (precursors of endothelial cells), were not detected on the 4-week-old cells. Immunoreactivity of cells for chymase in progenitor MC cultures was confirmed (see Fig E2 in this article's Online Repository at www.jacionline.org). The following results were focused on c-kit⁺CD13⁺ PrMCs. By 8 to 9 weeks of culture, the number of positive chymase cells increased (see Fig E2 in this article's Online Repository), and a single cell population of mature MCs was obtained (SSC^{high}c-kit⁺CD13^{high}IL3R α ⁻Fc ϵ RI⁻; see Fig E1, B, in this article's Online Repository at www.jacionline.org).

The migration of human MCs toward CXCL12 was evaluated in a Transwell assay. CXCL12 was chemotactic for HMC1 cells. CXCL12 at 10, 100, and 1000 ng/mL induced the migration of 3040 ± 577 ($P = .010$), 7235 ± 1473 ($P = .003$), and 5418 ± 1182 ($P = .016$) HMC1 cells, respectively, compared with 1070 ± 284 cells for the controls ($n = 7$). PrMCs, as well as mature MCs, migrated in response to 100 ng/mL CXCL12 ($188\% \pm 26\%$ migration over control values [$P = .040$] and $283\% \pm 51\%$ migration over control values [$P = .045$], respectively [$n = 5$]).

Histamine synergized the CXCL12-driven migration of PrMCs

A 1.5- to 2-fold increase in HMC1 cell and PrMC migration was observed when histamine and CXCL12 were used in combination compared with use of CXCL12 alone (Fig 1, A and B). By contrast, histamine had no significant effect on the CXCL12-driven migration of mature MCs (data not shown).

The synergy between CXCL12 and histamine was preserved when histamine was added to both the upper and lower chambers, but it was lost when CXCL12 was also present in both chambers (Fig 1, C; $n = 5$). Therefore no histamine gradient is needed for this synergy. A histamine-enhancing effect was also observed by first incubating cells in medium containing histamine (10^{-6} mol/L for 30 minutes) and then washing the cells and testing their migration toward CXCL12 (data not shown).

Inability of histamine to modulate other MC chemoattractants

SCF induced migration of HMC1 cells ($465\% \pm 115\%$ migration over control values, $P = .007$, $n = 6$), mature MCs ($564\% \pm 177\%$ migration over control values, $P = .025$, $n = 5$), and PrMCs ($140\% \pm 2\%$ migration over control values, $P = .045$). LTB₄ was efficacious as a chemoattractant on PrMCs (Fig 1, B; $130\% \pm 6\%$

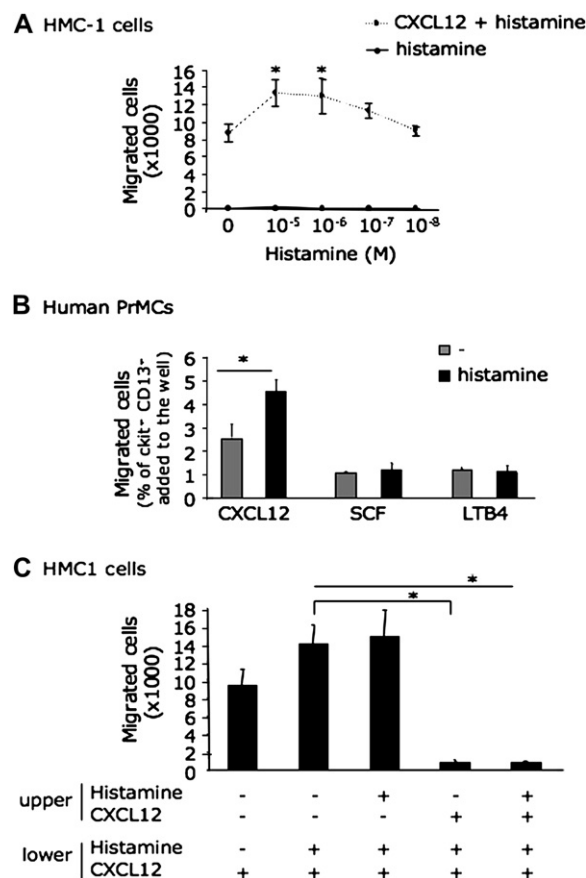


FIG 1. Histamine modulates CXCL12 activity on human PrMCs. **A**, Number of migrated HMC1 cells toward histamine alone or in association with CXCL12 ($n = 10$). **B**, Migration of PrMCs to CXCL12, SCF, and LTB₄ with or without histamine ($n = 5$). Migrations with histamine alone were $0.78\% \pm 0.21\%$ cells. **C**, Checkerboard analysis of HMC1 cell migration ($n = 5$). * $P < .05$.

over control values, $P = .044$, $n = 5$) but not on mature MCs, which is in agreement with the findings of Weller et al.⁶ With SCF used as the positive control, none of the following agents were chemotactic for PrMCs: TGF- β , CCL5, CCL11, CXCL2, and CX3CL1.

Histamine did not modulate the chemotactic activity of SCF and LTB₄ on PrMCs (Fig 1, B).

Effects of histamine are mediated through the H₄ receptor

The expression of H₁, H₂, and H₄ receptors of histamine was shown by using both RT-PCR (see Figure E3 in this article's Online repository at www.jacionline.org) and Western blotting in PrMCs, fully mature MCs, and HMC1 cells (Fig 2, A).

Cells were pretreated with antagonists of each receptor and used in Transwell assays to identify the histamine receptor or receptors involved in the synergy between histamine and CXCL12. As a readout, we evaluated the percentage of histamine activity on CXCL12-induced migration (Fig 2, B). The control values (100%)

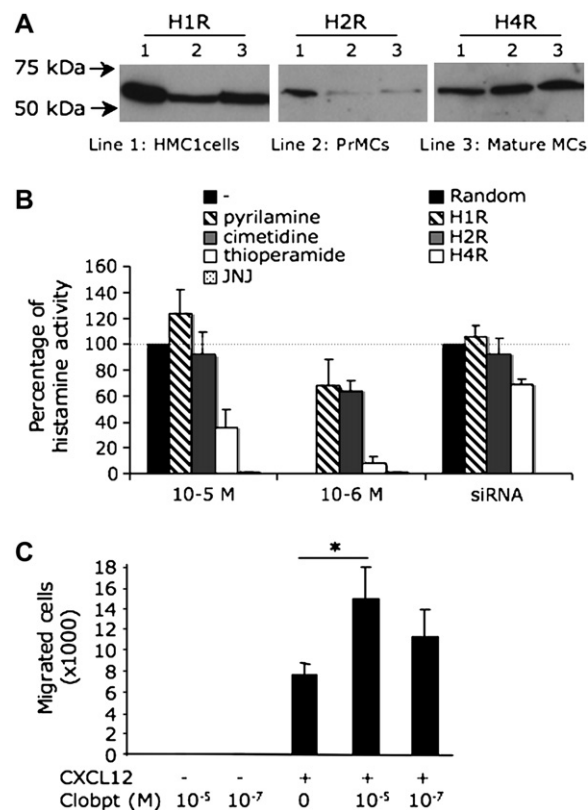


FIG 2. Signaling through the H₄ receptor (H₄R) enhances CXCL12-driven migration. **A**, Western blot analysis of histamine receptor expression in MCs. **B**, Migration of HMC1 cells to CXCL12 and histamine after incubation with 10^{-5} mol/L or 10^{-6} mol/L pyrilamine, cimetidine, thioperamide, or JNJ (left); inhibition of histamine receptor expression by specific siRNA (right). Results are expressed as percentage of histamine activity on CXCL12-driven migration ($n = 4$). **C**, Migration of HMC1 cells toward CXCL12 and clobenpropit (Clobpt; $n = 7$). * $P < .05$.

corresponded to cells pretreated with dimethyl sulfoxide (control vehicle). A pretreatment with pyrilamine (an H₁ receptor antagonist) or cimetidine (an H₂ receptor antagonist) had no effect on the synergistic effects of histamine on both HMC1 cells (Fig 2, B) and PrMCs. CXCL12-driven migration of PrMCs increased by $227\% \pm 40\%$ with histamine plus H₁ receptor antagonist and by $107\% \pm 3\%$ with histamine plus H₂ receptor antagonist ($n = 4$). After incubation of cells with thioperamide (an H₄ and H₃ receptor dual antagonist) or JNJ (a specific H₄ receptor antagonist), the synergy between histamine and CXCL12 was substantially inhibited in the first case and totally abrogated in the second case (Fig 2, B, and data not shown). Those results were confirmed by inhibiting expression of each histamine receptor with specific siRNA (Fig 2, B). We monitored the effects of clobenpropit (an H₄ receptor agonist) on the CXCL12-driven migration of MCs. Clobenpropit was not a chemoattractant on HMC1 cells (Fig 2, C), but it strongly potentiated the CXCL12-driven migration of HMC1 cells (Fig 2, C).

Histamine released by IgE-activated mature MCs enhances the CXCL12-driven migration of HMC1 cells

We tested whether histamine released by activated mature MCs could enhance the chemotactic activity of CXCL12. Mature MCs were passively sensitized with human IgE and activated with anti-IgE. Release of histamine by these activated cells reached 1469 ± 330 ng/mL ($n = 3$). Supernatants were tested in chemotaxis assays on HMC1 cells. Supernatants from activated MCs potentiated the CXCL12-driven migration of HMC1 cells, with a maximal response at a 1:100 dilution (Fig 3, A). The ability of IgE-activated MC supernatants to modulate CXCL12 activity was completely abrogated by pretreatment of HMC1 cells with thioperamide (Fig 3, B). No effect was observed with control supernatants from MCs sensitized with IgE but without anti-IgE cross-linking or from MCs pretreated with IgG and anti-IgG cross-linking (Fig 3, A). These supernatants contained 99 ± 29 ng/mL and 26 ± 2 ng/mL histamine, respectively ($n = 3$).

CXCL12-driven migration of CD4⁺ T cells and monocytes is not modulated by histamine

We tested whether histamine was able to enhance the migration toward CXCL12 of cell types other than PrMCs and mature MCs. The expression of histamine receptors (H_1 , H_2 , and H_4 receptors) was detected by using RT-PCR on CD4⁺ T cells and monocytes (see Fig E3 in this article's Online Repository at www.jacionline.org). CXCL12 strongly attracted CD4⁺ T cells (Fig 4, A; $160\% \pm 13\%$ of migrated cells over control values) and, to a lesser extent, monocytes (Fig 4, B; $114\% \pm 9\%$ of migrated cells over control values). Histamine was a chemoattractant for monocytes ($199\% \pm 44\%$ of migrated cells over control values) but not for CD4⁺ T cells. Histamine did not modulate the CXCL12-driven migration of CD4⁺ T cells and monocytes.

Histamine potentiates CXCL12-induced actin polymerization in HMC1 cells

We performed further experiments with HMC1 cells to gain information about the mechanisms underlying cofactor effects on CXCL12-induced migration. We tested whether histamine potentiated the ability of CXCL12 to reorganize the actin cytoskeleton in MCs. CXCL12 induced actin polymerization in HMC1 cells, with a maximal response at 15 seconds and a progressive decrease in time (Fig 5, A). Whereas histamine alone did not induce any actin polymerization in HMC1 cells (Fig 5, A), its association with CXCL12 enhanced the amount of polymerized F-actin compared with response to CXCL12 alone (Fig 5, A). The concomitant presence of histamine with CXCL12 was not required to exert its enhancing effect. Indeed, pretreatment of HMC1 cells with histamine significantly increased their subsequent response to CXCL12 (Fig 5, B).

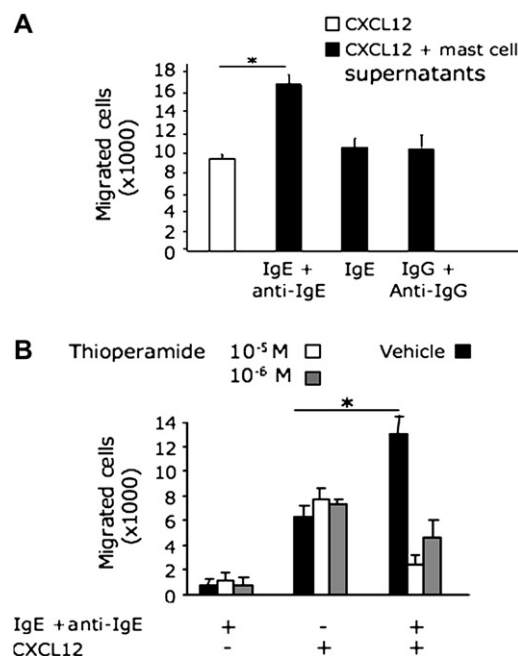


FIG 3. Culture supernatants from IgE-activated mature MCs modulate CXCL12 activity on HMC1 cells. **A**, Number of migrated HMC1 cells to CXCL12 combined with supernatants (1:100 dilution) obtained from cultures of FcεRI-activated MCs or their controls (IgE-sensitized MCs without anti-IgE, IgG control cross-linking; $n = 6$). **B**, The same experiment as described above was done with HMC1 cells previously incubated with thioperamide or control vehicle. * $P < .05$.

Histamine does not change the level of CXCR4 expression on MCs

No CXCR7 mRNA was detected in PrMCs, mature MCs, and HMC1 cells (Fig 6, A). High levels of CXCR4 mRNA were detected in those cells. Analysis of CXCR4 expression by means of flow cytometry revealed large cytoplasmic stores of the protein in MCs (data not shown). We tested whether histamine influences the distribution between pools of cytoplasmic and surface CXCR4. HMC1 cells were incubated with histamine for 30 minutes, 2 hours, and 4 hours. CXCR4 expression on these cells was determined by means of flow cytometry and compared with the expression on cells incubated in medium alone. Histamine did not release cytoplasmic CXCR4 to the cell surface after 30 minutes of incubation (Fig 6, B) or after 2 or 4 hours (data not shown).

In the next experiment we examined whether the enhancing effect of histamine on CXCL12-driven migration was mediated by an upregulation of CXCR4 recycling after engagement with its ligand. HMC1 cells were incubated with CXCL12 with or without histamine. Histamine did not modulate CXCR4 turnover on the cell surface (Fig 6, C).

DISCUSSION

MCs are major effector cells in allergic reactions. Their number increases in tissues under inflammatory conditions

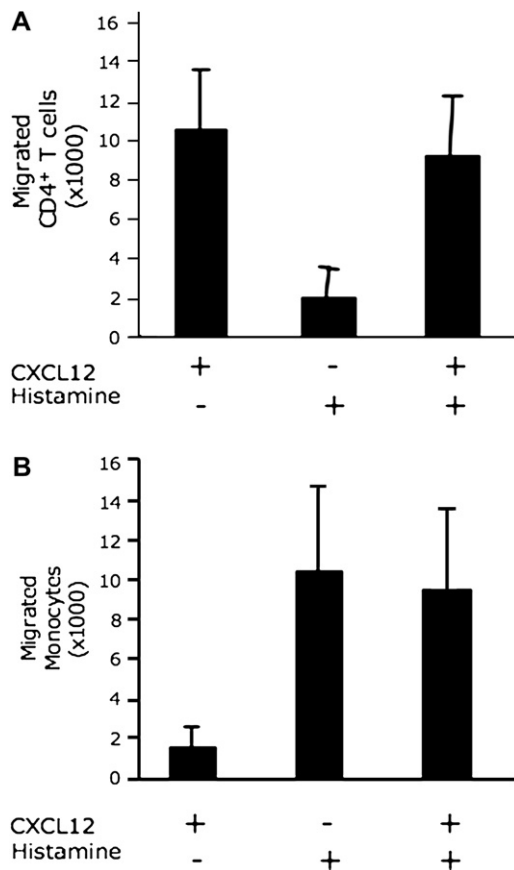


FIG 4. Histamine/CXCL12 synergy is specific of the MC population. Migration of human CD4⁺ T cells (**A**) or monocytes (**B**) to CXCL12 with or without histamine. Results are expressed as the number of migrated cells (n = 3).

because of their *in situ* proliferation and recruitment of their precursors from blood. CXCL12, a constitutive chemokine playing a key role in inflammatory allergic airways disorders, is a potent chemoattractant for both PrMCs and mature MCs.^{5,7} The pleiotropic effects of CXCL12 are optimized in the presence of cofactors, depending on the cell type.¹⁴⁻¹⁶ We hypothesized that histamine might interact functionally with CXCL12 to increase MC migration. This major product of MC degranulation is a key mediator in allergy. Histamine is mainly a vasoactive agent and a bronchial smooth muscle constrictor but also a proinflammatory factor able to activate MCs in an autocrine or paracrine manner.^{19,20} We demonstrated, using *in vitro* migration and actin polymerization assays, that histamine acted in synergy with CXCL12 to induce an optimal migration of PrMCs. Effects of histamine were significant for doses reachable in acute allergic situations.²¹ Histamine was a specific cofactor of CXCL12 for the PrMC subpopulation because it was unable to modulate the CXCL12-induced migration of CD4⁺ T cells or monocytes.

Activation of the H₄ receptor was required for an optimal recruitment of PrMCs by the association of CXCL12 and histamine. When the H₄ receptor was blocked by selective antagonists during migration assays, the effect

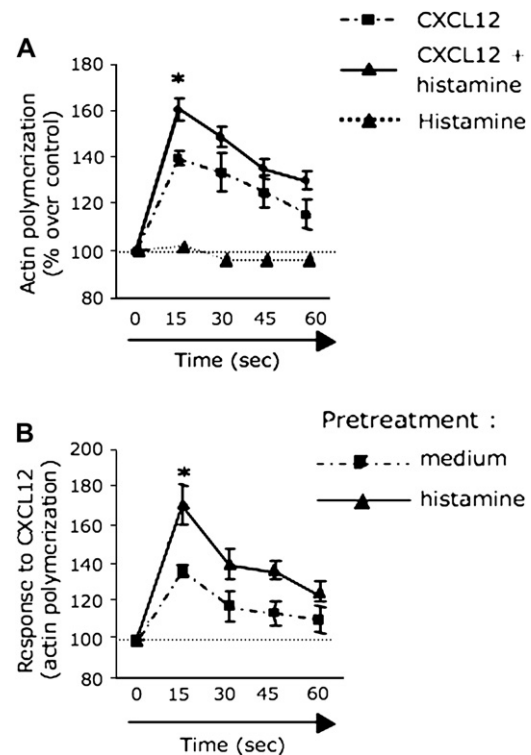


FIG 5. Effects of histamine on HMC1 cytoskeletal reorganization induced by CXCL12. Levels of polymerized actin (F-actin) were evaluated as a final readout of HMC1 cytoskeletal reorganization. **A**, Percentage of F-actin in response to CXCL12, histamine, or their combination (n = 5). **B**, Responses to CXCL12 after incubation (30 minutes) with histamine (10⁻⁶ mol/L) or medium (n = 5). *P < .05.

of histamine disappeared. These results suggest that H₄ receptor antagonists might have potential benefits for the treatment of allergic disorders in human subjects by reducing the number of tissue MCs. The role of the H₄ receptor in optimal recruitment of PrMCs was also defined in *in vitro* migration tests in which an H₄ receptor agonist enhanced the number of cells migrating toward CXCL12. When the H₁ receptor was blocked during migration of PrMCs, synergistic effects of histamine were markedly enhanced on CXCL12-induced migration. This finding implies that the H₁ receptor exerts a negative control in PrMC recruitment. Therapies containing H₁ receptor antagonists, despite neutralizing the acute effects of histamine, such as edema and vasodilatation, might also lead to an enhancement of PrMC recruitment from blood to airways and skin constitutively expressing CXCL12. The conflicting results as to the benefit of treatment of asthma with H₁ receptor antagonists are consistent with our data.

Histamine failed to modulate the migration of mature MCs toward CXCL12. No cofactors of CXCL12 were identified in this study for the mature MCs. Differential regulation of MC response to CXCL12 along their development is probably associated with the variable role of CXCL12 in their recruitment. During inflammatory allergic reactions, CXCL12 constitutively expressed in skin

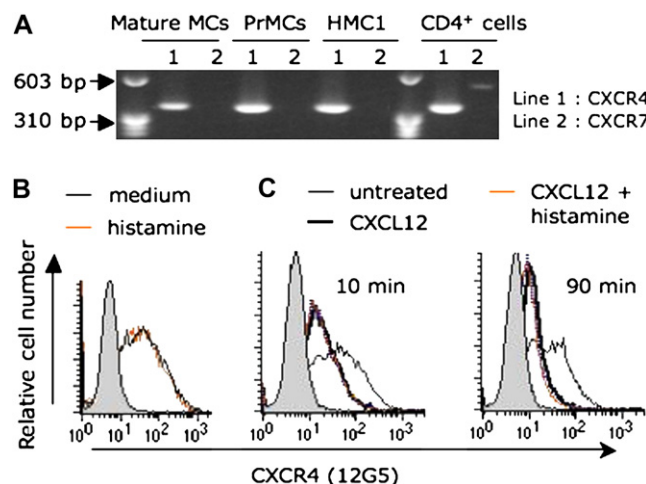


FIG 6. CXCR4 expression on the cell surface is not modulated by histamine. **A**, RT-PCR detection of CXCR4 and CXCR7 in MCs. **B**, Flow cytometric analysis (*empty histograms*, anti-CXCR4 mAb; *gray histograms*, control mAb) of CXCR4 expression in HMC1 cells incubated for 30 minutes with histamine. **C**, CXCR4 recycling in HMC1 cells incubated for 10 and 90 minutes with CXCL12 alone or with histamine. Results are representative of 3 experiments.

and airway epithelium might play a crucial role in PrMC recruitment from blood in the presence of histamine. This phenomenon could partially explain the crucial role of the CXCL12/CXCR4 axis in allergen-associated responses that has been previously described, although the underlying mechanism was not identified.^{12,13} In the absence of cofactors, other chemoattractants than CXCL12 control the local changes in mature MC tissue distribution. Accumulation of MCs in the airway smooth muscle of asthmatic patients is under the control of CX3CL1.^{8,22}

The concomitant presence of histamine and CXCL12 was not required to potentiate the migration of PrMCs. A short priming of cells with histamine before their use in functional assays was sufficient to obtain a better migration to a gradient of CXCL12. This sequential activation of PrMCs by histamine followed by CXCL12 reveals that a distinct spatial exposure with the 2 ligands is possible. Thus circulating PrMCs could be primed by histamine to enhance their response to CXCL12 constitutively expressed by endothelial cells⁹ and tissues.^{9,10}

To better reproduce physiologic conditions, we demonstrated that histamine released by IgE-activated mature MCs potentiated CXCL12 chemotactic properties. Supernatants from MCs collected after their activation through FcεRI enhanced the migration of HMC1 cells toward CXCL12. The combined effects of CXCL12 and supernatants from activated MCs on HMC1 migration were strictly imputable to histamine because they were totally abrogated by H₄ receptor antagonist.

CXCR7, a novel receptor for CXCL12, is expressed in human CD4⁺ T cells, monocytes, B cells, natural killer cells, and basophils.^{18,23} In our study mRNA encoding CXCR7 was detected neither in mature and PrMCs derived from cord blood progenitor cells nor in HMC1 cells. We detected large intracellular stores of CXCR4 in PrMCs,

mature MCs, and HMC1 cells. Signaling through histamine receptors was not accompanied by a redistribution of the cytoplasmic CXCR4 pool to the PrMC cell surface. CXCR4 recycling on the cell surface after its engagement by CXCL12 was not upregulated by histamine. We thus hypothesize that the H₄ receptor and CXCR4, which are both coupled with G proteins, shared signaling pathways in PrMCs. The convergence of multiple activating signals has been described in MCs. C-kit combined with α₄-integrin potentiates haptotaxis in primary MCs through both phosphatidylinositol-3 kinases and Rac pathways.²⁴ The signaling pathways implied in the synergistic effects of histamine on CXCL12-induced migration of human PrMCs are under study.

In conclusion, our data indicate that histamine produced by tissue MCs on allergen contact amplifies allergic reactions by enhancing PrMC recruitment to CXCL12-rich tissues promoting chronic allergic inflammation. In this process activation of the H₄ receptor by histamine is of particular importance. The restrictive expression of the H₄ receptor on hematopoietic cells and its role in chemotaxis of human eosinophils,²⁵ human monocyte-derived dendritic cells,²⁶ and PrMCs identifies this receptor as an attractive therapeutic target in allergic diseases. Effects of JNJ, a selective H₄ antagonist that completely abrogates the combined actions of histamine and CXCL12 on PrMC migration, supports this therapeutic potential.

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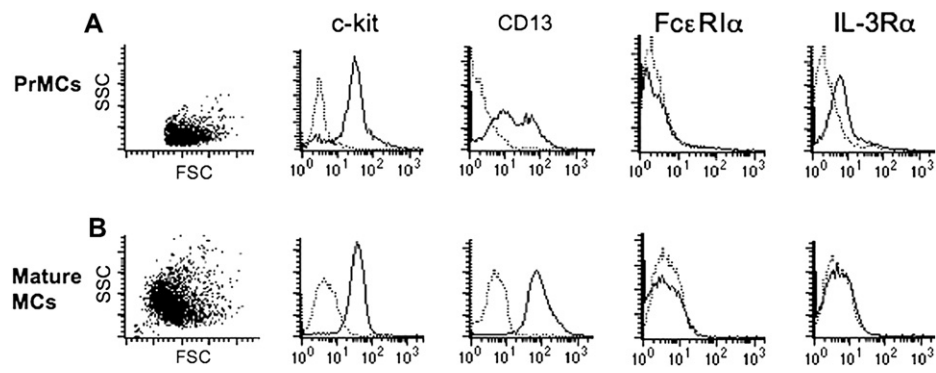


FIG E1. Phenotypic characteristics of human PrMCs and mature MCs. MCs were obtained by means of long-term culture of cord blood progenitor cells. Expression of c-kit, CD13, FcεRI, and IL-3Rα was analyzed by means of flow cytometry on PrMCs (4 weeks of culture) and mature MCs (9-week-old cells).

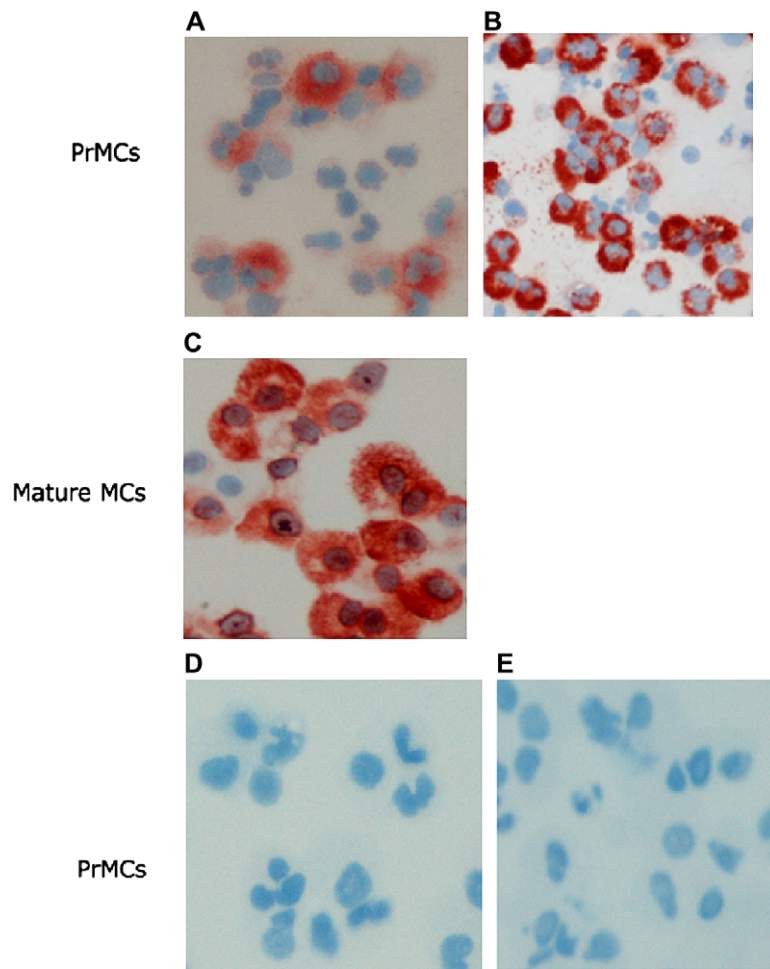


FIG E2. Chymase (A and C) and tryptase (B) immunoreactivity of human PrMCs and mature MCs. The negative control performed with PrMCs was omission of the primary antibody (D) and substitution of the primary antibody by the same isotype (E).

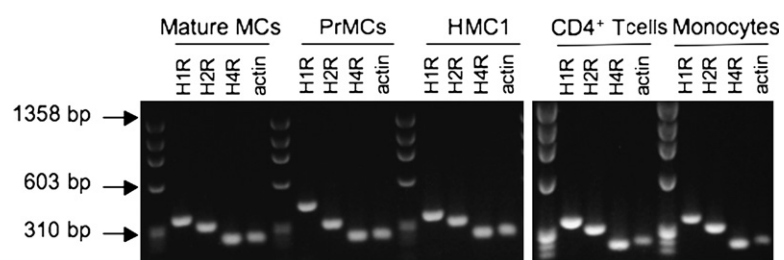


FIG E3. RT-PCR analysis of human H₁ receptor (*H1R*), H₂ receptor (*H2R*), and H₄ receptor (*H4R*) expression in human MCs, CD4⁺ T cells, and monocytes.

TABLE E1. PCR primers and PCR conditions used for human histamine and CXCL12 receptor expression studies

Gene	Primers	Amplification conditions	Amplified fragment size
<i>H1R</i>	F: 5'-GGCCAGCGAGATATCAGAGGA-3' R: 5'-ATGTAGCCCAGCCAGATGGTG-3'	40 cycles, 65°C	351 bp
<i>H2R</i>	F: 5'-CACCGCGTTTGTGTACCGTG-3' R: 5'-GCCGTGACTTCTGTCCCACTC-3'	40 cycles, 65°C	303 bp
<i>H4R</i>	F: 5'-TCGGAATGGTACATCCTTGCC-3' R: 5'-TGCAGGAACCTCTGTTCGATGC-3'	40 cycles, 62°C	231 bp
<i>CXCR4</i>	F: 5'-GACCGCTCACTGGCCATC-3' R: 5'-GGCAGCCAACAGGCGAAGA-3'	40 cycles, 62°C	345 bp
<i>CXCR7</i>	F: 5'- GTGGTCAGTCTCGTGCAGCAC-3' R: 5'- GCCAGCAGACAAGGAAGACC- 3'	33 cycles, 63°C	495 bp

F, Forward primer; R, reverse primer.