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Chemokine-like Receptor 1 (CMKLR1) and Chemokine (C-C motif) Receptor-like 2 (CCRL2); Two Multifunctional Receptors with Unusual Properties

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Abstract

Chemokine-like receptor 1 (CMKLR1), also known as ChemR23, and chemokine (C-C motif) receptor-like 2 (CCRL2) are 7-transmembrane receptors that were cloned in the late 1990's based on their homology to known G-protein-coupled receptors. They were previously orphan receptors without any known biological roles; however, recent studies identified ligands for these receptors and their functions have begun to be unveiled. The plasma protein-derived chemoattractant chemerin is a ligand for CMKLR1 and activation of CMKLR1 with chemerin induces the migration of macrophages and dendritic cells (DCs) in vitro, suggesting a pro-inflammatory role. However, in vivo studies using CMKLR-deficient mice suggest an anti-inflammatory role for this receptor, possibly due to the recruitment of tolerogenic plasmacytoid DCs. Chemerin/CMKLR1 interaction also promotes adipogenesis and angiogenesis. The anti-inflammatory lipid mediator, resolving E1, is another CMKLR1 ligand and it inhibits leukocyte infiltration and proinflammatory gene expression. These divergent results suggest that CMKLR1 is a multifunctional receptor.

The chemokine CCL5 and CCL19 are reported to bind to CCRL2. Like Duffy antigen for chemokine receptor (DARC), D6 and CCX-CKR, CCRL2 does not signal, but it constitutively recycles, potentially reducing local concentration of CCL5 and CCL19 and subsequent immune responses. Surprisingly, chemerin, a ligand for CMKLR1, is a ligand for CCRL2. CCRL2 binds chemerin and increases local chemerin concentration to efficiently present it to CMKLR1 on nearby cells, providing a link between CCRL2 and CMKLR1. Although these findings suggest an anti-inflammatory role, a recent study using CCRL2-deficient mice indicates a proinflammatory role; thus, CCRL2 may also be multifunctional. Further studies using CMKLR1- or CCRL2-deficient mice are needed to further define the role of these receptors in immune responses and other cellular processes.

Keywords

chemokine receptor; G-protein-coupled receptor; inflammation; leukocyte trafficking

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INTRODUCTION

Chemokine-like receptor 1 (CMKLR1), also known as ChemR23, and chemokine (C-C motif) receptor-like 2 (CCRL2) are 7-transmembrane (7-TM), G-protein-coupled receptors (GPCRs) cloned in the late 1990's. Recent studies identified ligands that specifically bind to these receptors and began to reveal their contribution to the regulation of immune responses and other cellular processes. Neither receptor is a functional chemokine receptor, yet they have the capacity to regulate the trafficking of dendritic cells (DCs) and contribute to the regulation of immune responses. Interestingly, CCRL2 has the capacity to bind the CMKLR1 ligand chemerin and presents it to CMKLR1 on nearby cells. Thus, these two receptors do not function independently and they collaborate with each other. Here we review the progress of studies involving these receptors and discuss their multifunctional roles in immune responses and other cellular processes.

CMKLR1/ChemR23

Cloning of the CMKLR1 gene

The human CMKLR1 gene was first cloned in 1996 using PCR with degenerate oligonucleotides based on conserved regions in the third and sixth transmembrane domain of the seven transmembrane G-protein linked somatostatin receptor subtypes 1–4. This gene encoded a putative 371-amino acid 7-TM receptor, but it was found to share only about 40% nucleotide sequence similarity to the somatostatin receptors 1–4 [1]. An identical gene was later cloned using a similar method and termed ChemR23 [2]. Structurally, CMKLR1 appears to be more closely related to chemoattractant receptors, such as anaphylatoxin C3a and C5a receptors and formyl peptide receptors rather than to the members of the CC- or CXC-chemokine receptor families [2]. The CMKLR1 gene is located on human chromosome 12q.24.1, whereas the genes for chemokine receptors are located on chromosome 2, 3, 6, 17, and X [3]. The murine orthologue of the human CMKLR1 (mCMKLR1) gene, originally termed DEZ, was cloned from mouse brain by PCR [4]. Although both CMKLR1 and ChemR23 are used as the term for this receptor, we use the term CMKLR1, the official symbol for the gene, in this review.

Cell types expressing CMKLR1

Expression of CMKLR1 mRNA was detected in a wide variety of tissues by Northern blotting. Its expression in hematopoietic tissues, such as thymus, bone marrow, spleen, fetal liver, and lymphoid organs, was consistent with its possible role in regulating leukocyte functions [1]. Among leukocyte populations, abundant CMKLR1 mRNA expression was detected by real time RT-PCR in monocyte-derived human macrophages and immature DCs (iDCs). In monocyte-derived iDCs, CMKLR1 mRNA expression was down-regulated in response to a low concentration of lipopolysaccharide (LPS, 10 ng/ml) but up-regulated in response to a high concentration of LPS (1 µg/ml). Low amounts of CMKLR1 mRNA were detected in unstimulated CD4⁺ T lymphocytes, but no transcript was detected in polymorphonuclear cells (PMNs), monocytes or B lymphocytes [5]. In another study, abundant CMKLR1 mRNA was detected by RT-PCR in blood monocytes and low levels in PMNs and T cells [6]. The expression of CMKLR1 mRNA in monocytes was further up-regulated in response to tumor necrosis factor (TNF) or interferon-γ (IFN-γ) [6]. CMKLR1 mRNA was detected in the monocytic THP-1 cell line in one study [6], but not in the other [2]. CMKLR1 mRNA was not detected in many cell lines, such as Raji (lymphoblastic), Jurkat (T lymphoblastic), KG-1A (promyeloblastic), HL-60 (promyelocytic), HEL 92.1.7 (erythroleukemia), MEG-01 (megakaryoblastic) and K-562 (myelogenous leukemia) [2].

By FACS analysis using a monoclonal Ab against this receptor, cell surface expression of CMKLR1 was detectable on monocyte-derived human macrophages and iDCs [7,8],

whereas monocytes did not express CMKLR1 [9] despite the detection of a high level of mRNA expression in these cells [6]. Consistent with the RT-PCR results, cell-surface CMKLR1 expression was down-regulated after maturation of monocyte-derived iDCs with LPS (10 or 100 ng/ml), CD40 ligand (CD40L), or influenza virus [5,7,9]. Examination of circulating leukocytes revealed that circulating plasmacytoid DCs (pDCs), especially immature pDCs, expressed CMKLR1 [7,9]. In pDCs matured by incubation with CpG oligonucleotides, CD40L plus interleukin (IL)-3, LPS, influenza virus, or LPS plus influenza virus, the expression of CMKLR1 was down-regulated [7,9]. CMKLR1 expression was detected on circulating myeloid-derived DCs (mDCs) in one study [7], but not detected in another study [9], suggesting possible variations in individual donor cells or in the sensitivity of the methods used. In addition to DCs, CD56⁺ natural killer (NK) cells also expressed CMKLR1 on the cell surface, but the expression on NK cells was down-regulated after in vitro culture with IL-2 or IL-15 [10]. CMKLR1 expression was lower in pDCs and mDCs from systemic lupus erythematosus patients compared with those from healthy donors [11]. By immunohistochemistry, DCs in dermis from normal skin were positive for CMKLR1, whereas Langerhans cells were negative [7]. In addition, cell surface expression of CMKLR1 was detected on human PMNs (J. Cash, personal communication), consistent with the detection of the transcripts in this cell type [6].

The expression of mCMKLR1 on mouse leukocytes was examined by two groups using two different rat monoclonal antibodies against mouse CMKLR1, clone BZ194 [8] or clone 489C [12]. Both groups detected mCMKLR1 on macrophages isolated from different tissues, including peritoneal cavities, pleural cavities and spleens. A recent study by another group detected the cell-surface expression of mCMKLR1 on peritoneal exudate macrophages using the clone BZ194 Ab [13]. Interestingly, the expression of mCMKLR1 on macrophages was down-regulated after “classical” activation with TLR ligands, such as LPS, poly(I:C) and CpG oligonucleotide, and cytokines, such as IFN- γ and TNF, whereas anti-inflammatory cytokines, such as TGF- β 1 and β 2, that deactivate macrophages [14] up-regulated mCMKLR1 expression [8]. This suggests an anti-inflammatory role for mCMKLR1. As described above, TNF and IFN- γ up-regulated the expression of CMKLR1 mRNA in human monocytes [6]; thus, these cytokines may have different effects upon maturation of monocytes to macrophages.

Conflicting results were obtained with regards to the expression of mCMKLR1 on DCs. Although mCMKLR1 was detected on DCs generated from BM cells or isolated from spleens by both groups, mCMKLR1 expression on pDCs was detected in one study [12], but not in the other study [8]. mCMKLR1 was also detected on NK cells in one study [12], but not in the other [8]. These conflicting results were likely due to the use of two different antibodies with different specificity and affinity. Additional studies with additional antibodies are necessary to conclude mCMKLR1 expression on murine pDCs and NK cells. In addition to leukocytes, CMKLR1 expression was detected in adipocytes [15–18], endothelial cells [19], oral epithelial cells [20], osteoclasts [21] and vascular smooth muscle cells [22] (Table 1).

The role of CMKLR1 in immune responses

A co-receptor for SIV and HIV entry—Since several chemokine receptors are utilized by HIV-1, HIV-2 and SIV as co-receptors for their entry into target cells, a panel of HIV-1, HIV-2 and SIV envelopes were tested in a fusion assay for their potential usage of CMKLR1 as a co-receptor. None of the HIV-2 strains tested used CMKLR1 as a co-receptor; however, several SIV clones and one HIV-1 strain were able to use CMKLR1 as a co-receptor [2].

Activation of CMKLR1 with chemerin—To identify the ligands for this receptor, more than 200 bioactive molecules, including chemokines, C5a, C3a, N-formyl-methionyl-leucyl-phenylalanine, bradykinin, PAF and leukotriene, were tested for activation of CMKLR1. None of them activated the receptor at concentrations higher (100 nM or 1 μ M) than those reported to activate their own receptors [5], requiring the search for its ligand in biological fluids. Wittamer et al. developed a screening assay, utilizing CHO-K1 cell lines coexpressing human CMKLR1, apoaquorin, and $G_{\alpha 16}$, thus allowing them to test fractions from human inflammatory fluids as well as the extracts of various porcine and human lymphoid organs. A biological activity, specific for CMKLR1-expressing cell lines, was detected in fractions resulting from the reverse phase HPLC fraction of human ascitic fluids (secondary to ovary or liver neoplasms), in arthritic synovial fluid as well as in extracts from human spleen. The active molecule was subsequently identified as the product of the human tazarotene-induced gene-2 (Tig-2) gene (truncated at the C-terminus), and was termed chemerin [5]. Meder et al. [23] and Zabel et al. [9] also identified the circulating form of Tig-2 gene product as a CMKLR1 ligand by screening a hemofiltrate peptide library or by directly purifying it from human serum, respectively.

Chemerin is produced as a 163-amino acid precursor protein - pro-chemerin (or Tig-2) [5,23,24] – and is activated when cleaved at its C-terminus by serine proteases of the coagulation, fibrinolytic or inflammatory cascades [24,25]. Active chemerin (137-amino acid, from residue 21 to 157) binds to CMKLR1 expressed on macrophages and DCs, and induces cell migration [5,9]. A nonapeptide (chemerin-9) corresponding to the C-terminus of the processed form of human chemerin (from residue 149 to 157) was identified as a potent agonist for CMKLR1 by an intracellular Ca^{2+} release assay, indicating the importance of the protein's C-terminus for receptor binding and chemotactic activity [26]. The bioactive form identified in the circulation was residue 21 to 154 of pro-chemerin [23].

As apparent from its expression pattern, a key result of the CMKLR1/chemerin interaction appears to be in directing the trafficking of pDCs [7,27]. Psoriasis is one of the most common chronic T cell-mediated diseases in humans. Among the most proximal event in the innate immunity cascade driving psoriatic inflammation is the secretion of type I interferon (IFN) by activated pDCs [28]. Prepsoriatic skin adjacent to active lesions and early psoriatic lesions were characterized by an elevated expression of chemerin in the dermis and by the presence of $CD15^{+}$ neutrophils and $CD123^{+}/BDCA-2^{+}/CMKLR1^{+}$ pDCs. In contrast, skin from chronic plaques showed low chemerin expression, segregation of neutrophils to epidermal microabscesses, and few pDCs in the dermis. Chemerin expression was localized mainly in fibroblasts, mast cells, and endothelial cells. Fibroblasts cultured from the skin of psoriatic lesions expressed higher levels of chemerin mRNA and protein than fibroblasts from uninvolved skin of psoriasis patients or skin of healthy donors. Supernatants of fibroblasts from psoriatic lesions promoted pDC migration in vitro in a chemerin-dependent manner. Therefore, chemerin expression specifically marks the early phases of evolving skin psoriatic lesions and is temporally strictly associated with pDCs. These results support a role for the chemerin/CMKLR1 interaction in the early phases of psoriasis development [29,30].

Activation of CMKLR1 with a chemerin-derived peptide, C15—Since chemerin activates CMKLR1 and induces migration of CMKLR1-expressing antigen presenting cells, this receptor is considered to play a proinflammatory role. However, this receptor may play an anti-inflammatory role. Cash et al. reported that pretreatment of elicited mouse peritoneal macrophages with chemerin inhibited their production of inflammatory mediators in response to LPS and IFN- γ [31]. This inhibitory effect required processing of chemerin by cysteine proteases and was in marked contrast to the proinflammatory properties of active chemerin produced by serine protease cleavage [25]. Since the biological activity of chemerin depends upon C-terminal processing, peptides released upon C-terminal

processing might be responsible for the inhibition. As speculated, one peptide, chemerin 15 (C15, from residue 140 to 154 of mouse chemerin), possessed potent anti-inflammatory effects at surprisingly low picomolar concentrations. Intraperitoneal administration of C15 to mice prior to zymosan challenge suppressed the recruitment of PMNs and monocytes with a concomitant reduction in the expression of pro-inflammatory mediators. C15 appeared to deliver a negative signal through CMKLR1, as it had no inhibitory effect in CMKLR1-deficient mice. Administration of neutralizing antibody against chemerin to mice before zymosan challenge markedly increased the numbers of intraperitoneally infiltrating PMNs and macrophages, suggesting that chemerin-derived inhibitory peptides are physiologically generated and may play an important role in controlling the severity of inflammatory responses. Thus, depending on the class of protease which cleaves prochemerin, CMKLR1-binding peptides with either pro- or anti-inflammatory effects could be produced, although the pro-inflammatory role of chemerin is yet to be demonstrated in vivo.

To analyze the mechanisms by which C15 acts as an anti-inflammatory peptide, Cash et al. examined the effect of C15 on macrophage phagocytosis [32]. In vitro, C15 enhanced macrophage phagocytosis of zymosan by up to 360% at low picomolar concentrations (optimal at 10 pM). In agreement with its in vivo anti-inflammatory effect described above, C15-treated macrophages released lower levels of proinflammatory cytokines and chemokines, including TNF, IL-6, IL-12 p40 and MCP-1/CCL2, in comparison with untreated macrophages which also phagocytized zymosan. C15-treated macrophages also exhibited enhanced phagocytosis of apoptotic Jurkat cells at an optimal dose of 1 pM. They again released lower levels of TNF and MCP-1, but higher levels of the anti-inflammatory cytokine TGF- β . These in vitro results are consistent with the anti-inflammatory role of C15 observed in vivo.

The prophagocytic effect of C15 was associated with increased actin polymerization and localization of F-actin to the phagocytic cup. Pharmacological inhibition of Syk activity completely abrogated the prophagocytic activities of C15 and associated changes in actin polymerization and phagocytic cup formation, suggesting that C15 promotes phagocytosis by facilitating phagocytic cup development in a Syk-dependent manner.

Surprisingly, chemerin, the parent molecule of C15, failed to enhance macrophage zymosan ingestion, but was able to promote phagocytosis of apoptotic cells at an optimal dose of 0.1 pM. This prophagocytic activity of chemerin was abolished when it was administered with leupeptin that inhibits both serine and cysteine proteases. Thus, it appears that proteases that cleave chemerin to generate peptides with an anti-inflammatory and prophagocytic activity were released by either macrophages or apoptotic cells during phagocytosis of apoptotic cells, but not of zymosan. Such enzymes are presumably released from apoptotic cells because chemerin did not exert any prophagocytic effects on the phagocytosis of zymosan [32]. Identification of proteases responsible for the generation of such anti-inflammatory peptides will help us define the role for CMKLR1 in inflammatory responses.

Activation of CMKLR1 with a lipid mediator resolving E1—Resolvin E1 (RvE1), a bioactive oxygenated product of the essential fatty acid, eicosapentaenoic acid, is another ligand of CMKLR1. At nanomolar levels, RvE1 reduced leukocyte infiltration in a TNF-induced air pouch or zymosan-induced mouse peritonitis model by 50 to 70%, and protected mice from sulfonic acid-induced colitis by reducing the degree of leukocyte infiltration in the colon [6,33]. CMKLR1 was identified as a receptor for RvE1 by expressing each human GPCR in HEK293 cells and monitoring the ability of RvE1 to inhibit TNF-induced NF- κ B activation in transfected cells. RvE1 inhibited IL-12p40 production by splenic DCs activated by *Toxoplasma gondii* soluble tachyzoite antigen (STAg). In vivo treatment with RvE1 also

blocked STAg-induced IL-12 production as well as DC migration into T cell areas of the spleen [6]. RvE1 also promoted clearance of PMNs from mucosal surfaces via induction of anti-adhesive molecule CD55 (decay accelerating factor) on CMKLR1-expressing epithelial cells and contributed to the resolution of inflammation [20].

RvE1 binds to another GPCR, the leukotriene B₄ (LTB₄) receptor BLT1, and it attenuates LTB₄-induced proinflammatory signals as a nonphlogistic ligand [34]. In a zymosan-induced mouse peritonitis model, intravenous injection of RvE1 blocked PMN infiltration by 38% 2 h after zymosan injection in wild type mice. This effect was not observed in BLT1-deficient mice, suggesting that the inhibitory effect of RvE1 was solely through inhibition of RvE1/BLT1 interaction; thus, contradicting the previous claim by the same authors that RvE1 reduced leukocyte infiltration, including PMN infiltration, via CMKLR1 [6]. Additional studies are required to evaluate the role of CMKLR1 and BLT1 in RvE1-mediated anti-inflammatory effects.

Interaction of RvE1 with CMKLR1 enhanced phagocytosis of fluorescent serum-treated zymosan with an optimal concentration of 10 nM. This RvE1 effect is similar to that of C15 but the required concentration for RvE1 was much higher than that for C15. Activation of CMKLR1-transfected CHO cells with RvE1 resulted in the phosphorylation of Akt and its downstream target, ribosomal protein S6 (rS6), a translational regulator, which was inhibited by the phosphatidylinositol 3-kinase inhibitor wortmannin and the ERK inhibitor PD98059, but not by the p38-MAPK inhibitor SB203580. RvE1-induced phosphorylation of rS6 was also detected in human macrophages [35].

Some of the effects of CMKLR1/RvE1 interaction, including the reduction in cytokine and chemokine production and phagocytosis, are similar to those of the CMKLR1/C15 interaction. However, C15 and RvE1 appear to activate different signaling pathways. In the case of C15, Syk was the important downstream signaling molecule activated upon CMKLR1 activation [32]. Syk is a tyrosine kinase that has been shown to be critical for various immune cell functions, including cytoskeletal rearrangements and phagocytosis [36]. It is not clear at present whether CMKLR1/RvE1 interaction activate Syk. It will be interesting to examine whether RvE1-induced, enhanced macrophage phagocytosis is also dependent on Syk.

An anti-inflammatory role of CMKLR1 in LPS-induced lung injury—The role of CMKLR1 was recently evaluated by Luangsai et al. using CMKLR1-deficient mice [12]. In a model of acute lung inflammation induced by LPS, intra-tracheal co-administration of recombinant chemerin with LPS reduced infiltrating PMN counts by 70% 12 h after challenge and this effect persisted at 24 h and 72 h within the resolution phase in WT mice. This reduced PMN infiltration was associated with reduced concentrations of KC/CXCL1, IL-6, TNF and IL-1 β in bronchoalveolar lavage fluids (BALFs). In CMKLR1-deficient mice, LPS induced a greater PMN infiltration and chemokine and cytokine release in BALFs as compared with WT mice, and chemerin treatment had no effect on the amount of chemokines and cytokines released into BALFs following LPS challenge. In contrast to PMNs, the number of macrophages in BALFs was increased after chemerin treatment in LPS-injected WT mice, whereas no effect was seen in CMKLR1-deficient mice. These findings suggested that macrophages recruited and perhaps activated via chemerin/CMKLR1 interaction were responsible for the reduced inflammatory response in WT mice. The data on the recruitment of pDCs, which are a major CMKLR1-expressing cell type, was not available.

Although studies by Cash et al. using the C15 chemerin peptide [31,32] and Luangsai et al. using CMKLR1-deficient mice [12] both demonstrated an anti-inflammatory role for

CMKLR1, there is a question as to the activity of the C15 peptide. First, the C15 peptide lacks a phenylalanine residue at the position 156 (¹⁵⁶F) that is essential for the activity of chemerin-derived peptides on CMKLR1 both in humans and mice [26]. Second, the C15 peptide functions in a picomolar concentration range which is much lower than that for active chemerin to function. Luangsai et al. tested the functional activity of the C15 peptide in various in vitro biological assays but they were not able to detect any activity of this peptide [12]. On the other hand, some of the biologically active forms of chemerin isolated from serum or hemofiltrate did not have ¹⁵⁶F [23,24]. Thus, ¹⁵⁶F may not be the determining factor for CMKLR1 activation after all. Additional studies are necessary to resolve the underlying molecular mechanisms leading to an anti-inflammatory effect shown by the interaction of CMKLR1 with chemerin or chemerin peptides, such as C15.

The role of CMKLR1 in adipogenesis and angiogenesis

The biological consequence of CMKLR1 activation may not appear to be limited to immune responses. High level expression of CMKLR1 and chemerin was detected in mouse and human adipocytes [15–18]. The level of CMKLR1 expression was significantly higher in adipose tissue of obese and type 2 diabetic *Psammomys obesus* compared with lean normoglycemic *Psammomys obesus*. In vitro, cultured 3T3-L1 adipocytes secreted chemerin protein, which triggers CMKLR1 signaling in adipocytes in an autocrine manner. Adenoviral small hairpin RNA targeted knockdown of chemerin or CMKLR1 expression impaired differentiation of 3T3-L1 cells into adipocytes, reduced the expression of adipocyte genes involved in glucose and lipid homeostasis, and altered metabolic functions in mature adipocytes. These results suggest that chemerin/CMKLR1 interaction results in a novel signaling mechanism that regulates adipogenesis and adipocyte metabolism.

CMKLR1 expression was also detected on human endothelial cells (ECs) and it was significantly up-regulated by pro-inflammatory cytokines, such as TNF, IL-1 β and IL-6. Chemerin was potently angiogenic and dose-dependently induced gelatinolytic (MMP-2 & MMP-9) activity of ECs. Furthermore, chemerin dose-dependently activated Akt and MAPKs pathways, such as ERK1/2 and p38 MAPK, key angiogenic and cell survival cascades in ECs. These results suggest a stimulatory role of CMKLR1 in angiogenesis [19]. The contributions of chemerin/CMKLR1 interaction to adipogenesis and angiogenesis are indicative of its multifunctional roles. These roles need to be further evaluated in vivo using CMKLR1-deficient mice.

Ligand-specific activation of CMKLR1

Classically, it was thought that ligand binding to 7-TM GPCRs stimulates or inhibits all receptor functions to an equal extent (called “correlated efficacies”) [37,38]. Although this paradigm explains most of the cellular responses to GPCR activation, accumulating evidence supports a new paradigm that ligands have the ability to selectively stabilize receptor conformations and stimulate or inhibit subsets of receptor activities (called “ligand bias”) [38–40]. This ligand-biased signaling was observed for a number of GPCRs, including chemokine receptors CCR7 [41] and CXCR4 [42]. In the case of CCR7, both CCL19 and CCL21 induced G protein activation and calcium mobilization with equal potency. However, only CCL19 promoted robust desensitization of CCR7. Activation of CCR7 by either CCL19 or CCL21 led to signaling to the ERK1/2 pathway, but CCL19 promoted 4-fold higher ERK1/2 phosphorylation than did CCL21. This was dependent on β -arrestin-2, suggesting that CCL19 and CCL21 place CCR7 in functionally distinct conformations that are independent of their G protein-coupling potency [41].

As described above, activation of CMKLR1 with its ligands, chemerin, C9, C15, and RvE1, activates different signaling molecules with various biological consequences (Fig. 1).

Recently, chemerin was found to promote macrophage adhesion to the extracellular matrix protein fibronectin and the adhesion molecule VCAM-1 [13]. The murine C-terminal peptide C9 exhibited 58.6% of the adhesive activity induced by intact chemerin, whereas C15 was unable to promote adhesion of CMKLR1-transfected cells. Thus, chemerin, chemerin-derived peptide C9 and C15, and RvE1, appear to bind to different subsets of receptor conformations, resulting in activation of a full or a partial set of signaling pathways downstream of CMKLR1.

CCRL2

Cloning of the CCRL2 gene

A cDNA coding for human CCRL2 was identified in a PMN cDNA library as an express sequence tag (EST) clone (termed human chemokine receptor; HCR) [43]. CCRL2 is also referred to as CRAM or CKRX in a database. The CCRL2 gene is located on chromosome 3p21 in close proximity to other chemokine receptors, including CCR5, CCR2, CX3CR1, CCR3 and CCR8. CCRL2 shares over 40% amino acid identity with CCR1, CCR2, CCR3 and CCR5 [44].

There are two transcript variants of CCRL2; the variant 1 (previously known as CRAM-B) and 2 (CRAM-A). The variant 1 encodes a 344-amino acid protein, whereas the variant 2 differs in the 5' UTR and coding region, compared to the variant 1, resulting in a longer 356-amino acid protein with a distinct N-terminus. LPS-inducible CC chemokine receptor (L-CCR) is considered to be the mouse orthologue of human CCRL2 (mCCRL2) [45].

Cell types expressing CCRL2

The expression of CCRL2 was detected on almost all human hematopoietic cells including monocytes, macrophages, PMNs, T cells (both CD4⁺ and CD8⁺), monocyte-derived iDCs, NK cells, and CD34⁺ progenitor cells [44,46,47] (Table 2). The level of CCRL2 on T cells was increased in response to stimulation with anti-CD3 or IL-2. The level of CCRL2 on monocyte-derived iDC was also increased in response to LPS, LPS + IFN- γ , poly (I:C), or CD40L [44]. In mouse bone marrow-derived iDCs, mCCRL2 mRNA was barely detectable, but LPS rapidly induced mCCRL2 mRNA expression, starting at 30 min and reaching a peak at 2 h, and decreased thereafter. The expression of mCCRL2 preceded that of CCR7 [48]. CCRL2 was not constitutively expressed on CD11c⁺ DCs purified from LN, spleen, thymus, bone marrow and lung. However, it was readily induced in spleen CD11c⁺ DCs by 6 h following LPS administration [48].

The expression CCRL2 on human PMN was examined in detail [47]. CCRL2 mRNA was not detectable in freshly isolated PMNs by Northern blotting. Stimulation of PMNs with LPS or TNF resulted in a dramatic up-regulation of CCRL2 mRNA expression after 4 h. Neither IL-1 nor IL-4 nor GM-CSF alone up-regulated CCRL2 mRNA expression. TNF in combination with IFN γ and/or GM-CSF synergistically induced CCRL2 mRNA expression. The effect of LPS on CCRL2 mRNA expression was variable, which may have been due to the induction of TNF in PMNs. Immunoprecipitation and Western blotting with anti-CCRL2 antibody confirmed the expression of a 39-kD protein in stimulated, but not in freshly isolated PMNs, at 16 h.

A study of joint fluids collected from 5 patients with active, chronic arthritis revealed that PMNs were the predominant cell type in the synovial fluids of all patients (average 79.04%). All of the PMNs examined were positive for CCRL2 expression, as determined by immunocytochemical staining. Positive CCRL2 staining was also observed in macrophages. Infiltrating lymphocytes did not show detectable CCRL2 expression [47], although other studies detected CCRL2 protein or mRNA expression in lymphocytes [44,46].

On B cells, the expression of CCRL2 was initially not detected [44]; however, a recent study detected CCRL2 transcripts for only the variant 1 (CRAM-B), in human B cells of healthy donors [49]. The pre-B acute lymphoblastoid leukemia cell lines, Nalm6 and G2, were also positive for CCRL2 [49].

In addition to hematopoietic cells, mouse astrocytes and microglia were shown to express mCCRL2 [50] and the mCCRL2 mRNA expression was rapidly up-regulated in response to LPS both in vitro and in vivo [50]. mCCRL2 mRNA expression in astrocytes and microglia was also demonstrated in an EAE model [51]. In addition, mCCRL2 mRNA was detected in bronchial epithelium in OVA-induced airway inflammation [52] (Table 2).

The role of CCRL2 in DC trafficking

The over 40% amino acid identity between CCRL2 and other functional chemokine receptors and the close proximity of the CCRL2 gene with other chemokine receptor genes strongly suggest that CCRL2 belongs to the family of signal transducing chemokine receptors. Previous studies reported that CCRL2 was activated by MCP-1, 2, 3, RANTES and joint fluid from rheumatoid arthritis patients [47,50,53]. However, CCRL2 may not function as a typical chemokine receptor. Human and mouse CCRL2 lack the conserved DRYLAIV motif in the second intracellular loop that is required for signaling of functional chemokine receptors. Other related GPCRs, such as DARC (Duffy antigen), D6 and CCX-CKR (Chemocentrin chemokine receptor), also lack this motif. Interestingly, these GPCRs bind to chemokines but do not transduce signals for cell migration or intracellular calcium mobilization. These non-functional (“silent” or “decoy”) receptors act as a sponge to absorb, internalize and clear chemokines, helping to dampen inflammation. Indeed, mice deficient in DARC or D6 displayed exacerbated inflammatory responses [54,55].

The expression of CCRL2 mRNA and protein was detected on human pre-B cell lines, Nalm6 and G2 as described above [49]. Interestingly, surface expression of CCRL2 was up-regulated in response to the chemokine RANTES/CCL5. Although Nalm6 cells did not express any of the known RANTES-binding receptors, such as CCR1 and CCR5, actin polymerization and the phosphorylation of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) were detected upon RANTES stimulation, suggesting that RANTES activated CCRL2. Neither calcium mobilization nor migration was induced. Thus, actin polymerization and ERK activation might reflect ligand-induced receptor cycling events rather than typical chemokine receptor activation. ERK1/2 phosphorylation was not inhibited by pertussis toxin, suggesting that CCRL2 does not couple to Gi proteins [49].

Although the binding of RANTES to CCRL2 was not demonstrated, the binding of the homeostatic chemokine MIP-3 β /ELC/CCL19 to CCRL2-expressing CHO-K1 cells was clearly shown. CCRL2 stimulation by CCL19 did not result in typical chemokine receptor-dependent cellular activation, such as calcium mobilization or migration. Instead, CCRL2 constitutively recycled via clathrin-coated pits and was able to internalize CCL19. Since CCL19 is critically involved in lymphocyte and DC trafficking, CCL19-binding competition by CCRL2 might affect the trafficking of these cells in disease states [56].

The role of CCRL2 in the trafficking of DCs was carefully examined by comparing the airway hypersensitivity reaction induced in WT and mCCRL2-deficient mice immunized with OVA plus alum [48]. The total number of DCs recruited to the lung of CCRL2-deficient mice in response to OVA challenge was normal. Interestingly, however, when FITC-OVA was used as an antigen, the migration of FITC⁺, antigen-loaded, lung DCs to mediastinal lymph nodes was significantly reduced in CCRL2-deficient mice. This resulted in defective lymphocyte priming in mediastinal lymph nodes, and subsequent reduction in airway inflammatory responses, including leukocyte infiltration and Th2 cytokine and chemokine

production. Since only antigen-loaded DCs expressed CCRL2, the migration of antigen-loaded DCs to mediastinal lymph nodes appeared to be dependent on CCRL2. There was no difference between WT and CCRL2-deficient mice when a Th1 response was induced by OVA plus LPS. Thus, CCRL2 plays a non-redundant role in the trafficking of activated, antigen-loaded DCs to the secondary LNs only in a Th2 response and CCRL2 acts as a proinflammatory receptor. However, the CCRL2 ligand contributing to this process remains unknown.

The role of CCRL2 in *Pneumocystis carinii* pneumonitis (PCP)

It is well established that CXCR4 and CCR5 are primary co-receptors for immunodeficiency virus (HIV)-1. Studies focusing on the association between human genotypes with HIV-1 infection or acquired immunodeficiency syndrome (AIDS) progression indicated that certain genotypes, such as CCR5-Δ32 or CCR2-641, protect the host from HIV-1 infection or delay the progression of AIDS, respectively [57].

There are two CCRL2 genotypes in humans, ¹⁶⁷Y genotype (T allele) and ¹⁶⁷F genotype (A allele). The ¹⁶⁷Y genotype codes for CCRL2 containing tyrosine at the residue 167 in the 2nd extracellular loop [43], whereas in the protein coded by the ¹⁶⁷F genotype, the tyrosine residue is substituted by phenylalanine. Similar allele frequencies were found in samples of all three tested ethnic groups, European American, African American and Han Chinese [58]. The impact of two CCRL2 genotypes on the progression of AIDS was compared [58]. Interestingly, AIDS patients with the ¹⁶⁷F genotype were found to be protected from *Pneumocystis carinii* pneumonitis (PCP). This finding supports the hypothesis that this allele change has a biological consequence and ¹⁶⁷F-CCRL2 may be a functional receptor.

When the amino acid sequences of CC chemokine receptors, CCR1 through 10, were aligned with non-functional chemokine binding proteins, DARC and D6, and human ¹⁶⁷F-CCRL2, CCR3, CCR4 and CCR6 were found to contain phenylalanine at the position corresponding to the residue 167 of human CCRL2. Furthermore, all functional chemokine receptors contained a non-polar amino acid at the position, whereas DARC, D6 and ¹⁶⁷Y-CCRL2 contained a polar amino acid at the position. This finding leads to the hypothesis that the presence of a non-polar amino acid at this position in each chemokine receptor may be important for their function and replacing phenylalanine in CCR3, CCR4 or CCR6, with tyrosine may reduce their responsiveness to the ligands. In fact, our results indicated that the replacement of phenylalanine with tyrosine in human CCR3 resulted in reduced chemotactic response of HEK293 cells to the CCR3 ligand eotaxin (Takahashi and Yoshimura, unpublished). It will be interesting to examine whether chemerin induces calcium mobilization and migration of cells expressing ¹⁶⁷F-CCRL2 as evidence that this receptor variant transduces signals. It should be noted that among three human CCRL2 cDNA clones, HCR, CRAM and CKRX, HCR and CKRX coded for ¹⁶⁷Y-CCRL2 whereas CRAM coded for ¹⁶⁷F-CCRL2. It is not clear which CCRL2 variant was used to examine its biological activity in previous studies.

The role of CCRL2 in CMKLR1 activation

The most intriguing recent finding is the co-operation between CCRL2 and CMKLR1. As described above, chemerin is a ligand for CMKLR1, but it also binds to CCRL2 [59,60]. In the study by Zabel et al., the authors showed that mouse CCRL2 was constitutively expressed on mast cells. To examine whether CCRL2 plays a role in the inflammatory response, they used mCCRL2-deficient mice. The absence of mCCRL2 did not affect basic in vitro mast cell functions or T cell-mediated contact hypersensitivity in vivo. The authors then examined a mast-cell-dependent model of atopic allergy, the IgE-dependent passive cutaneous anaphylaxis (PCA) reaction. Both WT and mCCRL2-deficient mice developed

marked local inflammation when they were sensitized with a high dose of DNP-specific IgE (150 ng/ear) and challenged with antigen intravenously. However, when a lower sensitizing dose was used (50 ng/ear), the mCCRL2-deficient mice had significantly reduced PCA reactions, suggesting that mCCRL2 ligation normally amplifies the inflammatory response. The amplified inflammatory response was due to mCCRL2 expression on mast cells, because mast cell-deficient mice engrafted with mCCRL2-deficient bone marrow progenitor cells had less ear swelling than did those engrafted with WT cells.

In an attempt to identify the ligand for CCRL2, a number of known available chemokines were screened using HEK293 transfectants expressing mCCRL2, but none of them stimulated chemotaxis of mCCRL2-transfected cells. Surprisingly, however, chemerin blocked the binding of anti-mCCRL2 antibody to mouse peritoneal mast cells. Despite binding to mCCRL2 (also to human CCRL2), with high affinity, chemerin elicited no functional response from either mCCRL2- or huCCRL2-expressing cells, including intracellular calcium mobilization, chemotaxis or CCRL2 internalization. Instead, incubating mCCRL2-transfected cells with chemerin caused a time-dependent increase in surface-bound chemerin. These chemerin-loaded cells then triggered calcium flux in responder cells expressing CMKLR1. Thus, CCRL2 seems to concentrate bioactive chemerin at inflammatory sites and facilitate its presentation to CMKLR1 on adjacent cells, unlike any other known chemokine receptor [59,60].

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Considerable progress has been made in our understanding of the functions of CMKLR1 and CCRL2. CMKLR1 is a signaling receptor activated by two ligands, chemerin and resolving E1, and this receptor appears to play an anti-inflammatory role. Chemerin/CMKLR1 interaction also facilitates adipogenesis and angiogenesis (Fig. 1). CCRL2 does not appear to be a signaling receptor, but it may play an anti-inflammatory role by capturing and internalizing CCL19 or by presenting chemerin to CMKLR1. In addition, CCRL2 is critical for the development of Th2 responses (Fig. 2). These findings are novel and appealing; however, some contradictory results were reported and important questions remain unanswered. 1) As described above, the chemerin peptide C15 and some of the endogenous, active chemerin lack ¹⁵⁶F which is demonstrated to be critical for CMKLR1 binding. How do they activate CMKLR1 and provide an anti-inflammatory effect? 2) Do all reported CMKLR1 ligands bind to the same CMKLR1 conformations? 3) What is the mechanism by which the recruitment of pDCs results in a reduction in inflammatory responses? Is this due to a tolerogenic activity of pDCs? In fact, increased expression of CMKLR1 and chemerin in inflammatory sites might be a feedback response to limit ongoing inflammatory responses. 4) CCRL2 is expressed not only on basophils, but also on activated PMNs and macrophages. What is the role of CCRL2 expressed on these cell types? 5) CCRL2 was required for the development of Th2, but not of Th1 response. How does CCRL2 regulate only Th2 responses? 6) Why does activation of CMKLR1 via CCRL2 result in an increased immune response? This in vivo finding contradicts the concept that CMKLR1 is an anti-inflammatory receptor. 7) In addition to CMKLR1 and CCRL2, chemerin binds to another GPCR, GPR1, and induce calcium mobilization [61,62]. How do these three receptors regulate chemerin activities? 8) What is the mechanism by which AIDS patients with ¹⁶⁷F-CCRL2 are protected from *Pneumocystis carinii* pneumonitis? Is ¹⁶⁷F-CCRL2 a signaling receptor? Further studies, especially those using CMKLR1- or CCRL2-deficient mice, will define the role of these receptors in immune responses and other cellular processes, and may provide us with novel therapeutic approaches for the treatment of inflammatory diseases.

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Figure 1. Multifunctional effects of CMKLR1 activation. A. Interaction of chemerin or chemerin peptides with CMKLR1 expressed on macrophages, DCs, adipocytes or ECs induces the activation of intracellular signaling molecules, such as Syk, ERK1/2, p38MAPK, Akt or PI3-K, leading to reduced immune responses and enhanced adipogenesis and angiogenesis. B. Similar to chemerin, resolvin E1 down-regulates cytokine/chemokine production in macrophages by reducing the activation of ERK1/2 and NF-κB. However, RvE1 appears to activate a distinct signaling pathway to stimulate nonphlogistic macrophage phagocytosis via CMKLR1.

Figure 2.

Biphaseic regulation of immune response by CCRL2. A. CCRL2 is reported to bind chemokines, such as CCL5 and CCL19. Internalization of these chemokines potentially reduces local concentration of these chemokines without CCRL2 activation, resulting in reduced immune responses. B. CCRL2 expressed on mast cells binds to chemerin and presents this ligand to CMKLR1. This leads to the activation of CMKLR1 and development of IgE-dependent, mast cell-dependent cutaneous anaphylaxis. This activity is detected only at a low concentration of antigen. C. In CCRL-2 deficient mice, the migration of antigen-loaded DCs from the lung to the secondary LN is impaired, resulting in the development of a Th2 response, but not of a Th1 response.

Table 1

Expression of CMKRL1

Cell types	Regulators	References
Monocytes	TNF, IFN- γ ↑	[6]
Macrophages	LPS, poly(I:C), CpG↓, TGF- β 1, β 2↑	[5,7,8,12,13]
Monocyte-derived iDCs	LPS, CD40L, influenza virus↓	[5,7–9,12]
Circulating immature pDCs	CpG oligonucleotides, CD40L+IL-3↓ LPS, influenza virus↓	[7,9]
Circulating mDCs		[7]
Skin DCs		[7]
PMNs		[6]
T lymphocytes		[6]
NK cells	IL-2, IL-15↓	[10]
Adipocytes	Differentiation↑ or ↓, obesity↑	[16,18]
Endothelial cells		[16,19]
Oral epithelial cells	TNF↓, TGF- β 1↑	[20]
Osteoclasts		[21]
Vascular smooth muscle cells		[22]
Glioma (DBTRG-05MG)		[6]
THP-1		[6]

Table 2

Expression of CCRL2

Cell types	Regulators	References
PMNs	LPS, TNF↑	[44,47]
Macrophages		[44,47]
T cells		[44]
Pre-B cells		[49]
DCs		[44,48]
Mast cells		[59]
CD34 ⁺ progenitors		[44]
Astrocytes	LPS↑	[50,51]
Microglia	LPS↑	[50,51]
Lung epithelial cells	OVA↑	[52]