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Chemokines and their receptors: new insights in cancer inflammation

Gwendal Lazenec ^{1¶} and Ann Richmond ^{2,3}

¹ INSERM, U844, Site Saint Eloi - Bâtiment INM - 80 rue Augustin Fliche , Montpellier, F-34091, France ; University of Montpellier I, F-34090, France.

² Veterans Affairs Medical Center, and ³ Department of Cancer Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA.

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Abbreviations:

AR: androgen receptor, CAF: cancer associated fibroblast, CTC: circulating tumor cell, ECM: Extracellular matrix, ER: estrogen receptor, GAG: glycosaminoglycan, MSC: mesenchymal stromal cell, PCa: prostate cancer, TAM: tumor associated macrophage, TAN: tumor associated neutrophil

¶ Corresponding Author:

Dr Gwendal Lazenec
INSERM, U844,
Site Saint Eloi - 80, rue Augustin Fliche -
34295, Montpellier, France
Tel: (33) 4 99 63 60 27
Fax: (33) 4 99 63 60 20
E-mail: gwendal.lazenec@inserm.fr

ABSTRACT

Chemokines are involved in the interactions and tropism of cells in pathological situations frequently associated with inflammation. The purpose of this review is to update the recent progress in the roles of chemokines and chemokine receptors in carcinogenesis. In particular, increasing evidence suggest that chemokines are produced by tumor cells but also by the components of tumor microenvironment comprising cancer-associated fibroblasts, mesenchymal stem cells, endothelial cells, tumor-associated macrophages and more recently the tumor-associated neutrophils. In addition to their classical effects on proliferation, angiogenesis and metastasis, chemokines appear to modulate senescence and cell survival. The review discusses the changes in the general scheme of chemokine action in cancer that will serve to develop novel therapies in the future.

Introduction

Chemokines are chemotactic cytokines with a molecular mass of around 8 to 17 kDa, with the ability to bind G protein-coupled receptors (Box 1). Chemokines were originally identified as potent attractants for leukocytes such as neutrophils and monocytes, and therefore were generally regarded as mediators of acute and chronic inflammation (inflammatory chemokines). Later, several chemokines have been found to be constitutively expressed in lymphoid tissues. Moreover, leukocytes also express specific chemokines and their receptors. Accumulating evidence suggest that in addition to inflammation, chemokines are important regulators in development, homeostasis, and in physiopathological situations including osteoporosis ¹, obesity and insulin resistance ², viral infections ^{3, 4}, immune response ^{5, 6}, mobilization of progenitors to the bone marrow ⁷, and autoimmune encephalomyelitis ⁸.

More recently, chemokines and their receptors have been identified as mediators of chronic inflammation, which plays a key role in the initiation or progression of cancers of the lung, colon, liver, breast, cervix, prostate, bladder, ovary, esophagus, skin and lymphatics ⁹⁻¹². Tumor growth and dissemination is the result of dynamic interactions between tumor cells themselves, but also with components of the tumor environment. In this regard, chemokines are now emerging as key mediators not only in the homing of cancer cells to metastatic sites but also in terms of recruitment of a number of different types of cells to the tumor microenvironment. This includes infiltrating cells such as tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs) and lymphocytes, cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs) and endothelial cells.

There are now extensive reports that cancer cells express chemokine receptors which mediate metastasis to target organs expressing chemokines for the chemokine receptors expressed on the

tumor cells. This review will focus on the recent concept that chemokines are produced by epithelial cancer cells themselves, leading to the recruitment of TAMs, TANs, lymphocytes, CAFs, MSCs, and endothelial cells into the tumor microenvironment. These infiltrating cells will provide a secondary source of chemokines that will affect tumor growth, cell survival, senescence, angiogenesis, and metastasis. These novel findings provide a rationale for developing therapies that targeting chemokines as well as their receptors.

Source of chemokines and chemokine receptors in the tumor

Early work has shown that cancer cells from a variety of types of solid cancers were expressing higher levels of the chemokine receptors CXCR4, CCR7, CCR9 and CCR10¹¹⁻¹³ (Table 1). This could thus define the metastatic tropism of each type of cancer, depending on the receptor present at the surface of cancer cells and on the chemokines produced by the sites of metastasis. Indeed, the ligand of CXCR4, CXCL12, is expressed at high levels in various organs, including the lung, liver, and lymph nodes, which are frequently involved in tumor metastases. In a similar manner, CCL21, the ligand of CCR7 is produced by lymph nodes; CCL27, the ligand of CCR10 is secreted by the skin¹⁴. The picture became more complex when increasing evidence revealed that cancer epithelial cells were producing higher levels of a number of chemokines compared to normal epithelial cells and were also expressing high levels a series of chemokine receptors, to establish a tumor-promoting microenvironment, facilitating tumor-associated angiogenesis and metastasis. (Table 1). Release of these factors produces a 'cytokine storm' that amplifies the inflammatory response by recruiting additional inflammatory cells, including macrophages, neutrophils, and lymphocytes¹⁵. This is in particular the case of infiltrating leukocytes, bearing chemokine receptors such as CXCR1, 2 CCR2, 4 and 5, but also endothelial cells and CAFs (Table 1). These cells present in the stromal compartment of the tumor will constitute another

source of chemokines (Table 1), which will alter the growth, angiogenesis, metastasis and tumor microenvironment. We will detail in the following sections the recent advances in these topics.

Tumor growth, cell survival, senescence

Previous work has shown that CXCR4/CXCL12 constitutes one of the most efficient chemokine/chemokine receptor pairs in terms of enhancing cell growth¹¹⁻¹³. An intriguing finding was the recent discovery of CXCR7, a novel chemokine receptor that binds both CXCL12 and CXCL11^{16, 17}, two chemokines which frequently exhibit opposite roles. CXCL12 is reported to display angiogenic properties, whereas CXCL11 is angiostatic and was originally discovered as a ligand for CXCR3. Surprisingly, despite high affinity binding of CXCL11 and CXCL12 to CXCR7 and internalization of CXCR7 upon CXCL12 binding, CXCR7 does not appear to induce intracellular signals and does not induce cell migration^{16, 17}. CXCR7 is expressed by tumor epithelial cells and endothelial cells and may serve as a ‘decoy receptor’ by competing with CXCR4 for CXCL12¹⁸. Despite these features, stable expression of CXCR7 in breast cancer cells increases the survival of breast cancer cells *in vitro*, without affecting their *in vitro* proliferation¹⁷. CXCR7 also stimulates cell adhesion. On the other hand, the CXCR7 antagonist CCX754 reduces tumor growth¹⁷. Furthermore, CXCR7 knock-down in breast or lung cancer cells reduces both tumor growth and lung metastasis¹⁹. However, it might not be possible to generalize these observations to all types of cancer cells, as data from other cell lines indicated that the proliferative effects of CXCL12 were mediated by CXCR7 and there was no reported effect on *in vivo* tumor growth when knocking down CXCR7²⁰. So, at the present stage, the role of CXCR7 in cancer remains controversial and one can say that if confirmed by other studies, the action of CXCR7 on cell growth and survival relies on non-conventional chemokine receptor signaling.

The role of another chemokine receptor, CXCR2, the receptor for chemokines CXCL1, 2, 3, 5, 6, 7 and 8, has been recently reevaluated. CXCR2 had been mainly studied in light of its roles in angiogenesis, proliferation and invasion. But, progression to malignancy could be also correlated to an impaired senescence, with a loss of the limit of proliferative life span. Senescence, which is mostly determined by the shortening of the telomere ends of chromosomes, can be triggered by a variety of signals including DNA damage and cellular stress. In this regard, Acosta and colleagues have reported that knock-out of the gene encoding CXCR2 in fibroblasts alleviates both replicative and oncogene-induced senescence²¹ and reduces the DNA damage response, as shown by an increase of the levels of the active phosphorylated form of ataxia telangiectasia mutated (ATM). Induction of senescence in wild-type cells leads to an increased expression of a number of chemokines including all CXCR2-binding ligands (CXCL1, 2, 3, 5, 6, 7, 8) and also CCL2, CCL13, CCL20²¹. *In vitro* experiments showed that NF- κ B and CEBP activation triggered by oncogene-induced senescence are responsible for the higher expression of CXCR2 binding ligands. Interestingly, the expression of CXCR2 is increased in prostate intraepithelial neoplasia (PIN) and prostate cancer compared with normal prostate²¹. In breast cancer, similar observations were obtained with a coordinated increase of the levels of CXCR2 ligands in cancer tissues, and also in estrogen receptor (ER)-negative breast cancers compared with ER-positive breast cancers^{11, 22, 23}. This might be explained by the fact that the genes encoding these chemokines are present in the same narrow cluster on chromosome 4q13. So, CXCR2 ligands must certainly have different properties depending on the stage of the disease. In early tumorigenesis, CXCR2 ligands function as gatekeeper of tumor growth by increasing senescence. When the disease progresses to a malignant state, chemokines might be ineffective to enhance cell death as mutations have occurred in neoplastic cells that disable senescence. In addition, CXCR2 ligands will affect the microenvironment and generate a favorable soil for tumor growth.

In addition to direct effects on cell growth, it is possible that chemokines also modulate cell survival. Indeed, CCL2 protects prostate cancer cells from autophagic death in conditions of serum starvation, at least in part by delaying the decay of survivin levels, a member of the inhibitor of apoptosis (IAP) family of antiapoptotic proteins. This protection is alleviated by the PI3K inhibitor LY294002. CCL2 can also partially counteract autophagic death induced by the immunosuppressant rapamycin²⁴.

What emerges from the recent literature is the role of a novel chemokine receptor, CXCR7, in cell proliferation and also the reinvestigation of the roles of CXCR2 and CCL2, which are also potent modulators of senescence and cell survival. But tumor growth cannot take place indefinitely without a strong support in terms of nutrients. Blood vessel formation appears as an essential step in tumor development, once the tumor has reached a certain size.

Tumor angiogenesis

Blood vessel density is correlated with a higher incidence of metastasis and a more rapid recurrence of the disease^{25, 26}. Most of the chemokines described as promoters of tumor angiogenesis are CXCR2 ligands, namely CXCL1, CXCL2 and CXCL8²⁷. The recent advances in the roles of chemokines in angiogenesis have focused on the understanding of the signals controlling the expression of previously known pro-angiogenic chemokines or chemokine receptors and the discovery of novel chemokine or chemokine receptors modulating angiogenesis.

Concerning previously identified chemokines and chemokine receptors, a novel link between chemokines and Prostaglandin E2 (PGE2) has been discovered. PGE2 is involved in chronic inflammation and in the promotion of colon cancer. In addition, CXCL1 and its receptor CXCR2 are overexpressed in colorectal tumors and adenomas from Apc^{Min} transgenic mice (a model of colorectal cancer)²⁸. Xenograft experiments showed that PGE2 increases tumor growth through

an increased angiogenesis²⁸. This occurs through an induction of CXCL1 expression by PGE2 in a MAPK-dependent manner that favors endothelial cell migration and tube formation *in vitro*. Blocking its action *in vivo* with specific antibodies can counteract tumor growth enhancement by PGE2²⁸. In the same line, Chan and colleagues have reported that prolylhydroxylase (PHD)2 anti-angiogenic properties were linked to the down-regulation of CXCL8²⁹. Indeed, PHD2 levels were lower in colon adenocarcinoma compared to normal adjacent colon tissue²⁹. Moreover, knocking-down PHD2 in colon cells increases tumor growth and angiogenesis. The effects of the down-regulation of PHD2 are mediated by an increased NF-κB activity as well as by the induction of CXCL8 and angiogenin²⁹.

In the search of less conventional chemokines and chemokine receptors involved in angiogenesis, recent attention has been directed to chemokine decoy receptors such as D6 and DARC, which are involved in post-inflammation clearance of chemokines³⁰. These decoy receptors bind a number of chemokines, without enabling conventional cell signaling and migration (Fig. 1) and thus inhibit chemokine action through conventional receptors. So far, decoy receptors had been studied in a limited manner, especially with regard to their role in cancer. D6 had been previously shown to reduce CCL chemokine recruitment in a mouse model of skin inflammation³¹. The use of knock-out mice for D6 has provided evidence that this receptor could control susceptibility to cutaneous tumors induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) in mice treated with 7,12-dimethylbenz(a)anthracene (DMBA)³². On the other hand, transgenic expression of D6 in the epidermis suppresses papilloma formation^{32, 33}. D6 overexpression in the skin is associated with a decreased number of epidermal CD3+ T cells and mast cells, compared to wild-type mice in TPA treated animals, which prevents tumor development.

In the model of carcinogen induced colitis associated cancer, D6 has also shown a protective role ³⁴. Crohn's disease and ulcerative colitis are the major forms of inflammatory bowel disease (IBD). Several studies have reported a possible link between IBD and increased risk of colon cancer ³⁵. In the experimental model of dextran sulfate sodium (DSS) induced IBD, D6 expression is increased in the colonic mucosa ³⁴. In addition, D6 Ko animals show an increased susceptibility to colitis ³⁴. Moreover, when colitis associated cancer was triggered by administration of the carcinogen azoxymethane to DSS treated mice, the incidence of the severity of colon tumors increased in D6 KO animals compared to wild-type animals ³⁴. This is concomitant with an increased leukocyte infiltration composed of CD3+ T cells, macrophages, dendritic and B cells in the cancer mucosa of D6 KO compared to wild-type animals, that sustains tumor growth ³⁴.

DARC, another decoy receptor, has been shown to modulate angiogenesis in breast and prostate cancer. DARC expression is inversely correlated to microvessel density, lymph node status and distant metastasis. The overexpression of DARC in metastatic breast cancer cells inhibits tumor growth, probably through inhibition of angiogenesis ³⁶. In the TRAMP transgenic model of prostate cancer, breeding of TRAMP mice that are null for DARC exhibit increased tumor growth and vascularization compared to TRAMP mice that express DARC ³⁷, probably due to a defect in the clearing of angiogenic chemokines in DARC null TRAMP mice.

With the recent advances in the involvement of chemokines in angiogenesis, a new picture arises with a double face, creating an impaired balance of pro- and anti-angiogenic factors between normal and cancer tissues. Indeed, not only pro-angiogenic chemokine levels increase for which a few tracks of the upstream signals for their expression have been studied; but in the meantime, there is also a decrease in the levels of decoy chemokine receptors, further favoring an angiogenic switch. This in turn will also constitute a favorable environment for further

progression of the disease, with an increased possibility for cancer cells to migrate away from the primary tumor site, reach blood vessels and metastasize to distant organs.

Tumor metastasis

Early work in the chemokine and cancer field had essentially focused its attention to the gradient of chemokines produced by the sites of metastasis, which in turn would attract cancer cells to these locations. This was important to explain why different cancers show distinct tropism for metastatic sites. Classically, this is the case for the pairs CXCR4/CXCL12 involved in bone metastasis, CCL19-CCL21/CCR7 involved in lymph node metastasis and CCL27/CCR10 involved in skin metastasis ¹⁴. We will now expand this biological role for chemokines in metastasis through identification of novel factors that further refine the understanding of metastasis tropism.

Recently, the case of pancreatic ductal adenocarcinoma (PDA) has high-lighted the role of another receptor, CX3CR1, in metastasis. PDA cells have the particularity of infiltrating intrapancreatic and extrapancreatic nerves. Interestingly, Marchesi and colleagues reported that PDA cells express high levels of CX3CR1 and migrate towards a gradient of its ligand, CX3CL1, produced by neurons and nerve fibers ³⁸. Clinical studies show also that CCR9-expressing human melanomas have a very high probability of metastasizing to the small intestine ³⁹. CCR9 is thought to be a receptor that “homes” melanoma metastasis to the small bowel. CCL25, the ligand for CCR9 is highly expressed in small bowel and thymus ³⁹. Studies with CXCR2 knock-out mice provide also evidence that CXCR2 expression on non-melanoma cells is important for melanoma metastasis to the lung ⁴⁰. By comparing gene signatures of breast cancer cell lines with weak or strong metastatic tropism for the lung, Massagué and colleagues have remarkably shown that chemokine CXCL1, one of the ligands for CXCR2, is one of the gene products promoting lung metastasis ⁴¹.

The involvement of CCL19-CCL21/CCR7 in metastasis has also been revisited recently to uncover why CCR7 is overexpressed in cancer cells. These studies also more surprisingly showed that the sites of metastasis were not the only producers of CCL19. Indeed, T-cell acute leukemia (T-ALL), a blood malignancy, shows a high risk of relapse to central nervous system (CNS). T-ALL tropism for CNS involves activation of Notch1, as shown by the use of a model of transplantation hematopoietic progenitors expressing the oncogenic intracellular Notch1 fragment (Notch1-IC) or transgenics for Notch1-IC⁴². In turn, Notch1 up-regulates CCR7 levels. Silencing of CCR7 or of its ligand, CCL19, is sufficient to inhibit CNS metastasis⁴². CCR7 is also involved in lymph node metastasis, the main route of dissemination for many cancers. Previously, the classical dogma had suggested that CCL19 and CCL21, the ligands of CCR7, are produced by lymphatics vessels and in turn attract CCR7-bearing tumor cells¹⁴. Recent findings, however, suggest that the tumor cells themselves generate a gradient of CCL19/CCL21 chemokines, which creates a continuous circle of recruitment⁴³. Production of CCR7 ligands by tumor cells is observed when cells are under the influence of slow interstitial flow, towards draining lymphatics. *In vitro* chemotaxis assays demonstrate that cancer cells migrate towards lymphatic endothelia in a CCR7-dependent manner and this is enhanced in flow conditions⁴³. These data further question the concept of the sole production of chemokines by the sites of metastasis and raise the possibility that cancers are actively promoting their own metastasis and tropism. One can envision that chemokines produced by cancer cells will affect the overall expression of surface molecules such as integrins or selectins⁴⁴ that in turn will control the rolling capacity of cancer cells and enable the extravasation to specific organs.

Though the identification of an involvement of chemokines and chemokine receptors in metastasis tropism offered a plausible explanation for site specific metastasis, it was still difficult to understand how cancer cells could survive the stress of leaving the primary tumor and

remaining for a long time in an unfavorable soil. Further progress has been made by the group of Massagué to elucidate the mechanisms responsible for latent bone metastasis arising in breast cancer. Indeed, depending on the type of cancer, metastasis can occur rapidly after the onset of tumor development or several years after the initial tumor arises. This supposes that cancer cells need to remain in a dormant state or to form indolent micrometastases during this period. They have shown that latent bone relapse of estrogen receptor (ER)-negative breast cancers is strongly associated with a Src-responsive signature⁴⁵. Src is not involved in the primary growth of such tumors or in their metastasis to the lung, but rather helps indolent breast cancer cells to survive in the bone marrow. These effects are mediated by CXCL12, which is regulated by Src and displays a higher expression in bone metastasis compared with other sites of metastasis. CXCL12 is able to increase the survival and resistance to TRAIL death signals by up-regulating the Akt/PKB pathway⁴⁵. Overall, these data provide an explanation for the mechanisms enabling dormant cancer cells to survive for a long period in the bone marrow during the latent metastasis period and could constitute another level of targeting to prevent dormant cells from being a source of future metastasis in the bone marrow. In the same line, the escape of tumor cells from the primary tumor is a stressful process for cancer cells, which requires leaving a favorable environment. Many cells won't survive this step and will undergo anoikis (detachment-induced cell death). Recent work suggests that CCR7 and CXCR4 could prevent anoikis, through the down-regulation of the pro-apoptotic Bcl2 modifying factor (Bmf)⁴⁶. In turn, overexpression of Bmf in cancer cells alleviates *in vitro* anoikis prevention by CXCL12 and CCL21 (the ligands of CXCR4 and CCR7 respectively) and *in vivo* reduces lung metastasis of breast cancer cells in a xenograft mouse model. This gives novel indications on the pathways that use cancer cells to survive the metastatic switch, where chemokines have also a role to play.

Cancer patients usually die from metastatic dissemination as opposed to the growth of the primary tumor. If one wants to reduce cancer mortality, it thus appears that a better understanding of the chemokines and chemokine receptors triggering not only overall metastasis, but also site specific metastasis, is essential. In addition to the previously described set of chemokines and chemokine receptors involved in tumor growth and metastasis, novel players are also dictating metastatic tropism. These novel chemokines/chemokine receptors could constitute further targets. In addition, chemokines appear to be involved in the survival of cancer cells when they escape the primary tumor as well as when they remain dormant or as indolent micrometastases, which will represent novel therapeutic opportunities. Upstream of the metastasis step, numerous reports suggest that cancer cells will modify tumor microenvironment, by affecting stromal cells properties or recruiting different types of cells. Due to the complexity of such findings, the following section has been dedicated to these aspects.

Role of the tumor microenvironment

Cancer cells participate in the creation of a favorable micro-environment by interacting with stromal cells and triggering the homing of a variety of cells to the tumor site. Among the cells which are affected by cancer cells, CAFs, which can have both a fibroblastic or mesenchymal stromal cell (MSC) origin^{47, 48}, are suspected to promote carcinogenesis. Weinberg's group nicely demonstrated that CAFs isolated from breast tumors secrete different types of chemokines including CXCL12, which in turn acts on cancer cells by promoting their proliferation, whereas normal fibroblasts isolated from a non cancerous region of the breast had only a moderate effect on tumor growth⁴⁹. CAFs promote tumor growth, through a direct stimulation of cancer cell proliferation and by increasing angiogenesis, through the recruitment of endothelial cells into carcinomas⁴⁹. Similarly, CXCL12, secreted by CAFs, stimulates the *in vivo* growth of benign prostate hyperplasia (BPH)⁵⁰. The action of CXCL12 occurs through CXCR4, which is

expressed by epithelial cells in BPH tissues. Moreover, the levels of CXCR4 increase in epithelial cells upon stimulation by the TGF β 1 that is produced by CAFs⁵⁰. However, it must be pointed out that CXCL12 overexpression by CAFs might not be generalized. Indeed, Crawford and colleagues have reported that CAFs isolated from EL4 (resistant to anti-VEGF treatment) or TB6 (sensitive to VEGF treatment) tumors express lower levels of CXCL12 than normal skin fibroblasts⁵¹. Moreover, angiogenesis triggered by EL4 CAFs was mediated by platelet-derived growth factor C (PDGF-C) and VEGF-A, PDGF-C increasing the migration of endothelial cells⁵¹. Still, CAFs definitely constitute a non-negligible source of chemokines, as CAFs isolated from breast cancer tumors or melanoma also produce significant levels of CXCL 1, 2, 3 and 8^{22, 52}. Recent work has also shown that MSCs that have overlapping properties with fibroblasts, constitute also a source of chemokines when they come in contact with breast cancer cells^{53, 54}. This occurs through the induction of the chemokine CCL5 by MSCs upon contact with breast cancer cells. Released CCL5 will then promote metastasis to the lung by acting on CCR5 present at the surface of tumor cells, without affecting significantly the development of the primary tumor⁵³.

In addition to the modification of stromal cells properties, cancer cells will also recruit circulating cells to the tumor. Colmone and colleagues have shown that leukemic cells have the ability to disturb the bone marrow environment and create novel niches⁵⁵. These niches, which are distinct from the osteoblastic niche, attract CD34+ hematopoietic progenitor cells (HPCs). The behavior of HPCs is altered as they migrate towards CXCL12-negative regions of the tumor, whereas in control mice, CD34+ cells migrate to CXCL12-positive vascular niches. The migration of HPCs is not dependent on chemokines but, rather, on stem cell factor (SCF) secreted by tumor cells, an HPC growth factor and chemoattractant believed to be involved in hematopoietic stem cell localization to endosteal niches⁵⁵. These data raise the possibility that leukemic cells could reorganize the bone marrow microenvironment, even though one cannot

exclude the possibility that the creation of a new niche represents a side effect of leukemia, that is not necessarily affecting tumor growth..

Among the different factors involved in cancer progression, TGF β has attracted a lot of attention and could also play a role in cell recruitment. Several studies have shown that loss of the TGF β response in breast cancer is linked to a poor prognosis ⁵⁶, even though this issue is controversial, as TGF- β could also switch from tumor suppressor to tumor promoter role, notably through the control of the expression of a number of chemokines, such as CXCL1, CXCL5 and CCL20 in cancer cells themselves ⁵⁷. But TGF β could also modulate the recruitment myeloid cells. In the model of colon cancer in which TGF β signaling is deficient (Apc^{+D1716} Smad4^{+/-}), an increased number of immature myeloid cells (iMC) expressing CCR1 is recruited from the bone marrow to the invasive front. iMC migrate towards a gradient of CCL9, one of the ligands of CCR1, which is produced in high amounts by tumor epithelium of the polyps in Apc^{+D1716} Smad4^{+/-} animals compared to Apc^{+D1716} Smad4^{+/-} animals ⁵⁸. These data support the idea that TGF- β signaling in tumor epithelial cells triggers the recruitment of imC cells expressing CCR1 that promote tumor invasion in early stages of intestinal adenocarcinomas.

Among circulating cells, exciting results come from the description of the newly identified tumor-associated neutrophils (TANs) and the cross-talk with TGF β , with a parallel to TAMs ⁵⁹, ⁶⁰. TAMs were defined as two populations: M2 macrophages promoting tumorigenesis, while M1 macrophages are thought to be more anti-tumorigenic ³⁰. Fridlender and colleagues report that, in a lung cancer model, TGF β blockade not only activates CD8+ T cells and macrophages but also increases the recruitment of hypersegmented neutrophils, their N1 polarization (high expression of TNF α , ICAM-1, FAS) and their anti-tumor activity ⁵⁹. By contrast, TGF β stimulation polarizes neutrophils to the so-called N2 state with an increased expression of arginase and chemokines such as CCL2 and CCL5. As defined with M1 and M2 TAMs, NI

TANs are tumor cytotoxic, whereas N2 TANs display pro-tumoral properties⁵⁹. N1 neutrophils produce T-cell-attracting chemokines including CCL3, CXCL9 and CXCL10. Accordingly, following TGF β blockade, depletion of neutrophils counteracts the anti-tumor properties of the TGF β R1 kinase inhibitor SM16 and reduces the activation of CD8+ T cells. TGF β inhibition leads to a shift to N1 neutrophils with anti-tumor activity and concomitant decreased expression of CCL2 and CCL5. On the other hand, in the presence of active TGF β signaling, neutrophil depletion leads to reduced growth and enhanced presence of CD8+ T cells⁵⁹. In the context of breast cancer and using TGF β R2 knock-out mice, Yang et al. have also reported that the abrogation of TGF β signaling increases the recruitment of Gr-1+CD11b+ myeloid cells to the tumor⁶¹. This recruitment of myeloid cells towards the tumor elevates TGF β 1 levels in tumor tissues of TGF β R2 KO animals. Very interestingly, tumor cells deficient for TGF β R2 produce high amounts of CXCL5, which in addition to CXCL12 present in the tumor microenvironment is responsible for myeloid cell recruitment. In turn, myeloid cells are located at the invasive front and, by producing high levels of metalloproteinases such as MMP2, MMP13 and MMP14 will promote tumor invasion⁶¹.

The chemokine CCL2 has also recently received a lot of attention concerning its involvement in the recruitment of infiltrating cells. Mice injected with the B16 melanoma cells transfected with a shRNA against CCL2 exhibit reduced malignant pleural effusions and enhanced survival, together with a reduced number of macrophages and blood monocytes compared with control shRNA transfectants⁶². By contrast, when human cytotoxic lymphocytes (CTLs) are adoptively transferred to nude mice with human melanoma xenografts that do not express CCL2, this leads to reduced T cell homing to the tumor. These data argue that CCL2 produced by tumor cells recruits CTLs to the tumor microenvironment to suppress tumor growth⁶³. This type of dichotomy in the role of chemokines on the immune system may be tightly linked to the tumor microenvironment. There will be different outcomes depending upon whether the T cells that are

recruited are capable of tumor cell killing, or whether they promote tumor metastasis through release of factors that facilitate intravasation of tumor cells into the vascular system. In the same line, CCL2 is a potent macrophage chemoattractant that is associated with the accumulation of associated macrophages and tumor stage⁶⁴. Depending upon whether the macrophages recruited are tumor progression protective (M1) or tumor progression promoting (M2), CRC progression may be enhanced or stimulated. Pro-tumorigenic M2 macrophages release CCL2, which can stimulate metalloproteinase production.

What emerges from the recent studies on microenvironment, is a reciprocal dialog between cancer cells and CAFs which is mediated by chemokines and a pivotal role of TGF β signaling in modulation of tumor microenvironment and cell recruitment; the creation of specific niches by cancer cells to attract circulating cells; the definition of TANs and their chemokine profiles, and the identification of novel chemokines such as CCL2 controlling leukocyte infiltration (Fig. 2). These data could also provide the basis for the novel targets of interest in therapeutic applications that we will discuss in the following section.

Therapeutic implications

Based on the early demonstration of the roles of chemokines and their receptors in tumor growth, angiogenesis, and metastasis, and upon the availability of drugs targeting these molecules in other diseases, several clinical trials have been launched (Table 2). Compared to clinical trials targeting chemokines or chemokine receptors for other diseases such as arthritis or asthma, the trials targeting chemokines or their receptors in cancer remain quite limited. These clinical trials have targeted mainly CXCR4, and at a lesser extent CCR4, CCR5 and CCL2⁶⁵⁻⁶⁹. The drugs used either were small molecule inhibitors, peptide antagonists or antibodies. Receptor antagonists correspond mainly to N-terminal truncations of the ligands that will impair

downstream signaling but not high affinity binding of the ligand. Small molecule inhibitors are also targeting the signaling of the receptor rather than blocking ligand binding. Most of these drugs were not specifically designed for cancer therapy but were originally developed for autoimmune and inflammatory diseases such as rheumatoid arthritis, psoriasis, multiple sclerosis and asthma ⁷⁰. It is not surprising to note that 4 different drugs were tested to target CXCR4, as this is one of the first chemokine receptors involved in metastasis. Based on the central role of CCR5 in the dialog between MSCs and cancer cells ⁵³, the targeting of this receptor is very interesting. This is reinforced by the fact that the CCR5 antagonist Maraviroc has been recently approved by the FDA for the treatment of HIV infected patients. So far, we are still waiting for the results of most of these trials.

The short terms novel approaches are likely to include drugs which have already been tested in preclinical settings. This will be the molecules which will antagonize protumorigenic chemokines, namely CXCR2 binding chemokines such as CXCL1 and CXCL8 which are involved in tumor growth, angiogenesis, metastasis and inflammatory infiltration. Humanized antibodies to CXCL8 (ABX-IL8) have been shown to inhibit melanoma tumor growth, angiogenesis and metastasis ⁷¹. Another very promising target is the angiogenic chemokine receptor, CXCR2, and antagonists for this receptor are under consideration for melanoma therapy. Schering Plough, AstraZeneca and Glaxo-Smith-Kline have developed CXCR2 antagonists that show some promise for cancer treatment ⁷². One can also envision that the CXCR7 antagonist CCX754 developed by Chemocentryx which inhibits tumor growth in mice ¹⁷ will also be tested in clinical trials. Another very attractive target is CCR9. As mentioned earlier, CCR9 is not only involved in IBD, but also in the metastasis of melanoma to small bowel ³⁹. Currently, clinical trials involving the CCR9 antagonist are in phase III for Crohn's disease ⁷³, which generates a lot of hope for cancer. Despite its involvement in lymph node and brain

metastasis, CCR7 has not received a lot attention, certainly by the fact, that there are currently no available drugs to target this receptor.

Conclusion

What are now the future tracks of development of the field? Chemokines are produced by metastatic sites and this could explain the tropism of cancer cells for specific organs, but they are also secreted by epithelial cells themselves and by stromal components such as fibroblasts, mesenchymal stem cells, inflammatory infiltrating cells or endothelial cells in the primary tumor (Fig. 2). Thus, multiple chemokines are certainly involved in all steps of tumor development including tumor cell proliferation and apoptosis, tumor angiogenesis, invasion of the peripheral tissues and specific homing to metastatic sites (Fig. 3). Future questions will address not only the fundamental involvement of chemokines and chemokine receptors at each particular stage of the cancer, but also the future therapy modalities. Due to the diversity of cancers, it will certainly not be possible to generalize these findings to all types of cancer. Moreover, one "black box" remains in the current scheme of action of chemokines and their receptors in cancer. Though we have some understanding of the roles chemokines and their receptors play at the the primary tumor site or at the metastatic sites, we have little information on the role of chemokines and their receptors between growth of the primary lesion and establishment of a secondary metastatic lesion. In particular, we can wonder what types of chemokines or chemokine receptor will influence circulating tumor cells (CTC), affect their rolling properties and enable or not cancer cells to seed at a particular sites. Moreover, the upstream signals that will dictate the elevels of expression of chemokines and their receptors and their turnover in cancer have only just begun to be understood. Much more effort is needed to clarify the role of chemokines/chemokine receptors in the intravasation, extravasation, and adaption of tumor cells to the metastatic niche and these events offer potential sites for therapeutic intervention.

Concerning therapy, the trials targeting chemokines and their receptors in cancer might face the same difficulties as the one targeting chemokines or chemokine receptors in autoimmune and inflammatory diseases. One obvious limitation is certainly linked to the inherent redundancy of chemokines and their receptors (Fig. 1). Most of the trials, which have failed so far, have targeted only one receptor ⁷⁴. An alternative to solve this problem might be polypharmacology, by the use of promiscuous drugs that target several receptors. This could use the similarities between some chemokine receptors such as CCR1 and CCR3 or CCR2 and CCR5 that show 59% and 72% of sequence identity, respectively. Such compounds have been developed, like TAK-652 from Takeda, which targets both CCR2 and CCR5 ⁷⁵. But one could also imagine antagonists for unrelated receptors, as this has been demonstrated for CXCR2 and CCR2, which display no more than 20% of homology ⁷⁶. Other tracks for drug development could be tested, notably the modification of chemokines themselves to constitute pure chemokine receptor antagonists. In particular, chemokines could be mutated to abolish chemokine receptor signaling and in the meantime increase the affinity of the chemokine for GAGs. Finally, learning from nature, proteins produced by parasites or viruses to divert the chemokine pathway of the host could be of interest ⁷⁷.

In addition to drug design, one can also question how these drugs are tested and also to which patients they are administered. First, it is difficult to develop a novel drug with a specificity limited to human targets, as the efficacy of these drugs will need to be tested in mouse models. Second, the populations of patients enrolled so far in most of clinical trials targeting chemokines or their receptors have not been stratified according to their expression levels of the target. This might reduce considerably the overall efficacy of the drug. With the emergence of the concept of personalized therapy for each patients (one of the best example being certainly the use of

Herceptin in HER2-positive breast cancer patients), it will certainly be essential to enroll patients with a presumably favorable profile.

It is stimulating to see that the number of possible chemokine and chemokine receptor targets is still increasing, which reinforces the idea that this pathway is essential in cancer. These targets offer potential for the developments of a variety of drugs to treat cancer. Based on the failures of previous clinical trials, we certainly need to rethink the strategies to target chemokines, but this could definitely constitute the therapies of tomorrow.

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Box 1: Chemokine families

Chemokines and their receptors are involved in neutrophil and monocyte cell trafficking^{78, 79} and are classified on the basis of the presence of variations on a conserved cysteine motif in the mature sequence of the proteins^{11, 12} (Fig. 1). The first group of chemokines, named CC subfamily (so-called because of the juxtaposition of the first two cysteine residues), is composed of 28 members, whereas the CXC subfamily (which possess a single variable amino acid between the first two cysteines) comprises 17 members. Two smaller subfamilies (1 member each) are represented by CX3C family (three amino acids between the first two cysteines) and the XC family, which lacks the first and third cysteines. The CXC chemokines can be further classified into ELR⁻ and ELR⁺ subgroups based on the presence or absence of the motif ‘glu-leu-arg (ELR)’. ELR⁺ CXC chemokines (CXCL1, 2, 3, 5, 6, 7, 8) are angiogenic factors, whereas ELR⁻ members (except CXCL12) function as angiostatic factors to inhibit the formation of blood vessels⁸⁰. Chemokines bind to the chemokine receptor subfamily of class A G-protein-coupled receptors (GPCRs). There are 10 CCR family members and 7 CXCR family members in addition to XCR1 and CX3CR1 (Fig. 1). D6, Duffy antigen receptor for chemokines (DARC) and CCX-CKR (ChemoCentryx, chemokine receptor) represent decoy receptors which bind ligands with high affinity but do not elicit signal transduction³⁰. Many chemokines bind multiple receptors and most receptors bind multiple chemokines (Fig. 1), suggesting the possibility of functional redundancy, even though this is certainly modulated by both spatial and temporal control of their expression. Several enzymes, in particular proteases, have been described to process chemokines at specific sites generating chemokine isoforms, with sometimes higher activity than the full-length protein⁸¹. In addition to GPCRs, chemokines also interact with glycosaminoglycans (GAGs), and, although this interaction is not required for *in vitro* chemotactic activity, GAG binding is essential for presentation of chemokines on endothelial layers and for leukocyte migration *in vivo*⁸². Chemokine GPCRs signal through

heterotrimeric G-proteins, which in turn regulate a diversity of signal transduction pathways involved in chemotaxis, including a cascade of signals through mitogen-activated protein (MAP) kinases, phospholipase-C β , phosphoinositide 3-kinase (PI3K) and RAS or Rho GTPases⁸³. It is interesting to note that chemokine receptors are themselves subjected to dynamic phosphorylation events, which could be crucial for their action and constitute another level of control.

Box "Future questions"

- Are there other chemokines or chemokines receptors involved in cancer?

If many progresses have been during the last decade in the identification of chemokine or chemokine receptors displaying an altered expression in cancer, other actors remain to be identified. In particular, chemokines with low level of expression might also be crucial even though not identified by conventional screens.

- What are the roles of chemokines and chemokine receptors in the migration of circulating tumor cells?

This is certainly one of the aspect lacking proper evaluation in the field, due to the dynamic and complexity of the process and difficulty to isolate correctly CTC.

- Which upstream signals control the expression of chemokines and chemokine receptors?

It is still a rarely studied question. Do modifications of chemokine and chemokine receptor levels come from epigenetic events, transcriptional events? What are the factors produced by the microenvironment which dictate their expression?

- Which patients should benefit from chemokine based therapy?

To improve the efficacy of current treatments, should we enroll patients with a better responsive profile? What should be the criteria to select these patients?

- Is the use of combined drugs or promiscuous drugs the next step in chemokine and chemokine receptor therapy for cancer?

In cancer field, the concept of multiple associated treatments is generalizing. We believe that the same will be for chemokine and chemokine receptor targeted treatment that should certainly hit different members, but could also be associated with more conventional treatments such as chemotherapy.

Figure Legends

Fig. 1 (embedded in Box 1): Chemokines and chemokine receptors family

Chemokines interact mostly with multiple receptors and a single receptor can interact with multiple chemokines. This is the case for most CC (red) and CXC (green). Decoy receptors (black) bind also multiple chemokines. On the other hand, a minority of receptors (blue) have only one ligand.

Fig. 2: Repertoire of chemokines and chemokine receptors expressed in cancer tissues

A fine interaction occurs between cancer cells and cells of the tumor microenvironment, including endothelial cells, CAFs, MSCs, myeloid cells and TAN. Cancer cells produce a variety of chemokines which can modulate not only their own properties but also the one of associated stromal cells. Cells of the tumor microenvironment constitute also a non negligible source of chemokines which can alter the function of cancer cells.

Fig. 3: Multiple chemokines affect angiogenesis, proliferation, invasion, apoptosis, and senescence

Chemokines can modulate cancer cell proliferation, apoptosis, senescence and invasion and participate in tumor angiogenesis as well as leukocyte infiltration. Recent findings suggest that CXCR7 could be a novel receptor involved in the control of proliferation. CXCR7 could also inhibit apoptosis, whereas CCL2 impedes autophagic death. Senescence is also tightly regulated by CXCR2 and its ligands CXCL1 and CXCL8. Decoy receptors such as DARC and D6 could regulate angiogenesis negatively. Other chemokines such as CXCL1, CXCL8 and their receptor CXCR2 promote angiogenesis. CXCL1 is induced by PGE₂, whereas CXCL8 is negatively regulated by PHD2. Homing of cancer cells to specific metastatic sites is tightly controlled by chemokines CXCL1,8, 12, CCL5, CCL19, 21, 25, 27 and chemokine receptors CCR5, 7, 9, 10 and CX3CR1. CXCL12 levels are induced by Src and in turn can modulate metastasis. CCR7, whose expression is controlled by Notch1 and interstitial flow, plays an active role in metastasis. Several reports show the central role played by TGF β which can induce CXCL12 expression, but are also revealing roles for CCL2, 5, CXCR2 and CXCR4. In turn, these chemokines and chemokine receptors favor inflammatory cell infiltration. By contrast, TGF β down regulates CXCL1, 5, 8, CCL9 and CCR1 levels, which acts to further modulate leukocyte infiltration.

Fig. 4: Current and future intervention points for chemokine and chemokine receptor based therapies in cancer.

In black are indicated the chemokines or chemokine receptors currently targeted in clinical trials. In red, the novel targets for future therapies, based on the recent advances in their action in different steps of cancer development.

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Figure1

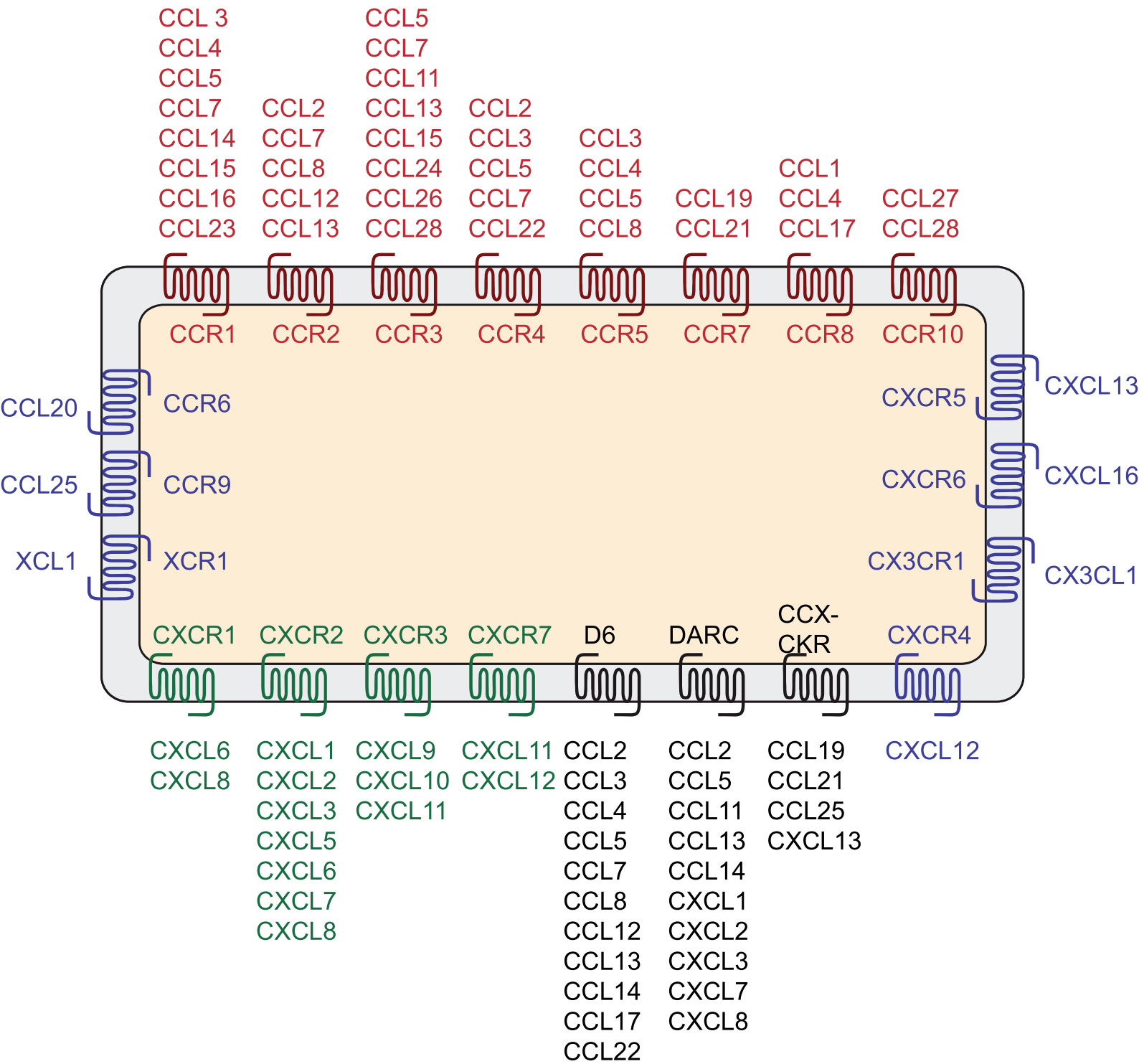


Fig. 1

Figure2

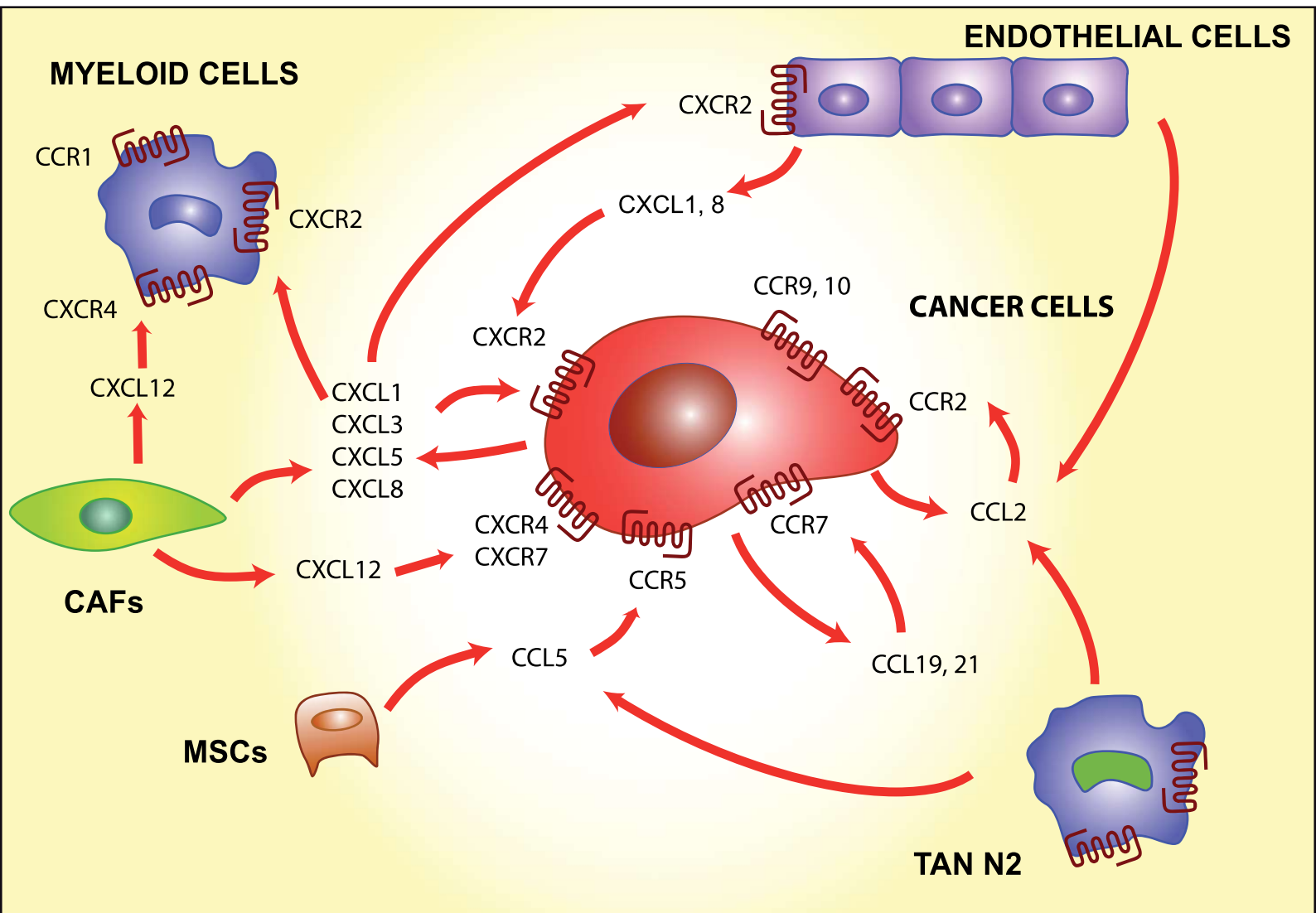


Fig. 2

Figure3

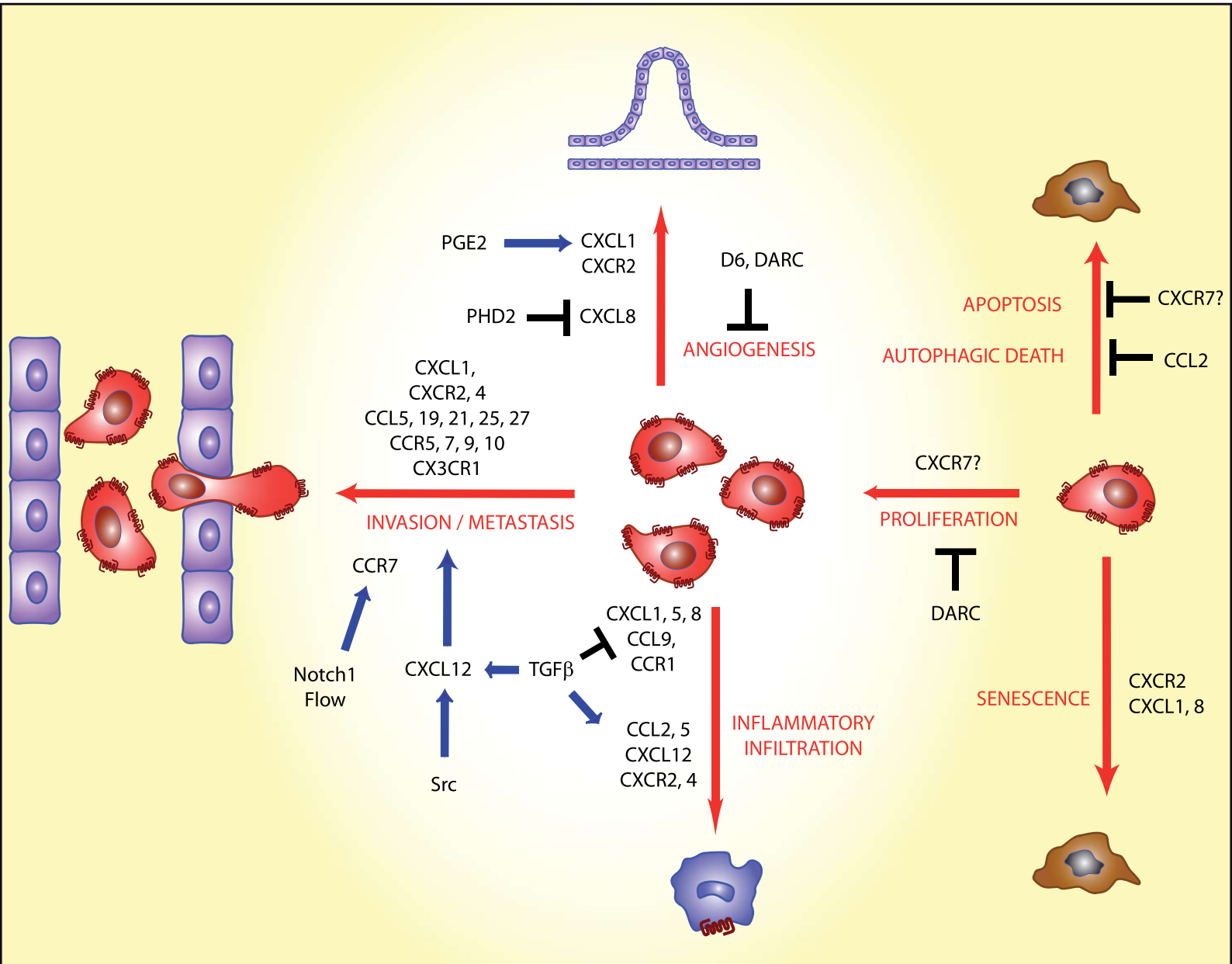


Fig. 3

Figure4

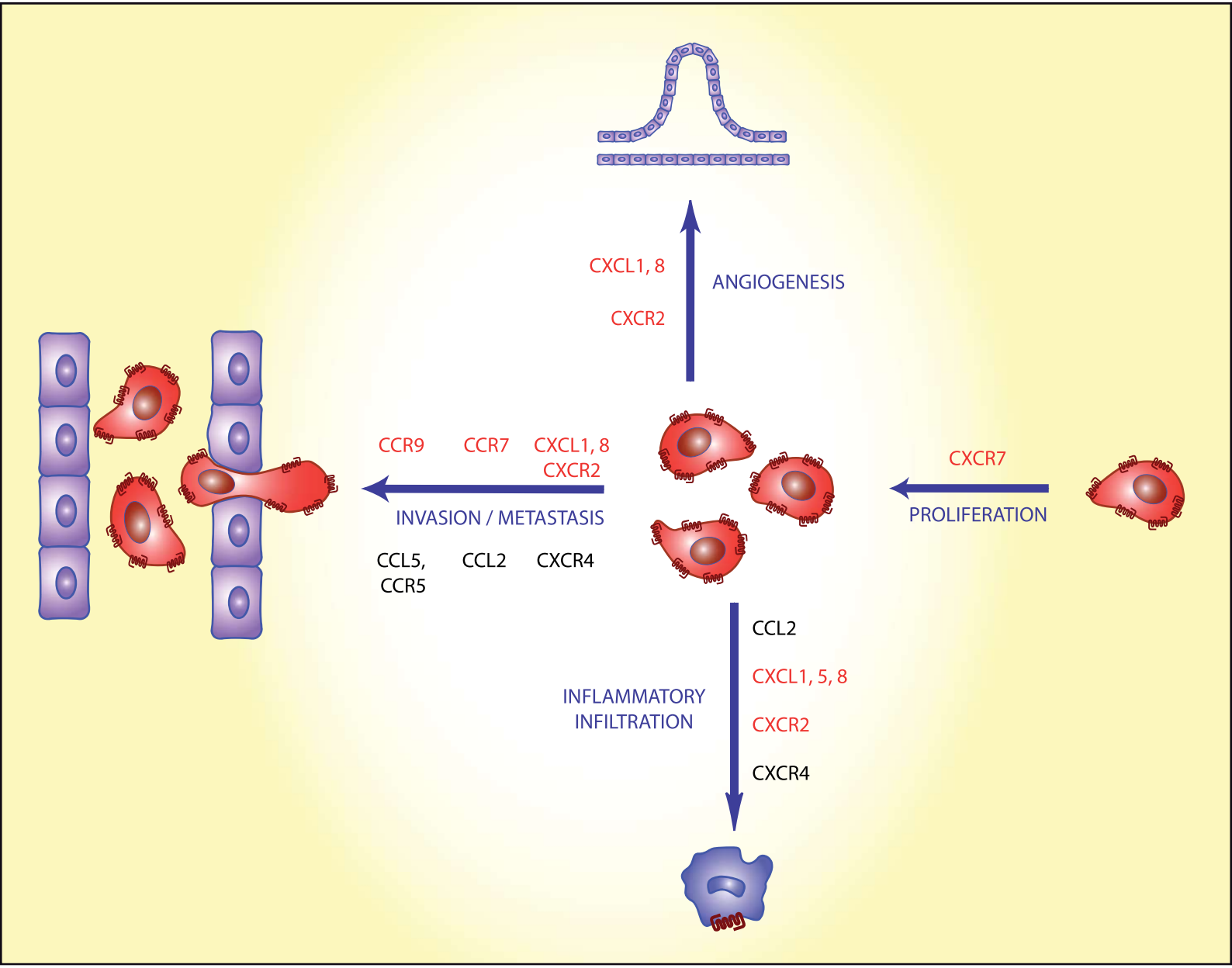


Fig. 4

	Ligands	Receptors
Epithelial cells	CXCL1, 3, 5, 6, 8, 10 CCL2, 4, 5 CX3CL1	CXCR1, 2, 4, 6, 7 CCR1, 2, 5, 6, 7, 9, 10 CX3CR1
Cancer associated fibroblasts	CXCL1, 2, 5, 6, 8, 12	CCR5
Endothelial cells	CXCL1, 2, 3, 8 CCL2 CX3CL1	CXCR2, 3, 4, 7
Infiltrating leukocytes	CXCL5, 8 CCL2, 3, 4	CXCR1, 2 CCR2, 4, 5

Table 1: Summary of the chemokines and chemokine receptors in cancer

Are indicated here only chemokines or chemokine receptors expressed at higher levels in epithelial cells compared to normal tissues, or expressed by cancer associated fibroblasts, endothelial cells and infiltrating leukocytes.

Target	Drug	type	Company	clinical_phase	Indication	source
CXCR4	AMD3100	small molecule inhibitor	Genzyme	Phase II/III	Multiple myeloma; acute myeloid leukemia; solid tumors	CT, 65, 66
	MDX-1338	Antibody	Medarex	Phase I	Acute myeloid leukemia	65
	BKT140	small molecule inhibitor	Biokine Therapeutics	Phase I/II	Multiple Myeloma	65,CT
	CTCE-9908	peptide antagonist	Chemokine therapeutic Corp.	Phase I/II	Solid tumors	CT
	MSX-122	small molecule inhibitor	Metastatix Inc	Phase I suspended	Solid tumors	66
CCR4	KW0761	Antibody	Kyowa Hakko Kogyo Co	Phase II	Adult T-Cell leukemia and lymphoma, peripheral T-Cell leukemia	65, CT
CCR5	Sch-C	Small molecule inhibitor	Schering-Plough	Phase I	Cancer	69
CCR9	CCX282	small molecule inhibitor	ChemoCentryx	Phase III	Crohn's disease	67,68,73
CCL2	CNTO 888	Antibody	Centocor	Phase I	Solid tumors	65
	MLN1202	Antibody	Millenium	Phase II	Bone metastasis	CT

Table 2: Survey of the clinical trials targeting chemokines in cancer

Sources: CT: clinicaltrials.gov and references of the literature