

## CCL17 and CCL22 attenuate CCL5-induced mast cell migration

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### Summary

**Background** Mast cells (MCs) accumulate at sites of allergic mucosal inflammation where they act as central effectors and regulatory cells. Chemokines are believed to be crucial for the recruitment of MCs to sites of inflammation. We recently reported that human umbilical cord blood MCs (CBMCs) expresses the CC chemokine receptors, CCR1 and CCR4. We found a unique response profile to ligands of the respective receptors in which, of all tested ligands, only CCL5/RANTES-induced migration.

**Objective** To further investigate the function of CCR4 in MCs.

**Methods** CBMCs were used for competition binding experiments, migration, and intracellular calcium mobilization and release response studies.

**Results** The natural ligands for CCR4, CCL17/TARC and CCL22/MDC could both compete for binding with radiolabelled CCL5. Further, both CCL17 and CCL22 act as CCR4 antagonists by inhibiting CCL5-induced migration. Although both CCL17 and CCL22 caused mobilization of intracellular calcium, none of them induced migration or histamine release.

**Conclusions** These results suggest that CCL5-induced migration of MCs via CCR4 can be regulated by the natural agonists CCL17 and CCL22, which are up-regulated at sites of allergic inflammation.

**Keywords** calcium flux, CCL5, CCL17, CCL22, CCR4, chemotaxis, mast cells

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### Background

Mast cells (MCs) are of primary importance in many pathological inflammatory disorders such as allergy, asthma and arthritis [1, 2]. Mature MCs are normally found interspersed in the tissues, but are known to accumulate at sites of both acute and chronic inflammation where they can act as initial effector cells [3]. This relocation of tissue MCs is believed to be mainly because of chemotactic factors such as chemokines. Until now, five CXC-chemokines, CXCL1, CXCL5, CXCL7, CXCL8 and CXCL12, but only two CC-chemokines, CCL5 and CCL11, have been reported as functional chemokines for human MCs [4–9].

We recently described the expression of two CC-chemokine binding receptors, CCR1 and CCR4, on human cord blood-derived MCs (CBMC) [10]. The migratory response of CBMC towards respective ligands of these receptors exhibited a very restricted and unique pattern. Of the five CCR1 ligands, CCL2, 3, 5, 7 and 14, only CCL5 induced migration. Surprisingly, CCL5 could also induce migration through CCR4, whereas the recognized CCR4 ligands, CCL17 and CCL22, did not. The expression of CCR4 on MCs is particularly interesting since recent reports claim that the CCR4 ligands CCL17 and CCL22 are up-regulated and play dominant roles in T helper 2-type disease conditions such as bronchial asthma [11–13].

The already complex chemokine system has been revealed to be even more intricate with reports of the antagonistic effects of chemokines [14]. In addition to their function as natural agonists, some chemokines have recently been described as natural antagonists, binding to receptors without inducing receptor activation. Subsequently, the binding of natural agonists is inhibited and cellular responses absent. The finding that CCL5 can act as an agonist for CCR4, inducing migration, and the lack of migratory response to CCL17 and CCL22, which are established ligands for CCR4 [15, 16], prompted further investigation of CCR4 expression and function in human MCs.

In this study, we demonstrate that CCL17 and CCL22 can compete with CCL5 for binding to CCR4, thereby acting both as CCR4 antagonists for CCL5-induced migration, and as agonists inducing intracellular calcium mobilization but not migration. CCL5, CCL17 and CCL22 seem not to cause degranulation of CBMCs since no histamine release was detected after chemokine treatment. These results imply that CCL17 and CCL22 can fine-tune the migratory response of MCs to CCL5 at sites of allergic inflammation, in which CCL5, CCL17 and CCL22 are produced.

### Methods

#### Cells

Cord blood samples were aspirated into heparinized tubes from the umbilical vein at normal delivery. Fully informed consent was obtained from the mothers of all neonates before

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harvesting the specimens. The study was approved by the Ethical Review Board at the University Hospital, Uppsala. Mononuclear cells were separated from the cord blood by Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden) gradient centrifugation. The cells were washed twice in phosphate-buffered saline (PBS) and resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 50  $\mu$ M 2-mercaptoethanol, 10 mM HEPES, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/mL penicillin and 50  $\mu$ g/mL streptomycin (Life Technologies, Renfrewshire, Scotland). The cells were seeded at a density of  $10 \times 10^6$  cells per 10 mL in flasks (Falcon, Oxnard, CA, USA) and grown for at least 8 weeks in the presence of recombinant human SCF (rhSCF) at 50 ng/mL and rhIL-6 at 10 ng/mL (Amgen, Thousand Oaks, CA, USA). The medium was replaced once a week. Development of MCs was assayed by staining for the MC-specific serine proteinase tryptase as previously described [6]. The cultures contained 95–100% tryptase-positive cells when used in the different assays. The study is based on results obtained from 12 different CBMC populations derived from individual cord blood collections.

#### *Competition of CCL5-binding and migration with CCL17 and CCL22*

Samples of  $1 \times 10^5$  CBMC in 200  $\mu$ L RPMI-1640 medium containing 1% bovine serum albumin (pH 7.2–7.4) were incubated with 0.25 nM  $^{125}$ I-labelled CCL5 in the presence of 100 nM unlabelled CCL5, CCL17, CCL22 or CXCL10, respectively, in Eppendorf tubes. After incubation for 1 h at room temperature, the CBMCs were centrifuged through a 10% sucrose/PBS cushion for 1 min at top speed in an Eppendorf centrifuge, and the supernatant was removed. After washing five times with PBS (0.5% NaCl), the tips of the Eppendorf tubes were cut and the radioactivity in the pellets was counted in a  $\gamma$  counter. The results were expressed as the percentage of  $^{125}$ I-labelled CCL5 bound. Experiments in which CCL5-induced migration was blocked were done by treating CBMC with CCL22 (100 ng/mL), CCL17 (50 ng/mL) and CXCL10 (100 ng/mL) (PeproTech, London, UK) 15 min before cells were allowed to migrate towards CCL5 (10 ng/mL). The migration was tested using a modified 48-well Boyden chamber (Neuroprobe Inc., Cabin John, MD, USA) as previously described [6]. In brief, 70 000 CBMCs in 50  $\mu$ L was added to the upper chamber and the cells were allowed to migrate into the filters for 2.5 h at 37 °C and 5% CO<sub>2</sub>. Cell chemotaxis was assayed as the migration of the two farthest migrating cells visible in focus of one high-power field ( $\times 200$ ). The migrating distance in each filter was calculated as the mean of the readings of three different areas of the filter, and the results are presented as percentages of control, in which the control is migration toward medium alone.

#### *Intracellular [Ca<sup>2+</sup>]<sub>i</sub> measurements*

[Ca<sup>2+</sup>]<sub>i</sub> in CBMC was determined using FURA-2 (Molecular Probes, Eugene, OR, USA). CBMCs ( $5 \times 10^6$ /mL) were incubated in Hanks' balanced salt (HBSS) (Life Technologies) with 1% FCS, containing 2.5  $\mu$ mol/L FURA-2 acetoxymethyl ester for 60 min at 30 °C. The cells were washed and

resuspended at  $1 \times 10^6$  cells/mL in HBSS with Ca<sup>2+</sup> and Mg<sup>2+</sup> and 1% FCS. A total of 350  $\mu$ L of the cell suspension was placed in a quartz microcuvette in a thermostat cell holder at 37 °C. The cells were continuously stirred during the experiments with a magnetic stirrer. Fluorescence measurements were performed with a Hitachi F-2000 fluorescence spectrophotometer (Tokyo, Japan) with the dual-wavelength optional function at the wavelengths 340/380 nm (excitation) and 505 nm (emission). Data were collected every 500 ms and presented as the relative ratio of fluorescence excited at 340 and 380 nm. Cytokines analysed for their ability to induce [Ca<sup>2+</sup>]<sub>i</sub> were: CCL5, CCL17, CCL22 and SCF (PeproTech).

#### *Treatment with pertussis toxin*

Pertussis toxin (Sigma, St Louis, MO, USA) treatment was performed by incubating  $2.5 \times 10^6$  cells/mL for 90 min at 37 °C with 1  $\mu$ g/mL of the toxin in complete medium. After incubation, cells were washed and resuspended in fresh medium before use.

#### *Histamine release assay*

CBMCs were counted, washed and seeded at a density of  $5 \times 10^5$  cells/mL in culture medium for 12 h in the presence of CCL5, CCL17 and CCL22 (100 ng/mL). As a positive control, cells were sensitized with human myeloma IgE (ND) (1  $\mu$ g/mL) for 12 h, and cells cultured for 12 h without additive served as negative control. After 12 h, cells were washed twice and IgE sensitized cells were activated by IgE crosslinking using an rabbit-anti-human IgE Ab (provided by Dr Torbjörn Karlsson, Pharmacia Diagnostics AB, Uppsala, Sweden) (2  $\mu$ g/mL). Histamine released into supernatants was measured by a commercial ELISA-kit (Coulter-Immunotech, Krefeld, Germany) and expressed as specific histamine release according to the following formula: histamine release after chemokine or IgE crosslinking/total cellular histamine content as determined after cell lysis by Triton-100 treatment.

## **Results**

### *CCL17 and CCL22 can inhibit CCL5-mediated migration*

To investigate the receptor usage in CCL5-induced migration of CBMCs and examine specificity of CCL5 for CCR4, competition-binding experiments using  $^{125}$ I-labelled CCL5 were carried out. The binding of  $^{125}$ I-CCL5 was significantly competed by cold CCL5 (80% inhibition) (Fig. 1). CCL17 and CCL22 also showed significant competition with  $^{125}$ I-CCL5, although to a lesser extent than CCL5 (55% and 66% inhibition, respectively) (Fig. 1). An unrelated CXCL-chemokine, CXCL10, which binds CXCR3 showed less competitive effect on  $^{125}$ I-CCL5 binding (33% inhibition) (Fig. 1). Since CCL17 and CCL22 could compete for the binding of CCL5, we next investigated whether CCL17 and CCL22 could act as antagonists for CCL5-induced migration. CBMCs were pre-treated with CCL17 (50 ng/mL) or CCL22 (100 ng/mL) 15 min before the cells were allowed to migrate towards CCL5 (10 ng/mL). Both CCL17 and CCL22 significantly inhibited the migration towards CCL5 by 54% and 74%, respectively (Fig. 2a). Notably, this inhibition was in the same

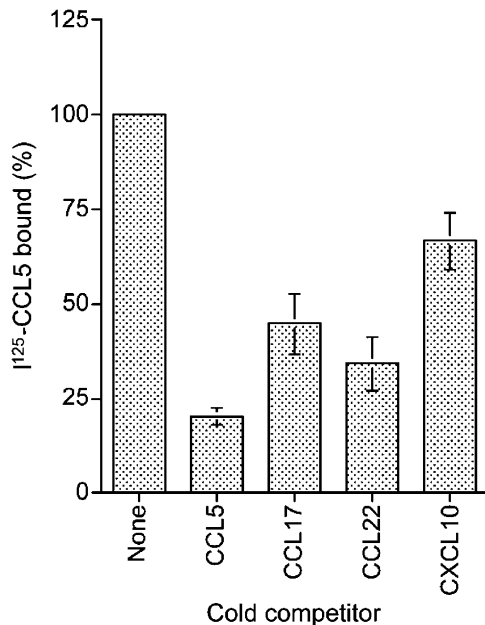


**Fig. 1.** Inhibition of CCL5-binding cord blood-derived mast cell ( $1 \times 10^5$  cells) were incubated with 0.25 nM [ $^{125}$ I]-labelled CCL5 in the presence or absence of 100 nM unlabelled CCL5, CCL17, CCL22 and CXCL10. Results are expressed as the mean  $\pm$  SEM percentage of inhibition of specific binding from three separate experiments.

range as the use of a blocking monoclonal antibody against CCR4 [10]. In contrast, treatment with CCL2, CCL3, CXCL10 or CXCL12 did not affect the CCL5-induced migration (Fig. 2 and data not shown). Furthermore, CCL17 and CCL22 did not affect CXCL12-induced MC migration (Fig. 2b).

#### *CCL17 and CCL22 but not CCL5 elicit intracellular calcium mobilization*

The finding that both CCL17 and CCL22 could inhibit CCL5-induced migration of CBMCs indicated that they act as antagonists for CCL5-mediated migration induced via CCR4. To explore whether CCL17 and CCL22 also act as agonists on CCR4 expressed on CBMCs (although not inducing a migratory response), we investigated whether

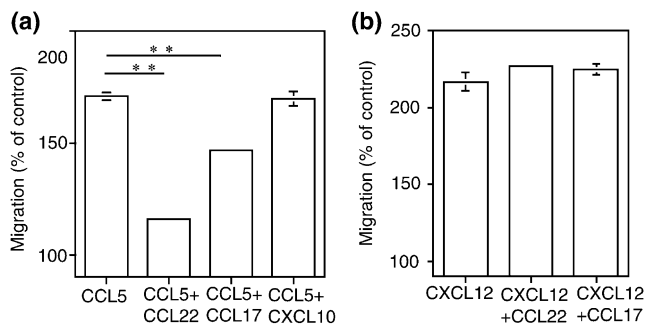


**Fig. 2.** Inhibition of CCL5-induced migration by CCL17 and CCL22 (a) cord blood-derived mast cell (CBMC) were incubated with CCL17 (50 ng/mL), CCL22 (100 ng/mL) or CXCL10 (100 ng/mL) 15 min before the cells were allowed to migrate towards CCL5 (10 ng/mL). Results are presented as percentage of control (mean  $\pm$  SEM,  $n = 6$ ) (b) CBMC were incubated with CCL17 (50 ng/mL) or CCL22 (100 ng/mL) 15 min before the cells were allowed to migrate towards CXCL12 (1000 ng/mL). Results are presented as percentage of control (mean  $\pm$  SEM,  $n = 3$ ).

CBMCs mobilized intracellular calcium upon stimulation with the CCR4 ligands. In CBMCs loaded with the fluorescent  $\text{Ca}^{2+}$  indicator Fura-2, both CCL17 and CCL22 induced a rapid and transient increase in  $[\text{Ca}^{2+}]_i$  at a concentration of 100 ng/mL (Fig. 3a). In contrast, CCL5, CCL3, CCL7 and CCL14 did not induce calcium mobilization at 10 or 100 ng/mL (Fig. 3a and data not shown). Sequential stimulation of CBMCs with 100 ng/mL CCL22 followed by 100 ng/mL CCL17, or vice versa, revealed that CCR4 became desensitized for a second stimulus in both cases (Fig. 3b). However, pre-treatment with 100 ng/mL CCL5 did not desensitize the CCL17 or the CCL22 induced calcium response (Fig. 3c). By treating CBMCs with pertussis toxin, the CCL17- and CCL22-induced calcium mobilization was completely blocked (Fig. 3d). As a control, SCF-induced calcium mobilization was used, which was not inhibited by similar treatment with pertussis toxin. Together with previous results, the current results suggest that CCL17 and CCL22 induce  $[\text{Ca}^{2+}]_i$  in CBMCs without eliciting migration, while CCL5 can induce migratory responses of CBMCs in the absence of intracellular  $\text{Ca}^{2+}$ -mobilization.

#### *CCL17 and CCL22 do not trigger degranulation in cord blood-derived mast cells*

After stimulation with CCL17 and CCL22, the CBMCs were activated, as shown by the increase in  $[\text{Ca}^{2+}]_i$  (Fig. 3a). In the

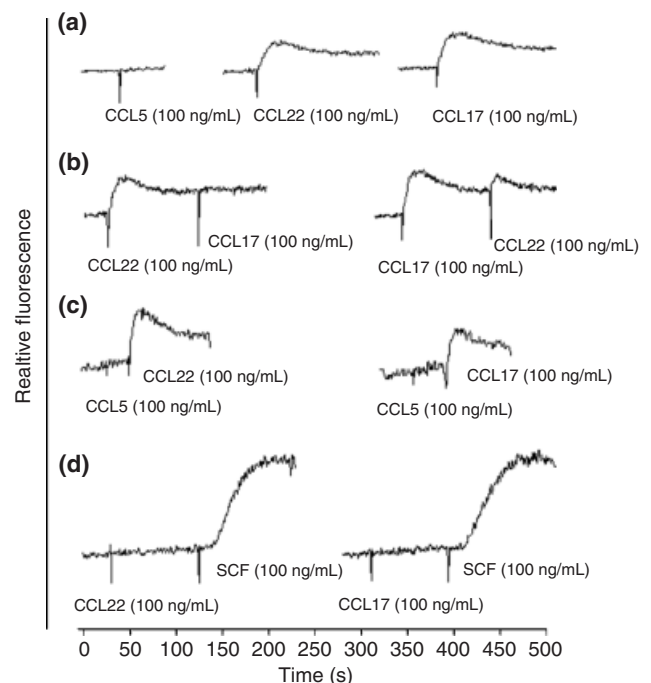


**Fig. 3.** Calcium mobilization by CCL17 and CCL22 but not CCL5 in cord blood-derived mast cell (CBMC). CBMCs loaded with FURA-2AM were used to study intracellular calcium mobilization. (a) CCL5, CCL17 and CCL22 were added at the time-point indicated by the arrow. (b) Receptor desensitization was determined by monitoring  $[\text{Ca}^{2+}]_i$  changes in response to sequential stimulation with CCL17 and/or CCL22. (c) Pre-treatment with CCL5 before stimulation with CCL17 and CCL22. (d) Inhibition of CCL17- and CCL22-induced calcium mobilization in CBMCs. CBMCs were pre-treated with pertussis toxin before stimulation with CCL17 or CCL22. SCF was used as a positive control. Results shown are from one representative experiment of three performed.

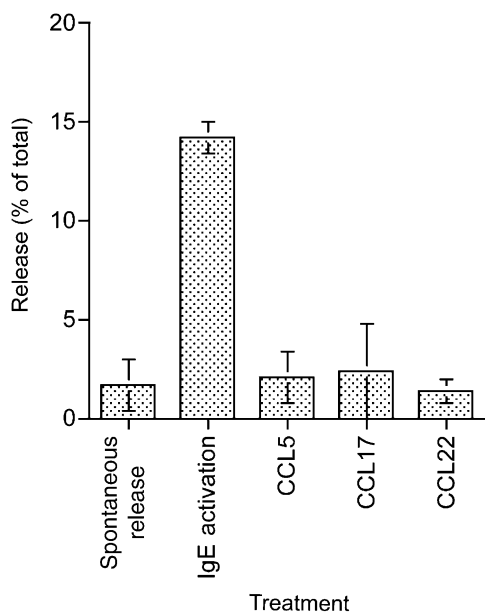


**Fig. 4.** Absence of histamine release after stimulation of cord blood-derived mast cell (CBMC) with CCL5, CCL17 and CCL22. CBMCs were stimulated for 12 h with CCL5, CCL17 and CCL22 (100 ng/mL). IgE-crosslinked CBMCs were used as positive controls. Results are expressed as percentage of total histamine content. The results represent the mean  $\pm$  SEM of three independent experiments performed in triplicate.

absence of a migratory response towards CCL17 and CCL22 as reported earlier [10], we addressed the possible cellular effects that the triggered activation of CBMCs by these chemokines could have. One obvious cellular response of MCs is the capacity to degranulate. To address this issue further, we analysed histamine release from CBMCs after treatment for 12 h with CCL5, CCL17 and CCL22 (100 ng/mL). CBMCs from three different donors showed no significant histamine release after stimulation with CCL5, CCL17 or CCL22 compared with spontaneous release (Fig. 4). This indicates that CCL17 and CCL22 induced activation of CBMCs, as demonstrated by mobilization of  $[Ca^{2+}]_i$ , does not lead to MC degranulation.

## Discussion

In our present study we followed up previous results on CCR1 and CCR4 expression on human MCs by demonstrating that the ligands for CCR4, CCL17 and CCL22, acted as antagonists by competing for binding with CCL5 and inhibiting CCL5-induced migration in CBMCs. Despite the lack of migratory response, both CCL17 and CCL22 activated CBMCs by inducing  $[Ca^{2+}]_i$  mobilization, which was absent in response to CCL5. Although the apparent response of CBMC to CCL5, CCL17 and CCL22, none of them caused degranulation and release of histamine.

The current results indicate that CCL17 and CCL22 may have further potential effects on MCs apart from regulation of recruitment. In a recent study we demonstrated the lack of migratory response of CBMCs to both CCL17 and CCL22 in

concentrations ranging from 1 to 1000 ng/mL [10]. Here we show that both CCL17 and CCL22 compete for binding with CCL5, and inhibit CCL5-stimulated migration of CBMCs. At the same concentration, both CCL17 and CCL22 induced  $[Ca^{2+}]_i$  mobilization (Fig. 3a). The fact that CCL17 and CCL22 only partially inhibited CCL5-induced migration could be a consequence of CCL5-signalling through CCR1 in addition to CCR4 [10].

There are several possible explanations for the antagonistic effects of CCL17 and CCL22. In recent years, there has been increasing evidence that dimerization of chemokine receptors occur upon interaction with ligands [17, 18]. Recent results reported by Mellado et al. [19], suggest that chemokine receptors can form heterodimers. If so, cooperative interactions between CCR1 and CCR4 or in combination with some other chemokine receptor expressed on MCs may affect ligand specificity or cellular responses. We cannot exclude the possibilities of cross-desensitization between different chemokine receptors, which has been shown for CCR1 and CXCR2-receptors [20], as an explanation for our results. This is currently under investigation using transfected cells. Furthermore, uncoupling of chemokine receptors has been reported upon inflammatory conditions [21] but is most likely not occurring in our experiments because of the steady state culturing conditions.

Altogether our results imply that CCL17 and CCL22 can decrease the migration of MCs to CCL5, but not in a true antagonistic manner since they have the capacity to activate cells upon binding to CCR4. However, the cellular response after activation of MCs by CCL17 and CCL22 via CCR4 does not induce degranulation (Fig. 4). Human MCs seem to be very restricted in their release responses after stimulation with chemokines as reported earlier [22–25]. CCL17 and CCL22 have been shown to induce integrin-dependent T cell adhesion to endothelial cells [26, 27] and we are currently investigating if CCL17 and CCL22 affects adhesion responses in CBMC.

Although CCL5 induced migration of CBMCs, it did not increase intracellular calcium levels. In contrast, both CCL17 and CCL22 caused calcium flux in CBMCs, which could be inhibited by pertussis toxin treatment. This indicates that CCR4 expressed on MCs is triggered by these ligands through a G-protein-dependent pathway (Fig. 3d). The demonstration of chemotaxis in populations of cells without detectable calcium flux, and vice versa, is in line with several reports suggesting that chemotaxis and calcium flux can be independent phenomena [28, 29]. In line with our results, Turner et al. [30] have reported that CCL5 activation of T lymphocytes induces chemotaxis in the absence of  $[Ca^{2+}]_i$  mobilization, and that the chemotactic response is dependent on PI3K activation. Further, calcium flux has been shown to be neither necessary nor sufficient for stimulation of actin association with cytoskeleton [31], or for chemotaxis, in standard assays using a variety of chemotactic factors [29, 32, 33].

In conclusion, our results suggest a potential mechanism by which MC migration to CCL5 can be regulated by CCL17 and CCL22. These findings are important for understanding how chemokines recruit MCs to sites of inflammation in allergic diseases such as asthma, and how this process can be fine-tuned by chemokines themselves.

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