Molecular Mechanisms of Atypical Chemokine Receptor 4 Ligand Interaction and Signalling

Doctoral thesis for obtaining the

academic degree Doctor of

natural sciences (Dr.rer.nat.)

submitted by

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at the

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Konstanz, 2020

Dissertation at the University of Konstanz
Date of the oral examination: 11. December 2020

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A journey of a thousand miles begins with a single step.

— Laozi

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Summary

Members of the chemokine receptor family have been known to regulate various processes such as proliferation, differentiation, and migration. The chemokine receptors are heptahelical membrane spanning proteins. They belong to the family of G-protein coupled receptors (GPCRs), which primarily elicit their signalling after chemokine binding via the activation of intracellular heterotrimeric Gi-proteins and β-arrestins. Among the chemokine receptor family, a second branch stands out, the atypical chemokine receptors (ACKRs). They have been shown to be unable to induce classical G-protein mediated signalling and to scavenge their ligands or to transcytose them from the basal to the apical side of cell layers. In immune cell migration, the lymph node homing chemokine receptor CCR7 together with its CC chemokine ligands (CCL) 19 and CCL21 have been shown to guide matured, activated dendritic cells (DCs) and matured, naïve T cells into lymph nodes (LNs), where they can interact and the T cells can be activate for the adaptive immune response. This migration is dependent on directed gradients of the two chemokines, which are established by the atypical chemokine receptor 4 (ACKR4). In detail, ACKR4 scavenges CCL19 in the periphery, thus skin derived DCs can reach the lymph vessels. ACKR4 further scavenges CCL21 at the subcapsular sinus of the LN thereby DCs arriving through the afferent lymph vessels sense the entry route into the LNs. One convenient way to study the mechanism of chemokine scavenging and

internalisation is using fluorescent chemokine ligands. We achieved this by developing suitable eukaryotic and prokaryotic expression systems, yielding chemokines tagged with either a fluorophore or enzymatically labelled ones with a fluorescent dye. How ACKR4 elicits its function has been a matter of debate, especially how β-arrestins interact and guide receptor function. As ACKR4 is broadly expressed along various immunological barrier sites and a role in several disease has been implicated, we wanted to investigate its function and intracellular interaction partners. Therefore, we developed various biosensors for G-proteins and G-protein-coupled receptor kinases (GRKs). We found clear recruitment of GRK3 and to a lesser extent GRK2 to activated receptor, which preceded the recruitment of β-arrestins. Further, we provide clear evidence, that β-arrestins are dispensable for receptor scavenging, however that they can significantly boost the efficacy of ligand scavenging by ACKR4. In addition, not all chemokines have been associated with an ACKR. Therefore, we used bioinformatic alignments and modelling approach to find putative ligands for ACKR4. This revealed CCL20 as a suitable candidate, which we further validated. β-arrestin recruitment and uptake of fluorescently labelled human CCL20 confirmed CCL20 as a ligand of human ACKR4. Moreover, using fluorescently labelled mouse CCL20, we validated the interaction of mouse ACKR4 and mouse CCL20 as well. CCL20 was scavenged by ACKR4 positive cells in the subcapsular sinus of LNs as was published for CCL21. To sum up, our findings shed light on the function of ACKR4 and indicate new roles for ACKR4 in CCL20 linked autoimmune disease. Together, our findings provide the foundation of future studies on the exciting atypical chemokine receptor 4.

Zusammenfassung

Viele Prozesse wie Proliferation, Differenzierung und Migration werden durch Mitglieder der Chemokinrezeptor-Familie gesteuert. Chemokinrezeptoren sind Proteine bestehend aus sieben membrandurchquerenden Helices. Sie gehören zur Familie der G-protein gekoppelten Rezeptoren, welche ihre Signalübermittlung nach der Bindung von Liganden durch die Aktivierung von intrazellulären Gα-Proteinen und β-Arrestinen ausüben. Innerhalb der Chemokinrezeptor-Familie gibt es eine zweite Gruppe, die der atypischen Chemokinrezeptoren (ACKRs). Es zeigte sich, dass ACKRs nicht in der Lage sind, typische G-Protein induzierte Signalübermittlung durchführen. Jedoch internalisieren ACKRs ihre Liganden, oder sie transportieren sie von der basalen zu der apikalen Seite der Zelle (Transzytose). In der Immunzellmigration, kommt dem lymphknotensuchenden Chemokinrezeptor CCR7 und seinen beiden CC Chemokinliganden (CCL) 19 und CCL21 eine besondere Rolle zu. Zusammen führen sie reife, aktivierte dendritische Zellen und reife, naive T-Zellen in Lymphknoten, welche als immunologische Treffpunkte dienen und zu Interaktionen zwischen den Zellen führen, wodurch T Zellen aktiviert werden und schlussendlich die adaptiven Immunantwort ausgelöst wird. Diese Migration hängt ab von gerichteten Gradienten der zwei Chemokine, welche durch den atypischen Chemokinrezeptor 4 (ACKR4) aufgebaut werden. Eine sehr geeignete Methode, um diese Chemokinaufnahme und -internalisierung zu studieren, sind fluoreszierende Chemokine. Wir haben dies durch die Expres-

sion von entsprechenden Chemokinliganden in eukaryontischen wie auch in prokaryotischen Zellen erreicht. Entweder produzierten wir Chemokine, welche direkt mit einem Fluorophor verbunden sind oder solche, welche mittels einer enzymatischen Reaktion nachträglich kovalent an einen Farbstoff gebunden werden. Wie genau ACKR4 seine Funktion ausübt war umstritten, im Besonderen wie β-Arrestine mit dem Rezeptor interagieren und seine Funktion steuern. ACKR4 ist an diversen immunologischen Grenzseiten exprimiert und dem Rezeptor wurde eine Rolle in verschiedenen autoimmunologischen Krankheiten zugewiesen, weswegen wir seine Funktion und seine intrazellulären Interaktionspartner genauer untersuchen wollten. Um diese Ziele zu erreichen, haben wir Biosensoren für G-Proteine und G-Rezeptorkinasen (GRKs) entwickelt. Wir entdeckten die Rekrutierung von GRK3 und leicht abgeschwächt die Rekrutierung von GRK2 zum aktivierten Rezeptor, welche der Rekrutierung von β-Arrestin vorausging. Des Weiteren haben wir klare Beweise, dass β-Arrestine zwar entbehrlich sind für die Internalisierung von ACKR4 Liganden, jedoch β-Arrestine signifikant die Aufnahme von Chemokinen durch ACKR4 verstärken. Da bis heute noch nicht alle Chemokine einem ACKR zugewiesen sind, wollten wir herausfinden, ob noch weitere Chemokine durch den ACKR4 aufgenommen werden können. Wir verwendeten bioinformatische Ansätze wie Sequenzabgleiche und Liganden:Rezeptor Modelle um neue Liganden zu finden, wodurch wir CCL20 entdeckten. Durch die Aufnahme von fluoreszierenden humanem CCL20 und der Verwendung von β-Arrestin Rekrutierung zeigten wir, dass CCL20 ein neuer Ligand für den humanen ACKR4 ist. Des Weiteren stellten wir fluoreszierendes murines CCL20 her, womit wir zeigten, dass CCL20 und ACKR4 ebenfalls im murinen System ein Paar bilden. CCL20 wurde durch Zellen im Subcapsular Sinus der Lymphknoten, die ACKR4 exprimieren, aufgenommen, so wie es bereits für CCL21 gezeigt wurde.

Zusammenfassend fanden wir durch unsere Arbeiten neue Funktionen und Interaktionspartner für ACKR4 und weisen auf eine neue Rolle für ACKR4 in CCL20 abhängigen Autoimmunkrankheiten hin. Die Erkenntnisse aus unserer Forschung dient als Grundlage, für neue Studien über den spannenden, atypischen Chemokinrezeptor 4.

Acronyms

A

ACKR atypical chemokine receptor.

B

BBB blood-brain barrier.

BRET bioluminescence resonance energy transfer.

C

cCKR conventional chemokine receptor.

CCL CC chemokine ligand.

CCR CC chemokine receptor.

CD Crohns Disease.

CK2 casein kinase 2.

CNS central nervous system.

CoA coenzyme A.

COPD chronic obstructive pulmonary disease.

CRS chemokine recognition site.

cTEC(s) cortical thymic epithelial cell(s).

CXCL CXC chemokine ligand.

CXCR CXC chemokine receptor.

D

DC(s) dendritic cell(s).

DMR Dynamic Mass Redistribution assay.

E

ECL extracellular loop domain.

EGFP (enhanced) green fluorescent protein.

ERM Ezrin/Radixin/Moesin.

EYFP enhanced yellow fluorescent protein.

F

FACS Fluorescence-activated cell sorting.

FRC fibroblastic reticular cells.

G

GAG glycosaminoglycans.

GDP guanosine diphosphate.

GPCR G-protein coupled receptor.

GRK G-protein-coupled receptor kinases.

GTP guanosine triphosphate.

H

h human.

HER2 human epidermal growth factor receptor 2.

HEV high endothelial venule.

HPLC high-performance liquid chromatography.

I

IBD inflammatory bowel disease.

ICL intracellular loop domain.

J

JNK c-Jun N-terminal kinase.

L

LN(s) lymph node(s).

M

m mouse.

MMPs matrix metalloproteinases.

mRFP monomeric red fluorescent protein.

MS multiple sclerosis.

mTEC(s) medullary thymic epithelial cell(s).

N

NK cell(s) natural killer cell(s).

NLuc NanoLuc luciferase.

NMR nuclear magnetic resonance.

NSCLC) non-small-cell lung carcinoma.

P

PGE2 prostaglandin E_2.

PI3K phosphoinositide 3-kinase.

PKA cAMP-dependent protein kinase.

PKC protein kinase C.

PKG cGMP-dependent protein kinase.

R

RA retinoic acid.

S

S1P sphingosine-1-phosphate.

SAXS small-angle X-ray scattering.

SCS subacapsular sinus.

SLO secondary lymphoid organ.

T

TH cell T helper cell.

TLO tertiary lymphoid organ.

TM transmembrane domain.

TNBC triple-negative breast cancer.

U

UC ulcerative colitis.

W

WT wild-type.

X

XL-MS cross-linking mass spectrometry.

Y

Y2H yeast two-hybrid screening system.

1 Introduction

1.1 A brief Introduction Into Migration and the Immune System

Migration in the human body is a highly regulated process which plays a role during the whole life cycle of an organism. It starts with the migration of stem cells and precursors during embryogenesis. And further continues up to the adult animal, where immune cells patrol the organism for surveillance and specifically migrate towards sites of infection during an adaptive immune response. A sophisticated system has been shown to be crucial to guide migration of leukocytes for a protective immune surveillance and pathogen clearance as well as in a destructive manner causing autoimmune reactions. This system is the chemokine system. It consists of chemotactic cytokines, termed chemokines, which act as ligands for chemokine receptors, as outlined later. (Bachelerie et al., 2014a) The migration and circulation of immune cells is guided in tissue, along the blood system as well as the lymph system. The lymph systems are a vessel network, parallel to the blood system connecting the periphery with primary and secondary lymphoid organs. The lymph system collects immune cells from the periphery and guides them through a network of secondary lymphoid organs. (Kerjaschki, 2014; Oliver, 2004) Those are cellular hubs, where immune cells can interact and activate each other, lead-

ing to proliferation and directed immune responses. Once a pathogen enters the body, such as through a scratch in the skin, the innate immune system fights off pathogens, generating debris of those which is taken up by antigen presenting cells, such as dendritic cells (DC). Thereby, DCs undergo a maturation process upon which they start to upregulate the lymph homing CC chemokine receptor (CCR) 7, guiding them along a signal gradient made of CC chemokine ligand (CCL) 19 and CCL21 to the nearest lymph vessels and then to the draining lymph nodes (LNs). (Johnson & Jackson, 2014) LNs are made up of various zones, in which specific leukocyte subsets are localised. LNs are surrounded by an epithelial sheet forming the subcapsular sinus which is connected to the afferent lymphatics, where cells and molecules from the periphery are coming from. (Munoz et al., 2014) Further, LNs are connected to the blood system, which enters through hilar arterioles and venules. During homeostasis, DCs enter through the subcapsular sinus, whereas T and B cells enter through the high endothelial venules (HEV), surrounding blood vessels. (Förster et al., 2008) T cells arrive as matured, naïve $CD4^+$ or $CD8^+$ cells from the thymus, where they mature and differentiate, whereas B cells arrive from the bone marrow. (Kumar et al., 2018; Seifert & Küppers, 2016) Once the DC has entered the LN T cell zone, it attempts to activate T cells recognising the previously taken up and processed antigen. (Förster et al., 2008) During the activation, the presented antigen together with a specific cytokine cocktail leads to the differentiation into one of several T helper (TH) cell subsets. The most prominent ones are TH1, TH2 and TH17 cells. Of those, the TH1 cells are known to mediate cell driven immune responses, TH2 cells coordinate humoral immune responses and TH17 cells are known to drive the induction of inflammation together with other cells of the immune system and TH17 cells have been associated with various autoimmune diseases. (Saito

et al., 2010) Autoimmune diseases are characterised by an imbalance of immune cell proliferation, often leading to permanent host cell and tissue damage. Examples are multiple sclerosis (MS), Sjorgens disease, chronic obstructive pulmonary disease (COPD) or inflammatory bowel disease (IBD). In MS, the myelin sheets of axons are degraded, leading to nerve loss. (Sawcer et al., 2011) In Sjorgens syndrome, the immune system attacks glands generating tears and saliva, but can also target other organs like kidney or liver. Ultimately, the result is a decrease in substances secreted by the affected glands and organs. (J. Wang et al., 2020) In COPD, the inflammation of either bronchial tubes or the destruction of alveoli at the end of the bronchioles causes obstructed airflow and breathing problems. (Martinez et al., 2011) IBD is an umbrella term for two diseases affecting the gut and intestines. One is Ulcerative colitis (UC), where the inner lining of the large intestine is affected and the other is Crohns Disease (CD), where the digestive tract as well as its surrounding tissue is affected. Mainly, IBD are diseases causing lesions in patches (like in UC) or continuous patches (like in CD) along the bowel tract. (Nishimura et al., 2009) Coming back to the newly activated T cells in the LN. The T cells upregulate tissue specific chemokine receptors, which guide them e.g. to gut mucosal sites or as in our starting example to the skin. After successful elimination of the pathogens, most T and B cells undergo apoptosis, whereas a small portion remains and differentiates further into the memory subtypes. (Kumar et al., 2018; Seifert & Küppers, 2016) Those cells circulate the body and remain in LNs or in healthy peripheral tissue over a long period of time, providing a fast response if the pathogen enters the body for a second time. All those processes are dependent on guided migration, which itself is dependent on signals and receptors. (Bachelerie et al., 2014a) Even though receptors can be up or downregulated by the migrating cell, other mechanisms

must be in place to generate directed movement. Already in 1970, Francis Crick postulated the existence of gradients formed by the interplay of a morphogen producing source and a morphogen destroying sink. (Crick, 1970) By now, it has been established that gradients of chemokines are present in various sites and are formed by atypical chemokine receptors. (Hughes & Nibbs, 2018) The presented thesis is going to focus on the function of the atypical chemokine receptor 4, how it binds ligands, transduces signal upon activation and thereby regulates chemokine gradients.

1.2 Chemokines

1.2.1 General

The driving force of the chemokine system are chemotactic cytokines, so-called chemokines, which are members of a large family of cytokines. They are mostly secreted by various cells and able to induce migration of cells expressing the cognate conventional chemokine receptors (cCKRs). (Rot & von Andrian, 2004) In most cases, chemokines are not only restricted to one but can interact with several cCKRs. (Bachelerie et al., 2014a) Chemokines are characterised based on their structural position of the first two cysteines in the sequence, into CC, CXC, CX3C and C as well as XC chemokines, e.g. CC chemokine ligand 19 is abbreviated as CCL19. (Zlotnik & Yoshie, 2000) Figure 1.1 The typical chemokine structure consists of an unstructured N-terminus of variable length, followed by the cysteine motif. This motif is linked through an N-loop with the globular chemokine core. The core itself consist of a 3_{10} helix, three antiparallel beta strands and an alpha helix leading to the C-terminus. (Crump et al., 1997) Depending on the chemokine, a prolonged C-terminal tail exists, which can ei-

ther be stabilized by an additional disulphide bond as in CCL21 (Love et al., 2012) or be unstructured and highly flexible. The two distinct disulphide bonds of the first two cysteines link to the loop between the first and second beta strand and to the third beta strand. This stabilises the chemokines and is crucial for their functionality. Figure 1.1 Of the four chemokines (CCL19, CCL20, CCL21 and CCL25) focussed in this work, two are known to be endogenously modified. Cleavage modifications by matrix metalloproteinases (MMPs) or e.g. by dipeptidyl peptidase IV (Metzemaekers et al., 2016) are often restricted to tissues or exerted by certain cell types only, leading to a specific tissue bias of chemokines. CCL20 was shown to be C-terminally processed by proteases once released from Monocytes. (Schutyser et al., 2000) In addition, it has an alternative splice, leading to an N-terminal alanine truncation and an even shorter N-terminus. (Power et al., 1997) Interestingly, both modifications did not abrogate chemokine function. Similarly, CCL21 exists in a natural protease cleaved version, CCL21trunc, which behaves more like CCL19 in terms of receptor activation. (Schumann et al., 2010) Chemokines are either categorized as being homeostatic or inflammatory. (Bachmann et al., 2006) Homeostatic chemokines such as CCL19 or CXCL13 are constitutively expressed and regulate cell movement during homeostasis. However, inflammatory chemokines are mainly upregulated under inflammatory conditions guiding immune cells to site of infection. (Rot & von Andrian, 2004) The ligands for ACKR4 have been associated with the group of homeostatic chemokines as well as being upregulated during inflammation, therefore being ambivalent. Moreover, deregulation of the designated chemokine:cCKR pairings often lead to autoimmune diseases or defunct clearing of pathogens. (Bachelerie et al., 2014a) To fully understand the importance of ACKR4 function and its ligand repertoire, the chemokine ligands of ACKR4 as well as their

respective receptors will be described in more detail. Those include CCL19 and CCL21 together with CCR7, CCL25 and its cCKR CCR9 and the hereby newly described ligand CCL20 with its receptor CCR6. (Gosling et al., 2000; Matti et al., 2020a; Meyrath et al., 2020a; Townson & Nibbs, 2002) Further, as we use several sophisticated methods to generate our engineered chemokines for studies, the main principles will be introduced at the end of this chapter.

1.2.2 CCL19 (ELC) and CCL21 (SLC)

CCL19 and CCL21 are both expressed in various tissues and by a certain subset of cells of the haematopoietic linage as well. They, for example, have been shown to be expressed in the skin (Christopherson et al., 2003), the lung (Lo et al., 2003), the blood brain barrier and the spinal cord barrier. (Alt et al., 2002; Reboldi et al., 2009) CCL21 was shown to be expressed along afferent lymphatics (Luther et al., 2000), as well as in the LN in the T cell zone by fibroblastic reticular cells (FRCs) (Luther et al., 2000) (Carlsen et al., 2005) and around the HEV in mice. (Luther et al., 2002) In addition to all those non-haematopoietic sources of CCL21, CCL19 is expressed by activated DC in LNs (Sallusto et al., 1999) thereby prolonging the DC:T cell interaction in LNs. (Kaiser et al., 2005) (Muthuswamy et al., 2010) (Y. Wang & Irvine, 2013)

Regarding the chemokine structure, already a lot of work was put into CCL19 and CCL21. Despite having a similar affinity for CCR7 (Yoshida et al., 1998), their signalling pattern differ. As such, they result in differential CCR7 phosphorylation (Zidar et al., 2009) and induce internalisation at different concentrations. (Bardi et al., 2001; Kohout et al., 2004; Otero et al., 2006) For the multistep process of chemokine:receptor binding and interaction, the N-terminus of the

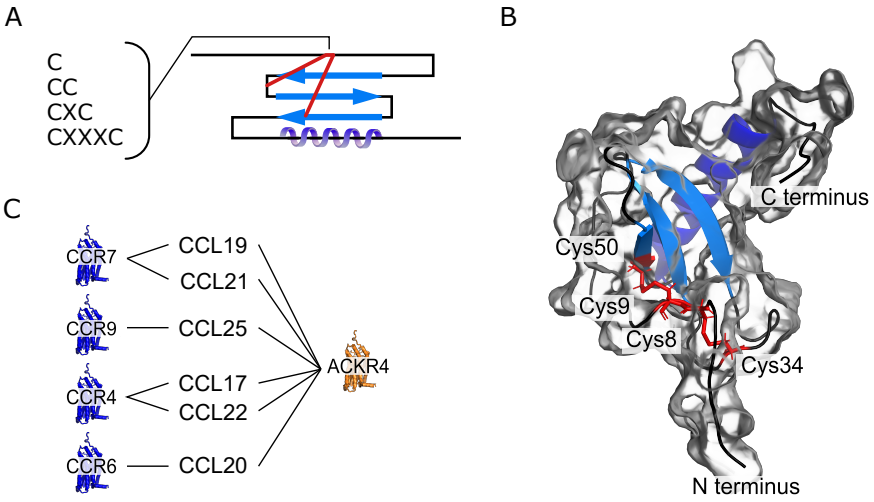

Fig. 1.1: A) Typical chemokine sequence, with the location of the two distinct disulphide bridges (red) and the location of the first cysteine after which chemokines are grouped. B) Illustration of the characteristic chemokine structure, with the disulphide bridges (red), the antiparallel beta sheets (marine blue) and the alpha helix (blue) leading to the C-terminus. Illustration is based on the human CCL19 structure [PDB:2MP1] (Veldkamp et al., 2015) C) All known ligands of ACKR4, including the ones discovered during this work together with their conventional chemokine receptors.

chemokine is of special interest (see Chapter 1.4.1). In terms of CCL19 and CCL21, despite having different amino acid compositions, their N-termini are interchangeable leading to similar CCR7 activation behaviour. (Ott et al., 2006) CCL21 and CCL19 size and structure mainly differ by the additional 32 amino acids forming the elongated CCL21 tail. The tail is the main driver of the formation of sticky, steep, haptotactic CCL21 gradients, whereas CCL19 gradients are soluble. The main binding partner to form CCL21 gradients are glycosaminoglycans (GAGs), as heparinase treatment abolished CCR7 mediated DC migration in the perilymphatic intersititum. (Barmore et al., 2016; Hirose et al., 2002; Tal et al., 2011; Weber et al., 2013). Further, the negatively charged CCL21 tail binds to Collagen IV(Tal et al., 2011; B.-G. Yang et al., 2007) and polysialic acid (Kiermaier et al., 2016). In addition, CCL21's tail can be truncated via the cleavage by plasmin and dendritic cell-secreted protease in vivo. (Lorenz et al., 2016; Schumann et al., 2010) Thereby, CCL21trunc gains enhanced CCR7 agonist activity, indicating a putative auto inhibition role by the tail on CCL21, which might be influenced by GAG binding as well. (M. A. Hauser et al., 2016; Hirose et al., 2002; Kiermaier et al., 2016; Moussouras et al., 2020)

1.2.3 CCL25 (TECK)

Compared to CCL19 and CCL21, CCL25 is less broadly expressed. It is mainly found in the small intestine and some minor expression sites around the stomach and colon. (Kunkel et al., 2000; Papadakis et al., 2001; Papadakis et al., 2000) TH1 cells use the distinct CCL25 pattern to migrate to the lamina propria and follicles in and along the intestine (Papadakis et al., 2003). Similarly to CCL19 in LNs, thymic DC secrete CCL25, which can help with DC:T cell interaction. (Vicari et al., 1997)

Besides its role in homeostasis, CCL25 is associated with IBD. (Keshav & Wendt, 2015) The inflammatory activity in IBD was correlated with increased CCL25 levels. (Trivedi et al., 2016) Furthermore, liver endothelial cells expressed CCL25 during IBD, attracting long-lived memory gut homing T cells and inducing inflammation at a distant site from where T memory cells were initially aimed at. (Eksteen et al., 2004) In terms of structure, CCL25 has not been solved yet. The sole study investigating CCL25's structure revealed it to have similar, although reduced GAG binding pattern like CCL21, indicating it to be a rather sticky chemokine and mainly forming haptotactic gradients. (de Paz et al., 2007)

1.2.4 CCL20 (MIP-3α)

CCL20 is constitutively expressed by isolated lymphoid follicles, Peyers patches, mesenteric LNs and other areas of secondary lymphoid organogenesis. There it is important for the chemotaxis of immature DCs (Anderle et al., 2005; Cook et al., 2000). Like CCL21, levels of CCL20 are greatly increased on inflamed lymphatic endothelial cells (Johnson et al., 2006; Kriehuber et al., 2001) as well as on lymph vessels within inflamed skin. (Johnson & Jackson, 2013) Like CCL19, CCL20 can be produced and secreted by immune cells during inflammation, such as by neutrophils in the gut to attract TH1 and TH17 cells to sites of inflammation (Pelletier et al., 2010) or by alveolar macrophages in the lung. (Facco et al., 2007) Its pathological role, as for CCL25, was so far shown in autoimmune diseases in the lung, the gut, the skin, and the CNS. CCL20 was further shown to be increased in patients with COPD (Demedts et al., 2007), thereby worsen disease outcome by attracting monocyte derived DCs into the small airways in two out of three studies. (Arellano-Orden et al., 2016; Demedts et al.,

2007; Willart et al., 2012) As for its role in the gut, CCL20 - like CCL25 - is upregulated in IBD patients. (Skovdahl et al., 2015) CCL20 upregulation is a designated risk factor for UC and CD, as the former attracts Th17 cells via CCR9 and the latter TH1 via CCR6. (Keshav & Wendt, 2015) Thereby, those TH cell subtypes worsen autoimmune diseases by recruiting further neutrophils and macrophages. (Weaver et al., 2013) Besides autoimmune reactions in the gut, CCL20 expressed in skin keratinocytes during diseases such as psoriasis and cutaneous T cell Lymphoma (Nestle et al., 2009) attracts Langerhans cell precursors. (Dieu-Nosjean et al., 2000) Finally, together with CCL19 and CCL21, CCL20 was shown to be expressed in epithelial cells of the choroid plexus (Reboldi et al., 2009), further upregulated in mouse models of MS (Wojkowska et al., 2014) and inflamed MS lesions, by astrocytes and macrophages. (Ambrosini et al., 2005)

1.2.5 Engineered Chemokines

Besides natural occurring chemokines, various modifications have been made to study chemokine function further. (Moepps & Thelen, 2016; Veldkamp et al., 2016) For example, the tail of CCL21 was fused to CCL19, to further proof its GAG binding defining characteristics. (Barmore et al., 2016; Jørgensen et al., 2018) In addition, the ability to add fluorescent proteins or tags for labelling slowly replaced older methods. Figure 1.2 Previously, the chemokine binding or scavenging was often studied using radiolabelled chemokines (Borroni et al., 2013; Gosling et al., 2000), whereas nowadays the possibility to engineer modified chemokines in house opens new options using specific fluorescent chemokines. (Volpe et al., 2012) Figure 1.2 The chosen methods are either long-chain peptide synthesis (Clark-Lewis, 2000) which is applied e.g. by companies like Almac (Craigavon, UK) or recombi-

nant expression in bacterial, insect or mammalian cells. (Moepps & Thelen, 2016) As the chemokine structure contains crucial disulphide bridges, the producing organisms must be able to form them during translation. If this is not the case, sophisticated refolding of isolated, denatured protein takes place. Thereby, specific oxidative refolding conditions have to be applied as described in (S. Yamaguchi et al., 2013). In addition, aggregation inhibitors and stabilisers can have a positive effect on the yield of such refolding reactions. (H. Yamaguchi & Miyazaki, 2014; S. Yamaguchi et al., 2013) In contrast, the isolation of functional chemokine produced from mammalian or insect cells is often more straight forward, as the functional secreted proteins of interest are collected from cell supernatant. (Moepps & Thelen, 2016; Purvanov et al., 2018) Further, those chemokines already possess the processed N-terminus, whereas the ones from prokaryotic expression systems have to be modified first. (Moepps & Thelen, 2016) If the chemokines are not fused to a fluorophore protein per se, the use of an enzyme specific tag such as the YbbR13 or the S6 tag are to be favoured. (Yin et al., 2005; Z. Zhou et al., 2007) Thereby, a phosphopantheteinyl transferase such as AcpS or Sfp recognises the specific amino acid sequence of the tag and transfers coenzyme A (CoA) derivatives onto the serine residue of the tag, generating a specific covalent bound labelled chemokine. (Lotze et al., 2016) Figure 1.2 Especially the paucity of antibodies recognising endogenous receptors for in vivo studies can be avoided using fluorescently labelled chemokines. The highly interweaved chemokine receptor interactions however can lead to a multitude of binding possibilities when using native, fluorescently labelled chemokines. An alternative is the generation of chimeric chemokines based on two known ligands of one receptor. The principle behind this is the fact that chemokine receptors are less stringent when it comes to the N-terminus of their des-

tined chemokines. (Ott et al., 2006) As a prime example, a chimera of CXCL11 and CXCL12, using the N-terminal sequence of the former with the core structure of the latter resulted in a specific atypical chemokine receptor 3 (ACKR3) ligand, which does not bind to either CXCR3 or CXCR4. (Ameti et al., 2018) Such synthetic chemokines can be used to detect chemokine receptor localisation and function, with extremely low background signal.

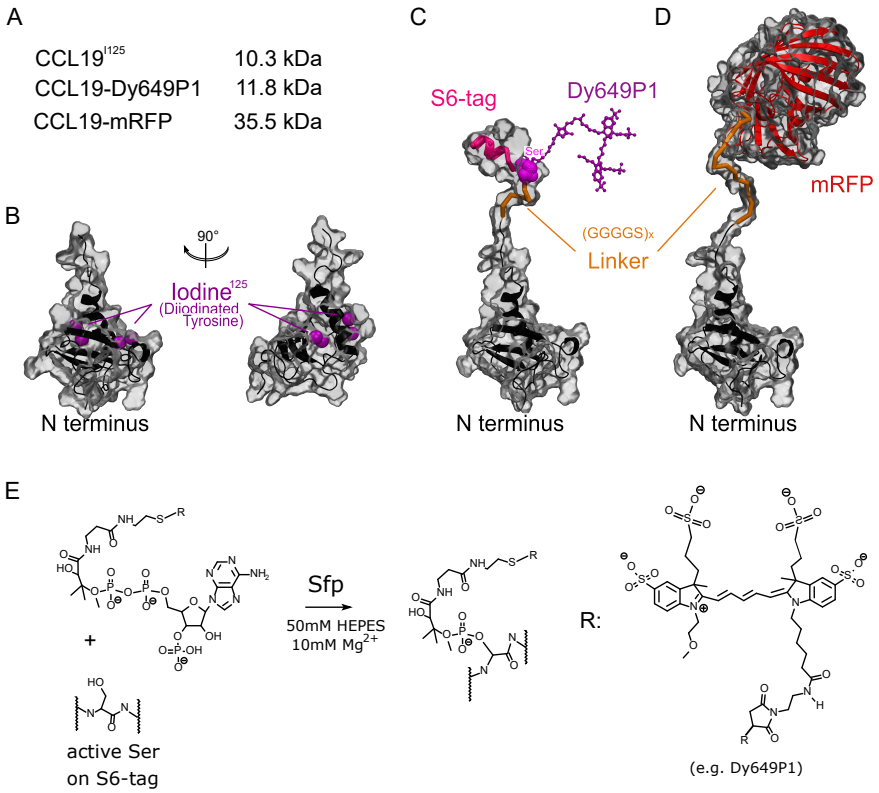

Fig. 1.2: Illustration of the different modifications introduced into chemokines, based on mRFP [PDB:2VAD] and CCL19 [PDB:2MP1] structure. (Strongin et al., 2007; Veldkamp et al., 2015) A) Basic information on the different chemokines. B) Iodinated CCL19, depicted with the two tyrosines to be diiodinated (Purple). The addition of a fluorophore C) or a labelled tag D) leaves the core structure intact, however adds more putative interaction surfaces at the tail of the chemokine. E) The CoA derivate transfer elicited by Sfp on the active serine of the S6-tag.

1.3 Chemokine Receptors

1.3.1 General

Chemokine receptors are seven transmembrane domain spanning membrane proteins. They are expressed in the plasma membrane and in intracellular vesicles and belong to the family of G-protein coupled receptors (GPCRs). GPCRs are divided based on evolutionary and sequence conservation into a total of six subfamilies, including class A (Rhodopsin like), to which all chemokine receptors belong. The other classes are B1 (Secretin receptor like), class B2 (adhesion receptors), class C (metabotropic glutamate receptor like), class F (Frizzled-like) and the taste 2 sensory receptor subfamily. (Venkatakrishnan et al., 2013) Chemokine receptors can be further divided into two groups: The conventional chemokine receptors (cCKR), which signal through heterotrimeric G_i proteins and drive migration, and atypical chemokine receptors (ACKR) which have been known to be unable to induce cell migration however to shape and modulate chemotactic gradients and chemokine concentrations. (Bachelerie et al., 2014a)

How exactly chemokine receptors recognize and interact with ligands and how the signal is transduced across the membrane on to intracellular signalling partners will be elucidated in section 1.4. To fully understand the scope of ACKR4 function and its relevance in immune system function one must look at its cCKRs counterparts and their role in migration of immune cells during homeostasis and inflammation.

1.3.2 CCR7

CCR7, the cCKR for CCL19 and CCL21, is expressed on various leukocytes such as thymocytes, several T cell subsets, naïve B cells, NK cells, neutrophils and semi-mature and mature DCs, as well as

on some tumour cell types (Comerford et al., 2013; M. A. Hauser et al., 2016; Laufer et al., 2019a) Besides chemoattraction (Kellermann et al., 1999; Sánchez-Sánchez et al., 2006) and related effects such as migratory speed (Braun et al., 2011; Riol-Blanco et al., 2005) and adhesion of DCs and T cells (Eich et al., 2011; Laufer et al., 2019a), CCR7 also regulates survival (Sanchez-Sanchez, 2004) and differentiation (Marsland et al., 2005) of DCs. The correct interplay between all those CCR7 functions is most likely needed for a more effective DC recruitment and an improved adaptive immune response in general. (López-Cotarelo et al., 2017) It is of special interest to understand the different migration routes steered by CCR7. Thanks to the creation of the CCR7 deficient mouse and the plt mouse (paucity of lymph node T cells (plt) mutation), which lacks CCL21 in lymphoid tissues and CCL19 completely, the effects of CCR7 could be investigated. (Förster et al., 2008) As CCR7 on T cells and on DC guides their homing into lymph nodes (LNs), the LN architecture in $CCR7^{-/-}$ animals is disrupted. (Kocks et al., 2007; Schneider et al., 2007; Woolf et al., 2007; Worbs et al., 2007) Especially, as those cells do not only populate LNs, but also constitute to the HEV formation in LNs by supplying growth factors such as VEGF-D. (Wendland et al., 2011) In an inflammatory context, matured skin DC migration into draining LN as well as T cells positioning in the T cell zone were shown to be CCR7 dependent. (Förster et al., 1999) The plt mouse revealed CCL21 from fibroblastic reticular cells to be the main guidance cue of the two CCR7 ligands guiding the T cell migration into LNs. (Link et al., 2007) Further, it was shown that CCL21 can mediate $CD4^+$ T cell co-stimulation by lowering the threshold for successful activation. (Gollmer et al., 2009)

Similar to its effect in LN populations, CCR7 together with its ligands were shown to be important in the formation of tertiary lymphoid

organs (TLOs). TLOs are often a sign of autoimmune inflammation and they are formed in areas of distress. It has been reported that ectopic expression of CCL21 in the pancreas and thymus is sufficient to induce TLO formation. (S.-C. Chen et al., 2002; Luther et al., 2002; Marinkovic et al., 2006) Conversely, the deletion of CCR7 inhibited the TLO development in an arthritis model. (Wengner et al., 2007) It should be mentioned however that TLOs can develop to a lesser extent in a CCR7 independent manner as well. (Davalos-Misslitz et al., 2007; Höpken et al., 2007) Besides guiding leukocytes, CCR7 expression correlates with the formation of metastases into secondary lymphoid organs in certain types of cancer, such as human colon cancer (J. Li et al., 2011), B cell chronic lymphocytic leukaemia (Redondo-Muñoz et al., 2008), non-small-cell lung carcinoma (NSCLC) (Sun et al., 2015), and breast cancer. (Legler et al., 2014; Müller et al., 2001) CCR7 can activate various intracellular signalling pathways, which have been extensively studied and summarized in detail recently by Rodriguez-Fernandez et al. and Yan et al. (Rodríguez-Fernández & Criado-García, 2020; Yan et al., 2019) Although they all originate from CCR7, the different cellular effects are induced by different intracellular pathways, which do not influence each other. (Ardeshna et al., 2000; Iijima et al., 2005) A highly regulated network of kinases transmits the signal in the cell leading to e.g. transcription or cytoskeletal rearrangements. (Rodríguez-Fernández & Criado-García, 2020) Most prominently, the survival pathway of CCR7 is induced via the Gβγ subunits, to the Phosphoinositide 3-kinases (PI3K) and Protein kinase B (Akt), to activate the transcription factor NFκb and mTORC. (Escribano et al., 2009; López-Cotarelo et al., 2015; Sanchez-Sanchez, 2004; Scandella et al., 2004) Alongside, CCR7 dependent chemotaxis is largely governed via rapidly accelerated fibrosarcoma (RAF) kinase activation downstream of mitogen-activated protein kinases MEK1/2,

ERK1/2, p38 and the c-Jun N-terminal kinase (JNK). (Iijima et al., 2005; Riol-Blanco et al., 2005; Scandella et al., 2004) However, as complete inhibition of the MAPK pathway does not abrogate the migration, most likely additional pathways contribute to the directed migration of $CCR7^+$ cells. As in many other migrating cells, CCR7 stimulation leads to a change in the cytoarchitecture, which is mediated via the RhoA pathway (Riol-Blanco et al., 2005) as well as CDC42 and Rac, however it has not been elucidated yet how these proteins interact with each other depending on species, cell type or maturation status. (Yanagawa & Onoé, 2002; 2003)

1.3.3 CCR9

Most peripheral blood and intestinal CCR9 positive cells are TH1 effector cells (producing IFNy) with a smaller proportion being TH17 cells and a minority acting as Treg and TH2. (Papadakis et al., 2003; Papadakis et al., 2000; Saruta et al., 2007) CCR9 is further expressed on plasmacytoid DCs (Hadeiba et al., 2012; Keshav & Wendt, 2015; Wendland et al., 2007), macrophages (Nakamoto et al., 2012), pro-inflammatory monocytes subsets in IBD (Linton et al., 2012) on B cells and various other T cells. (Bowman et al., 2000; Cassani et al., 2011; Pabst et al., 2004) Interestingly, CCR9 can be induced in lymphocytes with retinoic acid (RA), a vitamin A metabolite. (Iwata et al., 2004) RA itself is produced by PGE2 matured DCs and, upon secretion, induces CCR9 expression in proximal T cells. (Stock et al., 2011) Those T cells thereby become Treg cells with gut homing properties. (Bakdash et al., 2015) Besides the migration of DCs and T cells to the gut in homeostasis and upon inflammation (Mizuno et al., 2012; Wendland et al., 2007; M. A. Wurbel et al., 2011), the CCL25/CCR9 axis is critical in establishing an early thymocyte colonisation and the selec-

tive maturation of tolerogenic T cells. (Hadeiba et al., 2012) A proper colonisation of the thymus during development and in adult stages is important to ensure a central tolerance of the immune system and prevent autoreactive immune responses as T cells mature in the thymus and are negatively selected for host antigens. Furthermore, CCR9 is important in the seeding of the adult thymus together with CCR7 for hematopoietic progenitors. (Krueger et al., 2010) Despite those important functions, CCR9 or CCL25 deficient mice are healthy, grow to adulthood and are capable of breeding. (M.-A. Wurbel et al., 2007) The defective thymus development is still reflected in less $CD8^+$ T cells during homeostasis and less $CD8^+$ and $CD4^+$ T cells homing to the small intestine upon antigen challenge. (Stenstad et al., 2006; M.-A. Wurbel et al., 2007) Similar to CCR7, CCR9 expression in cancer is correlated with enhanced migrations and poorer outcome such as in the $CCR9^+$ melanoma (Kühnelt-Leddihn et al., 2012), in lung adenocarcinomas (Lu et al., 2020) and in NSCLC. (Niu et al., 2020) Interestingly, CCR9 leads to a survival advantage through the upregulation of drug exporter, e.g. making prostate cancer resistant against etoposide (Sharma et al., 2010), breast cancer cells against cisplatin-induced apoptosis (Johnson-Holiday et al., 2011) and pancreatic cancer cell lines against gemcitabine. (Lee et al., 2015) An intersting role for CCL25 was recently discovered in triple-negative breast cancer (TNBC), which are characterized by the lack of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) and cause often an early relapse and have a high mortality rate. Strikingly, TNBC cells do not express CCL25, and therefore the intratumoral delivery of CCL25 as a novel treatment can lead to $CD8^+$ T cell recruitment and to an antitumor effect. (H. Chen et al., 2020) Like CCL25, CCR9 is associated with autoimmune diseases related to gut organs. CCR9 expressing T cells with either a TH1 memory or T

follicular helper cell phenotype are as a result misguided from the gut and its associated lymphoid tissue to other organs such as the pancreas and salivary gland and even the blood of most Sjoergens syndrome patients. (Cosorich et al., 2019; McGuire et al., 2011) The misguided CCR9 positive T helper cells can promote expansion of $CD8^+$ T cells and thereby boost the immune reaction. (McGuire et al., 2011) Concerning CCR9's signalling pathway, less has been elucidated than compared to the signalling pathway of CCR7. To date, CCR9 has been shown to activate calcium flux and Akt activation via PI3K. (Youn et al., 2001; Zabel et al., 1999) As far as its migration effects on the cell cytoskeleton are concerned, CCR9 acts via the RhoA/ROCKL/MLC signalling pathway (L. Zhang et al., 2011) and influences Ezrin/ Radixin/ Moesin (ERM) translocation to the plasma membrane. (B. Zhou et al., 2010)

1.3.4 CCR6

CCR6 is expressed on most B cells (Bowman et al., 2000; Krzysiek et al., 2000), subsets of $CD4^+$ and $CD8^+$ T cells (Liao et al., 1999), Tregs (K. J. Chen et al., 2011) NKT cells (Kim et al., 2002), central and memory effector cells which co-express CCR7 (Förster et al., 2008) and mainly immature but also mature DCs. (Charles et al., 2010; Greaves et al., 1997; Iwasaki & Kelsall, 2000; Kucharzik et al., 2002; Regamey et al., 2007) Whereas, matured and activated DCs clearly express CCR7, the expression of CCR6 appears to be transient and dependent on the DC microenvironment. Immature DCs use CCR6 to migrate to sites of inflamation, where upon maturation, they downregulate CCR6 and are guided towards LNs by CCR7. (Dieu-Nosjean et al., 1999; D. Yang et al., 1999) Of note, CCR6 has been shown to be a specific marker for TH17 cells (Hirota et al., 2007; Kleinewietfeld et al.,

2005) On most cells CCR6 is expressed, it mediates several homeostatic migration patterns. As such, CCR6 localises innate-like lymphocytes to survey the LN subcapsular sinus (SCS) for pathogen entry. Further CCR6 is needed for the efficient induction of IL17 in those patrolling cells upon antigen encounter. (Y. Zhang et al., 2016) CCR6 also guides the migration of DC towards mucosal epithelial sites and mucosal lymphoid tissues. (Ito et al., 2011) As a consequence, CCR6 deficient mice exhibit significant reduction on the DC and macrophage populations in the peritoneal cavity, while no other lymphoid populations are significantly modulated. (Wen et al., 2007) Further, the CCR6 deficient mice have an impaired antibody response in the gut when immunized orally. (Cook et al., 2000) As for CCR9, deregulated CCR6 migration is often associated with autoimmune diseases. Interestingly in IBD models, CCR6 deficient mice have either a less severe or a more severe outcome, depending on the type of chemical used to induce the inflammation. A myeloid, non-specific inflammation by dextran sulfate sodium (DSS) leads to a less severe outcome, whereas a lymphoid antigen driven inflammation by 2,4,6-trinitrobenzene sulfonic acid (TNBS) leads to a severe outcome, possibly reflecting the cell population shifts induced by CCR6 deficiency. (Varona et al., 2003) However, CCR6 deregulation has a straight negative impact on airway and MS inflammations. On one hand, CCL20/CCR6 is responsible for the infiltration of DCs into COPD patient's airways. (Bracke et al., 2006; Demedts et al., 2007) Further it was shown that the immune reaction in an allergic airway inflammation model is mediated via CCL20 guided cDC migration and TH2 cell activation. (Lundy et al., 2005) On the other hand, the TH17 cells as well as neutrophils which are present inside the CNS in MS models were found to be $CCR6^{+}$ TH17 cells (Reboldi et al., 2009; Wojkowska et al., 2014) and able to migrate across the BBB. (Arima et al., 2012) Conversely, CCR6 deficient mice were

highly resistant to induced MS onset, but interestingly not to disease progression. (Reboldi et al., 2009) As can be deduced from its homeostatic functions and guidance preferences, CCR6 is found in gastric cancer tissues (Y. Zhang et al., 2016) where it was associated with tumour size, number, invasion, and overall poor outcome. Further, CCR6 and its ligand CCL20 are highly expressed in lung metastases and associated primary cancers. (Raynaud et al., 2010) As a consequence, blocking of CCL20 with specific antibodies led to significantly reduced tumour angiogenesis and reduced growth (He et al., 2017) In line, overexpression of CCL20 promoted the proliferation, growth and invasiveness of triple-negative cancer cells in xenograft models (W. Chen et al., 2018) Like CCR9, CCR6 with CCL20 can make tumours drug resistant. CCL20 overexpression enhanced the chemotherapeutic resistance of breast cancer cells by upregulating ABCD1, which is responsible for drug efflux. (W. Chen et al., 2018) As for CCR9, not that many signalling pathways downstream of CCR6 have been studied to date. The cell invasion and migration is induced via PKCα, which activates Src, which in turn leads to Akt, JNK and NFκB pathways. (Marsigliante et al., 2016) Moreover active CCR6 was recently co-crystallised with bound CCL20 and engineered Gαo. (Wasilko et al., 2020)

1.3.5 ACKR4

Given the importance of the three mentioned receptor ligand combinations (CCR7 with CCL19 and CCL21, CCR9 with CCL25 and CCR6 with CCL20) in the numerous immune responses, it is crucial that those processes are regulated. Not only has this to happen on a transcriptional but in a localised and highly dynamic fashion as well. ACKR4 fulfils this function in various anatomical niches, as described

below. ACKR4's, formerly known as CCRL1 and CCX-CKR, gene locus is within the CCR chemokine cluster on human chromosome 3p21 (Gosling et al., 2000), respectively on chromosome 9 in mice. In addition, a pseudogene of ACKR4 exists on the human chromosome 6q24.1. (Townson & Nibbs, 2002) The two expressed mRNA transcripts lead to the same protein, only differing in a longer 5' untranslated region. Interestingly, the longer transcript is exclusively transcribed in brain and testis, however no function has been determined so far. (Townson & Nibbs, 2002) ACKR4 expression has been detected in a wide range or peripheral and mucosal tissues via RT-PCR (Townson & Nibbs, 2002), however more specific in lymphatic endothelial cells (LECs) in LNs, (Heinzel et al., 2007; Ulvmar et al., 2014), in cTECs (Bunting et al., 2013) and a small portion of medullary thymic epithelial cells (mTECs) (Lucas et al., 2015), in skin keratinocytes (Bryce et al., 2016), in a subpopulation of intestinal submucosal fibroblasts (Thomson et al., 2018) and on epithelial cells of the endometrium. (Han et al., 2019) Although it was suggested to be expressed in immune cells (Gosling et al., 2000), analysis of a GFP reporter mouse indicated its expression only in stromal lineages. (Heinzel et al., 2007) The systemic influence of ACKR4 became apparent when analysing the ligand levels in ACKR4$^{-/-}$ mice. CCL19 and CCL21 amounts were increased in tissues, in LNs and even the levels of soluble CCL21 in the serum. More specifically, a 5-fold increase in the level of CCL21 in the blood and a 2- to 3-fold increase in CCL19 and CCL21 in peripheral LNs was observed. (Comerford et al., 2010) Nevertheless, those mice are generally healthy and viable. (Comerford et al., 2010; Heinzel et al., 2007) But it was shown that the ACKR4$^{-/-}$ mice strain (used in Bunting et al., 2013; Comerford et al., 2010; Kara et al., 2018; Lucas et al., 2015; Whyte et al., 2020) have a defect in B cell hyperproliferation, and therefore must be taken with caution. In contrast,

the ACKR4$^{GFP/GFP}$ knock-in mice (used in Bryce et al., 2016; Heinzel et al., 2007; Ulvmar et al., 2014) behave like wildtype C57BL/6 mice in terms of B cell hyperproliferation. (Eckert et al., 2019) Indications such as CCL21 expression in early stages of thymic development and CCL19 and CCL21 expression by mTECs in the medulla hint at a role for CCR7 in thymus development. (C. Liu et al., 2006) However, ACKR4 deficient mice have a normal adult thymus size, cellularity and similar rates of thymocyte migration into the blood, providing evidence against a major role for ACKR4 in thymus development and function. (Lucas et al., 2015) Similarly, despite its ligand playing roles in organising T and B cell zones in spleen and LNs, they are intact in ACKR4 KO mice (Comerford et al., 2010; Heinzel et al., 2007), indicating ACKR4 to have no crucial role in secondary lymphoid organ (SLO) development. ACKR4 plays its role rather during migration under homeostatic and inflammatory conditions. ACKR4 was shown to scavenge CCL19 in the skin, especially once it is upregulated during inflammation, still enabling the migration of activated Langerhans cells, i.e. dermal DCs, into the lymph vessels and subsequently to the LNs. (Bryce et al., 2016) Despite the abundance of CCL19 in the skin, CCL21 and its gradient formed by ACKR4 are the main driver for LN homing from the skin. Therefore, Langerhans cells and T cells still homed to LNs in CCL19 deficient mice. (Britschgi et al., 2010) Once reaching the LN, ACKR4 scavenging of CCL21 along the SCS is crucial for the intranodal positioning of DCs and T cells. Thereby ACKR4 is present on lymphatic endothelial cells lining the ceiling of the SCS, but not on those lining the floor to create a specific determined gradient of 40-60µm. (Ulvmar et al., 2014) Moreover, ACKR4 in the SCS does not discriminate where its ligands are originating, i.e. from the LN parenchyma or from the periphery. (Ulvmar et al., 2014) Despite its function in regulating DC migration from skin to draining LNs, no in-

fluence of ACKR4 deficiency of CCR7 dependent trafficking from the small intestine to mesenteric LNs was found. (Thomson et al., 2018) Like CCR7 deficient mice, ACKR4 deficient mice can spontaneously develop a similar autoimmune like pathology to Sjorgens syndrome. (Bunting et al., 2013) Moreover, ACKR4 deficient mice alter the T cell response in an MS model. In these mice, the T cell response is skewed towards a TH17 response and the disease onsets earlier and shows stronger symptoms. (Comerford et al., 2010) Due to the fact that all ACKR4's ligands have been involved in tumour growth and metastasis, ACKR4's function in cancer has been studied on several occasions. Overall, ACKR4 has been attributed to be a negative regulator of cancer growth and metastasis. Several studies analysed the expression of ACKR1-4 and cancer prognosis, linking ACKR expression with better outcome and survival in breast cancer (Feng et al., 2009; Zeng et al., 2011), cervical cancer (Hou et al., 2013), gastric cancer (Z. Zhu et al., 2013), colorectal cancer (Y. Zhu et al., 2014) and hepatocellular carcinoma. (J.-Y. Shi et al., 2015) Those effects were mainly attributed to the sequestration of ACKR ligands and thereby influencing other chemokine receptor signalling axes. In two studies, ACKR4 expression was inversely correlated with LN status in patients. (Hou et al., 2013; Y. Zhu et al., 2014) Besides, three studies have examined the effect of ACKR4 expression in mouse models and cell lines in detail. In one of them, ACKR4 expression was correlated with a better outcome, mainly due to less intratumoral neovascularity. (Feng et al., 2009) However, in another ACKR4 deficiency let to the retention of DCs in tumour tissue and enhanced priming and on-site proliferatioin of tumor-specific $CD8^+$ T cells, reducing tumour growth. (Whyte et al., 2020) It has to be mentioned, that the latter study employed the $ACKR4^{-/-}$ mice, which have a B cell hyperactivation. How this influenced the tumour growth and metastasis inhibition was not further

analysed. In a third study, ACKR4 expression in mouse mammary carcinoma cells was studied. There, the cancer cells administered to mice, ACKR4 promoted epithelial to mesenchymal transition, thus reducing the cancer cell adhesion to each other and to extracellular matrix proteins. In addition, those cancer cells had a higher resistance to anoikis (anchorage dependent cell death). (Harata-Lee et al., 2014) Whereas other atypical chemokine receptors like ACKR2 act on inflammatory chemokines, ACKR4 is internalising and scavenging mainly homeostatic chemokines. (Comerford et al., 2006) Specifically, its ligands have been accepted to be CCL19, CCL21, CCL25 for human and mice (Gosling et al., 2000; Heinzel et al., 2007; Townson & Nibbs, 2002) and CCL9 in mice. (Gosling et al., 2000) Their uptake has been postulated to be β-arrestin independent and internalised via dynamin engagement and caveolae instead of the traditional clathrin dependent route. (Comerford et al., 2006) Further, using binding assays with varying pH, a rapid ligand release from ACKR4 was shown upon pH shift to acid conditions, indicating the deposition of radiolabelled CCL19 in lysosomes where acid conditions prevail. This finding was further substantiated as radiolabelled CCL19 was rapidly and constantly removed from the supernatant by ACKR4 expressing cells and not anymore traceable afterwards. (Comerford et al., 2006) In contrast to Comerford et al., 2006, where CCL19 uptake into vesicular structures lacking β-arrestin was reported, Watts et al., 2013 showed the uptake of fluorescent CCL19 into β-arrestin-GFP positive vesicles. Further they provided evidence for a dose dependent β-arrestin recruitment to ACKR4 upon stimulation with CCL19, CCL21 and CCL25 and confirmed interaction of ACKR4 with CCL9. As studies on ACKR4 signalling compared with the cCKRs is rather sparse and contradictory, the next chapter is focussed on specific receptor activation and signal transmission, comparing ligand and intracellular protein interac-

tions, using modelling to get an overview on the possibilities in ACKR and cCKR signalling.

1.4 Receptor Signalling

ACKRs and cCKRs have been shown to differ in their signaling capabilities. Whereas cCKR signalling has already been extensively studied, ACKR signalling is barely understood. The following section focuses first on general conformational changes, then the focus shifts to more detailed view on ligand receptor interactions, followed by signal transmission and then on activation of downstream effectors such as G-proteins or G-protein coupled receptor kinases (GRK). Currently around 30% of the approved drug targets are on GPCRs. (A. S. Hauser et al., 2017; Sriram & Insel, 2018) This alone makes it remarkably interesting to understand and analyse the different mechanism leading to GPCR signalling. In addition, as ACKRs are regulating various aspects of adaptive immunity, it is of great interest to further understand ACKR signalling and be able to target them in future immunosuppressive therapies. Thereby, the scope lies on the differences and similarities of ACKRs and cCKRs, putatively explaining the different outcomes.

1.4.1 Chemokine Receptor Conformation Changes

GPCR signalling is determined through the dynamic interchange between several receptor states. The ligand free state is called the apo-state, after which an active state is stabilised by ligand binding. The now accepted model of GPCR activation consists of a proportionally induced signalling, considering the pluridimensional interactions between receptor and transducers, which can be further modulated by

the different receptor conformations stabilised by different ligands. (Smith et al., 2018; Wootten et al., 2018) The signalling is affected by various mechanisms, from transcriptional regulation, to posttranslational modifications (PTMs) such as sulfation and glycosylation and finally their membrane localisation and interaction with intracellular modulators. (Dunn & Ferguson, 2015; Ellisdon & Halls, 2016; Komolov & Benovic, 2018; Wootten et al., 2018) Here, the focus lies on the putative transmembrane signalling transmission as well as the interaction with subsequent intracellular signalling and scaffold proteins. The chemokine receptor activation is believed to follow a three-step binding mechanism: During the first step, the receptors N-terminal tail interacts with the globular core of the chemokine, leading to the binding of the chemokine to the extracellular loops (ECLs) of the receptor (this process is termed chemokine recognition site 1, CRS1). Newer data based on the CCR5/5P7/CCL5 complex and experiments with CCR1 added an additional step (CRS1.5) between the previous two step binding mechanism. During this intermediate, now second step, the conserved receptor motif consisting of proline and cysteines is packed against the chemokine disulphide bond and leading to a conformational rearrangement of the interactions to move the chemokine into the second recognition site. The third step, in which the chemokines' N-terminus intercalates into the 7TM bundle (termed chemokine recognition site 2, CRS2) leads to transmembrane domain (TM) rearrangement, stabilisation of the receptor in an active conformation and signal transmission by the receptor. (Arimont et al., 2019; Sanchez et al., 2019; Zheng et al., 2017) It was previously unknown whether monomeric or dimeric forms of chemokines induce different modes of TM rearrangement, recent study provided structural evidence that monomeric CXCL8 and dimeric CXCL8 can both bind and activate CXCR2 in a conventional fashion as described below. (K. Liu et al., 2020)

As can be concluded form several available structures and modelling approaches, general activation of GPCR through ligand binding leads to a conserved outward movement of TM6. (K. Liu et al., 2020; Wasilko et al., 2020; Wootten et al., 2018) Due to the extracellular face contraction induced by ligand binding, TM5 is laterally displaced and the TM3, TM6 and TM7 are rearranged, thus open the intracellular G-protein contacting residues. (K. Liu et al., 2020; Venkatakrishnan et al., 2016; Wasilko et al., 2020) As for cCKRs, a similar mode of membrane translocation and signal transmission was proposed for ACKRs based on studies on ACKR3 interacting with CXCL12. (Gustavsson et al., 2017) The rearrangement of a GPCR is associated with the interplay of up to 41 residues in the case of CXCR4 (the cCKR for CXCL12), which are responsible for chemokine engagement, signal initiation, signal propagation, microswitch activation and G-protein coupling. (Wescott et al., 2016) Those can then interact with the G-protein subunit, but also overlap with putative GRK interaction sites, as described later. (Komolov & Benovic, 2018) The strength of the signalling is therefore not only dependent on ligand interaction alone, but also on the way the transducer interacts and stabilises such conformations, therefore the process is called ”allosteric coupling”, describing the loose dynamics of receptor changing from inactive, intermediate and active like states, stabilised by transducers as well as ligand binding. (Wootten et al., 2018) Analogous, it was shown that transducer binding can render a receptor for activation. (Gupte et al., 2017; Malik et al., 2017)

1.4.2 Ligand Interaction

As mentioned before, the ligands interact first with the N-terminal tail of the receptor, before getting into closer proximity. The extracellular

side of the receptors is formed by three ECLs as well as two binding pockets. The extracellular surface is thereby stabilised by up to two disulphide bridges. The disulphide bridge from the TM3 to ECL2 is highly conserved among GPCRs and was previously shown to be important for proper GPCR folding and membrane integration. (Rader et al., 2004) Moreover, an additional cystine bridge exists between the N-terminus close the TM1 and the ECL3. This is the case for ACKR3, where the bridge from cysteine 34 and cysteine 287(7.25) is crucial for receptor and ligand interaction signalling. (Benredjem et al., 2017) Further, this disulphide bridge can tether the N-terminus to the ECL3 and form an additional ligand binding surface. (Wasilko et al., 2020)

Whereas the chemokine core structure determines how it interacts with ECLs of the receptor and the receptor N-terminus, the penetration depth of chemokines is dependent on their respective N-terminal length. The chemokines can hence be rendered inactive, such as by CD26 cleavage. CD26 shortens and produces N-terminal processed forms of chemokines, which are not any more recognised by cCKRs and ACKRs. (Bonecchi et al., 2004; Savino et al., 2009) Nevertheless, even CCL20 being the shortest native chemokine (Riutta et al., 2018) can activate its receptor. In contrary to previously shown structures of chemokines with receptors, CCL20 activates CCR6 by interacting with a site above of the conventional transmission switch in the TM core, highlighting the diverse mechanism of cCKR activation. (Wasilko et al., 2020) As explained, the ligand enters the CRS2 in the final step of chemokine receptor activation. In detail, once the ligand entered CRS2, the interaction occurs with the major and minor binding pocket. Those pockets are characterised by highly conserved residues and formed by TM1,TM2, TM3 and TM7 surrounding the minor and TM3, TM4, TM5, TM6 and TM7 surrounding the major pocket. Analysing several known receptor ligand structures, residue W2.60 in the minor

pocket is involved in all presently known binding studies forming either hydrophobic interactions with aliphatic amino acids or edge to face interactions with phenyl rings otherwise. In addition, E7.39 is interacting with most of the ligands, forming ionic and/or hydrogen bond interactions. (Arimont et al., 2017) The second group of interactions, known as major pocket, is more diverse. Depending on the investigated receptor ligands, several hydrogen bonds and hydrophobic interactions are present. Still, D6.58 is a conserved amino acid, which is shown to form salt bridges with bound ligands. (Arimont et al., 2017) Of note, most of those interactions are receptor:chemokine dependent, and despite each individual one having only a minor role in chemokine receptor binding, mutations in those residues have a high impact in receptor activation. (Arimont et al., 2017)

1.4.3 G-Proteins

Once the chemokine is bound in CRS2 of the receptor, it induces the TM rearrangement and leads to transducer activation. cCKRs traditionally exert their migratory effect through the activation of downstream heterotrimeric G-proteins, which consist of Gα-, Gβ-, and Gγ-subunits. In humans, 16 Gα, 5 Gβ and 13 Gγ subunits can combine. (Wootten et al., 2018) The Gα are further divided into four subfamilies, of which most GPCRs interact with proteins from the Gs, Gi/o, and Gq/11 family. In the inactive state, the Gα-subunit is bound to guanosine diphosphate (GDP) and builds heterotrimers with the Gβγ subunit. Whereas the Gβγ are obligate heterodimers and tethered to the membrane by the Gγ subunit (O'Neill & Gautam, 2014), the Gα-subunits dislocate from them upon activation through the GPCR and the induced exchange of GDP to guanosine triphosphate (GTP). The interaction of Gα with GPCR is dynamic, involving the Gα's α5 helix

to interact with the receptor's core and it's intracellular loop 2 (ICL2) of the receptor, both interactions needed for a full Gα activation. (Du et al., 2019) The α5 helix adopts a more helical structure upon receptor binding, thereby tightly and specifically interacting with the receptor intracellular core. (Dror et al., 2015; Flock et al., 2017) Interestingly, the release of GDP precedes the helical ordering, indicating a very dynamic process of Gα activation. (Dror et al., 2015) Of the receptor's ICL2, the DRY motif is believed to be crucial for the G-protein activation. (Arimont et al., 2017) In addition to the ICL2, part of the ICL3 and the into the cytoplasm extended TM5 is interacting with Gα protein. (Flock et al., 2017) Interestingly, there is evidence of a GPCR barcode system over the 800 known GPCRs. The barcode includes several conserved residues important for all Gα interactions, whereas some are specific to certain Gα subunits only. The barcodes of related receptors are like each other, whereas evolutionary distinct groups of GPCRs have different barcodes. Still both groups can interact act with the same G protein, having different patterns of grooves and ridges in their GPCR:G-protein interaction interface. As some GPCRs can signal through multiple Gα, their downstream pathways are further regulated by transcriptional availability depending on cell type and maturation state. (Flock et al., 2017)

1.4.4 GRKs

Following G-protein activation, cCKRs can be phosphorylated by various kinases (Busillo et al., 2010), such as those of the GRK family (Komolov & Benovic, 2018), upon which β-arrestins bind to GPCRs. To date, seven human GRKs have been identified, of which two, namely GRK1 and GRK7, are restricted to retinoid expression. (Komolov & Benovic, 2018) GRKs belong to the serine/threonine protein kinase

AGC subfamily, which is named after the three original families, the cAMP-dependent protein kinase (PKA), the cGMP-dependent protein kinase (PKG) and the protein kinase C (PKC) families. Besides a regulatory and a ligand binding domain, GRKs further possess a variable C-terminal lipid binding region to mediate membrane localisation via either palmitoylation, prenylation or direct lipid binding via pleckstrin homology or a polybasic/hydrophobic domain. (Komolov & Benovic, 2018) Interestingly, a complex of $G\alpha_q G\beta\gamma$ and GRK2 has been crystallised, indicating a putative orchestrated co-activation by activated receptors. (Tesmer, 2005) Although no specific phosphorylation site for GRKs is known to date, it was shown that GRK1/GRK2 prefer to phosphorylate Ser/Thr on sequences containing Ser/Thr with either a carboxy or amino terminal acidic residue. (Onorato et al., 1991) Further, peptides derived from the ICL1 and less from ICL2 and ICL3 of the β2-adrenergic receptor were able to reduce GRK2/3 phosphorylation. (Winstel et al., 2005) This together with mutation experiments (W. Shi et al., 1995), showed GRK to be interacting with similar receptor regions as Gα proteins do. Recent crosslinking studies between GRK5 and the β2-adrenergic receptor supported those findings. (Komolov et al., 2017) In addition, the proximity of ICL3 and the GRK kinase domain provides an explanation how bound GRKs can phosphorylate sites on the ICLs as well, which was previously observed. (Eason et al., 1995) Like the activation of different Gα by different ligands on the same receptor, different ligands can induce specific phosphorylation patterns. (Butcher et al., 2011; Nobles et al., 2011) This is achieved by the recruitment of specific GRKs for each ligand, which then leads to different degrees of β-arrestin recruitment as shown with CCL19 and CCL21 on CCR7. (Zidar et al., 2009)

1.4.5 β-arrestins

The arrestin family is composed of four cytosolic proteins, of which two, arrestin 1 and arrestin 4, are restricted to photoreceptors cells, whereas the other two arrestins, 2 and 3, are ubiquitously expressed and can bind to hundreds of receptors. Further, β-arrestin1 (arrestin 2) and β-arrestin2 (arrestin 3) can often compensate for each other, even though some receptors prefer one over the other. (Gurevich & Gurevich, 2019) β-arrestins are known to lead to ”desensitisation” of activated GPCRs. On one hand by binding into the receptor core which is otherwise occupied by inactive Gα that can be thereafter activated. On the other hand, by the internalisation of ligand bound GPCRs, and therefore rendering those receptors unavailable for further ligand stimulation. (Wootten et al., 2018) Arrestins intercalate with active receptor cores like Gα proteins and in addition arrestin's C-domain interacts with the phospholipid bilayer stabilising receptor arrestin interactions. (Staus et al., 2020) Further, arrestins respond to phosphorylated residues on the receptor tail. Not only does the phosphorylation pattern determine the arrestin activity, but also the interaction of arrestin with the receptor core. Still, the tail is not the sole determiner for arrestin receptor interaction, as was nicely shown using standardised phosphopeptides, which were ligated to several receptors and still lead to different degrees of arrestin recruitment. (Staus et al., 2018) Moreover, the first interaction with the receptor's tail still enables simultaneous G-protein coupling and putative signalling. (Kumari et al., 2016; Thomsen et al., 2016) Moreover, this tail-only interaction with GPCRs leads to receptor internalisation and subsequent signalling as shown for CCR7 from endosomal compartments. (Laufer et al., 2019b) The second interaction of β-arrestin with the receptor core however is responsible for desensitisation. (Cahill et al., 2017; Kang et al., 2015;

X. E. Zhou et al., 2017)

Newer molecular dynamics and in vitro studies confirmed that the receptor core as well as the phosphorylated tail can both alone stabilise β-arrestin in an active conformation. Moreover, they enhance it even further once the core and the tail interaction are combined. In addition, the intercalation of the finger loop of arrestin into the receptor core is not causing arrestin activation, but rather a consequence thereof and further stabilising arrestin in an active conformation. (Latorraca et al., 2018) Activated arrestin then localises to clathrin coated structures, where it accumulates and internalises attached to vesicles. This internalisation process occurs with as well as without loaded receptor, enabling β-arrestin to exert functions distant from the initial activation. (Eichel et al., 2018) Besides leading to the internalisation of receptors, β-arrestins were shown to act as scaffold proteins. Examples therefore are the pooling of components of the ERK cascade (Lefkowitz, 2005) or of E3 ligases. (Lee et al., 2019; Lefkowitz, 2005) For those functions, the interaction of β-arrestin with the receptor core is not required (Kumari et al., 2016), enabling signalling by the receptor and enhancing the downstream signalling by bringing relevant proteins together. For a long time therefore, it was believed that β-arrestins itself induce signalling along the ERK cascade. This dogma was challenged by a study from Grundmann et al., 2018. By using G-protein null cells, they showed that β-arrestin recruitment to model class A GPCRs (β2AR, AT1R, V2R) was G-protein independent, however, those cells didn't exert any ERK signalling even with β-arrestin biased ligands. (Grundmann et al., 2018) However, the role of β-arrestin as scaffold for ERK kinases was affirmed, as the EC50 for ERK signalling were higher in β-arrestin KO cells. (Grundmann et al., 2018)

1.5 Signalling Switches

As became apparent in the previous text, several sites on the receptor deserve a closer look, especially if one wants to find differences in signal transmission between ACKRs and cCKRs. To aid the understanding, the Ballesteros-Weinstein nomenclature was applied and sequence information for ACKR4 and its related cCKRs pulled from the GPCRdb.(Isberg et al., 2015; Pándy-Szekeres et al., 2018; van der Kant & Vriend, 2014) The Ballesteros-Weinstein numbering scheme consists of two numbers, where the first denotes the TM domain and the second the relation towards the most conserved residue, which receives the number 50. (Ballesteros & Weinstein, 1995)

1.5.1 Transmission/toggle switch or PIF Motif (3.40 5.51 6.44 6.48)

Upon ligand binding in several structures of GPCRs, a movement of conserved hydrophobic residues in the receptor core was observed. Mainly 6.48 and 6.44 moved towards 5.51 and 5.50, and 3.40 away from 5.50, and was shown in the recent CXCL8:CXCR2 structure as well. (K. Liu et al., 2020; Venkatakrishnan et al., 2013) The main residues of this so called PIF (or PMF depending on cCKR) switch are present in ACKR4, such as F5.51, P5.50, M3.40 and F6.44, but not Q6.48. However, as some cCKRs related to ACKR4 possess the same alterations in the transmission switch, the switch itself might still be functional. In addition, the structure of CCR6 as well as the inactive structure of CCR7 revealed an additional stabilisation of the TM3 and TM6 between the residues N6.52, Y3.32 and Q6.48, which are present in ACKR4 as well. (Wasilko et al., 2020)

Na+ Binding Pocket							
N1.50	L2.46	D2.50	S/G 3.39	L3.43	4.48	N (S/H) 7.45	S (C/A) 7.46

Alternative Switch			
	Y3.32	Q6.48	N6.52
ACKR4	Y119	Q253	N257
CCR6	Y125	Q267	N271
CCR7	Y136	Q276	N280
CCR9	Y126	Q267	N271

Transmission Switch					
	I/M 3.40	P5.50	L5.51	F6.44	W6.48
ACKR4	M127	P212	F213	F249	Q253
CCR6	M133	P226	L227	F263	Q267
CCR7	M144	P235	L236	F272	Q276
CCR9	V134	P226	F227	F263	Q267

DRYLAIV motif							
	D3.49	R3.50	Y3.51	L3.52	A3.53	I3.54	V3.55
ACKR4	D136	R137	Y138	V139	A140	V141	T142
CCR6	D142	R143	Y144	I145	A146	I147	V148
CCR7	D153	R154	Y155	V156	A157	I158	V159
CCR9	D143	R144	Y145	I146	A147	I148	A149

NPxxY Motif				
N7.49	P7.50	x7.51	x7.52	Y7.53

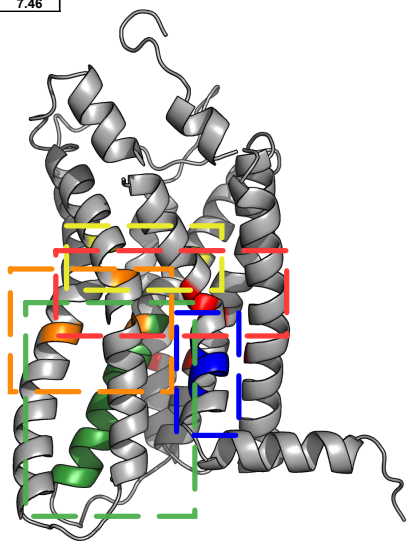

Fig. 1.3: The locations of the known signalling switches modelled onto an model of an inactive ACKR4 based on information from GPCRdb (Pándy-Szekeres et al., 2018) and CCR9 crystal structure [PDB:5LWE] (Oswald et al., 2016). In addition, the amino acids of ACKR4's relatives are noted, with the consensus sequence with Ballesteros-Weinstein nomenclature. The variations in the ionic lock are various, therefore no sequence is shown, further, the NPxxY motif is identical in all four receptors. Bold green lettering illustrates mutations compared to the consensus sequence.

1.5.2 CWxP Motif (6.47 6.48 6.50)

The CWxP motif is located at the bottom of the major pocket in the CRS2 site. Its activation occurs through a rotation of W6.48 towards TM5, where it interacts with a F5.47 and is trapped in an aromatic lock, stabilising the active confirmation. (Holst et al., 2010) The CWxP motif was also shown to be important in CXCR4 signalling. (Wescott et al., 2016) However as the PIF motif, the CWxP is not present in ACKR4 (TQLP) and not in all its related cCKRs.

1.5.3 DRY (or E/DRY) Motif (3.49-3.51) and ICL2

One of the most prominent features of ACKRs is the inability to induce migration. This is attributed to their natural mutations in the DRYLAIV motif. Initially the DRY motif was described as crucial for G-protein signalling and mutations thereof either forcing a constitutively active or ground state receptor. (Rovati et al., 2007) The alterations in the DRYLAIV motif in ACKRs are various, however most prominent is either a complete absence of the motif or the introduction of at least one charged amino acid residue (glutamic acid in ACKR2, serine and threonine in ACKR3 and threonine in ACKR4). Most studies on G-protein DRYLAIV motif either focussed on complete exchange thereof or on exchange of DRY residues. Among those, the R3.50 of the DRY motif was shown to directly interact with several G-protein α5 helices, as well as with engineered mini G-proteins and with residues of β-arrestin finger loop. (Arimont et al., 2017) Interestingly, the interaction of the R3.50 is not restricted to charged residues such as for the β2-adrenoceptor with the tyrosine 391 in the $G\alpha_s$-protein but in the case of rhodopsin can interact with glycine 2076 and leucine 2077 of visual arrestin. (Kang et al., 2015; Rasmussen et al., 2011) Despite the

interaction of the R3.50 with G-proteins, even a mutated, charge neutralised DRY to AAY was able to induce, although reduced, G-protein dependent signalling. (Pietraszewska-Bogiel et al., 2020) Supporting the fact that not the DRY motif alone is responsible for G-protein mediated signalling, exchanging ACKR4's ICL2 (including the DRYVAVT motif) with the one from CCR7 or CCR9 respectively did not result in cAMP activity as expected. (Watts et al., 2013) In line, replacement of the ACKR3 DRY motif with the one from CXCR4 did not restore G-protein activation either. (Hoffmann et al., 2012)

1.5.4 Ionic Lock (3.50 6.30)

For the β-adrenergic receptor and rhodopsin receptor an ionic lock was proposed. The interaction of charged residues for that matter stabilise a non-signalling conformation. Thereby, R3.50 interacts with a negatively charged residue, aspartic acid or glutamic acid, at 6.30 in the ground state, stabilising an intracellularly closed conformation. (Trzaskowski et al., 2012) As soon as then the receptor is activated and the TMs switch, the lock is broken and the residues 3.50 and 6.30 form other interactions with residues on TM5 and TM6. Thereby, the receptor core can be accessed by transducers such as Gα proteins. However, the ionic lock in this form is only present in 25% of all GPCRs and in none of the cCKRs or ACKRs studied here. Instead, ACKR4 and its related cCKRs possess positively charged residues around 6.30, which instead of R3.50 might form a lock with D3.49. Of note, CCR7 and CCR4 still possess a glutamic acid towards the end of ICL3.

1.5.5 NPxxY (7.49-7.53)

The NPxxY was first described on a signalling scale by Audet and Bouvier, 2012. The NPxxY motif is located towards the end of the TM7. The importance of this domain as an alternative signalling inducer was supported by spectroscopy studies of the β2-adrenergic receptor and employing agonists with distinct arrestin or G-protein bias. Interestingly, ligands favouring β-arrestin signalling altered only TM7, whereas ligands inducing G-protein and arrestin signalling altered TM6 and TM7. (W. Liu et al., 2012) NPxxY thereby stabilised G-protein signalling by the charged tyrosine of NPxxY which then prevented the reverse movement of TM6 into an inactive state. Of the ACKRs, only ACKR1 and ACKR2 have alterations in the NPxxY motif. Whereas N7.49 is exchanged with serine in ACKR2, the motif is completely altered in ACKR1.

1.5.6 Sodium Binding Site (2.50 3.39 4.48 7.45 7.46)

It was proposed that Na^+ is transported across the membrane and/or that it influences ligand binding to class A GPCRs. Na^+ is supposedly binding to D2.50 and S3.39 (W. Liu et al., 2012) during the inactive state and is then translocated while the receptor is activated. Although D2.50 is highly conserved, S3.39 is a glycine in 48% of chemokine receptors, such as in several ACKRs and cCKRs related to ACKR4. (Pándy-Szekeres et al., 2018) Despite structural differences in the Na^+ pocket, Na^+/H_2O was found in crystallographic experiments and is supposedly playing a role in allosteric receptor activation. (Katritch et al., 2014)

1.5.7 The 8th Helix and the Membrane Localisation

Even the membrane localisation and composition can influence the efficacy of signalling of GPCRs and other membrane receptors. It was shown that lipids and small molecules such as cholesterol can influence the oligomerisation, raft localisation and the allosteric activation. (Dawaliby et al., 2016a; 2016b; Thelen & Legler, 2018; Zocher et al., 2012) Moreover, modifications such as palmitoylation on the receptors amphipathic 8tth helix can influence the localisation and dimerization of receptors to specific membrane rafts. (Seno & Hayashi, 2017; X. Zhang & Kim, 2016) Further, membrane rafts properties such as tension and curvature play a role. (Milligan et al., 2018) Interestingly, the modification of receptors and the localisation to specific rafts and interaction with molecules therein such as cholesterol can be crucial for correct receptor function. (Hanson et al., 2008; Tiu et al., 2020) However, as the complexity of plasma membranes with up to 1000 different types of lipids, (Harayama & Riezman, 2018) the scope of such interactions are still far from being understood.

1.6 Atypical Chemokine Receptors and Signalling

Currently, there are 6 putative ACKRs, of which 4 have been accepted and two numbers are reserved for promising ACKR candidates which will be introduced later on. (Bachelerie et al., 2014b) Ancestry analysis revealed the evolution of initially two subsets of ACKRs, the subgroup for ACKR1 and for ACKR2, of which the latter diverged into ACKR2, ACKR3 and ACKR4. (Pan et al., 2018) Two of those stand out, on one hand ACKR1 which behaves completely different than other accepted ACKRs, and ACKR3 which binds the highly studied chemokine CXCL12 (SDF-1) on the other hand. Therefore, most studies on ACKR

interaction and signalling to date have been performed on ACKR3. The next section focusses on the ACKRs 1 to 6, except ACKR4, which has already been described in the subsection 1.3.5.

1.6.1 ACKR1

ACKR1, previously known as Duffy antigen receptor for chemokines (DARC), is the ACKR which lacks the characteristic DRYLAIV motif entirely. (Neote et al., 1994) Further it has been the oldest known chemokine receptor (since 1950) and is able to bind over 20 different chemokines from the CC and CXC subfamilies. (Bachelerie et al., 2014a) Despite its broad expression on endothelial cells on venules and veins, as well as erythrocytes, it cannot be found on arteries. (Rot, 2005) Its function ranges from being a putative reservoir of chemokines in erythrocytes and equilibrating the chemokine levels in the blood (Mei et al., 2010), to presenting chemokines on vein endothelial cells, where it is able to transport chemokines from the basolateral to the apical membrane. (Hub & Rot, 1998; Middleton et al., 1997; Rot, 1992) Interestingly, ACKR1 binding and internalisation does not lead to lysosomal degradation of the chemokines. (Pruenster et al., 2009) So far, no studies have been done on intracellular signal activation by ACKR1, and its various mutations in the known signal transmission motifs in its TMs indicate a completely different regulation of receptor function.

1.6.2 ACKR2

ACKR2, previously termed D6, binds all inflammatory chemokines of the CCL family. As other ACKRs, it is broadly expressed along ”barrier” sites, such as placenta, lung, skin, and gut. (Bachelerie et al., 2014a) In addition, it is also present on leukocyte subsets, like DCs

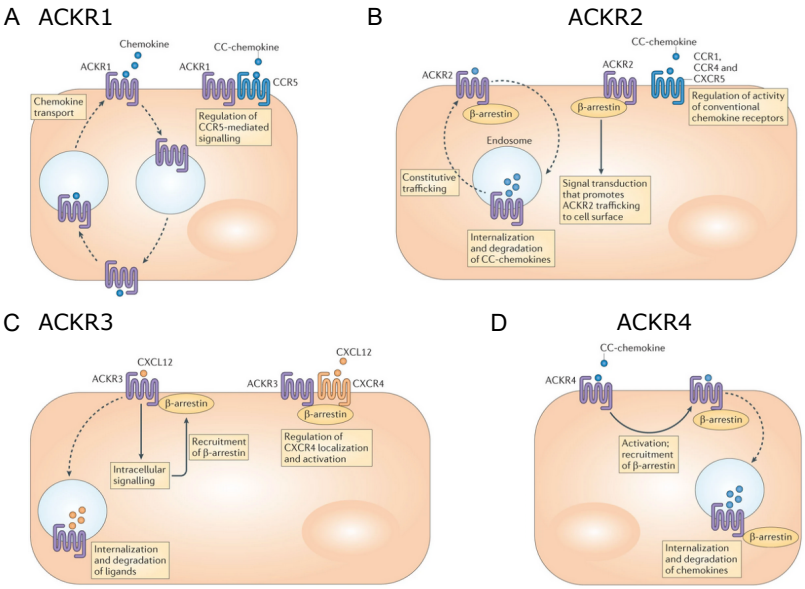

Fig. 1.4: A) ACKR1 can transport chemokines across polarized cells to present them to leukocytes. Further, it forms heteromers with CCR5 to influence CCR5's the signalling properties. B) ACKR2 traffics back and forth to the cell surface and captures chemokines bound at the cell surface. Thereafter, those are internalised and released into endosomes and degraded. ACKR2 is constitutively associated with β-arrestins and can regulate functions of cCKRs if bound as heteromer as with CCR1, CCR4 and CXCR5. C) ACKR3 internalises its ligands. Upon activation by CXCL12, it induces intracellular signaling and relocalises β-arrestins. Further, it regulates trafficking, subcellular localisation and signalling of CXCR4. D) ACKR4 can interact with β-arrestins and ACKR4 mediates the internalisation and degradation of its ligands. Modified from Nibbs and Graham, 2013 with the licence number: 4876711114999

and innate-like B cells. (Hansell et al., 2011; McKimmie et al., 2008) ACKR2, as most ACKRs, is mainly present in vesicles (Bonecchi et al., 2008; Galliera et al., 2004; Weber et al., 2004) and only low amounts are on the cell surface. (Blackburn et al., 2004) As one of the first evidence of downstream ACKR signalling, it was shown that ACKR2's scavenging and redistribution activity upon ligand binding was mediated via β-arrestin1, which in turn led to the phosphorylation of cofilin through the Rac1-p21 activated LIM kinase 1 cascade. (Borroni et al., 2013) Interestingly, in contrast to cCKRs which are phosphorylated via GRK upon ligand encountering (Zidar et al., 2009), ACKR2 appears to be constitutively phosphorylated on its intracellular C-terminal domain. (McCulloch et al., 2008)

1.6.3 ACKR3

ACKR3, formerly known as CXCR7, shares its ligands CXCL12 with CXCR4 and CXCL11 with CXCR3. Together with the myriad of interacting proteins already described in the literature (reviewed in Fumagalli et al., 2019), ACKR3 remains the most studied ACKR in terms of interaction partners and signalling. In contrast to ACKR1, ACKR2 and ACKR4 KO mice, ACKR3 deficiency leads to a lethal condition. this is in line with the lethality caused by a lack of CXCR4 or CXCL12. However, ACKR3$^{-/-}$ embryos have defects in all four heart valves. As this condition is not present in CXCR4 KO mice, the first indication on ACKR3 function aside scavenging is given, especially as the mice were CXCL11 null. (Sierro et al., 2007; Yu et al., 2011) As for ACKR2, ACKR3 cycles between the cytoplasm and the plasma membrane, and this cycling is attenuated in the presence of ligand. (Naumann et al., 2010) To date, the exact signalling pathway induced by ACKR3 remains unclear. (Gravel et al., 2010; Zabel et al., 2009) It is accepted

by now, that ACKR3 does not trigger typical chemokine receptor signal pathways via G-proteins, even when the only slightly altered DRYLAIV motif is restored to the canonical sequence. (Hoffmann et al., 2012) Due to the arrestin dependent internalisation, a putative ERK activation (Rajagopal et al., 2010) as observed in cortical GAGergic interneurons (J. Wang et al., 2011) might be possible where ACKR3 and CXCR4 are co-expressed. Interestingly, depending on the cell type, different downstream effectors of CXCL12 stimulated ACKR3 were activated. In astrocytes, signalling was transduced via ERK1/2 and Akt in the absence of CXCR4. However, in Schwann cells both receptors had to be present to induce ERK1/2, Akt and p38 signalling. (Ödemis et al., 2010) In contrary, newer data underlined a lack of G protein activation and no β-arrestin dependent ERK activation downstream of ACKR3. (Saaber et al., 2019) The link between ligand activation and phosphorylation was made in 2019, when GRK2 together with GRK3 were shown to be the main GRKs for ACKR3, not ruling out minor contributions of others like GRK5. (Saaber et al., 2019) interestingly, the generated phosphorylation deficient ACKR3$^{ST/A}$ knock-in mice were viable in contrary to the ACKR3$^{-/-}$ mice. Still, the interneuron migration in those mice was defective in contrast to a lack of β-arrestins together with wildtype ACKR3. Thereby, the phosphorylation of ACKR3 was shown to be crucial compared to a dispensable role for β-arrestin2 in ACKR3 scavenging and correct CXCR4 mediated interneuron migration during embryogenesis. (Saaber et al., 2019) A recent study finally added more diverse ligands to the ACKR3 repertoire. Those are not conventional chemokines, however all opioid ligands, making ACKR3 the atypical opioid receptor and the only of the ACKR family to recognise and efficiently scavenge such ligands. Further, it was shown that opioid ligands do not induce any G protein activation or arrestin dependent ERK activation. (Meyrath et al., 2020a)

1.6.4 ACKR5

The putative ACKR5 is CRAM (A and B), also known as CCRL2. Initially postulated to bind various chemokines (Biber et al., 2003; Zabel et al., 2008), among those CCL19 (Zabel et al., 2008), and inducing signalling and chemotaxis via calcium flux and ERK activation (Biber et al., 2003; Hartmann et al., 2008), only chemerin has been independently confirmed as a ligand. (Mazzotti et al., 2017) Interestingly, ACKR5 expression differs between the subsets of leukocytes of humans and mice and can be upregulated by various inflammatory stimuli. (Bachelerie et al., 2014a) As ACKR4 KO mice, the CCRL2 mice are healthy and viable (Zabel et al., 2008), however showing higher ligand levels of its ligands, chemerin, in the blood. (Hartmann et al., 2008; Monnier et al., 2012) However, ACKR5 was shown to neither activate G proteins nor to recruit β-arrestins, therefore its mechanisms are still unknown. (De Henau et al., 2016) Moreover, ACKR5 was shown to neither be rapidly recycling nor to scavenge chemerin, two of the main characteristics of ACKRs and is therefore suggest being removed from the ACKR family. (Mazzotti et al., 2017)

1.6.5 ACKR6

The putative ACKR6 is C5L2, which has so far not been shown to bind a chemokine. It was shown to bind C5a and C5a des-arg of the complement system, which both act as chemotactic agents, (Abdelbaset-Ismail et al., 2017; Cain & Monk, 2002) and is thereby weakly phosphorylated. (Okinaga et al., 2003) As one can expect from the altered DRLAIV motif of C5L2, ligand binding does not lead to calcium flux, chemotaxis, or MAPK activation. However, restoring the DRY motif weakly restored the receptors function in term of calcium flux.

(Scola et al., 2009) Further, also the NPxxY motif is altered, and its restoration together with the restoration of the DRY motif further partially restored its function, indicating further suppressive or altering mechanisms present. Interestingly, C5La is mainly present in vesicular structures and interacts with β-arrestin, negatively regulating C5Ra signalling. (Bamberg et al., 2010; Van Lith et al., 2009) As can be expected C5L2 greatly affects the immune response, modulating the local inflammatory responses triggered by C5aR. (Bachelerie et al., 2014a; X. X. Li et al., 2019) This function is not only achieved by scavenging but also by heteromerization with the native C5a ligand, C5R1, and thereby directly enhancing the C5R1 internalisation and desensitisation. (Croker et al., 2014)

2 Results

During the course of this PhD thesis, I focused on three main topics. First, to generate suitable tools for the tracking and studying of ACKR4, which could also be applied to CCR7, as both share the ligands CCL19 and CCL21. As traceable ligands are an ideal tool to study receptor function, the focus laid on the generation of modified chemokines. The presented publication 1 was a collaborative effort with Dr.rer.nat. Vladimir Purvanov who led the exploration of the chemokine production by transduced human cell lines. Secondly, I aimed to expand and bring together the so far dispersed knowledge on ACKR4 signalling. Thereby we modified and generated new GRK and G-protein sensors and first described the degree of influence of GRKs, arrestins and G-proteins on ACKR4's mode of action, as seen in the second publication 2 of this results chapter. Finally, as there are still lots of chemokines without designated scavenging receptors, we wondered which ones might be suitable ACKR4 ligands. Therefore, we employed bioinformatic approaches to discover CCL20 as a suitable ligand for ACKR4. In this great collaboration with the group of Prof. Dr. Marcus Thelen at the Institute for Research in Biomedicine (Switzerland), we have proven the CCL20 scavenging by ACKR4 in human as well as in mice as seen in the third publication 3 of the results part.

The following three publications (Matti et al., 2020a; Matti et al., 2020b; Purvanov et al., 2018) are all reprinted based on the Attribution-Non-Commercial 4.0 International (CC BY-NC 4.0) license.

Article

Fluorescently Tagged CCL19 and CCL21 to Monitor CCR7 and ACKR4 Functions

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Received: 26 October 2018; Accepted: 3 December 2018; Published: 4 December 2018

Abstract: Chemokines are essential guidance cues orchestrating cell migration in health and disease. Cognate chemokine receptors sense chemokine gradients over short distances to coordinate directional cell locomotion. The chemokines CCL19 and CCL21 are essential for recruiting CCR7-expressing dendritic cells bearing pathogen-derived antigens and lymphocytes to lymph nodes, where the two cell types meet to launch an adaptive immune response against the invading pathogen. CCR7-expressing cancer cells are also recruited by CCL19 and CCL21 to metastasize in lymphoid organs. In contrast, atypical chemokine receptors (ACKRs) do not transmit signals required for cell locomotion but scavenge chemokines. ACKR4 is crucial for internalizing and degrading CCL19 and CCL21 to establish local gradients, which are sensed by CCR7-expressing cells. Here, we describe the production of fluorescently tagged chemokines by fusing CCL19 and CCL21 to monomeric red fluorescent protein (mRFP). We show that purified CCL19-mRFP and CCL21-mRFP are versatile and powerful tools to study CCR7 and ACKR4 functions, such as receptor trafficking and chemokine scavenging, in a spatiotemporal fashion. We demonstrate that fluorescently tagged CCL19 and CCL21 permit the visualization and quantification of chemokine gradients in real time, while CCR7-expressing leukocytes and cancer cells sense the guidance cues and migrate along the chemokine gradients.

Keywords: fluorescent chemokines; chemokine receptors; CCL19; CCL21; CCR7; ACKR4; cell migration; leukocytes; cancer metastasis

1. Introduction

Chemokines are best known for their central role in orchestrating cell migration. Chemokines and their cognate receptors not only regulate leukocyte trafficking during homeostasis and inflammation; they also guide and position cells in organogenesis and control the growth and spread of numerous tumors [1–3]. Chemokines constitute a family of about 50 small proteins of 8–12 kDa with only little sequence homology. However, they share a common three-dimensional structure termed the chemokine fold that is stabilized by two characteristic disulfide bridges between mainly four conserved cysteine residues [4]. Depending on the arrangement and spacing of the first two cysteines, they are divided into CC, CXC, CX_3C, and XC chemokine subfamilies. Typical chemokine receptors are heptahelical class A G-protein coupled receptors (GPCRs) that signal through G_i-proteins for guiding cell migration [5]. Ligand binding to chemokine receptors leads to the GDP/GTP exchange of coupled heterotrimeric G-proteins and the subsequent dissociation of the $\beta\gamma$-subunits from the α-subunit. The $\beta\gamma$-subunits then activate phospholipase C and phosphoinositide 3-kinase, leading to further activation of kinase cascades, including the extracellular signaling regulated kinases ERK-1/2, and

to the generation of second messengers such as calcium from intracellular stores [5]. In addition, chemokine receptors can activate Src kinases and downstream signaling molecules such as the tyrosine phosphatase SHP2 [6]. The activity of chemokine receptors is commonly downregulated through phosphorylation of C-terminal serine and threonine residues, resulting in receptor desensitization, β-arrestin recruitment and receptor internalization [5].

The homeostatic CC chemokines CCL19 and CCL21 are constitutively expressed primarily by stroma cells in lymphoid organs [7–9]. In addition, CCL21 is produced by lymphatic endothelial cells. Its cognate receptor CCR7 is expressed on various subsets of immune cells including subpopulations of T cells and antigen-presenting dendritic cells (DCs). CCR7 and its ligands CCL19 and CCL21 play a crucial role in guiding antigen-bearing DCs and lymphocytes to lymphoid organs, thereby launching adaptive immune responses [8]. Misguidance of these leukocytes results in defective adaptive immune responses and can lead to autoimmune diseases, as shown in gene-targeted mice lacking CCR7 or its ligands [10]. Furthermore, if expressed by cancer cells, CCR7 facilitates cancer cell dissemination, migration and metastasis formation in lymphoid organs [11]. Interestingly, besides common signaling pathways of CCR7 by the two ligands, only CCL19 was shown to efficiently promote β-arrestin recruitment resulting in CCR7 internalization through clathrin-coated pits, whereas CCL21 is more proficient in inducing cell adhesion than CCL19 [9,12–14].

CCR7-guided cell migration is well studied for DCs. Upon pathogen encountering in peripheral tissues, they capture antigens and induce the expression of CCR7. DCs then migrate in a CCR7-dependent and haptotactic manner along an immobilized CCL21 gradient towards lymphatic vessels from where they are flushed into the sinus of the draining lymph node [15–17]. Although information on how a chemokine gradient is formed in vivo is scarce, it is well accepted that CCL21 binds to glycosaminoglycans (GAGs). For CCL21, this occurs through its unique extended C-terminal tail that includes stretches of basic amino acids and is therefore negatively charged [18–20]. The interaction of CCL21 with GAGs on lymphatic endothelium is critical for restricting the chemokine distribution and thus contributes to shape immobilized chemokine gradients [15]. Notably, CCL19 lacks high affinity binding to GAGs and hence is considered to diffuse or form soluble gradients. In vitro, it has been shown that the migration of DCs on homogenously immobilized CCL21 can be directed by applying soluble CCL19 locally [13]. Exploiting different microfluidic devices revealed that DCs differentially respond to competing chemokine gradients and that cell guidance varies depending on whether chemokine gradients are soluble or immobilized [21,22].

The generation and maintenance of local chemokine gradients is critical for efficient leukocyte trafficking. A family of atypical chemokine receptors (ACKRs) was recently identified to internalize and degrade chemokines, thereby ACKRs regulate the local availabilities of chemokines and shape chemokine gradients [23,24]. ACKRs share ligands and structure of typical chemokine receptors, but do not couple to G_i-proteins for signal transduction and hence fail to induce cell migration. However, ACKRs scavenge chemokines and deliver them for lysosomal degradation [25]. ACKR4 (formerly also known as CCRL1 or CCX-CXR) is expressed by lymphatic endothelial cells lining the ceiling of the subcapsular sinus of lymph nodes and scavenges the CCR7 ligands CCL19 and CCL21, as well as CCL25 [26]. In fact, ACKR4 has been shown to create CCL21 gradients across the sinus floor of lymph nodes and thereby enables directional migration of DCs [26].

So far, quantification of chemokine gradients was very challenging and had profound limitations: (i) quantification was often restricted to fixed specimen for staining purposes of the chemokines using antibodies, (ii) chemokines had to be printed as gradient to a substrate using highly sophisticated methods, or (iii) chemokine gradients were simply estimated by concurrently applying dyes, e.g., FITC-dextran, with the chemokine. Moreover, the scavenging activity of chemokine receptors was routinely determined by measuring liberated ^{125}I from iodinated chemokines upon receptor-mediated internalization and lysosomal degradation [27]. Here, we designed and generated functional fluorescently labeled chemokines by fusing CCL19 and CCL21 to monomeric red fluorescent protein

(mRFP), revealing CCL19-mRFP and CCL21-mRFP, as powerful tools to visualize and characterize CCR7 and ACKR4 functions in a spatiotemporal manner using time-lapse video microscopy.

2. Results

2.1. Design of Fluorescently Tagged CCL19 and CCL21

The availability of fluorescently labeled chemokines is highly desired but very limited. Chemokines can be expressed and produced in inclusion bodies of bacteria as recombinant proteins, but the denatured purified chemokine must be properly refolded and contaminating LPS derived from host cells needs to be carefully removed [4,27,28]. Moreover, the N-terminus of recombinant chemokines must be processed and freely available to render the chemokine functional. We decided to elaborate a strategy to easily and economically produce native fluorescently tagged chemokines in a mammalian cell expression system, such as HEK293 cells. To achieve this we designed an expression system in which we fused the cDNA coding for the mature, full-length forms of human CCL19 (amino acids 22–98) and CCL21 (amino acids 24–134) at their C-terminus to a flexible short linker sequence (coding three times for the amino acids: GGGGS, single letter code) followed by mRFP (Figure 1A). In addition, fusing an N-terminal signal peptide (derived from mouse IgGκ) followed by a His_6-SUMO double tag to the mature form of the chemokines entails three advantages: (i) the chemokine fusion protein is secreted as properly folded protein upon overexpression in mammalian cells. (ii) the His_6 tag enables easy affinity purification of the fusion protein over a Ni^{2+}-column. (iii) the SUMO3 tag renders the recombinant protein soluble and can be simply and precisely cleaved off with an impeccable specificity and efficiency using the SUMO protease 2 (SUMOstar™), liberating the native chemokine-mRFP with its correct mature N-terminus [4,28].

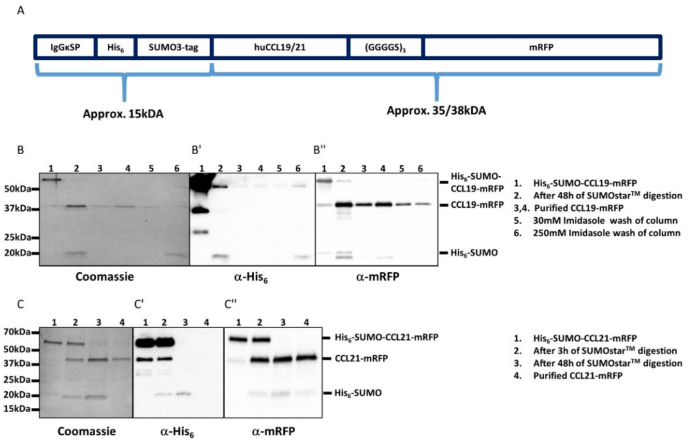

Figure 1. Design and purification of CCL19-mRFP and CCL21-mRFP. (**A**) Graphic scheme of the constructs used to generate fluorescently tagged full-length chemokines. (**B**,**C**) Coomassie-stained SDS-PAGE gels (left) and corresponding Western blots (middle and right) of samples collected during the purification steps of CCL19-mRFP (**B**) and CCL21-mRFP (**C**). Secreted His_6-SUMO-CCL19-mRFP was collected from the supernatants of transfected HEK293 cells and affinity purified over a Ni^{2+} column (**B**, line 1). The His_6-SUMO tag was cleaved off by incubation for 48 h with SUMOstar™ protease at 8 °C (**B**, lane 2) and applied again on the Ni^{2+} column to remove the His_6-SUMO tag. The purified CCL19-mRFP was collected as flow-through (**B**, lanes 3,4). The column was washed with 30mM imidazole (**B**, lane 5) and 250 mM imidazole to completely remove the His_6-SUMO tag from the column for reuse (**B**, lane 6). Similarly, secreted His_6-SUMO-CCL21-mRFP (**C**, lane 1), was digested with SUMOstar™ protease for 3 and 48 h (**C**, lanes 2,3) and applied to a Ni^{2+} column. Purified CCL21-mRFP was collected as flow-through (**C**, line 4). Western blots of the same samples using anti-His tag (**B′**,**C′**) or anti mRFP (**B″**,**C″**) antibodies are shown.

2.2. Purification and Functional Characterization of CCL19-mRFP and CCL21-mRFP

We transiently transfected HEK293 cells with either pHis$_6$-SUMO-CCL19-mRFP or pHis$_6$-SUMO-CCL21-mRFP and collected the supernatants after three and five days of culture, as described in Section 4. The secreted chemokine fusion proteins were purified in a single step over a Ni^{2+}-column. The His$_6$-SUMO tag was cleaved off the chemokine and removed by a subsequent affinity purification step using the same Ni^{2+}-column as before, yielding about 25-50 µg pure, native and mature human CCL19-mRFP or CCL21-mRFP per T175 cell culture flask (Figure 1B,C).

We tested the activity and functionality of the purified CCL19-mRFP and CCL21-mRFP by first assessing chemokine-mediated calcium mobilization. To this end, we used 300-19 pre-B cells stably expressing human CCR7 [19,29–31]. Stimulation of CCR7-expressing cells with CCL19-mRFP or CCL21-mRFP, as well as with CCL19 or CCL21, elicited transient elevations of $[Ca^{2+}]_i$ typical for ligand-induced chemokine receptor activation (Figure 2A). No chemokine-mediated calcium mobilization was observed in parental 300-19 cells that do not express CCR7 (not shown). Next we tested the fluorescently tagged chemokines for their ability to induce migration of human mature monocyte-derived DCs (MoDCs) using classical 2D-Transwell chemotaxis assays. Both fluorescently tagged chemokines recruited human mature MoDCs, although with a slightly reduced efficacy as compared to non-tagged versions of the chemokines (Figure 2B). Notably, at physiological concentrations of 50 nM, both CCL19-mRFP and CCL21-mRFP specifically attracted human mature MoDCs. In addition, stimulation of human MoDCs with 50 nM of fluorescently tagged and untagged chemokines resulted in the activation of ERK-1/2 (Figure 2C). Pre-treating human MoDCs with pertussis toxin (PTx) to inhibit G_i protein-coupling to CCR7 abolished chemokine-mediated ERK1/2 phosphorylation (Figure 2C). Consistent with ligand-biased CCR7 signaling [9,12,13], only CCL19 and CCL19-mRFP (at 50 nM each) efficiently recruited β-arrestin to CCR7 (Figure 2D).

These data clearly indicate that CCL19-mRFP and CCL21-mRFP are functional fluorescent chemokines with preserved biased signaling capabilities.

2.3. CCL19-mRFP Is Internalized by CCR7 and ACKR4, whereas CCL21-mRFP Is Preferentially Scavenged by ACKR4

In order to exploit our fluorescently tagged chemokines to study endocytosis, we performed time-lapse video microscopy using HEK293 cells as a model system that transiently express either CCR7-eGFP or ACKR4-eGFP. Applying CCL19-mRFP to the medium of transiently transfected HEK293 cells resulted in chemokine binding to cells expressing CCR7-eGFP, but not to untransfected cells. CCL19-mRFP subsequently, within seconds, was internalized together with the receptor and accumulated within a few minutes in vesicular structures where the chemokine and the receptor co-localized (Figure 3A).

ACKR4 is known to steadily cycle between the plasma membrane and endomembrane compartments even in the absence of ligands. Addition of CCL19-mRFP to HEK293 cells transiently transfected with ACKR4-eGFP was readily internalized exclusively by those cells expressing the atypical chemokine receptor (Figure 3B and Supplementary Video 1). Similar results were obtained in HeLa cells expressing high amounts of ACKR4-eGFP, which consequently scavenged CCL19-mRFP in an impeccably efficient manner (Supplementary Video 2).

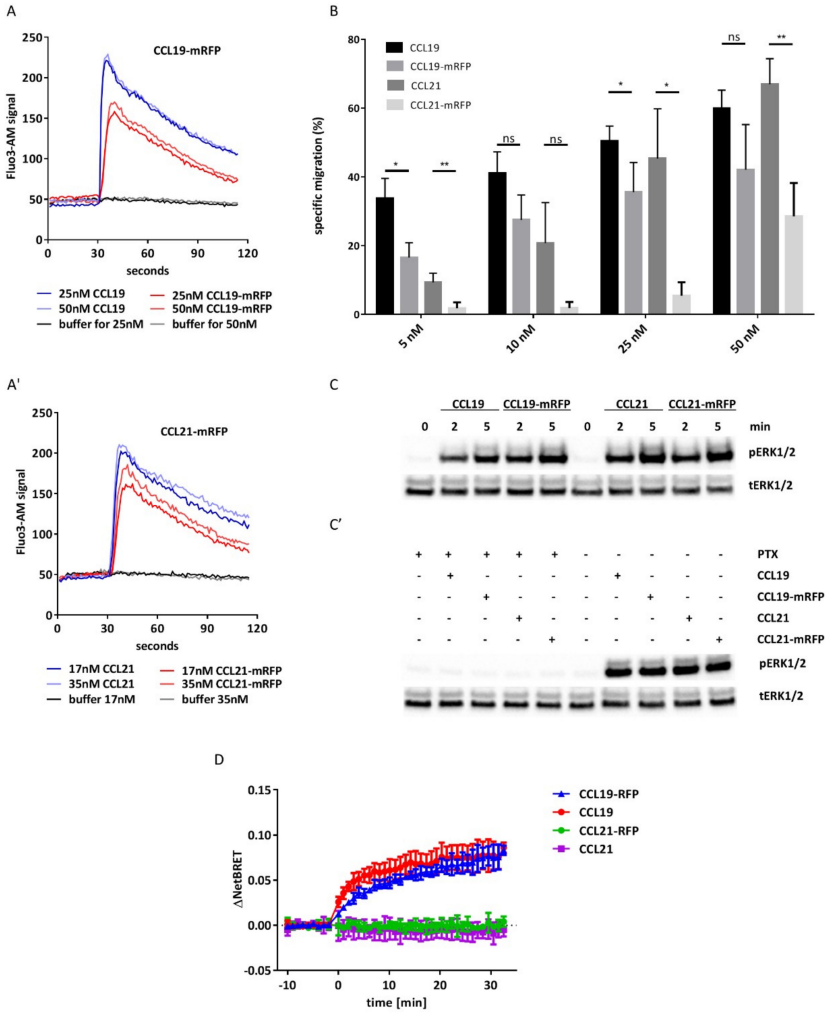

Figure 2. CCL19-mRFP and CCL21-mRFP are functional chemokines. (**A**) Real-time changes in $[Ca^{2+}]_i$ concentrations in CCR7 transfected 300-19 cells in response to graded concentrations of CCL19 and CCL19-mRFP (A) and CCL21 and CCL21-mRFP (**A′**). A representative experiment out of three is shown. (**B**) Transwell chemotaxis of human mature MoDCs in response to chemokines. Human mature MoDCs were allowed to migrate in response to gradient concentrations of chemokines for 180 min. Migrated cells were counted and percentages of specifically migrated cells relative to the input were calculated. Mean values and SEM of duplicates derived from three independent experiments are shown. (**C**) Stimulation of human mature MoDCs with CCL19-mRFP and CCL21-mRFP results in the phosphorylation of ERK1/2. Human mature MoDCs were stimulated with 50 nM of CCL19, CCL19-mRFP or CCL21-mRFP for the indicated time points. ns: statistically not significant; * $p < 0.05$; ** $p < 0.005$. (**C′**) MoDCs were pre-treated with 200 ng/mL PTx for 3 h and subsequently stimulated with 50 nM of chemokines for 5 min. Phosphorylation of ERK1/2 (pERK1/2) was determined by Western blotting. Re-probing the blots for total ERK1/2 (tERK1/2) served as a control for equal protein loading. (**D**) Chemokine-induced β-arrestin recruitment to CCR7. β-arrestin recruitment was determined by BRET upon stimulation with 50 nM of indicated chemokine. Mean values ±S.D. of 2 (CCL21, CCL21-mRFP) or 3 (CCL19, CCL19-mRFP) independent experiments with technical duplicates are shown.

Addition of CCL21-mRFP to HEK293 cells expressing CCR7-eGFP bound to surface CCR7, but was barely internalized (Figure 4A), as expected and previously described for untagged CCL21 [12]. In contrast, CCL21-mRFP was rapidly internalized by HEK293 cells expressing ACKR4-eGFP (Figure 4B). These data illustrate that CCL19-mRFP and CCL21-mRFP are versatile tools to spatiotemporally study chemokine internalization and receptor trafficking.

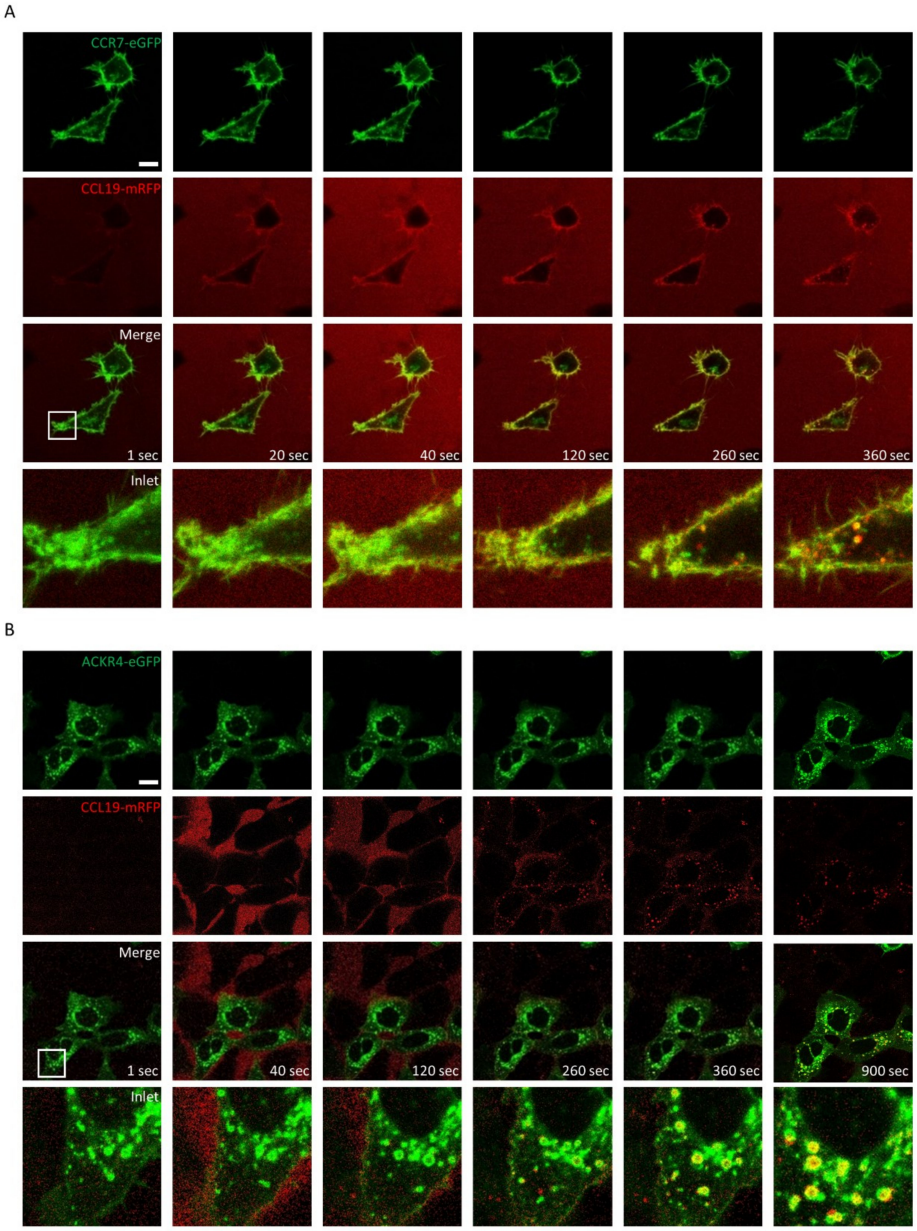

Figure 3. Internalization of CCL19-mRFP by CCR7-eGFP and ACKR4-eGFP. 100 nM of CCL19-mRFP was added to HEK293 cells transiently transfected with CCR7-eGFP (**A**) or ACKR4-eGFP (**B**) and CCL19-mRFP internalization was recorded by time-lapse video microscopy. Series of images captured at indicated time points are illustrated (scale bar: 10 μm). Frames: magnification of images.

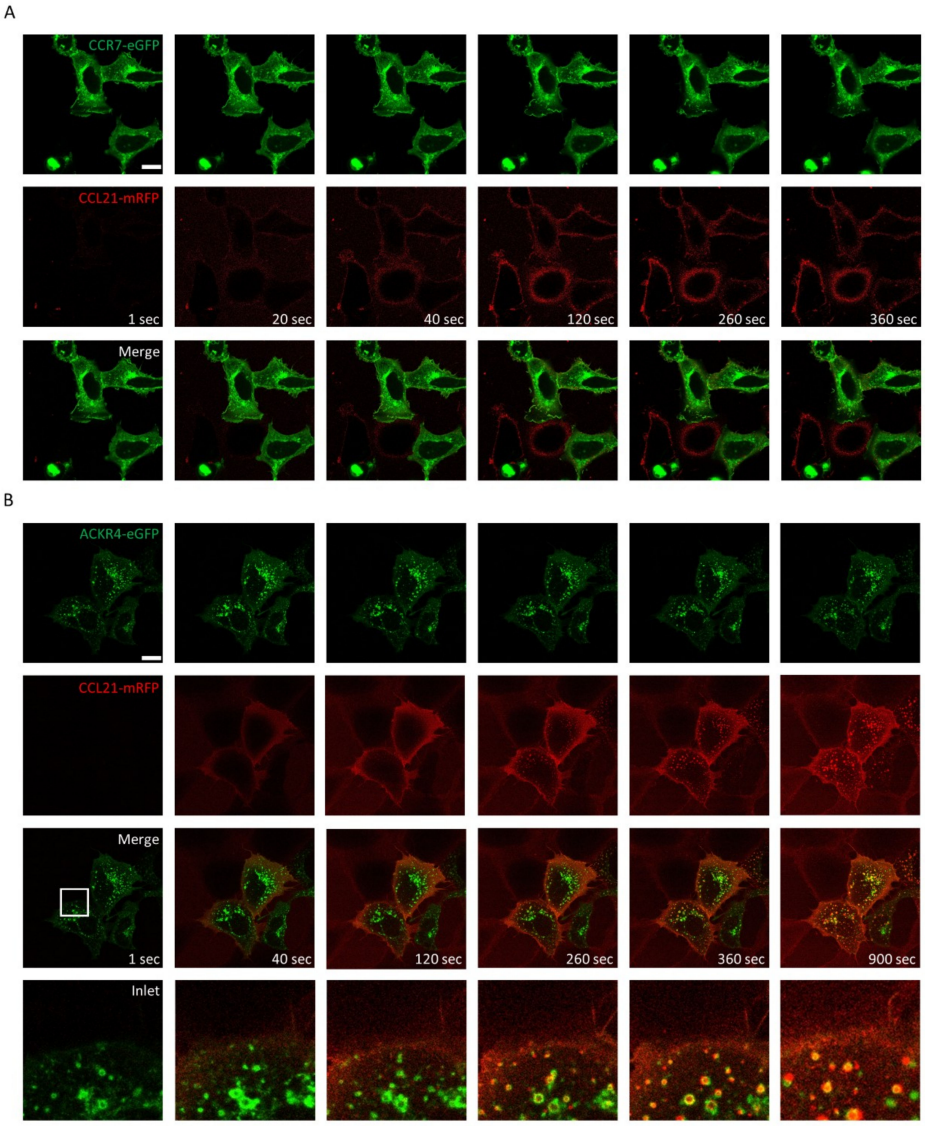

Figure 4. Monitoring CCL21-mRFP binding to CCR7-eGFP and internalization by ACKR4-eGFP. 100nM of CCL21-mRFP was added to HEK293 cells transiently transfected with CCR7-eGFP (**A**) or ACKR4-eGFP (**B**) and CCL21-mRFP binding and receptor trafficking was recorded by time-lapse video microscopy. Series of images captured at indicated time points are depicted (scale bar: 10 μm). White frames: magnification of images.

2.4. Monitoring DC Migration in 3D Collagen along Gradients of CCL19-mRFP or CCL21-mRFP by Time-Lapse Video Microscopy

Next, we addressed whether we can monitor DC migration through a 3D collagen matrix along gradients of fluorescently tagged chemokines by time-lapse video microscopy. To achieve this, we used commercially available μ-slides chemotaxis chambers (from Ibidi) where mouse mature bone-marrow-derived DCs are imbedded into a 3D collagen matrix within a narrow observation area connected to two larger reservoirs to one of which we applied 100 nM of chemokine to establish a gradient that is stable for at least 48 h based on the manufacturer's information. Migrating DCs were

recorded by time-lapse video microscopy, tracked and analyzed using the 'chemotaxis and migration tool' provided by the manufacturer. Figure 5A depicts directional migration patterns of DCs as spider and rose diagrams. DC migration through 3D collagen along gradients of CCL19, CCL19-mRFP, CCL21, or CCL21-mRFP was comparable as equal velocity was recorded (Figure 5B). Simultaneously, gradients of the fluorescently tagged chemokines were visualized and the intensity of the mRFP signal was enumerated (Figure 5C). These data suggest that although the same concentration of chemokine was applied in the reservoir, CCL21-mRFP formed steeper gradients than CCL19-mRFP, at least near the reservoir, which can be explained by the stickiness of CCL21 and its local immobilization.

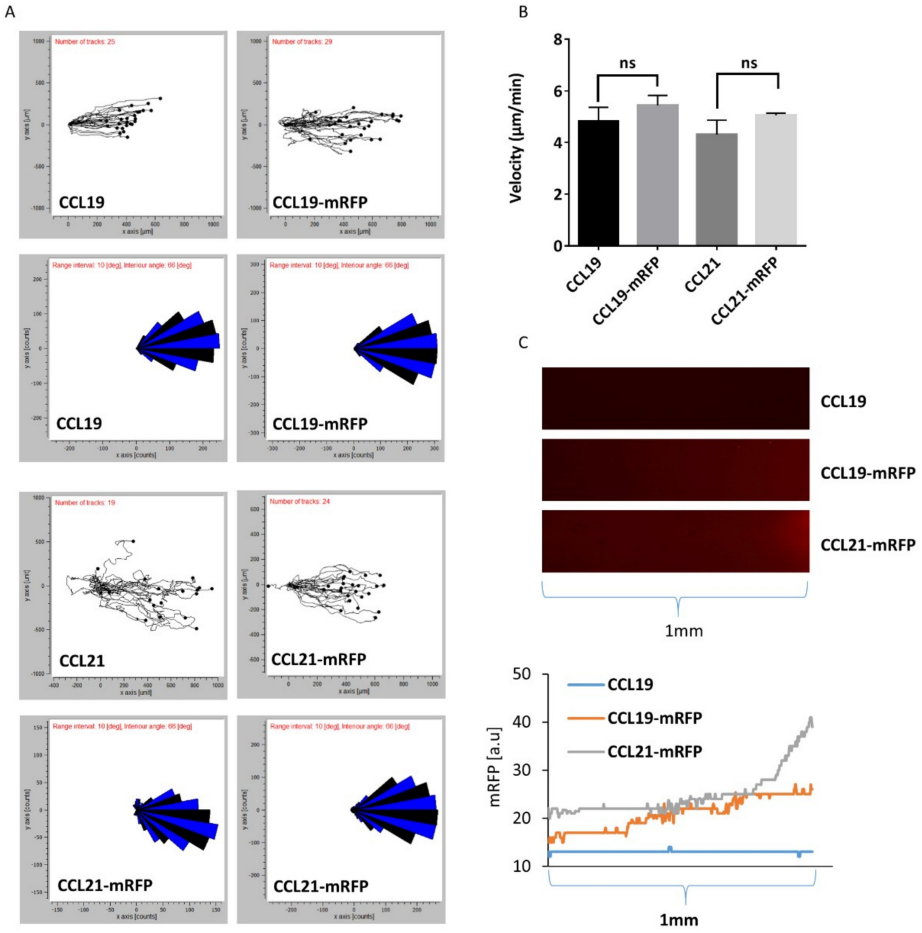

Figure 5. DC migration in 3D collagen along gradients of CCL19-mRFP and CCL21-mRFP. Mouse mature bone-marrow-derived DCs were imbedded in 3D collagen in µ-slide chemotaxis chambers. CCL19, CCL19-mRFP, CCL21, or CCL21-mRFP at 100 nM concentration was applied to the right reservoir. (**A**) Combined rose diagrams and tracks of single cells migrating towards higher concentrations of chemokines from a representative experiment are shown. (**B**) Mean velocity of individually migrating cells derived from three independent experiments were quantified. ns: statistically not significant. (**C**) Gradients of CCL19-mRFP and CCL21-mRFP from (**A**) were visualized by fluorescence microscopy and enumerated by determining the fluorescent intensity derived from the corresponding mRFP signal.

2.5. Monitoring CCR7-Dependent Cancer Cell Migration in 3D Collagen along a CCL19-mRFP Gradient

As cancer cells can utilize CCR7 to metastasize in lymph nodes [11], we used the human non-small cell lung carcinoma cell line H1299 expressing CCR7-eGFP or not as model to study cancer cell migration. H1299 cells imbedded in 3D collagen migrated only along CCL19 gradients if they express CCR7 (Figure 6A and Supplementary Videos 3 and 4). Preventing G_i-protein signaling by incubating H1299 cells expressing CCR7-eGFP with PTx abolished directional migration towards CCL19 (Figure 6B). Finally, we exposed H1299 cells expressing CCR7-eGFP imbedded in 3D collagen to a gradient of CCL19-mRFP. CCR7-eGFP expressing H1299 cells persistently migrated along the CCL19-mRFP gradient (Figure 6C and Supplementary Video 5).

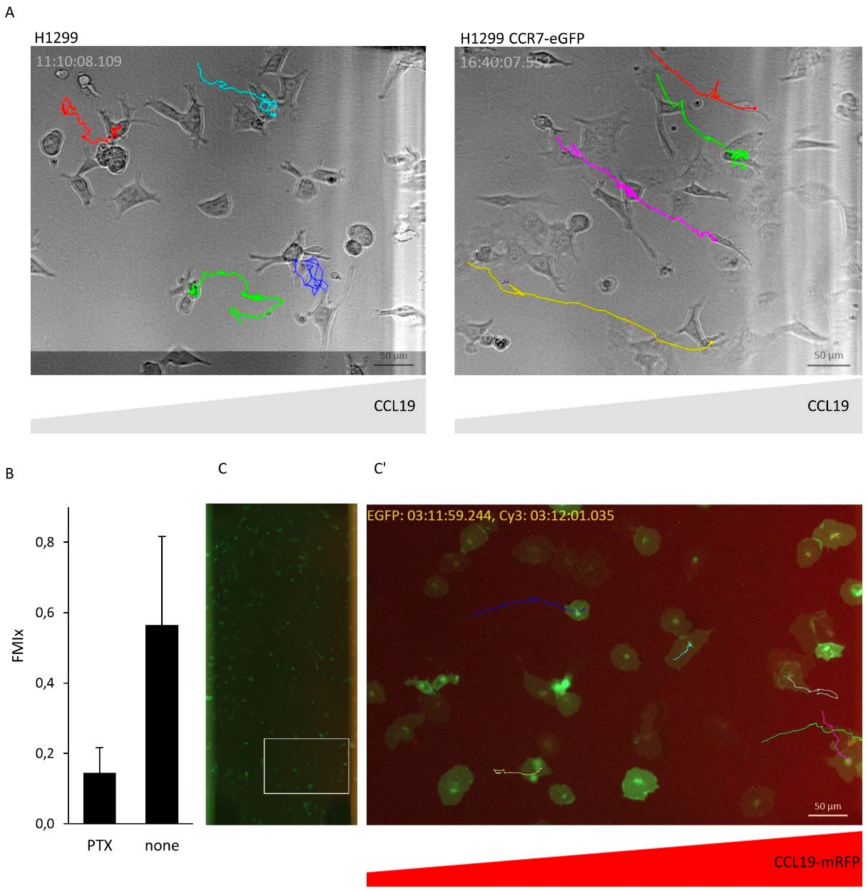

Figure 6. CCR7-dependet migration of H1299 cancer cells along CCL19 and CCL19-mRFP gradients. (**A**) H1299 wild-type cells and H1299 cells expressing CCR7-eGFP were imbedded in 3D collagen in µ-slide chemotaxis chambers. CCL19 (100 nM) was added to the right reservoir and cell migration monitored by time-lapse video microscopy. Colored tracks of individual cells are depicted. (**B**) H1299 cells expressing CCR7-eGFP were untreated or treated with PTx to inhibit G_i-dependent chemokine signaling and subjected to cell migration assays as described in (**A**). Mean forward migration index (FMIx) and S.D. of individually migrating cells derived from two independent experiments are indicated. (**C**) H1299 cells expressing CCR7-eGFP were imbedded in 3D collagen in µ-slide chemotaxis chambers, CCL19-mRFP was added to the right reservoir. Cancer cell migration was monitored by time-lapse video microscopy by depicting tracks of individual cells. CCL19-mRFP gradient was visualized simultaneously by its fluorescence (scale bar: 10 µm).

Taken together, we demonstrated in this study that CCL19 and CCL21 can be produced as functional fluorescently tagged proteins in mammalian cells. Purified CCL19-mRFP and CCL21-mRFP therefore represent versatile tools to study chemokine and receptor functions, such as receptor trafficking and chemokine scavenging, in real-time using time-lapse video microscopy. Moreover, the fluorescent tags fused to CCL19 and CCL21 permits visualization of chemokine gradients in vitro without perturbing the chemokine functions, while cells are sensing them and migrate along the gradients.

3. Discussion

In the present study, we demonstrated that the homing chemokines CCL19 and CCL21 can be functionally expressed in and secreted from mammalian cells as fluorescently tagged fusion proteins. We provided experimental evidence that CCL19-mRFP and CCL21-mRFP induce typical chemokine receptor signaling pathways such as mobilization of calcium or activation of the ERK-1/2 pathway in cell lines, as well as in primary cells. Moreover, the fluorescently tagged chemokines were perfectly suitable to monitor chemokine internalization by CCR7 and ACKR4, as well as receptor trafficking in real-time as assessed by time-lapse video microscopy. Furthermore, measuring the fluorescent intensity signal derived from the mRFP directly fused to the chemokine allowed us to visualize and quantify the CCL19 and CCL21 gradients in real time while cells were exploring the presented guidance cue. We observed that under the same experimental condition the CCL21-mRFP gradient is steeper than the CCL19-mRFP one, at least close to the reservoir releasing the chemokine. Such exponential CCL21 gradients over about 90 μm were also found in vivo, namely in the mouse skin where the CCL21 gradient was establish between the perilymphatic interstitium towards the chemokine-producing lymphatic endothelial cells forming lymph vessels [15]. Notably CCL19-mRFP gradients seem to be rather linear reflecting the soluble biochemical properties of CCL19. This observation is in line with the diffusion based 10 kDa-FITC-dextran gradient [22] or the rhodamine and fluorescein based gradients [21] that were used to predict soluble CCL19 gradients in previous studies. Our data suggest that estimating chemokine gradients in in vitro assays using FITC-dextran or other soluble molecules and/or dyes may only give limited and non-precise information on the nature of the real chemokine gradient. The use of fluorescently tagged chemokines, such as CCL19-mRFP or CCL21-mRFP, will now allow accurate measurements and quantification of chemokine gradients under various experimental conditions.

Furthermore, these fluorescently tagged chemokine can be used to study the endocytosis routes taken by classical and atypical chemokine receptors. CCL19 is known to be internalized together with CCR7 via clathrin-coated pits [12]. It is also known that internalized CCR7 recycle back to the plasma membrane, whereas co-internalized CCL19 becomes sorted to lysosomal degradation [12]. How and where the chemokine and its receptor are separated to be differentially sorted remains to be investigated. Remarkable, CCL21 is barely internalized by CCR7. Both ligands have similar binding affinities to CCR7 [31], but induce common as well as biased signaling [9]. The molecular details how this works are still largely unknown. The fluorescently tagged chemokine may permit to address the unanswered fundamental questions.

Interestingly, migrating monocytes were shown to scavenge CCL2-mCherry released from a microinjection needle [32]. Whether CCL19 is also internalized by migrating cells remains to be addressed. The fluorescently tagged CCL19 and CCL21 will allow future investigation to address the question whether such potential chemokine scavenging during migration modulates gradients and whether scavenging contributes to directional migration. These new tools will in addition permit to investigate potential differences in amoeboid single cell modes of migration used by leukocytes and mesenchymal-type collective cell migration of cancer cells. In vivo, cells are exposed to different chemokines and it remains largely unknown how cells navigate in the presence of different guidance cues. In vitro systems, including microfluidic devices, in combination with differentially fluorescently labeled chemokines will permit to address such fundamental cell biological concepts in future.

4. Materials and Methods

4.1. Reagents and Antibodies

The following reagents were purchased as indicated: recombinant human CCL19 and CCL21 (PeproTech, Rocky Hill, CT, USA), monoclonal anti-polyhistidine antibodies conjugated to HRP (Sigma, A7058-1VL, St. Louis, MO, USA), rabbit-anti-mRFP (PM005, MBL International, Woburn, MA, USA), anti-phospho-p44/42 MAPK (ERK1/2; pThr202/Tyr204; Cell Signaling Technology #4370, Danvers, MA, USA), anti-p44/42 MAPK (ERK1/2) Antibody (Cell Signaling Technology #9102), peroxidase conjugated AffiniPure goat-anti-rabbit IgG (Jackson Immunoreseach, West Grove, PA, USA), Fluo-3 AM (Molecular Probes, Eugene, OR, USA), Pertussis Toxin (Tocris, Bristol, UK).

4.2. Cloning, Expression, and Purification of CCL19-mRFP and CCL21-mRFP

The signal peptide of IgGκ (coding for the amino acids: METDTLLLWVLLLWVPGSTG) was ligated to the N terminus of the cDNA encoding His_6-SUMO3 followed by the human mature codon optimized cDNA of CCL19 (amino acids 22-98) and sub-cloned in pcDNA3-mRFP with a $(GGGGS)_3$ flexible linker revealing $pHis_6$-SUMO-CCL19-mRFP plasmid. To clone $pHis_6$-SUMO-CCL21-mRFP, a second BamHI restriction site was introduced by site directed mutagenesis into $pHis_6$-SUMO-CCL19-mRFP using the primers 5′-GCAGATCGGCGGATCCACCAACGACGCCGA GGATTG and 5′-CAATCCTCGGCGTCGTTGGTGGATCCGCCGATCTGC. The cDNA fragment of CCL19 was replaced by a PCR fragment coding for human mature CCL21 (amino acids 24–134) using the primers 5′-GACCCAAGGATCCGATGGAGGGGCTCAGGACTG and 5′-GTCGACGGATCCGAA TTCTGGCCCTTTAGGGGTCTG.

HEK293 cells were grown in DMEM medium supplemented with 10% FCS (Lonza) and 1% penicillin/streptomycin (Biowest, San Marcos, TX, USA) in T175 flasks. Cells were transfected with either $pHis_6$-SUMO-CCL19-mRFP or $pHis_6$-SUMO-CCL21-mRFP using FuGENE® 6 transfection reagent (Promega, Madison, WI, USA). The next day medium was changed to DMEM supplemented with only 5% FCS and cultured for another three days. The supernatants containing the secreted chemokines were collected and cells were grown for an additional two days in fresh medium before harvesting it again. Pooled supernatants were centrifuged to remove cell debris and supplemented with 20 mM imidazole before applying on 1 mL Ni^{2+} agarose matrix packed in a gravity column previously equilibrated with binding buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, and 20 mM imidazole). Ni^{2+} columns were washed with 10 column volumes of washing buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, and 30 mM imidazole) and the total captured protein was collected by two sequential elutions with an elution buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, and 250 mM imidazole). The column was re-equilibrated with washing buffer for the second purification step. The eluted His_6-SUMO-chemokine-mRFP proteins were applied on Vivaspin 20 cross-flow spin columns (Sartorius, Göttingen, Germany) with a cutoff of 10 kDa for chemokine concentration and to exchange for the washing buffer. His_6-SUMO-chemokine-mRFP proteins were digested using SUMOstar™ protease (LifeSensors, Malvern, PA, USA) for up to 48 h at 8 °C. The digested material was applied onto the re-equilibrated Ni^{2+} column to retain the His_6-SUMO tag and the impurities of the first purification step. The flow-through containing the pure CCL19-mRFP or CCL21-mRFP, respectively, was collected. The chemokine-mRFP containing fractions were concentrated and the buffer was exchanged to PBS on Vivaspin 20 cross-flow spin columns. The last flow-through fraction was collected as negative control buffer solution.

4.3. Cloning and Expression of Chemokine Receptors

Human ACKR4 cDNA was amplified by PCR using the primers 5′-GAACAAGCTTCATTACGG CCGCTTTGGA and 5′-CTACCTCGAGCCCCAATAGAGAAGGTAGAAGT using a synthetic cDNA (ID: 11AA4G6P; Life Technologies) as template. The PCR product was inserted into the HindIII and XhoI restriction sites of pcDNA3. Site-directed mutagenesis was performed to re-introduce a

methionine at position 1 using the primers 5′-CCCAAGCTTCATTACGATGGCTTTGGAACAAAATC and 5′-GATTTTGTTCCAAAGCCATCGTAATGAAGCTTGGGTC. Next, ACKR4 was amplified using 5′-GGAGACCCAAGCTTCATTACGATGGC (Primer-A) and 5′-GAGCTCGAGTCCACCAATAGAGA AGGTAGAAGTAGGTTCAGTTGGACC. In addition, eGFP was amplified from CCR7-eGFP [12] and a GGLES$(GGGGS)_3$ linker introduced using the primers 5′-GGACTCGAGAGCGGAGGTGGCGGT TCTGGTGGTGGCGGTTCCGGCGGTGGCGGTAGCGTGAGCAAGGGCGAGGAGCTG and 5′-GCC CTCTAGACTACTTGTACAGCTCGTCC (Primer-B). Both PCR products were digested with XhoI, ligated and PCR amplified with Primer-A and Primer-B, digested with HindIII and XbaI, and inserted into pcDNA3.

CCR7-eGPF and ACKR4-eGFP were transiently expressed in HEK293, HeLa, or stably expressed in H1299 cells. HEK293 and H1299 cells were grown in a DMEM medium supplemented with 10% FCS and 1% penicillin/streptomycin, whereas HeLa cells were cultured in RPMI1640 medium with 10% FCS and 1% penicillin/streptomycin.

4.4. Preparation of Monocyte-Derived and Bone-Marrow-Derived DCs

Human monocyte-derived DCs were generated and matured with a cocktail of inflammatory cytokines for 48 h as described previously [6]. Mouse-bone-marrow-derived DCs were generated and matured with LPS for 24 h as described before [33].

4.5. Chemotaxis Assays

Two-dimensional chemotaxis assays were performed in duplicate in 24-well Transwell™ chemotaxis chambers (Costar® Permeable support, 3421, Washington, DC, USA) with 5 µm pore-sized polycarbonate membranes. Mature MoDC from different donors (10^5 per well) in medium were allowed to migrate to the lower well containing graded concentrations of chemokines for 180 min at 37 °C in a 5% CO_2 atmosphere. Migrated cells were harvested and enumerated by flow cytometry (LSRII, BD Biosciences, San Jose, CA, USA). The number of cells that migrated spontaneously to the lower compartment in the absence of chemokines was subtracted and the percentage of migrated cells was calculated relative to the input of cells.

Three-dimensional migration through collagen was performed in µ-slide chemotaxis chambers purchased from Ibidi (Martinsried, Germany) according to the manufacturer's protocol. Briefly, mature bone-marrow-derived DCs or H1299 cells were collected and re-suspended in RPMI1640 or DMEM supplemented with 10% FCS and 1% penicillin/streptomycin, respectively, to 10^7 cells/mL. Twenty microliters 10× DMEM, 10 µL 7.5% $NaHCO_3$, 2 µL 10N NaOH, and 150 µL PureCol collagen I (Advanced Biomatrix) were premixed, added to 90 µL cell suspension, and mixed carefully. The mixture was applied to µ-slide chemotaxis chambers and mounted as described in the manufacturer's instructions. Chemokines were applied to the right reservoir, whereas control buffer from the chemokine purification was added to the left reservoir. Cell migration was monitored by time-lapse video microscopy on a Zeiss Axiovert 200M (Oberkochen, Germany) equipped with a Tokai Hit INU (Shizuoka Japan) incubation system for at least 3 h for DCs or overnight for H1299 cells. Cell migration was analyzed and quantified using the 'chemotaxis and migration tool' software provided by Ibidi. At least 24 cells per condition for every experiment were analyzed. The fluorescent mRFP gradient was stable and recorded before and after the migration assay.

4.6. Calcium Flux

Changes in concentrations of intracellular free calcium was determined in 300-19 pre-B cells expressing CCR7 loaded with Fluo-3-AM and recorded as previously described [12]. As a negative control, a solution from the last chemokine purification step was used.

4.7. β-Arrestin Recruitment Determined by Bioluminescence Resonance Energy Transfer (BRET)

BRET was determined essentially as described previously [34]. Briefly, HEK293 cells were transiently transfected with CCR7-EYFP or -HA and β-arrestin2-hRLuc at a ratio of 3:1 and were grown for 24 h. Cells were washed with PBS and re-suspended in PBS containing 5 mM glucose (PBS-G). The cell suspension was inoculated in a white 96F bottom half-well plate (Perkin Elmer, Waltham, MA, USA) in the presence of 10 µM coelenterazine H (Cayman Chemical, Ann Arbor, MI, USA) at 37 °C. Fluorescence and luminescence were recorded on a Spark 10M multimode reader (Tecan, Männedorf, Switzerland). Cells were stimulated after 9 min of recording with 50 nM of chemokines. EYFP fluorescence (505–590 nm, 350 ms) and luciferase bioluminescence (430–485 nm, 350 ms) were recorded over several subsequent cycles. BRET was calculated by dividing the EYFP emission by the luciferase emission, NetBRET by subtracting the BRET of EYFP-transfected wells from the BRET of HA-transfected wells, and ΔNetBRET by subtracting the NetBRET signal from the basal NetBRET.

4.8. Confocal Live-Cell Imaging

Live-cell confocal imaging was performed on a TCS SP5 II microscope (Leica, Wetzlar, Germany) equipped with a temperature-controlled chamber. The videos were routinely recorded with a 63× objective. Live cell imaging was conducted in 35 mm Mattek No. 1.5 dishes in a medium supplemented with 20 mM HEPES buffer pH 7.5.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/12/3876/s1.

Author Contributions: Conceptualization, V.P. and D.F.L.; designed and performed experiments, V.P., C.M., G.P.B.S. and I.K.; analyzed data, V.P., C.M., G.P.B.S., I.K. and D.F.L; writing—original draft preparation, V.P. and D.F.L.; writing—review and editing, C.M., G.P.B.S. and I.K.; supervision, D.F.L.; project administration, D.F.L.; funding acquisition, D.F.L.

Funding: This research was funded by the Swiss National Science Foundation through project grant 31003A_169936 and sinergia grant CRSII3_160719, the Thurgauische Stiftung für Wissenschaft und Forschung, and the State Secretariat for Education, Research and Innovation.

Acknowledgments: The authors thank the Legler lab for helpful discussions and Michel Bouvier for kindly providing the plasmid encoding β-arrestin2-hRLuc.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

ACKR4	Atypical chemokine receptor 4
CCL19	CC chemokine ligand 19
CCL21	CC chemokine ligand 21
CCR7	CC chemokine receptor 7
DCs	Dendritic cells
ERK	Extracellular signaling regulated kinase
GAGs	Glycosaminoglycans
GPCR	G-protein coupled receptors
MoDCs	Monocyte-derived DCs
mRFP	Monomeric red fluorescent protein
PTx	Pertussis toxin

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frontiers in Immunology

ORIGINAL RESEARCH
published: 22 April 2020
doi: 10.3389/fimmu.2020.00720

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

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Specialty section:
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

Received: *31 January 2020*
Accepted: *30 March 2020*
Published: *22 April 2020*

Citation:
Matti C, Salnikov A, Artinger M, D'Agostino G, Kindinger I, Uguccioni M, Thelen M and Legler DF (2020) ACKR4 Recruits GRK3 Prior to β-Arrestins but Can Scavenge Chemokines in the Absence of β-Arrestins. Front. Immunol. 11:720. doi: 10.3389/fimmu.2020.00720

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

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Bioinformatics

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

Generation of Expression Plasmids

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

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

TABLE 1 | General plasmids and primers.

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

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

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

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

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

TABLE 2 | GRK related plasmids and primers.

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

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

TABLE 3 | Phosphorylation site mutations in ACKR4-EYFP.

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

Chemokine Production

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

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

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

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

TABLE 5 | Templates for Gα and Nluc amplification.

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

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

Cell Culture and Transfection

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

Chemokine Mediated Erk1/2, Akt, and Src Activation

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

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

BRET Measurements

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

Chemokine Uptake Assay

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

ACKR4 Receptor Staining and Chemokine Binding

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

Confocal Fluorescence Microscopy

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

Data Analysis

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

RESULTS

ACKR4 Does Not Elicit Canonical Chemokine-Mediated Signal Transduction Pathways

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

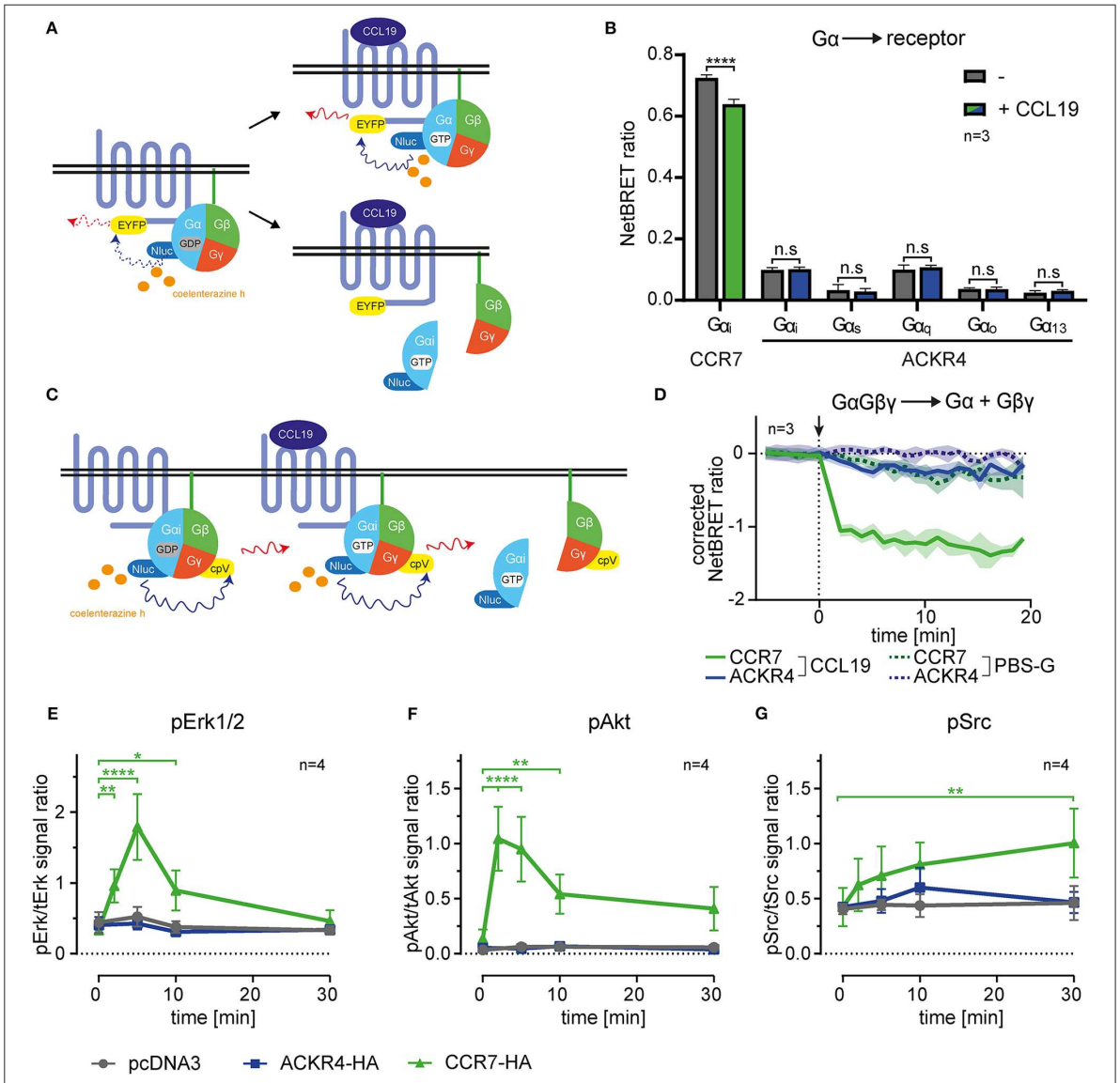

FIGURE 1 | ACKR4 does not interact with or activate G-proteins. **(A)** Schematic representation of BRET-based G-protein interaction, recruitment and dissociation assays. **(B)** HeLa cells were transiently transfected with either human CCR7-EYFP or human ACKR4-EYFP conjointly with Nluc fused to either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

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

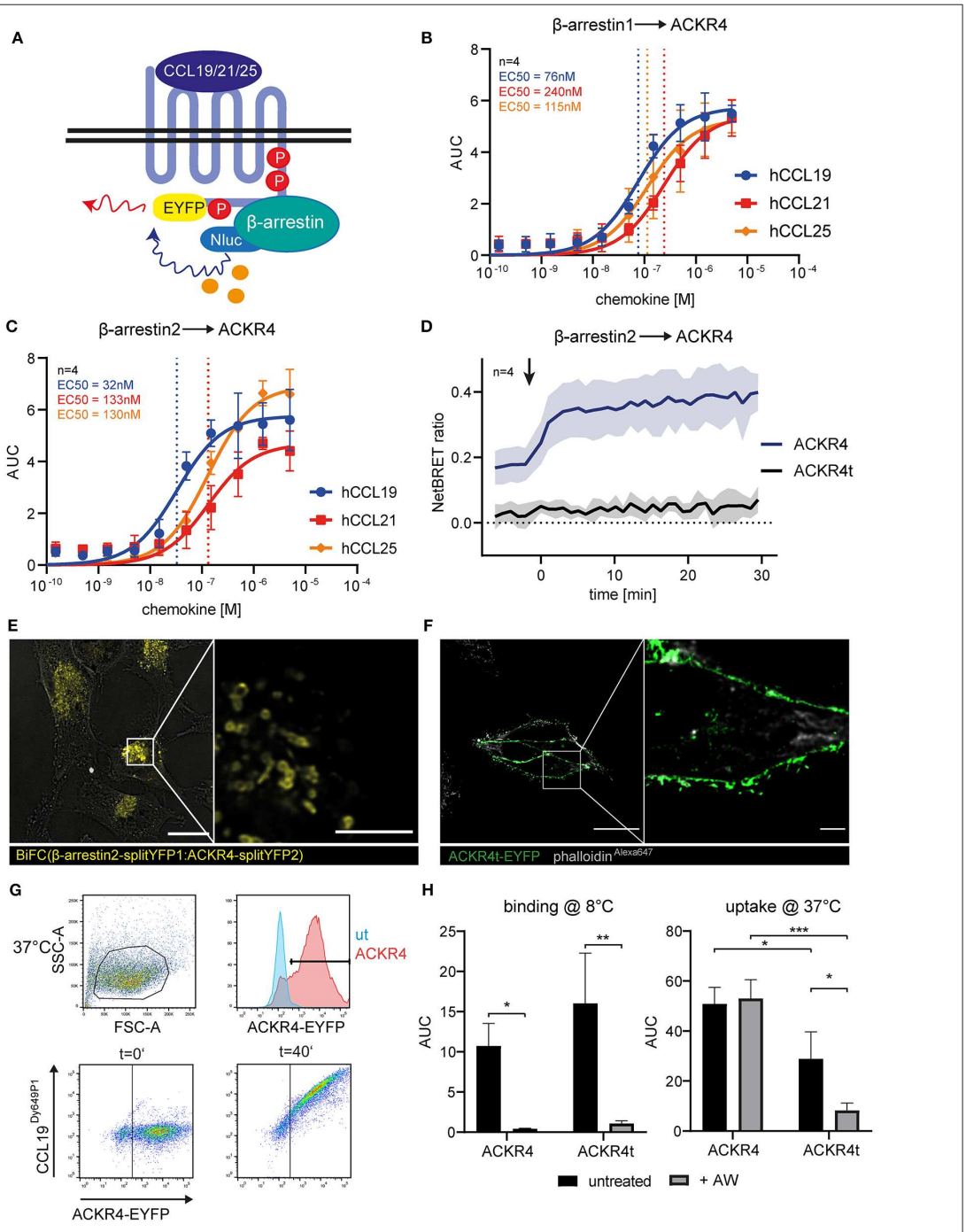

FIGURE 2 | The C-terminus of ACKR4 is critical for β-arrestin recruitment and chemokine uptake. **(A)** Schematic representation of BRET-based β-arrestin recruitment assay. HeLa cells co-transfected with ACKR4-EYFP and β-arrestin1-Nluc **(B)** or β-arresin2-Nluc **(C)** were stimulated with graded concentrations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

(Continued)

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

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

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

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

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

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

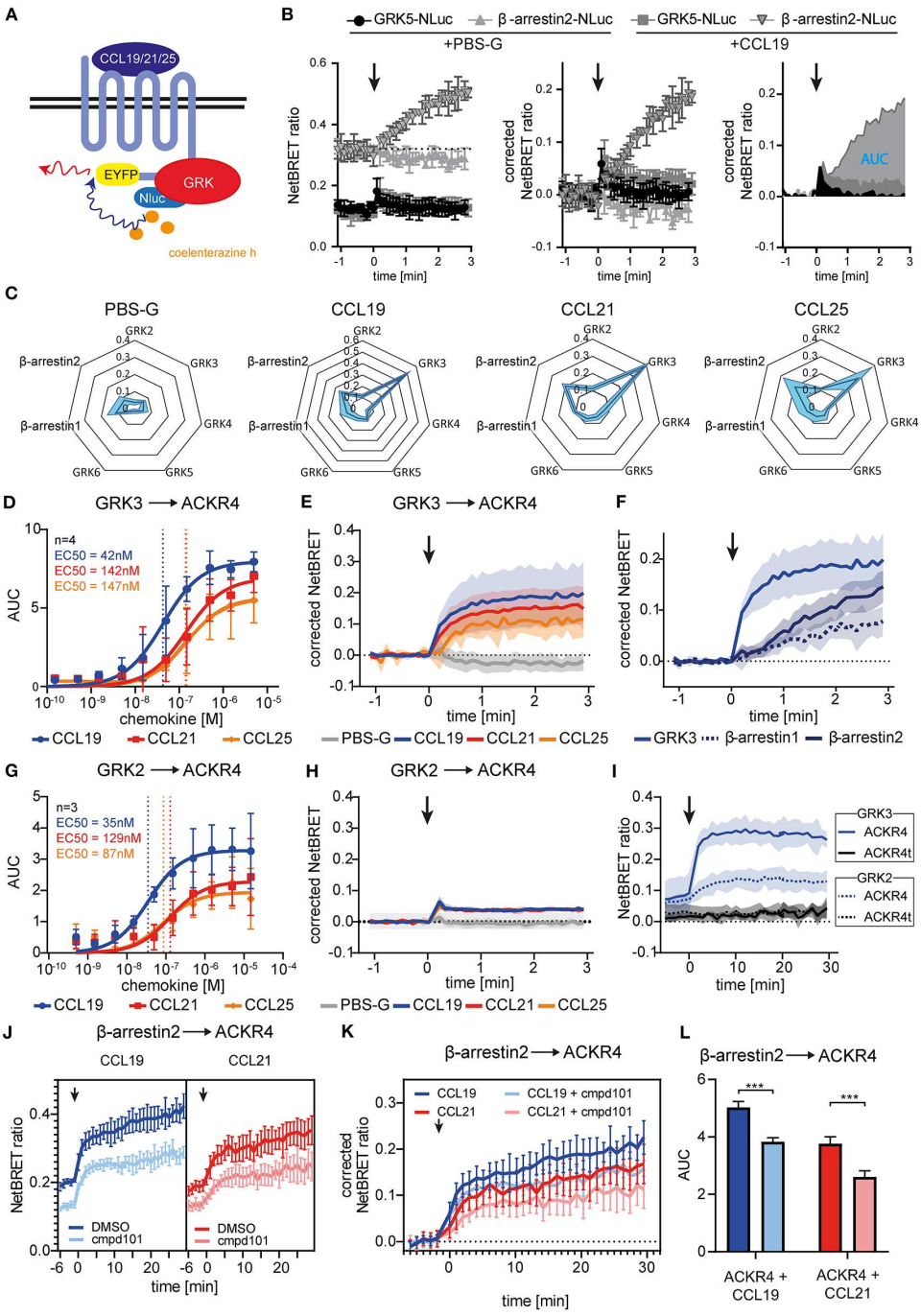

FIGURE 3 | Chemokine stimulation selectively recruits GRK3 and GRK2 to ACKR4. **(A)** Scheme of chemokine-mediated GRK recruitment determined by BRET. **(B)** HeLa cells were co-transfected with ACKR4-EYFP and GRK5-Nluc or β-arrestin2-Nluc and stimulated with CCL19. Chemokine-mediated GRK5 or β-arresin2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

(Continued)

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

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

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

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

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

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

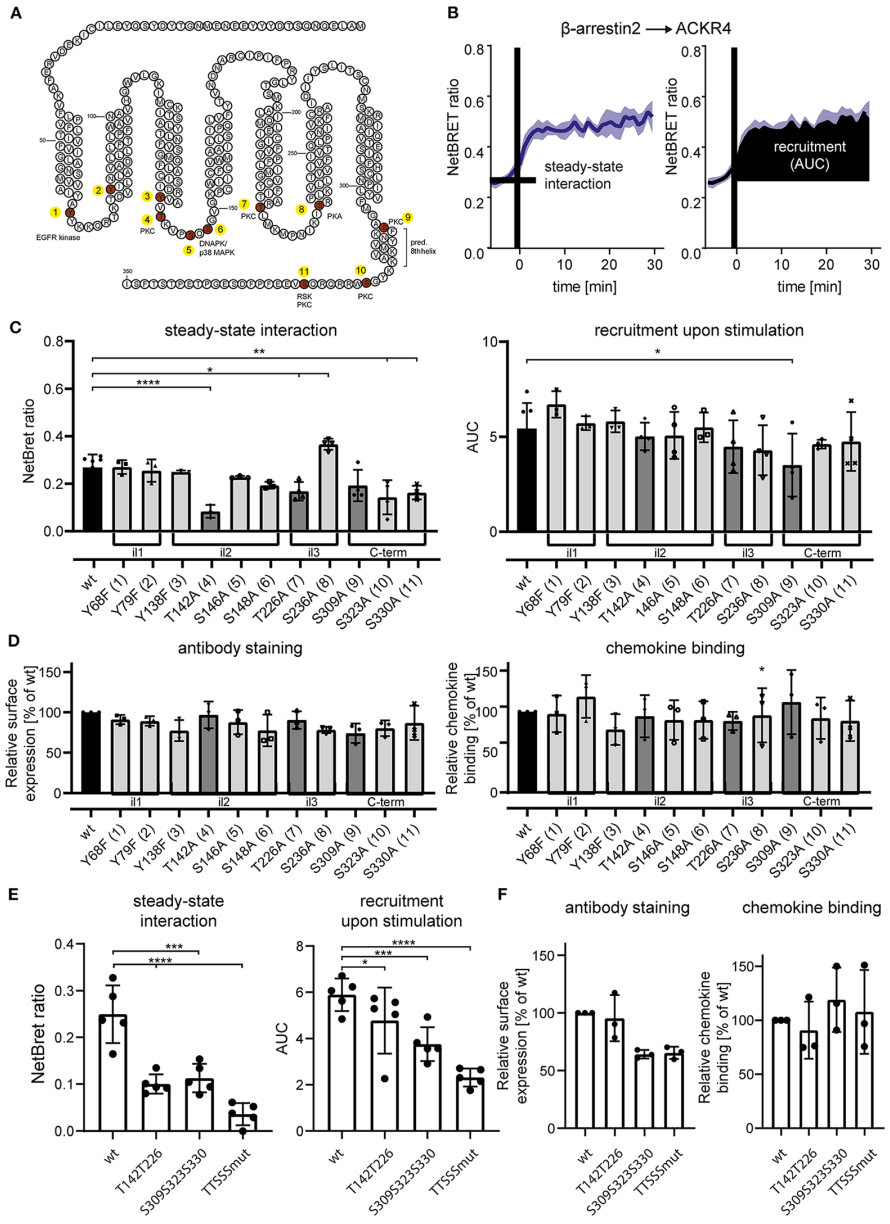

FIGURE 4 | Site-specific mutation analysis of ACKR4 for putative kinase interaction sites. **(A)** Schematic representation of ACKR4. *in silico* predicted phosphorylation sites are highlighted in red and putative kinase for site-specific tyrosine or serine/threonine phosphorylation are depicted. Numbers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

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

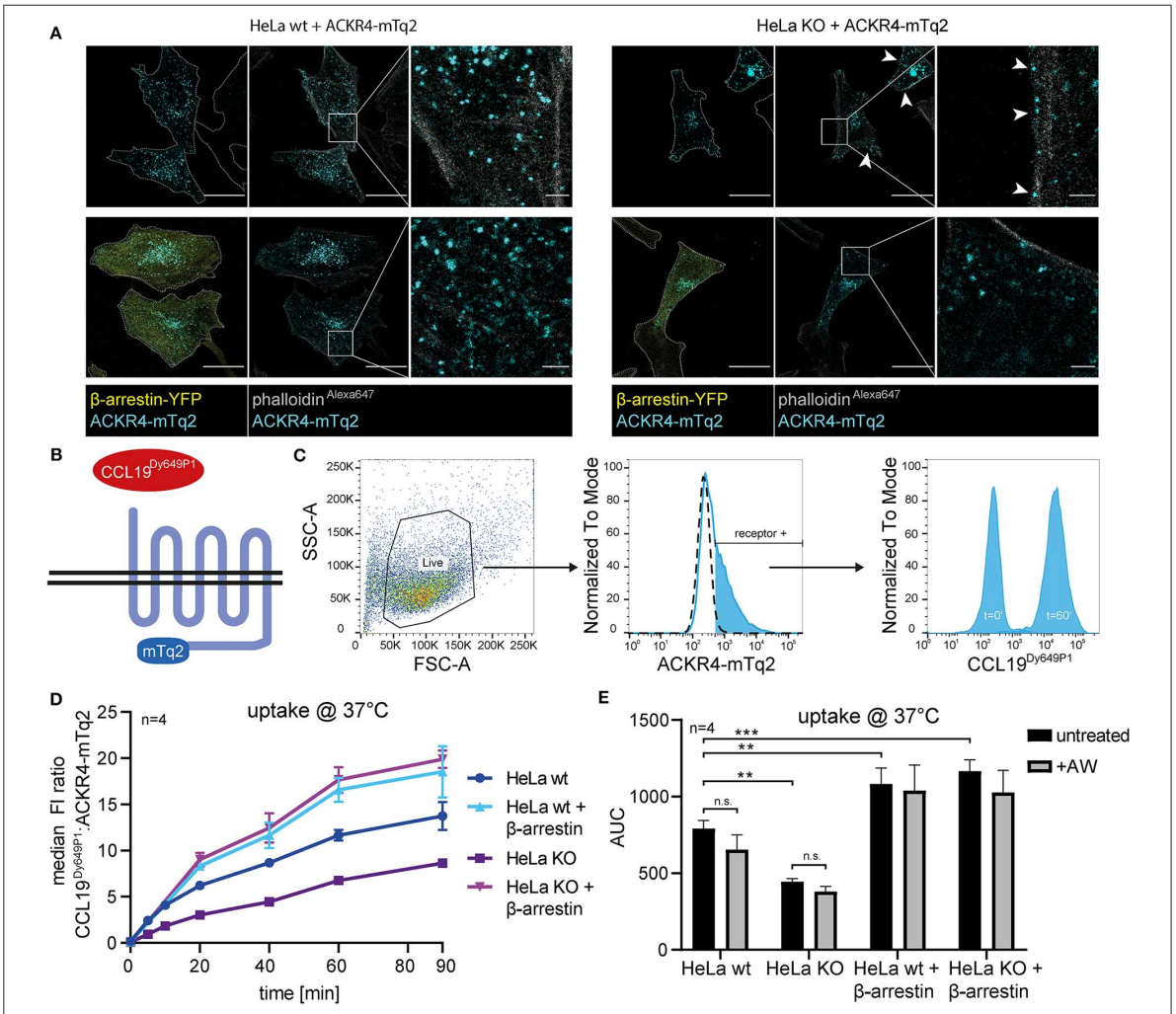

FIGURE 5 | β-arrestins contribute to, but are dispensable for CCL19 uptake. **(A)** Wild-type (wt) HeLa cells or β-arrestin1 and β-arrestin2 double gene targeted HeLa cells (KO) were co-transfected with ACKR4-mTq2 and β-arrestin2-YFP or empty vector, fixed, stained with phalloidin^{Alexa647} and analyzed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

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

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

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

DISCUSSION

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

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

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

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

This work was supported by the Swiss National Science Foundation [Sinergia CRSII3_160719 (to MT and DL)], the Helmut Horten Foundation (MU and MT), the Konstanz Research School Chemical Biology (KoRS-CB), the Crescere Stiftung Thurgau, the Thurgauische Stiftung für Wissenschaft und Forschung, and the State Secretariat for Education, Research and Innovation (DL).

SUPPLEMENTARY MATERIAL

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

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ARTICLE

JLB JOURNAL OF LEUKOCYTE BIOLOGY

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

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Abstract

The chemokine CCL20 is broadly produced by endothelial cells in the liver, the lung, in 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 CX_3C 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 extra- and intracellular loops, and signal through heterotrimeric G_i-proteins.[5]

In addition to the typical chemokine receptors, chemokines also bind to a subfamily of atypical chemokine receptors (ACKRs), which are

Abbreviations: ACKR, atypical chemokine receptor; BRET, bioluminescence resonance energy transfer; CCR, CC chemokine receptor; CoA, coenzyme A; EYFP, enhanced yellow fluorescent protein; h, human; m, mouse; NLuc, NanoLuc luciferase.

Received: 6 December 2019 | Revised: 28 April 2020 | Accepted: 30 April 2020

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 scavenge 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,[20] 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.[8] 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-arrestin2i1-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 *Hin*dIII and *Xba*I sites of pcDNA3. The construct includes a flexible GSI$(GGGGS)_3$ 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 *Hin*dIII/*Xho*I and *Xho*I/*Xba*I, respectively. Exceptions are pcDNA3 hCCR6-HA and pcDNA3 hCCR9-HA, where 2 oligos coding for GGLES$(GGGGS)_3$-HAtag were annealed and cloned into the *Xho*I and *Xba*I 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 *Xho*I and *Bsa*I restriction sites of pET-His_6-SUMO.[36] In addition, for hCCL20s a SGGGGS-S6tag[35] was added to the C-terminus of hCCL20. pET-His_6-SUMO hCCL21 has been described elsewhere.[36]

2.3 | Chemokine production

Recombinant human chemokines fused to a His_6-SUMOtag and a SGGGGS-S6tag were purified from BL21 (DE3) *Escherichia coli*, refolded via infinite dilution at pH 8.5 and the His_6-SUMOtag was cleaved by incubation with the Ulp-1 protease for 1–5 h and removed during the final purification step.[36-38] 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 hCCL20s^{Dy649P1} was generated by labeling purified hCCL20s with CoA-Dy649P1 (649P1-03; Dyomics GmbH) at 37°C for 2 h using the phosphopantetheinyl transferase Sfp (P9302S; 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,

TABLE 1 Overview of plasmids and primers

Construct	Template (if not synthesised); amplified insert in bold; [reference]	5′-Forward primer	5′-Reverse primer	Linker
pcDNA3 β-arrestin2i1-NLuc	pcDNA3 ***β*-arrestin2i1**-Y2[40]	GGTGGAAAGCTTATGGGGAGAAACCCG	GCATCGATCCACCGCAGAGTTG ATCATCATAGTC	[a]
	pAAVS1P-iCLHN **NLuc** (Addgene plasmid # 66579)[64]	CAATCGATCCACCGCTACCGCCACCGCCG GAACCGCCACCACCAGAACCGCCACC TCCGCCCGCCAGAATGCGTTC	GACCCAAGCTTGCCACCATGGTCTTCACA CTCGAAGATTTCGTTGG	
pcDNA3 β-arrestin1A-NLuc	***β*-arrestin1A** RC201279 (Origene)	GGTGGAAAGCTTATGGGCGACAAAGGG ACCCG	GCATCGATCCACCTCTGTTGTTGAGCT GTGGAGAGCC	[a]
pcDNA3 hACKR4-EYFP	pcDNA3 CCR7-**EYFP**[65]	GGACTCGAGAGCGGAGGTGGCGGTTCTG GTGGTGGCGGTTCCGGCGGTGGCGGTA GCGTGAGCAAGGGCGAGGAG	GAATAGGGCCCTCTAGACTACTTGTACA GCTCGTCCATGC	[b]
pcDNA3 hACKR4-YPet	PL-452 N-**YPet** (Addgene plasmid #19172)[66]	GCAGACTCGAGAGCGGAGGTGGCGGTT CTGGTGGTGGCGGTTCCGGCGGTGGCG GTAGCATGGTGAGCAAAGGCGAAGAG	GCAGGTCTAGATTACTTATAGAGCTCG TTCATGCCCTC	[b]
pcDNA3 hACKR4-mScarlet	p**mScarlet**_C1, (addgene: #85042)	GCAGACTCGAGAGCGGAGGTGGCGGTT CTGGTGGTGGCGGTTCCGGCGGTGGCG GTAGCATGGTGAGCAAGGGCGAGGC	GCAGGTCTAGATTACTTGTACAGCTCG TCCATGCCG	[b]
pcDNA3 hACKR4-mScarlet-I	SDM on pcDNA3 hACKR4-mScarlet replacing Thr74Ile on mScarlet	GGCTCCAGGGCCTTCATCAAGCACCCC GCCGAC	GTCGGCGGGGTGCTTGATGAAGGCCCT GGAGCC	[b]
pcDNA3 hACKR4-HA	pcDNA3 **ACKR4**-EGFP[22]	GGAGACCCAAGCTTCATTACGATGGC	CTACCTCGAGCCCCAATAGAGAAGGT AGAAGT	[c]
pcDNA3 hACKR3-HA	pcDNA3.1 PCP-**CXCR7**[67]	CCAAGCTTCATTACATGGACCTGCATCT CTTCGACTACTCAGAGC	CAGCTCGAGCCCCTTTGGTGCTCTGCTCC AAGGC	[c]
pcDNA3 hACKR3-EYFP	pcDNA3 hACKR3-HA	CCAAGCTTCATTACATGGACCTGCATCT CTTCGACTACTCAGAGC	CTGCTGGCTCGAGTCCACCTTTGGT GCTCTGCTCCAAGGC	[b]
pcDNA3 hCCR7-EYFP	pcDNA3 **CCR7**-HA[34]	GACCCAAGCTTGGTACCGAGCTCGGATC	GTAGCTCGAGTCCACCGGAGAAGGT GGTGGTGGTCTCG	[b]
pcDNA3 hCCR6-EYFP	pANT7_cGS **CCR6a** (DNASU HsCD00731816)	GCTGCGAAGCTTGCCACCATGAGCGGGG AATCAATGAATTTCAGC	GTCTCGCTCGAGTCCACCCATAGTGAA GGACGACGCATTGTCGTTATC	[b]
pcDNA3 hCCR6-HA	oligo annealing	GCCGCTCTCGAGAGCGGCGGCGGCGGC AGCGGCGGCGGCGGCAGCGGCGGCG GCGGCAGC	GGCCCTCTAGATCAGGCGTAGTCGGGCA CGTCGTAGGGGTAGCTGCCGCCGCCG CCGCTGC	[b]
pcDNA3 hCCR9-EYFP	pCEP4 **CCR9a**[68]	GTGCTGGAATTCCGTCATGACACCCACA GACTTCAC	CTCTGCCTCGAGTCCACCGAGGGAGAGTG CTCCTGAGGTTGTC	[b]
pcDNA3 hCCR9-HA	oligo annealing	GCCGCTCTCGAGAGCGGCGGCGGCGGC AGCGGCGGCGGCGGCAGCGGCGGCG GCGGCAGC	GGCCCTCTAGATCAGGCGTAGTCGGG CACGTCGTAGGGGTAGCTGCCGCCGCCG CCGCTGC	[b]
pSUMO hCCL19	pCR3-**hCCL19**-Fc[34]	GGTGCTCGAGTTAACTGCTGCGGCGCTTC	GACTAGGTCTCCGGTGGGGGCACCAATGA TGCTGAAGACTG	–
pSUMO mCCL19		GACTAGGTCTCCGGTGGGGGCGCGAACG ATGCGGAAG	GTGGTGCTCGAGTTAGCTCACCGGGCTG CGGCG	–
pSUMO hCCL20	pDONR221 **hCCL20** (DNASU HsCD00042527)[69]	GACTAGGTCTCCGGTGGGGCAAGCAAC TTTGACTGCTGTCTTGGATAC	GTGCTCGAGTTACAACATGTTCTTGACTT TTTTACTGAGGAGACGCAC	–
pSUMO mCCL20	pHis-Entero **mCCL20**ybbR13	GACTAGGTCTCCGGTGGGGCAAGC AACTACGACTGTTGCCTCTC	GTGCTCGAGTTACATCTTCTTGACTC TTAGGCTGAGGAGG	–
pSUMO hCCL21	[36]			–
pSUMO mCCL21		GACTAGGTCTCCGGTGGGAGTGATGGA GGGGGTCAG	GTGGTGCTCGAGTCATCCTCTTGAGGGC TGTG	–
pSUMO hCCL25	**hCCL25** RC222128 (Origene)	GACTAGGTCTCCGGTGGGCAAGGTGTC TTTGAGGAC	GTGCTCGAGTTACAGTCCTGAATTAGCT GATATCAGGAGGG	–
pSUMO mCCL25	pHis-Entero **mCCL25**ybbR13	GACTAGGTCTCCGGTGGGCAAGGTGCC TTTGAAGACTGCTG	GTGCTCGAGTTAATTGTTGGTCTTTCTGGG CATCATCAC	–
pSUMO hCCL20-S6	pSUMO **hCCL20**	GACTAGGTCTCCGGTGGGGCAAGCAAC TTTGACTGCTGTCTTGGATAC	GTGCTCGAGTTAGTTCAGCAGGCGCAGCA GCCAGCTCAGGCTATCGCCGCTGCCGCC GCCGCCGCTCATGTTCTTGACTTTTTT ACTGAGGAGAC	[d]

Linker sequence used between protein and tag:
[a]GSI$(GGGGS)_3$
[b]GGLES$(GGGGS)_3$
[c]GARA
[d]SGGGGS

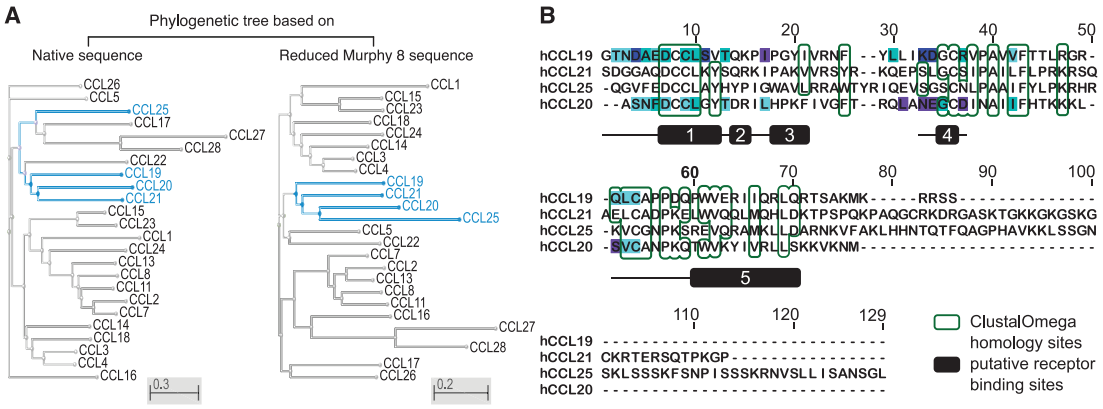

FIGURE 1 **In silico analysis of known and putative human ACKR4 ligands.** (**A**) Phylogenetic tree based on the native (left) and reduced Murphy 8 (right) amino acid sequence of human CC chemokines. (**B**) ClustalOmega sequence alignment of hCCL19, hCCL21, hCCL25, and hCCL20. Green frames represent similar amino acids based on the reduced Murphy8 amino acid sequence. Black boxes indicate regions of putative receptor binding sites predicted by InterPro; adjacent black lines illustrate extended interaction sites based on structural modeling shown in (**C**).

(Continues)

Glattbrugg, Switzerland). Recombinant mouse CCL20-ybbR13 (mCCL20y) and CCL25_19-ybbR13 (CCL25_19y) were labeled with either AlexaFluor647 (AF647; Thermo Fisher Scientific) or SiR700 (Spirochrome, Stein am Rhein, Switzerland) conjugated CoA and repurified by reverse phase HPLC.[37] The preparation of hCCL19-mRFP has been described previously.[22]

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 50µM 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 200*g*. 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 was added to the cells for indicated times. At $t = 0'$, all cells were washed twice with PBS; acidic wash (100 mM NaCl, 50 mM glycine HCl, pH = 3.0) was applied to the designated wells for 45 s, followed by 2 washings with PBS. Cells were detached using PBS based Gibco™ cell dissociation buffer and subsequently measured on a BD LSR II flow cytometer and FACSDiva™ software (BD Biosciences, Allschwil, Switzerland). Data were analyzed using FlowJo™ 10.7 software. Mouse 300–19 pre-B cells stably expressing mACKR4, mCCR7, and mCCR9 and GFP were plated on 96-well plates (10^5 cells in 100 µl) and incubated with 50 µl 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.

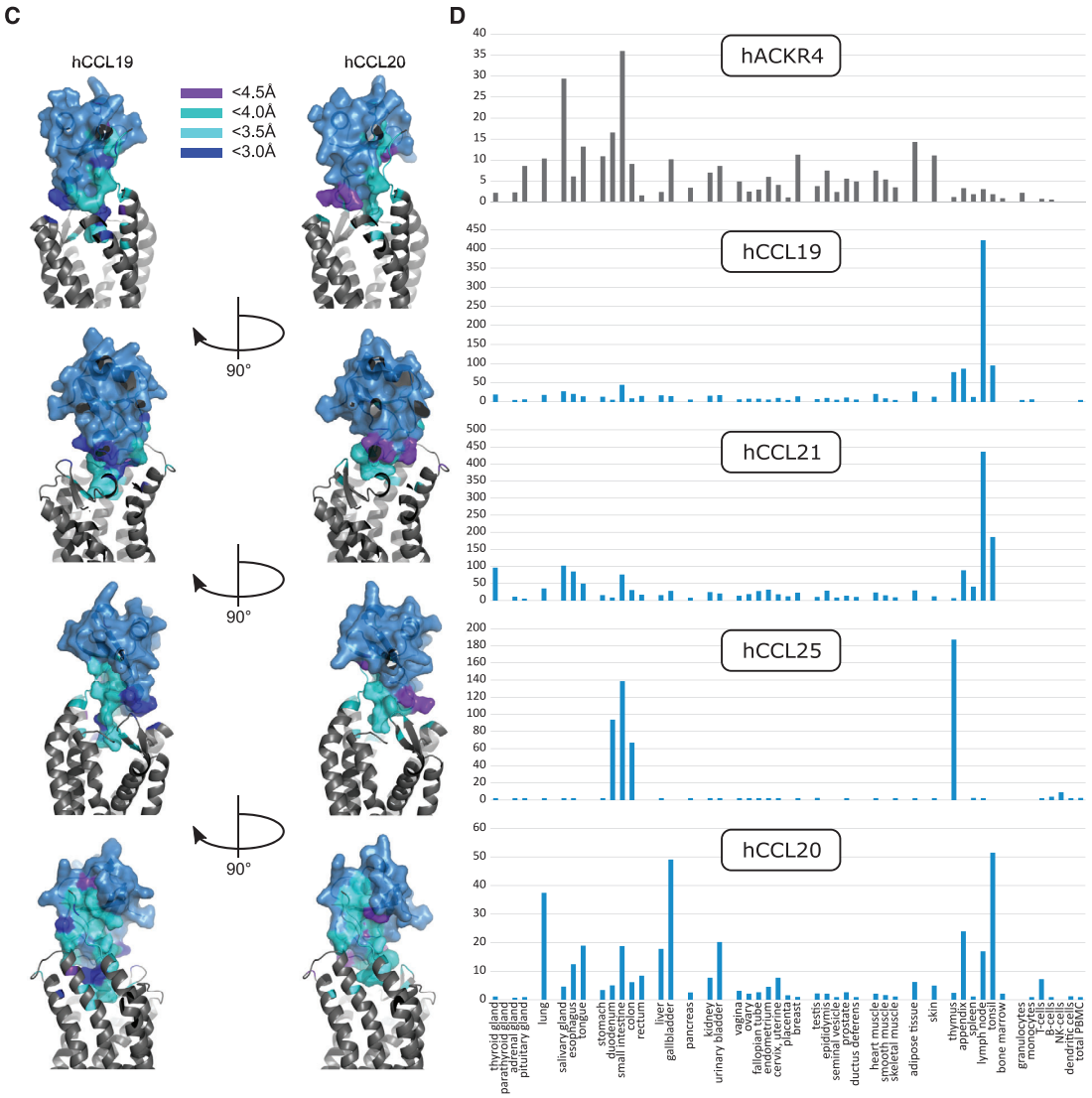

FIGURE 1 (Continued) (**C**) Pymol modeling of hACKR4 (gray) interacting with hCCL19 (amino acids T2-S69, left panel) and hCCL20 (S2-V67, right panel) based on the solved structure of CXCR4/Endolysin chimeric protein with vMIPII (PDB: 4RWS.1). Distances between chemokine and receptor amino acid side chains are color coded: < 4.5 Å (purple-blue), < 4.0 Å (teal), < 3.5 Å (cyan), < 3.0 Å (blue). (**D**) Normalized RNA expression profiles of hACKR4, hCCL19, hCCL21, hCCL25, and hCCL20 across various tissues retrieved from the human protein atlas

2.7 | In vivo uptake of mCCL20y^{AF647}

mCCL20yAF^{647} (15 µl, 30 nM in PBS) was injected into the food pad of wild-type C57BL/6 mice, CCR6^{ko} C57BL/6 mice,[41] kindly provided by Sergio Lira, or heterozygous (ACKR4$^{GFP/wt}$) and homozygous (ACKR4$^{GFP/GFP}$) ACKR4-EGFP knock-in reporter mice.[42] 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[30] 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 Fischer 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×

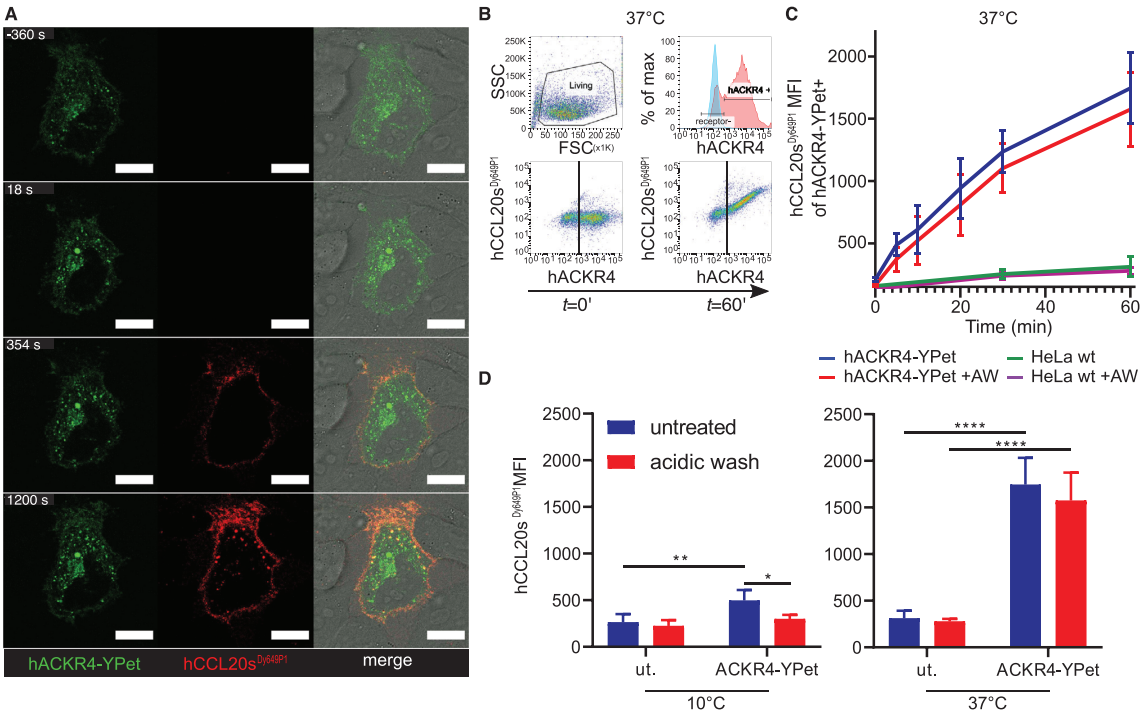

FIGURE 2 **Human ACKR4 is a scavenger for human CCL20.** (**A**) Uptake of fluorescently labeled hCCL20s^{Dy649P1} by hACKR4-YPet. HeLa cells were transiently transfected with hACKR4-YPet (green), stimulated at $t = 0$ s with 18 nM hCCL20s^{Dy649P1} (red) and chemokine uptake was monitored over time 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 hCCL20y^{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 hCCL20y^{Dy649P1} on hACKR4-YPet positive cells was determined as in (**B**). $n = 4$. (**D**) Quantification of hCCL20^{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,[43] 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 ACKR4 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 see[45] 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

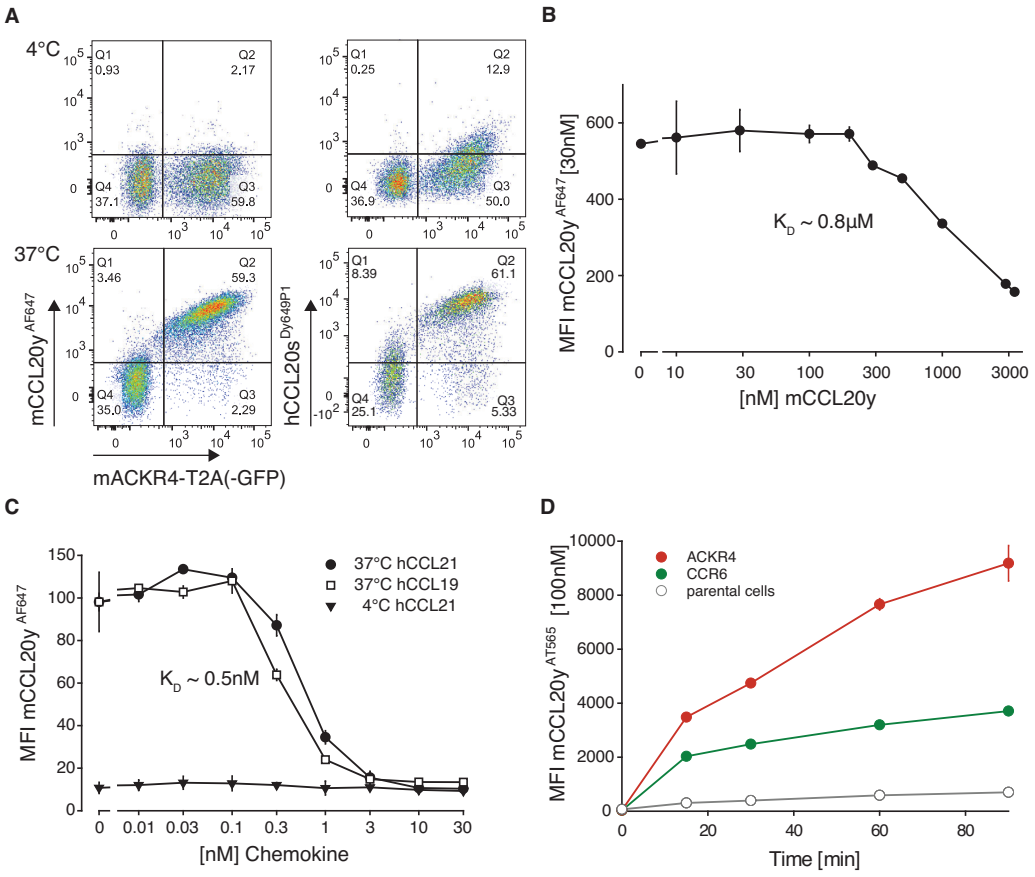

FIGURE 3 **Mouse ACKR4 is a scavenger for mouse CCL20.** (**A**) Binding and uptake of fluorescently labeled mCCL20y^{AF647} and hCCL20s^{Dy649P1} by mACKR4-T2A. 300–19 pre-B cells expressing mACKR4-T2A(-GFP) were incubated with 30 nM mCCL20y^{AF647} (left panel) or hCCL20s^{Dy649P1} (right panel) at 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 mCCL20y^{AF647} 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 mCCL20y^{AF647} 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 mCCL20y^{AF647} 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 mCCL20y^{AF647} 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 mCCL20y^{AT565} by mACKR4-T2A. Parental 300–19 pre-B cells and mACKR4-T2A(-GFP) or hCCR6 transfectants were incubated with 100 nM mCCL20y^{AT565} 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.[23,24] Further analysis of reduced sequences using ClustalOmega[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.[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[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

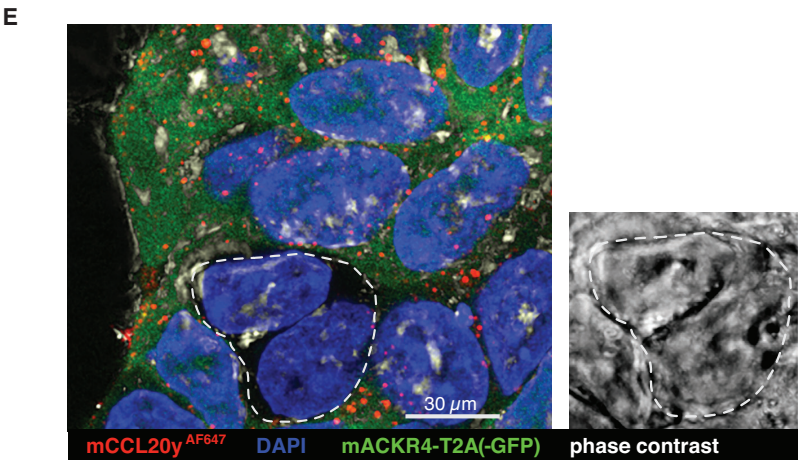

FIGURE 3 (Continued) (**E**) Uptake of mCCL20y^{AF647} by mACKR4-T2A. Confocal image of mCCL20y^{AF647} uptake by HEK293 cells expressing mACKR4-T2A(-GFP). HEK293 stably transfected with ACKR4-T2A(-GFP) were seeded together with parental HEK293 cells (circled with dotted line) on glass bottom dishes and incubated with 50 nM mCCL20y^{AF647} for 40 min at 37°C. Images were recorded with 40× magnification with a laser scan confocal microscope. mCCL20y^{AF647} (red) 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

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)[49] 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.[30,35,37,50] Sfp adds the fluorescent dye conjugated phosphopantetheine moiety of CoA[51] 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^{Dy649P1} 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^{Dy649P1} 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^{Dy649P1} 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^{Dy649P1} 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^{AF647} or hCCL20s^{Dy649P1} 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^{AF647} or hCCL20s^{Dy649P1} by mACKR4-T2A (Fig. 3A). 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^{AF647} binding to mACKR4-T2A (Fig. 3B). This observation is consistent with previous findings where 200 nM CCL20 essentially failed to outcompete ^{125}I-CCL19 on human and mouse ACKR4.[20,24] Nevertheless, at 37°C addition of increasing concentrations of unlabeled hCCL19 and hCCL21 efficiently outcompeted the uptake of 30 nM of mCCL20y^{AF647} (Fig. 3C), reflecting

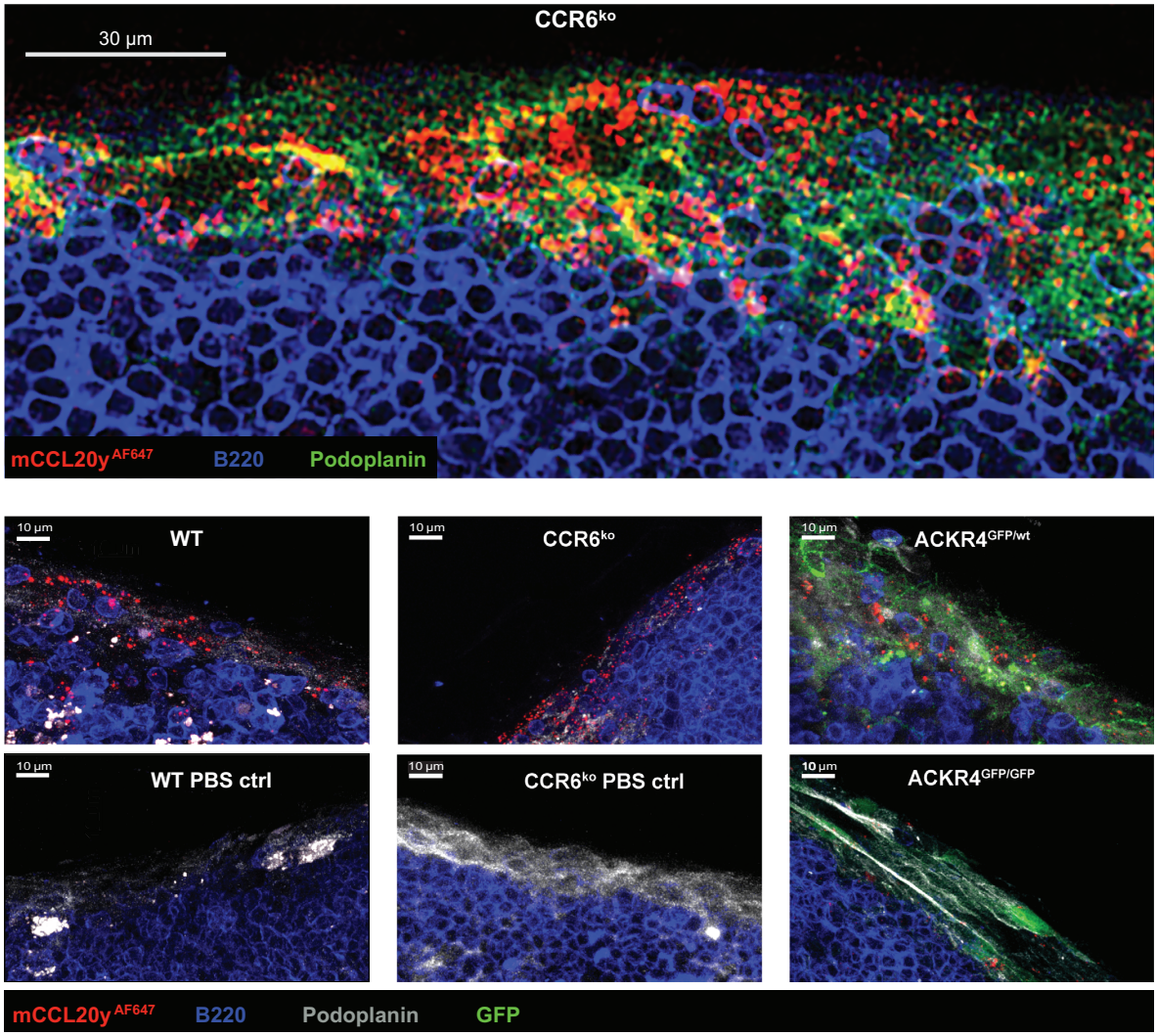

FIGURE 4 **In vivo uptake of fluorescently labeled mouse CCL20 by ACKR4.** mCCL20y^{AF647} (15 µl, 30 nM in PBS) or PBS as solvent control (ctrl) was injected into the food pad of wild-type (WT) and CCR6^{ko} mice, as well as of heterozygous ACKR4$^{GFP/wt}$ and homozygous ACKR4$^{GFP/GFP}$ reporter mice. Upper panel: zoomed imaged from a different CCR6^{ko} mouse. Mice were sacrificed after 15 min, the draining popliteal lymph node removed, fixed and stained with anti-podoplanin and anti-B220 antibodies, and imaged by confocal microscopy. Representative images derived from 3 mice per group injected at different days per condition are shown

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^{AT565} 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.[8] 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$^{GFP/wt}$ and homozygous ACKR4$^{GFP/GFP}$ (ACKR4^{ko}) reporter mice. As shown in Fig. 4, mCCL20y^{AF647} was taken up at the subcapsular sinus of heterozygous ACKR4$^{GFP/wt}$ 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

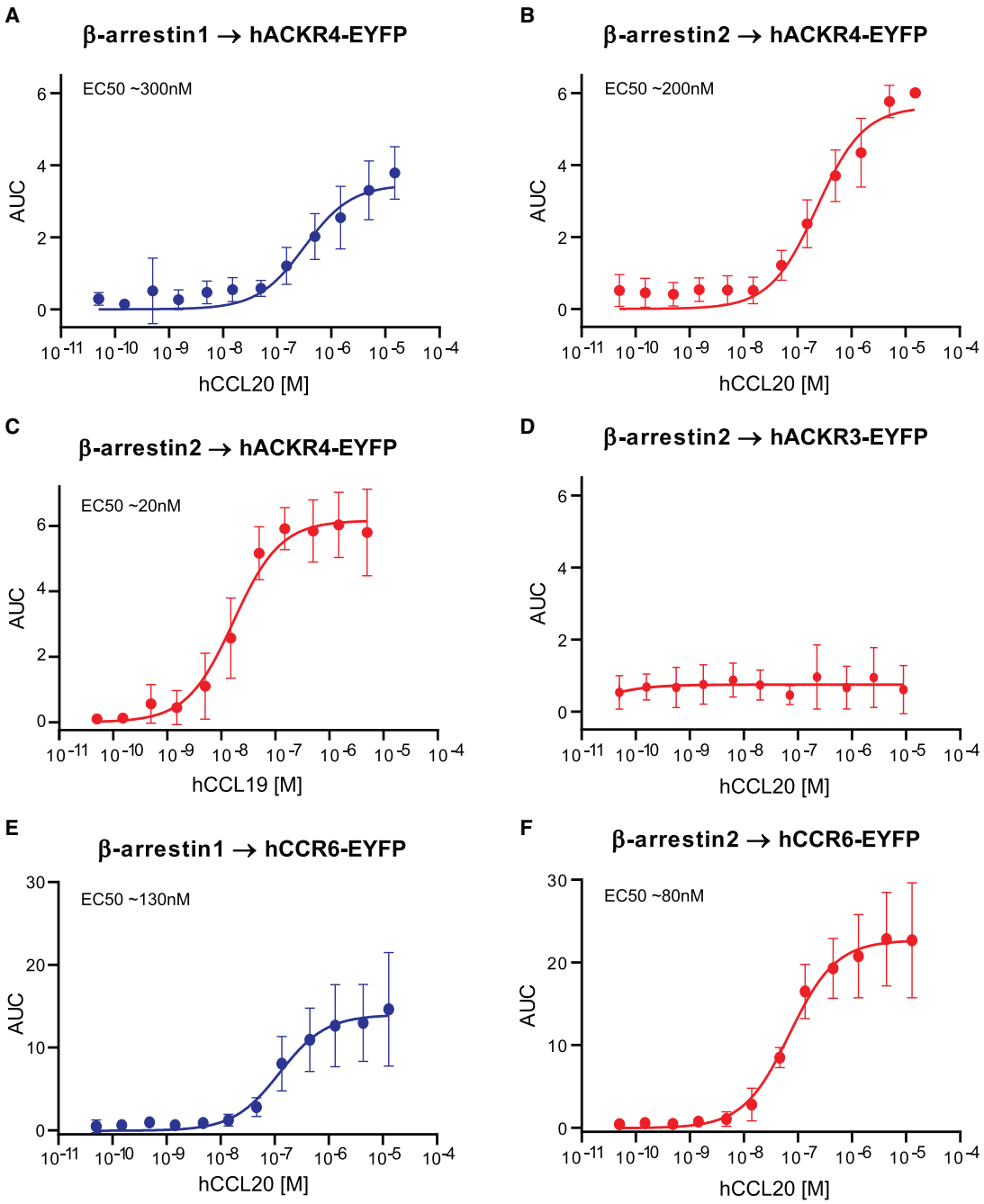

FIGURE 5 **hCCL20 recruits β-arrestins to hACKR4.** HeLa cells were transiently cotransfected with constructs for human receptor tagged with EYFP and β-arrestins fused to NLuc. At least 30 h after transfection, cells were incubated with the luciferase substrate coelenterazine H and subsequently stimulated 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 $\pm$ SD are depicted

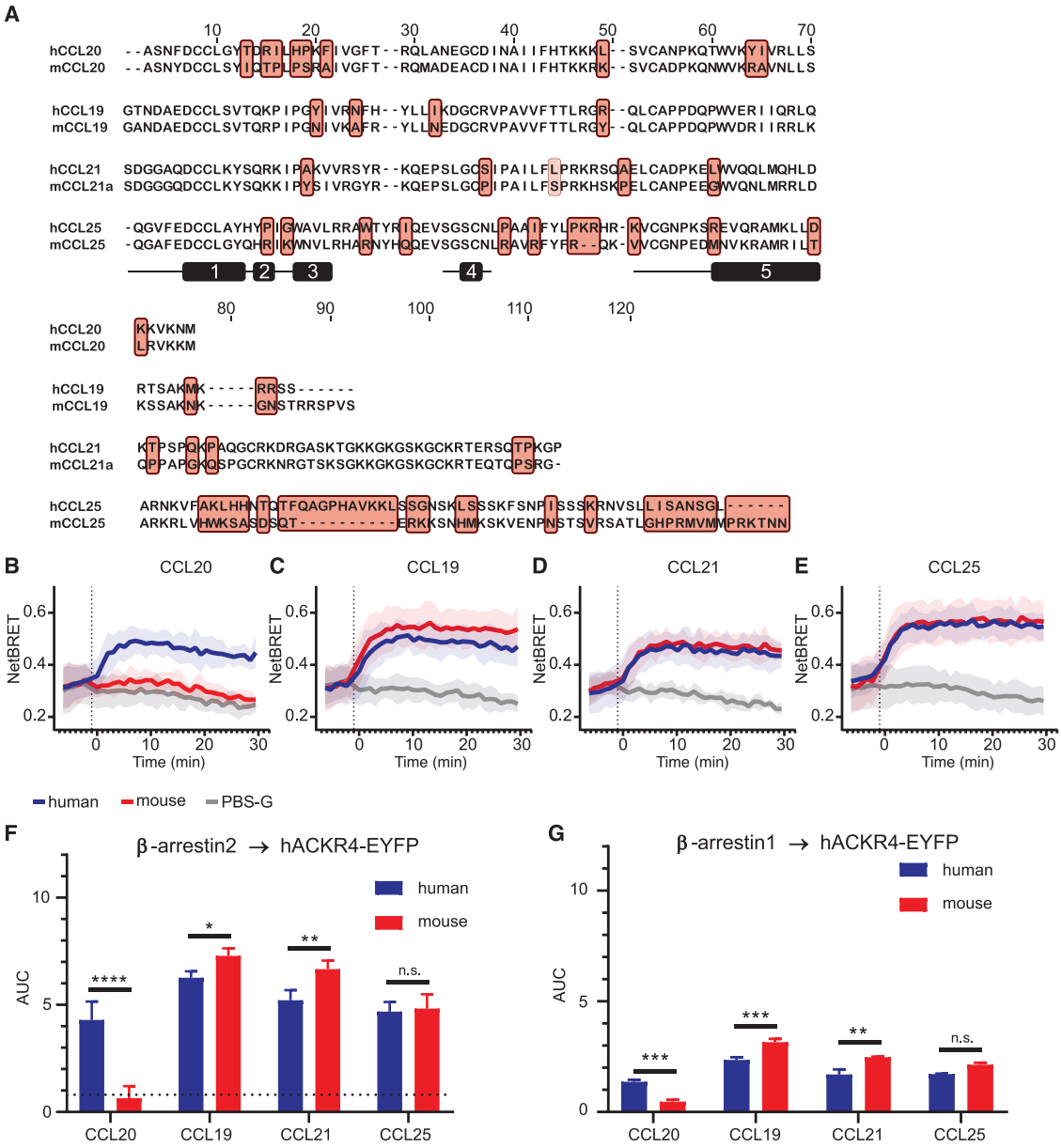

FIGURE 6 **Cross-species analysis of CCL20 and ACKR4.** (**A**) ClustalOmega sequence alignment of human and mouse chemokines. Differences in the amino acid sequence, based on the reduced Murphy8 amino acids, are highlighted with red boxes, putative receptor interaction sites are indicated with black lines and boxes. Chemokine-driven β-arrestin2-NLuc (**B–F**) or β-arrestin1-NLuc (**G**) recruitment to hACKR4-EYFP was determined by BRET. HeLa cells transiently coexpressing hACKR4-EYFP and β-arrestin2-NLuc (**B–F**) or β-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 β-arrestin2-NLuc recruitment to hACKR4-EYFP upon stimulation with CCL20 (**B**), CCL19 (**C**), CCL21 (**D**), or CCL25 (**E**). Chemokine-driven β-arrestin2-NLuc (**F**) or β-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 ± SD. $n = 3$.

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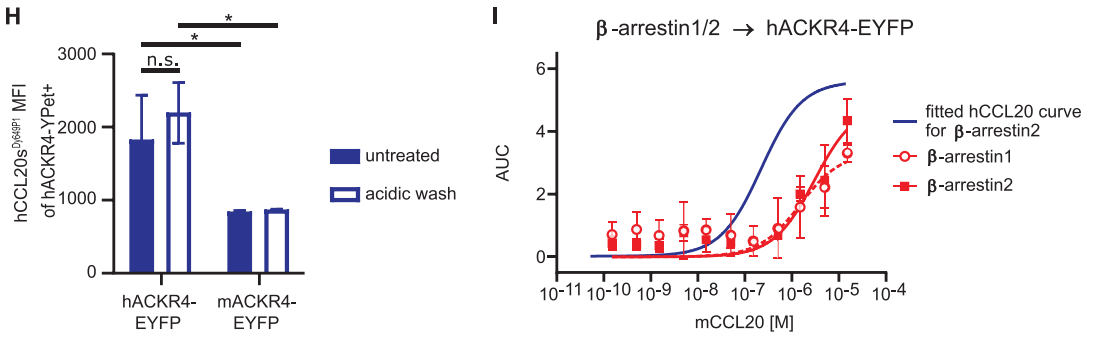

FIGURE 6 (Continued) (**H**) Uptake of hCCL20s^{Dy649P1} by human and mouse ACKR4-EYFP. HeLa cells transiently expressing either hACKR4-EYFP or mACKR4-EYFP were incubated at 37°C for 60 min with 18 nM hCCL20s^{Dy649P1}. Cells were left untreated or exposed to a brief acidic wash and chemokine uptake on EYFP-positive cells was determined by flow cytometry. Mean ± SD. $n = 3$. (**I**) HeLa cells were transiently cotransfected with hACKR4-EYFP and β-arrestin1-NLuc (dotted line) or β-arrestin2-NLuc (solid lines), stimulated with graded concentrations of mCCL20 and chemokine β-arrestin recruitment to the receptor was determined by BRET as described in Fig 5. For comparison, the fitted dose–response curve obtained in Fig 5B on hCCL20-mediated β-arrestin2 to hACKR4 is shown as blue solid line

mCCL20y^{AF647} can be used to study ACKR4 functions in vitro and in vivo. Taken together, these data demonstrate that ACKR4 can actively take-up CCL20 albeit with a lower affinity than CCL19 and CCL21.

3.3 | CCL20 recruits β-arrestins to ACKR4

Although information on ACKR signaling is sparse, ACKR2,[53] ACKR3,[54,55] and hACKR4[28] are described to recruit β-arrestins upon chemokine binding. We used a BRET-based assay to measure chemokine-induced β-arrestin recruitment to cognate receptors. We tagged β-arrestin1 and β-arrestin2 with the NLuc and hACKR4, hACKR3, and hCCR6 with the EYFP and expressed them in HeLa cells. Untagged, native hCCL20 in a dose dependent manner recruited both β-arrestin1-NLuc (Fig. 5A) and β-arrestin2-NLuc (Fig. 5B) to hACKR4-EYFP with a comparable potency. As noted for other chemokine/chemokine receptor pairs,[28] hCCL20 recruited β-arrestin2-NLuc slightly more potent to hACKR4-EYFP than β-arrestin1-NLuc (Figs. 5A and 5B). Consistent with the competition binding data, hCCL19 more efficiently recruited β-arrestin2-NLuc to hACKR4-EYFP than hCCL20 (Fig. 5C). By contrast, hCCL20 did not recruit β-arrestin2-NLuc to hACKR3-EYFP (Fig. 5D). When tested on its unique cognate typical receptor, hCCL20 also recruited β-arrestin1-NLuc (Fig. 5E) and β-arrestin2-NLuc (Fig. 5F) to hCCR6-EYFP in a dose dependent manner. These data provide clear evidence that hCCL20 is a ligand for hACKR4, but not for hACKR3.

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).

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).

3.5 | The chimeric chemokine CCL25_19 is a ligand for mACKR4 and hACKR4

All chemokines that bind to an ACKR share at least one typical 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

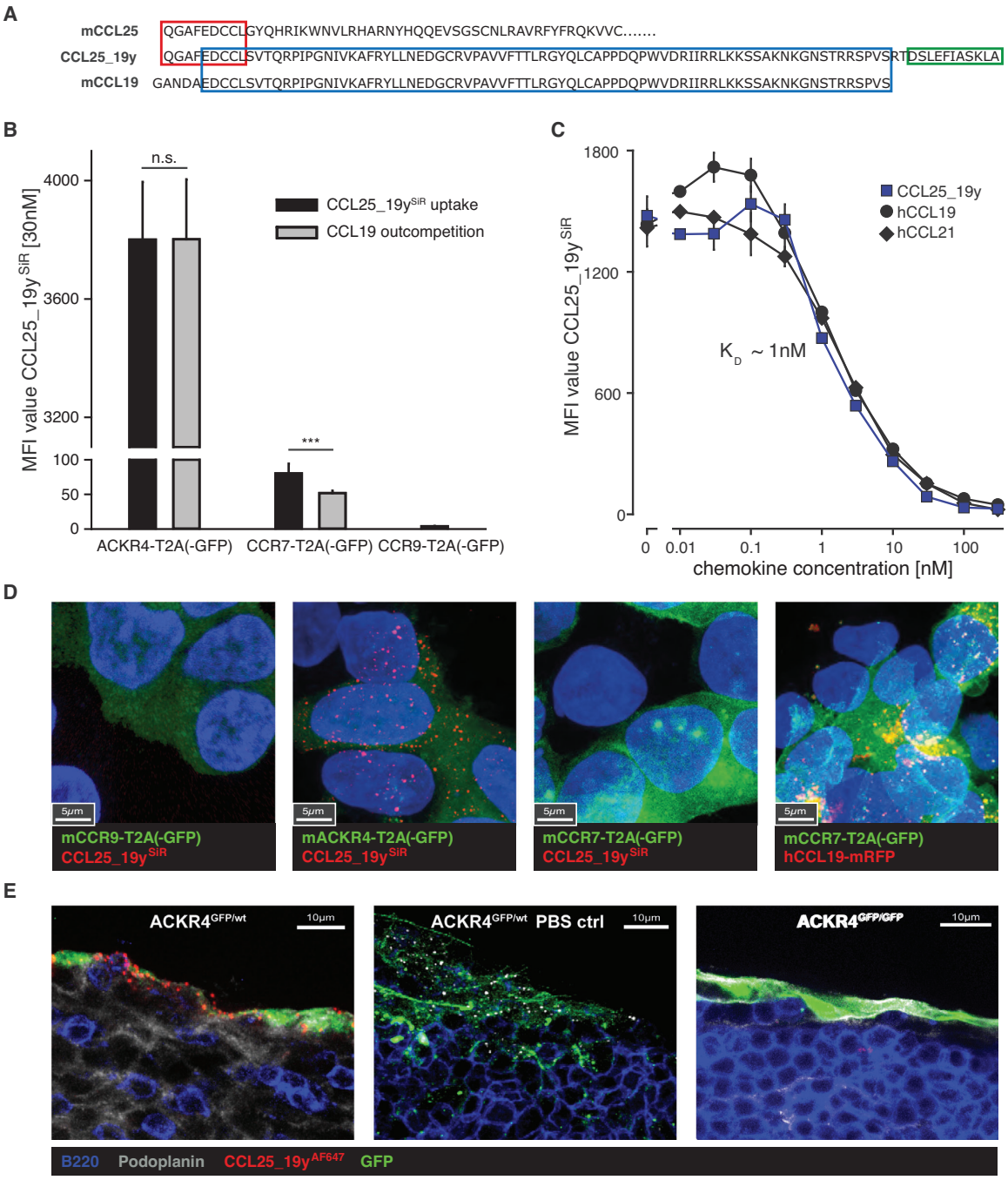

FIGURE 7 **The chimeric chemokine CCL25_19 is a ligand for mACKR4.** (**A**) Sequence alignment of mCCL25 (red rectangle), mCCL19 (blue rectangle), and the chimeric chemokine CCL25_19. The green frame indicates the ybbR13-tag fused to the C-terminus of CCL25_19y. (**B**) CCL25_19y^{SiR} is a ligand for mACKR4-T2A(-GFP), but not for mCCR9-T2A(-GFP) or mCCR7-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_19y^{SiR} 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_19y^{SiR} by mACKR4-T2A. Stably transfected 300–19 pre-B cells expressing mACKR4-T2A(-GFP) were incubated at 37°C for 45 min with 2 nM CCL25_19y^{SiR} and graded concentrations of unlabeled CCL25_19y, hCCL19, or hCCL21. Uptake of CCL25_19y^{SiR} 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_19y^{SiR} 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_19y^{SiR} 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_19y^{SiR} 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_19y^{SiR} interaction with mCCR7-T2A, but not with mACKR4-T2A (Fig. 7B). This indicates that CCL25_19y^{SiR} 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_19y^{SiR} from mACKR4-T2A(-GFP) expressing cells and hence has been internalized (Fig. 7B). CCL25_19y^{SiR} 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 ($K_d \sim 1$ 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_19y^{SiR}. Confocal imaging revealed that CCL25_19y^{SiR} is taken up solely by mACKR4-T2A(-GFP) transfected cells (Fig. 7D). By contrast, mCCR9-T2A(-GFP) or mCCR7-T2A(-GFP) expressing cells did not internalize CCL25_19y^{SiR} (Fig. 7D). To corroborate these findings, we expressed mACKR4-T2A (-GFP) or mCCR7-T2A(-GFP) in HEK293 cells and visualized CCL25_19y^{SiR} uptake by time-lapse imaging. Of note, CCL25_19y^{SiR} 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_19y^{SiR} (or PBS as control) into the food pads of heterozygous ACKR4$^{GFP/wt}$ reporter mice and homozygous ACKR4$^{GFP/GFP}$ that lack ACKR4. As shown in Fig. 7E, CCL25_19y^{SiR} was readily taken-up by GFP-positive subcapsular sinus endothelial cells of the draining lymph nodes of ACKR4$^{GFP/wt}$ reporter mice, but not by GFP-positive, ACKR4-deficient (ACKR4$^{GFP/GFP}$) cells.

Next, we tested whether the mouse-sequence derived chimeric chemokine CCL25_19 is also scavenged by hACKR4. CCL25_19y^{SiR} 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_19y^{SiR} uptake by time-lapse imaging (Supplementary Video 5). Notably, CCL25_19y^{SiR} 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 CCL25.[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

uptake of CCL25_19y^{SiR} by HEK293 cells expressing mACKR4-T2A(-GFP). HEK293 stably expressing mCCR9-T2A(-GFP), mACKR4-T2A(-GFP) (left panels), or mCCR7-T2A(-GFP) (right panels) were seeded together with parental HEK293 cells (ratio 1:1) on glass bottom dishes. Cells were incubated with CCL25_19y^{SiR} (the leftmost panels) or hCCL19-mRFP (right panel) for 40 min at 37°C to permit chemokine uptake, rinsed with PBS, fixed and analyzed with 63× magnification with a laser scan confocal microscope (Leica SP5). Nuclei of all cells were visualized with DAPI. (**E**) In vivo uptake of fluorescently labeled mCCL25_19y^{AF647}. CCL25_19y^{AF647} (15 µl, 30 nM in PBS, red), or PBS as control, was injected into the food pads of heterozygous ACKR4$^{GFP/WT}$ reporter mice (left and middle panel) or homozygous ACKR4$^{GFP/GFP}$ mice lacking ACKR4 (right panel). Mice were sacrificed after 15 min, the draining popliteal lymph node removed, fixed and stained with anti-podoplanin (gray) and anti-B220 antibodies (blue) and imaged by confocal microscopy. Middle panel shows a control lymph node without chimeric chemokine injection. Representative images obtained from 3 mice injected at different days are depicted

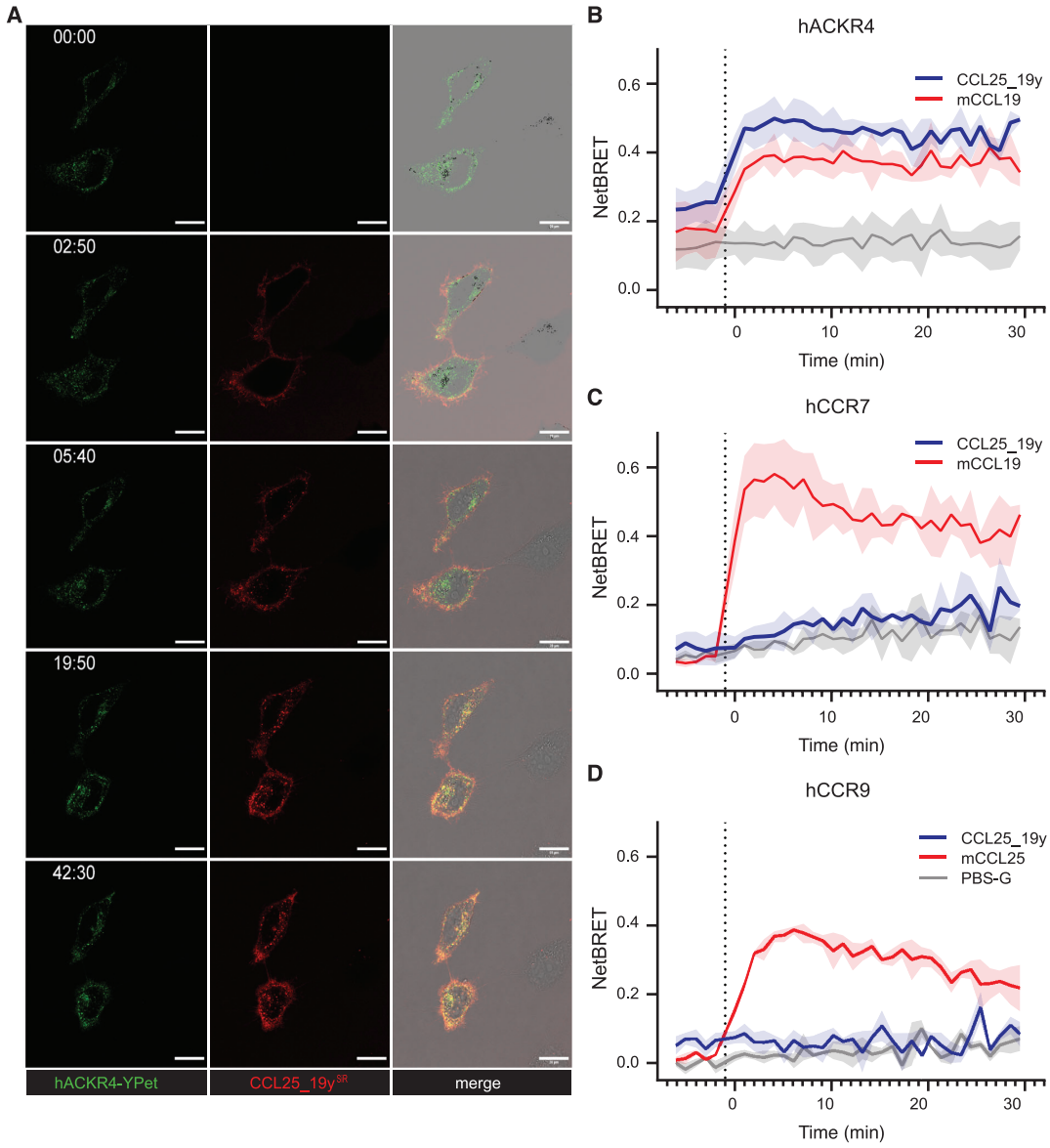

FIGURE 8 **CCL25_19 is a specific ligand for hACKR4.** (**A**) Uptake of fluorescently labeled CCL25_19y^{SiR} by hACKR4-YPet. HeLa cells were transiently transfected with hACKR4-YPet (green) and stimulated with 25 nM CCL25_19y^{SiR} (red) at $t = 2'$ and chemokine uptake was monitored over time by confocal 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.[2,58,59] 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,[53] ACKR3,[54,55] and hACKR4[28] 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.[28] 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 ACKR4 both in vitro and in vivo. Despite a relatively moderate binding affinity in vitro, uptake of CCL20 by ACKR4 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 ACKR4,[60] 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[61] 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 in 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 CRSII3_160719 (M.T. and D.F.L.)], the Helmut Horten Foundation (M.T.), the Konstanz Research School Chemical Biology (KoRS-CB), 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., S.T., V.P, 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.

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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

3 Discussion and Outlook

The presented work spanned topics from ligand engineering and production, to the discovery of new ligands based on sequence similarity and how activated ACKR4 transduces signals into the cell. Therefore, the discussion will be organised into subparagraphs. First, the possibilities of the engineered ligands will be highlighted, second, ACKR4, GRK and β-arrestin interactions will be elucidated, third ACKR4's role as scavenger of CCL20 and its interaction with its four ligands will be explained and finally ACKR4's impact in several CCL20 related diseases will be discussed.

3.1 The Plethora of Possibilities of Engineered Chemokines

As mentioned in the beginning, chemokines can be produced in various cell types. (Moepps & Thelen, 2016) We have applied mammalian (Purvanov et al., 2018) as well as eukaryotic expression systems (Matti et al., 2020a; Matti et al., 2020b) with both advantages and disadvantages. Both workflows had the employment of a His_6-SUMO tag in common. In the eukaryotic system, it helped to purify and efficiently separate the chemokine from other secreted proteins in the supernatant and in the prokaryotic system it helped to isolate the misfolded chemokines present in inclusion bodies. With both meth-

ods, we obtained good amounts (>0.5mg per batch) of recombinant chemokine, which we used to visualise gradients (with CCL19-mRFP and CCL21-mRFP) as well as for binding and internalisation studies (with dye-labelled chemokines). An advantage of the prokaryotic system was the generation of our S6 or YbbR13 tagged chemokines for specific labelling, as we did not rely on the fluorescent tag during the production. Thus, one is very flexible in choosing an appropriate fluorescent dye for experiments, without having to re-produce the entire chemokine batch. The flexibility is beneficial for future experiments, for whose the suitable dye in combination with extracellularly labelled or luciferase-tagged receptors can be used for more elaborate binding and receptor interaction studies as was done for e.g. β_2 adrenergic receptor and adenosine receptors. (Stoddart et al., 2015; Sykes et al., 2019) Therefore, we applied this approach to generate a CCL19 and CCL25 chimera, which is designed to specifically bind to and be scavenged by ACKR4 only. The use of a fusion chemokine based on CXCL12 core with CXCL11 N-terminus was previously published as mouse CXCL11_12 chimera, which is specific for mouse ACKR3 but neither for CXCR4 nor CXCR3. (Ameti et al., 2018) Analogue, our CCL25_19 is specific for ACKR4 but neither for CCR7 nor CCR9. We proved the chimera to be taken up in vivo as well as in vitro and determined a ligand dissociation constant (K_D) for ACKR4 in the nanomolar range, similar to the K_D of CCL19. In addition, we showed our chimera to be usable in the human as well as in the mouse system. For future studies, this chimera can be used to specifically identify ACKR4 positive cells in wild type animals. Depending on the cell localisation, these ACKR4$^+$ cells can further be isolated and characterised in more detail employing ex vivo and in vitro methods. For such experiments, one can apply the broad spectrum of fluorescent ACKR4 ligands generated to date, which are based on CCL19, CCL21 and CCL20. Interesting

aspects to examine would be whether ACKR4 scavenges all its ligands no matter where it encounters them, or if post translational modifications, as discussed later, influence the scavenging in specific tissues. Moreover, certain cells have been shown to co-express ACKR3 and CXCR4, (J. Wang et al., 2011) whereas cells co-expressing ACKR4 and any of the corresponding cCKRs like CCR7 or CCR6 remain still to be discovered. Thus, a combination of antibodies and fluorescent chimeric ligands can shed new insights into expression patterns, and maybe find a role for ACKR4 during the embryonal development, despite ACKR4$^{-/-}$ mice being viable.

3.2 New Insights on ACKR4 Signalling

ACKR4 signalling has been a matter of debate over the last couple of years, especially the role β-arrestins play. (Comerford et al., 2006; Watts et al., 2013) In agreement with other chemokine receptors like CXCR4 (Saaber et al., 2019) and ACKR3 (Montpas et al., 2018; Saaber et al., 2019), Comerford et al. found ACKR4 mediated scavenging of CCL19 in absence of β-arrestins. However, later the interaction of ACKR4 with β-arrestin along with the internalisation of fluorescent CCL19 into β-arrestin positive vesicles in ACKR4 transfected cells was observed. (Watts et al., 2013) We now elucidated the role of β-arrestins in ACKR4 signalling and function. First, using a tailless mutant, lacking residues 305-350 (ACKR4t), we showed that wild type ACKR4 has a basal interaction with β-arrestin2, which can contribute to the steady state recycling of ACKR4. We found ACKR4 together with β-arrestin in vesicular structures in unstimulated cells, whereas ACKR4t was localised mostly at the plasma membrane. Interestingly, this effect cannot be regulated by β-arrestin alone, as in β-arrestin de-

ficient HeLa cells, we did not find the same but a similar phenotype. In detail, ACKR4 was localised at the membrane and in vesicles close to the membrane and less in intracellular vesicles. This hints at a second role for β-arrestin mediated signalling or trafficking, which is induced by ACKR4 binding. Using our self-produced fluorescent chemokine ligands, we successfully elucidated the effect of β-arrestin ACKR4 interaction in chemokine scavenging. In line with previous observations in mouse embryonic fibroblasts lacking β-arrestin, β-arrestin deficient HeLa cells did internalise substantial amounts of fluorescent CCL19. However, this uptake was less efficient compared to wild type HeLa cells, showing β-arrestins to be dispensable for ACKR4 function. Remarkably however, β-arrestin contributes to the effectiveness of ACKR4 uptake, which was shown by co-transfection of β-arrestin into wild type and β-arrestin deficient cells. In both cases, the CCL19 uptake by ACKR4 was enhanced to a similar level, even higher as under wild type conditions, clearly showing β-arrestins to have a boosting effect on ACKR4 scavenging. As it has been shown by various groups before, β-arrestins recognise phosphorylated residues on the tail but are sensitive to phosphosites in the intracellular loops as well. (Eason et al., 1995; Staus et al., 2018) By using a site directed mutagenesis approach, we were able to find a cluster of residues on the C-terminal tail as well as on intracellular loops, which clearly influenced the recruitment of β-arrestin to ACKR4 but had no effect on surface expression or chemokine binding. Nonetheless, which kinase is interacting with those sites in the end remains to be elucidated in future studies. The method of choice to that end would be state of the art cross-linking mass spectrometry (XL-MS), using membrane bound linkers or artificial amino acids to specifically cross-link interaction partners prior to analysis. Besides gaining information on which proteins are interacting, one also retains distance information, as the linker length is

known a priori. (Koukos & Bonvin, 2020) In brief, proteins are chemically cross-linked in their native state and then, after enzymatic digestion, the sequence of the crosslinked peptides is identified via tandem MS. An approach with different linker lengths was applied to study the interaction of the β-adrenergic receptor:GRK5 interaction. (Komolov et al., 2017) As ACKR4 possess a lysine in the 8th helix, one could apply bis(sulfosuccinimidyl) suberate (BS3) or a rather novel Azide-A-DSBSO as a linker. (Kaake et al., 2014; Leitner et al., 2016) By the use of a StrepTagII tagged receptor in addition, one has the possibility to enrich receptor bound complexes prior to enzymatic digestion and subsequent analysis. Due to the high motility of ACKR4 in steady state conditions, we assume it to be regulated by two distinct sets of protein interactors. One which leads to the steady state trafficking along with a basal phosphorylation such as in the case of ACKR2 (McCulloch et al., 2008) and steady state β-arrestin recruitment, as well as a second set, which enhances the uptake, possibly shifting it to a different recycling route. So far, only a clathrin and RAB5 independent route was proposed for ACKR4 trafficking (Comerford et al., 2006), which opens the possibility, that β-arrestin itself does not always lead to a clathrin dependent trafficking. (Luttrell & Lefkowitz, 2002) Besides, Comerford et al., 2006 calculated a ligand intake of 6-7 CCL19 molecules per ACKR4 per hour, resulting in an extremely fast turn over, which was further increased with growing ligand concentrations. This contrasts with e.g. CCR7, which, depending on cell type, starts to recycle back to the membrane roughly after 30 minutes up to one hour. (Schaeuble et al., 2012) How β-arrestins interact in this regard has to be further elucidated. β-arrestins itself have been shown to react to different phosphorylation patterns induced by different ligands via the specific recruitment of GRKs (Zidar et al., 2009). In the mentioned study, CCR7 stimulated with CCL19 led to GRK3 and GRK6 recruitment, whereas

CCL21 lead to the sole recruitment and subsequent phosphorylation of GRK6. As for ACKRs, GRK recruitment has not been broadly investigated. For ACKR3, GRK2 has been shown to be recruited, and analogue to GRK5 to be able to phosphorylate ACKR3. (Saaber et al., 2019) We employed nanoluciferase (NLuc)-based GRK biosensors to investigate putative GRK recruitment. Interestingly, GRK2 as well as GRK3 were recruited to ACKR4, with GRK3 being more responsive. ACKR3 was shown to elicit its function depending on the intracellular phosphorylation, however not on β-arrestin recruitment. (Saaber et al., 2019) They used ACKR3$^{ST/A}$ mutant knock-in mice, lacking all GRK phosphorylation sites to successfully separate phosphorylation from β-arrestin mediated processes. As we do not have the convenient tools to determine the exact phosphorylation-patterns introduced by GRK2 and GRK3, we were so far unable to conduct studies along that line. Nevertheless, as mutants only affecting residues on intracellular loops of ACKR already showed a tendency towards reduced β-arrestin interaction, we suppose a multisite phosphorylation by GRKs as proposed for GRK5 and β2-adrenergic receptor. (Komolov et al., 2017) Interestingly, ACKR3ST/A mice were viable in contrast to complete ACKR3 deficient mice, yet, their CXCL12 distribution was impaired and led to misguided interneuron migration during development. (Saaber et al., 2019) Whether Schwann cells of those mice are still able to induce signalling as observed by Ödemis et al., 2010 has not been investigated. This would lead to new insights into the ACKR mediated signalling, e.g. whether other proteins in their inactive conformation could lead to different signalling routes. ACKRs have been known not to signal through classical signalling cascades via the activation of Gα proteins. In this regard, no calcium flux or cAMP activation by ACKR4 was observed. (Gosling et al., 2000; Townson & Nibbs, 2002) However, ACKR4 was shown to induce cAMP signalling after being

treated with pertussis toxin over night. Therefore, Watts et al., 2013 concluded that ACKR4 most likely associates with $G\alpha_i$, and once they are removed from the system, signalling through Gαs or Gαq occurs. Thus, the CRE response element translocates as a downstream signal of cAMP activation. For this reason, we further investigated if ACKR4 can couple with members of any of the G-protein families. We generated functional biosensors by fusing the NLuc into the loop after the second helix of Gα, which is distant from receptor and Gβγ interaction sites. In a first approach, we verified the functionality with CCR7, detecting a clear activation of e.g. $G\alpha_i$ as well as a pre coupling. This is in line with previous structure findings showing pre coupling of G-proteins with inactive class A GPCRs in order to quickly respond to low amounts of ligands. (Civciristov et al., 2018) However, no recruitment or high basal pre coupling of G-proteins with ACKR4 was observed. Analogue, we did not detect any ACKR4 mediated signalling along the Akt or MAPK signal cascade. Overall, ACKR4 conformational changes and activation most likely follows a similar pattern as in cCKRs and as shown with ACKR3 (Gustavsson et al., 2017), as most switches in the structure of ACKR4 except the DRYLAIV motif are present, such as the transmission/toggle switch, a sodium binding site and the NPxxY motif. Other switches, like the CWxP or the ionic lock are not present in either of ACKR4's related cCKRs, which are still able to signal through G-proteins. Still, ACKR4 most likely opens to interact with GRKs and interacts with the finger loop of β-arrestin, as it is properly internalised into vesicular structures together with β-arrestin. Albeit the receptor having to open for GRK and β-arrestin binding, no activation of G-proteins or pre-association was observed. It is highly likely, that further mutations aside the DRYLAIV motif influence the G-protein binding barcode, as an exchange of ACKR4's ICL2 had no effect on G-protein signalling. (Watts et al.,

2013) Nevertheless, all its ligands supposedly lead to the same activation of GRKs and β-arrestins, which contrasts with other reports of its ligands on CCR7. (M. A. Hauser et al., 2016; Kiermaier et al., 2016; Zidar et al., 2009) Those reports were partially explained by different TM rearrangement by the different ligands (Gaieb & Morikis, 2017), which indicates a more uniform recognition of the different ligands by ACKR4 and an overall similar TM rearrangement. XL-MS, as well as small-angle X-ray scattering (SAXS) or NMR studies, as done for ACKR3, would give rise to a more detailed view on the movement of ACKR4's TMs. (Gustavsson et al., 2017; Koukos & Bonvin, 2020) It is a possibility, that the steady state interaction with β-arrestin stabilises ACKR4 to be primed already for fast ligand recognition, as β-arrestin was shown to stabilise ACKR2 as well. (McCulloch et al., 2008) The fact that ACKR4 is associated with β-arrestin, however is still able to function without, opens the possibility, that still unknown signal transducers or scaffold proteins regulate the different modes of ACKR4 function in steady state and induced conditions. Candidates regulating ACKR4 trafficking can either be further kinases, such as casein kinase 2 (CK2), which was found in an Y2H screen of the total human liver proteome. (J. Wang et al., 2011) Still, CK2 is a special kinase, as it has over 300 substrates and is a constitutively active kinase. Therefore CK2 is a candidate for the regulation of steady state ACKR4 trafficking. (Pinna, 2002) In addition, chemokine receptor trafficking and function has been regulated by ubiquitination. For example, the constitutive ubiquitination of CCR7 resulted in recycling to the membrane and continuous migration of transfected cells, whereas a ubiquitination deficient CCR7 was retained in the trans-Golgi network after initial ligand binding and uptake.(Schaeuble et al., 2012) Along that line, ACKR3 C-terminus is constitutively ubiquitinated and is indeed deubiquitinated in response to CXCL12 binding. (Canals et al.,

2012; Hoffmann et al., 2012) Moreover, ACKR4's gene locus is evolutionary liked with the locus for UBA5, ACAD11 and NAHP. (Pan et al., 2018) UBA5, the Ubiquitin Like Modifier Activating Enzyme 5, is an E1 enzyme with a smaller size than normal E1. (Bacik et al., 2010) The enzymatic cascade of ubiquitin transfer consists of three subsequent steps. However, besides the E3 ligases in the last step, certain E2's can already transfer ubiquitin to substrate themselves. (Rape, 2018) As there are over 40 E2 and 600 E3 enzymes, it remains highly speculative how UBA5 might play a non-redundant role in ACKR4 function. Nonetheless, whether ACKR4 ubiquitination is only occurring for regulation purposes, i.e. to label ACKR4 for degradation, or if ubiquitination influences ACKR4 function is of great interest. ACKR4 ubiquitination could either help to constitutively recycle ACKR4 or ubiquitination could occur once ACKR4 is binding ligands to switch the recycling pathway to a faster route. If, ubiquitination also has an influence on the binding of β-arrestin in this case, must be elucidated. Considering the effect of ACKR4 on cancer proliferation in mammary carcinoma, which could not have been attributed to its scavenger function, putative signalling pathways remain to be discovered. Another option for β-arrestin engagement would be to help as a scaffold for further signalling cascades. Due to the high dynamic nature of ACKR4, its signalling effects might be more subtle, especially when influencing transcription changes working on a long-term time scale. On the one hand, one could employ fluorescent antibodies and perform microscopic assays, as was done to discover site specific ACKR3 mediated ERK signalling. (Rajagopal et al., 2010) On the other hand, more transcriptomic studies of cancer cells expressing ACKR4 are of interest. As a preliminary approach to find cells affected by ACKR4 signalling, Dynamic Mass Redistribution (DMR) assays can be used to screen efficiently for suitable cancer cell lines. In DMR assays, overall changes

in cell mass rearrangement are monitored using biosensor-containing assay plates, without manipulating the cells of interest, besides the application of ligands. (Codd et al., 2011) Next, the comparison of ACKR4^{high} and ACKR4^{low} cells using e.g. mRNA analysis as done to compare the ACKR4$^{GFP/GFP}$ and the ACKR4$^{-/-}$ mouse can give new insights into ACKR4 mediated transcriptomic changes. (Eckert et al., 2019)

3.3 CCL20 as a Novel ACKR4 Ligand

As of today, several chemokines have not yet been associated with a corresponding ACKR. Based on sequence similarity, focusing on physicochemical traits we found CCL20 as a putative ACKR4 ligand. By using a modelling approach, we first discovered several short interaction distances between ACKR4 and CCL20 which are remarkably like ACKR4 and CCL19. Moreover, using the same approach, we proposed CCL22 to be another ligand. CCL22 was simultaneously found with CCL20 and CCL17 as ACKR4 ligands by Meyrath et al., 2020a. Second, using in mice (in vivo) as well as with transfected HeLa cells (in vitro), we confirmed CCL20 to be a ligand for ACKR4 but not ACKR3 in both organisms. Interestingly, we found a species selectivity for human ACKR4, which recruits β-arrestin much stronger when stimulated with human than with mouse CCL20. This species difference was not found in any of the other three investigated ligands (CCL19, CCL21 and CCL25). CCL20 binds less deep into the receptor pocket and interacts with less amino acids to induce a conformation change as seen in the CCR6 CCL20 structure. (Wasilko et al., 2020) Therefore, we assume that mutations in CCL20 core structure have a more pronounced effect on receptor activation. Besides, the species-

specific mutation percentage was higher in CCL20 compared to the other three ligands. Nevertheless, ACKR4 was still able to scavenge CCL20, no matter the species. This again is in line with our previous publication, where we determined an enhancing but not a crucial role for β-arrestin in receptor scavenging. (Matti et al., 2020b)

The chemokine receptor and ligand binding can be further influenced by glycosylation or sulfation of the receptor N-terminus. (M. A. Hauser et al., 2016; Simpson et al., 2009) How this influences the interaction of ACKR4 with its ligands has not been studied and was not taken into account by our modelling approach. As the binding of CCL21 to CCR7 is influenced by glycosylation as well as sulfation, similar effects are possible for ACKR4. (M. A. Hauser et al., 2016; Phillips et al., 2017) ACKR4 possesses several asparagines which are proposed to be glycosylated as they are situated in Asn-Xaa-Thr/Ser motifs. (Wheatley & Hawtin, 1999) Moreover, a triple tyrosine motif is very likely to be sulphated in ACKR4's N-terminus. (Monigatti et al., 2002) So far, various methods have been employed to study the influence of receptor PTMs, be it sulphated peptides derived from CCR7 N-terminus, which showed a higher affinity for CCL21 (Phillips et al., 2017) or to use a combination of site-directed mutagenesis to remove asparagine residues and employ metabolic labelling with sugar derivates to identify and characterise the glycosylation sites. (M. A. Hauser et al., 2016) Furthermore, the glycan structure of the receptor could be characterised by immunoprecipitation followed by PNGase F digestion to isolate the glycan, which are further characterised using HPLC, as was done for e.g. monoclonal antibodies. (Arlauckas et al., 2017) Of course, this would either require the possibility to use a knock-in mouse, or to transfect primary cells or a suitable cell line of e.g. kreatinocyte or fibroblastic origin. It is of high interest, if the PTMs of ACKR4 are another layer of receptor regulation besides the distinct

expression to certain cell locations. As a consequence, one could imagine specific glycosylation patterns, which, depending on the tissue localisation of ACKR4, would then favour the scavenging of either CCL19 or CCL21.

The less deep binding of CCL20 was reflected in the higher K_D compared to the other ACKR4 ligands. This again might be a reason, why CCL20 was not discovered in the initial ligand screening assays performed by Gosling et al., 2000. They used radioactive labelled CCL19 and competed its binding to ACKR4 with a broad range of native chemokines. Interestingly, they did observe an approximate 25% competition by CCL20, however determined it as to be too low to reach their threshold. Moreover, chemokines like CCL5, CCL7, CCL11, CCL18, CCL22, CXCL9 or CX3CL1 induced a stronger competition than CCL20. Of those, Meyrath et al., 2020a found a significant recruitment of arrestins to ACKR4 upon stimulation with CCL7 and CCL22, and a tendency by CCL5 and CCL17 among others. CCL5 would be a logical addition to the ACKR4 repertoire, as it shares the cCKR CCR4 together with CCL17 and CCL22. In addition, some CXC chemokines like CXCL13 and CXCL12 resulted in a minor fluctuation in the signal. If e.g. CXCL12 plays a similar role in chemokine cooperativity for ACKR4 ligands as CXCL13 does can be an interesting study direction, further diversifying the roles of CXCL12. Moreover, Meyrath et al., 2020a found recruitment upon stimulation with the viral chemokine vCCL2, known as vMIP-II and vCCL1, known as vMIP-I. vCCL2 competition has already been observed by Gosling et al. Human herpesvirus-8 infected cells produce vCCL2, which binds to a extended range of chemokine receptors and vCCL1, which binds exclusively to CCR8. (Dairaghi et al., 1999) Thereby, vCCL2 helps the virus to survive and escape the immune response. (Szpakowska & Chevigné, 2016) In addition, Gosling et al., 2000 showed a competition by

vCXCL1, which was not examined by Meyrath et al., 2020a. vCXCL1 is another viral chemokine produced by the human cytomegalovirus and attracts neutrophils mainly via its interaction with CXCR2, however is also able to bind to CXCR1 and CX3CR1. (Yamin et al., 2016) How exactly a scavenging receptor influences the efficacy of viral clearance has not been investigated. As ACKR4 is expressed along barrier sites and is able to interact with vCXCL1, vCCL1 and vCCL2, this definitively demands more research.

On a systemic scale, ACKR4 function and identifying all its ligands is of crucial importance. The dysregulation of CCR7, together with its ligands often leads to a malfunction of the immune system, such as the induction of overreaction of the immune response via the generation of TLOs. (Marinkovic et al., 2006) CCR9 together with its ligand CCL25 is known to play a big part in autoimmune disorders such as IBD. (Linton et al., 2012) Furthermore, ACKR4 ligands and their cCKRs can crosstalk with each other, creating a more diverse response. As an example, CCR4, which is the receptor for CCL22 and CCL17, can crosstalk with CCR7 in Langerhans cells and thereby promotes CCR7 dependent migration. (Stutte et al., 2010) All those interactions can be regulated by ACKR4. The newly discovered ligand CCL20 is important for diseases such as IBD. (Trivedi & Adams, 2018) Moreover, CCL20 together with its cCKR CCR6 have been shown to be responsible for TH17 recruitment. (Reboldi et al., 2009) TH17 cells are recruited to resolve a plethora of infections, such as fungi, virus, and bacteria. This brings a new importance to the role of ACKR4, not only being important for homeostasis but also for regulating trafficking of activated T cells. The discovery of CCL20 as a novel ligand for ACKR4 also explains previous findings with ACKR4 KO animals. Comerford et al., 2010 already observed an impairment of $CD4^+$ cells migrating into the LN in ACKR4 KO animals, using CCR6 route, and moreover an ear-

lier MS onset. Previously they attributed this effect solely to the T cell priming in the spleen, whereas we now suppose that in addition more TH17 were able to migrate into the central nervous system (CNS) due to the higher CCL20 occurrence. Moreover, the role of TH17 cells in autoimmune diseases such as MS brings a new focus on the expression of ACKR4 along the CNS and how it regulates immune cell patrol functions. In contrast to ACKR3 which was shown to further regulate the abundance of opioid neurotransmitters, no such role has been discovered for ACKR4 so far. (Meyrath et al., 2020b) However, as ACKR4 is activated by CCL20, which utilises a receptor mode more reminiscent of muscarinic receptors without activating the switch in the TM7 core, it is highly interesting, to look for additional scavenging functions of ACKR4 in the CNS. Moreover, the specialised localisation and expression of ACKR4 was shown to lead to a specific expression of endothelial cell regulators. (Thomson et al., 2018) Therefore, it is interesting to see, if ACKR4 is also regulating its environment in other barrier sites such as along lymph vessels or the subcapsular sinus of the LN or along the blood-brain barrier (BBB). Moreover, it is possible that this expression can be further guided by inflammatory stimuli or ACKR4 ligand interaction. On the one hand, extended screening for other ligands such as non-Opioid neurotransmitters might be highly interesting. To this end, BRET experiments with β-arrestin recruitment to ACKR4 as a read-out on a high scale using 384 well plates and compound libraries could be a very efficient starting point for future studies. On the other hand, the isolation of ACKR4 expressing cells using the developed CCL25_19 chimera from sites along the BBB and performing transcriptomic analysis in line with Thomson et al., 2018 might be a promising approach.

3.4 Conclusion

Overall, we set out to investigate ACKR4's signalling and regulation function. On this journey, we successfully developed new tools to study receptor function, such as various fluorescent ligands for ACKR4, CCR6 and CCR7 and biosensors for GRKs and G-proteins. Further, we extended ACKR4's ligand repertoire with CCL20 and provided some modelling insight into the interaction of ACKR4 with its ligands. Finally, we cleared up the mist of β-arrestin's role in ACKR4 scavenging and thoroughly investigated possible interactions with G-proteins and GRKs. This resulted in the new finding, that GRK3, and to a minor extent GRK2, are recruited to ACKR4 prior to β-arrestins and that β-arrestins are boosting ACKR4 ligand uptake.

Appendix

1 Author Contributions

In this section I delineate what I contributed to the publications included in my PhD dissertation.

Fluorescently Tagged CCL19 and CCL21 to Monitor CCR7 and ACKR4 Functions	I contributed to discussion and writing of the project and performed some of the experiments.
ACKR4 Recruits GRK3 Prior to β-Arrestins but Can Scavenge Chemokines in the Absence of β-Arrestins	I designed, performed, and analysed large parts of the study and co-authored the manuscript.
CCL20 is a novel ligand for the scavenging atypical chemokine receptor 4	I designed, performed, and analysed the majority of the study and co-authored the manuscript.

2 List of Publications

Besides the three publications which are in focus of this PhD thesis, I contributed to several projects associated within the lab of Prof. Dr. Daniel F. Legler and which are herein listed.

Legler, D. F., **Matti, C.,** Laufer, J. M., Jakobs, B. D., Purvanov, V., Uetz-von Allmen, E., & Thelen, M. (2017). Modulation of Chemokine Receptor Function by Cholesterol: New Prospects for Pharmacological Intervention. Molecular Pharmacology, 91(4), 331–338.

Purvanov, V., **Matti, C.,** Samson, G. P. B., Kindinger, I., & Legler, D. F. (2018). Fluorescently Tagged CCL19 and CCL21 to Monitor CCR7 and ACKR4 Functions. International Journal of Molecular Sciences, 19(12), 3876.

Jakobs, B. D., Spannagel, L., Purvanov, V., Uetz-von Allmen, E., **Matti, C.,** & Legler, D. F. (2019). Engineering of Nanobodies Recognizing the Human Chemokine Receptor CCR7. International Journal of Molecular Sciences, 20(10), 2597.

Matti, C., Salnikov, A., Artinger, M., D'Agostino, G., Kindinger, I., Uguccioni, M., Thelen, M., & Legler, D. F. (2020). ACKR4 Recruits GRK3 Prior to β-Arrestins but Can Scavenge Chemokines in the Absence of β-Arrestins. Frontiers in Immunology, 11(April), 1–16.

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Matti, C., & Legler, D. F. (2020). CCR5/CCR5Δ32: resistant to HIV infection at the cost of curtailed $CD4^+$ T cell memory responses. The EMBO Journal, 5–7.

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Acknowledgements

At this point I would like to express my gratitude towards all those who supported me during my PhD.

First, I want to thank my thesis supervisor Prof. Dr. Daniel F. Legler. He gave me the opportunity to perform my PhD thesis in this extraordinary environment with this very exciting topic. I greatly appreciated Daniel's literature discussions during my PhD and his patience in discussing my progress. Moreover, I am very thankful for his support during the last year when we had to finalise several papers and revisions simultaneously.

I also would like to thank in advance Prof Dr. Martin Scheffner and Prof. Dr. Ralf Stumm (Universitätsklinikum Jena) for being the co-examiners of my thesis and defence.

Next, I would like to thank Prof. Dr. Marcus Thelen (Institute for Research in Biomedicine, Bellinzona) and Prof. Dr. Valentin Wittmann, the members of my thesis committee, for their helpful suggestions during my progress meetings.

Moreover, I would like to thank all collaborators of the Sinergia project for the scientific and the non-scientific discussions during our meetings, which always provided new inputs for the projects.

Furthermore, I would like to thank all members of the Konstanz Research School in Chemical Biology which I got to know over the years. I really appreciated the scientific and non-scientific exchange during the poster sessions, the interesting coursework, and the retreats. In

addition, I am incredibly grateful to have received the possibility to be representative of the doctoral students for one year together with Eva, which extremely boosted my organisational and communication skills.

All current and past members of the BITg made my work here an enjoyable experience, even when things did not look as good as they should. I like to thank Nico, Guerric, Verena, Anni, Pascal, Johanna, Julia, Ilona, Vladimir and especially Cris, Marc and Oli. All of those are great co-workers and friends, with whom I enjoyed performing experiments, drinking a coffee to get some new ideas and laughing once upon a time or dance all night long.

I like to thank all students during their theses or practical courses which I could hopefully teach loads of new things and show them how exciting and fun science can be! This goes to Marc, Tobias, Jakob, Oli, Angela, Lisa, Michail and Janine.

In addition, I highly appreciated the support received by Daniel, Daniel F., Leo, Marc and Oli while writing my thesis.

Finally, I want to thank my family and my close friends for their endless love and support! You cheered my up during the thesis when I found myself lost and needed some motivation. You have my greatest thanks for being patient when I needed to focus on my work and supporting me as good as you all could!