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Clinical utilization of chemokines to combat cancer: the double-edged sword

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Abstract

Chemokines are a small group of related chemoattractant peptides that play an essential role in the homeostatic maintenance of the immune system. They control the recruitment of cells needed for the induction and activation of innate and adaptive immune responses. However, tumors also utilize chemokines to actively progress and evade immunosurveillance. In fact, chemokines are involved directly or indirectly in almost every aspect of tumorigenesis. They mediate survival and metastatic spread of tumors, promote new blood vessel formation (neovascularization) and induce an immunosuppressive microenvironment via recruitment of immunosuppressive cells. As a result, a number of therapeutic strategies have been proposed to target almost every step of the chemokine/chemokine receptor involvement in tumors. Yet, despite occasional success stories, most of them appear to be ineffective or impractical, presumably due to 'nonspecific' harm of cells needed for the elimination of tumor escapees and maintenance of immunological memory. The strategy would only be effective if it also promoted antitumor adaptive immune responses capable of combating a residual disease and tumor relapse.

Keywords

angiogenesis; cancer; chemotaxis; metastases; tumor escape

The last few decades have been marked with a number of exciting findings that have deepened our understanding of multistage carcinogenic processes (FIGURE 1). The importance of immunomodulatory cytokines and specifically chemoattractant cytokines, designated chemokines, cannot be overlooked in this multifactorial process. This, in turn, has changed our simplistic view of chemokines as by hitherto recruiter and regulator of the directional migration (chemotaxis) of immune cells. Tumor progression is directly affected by pleiotropic effects of chemokines on survival, proliferation and metastatic spread of malignant cells [1–3]; and indirectly modulated inducing angiogenesis [4,5] or recruiting immunosuppressive cells that lead to escape from immunosurveillance [6–9]. It is striking that these complex functions are controlled by the 8–15 kDa (except fractalkine/CX3CL1)-related peptides with a simple

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structure. To date, the chemokine family includes approximately 50 secreted peptides that are classified into four super families (two major types CC and CXC and two minor C and CX3C chemokines) on the basis of the first two cysteine residues [10]. Although many other peptides and factors can have chemotactic potency, chemokines are certainly the masterminds that govern cell trafficking and migration. They act through binding and signaling with approximately 18 cell-surface G-protein-coupled 7-transmembrane receptors, designated chemokine receptors.

The differential and balanced regulation of chemokine and chemokine receptor expressions are an essential part of normal immune function. Impairments in chemokine or chemokine receptor expressions lead to various immunological disorders, including autoimmune diseases and cancer [11,12]. For example, aberrant expression of CCL22 induces prosurvival signaling of the CCR4-expressing tumors and, at the same time, promotes suppression of antitumor immune responses via recruitment of CCR4-expressing suppressive immune cells [13,14]. As a result, overexpression of CCL22 is often associated with severity and a poor disease outcome of cancer patients. This has made chemokines and chemokine receptors attractive targets for cancer therapy [1,15]. However, a genuine and significant interest, particularly from industry, was initiated by the seminal finding that chemokine receptors, such as CCR5 and CXCR4, were also utilized as HIV-1 coreceptors for infection of human $CD4^+$ T cells [16,17]. As a result, ample strategies continue to be reported in the literature, making it almost impossible to overview all of them owing to the limitations of the journal format. Herein, we have tried to summarize and analyze the most 'interesting' approaches proposed for cancer immunotherapy, including the use of pharmacological inhibitors, chemokine antagonists and antibodies to block chemokine/chemokine receptor activity. An increasing number of strategies aimed at inhibiting growth and metastastatic spread of tumors are being developed and even tested in humans with intentions either to indirectly affect tumor microenvironment and blood vessel formation, or to directly reduce survival, apoptosis, migration and adhesion of malignant cells. However, these strategies are not exclusively specific for tumors and will be partially effective without elimination of a residual disease. The approach will be partially successful unless it is also supported by induction of adaptive antitumor immune responses, particularly involving $CD4^+$ and $CD8^+$ T cells. In this respect, chemokines have been explored to improve the efficacy of cell-based vaccines by expressing them in tumors to recruit cytotoxic, phagocytic and antigen-presenting cells (APCs) and to elicit potent antitumor adaptive immune responses [18]. Alternatively, chemokines are utilized as carriers for nonimmunogenic tumor antigens to enable their preferential uptake and presentation by the professional APCs [19, 20]. However, cancer immunotherapy or vaccines will presumably be most effective on minimal residual disease, since tumor-specific cytotoxic T lymphocytes can be neutralized once they encounter the immunosuppressive microenvironment of bulky tumors or their immunosuppressive macrophages, natural killer cells (NK), natural killer T (NKT) cells, Tregulatory cells (Tregs) and immature dendritic cells (iDCs) [6,13,21,22]. Therefore, the success of chemokine-based therapy depends on a proper engagement of the delicate and 'double-edged' balance of activating and suppressive factors and induction of potent adaptive antitumor immune responses.

Chemotactic cytokine–chemokine

Chemokines, originally known as chemotactic cytokines, are secreted and related 8–15-kDa peptides (except fractalkine/CX3CL1) with very simple structures. They are considered master controllers that regulate migration and recruitment of various subsets of immune cells and even malignant cells. To date, the chemokine family consists of approximately 50 chemokines that are classified into four groups, designated CC, CXC, C and CX3C, based on the way the first two conserved cysteines are arranged; and most known chemokines belong to CC and CXC families [10]. There are two nomenclatures used in the literature: one based on given name at

the time of chemokine discovery, such as interferon-inducible 10-kDa protein (IP-10) or thymus and activation-regulated chemokine (TARC), and recently adopted a systematic nomenclature that combines structural motifs (CC, CXC, XC and CXC3C) with 'L' (for ligand) and the number of the respective gene, such as *CXCL10* for IP-10 and *CCL17* for TARC (a detailed list is described in [301]). Functionally, chemokines are distinguished as inflammatory (inducible) or homeostatic (constitutive), based on their pathophysiological activities [3]. Inflammatory chemokines are expressed during infection or tissue damage by resident and infiltrated leukocytes. By contrast, homeostatic chemokines are usually produced constitutively in discrete microenvironments and they are involved in maintaining the physiological trafficking of immune cells [3]. The simplistic view of chemokines as recruiters of immune cells and regulators of the directional migration (chemotaxis) is changing owing to the important role they play in the innate and adaptive immune responses [23]. It is also becoming clear that the chemokine network, as an essential part of an intricate system of immunosurveillance, affects and regulates either directly or indirectly, the growth and metastatic spread of malignant cells. Based on the role of chemokines in promotion and suppression of neovascularization, they are also described as angiogenic and angiostatic, respectively.

Structurally, chemokines contain a high-affinity chemokine receptor-binding site (usually at the N-terminus) and a low-affinity glycosaminoglycan (GAG)-binding region (either distinct or overlapping with a receptor-binding site) [24–27]. GAGs consist of a linear polysaccharide chain linked covalently to protein cores, forming proteoglycans, which are abundantly expressed key components of the endothelial cell surface and extracellular matrix. It has been proposed that, to elicit chemotaxis *in vivo*, chemokines must be immobilized on endothelial cells or extracellular matrix surfaces by interacting with negatively charged GAGs, which allows increased concentrations of chemokines near the initiating inflammatory or trafficking stimulus [28]. Chemokines on the luminal endothelial surface can activate chemokine receptors of the rolling cells, thus triggering integrin activation. This results in arrest, firm adhesion and transendothelial migration into tissues towards chemokine gradients. Chemokines are often detected in various pathological sites associated with GAGs, for example, interleukin (IL)-8/CXCL8 is found to be complexed with endothelial syndecan-3 in rheumatoid arthritis synovium [29]. The binding with GAGs may not only facilitate a solid-phase chemokine concentration gradient required for directional migration of leukocytes *in vivo* [30,31] but also modulate activities of CXCL8 and CXCL10 [32,33]. GAGs induce oligomerization of monocyte chemotactic protein (MCP)-1/CCL2, macrophage inflammatory protein (MIP)-1β/CCL4, and regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5 affecting their activity [34,35]. Moreover, association with GAGs, particularly heparin sulphate, is shown to protect stromal cell-derived factor (SDF)-1 from the proteolytic inactivation by CD26/dipeptidyl peptidase IV [36]. Interestingly, binding with GAGs can also result in unexpected activities, such as induction of apoptosis in human T-cell lymphoblastoid cell lines expressing CCR5 [37]. The extra-cellular matrix-bound RANTES and MIP-1β are able to stimulate T-cell adhesion [38], while organ-specific differential expression of GAGs sequester SDF-1/CXL12 affecting metastatic spread of cancer cells [39]. These observations led to proposals to target GAGs, for example using soluble antagonists, to control aberrant inflammation and cancer metastasis [36,39]. Alternatively, the efficacy of dominant-negative antagonists of CCL5 as an anti-inflammatory reagent can be improved by mutating the GAG-binding sites to inhibit formation of oligomers [35].

[301]Website
Cytokine Family Database http://cytokine.medic.kumamoto-u.ac.jp

Chemokines that affect tumor angiogenesis

The salient feature of all solid tumors is their strict dependence on neovascularization and development of new blood vessels through angiogenesis. Access to blood vessels for a sufficient supply of oxygen and nutrients is not only necessary for maintenance of tumor growth, but also for tumor dissemination and metastasis; it is assumed that tumors cannot grow beyond 2 mm in the absence of local angiogenesis. The microenvironment of tumor tissues can be viewed as an important cofactor of the carcinogenic process [40]. The invasive behavior and selection of neoplastic cells are affected by interactions between tumors and the activated microenvironments. The malignant cells recruit vasculature and inflammatory cells through a network of chemokines, cytokines and growth factors. Tumor cells or tumor microenvironments express specific chemokines regulating angiogenesis, growth and metastasis of malignant cells via recruitment effector cells, such as macrophages and lymphocytes (TABLE 1). Angiogenesis is a finely tuned process regulated through a delicate and complex balance between the collective action of proangiogenic (e.g., vascular endothelial growth factor, [VEGF]) and angiostatic (angiogenic inhibitors, such as thrombospondin-1) factors [41]. Since Judah Folkman's original idea in 1971, a surge of strategies to inhibit tumor angiogenesis has been developed. In particular, anti-VEGF therapy is effective in several preclinical models and Phase I/II or even Phase III human clinical trials [42,43]. This half century-long and never-ending battle continues, to date, by focusing on the improvement of specificity and the reduction of potentially harmful side effects. In this respect, chemokines attract significant interest, as a number of CXC and CC chemokines (CCL1, CCL2 and CCL11) and fractalkine/CX3CL1 modulate angiogenesis both *in vitro* and *in vivo* [44–47]. In particular, the so called ELR (Glu–Leu–Arg at the N-terminus) motif containing CXC chemokines, such as CXCL8, and CXCL1–3 (growth-regulated oncogene-[GRO]α, -β and -γ) and epithelial neutrophil activating peptide (ENA)-78/CXCL5, induce angiogenesis directly [44].

In pathological angiogenesis, the angiogenic switch is shifted toward the angiogenic factors and, if the imbalance continues, it results in irregular tumor vessel growth. Tumor growth and angiogenesis are often closely linked and associated with a worse disease prognosis for cancer patients [1,48–50]. Tumor growth results in hypoxia that, in turn, induces a profound change in gene expression, particularly angiogenesis-promoting genes, such as *VEGF*, chemokines (*IL-8*/*CXCL8*, *MIP-3α*/*CCL20*, *MCP-2*/*CCL8*, myeloid progenitor inhibitory factor (*MPIF*)-*1*/*CCL23* and human chemokine (*HCC*)-*2*/*CCL15*) and chemokine receptors (*CCR1*, -*2* and -*5* and *CXCR4*) [51–53]. Of note, this also means that, even if angiogenic factors and chemokines are not detected in *in vitro* cultured cells, they could be expressed once cells encounter the hypoxic conditions of solid tumors. Thus, angiogenesis can be further enhanced by chemokines (CCL2, for example) produced at the tumor site that, in turn, leads to the recruitment and retention of macrophages and monocytes that produce a variety of angiogenic factors. This has been documented in a number of different human tumors, such as gastric carcinoma, esophageal squamous cell carcinoma and breast carcinoma [54,55].

Expression of chemokines is positively associated with a poor disease outcome, indicating their useful value as prognostic biomarkers of disease outcome [56] and attractive therapeutic targets. For example, neutralizing anti-ENA78/CXCL5 antibody may be used for reduction of tumor vascularity and angiogenesis associated with CXCL5-expressing human nonsmall-cell lung cancer (NSCLC), as it was able to significantly inhibit tumor growth, vascularity and metastasis of NSCLC cells in severe combined immunodeficiency (SCID) mice [57,58]. Similarly, human cancers, including esophageal tumors and melanoma, highly express GROs/CXCL1–3 [59]; and their vascularization in SCID mice was reduced successfully by the depletion of GROs [60]. Advanced disease stage and poor survival of patients with melanoma, ovarian cancer, prostate and lung cancer is associated with augmented serum levels of CXCL8 [61,62]. Therefore, depletion of CXCL8 appears to be appealing, as it affected growth of

various tumors in SCID mice inhibiting tumor-associated vascular density and angiogenic activity [57,58,62].

Chemokines, particularly CXC chemokines (CXCL9, CXCL4 and CXCL10) that do not contain the ELR motifs can exert antiangiogenic/angiostatic properties inhibiting the angiogenic effects of the ELR^+ CXC chemokines [44]. Increased levels of IP-10 were detected in fresh specimens of human squamous cell carcinoma and were inversely correlated with tumor growth rate. Intratumorally administered CXCL10 was able to inhibit tumorigenesis and metastasis of NSCLC cells in SCID mice [63,64]. A positive association between expression of IP-10 and improved disease outcome can also be explained by its ability to inhibit production of monocyte-derived CXCL8, CXCL1–3 and CXCL5 [63,65,66]. However, CXCL10 recruits other important effector cells of the adaptive immune system, such as the T helper (Th)-1 type $CD4^+$ T cells and NK cells, that also produce interferon (IFN)-γ and activate keratinocytes, fibroblasts, endothelial cells and mononuclear phagocytes to produce CXCL10. Therefore, CXCL10 is an attractive chemokine to combat tumorigenesis and neovascularization through the shift of a local balance toward angiostatic chemokines. These strategies are potent and safe, although they would only transiently control angiogenesis unless combined with the induction of additional antitumor cellular responses.

Chemokine receptors

Chemokines transmit their signaling via binding with 18 cell-surface G-protein-coupled 7-transmembrane receptors, so-called chemokine receptors, designated CXCR1 through CXCR7, CCR1 through CCR11, CXCR1 and CX3CR1 (based on their specific preference for certain chemokines). Chemokine receptors share 25–80% amino acid homology of 340–370 residues with an acidic N-terminus, conserved ten amino acid sequence in the second intracellular loop, and one cysteine in each of the four extracellular domains. The chemokine binding site is complex and involves several noncontiguous sites, including the N-terminal segment. Although some chemokine receptors bind a single chemokine exclusively and *vice versa*, such as CXCR5 with BCA-1/CXCL13, CCR6 with MIP-3α/CCL20, CCR9 with TECK/CCL25, and CCR10 with CTACK/CCL27; most chemokine receptors appear to have a high promiscuity and induce redundant functions *in vitro* after binding with more than one member. For example, CCR7 binds equally well to ELC/MIP-3β/CCL19 and SLC/CCL21, or CCR4 with TARC/CCL17 and monocyte-derived chemokine (MDC)/CCL22, and CXCR3 with IP-10/CXCL10, monokine induced by interferon-γ (MIG)/CXCL9 and IFN-inducible T-cell α chemoattractant (I-TAC)/CXCL11. Despite the fact that the majority of chemokines and chemokine receptors are far more promiscuous (e.g., CCR3 can equally well bind eotaxin-1/CCL11, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13 and RANTES/CCL5), it appears that they are tightly controlled by differential and compartmentalized expression [67]. As a consequence, $CD4^+$ Th1 and Th2 cells can be separated by expression of CCR5 and CXCR3; and CCR4, CCR8 and CCR3, respectively. Moreover, CCR4, CCR8 and CCR10 are expressed on skin-homing $CD4^+$ T cells [68]. The ability of iDCs to migrate to proinflammatory chemokines MIP-1α/CCL3, MIP-1β/CCL4, MCP-1/CCL2, RANTES/CCL5, MCP-3/CCL7 and MIP-3α/CCL20 is associated with the expression of CCR1 [69–71], CCR2, CCR5 and CCR6 [72,73], unlike mature DCs that express CCR7. In fact, expression of CCR7 seems to be utilized by mature DCs to migrate to the secondary lymphoid organs to present processed antigens to the CCR7-expressing naive or central memory T cells, where MIP-3β/CCL19 and SLC/CCL21 are produced constitutively [73,74]. Interestingly, at least two reports suggest that some chemokine receptors may differentiate their ligands regulating their endocytosis. For example, internalization of CCR7 expressed on T cells was only detected after treatment with CCL19 but not CCL21 [75]. Similarly, CCL22, but not CCL17, downregulated expression of CCR4 on cutaneous and Th2-type $CD4^+$ T cells [76]. However, the extent of this type of differentiation remains unknown, as surface expression of chemokine receptors can be

regulated by their quick recirculation, or controlled by chemokine oligomerization with GAGs. It is also unclear whether this type of regulation is cell-type specific, as both CCL17 and CCL22 internalized CCR4 expressed on human lymphoblastoid cells equally well [BAATAR *ET AL*., UNPUBLISHED DATA].

Chemokine receptors & cancer metastasis

The formation of metastatic colonies is a nonrandom process that starts early during the growth of the primary tumor, probably increasing with time. The peculiar distribution of metastasis was first recognized by Paget in 1889, who called it 'seed and soil'. Since then, the idea has not been changed and it essentially indicates that different organs provide growth conditions optimal for specific cancers. Since a first association of breast-cancer metastasis with expression of CXCR4 and CCR7 [1], it is becoming accepted that chemokines and chemokine receptors play a key role determining the metastatic homing site (TABLE 2). It appears that tumors hijack normal trafficking pathways established for the proper function of the immune system by differentially expressing chemokine receptors. For example, expression of CXCR3 is documented on primary melanomas and various lymphomas; such as T-cell and NK-cell lymphomas, chronic lymphocytic leukemia/small lymphocytic lymphoma and splenic marginal zone B-cell lymphoma [77–80]. Similarly, pancreatic, gastric, prostate and NSCLC cancer cells express CCR6, CCR7, CXCR5 and CX3CR1 [81–84]. However, at least 23 different malignancies, including breast cancer, ovarian cancer, melanoma and prostate cancer, express CXCR4 [2]. This allows them to utilize an important network of the CXCL12- and CXCR4-mediated trafficking of hematopoietic progenitors and endothelial cells to preferentially home into lymph nodes [1,85,86]. High levels of CXCL12 or CCL19 and CCL21 not only promote adhesion but also stimulate CXCR4- or CCR7-mediated tyrosine kinases that promote growth of breast cancer cells [1]. It should be noted that, even if receptor expression was not detected in the primary tumors, its expression might be triggered by change in the stromal/tumor microenvironment at the primary or metastatic sites. The hypoxia-inducible factor is shown to upregulate the expression of CXCR4 via inhibition of suppressive effects of the von Hippel–Lindau tumor suppressor protein [87]. Furthermore, CXCR4, in turn, transactivates the metastasis-enhancing receptor tyrosine kinase HER2 that is found in almost a quarter of human breast cancers [88]. In turn, HER2 further upregulates expression of CXCR4 through the inhibition of its degradation [89]. As a result, overexpression of CXCR4 on tumors is often an indicator of poor overall survival of patients [90–92]. Lastly, the hypoxia not only induces expression of CXCR4, as well as CCR1, CCR2 and CCR5, but it also increases retention of the infiltrating leukocytes and tumor cells [51–53].

These reports indicate that tumor metastasis may be controlled if their chemokine receptors are blocked [71,93]. The idea has been further fueled by numerous resources created to combat transmission of HIV-1 via inhibition of CXCR4 and CCR5. As a result, therapeutic formulations, such as neomycin B hexa-arginine conjugate (NeoR) that protect human neuroblastoma cells from gp120-induced death, are finding ways to inhibit CXCR4-mediated cancer metastasis [94]. Similarly, antagonist-peptides T22 and T140 that block CXCR4 were shown to inhibit the CXCL12-induced migration of breast cancer cells, leukemia T cells, pancreatic cancer cells, squamous cell lung cancer (SCLC) cells, chronic lymphocytic leukemia B cells, pre-B acute lymphoblastic leukemia cells; promoting significant suppression of pulmonary metastasis of melanoma and breast cancer cells in mice [95,96]. Moreover, bisphosphonates that suppress CXCR-4 expression were also able to suppress tumor invasion both *in vitro* and *in vivo* [97,98].

Recent pioneering success in the treatment of people with B-cell lymphomas using a chimeric murine/human monoclonal antibody rituximab [99] ignited a surge of antibody-based strategies that target chemokine receptors. Rituximab binds with B-lymphocyte-restricted

differentiation antigen CD20, which is also expressed on the surface of most B-cell non-Hodgkin's lymphomas (NHL). To date, antibody therapy to a number of chemokines and their receptors is shown to be effective for the inhibition of both primary tumor growth and metastasis in SCID mice, such as anti-CXCL8 antibody against human NSCLC [57,58]; or anti-CXCR4 antibody against NHL [98]. Similarly with rituximab, anti-CCR4 antibody induced death of CCR4-expressing tumors in mice utilizing antibody-dependent cell-mediated cytotoxicity [15,100]. Of note, the clinical potency of antibody-dependent cell-mediated cytotoxicity can be affected by the host Fc receptor (FcR) polymorphism [101], as killing of the CCR4-expressing cells was not uniform *in vitro* [102]. Taken together, unlike traditional anticancer drugs, strategies that target chemokine receptors are not only less toxic, but also more specific and potent. Even inhibitors with broader specificity such as histone deacetylase inhibitors that downregulate *CXCR4* gene expression seem to be nontoxic both *in vitro* and Phase I clinical trials for acute lymphoblastic leukemia [103]. In addition, targets, such as CXCR4, promote additional benefits inhibiting phosphorylation and transactivation of HER2-*neu* [88] that, in turn, can downregulate expression of CXCR4 and lead to reduced metastasis and tumor growth [89].

Chemokines & tumor escape from immune surveillance

Aberrant expression of chemokines, particularly CCL2, CCL17, CCL22 and CXCL1–3, and CXCL5, CXCL6, CXCL8 and CXCL12, is often associated with a poor disease prognosis for cancer patients [1,48,49]. A number of chemokines activate chemokine receptors to signal, via two major pathways, the phosphoinositide 3-kinase (PI3K)/Akt and the extracellular signal-regulated (ERK)1/2 kinases [104–106], promoting prosurvival and antiapoptotic signals in various cells, including tumors [107,108]. For example, MIP-1γ/CCL9 has been associated with activation of nuclear factor (NF) κB ligand-induced osteoclast differentiation and survival [109], and CXCL8 with enhancement of proliferation and survival of CXCR1- and CXCR2-expressing endothelial cells [65,47,110]. Recently characterized novel chemokine receptor CXCR7 is shown to provide survival advantages and increased adhesion upon encounter with CXCL12 and CXCL11 [111]. CXCR7-expressing cells expanded more rapidly and were able to grow in suboptimal and serum-deprived tissue culture conditions. In this respect, it is tempting to speculate that an unfavorable disease outcome in patients with adult T-cell leukemia/lymphoma (ATLL), mycosis fungoides and Sézary syndrome associated with CCR4 may be due to the prosurvival and antiapoptotic effects of CCL17 and CCL22 [14,15,112]. Thus, tumors expressing the following chemokine receptors may acquire a higher survival and resistance to apoptosis: CCR1, CCR4 and CCR7, and CXCR1, CXCR2, CXCR4, CXCR5 and CXCR7. At least, their expression is correlated with a poor disease outcome in patients with primary melanoma colorectal cancer, head-and-neck cancer, and Hodgkin lymphoma and NHL [1,77,91,107].

On the other hand, tumors appear to utilize the same machinery as normal immune cells do to evade 'detection' or to eliminate abnormal and malignant cells. The evasion of host immune responses was originally documented for viruses, but it is now clear that it plays a significant role in tumor escape [113]. In this process, chemokines regulate recruitment of so-called tumor infiltrating CXCR3- or CCR4-expressing effector $CD8^+$ and $CD4^+$ T cells, leading to overall improvement of survival of patients with stage III melanoma [66]. However, aberrant expression of these chemokines may lead to immunosuppression and tumor progression. For example, overexpression of CXCL9 and CXCL10 in hepatocellular carcinoma (HCC) is shown to down-regulate CXCR3 on $CD4^+$ and $CD8^+$ T cells, leading to reduced T-cell infiltration and impaired host antitumor defenses [114]. Although chemokines are not usually cytotoxic *in vitro*, they may acquire proapoptotic properties after association with GAGs as shown for the RANTES-induced apoptosis of CCR5-expressing T-cell lines (so far only *in vitro*) [37]. Moreover, an excessive production of CXCL9, 10 and CXCL11 can be chemorepulsive for

DCs, monocytes and neutrophils, instead of chemotaxis [115,116]. Murine B16 melanoma increases its survival by overexpression of CXCL12 that repelled tumor-specific T cells [117]. Tumors also downregulate expression of chemokines that recruit APCs and iDCs, severely damaging their mobility and ability to induce antitumor immune responses [118]. For example, a loss of breast-and kidney-expressed chemokine (BRAK/CXCL14) is associated with reduced infiltration of iDCs and enhanced progression of a number of cancers, including prostate and head-and-neck squamous cell carcinoma [119–121]. However, even if antitumor cytolytic cells manage to infiltrate tumors, their activity can be modulated by the suppressive microenvironment generated by regulatory cells, such as macrophages and suppressive leukocytes, specifically Tregs, recruited to chemokine-expressing tumors. Chemokines participate in Th cell polarization [122]; and importance of activation of Th1 $CD4^+$ cells for induction of long-lasting effector and memory $CD8^+$ T cells that inhibit AIDS progression, eradicate intracellular pathogens or cancer cells has been documented widely [123–126]. However, Th1 responses can be suppressed by overexpression of CCL2 via induction of IL-4-mediated Th2-type cytokine polarization [127], which leads to inhibition of cellular antitumor responses [128,129].

Activity of naïve and effector $CD4^+$ and $CD8^+$ cells, or even DCs, can also be suppressed directly by Tregs via a cell-contact-mediated process [130–132]. Although Tregs comprise 5–10% of all $CD4^+$ T cells, they play a central role in regulation of self-tolerance, such as maternal tolerance to the fetus, autoimmunity and tumor survival [133]. Dysfunction or depletion of Tregs leads to spontaneous onset of various immune or autoimmune disorders, such as organ-specific autoimmune diseases, both in mice and humans [134–136]. Tregs have also been associated with a poor disease outcome in patients with a variety of malignancies [22,68,137, 138]. Migration and homing of Tregs are controlled by differential expression of chemokine receptors. For example, natural naive Tregs express CCR7 [139] to presumably home to secondary lymphoid organs [140]; while CXCR4 promotes retention of Tregs in bone marrow [141] and CCR6 in the recruitment of 'T regulatory effector/memory-like cells' into the skin [142]. Similarly, CCR4 and 8 are associated with the skin-homing of Tregs [22,68,69,143]. A recent comparison between CCR4-positive and -negative cases in 103 patients with ATLL indicated that, even in the absence of significant clinical characteristics at the time of diagnosis, expression of CCR4 was associated with skin homing. Moreover, an unfavorable disease outcome in ovarian carcinoma and ATLL [144] can also be explained by infiltration of Tregs towards high levels of CCL17 and CCL22 produced at tumor sites [13,14,22,136,145]. In addition, $CD4^+$ T cells recruited at tumor sites are shown to stimulate secretion of chemokines, leading to infiltration and accumulation of neoplastic cells [146]. Taken together, it is tempting to speculate that strategies that target CCL17 and CCL22 or their receptor CCR4 would enable control of the outcome of the disease acting at multiple steps: directly affecting survival and proliferation of tumors and indirectly controlling Tregs and improving antitumor cellular immune responses.

Tumor therapy using chemokines is an attractive but colossal task

The importance of chemokines in antitumor immune responses was demonstrated almost 25 years ago in the pioneering study of Luster and Leder who directly associated tumor eradication with IP-10-mediated recruitment of lymphocytes, neutrophils and monocytes [147]. Since then, a variety of chemokine-based immunotherapeutic strategies have been proposed to combat tumors (FIGURE 1). However, the majority were not translated successfully to the clinic, despite their obvious antitumor potency in inhibition of tumor-mediated neovascularization and metastasis. This is presumably because chemokines and their receptors are also expressed on normal immune cells. Thus, although tumor growth and metastasis can be blocked by targeting their chemokine receptors, normal immune cells, which are required for the clearance of residual tumor cells, would also be affected if they express the same receptors. The

importance of antitumor adaptive immune responses in the successful combat of the residual disease preventing relapse is well established. However, most of the reports do not evaluate this issue, since they used SCID mice that lack functional T cells. In addition, the efficacy of the treatments may be affected by the stability of the formulations in the patient's sera or in the tumor microenvironment. Tumors are shown to overexpress cathepsin-D that specifically cleaves a number of chemokines [148]. Moreover, chemokine activity is regulated by various peptidases, including CD26/dipeptidyl peptidase-IV, which degrade or modify their N- or C-terminal residues [149–151]. In this respect, some tumors, such as Sézary syndrome, cutaneous T-cell lymphoma cells downregulate their CD26/dipeptidyl peptidase-IV expression to enable migration to CXCL12 [149]. Nevertheless, the strategies that target chemokines or their receptors to reduce tumor burden and metastasis attract significant interest as a single treatment modality or in combination with other approaches. The anti-breast cancer effects of neutralizing CXCL8 antibody can be further enhanced in SCID mice when it was combined with anti-epidermal growth factor receptor (EGFR) antibody [152].

Taken together, tumor growth, angiogenesis and metastasis can be inhibited successfully by targeting chemokines and chemokine receptors, leading to reduction of disease severity. For example, since multiple myelomas secrete CCL3 that activates osteoclast stimulating factor [153] and RANKL-mediated bone destruction [154], inhibition of CCL3 was able to prevent bone loss in SCID mice, in addition to the reduction of tumor burden [155]. However, antichemokine or chemokine receptor treatments may suppress normal immune cells, specifically a small precursor pool of antitumor T cells present in cancer patients, thus hampering their beneficial and important therapeutic roles. Furthermore, the treatment will not be effective against tumors that do not express chemokine receptors. Although lung metastasis of CXCR3-expressing mammary tumors was inhibited by treatment with a small-molecular-weight CXCR3 antagonist AMG487, it did not affect local tumor growth and overall survival of mice [156]. It is tempting to speculate that the efficacy of the strategies discussed above would be impartial, unless they also induce robust adaptive anti-tumor immune responses. The task is usually assigned to the 'professional' cytolytic T cells working in concert with NK cells.

Chemokine–based cancer vaccines

A primary focus of cancer vaccines is to elicit tumor-specific adaptive immune responses, in particular to activate $CD8^+$ cytotoxic T lymphocytes and Th1 $CD4^+$ cells [125,126]. Cancer vaccines are therapeutic formulations designed to be most effective for the combat of a minimal residual disease, once the bulk of the tumor was reduced by other treatment modalities. However, they may also work as a prophylactic vaccine similarly to the human papillomavirus (HPV) vaccine that protects young women against oncogenic HPV-induced cervical infection and dysplasia [157]. Since human tumors are weakly immunogenic, the major issue is to render them immunogenic. In this respect, chemokines are also a useful tool to induce and modify tumor microenvironment and to promote antitumor adaptive immune responses. They are utilized widely to induce tumor-specific cellular immunity in mice, for example, by transfecting tumors with CXCL12 to recruit DCs [158]. However, chemokines should be used cautiously, since they may also lead to opposite results despite recruitment of DCs. For example, although murine lung carcinomas transduced with CCL22 were able to recruit DCs, NK cells and T cells, resulting in tumor regression [159], others reported only marginal protective effects of CCL17- or CCL22-transduced tumors [160]. In our hands, CCL22 was a very potent Th2-type chemokine that could not facilitate induction of antigen-specific $CD8^+$ T cells [20,161]. This is presumably owing to the fact that CCL22 can attract CCR4-expressing Tregs that suppress antitumor T-cell activity in human ovarian cancers and T-cell leukemia [13,14,68]. CCL3 is also a potent recruiter and activator of DCs [162], but it failed to reduce tumorigenicity CCL3-expressing cells in mice although it significantly increased recruitment of macrophages and neutrophils [163]. These results could also be explained by the recruitment of CCR5-expressing

Tregs [164]. Thus, to improve the potency of DC-based vaccines, others proposed to block the activity of the host CCR5, as the vaccine only worked in CCR5-knockout mice [165]. Taken together, chemokines can be used specifically to recruit iDCs and other APCs, such as macrophages, to improve their natural 'sampling' process, but their 'improper' use in a 'wrong' context would lead to immunosuppression through recruitment of various suppressive cells, such as macrophages, NK cells, NKT cells, Tregs and even iDCs [130–132,166].

Activated macrophages and professional APCs, including DCs, elicit tailored immune responses to pathogens and tissue abnormalities, controlling recruitment of Th1/2 polarized T cells via differential expression of chemokines. For example, myeloid DCs secrete CCL17 and CCL22 constitutively, while plasmacytoid DCs do not express them and, instead, produce CCL3 [167]. Thus, the two DCs can differentially recruit CCR4- or CCL5-expressing Tregs and Th2-type cells. This, in turn, may affect the activity of DCs by keeping them suppressed via contact-mediated processes with Tregs, or immunosuppressive cytokines, such as TGF-β and IL-10 and IL-13. The suppression affects an entire local area, including M2-skewed polarization of macrophages capable of downregulating Th1-type $CD4^+$ T-cell and $CD8^+$ T-cell responses [166]. It appears that the suppressive state of cells can be reversed, for example, by 'danger signals', which was postulated as a requirement for optimal recognition of self-tumor antigens and induction of proinflammatory rather than tolerogenic responses [168, 169]. This induces DC maturation upregulating expression of CD80 and CD86 costimulatory molecules and inducing production of various proinflammatory cytokines and chemokines [170].

Skin DCs and Langerhans' cells (LCs) constantly sample antigens and self-antigens as part of the normal immunosurveillance process, but do not induce neutralizing immune responses without activating stimuli to mature and express surface costimulatory molecules [171,172]. Tumor-associated antigens (TAAs) are taken up by iDCs and LCs to be processed into small peptides and presented utilizing MHC class I or II machinery. However, most extrinsic antigens are not efficiently taken up and presented to the MHC class I molecules (cross-presentation); instead, they are mostly delivered to the MHC class II-processing machinery. Although cross-presentation can be achieved by loading DCs with large quantities of extrinsic antigens (mg/ml) [173,174], skin DCs and LCs primarily deliver exogenous antigens to the MHC class II processing pathway, while cross-presentation was attributed to only $CD8^+$ DCs [175]. As a result, even if tumors express chemokines that recruit iDCs [93], their antigens may not be efficiently cross-presented to elicit cytolytic $CD8^+$ T cells. On the other hand, antigen presentation can be 100–10,000-fold increased if antigens are taken up using various endocytic cell-surface receptors [176,177]. Thus, we have hypothesized that presentation of weakly immunogenic tumor antigens can be enhanced when they are targeted to chemokine receptors, which may also promote necessary inflammatory conditions at the vaccination site by recruitment of various inflammatory cells [19]. As a result, otherwise nonimmunogenic tumor antigens were efficiently rendered immunogenic when used as a genetic fusion with chemokines, such as CCL7, CCL20 and CXCL10 [19,20]. In fact, we demonstrated that any chemokine, or even β-defensin (an antimicrobial peptide shown to bind CCR6 [178]), was suitable for this purpose as long as they acted via chemokine receptors expressed on iDCs (CCR1, CCR2, CCR5 and CCR6). The breadth of the strategy was apparent by its ability to elicit effector $CD4^+$ and $CD8^+$ T-cell-dependent antitumor immunity and eradication of established A20 lymphoma [19,20]. The response depended on the ability of chemokines to act as a carrier delivering fused TAA to chemokine receptors, indicating that the recruitment of APCs alone without antigen uptake is not sufficient. Although the mechanism of the carrier activity of inflammatory chemokines and defensins is not fully elucidated, we demonstrated recently that chemokines facilitated an efficient delivery of tumor antigens to MHC class I processing and presentation pathways (cross-presentation) [179]. The fate of the internalized chemokine-fused antigens is as follows: a certain proportion of them is degraded in endosomal/

lysosomal compartments (for subsequent presentation to MHC class II molecules) [180], while the rest escape to the cytosol where they are degraded by proteasomes to be cross-presented to MHC class I molecules. As a result, naive iDCs were able to stimulate both tumor-specific $CD8^+$ T cells and $CD4^+$ Th cells, despite being only 'loaded' with nmol (ng/ml) quantities of chemokine fused TAA. Although cross-presentation by DCs alone may induce tolerance in the absence of activation [172,181], it appears that necessary activating signals were properly engaged on DCs, as mice immunized with chemokine-fused with nonimmunogenic TAA elicited potent T-cell responses in the absence of adjuvants. Although chemokines are not known to induce DCs maturation directly, with the exception of murine β-defensin 2 [182], the chemokine constructs presumably stimulated APCs indirectly by recruitment of proinflammatory cells at the vaccination site.

Taken together, although utilization of chemokines for tumor therapy is a colossal task, our recent understanding of chemokine biology and their role in malignant diseases give rise to exciting hopes and novel ideas. Cancer is a multifactorial disease that requires combination of different strategies and, as such, it would require combination of various strategies. Success of chemokine-based strategies will also depend on their custom tailored use targeting specific stages and particular microenvironment of the disease. However, complete cancer cure may only be possible if the treatment also induces neutralizing adaptive immune responses. This can be achieved by utilizing chemokines as a carrier for TAAs. Tumor protection experiments in murine tumor models indicate that the strategy may have significant clinical value, specifically for eradication of human tumors on minimal residual disease.

Expert commentary & five-year view

The last few decades have been marked with a number of exciting findings that have deepened our understanding of the multistage carcinogenic processes. Identification of specific molecules involved in cancer contributed to the change of 'the dogma' for treatment of cancer, with the cytotoxic drugs used in chemotherapy to lose their dominance and to be used alongside targeted therapies, developed with the aim of targeting cancer cells more specifically. Thus, the identification of key molecules for cancer growth and progression represents an exciting and fast expanding area of cancer research. Numerous interesting strategies have been proposed, encouraged by preclinical and clinical results in recent successful treatments of hematological malignancies, utilizing approaches that exploit the therapeutic potential of tumor-specific antibodies and cellular immune effector mechanisms (vaccines). Overall, the concept has hardly left its preclinical testing stage and only a few are being tested successfully in clinics. For example, several promising immunotherapeutics have already reached the market, such as trastuzumab (Herceptin®), bevacizumab (Avastin®), ibritumomab (Zevalin®), tositumomab and iodine I-131 tositumomab (Bexxar®), Campath® and OncoVax®. Promising data are being reported on strategies that inhibit tumor angiogenesis. However, the next generation of treatments would probably be concentrated on their combined use with conventional therapeutic modalities. For example, bevacizumab, an anti VEGF-antibody used in association with chemotherapy, prolongs overall survival and progression-free survival in patients with metastatic colorectal cancer [183]. Efficacy of bevacizumab is also well known in human renal cell carcinoma [184]. Moreover, as underlined in this review, cancer growth and metastasis can also be controlled efficiently using chemotactic and immunomodulatory chemokines. Chemokines/chemokine receptors attract significant attention as anti-cancer strategies, since they are involved in almost every aspect of tumorigenesis and in the complex and multifactorial process of carcinogenesis. Chemokines are ideal targets for the inhibition of tumor angiogenesis, as ELR CXC chemokines exhibit angiogenic/angiostatic properties. The increased levels of IP-10/CCL10 detected in fresh specimens of human squamous cell carcinoma are inversely correlated with tumor growth rate [63,64]. To date, despite their obvious efficacy in the inhibition of tumor-mediated

neovascularization and metastasis, the chemokine-based approaches are yet to be translated to the clinic. In fact, this is a colossal task, as chemokines and chemokine receptors are also part of the normal functional immune system. It is tempting to speculate that this is presumably owing to the fact that 'passive' immunotherapy may not be fully effective unless it is accompanied with the induction of antitumor adaptive responses capable of clearing residual tumors. In this respect, the use of chemokines to render nonimmunogenic tumor antigens immunogenic by specifically delivering them to iDCs is potentially feasible, thus, attracting significant clinical interest for the development of vaccines against human cancers. Overall, recent advances in the therapeutic utilization of chemokines, fueled by seminal progress in the understanding of their biology and role in carcinogenesis, lead us to believe that chemokine-based strategies hold significant promise for combating cancer successfully.

Key issues

- Chemokines are a small group of related chemoattractant peptides that play a crucial role in the innate and adaptive immune responses.
- The chemokine network represents an essential part of the immunosurveillance system.
- Imbalanced and aberrant chemokine expression affects cancer progression and spread.
- Chemokines and chemokine receptors are an attractive target for cancer immunotherapy as they are involved in almost every aspect of tumorigenesis, including growth and metastatic spread of malignant cells, neovascularization and recruitment of immunosuppressive cells.
- cA variety of strategies targeting chemokines and chemokine receptors have been proposed, such as the use of pharmacological inhibitors, chemokine antagonists and specific antibodies.
- The task is colossal, as antichemokine or chemokine receptor treatments may also affect functions of normal immune cells, thus, hampering their potential therapeutic roles.
- The treatment strategy would be partial if it is not accompanied by induction of antitumor adaptive cellular immune responses capable of eradicating residual tumor cells and protecting from disease relapse.

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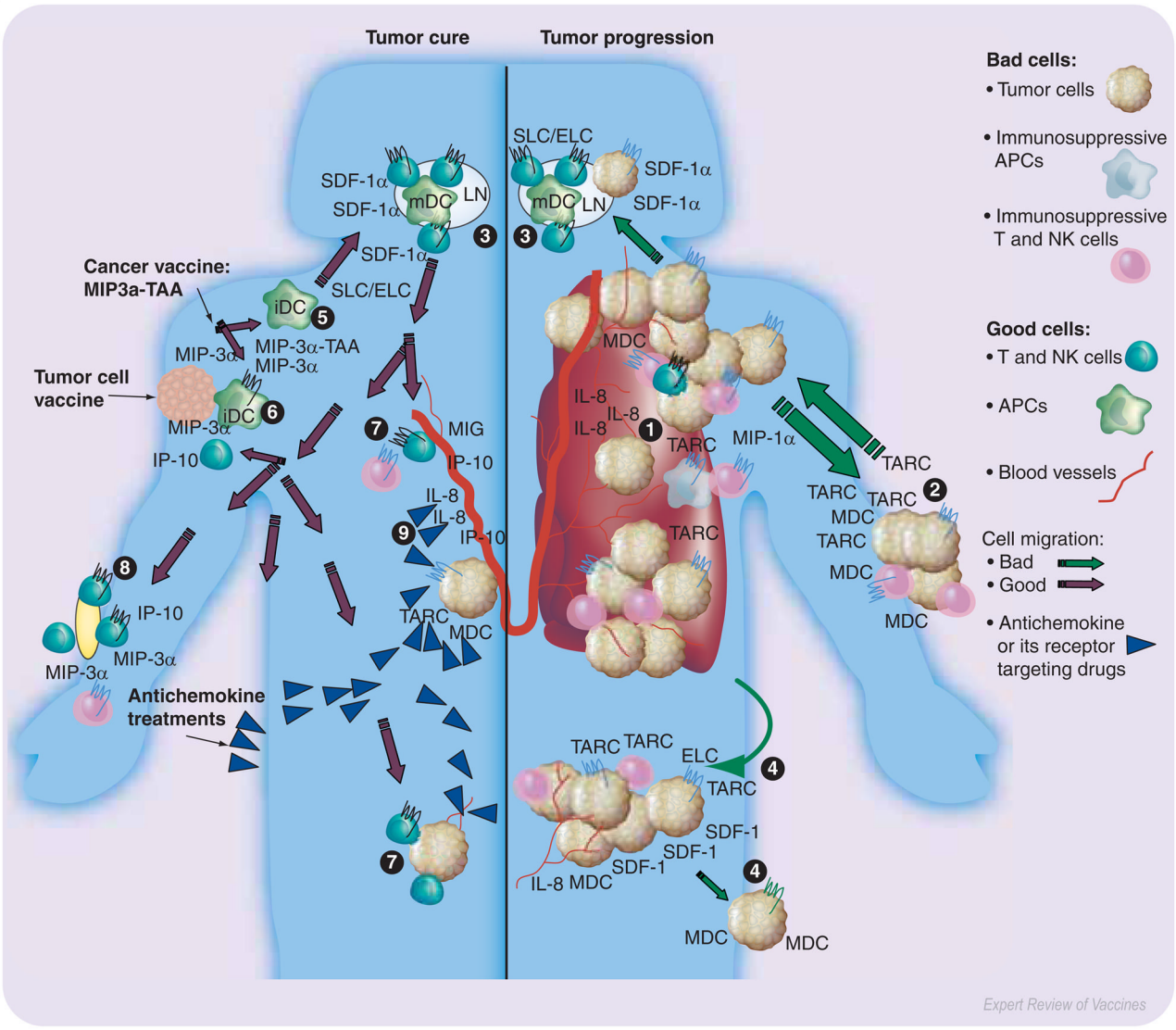

Figure 1. Chemokines are involved in almost every aspect of tumorigenesis and antitumor immunity

Primary tumors progress producing chemokines (IL-8/CXCL8, MIP-1α/CCL3, TARC/CCL17, MDC/CCL22) that promote neovascularization **(1)** and escape immunosurveillance via recruitment of immunosuppressive cells, such as T regulatory cells, NK T cells, macrophages and iDC. Tumors metastasize to distant organs, such as skin **(2)**, LN **(3)**, liver **(4)** and lung, that constitutively produce chemokines (SDF-1/CXCL12, ELC/CCL19 and SLC/CCL21; or TARC/CCL17 and MDC/CCL22). Therefore, to combat the disease and metastatic spread of tumor cells, use of various drugs and specific antibodies that target chemokines or their receptors were proposed, which inhibit neovascularization via expression of angiostatic chemokines (MIG/CXCL9 and IP-10/CXCL10, **9**) or depletion of angiogenic chemokines (CXCL8, **9**). However, these strategies cannot combat the disease efficiently, particularly relapse, unless they are accompanied with activation of antitumor adaptive immune responses. Immunizations with TAA fused with chemokines (MIP3α-TAA, **5**) or with tumor cells modified to express chemokines (MIP-3α/CCL20, IP-10/CXCL10, **6**) appear to be potent inducers of antitumor immunity. These vaccines recruit iDCs that take up and present TAAs leading to induction of tumor-specific T-cell responses in secondary lymphoid organs (LN,

3). The activated tumor-specific T cells can efficiently eradicate tumors in the skin **(8,2)** and other distant sites **(7,4)**. APC: Antigen-presenting cell; DC: Dendritic cell; ELC: EBI1-ligand chemokine; iDC: Immature dendritic cell; IL: Interleukin; IP: Interferon-γ inducible protein; LN: Lymph node; mDC: mature dendritic cellMDC: Monocyte-derived chemokine; MIG: Monokine induged by interferon-γ; MIP: Macrophage inflammatory protein; NK: Natural killer; SDF: Stromal cell-derived factor; SLC: Secondary lymphoid tissue chemokine; TAA: Tumor-associated antigen; TARC: Thymus and activation-regulated chemokine.

Table 1

Chemokines and tumour angiogenesis.

Chemokine	Chemokine receptor		Tumors involved	Ref.
	Promotes (+) angiogensis	Inhibits (–) angiogenesis		
CXCL8	CXCR1	+	Ovarian cancer, NSCLC, prostate cancer, glioblastoma, pancreatic cancer, gastric tumors, melanoma	[40,44,57, 61,185]
ENA-78 (CXCL5)	CXCR2	+	NSCLC	[44,58]
GRO-α (CXCL1)	CXCR2	+	Melanoma, gastric cancer	[44,59]
GRO-β (CXCL2)	CXCR2	+	Melanoma, esophageal cancer	[44,59]
GRO-γ (CXCL3)	CXCR2	+	Melanoma	[44,59]
I-309 (CCL1)	CCR8	+	-	[44,45]
Fractalkine (CX3CL1)	CX3CR1	+	NSCLC	[47,64]
IP-10 (CXCL10)	CXCR3b	-	Various cancers	[44,63]
MIG (CXCL9)	CXCR3b	-	-	[44]
ITAC (CXCL11)	CXCR3b	-	-	[44]
BRAK (CXCL14)	Receptor not known	-	Head and neck, prostate cancer	[121]

Chemokines affect tumor progression directly or indirectly via promoting neovascularization (angiogenic chemokines). However, some chemokines can inhibit angiogenesis (angiostatic chemokines). Only a representative list of chemokines is shown.

BRAK: Breast- and kidney-expressed chemokine; ENA: Epithelial neutrophil activating peptide; GRO: Growth-regulated oncogene; IP: Interferon-γ inducible protein; ITAC: Interferon-inducible T-cell α chemoattractant; MIG: Monokine induced by interferon-γ; NSCLC: Nonsmall-cell lung cancer.

Table 2

Chemokines and chemokine receptors in metastasis of solid tumors.

Primary tumor	Preferred metastasis site	Receptor	Ligand	Ref.
Melanoma	Melanocyte extravasation	CXCR1, CXCR2	CXCL8	[186,187]
	Lymph nodes, lung	CXCR3	IP-10, MIG, I-TAC	[188]
	Lymph nodes, liver	CXCR4	SDF-1	[2,189]
	Skin	CCR10	CTACK	[1,190]
	Lymph nodes	CCR7	SLC	[190]
Colon	Lymph nodes	CXCR4	SDF-1	[91]
	Lymph nodes, liver	CXCR5	BCA-1	[191]
	Lymph nodes	CCR7	SLC	[81,86]
	Liver	CCR6	MIP-3α	[81,192]
		CXCR1, CXCR2	CXCL8	[193]
Breast	Bone	CXCR1	CXCL8	[194]
		CXCR2	CXCL8	[195]
	Liver	CXCR4	SDF-1	[1,195]
	Brain	CX3CR1	CXCL8	[195]
	Pleural space	CCR6	MIP-3α	[195]
	Skin, lymph nodes	CCR7	SLC	[1,195]
	Lung	CXCR3	IP-10	[156]
Osteosarcoma	Lung	CXCR4	SDF-1	[2,196]
Pancreas	Liver, spleen, lymph nodes, stomach	CXCR5	BCA-1	[189,197]
	Lung, liver	CXCR4	SDF-1	[2]
	Liver	CCR6	MIP-3α	[82,192]
NSCLC	Pleural space	CXCR4	SDF-1	[198]
	Lymph nodes	CCR7	SLC	[199]
Nasopharyngeal	Lymph nodes	CXCR4	SDF-1	[200]
Prostate		CXCR1, CXCR2	CXCL8	[97,201–206]
Head and neck	Lymph nodes	CCR7	SLC and ELC	[83]

BCA: B cell-attracting chemokine; CTAK: Cutaneous T-cell attracting chemokine; ELC: EBI1-ligand chemokine; IP: Interferon-γ inducible protein; I-TAC: Interferon-inducible T cell α chemoattractant; MIG: Monokine induced by interferon-γ; NSCLC: Nonsmall-cell lung cancer; SDF: Stromal cell-derived factor; SLC: Secondary lymphoid tissue chemokine.