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Alexander Birbrair *Editor*

Tumor Microenvironment

The Role of Chemokines – Part A

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Preface

This book's initial title was *Tumor Microenvironment*. However, due to the current great interest in this topic, we were able to assemble more chapters than would fit in one book, covering tumor microenvironment biology from different perspectives. Therefore, the book was subdivided into several volumes.

This book *Tumor Microenvironment: The Role of Chemokines – Part A* presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of different chemokines in the tumor microenvironment during cancer development. Further insights into these mechanisms will have important implications for our understanding of cancer initiation, development, and progression. The authors focus on the modern methodologies and the leading-edge concepts in the field of cancer biology. In recent years, remarkable progress has been made in the identification and characterization of different components of the tumor microenvironment in several tissues using state-of-the-art techniques. These advantages facilitated the identification of key targets and definition of the molecular basis of cancer progression within different organs. Thus, the present book is an attempt to describe the most recent developments in the area of tumor biology, which is one of the emergent hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about the chemokines in the tumor microenvironment in various tissues. Eight chapters written by experts in the field summarize the present knowledge about distinct chemokines during tumor development.

Melissa J. Conroy and Joanne Lysaght from Trinity College Dublin discuss the role of CX3CL1 in the tumor microenvironment. Evangelos Terpos and colleagues from the University of Athens School of Medicine describe CCL3 signaling in the tumor microenvironment. Naofumi Mukaida and colleagues from Kanazawa University compile our understanding of CCL4 signaling within the tumor. Yeo Song Lee and Yong Beom Cho from Sungkyunkwan University update us with what we know about CCL7 signaling in the tumor microenvironment. Neo Shi Yong and Lundqvist Andreas from Karolinska Institutet summarize current knowledge on the multifaceted roles of CXCL9 within the tumor microenvironment. Suling Liu and colleagues from Fudan University address the importance of CCL20 signaling in the tumor microenvironment. Sherven Sharma and colleagues from David Geffen School of

Medicine at UCLA focus on how CCL21 programs immune activity in the tumor microenvironment. Finally, David Anz and colleagues from Ludwig Maximilian University of Munich give an overview of CCL22 signaling in the tumor microenvironment.

It is hoped that the articles published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deep gratitude to my wife Veranika Ushakova and Mr. Murugesan Tamilselvan from Springer, who helped at every step of the execution of this project.

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CX3CL1 Signaling in the Tumor Microenvironment

1

Melissa J. Conroy and Joanne Lysaght

Abstract

CX3CL1 (Fractalkine) is a multifunctional inflammatory chemokine with a single receptor CX3CR1. The biological effects elicited by CX3CL1 on surrounding cells vary depending on a number of factors including its structure, the expression pattern of CX3CR1, and the cell type. For instance, the transmembrane form of CX3CL1 primarily serves as an adhesion molecule, but when cleaved to a soluble form, CX3CL1 predominantly functions as a chemotactic cytokine (Fig. 1.1). However, the biological functions of CX3CL1 also extend to immune cell survival and retention. The pro-inflammatory nature of CX3CR1-expressing immune cells place the CX3CL1:CX3CR1 axis as a central player in multiple inflammatory disorders and position this chemokine pathway as a potential therapeutic target. However, the emerging role of this chemokine pathway in the maintenance of effector memory cytotoxic T cell populations implicates it as a key chemokine in anti-viral and anti-tumor immunity, and therefore an unsuitable therapeutic target in inflammation. The reported role of CX3CL1 as a key regulator of cytotoxic T cell-mediated immunity is supported by several studies that demonstrate CX3CL1 as an important TIL-recruiting chemokine and a positive prognostic factor in colorectal, breast, and lung cancer. Such reports are conflicting with an overwhelming number of studies demonstrating a pro-tumorigenic and pro-metastatic role of CX3CL1 across multiple blood and solid malignancies.

This chapter will review the unique structure, function, and biology of CX3CL1 and address the diversity of its biological effects in the immune system and the tumor microenvironment. Overall, this chapter highlights how we have just scratched the surface of CX3CL1's capabilities and suggests that further in-depth and mechanistic studies incorporating all CX3CL1 interactions must be performed to fully appreciate its role in cancer and its potential as a therapeutic target.

Keywords

Chemokines · CX3CL1 · CX3CR1 · Cancer · Inflammation · T cells · Natural killer (NK) cells · Tumor-associated macrophages (TAMs) · Metastasis · Tumor microenvironment · Cell adhesion · Migration

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1.1 The CX3CL1:CX3CR1 Axis

C-X3-C motif chemokine ligand 1 (CX3CL1) is a unique chemokine functioning in both a transmembrane and soluble form, unlike other chemokines which are solely expressed as soluble proteins [1]. It is a protein of 373 amino acids forming three main domains: chemokine, mucin-like stalk, and transmembrane [1]. In its transmembrane form CX3CL1 functions in immune cell adhesion in an integrin-independent manner (Fig. 1.1) [2, 3]. The mucin-like stalk facilitates cleavage of CX3CL1 by metalloproteases: ADAM metallopeptidase domain 10 (ADAM10) under homeostatic conditions and ADAM metallopeptidase domain 17 (ADAM17) under inflammatory conditions [4, 5]. Such cleavage releases the soluble glycoprotein form of CX3CL1, in which it primarily functions as a chemotactic cytokine [1]. CX3CL1 has also been shown to mediate immune cell survival and has been recently described in the maintenance of memory populations of cytotoxic T cells [6–11]. The distinct biological effects of CX3CL1 are mediated through its sole receptor CX3CR1, which is expressed by $CD8^+$ T cells, natural killer (NK) cells, B cells, monocytes, macrophages, neurons, microglia, smooth muscle cells, and even tumor cells in malignancies such as pancreatic cancer, multiple myeloma, B-chronic lymphocytic leukemia (B-CLL), and metastatic

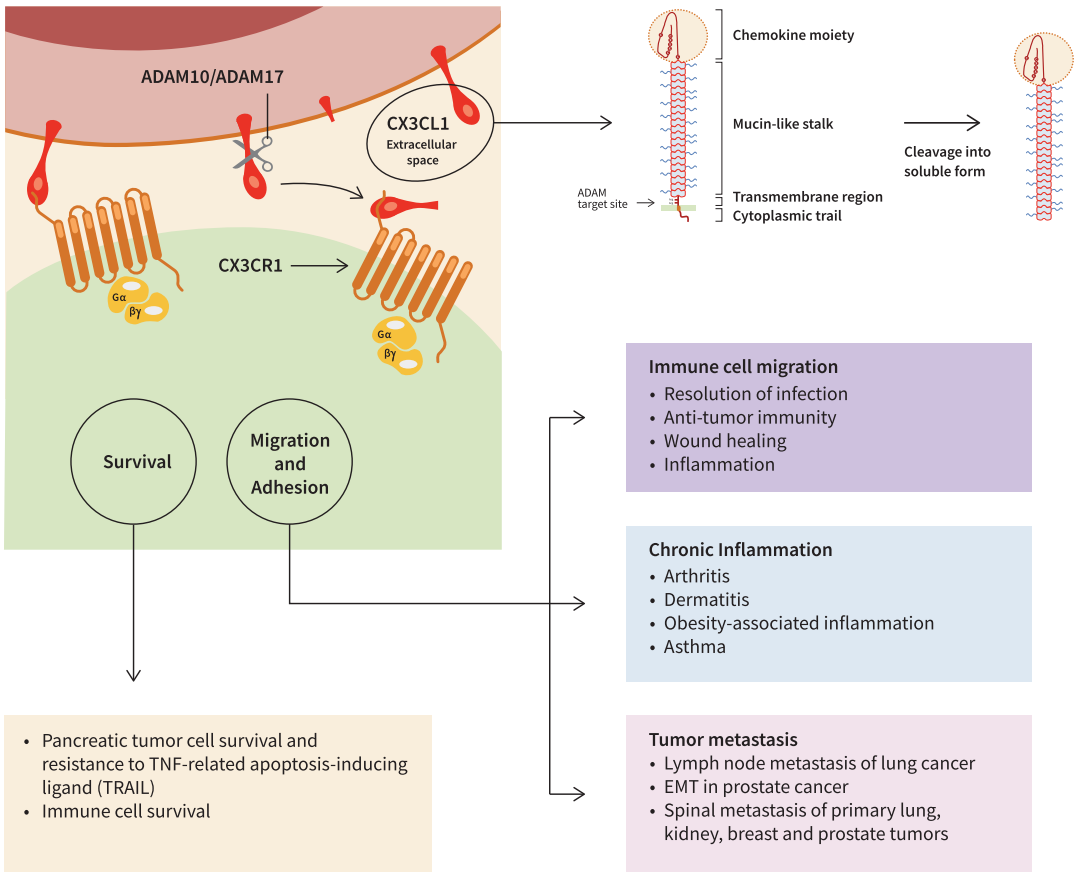

Fig. 1.1 The diverse biological functions of CX3CL1. CX3CL1 is expressed as a transmembrane protein which can be cleaved into a soluble form and secreted into the extracellular space. In its transmembrane form, it predominantly functions in adhesion. In a soluble form, it functions as a survival factor and a chemotactic cytokine. Its biological effects on cell migration can enable crucial immune responses but have also been implicated in chronic inflammation and tumor metastasis [1–5, 8, 9, 11, 16, 24, 27, 30, 50–54]

prostate cancer [1, 3, 12–17]. The biological effects of CX3CL1 are also determined by the differential expression of CX3CR1, which can be epigenetically regulated by immune cells [10]. Specifically, higher CX3CR1 expression by IL-7Rαl^{ow} effector memory (EM) $CD8^+$ T cells is accompanied by higher levels of DNA methylation of the CX3CR1 gene promoter and this confers increased migratory capacity toward CX3CL1, in contrast to the functionally distinct IL-7Rα^{high} EM $CD8^+$ T cell subset [10]. Therefore, since IL-7Rα^{low} EM $CD8^+$ T cells have higher levels of perforin, it is likely that they have greater cytotoxic potential than IL-7Rα^{high} EM $CD8^+$ T cells and this implicates CX3CL1 as a pivotal player in anti-tumor and anti-viral immunity [10]. Moreover, CX3CL1 has been shown to differentiate $CX3CR1^+$ pro-inflammatory macrophages in the liver implicating it in macrophage-mediated hepatic inflammation [18].

The redundancy of the chemokine system is an essential fail-safe for the functionality of the immune system and may present challenges for effectively therapeutically targeting specific pathways. For a long time, it was believed that the CX3CL1:CX3CR1 axis lacked this redundancy and comprised an exclusive ligand and receptor pair, which increased the attractiveness of this pathway for therapeutics [3]. However, in 2010 Nakayama et al. unexpectedly discovered that the chemokine CCL26 is a functional agonist for CX3CR1 [3, 12]. CCL26 was already established as an antagonist for chemokine receptors CCR1, CCR2, and CCR5, and a low-affinity agonist for CCR3 with 10-fold less affinity than CCL11 [19–21]. Therefore, CCL26 is regarded as an interesting and widely interacting chemokine, albeit a low-affinity chemokine; it has dual agonist and triple antagonist capabilities. CCL26 is classically regarded as a pivotal player in allergy and its ability to recruit CX3CR1-expressing cells adds another layer of complexity to the CX3CL1:CX3CR1 axis. In fact, it has been shown to work together with CX3CL1 to recruit NK cells to allergic nasal tissue [22]. This highlights the need for consideration of CCL26 in any future studies scrutinizing the role of CX3CL1 in cancer.

While the CX3CL1:CX3CR1 pathway is important in the recruitment of key anti-tumor immune cells such as NK cells and T cells, it has been implicated in the progression and metastasis of both hematological and solid malignancies [1–3, 13–16, 23–31]. Here, we review key studies uncovering this chemokine pathway to evaluate whether CX3CL1 is a friend or foe in tumorigenesis.

1.2 Pro- and Anti-Tumor Immune Roles of CX3CL1

There are a number of conflicting studies on the role of CX3CL1 in anti-tumor immunity. Numerous reports implicate CX3CL1 as a potent recruiter of NK cells and T cells into the tumor microenvironment and a crucial promoter of strong anti-tumor activity [25, 32–35]. Recent studies have identified CX3CR1 as a novel marker of effector memory $CD8^+$ T cells and a potential marker of PD-1 therapy-responsive and chemotherapy-resistant $CD8^+$ T cell subsets, implicating the importance of this chemokine pathway in successful anti-tumor immune responses and response to immunotherapy [7, 36]. In a case study of 158 patients with T2/T3 stage gastric adenocarcinoma, higher CX3CL1 levels were shown to correlate with higher infiltrations of $CD8^+$ T cells and NK cells and were identified as independent prognostic factors for disease-free survival [37]. Similar results were reported in a cohort of 80 colorectal cancer patients in which intratumoral CX3CL1 positively correlated with better prognosis and higher density of tumor-infiltrating lymphocytes (TILs) [38]. Similarly in 204 invasive breast carcinoma patients, intratumoral CX3CL1 levels positively correlated with higher tumor infiltrations of NK cells, T cells, and dendritic cells (DCs) and were identified as independent prognostic factors for disease-free and overall survival [39]. Interestingly, high intratumoral CX3CL1 expression was associated with smaller tumor size and lower tumor stage regardless of receptor status, i.e., estrogen receptor (ER^+ and ER^-), progesterone receptor (PR^+ and PR^-), and human epidermal

growth factor receptor 2 ($HER2^+$ and $HER2^-$) [39]. Moreover, high intratumoral CX3CL1 was more prevalent in patients without lymph node metastasis further supporting a positive role for this chemokine in controlling breast cancer progression [39]. Unsurprisingly, intratumoral CX3CL1 positively correlated with higher tumor-infiltrating lymphocytes (TILs) in a second breast cancer study [40]. However in contrast to the Park et al. study, this study revealed that high CX3CL1 levels observed in 252 of the 757 patients corresponded with reduced survival and poorer clinical outcomes such as lymph node involvement, high Ki67, α-B crystallin expression, and luminal B (worse prognosis luminal cancers) subtype [40]. Such adverse clinical outcomes were mostly observed in the patients with the lowest TILs suggesting that the complex and multifaceted functions of CX3CL1 in breast carcinoma requires further scrutiny and more in-depth immune profiling among multiple cohorts using comparable measures of high and low CX3CL1 expression before definitive conclusions can be drawn [40]. As we will discuss later, other studies have focused on the pro-tumorigenic role of CX3CL1 in breast cancer, which may negate any positive effects this chemokine has on anti-tumor immunity [26, 27, 31, 40].

To address the concept of CX3CL1 as a pro-tumor chemokine, it must first be noted that several studies have identified it as a key recruiter of tumor-associated macrophages (TAMs) into the tumor microenvironment and recent reports have uncovered soluble and molecular regulators of these processes [23, 41]. Zhou et al. reported that CX3CL1 correlates with increasing tumor stage in a cohort of 38 lung cancer patients, comprising 23 cases of squamous cell carcinoma and 15 cases of adenocarcinoma ranging in stage from I to IV. Schmall et al. demonstrated that this could potentially be mediated via the recruitment of TAMs into non-small cell lung carcinomas by CX3CL1 and CCL2. These data revealed that macrophage-derived IL-10 within the tumor microenvironment mediated the upregulation of CX3CR1, while tumor-derived CX3CL1 and CCL2 expression was driven by CCL1, granulocyte colony-stimulating factor (G-CSF), and CCL3 [41]. Furthermore, genetic ablation of CX3CL1 and CCL2 inhibited tumor growth and metastasis, shifted TAMs toward an anti-tumor M1 phenotype, and suppressed tumor vessel growth suggesting that blockade of these chemokine pathways might have therapeutic potential to block TAM-mediated immune suppression in lung cancer [41]. Furthermore, this study demonstrated that the prevalence of intratumoral $CCR2^+$ TAMs correlated with tumor stage in a cohort of 72 non-small cell lung cancer patients providing further evidence that CX3CL1 and CCL2 contribute to lung tumor progression [41]. While the previous studies provide insights into CX3CL1's role in lung cancer progression, a larger study examining datasets for 2443 patients across France, Sweden, Canada, and the USA was reported using data from The Gene Expression Omnibus database and The Cancer Genome Atlas and concluded that higher levels of intratumoral CX3CL1 mRNA was associated with improved overall survival in lung adenocarcinoma but not lung squamous cell carcinoma [42]. Such CX3CL1 expression was associated with genes regulating cell adhesion, leukocyte migration, T cell activation, and NK cell-mediated cytotoxicity in lung adenocarcinoma [42]. These reports present conflicting conclusions on the immune repertoires recruited to CX3CL1-expressing lung tumors and its divergent implications on patient outcomes might be impacted by other chemokines in the tumor microenvironment. For instance, CX3CL1 alone might promote anti-tumor immune responses against lung tumors, but in the presence of CCL2, it may enhance intratumoral TAM accumulation. In a testicular germ cell cancer study, Batool et al. demonstrated that the miRNA miR-125b is epigenetically repressed and that its restoration can reduce CX3CR1 expression and TAM recruitment in vivo, suggesting a possible means through which TAM accumulation in the tumor microenvironment might be regulated [23]. However, the role of miRNA miR-125b in the recruitment of $CX3CR1^+$ $CD8^+$ T cells and NK cells would need to be assessed before this could be considered therapeutically.

CX3CL1-mediated recruitment of myeloid derived suppressor cells (MDSCs) into the tumor

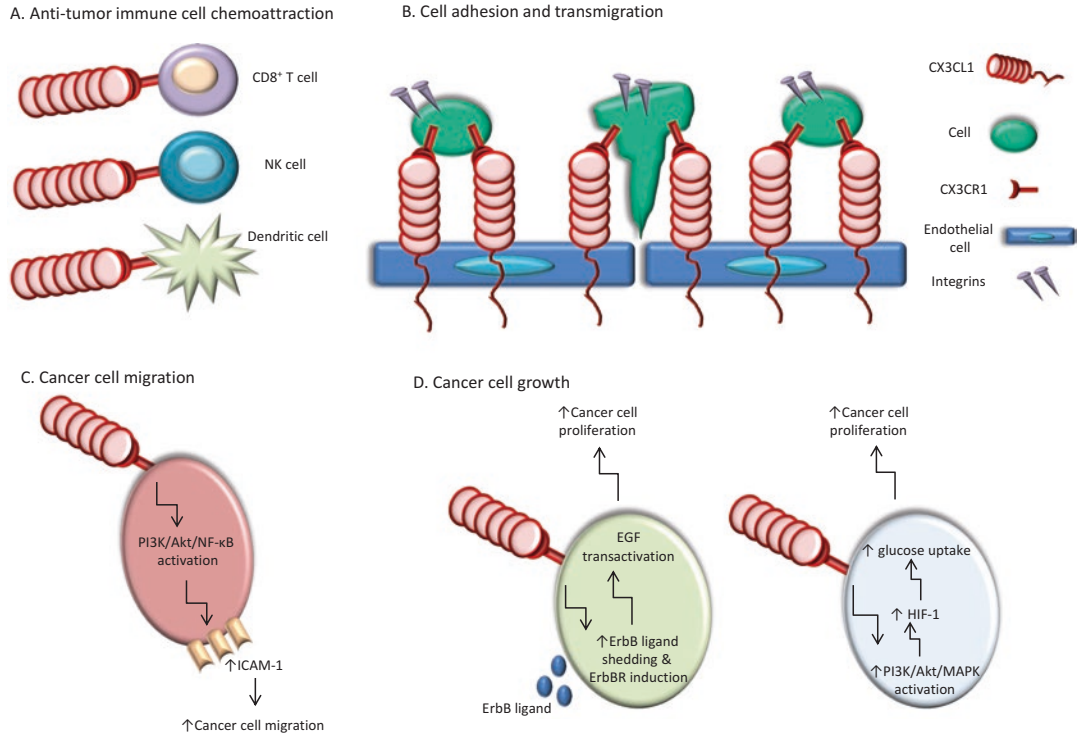

Fig. 1.2 The anti-tumor and pro-tumor roles of CX3CL1. Schematic illustrating the biological effects of CX3CL1 exerted on immune cells and tumor cells. (**a**) CX3CL1 facilitates anti-tumor immune responses via chemoattraction of $CD8^+$ T cells, natural killer (NK) cells, and dendritic cells (DC). (**b**) In its transmembrane form, CX3CL1 facilitates cell adhesion and transendothelial migration. (**c**, **d**) CX3CL1 has been shown to promote osteosarcoma cell migration via intercellular adhesion molecule-1 (ICAM-1) upregulation, breast cancer cell growth via transactivation of the epidermal growth factor (EGF) pathway, and pancreatic cancer cell growth via hypoxia-inducible factor (HIF)-1α [2, 3, 31, 39, 48]

microenvironment is another means through which this chemokine compromises anti-tumor immunity and facilitates tumor progression (Fig. 1.2) [43]. A recent study has pinpointed the mechanisms through which CX3CR1 expression on MDSC is regulated and a potential means of attenuating their CX3CL1-mediated recruitment to tumor [43]. In brief, the cyclin-dependent kinase (CDK) inhibitors p16INK4 and p21Cip1/Waf1 are highly expressed by monocytic MDSCs (Mo-MDSCs) and stimulate CX3CR1 chemokine receptor expression by preventing CDK-mediated phosphorylation and inactivation of SMAD3 [43]. This study demonstrated that deletion of p16INK4 and p21Cip1/Waf1 reduces CX3CR1 expression on Mo-MDSC and significantly reduces their recruitment to CX3CL1-expressing tumors, thus suppressing tumor advancement and offering therapeutic potential to prevent MDSC-mediated immune suppression in the tumor microenvironment [43]. It must be noted that other chemokines such as CCL2, CXCL12, and CXCL8 have also been shown to enhance MDSC recruitment to the tumor microenvironment in gastric and ovarian cancers, and therefore they may offer additional targets for use with CX3CL1-targeted therapy to block migration of tumor-promoting immune cells [44, 45]. Furthermore, prostaglandin E2 (PGE2) has been shown to modulate production of some of these chemokines, which presents further therapeutic opportunities through antagonism of the PGE2 G-protein coupled receptors [45].

With a diverse range of reported immune profiles and clinical outcomes accompanying high intratumoral CX3CL1, it appears that the biological ramifications are dependent on several

factors including the tumor type and the conditions within the tumor microenvironment. In the absence of thorough immune profiling studies and at a time when all biological functions of CX3CL1 are not completely understood, it is difficult to define the role of CX3CL1 in cancer and future studies must consider a wider array of factors to truly uncover whether this multifaceted chemokine is an enemy or ally of the anti-tumor immune response [23, 39, 40].

1.3 CX3CL1 Exerts Direct Biological Effects on Tumor Progression

As previously described, CX3CL1 is a potent driver of NK cell and $CD8^+$ T cell migration and this in itself should make it a formidable component in the immune system's arsenal against tumor growth. While several studies have demonstrated that high intratumoral CX3CL1 levels correspond to high TIL prevalence, there is emerging evidence that this chemokine also plays a role in shaping the immunosuppressive features of the tumor microenvironment [23, 25, 37–39, 41]. Aside from governing aspects of the immune control of tumor growth, CX3CL1 has also been shown to elicit direct pro-tumorigenic effects on tumor cells through its receptor CX3CR1 and these will be discussed in this section.

Pancreatic ductal adenocarcinoma (PDAC) is a malignancy plagued with dismal survival rates of ~5% and high levels of treatment resistance. There is emerging evidence that high expression of CX3CR1 and CX3CL1 correlates with poor prognosis in PDAC and that CX3CL1 can directly and indirectly promote PDAC tumor growth [17, 29, 46]. This has been the focus of several studies; a study by Huang et al. revealed significantly elevated CX3CL1 and CX3CR1 expression in PDAC tumors with highest levels associated with metastasis and severity of disease [17]. Further analysis demonstrated that CX3CL1 could directly enhance both growth and migration of PDAC cells and that such growth was regulated through the activation of the JAK/STAT signaling pathway [17]. In support of this, a more recent study has demonstrated that CX3CL1 can promote motility, invasion, and contact-independent growth of PDAC cells and that this can be reduced by the CX3CR1 inhibitor JMS-17-2 in an AKT-dependent manner [29]. These data suggest that the CX3CL1:CX3CR1 axis might be therapeutically targeted to limit PDAC tumor cell growth and metastasis [29]. Recent evidence demonstrates that CX3CL1 also promotes PDAC tumor cell survival and resistance to TNF-related apoptosis-inducing ligand (TRAIL) in an indirect manner via the recruitment of inflammatory cells, which in turn induces apoptosis resistance in PDAC cells, which is mediated at least in part by a RelA-CX3CL1 paracrine pathway [24]. This study showed that TRAIL-resistant pancreatic cancer cell lines exhibit enhanced NF-κB activity compared to sensitive cell lines, and TRAIL treatment induces CX3CL1 expression in these cells via the RelA-centered NF-κB signaling pathway [24]. In summary, these data reveal that the augmented migration of inflammatory cells conferring TRAIL resistance to PDAC tumors is facilitated by TRAIL-mediated activation of RelA/NF-κB and subsequent CX3CL1 expression in such tumors (Fig. 1.1) [24].

CX3CL1-elicited effects via NF-κB activity is not confined to TRAIL resistance and has been shown to govern tumor progression and metastasis in osteosarcoma through the regulation of intercellular adhesion molecule-1 (ICAM-1) [47]. This study showed that CX3CL1-induced human osteosarcoma cell migration, via upregulation of intercellular adhesion molecule-1 (ICAM-1) expression, was regulated through the CX3CR1/PI3K/Akt/NF-κB pathway (Fig. 1.2) [47]. Furthermore, intratumoral levels of CX3CL1 and ICAM-1 were associated with tumor stage in osteosarcoma patients and further in vivo studies revealed that lung metastases of osteosarcomas are governed at least in part by CX3CL1 [47]. Interestingly, such CX3CL1-mediated regulation of ICAM-1 was not observed in a separate study on circulating $CD8^+$ T cells suggesting that the effects of CX3CL1 are dependent on the cell type [6].

It is well established that tumor cells must alter their metabolic processes to survive in the hypoxic and nutrient-depleted tumor microenvironment. A study by Ren et al. has revealed that CX3CL1 stimulated Hypoxia-Inducible Factor (HIF)-1α expression in PDAC through the PI3K/Akt and MAPK pathways subsequently facilitating enhanced glucose uptake, thus identifying CX3CL1 as a promoter of PDAC tumor cell growth and survival in the challenging tumor microenvironment (Fig. 1.2) [48].

While the last section highlighted CX3CL1-mediated effects on breast cancer progression via its cross talk with the immune system, this complex chemokine has also been shown to directly exert biological effects on breast cancer cells. For instance, a recent study demonstrated that CX3CL1 transactivates ErbB receptors in human breast cancer cells and triggered proliferation of such cells through the proteolytic shedding of an ErbB ligand (Fig. 1.2) [31]. The group concluded that it is likely that CX3CL1 acts as a specific tumor promoter for ErbB2-expressing mammary carcinomas [31].

The CX3CL1-mediated promotion of tumor progression is not limited to solid malignancies and has been shown in several hematological malignancies. It is well established that CXCR4 and its ligand CXCL12 are players in the migration of myeloma cells to bone marrow and studies are uncovering a role for the CX3CL1:CX3CR1 axis in cross talk between multiple myelomas and their bone microenvironments [14]. In *B*-cell *chronic lymphocytic leukemia* (B-CLL), a study by Corcione et al. reported that CX3CL1 binding to CX3CR1 on B-CLL cells leads to the upregulation of CXCR4 and increased migration of B-CLL cells to monocyte-derived nurse like cells (NLCs) expressing CXCL12 in the tumor microenvironment [15].

It is clear that CX3CL1 elicits biological effects outside its defined function of an immune cell adhesion and chemotactic protein and that its role within the tumor microenvironment is shaped by complex multicellular and molecular cross talk.

1.4 CX3CL1 in the Metastatic Niche

Emerging evidence suggests that CX3CL1 governs spinal metastasis of primary lung, kidney, breast, and prostate tumors [16, 27]. In a study by Liu et al., a lack of CX3CR1 expression in such metastatic tumors was reported and this is likely due to sustained reduction of the receptor on the cell surface following ligand binding [6, 27]. So far, such CX3CL1-mediated internalization of CX3CR1 has only been reported on $CD8^+$ T cells and further work is warranted to determine whether this also occurs in cancer cells and to confirm the biological ramifications [6]. However, others have shown that both CX3CL1 and CX3CR1 expression is higher in prostate metastatic tumors in spine compared to the primary tumor site and that CX3CR1 overexpression leads to more spinal metastasis in mice [16, 27]. Further analyses revealed that CX3CL1 elicited its effects on prostate tumor metastasis via activation of the Src/FAK pathway in an epidermal growth factor receptor (EGFR)-dependent manner [16]. The inhibitors of these kinases repressed the cell migration induced by CX3CL1 or CX3CR1 overexpression and therefore may represent potential targets for preventing spinal metastasis in prostate cancer [16]. Others have also implicated CX3CL1-mediated spinal metastasis of breast tumors via the Src/FAK pathway and have demonstrated that the Src inhibitor Bosutinib and FAK inhibitor PF-00562271 can reduce CX3CL1-driven migration further, indicating that these are targetable pathways in the prevention of spinal metastasis of solid tumors [26]. In PDAC, tumor perineural dissemination is a common mechanism through which tumor recurrence occurs following surgery and the CX3CL1:CX3CR1 pathway is believed to drive the migration of the PDAC cells to local peripheral nerves [28]. Of course, CX3CL1-mediated metastasis is not restricted to the spine and the chemokine has also been implicated in lymph node involvement [40, 49]. Lymph node metastasis was promoted by CX3CL1 via activation of JNK and MMP2/MMP9 activity in lung cancer

cells [49]. Furthermore CX3CL1 was shown to promote epithelial-to-mesenchymal transition through the TACE/TGF-α/EGFR pathway and subsequent upregulation of Slug expression in prostate cancer cells [30].

Overall, it appears that the CX3CL1 can govern cancer metastasis, either via CX3CR1 overexpression by metastatic tumor cells to facilitate spread to the CX3CL1-enriched microenvironments or via direct effects on signaling pathways affecting the EMT pathways of cancer cells [30, 42].

1.5 CX3CL1 in Inflammation-Driven and Obesity-Associated Cancer

As a key regulator of pro-inflammatory cell migration, it is now well established that CX3CL1 plays a central role in multiple inflammatory conditions such as asthma, obesity, dermatitis, diabetes, and neuropathic pain [6, 9, 50–53]. In neuroinflammation, CX3CL1 mediates its effects via activation of CX3CR1 on microglial cells and subsequent phosphorylation of p38 MAPK [50]. Furthermore, CX3CL1-mediated activation of satellite glial cells leads to TNFα, IL-1β, and prostanoid expression to maintain the inflammatory-associated pain [52]. In airway inflammation, CX3CL1 serves to promote survival of pro-inflammatory T cells in the inflamed lungs of asthma patients [9]. The involvement of CX3CL1 in inflammatory disorders is further facilitated through its induction by inflammatory cytokines such as TNF-α and IFN-γ which are often expressed by the CX3CL1-responsive lymphocytes and myeloid cells, and this can provide a pathological feedback loop in maintaining the chronicity of the inflammation and in the exacerbation of disease [54]. For instance, the inflammatory subtype of monocytes/macrophages expressing intermediate levels of CX3CR1 ($CX3CR1^{INT}$) and producing TNF-α, IL-1 IL-6, and CCL2 are enriched in inflamed colon, where they undoubtedly contribute to tissue inflammation and further recruitment of inflammatory cells [55].

Inflammation-driven and obesity-associated malignancies represent a unique challenge for the immune system in that the desired immune response should facilitate tumor eradication without exacerbating tumorigenic inflammation derived from obese adipose tissue, and this presents challenges for cancer immunotherapy. Given the involvement of CX3CL1 in a plethora of inflammatory disorders, one would assume that it plays a role in the pathogenesis of inflammation-driven malignancies [9, 50–53, 56]. However, studies have shown that CX3CL1 is associated with better outcomes in the inflammatory-driven and obesity-associated colorectal cancer [38]. Moreover, a study by Erreni et al. indicates that this may be facilitated by the adhesive and retention functions of CX3CL1, which prevents migration of primary tumor cells and limits metastatic progression [57]. This is in striking contrast to the pro-metastatic role of CX3CL1 in breast, lung, kidney, pancreatic, bone, and prostate cancer described earlier.

While CX3CL1 has been identified as a key player in adipose tissue inflammation in obesity, its role in the tumorigenesis of obesity-associated cancers such as oesophageal adenocarcinoma (OAC) is poorly understood. OAC is an exemplar model of inflammation-driven cancer and has one of the strongest associations with obesity of all malignancies [58]. Several studies have revealed that the immune system is dysregulated and several chemokine pathways are altered in OAC patients and this undoubtedly contributes to chronic inflammation and compromised anti-tumor immunity in this cohort [6, 59–62]. One study by Conroy et al. has identified that CX3CL1 is a key regulator of $CD8^+$ T cells in OAC and that its abundance in the visceral adipose tissue (VAT) strongly correlates with markers of meta-inflammation such as CRP in these patients [6]. This is not surprising since there is an abundance of T cells expressing CX3CL1-inducing cytokines IFN-γ and TNF-α in these tissues [59, 60]. However, the role of CX3CL1 in anti-tumor immunity at the tumor site and its effects on tumor metastasis and patient outcomes warrant further investigation in these patients. It is known that CX3CL1 can alter the CX3CR1 and

L-selectin expression of cytotoxic T cells in OAC and this is likely to have significant ramifications for their anti-tumor activities in this cohort [6]. Inflammation-triggered changes in CX3CR1 expression by monocytes/macrophages have also been observed in inflamed colon leading to the accumulation of the most inflammatory $CX3CR1^{INT}$ populations of these cells in inflamed tissue, in comparison to the $CX3CR1^{HI}$ phenotype observed in normal colon [55]. Other phenotypic and transcriptional alterations observed in this study suggested that this altered CX3CR1 expression represents impaired monocyte-to-macrophage differentiation in inflamed colon, but the role of CX3CL1 in this cross talk was not elucidated [55]. However, given its regulation of CX3CR1 expression by T cells, CX3CL1 is a likely candidate in the inflammatory shift of gut-homing monocytes in intestinal inflammation [6, 55].

At a time when inflammation-driven and obesity-associated malignancies such as CRC and OAC are increasing in prevalence, it is becoming more urgent to delineate the role of CX3CL1 in the chronic inflammation and anti-tumor immune responses of such immunologically complex patients.

1.6 Challenges of Therapeutically Targeting the CX3CL1:CX3CR1 Pathway

The complex functionality of CX3CL1 as a regulator of migration, adhesion, and survival of both anti-tumor and pro-tumor immune cells and a facilitator of cancer growth and metastasis cloud its potential as an anti-cancer therapeutic. While the blockade of the CX3CL1:CX3CR1 chemokine pathway might limit tumor progression, it is also likely to attenuate NK cell and cytotoxic T cell recruitment to the tumor microenvironment, potentially disrupting effective anti-tumor immunity. Moreover, CX3CL1's reported role in limiting metastasis of colorectal tumors and in the maintenance of crucial T cell populations would also be compromised if its signaling through CX3CR1 was antagonized. While its biological effects vary between cancer types and cell types, the ability of CX3CL1 to recruit NK cells and T cells remains constant which should be taken into account when considering its suitability as an immunotherapeutic target. While the CX3CR1 antagonist JMS-17-2 shows great promise to disrupt breast cancer metastasis and might present a feasible approach in other malignancies, such antagonism might have negative effects on CX3CL1's governance of T cells and NK cells and this must be heavily scrutinized before such therapeutics are considered [6, 7, 25, 32, 63]. The multifaceted functionality of CX3CL1 in the context of the immune system alone is fascinating and complex, from a recruiter of MDSCs and TAMs to a potent orchestrator of anti-tumor immune cell migration and phenotype, and a driver of pathological inflammation [6, 17, 23, 25, 32, 43, 51, 56]. However, the doubled-edged nature of CX3CL1's biological effects present it as a more challenging therapeutic target and therefore more in-depth in vivo studies are warranted to gain a deeper understanding of its role in the progression of cancer and governance of the immune system (Table 1.1).

Table 1.1 Pro- and anti-tumorigenic functions of CX3CL1

Cancer type	Pro-tumorigenic functions	Anti-tumorigenic functions	Reference
Breast	CX3CL1 governs spinal metastasis of breast tumors via the Src/FAK pathway. CX3CL1 induces ErbB receptors in human breast cancer cells and triggered proliferation of such cells through the proteolytic shedding of an ErbB ligand and transactivates EGF pathway.	High intratumoral CX3CL1 associated with higher TILs, less lymph node involvement, lower tumor stage and size.	[26, 31, 39]

(continued)

Table 1.1 (continued)

Cancer type	Pro-tumorigenic functions	Anti-tumorigenic functions	Reference
Pancreatic	High CX3CL1 and CX3CR1 associated with metastasis and disease severity. CX3CL1 promotes invasion and growth of pancreatic cancer cells and drives their migration to local peripheral nerves. TRAIL resistance in pancreatic cancer is conferred via RelA-CX3CL1 paracrine pathway. CX3CL1 stimulates HIF-1α expression via PI3K/Akt and MAPK pathways facilitating enhanced glucose uptake and pancreatic tumor cell growth.		[17, 24, 28, 29, 48]
Colorectal		High CX3CL1 correlates with higher TILs.	[38]
Gastric		High CX3CL1 correlates with higher TILs.	[37]
Lung	CX3CL1 correlates with increasing tumor stage and contributes to TAM accumulation in lung cancer. CX3CL1 promoted lymph node metastasis via JNK and MMP2/MMP9 pathway in lung cancer cells.	High CX3CL1 expression associated with leukocyte migration, T cell activation and NK cell-mediated cytotoxicity and better prognosis in lung adenocarcinoma.	[41, 42, 49]
Osteosarcoma	CX3CL1 induces osteosarcoma cell migration, by enhancing ICAM-1 via the CX3CR1 /PI3K/Akt/NF-κB pathway. CX3CL1 governs lung metastasis.		[47]
B-CLL	CX3CL1 enhances B-CLL cell migration.		[15]
Multiple Myeloma (MM)	CX3CL1:CX3CR1 axis plays a role in MM cell migration to their bone microenvironment.		[14]
Prostate	CX3CL1 and CX3CR1 govern spinal metastasis of prostate tumors via the Src/FAK pathway. CX3CL1 promotes EMT transition in prostate tumors via TACE/TGF-α/EGFR and upregulation of Slug.		[16, 27, 30]

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2 CCL3 Signaling in the Tumor Microenvironment

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Abstract

Within the tumor microenvironment, chemokines play a key role in immune cell trafficking regulation and immune landscape formulation. CCL3 or macrophage inflammatory protein-1α (MIP-1α), an important chemokine implicated in both immune surveillance and tolerance, has emerged as a prognostic biomarker in both solid and hematological malignancies. CCL3 exerts both antitumor and pro-tumor behavior which is context dependent highlighting the complexity of the underlying interrelated signaling cascades. Current CCL3-directed therapeutic approaches are investigational and further optimization is required to increase efficacy and minimize adverse events.

Keywords

CCL3 · Macrophage inflammatory protein-1 alpha · MIP-1a · Chemokines · Tumor microenvironment · Chemokine · T cell · Lymph node · Immune cell · CCL2 · CCR1 · CCR4 · CCR5 · Tumor-associated macrophages · Metastasis-associated macrophages

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2.1 Introduction

Chemokines belong to the cytokine family and are small proteins (7–15 kDa) that bind to G-protein coupled receptors and induce intracellular cascades that ultimately regulate the trafficking of immune cells. They are structurally homologous to the conserved N-terminal cysteine motifs and are subdivided into four classes; C-X-C-, C-C-, C-, and C-X_3-C-, where X signifies the presence of an amino acid. Chemokine receptors are named from the corresponding chemokines as follows: CXCR-, CCR-, CR-, and CX_3CR-. Interestingly, more than one chemokine binds to each receptor and each chemokine may bind to more than one receptors. As a result, this redundancy leads to complex agonistic and antagonistic interactions [1, 2]. Thus, determining the isolated functions of chemokines and chemokine receptors and the effects of targeted interventions is complicated.

Chemokines play a key role in inflammation as cardinal regulators of immune cell trafficking and interactions among chemokines and their receptors orchestrate the immune landscape in the tumor microenvironment [3]. In analogy to inflammation, chemokines mediate the trafficking of immune cells to the tumoral niche and exert direct and indirect effects on tumor cells (Table 2.1) [2]. Major sources of chemokines in the tumor microenvironment

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Table 2.1 The multifaceted role of chemokines in modulating the tumor microenvironment

Chemokine	Chemokine receptor(s)	Regulation of immune cells	Regulation of tumor cells	Overall effect on tumor development
CCL2	CCR2, CCR5	Recruiting monocytes, NKT cells, and monocytic MDSCs	Promoting tumor cell proliferation, stemness, survival, tumor vascularization, extravasation, and metastasis	+
CCL3	CCR1, CCR4, CCR5	Recruiting monocytes and macrophages	Promoting extravasation	+
		Recruiting DCs, mediating CD4+-DC and naïve CD8+ T cell interactions		–
CCL5	CCR1, CCR3, CCR4, CCR5	Recruiting monocytes and macrophages	Promoting invasion and metastasis	+
CCL18	CCR8	ND	Promoting invasion and metastasis	+
CCL25	CCR9	ND	Promoting chemoresistance, invasion, and metastasis	+
CXCL8	CXCR1	Recruiting neutrophils and granulocytic MDSCs	Promoting stemness, invasion, migration, apoptosis, resistance to hypoxia, premature senescence, and angiogenesis	+
			Increasing tumor immunogenicity	–
CXCL9	CXCR3	Recruiting T and NK cells	Inhibiting angiogenesis	–
CXCL10	CXCR3	Recruiting T and NK cells	Inhibiting angiogenesis	–
CXCL12	CXCR4	Recruiting B cells, pDCs, and T_{reg} cells	Promoting proliferation, survival, invasion, metastasis, stemness, angiogenesis	+
CXCL14	ND	Recruiting DCs	Promoting invasion and motility	+
			Inhibiting proliferation, invasion, metastasis, promoting apoptosis	–
CXCL17	ND	Recruiting granulocytic MDSCs	Promoting angiogenesis	+

+ favorable, – unfavorable, *CCL* CC-chemokine ligand, *CXCL* CXC-chemokine ligand, *CXCR* CXC-chemokine receptor, *DC* dendritic cell, *MDSC* myeloid-derived suppressor cell, *NK* natural killer, *NKT* natural killer T cell, *pDC* plasmacytoid DC, *Treg cell* regulatory T cell, *ND* not determined

include immune cells such as neutrophils and myeloid cells, malignant cells, stromal cells, mesenchymal stem cells, endothelial cells, cancer-associated fibroblasts, and macrophages [3, 4].

The aim of this chapter is to provide insights into the key role of chemokines in the tumor microenvironment with focus on the role of CCL3 signaling and its potential clinical implications via the enhancement of immune-mediated antitumor responses.

2.2 CCL3 Basic Concepts

CCL3 belong to the C-C motif family of chemokines as no amino acid separates the cysteine residues. CCL3 is also known as macrophage inflammatory protein-1α (MIP-1α) due to its role in inflammation, which was first demonstrated in preclinical studies involving stimulated macrophages that secreted CCL3 [5]. Cellular sources of CCL3 include a great range of cell

Table 2.2 CCL3 cellular sources and targets

Cellular source	CCL3	Target receptor	Target cell type
Immune cells (macrophages, monocytes, B and T lymphocytes, NK cells, neutrophils, dendritic cells)		CCR1	Monocytes and immature myeloid cells
Epithelial cells, fibroblasts, osteoblasts, mesangial cells, vascular smooth muscle cells		CCR4	Th2 T cells and Tregs
Platelets, basophils, eosinophils, bone marrow CD34+ cells		CCR5	Th1 T cells, NK cells, and plasmacytoid dendritic cells
Glial cells, astrocytes			

Table 2.3 The role of CCL3 in regulating immune microenvironment

1. CCL3 promotes leukocyte trafficking to inflammatory sites.
2. CCL3 mediates memory CD8+ T cell response.
3. CCL3 regulates immune compartmentalization by driving trafficking of myeloid precursor cells from the bone marrow into the periphery.
4. CCL3 promotes dendritic cell homing in lymph nodes.
5. CCL3 gradient drives interactions between dendritic cells and CD4+ and naïve CD8+ T cells.
6. CCL3 promotes immune cell trafficking (tumor antigen-specific CD8+ T cells, macrophages) to niches of malignancy.

types ranging from immune cells (macrophages, monocytes, lymphocytes, NK cells, neutrophils) to epithelial cells, fibroblasts, osteoblasts, and glial cells [6–8]. CCL3 binds to three distinct receptors and, thus, it exerts divergent actions in multiple immune cell subtypes. CCR1 is found on monocytes and immature myeloid cells, CCR4 on Th2 T cells and Tregs, and CCR5 is expressed on Th1 T cells, NK cells, and plasmacytoid dendritic cells (Table 2.2) [9]. CCL3 is a key regulator of immune microenvironment and primarily mediates the trafficking of immune cells in both inflammation and cancer (Table 2.3) [10]. Preclinical in vitro and in vivo studies have demonstrated the chemotactic action of CCL3 on neutrophils and monocytes to inflammatory sites [11]. The binding of monocytes to the intercellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells is the main inducer of CCL3 production [12]. Knockout studies have also confirmed that CCL3 is a major regulator of monocyte and myeloid precursor cell trafficking [13, 14]. Recent studies have also demonstrated involvement in adaptive immunity. Cytotoxic T cells are the main effectors of the immune response against tumor cells, viruses, and intracellular pathogens. The activation of CD8+ cytotoxic T cells results in chemokine secretion including CCL3 that amplifies the immune response by recruiting additional leukocytes against the target cell [15, 16]. Interestingly, the release of CCL3 from memory CD8+ T cells promotes the secondary immune response to pathogens, which is antigen specific [17].

2.3 CCL3 Signaling in the Tumor Microenvironment

2.3.1 Antitumor Activity of CCL3

Dendritic cells play a key role in cancer immunology and are also involved in the tumoricidal and abscopal effects of chemotherapy and radiotherapy [18]. Exclusion of dendritic cells from the tumor microenvironment and inhibition of their differentiation are two common mechanisms of cancer immune evasion. Therefore, the chemotactic effect of CCL3 can enhance antitumor immunity by favoring the homing of dendritic cells in the tumor microenvironment [18]. More specifically, CCL3 exerts a cardinal effect on homing dendritic cells in lymph nodes. A major contributor to this concept was the study by Trifilo and Lane [19] that provided a proof of concept regarding the chemotactic function of CCL3. Increased CCL3 levels as a host response to mouse hepatitis viral infection of the central nervous system induced CD11c(+)CD11b(+)

CD8a(−) dendritic cell maturation, activation, and migration to draining cervical lymph nodes [19]. Furthermore, lack of CCL3 resulted in suboptimal dendritic cell trafficking to lymph nodes, impaired antigen presentation and reduced levels of IFN-γ by antigen-specific T cells and, consequently, reduced cytotoxic activity. Preclinical studies of HIV vaccination models have shown that CCL3 can be used as an adjuvant to DNA vaccines in order to enhance the immune response by driving the migration of dendritic cells and the antigen presentation to cytotoxic CD8(+) T cells [20]. The enhanced priming of cytotoxic T cells resulted in an efficient immune response upon antigen rechallenge. Importantly, this effect was CCL3-dependent and was not seen with other chemokines such as CCL19 [20]. Similarly, CCL3 exerts a systemic effect favoring the homing of dendritic cell vaccines to regional lymph nodes [21]. Regional induction of CCL3 production by tetanus-diphtheria toxoid injection at a single site led to a global increase in CCL3 levels, which enabled the migration of mRNA-loaded dendritic cells vaccines to draining lymph nodes independently of the injection site. This effect was evident in both humans and mouse models [21]. Regarding cancer vaccination, CCL3 has been evaluated as an adjuvant to antigen-specific vaccination. CCL3 enhances the trafficking of dendritic cells to the injection site, resulting in more potent T cell-mediated cytotoxic effects and antitumor activity [22, 23]. CCL3 has also been administered during vaccination with IL-2 or GM-CSF in hematological cancer models. Both approaches resulted in a significant antitumor response, but distinct immune cell subsets were implicated. CCL3 with IL-2 favored CD8-positive T cell and NK cell cytotoxicity, whereas CCL3 with GM-CSF resulted in both CD8- and CD4-positive antitumor response [24]. In addition, CCL3 may boost antigen recognition by antigen-presenting cells following ablative therapies such as radiofrequency ablation and chemoablation that result in tumor-associated antigenic release [25].

Furthermore, CCL3 promotes the induction of T cell responses specific to particular antigens [26]. The antigen recognition process during a primary immunological response in draining lymph nodes includes engagement of CD4-positive T cells with dendritic cells carrying the target antigen and, subsequently, binding of naïve CD8-positive T cells to dendritic cells, which initiates the CD8-positive T cell priming (Fig. 2.1). Interestingly, high levels of CCL3 and CCL4 have been detected upon dendritic cell and CD4-positive T cell contact. Importantly, it has been shown that naïve CD8-positive T cells prior to antigen recognition express high levels of CCR5. Therefore, CCL3-CCR5 interaction guides immature CD8-positive T cells to the site of dendritic cell engagement with CD4-positive T cells and, thus, favors the cytotoxic T cell response against the tumor antigenic epitope that is presented on the surface of the dendritic cell [26]. CCL3-mediated NK cell recruitment is also crucial for further enhancing CD8-positive antitumor response [27]. Furthermore, it has been demonstrated that the expansion of memory CD8-positive T cells, which are vital for sustaining the antigen-specific T cell response, is feasible only in the presence of CCL3 [26]. From a therapeutical perspective, CCL3-targeted therapeutics have shown promising results in preclinical studies. Genetic transfection of tumor cells with CCL3 increased intratumoral CCL3 levels and favored the recruitment of both antigen-presenting cells and T cells that resulted in enhanced antitumor immune response. Interestingly, high levels of CCL3 recruited activated neutrophils that exerted a cytotoxic function against tumor cells through increased TNF-α levels, reactive oxygen species, and paracrine protease-mediated cell killing [28]. Furthermore, T cells transfected to express CCR5 were subsequently infused intratumorally along with exogenous CCL3. This approach resulted in both tumor-specific cytotoxic T cell activity and tumor-specific T cell memory capable to initiate an immune response upon tumor rechallenge [29].

2.3.2 Pro-tumorigenic Effect of CCL3

The chemoattractive role of CCL3 may be multifaceted. Apart from regulating dendritic and cytotoxic T cell trafficking, CCL3 gradients can be manipulated by tumor cells and attract immunosuppressive cells to the tumor bed [30]. Tumor-derived chemo-

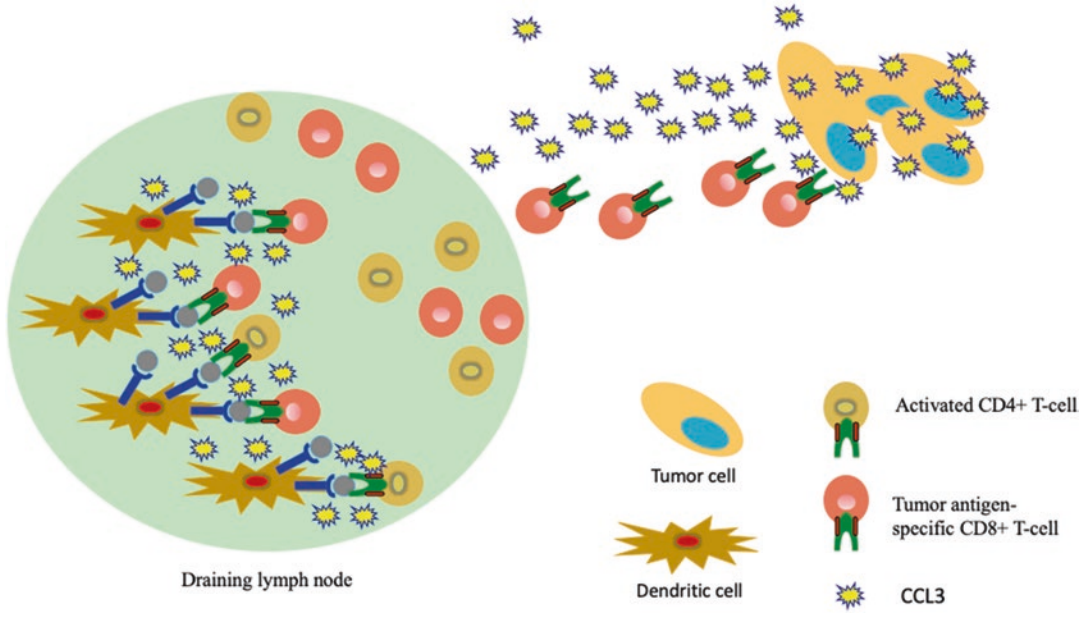

Fig. 2.1 Overview of the antitumor activity of CCL3. CCL3 enhances the trafficking of dendritic cells into draining lymph nodes from sites of peripheral inflammation. CCL3 secretion within the draining lymph node is the result of dendritic cells engagement with antigen-specific CD4-positive T cells. The resultant CCL3 gradient guides the CCR5-positive naïve CD8-positive T cells for antigen-specific activation. Furthermore, a CCL3 gradient promotes immune cell trafficking to the tumor bed

kine-containing microvesicles may also contain CCL3 and orchestrate a tumor-promoting microenvironment [31]. Preclinical studies have shown that high levels of CCL2 and CCL3 are associated with increased intratumoral infiltration of regulatory T cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) that allow tumor cells to evade the immune surveillance [32–35]. The cytokine and chemokine landscape in the tumor microenvironment can skew macrophages to obtain either a pro- or an antitumorigenic phenotype [4]. Importantly, high concentration of TAMs has been negatively correlated with disease prognosis [34]. It has also been reported that defective intracellular signaling of TAMs, such as in the NF-κβ cascade, may lead to decreased secretion of CCL3 and other chemokines including CCL4, TNF-α, and IL-6 [36–38].

Furthermore, CCL3-driven TAM recruitment has been recognized as a cardinal event in metastatic niches. Importantly, high levels of CCL2 binding to CCR2 on metastasis-associated macrophages (MAMs) induce CCL3 secretion and potentiate MAMs accumulation and their interaction with tumor cells [39]. Inversely, CCL2 blockade results in reduced CCL3 levels and macrophage recruitment [40]. In early stages of metastasis, CCL3-driven MAMs recruitment is mediated by CCR1, whereas in later stages of colonization of metastatic foci MAMs recruitment is based primarily on CCL3-CCR5 interactions [40, 41]. Therefore, CCL3 sustains MAMs-mediated support to the metastatic cancer cells, whereas absence of CCL3 impairs the metastatic spread [40–42]. Recent studies have shown that mast cell activation by IL-33 is another mechanism of increasing CCL3 levels and recruiting TAMs [43]. It is possible that increased levels of certain chemokines (CCL2, CCL5, CCL18) in the tumor microenvironment initiate the recruitment of monocyte-lineage cells and induce a de novo expression of additional chemokines (CCL3, CCL8, CCL22) that further support the trafficking of MAMs and Tregs [44]. Importantly, the net effect of CCL3 on tumor kinetics seems to be context related. In patients with oral squamous

cell carcinoma, high CCL3 levels were detected in metastatic lymph nodes compared with those which were disease free. On the other hand, high CCL3 levels within the primary tumor bed were associated with improved survival [45]. In this context, it seems that CCL3 receptors mediating recruitment of MAMs, such as CCR1, CCR2, and CCR5, constitute a suitable therapeutic target. Simultaneously, the presence of other chemokine receptors such as CXCR3 ensures the trafficking of cytotoxic T cell and NK cells to the tumor field [46–49].

In addition, CCL3 is actively implicated in leukemogenesis and myelomatogenesis [50–52]. Although CCL3 is a negative regulator of hematopoietic stem cells and progenitor cells, leukemia-initiating cells are nonresponsive to CCL3-mediated inhibition of proliferation. This may be attributed to decreased expression and/or dysfunctional CCL3 receptors [53–55]. As aforementioned, CCL3 promotes the accumulation of tumor-associated macrophages in the leukemic and myeloma niches, as well [42]. Moreover, leukemic cells may secrete CCL3 that impairs osteoblastic activity and deregulates bone metabolism in favor of a leukemic shift of hematopoiesis [56]. CCL3 favors decreased osteoblast activity coupled with increased osteoclast function, which constitutes the hallmark of myeloma bone microenvironment [57, 58]. Increased CCL3 levels derived from the neoplastic cells in bone marrow biopsies have been detected in patients with multiple myeloma and Waldenström's macroglobulinemia and CCL3 [59, 60]. Autocrine and paracrine CCL3 may also directly sustain survival and proliferation of myeloma cells [61]. However, taking into consideration the role of CCL3 in regulating myeloid differentiation and hematopoietic stem cell population, administration of a universal CCL3 inhibitor may not restore hematopoietic homeostasis [62].

2.4 Future Trends

CCL3 is a major regulator of immune cell trafficking in both homeostasis and disease. CCL3 in the tumor microenvironment may determine tumor cell fate by recruiting cytotoxic or tumor-promoting immune cell subsets. Therefore, the therapeutic manipulation of CCL3 and its targets is currently under clinical investigation. Importantly, CCL3 targeting should be evaluated in the context of the chemokine landscape, taking also into consideration potentially compensating mechanisms in each disease context separately. A promising therapeutic approach is CCL3 receptor inhibition. [63]. Although CCL3 has been assessed as an adjuvant to tumor vaccines, further research is essential before anticipating clinical efficacy. Optimization of the delivery systems should aim at overcoming stability issues and the half-life of this small molecule. Precision is another challenge, since CCL3-directed therapeutics should avoid the positive feedback to mechanisms of immune tolerance including MDSCs, MAMs, and Tregs, along with preventing the emergence of autoimmune adverse events.

Furthermore, CCL3 has emerged as a useful biomarker for response and immune activation during the recent years. Indeed, evaluation of CCL3 levels has been included in several clinical trials as a secondary endpoint (Clinicaltrials.gov numbers: NCT00319748, NCT00824733, NCT00626405, NCT00088855, NCT02943473, NCT03475628). In melanoma patients, a chemokine expression pattern including CCL3 has been associated with the presence of tertiary lymph node structures and patient prognosis [64]. High serum levels of CCL3 and CCL4 have been associated with dismal prognosis among patients with colorectal cancer [63]. Regarding clinical studies in hematological malignancies, high CCL3 plasma levels have been associated with increased risk of progression in chronic lymphocytic leukemia [65], shorter progression-free and overall survival in diffuse large B cell lymphoma [66], inferior progression-free survival and abnormal bone remodeling in Waldenström's macroglobulinemia [67, 68], and extent of bone disease and survival in multiple myeloma [69]. Therefore, CCL3 levels have an important prognostic value, whereas changes in CCL3 levels may be a valuable surrogate marker of response to therapy, which has to be investigated in future studies.

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CCL4 Signaling in the Tumor Microenvironment

3

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].

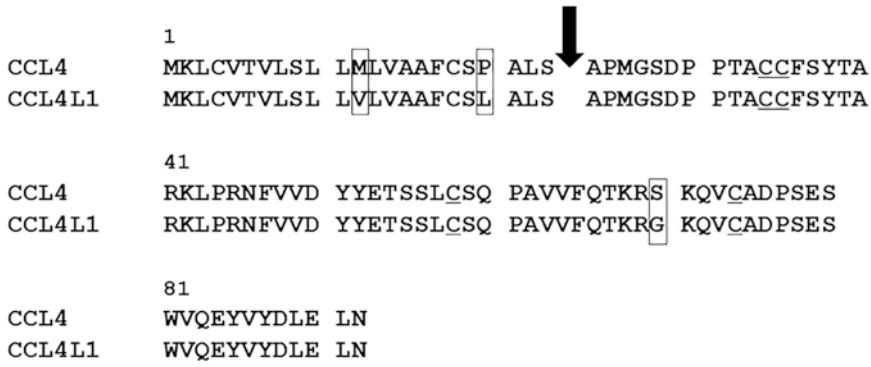

Fig. 3.1 Amino acid sequences of human CCL4 and CCL4L1. The arrow indicates the starting site of mature proteins while boxes indicate the sites at which the amino acid differs between the proteins. Well-conserved four cysteine residues are underlined. The numbers indicate the amino acid residue numbers

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].

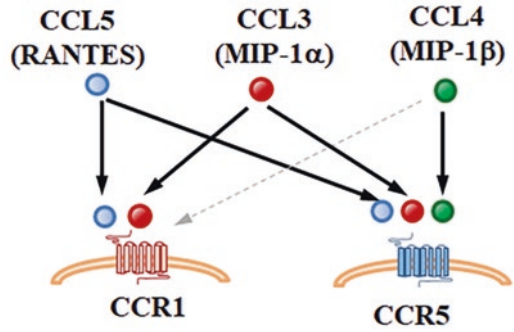

Fig. 3.2 Redundant use of CCR1 and CCR5 by the chemokines. CCR1 binds CCL3 and CCL5 but not CCL4 with high affinity, whereas CCR5 binds CCL3, CCL4, and CCL5 with high affinity

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-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

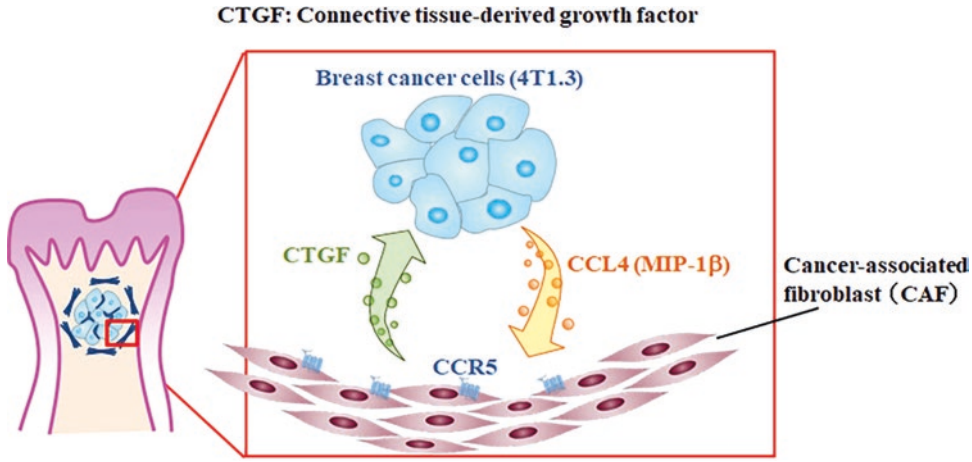

Fig. 3.3 Presumed roles of the CCL4/CCR5-mediated interaction between cancer cells and fibroblasts during the breast cancer metastasis to bone. Cancer-derived CCL4 attracts and activates CCR5-expressing fibroblasts, which can release connective tissue growth factor (CTGF) to promote cancer cell survival 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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CCL7 Signaling in the Tumor Microenvironment

4

Yeo Song Lee and Yong Beom Cho

Abstract

The tumor microenvironment is the primary location in which tumor cells and the host immune system interact. There are many physiological, biochemical, cellular mechanisms in the neighbor of tumor which is composed of various cell types. Interactions of chemokines and chemokine receptors can recruit immune cell subsets into the tumor microenvironment. These interactions can modulate tumor progression and metastasis. In this chapter, we will focus on chemokine (C-C motif) ligand 7 (CCL7) that is highly expressed in the tumor microenvironment of various cancers, including colorectal cancer, breast cancer, oral cancer, renal cancer, and gastric cancer. We reviewed how CCL7 can affect cancer immunity and tumorigenesis by describing its regulation and roles in immune cell recruitment and stromal cell biology.

Keywords

CCL7 · Tumor microenvironment · CCRs · Pro-tumor effect · Antitumor effect · Cancer-associated fibroblast · Cancer-associated adipocytes · Proliferation · Metastasis · Immune cell infiltration · Tumor-associated macrophage · Prognosis · Immunotherapy · Combination treatment · Clinical trials

4.1 Physiological Roles of CCL7

Chemokines comprise a large superfamily of at least 46 cytokines initially described based on their ability to bind to seven transmembrane domain G protein-coupled receptors to induce directed migration of leukocyte subsets to sites of inflammatory site or tumor microenvironment (TME) [1]. Their ligands can bind to extracellular N-terminus of receptors and lead to phosphorylation of serine/threonine residues on their cytoplasmic C-terminus, causing signaling and receptor desensitization [1]. Chemokine and chemokine receptor pairs not only mediate cellular migration but also affect many cellular functions, including survival, adhesion, invasion, and proliferation by regulating chemokine levels [2, 3]. Chemokines are classified into four groups (CXC, CC, C, and CX3C) based on the position of the first two cysteines [4, 5]. CXC chemokines act predominantly on neutrophils and T lymphocytes while CC chemokines are active on

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various cell types, including monocytes and lymphocytes [6–8]. Chemokine (C-C motif) ligand 7 (CCL7), also known as monocyte chemotactic protein 3 (MCP-3), is a member of chemokine ligand subfamily first characterized from osteosarcoma supernatant [9]. CCL7 is expressed in various types of cells (including stromal cells, immune cells, and airway smooth muscle cells) under physiological conditions and tumor cells under pathological conditions. CCL7 is a potent chemoattractant for a variety of leukocytes, including monocytes, eosinophils, basophils, dendritic cells (DCs), natural killer (NK) cells, and activated T lymphocytes [10, 11]. CCL7 is also highly expressed in advanced renal cancer, gastric cancer, colorectal cancer, and squamous cancer cells [12–15]. Ligands for chemokine receptors CCR1, CCR2, CCR3, and CCR5 can recruit macrophages to the TME [16]. Neutrophils and myeloid-derived suppressor cells (MDSCs) are recruited to the tumor through ligands for CCR2, CCR3, CXCR1, CXCR2, and CXCR4. Tregs express chemokine receptors CCR2, 3, 4, 6, 7, 8, and 10, CXCR3, and CXCR4 [17–25]. Among these receptors, CCR1, CCR2, CCR3, and CCR5 are widely known as the main functional receptors of CCL7 [26–28]. In this chapter, we will describe the role of CCL7 and its receptors in TME. We will also review how CCL7 and its receptors can affect cancer immunity and tumorigenesis in various types of tumor.

4.2 Opposite Role of CCL7 Signaling in the Tumor Microenvironment

Cross talk between tumor cells and their environment in peripheral TME is an important factor that affects tumor progression. Stromal cells including fibroblasts, macrophages, adipocytes, and others are components of the TME [29, 30]. Interactions between stromal cells and tumor cells are formed by a variety of soluble factors including inflammatory cytokines, growth factors, and chemokines secreted by tumor cells or stromal cells [31, 32]. CCL7 is an important molecular regulator in the reciprocal interaction between stromal cells and tumor cells. It not only participates in tumorigenesis (Table 4.1) but also exerts antitumor responses in particular contexts [33].

Table 4.1 Pro-tumoral effect of CCL7 in the tumor microenvironment

Cancer type	Cancer cell types	Producer (stimulator)	Recipient/signaling type (receptor)	Physiological effects	Ref.
Colorectal cancer	Patient sample	Colorectal cancer cells	Autocrine (CCR1, 2, 3, 5)	Enhances liver metastasis	[14]
Colorectal cancer	HCT116, HT29	Colorectal cancer cells	Autocrine (CCR3)	Enhances proliferation and promote migration, metastasis	[35]
Colorectal cancer	LS174T, CL-188	Kupffer cells	Hepatic stellate cells	Enhances liver metastasis	[46]
Oral cancer	YD-10B, YD-38, HSC-2, HSC-3	Cancer-associated fibroblast	Oral cancer cells (CCR1,3)	Enhances invasion	[12]
Oral cancer	YD-10B, YD-32, YD-38, HSC-2	Cancer-associated fibroblast	Oral cancer cells	Enhances cancer progression	[59]
Renal cancer	Patient sample	Renal cancer cells	Tumor-associated macrophage (CCR2)	Enhances brain metastasis	[13]
Renal cancer	A498, 769P, 786O, Caki-1, Caki-2	Renal cancer cells	Autocrine, endocrine	Enhances macrophage infiltration, tumor growth, metastasis	[34]
Gastric cancer	N.D.	Gastric cancer cells	Autocrine	Enhances lymph node metastasis and poor prognosis	[15]
Gastric cancer	C57BL6 mouse tissues	Adipose tissue	Paracrine, endocrine	Enhances macrophage recruitment	[67]

(continued)

Table 4.1 (continued)

Cancer type	Cancer cell types	Producer (stimulator)	Recipient/signaling type (receptor)	Physiological effects	Ref.
Breast cancer	Cal51, mda-mb-231, HFFF2	Cancer-associated fibroblast	Breast cancer cells (CCR1)	Enhances proliferation	[58]
Breast cancer	MDA-MB-231	Astrocytes	Breast cancer (CCR1)	Enhances self-renewal of tumor-initiating cells	[68]
Prostate cancer	LNCaP, C4-2B, Du-145, PC-3	Cancer-associated adipocytes	Prostate cancer cells (CCR3)	Enhances migration	[66]
Lung cancer	H1650	Neutrophils	Lung cancer cells	Enhances tumorigenic properties	[48]
Hepatocellular carcinoma	Huh-7, PLC	Cancer-associated fibroblast	Hepatocellular carcinoma cells (CCR1,2,3,5)	Enhances metastasis	[56]
Melanoma	B16-F0	Tumor cell-derived exosomes-educated mesenchymal stromal cells	Melanoma cells (CCR2)	Enhances macrophage recruitment and tumor growth	[71]
Laryngeal squamous cell carcinoma	Patient sample	Cancer-associated fibroblast	Laryngeal carcinoma cells (CXCR4)	Enhances tumor-supporting	[57]

Table 4.2 Antitumoral effect of CCL7 in the tumor microenvironment

Cancer type	Cancer cell types	Producer (stimulator)	Physiological effects	Ref.
Cervical carcinoma	HeLa	Cervical carcinoma cells	Enhances infiltration of macrophage, dendritic cells, and NK cells	[73]
Mastocytoma	P815	Mastocytoma cells	Enhances T cell activation and tumor rejection	[74]
Melanoma	B16, K1735	Melanoma cells	Enhances depletion of CD4, CD8, and NK cells	[75]
Pancreatic cancer	Panc-1	Pancreatic cancer cells	Enhances NK cell infiltration	[76]
Colorectal cancer	CMT93	Colorectal cancer cells	Enhances immune cell infiltration	[77]

Increased CCL7 levels can recruit monocytes to sites at the tumor periphery. This helps in the formation of an environment suitable for carcinoma progression and promotes monocytes to complete phenotypic transformation. CCL7 can also recruit leukocytes and activate antitumor immune responses (Table 4.2). Here, we will focus on the opposite roles of CCL7 based on original cells of CCL7 in various tumor types.

4.2.1 Pro-tumor Effect of CCL7

4.2.1.1 Signaling Induced by Tumor-Derived CCL7

CCL7 can act as a tumor-induced factor that can promote tumor growth, invasion, and metastasis by autocrine in metastatic renal cell carcinoma (RCC) rather than in primary RCC [13]. High CCL7 expression in RCC evokes the recruitment of tumor-associated macrophages (TAMs) that present CCR2 on their surface membrane, thus increasing vascular permeability. RCC cells can cross through blood-brain barriers to brain tissues [13]. microRNA Let-7d can specifically bind to the 3′UTR (untranslated region) of CCL7 mRNA and modify the expression of CCL7 in a negative feedback manner. The expression of let-7d is reduced in RCC, resulting in a large amount of CCL7 [34]. As a result, CCL7 plays an indirect role in RCC metastasis through the let-7d-CCL7-TAM axis. Pro-tumorigenic properties of CCL7 have also been confirmed in colorectal cancer (CRC) cells [14]. In vitro and in vivo CCL7 overexpression by lentiviral transduction can increase the proliferation, migration, and invasion of CRC cells [35]. In addition, by

binding to CCR3, CCL7 overexpression can activate the ERK/JNK signaling pathway that converges on downstream pathways of the MAPK cascade, thereby participating in the epithelial-mesenchymal transition (EMT) process that is sufficient to enhance cancer cell metastasis. Clinical studies have shown that CCL7 expression is higher in liver metastatic tumor tissues compared to primary CRC tissues, suggesting that CCL7 can promote CRC liver metastasis [14, 35]. In prostate cancer, PC3 cells can secrete more pro-metastatic factors, including CCL7 and TGF-β, thus accelerating the growth of prostate cancer and the rate of bone metastasis [36].

4.2.1.2 Signaling Induced by Immune Cells-Derived CCL7

Immune cells in the TME can promote tumor angiogenesis and suppress antitumor reaction of several activated immune cells, thus positively affecting tumor development process. Chemokine CCL7 was initially identified as a cytokine in mononuclear cells [37]. It can act on a variety of target cells, including neutrophils, eosinophils, basophils, NK cells, T lymphocytes, other inflammatory cells, DCS, and mononuclear cells, particularly monocytes [37–39]. In the last decade, many studies have shown that TAMs are closely related to tumor progression [40]. It has been found that TAMs differentiated through interaction with tumor cells are involved in immunosuppression, migration, and metastasis [41, 42]. Consistently with these functions of TAMs, studies using human tumor samples have shown that high density of TAMs with M2 phenotypes is closely linked to worse clinical prognosis, especially in many types of malignant tumors such as lung cancer, breast cancer, ovarian cancer, and bladder cancer [40–43]. Polarization signaling of TAM and TAM itself are new immunotherapeutic targets for malignant tumor treatment [44, 45]. Alcoholic liver damage is considered a high risk factor for colorectal cancer liver metastasis (CRLM) [46]. Overexpression of CCL7 in Kupffer cells (KCs), human liver macrophages, can create a favorable microenvironment for CRLM. The cascade begins with CCL7 and alcohol-stimulating KCs which express anti-inflammatory cytokine [46]. These stimuli can promote the potential ability of hepatic stellate cells (HSC) and enable the liver to become an important component of the metastatic niche. In pancreatic cancer, CCL7 mRNA levels are markedly increased after stimulating monocytes with thymic stromal lymphopoietin which is produced by activated cancer-associated fibroblast (CAF) [47]. CCL7 secretion by monocytes contributes greatly to the recruitment of basophil to tumor-draining lymph nodes (TDLN). Neutralizing antibody of CCL7 can partially block the recruitment of basophils to the TDLN. IL-4-positive basophils show greater accumulation in TDLNs than in non-TDLNs. This is relevant to Th2 inflammatory responses, indicating poor prognosis in pancreatic cancer patients with high proportion of basophils [47]. CCL7 is also secreted irregularly by neutrophils. It can increase the movement of human non-small cell lung cancer (NSCLC) cells so that cancer cells can metastasize to bone tissues [48, 49].

4.2.1.3 Signaling Induced by CAF-Derived CCL7

Cancer cells participate in the creation of a favorable microenvironment by interacting with stromal cells and triggering the homing of a variety of cells to the tumor site. Among cells affected by cancer cells, CAFs have both fibroblastic and mesenchymal stromal cell (MSC) origin [50, 51]. CAFs can promote tumor growth through direct stimulation of cancer cell proliferation, increasing angiogenesis, and recruitment of immune cells into TME [52]. Via interacting with tumor cells, activated CAFs can enhance the secretion of matrix metalloproteinase (MMP), chemokines, and growth factors to promote tumor migration [53, 54]. Compared to normal fibroblasts, CAFs are more numerous. In addition, they express higher quantities of mesenchymal markers such as E-cadherin. Furthermore, CAFs can significantly increase hepatocellular carcinoma (HCC) cell migration by inducing epithelial mesenchymal transition (EMT) in HCC cells in vitro [55]. CAFs also have powerful effects on HCC metastasis in vivo. CCL7 can activate the

TGF-β pathway by enhancing Smad2 phosphorylation. Blocking the TGF-β pathway markedly can inhibit effects of CCL7 on HCC tumor migration and invasion [56]. This study has highlighted the role of CCL7 in regulating tumor progression by influencing the TME via the TGF-β pathway [56]. In a coculture system of CAF and laryngeal squamous cell carcinoma, CCL7 protein levels are elevated, accompanied by rapid tumor cell proliferation with increasing CXCR4 expression [57]. A further study showed that CAF-derived CCL7 mainly promoted breast cancer cell proliferation by binding to its receptor CCR1 [58]. IL-1α secreted by oral squamous cell carcinoma (OSCC) can induce CCL7 release from activated stromal fibroblasts and stimulate CAF proliferation [59]. At the same time, CCL7 generated by CAF is the main promoter of OSCC cell migration and invasion. It guides cytoskeletal transformation and enhances cell dissemination and membrane disarrange [12, 59].

4.2.1.4 Signaling Induced by CAAs-Derived CCL7

It has been previously thought that obesity can serve as a risk factor of cancers such as breast cancer, prostate cancer, renal cancer, and gastrointestinal cancer [60]. Many studies have shown that cancer-associated adipocytes (CAAs) can produce cytokines, adipokines, chemokines, and MMP that can promote tumor initiation, progression, and metastasis [61, 62]. Furthermore, obese cancer patients show poor survival outcomes in prostate cancer, breast cancer, and CRC [63–65]. Inhibition of CCL7/CCR3 axis blocks the ability of adipocytes to enhance tumor cell migration. This means that CCL7/CCR3 interaction plays a crucial role in obese prostate cancer progression [66]. Increased expression of CCL7 can positively enhance proinflammatory reaction feedback loop and modulate immature monocytic myeloid cells mobilization in gastric TME [67]. Furthermore, in adipose tissue of obese mice, *Helicobacter felis* infection can induce macrophage accumulation and expression of CCL7 [67].

4.2.1.5 Signaling Induced by Other Cells-Derived CCL7

Astrocytes can secrete high levels of CCL7 when they are stimulated with cyclooxygenase 2 (COX2) and MMP-1 [68]. The axis of COX2-MMP-1/CCL7 can promote self-renewal of breast cancer and its brain metastasis [68]. Truncated CCL7 cleaved by MMP-13 can eliminate the action of its corresponding receptors. The cleaved CCL7 becomes part of the negative feedback loop, which in turn increases MMP-13 and osteolysis. Thus, malignant breast cancer MDA-MB-231 cells are easy to move to the bone [69]. CCL7 produced by bone marrow (BM) stromal cells can act as a chemoattractant for human multiple myeloid cells via CCL7/CCR2 interaction [70]. CCL7 plays a pivotal role in the recruitment of macrophages by tumor cell-derived exosome-educated mesenchymal stromal cells (MSCs) via binding to CCR2 on melanoma cells [71]. Overexpression of CCL7 by MSCs increases its interaction with neighboring immune cells and facilitates macrophage infiltration, making tumor microenvironment suitable for tumor self-renewing [72]. As shown in Fig. 4.1, CCL7-related signaling plays a pivotal role in tumor microenvironment to enhance tumorigenesis.

4.2.2 Antitumor Effect of CCL7

CCL7 is generally recognized as inflammatory cytokine. T lymphocytes and DCs activated by CCL7 play an important role in mobilizing immune responses to resist tumor growth. Transduced model of CCL7 using parvovirus which overexpresses CCL7 in cervical cancer tumor shows tumor regression and immune cell infiltration such as NK cells and macrophages in xenograft model [73]. Furthermore, CCL7 overexpression increases recruitment of leukocytes and triggers type I T cell-dependent reactions, evoking an antitumor cascade [74]. CCL7 gene transfer to mastocytoma cells causes reduced tumorigenicity, enhanced neutrophil recruitment to the tumor, and DC infiltration in peritumoral

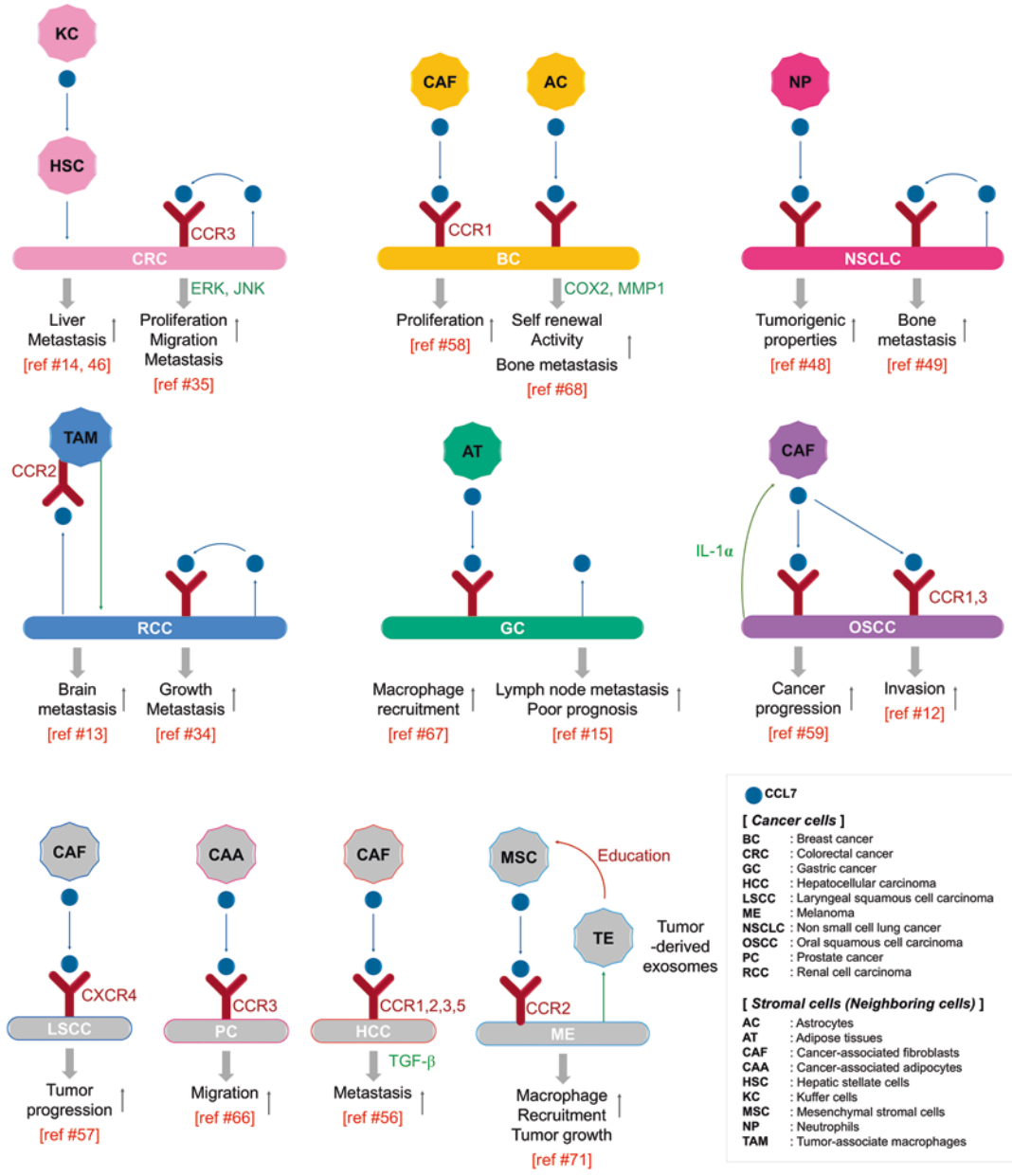

Fig. 4.1 The role of CCL7 signaling in tumor microenvironment

tissue [74]. CCL7-transduced melanoma cells also show strongly inhibited tumor growth in mice [75]. Such tumor regression is partly mediated via recruited CD4, CD8 T cells, and NK cells [75]. Another parvovirus-mediated CCL7 overexpression model of PDAC study has shown that CCL7 can activate and recruit NK cells and monocytes to enhance antitumor responses [76]. In addition to controlling tumor growth, CCL7 also impedes tumor metastasis in a mouse colon cancer model [77]. In brief, parvovirus-mediated transduction of CCL7 to cancer cells can reduce tumor progression through activated immune cell infiltration. In other words, CCL7 might be a strong activator of immune surveillance via recruiting immune cells to the TME.

4.3 Immunotherapy Landscape and Future Direction

As described earlier, the role of CCL7 in the tumor microenvironment has been consistently investigated over the last three decades since it was first identified in osteosarcoma cell (MG-63) supernatant [9]. Because CCL7 is derived from various normal cells [39, 78] as well as tumor microenvironmental cells, its physiological roles in the living body are profoundly complicated. Since CCL7 can bind to multiple seven-transmembrane receptors in normal physiology and plays a crucial role, anti-CCL7 antibody or such ligand targeting may inhibit other signaling pathways that are crucial for sustaining normal homeostasis. Therefore, rather than targeting chemokine itself, targeting chemokine receptors is an ideal immunotherapeutic strategy. Many preclinical models and clinical trials have been performed to validate their roles in actual patient's survival. In fact, anti-CCR4 monoclonal antibody and CXCR4 antagonist are already in the stage of clinical practice for various tumors [79–81]. Regarding CCL7 receptors, CCR1, CCR2, CCR3, and CCR5 have been investigated in preclinical studies. Clinical trials on CCR1, CCR2, and CCR5 in multiple myeloma, colorectal cancer, pancreatic cancer, and breast cancer have also been performed [82–86]. Although the role of CCR3 as a receptor for CCL7 has been revealed in many cancer types, clinical trials on CCR3 have not been reported yet. Because inhibiting chemokine receptor shows potential clinical value itself or when it is combined with immune checkpoint inhibitors, studies on chemokine receptor such as CCR3 might give encouraging results. Therefore, strategies targeting CCL7 receptors might be useful in the future to overcome poor survival outcomes of patients.

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The Multifaceted Roles of CXCL9 Within the Tumor Microenvironment

5

Shi Yong Neo and Andreas Lundqvist

Abstract

Chemokines are soluble proteins that orchestrate cell migration in a regulated concentration gradient. During early stages of tumor development, chemokines shape the immune landscape of tumor microenvironment. CXCL9, also known as monokine induced by gamma-interferon (MIG), can be produced during inflammatory conditions by myeloid cells within the tumor microenvironment. It attracts cells expressing the CXCR3 receptor including activated T and NK cells and has been shown to play a role in responses to immune checkpoint therapy. Overexpression of CXCL9 has also shown to reduce tumor progression and metastasis via the inhibition of angiogenesis. Conversely, CXCL9 can act directly on tumor cells expressing the CXCR3 receptor to promote cell migration and epithelial mesenchymal transition. In this chapter we discuss the anti- and pro-tumoral features of CXCL9 within the tumor microenvironment.

Keywords

CXCL9 · MIG · CXCR3 · Chemokines · Tumor microenvironment · Inflammation · T cells · NK cells · Tumors · Migration · Epithelial mesenchymal transition · Metastasis · Angiogenesis · CXC chemokines · Biomarker

5.1 Introduction

Chemokines are *chemo*tactic soluble proteins that orchestrates cell trafficking and localization by its presence in a regulated concentration gradient. Chemokines mainly bind to the seven-transmembrane spanning G protein-coupled chemokine receptors (GPCR) and non-G protein-coupled seven-transmembrane spanning receptors called atypical chemokine receptors (ACKR). Till date, 48 chemokines are characterized and can be classified into 4 subgroups based on the position of the first two N-terminal cysteine residues. The 4 groups of chemokines include the CC, CXC, CX3C, and XC subfamilies. The coordinated movement of immune cells is dependent on dynamic chemokine gradients to provide appropriate homing of different type of immune cells to mount an immune response. Often, the failure to maintain a tightly regulated chemokine

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gradient would implicate a broad range of inflammatory diseases including cancer [1, 2].

In the dynamic ecology of the tumor, the spatial expression of chemokines coordinates a variety of cell-cell communication and the concerted processes of tumor development such as stromal remodelling and metastasis. In early stages of tumor formation, chemokines shape the immune landscape of tumor microenvironment (TME), contributing to the concept of "hot and cold" tumors that often determines whether a patient would get a favorable response with immunotherapy. While the complex regulation of chemotaxis remains poorly understood, it has continued to be a barrier to overcome for successful treatment of solid tumors. In cases of "cold" tumors, cytotoxic lymphocytes often accumulate at the peritumoral stroma—an observation that correlated with poor prognosis in multiple tumor types.

In this chapter we will discuss the biological roles of CXCR3 ligands, in particular CXCL9, and how CXCL9 play a role in angiogenesis and epithelial mesenchymal transition and as a prognostic biomarker in cancer potentially exploited for clinical applications (Fig. 5.1).

5.2 Antitumoral Role of the CXCL9-CXCR3 Pathway

CXCR3 and its ligands (CXCL9, CXCL10, and CXCL11) are highly expressed on cells in inflamed tissue microenvironments, and hence commonly associated with several autoimmune diseases. The regulation of CXCR3 expression varies in different types of immune cells. Naïve T cells in general do not express CXCR3 until activation and priming mediated by dendritic cells. The majority of CXCR3-expressing cells are in fact CD8 memory T cells followed by CD4 T cells. The expression of the receptor is required for the essential homing to a localized inflammatory response. Similarly, CXCR3 can be expressed on innate lymphoid cells such as natural killer cells (NK cells), NKT cells, and also plamacytoid dendritic cells (pDCs) and B cells [3]. CXCR3 ligands are generally found in low baseline concentrations under normal homeostasis. CXCL9 (also known as MIG-monokine induced by gamma-interferon) can be highly produced by dendritic cells and macrophages in the tumor microenvironment. Tumor regions that

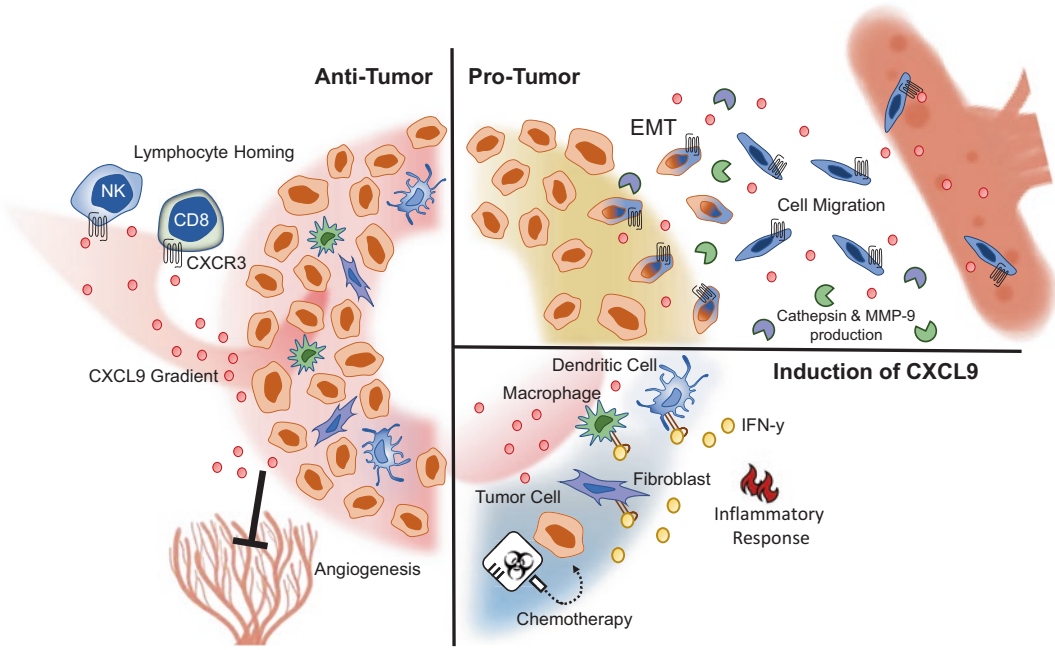

Fig. 5.1 Anti- and Pro-tumoral roles of CXCL9

highly expressed CXCL9 are found with marked infiltration of T cells that could be essential for the control of tumor growth via the IFN-γ-dependent pathway [4]. Dangaj et al. demonstrated that IFN-γ triggers CXCL9 production in myeloid cells to amplify production of CCL5 within the TME. Tumors expressing high levels of both CCL5 and CXCL9 was shown to be more immune reactive and higher likelihood of responding to immune checkpoint blockade [5]. Interestingly, tumors that are deficient for CXCL9 can still produce CXCL10 but could not recruit cytotoxic CD8 T cells resulting in tumor accelerated tumor growth [6].

5.3 CXCL9 as a Negative Regulator of Cancer Angiogenesis

The formation of new blood vessels, or angiogenesis, is a distinct feature for successful tumor growth of solid tumors. Much of the tumor angiogenesis research has focused on the contribution of the VEGF family [7]. However, CXC chemokine-mediated regulation of angiogenesis has been shown to play a critical role in solid tumors. While ELR-containing CXC chemokines are potent promoters of angiogenesis, the IFN-inducible non-ELR-containing CXC chemokines, including CXCL9, are potent inhibitors of angiogenesis. In humans, the CXCR3 receptor exists in at least three distinct mRNA splice variants: CXCR3A, CXCR3B, and CXCR3-alt [8, 9]. Lasagni et al. found that CXCR3A and CXCR3B transfected human microvascular endothelial cell line-1 (HMEC-1) bound to CXCL9, CXCL10, and CXCL11. Overexpression of CXCR3-A resulted in increased cell viability and proliferation, and increased ability of vessel formation in vitro. In contrast, overexpression of CXCR3-B resulted in altered growth properties and massive apoptotic cell death [9].

Most human malignancies express several of the CXC chemokine receptors including CXCR4, CXCR3, and CXCR7 [10]. While many studies have investigated the role of ELR-containing CXC chemokines as promoters of angiogenesis, fewer studies have investigated the angiostatic effects of non-ELR-containing CXC chemokines, and in particular CXCL9. Zhang et al. showed that combination of CXCL9 gene therapy and low-low chemotherapy resulted in inhibition of angiogenesis and the induction of tumor apoptosis in two separate murine models of cancer [11]. Addison et al. showed that overexpression of the CXCL9 in non-small cell lung cancer cells resulted in the inhibition of tumor progression and metastasis via a decrease in tumor-derived vessel density [12]. In addition to contributing to antitumor defenses by inhibition of angiogenesis, CXCR3 and its ligands can promote Th1-dependent immunity through recruitment of CXCR3-expressing T and NK cells [3]. In animal models of non-small cell lung cancer, Sharma et al. showed that CCL19 promoted the recruitment of dendritic cells and T cells leading to a reduction in tumor progression via increased expression of CXCL9 and CXCL10 [13]. Similarly, intratumoral injection of CCL21 resulted in complete tumor eradication which was attenuated by inhibiting CXCL9 and CXCL10 [14]. Other studies have shown that the combined administration of IL-2 with intratumoral CXCL9 resulted in reduced tumor progression and angiogenesis [15]. In contrast, Patil et al. found that IL-17-producing γδ T cells migrate toward tumors using CXCL9-CXCR3 axis in gallbladder cancer. These T cells contributed to tumor progression by producing vascular endothelial growth factor [16]. Another study by Tan et al. showed that CXCL9 promoted prostate cancer progression through inhibition of IL-6 and TGF-β by T cells [17].

5.4 CXCL9 Promotes the Invasiveness of Cancer

Metastasis is a concerted process whereby certain clones of tumor cells in the primary lesion acquire "mesenchymal" phenotype and enhanced migratory capacities to invade through the tumor margin to colonize a favorable tissue microenvironment distant from the primary tumor [18]. While we know that chemokines regulate the

trafficking of hematopoietic cells, it is currently well documented that tumor cells can make use of multiple chemokine systems during the process of metastasis [19].

Since tumor cells could also express CXCR3 on the cell surface, it is plausible that CXCL9 can promote cancer migration via chemotaxis which was demonstrated in melanoma models by Amatschek et al. [20]. Another recent study by Li et al. demonstrated that adding exogenous CXCL9 to CXCR3-expressing tongue squamous carcinoma cell line (Cal-27) promoted cell migration and epithelial mesenchymal transition [21]. Cathepsin B is a lysosomal protease that can be secreted by tumor cells, contributing to the remodelling of the extracellular matrix for cell invasion and migration. While cathepsin B was found to be able to cleave CXCR3 ligands, it was also found that CXCL9 and CXCL10 can induce upregulation of cathepsin B in breast cancer cell lines MCF-7 and MDA-MB-231 which are both expressing CXCR3 [22]. Exogenous CXCL9 also stimulates liver cancer cell lines expressing CD133 to activate p-ERK1/2-MMP2/MMP9 pathway, promoting cell migration and invasion as demonstrated in transwell and wound healing assays [23]. As discussed earlier that tumor cells can be triggered to produce CXCL9, it was also found in another study that CD4 T cells recruited by CXCL9 may in turn enhance the metastatic potential of the tumor cells in which it was proposed that CD4 T cells suppress androgen receptor-mediated signalling pathway and upregulate MMP-9 expression in prostate cancer cells [24].

5.5 CXCL9 as Biomarker in Immunotherapy

The function of CXCR3-CXCL9 binding may not be limited to the homing of T cells into the tumors. Chow et al. have recently demonstrated that CXCR3 may not be critical for infiltration of CD8 T cells into the tumor but rather to promote the interaction of these cytotoxic T cells with CXCL9-producing dendritic cells within the microenvironment. While the underlying mechanisms are interesting and not fully uncovered, it was also demonstrated that the measurement of CXCL9 in the patient serum may serve as a biomarker to indicate response to anti-PD-1 therapy [25]. In a 6-year follow-up cohort of non-small cell lung cancer (NSCLC) patients with tumor resections, patients with no recurrence (n = 30) had higher concentrations of CXCL9 in tumor blood than peripheral blood prior to surgery while patients with relapses (n = 27) had higher concentrations of CXCL9 in peripheral blood as compared to tumor blood. Even though it was observed that there was high variability in the data, the study of CXCL9 gradients may be a good indicator of clinical outcomes [26]. Another NSCLC study (n = 50) also observed that higher protein levels of CXCL9 in fresh tumor resections positively correlated with progression-free survival [27]. In a retrospective study of paraffin-embedded core biopsies, CXCL9 gene expression is significantly 3 times higher in breast cancer patient group with pathological complete response to neoadjuvant chemotherapy (n = 134) [28].

On the contrary, higher expression of CXCL9 may not be favorable in cancers with tumor cells overexpressing CXCR3. From hepatocellular carcinoma (HCC) tissues stainings (n = 89 paired comparisons of cancer tissue versus non-cancerous adjacent tissues), CXCR3 upregulation correlated with tumor size, differentiation, and metastasis [23]. It was observed in tongue squamous cell cancers (TSCC) that higher expression levels of CXCR3 and CXCL9 positively correlated with the presence of lymph node metastasis—an indication that CXCL9 could be a marker for tumor progression. Moreover, in a breast cancer cohort of 81 patients, higher tumoral expression of CXCR3 correlated with poorer overall survival [22]. Zhi et al. studied a cohort of 1057 of cervical cancer patient cohort and found that serum levels of CXCL9 correlated with disease progression together with other soluble factors such as C-reactive protein, CA125, and carcinoembryonic antigen (CEA) [29].

5.6 Modulating Chemokine Signalling in Cancer Immunotherapy

Primary strategies to improve T cell infiltration revolves around modulating the immunogenicity of solid tumors by introducing mainstay targeted therapies in combination and novel compounds. Given the important roles of CXCL9 in tumor angiogenesis, growth, aggressiveness, and metastasis, it is of importance to understand how its expression can be modulated to develop novel therapies for a wide range of human malignancies. While CXCL9, 10, and 11 are well known to be induced by IFN-γ, a few studies have explored other means to modulate the expression of CXCL9. Zhang et al. recently showed that treatment with β-glucan resulted in an induced expression of pro-inflammatory cytokines including CXCL9, leading to an inflamed tumor microenvironment and delayed primary and lung metastatic experimental melanoma tumors [30]. Prost et al. showed that increased levels of CXCL9 by IFNγ and TLR3 agonists by double-stranded RNA (dsRNA) induced the expression of CXCL9 and CXCL11 in leukocytes and skin-muscle fibroblasts, whereas ligation of TLR2, TLR4, TLR5, and TLR9 did not. In fact, TLR2 and TLR4 agonists inhibited IFNγ-induced CXCL9 expression in leukocytes [31]. Doorduijn et al. showed that the TLR-7/8 agonist imiquimod, but not the TLR3 or TLR9 agonists, mediated an effective antitumor response in experimental melanoma and lymphoma models. TLR7 agonists activated NK cells to kill tumor cells, resulting in release of tumor antigens and induction of tumor-specific CD4 T cells. These T helper cells provoked a strong induction of CXCL9 and CXCL10 in the tumor environment. Simultaneously, TLR7 agonists induced the expression of CXCR3 on peripheral lymphocytes [32]. Shimauchi et al. analyzed serum markers in patients with mycosis fungoides after treatment with the combination of IFN-gamma and narrow band-UVB phototherapy. While no significant changes were observed in the levels of CCL17 and CCL22, CXCL9 and CXCL10 were significantly elevated by the combination of IFN-gamma and narrow band-UVB phototherapy [33].

Conventional chemotherapeutic agents such as dacarbazine, temozolomide, and cisplatin can induce intratumoral expression of these CXCR3 ligands to promote immune infiltration and surveillance in "cold" and unresponsive tumors [34]. This strategy can also be applied to improve the efficacy of cell therapy. Hu et al. demonstrated in a tumor-bearing NSG mouse model that IL-12 and doxorubicin in combination upregulated the IFN-γ pathway and the production of CXCL9 and CXCL10. Using this drug combination treatment to prime the tumors one day prior to T cell infusion, they were able to achieve a marked increase of infiltration into the tumors [35].

Another approach to improve tumor homing of cell therapy infusions would be to engineer cells with chemokine receptor constructs. More than a decade ago, Kershaw et al. had transduced CXCR2 on T cells to improve homing into tumors expressing its ligand CXCL1 [36]. Phase 2 trial NCT 01740557 currently evaluates the efficacy of infusing T cells genetically modified to co-express nerve growth factor receptor (NGFR) and CXCR2 to treat stage III and stage IV metastatic melanoma.

The same concept can also be applied in the setting of adoptive NK cell therapy. While Wendel and Cerwenka demonstrated that the CXCL10-CXCR3 axis is required for intratumoral retention of NK cells, our group observed that ex vivo-expanded NK cells resulted in a 10-fold expression of CXCR3 compared to resting NK cells. By infusing large amounts of these NK cells into CXCL10+ melanoma-bearing mice, we were able to prolong survival and reduce tumor burden of these mice. As similarly shown in Wendel's study, the local administration of IFN-γ directly into the tumor could induce CXCL10 production for adequate homing of these freshly infused NK cells for antitumoral responses [37, 38]. Efficacy of adoptive cell therapy can also be improved by gene editing and modification of cellular products such as chimeric antigen receptor (CAR) T cells and CAR-NK cells. By engineering NK cells to express CXCR2 using a retroviral system, our group has demonstrated that CXCR2 positive NK cell infusion could potentially better infiltrate solid tumors and at the same time stronger adhesion properties to form conjugates with tumor targets [39].

5.7 Conclusion

As discussed in this chapter, the multifaceted roles of CXCL9 could contribute to both anti- and pro-tumoral events within the TME. While efforts are currently made to promote immune surveillance by CXCL9 and IFN-γ pathways, there could be more unknown mechanisms in which CXCL9 interplay in tumor progression.

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CCL20 Signaling in the Tumor Microenvironment

6

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Abstract

CCL20, as a chemokine, plays an important role in rheumatoid arthritis, psoriasis, and other diseases by binding to its receptor CCR6. Recent 10 years' research has demonstrated that CCL20 also contributes to the progression of many cancers, such as liver cancer, colon cancer, breast cancer, pancreatic cancer, and gastric cancer. This article reviews and discusses the previous studies on CCL20 roles in cancers from the aspects of its specific effects on various cancers, its remodeling on tumor microenvironment (TME), its synergistic effects with other cytokines in tumor microenvironment, and the specific mechanisms of CCL20 signal activation, illustrating CCL20 signaling in TME from multiple directions.

Keywords

CCL20 · Tumor microenvironment (TME) · CCR6 · NF-κB · Chemokine · Cytokine · Cancer · Dendritic cells (DCs) · Regulatory T cells (Tregs) · T helper 17 cells (Th17 cells)

6.1 Introduction

The growth process of tumor is regulated not only by its own internal signals but also by many external factors. These external regulatory factors come from the tumor microenvironment (TME) in which cancer cells are located [1–5]. More and more studies have shown that tumor growth is not an independent development process, but a complex regulatory process influenced the tumor microenvironment. These regulatory processes further affect the initiation of cancer, tumor growth, metastasis, drug resistance, recurrence of cancer, and so on. Tumor microenvironment is often a kind of tissue environment which has been reconstructed by tumor cells. That is to say, it often promotes the progress of cancer. What is more interesting is that some immune cells with normal functions gradually weaken or even lose their antitumor ability after they infiltrate into the tumor tissues, which further proves the reprogramming ability of tumor cells to the microenvironment [6–11]. Therefore, if the microenvironmental factors can be taken into account in studying the mechanism of cancer

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development, more useful and feasible anticancer strategies may be discovered. Recent findings suggest that in the course of anticancer treatment (such as chemotherapy and radiotherapy), some measures targeting the altered TME can be taken to improve the therapeutic effect of tumors. Recurrence of cancer is a process in which a small number of residual cancer cells recover their proliferative ability and lead to the growth of tumor again under the support of specific environment after treatment. Why can't the body's own immune system recognize and clean up the remaining cancer cells? If the normal tissue environment (such as normal antitumor immune function) can be effectively restored in the lesion site, the re-proliferation of cancer cells may be inhibited.

However, targeting the tumor microenvironment is not very easy. Tumor microenvironment is highly heterogeneous [12–15]. Firstly, the components of tumor microenvironment are complex, including tumor cells, fibroblasts, T cells, B cells, NK cells, macrophages, DC cells, pericytes, endothelial cells, mesenchymal stem cells, adipocytes, red blood cells, and other components, such as the exosomes and cytokines produced by these cells and extracellular matrix. Moreover, some cells are in different states induced by different environments. Tumor-associated macrophages (TAM) can be further classified into type I, type II, and other types due to their different differentiation, and the effects of different subtypes of macrophages on cancer are diverse [16–20]. These various cells in the microenvironment can regulate each other, thus forming a complex network related to cancer progression. Secondly, tumors occurring in various organs and tissues differ greatly because of their specific physiological environments. For example, chronic inflammation may exist before cancer initiation such as colon cancer, stomach cancer, and liver cancer, but rarely in glioma and breast cancer. Thirdly, the composition of tumor microenvironment is also different during the distinct stages of cancer development. The infiltration of multiple immune cells may be different between early- and late-stage tumors. It is possible that as tumors progress, the types and numbers of cells recruited and infiltrated into the microenvironment become more complex, and treatment becomes more difficult. In addition, after cancer cells are stimulated differently (e.g., with different therapeutic drugs), they may become resistant to treatment. And drugs can also lead to changes in the microenvironment, and the ultimate result may be to further enhance the resistance of cancer cells to drugs. In the treatment of cancer patients, the tumor microenvironment is not constant, but a dynamic process. All in all, the above factors may affect the complexity of tumor microenvironment and lead to its high heterogeneity.

Cytokines, produced by stromal cells or cancer cells, are an indispensable factor in tumor microenvironment formation [21–26]. Because cytokines can be secreted outside the cell, and can reach to a distant location, even as the circulatory system spreads, its influence range is extensive through the mode of autocrine or paracrine, establishing plenty of connections between tumor cells, or tumor cell and stromal cells [27–30]. Cytokines can transfer stimuli from the environment to cancer cells and promote their tolerance to environmental stress. In turn, cancer cells can create favorable conditions by secreting specific cytokines to act on a variety of microenvironmental stromal cells expressing the corresponding receptors. If we call cytokines in microenvironment as messengers, then they play the role of transmitting different signals in the environment. Tumor cells and stromal cells in the environment are the recipients of this information. For example, many studies have shown that IL-6, IL-8, TGF-beta, and other factors are closely related to the malignancy of multiple cancers. Chemokine, as the subfamily of cytokines, can recruit specific cell types, mainly those related to immune function. The presence of chemokines provides a vital condition for the infiltration of immune cells in tumor microenvironment [31–33]. Therefore, chemokines are very important for the formation and remodeling of tumor microenvironment, and also have a great impact on cancer progression. More importantly, chemokines not only have the

ability of recruiting specific cells, but also have the function of signal transduction (ligand-receptor binding). In other words, through chemokines, cancer cells can not only recruit specific cells, but also transmit "friendly" signals to these cells, further reprogramming the microenvironment to be conducive to their survival.

CCL20 is a member of chemokine family. It has antimicrobial activity and is related to arthritis, psoriasis, and other diseases in pathology [34–37]. Recent studies have shown that high levels of CCL20 are associated with malignancies of various cancers. Importantly, CCL20 can recruit immune cells such as DC cells and Treg cells, which further links CCL20 with tumor microenvironment. This chapter will review and discuss the regulation of CCL20 on various cancers, the remodeling of tumor microenvironment mediated by CCL20, the synergistic regulation of CCL20 and other factors on microenvironment, and the activation and transmission of CCL20 signaling in tumor microenvironment.

6.2 CCL20 Signaling

Chemokines are a class of inducible secretory proteins with a molecular weight of 8–10 kDa produced by a variety of cells and participate in the activation, chemotaxis, and migration of white blood cells, and play an important role in inflammation and immune response. Chemokines also have a positive impact on angiogenesis, hematopoiesis, and organogenesis. According to the arrangement of conservative cysteine at N-terminal, chemokines can be divided into four subfamilies, C, CC, CXC, and CX3C. CCL20, belonging to the CC subfamily of chemokines, also known as macrophage inflammatory protein 3 alpha (MIP3α), Liver and Activation Regulated Chemokine (LARC), and Exodus-1, was first discovered and characterized in hepatocytes [38]. CCL20 gene contains four exons and three introns. Due to the alternative splicing between the first intron and the second exon, two kinds of mRNA encoding CCL20 are produced. After translation and signal peptide removing, CCL20 contained only 70/69 amino acid residues. Since the initial discovery of CCL20 (a small ~8 kDa protein) in the early 1990s, it was gradually demonstrated that CCL20 is primarily expressed in the liver, colon, prostate, cervix, and skin. CCL20 is related with rheumatoid arthritis and human immunodeficiency virus infection and also linked to malignancies such as hepatocellular, colorectal, and breast cancers.

The binding receptors of chemokine are G protein-coupled receptors (GPCR) with seven transmembrane regions, mainly expressed in endothelial cells, immune cells, and some tumor cells. Unlike many other cytokines, CCl20 only binds CCR6 [39–41]. Under non-pathological conditions, the expression level of CCR6 was low in most tissues, but highest in intestinal mucosa, lung mucosa, and lymphoid tissue [42, 43]. At the cellular level, CCR6 was mainly expressed in B cells, memory and effector T cells, Th17, Tregs, and immature DCs [44–48].The basic expression of CCR6 on immune cells induces cells homing to ligand secretion sites [35, 49–51]. CCR6 was significantly upregulated in inflammatory bowel disease, rheumatoid arthritis, and other pathological autoimmune diseases [36, 52–54]. Interestingly, some studies have shown that CCR6 is highly expressed in cancer cells than in normal tissues and is associated with malignancy, making it a potential prognostic biomarker and therapeutic target. The expression of CCR6 in colon cancer cells was higher than that in normal adjacent tissues, and was related to lymph node status and distant metastasis. CCR6 expression was higher in colon cancer cells of node-positive cases, and highest in cases with metastasis [55]. In gastric cancer tissues, CCR6 was upregulated compared with that in adjacent noncancerous gastric tissues. High CCR6 expression was determined in 56.5% (210/372) of the samples, which was significantly correlated with recurrence and poor overall survival of gastric cancer [56].

CCL20 functions through binding to its receptor CCR6, and their high expression in some tumors further demonstrates the vital role of CCL20 signaling in the development of cancer.

6.3 Roles of CCL20 in Multiple Cancers

There is growing evidence that CCL20 is associated with a variety of cancers, including hepatocellular cancer, breast cancer, colorectal cancer, pancreatic cancer, gastric cancer, lung cancer, etc.

6.3.1 Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma (HCC) is primary liver cancer with high mortality, and it is the most common malignant tumor in the world. HCC is the main histological subtype of liver cancer, accounting for 90% of primary liver cancer. Heredity, epigenetic changes, chronic hepatitis B, hepatitis C virus infection, aflatoxin exposure, smoking, obesity, and diabetes are the main risk factors for HCC. The poor prognosis of HCC is due to the high recurrence and metastasis rate. Although there have been some new technologies and methods in the diagnosis and treatment of HCC in recent decades, the 5-year overall survival of patients is very low, still at 3–5%.

Chemokines and their receptors play a complex role in the progression of HCC. At present, chemokines and their receptors, such as CXCL12-CXCR4 axis, CX3CL1-CX3CR1 axis, and CCL20-CCR6 axis, have attracted extensive attention [57]. A large number of studies have shown that these signal axes are closely related to hepatocellular carcinoma. Ding et al. revealed the prognostic significance of CCL20 in patients with hepatocellular carcinoma after curative resection [58]. They found that CCL20 expression evaluated by immunohistochemistry in tumors was associated with tumor size, tumor number, vascular invasion, tumor differentiation, and recurrence. The recurrence-free survival rate and overall survival rate of patients with high expression of CCL20 were lower than those with low expression of CCL20. Multivariate analysis showed that CCL20 expression was an independent predictor of tumor recurrence, recurrence-free survival, and overall survival [58].These results suggested that CCL20 expression was associated with tumor recurrence and survival of HCC patients. Through determining secreted CCL20 levels in serum of two HCC cohort patients ($n = 95$, $n = 85$, respectively) and observing the mRNA expression of CCL20 and CCR6 in 41 paired HCC tumor and adjacent non-tumor tissues, researchers showed that pretherapy serum CCL20 was elevated in HCC patients and CCR6 expression was increased in HCC tissues, of which both were closely associated with tumor metastasis and disease poor prognosis [59]. Moreover, in HCC tissues, CCL20 expression was positively correlated with CCR6. Importantly, the neutralization of CCL20 activity could reduce the incidence of tumors and inhibit tumor outgrowth and distal metastasis. After blocking CCL20 activity, tumor angiogenesis was significantly inhibited [59]. This further suggests that CCL20 may be a new target for the treatment of HCC.

6.3.2 Breast Cancer

Breast cancer is an important factor threatening women's health, and it is the most common type of cancers in women. Breast cancer can be classified into different molecular subtypes, namely luminal A, luminal B, HER2+, and basal-like, according to the expression of specific genes in cancer cells, i.e., estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and nucleoprotein antigen (ki67). Patients of some receptor-positive subtypes, such as luminal A, luminal B, and HER2+ subtypes, are commonly treated with strategies such as surgery, chemotherapy, radiotherapy, endocrine therapy, and targeted therapy. Breast cancer without expression of the three receptors ($ER^-PR^-HER2^-$), also known as triple-negative breast cancer (TNBC) and predominantly identified as basal-like subtype, is the most malignant subtype of breast cancer, prone to recurrence, metastasis, and drug resistance.

Many cytokines play a specific role in the occurrence and development of breast cancer. Some may promote the progress of breast cancer by regulating the proliferation and invasion of

cancer cells, others affect the growth of breast cancer by regulating the self-renewal of breast cancer stem cells (BCSC), and some cytokines regulate the cancer process by regulating the microenvironment of breast cancer.

The overall survival and metastasis-free survival of breast cancer patients with high CCL20 expression were decreased. Intraperitoneal injection of anti-CCL20 antibody can inhibit bone metastasis of osteolytic breast cancer cells in mice. CCL20 treatment significantly promoted cell invasion and MMP-2/9 secretion in basal-like TNBC cells, further confirming the role of CCL20 in bone metastasis of breast cancer [60]. Other studies have shown that overexpression of CCL20 promotes the proliferation and invasiveness of triple-negative breast cancer cells and accelerates the growth of tumors in xenograft models [61]. Moreover, CCL20 promotes the self-renewal of breast cancer stem cells and enhances the expansion ability of cancer stem cell population, suggesting that CCL20 may regulate the growth of breast cancer by affecting cancer stem cells. Interestingly, CCL20 can also enhance the chemotherapeutic resistance of breast cancer cells. It activates the NF-kappa B pathway through PKC and p38 MAPK, promotes the expression of ABCB1, the protein responsible for drug efflux, and then enables taxanes to be continuously pumped out of cells, thereby enhancing the drug resistance of cancer cells. In addition, paclitaxel chemotherapeutic drugs can significantly induce the production of CCL20, which also explains why cancer cells appear to be resistant to taxanes in the treatment of breast cancer: drugs induce the production of CCL20, and CCL20 promotes chemotherapeutic resistance [61]. Therefore, targeting CCL20 or the downstream pathway (such as NF-kappa B) activated by CCL20 can significantly improve the therapeutic effect of breast cancer patients, especially triple-negative breast cancer.

Communication between tumor cells and surrounding cells is helpful to drive the development of tumors. Chemokine CCL20 in breast cancer microenvironment regulates the physiology of healthy breast epithelial cells in areas adjacent to the tumor. Breast cells of primary cultures of mammary cells taken from normal peritumoral areas expressed CCR6 [62]. CCR6 activates various signal kinases that participates in the proliferation and migration of breast cells induced by CCL20. Moreover, different concentrations of CCL20 may have different effects on breast epithelial cells. CCL20 (10 ng/ml) induces cell migration while 15–25 ng/ml CCL20 promotes cell proliferation [62]. In mechanism, CCL20 may promote cell invasion by PKC-alpha that activated Src, which may also lead to activation of downstream Akt, JNK, and NF-kB pathways [62]. Furthermore, CCL20 modulated the epithelial-mesenchymal transition (EMT) of primarily cultured healthy breast epithelial cells in areas adjacent to the tumor through downregulating E-cadherin and ZO-1 and upregulating N-cadherin, vimentin, and Snail expressions [63].

6.4 Tumor Microenvironment Alteration Mediated by CCL20 Signaling

Infiltration of specific type of cells is one of the most important aspects of CCL20 impacts on the tumor microenvironment. After being recruited into the tumor niche, these particular cells may undergo diverse alterations of themselves when challenged with the specific stimulus from tumor cells, in turn they will reprogram the tumor microenvironment to benefit cancer cell survival, leading to poorer prognosis of patients in the majority of cases. The following is the representative of recruited cell type via CCL20 signaling reported in recent studies.

6.4.1 Dendritic Cells (DCs)

DCs are the central cells in the development of antitumor immune response, but the number and function of these cells will change in microenvironment of various cancers. CCL20 functions in the recruitment of inflammatory cells by binding to CCR6 expressed on DCs which are critically linked to initiation of immunity to antigens and recruited to certain sites through CCL20-CCR6

interaction [64]. In colon cancer, the binding of lipopolysaccharide (LPS) to TLR4 activated ERK and NF-kappa B signals in colon cancer cells and promoted the production of CCL20. The chemoattractant ability to immature DCs of colon cancer cells after treatment with LPS was increased significantly. If CCL20 was neutralized, this recruitment effect was significantly weakened, indicating that CCL20 mediated the recruitment of immature DCs by LPS-treated colon cancer cells [65]. In addition, plasmacytoid DCs (PDCs) from patients with melanoma expressed higher CCR6 levels than those from controls, and can migrate to CCL20 sites. CCR6-expressing PDCs were present in melanoma primary lesions, and CCL20 was also produced in melanoma tumors, indicating that PDCs may contribute to the diagnosis of melanoma, and CCL20 may be involved in the recruitment of PDCs from blood to tumors [66].The infiltration of DC cells mediated by CCL20 signaling into the microenvironment of colon cancer and melanoma reprogrammed the tumor microenvironment, influenced the characteristics of cancer cells, and ultimately affected the survival and prognosis of cancer patients.

6.4.2 Regulatory T Cells (Tregs)

Regulatory T cells (Tregs) are a subset of T cells that control autoimmune response. Dysfunction or abnormal expression of Tregs are closely correlated with the occurrence of autoimmune diseases [67–69]. Tregs are very common in cancer tissues and can inhibit effective antitumor immune response. However, the reasons for the increase in infiltrated Tregs in tumors and its impact on cancer progression are not well demonstrated. High expression of CCR6 on circulating Tregs and their directional migration to CCL20 accounted for the selective recruitment of tumor-infiltrating Tregs though CCL20-CCR6 axis, which was also confirmed by the correlation of expression and distribution between intratumoral CCL20 and tumor-infiltrating Tregs [70]. The number of Tregs infiltrated into tumors was associated with cirrhosis background and tumor differentiation, and was an independent prognostic factor for disease-free survival and overall survival [70]. Increasement of Tregs infiltration indicated poor prognosis in patients with hepatocellular carcinoma.

More interestingly, CCL20 signal influencing Tregs infiltration in the tumor microenvironment may originate from stromal macrophages besides tumor cells. In colorectal cancer (CRC), Tregs significantly infiltrated into tumors, and expressed CCR6 [71]. In the in vivo and in vitro experiments, colon cancer cells (CMT93) and macrophages can produce a large number of CCL20. In the colon cancer graft model, injecting recombinant mouse CCL20 protein into the tumor site could significantly promote tumor progression and increase Tregs recruitment [71]. In addition, conditional macrophage deletion can significantly reduce CCL20 level, suppress Tregs recruitment, and then inhibit the growth of colorectal cancer [71]. CCl20 signaling from tumor cells or stromal macrophages mediates Tregs infiltration into tumor microenvironment through binding to CCR6 expressed in Tregs, which leads to cancer progression and poor prognosis in HCC and CRC patients.

6.4.3 T Helper 17 Cells (Th17 Cells)

Th17 cells are a subset of T cells secreting interleukin 17 (IL-17), which plays an important role in autoimmune diseases and body defense response. Th17 cells can produce and secrete IL-17, IL-17F, IL-6, tumor necrosis factor α (TNF-α), etc. These cytokines can motivate, recruit, and activate neutrophils collectively, thus effectively mediating inflammation in tissues. The main physiological roles of Th17 cells are to promote host defense against infectious agents, including certain bacteria, fungi, viruses, and protozoa and to maintain barrier immunity at mucosal surfaces, such as the gut and lungs, as well as in the skin [72–75]. Th17 cells are associated with the development of multiple cancers and the prognosis of patients, such as colorectal

cancer, bladder carcinoma, breast cancer, and cervical cancer [76–80].

In cervical cancer, there was obvious aggregation of Th17 cells in the tumor tissue, and this kind of Th17 cells were an activated phenotype, accompanied by a significant increase in CCR6 expression [81]. The expression level of CCL20 in tumor tissue was significantly higher than that in normal control tissue, and there was a positive correlation between CCL20 and Th17 cells. In addition, CCL20 can effectively chemoattract Th17 cells in in vitro migration experiments [81]. In cervical squamous cell carcinoma, level of CCAAT/enhancer-binding protein beta (C/EBPbeta) in cervical fibroblast was increased after the stimuli of IL-6 produced by cervical cancer cells [80]. Subsequently, the instructed fibroblast produced high level of CCL20 which attracted CD4/IL-17/CCR6-positive cells in a CCL20/CCR6-dependent manner. This study demonstrated a novel mechanism through which cervical cancer cells reprogram the tumor microenvironment with the recruitment of Th17 cells via interplay between cancer cell and cervical fibroblast [80]. And the signaling axis IL-6/C/EBPbeta/CCL20 may be considered as the novel targets for new strategies for cervical squamous cancer treatment.

6.5 Synergistic Tumor Regulation of CCL20 with Other Cytokines in Tumor Microenvironment

There exists the cytokine network in tumor microenvironment to collectively regulate tumor progression [25, 82–85]. During the process that CCL20 functions in tumor microenvironment, other cytokines such as CXCL8 and IL-6 may participate in the modulation. Analysis based on 213 colorectal cancer patients who underwent surgery identified CCL20 and CXCL8 as the prognostic factors of CRC patients [86]. More interestingly, CCL20 and CXCL8 could collaboratively induce epithelial-mesenchymal transition (EMT) in CRC cells but CCL20 or CXCL8 alone could not. EMT was required to maintain cell migration and invasion of colorectal cancer. In addition, concomitant expression of CCL20 and CXCL8 was negatively correlated with E-cadherin expression in CRC tissues, confirming the synergistic role of the two in mediating EMT in CRC cells. Liver metastases more likely occurred in patients with coexpression of CCL20 and CXCL8. Besides, high expression of both CCL20 and CXCL8 predicted the poorest overall survival and disease-free survival of CRC patients, making the two designated as the independent high-risk factor for CRC [86]. However, this study did not clearly demonstrate the interaction manner between CCL20 and CXCL8 mechanistically.

6.6 Input and Output of CCL20 Signaling

The factors that can activate CCL20 signaling are diverse, and vary in different cancers. This may be due to differences in the characteristics of different types of cancer, such as the microenvironment in which they live. If we call these molecules that activate CCL20 pathways as input signals, then the downstream pathway activated by CCL20 is the output signal of CCL20 (Table 6.1). Unlike input, the output of CCL20 signaling is relatively fixed, and most studies have shown that CCL20 can activate the NF-κB pathway to promote different cancer progression (Table 6.1). It is worth mentioning that, a study revealed that the positive-feedback loop between CCL20 and NF-κB contributed to the promotion of taxane resistance in triple-negative breast cancer [61], indicating NF-κB as both input and output of CCL20 signaling (Fig. 6.1).

6.7 Conclusions and Perspectives

Although CCL20 receptor is only CCR6, its effects in TME are diverse due to the following reasons: firstly, as a cytokine, it acts in a

Table 6.1 Inputs (factors that promote CCL20 signaling) and outputs (pathways that CCL20 activates) of CCL20 signaling

	Molecules/ factors	Cancer type	Description	Ref.
Input	HCV	HCC	HCV induced CCL20 protein expression and secretion in hepatoma cells.	[87]
	NNK	Lung cancer	Tobacco carcinogen nicotine-derived nitrosaminoketone (NNK) induces production of chemokine CCL20 to promote lung cancer.	[88]
	CD105	Oral cancer	CCL20 expression participated in CD105-elicited cell motility in oral cancer cells.	[89]
	CXCR4	Prostate cancer	CXCR4 upregulates CCL20 mRNA and protein expression in prostate cancer cells.	[90]
	C/EBPβ	Cervical Cancer	Cervical cancer cells instructed primary cervical fibroblasts to produce high levels of CCL20 through C/EBPβ pathway.	[80]
	ENO1	Head and neck cancer	ENO1-mediated cell transformation partly via CCL20 upregulation.	[91]
	HuR	Breast cancer	HuR regulates CCL20 production by directly binding to the 3′-UTR of CCL20 mRNA and stabilizing it.	[60]
	lncRNA u50535	Colorectal cancer (CRC)	LncRNA-u50535 promotes CRC growth and metastasis via activation of CCL20 signaling.	[92]
	NF-κB	PDAC	CCL20 represents the strongest TRAIL inducible direct RelA target gene in PDAC cells.	[93]
	NF-κB	Ovarian cancer	The CCL20 promoter activity was regulated by NF-κB dependent pathways.	[94]
	NF-κB	TNBC	CCL20 production in breast cancer cells could be abolished by inhibition of p65 NF-κB.	[61]
	RBPJK	Breast cancer.	Silencing of RBPJK in MDA-MB-231 cells significantly decreased CCL20 mRNA while protein levels were not significantly altered.	[95]
	RANK/ RANKL	Endometrial cancer	CCL20 was dramatically enhanced in RANKL-treated RANK over-expressed EC cells.	[96]
	STAT3	HCC	Tumor cells transfected with STAT3 siRNA presented significantly lower CCL20 expression than tumor cells transfected with control siRNA.	[97]
	TLR4	Colon cancer	TLR4 ligation by LPS significantly promotes CT-26 colon cancer cells to produce chemokine CCL20 via activation of TLR4 signaling pathways.	[65]
Output	NF-κB	Thyroid cancer	CCL20/CCR6 interaction induced the activation of NF-κB, and stimulated the expression and secretion of MMP-3.	[98]
	AwKT, NF-κB	GCTB	CCL20 recruited mononuclear cells and induced osteoclastogenesis by overactivating the AKT and NF-κB signaling pathways.	[99]
	PKC/ p38-NF-κB	TNBC	CCL20 activated the axis of PKC/p38-NF-κB-ABCB1 in TNBC to promote taxane resistance.	[61]
	ABCB1	Ovarian cancer	CCL20 activated NF-κB signal pathway to promote ABCB1 expression.	[100]
	CrkL	Gastric cancer	CCL20 activated the expression of p-CrkL in MGC803 cells in a dose-dependent manner.	[101]
	p38	Laryngeal cancer	P38 was activated significantly when the cells were treated with CCL20 at 100 ng/ml.	[102]
	ERK1/2, PI3K	Lung cancer	CCL20 promoted lung cancer cells migration and proliferation in an autocrine manner via activation of ERK1/2-MAPK and PI3K pathways.	[103]

NNK nitrosaminoketone, *PDAC* pancreatic ductal adenocarcinoma, *TNBC* triple-negative breast cancer, *EC* endometrial cancer, *LPS* lipopolysaccharide, *GCTB* giant cell tumor of bone

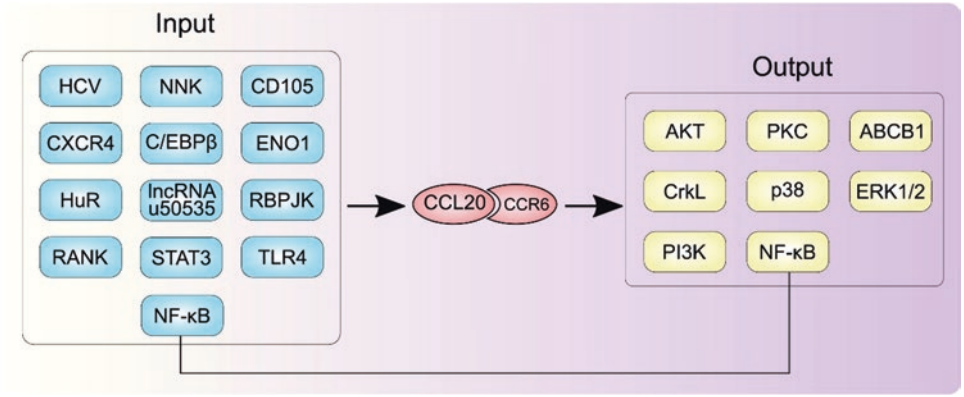

Fig. 6.1 Illustration of input and output of CCL20 signaling. It is the abridged general view of CCL20 signaling. Inputs originated from cancer cells or stromal cells in the tumor microenvironment or even from tumor matrix are the factors that activate CCL20 signaling. Outputs are the pathways that active CCL20 enhances. Noticeably and interestingly, NF-κB was proved to be both input and output of CCL20 signaling

variety of ways, including autocrine, paracrine, and endocrine; secondly, the input signals that can activate CCL20 in microenvironment are diverse, which makes it more complicated of CCL20 activation in tumor niche. In addition, by binding to CCR6, CCL20 recruits different types of cells to infiltrate into TME, which further complicates the signaling network in the microenvironment. These reasons, but not limited to them, make CCL20 regulate the malignant progression of multiple cancers.

However, so far, there are still some problems to be solved or worth exploring. Does CCL20 have potential clinical value as a target for cancer treatment? This requires more in vivo and even clinical experiments to illustrate. What is the importance of CCL20 signal in tumor microenvironment? Will its deletion cause great changes in tumor microenvironment and subsequent effects on tumor growth and even cancer treatment efficacy? CCL20-knockout mice model may be helpful to this problem. In addition, changes in the metabolic pattern of cancer cells cannot be ignored in the process of cancer development, so does CCL20 signal also have an impact on it? Therefore, more experiments and efforts are needed to bring us closer to the true face of CCL20 in tumor microenvironment.

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CCL21 Programs Immune Activity in Tumor Microenvironment

7

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Abstract

CCL21 promotes immune activity in the tumor microenvironment (TME) by colocalizing dendritic cells (DC) and T cells programing ectopic lymph node architectural structures that correlate with cancer prognosis. Innovative strategies to deliver CCL21 in cancer patients will reactivate the downregulated immune activity in the TME. Immune escape mechanisms are upregulated in the TME that promote tumor immune evasion. CCL21 combined with inhibition of dominant pathways of immune evasion will aid in the development of effective immunotherapy for cancer.

Keywords

CCL21 · Tumor microenvironment · T cells · Dendritic cells · Antigen-presenting cells · Activated T cells · Immune activity · Programmed cell death protein 1 (PD-1) · Immune checkpoint blockade · Immune suppression · Immunotherapy

7.1 Introduction

Lung cancer remains a challenging health problem with more than 1.1 million deaths worldwide annually. With current therapy, the long-term survival for the majority of lung cancer patients remains low, and thus new therapeutic strategies are needed. One such strategy would be to develop immune therapy for lung cancer. Immune approaches remain attractive because although surgery, chemotherapy, and radiotherapy alone or in combination produce response rates in all histological types of lung cancer, relapse is frequent. Strategies that harness the immune system to react against tumors can be integrated with existing forms of therapy for optimal responses toward this devastating disease. Antigen-presenting cell (APC) and T cell activities are reduced in the lung tumor microenvironment (TME). In this review we discuss our experience with efforts to restore host APC and T cell activities in lung cancer microenvironment by intratumoral administration

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of dendritic cells (DC) expressing the CCR7 receptor ligand CCL21 (secondary lymphoid chemokine, SLC). Based on the results demonstrating that CCL21 is an effective anticancer agent in the preclinical lung tumor model systems, a phase I clinical trial was initiated using intratumoral injection of CCL21 gene-modified autologous DC in lung cancer. Results from the trial reveal tolerability, immune enhancement, and tumor shrinkage via this approach. Although CCL21 induces immune responses, an immune-tolerant TME promotes immune evasion. Combining CCL21 and downregulating immune evasion pathways will program durable antitumor immune responses. One immune escape mechanism is dependent on immune inhibitory molecules that are upregulated on T cells in tumors causing a downregulation of antitumor activity. The programmed cell death protein 1 (PD-1, also known as CD279) is an inhibitory receptor that regulates immune responses. The PD-1 receptor interaction with the PD-L1 and PD-L2 ligands deliver inhibitory signals that regulate the balance between T cell activation and tolerance. We recently completed the first-in-man phase I clinical trial with CCL21 in which patients with advanced stage NSCLC received intratumoral administration of autologous DC overexpressing CCL21 (CCL21-DC). We observed CD8 T cell infiltration of the tumor and induction of systemic immune responses as evidenced by PBL IFNγ production in response to autologous tumor antigens [1]. However, our observation of T cell immune regulatory checkpoint expression in the TME of these patients suggests that tumor-mediated impairment of T cell function may be forestalling a more robust CCL21-mediated antitumor response. Combining CCL21 with PD-1 inhibition can augment host antitumor immunity and capture more NSCLC patients to be responsive to PD-1 checkpoint blockade therapy.

7.2 Requirements for Effective Antitumor Immune Activity

One of the challenges in developing immunotherapy for cancer is enlisting the host response to recognize poorly immunogenic tumors. Effective antitumor responses require antigen-presenting cells (APC), lymphocytes, and natural killer (NK) effectors. Although lung cancer cells express tumor antigens, limited expression of MHC antigens, defective transporters associated with antigen processing, and lack of costimulatory molecules make them ineffective APC. Both APC and T cell activities are reduced in lung cancer [2, 3], and intratumoral infiltration by relatively high numbers of activated T lymphocytes [4, 5] and APC [6] leads to better prognosis in lung cancer patients. Utilizing preclinical models of lung cancer, we are evaluating intratumoral delivery of immune-potentiating CCL21 chemokine via DC and stromal cell-based approaches for effective recruitment and activation of APC and T cells for the promotion of antitumor activity in lung cancer. The preclinical findings demonstrate that effective anticancer immunity can be achieved by CCL21-mediated recruitment of professional host APC for tumor antigen presentation to promote specific T cell activation [7–10]. Results from a phase I trial of intratumoral administration of autologous DC expressing CCL21 to lung cancer patients meets the objectives of the study in terms of safety and the induction of antitumor immune responses [1].

7.3 Rationale for CCL21 Chemokine in Cancer Therapy

Chemokines, a group of homologous, yet functionally divergent proteins, directly mediate leukocyte migration and activation and play a role in regulating angiogenesis. They also function in maintaining immune homeostasis and secondary lymphoid organ architecture. CCL21 has been identified as a lymphoid chemokine that is predominantly and constitutively expressed by high endothelial venules in lymph nodes and Peyer's patches, lymphatic vessels and stromal cells in spleen and appendix [11]. CCL21 binds to the chemokine receptor CCR7 and is a chemoattractant for mature DC and naive and memory T cells. Acting through the G-protein-coupled CCR7 transmembrane receptor, CCL21 mediates

the recruitment and colocalization of naive lymphocytes and antigen stimulated DC into T cell zones of secondary lymphoid organs, facilitating T cell activation. T cell activation in vivo occurs in a lymphoid milieu that presents chemotactic and T cell receptor signals concurrently. The T cell zone chemokines such as CCL21 are bound to the surface of lymph node DC. Contact with antigen-presenting cells bearing CCL21 chemokine co-stimulates T cells by a two-step contact mechanism. T cells initially form an antigen-independent "tethered" adhesion on CCL21-bearing antigen-presenting cells. The formation of these tethers supersedes T cell receptor signaling and immunological synapse formation. However, chemokine-tethered T cells are hyperresponsive to subsequent contacts with antigen-presenting cells. Thus, T cells are co-stimulated "in trans" and sequentially after initial engagement with their CCL21-rich environment [12]. This chemokine, along with CCL19, is required for normal lymphoid tissue organization that is ultimately essential for effective T cell-DC interactions. DC are uniquely potent APCs involved in the initiation of immune responses. Serving as immune system sentinels, DC are responsible for Ag acquisition in the periphery and subsequent transport to T cell areas in lymphoid organs where they prime specific immune responses. Thus, chemokines that attract both DC and lymphocyte effectors into the tumor can serve as potent agents in immunotherapy. In addition to inducing chemotactic migration, CCL21 co-stimulates expansion of CD4+ and CD8+ T cells and induces Th1 polarization. The immune suppressor cell population, CD4+CD25+ regulatory T cells are hyporesponsive to CCL21-induced migration and unresponsive to CCL21 co-stimulation [13]. These functions of CCL21 to both attract naïve T cells and co-stimulate their proliferation, differentiation, and activation suggest that CCL21 is a pivotal molecule for priming T cell responses and has therapeutic implications for local delivery of CCL21. The antitumor effectors NK and NKT cell subsets also express the CCR7 receptor and are chemoattracted by CCL21. The recruitment of NK and NKT cells is advantageous because these effectors can recognize tumor targets in the absence of MHC expression. The use of chemokines to attract DC, lymphocyte, NK, and NKT effectors into tumors can serve as an effective antitumor strategy. In addition, CCL21 has potent angiostatic effects, thus adding further support for its use in cancer therapy. CCL21 priority ranking is 13 among the list of 20 National Cancer Institute ranked biological agents with high potential for use in cancer therapy. Generation of an antitumor immune response requires the coordinate interaction of NK, T, and DC effectors. There is a paucity of these effectors in the tumor. One regimen to initiate antitumor responses is through the use of chemokines that induce both efficient recruitment and strong activation of effector cells in the tumor mass. The rationale for the use of CCL21 for immune therapy against solid tumors is that CCL21 sculpts host immune responses by recruiting and colocalizing NK, DC, and T cell effectors to mediate potent antitumor activity.

7.4 CCL21 Induction of Antitumor Immune Responses

The development of intratumoral therapies to effectively augment local and systemic antitumor immunity in lung cancer can lead to a paradigm shift in the current forms of therapy. In preclinical model systems, intratumoral administration of DC led to both local and systemic antitumor responses [14]. This form of therapy can be augmented by utilizing intratumoral administration of genetically modified DC overexpressing certain cytokine genes [15]. Congruent with this overall concept, the intratumoral administration of recombinant CCL21 mediated T cell-dependent antitumor responses [7]. In immune competent mice, intratumoral CCL21 injection led to a significant increase in CD4 and CD8 T lymphocytes and DC infiltrating both the tumor and draining lymph nodes. Studies performed in CD4 and CD8 T cell knockout mice revealed a direct therapeutic requirement for both CD4 and CD8 T cell subsets for CCL21-mediated tumor regression. These findings were the first demon-

stration of effective antitumor responses mediated by CCL21 [7]. CCL21-mediated antitumor responses exhibited an increased influx of CD4 and CD8 T cell subsets as well as DC at the tumor sites. Accompanying this cell infiltrates were increases in IFNγ, MIG/CXCL9, IP-10/CXCL10, GM-CSF, and IL-12, but a concomitant decrease in the immunosuppressive molecules PGE-2 and TGFβ. Lymphocytes from CCL21-treated tumor-bearing mice demonstrated enhanced specific responses against autologous tumors suggesting the generation of systemic immune responses [7, 9]. The importance of IFNγ, MIG/CXCL9, and IP-10/CXCL10 in CCL21 therapy was assessed. In vivo depletion of IP-10/CXCL10, MIG/CXCL9, or IFNγ indicates that the full potency of CCL19 or CCL21-mediated antitumor responses requires the induction of IFNγ, MIG/CXCL9, and IP-10/CXCL10 in concert in this model. Neutralization of any one of these cytokines led to a decrease in the frequency of CXCR3+ve T cells and CD11c+ve DC in the tumor [9, 16]. Based on these results, experiments were performed to evaluate the tumorigenicity of CCL21 gene-modified murine lung cancer cells. In all three tumor models, subcutaneous implantation of retroviral-mediated CCL21 gene-modified lung cancer cells led to T cell-mediated tumor eradication. Following our initial description of the antitumor activities of CCL21, several groups have reported that CCL21 has potent antitumor properties in a variety of model systems [17–21]. In all models, CCL21 demonstrated potent regression of tumors, which was shown to be dependent on host T cell immunity. All these studies reaffirmed the antitumor efficacy of CCL21, further supporting the rationale to proceed with clinical investigations of this chemokine.

7.5 CCL21 Gene-Modified DC Therapy for Lung Cancer

Our studies demonstrate that intratumoral administration of recombinant CCL21 reduced tumor burden in murine lung cancer models [7]. However the antitumor activity induced by recombinant CCL21 required high and frequent dosing because proteins administered intratumorally are not retained locally for prolonged periods. Although these studies delineated the role of CCL21 as an effective antitumor agent, frequent high dose intratumoral administration in the lung is invasive and clinically limiting with the potential of unnecessary systemic toxicity. Based on the limitations of this approach, we examined the use of DC for intratumoral CCL21 delivery [9, 10]. The intratumoral approach utilizes in situ tumor as a source of antigen. In contrast to immunization with purified peptide Ag, autologous tumor has the capacity to provide the DC administered in the tumor site access to the entire repertoire of available antigens in situ. This increases the likelihood of a response and reduces the potential for tumor resistance due to phenotypic modulation. To achieve in situ tumor antigen uptake and presentation, intratumoral administration of *ex vivo*-generated CCL21 gene-modified murine bone marrow-derived DC was utilized in a subcutaneous murine lung cancer model [9]. To determine if a cell type other than DC expressing CCL21 could also induce tumor reduction in this model, fibroblast cells were also evaluated as a delivery vehicle. In addition to fibroblast cells' ability to process and present antigens, the use of fibroblasts represents a promising treatment approach for lung cancer. These cells contribute to the formation of tumor-associated stroma [22], and the tumor microenvironment preferentially promotes their engraftment as compared with other tissues [23], making them an ideal system for tumor-selective delivery. Our data also support that reprogramming the TME with fibroblasts modified to express CCL21 alters the inflammatory infiltrates in the TME and promotes antitumor activity. The advantages of using transduced fibroblast cells for paracrine secretion of CCL21 are that fibroblasts (1) produce physiologically relevant levels of CCL21 after transduction, (2) are readily available for culture and expansion, (3) provide a platform for the development of CCL21-based antitumor strategies, (4) can process and present antigens to T cells, and (5) potentiate the activities of immune and innate

effectors in the TME. For translation to lung cancer patients we have the option of utilizing bone marrow-derived MHC-matched GMP-grade genetically modified donor stromal cells from a tissue bank that will circumvent autologous DC preparation, minimize batch-to-batch variability, and allow for comparability and standardization. DC or fibroblasts were transduced with an adenoviral vector expressing secondary lymphoid tissue chemokine (CCL21/SLC) to attract mature host DC and activated T cells in the tumor site. Established palpable tumors were treated with intratumoral DC-AdCCL21, Fib-AdCCL21, or controls. Intratumoral therapy with 10^6 DC-AdCCL21 (7–10 ng/ml/10^6 cells/24 h of CCL21) at weekly intervals for 3 weeks showed tumor eradication in 60% of the mice whereas therapy with 10^6 fib-AdCCL21 had complete resolution of tumors in 25% of mice because an optimum dose of Fib-AdCCL21 may not have been utilized for these studies [9]. Further investigation is required to determine the dose of fib-AdCCL21 that will perform as effectively as CCL21 gene-modified DC. In contrast, only 12% of the mice treated with unmodified or control vector modified DC (DCAdCV) showed complete tumor eradication. Intratumoral injection of AdCCL21 also led to tumor reduction, though at the dose tested it was not as effective as DC-AdCCL21 [9]. In addition, circulating neutralizing antibodies against adenovirus in patients will be a limiting factor for the use of adenovirus-based vectors to deliver CCL21. In the tumor model tested, intratumoral DC-AdCCL21 administration led to increases in the CD4+, CD8+, and CD3+CXCR3+ T cells as well as DC expressing CD11c+ and DEC205+ but decreases in CD4+CD25+ T regulatory cells infiltrating the tumors. Accompanying the tumor cellular infiltrates were enhanced elaboration of GM-CSF, IFNγ, MIG/CXCL9, IP-10/CXCL10, and IL-12 but decreases in the immunosuppressive mediators TGFβ and PGE2. DC-AdCCL21-treated tumor-bearing mice showed enhanced frequency of tumor-specific T lymphocytes secreting IFNγ and induced protective immunity [9]. The reduction in tumor growth may be explained by an increase in the frequency of activated T effector cell-mediated tumor apoptosis and/or T IFNγ-mediated anti-angiogenesis. In vivo depletion of IP-10/CXCL10, MIG/CXCL9, or IFNγ significantly reduced the antitumor efficacy of DC-AdCCL21 [9]. Based on these observations, we determined the antitumor effects of DC-AdCCL21 in a clinically relevant model of lung cancer. We utilized transgenic mice in which the adenocarcinomas develop in an organ-specific manner and have an average life span of 4 months. DC AdCCL21 (10^6 cells) or controls (diluent, DC (10^6 cells) and DC–AdCV (10^6 cells), AdCV (10^6 pfu) and AdCCL21 (10^6 pfu)) were administered once into the lungs of 3-month-old transgenic mice. When evaluated at 4 months of age, there was reduced tumor burden in DC-AdCCL21-treated CC-10 mice compared with the control groups. Median survival was 18 ± 2 weeks for all control-treated mice. In contrast, mice treated with DC-AdCCL21 had a median survival of 24 ± 1 weeks ($p < 0.01$ for DC-AdCCL21 compared to controls). In addition to marked tumor reduction, histological examination revealed areas of distinct mononuclear infiltration in remaining tumor [10].

7.6 Clinical Translation of DC-AdCCL21 Therapy to Lung Cancer Patients

Based on the results in the preclinical model systems, a clinical trial was initiated using intratumoral injection of CCL21 gene-modified autologous DC in lung cancer. The intratumoral route of DC administration is used to activate specific immune responses within the TME and, in addition, to generate systemic immunity. Several studies reveal [14, 24] that intratumoral DC administration may be particularly effective as an antitumor strategy. Lung cancer patients have decreased numbers of circulating competent DC; thus, injecting DC within the lung tumor site may be a particularly effective approach. A correlation exists between the number of tumor-infiltrating DC and survival in cancer patients. In fact, there is a relationship between tumor-infiltrating DC aggregation and

apoptosis in situ in human NSCLC. This is consistent with recent studies indicating that attraction and activation of DC at the site of tumor elicits potent antitumor immunity [25]. Dieu-Nosjean et al. [6] have identified ectopic lymph node or tertiary lymphoid structures within human NSCLC specimens and demonstrated a correlation of their cellular content with clinical outcome. These structures have been referred to as tumor-induced bronchus-associated lymphoid tissue, which are follicle-like and contain germinal centers, similar to those in secondary lymphoid follicles of lymph nodes. The density of DC-Lamp, mature DC within these structures is a predictor of long-term survival in lung cancer patients [6]. These findings reveal that tumor-induced bronchus-associated lymphoid tissues have clinical relevance and participate in the host's antitumor immune response, and they are consistent with previously reported preclinical and clinical data [26, 27]. For example, in murine tumor models, Mulé reported that DC genetically modified to secrete CCL21 can produce lymphoid cell aggregates and, importantly, prime naive T cells extranodally within a tumor mass, resulting in the generation of tumor-specific T cells and subsequent tumor regression [17, 27]. Thus, the intratumoral approach may achieve tumor antigen presentation by using the tumor as an in vivo source of antigens for DC. In contrast to immunization with purified peptide antigen(s), autologous tumor has the capacity to provide the activated DC administered at the tumor site access to the entire repertoire of available antigens in situ. This increases the likelihood of a response and reduces the potential for tumor resistance because of phenotypic modulation. CCL21 is distinctly advantageous because of its capacity to elicit a type 1 cytokine response in vivo that promotes antitumor activity. Intratumoral infiltration of T lymphocytes and DC in lung cancer has been shown to be associated with a better patient outcome. In accord with this observation in lung cancer, a recent study demonstrates that the presence of lymph node (LN)-like vasculature in tumors, characterized by expression of peripheral node addressin and chemokine CCL21, is correlated with T cell infiltration and positive prognosis in breast cancer and melanoma patients [28]. The authors further demonstrated that LN-like vasculature is present in murine models of melanoma and lung carcinoma. LN-like vasculature enables infiltration by naive T cells that significantly delay tumor outgrowth after intratumoral activation. The mechanisms contributing to the development of this vasculature is attributed to effector CD8 T cells and NK cells that secrete LTα3 and IFNγ. LN-like vasculature is also associated with organized aggregates of B lymphocytes and gp38(+) fibroblasts, which resemble tertiary lymphoid organs that develop in models of chronic inflammation. The results of this study establish that LN-like vasculature as both a consequence of and key contributor to antitumor immunity [28]. On the basis of preclinical results, a phase I clinical evaluation was initiated at University of California Los Angeles (in collaboration with the National Cancer Institute—Rapid Access to Intervention Development program now NCI Experimental Therapeutics Program) in patients with advanced stage NSCLC. The safety and clinical activities of the intratumoral administration of autologous DC transduced with a replication deficient adenoviral vector to express CCL21 in patients with pathologically confirmed and radiographically measurable NSCLC (Stage IIIB/IV) who have tumor accessible by CT-guided or bronchoscopic intervention and are refractory to standard therapy were selected. A GMP-grade AdCCL21 replication-deficient virus [29] was made available through the RAID program to conduct the Phase I clinical trial. Human DCs transduced with advenovirus-CCL21 produce CCL21 to attract T cells and DCs. Results demonstrate tumor-specific systemic immune responses as assessed by the IFNγ T cell ELISPOT. Multiplex assessment of plasma cytokines before and after therapy in these patients revealed induction of IL-2, γ, IFN IL-12, and CXCL10. Immunohistochemistry of post-tumor biopsies revealed an influx of CD4-expressing tumor-infiltrating lymphocytes.

Results indicate that vaccination is safe with no associated adverse reactions at the DC-AdCCL21 (1×10^6, 5×10^6, 1×10^7, or 3×10^7 DC-AdCCL21 cells/injection) doses administered (days 0 and 7) and antitumor immune responses can be elicited particularly in higher doses.

7.7 Polymer-Based CCL21 Delivery

The science of biomaterial engineering for drug delivery has evolved considerably for the past 30 years. Novel technology allows to design functional, biocompatible, and biodegradable polymer vehicles, such as poly-ε-caprolactone (PCL), poly (lactide-co-glycolide) (PLG), as well as alginate and fibrin hydrogel, for molecular and cellular delivery in cancer immunotherapy [30]. Three-dimensional porous polymer scaffolds exhibit great ability to deliver cytokine molecules and immune cells with spatiotemporal specificity, to promote cell-cell interaction in matrix, and to direct cell function [30]. This ability forms the rationale for polymer-based CCL21 cancer immunotherapy for programming host immune cells in vivo. These materials can be further integrated with other anticancer treatments in the design of next-generation therapy against cancer [31]. PCL/PLCL copolymer loaded with DC-CCL21 or chemotherapy drug cisplatin has been tested in an animal model of head and neck squamous cell carcinoma (HNSCC) to prevent cancer recurrence [32, 33]. HNSCC is difficult to resect completely by surgery due to complicated context and therefore exhibits high recurrence rate in the patients [34]. A drug delivery platform with spatiotemporal specificity is in demand for anti-recurrence therapy. In order to accomplish these requirements, a polymer platform was made from a mixture of a ratio of 70:30 of PCL to PLCL with relevant amount of CCL21 and/or cisplatin and was spread on a glass to form a thin sheet. The final product is a flexible sheet that exhibits nice drug release kinetics and can adhere to the surgical resected tissue contours [32]. In the initial animal study, cisplatin-loaded PCL/PLCL polymer was applied intraoperatively to the surgical bed after partial tumor resection, replicating the difficult situation seen in patients. The cisplatin-secreting polymer effectively reduced tumors by over 16-fold as compared to control plain polymer and intratumoral cisplatin injection groups. When combined with radiation, polymer therapy led to a statistically significant lower tumor weight compared to the radiation alone group and the control group [32]. Based on the above data, the PCL/PLCL scaffold was later tested for antitumor efficacy of DC-CCL21 therapy. In order to improve DC culture condition for immunotherapy, a thin layer of fibrin hydrogel with 10^6 DCs seated inside was added to the surface of PCL/PLCL polymer [33]. The component of hydrogel and polymer was optimized for the maximum production of bioactive CCL21. After implantation to the partially resected tumor, the gradient of local CCL21 that resulted from its sustained and localized release led to the recruitment of CD4+ T cells and CD11c+ DCs into the tumor, while tumor-infiltrating Treg cells were decreased. Overall, DC-CCL21 polymer treatment significantly reduced tumor burden, compared to control DC group or recombinant CCL21 injection group [33]. Currently, antitumor efficacy of polymer loaded with recombinant CCL21 is being further evaluated with combination of cisplatin chemotherapy, immune checkpoint blockade, and radiation therapy. In addition to cytokines and immune cells, tumor-associated antigen can also be loaded in polymer to activate DCs. Subcutaneous implantation of PLG polymer loaded with cytokine GM-SCF, TLR agonist CpG, and tumor lysate as antigen led to host DC recruitment, activation, and subsequent homing to lymph nodes [35]. This vaccine induced 90% prophylactic tumor protection and therapeutic protection. The polymer scaffold also displayed long-term activity for months postimplantation, which is superior to all soluble administration methods to date [35]. Other biomaterials, such as vault nanoparticles, were investigated for intratumoral CCL21 delivery. In a well-characterized Lewis lung cancer model, CCL21-vault nanoparticle system showed effective antitumor efficacy.

A single intratumoral injection of CCL21-vault nanoparticles was able to recruit antitumor effectors that induced potent antitumor activity and inhibit tumor growth [36]. The nanoparticle system can be further designed for target delivery and specific payloads to prime the immune system.

7.8 Diagnostics and Prognostic Monitoring of CCL21 and Effects

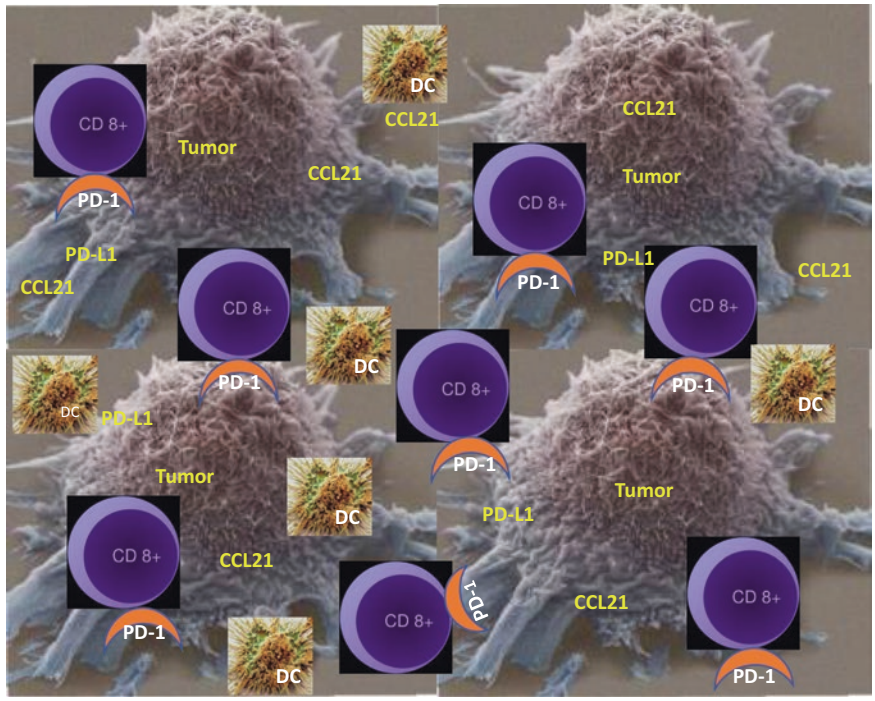
Diagnostic tests for CCL21 expression and protein concentrations in samples are performed by RT-PCR and ELISA. Tissue expression of CCL21 is assessed by immunocytochemistry and flow cytometry. Based on the preclinical data, high levels of CCL21 expression in tumors may be indicative of immune reactivity and serve as a prognostic marker for patient survival. Immune effects of CCL21 is monitored by antigen-specific IFN-γ T lymphocyte ELISPOTS, ELISA, or RT-PCR for Th1 cytokines and immunocytochemistry for T lymphocytes, NK and DC effector cell infiltrates. CCL21-mediated T cell activation can be monitored by immunocytochemistry for perforin and granzyme B-secreted activated T cells (Fig. 7.1).

7.9 Therapeutics

CCL21 is being developed as an anticancer therapeutic agent. The phase I clinical trials were in lung cancer and melanoma, but as the preclinical data warrants in other tumor models, this form of therapy may be extended to include other solid cancers. There is a strong rationale to combine CCL21 with immune checkpoint blockade therapy to increase T cell infiltrates in the tumor of patients who have minimal response to immune checkpoint blockade therapy. Recent groundbreaking studies in lung cancer immunotherapy reveal robust antitumor activity and durable responses in previously treated patients with progressive locally advanced or metastatic NSCLC. Immune inhibitory molecules are upregulated on T cells in tumors causing a downregulation of antitumor activity. The programmed cell death protein 1 (PD-1; also known as CD279) is an inhibitory receptor that regulates immune responses. The PD-1 receptor interaction with the PD-L1 and PD-L2 ligands delivers inhibitory signals that regulate the balance between T cell activation and tolerance. Recent studies reveal responses in approximately 20% of NSCLC patients treated with inhibitors of the PD-1 checkpoint. This includes robust and durable responses in previously treated patients with progressive locally advanced or metastatic NSCLC [37–42]. Studies in NSCLC and melanoma patient-derived tumor specimens reveal that responses to checkpoint blockade rely on tumor infiltration of activated T effector cells [38–41, 43]. It has been suggested that among patients who are nonresponsive or respond poorly to checkpoint blockade immunotherapies, there will be individuals who lack preexisting antitumor T cell responses [44]. This group appears to be comprised of patients who have absent or very limited immune responsiveness prior to initiation of therapy, and thus have limited CD8 T cell infiltration of the tumor and/or PD-L1 expression by tumor or TME. This situation appears to occur in approximately 50–60% of NSCLC cases [45]. Thus, it has been suggested that this deficit could be addressed with regimens that increase T cell infiltration combined with checkpoint inhibitors. Congruent with this concept, in a recent study, CCL21 enhanced the antitumor activity of PD-1 in a murine model of lung cancer [46].

7.10 Future Prospects

The results of the phase I studies in lung cancer and melanoma are promising. CCL21 is important in the formation of tertiary lymphoid structures and their presence in tumors is associated with favorable immune responses. Immunogenic tumors, with immune response positive gene signatures and/or increased TIL, have a better prognosis. Based on the findings on CCL21, it is



Control **CCL21-DC Peptide Vaccine + Anti-PD-1**

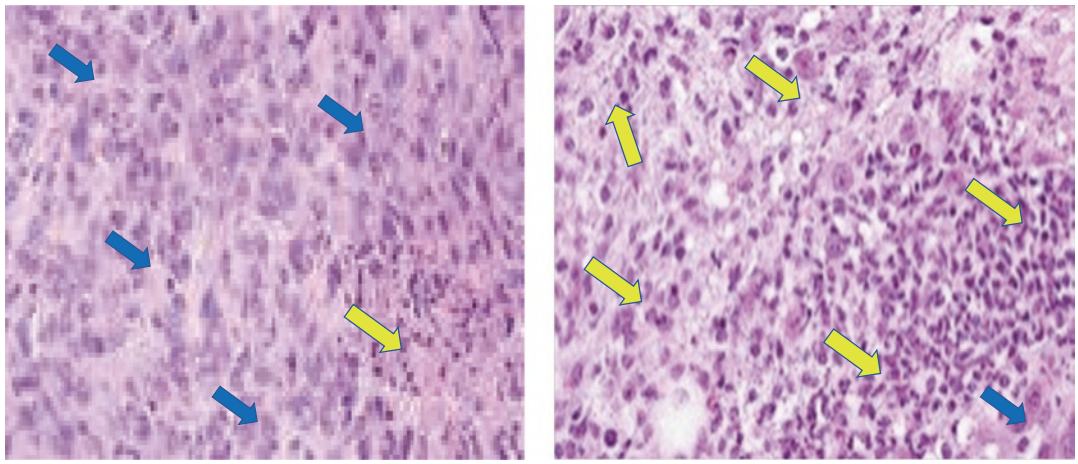


Fig. 7.1 (**a**–**d**) CCL21 programs PD-1 blockade antitumor activity. (**a**) CCL21-based strategies increase CD8 T and dendritic cells (DC) in the TME. PD-1 expression is increased on recruited T cells that makes them tolerant to the tumor. CCL21 combined with PD-1 blockade induces durable antitumor immunity leading to tumor regression/eradication. (**b**) CCL21-DC peptide vaccine combined with PD-1 blockade enhances activated T cells in the TME resulting in lung tumor destruction. Blue arrows indicate tumor and yellow immune infiltrates. *Magnification 60× objective.* (**c**) CCL21-DC peptide vaccine combined with PD-1 blockade increases CD3 T cells in the TME. Brown indicates CD3 T cells staining by immunohistochemistry. *Magnification 60× objective.* (**d**) CCL21-DC peptide vaccine combined with PD-1 blockade enhances activated T cells that secrete granzyme B in the TME resulting in lung tumor destruction. Brown indicates granzyme B staining by immunohistochemistry. *Magnification 60× objective*

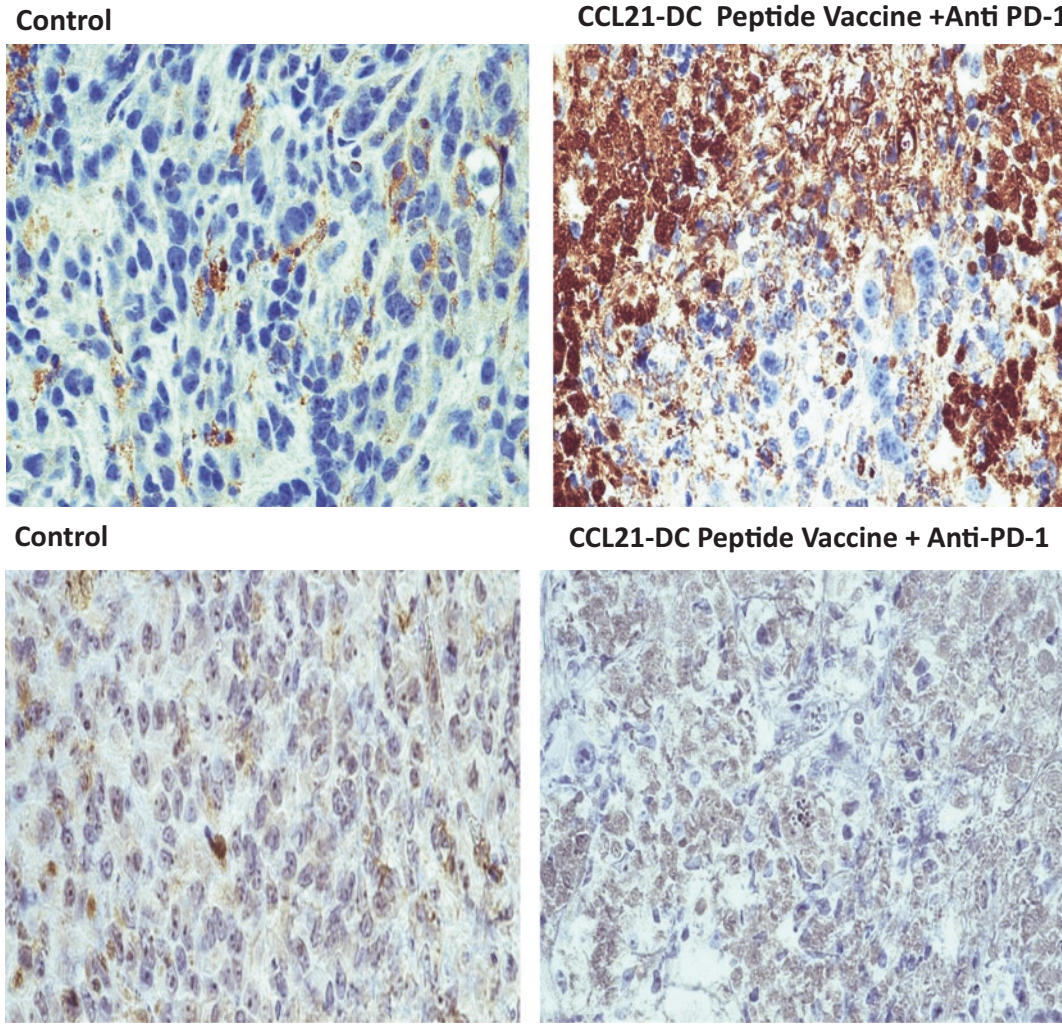


Fig. 7.1 (continued)

anticipated that the rational combination with immune checkpoint blockade therapy will improve the antitumor benefit of this chemokine in a broad range of solid tumors with low TIL frequency. Future studies could assess the combined efficacy of CCL21-based regimens with immune checkpoint blockade therapy in various solid tumors. CCL21-based therapeutic vaccination approaches will prove beneficial for tumors that are not accessible to intratumoral administration of CCL21 or that require multiple dosing. CCL21 in combination with PD-1 inhibition will capture more NSCLC patients that are not responsive to PD-1 blockade monotherapy. Furthermore, material and nanoparticle engineering provide several attractive strategies to design more potent CCL21 immunotherapy for cancer.

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CCL22 Signaling in the Tumor Environment

8

Natascha Röhrle, Max M. L. Knott, and David Anz

Abstract

T cell-mediated elimination of malignant cells is one cornerstone of endogenous and therapeutically induced antitumor immunity. Tumors exploit numerous regulatory mechanisms to suppress T cell immunity. Regulatory T cells (T regs) play a crucial role in this process due to their ability to inhibit antitumoral immune responses and they are known to accumulate in various cancer entities. The chemokine CCL22, predominately produced by dendritic cells (DCs), regulates T reg migration via binding to its receptor CCR4. CCL22 controls T cell immunity, both by recruiting T regs to the tumor tissue and by promoting the formation of DC-T reg contacts in the lymph node. Here, we review the current knowledge on the role of CCL22 in cancer immunity. After revising the principal mechanisms of CCL22-induced immune suppression, we address the factors leading to CCL22 expression and ways of targeting this chemokine therapeutically. Therapeutic interventions to the CCL22-CCR4 axis may represent a promising strategy in cancer immunotherapy.

Keywords

CCL22 · CCR4 · Regulatory T cells · Dendritic cells · Chemokines · T cell immunity · Immune checkpoint · Immunosuppression · Tumor environment · Lymph node · Antitumor immunity · Immune escape · Immunotherapy · IL-1 α · Prostaglandin E_2

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8.1 Introduction

Chemokines are a large group of structurally related cytokines that are commonly known for their role in directed migration, homing, and retention of immune cells [1, 2]. In the context of tumor immunity, chemokines play a crucial role in shaping the tumor microenvironment. They are secreted by both tumor cells and other components of the tumor microenvironment, such as

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immune cells and stromal cells. Subsequently, distinct immune cell populations are recruited into the tumor and draining lymph nodes in a spatiotemporal and well-orchestrated manner. Hence, chemokines can regulate tumor immunity by determining the composition of tumor-infiltrating immune cells [3–6]. The chemokine CCL22 is not only constitutively expressed in immune homeostasis but also inducible in inflammatory conditions [7] and cancer [8]. The interaction of CCL22 with its receptor CCR4 creates and maintains an immunosuppressive tumor environment. Thus, targeting the CCL22-CCR4 axis represents an interesting strategy for cancer immunotherapy. However, a profound understanding of the regulation of CCL22-mediated pathways is indispensable to make them accessible to therapeutic interventions. In this chapter, we will focus on the differential regulation of CCL22 in homeostasis and cancer, the role of CCL22 in shaping the tumor environment, and ways to exploit such pathways for novel therapeutic approaches in cancer.

8.2 The Chemokine CCL22 in Homeostasis and Immune Activation

The chemokine CCL22 was first discovered in humans by Godiska and colleagues in 1997 [9]. The murine equivalent was identified shortly thereafter [10] sharing 64% identity and 86% similarity with its human counterpart [11]. Initially, CCL22 was found to be primarily expressed and secreted by macrophages, hence its designated name macrophage-derived chemokine (MDC) [9]. Later reports identified DCs as predominant producers of CCL22 in homeostatic conditions [12]. In the setting of cancer, both DCs and macrophages were found to be the main producers of CCL22 [13]. Moreover, different DC subtypes express diverging levels of CCL22. In vivo, immature DCs in lymph nodes express high levels of CCL22 [12], while immature DCs in other tissues such as the skin only express moderate levels [14]. CCL22 expression by DCs is also dependent on their activation status: in vitro, immature DCs secrete only minimal amounts of CCL22, while they strongly upregulate CCL22 during maturation [14, 15]. Macrophages, T cells, and B cells can also express CCL22 upon activation, albeit at lower levels [16, 17]. During inflammation, stimuli such as LPS, IL-4, IL-13, and T cell receptor (TCR) stimulation are well-described inducers of CCL22 [18]. Furthermore, CCL22 has been implicated in various inflammatory conditions—for instance in allergy, where elevated CCL22 levels mediate the migration of CCR4-expressing T helper 2 cells (Th2), which maintain the allergic process [19]. So far, CCR4 is the only known receptor for CCL22. Importantly, in the absence of inflammation, CCR4 is highly and almost exclusively expressed on T regs and facilitates their migration in vitro and in vivo [18, 19]. Overall, CCL22 is both constitutively expressed under homeostatic conditions and inducible upon inflammation. In both mice and humans, CCL22 is found at very high levels in many lymphoid organs, such as lymph nodes and thymus [9, 10, 14, 20]. While the underlying biology of CCL22 regulation in inflammation and cancer is well studied, the physiological significance of this high CCL22 expression in homeostasis has only recently been unraveled. Data from our group and others support a role of CCL22 as a checkpoint within the immune system to preserve the delicate balance between immune activation and tolerance. Mechanistically, CCL22 is produced by DCs and facilitates their interaction with T regs via their receptor CCR4 in secondary lymphoid organs. In the setting of CCL22 deficiency, DC-T reg contacts are significantly reduced, leading to enhanced T cell immune responses, including augmented vaccination efficacy due to higher numbers of antigen-specific T cells, excessive inflammation, as well as reduced tumor growth and prolonged survival upon tumor vaccination [20] (Fig. 8.1). Consistent with these findings, Vitali and colleagues identified a crucial role for CCL22 in retaining induced T regs within

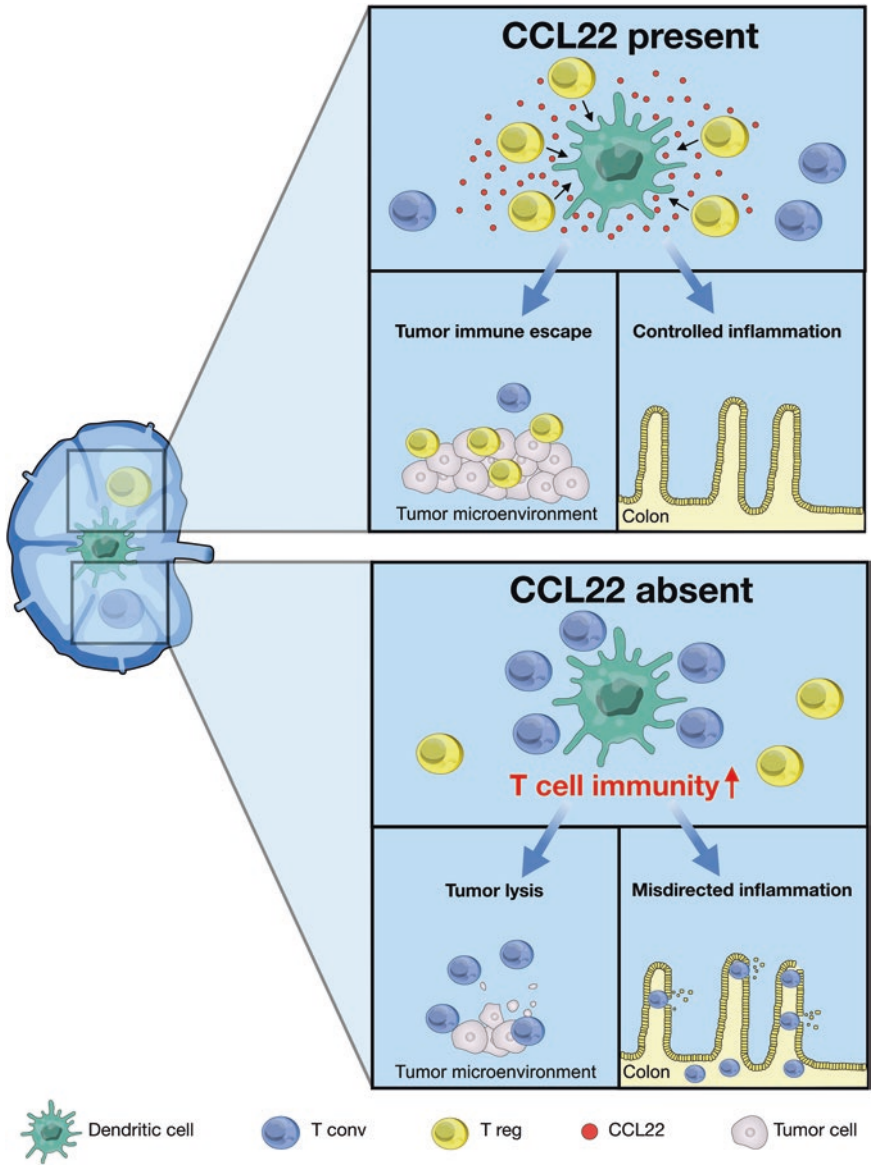

Fig. 8.1 CCL22 in homeostasis and disease. In homeostasis, CCL22 is expressed at high levels in lymph nodes by dendritic cells (DC) [9, 14] and DC-derived CCL22 promotes contacts between DCs and regulatory T cells (T regs) [20]. DC-T reg cell contacts are important for T reg-mediated immunosuppression [109] and favor the retention of induced T regs [21]. T cell immunity is controlled in the presence of high levels of DC-derived CCL22 in lymph nodes. Consequently, CCL22 can contribute to immune escape in the setting of cancer, but also to prevention of excessive and misdirected immunity. By contrast, CCL22 deficiency enhances T cell immunity as a result of reduced DC-T reg contacts, leading to increased tumor control in the setting of cancer, but also susceptibility to autoimmunity [20]

lymph nodes, thereby preventing autoimmunity by counteracting the activation of autoreactive T cells [21]. Altogether, CCL22-mediated DC-T reg communication is eminently important for the control of adaptive immunity. Thus, CCL22 most likely represents a central immune checkpoint controlling T cell immunity.

8.3 The Tumor Immune Environment

8.3.1 Antitumor Immunity and Immune Escape

Mechanisms that fundamentally control the immunological balance during homeostasis are prone to be hijacked by tumors to propagate conditions that favor immune escape. Conceivably, CCL22 not only coordinates the spatial distribution of T regs in homeostasis but also in cancer, where prevailing immunosuppression is often observed. In an attempt to develop novel therapeutic approaches targeting the tumor environment, it is imperative to understand immunological elements that compromise antitumor immunity. Successful tumor rejection is predominantly mediated by antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTL) that recognize and subsequently kill tumor cells [22]. However, when tumors become clinically apparent, they have usually already escaped many of the control authorities of the immune system [22]. Favorably, tumors utilize mechanisms designated to support tolerance, among which the induction and recruitment of T regs play a central role. Yet, another immunoregulatory program that is often exploited by tumors is the programmed cell death protein 1 (PD-1)/programmed cell death protein ligand 1 (PD-L1) axis—with important clinical implications [23]. Targeting the tumor immune environment with immunoregulatory drugs has moved into the spotlight particularly due to the recent success of clinical trials on PD-1/PD-L1 axis blockade in solid tumors [24]. PD-1 is a natural checkpoint molecule that controls adaptive immunity and prevents autoimmunity by limiting excessive T cell responses [25]. It is primarily expressed on T cells and is strongly upregulated upon T cell activation [26]. The ligands PD-L1 and PD-L2 are predominately expressed on antigen-representing cells (APC) and mediate immune cell inactivation upon interaction with PD-1 [25]. Intriguingly, tumor cells can also upregulate PD-L1 to very high levels [27], thereby inducing T cell "exhaustion" [28] and apoptosis [29] and thus presumably protecting tumors from CTL-mediated tumor cell lysis. Overall, the PD-1 pathway and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are known as "immune checkpoints" that are often hijacked by tumor cells and can be targeted therapeutically in cancer. Yet, anti-PD-1 therapy is based on entirely different principles than anti-CTLA-4 therapy. CTLA-4 appears to be critical for preventing T cell immune responses against self-antigens by coordinating T reg activity [30]. Moreover, it is induced on effector T cells and T regs upon stimulation, with delay, but proportionate to activation. Subsequently, CTLA-4 binds and blocks B7-1 (CD80) and B7-2 (CD86) with higher affinity than CD28, resulting in abrogated T cell activation by APC [31]. Furthermore, DCs upregulate the expression of indoleamine-2,3-dioxygenase (IDO) upon interaction with CTLA-4 [32], an enzyme which has been associated with $CD8^+$ T cell exhaustion in cancer [33]. T reg-mediated immunosuppression in cancer was indeed shown to be dependent on CTLA-4 expression [30]. Targeting CTLA-4 exerts potent T cell immunity by blocking CTLA-4 on T effector cells, but also on T regs, suggesting a synergistic effect of increased T effector cell function and reduced T reg-mediated immunosuppression [34, 35]. Nevertheless, interest in T reg-mediated tumor immunosuppression first emerged from a different study in the early 2000s, demonstrating that depletion of T regs in murine tumor models improved tumor rejection [36] and boosted antigen-specific antitumor immunity [37]. Today, it is generally accepted that tumor-infiltrating T regs can suppress tumor-specific cytotoxicity of CTLs in vitro and in vivo [8]. Understanding the regulatory programs of T regs that are exploited

by tumor cells and mechanisms by which they are recruited may have a significant impact on the development of novel therapeutic strategies to overcome tumor immune tolerance and will thus be further discussed in this chapter.

8.3.2 Regulatory T Cells in the Tumor Immune Environment

While T regs are essential for the suppression of conventional T cells (T conv) to prevent misdirected immune responses that can be harmful to the host, their suppressive capacity is unwanted in the setting of cancer. It has been shown for decades that tumors possess immunogenic properties to various degrees. Further, tumor-associated antigens most often comprise self-constituents that either trigger an immune response or induce tolerance, depending on the antigen and the environment [6]. To some extent, tumor immunity can be viewed as a subtype of autoimmunity, in which T regs play a key role as they try to maintain immunological tolerance [38]. In homeostasis, the majority of T regs are derived from thymic progenitor cells and are commonly designated $CD4^+$ $CD25^+$ $FoxP3^+$ T regs. Tolerance to innocuous antigens, such as commensal, food, and environmental antigens, is mediated by T regs that are generated through peripheral conversion of T conv and represent a small but important part of the $FoxP3^+$ T reg pool [39]. Peripheral conversion from T conv to T regs is often associated with the presence of high levels of TGF-β, in homeostasis [40] as well as in the setting of cancer [41]. Notably, both T reg populations have been shown to contribute independently to an immunosuppressive tumor microenvironment [42]. T regs facilitate immune escape through both contact-dependent and soluble mechanisms [43]. The mechanisms by which T regs exert immunosuppression range from the production of membrane-bound TGF-β [44], secretion of adenosine [45], direct infusion of cAMP via gap junctions [46], and downregulation of costimulatory molecules on both T cells and DCs via CTLA-4 [47] to the perforin/granzyme pathway [48]. Intriguingly, T reg-DC interactions directly at the tumor site appear to be key prerequisites for their local suppressive effector functions [49]. Bauer and colleagues showed that T regs transiently make contact with tumor-infiltrating DCs in an antigen-specific manner, leading to a rapid downregulation of the costimulatory molecules CD80/86 and subsequently to the development of dysfunctional CTL [49]. Noncontact-dependent mechanisms of T reg immunosuppression are based on soluble factors produced by T regs and include consumption of IL-2 and secretion of cytokines such as IL-10 and IL-35 [50]. Both contact-dependent and soluble mechanisms favor the development of an immunosuppressive tumor microenvironment. Comprising only a small proportion of T cells under physiologic conditions, T regs expand and show an altered tissue distribution in cancer patients. Progressive accumulation of T regs has been detected in many cancer patients, not only in the tumor mass itself but also in lymphoid organs and the blood [8, 51–55]. The extent of the intratumoral accumulation often correlates with impaired antitumor immune response and a poor clinical prognosis in many types of cancer, particularly when accompanied by a decreased $CD8^+$ T cell to T reg ratio [8, 51, 56–59]. Addressing the questions of how and why T regs increase and accumulate in tumors is important with respect to a better understanding of the underlying biology and its potential applications for cancer immunotherapy. Various mechanisms have been described by which such accumulation is facilitated, including the conversion of Tconv into peripheral T regs at the tumor site [51, 60], local expansion of tissue-resident T regs [61–63], and the selective recruitment of T regs into the tumor via different chemokines. In particular, CCL22 [8, 51, 64–66], CCL28 [67], and CCL1 [61] have been associated with T reg trafficking into tumors. Among those T reg-attracting chemokines, CCL22 has been most extensively linked to T reg recruitment and retention in the tumor tissue and will be further elaborated on in this chapter.

8.4 The Role of CCL22 in Cancer

8.4.1 Cancer-Associated CCL22 Expression in Tumors

The immunosuppressive properties of T regs make them ideal tools for tumors to promote immune evasion. T regs are actively recruited to the tumor, mainly via the CCL22-CCR4 axis [38]. High intratumoral levels of CCL22 have been described in many types of both murine [13, 68] and human tumors [51, 66, 69–73]. Consistent with findings in homeostasis, elevated levels of CCL22 in tumors are associated with a high T reg infiltration. For instance, CCL22 levels in patients with ovarian cancer were found to be strongly elevated in the tumor mass and malignant ascites [8]. In an elegant study, Curiel and colleagues provide evidence that $CCR4^+$ T regs migrate to and accumulate in the tumor tissue in a CCL22-dependent manner, contributing to an immunosuppressive environment [8]. This was the first study to directly link increased levels of intratumoral CCL22 with high T reg infiltration and was later recapitulated in a gastric cancer model [66]. It is noteworthy that T regs isolated from malignant effusions, peripheral blood, or the solid tumor itself display equal suppressive capacities in vitro [8], suggesting that T regs derived from the tumor mass do not show superior suppressive abilities than those derived from the blood. Accordingly, tempered antitumor immunity is most likely not a consequence of T regs equipped with enhanced suppressive capacities, but instead, a result of increased accumulation in the tumor, presumably mediated by CCL22. Intriguingly, overexpression of CCL22 in human tumors is indeed associated with a high infiltration of T regs into the tumor, along with augmented tumor growth and poor prognosis in several types of human tumors, including breast [51, 64], gastric [51, 66], liver [74, 75], and ovarian cancer [8].

Yet, the cellular source of intratumoral CCL22 has been unknown for a long time. Early studies argued that the tumor cells themselves were the main source of intratumoral CCL22, a finding that was predominately based on a study in a breast cancer model [76]. However, other reports suggested that in different breast cancer models DC-shaped tumor-infiltrating immune cells also secreted high amounts of CCL22 [51, 76]. A dual role for tumor and immune cells, such as DCs, macrophages, and monocytes, in the induction of CCL22 in cancer has further been suggested for Lewis lung carcinoma [72], gastric [66], head and neck [77], and ovarian cancer [8]. Nevertheless, more recent data provide evidence that in many types of cancer, immune cells such as DCs and macrophages are the exclusive producers of CCL22 [13, 78]. Yet, future studies are necessary to clarify the exact role of individual cell types in CCL22 induction in distinct tumor entities.

8.4.2 Mechanisms of CCL22 Induction in Solid Tumors

Tumor cell-derived IL-1α induces CCL22 in dendritic cells. To understand the underlying mechanisms of CCL22 induction in tumors, it is important to appreciate the similarities between cancer and inflammation, a concept which has been extensively reviewed elsewhere [3]. Tumors exploit numerous immunosuppressive mechanisms that are also an integral part of controlling an inflammatory processes during homeostasis. As CCL22 is inducible upon inflammation [79], it is conceivable that the same underlying regulatory processes might also be involved in cancer-associated inflammation. The cytokine IL-1 is an important mediator in both inflammation and cancer. Importantly, IL-1 has been shown to potently induce CCL22 in inflammation [17]. Interestingly, this finding might also extrapolate to cancer: IL-1α was shown to be produced by tumor cells of certain tumor entities such as pancreatic adenocarcinoma and to induce CCL22 in human DCs, subsequently facilitating T reg recruitment in vitro [78] (Fig. 8.2). Furthermore, high intratumoral levels of IL-1α are found in many tumors and

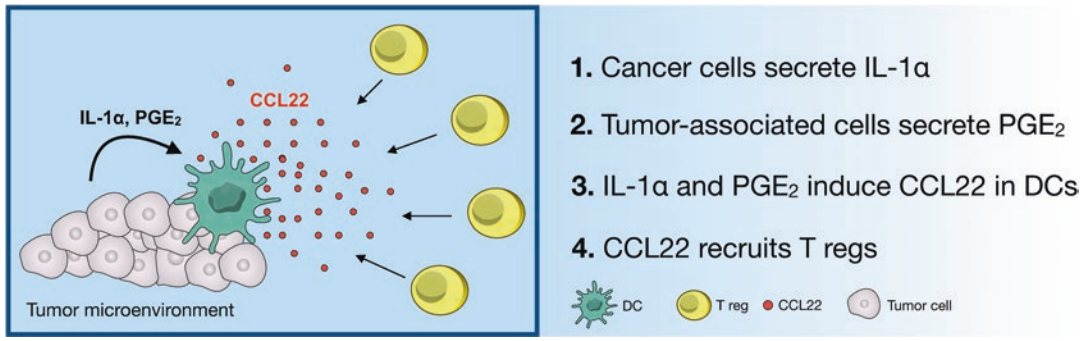

Fig. 8.2 Mechanisms of CCL22 induction in cancer. Cancer-cell derived IL-1α induces CCL22 in tumor-infiltrating dendritic cells (DCs) [78]. Tumor-associated prostaglandin E2 (PGE_2) has also been shown to upregulate CCL22 in DC [86]

have been linked to progressed disease [80] and metastasis [81–83]. Intriguingly, IL-1α-mediated migration of T regs can be abrogated by the IL-1 receptor (IL-1R) antagonist anakinra in vitro [78], potentially contributing to the antitumoral effect of IL-1R antagonists observed in a recent clinical trial [84, 85]. Taken together, IL-1α-mediated induction of CCL22 in cancer might be a novel mechanism of tumor immune evasion.

Tumor-derived prostaglandin E2 induces CCL22 in dendritic cells. Inflammatory mediators other than IL-1α also contribute to CCL22 induction in cancer. For instance, prostaglandin E2 (PGE_2) was found to substantially contribute to CCL22 secretion by DCs [86]. PGE_2 has long been implicated in the pathogenesis of chronic inflammation and cancer [87, 88]. PGE_2 has been shown to activate DCs and promote inflammation, but also to enhance T reg functionality and accumulation. While superficially paradoxical, this dual role can be explained by the sequential order of its effects: it is generally accepted that PGE_2 is a potent pro-inflammatory mediator in the early phase of inflammation, advocating local vasodilatation as well as the attraction and activation of distinct immune cell subsets such as neutrophils, macrophages, and DCs [87, 89–92]. At later stages, however, it is associated with the suppression of pro-inflammatory cytokines as well as the recruitment of immunosuppressive T regs, supposedly limiting unwanted or excessive inflammation [88, 93]. Notably, PGE_2 is overexpressed in many tumor entities and has been linked to the attraction of $FoxP3^+$ T regs [86, 94]. Mechanistically, maturing DCs are primed by PGE_2 to adopt a stable propensity to interact with T regs, a process which is facilitated by a strong upregulation of CCL22 [86] (Fig. 8.2). The proposed concept that PGE_2 instructs DCs to attract T regs via CCL22 adds to the already established notion that PGE_2 induces the de novo generation of suppressive T regs [95, 96]. Interestingly, inhibitors of PGE_2 synthesis, such as COX inhibitors, exhibit potent therapeutic efficacy in inflammation and have also been utilized for chemoprevention of certain types of cancer [97]. COX inhibitors have been shown to suppress the induction of CCL22 with a subsequent reduction of T reg migration to human tumors ex vivo [98]. Future studies are necessary to evaluate individual pathways of PGE_2-mediated immunosuppression and particularly the role of CCL22 induction. Furthermore, it will be interesting to analyze the relative contributions of the CCL22-inducing mediators IL-1α and PGE_2, in order to exploit these mechanisms for the development of novel therapeutic strategies in cancer.

8.4.3 CCL22 Expression in Peripheral Lymph Nodes and Its Implications in Cancer

In tumor-bearing individuals, CCL22 was not only found to be elevated in the tumor tissue itself but also in the serum and lymphatic organs [99], a finding which might contribute to an immunosuppressive environment. Of note, effective antitumor immunity is carried out by the coordinated interactions of immune cells not only within the tumor microenvironment but also within lymphoid structures [100]. Trafficking of immune effector cells within and between lymph nodes and the tumor tissue is orchestrated by chemokines and their binding affinities to their cognate receptors. The process of priming and activation of $CD8^+$ T cells by DCs in response to an infection shares many similarities with the immune response against tumor cells [3]. Naïve T cells continuously scan DCs for their cognate antigen by trafficking through secondary lymphoid organs, a process mediated by the interaction of constitutively expressed CCL19 and CCL21 with their respective receptor CCR7 on the surface of T cells [101]. However, antigen-loaded DCs that correspond to the antigen-specific $CD8^+$ T cell are scarce; thus, efficient and directed motility of $CD8^+$ T cells is vital within lymphoid organs [102]. Intriguingly, $CD4^+$ T cells provide help in this process by stimulating DCs to release CCL4 and CCL5 allowing CCR5-mediated guidance of $CD8^+$ T cells towards DC-$CD4^+$ T cell pairs with subsequent formation of a tricellular cluster [103, 104]. The concept of tricellular clusters is not exclusive: Semmling and colleagues put forth that cross-priming of CTLs can be facilitated by the coordinated interaction of CTLs with natural killer T (NKT) cell licensed DCs in a CCL17-CCR4-dependent manner [105]. Importantly, T cell priming is tightly regulated to prevent autoreactive T cells from being activated, thereby potentially harming the host. Herein, T regs play a central role. In the setting of cancer, this limiting step is unfortunate as T regs counteract the initiation of an effective antitumor immune response by inhibiting stable DC-T effector cell interactions [106]. DC-T reg interactions have been described to not only occur inside the tumor tissue [49], but also in tumor-associated tertiary lymphoid structures [107] and most importantly in the lymph node [108]. Within lymph nodes, the CCL22-CCR4 axis might play a crucial role in shaping the process of T cell priming and induction of antitumor immunity [20]. It is important to appreciate that T regs are dependent on direct cell-to-cell interactions with antigen-presenting DCs to effectively exert functions of immune regulation [109]. The T reg-attracting chemokine CCL22 promotes these DC-T reg contacts, hence enabling T reg-mediated regulation of immunity in lymph nodes [20]. In homeostasis, DCs produce high amounts of CCL22 that may help to control excessive T cell immunity. In the setting of cancer, elevated levels of CCL22 in lymph nodes could potentially attenuate T cell activation by promoting the formation of DC-T reg clusters. Indeed, DCs show a more suppressive phenotype after DC-T reg crosstalk due to CTLA-4-mediated IDO expression [110]. Moreover, in the setting of CCL22 deficiency, the number of antigen-specific T cells was strongly increased upon vaccination, most likely a consequence of a modulated T cell priming in the lymph node. Accordingly, vaccinated tumor-bearing and CCL22-deficient mice showed prolonged survival with a concomitant increase of tumor-antigen-specific T cells [20]. Notably, the percentage of intratumoral T regs in CCL22-deficient mice was not reduced compared to wild-type mice. Thus, CCL22 seems to indeed control T cell-mediated antitumor immunity by abrogating T cell priming in the lymph node, emphasizing the detrimental effects of DC-Treg clustering in cancer [20]. Preventing T regs from migrating into areas where antitumor immunity is primed might be equally important as preventing them from entering the tissue where it is finally exerted (Fig. 8.3).

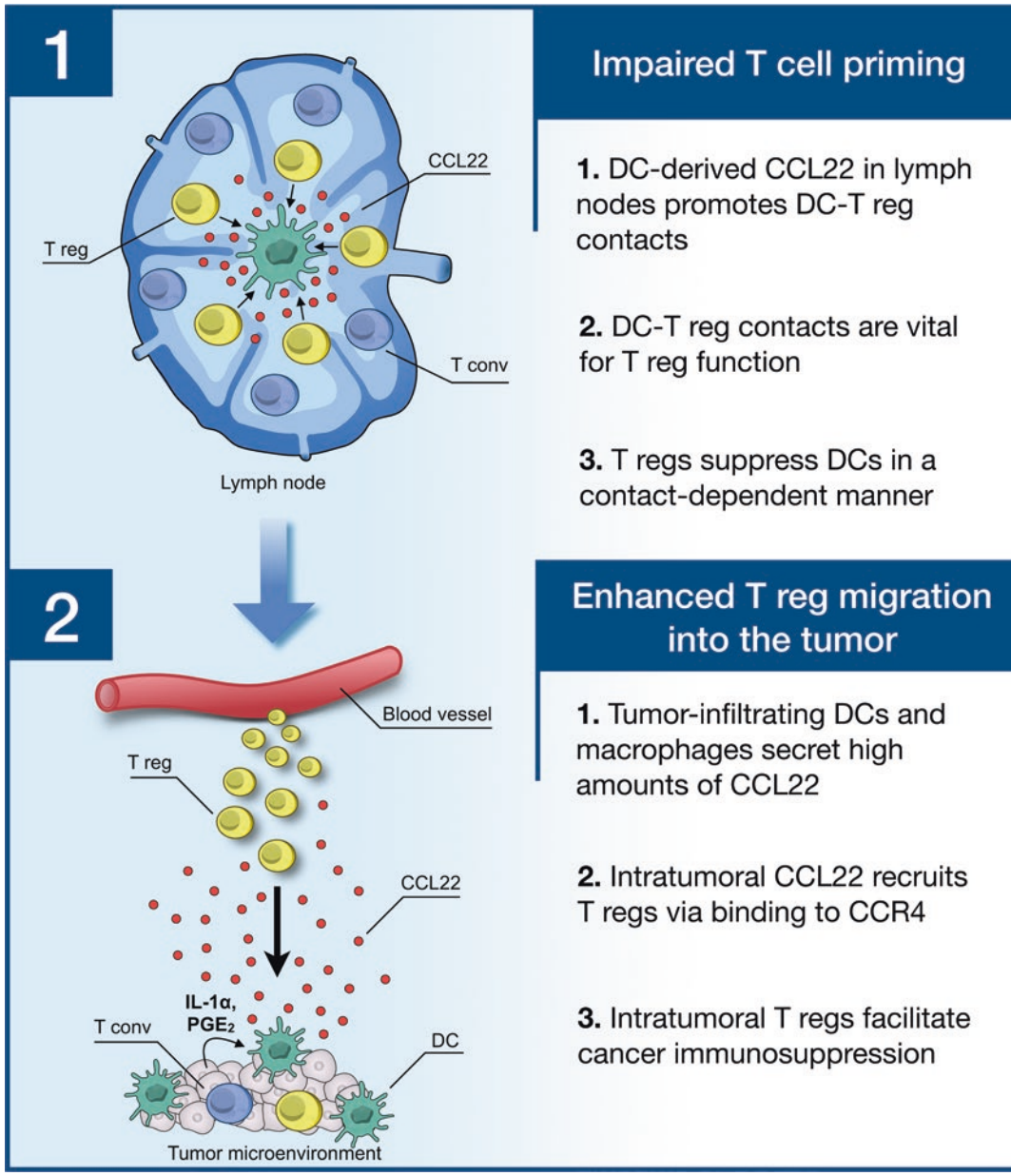

Fig. 8.3 CCL22-mediated immunosuppression in cancer. (1) In lymph nodes, excessive DC-derived CCL22 abrogates T cell priming by enhancing DC-T reg contacts. Subsequently, T regs suppress DC in a contact-dependent manner, thus hindering T cell priming that is necessary for effective antitumor immunity [20]. (2) Locally within the tumor microenvironment, DC-derived CCL22 preferentially recruits T regs into the tumor [8]. Subsequently, intratumoral T regs inhibit cytotoxic T cell function [8] and downregulate costimulatory molecules on DCs [49], thereby synergistically limiting antitumor immunity

8.5 Targeting the CCL22-CCR4-Regulatory T Cell Axis for Cancer Immunotherapy

CCL22 expression, both in the tumor tissue and lymph nodes, contributes to T reg recruitment and immunosuppression. Therefore, the CCL22-CCR4 axis serves as an interesting therapeutic target for cancer therapy. Evidence for the substantial impact of T regs in cancer immune subversion emanates from a study in the early 2000s that demonstrated tumor eradication after administering an anti-CD25 cell-depleting monoclonal antibody, primarily targeting T regs [36, 111]. Marked antitumor immune responses with CD25-depleting antibodies have been confirmed in several tumor models thereafter [37, 112–114]. This finding opened the field not only for a deeper understanding of T reg biology in cancer but also for evaluating T regs as potential targets for cancer immunotherapy. Subsequent studies further encouraged T reg-targeting strategies: denileukin difitoxin (ONTAK), a fusion protein comprising an immunotoxin conjugated to IL-2 that preferentially depletes T regs, resulted in augmented antitumor immunity and reduced tumor growth [115, 116]. Further, intratumoral injection of T reg-depleted $CD4^+$ T cells potently enhanced antitumor immune responses compared to adoptive transfer of T reg-containing $CD4^+$ T cells in mice, providing evidence for an inverse correlation between T regs and effective antitumor immunity [117]. In consideration of their central role in cancer immunosuppression, depleting T regs has been subject of numerous studies [118]. However, this approach has experienced limited clinical success [119], most likely due to a lack of T reg-specific target molecules [120]. Indeed, there was no clinical benefit for patients with metastatic melanoma treated with denileukin difitoxin, instead severe autoimmunity was observed [115]. Thus, alternative strategies are needed to target T regs in cancer patients. In this regard, it is noteworthy that effective T reg-mediated immunosuppression is dependent on DC-T reg interactions. Thus, targeting pathways involved in DC-T reg contact formation represents a promising novel approach. A breakthrough in the field of immunooncology was the development of immune checkpoint blockade antibodies like ipilimumab, a monoclonal antibody directed against CTLA-4, which has shown remarkable clinical successes in a subset of patients [121–123]. Ipilimumab enhances T cell immunity and prevents T reg-mediated immunosuppression [34, 35]. Later, it has been proposed that an important mechanism by which ipilimumab exerts its antitumor effects is antibody-mediated and Fc-dependent depletion of T regs in the tumor microenvironment [124]. Intriguingly, in a melanoma mouse model, CTLA-4 antibody binding to T regs contributed to the antitumor effects independent of T effector cell binding, thereby maximizing therapeutic efficacy of this molecule [34]. Nevertheless, induction of unwanted and potentially life-threatening autoimmunity is an important limiting aspect of CTLA-4-directed checkpoint blockade [125], presumably also due to the antibody-mediated depletion of T regs. Thus, targeting solely T reg migration, for instance by manipulating the CCL22-CCR4 axis, represents a promising alternative strategy, an approach which has already undergone extensive preclinical and also clinical evaluation.

8.5.1 Direct Targeting of the CCL22-CCR4 Axis

The cell-depleting monoclonal CCR4-antibody mogamulizumab (KW-0761) was the first drug to target the CCL22-CCR4 axis and quickly gained interest in the field (Fig. 8.4). It was approved in Japan in 2014 for the treatment of cutaneous T cell lymphoma (CTCL) and CCR4-positive peripheral T cell lymphoma (PCTL) [126]. A multicenter phase III clinical trial [127] was subsequently conducted, which showed significant clinical improvements for patients with CTCL receiving mogamulizumab compared to the U.S. Food and Drug Administration (FDA)-approved treatment vorinostat, in terms of progression-free survival and overall response rate [127]. Based on this clinical evaluation, the FDA and the European Medicines Agency (EMA) approved mogamulizumab in 2018 for the treat-

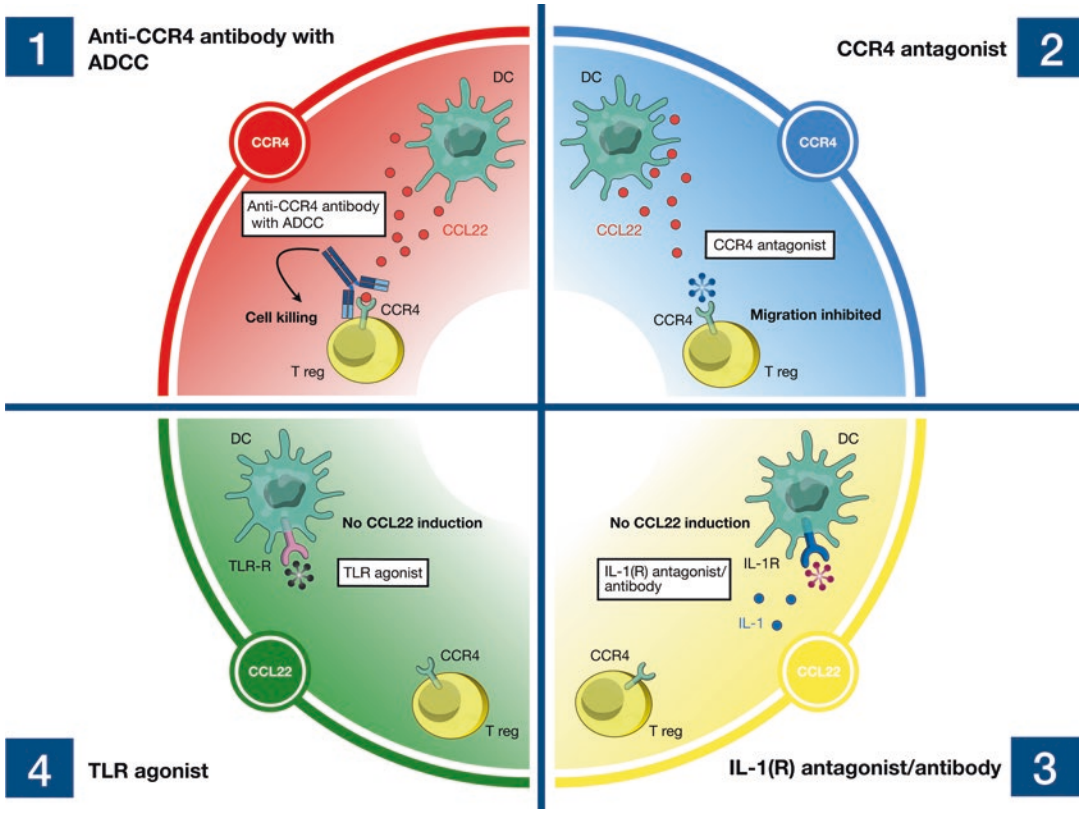

Fig. 8.4 Targeting the CCL22-CCR4 axis in cancer. Anti-CCR4 antibodies with antibody-dependent cellular cytotoxicity (ADCC) target CCR4-expressing cells with subsequent elimination of the target cell [71]. CCR4 antagonists inhibit the migration of CCR4-expressing cells without depleting them [134]. A different approach interferes with pathways that regulate CCL22 expression and secretion. Both IL-1α (R) antagonists and several TLR agonists inhibit CCL22 expression by dendritic cells (DCs) [78]

ment of adult patients with relapsed or refractory Sézary syndrome or mycosis fungoides, two subtypes of CTCL [128]. This is the first FDA- and EMA-approved drug to directly target CCR4. Of note, mogamulizumab does not directly interfere with CCR4-mediated T reg migration, but instead exerts its effect by promoting antibody-dependent cell-mediated cytotoxicity (ADCC) after binding to its target receptor CCR4, subsequently depleting CCR4-expressing cells, including CTCL cells but also T regs. Thereby, anti-CCR4 antibodies with ADCC have a dual role in that they not only directly kill CCR4-expressing cancer cells, but also have the potential to overcome an immunosuppressive tumor microenvironment by tempering the infiltration with $CCR4^+$ T regs [129, 130]. Consequently, mogamulizumab-induced T reg depletion bears again the risk of misdirected immunity [131, 132]. Indeed, serious autoimmune side effects have been observed in some cases [133].

Small molecules targeting CCR4 constitute interesting alternatives as they lead to impaired T reg migration without depletion (Fig. 8.4). Efforts in designing small molecules, preferentially with oral bioavailability, have led to the development of a set of CCR4 antagonists, some of which have subsequently undergone preclinical evaluation with promising results [134–137]. For instance, FLX475 is an orally available small molecule antagonist of CCR4 that inhibits T reg migration into the tumor, prompting potent antitumor immunity and tumor regression in several murine tumor models [134]. A phase I clinical trial with FLX475 has recently been successfully completed, enabling

an accelerated phase I/II study, for which patients are currently being enrolled [138]. Further studies evaluating the efficacy of small molecule CCR4 antagonists are necessary, in special consideration of their potential beneficial side effect profile.

8.5.2 Indirect Targeting of the CCL22-CCR4 Axis

A different strategy for manipulating the CCL22-CCR4 axis in cancer treatment is to target it indirectly, either by suppressing CCL22 expression by DCs, or by interfering with mechanisms that induce CCL22 in cancer. This requires the knowledge of the relevant pathways that regulate the expression of CCL22.

8.5.2.1 IL-1(Receptor) Antagonism

IL-1R antagonists have been implicated in the treatment of conditions such as rheumatoid arthritis for decades [139]. However, their promising potential in cancer therapy has only recently been appreciated and evidence for its efficacy is growing [83, 139–141]. Increased levels of IL-1 have been found in many types of human cancer, including breast, colon, lung, and head and neck cancers as well as melanoma and are often associated with a poor prognosis [142]. Mechanistically, IL-1 has been shown to promote tumor metastasis and angiogenesis, thus increasing tumor burden [143]. The pro-tumorigenic effects of IL-1 seem to depend on multiple pathways including increased expression of metalloproteinases, VEGF, growth factors, TGF-β, and chemokines, reflecting the pleiotropic nature of IL-1 [143]. Interestingly, IL-1 naturally competes with the endogenous IL-1 receptor antagonist (IL-1Ra) for binding to the IL-1R. However, IL-1Ra binds the IL-1R non-productively, thus preventing effective IL-1 signaling [144]. In analogy, anakinra, a recombinant form of this antagonist, has been developed to therapeutically block IL-1 signaling in many disease entities, including cancer [145]. Moreover, a recent phase III study evaluating the use of xilonix, an IL-1α neutralizing antibody, in combination with chemotherapy, showed increased overall survival in patients with end-stage metastatic colorectal cancer [84]. Intriguingly, CCL22 has been shown to be induced by tumor cell-derived IL-1α in vitro [78], fostering tumor infiltration by T regs. In this study, the administration of anakinra was able to significantly attenuate T reg migration [78]. Hence, blocking intratumoral CCL22 secretion by DCs through antagonism of IL-1 might account for some of the antitumoral effects of IL-1 antagonizing drugs, presumably by reducing the numbers of intratumoral T regs (Fig. 8.4). However, the detailed contributions of CCL22 to the antitumor effects of IL-1 antagonists still remain to be determined in vivo.

8.5.2.2 Toll-Like Receptor Agonists

Another concept of antitumor immunotherapy is the use of Toll-like receptor agonists (TLR). The principle cellular targets for TLR agonists are APCs, particularly DCs, but also NK cells and monocytes, with subsequent upregulation of the pro-inflammatory mediators IFN-α, IFN-γ, and TNF-α [146]. Several TLR ligands have been extensively studied for their potential application in cancer therapy, some of which indeed show promising therapeutic efficacy [147–149]. Imiquimod, a TLR7 agonist, is currently approved for the local treatment of superficial basal cell carcinoma [150]. Intriguingly, patients with squamous cell carcinoma treated with Imiquimod show altered proportions of T cell subsets in the tumor with an increased $CD8^+$ T cell to T reg ratio [151, 152]. Along the same line, CpG, a TLR9 agonist, exhibits a similar profile of T cell subsets within the tumor microenvironment in mice, facilitating increased numbers of T effector cells [153]. Furthermore, TLR ligands have influence on T reg-mediated immunosuppression itself. Ligation of TLR7 or TLR9 agonists to their respective receptors renders T effector cells unresponsive to suppression by T regs [154, 155], an effect which was shown to be dependent on TLR-induced INF-α, but also on TLR-2 and MDA-5 ligands [156, 157]. Another concept implies that CpG and other TLR ligands, such as Poly I:C, inhibit $FoxP3^+$ T reg migration into the tumor by suppressing CCL22 expression in DCs [13, 86]. The observed CCL22 suppression was mediated by TLR-induced expression

of type I interferons. Interestingly, IFN-α itself has antitumor properties and is an established therapeutic agent in melanoma [158]. Thus, TLR agonists represent promising therapeutic agents in tumor entities, in which CCL22 levels are strongly elevated (Fig. 8.4).

8.6 Conclusion

Our understanding of T reg biology and T reg-mediated immunosuppression in cancer is rapidly evolving. At the same time, novel concepts such as CCL22-mediated coordination of T reg migration in homeostasis and cancer are increasingly being studied. Targeting CCL22 or its receptor CCR4 in cancer represents a promising strategy to overcome an immunosuppressive tumor environment not only by reducing T reg-infiltration into the tumor but also by allowing effective T cell priming in the lymph node. Since CCL22 influences T cell priming, CCR4 antagonists and antibodies need to be carefully evaluated with respect to their potential to not only hamper T reg migration into the tumor but also to lymphoid tissues and thereby augment T cell activation. Nevertheless, it might turn out to be equally beneficial to tackle the CCL22-CCR4 axis at earlier steps with IL-1(R) antagonists or TLR agonists. Yet, many important questions still remain to be addressed. For instance, we lack reliable biomarkers to determine who would benefit from CCL22-targeting therapeutic approaches in cancer. CCL22 expression levels in the tumor tissue or even the lymph nodes or serum of patients could be an interesting criterion to select patients that might benefit from a CCL22/CCR4-targeting therapy. Thus, elaborate studies assessing CCL22 as a biomarker are required to answer these emerging questions.

Anti-CCR4 antibodies with antibody-dependent cellular cytotoxicity (ADCC) target CCR4-expressing cells with subsequent elimination of the target cell [71]. CCR4 antagonists inhibit the migration of CCR4-expressing cells without depleting them [134]. A different approach interferes with pathways that regulate CCL22 expression and secretion. Both IL-1α (R) antagonists and several TLR agonists inhibit CCL22 expression by dendritic cells (DCs) [78].

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