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Chemokine-like factor 1 is a functional ligand for CC chemokine receptor 4 (CCR4)

Ying Wang a,1, Yingmei Zhang a,1, Xue Yang b, Wenling Han a, Yanan Liu b, Qianmei Xu a, Rui Zhao b, Chunhui Di a, Quansheng Song a, Dalong Ma a,b,*

a Center for Human Disease Genomics, Peking University, 38 Xueyuan, Beijing 100083, P.R. China
b Laboratory of Medical Immunology, School of Basic Medical Science, Peking University Health Science Center, Beijing 100083, P.R. China

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Abstract

Chemokine-like factor 1 (CKLF1) exhibits chemotactic effects on leukocytes. Its amino acid sequence shares similarity with those of TARC/CCL17 and MDC/CCL22, the cognate ligands for CCR4. The chemotactic effects of CKLF1 for CCR4-transfected cells could be desensitized by TARC/CCL17 and markedly inhibited by PTX. CKLF1 induced a calcium flux in CCR4-transfected cells and fully desensitized a subsequent response to TARC/CCL17, and TARC/CCL17 could partly desensitize the response to CKLF1. CKLF1 caused significant receptor internalization in pCCR4-EGFP transfected cells. Taken together, CKLF1 is a novel functional ligand for CCR4.

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Introduction

Chemokines comprise a large family of structurally homologous small cytokines. The complexity and functions of the chemokine family have become increasingly diverse as new members have been identified and characterized. The classification of chemokines is based on the distance between the first two of four to six conserved cysteine residues. Four subfamilies of chemokines have been discovered to date: CXC(α), CC(β), C(γ), and CX3C (Bagginioli and Dahinden, 1994; Kelner, et al., 1994; Bazan, et al., 1997). All chemokines mediate their activities through G-protein coupled receptors, have a characteristic seven-transmembrane structure, bind specific ligands with high affinity and transduce signals to the inside of the cell through heterotrimeric G-proteins (Rollins, 1997) that are grouped into two families depending on the sensitivity to pertussis toxin (PTX). Gi/o family G proteins are sensitive to PTX whereas Gq family G proteins are known to be insensitive to this toxin (Wu, et al., 1993; Venkatakrishnan, et al., 2000).

The chemokine-like factor 1 (CKLF1) is a novel human cytokine isolated from PHA-stimulated U937 cells, which occurs as at least three isoforms, CKLF2, CKLF3 and CKLF4. The open reading frame of CKLF1 cDNA encodes a highly basic and hydrophobic polypeptide of a total of 99 residues with a calculated molecular mass of 10.9 kD. CKLF1, secreted by cells transfected with CKLF1 expression plasmid, displays chemotactic activities in a wide spectrum of leukocytes and CKLF1 also shows a capability for stimulating the proliferation of murine skeletal muscle cells (Han, et al., 2001) and the proliferation of human bone marrow hematopoietic progenitor cells and colony formation (Ke, et al., 2002). CKLF1 has a CC motif and the key amino acids around the motif are identical with those of TARC/CCL17 (Thymus- and activation-regulated chemokine) and MDC/CCL22 (Macrophage Derived Chemokine), though lacking the additional C-terminus cysteines as compared to other CC subfamily members (Han, et al., 2001). According to pair-wise sequence comparisons of conserved regions using Needle software (HGMP, Hinxton, Cambridge,
UK) (Han, et al., 2003), the sequence identities and similarities between the local conserved sequences around the CC motif of CKLF1 and TARC/CCL17 (67 amino acids were compared) or MDC/CCL22 (87 amino acids were compared) are 28.4%(19/67) and 41.8%(28/67), or 25.3%(22/87) and 46%(40/87), respectively. The gene encoding CKLF is located on chromosome 16q22, and the TARC/CCL17 and MDC/CCL22 are the only two CC chemokine genes located on chromosome 16. TARC/CCL17 and MDC/CCL22 have been found to be highly specific ligands for CCR4 (Imai et al., 1997, 1998). Because of the similarities between CKLF1 and TARC/CCL17 and MDC/CCL22, this study was undertaken to determine if CKLF1 interacts with CCR4, the receptor for TARC/CCL17 and MDC/CCL22. The results confirm that CKLF1 is a novel functional ligand for CCR4.

Materials and methods

Materials

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. BSA was from Sigma. PTX was from ALEXIS Biochemicals Corporation. Fluo-3, AM ester was from Biotium, Inc. Recombinant TARC/CCL17 and MIP-3α/CCL20 (macrophage inflammatory protein 3α) were from Peprotech. PE-conjugated monoclonal anti-CCR4 antibody and FITC-conjugated monoclonal anti-CCR6 antibody were from BD Pharmingen. Monoclonal anti-Myc antibody and horseradish peroxidase (HRP)-labeled goat anti-mouse antibody were from Sigma. Three Step Stain Set was from Richard-Allan Scientific.

Cell Culture

HEK293 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). 4 x 10⁶ HEK293 cells in 400μl were transiently transfected by electroporation with 10 μg of the expression plasmid at 120 V, 20 ms using an electric pulse generator (Electro Square Porator ECM 830, BTX, San Diego, CA) and calcium mobilization and chemotaxis assays were performed 48 h later (Zhu et al., 2001).

Production of CKLF1 in a drosophila expression system

Recombinant CKLF1 was prepared using the Drosophila expression system (Invitrogen Life Technologies, Inc.) following the protocol recommended by the manufacturer. Briefly, the CKLF1 cDNA containing the entire coding region was excised with EcoRI+XhoI and subcloned into the EcoRI+XhoI site of the pMT/V5-Myc-His A vector downstream of the metallothionein promoter. The resulting plasmid, pMT/V5-CKLF1-Myc-His, was co-transfected with the hygromycin B resistance vector pCoHygro into Drosophila Schneider 2 (S2) cells using a Calcium Phosphate Transfection Kit (Invitrogen Life Technologies, Inc.) and stable clones were selected with 300 μg/ml hygromycin B in Schneider’s Drosophila Medium plus 10% FBS. Transfected S2 cells grew in serum-free DES expression medium and were induced to produce recombinant CKLF1 with CuSO₄. The cell supernatant was dialyzed in phosphate-buffered saline (PBS) and loaded onto a Ni-NTA (Qiagen) chelating sepharose fast flow column (equilibrated in dialysis buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 5 mM imidazole (pH 8.0) and 5 mM β-mercaptoethanol). The column was then washed in PBS containing 10 mM imidazole (pH 8.0) and 10% glycerol. CKLF1 eluted in 50–500 mM imidazole. The sample was then dialyzed and was again loaded onto a Ni-NTA (Qiagen) chelating sepharose fast flow column and the CKLF1-Myc-His purified further. Fractions containing recombinant CKLF1 were stored frozen directly or after dialysis in PBS.

Western blot analysis

Protein samples were separated by SDS-PAGE (15%) and transferred to nitrocellulose filters. The blots were incubated with monoclonal anti-Myc antibody and secondary HRP-labeled goat anti-mouse antibody, and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Construction of receptor expression plasmids

The cDNA fragments covering the open reading frames for various chemokine receptors were obtained as follows. CCR4 and CCR6 receptors were cloned from a K562 cell cDNA library by polymerase chain reaction (PCR). The primers were designed using the sequences from the following GenBank™ submissions: CCR4 (NM_005508.2) and CCR6 (AY242126). The fragments were cloned into an expression vector pcDI (Liu et al., 1999) or pEGFP-N1 for efficient expression in HEK293 cells.

FACS analysis

5 x 10⁵ transfected HEK293 cells were cultured in RPMI 1640. Cells were harvested and washed with PBS buffer containing 2% BSA and were first Fc-blocked by treatment with 1 μg of human IgG/10⁵ cells for 15 min at room temperature in accordance with the supplier’s instructions. Then cells were incubated at 4°C for 30 min with anti-CCR4-PE antibody or anti-CCR6-FITC antibody. Finally, the cells were washed and analyzed on a FACSCaliber™ flow cytometer (Becton Dickinson).

Chemotaxis assay

The chemotaxis assay was performed using a 48-well microchemotaxis chamber (Neutroprobe; Cabin John, MD, U.S.A.) as described previously (Sarafi et al., 1997). In brief, chemoattractants were diluted in HEPES-buffered RPMI 1640 medium supplemented with 0.5% BSA and placed in the lower wells (28 μl/well). HEK293 cells mock-transfected or...
transfected with pcDI-CCR4 or pcDI-CCR6 were resuspended in the same medium at $2 \times 10^6$ cells/ml and added to the upper wells (50 µl/well), which were separated from the lower wells by a polyvinylpyrrolidone-free polycarbonate filter with 8-µm pores. The chamber was incubated for 2 h at 37 °C in 5% CO$_2$ and 95% air. Filters were removed from the chamber, washed, fixed, and stained with Three Step Stain Set. Cells that migrated were counted in 5 randomly selected high power fields (400×) per well. HEK293 cells transfected with pcDI (mock) vector used as a control. All samples were assayed twice. The chemotactic index (CI) was calculated from the number of cells that migrated to the control. Significant chemotaxis was defined as CI >2. In some experiments, cells were pretreated with 100 nM TARC/CCL17 for 30 min at 37 °C, or 100 ng/ml PTX for 6 h prior to CKLF1 stimulation.

Confocal microscopy of intracellular calcium flux

Measurements of calcium flux, were performed as described previously (Billstrom et al., 1998). Briefly, HEK293 cells transfected with pcDI-CCR4, pcDI-CCR6 or with a mock vector were grown in specialized glass-bottom micro-well dishes (MatTek Corporation, U.S.A.) and loaded with 10 µM fluo-3/AM in HEPES-buffered saline at 37 °C for 1 h in the dark. The cells were rinsed with HEPES-buffered saline and stimulated with 100 nM CKLF1 and 100 nM TARC/CCL17, or with 100 nM CKLF1 and 100 nM MIP-3α/CCL20, a cognate ligand for CCR6, respectively. Fluorescence was monitored at 488 nm (excitation wavelength) and 530 nm (emission wavelength) every 5 s using a Leica TCS-NT confocal fluorescence microscope with a ×40 oil immersion lens (Wetzler, Heidelberg, Germany). The measurement was completed at room temperature and each field of cells was selected at random. Images were collected at 5 s intervals for 405 s. The images were analyzed for relative fluorescence using Leica confocal software. The relative fluorescence was determined using Microsoft Excel. All calcium flux assays were performed in the presence of extracellular calcium (in the absence of EDTA or EGTA) in the assay buffers. Therefore, both intracellular calcium release and extracellular calcium influx were analyzed.

Receptor internalization

Receptor internalization was performed as described previously (Zhu et al., 2001). pCCR4-EGFP or pCCR6-EGFP transiently expressing HEK293 cells were cultured in glass-bottom dishes in RPMI 1640 with 10% FBS. After 16–24 h serum starvation, cells were treated with different ligands at 37 °C for 2 h. Cells were washed with cold PBS and fixed with 2% paraformaldehyde in PBS. The subcellular localization of CCR4-EGFP or CCR6-EGFP protein was visualized using a Leica TCS-NT confocal fluorescence microscope with a ×63 oil immersion lens (Wetzler, Heidelberg, Germany). The excitation and emission wavelengths were 488 and 515–540 nm, respectively.

Results

Production of recombinant CKLF1 protein

To obtain recombinant CKLF1, S2 cells were transfected with recombinant plasmid pMT/V5-CKLF1-Myc-His. Recombinant CKLF1 was purified from pooled culture supernatants by affinity chromatography on metal-chelating resins and was eluted from the column as a major peak at an imidazole concentration of 500 mM (Fig. 1). The CKLF1 protein, S2-transfected cell supernatant and cell lysate were collected for CKLF1-Myc-His detection by Western blot with anti-Myc monoclonal antibody. A specific band with a molecular weight of 10 kDa appeared in the supernatant and a band with 14 kDa in cell lysate, which is consistent with the predicted molecular size of CKLF1-Myc-His. This validated CKLF1 is a secreted protein in agreement with its expression in human cell lines (Han et al., 2001).

Chemotactic activity

HEK293 cells were transiently transfected by electroporation with pcDI-CCR4, pcDI-CCR6 or mock, and CCR4 or CCR6 expression was estimated 48 h later (Fig. 2D). We examined the ability of CKLF1 to induce migration of CCR4 or CCR6-transfected HEK293 cells. CKLF1 and TARC/CCL17 induced migration of CCR4-transfected HEK293 cells, as shown in Fig. 1. Production of CKLF1 in the Drosophila expression system. Recombinant CKLF1 was prepared using the Drosophila expression system. The cell supernatant was purified in a Ni-NTA (Qiagen) Chelating Sepharose Fast Flow column and CKLF1 eluted in 500 mM imidazole. Then the sample was dialyzed, loaded onto a Ni-NTA (Qiagen) Chelating Sepharose Fast Flow column and further purified. Protein samples were separated by SDS-PAGE (15%) and transferred to nitrocellulose filters. The blots were incubated with monoclonal anti-Myc antibody and secondary HRP-labeled goat anti-mouse antibody and developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). A pMT (mock)-transfected supernatant was purified following the same procedure.
2A. Both chemokines produced classic bell-shaped migration responses with maximal migration at 20 nM. However, HEK293 cells transfected with pcDI-CCR6 failed to migrate when treated with CKLF1 (Fig. 2C). The pretreatment with 100 nM TARC/CCL17 at 37 °C for 30 min can desensitize CCR4 to subsequent stimulation with CKLF1. It is known that CCR4 couples to PTX-sensitive Gi/o family proteins (Oh et al., 2002; New and Wong, 2003). To examine whether CKLF1’s action required such G protein, CCR4-transfected HEK293 cells was treated with PTX (100 ng/ml, 16 h pretreatment) before the stimulation of CKLF1. The chemotaxis induced by CKLF1 was completely abolished by PTX, suggesting the involvement of a Gi/o pathway, as shown in Fig. 2B. These results further confirm that CKLF1 is a ligand for CCR4.

**CKLF1 induces calcium flux in CCR4-transfected cells**

To examine the interaction of CKLF1 with CCR4, we measured CKLF1-induced calcium mobilization in HEK293 cells transfected with pcDI-CCR4, pcDI-CCR6 or in a mock transfection. TARC/CCL17 induced calcium flux in HEK293 cells expressing CCR4, as shown in Fig. 3. CKLF1 also induced calcium flux specifically in CCR4-transfected HEK293 cells, but not in CCR6 or mock-transfected cells. CKLF1 completely desensitized CCR4 transfectants to subsequent TARC/CCL17 treatment when both were added at 100 nM. However, pretreatment with TARC/CCL17 did not completely desensitize the receptor to subsequent stimulation with CKLF1 and the signal was of lower intensity than that produced by initial CKLF1 stimulation. These results clearly demonstrate that CKLF1 is a specific functional ligand for CCR4.

**Internalization of CCR4 induced by CKLF1**

G-protein coupled receptors undergo agonist-dependent desensitization and internalization (Ferguson et al., 1996; Mukherjee et al., 1997; Bohm et al., 1997; Zhu et al., 2001).
Fig. 3. Calcium flux of CCR4-transfectants stimulated with CKLF1 or TARC/CCL17 and desensitization of the intracellular calcium flux in response to subsequent stimulation of TARC/CCL17 or CKLF1. A–D. Monolayers of HEK293 cells transfected with CCR4 were grown in specialized glass-bottom microwell dishes and loaded with fluo-3/AM. CCR4-transfected HEK293 cells were stimulated with either 100 nM CKLF1 or 100 nM TARC/CCL17, and then desensitization to subsequent stimulation was measured. Cells were stimulated with 100 nM CKLF1, followed by the addition of the ligand, TARC/CCL17, at 100 nM (A, C), or 100 nM TARC/CCL17, followed by the addition of 100 nM CKLF1 (B, D) and a single field was analyzed by confocal microscopy for calcium flux after stimulation. A–B. Images were captured before first stimulation (a) and after first stimulation (b), and before second stimulation (c) and after second stimulation (d). C–D. Time course of the calcium flux in CCR4-transfected (closed circle) and mock-transfected HEK293 cells (open circle). The relative fluorescence from the images was analyzed using Leica confocal software. The relative fluorescence was processed using Microsoft Excel (version 2000). E–F. Time course of the calcium flux in CCR6-transfected (closed circle) and mock-transfected HEK293 cells (open circle). The relative fluorescence from the images was analyzed for relative fluorescence using Leica confocal software. The relative fluorescence was processed using Microsoft Excel (version 2000). C–F represented the signal derived from a single cell. Each experiment was repeated twice. All calcium flux assays were performed in the presence of extracellular calcium (in the absence of EDTA or EGTA) in the assay buffers. Therefore, both intracellular calcium release and extracellular calcium influx were analyzed.
When HEK293 cells were transfected with the pEGFP-N1 vector, EGFP protein was expressed in the cytosol of the cells (Xu et al., 2000). The CCR4 or CCR6-EGFP fusion protein, on the other hand, was expressed only at the plasma membrane (Fig. 4) and 500 nM CKLF1 induced internalization of CCR4-EGFP at 37 °C, but not CCR6-EGFP.

Discussion

Classical chemokines have a CXC, CC, C or CX3C motif. However, there is another group of proteins unrelated structurally to chemokines but having chemokine-like functions, basically an ability to recruit and activate leukocytes, such as urokinase, macrophage migration inhibitory factor and interleukin-6, anaphylatoxin C5a, ribosomal protein S19, nuclear protein HMGB1, and thioredoxin (Trx). These chemokine-like factors, classified into CLF (chemokine-like functions) class (Degryse and Virgilio, 2003), are multifunctional proteins that exert various well-defined primary roles, while under special circumstances can exhibit the chemokine-like functions as secondary roles. Like chemokines, elevated levels of Trx have been shown to inhibit neutrophil and monocyte recruitment induced by the murine chemokines KC/GROalpha, RANTES (regulated upon activation, normal T cell expressed and secreted), and MCP-1 (monocyte chemoattractant protein-1) (Nakamura et al., 2001). However, the mechanism of desensitization is unclear because Trx does not act through a 7TM-R and/or G proteins. In addition, like chemokines, uPA/uPAR-dependent signaling (Degryse et al., 1999, 2001b; Degryse, 2003) and the chemotaxis of HMGB1 (Degryse et al., 2001a) are PTX sensitive, suggesting the involvement of a heterotrimeric G-protein, but the complete mechanism of which are not known.

CKLF1 do not have the classical CC chemokine structure and has multiple activities including chemotactic activities in a wide spectrum of leukocytes and an ability to stimulate the proliferation of murine skeletal muscle cells (Han et al., 2001) and the proliferation of human bone marrow hematopoietic progenitor cells and colony formation (Ke et al., 2002). Therefore, CKLF1 shares structural and multifunctional properties with the CLF proteins mentioned above. On the other hand, CKLF1 is distinguished from these CLF proteins due to the similarity with TARC/CCL17 and MDC/CCL22, two members of the CC chemokine family and CCR4 is their common functional receptor, and due to the identical CC motif and key amino acids around the motif between CKLF1 and TARC/CCL17 and MDC/CCL22 (Han et al., 2001), though CKLF1 lacks the additional C-terminus cysteines comparing with other CC subfamily members. In addition, CKLF1 can induce migration of lymphocytes and shows high-level expression in thymus (Han et al., 2001), which is similar with TARC/CCL17 and MDC/CCL22. We therefore speculate that CKLF1 is a chemokine-like factor between classical chemokines and chemokine-like proteins (unrelated structurally to classical chemokines), and CKLF1, TARC/CCL17 and MDC/CCL22 may have overlapping receptor specificity and therefore compared the receptor activation by TARC/CCL17.

In this work, the potential interaction of CKLF1 with the chemokine receptors was investigated. CKLF1 is a potent agonist for CCR4, as demonstrated by calcium mobilization, chemotaxis and internalization experiments. CKLF1 was able to induce transmigration of HEK293 cells expressing CCR4, but not CCR6 or mock-transfected cells, while pretreatment with TARC/CCL17 can desensitize the receptor to subsequent stimulation with CKLF1 (Fig. 2). The CKLF1 dose–response curve for chemotaxis was bell-shaped, which is typical for responses to chemokines. It is known that CCR4 couples to the Gi/o family proteins (Oh et al., 2002; New and Wong, 2003), which is sensitive to PTX. Furthermore, these actions of CKLF1 were markedly inhibited by treatment of the cells with PTX. We were also able to show that CKLF1 or TARC/CCL17 is capable of specifically inducing transient calcium mobilization in CCR4-expressing HEK293 cells (Fig. 3). CKLF1 completely desensitized these cells to TARC/CCL17 and TARC/CCL17 partially desensitized CCR4-expressing HEK293 cells to CKLF1 (Fig. 3), suggesting the possibility that there is a higher affinity of CKLF1 compared to TARC/CCL17 for CCR4. CKLF1 specifically induced internalization of CCR4-EGFP (Fig. 4). Taken together, our data indicate that CKLF1 is a functional ligand for CCR4.
A recent study demonstrated that a electrophoretical transfer of naked plasmid with CKLF1 cDNA into skeletal muscle of BALB/c mice caused dramatic pathological changes to the lung, including peribronchial leukocyte infiltration, thickening of alveolar walls, epithelium shedding, subepithelial deposition of collagen, proliferation of bronchial smooth muscle cell and fibrosis of the lung parenchyma (Tan et al., 2004). The sustained morphological abnormalities of the bronchial and bronchiolar wall, induced by CKLF1, mimicked those in chronic persistent asthma. Acute pneumonitis and interstitial pulmonary fibrosis induced by CKLF1 is similar to acute respiratory distress syndrome (ARDS) as well as severe acute respiratory syndrome (SARS), suggesting that CKLF1 may play an important role in the pathogenesis of these diseases. A series of studies have showed the involvement of CCR4 in allergic lung inflammation (Gonzalo et al., 1999; Lloyd et al., 2001; Panina-Bordignon et al., 2001). Therefore, we speculate that CKLF1 selectively recruits CCR4 expressing cells, such as T-helper type 2 cells, as an important mechanism in allergic inflammation, which may constitute a new drug target for the development of antagonists, humanized monoclonal anti-CKLF1 antibody being an attractive candidate.

CKLF1 may also interact with other receptors different from CCR4. Besides high expression in thymus, CKLF1 has extensive tissue distributions, which is different from TARC/CCL17 and MDC/CCL22 abundantly expressed in the thymus with very little expression in other tissues (Power et al., 1995; Imai et al., 1996; Godiska et al., 1997; Imai et al., 1998). By contrast, CKLF1 also has high expression levels in human spleen, lung, testis, ovary, peripheral blood leucocytes, placenta, pancreas, fetal brain, fetal skeletal muscle, fetal thymus and fetal heart (Han et al., 2001). CKLF1 may possess other relevant biological functions. Previous results have demonstrated that CKLF1 is a potent chemoattractant for neutrophils, monocytes and lymphocytes (Han et al., 2001) and can increase the proliferation of human bone marrow hematopoietic progenitor cells and colony formation (Ke et al., 2002), it also has promoting effects on the proliferation and differentiation of skeletal muscle cells (Han et al., 2001). It is possible that CKLF1, TARC/CCL17 and MDC/CCL22 may play both similar as well as distinct physiological and pathological roles. CCR4 is selectively expressed on dendritic cells, basophils, T cells (Th2, Treg, skin-homing), and platelets (D’Ambrosio et al., 2003), suggesting CKLF1 may also act on other receptors different from CCR4. To date, about 50 chemokines and 20 receptors have been identified which suggests that a number of chemokines must bind to the same receptor and that one chemokine can bind several receptors (Bajetto et al., 2002). We also found that a chemically synthesized peptide, corresponding to the sequence of CKLF1, can interact with other receptors besides CCR4 (data not shown).

A recent study shows that GC-Th cells significantly upregulated the expression of CXCL13, a chemokine critical for B cell entry to lymphoid follicles, and CKLF1 and ICOS, important co-stimulation molecules in the formation of humoral immune response and germinal center formation (Kim et al., 2004). Thus, CKLF1 may also play a role in the humoral immune response and germinal center formation via CCR4 or other chemokine receptors.

In summary, our results indicate that CKLF1 is a novel high affinity ligand for CCR4 and represents a chemokine-like factor between classical chemokines and chemokine-like proteins (unrelated structurally to classical chemokines), a finding that should promote understanding of CKLF1-mediated physiological and pathophysiological phenomena. Further studies may be usefully directed to determining whether CKLF1 has affinity for other receptors and the respective functioning. Overall this study should aid in our understanding of inflammatory diseases and development of tissue cells.

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