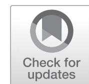

3 CCL4 Signaling in the Tumor Microenvironment

Naofumi Mukaida, So-ichiro Sasaki, and Tomohisa Baba

Abstract

CCL4, a CC chemokine, previously known as macrophage inflammatory protein (MIP)-1β, has diverse effects on various types of immune and nonimmune cells by the virtue of its interaction with its specific receptor, CCR5, in collaboration with related but distinct CC chemokines such as CCL3 and CCL5, which can also bind CCR5. Several lines of evidence indicate that CCL4 can promote tumor development and progression by recruiting regulatory T cells and pro-tumorigenic macrophages, and acting on other resident cells present in the tumor microenvironment, such as fibroblasts and endothelial cells, to facilitate their pro-tumorigenic capacities. These observations suggest the potential efficacy of CCR5 antagonists for cancer treatment. On the contrary, under some situations, CCL4 can enhance tumor immunity by recruiting cytolytic lymphocytes and macrophages with phagocytic ability. Thus, presently, the clinical application of CCR5 antagonists warrants more detailed analysis of the role of CCL4 and other CCR5-binding chemokines in the tumor microenvironment.

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Keywords

Arrestin · CCR5 · Chemokine · Chemotaxis · Cytolytic lymphocyte · Endothelial cell · Fibroblast · Macrophage inflammatory protein · Trimeric G protein · Human immunodeficiency virus · Macrophage · Metastasis · Myeloid suppressor cells · Neutrophils · Regulatory T cell

3.1 Introduction

Chemokines are defined as *chemo*tactic cyto*kines* that control the migration and positioning of immune cells and include more than 40 distinct molecules [1]. Chemokines display a low overall similarity at the amino acid level but have 4 cysteine residues at their well-conserved positions; they, thereby, adopt a similar three-dimensional structure by forming triple-stranded β-sheet structures arising from two intramolecular disulfide bonds formed between the first and third cysteines and between the second and fourth cysteines [2]. Additionally, they are divided into four subgroups, namely, CC, CXC, CX3C, and C, according to the positions of the first and second cysteines [2]. Chemokine receptors are trimeric G protein-coupled receptors with 4 extracellular, 7 transmembrane, and 4 intracellular portions. Chemokine receptors were initially presumed to be expressed solely by immune cells

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such as granulocytes, monocytes/macrophages, and lymphocytes. However, subsequent studies have revealed that some of the chemokine receptors are also expressed by nonimmune cells including endothelial cells, epithelial cells, fibroblasts, and cancer cells [3]. Thus, chemokines exert a wide variety of effects on myriads of target cells by interacting with their cognate receptors expressed on these target cells [3].

A doublet protein with an inflammatory activity was purified from the supernatant of endotoxin-stimulated mouse macrophages and was designated as macrophage inflammatory protein (MIP)-1 [4]. Further analysis revealed that MIP-1 consisted of two distinct molecules, MIP-1α and MIP-1β, which shared 68% identical amino acid sequence and displayed a characteristic feature of CC chemokines [5]. Mouse MIP-1α was eventually identified to be the mouse counterpart of human LD78α, which was previously cloned from stimulated human lymphocytes [6]; human equivalent of MIP-1β cDNA has been cloned by several independent groups [7–9]. According to the present nomenclature system, MIP-1α/LD78α and MIP-1β have been renamed as CCL3 and CCL4, respectively [10]. CCL4 utilizes CCR5 as its specific receptor. However, CCR5 is also used by CCL3 and CCL5 (synonym of RANTES), which are other related but distinct chemokines that can also bind to CCR1.

Here, we will mainly discuss CCL4 in the context of tumor microenvironment, but would also mention other CCR5-binding chemokines, viz. CCL3 and CCL5, if necessary.

3.2 Biochemistry of CCL4

Human *CCL4* gene is located in chromosome 17q12. Further, humans, but not the rodents, possess CCL4-related gene, presently named as *CCL4L1* gene, close to *CCL4* gene locus [11]. Both *CCL4* and *CCL4L1* genes can express alternatively spliced variants lacking exon 2, which seem to be deficient in biological activities [12]. *CCL4* gene has a single copy number, but *CCL4L1* gene has varied copy numbers ranging from 0 to 5 copies per diploid genome in Caucasians [13], with undetermined pathophysiological significance of its copy number variation. Additionally, *CCL4L1* gene is polymorphic with a nucleotide change in the intron 2 acceptor sites, thereby creating another allelic variant, *CCL4L2*, which can generate 9 new mRNAs and result in reduced CCL4L expression [12].

Human CCL4 protein is secreted in its mature form consisting of 69 amino acids after its signal peptide with 23 amino acids is removed from its precursor form (Fig. 3.1) [5]. Human mature CCL4 and CCL4L1 proteins exhibit a strikingly high amino acid identity with a single amino acid difference (Fig. 3.1) and are devoid of apparent N-linked glycosylation sites [5, 11]. Nuclear magnetic resonance (NMR) analysis has defined the three-dimensional structure of human CCL4 [14]. Under the specific analysis condition (pH 2.5), human CCL4 exists as a dimer; it aggregates at pH value of above 3.5 [14]. The amino-terminus comprises an irregular strand followed by a long loop extending from residues 12 to 20 and four-residue helical turn (residues 21 to 24). Like other chemokines [2], the helical turn leads to the formation of a triple-stranded antiparallel β sheet from residues 26 to 52, on which lies an α helix (residues 57 to 68). Hydrogen bonding and hydrophobic interactions induce the formation of CCL4 dimer, which is elongated and cylindrical [14]; this is in contrast to CXCL8 dimer, which is globular [2]. A subsequent study demonstrated that CCL4 and CCL3 monomers are sensitive to the degradation by proteases such as insulin-degrading enzyme and that their polymerization can prevent their degradation by this enzyme [15]. Concomitantly, the polymerization can bury the receptor-binding sites of these chemokines, thereby reducing their chemotactic activities. However, the molecular dynamics of these CC chemokine structures in various body fluids remain elusive.

CCL4, similar to the other chemokines, can tightly bind glycosaminoglycans (GAGs) such as heparin, heparin sulfate, and chondroitin sulfate [2]. CXCL8 binds heparin at its C-terminal α-helix and loop connecting the elongated N-terminal region with the first β-strand [16]. On the contrary, CCL4 binds heparin at three residues, viz. R18, K45, and R46, that are outside of its α helix portion [17].

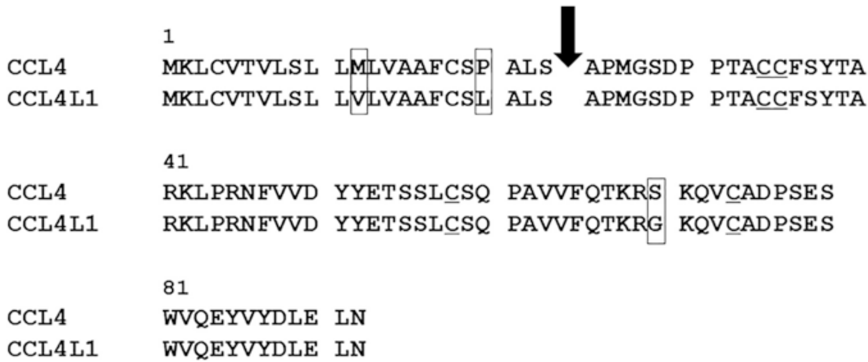

Fig. 3.1 Amino acid sequences of human CCL4 and CCL4L1. The arrow indicates the starting site of mature proteins while boxes indicate the sites at which the amino acid differs between the proteins. Well-conserved four cysteine residues are underlined. The numbers indicate the amino acid residue numbers

Among these, R46 is crucial for the interaction of CCL4 with, both, heparin and heparan sulfate under physiological conditions. Nevertheless, through the interaction with GAGs, secreted chemokines can be immobilized on luminal surface of endothelium, which is rich in GAGs.

3.3 Physiology of CCL4 and Its Receptor

Various types of blood cells can produce CCL4 in vitro [18]. Lipopolysaccharide (LPS) and interleukin (IL)-7 induce human peripheral blood monocytes to express CCL4 and the induction was suppressed by IL-4 [19]. Although *CCL4* gene possesses glucocorticoid-responsive elements in its 5′-regulatory gene, a glucocorticoid had no effects on CCL4 expression [19]. Anti-CD3 treatment induced peripheral $CD8^+$ T cells to abundantly produce CCL4 and CCL3 [20]. Additionally, $CD8^+$ T lymphocytes displayed robust CCL4 expression upon challenge with human immunodeficiency virus (HIV) or *Brucella abortus* [21, 22]. Moreover, activated natural killer cells [20] and activated B cells [23] could produce CCL4 and CCL3. Dendritic cells also produced CCL4 and CCL3 rapidly, but transiently, after they were stimulated with LPS, tumor necrosis factor (TNF)-α, or CD40 ligand [24]. Furthermore, neutrophils could produce CCL4 and CCL3 when they were stimulated with outer membrane vesicles from *N. meningitides* [25]. In addition to the blood immune cells, pulmonary vascular smooth cells produced CCL4 as well as CCL3 when stimulated with IL-1β, TNF-α, IL-4, IL-10, and interferon (IFN)-γ [26]. Collectively, CCL4 and CCL3 can simultaneously be produced by a variety of activated cells, except human brain microvessel endothelial cells which expressed CCL4 and CCL5 but not CCL3, when stimulated with LPS, IL-1β, TNF-α, and IFN-γ [27].

Samson and colleagues have identified a chemokine receptor, presently denoted as CCR5, as a specific receptor for CCL3, CCL4, and CCL5 based on their observations that these chemokines were active at their physiological concentrations [28]. Another chemokine receptor, presently denoted as CCR1, was proposed to be a specific receptor for CCL4 as well as CCL3 and CCL5; however, this receptor exhibits approximately 100-fold lower affinity for CCL4 than that for CCL3 and CCL5 [29] (Fig. 3.2). Thus, CCR5, but not CCR1, can be a specific receptor for CCL4 under physiological conditions. Additionally, CCR5 has been identified as a co-receptor for the entry of HIV, particularly the macrophage-tropic type, and hence, as a consequence, its 32-base pair deletion allele is associated with resistance to HIV infection as the deletion results in premature stop codon generation [30, 31].

Similar to other chemokine receptors, CCR5 is coupled with αβγ heterotrimeric G proteins [32].

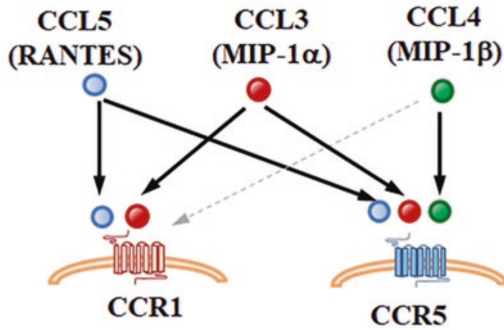

Fig. 3.2 Redundant use of CCR1 and CCR5 by the chemokines. CCR1 binds CCL3 and CCL5 but not CCL4 with high affinity, whereas CCR5 binds CCL3, CCL4, and CCL5 with high affinity

Once activated, both, the Gα and Gβγ subunits, can activate downstream signaling pathways including phosphatidylinositol 3 kinase (PI3K), phospholipases A, C, and D, and mitogen-activated protein (MAPK). Ligand stimulation simultaneously leads to CCR5 phosphorylation at its four C-terminal residues by G protein-coupled receptor kinase (GRK)2 and GRK3 [33]. Phosphorylated CCR5 recruits β-arrestin, which can activate signaling molecules such as Akt and MAPK, and can induce clathrin-dependent endocytosis, thereby leading to CCR5 internalization [34]. Thereafter, CCR5 accumulates in the trans-Golgi network and is recycled back to the cell surface when a bound ligand dissociates from CCR5 [35].

CCR5 is expressed by hematopoietic cells such as a subpopulation of lymphocytes and monocyte/macrophages in blood; primary and secondary lymphoid organs; and non-hematopoietic cells including epithelium, endothelium, and fibroblasts [36]. Among immune cells, T helper 1 and 2 lymphocytes [37], immature dendritic cells [24], regulatory T cells (Tregs) [38], and natural killer cells [39] express CCR5. In the central nervous system, neurons, astrocytes, and microglia express CCR5 [36].

CCR5 expression in the hematopoietic cells provides CCL4 with profound biological effects on hematopoietic cells present in the tumor microenvironment. CCL4 can in vitro induce chemotaxis and adhesion of T cells to the vascular cell adhesion molecule (VCAM)-1 [40, 41]. The responding T cell population includes T helper 1 and 2 cells [37], and Tregs [38]. CCL4 can induce chemotaxis of immature but not mature dendritic cells [42]. It can also induce chemotaxis and activation of both NK cells and monocytes; however, its effects are far less than that of CCL3 [39, 43]. Thus, CCL4 and another CCR5-binding chemokine, CCL3, exhibit similar biological effects on most types of hematopoietic cells. Nonetheless, CCL4 can antagonize CCL3-mediated suppression of hematopoietic stem/progenitor cell proliferation [44].

CCL4 can increase intracellular calcium concentration and tissue factor activity in vascular smooth muscle cells [45]. Endothelial cells can exhibit chemotactic and constrictor responses to CCL4 [46, 47]. We have previously revealed that CCL4 can induce collagen and growth factor gene expression in a fibroblast cell line [48]. Collectively, CCL4 can modulate the composition and functions of the cells present in the tumor microenvironment.

3.4 CCL4 in the Tumor Microenvironments

The effects of CCL4 gene polymorphisms on various carcinogenesis were recently reported. Subjects with A/G heterozygotes of the *CCL4* gene rs10491121 polymorphism had significant 0.665-fold lower risk of developing hepatocellular carcinoma (HCC) as compared to those with A/A genotype [49]. Similarly, patients with oral cancer who had rs10491121 A/G genotype showed a lower risk for an advanced tumor size as compared to those patients with AA genotype, whereas the T/T homozygotes of *CCL4* gene rs1634507 were associated with oral-cancer susceptibility [50]. Moreover, among patients with luminal A or luminal B subtype breast cancer, those with A/G genotype at rs10491121 were less likely to develop lymph node metastasis as compared with patients with the AA genotype [51]. Additionally, the patients carrying AG or GG genotype at rs10491121 were at lower risk of developing distant metastasis [51]. However, all these studies were conducted on the cohorts consisting solely of Chinese ethnicity without func-

tional studies such as the determination of CCL4 expression levels. Therefore, additional studies are warranted to clarify the validity and the functionality of *CCL4* gene polymorphisms.

CCL4 expression was found to have enhanced in human colon cancer tissues as compared with adjacent normal tissues; however, the magnitude of its enhancement were smaller than those of other chemokines such as CCL3 and CXCL8 [52]. Nishikawa and colleagues reported that bone marrow-derived mesenchymal stem cells in tumor sites could act as an additional source of CCR5-binding chemokines including CCL4, CCL3, and CCL5 in human colorectal cancer tissues [53]. They further demonstrated that CCL5 enhanced the in vitro proliferation of human colon cancer cell lines and assumed that these chemokines could directly augment colon cancer cell proliferation, although these cell lines expressed CCR5 intracellularly and not on their cell surface. Evidence is accumulating to indicate that colorectal cancer cells are a major source of CCL4 [54, 55]. Supporting this notion, CCL4 gene expression in colon cancer cell lines can be enhanced by β-catenin activation mutation, a frequently observed gene mutation in colorectal cancer [56]. However, the roles of CCL4 in colon carcinogenesis remain controversial. High serum CCL4 levels were reported to be associated with improved disease-free survival together with the increased densities of peritumoral $CD68^+$ macrophages [57]. On the contrary, tumor cell-derived CCL4 can promote human colon cancer progression by inducing pro-tumorigenic macrophage infiltration together with other chemokines such as CCL2 and CCL3 [54] or by recruiting Tregs to suppress tumor immunity [55]. Thus, it may be plausible that CCL4 has distinct roles in colon carcinogenesis in a context-dependent manner.

CCL4-mediated Treg infiltration was also observed in mice bearing lymphoma [38]. In these mice, myeloid-derived suppressor cells present in tumor tissues produced CCR5-binding chemokines, namely, CCL3, CCL4, and CCL5, to induce Treg infiltration. The pathogenic roles of CCR5-binding chemokines were further demonstrated by the observations that intratumoral injection of CCL4 or CCL5 increased tumor-infiltrating Tregs and that CCR5 deficiency delayed tumor growth with depressed intratumoral Treg numbers [38].

The expression levels of CCL4 and CCL2 were also enhanced in human lung adenocarcinoma cancer (LUAD) tissues as compared to human lung squamous cancer (LUSD) tissues; LUAD patients with higher CCL4 levels had shorter overall survival [58]. Moreover, macrophage infiltration in lung tumors was inversely correlated with its expression of a T-box transcription factor Brachyury [59]. In addition, Brachyury inhibited CCL2 and CCL4 expression in human lung tumors, thereby suppressing macrophage infiltration [59]. Thus, the expression of macrotropic chemokines, CCL2 and CCL4, might be negatively regulated by a transcription factor, Brachyury, in lung tumor tissues.

In *N*-diethylnitrosamine (DEN)-induced mouse HCC model, deficiency of another transcription factor, C/EBP homologous protein (CHOP), markedly attenuated the tumor load [60]. Simultaneously, CHOP deficiency reduced macrophage infiltration with depressed CCL4 and IFN-γ expression in liver. Thus, CHOP can regulate CCL4 gene expression during hepatocarcinogenesis, thereby inducing macrophage infiltration and subsequent tumorigenesis.

CCL4 can also be produced by immune cells in the tumor microenvironment. A human head and neck squamous cell cancer (HNSCC) line, FaDu-derived culture supernatant, induced CCL4 production by human neutrophils, the cells which are abundantly present in HNSCC tissues [61]. Hypoxia could in vitro induce CCL4 expression in a human macrophage cell line and CCR5 expression in a human glioblastoma cell line as well as the CCL4-CCR5 interaction could enhance in vitro invasion ability of a human glioblastoma cell line [62]. However, pathological roles of immune cell-derived CCL4 will require additional in vivo investigation.

CCL4 can also be produced by nonimmune resident cells present in the tumor microenvironment. Endothelial cells obtained from breast cancer-bearing mice expressed CCL4 constitutively and abundantly [63]. However, the molecular mechanisms underlying constitutive CCL4

expression in tumor-derived endothelial cells remain elusive. Fibroblasts in human pancreatic cancer tissues also displayed enhanced expression of several chemokines such as CCL3, CCL4, and CXCL10, as well as ETS2, which is a transcription factor [64]. Deletion of *ETS2* gene in fibroblasts decreased $K\text{-}ras^{G12D}$-driven acinar-to-ductal metaplasia with reduced CCL3, CCL4, and CXCL10 expression in fibroblasts, suggesting the contribution of fibroblast-derived chemokines to acinar-to-ductal metaplasia of pancreatic cancer cells.

CCL4 expression was detected in human oral squamous cell carcinoma (OSCC) tissues [65]. Moreover, CCL4 can in vitro induce cancer cells to express vascular endothelial growth factor (VEGF)-C, which is crucially involved in lymphangiogenesis. Attenuation of CCL4 gene expression in a human OSCC cell line, SAS, suppressed VEGF-C expression and lymphangiogenesis when the cells were implanted into nude mice. It is tempting to speculate that reduced lymphangiogenesis in depressed CCL4 expression may be linked to the reported association of CCL4 gene polymorphisms with lymph node metastasis in breast cancer [51].

Pervaiz and colleagues reported increased circulatory levels of CCR5-binding chemokines, CCL3, CCL4, and CCL5, in patients with breast cancer [66], although the origin of these circulating chemokines has not been precisely determined. They further demonstrated that a CCR5 inhibitor, maraviroc, directly reduced in vitro proliferation of human breast cancer cells and significantly decreased bone metastasis in nude rats implanted with a human breast cancer cell line, MDA-MB-231. We observed similar but distinct roles of the CCR5 axis in breast cancer metastasis to bone by using a 4T1.3 clone with a high capacity to metastasize to bone; this clone was established from a mouse breast cancer cell line, 4T1 [48]. This clone metastasizes to the bone cavity due to its higher capacity to survive in it. Moreover, 4T1.3 clone constitutively and abundantly expressed CCL4 and the deletion of CCL4 abrogated its enhanced bone metastasis ability. CCR5 was detected in type I collagen-positive fibroblasts in bone cavity but not in 4T1.3 cells. CCL4 could in vitro induce fibroblasts to express α-smooth muscle actin, a marker of myofibroblasts, and connective tissue growth factor (CTGF), which can promote the growth of 4T1.3 cells under hypoxic conditions. These observations prompted us to assume the crucial involvement of the interaction between cancer cell-derived CCL4 and CCR5-expressing fibroblasts in bone metastasis [48] (Fig. 3.3).

CCL4 can enhance tumor immunity under some conditions. In human esophageal cancer tissues, CCL4 expression in tumor cells was associated with a higher number of intratumoral $CD8^+$ T cell and granzyme B^+ cell numbers as well as longer overall survival, probably due to the capacity of CCL4 to attract $CCR5^+$ cytolytic lymphocytes [67]. In a humanized mouse B cell lymphoma model, cyclophosphamide induced the tumor cells to release CCL4, CXCL8, VEGF, and TNF-α, the cytokines that can induce macrophage infiltration and enhance the phagocytic activity of macrophages in bone marrow, thereby efficiently removing the cancer cells damaged by cyclophosphamide treatment [68]. In melanoma-bearing mice, Treg depletion abrogated tumor formation in $CD8^+$ T cell-dependent manner and simultaneously induced basophil infiltration into tumor tissues [69]. Infiltrating basophils were crucially involved in tumor rejection by abundantly producing CCL3 and CCL4, which cooperatively induced $CD8^+$ T cell infiltration and subsequent tumor rejection. Thus, CCL4 may have distinct roles in the tumor microenvironment in a context-dependent manner.

3.5 Conclusion and Perspective

CCR5 antagonist therapy has been proposed to battle cancer; this is based on the presumed roles of CCR5-binding chemokines including CCL4, CCL3, and CCL5 in caner development and progression [32]. The proposition is further supported by the lack of apparent pathological phenotypes in CCR5-deficient humans [30, 31], which suggest that CCR5 antagonist therapy cannot induce severe adverse effects. Clinical application, however, warrants further investigations regarding

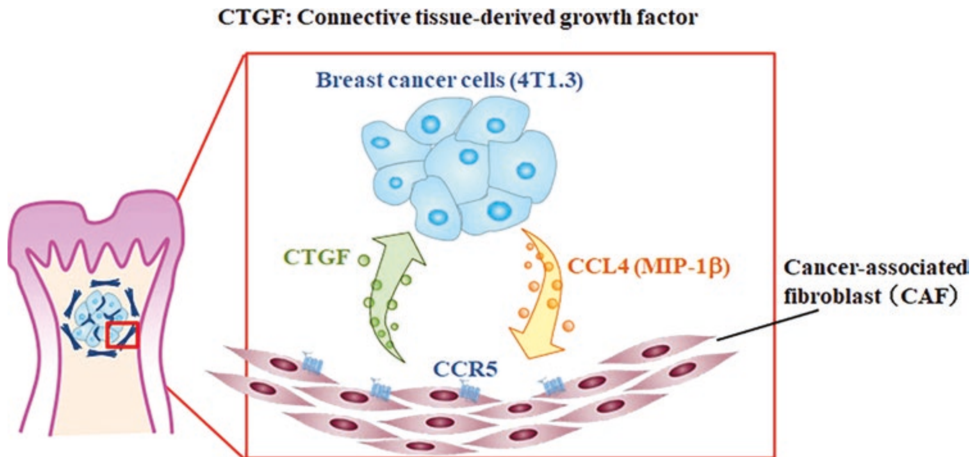

Fig. 3.3 Presumed roles of the CCL4/CCR5-mediated interaction between cancer cells and fibroblasts during the breast cancer metastasis to bone. Cancer-derived CCL4 attracts and activates CCR5-expressing fibroblasts, which can release connective tissue growth factor (CTGF) to promote cancer cell survival and growth in the bone cavity [48]

the pathophysiological roles of these CCR5-binding chemokines. Noteworthily, CCL3 and CCL5 can bind another chemokine receptor, CCR1, with high affinity and, as a consequence, blocking CCR5 may lead to compensated and exaggerated interaction between CCL3/CCL5 and CCR1. Accordingly, the precise determination of each CCR5-binding chemokine expression may be required under each carcinogenesis condition. Additionally, under most situations, CCL4 and CCL3 can have pro-tumorigenic activity; nonetheless, they can also enhance tumor immunity under several conditions [67–69]. Consequently, it will be necessary to determine the pathophysiological activities of these chemokines under every specific condition. Although CCL4 and CCL3 have similar biological effects on most types of cells, CCL4 can antagonize CCL3-mediated suppression of hematopoietic stem/progenitor cell proliferation [44]. Thus, it may be necessary to elucidate the intricate balance between CCL4 and CCL3 expression in each carcinogenesis. Further clarification on these aspects can advance anticancer strategies targeting CCL4 and other CCR5-binding chemokines.

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