Cell. Mol. Life Sci. (2011) 68:2811–2830
DOI 10.1007/s00018-011-0677-7

Cellular and Molecular Life Sciences

REVIEW

The role of chemokines and their receptors in angiogenesis

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Received: 1 December 2010 / Revised: 9 March 2011 / Accepted: 22 March 2011 / Published online: 9 April 2011

Abstract Chemokines are a vertebrate-specific group of small molecules that regulate cell migration and behaviour in diverse contexts. So far, around 50 chemokines have been identified in humans, which bind to 18 different chemokine receptors. These are members of the seven-transmembrane receptor family. Initially, chemokines were identified as modulators of the immune response. Subsequently, they were also shown to regulate cell migration during embryonic development. Here, we discuss the influence of chemokines and their receptors on angiogenesis, or the formation of new blood vessels. We highlight recent advances in our understanding of how chemokine signalling might directly influence endothelial cell migration. We furthermore examine the contributions of chemokine signalling in immune cells during this process. Finally, we explore possible implications for disease settings, such as chronic inflammation and tumour progression.

Keywords Chemokines · Angiogenesis · Seven-transmembrane receptors · Embryonic development · Immune response · Tumour · Cancer

Introduction

Angiogenesis is commonly defined as the sprouting of new blood vessels from pre-existing ones [1]. It is extensive in the developing embryo, in which the newly forming organs need to be vascularized. Work over the last decade has identified several key players important for proper angiogenesis to occur. Members of the vascular endothelial growth factor (VEGF) family have been shown to control many aspects of endothelial biology, including proliferation, differentiation, migration and survival [2]. Loss of VEGF function in the mouse [3, 4] and in the zebrafish [5] leads to severe vascular defects and reduced angiogenic sprouting. Mutants in components of the Notch signalling pathway also show severe vascular defects [6]. However, these are associated with an increase in angiogenic sprouting. In addition to these genetic pathways, changes in haemodynamics [7] and tissue hypoxia [8] are also important regulators of angiogenesis. In contrast to the embryo, angiogenesis in the adult is limited and is often associated with pathological situations. Accordingly, many disease settings, such as cancer, stroke and diabetes have a central vascular component [9]. Because of this, targeting newly forming blood vessels pharmacologically in order to slow disease progression has received increasing attention and several antiangiogenic strategies have been approved for human treatment. For instance, anti-VEGF therapy has proven beneficial in treating metastatic colorectal cancer [10] and age-related macular degeneration [11]. While many parallels exist between the mechanisms and molecules regulating angiogenesis in the early embryo and the adult, several differences have been elucidated recently. During early stages of blood vessel development, endothelial cells respond to proangiogenic factors present in the surrounding tissue. Later, mobile cells of the immune system appear to play an important role during blood vessel formation. They regulate angiogenesis in diverse settings, such as the embryonic brain, where they mediate fusion events between endothelial tip cells [12]. In pathological

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situations, such as wound healing [13] and tumour progression [14], immune cells secrete proangiogenic factors that act on the surrounding endothelium. They furthermore control the separation between blood endothelial cells and the forming lymphatic vasculature [15, 16]. Therefore, understanding the signals and molecules that mediate the interaction between endothelial and immune cells would be of great importance for our further understanding of the mechanisms controlling angiogenesis.

One class of molecules that has received increasing attention due to their action on both endothelial cells and cells of the immune system are chemokines (chemotactic cytokines). They were originally identified due to their role in directing cell migration and proliferation during the immune response. So far, 47 members have been described in humans, which can be categorized into four subgroups depending on the presence and position of conserved cysteine residues [17]. Typically, chemokines possess four cysteine residues that mediate the formation of intramolecular disulphide bonds. Members of the CXC group are characterized by the presence of a single amino acid between the first two N-terminal cysteines, while members of the CC class lack this amino acid. CXC chemokines can be further subdivided into ELR− and ELR+ classes, based on the presence of a glu-leu-arg (ELR) amino acid sequence at their N-terminus. The two other subfamilies consist of only one (CX3C) or two (XC) members each. In CX3C chemokines, three amino acids are present between the first two cysteines. The fourth class, XC chemokines, lack cysteines one and three of the typical chemokine structure [17].

Chemokines bind to chemokine receptors, which belong to the G-protein coupled receptor family. So far, ten CCR family receptors and seven CXCR family receptors in addition to CX3CR1 and XCR1 have been identified. In addition, several decoy receptors exist that bind chemokine ligands without eliciting signal transduction. These include D6, DARC and CCX-CKR [18]. While there is a high degree of promiscuity between ligands and receptors, the biological relevance of this is poorly understood. Differential ligand/receptor interactions might regulate receptor trafficking and thereby signalling outcomes (see below). Upon ligand binding, receptors signal via heterotrimeric G-proteins, leading to the activation of several downstream signalling pathways, such as the MAP (mitogen-activated protein) kinase, RAS and Rho GTPases and phosphoinositide 3-kinase (PI 3-kinase) signalling cascades, which directly influence cell migration and proliferation [19]. In this review, we discuss the different classes of chemokines and illustrate how they influence angiogenesis in several settings, both in the embryo and the adult. We furthermore examine the autonomous effects of chemokines on endothelial cells and compare them with non-autonomous effects elicited via the recruitment of immune cells (Fig. 1). We conclude by exploring the implications of these findings for disease treatment.

Angiogenic versus angiostatic chemokines

The aforementioned distinction between the CXC chemokines ELR+ and ELR− chemokines is also a functional one (Table 1). All identified ELR+ chemokines mediate angiogenesis [20]. CXCR2 is the main ELR+ chemokine receptor expressed on endothelial cells and shows promiscuity in its ligand binding [21]. In humans, CXCR1 has been identified as a second CXC receptor expressed on endothelial cells and mediating angiogenic responses. It selectively binds to CXCL8/IL-8 and CXCL6/GCP-2 [22]. Blocking either CXCR1 or CXCR2 function in human microvascular dermal endothelial cells strongly affects chemotaxis [23]. This effect is further enhanced by simultaneous application of antibodies blocking both receptors. Further studies on the function of each receptor suggest that CXCR1 affects early steps during chemotaxis, such as the appearance of stress fibres, while signalling through CXCR2 is involved in the later occurring accumulation of cortical actin and cell retraction [24]. Interestingly, human intestinal microvascular endothelial cells exclusively express CXCR2 [25]. Here, CXCR2-neutralizing antibodies similarly affect stress fibre assembly and chemotaxis. These findings suggest tissue specificity in the expression of CXCR1 in different endothelial cell populations. They also argue that CXCR2 is the major ELR+ chemokine receptor mediating angiogenic responses.

This is further supported by studies analysing CXCR2 function in mice. Angiogenesis in settings such as the cornea micropocket assay [21] and during wound repair [26] is compromised in CXCR2 knockout mice. When Addison et al. applied Hydron pellets containing the CXCR2 ligand CXCL8/IL-8 in the cornea micropocket assay, they observed a robust angiogenic response [21]. This response was strongly reduced in mice either treated with CXCR2 blocking antibodies or in CXCR2 homozygous mutant mice. The same was true for pellets containing CXCL2/MIP-2. In contrast to this, bFGF-driven angiogenesis was unaffected by loss of CXCR2 function. Thus, CXCL8/IL-8 and CXCL2/MIP-2 stimulate angiogenesis in a CXCR2-dependent manner. During wound healing, CXCR2 is necessary for epithelialization and neovascularization [26]. Loss of CXCR2 affects the recruitment of neutrophils and macrophages. Fewer neutrophils are recruited to the wound, while the number of macrophages is increased. Consistently, the expression profiles for several cytokines, such as interleukin-1beta (IL-1β) are

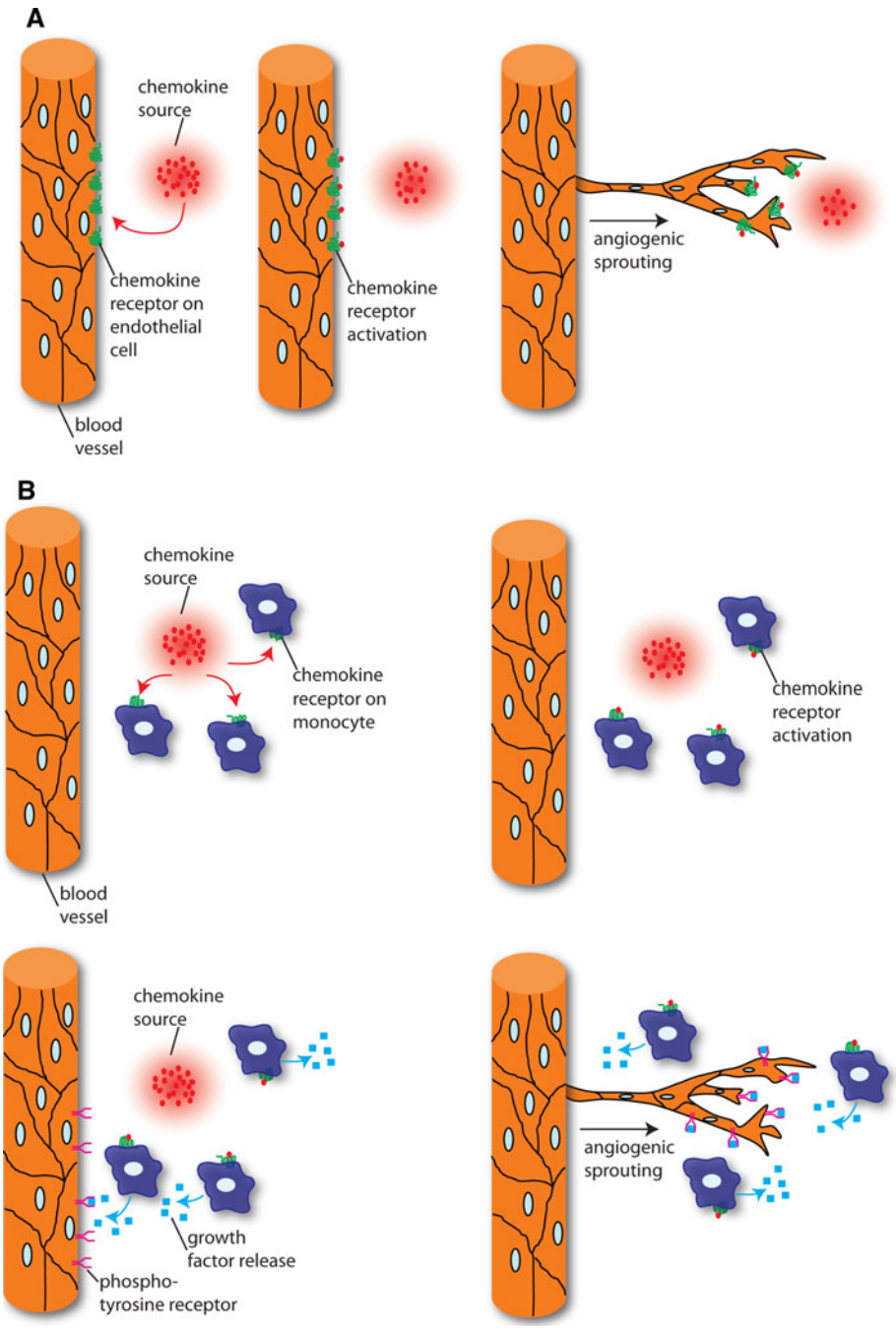

Fig. 1 Endothelial autonomous and non-autonomous roles of chemokines during angiogenesis. **a** Chemokines act directly on endothelial cells expressing chemokine receptors and thereby influence endothelial migration and angiogenesis. **b** Chemokines indirectly influence endothelial cell behaviours by attracting chemokine receptor-expressing leucocytes. These subsequently secrete proangiogenic factors, such as VEGF, that act on endothelial cells and initiate angiogenesis

changed. In addition, in vitro experiments using cultured keratinocytes have shown a decreased extent of wound closure after inducing a round wound in confluent cultures lacking CXCR2. These results suggest that CXCR2 function is necessary in different cell populations during wound closure.

It would be of interest to determine if the effect on wound neovascularization is due to an autonomous role of CXCR2 in endothelial cells or due to impaired neutrophil and macrophage recruitment (see Fig. 1). Analysis of endothelial specific CXCR2 knockout mice will help answer this question. Interestingly, CXCR2 knockout mice are outwardly healthy and viable, suggesting no major influence of this chemokine receptor during developmental angiogenesis [27]. This raises the interesting possibility that the mechanisms regulating angiogenesis in the embryo and during pathological conditions in the adult might differ in the set of chemokine receptor/ligands used. However, a detailed examination of embryonic angiogenesis in CXCR2 knockout mice has not been performed so far.

Table 1 Angiogenic and angiostatic chemokines and their receptors

Systematic nomenclature	Old nomenclature	ELR motif	Receptor	Reference
Angiogenic				
CXCL1	Gro-α	+	CXCR2	[206]
CXCL2	Gro-β	+	CXCR2	[207]
CXCL3	Gro-γ	+	CXCR2	[208]
CXCL5	ENA-78	+	CXCR2	[209]
CXCL6	GCP-2	+	CXCR1, 2	[210]
CXCL7	NAP-2	+	CXCR2	[211]
CXCL8	Il-8	+	CXCR1, 2	[212]
CXCL12	SDF-1	–	CXCR4, 7	[80]
CCL2	MCP-1	N/A	CCR2, 4	[170]
CCL11	Eotaxin	N/A	CCR3	[213]
CCL16	HCC-4/LEC	N/A	CCR1	[166]
Angiostatic				
CXCL4, CXCL4L1	PF-4, PF-4_{var}	–	CXCR3B	[37, 41]
CXCL9	Mig	–	CXCR3B	[52]
CXCL10	IP-10	–	CXCR3B	[214]
CXCL11	I-TAC	–	CXCR3B, 7	[52]
CXCL14	BRAK	–	Unknown	[215]

N/A non applicable

An exception to the rule is the ELR− chemokine CXCL12/SDF-1, which has been shown to induce angiogenesis via binding to its receptors CXCR4 [28] and CXCR7 [29]. It plays important roles during embryonic vascular development and during tumour growth and metastasis (see below). Stimulation of endothelial cells with VEGF leads to the induction of CXCR4 expression, while stimulating human umbilical vein endothelial cells (HUVECs) with TNF-α or IL-1β induces CXCR7 expression [29], suggesting that activation of endothelial cells is necessary for the expression of both CXCL12 receptors. These findings are in line with a recent study showing that in the adult tumour endothelial cells upregulate CXCR7 expression, while normal endothelial cells do not express CXCR7 [30]. Interestingly, while CXCR7 mRNA can be found in cells of many transformed and non-transformed tissues, CXCR7 protein is mainly detected in transformed tissues [29]. This suggests that CXCR7 expression is post-transcriptionally regulated.

The mode of action of both CXCL12 receptors is not clear to date. CXCR4 acts as a canonical chemokine receptor, since CXCL12 binding to CXCR4 induces calcium release and chemotaxis in various cell types, such as neurons [31] and lymphatic cells [29]. CXCL12 binding to CXCR7 does not induce these cellular responses, but leads to an increase in cell survival and cell adhesion [29]. These distinct cellular responses might result from the differential activation of downstream signalling events. Rajagopal et al. recently showed that CXCR7 activation preferentially results in signalling via β-arrestin, and not via canonical G-protein signalling [32]. In addition to the activation of different signalling pathways via CXCR4 and CXCR7, previous studies have suggested that CXCR7 might modulate CXCR4 function by forming heterodimers [33, 34]. Levoye et al. have proposed that in this setting CXCR7 modulates the activation of CXCR4 downstream signalling components, such as different G-proteins. Work in zebrafish has furthermore shown that somatic cells expressing CXCR7 can act as a sink for CXCL12 and thereby shape the CXCL12 gradient [35, 36]. Thus, in addition to directly acting via β-arrestins, CXCR7 can influence CXCL12-CXCR4 signalling by influencing ligand distribution and receptor downstream signalling.

ELR− chemokines are angiostatic and include CXCL4/PF-4, CXCL4L1/PF-4_{var}, CXCL9/Mig, CXCL10/IP-10, CXCL11/I-TAC and CXCL14/BRAK. The first angiostatic chemokine described was CXCL4/PF-4 [37, 38]. CXCL4/PF-4 is, besides β thrombomodulin (CXCL7/β-TG), the second major platelet chemokine [39]. It is stored along with other secretable platelet proteins and notably the proangiogenic chemokines CXCL7/NAP-2 and CXCL12/SDF-1 in α-granules (see [40] for a recent review). CXCL4/PF-4 exists as two non-allelic variants, CXCL4/PF-4 and CXCL4L1/PF-4_{var}, which differ from each other in three amino acids. Studies addressing human microvascular endothelial cell chemotaxis showed that CXCL4L1/PF-4_{var} inhibits both bFGF and CXCL8/IL-8 driven angiogenesis more efficiently than CXCL4/PF-4 [41]. The same is true for bFGF-induced angiogenesis in the rat cornea micropocket assay. The mode of action of

CXCL4/PF-4 or CXCL4L1/PF-4_{var} during angiogenesis is not well understood. In addition to influencing chemotaxis, CXCL4/PF-4 has also been shown to inhibit cell cycle progression in endothelial cells [42]. Both effects are mediated in part by the ability of CXCL4/PF-4 to inhibit binding of the two proangiogenic factors VEGF and bFGF to their respective receptors [43–45]. For instance, CXCL4/PF-4 can heterodimerize with bFGF, preventing bFGF homodimerization necessary for receptor binding [45, 46]. Similarly, CXCL4/PF-4 inhibits binding of $VEGF_{165}$ to VEGF receptors on endothelial cells and to soluble VEGF receptors [44]. In contrast to this, receptor binding of the $VEGF_{121}$ isoform is not inhibited by CXCL4/PF-4. The $VEGF_{165}$ and $VEGF_{121}$ isoforms differ in their ability to bind surface heparan sulphates. While $VEGF_{161}$ efficiently associates with heparan sulphates and therefore remains bound to the extracellular matrix, $VEGF_{121}$ does not [47]. This might indicate that CXCL4/PF-4 specifically inhibits $VEGF_{165}$ receptor binding by interfering with heparan sulphates. However, CXCL4/PF-4 mutants lacking heparin-binding capacity still inhibit angiogenesis [44]. In addition, even though CXCL4/PF-4 does not bind $VEGF_{121}$, it still inhibits $VEGF_{121}$-induced endothelial proliferation. These findings argue that CXCL4/PF-4 can inhibit VEGF signalling via a mechanism that does not involve direct binding to either VEGF ligands or receptors.

The main receptor for angiostatic CXC ELR− chemokines is CXCR3. It can bind to CXCL4/PF-4, CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC [48]. It exists in three different splice isoforms, CXCR3A, CXCR3B and CXCR3-alt. While CXCR3A is primarily responsible for the recruitment of lymphocytes [49, 50], CXCR3B is expressed on endothelial cells [23, 51, 52]. CXCR3B mediates the angiostatic effect of CXCL10/IP-10 in human melanoma progression [53]. Of interest, a recent study has provided evidence that CXCR3B does not exist in mice and that CXCL10/IP-10 can inhibit endothelial proliferation independently of CXCR3 function [54]. Together, these studies illustrate that the mode of action of the antiangiogenic chemokine CXCL10/IP-10 is context- and organism-dependent.

Chemokine receptor signalling

The pro- and antiangiogenic functions of the different chemokine classes are probably due to differential downstream signalling events elicited after receptor activation. Chemokine receptors are G-protein-coupled seven-transmembrane-spanning cell surface receptors (GPCRs) that belong to the family of class A rhodopsin-like GPCRs. Reminiscent of the mode of activation reported for receptor tyrosine kinases, GPCRs have been demonstrated to undergo homo- or heterodimerization following ligand engagement (reviewed in [55]). More recently, receptor trafficking has taken centre stage in the analysis of GPCR signal regulation and initiation. Endocytosis via a classic clathrin- and dynamin-dependent pathway (clathrin-coated pits) has been described for a number of chemokine receptors, including CXCR1, CXCR2, CXCR4 and CCR7. Other receptors, including DARC and CCX-CKR, are internalized via a caveolin-dependent pathway and a third group that includes CCR2, 4 and 5 appears to use both routes (reviewed in [56]). Interestingly, different chemokines can cause differential internalization of identical receptors. For instance, the receptor CCR4 is efficiently internalized after CCL22/MDC binding, but only poorly after CCL17/TARC binding [57]. Similarly, interaction of CCL3/MIP-1α and CCL5/RANTES with CCR5 results in rapid internalization, while binding of CCL4/MIP-1β to CCR5 does not [58]. Such differential effects can go along with and perhaps even be caused by differential activation of intracellular effectors, such as G-protein-receptor-coupled kinases (GRKs). This has been demonstrated for the interaction of CCR7 and CCL19/ELC, which in contrast to the interaction of CCR7 and CCL21/SLC, induces strong activation of GRK3 and GRK6 as well as β-arrestin recruitment [59]. For the vast majority of receptors, however, the molecular mechanisms underlying differential ligand-induced effects remain unknown.

As are immunoreceptors, for example, GPCRs are not only internalized following receptor engagement in an agonist-dependent fashion, but are also subject to constitutive uptake and recycling. Different ligands not only have divergent effects on receptor uptake, but they can also regulate the extent to which a receptor is internalized and degraded versus how much receptor is being recycled to the surface. For example, CCL5/RANTES induces efficient internalization of the three receptors CCR1, CCR3 and CCR5. However, while CCR5 is completely recycled to the surface, CCR3 is partly degraded and CCR1 is entirely degraded [60–62]. Thereby, different chemokines fine-tune the amount of receptor present on the surface of a target cell. In addition, ligand-induced GPCR internalization is not only a means to negatively regulate GPCR signalling by removal of active receptor from the surface. Signalling may also occur from endosomes, as internalized receptors continue to transmit signals initiated at the plasma membrane. Or receptors may even generate novel or stronger signals as they assemble “en route” newly formed complexes intracellularly (reviewed in [63, 64]). Taken together, these mechanisms, in addition to the large number of chemokines and receptors and the receptor promiscuity, add a further level of complexity to the regulation of cellular behaviour by chemokines.

Intracellularly, signalling through GPCRs, including chemokine receptors, is tightly coupled to the activation of an associated heterotrimeric G-protein. The heterotrimer of Gα, Gβ and Gγ, in its resting state, binds the guanine nucleotide GDP. Upon receptor occupancy, GDP is released from the Gα subunit, which now binds GTP. The resulting allosteric change causes dissociation of the complex into a βγ dimer and the GTP-bound α monomer. Rapid hydrolysis of GTP is followed by re-association of the heterotrimer and the receptor, functionally causing cessation of receptor signalling. Chemokine receptors preferentially associate with $G\alpha_i$ subunits, which inhibit adenylate cyclase and render chemokine receptors susceptible to pertussis toxin. Recent data, however, suggest that at least signalling through the CXCR4 receptor may also utilize other Gα proteins including $G\alpha_q$, $G\alpha_0$ and $G\alpha_s$ [65].

The preeminent function of chemokines is the induction of directed cell migration along a concentration gradient towards the source of the chemokine. Cell migration relies on prior cell polarization, leading to functionally distinct processes happening at the front (leading edge) and the rear (uropod) of the cell. Several classes of molecules are essentially involved in this process, including PI 3-kinases, Rho-family members, integrins, cytoskeletal elements and vesicular transporters. In particular, PI 3-kinases, that are divided into class I and class II enzymes, regulate various aspects of the migratory machinery, including actin reorganization and gradient sensing [66]. Class I PI 3-kinases have a broad substrate specificity and their 3′ phosphorylated products (phosphatidylinositol PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_2) provide docking sites for lipid binding effector proteins containing pleckstrin homology and phox homology domains (reviewed in [67]). In leucocytes, the p110γ PI 3-kinase isoform is essential for myeloid chemotaxis [68], while it is dispensable for T-cell chemotaxis [69, 70], indicating a high degree of cell-type specificity. Class I PI 3-kinase activity is counteracted by the lipid phosphatases PTEN and SHIP. In neutrophils, the PI 3-kinase product PtdIns(3,4,5)P_3 localizes to the leading edge, because in response to a chemokine gradient, PI 3-kinases concentrate at the leading edge, while PTEN is localized to the side and rear of the cell [71]. Class II PI 3-kinases are associated with membranes and nuclei. Chemokines efficiently stimulate class II PI 3-kinases, however, their activation mechanism and biological functions are less well investigated.

An important outcome of PI 3-kinase activation is the activation of protein kinase B (PKB/AKT), which shows antiapoptotic activity and fosters survival in many cell types, including cancer cells [72]. In addition, chemokines can cause the upregulation of genes associated with cell survival [73]. This is of particular relevance for protumorigenic chemokines.

Chemokines in embryonic angiogenesis

The earliest chemokine/receptor pair expressed during embryogenesis is CXCL12/SDF-1 and CXCR4, which starts to be expressed at around E7.5 in the mouse. Expression domains include embryonic and extraembryonic mesodermal and endodermal tissues [74]. Later, between E10.5 and E12.5, neural as well as cardiac and endothelial cells express CXCR4. Other chemokine receptors start to be expressed at around E12.5, at the onset of definitive haematopoiesis. In the zebrafish, two CXCR4 homologues are present, both of which are maternally provided [75]. Of these, only CXCR4A has been shown to be expressed in endothelial cells [75, 76]. These expression data from the mouse and the zebrafish suggest that CXCR4 is the only chemokine receptor acting autonomously in endothelial cells during early embryonic development. In line with this, mice deficient in either CXCL12/SDF-1 or CXCR4 show defects during embryonic blood vessel development [77]. In particular, vascularization of the gastrointestinal tract is affected, suggesting that CXCR4 plays a role during organ-specific blood vessel formation. An identical vascular phenotype has been observed in an endothelial specific knockout of CXCR4 [78]. The observed defects in the gastrointestinal tract vasculature were shown to be due to the absence of small interconnecting blood vessels between the superior mesenteric artery and the venous network. Recently, Takabatake et al. also found defects in the renal vasculature in mice mutant for CXCR4 [79]. Here, glomeruli in CXCR4 knockout mice exhibited aneurismal dilation of the capillaries, leading to a loss of the complex capillary tuft patterning. Work in the zebrafish has further revealed a function of CXCR4A during the formation of the first major artery in the body, the lateral dorsal aorta [76]. In this setting, only the anterior, bilateral aortae are affected in mutant embryos, while development of the single aorta in the tail proceeds normally. Using time-lapse microscopy, the authors also showed that endothelial migration per se is not affected in mutant zebrafish, but that mutant endothelial cells now migrate into ectopic locations within the embryo. Therefore, CXCR4 signalling appears to be crucial for the proper pathfinding of endothelial cells and not for their motility per se. Of interest, despite also being expressed in the forming intersomitic blood vessels in zebrafish [76] and mice [74], these vessels form normally in mutant embryos [76], suggesting the presence of compensatory mechanisms in these vascular beds. Together, these findings underscore the organ- and region-specific function of CXCR4 in the endothelium.

In addition to the specific defects in organ vascularization, CXCR4 and CXCL12 mutants also show defects in heart development, including ventricular septal defects [77, 80]. Similar defects in heart development have also

been reported for mutants in CXCR7 [34, 81]. Of interest, CXCR7 is strongly expressed in cardiac microvessels. An endothelial specific knockout reproduced the observed cardiac defects [34], suggesting that CXCR7 function is specifically needed in endothelial cells during heart development. In contrast to these similarities between the knockout phenotypes for CXCR4, CXCL12 and CXCR7, both studies on CXCR7 failed to detect defects in organ-specific vascularization. Therefore, CXCR7 function in the endothelium might be more crucial for heart development than it is for other organs. Further research is needed to clarify the precise cardiac vascular phenotype of CXCR7 mutants and the contribution of the vasculature to the observed heart defects. Knockdown of CXCR7 in zebrafish embryos by injection of morpholino (MO) antisense oligonucleotides also causes heart defects [30]. The authors also observed defects in the sprouting of intersegmental blood vessels in the trunk of MO injected embryos, suggesting that in the zebrafish CXCR7 might play a role during angiogenesis. However, the authors monitored intersegmental blood vessel formation by performing angiography with fluorescent dextrans. Since CXCR7 MO-injected embryos display a strong cardiac defect, distribution of the fluorescent dye might be compromised and not fill intersegmental vessels properly. As discussed previously, CXCR4 mutant embryos form intersegmental blood vessels normally [76]. If CXCR7 and CXCR4 play independent roles in intersegmental blood vessel formation remains an interesting question for future research.

About half of the mouse mutants for CXCR4 or CXCL12/SDF1 die at around E18.5 and neonates die within 1 h [77, 80]. In zebrafish, about half of the CXCR4A mutants die during embryogenesis, while the rest of the homozygous mutants survive to adulthood and are fertile [76]. This is in contrast to mutants in other signalling pathways implicated in vascular development, such as VEGF or Notch, which die at earlier developmental stages. Mice mutant for VEGF-A show vascular abnormalities starting at E8.5 and die at E10.5 [3]. Mutants in Notch pathway components die at comparable stages [82]. This indicates that mutants in CXCR4 and CXCL12/SDF1 might either affect later aspects of endothelial development or coordinate the fine-tuning of the developing vasculature. In addition, compensatory mechanisms might be in place to balance out the defects in endothelial cell migration observed in CXCR4 and CXCL12/SDF1 mutants. So far, early embryonic vascular defects in chemokine mutant mice or zebrafish have only been described for CXCL12/SDF1. In addition, defects have only been reported in embryonic blood vessels expressing the CXCL12/SDF1 receptor CXCR4, arguing for an autonomous role of chemokine signalling during early embryonic blood vessel formation.

A recent report has highlighted the potential role of macrophages during the anastomosis of angiogenic tip cells in mouse and zebrafish embryos [12]. Mice lacking tissue macrophages display a reduction in the number of fusion events between neighbouring tip cells, leading to a reduction of intersections between brain blood vessels. These findings highlight the potential role of myeloid cells in regulating embryonic angiogenesis. Although a role for chemokine signalling has not been reported, it is tempting to speculate that macrophages might be recruited to sites of anastomosis via chemokine signalling. Of interest, Strasser et al. reported CXCR4 expression in the tips of sprouting retinal blood vessels, while CXCL12/SDF1 expression was restricted to endothelial cells of the larger vessels and to the underlying astrocyte layer [83]. When the authors blocked CXCR4 signalling in the retina, they observed an absence of interconnections between endothelial cell sprouts, similar to what was observed in macrophage-deficient retinae [84]. Thus, CXCL12/SDF1 could serve a double role by guiding the migration of endothelial tip cells and via recruitment of accessory cells to facilitate anastomosis. Further experiments are required to address this relationship.

Regulation of CXCR4 and CXCL12/SDF1 expression

Several signalling pathways which are important for regulating angiogenesis have been shown to influence CXCR4 expression. The Notch signalling pathway is necessary to control endothelial responses to the proangiogenic factor VEGF. In this setting, Notch acts to downregulate the expression of VEGF receptors and thereby attenuates angiogenic sprouting [85]. In sprouting blood vessels, tip cells are characterized by low Notch signalling pathway activation corresponding with increased angiogenic behaviour, while stalk cells with activated Notch signalling show decreased angiogenic responses [86]. Williams et al. found that activating the Notch signalling pathway by expressing the Notch ligand *dll4* negatively influences CXCR4A expression and thereby inhibits migration of endothelial cells towards CXCL12/SDF1 [87]. These observations are in agreement with the reported expression of CXCR4 in endothelial tip cells in the mouse retina [83]. Therefore, Notch signalling might negatively influence angiogenic sprouting by simultaneously downregulating receptor expression of two promigratory pathways. Curiously, in addition to tip cells, expression of CXCR4 is highest in arterial endothelial cells [76, 78, 83]. This is hard to reconcile with the reported high activation of Notch signalling in arterial endothelial cells and the requirement of Notch signalling to induce arterial gene expression [82]. Therefore, additional mechanisms must exist in arteries

that allow the expression of CXCR4 in the presence of high Notch signalling.

Importantly, CXCR4 has also been shown to be responsive to changes in blood flow dynamics. Packham et al. showed by gene expression profiling that CXCR4A mRNA becomes downregulated after the onset of blood flow, suggesting that blood flow negatively regulates CXCR4A expression [88]. Accordingly, blocking blood flow in zebrafish embryos leads to an upregulation of CXCR4A expression in the vasculature. The same is true for embryos mutant for *gridlock* (*hey-2*), in which the distal artery is occluded. Importantly, this upregulation of CXCR4A expression is only observed in arterial endothelial cells. Similar results have been obtained in HUVECs. Exposing HUVECs to high laminar shear stress leads to a significant downregulation of CXCR4A expression [89]. Whether shear stress and Notch independently regulate CXCR4A expression remains an important unanswered question.

Chemokines during lymphatic development

In addition to their effects on blood endothelial cells, chemokines also influence the formation of the lymphatic system. Lymph endothelial cells differentiate in the mouse (at embryonic day 9.5) and human fetuses (around 6–7 weeks) from venous endothelium after the primitive cardiovascular system is formed and circulation has started [90]. The first lymphatic progenitors are detected in the anterior cardinal vein and are characterized by expression of the homeobox transcription factor PROX1 [91]. Soon after their appearance, PROX1-positive cells bud from the vein and migrate away in a polarized fashion to form the primary lymph sacs. This migration is largely directed by a gradient of VEGF-C [92]. However, additional locally provided spatial cues that govern the positioning of the primary vascular plexus in mammals have yet to be defined. In the zebrafish, arteries have recently been reported to provide indispensable guidance functions for lymphatic endothelial cells [93].

In the mouse, platelets are involved in the process of initial separation of the blood and lymphatic circulation [94–98]. While it is clear that recognition of the endothelial surface glycoprotein podoplanin (PDPN) by the platelet c-type lectin receptor 2 (CLEC-2) is essential for this process, the exact action of platelets remains to be elucidated. Genetic mouse mutants in PDPN, CLEC-2 or its downstream signalling components Syk, SLP-76 and PLC-γ suffer from defects in separation of the blood and lymphatic circulation [94, 96, 99]. In addition, Syk-deficient mice have been demonstrated to develop shunts between the otherwise strictly separated peripheral blood and lymphatic vessels [15]. Such illegitimate connections are the consequence of a strong lymphatic hyperplasia that is caused by a CCL2/MCP-1-driven accumulation of highly prolymphangiogenic macrophages in the skin of Syk-deficient mice. At sites of intimate contact between blood and lymphatic vessels, peripheral shunt formation between previously separated vessels is frequently observed [15]. The inappropriate expression of the inflammatory chemokine CCL2/MCP-1 in Syk-deficient monocytes may be a consequence of a lack of inhibitory environmental signals that are mediated by receptors, which signal via immune receptor tyrosine-based activation motifs [100].

Once the lymphatic sacs are established in the fetus, a primary lymphatic plexus that is subject to intense remodelling forms by sprouting lymphangiogenesis [101]. Cells expressing CD45, CD4, IL-7 receptor α (IL-7Rα), but not CD3, which have not undergone T-cell receptor (TCR) rearrangement and hence do not express a TCR, appear at the site of future lymph node formation. These lymphoid tissue inducer cells (LTIs) are probably of haematopoietic origin and share the expression of many surface proteins found on natural killer cells [102]. They differentiate from $CD45^{pos}$, $CD4^{pos}$ and $CD3^{neg}$ precursor cells in response to tumour necrosis factor-related activation-induced cytokine (TRANCE) in a process that is absolutely dependent on the retinoid-related orphan receptor (ROR) γt [103, 104]. Mice deficient for both CXCL13/BCA-1 and the IL-7Rα lack the development of all lymph nodes. CXCL13/BCA-1 has been demonstrated to be induced by retinoic acid and neuronal stimulation and to be essential for lymph node initiation [105]. Furthermore, CCL21/SLC and CCL19/ELC have been shown to function in the development of facial, cervical, brachial, and axillary lymph nodes [106].

Therefore, CXCL13/BCA-1 and CCR7 ligands, in cooperation with IL-7Rα, likely promote the recruitment and accumulation of LTIs to the sites of emerging lymph nodes. This recruitment is mostly driven via the chemokine receptor CXCR5, which is present on LTIs. In the absence of CXCR5, LTI cells in mesenteric lymph nodes lack activation of β1 integrins, suggesting that CXCL13/BCA-1 signalling is crucial for the initiation of tight interactions with stromal cells [107]. Further lymph node development is coordinated by a set of complex interactions between LTIs and local stromal cells also referred to as organizer cells [108]. Signals through the IL-7R and TRANCE receptor on LTIs cause high level expression of the surface lymphotoxin (LT) $\alpha 1\beta 2$, which engages the LTβ receptor (LTβR) on adjacent stromal cells [109]. Stromal cell activation through LTβR ligation causes a profound upregulation of the adhesion molecules ICAM-1 and VCAM-1 [110]. ICAM-1 and VCAM-1 on stromal cells interact tightly with the integrin $\alpha 4\beta 1$ expressed on LTIs (reviewed in [111]). In addition, activation of stromal cells

through the LTβR results in subsequent expression of the lymphoid chemokines CXCL13/BCA-1, CCL21/SLC and CCL19/ELC [112, 113]. This supports the recruitment of further LTIs to the site of the emerging secondary lymphatic organ and initiates the segregation of T and B cell areas [114]. Absence of CCL21/SLC and CCL19/ELC in mice deficient for CXCL13/BCA-1, results in failure to form any peripheral lymph nodes, while mesenteric lymph nodes can still form [106]. Related defects were detected in the absence of CXCR5 and CCR7, suggesting a highly cooperative function of the chemokines CXCL13/BCA-1, CCL19/ELC and CCL21/SLC during lymph node formation [115]. Similar mechanisms involving CXCL13/BCA-1 and its receptor CXCR5 are active during the formation of tertiary lymphoid organs at sites of chronic inflammation [116].

Chemokines in tumour angiogenesis and metastatic spread

The capacity to usurp and abuse normal cellular control mechanisms is a hallmark of malignantly transformed cells. Given the central role of chemokines in the regulation of tissue homeostasis and cell migration, it does not come as a big surprise that chemokines play essential roles during tumour progression and metastasis. Important steps in tumour development include the so-called "angiogenic switch" that describes the transition from a dormant hyperplasia to a vascularized, growing tumour [117] and the metastatic spread to distant sites. Several modes of action of chemokine subversion leading to tumour progression have been defined. Here we discuss four aspects: (1) tumours may express chemokines that attract leucocytes which are converted by the tumour environment into proangiogenic mediators. This is probably the most relevant action of chemokines in tumour angiogenesis; (2) tumour cells may produce chemokines that act directly on endothelial cells to stimulate angiogenesis; (3) tumour cells may produce chemokines that act in a paracrine fashion and thereby foster tumour growth and progression ultimately leading to an angiogenic outcome; and (4) tumour cells may express receptors for homeostatic chemokines that enable them to spread via access to the tumour vasculature.

Tumours may express chemokines that attract leucocytes Tumours can utilize inflammatory chemokines to attract leucocytes and control the extent and nature of the infiltrate. It has long been appreciated that chronic inflammation is a predisposing risk factor to cancer [118]. Microbial infections, such as *Helicobacter pylori* infection, are associated with gastric cancer and mucosal lymphoma, while autoimmune disease of the digestive tract can cause colitis-associated cancer. Indeed, the presence of smouldering inflammation in the tumour environment has been considered the seventh hallmark of cancer [119] and tumour-associated macrophages (TAMs) constitute a major fraction of the leucocyte infiltrate (Fig. 2a). Production of CCL5/RANTES by mammary carcinoma cells correlates with macrophage infiltration and lymph node metastasis [120]. Similarly CCL2/MCP-1 expression in oesophageal carcinoma fosters macrophage infiltration, and tumour angiogenesis and spread [121]. Blockade of CCL2/MCP-1, on the other hand, reduces chronic colitis-associated carcinogenesis in mice [122]. Interestingly, the finding that the D6 decoy and scavenger receptor for inflammatory cytokines exerts a protective role against colon and skin cancer in mice provides direct genetic evidence for a protumorigenic action of inflammatory leucocytes [123–125]. Besides chemokines, other factors including colony-stimulating factor-1 (reviewed in [126]) and VEGF-A (reviewed in [127]) contribute to the recruitment of TAMs into tumours (Fig. 2).

The overwhelming majority of clinical studies have shown protumorigenic effects associated with increased macrophage tumour infiltration, but there is also evidence for tumoricidal activity of TAMs [128]. The disparate activities of TAMs raises the necessity to characterize functionally different subsets of this heterogeneous population. Macrophages are a highly diverse class of mononuclear phagocytic cell and a successful classification refers to their mode of activation and immunological response capacity (reviewed in [127, 129]). Classically *activated* macrophages respond to type 1 helper cell (Th1) stimulation, show strong proinflammatory activity, cause profound tissue destruction and are characterized by expression of the proinflammatory marker myeloperoxidase, high IL-1, IL-12 and IL-23 levels and the generation of toxic intermediates (NO, reactive oxygen species). This M1 phenotype is also characterized by profound NFκB-driven responses and the expression of opsonic surface receptors and inflammatory mediators such as TNFα and the chemokine CCL2/MCP-1. At the opposite side of the spectrum, there are alternatively *activated* or M2 macrophages that differentiate in response to IL-4 and IL-13 during Th2-driven responses and are low in IL-12 production, but high in IL-10. Alternatively activated macrophages are rich in angiogenic mediators such as VEGF and the cyclooxigenase-2-derived prostaglandin E2. The non-inflammatory M2 phenotype is characterized by the presence of non-opsonic surface receptors and high metalloprotease expression. Biologically, M2 macrophages are believed to induce tissue morphogenesis and wound healing, show proangiogenic activity and support high cell turnover. M2 macrophages are probably similar to tissue-resident

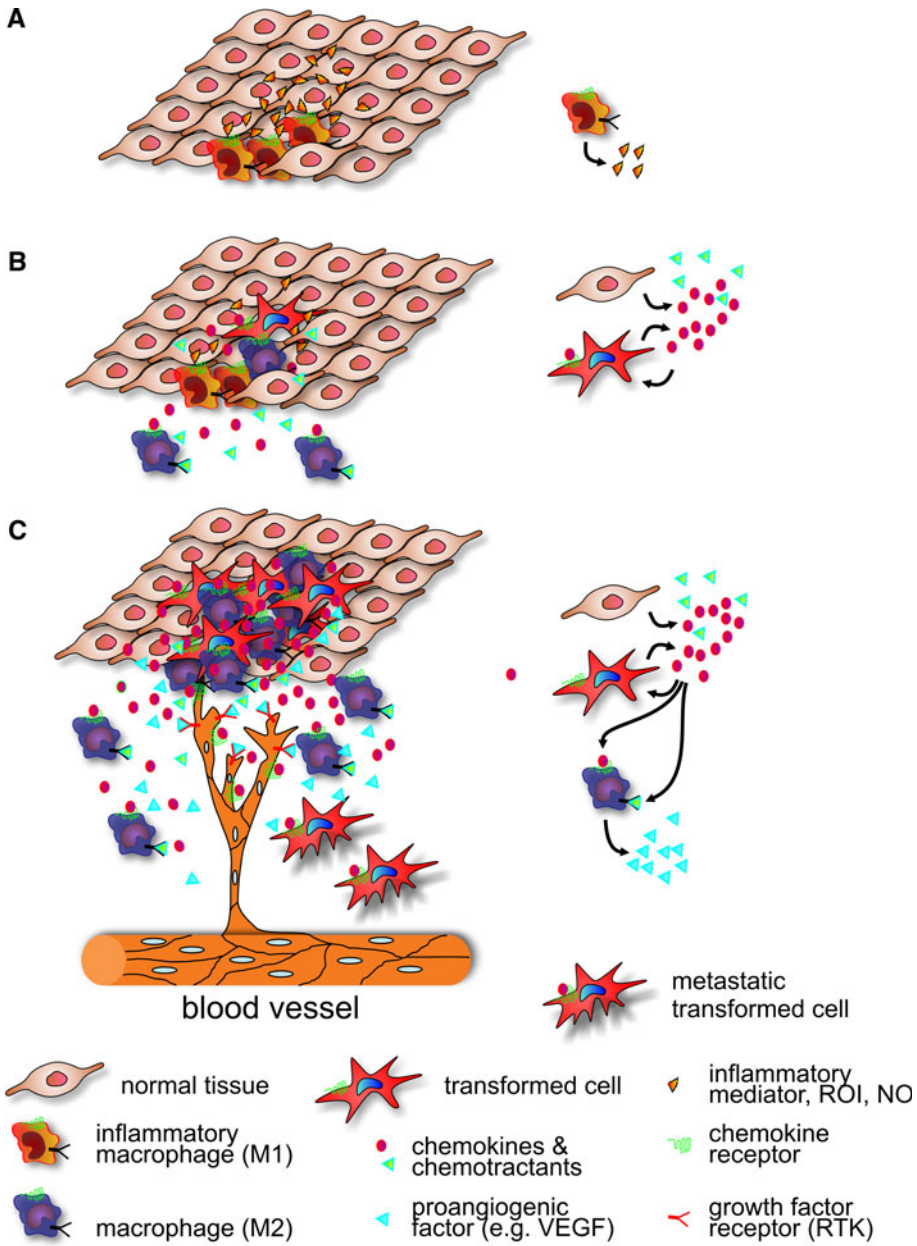

Fig. 2 The role of chemokine signalling during tumour formation. **a** Immune evasion. Chronic local inflammation caused by irritants or pathogens is accompanied by persistent leucocyte infiltration. Activated inflammatory macrophages secrete abundant growth factors and produce reactive oxygen and nitrogen species, which generate a mutagenic environment that ultimately initiates transforming events. **b** Angiogenesis. The forming hyperplastic tissue is a source of chemoattractants, including CCL2/MCP-1 and CSF-1, which recruit circulating monocytes. As these monocytes colonize the tumour, they are instructed by the tumour environment to differentiate into M2 type TAMs. Tumour cells may also express chemokine receptors and respond to autocrine and paracrine stimulation. **c** Tissue destruction/remodelling. Tumour growth is associated with accumulation of M2-type TAMs, which not only produce additional chemoattractants, but also actively suppress inflammatory and cytotoxic responses to the tumour. TAMs secrete proangiogenic factors that in addition to proangiogenic chemokines support tumour vascularization. TAMs also provide proteolytic activities that help the tumour to overcome constraining tissue borders and enhance metastasis. Throughout the entire development, new macrophages are recruited and subverted to serve the major objectives of the developing tumour. The major chemoattractant/growth factor producing cell types are highlighted on the right

macrophages found in the absence of inflammation or the trophic macrophages involved in developmental processes.

From their gene expression profile and biological activity, M2 macrophages would be well equipped to support tumour growth, progression and expansion into the

surrounding tissue, and indeed TAMs associated with actively growing tumours nearly exclusively appear to be polarized towards the M2 phenotype [130]. Tumour cell products such as CSF-1, IL-10 and extracellular matrix compounds not only activate macrophages, but also induce differentiation towards the M2 phenotype [131]. In human lung cancer, cancer-derived versican, by macrophage activation through TLR2 and its co-receptors TLR6 and CD14 and induction of TNFα secretion, strongly enhances metastatic growth [132]. But also the chemokines CCL17/TARC and CCL22/MDC that bind the receptor CCR4 have been shown to promote M2 polarization [127]. The inflammatory chemokine CCL2/MCP-1 not only attracts tumour-promoting macrophages to carcinomas, but also promotes survival and surprisingly M2 polarization of this cell type [133]. M2 macrophages express not only low levels of opsonic surface receptors, but also major histocompatibility class II proteins, resulting in reduced antimicrobial and tumoricidal activity [134]. At least a fraction of TAMs express the receptor tyrosine kinase Tie2 [135]. These Tie2-expressing, tumour-infiltrating monocytes (TEMs), which appear particularly closely related to Tie2-expressing embryonic/fetal macrophages and tissue resident monocytes, share many properties with M2-polarized macrophages and may actually represent largely overlapping populations. While the exact relationship between the two populations remains to be determined, TEMs are also excellently equipped to execute physiological, proangiogenic and tissue-remodelling programs and their profound capacity to change tissue architecture has apparently been co-opted by tumours. Therefore, in the tumour microenvironment, chemokines influence angiogenesis by acting on non-endothelial cell populations, mainly TEMs.

Although the M1/M2 paradigm has been very successfully applied to TAMs, the M1 and M2 phenotypes represent extreme polarizations of macrophage identity [127]. TAMs are a highly heterogeneous cell population that is likely to contain cells of various stages between the M1/M2 extremes. Macrophages are extremely plastic and are able to adapt their responses to a multitude of microenvironmental conditions [136]. Consequently, their role in cancer development is complex and can even be ambivalent. During tumour initiation, the proinflammatory properties of M1 macrophages support a mutagenic and tumour growth-promoting environment (Fig. 2a). Subsequent polarization towards the M2 phenotype during tumour progression results in synergistic interactions between tumour cells and TAMs (Fig. 2b, c). While most TAM populations apparently have been subverted to support tumour growth [137], from regressing tumours, TAMs can be isolated that display M1 polarization, possess the hallmarks of inflammatory macrophages and are probably tumoricidal.

Similar populations probably exist in human tumours, in which expression of CCL2/MCP-1 by pancreatic carcinomas positively correlates with macrophage infiltration. Patients with high circulating levels of CCL2 have a significantly higher survival rate than those with low CCL2 levels and interestingly a direct effect of CCL2 on tumour proliferation and apoptosis has been excluded [138]. Certainly, M2-polarized TAMs, which display defective NFκB activation in response to proinflammatory signals [139], are an attractive target for tumour therapy. It has been speculated that dynamic changes of the tumour microenvironment drive a switch of associated TAMs from an initial M1 state to the M2 state, which is reflected by the inhibition of NFκB responses in infiltrating myeloid and lymphoid cells during advanced tumour stages [140]. M2-polarized TAMs show a massive nuclear accumulation of homodimers of the inhibitory NFκB family member p50, which appears to be responsible for the maintenance of the unresponsive phenotype [141, 142]. This raises the question as to whether it is possible to convert TAMs during tumour development from a pro- to an antitumorigenic action. The first results obtained by blockade of the NFκB pathway in a mouse model of ovarian cancer are promising [143]. In this model, either genetic deletion or inhibition of the NFκB-activating kinase IKKβ in TAMs results in the loss of their IL-10^{high}/IL-12^{low} status, which is a sign of the loss of M2 polarization. Increased expression of IL-12 and NOS-2 indicates a repolarization towards the M1 phenotype that goes along with a significantly enhanced tumoricidal activity. While reprogramming of M2-polarized TAMs and reactivation of associated innate immune functions might be the ultimate therapeutic goal, the intrinsic protumorigenic function associated with TAMs in many human cancers has led to approaches that rather aim at blocking their recruitment altogether. Strategies that have shown promising results include the targeting of chemokines and CSF-1 or the direct inhibition of TAMs [144, 145].

While the majority of studies on myeloid cells in tumour development have focused on TAMs, in patients with melanoma or colon carcinoma, CD15+ IL4 receptor-expressing polymorphonuclear neutrophils also functionally suppress the antitumour immune response [146]. In patients with advanced renal cell cancer or pancreatic cancer, granulocytes appear to dominate the tumour-infiltrating myeloid population [147]. Following the M1/M2 paradigm, the existence of N1 and N2 polarized tumour-associated neutrophils (TANs) has been proposed [148]. Depletion of N2 TANs from untreated tumours increases CD8+ T cell activity, suggesting that the tumour environment converts neutrophils into immunosuppressive cells. After pretreatment of tumours by TGF-β blockade, neutrophil depletion rather results in a decreased activation

status of intratumoral CD8+ T cells, suggesting that the removed N1 TANs are predominantly immunostimulatory. Proinflammatory N1 TANs promote CD8+ T-cell recruitment by producing CCL3/MIP-1α, CXCL9/MIG and CXCL10/IP10 [149], while N2 TANs lack high levels of proinflammatory agents, but produce large amounts of arginase 1 that probably serves to inactivate T-cell effector functions [150].

Under certain conditions inflammation is protective against tumour development and it certainly contributes to the efficacy of chemotherapy [151]. Antigens from dying tumour cells are probably presented by inflammatory macrophages and dendritic cells (DCs) resulting in inflammasome activation, IL-1β production and the generation of protective immunity [152]. In breast carcinoma, mature DCs are restricted to peritumoral areas, while immature DCs are preferentially found in the tumour bed, where expression of CCL20/MIP-3α is high. Maturation and activation of these DC populations might be a route to positively influence the clinical outcome.

Tumour cells may produce chemokines that act directly on endothelial cells A more direct mechanism by which tumour cells foster angiogenesis and thereby support their own growth and survival is the production of proangiogenic chemokines by the tumour that can act on endothelial cells. Candidate receptors on endothelial cells are the CXCL8/IL-8 receptors CXCR1 and CXCR2. These appear to be differentially regulated in different endothelial cell types. While HUVECs apparently do not express CXCR1, 2 and 3, human microvascular dermal endothelial cells (HMECs) consistently express high levels of CXCR1 and CXCR4 and lower levels of CXCR2 and CXCR3 [23]. Increased expression of the ELR+ CXC chemokine CXCL8/IL-8 has been reported in various tumour settings including aggressive ovarian cancer [153]. Upon intraperitoneal transplantation of human ovarian carcinoma cells into nude mice, CXCL8 levels produced by the tumour directly correlate with the extent of neoangiogenesis and inversely correlate with survival [154]. Non-small-cell lung cancer cell (NSCLC) lines that express elevated levels of CXCL8/IL-8 display increased angiogenic activity in mice [155] while inhibition of CXCL8/IL-8 attenuated NSCLC development [156, 157]. Besides CXCL8/IL-8, CXCL5/ENA-78 which also engages the CXCR2 receptor is upregulated in NSCLC, and tissue levels directly correlate with the extent of tumour angiogenesis and even clinical outcome [158]. It should be pointed out, that in patients with NSCLC high CXCL8/IL-8 levels are also associated with extensive TAM infiltration. Also in prostate cancer, upregulation of ELR+ CXC chemokines results in angiogenesis-mediated tumour progression [159]. Here suppression of ELR+ CXC chemokine production by the tumour inhibited prostate cancer growth in SCID mice, which was largely attributed to inhibition of tumour angiogenesis [160]. Similar to the D6 decoy receptor, absence of the Duffy receptor for chemokines (DARC) in a mouse model for prostate cancer results in larger more aggressive tumours with higher vascularization [161]. Finally, elevated levels of CXCR2 ligands have been found in renal cell carcinoma biopsies, whereas CXCR2 has been found to be expressed on endothelial cells within the tumours. Again, elevated plasma levels of the CXCR2 ligands CXCL1/Groα, CXCL3/Groγ, CXCL5/ENA-78, and CXCL8/IL-8 correlate with a high metastatic spread in patients [162].

In addition to CXC chemokines, the CC chemokines CCL2/MCP-1, CCL11/eotaxin and CCL16/LEC have also been implicated in direct stimulation of angiogenesis (reviewed in [163]) based on the expression of CCR1 and CCR2 in endothelial cells [164–166]. CCL-2/MCP-1 has also been found to be upregulated in human breast cancer, where it is associated with neovascularization and survival [167, 168]. CCL2/MCP-1 is highly efficient in provoking neovascularization [169–171]. A recent study of the cytokine expression profile of breast cancer samples found an inverse correlation between oestrogen receptor status and the expression of a large number of cytokines [172]. In particular, CCL2/MCP-1, CCL4/MIP-1ß and CCL8/IL-8 were found to be elevated in high-grade tumours and to be associated with massive infiltration of proangiogenic macrophages.

Hence, due to the potent activity of chemokines on leucocytes and the simultaneous upregulation of multiple factors during tumour progression, it is impossible to distinguish in vivo between the direct effects of chemokines on endothelial cells and the proangiogenic activity of tumour-associated leucocytes.

Tumour cells may produce chemokines that act in a paracrine fashion Production of chemokine receptors by tumour cells allows chemokine-driven support of tumour growth in an either autocrine or paracrine fashion. A clear pathophysiological role for CXCL8/IL-8 has been proposed for human melanoma [173]. However, in these studies, tumour cells in addition to the chemokine also expressed the corresponding receptors CXCR1 and CXCR2 [174–176]. Autocrine signalling via CXCR2 is suspected to induce cell growth in a number of tumour types (reviewed in [177]). Interestingly, tumorigenic viruses such as Kaposi's sarcoma-associated herpes virus 8 (HHV-8) also encode G-protein-coupled receptors that bind CXCR2 ligands. Transgenic mice that express the HHV-8-encoded receptor in haematopoietic cells develop lesions that resemble Kaposi's sarcoma [178]. The development of angioproliferative defects in this transgenic model suggests that the CXCR2-like viral receptor is involved in oncogenic cellular transformation. Furthermore, in mouse melanoma cells that

constitutively express the chemokine receptor CXCR3, and its ligands CXCL9/Mig, CXCL10/IP-10 and CXCL11/I-TAC, induce cell survival, actin polymerization, migration and invasion [179].

Another chemokine receptor, CXCR4, is particularly well studied due to its essential role as a coreceptor for HIV entry [180]. The CXCR4 C-terminal domain plays a major role in receptor regulation, which appears to augment the process of epithelial-to-mesenchymal transition during breast cancer formation [181]. CXCR4 expression has also been detected on human glioma cell lines, where the corresponding ligand CXCL12/SDF-1α not only mediates AKT phosphorylation and resistance against serum withdrawal, but also chemotaxis [182]. CXCR4 is a prognostic marker in a number of cancer types including leukaemia, breast cancer and prostate cancer. Furthermore the SDF1/CXCL12–CXCR4 axis contributes to tumour progression (reviewed in [183]). Enhanced VEGF-A and NF-κB signalling increases CXCR4 expression during breast cancer progression and thereby promotes invasion and metastasis [184, 185]. It is interesting in this context that CXCR4 expression is increased in ductal carcinoma in situ (DCIS) lesions as compared to normal breast tissue and is further increased in invasive carcinoma [186].

Tumour cells may express receptors for homeostatic chemokines The potent chemoattractant activity of chemokines and the capacity of chemokine receptors to mediate leucocyte migration during homeostasis and inflammation enables chemokine receptor-expressing tumour cells to respond to chemokine gradients and the use certain chemokines as tissue-specific attractants. Conceptually, it is tempting to postulate that tumours misuse stem cell and leucocyte homeostatic chemokines to metastasize to lymph nodes and the bone marrow. Indeed, CXCR4 is found more highly expressed in mammary carcinoma tissue than in normal mammary tissue [187]. The corresponding ligand CXCL12/SDF-1α is expressed in lymph nodes, bone marrow, liver and lungs, where it induces homeostatic homing of haematopoietic cells [188, 189]. These are exactly the tissues to which mammary carcinoma cells preferentially metastasize and indeed a functional axis between CXCL12/SDF-1 and its receptor CXCR4 has been demonstrated for breast cancer metastasis [187]. CXCR4 is expressed in a large variety of human tumours including prostate cancer, leukaemia, brain tumours, breast cancer, Wilms' tumour, retinoblastoma, hepatoblastomas, ovarian cancers and cervical cancers [190]. The fact that CXCR4 is the chemokine receptor most commonly found in human tumours may provide an explanation for the frequent metastasis formation to CXCR4 ligand-expressing tissues [191]. Due to their diverse biological activity in many aspects of tumour formation, chemokines are attractive targets for the inhibition of tumour growth and metastasis formation.

The manifold actions of chemokines are also reflected in the higher efficacy of receptor neutralization in comparison to ligand neutralization. Monoclonal antibodies against CXCR4 inhibit growth and metastasis formation of human mammary carcinoma cells to the lymph nodes in SCID mice [187]. The growth of non-Hodgkin's lymphomas in immunodeficient mice is severely impaired by anti CXCR4 antibody treatment [192]. The CXCR4 small molecule inhibitor AMD3100, which was originally designed to block entry of human immunodeficiency virus, shows good efficacy in inhibiting brain tumours [193]. Besides CXCR4, CCR7, the receptor for the major T zone chemokines CCL21/SLC and CCL19/ELC, has also been implicated in tumour metastasis to the lymph nodes [194]. Also CCR7 has been shown to be highly expressed by human breast cancer cells and induces actin polymerization, chemotaxis and invasion [187]. In line with these findings, CCR7 is a biomarker predicting axillary lymph node metastasis in T1 breast cancer [195] and the CCR7 ligands CCL19 and CCL21 are significantly higher in lymph node-positive than lymph node-negative patients [196]. The CCR7-dependent lymphatic migration of tumour cells resembles the physiological migration of DCs into the draining lymph nodes [197]. CCR7-expressing gastric carcinoma cells metastasize with a high frequency to the lymph nodes and patients with CCR7-expressing tumours have a significantly poorer prognosis [198]. Apparently, this also holds true for oesophageal squamous cells carcinomas, where CCR7 expression directs oesophageal lymphatic permeation, lymph node metastasis, tumour depth and tumour-node-metastasis stage and is associated with a poor survival [199].

The chemokine receptors CCR10 and CCR4 have been implicated in metastasis formation of melanoma cells and localization of T cell leukaemia to the skin [200]. The CCR10 ligand CCL27/ESkine is constitutively expressed in the keratinocytes of normal skin [201]. In melanoma, CCR10 expression appears to have a dual role in conferring immune resistance and being associated with lymph node metastasis [202, 203]. CCR4, the second skin-homing chemokine receptor is frequently coexpressed with CCR10 in T-cell leukaemia/lymphoma and both receptors together are probably responsible for the invasion of acute T-cell leukaemia/lymphoma into the skin [204]. Finally, CCR3 has been found to be expressed in tumour samples and tumour cell suspensions from patients with CD30+ T-cell lymphoma, where together with its ligand CCL11/eotaxin CCR3 appears to play a role in the recruitment and retention of malignant T cells in the skin [205].

At first glance one might be led to believe that chemotaxis is the dominant mechanism for organ-specific

metastasis. However, metastasis involves many different aspects and types of molecules. It is therefore most likely that the attractive forces exerted by chemokines need the cooperation of many additional players during tumour dissemination. Still, the chemokine/chemokine receptor axis will remain an important target for antitumour therapy. This will hold true irrespective of the mechanism of anti-chemokine-receptor efficacy, to which true receptor inhibition could contribute as much as Fc-receptor-mediated killing and presentation of opsonized travelling tumour cells by the innate immune system.

Concluding remarks

The formation of a functional vasculature via angiogenesis plays an important part in embryonic development and during disease progression. Work over the last several years has unravelled a complex interplay between endothelial cells and accessory cells of the immune system, such as macrophages, in controlling angiogenic processes. Chemokines play an important role in coordinating this interplay via direct mechanisms, acting on endothelial cells, and by influencing immune cells to secrete proangiogenic factors. Deeper insights into these interactions will help us in understanding the mechanisms at play during situations where an immune response needs to be coordinated with the formation of new blood vessels, for instance during wound healing or tumour formation. Applying these insights in the clinic will also provide new avenues for disease treatments.

Acknowledgments The authors would like to thank their colleagues for stimulating discussions. This work was funded by the Max Planck Society, a DFG grant (SI-1374/3-1; A.F.S.), an ERC starting grant (260794-ZebrafishAngio; A.F.S.) and the DFG special priority program 629 (F.K.).

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