CCL20 is a novel ligand for the scavenging atypical chemokine receptor 4

Christoph Matti1 | Giulia D‘Uonnolo2 | Marc Artinger1 | Serena Melgrati2 | Angela Salnikov1 | Sylvia Thelen2 | Vladimir Purvanov1 | Tobias D. Strobel1 | Lisa Spannagel1 | Marcus Thelen2 | Daniel F. Legler1,3,4

1Biotechnology Institute Thurgau (BITg), University of Konstanz, Kreuzlingen, Switzerland
2Institute for Research in Biomedicine, Università della Svizzera italiana, Bellinzona, Switzerland
3Faculty of Biology, University of Konstanz, Konstanz, Germany
4Theodor Kocher Institute, University of Bern, Bern, Switzerland

Correspondence
Daniel F. Legler, Biotechnology Institute Thurgau (BITg), University of Konstanz, Unterseestrasse 47, CH-8280 Kreuzlingen, Switzerland. Email: daniel.legler@bitg.ch
Marcus Thelen, Institute for Research in Biomedicine (IRB), Università della Svizzera italiana, Via Vincenzo Vela 6, CH-6500 Bellinzona, Switzerland. Email: marcus.thelen@irb.usi.ch

Abstract
The chemokine CCL20 is broadly produced by endothelial cells in the liver, the lung, lymph nodes and mucosal lymphoid tissues, and recruits CCR6 expressing leukocytes, particularly dendritic cells, mature B cells, and subpopulations of T cells. How CCL20 is systemically scavenged is currently unknown. Here, we identify that fluorescently labeled human and mouse CCL20 are efficiently taken-up by the atypical chemokine receptor ACKR4. CCL20 shares ACKR4 with the homeostatic chemokines CCL19, CCL21, and CCL25, although with a lower affinity. We demonstrate that all 4 human chemokines recruit β-arrestin1 and β-arrestin2 to human ACKR4. Similarly, mouse CCL19, CCL21, and CCL25 equally activate the human receptor. Interestingly, at the same chemokine concentration, mouse CCL20 did not recruit β-arrestins to human ACKR4. Further cross-species analysis suggests that human ACKR4 preferentially takes-up human CCL20, whereas mouse ACKR4 similarly internalizes mouse and human CCL20. Furthermore, we engineered a fluorescently labeled chimeric chemokine consisting of the N-terminus of mouse CCL25 and the body of mouse CCL19, termed CCL25_19, which interacts with and is taken-up by human and mouse ACKR4.

KEYWORDS
ACKR4, atypical chemokine receptor, CCL19, CCL20, CCL21, CCL25, chemokine scavenging, β-arrestin

1 INTRODUCTION

The role of the chemokine system in regulating leukocyte trafficking is well established, however, it also controls directed cell migration in embryogenesis and organogenesis.1,2 The chemokine family arose early in vertebrate development,3 consisting of approximately 45 proteins in human and mice, and is defined based on a conserved cysteine motif.4 Chemokines share a conserved tertiary structure that is maintained by 2 characteristic disulfide bridges established between cysteine residues. Depending on the positioning of the first 2 cysteine residues, chemokines are classified into CC, CXC, XC, and CX3C subfamilies. Functionally, chemokines can be grouped into primarily inflammatory or homeostatic chemokines. Chemokines guide leukocyte migration through the interaction with cognate chemokine receptors expressed on the surface of their target cells. Typical chemokine receptors belong to the class A of G protein-coupled receptors. They consist of 7 transmembrane domains that are connected by extracellular loops, and signal through heterotrimeric Gα-proteins.

In addition to the typical chemokine receptors, chemokines also bind to a subfamily of atypical chemokine receptors (ACKRs), which are
predominantly expressed by stroma and endothelial cells. 6,7 ACKRs appear to be unable to transduce signals required for directed cell migration, but internalize and scaveng chemokines and thereby control their availability. Hence, ACKRs are important to limit local and systemic chemokine concentrations. 8-11 In addition to scavenging, ACKR1 transcytoses and presents over 20 different CC and CXC chemokines in endothelial cells 12 and regulates neutrophil hematopoiesis in nucleated erythrocytes. 13 Besides ACKR1, ACKRs cycle between the plasma membrane and endosomes either spontaneously or ligand induced, thereby taking-up chemokines from the environment and sort them for lysosomal degradation. ACKR2 internalizes and sorts for degradation essentially all inflammatory chemokines of the CC chemokine subfamily and consequently plays a key role in resolving inflammatory responses. 14 ACKR3 is the scavenging receptor for the chemokines CXCL12 and CXCL11, and plays a crucial role in development and in the neuronal context. 15-19 ACKR4 scavenges the mainly homeostatic chemokines CCL19, CCL21, and CCL25. 20-22 Although CXCL13 was originally described to displace CCL19 binding with low efficiency on human ACKR4, 23 this was later shown to occur through cooperative GAG binding rather than directly interacting with ACKR4. 23 Consistent with this, CXCL13 was reported not to bind to mouse ACKR4. 24 ACKR4 is best known for its scavenging activity for CCL21 by subcapsular sinus lymphatic endothelial cells forming local CCL21 gradients in lymph nodes in vivo. 5 This CCL21 gradient is sensed by CC chemokine receptor (CCR)7-expressing dendritic cells transporting pathogen-derived antigens from peripheral tissues to draining lymph nodes where they initiate an adaptive immune response. 25,26

ACKRs do not couple to G-proteins, but ACKR2-4 were shown to undergo ligand-induced or constitutive interaction with β-arrestins, whereas the interaction of ACKR1 with this scaffold protein family is not clear. 27 Human ACKR4 was found to interact with β-arrestin1 and β-arrestin2 in a ligand-dependent manner with CCL19 displaying slightly higher efficiency than CCL21 and CCL25. 28

The chemokine CCL20 is expressed by endothelial cells of several tissues, such as the liver, the lung, lymph nodes, and mucosal lymphoid tissues, 29 and recruits CCR6 expressing cells, including T cell subsets, mature B cells, and dendritic cells. 1,2 So far, it remains unknown how CCL20 is locally and systemically scavenged. Here, we identify that ACKR4 is a specific scavenger for CCL20. Moreover and in accordance with the chimeric chemokine CXCL11_12, 30 we engineered a (fluorescent) chimeric chemokine consisting of the N-terminus of CCL25 and the body of CCL19, that interacts with and is taken-up by ACKR4.

2 MATERIALS AND METHODS

2.1 Bioinformatic analysis

Python 3.7.4 (http://www.python.org) with Biopython package 31 was used for alignments with ClustalOmega and reduction of amino acid sequences. Phylogenetic trees were built using NCBI Genome-Workbench 3.0.1. Interaction site predictions were retrieved from InterPro (http://www.ebi.ac.uk/interpro/). Modelling was performed using the SWISS-MODEL Workspace, 32 fitting either the hCCL19 sequence or hCCL20 sequence and hACKR4 onto the solved crystal structure of hCXCR4/endolysin chimeric protein with vMIP2 (PDB: 4RWS.1). Pymol V.2.4 was used for visual representation of the models; for distance representations between sidechains pairwisedistances.py script 33 was used.

2.2 Cloning of plasmids

Reagents for molecular biology were purchased from Thermo Fisher Scientific (Basel, Switzerland); custom-designed primers from Microsynth (Balgach, Switzerland). An overview of constructs, including the corresponding primer sequences used for cloning are listed in Table 1. Briefly, pcDNA3 β-arrestin2-NanoLuc luciferase (NLuc) was generated by amplifying human β-arrestin2 and NLuc separately, ligating the 2 PCR products over a common ClaI restriction site and cloning it conjointly into the HindIII and Xbal sites of pcDNA3. The construct includes a flexible GSI(GGGGS)4 linker between the 2 proteins. Chemokine receptor constructs were cloned using pcDNA3 ACKR4-EGFP 22 and pcDNA3 CCR7-HA 34 as template, where the receptor or the tag can be exchanged using HindIII/Xhol and Xhol/Xbal, respectively. Exceptions are pcDNA3 hCCR6-HA and pcDNA3 hCCR9-HA, where 2 oligos coding for GLLE3(GGGGS)3-HAtag were annealed and cloned into the Xhol and Xbal restriction sites of pcDNA3 hCCR6-enhanced yellow fluorescent protein (EYFP) or pcDNA3 hCCR9-EYFP, exchanging linker-EYFP for linker-HAtag. 35 Chemokines were amplified by PCR and cloned into the Xhol and BsoI restriction sites of pET-His6-SUMO. 36 In addition, for hCCL20s a SGGGGS-S6tag 35 was added to the C-terminus of hCCL20, pET-His6-SUMO hCCL21 has been described elsewhere. 36

2.3 Chemokine production

Recombinant human chemokines fused to a His6-SUMOtag and a SGGGGS-S6tag were purified from BL21 (DE3) Escherichia coli, refolded via infinite dilution at pH 8.5 and the His6-SUMOtag was cleaved by incubation with the Ulp-1 protease for 1–5 h and removed during the final purification step. 26-28 Minor changes regarding CCL25 with additional arginine [0.2 M], glutamine [0.2 M], and 0.1% Triton X-100 as refolding additives were made. Mouse chemokines were prepared as described 37 and tagged with a ybbR13tag. 35 The final purification of all chemokines was performed by reverse phase HPLC on C18 columns. Coenzyme A (CoA)-conjugated (C3144-25MG; Sigma, Buchs, Switzerland) dyes were made as described. 37 Fluorescently labeled hCCL20sDy649P1 was generated by labeling purified hCCL20s with CoA-Dy649P1 (649P1-03; Dyomics GmbH) at 37°C for 2 h using the phosphopantetheinyl transferase Sfp (P93025; New England Biolabs, Ipswich, MA, United States) as described. 37 Excess of substrate was removed from fluorescently labeled human chemokines using a PD 10 desalting column (17085101; GE Healthcare Life Sciences,
| Construct | Template (if not synthesised); amplified insert in bold; [reference] | 5′-Forward primer | 5′-Reverse primer | Linker |
|-----------|-------------------------------------------------|------------------|------------------|--------|
| pcDNA3 β-arrestin2i1-NLuc | pcDNA3 β-arrestin2i1-Y240 | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | a |
| pcDNA3 β-arrestin1A-NLuc | β-arrestin1A RC201279 (Origene) | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | a |
| pcDNA3 hACKR4-EYFP | pcDNA3 CCR7-EYFP | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pcDNA3 hACKR4-mScarlet | pcDNA3 hACKR4-mScarlet I SDM on pcDNA3 hACKR4-mScarlet replacing Thr74Ile on mScarlet | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pcDNA3 hACKR3-HA | pcDNA3 hACKR3-HA | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pcDNA3 hCCR7-EYFP | pcDNA3 hCCR7-EYFP | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pcDNA3 hCCR6-EYFP | pCEP4 cGS | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pcDNA3 hCCR9-EYFP | pCEP4 cGS | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pSUMO hCCL19 | CR3 hCCL19-Fc | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pSUMO mCCL19 | | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pSUMO hCCL20 | pDONR221 mCCL20 (DNASU HsCD00042527) | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pSUMO mCCL20 | pHis-Entero mCCL20 ybbR13 | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pSUMO hCCL21 | | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pSUMO mCCL21 | | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pSUMO hCCL25 | hCCL25 RC222128 (Origene) | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pSUMO mCCL25 | pHis-Entero mCCL25 ybbR13 | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |
| pSUMO hCCL20-S6 | pSUMO hCCL20 | GGTGGAAGCTTATGGGGAGAACCCG | GCATCGTACCCACCGAGGTTG | |

Linker sequence used between protein and tag:

- GS1(GGGGS)$_3$
- GGLES1(GGGGS)$_3$
- GARA
- SGGGGS
2.4 | Cell culture and transfection

HeLa and HEK293 cells were cultured in DMEM (P04-04510; Pan Biotech, Aidenbach, Germany), containing 1% penicillin/streptomycin (P06-07100; Pan Biotech) and 10% FBS (10270-106; Thermo Fisher Scientific). Cells were transfected at least 30 h prior to the experiments using the 100 µl Neon® Transfection System (MPK10096; Thermo Fisher Scientific) according to the manufacturer’s protocol, transfecting 5 × 10^5 cells with 10 µg total plasmid DNA. For bioluminescence resonance energy transfer (BRET) experiments, the DNA ratio of fluorophore to luciferase construct was 3:1. Mouse 300–19 pre-B cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1% nonessential amino acids, 1% glutamax, and 50 µM β-mercaptoethanol as described.30,39

2.5 | 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 3 min. Cells were collected in twice the volume of dissociation buffer with DMEM containing 10% FBS before centrifugation for 2 min at 200g. Cells were washed and resuspended in PBS containing 5% (w/v) glucose (PBS-G). Aliquots of 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, Thal, Switzerland) and stimulated with various concentrations of chemokines. Ratiometric BRET measurements were performed using a Tecan Spark™ 10 M 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.40 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. Area under the curve analysis (AUC) was performed using the first 5 measurements as baseline and integrating the peak starting from 0 min until the end of measurement at 29.5 min.

2.6 | 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 HEPES-buffered, high glucose DMEM without phenol red (#21063045; Thermo Fisher Scientific) at 37°C or 10°C, respectively. Fifty microliters of chemokine solutions (for competition experiments) or 100 µl (for uptake experiments) for 45 min at 37°C in medium at the indicated concentrations. When indicated cells were subjected to a brief acidic wash. Reactions were stopped with medium containing 0.1% azide and cell suspensions measured with a BD Fortessa flow cytometer.
mCCL20yAF647 (15 µl, 30 nM in PBS) was injected into the food pad of wild-type C57BL/6 mice, CCR6ko C57BL/6 mice, kindly provided by Sergio Lira, or heterozygous (ACKR4 GFP/wt) and homozygous (ACKR4 GFP/GFP) ACKR4-EGFP knock-in reporter mice. After 15 min animals were sacrificed, the draining popliteal lymph node removed and fixed with formaldehyde by placing the specimens in 4% paraformaldehyde for 5 h. Vibratome sections were prepared as described and stained with anti-podoplanin-PE (clone eBio8.1.1; eBioscience, San Diego, CA, United States) and anti-B220-biotin (clone RA3-6B2; BD PharMingen, Allschwil, Switzerland)/streptavidin pacific blue (Thermo Fisher Scientific). Mice were treated in accordance with guidelines of the Swiss Federal Veterinary Office and experiments were approved by the Dipartimento della Sanità e Socialità.

2.8 Confocal fluorescence microscopy

Transfected HeLa or HEK293 cells were seeded in 1 ml of HEPES-buffered, high glucose DMEM without phenol red (#21063045; Thermo Fisher Scientific) in 35 mm glass bottom imaging dishes (Ibidi or Mattek). A Leica TCS SP5 II confocal microscope with a 40× or 63×
FIGURE 2  Human ACKR4 is a scavenger for human CCL20. (A) Uptake of fluorescently labeled hCCL20s Dy649P1 by hACKR4-YPet. HeLa cells were transiently transfected with hACKR4-YPet (green), stimulated at t = 0 s with 18 nM hCCL20s Dy649P1 (red) and chemokine uptake was monitored over time by confocal microscopy. Representative images from a time-lapse video out of 3 are shown. Scale bar = 15 µm. (B) Gating strategy and FACS profile of hCCL20s Dy649P1 uptake by hACKR4-YPet. Parental HeLa cells (blue histogram) and transiently transfected HeLa cells expressing hACKR4-YPet (red histogram and dot-plots) were stimulated at 37°C with 18 nM hCCL20s Dy649P1 for 0 or 60 min and hACKR4-YPet and hCCL20s Dy649P1-associated fluorescence (lower panel) was determined by flow cytometry. (C) HeLa cells were transiently transfected or not with hACKR4-YPet and stimulated at 37°C with 18 nM hCCL20s Dy649P1 for indicated times. Cells were subsequently exposed or not to a brief acidic wash (AW) for 45 s and the mean fluorescent intensity (MFI) ± SD of hCCL20s Dy649P1 on hACKR4-YPet positive cells was determined as in (B). n = 4. (D) Quantification of hCCL20s Dy649P1 uptake by hACKR4-YPet positive cells at t = 60 min of the same data sets shown in (C) are depicted as bar graph. MFI ± SD, n = 4

Oil-immersion objective was used. For time-lapse imaging 6 sec intervals between frames were chosen. Five minutes after initial recording, 100 µl medium containing the chemokine was added and uptake was monitored over time. Acquired images were processed using Fiji, ImageJ2,44 and Imaris V9 (Bitplane, Zurich, Switzerland).

2.9  Data analysis

Data analysis and presentation was performed using GraphPad Prism V.7 or SigmaPlot V14. Data represent an “n” number of independent experiments. EC50 values were calculated fitting a 3 parameter [Agonist] versus response curve. For experiments using 1 variable, Students t-test was performed (Fig. 7B) P < 0.001 (**). For experiments using 2 variables, 2-way ANOVA with Sidak’s multiple comparisons test with a single pooled variance (Figs. 2D and 6F) or 2-way ANOVA with Sidak’s multiple comparisons test with individual variances computed for each comparison (Fig. 6G) or mixed-effects model with Sidak’s multiple comparisons test with a single pooled variance (Fig. 6H) were performed. *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.0001.

3  RESULTS

3.1  CCL20 is closely related to ACKR ligands

The chemokine CCL20 recruits CCR6 expressing leukocytes, including mature B cells, subpopulations of T cells and dendritic cells.2-7 So far, no scavenging ACKR has been identified for CCL20. Sequence relationship analysis of human chemokines revealed a close relationship among CCL20, CCL19, and CCL21 (Fig. 1A), the latter 2 ligands share the typical chemokine receptor CCR7 and the atypical chemokine receptor ACKR4. As chemokines in general show relatively little sequence homology, we build a phylogenetic tree based on the physicochemical traits using the Murphy8 amino acid table.45 The Murphy8 alphabet reduces the 20 amino acids to a smaller 8-letter alphabet based on correlations between amino acid pairs with high similarity scores (for details see45 and https://biopython.org/DIST/docs/api/Bio.Alphabet.Reduced-module.html). This resulted in a phylogenetic cluster of CCL20 with the known ACKR4 ligands CCL19, CCL21, and CCL25 (Fig. 1A). Human CXCL13
FIGURE 3 Mouse ACKR4 is a scavenger for mouse CCL20. (A) Binding and uptake of fluorescently labeled mCCL20yAF647 and hCCL20sDy649P1 by mACKR4-T2A. 300–19 pre-B cells expressing mACKR4-T2A(-GFP) were incubated with 30 nM mCCL20yAF647 (left panel) or hCCL20sDy649P1 (right panel) at 4°C (to determine binding, upper panels) or 37°C (to measure uptake, lower panels). Chemokine uptake on GFP-positive cells was measured by flow cytometry. (B) Competition uptake of mCCL20yAF647 by mACKR4-T2A. Stably transfected 300–19 pre-B cells expressing mACKR4-T2A(-GFP) were incubated at 37°C for 45 min with 30 nM mCCL20yAF647 and graded concentrations of unlabeled mCCL20y. Uptake was measured by flow cytometry. Duplicate measurements of a typical experiment out of 3 independent determinations made with different chemokine preparations. (C) hCCL19 and hCCL21 outcompete binding and uptake of mCCL20yAF647 by mACKR4-T2A. 300–19 pre-B cells stably expressing mACKR4-T2A(-GFP) were incubated at 37°C or 4°C for 45 min with 2 nM mCCL20yAF647 and graded concentrations of unlabeled and untagged hCCL19 or hCCL21. Uptake was measured by flow cytometry and mean fluorescence intensities obtained as shown in (A). Duplicate measurements of a typical experiment out of 3 independent determinations. (D) Progressive uptake of mCCL20yAT565 by mACKR4-T2A. Parental 300–19 pre-B cells and mACKR4-T2A(-GFP) or hCCR6 transfectants were incubated with 100 nM mCCL20yAT565 over time and chemokine uptake was determined by flow cytometry. Duplicate measurements of a typical experiment out of 3 independent determinations.

(Continues)

did not fall into the same cluster (Supplemental Fig. 1A), supporting the notion that CXCL13 is not a ligand for ACKR4.\textsuperscript{22,24} Further analysis of reduced sequences using ClustalOmega\textsuperscript{46} identified sequence homology sites (green frames) among the 4 human CC chemokines (Fig. 1B), but less with CXCL13 (Supplemental Fig. 1B), that matched with putative receptor interaction sites (black boxes) (Fig. 1B) predicted by InterPro.\textsuperscript{47} Next, we fitted the human (h) ACKR4, hACKR4, sequence together with either hCCL19 or hCCL20 onto the solved crystal structure of the hCXCR4/vMIP2 complex\textsuperscript{48} using the SWISS-MODEL workspace and color-coded residues of hCCL19 and hCCL20 predicted to be closer than 4.5 Å to the receptor (Fig. 1C). This approximation suggests that hCCL19 has more short interaction distances and penetrates deeper into the binding pocket of hACKR4 than hCCL20, suggesting higher binding affinity for hCCL19.

Combining these modeling insights with the ClustalOmega sequence alignment permits to extend the predicted interaction regions by several amino acids illustrated by black lines in Fig. 1B. Thereby, the first half of region 1 of the chemokine interplays with the receptor pocket, where the second half of region 1 together with regions 2, 3, and 5 of the chemokine are predicted to interact with the receptor’s N-terminus. Region 4 and the N-terminus of the chemokine appear to be engulfed by the binding pocket of the receptor. These in silico
analysis suggest that hCCL19, hCCL21, and hCCL25 might share their scavenging receptor hACKR4 with hCCL20, but with potentially different affinities. Inspection of the human protein atlas (http://v19.proteinatlas.org) revealed complementary transcript expression of hACKR4, its ligands, as well as hCCL20 in lymphoid tissues and the gastrointestinal tract. In addition, hCCL20 is frequently expressed in the lung, liver, kidney, gallbladder, and urinary bladder, where hACKR4 is also expressed, but to a much lower extent than the so far known ligands hCCL19, hCCL21, and hCCL25 (Fig. 1D), or hCXCL13 (Supplemental Fig. 1C). These bioinformatic approaches conjointly suggest CCL20 as a novel ACKR4 ligand.

3.2 ACKR4 is the scavenger for CCL20

To assess whether ACKR4 binds and scavenges CCL20, we engineered fluorescently labeled versions of human and mouse CCL20. To this end, we fused short peptide tags (S6, abbreviated as (s) for human (h) and ybbR13 (y) for mouse (m) chemokines) to the C-terminus of chemokines, revealing hCCL20s and mCCL20y, respectively, for site-specific protein labeling with the phosphopantetheinyl transferase Sfp. Sfp adds the fluorescent dye conjugated phosphopantetheine moiety of CoA to one specific serine residue of the tags. To monitor chemokine uptake, we imaged HeLa cells transiently transfected with human ACKR4 fused at the C-terminus to the yellow fluorescent protein YPet (hACKR4-YPet) using confocal microscopy. We observed spontaneous trafficking of hACKR4-YPet by time-lapse imaging. Successively exposing the cells to fluorescently labeled hCCL20s or mCCL20y revealed specific binding and subsequent uptake of the chemokine by hACKR4-YPet expressing cells, but not by neighboring cells lacking the receptor (Fig. 2A and Supplemental Video 1). To further substantiate and quantify the specific uptake of hCCL20 by hACKR4, we stimulated HeLa cells expressing hACKR4-YPet with hCCL20s for various times. We measured chemokine binding (by incubating cells at 10°C to prevent internalization) and uptake (at 37°C) by flow cytometry. hCCL20s specifically bound to hACKR4-YPet expressing cells at 10°C, but not to untransfected control cells (Figs. 2B–2D). A brief acidic wash removed surface receptor bound chemokine (Figs. 2B–2D). By contrast, at 37°C hCCL20s was continuously internalized by hACKR4-YPet-expressing cells over time and consequently an acidic wash barely reduced the accumulated fluorescence intensity (Figs. 2C and 2D). Next, mouse ACKR4 fused via a self-cleaving peptide to green fluorescent protein (mACKR4-T2A(-GFP)) was expressed in 300–19 pre-B cells. The T2A sequence, after translation, splits the 2 proteins and marks cells that expressing mACKR4 tagged with a small peptide (mACKR4-T2A) at the receptor’s C-terminus proportionally with liberated GFP.52 This allows the distinction of mACKR4 expressing from untransfected cells or cells that have lost receptor expression. Incubating the cells with 30 nM fluorescently labeled mCCL20y or hCCL20s at 4°C revealed poor binding for both chemokines to mACKR4-T2A (Fig. 3A). Similar to the human ACKR4, incubation at 37°C for 45 min led to a marked uptake of either mCCL20y or hCCL20s by mACKR4-T2A (Fig. 3B). Uptake was slightly more pronounced for the mouse chemokine, suggesting a possible moderate species preference. Competition uptake at 37°C revealed a medium/low affinity for mCCL20y binding to mACKR4-T2A (Fig. 3B). This observation is consistent with previous findings where 200 nM CCL20 essentially failed to outcompete I-CCL19 on human and mouse ACKR4. Nevertheless, at 37°C addition of increasing concentrations of unlabeled hCCL19 and hCCL21 efficiently outcompeted the uptake of 30 nM of mCCL20y (Fig. 3C), reflecting

---

**FIGURE 3** (Continued) **(E)** Uptake of mCCL20y by mACKR4-T2A. Confocal image of mCCL20y uptake by HEK293 cells expressing mACKR4-T2A(-GFP). HEK293 stably transfected with ACKR4-T2A(-GFP) were seeded together with parental HEK293 cells (circled with dotted line) on glass bottom dishes and incubated with 50 nM mCCL20y for 40 min at 37°C. Images were recorded with 40× magnification with a laser scan confocal microscope. mCCL20y internalized by mACKR4-T2A (green) cells coexpressing GFP. Nuclei of all cells were visualized with DAPI. Right panel depicts phase contrast of the area with parental cells to identify cell borders.
the high affinity of these ligands for ACKR4 and suggests that the small T2A-tag does not interfere with receptor function (Fig. 1C). As expected for a scavenging receptor, 100 nM of mCCL20y AF647 was progressively taken up by 300–19 cells expressing mACKR4-T2A-GFP) over time, whereas uptake by hCCR6 was observed at early time-points, but did not profoundly increase over time (Fig. 3D). Importantly, HEK293 cells transfected with mACKR4-T2A(-GFP) readily internalized mCCL20y AF647, whereas untransfected cells did not (Fig. 3E). Actively scavenging ACKR4 is expressed on endothelial cells of the subcapsular sinus of lymph nodes. Therefore, to test CCL20 uptake in vivo, we injected mCCL20y AF647 into the foot pads of wild-type and CCR6 ko mice. Within 15 min, mCCL20y AF647 was readily found and taken up by podoplanin-positive supcapsular sinus endothelial cells of the draining popliteal lymph node in wild-type and CCR6 ko mice (Fig. 4). In addition, we injected mCCL20y AF647 in the food pads of heterozygous ACKR4 GFP/ GFP reporter mice. As shown in Fig. 4, mCCL20y AF647 was taken up at the subcapsular sinus of heterozygous ACKR4 GFP/ GFP reporter mice, but not by homozygous ACKR4 GFP/ GFP mice lacking ACKR4. These data indicate that ACKR4 acts as a scavenger for CCL20 at the subcapsular sinus and that ACKR4 is the main scavenger in this location. Moreover, we also provide evidence that
hCCL20 recruits β-arrestins to hACKR4. HeLa cells were transiently cotransfected with constructs for human receptor tagged with EYFP and β-arrestins fused to NLuc. At least 30 h after transfection, cells were incubated with the luciferase substrate coelenterazine H and subsequently stimulated with graded concentrations of untagged human chemokines. Chemokine-driven β-arrestin recruitment to the receptor was determined by BRET. The area under the curve (AUC) was determined after stimulation for a total of 29.5 min, the initial 5 min before stimulation served as baseline. (A) hCCL20-induced β-arrestin1-NLuc recruitment to hACKR4-EYFP, n = 4. (B) hCCL20-induced β-arrestin2-NLuc recruitment to hACKR4-EYFP, n = 4. (C) hCCL19-induced β-arrestin2-NLuc recruitment to hACKR4-EYFP, n = 4. (D) hCCL20 does not recruit β-arrestin2-NLuc to hACKR3-EYFP, n = 3. (E) hCCL20-induced β-arrestin1-NLuc recruitment to hCCR6-EYFP, n = 4. (F) hCCL20-induced β-arrestin2-NLuc recruitment to hCCR6-EYFP, n = 4. Mean ± SD are depicted.
FIGURE 6  Cross-species analysis of CCL20 and ACKR4. (A) ClustalOmega sequence alignment of human and mouse chemokines. Differences in the amino acid sequence, based on the reduced Murphy8 amino acids, are highlighted with red boxes, putative receptor interaction sites are indicated with black lines and boxes. Chemokine-driven $\beta$-arrestin2-NLuc (B–F) or $\beta$-arrestin1-NLuc (G) recruitment to hACKR4-EYFP was determined by BRET. HeLa cells transiently coexpressing hACKR4-EYFP and $\beta$-arrestin2-NLuc (B–F) or $\beta$-arrestin1-NLuc (G) were incubated with the luciferase substrate coelenterazine H and subsequently stimulated with a saturating concentration (1.5 µM) of untagged human (blue) or mouse (red) chemokine. NetBRET over time of $\beta$-arrestin2-NLuc recruitment to hACKR4-EYFP upon stimulation with CCL20 (B), CCL19 (C), CCL21 (D), or CCL25 (E). Chemokine-driven $\beta$-arrestin2-NLuc (F) or $\beta$-arrestin1-NLuc (G) recruitment to hACKR4-EYFP. The area under the curve (AUC) was determined after stimulation for a total of 29.5 min, the initial 5 min before stimulation were used as a baseline (dotted line). (B–G) Mean $\pm$ SD. n = 3.

(Continues)
ACKR4 can actively take-up CCL20 albeit with a lower affinity than CCL19 and CCL21. Taken together, these data demonstrate that ACKR4 can actively recruit CCL20, with only 81% homology compared with 98% (CCL19), 93% (CCL21), and 88% (CCL25).

We addressed putative ligand species specificity by exploiting our BRET-based β-arrestin recruitment assay. We transiently coexpressed HeLa cells with hACKR4-EYFP and β-arrestin2-NLuc, and stimulated cells with the same concentration of either human or mouse untagged chemokines (Figs. 6B–6F). At saturating conditions (1.5 µM), all 4 human chemokines comparably recruited β-arrestin2-NLuc to hACKR4-EYFP. Interestingly, mCCL19, mCCL21, and mCCL25 comparably activated hACKR4-EYFP. By contrast, the same concentration of mCCL20 hardly recruited β-arrestin2-NLuc to hACKR4-EYFP (Figs. 6B–6F). Similarly, 1.5 µM of h/mCCL19, h/mCCL21, h/mCCL25, but solely hCCL20 efficiently recruited β-arrestin1-NLuc to hACKR4-EYFP (Fig. 6G). Next, we measured uptake of fluorescently labeled hCCL20s Dy649P1 by HeLa cells expressing either hACKR4-EYFP or mACKR4-EYFP. As expected, hACKR4-EYFP efficiently took up 18 nM hCCL20s Dy649P1 (Fig. 6H). Interestingly, mACKR4-EYFP also internalized hCCL20s Dy649P1 although less efficient (Fig. 6H), which is in line with the finding that mACKR4-T2A(-GFP) is able to take-up both mouse and human CCL20 (Fig. 3A). These results prompted us to re-assess β-arrestin-NLuc recruitment to hACKR4-EYFP by higher concentrations of mCCL20. Dose–response curves revealed that mCCL20 was able to recruit β-arrestin1-NLuc and β-arrestin2-NLuc to hACKR4-EYFP although at much higher concentrations (>5 µM) than hCCL20 (Fig. 6I).

3.4 | Cross-species analysis of CCL20 and ACKR4

To assess species specificity of the 4 ACKR4 ligands to its receptor, we aligned human and mouse orthologue chemokines using ClustalOmega. Based on the reduced Murphy8 amino acids the orthologue sequence alignment revealed higher similarity for CCL19 and CCL21, than for CCL20 and CCL25 (Fig. 6A). Subsequent putative receptor binding site prediction using InterPro did not envisage differences for CCL19 and CCL21 in receptor binding (Fig. 6A).

The highest variability in the predicted binding regions of all chemokine pairs is present in CCL20, with only 81% homology compared with 98% (CCL19), 93% (CCL21), and 88% (CCL25).

To assess species specificity of the 4 ACKR4 ligands to its receptor through which they induce chemotaxis. To obtain specific ligands for ACKR3, we have previously engineered the chimeric chemokine CXCL11_12. It consists of the N-terminus of CXCL11 and the body of CXCL12, both are ligands for the atypical receptor, but bind...
FIGURE 7  The chimeric chemokine CCL25_19 is a ligand for mACKR4. (A) Sequence alignment of mCCL25 (red rectangle), mCCL19 (blue rectangle), and the chimeric chemokine CCL25_19. The green frame indicates the ybbR13-tag fused to the C-terminus of CCL25_19y. (B) CCL25_19ySiR is a ligand for mACKR4-T2A(-GFP), but not for mCCR7-T2A(-GFP) or mCCR9-T2A(-GFP). 300–19 pre-B cells expressing either mACKR4-T2A(-GFP), mCCR7-T2A(-GFP), or mCCR9-T2A(-GFP) were incubated with 30 nM CCL25_19ySiR at 37°C for 45 min. Cells expressing mACKR4-T2A(-GFP) or mCCR7-T2A(-GFP) were washed and incubated with 100 nM unlabeled CCL19 for 30 min on ice to replace surface bound labeled chemokine. Chemokine-associated fluorescence on GFP-positive cells was determined by flow cytometry. CCL25_19 did not bind to CCR9-T2A expressing cells. (C) Competition uptake of CCL25_19ySiR by mACKR4-T2A. Stably transfected 300–19 pre-B cells expressing mACKR4-T2A(-GFP) were incubated with 2 nM CCL25_19ySiR at 37°C for 45 min with 2 nM CCL25_19ySiR and graded concentrations of unlabeled CCL25_19y, hCCL19, or hCCL21. Uptake of CCL25_19ySiR was measured by flow cytometry. Duplicate measurements of a typical experiment out of 3 independent determinations. (D) Specific (continued on the next page)
exclusively the typical receptors CXCR3 and CXCR4, respectively.30 Hence, we reasoned that a chimeric chemokine consisting of the N-terminus of mCCL25 and the body of mCCL19 could be a ligand for mACKR4. We expressed the recombinant CCL25_19 chimeric chemokine fused to the ybbR13 tag (Fig. 7A) in E. coli.37 Fluorescently labeled CCL25_19ySiR is readily taken up at 37°C by 300–19 pre-B cells expressing mACKR4-T2A(-GFP), but not by cells expressing mCCR9-T2A(-GFP) (Fig. 7B and Supplemental Fig. 2). CCL25_19ySiR weakly interacted with 300–19 pre-B cells expressing mCCR7-T2A(-GFP) at 37°C (Fig. 7B). We therefore incubated mCCR7-T2A(-GFP) cells with 30 nM CCL25_19ySiR at 37°C for 45 min, followed by an incubation in the presence of 100 nM unlabeled hCCL19 on ice. This procedure outcompeted 30 nM CCL25_19ySiR interaction with mCCR7-T2A, but not with mACKR4-T2A (Fig. 7B). This indicates that CCL25_19ySiR slightly binds to mCCR7-T2A(-GFP) expressing cells, but is barely internalized as it can be outcompeted with unlabeled hCCL19. By contrast, unlabeled hCCL19 did not outcompete CCL25_19ySiR from mACKR4-T2A(-GFP) expressing cells and hence has been internalized (Fig. 7B). CCL25_19ySiR binding to mACKR4-T2A was of high affinity as determined by competing binding with unlabeled CCL25_19y, which was comparable with the affinities of hCCL19 and hCCL21 for mACKR4-T2A (Kd ~ 1 nM) (Fig. 7C). To confirm uptake of the chimeric chemokine, we expressed mACKR4-T2A(-GFP), mCCR9-T2A(-GFP), or mCCR7-T2A(-GFP) in HEK293 cells and treated the cells with CCL25_19ySiR. Confocal imaging revealed that CCL25_19ySiR is taken up solely by mACKR4-T2A(-GFP) transfected cells (Fig. 7D). By contrast, mCCR7-T2A(-GFP) or mCCR9-T2A(-GFP) expressing cells did not internalize CCL25_19ySiR (Fig. 7D). To corroborate these findings, we expressed mACKR4-T2A(-GFP) or mCCR7-T2A(-GFP) in HEK293 cells and visualized CCL25_19ySiR uptake by time-lapse imaging. Of note, CCL25_19ySiR was specifically taken up by cells expressing mACKR4-T2A(-GFP) (Supplementary Video 2) but not by mCCR7-T2A(-GFP) expressing cells (Supplemental Video 3). We did not determine surface expression of the different receptors in these experiments or examine the ability of mCCR9-T2A(-GFP) to internalize fluorescent mCCL25 to formally exclude conceivable differences between the receptors. However, we show that mCCR7-T2A(-GFP) expressing cells readily internalized hCCL19-mRFP (Fig. 7D). To test in vivo uptake of the chimeric chemokine, we injected CCL25_19ySiR (or PBS as control) into the food pads of heterozygous ACKR4GFP/wt reporter mice and homozygous ACKR4GFP/GFP that lack ACKR4. As shown in Fig. 7E, CCL25_19ySiR was readily taken-up by GFP-positive subcapsular sinus endothelial cells of the draining lymph nodes of ACKR4GFP/wt reporter mice, but not by GFP-positive, ACKR4-deficient (ACKR4GFP/GFP) cells.

Next, we tested whether the mouse-sequence derived chimeric chemokine CCL25_19 is also scavenged by hACKR4. CCL25_19ySiR was readily and specifically taken up by HeLa cells expressing hACKR4-YPet, but not by neighboring cells lacking the receptor (Fig. 8A and Supplementary Video 4). We then mixed transiently transfected HeLa cells expressing either hACKR4-mScarletI or hCCR7-YPet in a 1:1 ratio and visualized CCL25_19ySiR uptake by time-lapse imaging (Supplementary Video 5). Notably, CCL25_19ySiR was readily taken up by hACKR4-mScarletI, but not by hCCR7-YPet expressing cells. Finally, CCL25_19y specifically recruited β-arrestin2-NLuc to hACKR4-EYFP (Fig. 8B), but not to hCCR7-EYFP (Fig. 8C) or hCCR9-EYFP (Fig. 8D).

In summary, with the presented data we identified CCL20 as ligand for ACKR4. We found that fluorescently labeled human and mouse CCL20 bind to the ACKR4 with a relatively low affinity but, nonetheless, are efficiently taken-up by the scavenger receptor, with whom it shares the ligands CCL19, CCL21, and CCL25. We further demonstrated that β-arrestin1 and β-arrestin2 were recruited to ACKR4 by all 4 human chemokines. Interestingly, we noted a certain species preference for CCL20, particularly in terms of β-arrestin recruitment to human ACKR4. Finally, we present the engineered chimeric chemokine CCL25_19 as ligand for human and mouse ACKR4.

4 | DISCUSSION

The family of ACKR is emerging as key regulator of the chemokine networks in a wide range of developmental, physiologic, as well as pathologic context.6,7 For instance, ACKR3 plays an essential role in development and in the neuronal context by scavenging CXCL12, limiting its systemic concentrations,15-17,19 whereas ACKR2 contributes to the resolution of inflammation by removing inflammatory CC chemokines.14 So far, ACKR4 has been described to scavenge the homing and homeostatic chemokines CCL19, CCL21, and CCL20,20-22,56 Particularly, ACKR4 on subcapsular sinus lymphatic endothelial cells scavenges CCL21 to shape functional chemokine gradients that enable directed dendritic cell migration.8 Interestingly, others reported an accumulation of CCL20 in subcapsular sinuses of LN, however without distinguishing between CCL20 production by endothelial cells and potential scavenging by the ceiling cells.57 Here, we identified that ACKR4 in addition serves as scavenging receptor for CCL20. The CCL20/CCR6 axis is known to contribute on the one hand to effective humoral and memory immune responses, and on
FIGURE 8  CCL25_19 is a specific ligand for hACKR4. (A) Uptake of fluorescently labeled CCL25_19ySiR by hACKR4-YPet. HeLa cells were transiently transfected with hACKR4-YPet (green) and stimulated with 25 nM CCL25_19ySiR (red) at t = 2′ and chemokine uptake was monitored over time by confocal microscopy. Representative images from a time-lapse video out of 3 are shown. Scale bar = 20 µm. (B–D) CCL25_19y recruits β-arrestin2-NLuc to hACKR4-EYFP, but not hCCR7-EYFP or hCCR9-EYFP. HeLa cells were transiently cotransfected with β-arrestin2-NLuc and hACKR4-EYFP (B), hCCR7-EYFP (C), hCCR9-EYFP (D). At least 30 h after transfection, cells were incubated with the luciferase substrate coelenterazine H and stimulated with 1 µM CCL25_19y (blue line), control chemokines (red lines), or PBS-G (gray line). Chemokine-driven β-arrestin2-NLuc recruitment to the receptor was determined by BRET. n = 3

the other hand to Th17 cell-related inflammatory and autoimmune diseases. Identifying ACKR4 as scavenger for CCL20 opens the perspective for new approaches to develop therapeutics to treat various inflammatory diseases.

In general, information on ACKR signaling is sparse, but ACKR2, ACKR3, and hACKR4 are described to recruit β-arrestins upon ligand binding. More precisely, Watts and colleagues previously reported that CCL19, CCL21, and CCL25 recruited β-arrestin1 and β-arrestin2 to hACKR4, which correlated with internalization of fluorescently labeled CCL19. Here, we confirm this study and additionally show that hCCL20 recruited β-arrestins to hACKR4. Moreover, we also show that fluorescently labeled CCL20 is readily taken up by hACKR4 both in vitro and in vivo. Despite a relatively moderate binding affinity in vitro, uptake of CCL20 by hACKR4 readily occurs with low
nanomolar concentrations of the chemokine. In the light of the recent discovery that β-arrestins are dispensable for CCL19 scavenging by ACKR3, additional and more quantitative experiments are required to more rigorously characterize uptake of fluorescently labeled CCL20 (and CCL25_19) by ACKR4 in vitro and in vivo.

A major challenge in studying classical and atypical chemokine receptors is the poor availability of validated, high quality antibodies. As an alternative, fluorescently labeled chemokines are becoming commercially available or can be made as recombinant proteins, either as GFP/RFP-fusion protein, as Fc-tagged protein or as peptide-tagged protein that can be fluorescently labeled.22,34,37,50,62,63 The drawback of these fluorescently labeled chemokines is that they often interact with more than 1 receptor. By contrast to classical chemokine receptors, ACKRs seem to be remarkably tolerant to alternative N-termini of chemokines. Our silico analysis of ligand binding to ACKR4 further supports this notion. This property has recently been exploited to engineer a chimeric CXCL11_12 chemokine that selectively interacted with ACKR3.30 Here, we used a similar strategy to design a selective chimeric chemokine ligand for ACKR4. To achieve this, we engineered CCL25_19, which consists of the N-terminus of mouse CCL25 and the body of mouse CCL19. Fusing a short peptide tag to the C-terminus of CCL25_19 facilitated site-specific labeling with a fluorescent dye of choice.51 We demonstrated that fluorescently labeled CCL25_19 was efficiently and specifically internalized by ACKR4 expressing cells. Using this methodology, ACKR4-positive cells can be identified and labeled with fluorescent CCL25_19 (or CCL20). This approach will help to identify and isolate ACKR4 expressing cells from tissue and to study receptor functions in vitro and ex vivo.

ACKNOWLEDGMENTS
We thank Nicola Catone, Oliver Gerken and Ilona Kindinger for technical assistance with chemokine preparation and Joshua Farber for kindly providing the hCCR9 plasmid. This work is supported by the Swiss National Science Foundation [SInergia CRSIII_160719 (M.T. and D.F.L.)], the Helmut Horten Foundation (M.T.), the Konstanz Research School Chemical Biology (KoRSc-B), the Crescere Stiftung Thurgau, the Thurgauische Stiftung für Wissenschaft und Forschung, and the State Secretariat for Education, Research and Innovation (D.F.L.).

AUTHORSHIP
C.M., M.T., and D.F.L. designed the studies and wrote the manuscript. C.M., G.D’U., M.A., S.M., A.S., ST., VP, T.D.S., and L.S. performed the experiments. C.M., M.A., M.T., and D.F.L. analyzed the data. M.T. and D.F.L supervised the overall study. G.D’U. and M.A. contributed equally to this work. M.T. and D.F.L. are joint senior and corresponding authors. D.F.L. is the lead author.

DATA AVAILABILITY
Datasets for this study are deposited on Zenodo and are publicly available under a Creative Commons Attribution 4.0 International license, https://doi.org/10.5281/zenodo.3709160

DISCLOSURES
The authors declare no conflicts of interest.

ORCID
Daniel F. Legler https://orcid.org/0000-0001-8610-4764

REFERENCES
1. Zlotnik A, Yoshie O. The chemokine superfamily revisited. Immunity. 2012;36:705-716.
2. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol. 2014;32:659-702.
3. Nomiyama H, Osada N, Yoshie O. The evolution of mammalian chemokine genes. Cytokine Growth Factor Rev. 2010;21:253-262.
4. Legler DF, Thelen M. Chemokines: chemistry, biochemistry and biological function. Chimia. 2016;70:856-859.
5. Legler DF, Thelen M. New insights in chemokine signaling. F1000Research. 2018;7:95.
6.Nibbs RJ, Graham GJ. Immune regulation by atypical chemokine receptors. Nat Rev Immunol. 2013;13:815-829.
7. Bachelerie F, Ben-Barchu A, Burkhart AM, et al. International Union of Pharmacology. LXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. Pharmacol Rev. 2014;66:1-79.
8. Ulvmar MH, Werth K, Braun A, et al. The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in lymph nodes. Nat Immunol. 2014;15:623-630.
9. Di Liberto D, Locati M, Caccamo N, et al. Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in Mycobacterium tuberculosis infection. J Exp Med. 2008;205:2075-2084.
10. Wang H, Beaty N, Chen S, et al. The CXCR7 chemokine receptor promotes B-cell retention in the splenic marginal zone and serves as a sink for CXCL12. Blood. 2012;119:465-468.
11. Berahovich RD, Zabel BA, Lewen S, et al. Endothelial expression of CXCR7 and the regulation of systemic CXCL12 levels. Immunology. 2014;141:111-122.
12. Pruenster M, Mudde L, Bombosi P, et al. The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. Nat Immunol. 2009;10:101-108.
13. Duchene J, Novitszky-Basso I, Thiriot A, et al. Atypical chemokine receptor 1 on nucleated erythroid cells regulates hematopoiesis. Nat Immunol. 2017;18:753-761.
14. Jamieson T, Cook DN, Nibbs RJ, et al. The chemokine receptor D6 limits the inflammatory response in vivo. Nat Immunol. 2005;6:403-411.
15. Burns JM, Summers BC, Wang Y, et al. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. J Exp Med. 2006;203:2201-2213.
16. Gerrits H, van Ingen Schenau DS, Bakker NE, et al. Early postnatal lethality and cardiovascular defects in CXCR7-deficient mice. Genesis. 2008;46:235-245.
17. Sierra F, Biben C, Martinez-Munoz L, et al. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. Proc Natl Acad Sci USA. 2007;104:14759-14764.
18. Naumann U, Camerini E, Pruenster M, et al. CXCR7 functions as a scavenger for CXCL12 and CXCL11. PLoS One. 2010;5:e9175.
19. Saaber F, Schutz D, Miess E, et al. ACKR3 regulation of neuronal migration requires ACKR3 phosphorylation, but not beta-arrestin. Cell Rep. 2019;26:1473-1488.e9.

20. Gosling J, Dairaghi DJ, Wang Y, et al. Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. J Immunol. 2000;164:2851-2856.

21. Comerford I, Milasta S, Morrow V, Milligan G, Nibbs R. The chemokine receptor CCX-CKR mediates effective scavenging of CCL19 in vitro. Eur J Immunol. 2006;36:1904-1916.

22. Purvanov V, Matti C, Samson GPB, Kindinger I, Legler DF. Fluorescently tagged CCL19 and CCL21 to monitor CCR7 and ACKR4 functions. Int J Mol Sci. 2018;19:E3876.

23. Verhaar MA, van Offenbeek J, van der Lee MM, et al. Chemokine cooperation is caused by competitive glycosaminoglycan binding. J Immunol. 2014;192:3908-3914.

24. Townson JR, Nibbs RJ. Characterization of mouse CCX-CKR, a receptor for the lymphocyte-attracting chemokines TECK/mCCL25, SLC/mCCL21 and MIP-3beta/mCCL19: comparison to human CCX-CKR. Eur J Immunol. 2002;32:1230-1241.

25. Forster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol. 2008;8:362-371.

26. Hauser MA, Legler DF. Common and biased signaling pathways of the atypical chemokine receptor family. J Leukocyte Biol. 2000;67:223-233.

27. Vacchini A, Locati M, Borroni EM. Overview and potential unifying themes of the atypical chemokine receptor family. J Leukocyte Biol. 2013;94:512-523.

28. Watts AO, Verhaar F, van der Lee MM, et al. beta-Arrestin recruitment and G protein signaling by the atypical human chemokine decoy receptor CCX-CKR. J Biol Chem. 2013:288:7169-7181.

29. Hieshima K, Imai T, Opdenakker G, et al. Molecular cloning of a novel human CC chemokine receptor that binds dendritic cell- and T cell-localization, lymphocyte homeostasis, and immune responses in mucosal tissue. Immunity. 2000;12:495-503.

30. Ameti R, Melgrati S, Radice E, et al. Characterization of a chimeric receptor CCX-CKR. J Leukocyte Biol. 2000;67:223-233.

31. Waterhouse A, Bertoni M, Bienert S, et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 2018;46:W296-W303.

32. Pietro G-L, (2014) Pymol script: pairwisedistances.py.

33. Otero C, Groettrup M, Legler DF. Opposite fate of endocytosed chemokines: CCL19 and CCL21 to monitor CCR7 and ACKR4 function. J Immunol. 2014;192:3908-3914.

34. Waterhouse A, Bertoni M, Bienert S, et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 2018;46:W296-W303.

35. Pietro G-L, (2014) Pymol script: pairwisedistances.py.

36. Hauser MA, Kindinger I, Laufer JM, et al. Distinct CCR7 glycosylation pattern shapes receptor signaling and endocytosis to modulate chemotactic responses. J Leukocyte Biol. 2016;99:993-1007.

37. Moepps B, Thelen M. Monitoring scavenging activity of chemokine receptors. Methods Enzymol. 2016;570:87-118.

38. Veldkamp CT, Koplinksi CA, Jensen DR, et al. Production of recombinant chemokines and validation of refolding. Methods Enzymol. 2016;570:539-565.

39. Schaueble K, Hauser MA, Rippl AV, et al. Ubiquitylation of the chemokine receptor CCR7 enables efficient receptor recycling and cell migration. J Cell Sci. 2012;125:4463-4474.

40. Laufer JM, Hauser MA, Kindinger I, Purvanov V, Pauli A, Legler DF. Chemokine receptor CCR7 triggers an endomembrane signaling complex for spatial Rac activation. Cell Rep. 2019;29:995-1009.e6.

41. Cook DN, Prosser DM, Forster R, et al. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. Immunity. 2000;12:495-503.

42. Heinzel K, Benz C, Bleul CC. A silent chemokine receptor regulates steady-state leukocyte homing in vivo. Proc Natl Acad Sci USA. 2007;104:8421-8426.

43. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9:676-682.

44. Rueden CT, Schindelin J, Hiner MC, et al. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics. 2017;18:529.

45. Murphy LR, Wallqvist A, Levy RM. Simplified amino acid alphabets for protein fold recognition and implications for folding. Protein Eng. 2000;13:149-152.

46. Madeira F, Park YM, Lee J, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 2019;47:W636-W641.

47. Mitchell AL, Attwood TK, Babbitt PC, et al. InterPro in 2019: improving coverage, classification and access to protein sequence annotations. Nucleic Acids Res. 2019;47:D351-D360.

48. Qin L, Kufareva I, Holden LG, et al. Structural biology. Crystal structure of the chemokine receptor CXCR4 in complex with a viral chemokine. Science. 2015;347:1117-1122.

49. Uhlen M, Fagerberg L, Hallstrom BM, et al. Proteomics. Tissue-based map of the human proteome. Science. 2015;347:1260419.

50. Kawamura T, Stephens B, Qin L, et al. A general method for site-specific fluorescent labeling of recombinant chemokines. PloS One. 2014;9:e81454.

51. Yin J, Lin AJ, Golan DE, Walsh CT. Site-specific protein labeling by Sfp phosphopantetheinyl transferase. Nat Protoc. 2006;1:280-285.

52. Liu Z, Chen O, Wall JB, et al. Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. Sci Rep. 2017;7:2193.

53. Borroni EM, Cancellieri C, Vacchini A, et al. Beta-arrestin-dependent activation of the coiflin pathway is required for the scavenging activity of the atypical chemokine receptor D6. Sci Signal. 2013;6:ra30 1-11, S1-3.

54. Rajagopal S, Kim J, Ahn S, et al. Beta-arrestin- but not G protein-mediated signaling by the “decoy” receptor CXCR7. Proc Natl Acad Sci USA. 2010;107:628-632.

55. Szpakowska M, Meyrath M, Reymers N, et al. Mutational analysis of the extracellular disulphide bridges of the atypical chemokine receptor ACKR3/CXCR7 uncovers multiple binding and activation modes for its chemokine and endogenous non-chemokine agonists. Biochem Pharmacol. 2018;153:299-309.

56. Schweickart VL, Epp A, Raport CJ, Gray PW. CCR11 is a functional receptor for the monocYTE chemoattractant protein family of chemokines. J Biol Chem. 2000;275:9550-9556.

57. Zhang Y, Roth TL, Gray EE, et al. Migratory and adhesive cues controlling innate-like lymphocyte surveillance of the pathogen-exposed surface of the lymph node. elife. 2016;5.

58. Reboldi A, Coisne C, Baumjohann D, et al. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. Nat Immunol. 2009;10:514-523.

59. Lee AYS, Korner H. The CCR6-CCL20 axis in humoral immunity and T-B cell immunobiology. Immunobiology. 2019;224:449-454.

60. Matti C, Salnikov A, Artinger M, et al. ACKR4 recruits GRK3 prior to β-arrestins but can scavenge chemokines in the absence of β-arrestins. Front Immunol. 2020;11:720.

61. Le Broc ML, Fraser AR, Cotton G, et al. Chemokines as novel and versatile reagents for flow cytometry and cell sorting. J Immunol. 2014;192:6120-6130.

62. Allen SJ, Hamel DJ, Handel TM. A rapid and efficient way to obtain modified chemokines for functional and biophysical studies. Cytokine. 2011;55:169-173.

63. Volpe S, Camerini E, Moepps B, Thelen S, Apuzzo T, Thelen M. CCR2 acts as scavenger for CCL2 during monocyte chemotaxis. PloS One. 2012;7:e37208.

64. Cerbini T, Funahashi R, Luo Y, et al. Transcription activator-like effector nulease (TALEN)-mediated CLYBL targeting enables enhanced transgene expression and one-step generation of dual reporter human
induced pluripotent stem cell (iPSC) and neural stem cell (NSC) lines. PLoS One. 2015;10:e0116032.

65. Hauser MA, Schaeuble K, Kindinger I, et al. Inflammation-induced CCR7 oligomers form scaffolds to integrate distinct signaling pathways for efficient cell migration. Immunity. 2016;44:59-72.

66. Venken KJ, Kasprwicz J, Kuenen S, Yan J, Hassan BA, Verstreken P. Recombineering-mediated tagging of Drosophila genomic constructs for in vivo localization and acute protein inactivation. Nucleic Acids Res. 2008;36:e114.

67. Humpert ML, Tzouros M, Thelen S, et al. Complementary methods provide evidence for the expression of CXCR7 on human B cells. Proteomics. 2012;12:1938-1948.

68. Yu CR, Peden KW, Zaitseva MB, Golding H, Farber JM. CCR9A and CCR9B: two receptors for the chemokine CCL25/TECK/Ck beta-15 that differ in their sensitivities to ligand. J Immunol. 2000;164:1293-1305.

69. Seiler CY, Park JG, Sharma A, et al. DNASU plasmid and PSI:biology-Materials repositories: resources to accelerate biological research. Nucleic Acids Res. 2014;42:D1253-60.

SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Matti C, D’Uonnolo G, Artinger M, et al. CCL20 is a novel ligand for the scavenging atypical chemokine receptor 4. J Leukoc Biol. 2020;107:1137–1154. https://doi.org/10.1002/JLB.2MA0420-295RRR