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Novel Human Cytomegalovirus Viral Chemokines, vCXCL-1s, Display Functional Selectivity for Neutrophil Signaling and Function

Jinho Heo,*† Pranay Dogra,* Tom J. Masi,* Elisabeth A. Pitt,* Petra de Kruijf,† Martine J. Smit,‡ and Tim E. Sparer*

Human CMV (HCMV) uses members of the hematopoietic system including neutrophils for dissemination throughout the body. HCMV encodes a viral chemokine, vCXCL-1, that is postulated to attract neutrophils for dissemination within the host. The gene encoding vCXCL-1, UL146, is one of the most variable genes in the HCMV genome. Why HCMV has evolved this hypervariability and how this affects the virus’ dissemination and pathogenesis is unknown. Because the vCXCL-1 hypervariability maps to important binding and activation domains, we hypothesized that vCXCL-1s differentially activate neutrophils, which could contribute to HCMV dissemination, pathogenesis, or both. To test whether these viral chemokines affect neutrophil function, we generated vCXCL-1 proteins from 11 different clades from clinical isolates from infants infected congenitally with HCMV. All vCXCL-1s were able to induce calcium flux at a concentration of 100 nM and integrin expression on human peripheral blood neutrophils, despite differences in affinity for the CXCR1 and CXCR2 receptors. In fact, their affinity for CXCR1 or CXCR2 did not correlate directly with chemotaxis, G protein-dependent and independent (β-arrestin-2) activation, or secondary chemokine (CCL22) expression. Our data suggest that vCXCL-1 polymorphisms affect the binding affinity, receptor usage, and differential peripheral blood neutrophil activation that could contribute to HCMV dissemination and pathogenesis.

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Materials and Methods

Materials

DMEM, penicillin, and streptomycin were obtained from Hyclone Laboratories (Logan, UT). FBS was purchased from Mediatech (Manassas, VA). DMEM containing 25 mM HEPES and t-glutamine, OPTI-MEM, Hygromycin B, and Geneticin were obtained from Invitrogen (Paisley, U.K.). BSA Fraction V (BSA) was purchased from Roche (Mannheim, Germany). Polyethylenimine was obtained from Polysciences (Warrington, PA).

Clinical isolates used for cloning of the vCXCL-1 ORFs were provided by Dr. James Bale (University of Utah School of Medicine), Dr. Sunwen Chou, (Oregon Health and Science University), and Dr. Gail J. Demmler (Texas Children’s Hospital) as described previously (17).

Cell culture and CXCR2 transfection

Insect cells (serum-free adapted SF9 cells; Invitrogen, Carlsbad, CA) were grown at 28°C in serum-free SF-900 II SFM medium (Invitrogen). Hi5 cells
Peripheral blood neutrophils (PBNs) were isolated from EDTA-treated blood from healthy human volunteers using dextran sedimentation and density gradient centrifugation as previously described (19). Erythrocytes were removed with hypotonic lysis in 0.2% NaCl. Neutrophils were resuspended in the buffers for the individual assays. Viable neutrophils were quantified with trypan blue exclusion using a hemacytometer. The use of human subjects has been approved by the University of Tennessee Institutional Review Board (IRB no. 6476B).

Production of recombinant vCXCL1-proteins

The vCXCL1-gene UL146 was PCR amplified from HCMV DNA from each of the 11 clades. Amplicons were cloned into the baculovirus transfer plasmid Bac3 (Invitrogen), which contains homologous regions for recombination into the baculovirus genome. PCR primers were designed to include the ORF and with an additional two to four glycines and six histidines on the C terminus of the proteins for purification. For generation of baculoviruses, SP9 cells were transfected with the 1392/UL146 ORF plasmid construct and linearized AcNPV DNA (Sapphire Baculovirus DNA; Orbigen) using transfection reagent Cellfectin (Invitrogen). Recombinant baculoviruses containing the UL146 gene were titrated and used to infect Hi5 cells for optimum protein expression. Forty-eight hours after infection, cells and supernatants were harvested. Recombinant protein was isolated from the supernatants using Ni-NTA agarose beads (Qiagen, San Diego, CA) and resuspended in PBS. Protein concentration was quantified using silver staining of SDS-PAGE gel using lysozyme as a standard and analyzed using Quantity One software (Bio-Rad, Hercules, CA). MALDI-TOF was used to confirmed protein purity and the correct m.w.

Intracellular calcium mobilization assays

Release of calcium from intracellular calcium stores was determined on freshly isolated PBNs resuspended in MEM. PBNs at 5 × 10⁶ cells/ml were loaded with 4 μM Fluo-4 AM (Molecular Probes, Invitrogen) for 60 min at 37°C. Cells were then washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were resuspended in 106 cells/ml in MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once with MEM and incubated for an additional 30 min for completion of the esterification process. Finally, the cells were washed once

β2 integrin staining

PBNs (1 × 10⁶ cells) were resuspended in RPMI 1640 with 1% FBS and exposed to 100 nM of chemokines for 2 h at 37°C. Cells were washed with PBS and blocked with 1% goat serum. PBNs were incubated with fluorescently conjugated CD11a, CD11b, and CD11c Abs (Caltag; Invitrogen) on ice for 30 min and fixed with 4% paraformaldehyde. Cells were analyzed with flow cytometry (FACSCalibur; BD Biosciences).

Human PBN chemotaxis assays

Chemotaxis assays were performed on freshly isolated PBNs resuspended in HBSS with 0.1% BSA and 10 mM HEPEs. Assays were performed in triplicate in 96-well chemotaxis plates. Thirty microliters of chemokines were loaded at varying concentrations (100 and 500 mM) into the lower wells of the modified Boyden chamber (Neuroprobe, Gaithersburg, MD) and fitted with a 5-µm filter. PBNs were labeled with 1:1000 CalceinAM (Invitrogen) for 1 h on a rotating wheel at 37°C. Cells were washed with PBS and resuspended to 5 × 10⁶ cells/ml; 20 µl of cells was added to the upper well. The PBNs were allowed to migrate for 2–3 h at 37°C. The number of PBNs that migrated to the chemokines was measured on a fluorescent plate reader (Synergy 2; Bioteck) minus the fluorescence from the buffer-only control wells.

Receptor binding analysis

The ability of vCXCL1s to compete for binding to either CXCR1 or CXCR2 was evaluated as described previously (6). Briefly, 1 × 10⁶ to 3 × 10⁶ HEK293 cells stably expressing CXCR1 or CXCR2 were incubated with 100 pM [³²S]-labeled CXCL8 (MP Biomedical) and increasing concentrations of unlabeled chemokines for 1 h at room temperature. Cells were collected on glass filters and washed twice, and bound radioactivity was measured with liquid scintillation counting. The graph was plotted, and competition constants (IC₅₀) were analyzed using GraphPad Prism 5 for Windows.

³²S-GTPγS binding assay

Two days after transfection with CXCR2 expression constructs, HEK293T cells were detached from the plastic surface using ice-cold PBS and centrifuged at 1500 × g for 10 min at 4°C. The pellet was resuspended in ice-cold PBS and centrifuged. Cells were resuspended in ice-cold membrane buffer (15 mM Tris, 1 mM EGTA, 0.3 mM EDTA, and 2 mM MgCl₂, pH 7.5), followed by homogenization using a Teflon-glass homogenizer and sonication. The membranes were subjected to two freeze-thaw cycles using liquid nitrogen, followed by centrifugation at 40,000 × g for 25 min at 4°C. The pellet was resuspended with ice-cold Tri-s-sucrose buffer (20 mM Tris and 250 mM sucrose, pH 7.4) and subsequently resuspended in the same buffer and stored at −80°C. Protein concentration was determined using a BCA-protein assay (Thermo Scientific).

Membranes (2.5 µg/well) were incubated in 96-well plates in assay buffer (50 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, pH 7.2, with 5 µg saponin/well, 3 µM GDP, and ~500 pM³²S-GTPγS added) and the indicated concentrations of CXCL8 or vCXCL-1 to a final volume of 100 µl. The reaction mixtures were incubated for 1 h at room temperature, harvested with rapid filtration through Unifilter GF/B 96-well filter plates (PerkinElmer), and washed three times with ice-cold wash buffer (50 mM Tris-HCl and 5 mM MgCl₂, pH 7.4).³²S-GTPγS incorporation was determined using a Microbeta scintillation counter (PerkinElmer). Functional data were evaluated using nonlinear curve fitting using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

β-Arrestin recruitment assay

PathHunter HEK293-CXCR2 cells were plated out overnight at 1 × 10⁶ cells/well in a 384-well plate in 20 µl OPTI-MEM. A preincubation with vehicle (PBS plus 0.1% BSA) of 30 min at 37°C and 5% CO₂ was followed by treatment with CXCL8 or vCXCL1 stimulation at 37°C and 5% CO₂. PathHunter Detection Reagent (12 µl; DiscoveRx) was added. After 60 min of incubation at room temperature, β-galactosidase, as an indicator of β-arrestin–CXCR2 interaction, was measured for 0.3 s in a Victor® 1420 Multilabel Reader. Functional data were evaluated using a nonlinear curve fitting using GraphPad Prism 4.0.

Quantitative real-time PCR of CCL22 expression

HL-60 T2 cell transfectants overexpressing CXCR2 were differentiated and labeled with 1.3% DMSO to chemokinesis. Medium was exchanged with HBSS and incubated with viral chemokines at a final concentration of 100 nM for 4 h at 37°C. Total RNA was isolated with TriReagent (Sigma, St. Louis, MO) and reverse transcribed using TranscriptoM M-MuLV first-strand cDNA synthesis kit (New England Biolabs, Ipswich, MA). Real-time PCR was performed using iQ5 Real-Time PCR Detection System (Bio-Rad) with a reaction mixture volume of 25 µl containing 2× SYBRgreen (New England Biolabs) DyNaMo SYBR green qPCR kit), 300 nM of each primer, and ~25 ng cDNA. Primers for CCL22 were purchased from SA Biosciences (catalog no. PPH00697E). The reaction conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The results were analyzed with the iQ5 Optical System Software (Bio-Rad). The relative gene expression levels were calculated as the fold change using the formula: 2−ΔΔCT, where ΔΔCTtarget = Threshold cycle (CT) of the control gene (ACT1) − CT of the target gene (CCL22), and ΔΔCT = ΔCTreference − ΔCTtarget (20). The housekeeping gene encoding actin (ACT1) was used as a reference control. Primers for ACT1 were 5′-TGAGATGCTAGTTGACGAA-3′ (forward

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and 5′-CACGAAAGCAATGCTATCAC-3′ (reverse) generating a 120-bp product.

**Results**

**Amino acid sequence alignments**

Previously, we sequenced the UL146 gene from 51 clinical isolates and showed that it comprised 11 genetic clades (17). Representative isolates from the 11 clades were aligned with vCXCL-1 from the Toledo strain (vCXCL-1\textsubscript{Toledo}; Fig. 1). The percent identities of the mature forms of the vCXCL-1s, without the signal sequences, vary between 23.7 and 61.2% compared with vCXCL-1\textsubscript{Toledo}. The vCXCL-1s contain ~20 additional residues on the C terminus compared with host chemokines CXCL1 and CXCL8, but the function of these extra residues is unknown. Alignment of the vCXCL-1s and the host chemokines show seven conserved residues, including the arginine (R) in the ELR motif, two cysteines (C) in the N terminus (part of the CXC motif), a proline (P) at position 32, cysteines at position 35 and 55, and a leucine (L) at position 56. Furthermore, all vCXCL-1s contain a glycine (G), valine (V), histidine (H), tryptophan (W), and proline (P) at positions 21, 54, 60, 65, and 87, respectively, which are lacking in the host chemokines. The ELR motif was conserved in all except vCXCL-1\textsubscript{TX15}. The variability in the N-loop region (21), C terminus (22, 23), and even in the ELR motif (24) led us to evaluate differences in chemokine receptor binding and functional responses (25).

**vCXCL-1 production using the baculovirus expression system**

To address functional differences between the vCXCL-1s, we generated recombinant vCXCL-1s using the baculovirus protein expression system. Unlike protein production from prokaryotes, baculovirus expression provides mammalian signal-sequence cleavage, eukaryotic glycosylation patterns, and protein folding. Because some vCXCL-1s contain multiple predicted signal cleavage (6) and glycosylation sites, and differences in recombinant protein refolding conditions, we chose to express and purify them using the baculovirus system. All vCXCL-1s were 6 His-tagged and purified using Ni-NTA agarose beads. Purity was confirmed with MALDI-TOF and resulted in the predicted molecular weights (11–15 kDa).

**vCXCL-1s stimulate calcium release in PBNs**

Release of intracellular calcium is a common indicator of chemokine activation of PBNs (6, 15). Although there were no differences in calcium flux and integrin expression, these readouts could have a lower threshold for activation compared with a more complex PBN function such as migration. We quantified the PBNs that chemotaxed to different vCXCL-1 concentrations and found differences in their migratory ability (Fig. 4). All vCXCL-1s except vCXCL-1\textsubscript{TX24} and vCXCL-1\textsubscript{TX15} induce migration at 500 nM, whereas at 100 nM only vCXCL-1\textsubscript{C952}, vCXCL-1\textsubscript{E760}, vCXCL-1\textsubscript{Toledo}, vCXCL-1\textsubscript{100751}, and vCXCL-1\textsubscript{C956} could stimulate migration. To our knowledge, this is the first time that differences between the different vCXCL-1s were observed in a functional assay.

All vCXCL-1s induced calcium flux at a concentration of 100 nM, including 100751, which is not shown in Fig. 2. However, they differ in their ability to induce a calcium flux at other concentrations tested (Fig. 2). This demonstrates that although the viral chemokines can induce calcium mobilization in PBNs, the different vCXCL-1s have differing sensitivities for calcium signaling that may induce different downstream activation of PBNs.

**vCXCL-1s upregulate CD11b and CD11c**

β2 integrins are receptors that form heterodimers composed of an α component, such as CD11a, CD11b, and CD11c, and a β component, such as CD18. They are present on circulating leukocytes and, once the cell is activated, initiate adhesion to endothelial cells and subsequent transmigration across the endothelium (28). Host chemokine, CXCL8, upregulates CD11b and CD11c expression (29, 30). Moreover, vCXCL-1\textsubscript{Toledo} and the vCXCL-1 from chimpanzee CMV also increases integrins on PBNs (26). In this study, we tested the ability of vCXCL-1s to alter the surface expression of these receptors on PBNs (Fig. 3). Exposure to the vCXCL-1s or host chemokines, CXCL1 and CXCL8, does not change cell surface expression levels of CD11a. However, CD11b and CD11c levels are increased upon exposure to either the vCXCL-1s or host chemokines. The percent change in the mean fluorescent intensity of CD11b was 57–91% for the viral chemokines, which is similar to CXCL1 upregulation (82%) but less than then CXCL8 (143%). Likewise, the percent change of CD11c varied from 35% to 55% for the vCXCLs, which is similar to CXCL1 (43%) but lower than CXCL8 (80.3%). These results demonstrate that the viral chemokines selectively induce β2 integrin (CD11b and CD11c) upregulation, but without significant differences between them at 100 nM.

**Differential migration of human PBNs**

Both CXCL8 and vCXCL-1\textsubscript{Toledo} are potent chemoattractants for PBNs (6, 15). Although there were no differences in calcium flux and integrin expression, these readouts could have a lower threshold for activation compared with a more complex PBN function such as migration. We quantified the PBNs that chemotaxed to different vCXCL-1 concentrations and found differences in their migratory ability (Fig. 4). All vCXCL-1s except vCXCL-1\textsubscript{TX24} and vCXCL-1\textsubscript{TX15} induce migration at 500 nM, whereas at 100 nM only vCXCL-1\textsubscript{C952}, vCXCL-1\textsubscript{E760}, vCXCL-1\textsubscript{Toledo}, vCXCL-1\textsubscript{100751}, and vCXCL-1\textsubscript{C956} could stimulate migration. To our knowledge, this is the first time that differences between the different vCXCL-1s were observed in a functional assay.
Affinities for CXCR1 and CXCR2

Because some CXC chemokines such as CXCL8 bind to both CXCR1 and CXCR2, and these receptors are important for chemotaxis (31–33), we investigated receptor usage and affinity of the different chemokines for CXCR1 and CXCR2. Competition binding assays using the vCXCL-1s to displace $^{125}$I-CXCL8 on HEK293 cells expressing either CXCR1 or CXCR2 (Fig. 5) showed IC$_{50}$ concentrations that ranged from 2.6 to 148.7 nM for CXCR2 and 3.3 nM to $10^4$ nM (i.e., no competition) for CXCR1. Using cluster analysis of the averages of the different IC$_{50}$'s, we divided the chemokines into high-, medium-high-, medium-low-, and low-affinity binders for CXCR2 (Fig. 5A). The group of high-affinity binders (2.6–3.6 nM) along with CXCL8, are vCXCL-1$_{Toledo}$ and vCXCL-1$_{C952}$. Medium-high-affinity binders (11.3–18.6 nM) are vCXCL-1$_{Tx11}$, vCXCL-1$_{E760}$, vCXCL-1$_{C956}$, and vCXCL-1$_{100751}$, and medium-low members are vCXCL-1$_{102410}$, vCXCL-1$_{Tx24}$, and vCXCL-1$_{C954}$ (32.7–55.5 nM). The low-affinity group (>141 nM) contains only two members: vCXCL-1$_{Towne}$ and vCXCL-1$_{Tx15}$. Interestingly, the viral chemokines with high affinity for CXCR2 (vCXCL-1$_{Toledo}$ and vCXCL-1$_{C952}$) have weak binding to CXCR1 compared with the host chemokines. Generally, the higher the affinity for CXCR2, the more likely the viral chemokines will bind to CXCR1 (Fig. 5B). The medium-high CXCR2 binders generally do not bind to CXCR1 except for vCXCL-1$_{E760}$. These data indicate that the viral chemokines bind with differing affinities for CXCR2 with

**FIGURE 2.** CXCL8, CXCL1, and the different vCXCL-1s induce intracellular calcium mobilization on human PBNs. Changes in fluorescence were measured over time after exposure to different concentrations of chemokines (after 20 s at baseline, as indicated with an arrow). Data shown are representative figures of three independent experiments.
All vCXCL-1s, regardless of their affinity for CXCR1 or CXCR2 (except vCXCL-1 TX24 and vCXCL-1 TX15), induce migration above the limit of detection at 500 nM. At the lower concentration (100 nM), only the high-affinity or the select medium-high-affinity binders (i.e., vCXCL-1E760, vCXCL-1 100751, and vCXCL-1C956) could induce migration (Fig. 4). These data imply that affinity for CXCR2 (i.e., high affinity equals high migration) or CXCR1 usage are potential factors in PBN migration (32, 33). Because it is not strictly correlated with affinity, differential agonist activation signals could also contribute to PBN migration as well.

**vCXCL-1s induce differential 35S-GTPγS binding and β-arrestin-2 recruitment**

Chemotactic responses can be mediated via G protein–dependent or G protein–independent signaling, or both. Berger et al. (30) demonstrated that CXCL8-induced β2 integrin CD11b upregulation and migration of neutrophils is G_{o} dependent. Chemokine-induced

\[ \text{Chemokines} \mid \begin{array}{c|ccc} & \text{percent change in the mean fluorescent intensity} \\
& CD11a & CD11b & CD11c \\
\hline
CXCL8 & 6.8 & 142.6 & 80.3 \\
CXCL1 & -5.0 & 82.4 & 42.8 \\
C952 & 10.5 & 91.2 & 55.3 \\
E760 & 8.2 & 71.1 & 39.1 \\
Toledo & 12.3 & 80.7 & 50.5 \\
TX11 & 11.4 & 57.7 & 43.7 \\
100751 & 6.8 & 71.9 & 50.3 \\
C956 & 11.8 & 91.2 & 54.9 \\
TX24 & 8.6 & 81.4 & 55.3 \\
Towne & 10.5 & 57.0 & 34.9 \\
102410 & 6.4 & 64.5 & 47.8 \\
C954 & 7.3 & 73.7 & 37.0 \\
TX15 & 13.2 & 62.5 & 36.2 \\
\end{array} \]

**FIGURE 3.** vCXCL-1s elicit changes in surface expression of CD11a and CD11b. PBNs were incubated with 100 nM viral or host chemokine for 2 h. The shaded curve represents expression levels of integrins on unstimulated PBNs. The table below lists the percentage change in mean fluorescence intensity (Chemokine stimulated mean fluorescence intensity/Unstimulated mean fluorescence intensity × 100). Graphs are representative of three independent experiments.

**FIGURE 4.** Differential chemotaxis of PBNs to vCXCL-1s partially correlates with affinity. Chemotactic response of human PBNs to 500 and 100 nM of CXCL8, CXCL1, or vCXCL-1s. The chemotactic response was measured as fluorescence intensity of migrated PBN labeled with CalceinAM. Background chemotaxis was subtracted from all samples. Data shown are representative data of three independent experiments performed in triplicate.
calcium flux involves Gαi proteins as well (34, 35). Based on these studies and observation of differences in migration and binding, we investigated whether vCXCL-1s display differences in G protein–dependent and independent signaling that could explain the differences in migration. 35S-GTPγS binding experiments were performed on HEK293T membranes expressing human CXCR2 (Fig. 6A, 6B). The pEC50 value of CXCL8 in this assay is 6.9. Only CXCL1 and vCXCL-1Toledo are able to reach a maximal response equivalent to 1 μM CXCL8. vCXCL-1Toledo is a high-affinity CXCR2 agonist capable of inducing migration (Fig. 4), and it uses G proteins (Fig. 5A). Surprisingly, vCXCL-1C952, another high-affinity binder of CXCR2 that induces PBN migration, does not induce a G protein response. All those with medium affinity for CXCR2, except for vCXCL-1TX11, have medium potency for G protein binding, regardless of their ability to induce migration. vCXCL-1TX11 has a medium-high affinity for CXCR2 that induces PBN migration, does not induce a G protein response. All those with medium affinity for CXCR2, except for vCXCL-1TX11, have medium potency for G protein binding, regardless of their ability to induce migration. vCXCL-1TX11 has a medium-high affinity for CXCR2 and induces PBN migration, but does not use G proteins for inducing this response. As expected, those with low affinity for CXCR2 had no GTP binding (vCXCL-1Towne and vCXCL-1TX15). Based on the dose response curves, we propose a potency order of the chemokines for CXCR2: CXCL8 (pEC50 = 9.1 nM) [high affinity for CXCR2] > CXCL1 (pEC50 = 8.3 nM) [high affinity for CXCR2] > Toledo (pEC50 = 8.4 nM) [high affinity for CXCR2] > E760 (pEC50 = 8.1 nM) [high affinity for CXCR2] > TX24 (pEC50 = 7.5 nM) [high affinity for CXCR2] > C952 (pEC50 = 8.4 nM) [high affinity for CXCR2] > C956 [med-high affinity for CXCR2] > TX15 (pEC50 = 6.3 nM) [med-low affinity for CXCR2] > TX11 (pEC50 = 6.2 nM) [med-low affinity for CXCR2] > C954 (pEC50 = 6.0 nM) [low affinity for CXCR2].

Traditionally, β-arrestin proteins were thought to function only to desensitize activated G protein coupled receptors. However, in the last decade β-arrestins were shown to induce intracellular signaling as well (36, 37). The involvement of β-arrestins in chemokine-induced chemotaxis was first described for the CXCR4/CXCL12 axis (38) and includes the p38 MAPK pathway (39). Following from these studies, a role for β-arrestin-2 in CXCR2 directed chemotaxis was shown (23, 40, 41). β-arrestin-2–induced chemotaxis could explain the differences seen with the different chemokines (Fig. 4). To measure chemokine-induced β-arrestin-2 recruitment, we used the PathHunter-HEK293-CXCR2 indicator cell line, which produces a functional β-galactosidase in response to β-arrestin-2 (42). The pEC50 value of CXCL8 in this assay is 9.1. CXCL8, CXCL1, vCXCL-1 Toledo, vCXCL-1 C952, and vCXCL-1E760 make full dose-response curves (Fig. 6C, 6D), whereas the other viral chemokines display incomplete curves or no β-arrestin-2 signaling. Based on these data, the potency order of the vCXCL-1s for β-arrestin-2 activation is: CXCL8 (pEC50 = 9.1 nM) [high affinity for CXCR2] ≥ CXCL1 (pEC50 = 8.3 nM) [high affinity for CXCR2] ≥ Toledo (pEC50 = 8.4 nM) [high affinity for CXCR2] ≥ E760 (pEC50 = 8.1 nM) [med-high affinity for CXCR2] ≥ C952 (7.5 nM) [high affinity for CXCR2] ≥ C956 [med-high affinity for CXCR2] ≥ TX24 [med-low affinity for CXCR2] ≥ TX15 [low affinity for CXCR2].

FIGURE 5. vCXCL-1s have different binding affinities for human CXCR1 or CXCR2. Displacement of 125I-CXCL8 binding to HEK293 cells stably expressing human CXCR2 (A) or CXCR1 (B). Cells were incubated with indicated concentration of vCXCL-1s and 200 pM 125I-CXCL8 for 1 h at room temperature. For simplicity, curves for TX11, C956, 102410, and C954 are not shown. (C) The average IC50 ± SE for all vCXCL-1s for either CXCR2 or CXCR1 (n = 3–12). Those chemokines with incomplete competition curves are indicated with a > sign in front of the predicted IC50. Chemokines with no competition at all concentrations tested are listed as >1000 IC50.
affinity for CXCR2] ~ TX11 [med-high affinity for CXCR2] ~ Towne [low affinity for CXCR2]. For the most part, high affinity for CXCR2 or CXCR1 tracks with β-arrestin-2 activation. There are a few exceptions. A medium-high–affinity binder, vCXCL-1C956, did not induce β-arrestin-2, while the low-affinity vCXCL-1Towne did signal. These data point to differential signaling or “biased agonism” that leads to differential G protein activation and β2-arrestin-2 potencies not directly correlated with receptor affinity (43).

vCXCL-1s differentially induce secondary chemokine production (CCL22)

We have observed differences in migratory ability, G protein activation, and β-arrestin-2 recruitment, but how could these phenotypes affect HCMV dissemination or pathogenesis? HCMV productively infects macrophages and dendritic cells and may have evolved vCXCL-1s to increase the recruitment of these cell types via neutrophil activation. Macrophage-derived chemokine CCL22 recruits multiple immune cells, such as monocytes, dendritic cells, natural killer cells, and the Th2 subset of T cells (44). The induction of CCL22 could have profound effects on the recruited cell types and the immune response to CMV. Not only could these cells increase dissemination or CMV replication, CCL22 could also lead to an increase in the Th2 response and a downregulation of Th1 responses (45, 46). In fact, another UL/b’ protein, UL144, upregulates CCL22 and has been implicated in immune modulation (i.e., recruitment and activation of Th2 and regulatory T cells) (47). To address whether the vCXCL-1s induce CCL22, we performed quantitative real-time PCR for CCL22 expression on a neutrophil-like cell line that overexpresses CXCR2 (Fig. 7). vCXCL-1Toledo, vCXCL-1E760, and vCXCL-1C952, had the highest induction of CCL22, which is similar to CXCL1. vCXCL-1Toledo and vCXCL-1C952 are high-affinity CXCR2 binders, whereas vCXCL-1E760 belongs to the medium-high group. Others, in the medium-high binding group, except vCXCL-100751, induce CCL22. In contrast, all the members in the medium-low (i.e., vCXCL-102410 and vCXCL-1C954) or low-affinity group (i.e., vCXCL-1TX15 and vCXCL-1Towne) except for vCXCL-1TX24 do not induce CCL22. As seen in PBN migration and G protein and β-arrestin-2 usage, high-affinity binders activate downstream signaling and functional outcomes. Whereas the medium binders are variable in their activation and low-affinity binders are not activating PBNs except for calcium flux and integrin upregulation (Table I).

Discussion

Our findings contribute to our understanding of the functions of the HCMV viral chemokines and their agonist activation of CXCR2. In trials in which different HCMVs were inoculated into volunteers,

![FIGURE 6](http://jimmunol.org/)

**FIGURE 6.** G protein activation and β-arrestin-2 signaling correlates with CXCR2 affinity. (A and B) vCXCL-1 chemokine induction of 35S-GTP binding to HEK293T membranes expressing CXCR2. Data are corrected for basal [35S-GTPyS binding (n = 3–4). (C and D) vCXCL-1 β-arrestin-2 recruitment in PathHunter indicator cells. Data are expressed as percentage of β-galactosidase activity, in which the response to 1 μM CXCL8 is set to 100% (n = 3–4).
the role of the viral chemokines was suggested in human disease. For example, the Towne strain of HCMV was less virulent than the Toledo virus in humans. Towne differs in the ULb region, which contains the UL146 and UL147 viral chemokine genes (48–50). In this study, we have shown that Towne produces a vCXCL-1 with a low affinity for CXCR2 and induces a lower calcium flux (with no induction at 1nm), minimal ability for chemotaxis, and no signaling compared with the more virulent Toledo strain vCXCL-1 (Table I). Although this is only circumstantial evidence and one of several differences between the Toledo and Towne strains, vCXCL-1 differences in PBN activation are potentially a contributing factor to the HCMV virulence observed in these studies. Other animal models of HCMV pathogenesis provide a more direct link between viral chemokines and pathogenesis. The guinea pig CMV chemokine homolog functionally signals through the CCR1 receptor and plays a role in viral dissemination in vivo (51–53). Furthermore, this virally induced inflammation contributes to CMV-related inner ear injury (i.e., auditory pathology) (54). Whether the differences in the vCXCL-1s contribute to HCMV virulence or dissemination, or both, in a similar manner to this animal model remains to be tested. The role of vCXCL-1s in human pathogenesis is especially difficult without knowing the concentrations of these chemokines during an active HCMV infection in vivo.

We propose two nonexclusive models for how HCMV vCXCL-1s could function in vivo. One model for HCMV dissemination is the “neutrophil shuttle model.” In this model, the neutrophil functions as a vehicle for HCMV dissemination (55). PBNs pick up HCMV during neutrophil transendothelial migration and subsequently transmit infectious virus to fibroblasts (56, 57). We analyzed PBN induction of calcium flux and adhesion molecules upon vCXCL-1 treatment as indicators of neutrophil activation (Figs. 2, 3), which could affect subsequent cell-mediated viral dissemination (58). vCXCL-1–activated PBNs could transport virus and allow it to infect surrounding tissues or different cells. Ideally, we would address this shuttling effect directly with an antigenemia assay in which vCXCL-1–treated neutrophils are assayed for their ability to take up HCMV after migration through an infected monolayer (59). Unfortunately, potential differences in migration were masked by the large amount of the host chemokines that are secreted after HCMV infection of the fibroblast monolayer (data not shown). These “background” host chemokines conceal the effects of the vCXCL-1s in this in vitro model system. In the current study, although the binding affinities to CXCR2 and CXCR1 were variable (Fig. 5), all vCXCL-1s induce intracellular calcium mobilization in PBNs, albeit to different degrees at the concentrations tested (Fig. 2) and upregulate β2 integrins on the surface of PBNs (Fig. 3) similar to levels induced with human CXCL1 or CXCL8. We speculate that vCXCL1s from all the clades activate PBNs to increase contact with the endothelium. After activation and adhesion neutrophils could be induced to migrate to the site of HCMV infection. To investigate this possibility further, we measured vCXCL-1–induced migration.

The resulting chemotaxis profile did not correlate directly with receptor affinity (Fig. 2C). Although the majority of medium affinity vCXCL-1s had migration only at 500 nM, others had none at all (vCXCL-1TX24) or at lower concentrations (100 nM; vCXCL-1C954) (51). This leads us to conclude that CXCR2 binding affinities do not correlate directly with subsequent PBN activation, integrin upregulation, or chemotaxis patterns. This may not be too surprising, as others have observed decreases in CXCR2 affinity while still inducing a calcium flux (60, 61) and elastase production (21). Others have observed a complex relationship between binding and activation similar to our observations with our medium-affinity vCXCL-1s (62). These data illustrate the complexity of the CXCR2 response to agonist stimulation and its relationship with affinity.

A complementary, or alternate, model to explain the relationship between the vCXCL-1s, PBNs, and HCMV is the “neutrophil amplifier model.” This model focuses on vCXCL-1 induction of exocytosis of neutrophilic granules or secretion of specific cytokines and chemokines. These inflammatory mediators could increase inflammatory responses that subsequently recruit other immune cells (63). These infiltrating immune cells would provide a better vehicle for HCMV spread. Macrophages and dendritic cells are better targets for HCMV infection because HCMV can productively infect them (64–67), whereas PBN infections are nonproductive (57). The attraction and differentiation of myeloid cells could provide a means to infect a cell type that allows for more efficient virus production or dissemination within the host (65). The vCXCL-1s induce differential CCL22 production that could have effects on myeloid cell chemotaxis. In our studies, the upregulation of CCL22 correlates with the vCXCL1s’ affinity for CXCR2 (Fig. 7). Fifty-seven percent of medium-affinity vCXCL-1s induce CCL22 expression (vCXCL-1TX24, vCXCL-1C954, vCXCL-1C956, and vCXCL-1C952), whereas others did not (vCXCL-1C954, vCXCL-1C956, and vCXCL-1C952). The neutrophil amplifier model would predict that viruses that do not induce CCL22 in PBNs would be less pathogenic, but we have no in vivo data for this prediction. In comparisons of the sequences from HCMV congenitally infected infants, the vCXCL-1s that induce CCL22 do not correlate with clinical outcomes (17). Our interpretation of these data cannot completely exclude the shuttle model. Our study measured only a single inflammatory chemo-

Table I. Summary of vCXCL-1 functional outcomes

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Ca** Flux</th>
<th>Integrin Expression</th>
<th>Migration</th>
<th>G Protein Binding</th>
<th>β-Arrestin-2</th>
<th>CCL22 Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>High affinity</td>
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</tr>
<tr>
<td>CXCL8</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Toledo</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Low affinity</td>
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<tr>
<td>Towne</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>TX15</td>
<td>++</td>
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</tr>
</tbody>
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+++ highest activation; ++, high activation; +, activation; +/-, weak activation; -, no activation.
kine. Other chemokines and cytokines such as CCL2, CCL3, and CCL7 that were not measured could have a role in congenital sequelae.

To our knowledge, this study is the first to examine how the natural variation in the vCXCL-1s affects binding and PBN function. These variants provide an opportunity to assess how changes within CXC chemokines affect signaling as a “biased agonist.” Biased agonists stimulate G protein coupled receptors with differential signaling and functional outcomes (43). The activation of CXCR2 initially appears to be redundant. Host CXC1, CXC2, CXCL3, CXCL6, and CXCL8 all bind and activate CXCR2. Recently, Rajagopal et al. (68) measured β-arrestin-2 recruitment, cAMP signaling, and internalization with the differential ligands on CXCR2. These related chemokines displayed a biased agonism for cAMP and β-arrestin-2 activation. Our study found that high affinity for CXCR2 leads to activation of G protein dependent and independent signaling (Fig. 6). As expected, vCXCL-1s with low affinity for CXCR2 do not initiate detectable signaling. The chemokines with medium range affinity are more complex. Some have moderate G protein signaling without β-arrestin-2-activated (vCXCL-1C968) or no G protein activation with only β-arrestin-2 signaling (vCXCL-1TX11). Our data suggest a complex robustness to the viral chemokine response that only partially correlates with affinity.

In conclusion, our data suggest that polymorphisms in the vCXCL-1s elicit differential affinity to CXC chemokine receptors, which generates varying cellular responses or differential activation and triggering of diverse downstream signals. High affinity for CXCR2 leads to activation of G protein dependent and independent signaling with full activation of calcium flux, integrin expression, and CCL22 transcription (Table I). Those with low affinity for CXCR2 still induce calcium flux and integrin expression while not initiating detectable signaling or CCL22 expression and modest PBN migration. These data point to different thresholds for the different neutrophil functions. Calcium flux and integrin expression have low thresholds where any degree of stimulation will activate them (69). Other functional outcomes (i.e., migration, signaling, or CCL22 expression) are more complex. Generally, the extremes in affinity (i.e., high or low) correlate with signaling, migration, and CCL22 production. Those with medium-range affinity are more complex and result in varying degrees of activation (70). This nuanced response points to the biased agonism of these novel vCXCL-1s that could affect neutrophils, and we speculate an effect on subsequent HCMV dissemination or virulence.

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Disclosures

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References


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