Chapter 6
The Versatile World of Inflammatory Chemokines in Cancer

Tal Leibovich-Rivkin, Yaeli Lebel-Haziv, Shalom Lerrer, Polina Weitzenfeld and Adit Ben-Baruch

Abstract Until recently, inflammatory chemokines were viewed mainly as indispensable "gate keepers" of immunity and inflammation. However, updated research indicates that members of this chemokine sub-family are important constituents of the tumor microenvironment, having multifaceted tumor-promoting roles in cancer. A very large number of studies indicate that many of the inflammatory chemokines are exploited by the tumor cells for their own benefit, and are actually skewed to the pro-malignancy phenotype. The different chemokines may be simultaneously expressed at the tumor site, having overlapping but also distinct tumor-promoting impacts. In general (except for the axis of CXCR3 and its ligands, that acts as a "double-edged sword"), the inflammatory chemokines induce immune imbalance at primary tumors and metastatic sites, doing so by promoting the presence and activation of tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC) and/or T regulatory cells (Treg). In parallel, immune suppression is ensued due to inhibition of Th1 cells and cytotoxic T lymphocytes (CTL). The chemokines also elevate metastasis-related processes, such as angiogenesis and osteoclastogenesis in the bone. Furthermore, they act directly on the tumor cells, promoting their proliferation, migration and invasion properties. Obviously, not all chemokines have the same pro-malignancy roles; however, chemokines that share the same receptor tend to have much in common in terms of their pro-cancerous activities. Accordingly, we will describe the roles of inflammatory chemokines in malignancy by using a receptor-based categorization: (1) CXCR1 and CXCR2 with their ELR^+ CXC

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chemokine ligands, primarily CXCL8 (IL-8) but also CXCL1 (MGSA, GROα) and CXCL5 (ENA-78); (2) CXCR3 with its non-ELR CXC chemokine ligands: CXCL9 (Mig), CXCL10 (IP-10) and CXCL11 (I-Tac); (3) CCR2 and its ligands, mainly CCL2 (MCP-1); (4) CCR5 and its corresponding chemokines, with major emphasis on CCL5 (RANTES) and CCL3 (MIP-1α). Based on the findings obtained so far, we propose that inflammatory chemokines and their receptors are attractive therapeutic targets in malignancy, and discuss the expected difficulties in translating such approaches in the clinic.

Keywords Inflammatory chemokines · CXCR1/CXCR2 ligands · CXCR3 ligands · CCR2 ligands · CCR5 ligands, TAM, MDSC, T cells

6.1 Introduction

The tumor microenvironment is a complex entity that brings together several sub-environments, interacting and affecting each other. One of the most influential tumor sub-environments is the immune system, containing diverse cell populations and soluble factors. These components may exert immune surveillance functions and thus have the ability to protect the host against the developing tumor; however, very often they are skewed by the cancer cells to a pro-tumor phenotype. Under such circumstances, cells and soluble mediators of the immune system are exploited by the cancer cells, leading to formation of a tumor immune-environment that is favorable for the malignant cells, promoting their propagation and spreading capabilities.

Under physiological conditions, leukocytes, cytokines and chemokines cooperate, eventually mounting inflammatory reactions that protect the host against invading pathogens. These same elements join forces also in many pathological conditions, including cancer. Extensive research of the last two decades suggests that tumors are inflammatory organs, in which the tumor immune-environment has been diverted to a cancer-supporting entity (Balkwill and Mantovani 2011; Hanahan and Weinberg 2011; Hagemann et al. 2007; Joyce and Pollard 2009).

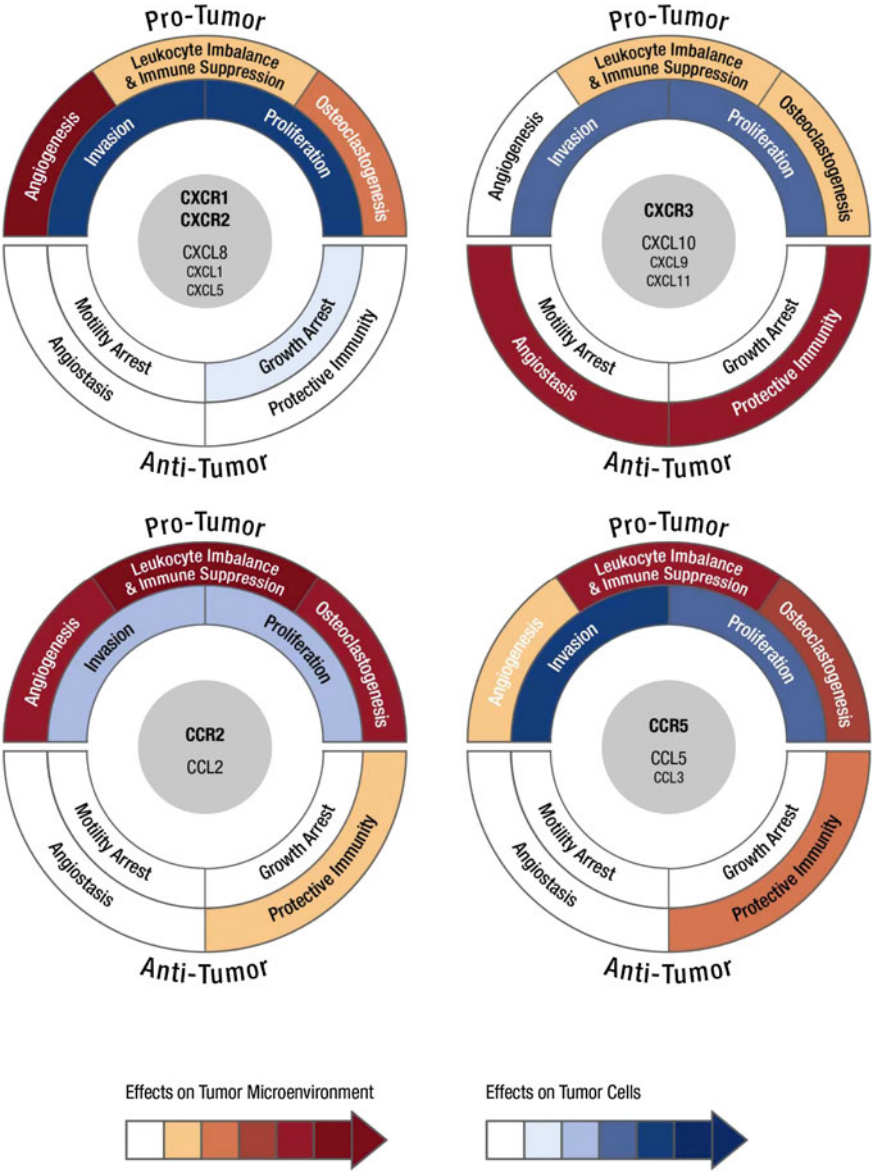
In this context, major roles have been attributed to inflammatory chemokines in cancer. The term “Inflammatory chemokines” denotes a functional categorization, describing chemokines that attract leukocytes to infected and damaged sites, playing key roles in the fight against foreign entities and enabling tissue repair. At such inflammatory sites, these chemokines are inducibly expressed in response to exposure of the tissue to inflammatory insults. In parallel, other chemokines known by the name “homeostatic chemokines” attract leukocytes to primary and secondary lymphoid organs, and are therefore involved in normal hematopoietic processes (Allen et al. 2007; Mantovani et al. 2006; Zlotnik et al. 2006).

Both these two functional sub-groups of chemokines include many members, divided by structural criteria to the CXC, CC, C and CX_3C sub-groups. The structural categorization is based on the number and the location of conserved cysteine residues in the N' terminus of the chemokine molecules. The CXC structural sub-group is further divided to chemokines expressing an ELR motif prior to the CXC sequence and therefore are termed "ELR^+ CXC chemokines", and to those that do not express such a motif, and are known by the name "non-ELR CXC chemokines" (Allen et al. 2007; Mantovani et al. 2006; Zlotnik et al. 2006).

The chemokine family includes around 50 different proteins in human, sharing the fundamental activity of chemoattracting leukocytes. In immune related-activities, chemokines that are presented by endothelial cell-expressed glycosaminoglycans (GAG) to leukocytes, activate high affinity heterotrimeric G protein-coupled receptors (GPCR) that are expressed by target immune and inflammatory cells. Many of the chemokines bind with high affinity several receptors, and similarly most of the chemokine receptors are activated by several chemokines. Very often, those chemokines that bind the same receptor have much in common, in terms of target cell preference and impact on immune and inflammatory activities (Allen et al. 2007; Mantovani et al. 2006; Zlotnik et al. 2006).

To follow on the above, in this chapter we will discuss the roles of inflammatory chemokines in cancer, using a receptor-based classification: (1) The receptors CXCR1 and CXCR2 and their ELR^+ CXC chemokines; (2) The CXCR3 receptor and its non-ELR CXC chemokines; (3) CCR2 and its chemokine ligands; (4) CCR5 and its corresponding chemokines. In each sub-section, the chapter will provide a general overview of the activities of the relevant receptors and their chemokines in cancer, their impact on events taking place at the tumor microenvironment, and their direct effects on tumor cells (summarized in Fig. 6.1).

Prior to addressing the four chemokine receptor sub-groups indicated above, it is important to put things in a broader perspective. Generally speaking, different inflammatory chemokines may be simultaneously expressed by the tumor cells and by stroma cells in their vicinity. The receptors corresponding to these chemokines are obviously expressed by specific leukocyte sub-types, but in addition functional chemokine receptors are expressed also by many different types of tumor cells. As expected from their roles in immune-related activities, inflammatory chemokines induce the migration of inflammatory cells such as monocytes and neutrophils to tumor sites, and also of cells exerting acquired immune activities, of which the most relevant ones to cancer are T cells. Therefore, there are specific circumstances in which these inflammatory chemokines mount protective immune activities against the tumor cells, and are thus beneficial for the host, as has been well documented for CXCR3 and its ligands. However, in the majority of cases, under the influence of the tumor cells and their products, most of the inflammatory chemokines are diverted towards the tumor-promoting phenotype, including the same CXCR3 ligands mentioned above. In general, the inflammatory chemokines lead to positioning of leukocytes with detrimental effects in the tumors, and this activity is fundamental to the way the chemokines affect cancer development and


Pro-Tumor
Leukocyte Imbalance & Immune Suppression
Angiogenesis
Invasion
Proliferation
Osteoclastogenesis
CXCR1
CXCR2
CXCL8
CXCL1
CXCL5
Motility Arrest
Angiostasis
Growth Arrest
Protective Immunity
Anti-Tumor
Pro-Tumor
Leukocyte Imbalance & Immune Suppression
Angiogenesis
Invasion
Proliferation
Osteoclastogenesis
CXCR3
CXCL10
CXCL9
CXCL11
Motility Arrest
Angiostasis
Growth Arrest
Protective Immunity
Anti-Tumor
Pro-Tumor
Leukocyte Imbalance & Immune Suppression
Angiogenesis
Invasion
Proliferation
Osteoclastogenesis
CCR2
CCL2
Motility Arrest
Angiostasis
Growth Arrest
Protective Immunity
Anti-Tumor
Pro-Tumor
Leukocyte Imbalance & Immune Suppression
Angiogenesis
Invasion
Proliferation
Osteoclastogenesis
CCR5
CCL5
CCL3
Motility Arrest
Angiostasis
Growth Arrest
Protective Immunity
Anti-Tumor
Effects on Tumor Microenvironment
Effects on Tumor Cells

◀ **Fig. 6.1** The versatile world of inflammatory chemokines in cancer. The tumor microenvironment contains simultaneously a large variety of inflammatory chemokines, which may have overlapping but also distinct activities, as illustrated in the figure. Here, we demonstrate the roles of inflammatory chemokines in cancer, using a receptor-based categorization according to the following receptors: CXCR1/CXCR2, CXCR3, CCR2 and CCR5. An individual chart has been ascribed to each of the receptors, describing the impact of the receptor/s and their most relevant chemokines on cancer. As has been indicated in the chapter, inflammatory chemokines may exert anti-tumor activities (demonstrated in the lower half of each chart, termed *Anti-Tumor*), primarily mediated by recruitment of leukocytes with protective activities to the tumor site. However, generally-speaking, the effects of the inflammatory chemokines in malignancy are dominated by their pro-tumoral functions (upper half of the charts, termed *Pro-Tumor*). The activities of the chemokines are exerted on cells of the tumor microenvironment (external circle of each receptor's chart, carrying red color code; each activity has been given a specific intensity value, according to its relative contribution to the activities of the chemokines and their receptors) and on the tumor cells themselves (Internal circle, carrying blue color code, intensified according to the same guidelines as in the red color code). The shift in equilibrium towards the pro-tumoral direction is manifested in the figure by (1) Strong colors dominating the upper, pro-malignancy part of the receptor charts; (2) Minimal coloring in the lower, anti-tumor part of the charts, indicating that such activities are actually hardly exerted by the chemokines (except for the specific case of CXCR3 ligands). In the receptor-based charts, the activities of the chemokines were summed up by using brief terms of functional categorization. Each categorization includes several functions, of which all or only some are exerted by the chemokines/receptors that are included in each chart. The categorizations include the following functions: (1) At the upper part of the receptor charts, describing *Pro-Tumor* activities: *Leukocyte Imbalance and Immune Suppression* = Induction of high TAM levels at the tumor site, elevated MDSC and Treg presence and activities, reduced localization of Th1 cells and lower tumor cell killing by CTL and/or shift in neutrophil balance (due to yet undefined roles of neutrophils in cancer, aspects related to neutrophils were taken into account only when the chemokines had direct impacts on neutrophil levels or functions); *Angiogenesis* = Increased proliferation and migration of endothelial cells, tube formation and neovascularization; *Osteoclastogenesis* = Elevated processes of bone osteolysis and resorption; *Proliferation* = Increased tumor cell survival, proliferation and/or cancer stem cell-related functions; *Invasion* = Induction of tumor cell adhesion, migration and/or invasion, that may lead to metastatic spread in remote organs. (2) At the lower part of the receptor charts, describing *Anti-Tumor* activities: *Protective Immunity* = Promotion of immune surveillance, exerted by CTL and NK cells (for neutrophils the considerations were as above, in the *Pro-Tumor* part). *Angiostasis* = Inhibition of angiogenic processes; *Growth Arrest* = Induction of apoptosis or senescence in response to stress; *Motility Arrest* = Prevention of tumor cell adhesion, migration and invasion, that may limit metastasis formation. For more details on the specific functions that adhere to each of the receptor-chemokine axes, the readers are referred to the relevant sections of the chapter

progression. In addition, these chemokines can act on stroma cells and promote angiogenic processes and osteoclastogenesis, while in parallel they can also induce processes of tumor cell proliferation and invasion.

On the whole, the activities of inflammatory chemokines and their receptors are mostly diverted towards tumor promotion and progression. Here, each of the receptors—CXCR1/CXCR2, CXCR3, CCR2 and CCR5—and the corresponding chemokine ligands, has its own "flavor" and preferable mode of action, by which it impacts malignant processes (Fig. 6.1). In this chapter we will describe those features that are the most characteristics for each receptor and for its most effective ligands. However, due to space and length limitations, not all the relevant studies

would be mentioned; rather, in each sub-section we will refer the readers to recent review papers, and will provide selected specific references, mainly of publications of the last several years.

6.2 Inflammatory Chemokines in Cancer

6.2.1 CXCR1 and CXCR2 Ligands

In human, CXCR1 and CXCR2 are the prototype receptors for ELR$^+$ CXC chemokines. In immune-related inflammatory conditions they are known as powerful attractants of neutrophils to sites of acute inflammation. Accordingly, the highest levels of constitutive expression of CXCR1 and CXCR2 are denoted on neutrophils, but both receptors are also expressed by monocytes and by other specific leukocyte subtypes (Allen et al. 2007; Mantovani et al. 2006; Zlotnik et al. 2006).

CXCR1 and CXCR2 show 77 % identity at the amino acid level, they have many shared characteristics and both bind CXCL8 with high affinity. However, these two receptors diverge in the spectrum of other chemokines they bind, and thus show some differences in their in vivo activities. CXCR2 is a promiscuous receptor also for the ELR$^+$ CXC chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7, whereas CXCR1 binds with high affinity also CXCL6 and possibly CXCL7 (Allen et al. 2007; Mantovani et al. 2006; Zlotnik et al. 2006). The different ELR$^+$ CXC chemokines and their two receptors exemplify a complex net of interactions that is fundamental to the immune integrity of the host. Extending beyond the immune context, the activation of CXCR1 and CXCR2 has been shown to regulate additional physiological and also pathological conditions, of which malignancy is a major one.

Based on extensive research, ELR$^+$ CXC chemokines and their CXCR1 and CXCR2 receptors, are considered powerful pro-tumorigenic components in many cancer diseases [reviewed in (Dhawan and Richmond 2002; Ijichi 2012; Singh et al. 2010a; Vandercappellen et al. 2008; Waugh and Wilson 2008)]. The chemokines act on leukocytes and endothelial cells (EC) in manners that promote tumor development and metastasis, and in addition they induce tumor cell proliferation and invasion. The number of reports opposing this view is rather small, and there is a general consensus that the axis of CXCR1/CXCR2-ELR$^+$ CXC chemokines has detrimental pro-tumoral impacts in a very large number of malignancies. The effects of this axis in cancer will be described below, but there is a need to indicate that in one specific aspect, which is the process of senescence that takes place in response to stresses, this axis protects the cells against progression to a malignant state. It has been recently found by several investigators that CXCR2 and its ligands reinforce senescence early in cancer (Acosta et al. 2008; Ruan et al. 2012; Acosta and Gil 2009). These findings indicate that the CXCR1/CXCR2-ELR$^+$ CXC chemokine axis exerts pro-malignancy effects on one

hand but pro-senescent functions on the other, emphasizing the need to identify the mechanisms involved in such opposing activities of these factors.

Going back to the tumor-promoting roles of ELR^+ CXC chemokines and their receptors, most of the research on human cancers has focused on the powerful chemokine CXCL8, and its two receptors, CXCR1 and CXCR2. The studies on human cancer biopsies and tumor extracts have been relatively limited; although the different studies have exemplified a general trend for associations between elevated expression of the receptors and of ELR^+ CXC chemokines with disease [for example (Grepin et al. 2011; Xu et al. 2012; Dimberg et al. 2012; Sunaga et al. 2012; Huang et al. 2010; Yang et al. 2010)], there are human malignancies in which the results were not fully conclusive, such as breast cancer (Green et al. 1997; Rody et al. 2011; Snoussi et al. 2010; Zuccari et al. 2012). However, determination of such chemokines and primarily of CXCL8 in patient serum yielded more definite findings, showing significant associations between high CXCL8 serum levels and increased tumor load, metastasis, disease progression and reduced survival. This pattern has been demonstrated in the past for example in melanoma, colon cancer and breast cancer (Benoy et al. 2004; Yokoe et al. 1997; Singh et al. 2010a; Ning et al. 2011).

Furthermore, the roles of ELR^+ CXC chemokines in malignancy have been extensively addressed in murine model systems, with two important limitations: the first is that the existence of a functional murine CXCR1 has been controversial and non-conclusive, and the second is that a mouse counterpart to human CXCL8 was not identified. Therefore, studies of leukocyte/stroma receptors in the mouse have actually addressed only CXCR2, and this was done in parallel to targeted reduction or over-expression of both CXCR1 and CXCR2 in human tumor cells. Also, CXCL8 was the subject of research when human tumor cells were analyzed in immune-deficient mice, but when murine host chemokines were studied, mainly CXCL1 and CXCL5 were addressed.

Investigations in animal model systems have provided a strong support for the causative tumor-promoting roles of ELR^+ CXC chemokines and their receptors in malignancy. Modalities such as neutralizing antibodies, siRNA, pharmacological inhibitors of CXCR1 and CXCR2, and approaches of over-expression, have shown direct roles for these chemokines and for CXCR1 and CXCR2 in promoting tumor growth and/or metastasis in many malignant diseases [several examples out of many: (Matsuo et al. 2009; Merritt et al. 2008; Rolny et al. 2008; Shamaladevi et al. 2009; Yamamoto et al. 2008; Grepin et al. 2011; Agarwal et al. 2010; Bandapalli et al. 2012; Huh et al. 2010; Chen et al. 2011; Varney et al. 2011; Vegran et al. 2011)]. Particular emphasis was put on melanoma, where extensive research has identified roles for tumor cell-expressed CXCL1, CXCL8, CXCR1 and CXCR2, as well as for host CXCR2 in advancing disease course (Dhawan and Richmond 2002; Haghnegahdar et al. 2000; Luan et al. 1997; Singh et al. 2009a; Singh et al. 2009b; Singh et al. 2009c; Gabellini et al. 2009; Huh et al. 2010; Singh et al. 2010b). Furthermore, direct activities of the chemokines were identified in the bone, where CXCL1 has elevated tumor cell adhesion in the bone matrix (Li et al. 2012), and both CXCL1 and CXCL8 stimulated osteoclastogenesis and bone resorption

(Bendre et al. 2003; Li et al. 2012; Pathi et al. 2010). Together, such chemokine-induced mechanisms may lead to increased bone metastasis and osteolysis.

The pro-tumoral activities of ELR^+ CXC chemokines are exerted on many different leukocyte, stroma and tumor cell properties. In view of the powerful chemotactic roles of ELR^+ CXC chemokines on neutrophils in physiological inflammatory conditions, studies of the last several years have questioned the effects of these chemokines on neutrophil recruitment and activation in cancer. In general, the roles of neutrophils in malignancy are debatable, with findings showing that these cells can inhibit or promote disease course, depending on the circumstances (Fridlender et al. 2012; Gregory et al. 2011; Piccard et al. 2012). For example, a recent study by Granot et al. 2011 has shown that neutrophils inhibited the metastatic seeding of breast tumor cells in the lungs, and that they did so by generating H_2O_2 (Granot et al. 2011). Alongside with that study, Fridlender and his colleagues have demonstrated that the activities of neutrophils in cancer are regulated by transforming growth factor beta (TGFβ) (Fridlender et al. 2009), and that after TGFβ blockade tumor-associated neutrophils acquired an anti-tumor phenotype. This latter study has also shown that ELR^+ CXC chemokines were expressed at the tumor site, and that recruitment of neutrophils to the site was followed by oxygen-mediated cytotoxicity mechanisms (Fridlender et al. 2009). However, the same study has found that a process largely driven by TGFβ has led to pro-cancer activities of neutrophils, illustrated by experiments in control tumors, where neutrophil depletion decreased tumor growth and resulted in more activated $CD8^+$ T cells in the tumors (Fridlender et al. 2009).

In parallel to these latter observations, many past studies demonstrated pro-malignancy roles for neutrophil-mediated processes that take place in the context of ELR^+ CXC chemokines (De Larco et al. 2004; Strell et al. 2010; Huh et al. 2010), including promotion of tumor cell motility, angiogenesis and mutagenesis through reactive oxygen species (De Larco et al. 2004). Along the same lines, it was demonstrated that following CXCL1/CXCL8-induced recruitment of neutrophils to tumors, the neutrophils interacted with the tumor cells, leading to ICAM-1-mediated motility of the cancer cells (Strell et al. 2010). In another study, following their CXCL8-induced migration to tumors, neutrophils interacted with tumor cells, eventually potentiating the anchoring of the cancer cells to vascular endothelium, again in an ICAM-1-dependent process (Huh et al. 2010).

The activities described above for ELR^+ CXC chemokine-affected neutrophils may account for elevated malignancy and metastasis, and they are complemented by the very powerful angiogenic effects of these chemokines. Chemokines and their receptors are important regulators of neovascularization in inflammatory processes and wound healing, as well as in diverse pathological conditions including cancer (Keeley et al. 2008; Keeley et al. 2010). Actually, this field of research has been overloaded with studies demonstrating that ELR^+ CXC chemokines, mainly CXCL8, are correlatively and causatively linked with high vascularization in cancers, and this is a major mode by which they contribute to tumor growth and metastasis [e.g. (Merritt et al. 2008; Keeley et al. 2008; Yoo et al. 2008; Agarwal et al. 2010; Vegran et al. 2011; Keeley et al. 2010; Wang et al. 2012c)].

The angiogenic effects of ELR$^+$ CXC chemokines are exerted by direct activity on EC, mainly through CXCR2. Although it was shown that both CXCR1 and CXCR2 can mediate the vascularizing activities of the chemokines on EC [migration and/or survival; (Gabellini et al. 2009; Li et al. 2005)], similar effects could be induced on EC via CXCR2 only (Keeley et al. 2008; Keeley et al. 2010). Moreover, in addition to CXCL8, CXCL1-3 and CXCL5 were found to have vascularizing activities [e.g. (Matsuo et al. 2009; Haghnegahdar et al. 2000; Luan et al. 1997; Xu et al. 2012)]; because these latter chemokines are not acting through CXCR1, it is assumed that CXCR2 is the major receptor mediating the angiogenic activities of ELR$^+$ CXC chemokines. Supporting this view are many studies showing that inhibition of CXCR2 activities has given rise to reduced vascularization in tumors (Matsuo et al. 2009; Singh et al. 2009c; Addison et al. 2000; Ning et al. 2012).

CXCL8 and the other ELR$^+$ CXC chemokines directly induce a large array of angiogenic functions in EC, including migration, proliferation, tube formation and the release of vascular endothelial growth factor (VEGF) [for example: (Matsuo et al. 2009; Yoo et al. 2008; Martin et al. 2009; Xu et al. 2012; Agarwal et al. 2010)]. In parallel, CXCL8 was shown to promote neovascularization indirectly, for instance by inducing VEGF production by the tumor cells (Li et al. 2008; Yang et al. 2010; Wang et al. 2012c). Thus, by acting on EC and also on the tumor cells, CXCL8 and other members of the ELR$^+$ CXC sub-family of chemokines contribute to the very important aspect of angiogenesis, which is fundamental to increased growth of tumors, and to their ability to spread to remote organs.

To follow on the observations showing that CXCL8 up-regulated VEGF production by tumor cells, it is important to indicate that ELR$^+$ CXC chemokines are very potent inducers of tumor-promoting functions in the cancer cells themselves, including invasion-related properties and proliferation. In this context, mainly CXCL8 but also other ELR$^+$ CXC chemokines (e.g. CXCL1, CXCL5) were shown to induce adhesion of tumor cells to EC, extracellular matrix and bone matrix (Warner et al. 2008; Huh et al. 2010; Li et al. 2012; Ju et al. 2012). The chemokines also potently promoted tumor cell migration and invasion [for example: (Araki et al. 2009; Neiva et al. 2009; Singh and Lokeshwar 2009; Bandapalli et al. 2012; Wang et al. 2012c; Ju et al. 2012; Yeudall et al. 2012; Lee et al. 2011; Halpern et al. 2011; Kuai et al. 2012; Nieman et al. 2011; Welte et al. 2012)], and amplified the expression of ICAM-1 in the tumor cells, as well as of other molecules involved in adhesive/migratory processes, such as VCAM-1, CD44 and the integrin $\alpha V\beta 3$ (Lee et al. 2012a; Kuai et al. 2012; Ju et al. 2012). In parallel, CXCL8 was shown to induce epithelial-to-mesenchymal transition in tumor cells (Bates et al. 2004; Fernando et al. 2011; Li et al. 2012) and elevations were found in the expression levels of matrix metalloproteinases (MMP) (Merritt et al. 2008; Wang et al. 2012c; Ju et al. 2012).

Very often, the migratory and invasive properties of tumor cells that were induced by the chemokines have been causatively linked to increased tumor load and metastasis formation. Depending on the cancer cell type and the experimental system, these functional events have been mediated by tumor cell expressed

CXCR1, CXCR2 or both receptors together (Singh et al. 2009a; Singh et al. 2009b; Gabellini et al. 2009; Warner et al. 2008; Bates et al. 2004; Varney et al. 2011; Singh et al. 2010b; Lee et al. 2011; Nieman et al. 2011; Fernando et al. 2011). Often, interactions between the tumor cells and stroma cells were stimulating tumor cell migration. For example, adipocytes were shown to release CXCL8, whose activities have led to adhesion of ovarian tumor cells to human omentum, and to migration towards human omental adipocytes and towards mouse omentum in vivo, in a process mediated by CXCR1 (Nieman et al. 2011). Cross-talks were also observed between tumor cells and mesenchymal stem cells [MSC, known as precursors to deleterious cancer-associated fibroblasts, CAF (Kalluri and Zeisberg 2006; Mishra et al. 2011; Shimoda et al. 2010)] or adipose tissue derived stromal cells. These interactions involved processes mediated through ELR^+ CXC chemokines and CXCR2, leading to tumor cell migration (Halpern et al. 2011; Welte et al. 2012), and such events could take place after the tumor cells attracted the stroma cells in their direction (Welte et al. 2012). Additional interactions were denoted between EC and the tumor cells, in which co-culturing of these two cell populations has induced CXCL8, and furthermore, Bcl-2 has induced in EC the release of CXCL8, leading to increased migration of the tumor cells (Neiva et al. 2009).

As already mentioned above, one additional very important activity of CXCL8, and in specific malignancies such as melanoma also of CXCL1, is induction of proliferation, anchorage-independent growth, increased survival and lower levels of apoptosis in the tumor cells. Several studies have shown that ELR^+ CXC chemokines, of which CXCL8 is the most prominent, promote proliferation and expression of cyclins such as cyclin D and E1, modifies cell cycle control, and shifts the balance between pro-apoptotic and anti-apoptotic proteins, eventually leading to inhibition of apoptotic processes. Moreover, these activities were detected not only by in vitro tests, but also in vivo, in tumors that have developed in mice (Dhawan and Richmond 2002; Shamaladevi et al. 2009; Singh and Lokeshwar 2009; Yang et al. 2010; Bandapalli et al. 2012; Wang et al. 2012c; Welte et al. 2012). As with other effects mediated by these chemokines, depending on the tumor context and experimental design, the direct activities of the chemokines were mediated by tumor cell-expressed CXCR1 or CXCR2, and in some cases by both receptors together (Shamaladevi et al. 2009; Singh et al. 2009a; Singh et al. 2009b; Gabellini et al. 2009; Zhong et al. 2008; Yang et al. 2010; Varney et al. 2011; Singh et al. 2010b; Ning et al. 2012; Singh et al. 2010b).

To follow on the above findings, it is interesting to note that CXCL8 and its proliferation-inducing functions have been shown to be of major relevance to cancer stem cells (CSC) [otherwise termed tumor-initiating cells (TIC)]. These cells were shown to express CXCL8, and the chemokine was up-regulated by elements such as neurotensin and the EMT-related protein snail (Tang et al. 2012b; Hwang et al. 2011), or after exposure to chemotherapy (Levina et al. 2008). Several studies reported the expression of CXCR1 and/or CXCR2 by these cells (Levina et al. 2008; Ginestier et al. 2010); CXCR1 blockade in breast CSC has led

to massive apoptosis in the bulk tumor population via FasL/Fas signaling, has reduced CSC in the tumors and has inhibited metastasis formation (Ginestier et al. 2010). Evidently, the production of CXCL8 by CSC was required for self renewal of these cells, and has contributed to tumor growth and angiogenesis (Tang et al. 2012b; Hwang et al. 2011).

The major tumor-promoting roles that were identified for ELR^+ CXC chemokines and their receptors in malignancy have been the basis for studies on the regulation of this axis. In the limits of the present chapter, we would illustrate the complexity of this issue by providing several representative examples only. First, an important aspect is the control of CXCL8 expression and activities by cells of the tumor microenvironment, to which several examples have been already given above. In addition, fibroblasts are major regulators of CXCL8 expression and activities. For example, co-culturing of tumor cells with fibroblasts (as is the case also for co-culturing with macrophages and EC), has given rise to substantial elevations in the release of CXCL1 and CXCL8 [depending on the cell system; (Zhong et al. 2008; Knowles et al. 2009; Tjomsland et al. 2011)]. Increased CXCL8 expression could be induced also through the activation of tumor-expressed c-Met by fibroblast-derived HGF (Knowles et al. 2009). The tumor-promoting activities of fibroblasts were mediated through CXCR2-dependent mechanisms (Ijichi et al. 2011).

Being inflammatory mediators that are regulated in the immune system by inflammatory cytokines, the expression of ELR^+ CXC chemokines by malignant cells was induced by cytokines such as tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β). It has been shown in the past that these two cytokines promoted the release of CXCL8 by tumor cells, and that TNFα acted on the tumor cells through CXCR1- and CXCR2-mediated autocrine loops (Kreeger et al. 2009; De Larco et al. 2001; Pantschenko et al. 2003). Furthermore, it was recently described by Massague's group that paracrine interactions, mediated via CXCR1, promoted resistance to chemotherapy and metastasis in breast tumors. In that study, genotoxic agents have limited the survival of cancer cells but also increased the production of TNFα by EC, which then has enhanced CXCL1 and CXCL2 expression in cancer cells. The chemokines recruited $CD11b^+$ $Gr1^+$ myeloid cells that expressed CXCR2, which in turn enhanced the viability of the cancer cells through S100A8/9 (calcium binding proteins that are associated with chronic inflammation and cancer) (Acharyya et al. 2012). In addition, another recent study suggested that induction of HIF-1α by IL-1β has led to increased tumor cell migration in a process possibly mediated by the CXCL8-CXCR1 pathway (Naldini et al. 2010).

Another interesting regulatory mode of CXCL8 which was characterized in breast cancer, is the associations between high CXCL8 expression and reduced expression of estrogen receptor α [(ERα); (Lin et al. 2004; Freund et al. 2003)], an indicator for poor prognosis in this disease. In contrast to ERα, estrogen is a powerful tumor-promoting factor in breast cancer, inducing tumor cell proliferation. Estrogen was shown to promote CXCL8 release by breast cancer cells (Yang et al. 2009; Bendrik and Dabrosin 2009; Haim et al. 2011), doing so in

cooperativity with epidermal growth factor (EGF) through combined activation of ERα and AP-1 (Haim et al. 2011). Furthermore, the EGF-signaling receptor ErbB2 induced the expression of ERβ in breast tumor cells, which has then led to elevations in CXCL8 (Chen et al. 2011).

In addition, it was found that constitutive activation of EGF receptor (EGFR, ErbB1) and the activation of Ras induced the expression of CXCL8 in tumor cells, as indicated in several research systems [e.g. (Cataisson et al. 2009; O'Hayer et al. 2009; Bonavia et al. 2012; Kim et al. 2011)]. Accordingly, inhibition of EGFR has potentiated the anti-tumor activities of antibodies against CXCL8 in a murine model of breast cancer (Salcedo et al. 2002). However, it was revealed that in non-transformed cells, constitutively activated Ras could induce the release of CXCL8 only when the protective activities of p53 were diminished (Leibovich-Rivkin et al. 2012). In addition, while in the non-transformed cells, down-regulation of p53 alone did not induce the release of CXCL8 in the absence of Ras hyperactivation (Leibovich-Rivkin et al. 2012), p53 mutant having gain-of-function characteristics has obtained powerful abilities to promote the expression of the chemokine in tumor cells (Fontemaggi et al. 2009; Yeudall et al. 2012).

To conclude, the information that has been obtained on the CXCR1/CXCR2-ELR$^+$ CXC chemokine axis in cancer strongly points to very prominent tumor-elevating roles of these components (Fig. 6.1), mediated mostly by increased angiogenesis and induction of tumor cell proliferation and invasion. Thus, this axis is an attractive target for inhibition, possibly with very many inhibitors that are now being developed. Here, in view of its being shared by all ELR$^+$ CXC chemokines and its roles in angiogenesis, CXCR2 may be a good candidate for inhibition. However, it is essential to further elucidate the mechanisms regulating the activities of this axis, and to find out whether its pro-senescence activities could be used in favor of the host, for the prevention of cancer progression.

6.2.2 CXCR3 Ligands

In response to chemokines of the non-ELR CXC subfamily, CXCR3-expressing leukocytes are recruited to infected and inflamed sites, including primarily Th1 and natural killer (NK) cells (Vandercappellen et al. 2008; Lacotte et al. 2009; Groom et al. 2010). In addition to the CXCR3 receptor that was originally identified, now termed CXCR3-A, two additional variants have been characterized in human cells but not in mice: CXCR3-B that has a longer N'-terminal extracellular domain due to alternative splicing (Lasagni et al. 2003), and CXCR3-alt that is truncated at the carboxyl terminus and is predicted to have four or five transmembrane domains (Ehlert et al. 2004) instead of the seven domains characterizing other GPCR.

CXCR3 binds the IFNγ-induced non-ELR CXC chemokines CXCL9, CXCL10 and CXCL11, which are typical inflammatory chemokines (Vandercappellen et al. 2008; Lacotte et al. 2009). In line with the high promiscuity of the chemokine

world, other interactions also exist: CXCL11 is a functional ligand of CXCR7 (Burns et al. 2006), and CXCL4 was proposed to signal through CXCR3-B (Lasagni et al. 2003). However, within the limits of this chapter, emphasis will be given to CXCR3 and its three predominant ligands—CXCL9, CXCL10, CXCL11 (to be called herein CXCL9-11) - which have been well studied in many malignancies [reviewed in (Vandercappellen et al. 2008; Keeley et al. 2008; Lacotte et al. 2009; Fulton 2009; Ben-Baruch 2007; Keeley et al. 2010; Groom et al. 2011)].

When one considers the roles of the CXCR3-CXCL9-11 axis in cancer, a very complex picture is obtained, demonstrating a typical "double-edged sword" mode of action. To provide a general view of the activities of CXCL9-11 in malignancy, we will first describe their potent anti-tumor properties, and then will discuss their opposite effects through which they support growth and progression of tumors.

Early studies on this axis in cancer have given rise to the "Immunoangiostasis" theory, based on observations showing that the three CXCR3 ligands have powerful anti-tumorigenic functions due to two complementing activities: The first is promotion of immune responses which are mediated mainly by CXCR3-expressing Th1 cells and NK cells, and the second is inhibition of angiogenesis (Keeley et al. 2008; Keeley et al. 2010). These two distinct activities of the CXCR3-binding non-ELR CXC ligands have been documented in a large number of malignant diseases, and were found to be pivotal in protection against tumor growth and metastasis, as has been previously reviewed (Vandercappellen et al. 2008; Keeley et al. 2008; Lacotte et al. 2009; Fulton 2009; Ben-Baruch 2007; Keeley et al. 2010; Groom et al. 2011). The prominent roles of CXCR3 and its ligands in anti-tumor activities were proven in many in vivo studies, using a number of inhibiting modalities or neutralizing antibodies. In parallel, studies of the last several years have revealed additional facets of the anti-tumor CXCR3-CXCL9-11 axis in cancer, showing that it is regulated by other factors of the tumor microenvironment, and by a complex net of interactions between many cells of the immune system, to be illustrated below.

Fundamentally, tumor cells are an important source for the chemokines, expressing them in response to IFNγ but also independently of this cytokine [e.g. (Wendel et al. 2008; Przewoznik et al. 2012; Zhu et al. 2010; Andersson et al. 2011; Bronger et al. 2012)]. Recent studies indicate that the ability of CXCR3 ligands to induce protective anti-tumor responses requires, in addition to infiltration of Th1 and NK cells to the tumors (Wendel et al. 2008; Fujihara et al. 2008; Przewoznik et al. 2012), the recruitment of $CD8^+$ T cells and CTL activities (Andersson et al. 2009; Zhu et al. 2010; Andersson et al. 2012; Hong et al. 2011; Wang et al. 2011). In addition, an important role was found to dendritic cells (DC) and antigen-presenting cells (APC) (Fujita et al. 2009; Andersson et al. 2012; Tanese et al. 2012). For example, in a murine glioma model, antigen-loaded Th1-polarizing DC were shown to induce CTL responses in a CXCL10-depenent mechanism, resulting in strong anti-tumor effects (Fujita et al. 2009). Moreover, several reports have suggested a role for CXCL9 and CXCL10 in reducing Treg (Andersson et al. 2009; Fujita et al. 2009; Muthuswamy et al. 2012), thus possibly

shifting the balance in favor of protective mechanisms. The importance of T cell infiltration was reinforced by recent studies showing that in melanoma patients, CXCL9 and CXCL10 were up-regulated in chemotherapy-sensitive lesions, and they correlated with T cell infiltration, improved tumor control, and improved patient survival (Hong et al. 2011).

The findings described above illustrate the impact of CXCR3 and its ligands on the balance between different leukocyte populations, and on their consequent ability to mount an effective immune response against tumors. However, recent studies suggest that in contrast to the inflammatory nature of CXCR3 ligands and their participation in physiologically-related inflammatory processes (Lacotte et al. 2009; Groom et al. 2011), these chemokines are suppressed at the setting of cancer-related inflammation. Thus, the inflammatory conditions that prevail in many tumors and are usually leading to enhancement of disease course, may lead to inhibition of anti-tumor immune activities. For example, the cyclooxygenase (COX) system that synthesizes prostaglandins (PGE), contributed to the inflammatory immune suppressive nature of the tumor microenvironment. Recent studies indicate that PGE2 reduced the release of CXCL9 and CXCL10 from tumor cells, and that inhibition of COX has led to up-regulation of these two chemokines, giving rise to increased attraction of T effector cells and reduced migration of Treg to the tumors (Bronger et al. 2012; Muthuswamy et al. 2012). The clinical relevance of these observations was exemplified by findings showing inverse correlation between COX-2 over-expression and CXCL9 levels in biopsies of breast cancer patients (Bronger et al. 2012). Also, the production of nitric oxide (NO) by iNOS—which are key molecules in the inflammatory nature of many malignancies—was found to inhibit the expression of CXCL10 in melanoma, and thus was suggested to lead to a pro-cancerous tumor milieu and to poor prognostic outcome (Tanese et al. 2012).

Being the second fundamental element of the immunoangiostatic activity of CXCR3 ligands, inhibition of angiogenesis has been shown in many tumor types [reviewed in (Vandercappellen et al. 2008; Keeley et al. 2008; Lacotte et al. 2009; Fulton 2009; Ben-Baruch 2007; Keeley et al. 2010; Groom et al. 2011)]. Using human EC, it was found that these chemokines have induced angiostatic effects by acting through CXCR3-B (Lasagni et al. 2003). A study of CXCR3-B transfected cells has revealed that the p38 MAPK pathway was a downstream effector of CXCR3-B, mediating the angiostatic action of this chemokine receptor (Petrai et al. 2008). Moreover, studies with CXCL10 mutants in human cells suggested that binding to CXCR3 and not GAG, was essential for the tumor angiostatic activity of this chemokine (Yang and Richmond 2004). However, the involvement of GAG in the angiostatic activities of these chemokines is currently under debate, because another study has shown that the angiostatic functions of CXCL10 could take place independently of CXCR3, in a mechanism requiring GAG (Campanella et al. 2010). Such a mechanism may very well explain the anti-angiogenic activities of the non-ELR CXC chemokines on mouse EC, which do not express CXCR3-B. Here, it is important to note that hetero-dimerization of the chemokines (specifically CXCL4) with angiogenic factors (e.g. basic fibroblast growth factor

(bFGF)) have been also shown to take place, and were suggested to contribute to inhibition of the angiogenic properties of factors such as bFGF, CXCL8 and VEGF (Keeley et al. 2008). The connections of the non-ELR CXC chemokines and the VEGF pathway were also illustrated in a renal cancer model, where intratumoral injection of CXCL9 combined with anti VEGFR2 therapy resulted in delayed resistance to the anti-angiogenic therapy, and had a beneficial impact of restoring angiostasis (Bhatt et al. 2010).

The observations described so far have illustrated the immunoangiostatic anti-tumorigenic functions of non-ELR CXC chemokines; however, the activities of the CXCR3-CXCL9-11 axis have been lately revealed to be more complex than originally expected, because the members of this axis can be used by the tumor cells for their own needs, and may therefore be deleterious. The members of this axis can promote malignancy by acting on cells of the tumor microenvironment, but mainly through direct activities on the tumor cells. In terms of the tumor milieu, recent studies suggest that cancer cells deviate immune cells from the protective phenotype, towards the pro-malignancy type. This has been shown in epidermal carcinogenesis, where it was suggested that recruitment of CXCR3-expressing $CD4^+$ and $CD8^+$ cells to the skin promoted keratinocyte proliferation (Winkler et al. 2011). In addition, in melanoma CXCL9 and CXCL10 induced disruption of endothelial cell barrier, possibly paving the way towards more efficient transendothelial migration of the tumor cells (Amatschek et al. 2010), that might result in enhanced invasiveness and metastasis formation.

In parallel, the non-ELR CXC chemokines act very potently on the tumor cells, and accordingly CXCR3 is expressed by many types of cancer cells as well (Fulton 2009; Ben-Baruch 2007; Ma et al. 2009; Cambien et al. 2009; Murakami et al. 2013). This has been shown not only in malignant cell lines, but also in clinical samples of cancer patients, where CXCR3 expression by the tumor cells was correlated with poor survival, and CXCR3 was higher in metastatic foci within lymph nodes and liver compared to primary tumors [e.g. (Ma et al. 2009; Murakami et al. 2013)].

The ectopic expression of CXCR3 on tumor cells may endow them selective advantages, as they may passively sequester chemokines that are anti-tumorigenic in nature. But probably this is not the whole story, because a growing number of studies indicate that the non-ELR CXC chemokines actively stimulate pro-malignancy functions in CXCR3-expressing tumor cells, leading primarily to increased tumor cell proliferation and migration (Fulton 2009; Ben-Baruch 2007; Cambien et al. 2009; Pradelli et al. 2009; Murakami et al. 2013; Liu et al. 2011; Lee et al. 2012b; Shin et al. 2011). In line with such cancer-enhancing activities, studies in animal models have revealed a direct and causative role for the CXCR3-CXCL9-11 axis in promoting metastasis formation in many cancer types (Fulton 2009; Ben-Baruch 2007; Cambien et al. 2009; Pradelli et al. 2009; Walser et al. 2006; Murakami et al. 2013). Moreover, following the CXCL10-induced recruitment of cancer cells to the bone, chemokines of this subfamily were found to support osteoclast differentiation and to promote the formation of osteolytic bone metastases (Lee et al. 2012a). Also, it was found that the migration of the tumor

cells may be enhanced by cells of their intimate milieu. For example, monocytes "conditioned" by co-culturing with human B cell precursor acute lymphoblastic leukemia cells released CXCL10, which in turn acted back on the tumor cells, and promoted their migration and invasion (Lee et al. 2012b).

The direct activities of the chemokines on the tumor cells bring about an important issue, related to the types of CXCR3 receptors expressed by the tumor cells, and their potential implications. In EC, CXCR3-B is the receptor inhibiting growth, while CXCR3-A increases survival (Lasagni et al. 2003). Along the same lines, recent data suggest that CXCR3-B is an "inhibitory" receptor, while CXCR3-A has an "inducing" phenotype not only in EC but also in tumor cells. Several reports indicate that CXCR3-B has anti-tumorigenic effects, and that inhibition of its activities lead to increased proliferation and migration of the tumor cells (Datta et al. 2006; Datta et al. 2008; Gacci et al. 2009; Datta et al. 2010).

To conclude, in this part of the chapter we have enlightened the complex nature of the CXCR3-CXCL9-11 axis in malignancy, having opposing activities that are difficult to expect in advance (Fig. 6.1). In many cancer types, contradicting results have been obtained, showing tumor-inhibiting as well as tumor-supporting activities for this axis. Eventually, the impact of CXCR3 and its ligands on disease course reflects equilibrium between effects that are exerted on many different cell types, not only of the tumor microenvironment but also directly on the tumor cells themselves. This equilibrium may be dictated by the differential response of specific target cells to the chemokines, by the ability of the chemokines to amplify the immune-potentiating modalities, and by the type of receptor expressed by different cell types.

The emerging literature on this topic indicates that the type of receptor—be it CXCR3-A, CXCR3-B and/or CXCR3-alt—is a crucial determinant of the overall impact of the CXCR3-CXCL9-11 axis in cancer. This fact has major implications, because it raises the possibility that it would be difficult to implement the findings obtained in mouse models—where CXCR3-B and CXCR3-alt are not expressed—to human patients. Together with findings suggesting that CXCR3-A is the receptor mediating the recruitment of anti-tumor Th1, CTL and NK cells, it is possible that the equilibrium in mouse models is biased to the immune-potentiating arm of CXCR3-CXCL9-11 activities. If so, this would suggest that the cancer-promoting activities of CXCR3 ligands are more potent than currently assumed based on murine models.

The above information leaves us with uncertainty regarding the therapeutic implications of the CXCR3-CXCL9-11 axis in cancer. To date, studies in animal model systems have used a large diversity of approaches whose aim was to increase the activities of CXCR3 ligands in malignancy, believing that such modalities would strengthen immunoangiostasis. However, the pro-malignancy activities of this axis suggest that implementing such approaches in human clinical settings may pose tumor-promoting threats. Thus, the picture in this case is far from being resolved, emphasizing the need for improved research and understanding of the complex implications of this axis in cancer.

6.2.3 CCR2 Ligands

CCR2 is best known for its major roles in mediating the migration of monocytes in response to chemokines of the CC group, specifically those termed monocyte chemotactic proteins (MCP). These chemokines share structural and genetic properties and thus have much in common in terms of target cell specificity; however, their in vivo activities do not fully overlap, possibly due to their different expression patterns in the organism and because some of them use other receptors besides CCR2 (Allen et al. 2007; Mantovani et al. 2006; Zlotnik et al. 2006; Conti and Rollins 2004; Yadav et al. 2010).

Of the different members of this group—that includes the chemokines CCL2, CCL7, CCL8, CCL13 (and murine CCL12)—CCL2 is the most potent activator of CCR2. Accordingly, the CCR2-CCL2 pair is the one dictating many of the biological responses in which monocytes are involved. In parallel to its strong impact on monocyte migration, CCL2 induces chemotactic responses also of DC, NK cells and T lymphocytes (Conti and Rollins 2004; Deshmane et al. 2009; Yadav et al. 2010).

In view of their fundamental roles in regulating monocyte recruitment, CCL2 and other MCP chemokines are essential for mounting effective physiological inflammatory responses, but they are also strongly involved in diverse pathological conditions (Deshmane et al. 2009; Yadav et al. 2010). Specifically in cancer, elevated expression of MCP chemokines and mainly of CCL2 was denoted in different malignancies; the chemokines were associated with advanced disease course and metastasis, as was shown to be the case for example in breast, colorectal and gastric cancers, while chemokine expression was hardly detected in normal epithelial cells (Soria et al. 2008; Ben-Baruch 2012a; Soria and Ben-Baruch 2008; Fujimoto et al. 2009; Hu et al. 2009; Ueno et al. 2000; Soria et al. 2011; Hwang et al. 2012).

These observations have led many investigators to question the roles of the MCP chemokines in malignancy, and whether they exert anti-tumor responses as would have been expected from the leukocyte target cells they act on. In the malignancy context, most studies have focused on the CCR2-CCL2 pair because of its outmost effects on monocyte migration. Several studies revealed anti-malignancy activities for this pair, increasing the potency of tumor vaccines or chemotherapy (Huang et al. 1994; Manome et al. 1995; Rollins and Sunday 1991; Tsuchiyama et al. 2008; Berencsi et al. 2011); however, these investigations are outnumbered by numerous findings providing evidence to a strong pro-tumorigenic impact of CCR2 and CCL2 in cancer [reviewed in (Keeley et al. 2008; Yadav et al. 2010; Conti and Rollins 2004; Deshmane et al. 2009; Ben-Baruch 2012a; Soria and Ben-Baruch 2008; Craig and Loberg 2006; Yadav et al. 2010; Ben-Baruch 2012b; Verma et al. 2012)].

Improved understanding of the roles played by CCR2-CCL2 in malignancy was provided by studies in tumor model systems in mice. Here, the different investigations used knockout (KO) of CCR2 in the animals, CCR2 antagonists and to

other modalities that reduced CCR2 or CCL2, such as siRNA/shRNA or neutralizing antibodies [examples of the last several years: (Hart et al. 2009; Lu and Kang 2009; Pahler et al. 2008; Popivanova et al. 2009; Koga et al. 2008; Mizutani et al. 2009; Baba et al. 2012; Izhak et al. 2012; Leuschner et al. 2011; Nakasone et al. 2012; Wolf et al. 2012; Chiu et al. 2012; Cortez-Retamozo et al. 2012; Fridlender et al. 2011; Jin et al. 2010; Lesokhin et al. 2012; Park et al. 2012; Qian et al. 2011; Tsuyada et al. 2012; Wang et al. 2012a)]. Joined by over-expression approaches (Lu and Kang 2009; Mizutani et al. 2009; Stathopoulos et al. 2008), these studies pointed out to direct and causative roles for CCR2 and CCL2 in elevating malignancy and metastasis formation, and have revealed many of their mechanisms of action.

Based on murine studies and clinical investigations, it is now clear that the CCR2-CCL2 pair promotes malignancy primarily by shifting the immune balance towards leukocyte sub-populations that support tumor growth and metastasis. Acting primarily on myeloid cells, CCL2 has the strongest impact on disease course by recruiting and activating two myeloid sub-populations: the first is of monocytes that turn at the tumor site to tumor-associated M2 macrophages (TAM), which have been long ago characterized as cells capable of releasing a large variety of tumor-supporting factors (Allavena et al. 2008; Biswas and Mantovani 2010); the second sub-population is of MDSC, recently identified for their ability to down-regulate potential anti-tumor T cell activities (Murdoch et al. 2008; Greten et al. 2011; Youn and Gabrilovich 2010). The shift in immunological balance imposed by CCR2-CCL2 is achieved by elevated levels of these two cell populations, in parallel to reduced activities of CTL and additional modifications in other leukocyte sub-populations, as would be described below (for specific references—see below).

As indicated above, many lines of evidence indicate that the CCR2-CCL2 axis plays major roles in positioning of monocytes at tumor sites, and leads to their skewing to the M2 phenotype, eventually giving rise to high presence of TAM in the tumors and potentiating tumor growth (Fujimoto et al. 2009; Ueno et al. 2000; Pahler et al. 2008; Popivanova et al. 2009; Koga et al. 2008; Mizutani et al. 2009; Stathopoulos et al. 2008; Allavena et al. 2008; Leuschner et al. 2011; Nakasone et al. 2012; Wolf et al. 2012; Cortez-Retamozo et al. 2012; Fridlender et al. 2011; Biswas and Mantovani 2010; Diaz-Valdes et al. 2011). In addition, driven by the CCR2-CCL2 axis, monocyte recruitment is also fundamental to metastasis formation (Lu and Kang 2009; Qian et al. 2011). For example, the study by Pollard and his colleagues has shown that CCR2-expressing inflammatory monocytes infiltrated breast metastasis, in response to CCL2 synthesized by the tumor cells and by stroma cells (Qian et al. 2011). That study also indicated that the CCL2-recurited monocytes promoted the metastatic seeding of the tumor cells, doing so in a VEGF-mediated manner (Qian et al. 2011). Also, Part et al. have shown that following priming of the murine host with the chemotherapeutic agent cyclophosphamide, an abrupt expansion of myeloid cells has taken place in the bone marrow and the circulation, and has promoted metastasis formation. This process

was mediated by host-derived CCL2, whose inhibition significantly reduced the pro-metastatic effects of cyclophosphamide (Park et al. 2012).

Alongside with the above findings, much evidence has been recently provided on the infiltration of tumors by CCR2-expressing MDSC, whose activities have led to suppression of CTL responses against tumor cells. The recruitment and/or activation of MDSC were shown to be induced by CCL2, and also by tumor cell-derived β-defensin 3 (Hart et al. 2009; Huang et al. 2007; Umemura et al. 2008; Lesokhin et al. 2012; Gehad et al. 2012). A recent study of ovarian tumor progression has shown that two monocyte subsets, differing in their markers, were present at the peritoneum at different tumor stages. These two monocyte subpopulations had immune suppressive activities towards naïve $CD8^+$ and $CD4^+$ T cells. CCR2 was a critical factor in recruiting these suppressive cells to the ovarian tumor microenvironment, as indicated by genetic ablation of CCR2 in the mice, leading to lower tumor burden (Hart et al. 2009). Moreover, CCR2-expressing MDSC limited the efficacy of immune-therapy by down-regulating the migration of $CD8^+$ T cells to the tumor site (Lesokhin et al. 2012). Also, in parallel to inducing monocyte polarization to the M2 phenotype, CCL2 was connected to reduced levels of active $CD8^+$ CTL (Fridlender et al. 2011).

These recent studies have also shown that the inhibitory activities of MDSC were mediated through the cytokine TGFβ, and also via the activation of arginase-1 and production of NO (Hart et al. 2009; Umemura et al. 2008; Lesokhin et al. 2012; Gehad et al. 2012), which are important mediators of T cell inhibition (Greten et al. 2011; Youn and Gabrilovich 2010; Schaer et al. 2011). To follow on the above, the tumor-related activities of CCL2 were regulated by its nitration (Molon et al. 2011). The recent study by Viola's group has shown that reactive nitrogen species (RNS) induced nitration of CCL2, and that this form of the chemokine was ineffective in inducing T cell infiltration to tumor sites. Accordingly, prevention of RNS production has improved intratumoral T cell migration, and has enhanced tumor eradication through CTL-mediated responses (Molon et al. 2011).

Adding to the suppressive influence of MDSC on anti-tumor cell responses, the CCR2-CCL2 axis contributed to the generation of tolerized DC: Tumor-bearing mice deposited CCL2 in interlobular vascular-rich regions of the thymus, where Sirpα+ conventional DC have accumulated. The CCR2-CCL2 pair was involved in enhanced capacity of the DC to take up antigens, resulting in a shift to negative selection (Baba et al. 2012).

As expected from its inflammatory nature, CCL2 is also associated with the inflammatory phenotype of the tumor microenvironment. CCL2 was found to induce the recruitment to tumors of Th17 cells (Su et al. 2010), considered as cells that may contribute to the pro-malignancy and inflammatory nature of the tumor milieu. Interactions between CCL2 and inflammatory factors residing at the tumor microenvironment have also been revealed: TNFα and IL-1β were shown to promote the release of CCL2 by tumor cells [e.g. of the breast (Neumark et al. 2002; Neumark et al. 2003; Seeger et al. 2006; Seeger et al. 2008; Soria et al. 2011)]; furthermore, the ability of the CCR2 antagonist 7ND to inhibit tumor

growth and monocyte infiltration to tumors was accompanied by reduced expression of the inflammatory cytokines TNFα and IL-1α, presumably from monocytes (Koga et al. 2008). Further adding to the network that may exist between CCL2, TNFα and IL-1β are findings showing that in breast cancer patient biopsies, TNFα and IL-1β expression in the tumor cells was coordinated with high abundance of CCL2 throughout different stages of disease (Soria et al. 2011).

Relating further to the CCR2-CCL2-induced immune imbalance is the impact of this axis on neutrophil responses (Pahler et al. 2008; Granot et al. 2011; Cortez-Retamozo et al. 2012). As previously mentioned, the implications of neutrophils on malignancy are not yet fully resolved; however, improved insights to this issue were recently provided by the study of Granot et al. 2011 described above, indicating that CCL2 secretion has enhanced tumor growth at the primary site, but at the same time the chemokine took role in neutrophil entrainment, so that neutrophils exerted an anti-metastatic response that inhibited tumor cell seeding at distant sites (Granot et al. 2011).

Overall, modification of immunological balance is a major pathway through which the CCR2-CCL2 axis diverts the immune-environment towards the pro-malignancy phenotype. However, CCL2 has additional activities on the tumor microenvironment, mediated by inducing angiogenesis and bone osteolysis, thus contributing to metastasis formation. Several recent reviews and updated publications documented the strong angiogenic activities of CCL2 in many tumor systems [e.g. (Keeley et al. 2008; Conti and Rollins 2004; Stathopoulos et al. 2008; Salcedo et al. 2000; Goede et al. 1999; Niu et al. 2008; Verma et al. 2012; Izhak et al. 2010)]. Here, the chemokine was shown to act indirectly by promoting the abundance of TAM at tumor sites. These cells were correlated with increased angiogenesis due to their ability to release a large variety of angiogenic factors, such as VEGF (Conti and Rollins 2004; Stathopoulos et al. 2008; Allavena et al. 2008; Goede et al. 1999; Verma et al. 2012; Qian et al. 2011; Biswas and Mantovani 2010; Izhak et al. 2010). Also, it has been well established that EC express CCR2, and that CCL2 directly promotes angiogenesis by inducing the proliferation and migration of these cells (Keeley et al. 2008; Niu et al. 2008; Weber et al. 1999; Wang et al. 2012a; Roy and Kolattukudy 2012). These direct activities were mediated by the transcription factor MCP-1-induced protein (MCPIP) (Niu et al. 2008; Roy and Kolattukudy 2008). A recent study by Roy & Kolattukudy suggested that MCPIP induced EC differentiation via induction of oxidative stress that has led to endoplasmic reticulum stress, and thereafter to autophagy which was involved in tube formation (Roy and Kolattukudy 2008).

An additional important tumor-promoting mechanism induced by CCL2 is osteoclastogenesis and bone loss, demonstrated for example in breast cancer [reviewed in (Ben-Baruch 2012a; Soria and Ben-Baruch 2008; Craig and Loberg 2006)]. CCL2 was shown to regulate pathological conditions in the bone, to exhibit chemotactic activities towards osteoclasts and to be expressed by osteoblasts (Craig and Loberg 2006; Fritz et al. 2002; Kinder et al. 2008; Wright and Friedland 2004; Bussard et al. 2010a; Bussard et al. 2010b). These observations have led researchers to speculate that CCL2 activities may be connected to the fact

that the bone is a preferred metastatic site in breast cancer, which is highly colonized by the tumor cells. Indeed, studies performed in this direction have led to the conclusion that CCL2 is responsible for "conditioning" of the bone microenvironment (Kinder et al. 2008; Chen et al. 2009; Molloy et al. 2009; Zhu et al. 2007; Bussard et al. 2012a). The release of CCL2 by osteoblasts, in response to cancer cells, has led to osteoclast activation and to bone loss (Kinder et al. 2008; Zhu et al. 2007), thus creating a unique niche that favored breast tumor growth. This was indicated by the fact that metastasis in the bone was correlated with osteoclast formation and recruitment of osteoclasts to the bone, and that CCL2-expressing tumor cells engaged $CCR2^{+}$-expressing monocytic cells, including preosteoclasts and macrophages, leading to elevated tumor cell localization in the bone (Lu and Kang 2009; Mizutani et al. 2009). In addition, it was found that MSC that have matured to osteoblasts released CCL2 that has induced migration of breast tumor cells (Molloy et al. 2009), and it was proposed that the angiogenic activities of CCL2 potentiated vascularization at the bone (Wilson et al. 2010).

Overall, the findings described above illustrate the pro-cancerous effects of the CCR2-CCL2 axis on the tumor microenvironment, in primary tumors and metastatic sites. However, CCR2 is also expressed by the tumor cells. Accordingly, CCL2-mediated signals promoted tumor cell migration, invasion and MMP production [examples: (Kawai et al. 2009; Mestdagt et al. 2006; Nam et al. 2006; Youngs et al. 1997; Chiu et al. 2012; Tang and Tsai 2012a)]. Although such activities of CCL2 are important and may have substantial roles in elevating metastasis, the major effects of the CCR2-CCL2 pair are dictated by its ability to act on immune cells and stroma cells, and less so by its direct activities on the tumor cells (Fig. 6.1).

The above-mentioned findings set CCR2 and its MCP ligands—of which CCL2 is the most prominent—as attractive therapeutic targets in malignancy. To date, a large variety of approaches are available for inhibition of this axis, including CCR2 antagonists, antibodies and siRNA modalities that were used in animal model systems. The efficacy of these measures in some of the experimental systems was strong, but in others less pronounced, emphasizing the challenge that we are about to face when trying to introduce these applications to the clinic. In addition, in view of the major roles of CCR2 in regulating monocyte recruitment to inflammatory sites, it may be expected that inhibition of this receptor would yield undesired side effects on immune activities following exposure to pathogens.

6.2.4 CCR5 Ligands

CCR5 is a promiscuous receptor that binds several chemokines of the CC family, including CCL3, CCL4 and CCL5. CCR5 and these three ligands are only part of a more complex net of interactions existing between these chemokines, several additional chemokines and CCR5, CCR1 and CCR3 (Levy 2009; Oppermann 2004; Mueller and Strange 2004). However, most of the studies related to these

chemokines and their receptors in solid malignancies have focused on the CCR5-CCL5 pair. In parallel, in hematological cancers and mainly in multiple myeloma (MM), most findings were obtained with CCL3 (see below). Therefore, this chapter will discuss mainly the CCR5-CCL5 axis in cancer, and will also describe CCL3 roles in promoting MM.

CCR5 is expressed by a large number of leukocytes, primarily effector T cells, monocytes and macrophages. Accordingly, CCR5 and its ligands play important regulatory roles in infection and inflammation. CCR5 has been extensively studied for its impact on immune activities, and has been at the center of AIDS research because of its being an HIV co-receptor. Particular interest has been put on the mutated Δ32 CCR5 receptor which is not expressed at the cell surface, providing protection against R5 HIV viruses (Levy 2009; Oppermann 2004; Mueller and Strange 2004); by analyzing this mutation of CCR5 in cancer, efforts were made to decipher the roles of this receptor in malignancy, as will be discussed below.

The key roles played by CCL5-induced leukocyte chemotaxis in protective immunity have given rise to new research directions in malignancy. Based on the premises that the chemokine may be mediating anti-tumor effects but that such activities are actually suppressed in cancer, hope was raised that potentiation of the CCR5-CCL5 axis may inhibit tumor formation and metastasis. As a result, the chemokine, and its three receptors were studied in many cancer diseases, where their expression patterns, roles and modes of action have been partly identified thus far [reviewed in (Ben-Baruch 2012a; Soria and Ben-Baruch 2008; Soria 2009; Ben-Baruch 2012b; Lapteva and Huang 2010; Suffee et al. 2011)].

As expected, CCL5 and its receptors have been found to lead to protective anti-cancer immunity when appropriate stimulatory conditions were provided. Specifically, when the activities of DC and lymphocytes were boosted by a variety of manipulations, CCL5 acted as an adjuvant that has potentiated anti-tumor activities, primarily those mediated by T lymphocytes [(Lapteva and Huang 2010); specific examples: (Nesbeth et al. 2009; Song et al. 2009; Inoue et al. 2008; Nesbeth et al. 2010; Gonzalez-Martin et al. 2011)]. These findings suggest that conditioning of the tumor microenvironment can serve as a good platform in which CCL5 can strengthen anti-cancer activities; however, they also testify to "failure" of acquired immunity to act against arising tumors, possibly because of tumor-induced suppression of protective immune mechanisms. Along these lines, associations between high expression levels of CCL5 in tumors, protective immune infiltrates and improved disease course are not many, suggesting that in most cases the potential beneficial effects of the CCR5-CCL5 axis are not appropriately activated in cancer.

Rather, it has been revealed that very often the tumor cells take advantage of CCR5 and of its corresponding ligands. Dominating the field are studies on CCR5-CCL5-mediated responses, showing that this axis is skewed in cancer to the pro-malignancy phenotype, and that these two components actively promote tumor growth and spread (Ben-Baruch 2012a; Soria and Ben-Baruch 2008; Soria 2009; Ben-Baruch 2012b; Lapteva and Huang 2010; Suffee et al. 2011). Many studies indicate that cancer cells constitute a major source for the chemokine and that its

elevated levels are associated with poor prognosis and advanced disease [Reviewed in (Ben-Baruch 2012a; Soria and Ben-Baruch 2008; Soria 2009; Ben-Baruch 2012b); specific publications as example: (Soria et al. 2008; Sugasawa et al. 2008a; Sugasawa et al. 2008b; Tan et al. 2009; Wu et al. 2008a; Wigler et al. 2002; Azenshtein et al. 2002; Luboshits et al. 1999; Yaal-Hahoshen et al. 2006; Borczuk et al. 2008; Zhang et al. 2009; Soria et al. 2012; Su et al. 2010; Velasco-Velazquez et al. 2012; Chang et al. 2012a; Soria et al. 2011)]. In addition, many cells of the tumor microenvironment can contribute to high CCL5 load at the tumor site, including leukocytes, EC, fibroblasts and MSC [e.g. (Sugasawa et al. 2008a; Laubli et al. 2009; Karnoub et al. 2007; Su et al. 2010; Mi et al. 2011; Gallo et al. 2012)].

The direct and causative pro-malignancy functions of the CCR5-CCL5 pair were proven in a large number of studies using animal model systems. Here, when different approaches were taken to reduce the expression or activities of each of these two components, tumor load and metastasis formation were significantly inhibited, and survival was often increased. The CCR5/CCL5 inhibitory modalities that were used included siRNA/shRNA to CCR5 or CCL5, neutralizing antibodies against these components, the CCR1/CCR5 antagonist met-CCL5, CCR5 KO mice and different pharmacological inhibitors of CCR5 such as maraviroc and TAK-779 [representative studies, of the last several years: (Sugasawa et al. 2008a; Wu et al. 2008a; Borczuk et al. 2008; Laubli et al. 2009; Karnoub et al. 2007; Adler et al. 2003; Mi et al. 2011; Robinson et al. 2003; Velasco-Velazquez et al. 2012; Chang et al. 2012a; Chang et al. 2012b; Song et al. 2012; Cambien et al. 2011; Wang et al. 2012b)].

Together with extensive in vitro analyses and studies of patient samples, two non-mutually exclusive tumor-promoting pathways have been identified for the CCR5-CCL5 axis, one acting on cells of the tumor microenvironment and the other directly affecting the malignant cells. When the intimate milieu of the tumor cells is concerned, a major pro-tumoral activity of CCL5 is mediated by its chemotactic properties towards leukocytes. Evidently, CCL5 changes the balance between different types of leukocyte infiltrates, leading to predominance of cells whose activities support malignancy, rather than exerting anti-tumor immune activities.

Supporting such tumor-promoting roles of CCL5 are studies showing that the chemokine has led to increased accumulation of Treg in the tumors. These studies have also shown that Treg recruitment was dependent on CCR5 expression by these cells, and that inhibition of this process has given rise to reduced tumor growth (Tan et al. 2009; Chang et al. 2012a; Chang et al. 2012b). In parallel, apoptosis of $CD8^+$ T cells and suppression of their activities were denoted (Sugasawa et al. 2008a; Chang et al. 2012a; Chang et al. 2012b). In the specific case of colorectal cancer, CCR5-CCL5 signaling increased the synthesis of TGFβ in Treg cells, in turn enhancing the cytolysis by $CD8^+$ T cells (Chang et al. 2012a). Emphasizing the roles of the CCR5-CCL5 axis in down-regulating the potential anti-tumor activities of $CD8^+$ T cells are studies showing that inhibition of this pair leads to increased presence of cytotoxic T cells at tumor sites, and that

reduced suppression of $CD8^+$ cells was associated with lower tumor volume (Chang et al. 2012a; Song et al. 2012; Chang et al. 2012b).

Studies on tumor microenvironments indicate that immune suppression and inflammatory conditions are often connected in malignancy (Ben-Baruch 2006). Accordingly, the CCR5-CCL5 axis is involved not only in mediating immune suppression, but also is associated with tumor inflammation. By virtue of its strong chemotactic activities towards monocytes, the CCR5/CCR1-CCL5 axis actively induced high presence of TAM in the tumors, and elevated macrophage content was correlated with high malignancy [e.g. (Ben-Baruch 2012a; Soria and Ben-Baruch 2008; Wu et al. 2008a; Laubli et al. 2009; Adler et al. 2003; Robinson et al. 2003; Wu et al. 2008b)]. Moreover, CCL5 released from tumor cells and from tumor-derived fibroblasts has been shown to induce the migration of Th17 cells, and thus may contribute to the inflammatory nature of the tumor milieu by yet another aspect (Su et al. 2010). In addition, the expression of CCL5 by tumor cells was up-regulated by inflammatory cytokines such as TNFα and IL-1β [e.g. (Soria et al. 2008; Azenshtein et al. 2002; Ali et al. 2000)]. By doing so, the inflammatory cytokines promoted the abundance of CCL5 at the tumor site, and accordingly recent findings demonstrated associations between CCL5, TNFα and IL-1β along malignancy course in breast cancer (Soria et al. 2011).

The above findings testify for the ability of the CCR5-CCL5 pair to skew the immune balance towards immune/inflammatory activities that support malignancy. Recent studies show that CCL5, CCR5 and other chemokines/receptors associated with them, control additional cells and events at the tumor site. Recent findings point to novel and yet minimally addressed angiogenic functions of CCL5 (Wu et al. 2008a; Azenshtein et al. 2002; Suffee et al. 2011; Suffee et al. 2012). Specifically, CCL5 was shown to have angiogenic effects that were mediated by VEGF, in mechanisms depending on CCR5, CCR1 and GAG (Suffee et al. 2012).

Here, it is important to note that the studies of hematological malignancies have provided evidence to additional cancer cell-microenvironment interactions—specifically osteoclastogenesis—that were induced by CCL5, and also very strongly by CCL3. Particularly in MM, tumor cell-derived CCL3 was shown to act on osteoclasts and promote osteoclastogenesis (Reviewed in Soria 2009). Moreover, it was recently demonstrated that CCL3 repressed mineralization and osteocalcin production by primary human bone marrow stromal cells. A CCL5/CCL3-CCR5/CCR1-depenedent process promoted the migration of MM cells to the bone marrow (Reviewed in Soria 2009); more recent references (Dairaghi et al. 2012; Vallet et al. 2011). By acting in these two complementary pathways, CCL3, CCL5 and their corresponding receptors play major roles in potentiating disease course in MM.

The diverse tumor-promoting functions of CCL5 on the tumor microenvironment are complemented by direct stimulation of cancer cells, leading to increased tumor cell proliferation and of migratory/invasive functions that are required for metastasis. Although, in general, the extent to which CCL5 promotes tumor cell proliferation is not very high, such effects were reported in quite a large number of tumor cell types [e.g. (Sugasawa et al. 2008a; Aldinucci et al. 2008; Murooka et al. 2009;

Cambien et al. 2011; Zhang et al. 2010)]. CCL5 was also shown to increase the proportion of $CD44^+/CD24^-$ breast CSC (Zhang et al. 2009), thus revealing yet another important mode of activity that may support malignancy. Of the three CCL5 receptors, it was mainly tumor-cell expressed CCR5 that mediated the proliferating activities of the chemokine (Aldinucci et al. 2008; Murooka et al. 2009; Cambien et al. 2011; Zhang et al. 2010). In line with such roles of CCR5, melanoma tumors which have been developed in CCR5 KO mice were enriched with apoptotic proteins, whereas the expression of survival proteins was reduced (Song et al. 2012). Also, CCL5 activities have been shown to induce the mTOR pathway of increased translation, leading to elevated protein expression for cyclin D1, c-Myc and Dad-1, without affecting their mRNA levels (Murooka et al. 2009).

As already indicated, another key determinant of the pro-tumoral activities of CCR5-CCL5 is induction of tumor cell migration and invasion. This important mode of action has been established with respect to CCL5 already at the very initial stages of research on its roles in malignancy (Ben-Baruch 2012a; Soria and Ben-Baruch 2008; Soria 2009; Ben-Baruch 2012b). Recent studies provide further evidence to this CCL5 function, in many different model systems [e.g. (Zhang et al. 2009; Karnoub et al. 2007; Pinilla et al. 2009; Makinoshima and Dezawa 2009; Velasco-Velazquez et al. 2012; Mi et al. 2011; Gallo et al. 2012; Cambien et al. 2011; Wang et al. 2012b)]. In line with the above, CCR5- and CCL5-induced tumor cell migration and invasion have been shown to be causally linked with elevated metastasis in mice (Karnoub et al. 2007; Velasco-Velazquez et al. 2012; Mi et al. 2011; Cambien et al. 2011). From the mechanistic point of view, it was demonstrated that the roles of CCL5 in invasion were regulated by c-Myc, and roles for $\alpha V\beta 3$ integrins and MMP were found in such processes. Actually, MMP have been induced by CCL5 not only in tumor cells, but also in EC and leukocytes (Wu et al. 2008a; Azenshtein et al. 2002; Chuang et al. 2009; Cappellen et al. 2007; Wang et al. 2012b; Suffee et al. 2012). Here again, induction of migratory and invasive properties in the tumor cells was mostly mediated by tumor cell-expressed CCR5 (Borczuk et al. 2008; Velasco-Velazquez et al. 2012; Wang et al. 2012b). Of interest in this respect are recent findings showing that CCR5 expression and CCL5-induced migration were more prominent in $CD44^+/CD24^-$ breast CSC (Zhang et al. 2009).

Another important aspect related to CCL5-induced tumor cell invasion is that the process can be mediated not only by tumor cell-derived CCL5, but also by CCL5 produced by stroma cells, such as MSC. Following co-culturing with tumor cells, MSC have produced elevated levels of CCL5, that in turn has up-regulated the migratory properties of the tumor cells, in a process leading to increased metastasis formation (Karnoub et al. 2007; Pinilla et al. 2009; Mi et al. 2011; Gallo et al. 2012). In breast cancer, this enhanced metastatic ability was reversible and depended on CCL5 signaling through CCR5 (Karnoub et al. 2007). Another study of breast cancer has shown that osteopontin-CCL5 interactions between MSC and tumor cells contributed to metastasis formation (Mi et al. 2011).

Overall, CCL5 (and other CCR5 ligands) act on cells of the tumor microenvironment and on the tumor cells in many different mechanisms, leading to

elevation in malignancy-related properties. Specifically concerning CCR5, hopes were raised that the Δ32 mutation in this receptor will provide improved understanding of the roles played by CCR5 in malignancy. Many studies in this respect were performed in breast cancer; however, non-conclusive findings were obtained [reviewed in (Ben-Baruch 2012a; Soria and Ben-Baruch 2008)]. Nevertheless, evidence for dominance of CCR5 over CCR3 and CCR1 in mediating CCL5 activities in cancer has emerged from a large number of studies (some cited above), suggesting that CCR5 is indeed the receptor mediating most of the tumor-promoting activities of CCL5.

To conclude, the above summary suggests that the CCR5-CCL5 pair is diverted by tumor cells to entities with ability to improve tumor growth and metastasis. The potential anti-tumor activities of immune cells which may have been recruited to the tumor site are inhibited, and the activities of the CCR5-CCL5 pair skew the tumor microenvironment towards immune suppression and inflammatory nature (Fig. 6.1). These mechanisms are complemented to some extent by the ability of CCR5-CCL5 to promote angiogenesis and tumor cell proliferation, and are strengthened mainly by the pivotal roles of this axis in promoting tumor cell invasion (Fig. 6.1). Accordingly, the activities of CCL5 and CCR5 have been found not only to actively promote tumor growth, but also to advance the most important and devastating step of metastasis (Wu et al. 2008a; Borczuk et al. 2008; Karnoub et al. 2007; Velasco-Velazquez et al. 2012; Mi et al. 2011; Song et al. 2012; Cambien et al. 2011; Wang et al. 2012b; Zhang et al. 2010).

As a whole, these findings pose the CCR5-CCL5 axis, and also CCL3 as preferable therapeutic targets in cancer. This research direction is attractive because of the extensive efforts taken by pharmaceutical companies to produce CCR5 antagonists for use in AIDS, and because of the availability of maraviroc for clinical use (Wasmuth 2012). However, when the complex roles of CCL5 in malignancy are taken into account, it is expected that CCR5-CCL5/CCL3 shut-off would lead to inhibition of potential protective immune mechanisms that could have been possibly effective against the tumor, primarily following appropriate boosting of the immune system.

6.3 Concluding Remarks

This review has demonstrated the powerful cancer-promoting activities of inflammatory chemokines, and their multiple impacts on the tumor microenvironment and on the cancer cells. These factors, that in principle could have protected the individual against the arising and developing tumor, are being used by the tumor cells for their own propagation, motility and spread (Fig. 6.1). To translate these findings in the clinic, we need to develop improved manners for targeting the chemokine-related pathways. Here, it is important to remember that many chemokines are expressed simultaneously at the tumor site, thus we need to

shift the equilibrium between different chemokines and their receptors, so that several pathways will be affected concomitantly.

In view of the pro-tumoral impacts of the inflammatory chemokines in cancer, we face two major challenges. The first is to identify modes for skewing the immune balance back towards the anti-malignancy phenotype. To this end, it is essential to identify the tumor-chemokine networks operating in each tumor type. Based on this information, we need to develop means for potentiating the migration of Th1, CTL and NK cells to the tumors, while inhibiting inflammatory infiltrates of monocytes, MDSC, neutrophils and Treg that exert tumor-promoting functions. However, as is the case with many other components of the immune system, these goals are difficult to achieve. To give an example, potentiation of Th1 and NK activities might be achieved by elevating the expression of CXCR3 or CCR5 ligands; however, these same chemokines could act on the tumor cells, to induce their proliferation and migration.

The second challenge is to develop means that will block chemokine receptors on deleterious immune cells and on tumor cells. Because of the functional redundancies of chemokines belonging to the same sub-group, their receptors may be superior therapeutic targets. Here, we need to understand which of the chemokine receptors are mediating most potently the abilities of the chemokines to induce tumor cell proliferation and invasion, and what are the regulatory pathways controlling these events. But again, difficulties are expected in implementing such measures. For example, modalities that will block CXCR1/CXCR2-, CCR2- or CCR5-mediated responses may inhibit the pro-tumor activities of their ligands on the cancer cells and possibly inhibit the recruitment of detrimental myeloid cells to the tumor site; however, simultaneously they may prevent the functions of monocytes, neutrophils and T cells that may have potential protective activities.

Thus, changing the balance between different chemokine types or targeting specific chemokine receptors may prove beneficial in terms of tumor eradication and inhibition of metastasis, but may prove counter-productive if the same measures would inhibit anti-tumor processes. In addition, such interventions in immune activities may decrease the ability of the host to mount protective immune and inflammatory responses against pathogens. Here, it is possible that deleterious effects would be partly prevented due to the promiscuity and redundancy of the "chemokine world", because specific chemokine-receptor axes can be backed up by others. Nevertheless, the numerous difficulties expected in implementing chemokine-based therapeutics in cancer would require new approaches, which will enable direct targeting of chemokines or chemokine receptors in specific cells, thus increasing specificity and reducing undesired side effects.

To conclude, inflammatory chemokines are highly relevant factors in malignancy, they have most important impacts on disease course, and are potential targets for therapeutic implications. While our understanding of these factors has been dramatically improved over the last several years, much is still to be learnt about the tumor microenvironment-chemokine network. Moreover, in view of the complex and redundant nature of the "chemokine world" and of inflammatory chemokines in particular, there is a need for novel research directions that will be

based on a more comprehensive view of the interactions between the different chemokines, between them and other inflammatory factors at the tumor microenvironment, and of the impact that different therapeutic modalities may have not only on cancer cells, but also on the immune integrity of the host.

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