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SPOTLIGHT REVIEW

Chemokines as effector and target molecules in vascular biology

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Abstract Chemokines are key mediators of inflammation. In pathological tissues, the main roles of chemokines are to regulate leucocyte accumulation through the activation of oriented cell migration and the activation of limited programs of gene transcription. Through these activities, chemokines exert many crucial functions, including the regulation of angiogenesis. The 'chemokine system' is tightly regulated at several levels, such as the post-transcriptional processing of ligands, the regulation of the expression and function of the receptors and through the expression of molecules known as 'atypical chemokine receptors', proteins that function as chemokine scavenging and presenting molecules. Several experimental evidence obtained *in vitro*, in animal models and in human studies, has defined a crucial role of chemokines in cardiovascular diseases. An intense area of research is currently exploring the possibility to develop new effective therapeutic strategies through the identification of chemokine receptor antagonists.

Keywords Chemokines • Chemokine receptors • Atypical chemokine receptors • Angiogenesis • Vascular biology

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1. Introduction

The correct tissue distribution and positioning of leucocytes is a process required for the functioning of the immune response under both homeostatic and reactive conditions.[1,2] Chemotaxis (i.e. directional migration) is governed by the ability of the cell to sense a gradient of chemotactic factors.[3] Chemotactic factors include molecules of different origin, namely bacterial products, such as formylated peptides; alarmins and antimicrobial proteins, such as the high mobility group protein B1 (HMGB1), defensins, cathelicidins, and chemerin;[4,5] inflammatory-derived lipids, such as leucotriene B4 and platelet-activating factor; complement factors, such as C3a and C5a;[6,7] and proteins of different nature, among which chemokines represent the largest family.[8] Chemokines comprise about 50 proteins characterized by a relatively low molecular weight (8–10 kDa) and the presence of conserved cysteine residues. Chemokines are classified in four families according to the number and position of the first two N-terminal cysteines, namely the CC, CXC, CX3C, and C chemokines.[8] Chemokines were initially discovered as early inducible genes rapidly produced by activated cells; this defines chemokines as mediators of a large variety of reactive and pathological conditions. A limited number of chemokines is, however, produced in a constitutive manner and these proteins were shown to be involved in the homeostatic positioning of immune cells within lymphoid organs as well as in peripheral tissues.[9,10]

Chemokines interact with 23 seven-transmembrane spanning receptors,[8,11] 19 of which operates as G protein-coupled members and are responsible for cell migration, while four are unable to interact with G proteins and are devoid of chemotactic activity.[8,12] Since chemokines are basically charged proteins, they also undergo low affinity interactions with different glycosaminoglycans expressed on the surface of many cells, such as endothelial and epithelial cells. This process is critical for chemokine biology and relevant for the formation of the chemotactic gradient.[13] The significant degree of ligand promiscuity further amplifies the level of complexity of the chemokine–chemokine receptor system. Indeed, with few exceptions, chemokines observe the general rule that each protein binds more than one receptor and each chemokine receptor interacts with more than one protein. However, the degree of promiscuity is somewhat more limited for the so called 'homeostatic' chemokines than for the 'inflammatory' ones and the ligands for a given receptor are almost always restricted to the same structural subclass.[8] An additional level of complexity is arising from the finding that some of the chemokine receptors also bind ligands that do not belong to the chemokine family. This is true for both 'classic' and 'atypical' chemokine receptors. For instance, it was recently reported that the hormone peptide adrenomedullin is a ligand of the

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atypical chemokine receptor ACKR3[14] and more examples are available for classic chemokine receptors. This is the case for molecules released from damaged cells or tissues which bind CXCR4, such as the extracellular ubiquitin and the HMGB1/CXCL12 complex.[8,15] Furthermore, collagen breakdown products, macrophage migration inhibitory factor and the N9-terminal domain of human tyrosyl-tRNA synthetase were reported to bind and activate CXCR1 and CXCR2. The antimicrobial proteins β-defensins are functional ligands of CCR6 and CCR2[4] and oxysterols, products of cholesterol metabolism, were recently reported as new CXCR2 functional ligands.[16] Finally, several microbe-derived molecules were shown to bind and activate chemokine receptors. Indeed, many chemokine receptors function as HIV co-receptors, being the envelope protein gp120 of some HIV strains able to bind CXCR4 (the so called X4-tropic strains), CCR5 (R5-tropic strains), or other chemokine receptors. Even though a large number of chemokine receptors were reported to interact with HIV-1 viral strains, CCR5 is the only receptor demonstrated to play an essential role in HIV/AIDS pathogenesis and currently is pharmacologically targeted in HIV-positive patients with a low molecular weight antagonist. In addition to gp120, other viral proteins were reported to activate chemokine receptors, such as HIV-1 p17 (CXCR2);[17] some viral-encoded chemokines, such as the cytomegalovirus encoded vCXCL1 (CXCR1 and CXCR2); the Kaposi's sarcoma-associated herpesvirus (HHV8)-encoded vMIP-II (CCR8),[18] and the HHV6-encoded chemokines U12 and U51 (many CC chemokine receptors). The extensive interaction of microbial components with the chemokine system suggests that the manipulation of chemokine functions provides a survival advantage to viruses highlighting the relevance of chemokines in antimicrobial immune responses.

2. Regulation of chemokines and chemokine receptors

Chemokines and chemokine receptors represent a complex network responsible for the migration of various leucocyte subsets. Fine-tuning of the chemokine system is obtained through the regulation of both ligand and receptor functions. A first level of regulation of the chemokine system is represented by the *in vivo* differential spatio-temporal expression of chemokines and their cognate receptors.[9,19] A valid example is represented by CXCR3 and its ligands (CXCL9, CXCL10, and CXCL11) which are produced in anatomically restricted sites where they participate in the control of T cell trafficking in inflammation.[20] Post-translational modifications of mature proteins, such as enzymatic processing by proteases, like elastase, cathepsins, and dipeptidylpeptidases, result in the activation of precursor proteins or in the inactivation of mature proteins. In addition to proteolytic processing, citrullination and glycosylation represent additional important levels of positive and negative regulation.[21] All these mechanisms contribute to strongly reduce the apparent redundancy observed *in vitro* for many members of the chemokine system.[9]

Specific expression profiles characterize leucocyte subsets, or their state of activation, and are responsible for shaping cell response. For instance, T cell subsets (e.g. Th1, Th2, Th17, and Tregs) are characterized by the expression of specific chemokine receptor profiles and are selectively recruited during immune responses based on the local chemokine microenvironment.[9,10] In a similar manner, the expression of chemokine receptors identifies 'resident' and 'inflammatory' monocytes that express CX3CR1 and CCR2, respectively.[22,23] Finally, the expression profile of chemokine receptors characterizes the functional and activation state of many cells, such as dendritic cells,[24] NK cells, B and T lymphocytes.[10]

Receptor signalling represents a further level of regulation. Different adaptor proteins (e.g. GPCR kinases and β-arrestins) can be recruited upon ligand binding.[25] For instance, CXCL8 is able to bind and trigger the phosphorylation, desensitization, and internalization of both CXCR1 and CXCR2. However, CXCR1 mainly couples to GRK2, while CXCR2 couples with GRK6 which is responsible for the negative regulation of the receptor.[26] Another example is represented by the CCR7 ligands, CCL19 and CCL21. Both ligands induce G protein activation, Ca^{2+} flux and chemotaxis, but only CCL19 induces β-arrestin-mediated CCR7 internalization. This effect has been ascribed to the different GRKs usage, as CCL19 activates GRK3 and GRK6, while CCL21 only recruits GRK6.[27] The lack of receptor coupling to G protein signalling, such as that observed in IL-10 stimulated dendritic cells, may result in a non-signalling receptor endowed with scavenging properties which contributes to the resolution of inflammation by removing inflammatory chemokines.[28] These observations represent examples of functional selectivity of chemokine receptors. Indeed, emerging evidence indicates that multiple regulatory mechanisms, including spatio-temporal and tissue-specific expression of chemokine receptors, the presence of coexpressed proteins, and the activation of different intracellular pathways by chemokines that bind the same receptor, results in biased signalling properties of the receptors.[29–31] Multiple mechanisms thus operate and introduce functional selectivity to chemokine receptors. This represents a new level of complexity that requires a new conceptual change, from the receptor-single ligand approach to a more systemic approach of investigation.[29] Recently, it was also reported that different CC and CXC chemokines can induce leucocyte migration through a positive cooperation process. This effect is the result of multiple molecular mechanisms.[32] First, synergism may result from the simultaneous or sequential activation of two chemokine receptors by their respective ligands. For instance, cooperation between CXCR3 ligands and CXCL12 was reported for the migration of plasmacytoid dendritic cells and memory T cells.[33,34] Similarly, CCL2, CCL5, and CCL7 were shown to synergize with CXCL8 or CXCL12 in monocytes migration[35] and CCL3 synergizes with CXCL8 and CXCL12 for dendritic cell migration.[36] Alternatively, in the presence of high concentrations of the ligands, synergism may derive from the activation of a single chemokine receptor by homo- or heteromers of the ligand. Synergistic chemokine activation may also derive from the amplification of the receptor signalling cascade. For instance, MAPK phophorylation and increased intracellular calcium levels have been proposed as the mechanisms underlying the synergistic activation of monocytes and dendritic cells. Independently of the mechanism, synergism between chemokines has been clearly documented both *in vitro* and *in vivo* and is likely to be one of the mechanisms that finely tunes leucocyte recruitment in inflammation. A last proposed mechanism of cooperation is between chemokine receptors with the formation of homo- or hetero receptor dimers.[37] CXCR4 and CCR5 dimers were reported to promote T cell migration[38] and the homo- and heterodimerization of CCR2 and CCR5 was shown to promote cell adhesion.[39] Finally, a small subset of chemokine receptors, referred to as atypical chemokine receptors, were recently proposed to act as negative regulators of chemokine biology through their ability to scavenge chemokines.

3. Atypical chemokine receptors

A small subset of molecules, highly homologous to 'typical' chemokine receptors, binds chemokines but does not signal through G proteins. Members of this receptor subfamily lack structural determinants supporting $G\alpha_i$ activation, a key signalling event in cell migration.[40] For this reason, these receptors were originally believed to be 'silent' chemokine binding molecules. Subsequently, evidence has accumulated demonstrating the ability to transport chemokines to intracellular degradative compartments; for this reason they are now considered as chemokine scavengers or decoys receptors. Following the recent discovery of their ability to activate β-arrestin-dependent signalling pathways,[41–43] these molecules have been renamed atypical chemokine receptors (ACKRs). This distinct subfamily at present includes four members, represented by ACKR1 (Duffy antigen receptor for chemokines-DARC), ACKR2 (D6 or CCBP2), ACKR4 (CCRL1 or CCX CKR and CCR11), and ACKR3 (CXCR7 or RDC1). The name ACKR5 is still pending for the protein known as CCRL2 or HCR (*Figure 1*).[8]

ACKRs recognize distinct and complementary sets of chemokines and are strategically expressed in different cell types often of the non-haematopoietic lineage. In most of the cases, ACKRs are expressed on lymphatic and/or vascular endothelium. The main biological function of ACKRs is the regulation of chemokine concentration by means of chemokine scavenging, transport or presentation allowing the creation, maintenance, and regulation in time of tissue chemokine gradients. Recent evidence indicates that ACKRs operates as β-arrestin-biased chemokine receptors and although the underlying molecular mechanisms are still incompletely defined this signalling pathway is central to their ability to regulate ligand gradients in the tissue.[43]

ACKRs play a non-redundant role in inflammatory responses controlling leucocyte extravasation from the blood vessels to the inflamed tissue and in the correct establishment of the adaptive immune response trough the regulation of leucocyte trafficking to lymph nodes.[44,45] For instance, ACKR2 was shown to play an important role in limiting chemokine-induced inflammation and it is required for adequate cardiac remodelling in an experimental model of myocardial infarction.[46] Beside inflammation and immunity, shaping of chemokine gradients by ACKRs was reported to play an important role in controlling cardiovascular and neuronal development.[14,47] ACKR3 KO mice have serious defects in cardiovascular development and they die postnatally.[48] These mice also present defects in the development of lymphatic vasculature and it was recently demonstrated that both defects are due to the ability of ACKR3 to bind and control the bioavailability of adrenomedullin.[14] ACKR3, like its canonical counterpart CXCR4, was also reported to have a role in controlling vascular reactivity and in the regulation of haemodynamics in normal and pathological conditions.[49]

4. Chemokines in angiogenesis

Chemokines of the CXC family are key mediators of angiogenesis in both physiological and pathological conditions. Some of the CXC chemokines contain a glu–leu–arg (ELR) amino acid sequence at their N-terminus and are referred to as ELR^+ chemokines. All the ELR^+ chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8) are pro-angiogenic while the ELR^- members (CXCL4, CXCL9, CXCL10, CXCL11, CXCL14) are anti-angiogenic.[50,51] The only remarkable exception to this rule is the ELR^- chemokine CXCL12, which is currently considered as a pro-angiogenic protein (see subsequently).

In mice, all angiogenic ELR^+ chemokines signal via CXCR2, while in humans CXCL6 and CXCL8 also bind to CXCR1.[52] Both receptors are expressed by endothelial cells and were demonstrated to be able to induce chemotaxis. Of note, CXCR2 is also expressed by leucocytes and, consistently with this observation, the defect of neovascularization observed in CXCR2 deficient mice is paralleled by modifications in the inflammatory infiltrate.[53] It was recently reported that CXCR2 may also promote tumour neoangiogenesis through the interaction with tumour-derived oxysterols.[54] Moreover, pro-angiogenic chemokines, such as CXCL8 were shown to be directly produced by tumour cells[55,56] and to be part of an amplification loop in the neovascularization process induced by master angiogenic factors, such as FGF2 and osteopontin.[57] Despite the pro-angiogenic role of ELR^+ chemokines, CXCR2 seems dispensable for embryonic angiogenesis since CXCR2 knockout mice develop normally, except for the altered positioning of oligodendrocyte precursors.[58,59] Thus, it is likely that a different set of chemokines plays a role in embryo development.

CXCL12 is currently regarded as the only ELR^- pro-angiogenic chemokine. CXCL12 is the first chemokine to be expressed during embryogenesis and mice lacking CXCL12 or CXCR4 show defective embryonic blood vessel development in the intestine and kidney.[60] However, while mice lacking master angiogenic molecules, such as VEGF-A or Notch, die early during embryonic development, half of the CXCR4 mutants come to light,[60] supporting the existence of CXCL12/CXCR4 independent mechanisms. CXCL12 also binds ACKR3; both CXCR4 and ACKR3 are induced in activated endothelial cells.[61] ACKR3 may sequester CXCL12 or alter CXCR4 signalling forming CXCR4/ACKR3 heterodimers.[62] Another atypical receptor, namely ACKR1, may be involved in the regulation of angiogenesis sequestering CXCL1, CXCL5, and CXCL8.[63] When overexpressed in endothelial cells, ACKR1 decreased the pro-angiogenic properties of ELR^+ CXC chemokines,[64] whereas ACKR1 deficient mice showed increased levels of these chemokines as well as increased angiogenesis in a model of prostate adenocarcinoma.[65] Finally, CXCL17, a chemokine able to induce *in vitro* and *in vivo* macrophage migration, was shown to promote angiogenesis and tumour growth.[66–68] CXCL17 acts through the interaction with GPR35, a GPCR which also binds metabolites of tryptophan catabolism, such as kynurenic acid,[69] for which it was recently proposed the name CXCR8.[11] CXCR8 and CXCL17 show similar expression patterns in mucosal tissues, such as gastric mucosa and bronchoalveolar epithelia.[70,71]

Other GPCRs, not belonging to the chemokine receptor family, able to interact with short fatty acids were recently reported to regulate angiogenesis. GPR43, which binds propionate and butyrate, is highly expressed by neutrophils and mediates their migration *in vitro*[72] and *in vivo* in experimental models of intestinal inflammation.[73] GPR120 is a receptor for unsaturated long chain free fatty acids that mediates insulin signalling and anti-inflammatory effects.[74] Moreover, recent observations have shown that GPR120 is able to induce angiogenesis and migration in human colorectal carcinoma.[75] Also members of the CC chemokine family may have a role in angiogenesis. For instance, endothelial cells can express functional CCR2 and CCL2, its ligand, can promote blood vessel formation *in vivo*.[76]

An angiostatic role has been proposed for the ELR^- chemokines CXCL10, CXCL11, CXCL4, and CXCL14. CXCL9, CXCL10, and

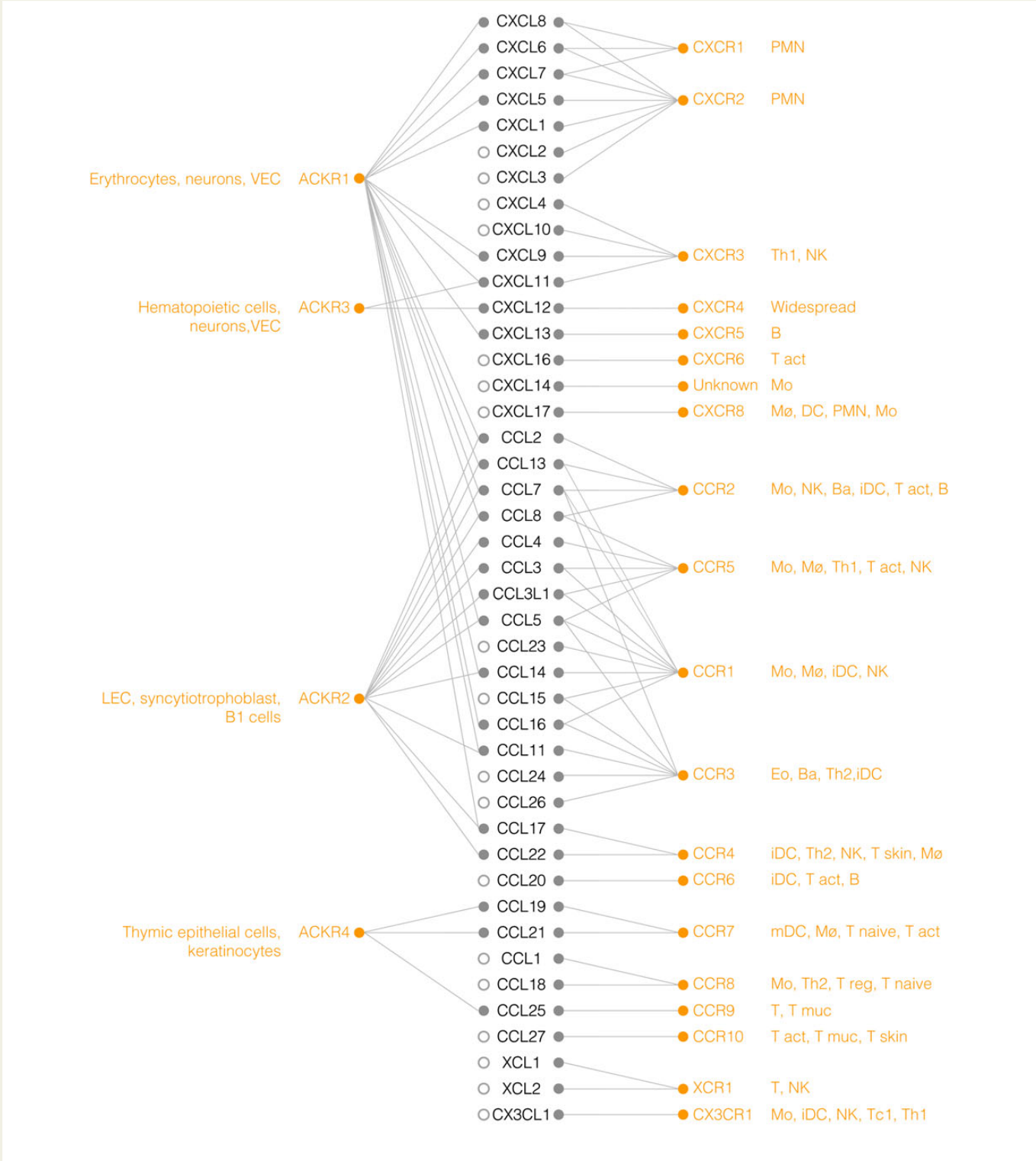

Figure 1 Leucocyte expression and ligand specificity of chemokine receptors. The ligand spectra of conventional and atypical chemokine receptors are shown. Ba, basophils; Eo, eosinophils; iDC, immature dendritic cells; mDC, mature dendritic cells; Mo, monocytes; Mø, macrophages; PMN, polymorphonuclear cells; NK; natural killer cells; T act, activated T-cells; Th1, type 1 helper T cells; Th2, type 2 helper T cells; T muc, mucosal-homing T-cells; T naive, naïve T-cells; T reg, regulatory T-cells; T skin, skin-homing T-cells; Tc1, cytotoxic 1T cells; VEC, vascular endothelial cells; LEC, lymphatic endothelial cells.

CXCL11 are induced by IFNs and bind CXCR3.[76] Their angiostatic functions are related to the inhibitory effects of this receptor on endothelial cell proliferation. In humans, CXCR3 exists in two main splicing variants, CXCR3A, expressed by lymphocytes, and CXCR3B, expressed by endothelial cells. CXCL4, one of the major platelet-derived chemokine, is the first angiostatic chemokine to be

described.[77] In humans, two non-allelic CXCL4 variants exist, CXCL4, which binds CXCR3B and CXCL4L1, which binds to both CXCR3A and CXCR3B. Of the two, CXCL4L1 was found to be more potent in inhibiting bFGF- and CXCL8-driven angiogenesis.[78] CXCL14 was also found to inhibit endothelial cell chemotaxis to CXCL8, VEGF, and bFGF *in vitro* and to potently inhibit angiogenesis *in vivo*.[79] CXCL14 was also shown to inhibit CXCL12-mediated angiogenesis, although the molecular mechanisms involved in this function are still unclear.[80,81]

5. Chemokines in pathology: focus on cardiovascular diseases

Chemokines, due to their involvement in orchestrating leucocyte trafficking are involved in almost all diseases. Here are summarized the evidence about the involvement of chemokines in cardiovascular diseases. The role of chemokines, in particular of CCL2, in the recruitment of circulating monocytes to the early atherosclerotic lesions, where they differentiate in foam cells, is very well established.[82] Boring *et al.*[83] provided the first evidence of the fundamental role of the CCL2–CCR2 axis in monocyte recruitment to the atherosclerotic plaque demonstrating that the lack of CCR2 decreases lesion formation in apoE$^{-/-}$ mice without effecting plasma lipid or lipoprotein concentrations.

CCL2 is one of the molecular links between oxidized low-density lipoproteins (LDLs) and foam cell recruitment because it is expressed by endothelial cells stimulated with oxidized LDLs.[84] In early plaques formation, there is also sustained production of CXCL10 and CCL5, both chemokines can recruit Th1 lymphocytes and monocytes. At more advanced stages, are instead produced CXCL1[85] and CX3CL1[86] which are induced by shear stress;[87] both chemokines are responsible for the recruitment of monocytes and involved in plaque rupture. This latter finding is supported by the evidence that chemokine inhibitors induce plaque size reduction and increase plaque stability.[88] CX3CL1 can also promote the recruitment of CX3CR1^{+} smooth muscle cells inside the plaques.[89] At later stages of the disease also CCL2 play a role in plaque rupture through its ability to induce macrophage activation[90] and CCR5 seems to be particularly important for the regulation of T cell infiltrate.[91] Consistently with these findings, Combadiere *et al.*[92] have demonstrated that the combined depletion of CCL2, CX3CR1, and CCR5 can almost abolish atherosclerosis in hypercholesterolaemic mice.

Chemokines produced by endothelial cells are also important mediators in ischaemia-induced myocardial injury (*Figure 2*). CXCL8 and CCL2 participate in the recruitment of neutrophils and inflammatory monocytes responsible of injury induction; targeting of both chemokines significantly reduces infarct size.[93] The importance of chemokine clearance for the correct cardiac repair after myocardial injury was also demonstrated by the use of atypical chemokine receptor ACKR2 KO mice that are more prone to cardiac rupture in a model of myocardial infarction.[46] On the contrary, CXCL12 and its receptor CXCR4 have a cardioprotective role after myocardial infarction because its ability in recruiting progenitor cells which promote tissue repair. However, the role of the CXCL12–CXCR4 axis in cardiovascular disease is not fully understood and complicated by the identification of another CXCR4 ligand, named MIF, and of an alternative receptor for CXCL12, the atypical chemokine receptor ACKR3.[94] MIF can be either cardioprotective, through the recruitment of endothelial progenitors,[95] or induce cardiovascular damage promoting myocardial infiltration of inflammatory cells.[96,97] On the contrary, the atypical chemokine receptor ACKR3 seems to play only cardioprotective effects. ACKR3 is expressed by endothelial progenitors and is necessary for endothelial repair.[98] In addition, ACKR3 possesses a cholesterol-lowering effect possibly reducing the hyperlipidaemia-induced monocytosis.[99]

Chemokines were also found to be involved in the development of congestive heart failure. These patients present increased circulating levels of both CC and CXC chemokines and *in vivo* experiments demonstrated that chemokines are induced as the result of mechanical overload, which attracts and activates monocytes and macrophages; these recruited cells will produce pro-inflammatory cytokines which contribute to the pathogenesis of heart failure.[100] In addition to the studies performed in animal models, several data obtained in humans propose a role for chemokines in cardiovascular diseases. CXCL8 expression is increased in carotid plaques excised from patients after ischaemic stroke compared with plaque specimens from asymptomatic subjects, suggesting a role for CXCL8 in plaque destabilization.[101] Referring to plasma chemokine levels, a negative correlation was found between CXCL12 and the risk of cardiovascular events.[102] The increased levels of CCL5 and CCL18 in patients with unstable angina[103] were proposed as predictors of cardiovascular risk but, at least for CCL5, a large case-cohort study excluded the possibility to use this chemokine as a coronary risk biomarker.[104] The importance of the chemokine system in cardiovascular diseases is highlighted by the altered risk of disease in patients carrying genetic polymorphisms. The G403A polymorphism in the promoter region of CCL5 correlates with higher risk of coronary artery disease,[105] while the loss of CCR5 expression observed in CCR5Δ32 subjects, the CX3CR1-I249 and CCR2-64I polymorphisms and the reduced expression conferred by the CCL2-2518A variant, all lead to protection against cardiovascular events.[106] Beside chemokines, increased risk of cardiovascular disease was also found in individuals carrying mutations in genes of the leucotriene pathway involved in the production of LTB4 a potent chemoattractant for different leucocyte subpopulations.[107]

6. Targeting chemokine receptors

Chemokine receptors constitute the largest branch of the γ GPCR subfamily, a receptor superfamily which overall represents the most successful target of small molecule inhibitors in modern pharmacology. As such, chemokine receptors have been considered valid candidates for the development of small molecular weight inhibitors, and in the last 15 years most chemokine receptors have been the target of numerous drug discovery programs developed by the major pharma companies. For some receptors, the progress is still at the preclinical stage, but for most receptors one or more inhibitors are presently under investigation. Disappointingly, several programs have proved unsuccessful in Phase II trials, and the only two inhibitors that have completed their path to the market so far have clinical indications not related to inflammation or immune responses, as the CCR5 inhibitor Maraviroc is used for HIV treatment and the CXCR4 inhibitor Plerixafor for stem-cell mobilization. A number of reasons have been put forward to explain the failure to develop chemokine receptor-targeted drugs. In some cases, the bias has been proved to be on the drug side, insufficient receptor coverage in particular, but some limitations are intrinsic to the biology of the chemokine system and could therefore prove to be hard to overcome. A major issue concerns the high level of redundancy characterizing the chemokine system, which has led to a more recent approach based on combined blockade of chemokine-receptor axes

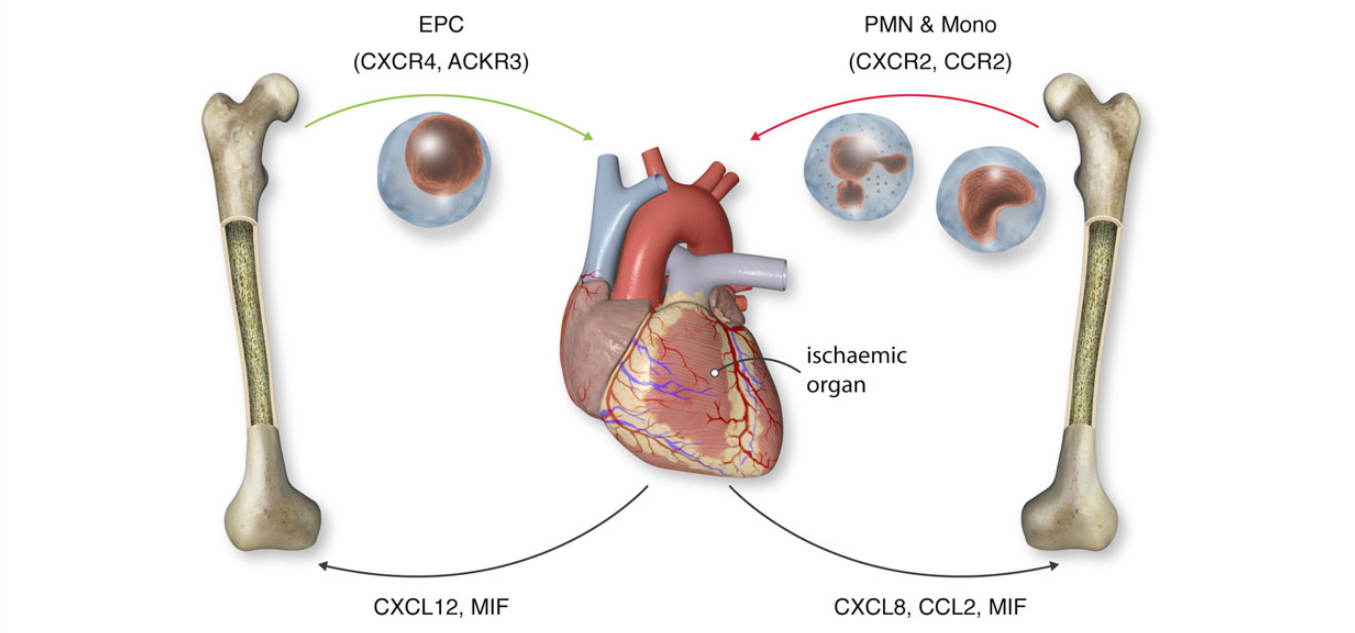

Figure 2 Role of chemokine-mediated bone-marrow cell mobilization in ischaemic myocardial injury. PMN and monocytes mobilized from the bone marrow by inflammatory CC and CXC chemokines released by the ischaemic organ drive leucocyte-mediated tissue damage. The ischaemic organ also releases CXCL12 and other mediators which act at the bone-marrow level releasing cardioprotective molecules. PMN: neutrophils; Mono: monocytes; MIF: macrophage migration inhibitory factor; EPC: endothelial progenitor cells. Green arrow means cardioprotection; red arrow means cardiotoxicity.

concomitantly supporting the pathogenesis of a given disease. A second limitation is represented by the growing evidence that chemokine receptors, as other GPCRs, are capable of biased signalling, a property that has not been taken into account upon developing chemokine receptors small molecule antagonists.[108,109] Evidence has also been provided that a given chemokine receptor inhibitor may regulate the functional properties of other receptors to which it does not bind directly, indicating that allosteric modulation of chemokine receptors represents a second neglected aspect of chemokine receptor biology with significant impact on drug development.[110] Thus, focus on the chemokine system in the search for suitable therapeutic targets is feasible, but recent setbacks in clinical testing of potential inhibitors of chemokines and their receptors reveal that this is more challenging than expected.

As mentioned, the chemokine system has a pivotal role in the progression of atherosclerosis. Different chemokine-receptor axes were demonstrated to recruit different leucocyte subtypes during successive stages of the disease. In particular, $CCR2^{+}/CX3CR1^{+}/Ly\text{-}6C^{hi}$ inflammatory and $CCR2^{-}/CX3CR1^{++}/Ly\text{-}6C^{lo}$ resident monocytes are both linked to progression of atherosclerotic plaques. CCR2 is required for bone-marrow mobilization of monocyte mobilization and their subsequent entry into the plaque and in combination with CCR5 and CX3CR1 sustain the efficient recruitment of $CCR2^{+}/Ly\text{-}6C^{hi}$ monocytes in the plaque, whereas $CCR2^{-}/Ly\text{-}6C^{lo}$ monocytes are recruited via CCR5 with less efficiency but are relevant for the development in $CD11c^{+}$ dendritic cell.[111] These chemokine/chemokine-receptor axes have been deleted or antagonized in different mouse models of atherosclerosis and results clearly illustrate their overlapping but non-redundant role in the pathogenesis.[92] Based on this experimental evidence and on the aforementioned information provided by genetic studies, several chemokine-receptor antagonists have also been assessed in different models of atherosclerosis. CCR5 blockade by TAK779 and NBL-74330 effectively reduced atherosclerotic lesion formation in mice by reducing recruitment of Th1 cells and increasing the number of Tregs,[112,113] while in rats CXCR2 blockade by SB517785-M significantly reduced angiotensin II-induced leucocyte recruitment in the arterioles and limited vascular damage preceding atherogenesis.[114] Alternatively, the infusion of M-T7, a secreted myxoma viral C, CC, and CXC chemokine-binding protein resulted in significant reduction of intimal hyperplasia in both rabbit and rat models of angioplasty injury.[115] Most antagonists mentioned earlier, however, have not reached clinical testing for atherosclerosis. The poor oral bioavailability of TAK779 prevented its entry into clinical trials as a treatment for HIV and, although this compound has been replaced with improved derivatives for the potential treatment of HIV, these have not been tested in models of atherosclerosis.[116] So far, the only approach assessing efficacy of targeting the chemokine system in atherosclerosis in humans has been a Phase II clinical trial with an anti-CCR2 mAb, and although the mAb was able to reduce CRP levels, the trial was not further developed. Finally, based on mechanistic evidence, benefits may be achieved through combined blockade of CCR2, CX3CR1, and/or CCR5, an approach that has been proved to be successful in mice,[92] though the risks of such extreme intervention on the immune system should be taken into account. Taken together, these results indicate that in principle chemokine receptors inhibition is a promising approach for the prevention and treatment of atherosclerosis, though the development of effective drugs for this clinical application may need the development of innovative strategies.

7. Concluding remarks

Chemokines are multifaceted mediators involved in many phases of the inflammatory process. Chemokines do not only play a role in the initial

phase of inflammation through the induction of directional leucocyte migration and activation of adhesion molecules, but are also important in the regulation of the composition and activation state of the inflammatory infiltrate. Among the several roles played in inflammation, chemokines are also potent regulators of angiogenesis. Angiogenesis is a crucial component of the resolution phase of inflammation and also characterizes the chronic phase of this process. Because of the ability to regulate angiogenesis, chemokines also contribute to many pathological processes, such as the formation of atherosclerotic lesions and the tissue damage that characterizes ischaemia-induced myocardial injury. In addition to the data generated in preclinical experimental models, the importance of chemokines in cardiovascular diseases is well supported by the altered risk of disease in patients carrying genetic polymorphisms. The dual pro- and anti-angiogenic role of chemokines in pathology deserves further investigations. The results coming from the search of low molecular weight molecules able to act as chemokine-receptor antagonists are encouraging and support the possibility to develop new effective therapeutic strategies in near future.

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