Kaposi Sarcoma-associated Herpes Virus Targets the Lymphotactin Receptor with Both a Broad Spectrum Antagonist vCCL2 and a Highly Selective and Potent Agonist vCCL3*

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Large DNA viruses such as herpesvirus and poxvirus encode proteins that target and exploit the chemokine system of their host. These proteins have the potential to block or change the orchestrated recruitment of leukocytes to sites of viral infection. The genome of Kaposi sarcoma-associated herpes virus (KSHV) encodes three chemokine-like proteins named vCCL1, vCCL2, and vCCL3. In this study vCCL3 was probed in parallel with vCCL1 and vCCL2 against a panel of the 18 classified human chemokine receptors. In calcium mobilization assays vCCL1 acted as a selective CCR8 agonist, whereas vCCL2 was found to act as a broad spectrum chemokine antagonist of human chemokine receptors, including the lymphotactin receptor. In contrast vCCL3 was found to be a highly selective agonist for the human lymphotactin receptor XCR1. The potency of vCCL3 was found to be 10-fold higher than the endogenous human XCL1 chemokine in respect to phosphatidylinositol turnover and calcium mobilization as well as chemotaxis. High expression of XCR1 was found in placenta and neutrophils by real-time PCR. These data are consistent with reports of different expression profiles for vCCL2 and vCCL3 during the life cycle of KSHV, indicate a novel, sophisticated exploitation by the virus of specifically the lymphotactin receptor by both agonist and antagonist mechanisms, and suggest a unique physiological importance of this (somewhat overlooked) chemokine receptor.

Kaposi sarcoma-associated herpes virus (KSHV)2 or human herpesvirus 8 (HHV8) was identified in 1994 (1). KSHV has great similarity to Epstein-Barr virus, and today these two viruses are the only members of the human gamma-herpesvirus family. KSHV has been found to be the agent responsible for Kaposi sarcoma (a vascular tumor), primary effusion lymphoma (a rare lymphoma of B-cell origin), and most cases of multicentric Castleman disease (a benign follicular hyperplasia in germinal centers of B-cell follicles) (2, 3). The genome of KSHV consists of at least 80 genes, and several of these encode viral homologues of human proteins (4).

KSHV belongs, along with Epstein-Barr virus, CMV, human herpes virus 6, human herpes virus 7, and the poxviruses molluscum contagiosum, vaccinia and variola, to a group of large human DNA viruses that encode proteins that exploit the human chemokine system. These proteins fall into three groups; chemokine proteins, 7 transmembrane G-protein-coupled receptors, some of which have been shown to bind chemokines, and chemokine binding proteins (5–9).

Chemokines are proteins of 70–80 amino acid with a characteristic three-dimensional fold, which are involved in guiding and activating distinct leukocyte subsets. Chemokines can be divided into four sub-families on the basis of the pattern and number of the conserved cysteine residues located near their N terminus, which are involved in disulfide binding formation; the CC-, CXC-, CX3C, and XC families, respectively. The XC chemokines have only cysteine in the N terminus. Chemokines act through seven transmembrane G-protein-coupled receptors of which we today know ten CC-chemokine receptors (CCR1–10), six CXC-receptors (CXCR1–6), one CX3C-receptor (CX3C-R1), and one XC-receptor (XCR1) (10, 11). The function of the lymphotactin receptor (XCR1) in the immune system is not well understood, because this receptor has received little attention from the research community.

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2 The abbreviations used are: KSHV, Kaposi sarcoma-associated herpes virus; CHO, Chinese hamster ovary cell; HHV8, human herpesvirus 8; IP3, inositol triphosphate; vMIP, viral macrophage inflammatory protein; XCL, lymphotactin; XCR1, lymphotactin receptor; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CMV, cytomegalovirus; HPLC, high-performance liquid chromatography; FACS, fluorescence-activated cell sorting; NK, natural killer cell. PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin.
The virally encoded chemokines have surprisingly different pharmacological phenotypes as some are of very broad spectrum, and others are highly selective; some are agonists, whereas others are antagonists (12–18). Thus, the first virally encoded chemokine to be characterized in detail, the CC-chemokine vCCL2 or vMIP-II from HHV8, was found to function as a broad spectrum chemokine antagonist, which efficiently blocked the CCR1, CCR2, CCR5, CX3CR1, XCR1, and CXCR4 receptors (15, 16). In accordance with this, vCCL2 has been shown to act as an efficient immunosuppressive agent in different murine and rat inflammatory models (19–21). Subsequently, another HHV8-encoded chemokine vCCL1 or vMIP-I was characterized as a selective CCR8 agonist (12, 14).

HHV8 is unique in encoding a total of three different chemokines. Besides vCCL1 and vCCL2, it also encodes vCCL3 or vMIP-III. In the present study we find that vCCL3 selectively activates the XCR1 receptor with even higher potency than the endogenous ligand lymphotactin, without affecting any of the other 18 chemokine receptor tested. Thus HHV8 targets the lymphotactin receptor, XCR1, both with a high potency, highly selective agonist, vCCL3, and with a broad spectrum antagonist, vCCL2. This highly sophisticated exploitation of specifically the lymphotactin receptor by HHV8 points to a unique importance of this receptor in our immune system, which has not previously been appreciated, besides apparently by the HHV8 virus.

EXPERIMENTAL PROCEDURES

Cloning of the KSHV K4.1 Gene—A biopsy was taken from a KSHV element from a patient, and a QiAamp tissue kit (Qiagen) was used to extract total DNA. Based on the nucleotide sequence deposited in GenBank™ (accession number U93872) the open reading frame K4.1 from HHV8 was amplified by PCR. The full-length coding sequence was inserted in the pTEJ-vector, which uses the ubiquitin UbC promoter (22). Nucleotide sequence analysis was performed on an ABI 310 Genetic analyzer (PerkinElmer Life Sciences).

Production of Recombinant vCCL3 Protein—To purify vCCL3, the method that had been successfully used for MC148 was applied with minor modifications (16). In brief, COS-7 cells were transiently transfected by a calcium phosphate precipitate method with addition of chloroquine. 2400 ml of serum-free medium was collected 24, 48, and 72 h after transfection. The medium was centrifuged at 1500 × g for 20 min, and the supernatant was adjusted to pH 7.2 and filtered through 0.22-µm filters (Nalgene, Rochester, NY). The medium was diluted with water 1:1 to reduce ionic strength and loaded onto cation exchange SP-Sepharose columns (Amersham Biosciences). The columns were washed with 0.2 m NaCl in a 50 mm sodium phosphate buffer, pH 7.2, and the protein was eluted with 0.8 m NaCl in the same buffer. The eluate was adjusted to pH 4 in trifluoroacetic acid, filtered, and loaded onto a C4 46 × 250-mm column (Vydac, Hesperia, CA) for reversed-phase HPLC from which the protein was eluted with a gradient of CH3CN from 0.1% in trifluoroacetic acid in water. The elution position of the recombinant vCCL3 protein as well as the purity was identified with MALDI-TOF mass spectroscopy using an Autoflex II (Bruker Daltonics, Bremen, Germany) and N-terminal sequence analysis using an ABI 494 protein sequencer (Applied Biosystems, Foster City, CA). The identity of each batch was verified with mass spectroscopy.

Chemo-Kines—Mass spectroscopy and HPLC of vCCL3 revealed a monomeric protein with the predicted molecular mass of 9670 Da. CCL2 and CCL7 were chemically synthesized as described previously (23). The HHV8-encoded chemokines, vCCL1 and vCCL2, and the human encoded chemokines CCL1, CCL5, CCL11, CCL16, CCL20, CCL21, CCL22, CCL28, CXCL8, CXCL12, CXCL13, CXCL16, C5a-C1, and XCL1, were bought from R&D (Minneapolis, MN), whereas CCL25 and CXCL11 were bought from PeproTech (Rocky Hill, NJ).

Stable Cell Lines—XCR1 was transfected into the murine pre-B cell line 300.19 by electroporation, and stable transfectants were obtained after limiting dilution and chemical selection with G418 and subsequently functionally selected by testing the clones for calcium response to XCL1. Likewise, stable clones were generated for CCR2, CCR9, CXCR1, and CXCR2 in 300.19 cells and CCR1, CCR5, CCR8, CCR10, and CXC6 in L1.2 cells. The L1.2 cell lines expressing CCR3 and CCR7 receptors were established at ICOS (Seattle, WA). The L1.2 cells stably expressing CCR4, CCR6, XCR1, and CXCR1 were a kind gift from Osamu Yoshie (Kinki University, Japan). Kuldeep Neote (Pfizer, Groton, CT) kindly provided 300.19 cells expressing the CXCR3 receptor, and Bernhard Moser (Theodor-Kocher Institute, Bern, Switzerland) kindly provided 300.19 cells expressing the CXCR5 receptor. The CHO cells stably expressing CCR1, CCR3, CCR5, and CXCR4 were provided by Tim Wells (Serono, Geneva, Switzerland). L1.2 and CHO cells were grown in 1640 RPMI supplemented with penicillin (180 µg/ml), streptomycin (45 µg/ml), and glucose (10 mM). 300.19 cells were grown in the same medium supplemented with 55 µM mercaptoethanol.

Cell Binding—125I-XCL1 and 125I-vCCL3 were prepared in-house, the first by both oxidative iodination as well as Bolton-Hunter iodination and the latter by Bolton-Hunter iodination prior to HPLC purification.

These iodinated proteins were used in an effort to establish a binding assay either by the method of Yoshiie using stably transfected L1.2 cells (24) or COS-7 cells transiently transfected with the XCR1 receptor as described previously (16).

Phosphatidylinositol Assay—COS-7 cells were transiently transfected as mentioned above. Briefly, 2 × 10⁶ COS-7 cells were transfected with 30 µg of cDNA encoding the promiscuous chimeric G-protein, Goα6q4myr (abbreviated as Gqi4myr), which allows the Gqi-coupled receptor to couple to the Gαq pathway (phospholipase C activation measured as phosphatidylinositol turnover) (25), with or without 20 µg of XCR1 cDNA. After transfection, COS-7 cells (2.5 × 10⁶ cells/well) were incubated for 24 h with 2 µCi of [3H]myo-inositol in 0.4 ml of growth medium per well. Cells were washed twice in 20 mM Hepes, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose, and 0.05% (w/v) bovine serum albumin; they were then incubated in 0.4 ml of the same buffer supplemented with 10 mM LiCl at 37 °C for 90 min. Cells were extracted by addition of 1 ml of 10 mM formic acid to each well followed by incubation on ice for 30 – 60 min.
**KSHV-encoded XCR1 Agonist and Antagonist**

The generated [3H]inositol phosphates were purified on AG1-X8 anion-exchange resin (Bio-Rad).

**Calcium Mobilization**—Cells stably transfected with various chemokine receptors were loaded with Fura-2AM (Molecular Probes, Eugene, OR) in RPMI with 1% fetal calf serum for 20–30 min, aliquots were made of 1 × 10^6 cells, and each aliquot was pelleted and resuspended in RPMI with 10 mM EGTA. Fluorescence was measured on a FluoroMax-2 spectrophotometer (Jobin Yvon Spex, Edison, NJ) as the ratio of emission at 490 nm when excited at 340 nm and 380 nm, respectively. For the screening of vCCL1, vCCL2, and vCCL3 on the panel of chemokine receptors, the following ligands and concentrations were used: CCR1, 10^{-8} M CCL5; CCR2, 10^{-8} M CCL2; CCR3, vCCL1 CCR3/CHO 10^{-7} M CCL7; vCCL2 and vCCL3, CCR3/L1.2 10^{-8} M CCL7; CCR4, 10^{-8} M CCL22; CCR5, 10^{-8} M CCL5; CCR6, 10^{-8} M CCL20; CCR7, 10^{-9} M CCL21; CCR8, 10^{-8} M CCL1; CCR9, 10^{-8} M CCL25; CCR10, 10^{-8} M CCL28; CXCR1, 10^{-8} M CXCL8; CXCR2, 10^{-7} M CXCL8; CXCR3, 10^{-9} M CXCL11; CXCR4, 10^{-8} M CXCL12; CXCR5, 10^{-8} M CXCL13; CXCR6, 10^{-8} M CXCL16; CXCR1, vCCL1 and vCCL3 10^{-7} M XCL1; vCCL2, 10^{-8} M XCL1; CXCR1, vCCL1 and vCCL3 10^{-8} M CX3CL1; and vCCL2, 10^{-9} M CX3CL1.

**Chemoattract**—Chemoattract was measured using 24 Transwell polycarbonate 3-μm membranes (Corning Costar, Cambridge, MA). Chemokines were diluted in 0.6 ml of chemoattract buffer (RPMI medium containing 0.5% bovine serum albumin) and added to the lower chemotaxis chamber. 1 × 10^6 XCR1/L1.2, neutrophils or PBMCs were resuspended in 0.1 ml of chemoattract buffer and added to the top chamber insert. Chemotaxis plates were then incubated for 30 min (neutrophils) or 4 h (PBMCs or L1.2/XCR1 cells) at 37 °C in a 5% CO2-humidified incubator. Following incubation, the cells from the bottom well were collected and counted manually or by a fluorescence-activated cell sorter (FACS Calibur, BD Biosciences).

**Real-time PCR Analysis of XCR1 Expression in Blood Leukocyte Subsets and Immunological Tissues**—Fresh buffy-coat cells were obtained from four healthy blood donors. Remaining erythrocytes were lysed by a 1:1 mixture of the buffy-coat cells with Hoffman reagent (0.16 μM NH4Cl, 0.10 mM Na2EDTA, and 10 mM NaHCO3), and the granulocytes were separated from PBMCs by density centrifugation using the Lymphoprep (AXIS-SHIELD, Oslo, Norway) according to the manufacturer’s recommendations. B cells, T cells, NK cells, and monocytes were purified from PBMCs using CD19+, CD3-, CD56-, and CD14-covered magnetic beads (Microbeads for Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, the PBMCs were mixed with the antibody-covered Microbeads (2 μl of Microbeads per 10^6 cells), and the mixtures were subsequently applied to MS columns (Miltenyi Biotec GmbH) that were attached to a MiniMac separator (Miltenyi Biotec GmbH). The purities of the different leukocyte subsets were determined by FACS analysis using perCP-Cy5.5 (peridinin chlorophyll protein cyanin), phycoerythrin, or fluorescein isothiocyanate-labeled CD5, CD14, CD20, and CD56 antibodies (DAKO, Denmark) and found to be 94–95% for neutrophils, 67–91% for monocytes, 93–98% for T-lymphocytes, 86–91% for B-lymphocytes, and 30–80% for NK cells. Total RNA was extracted from leukocyte subsets using the SV Total RNA Isolation system Z3100 (Promega, Madison, WI). Subsequently cDNA was synthesized from 200 ng of the purified RNA with the ImProm-II reverse transcriptase kit A3802 (Promega) according to the manufacturer’s recommendations. In addition the following cDNA panels were obtained from Clontech: the human immune system (catalog no. 636748/6010070) and the blood fractions (catalog no. 636750/6080050). The blood cell fractions were >95% pure. Activation of CD4+ cells had been done by using 5 μg/ml concanavalin A for 3–4 days, of CD8+ cells by 5 μg/ml PHA for 3 days, and of CD19+ cells by 2 μl/ml pokeweed mitogen for 4 days. Quantifications of the XCR1 transcript levels of the immunological tissues and leukocyte subsets were performed using the Sybr® Premix Ex Taq™ kit RR041A (Takara Bio Inc., Shiga, Japan). Expression of β2-microglobulin were used for normalization of input cDNA. Thymus cDNA was chosen as a calibrator to facilitate comparative analysis. The cDNA panels from Clontech were assessed three times in triplicates, whereas the cDNA samples made from donor RNA were measured at least two times in triplicate for each donor to calculate a mean expression value. The primer pairs used were XCR1 forward: 5’-CTGGAGTCCCTCAACCAACAT-3’; XCR1 reverse: 5’-CAGAGGAAGTCTCCACGCAC-3’; β2-microglobulin forward: 5’-TGA-CTTTGTTCAGCGCATAG-3’; β2-microglobulin reverse: 5’-AATCCAAATGCAGCATCCT-3’.

**Peripheral Blood Lymphocytes and Neutrophils for Chemoattract and Calcium Mobilization**—PBMCs and neutrophils were purified as mentioned above. Neutrophils were used for calcium mobilization and chemotaxis assays on the day of purification. PBMCs were grown overnight in 1640 RPMI supplemented with 180 units/ml penicillin, 45 μg/ml streptomycin, 10 mM glutamine, and 10% fetal calf serum. The following day adherent cells were removed, and the cells in suspension were used for assays or were activated with PHA (Sigma L4144) at 1 μg/ml for 3 days before calcium mobilization and chemotaxis assays.

**RESULTS**

**Production of Recombinant vCCL3 Protein**—The ORF K4.1 gene of HHV8 was cloned from a skin biopsy of a KSHV lesion in a patient and sequence analysis showed, that it was identical to the gene from the KSHV deposited in GenBankTM (accession number U93872). COS-7 cells were used for production of the vCCL3 chemokine protein, which was collected in conditioned serum-free medium, and was purified by cation exchange chromatography followed by HPLC purification. Production of the viral chemokine in the eukaryotic cells should ensure that the protein product corresponds to the chemokine normally secreted from cells infected by the virus. When the conditioned medium was run on HPLC a major novel protein peak eluting at 31.5% acetonitrile was demonstrated, which consisted of the protein product corresponds to the chemokine normally secreted from cells infected by the virus. When the conditioned medium was run on HPLC a major novel protein peak eluting at 31.5% acetonitrile was demonstrated, which consisted of the processed, secreted vCCL3 protein (Fig. 1) (26–28). Mass spectrometry and N-terminal sequence analysis revealed a single protein with a molecular mass of 9670 Da (theoretical 9670.4 Da) in accordance with the presence of three disulfide bridges and a cleavage site for the secretory signal-peptide between residues 26 and 27 of the precursor protein (Fig. 1). The yield
was \( 0.2 \mu g \) of purified protein per 175-cm\(^2\) flask per harvest. The HPLC-purified vCCL3 protein was used for in vitro receptor activation studies and in chemotaxis assays.

**Screening of Human Chemokine Receptors with vCCL1, vCCL2, and vCCL3**—The HHV8-encoded chemokine vCCL3 was characterized in calcium mobilization assays using a panel of cell lines individually expressing the eighteen known human chemokine receptors. For reference, the other two HHV8-encoded chemokines, vCCL1 and vCCL2, were also probed against this panel of chemokine receptors. vCCL1 was previously found to be a selective CCR8 agonist using a somewhat limited panel of human chemokine receptors (12, 14). When tested in the comprehensive panel covering all commonly accepted human chemokine receptors, no pharmacological activities beside activation of CCR8 was found for vCCL1, confirming that this HHV8-encoded ligand was indeed a highly selective CCR8 agonist (Fig. 2).

The panel of chemokine receptors, to which the HHV8-encoded, broad spectrum chemokine antagonist vCCL2 had been probed upon, was extended to include CXCR6. Using 100 nM vCCL2, the response of the endogenous human ligand was inhibited through CCR8 and blocked through CCR1, CCR2, CCR3, CCR5, CX3CR1, CXCR4, and importantly XCR1 (Fig. 2). These results are in agreement with what has been found earlier (16).

The panel of cell lines expressing chemokine receptors was screened with 100 nM of the recombinant vCCL3. vCCL3 was unable to elicit a calcium response through 17 of the 18 human chemokine receptors. However, in 300.19 cells stably transfected with XCR1, vCCL3 gave a robust calcium response similar in magnitude to the response elicited by XCL1, the endogenous XCR1 ligand (Fig. 2). In contrast, vCCL3 was not able to

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3 This result was confirmed using a batch of vCCL3 produced at ICOS (Seattle, WA) kindly provided by Johnny Stine and co-workers.
induce a calcium response in naïve 300.19 cells showing that the effect was mediated through XCR1 and not an endogenous receptor expressed in the 300.19 cell line (data not shown). vCCL3 did not act as an antagonist on any of the tested human chemokine receptors as pretreatment of the cell-lines with vCCL3 was unable to inhibit or block the response to the relevant endogenous chemokine through any of the human chemokine receptors tested (Fig. 2). However, in the XCR1-transfected 300.19 cells, an inhibition of the response to XCL1 was observed after pretreatment with 100 nM vCCL3, which, based on the observation that vCCL3 itself is an agonist, is interpreted to be caused by specific desensitization, which is observed with these receptors and is often used as evidence supporting the notion that these ligands act through a common receptor (Fig.

FIGURE 2. Effect of recombinant vCCL1, vCCL2, and vCCL3 on calcium mobilization on a panel of cell lines stably transfected with the human chemokine receptors. 10^(-2) M of vCCL1, vCCL2, vCCL3, or vehicle was first added to the cells followed by a sub-maximal dose of an appropriate endogenous human chemokine. The height of the response with the endogenous ligand only, the height of the response with the viral encoded chemokine only, and height of the endogenous ligand after addition of viral encoded chemokine were measured. Upper diagrams show the response of vCCL1, vCCL2, or vCCL3 compared with the response of endogenous ligand with ± S.E. as indicated. Lower diagrams show the inhibition with vCCL1, vCCL2, or vCCL3 of the response of endogenous ligand compared with the response with endogenous ligand only with ± S.E. as indicated (n = 2). For the chemokine and concentration used see “Experimental Procedures.”
2). Unfortunately, we could not determine the binding affinities of XCL1 and vCCL3, because we were unable to establish a binding assay for the XCR1 receptor with either iodinated XCL1 or vCCL3.

Signal Transduction Properties of vCCL3 and XCL1 on the Lymphotactin Receptor—To study the relative potencies and efficacies of the endogenous chemokine lymphotactin and the virally encoded vCCL3, dose-response studies were performed in COS-7 cells transiently transfected with the chimeric G-protein Gqi4myr, which enables Goçi-coupled receptors such as XCR1 to signal through the Gqi pathway as measured by phosphatidylinositol accumulation assays (25). In these cells, vCCL3 stimulated IP₃ accumulation in a dose-dependent manner and displayed a 10-fold higher potency for XCR1 (EC₅₀ = 4.3 nM, n = 3) than the endogenous chemokine ligand XCL1 (EC₅₀ = 56 nM, assuming Eₘₐₓ equal to that of vCCL3) (Fig. 3A). In control experiments with COS-7 cells transfected with Gqi4myr alone no effects of vCCL3 or XCL1 were observed upon the measured IP₃ accumulation demonstrating that the effect was mediated through XCR1 and not an endogenous receptor expressed in COS-7 cells (n = 2, data not shown).

In calcium mobilization assays vCCL3 also acted as a high potency agonist on the XCR1 receptor, because it induced a dose-dependent calcium response in 300.19 cells stably transfected with XCR1 with an EC₅₀ of 0.98 nM (Fig. 3B). In these cells the endogenous chemokine ligand, XCL1, was found to have an EC₅₀ of 6.4 nM. Furthermore, vCCL3 was able to cross-desensitize the response to 10 nM XCL1 in a dose-dependent manner, and XCL1 was able to cross-desensitize the effect of vCCL3 supporting the notion that the effect of both chemokine ligands was mediated through the same receptor, XCR1 (Fig. 3B).

Chemotactic Effects of vCCL3—The chemotactic activity of the three HHV8-encoded chemokines, vCCL1, vCCL2, and vCCL3, was compared with the endogenous chemokine XCL1 in the murine pre-B lymphocyte cell line L1.2 stably transfected with the human XCR1 receptor (Fig. 3C). As expected, vCCL1 and vCCL2 did not induce chemotaxis of these cells. In contrast vCCL3 induced chemotaxis and displayed a classic bell-shaped curve, which is a typical feature of such chemotactic responses (Fig. 3C). vCCL3 and XCL1 displayed similar efficacies, but again vCCL3 was found to have a 10-fold higher potency compared with XCL1. To probe whether the effect of vCCL3 on XCR1 cells was caused by chemotaxis or chemokinesis “checkerboard analysis” was performed. In short, either buffer or chemokine was added to the bottom and top wells making a total of four possible combinations. Migration of cells to the bottom well in the presence of chemokine in the top and bottom would have been measured.
be caused by chemokinesis, whereas migration of cells in the presence of chemokine in the bottom well only would be caused by chemotaxis. Using 1 nM vCCL3 or 10 nM XCL1 in this assay confirmed that the effect of both vCCL3 and XCL1 was due to chemotaxis and not chemokinesis (Fig. 3C).

vCCL2 Blocks Activation of the Lymphotactin Receptor—The HHV8-encoded chemokine vCCL2/vMIP-II acts as an antagonist for XCR1 as well as several other human chemokine receptors (Fig. 2). vCCL2, in a dose-dependent manner, was able to block the signal transduction response as measured in IP3 accumulation assays of not only XCL1 but also vCCL3 with EC50 values of 3.1 nM (using 10 nM XCL1) and 3.7 nM (using 1 nM of vCCL3), respectively (Fig. 4A). Thus 100 nM vCCL2 was able to reduce the IP3 turnover to the basal levels (Fig. 3A). In agreement with the results of the IP3 assays, vCCL2 was also able to block the response induced by 10 nM vCCL3 or 10 nM XCL1 in calcium mobilization experiments in XCR1-transfected 300.19 cells in a dose-dependent manner (Fig. 4B). In chemotaxis assays, vCCL2 in a dose-dependent manner inhibited the chemotactic response of XCR1-transfected L.1.2 cells, when increasing concentrations of vCCL2 were added in the lower well together with either 1 nM vCCL3 or 10 nM XCL1 (Fig. 4C).

Expression of XCR1 in the Immune System—XCR1 mRNA has previously been shown to be expressed in placenta at high levels, however comparative studies of expression in different leukocyte subpopulations have only been done to a limited extent. We decided to do real-time PCR of XCR1 cDNA from neutrophils, T cells, B cells, NK cells, and monocytes purified in-house using antibody-labeled magnetic beads as well as commercially obtained cDNA panels of immunological tissues and purified sub-populations of leukocytes. The relative expression of XCR1 was analyzed by real-time PCR and normalized to β2-microglobulin using thymus cDNA as the calibrator (Fig. 5 and 6).

As shown in Fig. 5, XCR1 was abundantly expressed in the placenta followed by thymus, spleen, lymph node, and tonsil. Fetal liver, bone marrow, peripheral blood leukocytes, and all the leukocyte subsets shown in Fig. 5 amplified outside of the linear standard curve. For these latter samples the signal was below the detection limit. However, we were able to quantify the signals from the leukocyte subsets purified in-house from four blood donors (Fig. 6). Surprisingly, the neutrophil fraction had a very high expression of XCR1 more than 50-times higher than that of the other cell types for donors 1–3 (Fig. 6). The patterns of expression of the lymphotactin receptor in the different leukocyte subsets were very similar for donors 1–3. Thus, a high expression of the lymphotactin receptor was found in neutrophils (Fig. 6), followed by lower expression in NK cells, B cells, and T cells and lowest to undetectable expression in monocytes (Fig. 6, inset). Donor 4 had a very high level of lymphotactin receptor expression for all cell populations. For example, B cells, NK cells, and neutrophils from this donor had a 3–10 times higher expression level of XCR1 than neutrophils from donors 1 and 2 (Fig. 6), whereas the expression level of XCR1 in monocytes from donor 4 was comparable to the expression level of XCR1 in neutrophils from donors 1 and 2.
We do not know the reason for this difference in expression patterns; it could be due to an artifact or possibly that the immune system of donor 4 in some way was activated.

To define XCR1-positive cell populations in greater detail and to confirm the results from the expression assays we bought the XCR1 antibody SP4618P (Acris Antibodies GmbH, Hidenhausen, Germany) to carry out FACS analysis. According to the notes of the manufacturer it had only been validated for immunohistochemistry. Unfortunately, this antibody was unable to bind to our stable XCR1 L12 and XCR1 300.19 cell lines in FACS analysis.

Chemotactic Properties of vCCL3 on Neutrophils and Lymphocytes—Having established that the lymphotactin receptor is expressed in neutrophils and lymphocytes, we then decided to see whether it would be possible to elicit a chemotactic or calcium response through the lymphotactin receptor in these primary cells (Fig. 7). CXCL12/SDF was able to chemoattract PBLs with high potency confirming that the cells were fully functioning and viable (Fig. 7). Interestingly, 100 nM vCCL3 or lymphotactin could not chemoattract this cell population whether it was activated with PHA or not (Fig. 7). Likewise 10 nM CXCL8/IL8 was able to chemoattract neutrophils with high potency showing that the cells were fully functioning. 100 nM vCCL3 was able to chemoattract neutrophils with almost the same efficacy as CXCL8 (n = 4). However, the potencies of XCL1 and vCCL3 were 10- to 100-fold lower, respectively, in chemotaxis assays using neutrophils compared with the XCR1-expressing L1.2 cells. When PBLs and neutrophils were tested in calcium mobilization experiments vCCL3 and XCL1 were consistently unable to elicit a calcium response in contrast to CXCL12 and CXCL8 (data not shown). It is concluded that neutrophils express XCR1 and that the XCR1 agonists vCCL3 and XCL1 are able to chemoattract these cells although with low potencies.

DISCUSSION

In the present study we demonstrate that the chemokine product of the K4.1 gene from HHV8 vCCL3 acts as a highly selective agonist for the human lymphotactin receptor XCR1. In contrast, another chemokine, vCCL2, also encoded by HHV8 is shown to act as an antagonist on the same receptor and is even able to block the effect of vCCL3. Different timing in the expression of vCCL2 and vCCL3 could explain the finding that the same virus encodes two chemokine proteins with opposite pharmacological properties targeting the same receptor. Thus KSHV seems to have evolved a way to exploit specifically the lymphotactin receptor by both agonist and antagonist mechanisms, which suggests a unique physiological importance of this chemokine receptor.
vCCL3 Is a Selective XCR1 Agonist—The K4.1 gene was discovered during the sequencing of the HHV8 genome almost a decade ago. Based on the deduced amino acid sequence it was classified as a member of the CC-chemokine subfamily (Fig. 1A). It is surprising that this CC chemokine does not act on any of the CC-chemokine receptors (CCR1–10) but instead selectively targets the receptor of another chemokine subfamily, XCR1. This suggests that CC- and XC-chemokine receptors, for practical purposes, are closely related, which is further supported by the fact that XCR1 lies in the middle of a major CC-chemokine receptor cluster on chromosome 3 (10).

The human ligand for XCR1 lymphotactin has only 2 cysteines forming a single disulfide bridge (Fig. 1, A and B). On the other hand, XCL1 has an extended O-glycosylated C terminus (Fig. 1, A and B), and it has been speculated that the C terminus compensates for the latent instability of a molecule with only one disulfide bridge instead of the two disulfide bridges found in other chemokines (29). In support of this, it has been shown that deletion of the 22 C-terminal amino acid residues of XCL1 abolishes the function of the protein (29) and that the de-glycosylated form of XCL1, which is the form that is commercially available, has less biological activity than the glycosylated form (30). In contrast, vCCL3 is likely to have greater stability with three disulfide bridges (Fig. 1A), which is supported by the mass spectroscopy data that predict 6 oxidized cysteines. Finally, the commercially available lymphotactin has an extra methionine attached at the N terminus due to its prokaryotic origin, and it is known from mutagenesis studies that chemokine receptor activation is sensitive to changes at the N terminus of their ligands (11). These structural differences could explain the observed 10-fold higher potency of vCCL3 compared with XCL1 (Figs. 3 and 4). Importantly, the higher potency and stability of vCCL3 is likely to make it a better tool for determining the functions of XCR1 compared with the currently available un-glycosylated form of lymphotactin.

XCL1 and vCCL3 do not have many amino acid residues in common that are not shared by other chemokines. An exception is the SL(S/T)(S/T) motif (Fig. 1, A and B) in the N-loop of XCL1 and vCCL3. It has been demonstrated for other chemokines that beside the N terminus the N-loop is important for receptor binding and activation (11). Future mutagenesis studies are needed to determine whether the SL(S/T)(S/T) motif is necessary for lymphotactin receptor activation.

vMIP-III/vCCL3 was in a previous report tested against CCR2, CCR3, CCR4, and CCR5 receptors and found to act with low (micromolar) potencies in CCR4 and CCR5 chemotaxis assays (31). In contrast, we were unable to demonstrate any calcium mobilization in L1.2 cells stably transfected with CCR2, CCR3, CCR4, and CCR5 using 100 nM vCCL3. It should be emphasized that we found vCCL3, in both calcium mobilization and chemotaxis assays on cells stably transfected with XCR1, to have potencies in the nanomolar range. Thus, if vCCL3 in fact does act as an agonist on the CCR4 and CCR5 receptors, this occurs with at least 100- to 1000-fold lower potency than it acts as an agonist on the lymphotactin receptor.

Expression Pattern of XCR1—The finding of a high expression level of the lymphotactin receptor in placenta compared with a moderate to low expression level in spleen and thymus (Fig. 5) is identical to the results reported by Yoshida et al. (24). The XCR1 expression patterns for donors 1–3 were surprisingly consistent. The findings that XCR1 are abundantly expressed in human neutrophils and that XCL1 and vCCL3 are able to chemoattract human neutrophils extend the results of earlier reports on lymphotactin receptor expression in murine neutrophils (32, 33). Thus, by reverse transcription-PCR the levels of mXCR1 in murine neutrophils were found to be equal or higher than the levels in murine T-lymphocytes (32, 33). Furthermore, lymphotactin was found to be able to chemoattract murine neutrophils in vitro (32, 33) and in vivo (32) in contrast to an earlier report (34). We are not able to explain why the potencies for vCCL3 and XCL1 in chemotaxis assays were lower for neutrophils than for XCR1-transfected L1.2 cells. However, the reason why we were unable to elicit a calcium response in neutrophils through the lymphotactin receptor could be due to a low receptor density at the cell surface compared with the transfected cell lines requiring a higher ligand concentration. In an earlier report, a concentration of 1 μM lymphotactin was required to induce a calcium flux in PBLs (35).

Several studies support the expression of XCR1 in NK cells, B cells, and T cells. First, by using reverse transcription-PCR XCR1 has been detected in murine B cells (33) and murine T cells (32, 33). Second, it has been demonstrated that lymphotactin could chemoattract human (36, 37) and murine NK cells (29) and murine B cells (33).

The majority of studies on the chemotactic response of XCL1 on lymphocytes has found an effect. Thus lymphotactin was able to chemoattract murine lymphocytes (29, 34) and human lymphocytes from peripheral blood (26, 35, 36, 38). Therefore it was unexpected that we found vCCL3 and XCL1 unable to chemoattract human PBLs. There are several possible explanations for this finding. First, the XCR1 receptors could have been internalized, second the receptor number on the cell surface may have been too low to elicit a chemotactic response, and lastly it could be that lymphocytes per se do not express XCR1 except certain minor subsets. In any case it should be noted that two reports found XCL1 unable to chemoattract PBLs (39, 40).

Real-time PCR of purified cell fractions as done in this study is a rather crude way to determine the expression of XCR1. Development of an anti-XCR1 antibody for FACS analysis would be an important future tool in defining XCR1-positive cell populations in greater detail, which along with a murine lymphotactin receptor knock-out model could contribute significantly to our understanding of the role played by this receptor in our immune system.

It should be noted that vCCL3 is not the only virus-encoded chemokine that can attract neutrophils, because vCXCL1 (a selective CXCR2 agonist) encoded by CMV also has this property (18). The neutrophil has a role in dissemination of CMV and neutrophil containing inflammatory infiltrates are seen in CMV diseases in AIDS patients such as retinitis, pneumonitis, and central nervous system complications (18). However, the role of the neutrophil in CMV biology has still not been defined, and even less so in KSHV pathogenesis, because this cell type has not been implicated in, for example, Kaposi sarcoma.
Expression patterns, receptor targets, and presumed cellular effects of the KSHV-encoded chemokine elements: vCCL1, vCCL2, and vCCL3. All except vCCL3 are expressed shortly after infection, whereas the expression of vCCL3 takes place 10 h after infection (47, 48). Three presumed target cell types are indicated, the monocyte/macrophage, the T cell/NK cell, and the neutrophil/granulocyte. Chemokine receptors presumed to be expressed by these cell types are shown (10) but only those that are presumed to be targets for the KSHV-encoded chemokines. The HHV8-infected cell could be an endothelial cell, a T cell, or a monocyte (2). A, receptor targets and the cellular effects of vCCL1 and vCCL2, which are expressed right after infection. B, in addition, the receptor targets and cellular effects of vCCL3, which are expressed 10 h after infection.
KSHV-encoded XCR1 Agonist and Antagonist

Why Has KSHV Chosen XCR1 as a Major Target?—In 1996, three open reading frames in the genome of KSHV were identified to encode CC-chemokine-like proteins. Initially, vCCL2/vMIP-II was found to be able to block several chemokine receptors, e.g. CCR1, CCR2, CCR5, XCR1, CX3CR1, and CXC5R4, and to inhibit other chemokine receptors as well (15, 16) (Fig. 2). Subsequently, vCCL1 was characterized as a selective CCR8 agonist (12, 14). The last of these chemokines, vCCL3, has in this report been found to act as a highly selective XCR1 agonist.

The notion that large DNA viruses target certain chemokine receptor pathways has become an emerging theme with the increasing number of pharmacologically characterized viral-encoded chemokine ligands, chemokine receptors, and chemokine-binding proteins. The CCR8 pathway is the most prominent example, because it is targeted by at least three viruses. First, molluscum contagiosum virus, a pox virus, which causes a local infection in human epithelial cells with a surprisingly small inflammatory response (41) encodes a selective CCR8 antagonist (16). Second, two viruses that establish systemic infections depend on activation of the CCR8 pathway. As demonstrated, KSHV encodes a selective CCR8 agonist (12, 14) (Fig. 2), whereas the pox virus Yaba-like disease virus, which infects monkeys and occasionally humans, encodes two G-protein-coupled receptors with high similarity to human CCR8 (42).

One of these viral receptors can be activated by the human CCR8 agonist (42). The role of the CCR8 pathway in the interaction between the virus and the host immune system has not been determined, but activation of CCR8 is known to rescue T cells from apoptosis (43–45). KSHV is known to infect CCR8-expressing cells such as endothelial cells and monocytes (2, 10, 46). Thus it could be speculated that Yaba-like disease virus-infected cells via a CCR8-like receptor and KSHV-infected cells via an autocrine loop (2, 10, 47, 48) (Fig. 8) are protected from apoptosis via the CCR8 pathway. In contrast, molluscum contagiosum virus by encoding a CCR8 antagonist can prevent the immune cells, recruited to the site of viral infection, rescue from apoptosis by blocking the CCR8 pathway.

The lymphotactin receptor pathway is another example. Besides KSHV, the orb virus, a pox virus that causes pustular dermatitis in sheep, goats, and humans, has been shown to encode a chemokine-binding protein that binds several inflammatory CC chemokines and lymphotactin (49), interestingly mimicking part of the chemokine-receptor pathways blocked by vCCL2. It is intriguing that two viruses target this pathway and suggests that XCR1 could have important but yet unknown functions in our immune system.

The most obvious explanation, as to why KSHV encodes two ligands with opposite pharmacological properties to XCR1, is that the virus needs to control the receptor over time. The reported gene expression profile for KSHV shows that vCCL1 and vCCL2 are primary lytic genes expressed within the first 10 h after infection, whereas vCCL3 is a secondary lytic gene expressed from 10 to 24 h after infection (47, 48) (Fig. 8). One explanation could be that during the lytic phase of the KSHV life cycle the virus needs to block the lymphotactin receptor along with other chemokine receptors to prevent recruitment of immune cells that could mount an antiviral response at a time point before viral particles are formed. At a later stage in the replication cycle when virus particles are being assembled KSHV could then be imagined to recruit certain XCR1-expressing cells from the immune system to ensure dissemination of the virus (Fig. 8). It should be noted that the agonistic effect of vCCL3 on XCR1 is rather easily able to overcome the antagonistic effect of vCCL2 (Fig. 4).

In conclusion we have shown that HHV8 encodes both a highly selective agonist for the lymphotactin receptor and a lymphotakin receptor antagonist. vCCL2 and vCCL3 have different expression profiles (47, 48) that could reflect the need of the virus to initially block recruitment of immune cells and later to reverse this process and attract certain cells, conceivably to infect them. vCCL3 may be a better pharmacological/immunological tool for determining the role of XCR1 compared with the commercially available un-glycosylated form of XCL1, which at present is the only agonist.

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