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MEETING ARTICLE

Characterization of a chimeric chemokine as a specific ligand for ACKR3

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Abstract

Chemokines, small chemotactic cytokines, orchestrate cell migration by binding to their cognate chemokine receptors. While chemokine-mediated stimulation of typical G-protein-coupled chemokine receptors leads to cell migration, binding of chemokines to atypical chemokine receptors (ACKRs) does not induce canonical signaling. ACKRs are considered important chemokine scavengers, that can create gradients which help direct cells to sites of inflammation or to their immunological niches. Synthetic chemokines have been used in the past to study and decode chemokine-receptor interactions. Characterizing specific chemokine-ACKRs interactions is challenging because the chemokines bind multiple receptors; for example, the ACKR3 ligands CXCL12 and CXCL11 bind to the canonical receptors CXCR4 and CXCR3, respectively. Here, we present the engineering of a chemokine-like chimera, which selectively binds to ACKR3. The addition of a ybbR13 tag at the C-terminus allows site specific enzymatic labeling with a plethora of fluorescent dyes. The chimera is composed of the N-terminus of CXCL11 and the main body and C-terminus of CXCL12 and selectively interacts with ACKR3 with high affinity, while not interfering with binding of CXCL11 and CXCL12 to their cognate receptors. We further provide evidence that the chimera can be used to study ACKR3 function in vivo.

KEYWORDS

Chemokine, chemokine receptor, ACKR3, synthetic chemokine

1 | INTRODUCTION

Chemokines and their cognate chemokine receptors constitute the chemokine system, which principally orchestrates leukocyte trafficking during homeostasis and inflammation. The system is also involved in development and has a key role in cancer growth and metastasis.[1] Typical pertussis toxin-sensitive chemokine receptors, members of the rhodopsin like family of G-protein coupled receptors (GPCRs), activate diverse intracellular signaling cascades and commonly trigger cell migration. In addition to the "typical" or "canonical" receptors, chemokines also bind to atypical chemokine receptors (ACKRs). ACKRs are heptahelical membrane proteins, structurally homologous and phylogenetically related to canonical chemokine receptors, but do not couple to G-proteins and do not activate the respective downstream signaling cascades, hence, do not induce cell migration.[2] The main activity of ACKRs is the scavenging of chemokines, thereby controlling their availability for typical receptors, modulating, and shaping chemotactic gradients. Growing evidence indicates that alternative signaling pathways, via arrestin in particular, may be triggered following chemokine ligation of ACKRs. Currently four ACKRs have been identified. ACKR3, also known as CXCR7, binds and scavenges the chemokines CXCL12 and CXCL11. The receptor can contribute to the overall activity of CXCR4, the cognate chemokine receptor of CXCL12. Soon after the discovery of ACKR3 as second receptor for CXCL12, it was shown in Zebrafish that CXCL12-directed CXCR4-dependent migration of primordial

Abbreviations: ACKR, atypical chemokine receptor; GPCR, G-protein coupled receptor; HPF, high power field; IMAC, immobilized-metal affinity chromatography; MFI, mean fluorescence intensity; PFA, paraformaldehyde

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germ cell requires ACKR3 expression.[3,4] The scavenging acitivity of ACKR3 expressed on stroma cells is required to maintain CXCL12 gradients for directed migration of the germ cells.[4,5] In addition, the cells of the primordium express CXCR4 and ACKR3 at the anterior and posterior side, respectively, creating CXCL12 gradients.[6,7] During mouse brain development ACKR3 controls the levels of CXCL12 to promote the CXCR4-dependent outgrowth of interneurons.[8] Under inflammatory conditions ACKR3 is upregulated at the basal site of brain microvessel endothelium scavenging perivascular CXCL12, thereby abolishing the perivascular retention of $CXCR4^+$ leukocytes and indirectly promoting their entry into the parenchyma.[9] Using a disseminated xenograft model, we recently reported that the diffuse large B cell lymphoma Val cells require ACKR3 expression for organ infiltration. In vitro ACKR3 expression by these cells was required for efficient CXCL12-induced CXCR4-dependent chemotaxis.[10] Ectopic expression of overexpressed ACKR3 and CXCR4 was suggested to result in heterodimerization, which altered CXCR4-dependent signaling.[11] Both receptors, ACKR3 and CXCR4, are broadly expressed on hematopoietic and mesenchymal cells.[1,12,13] The second ligand of ACKR3 is CXCL11, an IFN-γ inducible chemokine. CXCR3 binds CXCL11 with high affinity (apparent $K_d \sim 1$ nM)[14] and is expressed on various lymphocytes.[1] Targeted gene deletion of ACKR3 revealed a key role of the receptor in development, as deficient mice are not viable displaying a marked cardiac phenotype with stenotic defects in heart valves and vessels. Interestingly, the mouse strain used for target deletion C57BL/6 is deficient in CXCL11, hence the phenotype was ascribed to the abolished interaction of ACKR3 with CXCL12.[15]

One chemokine can bind several receptors and a receptor can bind multiple chemokines.[1] Given this promiscuity of the chemokine system, delineating the specific activity of a chemokine receptor pair in vivo is challenging. It is therefore valuable to engineer structurally related chemokines which specifically target a single receptor, in order to study ligand-receptor interactions in complex biological systems. About 50 human chemokines have been characterized. They are small proteins (8–10 kD) with highly diverse primary sequences, but share structural homology dictated by 4 conserved cysteines forming two characteristic disulfide bridges. According to the two step binding model[16] the rigid loop (also called N-loop) with an overall basic charge downstream of the first two cysteines mediates the initial binding to the negatively charged N-terminus of the receptors. Whereas the flexible N-terminus of chemokines inserts into the binding pocket of the receptors triggering conformational changes which translate in intracellular signaling. In line with this, modifications of the N-terminus often result in altered receptor activation leading to either loss of function or enhanced potency and/or efficacy. Three beta strands and the short helix present at the C-terminus of most chemokines harbor glycosaminoglycan (GAG) binding sites for presentation of chemokines on cell surfaces, which is required for haptotatic migration of cells.[17] Binding and retention of chemokines on cell surfaces or the extracellular matrix is critical for the formation of locally confined chemotactic gradients.[18] However, the interactions of chemokines with GAGs is not uniform and can fine-tune the function of chemokines.[19] CXCL12α possesses one GAG binding site in its first β-strand[20,21] and differs therein from CXCL12β and particularly CXCL12γ, which harbor additional GAG binding sites at their extended C-termini.[22,23]

In solution, CXCL12 can form dimers depending on the environmental conditions, such as pH and heparin.[24] Monomeric and dimeric forms of CXCL12 were shown to bind and to induce signal bias on CXCR4, where dimeric CXCL12 poorly recruits arrestin and essentially does not stimulate cell migration.[25,26] ACKR3 binds preferentially monomeric CXCL12[27] with about 10-fold higher affinity than CXCR4 (apparent K_d for ACKR3 $\sim$ 0.3 nM).[28,29] The affinity of CXCL11, the second chemokine ligand of ACKR3, is somewhat lower (2–5 nM).[29] CXCL11 and CXCL12 share at most 20% of their primary sequences including the 4 conserved cysteines. The N-termini preceding the first cysteine of both chemokines have the same length, but share only the amino acids at position 2 (proline; P, one letter code) and 8 (arginine; R). Currently no crystal structure of ACKR3 is available, however extensive radiolytic footprinting revealed contact points between CXCL12 and ACKR3. Comparing the sequences of CXCL11 and CXCL12 with the data from the radiolytic footprinting suggests that different domains of ACKR3 must be involved in the recognition of the 2 chemokines.[30] A recent study confirmed different modes of binding of CXCL11 and CXCL12 to ACKR3.[31] CXCL11 induced a faster ACKR3 internalization, but slower recycling than CXCL12. Moreover, the authors suggest that arrestin binding to ACKR3 is not required for chemokine scavenging.[31] The finding somewhat contrasts other observations where it was shown that CXCL11 and CXCL12 binding to ACKR3 enhances arrestin recruitment.[32–36]

A known drawback for the investigations of GPCRs is the paucity of antibodies which recognize endogenous receptors expressed at the cell surface. Alternatively, chemokine receptor surface expression and function was successfully measured with fluorescent labeled chemokines.[10,37–40] The specificity of this approach is limited by the promiscuity of the chemokine system, in addition chemokines are internalized through the scavenging activity of atypical receptors. Nevertheless, fluorescent chemokines can also be used in vivo to reveal receptor localization and function.[41] There are no selective chemokines for ACKRs because its cognate chemokines bind at least to one canonical receptor. Here, we describe the engineering of a novel chimeric chemokine combining CXCL11 and CXCL12, which is a selective ligand for ACKR3 and, by analogy to CXCL12, is monomeric and has reduced GAG binding ability. The chimera was in addition tagged at the C-terminus with an acyl carrier protein tag for site specific enzymatic labeling with fluorophores.[40,42,43] The high affinity of the chimera for ACKR3 allows to reveal sites of functional ACKR3 expression in vivo.

2 | MATERIALS AND METHODS

2.1 | Cells

Mouse 300.19 pre-B cells were cultured in B cell medium (RPMI-1640 supplemented with 10% FBS, 1% PenStrep, 1% nonessential amino acids, 1% Glutamax, and 50 μM β-mercapto ethanol (β-ME)). CEM cells were cultured in T cell medium (RPMI-1640 supplemented with 10% FBS, 1% PenStrep, and 1% Glutamax). HeLa cells were maintained in

high glucose DMEM (4.5 g/l) supplemented with 10% FBS and 1% PenStrep. All cell culture media and supplements were from Thermofisher. Transient transfections with plasmids containing an internal ribosomal entry side encoding for chemokine receptors CXCR4, CXCR3, or ACKR3 and EGFP were performed with 500 ng DNA added to 1 μl Lipofectamine LTX and 0.5 μl PLUS reagent (Thermofisher) per glass-bottom Petri dish (MatTek). Cells were left overnight and inspected for GFP expression prior to experimental usage. Bone marrow cells from WT and ACKR1-deficient mice were prepared as described elsewhere.[44]

2.2 | Chemokine expression and purification

Recombinant chemokines were expressed in *E. coli* as previously described.[42] Briefly, chemokine sequences were preceded by an enhancer GroE sequence, followed by an 8-H encoding sequence, 2 amino acids (LE), and the enterokinase recognition site (DDDDK).[45] When indicated to the C-terminus of the chemokine the ybbR13 (DSLEFIASKLA) sequence was fused to allow site specific labeling with phosphopantetheinyl transferase.[43] Chemokines were purified from inclusion bodies by immobilized-metal affinity chromatography (IMAC) and refolded under N_2 protection in an arginine containing buffer (80 mM Tris-Cl (pH 8.5), 100 mM NaCl, 0.8 M arginine, 2 mM EDTA, 1 mM cysteine, 0.2 mM cystine). After recovery and concentration the N-terminus was cleaved with enterokinase and chemokines purified by C_{18} reverse phase chromatography.

Chemokines were labeled with phosphopantetheinyl transferase (Sfp) (New England Biolabs), fluorescent labeled CoA.[42,46] Briefly, ybbR13-tagged chemokines (10 μM) were incubated overnight at room temperature in the dark in 20% glycerol, 100 mM NaCl, 10 mM $MgCl_2$, 50 mM HEPES, 1 μM Sfp, and 20 μM CoA conjugated fluorophore (Atto565, Alexa647, or Atto700). Labeled chemokines were purified by reverse phase chromatography.

2.3 | Chemokine binding and uptake assay by flow cytometry

Mouse 300.19 pre-B cells expressing either ACKR3, CXCR4, or CXCR3 were incubated at 4°C on ice or at 37°C (5% CO_2/humidified air) for 90 min in B cell medium containing 50 nM fluorescent labeled CXCL11, CXCL12, or CXCL11_12. Cells were washed once in PBS. To determine uptake at 37°C the cells were subjected to a short acidic wash for 1 min at 4°C to remove surface bound chemokines.[47] Surface bound and internalized chemokines were measured by flow cytometry (FACS Fortessa, BD Biosciences) and analyzed with FlowJo software.

Competition binding assays were performed with mouse pre-B 300.19 cells stably expressing human ACKR3.[37] Cells were incubated at 4°C for 90 min in RPMI, 1% human pasteurized plasma (PPL, CSL Behring AG) with 1 nM fluorescent labeled CXCL11_12_Alexa647. Competition was achieved with increasing concentrations of unlabeled CXCL11_12, CXCL11 and CXCL12. Cells were washed once with ice cold PBS containing 2% FBS. Binding of CXCL11_12 Alexa647 was measured by flow cytometry and quantified using geometric mean fluorescence intensity (MFI). Unspecific binding was measured using MFI values from displacement binding assays performed with parental 300.19 cells.

2.4 | Chemokine uptake assay by confocal microscopy

Uptake of fluorescent labelled chemokines was measured in transiently transfected HeLa cells incubated for 45 min at 37°C in OptiMem containing 50 nM CXCL11_Atto565, CXCL12_Atto565, and CXCL11_12_Atto565. Cells were washed in warm PBS, fixed in 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature, stained with DAPI (0.1 μg/ml PBS) for 5 min and washed with PBS. Cells were imaged with a laser scan confocal microscope (Leica SP5) and images analyzed using Imaris software (Bitplane).

Chemotaxis assays were performed using 48-well modified Boyden chambers (AP48, NeuroProbe) as previously described.[48] CEM cells (100,000/well) were allowed to migrate for 90 min at 37°C in an incubator (5% CO_2) toward increasing concentrations of chemokine (0.1–100 nM) added to the lower wells through 5 μm pored polycarbonate membranes (PVPF, NeuroProbe). Chemotactic assays using 300.19 pre-B cells stably expressing CXCR3 were performed as described above, in a modified chemotaxis buffer (RPMI supplemented with 25 mM HEPES and 1% PPL). Chemotaxis was quantified by counting the number of migrated cells per high power field (HPF).[48]

2.5 | In vivo uptake

ACKR3 GFP reporter mice[9] were anesthetized and injected i.v. with 150 μl of 6.5 μM CXCL11_12 H25R Gag^- Alexa647 in PBS. After 20 min, animals were sacrificed and flushed with PBS by injecting 20 ml PBS in the hearts. The organs were removed and placed in 4% PFA in PBS overnight at 4°C with slow agitation. Hearts were placed in fresh PBS and stored at 4°C until processing. For tissue processing the organs were placed into low melting agarose (2%) and sections of 150 μm were cut with a vibratome (Leica VT1200s) until valves became visible. Sections were stained with DAPI (0.1 μg/ml) in PBS for 30 min at RT, washed twice for 30 min with PBS, 1% FCS, 0.1% TritonX100, and 0.01%NaN_3. Tissue sections were imaged with a laser scan confocal microscope (Leica SP5) and analyzed using Imaris software (Bitplane).

3 | RESULTS

For determining the surface expression and scavenging activity of ACKR3 a specific endogenous ligand is missing. CXCL11 and CXCL12 both bind to ACKR3, but also to their cognate receptors CXCR3 or CXCR4, respectively, making it difficult to reveal the expression and function of the atypical receptor in the presence of the canonical receptors. AMD3100 was described as selective inhibitor of CXCR4, which blocks binding and CXCL12-mediated signaling[49,50] and could be used to discriminate between CXCR4 and ACKR3-dependent CXCL12 internalization. However, at full effective concentrations AMD3100 also targets ACKR3.[31,51] Binding of CXCL11 to

CXCR3 can be similarly abolished with small molecule inhibitors, however, ACKR1 also binds the chemokine potentially obscuring interactions with ACKR3.[1] In order to design a selective ligand for ACKR3, we hypothesized that a chimeric chemokine composed of domains derived from CXCL11 and CXCL12 could possess such characteristic. Modification or truncation of the N-terminus of CXCL12 markedly affects its affinity for CXCR4.[16] Exchange of proline[2] with glycine on CXCL12 (P2G-CXCL12) turns the chemokine into an antagonist that binds CXCR4 with similar affinity as the wild type counterpart, but does not induce signaling.[16,52] To test a possible effect of substitutions at the N-terminus of CXCL12 for its ability to bind to ACKR3 we took advantage of an in house available fluorescent fusion protein P2G-CXCL12-YFP, which bound to CXCR4 expressing MDCK cells, and as expected, did not induce signaling.[16,52] However, the fusion protein was readily internalized by MDCK cells transiently expressing ACKR3, suggesting that the substitution of the critical proline at the N-terminus did not abolish scavenger activity (not shown). On the other hand truncation of the N-terminus of CXCL12 was reported to markedly reduce its binding to ACKR3,[28] suggesting that the domain plays some role in chemokine recognition.

3.1 | Chimeric construct

Considering that ACKR3 binds CXCL12 and CXCL11, we designed a chimeric chemokine consisting of the 8 amino acids of the N-terminus of CXCL11 and the body of CXCL12α starting with the common arginine[8] preceding the first cysteine[9] of the CXC motif (Fig. 1). The first β-strand of CXCL12 contains a BBXB (B for basic amino acid and X any amino acid) motif comprising lysine (K)[24], histidine (H)[25], and lysine (K)[27] which promotes GAG binding[21,53] together with lysine (K)[41] located on the second β-strand.[54] In order to reduce GAG interactions, which could lead to background signals when ACKR3 binding is imaged in tissues, we substituted these residues by exchanging K[24], K[27], and K[41] for serine (S) at all positions. Replacing histidine[25] with arginine at the potential dimerization interface was shown to shift the equilibrium to the monomeric state of CXCL12α,[24] which is the favored state for the chemokine to bind ACKR3.[27] Figure 1 depicts the modifications in the first and second β-strand. We fused a tag for site specific enzymatic labeling to lysine (K)[68] located at the extreme C-terminus of CXCL12α, based on the notion that modifications of the C-terminus have minor effects on chemokine receptor binding.[40,42] We chose a short peptide tag for orthogonal protein labeling with phosphopantetheinyl transferase, which was fused to the palindrome sequence for BsiW1 (encoding for RT) allowing cassette cloning of chemokines. The 11 amino acid-long ybbR13 sequence (DSLEFIASKLA) was derived from *B. Subtilis* peptidyl carrier protein and is targeted by phosphopantetheinyl transferase Sfp from *B. subtilis*.[43,55] The affinity of the tag for the Sfp enzyme is 123 μM permitting an efficient modification of the serine residue at position 2 of the ybbR13 sequence with conjugated CoA as substrate.[43,56] We selected the ybbR13sequence because this consensus sequence is less hydrophobic compared with other motives and has a reasonably high affinity for the *B. Subtilis* enzyme Sfp.[43,57] Fusing more hydrophobic sequences with higher affinity for Sfp to the C-termini of chemokines caused their tendency to aggregate (not shown). The relative insensitivity of phosphopantethenyl transferases for modifications at the free sulfhydryl group of CoA allows to site specific label tagged chemokines with almost any low molecular weight fluorophore.[42,46] The full name of the chimeric chemokine, CXCL11_12 H25R Gag$^-$ ybb13, reconciles all these features, for convenience, we use here the short form CXCL11_12 followed by the fluorophore linked to the ybbR13 tag.

We expressed CXCL11_12 in *E.Coli* with an His-tag and the enterokinase consensus sequence at the N-terminus[42] and isolated the chimera from inclusion bodies by IMAC. After folding and purification by hydrophobic interaction chromatography the N-terminus was removed with enterokinase and the cleaved product purified by reverse phase HPLC. The final product migrated as single band on SDS-PAGE. Mass spectrometry revealed an average mass of 9361.98 which is in good agreement with the calculated average Mw 9366.95 of CXCL11_12 containing two disulfide bridges.

3.2 | Binding and specific uptake of CXCL11_12

First, we tested the binding of CXCL11, CXCL12, and CXCL11_12 to pre-B cells transfected with human CXCR3, CXCR4, and ACKR3 at 4°C. Under these conditions receptor endocytosis is blocked and chemokines bind only to cell surface expressed receptors. As expected, Supplementary Fig. 1 shows that CXCL11 bound to CXCR3 and ACKR3 expressing cells, but only marginally to CXCR4$^+$ cells. Contrariwise, CXCL12 only bound to CXCR4 and ACKR3 expressing cells. The chimera was found to associate with ACKR3 and CXCR4 expressing cells and marginally to CXCR3. Binding to CXCR4 is in agreement with the reported low affinity binding of CXCL12 variants with truncated N-termini.[16]

The selectivity of the chimeric chemokine for ACKR3 was tested through uptake by mouse pre-B 300.19 cells at 37°C. The parental cell line is known to not express either receptor. Figure 2A shows the results obtained with cells stably expressing either human CXCR3, CXCR4, or ACKR3. Cells were incubated with 50 nM of chemokine labeled with Atto-565 and prior to analysis by flow cytometry any surface bound chemokine was removed by a brief acidic wash.[47] CXCR3 expressing cells selectively internalize the cognate chemokine CXCL11, but neither the chimera nor CXCL12. Similarly, cells expressing CXCR4 did exclusively internalize CXCL12. By contrast, cells transfected with ACKR3 internalized both chemokines and the chimera. The findings endorse the hypothesis that ACKR3 may independently recognize the N-terminus of CXCL11 and the main body of CXCL12.

To test a potential interaction of CXCL11_12 with ACKR1, we isolated bone marrow from wild type and ACKR1-deficient mice. Cell suspensions were analyzed by flow cytometry gating for nucleated erythrocyte cells using transferrin (CD71) and the erythrocyte lineage marker Ter119.[58] The double positive cells from wild type animals express high surface levels of ACKR1.[44] Figure 2B shows that cells from wild type or ACKR1-deficient animals neither bound nor internalized CXCL11_12 during incubation at 37°C for 1 h.

To confirm the uptake of chemokines we transiently expressed either human or mouse CXCR3, CXCR4, and ACKR3 together with GFP in Hela cells and revealed uptake by confocal microscopy.

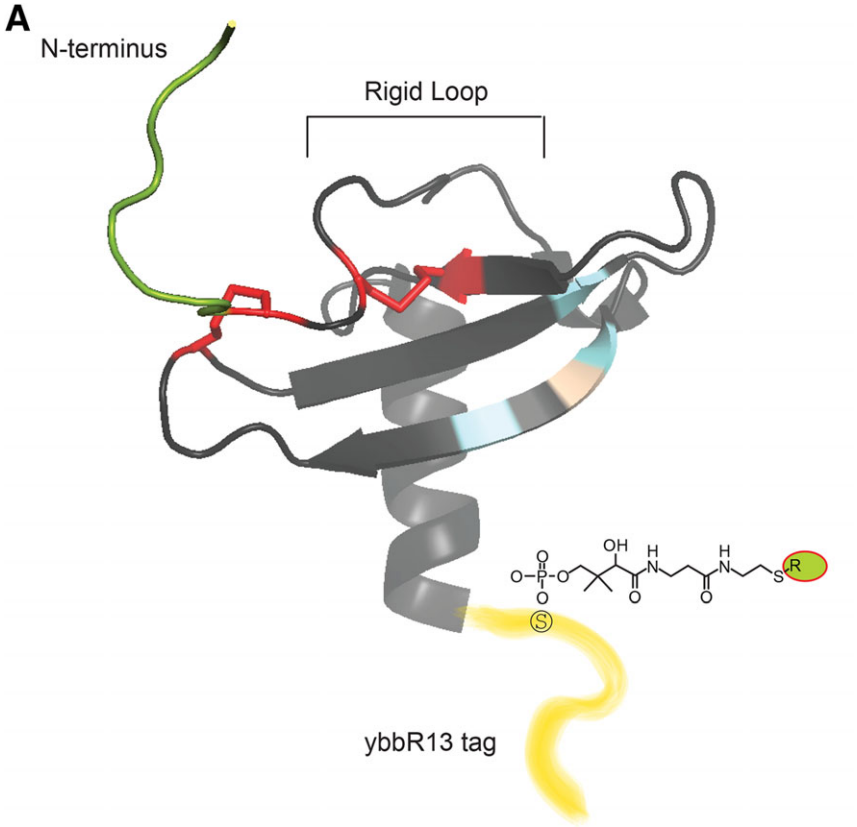


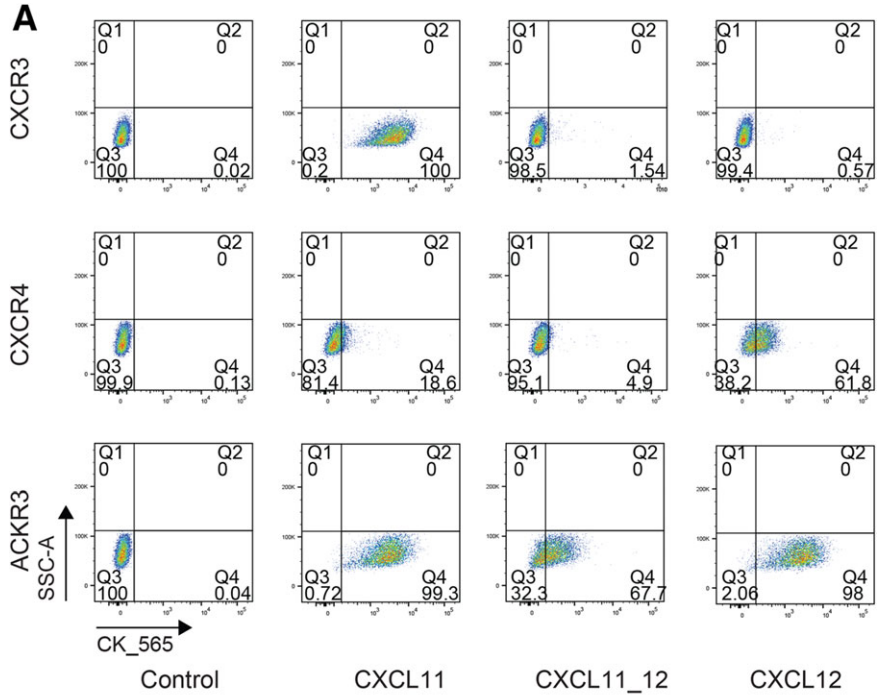
FIGURE 1 Structure of engineered CXCL11_12 H25R Gag- ybbR13. (A) The structure of CXCL11_12 was modeled onto the X-ray structure of CXCL12 (3GV3, 1.6 Å resolution). (B) Sequence alignment of human CXCL12, CXCL11, and CXCL11_12. Cyan indicates the position of the mutated GAG binding sites and beige the monomer inducing H25R point mutation. The ybbR13 sequence is marked in yellow. The circled S in the ybbR13 sequence becomes modified by phoshopantetheinyl transferase

Figure 3A show that Hela cells transfected with ACKR3 internalized all chemokines, CXCL11, CXCL12, and the chimera. Three dimensional rendering of image stacks reveals that the chemokines localized to endosomal structures in the cells (Fig. 3B). By contrast, cells transfected with CXCR3 or CXCR4 only internalized their cognate ligands. Taken together, our uptake data indicate that the chimera is a selective ligand for ACKR3. Similar results were obtained when MDCK cells were transfected with mouse ACKR3 (not shown).

We determined the affinity of the chimera for ACKR3 using competition binding for CXCL11_12 labeled with Alexa647 at 4°C, where no receptor internalization occurs. Figure 4 shows that unlabeled CXCL11_12 efficiently competed the binding of its fluorescent labeled homologue. The data best fitted with a single mode binding competition curve with a dissociation constant of $K_d \sim 1$ nM. The finding is in agreement with previous reports, showing that binding of chemokines to parental 300.19 cells is negligible (not shown).[59,60] Similarly, CXCL11 and CXCL12 competed binding of CXCL11_12 Alexa647 with similar kinetics. Consistent with the specificity of CXCL11 and CXCL12 for ACKR3 a highly promiscuous chemokine CCL5, which binds several chemokine receptors namely CCR1,2,3, 5[1] did not compete CXCL11_12 binding.

3.3 | Chemotaxis

We next tested possible effects of the chimera on CXCL11 and CXCL12-mediated chemotaxis through their cognate receptors CXCR3 and CXCR4, respectively. The T cell line CEM which expresses high surface levels of endogenous CXCR4 was used in Boyden chamber chemotaxis assays. Figure 5 depicts a typical bell-shaped dose response curve of CEM cell chemotaxis in response to CXCL12 with a peak migration at about 3 nM. An almost identical migratory behavior was observed, when we performed the assay in the presence of 10 nM CXCL11_12 in both, the upper and lower compartment, suggesting no interference of the chimera with CXCL12 binding to CXCR4. Similarly, we tested the chemotactic responsiveness of CXCR3-transfected



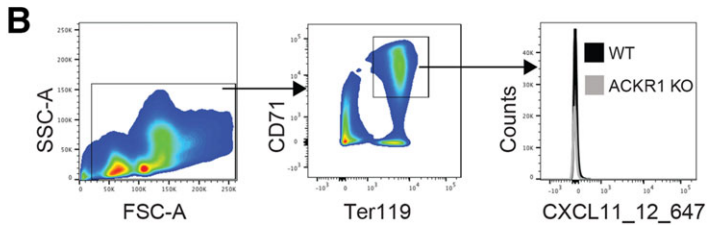


FIGURE 2 Measurement of binding and uptake of CXCL11_12 by FACS. (A) Pre-B 300.19 cells stably transfected with human CXCR3 (upper row), human CXCR4 (middle row), and human ACKR3 (bottom row) were incubated at 37°C for 90 min in the absence of chemokine (control) or in the presence of human CXCL11 (CXCL11), CXCL11_12 H25R Gag^{-} (CXCL11_12), and human CXCL12 (CXCL12). All chemokines were labeled with Atto565. After the incubation cells were subjected to a brief acidic wash to remove surface bound chemokines (see Methods). A typical experiment of three independent observations measured in duplicates is shown. (B) Bone marrow was flushed from femurs of WT and ACKR1 KO mice. Of 12×10^6 cells, each were incubated with 10 nM CXCL11_12Alexa647 in RPMI at 37°C and 5% CO_2 for 1 h, then washed. Cells were stained with antibodies against CD71 and Ter119 to identify the erythroblast population and analysed by flow cytometry. A typical experiment of 3 independent observations measured is shown

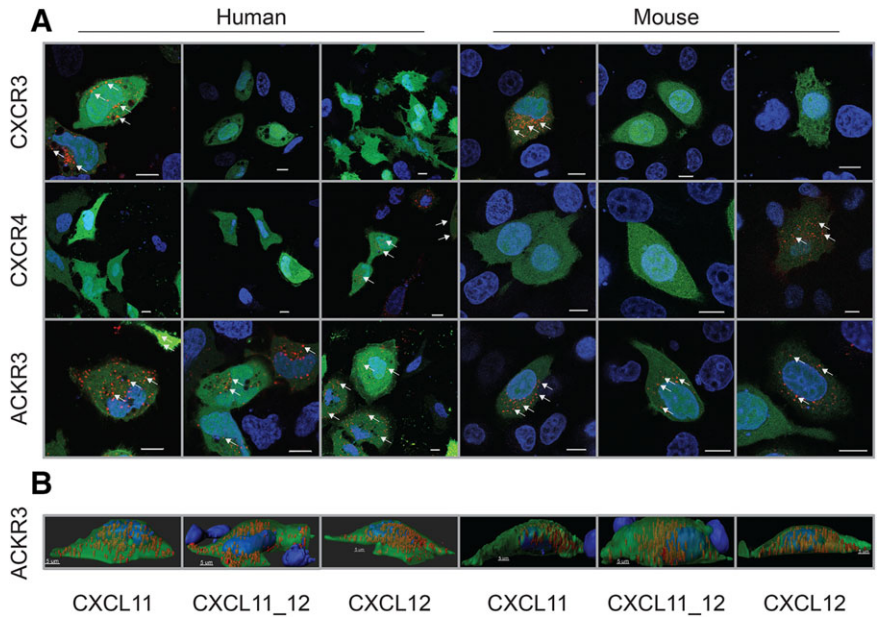


FIGURE 3 Uptake of CXCL11_12. (A) HeLa cells were transiently transfected with human (left panels) or mouse (right panels) CXCR3 (upper row), CXCR4 (middle row) and ACKR3 (bottom row). All plasmids expressed also GFP (green) from an IRES. Cells were incubated for 45 min with 50 nM of CXCL11_12, human CXCL11, and human CXCL12 (red), rinsed twice with PBS, fixed with 4% PFA, and counterstained with DAPI (blue). Arrowheads indicate intracellular chemokines. (B) Images stacks of cells transfected with human (left panels) or mouse (right panels) were rendered with Imaris software to reveal intracellular localization

pre-B 300.19 cells to CXCL11. Supplementary Fig. 2 shows the typical bell shape curve in response to increasing concentrations of CXCL11 with a peak of chemotactic activity at 10 nM. Addition of 100 nM chimera to the upper and lower compartment did not significantly affect CXCL11 stimulated chemotaxis. The data further confirm that the chimera does not interfere with CXCR3 and CXCR4-mediated responses stimulated by their cognate ligands.

3.4 | CXCL11_12 in vivo

ACKR3 is known to be expressed on vascular endothelium and to act there as a scavenger for CXCL12,[15,61] In fact systemic inhibition of ACKR3 with small molecules leads to elevated CXCL12 serum levels.[62] Expression of ACKR3 on heart endothelium appears to have a key role during embryonal development.[15] To test our chimera in

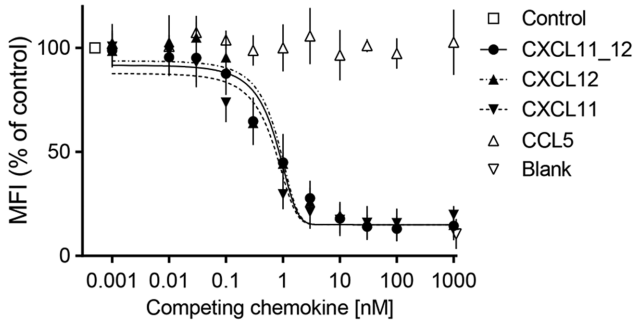


FIGURE 4 **Competition binding of CXCL11_12 on ACKR3.** Mouse 300.19 pre-B cells stably transfected with human ACKR3 were incubated at 4°C for 90 min with 1 nM CXCL11_12 labeled with Alexa647 alone (Control, □) or in the presence of increasing concentrations of unlabeled CXCL11_12 (●), CXCL12 (▲), CXCL11 (▼), and CCL5 (Δ). Surface binding was measured by flow cytometry and is expressed as geometric mean fluorescence (MFI). Blank denotes the MFI of non-transfected mouse 300.19 pre-B cells. Normalized data from five independent determinations performed in triplicates (CXCL11_12, CXCL12, CXCL11). Binding of the irrelevant chemokine CCL5 was tested in two instances with triplicates

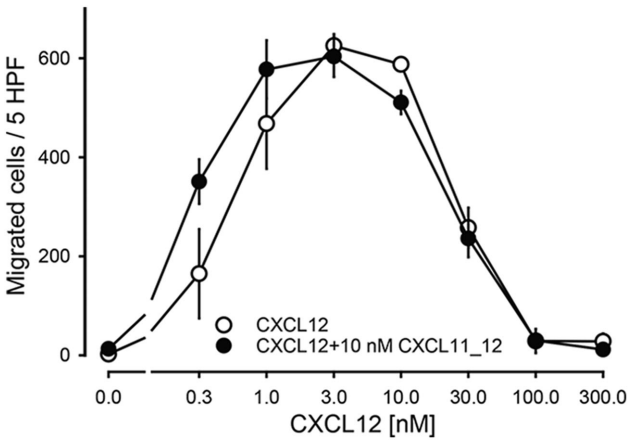


FIGURE 5 **Effect of CXCL11_12 on CXCL12 mediated chemotaxis.** Human CEM T cells expressing endogenous CXCR4 were allowed to migrate towards increasing concentrations of CXCL12 in modified Boyden chambers. When indicated (closed symbols) 10 nM CXCL11_12 was added to the upper and lower compartment. Migrated cells were counted from 5 randomly selected high power fields (HPF) of triplicates at 100x magnification. Typical experiment from 3 independent mesurements

vivo as ligand for ACKR3 we injected into reporter mice, where one allele of ACKR3 was replaced with GFP,[9] ~ 1 μmole of CXCL11_12 Alexa647. Animals were sacrificed after 20 min, hearts removed, and fixed in PFA. Figure 6 shows a maximal projection of stacked confocal images. The longitudinal section of the heart valve area reveals that the fluorescent chimera specifically associated with GFP positive endothelial cells. Three-dimensional rendering of the confocal planes confirms internalization by GFP/ACKR3 positive cells. The chimera is found in endosomal structures where ACKR3 is expected to deploy CXCL11_12 for lysosomal degradation. The results also indicate that CXCL11_12 is internalized by mouse ACKR3.

4 | DISCUSSION

Specific targeting of atypical chemokine receptors is challenging due to the promiscuity of their ligands which all bind also to at least one canonical chemokine receptor. The two ligands of ACKR3, CXCL11, and CXCL12, bind CXCR4 and CXCR3 respectively, induce their internalization and are therefore not specific for ACKR3. The atypical receptor does not couple to G-proteins and little is known about specific downstream signaling that could be used to reveal its activity in vivo using endogenous ligands. We engineered a selective fluorescent labeled chemokine-like ligand and show that it can be used to reveal receptor scavenging activity in vivo.

The promiscuity and lack of ligand-induced coupling to G-proteins suggest that atypical chemokine receptors are less sensitive than typical receptors to alterations of the N-terminus of chemokines. We exchanged the N-terminus of CXCL12 with the corresponding amino acids derived from CXCL11, driven by the notion that CXCR4 is particularly sensitive to modifications of the N-terminus of CXCL12.[16] A recent study suggests that chemokine binding and activation of ACKR3 differs significantly from that of the typical receptors CXCR3 and CXCR4.[36] In their study, the authors used peptides derived from the N-termini of CXCL11 and CXCL12, to show different binding and activation potencies depending of amino acid substitutions. However, contrarily to our chimeric chemokine, which has comparable affinity to ACKR3 as the natural ligands, the peptides have a markedly lower affinity (100–2000-fold) for ACKR3.[36] It is conceivable and in line with radiolytic footprinting analysis of CXCL12:ACKR3 complexes, that both the N-terminus and the body constitute together the high affinity binding to ACKR3.[30]

The putative GAG binding site in the rigid loop of CXCL11_12 is most likely not involved in ACKR3 binding. The assumption is again deduced from the radiolytic footprinting analysis of CXCL12:ACKR3 complexes where amino acids 21–26 were shown to not interact with ACKR3.[30] Hence, mutation of the GAG binding sites should reduce the retention of the chimera on proteoglycans, without altering binding to ACKR3. Similarly, the point mutation of histidine[25] to arginine, that was reported to render CXCL12 monomeric,[24] should not attenuate rather enhance the interaction with ACKR3, which preferably binds CXCL12 monomers.[27]

Competition of CXCL11_12 binding to ACKR3 revealed similar affinities for CXCL11 and CXCL12 and for the chimera itself (Fig. 4). The data appear to contrast the uptake of the chemokines by ACKR3 expressing cells (Fig. 2), where the chimera appears to be less efficiently internalized. It is plausible that chemokine uptake not only depends on their specific affinity, but also on the possible (biased) stimulatory capacity that modulates the scavenging activity.

Our FACS and microscopy analyses clearly indicate that CXCL11_12 is a selective chemokine-like ligand which binds ACKR3 with high affinity and is readily internalized by the scavenger. These properties render the chimeric chemokine an ideal tool to study expression and function of the receptor. Indeed i.v. injection of CXCL11_12 into live mice revealed its expected uptake by heart

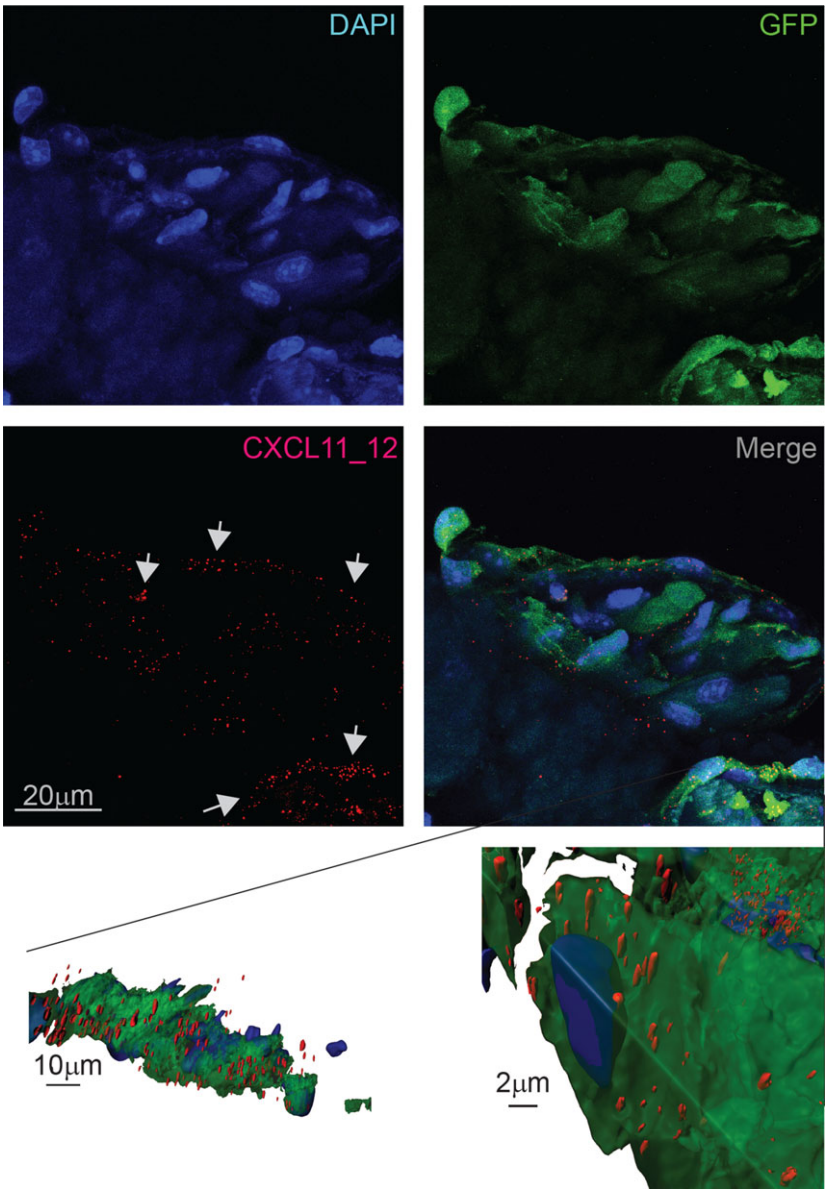


FIGURE 6 In vivo uptake of CXCL11_12 in mouse heart. Longitudinal section from a mouse heart. Upper panels maximal projections of 135 stacks. Arrows on the red panel indicate CXCL11_12 positive endosomal structures. Endogenous GFP (green) demarks ACKR3 positive cells and DAPI (blue) nuclei. Bottom row shows three-dimensional rendering of the 135 stacks at 2 electronic magnifications. Colors as in the upper panels. Images were taken at 0.29 μm distance with a 63× oil immersion objective

endothelium. This makes the chimera a particularly useful reagent for in vivo studies that is superior to the use of mouse monoclonal antibodies[29] which would interact with endogenous Fc-receptors. As long as specific high affinity antibodies either from different species or as Fab'$_2$ are not available the chimera remains an easily accessible alternative to study ACKR3 function. The high sequence homology between human and mouse CXCL12, with only 1 conservative substitution in the CXCL12-derived domain, and only 2 alterations in the sequence part derived from CXCL11 (proline^2/leucine and lysine^6/glutamine human/mouse, respectively) may explain the reactivity of our CXCL11_12 with ACKR3 from both species. The cross reactivity between species allows to use the chimera to determine the scavenging activity of ACKR3 in mice in the absence of functional antibodies. The finding that CXCL11_12 does not interfere with CXCL11 and CXCL12 signaling through their cognate receptors further suggest its application in complex biological systems. It remains to be established if a similar strategy can be used to engineer selective ligands for other atypical receptors like ACKR4, which binds CCL19, CCL21 (CCR7), and CCL25 (CCR9).

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DISCLOSURE

The authors declare that they have no conflict of interest.

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