Angiostatic and chemotactic activities of the CXC chemokine CXCL4L1 (platelet factor-4 variant) are mediated by CXCR3

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Short title: CXCL4L1 uses CXCR3 for angiostasis and chemotaxis

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Abstract

We investigated possible cellular receptors for the human chemokine platelet factor-4 variant/CXCL4L1, a potent inhibitor of angiogenesis. We found that CXCL4L1 has lower affinity for heparin and chondroitin sulphate-E than CXCL4 and showed that CXCL10 and CXCL4L1 could displace each other on microvascular endothelial cells. Labeled CXCL4L1 also bound to CXCR3A- and CXCR3B-transfectants and was displaced by CXCL4L1, CXCL4 and CXCL10. The CXCL4L1 anti-angiogenic activity was blocked by anti-CXCR3 Abs in the Matrigel and cornea micropocket assays. CXCL4L1 application in CXCR3+/− or in wildtype mice treated with neutralizing anti-CXCR3 Abs, resulted in reduced inhibitory activity of CXCL4L1 on tumor growth and vascularization of Lewis lung carcinoma. Furthermore, CXCL4L1 and CXCL4 chemoattracted activated T cells, human NK cells and human immature dendritic cells (DC). Migration of DC towards CXCL4 and CXCL4L1 was desensitized by pre-incubation with CXCL10 and CXCL11, was inhibited by pertussis toxin and neutralized by anti-CXCR3 Abs. Chemotaxis of T cells, NK cells and DC is likely to contribute to the anti-tumoral action. However, the in vivo data indicate that the angiostatic property of CXCL4L1 is equally important in retarding tumor growth. Thus, both CXCR3A and CXCR3B are implicated in the chemotactic and vascular effects of CXCL4L1.

Introduction

The chemokine family of chemotactic cytokines consists of rather small proteins (7-12 kDa) that signal via G protein-coupled receptors, designated CCR or CXCR, and regulate leukocyte recruitment to inflammatory sites, as well as leukocyte traffic between immunological compartments. Other target cells of chemokines include tumor cells and endothelial cells and consequently, chemokines play a role in tumor development.1,2 For example, the CXCR3 ligands, CXCL9, CXCL10 and CXCL11 are chemotactic for anti-tumoral lymphocytes and inhibit angiogenesis. However, some members of the chemokine family, e.g. the CXCR2 ligands CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8, favour tumor growth by attracting neutrophils, by stimulating the release of matrix metalloproteinases, by acting as growth factors and by promoting angiogenesis. One of the first chemokines that was investigated as an anti-cancer therapeutic is CXCL4 or platelet factor-4.1,2,3

The human CXC chemokine platelet factor-4 (CXCL4) is encoded by 2 genes, located side by side on chromosome 4 and probably arose through duplication.4,5 The two genes are indeed highly related and give rise to mature proteins that differ in only 3 amino acid residues in the COOH-terminal part. Analysis of conditioned media from thrombin-treated platelets revealed that both CXCL4 genes are translated into proteins.6 Afterwards, tumor cells and smooth muscle cells were identified as alternative cellular sources for CXCL4L1 but not for CXCL4, indicating that not every cell type which produces CXCL4, also releases CXCL4L1 and vice versa.7,8 The fact that tumor cells produce angiostatic CXCL4L1 provides a negative feed-back on tumor growth, since CXCL4L1 is more potent than CXCL4 in angiostatic assays.6 In this manuscript we confirm that the anti-tumoral activity of CXCL4L1 is exerted through inhibition of angiogenesis and demonstrate that this angiostatic activity is
CXCR3-dependent in human and mouse models. Furthermore, we also show for the first time that CXCL4L1 attracts human and mouse activated T lymphocytes, human dendritic cells (DC) and human natural killer (NK) cells and that CXCL4L1-induced chemotaxis of immature DC is mediated by CXCR3, coupling to a Gαi protein. In addition to CXCR3A and CXCR3B interaction, binding to glycosaminoglycans was explored as well.

**Methods**

An extended Methods section is available online (Supplemental data). All animal studies were approved by the institutional ethics committees of all participating universities.

**Chemokines and antisera**

Natural human CXCL4 was isolated from stimulated platelets as previously described.6 Recombinant human CXCL4L1 was produced in *E. coli* and purified by a 4-step chromatographical procedure.9 Human CXCL11 and CXCL12 were chemically synthesized.10 Monoclonal anti-human CXCR3/CD183 (Clone 1C6) and anti-human CXCR3/CD183 (Clone 49801) antibodies (Abs) and the corresponding isotype controls were purchased from BD Biosciences (San Diego, CA) and R&D Systems (Abingdon, UK), respectively. Recombinant human and mouse chemokines were ordered from PeproTech (Rocky Hill, NY). TAMRA [5(6)-carboxy-tetramethylrhodamine]-labeled CXCL10 was produced by Invitrogen (Eugene, OR). Polyclonal Abs were raised in goats or rabbits and were applied in several *in vitro* and *in vivo* assays.

**Tumorigenesis models**

The anti-tumoral effect of CXCL4L1 was tested in the human A549 adenocarcinoma and murine Lewis lung carcinoma (LLC) models as described previously11,12 using wild type and CXCR3−/− animals on a C57Bl/6 background.13

**Matrigel assay**

Human dermal microvascular endothelial cells (HMVEC-d) were plated on Matrigel-coated 48-well plates (Matrigel matrix from BD Biosciences with high growth factor content). Reorganization of the endothelial cells into tubular structures was followed using an Axiovert 200 M inverted microscope. Results are expressed as percentage inhibition compared to the control cultures containing growth medium alone.

**Rat cornea micropocket assay for angiogenesis**

*In vivo* angiogenesis was assessed using the rat cornea micropocket assay as previously described.6 Chemokines were diluted in Hydron casting solution and PBS plus 0.25% serum albumin to their final concentration with and without anti-murine CXCR3 or control antibody. Six days after implantation, corneas were harvested and photographed.
Chemotaxis
Human peripheral blood mononuclear cells were purified from buffy coats from healthy volunteers and cultured to generate human IL-2-activated T lymphocytes and immature DC. Boyden chamber chemotaxis assays with these cells were performed as previously described. Migration conditions for activated mouse T lymphocytes (generated from splenocytes by treatment with IL-2) were similar to conditions used for activated human T cells. Migration of human NK cells was evaluated using Transwell chambers.

Synthesis of fluorescently labeled CXCL4L1
Synthetic CXCL4L1, prepared by fluorenylmethoxycarbonyl solid phase peptide synthesis, was NH2-terminally labeled with TAMRA (Novabiochem, Hohenbrunn, Germany) on the peptide synthesizer. Subsequently, unlabeled and TAMRA-labeled CXCL4L1 were deprotected, cleaved from the resin and purified by RP-HPLC as described. Proteins with the correct Mr were folded and repurified by RP-HPLC. We demonstrated that chemical synthesis and addition of the TAMRA label to the NH2-terminus of CXCL4L1 did not affect its biological activity. Indeed, TAMRA-CXCL4L1 attracted immature dendritic cells (data not shown).

Binding and signaling assays
CHO-K1 cells were transfected with CXCR3A, CXCR3B or CXCR7 as described previously. These transfected cell lines were used in calcium and ERK signaling assays to identify the human CXCL4L1 receptor. Binding of TAMRA-CXCL4L1 to receptor-transfected cells was performed using confluent monolayers of CXCR3-transfected CHO cells seeded in 6-well plates. Cells were pre-chilled and incubated on ice for 2 h with 300 ng/ml TAMRA-CXCL4L1 in the absence or presence of competing unlabeled chemokines. Cell lysates were cleared by centrifugation and supernatants were collected. To visualize fluorescent chemokine in the lysates, samples were subjected to SDS-PAGE under reducing conditions and the gels were afterwards scanned using the Cy3 filterset (excitation 540/25 and emission 595/25) of an Ettan DIGE scanner (GE Healthcare, Uppsala, Sweden).

For endothelial cell binding tests with TAMRA-CXCL10 and TAMRA-CXCL4L1, HMVEC-L (human microvascular endothelial cells isolated from lung tissue) were seeded in black 96-well plates. After incubation with the labeled and unlabeled chemokines (1 h at 37°C), monolayers were washed and fixed with paraformaldehyde. Fluorescence (excitation 546 and emission 574) was quantified using a fluorescent plate reader (DYNEX Technologies, Chantilly, VA).

To determine the binding affinity of CXCL4 and CXCL4L1 for glycosaminoglycans (GAG) low molecular weight heparin (Sigma-Aldrich) or chondroitin sulphate-E (US Biologicals, Swampscott, MA) was immobilized on a GAG binding 96-well plate (BD Biosciences). After incubation with chemokines, biotinylated polyclonal rabbit anti-human CXCL4 or anti-human CXCL4L1 Ab was added. After removal of excess Ab, immune complexes were detected by streptavidin-coupled peroxidase.
Results

1. The anti-tumoral activity of human CXCL4L1 is mediated by inhibition of angiogenesis through CXCR3

In a previous study we have demonstrated that natural CXCL4L1 is a more potent inhibitor of endothelial cell migration and angiogenesis than CXCL4. Here, this was complemented in both a physiologically representative in vitro assay and a clinically relevant animal model of human non-small cell lung cancer. Indeed, after subcutaneous injection in SCID mice, human A549 adenocarcinoma cell growth was inhibited by intra-tumoral treatment with pure natural CXCL4 or recombinant human CXCL4L1 (0.1 µg; 3 times per week) for 8 weeks, as previously shown. The implication of an angiostatic effect in this model was evidenced for both chemokines by the reduced number of blood vessels present in the resected tumors. Indeed, the number of Factor VIII-related antigen positive cells in the tumors was significantly higher in control mice than in CXCL4- or CXCL4L1-treated mice, the effect of the latter being more pronounced (Supplemental Fig. 1).

In the Matrigel assay with human microvascular endothelial cells, CXCL4L1 at 20-100 ng/ml significantly reduced tube formation, whereas CXCL4 at 1000 ng/ml did not (Supplemental Fig. 2). Since CXCR3 has been reported to act as a functional receptor for CXCL4 and to be involved in the inhibition of angiogenesis by CXCL9 and CXCL11, we investigated the role of this receptor in the angiostatic and anti-tumoral effect of CXCL4L1. Indeed, when we pre-incubated (30 min, 37°C) the microvascular endothelial cells with neutralizing monoclonal anti-CXCR3 Abs before addition to the Matrigel assay, the endothelial cell network in the presence of CXCL4L1 was very similar to that of the cells incubated in assay medium without CXCL4L1 (Fig. 1A). Thus, the anti-CXCR3 Ab significantly reduced the inhibitory effect of CXCL4L1 on endothelial cell migration and tube formation. Following this in vitro observation, we applied anti-CXCR3 Abs in in vivo test systems. In the rat cornea micropocket assay, it was first confirmed that recombinant CXCL4L1 (80 ng/pellet) could inhibit the angiogenic activity of both CXCL8 (80 ng/pellet) and basic fibroblast growth factor (bFGF) (50 ng/pellet). Addition of Abs against mCXCR3 prevented the angiostatic activity of CXCL4L1 against CXCL8 and bFGF (Fig. 1B).

Further, in a syngeneic model of subcutaneous Lewis lung carcinoma (LLC), intra-tumoral injection of CXCL4L1 (0.1 µg; 3 times per week) reduced tumor growth (Fig. 2A, B and C). Indeed, a significantly smaller tumor size was observed for the CXCL4L1-treated compared to control animals. However, when mice were co-injected with CXCL4L1 plus anti-CXCR3 Abs, the LLC tumor growth was not retarded (Fig. 2A and C). There was no significant difference between control mice injected with either normal goat serum (NGS) or anti-CXCR3 Abs in the absence of CXCL4L1. In order to obtain additional evidence that CXCR3 is implicated in the anti-tumoral effect of CXCL4L1, its anti-tumoral effect was evaluated in CXCR3−/− animals injected subcutaneously with LLC cells. It was noticed that CXCL4L1 was no longer able to reduce tumor growth and size in CXCR3−/− animals (Fig. 2B and C). In addition, resected tumors were analyzed for changes in the number of microvascular cells. In the
syngeneic model of LLC, intra-tumoral injection of CXCL4L1 in wild-type mice reduced (p<0.05) the number of MECA32 positive cells (Fig. 2D). However, injection of both CXCL4L1 and anti-CXCR3 antibody reverted this inhibition to levels observed in control mice receiving no CXCL4L1. Similarly, no difference in Factor VIII-related antigen (Supplemental Fig. 3A) or MECA32 (Fig. 2D) positive cells was observed between CXCL4L1 injected or non-treated CXCR3−/− animals. Finally, when metastasis of tumor cells to the lungs was evaluated, absence or neutralization of CXCR3 increased the number of tumor cells in the lungs, as compared to control or wild-type CXCL4L1-treated animals (Supplemental Fig. 3B and C). Taken together, these data indicate that the anti-tumoral and angiostatic activities of CXCL4L1 in mice are at least in part mediated through binding and activation of CXCR3.

2. Comparison of the chemotactic potency of CXCL4 and CXCL4L1 for activated lymphocytes and NK cells

In spite of early reports, showing chemotactic and/or activating effects of CXCL4 on various human leukocytic cell types, including monocytes and neutrophils, the purity of the chemokine preparations used and the micromolar concentration of CXCL4 required to observe these effects compared to nanomolar concentrations required for other chemokines, allowed to question the physiological relevance of some of these observations.19,20,21 Nevertheless, in view of its potent anti-tumoral activity, we investigated the in vitro chemotactic capacity of CXCL4L1 for anti-tumoral leukocytes, i.e. activated T lymphocytes and NK cells (Fig. 3).

First, it was observed in the classical Boyden microchamber assay (Fig. 3A) that in vitro activated human T lymphocytes revealed a weak, but significant chemotactic activity at a micromolar (2.5 µg/ml) concentration of pure recombinant CXCL4L1 (index of 3.1 ± 0.2; p<0.05; n=5). The minimal effective concentration of the CXCR3 ligand and known lymphocyte chemoattractant CXCL10 was 100 ng/ml (index of 4.7 ± 0.6; n=4) in this test, confirming that CXCR3 was expressed and available for CXCL4L1 as well. Also activated mouse lymphocytes migrated in response to human CXCL4L1 at concentrations of 1000 ng/ml or more (Fig. 3B). At the highest dose tested, 3000 ng/ml, human CXCL4L1 attracted activated mouse T lymphocytes as efficiently as 100 ng/ml of murine CXCL11 (index of 2.9 ± 0.4 and 3.9 ± 0.8, respectively; n=5).

Next, human NK cells were investigated for their capacity to chemotactically respond to the PF-4 variants (Fig. 3C). A significant induction of NK cell migration was reached with an optimal concentration of 100 ng/ml for both CXCL4 (2.1 ± 0.5% migrated cells, n=5) and CXCL4L1 (2.7 ± 0.2% migrated cells, n=5) which is comparable to that obtained with CXCL8 at 10 ng/ml (2.7 ± 0.3%, n=5). Thus, in contrast to the rather high CXCL4L1 concentrations required for the recruitment of effector lymphocytes, NK cells were attracted at a 10-fold lower CXCL4L1 concentration.

3. Interaction of PF-4 forms with glycosaminoglycans

Since GAGs mediate some of the biological activities of CXCL4, e.g. activation of neutrophilic granulocytes inducing exocytosis,22 we investigated the affinity of both PF-4 forms for heparin and
chondroitin sulphate-E (Fig. 4). We observed strong interaction of authentic CXCL4 with both GAGs, since after addition of CXCL4 at concentrations as low as 8 ng/ml, immunoreactivity could still be detected. In contrast, high amounts (1000 ng/ml) of CXCL4L1 had to be added to heparin-coated plates, in order to detect bound chemokine, whereas no interaction was observed between CXCL4L1 and chondroitin sulphate-E. The observed differential binding of CXCL4 and CXCL4L1 to GAGs corresponded well with the affinity of both chemokines for the heparin-Sepharose matrix used to purify the PF-4 forms. For elution of CXCL4 from the heparin-Sepharose column, higher concentrations of NaCl (1.6 to 1.8 M) are required than needed for elution of CXCL4L1 (1 to 1.4 M), reflecting its high affinity for heparin.

4. Receptor binding of PF-4 forms
Although we provided evidence that CXCR3 is implicated in the angiostatic and anti-tumoral activity of human CXCL4L1 in mice (Fig. 2), the question remains how the PF-4 variants function in the human system. Indeed, human CXCR3B, a variant of CXCR3, has been designated as a functional receptor for CXCL4. To our knowledge, mice have no splice variants of CXCR3. In contrast to the human CXCR3A receptor that facilitates lymphocyte migration, CXCR3B should rather mediate the endothelial cell growth inhibiting capacity of CXCL4, as well as that of the previously known CXCR3 ligands CXCL9, CXCL10 and CXCL11. We therefore investigated whether CXCL4 and CXCL4L1 might bind to human CXCR3A, CXCR3B or CXCR7, a recently identified receptor for CXCL11 and CXCL12. It was found that CXCL4 and CXCL4L1 at 3 µg/ml did not compete for binding of 125I-CXCL11 to CHO-CXCR7 transfectants, whereas at 100 ng/ml cold CXCL11 and CXCL12 were able to displace 125I-CXCL11 for 67% and 97%, respectively (Supplemental Fig. 4). Interaction of CXCL4L1 with CXCR3A and CXCR3B was studied using fluorescently labeled TAMRA-CXCL4L1. TAMRA-CXCL4L1 bound to both CHO-CXCR3A and CHO-CXCR3B cells, because we observed the 8 kDa protein on fluorescence scanned SDS-PAGE gels loaded with the cell lysates (Fig. 5A, B and C). TAMRA-CXCL4L1 could be displaced from CHO-CXCR3A cells by cold CXCL4L1, CXCL4 or CXCL10 (Fig. 5A). The competition between labeled and cold CXCL4L1 on CXCR3A was dose-dependent, 3-fold excess of cold chemokine, but not equimolar concentrations, being able to displace 300 ng/ml of TAMRA-CXCL4L1 (Fig. 5B). Similarly, CXCL4L1 could dose-dependently prevent TAMRA-CXCL4L1 binding to CXCR3B-transfectants (data not shown). Furthermore, CXCL4L1, CXCL4 and CXCL10 could displace TAMRA-CXCL4L1 from the CXCR3B receptor (Fig. 5C). Fig. 5D demonstrates that the interaction of TAMRA-CXCL4L1 with these CXCR3-transfected cells was receptor specific. 300 ng/ml of TAMRA-CXCL4L1 was added to 3x10^6 mock-transfected, CXCR3A- or CXCR3B-transfected CHO cells under conditions reducing GAG-interaction (0.75M NaCl). The amount of fluorescence bound to CXCR3A- and CXCR3B-transfected cells was two-fold and three-fold higher, respectively, when compared to the signal obtained with mock-transfected cells.

Finally, we performed binding studies using TAMRA-CXCL4L1 and TAMRA-CXCL10 on HMVEC. For both chemokines we obtained a dose-dependent increase in binding of the labeled ligand to
HMVEC, and mutual competition between CXCL4L1 and CXCL10 was observed (Fig. 5E, F and G). These results suggest that CXCL4L1 shares its binding site on endothelial cells with CXCL10 and confirm the binding experiments with CXCR3B-transfected cells. Indeed, endothelial cells have been described to express exclusively CXCR3B. Based on the results, shown in Fig. 5G, the $K_d$ for interaction of CXCL4L1 with cellular receptors present on HMVEC was calculated to be equal to 0.28 nM.

5. CXCL4 and CXCL4L1 are chemotactic for immature dendritic cells via a pertussis toxin sensitive receptor mechanism

CXCL4, as well as CXCL4L1 provoked migration of immature DC derived from human peripheral blood monocytes in a dose-dependent manner (Fig. 6A). For CXCL4, the minimal effective concentration was 10 ng/ml (17 ± 3 migrated cells), whereas a maximal chemotactic response was reached at 100 ng/ml (38 ± 3 cells). The efficacy of CXCL4 on these cells was similar to that of CCL3 at 100 ng/ml (53 ± 6 cells). Furthermore, it was found that CXCL4L1 showed a different dose response curve on immature DC by reaching maximal chemotactic activity already at 10 ng/ml (23 ± 6 cells). It can be deduced that both PF-4 variants exert a physiologically relevant chemotactic effect on immature DC cells, which need to migrate to initiate immune responses. The participation of DC in the antitumoral activity of both angiostatic PF-4 variants can therefore not be excluded.

Further, it was evidenced that the DC chemotactic activity of CXCL4 and CXCL4L1 is mediated by signaling through a pertussis toxin sensitive G protein-coupled receptor mechanism (Fig. 6B). Indeed, in parallel with CCL3, the migratory effect of both PF-4 variants (at 100 ng/ml) for immature DC was completely abrogated in the presence of pertussis toxin. Finally, we wanted to confirm that CXCR3 is the tentative CXCL4 and CXCL4L1 receptor on human immature DC and, therefore, we tested whether DC migration towards the PF-4 variants was affected by pre-incubation of the cells with CXCR3 ligands or anti-CXCR3 Abs. Fig. 7A shows that pre-incubation of DC with CXCL10 (50 ng/ml) or CXCL11 (20 ng/ml) almost completely or partially inhibited migration of DC in response to CXCL4 (30 ng/ml) or CXCL4L1 (30 ng/ml), whereas the response to CCL3 (100 ng/ml) was not affected. Vice versa, CXCL4 (30 ng/ml) and CXCL4L1 (30 ng/ml) partially desensitized DC to subsequent CXCL10- or CXCL11-induced migration. In addition, DC chemotaxis in response to CXCL4, CXCL4L1, CXCL10 or CXCL11 was reduced after pre-incubation of the cells with neutralizing anti-human CXCR3 Abs (Fig. 7B). For CXCL4 and CXCL4L1, the neutralizing capacities of the monoclonal and polyclonal Ab preparations were similar. However, the monoclonal Ab fully abrogated the response to CXCL10 and CXCL11, which was only partially reduced by the polyclonal Abs. This suggests that compared to CXCL10 and CXCL11, CXCL4 and CXCL4L1 might have lower affinity for DC-expressed CXCR3 or distinct binding sites on this receptor. It has already been shown that compared to CXCL10, CXCL11 interacts differently with CXCR3. Taken together, we demonstrated that CXCL4 and CXCL4L1 share CXCR3 with CXCL10 and CXCL11 as a functional receptor on human DC. Furthermore, we showed that both PF-4 variants can chemoattract...
immunocompetent cells at relatively low concentrations, suggesting a role in the immune response to infection and cancer.

**Discussion**

CXCL4 is the first chemokine structure identified but its spectrum of biological activities is rather complex, because of the numerous molecules it is interacting with and because some of these partners are very different from the typical G protein-coupled chemokine receptors. One of the major physiological roles of CXCL4 is to influence blood coagulation, both pro-coagulant and anti-coagulant properties being reported. CXCL4 on the one hand neutralizes heparin-like molecules on the endothelial surface of blood vessels, thereby inhibiting local antithrombin III activity and promoting coagulation. On the other hand CXCL4 binds and induces conformational changes in protein C thereby enhancing generation of activated protein C, which is a potent anti-coagulant. In addition, by binding polysulfated GAGs CXCL4 can indirectly interfere with activation of receptors by growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), improve adhesion of neutrophils to the vessel wall and reduce proliferation of T cells. Furthermore, CXCL4 has been shown to directly interact with FGF-2, CXCL8 and CCL5. Finally, during the last decade evidence accumulated that also CXCL4 activates a typical chemokine receptor, namely a variant of CXCR3 (*vide infra*).

Since the first description of CXCL4L1, no receptor has been identified for this rather new chemokine variant. In this manuscript, we identified CXCR3 to be a functional CXCL4L1 receptor both in the human and the mouse systems. In humans several splice variants of CXCR3 have been described. For its angiostatic activity, CXCL4 is supposed to use CXCR3B, coupled to G<sub>ai</sub>, whereas its chemotactic activity for activated T lymphocytes is mediated via CXCR3 coupled to a G<sub>ai</sub> protein. However, the interaction with CXCR3 on activated T lymphocytes was of low affinity and Mueller *et al.*, could not dissect whether CXCL4 acted on this cell type via CXCR3A or CXCR3B or both CXCR3 variants. Furthermore, the data of Mueller *et al.* are in agreement with the hypothesis of Fleischer *et al.* that CXCR3 on activated T cells functions as a coreceptor that is activated after initial CXCL4 binding to proteoglycans. Also in our hands, assays with CXCL4 and CXCR-transfected cells were complicated because of the presence of GAG. We must thus conclude that proteoglycans remain major cellular mediators of CXCL4 activity. In this context, CXCL4L1 has lower affinity for heparin and chondroitin sulphate-E than CXCL4. Here, we demonstrated that fluorescent TAMRA-CXCL4L1 binds to both CXCR3A- and CXCR3B-transfectants and that bound TAMRA-CXCL4L1 can be displaced by other CXCR3 ligands. Also, chemotaxis of human immature DC towards CXCL4L1 can be desensitized by the CXCR3 agonists CXCL10 and CXCL11 and can be neutralized by anti-CXCR3 Abs. The fact that pertussis toxin inhibited migration of human immature DC towards CXCL4L1, suggests that CXCL4L1 attracts these cells via CXCR3, coupled to a G<sub>ai</sub> protein. Thompson *et al.* showed that CXCR3-mediated migration of murine activated T cells requires G<sub>in2</sub>. Because of its pertussis toxin sensitivity, probably the CXCR3A variant, and not CXCR3B, is activated by CXCL4L1.
on human leukocytes. Indeed, earlier reports indicated the presence of CXCR3 on DC, NK cells and activated T cells. CXCL4L1 shares with CXCL4 these target cells and similarly to CXCL4L1, CXCL4 attracts human DC via CXCR3. Only one other study described activity of CXCL4 on dendritic cells. More specifically, the differentiation of monocyte-derived dendritic cells in the presence of 10 µg/ml of CXCL4 led to decreased expression of CD1a and lower secretion of TNF-α and IL-12. We observed chemotaxis of immature monocyte-derived DC towards much lower concentrations of CXCL4L1 and CXCL4 (starting from 10 ng/ml). Also, chemotaxis of NK cells occurred at relatively low levels (100 ng/ml) of these platelet chemokines. CXCL4 reportedly stimulates release of CXCL8 by NK cells from 10 ng/ml onwards, but has until our study, never been shown to be chemotactic for these cells. We cannot exclude that the mode of action of CXCL4L1 is different on lymphocytes, NK cells, DC and endothelial cells, which might explain the different dose response curves. The finding that the chemotactic activity of CXCL4L1 for lymphocytes, DC and endothelial cells is mediated by CXCR3 shows that there is at least one common receptor, not excluding additional receptor binding (e.g. GAGs) and signaling mechanisms or components present in one (e.g. DC) but not another (e.g. lymphocyte) cell type.

Finally, we demonstrated that CXCR3 is also the key receptor involved in the angiostatic activity of CXCL4L1. Indeed, neutralizing CXCR3 Abs blocked the inhibitory effect of CXCL4L1 on network formation in the Matrigel assay with HMVEC and labeled CXCL4L1 and CXCL10 could displace each other on HMVEC. Further, the anti-tumoral activity for LLC of CXCL4L1 was completely abolished in the absence of CXCR3 or after neutralization of CXCR3. The lack of anti-tumoral activity in CXCR3+/− mice or antibody-treated mice concurred with enhanced tumor growth, a higher number of endothelial cells and more metastasis to the lungs. It can be deduced that for its anti-tumoral activity CXCL4L1 shares CXCR3 with CXCL4, CXCL9, CXCL10 and CXCL11. However, for CXCL4 several other mechanisms have been proposed to be involved in its inhibition of endothelial cell growth, i.e. interaction with cell surface proteoglycans and direct complex formation with growth factors. We can at present only speculate whether hetero-multimerization with growth factors occurs for CXCL4L1, but we demonstrated that the affinity of CXCL4L1 for heparin and chondroitin sulphate-E is lower compared to that of CXCL4. For this reason and because CXCL4L1 is angiostatic at much lower concentrations than CXCL4 we might speculate that the G protein-coupled CXCR3 is the major CXCL4L1 receptor. Indeed, chemokine-GAG interactions are mostly of lower affinity and often require chemokine dimerization or tetramerization (CXCL4). The three diverging amino acids of CXCL4 and CXCL4L1 are located in the COOH-terminal alpha helix and since a proline residue (P58 in CXCL4) is involved, the 3D structure of CXCL4L1 might be different, as well as its tendency to form homo-multimers. Taken together, our results provide evidence that both PF-4 forms can attract immature DC in a CXCR3 dependent manner. Moreover, the angiostatic and anti-tumoral effects of CXCL4L1 in vivo are also mediated through CXCR3.
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Author contributions

S.S. performed experiments, and wrote the paper; L.S. performed experiments with DC and NK cells; M.D.B. performed in vivo experiments and binding assays with endothelial cells; J.V. performed experiments with DC and receptor-transfected cell lines and the GAG-binding assay; M.G. performed signaling experiments; S.N. performed Matrigel experiments; P.P. made synthetic TAMRA-CXCL4L1 and performed binding experiments; G.O. designed/supervised experiments; C.G. developed CXCR3-/- mice; M.P. developed chemokine receptor-transfected cell lines; S.S. designed/supervised experiments; R.M.S. developed Abs and designed/supervised experiments; J.V.D. designed/supervised experiments and wrote the paper.

Conflict of interest disclosure: the authors declare no competing financial interests.
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Figure legends

Figure 1. The *in vitro* and *in vivo* angiostatic activity of human CXCL4L1 is CXCR3 dependent

HMVEC-d (Panel A) were seeded in 48-well plates on Matrigel in the presence of 100 ng/ml of CXCL4L1 and neutralizing monoclonal anti-CXCR3 Ab (2.5 µg/ml, Clone 49801, R&D Systems). After 12 h, rearrangement of the endothelial cells into tubular structures was evaluated by microscopy. The total length of tubes in each well was determined and results are expressed as the percentage inhibition of tube formation compared to control cultures stimulated with growth medium alone. Results (mean ± SEM of 5 independent experiments performed in duplicate or triplicate) were analyzed by the Mann Whitney test to detect differences between two conditions (* p<0.05, ** p<0.005).

Neovascularization (Panel B) was evaluated after implantation of Hydron pellets containing 50 ng of bFGF, 80 ng of CXCL8, 80 ng of CXCL4L1 plus 50 ng of bFGF or 80 ng of CXCL4L1 plus 80 ng of CXCL8, respectively, in rat corneas in the absence or presence of anti-CXCR3 Ab. Panels are at 40X magnification.

Figure 2. Function of CXCR3 in tumor growth inhibition of Lewis lung carcinoma after treatment with the angiostatic chemokine CXCL4L1

LLC cells were subcutaneously implanted in C57Bl/6 mice in two separate experiments with different settings (first experiment 40 wt and 20 CXCR3−/− mice, i.e. n=10 per group; second experiment 90 wt and 30 CXCR3−/− mice, i.e. n=15 per group). In the first experiment 6 treatment groups were included (Ctrl versus CXCL4L1 NGS-treated; Ctrl versus CXCL4L1 anti-CXCR3-treated and Ctrl versus CXCL4L1 in CXCR3−/− mice). In the second experiment two additional control groups of wt mice (WT Ctrl and WT CXCL4L1) were included that received no antibody treatment. Intra-tumoral chemokine treatment (vehicle control or 0.1 µg of human CXCL4L1 per injection) started at the time of tumor inoculation and was repeated 3 times a week. Neutralization of CXCR3 was obtained by intraperitoneal injection of 0.5 ml of polyclonal antiserum (4 mg Ig), 3 times a week, starting at the time of tumor inoculation. Tumor dimensions were measured every week (Panel A, B and C). Panel A and B represent mean tumor size (mm³) ± SEM per group from the second experiment. Panel C shows the statistical analysis of the tumor sizes after 3 weeks when data from both experiments were combined. The WT Ctrl and WT CXCL4L1 group are not shown, because these treatments were only tested in the
second experiment. To assess tumor vascularity, 5 tumors per group from both experiments were minced into single cell suspensions for flow cytometric analysis using Abs against the endothelial cell marker MECA32 (Panel D, n=10/group). Statistically significant differences between the indicated groups were determined by the Mann Whitney test (* p< 0.05; ** p< 0.01).

**Figure 3. CXCL4 and CXCL4L1 attract activated T lymphocytes and NK cells**

Chemotaxis experiments were performed to compare the chemotactic activity of CXCL4 and CXCL4L1 for human (h) IL-2-activated T lymphocytes (Panel A; n=4 to 5), murine (m) IL-2-activated T lymphocytes (Panel B; n=4 to 5) and unstimulated NK cells (Panel C; n=5). Statistically significant migration towards chemokine is indicated (* p< 0.05; ** p< 0.005; Mann Whitney test).

**Figure 4. Differential interaction of CXCL4 and CXCL4L1 with glycosaminoglycans**

Binding of CXCL4 and CXCL4L1 to immobilized low molecular weight heparin (Panel A) or chondroitin sulphate-E (CS-E; Panel B) was detected by biotinylated anti-CXCL4 or anti-CXCL4L1 Abs, respectively, as described in the Methods section. Results (mean ± SEM) shown are derived from 5 independent experiments. Statistical analysis testing for significant chemokine-GAG interaction is indicated (* p< 0.05; ** p< 0.01; *** p<0.001; Mann Whitney test).

**Figure 5. Receptor binding properties of CXCL4 and CXCL4L1 on endothelial cells and CXCR3 transfectants**

TAMRA-CXCL4L1 [0 ng/ml (no label) or 300 ng/ml (all other lanes)] was added to CXCR3A- or CXCR3B-transfected CHO cells in the absence (Co) or presence of 3000 ng/ml of cold CXCL4L1, CXCL4 or CXCL10 (Panel A and C). Cell-bound 8 kDa TAMRA-CXCL4L1 in lysates was analyzed by SDS-PAGE scanned for fluorescence. The dose-dependency of competition for binding to CHO-CXCR3A cells is demonstrated in Panel B. Competition for binding of TAMRA-CXCL4L1 to CHO-CXCR3B is shown in Panel C. To detect aspecific interaction, binding of 300 ng/ml of TAMRA-CXCL4L1 to mock-transfected cells (Panel D) was compared to binding to equal numbers of CXCR3A- and CXCR3B transfected cells. Results shown are representative of 2 (Panel C), 3 (Panels A and B) or 5 (Panel D) experiments. Confluent monolayers of HMVEC (Panels E, F and G) were
incubated with TAMRA-CXCL4L1 or TAMRA-CXCL10 in the presence or absence of competing unlabeled chemokines (CXCL4L1 or CXCL10). Cell-bound fluorescence present in the fixed cell cultures was quantified by a fluorescence plate reader. Either the concentration of labeled chemokine (Panels E and F) or cold chemokine (Panel G) was kept constant. Results shown are the mean of 2 independent experiments performed in triplicate (Panels E, F and G).

Figure 6. Chemotactic activity of PF-4 variants for immature dendritic cells

CXCL4L1 and CXCL4 were tested for their ability to induce chemotaxis of human immature dendritic cells derived from peripheral blood in the Boyden microchamber (panel A). * (p<0.05) indicates statistically significant migration towards chemokine (Mann Whitney test). The chemotactic response of CXCL4 (100 ng/ml), CXCL4L1 (100 ng/ml) and CCL3 (100 ng/ml) was reduced after treatment with 3 µg/ml pertussis toxin (Ptox; panel B) as determined by the Mann Whitney test (*p<0.05; ** p<0.005). Results represent the mean (± SD) number of migrated cells of 4 (panel A) or 3 (panel B) independent experiments.

Figure 7. The chemotactic activity of CXCL4L1 for human immature dendritic cells is mediated via CXCR3

To demonstrate the involvement of CXCR3 in immature DC chemotaxis, cells were pre-incubated with CXCR3 ligands [Panel A; 30 ng/ml CXCL4, 30 ng/ml CXCL4L1, 50 ng/ml CXCL10, 20 ng/ml CXCL11 or dilution buffer (Ctrl)] or CXCR3-neutralizing Abs [Panel B; dilution buffer (Ctrl), 10 µg/ml monoclonal antibody (mAb), 28 µg/ml polyclonal antibody (pAb) or 10 µg/ml isotype control antibody] before addition to the Boyden microchamber. As chemoattractants, CCL3 (100 ng/ml), CXCL4 (30 ng/ml), CXCL4L1 (30 ng/ml), CXCL10 (50 ng/ml) or CXCL11 (20 ng/ml) were added to the lower wells. Results shown are the mean ± SD from 4 (Panel A) or 3 (Panel B) independent experiments. In Panel A, statistically significant reduction of migration is indicated (*p<0.05; Mann Whitney test).
Fig. 1

A

CXCL4L1  + anti-CXCR3

CXCL4L1

Co  + anti-CXCR3

Co

network formation (%)

B

bFGF  bFGF + CXCL4L1  bFGF + CXCL4L1 + anti-CXCR3

CXCL8  CXCL8 + CXCL4L1  CXCL8 + CXCL4L1 + anti-CXCR3
Fig. 2

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

D

![Graph D](image)
**Fig. 4**

A. Heparin binding (OD$_{450\text{ nm}}$)

B. CS-E binding (OD$_{450\text{ nm}}$)

Concentration (ng/ml) vs. Heparin binding (OD$_{450\text{ nm}}$) and CS-E binding (OD$_{450\text{ nm}}$) for CXCL4 and CXCL4L1.

Key:
- ***: P < 0.001
- **: P < 0.01
- *: P < 0.05
Fig. 5

A. CXCR3A
- no label
- CXCL4L1
- CXCL4
- CXCL10

B. CXCR3A
- CXCL4L1 competition (ng/ml)

C. CXCR3B
- CXCL4L1
- CXCL4
- CXCL10

D. Fluorescence relative to CXCR3A (%)
- MOCK
- CXCR3A
- CXCR3B

E. Fluorescence (x10^6)
- 10 ng/ml TAMRA-CXCL10
- 30 ng/ml TAMRA-CXCL10
- 30 ng/ml TAMRA-CXCL4L1

F. Fluorescence (x10^6)
- 10 ng/ml TAMRA-CXCL4L1
- 30 ng/ml TAMRA-CXCL4L1
- 30 ng/ml TAMRA-CXCL10

G. Fluorescence (x10^6)
- 0 ng/ml CXCL4L1
- 10 ng/ml CXCL4L1
- 30 ng/ml CXCL4L1
- 30 ng/ml CXCL10

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Fig. 6

A

- A diagram showing the number of migrated cells in response to different concentrations of CCL3, CXCL4, and CXCL4L1.

B

- A bar graph comparing the number of migrated cells in the presence or absence of (Ptox) for CCL3, CXCL4, and CXCL4L1.

**Notes:**
- The y-axis represents the number of migrated cells.
- The x-axis represents the concentration (ng/ml).
- The graph includes error bars indicating the standard deviation.
Fig. 7

**A**

Desensitization by:
- **Ctrl**
- CXCL4
- CXCL4L1
- CXCL10
- CXCL11

**B**

Neutralization by:
- **Ctrl**
- mAb
- pAb
- Isotype

Chemotactic response (% of control)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>CCL3</th>
<th>CXCL4</th>
<th>CXCL4L1</th>
<th>CXCL10</th>
<th>CXCL11</th>
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Angiostatic and chemotactic activities of the CXC chemokine CXCL4L1 (platelet factor-4 variant) are mediated by CXCR3

Sofie Struyf, Laura Salogni, Marie D. Burdick, Jo Vandercappellen, Mieke Gouwy, Sam Noppen, Paul Proost, Ghislain Opdenakker, Marc Parmentier, Craig Gerard, Silvano Sozzani, Robert M. Strieter and Jo Van Damme