Chemokine Signaling Defines Novel Targets for Therapeutic Intervention

José Miguel Rodríguez-Frade, Carlos Martínez-A. and Mario Mellado*

Department of Immunology and Oncology, National Center of Biotechnology, CSIC, Campus Universitario de Cantoblanco, E-28049, Madrid, Spain

Abstract: Members of the human chemokine family are considered a suitable target for therapeutic intervention, as they have a fundamental role in several important human diseases. Here we outline potential new areas of intervention based on recent findings on chemokine receptor function.

I. CHEMOKINES AND CHEMOKINE RECEPTORS

Members of the family of low molecular weight pro-inflammatory cytokines termed chemokines, participate in an exceptional range of physiological and pathological processes [1, 2]. Nearly 50 chemokines have been described and are linked to the control of lymphocyte trafficking, regulation of T-cell differentiation, HIV-1 infection and development [3-6]. Despite their low amino acid sequence similarity, the distinct chemokine molecules have remarkably comparable three-dimensional structures [5, 6]. The original classification of chemokines based on structural criteria (C, CC, CXC and CX3C chemokines) is being abandoned, and replaced by a functional classification by which the chemokines are grouped in two main categories, constitutive and inducible [7]. As a general although not absolute rule, constitutive chemokines are regulated during development, whereas inducible chemokine expression is regulated during inflammatory processes. In addition, several viruses encode highly selective chemokine receptor ligands; these viral chemokine analogs function as agonists or antagonists, and may thus have a role in viral dissemination or evasion of the immune response [8, 9].

As chemokines direct leukocyte trafficking to inflammation sites and are thus involved in many disease states, attention soon focused on these molecules as possible targets of therapeutic intervention (reviewed in 10-12). Some of these early expectations were hampered by the relative promiscuity among chemokines and their receptors, and by the lack of selectivity in chemokine receptor expression by different cell types [13]. Despite this fact, interest in the chemokines has increased in recent years, as chemokine functions have been defined in physiological and pathological situations including angiogenesis, hematopoiesis, tumor rejection, cancer metastasis, and various diseases characterized by inflammation and cell infiltration [14, 15]. For example, the defects in cerebral structure, gastrointestinal tract development and hematopoiesis in CXCL12- and CXCR4-deficient (knockout, KO) mice indicate the importance of this ligand and its receptor during development [14-17].

Chemokines interact with extracellular matrix-associated cell surface glycosaminoglycans (GAG), which present chemokines to their receptors [18]. Chemokine interaction with GAG on endothelial surfaces is thought to facilitate high local chemokine concentrations, even under blood flow conditions [18]. Although it has long been known that chemokines can form dimers or higher-order oligomers, their relevance for chemokine function has not been clarified, since monomeric chemokines retain receptor binding and activation potential in *in vitro* studies [19]. Recent evidence suggests that GAG interaction promotes chemokine dimerization, an apparent prerequisite for *in vivo* chemokine activity [18].

Chemokines act by binding to seven-transmembrane, G protein-coupled receptors (GPCR) [20, 21]. These receptors are classified as CCR, CXCR, CX3CR and XCR, based on the ligand to which they bind [22, 23]. A single chemokine receptor usually interacts with several chemokines (redundant or shared receptors), although there are examples of receptors that interact with only a single known ligand (specific receptors) [13]. Complexity is further increased, as a single cell type may express more than one chemokine receptor simultaneously, or sequentially during its development [24, 25]. Non-signaling chemokine receptors have also been described that can bind CC or CXC chemokines, and whose function remains a matter of debate. In addition to their ability to bind chemokines in a specific and saturable manner, some chemokine receptors are used by HIV-1 for cell entry [26]. There is also evidence of virus-encoded chemokine receptors, which probably have an important role in viral dissemination.

Chemokines and chemokine receptors thus represent interesting targets for therapeutic intervention in an increasing number of diseases. Potential objectives might include chemokine sequestration, blockage of ligand-receptor interactions, alteration of receptor expression on the cell membrane or regulation of receptor function [10-12, 27, 28] (Fig. **1**).

II. CHEMOKINES AND CHEMOKINE RECEPTORS IN PHYSIOLOGY AND PATHOLOGY

Chemokine participation in the control of cell movement implicates these molecules in all situations, physiological and pathological, in which cells are recruited to specific sites. Extensive information has accumulated over the past two decades on the role of chemokines in many diseases [12, 29, 30]. Most involve an orchestrated recruitment of cell populations which correlate with the expression of specific chemokines. This has been defined precisely in animal models of asthma, in which the temporal and spatial patterns

*Address correspondence to this author at the Department of Immunology and Oncology, National Center of Biotechnology, Campus Universitario de Cantoblanco, E-28049, Madrid, Spain; E-mail: mmellado@cnb.uam.es

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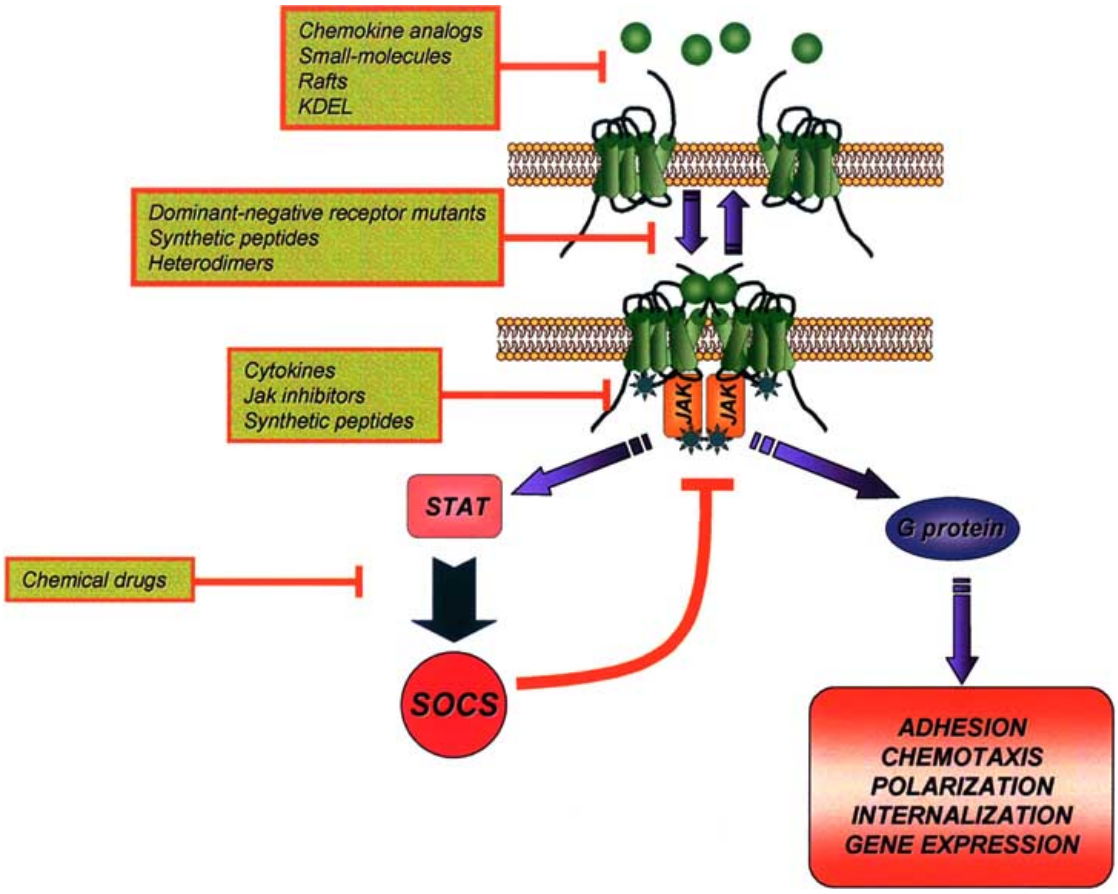

Fig. (1). Schematic model of the therapeutic potential of chemokines. The model shows the initial steps in chemokine signaling susceptible to targeting by external agents.

of chemokine expression during asthma development correlate precisely with the arrival and accumulation of inflammatory cell subsets in the lung [31]. Several chemokines and receptors have also been implicated in leukocyte migration to synovial tissue in rheumatoid arthritis [32, 33]. Some receptors (CCR1, CCR2, CCR3, CCR4 and CCR5) may be involved in monocyte recruitment from the circulation, others in T-cell accumulation in the synovium (CXCR4), and still others in leukocyte retention in the joint (CCR3 and CCR5). During transplantation injury and chronic rejection, chemokines follow a defined, time-dependent expression pattern, and their neutralization results in compromised recruitment of specific cell types [34, 35].

Other well-established examples in which the importance of chemokines and their receptors have been studied include experimental autoimmune encephalitis (EAE)[36], nephritis [37], inflammatory bowel disease [38, 39], multiple sclerosis [40], diabetes [41] and HIV-infection [26]. In the case of diabetes, several chemokine receptors (CXCR3, CCR4 and CCR5) are important during the course of the disease, suggesting that each of them is essential for the different migration stages that lead to lymphocyte infiltration of the islets [41-43]. Of special interest is the role of some chemokine receptors in HIV-1 infection. The initial observations that CXCR4 and CCR5 were the main HIV-1 coreceptors, brought a dramatic increase in research on the chemokines. A more recent development is the description of chemokine involvement in tumor progression and metastasis [44, 45], for which chemokines appear to act at different levels as growth factors, attracting dendritic cells to the tumor, as well as inducing angiogenesis, migration and invasion by increasing integrin expression or by inducing TNF, uPAR, PAI-1 and MMP1 expression [44, 45].

In several diseases, chemokine and receptor expression can be analyzed in biological fluids and tissue biopsies. CCR3 expression has been established in eosinophils obtained from bronchoalveolar lavage (BAL) of asthmatic individuals [46]. Upregulation of CCR5 and CXCR3, as well as of their ligands CCL3 and CXCL10, has been shown in multiple sclerosis lesions and in EAE [40]. CXCL8 has been also detected in sepsis and in adult respiratory distress syndrome [47].

The polymorphism of certain receptors and chemokines provides genetic evidence of their role under normal and pathological conditions, as well as in development. Individuals homozygous for ΔCCR5, a mutant form of the CCR5 receptor that is retained in cytoplasm, are resistant to HIV-1 infection by R5 strains, indicating that virus interaction with CCR5 is required for infection [48]. These individuals are physiologically normal, indicating that CCR5 blockade may ameliorate HIV-1 infection by R5

strains, without causing unwanted side effects [49, 50]. Additional polymorphisms described include a CCR2 point mutation, CCR2V64I, and another that affects the promoter regions of CXCL12, CCL5 or CCR2 [26].

Animal models are an important tool for the correlation of a chemokine with a specific disease, although conclusions from animals cannot always be extrapolated to man [31, 33, 51, 52]. Mice are the most common model; rabbits (transplantation), guinea pigs (asthma) and monkeys (asthma) [53] are also used, although the lack of species-specific reagents limits their utility. SCID mice have indicated the role of CXCR4 and CCR7 in metastasis and tumor progression [54]. The use of blocking antibodies in EAE showed the importance of CCL3 in the onset of symptoms, whereas CCL2 has a role in relapse. Several asthma models have been essential in delineating the sequential, highly controlled regulation of chemokine expression and leukocyte infiltration. Despite the wide variety of mouse models available, the lack of an efficient system for HIV-1 infection has hampered research significantly.

Mice have been developed that lack (KO) or overexpress (transgenic) nearly every chemokine and chemokine receptor described to date. Results are not always as informative as anticipated, as in many cases the animals show no clear physiological phenotype. CCR1 deletion in mice is associated with protection from lung injury [55]. CCR2 KO mice show normal hematopoietic development, although when backcrossed with apoE KO mice, they have defects in macrophage migration to inflammation sites [56]. In cases such as CCR8 KO mice, contrasting data for differentdisease models has caused debate [57, 58]. These approaches indicate that *in vivo* results may differ greatly from that predicted by *in vitro* assays. Diseases in which chemokines and their receptors have been implicated are summarized in Table **I**.

III. TARGETS FOR INTERFERENCE WITH CHEMOKINE RESPONSES

From correct receptor expression on the cell surface to its final internalization and degradation or recycling, there are numerous signaling events that mark opportunities for therapeutic intervention.

i. Chemokine Receptor Expression

A receptor that is not correctly expressed on the cell surface will not function. There are several examples of chemokine polymorphisms that affect chemokine receptors. Δ32CCR5 heterozygous individuals show diminished CCR5 expression on the cell surface, since the mutant receptor dimerizes with wild type CCR5 and retains it in the

Table I. Chemokines and their Receptors in Disease

Receptor	Ligand	Therapeutic indication	Reference
CCR1	CCL3, 5, 7	MS, RA, transplant, nephritis	[55, 59]
CCR2	CCL2, 7, 8, 13	MS, asthma, arthritis, glomerulonephritis	[56, 60, 61]
CCR3	CCL5, 7, 11, 13, 28	Asthma, dermatitis	[62, 63]
CCR4	CCL17, 22	Sepsis, asthma	[64]
CCR5	CCL3, 4, 5	MS, RA, diabetes, AIDS Transplant	[65, 60] [49]
CCR6	CCL20	Psoriasis Asthma Liver metastasis Skin inflammation Intestinal inflammation	[66] [67] [68] [69] [69]
CCR7	CCL19, 21	Liver metastasis EAE Cancer	[68] [70] [54]
CCR8	CCL1	Asthma, atopic dermatitis	[57]
CCR10	CCL27, 28	Ulcerative colitis, skin inflammation	[71]
CXCR1	CXCL6, 8	Lung reperfusion injury, psoriasis Cancer	[72] [73]
CXCR2	CXCL1, 2, 3, 5, 7, 8	Lung reperfusion injury, psoriasis, cancer, atherosclerosis	[72] [73]
CXCR3	CXCL9, 10, 11	MS, RA, transplant, cancer	[35, 74]
CXCR4	CXCL12	AIDS Cancer	[75] [54]

MS: multiple sclerosis
RA: rheumatoid arthritis

cytoplasm [48]. This observation has been used to develop several chemokine antagonist strategies. For example, chemokines fused to an endoplasmic reticulum retention signal (KDEL) confine the newly-synthesized chemokine and its receptor to the cell interior, where both are subsequently degraded. These intrakines act not only within the cell, but can also be secreted, and downregulate the cell surface receptor [76, 77].

Receptor localization in the cell membrane is of particular interest. Evidence shows that receptors and signaling molecules associate with rafts, which are lipid structures on the cell membrane. Membrane receptor association with these raft domains dictate receptor redistribution. Raft domains act as platforms for interaction between receptors and signal transduction molecules, thus increasing, restricting or modulating signaling efficiency [78]. As rafts are rich in cholesterol, altering their composition by cholesterol depletion has been shown effective in blocking receptor function. This strategy has been used to alter the course of HIV-1 infection, which requires interactions between the viral envelope and host cell receptors that partition in these cholesterol-enriched microdomains [79-81].

ii. Chemokine Sequestration: Binding Proteins and Decoy Receptors

Nature itself provides evidence on ways to manipulate chemokine biology. Virally-encoded proteins overcome chemokine redundancy by blocking several receptors simultaneously [8, 82-84]; this is the case of the broad-spectrum chemokine antagonist encoded by human herpesvirus 8 (HHV8), which blocks receptors from all four families. Other virally-encoded chemokine antagonists are highly selective, such as the CCR8-specific *Molluscum contagiosum*-encoded antagonist, MC148. Poxvirus family members encode chemokine-binding proteins that block chemokine glycosaminoglycan or receptor binding sites [85].

Other receptors interact with several chemokines from both the CC and CXC families. There are several hypotheses as to the role of these receptors in chemokine function. DARC and D6 are implicated in chemokine transport through the endothelial cell [86-88], but also as chemokine decoys [89-91]. It is of interest to note that these receptors do not induce calcium mobilization or chemotaxis in response to chemokine binding, although they are internalized and thus alter the responses of the chemokines that interact with them [90-92].

iii. Chemokine Antagonists: Antibodies and Small Molecule Antagonists

Ligand responses are generally modified by altering ligand-receptor binding, and many examples indicate that this is probably the most efficient design for antagonist molecules. Several laboratories have analyzed the amino acid residues involved in ligand-receptor interaction. The amino terminal and third extracellular loop regions of the receptor form part of the binding site [93-95]; drug screening using this information led to the development of several compounds with antagonist activity.

Monoclonal antibodies (mAb) have been described to chemokines and to receptors that act as antagonists. These mAb have allowed discrimination between receptor regions involved in ligand recognition and those involved in receptor function, for example, the CCR5 third extracellular loop defines the CCL5 binding region, whereas the N-terminus is required for function [96]. The use of mAb has several problems, since despite their specificity, they are unable to overcome the redundancy of the chemokine system. In addition, most mAb are of murine origin, impeding their clinical use in man, unless they are "humanized", although a humanized intact immunoglobulin or fragment may not retain the characteristics of its murine counterpart [97, 98].

Some modified chemokines and smaller molecules (such as peptides and chemical compounds) have been designed that mimic mAb mechanisms of action. These molecules block the chemokine binding site, but are virtually unable to trigger receptor functions. Some of these compounds act through yet-unknown mechanisms [27, 99], and may affect the chemokine signaling cascade at several different levels.

IV. INTERFERENCE WITH CHEMOKINE-ACTIVATED SIGNALING PATHWAYS

i. Receptor Dimerization

Chemokine receptors activate G protein-related signaling pathways. Most research on the chemokines has focused on developing drugs that block chemokine activity or impede HIV-1 interaction with its chemokine receptor. Analysis of chemokine receptor signaling has been limited in most cases to reproducing experiments that defined other members of the GPCR family [100-102]. For example, most chemokine-induced responses are blocked by pertussis toxin (PTx) treatment, indicating that a G_i protein is involved in signal transduction [103, 104], and the ligand-dependent association of $G\alpha_i$ proteins to chemokine receptors has been described [105]. More recent data nonetheless suggest much greater complexity in the mechanisms activated by this family of receptors.

Even though GPCR were originally considered to act as monomers, several observations described functional GPCR oligomers [106, 107]. The chemokine receptors have multiple conformational states that include monomers, homodimers, heterodimers and higher molecular weight oligomers [108, 109]. Availability of the appropriate ligand stabilizes the most favorable pre-existing conformation and initiates subsequent signaling cascades. Ligand-induced homodimerization has been demonstrated for CCR2, CCR5 and CXCR4 [109-111], and its functional relevance has been described. For example, the Δ32CCR5 mutant retains CCR5 in cytoplasm by forming receptor dimers [112]. Dimerization between CCR2V64I and CXCR4 may explain delayed AIDS progression in CCR2V64I individuals [113], and a CCR2 form (Y139F-CCR2), in which the tyrosine in the conserved DRY motif is mutated, has dominant negative behavior when co-expressed with the wild type receptor [109, 114].

To add further complexity, heterodimers between specific chemokine receptors also mediate specific functions. This is the case of CCR2 and CCR5, whose heterodimerization results in coupling of PTx-insensitive G proteins, reduced response threshold, and altered PI3K activation kinetics

[115, 116]. Definition of the dimerization motif residues is thus a critical step in the design of drugs to block or alter chemokine function by stabilizing distinct receptor conformations.

Chemokine receptors also interact with other GPCR, such as the opioid receptors. Opium-induced inhibition of chemotaxis was first reported over a century ago [117]. Other indications of this crosstalk were described more recently and include, increased opioid receptor ligand-mediated susceptibility to HIV-1 infection, as well as altered humoral or cell-mediated immune responses when both receptor types are co-expressed [118]. This crosstalk was previously explained as heterologous desensitization, a mechanism whose signaling pathways remain poorly defined. Opioid ligands also induce expression of pro-inflammatory chemokines and chemokine receptors [119, 120]. Responses to chemokines can thus be modulated in several ways, i.e., by action on cell surface GPCR, alteration of receptor expression, modification receptor function through heterologous desensitization, or by interference with homo- or heterodimer receptor formation.

ii. Inhibition of the JAK/STAT Pathway

The chemokine receptors initiate their responses by activating a G protein, in most cases of the G_i type [121], although members of the Janus family of tyrosine kinases (JAK) are also required in the initial steps of chemokine function. The conformational changes induced by chemokine binding to its receptor and by dimer formation, expose amino acid residues involved in JAK association and activation (114]. The complex formed by the chemokine, phosphorylated receptor dimers and activated JAK is needed to initiate G protein-associated signal transduction and other non-G protein-related signaling events [108]. Chemical inhibitors of JAK activity abolish chemokine responses. Neither JAK2-deficient cells nor those reconstituted with a kinase-dead JAK2 mutant, migrate or mobilize calcium in response to CXCL12 [122]. No $G\alpha_i$ association to CXCR4 is observed in either cell type, indicating that whereas kinase activity is not required for JAK association to the receptor, it is essential for function [122]. Analysis of signaling after chemokine binding also indicates that some pathways are activated in a G protein-independent manner. Even in PTx-treated cells, for example, chemokines promote translocation and activation of the signal transducer and activator of transcription (STAT) factors. As JAK/STAT pathways are triggered by cytokine/growth factors as well as chemokines, they could serve as an intracellular link between these families of mediators [111, 123].

This coalescence of signaling cascades can modify or regulate cell function. Chemokine signaling integrates GPCR-associated events with others linked to growth factor receptors, allowing cooperation or interference between these two fundamental pathways. Bovine growth hormone (bGH) transgenic mice show immune cell migration deficiencies, possibly the consequence of an imbalance between intracellular signaling pathways. JAK kinases bind constitutively to cytokine and to growth factor receptors, for which the consensus sequences involved have been defined [124-126]. In the GPCR, however, JAK association is a ligand-dependent mechanism that requires prior receptor activation and dimerization [114]; no JAK-interacting consensus sequences have been reported in GPCR, and adaptor protein involvement cannot be excluded [127, 128]. This distinct mode of JAK/STAT cascade initiation between chemokines and cytokines augurs well for the specific therapeutic modification of chemokine responses without affecting those mediated by cytokine.

JAK/STAT pathway activation by cytokine or chemokine receptors induces STAT-dependent upregulation of suppressors of cytokine signaling (SOCS) proteins. SOCS family members have been identified as feedback regulators of JAK/STAT activation through binding to JAKs or to chemokine/cytokine receptors [129, 130]. Cytokine-stimulated SOCS upregulation could thus interfere with both cytokine and chemokine signaling, allowing interference with chemokine function using cytokines or cytokine agonist molecules. There is evidence for signaling crosstalk between the GH receptor and CXCR4 in bGH transgenic mice, resulting in impaired immune cell migration. These defects in GH receptor-expressing cells are due to upregulation of SOCS3 levels in these cells, showing that modulated chemokine responses can act on cytokine signaling *in vivo* [123].

iii. Inhibition of G_i Protein Pathways

Most chemokine responses are inhibited by PTx treatment, indicating that G_i proteins are the primary receptors-associated transduction partners. Signaling studies of CC receptors in transfected HEK-293 cells revealed potent, agonist-dependent inhibition of adenylyl cyclase and mobilization of intracellular calcium, consistent with receptor coupling to $G\alpha_i$ [102]. In other studies, the calcium response was not completely blocked by PTx, suggesting that these receptors may couple other G proteins, such as G_q or G_{16} [131, 132].

Following activation by the chemokine receptor, the heterotrimeric $G\alpha\beta\gamma$ protein dissociates into the $\beta\gamma$ subunit complex and the GTP-bound $G\alpha_i$ subunit; the latter remains receptor-associated, probably through interaction with one or more regions of the intracellular loops [131]. Both receptor association and subunit dissociation then act on distinct effector molecules to initiate independent intracellular signaling responses. $G\beta\gamma$ also serves as a docking protein, providing a GPCR interface and facilitating its interaction in other signaling pathways. For example, $G\beta\gamma$ promotes G protein-coupled receptor kinase (GRK) interaction with the third intracellular loop of the M2-M3 muscarinic receptors [133]; $G\beta\gamma$ association with the activated receptor allows formation of a ternary complex with GRK2, which is required for effective receptor phosphorylation. The chemokine CCL2 also induces both $G\beta\gamma$ release and GRK2 association to the activated CCR2, allowing formation of a macromolecular complex that also includes arrestin [105].

Desensitization and recycling of chemotactic receptors appear to be mechanisms by which leukocytes maintain their ability to sense the chemoattractant gradient during an inflammatory response. GPCR internalization is generally believed to require agonist binding to the receptor, thus activating the events that initiate receptor endocytosis. This pathway is initiated by GRK phosphorylation on Ser/Thr residues in the GPCR intracellular carboxy-terminal domain;

this triggers recruitment of arrestin proteins, causing phosphorylated receptor sequestration into clathrin-coated pits [105, 134].

Synaptic vesicle recycling and endocytosis of many receptors, including GPCR, require the GTPase activity of dynamin [135]. After ligand activation, and as a consequence of Src-mediated tyrosine phosphorylation, dynamin is recruited to clathrin-coated pits, where it binds the appendage domain of α-adaptin, a component of the coated pits [136-138]. In endocytosis, dynamin catalyzes a GTP-dependent pinching-off of endocytic vesicles from the plasma membrane [135]. This process also takes place in CCL5-mediated CCR5 internalization, suggesting that dynamin-clathrin association is a critical step in normal CCR5 recycling [137].

In summary, it is clear that inhibitory G proteins (G_i) have a central role in chemokine receptor activation and internalization [108, 139, 140], and that rapid receptor desensitization involves agonist-promoted phosphorylation by GRK proteins [105, 141, 142]. Ser/Thr phosphorylation of residues in receptor intracellular regions increases affinity for arrestin-type proteins [105, 141]. The consequences of these events are the prevention of further coupling between the receptor and G proteins, as well as receptor internalization and recycling in a process that involves dynamin and clathrin vesicles [136, 137, 143]. Loss of recycling alters receptor expression, and thus, the ability of cells to respond to a continuous chemokine gradient signal. In addition, chemokine agonists have been described that produce greater receptor internalization than that induced by the natural ligand [144].

iv. Inhibition of Other Pathways: Kinases and Small GTPases

Signaling through chemokine receptors also involves kinases such as focal adhesion kinase (FAK) [145], extracellular signal-regulated kinases (ERK/MAPK) [100] and phosphatidylinositol 3-kinase (PI3K) [146, 147]. CCL5 generates T-cell focal adhesions and subsequent cell activation *via* a molecular complex formed by $p125^{FAK}$ and the T-cell tyrosine kinase zeta-associated protein (ZAP)-70 [145]. *Via* its SH2 domains, ZAP-70 binds to the phosphotyrosine in the TCR immunoreceptor tyrosine-based activation motif (ITAM) domains, in a process catalyzed by $p56^{lck}$ or $p59^{fyn}$ [148]. $p125^{FAK}$ and the ZAP-70 analogous Syk kinase are also activated in a monocytic cell line after stimulation with CCL2 (unpublished data). Signaling through the CCR5 receptor leads to phosphorylation and activation of the FAK protein kinase Pyk2 (also known as RAFTK or CAK-β) [149], resulting in downstream modulation of the JNK/SAPK kinase system. Through chemokine activation of the FAK kinases, the chemokine receptor is linked to the cytoskeletal protein paxillin in a functional molecular complex.

The MAPK/ERK kinases are activated by Tyr/Thr residue phosphorylation; they regulate several different proteins, including oncogenic transcription factors and protein kinases. MAPK activation of PLA2 and cytoskeletal elements suggest a role for this protein kinase signaling cascade in chemokine-induced cellular responses [150]. Stimulation of human neutrophils with the chemoattractant f-MLP activates Ras, which initiates the MAPK cascade by binding to the Ser/Thr kinase Raf [151]. Ras translocates Raf from the cytoplasm to the plasma membrane, where it is activated by interaction with members of the 14-3-3 protein family [152]. MAPK activation has also been described after CXCL8 stimulation of CXCR1- or CXCR2-transfected Jurkat cells [153].

In neutrophils, chemokine activation of the MAPK cascade is blocked by PTx treatment, indicating the mediation of G_i. Using COS-7-transfected cells and transient MAPK coexpression with GPCR, activated $G\alpha_i$ subunits do not mimic receptor stimulation of MAPK activity, evidence of an active role for Gβγ dimers in this signaling pathway [154]. Under distinct experimental conditions, Gβγ activation of MAPK requires neither PLC-β nor PKC activation, but is blocked by dominant interfering mutants of Ras [155], and Gβγ induces Ras accumulation in the GTP-bound, active form. Chemokine activation of MAPK may be involved in regulating gene expression.

PI3K activity is rapidly stimulated by chemoattractants [147, 156], but its role in chemotaxis depends on the cell line used to study the process. This may explain why, in some cases, PI3K inhibitors have no effect on chemotaxis [122]. A central role has been shown forchemokine-activated PI3K in integrin adhesiveness, cell migration and polarization [157]. PI3K is activated through GPCR stimulation, generating 3-phosphorylated lipids that act as second messengers for downstream effectors such as PKC, AKT, and for Ras pathways [158].

CCL4 triggers integrin activation and actin polymerization, which can be regulated through different pathways, including those of the G proteins, Tyr kinases, PKC, cAMP and PI3K [159]. Both PTx and wortmannin diminish F-actin polymerization and LFA-1 activation, and inhibit integrin-mediated adhesion; this adhesion is not blocked by Tyr kinases or PKC inhibitors. A CCL2, CXCL3, CCL5 and CXCL8-activated PI3K pathway is involved in integrin-mediated T-cell/neutrophil adhesion [160, 161]. Blocking with anti-chemokine antibodies or PI3K-specific antisense oligonucleotides diminishes LFA-1 activation and integrin-mediated adhesion, as does PTx. This indicates a role for chemokine receptor-dependent PI3K in the integrin activation necessary for migration and polarization [161].

Chemotaxis requires highly developed motile responses involving changes in cell shape, actin polymerization/depolymerization, and cell adhesion events mediated by the interaction of integrins with their ligands [162]. These processes are modulated by guanine nucleotides, and probably involve regulation by GTP-binding proteins, particularly the low molecular weight GTPases. Consistent with their role in regulating similar activities in other cells, it is not surprising that leukocytes have a large number of Ras-related GTP-binding proteins, including Ras, Rho, Rac, Cdc42 and Arf [163, 164].

Through their ability to modulate actin filament assembly, Rho and Rac probably have important roles in many leukocyte functions [165]. For example, IL-8 stimulation activates Rho, Rac and Cdc42, which regulate

focal adhesion and formation of lamellipodia and filopodia, respectively [166]. The link between chemokine receptors and these LMWG proteins remains unclear. After G protein activation, the Gβγ complex inhibits GTP[γS] binding to Rac and Rho, but not to Cdc42 [167]. Sequestration of free Gβγ might thus promote accumulation of GTP-bound forms of Rac and Rho, which could in turn inhibit endocytosis.

The ability of chemokines to activate both migratory and adhesive functions in leukocytes may depend on the relationship between chemokine receptors and LMWG [168].

V. CONCLUDING REMARKS

A cursory glance at chemokines and their receptors would suggest that their responses should be relatively simple to block, particularly considering that chemokine receptors belong to the GPCR family, among the most widespread and successful in today's armamentarium of therapeutic targets. Blocking chemokine action has nonetheless proved to be a complex task, and results of *in vitro* strategies often vary when applied *in vivo*. In some cases, murine chemokines and receptors differ from their human homologues in biological function or in expression under physiological or pathological conditions.

Another challenge is the redundancy of the system. In most cases, particularly that of the inducible chemokines, several chemokines or receptors may interact with the same target. This makes it difficult to evaluate the relative contribution of a single ligand or receptor, or to determine whether the absence or blockade of one receptor is compensated for by others.

In processes involving several chemokines and receptors, it is important to delimit their temporal interactions. In animal models of asthma and diabetes, for example, expression of several chemokines and receptors must be coordinated. Several laboratories have defined the importance of CCR4, CCR5 or CXCR3 in type I diabetes [41-43]. In asthma models, blocking CCL2 is as effective as blocking CCL11 (eotaxin) [169]. In both cases, distinct chemokines and receptors function at specific disease stages, and detailed knowledge of disease kinetics and of the cell types involved would help to determine the most appropriate target(s).

A further question is the stage at which therapeutic intervention would be most efficient. The search for new drugs relies heavily on mass screening, making appropriate assay design critical. Current strategies are based on compounds that block chemokine-receptor interaction, as determined by binding or functional assays. Compounds have nonetheless been identified that behave as antagonists, although they do not compete for binding; others may be considered partial agonists, as they induce some, but not all of the effects attributed to chemokines.

Recent approaches to therapeutic intervention have targeted signal transduction, analyzing receptor dimerization, JAK kinases, G proteins and SOCS. Except for the case of HIV-1 infection, intervention in chemokine-associated diseases requires modulation/regulation of responses rather than abrogation of chemokine function. Studying the signaling pathways activated by chemokines and by molecules that share a role in these diseases (cytokines, growth factors, integrins) will undoubtedly aid to design new drugs with greater therapeutic value.

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