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Alexander Birbrair *Editor*

Tumor Microenvironment

The Role of Chemokines – Part B

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This book is dedicated to my mother, Marina Sobolevsky, of blessed memory, who passed away during the creation of this volume. Professor of mathematics at the State University of Ceará (UECE), she was loved by her colleagues and students, whom she inspired by her unique manner of teaching. All success in my career and personal life, I owe to her.

My beloved mom Marina Sobolevsky of blessed memory (July 28, 1959–June 3, 2020)

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 B* 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 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. Nine chapters written by experts in the field summarize the present knowledge about distinct chemokines during tumor development.

Tracy O'Connor and Mathias Heikenwalder from Technical University of Munich discuss the role of CCL2 in the tumor microenvironment. Niradiz Reyes and colleagues from University of Cartagena describe CXCL3 signaling in the tumor microenvironment. Sahana Asokan and Obul Reddy Bandapalli from Heidelberg University compile our understanding on CXCL8 signaling in the tumor microenvironment. Qun Gao and Yi Zhang from Zhengzhou University update us with what we know about CXCL11 signaling in the tumor microenvironment. Weilong Chen and colleagues from Fudan University summarize current knowledge on the multi-faceted roles of CXCL12 signaling in the tumor microenvironment. Guang-Biao Zhou and colleagues from the Chinese Academy of Sciences address the importance of CXCL13 signaling in the tumor microenvironment. Sung-Jig Lim from

Kyung Hee University Hospital focuses on CCL24 signaling in the tumor microenvironment. Hina Mir and Shailesh Singh from Morehouse School of Medicine talk about the contribution of CCL25 signaling in the tumor microenvironment. Finally, Miguel Martínez-Rodríguez and Carlos Monteagudo from the University of Valencia give an overview of CCL27 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 Tamilsevan from Springer, who helped at every step of the execution of this project.

Belo Horizonte, Minas Gerais, Brazil Alexander Birbrair

Contents

CCL2 in the Tumor Microenvironment

1

Tracy O'Connor and Mathias Heikenwalder

Abstract

The C-C motif chemokine ligand 2 (CCL2) is a crucial mediator of immune cell recruitment during microbial infections and tissue damage. CCL2 is also frequently overexpressed in cancer cells and other cells in the tumor microenvironment, and a large body of evidence indicates that high CCL2 levels are associated with more aggressive malignancies, a higher probability of metastasis, and poorer outcomes in a wide range of cancers. CCL2 plays a role in recruiting tumor-associated macrophages (TAMs), which adopt a pro-tumorigenic phenotype and support cancer cell survival, facilitate tumor cell invasion, and promote angiogenesis. CCL2 also has direct, TAM-independent effects on tumor cells and the tumor microenvironment, including recruitment of other myeloid subsets and non-myeloid cells, maintaining an immunosuppressive environment, stimulating tumor cell growth and motility, and promoting angiogenesis. CCL2 also plays important roles in the metastatic cascade, such as creating a pre-metastatic niche in distant organs and promoting tumor cell extravasation across endothelia. Due to its many roles in tumorigenesis and metastatic processes, the CCL2-CCR2 signaling axis is currently being pursued as a potential therapeutic target for cancer.

Keywords

CCL2 · MCP-1 · Cancer · Tumor · Microenvironment · CCR2 · NFκB · Immunity · TAM · Macrophage · Angiogenesis · Extravasation · Metastasis · Immunosuppression · Invasion

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1.1 Introduction

1.1.1 CCL2

The C-C motif chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), was originally identified in a screen for platelet-derived growth factor (PDGF)-inducible genes in mouse fibroblasts [15]. CCL2

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was later discovered to be a potent monocyte chemoattractant produced by primate aortic smooth muscle cells [111], human gliomas [123], and human monocytic leukemia cells [67]. CCL2, whose expression is triggered by transcription factors such as nuclear factor κ-light-chain-enhancer of activated B-cells (NFκB) and Specificity protein 1 (Sp1), can be produced by a wide variety of cell types, typically in response to local inflammatory stimuli and tissue-resident innate immune cells producing tumor necrosis factor α (TNFα), interleukin-1 (IL-1), or interferon γ (IFNγ), thereby promoting rapid infiltration of inflammatory monocytes and other blood-resident, CCL2-reponsive cells into affected tissues. Although many chemokine receptors bind multiple chemokines, rendering the functions of single chemokines largely redundant, *Ccl2*$^{-/-}$ mice exhibit significant defects in monocyte recruitment during inflammatory responses, despite harboring normal levels of circulating lymphocytes and tissue-resident macrophages [64]. Thus, CCL2 is the main chemokine responsible for pro-inflammatory monocyte recruitment during inflammation, and other chemokines cannot fully compensate for its actions. CCL2 production and monocyte infiltration have been implicated in a number of inflammatory conditions in humans, such as multiple sclerosis [68, 97], ischemia [61], rheumatoid arthritis [44], epilepsy [118], atherosclerosis [73, 120], and lupus [56]. Moreover, infection of macrophages by human immunodeficiency virus (HIV) stimulates CCL2 production [70], thereby promoting recruitment and infection of CCR2^{+} monocytes and T-cells. CCL2 is also produced by astrocytes [84] and other cells in the brain under neuroinflammatory conditions in order to recruit microglia, the tissue-resident macrophages of the central nervous system, and neural progenitor cells [5] to sites of injury. CCL2 also enhances the permeability of brain endothelial cells (ECs) [100] to facilitate trafficking of blood-borne inflammatory monocytes across the blood-brain barrier.

1.1.2 CCR2

The receptor for CCL2 is C-C chemokine receptor type 2 (CCR2), also known as CD192. Two CCR2 isoforms (CCR2A and CCR2B) exist in humans [12], whereas a single CCR2 isoform exists in mice [7, 46]. Expression of CCR2 is highest in the hematopoietic compartment, particularly on monocytes, as well as activated memory T-lymphocytes, some B-cell subsets [10, 23, 25], immature dendritic cells (DCs) [99], natural killer (NK) cells [2], basophils [110], and microglia [6]. Besides CCL2, CCR2 ligands include CCL7 [16, 26], CCL8, CCL12 [92], CCL13 [29], and CCL16. Similar to *Ccl2*$^{-/-}$ animals, mice lacking CCR2 exhibit normal hematopoietic development but have defects in monocyte recruitment and host defense against bacterial pathogens [47]. Moreover, CCR2 can be utilized as a co-factor by HIV-1 to recruit and infect monocytes and T-cells [20].

1.2 CCL2 in the Tumor Microenvironment

1.2.1 Recruitment of Tumor-Associated Macrophages (TAMs)

The presence of so-called tumor-associated macrophages (TAMs) is a ubiquitous histological feature of a wide variety of tumor types in humans [3, 32, 36, 37]. Moreover, a correlation was already drawn early on between a high level of TAMs and local tumor progression [9]. A number of earlier studies had already identified CCL2 as a monocyte chemoattractant being produced by tumor cells [8, 67, 69, 122, 125], strongly implicating this chemokine as the effector of TAM recruitment in vivo. This idea is supported by human studies which have drawn a correlation between the density of TAMs in human primary tumors and levels of CCL2 in breast cancer [28, 90, 109] and ovarian cancer [72]. It was also recently reported that glioma cells directly trig-

ger CCR2 expression on TAMs, thereby sensitizing them to the effects of CCL2 through the aryl hydrocarbon receptor [105]. Thus, CCL2 plays an important role in TAM recruitment during the pathogenesis of a number of malignancies. Nevertheless, the exact mechanisms of TAM recruitment are complex and remain an area of active investigation [49]. Other chemokines also contribute to TAM recruitment, and some TAMs may be tissue-derived. Moreover, it is now clear that CCL2 has other effects on the tumor microenvironment that are unrelated to TAM recruitment. To date, the presence of CCL2 has been reported in a wide range of human cancers, including breast carcinoma, hepatocellular carcinoma, prostate cancer, cervical cancer, colon cancer, gastric cancer, medulloblastoma, and glioma [19, 54, 65, 98, 106, 109, 121, 131], most indicating a negative effect of CCL2 on patient outcomes (Table 1.1). For example, high CCL2 expression is associated with more advanced disease stage [98], early relapse [35, 109], as well as dissemination and metastasis in breast cancer [57]. Moreover, expression of a long non-coding mRNA which regulates CCL2 expression is correlated with an increased probability of brain metastasis in breast cancer patients [115]. CCL2 expression is positively correlated with tumor aggressiveness in prostate cancer [65] and the likelihood of liver metastasis and hence poor outcomes in colorectal cancer [121]. Higher CCL2 levels are associated with lower frequencies of relapse-free survival in cervical cancer patients [131] and a higher probability of metastasis in colon cancer patients [117]. Patients with high CCL2 levels in pancreatic tumors have significantly reduced survival times [91], and higher CCL2 levels are correlated with enhanced tumor vascularization in gastric cancer [77]. Finally, elevated CCL2 levels have recently been implicated in the metastasis of medulloblastoma to the leptomeninges [30].

1.2.2 Mechanisms of CCL2 Production by Tumor Cells

NFκB is an essential signaling pathway for the mobilization of the immune system in response to infections and tissue damage, allowing immune cells to rapidly proliferate and respond to inflammatory stimuli. Due to its central role in immune cell recruitment, CCL2 is a canonical transcriptional target of the NFκB signaling pathway [108]. NFκB is frequently co-opted by tumor cells, and mutations leading to constitutive activation of the NFκB signaling pathway are commonly observed in human cancers, which enhances tumor cell survival, proliferation, and metastatic potential. In addition to NFκB, signaling pathways controlling angiogenesis are associated with enhanced CCL2 expression. For example, vascular endothelial growth factor (VEGF) expression stimulates CCL2 transcription via the Activator protein-1 (AP-1) promoter region of the CCL2 transcript [119], and the CCL2 promoter contains binding sites for Sp1 [108]. Moreover, it has been reported that the CCL2 promoter contains binding sites for hypoxia-inducible factor 1α (HIF1α) [71], the primary transcription factor responsible for activating angiogenic expression programs in response to the low-oxygen conditions frequently encountered by rapidly dividing tumor cells.

Table 1.1 The effect of high CCL2 on the progression of different human cancers

Cancer	Effect of elevated CCL2	References
Breast cancer	Early relapse; enhanced dissemination and metastasis	Ueno [109], Heiskala [35], Linde [57], Wang [115]
Prostate cancer	Enhanced tumor aggressiveness	Lu [65]
Cervical cancer	Lower relapse-free survival	Zijlmans [131]
Colon cancer	High probability of metastasis	Wolf [117]
Pancreatic cancer	Reduced survival	Sanford [91]
Gastric cancer	Enhanced tumor vascularization	Ohta [77]
Medulloblastoma	Enhanced metastasis to the leptomeninges	Garzia [30]

AP-1 is also activated downstream of major cell survival signaling pathways, such as extracellular regulated kinase (ERK) and protein kinase B (PKB, aka Akt) which may explain why upstream effectors of Akt signaling, such as Kras mutations, also trigger CCL2 expression [1]. In addition, it was recently reported that CCL2 is expressed as a consequence of retinoblastoma (RB) gene inactivation – another major cell proliferation pathway [55]. CCL2 is also a target of the transcription factors Twist1 [62] and Snail [38], which are involved in epithelial to mesenchymal transition (EMT) in cancer cells. CCL2 expression was also recently shown to be influenced by polycomb repressor complex 1 [103], a repressor of developmental genes which is frequently dysregulated in cancer. Thus, CCL2 is transcriptionally regulated by a number of signaling pathways linked to tumorigenesis (Fig. 1.1), which explains why this chemokine is frequently upregulated in a number of different cancer cell types compared to their non-neoplastic counterparts, including breast carcinoma [112], hepatocellular carcinoma [54], prostate cancer [65], and melanoma [34, 74]. However, cancer cells are not the only cells in the tumor microenvironment capable of producing CCL2. In addition to tumor cells, CCL2 expression has been reported to emanate from a number of other cell types in the tumor microenvironment, including TAMs, ECs [112], tumor-associated fibroblasts (TAFs) [96], and T-cells [79].

1.2.3 Pro-tumorigenic Effects of TAMs

Intuitively, CCL2-mediated recruitment of immune cells to sites of tumorigenesis might be expected to facilitate the detection and elimination of tumor cells, and particular subsets of macrophages within the tumor microenvironment may do so. For example, a high density of macrophages at the invasive edge of colorectal tumors has been reported to correlate with a better prognosis and fewer hepatic metastases [24, 129]. Thus, TAMs may be phenotypically heterogeneous [78], with different macrophage populations exerting pro- and anti-tumorigenic effects simultaneously at different locations within the same tumor. Advances in single-cell expression analyses may help to shed further light on the

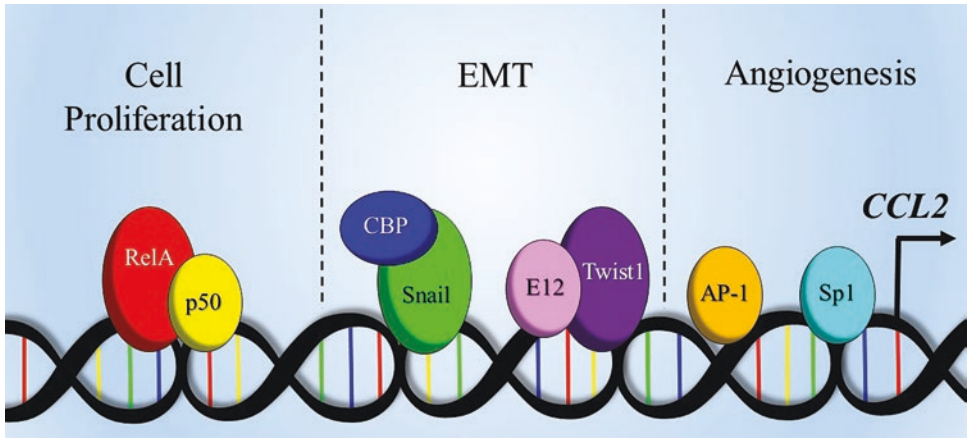

Fig. 1.1 Several biological processes associated with cancer progression induce CCL2 expression. CCL2 is a canonical transcriptional target of NFκB signaling, which is frequently upregulated in cancer and confers cell survival and proliferation advantages to cancer cells. Transcriptional repressors of the epithelial phenotype in cancer cells, such as Snail and Twist1, which facilitate the epithelial to mesenchymal transition (EMT) in cancer, also upregulate CCL2. Snail activates CCL2 transcription in cooperation with CREB-binding protein (CBP), whereas Twist1 is known to become a transcriptional activator in combination with E12 [48]. CCL2 expression is also enhanced by transcription factors associated with angiogenesis, such as Activator protein 1 (AP-1) and Specificity protein 1 (Sp1)

extent of in situ TAM heterogeneity in the future [66]. Nevertheless, the vast majority of in vivo and human data indicate that, in general, a high density of TAMs is associated with more aggressive malignancies, a higher probability of metastasis, and poorer outcomes in a variety of cancers, including colon cancer [4], lymphoma [102], breast cancer [50], and melanoma [107]. This is most likely due to the ability of tumor cells to coerce newly recruited monocytes to adopt a phenotype that supports tumor cell survival and proliferation. Many of the mechanisms by which TAMs promote tumorigenesis have been elucidated. TAMs are known to be recruited to hypoxic regions within tumors [51, 72] in a HIF1α-dependent manner [21] where they stimulate local angiogenesis [11, 82] through the secretion of a variety of factors, including VEGF, PDGF, thymidine phosphorylase (TP), as well as a number of chemokines and cytokines [18]. TAMs also produce matrix metalloproteinase-9 (MMP-9) and other extracellular matrix (ECM)-degrading enzymes, which further promote angiogenesis and facilitate tumor cell invasion [17, 21]. In addition, TAMs play an established role in suppressing cytotoxic T-cells through the expression or induction of the anti-inflammatory molecules programmed death-ligand 1 (PD-L1) [45], indoleamine 2,3-dioxygenase (IDO) [127], and arginine [95]. TAMs both produce and respond to transforming growth factor-β (TGF-β), which has immunosuppressive effects [101] and promotes EMT [126]. Moreover, TAMs have also been reported to recruit regulatory T-cells via CCL5 secretion, which further contributes to the immunosuppressive tumor microenvironment [93].

1.2.4 TAM-Independent Pro-tumorigenic Effects of CCL2

A number of studies have reported effects of CCL2 on the tumor microenvironment which may be as important if not more so than its role in TAM recruitment (Fig. 1.2). For example, CCL2 can recruit other myeloid cell subsets, such as myeloid-derived suppressor cells (MDSCs). In melanoma, CCL2 attracts $CCR2^+$ MDSCs, which promote tumor cell immune escape and accelerated tumor growth due to the creation of an anti-inflammatory microenvironment. Thus, depleting $CCR2^+$ MDSCs enhances cytotoxic $CD8^+$ T-cell recruitment and slows tumor growth [52]. A similar mechanism has been described in a mouse model of colorectal cancer [14]. CCL2 has also been implicated in the recruitment of mesenchymal stem cells to tumor sites [22], which enhance the motility and invasive capacity of cancer cells [41]. Some tumor cells, including prostate [65], bladder [13], and breast [39] cancer cells, express CCR2 themselves and can thus respond directly to CCL2. CCL2 directly augments cancer cell proliferation through activation of the phosphoinositide 3-kinase (PI3K)/Akt pro-survival pathway [59, 65, 89] and tumor cell motility and invasive capacity through activation of the Ras-related cytoskeletal regulatory GTPases, RhoA and Rac [60, 113]. Autocrine CCL2 also influences the migratory behavior of tumor cells through activation of protein kinase C (PKC) signaling and through phosphorylation of the focal adhesion adaptor protein, paxillin [13]. CCL2 influences the immunosuppressive tumor environment due to its ability to directly inhibit T-cell effector function in breast cancer models [114]. Moreover, investigators have shown that ECs express CCR2, ECs exhibit chemotactic activity toward CCL2, and CCL2 can directly promote angiogenesis in vivo independently of their role in TAM recruitment [31, 116]. Finally, CCL2 can also directly promote angiogenesis and support tumor cell invasiveness through induction of monocytic MMP-9 [85].

1.3 The Role of CCL2 in Cancer Metastasis

1.3.1 CCL2-Mediated Mechanisms in Metastasis

Consistent with the known roles of CCL2 in tumor cell invasiveness and angiogenesis, a strong association exists between CCL2 levels and the probability of tumor metastasis [76].

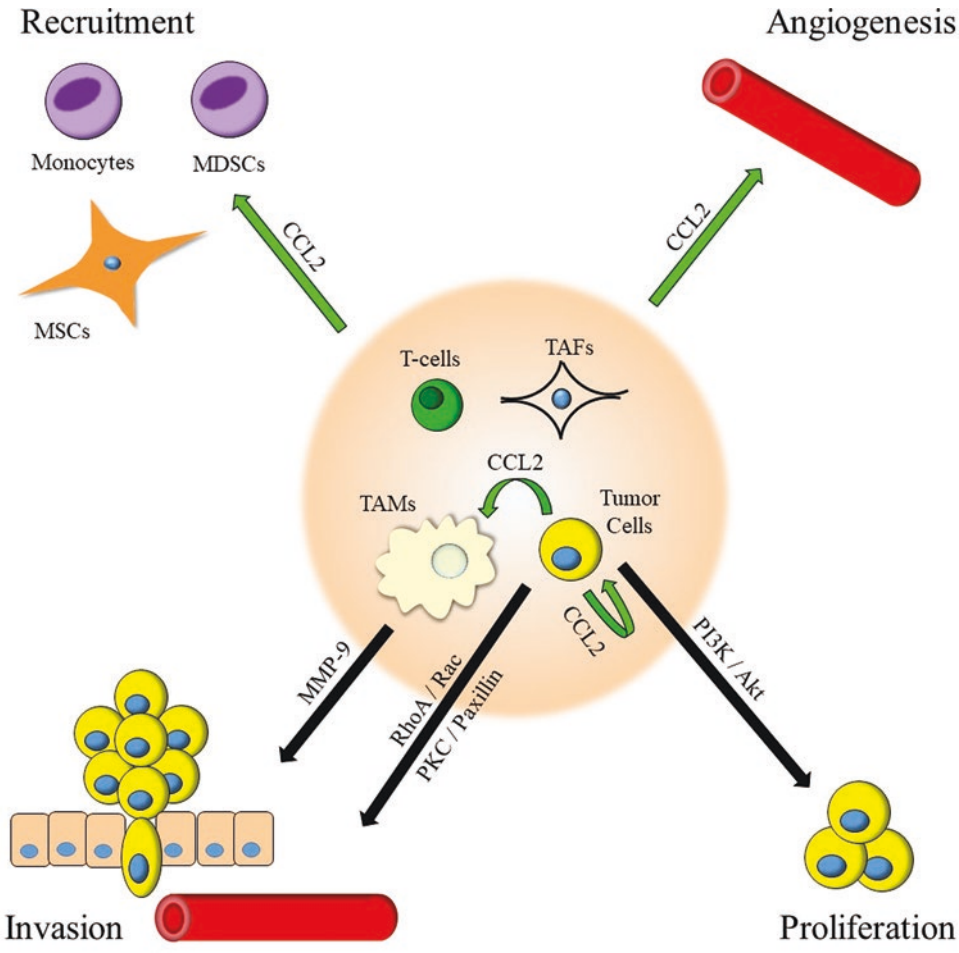

Fig. 1.2 The influence of CCL2 on the tumor microenvironment. CCL2 produced by tumor cells, tumor-associated macrophages (TAMs), tumor-associated fibroblasts (TAFs), leukocytes, and other local cells recruits myeloid cells, such as monocytes and myeloid-derived suppressor cells (MDSCs), and mesenchymal stem cells (MSCs), which support tumor cell survival, immunosuppression, and angiogenesis. CCL2 can also directly promote angiogenesis through engagement of CCR2 on endothelial cells. Stimulation of TAMs with CCL2 enhances metalloproteinase 9 (MMP-9) production, which promotes tumor cell invasion. Some tumor cells express CCR2 and can further enhance their own motility by activation of RhoA, Rac, protein kinase C (PKC), and paxillin and their own survival and proliferation through the PI3K/Akt pathway via autocrine CCL2 signaling

CCL2 appears to exert its pro-metastatic effects through a variety of mechanisms. For example, a role was identified for CCL2 in the metastasis of colon and lung carcinoma cells to the liver. Consistent with the idea that the primary function of CCL2 is to attract circulating immune cells, the effect of CCL2 on liver metastasis appeared to involve the recruitment of a specific myeloid cell subset to the liver with no role for the adaptive immune system identified [128]. However, the same study failed to identify this myeloid cell population in melanomas that had metastasized to the liver. In another study where CCL2 blocking antibody administered to mice with non-small-cell lung cancer slowed tumor growth and reduced the incidence of metastasis, the effect of CCL2 on monocyte recruitment was minimal. Rather, the main effect of CCL2 appeared to be on TAM polarization state, which ultimately determined the efficiency of $CD8^+$ T-cell recruitment [27]. Thus, the mechanisms operating within a particular metastatic cascade may, in

some instances, be specific to the type of malignancy and the metastatic site.

1.3.2 CCL2 in Tumor Cell Extravasation

Mechanistic studies have also identified a role for CCL2 in facilitating the extravasation of tumor cells across endothelia of potential metastatic sites. CCL2 derived from breast cancer cells can attract $CCR2^+$ monocytes to the lung and bone [63]. In turn, these recruited monocytes promote breast cancer cell extravasation via VEGF secretion [83] (Fig. 1.3). However, CCL2 also has myeloid cell-independent effects on the ability of tumor cells to traverse endothelia. For example, in a model of colon carcinoma metastasis to the lung, ablation of CCR2 on myeloid cells could not fully prevent lung metastasis, although CCR2 expression was required [117]. Moreover, deletion of CCR2 on endothelial cells was sufficient to significantly reduce the metastatic capacity of cancer cells [88]. CCL2 appears to directly engage CCR2 expressed on lung ECs, thereby enhancing vascular permeability [86, 87] through activation of JAK2/Stat5 and p38 signaling (Fig. 1.3) and EC contraction. Similar CCL2-driven mechanisms may drive transendothelial migration of multiple myeloma [40] and sarcoma cells [94]. However, consistent with reports that melanoma cells fail to recruit myeloid cells in liver metastases [128], melanoma cells appear not to require CCR2 signaling to metastasize to the lung [117], again highlighting the fact that

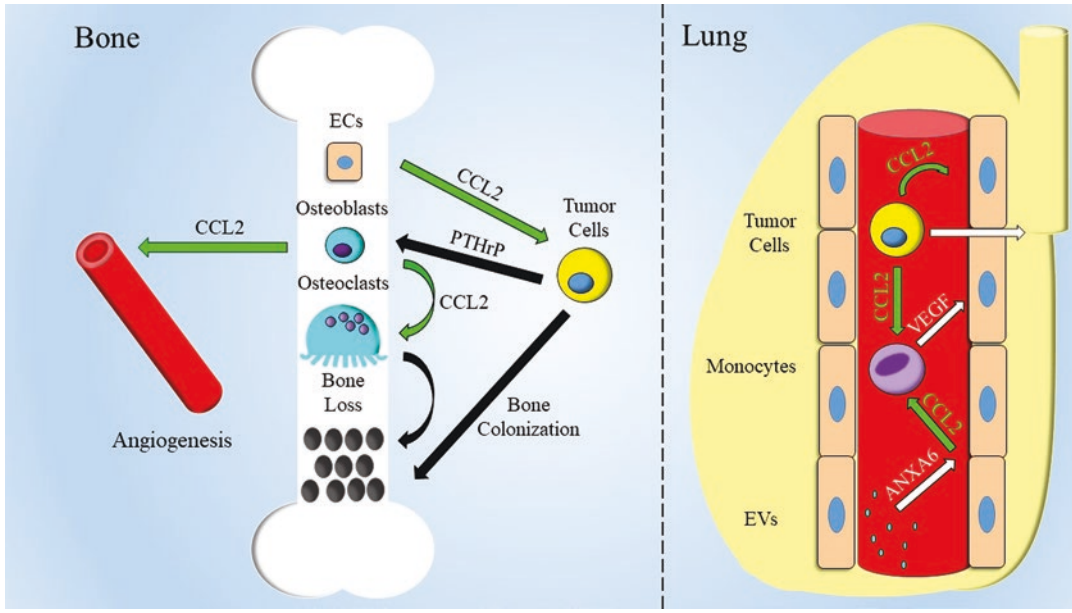

Fig. 1.3 Roles of CCL2 in cancer metastasis to the bone and lung. (Left) CCL2 secreted by bone marrow epithelial cells (ECs) stimulates parathyroid hormone-related peptide (PTHrP) production by tumor cells, which initiates CCL2 expression by osteoblasts. CCL2 produced by osteoblasts and ECs promotes angiogenesis and stimulates osteoclast activity. Enhanced osteoclast activity leads to bone resorption, which facilitates the colonization of bone by tumor cells. (Right) Tumor cells circulating in the lung blood vessels attract blood-borne monocytes via CCL2. Recruited monocytes promote tumor cell extravasation in the lung in a vascular endothelial growth factor (VEGF)-dependent manner. CCL2 secreted by tumor cells can directly engage CCR2 on ECs in the lung vasculature, thereby facilitating their own extravasation. In addition, extracellular vesicles (EVs) released from the primary tumor can stimulate CCL2 secretion from ECs and subsequent monocyte recruitment via annexin-A6 (ANXA6). The degree to which particular pathways and cell types are involved in the metastatic cascade likely depends on the type of malignancy and the tissue being colonized

different malignancies may utilize distinct mechanisms for metastasis.

1.3.3 Prostate Cancer

CCL2 signaling plays a particularly prominent role in the progression and metastasis of prostate cancer. As noted above, prostate cancer cells express elevated CCL2 compared to non-neoplastic prostate cells, and CCL2 enhances both the proliferation and invasiveness of prostate cancer cells [65]. In addition, parathyroid hormone-related protein (PTHrP) produced by prostate cancer cells can induce CCL2 production in osteoblasts, thus stimulating osteoclast activity and promoting angiogenesis [53]. Elevated osteoclast activity enhances bone resorption, thereby creating a "pre-metastatic niche" in which the likelihood that bone tissue becomes colonized by prostate cancer cells is increased (Fig. 1.3). CCL2 can also be produced by bone marrow ECs, which attracts prostate cancer cells and induces their proliferation [59].

1.3.4 Breast Cancer

Cancer metastasis is a complex, multi-step process. Therefore, studies at the organismal level that assess the contribution of an individual molecule or signaling pathway in specific cellular compartments are necessary to fully understanding the roles of these molecules at each location and stage of the metastatic cascade. For example, CCL2 has different effects on the metastasis of breast cancer cells, depending on the source of CCL2 and which stage in the metastatic cascade it is expressed. Non-tumor cell-derived CCL2 in the hematopoietic compartment seems to promote metastasis of breast cancer cells to the lungs [124], whereas tumor cell-derived CCL2 in the primary tumor inhibits metastasis of breast cancer cells to the lung and bone [104] through the recruitment of neutrophils to potential metastatic sites [33]. However, once breast cancer cells are in circulation, tumor cell-derived CCL2 can also contribute to metastatic seeding of distant organs [124]. Extracellular vesicles (EVs) shed from breast cancer cells at the primary tumor are also capable of initiating CCL2 expression at metastatic sites [42]. Breast cancer cells can also induce CCL2 secretion from osteoblasts [43, 130], promoting bone loss and colonization by breast cancer cells in a manner similar to that which has been described for prostate cancer.

1.4 CCL2 as a Therapeutic Target in Cancer Immunotherapy

Cancer immunotherapy is a rapidly advancing field of research, and drugs which attempt to boost T-cell responses against tumors, such as immune checkpoint inhibitors, are already in regular clinical use. TAM-targeted therapies are also being pursued, including drugs that aim to modulate the CCL2-CCR2 signaling axis. A monoclonal antibody against CCL2 (i.e., carlumab) was tested for the treatment of metastatic prostate cancer but was eventually discontinued due to lack of clinical benefit [81]. A number of small molecule CCR2 inhibitors are currently in clinical development for the treatment of pancreatic cancer [80] and have had some success in stabilizing disease progression as a combination therapy [75]. Finally, other therapies, such as histone deacetylase (HDAC) inhibitors, which are currently being pursued as cancer therapies, are thought to exert some of their anti-tumor effects via their ability to reduce endogenous CCL2 levels [58].

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CXCL3 Signaling in the Tumor Microenvironment

2

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Abstract

Cancer progression is driven, to a large extent, by the action of immune cells that have been recruited to tumor sites through interactions between chemokines and their receptors. Chemokines of the CXC subfamily are secreted by both tumor and non-tumor cells within the microenvironment of the tumor, where they induce either antitumor or protumor activity that fosters either clearance or progression of the tumor, respectively. Understanding the nature of these interactions is important to envisage novel approaches targeting the essential components of the tumor microenvironment, increasing the odds for favorable patient outcomes. In this chapter we describe the involvement of the chemokine (C-X-C motif) ligand 3 (CXCL3) in the human tumor microenvironment and its effects on immune and non-immune cells. Because of the limited data on the CXCL3 signaling in the tumor microenvironment, we extend the review to other members of the CXC subfamily of chemokines. This review also addresses the future trends or directions for therapeutic interventions that target signaling pathways used by these molecules in the tumor microenvironment.

Keywords

Tumor · Tumor microenvironment · Chemokine · Inflammation · CXC chemokine · Neovascularization · ELR motif · Chemokine receptor · Carcinogenesis · Stromal cells · Fibroblasts · Macrophages · Neutrophils · Extracellular matrix · CXCL3

2.1 Introduction

In addition to tumor cells, solid tumors also contain a variety of other cell types, and the multitude of communications and interactions among all these cells help to create the tumor microenvironment (TME) [1, 2]. It is widely recognized that the TME actively participates in the cancerous process, enhancing proliferation, survival, and migration of the tumor cells [3]. Crosstalk among the cells populating the microenvironment of the tumor is driven by a complex and dynamic network of signaling pathways activated by cytokines, chemokines, and growth factors and by the action of inflammatory molecules and matrix remodeling enzymes [1, 2]. Among the non-malignant cells present in this complex

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microenvironment, we can mention stromal cells (mainly fibroblasts), specialized cells such as endothelial cells and pericytes, and a number of infiltrating inflammatory cells (including neutrophils, macrophages, and lymphocytes). These non-malignant cells of the TME play a dynamic role at every stage of carcinogenesis and progression that eventually determines whether the primary tumor is cleared, undergoes apoptosis, senesces, grows, and/or metastasizes [4] (Fig. 2.1). Tumor cells and stromal cells, as well as the tumor-associated leukocytes and vascular endothelial cells, all contribute in different proportion to the local production of chemokines inside the tumor. They also become the targets of chemokines that affect tumor cell proliferation, invasiveness, and metastasis [1, 2].

Chemokines are a family of chemoattractant cytokines that signal through cognate seven-transmembrane G protein-coupled receptors to mediate trans-endothelial leukocyte migration to sites of inflammation and to secondary lymphoid organs through interactions with selectins and integrins expressed on leukocytes [5, 6]. The complex network created by the interaction between chemokines and their receptors controls leukocyte migration, not only during infection and inflammation but also in tumor-related processes, such as angiogenesis and chemoresistance [7, 8].

The chemokine family has been subclassified into four subfamilies based on the position of the conserved cysteines in the amino-terminal end of these molecules: CXC, CC, CX3C, and C chemokine ligands, where X represents any amino acid [9]. Among the factors produced by both the tumor cells and non-transformed cells that are known to regulate angiogenesis, increasing evidence supports the importance of the CXC chemokines in this critical process [10]. Most members of the CXC subfamily are induced by inflammatory stimuli, and they are further classified into two groups based on the presence of a conserved three amino acid motif (the "ELR motif": Glu-Leu-Arg) preceding the first conserved C [11, 12]. The ELR-containing

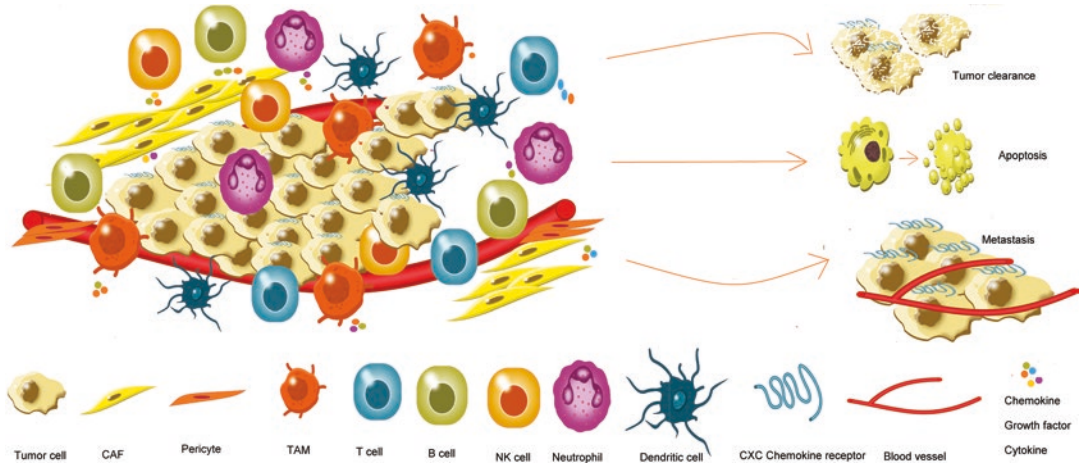

Fig. 2.1 Cellular crosstalk in the tumor microenvironment. Besides tumor cells, solid tumors also contain a variety of other cell types, and the multitude of communications and interactions among all these cells help to create the tumor microenvironment. Crosstalk among cells in the TME is driven by a complex and dynamic network of signaling pathways activated by cytokines, chemokines, and growth factors and by the action of inflammatory molecules and matrix remodeling enzymes. Among the non-malignant cells in this complex microenvironment are stromal cells (mainly fibroblasts, endothelial cells, and pericytes) and various infiltrating inflammatory cells (including neutrophils, macrophages, and lymphocytes). These non-malignant cells of the TME play a dynamic role at every stage of carcinogenesis and progression that ultimately determine whether the primary tumor is cleared, undergoes apoptosis, senesces, grows, and/or metastasizes. Tumor cells and stromal cells, as well as the tumor-associated leukocytes, all contribute in different proportion to the local production of chemokines inside the tumor. CAF (cancer-associated fibroblast), TAM (tumor-associated macrophage)

(ELR+) CXC chemokines are potent promoters of angiogenesis, a process that is essential for the growth of tumors [10]. ELR+ CXC chemokines include interleukin-8 (CXCL8), epithelial-derived neutrophil-activating protein 78 (CXCL5), granulocyte chemotactic protein 2 (CXCL6), neutrophil-activating peptide 2 (CXCL7), melanoma growth stimulatory activity alpha (GRO-α/CXCL1), melanoma growth stimulatory activity beta (GRO-β/CXCL2), and melanoma growth stimulatory activity gamma (GRO-γ/CXCL3) [11]. Chemokine action is also important for tumor initiation, promotion, and progression [5, 13] as breakdown in the control of leucocyte mobilization contributes to chronic inflammation, which in turn may favor the process of carcinogenesis by providing a microenvironment that is suitable for tumor cell development and growth [14, 15]. Inflammation is a promoter for neovascularization, also known as angiogenesis, a process that leads to formation of new vessels and that requires the action of angiogenic factors, which bind to specific receptors on the surface of endothelial cells of pre-existing blood vessels [16].

2.2 Cells and Chemokines in the Tumor Microenvironment

In addition to extracellular matrix components, tumors in general harbor both malignant and non-malignant cells. The tumor stroma contains specialized connective tissue cells, including fibroblasts, mesenchymal stromal cells, osteoblasts, and chondrocytes, and the extracellular matrix; it also includes other specialized cell types, such as endothelial cells, pericytes, adipocytes, and immune cells [17]. After years of intense research, it has become clear that non-malignant cells recruited from the local host stroma by the tumor cells themselves play a pivotal role in cancer development [18]. In addition to immune cells, non-malignant cells, such as CAFs and pericytes, are increasingly recognized as important cellular components of the tumor microenvironment [19]. Pericytes typically surround blood vessels and communicate with the endothelial cells by paracrine signaling and physical contacts [20, 21]. They actively participate in the inflammatory response playing a leading role in angiogenesis, promoting endothelial cell survival and migration [20]. In response to proinflammatory stimuli, such as LPS (lipopolysaccharide), these perivascular cells express a multitude of inflammatory mediators, including the ELR+ CXC chemokines CXCL3, CXCL1, CXCL2, and IL-8 [22]. The specific receptors for these chemokines, CXCR1 and CXCR2, are expressed by endothelial cells and different types of tumor cells (Fig. 2.2). Reciprocal interactions among malignant and non-malignant cells (including stromal and inflammatory cells) lead to the activation of cellular processes mediated by different growth factors, chemokines, and cytokines, which results in extracellular matrix remodeling, cell migration, neo-angiogenesis, invasion, drug resistance, and evasion of immunosurveillance [23]. Many cancers exhibit altered chemokine secretion profiles that favor the recruitment of protumorigenic immune cells while preventing the accumulation of anti-tumorigenic effector cells [24]. Chemokines in general attract different immune cells that express their cognate receptors, such as monocytes and neutrophils, inducing their migration into the microenvironment, where they differentiate, respectively, into tumor-associated macrophages (TAMs) or tumor-associated neutrophils (TANs); these two cell types then contribute to the regulation of the tumor immune responses in a spatiotemporal manner [13, 25]. Migration of neutrophils to tumor sites involves the interaction between CXCR2 expressed on the neutrophil surface and its ligands (CXCL1, 2, 3, 5, 6, 7, and 8) released by cells in the TME [26] (Fig. 2.2).

Although TANs make up a significant part of the cells present in the TME, TAMs and the cancer-associated fibroblasts (CAFs) outnumber them by far [27, 28]. In fact, TAMs are the most abundant immune cells in the microenvironment of solid tumors, comprising up to 50% of the tumor mass, and their abundance is associated

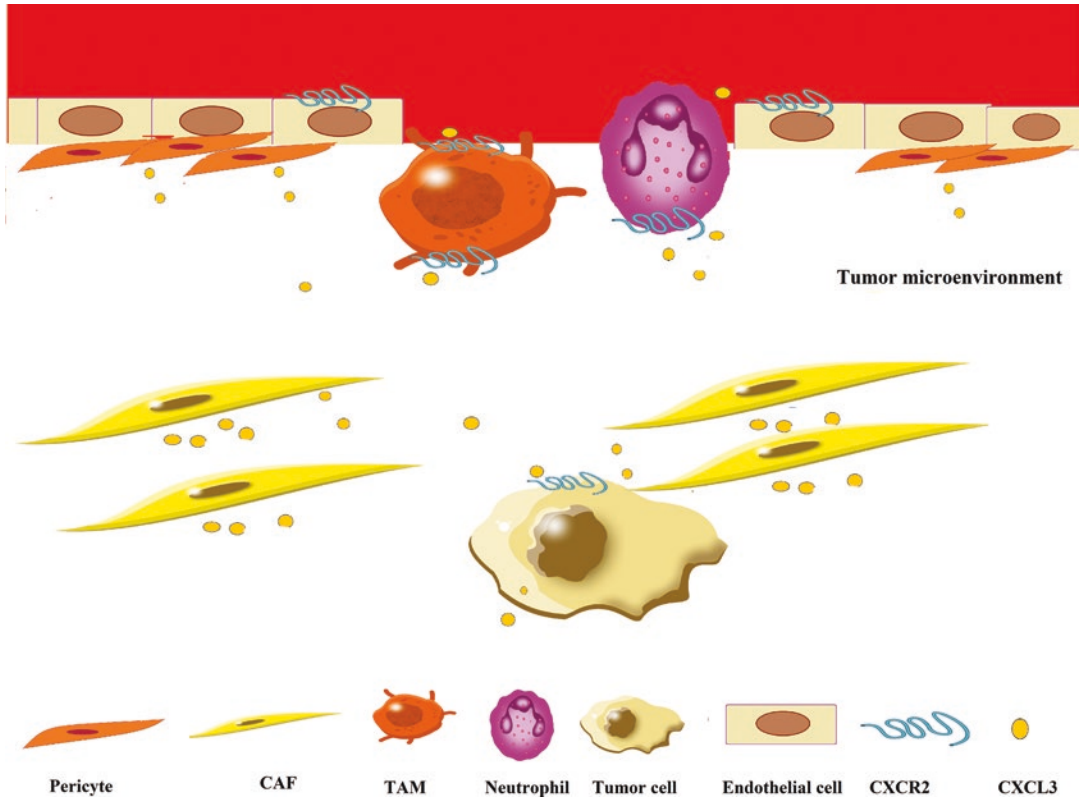

Fig. 2.2 CXCL3 and CXCR2 expression from cells in the TME. Tumor cells, macrophages, neutrophils, and endothelial cells express CXCR2 and other chemokine receptors. Tumor cells, CAFs, and pericytes secrete CXCL3 and other chemokines leading to leukocyte infiltration, including neutrophil and macrophage recruitment. Tumor cells interact with cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), neutrophils, and endothelial cells

with a poor clinical outcome in the majority of cancers [29].

During tumor development a number of phenotypically distinct TAM populations arise under the action of factors released by cancer cells, including cytokines, glucocorticoids, extracellular vesicles, and extracellular matrix components [30]. Most TAMs have the alternatively activated M2 phenotype and express multiple cytokines, proteases, and chemokines that promote tumor angiogenesis, in contrast to classically activated M1 macrophages that exhibit antitumor activities [29]. Several studies reported that TAMs release CXC chemokines that promote cancer growth and metastasis, such as CXCL1 and CXCL8, among others [31].

The other major cell type, CAFs, plays a pivotal role in tumorigenesis, actively participating in the growth and invasion of the tumor cells by sustaining a TME unique for each cancer type [27, 28]. CAFs are activated fibroblasts, and they comprise the major stromal component in the various types of malignancies [32]. Signaling crosstalk between CAFs and tumor cells may induce both types of cells to modify the surrounding tissue components, such as the extracellular matrix (ECM) and the basement membrane [27], favoring cancer progression [27]. CAFs originating from different cancers are highly heterogeneous among them [27], suggesting that cancer cells are able to induce specific subpopulations of CAFs that enhance growth and progression of the particular tumor [33]. CAFs

present in tumor regions are able to do this due to the variety of cell-cell interactions that they promote and the action of different secreted factors, including cytokines, chemokines, and various inflammatory mediators [19].

2.3 CXCL3 Signaling and Involvement in Cancer

CXC chemokines are secreted factors consistently shown to affect tumor progression and spread [10, 34]. CXCL3 is a member of the CXC chemokine subfamily, and it is subclassified as a Glu-Leu-Arg (ELR+) CXC chemokine [11, 12]. This chemokine, along with CXCL1 and CXCL2, was originally identified in the supernatants of melanoma cell lines in culture [35], and they are collectively referred to as GROs (growth-related oncogenes) [12, 36]. These three chemokines originated from gene duplication events during the course of chemokine evolution [37]. Different combinations of CXC chemokines, including CXCL3 and other soluble factors, are released by TAMs, CAFs, and tumor cells present in the TME [13, 38, 39].

The protein encoded by the CXCL3 gene is a secreted growth factor that signals through its cognate receptor CXCR2, a 7-transmembrane G protein-coupled receptor [40]. This receptor is present on endothelial cells [41] and in a variety of other cell types also present in the TME, including neutrophils, monocytes and macrophages, eosinophils, mast cells, and oligodendrocytes [42–45]. CXCR2 is also expressed by different types of cancer cells, including prostate [46], lung [47], and melanoma [48], among others.

Paracrine and autocrine interaction between CXC chemokines and CXCR2 triggers intracellular signaling pathways in cancer cells; upon ligand binding, the receptor becomes phosphorylated on serine/threonine residues at the C-terminus; it changes its conformation and activates the coupled heterotrimeric G protein [49]. Activation involves GTP binding to Gα subunit leading to disassociation from its Gβγ subunit partners, initiating the corresponding signaling pathways involving PI3K, p38/ERK, and JAK2/STAT3 pathways, leading to the regulation of cell survival and migration [40, 50–52]. CXCR2 also drives immune escape and chemoresistance in several human cancers [53–56]. Several studies performed with CXCR2 knockout mice in malignancies such as melanoma and prostate and renal cancers showed that these animals were less susceptible to spontaneous tumorigenesis [52, 57–61]; thus, this receptor can be viewed as an attractive target for therapy.

CXCL3, through its binding to CXCR2, has been shown to be a chemoattractant for neutrophils [62], and its involvement in metastasis, angiogenesis, and wound healing has been previously mentioned [63, 64]. The cancer types affected by the action of CXCL3 (along with CXCL1 and CXCL2) include prostate cancer, pancreatic cancer, melanoma, lung cancer, hepatocellular carcinoma, and gastric cancer [50, 65–69]. In vitro studies indicated that CXCL3 chemokine, besides CXCL1 and CXCL2, was expressed by prostate stromal cells in response to IL-1 secreted by epithelial cells, leading to the suggestion that these interactions might contribute to prostatic inflammation and progression at early stages of prostate cancer development [3]. The mechanism proposed for this effect implied that prostatic epithelial cells are able to secrete cytokines of the IL-1 family, which then activate the IL-1-signaling pathway in the stromal cells, triggering them to secrete this group of CXC chemokines. IL-1 has been previously shown to stimulate the NF-κB signaling pathway leading to the transcriptional activation of CXCL1, CXCL2, CXCL3 [70, 71], and CXCL8 [72], what is supported by the finding that the genes encoding CXCL1, CXCL2, and CXCL3 contain a conserved NF-κB-responsive element in their promoters [73].

In addition to stromal cells, epithelial cells from different cancer types, such as colon cancer [63], prostate cancer [69], and breast cancer [34], also display increased expression of CXCL3. In colon cancer, *Doll* et al. [63] reported increased transcript expression of CXCL3 along with CXCL2 and CXCL8 in resected colon carcinoma from a cohort of 97 patients. Compared to normal

colon tissue, colon cancer showed higher expression of these three chemokines; furthermore, evaluation of the three colon cancer cell lines (HT29, HCT116, and CaCO2) found that IL-1alpha strongly induced the expression of all the three chemokines by these cell lines, indicating a potential link to inflammatory processes [63]. Recent work also proposed that CXCL3 might serve as a novel biomarker in the diagnosis and prognosis of colon cancer based on the finding that high expression of CXCL3 was associated with considerably increased mortality in colon cancer patients [74].

In the prostate, studies have shown that prostate cancer cell lines and prostate cancer tissue overexpress both the CXCL3 and its receptor CXCR2, which may suggest a role for the CXCL3/CXCR2 axis in prostate cancer progression and metastasis [69]. This is supported by studies showing that overexpression of CXCL3 both in the aggressive PC-3 cell line and in prostatic cancer tissue correlated with metastasis [69, 75]. CXCL3 has been shown to function as a chemoattractant for neutrophils to areas of brain injury [76] and for cerebellar progenitor cells [77]; however, it is not clear whether this chemokine is chemoattractant or not for prostate cancer cells [69]. Decreased expression of CXCL3 has been found in PC-3 cells subjected to siRNA knockdown of the proteoglycan endocan (endothelial cell-specific molecule-1/ESM-1), a marker for angiogenesis [78].

This chemokine has also been suggested as a potential therapeutic target for hepatocellular carcinoma (HCC), since this cancer overexpresses this chemokine and HCC patients with higher CXCL3 expression correlated with a poorer prognosis [50]. Aggressive breast cancer cell lines also secrete high protein levels of this chemokine, and overexpression of its transcript levels has been identified in a large number of breast tumor samples, which prompted the suggestion of CXCL3 as a potential target for breast cancer metastasis [79].

Despite all the published studies that have reported the expression of CXCL3 in different tumor types, we still lack detailed information about the biological role of CXCL3 in the tumor microenvironment and the underlying mechanisms through which this chemokine affect tumor growth and spread. It is feasible that, after being released from different cells in the TME, CXCL3 acts in a paracrine and autocrine fashion by binding to both tumor and immune cells bearing the CXCR2 receptor on their surface. Ligand binding then leads to the activation of signaling pathways involved in processes associated to cell proliferation, migration, immune escape, and chemoresistance.

2.4 Future Trends in Tumor Research

Development of effective therapies against cancer has been hampered by the tumor heterogeneity that arises during the progression of cancer [80–83]. This heterogeneity paves the way for resistance to arise since the different subpopulations of cells within the tumor may express distinct molecular signatures with varied levels of treatment sensitivity [83]. Tumor heterogeneity is attributed in part to the dynamics of the surrounding microenvironment that greatly favors interactions of CAFs with tumor cells in proliferation, facilitating selection of those tumor cells better suited to that particular microenvironment [84]. The last few years have witnessed an increased interest in targeting the tumor microenvironment as a therapeutic approach in cancer.

CXC chemokines and their cognate receptors are among the factors present in the TME that significantly affect tumor development and progression. These molecules have been proposed as prognostic factors, as biomarkers of response to therapy, and as drug targets. The CXCLs/CXCR2 axis plays a vital role in the tumor microenvironment, and it is a target of therapeutic strategies for cancer and related diseases [85, 86]. Blockade

of the CXCLs/CXCR2 in the TME shows promise as a therapeutic approach against several cancer types [38, 87]. Several preclinical models [55, 88–91] and clinical trials [92] have been performed for CXCR2 receptor in different types of cancers. CXCR2 antagonists have been suggested as novel prophylactic or therapeutic anticancer treatments [55, 57].

An ideal target for therapy in cancer patients is TAMs, due to their ability to polarize toward different phenotypes [93, 94]. They are able to switch from proinflammatory and antitumorigenic (secreting cytokines to recruit T cells) to anti-inflammatory and protumorigenic (expressing transforming growth factor-β or secreting angiogenic vascular endothelial growth factors) [94].

Since approaches to eliminate protumorigenic TAMs from tumors have failed supposedly because of the concomitant elimination of antitumorigenic TAMs [95], the attention has focused toward the reprograming or re-education of TAMs. In this regard, a recent strategy that has been proposed to promote tumor inhibition is re-education of TAMs based on CXCR2 blockade [96]. Using either a selective antagonist of this receptor or an infusion of autologous CXCR2 knockout (KO) monocytes in tumor models in vivo has been shown to re-educate TAMs toward a TNFα-releasing pro-inflammatory phenotype, which resulted in induction of senescence and tumor inhibition [96].

Research has shown that members of the CXC family of chemokine ligands, known to bind the CXCR2 receptor, were increased in prostate cancer and that their levels correlate with cancer progression, lending support to previous evidence demonstrating that the CXCL-CXCR pathway plays a substantial role in tumor development [96]. Thus, effective therapies aimed to disrupt pathways involved in CXC chemokine signaling may be critical for tumor treatment in many cancer types.

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3 *CXCL8* Signaling in the Tumor Microenvironment

Sahana Asokan and Obul Reddy Bandapalli

Abstract

The tumor microenvironment represents a dynamic and complex cellular network involving intricate communications between the tumor and highly heterogeneous groups of cells, including tumor-supporting immune and inflammatory cells, cancer-associated fibroblasts, endothelial cells, tumor-associated macrophages, adipose cells, and pericytes. Associated with a variety of growth factors, chemokines, cytokines, and other signaling molecules, the interaction between the tumor microenvironment and the tumor cells empowers aggressiveness of tumor by enhancing its survivability. *CXCL8* (also known as Interleukin 8), a multifunctional proinflammatory chemokine that was initially classified as a neutrophil chemoattractant, recently has been found to be a key contributor in tumorigenesis. The upregulation of *CXCL8* at the tumor invasion front in several human cancers suggests its interplay between the tumor and its microenvironment rendering tumor progression by enhancing angiogenesis, tumor genetic diversity, survival, proliferation, immune escape, metastasis, and multidrug resistance. The autocrine and paracrine modulation of CXCL8 via the chemokine receptors CXCR1/2 promotes several intracellular signaling cascades that fosters tumor-associated inflammation, reprogramming, epithelial-mesenchymal transition, and neovascularization. Hence, decrypting the regulatory/signaling cascades of CXCL8 and its downstream effects may harbor prognostic clinical prospects of a tumor microenvironment-oriented cancer therapeutics.

Keywords

CXCL8 · Interleukin-8 · CXCR1/2 · Chemokines · Tumor microenvironment · Cancer · Invasion front · Tumor-related inflammation · Intracellular signaling cascade · Survival · Metastasis · Angiogenic switch · Epithelial-mesenchymal transition · Autocrine signaling · Angiogenesis

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3.1 Introduction

Tumor development and advancement relies on multiple genetic changes and parallelly can be largely defined by the variety of alterations in the surrounding normal tissues. These critical changes assure the survival of the cancer cells at the expense of the surrounding normal tissue. A suitable microenvironment is sculpted by reprogramming the surrounding cells, thereby facilitating the secretion of various cytokines, chemokines, and other favorable factors to sustain its growth and other nutritional requirements. A close resemblance has been observed between the changes that occur during tumor formation and healing of chronic inflammation. During inflammation, the initial ischemic condition results in the infiltration of the immune cells at the site which later induces the angiogenesis for nourishing the repaired tissues [1]. Similarly, it has been found that the tumor cells elicit an inflammatory response on the host which have been confirmed by the presence of immune cells in the cancerous and pre-cancerous lesions. The tumor cells escape these immune surveillances by immune selection and immune evasion. Recent studies illustrate the role of chemokines and cytokines as critical autocrine and paracrine factors in recruiting and activating various inflammatory cells, thus helping the formation of tumor-related inflammatory microenvironment to help the cancer cells to evade immune destruction [2]. Several experimental evidences with solid scientific data support the role of chemokines in attracting and maintaining the immune cells at the site of the tumor and inducing protumorigenic actions, including cytoproliferative, proangiogenetic, and pro-metastatic effects.

Chemokines are a family of cytokines with structural homogeneity of 8–10 kDa proteins with conserved cysteine residues which function by interacting with a subset of seven-transmembrane, G protein-coupled receptors (GPCRs). These are secreted as chemoattractants in diverse tissue environments which consequently signal for immune surveillance and thereby signal cell migration and inflammatory responses and play a key role in the development and maintenance of the homeostasis, thus providing the first-line host defense against microorganisms and tissue injury. Chemokines are identified based on their primary amino acid sequence and the orientation of the cysteine residues which form disulfide bonds, thus maintaining the monomeric chemokine structure. This conserved structure comprises of a central three stranded β-sheet, C terminus α-helix, and a small unstructured N-terminus for receptor activation [3]. Based on the precise variation of the configuration of the first two out of four sequentially conserved cysteine residues closest to the N-terminus, the chemokines are further grouped into four subfamilies: CC, CXC, CX_3C, and XC. For instance, in chemokine CC, the cysteines are directly juxtaposed; on the other hand, in chemokine CXC, a single variable amino acid is positioned in between the two cysteines. Similarly, in CX_3C chemokine, three amino acids are found between the two cysteine residues. The XC chemokines lack the first and third cysteines of the motif, and one form is found in mice and two in humans. Despite the initial functional nomenclature of the chemokines, this systematic nomenclature based on the cysteine position was introduced in the early 2000s. The nomenclature of the cytokine is generally written as subfamily designation (CC, CXC, CX_3C, and XC) followed by the letter L (referring to the ligand) and a number denoting the genes which code for the specific chemokine [4]. Based on their prominent function, they are classified under two major groups: homeostatic chemokines (*CCL14*, *CCL19*, *CCL20*, *CCL21*, *CCL25*, *CCL27*, *CXCL12*, and *CXCL13*) which are constitutively expressed and involved in homeostatic leukocyte trafficking and inflammatory chemokines (*CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, *CXCL7*, *CXCL8*, *CXCL9*, *CXCL10*, *CXCL11*, and *CXCL14*) which are induced by inflammation. Although they evolved to benefit the host, irregular chemokine/chemokine receptor expression, regulation, and utilization associate to or even cause array of pathological conditions and diseases [5].

The different chemokine receptor patterns have been exploited by the tumor cells for their

sustenance and maintenance of the metastatic potential to the target organs. It has been reported that the members of CXC family were among the first few chemokines identified to mediate tumorigenesis. Among the different members of the family, *CXCL8* is found to be a prototypical chemokine responsible for the recruitment and activation of granulocytes and neutrophils at the site of inflammation [6]. It is one of the dominant transcriptional targets of the inflammatory signaling-mediated activation of *nuclear factor-κB (NF-κB)*. *CXCL8* triggers its signaling by interacting with specific G protein-coupled receptors (GPCR), *CXCR1* and *CXCR2* [7]. This oncogenetic chemokine is found to mimic the vascular endothelial growth factor (VEGF) function in activating *VEGF-R2* for angiogenesis [8]. Studies have also found that *CXCL8* helps in the enhancement of the proliferation and survival of the endothelial cells and the matrix metalloproteases, *MMP-2* and *MMP-9* [9]. Furthermore, the upregulation of *CXCL8* expression at the invasion front of the tumor cells, multidrug resistance, and its role in metastasis open new avenues for targeting *CXCL8* as a potential therapeutic target.

Thus, the crosstalks between the proximal immune cells and the cancer cells and their reprogramming provides nourishment, thus fostering tumor growth, development, progression, proliferation, and metastasis. Hence, a clear understanding of the series of events occurring at tumor microenvironment and analysis of the role of *CXCL8* in the tumor microenvironment would provide great insights for the novel therapeutic approaches.

3.2 Tumor Microenvironment

Tumorigenesis is a complex and a multistep process, driven by oncogene activation and tumor suppressor gene inactivation caused by the successive mutations eventually resulting in the transformation of normal somatic cells into malignant tumor cells with enhanced proliferation and resistance to cell death. Decades of oncogenic studies have put forth several hallmarks that have been commonly observed in most human tumor types, which includes sustenance of proliferative signals, growth suppressor evasion, cell death resistance, ability to induce angiogenesis, enabling replicative immortality, genome instability and mutation, activation of invasion and metastasis, reprogramming energy metabolism, immune destruction avoidance, and tumor-induced inflammation [10, 11]. Recent studies have shown that, despite the traditional known driving force of tumor development and progression, the intercellular communication between the malignant and non-transformed cells, which forms the tumor microenvironment (TME), also plays a dynamic role in the tumor progression [12]. It has been found that the tumor microenvironment comprises of the resident fibroblasts, pericytes, endothelial cells, leukocytes, and extracellular matrix. The tumor cells can efficiently recruit stromal cells, immune cells, and vascular cells by secreting stimulatory cytokines, chemokines, growth factors, and inflammatory and remodelling enzymes, thereby building a suitable microenvironment [13–15]. Thus, new insights on tumor microenvironments would be potential targets for novel cancer therapeutic strategies.

3.2.1 Composition of Tumor Microenvironment

The composition and structure of the tumor microenvironment differ between the patients and also among different tumor types. Evidences from different studies have confirmed that the tumor cells recruit and reprogram the surrounding normal cells, mostly the fibroblasts, immune cells, and lymphatic and vascular cells, thus recruiting them at the primary tumor site building the tumor microenvironment, thereby contributing to tumor progression [16]. Based on the interactions, the tumor cells can change the nature of its microenvironment, and conversely, the tumor microenvironment can itself affect how the tumor grows and spreads.

3.2.2 Recruitment of Fibroblasts to the Tumor Microenvironment

A tumor is a highly complex tissue composed of stromal and neoplastic cells. Among the supporting cells, the fibroblasts represent the majority of the stromal cells in most types of human cancer. Activated fibroblasts inhibit the early stages of tumor progression with the help of simple gap junctions between *IL-6* production and fibroblasts [17, 18]. Later, these fibroblasts could be modulated by the tumor cells, thereby producing cancer-associated fibroblasts (CAFs). Among the non-immune components of the tumor microenvironment, the CAFs are responsible for the formation and remodelling of the extracellular matrix and promote growth of the tumor cells by constituently supplying the growth factors [19]. These can be identified by expression of various biomarkers like *vimentin*, *desmin*, *α-smooth muscle actin*, and *fibroblast-activated protein*. Yet, the primary source of CAFs still remains controversial as they are crucially involved in promoting growth and angiogenesis, remodelling of the extracellular matrix (ECM), and also directing the cell-cell interaction [20]. Frequently, inactivated or mutated *phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN)* and *p53* have been detected in CAFs around the primary tumor region [21]. The stimulated CAFs can secrete *stromal cell-derived factor 1 (SDF-1)* which facilitates the recruitment of circulating endothelial progenitor cells (EPCs) to stimulate angiogenesis into the tumor mass [22]. Certain studies also demonstrate that the CAFs also play a role in resisting the action of drugs such as tamoxifen in luminal breast cancer. Thus, further studies on the mechanism by which CAFs create a suitable tumor microenvironment would aid in new therapeutic approaches against tumor progression.

3.2.3 Recruitment of Vascular Cells to the Tumor Microenvironment

To meet the metabolic and nutrient needs for growth, cancer cells require the formation of vascular networks. This is facilitated by the activation of several pathways and factors that can induce angiogenesis such as platelet-derived growth factors (PDGFs), VEGFs, and fibroblast growth factors (FGFs). This can be correlated from different studies that have demonstrated high expression levels of *VEGF* in various human cancers, including breast, lung, kidney, bladder, and ovarian [23–25]. The cancer cells induce the production of the predominant angiogenic factor, *VEGF*, either directly or indirectly through the PI3K/AKT/mTOR (phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin) signaling pathway which leads to both *hypoxia-inducible factor 1(HIF-1)*-dependent and *hypoxia-inducible factor 1(HIF-1)*-independent VEGF secretions [26]. Advanced tumors also produce a range of other angiogenic factors as substituents for VEGF [27]. Recent studies also provide some insights on the essential roles of chemokine-stimulated endothelial cells and pericytes in the "turn on" mechanism of angiogenic switch, i.e., during neovascularization, which plays a major role in cancer growth [27, 28].

3.2.4 Recruitment of Immune Cells to the Tumor Microenvironment

The immune components of the tumor microenvironment play dual role in proregulatory and antitumor immune response. The tumor-infiltrating immune cells like the myeloid-derived suppressor cells (MDSC), cytotoxic lymphocytes, tumor-associated macrophages (TAM), and certain chemokines and cytokines comprise the immune components of the tumor microenvironment [29, 30]. Among these, the TAMs, representing the higher percentage, play critical roles in tumor-related inflammation. These are recruited at the sites with the help of VEGF, macrophage colony-stimulating factor (M-CSF), and monocyte chemotactic protein 1 (MCP-1) secreted by the tumor cells. The activation of toll-like receptor (TLR) pathways and the expression of immunosuppressive mediators like *transforming growth factor-β (TGF-β)*, *indoleamine 2,2-dioxygenase (IDO)*, *interleukin (IL)-10*,

VEGF, and *programmed cell death ligand 1 (PD-L1)* suppress antitumor immunity [31, 32].

Among the signaling molecules, the complex network of chemokine and chemokine receptors facilitates effective tumor cell metastasis. Among the CXC family, *CXCR4/CXCL12* pair is expressed in a variety of solid tumors like bladder, esophageal, head and neck carcinoma, lung, gastric, ovarian, colorectal, prostate and pancreatic cancer, melanoma, osteosarcoma, glioblastoma, neuroblastoma, and acute lymphoblastic leukemia [33]. It has been shown that *CXCL12* is involved in cancer cell trafficking to specific metastatic sites during the dissemination. The binding of ligands (CXCL6 and CXCL8) to CXCR1 has displayed well-established role in initiating and mediating breast, prostate, and lung cancer, melanoma, and colorectal carcinoma [34]. The binding of ligands (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8) to CXCR2 receptor mainly promotes angiogenesis, survival, invasion, and metastasis of lung, pancreatic, colorectal, renal, and prostate cancer [35]. In addition, the aberrant or upregulated expression of receptors of CC family has also been found to correlate with metastases in cervical, lung, prostate, and hepatocellular carcinoma, osteosarcoma, multiple myeloma, and T-cell leukemia [34]. Studies have shown that CXCL8 binds with high affinity to both CXCR1 and CXCR2 receptors, thus acting on leukocytes and endothelial cells promoting immune infiltration and angiogenesis. Thus, inspecting the role of *CXCL8* in the tumor microenvironment will provide more insights of survival and migration of the tumor cells from the primary site.

3.3 Role of *CXCL8* in Cancer

CXCL8, alternatively known as *Interleukin-8 (IL-8)* and *neutrophil-activating factor (NAF)*, belongs to the proinflammatory chemokines responsible for the recruitment and activation of neutrophil chemotaxis and degranulation. CXCL8 is secreted primarily by various cell types like the epithelial cells, endothelial cells, blood monocytes, alveolar macrophages, and fibroblasts, and its expression is induced by various cytokines (*CXCL12*, *IL-1*, *IL-6*, and *tumor necrosis factor α (TNFα)*) and other factors like hypoxia, environmental stresses, reactive oxygen species (ROS), bacterial particles, and also transcription factors like *NF-κB and AP-1* [7]. A simplified overview of the tumor microenvironment has been illustrated in Fig. 3.1.

Studies have shown the facilitation of *CXCL8* in different types of cancer, among which colorectal cancer and its liver metastasis show significant elevation of *CXCL8* signaling in the tumor microenvironment [36]. It has also been observed that *CXCL8* induces the epithelial-mesenchymal transition (EMT), thus aiding the tumor cells to escape host immune surveillance and thereby promoting colonization of the tumor cells at distant metastasis sites. Studies show the upregulation of the expression level of *CXCL8* at the invasion front of the tumor cells aids the colorectal liver metastasis and the corresponding expression of interfering (small hairpin) RNA (shRNA)-mediated *CXCL8* knockdown shows decreased proliferation, migration, and invasion in vitro [37].

3.3.1 Intracellular Signaling Pathways of *CXCL8*

Several well-characterized signaling pathways have found to be activated downstream of *CXCL8* receptors in the tumor microenvironment which emphasizes the importance of *CXCL8* in the progression of tumor. In this chapter, the key signaling pathways and its cellular responses have been described. It has been found that *CXCL8* profoundly induces the expression of phosphatidylinositol-3-kinase (PI3K) in most of its downstream intracellular signaling cascade which plays a critical role in modulating tumor cell survival, angiogenesis, and proliferation, by phosphorylating its substrate Akt [38]. Elevated *CXCL8*-induced *Akt* expression levels have been reported in androgen-independent prostate cancer (AIPC) cell lines [6]. *CXCL8* also activates the classical Raf-1/MAP/Erk cascade in both normal and cancer cells [39, 40]. Activation of

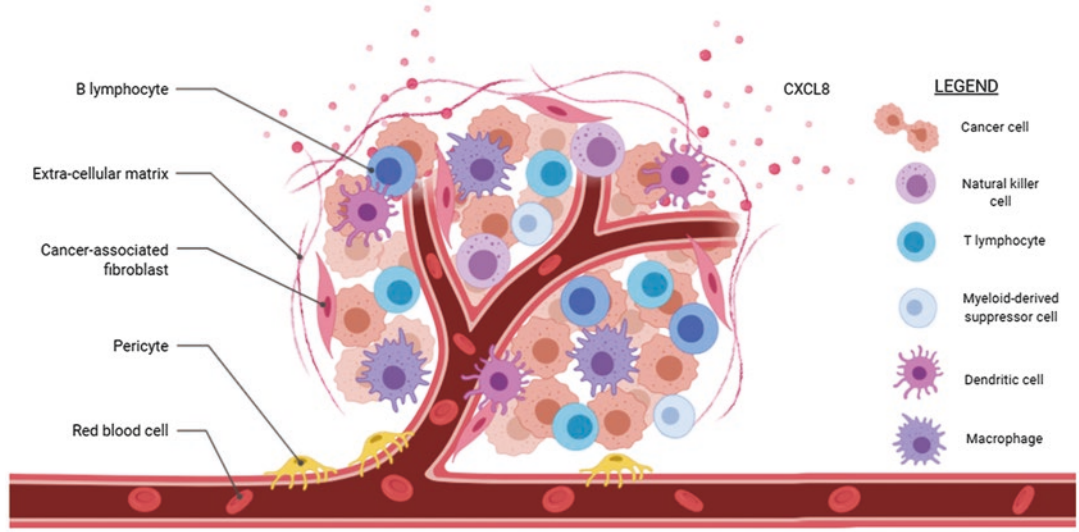

Fig. 3.1 Simplified overview of tumor microenvironment

MAPK signaling in neutrophils through PI3K and the transactivation of *epidermal growth factor receptor (EGFR)* by *CXCL8* result in the Ras-GTPase activation in lung and ovarian cancer cell lines [41, 42]. *CXCL8* activates the phospholipase C (PLC)-dependent protein kinase (PKC) signaling pathway, thereby stimulating the migration of cancer cells [43]. The overview of the various *CXCL8* signaling pathways has been illustrated in Fig. 3.2.

3.3.1.1 Activation of PI3K Cascade

Phosphatidylinositol-3 kinase (PI3K) is one of the key targets downstream of *CXCL8/CXCR1* and/or *CXCL8/CXCR2* stimulation in both neutrophils and cancer cells which consequently leads to the increased phosphorylation of its substrate PKB (protein kinase B, also known as Akt), a serine/threonine kinase [38, 44]. Phosphorylated Akt in turn phosphorylates a series of different proteins in the plasma membrane, nucleus, and cytosol which leads to cell proliferation, differentiation, and survival and also leads to other cellular responses. A major downstream effector of PI3K/AKT pathway is the activation of mTOR (mammalian target of rapamycin) which produces two complexes: mTORC1 and mTORC2. mTORC1 is found to be sensitive to rapamycin and is also activated by diverse stimuli like the growth factors, energy and stress signals, and other signaling pathways. Activated mTORC1 further activates S6K1 (ribosomal protein S6 kinase beta-1) and 4EBP1 (eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1) which are involved in mRNA translation, thereby controlling cell growth, proliferation, and inhibition of autophagy and thereby facilitating the survival of the cancer cells. mTORC2 is found to be insensitive to rapamycin and other nutrient and energy signals. It is found to activate *PKC-α (protein kinase C alpha)* and *AKT* and regulates actin cytoskeleton. *CXCL8*-stimulated activation of phospholipase C-dependent PKC signaling is found characteristic in different types of cancer [43]. *Phosphatase and tensin homolog (PTEN)* is found to be an important negative regulator of Akt signaling cascade functioning antagonistic to PI3K. Dysregulation of multiple elements like *PTEN* mutation, *PI3K* amplification/mutation, and *Akt*, *S6K1*, *4EBP1*, and *eIF4E* overexpression has been observed in different types of human cancers, especially in melanoma, where the alterations in major components of this pathway have led to significant effects on tumor progression [45]. Anomalies of *Akt* were found in different types of human cancers; gene amplifications of *Akt1* have been identified in glioblastomas, gliosarcomas, and gastric carcinoma, while

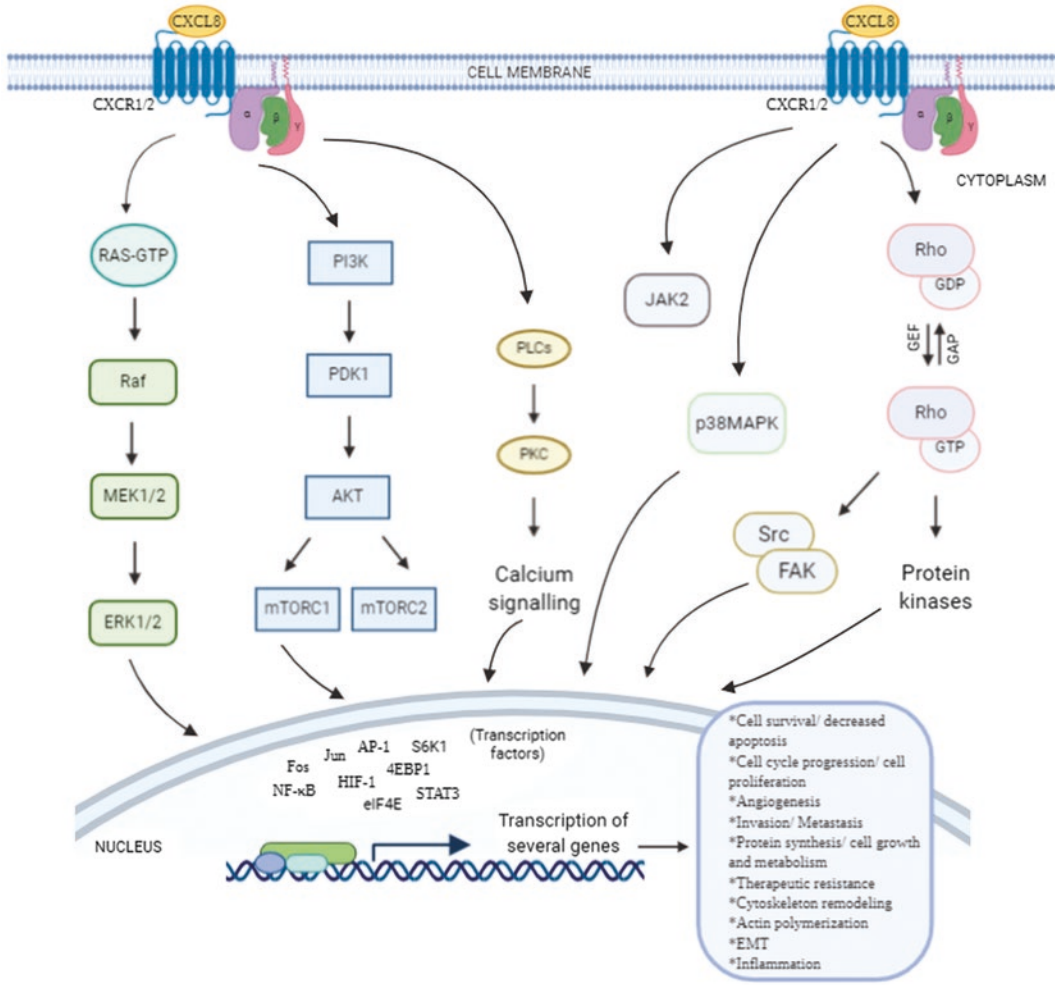

Fig. 3.2 Schematic figure illustrating various *CXCL8*-mediated signaling pathways

amplifications of *Akt2* have been reported in pancreatic, ovarian, and breast cancers and in head and neck squamous cell carcinoma [46]. Upregulation of *Akt3* has been described in studies of androgen-resistant prostate cancer cells, primary ovarian cancers, and also estrogen receptor-deficient breast cancer cells [47].

3.3.1.2 Activation of MAPK Cascade

The MAPK (mitogen-activated protein kinase) family consists of four major subfamilies of related kinases: *ERK1/2 (extracellular signal-regulated kinases 1/2)*, *JNK (c-Jun N-terminal kinases)*, *p38*, and *ERK5 (extracellular signal-regulated kinase 5)*. CXCL8 activates the classical MAPK signaling cascade constituting a number of serine/threonine kinases colocalized with their interacting scaffold proteins proximal to the cell-surface receptors. This leads to the substrate-specific activation of signaling through the RAF/MAP/ERK cascade, which is the best studied of the mammalian MAPK pathways. The signals from the cellular surface receptors like *EGFR (epidermal growth factor receptors)*, *GPCR (G protein-coupled receptors)*, and RTK (receptor tyrosine kinases) in response to the extracellular stimulus (like the growth factors, stress, hormones, etc.) activate the Ras and small GTPase. This complex (Ras-GTP) facilitates the formation of A-Raf, B-Raf, and C-Raf homodi-

mers or heterodimers by an intrinsic process which in turn activates MEK (MAPK and ERK kinase) (MEK1 and MEK2) and consequently catalyzes the activation of ERK (ERK1 and ERK2) by phosphorylation [48, 49]. This pathway involves a cascade of proteins which communicate by phosphorylating dozens of the neighboring cytosolic and nuclear proteins, thereby acting as an "on" or "off" switch and has been found in both neutrophils and cancer cells [38, 39]. Typically, both cytokines and growth factors have been found to bind to the tyrosine kinase receptor (TKR) consequently activating ERK1/2, thereby transducing signals upstream of Ras/Raf/MEK cascade which, depending on the cell type, regulates processes like proliferation, differentiation, migration, survival, angiogenesis, and chromatin remodelling and deregulation of these is common in cancer [50, 51]. Dysregulated MAPK signaling has been identified in a wide range of cancers via numerous mechanisms like mutations leading to abnormal expression of receptors, activation of receptors, and downstream signaling molecules by *CXCL8* and by other stimuli, which consequently leads to increased or uncontrolled proliferation of the cells and resistance to apoptosis, cell dissemination, and survival and confers resistance to chemotherapy, radiotherapy, and other targeted therapies [52].

The c-Jun N-terminal kinases (JNKs) belonging to the family of MAPK regulate several physiological processes including inflammatory response, cell proliferation, differentiation, morphogenesis, and cell survival and death and are produced in response to multiple stress factors like UV irradiation, osmotic shock, pathogens, growth factor deprivation, DNA-damaging agents, drugs, toxins, cytokines, and other stimulus [53]. CXCL8 signaling may also be regulated indirectly through the key regulators of signaling pathway and transcription factors (like HIF-1, AP-1, and NF-κB) responsible for CXCL8 and CXCL8 receptor regulation and expression [54, 55]. It has been found that the *CXCL8* expression is upregulated by the transactivation of NF-κB and JNK pathways and the *CXCL8* mRNA is stabilized by p38, another subfamily of MAPK consisting of four isoforms: α, β, γ, and δ [56]. Studies have shown that the crosstalk between the JNK and p38 pathways has emerged as a potent regulator of cellular responses and the expression of key inflammatory mediators, such as proteases and cytokines, which act as potent cancer enhancers [57]. Strategies targeting these signaling pathways and transcription factors can attenuate CXCL8 signaling in the cancer cells and thereby can function as a potent target to sensitize the cancer cells to conventional therapeutics.

3.3.1.3 Activation of Rho GTPase Pathway and Non-receptor Tyrosine Kinase Pathway

The Rho family of GTPases, a family of small signaling G proteins (range between 20 and 40 kDa in size), regulates the dynamics of the actin cytoskeleton, cell cycle progression, cell polarity, cell migration, metastasis, and invasion and aids in malignant transformation [58]. This family falls under the Ras superfamily and is divided into six subgroups: RhoA, RhoB, RhoC, Cdc42, Rac1, and Rac2. The Rho homologues were discovered earlier in the mammalian cells, while the later three, identified much later, were found to be functionally distinct yet shared a significant amino acid sequence homology with the other Rho proteins [59, 60]. The Rho GTPases switch between an inactive GDP-bound state and an active GTP-bound state which is regulated by GTPase-activating proteins (GAPs), guanine-nucleotide exchange factors (GEFs), and guanine-nucleotide dissociation inhibitors (GDIs) which interact with the cell membrane phospholipids and facilitate the GDP-GTP exchange activity [61]. GEFs accelerate the release of the bound GDP and substitute it by GTP to aid in activating the GTPase. GAPs inactivate the Rho GTPases by facilitating the hydrolysis of GTP, and the highly conserved arginine finger in the domain of 150 amino acids of the RhoGAPs has been identified to promote the catalysis of the GTPase activity [62]. GDIs monitor the cycling of certain Rho GTPases between

the cytosol and the membranes and also regulate the mechanism of activation and inactivation of the Rho GTPases. The interaction between the Rho GTPases and these molecules can also be post-translationally modified and regulated [63]. Altered expression levels of these proteins and their mutation have been observed in certain types of human cancers such as melanoma; glioblastoma; neuroblastoma; lung, liver, and colon cancer; androgen-resistant prostate cancer; testicular cancer; ovarian cancer; and estrogen and progesterone receptor-positive breast cancer [64, 65]. It has been observed that the chemotactic receptors *CXCR1* and *CXCR2* differentially activate the members of the Rho GTPase family. CXCR1 signaling promotes a quick induction of the Rho GTPase activity, while the CXCR2 signaling induces the activity via a delayed response [66]. Consequently, *CXCL8* activates the non-receptor tyrosine kinases like focal adhesion kinase (FAK) and Src by phosphorylating these protein kinases and thereby promoting cellular proliferation, motility, and invasion of the cancer cells and the facilitation of angiogenesis in them by activating the Rho-GTPase-induced polymerization of the actin cytoskeleton [6]. Also, studies have shown that CXCL8 induces the phosphorylation and re-localization of FAK and also the β-tubulin, thereby correlating directly with the CXCL8-mediated migratory response [67]. These activated Rho GTPases induce highly motile phenotypes by promoting cell transformation and several downstream effectors, thereby directly involving in tumor growth and metastasis, and their interaction with several signaling pathways aids in the regulation of extracellular matrix remodelling [68, 69]. Time-dependent regulatory studies of *CXCL8*-induced Rho GTPase activity on prostate cancer cells suggest the activation of their signaling pathway as essential promoters of CXCL8-mediated cancer cell motility, invasion, and metastasis [6]. Also, Rho GTPases and their corresponding regulators have been studied as potent targets, and several drugs have been demonstrated to target these molecules to aid in the inhibition of cell proliferation, metastasis, and invasion [70].

3.3.1.4 Activation of Phospholipase C Pathway

Phospholipase C consists of a family of enzymes that can directly modulate three distinct signals, phosphatidylinositol 4,5-bisphosphate (PIP_2), diacylglycerol (DAG), and inositol 1,4,5-trisphosphate (IP_3), and is classified into six different isoforms (β, γ, δ, ε, ζ, η) based on their structure. Of these DAG and IP_3 function as secondary messengers regulating diverse cellular processes and substrates for different signaling pathways. Further, these secondary messengers facilitate the calcium mobilization and thereby activate the protein kinase C (PKC). It has been found that *CXCL8* activates the PLC-dependent PKC signaling pathway and consequently promotes cancer cell migration when coupled with increased calcium concentration by regulating the actin cytoskeleton [43]. It has also been investigated that the CXCL8 signaling facilitates the translational regulation of *cyclin D* in androgen-independent prostate cancer by activating PKCε [40]. In most human cancers, the PLCγ signaling has been identified, thus confirming its key role in facilitating cellular migration and invasion and thus portraying itself as a potent therapeutic target for effective cancer treatment.

3.3.1.5 Activation of Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) is a dynamic process by which the epithelial cells acquire mesenchymal phenotype mostly during normal embryonic development, wound healing, tissue regeneration, and organ fibrosis. This phenomenon has been widely found in several human cancers which confers stemness to the cancer cell, thereby playing a major role in conferring resistance to the therapeutics. However, unlike EMT in embryonic development, the cancer cells exhibit a partial EMT which further enhances their mobility, invasiveness, proliferation, survival, resistance to anti-cancer drugs and other stresses, inhibition of senescence and anoikis, acquisition of cancer stem cell (CSC)-like features, and immunosuppression which have been confirmed by using specific markers like

E-cadherin, integrins, and cytokeratins as epithelial markers and vimentin, N-cadherin, and fibronectin as mesenchymal markers [71–74]. The cancer cells can also reverse the EMT by mesenchymal-to-epithelial transition (MET) which facilitates the cancer cells to develop a secondary tumor at a favorable metastatic niche.

A number of factors with complex underlying mechanisms have been identified to be associated with this process, such as *CXCL8*, *VEGF*, *SNAIL*, *MMP*, *TNFα*, *TGFβ*, *TWIST*, and many more. Several investigations have confirmed that overexpression of CXCL8 induces EMT by activating the PI3K/Akt/NF-κB pathway, thereby enhancing cell proliferation, invasion, and migration of the colon cancer cells [75]. It has been identified that an autocrine loop exists between *CXCL8* and EMT (Fig. 3.3) wherein the *CXCL8* induces EMT which consequently promotes CXCL8 secretion by activating the cytokine/growth factor cascade [76]. The *CXCL8*-induced EMT process has been identified in different cancer types, such as lung carcinomas and colon, nasopharynx, breast, prostate, and ovarian cancers [77–79]. Thus, blocking CXCL8 signaling could be a potential therapeutic strategy to inhibit EMT and related consequences.

3.3.1.6 Activation of Angiogenesis

Angiogenesis is a multistep complex process which involves the formation of novel blood vessels from the pre-existing blood capillaries regulated by the intricate balance between the pro-angiogenic and anti-angiogenic factor. It is an eminent physiological and pathophysiological mechanism which occurs during chronic inflammation, embryo development, and wound healing and is a striking feature in tumor growth. The "angiogenic switch" occurs at various stages of the tumor development and is activated by several factors like mechanical stress, hypoxia, hypoglycemia, and chronic inflammation [28]. This initial switching process involves various intricate transitions such as perivascular detachment and vessel dilation followed by angiogenic sprouting and new blood vessel formation and development and finally recruitment of the perivascular cells, thereby leading to tumor neovascularization. This process is mediated through several factors like increase in *VEGF* and reduction in *IFNγ (interferon-γ)* or through several other proteases degrading extracellular matrix (ECM). Angiogenesis plays a critical role during the cancer cell development to obtain nourishment and also for tumor invasion and metastasis. It has been found that the *VEGF* is highly expressed in several human cancers and is caused by several factors like inflammation, hypoxia, inflammatory cytokines (e.g., *CXCL8*), low pH, chemokines, activation of oncogenes, and inactivation of tumor suppressor genes. The tumor cells possess a paracrine VEGF action mechanism as they do not express cell-surface VEGF receptors and thereby depend on the endothelial cells, type 2 pericytes, and various other host cells like the platelets, tumor-associated stromal cells, and muscle cells to drive tumor angiogenesis [80, 81]. *CXCL8* has a ELR (Glu-Leu-Arg) motif at the NH_2 terminus and hence plays a major role as a potent promoter of angiogenesis unlike the ELR- molecules which act as potent angiostatic factors [82]. The role of *CXCL8* in angiogenesis has been determined in various studies, thereby portraying itself as a strong angiogenic factor on endothelial cells both in vivo and in vitro [83]. Among the chemokines, the potent angiogenic activity was first identified by implanting *CXCL8* into the rat cornea and was found to induce proliferation and chemotaxis of the HUVEC (human umbilical vein endothelial cells) [84]. It has been observed that besides the proinflammatory activity of the *CXCL8*, its expression has been highly correlated with vascularity in several carcinomas like the nasopharyngeal, lung hepatocellular, gastric, and colon cancers and its overexpression in hyperplastic mucosa adjoining the colon cancer reveals its direct and indirect angiogenic effects [85, 86]. Thus, *CXCL8* serves as a novel target for anti-angiogenetic therapies against various human cancers. Nevertheless, the intrinsic balance between the angiostatic and angiogenic CXC chemokines determines the degree of angiogenesis and hence regulates the tumor progression in the tumor microenvironment.

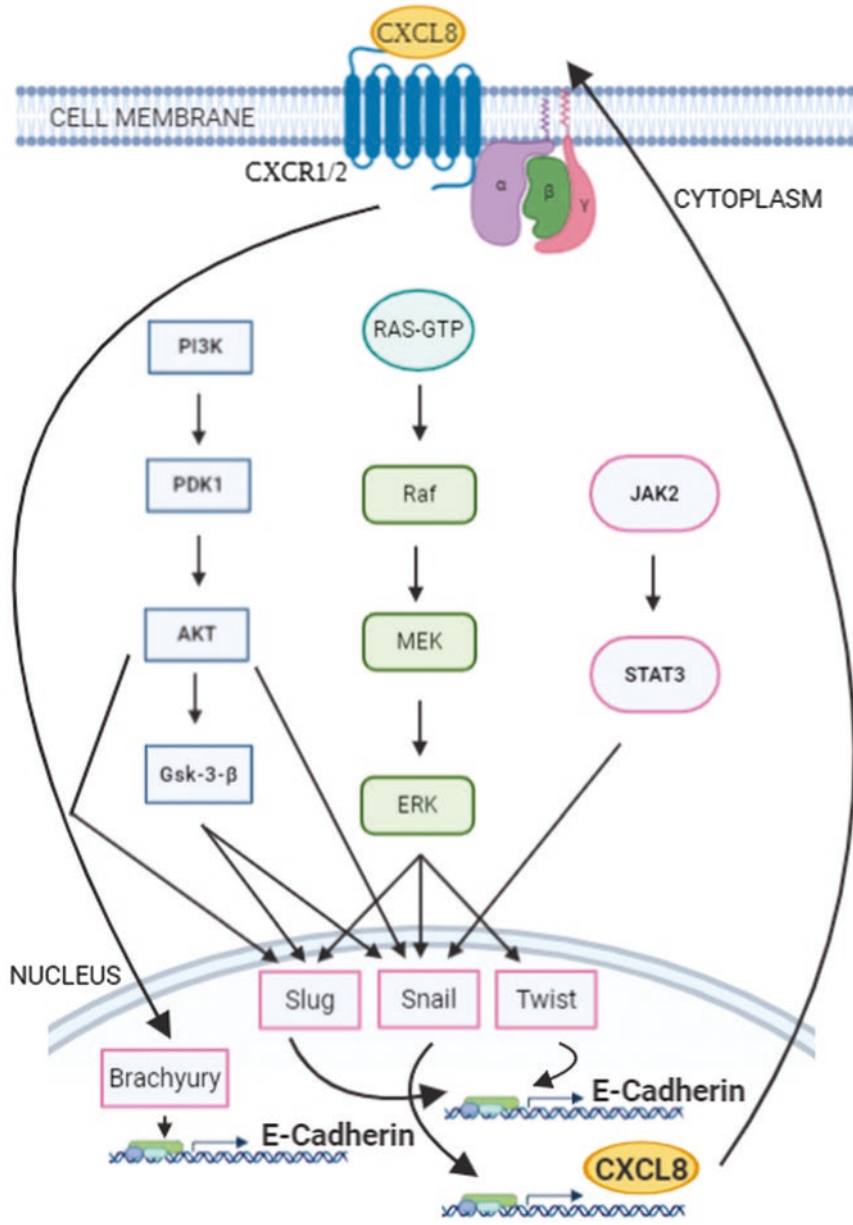

Fig. 3.3 Schematic diagram illustrating the autocrine *CXCL8* loop in EMT

3.4 Conclusion

CXCL8 and CXCR1/2 signaling networks play a vital role not only in the immunological perspective but also in promoting tumor progression and development of resistance against the therapeutics. As evident from several researches, *CXCL8*, a multifunctional chemokine, exerts multiple effects at various stages on the biological activity of the cancer cells including tumor growth, proliferation, development, and metastasis in an autocrine or paracrine fashion and also by regulating multiple signaling pathways, thereby portraying itself as a potent candidate for targeted therapeutic intervention. The high expression of *CXCL8* and its receptors in certain human can-

cers can be used as biomarkers for initial screening and evaluating the prognosis, drug efficacy, and drug responses in the patients. Further studies on the balance between the *CXCL8*, its receptor, and other signaling partners in a tumor microenvironment could make it possible to determine the fate of the tumor, thereby aiding in the development of therapeutics. Furthermore, the development of antagonists, analogs, and other effective strategies to interrupt the signaling pathways and other functions of *CXCL8* may serve as an attractive and a useful targeted cancer therapeutic approach.

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CXCL11 Signaling in the Tumor Microenvironment

4

Qun Gao and Yi Zhang

Abstract

CXCL11 which can bind to two different chemokine receptors, CXCR3 and CXCR7, has found a prominent place in current tumor research. In this chapter, we mainly discuss the current evidence on the role of the immune response of CXCL11 in tumor microenvironment (TME). The diverse functions of CXCL11 include inhibiting angiogenesis, affecting the proliferation of different cell types, playing a role in fibroblast directed carcinoma invasion, increasing adhesion properties, suppressing M2 macrophage polarization, and facilitating the migration of certain immune cells. In addition, we discussed the application of CXCL11 as an adjuvant to various mainstream anti-cancer therapies and the future challenges in the application of CXCL11 targeted therapies.

Keywords

CXCL11 · CXCR3 · CXCR7 · Immune cells · Cytotoxic T lymphocytes · Angiogenesis · Proliferation · Self-renewal · Tumorigenicity · Fibroblast migration · Cell adhesion · Polarization of immune cells · Immune cells migration · vvDD-CXCL11 · Tumor microenvironment

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4.1 Introduction

Chemokine (C-X-C motif) ligand 11(CXCL11), also termed IFN-inducible T cell a-chemoattractant (I-TAC) or interferon-gamma-inducible protein 9 (IP-9), is mainly expressed in the lung, pancreas, thymus, peripheral blood leukocytes, spleen, and liver and is expressed at lesser levels in the intestine, placenta, and prostate [10]. CXCL11 is located on human chromosome 4 and is mainly secreted by cancer cells, leukocytes, monocytes, dendritic cells, endothelial cells, and fibroblasts [27, 34, 44] (Fig 4.1a). CXCL11 is usually expressed at low levels in homeostatic conditions, but is upregulated during cancer or infectious disease processes. Certain research results have shown that cytokines can enhance the secretion of chemokines from different cells [15, 33]. Interferons have the ability to induce CXCL11 production among several cell lines including leukocytes, monocytes, endothelial cells, and fibroblasts. CXCL11 is mainly induced by IFN-γ and IFN-β and is weakly induced by IFN-α [32]. Monocytes, fibroblasts, endothelial cells, and cancer cells may secrete

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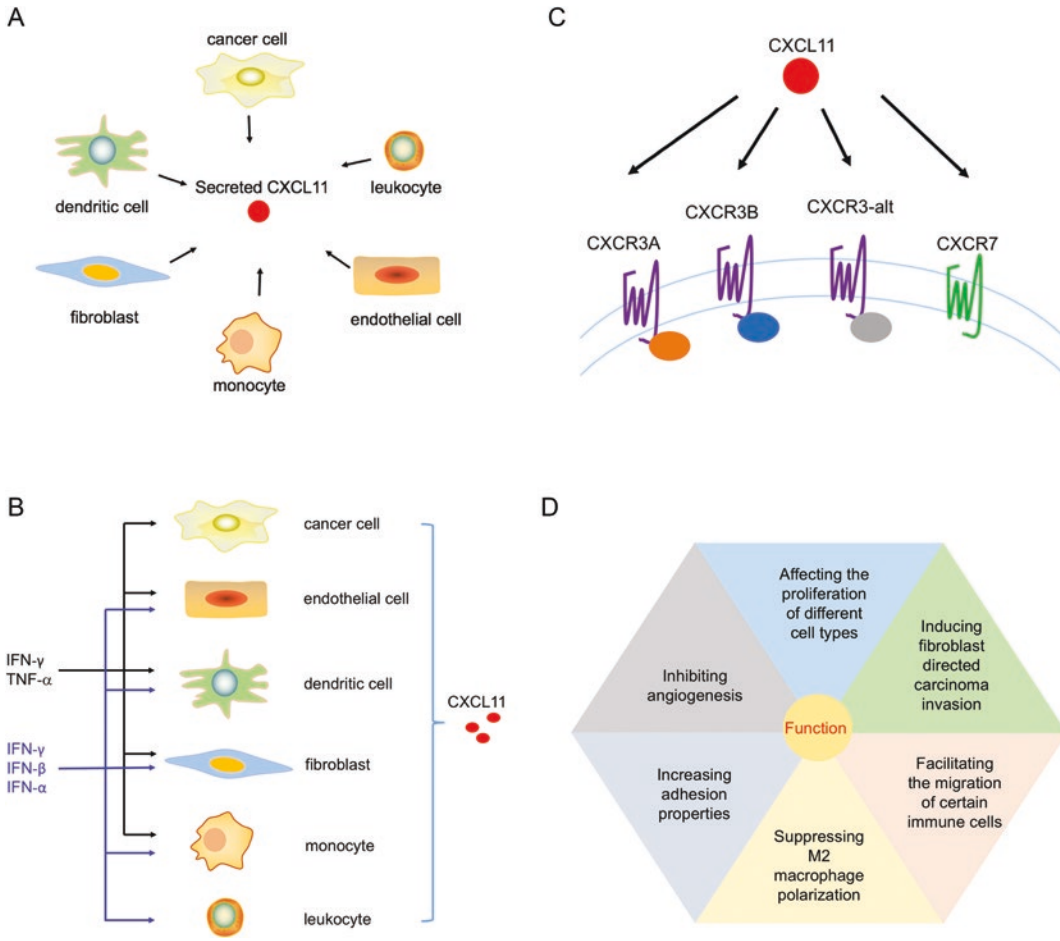

Fig. 4.1 (**a**) The different cells secrete CXCL11. (**b**) Cytokines induce CXCL11 production. (**c**) CXCL11 binds to different chemokine receptors. (**d**) CXCL11 has diverse functions

CXCL11 after the combined stimulation of IFN-γ and TNF-a [37]. In summary, the variety of cytokines that can induce CXCL11 production are shown in Fig. 4.1b.

CXCL11 exhibits multiple effects in tumor biology that go far beyond its originally described function in leukocyte chemotaxis. It is associated with pleiotropic functions including chemotactic migration, regulation of cell proliferation and self-renewal, increasing cell adhesion, and modulation of angiostatic effects. A further understanding of the functions of CXCL11 could provide a gateway to more effective strategies in the treatment of cancer.

4.2 CXCL11 and Its Receptors

CXCL11 binds to two different chemokine receptors, CXCR3 and CXCR7. CXCR3, a well-established receptor for CXCL11, has been reported to regulate tumor growth and metastasis in various solid tumors. CXCL9, CXCL10, and CXCL11 are three selective ligands that bind to CXCR3; however, CXCR3 binds to CXCL11 with higher affinity than CXCL9 or CXCL10 [10, 39]. The binding domain of CXCL11 and CXCR3 is located at different sites from the binding domains of CXCL9 and CXCL10 [11].

Interestingly, certain reports have shown that CXCL11 has the opposite effect on tumor proliferation and metastases [6]. This may be due to the different effects of the ligands on the variants of CXCR3 (CXCR3A, CXCR3B, and CXCR3-alt) (Fig. 4.1c). CXCR3-A is mainly expressed on epithelial cells, whereas CXCR3-B is primarily expressed on fibroblasts and epithelial cells. CXCR3-A and CXCR3-B play reciprocal roles in triggering distinct signal transduction pathways. The binding of CXCR3-A to its ligands results in promoting cell proliferation through AKT activation and Ca^{2+} flux. In contrast, CXCR3-B-ligand binding leads to the inhibition of growth or cell death by dissociation of heterotrimeric Gs into Gαs and Gβγ subunits and activation of AC-cAMP-p38 MAPK signaling cascade [20, 29, 35]. Furthermore, the high expression of CXCR3-A in prostate cancer has been associated with increased tumor cell migration and invasion, as well as the downregulation of inhibitory signals via CXCR3-B. This research provided a possible explanation on how chemokine CXCL11 may interact with CXCR3-A and suppress CXCR3-B expression through activating its downstream signaling cascades [20, 31]. CXCR3-B has been reported to mediate its inhibitory activity on human microvascular endothelial cell growth through CXCL11. In addition, CXCL11 may be used as a member of vascular suppression to damage established tumor-related vascular systems and thus promote extensive tumor necrosis [4, 16, 23, 43]. Therefore, CXCL11 is considered as a useful therapeutic target in anti-cancer therapy.

Increasing evidence suggests that CXCL11 can also bind with another chemokine receptor CXCR7 (RDC1), which is associated with invasiveness and reduces apoptosis of tumor cells. It has been previously reported that the chemokine receptor CXCR7 can also be engaged by CXCL12 (SDF-1, stromal-derived growth factor); however CXCR7 binds to CXCL11 with a 10- to 20-fold lower affinity than CXCL12 [5]. CXCL11 binds to CXCR7 and can also directly modulate CXCR4 signals via CXCR7/CXCR4 heterodimerization in colorectal cancer and influence the metastasis of colorectal cancer cells [30]. Another study confirmed that if CXCL11 interacts with CXCR7 or CXCR3-A, it promotes proliferative signals, whereas CXCL11 binding to CXCR3-B results in the inhibition of growth functions [20]. Therefore, chemokines and their receptors are highly pleiotropic; a single chemokine may bind to multiple chemokine receptors and thus result in various functions. CXCL11 may have opposite functions depending on its binding to either CXCR3 or CXCR7. These opposing functions of CXCL11 also provide challenges in regard to possible therapeutics.

4.3 CXCL11 and Its Diverse Functions

4.3.1 Inhibit Angiogenesis

In general, CXC chemokines are classified into two groups: one group has an ELR (tripeptide Glu-Leu-Arg) motif, and the other group does not have an ELR motif [36]. Those with the ELR motif have an angiogenic effect, whereas those without the ELR motif primarily inhibit angiogenesis. CXCL11 does not have an ELR (tripeptide Glu-Leu-Arg) motif and generally attenuates angiogenesis and thus leads to an antitumor effect.

4.3.2 Proliferation, Self-Renewal, and Tumorigenicity

CXCL11 and its receptor CXCR3 are significantly upregulated in basal cell carcinomas (BCC) as compared with normal skin epithelium. In culture, primary BCC-derived cells or HaCaT cells expressing CXCL11 and CXCR3 significantly increased in cellular proliferation [22]. In addition, CXCL11 and CXCR3 were reported to also be expressed in cells of the surrounding BCC stroma. Furthermore, CXCL11 and CXCR3 affect the proliferation of different cell types such as endothelial cells and vascular pericytes [20].

Reportedly, CXCL11 plays an important role in the development of hepatocellular carcinoma. It was found that the expression of CXCL11 in

hepatocellular carcinoma was significantly higher than that in matched normal tissues. CXCL11 expression depends on autocrine mechanism in upregulating HCC tumor-initiating cells (TICs), in which the overexpressed CXCL11 of alpha2 delta1$^+$ HCC TICs interacts with its receptor CXCR3 expressed on HCC TICs, inducing the activation of ERK1/2 and subsequently regulating its expression. Other signaling pathways, such as PI3K signaling pathway, may also participate in CXCL11/CXCR3 signaling [45].

4.3.3 Fibroblast Migration

CXCL11 secreted from CD11b$^+$ Gr1$^+$cells has been reported to have some effects on fibroblast migration. The deletion of TGFβ receptor II on CD11b$^+$ Gr1$^+$cells resulted in reduced fibroblast migration through downregulating CXCL11 expression. This study showed that CXCL11 plays a role in the fibroblast directed carcinoma invasion mediated by TGFβ signaling [19].

4.3.4 Cell Adhesion

The novel CXCL11-binding protein CXCR7 (the seven-transmembrane receptor RDC1) does not cause cell migration or Ca^{2+} mobilization like many other chemokine receptors. Membrane-associated CXCR7 is expressed on many kinds of cells, such as tumor cell lines, activated endothelial cells, and fetal liver cells. Burns et al. reported that CXCR7 confers a survival advantage to cells, a growth advantage, and increased adhesion properties [5].

4.3.5 Polarization of Immune Cells

CXCL11 decreases transcription of RORγ, leading to the polarization of type 1 regulatory T cells (Tr1) or type 2 helper T cells (Th2) from naive CD4$^+$ T cells by stimulating p70 kinase/mTOR pathways [1, 3]. Another study showed that the CXCL11/CXCR3 axis has an impact on the polarization of tumor-associated macrophages (TAMs), which play modulatory roles in the TME [26]. CXCL11, as well as CXCR3 agonists, can potentially suppress M2 macrophage polarization and thus lead to the regression of breast cancer tumors.

4.3.6 Migration of Immune Cells

Immune cells are regulated by many different chemokines and are trafficked in and out of tumor tissues depending on chemotactic gradients. Reportedly, CXCL11 promotes the recruitment of activated Th1 cells, CTLs, and NK cells in tumor tissues in vivo [7, 24]. All three CXCR3 variants (CXCR3A, CXCR3B, and CXCR3-alt) are expressed on T cells, so CXCL11 can elicit directional migration responses of T cells to the focal sites. CXCL11 increases frequency of tumor-infiltrating lymphocytes and inhibits tumor growth in both T cell lymphoma and breast cancer [9, 28, 40]. CXCL11 has potent antitumor activity in vivo through facilitating the infiltration of CD8$^+$ T lymphocytes. Reportedly, a positive correlation was seen between CXCL11 and tumor-infiltrating CD8$^+$ T cells in mice challenged with genetically modified CXCL11-EL4 T cell lymphoma cells. Depletion of CD8$^+$ T cells in vivo completely abrogated the antitumor effect of CXCL11. In addition, the increase in the production of CXCL11 in transduced EL4 cells enhanced the infiltration of total CD8$^+$ and CD8$^+$CXCR3$^+$ T lymphocytes and macrophages and had no effect on angiogenesis within EL4-CXCL11 tumors. This study provides the evidence that the local release of CXCL11 contributed to the induction of systemic tumor-protective immunity [18, 28]. Chheda et al. used a CXCR3 knock-out murine B16 melanoma model to demonstrate the critical role of CXCR3 in the recruitment of CTL cytotoxic T lymphocytes and revealed obvious tumor growth and decreased survival [8]. Furthermore, CXCL11 could attract Th1 cells and inhibit the migration of Th2 cells due to their ability to serve as one of three antagonists for CCR3 [41]. Interestingly, in a cutaneous T cell lymphoma model, benign T cells, characterized as

$CD40^+OX40^+$, could drive clinical skin inflammation partially through CXCL11 after Psoralen plus UVA (PUVA) treatment [38]. Hypoxia induced the expression of CXCL11 in CRC (colorectal cancer)-derived macrophages ($CD68^+$), thus enhancing the ability of CRC-derived $CD68^+$cells to recruit $Foxp3^+IL\text{-}17^+$T cells, which have the capacity to induce cancer-initiating cell development [42].

More recently, researchers have shown that CXCL11 induces FOXP3-negative regulatory T cells (Tr1 cells) via CXCR3 in autoimmune encephalomyelitis [46]. These findings have motivated researchers to further investigate the complex role of CXCL11 in tumors (Fig. 4.1d) (Table 4.1).

4.4 Anti-neoplastic Therapeutic Application of CXCL11

Hannesdottir et al. reported that doxorubicin and lapatinib can change the expression level of chemokine CXCL11 by affecting STAT1 pathway. Furthermore, they emphasized the role of the STAT1 pathway in the construction of an effective antitumor immune response, by affecting the migration and function of T cells. Downregulation of STAT1 will inhibit the expression of CXCL11, thus inhibiting the antitumor immune response [17]. The STAT1 level in TME links with differential response to chemotherapy in high-grade serous ovarian carcinoma (HGSC). Besides, the expression of CXCL11, STAT1 target genes which recruit intratumoral $CD8^+$T cells, as well as STAT1 were highly expressed in chemosensitive HGSC tumors [2]. Combining chemotherapy with immunotherapy has become a main trend in the applications of anti-neoplastic therapeutics. Another chemotherapy drug docetaxel (DOC) upregulated the expression of chemokine receptor ligand CXCL11 in lung cancer TME and subsequently increased the recruitment of $CD8^+$T cells. The mechanism of the release of CXCL11 was determined by HMGB1 via NF-κB signaling activation. The results demonstrated that DOC enhanced the recruitment of $CD8^+$ T cells to the TME by inducing the secretion of HMGB1 and CXCL11, therefore indicating that modulating the HMGB1-CXCL11 axis might improve the antitumor efficacy for non-small cell lung cancer (NSCLC) treatment [14].

Liu et al. armed a tumor-selective oncolytic vaccinia virus (vvDD) with CXCL11 (vvDD-CXCL11) and treated tumor-bearing mice in order to investigate if vvDD-CXCL11 would attract $CXCR3^+$ CTLs and possibly NK cells to the TME. vvDD-CXCL11 enhanced the local trafficking of tumor-specific T cells and to a lesser extent enhanced the local trafficking of NK cells. Furthermore, vvDD-CXCL11 reduced the expression of several suppressive molecules, COX2, TGF β, and CCL22 in a murine AB12 mesothelioma model, indicating the induction of antitumor immunity in TME [21].

Co-delivery of Fc-fused CXCL11 (CXCL11-Fc) enhanced vaccine antigen-specific $CD8^+$T cell frequencies and effector memory T cell ($CD44^{hi}CD62L^{lo}$) populations and was associated with $CD8^+$T cell proliferation. Taken together, these findings show that CXCL11 may be used as a strong genetic adjuvant to recombinant adenovirus-based vaccination and selectively improved antigen-specific $CD8^+$T cell immunity in tumor cells of mice [25].

Additionally, the modulation of chemokines in the TME enhanced the therapeutic efficiency of oncolytic virus for colorectal cancer. The combination of CKM (chemokine modulating drug cocktail), inducing a favorable chemokine profile, and vvDD-CXCL11, inducing functional CXCL11 secretion from infected cancer cells, elicited potent antitumor immunity and enhanced the recruitment of more tumor-specific $CD8^+$T cells and NK cells and enhanced antitumor efficacy in the TME of MC38-luc tumor-bearing mice [13].

The expression of the chemoattractive cytokine CXCL11 in colon cancer was also increased after protein inhibitor cobra venom factor (CVF) treatment in mice with colon cancer mice, and similar results were seen in complement inhibitor *Staphylococcus aureus* super antigen-like protein 7(SSL-7)-treated mice. The FSC results showed a higher percentage of $CD8^+$T cells, a decreased percentage of $CD4^+$T cells, and a reduced immunosuppressive environment shown by decreased myeloid-derived suppressor cells in CVF-treated mice [12] (Table 4.2).

Table 4.1 Summary of pleiotropic CXCL11-related functions

Receptor	Target cell	Type of cancer	Works for the axis (in TME)	Outcome	References
CXCR3	BCC-derived cells or HaCaT cells	Basal cell carcinomas	Increase their proliferation	Increase proliferation, self-renewal, and tumorigenicity	[22]
CXCR3	HCC tumor-initiating cells	Hepatocellular carcinoma	Positively regulate the stemness of a2δ1+ HCC TICs	Promote stem cell-like properties of HCC cells	[45]
–	Fibroblast	Mammary gland tumors	Increase fibroblast migration	Promote fibroblast directed carcinoma invasion	[19]
CXCR7	–	Breast tumor/lung carcinoma/lymphoma	Cell growth/survival and adhesion	Promote tumor growth	[5]
CXCR3	Macrophage	Breast cancer	Suppress M2 polarization	Inhibit tumor-promoting environment	[26]
CXCR3	CD8 + T cell	Lymphoma	Increase of tumor-infiltrating CD8 + CXCR3 + T cells	Protective antitumor immunity	[40]
CXCR3	CD4 + and CD8+ T cells	Breast cancer	Promote the proliferation of recruited T cells and sustain the tumor- specific T cells	Increase T cell recruitment and elicit a specific antitumor immunity	[9]
CXCR3	CD8 T cell	Melanoma	CTL migration	Enhance efficacy of CTL-based immunotherapies	[8]
–	CD40 + OX40+ benign T cells	Cutaneous T cell lymphoma	Th1 and benign T cell migration	Create a proinflammatory synapse in skin	[38]

Abbreviations: *CTLs* cytotoxic T lymphocytes, *NK cells* natural killer cells, *TME* tumor microenvironment, *BCC* basal cell carcinomas, *HCC TICs* hepatocellular carcinoma tumor-initiating cells

Table 4.2 Summary of anti-neoplastic therapeutic application of CXCL11

Receptor	Target cell	Type of cancer	Works for the axis (in TME)	Outcome	References
CXCR3	CD8 + T cell	Mammary tumors	CD8+ but not CD4+ T cell migration	Contribute to the building of an effective antitumor immunity in response to drug treatment	[17]
CXCR3	CD8 + T cell	HGSC	Intra-epithelial CD8+ T cell infiltration	Improve chemotherapy response	[2]
CXCR3	CD8 + T cell	NSCLC	Enhance CD8+ T cell infiltration	Improve the antitumor efficacy	[14]
CXCR3	CD8+ CTLs	Mesothelioma and colon cancer	Enhance local numbers of CD8+ CTLs	Enhance the therapeutic efficacy of oncolytic viruses and cancer vaccines	[21]
CXCR3	CD8+ CTLs	Lymphoma	Enhance CD8+ T cell proliferation, increase total and effector memory T cell frequencies	Increase antigen-specific CD8 T cell immunity elicited by vaccination	[25]
CXCR3	CD8+ T cells and NK cells	Colorectal cancer	Recruit tumor-specific CD8+ T cells and NK cells	Have great utility for oncolytic immunotherapy for cancer	[13]
–	CD8 + T cell	Colon cancer	Increase infiltration of CD8 + T cells	Increase the effective immune response to tumor and diminish the immunosuppressive effect	[12]

Abbreviations: *CTLs* cytotoxic T lymphocytes, *NK cells* natural killer cells, *TME* tumor microenvironment, *HGSC* high-grade serous ovarian carcinoma, *NSCLC* non-small cell lung cancer

4.5 Conclusion

In conclusion, CXCL11 is a complex cytokine with multiple functions in different tumors. In order to better understand the anti-cancer applications of CXCL11, we need to know more about its expression and its coordination with other members in the tumor microenvironment. CXCL11, CXCL9, CXCL10, CXCL12, and their receptors CXCR7 and CXCR3 are all expressed in the TME by tumor cells, fibroblasts, and other cells. However, the ultimate biological effect of CXCL11 is determined by the crosstalk outcome of the receptor-ligand. The abovementioned factors pose challenges in developing future CXCL11 targeted cancer treatments and should be closely considered in future therapeutics.

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CXCL12 Signaling in the Tumor Microenvironment

5

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Abstract

Tumor microenvironment (TME) is the local environment of tumor, composed of tumor cells and blood vessels, extracellular matrix (ECM), immune cells, and metabolic and signaling molecules. Chemokines and their receptors play a fundamental role in the crosstalk between tumor cells and TME, regulating tumor-related angiogenesis, specific leukocyte infiltration, and activation of the immune response and directly influencing tumor cell growth, invasion, and cancer progression. The chemokine CXCL12 is a homeostatic chemokine that regulates physiological and pathological process such as inflammation, cell proliferation, and specific migration. CXCL12 activates CXCR4 and CXCR7 chemokine receptors, and the entire axis has been shown to be dysregulated in more than 20 different tumors. CXCL12 binding to CXCR4 triggers multiple signal transduction pathways that regulate intracellular calcium flux, chemotaxis, transcription, and cell survival. CXCR7 binds with high-affinity CXCL12 and with lower-affinity CXCL11, which binds also CXCR3. Although CXCR7 acts as a CXCL12 scavenger through ligand internalization and degradation, it transduces the signal mainly through β-arrestin with a pivotal role in endothelial and neural cells. Recent studies demonstrate that TME rich in CXCL12 leads to resistance to immune checkpoint inhibitors (ICI) therapy and that CXCL12 axis inhibitors sensitize resistant tumors to ICI effect. Thus targeting the CXCL12-mediated axis may control tumor and tumor microenvironment exerting an antitumor dual action. Herein CXCL12 physiology, role in cancer biology and in composite TME, prognostic role, and the relative inhibitors are addressed.

Keywords

Cancer · Chemokines · Tumor microenvironment · Chemokine receptors · CXCL12 · CXCR4 · CXCR7 · CXCR4-CXCL12-CXCR7 axis · Metastasis · Tumor progression · Angiogenesis · Immunotherapy · Checkpoint inhibitors · CXCL12 antagonist · Antitumor immune response

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5.1 Introduction

Tumor microenvironment (TME) is the local environment of tumorigenesis and tumor growth, a dynamic space that determines cancer fate. It is composed of tumor and surrounding cells such as bone marrow-derived dendritic cells, mesenchymal stem/stromal cells, fibroblasts, pericytes, and immune cells [1, 2]. TME components support tumors, angiogenesis, and growth and represent the site of critical interactions mostly promoting tumor initiation, resistance, metastasis, and recurrence [3]. Chemokines, or chemotactic cytokines, are small chemoattractant secreted molecules regulating directed cell migration, proliferation, and survival with a role in physiological and pathological processes including cancer [4]. Structurally chemokines are classified on the basis of a specific cysteine motif at the N-terminal into CC, CXC, XC, and CX3C subfamilies: the CC chemokines have two adjacent conserved cysteine residues, XC chemokines have only one N-terminal cysteine residue, whereas CXC and CX_3C chemokines have one or three other amino acids in between their conserved N-terminal cysteine residue; they accordingly bind to their respective chemokine receptor subfamilies CCR, CXCR, XCR, and CX3CR [5] (Fig. 5.1). Chemokines can be divided into inflammatory and homeostatic based on their prominent functions: mainly inflammatory chemokines, that are expressed during inflammatory processes, are CXCL1, CXCL2, CXCL3, CXCL5, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, and CXCL14. On the other hand, homeostatic chemokines such as CCL14, CCL19, CCL20, CCL21, CCL25, CCL27, CXCL12, and CXCL13 are constitutively expressed and regulate homeostatic leukocyte trafficking [6]. The chemokine system is complex with several chemokines that bind the same receptor with similar affinities and receptors that bind the same ligand. Nevertheless, the system is not redundant as cells dynamically express receptors at the cell membrane simultaneously or during different stages of their life [7]. Chemokines are expressed by neurons, glia, and neural progenitor cells, the major cell types of the nervous system, and are induced by neuroinflammatory responses [8]. Combined with the normal and/or pathological nervous system expression of chemokine receptors, chemokines potentially initiate a cascade of events leading to neuroinflammation [9]. The chemokines generated in association with neuroinflammation are crucial for the migration of leukocytes into inflamed neural tissue, just as in other parts of the body [10]. Chemokines also regulate the migration of mesenchymal stem cells (MSCs) to tumor sites where they promote tumor development and differentiate to tumor-promoting cancer-associated fibroblasts (CAFs). Moreover, chemokines expressed in metastatic sites are key players in attracting tumor cells that express the corresponding receptors [11].

Twenty-three human chemokine receptors and about 50 chemokines [12] have been identified. Chemokine receptors are G protein-coupled/seven-transmembrane domain receptors. A new family of chemokine receptors named atypical chemokine receptors (ACKR) has recently emerged as important regulators of chemokine functions. The ACKRs are unable to trigger the canonical G protein-mediated signaling. Four chemokine receptors belong to ACKR: 1. ACKR1, previously called Duffy antigen receptor for chemokines (DARC); 2. ACKR2, also known as D6; 3. ACKR3, also called CXC-chemokine receptor 7 (CXCR7); and 4. ACKR4, previously called CC chemokine receptor-like 1 (CCRL1) [13]. In the TME chemokines can be expressed by tumor, immune, and stromal cells such as leukocytes, fibroblasts, pericytes, and endothelial cells crucial for tumor vascularization and metastatic spread [14].

CXCL12 is a homeostatic chemokine that binds CXCR4 and CXCR7 receptors and physiologically functions in hematopoiesis, leucocyte trafficking, cardiogenesis, and neurogenesis. CXCR4 (352 amino acids, 48 kDa) [15–17] is a G protein-coupled chemokine receptor encoded on chromosome 2.1 in human. CXCL12 binding to CXCR4 triggers multiple signal transduction pathways that regulate intracellular calcium flux, chemotaxis, transcription, and cell survival [18]. CXCR7 binds with high-affinity CXCL12 and with lower-

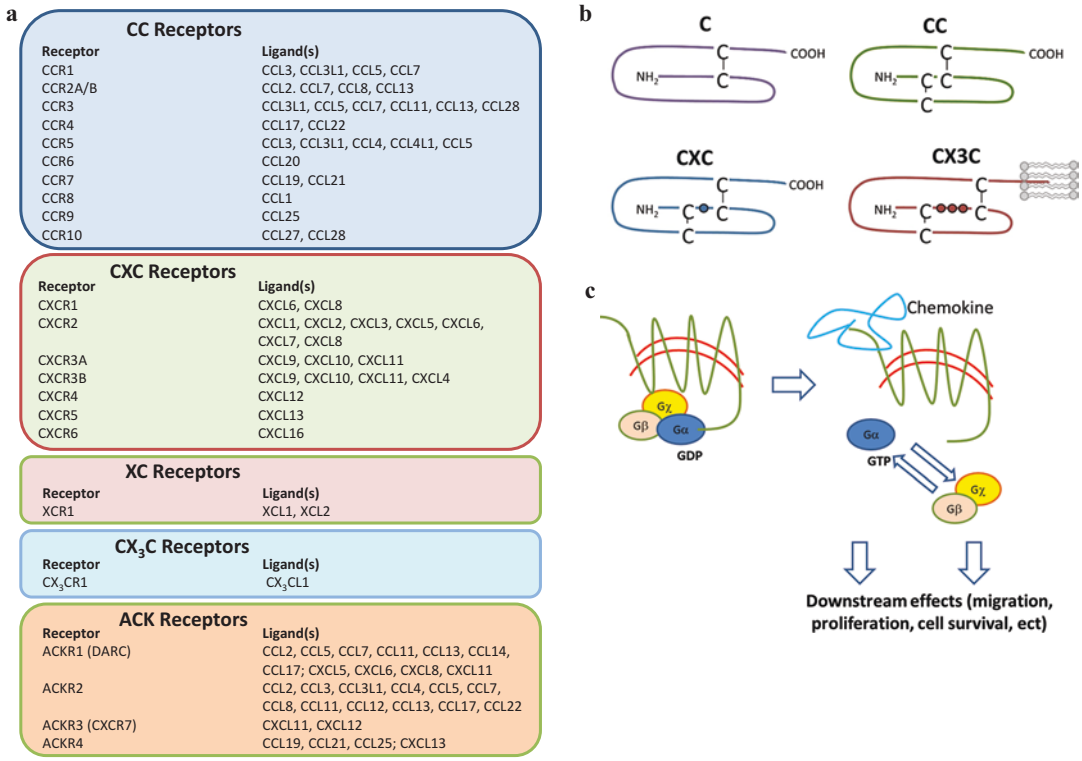

Fig. 5.1 The chemokine and chemokine receptor superfamily. (**a**) The chemokine receptors' subfamilies and ligands. (**b**) Schematic representation of chemokine structure according to cysteine residue position. (**c**) Schematic representation of chemokine and chemokine receptor interactions at cell surface. Heterotrimeric G proteins associate with the intracellular domains of chemokine receptors. Specific ligand-receptor interactions lead to triggering of the receptor and dissociation of the heterotrimeric G protein complex into the Gα and Gβγ subunits. These second messengers then play a critical role in activation of the various signal transduction cascades, leading to migration and other responses driven by chemokines. (Panel (b) adapted from *de Munnik S.* et al. *Modulation of cellular signaling by herpesvirus-encoded G protein-coupled receptors Front. Pharmacol., 2015*)

affinity CXCL11, which binds also CXCR3 [19]. Although CXCR7 acts as a CXCL12 scavenger through ligand internalization and degradation [20], it transduces the signal mainly through β-arrestin with a pivotal role in endothelial and neural cells.

5.2 The CXCR4-CXCL12-CXCR7 Axis

5.2.1 CXCL12

Initially known as stromal cell-derived factor-1α (SDF-1 α) or pre B-cell growth-stimulating factor (PBSF), CXCL12 is the most studied member of the chemokine family [21, 22]. CXCL12 gene in human is located on chromosome 10 (10q11.21) and recognizes seven isoforms deriving from alternative gene splicing (α, β, γ, δ, ε, θ) with α and β being the most studied [23] and three (CXCL12α to γ) in mice [24, 25] (Fig. 5.2). CXCL12α (89aa, 10 kDa) is the most common isoform produced in lymph nodes (LNs), brain, liver, colon, kidney, testis, lung, pancreas, skin, and placenta and by different cell types including stromal cells, osteoblasts, fibroblasts, dendritic cells, and monocytes, among others. CXCL12 is the major chemokine produced in the bone marrow (BM), where it regulates quiescence, retention, and differentiation of hematopoietic stem cells (HSC) [26]. CXCL12

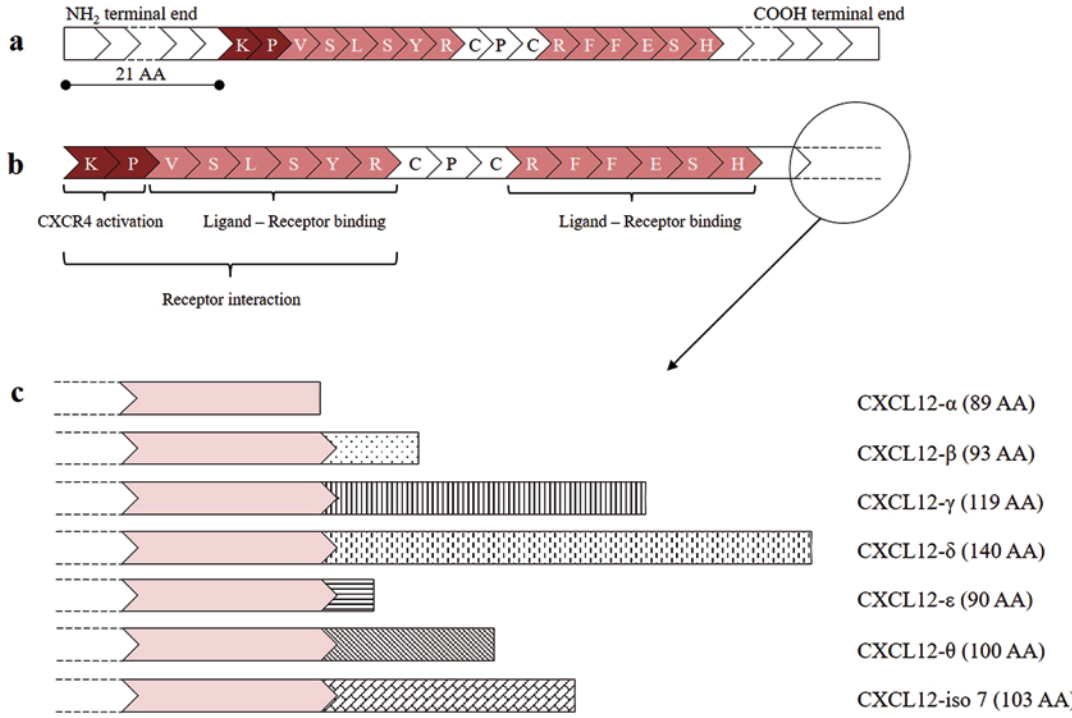

Fig. 5.2 CXCL12 isoforms. (**a**) The CXCL12 immature form, the propeptide, which includes the 21 amino acids at the N-terminal end that will be removed. (**b**) The mature CXCL12 form has undergone a proteolytic cut of 21 amino acids at the N-terminal end. The first eight amino acids of the mature CXCL12 allow the receptor interaction; in particular, the first two, lysine and proline, activate the CXCR4 receptor, while the other six are used for the receptor binding. In addition, the "RFFESH" sequence allows the ligand-receptor binding. (**c**) Representations of all CXCL12 isoforms are shown. They all have the same starting sequence, but each one differs from the others in the terminal region length. (Adapted from *Righetti A. et al CXCL12 and Its Isoforms: Different Roles in Pancreatic Cancer? Journal of Oncology* [8]*:1–13. (2019)*)

half life is approximately 26 minutes. CXCL12 is then degraded at the N-terminal by enzymes matrix metalloproteinase-2/9 (MMP-2/9) crucial to extracellular matrix (ECM) remodeling. CXCL12 is described classically as a homing chemokine as it exhibits chemoattraction of tumoral cells toward the target tissues [27]. CXCL12, although being homeostatic in classification, also takes inflammatory activities [28]. CXCL12 binds to glycosaminoglycans (GAGs) exposed on the surface of endothelial cells through a cluster of basic residues—the BBXB motif (B for basic amino acid and X any amino acid) generating its chemotactic gradients and promoting leukocyte/cancer cell migration [29–31]. CXCL12-α is not present in blood due to enzymatic degradation [23], but it is expressed in adult BM, where it accounts for progenitor cell retention and chemotaxis of leukemia cells [32]. Unlike CXCL12α, CXCL12β (93aa, 10.6 kDa) promotes angiogenesis [33], while CXCL12γ (119aa, 13.6 kDa) is highly expressed in less vascularized organs, such as the heart and brain [34]. CXCL12γ is poor chemotactic in vitro but the most active in vivo [35] and, due to a stable binding interaction, produces a powerful inflammatory reaction in in vivo mouse models [23]. Recently CXCL12γ has been found to interact with CXCR4 inducing cancer stem cell (CSCs), with neuroendocrine phenotypes and development of metastatic castration-resistant prostate cancer (mCRPC) [36].

5.2.2 CXCL12 Signaling

CXCL12 binds the seven α-helical transmembrane domains (7TM) G protein-coupled (GPCR) conventional receptor CXCR4, and the atypical receptor CXCR7 (also known as ACKR3) and plays a key role in physiological and pathological processes, including embryogenesis, hematopoiesis, angiogenesis, and inflammation, regulating the migration of hematopoietic progenitor and stem cells, endothelial cells, and leukocytes [7, 37, 38] (Fig. 5.3). CXCL12 main receptor, CXCR4, also binds macrophage migration inhibitory factor (MIF), a cytokine involved in the regulation of innate immunity [39]. MIF binds to the N-terminal tail of CXCR4 and to the exterior side of TM helices, but not inside the TM pocket [39, 40]. MIF also binds to other receptors, including CXCR2, CD74/CD44, and ACKR3 [41]. CXCL12 can also activate CXCR4 through heterodimers like HMGB1-CXCL12. High-mobility group box 1 protein (HMGB1) is the archetypal damage-associated molecular pattern (DAMP) released from dead or severely stressed cells to alert their microenvironment and the innate immune system. However, the conformational rearrangements of CXCR4 differ when triggered by CXCL12 alone or by HMGB1•CXCL12, and the complex is extremely more potent than CXCL12 alone in inducing cell migration [42]. Extracellular ubiquitin (eUb), also considered a DAMP, is a CXCL12 antagonist [41] that binds to CXCR4 inside the cavity delimited by TMs [43], but makes contact to CXCR4 residues that are not contributing to CXCL12 binding [44]. Beta-defensin-3 (HBD3) also competes with CXCL12 for CXCR4 binding and promotes CXCR4 internalization without inducing calcium flux, ERK phosphorylation, or chemotaxis. CXCL12-CXCR4 forms a complex with the $G\alpha_i$ subunit G protein, inhibiting the adenylyl cyclase-mediated cyclic adenosine monophosphate (cAMP) production and promoting mobilization of intracellular calcium. Dissociation of the $G\alpha_i$ subunit from Gβγ leads to activation of multiple downstream targets, including protein kinase B (Akt), c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MEK), and extracellular signal-regulated kinase-1 (ERK1/2) effectors [45]. In addition, Gα subunit activates Ras and Rac/Rho pathways, leading to the phosphorylation of ERK and P38 proteins, respectively [46]. CXCR4 homodimerization results in G protein-independent activation of the JAK/STAT pathway that, in conjunction with other signaling pathways, promotes polarization and chemotactic responses [47]. Recent evidence has shown CXCR4-dependent mTOR signaling in pancreatic, renal, and gastric cancer and T cell leukemia cells [18, 48]. CXCR7 homo- and heterodimerizes with CXCR4, and overall, the signaling properties of CXCR7 seem to be multifaceted and may be cell context-dependent. CXCR4-CXCR7 heterodimers regulate the subcellular distribution of CXCR4, recruit β-arrestins, and modify CXCL12-driven responses through CXCR4 [49]. Emerging evidence suggests that CXCR7 internalizes its ligands and is activated by CXCL12 to induce intracellular signaling and in particular Akt, MAPkinase (MAPK), and Janus kinase-signal transducer and activator of transcription (JAK/STAT3) through β-arrestin [19] or in heterodimers with CXCR4 [50, 51]. β-arrestin recruitment to the CXCR4/CXCR7 complex enhances downstream cell signaling (ERK1/2, p38, SAPK/JNK), which induces cell migration in response to CXCL12 [19]. CXCR4 is expressed by migrating cells, and CXCR7 acts by sequestrating CXCL12 from non-target areas, allowing the correct cell migration [20]. In the absence of CXCR7, migrating cells still respond to CXCL12, but their movement ends in undesirable sites due to the lack of a CXCL12 gradient required for a directional migration [67]. Continuous CXCL12 stimulation desensitizes CXCR4-expressing cells promoting CXCR4 endocytosis, as uncoupling from G proteins by GPCR kinase (GRK)-dependent phosphorylation and subsequent interaction of CXCR4 with β-arrestin, which mediates internalization of the receptor that is ubiquitinated and degraded in lysosomes [52, 53]. Activated YY1, a transcription factor, can inhibit CXCR4 expression favoring C-terminal phosphorylation of Src kinase in breast cancer cells [54, 55]. The histone deacetylase CREB3 and the

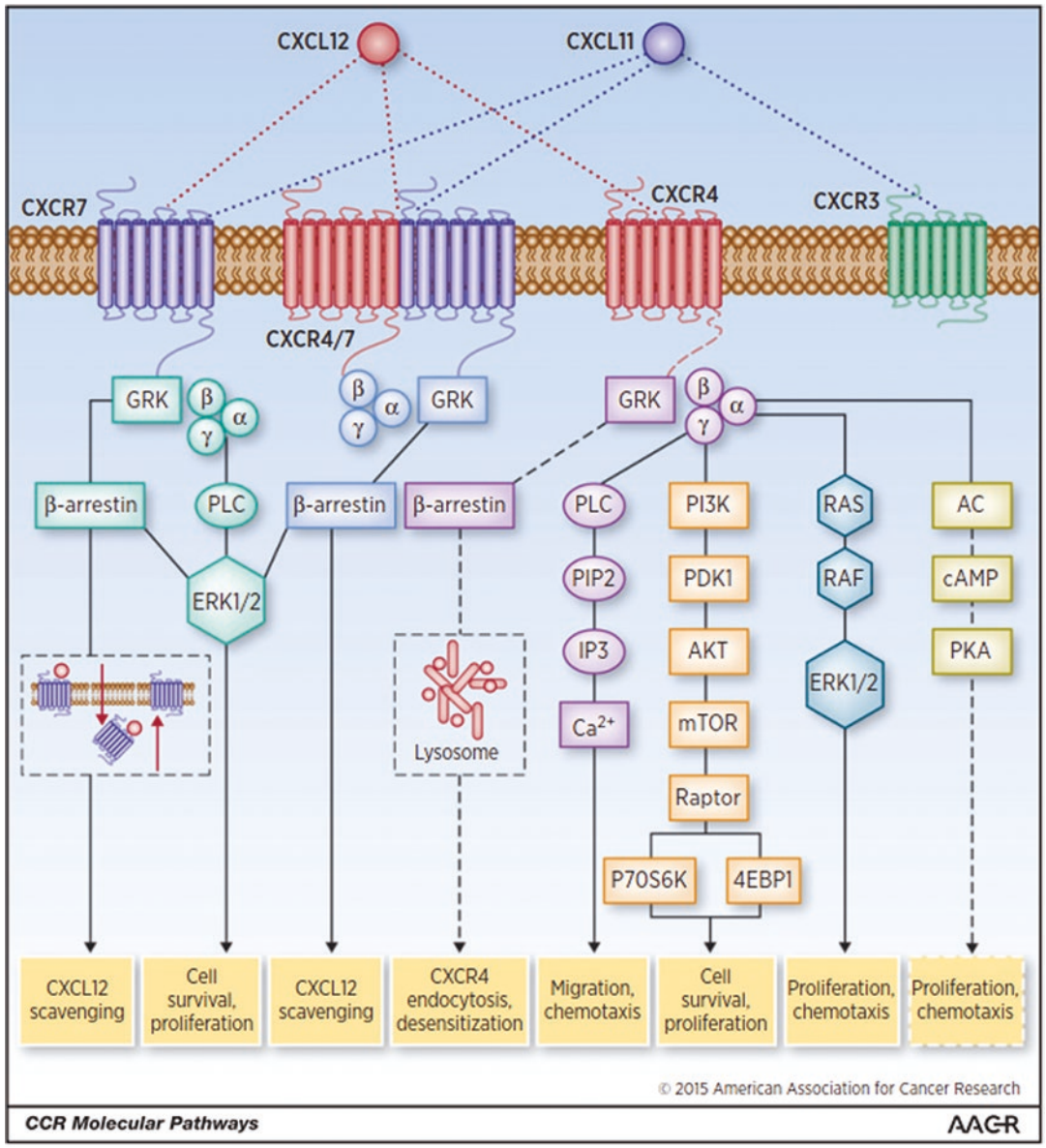

Fig. 5.3 The CXCR4–CXCL12–CXCR7 transduction pathway. CXCL12 acts on two distinct receptors, CXCR4 and CXCR7, which are seven-membrane GPCR receptors. CXCR4 and CXCR7 can form homodimers or heterodimers. CXCL12 shares CXCR7 binding with another chemokine, CXCL11, that is also a ligand for CXCR3. CXCR4 triggers preferentially G protein-coupled signaling, whereas activation of CXCR7 or the CXCR4-CXCR7 complex induces b-arrestin-mediated signaling. The Gai monomer inhibits the adenylyl cyclase activity regulating cell survival, proliferation, and chemotaxis. Although Gai triggers PI3K/AKT/mTOR and ERK1/2, the Gbg dimer triggers intracellular calcium mobilization through PLC. When CXCL12 binds CXCR7, the receptor signals through b-arrestin, inhibits G protein-coupled signaling, and activates the MAPK pathway. CXCR7 can also signal through PLC/MAPK to increase cell survival. The CXCR4-CXCR7 heterodimers-b-arrestin pathway can be activated through GRK-dependent phosphorylation to internalize CXCR4, scavenging CXCL12, and/or control cell survival through ERK1/2. CXCL12 also causes CXCR4 desensitization, uncoupling from G protein by GRK-dependent phosphorylation, and b-arrestin-dependent endocytosis. In contrast with CXCR4, when CXCL12 binds CXCR7, the interaction between b-arrestin and CXCR7 internalizes the receptor and subsequently recycles it to the cell membrane. Upon binding to CXCR4 or CXCR7, CXCL12 is internalized and subjected to lysosomal degradation. Activator signals are represented by straight lines. Inhibitory symbols are represented by dashed lines. (From *Scala S., Molecular Pathways: Targeting the CXCR4-CXCL12 Axis--Untapped Potential in the Tumor Microenvironment. Clin Cancer Res;21* [19]*:4278-85 (2015)*)

Kruppel-like factor 2 can inactivate CXCR4 [56, 57]. The oncogene Her2 can block CXCR4 ubiquitination and degradation after CXCL12 binding in breast cancer cells [58, 59]. CXCL12 is considered a key molecule for normal development as 50% of the CXCL12$^{-/-}$ knockout mice die before birth around day 18.5 of embryogenesis and neonates die within an hour. CXCL12$^{-/-}$ mice show severely reduced B cell progenitors in fetal liver and bone marrow and myeloid progenitors virtually absent in the bone marrow. In addition, the mutants have a cardiac ventricular septal defect [60]. Similar defect was reported for CXCR4$^{-/-}$ mice suggesting a strong relationship between CXCR4 and CXCL12 [61]; moreover, in the CXCR4$^{-/-}$ mice, nervous system defects were also observed with the cerebellum characterized by an irregular external granule cell layer, ectopically located Purkinje cells, and numerous chromophilic cell clumps of abnormally migrated granule cells within the cerebellar anlage [61]. CXCR4$^{-/+}$ knockout mice presents few mature B and T cells within the peripheral lymphoid organs, impaired vascularization in various organs such as the intestines, stomach, and heart, and ventricular septal defect that occurs during embryogenesis [62]. CXCR7 has a role in the development of the central nervous system [50], angiogenesis [63], neurogenesis [64], and cardiogenesis [65], while CXCR4 is involved in vascularization, homing of immune cells in the bone marrow [62], and neurogenesis [66]. CXCR7$^{-/-}$ knockout mice die perinatally due to semilunar heart valve malformation and ventricular septal defects and show furthermore disrupted lymph angiogenesis and cardiomyocyte hyperplasia, while their hematopoiesis remains normal.

5.3 The CXCL12-CXCR4/CXCR7 Axis in Cancer

The expression of CXCL12-CXCR4/CXCR7 axis is mostly reported in aggressive tumors [68] and correlates with tumor recurrence [69, 70], poor prognosis and patient survival [16, 71, 72]. CXCR4 is overexpressed in a wide range of tumors comprising prostate, brain, breast, lung, liver, gastric, colon, ovary, and pancreas [73–76]. CXCL12 confers several advantage to CXCR4-expressing cancer cells including a chemo-resistant phenotype via crosstalk with several pathways of survival, proliferation, tumorigenesis, epithelial to mesenchymal transition (EMT), and acquisition/maintenance of stem-like proprieties [36, 77–82]. In addition, CXCL12 hypermethylation was reported in gastric cancer [83], breast cancer [84, 85], colon cancer [86], lung cancer [87], as well as prostate cancer [88]. Within the TME, CXCL12/CXCR4 regulates trafficking of immune and tumor cells promoting tumor-related inflammation and metastasis [47, 89, 90]. In addition, endothelial cells express both CXCR4/CXCR7 and CXCL12 that facilitates intravasation and extravasation of cancer and immune cells as well as tumor angiogenesis [91]. CXCR4 facilitates angiogenesis by recruitment of endothelial progenitor cells or BM-derived accessory cells, while VEGF promotes sprouting angiogenesis by inducing tip cell filopodia and serving as an attraction cue [92]; the CXCL12/CXCR4 axis stimulates tip cells and migration in neovascular sprouting [93]. CXCR7 is highly expressed by most tumor-associated blood vessels of human breast and lung cancers as well as melanoma, but not by normal vasculature [49]. Overexpression of CXCR7 and its activation in the vascular endothelium enhance invasive and migratory ability toward breast, prostate, and lung cancer [94]. In ovarian cancer, estrogen induces CXCR7 expression that favors tumor cell migration and invasion through CXCL11 [95], while CXCL12-stimulated EMT depends on CXCR4 [70]. Although CXCR7 is not considered a chemotactic receptor, addition of CXCL12 enhances CXCR4+/CXCR7+ cancer cell trans-endothelial migration toward CCL19 and CXCL13, chemokines expressed by endothelial cells inside the lymph nodes [96]. Moreover, in ovarian cancer CXCL12 stimulation reduced the expression of ARH-GAP10, a member of Rho GTPase-activating proteins considered a tumor suppressor gene [97].

MicroRNAs have been reported to play critical roles in regulating tumor progression through

CXCL12/CXCR4 axis [98]. MiR-302a decreased the invasion and metastasis of breast cancer cells by reducing CXCR4 production [99]. MiR-9 reduced the proliferation of oral squamous cell carcinoma cells by the inhibition of CXCR4 via the Wnt/β-catenin signaling pathway [100]. MiR-146a down-modulated CXCR4 production in target cells [101]. CXCR4 was inhibited upon miR-451 treatment in lung cancer cells [102]. MiR-204-5p may function as an inhibitory RNA molecule in oral squamous cell carcinoma by targeting CXCR4 [103]. Artificial microRNA was demonstrated to effectively block invasion and metastasis of breast cancer cells by targeting CXCR4 [104]. MiR-126 may also act as a tumor suppressor by inactivating RhoA signaling via CXCR4 in colon cancer [105]. In addition, miR-101 was recently discovered to directly target CXCL12 in lung cancer cells [106].

CXCL12-CXCR4 axis mediates also chemotherapy-induced transition toward a mesenchymal/stem cell phenotype. Cisplatin induces upregulation of both CXCR4 and CXCL12 expression in NSCLC cells. In colon cancer, chemotherapy or chemoradiotherapy induces CXCR4 expression as part of a mesenchymal transition [107]. Chemotherapy- induced cytokines also have a direct effect on tumor cells. In vivo CXCL12 or S1p/S1PR1 inhibition prevented chemotherapy-enhanced metastasis in tumor-bearing mice. In glioblastoma (GMB) CXCR4 inhibition through Pep R impairs the metabolic activity and tumor growth of GMB cells in vitro and reduced tumor cellularity, promoted M1 features of TAMs and astrogliosis, and hindered intratumor vasculature in orthotopic GMB model [108].

5.4 CXCL12 as Prognostic Factor in Cancer

In a meta-analysis of 38 studies involving 5807 patients, high CXCL12 expression was associated with reduced overall survival in patients with esophageal, colorectal, gastric, pancreatic, ovarian, and lung cancer, while in breast cancer patients, high CXCL12 expression conferred an overall survival advantage [109]. In esophageal cancer patients' meta-analysis, high expression of CXCL12 and its receptors (CXCR4 and CXCR7), CXCL8 and its receptor (CXCR2), CCL21 and its receptor (CCR7), or CCL20 was associated with worse prognosis [110]. CXCL12γ was detected only in breast cancers from patients with advanced disease suggesting CXCL12γ as a prognostic marker for breast cancer [34]. CXCL12γ, mostly expressed by carcinoma-associated fibroblasts, confers to CXCR4-positive breast cancer cells the ability to metastasize to the bone marrow through the expression of the receptor activator of NFκB ligand (RANKL) [33]. High levels of CXCL12 and CXCR4 were reported in sinusoidal endothelial cells in hepatocellular carcinoma (HCC) specimens, breast tumors, metastatic lung cancer, bladder cancer, head and neck squamous cell carcinoma, glioblastoma, and pancreatic tumors [17, 100, 108, 111–115]. In metastatic germ cell tumors, CXCL12 is almost exclusively expressed in non-seminoma [116]. CXCL12/CXCR4 axis is centrally involved in ovarian cancer progression since CXCL12 induces ovarian cancer cell migration and invasion and was reported as prognostic factor in ovarian cancer [117]. In lung cancer patients, CXCL12 protein and mRNA expression levels were significantly higher in metastatic lymph nodes than in primary site. CXCL12 high expression in metastatic lymph nodes was associated with poor overall survival [118]. In another study on 63 patients undergoing surgical resection for lung adenocarcinoma, CXCL12 overexpression was a significant poor prognostic factor in patients with surgical resected lung adenocarcinoma. In a study including 596 patients, CXCL12 and relative CXCL12-CXCR4 expression was independent prognostic factors for 5-year DFS in TNM stage III colon cancer [119]. In renal cancer (RCC) patients with high expression of CXCR4, CXCR7, and CXCL12 had shorter overall survival and recurrence-free survival than those with low expression [120]. Moreover, CXCR4 and CXCR7 expression, alone and in combination, was prognostic in RCC [121]. Even if CXCL12 was not prognostic, CXCR4 and CXCR7

correlate with poor prognosis in melanoma, hepatocellular carcinoma (HCC), and colorectal cancer (CRC) [122–125].

5.5 The CXCL12-CXCR4/CXCR7 Axis in the Tumor Microenvironment

Tumor microenvironment is the tumor local environment composed of immune cells, fibroblasts, epithelial cells, extracellular matrix (ECM) proteins, blood and lymphatic vessels, metabolites, chemokines, and cytokines [1, 126]. TME is defined "hot" when it is highly infiltrated by T cell lymphocyte generating "inflamed" TME; in contrast, lack of T cells infiltrating the tumor characterizes "non-inflamed" or "cold tumors" in which other immune populations, like myeloid cells (mostly immunosuppressive), prevail. Tregs (regulatory T cells) and MDSC (myeloid-derived suppressor cells) prevail. Tregs and MDSCs (myeloid-derived suppressor cells) prevail in a COLD TME; moreover, there are few TH1, NK, and CD8+ T cells and few functional antigen-presenting cells (APCs) and it is enriched in immunosuppressive cytokines. Hot TME displays high PDL1 expression and is enriched in TH1-type chemokines, effector immune cells (TH1 cells, NK cells, and CD8+ T cells), and functional APCs [1, 126, 127]. Recent evidence showed that the CXCL12/CXCR4 axis can regulate the recruitment of specific immune cell populations within the TME (facilitating the access of immune cells with suppressive function and repelling immune effector cells) and drive the polarization of CXCR4-expressing immune cells toward an immunosuppressive phenotype (Fig. 5.4). CXCL12-CXCR4 signaling mediates plasmacytoid DC trafficking into tumors and Treg cells homing to the bone marrow microenvironment [128]; moreover it has an anti-inflammatory role by mediating T cell polarization toward Tregs [129, 130] and generating poorly functional DCs to stimulate antigen-specific T lymphocyte and macrophages expressing proangiogenic factors [131]. Cytokines produced by stromal cells such as TGF-β elicit epithelial CXCR4 expression that, activated by stromal CXCL12, mediates the activation of the Akt pathway in the epithelial cells promoting malignant progression [132]. CXCL12/CXCR4 signaling is central to the retention of neutrophils in the BM, mobilization from the bone marrow, and the homing back of senescent neutrophils [133]. Mice carrying a myeloid-specific deletion of CXCR4 (myeloid-specific knockout (MKO) mice) display a marked redistribution of neutrophils from the bone marrow to the blood and spleen [134]. In a melanoma mice model, disruption of CXCL12/CXCR4 signaling in myeloid cells via genetic knockout of CXCR4 inhibits the outgrowth of circulating B16 melanoma cells in the lung and inhibits tumor growth in an inducible BrafV600E/PTEN null melanoma mouse model as IL18 overexpression activates NK cells and enhances antitumor immunity [135]. BMDCs attracted at the tumor site produce CXCL12 that interacts with DC cells in an autocrine manner to promote DC maturation and survival [136, 137]. High numbers of plasmacytoid DCs have been observed in human ovarian carcinoma due to CXCL12 in malignant ascites that attracts DCs into the TME [138]. Intratumoral DC plays an important role in stimulating cytotoxic T cells and driving antitumor immunity. In metastatic ovarian tumor model, locoregional delivery of the CXCR4-antagonist-armed virus reduced the tumor load and the immunosuppressive network in the TME, leading to infiltration of CD103+ DC capable of phagocytic clearance of cellular material from virally infected cancer cells [139]. As natural and synthetic amines inhibit DC activation, CXCR4 has been identified as receptor used by amines to inhibit DCs [140]. Thus, CXCR4 was described as a potential "on-off" switch of DC activity with therapeutic potential [140]. Tregs are recruited at the tumor site by chemokines such as CXCL12 [141] and CXCR4 overexpression by Tregs is reported in advanced cervical cancers [142], malignant pleural mesothelioma [143], ovarian [144] and renal cell carcinoma [145]. The CXCR4 antagonist AMD3100 reduced intratumoral Treg by conversion of Treg cells into T-helper-like cells conferring survival

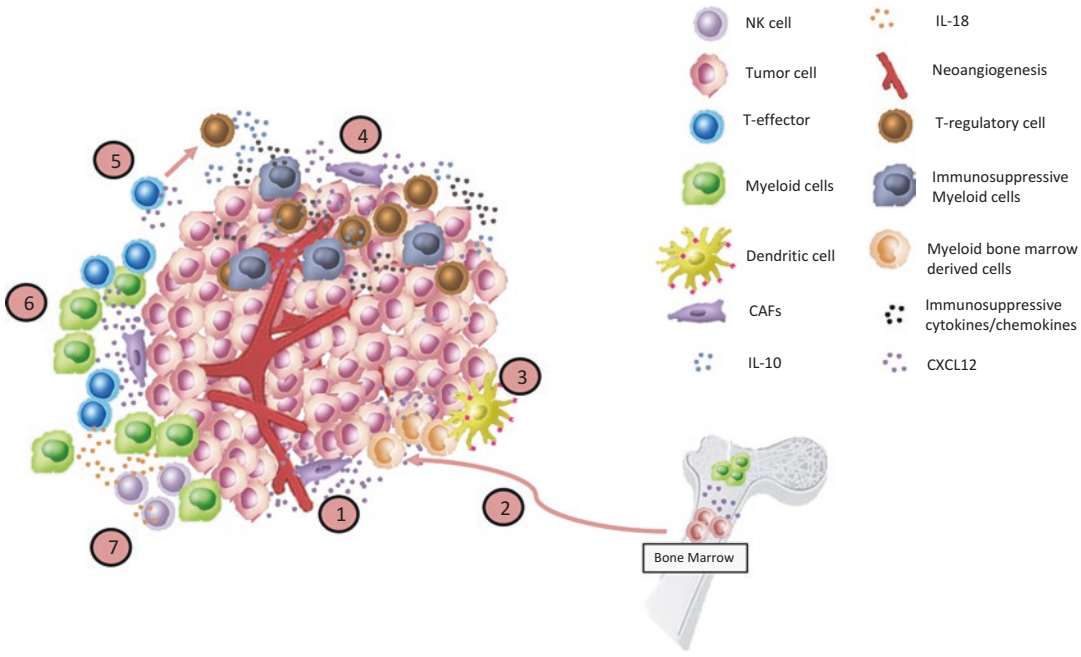

Fig. 5.4 CXCL12 in the tumor microenvironment. CAF-produced CXCL12 acts on different TME cells and regulates the recruitment of immune cells. 1. CXCL12/CXCR4 axis stimulates endothelial cells promoting neovascularization, tumor growth, and metastatic progression. 2. CXCL12/CXCR4 axis regulates trafficking and tissue localization of human HSC in the bone marrow. 3. CXCL12/CXCR4 axis recruits bone marrow-derived myeloid cells promoting DC maturation and survival. 4. CXCL12 recruits immunosuppressive cells and 5. excludes effector cells designing a "colder" TME that impairs immunotherapy response. 6. CXCL12 also redirects the polarization of effector Th1 cells into $CD4^{+}CD25^{-}Foxp3^{-}$interleukin (IL) 10^{high} regulatory T cells. 7. Neutrophils produce IL-18 which increases the NK cell population and their antitumor activity. Adapted from Luker et al: At the Bench: Pre-clinical evidence for multiple functions of CXCR4 in cancer (2021) J Leukoc Biol. 2021;109:969–989

advantage to the ovarian tumor [144]. In ovarian cancer CXCR4 antagonism potentiates the PD-1 blocking antibody activity impairing the recruitment of immunosuppressive cell components and increasing tumor-specific cell-mediated immune responses [146]. AMD3100 alone and in combination with a mesothelin-targeted, immune-activating fusion protein VIC-008 modulated immunosuppression in tumors inhibiting PD-1 expression on CD8+ T cells and promoting the conversion of Tregs into CD4+CD25–Foxp3+IL2+CD40L+ helper-like cells [147]. Ex vivo treatment with the new developed CXCR4 antagonist R29 suppresses Treg function and restores T effector cell proliferation without affecting Treg viability in RCC patients [145]. Nomura et. al demonstrated that transducing CXCL12 into two murine immunogenic tumor cells (fibrosarcoma and ovarian cancer, Meth A and HM-1) increased infiltration of CD4+ and CD8+ T cells and antitumor immune responses [148, 149]. However, CXCL12-overexpressing melanoma mice model (B16/OVA cells engineered to overexpress CXCL12) demonstrated that CXCL12 has a bimodal effect on CXCR4-expressing T effector cell migration attracting them at low concentrations and repelling them at higher concentrations of the chemokine [150]. This mechanism is termed chemorepulsion or fugetaxis [150–152] and contributes to the physiological process of T cell migration from the thymus, while repelling T effector cells inside the TME may represent a mechanism by which high CXCL12-expressing tumors evade the immune system [150–152].

Interestingly, recent studies are investigating the role of a novel component of TME: nerves. Nerves are gaining attention for their role in

cancer as their presence in the TME correlates to cancer metastasis and poor prognosis [153]. Cancer-nerve crosstalk is based on perineural invasion (PNI, cancer cells invading and migrating along the perineurium) and tumor innervation (nerve fibers recruited by neoplastic tissues); both mechanisms cause pain, poor prognosis, and higher risk for recurrence and depend on the chemokine network [153]. In a PNI-associated pancreatic cancer model, in vitro and in vivo analyses revealed that the CXCL12/CXCR4 signaling axis can promote PNI, which can be inhibited by AMD3100 or CXCR4 short hairpin RNA [154].

5.6 Targeting the CXCL12-CXCR4/CXCR7 Axis in Combination Therapy

Different CXCL12/CXCR4 antagonists have been developed and validated (Table 5.1), showing promising anti-cancer activities in several tumor types and can be divided in five major classes: (1) small modified peptides, including BKT140 [155], FC131 [156], T140 [157], and POL6326 [158]; (2) small molecules, including the bicyclam AMD070 [159], AMD3100 [160], AMD11070 [161], MSX-122 [162], GSK812397 [163], and KRH-3955 [164]; (3) antibodies, such as MDX-1338/BMS 93656 [165]; (4) modified agonists and antagonists for CXCL12 such as CTCE-9908 [166]; and (5) microRNAs, such as miR-302a [98], miR-9 [100], miR-204-5p [103], and miR-126 [105].

CTCE-9908 is a CXCL12 analogue which actually received the orphan drug status for osteogenic sarcoma treatment [166]; CTCE-9908 showed in vivo and in vitro antitumor activity in different tumor models such as osteosarcoma, prostate, esophageal, and breast cancer [167–171]. NOX-A12 (OLA-PEG) is a high-affinity anti-CXCL12 Spiegelmer which binds CXCL12 blocking its interaction with both CXCR4 and CXCR7. OLA-PEG antitumor activity has been evaluated in multiple myeloma, leukemia, colorectal cancer, and glioma and in association with immune checkpoint inhibitors in pancreatic cancer and colorectal cancer (NCT03168139, NCT01521533, NCT01486797, NCT04121455) [172–175]. Combinations of OLA-PEG with other chemotherapeutic agents were investigated [176]. Peptide R and Peptide R54 are CXCR4 inhibitors generated by rational design. Peptide R inhibits CXCL12-induced cell migration and lung metastasis development [177–180] and potentiates standard/immune therapy in colorectal cancer models in vivo [107, 181].

Several evidence showed that combinatorial blockade of CXCR4 and PD-1 greatly reduces specific cellular and functional elements within the immunosuppressive tumor microenvironment and augments tumor-specific cell-mediated immune responses. Combined treatment with the Pep R and anti-PD-1 reduced tumor progression in two syngeneic murine models, anti-PD-1 sensitive and resistant, increasing Granzyme+ and inhibiting Foxp3+ cell tumor infiltration. In addition Pep R54, a Peptide R derivative [182], synergizes with nivolumab in inhibiting the growth of the PD-1 expressing human PES43 melanoma xenograft [181]. Fibroblast activation protein-α (FAP)-positive CAF are the major source of CXCL12 in TME that regulates TME exclusion of T cells. The conditional depletion of the FAP+ CAF permits immune control effects of both anti-PD-L1and anti-CTLA-4; administering AMD3100 induced rapid T cell accumulation in this autochthonous model of pancreatic ductal adenocarcinoma (PDA) [183, 184]. Moreover, CXCL12 upregulation in HCC models increased hypoxia and the recruitment of immunosuppressive cells, PD-L1, regulatory T cells, and M2-type macrophages after treatment of sorafenib. PD-1 blockade combined with CXCR4 inhibition and sorafenib decreased HCC growth [185]. NOX-A12 in colon cancer spheroid increased numbers of T and NK infiltrating cells and reduces tumor growth in combination with anti-PD-1 compared with anti-PD-1 alone [172].

5.7 Future Trends or Directions

CXCL12 has two main interaction partners, specifically CXCR4 and CXCR7. CXCL12 is a crossroad molecule that modulates crucial

Table 5.1 CXCL12/CXCR4 axis antagonists in cancer clinical development

Drug name	Phase	Active indication	Combination therapy	Trial number
Small molecules				
Plerixafor (AMD3100)	Phase 2	Metastatic pancreatic cancer	Cemiplimab	NCT04177810
	Phase 2	Acute myeloid leukemia, acute lymphoid leukemia	Busulfan, cyclophosphamide	NCT02605460
X4P-001	Phase 1	Melanoma	Pembrolizumab	NCT02823405
	Phase 1/2	Renal cancer	Axitinib	NCT02667886
CX-01	Phase 1	Myelodysplastic syndromes, acute myeloid leukemia	Azacitidine	NCT02995655
	Phase 2	Acute myeloid leukemia	Idarubicin, cytarabine	NCT02873338
CXCL12 Spiegelmer				
NOX-A12	Phase 1/2	Glioblastoma	Radiotherapy	NCT04121455
	Phase 1/2	Metastatic colorectal cancer Metastatic pancreatic cancer	Pembrolizumab	NCT03168139
CXCR4 peptide antagonists				
Balixafortide	Phase 3	Metastatic breast cancer	Eribulin	NCT03786094
LY2510924	Phase 1	Leukemia	Idarubicin, cytarabine	NCT02652871
BL-8040	Phase 2	Metastatic pancreatic adenocarcinoma	Pembrolizumab	NCT02826486
	Phase 2	Malignant neoplasms of digestive organs, metastatic pancreatic cancer	Pembrolizumab	NCT02907099
	Phase 1/2	Pancreatic adenocarcinoma	PEGPH20, cobimetinib, atezolizumab, gemcitabine, Nab-paclitaxel, oxaliplatin, leucovorin, fluorouracil	NCT03193190
	Phase 1/2	Gastric adenocarcinoma or gastroesophageal junction adenocarcinoma	PEGPH20, linagliptin, paclitaxel, ramucirumab, 5-FU, leucovorin, oxaliplatin, atezolizumab, cobimetinib	NCT03281369
	Phase 1/2	Carcinoma, non-small cell lung	Gemcitabine, carboplatin, pemetrexed, CPI-444, tazemetostat, atezolizumab, cobimetinib, RO6958688, docetaxel	NCT03337698
Anti-CXCR4 antibodies				
Ulocuplumab (MDX-1338)	Phase 1/2	Waldenstrom's macroglobulinemia	Ibrutinib	NCT03225716

mechanisms such as proliferation and migration of tumor and tumor microenvironment cells. CXCL12 targeting affects tumor primary growth, mesenchymal transition, and migration but also shapes the TME toward immunoresponsive TME, potentiates the efficacy of checkpoint inhibitors targeting drugs, and interferes in building distant pre-metastatic niches. CXCL12-CXCR4 antagonists, although suboptimal, need deeper evaluation in terms of patient's selection, schedule, and combination therapies.

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CXCL13 Signaling in the Tumor Microenvironment

6

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Abstract

Chemokines have emerged as important players in tumorigenic process. An extensive body of literature generated over the last two or three decades strongly implicate abnormally activated or functionally disrupted chemokine signaling in liaising most—if not all—hallmark processes of cancer. It is well-known that chemokine signaling networks within the tumor microenvironment are highly versatile and context-dependent: exert both pro-tumoral and antitumoral activities. The C-X-C motif chemokine ligand 13 (CXCL13), and its cognate receptor CXCR5, represents an emerging example of chemokine signaling axes, which express the ability to modulate tumor growth and progression in either way. Collateral evidence indicate that CXCL13-CXCR5 axis may directly modulate tumor growth by inducing proliferation of cancer cells, as well as promoting invasive phenotypes and preventing their apoptosis. In addition, CXCL13-CXCR5 axis may also indirectly modulate tumor growth by regulating noncancerous cells, particularly the immune cells, within the tumor microenvironment. Here, we review the role of CXCL13, together with CXCR5, in the human tumor microenvironment. We first elaborate their patterns of expression, regulation, and biological functions in normal physiology. We then consider how their aberrant activity, as a result of differential overexpression or co-expression, may directly or indirectly modulate the growth of tumors through effects on both cancerous and noncancerous cells.

Keywords

Antitumoral · B-cell differentiation · BCA-1 · BLR1 · Cancer progression · Chemokines · CXCL13 · CXCR5 · GPCR · Immune responses · Lymphoid neogenesis ·

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Pro-umoral · Survival niche · TLSs · Tumor microenvironment

6.1 Background

Significant progress has been, and is being, made in understanding the complex mechanisms by which the tumor microenvironment regulates cancer development and progression [1]. Indeed, a number of versatile intercellular and molecular cross talks and the accompanying signaling events have been recognized, which enable malignant cells and the corresponding stromal cells to coevolve with time and, thus, establish dynamic loop networks aiding tumor growth and progression [1]. In this regard, the tumor-/stroma-derived chemokines, together with other extracellular mediators (growth factors, eicosanoids, etc.), have particularly gained attention in the last few years [2, 3]. Initially thought to orchestrate exclusively leukocyte recruitment, chemokines are now being regarded as central players in coordinating the complex tumor-stroma cross talks that dictate processes of cancer development and progression [2–4].

The term chemokines represents a large family of small-molecular-weight (~8 to 12 kDa) chemotactic ligands that are further classified into four subgroups (CC, CXC, XC, and CX3C) based on the varying pattern of two of the four conserved cysteine residues located near the N-terminus of the mature ligand (Fig. 6.1a) [5]. Chemokines act via seven transmembrane-spanning G-protein-coupled receptors (GPCRs) and are best known for their ability to regulate directed migration of diverse cells in both physiological and pathological conditions [3, 5, 6]. Functionally, they are either homeostatic or inflammatory depending upon the pattern of expression [7]. Chemokines play multiple roles in the normal physiology by mediating different cellular and biological processes, while their dysregulated or abnormal functioning may contribute to a variety of pathological disorders, including cancer [2, 4].

The research area concerning the fundamental roles of the chemokine system in cancer is very complex and dynamic [2, 7]. The existing data highlight the ability of many chemokine signaling pathways to express both pro-tumor and antitumor activities within the tumor microenvironment, although several are skewed toward cancer-promoting direction [2, 7]. The aim of this chapter is to specifically enlighten the role of chemokine C-X-C motif ligand 13 (CXCL13), and its cognate receptor CXCR5, in the tumor microenvironment while alluding its involvement in both pro-tumorigenic and antitumoral responses. We first elaborate on current knowledge concerning their gene cloning, structure characterization, expression patterns, and functioning in physiological context of expression. We then discuss how their aberrant activity as a result of differential overexpression or co-expression may directly or indirectly modulate the growth of tumors through effects on both cancerous and noncancerous cells. For some recent reviews on this topic, the readers may also see references [8, 9].

6.2 CXCL13 and CXCR5: Genes, Proteins, and Regulation

CXCL13 is a 109-amino-acid protein that, in humans, is encoded by *CXCL13* gene. The protein was initially termed B cell-attracting chemokine 1 (BCA-1) after its identification, in 1998, as the first chemokine to be selective toward human B-lymphocytes [10, 11]. The name BCA-1 originated from its unique property of preferentially inducing B-lymphocyte chemotaxis, though it was able to promote migration of small numbers of T cells and macrophages as well [10, 11]. The BCA-1 protein was later renamed as CXCL13 based on the facts that (1) its sequence contained four cysteine residues in a typical C-X-C chemokine pattern (Fig. 6.1b) and (2) the gene *CXCL13* was mapped on chromosomal location 4q21, a locus that is in close proximity to a cluster of other known CXC chemokine genes [12]. The main structural feature of CXCL13 is the typical “Greek key”

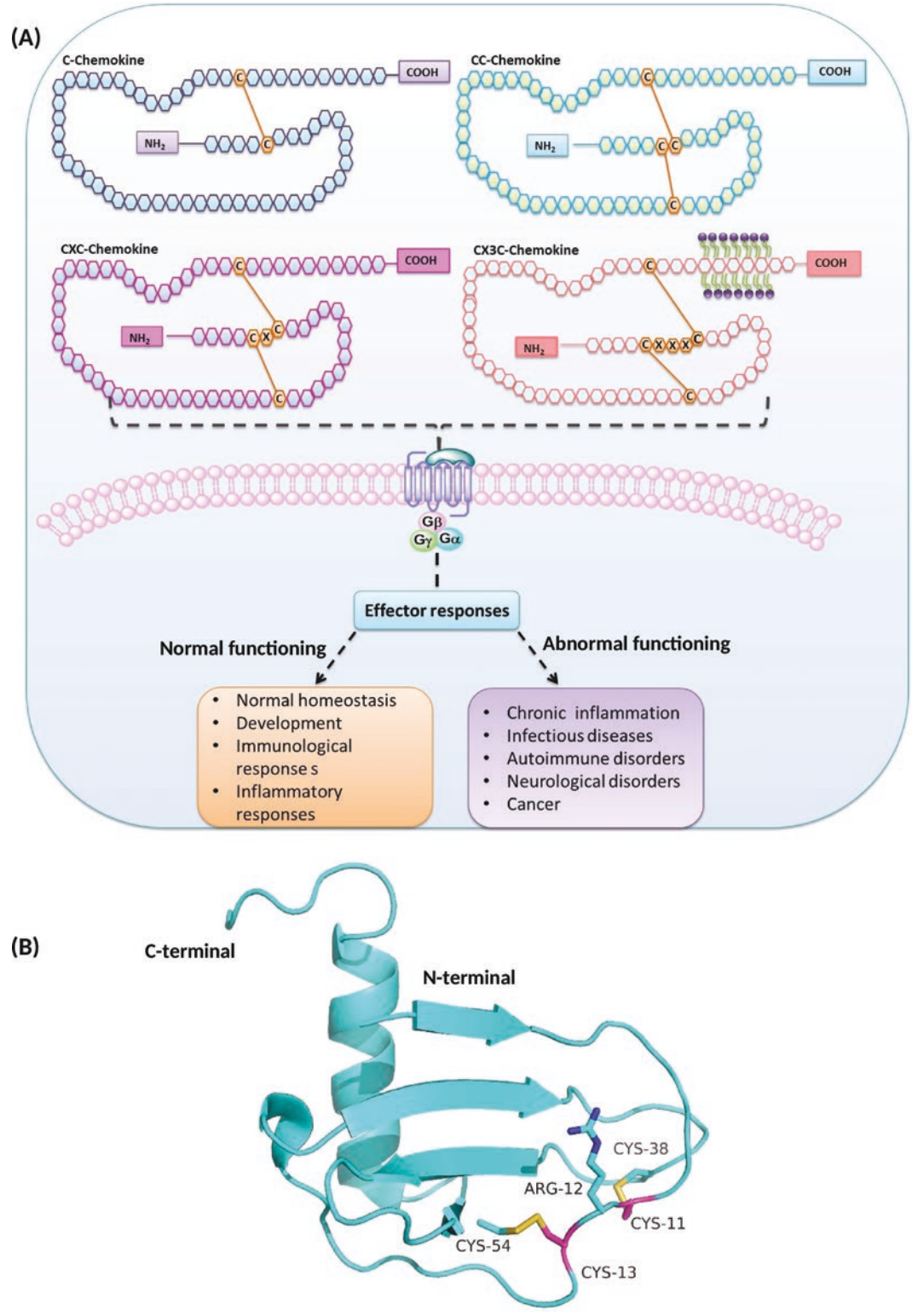

Fig. 6.1 (**a**) Schematic representation of the four subgroups in chemokine system. (**b**) Crystal structure of CXCL13 (PDB: 4ZAI). The four conserved cysteines are depicted in stick representation. The two cysteines of the typical C-X-C (CYS-11-ARG-12-CYS-13) motif are colored magenta

shape, in which a three-stranded β-sheet is overlaid by a C-terminal α-helix (Fig. 6.1b). Almost all chemokines share this structural feature owing to the presence of four conserved cysteine residues in their structure, which form disulfide bridges to stabilize this conformation.

CXCL13 is a homeostatic chemokine that is constitutively expressed by stromal cells in B cell-rich areas of secondary lymphoid tissues (follicles), such as the spleen, lymph nodes, and germinal centers in Peyer's patches [11]. Transcriptionally, CXCL13 expression within all secondary lymphoid organs is regulated by lymphotoxin-beta receptor (LTβR) signaling [13], which involves the cooperation of multiple intracellular signaling cascades, including the canonical (RelA-p50) and noncanonical (RelB-p52) nuclear factor-kappa B (NF-kB) pathways and protein kinase C (PKC) activity [14]. The proximal promoter region of *CXCL13* gene contains a binding site for Rel/NF-kB (for inducible expression) and another site for the binding of transcription factor Sp1 (for basic transcription) [15]. Some recent studies have shown that Sox4 [16], androgen receptor (AR) [17], and reciprocal activity of RelA and Nrf2 [18] may also control transcriptional expression of CXCL13 under disease conditions.

The receptor for CXCL13, CXCR5, was independently identified several years back in 1992 by the pioneering work of Dobner et al. [19], who isolated a cDNA encoding a novel member of the superfamily of GPCRs, originally designated BLR1 (Burkitt's lymphoma receptor 1). The researchers observed exclusively high mRNA expression of BLR1 in Burkitt's lymphoma cell lines and lymphatic tissues, but not in other cell lines either of the B-cell lineage (undifferentiated B-lymphocytes) or of other hematopoietic (myeloid, monocytic, erythroid, or T-lymphocytic) or non-hematopoietic origin [19]. In humans, the gene *BLR1* maps on chromosome 11, at 11q23.3, and the encoded protein sequence shares ~40% amino acid homology to CXCR1, a structurally well-characterized chemokine receptor for the cytokine interleukin (IL)-8 [19]. BLR1 was later renamed CXCR5 (after identification of CXCL13) in agreement with the nomenclature rules for chemokine receptors [10]. CXCR5 is remarkably expressed by mature recirculating B-lymphocytes [20], a subpopulation of follicular B helper T cells (T_{FH}) [21, 22], and antigen-bearing dendritic cells (DCs) [23], controlling their migration into secondary lymphoid organs toward the gradient of CXCL13 [10, 11, 23, 24]. With respect to transcriptional regulation, Raf-1/nuclear factor of activated T cells c3 (NFATc3) and NF-kB transcription factors have been found to control the *CXCR5* gene expression in different cell types [25–27].

The CXCR5 interactions with CXCL13 obey the classical GPCR activation paradigms, which normally involve coupling to an intracellular heterotrimeric G-protein complex that is composed of Gα, Gβ, and Gγ subunits [28–31]. Previously, it has been shown that CXCR5 characterizes unique structural constraints—particularly the presence of specific sequence motifs in the intracellular domains and probably the transmembrane spanning regions—that are of utmost importance not only for coupling to the heterotrimeric G-proteins but also for fine-tuning the cellular response toward the stimulus [32]. However, the precise nature of CXCL13-CXCR5 signal transduction mechanisms largely remains elusive. In the past, most of the functional studies of CXCL13-CXCR5 axis were carried out in cancer settings, characterizing its receptor coupling to divergent signaling events downstream of ligand binding (as will be discussed in later sections), which ultimately may induce a variety of responses in both cancer and immune cells.

6.3 Biological Functions of CXCL13-CXCR5 Axis

The primordial function of CXCL13-CXCR5 axis is the B-cell development and their architectural organization within lymphoid follicles (Fig. 6.2) [11, 20, 24, 33]. Lymphoid follicles represent B cell-rich compartments of lymphoid organs, which serve as sites of B-cell antigen encounter and differentiation [34]. Generally, lymph organs com-

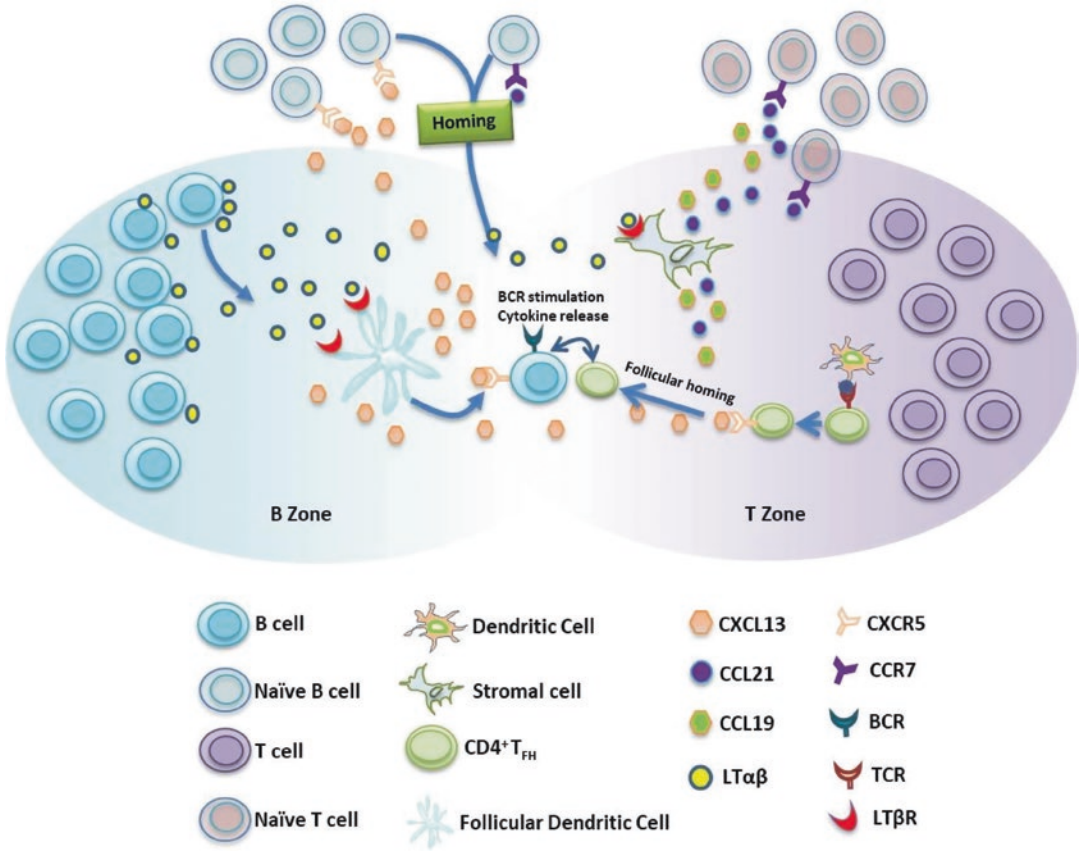

Fig. 6.2 Schematics of the CXCL13-CXCR5-derived architectural organization of B cells within lymphoid follicles. Together with other cytokines, such as chemokine (C-C motif) ligand (CCL19), CCL21, and members of the lymphotoxin/tumor necrosis factor (LT/TNF) family, CXCL13-CXCR5 axis guides the cellular arrangement of the follicular structure. Different gradients of CXCL13 and CCL19/CCL21 induce segregation of B- and T-cells into specific compartments

prise two types of B cell-rich compartment: follicles containing follicular dendritic cells (FDCs) and areas lacking such cells [35]. A prerequisite step for the formation of B-cell lymphoid follicles is the clustering of circulating naïve B cells in the proximity of FDCs [34, 35]. CXCL13-CXCR5 axis cooperates with members of the lymphotoxin/tumor necrosis factor (LT/TNF) family to devise the stromal cell-B-cell signaling cascades, which ultimately provide the underlying roads that circulating naïve B cells actively follow for localization within the specific anatomic compartments of the lymphoid follicles (Fig. 6.2) [10, 24, 35, 36]. In brief, the B cell-derived LTα and LTβ cytokines interact with LTβ receptor (LTβR) on surrounding stromal cells which, in turn, leads to increased secretion of CXCL13 by stromal cells including FDCs [35]. In addition to mediating chemoattraction, the signaling by stromal-derived CXCL13, via CXCR5, may reciprocally enhance the cell surface expression of LTα1β2 on B cells. The activated B cells with high LTα1β2 may then further induce expression of CXCL13 by stromal cells via LTβR signaling, eventually deriving the LTα1β2:CXCL13 positive feedback loop system that is crucial for the development of B-cell areas of secondary lymphoid tissues and homeostasis (Fig. 6.2) [10, 35].

Mice deficient in CXCR5 failed to form structured lymphoid organs, due to defective traffick-

ing of mature B cells to lymphoid follicles [24]. This suggested an additional role of CXCL13-CXCR5 axis in lymphoid organ development. Moreover, CXCL13-CXCR5 axis is also actively involved in lymphoid neogenesis, a process of events leading to the formation of organized lymphoid tissues or organs. The experimental data emerging from parallel mouse studies, involving LTα-, LTβ-, and CXCR5-deficient mice as well as the transgenic mouse models, indicate that LTα1β2 and CXCL13-CXCR5 act in a common pathway of lymphoid neogenesis [24, 37, 38].

Furthermore, CXCL13-CXCR5 axis mediates the development of B cell-mediated, T cell-dependent or T cell-independent immune responses [39, 40]. For instance, CXCL13-CXCR5-derived homing of B-1 B cells—a subset of recirculating B cells predominately found in peritoneal and pleural cavities [41]—to body cavities plays a dominant role in balanced development of IgM-based innate immune response against bacterial pathogens [39, 40]. Similarly, LT-expressing, B cell-derived CXCL13 has been shown to attract CXCR5+ DCs and CD4+ T cells to sites of immune priming, leading to optimal development of TH_2 effector cell responses against infection [23].

Altogether, CXCL13-CXCR5 axis plays important roles in B-cell terminal differentiation, maintenance of lymphoid tissue microarchitecture, and the development of B- and T-cell-mediated immune responses.

6.4 Tumor Microenvironment and CXCL13-CXCR5 Signaling Axis

Tumor microenvironment is the location in which cancer cells continuously interact with noncancerous cells, such as fibroblasts, immune cells, and endothelial cells, and thereby acquire proliferative and invasive properties that further facilitate tumor growth and metastatic spread [42]. Chemokines have the ability to affect both cancerous and noncancerous cells and, thus, may act as pro-tumoral or antitumoral regulators of malignancy [7]. Many of the chemokine signaling pathways are more typically considered as pro-tumorigenic, even if at times they can have antitumor effects [7].

Looking from the perspective of CXCL13 and CXCR5, the differential overexpression and co-expression of both proteins have been linked to aggressive cancer biology in many tumor tissues [8]. Over the last two decades, considerable amount of experimental and clinical data have emerged which strongly implicate the dysregulated CXCL13-CXCR5 signaling in the initiation and progression of several human malignancies [8, 9]. These data also characterize biochemical, molecular biology, and genetic approaches-based investigations to unravel the biological roles of CXCL13-CXCR5 signaling within the tumor microenvironment. Such investigative attempts have provided a good interpretation of the important CXCL13-CXCR5 functions, and the structural links of this pathway with other signaling cascades/proteins, in relevance to cancer and immune cell biology while manifesting that:

1. CXCL13-CXCR5 axis may directly modulate tumor growth by inducing proliferation of cancer cells and preventing their apoptosis [43–46];
2. CXCL13:CXCR5 interactions promote invasive phenotypes and may direct tumor cell movement required for metastasis [46, 47]; and
3. CXCL13-CXCR5 axis may also indirectly modulate tumor growth by regulating noncancerous cells, particularly the immune cells, within the tumor microenvironment [48, 49].

CXCL13-CXCR5 axis may indirectly assist growth of tumor cells by aiding their escape from T-effector cell immunity [48, 50, 51]. This effect is mediated by CXCL13-CXCR5-derived secretion of immunoregulatory cytokine IL-10 by tumor cells or recruitment of immunosuppressive myeloid-derived suppressor cells (MDSCs) and T-regulatory (Treg) cells within the tumor microenvironment (pro-tumoral activity). On the other hand, CXCL13-CXCR5

axis also has the potential ability to induce antitumoral responses through formation of tertiary lymphoid structures (TLSs) [52]. TLSs are often associated with tumoral tissues, where they shape local adaptive immune responses [53]. Because of its ability to recruit circulating CXCR5+ B-cell and CXCR5+ CD4+ TFH-cell populations to the site, CXCL13 is considered as the critical triggering factor of TLS formation in tumor tissues [54]. In addition, the presence or absence of CXCR5 receptor seems to be crucial for antitumor activity of CD8+ cytotoxic T-lymphocytes (CTLs) [55–57]. CD8+ CTLs are key elements of the tumor immunosurveillance which kill tumor cells by lysosome/protease system (the perforin/granzyme B) and also through secretion of the tumoricidal lymphokines, such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) [58].

In the later sections, we elaborate the role of CXCL13-CXCR5 axis within the tumor microenvironment of different human cancers while emphasizing its direct (on cancerous cells) and indirect (on noncancerous cells) effects to modulate tumor growth and progression.

6.5 CXCL13-CXCR5 Axis in Hematological Malignancies

Hematological malignancies mainly encompass the malignant tumors of the hematopoietic (leukemia) and lymphoid (lymphomas) tissues [59]. Differential overexpression of CXCR5 has been reported in B-cell lineage acute and chronic lymphocytic leukemia (B-ALL and B-CLL) cells [28, 45]. In addition, high CXCL13 serum levels were also found in CLL patients [28].

Imbalances in the CXCL13-CXCR5 axis may contribute to pathobiology of hematological malignancies. The leukemic cells, in particular, can take advantage of the CXCL13-CXCR5 signaling axis for their inappropriate proliferation and resistance to apoptosis. It is well established that CXCL13-CXCR5 axis is intimately involved in the microenvironmental regulation of B-cell chronic lymphocytic leukemia (B-CLL) cells [28, 43, 60]. Distinct microanatomical environments in the bone marrow as well as the secondary lymphoid organs serve as the sanctuary sites and protective niches of leukemic cell proliferation [61]. Given the fact that CXCL13-CXCR5 axis is involved in B-cell terminal differentiation and maintenance of lymphoid tissue microarchitecture (discussed in last section), it is not surprising that enhanced expression of CXCR5 on B-CLL cells, and its stimulation by stromal-derived CXCL13, may induce the recruitment of leukemic cells to the putative proliferation and survival niches within the secondary lymphoid organs [28, 43]. Using the murine Emu-Tcl1 CLL model, Hopken and coworkers [43] have shown that CXCL13-CXCR5-derived reciprocal stroma-leukemia cross talks may help in deriving a paracrine feedback loop network, in which CXCR5-dependent lodging of B-CLL cells stimulates resident mesenchymal stromal cells through LTβR activation and, thus, results in CXCL13 secretion and stromal compartment remodeling. By identifying the marginal zone (MZ)-specific factors involved in migratory and adhesive behavior of leukemic cells, they further outlined that CXCL13-CXCR5 interactions may facilitate the follicular tumor cell homing, shaping of a survival niche, and niche-specific retention and survival of B-CLL cells [43, 60].

Apart from inducing recruitment to survival niches, CXCL13-CXCR5 interactions may favor the leukemic cell resistance to apoptosis. Two earlier studies by Ticchioni et al. [44] and Chunsong et al. [45] manifested CXCL13-CXCR5 axis as one of the homeostatic chemokine signaling networks, which can aid leukemic cell survival via inactivation of apoptotic machinery. By using the patient-derived leukemic cells, Ticchioni et al. demonstrated that CXCL13 enhances cell survival mainly through the Akt-dependent inactivation of the proapoptotic transcription factor FOXO3a [44]. Mechanistically, CXCL13, along with other homeostatic chemokines (CXCL12, CCL19, and CCL21), induced the phosphorylation of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)1/2

and p90RSK, and Akt and its effectors GSK3 and FOXO3a [44]. Similarly, Chunsong et al. showed that CXCL13 and CCL19 together by means of frequent activation of CXCR5 and CCR7, respectively, upregulate the expression and function of paternally expressed gene 10 (PEG10), which subsequently stabilize caspase-3 and caspase-8 in B-ALL and B-CLL, and further rescue the cells from TNF-alpha-mediated apoptosis [45].

Elevated CXCL13 and/or CXCR5 expressions have also been implicated in the pathogenesis of different types of lymphomas, both of B- and T-cell origin. To name a few lymphoproliferative disorders in which aberrant overexpression of CXCL13 and/or CXCR5 has been linked to the tumorigenic process include gastric lymphoma [36, 62], cutaneous B- and T-cell lymphomas [63, 64], follicular lymphoma (FL) [65], primary intraocular lymphoma [66], non-Hodgkin lymphomas (NHL) [67], angioimmunoblastic T-cell lymphoma (AITL) [68–70], and primary central nervous system lymphoma (PCNSL) [71]. In PCNSL, cell type-specific expression and the microenvironmental interactions of CXCL13 and CXCR5 have been shown to facilitate tumor development and localization to CNS [71, 72]. Moreover, elevated expression of CXCL13 has also been proposed as a diagnostic marker for certain lymphomas, with strongest correlations reported for AITL [73], PCNSL [74], human immunodeficiency virus (HIV)-associated NHL (AIDS-NHL) [75], and extranodal natural killer (NK)/T-cell lymphoma [76].

Pro-tumoral Effects Collateral evidence indicate that CXCL13-CXCR5 axis may exert protumorigenic effects by inducing pleiotropic cytokine IL-10 secretion, as well as promoting the infiltration of immunosuppressive Treg cells within the tumor-immune microenvironment of both leukemic and lymphoproliferative disorders. IL-10 is an immunoregulatory cytokine whose increased expression may contribute to pathogenesis and progression of malignant cells via immunosuppression [77]. Previously, it has been shown that CXCL13-CXCR5 axis may synergize with CCL19-CCR7 pathway to regulate interactions between B-ALL CD23+ CD5+ B-cells and CD8+ T cells which, in turn, resulted in IL-10 overexpression and impairment of tumor-specific cytotoxicity of syngeneic CD8+ T cells [50]. Likewise, in DLBCL, IL-10 secretion by tumor cells also inhibited CXCR5-expressing circulating CD4+ and CD8+ T-cell-mediated cytotoxicity, which subsequently assisted the growth and survival of malignant cells [78, 79]. With respect to immune cell infiltration, FL Treg cells have been found to autoregulate their own chemotaxis, via autocrine CXCL13-CXCR5 signaling mechanism, and thereby localize and accumulate within the malignant lymph node, where they suppressed effector T-cell activity to further facilitate malignant cell growth and expansion [80]. Similarly, CD4+ CXCR5+ Foxp3+ follicular Treg cells suppressed the proliferation and cytokine expression by CD8+ T cells in the tumor microenvironment of DLBCL [81].

6.6 CXCL13-CXCR5 Axis in Solid Tumors

6.6.1 CXCL13-CXCR5 Axis in Lung Cancer

Lung cancer is the leading cause of cancer-related mortality around the world. It is broadly classified into small cell lung carcinomas (SCLCs) and non-small cell lung carcinomas (NSCLCs). CXCL13 is included among the cytokine profiles that are elevated in NSCLC [82, 83]. Some studies have also identified CXCL13 as a predictive factor for risk of early-stage lung adenocarcinoma [83, 84] and as a part of secreted phosphoprotein 1 (SPP1) upstream invasive network module [85]. Singh et al. [86] reported the elevated serum CXCL13 levels in lung carcinoma patients compared with healthy volunteers. They also found higher CXCR5 expression in human NSCLC tissues relative to nonneoplastic lung tissues. By using migration assays, they further revealed that a pro-migratory phenotype in NSCLC cells could be stimulated by CXCL13 [86], thus suggesting the possible involvement of CXCL13-CXCR5 axis in

the tumorigenic and metastatic phenotypes of primary lung tumors [86].

One study has previously implicated CXCL13-CXCR5 signaling axis in the benzo(a)pyrene (BaP)—a key carcinogen present in cigarette smoke and air pollution—induced lung carcinogenesis [87]. While comparing the levels of certain proteins (which are linked to inflammation) in lung cancer patients, the authors found a clear link between cigarette smoke and CXCL13 expression. BaP could induce CXCL13 expression in lung epithelial cells and cancer cells and in mice prior to development of detectable lung cancer, while CXCL13 or CXCR5 knockout significantly attenuated BaP-induced lung cancer in mice [87]. Further experiments showed that CXCL13 is a direct target gene of aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that can bind to a xenobiotic-responsive element (XRE) in the *CXCL13* gene, and BaP induces CXCL13 production by facilitating AhR translocation to the nucleus [87]. From mechanistic perspective, it was revealed that CXCR5 is highly expressed by CD68+ macrophages within the tumor site, and its interactions with overexpressed CXCL13 may induce the production of SPP1 by tumor-associated macrophages (TAMs) [87]. The CXCL13-CXCR5-induced SPP1 further established a positive feedback loop network via activation and nuclear localization of β-catenin in epithelial and cancer cells, in turn promoting endothelial mesenchymal transition (EMT) and lung cancer progression [87]. Dexamethasone, a synthetic glucocorticoid, inhibited CXCL13 production by epithelial cells and SPP1 production by TAMs, which resulted in inhibition of EMT and tumor burden in mice [87]. Overall, the work demonstrated the causal link between CXCL13-CXCR5 axis and lung tumor initiation and progression.

Antitumoral Effects A limited evidence also hints on the potential role of CXCL13-CXCR5 axis in devising antitumoral immune response against lung cancer. Indeed, CXCL13 is the known critical triggering factor of TLS formation, which can devise humoral and cell-mediated immune responses against tumors [54]. With functional TLSs in place, efficient antigen presentation and cell activation and differentiation occur for the development of both humoral and cell-mediated immune responses against tumors [53, 88]. Given this fact, CXCL13 has previously been characterized as an important component of specific gene expression signature that is associated with T cell presence in TLSs in human lung cancer [88]. Silina et al. [52] have recently identified a perivascular CXCL13-positive niche that supports TLS development in lung squamous cell carcinoma (LSCC) patients, with significant relevance for patient survival. Through gene expression analysis, they found that CXCL13 expression correlates with intratumoral TLS density, which further correlated with GC formation and expression of adaptive immune response-related genes [52]. Additionally, steroid treatment showed a negative impact on TLS development and specifically on GC formation in LSCC patients [52]. Similarly, CXCL13-secreting CD8+ lymphocyte populations with high PD-1 expression from NSCLC patients have been shown to play a critical role in the recruitment of immune cell subsets to the tumor microenvironment, suggesting their possible involvement in the formation of TLSs [89]. Together these data imply that further studies ought to be endorsed in order to distinctly define the role of CXCL13-CXCR5 axis in antitumor immunity against lung cancer.

6.6.2 CXCL13-CXCR5 Axis in Breast Cancer

Breast cancer is the most frequently diagnosed malignancy and the common cause of cancer death in women worldwide. Breast cancer exhibits one of the strongest relationships between CXCL13-CXCR5 axis and tumor progression. Many studies have reported significantly higher expressions of CXCL13 and/or CXCR5 in both tumor tissues and peripheral blood of breast cancer patients [47, 90–92] and also proposing their co-expression as a poor-prognosis marker [47]. Strong expressions of CXCL13 and CXCR5 have also been described in breast cancer cell lines such as MCF-7, MDA-MB-231, ZR-75, and

BT-20 [90]. The upregulation of CXCL13 and CXCR5 in breast tumors is governed by different mechanisms: CXCL13 expression is under the direct transcriptional control of RelA and Nrf2, while CXCR5 is epigenetically regulated (lack of CpG island methylation) within its promoter [18].

CXCL13-CXCR5 axis serves a pivotal role in breast cancer growth and lymph node metastasis (LNM). Tumorigenesis experiments, involving BALB/c mice inoculated with 4 T1 breast cancer cells, have demonstrated the involvement of CXCL13-CXCR5 signaling in tumor growth and progression via ERK-mediated production of inflammatory cytokines IL-1β and TNF [93]. Treatment of mice with an anti-CXCL13 antibody induced tumor cell apoptosis while attenuating the activation of CXCR5/ERK pathway [93]. In another recent study, anti-CXCL13 antibody also effectively suppressed the proliferation and induced apoptosis of MDA-MB-231 cells through blockade of CXCR5/ERK pathway [94]. CXCL13 blockage significantly downregulated p-ERK/ERK, IL-1, and TNF-alpha levels in tumor cells [94]. Together these findings provide an evidence that CXCL13-CXCR5/ERK axis is involved in breast cancer growth and progression. Apart from this, two genetic knockdown studies, involving lack of functional p53 family members, have also linked elevated CXCR5 expression to abnormal cell survival and CXCL13-directed migration in MCF-7 breast cancer cells [26, 95].

Similarly, a clear-cut evidence of CXCL13-CXCR5 involvement in LNM was provided by Biswas et al. [47], who demonstrated high expression of metastasis-associated mesenchymal markers (Vimentin, N-cadherin), EMT regulators (Snail, Slug), and matrix metalloproteinase-9 (MMP9) in CXCL13-stimulated breast cancer cells through CXCR5 signaling [47]. CXCL13-CXCR5 axis enhanced the expression of receptor activator of nuclear factor kappa-B ligand (RANKL), which is known to regulate MMP9 expression and, thus, induce breast cancer cell migration via c-Src-mediated activation of the ERK and Akt signaling pathways [47, 96]. By using p110α, PI3K, and Src kinase inhibitors, the authors confirmed that CXCL13-CXCR5-derived RANKL-Src axis may upregulate MMP9 and different EMT regulators in breast cancer cells, which eventually promote their migration and invasion [47]. This study also documented that CXCL13-CXCR5 co-expressing patients with LNM involvement displayed significant correlation to higher expression of various mesenchymal markers and regulators [47]. Nevertheless, the signaling events regulating CXCL13-CXCR5-induced RANKL expression in breast cancer cells still remain elusive.

Antitumoral Effects Tumor-derived CXCL13 may enhance the antitumorigenic B- and T-lymphocyte trafficking to the tumor microenvironment. An interesting evidence in this regard is the IRF5 (interferon regulatory factor 5)-induced expression of CXCL13 in mammary epithelial tumor cells where it orchestrated the recruitment of CD19+ CXCR5+ B cells and CD4+ CXCR5+ T cells to the tumor site [97]. These immune cells are critical to the formation of functional TLSs, which are known to confer better prognosis in breast cancer [53, 98, 99]. Likewise, tumor-infiltrating CXCL13-producing TFH cells (TFHX13) have been shown to potentially trigger TLS formation at tumor site and thereby generate GC B-cell responses in human breast cancer [98]. It was further suggested that TFHX13 cell differentiation may be of crucial importance in converting Treg-mediated immune suppression to de novo activation of adaptive antitumor humoral responses in the breast cancer microenvironment [98]. Moreover, a recent study, aimed to investigate the prognostic impact of CD4+ T-cell subsets in early-stage breast cancer patients, has described an association between high CXCL13 and distant disease-free survival, thus providing evidence that humoral immunity influences the survival outcomes in these patients [100]. Taken together, one may infer that CXCL13-CXCR5 axis may facilitate shaping of favorable immune microenvironment that probably would inhibit breast cancer progress. This notion is further corroborated by the fact that CXCL13 has been included in several immune-related molecular profiles which

indicate better prognosis in breast cancer [101–103].

6.6.3 CXCL13-CXCR5 Axis in Prostate Cancer

Prostate cancer is the second leading cause of cancer-associated mortality in men. CXCL13-CXCR5 axis promotes tumorigenesis, growth, malignant progression, and metastatic spread of prostate cancer cells [17, 29, 31, 46, 104, 105]. Significantly high levels of CXCL13 have been reported in the serum of prostate cancer patients [104]. In addition, CXCL13 has been found to be a better predictor of prostate cancer than prostate-specific antigen (PSA) [104]. The transcriptional upregulation of CXCL13 in prostate cancer tissues is driven by androgen-AR axis and noncanonical NF-κB pathway [17, 46].The putative sites for canonical androgen-responsive element and a noncanonical NF-κB pathway have been identified in the *CXCL13* gene promoter [46]. Differential expression of CXCR5 has also been described in prostate cancer tissues and cell lines [106, 107].

Prostate cancer represents a particular example of solid tumor malignancies where the CXCL13-CXCR5-derived microenvironmental cellular cross talks, and the downstream molecular events, have been elucidated more comprehensively in relation to tumor initiation and progression. Earlier this decade, a series of studies by El-Haibi et al. [29–31, 105] revealed the involvement of divergent intracellular molecular pathways, such as ERK1/2, PI3K/Akt, and stress-activated protein kinase (SAPK)/c-jun kinase (JNK), in CXCL13-CXCR5-derived invasive and proliferative responses in prostate cancer cells. The studies also identified specific G-protein isoforms regulating CXCR5 signal transduction following CXCL13 stimulation (see Fig. 6.3) [30]. Likewise, Garg et al. [46] further identified CXCL13 as a bona fide effector of protein kinase C epsilon (PKCε) in prostate cancer cells. Mechanistic studies revealed that PKCε overexpression cooperates with Pten loss (which leads to PI3K activation) to upregulate CXCL13 production by prostate tumor cells, via a noncanonical NF-kB pathway, which contributes to CXCR5 signal amplification and eventually results in a cell autonomous pro-migratory and tumorigenic autocrine loop [46]. Furthermore, CXCL13 produced by both cancer and stromal cells (specifically cancer-associated myofibroblasts) has been shown to create a pro-tumorigenic environment, leading to B-cell recruitment and evolution of castration-resistant prostate cancer after androgen-deprivation treatment [108]. From mechanistic perspective, it appears that hypoxia-inducible factor 1 (HIF-1)-derived autocrine transforming growth factor (TGF)-β/small mothers against decapentaplegic (SMAD) signaling may promote the activation of CXCL13-expressing myofibroblasts under hypoxic conditions (Fig. 6.3). The tumor-infiltrating B cells, that are recruited by overexpressed CXCL13, may then secrete LTα:β heterotrimers which further induce the stimulation of LTβR on prostate cancer cells, leading to nuclear translocation of IKKα and activation of STAT3, thereby enhancing tumor growth and progression [108, 109]. Blockade of CXCL13 expression by different treatments, including pharmacological inhibition of TGF-β signaling and immunodepletion of myofibroblasts, inhibited B-cell recruitment to the tumor tissues and prevented the emergence of more aggressive type of cancer [108].

Androgen/AR-induced overexpression of CXCL13 has also been found to promote cell cycle and cell phase (G2/M) transition in primary prostate cancer tissues [17]. CXCL13 appeared to be direct target of androgen/AR axis, and its increased expression cooperated with AR in androgen/AR axis-mediated prostate tumor cell growth, proliferation, EMT, migration, and invasion in androgen-dependent LNCaP cells [17].

6.6.4 CXCL13-CXCR5 Axis in Gastrointestinal Cancers

Prominent expressions of CXCL13 and/or CXCR5 have been found in gastrointestinal cancers, such as colon or colorectal cancer [110, 111], hepatocellular carcinoma [49, 112], gastric

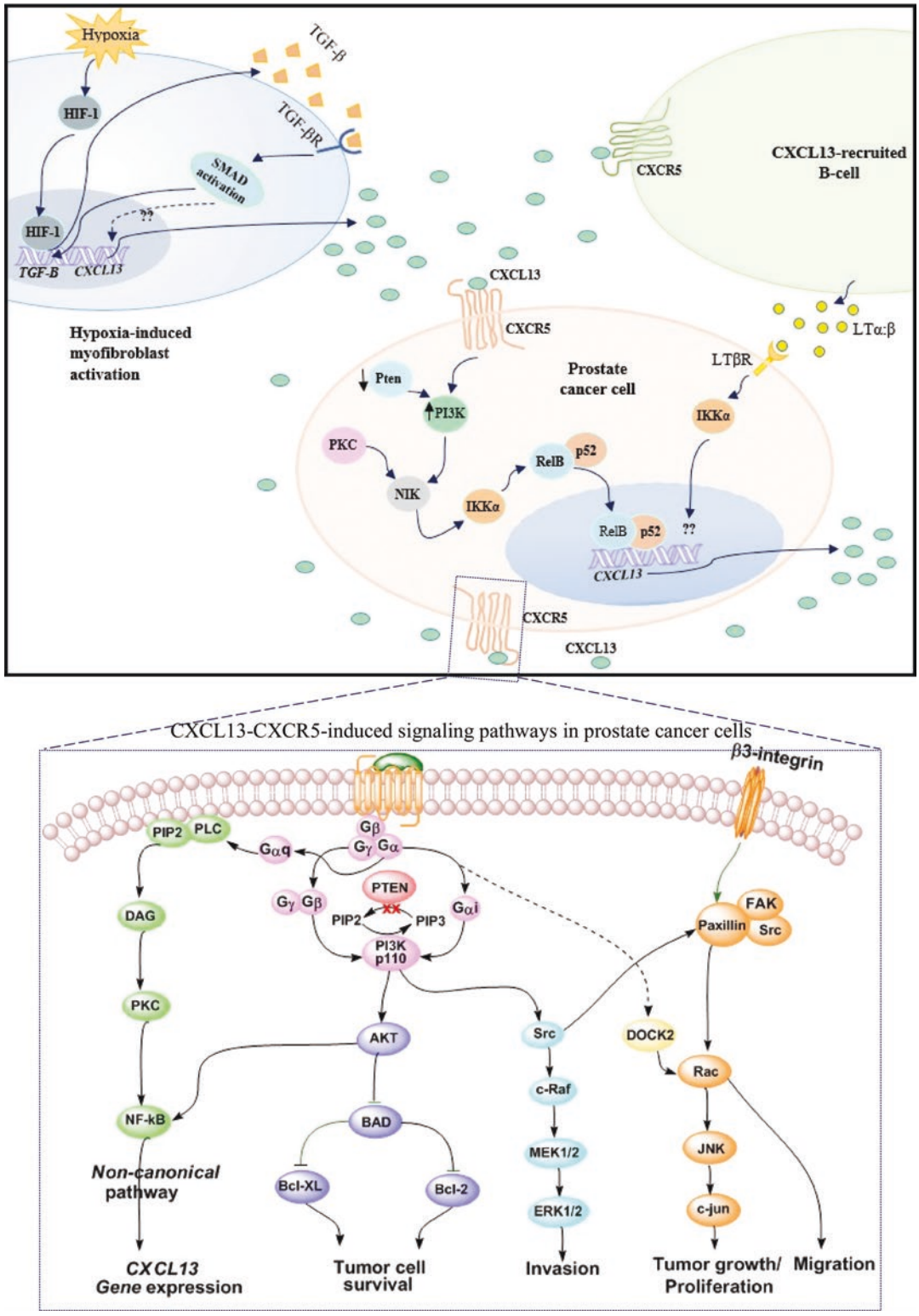

Fig. 6.3 CXCL13-CXCR5 signaling axis in prostate tumor microenvironment. CXCL13 can be produced both by tumor cells and by cells in the tumor microenvironment, such as hypoxia-activated myofibroblasts. In prostate cancer cells, upregulation of the protein kinase C epsilon (PKCε) and loss of the tumor suppressor Pten

cancer [113, 114], and pancreatic cancer [110]. Constitutively active noncanonical NF-κB pathway has been shown to induce CXCL13 expression in human pancreatic cancer cell lines [115].

High levels of CXCL13 and CXCR5 have demonstrated the correlation with tumor development, metastasis, and poorer survival in advanced colorectal cancer [111, 116]. A prior study highlighted that CXCL13 may promote the growth of CXCR5-expressing colon cancer cells in the liver [110]. Similarly, a molecular mechanism study by Zhu et al. [117] demonstrated that CXCL13-CXCR5-derived PI3K/Akt pathway may promote the migration and invasion of colon tumor cells via induction of MMP-13 production [117]. The genetic knockdown of CXCR5 and the pharmacological inhibition of PI3K/Akt pathway (by LY294002) both significantly suppressed the CXCL13-mediated growth, migration, and invasion of colon cancer cells [117]. Furthermore, Chen et al. [51] manifested that CXCL13-CXCR5 axis is essential in mediating the recruitment of Foxp3+ Treg cells by histidine decarboxylase(HDC)-expressing myeloid cells in the settings of colorectal carcinogenesis. HDC+ myeloid-derived CXCL13 may enhance the Foxp3 expression and cellular proliferation of CXCR5-expressing Treg cells through phospho-Stat3 pathway [51]. Both HDC+ myeloid cells and Foxp3+ Treg cells have the ability to suppress CD8+ T-cell tumoricidal immunity. Together these findings suggest that CXCL13-CXCR5 axis may promote colon cancer growth and progression in both direct (affecting cancer cells) and indirect (affecting noncancerous cells) ways.

Elevated CXCL13 serum levels have also shown correlation with tumor size, metastatic disease, advanced stages, and recurrence-free survival in hepatocellular cancer patients [49, 112]. A positive feedback loop network characterizing mutual interaction between CXCL13 and the Wnt/β-catenin pathway has been reported to induce progression of hepatocellular carcinoma [112].

In gastric cancer, intratumoral CXCL13 expression is associated with larger tumor diameter [118], and its increased levels have been proposed as independent prognostic marker for patients undergoing gastric cancer resection [118]. Ding et al. demonstrated that CXCL13-CXCR5-mediated recruitment of CD40+ MDSCs may stimulate gastric tumor growth, by enabling immune evasion, via inhibition of T-cell expansion within the tumor microenvironment [48]. They further revealed that CD40 critically regulates the recruitment and accumulation of MDSCs in gastric cancer settings by controlling CXCR5 expression in MDSCs [48].

6.6.5 CXCL13-CXCR5 Axis in Other Solid Tumor Malignancies

CXCL13 has been identified as a prognostic biomarker for clear cell renal cell carcinoma (ccRCC) [119]. Upregulated CXCL13 expression in ccRCC correlated with advanced disease stage and poor prognosis [119]. Functional and mechanistic studies revealed that CXCL13-CXCR5-mediated activation of PI3K/Akt/mTOR

Fig 6.3 (continued) lead to elevated activity of PI3K, which, in turn, results in sequential activation of the members of the noncanonical NF-κB pathway, ultimately inducing transcription of the *CXCL13* gene. CXCL13 produced in this autocrine manner, together with CXCL13 generated by activated myofibroblasts, not only recruits of B cells within the tumor microenvironment but also stimulates divergent intracellular molecular pathways that are involved in cell growth, survival, invasion, and migration of prostate cancer cells (inset figure). The signal transduction of CXCL13-CXCR5 interactions is mediated by G-protein heterotrimer comprising the typical Gα, Gβ, and Gγ subunits. Both Gαi and Gαq proteins have been implicated in CXCL13-CXCR5-derived molecular events. DOCK2, dedicator of cytokinesis 2; ERK, extracellular signal-regulated kinase; HIF-1, hypoxia-inducible factor 1; JNK, Janus kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3K, phosphoinositide-3-kinase; SMAD, small mothers against decapentaplegic; TGF, transforming growth factor; TGF-βR, TGF-beta receptor; LTα:β, lymphotoxin alpha and beta; and ??, underlying signaling events remain elusive

signaling pathway may promote the proliferation and migration of ccRCC cells [119]. Similarly, high CXCL13 expression levels have been reported in oral squamous cell carcinoma (OSCC) primary tumors [120–122]. OSCC are malignant tumors with a potent activity of local bone invasion/osteolysis. CXCl13-CXCR5-derived RANKL signaling has been implicated in the osteolytic process and metastasis of OSCC tumors [122]. RANKL induces high levels of MMP-9 expression in OSCC as well as in stromal/preosteoblast cells and thereby plays critical role in cancer invasion of the bone/osteolysis [122]. CXCL13-CXCR5 axis may upregulate JNK activity in OSCC tumor cells, which subsequently follows activation and nuclear translocation of NFATc3 transcription factor to enhance RANKL expression [123]. Nonetheless, in stromal/preosteoblast cells, c-Myc, rather than JNK, appears to be downstream target of the CXCL13-CXCR5 axis to stimulate NFATc3-mediated RANKL expression [121].

6.7 Concluding Remarks and Future Directions

To summarize, the findings discussed above highlight the fact that CXCL13-CXCR5 axis often leads to pro-cancerous consequences. Within the tumor microenvironment, CXCL13 interacts with CXCR5 in both autocrine and paracrine fashion and, thereby, may integrate different tumor-immune-stroma cellular cross talks that are critical for dictating several aspects of tumor development and progression, including growth, proliferation, invasion, metastasis, and survival. Nevertheless, a lot more basic investigations are still needed before translating this information in clinical settings. For instance, the downstream signaling regulating the biology of this pathway in cancer and immune cells is yet to be fully explored. In addition, the signaling events which regulate *CXCL13* and *CXCR5* expression in cancerous and noncancerous cells need much fuller investigation. Also, the data regarding immune-mediated pro- and anti-tumoral activities of this pathway is limited in scope and depth. Much remains to be done to grasp a more comprehensive and conclusive landscape of its immune-related effects under broader terms of immune surveillance and evasion mechanisms.

From therapeutic prospect, the data discussed above position CXCL13-CXCR5 pathway as a potential therapeutic target for cancer treatment. Figure 6.4 summarizes the potential impacts of direct targeting as well as blocking (upstream and downstream) the CXCL13-CXCR5 pathway in various cancers. However, the small-molecule inhibitors that could specifically target CXCL13 or CXCR5 are lacking so far. One possible reason for this caveat is probably still-to-solve crystal structure of CXCR5 that might have limited the drug discovery efforts against this pathway. With CXCL13 crystal structure already in place, solving the one of CXCR5, either alone or in complex with CXCL13, would definitely help elucidating the critical aspects of ligand-receptor interactions, which could be further exploited for drug discovery against these proteins. An alternative approach for specific targeting could be anti-CXCL13 and anti-CXCR5 antibodies, which previously have successfully been used to target this axis for investigative purposes (Fig. 6.4). Exploiting them for therapeutic purpose might open new avenues in cancer treatment. Indeed, antibodies targeting cell surface receptors have proven clinical utility in cancer therapy. Examples include Herceptin that targets HER2/Neu [124] and Erbitux that targets epidermal growth factor receptor (EGFR) [125]. Both prevent binding of the endogenous ligand to the respective receptors. Apart from this, it would also be interesting to consider the possibility of inhibiting upstream inducers, for example, the signaling proteins involved in transcriptional regulation of CXCL13 or CXCR5 [18, 46, 87], or, alternatively, blocking of downstream signaling molecules which mediate CXCL13-derived cancer pathobiology (Fig. 6.4). However, a major challenge to implement such approaches would probably be poor specificity. Further complicating the situation is the fact that cancer cells do not rely on a single chemokine or only one chemokine receptor [7]. Therefore, it will be imperative

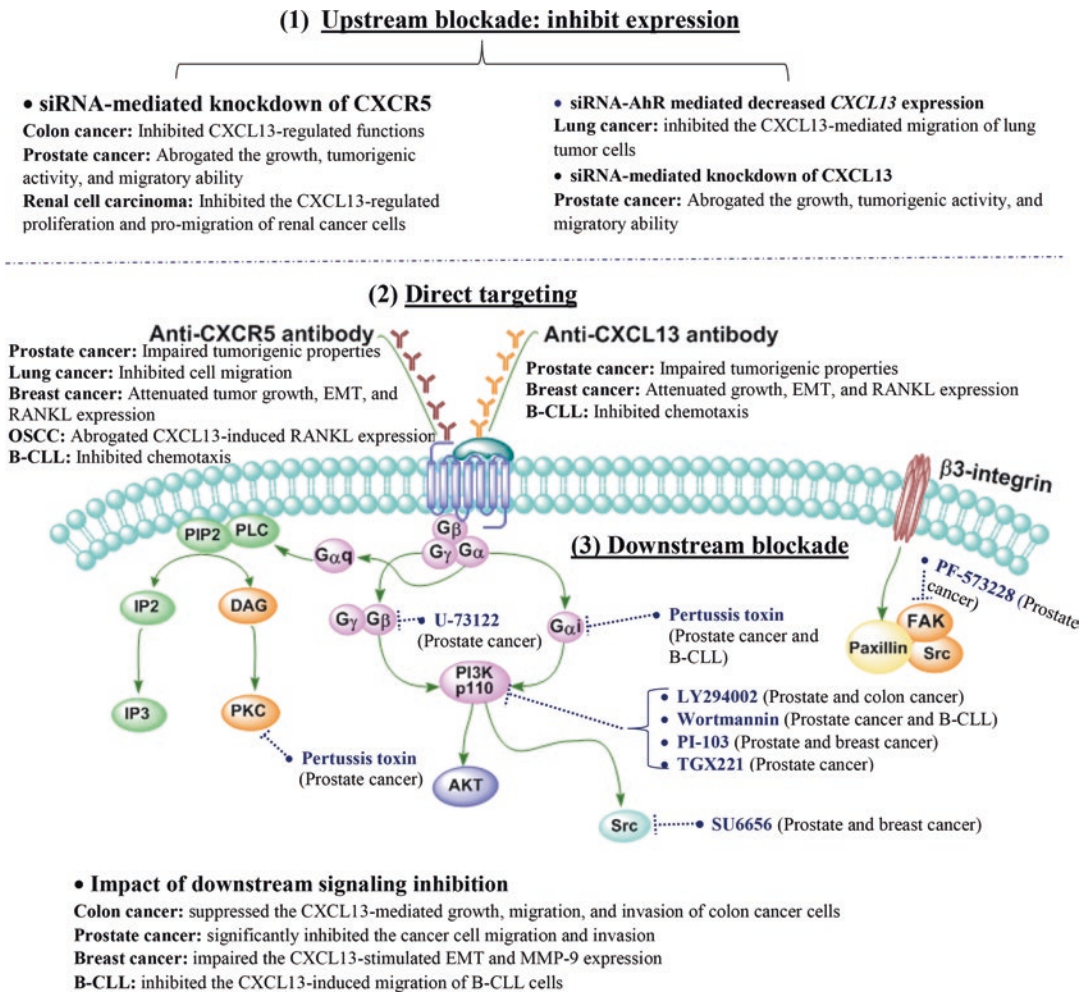

Fig. 6.4 Therapeutic implications of CXCL13-CXCR5 axis in cancer. The data originates from several selected studies (reported in this manuscript), which implemented these interventions for investigational purpose. The portrayed information deduces the feasibility of targeting CXCL13-CXCR5 axis in different cancers. In addition, it also infers strategies to block this pathway at different levels. (1) Neutralization of overexpressed CXCL13 or CXCR5 with small interference RNAs hints at inhibiting upstream events. (2) Direct inhibition with anti-CXCL13 or anti-CXCR5 antibodies in itself represents the strategy of choice while giving the reason to consider both proteins as potential targets for small molecule-based drug discovery. (3) Pharmacological inhibition of downstream signaling events might serve as an alternative approach for blocking CXCL13-mediated effects

to balance the positive and negative effects of enhancing or inhibiting the underlying signaling events that would result from the therapeutic targeting of this pathway. In short, the future of modulating this pathway for therapeutic purposes against cancer is very much dependent on efforts to elucidate its complex pro-tumor and antitumor roles in the tumor microenvironment.

Hopefully, future research efforts in this area will undoubtedly yield more exciting information that would pave the route to exploit this pathway for developing new treatment regimens against cancer.

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CCL24 Signaling in the Tumor Microenvironment

7

Sung-Jig Lim

Abstract

Chemokines with their network play an important role in cancer growth, metastasis, and host-tumor interactions. Of many chemokines, C-C motif chemokine ligand 24 (CCL24) has been shown to contribute to tumorigenesis as well as inflammatory diseases like asthma, allergies, and eosinophilic esophagitis. CCL24 is expressed in some tumor cells such as colon cancer, hepatocellular carcinoma, and cutaneous T cell lymphoma. CCL24 can be used as a potential biomarker in several cancers including colon cancer, non-small cell cancer, and nasopharyngeal carcinoma as the plasma level of CCL24 is increased. The various functions of CCL24 contribute to the biology of cancer by M2 macrophage polarization, angiogenesis, invasion and migration, and recruitment of eosinophils.

Keywords

CCL24 · Cancer · Tumor microenvironment · Eosinophils · Immunotherapy

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7.1 Introduction

C-C motif chemokine ligand 24 (CCL24), which also is called eotaxin-2, is a chemokine for eosinophils, basophils, and T cells and has been mostly investigated in asthma, allergies, and eosinophilic esophagitis. Recently, CCL24 has been shown to contribute to tumorigenesis, especially in cancer of the colon and liver. This chapter focuses on the biology of CCL24 in tumors and discusses its role in the tumor microenvironment and its potential as a target of immunotherapy.

7.2 Chemokines

Chemokines are chemotactic cytokines that cause directed migration of many cells and are induced by inflammatory cytokines, growth factors, and pathogenic stimuli [1–3]. Directed migration of cells that express the appropriate chemokine receptor occurs along a chemical gradient of ligand, allowing cells to move toward high local concentrations of chemokines [4]. Chemokines are principally divided into the four major groups—C, CC, CXC, and CX3C—based on the position of the first two cysteine residues in their biochemical structure. Together with their receptors, they play key roles in the immune defense by directing and controlling the migration, activation, differentiation, and survival of cells in the physiology of acute and chronic inflammatory

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processes as well as in pathological deregulation by attracting and simulating various subsets of specific leukocytes [5]. Different spatiotemporal expression patterns for different chemokines and their receptors in our body suggest distinct roles in vivo [6]. About 45 chemokines and 20 chemokine receptors have been identified [7].

7.3 Tumor Microenvironment and Chemokines

Tumors are not just masses of cancer cells; most solid tumors contain many nonmalignant various stromal components, including endothelial cells, pericytes, fibroblasts, various classes of leukocytes, extracellular matrix, and even autonomic and sensory nerves [8, 9]. Their stromal components have been reported to contribute to the growth and maintenance of numerous tissues [9]. Cancer is a complex disease involving a variety of interactions between tumors and the tumor microenvironment (TME) through direct contact and/or through paracrine signals [10]. It is critical to have proper knowledge of a tumor lesion and the associated microenvironment for complete understanding of tumorigenesis and development of effective preventive and interception strategies. The TME is an indispensable participant in the neoplastic process, fostering proliferation, survival, and migration of cells such as macrophages, lymphocytes, endothelial cells, fibroblasts, eosinophils, granulocytes, natural killer cells, and B cells. The numbers and types of cells that make up the stroma in solid tumors are related to local production of chemokines by the tumor cells and stromal cell themselves.

It is becoming increasingly clear that the chemokine network plays an important role in cancer through its effect on growth and metastasis of tumor cells as well as in host-tumor interactions [4].

7.4 CCL24 in Nonneoplastic Lesions

Eotaxin, a member of the CC chemokine family, has three forms, eotaxin-1/CCL11, eotaxin-2/CCL24, and eotaxin-3/CCL26.

C-C motif chemokine ligand 24 (CCL24) was cloned and described for the first time in 1997 [11]. It is located on chromosome 7q11.23 [12]. As its effects on human eosinophils and basophils are surprisingly similar to those of eotaxin-1 (CCL11), CCL24 also is called eotaxin-2 [11]. CCL24 contributes to atopic disorders, parasitic infections, and numerous systemic diseases, and then it has been mainly studied in asthma, allergies, and eosinophilic esophagitis [13–17]. CCL24 is secreted by many cells, including macrophages, epithelial cells, endothelial cells, and fibroblasts, and is stimulated predominantly by IL-4, IL-13, and/or TNF-α [14, 16, 18–20]. CCL24 is significantly elevated in the skin of $Smad3^{-/-}$ mice and facilitates target cell migration by upregulating adhesion to endothelial cells [15, 21]. CCL24 action occurs via binding to chemokine receptor CCR3, which is expressed mainly on eosinophils, basophils, and T_H2 cells [22]. Thus, typical targets of CCL24 are eosinophils, T cells, and basophils [23–25]. However, CCR3 can interact with multiple ligands, including eotaxin-1, eotaxin-2, eotaxin-3, monocyte-specific chemokine protein-2 (MCP-2)/CCL8, monocyte-specific chemokine protein-3 (MCP-3)/CCL7, monocyte-specific chemokine protein-4 (MCP-4)/CCL13, Regulated on Activation, Normal T Expressed and Secreted (RANTES)/CCL5, and macrophage inflammatory protein-5 (MIP-5)/CCL15 [26].

7.5 Expression of CCL24 in Cancer

Limited research has been published regarding CCL24 in tumors (Table 7.1) [27–30]. Thus, very little is known about the role of CCL24 in cancer.

CCL24 has been mainly studied in colorectal tumors. Colon cancer cells produce CCL24, and secretion of CCL24 can be depressed by IFN-γ and enhanced by T_H2-type cytokines such as interleukin-4 and interleukin-13 [31]. Both primary colorectal cancer and liver metastasis of colorectal cancer produce significant level of CCL24, whereas metastatic cancer from rhabdo-

Table 7.1 CCL24 in cancer

Tumor	Model system	CCL24	References
Colon	Human tissue, primary and hepatic metastasis	Increased	[30]
	Cell lines	Increased by IL-4 and IL-13 Decreased by IFN-γ	[30]
	Serum	Increased	[28]
	Human tissue	Decreased	[26]
Nasopharynx	Serum	Increased	[36]
Hepatocellular carcinoma	Human tissue	Increased	[27]
Melanoma	Mice, lung metastasis	Increased	[37]
Lung	Serum	Correlate with tumor metabolic burden	[34]
Osteosarcoma	Cell lines	Decreased by melatonin	[48]
Cutaneous T cell lymphoma	Human tissue	Increased in eosinophils	[29]

myosarcoma and breast, renal, and neuroendocrine tumors did not express a detectable level of eotaxin-2 [31]. These results suggest specific mechanisms for the production of CCL24 in colorectal cancer. Expression of CCL11 and CCL24 in tumor cells from adenoma with low-grade dysplasia to adenocarcinoma of the colon decreased significantly with tumor progression [27]. The reduced expression of CCL11 and CCL24 in advanced tumor cells could contribute to the immune-evasion mechanisms of colorectal adenocarcinoma by inhibiting recruitment of eosinophils that function as effector cells for the neoplasm [27]. Interestingly, stromal expression of CCL11 and CCL24 appeared to increase under these same conditions. The CCL11-/CCL24-secreting stromal cells are mostly mononuclear inflammatory cells, and increased expression of CCL11 and CCL24 in stromal cells might explain the elevated serum concentrations of those chemokines [27].

CCL24 was upregulated in hepatocellular carcinoma tissues and correlated with poor prognosis in hepatocellular carcinoma (HCC) patients, and CCL24 expression was associated with promoted proliferation, migration, and invasion [28].

High expression of CCL24 in eosinophils themselves was noted in cutaneous T cell lymphoma, showing an autocrine and/or paracrine origin of tissue eosinophilia [30]. In oral squamous cell carcinoma, eotaxin-1 was mainly derived from infiltrating eosinophils in autocrine and/or paracrine pathways [32].

Like nonneoplastic conditions, CCL24 expression can be upregulated by T_H2-type cytokines like IL-4 and IL-13 in colorectal cancer [31]. But T_H2-type cytokines failed to modulate CCL24 in HCC, suggesting that the TME of colorectal cancer might be more suitable for plantation and/or progress of cancer cells [28].

The ability to produce CCL24 was acquired in a human leukemic cell line through GATA-1 expression [33].

7.6 CCL24 as a Biomarker

There have been a few studies about CCL24 as a potential biomarker.

The plasma CCL24 level in colorectal cancer patients is very high and exclusively associated with colorectal-specific mortality [29]. These data suggest high plasma level of CCL24 as a potential biomarker of prognosis.

Radiation therapy affects living cells in tumor tissue, as well as the TME, indicating that TME has an important role in response to treatment [34]. Irradiation elicits a complex response involving cross talk between several actors in the TME as well as with tumor cells, which is possi-

bly reflected in the systemic levels of cytokines. Also, localized radiation therapy can trigger systemic antitumor effects [35]. Radiation therapy elicits immediate responses in the irradiated tissue, with increased expression of cytokines instrumental in generating free radicals and oxidative stress, as well as to restore homeostasis [36]. In non-small cell lung cancer, serum level of CCL24 was significantly correlated with tumor volume and total lesion glycolysis measured by 18F-FDGPET/CT before, during, and after radiation therapy [35]. Thus, serum level of CCL24 could be a putative indication of tissue radiation sensitivity. Cytokines such as CCL24 are released by macrophages and activating T-lymphocytes recruited by radiation therapy [24].

The level of CCL24 in the serum of nasopharyngeal carcinoma is significantly higher than that of controls, illustrating its role as a potential serum biomarker for diagnosis and prognosis of carcinoma [37].

7.7 Functions of CCL24 in Cancer

7.7.1 Immune Functions

There has been limited research on the role of CCL24 in tumor immune responses. In normal lung, commensal microbiota in the upper respiratory tract maintains alveolar macrophages with a low level of CCL24 production to generate antimetastatic tumor activity [38].

In lung melanoma tissue of an antibiotic-treated mouse model, alveolar macrophages showed M2 macrophage polarization with elevated expression of CCL24 and decreased γδ T cells, resulting in a defective antitumor response [38]. In contrast to CCL24-mediated recruitment of $CCR3^+$ immune cells, including eosinophils, basophils, and T_H2 cells during lung allergic inflammation, a high level of CCL24 failed to induce migration of $CCR3^+$ immune cells in melanoma lung tissue of the antibiotic-treated mouse model [38–40]. Furthermore, CCL24 neutralization by anti-CCL24 antibody promotes immune cell infiltration in the lung, particularly that of γδT17 cells, which might be a novel feature of alveolar macrophage-derived CCL24 in the tumor immune response [38]. More attention should be paid to the functions of CCL24 in the TME in terms of macrophages and microbiota for better understanding of CCL24 functions and interaction between CCL24 and macrophages in the immune responses of tumors.

Cancer has a hypoxic TME. Hypoxic cells need alternative energy sources to compensate the inhibition of oxidative metabolism [41, 42]. The response of cells to hypoxia is associated with changes in gene expression and modulation of mononuclear phagocytes. Dendritic cells (DCs) respond to a hypoxic condition by improving migratory function via upregulation of some chemokine receptors (CCR2, CCR3, CXCR4, and CX3CR1) and downregulation of others (CCL13, CCL14, CCL18, CCL23, CCL24, and CCL26) [43]. Hence by decreasing chemokines like CCL24 and increasing chemokine receptors in the hypoxic tissue, DCs in hypoxic tumor tissue have a tendency to exit the hypoxic tissue and migrate toward a normoxic area and finally to the lymph nodes to induce immune responses against the tumor [43].

7.7.2 Angiogenesis

Expression of CCL24 is increased in HCC tissues, and overexpression of CCL24 in HCC predicts shorter overall survival and higher recurrence rate [28]. CCL24 contributes to the malignant biological behavior of HCC through the RhoA-vascular endothelial growth factor A (VEGFA)-vascular endothelial growth factor receptor 2 (VEGFR2) angiogenesis pathways and is correlated with poor prognosis [28]. Studies have shown conflicting results regarding CCL24 and VEGF. CCL24 was upregulated with VEFGA in atopic dermatitis in one study, whereas VEGFA was reduced with increase of CCL24 concentration in plasma in myalgic encephalomyelitis/chronic fatigue syndrome [44, 45].

Eotaxin-1 has also been shown to directly mediate angiogenesis of $CCR3^+$ microvascular endothelial cells [46]. Melanoma with eotaxin

production has increased microvascular density and extensive thrombosis of the blood vessels of the tumor in a murine model system [47].

7.7.3 Metastasis of Cancer Cells

Chemokines contributes to cancer progression and metastasis as critical mediators in the TME [48]. CCL24 influences the motility, migration, and invasion of osteosarcoma cell lines, and these effects are mediated by the extracellular signal-regulated kinase (ERK)/c-Jun N-terminal kinase (JNK) pathway [49]. Metastatic potential of HCC cell lines was increased with the expression of CCL24 with promoted migration and invasion [28]. These results suggest the need for further studies of CCL24 as a potential target for anticancer therapy. Modulation of CCL24 expression in cancer cells to repress invasion, migration, and metastasis could be a target of anticancer therapies.

7.7.4 Chemokines for Eosinophils

Infiltration of eosinophils to sites of tumor of inflammation is a complicated process dependent on a combination of cytokines, adhesion molecules, and chemokines. Eosinophil recruitment is conducted by many chemokines, including RANTES (Regulated on Activation, Normal T Expressed, and Secreted or CCL5), eotaxin-1, eotaxin-2, eotaxin-3, IL-8 (CXCL8), macrophage inflammatory protein (MIP)-1α (CCL3), and monocyte-specific chemokine protein (MCP-3 or CCL7) [50]. Tumor cells or stromal cells in TME can secrete eotaxin-2 and can induce infiltration of eosinophils. There have been conflicting reports of tumor-associated tissue eosinophilia (TATE) as a prognostic indicator in solid tumors. TATE is associated with improved prognosis in colon tumors, esophageal SCC, nasopharyngeal carcinoma, penile carcinoma, and oral squamous cell carcinoma [51–55]. In contrast, TATE is associated with poor prognosis in Hodgkin's lymphoma and cervical carcinoma [56, 57]. However, oral SCC showed both favorable and unfavorable prognoses, with the discrepancy possibly related to differences in study methods and design [58]. Tumor-associated tissue eosinophils appear to be protective in general, but further studies are required to determine their exact mechanisms and their discrepant behaviors.

Protective effects of eosinophils on tumors are not completely understood. First of all, antitumor cytotoxic effects caused by degranulation of eosinophils are suggested by observation of granules in the local vicinity of tumors [59]. Eosinophils induce apoptosis and secrete granzyme A, resulting in tumoricidal activity toward a colon cancer cell line [60].

Secondly, eosinophils recruited into the TME secrete chemokines such as CCL5 that attract $CD8^+$ T cells to the tumor site, resulting in improvement of antitumor immunity [61].

7.8 Limitations and Future Considerations

Tumor microenvironment helps shape tumors and their progression and influences therapeutic responses. Chemokines are implicated in aggressive metastatic tumor progression, and a better understanding of their regulation could lead to new therapeutic targets for cancer.

While CCL11 biology and functions in tumor and TME are quite present, there has been a limited study about the role of CCL24 within the TME and tumor. Further extensive investigations on the biology, functions, and regulation of CCL24 in tumors are required.

Immune cells engineered to express the receptor for CCL24 (CCR3) effectively migrated in response to CCL24 protein. This suggests that immune cells undergoing gene modification to express a chemokine receptor may have improved abilities to localize to a tumor [31].

Melatonin attenuated CCL24 through inhibition of the JNK pathway to suppress motility, migration, and invasiveness of human osteosarcoma cell lines [49]. Future research on melatonin treatment for antimetastasis of cancer cells and its mechanism is needed. In addition, involve-

ment of the JNK pathway and downstream CCL24 in the metastasis of human osteosarcoma highlights the therapeutic potential of melatonin and JNK inhibitors for cancer metastasis [49].

CM-101 is a humanized monoclonal antibody that targets the human chemokine CCL24. Previous studies have shown that administration of CM-101, by binding CCL24 with high affinity, reduced pro-inflammatory responses in animal models of several diseases, including atherosclerosis, rheumatoid arthritis, multiple sclerosis, and pulmonary fibrosis [62–65]. CM-101 decreases migration of fibroblasts and attenuation of endothelial cell activation in CCL-24-expressing diseases, resulting in reducing tissue damage of both skin and lung [66]. Those studies support translation of an anti-CCL24 antibody not only to the skin and lung fibrotic diseases in the clinic but also to CCL-24-expressing neoplastic diseases.

In an experimental mouse model of age-related macular degeneration (AMD) induced by laser injury, CCL11 and CCL24 were expressed in the choroidal neovascular tissue and retinal pigmented epithelium, and administration of neutralizing antibodies against CCL11 or CCL24 suppressed the choroidal neovascularization (CNV), characteristic of AMD [66]. It is tempting to speculate that targeting CCL11 or CCL24 through an antiangiogenic effect might provide benefit in CCL11- or CCL24-secreting tumors.

In acute promyelocytic leukemia, treatment of all-trans-retinoic acid (ATRA) induced a differentiation syndrome with massive pulmonary infiltration, and expression of CCL24 and CCL2 was elevated by ATRA stimulation [67]. These results indicate that CCL24 and CCL2 are directly regulated by ligand-activated retinoic acid receptors [67]. CCL24 is a possible pharmacological target, and the chemokine receptor antagonists are candidates for therapy of CCL24-secreting tumors (Fig. 7.1).

CCL24 shows various effects on cancer cells by M2 macrophage polarization, angiogenesis, invasion, and migration and recruitment of eosinophils.

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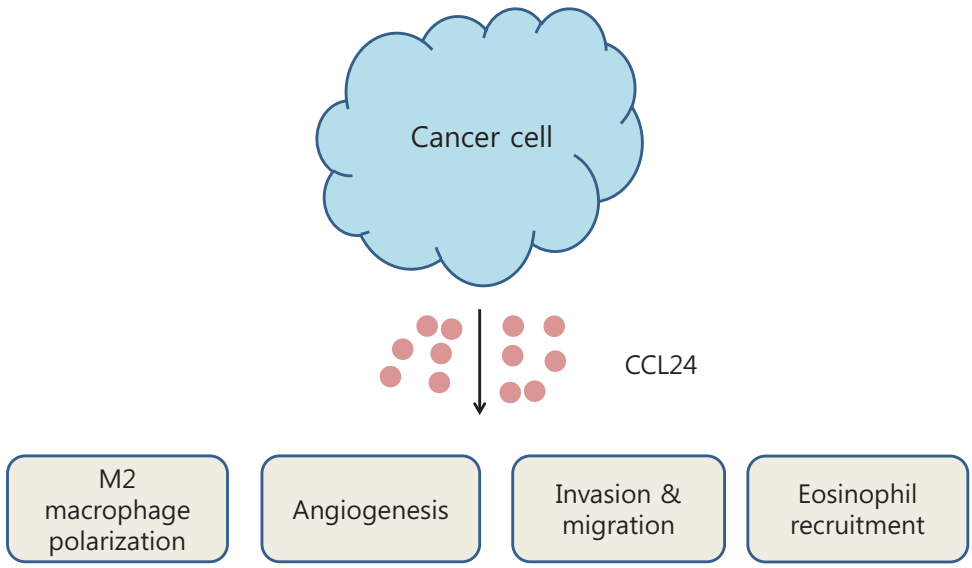

Fig. 7.1 Functions of CCL24 in cancer

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CCL25 Signaling in the Tumor Microenvironment

8

Hina Mir and Shailesh Singh

Abstract

Multiple checkpoint mechanisms are overridden by cancer cells in order to develop into a tumor. Neoplastic cells, while constantly changing during the course of cancer progression, also craft their surroundings to meet their growing needs. This crafting involves changing cell surface receptors, affecting response to extracellular signals and secretion of signals that affect the nearby cells and extracellular matrix architecture. This chapter briefly comprehends the non-cancer cells facilitating the cancer growth and elaborates on the notable role of the CCR9-CCL25 chemokine axis in shaping the tumor microenvironment (TME), directly and via immune cells. Association of increased CCR9 and CCL25 levels in various tumors has demonstrated the significance of this axis as a tool commonly used by cancer to flourish. It is involved in attracting immune cells in the tumor and determining their fate via various direct and indirect mechanisms and, leaning the TME toward immunosuppressive state. Besides, elevated CCR9-CCL25 signaling allows survival and rapid proliferation of cancer cells in an otherwise repressive environment. It modulates the intra- and extracellular protein matrix to instigate tumor dissemination and creates a supportive metastatic niche at the secondary sites. Lastly, this chapter abridges the latest research efforts and challenges in using the CCR9-CCL25 axis as a cancer-specific target.

Keywords

CCR9 · CCL25 · Tumor microenvironment · Dendritic cells · T cells · Macrophages · Chemoresistance · Angiogenesis · VEGF ·

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TGF-β · Extracellular matrix · MMP · Metastasis

8.1 Introduction

Tumors do not develop quickly in a healthy, immune-competent host because checkpoints at multiple stages warrant that no transformed cells propagate. At the cellular level, cell cycle checkpoints arrest the division of cells carrying altered or damaged DNA and divert them toward repair or death. In contrast, at the organismal level, the immune system carries out the surveillance and targets and clears the neoplastic cells. All these processes are integrated, ensuring the optimum health of the host. Transformed cells that prevail over the cellular checkpoints form a neoplastic lesion that further supersedes various surveillance mechanisms, undergoes a series of molecular and morphological changes and uncontrolled cell divisions to result in tumor development and cancer progression collectively. Therefore, the development of a tumor reflects a composite of multiple checkpoint failures. In this chapter, we outline the significance of the cellular composition of the tumor microenvironment (TME) and the role of chemokine signaling in determining overall TME characteristics, focusing on the CCR9-CCL25 axis. We also highlight current and future therapeutic ventures directed toward this chemokine axis to curb cancer.

8.2 Significance of Tumor Microenvironment

To gauge the impact of any phenomenon on cancer development, it is imperative to understand it at the local and the organismal scale. Teasing out carcinogenesis at the cellular level has shown several survival and apoptosis signaling pathways, mandatory for normal cell survival, are modulated to support cancer progression. The discrete cellular milieu around the neoplastic lesion, from now on termed as tumor microenvironment (TME), instigates cancer initiation and progression. Infiltration of many different cell types, including immune cells, stromal cells (connective tissues and blood vessels), fibroblasts, and nerve cells, and their secretions characterize and govern TME dynamics and blood vasculature. The heterogeneous composition of tumors in different malignancies and individuals varies; however, often, the nonmalignant component constitutes 80% of the tumor architecture with cancer cells contributing only 10–20%. These facts emphasize the significance of the supportive role of the nonmalignant cells in driving cancer progression. All cells communicate and influence each other's functional characteristics via the cytokines, chemokines, and metabolic by-products secreted in the TME [12, 52].

8.3 The Shaping of TME: The Nonmalignant Component

Nonimmune cells constituting TME: A significant proportion of TME is occupied by fibroblasts. These cancer-associated fibroblasts (CAFs), activated fibroblasts, myofibroblasts, or tumor-associated fibroblasts, actively participate in cancer progression [73]. They impact TME by cellular interactions with cancer cells and by secreting factors that modulate the extracellular matrix (ECM) to become a tumor conduit. CAFs contribute extracellular proteins like collagen [34], laminin [35], versican [120], and fibronectin [8, 33, 97] that serve as an ally to malignant characteristics of cancer. In addition, CAF-secreted extracellular enzymes like LOX, MMPs, ADAMs, TIMPs, and kallikrein [124] function to remodel the ECM and collectively increase tumor stiffness by cross-linking ECM proteins, facilitate metastasis by digestion of extracellular matrix proteins, reduce cancer cell immunogenicity by shedding surface antigens, and cleave cell surface receptors affecting various signaling mechanisms. Simultaneously, hormones and cytokines secreted by CAFs also impact the immune phenotype of TME. In fact, some CAFs are known to create immunosuppressive TME [49] by secretion of TGF-β [120]. A handful of

in vitro studies also demonstrated CAF-cancer cell contact-dependent invasion ability of tumor [26, 65, 85].

The unevenly distributed endothelial cells in the TME are no less important than CAFs in supporting the angiogenic switch of the tumor [61]. TME constituting endothelial cells are more pro-angiogenic and frequently upregulate MMP and receptors for VEGF. They are the significant source of angiogenic factors [14] for rapidly dividing neoplastic cells as well as they help creating a tolerant immune environment [29]. Under the influence of the angiogenic factors, endothelial cells downregulate the leukocyte adhesion proteins and so may reduce the infiltration of immune cells into the TME.

Nerve cells in TME: Research connecting the nervous system and cancer has drawn substantial attention recently. The previous impression of lack of nerve cells in the tumor was challenged with the evidence of perineural invasion (PNI) that show cancer cell proliferation surrounding the peripheral nerves. Like a blood vessel and lymphatic vessel, the nerve is apparently used as a route for dissemination by the growing cancer cells. The presence of PNI is a poor prognostic marker [9, 24, 39], and denervating tumor impacts cancer metastasis [27, 89–91]. Several chemokines are involved in influencing tumor innervation [24, 39, 127]. However, the involvement of CCR9 signaling has not yet been studied in this context.

Immune cells of TME: Both innate and adaptive immune cells reside and help shape the TME. Natural killer (NK) cells are the frontliners that discriminate and directly kill the neoplastic cells in developing and progressive tumors by secreting perforin or granzyme after activation. They also induce receptor (-TNF, FasL, and TRAIL)-mediated apoptosis of their target cancer cells. NK cell activation is a cytokine-dependent process that requires IFN-α, IFN-β, IL-2, IL-12, and IL-15 and follows a gauging of activating (NKG2D) and inhibitory (KIR) receptors on the target cells [103, 106, 113]. NK cells also control tumor proliferation by activating M1 macrophages (Mϕ) and DCs, as well as Th1 cells of the adaptive immune system by secreting IFN-ϒ and generating tumor-specific antigens (TSA) due to direct killing.

Dendritic cells (DC), in the TME, under the influence of cytokines like TNFs and IFNs released by other cells, are polarized into distinct effector phenotype [4, 50, 105]. They capture, process, and combine the TSAs with MHC-I/II molecules for antigen presentation, eliciting the adaptive arm of immunity. Precisely, antigen presented in this way signal clonal expansion of naïve CD4+ and CD8+ T cells and their differentiation to the helper (Th1, Th2, Th17, or T- follicular helper (Tfh, Treg)) cells and cytotoxic T cells (CTLs). Interactions of DCs also regulate B cell-mediated immunity.

Macrophages also recognize TSAs and phagocytose the cells [1]. Macrophages determine the immune response by significantly contributing to the TME cytokine and chemokine profile.

Cancer cells would be effectively eliminated if these cells could perform optimal immune surveillance. However, the TME with its immune evading and manipulating characteristics facilitates cancer progression. That is, cancer cells become less immunogenic [36, 93, 94, 112, 129] by shedding antigenic proteins from their cell surface as facilitated by upregulation of extracellular protease activities. These poorly immunogenic cancer cells undergo unrestricted clonal expansion and evade immune attack while simultaneously driving TME toward more immunosuppressive constitution by asserting the differentiation of myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), regulatory T cells (T-regs), and regulatory dendritic cells (reg-DCs) resulting in aggressive tumors. Mechanisms that allow for the evasion and immune suppression include the production of selective cytokines and upregulation of IDO, leading to increased kynurenine release that, in turn, affects the cytotoxicity of T cell, DC, and macrophages. In all, the combined activity of these immune suppressor cells regulates tumor growth, survival, migration, and invasion by changing the hormone, growth factor, and cytokine profile of TME.

Immature myeloid cells are precursors of DCs, macrophages, and granulocytes.

Unfortunately, in TME, very often, these otherwise anticancer immune cells attain immunosuppressive phenotype termed as MDSCs [20, 78, 79, 82]. These cells can deactivate innate and adaptive immune cells. Specifically, MDSCs, via factors like inducible nitric oxide synthase, generation of ROS, and arginase, inhibit T cell division and cytotoxicity, suppress CD8+ T cell response, favor Treg differentiation, and inhibit activation of NK cell [20, 82]. Additionally, the cytokine and chemokine composition of TME is not conducive for M1 macrophages (Mϕ) but favors polarization to M2 phenotype that is more immunosuppressive [76, 77]. Further, these TAMs considerably assist cancer progression through immune evasion, angiogenesis, and metastasis by secreting IL-10 and TGF-β [54, 76, 101, 104]. Treg cells support tumor progression by abating NK cell-, T cell-, and B cell-mediated immune functions [40, 116, 130]. This diminishing effect is achieved via secretion of suppressive cytokines and contact-dependent mechanisms [37].

8.4 Chemokine-Guided TME Infiltration

Infiltration of nonmalignant cells within the TME is mostly guided by a group of low MW (8–10 kDa) cytokines called chemokines. Primarily, chemokines function in bringing in specific GPCR bearing cells at their source site. These receptor-expressing cells are often immune cells that mark immune cell trafficking as the most common biological chore of chemokines. So, chemokine secretion pattern of a tumor (cancer and stromal cells) governs what immune cells populate the TME. For instance, CC chemokines released by breast, pancreatic, and cervical tumors, as well as those emanating from sarcomas and gliomas, cause infiltration of macrophages and lymphocytes [10, 18]. CC and CXC chemokine-mediated infiltration of macrophages and CD8+ T cells is observed in the case of ovarian cancer [84]. However, the hypoxic microenvironment in solid tumors, along with chemokine and pro-inflammatory cytokine surge from tumor cells, diminishes the antitumor immune response of these tumor-infiltrating leukocytes (TILs) by chemokine receptor downregulation on their surface [42, 99]. The emerging role of chemokines in suppressing host antitumor immunity has initiated lots of research in this field. CCL25-CCR9 signaling axis is one such signaling that is physiologically relevant but is also extensively abused in various malignancies. Following sections comprehend the role of CCL25 signaling locally in the tumor microenvironment and at the systemic level in the development of a tumor.

8.5 Role of CCR9-CCL25 Axis in Sculpting TME

CCR9, a GPCR that belongs to the A2 subfamily of Rhodopsin-like receptors, is a sole receptor for CCL25. CCL25, also called TECK (thymus-expressed chemokine), is typically expressed in the thymus. It is important for the development, homeostasis, and function of mucosal T lymphocytes wherein migration is augmented by TCR signaling. The prime role of CCR9-CCL25 signaling is in T lymphopoiesis. Maturation occurs as the lymphoid progenitor cells sequentially interact with the discrete stromal cells during their migration in the thymus from the cortex through the medulla [95, 96, 109, 110]. CCR9, together with CCR7, affects the ushering of the lymphoid progenitors to the thymus [62]. CD69-positive thymocytes, most αβ thymocytes, and about 50% of γδ TCR-positive thymocytes and T cells express CCR9 [22, 110]. The expression is highest in $CD4^+$ $CD8^+$ cells and is downregulated as they mature. $CD8^+$ cells maintain functional CCR9 even through their course of the journey to the secondary lymphoid organs, whereas the $CD4^+$ counterparts omit their CCR9 before egressing thymus [22]. In addition to T lymphocytes, CCR9 is expressed by a subset of DC and pre-pro B lymphocytes. It is also proposed to play a role in B cell development [19] and reactivity [17]. This chemokine receptor expression interestingly varies with age [22, 110]. CCL25 production also reduces by puberty as the thymus shrinks.

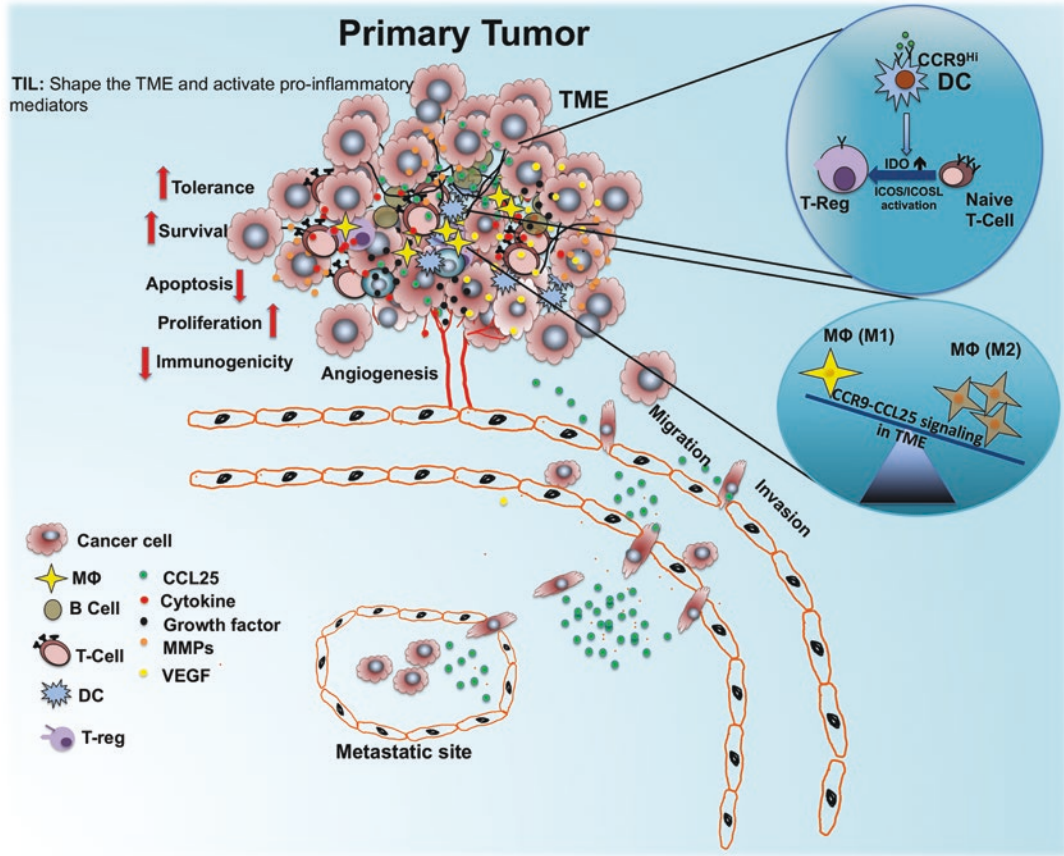

Fig. 8.1 *Shaping of the immunotolerant tumor microenvironment (TME) by CCR9-CCL25.* Tumor with higher CCL25 makes the microenvironment more tolerant by recruiting CCR9-positive immune cells such as dendritic cells (DC), macrophages, MDSC, and T cells. Once recruited, CCL25-CCR9 in TME promotes polarization of macrophages from M1 to M2 phenotype. Increased $CCR9^{Hi}$ DC in the TME induces differentiation of naive T cells to Treg. Elevated CCR9-CCL25 signaling induces MMPs, making cancer cells less immunogenic by shedding their cell surface antigens. In addition, these elevated MMPs will also increase angiogenic factors such as VEGF and TGF-β in the TME that dually support cancer progression by creating an immunosuppressive microenvironment and promoting angiogenesis

Understandably, CCR9 is induced in acute and chronic lymphocytic leukemia of T cells compared to normal $CD4^+$ T cells [92]. In solid malignancies, elevated CCR9-CCL25 axis is one of the most abused navigating mechanisms for tumor progression, and it could considerably influence the kind of cells infiltrating TME and TME dynamics. Although abundant evidence supports CCR9-CCL25 involvement in T cell recruitment at the inflamed tissues sites, studies directly correlating increased CCL25 levels and T cell homing in TME are surprisingly scarce. However, plentiful studies demonstrating CCR9 facilitated DC infiltration in TME signify its indirect impact on the T cell population in TME (Fig. 8.1). CCR9 expression in DC negatively correlates with the expression of costimulatory molecules and the capacity of DC to induce differentiation in naïve T cells. $CCR9^{Hi}$ DC poorly stimulates T cell division than $CCR9^{Lo}$ DC subset, which also reflects in reduced IL2 secretion [31]. Plasmacytoid DC (pDC) expressing CCR9 suppresses the immune response [44, 81] by reducing the naïve-Th1 and inducing Treg differ-

entiation. pDC leads to increased generation of IL-10-producing Treg cells via ICOS-ICOSL pathway or IDO induction [111]. These evidence imply that CCR9^{Hi} DC attracted by CCL25 producing tumors will bend the TME toward immunosuppression [111]. Accordingly, increased infiltration of pDCs in the tumor is a poor prognostic indicator in several cancer types [7, 28, 46, 66, 88, 102, 108] that also show elevated CCR9-CCL25 signaling.

Besides DCs, CCR9 activation is also involved in macrophage polarization (Fig. 8.1) in rheumatoid arthritis and hepatitis conditions; however, studies directly linking the CCR9-CCL25 axis to TAMs are also lacking. On the other hand, the expression of CCR9 by NK cells is controversial. CCR9 is expressed only in a subset of CD56^{bright} NK cells [13, 21]. CCL25 induces the infiltration of CCR9-positive MDSCs in the endometriosis microenvironment [107]. Although one needs to take heed of immune cell plasticity in TME before drawing concrete conclusions regarding the fate and role of a cell in cancer, it is clear that CCL25-CCR9 signaling promotes immunosuppressive TME by recruitment of MDSCs and polarization of T-helper cells toward Th2 and Treg differentiation facilitated by CCR9^{Hi} DC (Fig. 8.1).

In addition to the immune cell homing, CCR9 led navigation helps cancer cell dissemination and proliferation in a secondary niche. CCL25 is significantly expressed by the epithelial cells of the small and large intestine [110], and consequently, it is one of the main factors governing gut as the primary metastatic niche of cancer like melanoma [5, 70] and ovarian cancer. CCR9 overexpression in melanoma patients did not correlate with patient survival or prognosis [63]; however, metastasis of melanoma to the lung is assisted by CCR9-CCL25 signaling [55].

8.6 Involvement of CCR9-CCL25 Axis in Tumor Promotion: The Nonimmune Component

The role of chemokines is not merely limited to immune cell trafficking but expands to the regulation of cell differentiation, organ development, cell motility, and neuromodulation, among many others. Intracellular signaling cascades initiated after binding of chemokines to their GPCR regulate these biological processes. Therefore, the array of G proteins bound to receptor C terminal determines the intracellular signaling triggered by binding of a specific chemokine. Consequently, the significance of chemokines in promoting growth, survival, proliferation, and metastatic progression of cancer cells, mainly by promoting angiogenesis as well as the release of growth factors from cells in TME, has come to light [12, 25, 51, 52, 69, 75]. Chemokines work in autocrine or paracrine fashion to stimulate the rapid growth of a tumor that cannot be eliminated by host immune response. Inhibiting certain chemokine signaling results in control of tumor progression by inducing cancer cell death as well as overcoming the macrophage- and Th2-mediated immunosuppression. Our group has extensively demonstrated the significance of CCR9-mediated signaling in supporting the progression of several solid malignancies (Fig. 8.2). Partial elucidation of CCR9-CCL25 signaling indicates downstream activation of the PI3K/Akt pathway [59, 115] and its downstream mediators, β- catenin [68], FKHR, and GSK-3β [56].

Survival and chemoresistance: Stimulation with CCL25 augments cellular proliferation and survival by regulating β-catenin [64, 68], Livin, and caspases, among many other survival-apoptosis controlling proteins [72]. Consequently, CCR9 activation also underlies chemoresistance (Fig. 8.2). The deterring effects of amplified CCR9-CCL25 axis on the chemotherapeutic response have been shown using several cancer models [56, 59, 72, 98]. Rearrangement of the cytoskeleton by regulating P-glycoprotein interactions with F-actin and ERM (Ezrin-Radixin-Moesin) downstream to CCR9-CCL25 activation is also shown to determine the sensitivity of cancer cells to drugs [125].

Migration and invasion: Penetration of the basement membrane and invasion of the interstitial stroma that is a prerequisite for the cancerous cells to metastasize are achieved principally by cytoskeletal rearrangement and fine-tuning of the activity of MMP and their tissue inhibitors (TIMP). Activation of the CCR9-CCL25 axis

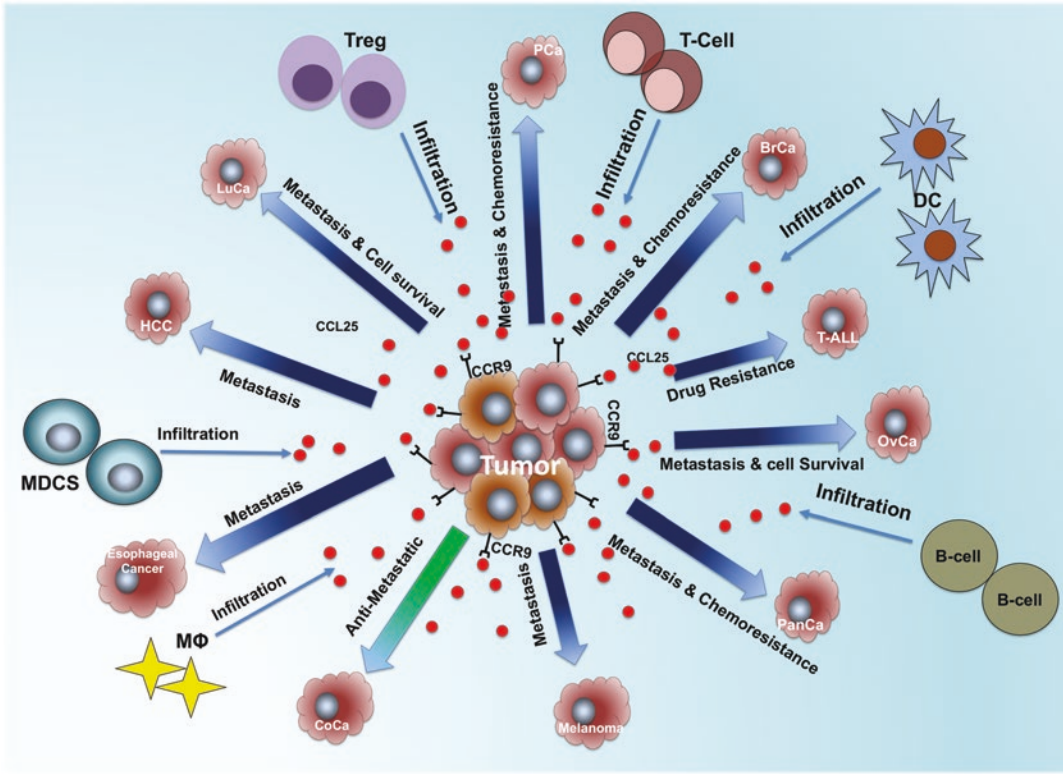

Fig. 8.2 *Role of CCR9-CCL25 axis in different malignancies.* CCR9-CCL25 axis activates signaling cascades that support cancer cell survival and proliferation and, inhibit apoptosis. Thus, this chemokine receptor axis, in addition to promoting various cancers, is also involved in developing chemoresistance. Higher CCL25 in the tumor vicinity promotes infiltration of CCR9-expressing lymphocytes, and these infiltrating immune cells support tumor dissemination and metastasis. The figure illustrates CCR9-CCL25 signaling induced processes and mechanisms as reported for various malignancies. Blue and green arrows indicate cancer promoting and anti-metastatic effects, respectively

could alter cellular adhesion by activation of β-catenin. It prompts the migration of cells via ERM (Ezrin-Radixin-Moesin) facilitated actin filament reorganization [128]. Specifically, CCL25 gradient induces migration of malignant ascites derived ovarian cancer cells [45].

The CCR9-CCL25 chemokine axis also strongly correlates with upregulated activity of MMP-TIMP family of proteins [16, 43, 57, 100, 114] which is commonplace in cancers. Aggressive ovarian cancer cells show modulation of MMP and TIMP family of proteins when stimulated with CCL25 correlating with faster basement membrane invasion. Similarly, the functional significance of the CCR9-CCL25 axis in migration and invasion is established for hepatocellular carcinoma and breast, prostate, and lung cancers (Fig. 8.2) [58, 86, 126]. Cancer cell produced CCL25 also attracts mesenchymal stem cells [118], which are associated with increased invasiveness of cancer.

CCR9-CCL25 led β-catenin signaling [64, 68], and MMP induction and activation [126] correlates with the modulation of EMT markers in cancer cells and could also be associated with angiogenesis [41, 71, 121] as discussed in the next section.

Angiogenesis: The infiltration of leukocytes to the tumor by chemokines is facilitated directly by their angiogenic activity and indirectly by the release of angiogenic factors from cells in the TME. Most of the chemokines secreted by tumor

cells are angiogenic. Furthermore, the extracellular protease activities induced by CCR9 signaling also shed surface proteins from cells in TME and from ECM, many of which are angiogenic cytokines like TGF-β, VEGF, and TNF-α.

CCL25-CCR9-induced elevation of MMP-2 and MMP-9 activity could increase the bioavailability of some angiogenic cytokines. MMP-2 and MMP-9 activate TGF-β [122]. This angiogenic cytokine supports immunosuppressive TME [119]. It inhibits expansion and cytolytic activity of CTLs and NK cells [3, 38], favors Th2 differentiation, enhances pro-angiogenic chemokine production by MDSCs [83], supports Tregs [32], and impedes DC functions [2]. TGF-β underlies versican upregulation by CAF in the TME and promotes ovarian cancer invasion [120]. It also enhances the EMT transition of cancer cells and is a potent malignant growth supporting cytokine [3, 15, 30, 48, 80, 123].

Recent evidence demonstrated a positive correlation between the expression of CCR9 and VEGF in tumor tissues. CCR9 activation induces VEGF-C and VEGF-D in lung cancer cells by modulating MMPs [86]. VEGF is the primary driver leading to tumor vascularization [61]. Proteolytic cleavage of VEGF from ECM regulates its angiogenic capacities. MMP-9 that is elevated by CCL25 [57, 58] is the primary protease involved in VEGF bioavailability [67]. An increase in VEGF reflects pro-inflammatory cancer-promoting microenvironment and implies activation of a positive feedback loop: TNF-α, IL-1β, IL-6, and IL-8 [47]. CCR9-induced VEGF could also enhance CXCL1 secretion [74] by epithelial cells and COX2 (cyclooxygenase2) expression in endothelial cells [6]. Thus, CCL25 induced elevation in VEGF could promote immunosuppressive TME.

Upregulated CCR9 signaling is also associated with increased IL-10 production [11, 117] in the ulcerative colitis model. IL-10, like TGF-β and VEGF, is highly immunosuppressive. Elevated IL-10 in TME is associated with infiltration of TAM, Treg differentiation, and downregulating pro-inflammatory cytokines, MHC class II molecules, and costimulatory proteins [60, 87].

Except for cancer of the ovary and colorectal origin, all other cancer primarily metastasize to the bone, with the liver being the secondary homing site for most malignant tumors. Colorectal cancers are also exceptional concerning the CCR9-CCL25 axis; unlike other malignancies, this axis negatively associates with metastasis of the colorectal tumors (Fig. 8.2). All these highlight the significance of the organ of origin in determining the role of the CCR9-CCL25 axis.

8.7 CCR9-CCL25 as a Potential Target for Cancer Treatment

Based on the biological and clinical significance of CCR9-CCL25 in TME, it is a potential biomarker and a therapeutic target. Serum CCL25 levels have diagnostic value for various malignancies. Small molecule inhibitor of CCR9, vercirnon or CCX282, was in phase III clinical trial as a potential treatment option for Crohn's disease, a chronic inflammatory condition of the gastrointestinal tract, and some inflammatory bowel diseases. It was also anticipated to improve the overall survival of patients suffering malignancies with elevated CCR9-CCL25 axis. However, the clinical trial data was less than promising. Sporadic reports using anti-CCR9, alone or as a fusion, to block CCL25 triggered signaling have shown encouraging results. However, most of these studies are focused on T cell lymphocytic leukemia [23, 53] and may not be as effective in solid tumor patients since targeted delivery of bulky therapeutic antibodies in TME would be a challenge. This leaves room for researchers to try novel strategies targeting this clinically significant axis to improve the therapeutic outcome and patient survival. Considering the limited access of the receptor (CCR9), targeting the easily accessible ligand -CCL25 would be a more practical approach. Systemic neutralization of CCL25 may not be associated with the usual systemic drug-led toxicities since CCL25 is mostly important during early developmental stages. Our lab is developing this approach, and past in vivo experiments carried out on this line

do not show any toxicity concern. On the other hand, an immune cell-based therapy similar to CAR-T or Sipuleucel-T, where the CCL25 signaling is targeted, may be useful in the tumor context. However, the immune cell plasticity in the TME may pose a challenge.

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CCL27 Signaling in the Tumor Microenvironment

9

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Abstract

Chemokines are a group of small proteins which play an important role in leukocyte migration and invasion. They are also involved in the cellular proliferation and migration of tumor cells.

Chemokine CCL27 (cutaneous T cell-attracting chemokine, CTACK) is mainly expressed by keratinocytes of the normal epidermis. It is well known that this chemokine plays an important role in several inflammatory diseases of the skin, such as atopic dermatitis, contact dermatitis, and psoriasis. Moreover, several studies have shown an association between CCL27 expression and a variety of neoplasms including skin cancer.

In this chapter, we address the role of chemokine CCL27 in the tumor microenvironment in the most relevant cancers of the skin and other anatomical locations. We also make a brief comment on future perspectives and the potential relation of CCL27 with different immunotherapeutic modalities.

Keywords

Chemokines · Chemokine receptors · CCL27 · CCR10 · Tumor microenvironment · Angiogenesis · Pericytes · Skin cancer · Melanoma, mycosis fungoides · Breast cancer · Colorectal cancer · Glioblastoma · Metastasis · Immunotherapy

9.1 Introductory Section

9.1.1 Introduction: Chemokine and Chemokine Receptors

Chemokines are a superfamily of small (8–14 kDa) secreted basic proteins that regulate relevant leukocyte migration and tissue invasion by interacting with their specific receptors, the latter belonging to the superfamily of seven-transmembrane domain G-protein-coupled receptors. Chemokines are able to attract specific immune cells: Their function has been demonstrated in inflammatory sites as well as in healthy lymphoid tissues [1, 2], and they also contribute to leukocyte extravasations and localization within peripheral sites [1, 3].

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Chemokine receptors are a group of membrane proteins about 350 amino acids in size. At least 19 chemokine receptors have been identified in mammals [4], and 10 members (CCR1-10) have been described in humans. CCR are receptors for ligands of the CC chemokine subfamily (containing at least 28 members, CCL1-28) [5]. Chemokine receptors can be subclassified into four subfamilies. Chemokine receptors, which are expressed on the cell surface, direct cell migration along guidance cues provided by their respective ligands. This process is called chemotaxis. The chemokine receptor-transduced signals have also been involved in several functions such as cellular survival, proliferation, and activation [5].

Interactions of chemokines with a variety of cell types present in the tumor microenvironment, particularly cutaneous cells, mesenchymal stem cells, and pericytes, have been described. Van den Broek et al. (2014) analyzed the variable response of different types of mesenchymal cells (adipose tissue-derived mesenchymal stem cells and dermal fibroblasts) as well as keratinocytes and how these cells would react to cutaneous burn wound [6]. In this study, they described the increased secretion of several chemokines by dermal fibroblasts and adipose tissue-derived mesenchymal cells in contact with burn wound exudates. This process could play a very important role in future therapies with stem cells [7].

A healthy vasculature is essential for normal and tumoral tissues. Appropriate blood vessels are responsible for the normal supply of oxygen and nutrients and for removing products of degradation [8]. Angiogenesis, the process by which new blood vessels form from existing vessels [9], is crucial to maintain an appropriate vasculature. Pericytes, fibroblast-like cells that are located around the endothelial cells in arterioles, capillaries, and venules, play an important role in angiogenesis. Type 2 pericytes have demonstrated angiogenic capacity both in vivo and in vitro [10]. Only type-2 pericytes located in the normal tissue involved by neoplasms contribute to tumor angiogenesis [10]. These findings could be important to develop different therapeutic strategies in cancer treatment and in ischemic processes. Pericytes also participate in the production of chemokine CCL12, which is involved in the maintenance of hematopoietic stem cells [11, 12]. Deletion of CCL12 from perivascular cells results in depletion of bone marrow hematopoietic stem cells [11].

9.1.2 Chemokine CCL27

Four families of chemokines (C, CC, CXC, CX3C) have been defined [13]. CCL27 (cutaneous T cell-attracting chemokine, CTACK) is one of the CC chemokines [14]. In humans and mice, this has been reported to be expressed mainly, but not exclusively, by keratinocytes [14]. CCL27 is also expressed in skin-derived Langerhans cells [14, 15].

In normal skin, CCL27 represents the dominant chemokine and is expressed by basal keratinocytes of the epidermis [16–21], and it has been shown to attract cutaneous lymphocyte antigen (CLA)-positive cells expressing the receptor CCR10 [17, 19, 20, 22]. CCL27 overexpression leads to the enhanced recruitment of CCR10+ cells, both of which have been shown to play an important role in T cell-mediated skin inflammation [16].

CCL27 has the potential to convert between different oligomeric states. This property makes it possible to accommodate multiple glycosaminoglycans-binding partners, providing a mechanism for cell specificity and regulation [23].

Therefore, CCL27 is a very important basic chemokine for T cell skin-homing [14]. In normal skin, suprabasal keratinocytes express only a minimum amount of CCL27. However, the lesions of human atopic dermatitis, contact dermatitis, and psoriasis usually demonstrate much stronger expression of CCL27 [16]. In addition, CCL27 can be found in the dermal extracellular matrix, fibroblasts, and endothelial cells of the superficial plexus, especially in inflamed skin [16]. Nevertheless, CCL27 mRNA expression has not been identified in fibroblasts and endothelial cells [22]. It has been suggested that CCL27 is secreted into the papillary dermis,

where it is immobilized in the extracellular matrix and displayed on the surface of endothelial cells [16]. It is not currently clear how the secreted CCL27 localizes on the surface of the endothelial cells and where it is involved in the activation of integrins. The presentation of CCL27 on endothelial cells probably occurs through transcytosis, a mechanism known to localize chemokines such as CXCL8 and CCL19 on endothelial cells [17, 24].

CCR10, the receptor for CCL27, is expressed by T cells, primary dermal microvascular endothelial cells, dermal fibroblasts, and melanocytes, but not keratinocytes [22]. In humans, CCL27 selectively attracts CLA+ memory T cells by interacting with CCR10 expressed in these lymphocytes [14, 22].

Fluctuations in CCL27 expression are seen in the skin lesions of patients with diseases such as atopic dermatitis, contact dermatitis, and psoriasis [25], diseases which have been correlated with increased CCL27 serum levels [26]. It has been reported that CCL27 expression is enhanced by tumor necrosis factor-alpha and interleukin beta during skin inflammation [27]. Increased levels of CCL27 have been detected in sera of patients with various skin inflammatory diseases, including diffuse or limited cutaneous systemic sclerosis, atopic dermatitis, and psoriasis vulgaris [16, 26, 28, 29].

In vitro studies have revealed that CCL27 markedly enhances wound repair of endothelial cells and fibroblasts, which suggests a role for CCL27-CCR10 interactions in tissue repair [21]. Basal keratinocytes of the neo-epidermis are usually strong producers of CCL27 throughout the reepithelialization period [21].

It has been speculated that ultraviolet exposure leads to the secretion of CCL27 from the epidermis into the dermis, mediating the recruitment of CCR10-positive skin-homing leukocytes into the sites of ultraviolet-induced injury [30]. One study found that ionizing radiation induced CCL27 secretion in vitro. After irradiation, a rapid increase in the levels of intracellular reactive oxygen species promoted the secretion of tumor necrosis factor alpha, which in turn induced signaling that further boosted reactive oxygen species production. The cross talk between reactive oxygen species and tumor necrosis factor alpha can further trigger skin immune and inflammatory reactions to irradiation through the induction of CCL27 secretion [31]. Cutaneous T cells activated in the presence of calcitriol express the chemokine receptor CCR10, attracting them to CCL27 expressed by epidermal keratinocytes, and then migrate from dermal layers to the epidermis under UV radiation [32].

CCL27 has a relatively restricted expression pattern in normal physiological conditions. While CCL27 transcripts are found in multiple tissues, they are predominantly expressed in the skin by keratinocytes [14, 22]. One study has shown that CCL27 is a major regulator of keratinocyte precursor cell migration from the bone marrow to the skin [33]. In this study, bone marrow-derived cells were seen to transdifferentiate into keratinocytes at the sites of skin wounds [33].

9.2 Main Text

Chemokines are involved in neoplastic transformation, tissue invasion, and metastasis, as well as in the host antitumor response in cancer [34]. Cancer progression is facilitated by the evasion of the immune system by tumor cells [34]. Chemokines induce chemotaxis for a variety of cell types [26]. It has been hypothesized that dissemination of tumor cells to specific organs may be influenced by the chemokine receptors expressed by tumor cells and by the expression of their ligands in the target organs [35]. Cancer cells are special cells which do not respond to normal homeostatic regulations. Therefore, understanding the recruitment and involvement of a large amount of effector cells into a tumor is difficult [16]. However, accumulated evidence suggests that tumor immunity can be initiated by providing pro-inflammatory mediators in solid tumors [36]. Tumor-infiltrating lymphocytes usually represent a favorable feature but require the presence of numerous T cells and natural killer cells [37]. Chemokines have an important role in mediating the recruitment of the appropriate

immune cells to the tumor and the acquisition of an effective antitumor immunity [37]. The important role of T cells in tumor rejection and their cytolytic activity against tumors have been known for decades [38, 39]. T cells circulating in the peripheral tissues are constantly screening for foreign and changed self-antigens expressed by malignant cells [40]. Subsequently, malignant cells are recognized and eliminated by tumor-associated antigen-specific T cells, thereby preventing tumor progression [41].

The acute stress response has been described as an essential survival mechanism [42]. CCL27 overexpression by short-term stress may induce recruitment of numerous T cells to sites of ultraviolet exposure and enhance an early effector function against damaged cells that are likely to develop into tumors [43–45]. The activation of short-term stress may mediate antitumor immune responses [46]. Mobilizing protective immune responses is critical during cancer therapy for eradicating immune-responsive tumor cells as well as providing protection against infection [46].

Although cancer cells produce a variety of chemokine ligands that may be involved in neoangiogenesis, attraction and retention of inflammatory cells, and tumor cell proliferation [47], neoplastic cells express a limited repertoire of chemokine receptors [35, 47]. These receptors play distinct roles in distant organ metastasis [35, 48].

CCL27 is overexpressed in certain types of squamous cell carcinoma [49] and expressed at various levels in metastatic prostate, breast, colorectal, and pancreatic cancer as well as in melanoma cell lines [50].

CCR10 and its ligand are expressed on various epithelia-homing or epithelia-originating cancer cells and might play an important role in their specific tissue location, survival, and metastasis. Skin lesions of adult T cell leukemia/lymphoma contain CCR10 and CCL27 transcripts, which suggests that they both play a role in T cell leukemia/lymphoma cell invasion into the skin. Consistent with this, cutaneous T cell lymphoma such as mycosis fungoides has extensive expression of CCR10 [51]. Human melanoma also has a high level of CCR10 expression that is associated with a lower survival rate and shorter time to progression [35]. CCR10 expression in melanoma cancer cells might promote their progression and immune escape [52]. In mouse experiments, ectopically expressed CCR10 on melanoma cell lines increased their survival through engagement with locally produced CCL27, allowing the melanoma cells to escape host immune antitumor killing mechanisms, possibly by increasing the expression of antiapoptotic molecules such as BCL-2 [48]. In squamous cell carcinoma, overexpression of CCR10 and CCL27 has been found to be associated with tumor progression [49]. Intriguingly, it has been reported that some human keratinocyte-derived skin tumors might downregulate the expression of CCL27 to prevent the attraction of the T cell-mediated antitumor immunity [53].

Lymphatic endothelial cells secreting CCL27 guide entry of CCR10-positive activated T cells to afferent lymphatic vessels [54]. CCL27 has the capacity to drive lymphatic endothelial cell migration and demonstrates a role for CCR10 in lymphatic vessel development and patterning. CCL27 cooperates with VEGFD to promote lymphangiogenesis [50].

Decreased interactions between CCR10 and CCL27 may play an important role in vaccination and induced inhibition of tumor metastasis [55], and blockade of CCR10/CCL27 interaction may improve cancer survival [55].

9.3 CCL27 in Different Cancer Types

The importance of CCL27 and its ligand CCR10 in tumor behavior and prognosis has been described in several studies. Here we summarize the most relevant features of these markers with regard to the most common tumors and described in relation to their anatomic location (Fig. 9.1).

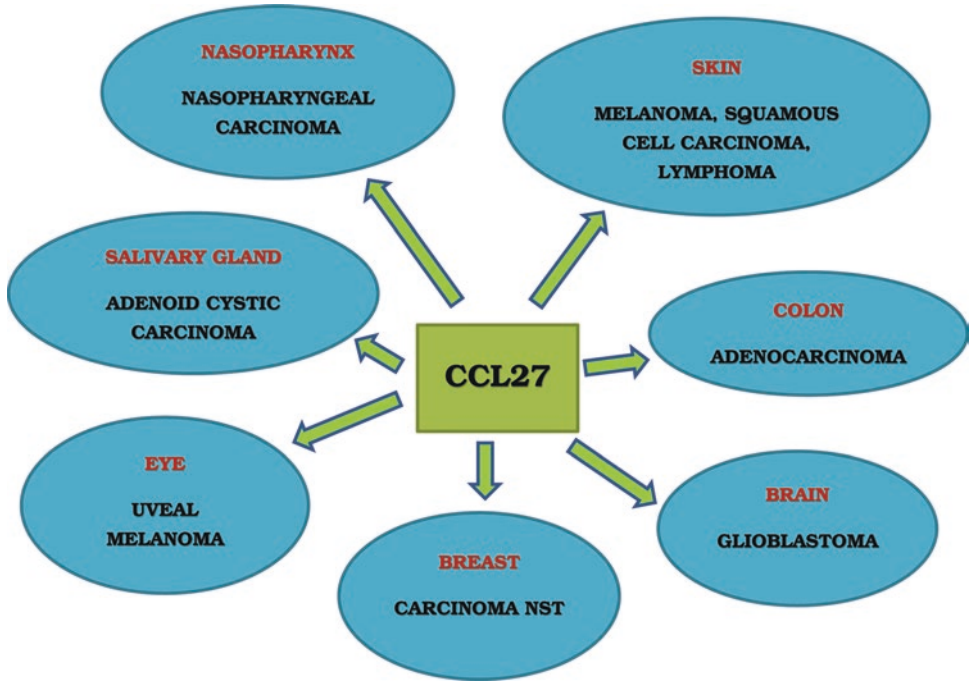

Fig. 9.1 The major human tissues in which CCL27 plays an important role in tumor progression and the main types of tumors which are influenced by CCL27

9.3.1 Skin Tumors

9.3.1.1 Melanoma

Cutaneous malignant melanoma is a potentially fatal tumor generated from activated or genetically modified melanocytes [56, 57] and is increasing in incidence [58]. The ability of tumor cells to avoid immune surveillance is probably central to the progression of melanoma and other cancers, and many mechanisms have been described which, in theory, enable cancers to escape immune-mediated cell death [34, 59].

Melanoma is considered a highly immunogenic tumor due to the numerous tumor-associated antigens identified [60], the well-known occurrence of spontaneous regression mediated by the host immune response [61], and the detection of antigen-experienced antitumor-specific T lymphocytes in vivo [62].

Tumor immunology embraces an extensive array of biological phenomena that include interactions between neoplastic cells and the innate and adaptive immune response [35]. It has been suggested that the expression of chemokines and chemokine receptors by melanoma cells may be involved in tumor immune escape [35]. CCL27 and chemokine receptor CCR10 in human melanomas may help tumor cells to grow, invade tissue, evade immune response, and spread to lymph nodes [34, 52, 63].

The implication of CCL27 in cutaneous melanomas is currently unresolved [64]. It has been proposed that CCL27 expression in melanomas may induce antitumoral immunity [64] and that CCL27 may suppress tumor growth, probably due to the local recruitment of T lymphocytes and natural killer cells [65].

The ability to internalize the CCL27 chemokine might be a feature of malignant melanocytes [48]. CCL27 internalization in malignant cells can mediate a number of biological effects, such as an increase in migratory competence by inducing actin cytoskeleton relaxation [66]. Internalization of CCL27 has been observed more frequently in melanomas than in benign lesions [48]. CCL27-positive melanomas tend to have a lower mean density of CD3 and CD8 T cells compared to melanomas not expressing

CCL27, suggesting that liganding CCR10 by CCL27 could make melanoma cells less susceptible to the host antitumor response [48, 52]. Moreover, cases with CCR10 and CCL27 co-expression tend to have a higher tumor thickness, suggesting that liganding CCR10 by CCL27 in melanoma cells could provide an advantage in tumor growth [48]. Since CCL27 can be regarded as a cellular response that recruits lymphocytes to the tumor site, CCL27 internalization in neoplastic melanocytes expressing CCR10 could, through CCL27/CCR10 interaction, lead to a sequestering of the chemokine in an attempt to withdraw lymphocyte recruitment [52]. CCR10 overexpression by melanoma cells might also be related to aggressive behavior and to their ability to escape immune control [52]. Likewise, CCL27 chemokine is downregulated in melanoma metastases compared with primary melanoma [67].

Some studies have demonstrated that CCL27 expression in the environment could play an important role, as, for example, in epidermal cells just covering the melanoma ("supratumoral epidermis") (Fig. 9.2). A higher expression of CCL27 in supratumoral epidermis has been associated with a longer progression-free interval and longer melanoma-specific survival [68]. However, patients with the histological subtype lentigo maligna melanoma did not fulfill these criteria [68]. Differences in CCL27 immunostaining in these cases could be explained by the fact that this melanoma subtype is located in

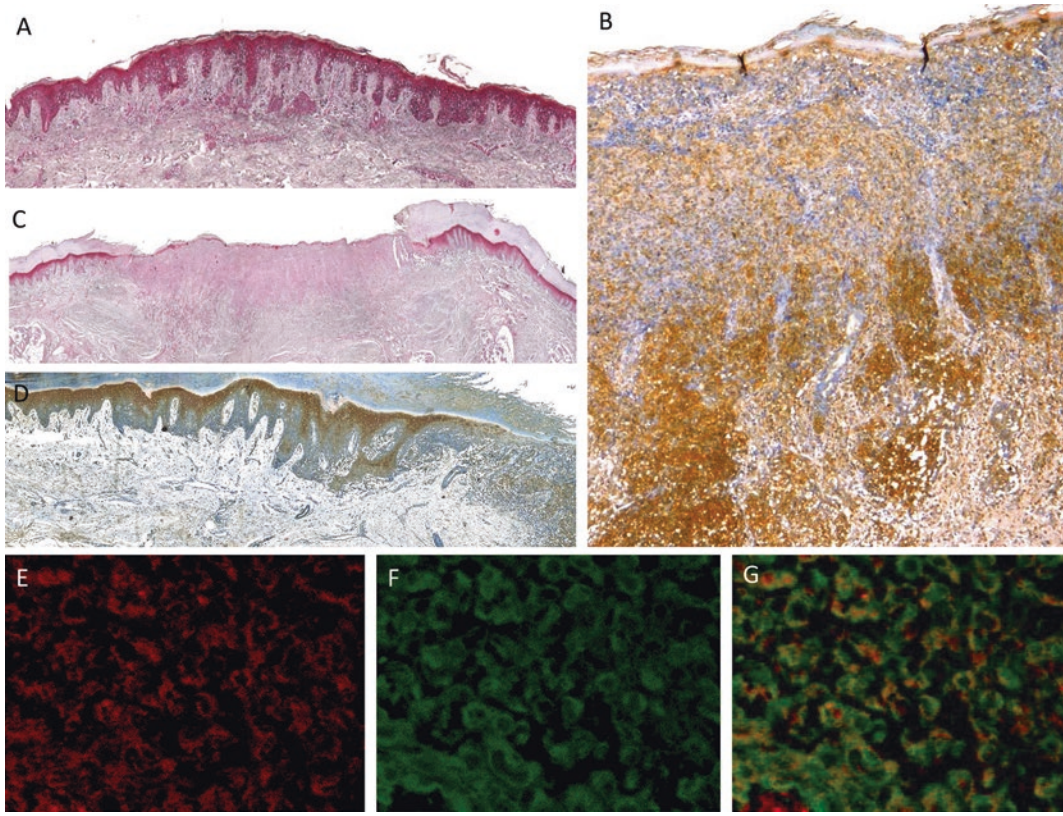

Fig. 9.2 Cutaneous malignant melanoma. Low-power view showing expression of CCL27 in supratumoral and peritumoral epidermis (**a**) or only in peritumoral epidermis (**c**, **d**); prominent CCL27 expression in melanoma tumor cells invading the reticular dermis (**b**). Co-expression of CCR10 (E, red) and CCL27 (F, green) and their fusion (G, orange) in melanoma cells (**a**, **c**, immunohistochemistry with Fast Red as chromogen; **b, d**, immunohistochemistry with DAB as chromogen; **e**–**g**, double indirect immunofluorescence labeled with Alexa Fluor 488 and Alexa Fluor 633, Invitrogen, UK)

areas of the body with high sun exposure, and solar radiation is known to downregulate CCL27 expression [53, 68]. Pivarcsi et al. (2007) [53] suggested that this downregulation could have originated from a Ras mutation. This finding is also consistent with the Whiteman et al. (2011) molecular classification of melanoma [69], in which chronic sun-damaged melanomas have common Ras mutations. Therefore, it may be possible to explain lower CCL27 expression in lentigo maligna melanomas solely as a result of chronic sun damage and not through the downregulation of the chemokine within the tumor [69].

Although CCL27 is constitutively synthesized in the epidermis, CCL27 protein is also present in the dermis [14]. Thus, CCL27 may affect tumor cells implanted into the dermis because of its ability to cross the basement membrane after synthesis by keratinocytes [35]. The presence of CCL27 in the skin may potentially explain why CCR10 expression could be advantageous to melanoma, which arises in the environs of the skin and frequently metastasizes to the skin [35].

The use of the ligand-receptor expression ratio has been described as a powerful tool in melanoma prognosis. A higher chemokine ligand-receptor expression ratio in thin versus thick primary melanomas (36 times for CCL27-CCR10 ratio) and in primary tumors versus melanoma metastasis (315 times for CCL27/CCR10) supports the implication of the chemokine ligand-receptor ratio in human melanoma progression [70]. Moreover, CCL27-CCR10 ratio in primary cutaneous melanoma is, combined with Breslow, the best predictor for the development of distant metastases [70], enhancing the prognostic predictive ability of Breslow tumor thickness and ulceration [70]. The fact that ligand-receptor ratios are absolutely independent of the control housekeeping gene employed for normalization provides outstanding additional value [70].

9.3.1.2 Carcinoma

Cutaneous squamous cell carcinoma is one of most prevalent nonmelanoma skin cancers [71]. It is more common in lightly pigmented than in heavily pigmented populations since pigmentation provides protection against harmful effects of ultraviolet radiation [73, 74]. This neoplasm and basal cell carcinoma of the skin represent the most common malignancies in countries with a predominantly white Caucasian population [72]. Ultraviolet-induced squamous cell carcinoma is generally common in countries with a mostly white population, whereas the number of ultraviolet-induced rates of squamous cell carcinoma cases is low in countries with a lower white population [75]. Aside from ultraviolet radiation, chronic scarring processes and areas of chronic inflammation are also important risk factors for the development of squamous cell carcinoma [73]. Therefore, expression and functional significance of CCR10 and CCL27 may differ according to the extent of ultraviolet exposure and chronic inflammation [49].

Acute stress induces higher levels of CCL27 gene expression. Exposure to short-term stress shifts the cytokine balance toward conditions that favor the development and maintenance of cell-mediated immunity, which is known to confer protection against skin cancer [76, 77]. Chronic stress increases susceptibility to skin cancer and shifts the balance from protective to suppressive immune responses [78]. On one hand, chronic stress suppresses type 1 cytokines and CCL27 gene expression, as well as CD4+ and CD8+ T cell infiltration at sites of tumor emergence and progression, while on the other, it increases the number of regulatory/suppressor cells at tumor sites and in circulation [78]. T cells have been implicated in both the regression [79] and rejection [80] of ultraviolet-induced squamous cell carcinoma. Therefore, downregulation of CCL27 gene expression may contribute to suppression of T cell infiltration and T cell driven antitumor immune responses [78]. Chronic stressors increase susceptibility to disease by mobilizing endogenous immunosuppressive mechanisms such as regulatory/suppressor T cells [78].

A study of homeostatic chemokine expression demonstrated that human cutaneous tumors can evade antitumoral immunity by downregulating CCL27 expression through epidermal growth factor receptor and Ras activation. The authors suggested that the progressive loss of the homeo-

static and skin-associated chemokine CCL27 during malignant transformation of keratinocytes represents a mechanism by which tumor cells evade the immune system. Furthermore, loss of CCL27 during cutaneous carcinogenesis was also found. Immunohistochemical analysis of skin samples showed that CCL27 expression was high in normal epidermis, lower in actinic keratosis, and almost absent in basal cell and squamous cell carcinomas. These results suggest a potential implication of a progressive loss of CCL27 expression during squamous cell carcinoma development [53].

In contrast, another study produced dissimilar results [49]. In normal skin, CCL27 was slightly expressed in basal but not in suprabasal epidermal cells, while strong cytoplasmic staining of the anaplastic tumor cells was found in squamous cell carcinoma (Fig. 9.3). CCL27 immunostaining was weak in Bowen's disease and sparse in basal cell carcinoma. The authors suggested population sampling (Caucasian versus Asian) as a reason for the difference, but the true reasons for the discrepancy remain unknown [49]. The study in the Asian population showed that in squamous cell carcinoma Clark level III or higher, strong CCL27 immunostaining was noticed in the cytoplasm of the anaplastic squamous cells within the tumor [49]. In squamous cell carcinoma Clark level II, actinic keratosis, bowenoid actinic keratosis, and seborrheic keratosis, CCL27 immunostaining was moderately recognized [49]. Only weak CCL27 immunoexpression was detected [49] in Bowen disease. CCL27+ tumor cells were scarce in seborrheic keratosis and basal cell carcinoma. Thus, CCR10 and CCL27 expression

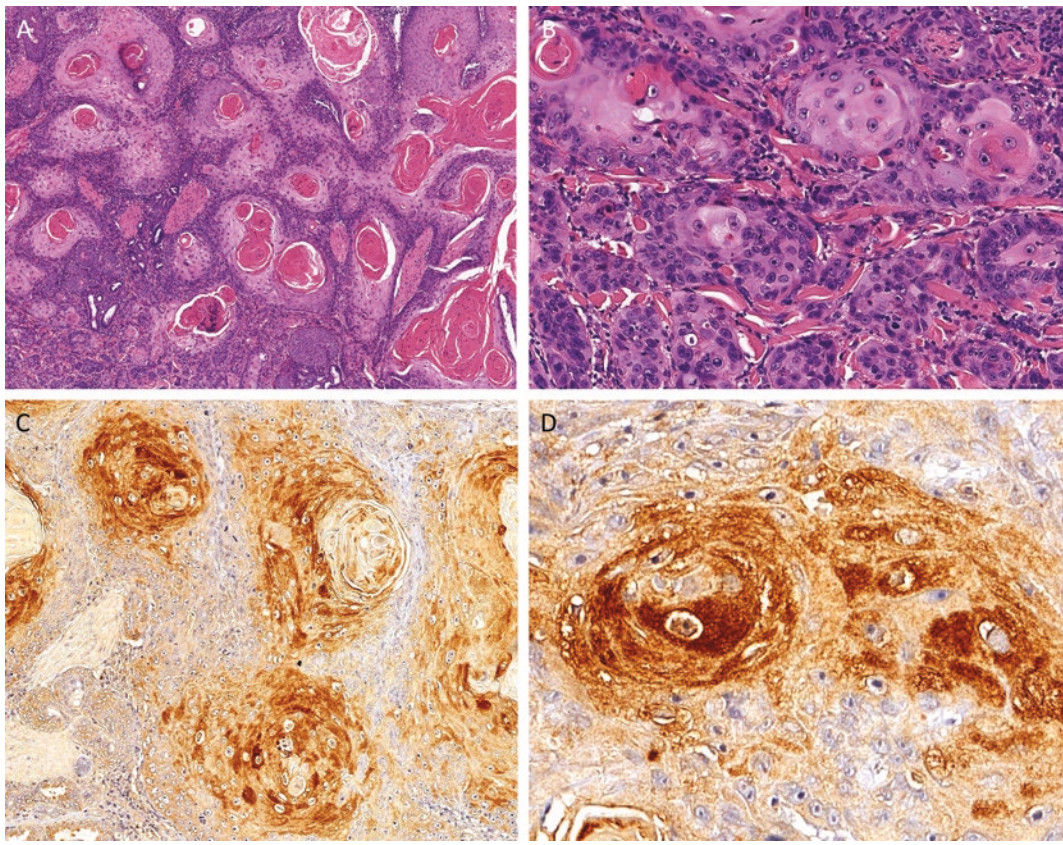

Fig. 9.3 Cutaneous squamous cell carcinoma. Histological features showing keratinizing atypical squamous cell nests (**a**, **b**) showing strong CCL27 cytoplasmic immunostaining (**c**, **d**) (**a**, **b**, H&E staining; **c**, **d**, immunohistochemistry with DAB as chromogen)

was upregulated in squamous cell carcinoma Clark level III or higher relative to Bowen's disease and basal cell carcinoma [49].

9.3.1.3 Cutaneous Lymphoma

Most cutaneous T cell lymphomas are low grade non-Hodgkin lymphomas that belong to the heterogeneous group of mature (peripheral) T cell neoplasms, characterized by clonal expansion of a epidermotropic and mature CD4-positive clone, putatively from a skin-homing subset of memory T cells [81, 82].

Theoretically, the origin of cutaneous T cell lymphoma cells seems to be a mature CD45RO + CD4+ memory T cell, which frequently lacks expression of CD26 or CD7. The clue to understanding the nature and progression of cutaneous T cells lymphoma would be to unravel the mechanisms involved in their migration to the skin [82].

The roles of CCR10 and CCL27 in adult T cell leukemia/lymphoma invasion of the skin may be related to the retention of adult T cell leukemia/lymphoma cells in the dermis besides their initial migration into the skin [83].

The T cell homing mechanism to the skin is not fully understood, but there is growing evidence that chemokines and their corresponding receptors play a significant role [84–86]. Chemokines and their receptors have been associated with tumor metastases [35], invasion of lymphatic vessels [87], and possibly trafficking of lymphoma cells [88].

According to WHO criteria, mycosis fungoides is the most common cutaneous T cell lymphoma. Mycosis fungoides have a classically slow clinical course that progresses over time through the patch, plaque, and tumor stages, followed by lymph node and visceral involvement [81]. Manifestations may be similar to psoriasis, due to skin-homing and proliferation of a malignant T cell clone [81, 89, 90]. In mycosis fungoides, although malignant T cells persist mainly in the skin and only few cells circulate in peripheral blood, some studies have revealed an aberrant T cell immunophenotype and circulating clonal cutaneous lymphocyte antigen-positive T cells in patient's blood [91–93].

Normal epidermal keratinocytes usually exhibit weak cytoplasmic expression of CCL27 [84]. Epidermal keratinocytes show stronger CCL27 expression in most cases of mycosis fungoides and adult T cell leukemia/lymphoma than in normal skin [84]. CCL27 may be expressed not only in the cytoplasm but also occasionally in the membrane of mycosis fungoides and adult T cell leukemia/lymphoma tumor cells [84]. The observations made in this study support the notion that CCL27 and its ligand CCR10 may contribute to infiltration of the skin by malignant T cells in mycosis fungoides and adult T cell leukemia/lymphoma [84, 94]. The serum concentrations of CCL27 in patients with mycosis fungoides is significantly increased, which suggests a dynamic interaction between basal keratinocytes and malignant T cells [95].

Similarly, increased CCL27 serum levels and epidermal expression in mycosis fungoides patients compared to normal controls has been documented, with the hypothesis that CCR10-CCL27 interactions play an early role in the evolution from patch to tumor stage [95]. Epidermal CCL27 overexpression has been seen in 70% of early mycosis fungoides lesions at diagnosis [96].

CCR10 is an important receptor involved in the pathophysiology of mycosis fungoides skin-homing and epidermotropism [95]. Increases in the number of CCR10+ peripheral blood cells and serum CCL27 levels may reflect the extent of increasing infiltration during the course of mycosis fungoides disease progression [95]. The increase in CCR10+ CD4+ cells in the peripheral blood mononuclear cells of patients with mycosis fungoides may be considered as further proof that malignant, clonal T cells may already be circulating from early stages of mycosis fungoides disease [95]. This would suggest a role for CCR10-CCL27 interactions during epidermotropism and skin-homing, not only in Sézary syndrome but also in mycosis fungoides by a subtly different pathway from other allergic skin reactions [95].

The WHO lymphoma classification system identifies Sézary syndrome as a distinct clinical entity [82, 90] with erythrodermia, lymphade-

nopathy, and a circulating malignant clone characterized by large cells with hyperconvoluted cerebriform nuclei [97] and is usually associated with poor prognosis [82]. CCR10 is expressed in circulating clonal cutaneous lymphocyte antigen-positive CD4+ cells in Sézary syndrome [98].

CCL27 in combination with CCL17 is capable of inducing extravasation of Sézary cells into the dermis, consistent with a nonredundant function of these chemokines in cutaneous T cell lymphoma accumulation in the skin. Other studies support the hypothesis that CCL27 is necessary for the transmigration of Sézary cells [99].

CCR10 is involved in skin-homing of the malignant T cell clone. CCR10 expression in Sézary syndrome may partially contribute to the aggressive clinical behavior in comparison with mycosis fungoides [51]. It has been found that tumor cell infiltration of the lymph node of patients with Sézary syndrome expressed CCR10 in a different pattern within the distinct lymphatic microenvironments [51]. Tumor cells within the sinusoidal area of the lymph node showed high expression of CCR10, whereas those in the follicular area showed no expression [51]. Since there was no evidence that CCL27 is expressed in lymphatic tissue, most probably CCR10 would not play a direct role in tumor migration into the lymph nodes [51].

9.3.2 Gastrointestinal Tumors

9.3.2.1 Carcinoma

Colorectal cancer is the third most common cancer worldwide [100] (Fig. 9.4). There is no single predominant risk factor attributed to colorectal cancer, but epidemiological studies have identified family history of colorectal cancer, smoking, excessive alcohol consumption, high intake of red and processed meat, obesity, physical inactivity, and diabetes as risk factors, in addition to age and male sex [101, 102]. Five chemokines, one of them CCL27, have been independently associated with risk of disease, and elevated levels of CCL27 have been associated with increased colorectal cancer risk [103].

The relationship between chemokines and inflammation (chronic and acute) is well known. Inflammatory bowel disease is a well-known risk factor for colorectal cancer, suggesting a relation between chronic inflammation and malignant transformation [104]. These findings are also supported by the lowered risk associated with regular use of aspirin and other nonsteroidal anti-inflammatory drugs [105].

To elucidate the role of chemokines in colorectal adenocarcinoma, some studies have been conducted using cell cultures of cancerous human cell lines. The tumor microenvironment in colorectal cancer cells plays an important role in antitumor effector cells. CCL27 is expressed in colorectal cancer cell line HCT-116 [106] and seems to play a critical role in the tumor microenvironment, suggesting that HCT-116 colorectal cancer cells secrete chemokines that attract effector cells toward the sites of tumor growth permitting them to perform their antitumor activity [106].

T cell recruitment and activation at tumor sites can be elicited by the intratumoral administration of CCR10 [107]. Intratumoral injection of CCL27 demonstrates that after stimulation with dimethyl fumarate or monomethyl fumarate, NK92 cells upregulate the expression of CCR10 and migrate toward CCL27 [106]. Calcium fluxes are also important to initiate the chemoattracting process [106].

Th22 cells (a subset of CD4+ T cells) could be increased in peripheral blood and tumor tissues in several types of gastrointestinal tumors [46]. Th22 cells have several chemokines as their corresponding ligands, one of which is CCL27 [108]. It has been demonstrated that the proportion of Th22 cells is much higher in tumor tissues than in paratumoral tissues [46]. Furthermore, colorectal tumor microenvironment expresses higher levels of CCL27 compared with those of the paratumoral tissues, suggesting that the accumulation of Th22 cells in tumor tissues may be mediated by chemotactic cytokines secreted by the tumor microenvironment [109]. In addition, a reduction in tumor growth by immune responses has been noted upon transfection of CCL27 into other tumors such as ovarian carcinoma cells

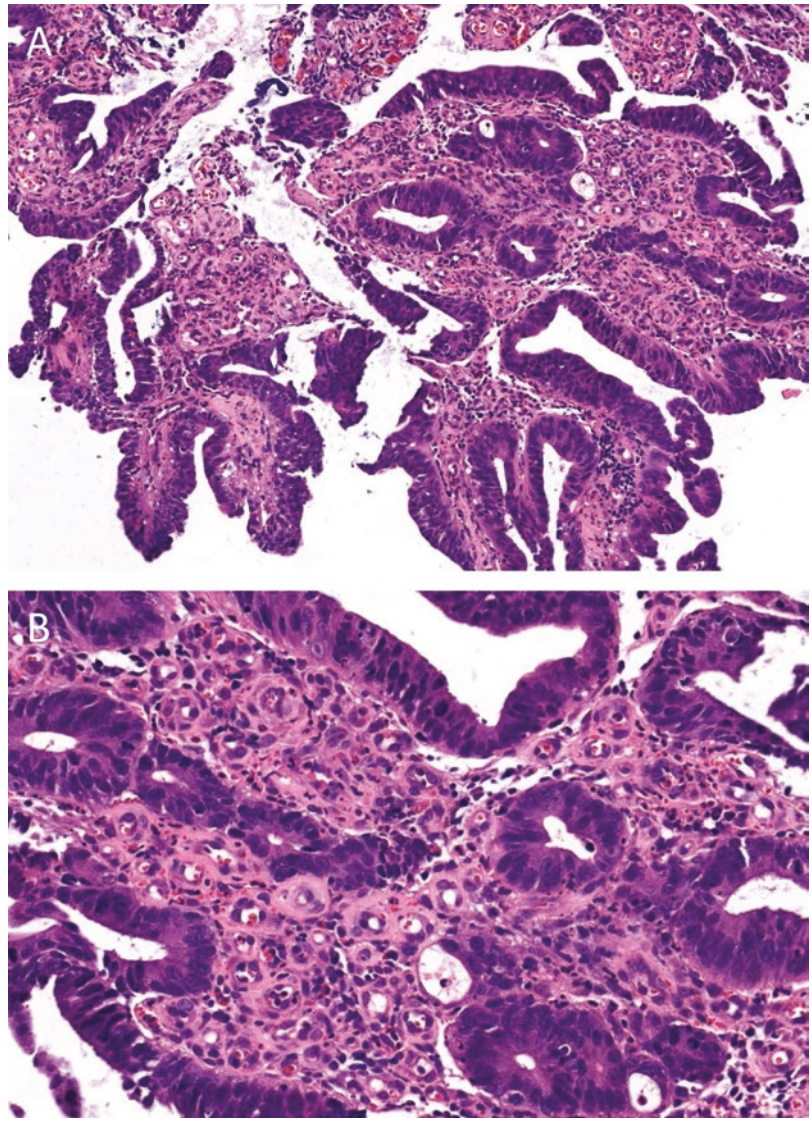

Fig. 9.4 Colonic adenocarcinoma with prominent pseudoglandular tubular differentiation (**a**, **b**, H&E staining)

[65]. In the same way, neutralization of CCL27 has been shown to cause a decline in leukocyte migration to cutaneous tumor sites, thus promoting tumor growth [53].

9.3.2.2 Metastasis from Cutaneous Lymphoma

Extracutaneous localizations of mycosis fungoides are rare and occur in long-standing mycosis fungoides with lymph node involvement [110]. Pancreatic metastases of mycosis fungoides have been described [111] in which chemokines seem to play a critical role.

A histological analysis of pancreatic tissue demonstrated that glucagon-secreting cells of the pancreatic islets expressed the CCL27 chemokine. Mycosis fungoides cells are known to express CCL27 receptor CCR10 [110]. Therefore, CCL27 chemokine could attract mycosis fungoides cells to the pancreatic tissue. It was also suggested that CCL27 may be abnormally regulated in this case [110].

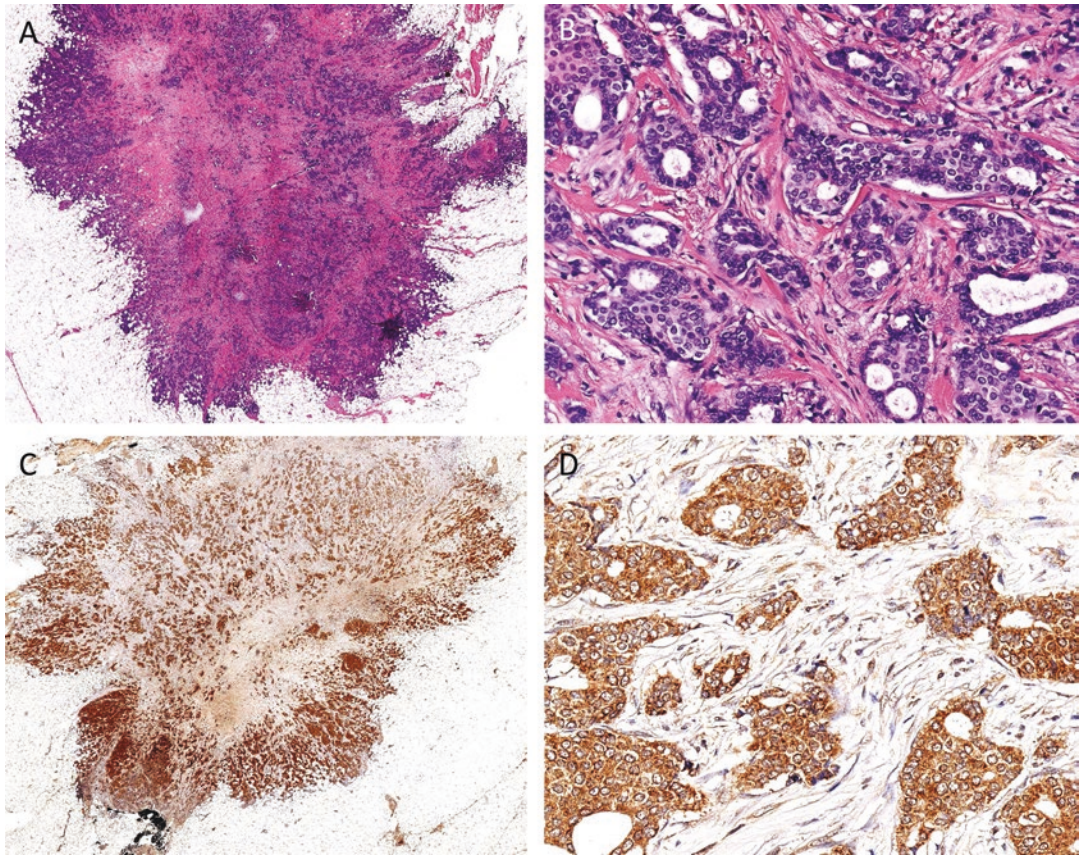

Fig. 9.5 Breast carcinoma. Histological features of an invasive adenocarcinoma with ductal differentiation showing tubular and cribriform features (**a**, **b**) and CCL27 immunostaining in most tumor cells (**c**, **d**) (**a**, **b**, H&E staining; **c**, **d**, immunohistochemistry with DAB as chromogen)

9.3.3 Breast Tumors

Breast cancer is one of the most common cancers in women worldwide and accounts for approximately 15% of newly diagnosed cancers in women [112, 113].

Breast adenocarcinoma cells express CCR10 [25], and some studies have shown that CCL27 is able to increase breast cancer cell invasion and migration (Fig. 9.5). However, knockdown of CCR10 attenuates CCL27-mediated cell invasion and migration [113] which demonstrates that CCL27 enhances breast cancer cell invasion and migration through CCR10 [113].

CCL27/CCR10 interaction dose-dependently induces ERK1/2 activation. The CCR10-mediated ERK1/2 activation increases MMP-7 production and subsequently enhances breast cancer cell invasion and migration [113], whereas knockdown of CCR10 inhibits the CCL27-mediated ERK1/2 activation [113].

CCR10 expression has been associated with tumor stage, lymph node metastasis, and capsular invasion, suggesting that CCR10 could be involved in breast cancer cell invasion and metastasis [113].

9.3.4 Nasopharyngeal Tumors

Nasopharyngeal carcinoma is one of the most common malignant neoplasms in South China and Southeast Asia. Its etiology includes Epstein-Barr virus infection, environmental and genetic factors, and dietary habits [114, 115].

CCL27 levels in serum could be important in the detection of nasopharyngeal carcinoma induced by Epstein-Barr virus infection. According to the literature, CCL27 levels seem to be significantly higher in Epstein-Barr virus-infected individuals than in uninfected normal subjects, whereas CCL27 is downregulated in nasopharyngeal carcinomas [116].

Epstein-Barr virus infection induces an immune response that, under normal conditions, would increase CCL27 levels for recruitment of T cells [117]; however, CCL27 concentrations may be lower in subjects with abnormal immune function [68].

It has been described that reduced levels of cytokines and chemokines, such as CCL27, could permit tumor cells to avoid the immune response [53]. Compared with normal skin, keratinocyte-derived cutaneous tumor cells may downregulate the expression of CCL27 via the epidermal growth factor receptor-Ras-MARK signaling pathway, thereby evading the T cell-dependent antitumor immune response [118].

Plasma CCL27 concentrations could effectively differentiate patients with nasopharyngeal carcinoma from the capsid antigen-specific-IgA (VCA-IgA)-positive healthy donors [116]. Moreover, CCL27 may also distinguish between early-stage nasopharyngeal carcinoma patients and the VCA-IgA-positive healthy donors [116]. It is suggested that CCL27 could be used as a biomarker to identify nasopharyngeal carcinoma patients and serve as the complement of VCA-IgA titers [116].

The combination of traditional viral markers and cellular markers (CCL27) could provide a new and effective method for diagnosing nasopharyngeal carcinoma, with CCL27 complementing the more traditional biomarkers [116]. CCL27 detection in plasma has the advantage that it can be achieved with good accuracy and reproducibility and does not require specialized equipment [116].

9.3.5 Salivary Gland Tumors

Adenoid cystic carcinoma of salivary gland is considered to be the second most frequent malignant tumor of the salivary glands, with a slow and relentless growth, local recurrence, and frequent distant metastasis, mainly to the lung. The involvement of regional lymph nodes is relatively rare, unlike perineural invasion, which has a high incidence and can increase the risk of recurrence [119].

The main cause of mortality in salivary adenoid cystic carcinoma is the development of distant metastasis [120, 121]. Primary treatment is complete surgical resection when possible and adjuvant radiotherapy. The role of chemotherapy is controversial. Due to a lack of specific targets for metastatic cells, treatment of adenoid cystic carcinoma remains a challenge [119].

Chemokines also play an important role in adenoid cystic carcinoma. Lower expression of chemokines, including CCL27, has been associated with lower recurrence and/or perineural invasion [122]. These findings suggest that CCL27 may play an important role in the development of tumor dissemination in salivary adenoid cystic carcinoma [122].

9.3.6 Brain Tumors

Glioblastoma is the most malignant primary brain tumor in humans (Fig. 9.6) and is characterized by invasion of normal brain structures, poor survival, and resistance to treatment [123]. Various chemokine receptors are expressed in glioblastoma, including CCR10 [124]. As in other tumors, interaction of chemokines and their receptors may guide tumor growth and invasion in these tumors [124].

It has been demonstrated that CCR10 activation by CCL27 stimulation could promote glioblastoma cell proliferation and invasion [124]. Moreover, a high expression of CCR10 in glioma is crucial for tumor proliferation, invasion, and progression [124]. These findings are also supported by the fact that CCR10 neutralization inhibited tumor growth in vivo [124].

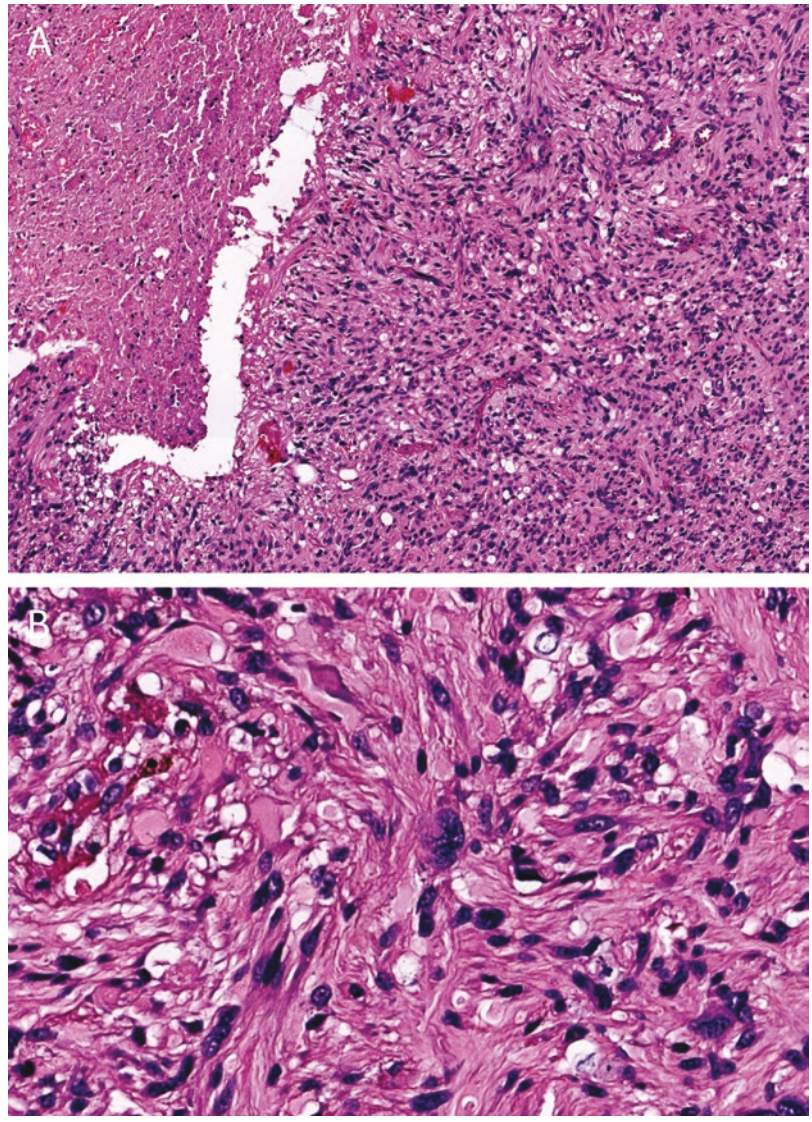

Fig. 9.6 Glioblastoma with extensive necrosis (**a**), spindle and pleomorphic tumor cells (**b**) (**a**, **b**, H&E staining)

9.3.7 Eye Tumors

Uveal melanoma is the most common intraocular malignant tumor [125]. Primary tumor treatment is relatively successful, although nearly half of patients with uveal melanoma will develop metastatic disease, mostly to the liver [126].

Weak expression of CCR10 and CCL27 has been described in the cytoplasm of uveal melanoma cells [127]. Furthermore, the role of CCR10 and CCL27 in cutaneous melanoma is well known [128]. CCL27 is a skin-specific chemokine and is expressed in primary metastatic destinations of melanoma as in lymph nodes, lung, liver and bone marrow. In this regard, high expression of both CCR10 and CCL27 in cutaneous melanoma cells might increase their ability to grow, invade tissues and lymph nodes, and escape the host immune response [52].

A relationship can be established between some chemokines and progression in the metastatic disease of uveal melanomas; however, in contrast to the results described in cutaneous melanomas, no clear association has been found between CCL27/CCR10 and metastatic spread of uveal melanomas to the liver [127].

9.4 Future Trends

Throughout this chapter, we have described the central importance of chemokines in the inflammatory response and tumor microenvironment. A wide range of therapies are now used in cancer treatment. Immunotherapy plays an important role, not only as rescue therapy in patients with no other possibilities but also sometimes as a first-line treatment. The challenge is to improve our understanding of the biology of chemokines and the role they may play in treatment.

The importance of chemokines in cancer immunotherapy is considered to be due mostly to their chemoattractant property for a variety of immune cells as well as their angiostatic activity. In addition, it is known that some tumor cells express a lower level of chemokines than normal cells [129].

Tumor-suppressive activity of several chemokines, including CCL27, has been observed in several experimental tumor models using different laboratory techniques such as the in vitro transfection method [64].

In general, three criteria are required for the immunologic destruction of established tumors: (a) Sufficient numbers of immune cells with highly avid recognition of tumor antigens must be generated in vivo, (b) these cells must traffic to and infiltrate the tumor stroma, and (c) the immune cells must be activated at the tumor site to manifest appropriate effector mechanisms such as direct lysis or cytokine secretion capable of causing tumor destruction [130].

Regarding this three rules, we can deduce that not only the accumulation but also the activation of immune cells in tumor tissue is very important in cancer immunotherapy and the use of chemokines could play an important role in this process. Several approaches combining chemokines with cytokines or costimulatory molecules have been studied and have resulted in the synergic enhancement of antitumor activity as compared with the application of chemokines alone [131, 132]. Therapy directed at specific chemokine receptor pathways may enhance antitumor immunity and may be a useful treatment. [48].

Immune chemokines, which primarily target lymphocytes and dendritic cells, could be useful molecules to improve the efficacy of cancer immunotherapy by augmenting tumor-infiltrating immune cells [133].

As chemokines regulate leukocyte migration and infiltration of local sites, they may play an important role in increasing tumor-infiltrating immune cells in cancer immunotherapy. Recent studies in several murine tumor models have provided experimental evidence that introduction of chemokines into the tumor environment results in the recruitment of relevant leukocyte subsets and decreases tumorigenicity of malignant cells [64, 65, 134].

However, most of these studies used ex vivo gene transfection methods that are not suitable for use within clinical settings, especially for the treatment of patients with established malignancies, and few reports have shown that direct in vivo transduction with chemokine genes alone could induce complete regression of a preexisting tumor mass [133].

Chemokine CCL27 is a strong recruiter for T cells into tumor tissue [133]. Cellular immune responses, including the activation of NK cells and cytotoxic T lymphocytes, play a more important role in the elimination of tumor cells by tumor immunity than tumor immune responses accompanied by antibody production from B cells [133]. The antitumor effects in the IL-12/CCL27 combinational therapy have shown T cell dependence with the effector activity of CD8+ cytotoxic T lymphocytes, rather than with the NK activity, mainly contributing to the regression of the preexisting tumors [133]. Some studies have shown that chemoattractant activity of CCL27 to NK is directed by ligand-receptor interaction [37].

The use of CCL27 in studies using the ex vivo transfection model [134] demonstrated that it could attenuate tumorigenicity of murine ovarian carcinoma cell line (OV-HM) by augmenting tumor-infiltrating T and NK cells to promote antitumor efficacy and improve the safety of IL-12 gene therapy for established tumors [134]. Tumors injected with the IL-12/CCL27 combination showed more enhanced accumulation of

CD3 T cells than those injected with RGD fiber-mutant adenoviral vector (AdRGD)-IL12 alone [133].

Compared to ex vivo experiments using CCL27 gene transferred OV-HM cells, in which about 70% of tumors regressed completely, antitumor activity was not achieved in vivo, although the intratumoral injection of AdRGD-CCL27 did induce a large accumulation of NK cells [37]. Similarly, recent studies provide experimental evidence that introduction of chemokines into the tumor environment results in the recruitment of relevant leukocyte subsets in vivo and decreases tumorigenicity of malignant cells [64]. Nevertheless, other results have suggested that the accumulation of immune cells in tumors does not induce notable tumor regression per se [37].

CCL27 may be an important mediator of vaccines against metastatic disease. It has been demonstrated that decreased levels and interactions between CCR10 and CCL27 may play an important role in vaccine-induced inhibition of tumor metastasis and survival improvement [55].

As previously documented, CCL27 chemokine could play a very important role, not only in immunotherapy but also as a marker to facilitate and provide new and effective methods in diagnosis as, for example, in nasopharyngeal carcinoma [116].

Chemokines seem to be an important factor in the tumor microenvironment and will constitute a potential and critical element in future immunotherapy treatments. In this chapter, we have discussed the importance of chemokine CCL27 in the tumor microenvironment, with specific focus on the most relevant subtypes of malignancies influenced by CCL27 in the development and progression of tumors. Further research into the CCL27 chemokine may open up new and interesting perspectives for more effective therapies against cancer in the future.

Conflict of Interest Statement The authors have no conflict of interest.

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