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I am submitting herewith a dissertation written by Jinho Heo entitled “Analysis of polymorphism in human cytomegalovirus (HCMV) chemokine, vCXCL-1, and its role in cellular activation” I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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ANALYSIS OF POLYMORPHISM IN HUMAN CYTOMEGALOVIRUS (HCMV) CHEMOKINE, VCXCL-1, AND ITS ROLE IN CELLULAR ACTIVATION

A Dissertation

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DEDICATION

I dedicate this dissertation to my family. Most importantly, I dedicate my dissertation to my wife, Sunmi, who equally shared all the emotional burdens with me through this process. There is no doubt in my mind that without your love and sacrifice I could not have done this. I also dedicate this to my daughter, Jiyoon, who fills me full of love and energy to proceed forward.

The last, but not the least, I dedicate this dissertation to my parents, Young-Chon and Young-Ja, who made all of this possible, for their endless support and patience. Father, this is my gift for your 70th birthday.

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ABSTRACT

The human cytomegalovirus (HCMV) viral chemokine gene, *UL146*, shows a high degree of variability in clinical isolates. The *UL146*-produced viral chemokine, vCXCL-1, has homology to CXC chemokines and is predicted to be an immune modulator that may contribute to the pathogenesis of HCMV infections. In the analysis of clinical isolates from congenitally infected infants, we found 11 distinct vCXCL-1 clades. Although the four cysteine residues that create two disulfide bonds providing the essential structure for CXC chemokines,are conserved, the N-loop region, which is important for receptor binding and activation, was hypervariable. One clade also contained a modified glutamic acid-leucine-arginine (ELR) motif (asparagine-glycine-arginine / NGR), which regulates binding to CXCR1 and CXCR2 receptors. Based on this sequence information, we hypothesize that these proteins differentially activate neutrophils, which may have a role in HCMV pathogenesis. To address these functional differences, we produced representative vCXCL-1 proteins from each of the 11 clades using a baculovirus protein expression system. Using competition binding assays, we have examined their binding affinities to either CXCR1 or CXCR2 expressed on HEK293 cells. All vCXCL-1s bound to CXCR2 with different binding affinities. Interestingly, only three vCXCL-1s bound to CXCR1 while the others demonstrated did not. We analyzed functional differences between the vCXCL-1s in calcium mobilization, adhesion molecule induction, and chemotaxis on human peripheral blood neutrophils (PBNs).

Although the binding affinities to CXCR2 and/or CXCR1 were variable, all vCXCL-1s were capable of inducing intracellular calcium mobilization in PBNs and upregulating adhesion molecules on the surface of PBNs to similar levels as human CXCL1. However, the potency of the vCXCL-1s in the chemotaxis of neutrophils varied and was affinity independent. We also examined secondary chemokine production upon vCXCL-1 treatment on neutrophil-like HL60 T2 cells using real-time PCR. The results showed CCL22 induction was affinity dependent. Taken together, these results provide insights into the potential role of vCXCL-1 in HCMV pathogenesis and how the variability in these chemokines can affect neutrophil function.

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Chapter 1

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus infecting 50-90% of the population with higher infection rates found in people with lower socioeconomic status. Generally, HCMV infection is generally asymptomatic or causing mononucleosis in immunocompetent individuals, but high levels of morbidity and mortality rates are observed in immunocompromised individuals following organ transplantation or untreated HIV infection. For example, 80% of kidney recipients acquire a primary CMV infection following transplantation [1] and an 84% mortality rate was observed in bone marrow recipients [2, 3]. Also, HCMV disease occurred in 44% of AIDS patients before the introduction of antiretroviral therapy [4]. In addition, HCMV is the most common congenitally acquired infection in the United States. Each year, about 1% of all newborns in the United States are infected with HCMV congenitally [5], reaching almost 40,000 newborns per year [6]. 0.1% of these infected infants display clinical manifestations at birth, such as microcephaly, hepatosplenomegaly, jaundice, petechiae, or chorioretinitis [7] and up to 80% of those develop long-term neurodevelopmental sequelae, such as lower intelligence/developmental quotient (IQ/DQ), hearing loss, or mental retardation [6, 8]. Newborns that are asymptomatic at birth also can develop permanent damage, which may not manifest until they are older [9]. Due to this high rate of disease development and lasting morbidity, the Institute of Medicine ranked the

development of a CMV vaccine as a top priority based on the overall cost of treatment and human suffering [10].

HCMV is a large dsDNA virus that comprises 165-252 kb [11, 12]. HCMV encodes over 200 viral proteins [13, 14], yet they have evolved survival mechanisms to overcome host immune responses that eventually contribute to HCMV pathogenesis and disease development [15]. Yu et al showed 41 HCMV ORFs in the laboratory-adapted strain, AD169, that are essential for viral replication in vitro, such as DNA polymerase, capsid, matrix and envelope proteins [16]. He also found 88 "nonessential" genes for efficient HCMV replication *in vitro*. Some of these nonessential HCMV genes are homologous to the host genes: class I MHC proteins [17-21], TCR gamma chain [22], IL-10 [23], TNF receptor [24], and chemokines [25]. Among those nonessential genes, the 15 kb UL/b' genomic region of the virulent Toledo strain contains at least 19 open reading frames (ORFs) which are not present in laboratory-adapted strains such as the AD169 strain [25, 26]. Two ORFs in this region show homology to host chemokines: *UL146* and *UL147* that encode vCXCL-1 (also called p*UL146*) and vCXCL-2 (also called pUL147) [25]. These chemokine homologs genes show hypervariability among different viral strains [27]. Moreover, the vCXCL-1 chemokine from the Toledo strain, vCXCL-1_{Tol}, induces neutrophil chemotaxis and calcium mobilization [28]. Therefore, evaluations of the function of vCXCL-1s will provide a better understanding of HCMV pathogenesis that may ultimately facilitate development of an effective vaccine or novel antiviral agents against CMV.

Chapter 2

LITERATURE REVIEW

I. Chemokines and Chemokine Receptors

Chemokines

Chemokines are small chemotactic cytokines (approximately 8-12 kDa in size) that mediate their signaling through G-protein coupled receptors [29, 30]. They are classified by the presence of conserved cysteines that define the protein structure. Chemokines are divided into C, CC, CXC, and CX_3C chemokines based on the number of conserved cysteine residues and the spacing of two cysteines at the N-terminus. For example, CXC chemokine (α subfamily) members have two cysteines separated by an amino acid residue while the CC chemokine (β subfamily) has two juxtaposed cysteine residues. The C chemokine family (γ subfamily) has only one cysteine at the N-terminus and the CX3C chemokine (δ subfamily) contains two cysteines separated by three amino acids [31]. CXC chemokines can be further subdivided into ELR or non-ELR CXC chemokine dependent upon the presence of a glutamate-leucine-arginine (ELR) motif directly preceding the CXC motif [32].

The ELR motif is critical to induce activation and migration of neutrophils [32]. The ELR CXC chemokines bind to CXCR1 and/or CXCR2, and it has been shown that CXCR2 activation is mainly responsible for neutrophil activation [33]. Single amino acid substitutions in the ELR motif showed all three residues,

especially arginine, are sensitive to modification and are critically important for CXCL8 (IL-8) function [34, 35]. A further demonstration of the importance of this motif is that the addition of ELR to the N-terminal domain of a non-ELR CXC chemokine, CXCL4 (PF4), transforms it into a neutrophil chemoattractant [34] although another non-ELR CXC chemokine, CXCL10 (IP-10), requires additional Gly^{31} and Pro^{32} changes in order to become a fully functional chemokine on human neutrophils [36]. ELR CXC chemokines also have angiogenic properties. CXCL8 (IL-8), CXCL7 (NAP-2), CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ) and CXCL5 (ENA-78) have been reported as proangiogenic agents through the recruitment of endothelial cells [37, 38]. Although, angiostatic activities of ELR CXC chemokines were also reported for CXCL1 (GRO-α), CXCL2 (GRO-β), and CXCL3 (GRO-γ) [39], the concentration was 1000-fold higher (1-10 uM) than what was used for its angiogenic activity (1-10 nM) [40, 41]. This implies that at high concentrations ELR CXC chemokines are angiostatic while at low concentrations they are angiogenic.

In contrast to ELR CXC chemokines, many non-ELR CXC chemokines have angiostatic properties and almost exclusively attract cells other than neutrophils [32, 40]. For instance, CXCL10 (IP-10) was reported to attract monocytes, T lymphocytes, and NK cells [42, 43]. CXCL9 (MIG) was shown to attract tumor-infiltrating T lymphocytes [44]. CXCL12 (SDF-1) stimulates the proliferation of B cell progenitors and was also termed PBSF (pre-B cell growth stimulating factor) [45]. Subsequent studies showed that synthetic CXCL12 also stimulates monocytes and peripheral blood lymphocytes through intracellular

calcium mobilization and chemotaxis [46, 47]. This illustrates the specificity that the ELR motif brings to chemokine function.

The CC chemokines are the largest family of chemokines and attract a variety of cell types including monocytes, basophils, eosinophils, and dendritic cells [48]. CCL2 (MCP-1) was the first CC chemokine to be biologically characterized and shown to attract monocytes but not neutrophils [49]. The monocyte chemotactic proteins (MCPs) are not only effective on monocytes [50-52], but also attract CD4+ and CD8+ T lymphocytes [53-55] and basophils [56-59]. CCL5 (RANTES) [60] and CCL11 (eotaxin) [61] were also shown to be powerful attractants of eosinophils.

The C and CX3C chemokines are regarded as two minor families because they contain few members and are highly specific for a few receptors. The CX3C family has three amino acids separating the initial pair of cysteines and has only one member, CX3CL1 (fractalkine) [62]. CX3CL1 is produced as a long protein (373 amino acid) with an extended mucin-like stalk and a chemokine domain on top [62]. CX3CL1 is bound to the surface of cells, such as endothelial cells and promotes strong adhesion of leukocytes to endothelial cells. This membrane-bound CX3CL1 can also be cleaved and become a soluble chemokine (85 or 95 kDa) that attracts T cells and monocytes [62-64].

Lastly, the C chemokine family is represented by two chemokines, XCL1 (lymphotactin α) and XCL2 (lymphotactin β). They lack two of the four cysteine residues that are characteristic of chemokines [65]. Lymphotactin is expressed in progenitor T cells, activated CD8+ T cells, and natural killer (NK) cells [65, 66].

It has been shown to be a potent chemoattractant for both CD4+ and CD8+ T cells, natural killer (NK) cells, but not monocytes [65, 67-69]. Lymphotactin has also been reported to be chemotactic for B cells and neutrophils through XCR1 receptor [70].

Chemokine Receptors

Chemokines mediate their signals through 7 transmembrane G-protein coupled receptors (GPCR). Since GPCRs are generally known to be promiscuous for their ligands, it is reasonable that there are fewer receptors than chemokines [71]. Complex and multiple downstream signaling events, however, are possible due to the large number of heterotrimeric G proteins, such as α, β, and γ subunits, and β-arrestin [72, 73]. To date, 17 Gα, 5 Gβ, and 12 Gγ proteins have been found [73, 74]. Chemokine binding to a GPCR induces conformational changes and triggers its function as a guanine-nucleotide exchange factor (GEF) that exchanges GDP for GTP on the Gα subunit. This exchange causes the dissociation of the Gα subunit from the G$\beta\gamma$ dimer, both of which activate several down stream effectors [75, 76]. In addition to GTP mediated signaling, G protein receptor kinases (GRKs) and β-arrestins also control signaling [77]. GRKs phosphorylates serine and threonine residues in the C-terminus allowing β-arrestin binding. β-arrestins then prevent association of the G protein with the GPCR (desensitization) [78]. Additionally, the GRK-arrestin system promotes clathrin-mediated internalization of the inactivated GPCR to endosomal compartments for subsequent degradation or recycling to

the cell surface [79, 80]. Interestingly, β-arrestins have also been shown to act as adaptor proteins mediating signaling events downstream of the GPCR, independently of G proteins activating mitogen-activated protein kinases (MAPKs), SRC, nuclear factor-κB (NF-κB) and phosphoinositide 3-kinase (PI3K) [78, 81, 82]. Therefore, various ligand binding with multiple combinations of G proteins and complex signaling pathways from G proteins and β-arrestins can generate numerous cellular effects from a similar ligands (Fig. 1).

Once the Gα subunit and the Gβγ dimer are dissociated, the Gα subunit remains in the activated GTP-bound state until hydrolysis of GTP to GDP leads to reassociation of the heterotrimer and termination of the signal by the regulator of G-protein signaling protein (RGS) [83]. The α subunits of G proteins are divided into four subfamilies: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. Each Gα protein activates several downstream effectors. Typically $G\alpha_s$ protein stimulates adenylyl cyclase (AC) and increases levels of cyclic AMP (cAMP) that subsequently activates mitogen-activated protein kinase (MAPK) signaling modules and protein kinase A (PKA) [84]. However, $G\alpha_{i/o}$ inhibits AC and reduces cAMP levels that subsequently activates G-protein-coupled inwardly rectifying potassium (GIRK) channels [85]. $G\alpha_{q/11}$ protein activates phospholipase Cβ (PLCβ), and $G\alpha_{12/13}$ subunit activates Rho guanine-nucleotide exchange factors (GEFs) [86]. 8 Gα proteins are ubiquitous or widely distributed, but 9 Gα proteins are expressed in selective cell types, such as neuronal cells, retinal cells, or haematopoietic cells, etc (supplementary information of [87]). In summary, GPCRs can induce differential signalings via activation of various G

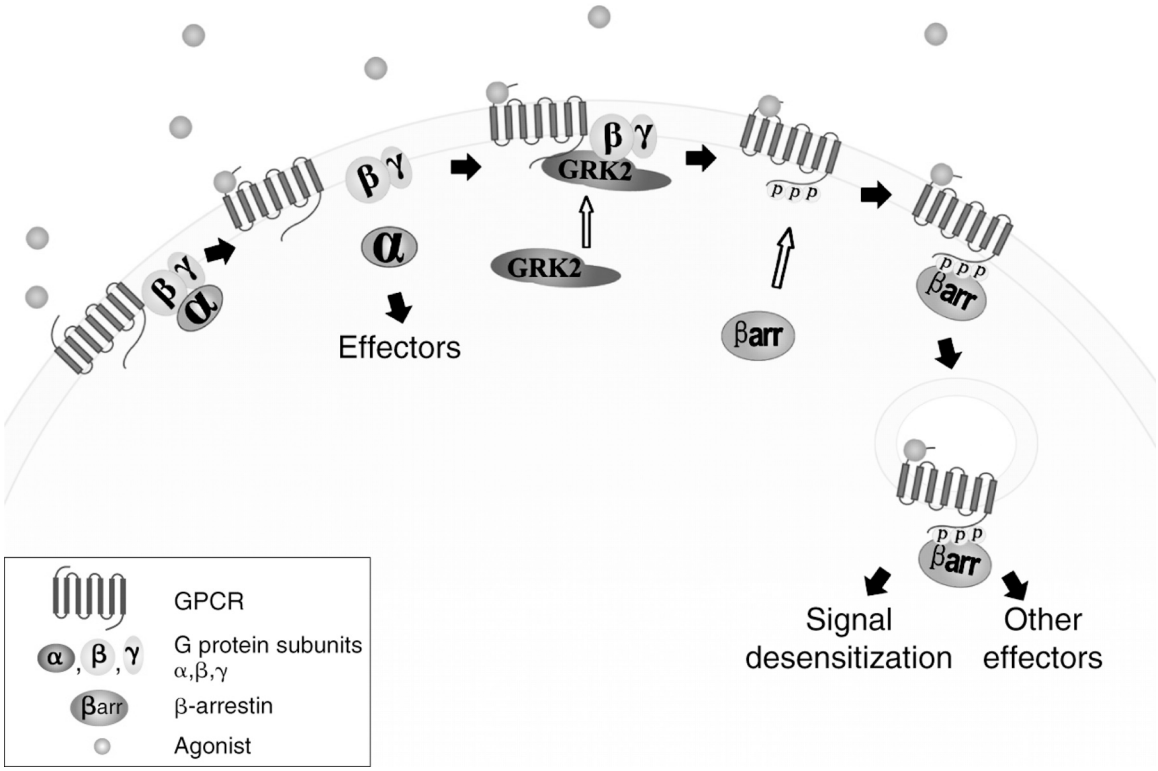

Figure 1. Basic scheme for GPCR activation and feedback of signaling.
Agonist exposure stimulates activation of GPCR, which leads to the dissociation of G proteins into activated subunit and dimers and triggers the activation of various effectors, such as adenylate cyclase and phospholipase C. The agonist-occupied GPCR is phosphorylated by GRKs, leading to signal desensitization, binding of β-arrestin to the activated, phosphorylated GPCR and subsequent endocytosis of the receptor. β-arrestin also initiate downstream signaling events. (adopted from Ma and Pei, 2007).

proteins that would also depend on the presence of specific G proteins in the cell as well as the right GPCR conformation to stimulate different G proteins.

Dissociated Gβγ subunits also regulate a number of signaling pathways. Gβγ mediates the phosphorylation of PLCβ as $G\alpha_q$ does [88, 89]. Activated PLCβ cleaves phosphotidylinositol 4,5-bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 elicits calcium mobilization from the endoplasmic reticulum (ER) into the cytoplasm, while DAG activates protein kinase C (PKC) [90]. Transient intracellular calcium mobilization is frequently used as a marker of cellular activation [91], whereas PKC activation is required to upregulate β2 integrins, such as CD11b/CD18, in neutrophils and eosinophils [92, 93]. In addition, Gβγ also stimulates Akt through phosphatidylinositol 3-kinase (PI3K) as $G\alpha_s$ does through PKA [74]. It has been demonstrated that Akt activation as well as ERK are responsible for chemotaxis of HL60 cells or CXCR2 transfected HEK293 cells [94, 95]. As there are various Gα proteins, there are 5 Gβ and 12 Gγ proteins that can compose numerous combinations [73, 87]. Gβ and Gγ proteins are widely distributed except 1 Gβ and 5 Gγ proteins are expressed in selective tissues, such as brain or taste buds (supplementary information of [87]). Therefore, overlapping signaling cascades through Gβγ can also increase the complexity of the signaling pathway.

CXC chemokine and chemokine receptor interaction

GPCR activation requires interaction with specific domains on the chemokines. The N-terminus of both CC and CXC chemokines preceding the

first cysteine is considered an important region for receptor binding and activation [30, 96-100]. For instance, N-terminal truncation mutants of the CXC chemokine CXCL12 (SDF-1) revealed the eight residues proceeding first cysteine are essential for receptor binding and the deletion of the first two residues generates a receptor antagonist [101]. For CC chemokines, the entire 10 residues preceding the first cysteine in CCL2 (MCP-1) are strictly required for full activity [97, 98]. Manipulation of the N-terminus of CCL7 (MCP-3) and CCL5 (RANTES) produced potent antagonists [99, 100]. This included a single methionine, which converts CCL5 (RANTES) from an agonist to an antagonist. Furthermore, in ELR CXC chemokines, mutations and domain swaps of CXCL1 (Groα) and CXCL8 (IL-8) revealed that the Glu-Leu-Arg (ELR) motif is a critical motif for receptor recognition and activation [102, 103]. Following the second cysteine is a flexible N-loop of approximately ten residues, which is followed by a single turn helix. The N-loop region has been shown to be responsible for receptor binding and specificity [30, 35, 96, 104]. For example, Skelton et al. showed that CXCL8 (IL-8) and CXCR1 N-terminus fragment, CXCR1-p1, are found to interact along the ELR and N-loop motifs of CXCL8 (IL-8) [105]. Furthermore, N-loop swaps between CXCL8 (IL-8) and CXCL1 (Gro-α) and mutations in this region demonstrated that the CXCL8 (IL-8) N-loop residues are critical for receptor specificity [106], indicating the ELR motif is necessary for receptor recognition and the N-loop region controls receptor specificity. Nevertheless, other regions within the chemokine also contribute to receptor interactions as synthetic

peptides comprising the N-terminus and the N-loop have reduced activities compared to their full-length counterparts [32].

Chemokines bind to glycosaminoglycans (GAGs) due to their cationic nature [107, 108]. CXCL8 (IL-8) binds to GAG through its C-terminal α-helix and shows enhanced chemotactic activity on neutrophils when complexed to heparan sulfate [109]. The GAG interaction on the cell surface or within the extracellular matrix is thought to facilitate the retention of chemokines and subsequently present it to the receptor expressed on the leukocyte cell surface, promoting cell adhesion and activation [110]. This will also create a solid support for chemokine gradients allowing the directed movement of cells under conditions of blood flow at the endothelial surface. Several studies have shown the importance of GAG binding on chemokine function. Gilat et al, using extracellular matrix (ECM)-coated culture dishes, showed that CCL4 (MIP-1β) and CCL5 (RANTES) leukocyte adhesion and chemotaxis is GAG-dependent [111]. In addition, Proudfoot et al have shown that mutations in GAG binding sites of CCL2 (MCP-1), CCL4 (MIP-1β), and CCL5 (RANTES) results in recruitment defects when injected into the peritoneum of mice while they retain chemotactic activity *in vitro*; thus, highlighting the importance of the chemokine-GAG interaction *in vivo* [112].

II. Viral immune evasion and pathogenesis

Viral immune evasion

Viruses replicate in a host cell pirating cellular resources. In order to survive against host immune responses that recognize and eliminate virus-infected cells, viruses have evolved strategies to overcome or manipulate the host immune system. For instance, large DNA viruses such as β- and γ-herpesviruses and some poxviruses have evolutionally acquired and modified host chemokines or chemokine receptors [113-115]. These virally encoded proteins are able to manipulate host immune responses, potentially leading to better viral dissemination and establishment of latency [114, 116, 117]. For dissemination purpose, viruses may attract immune cells through viral chemokines for infection and viral chemokine receptors to facilitate cell migration to sites of latency [114]. For immune system manipulation purposes, viruses may attract specific cell types, such as Th2 leukocytes, so that cytotoxic T cell (CTL) responses can be diminished [118, 119].

Viral pathogenesis

Herpesviruses are large double-stranded (ds) DNA viruses with 84–252 open reading frames (ORFs) [115, 120, 121]. Herpesviruses replicate in the nucleus of the cell and undergo lytic replication and can establish latency. Following a primary productive infection, the virus is not completely eliminated from the host, but instead establishes a lifelong latent infection expressing only

minimum viral proteins in the absence of detectable infectious virus [122, 123]. It can reactivate at a later stage to generate new infectious virus and spread to a new host. Herpesviruses are classified into three subfamilies, the α-, β-, and γ-herpesvirinae based on their genetic organization, homology, and tropism [124]. At least eight virus types are known to infect humans. The α-herpesviruses include herpes simplex virus 1 and 2 (HSV-1 and HSV-2; HHV-1 and HHV-2) and varicella zoster virus (VZV, HHV-3). These viruses remain latent in neurons. The β-herpesvirus subfamily includes cytomegalovirus (CMV, HHV-5), human herpesvirus 6 (HHV-6), and human herpesvirus 7 (HHV-7) and infects many cell types compared to α-herpesviruses. Human CMV DNA has been found in a wide variety of organs [125]. Cells containing latent HCMV DNA has been found in monocyte/macrophage lineages [126-129] and hematopoietic progenitor cells [130-135]. In Murine CMV, latent DNA has been detected in endothelial cells of diverse organs including heart, kidney, liver, lung, spleen, brain, salivary glands, and bone marrow, and macrophages in the lungs [136, 137]. The γ-herpesviruses include Epstein–Barr virus (EBV, HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8). They infect lymphocytes and endothelial cells and can induce cellular transformation [138].

Poxviruses are also large dsDNA viruses with a 130–360 kbp genome that encodes over 230 proteins (See www.poxvirus.org for more detail). In contrast to herpesviruses, poxvirus replication and the formation of progeny viruses take place in the cytoplasm, which is independent of the host nuclear machinery [117, 139]. Poxviruses generally infect a variety animal hosts and causes acute

infections with abrupt onset of fever and skin lesions without establishing latency. Some families of poxvirus, however, can cause life-threatening infections. Variola virus, the causative agent of smallpox, was one of the most virulent human pathogens, with a mortality rate as high as 40%. Vaccinia virus has been used as a vaccine and led to the 1979 eradication of smallpox worldwide [140]. Molluscum contagiosum virus (MCV) is the only poxvirus member that infects humans exclusively. MCV commonly spreads through skin-to-skin contact causing skin lesions that are dome-shaped and pearly in appearance. Infection is usually benign and painless. The Orf virus causes disease with papulo-vesicular lesions on the hand or face through direct contact with infected sheep and goats. Often times when poxviruses cross species, disease is generally much more mild compared to the original host. There are many more members of the poxvirus family, but are beyond the scope of this review. Importantly, this large DNA virus family that causes acute infection produces an array immune modulating proteins similar to herpesviruses.

III. Viral chemokine homologs

CXC chemokine homologs in CMV

Human CMV (HCMV) have two ORFs, *UL146* and *UL147*, encoding CXC chemokine homologs, vCXCL-1 and vCXCL-2, respectively [25] (Table 1). Recently a presentation at the International Herpesvirus Workshop (Salt Lake City, 2010) indicated that vCXCL-2 is not secreted and is able to downregulate

Table 1. Cytomegalovirus chemokine-like genes (adapted from Beisser et al. 2008).

CMV species	Gene (product name)	GenBank accession	References	Comments
HCMV	*UL128*	NC_006273	[141, 142]	Intact in AD169, disrupted in Merlin and Toledo
	UL146 (vCXCL–1)	NC_006273	[143]	Intact in Towne, Toledo, Merlin, lost in AD169
	UL147 (vCXCL–2)	NC_006273	[143]	Intact in Towne, Toledo, Merlin, lost in AD169
CCMV	*UL128*	AF480884	[141, 142]	Disrupted
	UL146 (vCXCL–1)	AF480884	[144]	
	UL146A	AF480884	[11]	UL146-like, not present in other CMVs
	UL147	AF480884	[11]	
	UL156	AF480884	[11]	Splice variant may encode CXC chemokine, not present in other CMVs
	UL157	AF480884	[11]	UL146-like, not present in other CMVs
RhCMV	*rhUL128*	DQ120516	[145]	Intact in RhCMV 180.92, not present in RhCMV68.1
	rh158	AY186194	[145]	Intact in 68.1, not present in 180.92
	rh156.2	DQ120516	[145]	
RCMV	*r129* (RCK–3/ ECK–3)	AF232689	[146]	
	r131 (RCK–2)	AF232689	[146]	
MCMV	*m131–129* (MCK–1; MCK–2)	U68299	[147]	
GPCMV	*GPCMV-MIP*	AF500307	[148, 149]	Unique, MIP–1-like
HHV–6	*U83A*	NC_001664	[150, 151]	

NK activation with cooperation of *UL142* [152]. Chimpanzee CMV (CCMV) also encodes homologs of *UL146* and *UL147* as well as the *UL146*-related gene *UL146a* and *UL157* that are not present in other CMVs [11]. Rhesus CMV (RhCMV) encodes one homolog of *UL147*, *rh158* [153]. It will be interesting to determine whether these homologues in other species have the same NK inhibitory functions as *UL147* from HCMV.

Yu et al showed that only 41 genes out of 150 ORFs in the lab-adapted AD169 strain are essential for efficient viral replication *in vitro*, which indicates many viral genes may be utilized for viral survival *in vivo* [16]. The virulent Toledo strain contains at least 19 open reading frames (ORF) in the 15 kb, UL/b' region, which are not present in AD169 and are not essential for virus replication *in vitro* [25, 26]. This has lead to speculation that these genes may have a role in HCMV pathogenesis. *UL146* and *UL147* chemokine genes reside in this UL/b' [25]. *UL146* ORF from the Toledo strain has been shown to produce a functional CXC chemokine called vCXCL-1_{Tol} [143]. vCXCL-1_{Tol} induced chemotaxis and intracellular calcium mobilization in human neutrophils at comparable levels to host chemokines. Initially, vCXCL-1_{Tol} was reported to bind exclusively to the human CXCR2 (hCXCR2) chemokine receptor [143]. However, Lüttichau et al recently showed that vCXCL-1_{Tol} can bind not only hCXCR2 but also hCXCR1 [154], similar to that of the host chemokine, CXCL8 (IL-8). A deletion mutant of *UL146-UL147* showed that it impairs viral passage to neutrophils while its tropism for other cell types is retained [142]. This result provides a potential role for vCXCL-1 in HCMV dissemination.

Interestingly, sequencing of the entire CMV genome has shown that the *UL146* gene is one of the most variable genes in the entire HCMV genome [26, 27, 155-160]. For instance, Dolan et al demonstrated HCMV genetic variability using a nucleotide divergence assay [27]. He showed high levels of variability in the *RL11* gene families at left ends of U_L and in the *UL139*, *UL144*, and *UL146* at right ends of U_L. The proteins encoded by the *RL11* gene family have potential roles in tropism [161]; a *UL9* mutant from Towne grows better in HFFs than wild type virus while a *UL10* mutant grows better in epithelial cells than wild type virus, although Yu et al did not find enhanced growth of *UL9* mutants from AD169 in HFFs [16], which is possibly due to the use of different parental viruses. The *UL139* protein shares sequence homology with human CD24 [162], a cell-surface sialoglycoprotein. It is expressed on immature cells modulating B-cell activation and on many cancers, such as non-small cell lung cancer, breast cancer, and prostatic cancer and correlates with tumor metastasis [163]. Qi et al, however, did not find an association between *UL139* genotypes and the clinical outcomes [162]. The *UL144* protein has limited homology to the herpes simplex virus entry mediator (HVEM), a member of the tumor necrosis factor receptor (TNFR) superfamily [164]. Research on an association between *UL144* genotypes and clinical outcomes showed no strong association [165-169] while Arav-Boger et al reported otherwise [156, 170]. Research regarding polymorphisms of *UL146* from clinical isolates showed 4% identity (5 residues) and 5% similarity (6 residues) to each other [160]. It was postulated that this variability might correlate with the severity of CMV disease however no clear

relationship in this regard has been identified [158, 159, 165] and the function of the *UL146* polymorphisms remains to be determined.

CC chemokine homologs in CMV

HCMV has one ORF with limited homology to CC chemokine, *UL128* [141], and *UL130* that encode a protein containing a putative C chemokine fold [171]. CCMV and RhCMV 180.92 also have a *UL128* ORF with homology to CC chemokines [141, 142, 145]. Murine CMV (MCMV) contains spliced genes, *m131* and *m129*, which produce the CC chemokine homolog, MCK-2 [147, 172]. Rat CMV (RCMV) also has both *r131* and *r129*, but *r131* has limited homology to either *m131* and *m129* [173]. Guinea pig CMV (GPCMV), also known as CavHV2_GP1, contains an ORF with limited homology to the CC chemokine MIP-1 [148, 174].

The HCMV *UL128* protein, however, is a non-functional chemokine due to a partial inversion or frame shift mutation in clinical HCMV strains such as Toledo and Merlin [141]. Although the *UL130* protein contains a putative C chemokine fold [171], functional data of *UL130* as chemokine is lacking. Interestingly, the *UL128* locus (*UL128L*) that comprises *UL128*, *UL130* and *UL131A* was found to be essential for growth in endothelial cells, dendritic cells and epithelial cells, and transmission to leukocytes [142, 175, 176]. The *UL128L* proteins form a complex with glycoprotein H and L (gH/gL) on the virion surface [177]. The gH/gL/UL128L complex mediates entry of CMV into endothelial and epithelial cells whereas the gH/gL/gO complexes mediate virus entry into fibroblasts [176, 177]. So even

though these have homology to chemokines, they more than likely have a role in entry.

MCMV *m131* and *m129* genes generate a spliced mRNA that encodes the CC chemokine homolog, MCK-2 [147]. A product of *m131*, MCK-1, was originally described as a functional chemokine that induces calcium mobilization in peritoneal exudate cells *in vitro* from MCMV-infected mice [178]. MCK-1 was soon re-characterized as a part of a CC chemokine including a long carboxy-terminal domain, which includes the entire *m129* ORF [147, 172]. *In vivo* studies with MCMV mutants that delete *m131*/129 or a mutated *m131* gene show a defect in dissemination to the salivary gland [179, 180] demonstrating that MCK-2 is important for viral dissemination *in vivo*. The mutant MCMV with a defective MCK-2 protein developed less inflammation at the site of inoculation and reduced dissemination to the salivary glands [179, 180]. MCK-2 can recruit myeloid progenitor cells [181] and monocytes [178], which not only contribute to MCMV dissemination to the salivary gland but could also provide a reservoir for establishing latency [130, 137, 182, 183].

As noted, RCMV contains the *r131* gene that shows homology to both MCMV *m131* and *m129* [173]. The RCMV *r131* ORF encodes a functional CC chemokine, pr131, which is essential for viral reproduction in the salivary glands [146]. When RCMV deletion mutants lacking *r131* were inoculated into immunocompromised rats, RCMV was not able to establish a high-titer infection in the salivary gland. In a rat footpad infection, this mutant showed significantly

reduced swelling than did wild type RCMV, which mimics what was shown for MCK-2 in MCMV.

GPCMV encodes a unique, MIP-1 CC chemokine homolog, GPCMV-MIP, which is not conserved in other animal CMVs [148]. This gene encodes an 11 kDa protein, which then yields an 8.6 kDa mature polypeptide. Phylogenetic analysis indicated GPCMV-MIP has homology to the macrophage inflammatory protein 1 (MIP-1α / CCL3) and hemofiltrate CC-chemokine 1 (HCC-1 / CCL14). MIP-1α and HCC-1 are known to enhance the proliferation of CD34+ myeloid progenitor cells, which could be used to harbor latent GPCMV or to disseminate the virus *in vivo* [184]. Functional assays showed that GPCMV-MIP mobilizes equivalent levels of intracellular calcium mobilization and chemotaxis in hCCR1 transfectants [149] as CCL3 (MIP-1α) stimulation [185]. However, GPCMV-MIP was not active on hCCR2 or hCCR5, which are the targets of CCL3 (MIP-1α). The intracochlear inoculation of a recombinant GPCMV lacking the GPCMV-MIP homolog resulted in less hearing loss compared to wild type GPCMV infection [186] indicating GPCMV-MIP has roles in CMV pathogenesis.

CC chemokine homologs in other herpesviruses

The U83 protein of human herpesvirus 6 (HHV-6) has a unique CXXCC sequence, which may be interpreted as a CC or/and CX3C chemokine [187]. However, the protein product of *U83* gene was designated vCCL4 because it causes intracellular calcium mobilization and chemotaxis exclusively through the CCR2 receptor [188]. The HHV-6A (variant A) *U83* gene encodes a 97 amino

acid protein whereas HHV-6B (variant B) encodes one of 113 amino acids [189]. As noted, vCCL4 of HHV-6B, also known as *U83B* while that of HHV-6A is *U83A*, is a selective but weak CCR2 agonist, inducing calcium mobilization and chemotaxis of THP-1 cells and CCR2 transfected L1.2 cells [187, 188]. CCR2 is expressed on monocytes, activated T cells, B cells, and immature dendritic cells [190, 191]. HHV-6 predominantly infects and replicates in CD4 T cells [192] and establishes latency in monocytes [193]. Furthermore, it has been shown that HHV-6 replication in immature monocyte-derived dendritic cells (MDDCs) is a prerequisite for viral transmission to CD4+ T cells [194]. Therefore, it seems sensible that vCCL4 attracts and HHV-6 utilizes those cells for viral dissemination or the establishment of latency. Although HHV-6A also has cellular trophism for CD4+ T cell, its vCCL4 homolog (U83A) showed a broad specificity for the chemokine receptors CCR1, CCR4, CCR5, CCR6, and CCR8 [151]. Because HHV-6A has been detected in skin biopsies and implicated in cases of multiple sclerosis (MS), the broad specificity of vCCL4 (U83A) suggests that its multiple cellular tropisms could contribute to pathogenesis of HHV6A. Interestingly, vCCL4 (U83A) can also manipulate the functions of host chemokines by delaying CCR5 internalization and blocking chemokine stimulation so this chemokine could be multi functional [151, 195].

Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8) is responsible for the development of Kaposi's sarcoma (KS, a cancer that causes patches of abnormal tissue to grow under the skin), primary effusion lymphoma (PEL, a rare aggressive type of B cell lymphoma), and multicentric Castleman's disease

(MCD, a rare benign follicular hyperplasia in germinal centers of B-cell follicles) [196, 197]. KSHV encodes three CC chemokine homologs, vCCL1, 2, and 3 (vMIP1, 2, and 3) [198-200]. KSHV chemokines antagonize or stimulate host chemokine receptors, induce angiogenesis, and reduce apoptosis as part of its immune manipulation. vCCL1 (vMIP-1) is an agonist of CCR8 [198, 201, 202], which is preferentially expressed on T_H2 cells [203]. By attracting Th2 cells the virus could limit activation of the Th1/CTL response allowing for increased survival. Both vCCL1 and vCCL2 are also potent chemoattractants for endothelial cells [204, 205], which appear to be required for angiogenesis and potentially contribute to tumor development [206]. Furthermore, vCCL1 and vCCL2 have anti-apoptotic effects *in vitro*. These viral chemokines enhanced the survival rate of endothelial cells and therefore promoted viral replication [207]. vCCL2 (vMIP-2) is a broad spectrum chemokine antagonist covering all four classes of chemokines (XCR, CCR, CXCR, and CX3CR), which are particularly found on Th1 cells [202, 208-210]. The efficient antagonistic activity of vCCL2 *in vivo* has been demonstrated in mouse and rat inflammatory models [209, 211, 212]. However, others have shown that vCCL2 can bind and activate cells through CCR8, CCR3, and CCR5 present on Th2 cells, eosinophils, or cell lines [204, 213-215]. In fact, KSHV lesions expressing vCCL2 have a predominance of Th2 polarized cells compared to Th1 type cells based on cytokine profiles within the lesion [214]. vCCL3 (vMIP-3) was originally found to act with low potencies in CCR4 and CCR5 chemotaxis assays [200]. However, Lüttichau et al. were unable to demonstrate any calcium mobilization in L1.2 cells stably

transfected with those receptors but showed that vCCL3 selectively activates the XCR1 receptor with higher potency than the endogenous ligand XCL1 (lymphotactin) [216].

CC chemokine homologs in poxviruses

In addition to herpesviruses, poxviruses also produce viral chemokines. Molloscum contagiosum virus (MCV) is a poxvirus that causes benign tumors of the skin or occasionally on mucous membranes. It causes infections with a higher incidence in children and is an opportunistic infection in immunocompromised individuals [217]. MCV encodes a CC chemokine homolog, MC148 [218]. Sequence analysis originally predicted MC148 to be an antagonist because the N-terminus of the protein, a region important for receptor activation, is shorter compared to cellular CC chemokines [219]. *In vitro* analysis demonstrated that MC148 inhibits the migration of monocytes, lymphocytes, and neutrophils in response to a range of chemokines and is not an agonist for any of the CC or CXC receptors tested [219, 220]. However, Lüttichau et al. compared the antagonism of MC148 with its analog, a broad-spectrum chemokine antagonist vCCL2 from HHV-8, and demonstrated that CCR8 is the sole receptor for MC148 [208, 221]. Interestingly, vCCL2 attracts CCR8 expressing cells for viral dissemination and survival [213] while MC148 acts as an antagonist. In fact, molloscum contagiosum lesions lack inflammatory infiltrates and MC148 might contribute to this feature of these lesions [222].

Summary and Statement of Research Aims

The development of a HCMV vaccine or other effective therapeutic treatment is upmost importance due to the high morbidity and mortality in HCMV infected newborns and immunocompromised individuals. However, no vaccine against CMV is currently available despite over three decades of effort. This is mainly due to the limited understanding of HCMV pathogenesis including viral dissemination. HCMV dissemination occurs via cell-associated viremia in cells of hematopoietic origin including monocytes and neutrophils. Large dsDNA viruses such as herpesviruses possess modified host chemokines that could alter the response of leukocytes during viral infection [84-86]. For example, MCMV, RCMV, and GPCMV encode CC chemokines such as MCK2, pr131, and GPCMV-MIP, respectively. MCK2 and pr131 induce inflammatory responses that enhance virus dissemination to the salivary gland *in vivo*, while GPCMV-MIP showed the induction of inflammatory responses in the inner ear that leads to hearing loss. The *UL146* ORF from the Toledo strain encodes a functional CXC chemokine called vCXCL-1_{Tol} [143]. Moreover, vCXCL-1s from different HCMV strains showed hypervariability, which could be a determinant of HCMV pathogenesis. Therefore, the analysis of the relationship between the vCXCL-1s polymorphism and the clinical manifestations may provide a key to developing a novel HCMV vaccine. In addition, evaluating the function of those vCXCL-1s will broaden our understanding of HCMV pathogenesis in relation to immune evasion strategies.

The hypothesis of this dissertation is that polymorphisms of the viral chemokine, vCXCL-1, elicit different functions or different levels of cellular activation, which could contribute to HCMV pathogenesis. To test this hypothesis, the following research objectives were performed:

1. Analysis of the relationship between polymorphisms within human cytomegalovirus chemokine, vCXCL-1, and clinical manifestations in congenitally infected children.

2. Expression of recombinant vCXCL-1s from different clinical isolates and evaluation of their functions as a chemokine *in vitro.*

Chapter 3

POLYMORPHISMS WITHIN HUMAN CYTOMEGALOVIRUS CHEMOKINE (UL146/UL147) AND CYTOKINE RECEPTOR GENES (UL144) ARE NOT PREDICTIVE OF SEQUELAE IN CONGENITALLY INFECTED CHILDREN

This chapter is a publication by the same title published in the journal Virology in 2008 by Jinho Heo, Susie Petheram, Gail Demmler, Jody R. Murph, Stuart P. Adler, James Bale, and Tim E. Sparer.

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My use of we in this chapter refers to my coauthors and myself. My primary contributions to this paper include (1) the majority of the lab work and (2) most of the data analysis, and (3) most of the writing of this paper.

Abstract

Human cytomegalovirus (HCMV) viral chemokine, *UL146*, and TNF α- like receptor *UL144* genes show a high degree of hypervariability in clinical isolates. These proteins are predicted to be immune modulators and may contribute to the pathogenesis of HCMV infections. We analyzed the *UL146* and *UL144* genetic

variation of 51 HCMV isolates from congenitally infected children and 13 isolates from children in childcare. There was no statistically significant correlation between *UL146* and *UL144* genotypes and HCMV disease and/or sequelae. However, there were some groups that had a relatively large proportion of asymptomatic outcomes. These included *UL146* group 8 (7/8 asymptomatic) and *UL146* group 10 (3/3 asymptomatic). *UL144* group B had 11/15 (73%) asymptomatic. *UL146* and *UL144* genes remained stable in serial isolates from children in daycare for intervals up to three years. These results indicate that most *UL146* and *UL144* genotypes do not predict clinical sequelae following congenital HCMV infections.

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus, congenitally infecting about 1% of all newborns in the United States [5]. 0.1% of these infected infants display clinical manifestations at birth such as microcephaly, hepatosplenomegaly, jaundice, petechiae, or chorioretinitis [7]. Of those with clinical symptoms at birth, most develop long-term neurodevelopmental sequelae. 100% of these children will have lower intelligence/developmental quotient (IQ/DQ), 65% will have some hearing loss, and 53% will be diagnosed with mental retardation [6, 8]. Even those newborns that are asymptomatic at birth can develop clinical outcomes of hearing loss and learning disabilities, which may not manifest themselves until they are older [9]. Due to this lasting morbidity, the Institute of Medicine recently ranked the

development of a CMV vaccine as a top priority based on the overall cost and human suffering [10]. To develop an adequate and effective vaccine or drug, a thorough understanding of the viral proteins that cause clinical disease are necessary.

The outcomes of congenital HCMV infections are determined by a variety of factors. Timing of infection, cell types infected at the placental interface [223, 224], and maternal antibody titers [225] are among the factors that may affect clinical outcomes. Several investigators have speculated that HCMV genes are important in the pathogenesis of human infections. HCMV possesses genes that are predicted to be involved in immune evasion that could function to increase viral survival or spread [141, 171, 226-232]. There are a large number of open reading frames (~62) in HCMV that are "non essential" for virus replication *in vitro* but may have a role in immune evasion *in vivo*. The 15 kb, UL/b' genomic region of the virulent Toledo strain contains at least 19 open reading frames (ORFs) which are not present in laboratory-adapted strains such as the AD169 strain [25, 26]. The products of two of these ORFs: vCXCL-1 (also called p*UL146* and vCXC-1) from the *UL146* gene and vCXCL-2 (also called p*UL147* and vCXC-2) from the *UL147* gene have limited homology to CXC chemokines [25]. *UL147* has only limited variability in the coding region of the mature protein. Variability is limited to the signal sequence portion of *UL147*. The vCXCL-1 chemokine from the Toledo strain, vCXCL-1_{Tol}, induces neutrophil chemotaxis and calcium mobilization [28]. Recently, numerous HCMV genomes from clinical isolates have been sequenced [26, 155, 157-159, 233, 234] and have shown that the

UL146 gene, along with the *UL139* [162] and *UL144* genes, are some of the most variable genes in the entire HCMV genome. The evolutionary reasons for this variability are unknown.

One hint of the role that vCXCL-1 plays during *in vivo* infection comes from early vaccine trials [235]. During this study, two strains, Toledo and Towne, were inoculated into naïve adult volunteers and the degree of febrile illness measured. The outcome of this study showed that the Toledo strain generated febrile illness at a tenfold lower dose than Towne, implying that Toledo is more virulent than Towne. When these genomes were sequenced, the *UL146* genes were shown to be significantly different. This example is commonly used as proof of the role that vCXCL-1 plays in HCMV pathogenesis.

Because of the variability in *UL146*, it has been suggested that vCXCL-1 proteins correlate with differences in long-term outcomes following congenital infections or during reactivation in immune compromised patients. Several groups have analyzed the association between disease outcomes and *UL146* variability [155, 157-159, 233]. These clinical specimens were isolated from immunocompromised adults or congenitally infected newborns within first three weeks of life. In adult renal transplant patients there was no correlation with clinical outcomes and *UL146* genotypes [159]. In the congenital studies, the analysis did not take into consideration long-term sequelae. The analysis of congenitally infected infants was based solely on symptoms at birth [155, 157-159, 233]. This could lead to an underestimate of the number of asymptomatic

children as HCMV infections leading to learning disabilities or mental retardation may not become apparent until months or years after birth [9].

Another member of UL/b' region, *UL144*, is also variable and a potential virulence factor [164]. This protein has limited similarity to the herpes simplex virus entry mediator (HVEM), a member of the tumor necrosis factor receptor superfamily (TNFRSF) [164]. The *UL144* protein, however, does not bind any known TNF-related ligands [24, 164, 166]. To date, only one ligand, the Ig superfamily member B and T lymphocyte attenuator (BTLA), has been shown to bind to the *UL144* protein [236]. By binding to BTLA, *UL144* could decrease lymphocyte responses to HCMV. Recently, *UL144* has been shown to activate TNFR-activated factor 6 (TRAF6) leading to NF_KB activation, a pleiotropic transcription factor [24]. NF_KB activation enhances expression of the chemokine CCL22, which attracts Th2 and regulatory T cells diminishing Th1 response. Therefore, the authors speculate that upregulation of CCL22 may help HCMV evade immune surveillance, resulting in increased viral spread. Data regarding the association between *UL144* genotypes and the outcomes of congenital infection is conflicting. While Arav-Boger et al reported an association [156, 170], others have not [165-169].

Our study addresses whether an association exists between specific *UL146*, *UL147*, and *UL144* genotypes and symptoms at birth in congenitally infected children. Many of these children that were asymptomatic at birth were followed for several years to ensure there were no long-term sequelae. In addition, serial isolates from children who shed HCMV over long periods of time

were analyzed to examine whether *UL146* hypervariability is driven via immune pressure.

Materials and Methods

Clinical subjects and specimens

HCMV isolates from symptomatic and asymptomatic congenitally infected children were obtained for phylogenetic analysis from two geographically distant locations at four facilities: 40 HCMV isolates from Iowa (four hospitals in Iowa City and Cedar Rapids, IA) and 11 isolates from Texas. Six isolates from the Texas clinic were from a previous study [165]. Together, there are 18 symptomatic (K.P., Sk, A.B., P.L., Ca, 100546, 101027, 102124, 102226, 102410, 300584, TX03, TX11, TX12, TX20, TX21, TX24, and TX30) and 33 asymptomatic children (100427, 100457, 100691, 100738, 100751, 101142, 101329, 101430, 101465, 102429, 200164, 200550, 201334, 201370, 300016, 300482, 300521, 300667, 301217, 301699, 302281, 400191, 400328, 400340, 400600, 400818, 400865, 401058, 401361, TX05, TX15, TX25, and TX32) (Table 2).

For the evolution of *UL146* over time, 13 serial viral isolates from six children attending six day care centers in Virginia were grown *in vitro* and sequenced (Table 3).

DNA Isolation

DNA was isolated from the Iowa and Texas clinical isolates as previously described [165]. HCMV infected MRC-5 cells from the Virginia Commonwealth University (VCU) study were maintained until 70 to 80% cytopathic effect (CPE) was attained. Cold Tris/EDTA buffer (0.5 ml) with 10% SDS (50 ul) and proteinase K (100 ug/ml) was added to the cell pellets and incubated for one hour at 65°C. Viral DNA was extracted using phenol/chloroform and precipitated with ethanol. The DNA pellets were washed with 70% and dried. DNA concentration was determined spectrophotometerically ($A_{260}/_{A280}$).

PCR Amplification

The primers for the *UL146* and *UL147* ORFs were designed for conserved regions that flank each of the ORFs. The primers 5'-GTCATGGACGCAGTTTTG-3' (forward) and 5'-GAACGATCTCGTCCGGTTC-3' (reverse) were identical to those used in Arav-Boger et al [158]. The size of the PCR product is 1.2-kb. For the *UL144* ORF, the primers 5'-CGTATTACAAACCGCGGAGAGGAT-3' (forward) and 5'-CTCAGACACGGTTCCGTAAAGTG-3' (reverse), which are located upstream or downstream of *UL144* ORF, generate a 740-bp product. The PCR conditions for *UL146* and *UL147* ORFs were 95ºC for 2 min, followed by 40 cycles of 95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 2 min. The reaction was extended at 72ºC for 10 min. The *UL144* PCR conditions were similar except the extension time was reduced to 1min.

DNA Sequencing

The amplicons were cloned into the pGEM-T Easy vector using the pGEM-T Easy kit (Promega) according to the manufacturer's instructions. At least three colonies were picked and sequenced in both directions using universal primers (SP6 and T7). For the serial isolates from VCU, if differences were identified in the *UL146* ORF, the entire PCR reaction was repeated to ensure that any differences were due to differences in viral DNA and not due to a PCR amplification error. All confirmed sequences were submitted to Genebank (Accession numbers: UL146: EU514891-EU514955; UL147: EU514794-EU514845; UL144: EU514846-EU514890)

Phylogenetic Analysis

The sequenced ORFs were converted to amino acids using Vector NTI 9 (Invitrogen). The predicted mature forms of vCXCL-1 and vCXCL-2 were generated with Signal P (www.cbs.dtu.dk/services/SignalP). Alignments of the mature forms of vCXCL-1, vCXCL-2, and *UL144* proteins were aligned using ClustalX 1.83. Neighbor-joining phylogenetic trees were generated using ClustalX 1.83 with 1000 bootstrap trials. The generated phylogenetic tree was viewed by TreeView (Win32) 1.6.6.

Statistical Analysis

Associations between genotype and whether any symptoms were observed were analyzed using Fisher's exact test. For each individual clade, we also calculated a 95% confidence interval for the proportion of asymptomatic individuals. All analyses were performed using SAS v9.1.

Results

vCXCL-1s from children with congenital HCMV infection can be divided into 11 distinct clades

To understand whether specific vCXCL-1s correlate with clinical outcomes, 51 samples were analyzed from 50 congenitally infected children (Table 2). TX11 and TX12 were from same child. Using primers upstream and downstream of the *UL146* and *UL147* genes [158] PCR products were obtained from the 51 clinical isolates. Each sample was PCR amplified twice, TA cloned, and two to five clones were sequenced. The accuracy rate was greater than 99.9% between the two separate PCR reactions. For reference sequences, we included the previously analyzed strains Toledo, Towne, C952, C954, C956, E760, ML1, TB40/E, FS, AL, 6397, KM, RK, Davis, NT, Merlin, and KSG [155, 234] in addition to the F strain from Prichard et al [26]. The *UL146* ORFs encode vCXCL-1 proteins containing between 115-121 amino acids. The mature forms without the signal sequence and the reference sequences contain between 93 and 103 residues that show 5% identity (6 residues) and 8% similarity

Table 2. Clinical isolates of congenital CMV infection

	Clinical manifestation							
Isolate ID	Low Birth Wt.	Microcephaly	Hepatosplen-omegaly	Jaundice	Petechiae/ Purpura	Chorioretinitis	UL146 Genotype	UL144 Genotype
A.B.	–	–	H	J	P	–	13	C
Ca	L	–	H	J	P	–	8	A
K.P.	–	–	H	J	–	–	13	
P.L.	–	–	H	J	P	–	11	B
Sk	–	–	H	J	P	–	13	B
100546	–	–	–	J	–	–	7	A
101027	–	–	–	J	–	–	13	C
102124	–	M	–	–	–	–	15	A
102226	L	M	H	J	–	–	1	A
102410	–	–	–	J	–	–	9	B
300584	–	–	–	J	–	–	13	B
TX03	–	–	H	J	P	–	1	
TX11	L	M	H	–	P	–	11	B*
TX12	L	M	H	–	P	–	11	A*
TX20	–	M	H	–	P	–	13	B
TX21	–	M	–	J	P	–	9	
TX24	L	M	H	J	P	C	12	AC
TX30	–	M	–	–	–	C	14	C
100427	–	–	–	–	–	–	8	B
100457	–	–	–	–	–	–	15	A
100691	–	–	–	–	–	–	12	A
100738	–	–	–	–	–	–	9	B
100751	–	–	–	–	–	–	10	AB
101142	–	–	–	–	–	–	7	A
101329	–	–	–	–	–	–	10	AB
101430	–	–	–	–	–	–	8	B
101465	–	–	–	–	–	–	11	B
102429	–	–	–	–	–	–	8	B
200164	–	–	–	–	–	–	9	B
200550	–	–	–	–	–	–	12	A
201334	–	–	–	–	–	–	1	C
201370	–	–	–	–	–	–	10	AB
300016	–	–	–	–	–	–	1 / 13	B
300482	–	–	–	–	–	–	12	A
300521	–	–	–	–	–	–	15	A
300667	–	–	–	–	–	–	12	A
301217	–	–	–	–	–	–	1	B
301699	–	–	–	–	–	–	12	A
302281	–	–	–	–	–	–	11	B
400191	–	–	–	–	–	–	8	A
400328	–	–	–	–	–	–	8	B
400340	–	–	–	–	–	–	8	B
400600	–	–	–	–	–	–	8	B
400818	–	–	–	–	–	–	11	B
400865	–	–	–	–	–	–	11	B
401058	–	–	–	–	–	–	13	B
401361	–	–	–	–	–	–	7	B
TX05	–	–	–	–	–	–	1	B*
TX15	–	–	–	–	–	–	6	C
TX25	–	–	–	–	–	–	11	B
TX32	–	–	–	–	–	–	13	B

* UL144 clades of TX11, TX12, and TX05 were identified by Bale et al (2001)

(9 residues) (Fig. 2). All sequences were assigned to 11 distinct vCXCL-1 clades (Fig. 3). The intravariability within each clade was less than 6%. The designated genotypic group numbers were assigned according to the 14 UL146 groups described by Dolan et al. [234]. No vCXCL-1 sequences were found in groups 3, 4, and 5 described by Dolan et al.

If the most distant reference group is removed (NT from Group 4, for which we did not assign any samples), the number of 100% consensus residues increases from 6 to 11. This includes the CXC chemokine motif and the four cysteine residues. This implies that this isolate is a very distant cousin of other vCXCL-1s. This number increases to 12 when all of the reference sequences are removed from the analysis. These conserved residues provide the essential structure for all α-chemokines [107]. The vCXCL-1 sequence of TX15 replaces the glutamic acid-leucine-arginine (ELR) with asparagine-glycine-arginine (NGR) motif, which is similar to the NRG containing chemokine found in the ML1 strain [234] (Fig. 2). Modification of the ELR motif (E → N and L → G) could have profound effects on chemokine function as the ELR motif is necessary for chemokine activity and receptor binding [237, 238]. To further identify possible functional differences that are reflected in their sequences, we clustered the groups based on their N-loop regions. The N-loop region, the residues between the second cysteine and the 3_{10}-helical turn (approximately 8-10 residues), is responsible for receptor engagement and function [36, 106, 107]. Analysis of these sequences divides the chemokines into the same 11 groups indicating that the N-loop region is mainly responsible for vCXCL-1 variability (Fig. 4). There is

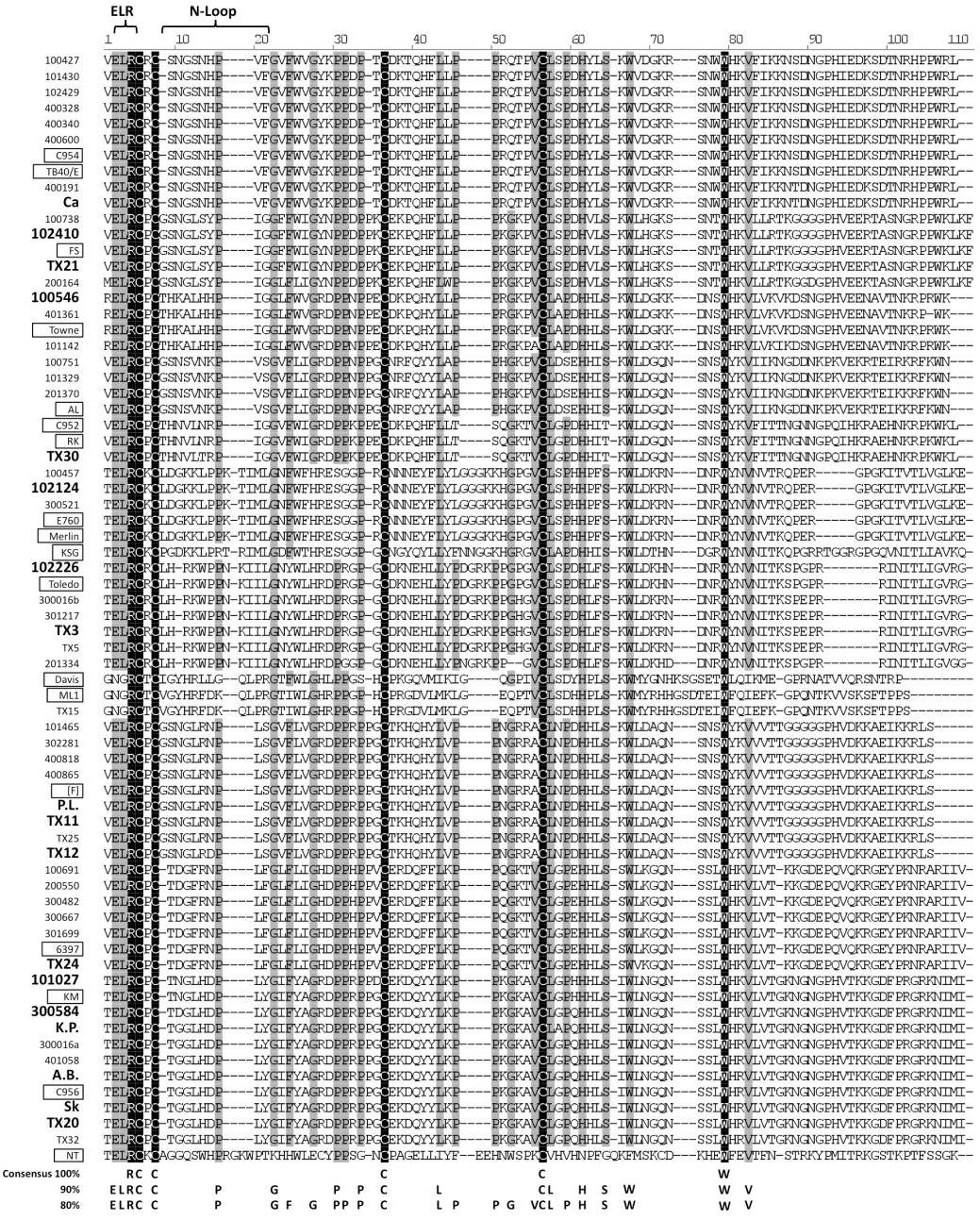

Figure 2. Amino acid alignments of the mature forms of vCXCL-1s from clinical isolates.

vCXCL-1 amino acid sequences from 51 clinical isolates were analyzed. Eighteen symptomatic clinical isolates are indicated in bold. The vCXCL-1 sequences from reference strains Toledo, Towne, C952, C954, C956, E760, ML1, TB40/E, FS, AL, 6397, KM, RK, Davis, NT, Merlin, and KSG (GenBank accession numbers AY681092, AY681095, DQ115733, DQ115734, DQ115735, DQ115754, AY446880, AY446866, AY446877, AY446887, AY446870, AY446893, AY446885, AY446868, AY446890, AY446894, and AY446889, respectively) plus strain [F] from Prichard et al. (2001) are boxed. The ELR motif and N-loop region, which are important in receptor binding and activation, are indicated at the top. Residues that are 100% conserved are shaded black and residues that are conserved in greater than 80% of the sequences are shaded in gray. Consensus sequences with 100%, 90%, and 80% conservations are shown at the bottom.

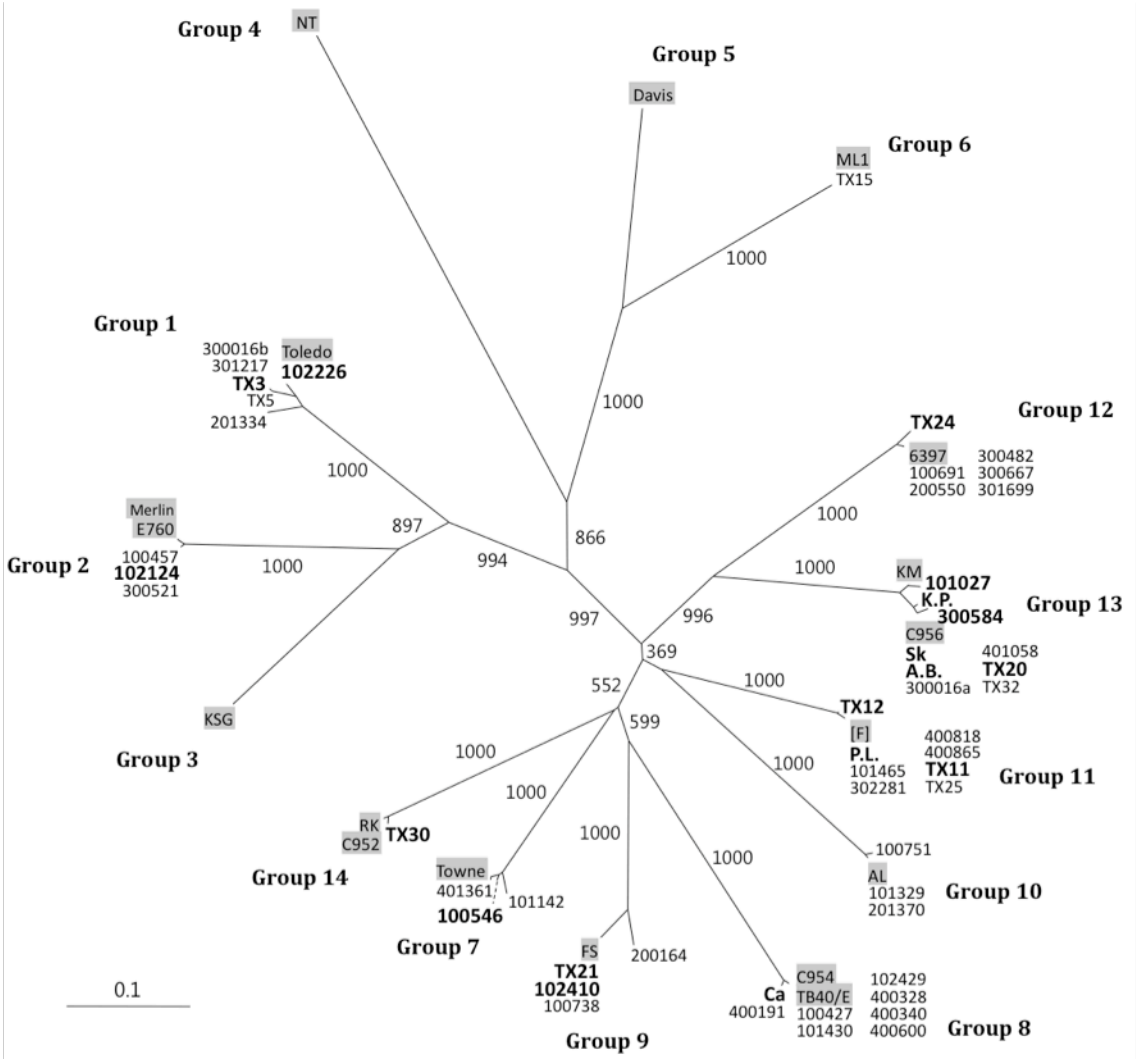

Figure 3. vCXCL-1 phylogenetic analysis.

The mature forms of vCXCL-1 from 51 clinical isolates with the reference sequences (shaded gray) were assembled into a phylogenetic tree using ClustalX. Eighteen symptomatic clinical isolates are indicated in bold. Bootstrap numbers are shown. Group designations correspond to those used by Dolan et al. (2004).

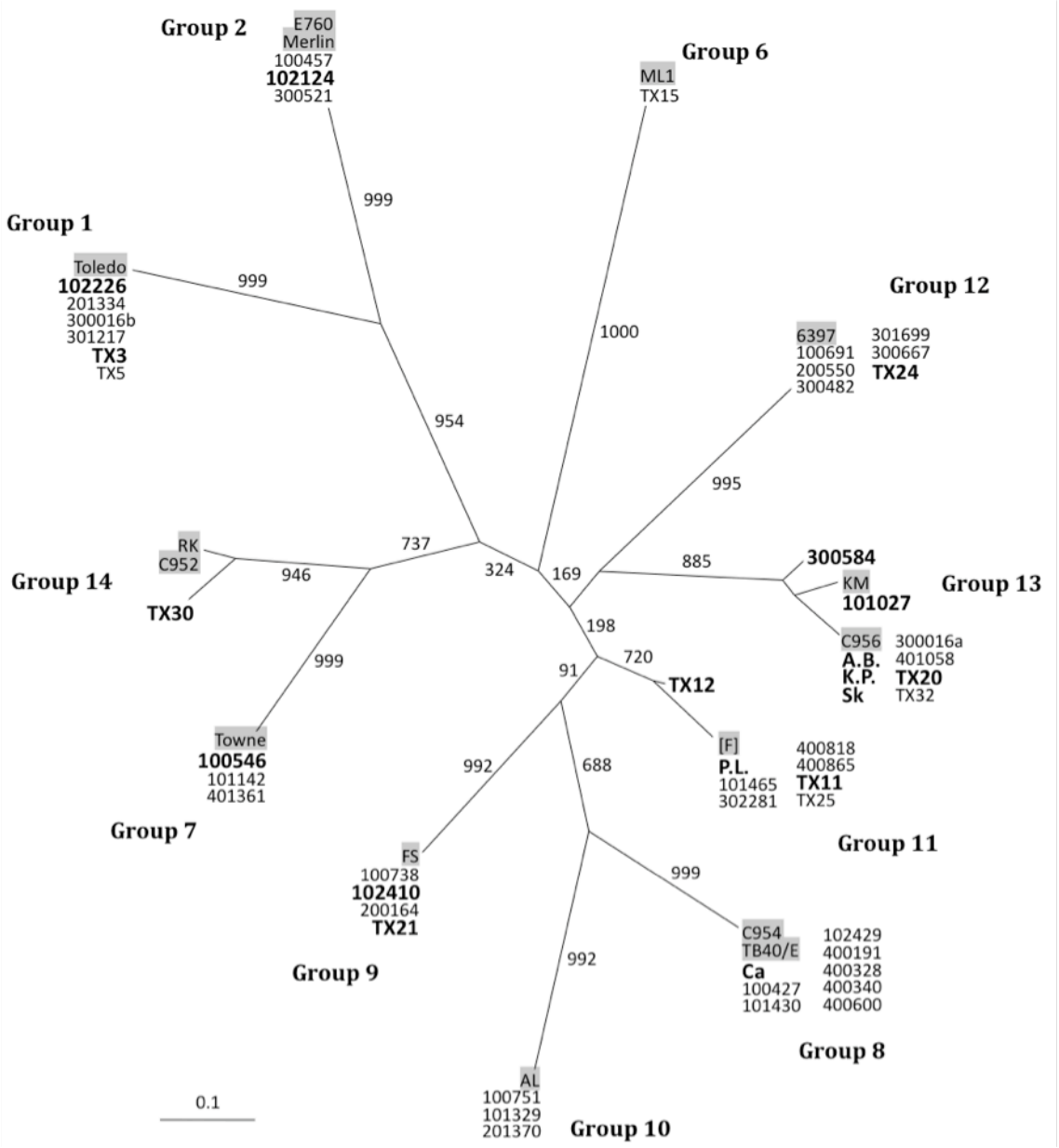

Figure 4. Phylogenetic analysis for the N-loop region of vCXCL-1.

The N-loop regions of vCXCL-1 from 51 clinical isolates with the reference sequences (in gray box) were analyzed using ClustalX. Eighteen symptomatic clinical isolates are indicated in bold. Bootstrap numbers are shown. Group designations correspond to those of the mature forms of UL146 amino acid (Fig. 2).

0% similarity within this region between the different clades. The fact that there is hypervariability within this region implies that these chemokines may have different affinities for the chemokine receptors and could trigger different downstream signaling and subsequent functions. Differences in affinity, apoptosis, and integrin expression have been noted for chimpanzee CMV vCXCL-1 compared to vCXCL-1_{Tol} [239].

The variability of vCXCL-1 does not show a definitive correlation between specific vCXCL-1 sequences and HCMV sequelae. Overall, there was not a statistically significant association between vCXCL-1 clades and symptoms at birth (p=0.37). However, examination of individual clades did reveal three clades with an unusually high proportion of asymptomatic children. These were group 8 with 7 of 8 (88%) asymptomatic (95% CI: 47% to 100%), group 10 with 3 of 3 (100%) asymptomatic (95% CI: 29% to 100%) and group 12 with 5 of 6 (83%) asymptomatic (95% CI: 36% to 100%).

One isolate, 300016, is assigned to two vCXCL-1 groups (group 1 and 13) (Fig. 3). After resequencing and analyzing 10 clones, these assignments still held. This indicates this child was infected with two different HCMV isolates as has been seen in both congenital infections and immune compromised patients [27, 240, 241].

vCXCL-2 from children with congenital HCMV infection has limited variability

The *UL147* ORF from the same set of clinical isolates encodes vCXCL-2 proteins comprised of 157–167 amino acids (Fig. 5). In contrast to the vCXCL-1 protein the mature form of the vCXCL-2 from the clinical isolates were all identical lengths (119 aa). The mature form of vCXCL-2 shows 87% identity (103 residues) and an additional 11% similarity (13 residues) overall. Because of the limited variability of *UL147* sequences from clinical isolates, the alignment of the mature vCXCL-2 proteins falls into a single group. As previously published, the only significant variability within vCXCL-2 occurs in the signal sequence [158], which is cleaved during protein maturation.

UL144 sequences fall into three genotypes

UL144 is a member of the TNF receptor family [164]. Arav-Boger et al showed a direct link between specific *UL144* genotypes and clinical outcomes [156, 170] while others have not [165-169, 242]. In order to establish whether specific clades of *UL144* are associated with clinical outcomes, 44 *UL144* sequences from the 51 clinical isolates were amplified and sequenced. We included the sequences of genotypes A, B, C, AB, and AC (GenBank accession numbers AF498086-AF498090) as reference sequences. The *UL144* ORF encodes a protein of 175–176 amino acids. The *UL144* mature form without the

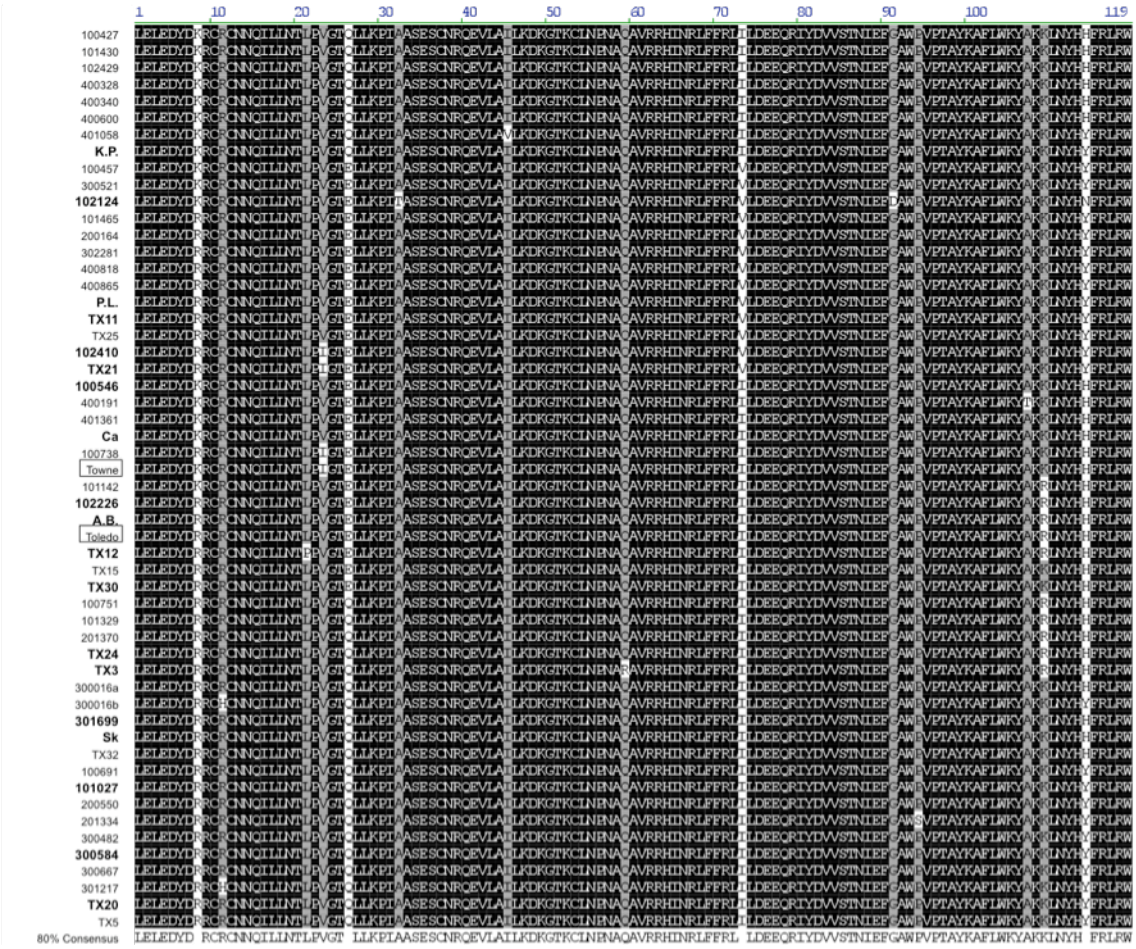

Figure 5. Alignment of the mature forms of vCXCL-2.

vCXCL-2 sequences from 51 clinical isolates were analyzed. 18 symptomatic clinical isolates are indicated in bold. The reference strains Toledo and Towne (GenBank accession # AY681092 and AY681095, respectively) were included and are boxed. Residues that are 100% conserved are shaded black and residues conserved in 80% to 99% of the sequences are shaded grey. The consensus residues with 80% conservation are shown at the bottom.

signal sequence is between 146–157 residues in length with 60% identity (95 residues) with an additional 27 similar residues (Fig. 6). These *UL144* amino acid sequences separate the isolates into five major genotypic groups (A, B, C, and subgroup AB and AC) (Fig. 7). Previously, Lurain et al and Arav-Boger et al reported the same groupings based on *UL144* sequences [166, 170]. We used the *UL144* classification of Arav-Boger et al for this study. Bale et al showed *UL144* variability from 16 congenitally HCMV infected children [165]. They found that clinical isolates TX11 and TX12, which were obtained from same child, showed different *UL144* group designations, group 3 (or B) and group 1 (or A). Once again this indicates a child with multiple HCMV infections. Interestingly, our results mapped the *UL146* genotypes of these two isolates to the same clade (group 11), which were confirmed by two separate PCR amplifications and sequencing of five clones each (Fig. 3). This implies divergence within *UL144* and that the two strains could have evolved under different environmental pressures.

The *UL144* protein consists of two cysteine-rich domains (CRD), one transmembrane domain (tm), and one cytoplasmic domain (cyto). Unlike the TNFR, *UL144* lacks an additional CRD3 region which may explain absence of binding to TNF-like ligands [164]. The alignments show that *UL144* CRD1, which is comprised of 34 residues, shows 50% identity (17 residues) and this percentage increases to 71% when similar residues are included. The CRD2 is slightly more conserved. Of the 36-37 residues, there is 57% identity (21 residues) and 81% similarity. BTLA binds to the CRD1 of HVEM, which is

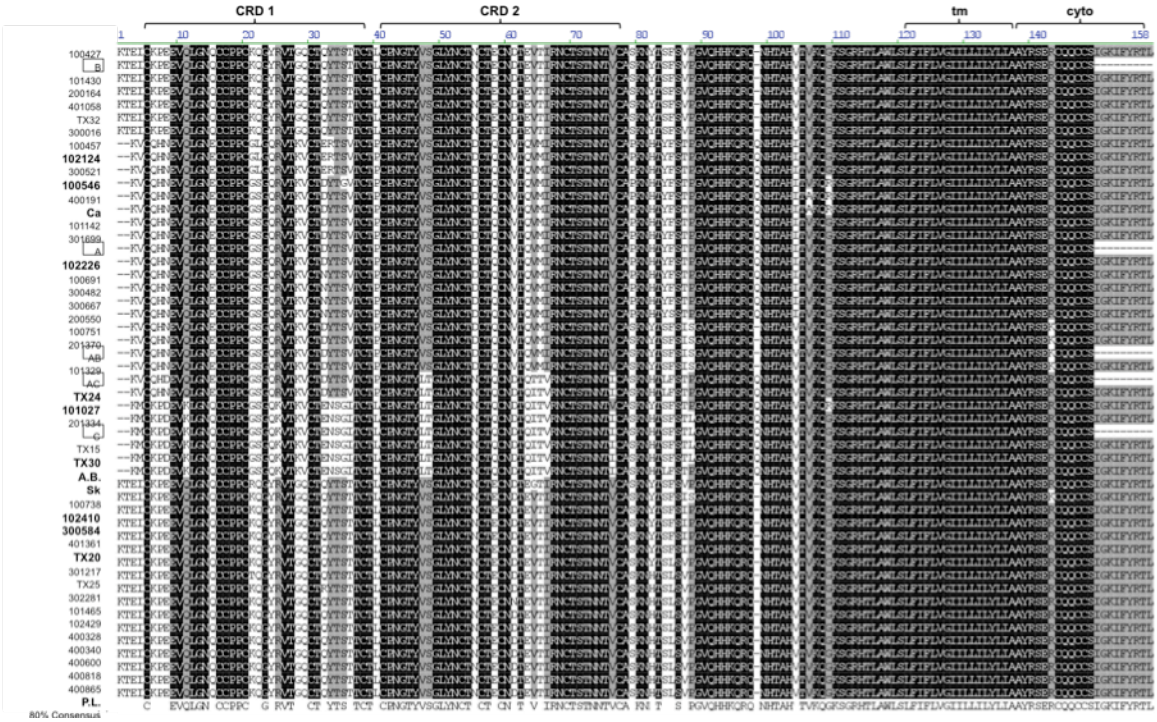

Figure 6. UL144 contains highly conserved domains.

UL144 amino acid sequences from 44 clinical isolates were analyzed. 13 symptomatic clinical isolates are indicated in bold. Representative sequences from genotype groupings A, B, C, AB, and AC (GenBank accession numbers AF498086-AF498090) are included as reference sequences and are boxed. Residues that are 100% conserved are shaded black and residues that are greater than 80% conserved are shaded grey. Consensus sequence with 80% conservation across all isolates are shown at the bottom. The cysteine rich domains (CRD), the transmembrane region (tm), and cytoplasmic tail (cyto) are indicated at the top.

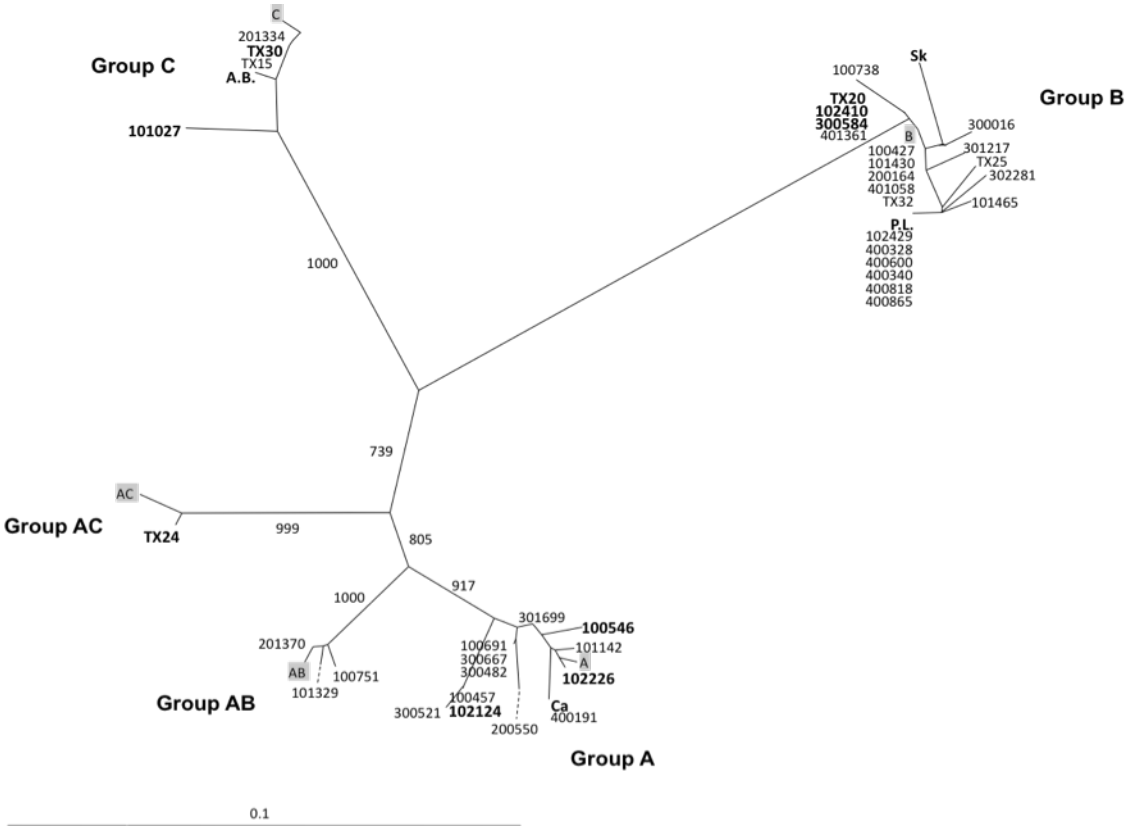

Figure 7. UL144 sequences fall into 5 groups.

The mature forms of UL144 amino acid sequences from 44 clinical isolates were analyzed by the ClustalX. 13 symptomatic clinical isolates are indicated in bold. Reference sequences are illustrated with grey boxes. Group designations correspond to those of Arav-Boger et al (2002).

homologous to *UL144* CRD1 [236]. As observed in previous studies there is a high level of conservation in the transmembrane and cytoplasmic domains of *UL144* between the different isolates [166, 170]. The variability in the CRD regions has been used to infer that the interaction between *UL144* and BTLA could differ between the different genotypes, leading to differential virus survival and/or clinical outcomes.

Using our clinical samples from congenitally infected children, we show that the *UL144* genotype does not correlate with HCMV sequelae. Using Fisher's exact test, there was no statistically significant association between *UL144* genotype and symptoms (p-value = 0.45). Group B in our sample did have 18 of 23 (78%) asymptomatic (95% CI: 45% to 92%) while groups A and C had lower percentages of asymptomatic newborns (Group A= 69% (9/13) and Group C=40% (2/5)). This data indicates that there is no direct correlation between UL144 genotypes and symptoms at birth, although the trend of more asymptomatic children in group B is similar to the findings of Arav-Boger et al [233].

***UL146* genes under immune selection pressure do not mutate**

One explanation for the variability in the *UL146* gene is that *UL146* evolves to avoid the immune response. To address whether the *UL146* gene accumulates mutations while under immune selection pressure, we studied serial HCMV isolates from children who shed HCMV over many years [243]. We cloned and sequenced the *UL146* ORF from 13 serial isolates from six children

from daycare centers in Virginia (Table 3). For up to three years, there was no change in vCXCL-1 (Fig. 8). However, multiple *UL146* sequences were identified from sequential isolates from two individuals. In child 5, isolates 30135 and 31099 were assigned to group 9 and 8, and in child 6, isolates 23914 and 24864 were assigned to group 8 and 9, respectively. In order to confirm these isolates resulted from multiple infections and not mutations of the original virus, we sequenced the *UL144* gene from these same isolates. The *UL144* sequences from isolates 23914 and 24864 were assigned to group A and B, respectively. This data indicates these children were infected with two different viruses and that the *UL146* gene did not accumulate multiple mutations leading to assignment to a new clade.

Discussion

There have been several studies addressing the effect of *UL146* variation on HCMV pathogenesis in immune compromised patients [155, 157-159, 233, 234]. The *UL146* ORF is one of the most variable genes in the HCMV genomes following analysis of isolates from AIDS or organ transplant patients [27, 244, 245]. These studies lead to the classification of *UL146* sequences into 14 groups. This variability, its role as a chemokine and its absence from lab attenuated strains, lead to the hypothesis that this gene could encode a virulence factor. Dolan et al, Hassan-Walker et al, and Arav-Boger et al investigated the link between specific *UL146* clades and symptomatic/asymptomatic congenital infections [159, 233, 234]. Arav-Boger et al showed that from 21 clinical isolates

Table 3. Serial isolates from Virginia Commonwealth University (VCU) day care center

child No.	Isolate ID	Sampling Date	UL146 Genotype
1	24534	12/2/1997	8
	27472	3/9/2000	8
2	27568	5/12/2000	10
	27716	7/12/2000	10
	29333	8/16/2001	10
3	26756	8/25/1999	11
	29524	10/10/2001	11
4	29140	6/25/2001	7
	30803	2/20/2003	7
5	23914	2/25/1997	8
	24864	4/1/1998	9
6	30135	4/25/2002	9
	31099	6/19/2003	8

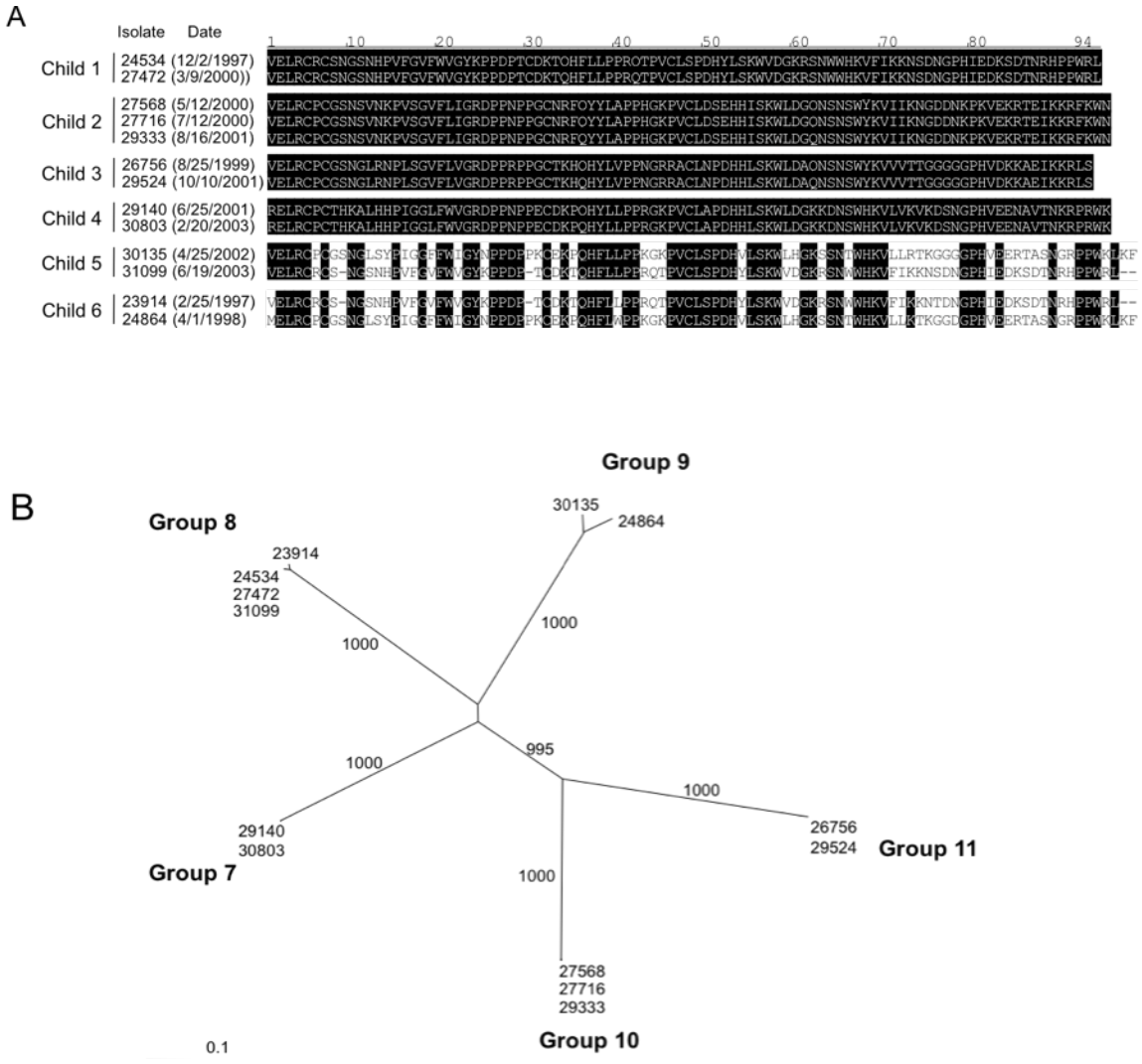

Figure 8. vCXCL-1 does not mutate in long term shedders.

vCXCL-1 from 13 serial isolates from 6 children for up to 3 years were analyzed. A) The alignments shown are from individual children over a span of one to three years. Residues that are 100% conserved are shaded black and grey regions residues indicate conservative substitutions. B) ClustalX was used for phylogenetic analysis of the different isolates.

no specific *UL146* genotype was associated with disease outcome [233]. Hassan-Walker et al and Dolan et al showed 4 and 14 genetic groups, respectively. However, due to the small sample size (14 and 7, respectively) they could not draw a direct link between genotypes and symptoms [159, 234].

Even though our study includes a relatively large sample size, we are unable to reach statistically significant conclusions about specific *UL146* clades and symptomatic outcomes. Our results demonstrate that out of the 14 groups, 11 of the clades did not exhibit a definitive association between UL146 genotypes and symptoms. Group 10 contained only asymptomatic children but had a sample size of 3. In group 8, only 1 out of 8 were symptomatic. From this data we can conclude that most of the *UL146* genotypes do not correlate with symptomatic births and two of these groups (Group 8 and 10) point towards asymptomatic outcomes but a statistically significant conclusion is limited because of sample size.

The other gene located within the UL/b' region, which has homology to the TNF receptor and is variable is *UL144*. Our *UL144* data in this study shows a lack of correlation between symptoms and *UL144* clades supporting the findings of several other studies [156, 165-170, 242]. Only Arav-Boger concluded that *UL144* is predictive of congenital infection outcomes [156, 170]. The major differences between these studies are the sample size and the source of the isolates. Arav-Boger et al suggested that groups A and C are associated with more serious outcomes upon analysis of 23 congenitally infected isolates comprised of 13 symptomatic neonates and fetuses. The B genotype was

associated with both asymptomatic (10 out of 10) and symptomatic (6 out of 13) children and the A and C genotypes were only found in the symptomatic children [170]. From this, they concluded that the A and C genotypes are associated with symptomatic outcomes. This group also showed a possible relationship between the C genotype and symptomatic diseases in newborns in a study in Italy. They showed a statistically significant association between the C genotype and termination of pregnancy, while the B genotype did not [156]. This study did suffer from enrollment biases as women were enrolled based on a higher risk for congenital CMV infections. In contrast, Bale et al examined 51 HCMV isolates from 44 patients containing 16 congenitally infected children to conclude that *UL144* genotype does not correlate with symptoms at birth [165]. Others have corroborated the Bale study. Mao et al analyzed 70 isolates from Chinese infants where 58 were from symptomatic children, but only five infants were determined to have acquired CMV from congenital infections [167]. An even larger Japanese study assessed the *UL144* sequences from 74 Japanese HCMV isolates where 10 samples were from congenitally infected newborns [168]. They also reported that *UL144* genotype does not correlate with symptoms. Analyzing HCMV congenitally infected fetuses, Picone et al concluded that there was no correlation between severity of infection and *UL144* genotype [242]. All of these studies divided the *UL144* genotypes into three major groups: 1, 2, and 3 (or A, C, and B, respectively) and did not find a statistically significant relationship between these genetic groups and specific clinical symptoms at birth. In our study, 23 of 44 samples had the B *UL144* genotype. Of these, 5 of 23

infants were symptomatic while 18 were asymptomatic. In contrast to the study of Arav-Boger, we observed that both A and C clades contain both asymptomatic and symptomatic children (Fig. 7). Therefore, our analysis of the *UL144* sequences agrees with previous findings of Bale et al.

Because of its high variability, *UL146* sequences can be used as an indicator of multiple HCMV infections. Multiple HCMV infections could be due to either incomplete protection after HCMV infection or coinfection simultaneously with different strains of HCMV. Multiple infections have been found in both congenitally infected patients [233], renal transplant patients [155], bone marrow transplant patients [155], and AIDS patients [234]. In immunocompetent children, Bale et al studied 11 serial isolates from five children for up to 2.5 years and found multiple sequences [165]. Based on the genetic stability of *UL146* sequence over time, multiple sequences are not due to random mutations. Therefore the multiple sequences were due to coinfection with another strain of HCMV. We have also found multiple sequences from children in daycare (Fig. 8A) indicating that coinfections with multiple strains of HCMV are possible.

Since *UL146* and *UL144* genes are variable, many studies have addressed the stability of *UL146* or *UL144* genotype upon serial isolation over many years [155, 157, 159, 168, 170, 234]. Lurain et al studied various organ transplant recipients for up to 5 years with no changes in *UL146* sequence [155]. Both *in vitro* [155] and in immune compromised hosts, *UL146* did not mutate over time [159]. A possible explanation of *UL146* and *UL144* hypervariability could be due to immune selection pressure within the host. Our study (Fig. 8) and Bale et

al analyzed immunocompetent children shedding virus over many years. This would have allowed for vigorous immune selection compared to the transplantation studies where the host is immune suppressed and are often treated with anti-CMV drugs. Immune selection could select for mutated, secreted vCXCL-1 protein that is recognized as foreign, because it is only ~20-30% homologous to host chemokines. Interestingly, Lurain et al showed no variation in *UL146* sequences even when different patients received organs from the same donor. These organs contained the same strain of HCMV as the donor but were transplanted into different hosts, which could mount different immune responses [155]. This shows the stability of *UL146* over time and in different immunological settings from different individuals.

Most immune competent individuals shed HCMV for about 9-12 months after acquisition. In contrast, primary CMV infection of neonates and infants results in continuous viral shedding into the urine and saliva for several years [246-248]. Our data are unique in that our samples were isolated from healthy children (i.e. not immunocompromised). If immune selection drives *UL146* variation, our analysis should identify it. Our data from healthy children also agrees with the studies from immunocompromised patients. We found 2 cases of coinfection, which fell into group 8 and 9, but no sequence changes were detected during two years, indicating that virus maintains its genetic integrity under healthy immune defense. If mutations are not occurring in this time frame, how and why are these genes so variable? One possibility is that changes in the genes accumulated during HCMVs' co-evolution with humans. The gradual

changes in *UL146* and *UL144* provide an evolutionary advantage, which only becomes dominant over millennia.

In conclusion, this study shows that specific *UL146* and *UL144* genotypes do not correlate with the sequelae of congenital infection. We also show that some children in daycare are infected with multiple strains of HCMV and that their HCMV *UL146* genotypes do not change over several years.

Acknowledgements

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Chapter 4

POLYMORPHISMS IN HCMV VIRAL CHEMOKINE, VCXCL-1, ELICIT DIFFERENTIAL ACTIVATION

Abstract

The human cytomegalovirus (HCMV) *UL146*-encoded viral chemokine, vCXCL-1, is hypervariable. In our previous analysis of clinical isolates from congenitally infected infants, we found 11 distinct vCXCL-1 genotypic clades. In these clades, the N-loop region, which is important for receptor-binding varied. One isolate also contained a modified ELR motif, which is vital for binding to CXCR1 and CXCR2 receptors. In this study, we produced and purified representative vCXCL-1 proteins from each of the 11 clades using a baculovirus protein expression system. We measured vCXCL-1's affinity for either CXCR1 or CXCR2 using competition binding assays. All vCXCL-1s bound to CXCR2 albeit with different affinities. Interestingly, we found a trend that vCXCL-1s with high affinity for CXCR2 receptors also bind CXCR1. We also examined functional differences between the vCXCL-1s using chemotaxis, integrin activation, and calcium mobilization assays on human neutrophils. All vCXCL-1s were able to activate human neutrophils. However, the potency of the vCXCL-1s to induce chemotaxis of neutrophils varied. Moreover, the vCXCL-1s varied in their ability to induce CCL22 production, which did not correlate with chemotaxis potencies.

Our data suggest that the vCXCL-1 polymorphisms elicit different binding affinities, receptor usage, and differential cellular activation, which potentially contribute to HCMV pathogenesis.

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that is well adapted to modulate host immune responses [113, 249]. HCMV contains genes for immune evasion that could function to increase viral survival, dissemination, or may contribute to pathogenesis [143, 250, 251]. There are a large number of open reading frames (~62) called the UL/b' region in HCMV that are non-essential for virus replication *in vitro* but may have a role in immune evasion *in vivo* [25, 245]. Among the UL/b' open reading frames (ORFs), *UL146* and *UL147* genes have limited homology to CXC chemokines [25]. The *UL146* ORF from the Toledo strain of HCMV called vCXCL-1_{Tol} has been shown to produce a functional CXC chemokine [143]. vCXCL-1_{Tol} binds to CXCR1 and CXCR2, induces neutrophil chemotaxis and calcium mobilization [154]. These data suggest that vCXCL-1s produced from CMV-infected endothelial cells recruit neutrophils and contribute to viral dissemination [143, 252]. Sequencing of the HCMV genomes and the *UL146* gene from numerous clinical isolates [27, 240, 241, 244, 245, 253] showed that the *UL146* gene is one of the most variable genes in the entire HCMV genome [27].

vCXCL-1s are members of the chemokine family. Chemokines are small chemoattractant cytokines between 70-130 amino acids in length. The two major families of chemokines (CC and CXC) contain four conserved cysteine residues that contribute to the overall structure. Whether an amino acid separates the amino terminal cysteines is used for dividing chemokines into the CXC or CC families. The N-terminus preceding the first cysteine is an important region for receptor binding and activation [30, 96]. The Glu-Leu-Arg (ELR) motif found in CXC chemokines is a critical motif for CXCR1 and/or CXCR2 receptor recognition and activation [102, 103]. Introduction of an ELR motif into the non-ELR CXC chemokine CXCL4 (PF4) converted it into a neutrophil attractant and activator [34], although another non-ELR CXC chemokine, CXCL10 (IP-10), required additional modifications to become a fully functional protein on human neutrophils [104]. Following the second cysteine is a flexible N-loop of approximately ten residues. The N-loop is responsible for receptor engagement and function [30, 35, 96, 104]. Skelton et al. showed that CXCL8 and CXCR1 N-terminus fragment, CXCR1-p1, interact along the ELR and N-loop motifs of CXCL8 [105]. Further, Lowman et al. demonstrated that CXCL1 (Gro-α) could be altered to bind both CXCR1 and CXCR2 when the N-loop region of CXCL8 (IL-8) was swapped into CXCL1 [96]. These data indicate that the ELR motif is necessary for receptor recognition and the N-loop region controls specificity for CXCR1 over CXCR2.

Previously, we sequenced the *UL146* gene from congenitally HCMV infected clinical isolates [160]. We found 11 clades of vCXCL-1 including one

non-ELR CXC chemokine, vCXCL-1_{TX15}. Furthermore, analysis of the N-loop divides the chemokines into the same 11 clades, indicating that the N-loop region is mainly responsible for vCXCL-1 variability [160]. Although the genetic variability of vCXCL-1 did not show a definite correlation with clinical symptoms, this hypervariability within the N-loop region suggests that these chemokines may have different affinities, receptor usage, and trigger different downstream signaling and functions. For example, eotaxin (CCL11) induces eosinophil migration exclusively via CCR3. CCR3 is known to be a promiscuous chemokine receptor, responsive to more than a dozen different ligands [254, 255]. Eotaxin-2 and eotaxin-3 bear less than 40% identity to eotaxin at the amino acid level (i.e. eotaxin vs. eotaxin-2 (39%); eotaxin-2 vs. eotaxin-3 (33%)), yet they also bind and function through CCR3. While eotaxin and eotaxin-2 bound equally, eotaxin-3 showed ~10-fold lower affinity than eotaxin [256]. Eotaxin-3 was less potent than eotaxin in eosinophil chemotaxis [257] while eotaxin-2 showed a similar potency to eotaxin [258]. In order to address functional variability of the vCXCL-1s, we generated recombinant vCXCL-1s from each clade and performed competition-binding assays and functional assays to assess how vCXCL-1 variability affects function.

Materials and methods

Cells and clinical isolates

Insect cells SF9 were purchased from Gibco and Hi5 cellls from Cellgro. HEK293 cells were a gift from Dr. Cynthia Peterson's lab (University of Tennessee). Human myelomonocytic HL-60 T2 cell transfectants over-expressing CXCR2 were a gift from Dr. Ann Richmond (Vanderbilt University). The 51 clinical isolates were provided by Dr. James Bale (University of Utah School of Medicine) and Dr. Gail J. Demmler (Texas Children's Hospital) as described previously [160].

Neutrophil isolation

Peripheral blood neutrophils (PBN) were isolated from EDTA-treated blood from healthy human volunteers using dextran sedimentation and density gradient centrifugation as previously described [259]. Erythrocytes were removed with hypotonic lysis in 0.2% NaCl. After removal 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# 6476B).

Production of recombinant vCXCL-1s

The vCXCL-1 gene, *UL146*, was PCR amplified from HCMV DNA of each of the 11 clades from a representative clinical isolate and cloned into the

baculovirus transfer plasmid 1392 (Invitrogen), which contains homologous regions for recombination into the baculovirus genome. PCR primers were designed to include the open reading frame (ORF) and included an additional 2-4 glycines and six histidines on the carboxyl terminus of the proteins for purification. For the generation of baculoviruses, SF9 cells were transfected with the 1392/*UL146* ORF plasmid construct and Sapphire linearized baculovirus DNA (Orbigen) according to the manufacturer's instructions. Recombinant baculovirus containing the *UL146* gene was titrated and used to infect Hi5 cells for optimum protein expression. 48 hrs after infection, cells and supernatants were harvested. Recombinant proteins were isolated from the supernatants using Ni-NTA agarose beads (Qiagen) and resuspended in PBS. Protein concentration was quantified using Coomassie blue staining of SDS-PAGE gel using lysozyme as a standard. Quantity One software (Bio-Rad) was used for protein quantification.

Receptor binding analysis

The ability of vCXCL-1s to compete for binding to either CXCR1 or CXCR2 was evaluated as previously described [143]. Briefly, 1-3x10^5 HEK293 cells over-expressing CXCR1 or CXCR2 were incubated with 0.2nM ^{125}I-labeled CXCL8 (Perkin Elmer) and increasing concentrations of unlabeled chemokines. Cells were collected via a 96-well 0.45 mm Durapore membrane (Millipore) filter format on a vacuum manifold, washed twice, and bound radioactivity was

measured with liquid scintillation counting. The competition curves were plotted and 50% effective concentration (EC_{50}) were determined using GraphPad Prism.

Intracellular calcium mobilization assays

Release of calcium from intracellular stores was determined on freshly isolated PBN resuspended in PBS. Cells were loaded with 3 μg/ml Indo-1-AM (Molecular Probes) for 60 min at 37 °C. Cells were washed with PBS and diluted to 1×10^6 cells/ml in Hanks' balanced salt solution (HBSS) containing Ca^{2+} and Mg^{2+} and 1% FBS (Hyclone). Chemokines were added to 2 ml of cells at a final concentration of 100 nM. All host chemokines were purchased from Peprotech (Rocky Hill, NJ) and endotoxin levels were less than 0.1 ng per μg (1 EU/μg). Calcium flux was measured using a Photon Technology International Spectrophotometer (New Jersey) at an excitation of 350 nm and FeliX32 software for analysis. Relative intracellular calcium levels were expressed as the ratio of emissions at 490 nm / 400 nm.

β2 integrins staining

1×10^6 PBN cells were resuspended in RPMI-1640 (Biowhittaker) 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. PBN were incubated with fluorescently conjugated CD11a, CD11b, and CD11c antibodies (Caltag) on ice

for 30 min. and fixed with 4% paraformaldehyde. Cells were analyzed with flow cytometry (FacsCalibur, BD Bioscience).

Human PBN chemotaxis assays

Chemotaxis assays were performed on freshly isolated human PBN resuspended in HBSS with 0.1% BSA and 10 mM HEPES. Assays were performed in triplicate in 96-well chemotaxis plates. 20 µl of chemokines were loaded at varying concentrations (0, 1, 10, 100, and 500 nM) into the lower well of the modified Boyden chamber (Neuroprobe) fitted with a 5 µm filter. 1×10^6 PBN in 30 µl were added to the upper well. The cells were incubated for 3 h at 37 °C. Migration of PBN was measured by direct counting of migrated cells into the lower chamber. The chemotactic index was calculated as the number of migrated cells in the presence of chemokine divided by the number of migrated cells with buffer only.

Quantitative real-time PCR of CCL22 expression

HL-60 T2 cells over-expressing CXCR2 were differentiated for 7 days with 1.3% DMSO prior to chemokine treatment. Media was exchanged to HBSS and incubated with viral chemokines at a final concentration of 100 nM for 4 h at 37 °C. Total RNA was isolated with Tri-Reagent (Sigma) and reverse transcribed by the ProtoScript M-MuLV first strand cDNA synthesis kit (NEB). Real-time PCR was performed using iQ5 Real-Time PCR Detection System (Bio-Rad) with a

reaction mixture volume of 25 µl containing SYBR green (NEB DyNAmo SYBR green qPCR kit), 300 nM of each primer, and ~25ug of cDNA. Primers for CCL22 were purchased from SABiosciences (Cat # 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. Data analysis used iQ5 Optical System Software (Bio-Rad). The relative gene expression levels were calculated as fold changes using the formula Ratio = $2^{-\Delta\Delta CT}$, where ΔCT_{target} or $_{reference}$ = threshold cycle (CT) of the control gene (ACT1) − CT of the target gene (CCL22), and $\Delta\Delta CT = \Delta CT_{reference} - \Delta CT_{target}$ [260]. The housekeeping gene encoding actin (ACT1) was used as a reference control.

Results

Amino acid sequence alignment

Previously we sequenced the *UL146* gene from 51 clinical isolates and showed that it comprised 11 genetic clades [160]. From 11 clades, representative isolates were selected and aligned with vCXCL1 from the Toledo strain (Fig. 9A). The percent identities of the vCXCL-1s ranged from 23.7 – 61.2% with 11 conserved amino acids: including the R of ELR motif, the four cysteines, a glycine at position 21, two prolines at positions 32 and 87, a leucine at position 56, a histidine at position 60, and a tryptophan at position 65 (Fig. 9A). The Gly21 generates a single-turn 3_{10} helix that finishes the N-loop region. The

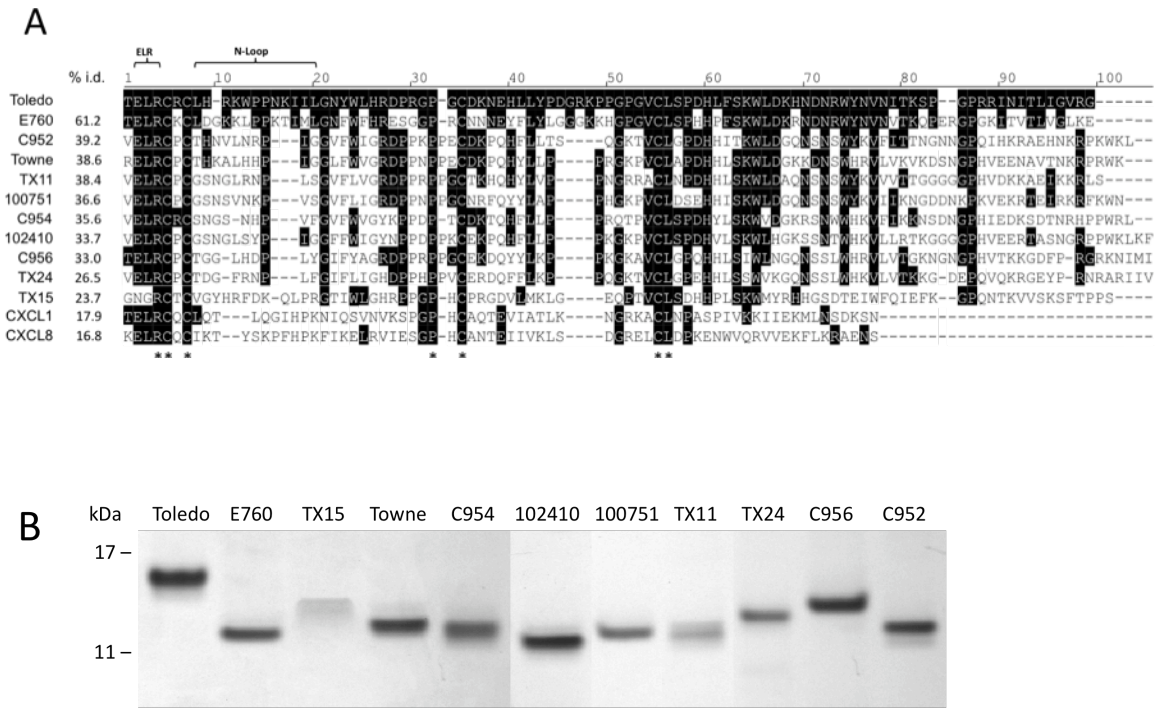


Figure 9. Amino acid alignment and recombinant vCXCL-1 production.

(A) Amino acid alignment of the mature forms of vCXCL-1s and host chemokines (CXCL1 and CXCL8). Seven amino acids are conserved including four cysteines. The ELR motif is conserved except for vCXCL-1_{TX15}. The variable N-loop region is located after second cysteine. (B) Recombinant vCXCL-1s production in baculovirus protein expression system. 6His-tagged protein eluted from nickel beads was run on a 15% SDS PAGE gel and stained with Coomassie blue.

Pro32 is located in the 30s-loop between the β1-sheet and β2-sheet. Previously, the Gly31-Pro32 motif in CXCL8 was shown to be essential for determining the structure of the 30-35 region and possibly the 5-35 disulfide bond [29, 97, 104]. The Trp65 and Pro87 are in the C-terminal α-helix, which is followed by His60. Furthermore, Leu25 and Val27 in CXCL8 that creates a hydrophobic environment for dimer formation was not conserved in the vCXCL-1s [261, 262]. Also, mutation of Leu25 to tyrosine introduces monocyte chemoattractant activity into CXCL8 [263]. Therefore, different β1-sheet amino acid formation can possibly contribute to determining cell type selectivity via selective chemokine receptor binding.

The mature forms of the viral chemokines, without the signal sequences, contain between 93-98 residues; whereas human chemokines, such as CXCL1 (Gro-α) or CXCL8 (IL-8), contain 70-71 residues. What affect these additional 20 residues of vCXCL-1s have on function is unknown. However, it has been shown that glycosaminoglycans (GAGs) can bind to the C-terminal α-helix of CXCL8 enhancing chemokine activity on neutrophils [264]. When vCXCL-1s are compared to human chemokines, the scope of the percent identities was lowered to 16.8% with 7 conserved residues: including the R of ELR motif, the four cysteines, a proline at position 32, and a leucine at position 56. The ELR motif was conserved except vCXCL-1_{TX15} which replaces ELR with an Asn-Gly-Arg (NGR) motif. Single amino acid substitutions in this motif showed that all three residues are sensitive to modification although the arginine is critically important for CXCL8 function [34, 35]. Therefore, the modified ELR motif of vCXCL-1_{TX15}

could have profound effects on chemokine function or receptor binding [102, 103]. The variable N-loop region that is mainly responsible for vCXCL-1 variability is followed by the second cysteine [160]. Taken together, from the high degree of variability in the N-loop, C-terminus, and even the ELR motif, we hypothesized that there would be differences in function and even receptor usage based on the modified ELR motif.

vCXCL-1 production using the baculovirus expression system

In order to address functional differences between the vCXCL-1s, we generated recombinant vCXCL-1s using the baculovirus protein expression system. Baculovirus expression provides mammalian signal-sequence cleavage, generates eukaryotic glycosylation patterns and protein folding. vCXCL-1s were 6 His-tagged and purified using Ni-NTA agarose beads. A Coomassie blue stain of the isolated proteins is shown in Fig. 9B. The sizes ranged from approximately 11 kDa to 15 kDa. The actual molecular weights of vCXCL-1s were confirmed by Matrix-assisted laser desorption/ionization (MALDI) (Table 4).

vCXCL-1s vary in their affinities for CXCR1 or CXCR2

The conserved ELR motif suggests that the viral chemokines bind to CXCR1 and/or CXCR2. However, the hypervariable N-loop binding region of the vCXCL1s and non-ELR motif of vCXCL-1_{TX15} could result in differences in receptor binding affinity. To test this possibility, competition-binding assays were

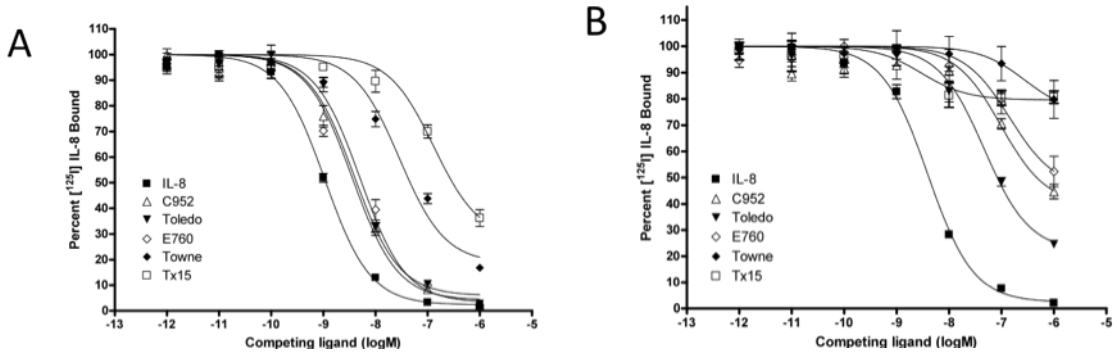
Table 4. Molecular weight of vCXCL-1s by Matrix-assisted laser desorption/ionization (MALDI) analysis

Chemokine	MW by MALDI
Toledo	14137
E760	11077
TX15	12032
Towne	11960
C954	11961
102410	11880
100751	11853
TX11	11157
TX24	13036
C956	12912
C952	11959

used to evaluate the ability of vCXCL-1s to compete for binding to human CXCR1 and/or CXCR2. All vCXCL-1s were able to compete with [^{125}I]-CXCL8 for binding to CXCR2. 50% effective concentrations (EC_{50}) of vCXCL-1s were higher than CXCL8 (EC_{50} = 1.1 nM) (Fig. 10A). This indicates that most vCXCL-1s bind to CXCR2 with a lower affinity than CXCL8. Among them, three vCXCL-1s showed a high level of affinity for CXCR2 (vCXCL-1_{C952} EC_{50} = 3.5 nM, vCXCL-1_{E760} EC_{50} = 3.8 nM, and vCXCL-1_{Tol} EC_{50} = 5.1 nM). Interestingly, the non-ELR CXC chemokine, vCXCL-1_{TX15}, still bound to CXCR2 albeit with very reduced affinity (EC_{50} > 131.4 nM). We also examined vCXCL-1 binding affinity to CXCR1 to evaluate an idea of whether these chemokines mimic CXCL1, which binds only to CXCR2, or CXCL8, which binds to both CXCR1 and CXCR2 (Fig. 10B). Although the binding affinities were very low, those with high affinity binding to CXCR2 also showed binding to CXCR1 while the others demonstrated no binding (vCXCL-1_{Tol} EC_{50} > 46.7 nM, vCXCL-1_{C952} EC_{50} > 97.7 nM, vCXCL-1_{E760} EC_{50} > 143.3 nM). This points to a trend that vCXCL-1s with high affinity to CXCR2 receptors can also bind CXCR1.

vCXCL-1s stimulate calcium release in human PBN

Release of intracellular calcium is a common indicator of chemokine activation of PBNs [143, 144]. vCXCL-1s were added to 2 ml of freshly isolated human PBN to a final concentration of 100 nM. All vCXCL-1s showed similar activation potential (Fig. 11) demonstrating that recombinant vCXCL-1s are capable of inducing cellular activation of human PBNs and any differences in



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Chemokine	50% effective concentration (EC50) to	
	CXCR2	CXCR1
CXCL8	1.1	3.9
C952	3.5	> 97.7
E760	3.8	> 143.3
Toledo	5.1	> 46.7
TX11	> 8.3	> 1000
100751	> 11.9	> 1000
C956	>12.8	> 1000
TX24	> 25.2	> 1000
Towne	> 29.0	> 1000
102410	> 30.1	> 1000
C954	> 82.2	> 1000
TX15	>131.4	> 1000

Figure 10. vCXCL-1s display variable affinities for CXCR1 and CXCR2.

(A) vCXCL-1s bind to CXCR2 with varying affinities. Representative vCXCL-1s are shown. HEK293 cells stably expressing only CXCR2 were incubated with a constant amount (0.2nM) of [^{125}I]-CXCL8 and increasing amounts of unlabeled chemokines as cold competitor. (B) Three vCXCL-1s bound to CXCR1 with various binding affinities whereas other vCXCL-1s did not bind (>1000). (C) The table summarizes the calculated EC50 values of all vCXCL-1s binding to CXCR1 and CXCR2 (nanomolar (nM)). vCXCL-1s that do not displace greater then 90% of total counts are indicated with a > sign; therefore we are unable to calculate accurate EC_{50} values with confidence. The data was analyzed in Prism 4.0. This is combined data of three experiments.

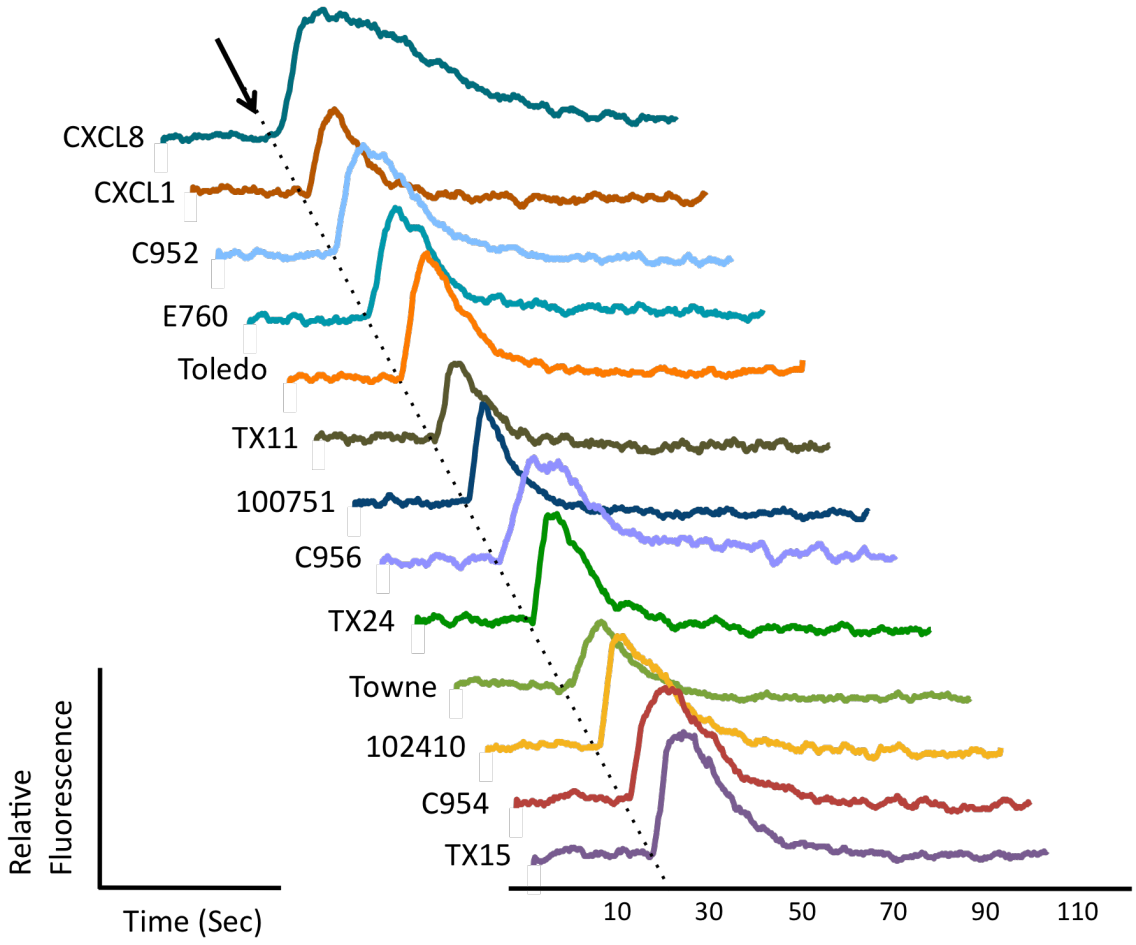


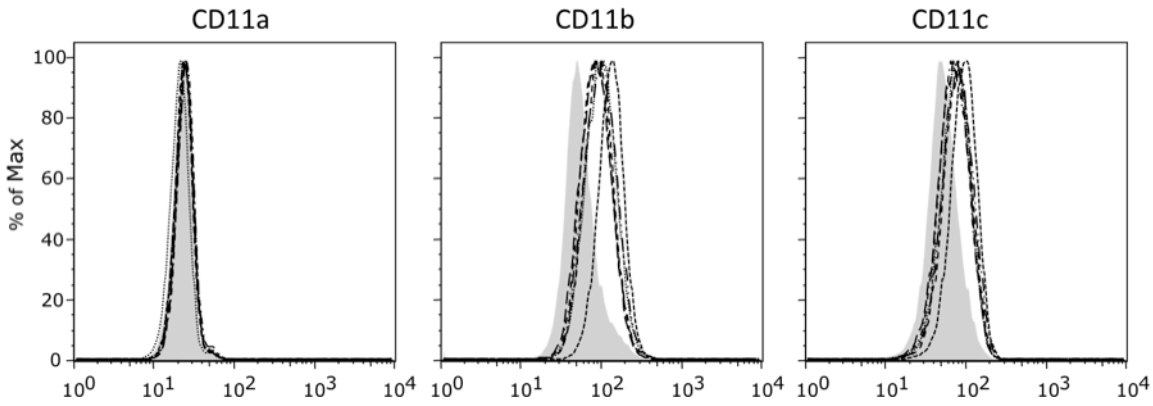
Figure 11. vCXCL-1s, regardless of affinity, mobilize intracellular calcium.

Recombinant vCXCL-1s have similar activation thresholds as host chemokines. Changes in fluorescence were measured over time after exposure of human PBN to 100nM of vCXCL-1s. Arrows indicate the time of addition of the chemokine. Data is representative of three experiments.

CXCR2 affinities do not affect calcium mobilization. Interestingly, 500 nM of the non-ELR CXC chemokine, vCXCL-1_{TX15}, did not show calcium mobilization in the presence of 1 uM the CXCR2-specific inhibitor SB225002 while reduced activation was observed when 100 nM CXCL8 was treated in the presence of the inhibitor. This data indicates that even though vCXCL-1_{TX15} has a non-ELR CXC motif, it still activates PBNs exclusively through CXCR2 (data not shown).

vCXCL-1s upregulate CD11b and CD11c on the surface of PBN

β2 integrins consist of an α component, such as CD11a, CD11b, and CD11c, and a β component, CD18. They are present on circulating leukocytes and mediate leukocyte adhesion and transmigration across the endothelium [265]. Previous studies demonstrated the ability of CXCL8 to upregulate CD11b expression [266]. Moreover, vCXCL-1_{Tol} and a viral chemokine from chimpanzee CMV (vCXCL-1_{CCMV}) also increased CD11b and CD11c expression on the cell surface of PBNs [144]. We tested the ability of vCXCL-1s stimulation to alter surface expression of these receptors on PBNs. Exposure of human PBNs to vCXCL-1s resulted in no change in the level of CD11a expressed on the cell surface (Fig. 12). However, CD11b and CD11c levels increased upon exposure to vCXCL-1s. The percent changes in the mean fluorescent intensity of CD11b were 57-91.2 %, which were similar to CXCL1 (82.4 %) but less than CXCL8 (142.6 %). Likewise, the percent changes of CD11c were 34.9-55.3 %, which were similar to CXCL1 (42.8 %) but still less than CXCL8 (80.3 %). These results imply that the viral chemokines selectively induce adhesion molecule



Chemokines	percent change in the mean fluorescent intensity		
	CD11a	CD11b	CD11c
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

Figure 12. vCXCL-1s differentially increase β2 integrin expression on the surface of neutrophils.

(A) Human PBN were incubated in the presence of 100 nM of different vCXCL-1s and labeled with fluorescently-conjugated antibodies against CD11a, CD11b, and CD11c. Representative vCXCL-1s are shown. The shaded curve represents expression levels of unstimulated neutrophils. (B) The table summarizes the flow cytometry data and is expressed as percent change in the mean fluorescent intensity compared to non-stimulated PBNs. This is representative experiment of three experiments.

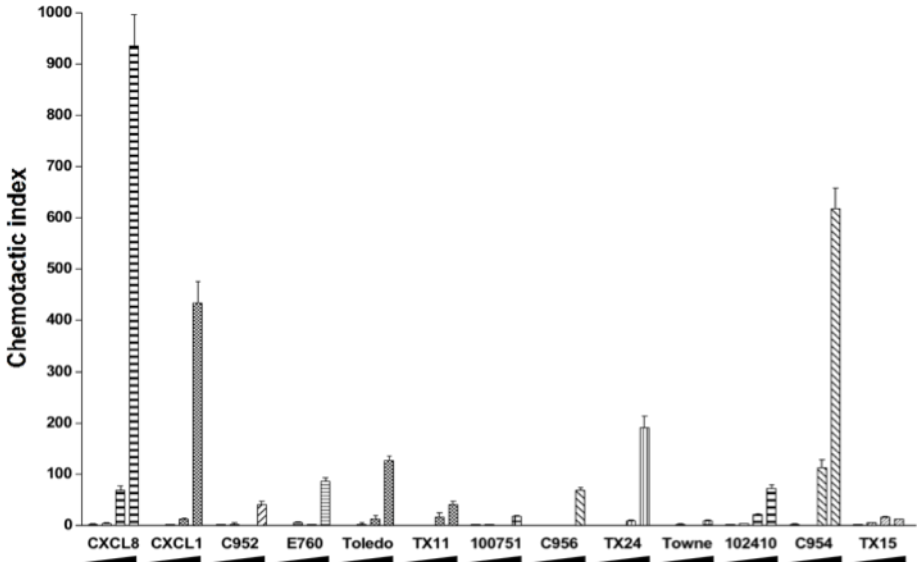
upregulation, but there were no significant differences in expression levels between different viral chemokines.

vCXCL-1s induce differential migration of human PBN

CXCL8 and vCXCL-1_{Tol} are potent chemoattractants of neutrophils [102, 143]. The ability of the HCMVs to alter PBN migration could have a profound effect on HCMV dissemination and subsequent pathogenesis. In order to address whether vCXCL-1 variation results in chemotaxis of PBNs, each chemokine was used as a chemoattractant in a modified Boyden chamber assay. vCXCL-1s showed different potencies in induction of neutrophil migration (Fig. 13). We categorized chemokines as low, intermediate and high responders based on a ten-fold increase above random migration. vCXCL-1_{C954} showed the highest chemotactic index among vCXCL-1s while the non-ELR CXC chemokine, vCXCL-1_{TX15}, was a low responder. Interestingly, the differences in potency of neutrophil migration did not correlate with the differences in affinity for CXCR2 or CXCR1 binding (Fig. 10).

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

In order to examine secondary effects of vCXCL-1 exposure, the neutrophil-like cell line, HL60T2, was stimulated with vCXCL-1_{Tol}, host human chemokine CXCL1, or PBS control and the change in transcription levels were measured



Response category	Chemokine	Means of 500nM treatment
No response (less than 1±0.236)		
Low response (2~20x)	Towne	8.16
	TX15	15.09
	27716	16.16
Intermediate response (20~200x)	C952	39.39
	TX11	39.92
	C956	67.86
	102410	71.54
	E760	84.85
	Toledo	125.75
	TX24	189.62
High response (>200)	CXCL1	433.57
	C954	617.72
	CXCL8	934.73

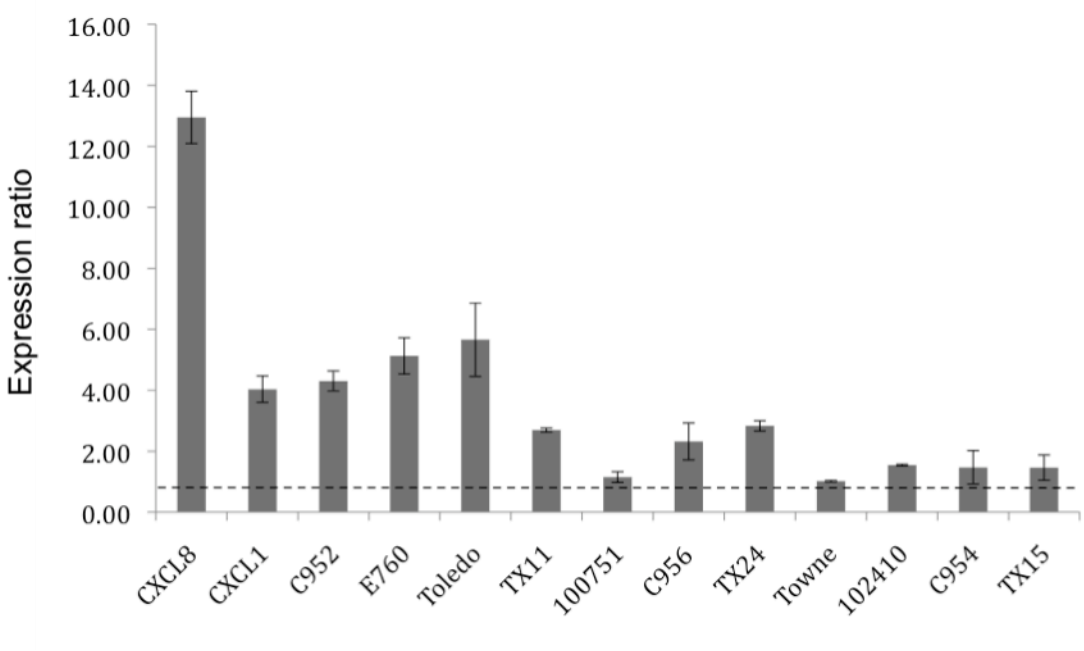
Figure 13. vCXCL-1s induce variable chemotaxis patterns of human neutrophils.

Chemotaxis assays were performed by loading neutrophils into the upper chamber of a 96-well plate fitted with a 5 μm filter with increasing amounts of chemokine loaded in the lower chamber (1, 10, 100, and 500nM). Results are reported as a chemotactic index of the number of cells migrated divided by the random migration ±S.D. Low responders are 20 fold greater than background. Intermediate responders are 20-200 fold greater. High responders are more than 200 fold greater. Data is representative from 10 experiments.

using Affymetrix whole human genome array. One of the genes that was up regulated upon exposure to either host or viral chemokines was CCL22 (macrophage derived chemokine (MDC) (data not shown)). CCL22 recruits multiple immune cells, such as monocytes, dendritic cells, NK cells, and the Th2 subset of T cells [267]. Because HCMV can productively infect macrophages and dendritic cells and an increase in Th2 responses will down-regulate Th1 responses, inducing CCL22 could increase HCMV dissemination while diminishing CTL responses [118, 119]. We measured mRNA expression levels of CCL22 upon exposure to the different vCXCL-1s (Fig. 14). We found that vCXCL-1s differentially induce CCL22 production *in vitro*. For example, vCXCL-1_{Tol} showed the highest induction of CCL22 similar to or slightly higher than host CXCL1, whereas the strains of Towne, TX15, 100751, 102410, and C954 did not induce CCL22 expression. Interestingly, CCL22 induction correlated with affinity, unlike calcium flux or integrin expression (Fig. 10). vCXCL-1_{C952}, vCXCL-1_{E760}, and vCXCL-1_{Tol} showed high affinity for CXCR2 and the highest CCL22 production. These patterns, however, do not correlate with their chemotaxis profiles. This shows the complexity of signaling for the different phenotypic readouts following chemokine exposure and how these chemokines can functionally select different pathways.

Discussion

In order to investigate the relationship between the hypervariable vCXCL-1s and clinical outcomes, we previously cloned and analyzed *UL146* ORF from



Chemokine	Mean	STDEV
CXCL8	12.96	0.86
CXCL1	4.04	0.44
C952	4.31	0.33
E760	5.13	0.59
Toledo	5.66	1.20
TX11	2.69	0.06
100751	1.16	0.18
C956	2.32	0.60
TX24	2.83	0.17
Towne	1.02	0.03
102410	1.55	0.03
C954	1.47	0.55
TX15	1.47	0.41

Figure 14. Secondary chemokine production (CCL22) various upon vCXCL-1 stimulation.

Neutrophil-like HL-60 T2 cells over-expressing CXCR2 were incubated in the presence of 100 nM of different vCXCL-1s. Total RNA was extracted and reverse transcribed for real time RT-PCR. A ratio of 1, indicated with a dashed line, means no change in expression compared to the negative control with no chemokine treatment. This is representative data from three experiments using separately stimulated HL-60 T2s each time.

congenitally CMV infected children [160]. We did not find a statistical correlation between a specific *UL146* genotype and congenital infection outcome, which perhaps was due to the limited sample size. However there were specific clades that showed a tendency toward asymptomatic outcomes. This led us to the current investigation of chemokine function to address whether specific vCXCL-1 clades have distinct functions or at least different levels of activation. Alignment of the mature vCXCL-1s showed the highly conserved ELR motif and placement of the conserved cysteines, whereas the N-loop region was hypervariable with very little homology (Fig. 9). Structure-function studies on chemokine and chemokine receptor interactions have suggested a two-site binding model [101, 268, 269]. This model introduces two interaction sites: the N-loop of the chemokine to the N-terminus of receptor (Site-I) and the N-terminus including the ELR motif to the extracellular/transmembrane part of the receptor (Site-II). Site-I binding serves as an initial docking step, and then Site-II interaction induces conformational changes in receptor transmembrane helices that allow signaling of cellular function [270]. We have shown that two additional viral chemokines (vCXCL-1_{C952} and vCXCL-1_{E760}) bind to CXCR1 and this correlated with high affinity binding to CXCR2. The evolutionary advantage of binding to CXCR1 could be an induction of neutrophilic responses, such as increased chemotaxis, cytokine production or ”functionally selective” cellular responses.

There are two nonexclusive models for the role of viral chemokines in HCMV dissemination. One model is the neutrophil shuttle model. This model proposes that the neutrophil is the main vehicle for HCMV dissemination [271]. It

has been reported that HCMV abortively infects neutrophils during neutrophil transendothelial migration across infected endothelium and subsequently transmits HCMV to fibroblasts [252, 272]. This would allow HCMV to infect the surrounding tissues or different cell types. The guinea pig CMV (GPCMV) MIP homolog functions through the CCR1 receptor and appears to play a role in viral dissemination *in vivo* [149, 273]. Furthermore, viral-related inflammation also likely contributes to cytomegalovirus-related inner ear injury (auditory pathology) [274]. We analyzed the cell activation level and induction of adhesion molecules upon vCXCL-1 treatment, which could affect neutrophil diapedesis from the vasculature and subsequent cell-mediated viral dissemination. In this study, although the binding affinities to CXCR2 and/or CXCR1 were variable, all vCXCL-1s were capable of inducing intracellular calcium mobilization in PBNs (Fig. 11) and upregulating β2 integrins on the surface of PBNs to similar levels to human CXCL1 (Fig. 12). Chemotaxis of circulating neutrophils is dependent on adhesion to and extravasation across the endothelium. Therefore, we conclude that vCXCL-1s increase PBN contact with the endothelium and could then use PBN as a vehicle for dissemination. Interestingly, albeit all tested vCXCL-1s showed similar activation patterns in both calcium flux and β2 integrin assays, they have different potencies to recruit human PBN (Fig. 13). This response was independent of receptor binding affinity. For example, vCXCL-1_{C952} has high affinity but low chemotaxis and vCXCL-1_{C954} showed low affinity but high chemotaxis. This leads us to conclude that different receptor binding affinities do not directly correlate with subsequent PBN activation, integrin upregulation or

chemotaxis pattern. This has recently been shown in a mutagenic study of CXCL8 where mutations of the CXC motif diminished affinity while not affecting function [268].

Another model for the relationship between the viral chemokine and neutrophil is the neutrophil amplifier model. The focus of this model is more of the downstream effects of neutrophil activation. This model proposes that vCXCL-1 leads to exocytosis of neutrophilic granules or induces expression of specific cytokines or chemokines that then affect the next wave of the inflammatory response that would then contribute to HCMV dissemination. For example, macrophages and dendritic cells would provide better targets for HCMV because HCMV can productively infect these cells [275-277] while infection of PBNs is abortive [272]. Moreover, HCMV infection promotes monocyte-to-macrophage differentiation as a strategy to promote viral replication [277]. At the same time, secretion of chemokines or cytokines from the infiltrating cells could also alter the immune response. This has been proposed for the Kaposi sarcoma-associated herpes virus (KSHV)-encoded CC chemokine vMIP-III. It is a CCR4 agonist that selectively attracts Th2 cells [278]. Since the host response to viral infection is mainly Th1-mediated [279], the recruitment of Th2 cells and down-regulation of Th1 responses would be beneficial for HCMV [118, 119].

In order to examine the amplifier model, we measured CCL22 levels following vCXCL-1 exposure (Fig. 14). CCL22 is a potent chemoattractant for monocytes, dendritic cells, NK cells, and the Th2 cells [267]. HCMV induction of CCL22 could lead to an influx of cells that could then be used as a vehicle for

dissemination and/or alter the Th status allowing for delayed viral clearance. Analysis of CCL22 transcript levels found that vCXCL-1s can induce CCL22 production (Fig. 14). vCXCL-1_{Tol}, vCXCL-1_{E760}, and vCXCL-1_{C952} showed high induction of CCL22 similar to CXCL1. Interestingly, CCL22 levels paralleled vCXCL-1 affinity for CXCR2 and ability to bind to CXCR1. In the case of secondary chemokine secretion, affinity does correlate with CCL22 production.

Unlike CCL22 induction, calcium flux, integrin expression and chemotaxis patterns did not correspond with chemokine receptor affinity. For example, vCXCL-1_{Tol}, vCXCL-1_{C952}, and vCXCL-1_{E760}, the intermediate responders in chemotaxis, showed high binding affinities to CXCR2 (and the ability to bind to CXCR1) and high levels of CCL22 induction. Conversely, vCXCL-1_{C954}, a high responder in chemotaxis, showed low binding affinity to CXCR2 and low induction of CCL22. vCXCL-1_{TX24} also showed similar results to vCXCL-1_{C954}. Only vCXCL-1_{TX15}, a non-ELR CXC chemokine, showed a correlation between these assays: low binding affinity, low response in chemotaxis, and no change in CCL22 induction. But vCXCL-1_{TX15} also showed similar calcium flux and integrin upregulation patterns as well other vCXCL-1s. It is possible that vCXCL-1_{TX15} has different functions *in vivo* through calcium flux and integrin induction without complete neutrophil activation.

Discrepancy in the results between binding affinity, chemotaxis, and CCL22 induction profiles could be explained through functional selectivity of a receptor [72, 280]. G protein-coupled receptors (GPCR) are allosteric proteins that are capable of adopting different conformations, which can lead to changes

in downstream effector proteins such as the G-proteins or β-arrestins. Different active conformations are stabilized with the different chemokines leading to stimulation of down stream signaling. For example, a high affinity agonist of the 5-HT_{2C} serotonin receptor, a GPCR in same class as the chemokine receptors, bufotenin [281] preferentially activates the phospholipase A2 (PLA2)-dependent arachidonic acid (AA) release. While another high affinity agonist, quipazine [282], favors the phospholipase C (PLC)-mediated inositol phosphate (IP) accumulation pathway [283]. This model of GPCR activation supports that different vCXCL-1s activation of CXCR2 (and CXCR1) promotes conformational changes leading to specific signaling outcomes.

In conclusion, our data suggest that the polymorphisms in the HCMV viral chemokine, vCXCL-1, bind to CXC chemokine receptors with different affinities and elicit various degrees of cellular responses through triggering of diverse downstream signals. This could potentially contribute to HCMV pathogenesis by accelerating viral dissemination and/or manipulating the host immune responses.

Acknowledgments

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Chapter 5

SUMMARY AND CONCLUSIONS

Human cytomegalovirus contains a CXC chemokine homolog gene, *UL146*, encoding vCXCL-1. Previously, vCXCL-1 from the Toledo strain, vCXCL-1_{Tol}, was shown to be a functional chemokine for chemotaxis and intracellular calcium mobilization in human neutrophils with equivalent potency to host chemokines [143]. vCXCL-1_{Tol} can bind both CXCR1 and CXCR2 that are preferentially expressed on neutrophils [284] although it was initially reported to bind only CXCR2 [143]. Again, the functional ability of vCXCL-1_{Tol} to both receptors is similar to that of the host chemokine, CXCL8 (IL-8), even though its affinity for each receptor is not as good as CXCL8 (IL-8). Because HCMV can infect and replicate abortively in PBN, vCXCL-1's recruitment of PBN could be beneficial for HCMV [285]. Gerna et al showed viral transmission from HCMV-infected endothelial cells to PBN *in vitro* after as little as 1 hour of coculture [285]. HCMV transmission was prevented by lack of contact between the two cell populations or by the use of monoclonal antibodies against adhesion molecules CD18 and/or ICAM-1. Therefore, they suggested transient microfusion events as a major viral transfer mechanism. The PBN life cycle is believed a one-way trip that ends with apoptosis and/or necrosis in a tissue [286, 287], we believe PBN can pick up HCMV from endothelial cells through transitory microfusion events and disseminate HCMV through the bloodstream without performing transendothelial migration [252] (Fig. 15).

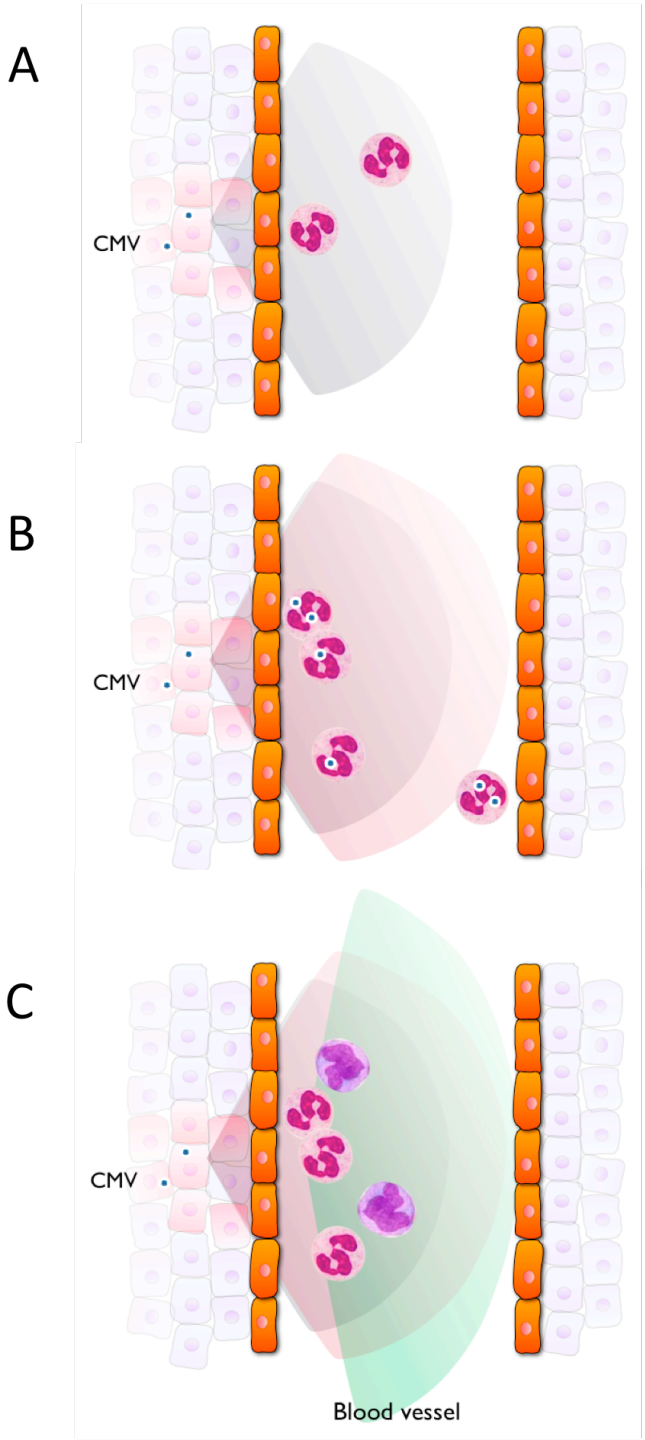

Figure 15. PBN associated viral dissemination models.

(A) PBNs are attracted by host chemokines. (B) PBN shuttle model: vCXCL-1s attract more PBNs. PBNs are used for viral dissemination. (C) PBN amplifier model: vCXCL-1s induce chemokine production from PBN for attraction of other leukocytes such as monocytes, dendritic cells, or Th2 cells.

There are two models for the role of vCXCL-1s in HCMV dissemination (Fig. 15). First, the neutrophil shuttle model proposes that the neutrophil is the main vehicle for HCMV dissemination [271]. The receptors of vCXCL-1s, CXCR1 and/or CXCR2, are expressed to high levels on neutrophils. Therefore, neutrophils can be attracted by vCXCL-1s and can be used a vehicle for HCMV dissemination. In addition to neutrophils, however, CXCR1 and/or CXCR2 have been detected on monocyte/macrophages, eosinophils, basophils, CD8 T cells, mast cells, and dendritic cells [288-295]. HCMV infection in monocytes/macrophages, dendritic cells, CD4 T cells and CD8 T cells were reported [252, 275-277, 296]. Therefore, although functional roles of CXCR1 and CXCR2 in theses cell types *in vivo* have not been fully understood, HCMV could utilize these cells for possible means for dissemination.

The second model for the relationship between the viral chemokine and neutrophil is the neutrophil amplifier model. This model focuses the downstream effects of neutrophil activation. In this model, vCXCL-1 leads to neutrophil degranulation or expression of cytokines and/or chemokines that promote a second inflammatory response that would contribute to HCMV dissemination. Either of these scenarios is possible and our data suggests that perhaps both are feasible.

Interestingly, the vCXCL-1s from different HCMV strains are hypervariable [26, 27, 155-160] and single nucleotide polymorphisms (SNPs) has been found in both CXCR1 and CXCR2 [297, 298]. While the strong association was not observed between identified SNPs in CXCR1 and/or CXCR2 from patients with

various diseases, such as rheumatoid arthritis, Behçet's disease, or Kawasaki disease [297, 299, 300], recently SNPs in CXCR2 can increase their susceptibility to periodontitis [301]. Therefore, their variability with in the CXCR receptor could lead to combinatorial triggering of different signal transduction pathways that could be related to clinical manifestations presented in the patients. Although the hypothesis of this research is that polymorphisms in the HCMV chemokine, vCXCL-1, elicit different functions or different levels of cellular activation that result in determinant factors of HCMV pathogenesis, the given SNP in the receptors could also play role in pathogenesis.

To address this, we initially compared the amino acid sequences of vCXCL-1s from 51 clinical isolates from 50 congenitally infected children [160]. 18 isolates displayed clinical manifestations such as low birth weight, microcephaly, hepatosplenomegaly, jaundice, petechiae, or chorioretinitis. The mature forms without the signal sequence contained between 93 and 98 residues with 5% identity (6 residues) (Fig. 2). This includes the four cysteine residues that provide the essential structure for CXC chemokines [107]. All sequences were assigned to 11 distinct vCXCL-1 clades (Fig. 3). We found no statistical significance between the polymorphisms of vCXCL-1 sequences and HCMV sequelae ($p=0.37$), although three clades showed a high proportion of asymptomatic patients: group 8 (88%), group 10 (100%), and group 12 (83%). To address whether the vCXCL-1 accumulates mutations under immune selection pressure, we analyzed serial HCMV isolates and found no changes in vCXCL-1 sequences for up to three years (Fig. 8).

Among the differences in alignment, the vCXCL-1_{TX15} replaces the glutamic acid-leucine-arginine (ELR) with the asparagine-glycine-arginine (NGR) motif while the other 10 clades have a conserved ELR motif (Fig. 2). Because the ELR motif is important for receptor binding and activation [237, 238], modification of the ELR motif could have profound effects on chemokine function. Another important receptor binding site, the N-loop region, was also analyzed [36, 106, 107]. The N-loop region resides between the second cysteine and the 3_{10}-helical turn (approximately 8-10 residues). Analysis of N-loop sequences divides the chemokines into the same 11 clades indicating that the N-loop region is mainly responsible for vCXCL-1 variability (Fig. 4). Since hypervariability occurs within binding motifs, such as ELR and N-loop, vCXCL-1s may have different affinities for the chemokine receptors and could trigger different downstream signaling and subsequent functions.

In order to address functional variability of the vCXCL-1s, we generated recombinant vCXCL-1s from each clade and measured binding affinities and performed several functional assays to assess how vCXCL-1 variability affects function. Since the majority of vCXCL-1s contain the conserved ELR motif and vCXCL-1_{Tol} binds to both CXCR1 and CXCR2 [28], we measured binding of vCXCL-1s to these receptors. All vCXCL-1s were able to compete with radio-labeled CXCL8 for binding to CXCR2 with lower affinities compared to CXCL8 (Fig. 10A). Nevertheless, three vCXCL-1s, vCXCL-1_{Tol}, vCXCL-1_{C952}, and vCXCL-1_{E760}, showed a higher affinity compared to others. Interestingly, the non-ELR CXC chemokine, vCXCL-1_{TX15}, also bound to CXCR2 albeit with very

reduced affinity. When we examined vCXCL-1 binding affinity to CXCR1, we found a trend that vCXCL-1s with high affinity to CXCR2 receptors can also bind CXCR1 (Fig. 10B), which is similar to CXCL8 which binds to both CXCR1 and CXCR2.

We measured calcium mobilization to evaluate vCXCL-1s ability to activate neutrophils and induction of adhesion molecules that may contribute to cell-mediated viral dissemination. All vCXCL-1s showed similar activation potentials (Fig. 11) indicating that recombinant vCXCL-1s are capable of inducing PBN activation and receptor affinities do not affect calcium mobilization. vCXCL-1_{TX15} also activated PBNs at equivalent levels compared to other vCXCL-1s, but was inhibited by a CXCR2-specific inhibitor suggesting vCXCL-1_{TX15} activates PBNs exclusively through CXCR2. β_2-integrins are important in the adhesion and extravasation of circulating neutrophils across the endothelium. All vCXCL-1 differentially upregulated CD11b and CD11c at equivalent levels to host chemokine CXCL1, although any distinct differences in activation potentials among the vCXCL-1s were not detected (Fig. 12).

Based on the binding information of CXCR1 and CXCR2, we analyzed chemotactic functions of vCXCL-1s on human PBNs. Because CXCL8 (IL-8) and vCXCL-1_{Tol} are potent chemoattractants of neutrophils [102, 143] and there were little differences in PBN activation between vCXCL-1s (Fig. 11), it was surprising when the vCXCL-1s showed different potencies in the induction of neutrophil migration (Fig. 13). Interestingly, the differences in neutrophil migration potency did not correlate with the differences in affinity for CXCR2 or CXCR1

(Fig. 10). For example, vCXCL-1_{C954} showed the highest chemotactic index among vCXCL-1s, but had one of the lowest affinities (EC_{50} > 82.2 nM).

Another functional difference was observed in the induction of CCL22 (macrophage-derived chemokine (MDC)) production of neutrophil-like HL60 T2 cells upon vCXCL-1 treatment (Fig. 14). CCL22 (MDC) attracts multiple immune cells, including monocytes, dendritic cells, and the Th2 cells [267]. Although neutrophils are the main target for vCXCL-1s and can be used as a vehicle for dissemination, the life span of a neutrophil is limited: only a few hours to a few days, and they do not multiply. Monocytes and dendritic cells, on the other hand, can survive for months and can multiply. Therefore, the induction of CCL22 (MDC) can help HCMV dissemination. Furthermore, HCMV can productively infect macrophages and dendritic cells while they infect neutrophils only abortively [252, 272, 302]. An increase in Th2 responses also can help HCMV pathogenesis by diminishing CTL responses [118, 119]. From the real-time PCR analysis, we found that vCXCL-1s differentially induce CCL22 (MDC) production *in vitro*. Interestingly, CCL22 (MDC) induction correlated with affinity, unlike chemotaxis analysis (Fig. 10). For example, vCXCL-1_{C952}, vCXCL-1_{E760}, and vCXCL-1_{Tol}, showed high affinity for CXCR2 and the highest CCL22 production.

In conclusion, this study is the first to characterize 11 different HCMV viral chemokines, vCXCL-1s. We showed they are functionally similar to each other and to CXCL1 in intercellular calcium mobilization and adhesion molecule induction. There was no correlation between the neutrophil functional data and the three clades that trended towards asymptomatic outcomes. However, we

also showed they are functionally different to each other in chemotaxis in an affinity independent manner and secondary chemokine production (CCL22) in an affinity dependent manner. There may be other chemokine functions that did not show high activity in chemotaxis and CCL22 (MDC) production because all vCXCL-1s activate PBN at comparable levels of host chemokines. Future work such as the investigation of signal transduction pathways will clarify the polymorphisms of vCXCL-1 at the molecular level and provide a better understanding of vCXCL-1 and its role in HCMV pathogenesis.

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Appendix

Protein production of other viral chemokines

Introduction

GPCMV encodes a unique, MIP-1 CC chemokine homolog, GPCMV-MIP [148]. This gene encodes an 11 kDa protein, which then yields an 8.6 kDa mature polypeptide. Functional assays showed that GPCMV-MIP mobilizes intracellular calcium mobilization and chemotaxis via CCR1 receptor exclusively [149]. The intracochlear inoculation of a recombinant GPCMV lacking the GPCMV-MIP homolog resulted in less hearing loss compared to wild type GPCMV infection [186] indicating GPCMV-MIP has roles in CMV pathogenesis.

The *UL147* ORF in HCMV encodes CXC chemokine homolog, vCXCL-2 [25]. Functional assays of vCXCL-2 are lacking because *UL147* is less variable than *UL146* (i.e. not that many differences between strains) [160] and protein production has not been successful. Recently, it was suggested that vCXCL-2 is not secreted and is able to reduce NK activation by downregulating a NKG2D receptor ligand, ULBP3 (IHW workshop 2010).

Previously, viral chemokine productions of GPCMV-MIP and vCXCL-2 were attempted in our lab but was unsuccesful. GPCMV-MIP ORF was cloned into the baculovirus transfer plasmid pVL1392 and UL147 ORF was cloned into TA vector. This appendix is follow-up study for those viral chemokine productions.

Materials and Methods

Checking the sequence integrity in vectors

Previously, the *UL147* ORF with an additional 6-His tagged sequence was cloned into the baculovirus transfer plasmid pVL1392 (Invitrogen), which contains homologous regions for recombination into the baculovirus genome. To check its integrity, the forward and reverse polyhedrin primers were purchased (Invitrogen) and used for the DNA sequencing. The polyhedrin primer regions are located outside of the multi cloning site (MCS). The GPCMV-MIP ORF with 6-Hig tag was cloned into the TA vector. To check its integrity, SP6 and T7 primer were used for DNA sequencing.

Site-Directed Mutagenesis

To fix a single nucleotide substitution of *UL147* in pVL1392 vector, the site-directed mutagenesis technique was conducted using primers (UL147_Fix_Foward: 5'-GAAGTGCTGGCTATTTTAAAGGACAAGGGAACCAAG -3'; UL147_Fix_Reverse : 5'-CGTTAGGATTGAGACACTTGGTTCCCTTGTC-3'). The PCR condition was 95 °C for 15 min, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 18 min. The reaction was extended at 72 °C for 15 min. DpnI treatment was used to elimnate the original (unmutated) DNA strand. PCR was cleanuped using a QIAquick PCR purification kit (QIAGEN) and transformed into MAX Efficiency DH5α competent cells (Invitrogen). After 16 hr incubation the colonies were picked and miniprepped for sequencing.

Transfer of GPCMV-MIP from TA to pVL1392

To clone out GPCMV-MIP ORF from TA vector and to delete a redundant 5' sequence preceding the start codon, PCR amplification was performed using primers (GP526_Fix_Forward: 5'-GCGCTGCAGATGAGAAAGATGAGAGCTTA TCTTGGCACAG-3'; GP526_Fix_Reverse: 5'-TCGGAATTCTTAGTGGTGGTGG TGGTGGTGTCCTCCGGCCTG-3'). The forward primer has PstI restriction enzyme site and the reverse primer has an EcoRI site to give them the correct orientation in pVL1392. The PCR condition was 95 °C for 15 min, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 5 min. The reaction was extended at 72 °C for 10 min. The PCR-amplified GPCMV-MIP was cloned into the pVL1392 vector (Invitrogen) using PstI and EcoRI double digestion. To check the gene integrity, DNA sequencing was performed using the polyhedrin primers (Invitrogen).

Production of recombinant viral chemokines

For the generation of baculoviruses, SF9 cells were transfected with the 1392/viral chemokine ORF plasmid construct and Sapphire linearized baculovirus DNA (Orbigen) according to the manufacturer's instructions. Recombinant baculovirus containing the viral chemokine gene was titrated in the small scale (2 ml) of Hi5 cells for optimum protein expression. Based on the small scale titration, the large scale (350-400 ml) of Hi5 cells were used for protein production. 48 hrs after infection, supernatants were harvested and

recombinant proteins were isolated from the supernatants using Ni-NTA agarose beads (Qiagen) for 2 hrs incubation in cold room. Harvested Ni-NTA agarose beads were washed 3 times with PBS. Proteins were eluted from the beads using 0.5 M imidazole for 2 times of 10 min incubation at cold room on the rocker. Eluted proteins were concentrated by Amicon Ultra Centrifugal Filter Units (Millipore) with 3 kDa molecular weight cut off.

Result and Discussion

GPCMV-MIP protein production

GPCMV-MIP ORF was cloned into TA vector. DNA sequencing revealed that it has a redundant nucleotide preceding start codon (Fig. 16A). Furthermore, restriction enzyme sites flanking the ORF were EcoRI, which does not provide an orientation upon cloning into pVL1392. Therefore, when we clone out this ORF from the TA vector and put it into pVL1392, we designed primers containing different restriction enzyme sites, PstI and EcoRI. Expected PCR product, a 348 bp band, was observed (Fig. 17A). After the PCR product was extracted from the gel, both pVL1392 vector and PCR product were double digested by PstI and EcoRI (Fig. 17B). To get rid of any non-specific DNA fragment, digested bands

Figure 16. The sequences of GPCMV-MIP and *UL147* ORF from previous work.

The orange color region is the actual ORF sequences. The start and stop codons are boxed. (A) GPCMV-MIP ORF cloned into the TA vector showed a additional 5'-sequences preceding start codon. In addition, all restriction enzyme sites were EcoRI. (B) *UL147* ORF cloned into pVL1392 showed a nonsynonymous substitution.

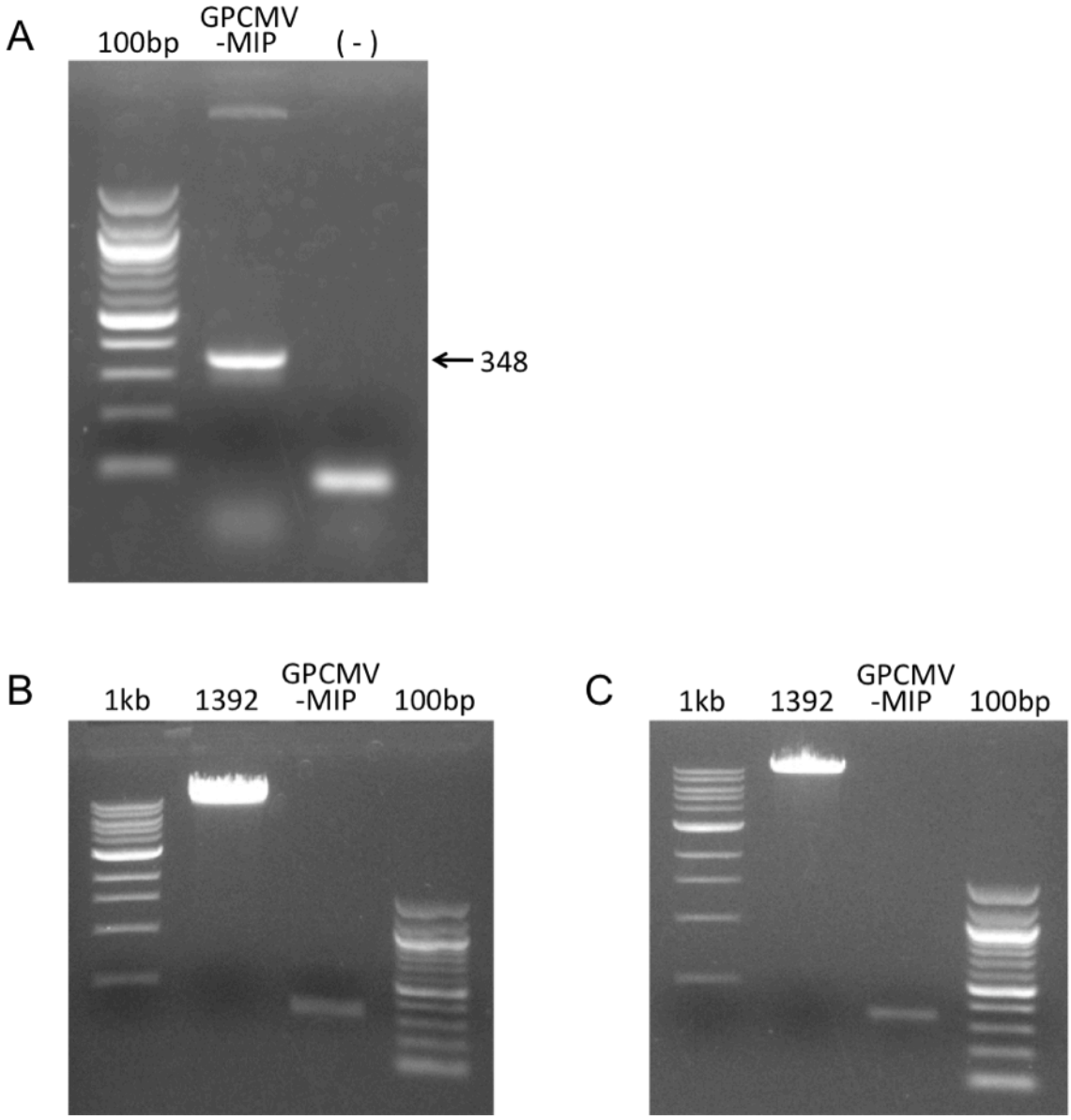

Figure 17. PCR and Cloning of GPCMV-MIP into pVL1392

(A) Primers with PstI or EcoRI enzyme sites produced 348 bp PCR product. (B) 348 bp band was extracted and double digested with PstI or EcoRI. pVL1392 vector also double digested with the same restriction enzymes. (C) To remove non-specific DNA fragments, double digested DNAs were extracted from the gel.

were extracted (Fig. 17C). Then, the PCR product was ligated into pVL1392 vector. After bacterial amplification of 1392/GPCMV-MIP ORF plasmid construct, DNA sequencing confirmed its integrity again (data not shown).

Transfection/infection into SF9 cells was performed to produce GPCMV-MIP ORF-containing baculovirus. When cytopathic effect (CPE) was observed in over 90% of SF9 cells, the supernatant containing baculovirus was harvested. Small scale of Hi5 cells (2 ml) was used to titrate the baculoviral stock for optimum protein expression. 900ul of Hi5 supernatants, 100ul of 10xHBS and 50ul of Ni-NTA agarose beads (Qiagen) were incubated for 2 hrs and the amount of purified protein confirmed on a silver stained SDS PAGE gel. The expected 9.4 kDa protein was detected in supernatants from Hi5 cells infected with 10 ul input of baculoviral stock (Fig. 18A). The following large scale production was also successful. Interestingly, another 16kDa band appeared in large-scale production (Fig. 18C). There is one N-glycosylation site (N79) in GPCMV-MIP, we suspect the 16kDa protein is a glycosylated form of GPCMV-MIP. PNGaseF treatment confirmed it is a glycosylated form as it is deacreased to the 9.4kDa upon treatment (Fig. 19).

vCXCL-2 protein production

UL147 ORF was already cloned into pVL1392 vector. DNA sequencing with polyhedrin primers revealed that it contained a nonsynonymous substitution (A → G), which changes lysine to glutamic acid (Fig. 16B). Therefore, we performed site-directed mutagenesis PCR to fix the substitution. This

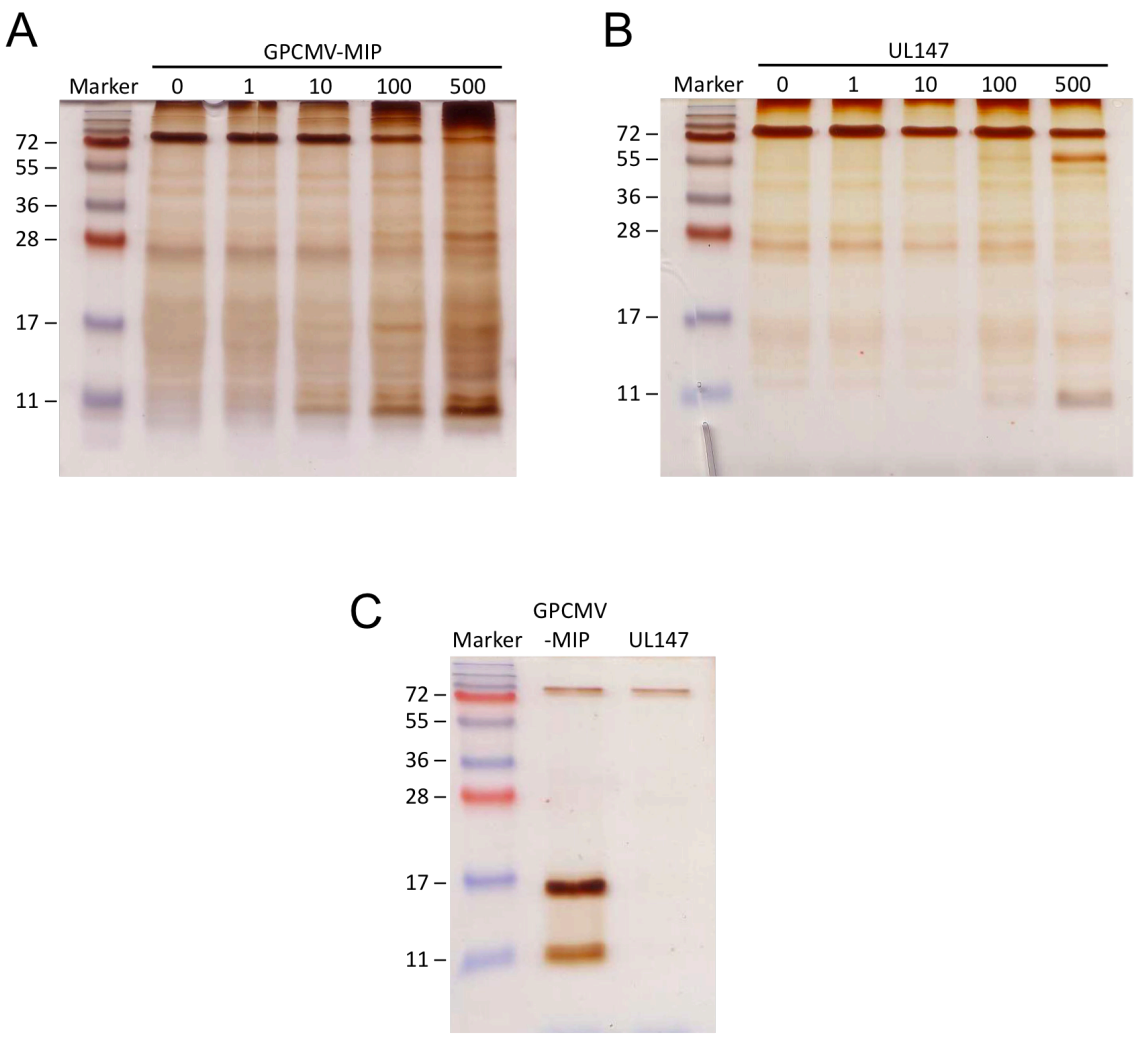

Figure 18. GPCMV-MIP and vCXCL-2 production.

(A) Small scale baculoviral stock titration for GPCMV-MIP production. The expected 9.4 kDa band begins to appear at 10 ul input supernatant. (B) Baculoviral stock titration for vCXCL-2 in small scale. Expected 8.8 kDa band started to appear in 100 ul input supernatant. (C) Large scale protein production. Two distinct bands were observed in GPCMV-MIP while no protein product in vCXCL-2 production.

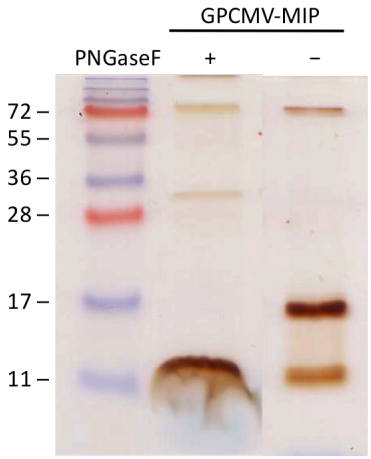

Figure 19. PNGaseF confirms GPCMV-MIP glycosylation.

PNGaseF treatment confirms that a 16 kDa protein is a glycosylated GPCMV-MIP as it is reduced in size upon treatment.

mutagenesis was successfully performed and confirmed by DNA sequencing (data not shown).

Transfection/infection of SF9 cells was performed to produce *UL147* ORF-containing baculovirus. When cytopathic effect (CPE) was observed in over 90% of SF9 cells, the supernatant containing baculovirus was harvested. Small scale infection of Hi5 cells (2 ml) was used to titrate the baculoviral stock for optimum protein expression. The expected 8.8 kDa protein was observed in a 100 ul infection of baculoviral stock (Fig. 18B). Following large-scale production, however, no protein was isolated (Fig. 18C).

Penfold et al showed GPCMV-MIP is a functional CC chemokine as it induces chemotaxis in hCCR1 expressing L1.2 cells [149]. However, multiple minor bands were shown in their SDS-PAGE gel. They speculated those minor bands might be multimers of the 18 kDa species because they did not see any mobility of the proteins with endoglycosylase PNGase F. In contrast, we only had two bands in SDS-PAGE and 16 kDa band was disappeared with PNGase F treatment. Therefore, we believe our GPCMV-MIP has much higher purity, which could generate different results in chemokine function in the same hCCR1 expressing L1.2 cells or guinea pig cells expressing CCR1 such as neutrophils or monocytes.

Interestingly, *UL147* ORF-containing baculovirus expresses protein in a small scale but not in a large scale. To confirm whether the protein in a small scale is actually vCXCL-2, western blotting with an antibody against the 6 His tag could be carried out. If it is right protein, then protein production on a large scale

could be followed. The possible reasons for why we were not able to purify protein on a large scale may be the incorrect volume of viral inoculation or simply non-optimal growth conditions for the cells. If we are able to purify soluble vCXCL-2, we can test it all the functional assays for chemokines, such as binding assay, calcium mobilization, and chemotaxis. Because vCXCL-2 protein production in soluble form has been unsuccessful, it will certainly help to build our knowledge about HCMV pathogenesis as well as being a potential novel chemokine.

Vita

Jinho Heo was born in September 13, 1974 in South Korea. He attended GyeongSang National University in Jinju, South Korea where he received his B.S. in Science in February 2000. After that, he began his master study at the Yonsei University in Seoul, South Korea where he received his M.S. in Biology in February 2003. He started his doctoral program in the University of Tennessee-Knoxville in August 2005 and completed in August 2010. His diploma (Doctor of Philosophy in Microbiology) will be issued in December 2010.