

Cytomegalovirus Expresses the Chemokine Homologue vXCL1 Capable of Attracting XCR1⁺ CD4⁻ Dendritic Cells

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Cytomegaloviruses (CMV) have developed various strategies to escape the immune system of the host. One strategy involves the expression of virus-encoded chemokines to modulate the host chemokine network. We have identified in the English isolate of rat CMV (murid herpesvirus 8 [MuHV8]) an open reading frame encoding a protein homologous to the chemokine XCL1, the only known C chemokine. Viral XCL1 (vXCL1), a glycosylated protein of 96 amino acids, can be detected 13 h postinfection in the supernatant of MuHV8-infected rat embryo fibroblasts. vXCL1 exclusively binds to CD4⁻ rat dendritic cells (DC), a subset of DC that express the corresponding chemokine receptor XCR1. Like endogenous rat XCL1, vXCL1 selectively chemoattracts XCR1⁺ CD4⁻ DC. Since XCR1⁺ DC in mice and humans have been shown to excel in antigen cross-presentation and thus in the induction of cytotoxic CD8⁺ T lymphocytes, the virus has apparently hijacked this gene to subvert cytotoxic immune responses. The biology of vXCL1 offers an interesting opportunity to study the role of XCL1 and XCR1⁺ DC in the cross-presentation of viral antigens.

Chemokines are small chemotactic cytokines that are classified into the CXC, CC, C, and CX₃C subfamilies based on the positions of conserved cysteine residues at their N terminus. The C chemokine subfamily is characterized by only one cysteine at the N terminus and contains only one member, XCL1. In humans, two variants (XCL1 and XCL2), which differ by two amino acids, have been reported (1). XCL1, a glycosylated 93-amino-acid mature protein (2), has been shown to be secreted by activated NK cells (3) and CD8⁺ T cells (4). Murine and human XCL1 chemokines specifically chemoattract a particular subset of dendritic cells (DC) that express XCR1, the only receptor for XCL1 (5, 6). XCR1⁺ DC excel in antigen cross-presentation, a process in which extracellular antigens are presented by major histocompatibility complex class I (MHC-I) molecules to CD8⁺ T cells (7–9). Antigen cross-presentation is thought to play a major role in the immune defense against viruses that do not directly infect DC (10, 11). The XCL1-XCR1 interaction facilitates the communication between XCR1⁺ DC and activated CD8⁺ T cells or NK cells secreting XCL1 during infection and thereby promotes the differentiation of CD8⁺ T cells into cytotoxic effector cells (reviewed in reference 12).

Cytomegaloviruses (CMV) belong to the *Herpesviridae*, a family of large double-stranded DNA viruses that infect a broad spectrum of species and cause lifelong infections in their respective hosts (13). In order to survive successfully and establish latency, CMV have developed various strategies to escape different immune defense mechanisms. One strategy involves the expression of virus-encoded chemokines that interfere with the host chemokine network. It has been speculated that these genes were obtained from the host genome during coevolution and that they contribute to viral dissemination and maintenance (14).

So far, CXC and CC chemokines have been described for rodent, primate, and human CMV (HCMV). Three chemokine-like genes have been described in the HCMV genome: *UL128*, *UL146*, and *UL147* (15, 16). *UL146* and *UL147* encode the proteins vCXC-1 and vCXC-2, respectively. Only vCXC-1 has been shown

to represent a functional chemokine since it binds to the chemokine receptors CXCR1 and CXCR2 and induces migration of neutrophils to the site of infection (17), a process that has been suggested to facilitate viral dissemination. Whereas one study showed that the *UL128* gene product pUL128 blocked migration and induced downregulation of chemokine receptors in monocytes (18), another report demonstrated an opposite effect for pUL128, as it induced migration of peripheral blood mononuclear cells (19), a discrepancy that might be due to N-terminal modifications of the chemokines resulting in different chemotactic behavior. It has also been reported that murid herpesvirus 2 (MuHV2) r129 induces migration of immune cells (20). *UL146* and *UL147* are restricted to genomes of primate CMVs and have no sequence counterparts in rodent CMV.

CC chemokines have been characterized in rodent CMV, e.g., Maastricht rat CMV (RCMV; MuHV2)-encoded *rck-3* (21) and *rck-2* (22), murine CMV (MCMV; MuHV1)-encoded chemokine *mck-2* (23), and English RCMV (MuHV8)-encoded *eck-2* (24). Among these chemokines, MCK-2 is the most extensively studied viral chemokine. MCK-2 has been shown to enhance recruitment of inflammatory monocytes to the site of infection (25). Attracted monocytes inhibit CD8⁺ T cell activation and cytotoxicity, which results in slower viral clearance (26).

Our analysis of the MuHV8 genome (27) revealed the presence of a C chemokine, located at nucleotide positions 186261 to 186608 toward the right terminus, which to our knowledge is the first viral C chemokine to be reported. The gene product, desig-

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nated vXCL1, shares extensive homology with the C chemokine XCL1 of rat, mouse, and human. Here, we show that vXCL1 carries a cleavable N-terminal signal sequence that allows secretion from infected cells. Furthermore, vXCL1 functionally resembles host XCL1 since it binds to XCR1⁺CD4⁻ DC and selectively chemoattracts this particular cell subset. Since murine and human XCR1-expressing DC excel in antigen cross-presentation, vXCL1 might attract this rat DC subset in order to manipulate and disable this important branch of the immune defense.

MATERIALS AND METHODS

Viruses and cell culture. MuHV8 was propagated on rat embryo fibroblasts (REF) maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum, 2 mM L-glutamine, and 100 µg/ml penicillin/streptomycin. To determine vXCL1 mRNA and protein expression kinetics, REF were treated 2 h prior to infection or 3 h prior to harvest with 100 µg/ml cycloheximide (Sigma-Aldrich, Taufkirchen, Germany) or 5 µg/ml brefeldin A (Sigma-Aldrich), respectively. In order to generate a MuHV8 mutant lacking *vxcl1*, a shuttle vector was generated, containing 1.5 kbp of upstream and downstream sequences of *vxcl1* using e155 forward (5'-TAGCCGAGACTTCGCACTTC-3') and reverse (5'-GCCGAGAGGTGTTTGGATTC-3') primers, and e159 forward (5'-TTCACTACCGAGTGGAACTG-3') and reverse (5'-GTTCCGCAACGAGACCCTCAG-3') primers, respectively. The *vxcl1* open reading frame (ORF) in this shuttle vector was exchanged by a green fluorescent protein (GFP) expression cassette flanked by two LoxP sites. To knock out the *vxcl1* ORF within the MuHV8 genome, REF were cotransfected with 2 µg of linearized shuttle vector and 2 µg MuHV8 virion DNA using Polyfect according to the manufacturer's recommendations. The *vxcl1* knockout virus was identified by GFP fluorescence and isolated by limiting dilution.

RNA isolation, cDNA synthesis, PCR, and 3' and 5' rapid amplification of cDNA ends (RACE). Isolation of viral and cellular RNA was carried out with the RNeasy Mini Isolation kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Two million cells were used per RNA isolation column, and remaining DNA contaminations were removed by a 30-min digestion with 20 units of Turbo-DNase (Ambion, Darmstadt, Germany) on the column. For cDNA generation, 1 µg of RNA was incubated for 1 h at 45°C with the following components: 200 U RevertAid H minus reverse transcriptase, 5 µM oligo(dT)₁₈ primer, 1× reaction buffer, 1 mM deoxynucleoside triphosphate (dNTP), and 20 U RiboLock RNase inhibitor (Fermentas, St. Leon-Rot, Germany). To exclude DNA contaminations present in the RNA preparation, cDNA synthesis was additionally carried out with the same components except reverse transcriptase. The reaction was terminated by heating the mixture for 10 min at 70°C. A 1-µl volume of the reaction mixture was applied in a PCR using the Platinum *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany) to amplify cDNA with gene-specific primers according to the manufacturer's recommendations. The primer pair vXCL1 universal fwd (5'-ATGCGAGCGGTAATCTTTG-3') and vXCL1 universal rev (5'-CAGGAACCTGCGTGGGAATA-3') was used for the amplification of vXCL1 mRNA, the primer pair *c-myc* fwd (5'-GCCAGAGGAGGAACGAGCT-3') and *c-myc* rev (5'-GGGCCTTTTCATTGTTTTCCA-3') for the amplification of *c-myc* mRNA, and the primers GAPDH fwd (5'-GGTCGGTGTGAACGGATTTG-3') and GAPDH rev (5'-GTGAGCCCA GCCTTCTCCAT-3') for the amplification of glyceral-3-phosphate dehydrogenase (GAPDH) mRNA.

The 3'-untranslated region (UTR) and the 5'-UTR of vXCL1 were determined with the FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's instructions. *vxcl1* gene-specific primers 3'-UTR vXCL1 (5'-CACGAAACCATCTGCGTAAG-3') and 5'-UTR vXCL1 (5'-AGGAACCTGCGTGGGAATAACTG-3') were used for amplification of vXCL1-specific mRNA.

Generation of vXCL1-specific MAb vXCL1.11. For overexpression, the complete coding region of vXCL1 was cloned into the BamHI site of vector pQE-30 (Qiagen) and transformed into *Escherichia coli* host strain

M15 [pREP4] (Qiagen). BALB/c mice were immunized subcutaneously with recombinant protein (50 µg) in complete Freund's adjuvant. Two weeks later, mice were boosted with the same protein in incomplete Freund's adjuvant by injecting two-thirds of the volume subcutaneously and one-third of the volume intraperitoneally (i.p.). After 2 weeks, sera of immunized mice were screened for antibody titer against the immunogen by enzyme-linked immunosorbent assay (ELISA). The best responders were additionally boosted i.p. with the immunogen dissolved in phosphate-buffered saline (PBS). Three days later, spleen cells were collected and fused with SP2/0 myeloma cells at a ratio of 1:1 after lysis of red blood cells. Cells were seeded on 96-well tissue culture plates in 20% RPMI 1640 medium containing hypoxanthine, aminopterin, and thymidine for hybridoma selection. Cultures were screened for monoclonal antibodies (MAbs) reactive against immunogens by ELISA. Positive mother wells were expanded and cloned. Mice used for immunization were bred and maintained under specific-pathogen-free (SPF) conditions at the Laboratory Mouse Breeding and Engineering Centre (LAMRI) at the Faculty of Medicine, University of Rijeka. All experimental procedures were approved by the Ethics Committee of the University of Rijeka.

Other antibodies, flow cytometry, and recombinant chemokines. Antibodies recognizing CD45RA (OX-33) and MHCII (OX-6) were from BD Pharmingen, anti-CD4 (W3/25) antibodies from AbDSerotec, and anti-CD103 (OX62) antibodies from Biologend. Mouse XCR1-specific monoclonal antibody (MARX10 [9], cross-reacting with rat XCR1) and goat serum directed against murine XCL1 (cross-reacting with vXCL1) were generated in the laboratory of R.A.K. Polyclonal rabbit anti-goat immunoglobulin-horseradish peroxidase (HRP) and goat anti-mouse immunoglobulin-HRP were obtained from Dako. Flow cytometry data were acquired on an LSR II or FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using Facsdiva or Cell Quest Pro software (BD Biosciences), respectively. Final examination and compensation of the data were carried out using FlowJo software (Tree Star, Ashland, OR, USA). vXCL1 and rat XCL1 containing an affinity tag at their respective carboxy termini were cloned into the expression vector pRmHa-3 (28) and expressed in stable *Drosophila* SL-3 cell transfectants (L. Voss, H.W. Mages, and R.A. Kroczeck, unpublished data).

Sandwich ELISA. Maxisorp 96-well microtiter plates (Nalgene Nunc, Roskilde, Denmark) were coated with 100 µl MAb vXCL1.11 (final concentration, 1 µg/ml) diluted in coating buffer (0.1 M NaHCO₃, Na₂CO₃, pH 9.5) overnight at 4°C. Wells were washed five times with washing buffer (1× PBS containing 0.1% [vol/vol] Tween 20) and blocked with blocking buffer (1% bovine serum albumin [BSA], wt/vol, in washing buffer) for 2 h at room temperature. After five washes with washing buffer, 100 µl of test sample and serial dilutions of recombinant vXCL1 were applied per well and incubated for 2 h at room temperature. Unbound proteins were removed by rinsing five times with washing buffer, followed by 1 h of incubation with cross-reactive goat serum directed against murine XCL1 (1:500 dilution) in blocking buffer at room temperature. Polyclonal anti-goat immunoglobulin-HRP was diluted in blocking buffer (final concentration, 0.2 µg/ml) and added after rinsing five times with washing buffer for 1 h at room temperature. After 10 additional washes, 50 µl of TMB Plus (Kem En Tec, Taastrup, Denmark) was added to each well and incubated for 5 min in the dark. The reaction was stopped by adding 50 µl of 0.5 N H₂SO₄, and absorbance was determined at 450 nm using a Spectrafluor Plus (Tecan, San Jose, CA, USA) microplate reader.

SDS-PAGE, immunoblotting, and posttranslational modification analysis. To analyze N-glycosylation of vXCL1, lysates of MuHV8-infected REF (20 µg of total protein of REF infected at a multiplicity of infection [MOI] of 10) were denatured for 10 min in 1× glycoprotein denaturing buffer (NEB, Ipswich, MA, USA) and then digested with 1 U PNGaseF (NEB) for 1.5 h at 37°C using 1× G7 reaction buffer (NEB) and 1% Nonidet P-40. The digested protein lysate was then mixed with 4× SDS loading buffer (0.3 M Tris, 12% SDS, 40% [vol/vol] glycerol, 0.3 M dithiothreitol [DTT], 0.02 bromophenol blue) and separated electrophoretically on a 15% Tris-Tricine SDS gel using the prestained PageRuler

ladder (Fermentas). Afterwards, the gel was blotted onto a Hybond ECL nitrocellulose membrane (GE Healthcare, Munich, Germany) with 0.8 mA per cm² and stained with MAb vXCL1.11 using the ECL Western blotting reagents (GE Healthcare).

Mass spectrometric peptide analysis. vXCL1 produced in insect cells was purified by heparin Sepharose affinity chromatography. Peptides from the gel-purified protein were obtained by trypsin and endoprotease Glu-C in-gel digestion as described previously (29), and peptide masses were analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF/MS) using an Ultraflex-II TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a 200-Hz solid-state Smart beam laser. The mass spectrometer was operated in the positive reflector mode; mass spectra were acquired over an *m/z* range from 600 to 4,000. CHCA (α -cyano-4-hydroxycinnamic acid) was used as the matrix, and samples were spotted using the dried-droplet technique. Tandem MS (MS/MS) spectra of selected peptides were acquired in LIFT mode (30).

Cell isolation. Splens of Lewis rats were cut into small pieces and digested with 500 μ g/ml collagenase D (Roche, Penzberg, Germany) and 20 μ g/ml DNase I (Roche) in RPMI 1640 containing 2% fetal calf serum (FCS; Pan Biotech GmbH, Regensburg, Germany) for 25 min at 37°C and shaking at 200 rpm. After addition of 10 mM EDTA and incubation for 5 min at 37°C, cells were filtered through a 100- μ m nylon sieve (BD Pharmingen) followed by Nycoprep density gradient (1.073 g/ml) for 10 min at 1,700 \times g and 4°C. The DC-enriched interphase was recovered and used for chemokine binding assays. For chemotaxis experiments, rat DC were enriched by magnetic cell sorting using CD103 microbeads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

Chemotaxis assay. Migration of CD103-enriched splenocytes was analyzed in a migration assay as described elsewhere (31). In brief, 1×10^5 to 5×10^5 CD103-enriched rat splenocytes were resuspended in chemotaxis medium (RPMI 1640, 1% BSA, 50 μ M β -mercaptoethanol, 100 μ g/ml penicillin-streptomycin) and placed in the upper chamber of a 24-well 6.5-mm transwell system (Corning, Salt Lake City, UT, USA). The lower chamber was filled with chemotaxis medium containing the chemokine of interest, no chemokine as a control, or supernatant of infected REF. Cells were incubated for 2.5 h at 37°C and 5% CO₂. To examine the effect of pertussis toxin (PTX) on cell migration, CD103-enriched splenocytes were treated with 100 ng/ml PTX (Sigma-Aldrich, Taufkirchen, Germany) for 2 h at 37°C and 5% CO₂ and then washed three times with chemotaxis medium prior to migration analysis. Cells migrating into the lower chamber were analyzed by flow cytometry. T cells (CD3⁺, MHCII⁻), B cells (CD45RA⁺, MHCII⁺), CD4⁺ DC (MHCII⁺, CD103⁺, CD4⁺), and CD4⁻ DC (MHCII⁺, CD103⁺, CD4⁻) were identified by flow cytometry using the indicated markers. Numbers of migrated cells and input cells were determined by counting cells over a time frame of 300 s in a defined volume. The calculation of the percentage of migrated cells was based on the number of input cells: (number of migrated cells/number of input cells) \times 100.

RESULTS

MuHV8 encodes a C chemokine homologue. Genome analysis of MuHV8 identified a 348-bp ORF between viral genes *e155* and *e159* encoding a putative 115-amino-acid protein. BLAST analysis at the protein level revealed 64% identity (73% homology) with the C chemokine XCL1 of *Rattus norvegicus*, 58% identity with mouse XCL1, and 46% identity with human XCL1 (Fig. 1A). Given the high degree of identity and homology to XCL1 and the conserved cysteines at positions 30 and 67, characteristic of C chemokines, this putative protein was designated viral XCL1 (vXCL1). The highest divergence between vXCL1 and XCL1 was observed at the C terminus, whereas the N terminus and the core of the protein were conserved.

Rat, murine, and human *xcl1* each contain three exons that are transcribed into a single mRNA (1, 32). In contrast, vXCL1 mRNA is unspliced. To investigate vXCL1 expression, mRNA of MuHV8-infected rat embryo fibroblasts (REF) was harvested and the 3'-UTR and 5'-UTR were determined by rapid amplification of cDNA ends (RACE). MuHV8 *vxcl1* encodes a transcript of 1,689 bp consisting of a 99-bp 5'-UTR, a 348-bp ORF, and a 1,242-bp 3'-UTR (Fig. 1B). Computational analysis detected four AU-rich elements (ARE), one Musashi binding element (MBE) in the 3'-UTR, and one MBE motif in the 5'-UTR of vXCL1 mRNA.

If vXCL1 were to be involved in immune evasion, precise timing of viral gene expression to bypass certain host immune responses would be expected; we therefore analyzed *vxcl1* expression kinetics. Semiquantitative reverse transcription (RT)-PCR revealed that vXCL1 mRNA is already expressed 2 h postinfection and follows early kinetics (Fig. 1C). To clarify if vXCL1 mRNA is produced immediately early or early, REF were treated with cycloheximide prior to infection. vXCL1 mRNA was not detected upon treatment with the protein synthesis inhibitor (Fig. 1D), indicating that *vxcl1* is expressed early after infection in a protein-dependent fashion.

vXCL1 is a posttranslationally modified, secreted protein. To examine if and at what time postinfection vXCL1 is secreted, flow cytometry and sandwich ELISAs were carried out using a monoclonal antibody directed against vXCL1 (MAb vXCL1.11). As shown in Fig. 2A, vXCL1 could be detected in infected cells by flow cytometry at 13 h postinfection and accumulated thereafter in the presence of brefeldin A. A quantitative comparison of vXCL1 protein in lysates and supernatants of infected cells by ELISA revealed that vXCL1 is enriched over time in the supernatant of infected REF (Fig. 2B). Only small amounts of viral protein were detected throughout the course of infection in cell lysate.

Chemokines are usually generated as precursor proteins containing an N-terminal signal peptide of approximately 20 residues (e.g., in human XCL1 [2]). Cleavage of the signal peptide yields the mature protein, which is secreted. In order to characterize the N terminus of MuHV8 vXCL1 experimentally, the viral protein was expressed in insect cells and harvested from the supernatant by heparin affinity chromatography. Following purification, recombinant vXCL1 was analyzed by peptide mass fingerprinting. MALDI mass spectrometric analysis of secreted vXCL1 detected isoleucine₂₀ (I₂₀) as the N-terminal amino acid, indicating that the precursor protein is cleaved between serine₁₉ (S₁₉) and isoleucine₂₀ (I₂₀), resulting in a 96-residue mature protein. Additionally, the analysis revealed the presence of another mature protein starting with isoleucine₂₂, although this form was less abundant. Figure 2C summarizes the structures of the two mature protein variants.

Chemokine glycosylation has been implicated in chemokine folding and stability, thereby influencing the interaction with the respective receptor (33). Glycosylation was found to modify human XCL1 in such a way that different molecular sizes become apparent in Western blot analysis (2). To clarify whether different posttranslationally modified versions of vXCL1 exist, lysates of REF infected with MuHV8 were analyzed by Western blotting after digestion with PNGaseF, an amidase cleaving N-linked oligosaccharides from asparagines. As shown in Fig. 2D, PNGaseF treatment led to a size shift of vXCL1, indicating the presence of N-linked sugars attached to the viral protein. These data were corroborated by mass spectrometric analyses. PNGase F treat-

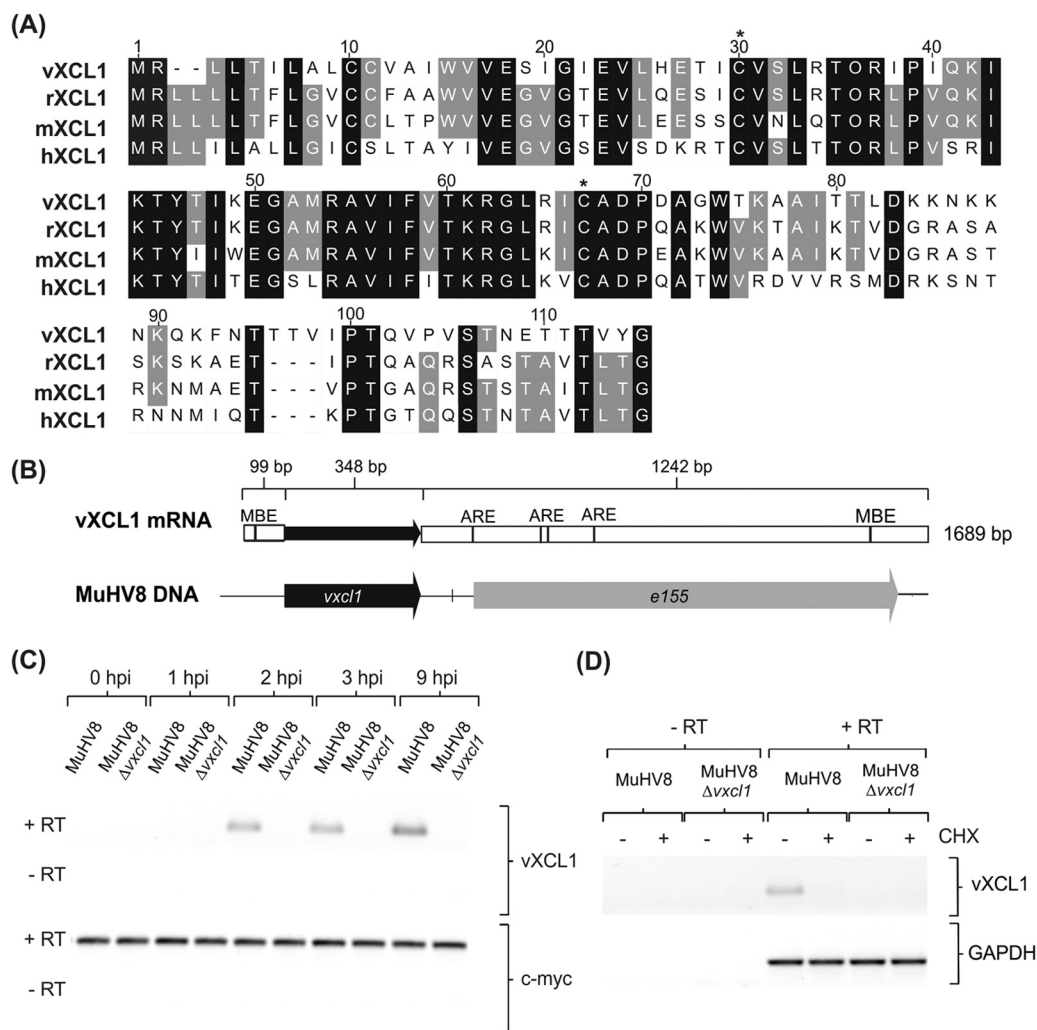


FIG 1 Characterization of MuHV8-encoded C chemokine vXCL1. (A) Muscle alignment (Geneious version 5.5.7) of amino acid sequences of vXCL1 and rat, mouse, and human XCL1. Identical amino acids are indicated by a black background and similar amino acids by a gray background, and lack of similarity is indicated by a white background. Cysteine positions characteristic of C chemokines are indicated by asterisks. Numbers refer to the amino acid positions of the viral chemokine. Accession numbers for the sequences depicted are JX867617.1 (vXCL1, MuHV8), NP_599188 (rXCL1, *Rattus norvegicus* XCL1), NP_032536 (mXCL1, *Mus musculus* XCL1), and NP_002986 (hXCL1, *Homo sapiens* XCL1). (B) Analysis of vXCL1 mRNA. Total RNA from MuHV8-infected REF was isolated 24 h postinfection (hpi) and 3'- and 5'-UTRs were determined. Musashi binding elements (MBE) were detected using UTRScan, and AU-rich element (ARE) were identified by scanning for an AUUUU element with CloneManager (Sci-Ed software, Cary, NC, USA). (C) Time course of MuHV8 vXCL1 expression. Total RNA from wild-type MuHV8 and MuHV8 $\Delta vxc1$ -infected cells was harvested at indicated time points, reverse transcribed with oligo(dT) primers, and amplified with *vxc1*-specific primers. (D) Cycloheximide (CHX) was used to discriminate between immediate early and early gene expression. MuHV8 $\Delta vxc1$ was included as a negative control. The experiment was carried out at least twice with similar results. c-myc and GAPDH served as controls. RT, reverse transcriptase.

ment resulted in a shift of the main peak from 12,778 to 10,701, indicating that vXCL1 is N-glycosylated (Fig. 2E). The observed mass shift of 2,077 could best be explained by attachment of a $\text{Man}_3\text{GlcNAc}_2\text{Fuc}$ moiety ($m = 1,039$) to each of the two potential N-glycosylation sites at N94 and N108. After removal of N-linked oligosaccharides, the mass heterogeneity persisted, presumably due to heterogeneous O-glycosylation. In the spectrum of the N-deglycosylated sample, the mass peak at 11,067 pointed to an additional HexNAc-Hex, compatible with a mucin-type O-glycan core structure. This peak could be eliminated by O-glycosidase, also known as endo- α -N-acetylglactosaminidase, an enzyme that preferentially catalyzes the removal of O-linked disaccharides from glycoproteins, while it does not attack more-complex O-gly-

can structures. These findings suggest that vXCL1 is additionally O-glycosylated (Fig. 2F).

vXCL1 is a chemoattractant for CD4⁻ DC, but not for CD4⁺ DC, T cells, or B cells. Murine and human XCL1 chemokines exert their biological function by attracting XCR1-expressing DC. We therefore tested vXCL1 for chemotactic capacity on rat DC, which were defined as CD103⁺MHCII⁺ (34), and used rat XCL1 as a positive control. DC were obtained by collagenase digestion of splenic tissue followed by Nycoprep density gradient centrifugation and magnetic sorting of CD103⁺ cells. The enriched (60%) DC population (composed of 36% CD4⁺ and 62% CD4⁻ DC; Fig. 3A, input) was placed in the upper chamber of a transwell system. Only very low background migration was observed with CD4⁻

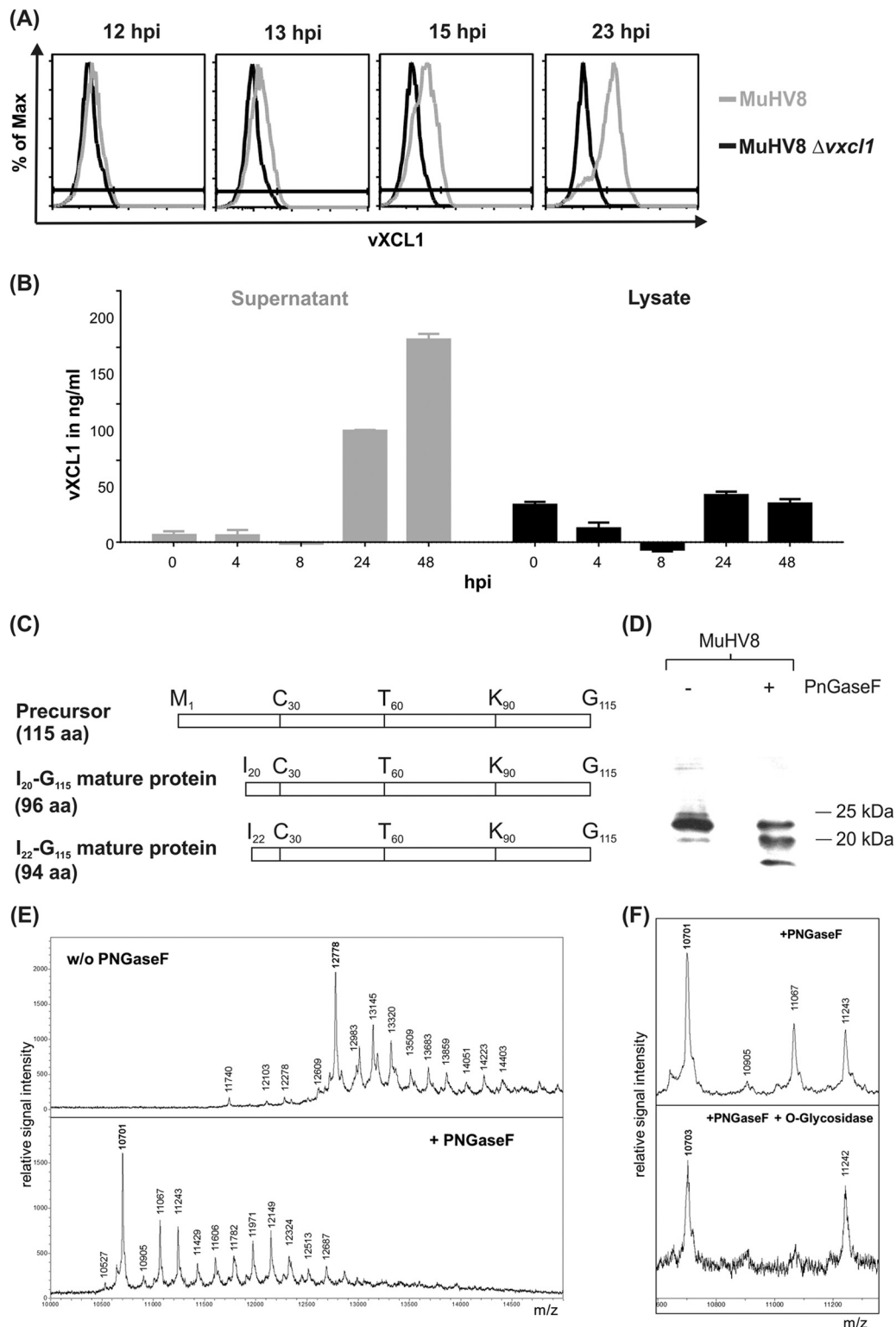


FIG 2 Analysis of vXCL1 protein expression and posttranslational modification. (A) Protein expression kinetics of vXCL1. MuHV8-infected REF were treated 3 h before harvest with brefeldin A, harvested at indicated time points, and analyzed by flow cytometry using MAb vXCL1.11, which specifically recognizes vXCL1 (gray line). REF infected with MuHV8 $\Delta vxcl1$ served as a control (black line). (B) Secretion of vXCL1. Supernatants (gray bars) and lysates (black bars) of infected REF were collected at indicated time points and tested for the presence of vXCL1 by sandwich ELISA. Values were normalized by subtracting the mean of the value obtained with MuHV8 $\Delta vxcl1$ -infected cells. Results shown are from two independent experiments. Error bars represent means \pm standard deviations (SD). (C) Schematic overview of the precursor protein and the two variants of mature vXCL1 determined by mass spectrometric analysis. aa, amino acids. (D) N-glycosylation variants of vXCL1. Lysates of MuHV8-infected REF were digested for 1.5 h in the presence or absence of PNGaseF and separated electrophoretically on a 15% Tris-Tricine polyacrylamide gel, followed by blotting and detection using MAb vXCL1.11. (E) Mass spectrometric analysis of vXCL1 N-glycosylation. PNGase F treatment resulted in a main peak shift from 12,778 to 10,701, the expected mass of the vXCL1 protein. (F) Mass spectrometric analysis of vXCL1 O-glycosylation. The mass peak at 11,067 in the N-deglycosylated sample (top panel) could be eliminated by O-glycosidase (bottom panel).

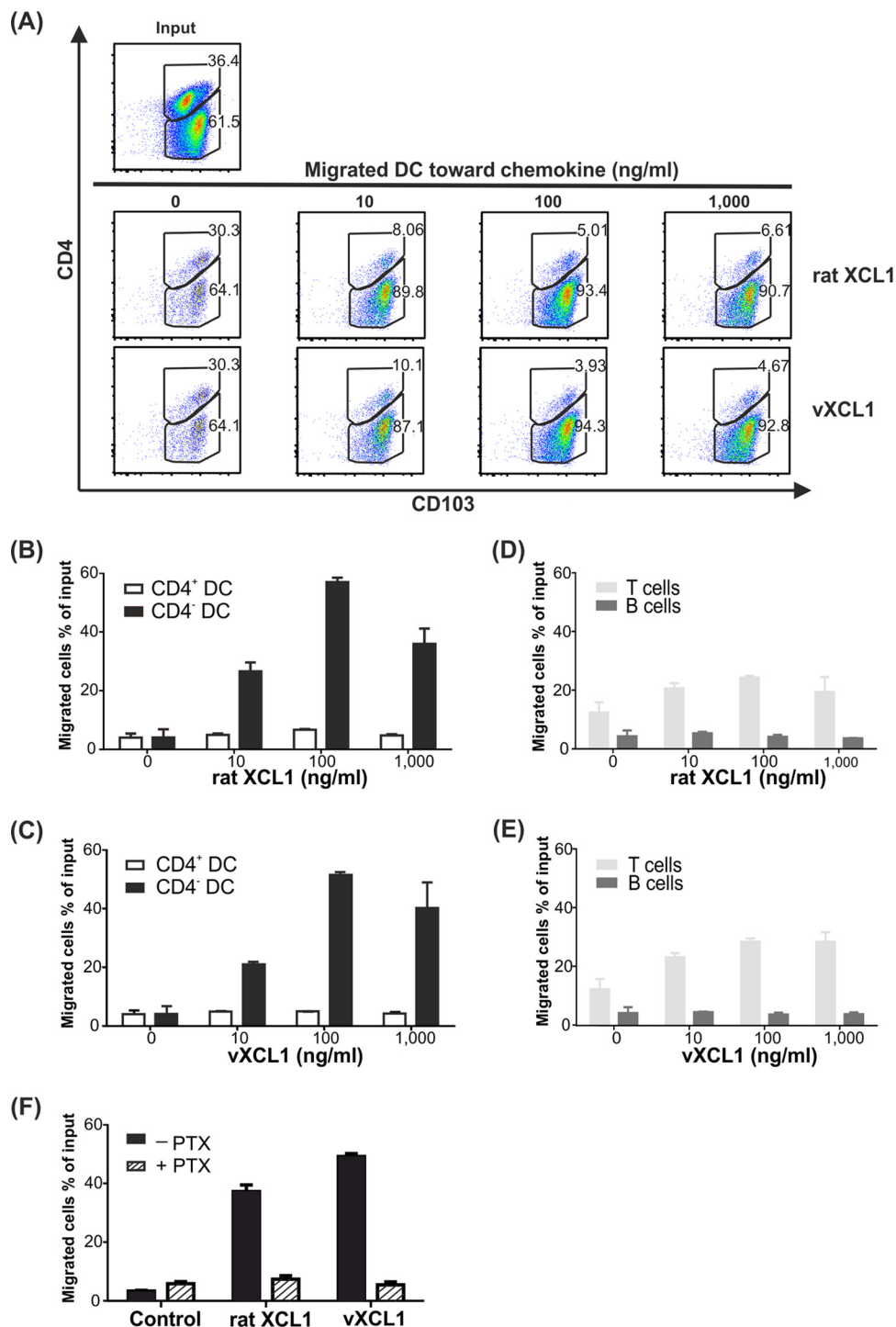


FIG 3 Analysis of vXCL1 chemotactic activity. (A) Magnetically sorted splenic DC were tested for migration toward different concentrations of vXCL1 and rat XCL1 (10 to 1,000 ng/ml) in a transwell assay. DC subpopulations were defined based on expression of CD103 and CD4. The dot plots represent the numbers of CD4⁻ DC and CD4⁺ DC that have migrated into the lower chamber. (B and C) Proportions of migrated CD4⁻ and CD4⁺ DC in response to different concentrations of rat XCL1 and vXCL1. (D and E) Proportions of migrated T and B cells toward rat XCL1 and vXCL1. (F) Pertussis toxin (PTX) blocks migration of CD4⁻ DC toward rat XCL1 and vXCL1. No migration is detected in the absence of chemokine (control). All experiments were performed twice. Error bars represent means \pm standard errors of the means (SEM).

DC and CD4⁺ DC fractions (less than 5% of input cells) in the absence of rat XCL1 or vXCL1. With increasing concentrations of rat XCL1 and vXCL1, selective migration of CD4⁻ DC occurred while CD4⁺ DC essentially failed to migrate (Fig. 3B and C). A

concentration of 100 ng/ml of either vXCL1 or rat XCL1 yielded the strongest migration, approximately 60% of CD4⁻ DC. vXCL1 and rat XCL1 did not induce migration of T or B cells (Fig. 3D and E). These results demonstrate that vXCL1 is a bona fide chemo-

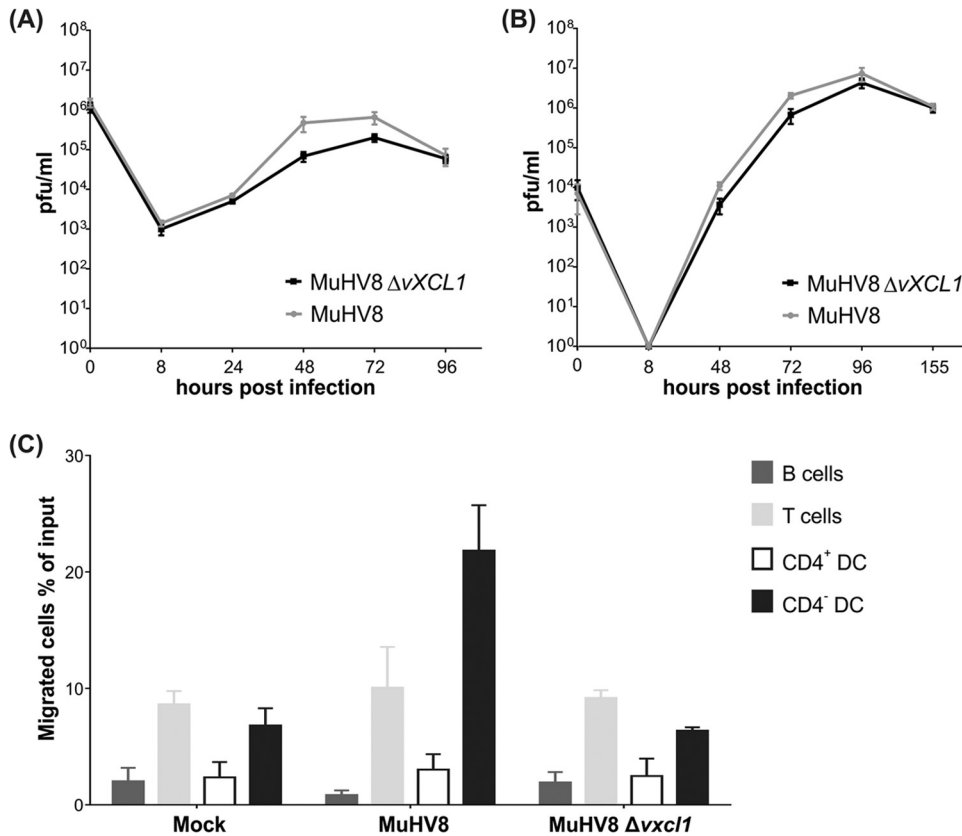


FIG 4 vXCL1 is dispensable for viral growth in cell culture and attracts CD4⁻ DC. Growth of wild-type MuHV8 and MuHV8 $\Delta vXCL1$: REF were infected at MOIs of 5 (A) and 0.05 (B), respectively, and titers were determined at the indicated time points by plaque assay. Results were obtained from three independent experiments. (C) The ability of vXCL1 to induce chemotaxis of different rat cell subsets was analyzed with supernatants of mock-infected, wild-type MuHV8-infected, and MuHV8 $\Delta vXCL1$ -infected REF. Results were obtained from two independent experiments. Error bars represent means \pm SD.

kine selectively attracting CD4⁻ DC but not CD4⁺ DC. Since chemokine receptors have been shown to signal via PTX-sensitive $G\alpha_i$ proteins (35), we investigated if the latter play a role in rat XCR1 signaling. DC were preincubated with 100 ng/ml PTX and analyzed for their ability to migrate toward vXCL1 or rat XCL1 (Fig. 3F). Migration of CD4⁻ DC toward vXCL1 and rat XCL1 was blocked by PTX, indicating that XCR1 signaling involves $G\alpha_i$ protein.

MuHV8-infected cells secreting vXCL1 attract CD4⁻ DC. Since recombinant vXCL1 induced migration of DC, it was expected that supernatant harvested from cells infected with wild-type virus but not virus with deletion of *vxcl1* ($\Delta vxcl1$) would also result in DC migration. However, to rule out that differences in replication kinetics between wild-type and $\Delta vxcl1$ MuHV-8 cause variations in DC migration, the growth behaviors of the viruses were analyzed and found to be similar (Fig. 4A and B).

Forty-eight hours postinfection, supernatants of mock-infected, MuHV8 wild-type-infected and $\Delta vxcl1$ mutant-infected REF were analyzed in chemotaxis assays (Fig. 4C). Supernatants of cells infected with $\Delta vxcl1$ MuHV8 failed to induce migration of CD4⁻ DC above the background compared with supernatants obtained from mock-infected REF. In contrast, supernatants of MuHV8 wild-type-infected REF induced chemotaxis in more than 20% of CD4⁻ DC. None of the supernatants induced migration of T or B cells. Taken together, these data indicate that vXCL1 generated during infection selectively attracts CD4⁻ DC.

Both vXCL1 and rat XCL1 bind to XCR1⁺CD4⁻ DC. In the mouse and humans, expression of XCR1 is restricted to a subset of DC (9, 31, 36). Since XCR1 is the only receptor for murine and human XCL1, we tested whether vXCL1 and rat XCL1 interact with rat XCR1. For these experiments, we used MAb MARX10, which specifically recognizes murine XCR1 (9) in a nonblocking fashion and cross-reacts with rat XCR1 (E. Hartung, H. Geyer, S. Voigt, and R. A. Kroczeck, unpublished data). CD4⁻ and CD4⁺ splenic DC were stained with MARX10 and costained with either rat XCL1 or vXCL1 (both tagged with a fluorochrome). As shown in Fig. 5, vXCL1 bound exclusively to CD4⁻ DC expressing XCR1 (bold square). Neither CD4⁺ DC nor T or B cells exhibited any binding of the viral chemokine. Rat XCL1 was tested in parallel and demonstrated a very similar staining pattern.

Since rat XCL1 and vXCL1 are highly similar in their amino acid sequence and induced chemotaxis and are bound to the same cell subset, we tested whether they share the same receptor, XCR1. To address this question, enriched DC were incubated with rat XCL1-APC in the presence of increasing concentrations of unlabeled vXCL1 and *vice versa*. Figure 6A shows that binding of rat XCL1-APC to CD4⁻ DC was effectively competed by unlabeled vXCL1. Analogous results were obtained when DC were incubated with vXCL1-APC in the presence of unlabeled rat XCL1 (Fig. 6B). These findings indicate that both chemokines bind to the same receptor on CD4⁻ DC.

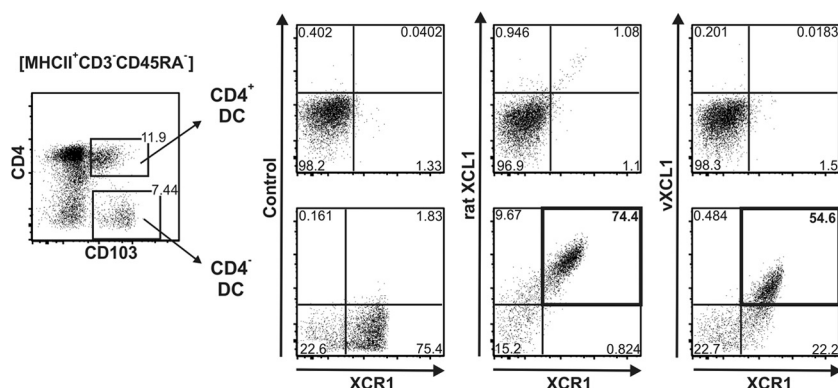


FIG 5 Analysis of rat XCL1 and vXCL1 binding to XCR1. Splenic rat DC (MHCII⁺CD103⁺CD3⁻CD45RA⁻) were gated into CD4⁺ (upper panels) and CD4⁻ (lower panels) DC subsets and analyzed for binding of XCL1, vXCL1, and MAb MARX10. Both rat XCL1 and vXCL1 selectively bound to DC expressing XCR1 (bold frames); the inset numbers give the proportion of double-positive cells. One representative experiment of three is shown.

DISCUSSION

CMV encodes several proteins that interfere with the chemokine network of the host. These proteins include molecules that act as chemokines (21), as chemokine-binding proteins (37), or as chemokine receptors (38, 39). In our study, we identified and characterized a C chemokine encoded by MuHV8, vXCL1, to our knowledge the first reported viral C chemokine. We could not identify sequence homologues in any other virus by database screening.

BLAST analysis of the vXCL1 amino acid sequence revealed high similarity to the only known C chemokine expressed in rat, mouse, and humans, XCL1. XCL1 in various species and vXCL1 have a similar length, of 114 to 115 amino acids, in their immature form. Both mouse and rat *xcl1* have three coding exons. Rat *xcl1* (GenBank Gene ID, 171371) has a size of 3.44 kbp with a transcript length of 345 bp, and mouse *xcl1* (GenBank Gene ID, 16963 [40]) has a size of 3.88 kbp with a transcript of 523 bp. The *vxcl1*

transcript of 1.6 kbp (including a 1.2-kbp 3'-UTR) originates from a continuous viral gene segment and contains one MBE motif and various AU-rich elements. In contrast to *vxcl1*, the MuHV8-encoded CC chemokine *eck-2* was acquired as an unspliced transcript with intron/exon boundaries similar to *mck-2* in MCMV (24). Possibly, acquisition of unspliced transcripts enables different levels of regulation of the CC chemokines, whereas this might not occur in the case of *vxcl1*.

Expression of immunomodulatory CMV genes is precisely timed to compromise cellular defense strategies (41). We therefore carefully analyzed both vXCL1 mRNA and protein expression kinetics. Semiquantitative RT-PCR revealed that vXCL1 mRNA is expressed 2 h postinfection and therefore follows early kinetics. In contrast to these results, intracellular vXCL1 protein could be detected only 13 h after infection. Since the vXCL1 transcript contains various RNA-binding protein motifs, it is conceivable that vXCL1 mRNA translation is inhibited early during infection

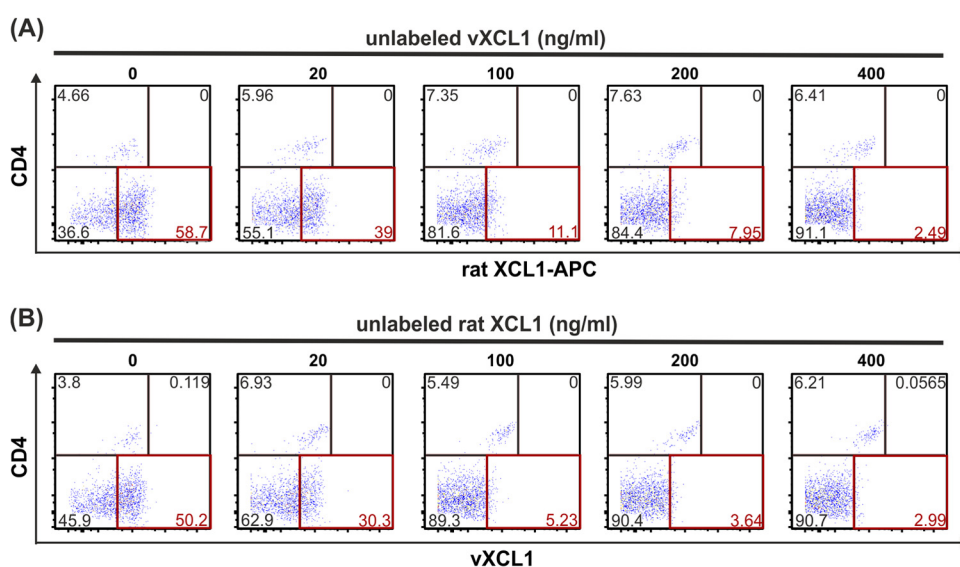


FIG 6 Competitive binding of vXCL1 and rat XCL1 to rat CD4⁻ DC. NycoPrep gradient-enriched rat DC were incubated with 400 ng/ml rat XCL1-APC in the presence of different concentrations of unlabeled vXCL1 (A) or with 400 ng/ml vXCL1-APC in the presence of different concentrations of unlabeled rat XCL1 (B), washed, and analyzed for binding of the respective fluorophore-tagged chemokine to rat DC (CD103⁺MHCII⁺). In both instances, binding of the labeled chemokine was effectively competed by the unlabeled chemokine on CD4⁻ DC (lower right quadrants). One representative experiment of two is shown.

through the interaction with RNA-binding proteins. Moreover, vXCL1 mRNA contains more AU-rich elements and also other potential binding sites for proteins and microRNAs than host rat XCL1. These sites might influence translation of vXCL1 mRNA and could serve as an explanation for the discrepancy between mRNA production and the onset of protein expression.

Correct cleavage of the signal peptide in the conversion of an immature chemokine into its mature form is critical for receptor binding and function (42). Since vXCL1 was secreted and enriched in the supernatant of infected REF, we performed mass spectrometric analysis to determine the N terminus of the mature protein. The supernatants contained two variants of vXCL1: an abundant mature protein spanning from isoleucine₂₀ to glycine₁₁₅ and a less abundant truncated protein ranging from isoleucine₂₂ to glycine₁₁₅. In addition, vXCL1 was shown to contain N-linked sugars since PNGaseF treatment resulted in a size shift as detected by Western blotting and mass spectrometric analyses. The vXCL1 amino acid sequences possess two N-glycosylation motifs at which N-glycosylation could take place: Asn₉₄-Thr₉₅-Thr₉₆ and Asn₁₀₈-Glu₁₀₉-Thr₁₁₀. The N-linked-sugars of vXCL1 might improve interaction with the chemokine receptor, increase the stability and thus the biological activity of the chemokine, or help to mask antigenic sites of the viral molecule. Interestingly, human and murine XCL1 presumably carry O-linked sugars at their respective C termini (2, 43). NetOGlyc analysis predicted several potential O-glycosylation sites for vXCL1 at the C terminus, and mass spectrometric analysis confirmed that vXCL1 possesses an O-linked sugar.

Murine and human XCL1 have been shown to act as chemoattractants on cross-presenting DC (9, 31, 36), while there are no data available for rat XCL1. We therefore compared vXCL1 and rat XCL1 in their ability to induce migration of CD4⁻ DC, CD4⁺ DC, B cells, and T cells. Our data demonstrate that only CD4⁻ DC migrate in the presence of either the host or the viral chemokine. Similar findings were obtained when supernatants of infected REF were used in chemotaxis assays: only supernatants of MuHV8-infected REF but not those of the $\Delta vxcl1$ mutant-infected cells attracted CD4⁻ DC. A revertant virus was not employed in these assays because complete genome sequencing of the MuHV8 $\Delta vxcl1$ knockout virus and comparison with wild-type MuHV8 showed the desired sequence divergence only in the *vxcl1* region but not elsewhere in the genome. Rat CD4⁻ DC are thought to be the equivalent of murine CD8⁺ DC (44, 45) since they both produce large amounts of interleukin 12 (45), efficiently phagocytose apoptotic cells, and are located in the red pulp and T cell area of the spleen (46). The finding that both rat XCL1 and vXCL1 selectively induce migration of only CD4⁻ DC further supports the concept that these DC are the equivalents of human (31, 47) and murine (9) cross-presenting DC.

The G protein-coupled receptor XCR1 was shown to be expressed only on cross-presenting DC in mice (9) and was identified as the only interaction partner of XCL1 in the human and murine systems. Thus, XCR1 is the most prominent candidate as a vXCL1 and rat XCL1 interaction partner. The monoclonal antibody MARX10 directed against mouse XCR1 (9) cross-reacts with rat XCR1 and therefore served as a valuable tool to analyze the correlation between chemokine binding and receptor expression. Approximately 80% of rat CD4⁻ DC expressed XCR1, which correlates with the murine system, where 70 to 85% of spleen-derived CD8⁺ DC were shown to express XCR1 (9, 36). Flow cytometry

studies revealed that vXCL1 and rat XCL1 bound selectively to CD4⁻, XCR1-expressing DC. Chemokine binding to CD4⁺ DC was not observed, which is in accordance with our chemotaxis data.

Upon ligand binding, intracellular G proteins are dissociated into GTP-bound subunits, and a signal cascade that results in cell migration is initiated. Since little is known about rat XCR1-mediated signaling, we addressed the question if vXCL1- or rat XCL1-mediated migration can be inhibited by treatment with PTX, an agent that modifies G α_i proteins, and if this would prevent G α_i protein interaction with XCR1 and abolish DC migration. Indeed, preincubation with PTX inhibited CD4⁻ DC migration, suggesting that rat XCR1 is linked with a G α_i protein.

Since both rat XCL1 and vXCL1 bound to XCR1⁺CD4⁻ DC, it was likely that the two chemokines shared the interaction partner. However, it was not clear if XCR1 is the only interaction partner or if another receptor coexpressed with XCR1 is targeted. To address this question, we examined binding of rat XCL1-APC to rat DC subsets in the presence of different concentrations of unlabeled vXCL1 and *vice versa*. Competitive binding studies revealed that binding of APC-labeled rat XCL1 was abolished in the presence of unlabeled vXCL1 and *vice versa*, implying that vXCL1 binds to the same surface molecule, i.e., XCR1.

The data presented here show that vXCL1 (i) has chemokine-like properties, (ii) binds to and attracts CD4⁻ rat DC that express XCR1, and (iii) functionally resembles rat XCL1. At first glance, the attraction of XCR1⁺ DC seems rather unfavorable for the virus. In mice and humans, XCR1-expressing DC are capable of antigen cross-presentation and thus possess a key role in controlling viruses (10, 11). In the mouse model, CD8⁺ cross-presenting DC could be infected with MCMV at a low percentage (48, 49). Infection of DC was shown to be accompanied by reduced surface expression of MHC-I and -II molecules (50–52), altered cytokine and chemokine receptor expression (53), and downregulation of molecules required for T and NK cell proliferation (48, 50, 52). Since DC are important for regulating and controlling T and NK cell responses (54, 55) and the latter play a major role in controlling CMV infection (56–60), MuHV8 might infect and functionally paralyze this DC subset by vXCL1 attraction in order to impair antiviral responses. Further, immature DC have been shown to be an important reservoir of latent CMV from which reactivation can occur (61, 62), but it remains to be determined if vXCL1 has a role in the establishment of a latent infection.

Alternatively, it is possible that cross-presenting DC regulate T cell activation and cytotoxicity without being infected and that vXCL1 functions in a manner analogous to that of the MCMV-encoded CC chemokine MCK-2. MCK-2 attracts inflammatory monocytes to the site of infection, where they compromise virus-specific CD8⁺ T cells and thus contribute to viral persistence (26). Possibly, vXCL1 attracts XCR1⁺ DC to the site of infection and locally disturbs cooperation with CD8⁺ T cells, thereby compromising the adaptive immune response. However, it remains to be shown if the attracted XCR1⁺ DC subset is able to cross-present antigen. Eventually, the influence of MuHV8 on this potentially cross-presenting DC subset and possible vXCL1 interference with the host immune system will have to be evaluated in *in vivo* studies.

So far, all CMV-encoded chemokines have been classified as either CXC or CC chemokines and have been shown to attract neutrophils (17), monocytes (25), macrophages (63), and CD4⁺

T cells (20). vXCL1 is the first reported CMV-encoded C chemokine that targets XCR1⁺CD4⁻ DC. This might imply an important physiological function, since Kaposi's sarcoma-associated herpesvirus (KSHV) has also been shown to encode two chemokines, vCCL2 and vCCL3, which likewise target XCR1 at different time points during infection (64, 65). The observation that both MuHV8 and KSHV exploit XCR1 with different virally encoded chemokines suggests a substantial importance of this receptor in the defense against herpesviruses.

Since MuHV8 is so far the only known virus to encode an XCL1 homologue, the MuHV8-rat model appears to be particularly useful in analyzing the biological function of vXCL1 and its interference with the endogenous XCL1 molecule. Thus, this model offers a unique opportunity to study the involvement of XCL1 in antiviral defenses and might be promising to provide clues for novel antiviral strategies.

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