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Synergy between co-produced CC and CXC chemokines in monocyte chemotaxis through receptor mediated events*

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Running title: CXC and CC chemokines cooperate in monocyte recruitment

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Abbreviations: GAG, glycosaminoglycans; IFN; interferon; LPS, lipopolyssaccharide; IL-, interleukin; MCP-, monocyte chemotactic protein-; CCR, CC chemokine receptor; SDF, stromal cell-derived factor; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GCP-2, granulocyte chemotactic protein-2; PBMC, peripheral blood mononuclear cells; dsRNA, double-stranded ribonucleic acid; PIC, polyriboinosinic-polyribocytidylic acid; PMA, phorbol myristate acetate; FCS, foetal calf serum; ATP, adenosine triphosphate; AKT, protein kinase B; ConA, concanavalin A; GPCR, G protein-coupled receptor; PI3K, phosphoinositide-3-kinase; MDC, macrophage-derived chemokine

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ABSTRACT

CC and CXC chemokines co-induced in fibroblasts and leukocytes by cytokines and microbial agents determine the number of phagocytes infiltrating into inflamed tissues. Interleukin-8/CXCL8 and stromal cell-derived factor-1/CXCL12 significantly and dose-dependently increased the migration of monocytes, expressing the corresponding CXC chemokine receptors CXCR2 and CXCR4, towards suboptimal concentrations of the monocyte chemotactic proteins CCL2 or CCL7. These findings were confirmed using different chemotaxis assays and monocytic THP-1 cells. In contrast, the combination of two CC chemokines (CCL2 plus CCL7) or two CXC chemokines (CXCL8 plus CXCL12) did not provide synergy in monocyte chemotaxis. These data show that chemokines competing for related receptors and using similar signaling pathways do not synergize. Receptor heterodimerization is probably not essential for chemokine synergy as shown in CXCR4/CCR2 co-transfectants. Interestingly, CCL2 mediated extracellular signal-regulated kinase (ERK)1/2 phosphorylation and calcium mobilization was significantly enhanced by CXCL8 in monocytes, indicating cooperative downstream signaling pathways during enhanced chemotaxis. Moreover, in contrast to intact CXCL12, truncated CXCL12(3-68), which has impaired receptor signaling capacity but can still desensitize CXCR4, was not able to synergize with CCL2 in monocytic cell migration. Furthermore, AMD3100 and RS102895, specific CXCR4 and CCR2 inhibitors, respectively, reduced the synergistic effect between CCL2 and CXCL12 significantly. These data indicate that for synergistic interaction between chemokines binding and signaling of the two chemokines via their proper receptor is necessary.

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Tissue infiltration by leukocytes is an important phenomenon of a variety of normal as well as pathological processes including leukocyte homing, inflammation and cancer (Murphy et al., 2000; Strieter et al., 2006). This leukocyte recruitment is tightly regulated by the interplay between endothelial cells and leukocytes, a process in which G protein-coupled receptor (GPCR) agonists, including complement factor C5a, bacterial peptides (e.g. fMLP) as well as chemokines, play a central role. Chemokines have been detected during inflammation in many tissues, suggesting that most, if not all, cell types can secrete chemokines, after induction by appropriate stimuli (Gouwy et al., 2005). Thus, it is likely that, at the site of inflammation, more than one chemoattractant is present. These co-induced chemokines may cooperate to attract leukocytes to the site of infection, thereby enhancing the outcome of an inflammatory response. Many different ways exist to enhance the cell influx mediated by chemokines. One possibility is the synergistic interaction between cytokines to induce chemokines followed by subsequent cooperation amongst co-induced chemokines to further increase leukocyte recruitment (Gouwy et al., 2005). In addition, chemokines can bind to glycosaminoglycans (GAG) to positively sustain a stable chemotactic gradient and hence leukocyte influx (Johnson et al., 2005). Alternatively, dampening inflammation prevents excessive tissue damage and can be mediated through posttranslational modification of chemokines by proteases, resulting in impaired receptor binding and signaling capacities (Struyf et al., 2003). Furthermore, inflammatory chemokines can be trapped by chemokine decoy receptors with seven transmembrane domains and internalized by these non-signaling receptors followed by intracellular degradation of the ligands (D'Amico et al., 2000; Mantovani et al., 2001; Nibbs et al., 2003). Finally, the expression level of functional chemokine receptors can be

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directly up- or down-regulated by endogenous (IFN-γ) or exogenous (LPS) inflammatory mediators, thereby affecting the degree of leukocyte infiltration (Sica et al., 1997; Bonecchi et al., 1999).

In an initial study, we found that CC chemokines constitutively circulating in the blood can cooperate with the inflammatory chemokine interleukin-8 (CXCL8/IL-8) in various neutrophil activation and migration tests (Struyf et al., 2001; Gouwy et al., 2002). Subsequently, the inflammatory CC chemokine monocyte chemotactic protein-3 (CCL7/MCP-3), which is a weak neutrophil chemoattractant, was found to dose-dependently enhance the neutrophil influx towards a suboptimal concentration of CXCL8. Also other CC chemokines (CCL2/MCP-1 and CCL8/MCP-2), yielded a statistically significant enhancement of the neutrophil chemotactic response to low doses of CXCL8 by binding to CCR1 and/or CCR2, two receptors which are expressed on neutrophils (Gouwy et al., 2004). Moreover, we demonstrated that the constitutively circulating chemokine regakine-1, as well as the inducible CC chemokine CCL7, cooperates with inflammatory CXC chemokines such as granulocyte chemotactic protein-2 (CXCL6/GCP-2) to enhance the recruitment of neutrophils into the peritoneal cavity in mice (Struyf et al., 2005).

In this study, we investigated whether the synergistic interactions between chemokines can be extended to activation of other leukocyte cell types e.g. monocytes. In particular, we describe the synergy between CC chemokines (e.g. CCL7 or CCL2) and the CXC chemokines CXCL8 or CXCL12 to chemoattract freshly isolated peripheral blood

monocytes as well as the monocytic THP-1 cell line in various migration assay systems. Several lines of evidence are provided that exclude the implication of chemokine or chemokine receptor dimerization, as postulated by others for other experimental settings (Mellado et al., 2001; Paoletti et al., 2005), indicating that this synergistic effect in monocytes rather occurs at the level of intracellular signal transduction. Indeed, chemotaxis experiments using truncated CXCL12 with impaired signaling capacity as well as chemokine receptor antagonists, indicate that each chemokine has to bind and signal through its proper receptor to cause synergistic interaction. However, evidence for post-receptor events to explain synergy remains limited, although it is shown here that, in contrast to receptor transfected CHO cells, CC and CXC chemokines did cooperate to enhance the phosphorylation of ERK1/2 and calcium signaling in monocytes.

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MATERIALS AND METHODS

Reagents

Natural human CXCL8 and CCL2 were purified to homogeneity from monocyte-derived, conditioned medium (Van Damme et al., 1989; Van Damme et al., 1997). Recombinant human CXCL12 and human CXCL8(6-77), used in the ERK phosphorylation assay, were obtained from Peprotech (Rocky Hill, NJ, USA). Synthetic CXCL12(1-68) and CXCL12(3-68) and the CC chemokine CCL7 were synthesized by solid-phase peptide synthesis using fluorenylmethoxy-carbonyl (Fmoc) chemistry and were purified as previously described (Struyf et al., 2001). The bacterial chemotactic peptide fMLP was obtained from Sigma (St. Louis, MO, USA). To measure chemokine production by fibroblasts, THP-1 cells and peripheral blood mononuclear cells (PBMC), cells were stimulated with a diverse set of inducers: recombinant human interferon-γ (IFN-γ) and IL-1β (both from Peprotech), concanavalin A (Con A; Calbiochem, La Jolla, CA, USA), LPS from *Escherichia coli* (0111:B4; Difco Laboratories, Detroit, MI, USA), the dsRNA polyriboinosinic:polyribocytidylic acid (polyrI:rC or PIC) and phorbol myristate acetate [(PMA) both purchased from Sigma].

The CCR2 and CXCR4 antagonists, RS102895 and AMD3100, respectively, were obtained from Sigma.

Cells

Blood was collected upon heparin and PBMC were isolated from granulocytes and erythrocytes by density gradient centrifugation (400 *g*, 30 min, 15°C) on Ficoll-sodium

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diatrizoate (Lymphoprep; Invitrogen, Groningen, The Netherlands). Erythrocytes in the granulocyte pellet were removed by sedimentation for 30 min at 37°C in hydroxyethyl-starch solution (Plasmasteril; Fresenius AG, Bad Homburg, Germany). The remaining erythrocytes were lysed by hypotonic shock (30 s) in bidistilled water. The monocytic THP-1 cell line [American Type Culture Collection (ATCC), Manassas, VA] was grown in RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% foetal calf serum (FCS; Sigma). The CHO cell line transfected with CCR2 or CXCR4 was cultured in Ham's F-12 growth medium (Cambrex Bio Science) enriched with 10% FCS, 400 µg/ml G418 and 250 µg/ml zeocin (Invitrogen). The double-transfected CCR2/CXCR4/CHO cell line was cultured in Ham's F-12 growth medium enriched with 10% FCS, 400 µg/ml G418, 250 µg/ml zeocin and 5 µg/ml blasticidin (Invitrogen) (Sohy et al., 2007). Human diploid skin/muscle-derived fibroblasts (E1SM) were grown in minimal essential medium (MEM; Invitrogen) containing 10% FCS.

Chemotaxis

Cell migration was measured in two different chemotaxis assay systems. For the classical Boyden microchamber technique (Neuro Probe, Cabin John, MD, USA), cell fractions and samples were diluted in Hank's Balanced Salt Solution (HBSS; Invitrogen) supplemented with 1 mg/ml human serum albumin (HSA; Belgian Red Cross) and tested in triplicate. The upper wells of the chamber were filled with a PBMC (2×10^6 cells/ml) suspension and separated from the lower wells by a 5-µm pore-size polyvinyl pyrrolidone- (PVP) membrane (GE Osmonics, Minnetonka, MN, USA). Monocytes were allowed to migrate for 2 h at 37°C. After migration the filters were fixed and stained

using Hemacolor solutions (VWR, International, Leuven, Belgium). The cells that migrated through the pores and adhered to the lower surface of the membrane were counted microscopically (500x magnification). A chemotactic index (CI) was used to express chemotactic activity and was measured by calculating the number of cells migrated to the chemokine dilution, divided by the number of cells that migrated spontaneously to the chemotaxis buffer. Synergy experiments were performed by adding two different chemokines together to the lower wells of the chamber.

The Boyden chemotaxis assay, as used in our laboratory to study the synergy between CC and CXC chemokines, is a very labour-intensive assay system, involving manual microscopic cell counting. Since, THP-1 cells are readily available in large numbers compared to blood monocytes, we applied a new cell migration assay with an enzymatic read out, namely the Multiscreen chemotaxis assay, which makes it possible to screen the synergistic effect between chemokines on a larger scale and in a more automatic way. The Multiscreen plate (Millipore Corporation, Billerica, MA, USA) is a disposable device with a 96-well filter plate (5 µm pore size) and a 96-well receiver plate. Cell migration occurs through the 96-well filter plate in response to a chemotactic gradient. The THP-1 cell suspension (100 µl in 96-well filter plate at a concentration of 3.5×10^6 cells/ml) and test samples (150 µl in 96-well receiver plate) were diluted in RPMI medium without phenol red and L-glutamine (Cambrex Bio Science) supplemented with 0.1% BSA (endotoxin free, Sigma). To study the effect of chemokine receptor antagonists (RS102895 and AMD3100), THP-1 cells were mixed with the antagonists before loading in the upper wells of the Multiscreen plate. After 3 h migration at 37°C, the upper 96-well filter plate was removed and the cells in the lower receiver plate were

quantified using the luminescence ATP detection assay system (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Adenosine triphosphate (ATP) is present in all metabolically active cells and the concentration is a value for the number of cells. This highly sensitive enzymatic ATPlite assay measures the production of light caused by the reaction of ATP, derived from the THP-1 cells after cell lysis, with added luciferase and D-luciferin. The emitted light is measured in a luminescence reader (FL600 microtiterplate fluorescence reader, Biotek Instruments, Vermont, USA) and is proportional to the ATP concentration and the number of cells. The chemotactic activity (CI) was calculated by dividing the luminescence value of the test sample through the luminescence value of the control buffer.

Statistically significant induced chemotaxis compared to buffer as determined by the Mann-Whitney U test is indicated († $p<0.05$; ‡ $p<0.01$). Statistically significant differences in chemotactic indices between the combination of two chemokines and the sum of the indices obtained for the chemokines alone, determined by the Mann-Whitney U test, are indicated by asterisks (*$p<0.05$; **$p<0.01$).

ERK phosphorylation

For the single- and double-transfected CHO cells, 0.5 x 10^6 cells (in 2 ml) were seeded in a 6-well plate (9 cm², Techno Plastic Products AG, Trasadingen, Switzerland) in their corresponding Ham's F-12 growth medium. After 24 h, the growth medium was removed and the cells were cultured overnight in serum-free starvation medium. Monocytes were enriched by seeding PBMC at 2.5 x 10^6 cells/ml (2 ml/well) in 6-well plates in MEM without serum (starvation medium), followed by a 2 h adhesion period at 37°C. Before

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stimulation, the starvation medium was removed from these adherent cells (90% pure monocytes) and 900 µl Ham's F-12 medium or MEM supplemented with 0.5% BSA was added to each well. The cells were preincubated at 37°C for 15 min before stimulation with the test sample (diluted in 100 µl Ham's F-12 medium or MEM supplemented with 0.5% BSA). After two minutes, signal transduction was stopped by chilling the cell culture plates on ice and adding ice-cold PBS. Afterwards, cells were washed twice with ice-cold PBS and cell lysis was performed in PBS containing 1 mM ethylenediamine tetraacetic acid, 0.5% Triton X-100, 5 mM NaF, 6 M urea, protease inhibitor cocktail for mammalian tissues and phosphatase inhibitor cocktails 1 and 2 (Sigma) (150 µl/well). After 10 min cells were scraped off, and the lysate was collected, incubated for 45 min on ice and clarified (10 min, 1200 *g*). The protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The amount of extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in the supernatant (pg phosphor-ERK/mg total protein) was determined using a ELISA for phospho-ERK1 (T202/Y204) plus phospho-ERK2 (T185/Y187) (R&D Systems).

Ratiometric imaging of fura-2 loaded monocytes using microscopy

Freshly purified PBMC, suspended in MEM containing 2 % FBS, were allowed to adhere to Poly-L-Lysine (Sigma) precoated 4-well Lab-Tek plates (Nalge Nunc International, Rochester, NY, USA) for 2 h at 37°C and 5 % CO_2 (2 x 10^6 cells/well). Fura-2 (Invitrogen) was added at a final concentration of 2.5 µM, 30 min before PBMC were washed to enrich for adherent monocytes. Afterwards, the monocytes were washed twice

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with medium, twice with calcium buffer (HBSS, 1 mM Ca^{2+}, 10 mM HEPES and 0.1 % FCS; pH 7.4) and finally 500 µl of calcium buffer was added as working volume.

Calcium fluxes were monitored by ratiometric imaging using an Axiovert 200 M inverted microscope (Zeiss, Göttingen, Germany) equipped with a Plan-Apochromat dry objective 20x/0.8 in combination with the Zeiss fura-2 filter set. Temperature was controlled via an XL-3 incubator. Excitation light of the high speed filter changer Lambda DG-4 (Sutter instrument Company, Novato, USA) was switched between two filters selecting the wavelengths 340 nm and 380 nm corresponding to the maximal excitation wavelengths of calcium bound and calcium free fura-2, respectively. Emission light of 510 nm was recorded using an Axiocam MRm camera. The exposure times were fixed at 300 ms and 50 ms for the 340 nm and 380 nm excitation wavelengths, respectively, and interval settings were set at maximal speed. In each time lapse experiment, cells were stimulated after 2 min with 250 µl of preheated (37°C) calcium buffer solution containing chemokine.

Ratio analysis on single cell level was performed with the Axiovision software release 4.6.3. Regions of interest (ROI) were drawn around 49 randomly chosen cells. After background correction, the mean ratio value for each ROI was calculated by dividing the mean pixel value of the calcium-bound fura-2 form (excitation wavelength of 340 nm) by the mean pixel value of the calcium-free fura-2 form (excitation wavelength of 380 nm). In each experiment the ratio value of each condition was normalized against the mean ratio value after stimulation of the monocytes with 3 ng/ml CCL2 (100 %). Four independent experiments (different donors) were performed. In each experiment every condition was tested at least in triplicate.

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Induction experiments

Fibroblast monolayers were grown to confluency in 24-well plates in MEM containing 10% FCS. Fibroblasts were stimulated for 48 h with different doses of IL-1β (1, 10 or 100 U/ml) or IFN-γ (2, 20 or 200 ng/ml) or were left untreated (control). PBMC were seeded in 24-well plates in RPMI supplemented with 10% FCS at a concentration of 2 x 10^6 cells/ml (1 ml/well) and induced for 48 h with different doses of Con A (1 or 10 µg/ml), LPS (0.5, 5 or 50 µg/ml), PIC (10 or 100 µg/ml), IL-1β (1, 10 or 100 U/ml) or IFN-γ (2, 20 or 200 ng/ml) or were left untreated (control). Levels of human CXCL8 and CCL2 were quantified by specific sandwich ELISAs developed in our laboratory as previously described (Gijsbers et al., 2005).

FACS analysis

THP-1 cells and PBMC were incubated and washed twice with ice-cold FACS buffer (PBS supplemented with 2% FCS). Subsequently, cells (0.3 x 10^6 cells) were labeled with 50 µg/ml anti-CXCR4 antibody clone 12G5 (BD Pharmingen, Heidelberg, Germany), 50 µg/ml anti-CXCR1 clone 5A12 (BD Pharmingen) or 50 µg/ml anti-CXCR2 antibody clone 48311(R&D Systems), 50 µg/ml anti-CCR2 antibody clone 48607 (R&D Systems) or buffer for 30 min on ice. After washing, cells were incubated with 1.3 µg/ml PE-conjugated goat anti-mouse IgG polyclonal antibody (BD Pharmingen) for 30 min on ice in the dark. Finally, cells were washed three times with ice-cold FACS buffer, fixed in FACS buffer containing 4% paraformaldehyde and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Monocytes were gated by their forward

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scatter (FCS)/ side scatter (SSC) profile. It was previously confirmed that all cells within this gate are $CD14^{+}$.

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RESULTS

1. CXCL8 synergizes with CCL2 and CCL7 in monocyte migration

Previous findings demonstrated that CXC and CC chemokines synergized to chemoattract neutrophils in the Boyden chamber assay. This phenomenon was further investigated in monocytes, using the same chemokines but at inverse concentration ratios. Table 1 shows that in the Boyden microchamber assay a biologically active concentration (50 ng/ml) of CXCL8 significantly increased the monocyte chemotactic activity of CCL7 (3 and 10 ng/ml) above the additive effect of the individual chemokines. Moreover, CXCL8 dose-dependently increased the monocyte chemotactic activity of the CCR2 ligand CCL2 at suboptimal concentration (0.3, 1 and 3 ng/ml) in the microchamber assay (Fig. 1A**)**. CXCL8 alone at moderate concentration (30 ng/ml) had a weak monocyte chemotactic activity, indicating that this molecule functionally recognizes receptors expressed on monocytes. When active concentrations of CXCL8 (30 and 100 ng/ml) were added together with low concentrations of CCL2 (0.3, 1 and 3 ng/ml), the number of migrating monocytes was significantly increased above the sum of that reached with the individual chemokines (Fig. 1A**)**. Thus, a suboptimal concentration of CC chemokine can provide a maximal monocyte influx in the abundant presence of a weakly active CXC chemokine. This cooperation between these chemokines is relevant because of the co-expression of CCR2, CXCR1 and CXCR2 on monocytes (Bonecchi et al., 2000; Gerszten et al., 1999). Indeed, FACS analysis demonstrated the presence of both CCR2 and CXCR1,2 on freshly isolated monocytes used for the chemotaxis assay (Fig. 1C). Under pathological conditions CXC and CC chemokines can be co-induced by TLR ligands

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allowing synergy between these chemoattractants. Since this up-regulation can be evoked either directly by the TLR ligands (e.g. LPS, ConA or PIC) or indirectly via TLR ligands induced cytokines (e.g. IL-1β), it is possible that chemokines are simultaneously present *in vivo*, some at high and others at low concentrations. Indeed, CXCL8 and CCL2 are often co-expressed in monocytes and fibroblasts upon stimulation with the same inflammatory mediators such as cytokines (IL-1β and IFN-γ) or TLR ligands (ConA, PIC and LPS) (Fig. 2). Moreover, CXCL12, another CXC chemokine and weak monocyte agonist, when applied at high concentrations (30, 100 and 300 ng/ml) synergized with suboptimal concentrations of CCL2 (0.3 and 1 ng/ml) to chemoattract monocytes. This is in agreement with the finding that the CXCL12 receptor CXCR4 is also expressed on monocytes (Fig. 1B and C)**.**

2. Synergy between CC and CXC chemokines in monocytic THP-1 cell migration using different chemotaxis assay systems

Next, we tested the synergy between CC and CXC chemokines using the monocytic cell line THP-1. For comparison with monocytes expressing both CCR2 and CXCR4, we first confirmed the presence of these receptors on THP-1 cells by FACS analysis and calcium signaling experiments (Fig. 3A and data not shown). Both CCL2 and CXCL12 dose-dependently induced THP-1 cell chemotaxis using the Boyden chamber (n=2) but higher concentrations (10 to 100 ng/ml) of CXCL12 were required compared to CCL2 (1 to 10 ng/ml) (Fig. 3B)**.** Furthermore, recombinant CXCL12 augmented the migration of THP-1 cells to suboptimal concentrations of CCL2 but to reach statistically significant effects more experiments needed to be performed (Fig. 3B). To reach that goal we did not use

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the labour-intensive Boyden chemotaxis assay, but a new cell migration assay with an enzymatic read out, namely the Multiscreen chemotaxis assay, that makes it possible to screen the synergistic effect between chemokines on a larger scale. In order to test the synergy between CCL2 and CXCL12 on THP-1 cells, we repeatedly combined different concentrations of CCL2 (0.3, 1 and 3 ng/ml) together with multiple concentrations of CXCL12 (0.3, 1, 3, 10 and 30 ng/ml) in the lower compartment of this enzymatic 96-well plate chemotaxis assay**.** Figure 3C shows that in this test system, recombinant CXCL12 is almost as potent as CCL2 to chemoattract THP-1 cells**.** In addition, suboptimal concentrations of CXCL12 (0.3 and 1 ng/ml) significantly increased the chemotactic response of the $CXCR4^{+}/CCR2^{+}$ THP-1 cells towards a suboptimal concentration of CCL2 (0.3 and 1 ng/ml) (Fig. 3C). This indicates that the phenomenon of chemokine synergy for monocyte chemotaxis can be confirmed with an alternative migration test system using a monocytic cell line. By FACS analysis we demonstrated that during the chemotaxis assay period (3 h), CCL2 or CXCL12 did not alter the expression of CXCR4 and CCR2, respectively. As expected the chemokine CCL2 down-regulated its own receptor CCR2 on THP-1 cells (data not shown).

3. Lack of synergy between two CC chemokines or between two CXC chemokines in monocyte chemotaxis

In a further attempt to precisely delineate the spectrum of synergy between chemokines for monocytic chemotaxis, the two CC chemokines CCL2 and CCL7 were evaluated for their synergistic capacity in THP-1 chemotaxis, using the enzymatic migration assay system (Fig. 4A). The CCR2 agonist CCL2 and the CCR1, CCR2 and CCR3 agonist

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CCL7 alone have already detectable THP-1 cell chemotactic activity at 0.3 ng/ml (e.g. CI ± SEM of 1.6 ± 0.3 and 2.1 ± 0.4, respectively). However, these two THP-1 cell agonists were not able to synergize in the Multiscreen chemotaxis assay when tested at various concentrations (0.3, 1 and 3 ng/ml) (Fig. 4A). Even, a less than cumulative effect was observed when CCL2 and CCL7 were combined, maybe because of competition for CCR2 (Combadière et al., 1995). Furthermore, the CXC chemokines CXCL8 and CXCL12 were also not able to synergize in THP-1 cell chemotaxis (Fig. 4B). The fact that 300 ng/ml of CXCL8 and 3 ng/ml CXCL12 induced a weak but statistically significant THP-1 cell chemotactic activity confirms the expression of their receptors on these cells (Fig. 3A). These data show that chemokines competing for receptors using similar signaling pathways or receptors cross-regulating the functions of each other do not synergize for chemotaxis. We therefore investigated the phenomenon of synergy at the level of signal transduction.

4. CC and CXC chemokines synergize in signal transduction pathways in normal monocytes but not in chemokine receptor double-transfected CHO cells

To further elucidate the mechanisms through which synergy among CC and CXC chemokines on monocytes occurs, direct cellular responses important for cell migration were investigated. Since it has been reported that ERK1/2 activation is involved in CCL2-mediated monocyte migration (Yen et al., 1997), we examined the involvement of this pathway in the synergistic effect between CCL2 and CXCL8 on monocytes. Figure **5** shows that PBMC-derived adherent monocytes incubated for 2 min with CCL2 at 1 and 10 ng/ml induced significant phosphorylation of ERK1/2 protein compared with buffer-

treated cells (p= 0.007 and p= 0.0002, respectively). In contrast to CCL2, treatment with recombinant CXCL8(6-77) (50 and 500 ng/ml) did not lead to a significant ERK1/2 activation in monocytes. Furthermore, when monocytes were incubated for 2 min with combinations of different concentrations of CCL2 (0.1, 1 and 10 ng/ml) and CXCL8(6-77) (50 and 500 ng/ml) synergy was observed in ERK1/2 phosphorylation, in agreement with the fact that CCL2 synergizes with CXCL8 in monocyte migration (Fig. 1A). Indeed, the combination of 50 ng/ml CXCL8(6-77) with 10 ng/ml CCL2 significantly (p= 0.043) enhanced the phosphorylation of ERK1/2 compared with the ERK1/2 phosphorylation induced by CCL2 alone. Next, we investigated whether at other time points (5, 10 and 20 min) CCL2 and CXCL8 were also able to synergize to induce ERK phosphorylation in monocytes. In contrast to CXCL8, CCL2 induced significant phosphorylation of ERK1/2 in monocytes after incubation of the cells during 5, 10 and 20 min, compared to buffer-treated cells. Incubation of the monocytes with a combination of CCL2 (10 ng/ml) and CXCL8(6-77) (50 ng/ml) for 5 min also provided enhanced ERK phosphorylation as compared to incubation of the monocytes with CCL2 alone. However, when the monocytes were incubated during 10 and 20 min with a combination of CCL2 and CXCL8, no enhancement in ERK phosphorylation was observed compared to CCL2-treated cells. We can conclude that the synergistic effect on ERK phosphorylation is time point dependent (data not shown). Furthermore, we investigated whether a combination of CCL2 and CXCL12 can enhance the ERK1/2 phosphorylation in non-hematopoietic cells transfected with a single chemokine receptor (i.e. CHO/CXCR4 or CHO/CCR2) or in double-transfected CXCR4/CCR2/CHO cells (Fig. 6 and data not shown). CXCL12 (3, 30 and 300 ng/ml) and CCL2 (1, 10 and 100 ng/ml) induced significant ERK1/2

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phosphorylation within 5 min in CHO/CXCR4 and CHO/CCR2 cells, respectively, compared to buffer-treated cells. However, no synergy in the ERK1/2 signaling pathway could be observed in the cell lysates of CHO/CXCR4 and CHO/CCR2 cells when the cells were incubated during 5 min with different concentrations of CXCL12 in combination with CCL2 (Fig. 6A and data not shown)**.** Moreover, CXCL12 (0.3, 3, 30 and 300 ng/ml) and CCL2 (0.3, 3, 30 and 300 ng/ml) did not cooperate to enhance the phosphorylation of ERK1/2 in double-transfected CXCR4/CCR2 CHO cells (Fig. 6B). We can conclude that ERK1/2 phosphorylation induced by CCL2 can be significantly enhanced by CXCL8 in monocytes. In contrast, the synergistic effect in ERK1/2 phosphorylation observed on monocytes does not occur in single- or double-transfected CHO cells. This discrepancy can be due to the fact that GPCR transfected non-hematopoietic cells might miss essential mediators to allow synergy in one or more signaling pathways.

5. CC and CXC chemokines synergize in calcium signaling in monocytes

Many chemokines induce a rapid elevation of the cytosolic calcium level after binding to their GPCR. Using the single cell calcium assay, we demonstrated that CCL2 (3 and 10 ng/ml) was capable to trigger a detectable calcium signal in adherent monocytes. Indeed, the mean ratio value after stimulation of the monocytes with 3 and 10 ng/ml CCL2 was 0.07 and 0.12, respectively (Fig. 7A and data not shown). To investigate the synergy between CCL2 and CXCL8 in the single cell calcium assay on monocytes, we normalized in each experiment the ratio value of each condition against the mean ratio value after stimulation of the monocytes with 3 ng/ml CCL2 (=100 %) (Fig. 7B).

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Simultaneous stimulation of monocytes with CCL2 (3 ng/ml) and CXCL8 (100 ng/ml) significantly enhanced the single cell intracellular calcium response compared to CCL2 alone, indicating that this signaling pathway is involved in the synergy between CC and CXC chemokines in monocyte chemotaxis. Indeed, the mean ratio value after stimulation of the monocytes with a combination of CCL2 (3 ng/ml) and CXCL8 (100 ng/ml) was 211 %, whereas the ratio value after stimulation of the cells with CCL2 and CXCL8 alone was 100 % and 40 %, respectively (Fig. 7B).

6. Effect of proteolytic processing of chemokines on synergy in monocyte chemotaxis

In order to further elucidate the implication of chemokine specific receptor signaling in the synergy between GPCR ligands in chemotaxis, experiments were performed using intact and NH_2-terminally truncated chemokines with impaired receptor signaling capacity. For this purpose, either intact CXCL12 or CXCL12 lacking the NH_2-terminal dipeptide was used in combination with CCL2. In contrast to intact CXCL12, the truncated form did not induce a calcium response in CHO/CXCR4 cells (Table 2) (Gouwy et al., 2004). Moreover, truncated CXCL12(3-68) only partially desensitized the calcium mobilizing capacity of intact CXCL12 indicating its impaired CXCR4 signaling capacity. Next, it was shown that the truncated CXCL12(3-68) was not able to synergize with CCL2 in the THP-1 cell Multiscreen migration test, whereas intact synthetic CXCL12 did (Fig. 3C and Fig. 8). These data suggest that, to obtain synergy, two chemokines must bind and signal via their proper receptor (Gouwy et al., 2004).

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7. The synergistic effect between CCL2 and CXCL12 in THP-1 cell chemotaxis is inhibited by receptor specific antagonists.

To demonstrate that the synergy between CCL2 and CXCL12 in THP-1 cell chemotaxis implies receptor mediated events, the combination of CCL2 and CXCL12 was evaluated in the Multiscreen chemotaxis assay in the presence of their receptor specific antagonists RS102895 (CCR2) and AMD3100 (CXCR4) (Fig. 9). The chemotactic effect of CCL2 (1 ng/ml) and CXCL12 (2 ng/ml) alone was blocked (mean % inhibition ± SEM of 36.3 ± 13.5 and 47.3 ± 8.6, respectively) in the presence of RS102895 and AMD3100, respectively. RS102895 (1 μM) as well as AMD3100 (1 μg/ml) significantly reduced ($p=0.02$ and $p=0.0064$, respectively) the synergistic effect between CCL2 (1 ng/ml) and CXCL12 (2 ng/ml) observed in the absence of receptor antagonist. Thus, it seems that both chemokine receptors are implicated in the synergistic effect of chemokines on monocytic cell migration.

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DISCUSSION

Chemokines constitute a family of GPCR binding proteins, each acting on a selective set of target cells, predominantly leukocytes, defined by their receptor specificity. *In vitro* Induction of chemokines by cytokines or TLR ligands has been reported for various cell types. This induction pattern can be chemokine specific depending on both the cell type and the precise stimuli (Gouwy et al., 2005). *In vivo* microbial infection triggers production of many chemokines simultaneously either directly in response to TLR ligands or indirectly to induced inflammatory cytokines, which might act synergistically. As an additional dimension, cooperation between chemokines to enhance the inflammatory response should also be considered. Indeed, we previously showed that suboptimal concentrations of the neutrophil chemoattractant CXCL8 synergized with high concentrations of monocyte attracting CC chemokines (e.g. CCL2 or CCL7) to enhance neutrophil migration (Gouwy et al., 2004). In this study, we show that CXCL8 and CXCL12 significantly increase the chemotactic response of monocytes towards a suboptimal concentration of CCL2 or CCL7. In contrast, the combination of two CC chemokines i.e. CCL2 and CCL7 or two CXC chemokines i.e. CXCL8 and CXCL12 did not provide synergy in monocyte chemotaxis. These data show that chemokines competing for identical receptors or for different GPCR but using similar signaling pathways (e.g. CCL2 and CCL7) or for receptors cross-regulating the functions of each other (e.g CXCR1 and CXCR4) do not synergize for chemotaxis. Indeed, CCL7 probably utilizes the same receptor CCR2 and signal transduction pathways as CCL2 in monocytes, because CCL2 cross-desensitizes the calcium and chemotactic response of CCL7 and vice versa in these cells (Sozzani et al., 1994; Sozzani et al., 1995). Moreover,

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CXCL8 activation of CXCR1 cross-phosphorylates CXCR4 and cross-desensitizes the responsiveness of monocytes to CXCL12 (Richardson et al., 2003). This desensitization between chemokine receptors could explain the lack of synergy between CCL7 and CCL2 or between CXCL8 and CXCL12 in monocytic cell migration. From these observations, it must be deduced that synergy between chemokines is not a general phenomenon and only exists for specific chemokine pairs on specific leukocyte types.

The cellular mechanisms involved in chemokine synergy to attract monocytes have not been investigated in detail and the suggested mode of action for synergy to attract other leukocytes or receptor-transfected cells are not concurrent. Some studies claimed that chemokine or receptor dimerization may be implicated in the synergistic effect between chemokines (Mellado et al., 2001; Paoletti et al., 2005, Sebastiani et al., 2005). Simultaneous stimulation of PBMC with CCL2 and CCL5 induced the formation of CCR2/CCR5 heterodimers and enhanced the calcium signal and chemotactic response when compared to addition of these chemokines alone (Mellado et al., 2001). In contrast, El-Asmar et al. (2005) did not observe such a synergistic effect between these chemokines in calcium signaling upon co-stimulation of CCR5 and CCR2 expressed in CHO cells, whereas Springael et al. (2006) rather found a negatively binding cooperativity. We observed that simultaneous stimulation of monocytes with CCL2 and CXCL8 did enhance the intracellular calcium signal monitored at single cell level, indicating that this signaling pathway is involved in the synergy between CC and CXC chemokines. Sohy et al. (2007) observed GPCR heterodimers formed by CCR2 and CXCR4 in primary leukocytes. However, chemotaxis experiments with activated $CD4^+$ T lymphocytes showed that migration towards CXCL12 or a combination of CXCL12 and

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CCL2 at equimolar concentrations was similar (Sohy et al., 2007). In order to further elucidate the mechanism of the synergistic effect between CCL2 and CXCL12 at the receptor binding and signaling level, we performed chemotaxis experiments using intact CXCL12 or CXCL12(3-68) lacking the NH_2-terminal dipeptide, hence exerting weak chemotactic activity. In contrast to the intact CXCL12, the truncated CXCL12(3-68) was not able to synergize with CCL2 in THP-1 cell migration. Since CXCL12(3-68) has impaired signaling capacity through CXCR4 compared to CXCL12, these data corroborate with our previous findings that, to obtain neutrophil synergy, binding and signaling of the two chemokines to their proper receptor is most likely necessary (Gouwy et al., 2004). These findings are different from those obtained with a CCR4- or CCR7-transfected murine pre-B cell line, in which a single receptor type was reported to be sufficient for synergy between two chemokines recognizing different receptors (Sebastiani et al., 2005; Paoletti et al., 2005). Furthermore, the specific CCR2 and CXCR4 inhibitors RS102895 and AMD3100, inhibited the synergistic effect between CCL2 and CXCL12 indicating that this phenomenon implies receptor mediated events.

Another possible mechanism of the cooperation between chemokines is synergy between these mediators at the level of intracellular signal transduction. Chemokines bind to their GPCR to trigger multiple independent signal transduction pathways (Neel et al., 2005). Although, several reports demonstrated that CCL2 can affect several secondary messengers (Dubois et al., 1996; Cambien et al., 2001), data concerning the exact relationships between these different signaling cascades and their impact on the migratory response are conflicting (Ashida et al., 2001; Jimenez-Sainz et al., 2003). For instance, Yen et al. (1997) demonstrated that MCP-1 induced rapid and transient activation of

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ERK1/2 in human monocytes and in CHO cells expressing CCR2 and that an inhibitor of ERK1/2, i.e. PD980959, impaired the CCL2-induced chemotaxis of these cells. The same inhibitory effect on cell migration was observed for the PI3K inhibitor wortmannin, although this compound was not effective in the prevention of ERK1/2 activation. These data were further confirmed by Wain et al. (2002) who have shown that an inhibitor of MAPK-kinase activation significantly inhibited the CCL2 mediated chemotaxis. Moreover, wortmannin also partially inhibited the CCL2-induced chemotaxis, although this inhibitor did not significantly inhibit ERK1/2 activation. These data suggest the existence of a coordinated action of multiple independent signal transduction pathways to induce chemokine-mediated chemotaxis (Cambien et al., 2001). On the contrary, according to Fine et al. (2001) neither PI3K nor ERK1/2 activity was required for monocyte migration towards CCL2, since pretreatment of monocytes with wortmannin or PD980959, respectively, had no effect on the chemotactic response. In this study we confirmed that, CCL2 induces phosphorylation of ERK1/2 in monocytes. Moreover, ERK1/2 phosphorylation induced by CCL2 can be significantly enhanced by CXCL8 in monocytes. In contrast, the synergistic effect in ERK1/2 phosphorylation observed in monocytes does not occur in single CCR2- or CXCR4- or in CCR2/CXCR4 double transfected CHO cells treated with a combination of CCL2 and CXCL12. This suggests that the synergy in chemotaxis is dependent on post-receptor binding signaling pathways, not functional in these transfectants. Indeed, this may be due to the different availability of certain G protein subunits or the expression level of other downstream mediators in different cell types. Arai et al. (1996) demonstrated that in transfected COS-7 cells, CCR2 was coupled to $G_{\alpha i}$, $G_{\alpha q}$ and $G_{\alpha 16}$, whereas in transfected HEK-293 cells, CCR2

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was coupled to $G_{\alpha q}$ but failed to couple to $G_{\alpha 16}$. Nevertheless, Sebastiani et al. (2005) observed such a synergistic effect in ERK1/2 phosphorylation after co-stimulation of single CCR4-transfected murine pre-B cell line with the CCR4 ligand CCL22 in combination with CXCL10. Moreover, CXCL13, which on its own did not lead to ERK1/2 activation in CCR7-transfected murine pre-B cells, synergistically augmented the phosphorylation of ERK1/2 induced by suboptimal concentrations of CCL21 (Paoletti et al., 2005). It needs to be emphasized that these latter studies used a receptor expression system. Physiologically more relevant, the synergistic effect between C3a and CXCL12 to promote the homing of hematopoietic progenitor cells to the bone marrow was not mediated by the CXCL12-dependent activation of the ERK1/2 or PI3K signal transduction pathway (Reca et al., 2003). Finally, it cannot be excluded that minimally enhanced multiple signaling pathways each in part contributes to provoke in concert a synergistic chemotactic response.

Chemokines play a complex role in various inflammatory diseases and the apparent redundancy in their expression requires improved concepts defining the cooperation of chemokines in regulating the recruitment of mononuclear cells (Weber et al., 2004). Both, the chemokine concentration and the presence of a cooperating chemokine determine the number of mononuclear cells that will infiltrate the inflamed tissue. It can be concluded that the receptor signal transducing capacity must remain unaffected to guarantee chemokine synergy at inducing cell migration. As a consequence, proteolytic processing of chemokines during the inflammatory response not only leads to impaired chemotaxis, but also to incapability to synergize with other chemokines. This double negative feed back loop is even further reinforced by the fact that by their residual

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receptor binding capacity, truncated chemokines antagonize intact chemokines by competing for receptor binding. The synergy between chemokines in leukocyte migration may enhance an inflammatory response. As a consequence, antagonization of a single chemokine may down-modulate immune responses, due to the inhibitory effect on its synergy with other chemokines.

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FOOTNOTES

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LEGENDS OF FIGURES

Figure 1: Synergy between CXC (CXCL8 or CXCL12) and CC chemokines (CCL2) in monocyte migration using the Boyden microchamber

Panel A: Natural CXCL8 (30, 100 and 300 ng/ml) was combined with different concentrations of CCL2 (0.3, 1 and 3 ng/ml) in the lower compartment of the microchamber to measure monocyte chemotaxis. The chemotactic response is expressed as the mean chemotactic index, derived from 4 to 13 independent experiments. Panel B: CXCL12 (30, 100 and 300 ng/ml) was combined with different concentrations of CCL2 (0.3, 1 and 3 ng/ml) in the lower compartment of the microchamber to measure monocyte chemotaxis. The chemotactic response is expressed as the mean chemotactic index, derived from 3 to 17 independent experiments. Symbols (∗ and †) are explained in the Materials and Methods section. Panel C: FACS analysis for chemokine receptor expression on PBMC-derived monocytes was performed as described in Materials and Methods. One representative experiment out of 3 is shown. The filled curves represent the staining by the chemokine-receptor specific monoclonal antibody. The open curves represent the background staining with secondary antibody only.

Figure 2: Co-production of CCL2 and CXCL8 in mononuclear leukocytes and fibroblasts after induction by inflammatory mediators

PBMC (panel A and B) or confluent fibroblast monolayers (panel C) were stimulated for 48 h with different doses of Con A (1 or 10 µg/ml), LPS (0.5, 5 or 50 µg/ml), PIC (10 or 100 µg/ml), IL-1β (1, 10 or 100 U/ml) or IFN-γ (2, 20 or 200 ng/ml) or were left

untreated. Results represent the mean CXCL8 and CCL2 protein concentration from 3 to 8 independent experiments, measured in the culture supernatant by ELISA. Significant chemokine induction is indicated by asterisks (*p<0.05; **p<0.01).

Figure 3: Synergy between CXCL12 and CCL2 in THP-1 cell migration using different chemotaxis assay systems

Panel A: FACS analysis for chemokine receptor expression in THP-1 cells was performed as described in Materials and Methods. One representative experiment out of 3 is shown. The filled curves represent the staining by the chemokine receptor-specific monoclonal antibody. The open curves represent the background staining with secondary antibody only. Panel B: Recombinant CXCL12 (0-300 ng/ml) was combined with different concentrations of CCL2 (0-10 ng/ml) in the lower compartment of the Boyden microchamber to measure THP-1 cell chemotaxis. The chemotactic response is expressed as the mean chemotactic index (CI), derived from 2 independent experiments. Panel C: Different concentrations of CCL2 (0-3 ng/ml) were combined with multiple concentrations of recombinant CXCL12 (0-30 ng/ml) in the lower compartment of the enzymatic Multiscreen chemotaxis assay to measure THP-1 cell chemotaxis. The chemotactic response is expressed as the mean chemotactic index (CI), derived from 3 to 10 independent experiments. Symbols (∗ and †) are explained in the Materials and Methods section.

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Figure 4: Lack of synergy between CC (CCL2 and CCL7) chemokines or between CXC (CXCL8 and CXCL12) chemokines in monocytic THP-1 cell chemotaxis

CCL7 (0.3, 1 and 3 ng/ml) and CCL2 (0.3, 1 and 3 ng/ml) (panel A) or CXCL8 (0.3, 3, 30 and 300 ng/ml) and CXCL12 (0.3, 1 and 3 ng/ml) (panel B) were combined in the lower compartment of the enzymatic Multiscreen chemotaxis assay to measure THP-1 cell chemotaxis. The chemotactic response is expressed as the mean chemotactic index (CI), derived from 5 to 9 independent experiments. Symbols (∗ and †) are explained in the Materials and Methods section.

Figure 5: Synergy between CC and CXC chemokines in the ERK1/2 pathway in monocytes

PBMC-derived adherent monocytes were stimulated with different concentrations of CCL2 (0.1, 1 and 10 ng/ml), recombinant CXCL8(6-77) (50 and 500 ng/ml) or a combination of CCL2 and CXCL8(6-77). The level of ERK1/2 phosphorylation in the cell lysate was determined by ELISA for phosphoERK1/2. The mean values and standard errors are derived from 5 to 12 independent experiments. Statistically significant ERK1/2 phosphorylation induced by CCL2 compared to medium-treated cells determined by the Mann-Whitney U test is indicated (‡ $p<0.01$). Statistically significant differences in ERK1/2 phosphorylation between the combination of CCL2 and CXCL8(6-77) and the ERK1/2 phosphorylation induced by CCL2 alone, determined by the Sign test, are indicated by asterisks (*$p<0.05$). Symbols (∗ and †) are explained in the Materials and Methods section.

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Figure 6: Failure of CC and CXC chemokines to synergize in ERK1/2 pathway in CHO/CXCR4 or CHO/CCR2/CXCR4 cells

Serum-starved CXCR4 (panel A) or CCR2/CXCR4 double-transfected (panel B) CHO cells were stimulated with different concentrations of CXCL12 or CCL2 or a combination of CXCL12 and CCL2. The level of ERK1/2 phosphorylation in the cell lysate was determined by ELISA for phosphoERK1/2. The mean values and standard errors are derived from 1 to 4 independent experiments.

Figure 7: CCL2 and CXCL8 chemokines synergize in calcium signaling in monocytes.

Adherent PBMC-derived monocytes were loaded with FURA-2, washed and stimulated with CCL2 (3 ng/ml), CXCL8 (100 ng/ml) or a combination of CCL2 (3 ng/ml) and CXCL8 (100 ng/ml). Panel A: One representative experiment is shown out of four independent experiments. The ratio value for each ROI was calculated by dividing the mean pixel value of the calcium bound FURA-2 form by the mean pixel value of the calcium free FURA-2 form. Panel B: Data are represented as box and whisker plots in which the small squares indicate the median value of a group. The box is outlined by the first quartile at the bottom and the third quartile at the top and encloses the middle 50 % of the data (interquartile range). Whiskers extend to the non-outlier range of the data set. Circles depict outliers, with a value > 1.5 times the interquartile range. The ratio value was calculated for each condition and normalized against the ratio value after stimulation of the monocytes with 3 ng/ml CCL2 (100 %). Four different experiments were performed. Statistically significant differences between the calcium increase after

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stimulation with the combination of CCL2 and CXCL8 and the calcium increase induced by CCL2 alone, determined by the Sign test, are indicated by asterisks (*p<0.05).

Figure 8: NH_2-terminal processing of CXCL12 impairs synergy with CCL2 in monocyte chemotaxis

Different concentrations of CCL2 (0.3, 1 and 3 ng/ml) were combined with multiple concentrations of synthetic CXCL12(1-68) (1 or 10 ng/ml) or synthetic CXCL12(3-68) (1, 30 or 300 ng/ml) in the lower compartment of the enzymatic Multiscreen chemotaxis assay to measure THP-1 cell chemotaxis. The chemotactic response is expressed as the mean chemotactic index (CI), derived from 4 independent experiments. Symbols (∗ and †) are explained in the Materials and Methods section.

Figure 9: Inhibition of the synergistic effect between CCL2 and CXCL12 by chemokine receptor antagonists.

CCL2 (1 ng/ml), CXCL12 (2 ng/ml) and CCL2 (1 ng/ml) plus CXCL12 (2 ng/ml) were added in the lower compartment of the enzymatic Multiscreen chemotaxis assay to measure THP-1 cell chemotaxis. RS102895 (1 μM), AMD3100 (1 μg/ml) or buffer were added to the cells just before loading in the upper compartment of the Multiscreen plate. The chemotactic response is expressed as the mean chemotactic index (CI), derived from 6 independent experiments. The combination of CCL2 and CXCL12 resulted in a significant (p=0.03) increase in chemotactic index. The statistically significant reductions in synergy between CCL2 and CXCL12 in the presence of RS102895 and AMD3100 are determined by the Mann-Whitney test and are indicated by an asterisk (*p<0.05;

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**p<0.01). The statistically significant inhibition of the chemotactic activity of CCL2 or CXCL12 in the presence of RS102895 or AMD3100 are determined by the Mann-Whitney test and are indicated as † p<0.05 and ‡ p<0.01.

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Table 1: CXCL8 synergizes with CCL7 in monocyte migration

CXCL8[(a)] (ng/ml)	CCL7[(a)] (ng/ml)	CI ± SEM [(b)]	p-value [(c)]
0	3	2.7 ± 1.4	
0	10	5.4 ± 1.6	
0	30	19.3 ± 8.8	
50	0	4.3 ± 0.3	
50	3	13.2 ± 2.1	0.02
50	10	13.7 ± 1.1	0.02
50	30	27.6 ± 5.6	0.38

[(a)] CCL7 (3, 10 and 30 ng/ml) was combined with buffer or 50 ng/ml CXCL8 in the lower compartment of the Boyden microchamber to measure monocyte chemotaxis.

[(b)] The chemotactic response is expressed as the CI ± SEM, derived from 4 to 8 independent experiments.

[(c)] Statistical analysis, see Materials and Methods

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Table 2: Desensitization of Ca^{2+} response induced in CHO/CXCR4 cells by synthetic CXCL12/SDF-1(1-68)

First stimulus		Second stimulus		
Chemokine (ng/ml)	Increase in $[Ca^{2+}]_i$ (nM)[(a)]	CXCL12(1-68) (ng/ml)	Increase in $[Ca^{2+}]_i$ (nM)[(a)]	% inhibition[(b)]
CXCL12(1-68) 10	304	10	63	79
CXCL12(3-68) 100	0	10	323	0
CXCL12(3-68) 300	0	10	368	0
CXCL12(3-68) 1000	0	10	166	45
CXCL12(3-68) 3000	0	10	72	76

[a] results shown are the mean of two experiments, intracellular calcium responses were determined as described in Gouwy et al., (2004).

[b] the percentage inhibition of the second stimulus by the first stimulus.

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Fig 1

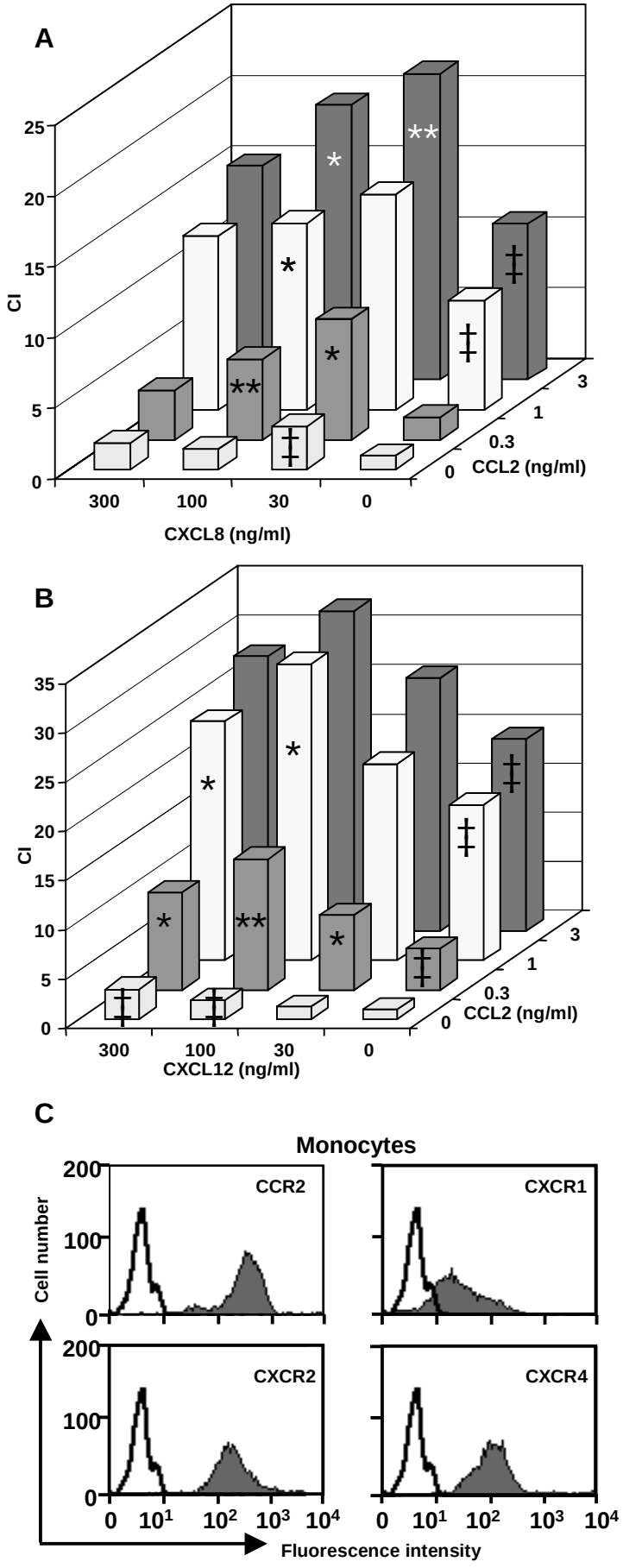

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Fig 2

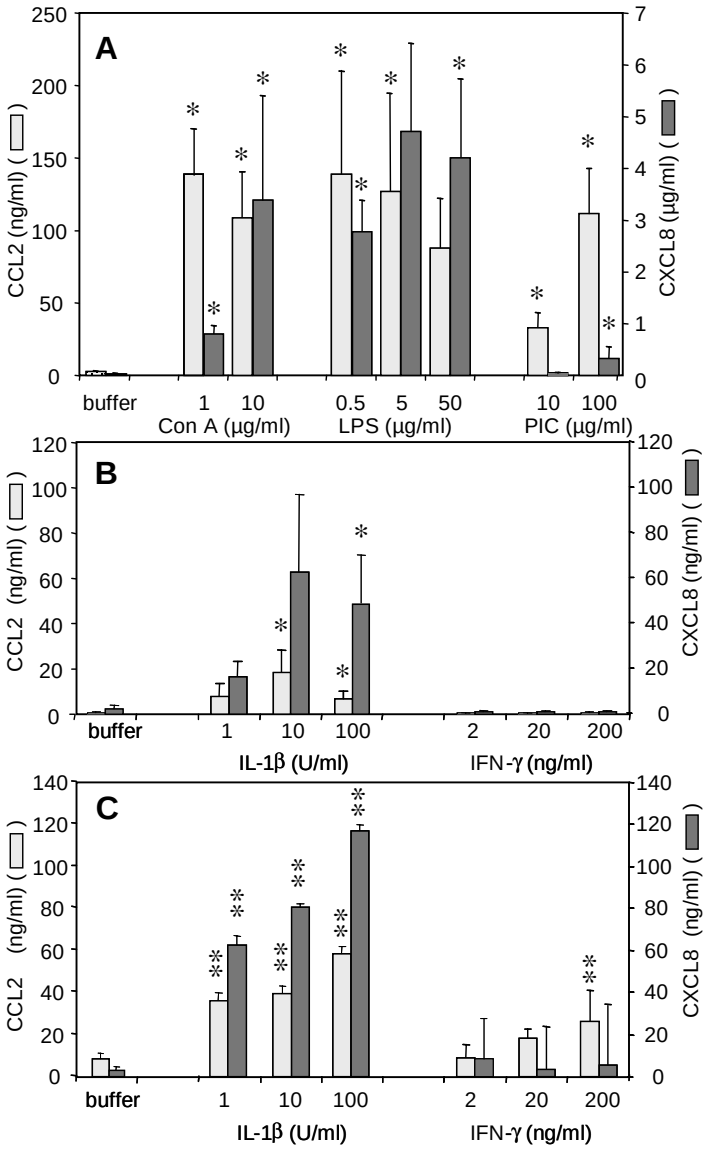

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Fig 3

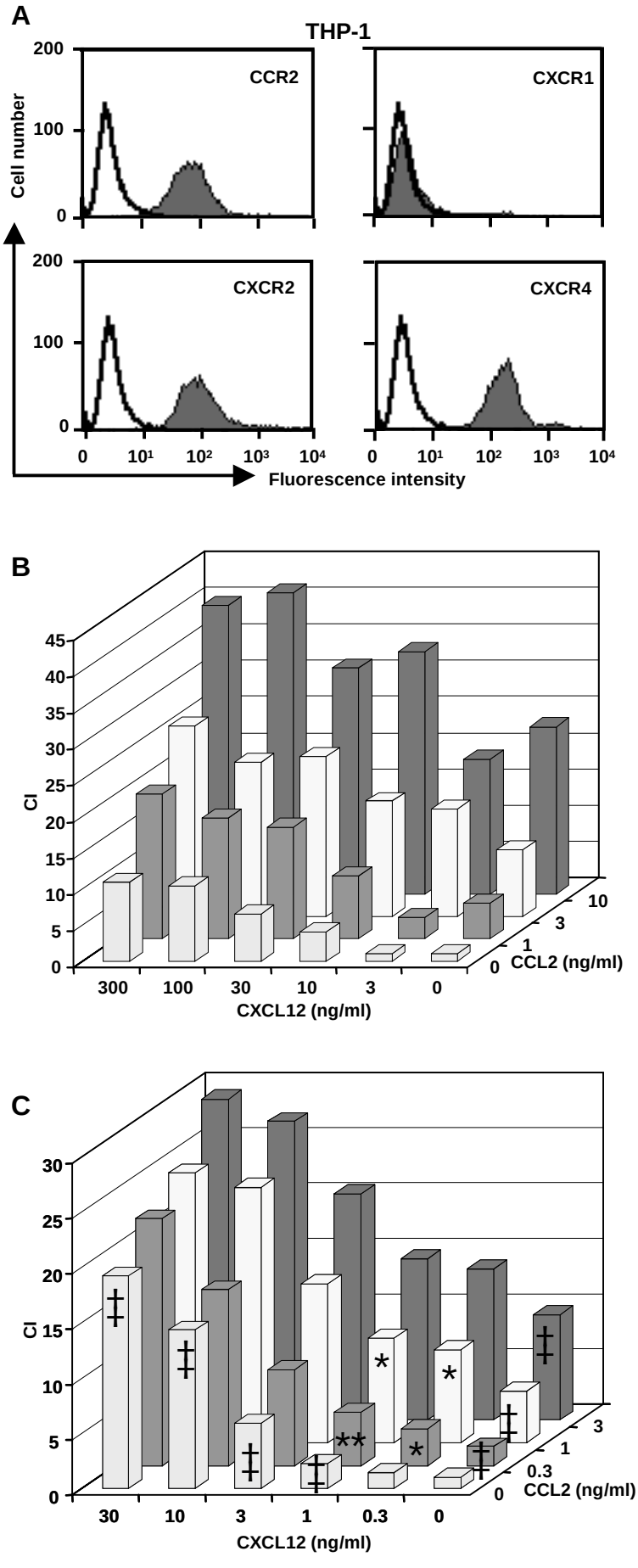

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Fig 4

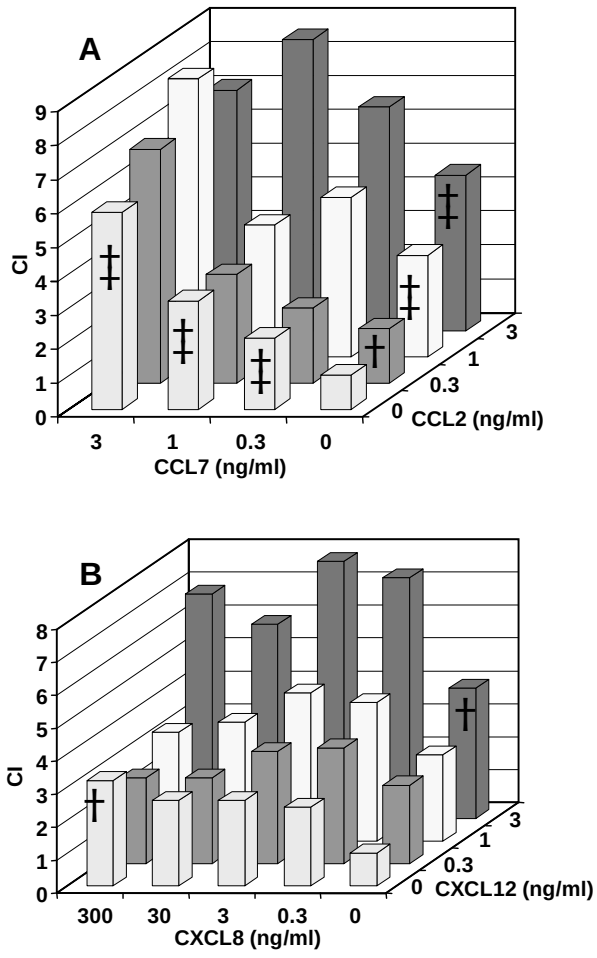

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Fig 5

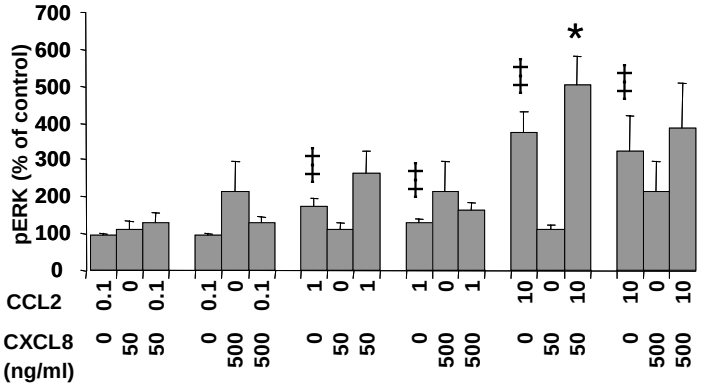

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Fig 6

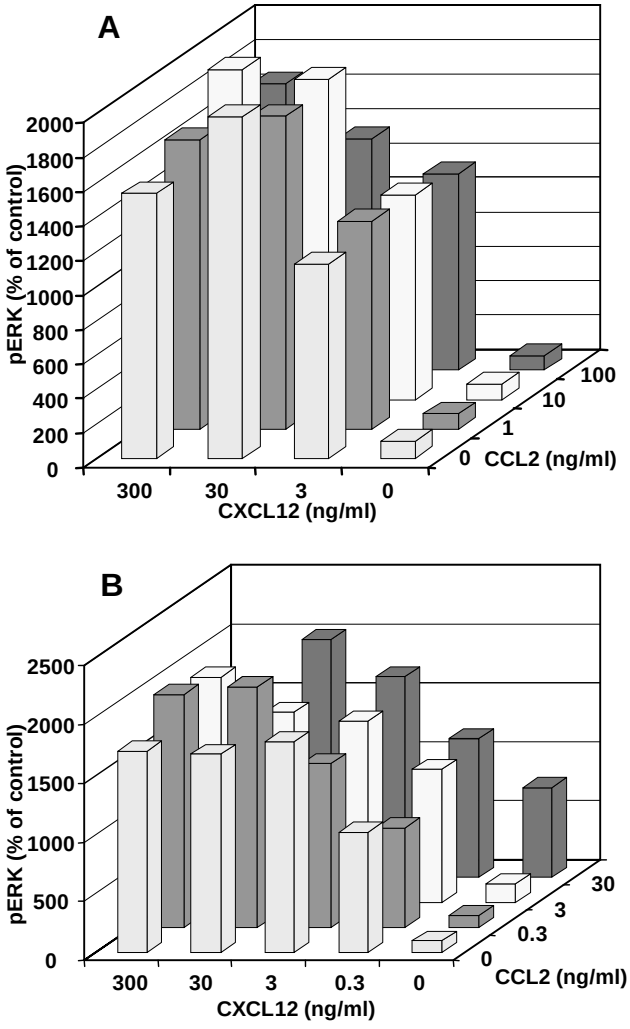

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Fig 7

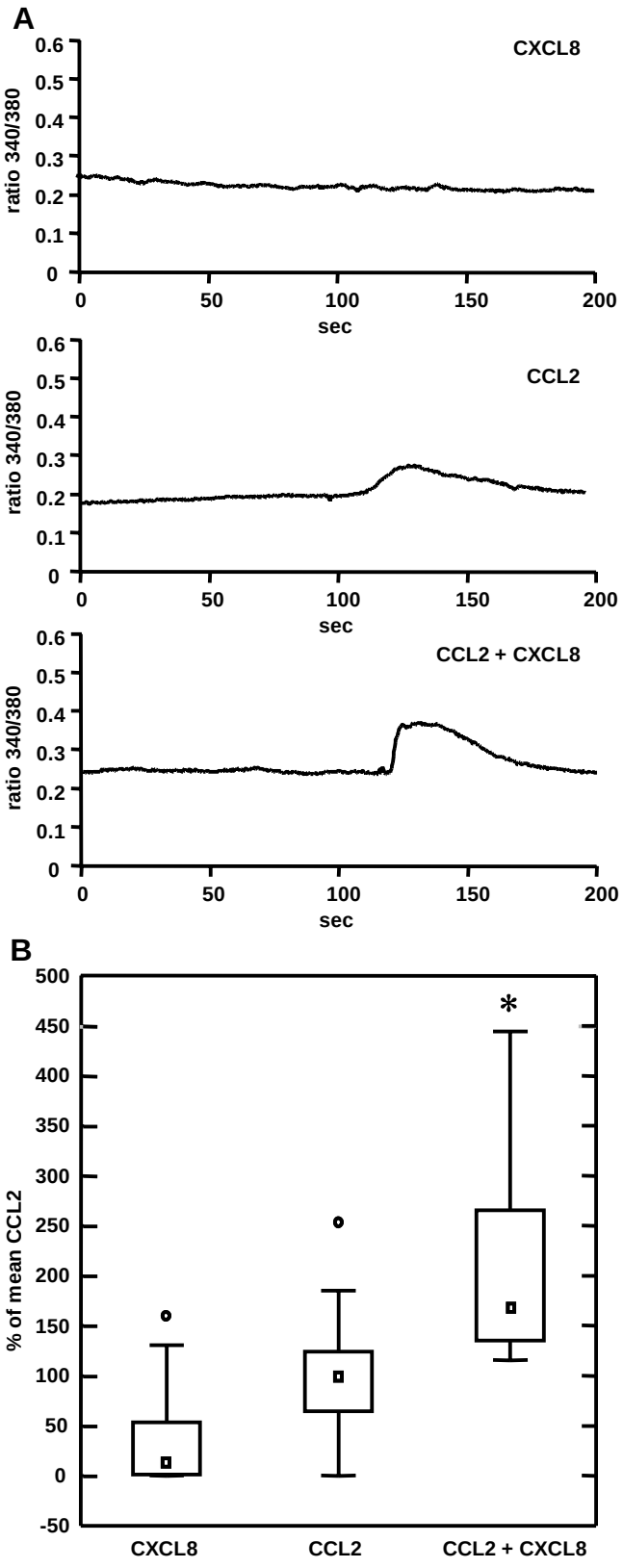

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Fig 8

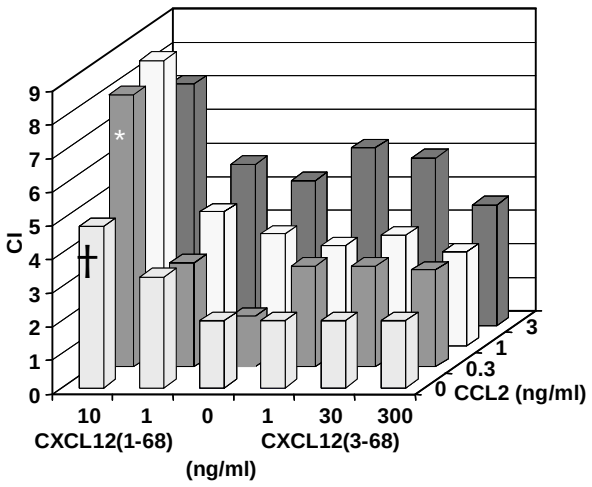

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Fig 9