

## BRIEF CONCLUSIVE REPORT

# Systematic reassessment of chemokine-receptor pairings confirms CCL20 but not CXCL13 and extends the spectrum of ACKR4 agonists to CCL22

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## Abstract

Atypical chemokine receptors (ACKRs) have emerged as important regulators or scavengers of homeostatic and inflammatory chemokines. Among these atypical receptors, ACKR4 is reported to bind the homeostatic chemokines CCL19, CCL21, CCL25 and CXCL13. In a recent study by Matti et al., the authors show that ACKR4 is also a receptor for CCL20, previously established to bind to CCR6 only. They provide convincing evidence that, just as for its other chemokine ligands, ACKR4 rapidly internalizes CCL20 both in vitro and in vivo. Independently of this discovery, we undertook a screening program aiming at reassessing the activity of the 43 human chemokines toward ACKR4 using a highly sensitive  $\beta$ -arrestin recruitment assay. This systematic analysis confirmed CCL20 as a new agonist ligand for ACKR4 in addition to CCL19, CCL21, and CCL25. Furthermore, CCL22, which plays an important role in both homeostasis and inflammatory responses, and is known as a ligand for CCR4 and ACKR2 was found to also act as a potent partial agonist of ACKR4. In contrast, agonist activity of CXCL13 toward ACKR4 was disproved. This independent wide-range systematic study confirms the pairing of CCL20 with ACKR4 newly discovered by Matti and co-authors, and further refines the spectrum of chemokines activating ACKR4.

## KEYWORDS

ACKR2, ACKR3, ACKR4, CCL20, CCL22, CCR4, CCR6, CCR7, CCX-CKR

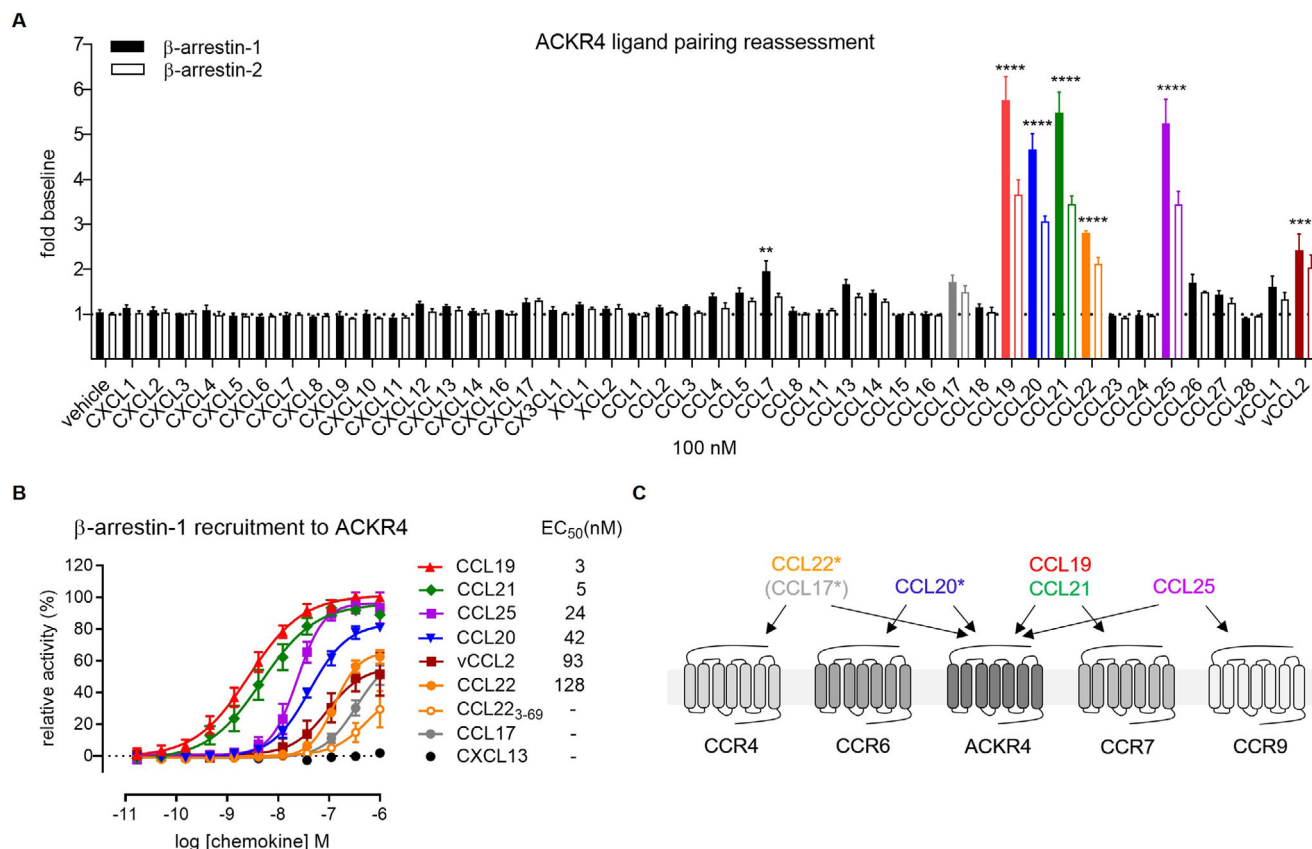
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Atypical chemokine receptors (ACKRs) form a subfamily of 4 chemokine receptors unable to trigger G protein-dependent signaling or to directly induce cell migration in response to chemokines.<sup>1</sup> ACKRs play however a crucial role in chemokine biology by capturing, scavenging, or transporting chemokines, thereby regulating their availability and signaling through classical chemokine receptors.<sup>2,3</sup> Among ACKRs, ACKR4, formerly known as CCX-CKR, CCR11, or CCRL1, which is expressed on keratinocytes, astrocytes, lymphatic endothelial cells and on thymic epithelial cells, is an important regulator of homeostatic chemokines.<sup>4–6</sup>

Abbreviations: ACKR, atypical chemokine receptor; CD, cluster of differentiation; DC, dendritic cell; HHV-8, human gammaherpesvirus 8; vMIP-II, viral macrophage inflammatory protein II.

Human ACKR4 was orphanized in 2000 based on competition studies with radiolabeled CCL19.<sup>7</sup> It was initially proposed to bind CCL19, CCL21, CCL25, and CXCL13, which are the ligands for CCR7, CCR9, and CXCR5, respectively.<sup>7,8</sup> Of note, CXCL13 interaction with the mouse ACKR4 could not be confirmed<sup>5</sup> and the human CXCL13-ACKR4 pairing inferred from binding competition studies<sup>7</sup> was later reevaluated and the observations reattributed to cooperative GAG binding rather than direct receptor interactions.<sup>9</sup>

By scavenging its chemokine ligands, ACKR4 was shown to regulate mainly the trafficking and positioning of T-cells and dendritic cells (DCs).<sup>6,10</sup> ACKR4 is best known for its role in shaping the gradient of CCL19 and CCL21 for CCR7-expressing DCs in the subcapsular sinuses of lymph nodes during the initiation phase of the adaptive immune response.<sup>11,12</sup> ACKR4 is also involved in antitumor immunity and modulates epithelial-mesenchymal transition and metastasis,<sup>13–15</sup> and studies with ACKR4-deficient mice in an EAE model also demonstrated the receptor implication in autoimmune diseases notably by



**FIGURE 1** Systematic reassessment of human ACKR4 activation by chemokines using a highly sensitive  $\beta$ -arrestin recruitment assay based on NanoBIT technology. (A)  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2 recruitment to ACKR4 in response to all known human and 2 viral chemokines (100 nM). Experiments were conducted in U87 cells as previously described.<sup>20</sup> For statistical analysis, 1-way ANOVA with Dunnett's multiple comparison test with vehicle as reference was performed. \*\* $P < 0.01$  ( $\beta$ -arrestin-1 only), \*\*\*\* $P < 0.0001$  (in both  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2 recruitment assays). (B)  $\beta$ -arrestin-1 recruitment to ACKR4 by the previously reported and newly identified chemokines CCL17, CCL19, CCL20, CCL21, CCL22, CCL25, CXCL13, and vCCL2, showing the concentration-response relationship.  $EC_{50}$  values are indicated (nM). (A and B) Data are represented as mean  $\pm$  SEM of at least 3 independent experiments. (C) Schematic representation of the interactions between ACKR4 and CC receptors CCR4, CCR6, CCR7, and CCR9, and their shared endogenous chemokines. The newly identified ligands are indicated with an asterisk

accelerated disease onset and more severe symptoms attributable to increased Th17 response.<sup>4,16</sup> Of note, 2 ACKR4-deficient mouse strains (ACKR4<sup>-/-</sup> and ACKR4<sup>GFP/GFP</sup>) are available and display distinct phenotypes. While ACKR4<sup>-/-</sup> mice show a strong accumulation of plasma blasts in mesenteric lymph node and spleen as well as increased B cell proliferation after in vitro activation,<sup>17</sup> B cells from ACKR4<sup>GFP/GFP</sup> mice exhibit a phenotype comparable to wild-type cells,<sup>18</sup> suggesting that the results from the above-mentioned ACKR4-deficient mouse studies should be interpreted cautiously.

In a recent study, Matti and co-authors reported CCL20, previously established to bind to CCR6 only, as a novel chemokine ligand for ACKR4.<sup>19</sup> The authors had predicted the existence of this interaction based on CCL20 sequence and expression similarities with CCL19 and CCL21. They showed that CCL20 induces  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2 recruitment to ACKR4, and is rapidly internalized and scavenged by ACKR4-expressing cells, both in vitro and in vivo. They proposed that by scavenging CCL20, ACKR4 regulates its availability for the classical signaling receptor CCR6 and thereby plays a role in the positioning of CCR6-positive leukocytes within

secondary lymphoid tissues to initiate effective humoral and memory immune responses.<sup>19</sup>

Independently of this discovery, we undertook a systematic screening program aiming at reassessing the agonist activity of the 43 human chemokines (24 CCLs, 16 CXCLs, 2 XCLs, and 1 CX3CL) and 2 viral chemokines (vCCL1 and vCCL2) toward ACKR4, by monitoring  $\beta$ -arrestin recruitment to the receptor using a highly sensitive NanoLuciferase complementation-based assay (NanoBIT) (Fig. 1A).<sup>20-22</sup> In agreement with the initial description by Matti and co-authors, our systematic analysis also identified CCL20 as an ACKR4 ligand capable of inducing  $\beta$ -arrestin-1 and  $\beta$ -arrestin-2 recruitment to the receptor and behaving as a partial agonist (80% efficacy) with a potency ( $EC_{50}$  = 42 nM) somewhat lower than potencies observed for CCL19 ( $EC_{50}$  = 3 nM), CCL21 ( $EC_{50}$  = 5 nM), and CCL25 ( $EC_{50}$  = 24 nM) (Fig. 1B).

Besides CCL19, CCL20, CCL21, and CCL25, our systematic reassessment of chemokine-receptor interactions revealed that CCL22, known to bind to CCR4 and ACKR2, is also a ligand for ACKR4. CCL22 was slightly less potent ( $EC_{50}$  = 128 nM) than the other ligands

in inducing  $\beta$ -arrestin-1 recruitment to ACKR4 and acted as a partial agonist, displaying about 60% of the maximum efficacy observed with CCL19. Interestingly, the CCL22 variant lacking the first 2 N-terminal residues (CCL22<sub>3-69</sub>) and hence mimicking the dipeptidyl peptidase 4 (DPP4 or CD26)-cleaved chemokine, retained significant activity toward ACKR4, which contrasts with its absence of activity toward ACKR2<sup>23</sup> but is reminiscent of the agonist effect of processed CXC chemokines toward ACKR3<sup>20,24</sup> (Fig. 1B).

CCL22 was not the only CCR4-related chemokine acting on ACKR4. CCL17, the second ligand of CCR4, showed detectable activity toward ACKR4 but statistical significance was not reached for this pairing. A similar low activity was also observed for several other human CC chemokines including CCL7, CCL13, CCL14, CCL26, and CCL27, which are ligands for CCR1, CCR2, CCR3, and CCR10. Moreover, the viral broad-spectrum antagonist chemokine vCCL2/vMIP-II encoded by the Kaposi sarcoma-associated herpes virus HHV-8,<sup>25</sup> known to bind to ACKR4,<sup>7</sup> and to activate ACKR3,<sup>26</sup> also behaved as a partial agonist of ACKR4 (EC<sub>50</sub> = 93 nM) inducing 50% of the maximum efficacy observed with CCL19. In contrast, CXCL13, for which discordant observations have been reported regarding its interaction with ACKR4,<sup>7,9</sup> did not induce  $\beta$ -arrestin recruitment to ACKR4 in our assay, narrowing down ACKR4 specificity to CC chemokines only. In a similar screening conducted with all 45 chemokines (100 nM) in antagonist mode, that is, in the presence of CCL19 at a concentration equivalent to its EC<sub>50</sub> (3 nM), no chemokine other than the above-mentioned agonists was able to modulate CCL19-induced  $\beta$ -arrestin recruitment to ACKR4 (data not shown).

A phylogenetic analysis based on amino acid sequences shows that CCL22 clusters together with all other ACKR4 ligands, including CCL20, further supporting its pairing with ACKR4. Moreover, CCL22 is constitutively expressed in lymphoid tissues, the intestine, lung, and skin, where ACKR4 is also expressed.<sup>4,10</sup> CCL22 acts as a potent agonist for CCR4, primarily expressed on Th2 cells<sup>27,28</sup> but it has also been described to be involved in self-tolerance and mediate DC-Treg interaction. Indeed, deletion of CCR4 or CCL22 in mice showed a disruption of T-cell immunity, which caused an accumulation of autoreactive T-cells and, as a result, autoimmune diseases.<sup>29,30</sup> Therefore, together with the reported acceleration of CD4<sup>+</sup> T-cell skewing toward Th17 in ACKR4-deficient mice,<sup>4</sup> one could postulate the involvement of ACKR4 in the CCR4/CCL22-axis to mediate effective T-cell immunity. The relevance of this newly identified interaction remains nevertheless to be established, especially considering the crosstalk of CCL22 with CCR4 and ACKR2. It also needs to be determined whether the interaction between CCL22 and ACKR4 holds true for the murine homologues.

Altogether, this systematic reassessment of chemokine-receptor interactions confirmed the pairing between CCL20 and ACKR4 in a different cellular background and using a different readout,<sup>31</sup> reinforcing the initial description by Matti and co-authors.<sup>19</sup> In addition, it identified another ligand for ACKR4, CCL22. These novel pairings add a level of complexity to ACKR4 interactions within the chemokine-receptor network and extend its regulatory functions

to the CCL20/CCR6 and CCL22/CCR4 axes (Fig. 1C). Nevertheless, complementary pharmacological experiments, including binding and scavenging or internalization assays, remain to be performed to validate these results. The importance of these novel interacting partners for ACKR4 in pathophysiological context also remains to be elucidated. Finally, our study focused exclusively on chemokine-induced  $\beta$ -arrestin recruitment and thus chemokines with different mode of action toward ACKR4 may have been overlooked.<sup>32</sup> Indeed, although ACKR4-driven chemokine scavenging is generally considered as dependent on  $\beta$ -arrestins, recent studies indicated that they are not essential for this activity, suggesting that further investigations are necessary to fully understand this still enigmatic receptor.<sup>8,33</sup>

## AUTHORSHIP

M.M., N.R.: data acquisition, analysis and interpretation, critical revision of the manuscript. T.U.: critical revision of the manuscript. A.C.: study conception, data analysis and interpretation, manuscript drafting and critical revision of the manuscript. M.S.: study conception, analysis and interpretation, manuscript drafting and critical revision of the manuscript. M.M., N.R., A.C., M.S. contributed equally to this work.

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## DISCLOSURE

The authors have declared no conflict of interest.

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