ATYPICAL FUNCTIONS OF LEUKOCYTE CHEMOATTRACTANT RECEPTORS

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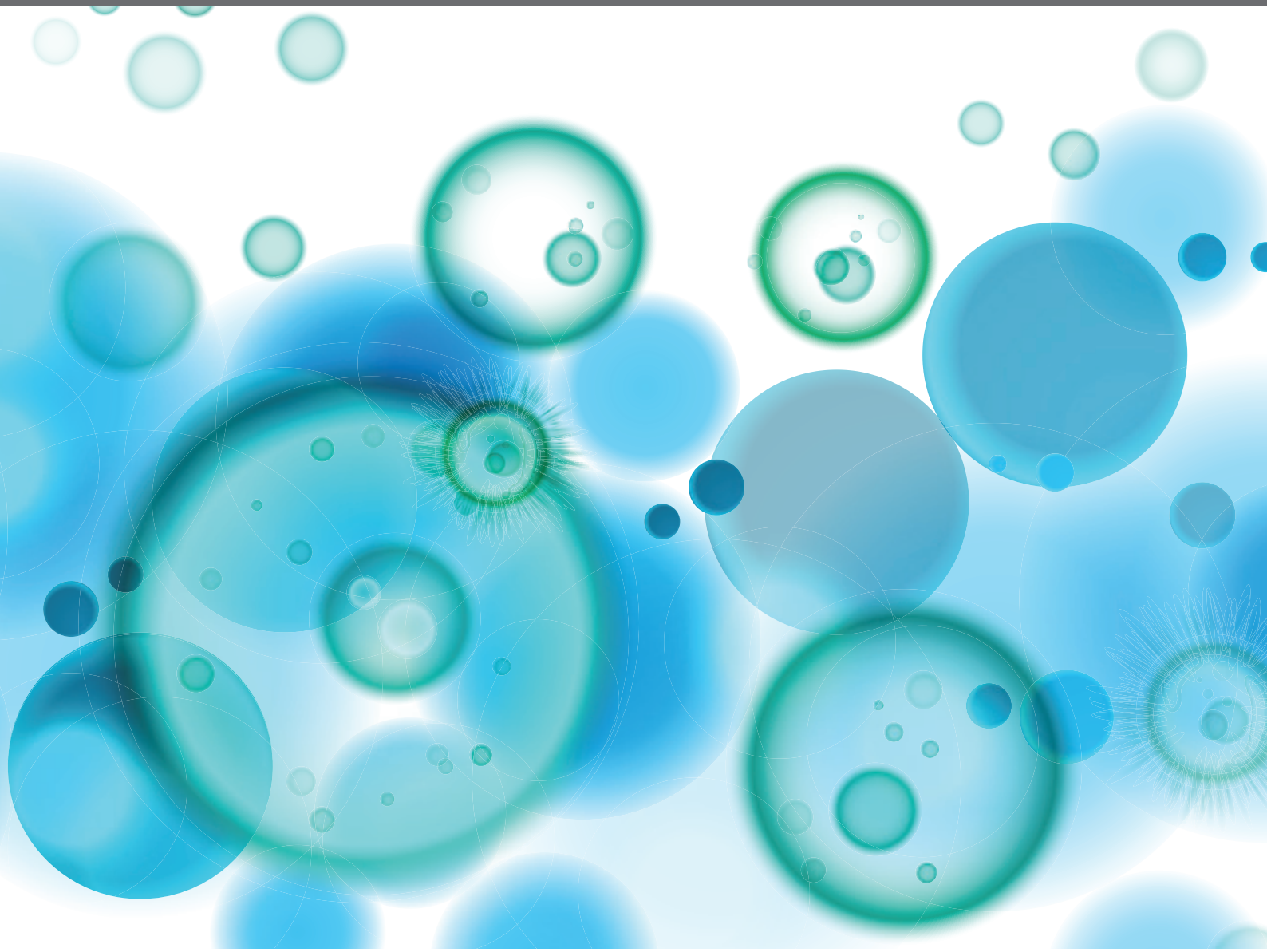

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ATYPICAL FUNCTIONS OF LEUKOCYTE CHEMOATTRACTANT RECEPTORS

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Editorial: Atypical Functions of Leukocyte Chemoattractant Receptors

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Editorial on the Research Topic

Atypical Functions of Leukocyte Chemoattractant Receptors

Chemoattraction is a process that involves directed cell movement toward extracellular chemical stimuli. It is a fundamental feature of all biological systems that was discovered in the late nineteenth century, when the word "chemotaxis" was coined by the German biologist Wilhelm Pfeffer. Metchnikoff's 1882 description of macrophages from starfish larvae migrating toward an implanted rose thorn irritant represents a seminal observation of chemoattraction *in vivo*. Since then immunologists have described many chemoattractants, including nucleotides, adenosine, lipids (e.g., leukotrienes, prostaglandins, and platelet-activating factor), proteolytic fragments of complement proteins (e.g., C3a and C5a), small peptides (e.g., *N*-formyl peptides), and small proteins (e.g., chemokines, non-chemokine cytokines, and defensins). Chemokines comprise the largest known group of chemoattractants (1, 2).

Despite their structural diversity, chemoattractants typically act by binding to the same class of G-protein-coupled receptors. Chemoattractant system acts appropriately to mediate host defense and tissue repair but can become dysregulated and act inappropriately to mediate immunopathology in the context of autoimmunity and chronic inflammatory disease, including allergic inflammation (3).

The term chemoattractant does not convey the protean activities possessed by many of these molecules that may regulate survival, cytoarchitecture, migratory speed, cell adhesion, reactive oxygen species (ROS) generation, degranulation, endocytosis, differentiation, chemorepulsion, stimulation of immune synapse formation, and release of neutrophil extracellular traps (NETosis), among others (4–6). Chemoattractant multitasking can be viewed as a parsimonious molecular solution to the problem of directing activated immune effector cells to sites of infection and inflammation.

Work on the earliest chemoattractants such as fMet-Leu-Phe and C5a and the first chemokines already showed multiple immunoregulatory activities beyond chemoattraction (7). This was conveyed in the original names that were assigned to some of these chemoattractants, although they were later replaced in the case of the chemokines by a systematic nomenclature (8). For example, neutrophil-activating factor (now CXCL8), macrophage inflammatory protein-1α (now CCL3), and scavenger receptor for phosphatidylserine and oxidized LDL (now CXCL16). This multifunctionality is apparently ancient since it has been observed in unicellular organisms such as *Dictyostelium discoideum* (9). Why then the volume of chemoattractant research has been largest for understanding cell migration?. One explanation is that this is the sole shared property for all of these molecules, which elevates it to a position of general importance. Moreover, cell trafficking is complex with certain general rules, but many exceptions that must be sorted out for each leukocyte subtype, each organ and each tissue barrier.

A fundamental division in the biochemical properties of chemokine receptors has highlighted the division of labor subserved by chemoattractants. The 18 conventional chemokine receptors (cCKRs) drive cell responses by triggering heterotrimeric G protein signaling pathways, whereas a smaller group of atypical chemokine receptors (ACKRs) largely behave as scavengers of chemokines and can use β-arrestins, instead of G proteins, to promote endocytosis and the transport of chemokines to intracellular degradative compartments. The two classes of receptors are oppositely directed and presumably act coordinately to strike a balanced and regulated immune response to prevent a positive feedback loop and runaway inflammation (10).

The study of non-chemotactic functions with modern molecular tools may reveal new insights into how leukocytes transduce simple molecular signals into an integrated and effective immune response in the right place and time. Importantly, chemoattractant receptors may ideally trigger activation as the cells arrive at a focus where the chemoattractant concentration may be highest and above the threshold needed for effector responses. The information gained through the analysis of diverse activities controlled by chemoattractants in leukocytes and the signaling mechanisms involved may be useful to develop strategies to modulate the immune response. The articles of this Research Topic highlight the importance of the non-chemoattractant functions of chemoattractant receptors in cell regulation.

Abouelasrar Salama et al. show that a highly homogeneous rSAA1 [receptor: formyl peptide receptor 2 (FPR2)] induces chemoattraction in leukocytes, synergy with other chemoattractants and monocyte survival. However, they show that commercial SAA1, promotes additional activities in leukocytes caused by contaminants. Their paper represents a note of caution when using commercial ligands to analyze the functions of chemoattractant receptors.

Bianchi and Mezzapelle analyze the role of CXCL12 (receptor: CXCR4) in heart, liver, lung, and peripheral nerve regeneration. They discuss the role of the CXCL12-HMGB1 complex (receptor: CXCR4) in skeletal muscle and bone regeneration. Authors also discuss CXCR4-dependent signaling pathways involved in the regulation of vascular, progenitor, and tumor cell proliferation.

Capucetti et al. analyze the non-chemotactic functions controlled by different chemokine receptors expressed in neutrophils, including CXCR4, CXCR1, CCR1, CCR2, CCR5, and CCR7. The authors analyze the functions regulated by these receptors in different subsets and maturation stages of the neutrophils that are found in multiple physiological and pathological conditions, for example in sites of infection, during autoimmunity, and cancer.

Karin analyses the role of the chemokines CXCL9, CXCL10, and CXCL11 (receptor: CXCR3) and discusses how biased signaling by these ligands affects T cell differentiation and lineage development and may modulate cancer progression and autoimmunity. It is also suggested that CXCL10 and CXCL9 may inhibit cancer cell growth and promote anti-tumor immunity.

Matti et al. focus on the atypical chemokine receptor ACKR4 (ligands: CCL19, CCL20, CCL21, CCL25). The authors show that in the absence of chemokines, β-arrestins interact with ACKR4 and controls the steady-state trafficking of this receptor. Notably, cells lacking β-arrestins can take up CCL19, indicating that these proteins are dispensable for ACKR4-mediated chemokine scavenging.

Morein et al. discuss the effects that the non-conventional activities controlled by chemokine receptors exert on cancer cells. They show that chemokine receptors affect tumor progression by promoting cell proliferation, survival, senescence, enrichment of tumors with cancer stem cells, promotion of metastasis-related functions, such as epithelial-to-mesenchymal transition and increasing the expression of matrix metalloproteinases.

Rodríguez-Fernández and Criado-García analyze the signaling pathways underpinning the different functions controlled by the chemokine receptor CCR7 in dendritic cells. They indicate that CCR7-regulated functions are mediated by highly independent and biased signaling modules. The authors also suggest that other chemoattractant receptors could use a similar strategy to regulate leukocyte functions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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The Chemokine Receptor CCR7 Uses Distinct Signaling Modules With Biased Functionality to Regulate Dendritic Cells

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Chemotaxis is a molecular mechanism that confers leukocytes the ability to detect gradients of chemoattractants. Chemokine receptors are well-known regulators of chemotaxis in leukocytes; however, they can regulate several other activities in these cells. This information has been often neglected, probably due to the paramount role of chemotaxis in the immune system and in biology. Therefore, the experimental data available on the mechanisms used by chemokine receptors to regulate other functions of leukocytes is sparse. The results obtained in the study of the chemokine receptor CCR7 in dendritic cells (DCs) provide interesting information on this issue. CCR7 guides the DCs from the peripheral tissues to the lymph nodes, where these cells control T cell activation. CCR7 can regulate DC chemotaxis, survival, migratory speed, cytoarchitecture, and endocytosis. Biochemical and functional analyses show: first, that CCR7 uses in DCs the PI3K/Akt pathway to control survival, the MAPK pathway to control chemotaxis, and the RhoA pathways to regulate actin dynamics, which in turn controls migratory speed, cytoarchitecture, and endocytosis; second, that these three signaling pathways behave as modules with a high degree of independence; and third, that although each one of these routes can regulate several functions in different settings, CCR7 promotes in DCs a functional bias in each pathway. The data uncover an interesting mechanism used by CCR7 to regulate the DCs, entailing multifunctional signaling pathways organized in modules with biased functionality. A similar mechanism could be used by other chemoattractant receptors to regulate the functions of leukocytes.

Keywords: C–C chemokine receptor 7, signaling, leukocyte, MAPK pathway, RhoA pathway, PI3 K/Akt pathway, Chemotaxis (MeSH ID D002633)

"Divide and rule"
Attributed to Philip II of Macedon

INTRODUCTION

Chemokine receptors regulate chemotaxis, a process that allows cells to detect gradients of chemoattractants. Based on this property, chemokine receptors, together with their ligands, serve as "address codes" that guide leukocytes to specific sites in the organism (1–3). Although chemoattraction is an important activity controlled by these receptors, they can regulate additional functions of leukocytes (4–6). This fact has been largely overlooked, probably due to the capital

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importance of chemoattraction in biology; consequently, the information on other functions of chemokine receptors is sparse (6). It is expected that non-chemotactic functions regulated by chemokine receptors may contribute to the efficient functioning of leukocytes in the immune system. Therefore, getting insight into the molecular mechanisms used to regulate these functions may allow the identification of novel targets to modulate the immune response.

C–C chemokine receptor 7, like all chemokine receptors, is included in the G protein-coupled receptor superfamily (6). CCR7 (ligands CCL19 and CCL21) is one of the chemokine receptors on which more functional information is available (6–8). We have studied the signaling pathways controlling CCR7-mediated functions in dendritic cells (DCs). It was found that, to control specific cellular functions of DCs, this receptor uses well-known signaling pathways that organize as signaling modules with biased functionality and limited crosstalk among them (9–13). Herein we describe the signaling components of these modules and discuss how they may regulate the functions of DCs.

CCR7-CONTROLLED NON-CHEMOTACTIC ACTIVITIES MAY CONTRIBUTE TO THE EFFICIENCY OF DENDRITIC CELLS IN THE IMMUNE SYSTEM

Dendritic cells are leukocytes that are found in peripheral tissues in a differentiation state called immature, during which they display a high ability to detect, capture, and process antigens (14). After exposure to danger signals, including pathogens, toxic agents, or inflammatory cytokines, immature DCs undergo a complex differentiation program that transforms them into mature DCs (maDCs), which migrate to the lymph nodes (LNs), where they present antigens captured in the immature stage to antigen-specific T cells. As part of their differentiation program, the maDCs upregulate the expression of the chemokine receptor CCR7 that guides the maDCs to the LNs, attracted by CCL21 which is expressed in the afferent lymphatics vessels and by CCL19 and CCL21 which are expressed in stromal cells in the LNs (15, 16). CCR7 is crucial to guide the maDCs to the LNs, implying that its correct expression and function is important for adequate adaptive immune response (15, 17–21). Apart from chemoattraction (8, 22, 23), CCR7 regulates cytoarchitecture (13, 24), endocytosis (13, 25), survival (12), migratory speed (11, 26), adhesion (27), and differentiation in maDCs (28). Predictably, these non-chemotactic activities regulated by CCR7 contribute to the correct functionality of the maDCs in the immune system (6). It is expected that the enhanced survival, migratory speed, and differentiation may increase the number of antigen-loaded maDCs that reach the LNs. The increment in endocytosis may confer the maDCs migrating through the afferent lymphatic vessels, or located in the LNs, the ability to endocyte antigens, e.g., viral particles that can be subsequently presented to T cells (29, 30). Enhanced adhesion facilitates the migration of the maDCs through the afferent lymphatic vessels (27, 31). The induced changes in actin cytoarchitecture can regulate the motility of the maDCs that migrate toward the LNs and confer these cells their dendritic morphology (11, 13, 24). Pseudopod extensions increase the surface-area-to-volume ratio of the maDCs when compared with a spherical cell of equal volume (32), increasing the possibilities of contact with T cells. In summary, the different functions controlled by CCR7 may predictably contribute to a more effective maDCs in the immune system and to a better adaptive immune response (6). An important issue is the identification of the mechanisms used by CCR7 to regulate the cellular activities of the maDCs. In the following sections, we discuss recent experimental data that provide information on the molecules and the signaling mechanism involved in this process.

CCR7-DEPENDENT SURVIVAL IS GOVERNED BY A PI3K/AKT-CONTROLLED SIGNALING MODULE

When maDCs are kept in serum-free medium, they initiate an apoptotic program that leads to their demise (12). This simple experimental setting is useful to identify the receptors that inhibit cellular apoptosis and the intracellular pathways involved (12, 33). The stimulation of maDC kept in serum-free medium with any of the ligands of CCR7, CCL19, or CCL21 slows down the apoptosis of these cells, indicating that this receptor induces anti-apoptotic intracellular signaling (12). Using this experimental strategy, it was also found that the kinases AMPK and GSK3β played pro-apoptotic roles *in vitro* and *in vivo* because a forced increase or decrease of their activities enhanced or impaired apoptosis in maDCs (9, 10, 34) (**Figure 1**). Moreover, it was found that AMPK promotes apoptosis in maDCs by inhibiting the mechanistic target of rapamycin complex 1 (mTORC1), a kinase complex that promotes survival in maDCs (see below) (10). Both AMPK and GSK3β induce the activation of the transcription factors FOXO1/3, which controls the pro-apoptotic Bcl2 family member Bim (9, 10, 12, 35). Moreover, active GSK3β also prevents the activation of anti-apoptotic transcription factor NFκB, which controls the transcription of the anti-apoptotic Bcl2 family member Bcl_{xl} (9) (**Figure 1**). The balance between pro-apoptotic and anti-apoptotic Bcl2 family members determines whether a cell becomes apoptotic or survives (36). An excessive increase in pro-apoptotic (e.g., Bim) over pro-survival (e.g., Bcl_{xl}) Bcl2 members induces the activation of the mitochondria gatekeepers Bax/Bak (36, 37), resulting in mitochondrial outer membrane permeabilization and liberation of cytochrome c from the intermembrane space of the mitochondria, which leads to caspase activation and apoptosis (**Figure 1**) (36).

When CCR7 was stimulated in the maDCs that were in serum-free medium, it was observed that the pro-apoptotic signaling described above was turned off because this receptor induced the activation of the signaling axis PI3K/Akt (9, 10, 12, 19, 38, 39) which, as shown below, is a core component of a pro-survival pathway in these cells (**Figure 1**). The Gi family of G proteins and, particularly, the Gβγ complex (12), a

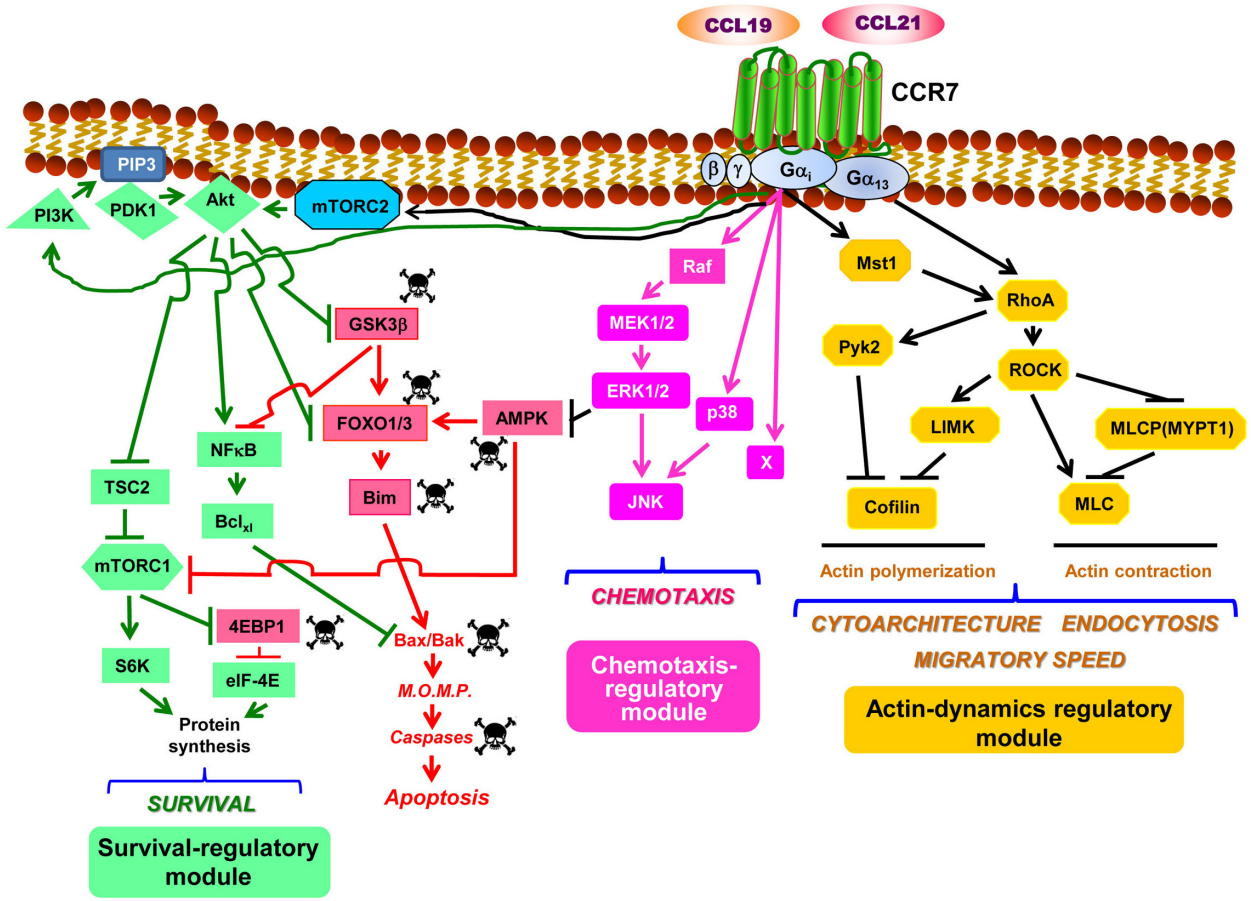

FIGURE 1 | CCR7 uses signaling modules to regulate the functions of dendritic cells. Considering the sparse data within this field, the figure should be considered as a tentative model to be completed as additional experimental information becomes available. The model is largely based on the data presented in **Table 1**. Downstream of CCR7, the PI3K/Akt, the MAPKs, and the RhoA pathways organize as signaling modules that regulate survival, chemotaxis, and actin dynamics, respectively (see text for details). Abbreviations used: Akt, also known as Protein kinase B (PKB); AMPK, AMP-activated protein kinase; Bcl_{xl}, B-cell lymphoma extra-large; Bim, Bcl-2-interacting mediator of cell death; 4EBP1, eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1; MEK1/2, MAPK/ERK kinase 1 and 2; ERK1/2, extracellular signal-regulated kinase 1 and 2; mTORC1, mechanistic target of rapamycin (mTOR) complex 1; FOXO1/3, forkhead box protein O1 and O3; GSK3β, glycogen synthase kinase 3β; JNK, c-Jun N-terminal kinase; LIMK, LIM domain kinase; mTORC2, mTOR complex 2; MLC, myosin light chain; M.O.M.P., mitochondrial outer membrane permeabilization; Mst1, mammalian sterile 20-like kinase 1; MYPT1, myosin phosphatase target subunit 1; NFκB, nuclear factor-κB; PDK1, phosphoinositide-dependent kinase-1; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PI3K, phosphatidylinositol 3-kinase; Pyk2, proline-rich tyrosine kinase 2; RhoA, Ras homolog family member A; ROCK, Rho-associated protein kinase; S6, ribosomal protein S6; S6K, S6 kinase; TSC2, tuberous sclerosis complex 2. The skull and crossbones symbol indicates molecules that promote apoptosis.

dimer associated to the Gα subunit to form a heterotrimeric G protein, mediate the CCR7-dependent activation of the PI3K/Akt pathway (10, 12). This pathway induces survival in multiple cell types due to the ability of its signaling components, particularly Akt, to switch off pro-apoptotic molecules and turn on pro-survival signals (40, 41). Upon the CCR7-dependent activation of Akt in maDCs, this kinase directly phosphorylates and inhibits the transcription factor FOXO1/3 which, as mentioned above, controls the expression of pro-apoptotic Bim (9). Active Akt further prevents apoptosis by phosphorylating/inhibiting GSK3β which, as mentioned before, promotes the activation of pro-apoptotic FOXO1/3 and also the inhibition of pro-survival NFκB (9) (**Figure 1**). Akt also induces the activation of the transcription factor NFκB, which regulates Bcl_{xl} (9, 12) that, as indicated above, protects the cells from apoptosis by opposing pro-apoptotic Bim (**Figure 1**). Akt can also enhance cell survival by inducing the activation of mTORC1, which stimulates translation, a process that promotes survival in maDCs (42) (**Figure 1**). Active mTORC1 stimulates translation by inducing phosphorylation/inactivation of the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), which retains eIF-4E inhibited. After the phosphorylation of 4E-BP1, eIF-4E is released and becomes part of the translation initiation complex (10, 42). Moreover, mTORC1 also activates translation by phosphorylating/activating ribosomal S6 kinase (S6K), which subsequently phosphorylates the protein targets involved in translation, including the ribosomal protein S6 (**Figure 1**). The combined effects of the activation of Akt, which

leads to the up-regulation of Bcl_{xl}, the inhibition of Bim and, in addition, the activation of mTORC1, which promotes an increase in protein synthesis, contribute to extend the survival of the maDCs (10, 42). The prior results indicate that the PI3K/Akt pathway mediates CCR7-dependent survival. Regarding the contribution of other pathways to the regulation of survival, a modest contribution of the chemotaxis pathway was observed because the inhibition of MEK1/2/ERK1/2, two key regulators of chemotaxis (see below), reduced by ∼20% the pro-survival effects induced by CCR7 (10). MEK1/2/ERK1/2 may exert this moderate pro-survival effect by inhibiting the pro-apoptotic kinase AMPK (10) (**Table 1** and **Figure 1**).

The inhibition of the PI3K/Akt pathway strikingly failed to affect the CCR7-dependent MAPK pathway (**Table 1**), the RhoA pathway, or the functions regulated by these two routes in maDCs (**Table 1**) (9, 10, 12). A lack of effect of PI3K/Akt on CCR7-regulated chemotaxis (19, 39) or endocytosis (see below) has also been reported by other groups (39). These results suggest that the PI3K/Akt pathway constitute a signaling module that largely controls CCR7-induced survival, but no other CCR7-mediated functions in maDCs (see below). This is interesting because the PI3K/Akt pathway also regulates chemotaxis, proliferation, and metabolism in other cells (43, 44). This functional bias is also observed in the other two pathways controlled by CCR7 in maDCs (see below). In summary, the data indicate that CCR7 controls in maDCs a PI3K/Akt-controlled signaling module that regulates survival, but not chemotaxis or actin dynamics (see below).

CCR7-DEPENDENT CHEMOATTRACTION IS GOVERNED BY A MAPK- CONTROLLED SIGNALING MODULE

Experimental evidence indicates that CCR7-dependent chemotaxis is largely regulated by a signaling module formed by Raf and the MAPKs family members MEK1/2, ERK1/2, p38, and JNK in maDCs (11, 39). As shown for CCR7-induced survival, the activation of both the MAPK pathway and chemotaxis is regulated in maDCs by Gi proteins and Gβγ dimers (11). Previously, Gβγ dimers have been shown to regulate chemotaxis in other chemokine receptors and cells (45). Although it has not been experimentally analyzed if CCR7 induces the activation of Ras in maDCs, it is possible that this GTPase may mediate the effects of CCR7 on the MAPK pathway because Ras is an upstream regulator of the Raf-MEK1/2-ERK1/2 pathway (46), and different chemokine receptors can induce the activation of this GTPase (47–49). Moreover, upon G-protein coupled receptor activation, Gi and Gβγ dimers mediate, like in maDCs, the Ras-dependent activation of MAP kinase pathway (50, 51). In summary, the results indicate that the MAPK pathway controls CCR7-dependent chemotaxis in maDCs (11). However, the complete inhibition of ERK1/2, p38, and JNK does not abrogate chemotaxis, suggesting that additional regulator/s, denoted as "X" in **Figure 1**, may also contribute to the regulation of this pathway (11). The inhibition of key molecular components of the MAPK pathway does not affect dramatically the CCR7-dependent PI3K/Akt pathway and survival or the RhoA pathway and the function associated to it (actin-regulated functions, including cytoarchitecture, endocytosis, and migratory speed; see below) (**Table 1**). Other authors have also shown that the stimulation of CCR7 induces the activation of JNK and that the inhibition of this MAPK blocks chemotaxis, but not the endocytosis in maDCs (39), supporting the independence of the RhoA pathway (see below) of the chemotaxis regulatory module. In summary, the results suggest that the MAPK pathway may constitute a signaling module that displays a high degree of independence since it seems independent of the module that regulates CCR7-regulated actin dynamics and displays only a very modest contribution to the regulation of CCR7-controlled survival (**Figure 1**). Another interesting aspect that emerges from these results is the high degree of functional bias of the MAPK pathway, which seems to regulate mainly CCR7-controlled chemotaxis and only modestly survival in maDC, although it is a potent regulator of survival and proliferation in other contexts (52).

The data suggesting that the MAPK pathway mediates CCR7-dependent chemotaxis in maDCs is consistent with prior data showing that Ras, an upstream regulator of the MAPKs (46), is a regulator of chemotaxis in response to N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) in neutrophils (53, 54) and to cyclic-adenosine monophosphate (cAMP) in *Dictyostelium discoideum* (55, 56). Moreover, in *Dictyostelium*, Ras activation takes place independently of PI3K (55), which reminds of the observed independence between the CCR7-regulated activation of MAPK and the PI3K/Akt pathways described in maDCs. In summary, the results indicate that CCR7 controls in maDCs a MAPK-regulated signaling module, which selectively controls chemotaxis, independently of the survival and actin-regulatory modules (see below).

CCR7-DEPENDENT CHANGES IN ACTIN DYNAMICS IS GOVERNED BY A RhoA-CONTROLLED SIGNALING MODULE

Our studies indicate that the CCR7-induced stimulation of migratory speed, endocytosis, and changes in cytoarchitecture in maDCs is mediated by the RhoA pathway (11, 13). Thus RhoA, a key regulator of actin organization in multiple cells, including maDC (57–64), would govern the actin dynamic changes involved in the control of the aforementioned activities. It has been suggested that RhoA does not mediate CCR7-induced morphological changes and endocytosis in murine maDCs, which would be controlled instead by Cdc42 and Rac (24, 25). These discrepancies could be due to species differences [murine maDCs (24, 25) vs. human maDCs (11, 13)] or caused by the maturation stimulus used for the DCs [LPS (24, 25) vs. TNFα (11, 13)]. This issue will have to be settled in future studies. Unlike CCR7-dependent survival and chemotaxis, which depend largely on Gi proteins, CCR7-dependent changes in actin dynamics were

TABLE 1 | Experimental data support a high degree of independence between the different CCR7-regulated modules controlling the functions of the dendritic cells.

		Inhibition of the survival module		**Effects on the chemotaxis module**		**Effects on the actin module**	
Key proteins Inhibited in the survival module	**Inhibitor used**	**Target molecule: -control -inhibitor effect -[Fig (Ref)]**	**Target Function: -control -inhibitor effect -[Fig (Ref)]**	**Target Molecule: -control -inhibitor effect -[Fig (Ref)]**	**Target Function: -control -inhibitor effect -[Fig (Ref)]**	**Target Molecule: -control -inhibitor effect -[Fig (Ref)]**	**Target Function: -control -inhibitor effect -[Fig (Ref)]**
PI3K	LY 294002	PI3K: -Activated -Activation prevented -[Fig 2A (12)] Akt: -Activated -Activation prevented -[Fig 3A (11); Fig 2C (12)] GSK3β: -Inhibited -Inhibition prevented -(9) AMPK: -Inhibited -Inhibition not altered -[Fig 5B (10)]	Survival: -Increase -Increase inhibited (∼100%) -[Fig 2C (12)]	ERK1/2: -Activated -Activation not altered -[Fig 3C (11)]	Chemotaxis: -Increase -Increase not altered -[Fig 3B (11)]	Mst1: -Activated -Activation not altered -[Fig 2B (13)]	Migratory speed: -Increase -Increase not altered -[Fig 3B (11)]
Akt	Akti	Akt: -Activated -Activation prevented -[Fig 5D (10)] GSK3β: -inhibited -Inhibition prevented -[Fig. 4C (9)] S6K: -Activated -Activation prevented -[Fig 5D (10)] AMPK: -inhibited -Inhibition not altered -[Fig 5D (10)]	Survival: -Increase -Increase inhibited (∼100%) -[Fig 7C (10)]			Mst1: -Activated -Activation not altered -[Fig 2C (13)]	
GSK3β	LiCl	Akt: -Activated -Activation not altered -[Fig 4D (9)]		ERK1/2: -Activated -Activation not altered -[Fig 4F (9)]			
		Effects on the survival module		**Inhibition of the chemotaxis module**		**Effects on the actin module**	
Key proteins Inhibited in the chemotaxis module	**Inhibitor used**	**Target molecule: -control -inhibitor effect -[Fig (Ref)]**	**Target function: -control -inhibitor effect -[Fig (Ref)]**	**Target molecule: -control -inhibitor effect -[Fig (Ref)]**	**Target function: -control -inhibitor effect -[Fig (Ref)]**	**Target molecule: -control -inhibitor effect -[Fig (Ref)]**	**Target function: -control -inhibitor effect -[Fig (Ref)]**
MEK1/2	UO126	Akt: -Activated -Activation not altered -[Fig 4 (12)] GSK3β: -Inhibited -Inhibition not altered -[Fig 4E (9)] AMPK: -Inhibited -Inhibition prevented -[Fig 6B (10)]	Survival: -Increase -Increase inhibited (∼20%) -[Fig 7C (10)]	ERK1/2: -Activated -Activation prevented -[Fig 4E (9); Fig 2C (11)] p38: -Activated -Activation not altered -[Fig 2D (11)] JNK: -Activated -Activation prevented [Fig 2D (11)]	Chemotaxis: -Increase -Increase inhibited (∼50%) -[Fig 2G (11)]	Pyk2: -Activated -Activation not altered -[Fig 5G (11)]	Migratory speed: -Increase -Increase not inhibited -[Fig 2F (11)]

(Continued)

TABLE 1 | Continued

MEK1/2	PD 0325901	AMPK: -Inhibited -Inhibition prevented -[Fig 6C (10)]		ERK1/2: -Activated -Activation prevented -[Fig 2A (13)]		Mst1: -Activated -Activation not altered -[Fig 2A (13)]	
ERK1/2	CAY 10561	AMPK: -Inhibited -Inhibition prevented -[Fig 7A (10)]	Survival: -Increase -Increase inhibited (~20%) -[Fig 7C (10)]	ERK1/2: -Activated -Activation prevented -[Fig 7A (10)]			
p38	SB 203580	Akt: -Activated -Activation not altered -[Fig 4 (12)]	Survival: -Increase -Increase not inhibited -[Fig 4 (12)]	ERK1/2: -Activated -Activation not altered -[Fig 2D (11)] p38: -Activated -Activation prevented -[Fig 2C (11)] JNK: -Activated -Activation prevented -[Fig 2D (11)]	Chemotaxis: -Increase -Increase inhibited (~30%) -[Fig 2G (11)]		Migratory speed: -Increase -Increase not inhibited -[Fig 2F (11)]
JNK	SP 600125	Akt: -Activated -Activation not altered -[Fig 4 (12)]	Survival: -Increase -Increase not inhibited -[Fig 4 (12)]	ERK1/2: -Activated -Activation not altered [Fig 2D (11)] JNK: -Activated -Activation prevented -[Fig 2C (11)] p38: -Activated -Activation not altered -[Fig 2D (11)]	Chemotaxis: -Increase -Increase inhibited (~40%) -[Fig 2F (11)]		Migratory speed: -Increase -Increase not inhibited -[Fig 2F (11)]
		Effects on the survival module		**Effects on the chemotaxis module**		**Inhibition of the actin module**	
Key proteins Inhibited in the actin module	**Inhibitor used**	**Target molecule:** **-control** **-inhibitor effect** **-[Fig (Ref)]**	**Target function:** **-control** **-inhibitor effect** **-[Fig (Ref)]**	**Target molecule:** **-control** **-inhibitor effect** **-[Fig (Ref)]**	**Target function:** **-control** **-inhibitor effect** **-[Fig (Ref)]**	**Target molecule:** **-control** **-inhibitor effect** **-[Fig (Ref)]**	**Target function:** **-control** **-inhibitor effect** **-[Fig (Ref)]**
Mst1	siRNA	Akt: -Activated -Activation not altered -[Fig 3B (13)]	Survival: -Increase -Increase not altered -[Fig 3D (13)]	MEK1/2: -Activated -Activation not altered [Fig 3B (13)] ERK1/2: -Activated -Activation not altered -[Fig 3B (13)]	Chemotaxis: -Increase -Increase not altered -[Fig 3C (13)]	Mst1: -Activated -Activation prevented -[Fig 3A (13)] MYPT1: -Inhibited -Inhibition prevented -[Fig 5A (13)] MLC: -Activated -Activation prevented -[Fig 5A (13)] Cofilin: -Inhibited -Inhibition prevented -[Fig 5A (13)]	Actin cytoarchitecture: -Changes -Changes Inhibited (~75%) [Fig 7B (13)] Endocytosis: -Increase -Increase inhibited (~40%) -[Fig 7C (13)] Migratory speed: -Increase -Increase inhibited (~100%) -[Fig 7D (13)]
RhoA	C3			ERK1/2: -Activated -Activation not altered -[Fig 4C (11); Fig 5D (13)]	Chemotaxis: -Increase -Increase not inhibited -[Fig 4B (11)]	Mst1: -Activated -Activation not altered -[Fig 5D (13)] MLC: -Activated -Activation prevented -[Fig 5D (13)]	Migratory speed: -Increase -Increase inhibited (~100%) [Fig 4B (11)]

(Continued)

TABLE 1 | Continued

				Cofilin: -Inhibited -Inhibition prevented -[Fig 6B (11)] Pyk2: -Activated -Activation prevented -[Fig 5C (11)]	
Pyk2	PRNK	ERK1/2: -Activated -Activation not altered -[Fig 5F (11)]	Chemotaxis: -Increase -Increase not altered -[Fig 5E (11)]	Pyk2: -Activated -Activation prevented -[Fig 5F (11, 75)]	Migratory speed: -Increase -Increase inhibited (~100%) -[Fig 5E (11)]

*The experiments shown were performed with human-monocyte-derived dendritic cells (DCs) that were matured with TNFα (12, 74). The maDCs were largely stimulated with CCL21, but similar results were observed with CCL19. Only 2D chemotactic analyses were performed. The maDCs, pretreated or not with the indicated inhibitor (see below), were stimulated with CCL21, and the activity of the signaling molecules or the cell functions were analyzed (see below). In the two columns on the left-hand side of the table are shown, in red, the target proteins inhibited in each signaling pathway/module and the inhibitors used. The first row of the table is divided in three blocks, denoting, in red, the module that is inhibited and, in black, the effects of this inhibition on the other two modules. Below these three blocks are shown from up to down: (i) the "target molecule" and the "target function" analyzed in each module; (ii) the "control", which indicates the effect that the stimulation of CCR7 with CCL21 exerts on the activity of the "target molecule" ("activated" or "inhibited") or the "target function" ("increase" or "changes") analyzed; (iii) the "inhibitor effect" indicates whether the treatment with an inhibitor prevents or not the activation or inhibition observed in the "target molecule" or "target function" in the CCR7-stimulated "control". In the "target function," the percentage of inhibition of the function analyzed is also shown (between parentheses); and (iv) "Fig (Ref)", indicates the figure in the reference/s where the original data can be examined. When only the reference is included, this means that it was presented as a "results not shown". Abbreviations: C3, exoenzyme C3 that ADP-ribosylates and inhibits RhoA; PRNK, is a dominant negative fragment derived from the kinase Pyk2; siRNA, small interfering RNA. Mst1 activity was inhibited by reducing its level with siRNA (see other abbreviations in the legend of **Figure 1**).*

found to be regulated both by Gi and G_{13} family of G proteins (**Figure 1**). Interestingly, the kinase Mst1 connects Gi with RhoA, which is also downstream of G_{13} (13) (**Figure 1**). These data are consistent with prior results indicating that the $G_{12/13}$ proteins control RhoA (65). RhoA effects are mediated by a pathway that controls actin dynamics, including actin polymerization (ROCK-LIMK-cofilin) and contraction (ROCK/MLCP/MLC) (57, 59, 61) (**Figure 1**). It was also observed that, downstream of CCR7, RhoA controls the activation of the tyrosine kinase Pyk2 (11) (**Figure 1**), suggesting that this kinase can also mediate the effects of RhoA on the actin cytoskeleton. Accordingly, other authors have suggested that Pyk2 is activated downstream of G_{13} and that it is involved in the control of leukocyte motility and cytoarchitecture (66, 67). The selective blocking of the molecular components of the RhoA pathway in mDCs results in the inhibition of CCR7-dependent migratory speed, endocytosis, and alterations of the cytoarchitecture (11, 13), suggesting that RhoA-controlled actin mediates these functions (58, 61). In summary, CCR7 controls two axes, namely, CCR7/G_{13} and CCR7/G_i/Mst1, that converge on RhoA, which is upstream of a pathway that controls the actin dynamics involved in the regulation of migratory speed, endocytosis, and cytoarchitecture (**Figure 1**) (11, 13, 57, 60). As shown for the other modules, the inhibition of specific signaling components of this pathway failed to affect the chemotaxis or survival of the signaling components controlling these functions, supporting the independence of the CCR7-dependent RhoA-regulated signaling module (**Table 1**) (11, 13). Supporting that the actin dynamics regulatory module is independent of the chemotaxis module, it has been shown that the inhibition of the kinase ROCK fails to block the activation of the chemotaxis regulator JNK in maDCs (39). The results together suggest that the CCR7-regulated RhoA pathway behaves as a signaling module that displays a high degree of independence in maDCs. As shown with the other two modules, although in addition to actin dynamics, the RhoA pathway may regulate other cell functions, including survival and proliferation (62); however, CCR7 in maDCs apparently regulates largely actin dynamics. In summary, the RhoA-regulated module controls selectively CCR7-dependent actin dynamics and the cellular activities associated to it, including migratory speed, endocytosis, and cytoarchitecture.

Finally, the described independence between the chemotaxis and actin dynamics regulatory modules suggests that chemotaxis and motility are different functions. The following results further support this concept. Using microfluidic devices, it has been shown that perturbing actin dynamics with actin and myosin inhibitors in mouse maDCs affects the migratory speed, but not the chemotaxis in response to CCL19 (68). The actin-associated protein mDia, which regulates actin dynamics, mediates migratory speed, but it is dispensable for 3D chemotaxis in response to CCL21 in murine maDCs (69, 70). In response to the external gradients of cAMP, in *D. discoideum*, a polarized localization of Ras is observed, and in neutrophils exposed to the gradients of fMLP, a polarization of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) also takes place. However, in *Dictyostelium* and human neutrophils exposed to these chemoattractants, Ras and PIP_3 still polarize, even when the cells were immobilized either on highly adherent substrates or by disrupting their actin cytoskeleton with latrunculin (53, 55). It has been shown that, although the actin-associated leading edge protein Arp2/3, which regulates actin dynamics, is critical for lamellipodial formation and cell motility in fibroblast and cancer cell lines, it is, however, dispensable for chemotaxis (71, 72). These examples suggest that perhaps it is more appropriate to define chemotaxis as "chemoattractant sensing" to separate it from motility, which could be a different cell activity.

CONCLUDING REMARKS

Herein we discuss experimental findings indicating that CCR7 activates three signaling pathways in maDCs, namely, the PI3K/Akt, the MAPK, and the RhoA pathways, which largely regulate survival (12), chemotaxis (11), and actin dynamics (11, 13), respectively. The results obtained suggest a high degree of independence between these pathways, although it is not complete because at least the chemotaxis and the survival modules are connected, with the former controlling modestly the latter. Albeit each one of the three pathways can regulate several functions in different contexts (43, 44, 52, 62), CCR7 seems to select only one activity in maDCs. The molecular mechanisms supporting the independence and biased functionality of these pathways are not known. CCR7 regulates in maDCs other signaling molecules not analyzed in this review, e.g., cyclic AMP, calcium, phospholipase C, Src, and others (11, 19, 73). Future studies will determine their roles in the modules described or in others described in the future. Finally, the independent modular organization described could be one among several strategies used by chemokine receptors to regulate leukocyte functions because, for instance, the receptor CXCR4 uses redundant signaling to control survival and chemotaxis in maDCs (33). In summary, the information gathered point out an interesting mechanism that could be used by multifunctional chemokine receptors to regulate the functions of leukocytes.

AUTHOR CONTRIBUTIONS

JR-F conceived and wrote the manuscript. OC-G performed important contributions to the manuscript and the figure and designed the table as presented.

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ACKR4 Recruits GRK3 Prior to β-Arrestins but Can Scavenge Chemokines in the Absence of β-Arrestins

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Chemokines are essential for guiding cell migration. Atypical chemokine receptors (ACKRs) contribute to the cell migration process by binding, internalizing and degrading local chemokines, which enables the formation of confined gradients. ACKRs are heptahelical membrane spanning molecules structurally related to G-protein coupled receptors (GPCRs), but seem to be unable to signal through G-proteins upon ligand binding. ACKR4 internalizes the chemokines CCL19, CCL21, and CCL25 and is best known for shaping functional CCL21 gradients. Ligand binding to ACKR4 has been shown to recruit β-arrestins that has led to the assumption that chemokine scavenging relies on β-arrestin-mediated ACKR4 trafficking, a common internalization route taken by class A GPCRs. Here, we show that CCL19, CCL21, and CCL25 readily recruited β-arrestin1 and β-arrestin2 to human ACKR4, but found no evidence for β-arrestin-dependent or independent ACKR4-mediated activation of the kinases Erk1/2, Akt, or Src. However, we demonstrate that β-arrestins interacted with ACKR4 in the steady-state and contributed to the spontaneous trafficking of the receptor in the absence of chemokines. Deleting the C-terminus of ACKR4 not only interfered with the interaction of β-arrestins, but also with the uptake of fluorescently labeled cognate chemokines. We identify the GPCR kinase GRK3, and to a lesser extent GRK2, but not GRK4, GRK5, and GRK6, to be recruited to chemokine-stimulated ACKR4. We show that GRK3 recruitment proceded the recruitment of β-arrestins upon ACKR4 engagement and that GRK2/3 inhibition partially interfered with steady-state interaction and chemokine-driven recruitment of β-arrestins to ACKR4. Overexpressing β-arrestin2 accelerated the uptake of fluorescently labeled CCL19, indicating that β-arrestins contribute to the chemokine scavenging activity of ACKR4. By contrast, cells lacking β-arrestins were still capable to take up fluorescently labeled CCL19 demonstrating that β-arrestins are dispensable for chemokine scavenging by ACKR4.

Keywords: atypical chemokine receptor, ACKR4, CCL19, CCL21, CCL25, β-arrestin, GRK3

INTRODUCTION

Chemokines, a group of about 50 chemotactic cytokines, have fundamental roles in regulating immune responses, primarily by orchestrating leukocyte migration and controlling their localization (1, 2). The biological functions of chemokines are typically mediated by signaling through seven-transmembrane spanning, G-protein coupled receptors (GPCRs) (3, 4). The chemokines CCL19 and CCL21 are essential for guiding dendritic cells and subsets of T cells to lymph nodes by signaling through the cognate chemokine receptor CCR7 (5, 6) and thereby initiate adaptive immune responses. Notably, canonical CCR7 signaling by CCL19 and CCL21 is controlled by the G_i subfamily of G-proteins (7–9). In addition, chemokines bind to a small family of atypical chemokine receptors (ACKRs), which are structurally related to GPCRs but seem unable to elicit canonical, G-protein-dependent signal transduction pathways upon ligand binding (10, 11). However, ACKRs are emerging as crucial regulators for the availability of chemokines. Namely, ACKRs function as "decoy" or "scavenger" receptors that progressively internalize chemokines and sort them for lysosomal degradation to limit local and systemic chemokine concentrations (10, 12). The atypical chemokine receptor ACKR4, formerly also known as CCRL1 and CCX-CKR, is the scavenging receptor for CCL19, CCL21, and CCL25 (13–16). Notably, mice lacking ACKR4 systemically have a 5-fold increase in the level of CCL21 in the blood and a 2- to 3-fold increase in CCL19 and CCL21 in peripheral lymph nodes (17). Despite its expression on thymic epithelial cells where ACKR4 is supposed to scavenge the CCR7 and CCR9 ligands CCL19/CCL21 and CCL25, respectively, mice lacking ACKR4 seem to have a fairly normal thymic T cell lymphopoiesis (18). By contrast, ACKR4 expression by skin keratinocytes and a subset of dermal endothelial cells is critical for shaping functional CCL19/CCL21 gradients under steady-state and inflammatory conditions (17). These local CCL19/CCL21 gradients are essential for allowing dendritic cells to egress the skin and enter lymphatic vessels (19, 20). In addition, ACKR4 is present on lymphatic endothelial cells lining the ceiling of the subcapsular sinus, but not on those lining the floor, forming local CCL21 gradients in lymph nodes to guide dendritic cell homing in a CCR7-dependent manner (21). Consequently, the frequency of dendritic cells in the skin of ACKR4 deficient mice increases and dendritic cells fail to efficiently egress and migrate to draining lymph nodes (20, 21).

Although information on how ACKRs fulfill their scavenging function is limited, ACKR2-4 are known to spontaneously traffic between the plasma membrane and endosomes. Upon ligand binding, ACKRs internalize cognate chemokines and sort them for lysosomal degradation in a G-protein independent manner (10, 12, 16). The ability of ACKRs to scavenge chemokines has been linked to β-arrestins (22–26), which are universal intracellular adaptor proteins of GPCRs (27). In the case of classical GPCRs, β-arrestin recruitment depends on agonist-driven phosphorylation of serine/threonine residues situated at the receptor's C-terminus by GPCR kinases (GRKs) or other protein kinases and leads to clathrin-mediated receptor endocytosis (28). This general concept has recently been challenged, at least for ACKRs, as chemokine uptake by ACKR2 (29), ACKR3 (30, 31), and ACKR4 (16) was observed in cells lacking β-arrestins. By contrast, ligand-mediated β-arrestin recruitment to ACKR4 (26), ACKR3 (24), and ACKR2 (29), as well as subsequent β-arrestin-dependent activation of Erk1/2 through ACKR3 (24) was reported. These controversial data prompted us to in depth investigate early signal transduction pathways and chemokine scavenging activities of ACKR4.

In the present study, we provide evidence that ACKR4 neither interacts with nor activates heterotrimeric G-proteins. Chemokine binding to ACKR4 does also not activate canonical chemokine receptor kinases, such as Erk1/2, Akt, or Src. By contrast, chemokine triggering recruited β-arrestin1 and β-arrestin2 to ACKR4. Moreover, we identify GRK3, and to a lesser extent GRK2, as interaction partner of chemokine engaged ACKR4 and show that GRK3 recruitment precedes the recruitment of β-arrestins upon receptor triggering. We further demonstrate that the C-terminus of ACKR4 is critical for spontaneous receptor trafficking, β-arrestin recruitment and chemokine scavenging. Strikingly, overexpression of β-arrestins increased chemokine uptake by ACKR4, whereas in the absence of β-arrestins ACKR4 was still able to take up cognate chemokines although to a lesser extent, thus providing clear evidence that β-arrestins are dispensable for chemokine scavenging.

MATERIALS AND METHODS

Bioinformatics

Phosphorylation site and kinase interaction site predictions were performed using the native human ACKR4 sequence (uniport: Q9NPB9) and the webservers NetPhos Server 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) (32), NetphosK 1.0 (www.cbs.dtu.dk/services/NetPhosK) (33), and the ELM resource (34). Secondary structure predictions were made using NetSurfP-2.0 (35) and human uniprot sequences (GNAI: P63096, GNAO: P09471, GNAQ: P50148, GNAS2: P63092, GNA13: Q14344).

Generation of Expression Plasmids

Reagents for molecular biology were purchased from Thermo Fisher Scientific and custom-designed primers from Microsynth. An overview of chemokine, receptor and β-arrestin constructs with corresponding primer sequences used for cloning are listed in **Table 1**. Briefly, pcDNA3 β-arrestin2i1-NLuc was generated by amplification of human β-arrestin2 and NLuc and subsequent ligation of the two PCR products over a common ClaI restriction site, followed by subcloning the DNA conjointly into the HindIII and XbaI sites of pcDNA3. Chemokines were amplified by PCR and further cloned into the XhoI and BsaI restriction sites of pET-His_6-SUMO (41). SUMO-hCCL19-S6 was amplified by PCR and cloned into the XhoI and XbaI restriction sites of pET-His_6-SUMO (41).

Nluc-GRK2 was generated by amplifying human GRK2 and Nluc separately, ligating the PCR products over a common ClaI restriction site and cloning it conjointly into the HindIII and XbaI sites of pcDNA3. The other GRK constructs were prepared

TABLE 1 | General plasmids and primers.

Construct	Template (if not synthesized); amplified insert in bold; [reference]	5′-forward primer	5′-reverse primer	Linker
pcDNA3 β-arrestin2i1-Nluc	pcDNA3 **β-arrestin2i1**-Y2	GGTGGAAAGCTTATGGGGGAGAAACCCG	GCATCGATCCACCGCAGAGTTGATCATCATAGTC	*
	pAAVS1P-iCLHN **Nluc** (Addgene plasmid # 66579) (36)	CAATCGATCCACCGCTACCGCCACCGCCGGAACCGCCACCACCAGAACCGCCACCTCCGCCCGCCAGAATGCGTTC	GACCCAAGCTTGCCACCATGGTCTTCACACTCGAAGATTTCGTTGG	
pcDNA3 β-arrestin1A-Nluc	**β-arrestin1A** RC201279 (Origine)	GGTGGAAAGCTTATGGGCGACAAAGGGACCCG	GCATCGATCCACCTCTGTTGTTGAGCTGTGGAGAGCC	*
pEYFP β-arrestin2-EYFP	Published in (37)	-	-	
pcDNA3 hACKR4-EYFP	pcDNA3 CCR7-**EYFP** (9)	GGACTCGAGAGCGGAGGTGGCGGTTCTGGTGGTGGCGGTTCCGGCGGTGGCGGTAGCGTGAGCAAGGGCGAGGAG	GAATAGGGCCCTCTAGACTACTTGTACAGCTCGTCCATGC	**
pcDNA3 hACKR4t-EYFP	pcDNA3 **ACKR4**-EGFP (38)	GGAGACCCAAGCTTCATTACGATGGC	CTCTCGAGTCCACCAACGTACAAGATTGGGTTCAAACAAGAGTG	**
pcDNA3 hACKR4-mTq2	pcDNA3.1(-)Galphai1-**mTurquoise2** (39)	GCAGACTCGAGAGCGGAGGTGGCGGTTCTGGTGGTGGCGGTTCCGGCGGTGGCGGTAGCATGGTGAGCAAGGGCGAGG	GCAGGTCTAGATTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCG	**
pcDNA3 hACKR4-HA	pcDNA3 **ACKR4**-EGFP (38)	GGAGACCCAAGCTTCATTACGATGGC	CTACCTCGAGCCCCAATAGAGAAGGTAGAAGT	***
pcDNA3 hCCR7-EYFP	pcDNA3 **CCR7**-HA (40)	GACCCAAGCTTGGTACCGAGCTCGGATC	GTAGCTCGAGTCCACCGGAGAAGGTGGTGGTGGTCTCG	**
pcDNA3 hCCR7-HA	Published in (40)	-	-	***
pSUMO hCCL19	pCR3-**hCCL19**-Fc (40)	GGTGCTCGAGTTAACTGCTGCGGCGCTTC	GACTAGGTCTCCGGTGGGGGCACCAATGATGCTGAAGACTG	-
pSUMO hCCL21	Published in (41)			-
pSUMO hCCL25	**hCCL25** RC222128 (Origene)	GACTAGGTCTCCGGTGGGCAAGGTGTCTTTGAGGAC	GTGCTCGAGTTACAGTCCTGAATTAGCTGATATCAGGAGGG	-
pSUMO hCCL19-S6	p**SUMO hCCL19**	CCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGG	CAGGTGCTCGAGTTATTAGTTCAGCAGGCGCAGCAGCCAGCTCAGGCTATCGCCGCTGCCGCCGCCGCCGCTACTGCTGCGGCGCTTCATCTTGG	****

Linker sequence used between protein and tag.
**GSI(GGGGS)$_3$.*
***GGLES(GGGGS)$_3$.*
****GARA.*
*****SGGGGS.*
******(GGGGS)$_3$GGS.*

by replacing GRK2 with GRK3, GRK4, GRK5, and GRK6 using HindIII and ClaI or ClaI and XbaI, as listed in **Table 2**.

Site directed mutations of putative ACKR4 phosphorylation sites are listed in **Table 3**. Multiple site directed PCR were performed in consecutive cloning rounds to get ACKR4$_{TT}$, ACKR4$_{SSS}$, and ACKR4$_{TTSSS}$ mutants.

To generate the BRET constructs for G proteins, all redundant HindIII, ClaI, BamHI, XhoI, or XbaI sites were removed by introducing silent mutations as listed in **Table 4**. Then, a BamHI site encoded in a SGGGGS linker was introduced (**Table 5**, **Supplementary Figure 3**). Further, the modified Gα-subunits were amplified with adjacent HindIII and XbaI sites and cloned into pcDNA3. PCR amplified Nluc was introduced into the BamHI sites (**Table 5**). An exception is Gα_q, were the RLuc8 in Gα_q-RLuc8 was replaced via BamHI cutting and insertion of Nluc. To generate pIRES Gβ-2A-cpV-Gγ2 Gα-Nluc, a redundant HindIII was removed and a new one added after the IRES sequence using site directed mutagenesis. Then the mutated IRES sequence was amplified using the forward primer hybridizing at a SalI site and the reverse primer with a HindIII site, hybridizing to the one introduced beforehand, followed by an XbaI site at its end. The PCR-product was ligated into the original IRES plasmid, removing the Gαi2-mTurquoise2 sequence, after digesting both with SalI and XbaI. Gα_i-Nluc was cut out from pcDNA3 Gα_i-Nluc utilizing HindIII and XbaI and ligated into the modified IRES vector.

TABLE 2 | GRK related plasmids and primers.

Construct	Template (if not synthesized); amplified insert in bold; [reference]	5′-forward primer	5′-reverse primer	Linker
pRK5-hGRK4 missing XbaI site	pRK5-hGRK4 (addgene: #32690) (42)	GCTTTGCCATTAGATCTCGACAAGA ACATACATAC	GTATGTA TGTTCTTGTCGAGATCTAA TGGCAAAGC	
pRK5-hGRK4 missing HindIII and XbaI site	pRK5-hGRK4 missing XbaI site	GTGAAAGTGAGGAAGCCTTGCCAT TAGATCTCG	CGAGATCTAATGGCAAGGCTTCCTC ACTTTCAC	
pcDNA3 Nluc-GRK2	pWZL Neo Myr Flag **ADRBK1**(addgene: #20418) (43)	GTAGCGGTGGATCGATGGGGTCTT CAAAATCTAAACCAAAGGACC	GATAGGGCCCTCTAGATCAGAGGCC GTTGGCACTGCCGCGCTGGACCAG CGGCACCTTGCTCAGCTCCACCACG GGCGAG	*****
	pcDNA3 β-arrestin2i1-**Nluc**	GACCCAAGCTTGCCACCATGGTCT TCACACTCGAAGATTTCGTTGG	CAATCGATCCACCGCTACCGCCACC GCCGGAACCGCCACCACCAGAACC GCCACCTCCGCCCGCCAGAATGCG TTC	
pcDNA3 GRK2-NLuc	pcDNA3 Nluc-**GRK2**	GAGACCCAAGCTTCATTACGATGG CGGACCTGGAG	GCAGCATCGATCCACCGAGGCCGTT GGCACTGC	*
pcDNA3 GRK3-Nluc	pDNR-Dual **GRK3** (DNASU: HsCD00022400)	GAGACCCAAGCTTCATTACGATGG CGGACCTGGAGG	GCAGCATCGATCCACCGAGGCCGTT GCTGTTTCTGTG	*
pcDNA3 Nluc-Grk4	pRK5-**hGRK4** missing HindIII and XbaI site	GTGGCGGTAGCGGTGGATCGATG GAGCTCGAGAACATCGTGGCCAAC	GAATAGGGCCCTCTAGATTAGCATTG CTTGGGTTCCACTTCCTTCTC	*****
pcDNA3 GRK5-Nluc	pWZL Neo Myr Flag **GRK5** (addgene: #20495) (43)	GAGACCCAAGCTTCATTACGATGG AGCTGGAAAACATCGTG	GCAGCATCGATCCACCGCTGCTTCC GGTGGAGTTC	*
pcDNA3 GRK6-Nluc	Synthesized	CTATAGGGAGACCCAAGCTTATGG AGCTCGAGAACATCGTAGCG	CCTCCAATCGATCCACCCCGCCAAC TGCTGGTGGGGGCCTCGGGCTG	*

Linker sequence used between protein and tag.
**GSI(GGGGS)$_3$.*
******(GGGGS)$_3$GGS.*

TABLE 3 | Phosphorylation site mutations in ACKR4-EYFP.

Mutation	Nucleotide mutation	5′-forward primer	5′-reverse primer
ACKR4$_{Y68F}$	TA1103TT	GGTTGTTGCTATCTATGCTTTCTACAAGAAGCAAAG	CTTTGCTTCTTGTAGAAAGCATAGATAGCAA CAACC
ACKR4$_{Y79F}$	TA1136TT	GACCGATGTCTTCATTTTGAACTTGGCTGTTG	CAACAGCCAAGTTCAAAATGAAGACATCGG TC
ACKR4$_{Y138F}$	TA1313TT	CATCTCTATTGATAGATTCGTTGCTGTTACCAAGG	CCTTGGTAACAGCAACGAATCTATCAATAGA GATG
ACKR4$_{T142A}$	A1325G	GATACGTTGCTGTTGCCAAGGTCCCATCTC	GAGATGGGACCTTGGCAACAGCAACGTATC
ACKR4$_{S146A}$	TCT1337GCC	CTGTTACCAAGGTCCCAGCCCAATCTGGTGTTGG	CCAACACCAGATTGGGCTGGGACCTTGGTA ACAG
ACKR4$_{S148A}$	TCT1343GCC	CAAGGTCCCATCTCAAGCCGGTGTTGGTAAACCATG	CATGGTTTACCAACACCGGCTTGAGATGGG ACCTTG
ACKR4$_{T226A}$	ACT1577GCC	GCTACTTCATTACCGCTAGAGCCTTGATGAAGATGCC AAACATC	GATGTTTGGCATCTTCATCAAGGCTCTAGCG GTAATGAAGTAGC
ACKR4$_{S236A}$	T1607G	CAAACATCAAGATCGCCAGACCATTGAAGG	CCTTCAATGGTCTGGCGATCTTGATGTTTG
ACKR4$_{S309A}$	TCT1826GCC	CGTTTTTATGGGTGCCGCCTTCAAGAACTACG	CGTAGTTCTTGAAGGCGGCACCCATAAAAA CG
ACKR4$_{S323A}$	TCT1868GCC	GCTAAGAAGTACGGTGCCTGGAGAAGACAAAGACAA TC	GATTGTCTTTGTCTTCTCCAGGCACCGTACT TCTTAGC
ACKR4$_{S330A}$	T1889G	GAAGACAAAGACAAGCCGTTGAAGAATTCCC	GGGAATTCTTCAACGGCTTGTCTTTGTCTTC

Chemokine Production

Recombinant human chemokines fused to a His$_6$-SUMO-tag were purified from BL21 (DE3) *E. coli* and refolded by infinite dilution at pH 8.5. The His$_6$-SUMO-tag was cleaved off by incubation with the Ulp-1 protease for 1–5 h and removed (41, 46, 47). Chemokines were purified by RP-HPLC on C18 columns.

To generate fluorescently tagged CCL19^{Dy649P1}, human CCL19 fused to a His$_6$-SUMO-tag and a SGGGGS-S6-tag was

TABLE 4 | Templates for Gα and site directed mutations thereof.

Gα variant	Mutation effect	5′-forward primer	5′-reverse primer
$G\alpha_{i/o/q}$-RLuc8	A kind gift from Nevin Lambert (44)		
$G\alpha_s$	Synthesized		
$G\alpha_{13}$	A kind gift from B.Moepps. (45)		
pIRES Gß-2A-cpV-Gy2 GNAi3-mTq2	addgene #69625 (39)		
Gαo	– BamHI site	CGCAAGAAGTGGATTCATTGCTTCGAGGAC	GTCCTCGAAGCAATGAATCCACTTCTTGCG
GαS	– BamHI site	CATTGTGAAGCAGATGAGAATCCTGCATGTTAATGG	CCATTAACATGCAGGATTCTCATCTGCTTCA CAATG
	– BamHI site	GCCGCAAGTGGATACAGTGCTTCAACG	CGTTGAAGCACTGTATCCACTTGCGGC
	+ BamHI site	CCCCCCGTGGAGCTGTCAGGTGGCGGATCCCAGTT CAGAGTGG	CCACTCTGAACTGGGATCCGCCACCTGACA GCTCCACGGGGGG
Gα13	– BamHI site	CATATTCCCTGGTCAGGTGGCGGATCCGGAGACAAC TC	GAGTTGTCTCCGGATCCGCCACCTGACCAG GGAATATG
	– HindIII site	CTCGAGAGAAGCTCCATATTCCCTGGG	CCCAGGGAATATGGAGCTTCTCTCGAG
	+ BamHI site	CTATTTCCTAGAATTTGAAGGCGATCCCCACTGCTTA AGAGAC	GTCTCTTAAGCAGTGGGGATCGCCTTCAAAT TCTAGGAAATAG
pIRES	– HindIII site	AATGTCGTGAAGGAAGCAGTACCTCTGGTAGCTTCTT GAAGACAAACAAC	TTGTTTGTCTTCAAGAAGCTACCAGAGGTAC TGCTTCCTTCACGACATTC
	+ HindIII site	GTTTTCCTTTGAAAAACACGATGATAATAAGCTTTGCA CGTTGAGCGCCGAAGACAAGGCGG	CCGCCTTGTCTTCGGCGCTCAACGTGCAAA GCTTATTATCATCGTGTTTTTCAAAGGAAAAC

TABLE 5 | Templates for Gα and Nluc amplification.

Affected protein	Amplification of	5′-forward primer	5′-reverse primer
NLuc	BamHI-Nluc-BamHI	TCAGGTGGCGGATCCATGGTCTTCACACTCGAAGATT TCGTTG	GATGCCGGATCCTCCACCGCCAGAGCCCGCCAGAA TGCGTTCGCAC
$G\alpha_o$	HindIII-Gαo(1)-BamHI	GAGACCCAAGCTTCAGCCACCATGGGATGTACTCTG AGCGCAGAGGAG	GGATCCGCCACCTGACAAAGTGTCCATGGCCCGGAC GATGGCTGCCAGGGAC
	BamHI-Gαo(2)-XbaI	TCTGGCGGTGGAGGATCCGGCATCGAATATGGTGAT AAGGAGAGAAAG	CAGGGCCCTCTAGATCAGTACAAGCCGCCGCCCCG GAG
$G\alpha_i$	HindIII-Gαi(1)-BamHI	GGATCCGCCACCTGACAACCTCCCCATAGCCCTAAT GATAGCAATAATTGACTG	GAGACCCAAGCTTCAGCCACCATGGGCTGCACGCTG AGC
	BamHI-Gαi(2)-XbaI	TCTGGCGGTGGAGGATCCAAGATAGACTTTGGTGAC TCAGCCCG	GTATGCCTCTAGATCAAAAGAGACCACAATCTTTTAG ATTA
$G\alpha_{13}$	HindIII-$G\alpha_{13}$-XbaI	GACCCAAGCTTATGGCGGACTTCCTGCCGTC	CAGGGCCCTCTAGATTACTGTAGCATAAGCTGCTTGA GGTTGTC
$G\alpha_S$	HindIII-$G\alpha_S$-XbaI	GACCCAAGCTTATGGGCTGCCTC	CAGGGCCCTCTAGATTAGAGCAGCTCGTACTGACGA AGGTG
IRES sequence	amplification	GTTCGAAGTCGACAGATCTC	CATCGCTCTAGACGTACTAGCAAGCTTATTATCATCG TGTTTTTCAAAGG

expressed and purified as described above. CoA-conjugated (C3144-25MG, Sigma) Dy649P1 (Dy649P1-03, Dyomics GmbH) was prepared as described (46). Fluorescently labeled $CCL19^{Dy649P1}$ was generated by labeling purified CCL19-S6 with CoA-Dy649P1 at 37°C for 2 h using the phosphopantetheinyl transferase Sfp (P9302S, New England Biolabs) as previously described (46). Excess of substrate was removed from fluorescently labeled chemokine by reverse phase HPLC.

Cell Culture and Transfection

HeLa cells were cultured in DMEM (P04-04510, Pan Biotech), containing 1% penicillin/streptomycin (Pan Biotech), 10% FBS (Thermo Fisher Scientific). Cells were transfected at least 30 h prior to the experiments using the 100 µl Neon® Transfection System (Thermo Fisher Scientific) according to the manufacturer's protocol, transfecting 5×10^5 cells with 10 µg total plasmid DNA. For BRET recruitment experiments, the DNA ratio of fluorophore to luciferase construct was 3:1, for $G\alpha_i$ activation experiments, the ratio of pcDNA3 receptor-HA to pIRES Gα-Nluc Gβγ-cpVenus construct was 1:3.

Chemokine Mediated Erk1/2, Akt, and Src Activation

HeLa cells were transfected either with pcDNA3 ACKR4-HA, pcDNA3 CCR7-HA or empty pcDNA3. After 36 h, cells were

starved for 2 h with medium containing 0.5% serum before they were stimulated with 1 μg/ml (114 nM) human CCL19. Cells were lysed using NP-40 lysis buffer as described (9). Samples were separated by SDS-PAGE and phosphorylated (p) and total (t) amounts of signaling proteins detected by Western blotting using the following antibodies purchased from Cell Signaling Technology: tErk (#9102) pErk (#4370), tSrc (#2109), pSrc (#6943), tAkt (#9272), pAkt (#9271).

BRET Measurements

Transfected HeLa cells were grown in 6 well plates, washed with PBS, and detached using PBS based Gibco™ cell dissociation buffer (#13151014, Thermo Fisher Scientific) for a minimum of 3 min. Cells were collected in twice the volume of dissociation buffer with DMEM containing 10% FCS before being centrifuged for 2 min at 200 g. Cells were washed and resuspended in PBS containing 5% (w/v) glucose (PBS-G). Aliquots of around 8×10^4 cells in 40 μl were inoculated in white 96-flat-bottom half-well plates in the presence of 5 μM luciferase substrate coelenterazine H (#C-7004, Biosynth) and stimulated with various concentrations of chemokines. Ratiometric BRET measurements were performed using a Tecan Spark™ 10M multimode microplate reader, measuring luciferase bioluminescence (384–440 nm, 350 ms integration time) and EYFP fluorescence (505–590 nm, 350 ms integration time) to calculate the BRET ratio between both signals (48). For short term observations (−1 to 3 min), the integration time of both signals was decreased to 250 ms and an injector used for chemokine addition. To calculate NetBRET, BRET ratio of control wells containing luciferase and HA-tagged receptor instead of EYFP-tagged receptor was subtracted from the sample BRET ratio. For $G\alpha_i$ activation, the control wells contained cells transfected with pIRES Gα-Nluc Gβγ-cpVenus alone. Area under the curve analysis (AUC) was performed using the measurements before stimulation as baseline and integrating the peak starting from 0 min until the end of measurement. For data representation of GRK and G protein activation, baseline reduction was performed using the measurements before addition of ligands, which is referred to as "corrected NetBRET."

Chemokine Uptake Assay

Transfected HeLa cells were seeded at 4.5×10^4 cells per well in 24 well plates. Cells were washed with PBS and incubated for at least 10 min in 200 μl 50 mM HEPES-buffered, high glucose DMEM without phenol red (#21063045 Thermo Fisher Scientific) at 37 or 8°C, respectively. Fifty microliter of chemokine solution was added to the cells for indicated times. At $t = 0$ min, all cells were washed twice with PBS; acidic wash (100 mM NaCl, 50 mM glycine HCL, pH 3.0) was applied to the designated wells for about 45 s, followed by two PBS washes. Cells were detached by incubation with PBS based Gibco™ cell dissociation buffer and subsequently measured on a BD LSR II flow cytometer and FACSDiva™ software (BD Biosystems). Data were analyzed using the FlowJo™ 10.7 software.

ACKR4 Receptor Staining and Chemokine Binding

Transfected HeLa cells were seeded at 2.5×10^5 cells per well in 6 well plates. About 24–36 h post-transfection, cells were washed with FACS buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium phosphate, 5 mM HEPES, pH 7.5) and detached using Gibco Cell Dissociation Buffer (ThermoFisher). Cells were incubated with α-hACKR4 primary antibody (clone 13E11; #362102 Biolegend, dilution 1:750) at 8°C for 40 min followed by intense washing and incubation with goat α-mouse IgG coupled to Alexa647 (#A-21235 ThermoFisher, dilution 1:1000) for additional 20 min. To determine chemokine binding capacities to different ACKR4-EYFP mutants, transfected cells were incubated with 25 nM site specific labeled human CCL19 ($CCL19^{Dy649P1}$) at 8°C for 30 min. After washing, cells were analyzed by flow cytometry on a LSR II (BD Biosciences). Flow cytometry data were analyzed using FlowJo V10 (BD Biosciences). Medians of chemokine or antibody fluorescence of $EYFP^+$ cells were related to the median of EYFP to consider transfection efficiency.

Confocal Fluorescence Microscopy

Transfected HeLa were seeded in 6 well plates containing 18 mm 1.5H glass slides (#0117580 Marienfeld-Superior). After 36 h, cells were fixed using 4%formaldehyde and 1%glutaraldehyde and subsequently stained with phallodin-Alexa647 and mounted with DAPI Fluoromount-G (#0100-20, SouthernBiotech). A Leica TCS SP5 II confocal microscope with a 63x oil-immersion objective was used. Acquired images were processed using Fiji (49) and ImageJ2 (50). For deconvolution of 3D stacks, SVI Huygens Essential version 16.10.0p3 was used.

Data Analysis

Data analysis and presentation was performed using GraphPad Prism V.7 and V8. For statistics with one variable, RM one-way ANOVA or mixed-effects analysis, both with Dunnett's multiple comparisons test with a single pooled variance was performed (**Figure 4**). For statistics of Western blot ratio, mixed-effect model with Tukey's multiple comparisons test with a single pooled variance was performed (**Figure 1**). For experiments using two variables, ordinary two-way ANOVA with Tukey's multiple comparisons test, with individual variances computed for each comparison was performed (**Figures 2**, **5**). EC50 values were calculated fitting a three parameter [agonist] vs. response curve. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, $^{****}p < 0.0001$.

RESULTS

ACKR4 Does Not Elicit Canonical Chemokine-Mediated Signal Transduction Pathways

ACKRs, including ACKR4, were reported not to signal through heterotrimeric G_i-proteins manifested by the failure to induce cell migration or calcium mobilization (16, 26). However, it has been speculated that the G_i-protein might associate with ACKR4, hence sterically block G_s activation unless it

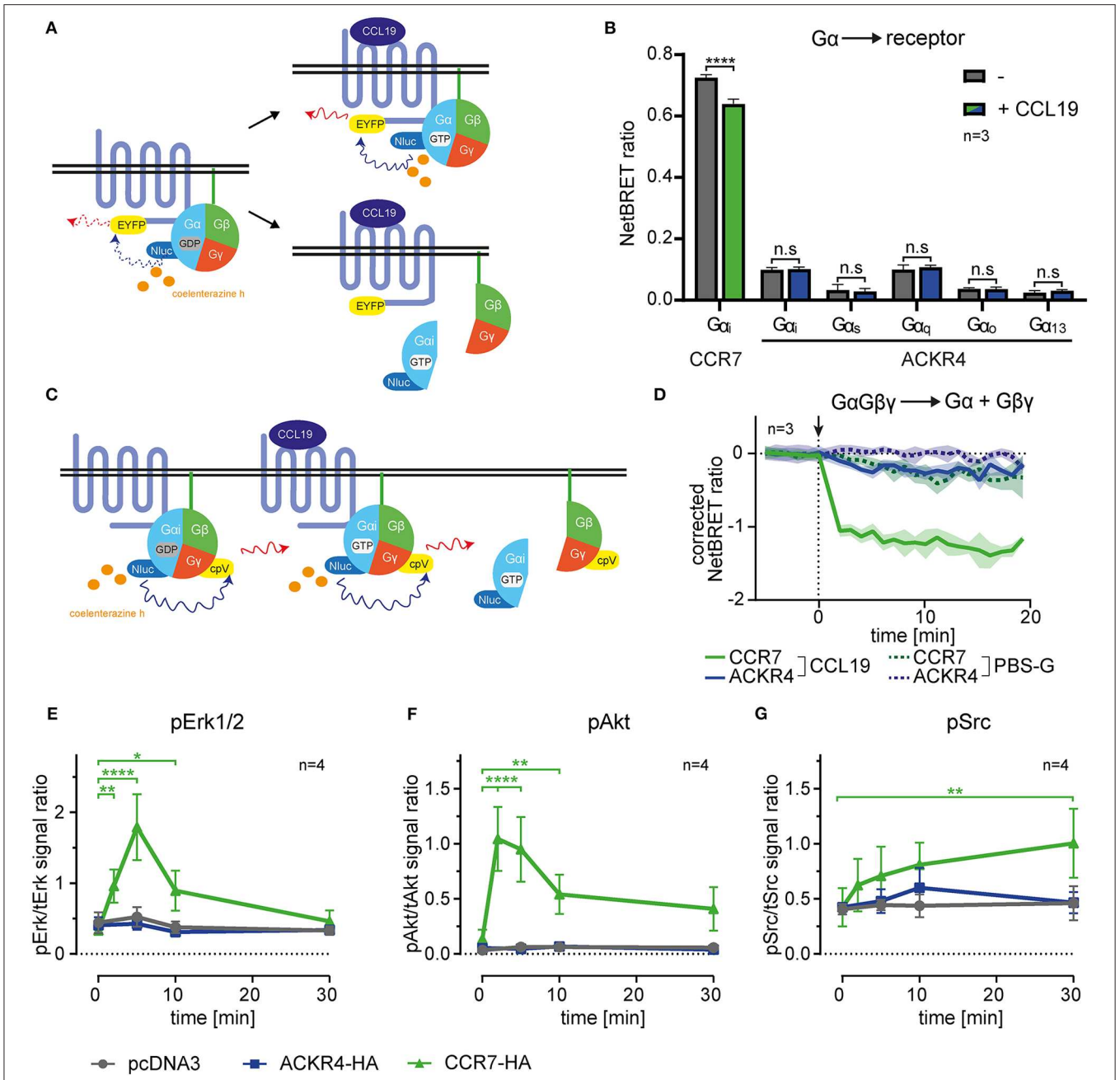

FIGURE 1 | ACKR4 does not interact with or activate G-proteins. **(A)** Schematic representation of BRET-based G-protein interaction, recruitment and dissociation assays. **(B)** HeLa cells were transiently transfected with either human CCR7-EYFP or human ACKR4-EYFP conjointly with Nluc fused to either $G\alpha_i$, $G\alpha_q$, $G\alpha_s$, $G\alpha_o$, or $G\alpha_{13}$. NetBRET was determined before and after stimulating cells with 1 μg/ml (114 nM) human CCL19. $n = 3$. **(C)** Schematic representation of BRET-based G-protein activation assays. **(D)** HeLa cells expressing either human CCR7 or human ACKR4 together with an IRES vector coding for $G\alpha_i$-Nluc and Gβγ-cpVenus were stimulated at $t = 0$ (indicated by an arrowhead) with 1 μg/ml CCL19 (solid lines) or PBS-G as control (dashed lines). G-protein activation was determined by measuring chemokine-mediated dissociation of the Gβγ- from the Gα-subunit manifested by a decrease in NetBRET. Mean values and SEM of 3 independent experiments are shown. **(E–G)** Mock transfected (pcDNA3) and ACKR4-HA or CCR7-HA transfected HeLa cells were stimulated with 1 μg/ml CCL19 and total vs. phospho-Erk1/2 **(E)**, total vs. phospho-Akt **(F)**, and total vs. phospho-Src **(G)** was determined by Western blot densitometric analysis. Mean values and SEM of 4 independent experiments are shown.

dissociates from the receptor (26). To address this possibility, we established bioluminescence resonance energy transfer (BRET)-based assays to measure G-protein activation and interaction with the receptor. We engineered human Gα-proteins ($G\alpha_i$, $G\alpha_q$, $G\alpha_s$, $G\alpha_o$, or $G\alpha_{13}$) where we introduced the Nano luciferase (Nluc) as luminescence donor into the unstructured region

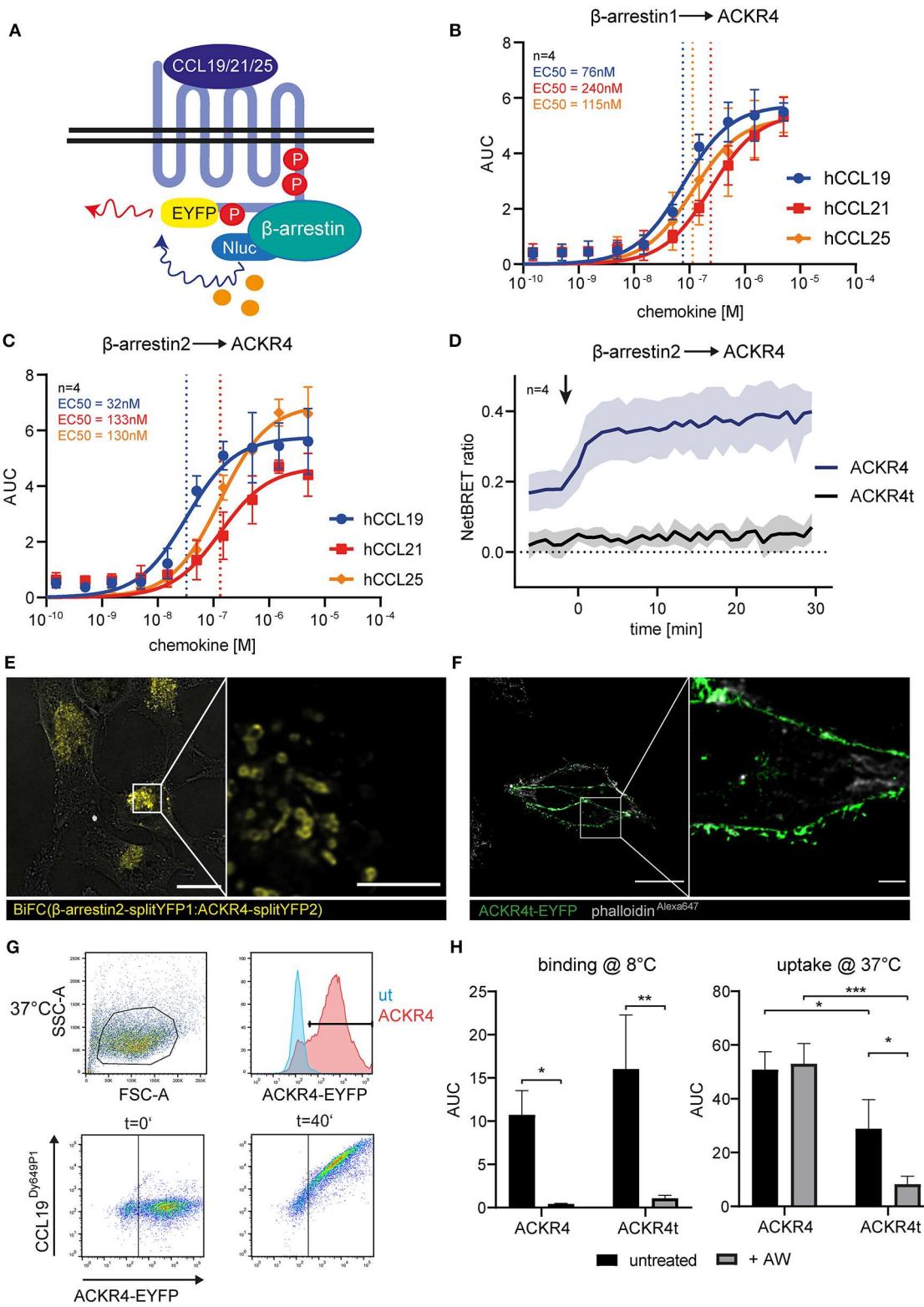

FIGURE 2 | The C-terminus of ACKR4 is critical for β-arrestin recruitment and chemokine uptake. **(A)** Schematic representation of BRET-based β-arrestin recruitment assay. HeLa cells co-transfected with ACKR4-EYFP and β-arrestin1-Nluc **(B)** or β-arresin2-Nluc **(C)** were stimulated with graded concentrations of either human CCL19, CCL21, or CCL25 and β-arrestin recruitment to ACKR4 determined by BRET. $n = 4$. **(D)** HeLa cells co-transfected with β-arrestin2-Nluc together with ACKR4-EYFP or tailless ACKR4t-EYFP and stimulated at $t = 0$ min (indicated by an arrowhead) with 1.5 μM CCL19 and mean NetBRET and SEM derived from four

(Continued)

FIGURE 2 | individual experiments are depicted over time before and after chemokine addition. **(E)** HeLa cells were co-transfected with ACKR4-splitYFP2 and β-arrestin2-splitYFP1. BiFC was visualized by confocal microscopy under steady-state conditions. Scale bar = 25 μm or 3 μm for deconvoluted, zoomed image. **(F)** HeLa cells were transfected with ACKR4t-EYFP and its subcellular localization determined by confocal microscopy. A representative deconvoluted image is shown. Scale bar = 25 μm or 2.5 μm for zoomed image. **(G)** HeLa cells (ut) or HeLa cells expressing ACKR4-EYFP (ACKR4) were incubated at 37°C with 10 nM fluorescently labeled $CCL19^{Dy649P1}$ for various time points. Receptor expression and chemokine uptake was determined by flow cytometry. **(H)** HeLa cells expressing ACKR4-EYFP or ACKR4t-EYFP were incubated with 10 nM fluorescently labeled $CCL19^{Dy649P1}$ at either 8°C to determine chemokine binding, or at 37°C to determine chemokine uptake by flow cytometry for up 40 min. Where indicated, cells were shortly exposed to an acidic wash to remove surface bound chemokine. Mean values and SD of three independent experiments are shown.

after the second helix (51). As luminescence-acceptor, we used human ACKR4, or human CCR7, fused to EYFP to measure steady-state association, chemokine-driven recruitment, as well as activation-dependent dissociation of the heterotrimeric G-protein from the receptor (**Figure 1A**). Co-expressing Gα-Nluc variants together with ACKR4-EYFP in HeLa cells revealed that none of these tested Gα-proteins associated with ACKR4 under steady-state conditions (**Figure 1B**). Stimulating cells with 1 μg/ml of human chemokine CCL19, known to elicit strong CCR7 responses, neither recruited one of the Gα-proteins to ACKR4, nor resulted in the dissociation of one of the G-proteins from the receptor (**Figure 1B**). By contrast, $G\alpha_i$ was found to pre-associate with the canonical chemokine receptor CCR7-EYFP and dissociated from the receptor upon CCL19 stimulation (**Figure 1B**), which is in line with the pre-association of the G_s-protein with the adrenergic receptor and its subsequent ligand-induced dissociation (51). Next, we used $G\alpha_i$-Nluc and Gβγ fused to cpVenus as luminescence-acceptor to monitor activation-induced dissociation of Gβγ from the $G\alpha_i$-subunit (**Figure 1C**). Whereas, CCL19 stimulation led to the dissociation of the $G\alpha_i$ from the Gβγ-subunit upon CCR7 triggering, this was not observed for ACKR4 (**Figure 1D**). Consistent with these findings, CCL19 triggering of CCR7, but not of ACKR4, lead to the phosphorylation and activation of the MAP kinase Erk1/2 (**Figure 1E, Supplementary Figure 1**) and protein kinase B/Akt (**Figure 1F, Supplementary Figure 1**) through the canonical G_i-signaling pathway (7–9). Moreover, CCL19 stimulation of CCR7 caused the phosphorylation of the kinase Src (**Figure 1G, Supplementary Figure 1**), which occurs through G-protein-independent signaling (9). Again, no CCL19-mediated Src phosphorylation was observed upon ACKR4 triggering (**Figure 1G, Supplementary Figure 1**). These data provide comprehensive evidence that ACKR4 does neither associate with and activate G-proteins, nor elicits canonical chemokine receptor signaling pathways involving Erk1/2, Akt or Src kinases.

The C-Terminus of ACKR4 Controls Interaction and Recruitment of β-Arrestins and Is Essential for Chemokine Uptake

As the role of β-arrestins in chemokine scavenging by ACKR4 is debated (16, 26), we determined β-arrestin1 and β-arrestin2 recruitment to ACKR4 by BRET (**Figure 2A**). We co-expressed EYFP-tagged human ACKR4 together with either Nluc-tagged human β-arrestin1 or β-arrestin2 in Hela cells and stimulated the cells with graded concentrations of the human ACKR4 ligands CCL19, CCL21, and CCL25. All three chemokines recruited β-arrestin1 (**Figure 2B**) and β-arrestin2 (**Figure 2C**) to ACKR4. CCL19 was the most potent agonist in recruiting β-arrestin1 (EC50∼76 nM) and β-arrestin2 (EC50∼32 nM). EC50 values for CCL21 were ∼240 nM (β-arrestin1) and ∼133 nM (β-arrestin2), those for CCL25 ∼115 nM and ∼130 nM, respectively (**Figures 2B,C**). As β-arrestin recruitment to a GPCR is controlled by phosphorylation of serine/threonine residues located at the receptor's C-terminus, we generated a tailless human ACKR4 variant by truncating the receptor directly after the conserved NPxxY motif ($ACKR4_{1-304}$; termed ACKR4t). As expected, ACKR4t failed to recruit β-arrestin1 or β-arrestin2 upon chemokine stimulation (**Figure 2D, Supplementary Figure 2**). Notably, ACKR4t already showed a markedly reduced steady-state interaction with β-arrestins before the chemokine was added compared to wild-type ACKR4 (**Figure 2D**). To confirm and visualize β-arrestin interaction with ACKR4 under steady-state conditions, we exploited a split-YFP based biomolecular fluorescence complementation (BiFC) assay (9, 48, 52). We found that BiFC between ACKR4-splitYFP2 and β-arrestin2-splitYFP1 was predominantly found in vesicular structures (**Figure 2E**), suggesting that β-arrestins might contribute to the steady-state trafficking of ACKR4. Consistent with this hypothesis, ACKR4t (fused to EYFP) was predominantly expressed at the plasma membrane (**Figure 2F**). To assess chemokine scavenging, we incubated HeLa cells expressing either ACKR4-EYFP or ACKR4t-EYFP with fluorescently labeled CCL19 ($CCL19^{Dy649P1}$) at either 8°C (to measure chemokine binding) or 37°C (to determine chemokine uptake; **Figure 2G**). At 8°C, both ACKR4 variants bound $CCL19^{Dy649P1}$ with ACKR4t being slightly, but not significantly, more efficient (**Figure 2H**). Surface bound $CCL19^{Dy649P1}$ was effectively removed by a short acidic wash (**Figure 2H**). Incubating ACKR4-EYFP expressing cells at 37°C resulted in a marked uptake of $CCL19^{Dy649P1}$ which resisted the acidic wash, indicating that the chemokine was indeed rapidly internalized (**Figure 2H**). By contrast, using the same conditions, $CCL19^{Dy649P1}$ bound to ACKR4t-EYFP, but was efficiently removed by an acidic wash (**Figure 2H**), revealing that ACKR4 lacking its C-terminus fail to efficiently take up CCL19.

Chemokine Triggering Recruits GRK3, and to a Lesser Extent GRK2, to ACKR4

To identify which GRK promotes putative receptor phosphorylation and subsequent β-arrestin recruitment we established BRET assays to measure recruitment of individual GRKs to engaged ACKR4 (**Figure 3A**). Therefore, we fused Nluc to all ubiquitously expressed human GRKs and co-expressed them individually with ACKR4-EYFP in HeLa

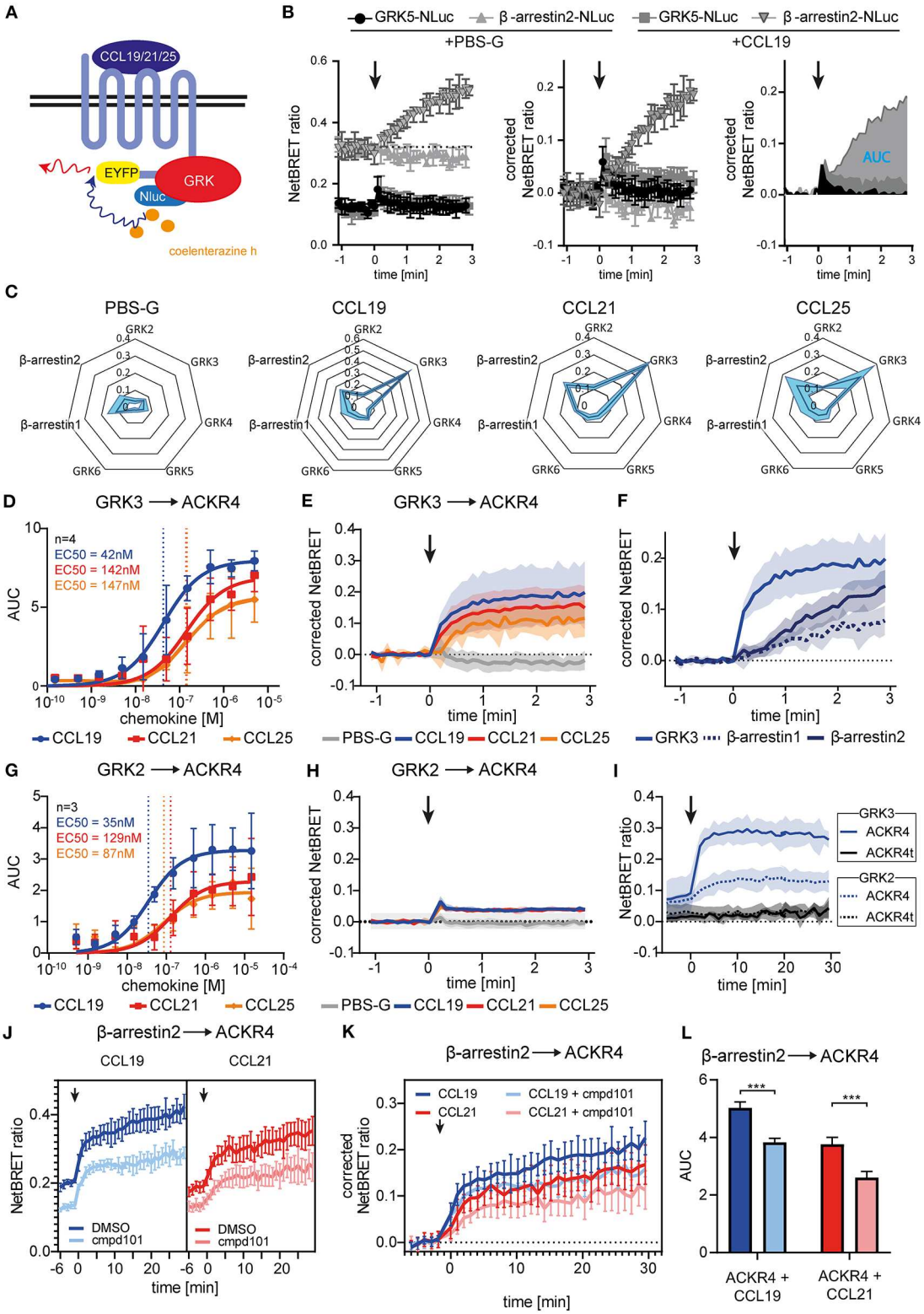

FIGURE 3 | Chemokine stimulation selectively recruits GRK3 and GRK2 to ACKR4. **(A)** Scheme of chemokine-mediated GRK recruitment determined by BRET. **(B)** HeLa cells were co-transfected with ACKR4-EYFP and GRK5-Nluc or β-arrestin2-Nluc and stimulated with CCL19. Chemokine-mediated GRK5 or β-arresin2 recruitment was determined by NetBRET (left). NetBRET values before chemokine stimulation (baseline) was subtracted for corrected NetBRET values (middle). Corrected NetBRET values over time after chemokine addition was integrated and depicted as area under the curve (AUC) values (right). Chemokine addition is

(Continued)

FIGURE 3 | indicated by an arrowhead. Data from one representative experiment out of three experiments is shown. **(C)** Spider diagram of PBS or chemokine-mediated GRK2, GRK3, GRK4, GRK5, GRK6, β-arrestin1, and β-arrestin2 recruitment measured as AUC over 3 min. Chemokines were used at a concentration representing about 3-times the EC50 value of β-arrestin2 recruitment, namely 114 nM for CCL19, 408 nM for CCL21, and 352 nM for CCL25. $n = 3$. **(D)** Dose-response curve of GRK3-Nluc recruitment to ACKR4-EYFP upon ligand stimulation. $n = 4$. **(E)** Time-resolved GRK3-Nluc recruitment to ACKR4-EYFP upon 114 nM chemokine stimulation (indicated by an arrowhead). $n = 3$. **(F)** Time-resolved recruitment of GRK3-Nluc from **(E)**, β-arrestin1-Nluc, or β-arrestin2-Nluc to ACKR4-EYFP upon stimulation (indicated by an arrowhead) with 114 nM CCL19. $n = 3$. **(G)** Dose-response curve of GRK2-Nluc recruitment to ACKR4-EYFP upon ligand stimulation. $n = 3$. **(H)** Time-resolved GRK2-Nluc recruitment to ACKR4-EYFP upon 114 nM chemokine stimulation (arrowhead). $n = 3$. **(I)** GRK2 (dashed lines) and GRK3 (solid lines) recruitment to ACKR4-EYFP or ACKR4t-EYFP upon 114 nM CCL19 stimulation (arrowhead) over 30 min. $n = 3$. **(J–L)** HeLa cells transfected with ACKR4-EYFP and β-arrestin2-Nluc were pretreated for 2 h with 30 μM of the GRK2/3 inhibitor cmpd101 or solvent (DMSO) and subsequently stimulated with either 114 nM CCL19 or 408 nM CCL21 (indicated by an arrowhead). NetBRET **(J)** corrected NetBRET **(K)** or AUC **(L)** are depicted. $n = 3$.

cells. As internal control we also determined β-arrestin2-Nluc recruitment to ACKR4-EYFP. Cells were stimulated with the three ACKR4 ligands at a concentration representing about 3-times the EC50 value of β-arrestin2 recruitment. To determine chemokine-mediated recruitment of signaling molecules, basal NetBRET values were subtracted for each condition and the area under the curve (AUC) for the first 3 min of stimulation were calculated as depicted in **Figure 3B**. A comprehensive analysis revealed that CCL19, CCL21, and CCL25 selectively and efficiently recruited GRK3 to ACKR4, whereas no interaction of ACKR4 with GRK4, GRK5, or GRK6 was observed (**Figure 3C**). Dose-response kinetic analysis revealed similar EC50 values for the recruitment of GRK3 (**Figure 3D**) by CCL19 (EC50∼42 nM), CCL21 (EC50∼142 nM), and CCL25 (EC50∼147 nM), as determined for the recruitment of β-arrestin2 by these ACKR4 agonists. Chemokine-mediated GRK3 recruitment to ACKR4 was fast, reaching its maximum within a minute (**Figure 3E**), and preceded the recruitment of β-arrestin1 and β-arrestin2 (**Figure 3F**). A less pronounced chemokine-mediated BRET signal was also observed between GRK2 and ACKR4 (**Figures 3C,H**). Subsequent dose-response kinetic analysis for GRK2 (**Figure 3G**) revealed EC50 values for CCL19 (EC50∼35 nM), CCL21 (EC50∼129 nM), and CCL25 (EC50∼87 nM) that are comparable to those for GRK3. The chemokine-mediated interaction between GRK2/3 and ACKR4 was not as transient as one could expect, which can be explained by the spontaneous trafficking of ACKR4 that continuously deliver receptor molecules to the plasma membrane that can interact with GRK2/3 over time and upon chemokine triggering. Notably, steady-state interaction of GRK2/3 with the tailless variant ACKR4t was abrogated and no chemokine-mediated recruitment of GRK2 or GRK3 to ACKR4t was observed (**Figure 3I**). To investigate the role of GRK2/3 in the recruitment of β-arrestin to ACKR4, we treated cells with cpmd101, a known GRK2/3 inhibitor (31). Treating cells with cpmd101 reduced both basal interaction of ACKR4 with β-arrestin2 (**Figure 3J**), as well as chemokine-mediated β-arrestin2 recruitment to the receptor (**Figures 3K,L**).

Notably, although cpmd101 treatment interfered with the recruitment of β-arrestin to ACKR4, the interaction was not completely abolished, suggesting that other kinase(s) contribute to potential ACKR4 phosphorylation and subsequent β-arrestin recruitment. To address this, we searched for putative serine/threonine phosphorylation sites of ACKR4. *In silico* studies using NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) and NetPhosK 1.0 server (www.cbs.dtu.dk/services/NetPhosK) predicted several putative phosphorylation sites or protein binding motifs for ACKR4 (**Figure 4A**). In order to validate these putative phosphorylation and kinase binding sites of ACKR4, we performed site-directed mutagenesis to exchange tyrosine residues for phenylalanine and serine/threonine residues for alanine and determined steady-state interaction and CCL19-driven recruitment of β-arrestin2 by BRET as depicted in **Figure 4B**. Whereas none of the tyrosine mutants affected steady-state interaction or stimulation-dependent recruitment of β-arrestin2 to ACKR4, a number of serine/threonine single point-mutants significantly reduced the interaction between β-arrestin2 and ACKR4 (**Figure 4C**). Most prominently, $ACKR4_{T142A}$ showed severely impaired steady-state interaction with β-arrestin2 without affecting the chemokine-driven β-arrestin2 recruitment. Importantly, none of these ACKR4 mutants showed significantly impaired surface expression or $CCL19^{Dy649P1}$ binding capabilities (**Figure 4D**). Additional sites affecting the steady-state interaction with β-arrestin2 include $ACKR4_{T226A}$, $ACKR4_{S309A}$, $ACKR4_{S323A}$, and $ACKR4_{S330A}$, which, together with $ACKR4_{T142A}$, are predicted as putative PKC phosphorylation sites (**Figures 4A,C**). Thus, we generated additional mutants, where the two threonine residues ($ACKR4_{T142T226}$), the three serine residues ($ACKR4_{S309S323S330}$) or the combination thereof ($ACKR4_{TTSSSmut}$) were replaced by alanines. Notably, steady-state interaction of $ACKR4_{TTSSSmut}$ with β-arrestin2 was profoundly reduced, whereas the other two mutants showed an intermediate phenotype (**Figure 4E**). Similarly, $ACKR4_{TTSSSmut}$ showed a significantly decreased ability to recruit β-arrestin2 upon CCL19 stimulation (**Figure 4E**), while retaining their surface expression and chemokine binding abilities (**Figure 4F**).

Taken together, these data demonstrate that chemokine triggering selectively recruits GRK3, and to a lesser extent GRK2, to ACKR4 and suggest that GRK2/3 and other serine/threonine kinases contribute to the recruitment of β-arrestins to the receptor. Although, mutating selected serine and threonine residues is not a direct proof that these residues are indeed phosphorylated by GRK2/3, our data provide evidence that these residues are critical for β-arrestin recruitment.

β-Arrestins Contribute to, but Are Dispensable for Chemokine Uptake

To assess the role of β-arrestins in steady-state trafficking and chemokine scavenging by ACKR4, we exploited wild-type HeLa (HeLa wt) and β-arrestin1/β-arrestin2-double deficient HeLa (HeLa KO) cells expressing mTurquoise2-tagged ACKR4

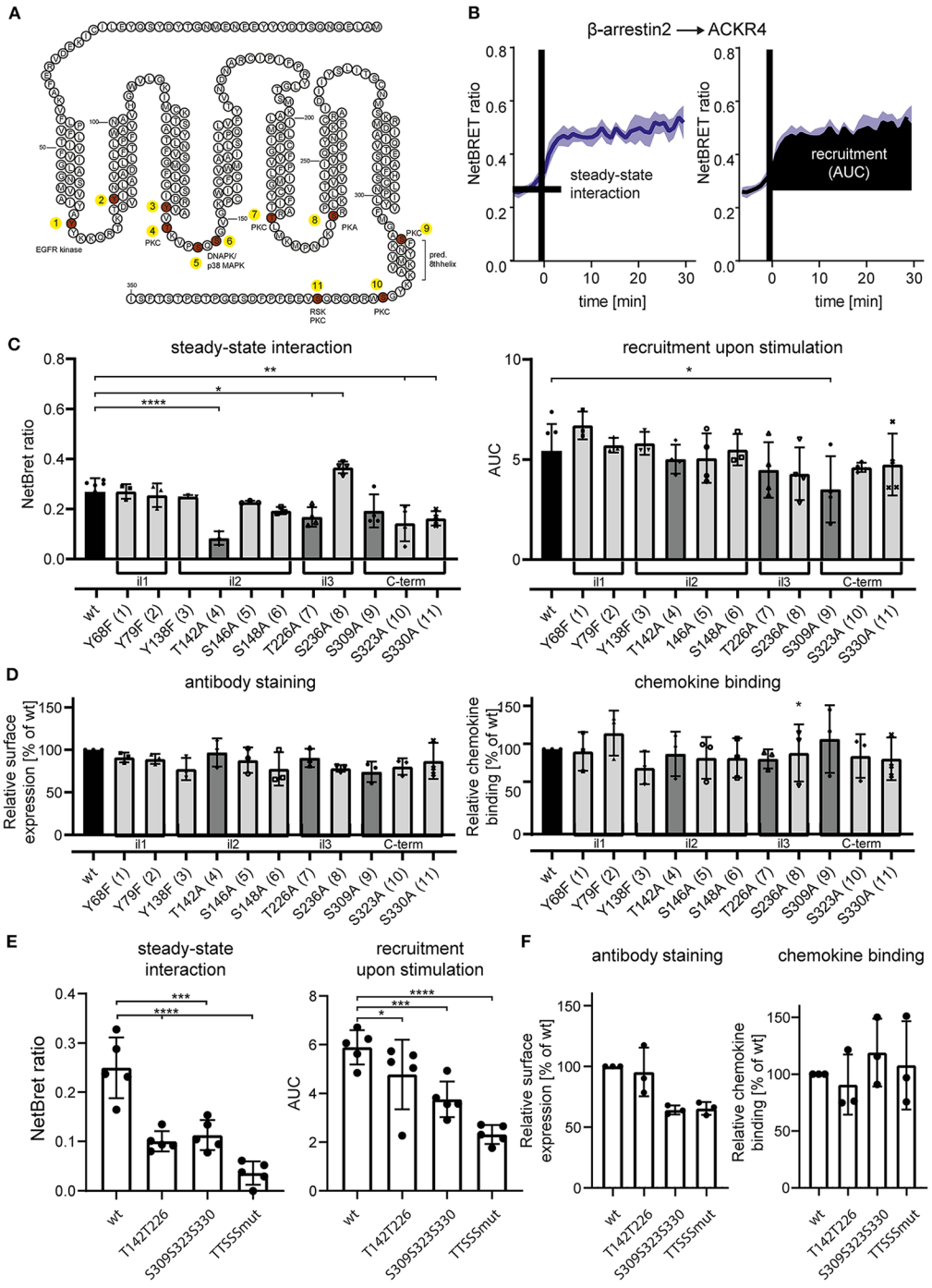

FIGURE 4 | Site-specific mutation analysis of ACKR4 for putative kinase interaction sites. **(A)** Schematic representation of ACKR4. *in silico* predicted phosphorylation sites are highlighted in red and putative kinase for site-specific tyrosine or serine/threonine phosphorylation are depicted. Numbers indicate individual mutant clones analyzed in **(C)**. **(B)** HeLa cells were transfected with ACKR4-EYFP and β-arrestin2-Nluc and stimulated with 1 μg/ml (114 nM) CCL19 and steady-state interaction before chemokine stimulation and chemokine-mediated recruitment determined by NetBRET of a representative experiment out of four is shown. **(C,E)** HeLa cells were transfected with mutants of ACKR4-EYFP together with β-arrestin2-Nluc and steady-state interaction **(C)**, as well as CCL19-mediated β-arrestin2 recruitment was determined as outlined in **(B)**. **(D,F)** HeLa cells were transfected with mutants of ACKR4-EYFP and surface expression using antibody staining or chemokine binding (25 nM $CCL19^{Dy649P1}$ at 8°C) was determined by flow cytometry. $n = 3–5$.

(ACKR4-mTq2). ACKR4-mTq2 showed the expected surface and mainly vesicular localization in HeLa wt cells (**Figure 5A**). By contrast, ACKR4-mTq2 predominantly associated with the plasma membrane in HeLa KO cells and was less present in vesicular structures (**Figure 5A**), similarly to ACKR4t-EYFP (**Figure 2F**). Reconstituting HeLa KO cells by

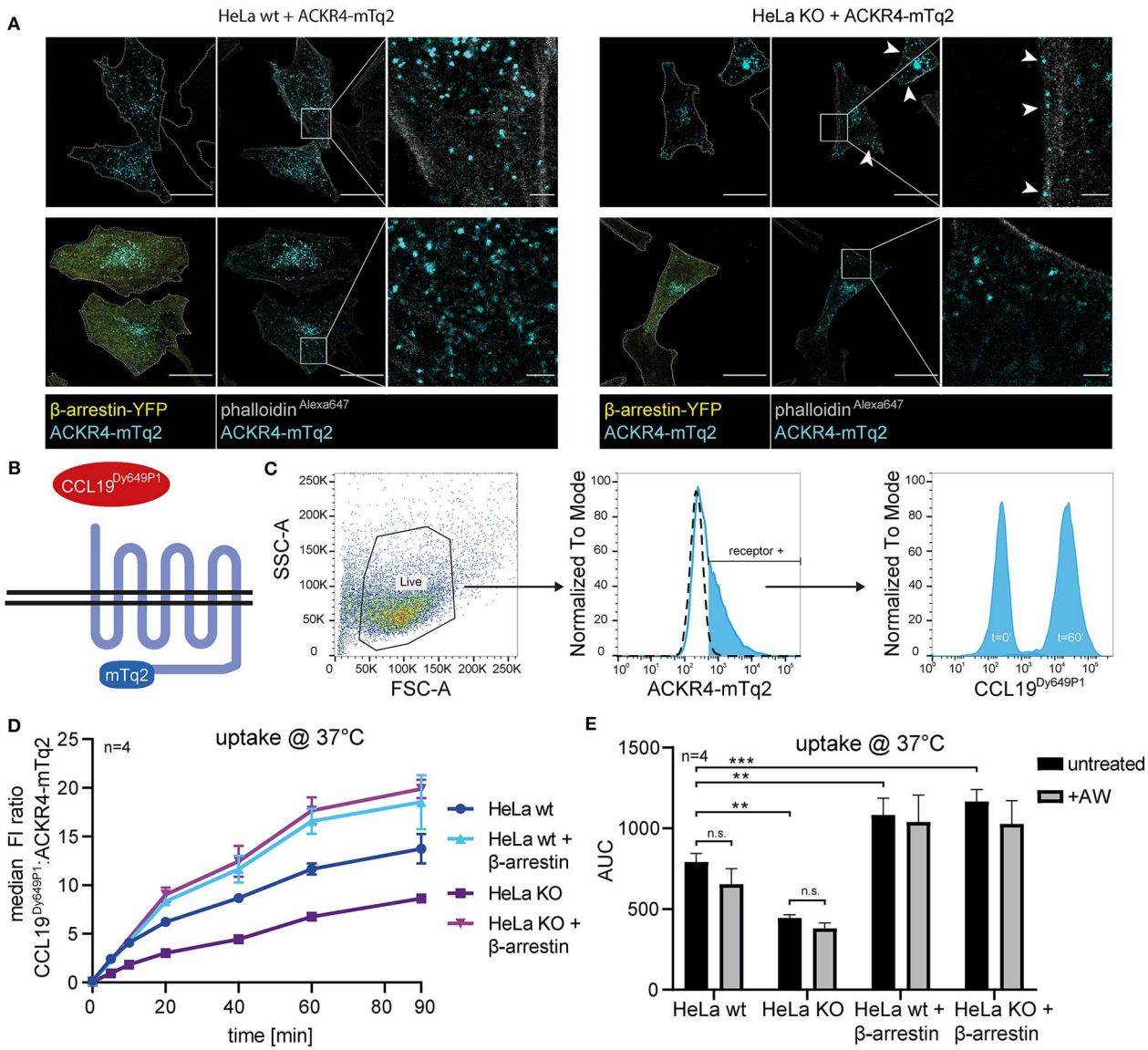

FIGURE 5 | β-arrestins contribute to, but are dispensable for CCL19 uptake. **(A)** Wild-type (wt) HeLa cells or β-arrestin1 and β-arrestin2 double gene targeted HeLa cells (KO) were co-transfected with ACKR4-mTq2 and β-arrestin2-YFP or empty vector, fixed, stained with phalloidin^{Alexa647} and analyzed by confocal microscopy. Cell shapes are marked with a dashed line (outer-left panels). Scale bar = 25 μm or 2.5 μm for zoomed images. **(B)** Scheme of CCL19^{Dy649P1} binding to mTq2-tagged ACKR4. **(C)** HeLa cells (ut, dashed line) were transfected with ACKR4-mTq2 (blue line) and incubated for 0 ($t = 0$) or 60 min ($t = 60$) at 37°C with 5 nM fluorescently labeled CCL19^{Dy649P1}. ACKR4-mTq2 expression and chemokine uptake (by receptor + cells) was determined by flow cytometry. One representative experiment including gating strategy is shown. **(D,E)** HeLa wt or HeLa KO cells over-expressing or not β-arrestin2 were transfected with ACKR4-mTq2 and incubated with 5 nM CCL19^{Dy649P1} for indicated times and chemokine uptake determined by flow cytometry. Mean values and SEM **(D)** or SD **(E)** are shown **(D)**. Cumulative chemokine uptake over time, as determined by the area under the curve (AUC) of the experiments shown in **(D)**. Where indicated, cells were exposed to a short acidic wash to remove surface bound, but not internalized CCL19^{Dy649P1}. $n = 4$.

reintroducing β-arrestin2-YFP, promoted the re-localization to predominantly vesicular and surface localization of ACKR4-mTq2 (**Figure 5A**), supporting the notion that β-arrestins control the steady-state trafficking of ACKR4. To asses ACKR4-mediated chemokine scavenging, we incubated transfected HeLa cells with fluorescently labeled CCL19^{Dy649P1} for various time points at 37°C (**Figures 5B,C**). CCL19^{Dy649P1} was steadily taken up over time by ACKR4 in HeLa wt cells (**Figure 5D**). Exposing cells to a short acidic wash hardly reduced chemokine-derived fluorescence, indicated that CCL19^{Dy649P1} was indeed internalized (**Figure 5E**). Remarkably, uptake of CCL19^{Dy649P1} was significantly reduced by roughly ∼40–50% in HeLa KO cells, but was not completely abolished (**Figures 5D,E**). Moreover, overexpression of β-arrestin2 in either HeLa wt or HeLa KO cells significantly enhanced CCL19^{Dy649P1} uptake (**Figures 5D,E**).

In summary, we show that β-arrestins interact with ACKR4 in the steady-state and contribute to the spontaneous trafficking

of the receptor. Furthermore, we demonstrate that β-arrestins enhance the scavenging activity of ACKR4, but are dispensable for chemokine uptake.

DISCUSSION

ACKR4 plays an important role in the regulation of immune cell migration by shaping local chemokine gradients (20, 21). The molecular mechanism how ACKR4 scavenges its cognate ligands remains poorly understood. Initially, biotinylated CCL19 was detected in vesicular structures of ACKR4 transfected MEFs derived from β-arrestin1/β-arrestin2 double-deficient mice, suggesting that chemokine uptake by ACKR4 is not critically dependent on β-arrestins (16). By contrast, CCL19 stimulation of an ACKR4 transfected osteosarcoma cell line was shown to result in the translocation of β-arrestin2-GFP to vesicular structures (26). In addition, chemokine stimulation recruited β-arrestin1 and β-arrestin2 to ACKR4 using slit-galactosidase and BRET assays in CHO cell transfectants (26). This later study is in line with the common concept of a β-arrestin-dependent receptor trafficking route taken by class A GPCRs (27). In the present study we show that CCL19, CCL21, and CCL25 effectively recruit β-arrestin1 and β-arrestin2 to engaged ACKR4, which confirms the study by Watts and colleagues (26). In addition to that study, we found that β-arrestin already interacts with ACKR4 prior to chemokine stimulation and that this steady-state interaction occurs at vesicular structures. Notably, an ACKR4 mutant lacking its C-terminus not only failed to interact with and recruit β-arrestins, it also lost its vesicular localization and showed an impaired capacity to take up chemokines. Interestingly, a C-terminally truncated variant of ACKR2 also fails to recruit β-arrestins, but was still able to scavenge chemokines (29), whereas a C-terminal deletion variant of ACKR3 (31, 53) showed a similar absence of chemokine scavenging behavior as ACKR4t. Together with the finding that overexpression of β-arrestin2 enhanced chemokine uptake, our data indicate that β-arrestins control steady-state trafficking of ACKR4 and contributes to an enhanced chemokine scavenging activity. However, we also provide experimental evidence that β-arrestins are dispensable for chemokine uptake by ACKR4, as β-arrestin1/β-arrestin2-double deficient HeLa cells are still able to internalize chemokines although less efficient that wild-type cells. Notably, CCL19 uptake by ACKR4 was shown to be partially reduced in HEK293 cells treated with methyl-β-cyclodextrin to deplete cholesterol or in cells overexpressing caveolin-1 or a dominant-negative form of dynamin, but not in cells overexpressing a dominant-negative form of Eps15 or Rab5 (16). These data conjointly suggest, that ACKR4 and likely other ACKRs utilize additional routes of endocytosis compared with canonical chemokine receptors.

Due to the lack of canonical G protein-dependent signaling, ACKRs were initially considered to be silent receptors. More recently, ACKR3 was described to execute a signaling bias toward β-arrestins leading to MAP kinase activation (24), an alternative signaling pathway for canonical class A GPCRs (27). β-arrestin signaling usually relies on GPCR kinase recruitment and subsequent receptor phosphorylation. Consistent with this concept, GRK2 (and partially GRK5) recruitment was shown to induce ACKR3 phosphorylation upon chemokine stimulation (31). Here, we identified that GRK3 and GRK2, but no other GRK, are selectively recruited to chemokine engaged ACKR4 and that GRK2/3 recruitment precedes the recruitment of β-arrestins, pointing to a remarkable specificity of GRKs for different ACKRs. Inhibiting GRK2/3 by cpmd101 partially, but significantly reduced steady-state interaction as well as chemokine-driven recruitment of β-arrestins to ACKR4. However, we did not find any experimental evidence for a β-arrestin-dependent or independent phosphorylation of Erk1/2 and Akt upon chemokine triggering of ACKR4. Our data are thus in line with a previous study on ACKR4 showing no Erk1/2 activation (26) and one on the adrenergic receptor showing that β-arrestins are dispensable for Erk1/2 phosphorylation (54).

In conclusion, it emerges that distinct GRKs are recruited to ACKRs (GRK2/5 for ACKR3; GRK2/3 for ACKR4) upon ligand stimulation, which phosphorylate C-terminal serine/threonine residues of the receptor (31) and thereby recruit β-arrestins. We herein further provide evidence that β-arrestins control steady-state trafficking of ACKR4 and promote chemokine uptake. However, it is becoming clear that β-arrestins are dispensable for chemokine scavenging by ACKR2 (29), ACKR3 (30, 31) and ACKR4 (16). The fact that GRKs are recruited to and phosphorylate the receptors strongly indicates that ACKRs are not silent receptors, but are able to elicit alternative, yet unknown signaling pathways. This is most convincingly supported by the fact that mice lacking ACKR3 die at birth with ventricular septal defects and semilunar heart valve malformation (55), while mice expressing a chemokine scavenging deficient ACKR3 are vital (31).

DATA AVAILABILITY STATEMENT

Datasets for this study are deposited on Zenodo and are publicly available under a Creative Commons Attribution 4.0 International license, doi: 10.5281/zenodo.3631895.

AUTHOR CONTRIBUTIONS

CM and DL designed the studies and wrote the manuscript. CM, AS, MA, and IK performed the experiments. GD'A and MU contributed HeLa KO cells. CM, AS, MA, MT, and DL analyzed the data. DL supervised the overall study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00720/full#supplementary-material

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Serum Amyloid A1 (SAA1) Revisited: Restricted Leukocyte-Activating Properties of Homogeneous SAA1

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Infection, sterile injury, and chronic inflammation trigger the acute phase response in order to re-establish homeostasis. This response includes production of positive acute phase proteins in the liver, such as members of the serum amyloid A (SAA) family. In humans the major acute phase SAAs comprise a group of closely related variants of SAA1 and SAA2. SAA1 was proven to be chemotactic for several leukocyte subtypes through activation of the G protein-coupled receptor FPRL1/FPR2. Several other biological activities of SAA1, such as cytokine induction, reported to be mediated via TLRs, have been debated recently. Especially commercial SAA1, recombinantly produced in *Escherichia coli*, was found to be contaminated with bacterial products confounding biological assays performed with this rSAA1. We purified rSAA1 by RP-HPLC to homogeneity, removing contaminants such as lipopolysaccharides, lipoproteins and formylated peptides, and re-assessed several biological activities attributed to SAA1 (chemotaxis, cytokine induction, MMP-9 release, ROS generation, and macrophage differentiation). The homogeneous rSAA1 (hrSAA1) lacked most cell-activating properties, but its leukocyte-recruiting capacity *in vivo* and it's *in vitro* synergy with other leukocyte attractants remained preserved. Furthermore, hrSAA1 maintained the ability to promote monocyte survival. This indicates that pure hrSAA1 retains its potential to activate FPR2, whereas TLR-mediated effects seem to be related to traces of bacterial TLR ligands in the *E. coli*-produced human rSAA1.

Keywords: SAA, neutrophils, FPR2, chemotaxis, chemokines, ROS, MMP-9, macrophages

INTRODUCTION

The serum amyloid A (SAA) proteins form a family that is highly conserved in a wide number of species ranging from fish to humans (1). The remarkable conservation of SAA throughout evolution points toward a rather important biological role. Humans have four distinct *SAA* genes giving rise to SAA1, SAA2, SAA3, and SAA4. SAA1 and SAA2 are upregulated during the acute phase response and are hence referred to as acute-SAA (A-SAA) (2). Expression of A-SAA primarily occurs in the liver in response to inflammatory cytokines such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor–α (TNF-α) (2). Under inflammatory conditions, SAA1 plasma levels rise

exponentially (3). In contrast to mouse, human *SAA3* has long been considered a pseudogene, a fact that was recently debated (4). The role of SAA4, which is constitutively expressed, has been scarcely studied.

A considerable amount of literature has been published on the numerous biological activities attributed to SAA1, most of which are of a pro-inflammatory nature. SAA1 has been reported to upregulate the expression of various inflammatory mediators such as cell adhesion molecules, cytokines, chemokines, matrix-degrading proteases, reactive oxygen species (ROS) and pro-angiogenic molecules in several cell types including leukocytes, fibroblasts, and endothelial cells (3, 5–9). In addition, SAA1 has been described to induce the recruitment of different cell types including various leukocyte subsets (6, 10–12). Furthermore, this acute phase protein has been suggested to possess antimicrobial activity (13–16). Interestingly, SAA has been indicated as a pleiotropic molecule owing to its capacity to also induce anti-inflammatory effects (17–20).

As a multifunctional protein, SAA1 has been reported to activate various receptors. The majority of SAA1 functions has been linked to toll-like receptors (TLRs) 2 and 4 (6, 21, 22). Nevertheless, SAA1 has also been described as a ligand for additional receptors. For instance, the G protein-coupled receptor, formyl peptide receptor 2 (FPR2) has been shown to relay the direct chemotactic signal of SAA1 on FPR2-transfected HEK293 cells, neutrophils and macrophages (23–25). In addition, SAA1 was shown to synergize with CXCL8 to enhance neutrophil recruitment through activation of FPR2 (6, 26). Although the capacity to synergize with CXCL8 is retained in the C-terminal fragment of SAA1, SAA1(58–104), generated by matrix metalloproteinase-9 (MMP-9), its direct chemotactic activity is lost as a result of proteolysis by MMP-9 (27). Furthermore, SAA1 has been linked to scavenger receptor class B type I (SR-BI), receptor for advanced glycation end products (RAGE) and the purinergic receptor P2X7 (28–31).

Burgess *et al*. recently demonstrated that the TLR2-activating capacity of SAA1 recombinantly expressed in *Escherichia coli* (*E.coli*) is in fact due to contaminating bacterial lipoproteins (32). The analysis of such commercially available recombinant SAA1 (rSAA1) revealed the presence of multiple bacterial proteins, some of which are probable bacterial lipoproteins. Treatment of SAA1 from a bacterial source with lipoprotein lipase provoked a dose-dependent decline in the cytokine-inducing capacity of rSAA1 in peripheral blood mononuclear cells (PBMCs) and neutrophils. In line with this, SAA1 expressed in mammalian HEK293T cells did not induce inflammatory cytokine expression. Furthermore, rSAA1 from a bacterial source induced Th17 polarization whereas HEK293T-expressed SAA1 displayed no effect on these cells (32). The majority of studies performed have utilized rSAA1 that has been expressed in bacteria. Thus, it is currently unclear which functions are intrinsic to SAA1 and which are due to contaminating bacterial products. The main purpose of this study is to provide a correct understanding of the biological activities that are inherent to SAA1. Therefore, we purified commercially available rSAA1, expressed in *E. coli*, to homogeneity using reversed phase-high performance liquid chromatography (RP-HPLC). Following purification, we carried out multiple biological assays to investigate the role of homogenous rSAA1 (hrSAA1) in leukocyte survival, activation and migration, chemokine and MMP-9 induction, ROS expression and macrophage polarization. We conclude that all FPR2-mediated effects of hrSAA1 remain intact, whereas the TLR-related activities are absent.

MATERIALS AND METHODS

Reagents

Recombinant human SAA1 (rSAA1) (300–353), CXCL8 (200–208M), IL-4 (200–204) and M-CSF (300–325) were purchased from Peprotech (Rocky Hill, NJ, United States). Lipopolysaccharide (LPS) derived from *E.coli* (0111:B4) and lipoprotein lipase (LPL) derived from *Pseudomonas* species (62335) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Pam3CSK4 (11B07-MM) was purchased from InvivoGen (San Diego, CA, United States). IFN-γ (285-IF) was obtained from R&D Systems (Minneapolis, MN, United States). The FPR2 agonist MMK-1 (33) was chemically synthesized based on N-9-(fluorenyl) methoxy-carbonyl (Fmoc) chemistry using an Activo-P11 automated solid-phase peptide synthesizer (Activotec, Cambridge, United Kingdom). The peptide was purified by C18 RP-HPLC and purity was confirmed on an Amazon-SL ion trap mass spectrometer (Bruker Daltonics; Bremen, Germany). The selective FPR2 antagonist WRW_4 was purchased from Calbiochem (San Diego, CA, United States).

Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) and Mass Spectrometry (MS)

To purify rSAA1, RP-HPLC (Higgins Analytical, Inc, Mountain View, CA, United States) coupled to MS was utilized. rSAA1 was purified on a C8 Aquapore RP-300 HPLC column (220 × 2.1 mm; PerkinElmer, Norwalk, CT, United States). The loading solvent consisted of 0.1% trifluoroacetic acid (TFA) in ultra-pure water. After loading rSAA1 onto the column, elution was achieved by a gradually increasing acetonitrile (ACN) gradient. UV absorbance was measured at 214 nm reflecting protein concentration. Following chromatographic separation, fractions containing rSAA1 were analyzed by ion trap MS. Fractions containing highly pure rSAA1 were pooled, underwent lyophilization and were reconstituted with PBS [supplemented with 1 mg/ml of human serum albumin (HSA; Belgian Red Cross, Brussels, Belgium)]. RP-HPLC-purified rSAA1 will be referred to as homogenous rSAA1 (hrSAA1) from here onward. To avoid that the activity of purified hrSAA1 would depend on batch to batch differences, the same preparation of hrSAA1 was used in chemokine and MMP-9 induction experiments, ROS production, macrophage polarization, monocyte survival, *in vitro* and *in vivo* chemotaxis, and shape change assays (*vide infra*).

Detection of LPS and LPL Treatment

The limulus amebocyte lysate (LAL) assay was utilized to determine the endotoxin level in rSAA1 preparations before

and after RP-HPLC purification. The endotoxin level in rSAA1 was measured at 2.90 EU per mg of rSAA1. Following RP-HPLC, the endotoxin level in hrSAA1 was <0.15 EU per mg of hrSAA1. The LAL assay was carried out using a specific kit as per the manufacturer's instructions (BioMérieux; Marcy-l'Étoile, France). To deactivate bacterial lipoproteins in the rSAA1 preparation, rSAA1 was pre-incubated with LPL for a period of 4 h at 37°C in $CD14^+$ monocyte culture medium prior to cell stimulation.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

hrSAA1 and rSAA1 were diluted in reducing loading buffer (10% beta mercaptoethanol, 0.02% bromophenol blue, 8% SDS, 40% glycerol in 0.25 M tris pH 6.8) and heated at 95°C for 5 min. Afterward, the samples were loaded onto a precast 16% tris-glycine gel (Invitrogen, Carlsbad, CA, United States) and run at 200 V in running buffer (192 mM glycine, 0.1% SDS in 25 mM tris pH 8.6). Following electrophoresis, the gel was stained using a silver stain kit as per the manufacturer's instructions (Invitrogen).

Signal Transduction Assay

FPR1- and FPR2-transfected HEK293 cells were kindly provided by Prof. J.M. Wang (NCI, Frederick, United States). Changes in intracellular calcium concentration were measured by fluorescence spectrometry as previously described (34). In brief, HEK293 cells were loaded with the ratiometric fluorescent dye Fura-2/AM (Invitrogen) and incubated for a period of 0.5 h at 37°C. Afterward, the cells were washed and resuspended in HBSS containing 1 mM Ca^{2+} and Mg^{2+} (Gibco, Thermo Fischer Scientific, Waltham, MA, United States), 0.1% fetal calf serum (FCS, Invitrogen), 10 mM HEPES and pH 7.4, at a concentration of 1.5×10^6 cells/ml. Fura-2 fluorescence was measured at 510 nm upon excitation at 340 and 380 nm.

Human Monocyte Isolation and Activation

Human $CD14^+$ monocytes were isolated from 1-day-old buffy coats, obtained from healthy donors (Belgian Red Cross, Mechelen, Belgium), via density gradient centrifugation and positive selection (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (26). $CD14^+$ monocytes were seeded in 48-well plates (2×10^6 cells/ml, 450 µl/well) in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 1 mg/ml HSA. Monocytes were stimulated for 24 h at 37°C and 5% CO_2. Cell supernatants were collected and stored at −20°C until chemokine and MMP-9 quantification.

Human Neutrophilic Granulocytes Isolation and Activation, Migration, and Cell Shape Assays

Human neutrophils were isolated from fresh blood, obtained from healthy donors via density gradient centrifugation as previously described (6). Neutrophil migration was determined in a 48-well Boyden microchamber assay (Neuro Probe, Gaithersburg, MD, United States). Chemoattractants were added in triplicate to the lower wells of the microchamber. Boyden buffer (HBSS supplemented with 1 mg/ml of HSA) served as negative control. Neutrophils (1×10^6 cells/ml, 50 µl/well) diluted in Boyden buffer were added to the upper compartment, which was separated from the lower compartment using a polyvinylpyrrolidone-free membrane (5 µm pore size; GE water & process Technologies, Manchester, United Kingdom). After an incubation period of 45 min at 37°C and 5% CO_2, the membrane was fixed and stained with Hemacolor solutions (Merck, Darmstadt, Germany). The migrated cells adhering to the bottom surface of the membrane were counted microscopically in 10 high power fields/well. The chemotactic potency was expressed in terms of the chemotactic index (CI). The CI was calculated by dividing the average number of migrated cells in response to chemoattractants by the average number of spontaneously migrated cells.

Shape change assays were carried out to determine the morphological changes that occur when neutrophils are stimulated with chemoattractants in suspension. Different concentrations of inducers (50 µl) were added to a flat-bottomed 96-well plate. The stimuli and neutrophils were diluted in pre-warmed (37°C) shape change buffer (HBSS, supplemented with 10 mM HEPES), which also served as the negative control. Neutrophils (50 µl/well) were then added to the plate at a concentration of 0.6×10^6 cells/ml. Following 3 min of stimulation, neutrophils were fixed with 100 µl of 4% formaldehyde in shape change buffer. One hundred cells per condition were counted microscopically and categorized as either active (blebbed and elongated cells) or resting/not activated (round). Synergy was defined as a response to the combination of two chemoattractants that exceeded the sum of the responses obtained for the individual chemoattractants.

CXCL8 and CCL3 Enzyme-Linked Immunosorbent Assay (ELISA)

Quantification of CXCL8 in monocyte supernatants was done by ELISA. The human CXCL8 ELISA was developed in our laboratory using monoclonal mouse anti-human CXCL8 (MAB208) and polyclonal goat anti-human CXCL8 (BAF208) antibodies from R&D Systems (6). Human CCL3 was measured with a specific ELISA Duoset kit as per the manufacturer's instructions (R&D Systems).

MMP-9 Zymography

MMP-9 release by activated $CD14^+$ monocytes was quantified as described by Vandooren *et al.* (35). Monocyte cell supernatants, diluted in a non-reducing loading buffer (0.02% bromophenol blue, 8% SDS, 40% glycerol in 0.25 M tris pH 6.8), were loaded onto 7.5% polyacrylamide gels containing 0.1% gelatin and run at 25 mAmp. Following electrophoresis, gels were washed with 2.5% triton-X 100 in ultrapure water. Afterward, the gels were incubated overnight in incubation buffer (10 mM $CaCl_2$ in 50 mM tris pH 7.4) for the development of enzyme activity. The gels were then stained with InstantBlue™ protein stain (Expedeon, Heidelberg, Germany) as per the manufacturer's instructions and destained for 1 h in destaining solution

(30% methanol and 10% acetic acid in ultrapure water). MMP-9 gelatinase activity was observed as unstained bands on a blue background. The obtained signals were quantified by computerized image analysis using ImageJ software.

Cellular ROS Assay

$CD14^+$ monocytes (2×10^6 cell/ml, 200 µl) were incubated with stimuli (100 µl) in pre-warmed RPMI-1640 medium (Lonza), supplemented with 2% FCS for a period of 1 h at 37°C and 5% CO_2. ROS generation was detected using 50 µM (in 100 µl) of 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) which was added for 20 min at 37°C following cell stimulation. The cells were placed on ice for 10 min to stop the reaction and subsequently washed with HBSS buffer. Afterward, the cells were fixed using 0.8% formaldehyde. Fluorescence intensity was measured using an LSRFortessa X-20 cell analyzer (BD Biosciences, Heidelberg, Germany) at 488 nm. Data were analyzed using FlowJo software (Tree Star, Ashland, OR, United States).

Macrophage Differentiation

$CD14^+$ monocytes were suspended at a concentration of 2×10^6 cells/ml in RPMI1640 medium, supplemented with 10% FCS and 50 µg/ml gentamycin, and cultured in 6-well plates (2 ml/well). To induce the differentiation of human monocytes into macrophages, M-CSF (100 ng/ml) was added on day 0. The stimuli were added on day 4. On day 6 of culture, macrophages were collected from the 6-well plates and processed for analysis of surface molecules via flow cytometry (*vide supra*). To exclude dead cells from the analysis, cells were incubated in Zombie Aqua viability dye (BioLegend, San Diego, CA, United States) for 15 min at room temperature. To block the Fc receptors, the cells were washed and incubated for 10 min at 4°C with FACS buffer (PBS + 2% FCS + 1 mM EDTA). Afterward, the cells were stained with the mouse anti-human antibodies mentioned hereafter (0.5 h at 4°C) and analyzed by flow cytometry (*vide supra*). Fluorescein Isothiocyanate (FITC)-labeled anti-TLR4 antibody (clone HTA125) was obtained from InvivoGen. FITC-labeled anti-HLA-DR antibody (clone LN3) and phycoerythrin (PE)-labeled anti-CD163 antibody (clone GHI/61) were obtained from eBioscience (San Diego, CA, United States). PE-labeled anti-IL-1RI (clone 150503) and anti-CCR7 (catalog number FAB269P) antibodies were obtained from R&D Systems. FITC-labeled anti-DC-SIGN (clone DCN46), PE-labeled anti-CD80 (clone L307.4), PE-labeled anti-CD86 (clone 2331), Brilliant Violet 421 (BV421)-labeled anti-TLR2 (clone 1167) and allophycocyanin (APC)-labeled anti-CD14 (clone M5E2) antibodies were obtained from BD Biosciences. The degree of polarization is expressed as percentage change of marker expression compared to control M-CSF-differentiated macrophages.

Monocyte Survival

Peripheral blood mononuclear cells were suspended in RPMI1640 supplemented with 1% FCS and seeded at a concentration of 1.5×10^6 cells/ml (100 µl/well) in a 96-well plate. Monocytes were allowed to adhere for 2 h at 37°C and then washed with PBS. Following a stimulation period of 24 h, monocyte survival was assessed using the ATPlite™ luminescence assay system kit as per the manufacturer's instructions (PerkinElmer). Results are expressed as percent of survival in comparison to buffer-stimulated monocytes (100%).

Actin Polymerization

To monitor changes in the cytoskeleton in response to stimuli, neutrophils were seeded in a U bottom 96-well plate at 1.5×10^6 cells/ml (70 µl/well) in pre-warmed (37°C) RPMI1640 + 0.5% HSA. After stimulation for 30 sec, cells were placed on ice, fixed and permeabilized using the BD Cytofix/Cytoperm™ kit (BD Biosciences) as per the manufacturer's instructions. Subsequently, cells were incubated (20 min on ice) with Alexa Fluor™ 555 Phalloidin (20 U/ml, Invitrogen), a dye which selectively stains F-actin. After washing, the cellular F-actin content was quantified by flow cytometry (*vide supra*). Results were expressed as relative mean fluorescence intensity (MFI), i.e. compared to the MFI of buffer-stimulated cells (100%).

Intra-Articular (i.a.) Knee Injections

The *in vivo* neutrophil chemotactic potential of hrSAA1 was determined in C57BL/6J male mice (Centro de Bioterismo of the Universidade Federal de Minas Gerais) via i.a. injections. The mice were first anesthetized through intraperitoneal (i.p.) injection of a mixture of 3.75% (w/v) of ketamine (Syntec, Santano de Parnaíba, Brazil) and 0.25% (w/v) of xylazine (Syntec) diluted in PBS. Afterward, the mice were injected i.a. with 10 µl of stimulus in one joint and, as a control, the other joint was injected with 10 µl of 0.9% sodium chloride. After 3 h, mice were sacrificed by a subcutaneous injection of a ketamine/xylazine overdose. Cells from the joint were collected and cytospins were prepared for differential cell counts. After drying, cells on the glass slides were stained with Panoptic solutions (Laborclin, PR, Brazil). The slides were microscopically counted (500× magnification) independently by 2 individuals. All procedures were approved by the animal ethics committee of the Federal University of Minas Gerais (295/2018).

Statistical Analysis

The data were first analyzed using the Kruskal–Wallis test for comparison of multiple groups. Afterward, the Mann-Whitney U-test or the Wilcoxon signed-rank test were utilized to perform pairwise comparisons. Statistical analysis was performed using GraphPad software (GraphPad Software Inc. La Jolla, United States). Unless indicated otherwise, results are expressed as mean + SEM. An alpha of 0.05 was used as the cutoff for significance.

RESULTS

Purification of rSAA1 to Homogeneity

To further eliminate bacterial components, such as lipoproteins, lipopolysaccharides, and formyl peptides from commercial rSAA1 expressed in *E.coli*, we performed RP-HPLC. rSAA1 eluted from the C8 column between 50.0% and 58.0% ACN in 0.1% TFA.

Following RP-HPLC purification, mass spectrometry was used to assess the purity and confirm the presence of homogeneous (h) rSAA1. **Figure 1A** shows the mass spectrum obtained following rSAA1 purification with ions containing 10–18 charges. The insert in the figure shows the deconvoluted relative molecular mass of hrSAA1 (11813.68) which aligns with the theoretical relative molecular weight of rSAA1 (11813.78). To further assess the purity of the preparation, hrSAA1 was analyzed by SDS-PAGE, which displayed no major contaminants (**Figure 1B**). In order to rule out the possibility that exposure to harsh solvents (50% ACN in 0.1% TFA) would alter the biological activity of hrSAA1, rSAA1 was incubated in 50% ACN in 0.1% TFA for a period of 1 h followed by lyophilization and reconstitution. Solvent-treated rSAA1 (ST rSAA1) retained biological function, including neutrophil chemotactic activity (**Figure 2A**). In addition, ST-rSAA1 induced calcium mobilization in FPR2-transfected HEK293 cells (**Figure 2B**) but did not significantly desensitize the calcium response induced by the FPR2 agonist WKYMVm (**Figure 2C**). Furthermore, ST rSAA1 retained the chemokine-inductive capacity in $CD14^+$ monocytes (data not shown). hrSAA1 was then utilized in multiple biological assays in parallel to rSAA1.

Absence of Formyl Peptide Contamination and Separation of Bacterial Lipoprotein From rSAA1 by RP-HPLC

Contamination with formyl peptides was excluded, as neither rSAA1 (**Figure 2D**), nor hrSAA1 (data not shown) induced calcium signaling in FPR1-transfected HEK293 cells. Furthermore, rSAA1 failed to desensitize calcium signaling in response to fMLF in FPR1-transfected cells, thus further confirming the lack of formyl peptides in the preparation (**Figures 2D,E**). Currently, there exists no method to detect and quantify low levels of contaminating bacterial lipoproteins. Therefore, to confirm that bacterial lipoproteins present in rSAA1 do not co-elute with rSAA1 during RP-HPLC purification, Pam3CSK4, a synthetic bacterial lipoprotein variant, was loaded onto a C8 Aquapore RP-300 HPLC column. Elution was carried out in a similar manner to that of rSAA1. The elution of Pam3CSK4 was achieved at a solvent composition of 76.6–100.0% ACN in 0.1% TFA. As previously mentioned, rSAA1 eluted at a solvent composition of 50.0–58.0% ACN in 0.1% TFA, indicating that rSAA1 does not co-elute with contaminating bacterial lipoproteins under the described conditions of RP-HPLC (data not shown).

hrSAA1 Fails to Induce Chemokine Production in $CD14^+$ Monocytes

rSAA1 has been reported to induce the expression of chemokines including CCL2, CCL3, and CXCL8 in monocytes and dendritic cells (11). As such, monocytes were stimulated with hrSAA1 in parallel to rSAA1 to determine whether rSAA1 retains the capacity to induce chemokines following purification. Following a stimulation period of 24 h, CCL3 and CXCL8 levels were determined in the cell supernatants. In comparison to rSAA1 (CCL3 4.6 ± 0.6 ng/ml and CXCL8 79.9 ± 3.4 ng/ml), hrSAA1 did not induce significant CCL3 nor CXCL8 expression in monocytes at 100 ng/ml (CCL3 was undetectable and CXCL8

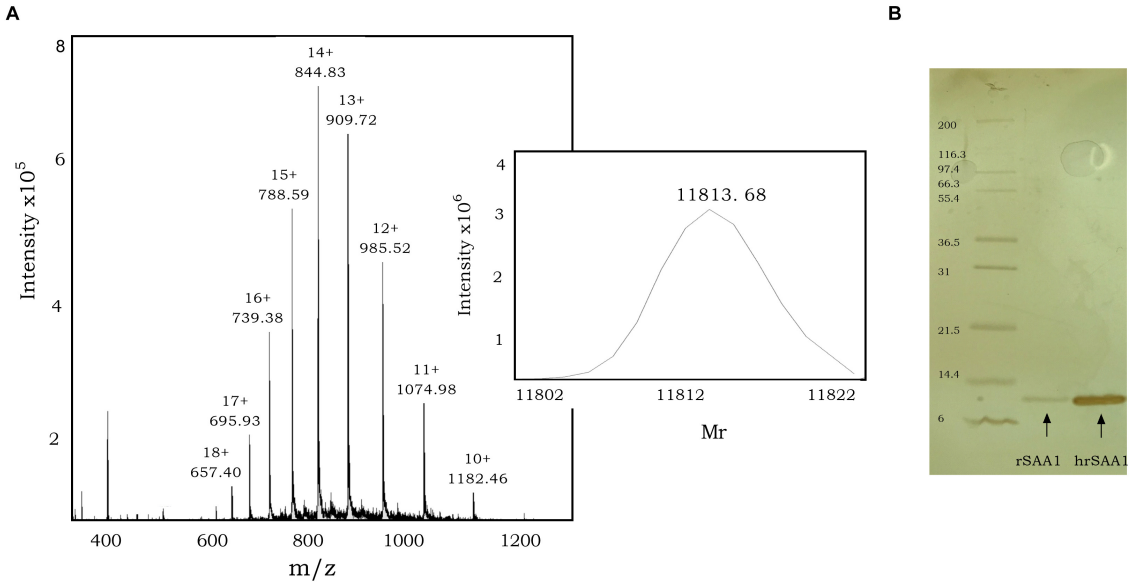

FIGURE 1 | Purification of rSAA1 using RP-HPLC to homogeneity. **(A)** Relative molecular mass determination of RP-HPLC-purified homogenous rSAA1 (hrSAA1) by mass spectrometry. The averaged mass spectrum of the pooled hrSAA1 fractions is shown with the ion intensities, the number of charges and the corresponding mass over charge ratio (m/z) for multiple charged ions. The experimentally determined deconvoluted mass spectrum of uncharged hrSAA1, as calculated by the Bruker deconvolution software, is shown as an insert at the right of the mass spectrum. **(B)** Homogenous rSAA1 (hrSAA1; 200 ng) was analyzed in parallel to rSAA1 (20 ng) using SDS–PAGE and silver staining. The molecular mass of the standard marker proteins is indicated in kilodalton.

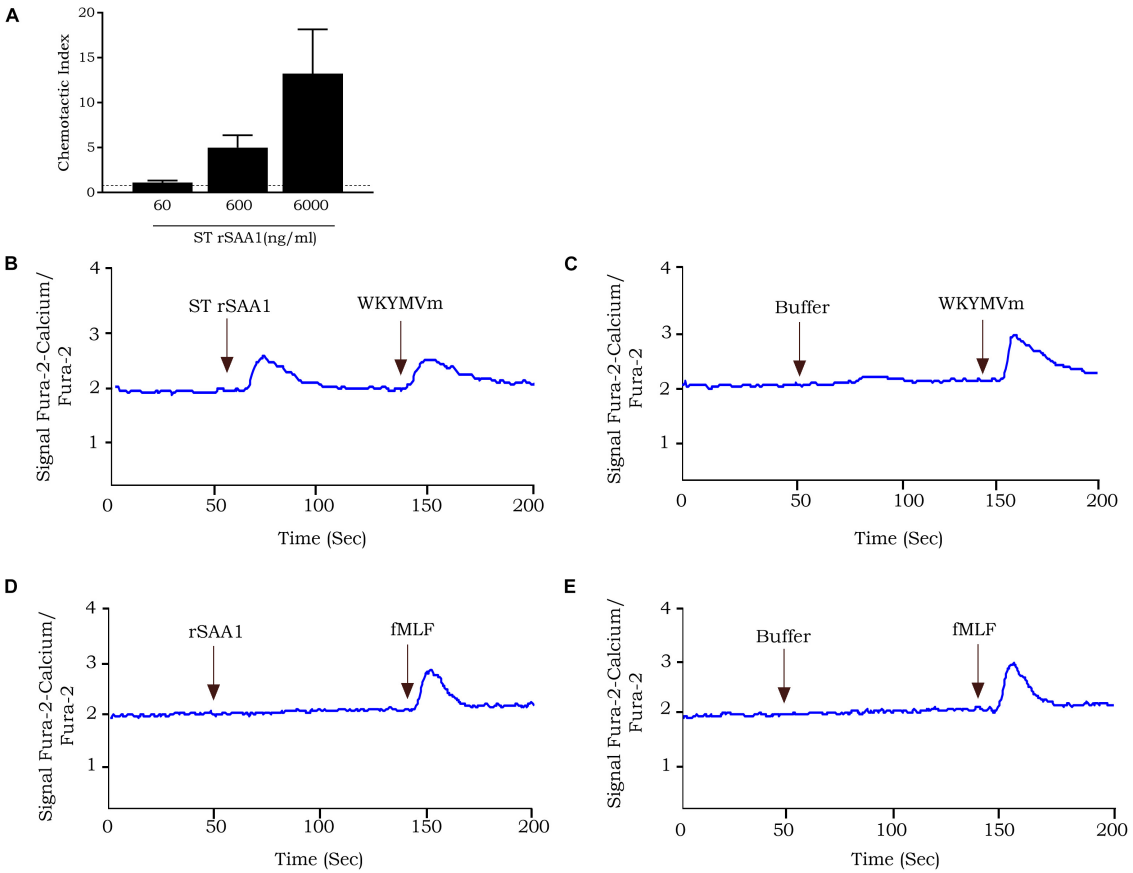

FIGURE 2 | rSAA1 treated with RP-HPLC elution solvents retains its biological activity. **(A)** The chemotactic activity of rSAA1 treated with 50% acetonitrile and 0.1% trifluoroacetic acid (ST rSAA1) was evaluated on human neutrophils in the Boyden microchamber assay. The chemotactic potencies are expressed as mean chemotactic index + SEM derived from three independent experiments. Control migration is indicated by a dashed line (---). **(B)** FPR2-transfected HEK293 cells were stimulated with rSAA1 (6000 ng/ml, 500 nM) treated with 50% acetonitrile and 0.1% trifluoroacetic acid. Afterward, the cells were stimulated with WKYMVm (10 ng/ml, 12 nM) as control. **(C)** FPR2-transfected HEK293 cells were stimulated with WKYMVm (10 ng/ml, 12 nM). **(D)** FPR1-transfected HEK293 cells were stimulated with rSAA1 (6000 ng/ml, 500 nM). Afterward, the cells were stimulated with fMLF (10^{-10} M) as control. **(E)** FPR1-transfected HEK293 cells were stimulated with fMLF (10^{-10} M). **(B–E)** Changes in intracellular calcium levels were monitored by spectrophotometry. Results are presented as the ratio of emission of calcium-bound fura over calcium-free fura. One representative experiment out of two independent experiments is shown.

2.3 ± 1.2 ng/ml) (**Figures 3A,C**). Furthermore, as shown in **Figures 3B,D**, treatment of rSAA1 (100 ng/ml) with LPL (2000 ng/ml) reduced CCL3 and CXCL8 expression in monocytes by 62.3 (*p*-value 0.0286) and 26.2% (*p*-value 0.0286), respectively, confirming the results of Burgess *et al.* (32). SAA shows a diverse set of functions which are conveyed at a wide range of varying concentrations from as low as 12.5 ng/ml to 50000 ng/ml (2). To confirm that hrSAA1 does not induce chemokine expression due to the lack of inherent capacity and not due to too low concentrations used (1–100 ng/ml), hrSAA1 was also added at higher concentrations (1200 ng/ml and 12000 ng/ml) to $CD14^+$ monocytes, but failed to induce CXCL8 production (**Figure 3A**).

hrSAA1 Fails to Induce MMP-9 Release by $CD14^+$ Monocytes

Connolly *et al.* demonstrated the expression of MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13 by fibroblast-like synoviocytes in response to rSAA1 stimulation (9). In addition, rSAA1 was also described to induce MMP-10 upregulation in human umbilical vein endothelial cells (36). In order to determine whether the MMP-inducing capacity of rSAA1 is endogenous to SAA1, monocytes were stimulated with rSAA1 and hrSAA1. Following a 24 h stimulation period, cell supernatants were collected and MMP-9 activity was determined using zymography. Significant expression of MMP-9 was not observed in response to hrSAA1 stimulation (band intensity 48.1 ± 11.4 × 10^3 versus control 33.5 ± 8.0 × 10^3) (**Figure 4**). On the other hand, rSAA1 induced significant MMP-9 expression at 100 ng/ml (band intensity 84.9 ± 1.8 × 10^3).

hrSAA1 Fails to Induce ROS Production in $CD14^+$ Monocytes

In response to rSAA1 stimulation, ROS expression by several cell types, such as glioma cells, fibroblasts and neutrophils, has been reported (37–39). In addition, rSAA1 has been described to activate NADPH-oxidase, thereby contributing to the production

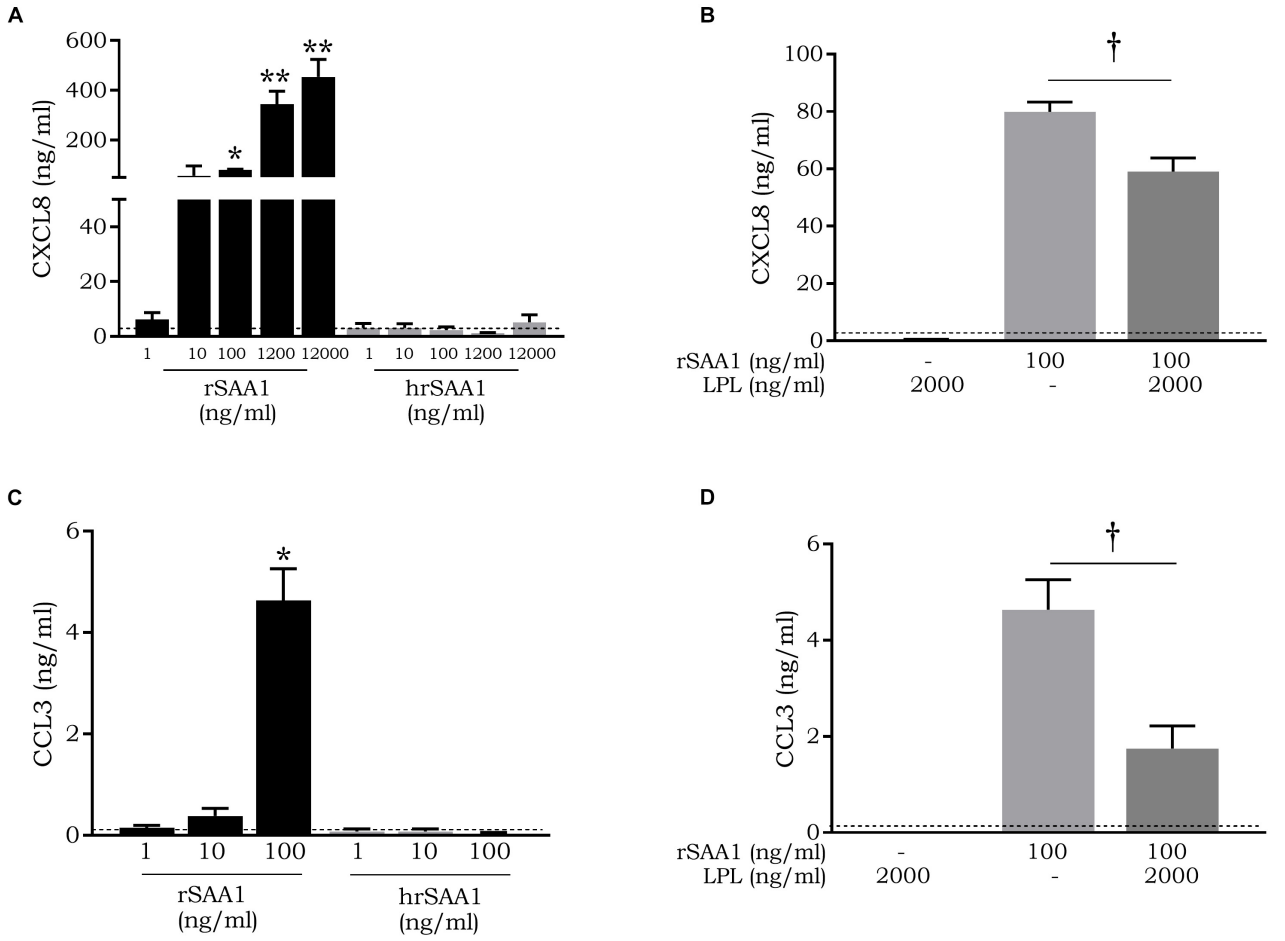

FIGURE 3 | hrSAA1 does not induce chemokine expression in CD14$^+$ monocytes and treatment of rSAA1 with LPL diminishes activity. **(A,C)** Freshly isolated monocytes were induced with homogenous rSAA1 (hrSAA1) (1–12000 ng/ml, 0.08–1000 nM) in parallel with rSAA1 (1–12000 ng/ml, 0.08–1000 nM). **(B,D)** rSAA1 (100 ng/ml, 8 nM) was pre-incubated with LPL (2000 ng/ml) for 4 h prior to stimulation of monocytes. Following an incubation period of 24 h, cell supernatants were collected and CXCL8 and CCL3 expression was determined via specific ELISAs. Expression in unstimulated cells is indicated by a dashed line (---). Results are represented as the mean chemokine concentration + SEM and are derived from four to six independent experiments. Significant upregulation in comparison to control is indicated by asterisks (Mann-Whitney *U*-test; **p*-value < 0.05, ***p*-value < 0.01). Significant reduction in inductive capacity following LPL treatment is indicated by a dagger (Mann-Whitney *U*-test; †*p*-value < 0.05).

of superoxide (40). Furthermore, rSAA1 has been shown to prime neutrophils to enhance ROS production in response to zymosan (39). To determine whether SAA1 possesses the capacity to induce ROS expression, monocytes were stimulated for 1 h with hrSAA1 in parallel with rSAA1 and ROS production was measured through DCFH-DA staining. In line with previously published results, rSAA1 induced a dose-dependent increase in ROS expression. At 1000 ng/ml, rSAA1 induced an 81 ± 36% increase in ROS expression in comparison to control (*p*-value 0.0006, **Figure 5**). In contrast, hrSAA1 did not induce notable ROS expression in monocytes (−3 ± 7% change at 1000 ng/ml).

hrSAA1 Lacks Macrophage Polarizing Capacity

Previous studies have explored the capacity of rSAA1 to regulate macrophage polarization. Indeed, Li *et al.* have observed the polarization of U937 cells into M2b macrophages in response to SAA stimulation (41). In line with this, Sun *et al.* demonstrated M2 polarization of human CD14$^+$ monocytes in response to rSAA (42). On the contrary, Anthony *et al.* demonstrated a mixed M1/M2 phenotype following stimulation of blood monocytes with rSAA1 (43). To compare the phenotype of hrSAA1-stimulated to rSAA1-stimulated macrophages, the expression of several surface markers (relative to M-CSF-differentiated macrophages) was determined. IFN-γ (100 ng/ml) combined with LPS (25 ng/ml) or IL-4 (20 ng/ml) were added as controls to skew toward the M1 or M2 phenotype, respectively. As anticipated, LPS combined with IFN-γ upregulated a number of M1 markers including CCR7 (183 ± 74%), CD80 (382 ± 36%), CD86 (236 ± 17%), and TLR4 (222 ± 72%) (**Figure 6A**). Similarly, IL-4 upregulated the M2 marker DC-SIGN (359 ± 34%). rSAA1 (100 ng/ml) induced the downregulation of a number of M1 markers including IL-1

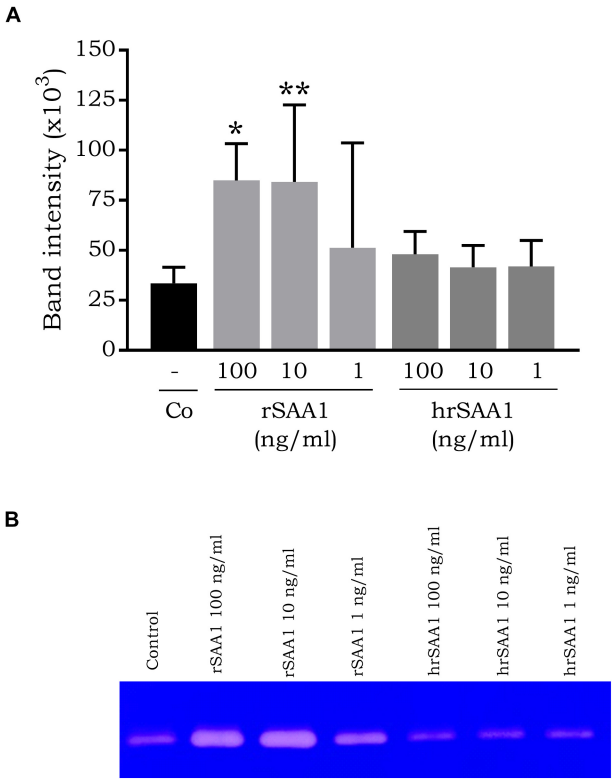

FIGURE 4 | hrSAA1 does not induce MMP-9 expression in CD14$^+$ monocytes. Freshly isolated monocytes were induced with homogenous rSAA1 (hrSAA1) (1–100 ng/ml, 0.08–8 nM) in parallel with rSAA1 (1–100 ng/ml, 0.08–8 nM). Following an incubation period of 24 h, cell supernatants were collected and MMP-9 expression was determined via zymography. **(A)** Results are represented as the mean band intensity ($\times 10^3$) + SEM and are derived from four to six independent experiments. Significant upregulation in comparison to control is indicated by asterisks (Mann-Whitney *U*-test; **p*-value < 0.05, ***p*-value < 0.01). **(B)** One representative zymography is shown.

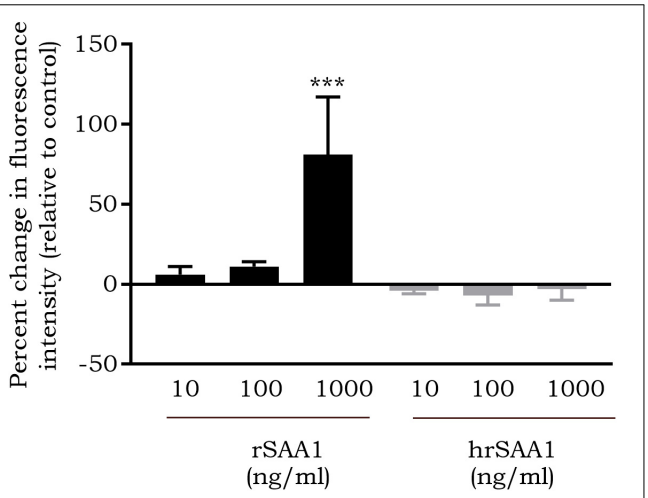

FIGURE 5 | hrSAA1 does not induce ROS production in CD14$^+$ monocytes. Freshly isolated monocytes were stimulated with homogenous rSAA1 (hrSAA1) (10–1000 ng/ml, 0.8–80 nM) in parallel with rSAA1 (10–1000 ng/ml, 0.8–80 nM). Following an incubation period of 1 h, staining with 2′,7′-dichlorofluorescein diacetate (50 μM) was carried out and flow cytometry was used to quantify ROS production. Results are presented as the mean percent change in fluorescence intensity in comparison to buffer-treated cells + SEM (i.e., 0% is equivalent to the same fluorescence intensity as obtained for buffer-treated cells) and are derived from four to seven independent experiments. Significant upregulation in comparison to control is indicated by asterisks (Mann-Whitney *U*-test; ****p*-value < 0.001).

receptor type I (IL-1RI) (−31 ± 8%), HLA-DR (−58 ± 6%) and TLR4 (−31 ± 8%), whereas it also upregulated a number of M1 markers including CD80 (131 ± 25%) and TLR2 (203 ± 16%). Furthermore, rSAA1 increased the CD14 expression level (107 ± 16%) (**Figure 6B**). In sharp contrast, hrSAA1 (100 ng/ml) did not exert any effect on the expression of M1 or M2 macrophage markers.

hrSAA1 Promotes Monocyte Survival

A number of studies have demonstrated the capacity of rSAA1 to promote the survival of leukocytes namely neutrophils via the activation of FPR2 (44, 45). More interestingly, also plasma-derived SAA, which lacks the inflammatory capacity displayed by its recombinant counterpart, was shown to promote neutrophil survival (46). To determine whether rSAA1 free from bacterial contaminants can promote leukocyte survival, monocytes were treated during 24 h with hrSAA1 (300 and 3000 ng/ml) in parallel with rSAA1 (300 and 3000 ng/ml) and M-CSF (20 ng/ml). Cell viability was assessed through the quantification of ATP in the cell lysates. Following a 24 h stimulation period, 300 and 3000 ng/ml of hrSAA1 (162.8 ± 12.7% and 201.5 ± 57%, *p*-value 0.0094 and 0.0136, respectively) promoted notable monocyte survival in comparison to medium treatment (100.0 ± 11.1%; **Figure 7**). Albeit slightly less effective than hrSAA1, rSAA1 at 3000 ng/ml also induced statistically significant monocyte survival (172.5 ± 36.3%, *p*-value 0.0136). Furthermore, M-CSF, an established monocyte survival factor, enhanced monocyte survival at 20 ng/ml (195.5 ± 45.2%, *p*-value 0.0268).

hrSAA1 Retains Its Leukocyte Recruiting Capacity *in vivo*

Various studies, utilizing recombinantly expressed SAA1, have demonstrated the chemotactic potential of SAA1. rSAA1 has been described as an *in vitro* chemoattractant for a wide range of cells including endothelial cells, fibroblasts, immature dendritic cells, mast cells, monocytes, neutrophils, smooth muscle cells, and T cells (6, 11, 12, 47–50). In addition, the *in vivo* chemotactic activity of rSAA1 has been well documented (10). To verify whether pure hrSAA1 retains its chemoattractant capacity, C57BL/6J mice were i.a. injected with hrSAA1 (100 ng/10 μl or 500 ng/10 μl) in parallel with rSAA1 (100 ng/10 μl). Injection of hrSAA1 (500 ng/10 μl i.a.) induced significant recruitment (28-fold increase, **Figure 8**) of neutrophils in comparison to control [11400 versus 400 cells per ml (median values); *p*-value 0.0002]. Furthermore, hrSAA1 (500 ng/10 μl) induced the recruitment of mononuclear cells into the joint cavity (109800 cells per ml) which is evidenced by an approximate threefold increase in the

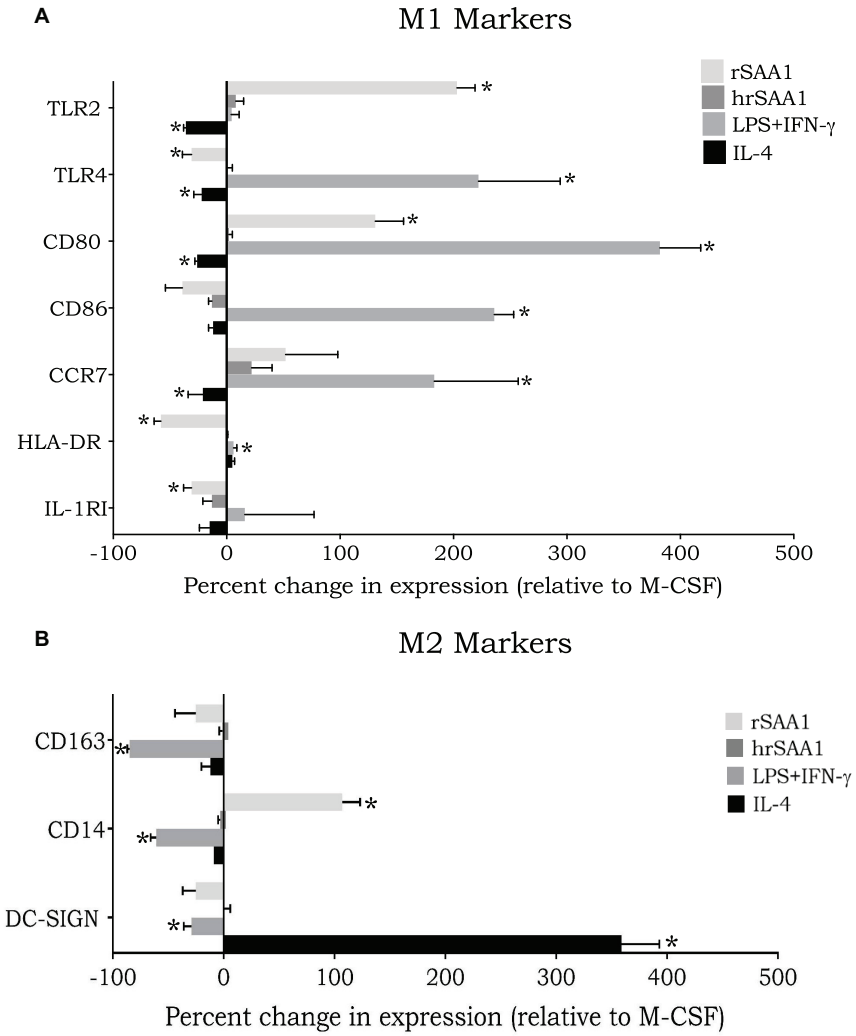

FIGURE 6 | hrSAA1 does not mediate macrophage polarization. Freshly isolated monocytes were treated with M-CSF (100 ng/ml, 2.7 nM) on day 0 to induce macrophage differentiation. On day 4 cells were treated with IFN-γ (25 ng/ml, 1.5 nM) plus LPS (100 ng/ml), IL-4 (20 ng/ml, 1.3 nM), rSAA1 (100 ng/ml, 8 nM), or homogenous rSAA1 (hrSAA1) (100 ng/ml, 8 nM) to promote macrophage polarization. On day 6 of culture, macrophages were collected and analyzed for expression of **(A)** M1 markers and **(B)** M2 markers using flow cytometry. Results are presented as the mean percent change in expression compared to M-CSF-treated cells + SEM (i.e., 0% is equivalent to the same expression level as M-CSF-treated cells) and are derived from seven independent experiments. Significant upregulation in comparison to control is indicated by asterisks (Wilcoxon signed-rank test; *p-value < 0.05).

number of mononuclear cells compared to control (38200 cells per ml; p-value 0.003). In a similar manner, rSAA1 induced the recruitment of neutrophils and mononuclear cells into the joint cavity (107500 and 187900 cells per ml, respectively). However, rSAA1 displayed a higher potency which could be attributed to contaminating bacterial products (p-value < 0.05; 100 ng/10 μl of rSAA1 versus both 100 and 500 ng/10 μl of hrSAA1).

hrSAA1 Synergizes With CXCL8 to Activate and Chemoattract Neutrophils *in vitro* via FPR2 Activation

De Buck *et al.* have previously reported a synergistic effect between SAA1 and CXCL8 in the recruitment of neutrophils (6, 26). To this end, we carried out chemotaxis experiments on neutrophils using combinations of CXCL8 and hrSAA1. hrSAA1 retained the capacity to synergize with CXCL8 in neutrophil recruitment which was evidenced by the enhanced chemotactic indices (CI) observed with the combination of hrSAA1 and CXCL8 (CI = 0.4 ± 0.1 for 3000 ng/ml hrSAA1, CI = 20.0 ± 4.0 for 10 ng/ml CXCL8 versus CI = 29.5 ± 4.0 for 3000 ng/ml hrSAA1 + 10 ng/ml CXCL8, p-value 0.0286; **Figure 9A**). Moreover, in line with previous studies reporting the usage of FPR2 by SAA1 during synergy with CXCL8 during neutrophil recruitment (6, 26, 27), we observed the inhibition of the synergistic effect between hrSAA1 and CXCL8 in response to the selective FPR2 antagonist WRW_4. Following treatment of neutrophils with WRW_4 (20 μg/ml), migration toward the FPR2

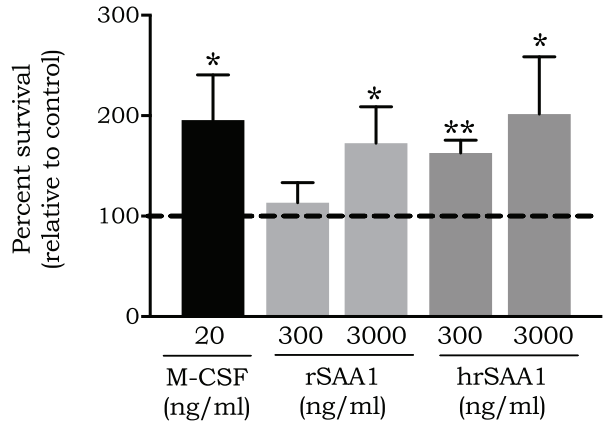

FIGURE 7 | hrSAA1 promotes the survival of monocytes. Freshly isolated monocytes were treated with hrSAA1 (300 or 3000 ng/ml, 25 or 250 nM), rSAA1 (300 or 3000 ng/ml, 25 or 250 nM), M-CSF (20 ng/ml, 0.54 nM) or left untreated (control) for a period of 24 h. Following stimulation, ATP levels were detected using firefly luciferase luminescence. Control survival is indicated by a dashed line (---). Results are presented as the mean percent of survival in comparison to control + SEM and are derived from five independent experiments. Significant survival in comparison to control is indicated by asterisks (Mann-Whitney *U*-test; **p*-value < 0.05, ***p*-value < 0.01).

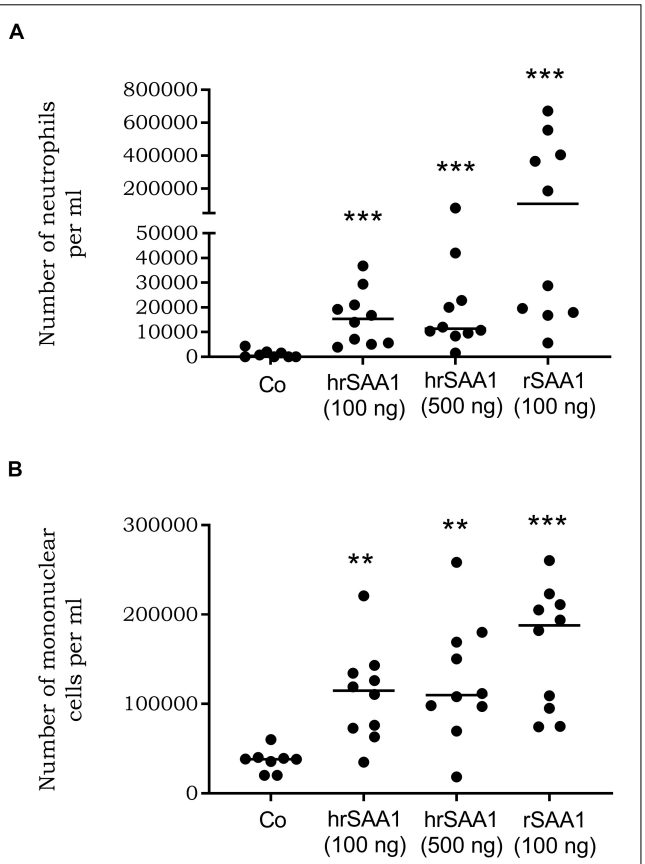

FIGURE 8 | hrSAA1 induces *in vivo* recruitment of neutrophils and mononuclear cells. C57BL/6J male mice (8–10 mice per group) were intra-articularly injected with homogenous rSAA1 (hrSAA1; 100 or 500 ng/10 μl), rSAA1 (100 ng/10 μl) or 0.9% sodium chloride (control; 10 μl). Following an incubation period of 3 h, cells from the joint were collected and cytospins were prepared. Each dot represents one mouse and the horizontal line indicates the median number of recruited **(A)** neutrophils and **(B)** mononuclear cells per ml. Data are derived from two independent experiments. Significant leukocyte recruitment in comparison to control is indicated by asterisks (Mann-Whitney *U*-test; ***p*-value < 0.01, ****p*-value < 0.001).

agonist MMK-1 was blocked. In addition, WRW_4 inhibited the synergistic effect between hrSAA1 (3000 ng/ml) and CXCL8 at 3 and 9 ng/ml by 32% and 53%, respectively ($n = 3$; **Figure 9B**) thus indicating that hrSAA1 retains its FPR2 binding capacity.

Shape change assays yielded further evidence of this synergistic effect. The net number of activated cells was 3.3-fold higher (*p*-value 0.0079) following stimulation with hrSAA1 (3000 ng/ml) and CXCL8 (3 ng/ml) simultaneously in comparison to the sum of the numbers obtained in response to either stimulus alone (**Figure 9C**). In addition, measurement of actin polymerization confirmed the findings observed with the Boyden chamber chemotactic assay and the shape change assay. Following stimulation with hrSAA1 and CXCL8 simultaneously, a statistically significant dose-dependent synergistic effect on actin polymerization was observed (relative MFI = 98.8 ± 3.1 for 3000 ng/ml of hrSAA1 and relative MFI = 131.2 ± 7.9 for 1 ng/ml of CXCL8 versus relative MFI = 207.8 ± 23.0 for 3000 ng/ml hrSAA1 + 1 ng/ml CXCL8; *p*-value 0.008; **Figure 9D**). It was concluded that in three different neutrophil activation assays hrSAA1 retained its synergizing capacity.

DISCUSSION

In the present study, we purified commercially available rSAA1 expressed in *E. coli*, which has been used in most studies dealing with its biological activities, via RP-HPLC to produce homogenous rSAA1 free of any residual bacterial contaminants. Several biological assays were carried out to determine whether hrSAA1 is a real inflammatory mediator. In contrast to rSAA1, we observed a lack of chemokine induction in response to stimulation of monocytes with hrSAA1 (**Table 1**). In addition, no ROS production, nor MMP-9 release by monocytes was detected in response to hrSAA1 stimulation. Furthermore, macrophages did not change their expression profile of M1 or M2 markers following incubation with hrSAA1. All these effects have been attributed to rSAA1 binding to TLR2. Nevertheless, hrSAA1 retained its capacity to synergize with CXCL8 in the activation and recruitment of neutrophils, an effect that has also been observed with COOH-terminal fragments of SAA1 (26, 27). This chemotactic activity is reportedly mediated by FPR2 (23–25). By the use of an FPR2 antagonist we previously demonstrated that also synergy in chemotaxis between rSAA1 and CXCL8 is dependent on FPR2 (6). We could confirm here that hrSAA1 still binds to FPR2, as we have observed desensitization of calcium signaling by the FPR2 agonist CCL23(46−137) (51) when FPR2-transfected HEK293 cells were pre-treated with hrSAA1 (data not shown). Moreover, the cooperative interaction between hrSAA1

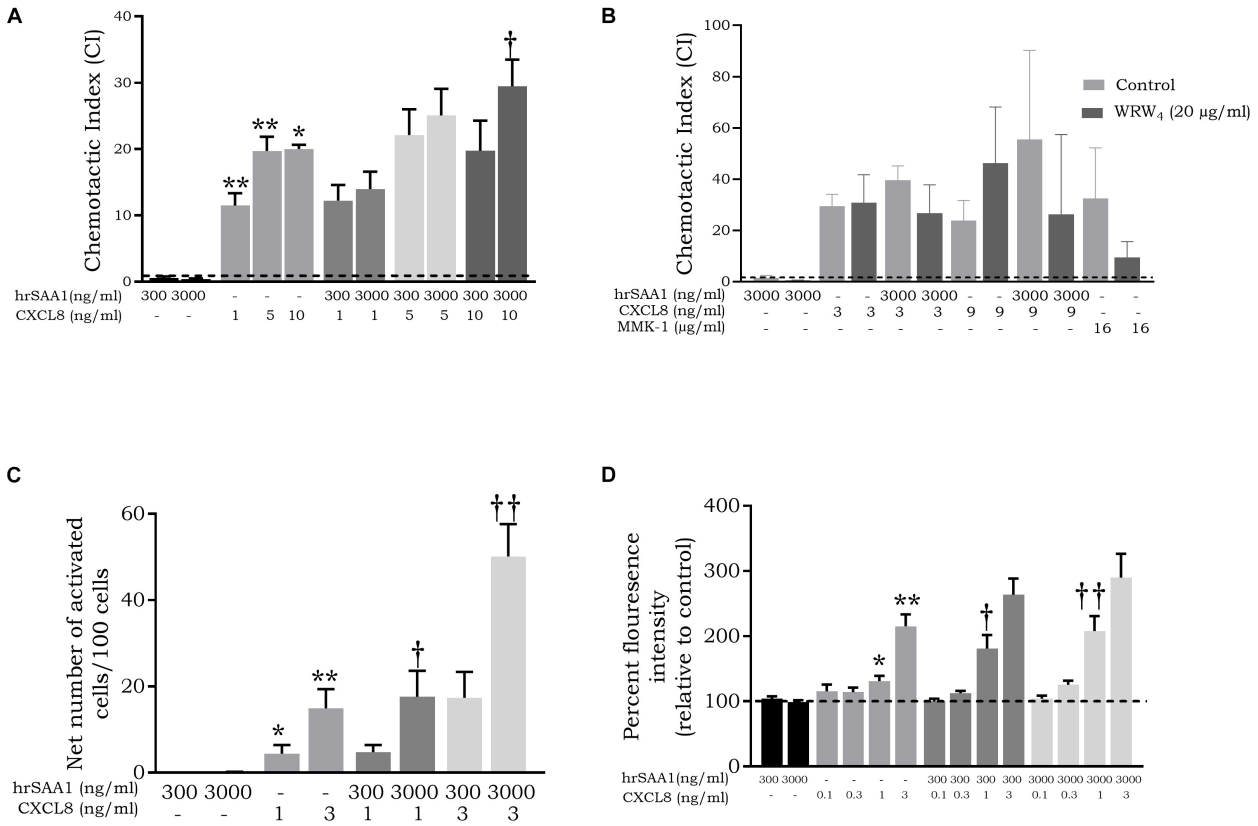

FIGURE 9 | hrSAA1 retains the capacity to synergize with CXCL8 in the *in vitro* attraction and activation of neutrophils. **(A)** Homogenous rSAA1 (hrSAA1, 300 or 3000 ng/ml, 25 or 250 nM) and CXCL8 (1–10 ng/ml, 0.12–1.2 nM) were added to the lower compartment of a Boyden microchamber either alone or in combination. Freshly isolated neutrophils were added to the upper compartment and allowed to migrate for 45 min. The chemotactic potencies are expressed as mean chemotactic index (+SEM) derived from six independent experiments. Control recruitment is indicated by a dashed line (---). **(B)** MMK-1 (16 µg/ml, 9930 nM), hrSAA1 (3000 ng/ml, 250 nM), CXCL8 (3 or 9 ng/ml, 0.36 or 1.07 nM) or a combination of hrSAA1 and CXCL8 were added to the lower compartment of a Boyden microchamber. Freshly isolated neutrophils in the presence or absence of the FPR2 antagonist WRW_4 (20 µg/ml, 18110 nM) were added to the upper compartment and allowed to migrate for 45 min. The chemotactic potencies are expressed as mean chemotactic index + SEM and are derived from three independent experiments. Control recruitment is indicated by a dashed line (---). **(C)** Neutrophils were stimulated (3 min) with homogenous hrSAA1 (300 or 3000 ng/ml, 25 or 250 nM), CXCL8 (1 or 3 ng/ml, 0.12 or 0.36 nM) or a combination of hrSAA1 and CXCL8. Data from six independent experiments are expressed as the net percentage of activated cells (blebbed and elongated cells) (+SEM). **(D)** Freshly isolated neutrophils were stimulated for 30 s with different concentrations of hrSAA1, CXCL8 or a combination of hrSAA1 and CXCL8. Following stimulation, cells were stained with Alexa Fluor 555 Phalloidin. Control actin polymerization is indicated by a dashed line (---). Results are presented as the mean percent florescence intensity relative to buffer-stimulated cells (+SEM) and are derived from six independent experiments. Significant neutrophil recruitment/activation in comparison to control is indicated by asterisks (Mann-Whitney *U*-test; **p*-value < 0.05, ***p*-value < 0.01). Significant synergy between hrSAA1 and CXCL8 in neutrophil activation/recruitment is indicated by daggers (Mann-Whitney *U*-test; †*p*-value < 0.05, ††*p*-value < 0.01).

TABLE 1 | Comparison of the biological activities of *E. coli*-expressed SAA1 before (SAA1) and after (hrSAA1) purification to homogeneity using RP-HPLC.

Activity	SAA1	hrSAA1
In vivo mononuclear cell attraction	x	x
In vivo neutrophil attraction	x	x
Synergy with CXCL8 in neutrophil migration	x	x
Desensitization of FPR2-mediated calcium signaling by CCL23(46–137)	x	x
Induction of MMP-9 release in monocytes	x	–
Induction of chemokine expression in monocytes	x	–
Induction of ROS production in monocytes	x	–
Monocyte survival	x	x
Macrophage polarization	x	–

x, biological activity demonstrated; – not active.

and CXCL8 in neutrophil chemotaxis is mediated by FPR2. The reported chemotactic activity of SAA1 is rather weak and requires rather high doses, 0.8 μM for monocyte chemotaxis as reported by Su *et al.* (23) and 4 μM for monocyte and neutrophil chemotaxis as reported by Badolato *et al.* (10, 52). Furthermore, the synergy between rhSAA1 and CXCL8 we describe in neutrophil actin polymerization already occurs at 300 ng/ml (25 nM) of hrSAA1 (**Figure 9D**). Therefore, the synergistic interaction of rhSAA1 with other chemoattractants is more relevant than its individual chemotactic property.

The previously reported TLR2-mediated activities of rSAA1, for instance induction or upregulation of cytokine production by rSAA1, are probably due to contaminating bacterial lipoproteins present in the recombinantly produced SAA. Indeed, similar to Burgess *et al.* (32), we have observed partial downregulation of chemokine induction by monocytes in response to rSAA1 following LPL treatment. Besides lipoproteins, rSAA1 might contain additional chemokine-inducing substances, e.g., non-lipopeptides, such as outer membrane protein A (ompA) or LPS, which were completely removed from rSAA1 by RP-HPLC, but remained unaffected by incubation of rSAA1 with LPL. Indeed, analysis of rSAA1 expressed in *E. coli* revealed the presence of of 91 proteins of bacterial origin. Some of these proteins may also play a role in the inductive capacity of rSAA1 (32). Nevertheless, He *et al.* have attributed rSAA1-induced CXCL8 production in neutrophils to the activation of FPR2, because the CXCL8 production was inhibited by pertussis toxin (53). On the other hand, we did not observe any CXCL8 production by monocytes treated with up to 1 μM hrSAA1. Our results provide an explanation for the reported discrepancies in the activity of rSAA1 and SAA derived from inflammatory plasma. In contrast to rSAA1, plasma-derived SAA was not found to possess characteristics of an inflammatory mediator (54). These findings are in line with the observations made by Christenson *et al.* indicating that SAA-rich inflammatory plasma derived from patients with arthritis failed to activate neutrophils (55).

It has long been known that SAA is a lipophilic protein as it can replace apolipoprotein A-I in high density-lipoprotein during an inflammatory insult (56). Taking into consideration the propensity of SAA1 to bind hydrophobic molecules could explain the difficulty encountered to purify rSAA1 from contaminating bacterial products, particularly LPS and lipoproteins. The capacity of SAA to bind hydrophobic molecules is largely intertwined with several of its functions, including its antimicrobial function. Via its interaction with the hydrophobic vitamin retinol, recombinant human SAA1 and recombinant mouse SAA1/3 indirectly regulate the immune response during acute infection (57). Via its binding to ompA of gram-negative bacteria, serum-derived SAA1 acts as an opsonizing agent thereby promoting phagocytosis (15). Through its hydrophobic interaction with LPS, SAA1 dampens the inflammatory response providing protection against excessive inflammatory tissue damage. In LPS-induced acute lung injury, SAA1-transgenic (Tg) mice displayed reduced neutrophil infiltration and lowered expression of inflammatory cytokines (e.g., IL-6 and TNF-α) (58). In a colitis mouse model, SAA1/SAA2 double knockout mice displayed increased weight loss, histological disease scores and TNF-α expression, suggesting that SAA1/SAA2 may provide protection at the intestinal epithelial barrier through its direct antibacterial properties (20).

The idea that SAA is an anti-inflammatory mediator is certainly one worth contemplating. Indeed, a few other studies have provided evidence that SAA mediates anti-inflammatory functions *in vivo*. Murdoch et al. demonstrated that although SAA is essential in zebrafish for the recruitment and maturation of neutrophils, it also plays a role in confining neutrophil-mediated inflammation via the reduction of neutrophil bactericidal activity and expression of inflammatory markers (19). The role of mouse SAA in relation to *in vivo* macrophage polarization was recently investigated in the context of carbon tetrachloride-induced hepatic injury where an increase in fibrogenesis was observed following SAA neutralization. It was found that SAA provided a protective effect via the polarization of macrophages toward an M2b phenotype (59).

Contrary to the observations made by Burgess *et al.*, using rSAA1 derived from an eukaryotic source (32), a number of studies have provided both *in vitro* and *in vivo* evidence regarding the role of SAA in the development of the Th17 response. Through the use of SAA1/2 double knock out mice, Sano *et al.* demonstrated the role of this acute phase protein in the expression of Th17 cytokines (IL-17A and IL-17F) and the proliferation of Th17 cells in the terminal ileum following segmented filamentous bacteria colonization. These findings were confirmed *in vitro* using recombinant murine SAA1 (rmSAA1). Indeed, the treatment of RORγt$^+$ CD4$^+$ T cells in suboptimal Th17 polarizing conditions with rmSAA1 induced the expression of both IL-17A and IL-17F. Furthermore, rmSAA1 was found to promote the differentiation of Th17 cells through the upregulation of the RORγt pathway (60). Lee *et al.* recently made similar observations using rmSAA1 which was found to promote Th17 differentiation of naïve murine CD4$^+$ T cells evidenced through the upregulation of several Th17 markers such as IL-23R and S100a4. *In vivo* evidence for the role of murine SAA in the Th17 response was provided using an autoimmune encephalomyelitis mouse model. In this model the Th17 response was diminished in the central nervous system of SAA3 knockout mice in comparison to control wildtype mice (61).

The true nature of SAA, first identified in the late 1970s, remains an enigma (62). Indeed, a whole spectrum of divergent biological activities has been attributed to SAA1 and many apparently contradictory data have been published. Nonetheless, the multiple biological activities attributed to SAA1 could possibly be explained by the fact that SAA1 is an intrinsically disordered protein and thus displays various conformations depending on environmental conditions such as pH or ligand concentration (63). Ji *et al.* investigated the role of SAA1 in T cell-mediated hepatitis through the use of SAA1-Tg mice; SAA1 was shown to promote hepatic injury through several pathways amongst which the upregulation of chemokine expression (64). This study and the data of Burgess

et al., strongly contest the TLR2- and TLR4-mediated *in vitro* activities ascribed to rSAA1. Although TLR activation by rSAA1 is currently rather excluded, it was not implausible. Indeed, several endogenous TLR2/4 ligands exist including proteins and peptides such as β-defensin 2, biglycan, fibronectin, S100 proteins, and high mobility group box 1 (HMGB1) to name a few (65). In contrast, the FPR2-mediated chemotactic activities were confirmed with hrSAA1 and were also observed with synthetic SAA1-derived COOH-terminal peptides which were free of any bacterial components (26, 27), suggesting that this COOH-terminal part of hrSAA1 is responsible for the chemotactic activity of homogenous intact SAA1. Similar to TLRs, FPR2 is activated by a diverse set of endogenous molecules including fatty acids (lipoxin A4), proteins (annexin 1) and peptides (humanin), including chemokine-derived peptides, such as CCL23(46−137) (66, 67).

Some remaining biological activities ascribed to SAA1, such as suppression of antibody production or inhibition of platelet activation and aggregation should be further investigated using homogenous SAA1 (2, 45). Alternatively, the *in vitro* use of rSAA1 produced by eukaryotic expression and the SAA1, SAA2, and SAA3 knock out and/or transgenic animals (19, 20, 58) are important tools to further establish the biological functions of the SAA family members.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available upon reasonable request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the animal Ethics Committee of the Federal University of Minas Gerais (295/2018).

AUTHOR CONTRIBUTIONS

SA, JV, and SS wrote the manuscript. SA, MDBo, NB, VO, MDBu, MG, and SS performed the experiments and analyzed the data. FA analyzed and supervised the *in vivo* experiments. PP and JV gave technical advice regarding the purification of rSAA1. FA, PP, JV, and SS designed the experiments and provided the funding. All authors corrected the manuscript and approved its final version.

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MINI REVIEW
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CXCR3 Ligands in Cancer and Autoimmunity, Chemoattraction of Effector T Cells, and Beyond

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CXCR3 is a chemokine receptor with three ligands; CXCL9, CXCL10, and CXCL11. CXCL11 binds CXCR3 with a higher affinity than the other ligands leading to receptor internalization. Long ago we reported that one of these chemokines, CXCL10, not only attracts CXCR3+ CD4+ and CD8+ effector T cells to sites of inflammation, but also direct their polarization into highly potent effector T cells. Later we showed that CXCL11 directs the linage development of T-regulatory-1 cells (Tr1). We also observed that CXCL11 and CXCL10 induce different signaling cascades via CXCR3. Collectively this suggests that CXCR3 ligands differentially regulate the biological function of T cells via biased signaling. It is generally accepted that tumor cells evolved to express several chemokine receptors and secrete their ligands. Vast majority of these chemokines support tumor growth by different mechanisms that are discussed. We suggest that CXCL10 and possibly CXCL9 differ from other chemokines by their ability to restrain tumor growth and enhance anti-tumor immunity. Along with this an accumulating number of studies showed in various human cancers a clear association between poor prognosis and low expression of CXCL10 at tumor sites, and vice versa. Finally, we discuss the possibility that CXCL9 and CXCL10 may differ in their biological function via biased signaling and its possible relevance to cancer immunotherapy. The current mini review focuses on exploring the role of CXCR3 ligands in directing the biological properties of CD4+ and CD8+ T cells in the context of cancer and autoimmunity. We believe that the combined role of these chemokines in attracting T cells and also directing their biological properties makes them key drivers of immune function.

Keywords: CXCR3, chemokines, CXCL10, CXCL9, EAE, cancer, tolerance

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INTRODUCTION

Chemokines are small (~8–14 kDa), structurally cytokine-like, secreted proteins that regulate cell trafficking through interactions with a subset of 20 different seven-transmembrane, G protein-coupled receptors (GPCRs) (1). These receptors could be divided into single (mono) receptors, and shared receptors in which a single receptor binds several chemokines. Different chemokines that bind a shared receptor may have different modes of interactions. They may either poses similar biological properties (may explain redundancy), or induce divers signaling cascades and thereby differ in biological properties. This type of biased signaling has been previously observed for beta2-adrenergic receptor (also GPCRs) by the Nobel prize winner Robert J. Lefkowitz (2) and by others (3). Our laboratory was the first to report that such biased signaling is also used by

chemokines to direct the biological properties of CD4+ T cells in controlling effector T cell function vs. tolerance to self (4, 5), and perhaps in controlling anti-cancer immunity (6). The current review focuses on the role of CXCR3 and its ligands: CXCL9, CXCL10, and CXCL11 on the biological function of CD4+ and CD8+ T cells and its translational implications.

Of the three CXCR3 ligands most of the attention has been drawn thus far to CXCL10, as a candidate for cancer immunotherapy. Only recently it has been suggested that that CXCL9 is also involved in directing the potentiation of CD8+ T cells in cancer, and that its activity differs from CCL10 (7). Not much is known about the role of CXCL11 in cancer diseases. As for autoimmunity, the role of CXCL10 and CXCL11 has been largely studied by several laboratories including ours, whereas the role of CXCL9 is still elusive (8).

CXCR3 AND ITS LIGANDS

CXCR3 is a chemokine receptor that is primarily expressed on CD4+ and CD8+ T cells, and to some extent by other cells, among them, epithelial cells (9). Within the CD4+ subset CXCR3 is mostly abundant on proinflammatory Th1 cells, but notably it is also expressed by FOXp3+ regulatory T cells (T_{regs}) (10–12). Mice express a single isoform of CXCR3 that exclusively bind CXCL9, CXCL10, and CXCL11. In human three isoforms were identified: CXCR3A that is reciprocal to the mouse CXCR3 and also binds CXCL9, CXCL10, and CXCL11, CXCR3-B that binds CXCL9, CXCL10, CXCL11 as well as an additional ligand CXCL4, and CXCR3-alt that only binds CXCL11 (13). The CXCR3 ligands share limited sequence homology. Yet, in their structural homology they are more similar to each other than to other non-ELR chemokines. Also all three chemokines are inducible by IFN-γ (14). Together this makes them a well-characterized subfamily of the non-ELR chemokines. CXCL11 is believed to be the dominant CXCR3 agonist, as it is more potent than CXCL10 or CXCL9 as a chemoattractant and in stimulating calcium flux and receptor desensitization (15).

BIASED SIGNALING VIA CXCR3 DIRECTS THE POLARIZATION OF CD4+ T CELL SUBSETS

Based on their cytokine profile FOXp3-negative CD4+ T cells fall into different subsets among them IFN-γ^{high}IL4^{low} Th1 cells IFN-γ^{low}IL4^{high} Th2 cells, IL17^{high} Th17 cells and IL10^{high} T regulatory-1 (Tr1) cells (16). It is generally accepted that the polarization of non-polarized CD4+ T cells (Thnp) into these subsets is directed by the cytokine milieu within their microenvironment (16). Not much attention has been drawn to the role of chemokines in T cell polarization.

Long ago we observed that along the development of two different experimental autoimmune diseases in Lewis rats: Experimental autoimmune encephalomyelitis (EAE), and adjuvant induced arthritis (AA) the immune system generate an autoantibody response (IgG isotype) to pro-inflammatory cytokines and chemokines that are likely to be involved in the pathogenesis of these diseases (17, 18). In these studies we also observed that amplification of these responses by targeted DNA plasmids may restrain the progression of these diseases (17, 18). We further investigated the mechanistic basis of this response and named it "beneficial autoimmunity" (19). While extending these studies to CXCL10 we learned that targeting the function of CXCL10 restrained the development of EAE or AA. *Ex vivo* analysis of CD4+ T cells subsets indicated for *in vivo* shift from Th1 to Th2 (20, 21). Independently, others observed that CXCL10 promotes the polarization of human CD4+ T cells into IFNγ^{high}IL4^{low} Th1 cells (22). The role of CXCL9 in directing effector T cell polarization is yet to be studied. Collectively, this suggests that CXCL10 promotes the polarization of Th1 cells, thus its targeted neutralization restrains autoimmunity. In our studies we could clearly record the effect of CXCL10 neutralization on the Th1/Th2 balance of antigen specific T cells in the periphery (17, 18), and suggested that along the dynamics of each disease these cells are recruited to the inflammatory site, to replace those that undergo apoptosis there (23). The possibility that these antibodies directly enter the CNS to affect T cell polarization there has not been detected.

While further exploring the interplay between CXCR3 ligands, particularly CXCL10 vs. CXCL11 and their role in directing CD4+ T cell polarization we observed that CXCL11 preferentially drives the polarization of IL10^{high} Tr1 cells (4, 5). The underlying signal cascade included signaling via p70 kinase/mTOR in STAT-3- and STAT-6-dependent pathways (4, 5). This differed from CXCL10 that signals via STAT1, STAT4, and STAT5 phosphorylation (4, 5). CXCL11 is believed to be the dominant CXCR3 agonist, as it is more potent than CXCL10 or CXCL9 as a chemoattractant and in stimulating calcium flux and receptor desensitization (15). This suggests that the interplay between CXCL11 and CXCL10 dominates the regulation of CD4+ T cell mediated responses, while favoring active tolerance over effector reactivity. C57BL/6 mice that lack functional CXCL11 due to a shift in the open reading frame of the CXCL11-encoding gene (insertion of two bases after nucleotide 39), resulting in the translation of a chimeric protein lacking the critical CXC motif (24), preferentially induce Th1 oriented response, are highly susceptible to the induction of various Th1-related autoimmune diseases. We observed that these mice are excellent responders to low doses CXCL11-Ig based therapy of EAE in comparison to SJL mice that do not display this open reading frame mutation (4).

The idea of different ligands that differ in their binding site to the same GPCRs receptor also induce different signaling cascade has been primarily investigated by Robert J. Lefkowitz and his team while exploring the Molecular mechanism of beta-arrestin-biased agonism (2, 25, 26). We have explored the relevance of this mechanism for chemokines and T cell regulation.

In summary, we suggest that CXCL11 and CXCL10 plays an opposing role in directing T cell polarization, and as CXCL11 has a higher affinity to CXCR3 it is likely to dominate immune regulation.

THE CONTRADICTIVE ROLE OF CXCR3-CXCL10 AXIS IN NEUROINFLAMMATION

It is largely accepted that CXCL10 promotes the activity of effector CD4+ and CD8+ T cells, and also their recruitment at inflammatory sites (also tumor site) and thus its targeted neutralization could be beneficial in treating various T cell mediated autoimmune diseases among them: psoriasis, rheumatoid arthritis (RA) (27, 28), Inflammatory Bowel Disease [IBD) (29), and type I diabetes (T1DM) (30, 31) (for a recent review also see (32)] (**Figure 1B**). The role of the CXCL10-CXCR3 axis in neuroinflammation is likely to more complex and controversial (37). The first record that systemic administration of polyclonal antibodies against CXCL10 suppress EAE came from the study of William Karpus and his group in 2001 (39). Independently, and shortly after we reported that targeted DNA vaccines encoding CXCL10 could amplify the production of neutralizing autoantibodies to CXCL10 that could also suppress EAE in Lewis rats (20). Both studies were limited in the use of polyclonal antibodies. Four years later Richard Ransohoff and his group reported that CXCR3 KO mice lacking the CXCR3-CXCL10 interaction develop more severe EAE then WT (40). The absence of CXCR3-CXCL11 interaction could not be taken in account as these were C57BL6 mice lacking functional CXCL11. Klein et al. examined the development of EAE in WT Vs CXCL10 KO mice and observed differences only during sub-optimal induction of disease (41). In another study, Iain Campbell and his group compared the development of EAE in WT and CXCR3KO mice and observed that along the later chronic phase of disease CXCR3KO mice develop a more severe EAE then WT, and that this has been associated with reduced number of FOXp3+ Tregs at the CNS (38). Campbell and his co-authors suggested that perhaps CXCL10 produced by astrocytes at the inflamed CNS mostly direct the recruitment of FOXp3+ Tregs that then suppress effector T cells function (37) (**Figure 1C**). Yet, the authors question the validity and relevance of using CXCL10KO mice, or CXCR3KO mice in EAE studies, as in the absence of CXCL10 produced by astrocyte migration of T cells to the CNS is very limited, and may not reflect the disease in WT mice, or MS patients (37). It should also be noted that vast majority of these experiments were conducted in C57BL/6 mice that lack CXCL11. Finally, Chung & Liao used an adoptive transfer system in which $CXCR3^{+}$ Th17 cells compared to $CXCE3^{-/-}$ Th17 cells were transferred during EAE to suggest that negative signaling via glial cells restrain the activities of Th17 cells within the CNS (42).

In summary, the role of CXCL10 in inflammatory autoimmunity, particularly in neuroinflammation is controversial and need to be further addressed discussed below.

HOW THE FIELD COULD MOVE FORWARD FROM THE CURRENT CONTROVERSY?

The controversy of the role of CXCL10 in neuroinflammation, particularly when comparing systemic administration of anti CXCL10 neutralizing antibodies vs. using CXCL10 KO mice should be further addressed, particularly if one would like to consider anti CXCL10 based therapy for autoimmunity. An essential set of experiments should be conducted on CXCR3 KO mice vs. WT and CXCL10 KO mice vs. WT subjected to the induction of different inflammatory autoimmune disease that are not associated with neuroinflammation. Particularly arthritis and IBD. Ideal models would be mice models that express functional CXCL11 (the only one that does not do so is the C57BL/6 mice). Systemic blocked of CXCL10 in various diseases (including neuroinflammation) should be addressed using anti CXCL10 mAbs with very high specificity. Finally, a set-up in which CXCL10 is selectively knocked down from astrocytes would also be helpful for addressing the role of astrocytes CXCL10 in neuroinflammation. An open-end question that should still be unresolved is that why would CXCL10 selectivity recruits FOXp3+ T cells to the CNS?

CANCER EVOLUTION AND CHEMOKINES-CHEMOKINE RECEPTOR INTERACTION

Chemokine-chemokine receptor interactions play a major role in cancer biology (43–48). The common deterministic dogma suggests along cancer evolution tumor cells evolved to express chemokine receptor and produce their ligands because these interactions support tumor growth by several mechanisms (47, 49–51): First, many of them function as growth/survival factors either by autocrine pathway, and/or by inducing growth factors production by epithelial cells and stromal cells within the tumor microenvironment. Second, several of them direct the recruitment of bone marrow derived cells that support tumor growth and suppress anti-tumor immunity. Third, chemokine—chemokine receptor interactions are involved in attracting tumor cells to metastatic sites. The key chemokine receptor pathways that directly support tumor development are the CCR2-CCL2 (52–56), CXCR4-CXCL12 (48, 57), and CCR5- CCL3/4/5 (58–62) (**Table 1**). All three pathways are also associated with the recruitment of bone marrow derives cells to the tumor site, and with direct attraction of tumor cells to form metastatic spread. An additional chemokine receptor that recently became of a major interest is CCR8 that is abundant of on FOXp3+ Tregs (63).

Aside of this axis many other chemokine-chemokine receptors are involved in different cancer diseases (for a recent review see (65)). However, the current mini review mostly focuses on CXCR3 and its ligands.

WHAT IS KNOWN ABOUT CXCL10 AND CXCL9 AND IN CANCER IMMUNITY?

Several studies showed that CXCL9 and CXCL10, particularly CXCL10 produced by tumor or host cells can recruit CXCR3+ tumor-infiltrating CD4+ T cells, CD8+ T cells and NK cells that are associated with tumor suppression (33, 66–74). Zumwalt et al. showed active secretion of CXCL10 and CCL5 from colorectal cancer microenvironments in human was associates

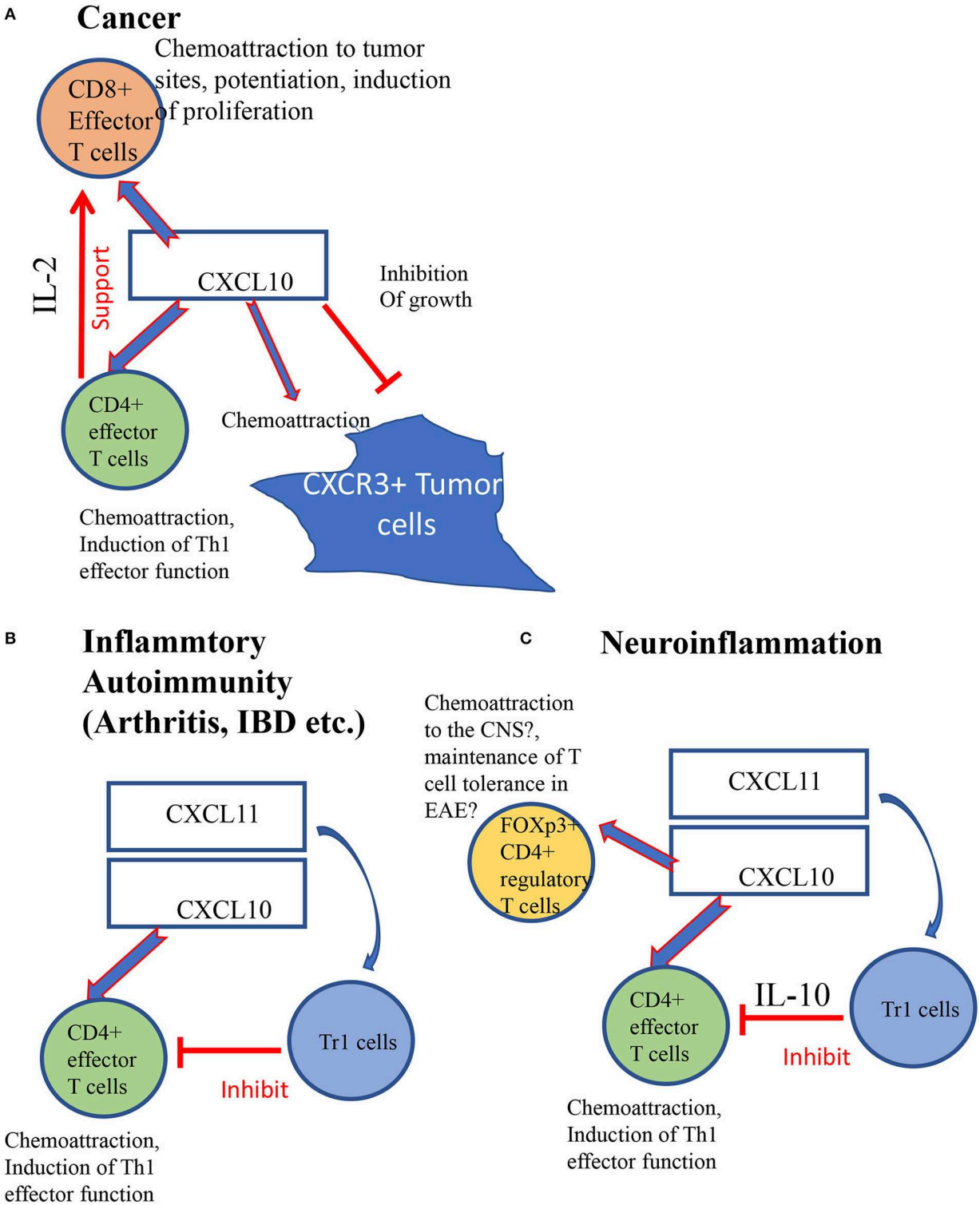

FIGURE 1 | *CXCL10* directs the biological function of CD4+ and CD8+ T cells in cancer and autoimmunity. **(A)** ***The role of CXCL10 in cancer diseases***: CXCL10 directs the accumulation of CXCR3+ effector T cells, in particular effector CD8+ T cells to the tumor site (33) and potentiates their anti-tumor activities, either directly or via the potentiation of effector CD4+ T cells to support their activity. As for tumor cells, it directly suppresses tumor growth (34, 35). Yet, for CNS metastatic spread it had been suggested that CXCL10 produced by astrocytes directs metastatic spread to the brain (36). **(B)** ***The role of CXCL10 and CXCL11 in inflammatory autoimmunity:*** CXCL10 is associated with chemoattraction and potentiation of effector T cells that commence the inflammatory process. Its activity is regulated, in part, by CXCL11 that induces T regulatory-1 (Tr1) cells (4). **(C)** Neuroinflammation: In neuroinflammation CXCL10 is likely to hold a duale function. Aside of chemoattraction of effector T cells it selectively induces the accumulation of FOXp3+ Tregs to restrain inflammation (37, 38).

TABLE 1 | Key chemokine receptor pathways that support tumor development.

Chemokine receptor -chemokine axis	Key pathways	References
CCR2-CCL2	Direct support of tumor growth, recruitment of tumor associated macrophages (TAMs) to support tumor growth and suppress anti-tumor immune reactivity	(52–56)
CXCR4-CXCL12	Direct support of tumor growth, metastatic spread, particularly to the bones	(48, 57)
CCR5- CCL3/4/5	Direct support of tumor growth, recruitment of polymorph nuclear myeloid derived suppressor cells and potentiation of their function at the tumor site.	(58–62)
CCR8-CCL1	CCR8+ Tregs function as master drivers of immune regulation and therefore are key drivers in tumor escape from immune destruction	(63, 64)

with Granzyme B+ CD8+ T-cell infiltration (75). It is likely that for CD8+ T cells the CXCR3-CXCL10 axis that is involved in directed migration of these cells to the tumor site also induces their potentiation and proliferation there (7, 33) (**Figure 1A**). What about CXCL9? Very recently Andy Luster and his group showed that anti PD-1 efficacy is reduced in CXCR3KO mice, and suggested that the interaction between CXCL9, largely produced by CD103+ dendritic cells (DC) at the tumor site, and CXCR3 on CD8+ T cells enhances anti PD-1 efficacy (7). The authors also extended this study to humans, suggesting that levels of CXCR3 ligands in the plasma may be used to predict success in anti PD-1 checkpoint therapy (7). It is yet to be explored whether CXCL9 and CXCL10 induce different signaling cascade via CXCR3 in CD8+ T cells.

WHAT IS KNOWN ABOUT CXCL10 BASED THERAPY OF CANCER DISEASES?

Nineteen years ago, Arenberg et al. showed that intra-tumoral injection of CXCL10 limits non-small-cell lung cancer (NSCLC) in SCID mice by a direct effect on tumor growth (76). Our collaborative study with Israel Vlodavsky was the first to show that systemic administration of CXCL10 (CXCL10-Ig) limits cancer in immunocompetent mice (34). One year later (2015) Peng et al. showed that treatment with epigenetic modulators that increase CXCL9/CXCL10 enhances effector T-cell tumor infiltration, and slows down tumor progression of ovarian cancer (77). At the same year, Barreiara da Silva et al. showed that Dipeptidylpeptidase 4 inhibition enhances endogenous CXCL10 levels and suppresses B16/F10 melanoma growth (78). This study also showed a highly effective effect of Dipeptidylpeptidase 4 based therapy if administered in combination with checkpoint blockers (78). It has recently been suggested that in the set-up of multiple myeloma CXCR3 receptor ligands CXCL9 and CXCL10, limits NK cell positioning into the bone marrow by interfering with CXCR4 function (79). It should also be noted that CXCR3 is expressed on T_{regs} and may be involved in directing their recruitment in cancer and transplantation (11, 12). Collectively this may vote for possible immune-regulating effect. Yet, it has been clearly shown that enhancement of CXCL10 in an *in vivo* set-up increases anti-tumor immunity and could be effectively used for cancer immunotherapy either as monotherapy, or in combined therapy with immune checkpoint inhibitors (78).

CXCL10 AND BRAIN CANCERS

As discussed above chemokines are involved in cancer diseases by several mechanisms among the direct and indirect effect on anti-cancer immunity, direct and indirect effect on cancer growth, and attracting cancer cells to tumor sites. It is generally accepted that CXCL10 enhances anti-cancer immunity, and by so doing limits cancer development. It has also been observed that CXCL10 directly limits cancer (melanoma) growth *in vivo* and *in vitro* (80). Collectively this applies for an anti-cancer property of CXCL10. As for directing metastatic spread Neta Erez and her team very recently suggested that CXCL10 produced by astrocytic cells participates in chemoattraction of tumor cells to the CNS (36). This may give rise to a possible tumor supporting function of CXCL10 in brain metastasis. Nevertheless, as described below many human studies clearly show that in various human cancer diseases low expression/transcription of CXCL10 at tumor sites indicate poor cancer prognosis, whereas high levels of this chemokine are associated with good prognosis.

In summary, CXCL10 is likely to hold anti-cancer propertied that include: 1. Direct effect on the immune system resulting in enhanced anti-cancer response, effect on epithelial cells surrounding the tumor and direct effect on tumor growth. Its tumor supporting role is by attracting tumor cells to form metastasis, as was recently suggested for brain tumors. We are now using CXCR3KO mice engrafted with CXCR3+ tumor cells to dissect the direct effect of CXCL10-Ig based therapy on tumor growth.

CXCL10 AND CANNER PROGNOSIS IN HUMAN

Ten years ago Jiang et al. reported that low transcription of CXCL10 shows poor prognosis in stage II and III colorectal cancer (81). Later Li et al. showed that in patients with rectal cancer that high expression of CXCL10 may predict better successes in chemoradiotherapy suggesting a synergistic beneficial effect of both (82). Rainczuk et al. showed that high levels of a CXCL10 antagonist in patients with high-grade, serous epithelial ovarian carcinoma (HGSOC) is associated with poor prognosis (83). As for Osteosarcoma (OS), Flores et al. showed better survival in patients with high level of CXCL10 (84). Finally, very recently Zhang et al. showed that in hepatocellular carcinoma (HCC) high levels of CXCL10 are associated with better prognostic and overall survival (85). Several publications challenged this concept (86–88). These studies focused on different cancers: breast cancer,

renal cancer and multiple myeloma (86–88). One is that the discrepancy between the studies is because the role of CXCL10 / CXCL9 varies between different cancer disease. If so this should be taken in account as a major criterion in candidate selection for a favorable disease for CXCL10/CXCL9 based therapy.

In summary, CXCL10 is likely to restrict cancer development in many cancers by inducing anti-cancer immune response, and by a direct effect on epithelial cells within the tumor microenvironment and by direct suppression of tumor growth. It is possible that CXCL10 and perhaps pro-cancer function is due to its chemotactic properties for cancer cells.

CHEMOATTRACTION AND BEYOND, CAN WE DIFFERENTIALLY ANALYZE THESE PROPERTIES?

It is clear that chemoattraction of CXCR3+ T cells, and other CXCR3+ cells, to sites of inflammation and tumor sites is an essential feature, and that inhibition of the CXCR3 dependent migration of CXCR3+ T cells to tumor site, or even their adhesion molecule dependent arrest, plays a major role in inflammation and cancer. For example, Mikucki et al. applied adoptive transfer experiments of T cells from CXCR3KO Vs WT mice in a cancer set-up to show that recruitment to the tumor site was markedly inhibited when donor cells came from CXCR3KO mice, and inhibition was comparable to the one achieved by using T cells form WT donors and pertussis toxin (PTX) (33). It is also clear that CXCL10, and probably CXCL9 signaling enhance the effector properties of these cells (7). We believe that what makes CXCR3 and its ligands drivers of immune function is the combination of chemotaxis and direct effect on the biological function (6, 89). Dissecting the direct effect of CXCR3 ligands on cells migration from their ability to affect the biological properties of these cells could be of interest when developing therapeutic tools, such as blocking antibodies or stabilized chemokines for immunotherapy.

CONCLUSIONS

The main take home message of this minireview is that few chemokine receptors, among them CXCR3, are key drivers in directing the immune response as aside of chemoattraction they also direct the biological function of immune cells that possess them. CXCR3 is of high interest as each of its three ligands differs in its biological properties via this receptor, and its ability to regulate the biological function of others. For example, CXCL11 with the higher affinity to CXCR3 is likely to hold anti-inflammatory properties and by leading to receptor internalization makes the receptor less accessible to others. Currently much attention is given to CXCL9 and CXCL10 and their role in the potentiation of anti-tumor CD8+ T cells.

Chemokine receptors support tumor development different complementary pathways: First, many of them function as growth/survival factors either by autocrine pathway, and/or by inducing growth factors production by epithelial cells and stromal cells within the tumor microenvironment. Second, several of them direct the recruitment of bone marrow derived cells that support tumor growth and suppress anti-tumor immunity. Third, chemokine—chemokine receptor interactions are involved in attracting tumor cells to metastatic sites. **Table 1** indicates the involvement of key chemokine receptors in these pathways.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Beyond Cell Motility: The Expanding Roles of Chemokines and Their Receptors in Malignancy

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The anti-tumor activities of some members of the chemokine family are often overcome by the functions of many chemokines that are strongly and causatively linked with increased tumor progression. Being key leukocyte attractants, chemokines promote the presence of inflammatory pro-tumor myeloid cells and immune-suppressive cells in tumors and metastases. In parallel, chemokines elevate additional pro-cancerous processes that depend on cell motility: endothelial cell migration (angiogenesis), recruitment of mesenchymal stem cells (MSCs) and site-specific metastasis. However, the array of chemokine activities in cancer expands beyond such "typical" migration-related processes and includes chemokine-induced/mediated atypical functions that do not activate directly motility processes; these non-conventional chemokine functions provide the tumor cells with new sets of detrimental tools. Within this scope, this review article addresses the roles of chemokines and their receptors at atypical levels that are exerted on the cancer cell themselves: promoting tumor cell proliferation and survival; controlling tumor cell senescence; enriching tumors with cancer stem cells; inducing metastasis-related functions such as epithelial-to-mesenchymal transition (EMT) and elevated expression of matrix metalloproteinases (MMPs); and promoting resistance to chemotherapy and to endocrine therapy. The review also describes atypical effects of chemokines at the tumor microenvironment: their ability to up-regulate/stabilize the expression of inhibitory immune checkpoints and to reduce the efficacy of their blockade; to induce bone remodeling and elevate osteoclastogenesis/bone resorption; and to mediate tumor-stromal interactions that promote cancer progression. To illustrate this expanding array of atypical chemokine activities at the cancer setting, the review focuses on major metastasis-promoting inflammatory chemokines—including CXCL8 (IL-8), CCL2 (MCP-1), and CCL5 (RANTES)—and their receptors. In addition, non-conventional activities of CXCL12 which is a key regulator of tumor progression, and its CXCR4 receptor are described, alongside with the other CXCL12-binding receptor CXCR7 (RDC1). CXCR7, a member of the subgroup of atypical chemokine receptors (ACKRs) known also as ACKR3, opens the gate for discussion of atypical activities of additional ACKRs in cancer: ACKR1 (DARC, Duffy), ACKR2 (D6), and ACKR4 (CCRL1). The mechanisms involved in chemokine activities and the signals delivered by their receptors are described, and the clinical implications of these findings are discussed.

Keywords: atypical chemokine activities in cancer, atypical chemokine receptors, breast cancer, chemokines, classical chemokine receptors

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INTRODUCTION

Leukocyte trafficking is the hallmark of immune integrity, directing the appropriate positioning of lymphocytes and myeloid cells in tissues during acquired immunity, inflammation, and immune homeostasis. These processes are controlled by a very large array of chemotactic molecules—chemokines and others—that act in an orchestrated manner to achieve accuracy, fine-tuning, and precise turn-on/turn-off signals in regulating leukocyte influxes (1–3).

In addition, chemotactic cues that are largely mediated by chemokines and their receptors are strongly involved in the dynamic processes of tumor development and progression. In line with their key roles in regulating leukocyte trafficking under physiological conditions, chemokines and their G protein-coupled receptors (GPCRs) are central players in dictating the types and amounts of leukocytes that are recruited to tumors and metastases (4–7). For example, at relatively early stages of the malignancy process, chemokines can induce the infiltration of lymphocytes that have the potential to raise anti-tumor activities. This is illustrated by Th1 cells, cytotoxic T cells (CTLs) and natural killer cells (NK). However, gradually, the leukocyte contexture at the tumor site is changed in chemokine-driven manner toward an immune-suppressive and pro-inflammatory type, where chronic inflammation turns into a deleterious force that was termed "The Seventh Hallmark of Cancer." Here, the cellular infiltrates can include inflammatory macrophages that are typically regarded as M1 macrophages, as well as M2 macrophages that constitute an important essence of tumor-associated macrophages (TAMs); they can also include neutrophils that are sub-divided to N1 and N2 types and myeloid-derived suppressor cells (MDSCs) of the monocytic (M-MDSCs) or granulocytic (G-MDSCs) subsets. In parallel, T regulatory cells (Tregs) can put their marks on the process, usually contributing to immune suppression (but in other cases they can also be beneficial by inhibiting chronic inflammation) (4–12).

With time, it was realized that other processes that depend on chemokine-induced cell motility can also take place in the tumor context. Well-known are the functions of chemokines in regulating the migration of endothelial cells and their progenitors during angiogenesis; these processes are typically induced by ELR+ CXC chemokines, by CXCL12 and by some of the CC chemokines, but can alternatively be inhibited by non-ELR CXC chemokines. Chemokines also regulate the migration of mesenchymal stem cells (MSCs) to tumor sites, where they can express a variety of pro-cancerous activities and differentiate to tumor-promoting cancer-associated fibroblasts (CAFs). Moreover, chemokines expressed in metastatic sites are key players in attracting to these organs tumor cells that express the corresponding receptors. This venue has been predominantly demonstrated by the CXCL12-CXCR4 axis but also by other chemokine-chemokine receptor pairs, mostly of the homeostatic sub-family. All of these aspects of chemokine activities in cancer have been broadly reviewed, and representative summarizing articles covering these different aspects are provided (4, 5, 7, 13–26).

At this point in time, research on chemokine activities in cancer—that are not directly mediated by cell migration, e.g., in response to chemotactic gradients—is rapidly growing, providing insights to atypical activities of different members of the family in many cancer types. In this review, we describe such non-conventional chemokine activities in cancer, exerted directly on the tumor cells and at the tumor microenvironment (TME). As will be described below, chemokines can promote cancer cell proliferation and survival, reduce their apoptosis and control their senescence; chemokines can also enrich the sub-population of cancer stem cells (CSCs) in tumors, facilitate tumor cell spreading by promoting epithelial-to-mesenchymal transition (EMT) and the release of matrix metalloproteinases (MMPs) in the cancer cells, and increase tumor cell resistance to therapy. In parallel, atypical chemokine-mediated effects can promote interactions between cancer cells and their microenvironment in a way that can also contribute to tumor progression: chemokine activities reduce the efficacy of immune checkpoint blockades (ICBs), induce bone remodeling processes that support the metastatic cascade and enhance the tumor-promoting interactions of cancer cells with stromal cells, such as MSCs and CAFs.

To exemplify the atypical activities of chemokines in cancer, we focus in this review on the effects of inflammatory chemokines that play causative tumor-promoting roles in many malignancies, and whose migration-related functions in cancer have been comprehensively described in many review articles [representative review articles are given as references (4, 5, 7, 13–26)]. In this context, emphasis is put mainly on ELR+ CXC chemokines that act through CXCR1/CXCR2 (e.g., CXCL1, CXCL5, CXCL8), CCL2 that signals mainly *via* the CCR2 receptor and CCL5 with its CCR5 receptor. In parallel, the review also addresses CXCL12—that can exert inflammatory and homeostatic activities—and its CXCR4 receptor, due to their major involvement at all stages of tumor progression. The major findings described herein are summarized in **Table 1**.

In the context of CXCL12 activities in cancer, the review also addresses the roles of CXCR7 which is the other CXCL12 receptor; here, we describe the functions of CXCR7 alone or in the context of CXCR4, in regulating non-conventional cancer-related effects. Although these two receptors can cooperate in mediating tumor-promoting effects, anti-tumor effects of CXCR7 were reported as well, possibly resulting from its being an atypical chemokine receptor (ACKR). Like CXCR7—known also as ACKR3—other ACKRs do not transmit intracellular signals through heterotrimeric G proteins, and regulate many aspects of tumor progression (2, 4, 27). Thus, to broaden the scope of atypical activities of chemokine receptors in cancer, a section of the review is dedicated to atypical roles of additional members of the ACKRs sub-group in malignancy: ACKR1, ACKR2, and ACKR4. A summary of the key findings that are described below on ACKRs in cancer is provided in **Table 2**.

Of the different malignant diseases, breast cancer has been the subject of intensive research that has addressed the way chemokines affect disease progression. Thus, we hereby use breast malignancy to exemplify the non-conventional effects of the above chemokines in the cancer setting. The different

TABLE 1 | Atypical chemokine functions in cancer, mediated by axes of chemokines and classical chemokine receptors.

Axis	ATYPICAL tumor-related activities induced *via* CLASSICAL chemokine receptors*	Effect
CXCR1/CXCR2 CXCL1 CXCL5 CXCL8	• Increases tumor cell proliferation, viability and anchorage independent cell growth • Reduces cancer cell apoptosis • Down-regulates tumor senescence; Increases senescence, which is accompanied by elevated pro-metastatic potential • Enriches the CSC sub-population • Elevates EMT properties and tumor cell invasion • Increases MMP production by cancer cells • Promotes chemoresistance and endocrine resistance of tumor cells • Elevates the expression of inhibitory immune checkpoints (PD-L1) by cancer cells and immune cells • Reduces the efficacy of immunotherapy • Promotes osteoclastogenesis and bone damage • Drives forward pro-cancerous tumor-stroma interactions	Pro-cancerous
CCR2 CCL2	• Increases breast tumor proliferation and survival • Reduces cancer cell apoptosis • Elevates tumor cell invasion (including *via* CCL2 that is released by senescent tumor cells) • Enriches the CSC sub-population • Elevates EMT properties and tumor cell invasion • Promotes endocrine resistance of tumor cells • Reduces the efficacy of immunotherapy • Promotes osteoclast differentiation and bone resorption • Drives forward pro-cancerous tumor-stroma interactions	Pro-cancerous
CCR5 CCL5	• Increases tumor cell proliferation (particularly in the context of hormonal stimulation) • Elevates tumor cell invasion (including *via* CCL5 that is released by senescent fibroblasts) • Enriches the CSC sub-population • Elevates EMT properties and tumor cell invasion • Elevates the expression of inhibitory immune checkpoints (PD-L1) by cancer cells • Reduces the efficacy of immunotherapy • Drives forward pro-cancerous tumor-stroma interactions - • Inhibits tumor cell proliferation • Promotes the efficacy of ICBs (*via* recruitment of T effector cells)	Mostly pro-cancerous
CXCR4 CXCL12	• Increases tumor cell proliferation • Induces EGFR transactivation in cancer cells • Elevates collective invasion and elevates survival of non-senescent cells (*via* CXCL12 released by senescent tumor cells) • Enriches the CSC sub-population • Elevates EMT properties and tumor cell invasion • Increases MMP production by cancer cells • Promotes endocrine resistance of tumor cells • Elevates the expression of inhibitory immune checkpoints (PD-L1) by cancer cells • Reduces the efficacy of immunotherapy • Promotes (together with TGFβ) fibroblast transition to CAFs and drives forward pro-cancerous tumor-stroma interactions	Pro-cancerous

*The Table summarizes the effects of axes established between chemokines and their classical receptors (that signal via heterotrimeric G proteins) on atypical cancer-related activities (that are not directly mediated by cell motility). *Most of these findings were obtained in breast cancer studies, as described in the text. CAFs, Cancer-associated fibroblasts; CSC, Cancer stem cells; EGFR, Epithelial growth factor receptor; EMT, Epithelial-to-mesenchymal transition; ICBs, Immune checkpoint blockades; MMPs, Matrix metalloproteinases; TGF, Transforming growth factor. The dashed line separates the pro-malignancy activities of CCL5, which mostly dominate its effects in cancer (above the line), from its anti-malignancy roles (below the line).*

published studies on chemokine roles in breast cancer addressed so far primarily two subtypes of disease: (1) The highly aggressive triple-negative (TNBC) subtype in which the tumors are negative for the expression of hormone receptors and lack HER2 amplification; these tumors commonly develop resistance to chemotherapy; (2) The luminal-A subtype in which the tumors express estrogen/progesterone receptors (but not amplified HER2) and are hormone-responsive; this disease subtype is treated by endocrine therapies and is considered as having the best prognosis out of all breast cancer subtypes (28, 29). Of note, some of the aspects are relatively newly investigated, thus not much information is available in breast cancer; in these cases the scope is expanded to other cancer types as well. Together, the findings presented in this review address the multifaceted impact that chemokines may have in cancer, through functions that are beyond the typical motility-mediated levels described so far.

ATYPICAL CHEMOKINE ACTIVITIES EXERTED ON CANCER CELLS

Tumor Cell Growth, Survival and Senescence

One of the first indications that chemokines can regulate tumor progression by acting directly on the tumor cells came from

TABLE 2 | Tumor-related activities, mediated by atypical chemokine receptors.

Receptor	Tumor-related activities induced *via* ATYPICAL chemokine receptors*	Effect/s
ACKR1 (DARC, Duffy)	• Inhibits tumor cell proliferation and increases tumor cell senescence • Interferes with CXCR2-induced STAT3 activation in cancer cells • Reduces MMP production by tumor cells • Leads to reduced microvessel density • Single nucleotide polymorphisms affect angiogenesis, tumorigenesis and lung metastasis	Anti-cancerous
ACKR2 (D6, CCBP2)	• Inhibits tumor cell proliferation • Reduces cancer cell invasion • Reduces the infiltration/activities of tumor-supporting leukocytes (in parallel to lower chemokine levels) • Restricts angiogenesis --- • Elevates EMT properties and tumor cell migration • Prevents anti-tumor activities of NK cells and neutrophils	Anti-cancerous; At times pro-cancerous
ACKR3 (CXCR7)	• Increases tumor cell proliferation, and reduces trail-mediated apoptosis • Induces EGFR activation • Enriches the CSC sub-population • Increases ERα stability and confers insensitivity to endocrine therapy • Leads to increased endothelial cell migration (angiogenesis) --- • Inhibits cell proliferation, possibly through CXCL12 sequestration • Antagonizes the ability of CXCR4-expressing tumor cells to degrade matrix	Mostly pro-cancerous; Anti-cancerous under certain settings
ACKR4 (CCRL1, CCX-CKR)	• Inhibits tumor cell proliferation • Reduces EMT properties and tumor cell migration • Sequesters CC chemokines in tumor xenografts --- • Increases resistance to anoikis • Elevates EMT in tumor cells and modifies tumor cell adhesion (cell-to-cell and to ECM)	Mostly anti-cancerous

The Table summarizes the effects of atypical chemokine receptors (ACKRs) in breast cancer as well as in other malignancies; the findings refer to non-conventional functions (not motility-related) and to other ACKR activities as well. ECM, Extracellular matrix; EGFR, Epithelial growth factor receptor; EMT, Epithelial-to-mesenchymal transition; ER, estrogen receptor; MMPs, Matrix metalloproteinases. For each of the ACKR (ACKR2, ACKR3, ACKR4), the dashed line separates the functions that dominate its effects in cancer (above the line) from its opposing roles (below the line).

early studies in melanoma, where ELR+ CXC chemokines were found to up-regulate tumor cell proliferation. By inhibiting the expression or activities of the chemokines, the different investigations indicated that CXCL1 (MGSA) and CXCL8 up-regulated the proliferation of different melanoma cells (30–33).

Along these lines, CXCL1 as well as CXCL8 have been found to promote the proliferation of breast cancer cells. These two chemokines share high affinity binding to CXCR2, but differ in their ability to activate the CXCR1 receptor; accordingly, in some of the studies inhibitors of both receptors or only of CXCR2 (e.g., repertaxin and SB225002, respectively) were used in order to determine the involvement of these two receptors in mediating such chemokine activities. In parallel, other inhibitory measures were used in order to down-regulate the chemokine/s or their receptors, and the opposite approach of over-expression was also used to determine the roles of these chemokine axes in breast cancer progression. Together, these publications indicated that ELR+ CXC chemokines—derived from autocrine or paracrine sources—induced signaling through CXCR1/CXCR2, leading to increased tumor cell proliferation, viability and anchorage independent cell growth; the chemokines also reduced the levels of tumor cell apoptosis, and inhibition of these chemokine pathways caused cell cycle arrest. In some of the studies, the chemokines were not potent in regulating such growth-related parameters when they acted alone but they have intensified the impacts of other regulators of cell growth, such as IL-6 and chemotherapy (34–40).

In essence, similar growth-stimulating regulatory modes were also reported for the inflammatory CC chemokines CCL2 and CCL5. Here, interesting connections were found between CCL2-CCR2 and estrogen responsiveness and activities: CCL2 activated estrogen receptor α (ERα) through PI3K/Akt/mTOR signaling to elevate breast tumor cell division (41); another facet of CCL2-estrogen interactions was revealed when stimulation of luminal-A breast tumor cells by estrogen has led *via* twist activation to elevated production of CCL2, then giving rise to increased proliferation of the cancer cells (42). Another study found that CCL2 binding to CCR2 has led through MEK and ERK activation to increased cancer cell survival, partly through activation of the Rho pathway (43). In parallel, CCL2 has elevated the levels of PCNA+ cancer cells and has also shifted the cell cycle from G2-M to G1-S in association with SRC and PKC activation in TNBC cells (44). The effects of the CCL2-CCR2 axis were noted not only on breast tumor cells of different subtypes (e.g., TNBC and luminal-A) but also in mammary intra-ductal injection models that mimicked the ductal carcinoma *in situ* (DCIS) stage of disease. In this system, CCL2 provided by fibroblasts has activated CCR2 that was expressed by transformed breast cells, leading to their increased proliferation and reduced apoptosis. The opposite result was obtained when CCR2 was down-regulated in the malignant cells. These changes were noted in cells within DCIS lesions, accompanied by reduced lesion size when CCR2 expression was reduced (45).

Parallel studies on CCL5 demonstrated its ability to induce small increases in breast tumor cell proliferation; in one of the research systems, such CCL5 activity was mediated by CCR5-dependent mTOR activation (46–48). CCR5, a major CCL5 receptor, was targeted in several studies by maraviroc, leading to controversial results in terms of tumor cell proliferation (48–51), which possibly reflect the use of different model systems and/or the ability of CCL5 to activate CCR1 and CCR3 in addition to CCR5. Cooperativity between CCR5-related pathways and other elements was revealed when maraviroc—that did not act alone to prevent tumor cell survival—potentiated the effect of IL-6-directed inhibition in reducing tumor cell proliferation. Of interest is the fact that in contrast to these culture experiments, maraviroc has led to significant inhibition of tumor metastasis in animal studies (49, 51), possibly reflecting the ability of CCR5 to promote breast malignancy by additional pro-tumorigenic properties, such as those that depend on cellular migration.

Increased tumor cell proliferation and growth were also found to be exerted by CXCL12 and its two receptors, CXCR4 and CXCR7/ACKR3, primarily in the context of hormonal stimulation. Studies of luminal-A breast cancer cells, that by definition are responsive to estrogen, demonstrated that the hormone induced the expression of CXCL12 and of CXCR4 in the tumor cells, leading to enhanced tumor cell growth, and also gave rise to EGFR transactivation and then to increased DNA synthesis (52–54). Along the same lines, following EGF stimulation a CXCR7/ACKR3-mediated process of EGFR activation was revealed (possibly through β-arrestin scaffold), leading to increased tumor cell proliferation (55). Additional research in this direction provided evidence to complex roles for CXCR7/ACKR3 and for its interactions with CXCR4 in regulating the proliferation and growth of breast tumor cells. On one hand, it was found that the expression of CXCR7/ACKR3 by breast tumor cells has provided growth advantages to luminal breast tumor cells (at times even when CXCR4 was not active in this respect), and reduced trail-mediated apoptosis in such cells (56, 57). Moreover, CXCR7/ACKR3-expressing cells increased the proliferation of CXCR4-expressing tumor cells (58), and silencing experiments of CXCR4 or CXCR7/ACKR3 demonstrated that each of the two receptors elevated tumor cell growth and that the joint impact of both receptors together was stronger than of each alone (59). However, another study demonstrated different roles for CXCR4 and CXCR7/ACKR3 in regulating estrogen-dependent growth of luminal breast tumor cells, where CXCR4 enhanced cancer cell growth and CXCR7/ACKR3 over-expression inhibited cell proliferation, possibly through CXCL12 sequestration (60).

A complementary subject that is related to tumor cell survival concerns the roles of chemokines in regulating cellular senescence; this process, in which cells cannot enter cell cycle and their proliferation is halted in a permanent manner, has major roles in controlling cancer progression (61, 62). Senescent cells are metabolically active and secrete many proteins, identified as senescence-associated secretory phenotype (SASP), which includes many pro-inflammatory factors, of which a predominant factor is CXCL8 (62–64).

Although chemokine-induced senescence of tumor cells may limit tumor growth, it is possible that such growth-restraining processes may be overcome by chemokine-induced pro-malignancy activities such as tumor cell growth or invasion. The dual roles of chemokines in the senescence context are nicely exemplified by a study on human pituitary tumor-transforming gene 1 (PTTG-1)-driven expression of CXCL1 and CXCL8 in breast tumor cells. In this study, it was demonstrated that activation of CXCR2 has induced senescence in luminal-A breast tumor cells and limited tumor growth and metastasis; but in parallel, the pro-metastatic potential of the cancer cells was elevated when they were co-injected with PTTG-1-over-expressing MCF-7 cells, by creating a metastasis-promoting TME (65). Whereas, this study indicated that signaling *via* CXCR2 has increased the senescence of luminal-A breast tumor cells, in another study opposite findings were found, demonstrating that CXCR2 down-regulated senescence of breast tumor cells, including of the luminal-A subtype (66). In this respect, it was found also that fibroblast-derived SASP induced EMT in non-aggressive breast tumor cells, with direct roles of CXCL8 + IL-6 in promoting tumor cell invasiveness (67). Similarly, CCL2 that was released by senescent melanoma cells increased tumor cell invasion (68) and CCL5 derived from age-senescent fibroblasts elevated the proliferation of prostate epithelial cells (69). Along these same lines, CXCL12 that was present in SASP of senescent papillary thyroid carcinoma (PTC) cells played key roles in inducing collective invasion of the cancer cells and in increasing the survival of non-senescent PTC cells, in a CXCR4-dependent manner (70).

Chemokines released by senescent cells can also impact the type of leukocytes entering the tumor site, thus dictating the effects of the immune contexture on tumor fate. For example, CCL2 produced by oncogene-induced senescent hepatocytes had the potential to induce the recruitment of immature myeloid cells that could differentiate to macrophages, which cleared senescent tumor cells; but when the cancer has been fully established, immature myeloid cells that were recruited by CCL2-mediated signals, inhibited the anti-tumor activities of NK cells and led to increased tumor growth (71). The connection between senescence, chemokines and NK cell activities was also demonstrated in a mouse model of liver carcinoma, when inducible p53 expression has increased tumor cell senescence *via* induction of CCL2, leading to recruitment of NK cells expressing anti-tumor functions (72).

Cancer Stem Cells

Stemness is an essential trait of malignancy, whereby a small proportion of cancer stem cells (CSCs; called also tumor-initiating cells) can generate a heterogeneous tumor cell population; the CSC sub-population is often increased following treatment and therefore is considered fundamental in development of therapy resistance (73, 74). In breast cancer, CSCs are usually defined by the $CD44^{+}/CD24^{-/low}$ phenotype, and/or as being positive for the activity of the ALDH1 enzyme which is recognized by elevated proportion of an ALDEFLOUR+ cell population; often, elevated extent/size of tumor spheroids (mammospheres) is also considered a potential marker for enrichment of CSCs (74, 75).

ELR+ CXC chemokines such as CXCL1 and CXCL8, as well as their CXCR1/CXCR2 receptors, have been demonstrated

to be significant factors in promoting CSC enrichment in breast cancer. In line with findings demonstrating that CXCL8 increased the ALDEFLOUR+ population and spheroid formation in breast cancer cells (76), blockade of CXCR1, *in vitro* or *in vivo* decreased the ALDEFLOUR+ population and reduced tumor growth and metastasis; this CXCR1-mediated effect on CSC viability depended on Akt activation (77). In parallel, CXCL1 arriving from TAMs was found to promote the $CD44^+/CD24^-$ sub-population and formation of tumor spheroids in human TNBC cells (78). From the mechanistic aspect, the cross-talk between chemokine receptors and the Erb-pathway may contribute to generation of CSCs in breast cancer. This possibility is exemplified by the fact that CXCR1/2 inhibition by the antagonist SCH563705 has given rise to inhibition of spheroid formation in HER2+ breast tumor cells, and inhibition of HER2-mediated signaling by lapatinib or siHER2 has led to inhibition of CXCR1/2-dependent CSC-spheroid formation (79).

With respect to clinical relevance, a recent study indicated that CXCL8 neutralizing antibodies abrogated the ability of paclitaxel and gemcitabine to elevate CSC levels in breast cancer (tumor spheroids and ALDH-expressing cells). Here, the induction of CXCL8 by chemotherapy was mediated by HIF signaling *via* ROS-dependent expression (80). Similar findings, supporting the roles of CXCL8 and its receptors in generating CSC when breast cancer cells are exposed to chemotherapy, were found when neutralizing antibodies to CXCL8 or the CXCR1/2 inhibitor reparixin inhibited the generation of $CD44^+/CD24^-$ cells, ALDH-expressing cells and spheroid formation following paclitaxel treatment. In this study, treatment of mice with reparixin decreased the number of tumor-initiating cells, which was originally increased in the tumor as a result of chemotherapy administration (81). Another study indicated that when CXCR1 was inhibited and has led to reduced generation of spheroids and their volume, paclitaxel has further augmented this effect (35).

The two inflammatory CC chemokines, CCL2 and CCL5 were also found to elevate the generation of CSCs. This was evidenced by a CCL2-promoted formation of primary and secondary tumor spheroids that contained more self-renewing CSCs (82), and by the fact that stimulation of breast tumor cells with CCL5 increased the $CD44^+/CD24^-$ sub-population (47). These CCL5-enriched CSCs expressed higher levels of the corresponding receptor CCR5 and were able to invade more than non-CSCs, ability that was abrogated by inhibition of CCR5 (47). In another study, CCR5-expressing breast cancer cells demonstrated higher potency in forming mammospheres *in vitro* and in initiating tumor formation *in vivo*, than cells not expressing the receptor (83).

Another important axis in this respect is CXCL12-CXCR4, as demonstrated in the luminal-A subtype of breast cancer. Overexpression of CXCL12 in breast cancer cells elevated the proportion of $CD44^+/CD24^-$ cells, of ALDH-expressing cells, as well as the expression of stemness markers such as Oct4, nanog and sox2 (84). Along these lines, CXCR4-expressing tumor cells demonstrated higher ability to form mammospheres than CXCR4-negative cells (85); like CXCR4, CXCR7/ACKR3 was found to play key roles in promoting the CSC sub-population, as indicated by reduced levels of $CD44^+/CD24^{low}$ cells, of ALDH-expressing tumor cells and of Oct4 and nanog expression following down-regulation of CXCR7/ACKR3 (56). Following co-culturing of the tumor cells with CAFs, a process that has led to increased production of CXCL12, CXCR4 inhibition has reduced the formation of spheroids that were enriched with $CD44^+/CD24^-$ cells (86). Additional findings connected chemokines with CSCs and resistance to therapy by demonstrating that CXCR4 signaling was required for the generation of cells with CSC characteristics out of tamoxifen-resistant luminal-A breast tumor cells (87).

Metastasis-Promoting Functions: EMT and MMPs

A major paradigm in the context of chemokine-directed site-specific metastasis is that in response to chemokines that are expressed at specific organs, tumor cells that express the corresponding receptors migrate and home to these sites. Such processes were well-exemplified for the CXCL12-CXCR4 pair, as well as for other chemokine axes in a very large number of malignant diseases [summarized for example in (23, 25, 26)]. In parallel, irrespective of directing cancer cells to defined organs in the course of metastatic spread, chemokine-induced cytoskeleton re-organization and tumor cell migration/invasion were reported in many tumor systems and were strongly connected to the ability of the cancer cells to acquire a more aggressive phenotype.

Within the scope of the current review article, we wish to expand the discussion beyond such direct chemokine activities that promote tumor cell migration and invasion, and elaborate on other chemokine-induced functions that can promote cancer cell spreading and metastasis, such as EMT and MMP release. Indeed, the arena of chemokine activities was expanded toward direct abilities of chemokines to promote in the tumor cells mesenchymal properties; the mesenchymal characteristics of cells undergoing EMT include properties such as elevated expression of vimentin and of specific transcriptional repressors (such as twist, snail, slug and zeb) alongside with reduced E-cadherin expression. As mesenchymal properties generally facilitate motility, often independently of chemotactic gradient-mediated processes, in some of the studies the elevated levels of EMT were connected to increased tumor cell migration and invasion.

For example, a recent study demonstrated that CXCL1 derived from TAMs elevated EMT properties in luminal-A and TNBC breast tumor cells, in a NF-κB-mediated process that has led to activation of SOX4 (88). Another study indicated that through the activities of the transcription factor Brachyury that has led to CXCL8 up-regulation in breast tumor cells, EMT processes were increased in adjacent cancer cells. Accordingly, CXCL8 induced tumor cell invasiveness through a Brachyury-dependent process (89). With relevance to obesity-related aspects of breast cancer, CXCL8 that was induced *via* the PI3K/Akt-mediated pathway was found to mediate the EMT-inducing effects of leptin and its ability to increase tumor cell invasion (90). Similar roles for CXCL1/CXCL8 and their receptors in inducing EMT were implicated in several other publications of breast tumor cells (66, 78, 91, 92).

In parallel, CCL2 activities *via* CCR2, as well as CCL5-induced signaling were demonstrated to contribute to increased EMT and twist expression, at times accompanied by increased tumor cell invasion in breast cancer cells (93–96). Similar findings were obtained for the CXCL12-CXCR4 axis, when over-expression of CXCL12 or constitutively active CXCR4 have led to reduced E-cadherin levels, accompanied with up-regulation of slug, vimentin and fibronectin or with switch toward elevated expression of cadherin 11 (84, 97, 98). Mechanistic analyses indicated that over-expression of CXCL12 in breast tumor cells has led to E-cadherin reduction through activation of the NF-κB pathway (84) and by up-regulation of β-catenin expression (98). As before, CXCL12-CXCR4-induced EMT-related properties in the cancer cells were often accompanied by increased tumor cell migration or invasion (84, 97, 98).

In parallel to the EMT-inducing properties of chemokines, they also were implicated in up-regulation of other processes that can promote metastasis, such as the release of MMPs that facilitate cancer cell spreading through extracellular matrix (ECM) components during extravasation or intravasation in the course of tumor cell dissemination. For example, twist up-regulated the expression of functional MMPs by non-transformed and transformed breast cells through CXCL8 and CCL5 activities (99–102). Other chemokines (CXCL1, CCL9) were also connected to induction of MMPs in breast tumor cells (102, 103). Elevated production of functional MMP2 and MMP9 was detected in breast tumor cells following CXCL12 stimulation, in the context of CXCR4 expression (104, 105). Addressing the roles of CXCR7/ACKR3, the other CXCL12 receptor, the study of murine breast tumor cells demonstrated that CXCL12 has induced the functional expression of MMP9 through CXCR7/ACKR3 *in vitro* and that CXCR7/ACKR3 inhibition has led to reduced tumor growth and MMP9 expression in tumors *in vivo* (106). In contrast, the research of rat mammary adenocarcinoma cells demonstrated that the ability of CXCR4-over-expressing cells to degrade matrix was antagonized by simultaneous co-expression of CXCR7/ACKR3 (107).

Chemoresistance and Endocrine Resistance

A major obstacle in cancer therapy is intrinsic resistance to therapy or resistance that is acquired due to many different mechanisms, some of which taking place in the cancer cells following their interactions with TME elements. Being a part of the TME, chemokine axes were found to increase chemoresistance and resistance to endocrine therapy. In line with the fact that CSCs often stand in the basis of resistance to therapy (75, 108, 109), chemokine activities that increase the CSC sub-population may eventually also reduce tumor cell response to treatments, and the two processes may thus be connected [as reported for example in (81)].

To date, key roles were identified in breast cancer for CXCR1/CXCR2 and their CXCL1/CXCL8 ligands in promoting resistance to chemotherapeutic drugs such as doxorubicin and paclitaxel. By taking different measures to modify the expression of chemokine receptors or of the chemokines themselves, evidence was provided to the ability of this chemokine axis to directly promote chemoresistance *in vitro* and in animal studies (34, 66, 110, 111). Actually, *in vivo* studies demonstrated the benefit of co-administration or sequential treatment by chemotherapy and by inhibitory measures directed to CXCR1/CXCR2 on the volume of breast tumors, on their ability to metastasize, on neovascularization and on repopulation of the tumors by drug-resistant cells (34, 66, 81, 110–112). Along these lines, a study by Massagués and colleagues demonstrated that CXCR2 inhibitors that were administered to mice prior and in the course of chemotherapy, sensitized the tumor cells to the cytotoxic effects of the drugs. This study has revealed a regulatory loop in which genotoxic stress created by chemotherapeutic drugs limited the survival of breast tumor cells, but the expression of tumor necrosis factor α (TNFα) was also increased and has led to elevated production of CXCL1/2 by the tumor cells; these chemokines recruited CXCR2-expressing CD11b+ Gr1+ myeloid cells which in turn acted *via* S100A8/9 factors to promote the viability of tumor cells that expressed CXCR2. Myeloid cells recruited by CXCL1/2 thereby enhanced viability and chemoresistance in the cancer cells (113). Other members of the chemokine receptor family, such as CCR5 and CXCR4, were also noted as chemoresistance-mediating factors in breast cancer, acting to increase DNA repair [CCR5; (83)] or to elevate tumor cell proliferation and reduce sensitivity to chemotherapeutic drugs through induction of interleukin 1 (IL-1) by MSCs [CXCR4; (114)].

In addition, chemokines were reported as potential regulators of endocrine therapy in breast cancer. It was recently demonstrated that CCL2 derived from TAMs has led to elevated endocrine resistance in luminal-A breast cancer cells, through the activation of the PI3K/Akt/mTOR cascade (115). Important roles for the CXCL12-CXCR4 axis in this aspect were also reported, demonstrating that CXCL12 has induced the activation of the two estrogen receptors—ERα and ERβ–and these processes were down-regulated when CXCR4 was inhibited (54). Moreover, this same study demonstrated that CXCR4 activation has led to increased ERβ activities in the presence of tamoxifen treatment, altogether suggesting that CXCL12-induced CXCR4 activation enabled ERβ to promote down-stream signaling that may overcome inhibition by endocrine therapy. Roles for CXCR4 in resistance to endocrine treatments were also demonstrated when CXCL12 administration has increased the volumes of tumors generated by luminal-A breast tumor cells in mice treated by the estrogen receptor antagonist Fulvestrant (116). Along these same lines, it was found that CXCR7/ACKR3 increased the stability of ERα and conferred insensitivity to tamoxifen in luminal-A breast cancer cells (117).

To conclude this part of the review, the findings presented above emphasize the significant involvement of chemokines in up-regulating multiple tumor-enhancing aspects, where they act directly on the cancer cells to promote many levels of the malignancy process. By promoting tumor cell proliferation and survival, CSC enrichment, EMT induction, MMP production and therapy resistance, chemokines can elevate cancer establishment at the primary site as well as tumor cell dissemination to remote organs and the generation of metastases.

ATYPICAL CHEMOKINE ACTIVITIES EXERTED AT THE TUMOR MICROENVIRONMENT

Immune Checkpoints and Their Blockade

As noted above, by virtue of their chemotactic properties toward leukocytes, chemokines have a strong impact on the content of immune and inflammatory cells at the TME, as has been broadly investigated and reviewed [e.g., (4–12)]. However, a relatively novel topic of research that is still in its early phases indicates that chemokines can impact immune activities not only by directly dictating the leukocyte landscape at tumor/metastatic sites but also by affecting aspects related to inhibitory immune checkpoints—such as the PD-1/PD-L1 axis—and their blockade.

In this respect, an interesting research aspect is the ability of chemokines to up-regulate or stabilize the expression of PD-L1 by tumor cells, thus indirectly reducing the efficacy of anti-tumor immune functions. For example, CXCL8, whose source was in gastric cancer-derived MSCs, has induced the expression of PD-L1 in gastric cancer cells. The process was mediated by STAT3 and mTOR activation, leading to tumor cell resistance against CD8+ T cell-mediated killing (118). Along these lines, CXCL5 that was secreted by CAFs promoted the expression of PD-L1 by several colorectal cancer cell lines; here, CXCL5 signals were transferred through CXCR2, leading to PD-L1 up-regulation *via* a PI3K-dependent process. In mouse models the potential relevance of these findings to tumor progression was supported by the fact that the expression of CXCR2 and p-Akt was coordinated with PD-L1 expression in the tumors, and by immune-suppressive activities of the CAFs (119). Evidence in the same direction was obtained in colorectal cancer, where macrophage-derived CCL5 acted through p65-STAT3 complexes that bound the COP9 signalosome promoter, giving rise to PD-L1 stabilization and up-regulation in the cancer cells. These CCL5-mediated activities have led to enhanced escape from T cell-mediated immune activities (120).

Similarly, chemokines can up-regulate the expression of inhibitory immune checkpoints by myeloid cells at the TME. For example, in gastric cancer CSF-2 elevated the production by macrophages of CXCL8, which then elevated PD-1 expression by TAMs, giving rise to inhibition of CD8+ T cell activities (121). Also, in a recent study it was demonstrated that CXCR2+ MDSCs that were recruited to mouse mammary tumors by ELR+ CXC chemokines such as CXCL1/2, up-regulated the expression of immune checkpoint molecules (e.g., PD-1, CTLA-4, LAG3) by CD4+ and CD8+ T cells; they have also induced T cell exhaustion, partly through interferon γ (IFNγ) (122).

The above findings demonstrate that chemokine activities can lead to elevated expression of molecules that participate in down-regulation of immune activities in cancer. This way, chemokines can reduce the efficacy of therapeutic approaches using ICBs in cancer; accordingly, it was suggested that inhibition of chemokine axes may potentiate the efficacy of ICBs and augment anti-tumor immune activities that restrain tumor growth and metastasis. Obviously, such chemokine/chemokine receptor-targeting modalities can affect not only immune checkpoint regulation by chemokines, but also the impact of chemokines on the leukocyte landscape at tumor/metastatic sites. Indeed, in gastric cancer tissue samples obtained following treatment by the CXCR1/2 inhibitor reparixin, reduced levels of proliferating tumor cells were noted, alongside with reduced presence of PD-L1+ macrophages and increased fraction of CD8+ T cells (121). In rhabdomyosarcoma, where MDSCs of the CXCR2+ CD11b+ Ly6G^{high} phenotype mediated local immune suppression, the efficacy of antibodies directed to PD-1 was augmented when tumor-bearing mice had myeloid cells deficient in CXCR2 (123). Essentially similar findings were noted in a mouse model of lung cancer, where PMN-MDSCs reduced T cell proliferation, and treatment of mice with antibodies to CXCL5—which is a key chemoattractant of such MDSCs—has reduced the proportion of PMN-MDSCs and elevated the efficacy of anti-PD-L1 in increasing the survival of mice (124).

Similar findings demonstrating the importance of chemokine-induced MDSC infiltration in regulating the efficacy of ICB activities were provided in a recent study of anti-PD-1-resistant gliomas. Here, the survival of mice was increased when the CCR2 antagonist CCX872 was used, and further improvement was obtained upon treatment with anti-PD-1 (125). Increased benefit in terms of tumor inhibition was also obtained by measures that down-regulated CCR1 or CCL5 activities, combined with ICBs directed to PD-1 or PD-L1; here again, major roles were revealed for TAMs and MDSCs as targets whose inhibition potentiates the activities of ICBs (126, 127).

In parallel, improved activities of ICBs upon chemokine/chemokine receptor inhibition, manifested by reduced presence of immuno-suppressive/myeloid cells and increased immune surveillance was noted when the CXCL12-CXCR4 axis was down-regulated. In a model of metastatic breast cancer, in which ICBs were combined with the CXCR4 inhibitor plerixafor (AMD3100), the drug had multiple effects including reduction of fibrosis and of Tregs alongside with increased infiltration of CTLs; also, the inhibition of CXCR4 by plerixafor increased the effect of dual treatment of mice by anti-PD-1 + anti-CTLA-4, in terms of metastatic inhibition and prolonged survival (128). Following their studies demonstrating that plerixafor decreased the intra-tumor infiltration of Tregs, Poznansky and colleagues have recently combined plerixafor with anti-PD-1 in ovarian cancer models. The joint inhibitory modality had higher efficacy than each measure alone in enhancing infiltration and function of effector T cells, increasing memory T cells, and reducing the presence of MDSCs in the tumors. Compared with treatment by each element alone, the combined therapy was more potent in inhibiting tumor growth and increasing survival of mice (129). Along the same lines, anti-PD-L1 synergized with the CXCR4-inhibiting drug plerixafor in killing tumor cells in a mouse pancreatic model (130). Additional reports have also provided evidence to the benefit provided by co-inhibition of the CXCL12-CXCR4 axis and ICBs in other animal model systems, through regulation of immune activities (131–133).

In this context, it is important to mention that chemokines can induce intra-tumor infiltration not only of deleterious leukocyte sub-population but also of immune cells that can exert anti-tumor activities. Under these circumstances, it is expected that the chemokines themselves, rather than their inhibition, will collaborate with ICBs and increase their potency. One such example was demonstrated by the cooperativity between CCL5—known as chemattractant of T effector cells (2)—and CXCL9 that can act through CXCR3 to recruit Th1 cells, CD8+ T cells and NK cells (2). In this study, it was found that tumor-derived CCL5 has recruited effector T cells to tumors; the release of IFNγ by T cells has increased the production of CXCL9 by macrophages, leading to increased immune surveillance of the tumors. Moreover, tumors that expressed CCL5 and CXCL9 were responsive to anti-PD-1 treatment, in contrast to tumors that did not (134). These findings illustrate the importance of non-ELR CXC chemokines such as CXCL9 and CXCL10 that act through CXCR3 to recruit anti-tumor immune cells and can also have anti-angiogenic activities. Although when CXCR3 is expressed by tumor cells its ligands may promote tumor growth (16, 135–137), many studies demonstrated that these chemokines exert immuno-angiostatic activities on the TME (16, 138, 139). Thus, it is expected that in different tumor systems, chemokines acting through CXCR3 would act alongside with ICB activities, as was suggested by several published reviews (16, 138, 139).

Bone Remodeling

The bone is a preferred metastatic site which generally marks poor prognosis in many malignancies, including breast cancer. Following tumor cell invasion to bones, their metastatic colonization at the site is accompanied by bone remodeling, reflecting an inappropriate balance between bone-forming osteoblasts and bone-resorbing osteoclasts that leads to bone destruction. This osteolytic process, driven by several mediators such as RANKL and others, serves well the needs of the metastasizing cancer cells and contributes to their outgrowth in this niche (140, 141).

Many members of the chemokine family were found to contribute to bone remodeling with and without connection to malignancy (140–144). In this context, under physiological conditions, CXCL8 can promote RANKL production by osteoblasts and collaborate with it to increase the generation of osteoclasts (141, 145–147). Thus, when cancer cells acquire the ability to express CXCL8, it is assumed that they will enhance osteoclastogenesis during the metastatic process. Indeed, several studies support such a scenario: when breast tumor cell-derived supernatants promoted osteoclastogenesis, as indicated by increased generation of TRAP+ cells out of peripheral blood mononuclear cells, the process was down-regulated by inhibitors of CXCL8 or its receptors (146, 148, 149). Moreover, CXCL8 produced by tumor cells or by CXCL8-transgenic mice gave rise to elevated osteolysis *in vivo*, whereas antibodies to CXCL8 prevented bone damage and elevated the survival of mice (146). It was also found that breast tumor cells produced semaphorin D, which has increased CXCL8 production by osteoblasts and the levels of TRAP+ expressing cells *in vitro*. In parallel, *in vivo* studies indicated that shRNA-mediated inhibition of semaphorin D expression in breast tumor cells has led to reduced levels of metastasis and longer survival, accompanied by reduced formation of osteolytic skeletal lesions (147). In this context, it is interesting to note that analysis of plasma from breast cancer patients identified significant correlation between increased CXCL8 levels and elevated degree of bone resorption as well as with bone metastasis, supporting key roles for CXCL8 in this setting (146).

In parallel, CCL2 was found to be expressed at the site of metastatic breast cancer localization in the bones (150) and breast cancer-derived CCL2 has acted through CCR2 to promote osteoclast differentiation and contributed to bone metastasis (151). Moreover, it was found that MAPK11 (p38β) activation in breast cancer cells has given rise to elevated CCL2 production, which then contributed to increased bone resorption (152).

The picture seems to be more complex in the case of axes including CCL5 and CCL3, and their shared receptors CCR1 and CCR5 in regulating bone remodeling in cancer (141, 144, 153). Information also is lacking regarding the roles of the CXCL12-CXCR4 pair in this context. This axis is of particular interest because CXCL12 was found to promote bone resorption under physiological conditions, and in parallel is a leading factor in driving tumor cell homing to the bones in a very large number of malignancies. In view of these dual roles of CXCL12, it is expected that the CXCL12-CXCR4 pair will be instrumental in regulating osteoclastogenesis and osteolysis in tumors, but currently this aspect was mainly investigated in multiple myeloma (141, 144) and needs to be extensively addressed in future studies.

Pro-cancerous Tumor-Stroma Interactions

MSCs and CAFs are major components of the tumor stroma that in many malignancy-related systems (including breast cancer), although not all, have been strongly connected to increased tumor-promoting functions. The activities of MSCs and CAFs at the tumor setting include induction of EMT, angiogenesis and more, and are affected by their interactions with the TME, primarily the pro-inflammatory TME (154–165).

The sources of CAFs are diverse, including resident fibroblasts, adipose MSCs and bone marrow-derived MSCs that differentiate to CAFs at the tumor site (155, 166–169). In addition to their roles as chemoattractants of MSCs to tumor sites, which have been reviewed previously [e.g., (155, 158, 170, 171)], chemokines can stand in the basis of tumor-stroma interactions that promote cancer progression. MSCs and CAFs can establish direct contacts with the cancer cells; in addition, the tumor cells and stromal cells can affect each other indirectly by the release of soluble mediators. In the scope of this article we hereby elaborate on studies demonstrating the roles of chemokines in regulating tumor-stroma interactions, which eventually affect the pro-malignancy functions of one or both cell types, or of the TME.

In this respect, our recent study indicated that interactions formed between TNBC cells and MSCs under the influence of the pro-inflammatory cytokine TNFα have given rise to increased lung metastasis in a breast cancer animal model system (159). In this system, we demonstrated that cell-to-cell contacts between the tumor cells and the stromal cells, as

well as soluble mediators, have led to increased production of inflammatory chemokines; this process was further promoted by stimulation of tumor-stroma co-cultures with TNFα and IL-1β. One of the key chemokines that was potently elevated due to such inflammation-driven TNBC-MSC cross-talks was CXCL8. Our findings indicated that following TNFα stimulation of tumor-stroma co-cultures, NF-κB activation has led to CXCL8 induction, partly through a Notch1-dependent process. Then, CXCL8 that was expressed at elevated levels played direct roles in promoting angiogenesis as well as tumor cell migration and invasion (159, 160). Here, it was interesting to note that similar elevations in CXCL8 production were not evident when the partners in the co-cultures were luminal-A breast tumor cells instead of TNBC cells (159). In another study of TNBC cells, Jin et al. demonstrated that in response to factors released by the tumor cells, CAFs and macrophages released CXCL8 that promoted the proliferation and migration of the cancer cells in a process that depended on CXCR2 activation (40). Other members of the ELR+ CXC chemokine family, CXCL1 and CXCL2 were found to be induced in normal mammary fibroblasts that gained a CAF phenotype in response to tumor cell-derived osteopontin, that also promoted tumor growth (172).

In breast cancer it was also demonstrated that CCL2 levels were higher in stromal cells derived from tumors compared to normal breast tissues and that fibroblast-derived CCL2 contributed to tumor growth and metastasis *in vivo* (82, 162). More so, physical as well as indirect interactions between breast tumor cells and cancer-associated stromal cells (and not normal mammary stromal cells) have contributed to elevated levels of CCL2 (82, 159, 161, 162). It was found that under such interactive settings, CCL2 has contributed to elevated tumor cell migration, generation of CSCs and angiogenesis (82, 161). CCL2 production in the tumor-stroma setting was connected to pro-inflammatory conditions: pro-inflammatory stimuli (TNFα and IL-1β) have strongly up-regulated the release of CCL2 by tumor-stroma co-cultures (159), and in parallel CCL2 has induced an inflammatory TME in mice, demonstrated by high localization of macrophages and increased stroma and collagen density in mice (173). As with CXCL8, a connection to the Notch pathway was revealed for CCL2 in mediating tumor-stroma interactions, when CCL2 produced by fibroblasts that were activated in the presence of breast cancer cells has elevated CSC levels in cancer cells by activating the Notch pathway, and has induced the expression of Notch1 by the cancer cells (82).

Strong interactions through the CCL5-CCR5 axis were also reported to exist between breast tumor cells and stromal cells, mainly MSCs. For example, osteopontin was found to be a key factor released by breast tumor cells, binding αVβ3 integrins expressed by MSCs and then giving rise to elevated levels of CCL5 production. This interactive loop gave rise to increased metastasis in mice that were administered with tumor-MSC co-cultures, through osteopontin and CCL5-dependent mechanisms (163). Also, the study by Weinberg and colleagues demonstrated that CCL5 released by MSCs has acted through CCR5 to promote breast tumor cell migration, invasion and metastasis in animal studies (164). In the same spirit, CCL5 produced by MSCs has acted on CCR5-expressing breast tumor cells, leading to the release of CSF-1 and then to increased accumulation of macrophages and MDSCs in tumors. Accordingly, CCR5 inhibition by siRNAs gave rise to reduced metastasis formation, accompanied by decreased levels of CSF-1-expressing macrophages and CD11+ Ly6C+ MDSCs (174). In another study, it was demonstrated that the conditioned medium (CM) of MSCs increased the expression levels of two CCL5 receptors, CCR5 and CCR1 by murine breast tumor cells; in line with these findings the inhibitor met-CCL5 inhibited the migration of the cancer cells in response to MSC-derived CM (102). Cooperativity of CCL5 with IL-6 was also noted when CM of MSCs promoted breast tumor cell migration (175).

When coming to address the roles of CXCL12 in mediating tumor-stroma interactions in breast cancer, the majority of studies indicated that this chemokine or CXCR4 stand in the center of tumor-promoting cross-talks between cancer cells and stromal cells. CAFs constituted a major source for CXCL12, and produced it in higher levels than normal fibroblasts or fibroblasts located in seemingly healthy tissues that were adjacent to patient tumors (176–178). Moreover, CXCL12 production was elevated when CAFs or MSCs interacted directly or indirectly with breast tumor cells (86, 179). Under such conditions, CXCL12- and CXCR4-mediated signaling elevated a large number of pro-cancerous characteristics and functions in breast cancer: tumor cell proliferation and invasion, generation of CSCs and angiogenesis (through attraction of endothelial progenitor cells), as well as tumor growth and metastasis *in vivo* (86, 176–180).

However, the roles of CXCR4 in mediating tumor-stroma networks that promoted breast malignancy were put to question in several other studies. In one of these works it was demonstrated that CM of MSCs elevated the proliferation of breast tumor cells not through CXCR4, but rather *via* CXCR7/ACKR3 (181). Another study indicated that CXCR7/ACKR3 expression by breast cancer cells was down-regulated by MSC-derived CXCL12 (possibly due to ligand-dependent receptor internalization), and under these conditions, metastasis was reduced. However, when TGFβ was introduced, CXCL12 production by the MSCs was reduced, CXCR7/ACKR3 expression levels remained intact and metastasis was elevated (182). Here, it is worth noting that unlike these findings, a positive feedback loop between TGFβ and CXCL12 was found in relation to CAFs, when TGFβ and CXCL12 up-regulated each other's expression in mammary CAFs, and both contributed to the gradual process of fibroblast transition to CAFs (177).

Overall, the research on the impact of chemokines at the TME has been largely expanded beyond their fundamental roles in regulating the migration of leukocytes, endothelial cells and stromal cells. Currently, it is becoming evident that chemokines affect the ability of immune cells to exert anti-tumor activities by regulating the expression of immune checkpoints and the activity of ICBs. Moreover, chemokines facilitate metastasis by remodeling bone structure and by mediating pro-tumorigenic interactions that take place between cancer cells and stromal cells. Evidently, all of these activities largely contribute to elevated tumor progression and may lead to reduced patient survival.

Activities of Atypical Chemokine Receptors in Cancer

Between others, in the previous sections of the article we described non-conventional activities of CXCL12, taking place *via* its two receptors, CXCR4 and CXCR7/ACKR3. Whereas, CXCR4 was characterized as a typical tumor-supporting receptor, many lines of evidence indicated CXCR7/ACKR3 can have pro-metastatic effects but in specific settings it can act in an opposite manner. The tumor-restricting activities of CXCR7/ACKR3 may be connected to the fact that unlike CXCR4, it is an atypical chemokine receptor (and thus was given the additional name ACKR3). ACKRs lack the classical heterotrimeric G protein-mediated signaling pathway, they control responses to a variety of CXC and CC chemokines and they are expressed by various cell types. This class of receptors was originally considered as "decoy receptors" that sequester chemokines from the microenvironment, thereby inhibiting the effects of chemokines at different settings. In parallel, recent studies indicate that ACKRs regulate cancer progression by their chemokine-sequestering functions, as well as by other mechanisms.

Aside from controlling motility-related aspects in cancer, such as tumor cell invasion, endothelial cell migration (angiogenesis) and eventually tumor progression *in vivo* (183–187), CXCR7/ACKR3 regulates non-conventional cancer-related activities. The array of atypical cancer-regulating functions of CXCR7/ACKR3—when it acted alone or in the context of CXCR4—were discussed in the previous sections of the article, as appropriate. The intriguing findings on CXCR7/ACKR3 illustrate the importance of the ACKR subgroup in general; thus in this section of the manuscript we discuss the atypical roles of additional key ACKRs in malignancy: ACKR1 (DARC, Duffy), ACKR2 (D6, CCBP2) and ACKR4 (CCRL1, CCX-CKR).

ACKR1 is a highly promiscuous receptor that binds a large number of chemokines, from the CC and CXC sub-families, mainly those of the inflammatory sub-group. Through internalization, ACKR1 plays key roles as depot of chemokines; accordingly, its constitutive expression by venular endothelial cells results in low availability of ELR+ CXC chemokines that promote angiogenesis (2, 4, 27). By sequestering ELR+ CXC chemokines as well other members of the family, and possibly also *via* other pathways, ACKR1 usually acquires anti-tumorigenic effects. Indeed, ACKR1 was strongly connected to improved outcomes in breast cancer as well as in several other malignancies, at times upon co-expression with ACKR2 or ACKR4 (188–192). Accordingly, ACKR1 was causatively linked to reduced tumor growth and metastasis in animal models, and ACKR1 single nucleotide polymorphisms that were related to chemokine sequestration affected angiogenesis, tumorigenesis and lung metastasis (193–195). The anti-tumor activities of ACKR1 were mediated not only by reducing microvessel density, but also by inhibiting atypical cancer-related activities, such as MMP9 production and tumor cell proliferation, as well as increasing tumor cell senescence (192, 194–196); from the mechanistic perspective, it was demonstrated that ACKR1 caused anti-tumor effects by interfering with CXCR2-induced STAT3 activation in pancreatic adenocarcinoma cells (192). Moreover, in prostate cancer ACKR1 expressed by vascular endothelial cells interacted with tetraspanin KAI1 (CD82) on tumor cells, leading to decreased DNA synthesis and induction of tumor cell senescence (195); in parallel, it was found that melanoma-expressed KAI interacted with endothelial cell-expressed ACKR1 preventing CXCL8-inudced gap formation in endothelial cells and leading to tumor cell senescence (197).

Very much like ACKR1, ACKR2 binds and internalizes inflammatory chemokines, leading them to degradation; however, unlike ACKR1, the activities of ACKR2 are limited mostly to CC chemokines that signal through CCR1 and CCR5 (2, 4, 27). By virtue of its expression by lymphatic endothelial cells and by tumor cells, ACKR2 plays key roles in preventing inflammatory conditions in a variety of settings, and was mainly referred to as tumor-restricting receptor (2, 4, 27). In breast cancer and in many other malignancies, ACKR2 expression was causatively linked to down-regulation of tumor growth and metastasis (198–202). In many cases, ACKR2 inhibited the infiltration of tumor-supporting leukocytes, angiogenesis or tumor cell invasion; often, these processes were accompanied by reduced levels of the relevant chemokines and competition with CCR2-mediated signaling (198–202). However, through the same mechanisms of CCL2/CCR2 inhibition, ACKR2 was also reported to prevent the activities of beneficial leukocyte sub-populations, such as NK cells and neutrophils that are cytotoxic against tumor cells (203, 204).

By functioning in these manners, the tumor-restricting but also the tumor-promoting activities of ACKR2 resulted from the expected, motility-related functions that are involved in cancer progression. However, in addition, ACKR2 was found to restrict tumor progression by regulating atypical chemokine activities at the tumor setting, such as cancer cell proliferation (198, 199). However, in this context of non-conventional cancer-related chemokine activities, it is possible that ACKR2 may also have pro-tumor effects. This is illustrated by a recent study demonstrating that fibroblast-derived CXCL14 acted in the context of tumor cell expressed ACKR2, activating the ERK pathway and inducing EMT, elevating migration and lung colonization by luminal-A breast cancer cells (205).

ACKR4 joins ACKR2 in sequestering CC chemokines, but with preference to homeostatic members of the family: CCL19, CCL21, CCL25 (and with lower affinity CCL13). Resembling ACKR1 and many of the functions of ACKR2, ACKR4 demonstrates predominantly tumor-restricting effects, and was positively correlated with patient survival rates in several cancers; at times, ACKR4 expression was inversely correlated with the expression of chemokines in patient materials (188, 191, 206–208). Supporting these findings are the results of a breast cancer report, demonstrating that ACKR4 overexpression by breast tumor cells inhibited tumor growth and lung metastases, and decreased the expression of mouse CCL19, CCL21, CCL25 and CXCL13 chemokines in xenografts (208). In this study, and in reports on hepatocellular carcinoma and nasopharyngeal carcinoma, the tumor-restricting effects of ACKR4 were mediated by inhibition of cancer cell proliferation, EMT and/or migration, through abrogation of the relevant chemokine axes

(207–209). Conversely, although smaller primary tumors were formed when CXCR4-over-expressing mouse TNBC cells were administered to mice, the cancer cells acquired increased ability to colonize the lungs; in this case, ACKR4 promoted EMT in the tumor cells, reduced adherence of the cancer cells to each other and to ECM proteins, and increased their resistance to anoikis (210).

The findings discussed above suggest that CXCR7/ACKR3 may have definite tumor-enhancing roles but also can acquire anti-malignancy effects in certain settings. In contrast, ACKR1, ACKR4 and in many cases also ACKR2 exert anti-tumor functions at conventional migration-related processes as well as at non-conventional aspects. Thus, specific ACKRs may have important implications toward chemokine-designed therapies in cancer.

DISCUSSION

The chemokine family includes a very large number of members, which regulate physiological and pathological conditions at many different levels. In cancer, specific members of the family that act under defined situations can exert anti-tumor activities, e.g., by recruiting cytotoxic immune cells to tumors or down-regulating angiogenesis. However, in many of the malignancies, a large number of the chemokines demonstrates the ability to promote tumor growth and progression and dominate the setting by giving rise to elevated tumor aggressiveness.

Their prime function as inducers of cellular motility has set chemokines and their receptors as major regulators of malignancy-related events that depend on cell migration in response to chemotactic signals (**Figure 1**—"Typical" chemokine activities in cancer). These events include primarily the following mechanisms: (1) Chemokines control in spatial and temporal manners the migration of different leukocyte subsets and their recruitment to tumors/metastases, thus having a strong impact on leukocyte content at these sites. Accordingly, the equilibrium between immune cells that recognize tumor antigens *vs.* pro-inflammatory/immune-suppressive cells has major roles in determining the fate of the developing tumor and of metastases; (2) Signals delivered by specific chemokines promote the migration of endothelial cells and their progenitors, thus supporting the essential process of angiogenesis; (3) Chemokines recruit MSCs from other tissues, primarily bones, to tumors/metastases; there, the MSCs can express many tumor-promoting activities on their own, and also after their transition to CAFs; (4) Cancer cells that express chemokine receptors respond to their corresponding chemotactic cues at remote sites, thus chemokines form an important venue that through directed tumor cell migration dictates site-specific metastasis.

At first, reports on chemokine activities that are beyond regulation of cell motility were sporadic; however, with time it became clear that chemokines influence cancer cells and the TME at many levels that are not directly connected to cell migration, eventually supporting the establishment of primary tumors and cancer cell spreading to metastatic sites. Such chemokine functions were exemplified in this article by focusing on the activities of several tumor-promoting chemokines of the CXC and CC sub-families—mainly of the inflammatory arm—in breast cancer (**Figure 1**—"Atypical" chemokine activities in cancer). We have illustrated such atypical roles of chemokines in promoting tumor cell proliferation and survival and in parallel in regulating the senescence of cancer cells; in enriching tumors with CSCs; in promoting the mesenchymal phenotype of cancer cells (EMT) and the release of MMPs; and in elevating resistance to chemotherapy and to endocrine therapy. In parallel, atypical chemokine activities lay out the basis for a tumor-supportive TME by modulating immune checkpoints and interfering with their blockade, by facilitating bone metastasis through osteoclastogenesis and bone resorption, and by mediating tumor-stroma interactions that promote the pro-cancerous potential of the tumor cells and of their adjacent milieu.

Very likely, the different levels affected by the chemokines are inter-connected, further amplifying disease progression. First and foremost is the strong connection of chemokines to immune activities: here, the ability of chemokines to regulate the immune and inflammatory contextures of tumors is joined by their ability to promote the expression of inhibitory immune checkpoints and to regulate the efficacy of their blockade. As a result of these joint activities, chemokines may strongly impact the efficacy of ICBs and of other immune-mediated anti-cancer therapies.

Another illustration of integrative chemokine effects at several levels simultaneously is provided when chemokines affect in atypical manners the cancer cells themselves. For example, tumor cell stemness is strongly connected to elevated EMT and to therapy resistance; accordingly, in some of the studies mentioned above chemokines were found to promote some of these processes concurrently. Similarly, when cancer cells acquire in response to chemokine activities a mesenchymal phenotype that is manifested by EMT-related properties, they often also gain increased ability to migrate and invade. Additional strong connections are revealed when chemokines stand in the center of tumor-stroma interactions. Such interactions, which are mediated by chemokines or lead to their increased production can eventually play key roles in promoting directly the aggressiveness of the cancer cells (proliferation, invasion, therapy resistance *etc.*) and the pro-tumor nature of the TME (for example, recruitment of inflammatory cells, angiogenesis and bone remodeling).

The research of some of these topics is only at its beginning, and evidence of novel aspects that are regulated by chemokines in the course of cancer development and progression are now emerging. These aspects include for example the ability of chemokines to elevate the levels of DNA repair (83), to alter tumor cell metabolism (48, 96, 211, 212), to regulate the localization and retention of dormant cancer cells in the bone marrow (213) and to promote vasculogenic mimicry by tumor cells (38).

The tumor-promoting roles of chemokines in malignancy—through conventional (motility-related) and non-conventional functions—should be carefully considered in the context of tumor heterogeneity. Malignant diseases differ considerably from each other in terms of cause and progression patterns; this is illustrated not only when different cancer types are compared

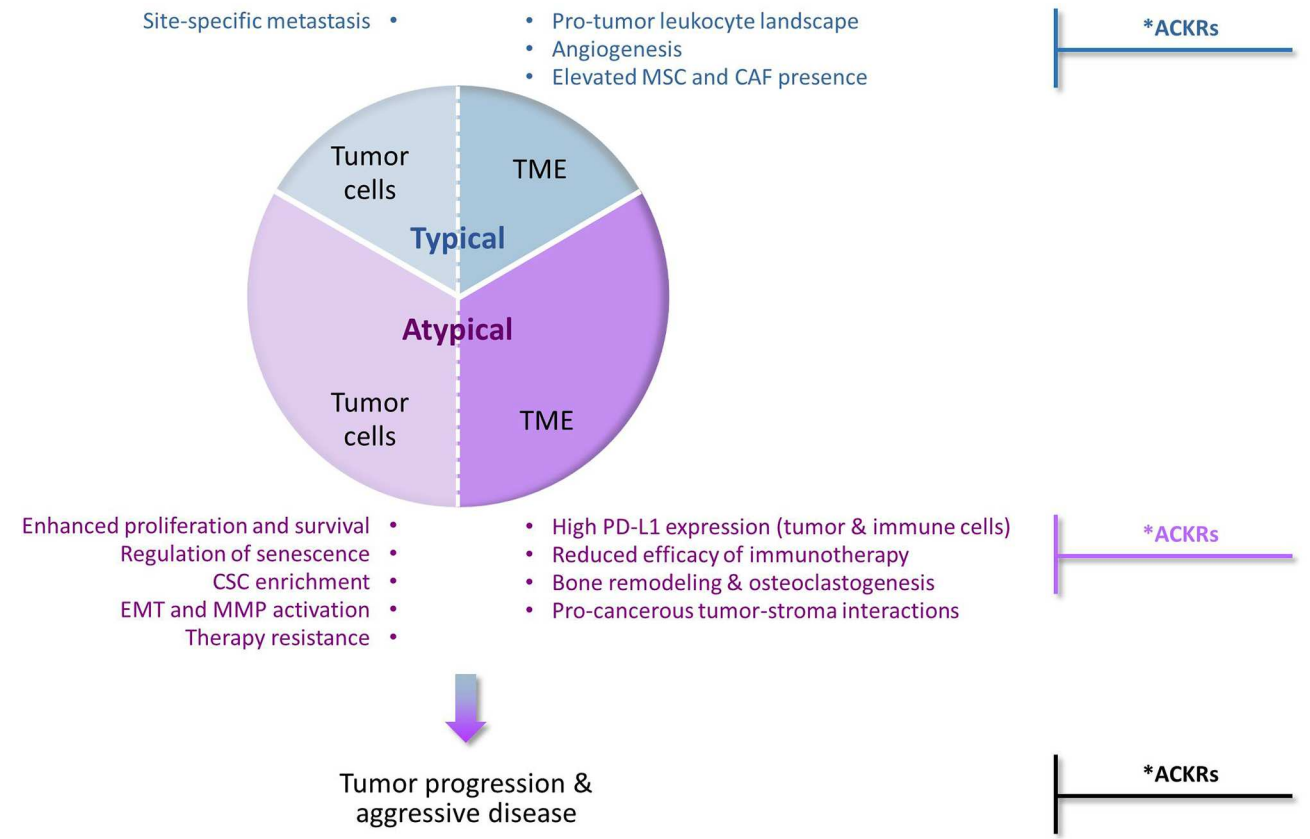

FIGURE 1 | Typical and atypical pro-tumor activities of chemokines and their receptors in cancer. The chemokine family contains many different members, some of which can limit tumor progression for example by inducing the recruitment of cytotoxic immune cells to tumors, or by inducing angiostasis (e.g., CXCR3). However, extensive investigations of chemokine roles in cancer indicate that chemokine activities that promote tumor development and progression are very common and often dominate the malignancy process. Being prime regulators of leukocyte migration in the immune context, chemokines are primarily considered as inducers of cellular motility. Accordingly, chemokine activities that promote tumor progression *via* induction of directional cell motility—of leukocytes, endothelial cells, stromal cells and cancer cells—are regarded in the scope of this review as "Typical". Very much like the "Typical" chemokine activities, also those that do not directly affect cellular motility and are thus termed herein "Atypical", can be exerted on the tumor cells or on the TME (tumor microenvironment). By addressing most of these aspects in breast cancer, we emphasize in this review article the atypical activities of chemokines in cancer (thus given a higher proportion in this drawing, but not necessarily so in the actual cancer setting). In the "Typical" part, we mention that typical chemokine-induced migration can lead to homing of cancer cells at specific metastatic sites and to remodeling of the tumor landscape by recruiting leukocytes, inducing angiogenesis through endothelial cell migration, and attracting MSCs that can then differentiate to CAFs. In parallel, in the "Atypical" part, we describe the roles of chemokines in reinforcing (1) the aggressiveness of the tumor cells, by elevating tumor cell proliferation and survival, regulating senescence, enriching tumors for CSCs, inducing EMT and MMP production and elevating resistance to chemotherapy and endocrine treatments; and (2) the pro-metastatic nature of the TME, by interfering with the activities of ICBs, remodeling the bone niche by elevating osteoclastogenesis and bone resorption, and promoting tumor-stroma interactions that contribute to elevated malignancy. Together, all of these chemokine effects—typical and atypical—eventually lead to elevated metastasis and worsening of disease course. *Anti-metastatic activities in cancer: Under specific settings, the pro-metastatic activities of chemokines can be inhibited by other chemokines that act through classical chemokine receptors (e.g., CXCR3) or by atypical chemokine receptors (ACKRs). Such tumor-inhibitory activities of ACKRs have been well-documented for ACKR1 and ACKR4, whereas ACKR2 was mostly reported as an anti-malignancy element, with pro-tumor activities reported as well. In contrast, CXCR7/ACKR3 is mainly characterized as tumor-enhancing factor, although its roles in malignancy are complex, can be anti-tumorigenic and often reflect its interactions with CXCR4, the other receptor that binds CXCL12.

but also within the same disease, as is the case in breast cancer (e.g., the TNBC *vs.* luminal-A subtypes). In addition, a very challenging aspect in this regard is intra-tumor heterogeneity which is observed in many tumor types (73). Obviously, when chemokine roles in cancer are investigated, these aspects of inter-tumor and intra-tumor heterogeneity need to be considered.

More so, chemokine roles in cancer and their relevance for therapy need to be regarded in the broader scope of "chemokine heterogeneity". Here, one needs to consider tumors and metastases as multi-chemokine organs, thus the impact of chemokines on tumor progression depends much on their relative amounts, temporal/spatial localization at the tumor site/metastatic organs and the expression of corresponding receptors by cancer cells, leukocytes, endothelial cells, and stromal cells. Eventually, these parameters will dictate to a large degree which of the chemokine/s will dominate the overall malignant setting, *via* its/their typical and atypical activities, affecting the tumor cells or the TME.

The aspect of "chemokine receptor heterogeneity" adds even more to the complexity of chemokine roles in cancer, by demonstrating the ability of ACKRs to control cancer-related activities. Whereas, CXCR7/ACKR3 has predominantly pro-metastatic roles in cancer, ACKR1 and ACKR4 demonstrate mainly tumor-restricting effects. Here, they very often sequester and thus prevent the activities of pro-metastatic chemokines at many levels, conventional and non-conventional. Thus, certain ACKRs may represent a balancing arm of the chemokine field in controlling cancer progression and in this regard, should be considered as a therapeutic tool in cancer.

To conclude, our understanding of the roles of chemokines in cancer progression has been largely expanded with time. There are circumstances in which chemokines can interfere with the malignancy cascade, as illustrated by the immune-angiostasis functions of non-ELR CXC chemokines and by the tumor-restricting activities of ACKRs in certain settings. However, often the motility-driven and atypical activities of chemokines dominate the scene, leading to enhanced disease course and poor prognosis. Pre-clinical and initial clinical studies suggest that inhibitors of defined chemokines or of their receptors may be effective as therapeutic measures in cancer, primarily when they are joined by other modalities such as chemotherapy or ICBs [as illustrated above and also discussed in (4, 20, 21, 214–219)]; however, to reach the point in which chemokines or their receptors are used as targets in cancer therapy, extensive research of their functions, typical and atypical, is needed in the broader context of tumor heterogeneity, chemokine heterogeneity, and chemokine receptor heterogeneity at the tumor bed and in metastases.

AUTHOR CONTRIBUTIONS

DM and NE are equal contributors in bibliography search and in preparing defined article sections. AB-B was responsible for the entire setup and structure design of the manuscript, and contributed to all stages of manuscript preparation.

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Multiple Roles for Chemokines in Neutrophil Biology

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Chemokines are recognized as the most critical mediators for selective neutrophil recruitment during inflammatory conditions. Furthermore, they are considered fundamental regulators of neutrophil mobilization from the bone marrow (BM) to the bloodstream and for their homing back at the end of their life for apoptosis and clearance. However, chemokines are also important mediators of neutrophil effector functions including oxidative burst, degranulation, neutrophil extracellular trap (NET)osis, and production of inflammatory mediators. Neutrophils have been historically considered as a homogeneous population. In recent years, several maturation stages and subsets with different phenotypic profiles and effector functions were described both in physiological and pathological conditions such as infections, autoimmunity, and cancer. The aim of this review is to give an overview of the current evidence regarding the role of chemokines and chemokine receptors in neutrophil biology, including their possible role in neutrophil maturation, differentiation, and in defining emerging neutrophil subsets.

Keywords: atypical chemokine receptors, chemokine receptors, chemokines, neutrophils, neutrophil subpopulations

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INTRODUCTION

Neutrophils are essential players of the innate immune response and their function is strictly dependent on their trafficking. Indeed, under homeostatic conditions they have a short half-life and a constant circulating neutrophil number has to be maintained to ensure their timely recruitment during inflammation. Neutrophils produced in the bone marrow (BM) are released in the bloodstream and, after the aging process they return to the BM, spleen, lungs, or liver for their clearance. During inflammation neutrophils extravasate quickly in the tissues and consequently there is an increased neutrophil release from the BM (1, 2).

Many studies have demonstrated that neutrophils can sense different classes of chemoattractants such as leukotrienes, anaphylatoxins, and formylated peptides that are primary activators during inflammation. Moreover, they express several chemokine receptors that finely tune their directional migration in homeostatic conditions and mediate their effector functions such as oxidative burst and neutrophil extracellular trap (NET) activation and release, once extravasated in tissues (3). The ability to respond to multiple chemokines represents a mechanism to finely control neutrophil recruitment and activation providing a first line defense (4).

It is now clear that several BM and circulating neutrophil subpopulations exist with different expression patterns of chemokine receptors (4, 5). In this review, the role of chemokines in neutrophil biology will be discussed, trying to dissect their role in neutrophil differentiation, heterogeneity, and activation.

CHEMOKINES ACTING ON NEUTROPHILS

Neutrophils respond to a multitude of chemokines via binding to their cell-surface receptors, called chemokine receptors belonging to a family of seven-transmembrane domain G protein–coupled receptors. Chemokines are divided into four structural groups (C, CC, CXC, and CX_3C) based on the spacing of two conserved cysteine residues at their N terminal (6).

Neutrophils are generally thought to be limited in expression of chemokine receptors, consisting predominantly of the CXC group. Indeed, neutrophils express high levels of the CXC chemokine receptors CXCR1 and CXCR2 that bind ELR^+-CXC chemokines (containing a glutamate–leucine–arginine motif before the amino-terminal CXC motif). hCXCR2 is a promiscuous receptor binding seven different chemokines CXCL1, 2, 3, 5, 6, 7, and 8 (7). hCXCR1 is very similar to CXCR2 (78% of sequence homology) but only binds CXCL6 and CXCL8 (8).

CXCR1, CXCR2, and their ligands were also identified in the murine system but there are many differences with their human counterpart (9, 10). First, in mice there are fewer ELR^+-CXC chemokines and the homolog of CXCL8 is missing. Its analogs in mice are CXCL1 (KC), CXCL2, and CXCL5 (LIX). Mouse CXCR1 was only recently cloned and shown to be a functional receptor for the mouse chemokines CXCL5/LIX and CXCL6/GCP-2 (11).

CXCR2 activates many G-protein–induced signaling cascades: PI3K/Akt inducing cell migration, PLC/PKC that affects cell function, and mitogen-activated protein kinase (MAPK)/p38 that promotes cell proliferation and survival (12). CXCR1 and CXCR2 signaling activates the NF-κB pathway, inducing the transcription of many cytokines among which are CXC chemokines that amplify neutrophil recruitment (13). Very interestingly, only CXCR1 activates phospholipase D (PLD), involved in radical oxygen species (ROS) generation. This difference is due to a slower rate of internalization of CXCR1 compared to CXCR2 (14, 15).

Recent results outline differential roles among ELR^+-CXC chemokines in neutrophil extravasation and migration. CXCL2 is almost exclusively produced by neutrophils and, in addition to CXCR1 and CXCR2, it binds the atypical chemokine receptor ACKR1. ACKR1, expressed by endothelial cell (EC) junctions on post capillary venules, works as a CXCL2 presenter guiding neutrophils to extravasation sites. Otherwise, CXCL1 mediates neutrophil adhesion and intraluminal crawling on inflamed ECs and sub-EC crawling on pericytes (16).

Neutrophils express also the CXC receptor CXCR4, essential for their life cycle. BM neutrophils express high levels of CXCR4, which is mainly intracellular because of high CXCL12 production by mesenchymal cells inducing its internalization (17). The interaction between CXCR4 and CXCL12 retains a large pool of neutrophils into BM and spleen (18). This is demonstrated by CXCR4 genetic deletion in murine myeloid cells that results in depletion of the BM pool and in concomitant increase of circulating neutrophils (19). On the contrary, WHIM (warts, hypogammaglobulinemia, infections, myelokathexis) patients, who bear a gain of function mutation in CXCR4, have a chronic neutropenia for increased neutrophil BM retention (20). Circulating neutrophils express low levels of CXCR4 that is upregulated in senescent neutrophils before apoptosis, promoting their homing back to the BM and other organs for clearance (21). Studies *in vitro* demonstrated that CXCR4 is downregulated by type I cytokines such as interferon-γ (IFN-γ), IFN-α, granulocyte-macrophage colony stimulating factor (GM-CSF), and granulocyte-colony stimulating factor (G-CSF) (22).

CC chemokine receptors are barely expressed by BM and circulating neutrophils. When neutrophils are activated by IFN-γ or GM-CSF, they upregulate the expression of CCR1 and CCR3 (23, 24). CCR1 was found necessary for neutrophil recruitment in a murine model of renal immunopathology (25) together with other CC receptors (CCR2, CCR3, CCR5) (26, 27).

CCR2, expression of which was previously supposed to be restricted to monocytes, is important also for neutrophils. It induces neutrophil BM mobilization (28), accumulation in joints of rheumatoid arthritis patients (29), and recruitment to metastatic sites (30, 31). A subpopulation of neutrophils with antigen presenting function expressing CCR6 and CCR7 was also described (32–34).

Finally, neutrophils express one atypical receptor named CCRL2 that, despite being very similar in structure to chemokine receptors, does not bind chemokines. CCRL2 forms dimers with CXCR2 regulating its membrane expression and function (35).

ROLE OF CHEMOKINES IN GRANULOPOIESIS

Neutrophil maturation follows a multistep process called granulopoiesis. The most immature progenitor, the hematopoietic stem cell (HSC), gives rise to multipotent progenitors, the common myeloid progenitors (CMPs) that stimulated with G-CSF give rise to granulocyte-macrophage progenitors (GMPs). In the classical granulopoiesis model, downstream of GMPs there are neutrophil committed progenitors called promyelocytes and myelocytes (36–39). These immature proliferating progenitors are now referred as neutrophil progenitors (NePs) and neutrophil precursors (preNeu) (40); they have been transcriptionally defined and can be identified by fluorescence-activated cell sorting (FACS) analysis (**Table 1**). These unipotent progenitors differentiate into non-proliferating immature neutrophils (previously called metamyelocytes and banded neutrophils) and mature neutrophils (41) (**Figure 1**).

The chemokine system is involved in several aspects of myelopoiesis and granulopoiesis. CXCL12 is constitutively produced by BM stromal cells and provides a retention signal for CXCR4-positive neutrophil committed progenitors and immature neutrophils. G-CSF mobilizes neutrophils through the cleavage of CXCL12 and CXCR4 (42). Beyond BM retention, it is not known if CXCR4 modulate proliferation of NePs as in the case of HSC (43).

CXCR2 signaling, interacting antagonistically with CXCR4, represents a second chemokine axis to regulate neutrophil release from the BM (44). The mobilization of neutrophils from the BM to the blood is determined by the downregulation of CXCR4 and

TABLE 1 | Expression signature of neutrophil progenitors and subpopulations.

	Mouse	Human
HSC	Lin^-, $CD117^+$, Sca-1^+, $CD34^+$, $CXCR4^+$	Lin^-, $CD34^+$, $CD38^-$, $CD45RA^-$, $CXCR4^+$
CMP	Lin^-, $CD117^+$, Sca-1^-, $CD34^+$, $CXCR4^+$, $CCR1^+$, $CCR2^+$	Lin^-, $CD34^+$, $CD38^+$, $CD45RA^-$, $CXCR4^+$, $CCR1^+$, $CCR2^+$
GMP	Lin^-, $CD117^+$, Sca-1^-, $CD34^+$, $CD16/32^+$, $CXCR4^+$, $CCR1^+$	Lin^-, $CD34^+$, $CD38^+$, $CD45RA^+$, $CXCR4^+$, $CCR1^+$
NeP	Lin^-, $CD117^+$, Ly6A/E^-, Siglec F^-, $Fc\varepsilon RI\alpha^-$, $CD16/32^+$, $Ly6B^+$, $CD11a^+$, $CD162^{lo}$, $CD48^{lo}$, $Ly6C^{lo}$, $CD115^-$, $Ly6G^-$, $CXCR4^+$	Lin^-, $CD117^+$, $CD66b^+$, $CD38^{hi}$, $CXCR4^+$
preNeu	Lin^-, $CD117^+$, $CD115^-$, Siglec-F^-, $Gr1^+$, $CD11b^+$, $Ly6G^{lo}$, $CXCR2^-$, $CXCR4^+$	Lin^-, $CD117^-$, $Siglec8^-$, $CD15^+$, $CD34^-$, $CD66b^{hi}$, $CD49d^+$, $CD101^-$, $CXCR2^-$, $CXCR4^+$
Immature neutrophil	Lin^- $CD117^-$ $CD115^-$, Siglec-F^-, $Gr1^+$, $CD11b^+$, $Ly6G^{lo/+}$, $CXCR2^-$, $CXCR4^{mid}$	Lin^-, $CD66b^+$, $CD15^+$, $CD33^{mid}$ $CD101^+$, $CD10^-$, $CD16^{lo/+}$, $CXCR2^-$, $CXCR4^-$
Mature neutrophil	Lin^-, $CD115^-$, $CD11b^+$, $Ly6G^+$, $CXCR2^+$, $CXCR4^-$	Lin-, $CD66b^+$, $CD15^+$, $CD33^{mid}$, $CD101^+$, $CD10^+$, $CD16^{hi}$, $CXCR2^+$, $CXCR4^-$
Aged neutrophil	$CD11b^+$, $CD16/32^+$,$CD62L^{lo}$, $CXCR2^{lo}$, $CXCR4^{hi}$	$CD11b^+$, $CD16^{hi}$, $CD62L^{lo}$, $CD10^+$, $CXCR2^{lo}$, $CXCR4^{hi}$

subsequent upregulation of CXCR2 receptor both in humans and in mice (17, 45).

Other chemokines and chemokine receptors have a role in the process of granulopoiesis and neutrophil release from BM. CCL3 induces the proliferation of CCR1-positive myeloid progenitors even if the *in vivo* relevance of this effect is not evident because CCR1 KO mice do not show significant differences in CMP and GMP proliferation compared to WT (46). CCR2 is expressed by CMPs and exerts a negative control on myelopoiesis (47). In addition, CCR2 mediates mobilization from BM to peripheral blood of myeloid populations such as monocytes and neutrophils (48).

Granulopoiesis is also affected by atypical chemokine receptors. ACKR1, expressed by BM nucleated erythroid cells (49, 50), and ACKR2, expressed by hematopoietic progenitors, control neutrophil differentiation (31). However, it is not known the mechanism by which their control of the chemokine system affects neutrophil differentiation.

Thus, in homeostasis the chemokine receptors CXCR4 and CXCR2 play an essential role in controlling neutrophil retention and release from the BM. CXCR4 and CC chemokine receptors are expressed by neutrophil progenitors, but further research is needed to better understand their role in granulopoiesis.

ROLE OF CHEMOKINES IN NEUTROPHIL HETEROGENEITY

Despite the previous belief that differentiated neutrophils were a homogeneous population, the existence of different circulating subsets was demonstrated in varied health and disease contexts, both in mice and humans (51) (**Figure 1**). A consensus on the phenotype of these subpopulations is still missing and under steady-state conditions heterogeneity may arise mainly from the aging process of circulating neutrophils (52). Indeed, neutrophils oscillate in a circadian manner in numbers, morphology, and phenotype (53, 54). This process is regulated by gut microbiota (55) and is controlled by neutrophils themselves through the circadian expression of the transcription factor Bmal1 that controls the production of CXCL2. In turn, CXCL2 acting on CXCR2 induces neutrophil aging (56).

During inflammatory conditions, increased levels of a neutrophil circulating population that shared characteristics with BM immature neutrophils was described both in mice and humans. These cells express low levels of CD16 and are $CD10^-$ (57–59). The functional properties of this subset are still controversial, they were described having either immunosuppressive activity (60) or promoting T-cell survival and proliferation (57).

Other circulating neutrophils subpopulations were described: olfactomedin 4 (OLFM4)-positive neutrophils in healthy donors (61), T-cell receptor (TCR)–based variable immunoreceptor neutrophils (62), and $CD177^+$ neutrophils during inflammatory diseases both in mice (63) and humans (64).

In addition, a reverse transendothelial migrating neutrophil subset (rTEM) was described in a murine model of sterile injury (65). These neutrophils are $CD54^{hi}$ and, in order to reverse transmigrate into vasculature, downregulate CXCR1. Concomitantly, they upregulate CXCR4 to go into the lungs, before being cleared in BM (66). This subset represents a phenotypically and functionally distinct population different from circulating neutrophils ($CD54^{lo}$ $CXCR1^{hi}$) and express vascular endothelial growth factor receptor (VEGFR) 1, indicating a possible role in angiogenesis (67, 68). Similar cells, with increased levels of CD54 and CD18 and downregulation of CD62L and CXCR1 and 2, were found in patients with chronic inflammatory diseases, suggesting a role of rTME neutrophils in the persistence of inflammation (67). Moreover, around 1% of circulating neutrophils after ischemia-reperfusion were found to be $CD54^{hi}$ and producing ROS into lungs (65). On the contrary, neutrophils that migrate away from the inflammation site in interstitial tissues are called reverse interstitial migration (rIM) neutrophils and are supposed to contribute to the resolution of inflammation. The role of chemokine receptors in this process is still not clear (69).

Finally, in circulation it is possible to identify aged or senescent neutrophils (54, 70, 71). *Ex vivo* aging experiments have shown that neutrophils kept in culture downregulate the expression of CXCR2 (44) and re-express CXCR4 in a

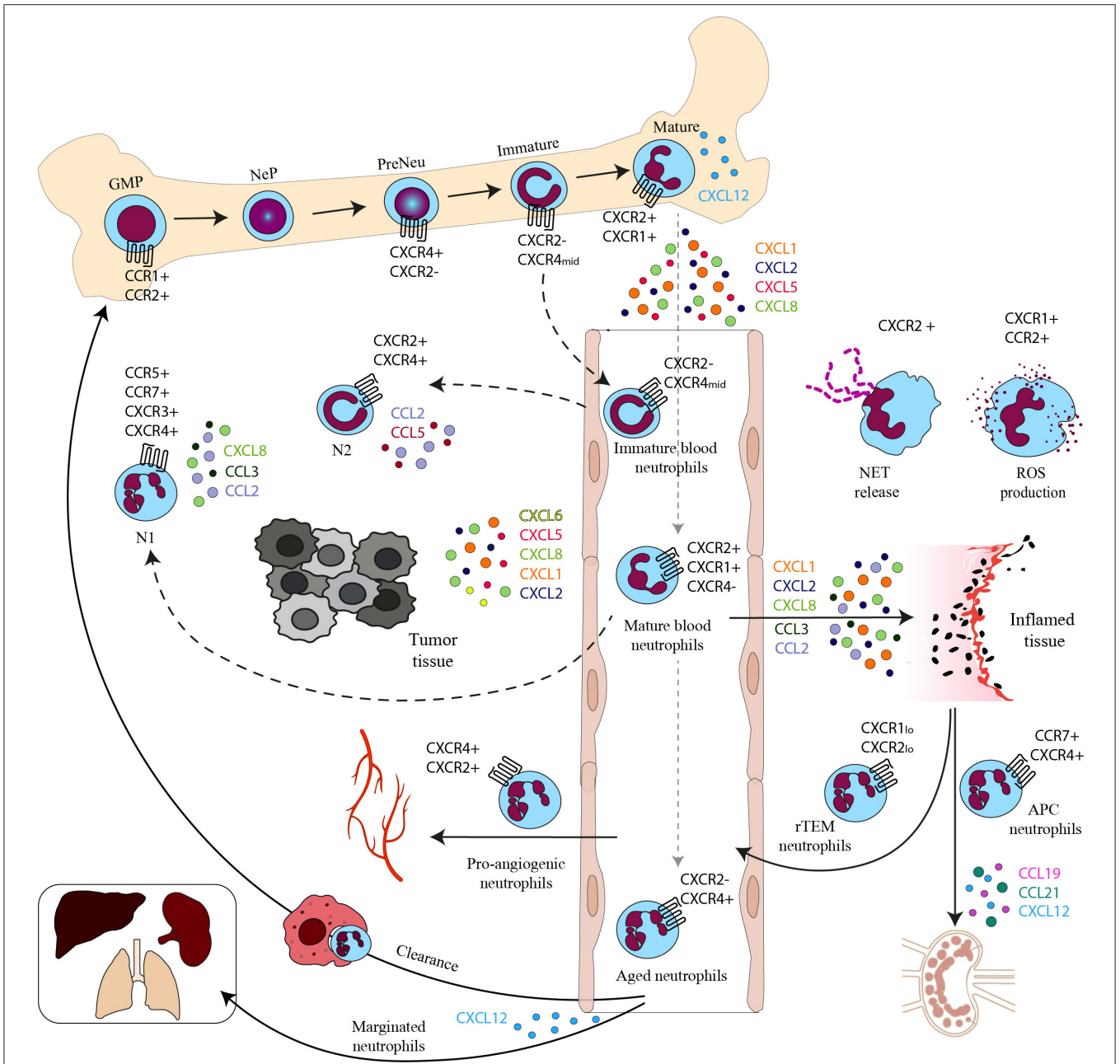

FIGURE 1 | Chemokines and chemokine receptors in the neutrophil life cycle. Neutrophil progenitor proliferation is regulated by CC chemokines binding to CCR1 and CCR2. Neutrophil subpopulations are retained in the BM by the CXCL12–CXCR4 axis. Mature neutrophils are released in the bloodstream upon the upregulation of CXCR2. In the bloodstream, besides mature neutrophils, there are immature neutrophils derived from immature BM neutrophils and senescent neutrophils that upregulate CXCR4 expression and follow the CXCL12 gradient to home back to the BM or other tissues for their clearance. CXCR4 can also drive senescent neutrophils to the lungs, spleen, and liver where they reside as a marginated pool. In the bloodstream there are also rTEM with low levels of CXCR1 and 2. In inflamed tissues activated neutrophils produce ROS on stimulation of CXCR1 and CCR2 and release NETs after engagement of CXCR2. Neutrophils with APC function migrate to draining LNs by the CCR7–CCL19/21 or the CXCL12–CXCR4 axis and proangiogenic neutrophils express high levels of CXCR4 that allows their migration to hypoxic areas. Mature and immature neutrophils migrate in the tumor, where they are referred to as N1 and N2 tumor associated neutrophils (TAN).

time-dependent way (22), suggesting a preferentially homing of senescent cells to the BM in response to CXCL12 (21). In mice aged neutrophils display circadian oscillations and, in addition to high levels of CXCR4, are characterized by an increased surface expression of CCR5 and decreased expression of CD62L (53, 72). CXCR4 upregulation seems involved not only in guiding neutrophils back to the BM but also in their migration within the marrow tissue in order to be engulfed with greater efficacy

by macrophages (17, 19, 53, 54, 72). CCR5 was reported to work as a chemokine scavenger promoting the resolution of the inflammatory response (73). Aged neutrophils were found in lungs, where pulmonary vasculature expresses CXCL12, and this could either supply the pool of circulating neutrophils or respond to injury (45, 68).

New data from single cell sequencing of murine circulating neutrophils confirm the presence of three transcriptionally different neutrophil subpopulations. The first expresses high levels of inflammatory genes and the highest levels of CXCR2 arising mainly from BM mature neutrophils. The second expresses interferon-stimulated genes and derives from BM immature neutrophils. Both populations mature in an aged subset CXCR4 positive with high phagocytic activity and still highly transcriptionally functional (41). The correlation of these subpopulations of neutrophils with the others described in the foregoing is still missing. In addition, the role of chemokines in the mobilization and function of these neutrophil subpopulations is not known. Of relevance, at least in mice, mobilization of immature neutrophils could be CXCR2 independent because they are referred to as CXCR2 negative (44).

Finally, neutrophil heterogeneity has been described in tumors where tumor-associated neutrophils (TANs) can exist in two different functional states: N1 proinflammatory and antitumoral subset and an antiinflammatory tumor promoting N2 population, distinguished for the expression of adhesion molecules, cytokines and inflammatory mediators, chemokines, and chemokine receptors (4, 74). N1 phenotype has been associated with IFN-β polarization both in mice and humans. These cells have an activated phenotype ($CD62L^{lo}$ $CD54^{+}$); express the chemokine receptors CCR5, CCR7, CXCR3, and CXCR4; and produce the proinflammatory chemokines and cytokines: CCL2, CXCL8, CCL3, and interleukin-6 (IL-6). Moreover, this subset has been associated with stimulation of T-cell responses and ROS production (4, 75, 76). In contrast, N2 neutrophils are induced by transforming growth factor- β (TGF-β) stimulation. Protumoral N2 neutrophils display high levels of CXCR4, VEGF, and matrix metalloproteinase 9 (MMP-9) (77), and produce high levels of CCL2, CCL5, neutrophil elastase (NE), cathepsin G (CG), and arginase 1 (78–80).

Therefore, results obtained in preclinical mouse models and in humans suggest that the interplay between CXCR2 and CXCR4 dictates not only BM neutrophil mobilization and retention but also neutrophil diversity in homeostasis. CXCR2 signaling promotes neutrophil aging and CXCR4 guides their homing back to the BM. Furthermore, diversity of tissue infiltrating neutrophils is also associated with a distinct pattern of chemokine receptors; in particular N1 neutrophils express inflammatory CC chemokine receptors important for their effector functions (see later).

ROLE OF CHEMOKINES IN NEUTROPHIL EFFECTOR FUNCTIONS

Neutrophils, once recruited to sites of infection, recognize and phagocytize microbes and then kill pathogens with different cytotoxic mechanisms. These include the production of ROS,

TABLE 2 | Clinical trials with CXCR1 and CXCR2 inhibitors.

Target	Inhibitor	Pathology	Clinical trials	Results
CXCR2	AZD5069	Asthma and bronchiectasis	NCT01704495 NCT01255592	Reduced neutrophils in sputum and lung tissue; no improvement in clinical outcomes
		Advanced solid and metastatic tumors (head and neck carcinoma, prostate cancer, pancreatic cancer)	NCT02499328 NCT03177187 NCT02583477	Not available
	Danirixin (GSK1325756)	COPD	NCT02130193 NCT03250689	Improvements in respiratory symptoms; reduced NET formation
		Viral disease (influenza)	NCT02469298	Termination for emergence of severe adverse events (cardiac failure and respiratory disease)
	SB-656933	Ulcerative colitis,	NCT00748410	No clinical benefit
		Cystic fibrosis	NCT00903201	Improved inflammatory markers in patients' sputum; no change in lung function
CXCR1 and CXCR2	Reparixin	Liver, lung, and kidney transplantation	NCT03031470 NCT00224406 NCT00248040	Attenuated inflammatory reaction and reduced tissue damage
		Islet transplantation in diabetes mellitus type 1	NCT01817959	No improvement in islet inflammation-mediated damage
		Metastatic breast cancer	NCT02370238 NCT02001974	Not available
	Navarixin (SCH 527123, MK-7123)	COPD	NCT01006616	Improved clinical outcomes
		Advanced/metastatic solid tumors (in combination with pembrolizumab)	NCT03473925	Not available
		Psoriasis	NCT00684593	No clinical benefit

the release of antimicrobial peptides, and the expulsion of their nuclear contents to form NETs. Moreover, neutrophils can also shape the immune response interacting with adaptive immune cells (1, 68) (**Figure 1**).

The chemokine system, fundamental for selective neutrophil recruitment in the tissues, also has an important role in the regulation of the effector functions of neutrophils. Engagement of both CXCR1 and CXCR2 induces neutrophil activation but the two receptors have distinct and non-redundant roles in inflammation and infection. Studies with knockout mice proved the importance of mCXCR2 in inflammatory diseases related to neutrophil infiltration and activation (30, 81). On the contrary, mCXCR1 appears dispensable for neutrophil transmigration while necessary for ROS production in *Pseudomonas aeruginosa* and degranulation in *Candida albicans* infections (82, 83). The fundamental role of CXCR1 in fighting infections is further confirmed in humans carrying a genetic variant of CXCR1 (CXCR1–T276) that have increased bacterial infections. Neutrophils taken from these individuals have impaired degranulation and fungal killing ability (83). On the contrary, ROS production induced by the CXCL8–CXCR2 axis on circulating neutrophils has a regulatory function. Indeed, it limits the rolling capability of neutrophils in an autocrine manner by inducing the shedding of CD62L (83).

In inflammatory conditions and after extravasation, neutrophils completely change their chemokine receptor repertoire. They downregulate CXCR2 levels and upregulate inflammatory CC receptors CCR1, CCR2, and CCR5. These receptors activate neutrophil phagocytic activity and ROS production (26, 27, 84). In murine models of breast lung metastasis, CCR2 expression on neutrophils promotes ROS production that kills cancer cells (31, 85).

Release of NETs is induced by CXCR2 activation via Src, extracellular signal-regulated kinase (ERK), and p38/MAPK signaling (86). The CXCL1–CXCR2 axis has been associated to NET formation and neutrophil degranulation in a model of deep vein thrombosis in mice (87) and in circulating and airway mucosal neutrophils of chronic obstructive pulmonary disease (COPD) patients. The use of CXCR2 antagonist in these patients had significantly improved their lung function, even if a direct effect in NETs inhibition was not proved (88). Clinical trials are ongoing for the use of CXCR2 inhibitors in COPD patients (**Table 2**). NETs role in cancer still remains controversial; indeed, they have been associated with both pro- and antitumoral functions (89). In diffuse large B-cell lymphoma, CXCL8-induced NETs promote tumor progression and blocking the CXCL8–CXCR2 axis delays cancer progression in preclinical models (90). CXCR1 and CXCR2 inhibitors show encouraging results in tumor preclinical models when they are used in combination with chemotherapy and checkpoint inhibitors and are now in use in several clinical trials (**Table 2**) (91).

Chemokines acting on neutrophils can also regulate angiogenesis in a direct and indirect way. CXCL1 induces VEGF-A production by neutrophils (92), and neutrophils with an aged-like profile (VEGFR1$^+$, CD49d$^+$, and CXCR4$^+$), recruited to hypoxic areas where CXCL12 is produced, promote angiogenesis by release of MMP9 that cleaves VEGF-A stored in the matrix (93–95).

Of relevance, neutrophils recruited at an inflammatory site orchestrate and polarize the immune response, producing many chemokines. ELR$^+$CXC and inflammatory CC chemokines amplify innate immune cell recruitment. Neutrophils can also promote the recruitment of lymphocytes producing the Th1 chemokines CXCL9, 10, and 11 and the B-cell attracting chemokine CXCL13. On the contrary, TANs exert their immunosuppressive function by producing the Treg attracting chemokine CCL17 (96). Moreover, a subset of activated neutrophils expressing CCR7 and CXCR4 can migrate to lymph nodes (LNs) and act as antigen presenting cells (APC) (97, 98).

According to these results, CXCR2 expression on neutrophils, besides being fundamental for extravasation, induces NET release, while CXCR1 together with CC chemokine receptors CCR1, CCR2, and CCR5 acquired by infiltrated neutrophils promote degranulation and ROS production. Chemokines produced by neutrophils in inflamed tissues amplify and polarize the immune response, and the expression of CCR7 by activated neutrophils promotes their migration to LN, where they can directly act as APC.

CONCLUDING REMARKS

Chemokines and their receptors play multiple and non-overlapping roles in the life span of a neutrophil. CXCR4 has a central role for BM retention of immature neutrophils and BM homing of aged neutrophils. On the contrary, CXCR2 induces neutrophil mobilization from the BM to the bloodstream and has a critical role in neutrophil extravasation, NET release, and the aging process. CXCR1 together with CCR1, CCR2, and CCR5 are important for degranulation and ROS production after extravasation. The production of inflammatory chemokines by neutrophils at an inflammatory site amplifies and polarizes the immune response, and CCR7 and CXCR4 expression guides neutrophil migration to draining LNs for antigen presentation.

Clinical trials using CXCR1 and CXCR2 inhibitors revealed that they are successful in treating patients with chronic diseases (e.g., COPD), whereas their use can be detrimental in patients with viral infection. Therefore, a better understanding of the role of chemokines not only in neutrophil migration but also in diversity, effector functions, and regulation of the immune response is required to develop successful therapeutic strategies.

AUTHOR CONTRIBUTIONS

All authors have equally contributed to this review by writing and critically evaluating the literature.

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MINI REVIEW
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The Chemokine Receptor CXCR4 in Cell Proliferation and Tissue Regeneration

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The CXCR4 receptor upon binding its ligands triggers multiple signaling pathways that orchestrate cell migration, hematopoiesis and cell homing, and retention in the bone marrow. However, CXCR4 also directly controls cell proliferation of non-hematopoietic cells. This review focuses on recent reports pointing to its pivotal role in tissue regeneration and stem cell activation, and discusses the connection to the known role of CXCR4 in promoting tumor growth. The mechanisms may be similar in all cases, since regeneration often recapitulates developmental processes, and cancer often exploits developmental pathways. Moreover, cell migration and cell proliferation appear to be downstream of the same signaling pathways. A deeper understanding of the complex signaling originating from CXCR4 is needed to exploit the opportunities to repair damaged organs safely and effectively.

Keywords: chemokine, cancer, tissue regeneration, CXCL12, HMGB1, CXCR4

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INTRODUCTION

The binding of chemokines to G protein-coupled receptors (GPCRs) typically directs cell movement and traffic in and out of specific tissues in developing embryos and adult animals. They are also involved in tumor metastasis and invasion, and in the extension of neurites and axons of neurons (a part of a cell moves, while the cell body stays put). How chemokines and their receptors recruit hematopoietic cells to injured sites and tumors has been intensely investigated, whereas their involvement in the control of cell proliferation is less explored (1). Among chemokine receptors, CXCR4 is the most widely expressed, and is involved in numerous physiological and pathological conditions. CXCR4 is expressed by most cells, including hematopoietic and endothelial cells (ECs), neurons and stem cells (embryonic and adult). Increased levels of CXCR4 are present in cancer cells compared to the normal cells (2, 3). The focus of this mini-review is the emerging role of CXCR4 and its ligands in tissue repair and regeneration, and its relation to cancer cell proliferation. The role of CXCR4 in differentiation, retention, mobilization, migration, and polarization of hematopoietic cells is covered by other excellent reviews (4, 5).

CXCR4 AND ITS LIGANDS

CXCR4 is a 352 amino acid rhodopsin-like GPCR, comprising an extracellular N-terminal domain, 7 transmembrane (TM) helices, 3 extra-cellular loops (ECL), 3 intra-cellular loops (ICL) and

an intracellular C-terminal domain (6). CXCR4 can exist in the plasma membrane as a monomer, dimer, higher-order oligomer or nanoclusters (7), although the partitioning and relevance of these different multimerization states has not been addressed *in vivo*. Several crystal structures of CXCR4 bound to agonists and small molecules are in accordance with the ability of CXCR4 to form homodimers via interactions of the TM5 and TM6 helices (6). TM6 is also implicated in nanoclustering (7). CXCR4 can also form heterodimers with ACKR3 (a related GCPR also known as CXCR7), which have distinctive signaling properties (8).

The canonical ligand of CXCR4 is CXCL12, also known as stromal cell-derived factor 1 (SDF-1) (9, 10). A single gene, CXCL12, codes for six protein isoforms in human (three in mouse), all deriving from alternative splicing of the fourth and final exon. The various forms are differentially expressed and have different affinities to glycosaminoglycans present on the cell surface and in the extracellular matrix (11). CXCL12α, an 89 amino acid protein, is the shorter and most expressed isoform (12, 13). Notably, CXCL12α can exist in monomeric and dimeric forms. CXCL12 only binds to chemokine receptors CXCR4 and ACKR3, itself a CXCR4 interactor; such a restricted receptor selectivity is unusual among chemokines.

The structure of the CXCR4/CXCL12 complex has not yet been determined; a model integrating homology modeling, experimentally derived restraints, and charge swap mutagenesis (14) highlights several contacts between the N-terminal tail of CXCR4 and CXCL12, and the interaction of the N-terminus of CXCL12 with the cavity delimited by the TM helices.

High mobility group box 1 protein (HMGB1) is the archetypal damage-associated molecular pattern (DAMP) molecule; DAMPs are released from dead or severely stressed cells to alert their microenvironment and the innate immune system. HMGB1 can form a heterocomplex with CXCL12 (HMGB1·CXL12) that also binds to CXCR4; of note, the conformational rearrangements of CXCR4 differ when triggered by CXCL12 alone or by HMGB1·CXCL12, and the complex is over one order of magnitude more potent than CXCL12 alone in inducing cell migration (15). Only the reduced form of HMGB1, where the pair of cysteines in the HMG-box domain A do not form a disulfide bond, binds CXCL12 and interacts with CXCR4 (16). However, a designer form of HMGB1 called 3S-HMGB1, where serines replace all three cysteines, binds to CXCR4 directly and is as effective as HMGB1·CXCL12 in promoting cell migration and muscle regeneration (17).

CXCR4 also binds macrophage migration inhibitory factor (MIF), a cytokine involved in the regulation of innate immunity (18). MIF binds to the N-terminal tail of CXCR4 and to the exterior side of TM helices, but not inside the TM pocket (18, 19). MIF also binds to other receptors, including CXCR2, CD74/CD44, and ACKR3 (20), which complicates the dissection of its activities.

Extracellular ubiquitin (eUb), also considered a DAMP, is a CXCL12 antagonist (21). Molecular modeling and mutagenesis suggest that it binds to CXCR4 inside the cavity delimited by TMs (22), but makes contact to CXCR4 residues that are not contributing to CXCL12 binding (23).

Beta-defensin-3 (HBD3) also competes with CXCL12 for CXCR4 binding, and promotes internalization of CXCR4 without inducing calcium flux, ERK phosphorylation, or chemotaxis (24).

Although the above list of actors is long, and multimeric complexes and multiple interactions increase complexity, genetics originated the widespread idea of CXCR4 and CXCL12 as a biunivocal couple: deletion in mice of either the *Cxcr4* or *Cxcl12* genes causes fetal lethality, defective B-cell lymphopoiesis, impaired bone-marrow myelopoiesis, and abnormal development of the cardiac septum and of the cerebellum (25, 26).

CXCR4 AND CXCL12 IN TISSUE REGENERATION

Mice lacking CXCL12 or CXCR4 were first generated in the 1990s; since both die *in utero*, their ability to regenerate injured tissues was not investigated until later. Depletion of either CXCR4 or CXCL12 with small interfering RNAs injected in injured muscle impairs its regeneration, as does local injection of the CXCR4 antagonist AMD3100 (27), consistent with the expression of both CXCR4 and CXCL12 in skeletal muscle (28), and with impaired myogenesis and depletion of satellite cells in CXCR4 deficient mice (29). Satellite cells are the direct targets of CXCL12 (27).

More recently, CXCR4 and CXCL12 have been shown to control the regeneration of multiple organs and tissues, including lung, heart, liver, and the nervous system.

Surgical removal of one lung or part of it (pneumonectomy, PNX) is compensated by alveolar regrowth/regeneration in the remaining lung. After PNX, activated platelets trigger lung regeneration by binding to pulmonary capillary endothelial cells (PCECs) and supplying CXCL12 to activate CXCR4 and ACKR3 on their surface (30). PCECs activate AKT, proliferate and express the membrane metalloproteinase MMP14, which releases ligands that promote the proliferation of progenitor type II alveolar epithelial cells, and eventually alveolar regrowth. Endothelial cells are direct targets of CXCL12 via CXCR4, since genetic silencing of *Cxcr4* and *Ackr3* in PCECs impairs lung regeneration.

The mammalian heart cannot regenerate in adults, but it can in neonate mice (31). In myocardial infarction (MI), coronary arteries get obstructed, and must regenerate to support continued heart function. A unique CXCR4/CXCL12-dependent process termed "artery reassembly" allows the formation of an alternative (collateral) artery network to bypass obstructed or severed coronary arteries (32). In the mouse, within a few days after ligation of the left coronary artery on day 2 after birth, individual arterial endothelial cells (ECs) migrate out of the existing arteries, proliferate and then coalesce with capillaries, forming collateral arteries that connect branches of the right and left coronary arteries. A similar process reconnects severed arteries after the resection of the apex of the neonatal heart. Artery reassembly does not occur in adult hearts, but injection of a single dose of CXCL12 in the infarcted area promotes collateral formation and functional recovery of the heart. Notably, deletion of *Cxcl12*

capillary ECs or *Cxcr4* in arterial ECs impairs artery reassembly; CXCL12 is not basally expressed in ECs, but hypoxia induces its expression. Thus, during artery reassembly different ECs are both source and target of CXCL12, via CXCR4.

Adult zebrafish hearts do regenerate, and coronary revascularization initiates within hours of injury. After cryoinjury, new coronaries regenerate both superficially around the injured area and intra-ventricularly toward the cardiac lumen, and act as a scaffold for proliferating cardiomyocytes (33). Epicardial cells express Cxcl12b after injury, as a consequence of hypoxia and HIF-1α activation. ECs in both superficial and intra-ventricular coronaries have a common origin and both express CXCR4, but inhibiting CXCR4 pharmacologically or deleting *Cxcr4* in the whole heart limits superficial, and not intra-ventricular, regeneration.

The liver is capable of continuous turnover and regeneration, which is overridden by fibrosis, cirrhosis and hepatic failure only after chronic or overwhelming injury. CXCL12 is constitutively expressed in healthy liver, and its expression increases following acute or chronic injury. Liver sinusoidal endothelial cells (LSEC) and hepatic stellate cells (HSC) are important sources of CXCL12 in liver disease. HSC and mesenchymal stem cells mainly respond via CXCR4, while LSEC express both CXCR4 and ACKR3. CXCL12 can activate HSC and recruit bone marrow mesenchymal cells, which promote liver fibrosis; in LSEC, CXCL12 signals via the physical association of CXCR4 and ACKR3 to activate eventually the transcription factor Id1, which orchestrates pro-regenerative responses, such as production of Wnt2 and hepatocyte growth factor (HGF) (34). Liver regeneration is abrogated by genetic silencing of either ACKR3 or CXCR4 in LSEC, or by chronic injuries that lead to excessive CXCR4 and reduced ACKR3 expression. *In vitro*, CXCL12 induces dose-dependent proliferation of human liver-derived stellate LX-2 cells, mediated by PI3K/Akt and Erk1/2 pathways (35).

The peripheral nervous system has retained throughout evolution the capability to regenerate. Recently, CXCL12 was found to promote the structural and functional recovery of the neuromuscular junction after degeneration of the motor axon terminal (36). CXCL12 is synthetized and released by peri-synaptic Schwann cells, and acts on CXCR4 re-expressed upon injury on the tip of the motor axon. CXCL12 also supports the functional and anatomical recovery of the sciatic nerve after crush injury; of special note, the small molecule NUCC-390, a CXCR4 agonist (37), also promotes nerve regeneration (38).

The central nervous system, in contrast, has a limited ability to regenerate, mostly dependent on neural progenitor cells (NPCs). Astrocytes are the main source of CXCL12 in the brain (39); CXCR4 is expressed on NPCs and CXCL12 appears to stimulate directly their *in vitro* proliferation and differentiation into neurons (40–42), via PI3K-Erk1/2 (43) and/or AKT/FOXO3α (44) activation. However, Li at al. (45) found no CXCL12-induced proliferation of NPC cells from E12 mouse embryos. CXCR4 activation by CXCL12 promotes the differentiation of human embryonic stem cells into neural stem cells (46) and then helps to maintain their stemness (47).

Overall, these studies implicate CXCR4 and CXCL12 in the regeneration of multiple organs, via CXCL12 release from various sources and CXCR4 activation on endothelial and progenitor cells, which then go on to proliferate; so far, a role of CXCR4 activation on parenchymal cells is not convincingly proven nor excluded. Hematopoietic and mesenchymal cells also contribute to tissue regeneration, but in this case the role played by the CXCL12/CXCR4 system appears limited to directing their chemotaxis to the damaged site.

THE HMGB1·CXCL12 COMPLEX

The existence of the HMGB1·CXCL12 complex was first inferred from the ability of HMGB1 to promote the migration of endothelial, hematopoietic and mesenchymal cells (15) via CXCR4; the complex was then biochemically characterized (48). The complex was also found to promote the regeneration of skeletal muscle, since the reduced HMGB1 expression in *Hmgb1*+/− mice delays muscle regeneration (49), whereas the injection of exogenous reduced HMGB1 accelerates muscle, bone and liver repair in mouse (17, 50). Several cell-specific responses are involved, including the proliferation of satellite cells, skeletal stem cells and hepatocytes. The requirement for HMGB1, as opposed to CXCL12 alone, is supported by several observations: injection of CXCL12 alone promotes abnormal bone regeneration, with a larger fracture callus without a concomitant increase in bone mineral density and mechanical strength (50); local injection of glycyrrhizin, a HMGB1 inhibitor (51), delays bone fracture healing; injection of 3s-HMGB1, a mutant form of HMGB1 that can bind to CXCR4 in the absence of CXCL12, mimics the biological effects of HMGB1·CXCL12, including the promotion of *in vitro* myogenesis (17).

Remarkably, systemic injection of fully reduced HMGB1 (frHMGB1) or 3S-HMGB1 predisposes muscle and bone to regeneration/repair even if injected 2 weeks before injury (50), by inducing the transitioning of resting stem cells to a dynamic state of the cell cycle, intermediate between G_0 and G_1, termed "G_{Alert}" (52). In contrast to deeply quiescent G_0 stem cells, G_{Alert} stem cells are more metabolically active, contain higher levels of ATP and mitochondrial DNA, are larger and poised to enter the cell cycle when exposed to activating signals. Activation mTORC1 is both necessary and sufficient for the transitioning to the G_{Alert} state (53), and rapamycin, an mTORC inhibitor, interferes with HMGB1-induced transitioning to G_{Alert} (54). Multiple stem cell types (SSCs, satellite cells and hematopoietic stem cells) in mice subject to bone fracturing or muscle damage transition to the G_{Alert} state, and this requires HMGB1·CXCL12, since stem cells in HMGB1-deficient mice do not transition to the G_{Alert} state after injury unless exogenous HMGB1 is provided.

Thus, HMGB1·CXCL12 has similar activities to those reported for CXCL12 in muscle regeneration, but is absolutely required in G_{Alert} transitioning of stem cells. In this context, two questions arise: is HMGB1·CXCL12 (as opposed to CXCL12 alone) responsible for the regeneration of most or all tissues? Does HMGB1·CXCL12 also promote the proliferation of ECs? Indeed, HMGB1 has been shown to promote the proliferation of

ECs of different origin, although the involvement of CXCR4 as the cognate receptor was not investigated (55).

CXCR4/CXCL12 IN CANCER GROWTH

Tumor is an illegitimate tissue that grows out of control because of an altered expression and behavior of pro-proliferative and pro-survival signals. Precisely because tumor tissue is out of balance with the surrounding legitimate tissues, it is also in a state of distress, similar to an injured tissue, and recruits inflammatory cells that support it. Famously, it has been said that a tumor is wound that never heals (56).

Chemokines and their receptors not only drive the trafficking of leukocytes inside the tumor mass but also contribute to most aspects of tumor cell biology (1). High expression of CXCR4 is observed in hematological malignancies (57–59) and in many types of solid tumors, including melanomas and kidney, lung, brain, prostate, breast, pancreas and ovarian tumors (2, 3), where it correlates with poor prognosis (59). Interestingly, the normal tissue adjacent to the CXCR4 overexpressing tumor shows normal or no CXCR4 expression (41), which suggests a differential response of cancer cells to microenvironmental conditions. Expression of CXCR4 and CXCL12 in cancer cells is also controlled by specific microRNAs: CXCL12 by miR-1 (60), miR-9 (61, 62), miR-126 (63), miR-146a (64), and miR-150 (65), whereas miR-200a can increase CXCR4 expression (66).

The expression of CXCR4/CXCL12 in tumors is partially dependent on the hypoxic tumor microenvironment, in a HIF-1α dependent manner (42). As a consequence of CXCL12 release, tumor-associated CXCR4-expressing ECs proliferate (67). CXCR4 is also expressed on putative cancer stem cells populations in various tumors, including renal (68), prostate (69) and non-small lung cancer (70), and affects their clonogenicity and spherogenicity, with adverse effects on prognosis. These CXCL12/CXCR4 effects are similar to the promotion of endothelial and stem cell proliferation in injured tissue.

Moreover, many reports indicate that binding of CXCL12 to CXCR4 on tumor cells of various types enhances their proliferation, both *in vitro* and *in vivo*, either via MAPK or PI3K/Akt pathways (54, 71, 72).

Table 1 lists a sample of reports on the role of CXCR4/CXCL12 in tumor cell proliferation (mostly tumor cell lines) (53, 69, 72–80). Targeting of CXCR4 with antibodies or specific inhibitors, most commonly AMD3100, has been intensely investigated; however, AMD3100/Plerixafor/Mozobil has been approved for bone marrow transplantation, but not as anti-cancer treatment.

CXCR4 SIGNALING

The preceding sections have highlighted that CXCR4 activation can drive both cell migration and cell proliferation, at least in vascular, progenitor and tumor cells. We will now review current information on the signaling involved.

Ligand binding to CXCR4 induces conformational changes that lead to the activation of multiple signaling pathways (**Figure 1**), originating proximally from the dissociation of heterotrimeric G proteins and from the phosphorylation of the C-terminal cytoplasmic tail of CXCR4. CXCR4 is mainly bound to heterotrimeric G_i proteins, although other G protein classes may transduce CXCR4 binding as well (81). Upon ligand binding, the G_i heterotrimer detaches from the CXCR4 intracellular loops and dissociates into GTP-bound α_i and βγ subunits (82, 83). The βγ subunits directly bind and activate phosphatidylinositol-3-OH kinases (PI3K) β or γ, which produce phosphatidylinositol triphosphate (PIP3), and phospholipase C β (PLC-β), which produces inositol-(1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG). The $G\alpha_i$ subunit induces calcium release from intracellular stores and indirectly activates the PI3K-AKT and MEK1/2-Erk1/2 axes (84). Via the production of PIP3, PI3Ks activate the serine-threonine kinase AKT, which can then can phosphorylate many target proteins, most notably glycogen synthase kinase 3 (GSK3), tuberous sclerosis 2 (TSC2), caspase 9 and PRAS40 (AKT1S1), which explains its wide spectrum of downstream effects in promoting cell proliferation, differentiation, apoptosis, angiogenesis, and metabolism (85).

CXCR4 ligand binding induces JAK/STAT activation in a Gα-independent manner (86). GPCR kinases (GRKs) phosphorylate multiple serines/threonines in the cytoplasmic tail of CXCR4. Phosphorylated CXCR4 recruits β-arrestin-1 and -2, which promote CXCR4 internalization (87). Thereafter, CXCR4 can be recycled back to the plasma membrane or sorted to the lysosomes for degradation (88). Of note, the recruitment of β-arrestins to CXCR4 also activates Erk signaling (89).

The binding of CXCL12 to CXCR4-ACKR3 heterodimers activates G protein-independent signaling cascades originating from β-arrestins that potentiate cell migration (8).

Overall, the activation of PI3Ks and Akt supports the proliferation and survival of both normal and cancer cells. mTORC activation underpins the anabolic metabolism that is required for cell growth; indeed, mTORC activation is also necessary for the transitioning of stem cells to the G_{Alert} state.

Notably, the CXCR4-activated pathways that direct cell movement and migration are exactly the same that are involved in cell proliferation, and both processes can be inhibited by the same small molecules. For example, rapamycin is an mTORC inhibitor that blocks cell proliferation, but it inhibits cell migration as well (90, 91). The same is true for PI3K inhibitors (92).

Although the various pathways originating from CXCR4 are known, there is ample scope for cell specificity. The human genome encodes 18 different Gα proteins, 5 Gβ proteins and 12 Gγ proteins, and multiple PI3Ks and PLCs, with ample variation of expression in different cell types. Moreover, signaling is enhanced or dampened by dozens of modulators, including scaffold proteins that facilitate the physical interactions of kinases and other enzymes that introduce post-translational modifications. We are unaware of studies that delineate the CXCR4-initiated signaling pathways in cell proliferation down to the specific isoforms and post-translational modifications of the signal transducers involved. Cancer is not the most amenable biological system, since

TABLE 1 | CXCR4/CXCL12 axis is involved in cancer cell proliferation.

Tumor type	Cancer cell lines	Pathway involved	References
Glioblastoma	Glioblastoma cell lines GB1690, 5GB, HTB-16	–	Sehgal et al. (72)
	Glioblastoma cell lines U87-MG, DBTRG-05MG	ERK; AKT	Barbero et al. (70)
Non-small cell lung cancer (NSCLC)	NSCLC cell lines L3, L4, A549	ERK	Wald et al. (73)
Malignant mesothelioma (MM)	MM cell lines H28, 211H, H2052, ms-1, H290, H513	AKT/mTOR	Li et al. (74)
Breast cancer	Breast cancer cell line MCF-7	–	Hall et al. (75)
Ovarian cancer	Ovarian cancer cell lines BG-1, SKOV3	–	Hall et al. (75), Guo et al. (76)
Colorectal cancer (CRC)	CRC cell lines HT-29, CaCo21, Colo320	PI3K/AKT	Ma et al. (77)
Pancreatic cancer	Pancreatic cancer cell lines AsPC-1, SW1990, BxPC-3	–	Gao et al. (78)
Esophageal squamous cell carcinoma (ESCC)	ESCC cell line EC9706 (*in vitro* and ESCC mouse xenograft model)	G0/G1 cell cycle arrest and apoptosis induction	Wang et al. (79)
Extrahepatic hilar cholangiocarcinoma (hilar-CCA)	Hilar-CCA cell line QBC939	–	Tan et al. (80)
Prostate cancer	Prostate cancer cell lines DU145 and PC3	PI3K/AKT	Dubrovska et al. (67)

Many primary tumors overexpress CXCR4 and CXCL12 compared to their normal cells. The activation of CXCR4/CXCL12 in tumor cells leads to cell proliferation. –, not known.

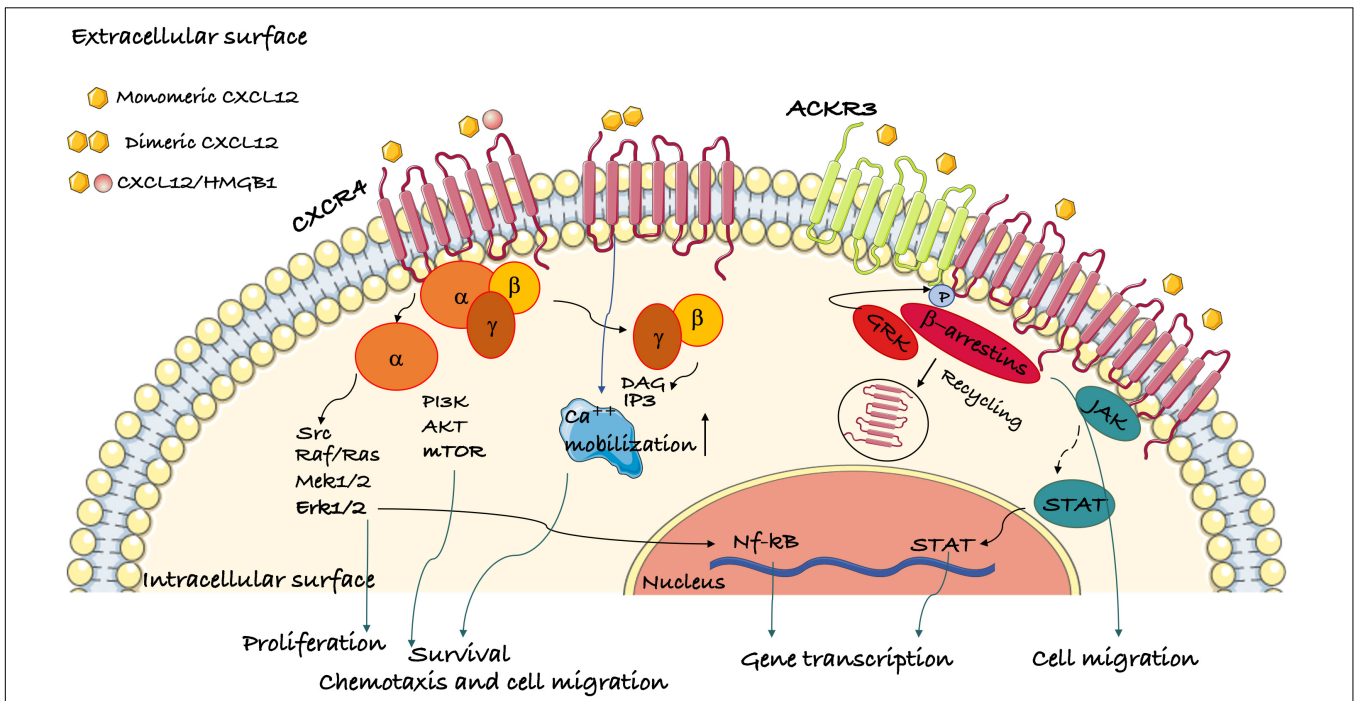

FIGURE 1 | Schematic representation of the signaling pathways activated by CXCR4. Ligand binding to CXCR4 activates G protein subunits and the downstream Ca^{2+} mobilization from intracellular stores and PI3K/Akt, PLC, and ERK1/2 pathways. This results in gene transcription, cell migration, proliferation and survival. CXCR4 oligomerization can also activate the G-protein independent JAK/STAT pathway. β-arrestins are recruited following GRK phosphorylation of CXR4 and mediate its internalization. ACKR3 is another receptor for CXCL12 that can induce β-arrestin-mediated signaling both by itself or as a heterodimer with CXCR4.

cancer cells have accumulated a number of genetic and epigenetic alterations, often including those of PI3Ks. Cell-specific conditional mutants could be used to investigate CXCR4-controlled proliferation following injury, and this would provide a list of parts in specific cells; even so, we would still miss mechanistic details such as the interaction with modifiers, possible feed-forward and feedback loops and time-dependent signal adaptations like those involving Rac (93) or oscillatory behaviors like those described for NF-κB and p53 (94, 95).

CONCLUSION

We have discussed several reports showing that CXCR4 can control cell proliferation in addition to directing cell retention

and movement, both in physiological processes, such as development and tissue regeneration, and in pathological ones, such as cancer growth. The mechanisms and pathways involved may be broadly similar in all cases, since regeneration often recapitulates developmental processes, and cancer often exploits developmental pathways.

Signal transduction pathways downstream CXCR4 eventually control both cell movement and cell proliferation, which are both dependent on PI3K-Akt and mTORC signaling; the details, however, may vary from cell to cell and in different settings.

So far, the interest has focused on cancer and on drugs that block CXCR4-initiated signaling; we suggest that small molecules that activate CXCR4 signaling or can dissect the effects on cell migration and proliferation may be as useful.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the writing of the review.

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