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Human herpesvirus 8-encoded chemokine vCCL2/vMIP-II is an agonist of the atypical chemokine receptor ACKR3/CXCR7

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ABSTRACT

The atypical chemokine receptor CXCR7/ACKR3 binds two endogenous chemokines, CXCL12 and CXCL11, and is upregulated in many cancers or following infection by several cancer-inducing viruses, including HHV-8. ACKR3 is a ligand-scavenging receptor and does not activate the canonical G protein pathways but was proposed to trigger β-arrestin-dependent signaling. Here, we identified the human herpesvirus 8-encoded CC chemokine vCCL2/vMIP-II as a third high-affinity ligand for ACKR3. vCCL2 acted as partial ACKR3 agonist, inducing β-arrestin recruitment to the receptor, subsequent reduction of its surface levels and its delivery to endosomes. In addition, ACKR3 reduced vCCL2-triggered MAP kinase and PI3K/Akt signaling through other chemokine receptors. Our data suggest that ACKR3 acts as a scavenger receptor for vCCL2, regulating its availability and activity toward human receptors, thereby likely controlling its function in HHV-8 infection. Our study provides new insights into the complex crosstalk between viral chemokines and host receptors as well as into the biology of ACKR3, this atypical and still enigmatic receptor.

1. Introduction

Chemokines are small (8–14 kDa) secreted proteins that play a central role in guiding directional migration (chemotaxis) of leukocytes in immunosurveillance and immune responses. They exert these functions by interacting and activating a family of seven-transmembrane domain G protein-coupled receptors (GPCRs). To date, 47 chemokines and 19 receptors have been identified in humans. Based on conserved cysteine motifs, chemokines are divided into four subfamilies: CC, CXC, XC, CX3C and the receptors are named according to the subfamily of chemokines they bind (CCR, CXCR, XCR and CX3CR) [1]. The chemokine-receptor network is highly intricate and a chemokine can bind one or many receptors, while a receptor usually recognizes several chemokines [2]. In addition, other receptors referred to as atypical chemokine receptors (ACKR1–4) can recognize chemokines and act as scavengers or signal through alternative pathways, further contributing to the complexity of the chemokine network [3,4].

Various pathogens have evolved ways to subvert and exploit the immune processes regulated by chemokines and their receptors in order to promote their survival and propagation. Viruses from different families (e.g. poxviruses, herpesviruses or retroviruses) encode chemokine-binding proteins, chemokine receptors as well as chemokine analogs or envelope proteins that hijack cellular chemokine receptors [5,6]. Human herpesvirus 8 (HHV-8), also known as the Kaposi's sarcoma-associated herpesvirus (KSHV), strikingly illustrates such molecular mimicry and its role

Abbreviations: ACKR, atypical chemokine receptor; CCKAR, cholecystokinin A receptor; CCL, chemokine (CC motif) ligand; CHO, Chinese hamster ovary; CXCL, chemokine (CXC motif) ligand; CX3CL, chemokine (CX3C motif) ligand; EBV, Epstein–Barr virus; ERK, extracellular receptor kinase; GFP2, green fluorescent protein 2; GPCR, G protein-coupled receptor; HCMV, human cytomegalovirus; HEK, human embryonic kidney; HHV-8, human herpesvirus 8; HTLV-1, human T-cell leukemia virus 1; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpesvirus; mAb, monoclonal antibody; MAP, mitogen-activated protein; MCD, multicentric Castleman disease; ORF, open reading frame; PEL, primary effusion lymphoma; PI3K, phosphoinositide-3 kinase; PRF-DMEM, phenol red-free Dulbecco's modified Eagle medium; PTX, pertussis toxin; Rab5, Ras-related protein 5; Th1/Th2, type 1 or 2 T helper cells; vCCL2/vMIP-II, viral CC motif chemokine 2/viral macrophage inflammatory protein II; XCL, chemokine (C motif) ligand.

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in virus pathogenesis. HHV-8 causes Kaposi's sarcoma (KS), a disease generally linked with immunodeficiency, but also two rare proliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman disease (MCD). HHV-8 infects endothelial cells in KS and is found in B lymphocytes from PEL and MCD lesions and the peripheral blood of KS patients [7]. The HHV-8 genome encodes one chemokine receptor, ORF74, and three CC chemokines, vCCL1/vMIP-I, vCCL2/vMIP-II and vCCL3/vMIP-III. Unlike vCCL1 and vCCL3, vCCL2 interacts with a broad spectrum of receptors and is the only chemokine reported so far capable of binding to receptors of the four classes [8]. Besides downregulating the activity of ORF74 [9], vCCL2 binds to host CC receptors (CCR1, CCR2, CCR3, CCR5, CCR8 and CCR10) but also to the two single representatives of XC and CX3C receptor families (XCR1 and CX3CR1) and to only one CXC receptor (CXCR4) [10,11]. Although vCCL2 is generally described as an antagonist chemokine, it also acts as an agonist toward two CC receptors, CCR3 and CCR8 [8,12,13]. Through its selective antagonist action on Th1-specific chemokine receptors (CCR1, CCR2, CCR5 and CX3CR1) and agonist effect on Th2-related CCR3 and CCR8, vCCL2 is proposed to play an important role in the skewing of the host immune system away from the Th1 cytotoxic response toward a Th2 response, which is less effective against intracellular pathogens [8,13]. Additionally, by activating CCR8, vCCL2 is proposed to contribute to virus pathogenesis through an autocrine pro-survival action or angiogenic effect on neighboring cells [13–17]. Up to now, the interaction of vCCL2 with the atypical chemokine receptors and especially with the most recently deorphanized chemokine receptor, CXCR7/ACKR3, has not been reported.

ACKR3, formerly CXCR7, is expressed in various cells such as B and T lymphocytes, neurons and endothelial cells and plays a crucial role in many processes including cardiovascular and neuronal development as well as in migration and homing of hematopoietic stem/progenitor cells [18–24]. An increasing number of studies point to the involvement of ACKR3 in many cancers. ACKR3 is expressed in various cancer cell types as well as on tumor-associated vasculature and accumulating evidence demonstrates its involvement in metastasis development [25–28]. ACKR3 was also shown to be upregulated upon infection by several cancer-inducing viruses including HHV-8, EBV, HTLV-1 and to play an important role in cell transformation and proliferation [29,30]. Due to its unusual biology, it has recently been classified as an atypical chemokine receptor [3,31]. Indeed, ACKR3 binds two endogenous chemokines, CXCL12 and CXCL11, which are also recognized by CXCR4 and CXCR3, respectively but unlike conventional chemokine receptors, ACKR3 does not activate the canonical G protein pathways and is proposed to trigger β-arrestin-dependent signaling. In addition, through its continuous cycling between the plasma membrane and endosomal compartments and its capacity to efficiently internalize and degrade chemokines, ACKR3 functions as a scavenger receptor regulating the availability of CXCL12 and CXCL11 for CXCR4 and CXCR3 [32–34]. Moreover, ACKR3 was proposed to modulate the activity of CXCR4 by forming heterodimers or competing for intracellular effector proteins involved in signal transduction [35–38]. However, the exact molecular basis of the interactions of ACKR3 with its ligands, its ability to signal and its crosstalk with other receptors is poorly understood and still a matter of debate. Besides, many questions remain unanswered regarding the physiological roles of ACKR3, how it partakes in the complex chemokine-receptor network and whether it can also be exploited by virus-encoded chemokines or the opposite, thwart their action.

In this study, we identified vCCL2, a chemokine encoded by HHV-8, as a new ligand for ACKR3. We showed that in addition to its unexpected agonist effect toward this atypical receptor, vCCL2 may also be subject to scavenging by ACKR3, regulating its activity on other human chemokine receptors that may be important for virus biology.

2. Methods

2.1. U87.ACKR3 cell line validation

U87 cells obtained through the NIH AIDS Reagent Program from Dr. Deng and Dr. Littman [39] were transfected using Lipofectamine (Life Technologies) with a pBABE-puro vector (Addgene) containing the ACKR3 sequence (Uniprot: P25106) optimized for mammalian expression or ACKR3 sequence C-terminally fused to GFP2. Stable U87.ACKR3 and U87.ACKR3-GFP2 cell lines were obtained following puromycin selection and subsequent single cell sorting. Chemokine receptor expression at the surface of these cell lines was analyzed by flow cytometry using mAbs specific for ACKR3 (clones 11G8 (R&D Systems) and 8F11 (BioLegend)), CXCR4 (clones 4G10 (Santa Cruz Biotechnology) and 12G5 (BD Biosciences)), CXCR3 (clone 1C6 (BD Biosciences)), and allophycocyanin-conjugated $F(ab')_2$ fragment of anti-mouse IgG (Jackson ImmunoResearch).

2.2. Binding competition with fluorescently labeled CXCL12

Alexa Fluor 647-labeled CXCL12 (40 ng/ml) (Almac) was mixed with unlabeled CXCL12, CXCL11, vCCL2, CCL3, CCL4, CCL15, CCL18, CXCL14 (Peprotech) or vCCL1 (R&D Systems) at concentrations ranging from 6 pM to 1 μM and incubated 90 min at 4 °C with U87.ACKR3 cells. CXCL10 (Peprotech) was used as negative control. Non-specific binding of CXCL12-AF647 was evaluated by adding a 250-fold excess of unlabeled CXCL12. Chemokine binding was quantified by mean fluorescence intensity on a BD FACS Canto cytometer (BD Biosciences).

2.3. Arrestin recruitment

Chemokine-induced β-arrestin-2 recruitment to ACKR3 was monitored by β-galactosidase complementation assay, using CHO cells stably expressing β-arrestin-2 fused to enzyme acceptor of β-galactosidase and ACKR3 fused to the β-galactosidase ProLink donor peptide (DiscoverX). Cells were seeded 48 h before the experiment in 96-well plates at a density of 5000 cells/well. Chemokines at concentrations ranging from 50 pM to 1 μM were then added and incubated 90 min at 37 °C. For kinetic analysis, reversible complementation of split firefly luciferase was used [40] in HEK cells expressing β-arrestin-2 fused at its N-terminus to residues 1–415 of firefly luciferase and ACKR3 fused at the C-terminus to residues 413–549 of firefly luciferase. Cells were treated for different times at room temperature with 100 nM chemokines prepared in phenol red-free DMEM. Chemiluminescent signal was generated through addition of β-galactosidase substrate (PathHunter Detection reagent) or D-luciferin (Synchem) and plates were read with POLARstar Omega or Centro XS3 LB 960 luminometer.

2.4. Chemokine-induced changes in ACKR3 cell surface levels

U87.ACKR3 cells were incubated 30 min at 37 °C in the presence of chemokines at concentrations ranging from 0.3 nM to 1 μM. Stimulation was stopped by placing cells on ice and surface-bound ligand was stripped by a brief glycine wash (50 mM glycine, 150 mM NaCl, pH 2.7). Cell surface levels of ACKR3 were then measured by flow cytometry using receptor-saturating concentration of the mAb 11G8 (R&D Systems) and allophycocyanin-conjugated $F(ab')_2$ fragment anti-mouse IgG (Jackson ImmunoResearch). Mean

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