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Chemokines and peptides that promote and inhibit CMV entry

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I am submitting herewith a thesis written by Elisabeth Anne Pitt entitled "Chemokines and peptides that promote and inhibit CMV entry." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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Chemokines and peptides that promote and inhibit CMV entry

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DEDICATION

I dedicate this work to my parents. You have encouraged, supported and celebrated my achievements, big and small.

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ABSTRACT

Human cytomegalovirus (HCMV) causes morbidity and mortality in congenitally infected newborns, transplant recipients, and AIDS patients. Currently, there is no approved CMV vaccine to address these issues. In an effort to develop an alternative treatment to CMV we tested our hypothesis that heparan sulfate binding D-peptides would be effective against multiple HCMV strains *in vitro* and it would be effective *in vivo* against murine CMV (MCMV) (Chapter 1). We show that the D-peptide is able to reduce CMV infection *in vitro* and *in vivo*. Another approach to combating CMV infections is to neutralize pathogenic factors that contribute to CMV spread and/or pathogenesis. HCMV contains genes with homology to human immune modulators. These genes have been implicated in establishing lifelong HCMV latency. One of these factors is an HCMV expressed viral chemokine (vCXCL-1). CXCL-1 binds to chemokine receptors expressed on host immune cells. In order to understand how vCXCL-1 is involved in CMV pathogenesis we used recombinant MCMV overexpressing chimpanzee CMV vCXCL-1 (vCXCL-1_{CCMV}) and murine CXCL-1 (KC). We hypothesized that CMV encodes vCXCL-1 to aid in viral dissemination (Chapter 2). In contrast we found that over expression of CXC chemokines recruits innate immune cells to the site of infection and primary dissemination organs leading to a dissemination blockade. vCXCL-1 is encoded by the HCMV *UL146* gene, which has shown variation among clinical isolates. This variation may attribute contribute to varying clinical outcomes of HCMV infections. We hypothesize that variation in vCXCL-1 leads to differential activation of neutrophils (Chapter 3). Throughout this thesis we have explored how the virus spreads, causes disease and a potential peptide that could be used to prevent the initial CMV infection.

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INTRODUCTION

Chemokines and chemokine receptors

Chemokines are small cytokine-like proteins involved in chemotaxis, positioning and activation of leukocytes. This function is important for immune cell development and the generation of humoral and cellular responses [1]. There are four chemokine classifications based on the terminal cysteine residue spacing: C (γ subfamily), CC (β subfamily), CXC (α subfamily), CX3C (δ subfamily). These chemokine bind to ~20 chemokine receptors which play a role in modulating the immune response by signaling, internalizing or degrading the chemokine ligands [2-4]. Chemokine signaling is mediated through rhodopsin class G-protein coupled receptors (GPCRs) and G-protein independent atypical chemokine receptors [1]. GPCRs are composed of seven-transmembrane helical regions connected by alternating extracellular loops and intracellular loops. Disulfide cysteine pairs connect the N-termini and the seventh α helix. The third α-helix and second extracellular loop are conserved across CXC chemokine receptors, which contributes to their CXC chemokine ligand specificity [5]. Residues at the N-terminus of the chemokine receptors are required for ligand binding and subsequent receptor signaling [6-11]. Truncations at the N-terminus of the receptor disrupt chemokine mediated signaling due to inefficient ligand binding [7, 8, 10-12]. Residue changes or deletions in distal parts of the receptor also alter binding specificity and affinity [13]. Variability within each class of chemokine receptors and ligands contribute to binding specificity and subsequent signaling pathways. These variations will be discussed below.

Within each chemokine classification there are specific ligands that bind to a cognate chemokine receptor on different cells. This specificity facilitates its ability to induce different chemokine-mediated signaling. Within the γ subfamily there are two C chemokines, XCL1 and XCL2, which are also known as lymphotactin α and β [14-17]. Lymphotactin α and β chemokines bind to XCR1 and XCR2, respectively. These chemokines attract neutrophils, T lymphocytes and B lymphocytes in inflammatory diseases such as Crohn's Disease [18].

There are 28 ligands within the CC chemokine subfamily. CC chemokines affect a wide variety of cell types including dendritic cells, monocytes, basophils, and eosinophils [13, 19]. CCL2 (monocyte chemoattractant protein; MCP-1) is a well characterized CC chemokine that

plays a role in monocyte mobilization [20-24]. Epithelial and endothelial cells can produce CCL2, especially during inflammation or tissue damage that occurs during atherosclerosis [25-27]. CCL2 also binds to CCR2 or CCR4 on CD4+ and CD8+ T lymphocytes, which elicits the adaptive immune response after the initial innate immune response [28-30]. Dendritic cells, macrophages and mast cells produce CCL3 and CCL4 (macrophage inflammatory protein 1- α and β; MIP-1 α and β) [31, 32]. CCL3 and CCL4 bind to CCR5 on monocytes, NK cells, and CD8+ T cells [33, 34]. TNF, released during inflammatory processes, induces CCL3 leading to an influx of neutrophils and macrophages to the site of inflammation [35, 36]. CCL5 (RANTES) also binds CCR5 on eosinophils and T lymphocytes [37]. More recent studies suggest CCL5 is associated with progression of breast cancer because it increases inflammation and eosinophilia in the tumor microenvironment [38]. Some CC chemokines like CCL19 and CCL21 are involved in T lymphocyte homing [39]. CCR7, which is upregulated on CD4+ and CD8+ T lymphocytes during inflammation, binds CCL19 and CCL21 to initiate the adaptive immune response [40-42]. The NH_2 terminus of CCR2, CCR3, and CCR5, is important for chemokine recognition of their respective CC chemokine ligand binding [43-47]. Another characteristic of CC chemokine receptors is extensive tyrosine glycosylations that increase CC chemokine affinity [48-54]. Heterodimerization can also affect chemokine receptor signaling. For example, CCR2 can heterodimerize with CCR5 if co-expressed in CD8+ T lymphocytes [55]. In this instance CCR5 ligands, CCL3 and CCL4, can block CCR2 signaling. Likewise, the CCR2 ligand can block signaling through CCR5. This points to the complexity of signaling from chemokine receptors and their ligands.

CXC chemokines fall into two categories, those with the amino acid motif of glutamic acid-leucine-arginine (ELR) and non-ELR [13]. The ELR motif is necessary to attract neutrophils [56]. The ELR CXC chemokines, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8, bind to either CXCR2 alone or both CXCR1 and CXCR2. These receptors are found on neutrophils, dendritic cells, NK cells and γδ T lymphocytes [13, 57-61]. CXCR2 has a high affinity for CXCL1 (Groα) and CXCL8 (IL-8) [62]. The CXCL1 ligand is expressed on the luminal surface of the endothelium and participates in neutrophil rolling and adhesion by binding to CXCR2 on those cells [63]. CXCL8 is important for neutrophil recruitment and sustainment during acute inflammation [64]. Furthermore studies have shown CXCL8

upregulates expression of β_2-integrins on neutrophils, which facilitate migration from the vasculature into inflamed tissues [65-67]. Non-ELR CXC chemokines include CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, and CXCL15. These chemokines are involved in the recruitment of a variety of inflammatory cells. CXCL9, (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC) bind to CXCR3 on monocytes, NK cells, NKT cells and T lymphocytes [68-73]. The expression of CXCR3, along with CCR5, on CD8+ T lymphocytes is a hallmark of memory cell generation [74-76]. It is thought that CXCR3 is used to traffic CD8+ T lymphocytes into infected or inflamed tissues [75]. Often during inflammation or infection IFN-γ produced by CD4+ T lymphocytes and NK cells stimulates CXCL9 and CXCL10 production which exacerbates the inflammatory response [71-73, 77, 78]. CXCL12 binds to CXCR4 on B lymphocytes during proliferation [79] then induces B lymphocyte migration into the secondary lymphoid organs [39]. Recently CXCR1 and CXCR4 were shown to be structurally similar [5, 80]. Both CXCR1 and CXCR4 contain charged residues near the membrane surface that bind to basic residues found in their ligands [43]. They differ from previously characterized GPCRs in that a kink causes a directional change of one of the transmembrane helices. However the CXCR1 receptor is longer than the CXCR4 receptor because of the H8 helix present right before the C terminus. The H8 helix contributes to the extension of CXCR1. This small difference in length could correlate to their binding affinities for their respective chemokines, i.e. CXCL-1 and CXCL-8 versus CXCL12. The NH_2-terminal extracellular loops are important for ligand specificity. Exchange of this region between CXCR1 and CXCR2 changes the ligand specificity between the two [9]. Although there are similarities between chemokine receptors within and among classifications, subtle differences in structure and residues alter ligand binding significantly.

CX3CL1 (fractalkine) is the only ligand in the CX3C subfamily. CX3CL1 is unique in that it contains a mucin-like stalk with a chemokine domain at its extracellular end. The stalk like structure of CX3CL1 allows leukocytes to adhere and travel along the endothelium [81, 82]. The chemokine domain can be cleaved from its stalk and activate monocytes and T lymphocytes [81-83]. CX3CR1 is expressed on patrolling, or anti-inflammatory monocytes, NK cells, and CD8+ T lymphocytes [58, 59, 84-86]. CX3CL1 is highly expressed in atherosclerotic plaques [87, 88] suggesting it causes an influx of monocyte/macrophage populations involved in plaque

formation. CX3CL1 is also associated with inflammation in adipose tissue and functions as an adipokine in obese and diabetic patients [89, 90]. Because CX3CL1 is a potent activator of monocytes it has the potential to induce an inflammatory response contributing to atherosclerotic plaque formation.

Chemokine Signaling

Upon ligand binding, GPCRs undergo a conformational change. These changes trigger G proteins inside the cell to begin the first intracellular signaling events. G proteins are made of hetero trimeric proteins with: α, β, γ, and sometimes δ, subunits [91, 92]. Conformational changes in GPCRs upon chemokine binding results in the exchange of guanine diphosphate (GDP) for guanine triphosphate (GTP) on the α-subunit. The GTP bound α-subunit dissociates from its βγ subunits and remains in an active state until the terminal phosphate is hydrolyzed and the α-subunit returns to its inactivate-GDP bound state [93-96]. The α-subunits are $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$. These subunits are important for transducing the external chemokine signal to the downstream signaling proteins in the cytoplasm. $G_{\alpha s}$ activates adenylate cyclase (AC), which acts on cyclic AMP (cAMP), leading to mitogen activated protein kinase (MAPK) and protein kinase A (PKA) signaling cascades [97]. MAPK activation is important for many immune responses, especially cytokine production. $G_{\alpha i/o}$ antagonizes $G_{\alpha s}$'s function. $G_{\alpha i/o}$ inhibits AC, reducing levels of cAMP, and activating potassium channels causing membrane polarization, which furthers the inflammatory response [98]. $G_{\alpha q/11}$ couples to phospholipase C (PLC) which hydrolyzes phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG) [99]. The cleavage of PIP_2 leads to a calcium flux in the cytoplasm and activation of protein kinase C (PKC). PKC is important for β2 integrin upregulation on neutrophils and eosinophils [100, 101]. Lastly $G_{\alpha 12/13}$ activates Rho guanine nucleotide exchange factors important for GTPase activation [99]. GTPases hydrolyze GTP and shut down signaling. Signaling through specific G_{α} subunits creates signaling patterns often unique to different cell types and specific for chemokine subsets [102]. Although $G_{\beta\gamma}$ subunits regulate different pathways than G_{α} signals, $G_{\beta\gamma}$ pathways ultimately lead to PLC activation [103, 104]. The βγ subunits activate serine/threonine kinase (Akt) through phosphatidylinositol 3- kinase (PI3K). Akt activation induces chemotaxis, cell cycle progression and apoptosis [105,

106]. The multiple G protein subtypes and combinations contribute to refined chemokine signaling responses.

Inhibition of GPCRs can be mediated by the enzyme CD26/dipetidylpeptidase IV (DPP-IV), which truncates the NH_2-termini of several chemokine receptors [107]. Other synthetic antagonists of GPCRs are small molecules that block chemokine-binding pockets on the NH_2-terminus of the GPCR [108]. Currently the chemokine receptor antagonists for CCR1, CCR2, CCR5, and CXCR3 are used to treat retroviral and auto-inflammatory diseases [109]. Other antagonists of GPCRs target GPCR kinases (GRKs) and β-arrestins [110]. GRKs halt GPCR signaling by phosphorylating serine and threonine residues at the C-terminus of the GPCRs. β-arrestins bind the C-terminal phosphorylated serine and threonine residues to induce GPCR conformational changes that no longer permit G protein binding [111]. This antagonist pathway also induces GPCR internalization through endosomal compartmentalization [112, 113]. This mechanism inactivates the GPCRs and recycles the receptor. Interestingly β-arrestins have been shown to act as adaptors in MAPK, SRC, nuclear factor-κB and phosphoinositide 3-kinase signaling [111, 114, 115]. Chemokine signaling can be mediated through many adaptors and pathways in addition to the ones listed above. This variability allows for diverse outcomes upon chemokine binding and subsequent signaling.

Cytomegalovirus encoded cytokine, chemokine and chemokine/cytokine receptor homologs

Human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus with an adult seroprevalence over 90% in many countries [116, 117]. HCMV primary infection is characterized by a systemic and productive viral replication in the human host [118]. HCMV enters latency in myeloid and bone marrow cells of immune competent hosts [117]. During latency viral replication is undetectable. Primary infection or reactivation from latency causes morbidity and mortality in newborns infected *in utero* and immune compromised individuals like organ transplant recipients and AIDS patients [117, 119-123]. The ability of HCMV to survive for the life of the host suggests that the virus encodes proteins that evade host immune responses during all phases of infection [124]. Within the HCMV genome, there are 88 genes that encode proteins that are non-essential for replication *in vitro* [125]. These genes may play a role in replication and persistence within the host (*in vivo*). At least 11 of these 88 genes encode chemokines [126],

cytokines [127, 128], and chemokine receptor [129, 130] homologs, including the UL21.5, US27, US28, UL33, UL78, UL111A, UL111A, UL128, UL144, UL 146, and UL147 genes, which are cytokine, chemokine, or chemokine/cytokine receptor homologs [124]. Virally encoded immune modulating proteins have also been extensively characterized in murine cytomegalovirus (MCMV). These viral proteins have diverse and unique functions compared to their host homologs.

Cytokine homologs in CMV

The HCMV gene *UL111A* encodes a human IL-10 (hIL-10) homolog, cmvIL-10 [127, 128]. During latent infection alternative splicing events result in the expression of another cmvIL-10 known as LAcmvIL-10 [131]. *UL111A* encodes a 175 amino acid protein during primary infection, while latent infection results in the truncated LAcmvIL-10 that is 139 amino acids long. LAcmvIL-10 is identical to cmvIL-10 for the amino terminal 127 amino acids but differs in the last 12 residues [131]. Although both LAcmvIL-10 and cmvIL-10 only share a 27% amino acid identity with hIL-10, they are capable of signaling through the hIL-10 receptor [127, 128, 132]. The use of recombinant proteins or deletion viruses, cmvIL-10 demonstrates inhibition of the production of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 from peripheral blood mononuclear cells and monocytes much like its human counterpart, hIL-10 [133-135]. Similarly hIL-10 and cmvIL-10 share the ability to decrease the expression of major histocompatibility complexes (MHC I and MHC II) on monocytes. Furthermore, cmvIL-10 inhibits the maturation of monocyte-derived dendritic cells (DCs) [136-138] and plasmacytoid DCs [139] and their subsequent effector functions. cmvIL-10 dampens microglial macrophage functions, which aid in the chemotaxis of activated T lymphocytes [140]. In these cases the cmvIL10 functions to dampen the immune responses. Besides immune modulation of myeloid cells, cmvIL10 contributes to the proliferation and differentiation of B lymphocytes [141] and monocytes [142] but not cytotoxic T lymphocytes. CMVs with a *UL111A* deletion does not alter their replication but causes a shift in the differentiation of monocytes to DCs rather than phagocytic macrophages [143]. It is not clear the role LAcmvIL10 plays in this scenario. MCMV shares many homologous genes with HCMV, but MCMV does not encode a direct homolog of hIL-10. MCMV exploits IL-10 during salivary gland infection for its survival *in*

vivo via CD4+ T lymphocytes immune regulation [144]. Perhaps this points to the alteration of pro-inflammatory responses that may allow HCMV to productively replicate during primary infection and persist within the host during latency.

Cytokine receptor homologs in CMV

HCMV *UL144* encodes a type I transmembrane glycoprotein which was originally identified as a homolog of tumor necrosis factor receptor (TNFR) superfamily members [145]. In spite of this homology the *UL144* protein (pUL144) does not function by binding TNF ligands [145, 146]. The pUL144 extracellular domain, binds to B- and T- lymphocyte attenuator (BTLA) [147, 148]. BTLA is an inhibitory receptor found on T lymphocytes that inhibits CD4+ T lymphocyte activation [147]. HCMV may utilize BTLA binding to alter CD4+ T lymphocyte responses *in vivo*. pUL144 also induces NF-κB activation and modulation of host gene expression through interactions with the TNFR associated factor 6 (TRAF6) and tripartite motif 23 protein (TRIM23) [146, 149]. Through this pathway, pUL144 induces expression of CCL22 [146, 150], which attracts regulatory T lymphocytes [151], and could dampen T-lymphocyte detection of CMV *in vivo*.

CXC chemokine homologs in CMV

Within the HCMV genome, there are two ORFs, *UL146* and *UL147* that encode CXC chemokine homologs [152]. *UL146* encodes viral CXCL-1 (vCXCL-1) and *UL147* encodes viral CXCL-2 (vCXCL-2). MCMV does not encode a CXC chemokine homolog but does have a CC homolog [153]. The amino acid makeup of vCXCL-1 varies among different HCMV strains [154]. Sequencing studies show that *UL146* is one of the most variable genes in the HCMV genome [155-163]. This variability could contribute to CMV pathogenesis [158, 160, 164]. For instance, the attenuated lab adapted strain, AD169, is missing these ORFs within the 15kb UL/b' region, but the virulent Toledo strain still maintains this region [126]. The absence of *UL146* and *UL147* from AD169 does not affect viral replication *in vitro*, thus the two gene products are hypothesized to play a role in survival *in vivo* [126, 155, 156]. Deletion of *UL146* from the Toledo strain limited HCMV's ability to infect fibroblasts and promote neutrophil chemotaxis [152]. Of the two viral CXC chemokine homologs, vCXCL1 has been extensively characterized.

It mediates chemotaxis through binding of CXCR2 and sometimes CXCR1 [152, 165]. Furthermore, vCXCL1 is capable of inducing calcium flux in isolated human neutrophils similar to CXCL8 [152, 166]. Different clinical isolates with variations in *UL146* show different binding affinities for CXCR2 which may contribute to differences in neutrophil activation.

CC chemokine homologs in CMV

HCMV encodes the *UL128* protein (pUL128), which forms part of a pentameric complex with glycoprotein H (gH; UL115), glycoprotein L (gL; UL75), pUL130, and pUL131A. This complex plays an important role in mediating HCMV entry into endothelial, epithelial cells, DCs and leukocytes [409, 423, 424]. Expression of the pentameric complex varies depending upon which cell type the virus was propagated [167-170]. In addition to its role in entry, pUL128 shows limited homology to CC chemokines [419]. pUL128 has two cysteines near the N-terminus, which is characteristic of the C-C motif found in human C-C chemokines [171]. This putative chemokine promotes chemotaxis of PBMCs similar to the human-encoded CCL3 (MIP-1α) [172]. pUL128 induces expression of the pro-inflammatory cytokines, TNF-α and IL-6, which may aid in the recruitment of cells important for dissemination or clearance of the virus [173]. The *UL128* gene does not encode a functional protein in clinical strains (Toledo and Merlin) due to a frameshift and partial inversion mutation [171]. This mutation likely contributes to survival of these strains *in vivo* by evading the host immune response because pUL128 induces expression of pro-inflammatory cytokines that may dampen viral infection. MCMV encodes a CC chemokine, which is expressed as a result of splicing of *m131* and *m129* genes in MCMV to produce MCK2 [174, 175]. Deletion of MCK2 results in defective viral dissemination to the salivary gland and less inflammation at the site of inoculation [176, 177]. MCK2 recruits myeloid progenitor cells [178, 179] and monocytes [180], which are involved in viral dissemination to the salivary gland. Therefore, it appears that MCK2 is involved in the recruitment of cells important for dissemination and these cell types can serve as a reservoir for latent infection [181-184]. Recent studies have shown that MCK-2 and gH/gL form a complex that promotes MCMV infection of monocytes and macrophages *in vitro* and *in vivo* [185] suggesting that MCK2 and HCMV pUL128 may function by promoting entry into specific cell types that determine CMV tropism.

Chemokine receptor homologs in CMV

There are four genes encoding chemokine receptor homologs in the HCMV genome: *US27, US28, UL33,* and *UL78* [129, 130]. Of these chemokine receptor homologs US28 is the most extensively characterized. The US28 protein (pUS28) is a potential oncogene and an immune modulator during HCMV infection [186-188]. pUS28 shows 30% amino acid identity with CCR1with some similarity to both CCR2 and CCR5 [189]. pUS28 is the only viral chemokine receptor that binds both CC and CX3C chemokines [189-191]. Infected cells expressing pUS28 signal through this virally encoded chemokine receptor to induce cellular responses similar to host CC chemokine receptors [189, 192-195]. Ligand binding to pUS28 induces cellular responses including MAPK activation, calcium flux, cellular migration and activation of transcription factors like the cAMP responsive element binding protein (CREB) and NF-κB [192-196]. In an independent, constitutive signaling pathway, pUS28 induces phospholipase C activation which leads to the production of secondary messengers, IP3, DAG and NF-κB activation [197-200]. Through these signaling pathways, it is proposed HCMV induces smooth muscle cell migration which accelerates atherosclerosis [193, 201]. pUS28 has been suggested to be an immunoevasin protein based on its ability to bind and internalize chemokines, which limits their chemotactic effects on surrounding cells [192, 202]. Furthermore, pUS28 inhibits CCL2 and CCL5 mediated chemotaxis of monocytes [204]. Cells infected with HCMV expressing pUS28 can bind a variety of host chemokines including CCL2, CCL7, CCL4 and CCL5 leading to a reduction in the host immune response in virally infected tissues [203]. HCMV has been implicated in human cancer, especially glioblastomas [205-208]. Whether this is causation or coincidence remains to be tested. In support of pUS28 being involved in transformation, NIH-3T3 murine fibroblasts expressing pUS28 experience increased growth, enhanced cell cycle progression, and vascular endothelial growth factor (VEGF) production [206]. Injection of these cells into nude mice resulted in rapid tumor formation [206, 207]. Transgenic mice overexpressing pUS28 leads to intestinal neoplasia that was linked to the dysregulation of cellular proliferation genes like survivin and cyclin-D1 and co-expression of CCL2 [209]. The increased proliferation in cells expressing pUS28 is related to NF-κB mediated upregulation of STAT/IL-6 [208]. MCMV encodes a chemokine receptor homolog, M33. When M33 is deleted, it limits dissemination, salivary gland replication and is associated

with reduced reactivation from spleen and lung explants [210, 211]. Studies that replace MCMV M33 with HCMV US28 showed partial complementation emphasizing the similar functions that these proteins have in their respective viruses and hosts [210].

US27 is expressed as an HCMV late gene. Currently, *US27* has not been shown to have constitutive signaling activity, nor does it bind any ligand [212]. The *US27* protein, pUS27, can be detected intracellularly in multivesicular bodies and also on the cell surface. Mutant viruses that do not encode *US27* have impaired extracellular spread in endothelial cells and fibroblasts [213]. However, *US27* deletion does not affect cell to cell spread. Although pUS27 does not trigger constitutive signaling, its heterodimerization with pUS28 allows the pair to constitutively signal [214].

The HCMV encoded UL33 gene is conserved across all CMV species including MCMV (M33). The UL33 and M33 proteins show constitutive signaling [199, 200, 215, 216], but they do not play a role in viral replication *in vitro* or viral dissemination *in vivo* [217]. pUL33 can form a complex with pUS28, but it does not alter pUS28's ability to signal through phospholipase C. It does, however, alter NF-κB induction [214]. Therefore, pUL33 may also play an immune modulatory role along with pUL128.

Like *UL33*, *UL78* is conserved among all β-herpesviruses, suggesting that both play a role *in vivo* because they are not required for replication *in vitro* [218]. pUL78 like the other previously described receptor homologs can complex with pUS28 [214, 219]. This complex formation does not alter pUS28 signaling. Furthermore, UL33 and UL78 can heterodimerize with host CCR5 and CXCR4 in THP-1 cells [220]. Because pUL33 and pUL78 can dimerize with CCR5 and CXCR4, these viral proteins can alter responses to their ligands, CCL5 and CXCL12 [220]. It appears that virally encoded chemokine receptors may skew the immune response to HCMV infection *in vivo* as well as promote viral pathogenesis.

The UL21.5 gene encodes the protein, pUL21.5, which is a secreted glycoprotein that is not vital for HCMV growth in fibroblasts [221]. pUL21.5 can bind CCL5 but not CCL2, CCL3 or CXCL-8, suggesting that the role of pUL21.5 is to sequester CCL5 [189, 205]. The mRNA for pUL21.5 is incorporated into the HCMV virion presumably so that it can be translated immediately upon virus entry [222]. Viruses without this gene may not fare well *in vivo* where

infection of cell types other than fibroblasts is required for productive and continued replication. Without sequestering CCL5 CMV would not be able to evade the host immune response.

CMV entry

All herpesviruses encode the glycoprotein complex consisting of glycoproteins H and L (gH/gL) and B (gB), which are essential for herpesvirus entry into host cells [223-225]. HCMV glycoproteins, gH/gL will not fuse with host cell membranes unless gB is present [226, 227]. Therefore HCMV virions that lack gB are able to assemble but not enter host cells [228]. Coincidentally gL deletions also inhibit viral entry [229]. The gH/gL complex can recognize and bind receptors for entry into host cells independently or by forming a complex with other glycoproteins [225].

CMV glycoprotein B

Together gB and glycoproteins M and N (gM/gN) mediate the initial adsorption of HCMV onto heparan sulfate glycosaminoglycans (GAGs) [230]. Cell-cell fusion is mediated through gB and gH/gL in epithelial, endothelial cells, and fibroblasts [170, 231]. This fusion event occurs only if both gB and gH/gL are present. While glycoprotein O (gO) and pUL128-131 form complexes with gH/gL, their expression does not increase fusion efficacy in any cell type [170]. A relatively small amount of gH/gL reach the membrane without forming a complex with gO or pUL128-131. This suggests that gB and gH/gL are collectively involved in cell-cell fusion events. Recent studies have indicated that gB acts downstream of gH/gL binding [231]. This demonstrated by trans- expression of HCMV gB on epithelial cells, which can initiate fusion events with other cells expressing gH/gL [232]. MCMV encodes a glycoprotein B that is functionally homologous to HCMV gB [233].

CMV gH/gO/gL trimeric complex

HCMV entry into fibroblasts is predominantly mediated by gH/gL assembled with glycoprotein O (gO) through a disulfide linkage [170, 234]. Extracellular CMV uses the trimeric gH/gL/gO complex to enter fibroblasts by fusion at the plasma membrane [235]. Previous studies have indicated that in the clinical strain ‘TR’, gH/gL does not form a complex with gO at the viral

envelope [236]. Instead gO is responsible for chaperoning gH/gL from the rough endoplasmic reticulum to the Golgi apparatus and trans Golgi network [232, 237]. While the lab adapted strain AD169 trafficked gO to and from the trans Golgi network to be incorporated in the viral envelope with gH/gL [238]. The differences in the function of gO between these two strains indicates that the lab adapted strain is more suitable for fibroblast entry while the clinical strains may preferentially use a different complex. An HCMV gO-null mutant is highly impaired in entry into fibroblasts, endothelial, and epithelial cells [232, 239-242]. Furthermore the TR gO-null mutant was unable to enter those cells even when normal virion particles were produced [232]. Previous studies have shown that gO-null mutants are not impaired in cell-associated focal virus spread in cell culture [243]. This indicates that the gH/gL/gO complex is important for extracellular CMV fusion into these different cells but not cell-to-cell spread. The gH/gL/gO complex of MCMV is functionally homologous to the HCMV gH/gL/gO complex *in vitro* [244]. Although the CMV gH/gL/gO complex is important for initial entry and infection of salivary gland tissue, it is not vital for cell-to-cell spread [245]. This cell to cell spread is important for infection *in vivo*.

CMV gH/gL/pUL128, 130, 131A pentameric complex

The gH/gL/pUL128, 130, 131A pentameric complex was first discovered in strains AD169 and Towne, which had been passaged several times in fibroblasts and lost the ability to infect epithelial and endothelial cells [126]. Subsequent sequencing of AD169 and Towne revealed that 22 genes within the HCMV genome were mutated. These mutations spanned from *UL128* to *UL151*. By repairing mutations only in the *UL128*, *UL130* and *UL131A* genes, infection of epithelial and endothelial cells was restored in the lab propagated strains [168, 246]. Subsequent characterization of the gH/gL/pUL128, 130, 131A complex revealed that it is found in the HCMV envelope and mediates infection of endothelial, epithelial, monocytic and dendritic cells [169, 244, 247-249]. This entry into epithelial and endothelial cells is via pH-dependent endocytosis and cell-to-cell spread [250]. The complex will not traffic to the envelope and serve its function in entry if any of the five proteins are missing [251]. Expression of the HCMV pentameric or trimeric complex (gH/gL/gO), affects its preferred entry mechanisms, which vary depending on the cell types in which they are propagated [167]. For instance endothelial cells

only release virus progeny that express low amounts of the gH/gL/pUL128, 130, 131A pentameric complex. Also progeny propagated in epithelial cells preferentially enter epithelial cells by fusion at the plasma membrane while HCMV propagated in fibroblasts enter epithelial cells through pH-dependent endocytosis [252]. As mentioned previously pUL128 shows limited homology to CC chemokines [171]. Thus far pUL128 has been characterized for both its chemokine and entry capabilities. In a parallel fashion, gH/gL from MCMV forms a complex with the gene products of the m131-129 ORFs [185]. The MCMV m131-129 ORFs encode MCK-2, a C-C chemokine homolog [174, 175]. The gH/gL/MCK2 trimeric complex mediates infection of macrophages *in vitro* and *in vivo* and enhances infection of epithelial cells *in vitro* [185]. These same characteristics are also attributed to the HCMV gH/gL/pUL128, 130, 131A pentameric complex, implying MCK2 functions as an entry protein besides being important for dissemination to the salivary gland [176, 177]. This information suggests that HCMV pUL128 and MCMV MCK-2 plays dual roles as chemokines and as part of a glycoprotein complex used for entry into specific cells.

CMV entry pathways and cellular receptors

Entry of both lab adapted strains (AD169) and clinical isolates (TR) into fibroblasts is mediated by fusion at the plasma membrane, and not by pH dependent endosomal entry [235, 250]. TR entry into epithelial and endothelial requires endosomes and the gH/gL/pUL128, 130, 131A pentameric complex [250]. This mechanism of entry is dependent upon the low pH in acidified endosomes. There are several cellular receptors that are involved in mediating HCMV entry: heparan sulfate proteoglycans (HSPGs), α/β-integrins, epidermal growth factor (EGFR), and platelet derived growth factor receptor-α [238]. When HS GAGs are bound to a protein core they are called heparan sulfate proteoglycans (HSPGs) [253-255]. The HS backbone is generated by alternating glucuronic acid (or iduronic acid) and N-acetylglucosamine. The HS chains are modified by adding sulfate groups at various positions [256-259]. HS chains are highly negatively charged and highly complex due to enzymatic modifications [260, 261]. HSPGs bind to cell adhesion molecules, growth factors, chemokines, and factors involved in angiogenesis and blood coagulation [262-265]. HSPGs have also been implicated in herpesviral, malarial and amyloid-related pathogenesis [230, 266-270]. HCMV gB and gM/gN bind to HS

on the cell surface [230]. This is the important first step in a multistep fusion event. CMV uses many receptors in order to facilitate fusion.

EGFR is expressed on fibroblasts and monocytes [243, 271, 272]. Each cell type is capable of HCMV infection. HCMV infection activates EGFR-dependent signaling which is important for initiating infection [271]. However HCMV triggered EGFR activation does not occur in fibroblasts, and blockade of EGFR-cytoplasmic signaling does not inhibit HCMV entry in these cells. These data leave the question open as to whether there is a correlation between EGFR expression and HCVM infection [273], suggesting there are other mechanisms for initiating infection. HCMV gB binds integrins: α2β1, α6β1, and αVβ3 integrins [274] and induces integrin signaling [275]. Peptides that mimic the gB disintigrin binding motif blocks HCMV and MCMV entry, pointing to the importance of integrins in virus entry [274]. Another growth factor receptor, PDGFRα, is induced by HCMV infection of fibroblasts [276]. Inhibition of PDGFRα signaling either by blocking receptor binding or the signaling pathway itself inhibits HCMV immediate early gene expression [276]. These results illustrate that several viral glycoprotein interactions with cell surface receptors are necessary to mediate HCMV entry. Disruption of these interactions hinders CMV entry and could point to potential targets for novel drugs that inhibit CMV entry.

CMV cell-to-cell spread

In addition to the attachment/fusion pathways described above, HCMV also infects cells through cell-to-cell spread. Anti-gB antibodies neutralize initial and extracellular virus entry into all cell types while anti-gH/gL/pUL128, 130, 131A pentameric complex antibodies only prevent entry into epithelial cells [277]. However anti-gB antibodies do not prevent CMV cell-to-cell spread meaning that gB is only involved in initial viral entry [213, 278, 279]. Deletion or modification of the gH/gL/pUL128, 130, 131A pentameric complex inhibits initial entry into epithelial cells and cell-to-cell spread of HCMV in epithelial cells [250]. Furthermore lab adapted strains appear to use both the extracellular fusion mechanisms described above and cell-to-cell spread while clinically isolated HCMV preferentially use cell-to-cell spread [280]. The gH/gL/pUL128, 130,131A appears to mediate cell-to-cell spread in epithelial cells which is useful *in vivo* where CMV encounters diverse cell types including epithelial cells.

Treatment of HCMV

HCMV vaccine development

While several vaccines are currently in human clinical trials [281], there are no currently approved vaccines for HCMV. An HCMV strain, Towne, was tested as a vaccine in renal transplant recipients [282]. Although Towne failed to prevent infection after disease, it reduced the severity of end organ disease (EOD). Subsequently live virus vaccines using a Towne background or an attenuated strain of Toledo have been tested in phase 1 clinical trials [283-285]. A proposed subunit vaccine using gB has reached phase 2 clinical trials [286, 287]. The results from this study show that the gB subunit vaccine protected ~50% of seronegative female patients from primary HCMV infection or reduced viral load in the kidney and liver of infected transplant patients [286, 287]. Another subunit vaccine containing both gB and the tegument protein pp65 reduced CMV reactivation in bone marrow transplant recipients [288, 289] Yet another proposed vaccine contains gB, pp65, and the major immediate-early proteins (MIEPs) [290]. This vaccine has yet to reach human trials. Another approach is the use of antibodies against gB or the HCMV pentameric complex, gH/gL/pUL128-131, to reduce HCMV infection in epithelial and endothelial cells [291, 292]. Recently, CD8+ T lymphocyte CMV peptides composed of multiple ORFs have been identified and are currently being tested [196]. So far over one hundred HCMV ORFs were shown to be immunogenic for CD4(+) and/or CD8(+) T cells. A vaccine was tested in rhesus macaques, which induces broadly neutralizing antibodies induced following overexpression of Rhesus CMV UL128 pentameric complex (RhCMV UL128C) [197]. Rhesus CMV (rhCMV) pUL128 is homologous to HCMV gH/gL/pUL128, 130, 131A pentameric complex. The RhCMV pUL128C is also important for entry into epithelial and endothelial cells. Administration of this complex inhibits entry of RhCMV into epithelial, endothelial and fibroblasts. However when the RhCMV gB is expressed it produces a substantially lower amount of neutralizing antibodies. These data suggest that the immune modulatory proteins like pUL128, can be exploited during vaccine development [197]. The development of vaccines against HCMV has been unsuccessful in part due to HCMV's ability to modulate and evade the host immune system [124, 292]. Superinfection of rhesus CMV *in vivo* requires evasion of CD8+ T lymphocyte immunity by the viral encoded inhibitors of major

histocompatibility complex class I (MHC-I) antigen presentation [293]. RhCMV glycoproteins (US2-11) promote immune evasion of CD8+ T lymphocytes *in vivo*. This process is one that complicates the development of preventive CMV vaccines. One characteristic of CMV is its ability to persist within the host even when faced with a strong immune response. For instance, in immune competent individuals up to 10% of their specific CD4+ and CD8+ T lymphocytes are specific for CMV but cannot prevent HCMV infection [124, 196]. Clearly the effects of the HCMV immune modulatory factors must be circumvented in order to develop a successful vaccine.

HCMV antivirals

Due to the lack of an HCMV vaccine, treatment of HCMV relies on antiviral therapeutics. Current anti-HCMV therapeutics include: ganciclovir (GCV), valganciclovir (oral prodrug of GCV; VGCV), foscarnet (FOS) and cidofavir (CDV) [294]. Current anti-HCMV therapeutics target the viral DNA chain elongation through the use of a nucleoside analogs, which mimic actual nucleotides. The nucleoside analog serves as a poor substrate for chain elongation because it lacks a hydroxyl group for addition of the next nucleotide [295, 296]. HCMV expressed kinase is necessary to activate GCV [295-299]. Therefore nucleoside analogs such as GCV, disrupt viral DNA synthesis. GCV has been used to treat congenital infections and HCMV disease in transplant recipients and AIDS patients [294]. Unfortunately the current anti-HCMV therapeutics, FOS and CDV are nephrotoxic [295]. Furthermore long-term use of anti-HCMV therapeutics has contributed to the evolution of HCMV resistance to these drugs. For organ transplant recipients prophylaxis or preemptive therapy is used for 100 to 200 days posttransplant [300]. This extended anti-HCMV treatment contributes to a 5%-10% chance of developing GCV resistant strains among transplant recipients and AIDS patients [294]. For example, it is estimated that up to 27.5% of circulating HCMVs are GCV resistant (GCV^R) [301, 302]. Mutations in the HCMV *UL54* DNA polymerase and *UL97* phosphotransferase gene confer resistance to GCV [119, 120, 303, 304]. Mutations in the HCMV *UL54* DNA polymerase gene confer resistance to GCV, CDV, and FOS [119, 294]. Development of novel anti-CMV therapeutics needs to address both the toxicity and development of antiviral resistance.

Summary and Research Aims

HCMV increases morbidity and mortality in congenitally infected newborns and immunocompromised individuals. There is an urgent need for a vaccine or effective therapeutic. Faced with the difficulties of vaccine development an effective therapeutic is an approach to minimize CMV pathology. Current HCMV therapeutics target viral DNA replication. Resistance to these antivirals is emerging. Therefore it may be beneficial to target other aspects of the virus life cycle. For instance, if a therapeutic drug inhibited HCMV entry it would alleviate the need for prolonged use of the current antivirals. Furthermore understanding of CMV pathogenesis may aid in developing effective vaccines that can counteract important HCMV encoded immune modulators. CMV encoded chemokines that aid in viral infection, dissemination, and the antiviral immune response. These chemokines are hyper variable which could factor into viral pathogenesis.

We begin to understand the CMV lifecycle and pathogenesis by testing HS binding peptides that affect entry, examine the role of viral chemokines on dissemination by using murine CMV viral recombinants that overexpress CXC chemokines and dissect the function of CXCL-1s using recombinant vCXCL-1 proteins. The following three hypotheses were tested and detailed in this thesis:

1. Heparan sulfate binding D-peptides reduce CMV infection *in vitro* and *in vivo.*
2. vCXCL-1 contributes to CMV viral dissemination.
3. Polymorphisms in vCXCL-1, lead to differences in neutrophil activation that contribute to HCMV pathogenesis.

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CHAPTER I
THE D FORM OF A NOVEL HEPARAN SULFATE BINDING PEPTIDES DECREASES CYTOMEGALOVIRUS INFECTION *IN VIVO* AND *IN VITRO*

A version of this chapter is being submitted for publication.

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The use of "we" in this chapter refers to my coauthors and me. My primary contributions to this paper include (1) researching the topic, (2) performing the experiments presented in each figure, (3) data analysis, and (4) primary author of the manuscript.

Abstract

Human cytomegalovirus (HCMV) infection in utero can lead to congenital sensory neural hearing loss and mental retardation. Reactivation or primary infection can increase the morbidity and mortality in immune suppressed transplant recipients and AIDS patients. The current standard of care for HCMV disease is nucleoside analogs, which can be nephrotoxic. In addition resistance to current treatments is becoming increasingly common. In an effort to develop novel CMV treatments, we tested the effectiveness of the D-form of a novel heparan sulfate binding peptide, $p5R_D$, at reducing infection of ganciclovir (GCV) resistant HCMVs *in vitro* and MCMV *in vivo*. HCMV infection was reduced by greater than 90% when cells were pretreated with $p5R_D$. Because $p5R_D$ acts by a mechanism unrelated to those used by current antivirals, it was effective at reducing GCV resistant HCMVs by 85%. We show that $p5R_D$ is resistant to common proteases and serum inactivation, which likely contributed to its ability to significantly reduced infection of peritoneal exudate cells and viral loads in the spleen and the lungs *in vivo*. The ability of $p5R_D$ to reduce HCMV infectivity *in vitro* including GCV resistant HCMVs and MCMV infection *in vivo* suggests that this peptide could be a novel anti-CMV therapeutic.

Introduction

Human cytomegalovirus (HCMV) infection or reactivation from latency causes severe disease in immune-suppressed transplant recipients, AIDS patients, and newborns [1]. Infection can lead to mononucleosis-like symptoms, interstitial pneumonia, gastroenteritis, retinitis, or

organ transplant rejection [2-6]. *In utero* infection can lead to microcephaly, hepatosplenomegaly and sensorineural hearing loss (SNHL) in newborns [7, 8].

Due to the limited success of HCMV vaccines [9, 10], HCMV treatment consists of antiviral drugs like ganciclovir (GCV), valganciclovir (i.e., an oral prodrug of GCV), cidofovir (CDV), and foscarnet (FOS). These antivirals are nucleoside analogs that target viral DNA synthesis [11]. Not only are FOS and CDV nephrotoxic [12], but long-term use of antivirals has contributed to the evolution of HCMV resistance [13, 14]. One approach to develop novel anti-CMV therapeutics is to target other aspects of the HCMV lifecycle other than viral DNA synthesis.

A potential target for anti-CMV therapeutics is the initial, critical attachment step during viral entry in which HCMV binds to cell surface heparan sulfate (HS) [15, 16]. HS consists of glucosamine and glucuronic acid or iduronic acid moieties that are N-acetylated as well as N- or O-sulfated, which allows for incredible diversity in the structures on different cell types [17-19]. Heparan sulfate proteoglycans (HSPGs) participate in physiological processes including embryonic development, binding of growth factors, chemokine transcytosis, cell adhesion and lipid metabolism [20]. CMVs utilize HSGPs for their initial attachment to the cell and subsequent initiation of infection [15]. Previous studies have shown that HCMV may preferentially bind to 6-O-sulfated or 3-O-sulfated heparan sulfate moieties during viral entry [21, 22]. The distribution of HS on host cells in addition to viral preference for specific subtypes of HS, make this structure a potential anti-CMV therapeutic target.

One approach to limit HS-mediated viral entry is through the use of peptides designed to preferentially bind HSPGs. Peptides offer several benefits as therapeutics. They can be readily synthesized, designed to be highly specific, easily modified to enhance biological activity, and less toxic because they are catabolized into amino acids [23]. However, their susceptibility to proteases and short serum half-life have historically limited the use of peptides as therapeutics [24]. Recently several HS-binding peptides have been tested *in vitro* and *in vivo* for their ability to inhibit herpesvirus infections. The HS reactive peptide, G2, a 10-mer derived from a phage display library, inhibits HSV-1 infection *in vivo* [25]. Additionally, a peptide known to bind to hypersulfated HS, p5+14, was shown to effectively inhibit HS-mediated entry of both murine and human CMV as well as HSV *in vitro* [26]. In this his current study we characterize a related

peptide, p5R, which is 14 amino acids shorter and has a higher propensity to form an α-helix. Furthermore, we hypothesized that D- form of peptide p5R, $p5R_D$, would still inhibit CMV infection *in vitro* and would be more efficacious *in vivo* due to its resistance to proteolytic cleavage. Finally, we tested whether $p5R_D$ inhibited GCV-resistant HCMVs *in vitro* in an effort to show its efficacy against clinical strains of HCMV.

Materials and Methods

Peptide synthesis

Using p5 as a template to design p5R and $p5R_D$ the lysine resides were replaced with arginine residues to yield p5R - GGGYS RAQRA QARQA RQAQR ARGAR Q. Both peptides have a positive net charge of +8 and a predicted alpha helical secondary structure according to ITASSER predictions [27, 28]. Peptides p5R and $p5R_D$ were purchased from Anaspec (Fremont, CA) and purified as previously described [26].

Cells and mice

All cells used in our experiments were low passage-number (less than 22 passages). Human normal lung fibroblasts (MRC-5) were cultured in MEM (Corning, Manassas, VA and HyClone, Logan, UT) supplemented with 10% Fetal Bovine Serum – Premium (FBS; Atlanta Biologicals, Atlanta, GA), and 2mM L-glutamine (HyClone, Logan, UT). Human foreskin fibroblasts (HFF; ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, Logan UT) with 2mM sodium pyruvate (Corning, Manassas, VA and HyClone, Logan, UT) supplemented with 10% FBS, and 2mM L-glutamine. Human aortic endothelial cells were cultured in EGM-2 Bullet Kit (Lonza, Walkersville, MD) supplemented with 6% FBS, and 2mM L-glutamine. Human pigment epithelial cells (ARPE-19) were cultured in DMEM:F12 medium (Corning, Manassas, VA and HyClone, Logan, UT) supplemented with 10% FBS. Mouse embryonic fibroblasts 10.1 (MEF 10.1) were cultured in DMEM (Lonza, Rockland, ME) supplemented with 10% Fetal Clone III serum (FCIII) (Hyclone, Logan, UT), 100U/ml Penicillin/Streptomycin and 2mM L-glutamine. All cells were grown at 37°C and 5.0% CO_2. Male and female BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in the University of Tennessee Laboratory Animal Facility. Eight to 12 week old mice were used in all experiments.

All mice were housed under specific pathogen-free conditions and the experiments were performed under the auspices of University of Tennessee IACUC-approved protocols.

Viruses

HCMV TB40E expressing luciferase under the control of the UL18 promoter was a gift from Drs. Christine O'Connor and Eain Murphy (University of Buffalo and FORGE Life Science, LLC). Virus was cultured on HFF cells and titered using plaque assay or luciferase/luminescent expression assay using a Synergy 2 plate reader (BioTek, Winooski, VT). Based on a standard curve, approximately fifteen plaques are equivalent to 1000 relative light units (RLU).

Recombinant, Wild Type and GCV resistant HCMVs (T3261, T3252, T3265 and T3429) were generated as described [29-32]. These recombinant viruses were a kind gift from Dr. Sunwen Chou (Oregon Health Sciences University). Recombinant viruses were titered using plaque assays and secreted alkaline phosphatase (SEAP) assay[29]. HCMV clinical isolates, CH19 and CH-13 [14], were a kind gift from Dr. Nell S. Lurain (Rush University). All viruses were titered using 0.5% agarose overlay in a plaque assay.

MCMV K181 [33] was cultured *in vitro* in MEF 10.1 cells. The viral titer was determined via plaque assay. All viruses were stored at -80°C until use.

Luciferase assay

Peptide reduction of HCMV TB40E infection was measured using a luciferase assay. Briefly, cells were seeded into 24-well plates. After the cells reached 80-85% confluency, peptide, suspended in 10% FBS/PBS was added. Control treatments included 10% FBS in PBS without peptide. After 30 minutes virus was added to cells at ~35-50 pfu or ~2500-3000 RLU and allowed to incubate at 37° C for 60 minutes. Following virus incubation, the peptide and virus was removed and fresh media was added. Plates were incubated at 37° C in 5% CO_2 for 3 days.

On day 3, medium was removed and cells were washed once with PBS. Cells were lysed using the passive lysis buffer (Promega, Madison, WI). Cell lysate was pelleted and luciferase assay reagent was combined with equal amounts of supernatant into an opaque 96-well microplate. Luminescence was measured as RLU using a Synergy 2 plate reader (BioTek). The RLU values for peptide-untreated and uninfected treatment served as a negative control and were

subtracted as background. Peptide-untreated virally-infected cells served as the positive control and normalized to 100%. Data was analyzed using Prism 5.0 (GraphPad Software, La Jolla, CA). Data was expressed as percent infection (100x (number of RLU after treatment/ RLU in the PBS treated cells)).

Proteolytic stability

Proteolytic stability was measured following incubation with proteases or serum. The peptides were incubated with trypsin (Lonza, Walkersville, MD) or elastase (Sigma, St. Louis, MO) at 100 μg/ml, or FBS, or human serum for 30 or 60 minutes then added to cells 30 minutes prior to infection with virus.

Plaque reduction assay

Peptides were screened for their ability to reduce infection of the BAC-derived recombinant GCV-resistant HCMV and clinically isolated GCV-resistant strains using a plaque reduction assay on MRC-5 and HFF cells. Cells were treated with peptide or PBS for 30 minutes prior to the addition of virus. After one hour of incubation, the mixture was removed and replaced with a 0.5% agarose in complete MEM (MRC-5 cells) or DMEM (HFF cells) medium. The plates were incubated at 37°C and 5.0% CO_2 for 7 to 10 days when plaques began to develop. Plaques were manually counted using a dissection microscope after staining with Coomassie blue. For MCMV *in vivo* experiments, MEF 10.1 cells were infected with serial dilutions of homogenized organs or infected peritoneal exudate cells (PECs). Following a 1 hr. incubation, the media was removed and fresh 2% carboxymethyl cellulose (Sigma Aldrich, St. Louis, MO; CMC) in complete DMEM was added. Cells were incubated at 37°C and 5.0% CO_2 for 5 days then stained with Coomassie stain and plaques counted.

Biodistribution of peptides

To determine the distribution of $p5R_D$ among the relevant organs, mice were injected i.v. in the lateral tail vein, with I^{125} labeled peptides (< 120 μCi, 20 μg of peptide). At 1, 2, 4 or 24 hr. post-injection mice were euthanized with isoflurane inhalation overdose and the spleen, liver, lung, and eight other tissues were harvested and the tissue radioactivity measured as previously

described [34]. The biodistribution of radiolabeled peptide was expressed as percent injected dose per gram of tissue (%ID/g).

Data and statistical analyses

All data were normalized to 100% using the peptide untreated and infected positive control data. The data are the combined from three or more independent experiments with at least three replicates in each experiment. Error bars represent the standard deviation (SD). Statistical significance was calculated using a Student's t test, Man Whitney, or 2 way ANOVA followed by Bonferroni posttests. The IC_{50} values were calculated using a linear regression sigmoidal dose dependent test. All analyses were performed using Prism 5.0 (GraphPad). A p-values less than 0.05 were considered statistically significant. Significant values are labeled as *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, NS=non-significant reduction in infection.

Results

Peptide efficacy *in vitro*

Previously we have shown that the p5-related peptides bind amyloid deposits via interactions with hypersulfated HS and amyloid fibrils and can acts as an inhibitor of herpesvirus entry [26, 34, 35]. The synthetic peptides, p5R and $p5R_D$, are comprised of the heptad repeat [AQRAQAR] in the L and D-forms, respectively. Both have an overall +8 net charge and reduce MCMV infection by >75% (data not shown)[26]. We also showed that the peptides do not affect cell viability (Supplemental Figure 1.S1). Furthermore, the peptides have a similar IC_{50} for blocking infection of human fibroblasts with a luciferase-expressing TB40 HCMV (48.5μM p5R and 60.2μM $p5R_D$). At a concentration >200 μM, both p5R and $p5R_D$ reduce HCMV infection by ≥90% (Figure 1.1A).

HCMV infects a wide range of host cells and its tropism varies depending on the cell types used for propagation [16, 36-42]. To examine the breadth of HCMV peptide inhibition, we tested peptide blockage of infectivity using fibroblasts from another tissue (HFF), endothelial cells (HAEC), and epithelial cells (ARPE-19). Both p5R and $p5R_D$, when added at least 30 minutes prior to infection, reduced HCMV infection by >80% in all cell types (Figure 1.1B). Although similar to each other, both were more efficient at preventing HCMV infection in

fibroblast cells (~10% more effective) than on other cell types. This is in contrast to the previously characterized p5+14, which was most effective on ARPE-19 cells [26]. This implies that these peptides could be binding different HS moieties on different cells. Previous data show that p5R has a higher affinity for heparin, amyloid, and blocks MCMV infection 2x more efficiently than p5 [26, 34, 35]. These data suggest peptides containing arginine in place of lysine may bind to an HS that is important for HCMV entry into different cell types. Our results demonstrate that both p5R and $p5R_D$ have similar IC_{50} values and efficiency to reduce HCMV infection in different cell types *in vitro*.

Proteolytic stability of $p5R_D$

D-form peptides are resistant to many of the body's proteases. This proteolytic resistance would prolong peptide survival within the host and potentially increase its efficacy [24]. To measure the proteolytic stability of $p5R_D$, we incubated both peptides with trypsin and elastase serine proteases. Peptides p5R and $p5R_D$ were pre-treated with the different proteases for 30 or 60 minutes. After which, the peptides were added to cells 30 minutes prior to the addition of virus. Peptide $p5R_D$ retained its ability to limit HCMV infection by ~90%, whereas p5R exhibited only a 20% inhibition after incubation with the serine proteases (Figure 1.2A and B). To mimic a biologically relevant environment, peptides were also incubated in human serum or FBS for 30 minutes or 60 minutes prior to addition to MRC-5 cells. Under these conditions, peptide $p5R_D$ reduced HCMV infection by 90% after incubation with FBS while p5R was no longer inhibitory (Figure 1.2C). Similarly, $p5R_D$ incubated with human serum reduced HCMV infection by ≥85%, while serum incubated p5R only achieved a 50% reduction (Figure 1.2D). The differences between human serum and FBS treatment could be due to differences in protease concentrations or other inhibitors of the peptide/HS interaction [43]. These data suggest the D-amino acids in p5R protect it from proteolysis making it a better option for *in vivo* evaluation.

Efficacy of $p5R_D$ *in vivo*

. Our data suggests that $p5R_D$ effectively reduces HCMV infection and was resistant to serum proteases and degradation when exposed to serum. Before evaluating the anti-viral efficacy in mice we characterized the peptide's biodistribution in healthy animals.

Biodistribution experiments of ^{125}I-labeled peptide in BALB/c mice showed that, ^{125}I-p5R$_D$ was retained at high levels in the spleen, liver, and kidneys of mice for at least 24 hours post injection (Table 1.1). In contrast, the majority of ^{125}I-p5R was excreted or degraded by 4 hours. Only radioiodide, which is liberated during renal and hepatic catabolism of the peptide, is visible in the stomach of the mice. To assess the anti-viral efficacy of p5R$_D$ *in vivo*, cohorts of mice were injected i.v. with 500 μg of p5R$_D$ or PBS at 1, 2, or 4 hour prior to i.p. administration of MCMV ($1x10^6$ pfu). The spleen and liver, which are sites where peptide p5R$_D$ was retained for >24 h, were harvested 4 days post infection (dpi) for titering of virus. The p5R$_D$-treated mice show reduced viral titers for both the liver and spleen when the animals were treated with peptide prior to infection (Figure 1.3). However, the greatest reduction occurred in the spleen (~25% reduction) when the peptide was administered 1 hour prior to infection (Figure 1.3C). There is a statistically significant reduction of the viral load in the spleen and liver with ~15 -25% decrease in viral titers between peptide treated and PBS treated mice at all timepoints.

To address whether peptide p5R$_D$ was capable of inhibiting initial MCMV entry but failing to inhibit the subsequent infection of new cells days after administration, we focused on the infection of peritoneal exudate cells (PECs) early after infection. 500 μg of peptide was administered i.v. 4 or 1 hr. prior to i.p. infection of MCMV. The PECs were harvested 2 hrs. post infection and washed to remove excess virus. An infectious centers assay was used to measure infected PECs. Our data show that administration of peptide 4 hrs. (Figure 1.4A) or 1 hr. (Figure 1.4B**)** prior to infection significantly reduced the number of infected PECs ($p = 0.007$ and 0.003, respectively), suggesting that p5R$_D$ likely inhibits the initial entry of MCMV into host cells.

Peptide efficacy against GCV^R HCMV

The long-term use of antivirals has contributed to the development of HCMV resistance to the current antivirals [13]. For example, it is estimated that between 2-27.5% of HCMVs are now resistant to GCV [44-47]. We next examined the efficacy of peptide p5R$_D$ as an inhibitor of infection using GCV^R HCMV. Bacterial artificial chromosome (BAC)-derived HCMVs with mutations in either the *UL54* gene (T3429) that encodes the DNA polymerase targeted by GCV or the viral *UL97* gene (T3252) that encodes the kinase required for phosphorylation of GCV

were tested against $p5R_D$. These point mutations are representative mutations found in clinical isolates and incorporated into these BAC derived strains [29-31, 48]. Notably mutations in *UL54* also confer resistance to CDV and FOS [13]. We tested the hypothesis that mutations that contribute to GCV resistance would not confer "resistance" to the inhibitory effects of the $p5R_D$ peptide treatment. First, we confirmed, using the SEAP assay, that T3429 and T3252 strains are resistant to high concentrations of GCV (Supplemental Table 1.S1) [31, 32]. To test the efficacy of peptide $p5R_D$ at inhibiting these GCV-resistant viruses, MRC-5 cells were treated with 200 μM peptide prior to infection. Using the SEAP assay to quantify infection as a surrogate for the conventional plaque assay, treatment with $p5R_D$ reduced the infection of MRC-5 fibroblasts by recombinant GCV^R HCMVs, (T3429 and T3252) by ~80% (Figure 1.5A).

GCV^R HCMV has been isolated from organ transplant recipients as well as other settings [2, 3, 13, 14, 49]. Given that peptide $p5R_D$ reduced the infectivity of recombinant GCV^R HCMV *in vitro*, we next tested whether this peptide was similarly effective on clinically isolated GCV^R HCMV. These strains, from patients CH-13 and CH-19, were isolated from seropositive recipients during lung transplantation [14]. The strain from patient CH-19 (CH-19) has a point mutation A594V making it GCV^R. Notably T3252 recombinant virus from the previous experiment (Figure 1.5A) contains the same point mutation, A594V. The CH-13 A isolate (CH-13 A) contains a deletion in codons 597-603 within the *UL97* gene which confers resistance to GCV [50]. Using the *in vitro* plaque assay, peptide $p5R_D$ reduced CH-13 A and CH-19 infection of MRC-5 cells by >84% (Figure 1.5B).

Synergistic effects of GCV and $p5R_D$

Prolonged use or high concentrations of GCV, FOS, and CDV leads to kidney damage [12]. Treatment of GCV^R HCMV requires 2 to 10 fold more GCV in order to inhibit viral replication [14, 49]. To examine the synergistic effect of both with $p5R_D$ and GCV treatment MRC-5 cells were incubated with either GCV, $p5R_D$, or a combination of GCV and $p5R_D$ at decreasing concentrations of GCV. Cells treated with 3 μM of GCV alone showed ~95% reduction in HCMV infection (Figure 1.6). Similarly, cells treated with 200 μM of $p5R_D$ showed ~90% reduction in HCMV infection (Figure 1.1 and 1.6). The combination therapy of $p5R_D$ (200 μM) and GCV, at either 1.5 μM or 0.75 μM lead to a ≥ 94% reduction in HCMV infection

(Figure 1.6). These data suggest that the IC_{50} value for GCV was reduced when cells are pre-treated with 200 μM of peptide $p5R_D$ prior to infection. The combination of the two anti-CMV treatments should reduce prolonged GCV usage, making the development of GCV^R HCMV less likely and limiting GCV's toxic side effects.

Discussion

Generation of an effective reagent, such as a synthetic peptide, that prevents cellular tethering of CMV and thereby hindering infection would benefit 20 to 60% of transplant patients that are at risk for developing HCMV disease [44]. Furthermore, an effective prenatal prophylactic anti-CMV peptide will benefit the 0.5% to 2% of newborns at risk for contracting HCMV *in utero* [5]. Novel treatments for HCMV must address host organ toxicity and antiviral resistance currently associated with GCV, CDV, and FOS treatments. The current study aims to address those concerns by testing the ability of a D-amino acid, proteolytically-resistant peptide, $p5R_D$, to reduce CMV infection. Our data show that p5R and $p5R_D$ reduce HCMV infection of not only fibroblasts, but also endothelial and epithelial cells *in vitro* (Figure 1.1). A broad spectrum CMV inhibitor is important as CMV has an expansive cell-type tropism for replication and latency [16]. While p5R and $p5R_D$ reduce HCMV infection in all cell types, there are subtle differences in the percent reduction that potentially reflects differences in HS expression on the cell. It is known that CMV uses a 6-O-sulfated and 3-O-sulfated heparan sulfate for entry [21, 22]. Our data suggest that $p5R_D$ binds to a specific subset of HS, that is different from HS targeted by the previously characterized p5+14 and the HSV-1 entry inhibitor peptide, G2 [25, 26]. This is supported by our previous observation that, in contrast to $p5R_D$, ^{125}I-labeled p5+14 is not retained in the kidneys, liver, and spleen of WT mice at high concentrations for >4 h post injection [51].

In order for $p5R_D$ to effectively reduce CMV infection *in vivo*, it must resist hydrolysis from host proteases. Our data indicates that the anti-CMV function of $p5R_D$, but not p5R, was unaffected after incubation with the serine proteases, trypsin and elastase, or with bovine or human serum (Figure 1.2). Using radiolabeled peptide, we also show that $p5R_D$ is retained in the spleen, liver, and kidney of WT mice for >24 h while the L-form, p5R, is rapidly dehalogenated

and/or excreted. These data suggest that the D-amino acids used to synthesize $p5R_D$ confer protection from hydrolysis and enhances its potential to reduce HCMV infection *in vivo*.

In our *in vivo* experiment, $p5R_D$ was administered i.v. before i.p. MCMV infection. Peptide was administered i.v. to mimic clinical administration of antiviral reagents. Additionally i.v. administration results in widespread biodistribution of $p5R_D$ (Table 1.1). MCMV i.p. injection results in a systemic infection that involves a variety of tissues and organs [52]. In a systemic infection model of MCMV infection followed by primary dissemination to the spleen and liver, we found that $p5R_D$ reduces infection in these organs at 4 dpi. There are several possible explanations for why the reduction was not as great as the *in vitro* reduction. One possibility is that CMV infects host cells by a variety of mechanisms including cell-to-cell spread or pH-dependent endosomal entry, which do not require cell surface HSPGs [16, 53-57]. MCMV intra-tissue spread is mediated by a specific glycoprotein complex (gH/gL/MCK-2) that differs from the glycoprotein complex (gH/gL/gO) used in initial MCMV entry [58]. The MCMV gH/gL/gO complex is responsible for entering fibroblasts through a fusion event, which presumably initially involves HSPG tethering [59]. The MCMV gH/gL/MCK-2 complex facilitates infection in macrophages via the endocytic pathway [37, 58]. It is not known whether this utilizes HSPGs for entry. If intra-tissue spread occurs without the use of HSPGs, $p5R_D$ would be ineffective at reducing infection of tissue cells *in vivo*. Another possible explanation for $p5R_D$'s lack of efficacy *in vivo* could be due to its lack of efficacy once infection has been initiated. At 4 dpi MCMV has likely undergone at least two rounds of exponential replication [60]. Even at 90% reduction of infection, two rounds of growth would only decrease the viral load by little more than a log, which is at the limit of detection of plaques assay. Whether this degree of reduction in viral load would result in reduced pathology in the humans, remains open to speculation.

Another factor that could affect $p5R_D$ efficacy is the turnover rate for the HS ligand that to which it binds. The turnover rate for HS and HSPGs is relatively rapid ($T_{1/2}$ <4 hours) for murine epithelial cells [61]. To examine whether $p5R_D$ limits initial MCMV entry, mice were injected with $p5R_D$ prior to MCMV infection of PECs early after infection [62]. We chose an i.v. treatment with $p5R_D$ in order to be consistent with previous data (Figure 1.3 and Table 1.1) and to mimic potential clinical utility. Peptides are small enough to penetrate blood vessel walls.

Therefore, we predicted $p5R_D$ would still be able to access the virus in the peritoneum [63]. An infectious centers assay revealed that $p5R_D$ significantly reduced the amount of infected PECs when administered 1 or 4 hours before MCMV infection (Figure 1.4). While ~50% of PECs are macrophages [64], we did not assay whether the peptide is active against macrophages, their subsets, or other cells found in peritoneal cavity. Thus, the timing of $p5R_D$ administration appears to be important *in vivo* which may be due to the relatively quick turnover rate of HSPGs on different cell types.

The increasing frequency of GCV^R HCMV has warranted the development of novel therapeutics and approaches to dealing with CMV infection [13, 14, 48, 65, 66]. The current HCMV antiviral agents target and prevent viral DNA replication [67]. Given this clinical need we hypothesized and demonstrated that $p5R_D$ could inhibit GCV^R HCMV infection of cells because the peptide is thought to inhibit HSPG-mediated viral entry instead of viral DNA replication (Figure 1.5). Often HCMV antiviral agents are used to treat organ transplant recipients for months following transplantation [13, 14, 48, 65]. Among transplant recipients and AIDS patients there is a 5%-10% rate of therapeutic resistance [14]. Therefore peptides such as $p5R_D$ and similar reagents that act by inhibiting the initial CMV-cell interaction may provide new benefits for these patients. In addition, we have demonstrated that $p5R_D$ may be effectively used in combination therapy with traditional anti-CMV nucleoside analogs, such as GCV. Pre-treatment using $p5R_D$ would require less GCV to achieve therapeutic benefit. This would decrease the likelihood of developing GCV-resistant strains of HCMV and minimize the organ toxicity associated with high concentrations of GCV. This peptide, and similar reagents, could provide the basis for a novel approach to treating CMV diseases.

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Appendix: Figures and Tables

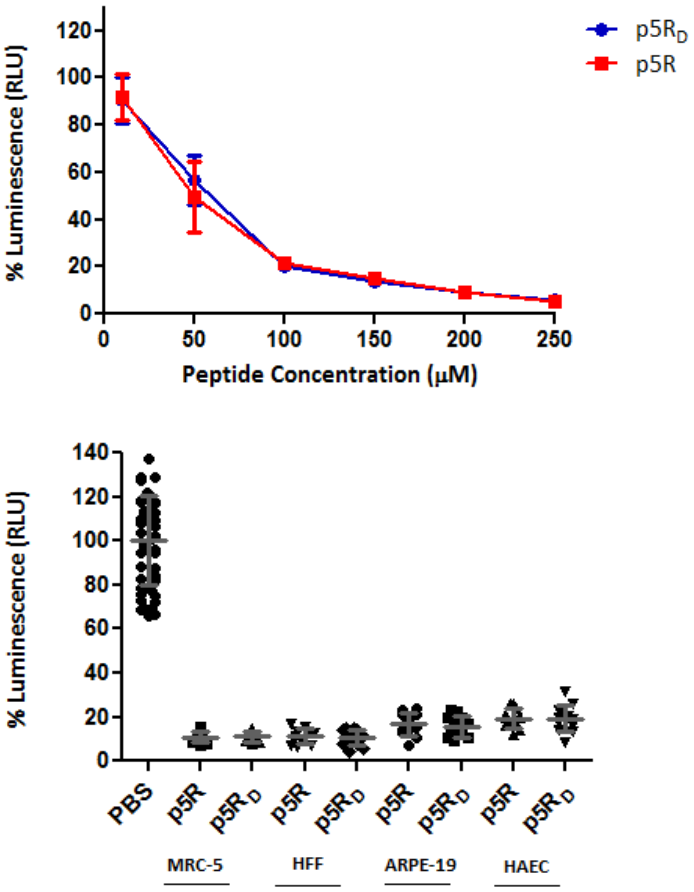

Figure 1.1. *in vitro* analysis of p5R and p5R$_D$. (A) The IC_{50} was determined by adding successive peptide concentrations (10µM, 50µM, 100µM, 200µM, and 250µM) to MRC-5 cells 30 minutes prior to the addition of virus. (B) 200µM of p5R and p5R$_D$, were added to either MRC-5, HFF, ARPE-19 or HAEC cells 30 min prior to the addition of virus . Data represents the average of the percent reduction compared to PBS-treated control from three separate experiments with three replicates in each experiment ±SD.

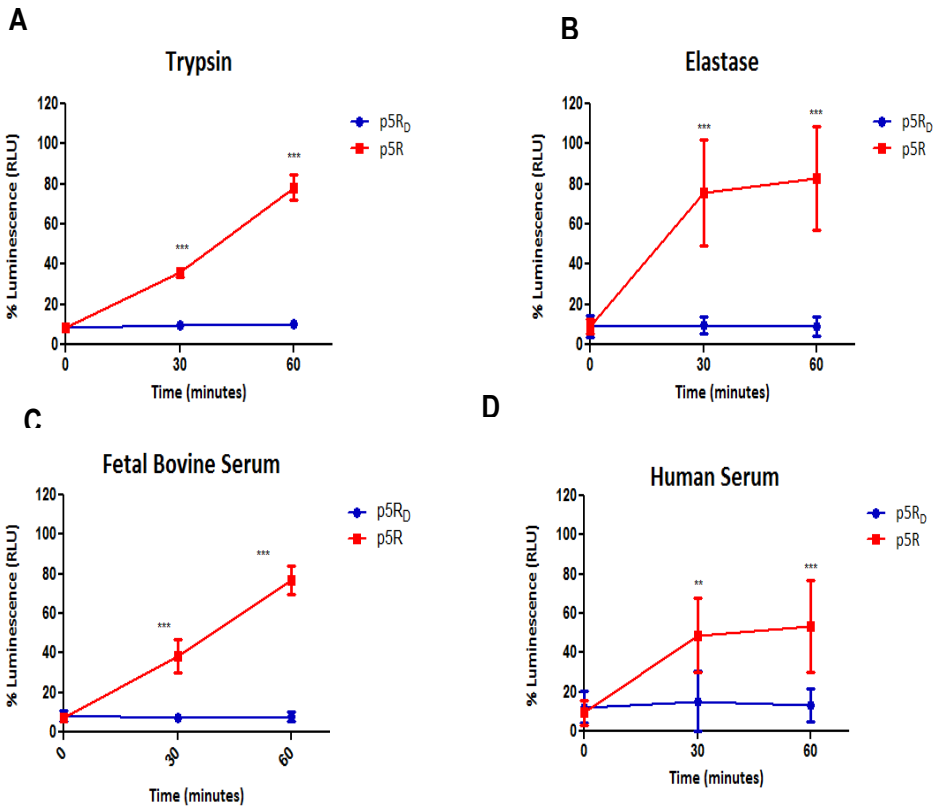

Figure 1.2. Proteolytic stability of p5R and p5R$_D$. 200µM of each peptide was pre-treated with 100µg/ml of trypsin, elastase, fetal bovine serum (FBS) or human serum for 30 minutes and 60 minutes. Protease treated peptides were added to MRC-5 cells 30 minutes prior to the addition of virus. Data represents the average of the percent reduction compared to PBS-treated control three separate experiments with three replicates in each experiment. Statistical significance determined by 2 way ANOVA with Bonferroni posttest: * p-value<0.05, ** p-value<0.01, *** p-value<0.001 ±SD.

Table 1.1 Biodistribution of p5R and $p5R_D$

p5RD						
balb/c mice 2hpi				balb/c mice 24hpi		
	Tissue distribution (%ID/g)				Tissue distribution (%ID/g)	
	AVERAGE	SD			AVERAGE	SD
muscle	0.17	0.03		muscle	0.07	0.01
liver	40.04	5.06		liver	35.69	1.28
pancreas	0.28	0.04		pancreas	0.12	0.01
spleen	7.86	1.50		spleen	6.87	0.60
L kidney	50.90	6.40		L kidney	46.29	2.08
R kidney	56.02	8.73		R kidney	48.76	4.68
stomach	1.64	0.27		stomach	0.51	0.14
up intestine	0.91	0.13		up intestine	0.44	0.00
low intestine	0.64	0.07		low intestine	0.30	0.02
heart	0.66	0.13		heart	0.32	0.01
lung	3.22	0.40		lung	1.73	0.30
p5R						
balb/c mice 1hpi				balb/c mice 4hpi		
	Tissue distribution (%ID/g)				Tissue distribution (%ID/g)	
	AVERAGE	SD			AVERAGE	SD
muscle	0.80	0.19		muscle	0.28	0.07
liver	2.96	0.58		liver	1.25	0.26
pancreas	2.64	0.77		pancreas	0.70	0.25
spleen	1.95	0.42		spleen	1.02	0.47
L kidney	3.72	0.17		L kidney	1.00	0.26
R kidney	2.97	1.41		R kidney	0.97	0.20
stomach	19.69	3.52		stomach	5.43	0.58
up intestine	2.12	0.66		up intestine	0.68	0.18
low intestine	1.68	0.26		low intestine	0.77	0.08
heart	1.85	0.43		heart	0.76	0.27
lung	3.03	0.45		lung	1.02	0.21

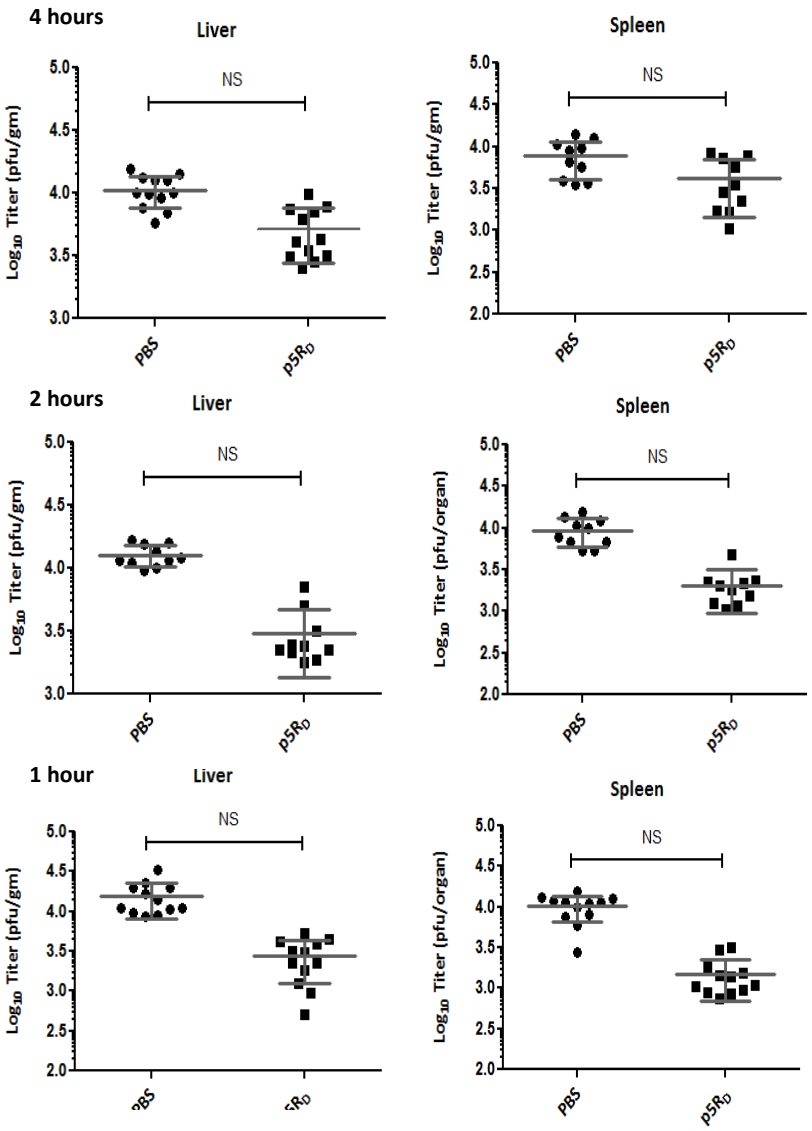

Figure 1.3. Viral load in spleen and liver of p5R$_D$ treated mice. Mice were pretreated with 500µg of p5R$_D$ or PBS intravenously 4 , 2 , 1 hours before infection with 1x10^6 pfu of WT MCMV (K181). Organs were harvested 4dpi. Data represents the average ± SD of peptide treated or PBS-treated control from three experiments with five (A and B) or six mices (C) each group. Statistical significance was determined by t-test. NS – non significant.

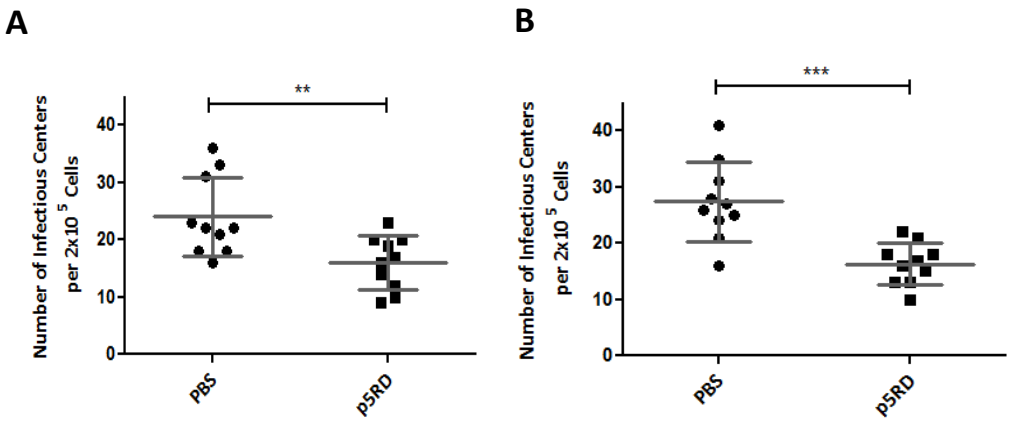

Figure 1.4. p5R$_D$ decreases PECs infection. Mice were pretreated with 500µg of p5R$_D$ or PBS intravenously 4 hours (A) or 1 hours (B) before i.p. infection with 1x10^6 pfu of WT MCMV (K181). PECs were harvested 4hpi. 2x10^5 PECs were placed over a confluent layer of MEF 10.1 cells. Data represents the average ± SD of peptide treated or PBS-treated control from two experiments with 5 mice each experiment. Statistical significance was determine by using t-test: * p-value<0.05, ** p-value<0.01, *** p-value<0.001.

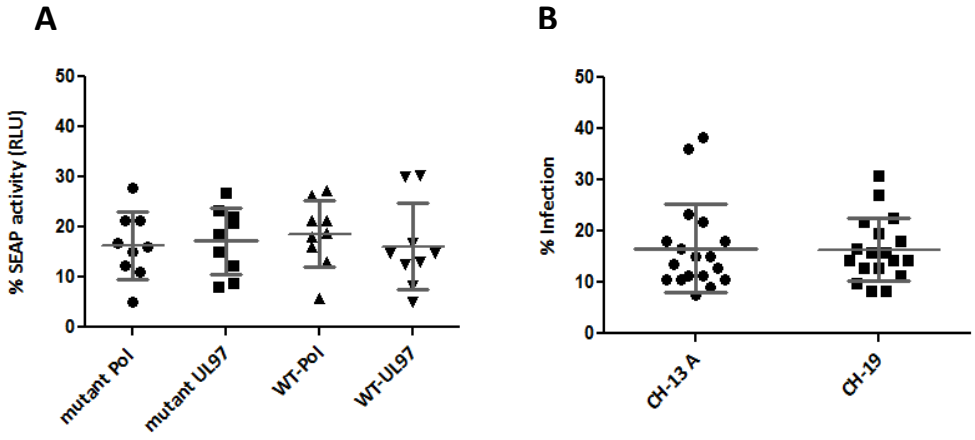

Figure 1.5. p5R and p5R$_D$ efficacy against GCV resistant HCMV. 200μM of p5R$_D$ were added to MRC-5cells 30 min prior to the addition of (A) BAC recombinant parental, recombinant GCV^R RLU determined via SEAP assay or (B) clinically isolated GCV^R HCMV determined via plaque assay. All data sets are representative of three independent experiments with at three to six repeats ±SD.

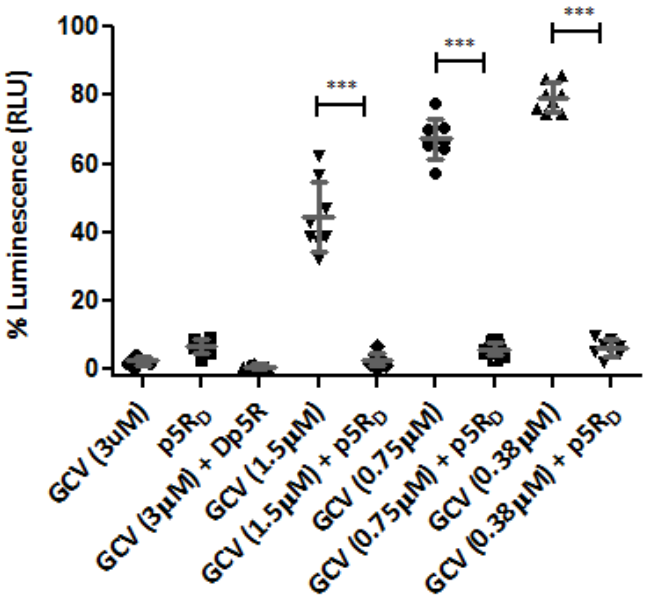

Figure 1.6. Combined effects of GCV and p5R$_{D.}$ MRC-5 cells were either treated with PBS (control) or 200μM of p5R$_{D}$ 30 min prior to the addition of virus. After infection, virus media was removed and cells were treated with four different GCV concentrations (3μM, 1.5μM, 0.75μM and 0.38μM) or no GCV (control). Data represents the average of GCV, peptide, GCV + peptide treated or PBS-treated control from four experiments with two replicates in each experiment. Statistical significance was determined by 2 way ANOVA with Bonferroni posttest: * p-value<0.05, ** p-value<0.01, *** p-value<0.001 ± SD.

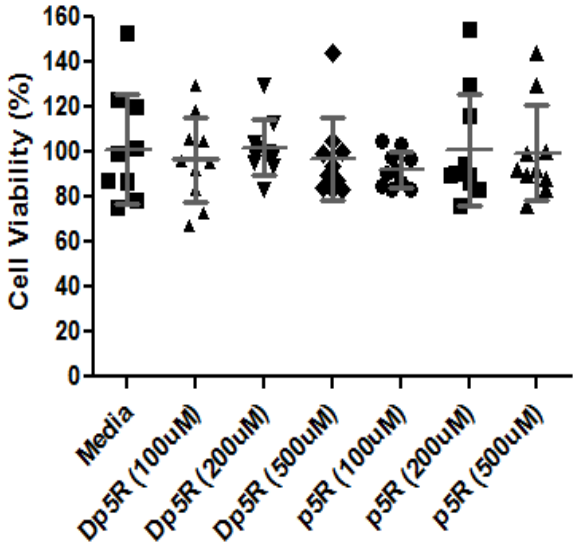

Figure 1.S1. Both p5R and p5R$_D$ are not toxic *in vitro.* 100µM, 200µM or 500µM of p5R and p5R$_D$, 0.1% Triton-x 100 (positive control) or media alone (negative control) was added to MRC-5 cells or 24 hours. Cell viability was determined using an MTS cell viability assay (Promega). All data sets are representative of two independent experiments with at least five replicates in each experiment ±SD.

Table 1.S1. EC_{50} for GCV treatment of BAC-derived recombinant HCMV mutants.

Virus name	EC_{50} (μM)	SD	No. of assays
WT Pol	2.01	0.15	3
WT UL97	1.92	0.10	3
Pol A987G	8.41	1.11	3
UL97 A594V	7.32	2.17	3
TB40E Luciferase	1.46	0.25	3

CHAPTER II
A LITTLE COOPERATION HELPS MCMV GO A LONG WAY–CO-INFECTION BETWEEN MCMVS RESCUES A VIRAL CHEMOKINE DISSEMINATION DEFECT

A version of this chapter was accepted for publication with revisions in 2016 in J. General Virology. Authors include: Pranay Dogra, Mindy Miller-Kittrell, Elisabeth Pitt, Tom Masi, Courtney Copeland, Shuen Wu, William Miller and Tim Sparer

My use of "we" in this chapter refers to my coauthors and myself. My primary contributions to this paper include (1) researching the topic and (2) performing experiments to generate data for figure 2.5, 2.6 and supplemental figures and data for the rebuttal to reviewer's comments.

Abstract

Cytomegaloviruses (CMVs) produce chemokines (vCXCLs) that have both sequence and functional homology to host chemokines. Assessment of vCXCL-1's role in CMV infection is limited to *in vitro* and *in silico* analysis due to CMV's species specificity. In this study, we used the murine CMV (MCMV) mouse model to evaluate the function of vCXCL-1 *in vivo*. Recombinant MCMVs expressing chimpanzee CMV vCXCL-1 (vCXCL-1_{CCMV}) or host chemokine, mCXCL1, underwent primary dissemination to the popliteal lymph node, spleen, and lung similar to the parental MCMV. However, neither of the recombinants expressing host or viral chemokines was recovered from the salivary gland (SG) at any time point. This implies that either the virus does not disseminate or grow in the SG. Eliminating the growth defect explanation, dissemination to the SG was restored upon co-infection with parental virus and infection of immune ablated mice (i.e., SCID, NSG, or cyclophosphamide treated). This recovery of SG dissemination could be explained by reduced levels of chemokine expression leading to a decrease in NK cells and inflammatory monocytes. The co-infection of recombinant and parental viruses reduces consititutive chemokine expression and restores dissemination of the recombinants to the SG. Therefore, we conclude that aberrant expression of the chemokines induces cells of the innate and adaptive immune system that curtail the dissemination of the recombinants to the SG.

Introduction

Human Cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus that is an important pathogen in immune compromised individuals and newborns [1-3]. It infects between 50% and

90% of the population resulting in largely asymptomatic infections [1]. However, primary or reactivated HCMV is a frequent cause of retinitis in AIDS patients [4] and increases the incidence of organ rejection and graft versus host disease in transplant recipients [5,6]. Central nervous system damage due to congenital HCMV infection affects 5,000-8,000 newborns in the U.S. each year [7]. As a result, HCMV is the leading cause of infectious hearing loss and non-hereditary mental retardation [1]. Understanding HCMV pathogenesis is important for the development of an effective vaccine or potential therapeutics.

CMVs encode numerous proteins that modulate the host immune system. HMCV infection alters the expression of host chemokines [8], but also encodes viral homologs of host chemokines and their receptors [9-12]. The virulent strain of HCMV, Toledo [13], produces a functional CXC chemokine, vCXCL-1_{Tol}, which binds the chemokine receptors CXCR1 and CXCR2 inducing chemotaxis and calcium flux in freshly isolated human peripheral blood neutrophils (PBNs) [14,15]. In our previous studies, we have shown that the viral chemokine from chimpanzee cytomegalovirus (vCXCL-1_{CCMV}), which is 22% identical and 52% similar to the vCXCL-1_{Tol} protein, triggers calcium release and chemotaxis of PBNs [16]. Both viral chemokines were shown to upregulate the expression of adhesion molecules on PBNs and downregulate neutrophil apoptosis, albeit with different potencies [16]. These findings provide circumstantial evidence for a role of vCXCL-1 in activating and recruiting neutrophils to facilitate CMV dissemination. Clinical evidence, including the recovery of HCMV from neutrophils of immunocompromised patients [17-19] and the presence of neutrophilic infiltrates in CMV associated retinitis [20], suggests neutrophils, as well as monocytes [21-23], are important in HCMV dissemination. While the species specificity of CMVs limits the direct evaluation of vCXCL-1 in HCMV dissemination, the function of vCXCL-1_{CCMV} may be representative of its HCMV homolog. In this study, we wanted to extend our previous *in vitro* findings with vCXCL-1_{CCMV} into an *in vivo* system.

MCMV is a well-established animal model of CMV infection and has similar cellular tropism and disease manifestations as HCMV [24]. MCMV expresses a chemokine homolog, MCK2, which has been shown to increase the inflammatory response in mice and enhance dissemination of MCMV to the SG [25,26]. Therefore, MCMV was chosen to characterize the role of vCXCL-1_{CCMV} *in vivo.* To test this in the mouse system, we generated recombinant

MCMVs expressing vCXCL-1_{CCMV} and host chemokine, mCXCL1. Our results show that expression both the host and viral chemokines is detrimental to the dissemination of the recombinants to the SG. We show that NK cells and inflammatory monocytes play a role in an innate immune system-mediated blockade of this dissemination. Surprisingly, co-infection with a non-chemokine-expressing strain of MCMV restored the chemokine expressing recombinants to the salivary gland by tipping the immune response to a more favorable environment for normal dissemination of the recombinants.

Materials and Methods

Cells and Viruses

Murine NIH3T3 and M210B4 cells (ATCC) were propagated in DMEM supplemented with 10% Fetal clone III (FCIII) (Hyclone), 1% penicillin/streptomycin (Hyclone), 1X NEAA, 1% sodium pyruvate (100mM), and 0.5 % HEPES (1M). MEF 10.1 cells (ATCC) were propagated in DMEM supplemented with 10% FCIII and 1% P/S and L-Gln. The parental MCMV strain used in this study was MCMV RM4511, [26] which has a 1.7 kb puromycin-green fluorescent protein (GFP) cassette inserted into the *ie2* region and a double point mutation in the *m131* gene resulting in a nonfunctional MCK2 protein (Figure S1A). RM4503 is similar to 4511 except with wild-type *mck2*. These virusese were obtained from Dr. Edward Mocarski, Emory University. For UV inactivation of the virus, 50µl of the stock virus were exposed to UV light in a UV crosslinker (Stratagene Stratalinker) at a setting of 1200 for 8 minutes. Complete inactivation was confirmed via a plaque assay.

Mice

Initially, mice that over express human CXCR2 (hCXCR2) or replace murine CXCR2 (mCXCR2) with human CXCR2 (hCXCR2) were used. There was concern that vCXCL-1_{CCMV} might not stimulate mCXCR2 as seen with vCXCL-1_{Tol} [27]. The hCXCR2 transgenic BALB/c mice express hCXCR2 under the control of the neutrophil-specific, human myeloid related protein-8 promoter [27]. In the co-infection experiments, mice that replace the hCXCR2 gene with the mCXCR2 gene were used [28]. These mice have normal expression levels of hCXCR2 in all the appropriate murine cell types. We have subsequently shown that vCXCL-1_{CCMV} can

function in normal mice (data not shown), which allowed us to use SCID and NSG mice with the appropriate parental controls. 3-4 week old BALB/c, NOD-NSG (NSG) mice were originally purchased from Jackson Laboratory (Bar Harbor, ME) and SCID/NCr were purchased from Taconic Labs. All mice were housed under specific pathogen free conditions at the University of Tennessee or the University of Cincinnati Medical School.

Plasmid Constructs

An EcoRI/PstI digested fragment containing the coding region for vCXCL-1_{CCMV} or host mCXCL1 (KC) was cloned into the EcoRI/PstI digested plasmid pcDNA3.1/Zeo immediately downstream of the HCMV immediate early promoter (HCMV IE). The 1.2kb HCMV IE-chemokine fragment was PCR amplified adding the flanking restriction sites and a C-terminal 6-His tag using the primers: MluI HCMV IE (5'-CGACGCGTCGATGTACGGGCCAGATATACGCCTTGACATTGATTAT-3') and SalI 6 His (5'ACGCGTCGACTGGTGGTGGTGGTGGTGACCTCCTCC-3'). After sequence verification, the HCMV IE-chemokine cassette was digested with MluI and SalI and cloned into the plasmid L120.1. L120.1 has 5' and 3' sequences from MCMV IE2 for homologous recombination and a *gpt* expression cassette used for selection of recombinant viruses (C. Meiering, unpublished data).

Generation of Recombinant Viruses

Recombinant viruses were generated using a transfection/infection strategy with subsequent selection for the loss of GFP along with *gpt* expression. NIH3T3 cells were transfected with Drd-linearized L120.1+vCXCL-1_{CCMV} or L120.1+mCXCL1 using Lipofectamine 2000 (Invitrogen). Three hours post-transfection, the cells were infected with MCMV RM4511 at an MOI=3. Transfectants were harvested and passaged twice under selection (mycophenolic acid (12.5 μg/ml) and xanthine (100 μg/ml)). Recombinant viruses were identified by the loss of GFP fluorescence and subjected to three rounds of plaque purification. For PCR verification, viral DNA was isolated by phenol-chloroform extraction and used for diagnostic PCR. The primers used were: RM4511 For (5'-CATTGACGTCAATGGTGGGAAAGTACATGGCG-3'),

RM4511 GFP Rev (5'-CCCGACGCGCGTGAGGAAGAGTTCTTGCAG-3'), and HCMV IE Rev (5'-GAACTCCATATATGGGCTATGAACTAATGACC).

In vitro Growth Assay

NIH3T3 or MEF 10.1 cells were plated in triplicate in a 6-well dish and infected with RM4511, RMvCXCL-1_{CCMV}, or RMmCXCL1 for either a multi-step (MOI=0.5) or single-step (MOI=5) growth analysis. Supernatants were collected at the indicated times post infection sonicated prior to tittering. Viruses were titered via plaque assay.

In vitro protein expression

Aliquots from the single-step growth assay were removed and used to verify chemokine expression. Ni-NTA agarose beads were used to isolated vCXCL-1_{CCMV} and mCXCL1 proteins from 100 μg of total protein. The eluted protein samples were subjected to Western blot analysis using the primary anti-6-His antibody (Qiagen) diluted 1:200 and secondary anti-mouse HRP antibody diluted 1:2000. Silver staining for the proteins in the supernatant was carried out using Pierce Silver Staining Kit (ThermoFisher Scientific) per manufacturer's instructions. Relative concentration of the protein was calculated using ImageJ on the captured images (NIH).

In vivo Growth of Parental and Recombinant Viruses

10^6 PFUs of parental or recombinant viruses were inoculated in the FP or intraperitoneally of hCXCR2 transgenic, Balb/c, NSG or SCID mice. At different times post infection, mice were euthanized and their footpads, spleens, liver, lungs, popliteal lymph node, and salivary glands were removed. Organs were individually weighed, homogenized, and clarified. Supernatants were titered.

Leukocyte Infectious Centers Assay

Assay was performed as described in [29]. Briefly, peripheral blood was harvested and red blood cells lysed. Leukocytes were plated onto NIH3T3 monolayers. After 6 hrs. cells were overlayed with CMC media. Plaques were counted after 7 days.

Plaque Formation Assay

Plaque formation assay on MEF 10.1 cells was used to determine viral titers in the organs. Briefly MEF 10.1 cells were plated in a 6 well dish. Organs were harvested and homogenized. The homogenate was serially diluted and added the MEF 10.1 cells and incubated for 1 hr. After incubation the diluted virus was removed and cells were overlayed with carboxy methyl cellulose (CMC) media and incubated for 7 days. At the end of the incubation period, CMC was removed and plates were stained with Coomassie blue and plaques counted.

Co-infection experiments

Mice were infected (either in the same foot pad or separate foot pads) or intra-peritoneally with 5 x 10^5 PFU each of chemokine expressing recombinants (RMmCXCL1/RMvCXCL1) and RM4511 or RM4503 for high titer inoculum experiments, or with 100 PFU of each virus for the low titer inoculum experiments. Mice infected separately with chemokine expressing recombinants or RM4511/RM4503 served as controls for these experiments. Salivary glands were harvested at 14 days p.i. from these mice and virus was titered. Expression of GFP was used to differentiate between RM4511/RM4503. Chemokine expressing recombinant virus plaques from RM4511/RM4503 were GFP+ and those from chemokine expressing recombinants were GFP negative.

Depletion of Cellular Subsets

In vivo depletion of cellular subsets was performed using antibodies one day prior to MCMV infection and then every three days until harvest. Neutrophils were depleted using 1A8 (anti-Ly6G) or RB6C (anti-Ly6G/C) antibody (1mg/ inoculation) (BioXcell, West Lebanon, NH). Flow cytometry was used to confirm depletion of GR-1hi, Mac-1hi neutrophils (Pharmingen).

Flow Cytometry

The following fluorochrome-conjugated antibodies were used to analyze the cellular subsets: anti-CD3 (17A2), anti-CD45.2 (104), anti-CD11c (N418), anti-Ly6G (1A8), anti-Ly6C (HK1.4) (all from Biolegend); anti-CD49b (DX5) from eBiosciences; and anti-CD11b (M1/70) from BD Pharmigen. Cells were analyzed on BD LSR II flow cytometer (BD Biosciences) and evaluated using FlowJo Mac software, version 8.7.

Statistical Analysis

Statistical significance was calculated using one tailed Student's *t* test or 1 way ANOVA followed by Tukey's or Bonferroni's Multiple Comparison Test in Prism5 (GraphPad) following the recommendations of Vaux *et al*. [30,31]. A p value < 0.05 was considered statistically significant.

Results

Construction of the chemokine expressing MCMVs

Strict species specificity of CMVs requires the generation of a recombinant MCMV to assess the function of viral chemokines in the mouse model. Therefore, we generated a recombinant MCMV expressing vCXCL-1_{CCMV} (RMvCXCL-1_{CCMV}) to study the role of this chimpanzee CMV viral chemokine in viral dissemination *in vivo*. MCMV expressing mCXCL1 (KC) was also generated as a control to evaluate specific effects of the viral chemokine on dissemination. mCXCL1 is the murine equivalent of human CXCL-1 (Gro-α) with a high affinity for CXCR2. MCMV RM4511 was the parental strain used for construction of these recombinants because RM4511 lacks a functional MCMV viral CC chemokine, MCK2. This will allow us to analyze the contribution of vCXCL-1_{CCMV} and mCXCL1 in the dissemination of MCMV in the absence of the endogenously encoded chemokine, MCK2 (Figure 2.S1A).

Recombinant plasmid, L120.1, was modified to contain either the vCXCL-1_{CCMV} or mCXCL1 coding sequence under the control of HCMV IE promoter (Figure 2.S1B). This promoter has been used to drive expression of other genes inserted in the MCMV genome and was chosen to ensure high levels of chemokine expression. We chose to insert the chemokine cassette into the *ie2* region as it is dispensable for growth of MCMV *in vivo* [32]. The chemokine cassette displaces the puromycin-GFP segment present in RM4511, such that loss of GFP expression allowed visual selection of recombinant viruses.

Following transfection of NIH3T3 cells with Drd-linearized L120.1+vCXCL-1_{CCMV} or mCXCL1 and subsequent infection with RM4511, recombinant viruses were passaged twice in medium containing mycophenolic acid and xanthine to select for recombinant viruses expressing *gpt*. The loss of GFP expression identified recombinant viruses and each virus was plaque

purified three times. Recombination and correct insertion of the chemokine cassette was confirmed using PCR (Figure 2.S1C).

Recombinant MCMVs have similar in vitro growth kinetics and chemokine expression

To determine whether insertion of the vCXCL-1_{CCMV} or mCXCL1 cassette affected replication or spread of the recombinant viruses in cell culture, we setup both single (MOI=5) and multi (MOI=0.05) step growth curves. RMvCXCL-1_{CCMV} and RMmCXCL1 replicated as well as RM4511 in both assays (Figure 2.S1D), indicating no deleterious effects of the insertion on growth of the viruses in cell culture. Immunoblotting Ni-NTA-concentrated supernatants from each time point of the single step growth curve was used to detect the temporal expression of the chemokines in the supernatants of virally infected cells. Both RMvCXCL-1_{CCMV} and RMmCXCL1 proteins were detected in the supernatants beginning at the second day post infection (p.i.) and continuing for the duration of the experiment (Figure 2.S1E).

Dissemination of virus in vivo

The contribution of vCXCL1 to the replication and dissemination of recombinant viruses *in vivo* was evaluated after infecting mice with RM4511, RMmCXCL1, and RMvCXCL1_{CCMV} in the foot pad (FP) and measuring virus in the organs with a plaque assay. Recombinants reached similar titers as parental virus in FP, and in organs of primary dissemination (i.e., lymph node, lung, and spleen) (Figure 2.1A). However, no recombinant virus was detected in SG at day 7 and 14 p.i. when the parental virus usually reaches peak titers (Figure 2.1A). To exclude the possibility that the dissemination of the recombinant virus to the SG was only delayed, viral load in the SG was also measured at day 21 p.i. No recombinant virus was detected even at this later time point (data not shown). Similar experiments were performed using i.p. infection to determine if the route of infection had any effect on viral dissemination. Mice infected i.p. also had no chemokine expressing recombinants in the SG (data not shown). The lack of dissemination of these recombinants to the SG was accompanied by an absence of viremia during secondary dissemination (Figure 2.1B). These data point to a dissemination defect affecting viremia and subsequent viral dissemination to the SG.

Co-infection rescues dissemination of chemokine expressing recombinants

Expression of the chemokine genes in the recombinants is under the control the HCMV MIEP promoter. This strong and constitutively expressed promoter [33] leads to an aberrant expression of the chemokines, adversely affecting the dissemination of the recombinants to the SG (Figure 2.1A). We hypothesized that during co-infection (i.e., parental + recombinant), the dissemination defect would dominate, leading to an inhibition or reduction in the dissemination of parental virus to the SG. To test this hypothesis, mice were infected in the same FP with a mixed inoculum (1:1 ratio of each parental and chemokine expressing recombinant virus). SGs from these mice were harvested 14 days p.i. and the viral load determined. Surprisingly, chemokine expressing recombinant viruses were recovered from the SG of mice infected with the mixed inoculum (Figure 2.2A). Dissemination of the chemokine-expressing recombinants to the SG was also rescued in mice that were infected i.p. with a mixed inoculum demonstrating that the route of infection was not critical (Figure 2.S2A). The rescue of dissemination to the SG is independent of MCMV's expression of its endogenous CC chemokine (MCK2), as both the CC expressing RM4503 and mutated MCK2 RM4511 rescue the dissemination of the recombinants to the SG (Figure 2.S2B). The rescue in dissemination was not due to higher replication of the virus in the FP, spleen, or lung post co-infection nor was it due to higher virus at the site of infection (Figure 2.S2C). In addition, the timing of the infections was critical for the rescue of dissemination. For example, no recombinants were found in the SG when the infection of the two viruses was separated by 2 or 7 days (Figure 2.S2D). Taken together, these data indicate that the adverse effects of aberrant chemokine expression is nullified during co-infection and the SG dissemination rescue is temporally restricted requiring the viruses to interact early during infection.

The requirement for interaction at the site of infection during co-infection

One possible explanation for the recovery of chemokine expressing viruses in the SG, is that parental MCMV "interferes" with chemokine expression. To explore how co-infection rescues the dissemination of recombinant viruses to the SG, mice were infected with the two viruses in separate footpads on the same day. This would eliminate the possibility of the viruses interacting at the site of infection, but still allow them interact at the sites of primary dissemination. Both the

viruses grew to similar levels at in the footpad and primary dissemination sites (Figure 2.S2C). However, no chemokine expressing recombinant viruses were recovered from the SG of these mice at 14 days p.i. (Figure 2.2B).

The requirement of the close proximity of the two viruses to mediate the rescue phenotype suggests that the viruses may co-infect the same cell at the site of infection. To test this possibility we infected mice with a low PFU mixed inoculum (1:1 ratio of 100 PFU each of RM4511 and RMmCXCL1). We only used RMmCXCL1 throughout these sets of co-infection experiments because both RMmCXCL1 and RMvCXCL1$_{CCMV}$ gave similar results both *in vitro* and *in vivo* (Figure 2.1 and 2.2A). Infection with a low PFU inoculum reduces the probability of co-infection by greater than 1000 fold and subsequently reduces the dissemination rescue (Figure 2.2C). In order to directly address whether the viruses co-infect cells at the site of infection, we performed infectious centers assay with plastic adherent leukocytes isolated from the FP at day 3 p.i. from mice infected with high titered mixed inoculum (1:1 mix of parental and recombinant), which is schematically described in Figure 2.2D. During the amplification step, we were able to detect GFP positive and negative plaques from ~ 49% of purified GFP positive plaques (Figure 2.2E). Purified GFP negative plaques yield all GFP negative plaques during the amplification step in all instances. These results indicate that the two viruses need to be at the same site of infection, where they co-infect the same cell.

Live parental virus is required to rescue the dissemination of chemokine expressing recombinant virus

One possible explanation for the rescue of dissemination of the recombinants to the SG during co-infection is that the presence of the parental virus particles, stimulates an immune response that overcomes the blockade of dissemination. To explore this possibility, we infected mice in the same FP with a mixed inoculum containing a 1:1 mixture of viable RMmCXCL1 or RM4511 and either UV inactivated RM4511 or RMmCXCL1, respectively. Infection of mice with an inoculum containing UV inactivated RMmCXCL1 + Rm4511 did not alter the dissemination of RM4511 to the SG (Figure 2.3). However, UV inactivation of RM4511 completely abolished the rescue of the dissemination of RMmCXCL1 to the SG (Figure 2.3). These data suggest that live-replicating parental virus is necessary to mediate the rescue of the chemokine expressing recombinants to the SG.

The need for recombinant and live parental virus to co-infect the same cells suggests that the chemokine expressing viruses might have undergone DNA recombination with the parental virus. This could lead to the loss of expression of the chemokine gene and subsequent dissemination. PCR and subsequent DNA sequencing of the chemokine gene from recombinants isolated from the SG following co-infection shows that they all carry the chemokine gene and that there are no point mutations in the ORF (Figure 2.S3A). Additionally, RFLP analysis of these isolates showed no overt recombination (Figure 2.S3B).

Co-infection reduces mCXCL1 production *in vitro*

In the absence of any recombination or mutation of the chemokine gene, rescue of the SG dissemination defect of the chemokine expressing recombinants result from the reduction in chemokine production from the chemokine expressing recombinants during co-infection. To test this possibility, we carried out an *in vitro* co-infection assay. Cells in culture were infected with recombinant or parental virus alone or with a mixed inoculum. Supernatants were harvested every 24 hrs and silver stained to measure chemokine protein levels. We observed a reduction in the relative expression of the chemokine during co-infection *in vitro* compared with recombinants alone (Figure 2.4A). In spite of these differences in chemokine expression, there was no difference in the viral titers (Figure 2.4B). These data suggest that during co-infection, less chemokine is produced *in vivo*, without any effect on viral load. This reduction could be sufficient for dissemination of the recombinants to the SG.

Co-infection alters the immune response to chemokine expressing recombinant viruses

Both vCXCL-1_{CCMV} and mCXCL1 bind and activate neutrophils via CXCR2 [16, 34]. The recruitment and activation of CXCR2 cells could lead to an inflammatory environment that prevents normal secondary viral dissemination. Although there was a slight increase in the number of neutrophils recruited to the site of inoculation at day 3 p.i. in mice infected with the chemokine expressing recombinants compared to RM4511, it was not statistically significant (Figure 2.5A). In addition, we observed more NK cells in the spleen but not the lungs of mice compared to RM4511 infected mice at day 4 p.i. (Figure 2.5B). There was a 2-3 fold increase in inflammatory and patrolling monocytes in the lungs of chemokine expressing recombinant viruses compared with RM4511 infected mice (Figure 2.5B). To see how this profile changes

following co-infection, mice were co-infected with chemokine expressing and parental viruses. Although this leads to a reduction in NK cells in the spleen and lung, these were not statistically significant (Figure 2.5C). At the same time there was also a reduction in the number of inflammatory monocytes in the lungs, but not the spleens of co-infected mice compared to singly infected (RMmCXCL1) mice (Figure 2.5C). Together these data suggest that infection with the chemokine expressing recombinant viruses leads to a higher infiltration of NK cells and monocytes compared to the parental virus at different sites of viral replication. These cell types may interfere with the normal secondary dissemination of the chemokine expressing recombinants to the SG. During co-infection, the reduction in the recruitment of these cells to sites of infection would then allow for dissemination of the chemokine expressing recombinants to the SG.

NK and T/B cell mediated blockade of dissemination of the chemokine expressing recombinants

The chemokine expressing recombinants induce differential recruitment of inflammatory monocytes and NK cells suggesting an immune mediated blockade of the dissemination of recombinants to the SG. To investigate the extent of this blockade, mice were administered cyclophosphamide (cyclo) to deplete immune cells prior to infection [35-37] and dissemination of the recombinants was measured in the different organs. We observed no difference in the primary dissemination between the chemokine expressing recombinants and the parental virus in untreated and cyclo treated mice (Figure 2.S4). However, the chemokine expressing recombinants were recovered from the SG of cyclo treated mice albeit to much lower titer compared with the parental (Figure 2.6A), with detectable viremia measured at day 4 p.i (Figure 2.6E left panel).

Data from the above experiment provides further evidence for an immune mediated blockade of recombinant virus dissemination to the SG. To parse out the contribution of the immune cell subsets in this blockade, we depleted neutrophils. Activated neutrophils can trigger an inflammatory response capable of clearing recombinant viruses and respond to both of the CXC chemokines overexpressed in these viruses [38]. Mice were depleted of neutrophils using anti-Ly6G antibody prior to infection with parental and recombinant virus, and viral load was

measured in the SG at day 14 p.i. Despite the chemokine expressing recombinants slight enhancement of recruitment of neutrophils to the site of infection (Figure 2.5A), neutrophil depletion did not restore their dissemination to the SG (Figure 2.6B). These results show that neutrophils are not responsible for increased clearance of the recombinants in the SG or the dissemination defect observed *in vivo*.

Switching to mice with known immune cell defects, we used a series of inbred strains that lack immune subsets. NK cells form an important arm of the innate immune response to MCMV infection [39-41]. To address their role in our model, we utilized the NSG mouse model, which lacks NK cells, T and B lymphocytes [42]. NSG mice were infected with the recombinant or parental viruses and the viral load in the SG at day 14 p.i. was measured. We recovered recombinant virus from the SG of NSG mice at day 14 p.i., albeit much less than the parental virus (~3 logs) (Figure 2.6C). Note that these viral titers are similar to those in the cyclo treated mice (Figure 2.6A). In addition we observed viremia for the chemokine expressing recombinants as well as parental virus in NSG mice (Figure 2.6E right panel). Data from this experiment suggests that NK cells play a partial role in preventing the dissemination of the recombinant virus to the SG.

Because NSG mice also lack T and B as well as NK cells, we wanted to address the role of the adaptive response plays in the lack of dissemination of chemokine expressing recombinants. In particular, T cells play an important role in controlling CMV infection. While CD8+ T cells effectively clear MCMV from organs in the periphery [43], viral clearance from the SG is dependent on CD4+ T cells [44,45]. To determine if the adaptive immune response could be clearing RMvCXCL-1_{CCMV} and RMmCXCL1 from the SG, SCID mice were infected with recombinant or parental virus and viral load was measured in the SGs at day 14 p.i. Here again, the chemokine expressing recombinants were recovered from the SG of SCID mice as seen in the NSG mice, although not at the same levels as parental virus (Figure 2.6D). This data suggests that the absence of RMvCXCL-1_{CCMV} and RMmCXCL1 from the SG may be a result of an amplified adaptive immune response to the recombinants. Together, data from these experiments suggests that the aberrant production of the chemokines by the chemokine expressing recombinants induces an innate (i.e., NK cell mediated) and adaptive (i.e., T/B cell

mediated) immune response that is responsible for the lack of dissemination of the chemokine expressing recombinants to the SG.

Discussion

In our previous study we characterized the CCMV chemokine homolog, vCXCL-1_{CCMV}, and demonstrated that it is a functional chemokine, activating and recruiting human neutrophils similar to the HCMV chemokine vCXCL-1_{Tol} [16]. Due to the species specificity of CMV, the *in vivo* function of vCXCL-1_{CCMV} is unknown. We have gained significant knowledge about HCMV dissemination using the MCMV mouse model [345, 352]. MCMV has similar tropism to HCMV [1,9] and both the viruses demonstrate a cell-associated viremia in cells of the myelomonocytic lineage such as neutrophils, monocytes, and their precursors [1, 8, 17, 46-48]. Although the mechanism and relative contribution of each of these cell types to MCMV dissemination *in vivo* has been studied in some detail, the role of host and viral chemokines on this dissemination remains unclear [1, 8, 44, 49]. The MCMV CC chemokine, MCK2, contributes to the dissemination of MCMV to the SG while spread to other organs is MCK2-independent [26]. In this study, we used MCMV RM4511, which does not express functional MCK2, to generate recombinant MCMVs expressing viral and host CXC chemokines in order to evaluate the impact of vCXCL-1_{CCMV} on viral dissemination

The primary dissemination pattern of RMvCXCL-1_{CCMV} and RMmCXCL1 was similar to RM4511 (Figure 2.1A). However, the chemokine expressing recombinant viruses were not recovered from the SG (i.e., secondary dissemination) (Figure 2.1A). The absence of the recombinants from the SG was not due to impaired viral growth. There was no difference in viral growth of the recombinants compared to the parental virus at the site of inoculation in the FP or the primary dissemination organs (i.e., popliteal lymph node, spleen, and lung (Figure 2.1A)). It is possible that our recombinants are unable to replicate in the SG and carry a mutation in the *sgg1* gene, which has been shown for other MCMV recombinants with a SG growth defect [50,51]. However, this is unlikely for several reasons. First, the independently generated recombinants show a similar dissemination phenotype (data not shown). Moreover, when the recombinants reach the SG, as is the case of SCID mice, NSG mice, cyclo treatment or co-

infected mice, they are able to replicate in the SG (Figure 2.6A, C, and D). However, we did observe defective viremia for the recombinants (Figure 2.1B). Therefore, the recombinants are capable of SG replication and show a dissemination defect.

It is possible that overexpression of the chemokine leads to an over active immune response against the recombinants, which results in their increased clearance from the SG. Viral clearance from the SG is CD4+ T cell mediated [44,45]. Chemokine expressing recombinants were found in the SG of SCID mice, lacking B and T lymphocytes, supporting this possibility (Figure 2.6D). Chemokine expressing recombinant viruses were also found in the SG in NSG mice and after systemic immune ablation with cyclo treatment, implicating both the innate/adaptive immune system for the SG dissemination defect/clearance (Figure 2.6 A, and C). Interestingly, restored viremia for the recombinants in both cases paralleled SG dissemination (Figure 2.6E). Thus, although the recombinants may be susceptible to adaptive immune mediated clearance in the SG, they also show an innate immune-mediated defect in SG dissemination.

While working with the co-infection model (i.e., parental + chemokine expressing recombinants), we made the serendipitous discovery that the recombinants were able to disseminate to the SG even in immune-competent mice (Figure 2.2A). The rescue of dissemination required the two viruses to infect simultaneously and at the same site, as separating the infection spatially or temporally did not rescue the dissemination to the SG (Figure 2B and Figure 2.S2D). This localization is required for the two viruses to infect the same cell, most likely a monocyte/macrophage population at the site of infection (Figure 2.2C, D and E). Our experiments also show that live replicating virus was required to rescue the dissemination of the recombinants to the SG, not just viral particles interfering with chemokine expressing recombinant dissemination (Figure 2.3). We show that these viruses do not undergo recombination, mutation, or deletion of the chemokine gene while replicating with parental virus infected cells (Figure 2.S3). It is also conceivable that the parental virus reduces the replication and/or production of the chemokine from the recombinant during co-infection. Our *in vitro* co-infection assay showed that co-infection did not affect viral growth. However we did observe a modest reduction in the chemokine levels produced (Figure 2.4A). Therefore, the reduced chemokine level seen during mixed infection is not due to less virus production, but to

intracellular resource competition [52-58]. We were unable to show this reduction in the chemokine levels *in vivo* due to the limits of chemokine protein detection. We speculate that similar to the *in vitro* set up, intracellular resource competition or gene expression suppression within the infected cells could also lead to reduced chemokine levels *in vivo* and subsequent dissemination of the chemokine expressing recombinants to the SG. Our data shows that over-produced chemokines may recruit or activate cells of innate immune system that are detrimental for the dissemination of the recombinants to the SG. A more physiological level of expression of the viral chemokine could allow for evaluation of the viral chemokine *in vivo* such as expression under the control of the MCK2 promoter, which expresses with late kinetics. These viruses are currently being constructed. These data also bring up the interesting observation that the immune system mediated blockade of dissemination of the chemokine expressing recombinants to the SG is not very stringent and a little reduction in chemokine levels seems to be sufficient to tip the scales in favor of normal dissemination to the SG.

Surprisingly, neutrophil depletion did not restore dissemination to the SG (Figure 6B). Therefore another innate cell type that expresses CXCR2 that is not depleted with the anti-Ly6G antibody could play a role in blocking dissemination of the chemokine expressing recombinants. For example, dendritic cells, a subset of monocytes, or NK cells can be induced to express CXCR2 and CXCR1 [59-62] and may be involved in this process. Although an exhaustive analysis of $vCXCL1_{CCMV}$ receptor usage is lacking, mCXCL1 receptor usage is well-characterized [63]. As both have the same phenotype in our experiments, this SG dissemination defect does not seem to be exclusively a $vCXCL1_{CCMV}$ phenomenon. NK cells play a major role in the antiviral response against MCMV [39-41]. BALB/c mice which do not induce NK cell activation via the m157-Ly49H axis are susceptible to MCMV infection and show much higher viral titers in peripheral organs [64-65]. In our experiments we observed higher numbers of NK cells recruited to the site of infection in mice infected with the chemokine expressing recombinants (Figure 2.5B). The activation of the NK cells prevents the dissemination of the recombinants. Evidence in support of this premise comes from the following observations (i) Co-infection is associated with a reduction of NK cells recruited to spleen and lung (Figure 2.5C), (ii) There was a significant reduction in the NK cells in mice treated with cyclo (Figure 2.S5), and (iii) We observed dissemination of the recombinant virus to the SG in NSG mice, which

lack, not only T and B cells, but also NK cells. The reduction or absence of NK cells correlates with reduced inflammation at the site of infection or around the foci during primary organ infection, which could benefit virus growth and escape from the organ.

Recently, Farrell *et al.* showed that FP inoculations probably spread via the lymphatics and that this infection/spread is multi-layered [66]. They show that without the subcapsular sinus macrophages, more fibroblasts are infected resulting in higher titers and increased spread. In our experiments, we hypothesize that an increase in inflammatory cells blocks the normal dissemination of MCMV to the SG. In mice infected with the chemokine expressing recombinants, we observed more inflammatory monocytes in the lungs compared to mice infected with the parental virus (Figure 2.5B). Co-infection reduced the number of inflammatory monocytes in the lung, while not affecting the number of patrolling monocytes (Figure 2.5C). This data supports our hypothesis that the monocytes at the site of infection or around the foci of infection are the wrong type when the chemokine is expressed during MCMV infection (i.e., more inflammatory rather than patrolling). Although this might not affect viral growth at the site of infection, it would adversely affect the dissemination of the virus during viremia. Preliminary experiments adoptively transferring leukocytes isolated at day 3 p.i. from the FP of mice infected with parental, recombinant, or a mixed inoculum show that we can recover virus from the SG of mice receiving cells from mice infected with parental or a mixed inoculum. However, no virus was recovered from the SG of mice that received cells from chemokine expressing recombinants (Figure 2.S6).

Therefore we propose a model where the virus is carried out of the FP to the organs of primary dissemination initially by patrolling monocytes. During infection with the chemokine expressing recombinants alone, the over expression of the chemokine in the organs of primary dissemination (i.e., spleen, liver, lung) causes an increased recruitment of NK cells to the site and an enhanced inflammatory state. This supports the differentiation of inflammatory monocytes to M1 macrophages at the site, which do not contribute to the dissemination of the virus to the SG. The increased number of inflammatory monocytes may also interfere with the ability of patrolling monocytes to gain access to virally infected cells. During co-infection, there is a reduction in chemokine levels without affecting viral titers. The reduced chemokine levels leads to less NK cells at the site of infection and a reduction in the inflammatory environment granting

patrolling monocyte access to the foci of infection, which then allows for the normal dissemination. This model is summarized in (Figure 2.S7).

Although our results may seem to contradict our original hypothesis (i.e., the expression of HCMV CXC chemokines aids dissemination), we have to consider the caveat that our chemokine expressing recombinants *over express* the chemokines. In reality, the expression of the vCXCL-1 gene in HCMV is tightly regulated and expressed with late expression kinetics [14, 67], and not constitutively, as in the case of our recombinants. CXC chemokines are capable of making monocytes adhere to the endothelium [68-70], which could allow them to be infected more efficiently. Not only can CMV infect monocytes but the infection increases their life span [71], allows them to re-circulate [72], and promotes their differentiation into macrophages [21-23]. This differentiation then allows for productive infection of the virus in these cell types [73-75]. Because monocytes play an important role in HCMV dissemination *in vivo* [70-72], it is conceivable that HCMV has evolved to express vCXCL-1 at the stage when the virus is budding from the infected cell. The chemokine-induced halted monocyte is at the right place at the right time to pick up the budding virus and once the infected the monocyte re-circulates, it carries the virus to distal sites spreading infection within the host.

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Appendix: Figures

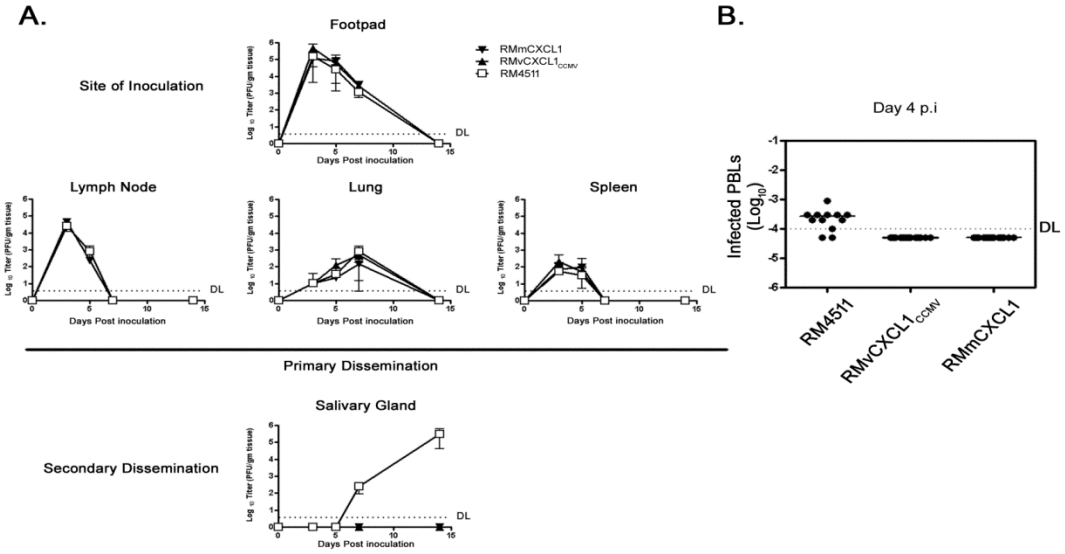

Figure 2.1. Overexpression of host or viral chemokine prevents MCMV dissemination to the SG. (A) hCXCR2 transgenic mice were inoculated in the footpad with 10^6 PFU of either RMmCXCL1 (▼), RMvCXCL-1_{CCMV} (▲), RM4511 (□). Organs were harvested at the indicated days p.i. and virus titers were determined via plaque assay. Each symbol represents the mean virus titer of 5-10 mice (+/- standard deviation). Data is representative of 2 experiments. (B) Viremia was measured on PBLs isolated from hCXCR2 transgenic mice. Data points represent % infected PBLs from individual mice and the horizontal line represents the mean from at least 3 mice for each experiment. The dashed line indicates the detection limit (DL) of the assay.

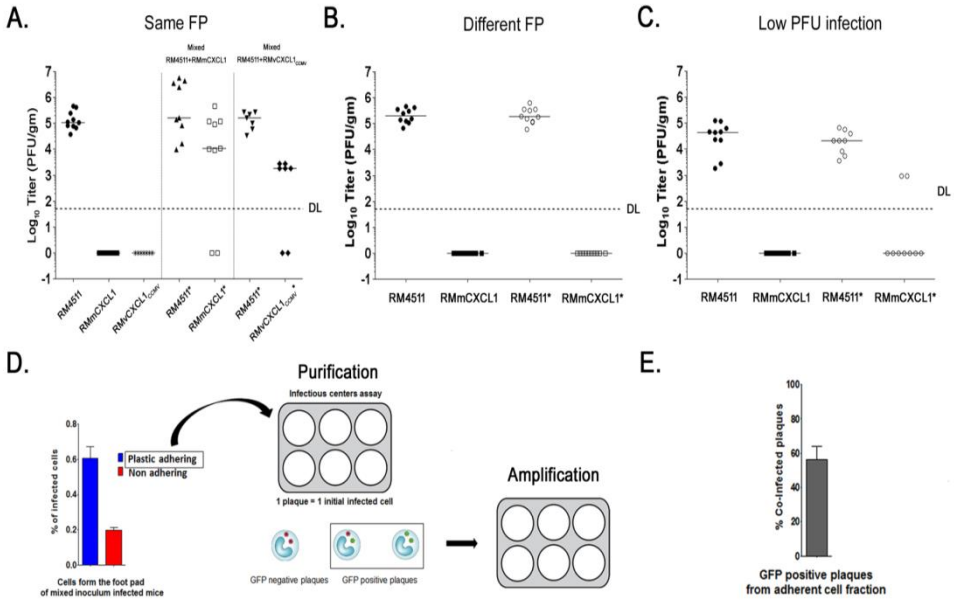

Figure 2.2. Rescue of the chemokine expressing recombinant virus dissemination to the SG upon co-infection with parental viruses requires interaction at the site of infection. (A) Mice were infected with 5 x 10^5 PFU of either parental (RM4511) or chemokine-expressing recombinant viruses (RMmCXCL1 or $RMvCxCL1_{CCMV}$) alone or in the same FP with a 1:1 mixed inoculum (5 x 10^5 PFU of each). Viral titer was measured in the SG at day 14 p.i. (B-C) Mice were infected with 5 x 10^5 PFU of either RM4511 or RMmCXCL1 virus alone, in different FPs (B) with 5 x 10^5 PFU of RM4511 and RMmCXCL1 or in the same FP (C) with 1:1 mixed inoculum (low titer) with 100 PFU each of RM4511 and RMmCXCL1. Viral titer in the SG was measured at day 14 p.i. The * indicates viral titers in the SG from mice infected with a mixed inoculum. The horizontal line is the median titer from the experiment. Each symbol represents the titer from an individual mouse. Dashed line indicates the DL of the plaque assay. (D) Schematic representation of the infectious centers assay performed on plastic adherent leukocytes isolated from the FP of mice infected with a 1:1 mixed inoculum at day 3 p.i. (E) GFP positive viruses were plaque purified from D and subjected to a round of amplification to ascertain the presence of GFP negative viruses within these plaques. Data is expressed as % co-infected plaques from total GFP positive plaques purified. Bar represents mean from 9 mice + SD.

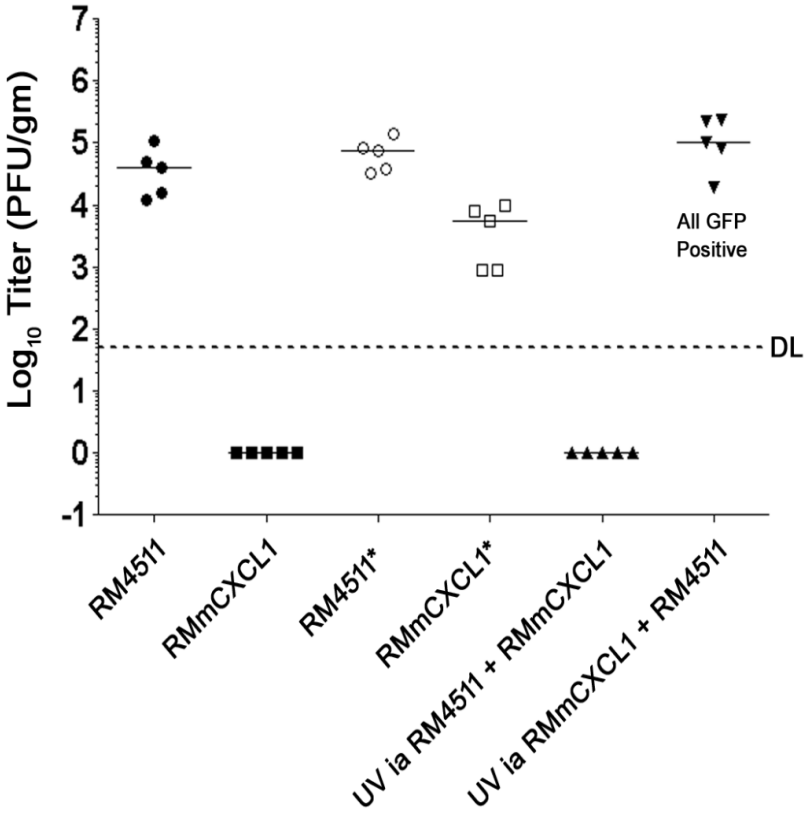

Figure 2.3. The rescue of the dissemination phenotype of the chemokine-overexpressing recombinants requires live virus.
Mice were infected with 5 x 10^5 PFU of either RM4511 or RMmCXCL1 virus alone, or a mixed inoculum (1:1 mix) of UV inactivated (UV ia) RMmCXCL1: live RM4511 or UV ia RM4511: live RMmCXCL1. Viral titer was measured in the SG at day 14 p.i. Each symbol indicates the titer from individual mice. The * indicates viral titers in the SG from mice infected with a mixed inoculum. The horizontal line represents the mean titer from the experiment. The dashed line indicates the DL of the assay.

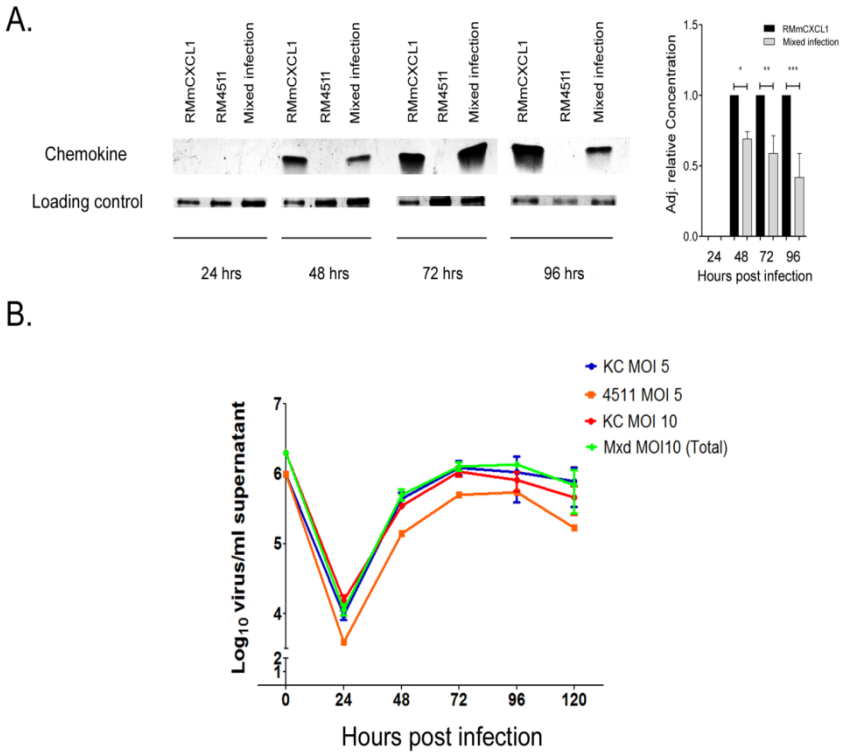

Figure 2.4. Co-infection *in vitro* reduces the recombinant MCMV chemokine levels without affecting viral growth. MEF cells were co-infected at an MOI of 5 of RMmCXCL1 and RM4511 or each one individually. The supernatant was collected at different time points p.i. After 6xHis-tagged chemokine enrichment, proteins were visualized on a silver stained SDS PAGE gel. Graph represents the relative concentration of chemokine to the loading control + SD from two experiments. One Way ANOVA followed by Bonferroni's multiple comparison test was used to compare the data. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$ (B) Viral titers were measured in the supernatant collected from the *in vitro* co-infection assays. Mixed infection (Mxd) was carried out at a MOI of 10 (i.e., MOI 5 of RM4511 + MOI 5 of RMmCXCL1). The data is representative from 3 experiments. Symbols represent the average titer +/- SD.

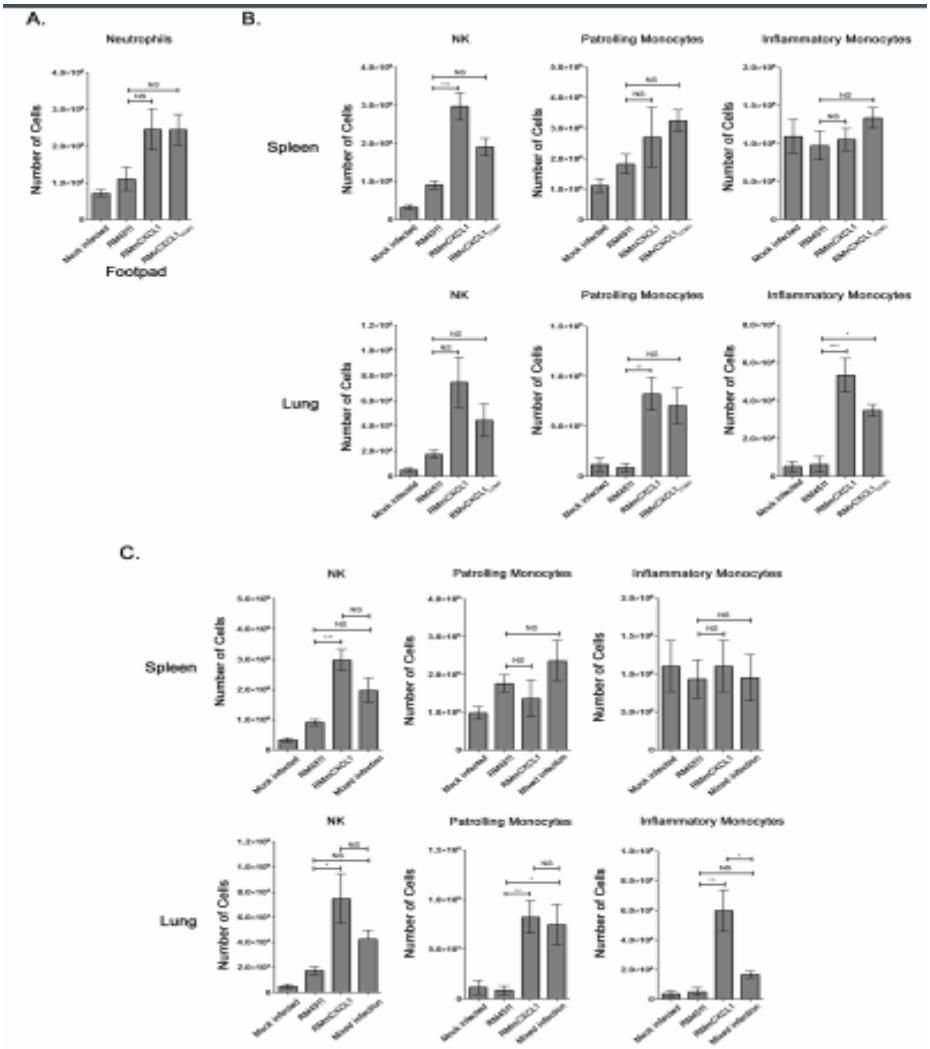

Figure 2.5. Co-infection alters the cellular infiltrate at the sites of infection to favor dissemination. Mice were infected in the FP with 1 x 10^6 PFU RM4511, RMmCXCL1, or RMvCXCL1. Flowcytometry was used to measure the number neutrophils infiltrating the FP at day 3 p.i. (A) and NK cells, patrolling and inflammatory monocytes recruited (B) into the spleen and the lung at day 4 p.i. (C) Mice were infected either singly or at 1:1 of each and analyzed with flowcytometry. Bars represent the average of the data from 6-9 mice per experiment +/- SEM. One Way ANOVA followed by Tukey's multiple comparison test was used to compare the data. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, NS= non significant.

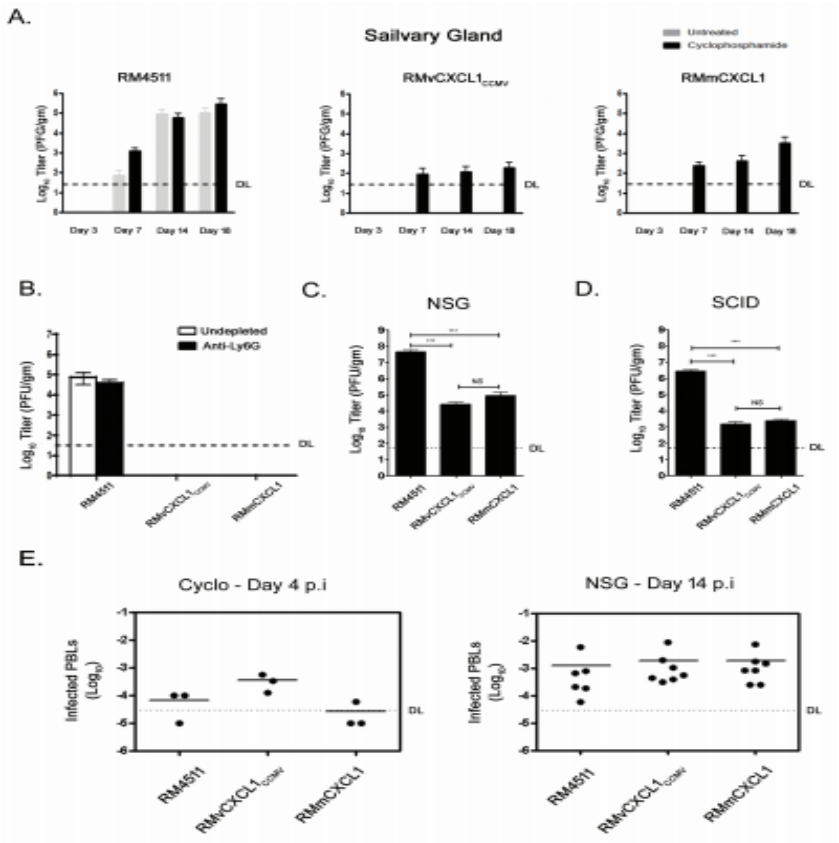

Figure 2.6. Absence of cellular subsets permits dissemination of chemokine expressing recombinants to the SG. (A) Mice were treated with cyclophosphamide for systemic immune ablation prior to infection with the parental and recombinant viruses alone. Viral titers in the SG were measured at days 3, 7, 14 and 18 p.i. (B) Mice were depleted of neutrophils using anti-Ly6G antibody and inoculated with parental and recombinant viruses alone. (C) NSG mice, which lack T, B, and NK cells or (D) SCID mice were inoculated with parental or recombinant viruses in the FP or i.p., respectively. For experiments in panel B-D the SG from the mice were harvested at day 14 p.i. and the virus titered. Results are from 5-10 mice per infection and are representative of 2 or more experiments. Bars represent the mean virus titer (+/- SD). One Way ANOVA followed by Tukey's multiple comparison test was used to compare the data. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$. (E) Viremia as measured on PBLs isolated from cyclophosphamide (Cyclo) treated BALB/c mice (left) or NSG mice (right). Data points represent the % infected PBLs and the horizontal line represents the mean of at least 3 mice for each experiment. The dashed line indicates the DL of the assay.

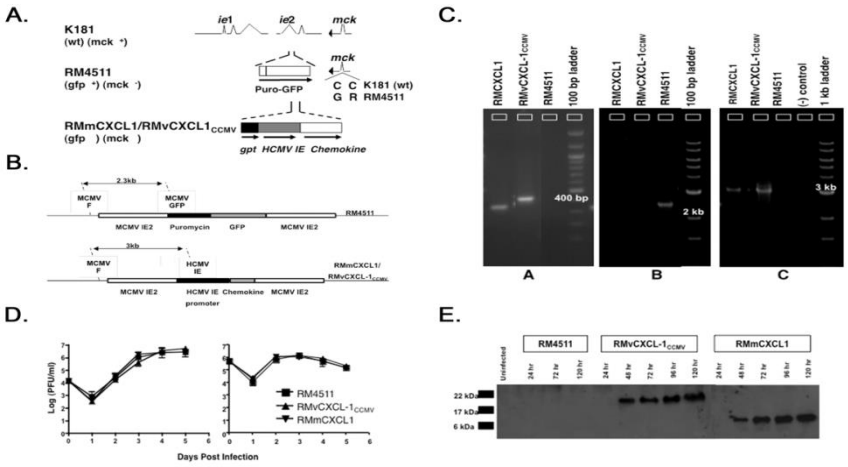

Figure 2.S1 Construction and characterization of wild type (WT) MCMV, parental MCMV (RM4511), RMmCXCL1, and RMvCXCL1CCMV viruses (A) The RM4511 recombinant MCMV contains a double point mutation resulting in a missense mutation that converts amino acids CC to GR in MCK2. This in effect creates an MCK2 negative strain. RM4511 also has a puromycin/green fluorescent protein expression cassette (puro-GFP) cassette in the ie2 locus. The mCXCL1 or vCXCL-1CCMV expression cassette containing a gpt selectable marker was used for selection and replaces the puro-GFP cassette. (B) Schematic representation of the puro-GFP and chemokine expression cassettes. The sizes of the diagnostic PCR products produced using the MCMV/GFP primers (2.5kb) and MCMV F/HCMV IE primers (3.0 kb) are shown. (C) Three PCR reactions were performed using viral DNA as the template. (i) Amplification of either the mCXCL1 or vCXCL-1CCMV gene using HCMV IE and 6 His primers. This generates a ~325 bp product (mCXCL1) and a ~425bp (vCXCL-1CCMV). (ii) Verification of the loss of the puromycin-GFP expression cassette using a primer flanking the 5' MCMV IE2 homologous region and a primer within the puromycin-GFP cassette (MCMV GFP). This generates a 2.3kb product. (iii) Verification of the correct insertion site of the mCXCL1 or vCXCL-1CCMV expression cassette using a primer flanking the 5' MCMV IE2 homologous region and a primer within the expression cassette (HCMV IE). This generates a 3kb PCR product. (D) NIH3T3 cells were infected in triplicate with RM4511, RMmCXCL1 or RMvCXCL1CCMV at a MOI of 0.05 (multi-step) or 5 (single-step). Supernatants were harvested daily for five days p.i. and assayed via a plaque assay. (D) Western blot analysis was used to analyze the expression of mCXCL1 and vCXCL1CCMV proteins. NIH3T3 cells were infected with RM4511, RMmCXCL1, or RMvCXCL1CCMV at a MOI of 5. Supernatants were harvested at the indicated times post infection. Recombinant proteins were enriched using Ni-NTA agarose beads. The eluted protein samples were subjected to Western blot analysis using anti-6-His antibody to detect the 6-His tagged chemokines.

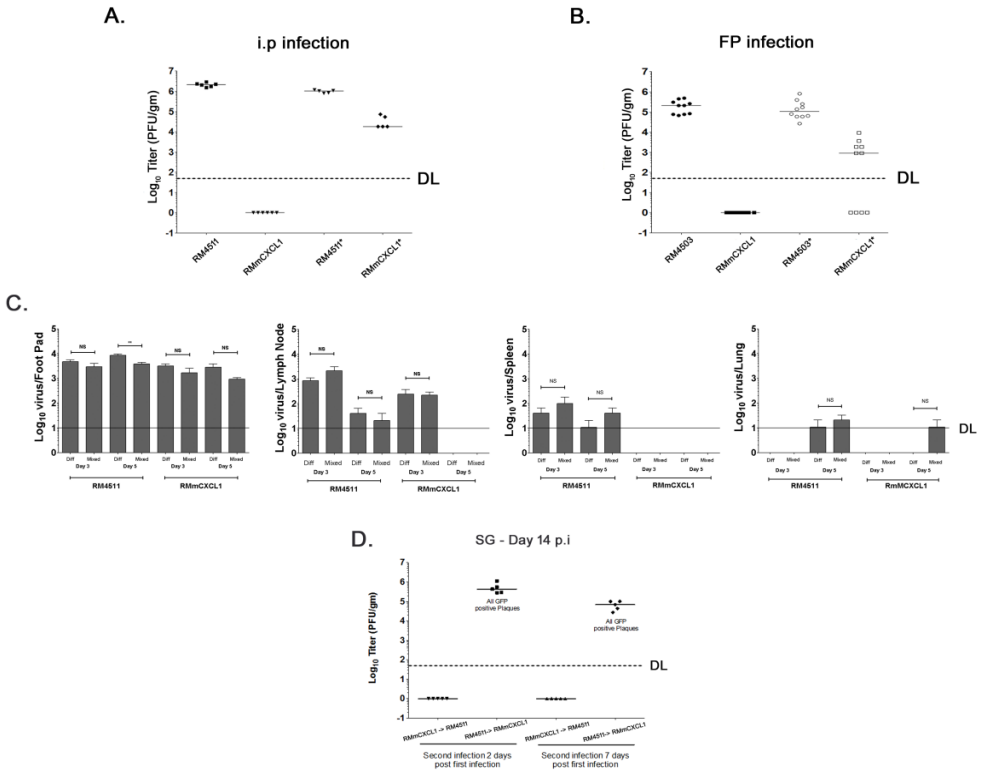

Figure 2.S2. Rescue of dissemination of recombinant viruses upon co-infection is not affected by route of infection and MCK2 chemokine expression. (A) Mice were infected i.p. with either the parental virus (RM4511) or recombinant virus alone, or a mixed inoculum (1:1 mix of 5 x 105 PFU each of parental and recombinant virus). Viral titer was measured in the SG at day 14 p.i. (B) Mice were infected with either RM4503 (i.e., MCK2+) or recombinant RMmCXCL1 virus alone or mixed inoculum (1:1 mix of 5 x 105 PFU of each Rm4503 and RmMCXCL1) in the FP. Viral titer was measured in the SG at day 14 p.i. (C) Mice were infected with a mixed inoculum (1:1 mix of 5 x 105 PFU each of parental and recombinant virus) in the FP (Mixed) or in different FPs with 5 x 105 PFU each of RM4511 and RMmCXCL1 (Diff). FP, popliteal lymph node, spleen and lungs were harvested at days 3 and 5 days p.i., homogenized and viral titer measured. Bars represent titer from 5 mice per group + SEM. (D) Mice were infected with 1 x 106 PFU of RM4511 and RMmCXCL1 in the same FP but delayed by 2 or 7 days. Viral titer was measured in the SG of these mice at day 14 p.i. after the second infection. RMmCXCL1->RM4511 = first infection RMmCXCL1 and second infection with RM4511, RM45111->RMmCXCL1 = first infection RM4511 and second infection with RMmCXCL1. In all experiments each symbol represents the titer from a mouse with the horizontal line representing the median titer for each group. Dashed line represents the detection limit (DL) for the plaque formation assay. The * indicates viral titers in the SG from mice infected with a mixed inoculum

A.

```
RMmCXCL1 parental   MIPATRSLLCAALLLLATSRLATGAPIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCT 60
RMmCXCL1 isolate #1 MIPATRSLLCAALLLLATSRLATGAPIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCT 60
RMmCXCL1 isolate #2 MIPATRSLLCAALLLLATSRLATGAPIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCT 60
RMmCXCL1 isolate #3 MIPATRSLLCAALLLLATSRLATGAPIANELRCQCLQTMAGIHLKNIQSLKVLPSGPHCT 60
                    ************************************************************

RMmCXCL1 parental   QTEVIATLKNGREACLDPEAPLVQKIVQKMLKGVPKGGGHHHHHQSTR 108
RMmCXCL1 isolate #1 QTEVIATLKNGREACLDPEAPLVQKIVQKMLKGVPKGGGHHHHHQSTR 108
RMmCXCL1 isolate #2 QTEVIATLKNGREACLDPEAPLVQKIVQKMLKGVPKGGGHHHHHQSTR 108
RMmCXCL1 isolate #3 QTEVIATLKNGREACLDPEAPLVQKIVQKMLKGVPKGGGHHHHHQSTR 108
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B.

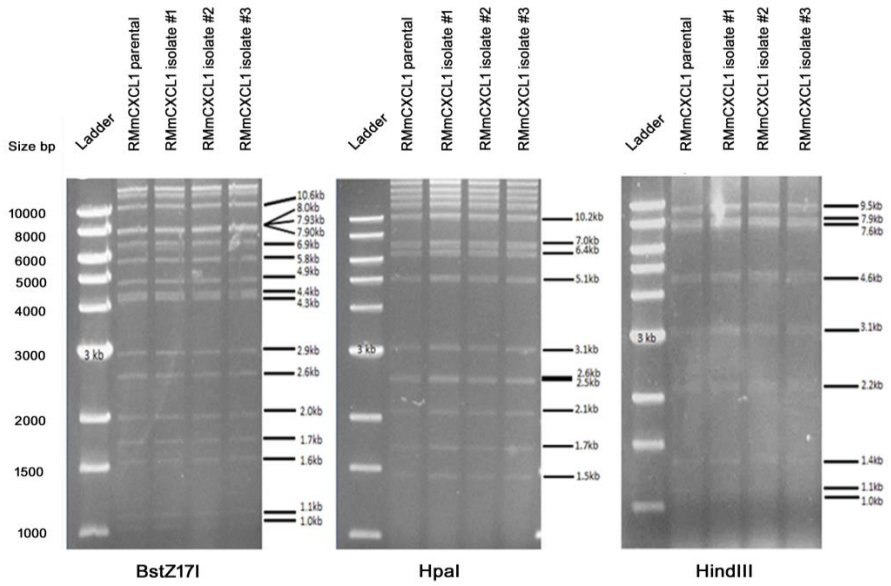

Figure 2.S3 Recombinant virus isolated from the salivary gland after co-infection with parental virus does not undergo recombination or mutation of the chemokine gene. RMmCXCL1 plaques were isolated from the SG of co-infected mice at day 14 p.i and grown in large scale cultures. (A) Phenol-chloroform extraction was used to isolate viral DNA and sequenced using HCMV IE and 6His primers described in materials and methods. The protein sequence generated from the resulting DNA sequence and aligned using Web based ClustalW (1, 2). Green is the signal peptide sequence, red is the CXC motif, blue is the His tag, * = 100% identity of the amino acid at the position among the sequences analyzed. (B) RFLP analysis was performed on viral DNA isolated from the RMmCXCL1 virus (RMmCXCL1 parental) and SG isolated virus following coinfection. Viral DNA was cut using BstZ17I, HpaI and HindIII restriction endonucleases. Predicted restriction fragment sizes of the digested viral DNA are indicated on right hand side of each gel.

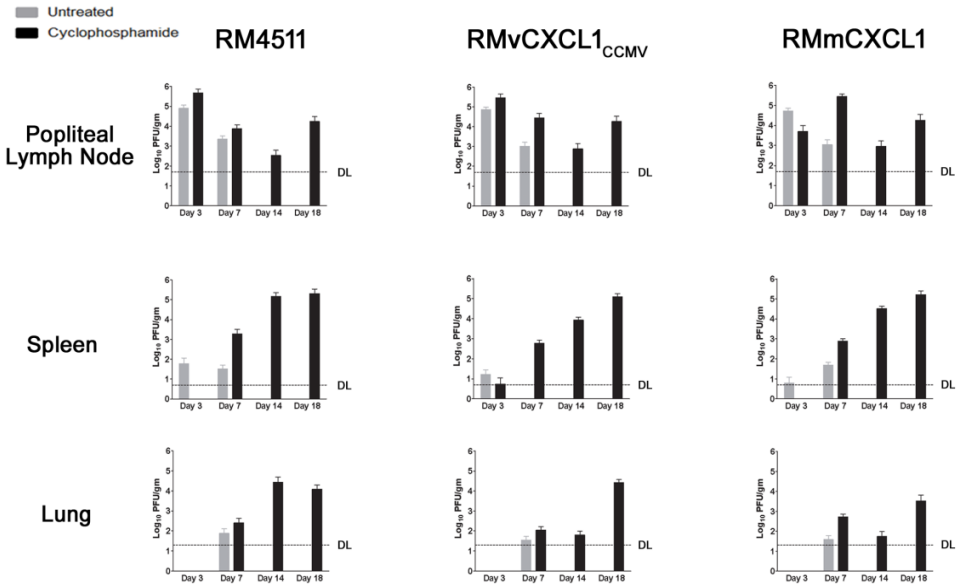

Figure 2.S4. Primary dissemination of virus in cyclophosphamide treated mice. Mice were treated with cyclophosphamide to deplete the immune cells prior to being infected in the foot pad with 1 x 106 PFU of the parental (RM4511) or chemokine expressing recombinant (RMvCXCL1CCMV or RMmCXCL1) virus. Untreated mice were use as control. Organs (popliteal lymph node, spleen and lung) were harvested at days 3, 7, 14, 18 post infection, homogenized and viral titer measured. Bars represent virus titer from at least 5 mice per time point + SEM. Dashed line represents the detection limit (DL) for the plaque assay.

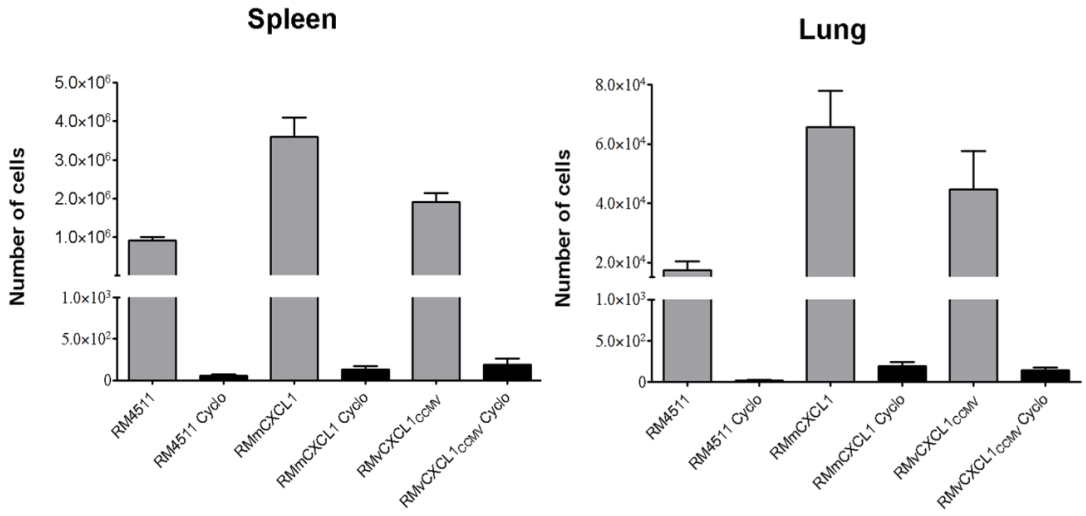

Figure 2.S5. NK cells are reduced upon cyclophosphamide treatment of mice. Mice were treated with cyclophosphamide to deplete immune cells prior to i.p. infection with the viruses. The spleen and lung were harvested at day 4 p.i., leukocytes were stained for flow cytometric analysis. The graph shows the number of NK cells in the spleen and lung of infected untreated and cyclo-treated mice. Bars represent the average number of NK cells from 5 mice per treatment group + SEM.

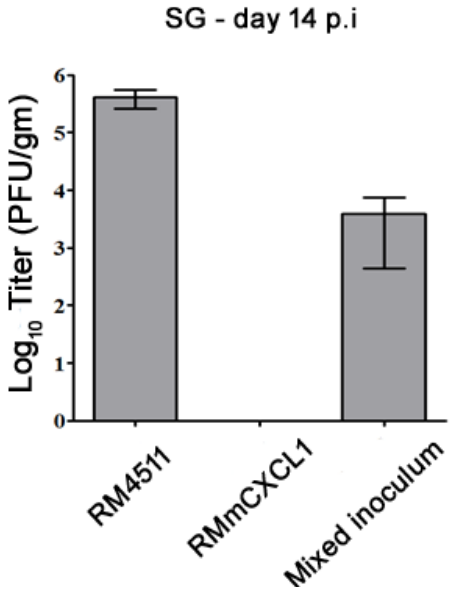

Figure 2.S6. Dissemination of virus after adoptive transfer of infected leukocytes. Mice were infected with 1x105 PFU of RM4511, RMmCXCL1, or a mixed inoculum consisting of a 1:1 mixture 1x105 PFU each of RM4511 and RMmCXCL1. Leukocytes were isolated from the FP at day 3 p.i. and adoptively transferred to mice via tail vein injection. SG from these mice was harvested at day 14 post transfer, homogenized, and virus titered. Bars represent average virus titer from 3 mice per group +/- SEM.

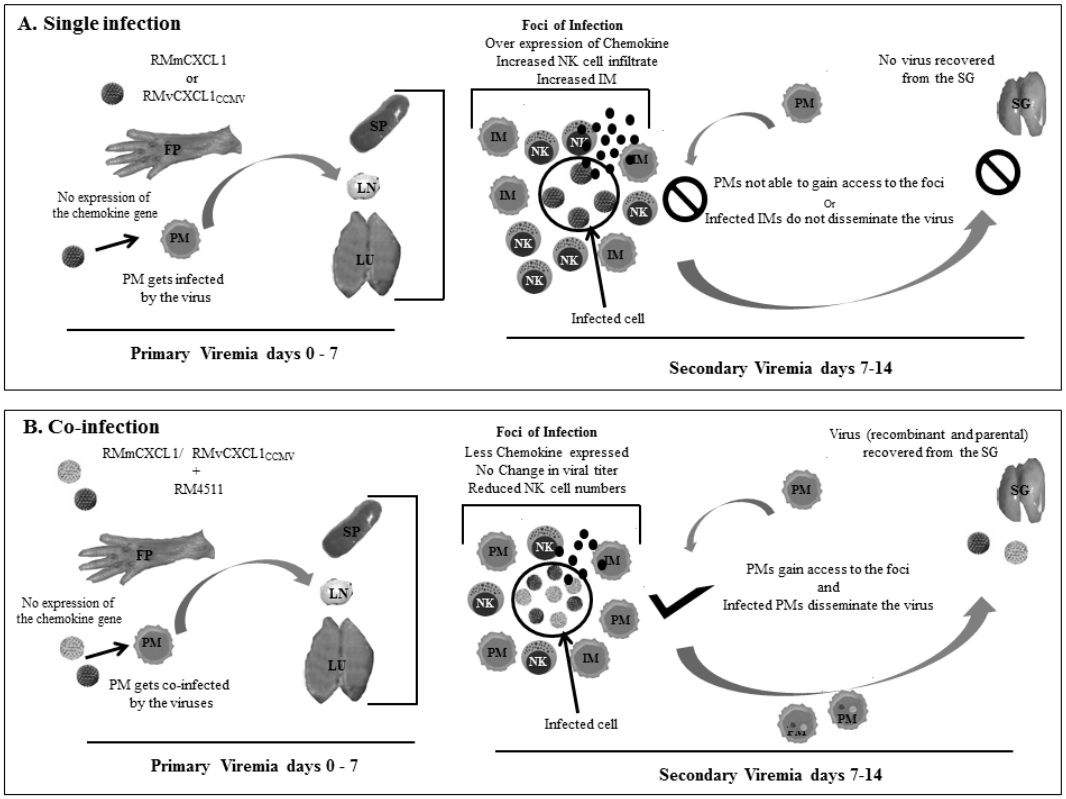

Figure 2.S7. A model of how co-infection rescues of the chemokine expressing recombinants dissemination. (A) During single infection, the overproduction of the chemokine at the site of infection in the primary organs of dissemination recruits inflammatory cells (NK and IM) to the site and interferes with normal secondary viraemia and subsequent salivary gland dissemination. (B) During co-infection, the reduction in chemokine levels in the primary organs reduces the number of inflammatory cells (NK and IM) to the site and allows PMs to gain access to the virus. The infected PMs then disseminate the virus to the SG. Figure 10 Key: IM = Inflammatory monocyte, PM = Patrolling monocyte, NK = Natural killer cells, FP = Foot pad, SP = Spleen, LN = Popliteal lymph node, Lu = Lung and Sg = Salivary gland, =Chemokine; Viruses: = RMmCXCL1/RMvCXCL1CCMV, = RM4511

CHAPTER III
NOVEL HUMAN CYTOMEGALOVIRUS VIRAL CHEMOKINES, VCXCLL-1S, DISPLAY FUNCTIONAL SELECTIVITY FOR NEUTROPHIL SIGNALING AND FUNCTION

This chapter is a publication by the same title published in the Journal of Immunology in 2015 by Jinho Heo, Pranay Dogra, Tom J. Masi, Elisabeth A. Pitt, Petra de Kruijf, Martine J. Smit, Tim E. Sparer and has been reproduced here with permission from the journal (appendix 6).

Heo J, Dogra P, Masi TJ, Pitt EA, de Kruijf P, Smit MJ, et al. Novel Human Cytomegalovirus Viral Chemokines, vCXCL-1s, Display Functional Selectivity for Neutrophil Signaling and Function. JI. 2015. Epub 2015/05/20. doi: 10.4049/jimmunol.1400291. PubMed PMID: 25987741.

My use of "we" in this chapter refers to my coauthors and myself. My primary contributions to this paper include (1) performing experiments to generate data for figure 3.2 and for the rebuttal to the reviewers, and (2) researching the topic.

Abstract

Human cytomegalovirus (HCMV) uses members of the hematopoietic system including neutrophils for dissemination throughout the body. HCMV encodes a viral chemokine, vCXCL-1, that is postulated to attract neutrophils for dissemination within the host. The gene encoding vCXCL-1, *UL146*, is one of the most variable genes in the HCMV genome. Why HCMV has evolved this hypervariability and how this affects the virus' dissemination/pathogenesis is unknown. Because the vCXCL-1 hypervariability maps to important binding and activation domains, we hypothesized that vCXCL-1s differentially activate neutrophils, which could contribute to HCMV dissemination and/or pathogenesis. In order to test whether these viral chemokines affect neutrophil function, we generated vCXCL-1 proteins from 11 different clades from clinical isolates from HCMV-congenitally infected infants. All vCXCL-1s were able to induce calcium flux at a concentration of 100 nM and integrin expression on human peripheral blood neutrophils (PBNs) in spite of differences in affinity for the CXCR1 and CXCR2 receptors. In fact their affinity for CXCR1 or CXCR2 did not directly correlate with chemotaxis, G protein-dependent and independent (β-arrestin2) activation, or secondary chemokine (CCL22) expression. Our data suggest that vCXCL-1 polymorphisms impact the binding affinity, receptor

usage, and differential PBN activation that could contribute to HCMV dissemination and/or pathogenesis.

Introduction

Human CMVs (HCMVs) are ubiquitous pathogens that are well adapted to modulate host immune responses [1, 2]. HCMV contains genes for immune evasion that function to increase viral survival and dissemination and that may contribute to pathogenesis [3]. There are a large number of open reading frames (ORFs; ~82) in HCMV that are nonessential for virus replication in vitro, but may have a role in immune evasion in vivo [4, 5]. In one of these regions, the UL/b' region, the ORFs UL146 and UL147 have limited homology to host CXC chemokines [5]. Yet, the UL146 protein from the Toledo strain of HCMV, vCXCL-1Toledo, acts as a functional CXC chemokine [6] that binds to CXCR1 and CXCR2 and induces neutrophil chemotaxis and calcium mobilization [7]. This gene is one of the most variable in the entire HCMV genome [8–12]. This variability is localized throughout the entire chemokine, including the N terminus and N-loop region, which are important for chemokine receptor binding and activation [13, 14]. Some strains even alter the GluLeu-Arg (ELR) prior to the CXC motif, which is critical for receptor recognition and activation [15, 16]. We hypothesized that hypervariable vCXCL-1s produced from HCMV-infected endothelial cells recruit neutrophils with alterations in binding, activation, and neutrophil functions that contribute to viral dissemination and possibly its pathogenesis. Eleven distinct vCXCL-1 clades were previously found in clinical isolates from congenitally infected infants [17]. In these groups the N-loop region was highly variable. In addition one isolate, vCXCL-1TX15, encoded a non-ELR CXC chemokine. Although the genetic variability of vCXCL-1 does not correlate definitively with congenital outcomes, the hypervariability within the N-loop region suggests that the vCXCL-1s may have different interactions with the chemokine receptors CXCR1 and CXCR2. To address functional variability of the vCXCL-1s, recombinant vCXCL-1s from each clade were generated. Competition binding, signaling, and neutrophil activation assays were used to assess the effect of vCXCL1 variability on chemokine function.

Materials and Methods

Materials

DMEM, penicillin, and streptomycin were obtained from Hyclone Laboratories (Logan, UT). FBS was purchased from Mediatech (Manassas, VA). DMEM containing 25 mM HEPES and L-glutamine, OPTI-MEM, Hygromycin B, and Geneticin were obtained from Invitrogen (Paisley, U.K.). BSA Fraction V (BSA) was purchased from Roche (Mannheim, Germany). Polyethylenimine was obtained from Polysciences (Warrington, PA). 125I-CXCL8 and 35S-GTPgS was obtained from PerkinElmer Life Sciences (Boston, MA). Clinical isolates used for cloning of the vCXCL-1 ORFs were provided by Dr. James Bale (University of Utah School of Medicine), Dr. Sunwen Chou, (Oregon Health and Science University), and Dr. Gail J. Demmler (Texas Children's Hospital) as described previously [18].

Cell culture and CXCR2 transfection

Insect cells, serum-free adapted SF9 cells (Invitrogen, Carlsbad, USA), were grown at 28°C in serum-free Sf-900 II SFM medium (Invitrogen, Carlsbad, USA). Hi5 cells (Invitrogen, USA) were grown in suspension at 28°C in serum-free Insect-XPRESS medium (Lonza, Switzerland). Both cells were grown in non-humidified, ambient air-regulated incubator.

PathHunter™ HEK293-CXCR2 cells (DiscoveRx, Fremont, USA), were grown at 5% CO_2 and 37°C in DMEM with 25 mM HEPES and L-glutamine supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 800 μg/ml Geneticin and 200 μg/ml Hygromycin-B.

HL-60 T2 cell transfectants over-expressing CXCR2 (a kind gift from Dr. Ann Richmond, Vanderbilt University, USA) were grown at 5% CO_2 and 37°C in RPMI-1640 Hyclone Laboratories (Logan, Utah) supplemented with 10% (v/v) fetal bovine serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 400 μg/ml G418 (Mediatech, Manassas, USA).

For ^{35}S-GTPγS experiments, HEK293T cells were grown at 5% CO_2 and 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. HEK293T were transiently transfected (per 10 cm

dish) with 2.5μg of cDNA encoding human CXCR2 supplemented with 2.5 μg of pcDEF3 by using linear polyethyleneimine (PEI) with a molecular weight of 25 kDa as previously described (18).

Neutrophil isolation

PBNs were isolated from EDTA-treated blood from healthy human volunteers using dextran sedimentation and density gradient centrifugation as previously described [19]. Erythrocytes were lysed with hypotonic lysis in 0.2% NaCl. Neutrophils were resuspended in the buffers for the individual assays. Viable neutrophils were quantified with trypan blue exclusion using a hemacytometer. The use of human subjects has been approved by the University of Tennessee Institutional Review Board (IRB# 6476B).

Production of recombinant vCXCL-1 proteins

The vCXCL-1 gene, *UL146*, was PCR amplified from HCMV DNA from each of the 11 clades. Amplicons were cloned into the baculovirus transfer plasmid 1392 (Invitrogen, Carlsbad, USA), which contains homologous regions for recombination into the baculovirus genome. PCR primers were designed to include the open reading frame (ORF) and with an additional 2-4 glycines and six histidines on the carboxyl terminus of the proteins for purification. For generation of baculoviruses, SF9 cells were transfected with the 1392/UL146 ORF plasmid construct and linearized AcNPV DNA (Sapphire Baculovirus DNA) (Orbigen USA) using transfection reagent Cellfectin (Invitrogen, Carlsbad, USA). Recombinant baculoviruses containing the *UL146* gene were titered and used to infect Hi5 cells for optimum protein expression. 48 hrs after infection, cells and supernatants were harvested. Recombinant protein was isolated from the supernatants using Ni-NTA agarose beads (Qiagen, San Diego, USA) and resuspended in PBS. Protein concentration was quantified using silver staining of SDS-PAGE gel using lysozyme as a standard and analyzed using Quantity One software (Bio-Rad, Hercules, USA). MALDI-TOF was used to confirmed protein purity and the correct m.w.

Intracellular calcium mobilization assays

Release of calcium from intracellular stores was determined on freshly isolated PBNs resuspended in Minimal Essential Medium (MEM). PBNs at 5 x 10^6 cells/ml were loaded with 4

μM Fluo-4, AM (Molecular Probes from Invitrogen, Carlsbad, USA) for 60 min at 37 °C. Cells were then washed once with MEM and incubated for another 30 min for completion of the esterification process. Finally the cells were diluted to 1 x 10^6 cells/ml in MEM and for the calcium flux assay. Chemokines were added to 2 ml of cells at a final concentration of 100, 10 and 1 nM. Calcium flux was measured using a Photon Technology International Spectrophotometer (Birmingham, NJ, USA) at an excitation of 494nm nm and emission of 516nm, FeliX32 software for analysis. Relative intracellular calcium levels post stimulation were expressed as change in fluorescence = fluorescence after stimulation – background fluorescence (Δ Fluorescence) for each of the chemokines tested.

β2 integrin staining

1×10^6 cells PBNs were resuspended in RPMI-1640 with 1% FBS and exposed to 100 nM of chemokines for 2 h at 37 °C. Cells were washed with PBS and blocked with 1% goat serum. PBNs were incubated with fluorescently conjugated CD11a, CD11b, and CD11c antibodies (Caltag Invitrogen, Carlsbad, USA) 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 PBNs resuspended in HBSS with 0.1% BSA and 10 mM HEPES. Assays were performed in triplicate in 96-well chemotaxis plates. 30 μl of chemokines were loaded at varying concentrations (100 and 500nM) into the lower well of the modified Boyden chamber (Neuroprobe, Gaithersburg, USA) and fitted with a 5 μm filter. PBNs were labeled with 1:1000 CalceinAM (Invitrogen, Carlsbad, CA) for 1hr. on a rotating wheel at 37°C. Cells were washed with PBS and resuspended to 5×10^6 cells/ml. 20 μl were added to the upper well. The PBNs were allowed to migrate for 2-3 hr. at 37°C. The number of PBNs that migrated to the chemokines was measured on a fluorescent plate reader (Synergy 2, Biotek, USA) minus the fluorescence from the buffer only control wells.

Receptor binding analysis

The ability of vCXCL-1s to compete for binding to either CXCR1 or CXCR2 was evaluated as previously described (6) [152] [152] [152]. Briefly, 1×10^5 - 3×10^5 HEK293 cells stably over-expressing CXCR1 or CXCR2 were incubated with 100pM ^{125}I-labeled CXCL8 (MP Biomedical) and increasing concentrations of unlabeled chemokines for 1 hour at room temperature. Cells were collected on glass filters, washed twice, and bound radioactivity was measured with liquid scintillation counting. The graph was plotted and competition constants (IC_{50}) were analyzed using GraphPad Prism 5 for Windows.

^{35}S-GTPγS binding assay

Two days after transfection with CXCR2 expression constructs, HEK293T cells were detached from the plastic surface using ice-cold phosphate-buffered saline (PBS) and centrifuged at 1500*g* for 10 min at 4°C. The pellet was resuspended in ice-cold PBS and centrifuged. Cells were resuspended in ice-cold membrane buffer (15 mM Tris, 1 mM EGTA, 0.3 mM EDTA, and 2 mM $MgCl_2$, pH 7.5), followed by homogenization using a Teflon-glass homogenizer and rotor. The membranes were subjected to two freeze-thaw cycles using liquid nitrogen, followed by centrifugation at 40,000*g* for 25 min at 4°C. The pellet was rinsed once with ice-cold Tris-sucrose buffer (20 mM Tris and 250 mM sucrose, pH 7.4) and subsequently resuspended in the same buffer and stored (-80°C). Protein concentration was determined using a BCA-protein assay (Thermo Scientific, Rockford, USA).

Membranes (2.5 µg/well) were incubated in 96-well plates in assay buffer (50 mM Hepes, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.2 with 5 µg saponin/well, 3 µM GDP and approximately 500 pM of ^{35}S-GTPγS added) and the indicated concentrations of CXCL8 or vCXCL-1 to a final volume of 100 µl. The reaction mixtures were incubated for 1 hour at room temperature, harvested with rapid filtration through Unifilter GF/B 96-well filterplates (PerkinElmer, USA) and washed three times with ice-cold wash buffer (50 mM Tris-HCl and 5 mM $MgCl_2$, pH 7.4). ^{35}S-GTPγS incorporation was determined using a Microbeta scintillation counter (PerkinElmer, USA). Functional data were evaluated by a non-linear curve fitting using GraphPad Prism 4.0 (GraphPad Software, inc., San Diego, CA).

β-arrestin recruitment assay

PathHunter™ HEK293-CXCR2 cells were plated out overnight at $1x10^4$ cells/well (384-wells format) in 20 µl OPTI-MEM. A pre-incubation with vehicle (PBS + 0.1 % BSA) of 30 min at 37°C and 5% CO_2, was followed by 90 min with CXCL8 or vCXCL-1 stimulation at 37°C and 5% CO_2. 12 µl PathHunter Detection Reagents (DiscoveRx, Fremont, USA) was added. After 60 min. incubation at room temperature, β-galactosidase, as an indicator of β-arrestin-CXCR2 interaction, was measured for 0.3 sec in a Victor^2 1420 Multilabel Reader. Functional data were evaluated using a non-linear curve fitting using GraphPad Prism 4.0 (GraphPad Software, inc., San Diego, CA).

Quantitative real-time PCR of CCL22 expression

HL-60 T2 cell transfectants over-expressing CXCR2 were differentiated for 7 days with 1.3% DMSO prior to chemokine treatment. Medium was exchanged with HBSS and incubated with viral chemokines at a final concentration of 100 nM for 4 h at 37 °C. Total RNA was isolated with Tri-Reagent (Sigma, St. Louis, USA) and reverse transcribed using ProtoScript M-MuLV first strand cDNA synthesis kit (NEB, Ipswich, USA). Real-time PCR was performed using iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, USA) with a reaction mixture volume of 25 µl containing SYBR green (NEB DyNAmo SYBR green qPCR kit), 300 nM of each primer, and ~25ng 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 sec. and 60°C for 60 sec. The results were analyzed with the iQ5 Optical System Software (Bio-Rad, Hercules, USA). The relative gene expression levels were calculated as the fold change 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 $\Delta\Delta CT = \Delta CT_{reference} - \Delta CT_{target}$ [20]. The housekeeping gene encoding actin (ACT1) was used as a reference control. Primers for ACT1 were 5'-TGAGATGCATTGTTACAGGA-3' (forward) and 5'-CACGAAAGCAATGCTATCAC-3' (reverse) generating a 120-bp product.

Results

Amino acid sequence alignment

Previously, we sequenced the *UL146* gene from 51 clinical isolates and showed that it comprised 11 genetic clades [161]. Representative isolates from the 11 clades were aligned with vCXCL-1 from the Toledo strain (vCXCL-1_{Toledo}) (Figure 3.1). The percent identities of the mature forms of the vCXCL-1s, without the signal sequences, vary between 23.7% - 61.2% compared to vCXCL-1_{Toledo}. The vCXCL-1s contain ~20 additional residues on the carboxyl terminus compared to host chemokines CXCL1 and CXCL8, but the function of these extra residues is unknown. Alignment of the vCXCL-1s and the host chemokines show seven conserved residues, including the arginine (R) in the ELR motif, two cysteines (C) in the N-terminus (part of the CXC motif), a proline (P) at position 32, cysteines at position 35 and 55, and a leucine (L) at position 56. Furthermore, all vCXCL-1s contain a glycine (G), valine (V), histidine (H), tryptophan (W) and proline (P) at position 21, 54, 60, 65 and 87, respectively, which are lacking in the host chemokines. The ELR motif was conserved in all except vCXCL-1_{TX15}. The variability in the N-loop region [21][409], C-terminus [22, 23], and even in the ELR motif [24], led us to evaluate differences in chemokine receptor binding and functional responses [25][410].

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. Unlike protein production from prokaryotes, baculovirus expression provides mammalian signal-sequence cleavage, eukaryotic glycosylation patterns and protein folding. Because some vCXCL-1s contain multiple predicted signal cleavage [6][152] and glycosylation sites, and differences in recombinant protein refolding conditions, we chose to express and purify them using the baculovirus system. All vCXCL-1s were 6 His-tagged and purified using Ni-NTA agarose beads. Purity was confirmed with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and resulted in the predicted molecular weights (11-15 kDa).

vCXCL-1s stimulate calcium release in PBNs

Release of intracellular calcium is a common indicator of chemokine activation of PBNs [6, 26][152, 354]. CXCL8 and CXCL1 were shown to induce calcium flux at similar concentrations via CXCR2 [27]. To investigate vCXCL-1's activation of PBNs, vCXCL-1s from the different strains were added to freshly isolated PBNs. All vCXCL-1s induced calcium flux at a concentration of 100 nM including 100751, which is not shown in Figure 3.2. However they differ in their ability to induce a calcium flux at other concentrations tested (Figure 3.2). This demonstrates that even though the viral chemokines can induce calcium mobilization in PBNs, the different vCXCL-1s have differing sensitivities for calcium signaling that may induce differential downstream activation of PBNs.

vCXCL-1s upregulate CD11b and CD11c

β2 integrins are receptors that form heterodimers composed of an α component, such as CD11a, CD11b, and CD11c, and a β component, CD18. They are present on circulating leukocytes and, once the cell is activated, initiate adhesion to endothelial cells and subsequent transmigration across the endothelium [28]. Host chemokine, CXCL8, upregulates CD11b and CD11c expression [29, 30]. Moreover, vCXCL-1_{Toledo} and the vCXCL-1 from chimpanzee CMV also increases integrins on PBNs [26]. In this study, we tested the ability of vCXCL-1s to alter the surface expression of these receptors on PBNs (Figure 3.3). Exposure to the vCXCL-1s or host chemokines, CXCL1 and CXCL8, does not change cell surface expression levels of CD11a. However, CD11b and CD11c levels are increased upon exposure to either the vCXCL-1s or host chemokines. The percent change in the mean fluorescent intensity of CD11b was 57-91% for the viral chemokines, which similar to CXCL1 upregulation (82%) but less than CXCL8 (143%). Likewise, the percent change of CD11c varied from 35-55% for the vCXCLs, which is similar to CXCL1 (43%) but lower than CXCL8 (80.3%). These results demonstrate that the viral chemokines selectively induce β2 integrins (CD11b and CD11c) upregulation but without significant differences between them at 100nM.

Differential migration of human PBNs

Both CXCL8 and vCXCL-1_{Toledo} are potent chemoattractants for PBNs [15, 26][152, 411]. Even though there were no differences in calcium flux and integrin expression, these readouts could have a lower threshold for activation compared to a more complex PBN function like migration. We quantified the PBNs that chemotaxed to different vCXCL-1 concentrations and found differences in their migratory ability (Figure 3.4). All vCXCL-1s except vCXCL-1_{TX24} and vCXCL-1_{TX15} induce migration at 500nM while at 100nM only vCXCL-1_{C952}, vCXCL-1_{E760}, vCXCL-1_{Toledo}, vCXCL-1_{100751}, and vCXCL-1_{C956} could stimulate migration. This is the first time that differences between the different vCXCL-1s were observed in a functional assay.

Affinities for CXCR1 and CXCR2

Because some CXC chemokines such as CXCL8 bind to both CXCR1 and CXCR2 and these receptors are important for chemotaxis [31-33], we investigated receptor usage and affinity of the different chemokines for CXCR1 and CXCR2. Competition binding assays using the vCXCL-1s to displace ^{125}I-CXCL8 on HEK293 cells expressing either CXCR1 or CXCR2 (Figure 1.5) showed IC_{50} concentrations that ranged from 2.6 - 148.7 nM for CXCR2 and 3.3 nM to > 1,000 nM (i.e. no competition) for CXCR1. Using cluster analysis of the averages of the different IC_{50}'s we divided the chemokines into high, medium-high, medium-low, and low affinity binders for CXCR2 (Figure 3.5A). The group of high affinity binders (2.6 - 3.6 nM) along with CXCL8, are vCXCL-1_{Toledo} and vCXCL-1_{C952}. Medium-high affinity binders (11.3 - 18.6 nM) are vCXCL-$1_{Tx11,}$ vCXCL-1_{E760}, vCXCL-$1_{C956,}$ vCXCL-1_{100751} and medium-low members are vCXCL-$1_{102410,}$ vCXCL-1_{Tx24}, vCXCL-1_{C954} (32.7 - 55.5 nM). The low affinity group (> 141 nM) contains only two members, vCXCL-1_{Towne} and vCXCL-1_{Tx15}. Interestingly, the viral chemokines with high affinity for CXCR2 (vCXCL-1_{Toledo} and vCXCL-1_{C952}) have weak binding to CXCR1 compared to the host chemokines. Generally the higher the affinity for CXCR2 the more likely the viral chemokines will bind to CXCR1 (Figure 3.5B). The medium-high CXCR2

binders generally do not bind to CXCR1 except for vCXCL-1_{E760}. These data indicate that the viral chemokines bind with differing affinities for CXCR2 with weak to no binding to CXCR1. All vCXCL-1s regardless of their affinity for CXCR1 or CXCR2 (except vCXCL-1_{TX24} and vCXCL-1_{TX15}) induce migration above the limit of detection at 500nM. At the lower concentration (100 nM) only the high affinity or the select medium-high affinity binders (i.e., vCXCL-$1_{E760,}$ vCXCL-1_{100751} and vCXCL-1_{C956}) could induce migration (Figure 3.4).

This data implies that affinity for CXCR2 (i.e., high affinity equals high migration) and/or CXCR1 usage are potential factors in PBN migration [32, 33][64, 412]. Because it is not strictly correlated with affinity, differential agonist activation signals could also contribute to PBN migration as well.

vCXCL-1s induce differential ^{35}S-GTPγS binding and β-arrestin2 recruitment

Chemotactic responses can be mediated via G-protein dependent and/or G-protein independent signaling. Berger *et al* [30] demonstrated that CXCL8-induced β2 integrin CD11b upregulation and migration of neutrophils is $G\alpha_i$ dependent. Chemokine-induced calcium flux involves $G\alpha_i$ proteins as well [34, 35]. Based on these studies and observation of differences in migration and binding, we investigated whether vCXCL-1s display differences in G-protein dependent and independent signaling that could explain the differences in migration. ^{35}S-GTPγS binding experiments were performed on HEK293T membranes expressing human CXCR2 (Figure 3.6A-B). The pEC_{50} value of CXCL8 in this assay is 6.9. Only CXCL1 and vCXCL-1_{Toledo} are able to reach a maximal response equivalent to 1 μM CXCL8. vCXCL-1_{Toledo} is a high affinity CXCR2 agonist capable of inducing migration (Figure 3.6) and uses G proteins (Figure 3.6A). Surprisingly, vCXCL-1_{C952}, another high affinity binder of CXCR2 that induces PBN migration does not induce a G protein response. All those with medium-affinity for CXCR2, except for vCXCL-$1_{TX11,}$ have medium potency for G protein binding, regardless of their ability to induce migration. vCXCL-1_{TX11} has a medium-high affinity for CXCR2 and induces PBN migration but does not use G proteins for inducing this response. As expected, those with low affinity for CXCR2 had no GTP binding (vCXCL-1_{Towne} and vCXCL-1_{TX15}). Based on the dose response curves, we propose a potency order of the chemokines for CXCR2: CXCL8 ~ CXCL1 ~

vCXCL-1_{Toledo} ≥ Intermediate: vCXCL-1_{E760}, vCXCL-1_{100751}, vCXCL-1_{C956}, vCXCL-1_{102410}, vCXCL-1_{TX24}, vCXCL-1_{C954} > vCXCL-1_{C952}. No response: vCXCL-1_{Towne}, vCXCL-1_{TX11}, vCXCL-1_{TX15}. These results illustrate that the different vCXCLs use G protein dependent mechanisms that correlate with affinity for CXCR2 except in two cases (vCXCL-1_{C952} and vCXCL-1_{TX11}).

Traditionally, β-arrestin proteins were thought to function only to desensitize activated GPCRs. However, in the last decade β-arrestins were shown to induce intracellular signaling as well [36, 37]. The involvement of β-arrestins in chemokine-induced chemotaxis was first described for the CXCR4/CXCL12 axis [413] and involves the p38 MAPK pathway [38]. Following from these studies, a role for β-arrestin2 in CXCR2 directed chemotaxis was shown [23, 40, 41]. β-arrestin2 induced chemotaxis could explain the differences seen with the different chemokines (Figure 3.6). To measure chemokine-induced β-arrestin2 recruitment, we used the PathHunter-HEK293-CXCR2 indicator cell line, which produces a functional β-galactosidase in response to β-arrestin2 [42]. The pEC_{50} value of CXCL8 in this assay is 9.1. Figure 3.6C-D shows that CXCL8, CXCL1, vCXCL-1_{Toledo}, vCXCL-1_{C952} and vCXCL-1_{E760} make full dose-response curves, whereas the other viral chemokines display incomplete curves or no β-arrestin2 signalling. Based on these data the potency order of the vCXCL-1s for β-arrestin2 activation is: CXCL8 (pEC_{50} = 9.1 nM) [high affinity for CXCR2] ≥ CXCL1 (pEC_{50} = 8.3 nM) [high affinity for CXCR2] ~ Toledo (pEC_{50} = 8.4 nM) [high affinity for CXCR2] ~ E760 (pEC_{50} = 8.1 nM) [med-high affinity for CXCR2] ≥ C952 (7.5 nM) [high affinity for CXCR2] ≥ C956 [med-high affinity for CXCR2] ~ 102410 [med-low affinity for CXCR2] ~ 100751 [med-high affinity for CXCR2] ~ C954 [med-low affinity for CXCR2] ~ TX24 [med-low affinity for CXCR2] ≥ C952 [med-low affinity for CXCR2] ≥ TX15 [low affinity for CXCR2] ~ TX11 [med-high affinity for CXCR2] ~ Towne [low affinity for CXCR2]. For the most part, high affinity for CXCR2 or CXCR1 tracks with β-arrestin2 activation. There are a few exceptions. A medium-high affinity binder, vCXCL-1_{C956}, did not induce β-arrestin2 while the low affinity, vCXCL-1_{Towne}, did signal. These data point to differential signalling or "biased agonism" that leads to differential G protein activation and β-arrestin2 potencies not directly correlated with receptor affinity [43].

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

We have observed differences in migratory ability, G protein activation, and β-arrestin2 recruitment, but how could these phenotypes affect HCMV dissemination or pathogenesis? HCMV productively infects macrophages and dendritic cells and may have evolved vCXCL-1s to increase the recruitment of these cell types via neutrophil activation. Macrophage derived chemokine (MDC), CCL22, recruits multiple immune cells, such as monocytes, dendritic cells, NK cells, and the Th_2 subset of T cells [44]. The induction of CCL22 could have profound effects on the recruited cell types as well as the immune response to CMV. Not only could these cells increase dissemination and/or CMV replication, CCL22 could also lead to an increase in the Th2 response and a down-regulation of Th1 responses [45, 46]. In fact, another UL/b' protein, UL144, upregulates CCL22 and has been implicated in immune modulation (i.e., recruitment and activation of Th_2 and T_{reg}) [47]. In order to address whether the vCXCL-1s induce CCL22, we performed quantitative real-time PCR for CCL22 expression on a neutrophil-like cell line that over expresses CXCR2 (Figure 3.7). vCXCL-1_{Toledo}, vCXCL-1_{E760}, and vCXCL-$1_{C952,}$ had the highest induction of CCL22, which is similar to CXCL1. vCXCL-1_{Toledo} and vCXCL-1_{C952} are high-affinity CXCR2 binders while vCXCL-1_{E760} belongs to the medium-high group. Others, in the medium-high binding group, except vCXCL-1_{100751}, induce CCL22. In contrast, all the members in the medium-low (i.e., vCXCL-1_{102410} and vCXCL-1_{C954}) or low affinity group (i.e., vCXCL-1_{Tx15} and vCXCL-1_{Towne}) except for vCXCL-1_{Tx24} do not induce CCL22. As seen in with PBN migration, G protein and β-arrestin2 usage, high affinity binders activate downstream signaling and functional outcomes while the medium binders are variable and low affinity binders do not except for calcium flux and integrin upregulation.

Discussion

Our findings contribute to our understanding of the functions of the HCMV viral chemokines and their agonist activation of CXCR2. In trials where different HCMVs were inoculated into volunteers, role of the viral chemokines was suggested in human disease. For example, the Towne strain of HCMV was less virulent than the Toledo virus in humans. Towne differs from the Toledo strain in the ULb' region which contains the *UL146* and *UL147* viral chemokine

genes [48-50]. Here we have shown that Towne produces a vCXCL-1 with a low affinity for CXCR2, induces a lower calcium flux (with no induction at 1nm), minimal ability for chemotaxis, and no signaling compared with the more virulent Toledo strain vCXCL-1 (Table 3.1). Although this is only circumstantial evidence and one of several differences between the Toledo and Towne strains, vCXCL-1 differences in PBN activation are potentially contributing factors to the HCMV virulence observed in these studies. Other animal models of HCMV pathogenesis provide a more direct link between viral chemokines and pathogenesis. The guinea pig CMV (GPCMV) chemokine homolog functionally signals through the CCR1 receptor and plays a role in viral dissemination *in vivo* [51-53]. Furthermore, this virally induced inflammation contributes to cytomegalovirus-related inner ear injury (i.e., auditory pathology) [54][414]. Whether the differences in the vCXCL-1s contribute to HCMV virulence and/or dissemination in a similar manner to this animal model remains to be tested. The role of vCXCL-1s in human pathogenesis is especially difficult without knowing the concentrations of these chemokines during an active HCMV infection *in vivo*..

We propose two non-exclusive models for how HCMV vCXCL-1s could function *in vivo*. One model for HCMV dissemination is the "neutrophil shuttle model". In this model the neutrophil functions as a vehicle for HCMV dissemination [55]. PBNs pick up HCMV during neutrophil transendothelial migration and subsequently transmit infectious virus to fibroblasts [56, 57]. We analyzed PBN induction of calcium flux and adhesion molecules upon vCXCL-1 treatment as indicators of neutrophil activation (Figure 3.2, 3.3), which could affect subsequent cell-mediated viral dissemination [58]. vCXCL-1 activated PBNs could transport virus and allow it to infect surrounding tissues or different cells. Ideally we would address this shuttling effect directly with an antigenemia assay where vCXCL-1-treated neutrophils are assayed for their ability to "take up" HCMVs after migration through an infected monolayer [59].

Unfortunately, potential differences in migration were masked by the large amount of the host chemokines that are secreted following HCMV infection of the fibroblast monolayer (data not shown). These "background" host chemokines conceal the effects of the vCXCL1s in this *in vitro* model system. In the present study, although the binding affinities to CXCR2 and/or CXCR1 were variable (Figure 3.5), all vCXCL-1s induce intracellular calcium mobilization in PBNs, albeit to different degrees at the concentrations tested (Figure 3.2) and upregulate β2

integrins on the surface of PBNs (Figure 3.3) similar to levels induced with human CXCL1 or CXCL8. We speculate that vCXCL1-s from all the clades activate PBNs to increase contact with the endothelium. After activation/adhesion neutrophils could be induced to migrate to the site of HCMV infection. To further investigate this possibility, we measured vCXCL-1-induced migration. The resulting chemotaxis profile did not directly correlate with receptor affinity (Figure 3.5C). Although the majority medium affinity vCXCL-1s had migration only at 500nM, others had none at all (vCXCL-1_{TX24}) or at lower concentrations (100nM) (vCXCL-1_{100751}). This leads us to conclude that CXCR2 binding affinities do not directly correlate with subsequent PBN activation, integrin upregulation or chemotaxis patterns. This may not be too surprising as others have observed decreases in CXCR2 affinity while still inducing a calcium flux [60, 61] and elastase production [21][409]. Others have observed a complex relationship between binding and activation similar to our observations with our medium affinity vCXCL-1s [62]. These data illustrate the complexity of the CXCR2 response to agonist stimulation and its relationship with affinity.

A complementary/alternate model to explain the relationship between the vCXCL-1s, PBNs, and HCMV is the "neutrophil amplifier model". This model focuses on vCXCL-1 induction of exocytosis of neutrophilic granules or secretion of specific cytokines/chemokines. These inflammatory mediators could increase inflammatory responses that subsequently recruit other immune cells [63]. These infiltrating immune cells would provide a better vehicle for HCMV spread. Macrophages and dendritic cells are better targets for HCMV infection because HCMV can productively infect them [64-67] while PBN infections are nonproductive [57]. The attraction/differentiation of myeloid cells could provide a means to infect a cell type that allows for more efficient virus production and/or dissemination within the host [65]. The vCXCL-1s induce differential CCL22 production that could have effects on myeloid cell chemotaxis. In our studies, the upregulation of CCL22 correlates with the vCXCL1s' affinity for CXCR2 (Figure 3.7). 57% of medium-affinity vCXCL-1s induce CCL22 expression (vCXCL-1_{TX11}, vCXCL-1_{E760}, vCXCL-1_{C956}, and vCXCL-1_{TX24}) while others did not (vCXCL-$1_{1000751}$, vCXCL-1_{102410}, and vCXCL-1_{C954}). The neutrophil amplifier model would predict that those viruses that do not induce CCL22 in PBNs would be less pathogenic, but we have no *in vivo* data for this. Comparisons of the sequelae from HCMV congenitally infected infants, the vCXCL-1's that

induce CCL22 do not correlate with clinical outcomes [17]. Our interpretation of this data cannot completely exclude the shuttle model our study only measured a single inflammatory chemokine and others chemokines/cytokines such as CCL2, CCL3, and CCL7 that were not measured could have a role in congenital sequelae.

This study is the first to examine how the natural variation in the vCXCL-1s affects binding and PBN function. These variants provide an opportunity to assess how changes within CXC chemokines affect signaling as a "biased agonist." Biased agonists stimulate GPCRs with differential signaling and functional outcomes [43]. The activation of CXCR2 initially appears to be redundant. Host CXCL1, CXCL2, CXCL3, CXCL6 and CXCL8 all bind and activate CXCR2. Recently *Rajagopal et al* [68][415] measured β-arrestin2 recruitment, cAMP signaling, and internalization with the different ligands on CXCR2. These related chemokines displayed a biased agonism for cAMP and β-arrestin2 activation. Our study found that high affinity for CXCR2 leads to activation of G protein dependent and independent signaling (Figure 3.6). As expected, those vCXCL-1s with low affinity for CXCR2 do not initiate detectable signaling. The chemokines with medium range affinity are more complex. Some have moderate G protein signaling without β-arrestin2 (vCXCL-1_{C956}) or no G protein activation with only β-arrestin2 signaling (vCXCL-1_{TX11}). Our data suggest a complex robustness to the viral chemokine response that only partially correlates with affinity.

In conclusion our data suggest that polymorphisms in the vCXCL-1s elicit differential affinity to CXC chemokine receptors, which generates varying cellular responses or differential activation and triggering of diverse downstream signals. High affinity for CXCR2 leads to activation of G protein dependent and independent signaling with full activation of calcium flux, integrin expression, and CCL22 transcription (Table 3.1). Those with low affinity for CXCR2 still induce calcium flux and integrin expression while not initiating detectable signaling or CCL22 expression and modest PBN migration. These data point to different thresholds for the different neutrophil functions. Calcium flux and integrin expression have low thresholds where any degree of stimulation will activate them [69]. Other functional outcomes (i.e., migration, signaling, or CCL22 expression) are more complex. Generally the extremes in affinity (i.e., high or low) correlate with signaling, migration, and CCL22 production. Those with medium range affinity are more complex and result in varying degrees of activation [70][416]. This nuanced

response points to the biased agonism of these novel vCXCL-1s that could affect neutrophils and we speculate an effect on subsequent HCMV dissemination or virulence.

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Appendix: Figures and Tables

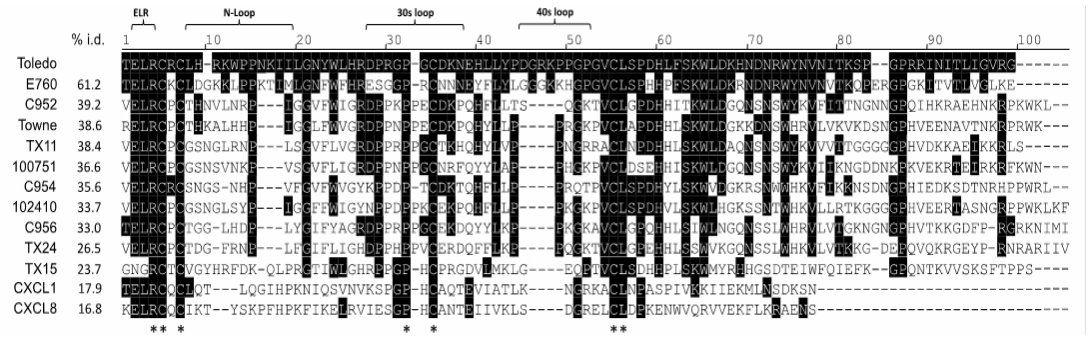


Figure 3.1 Amino-acid alignment of the mature forms of recombinant vCXCL-1s and the host chemokines CXCL1 and CXCL8 with vCXCL-1 toledo. Seven amino acid residues that are 100% conserved are indicated with an *. The important ELR, N-loop region, and 30s and 40s loops are indicated

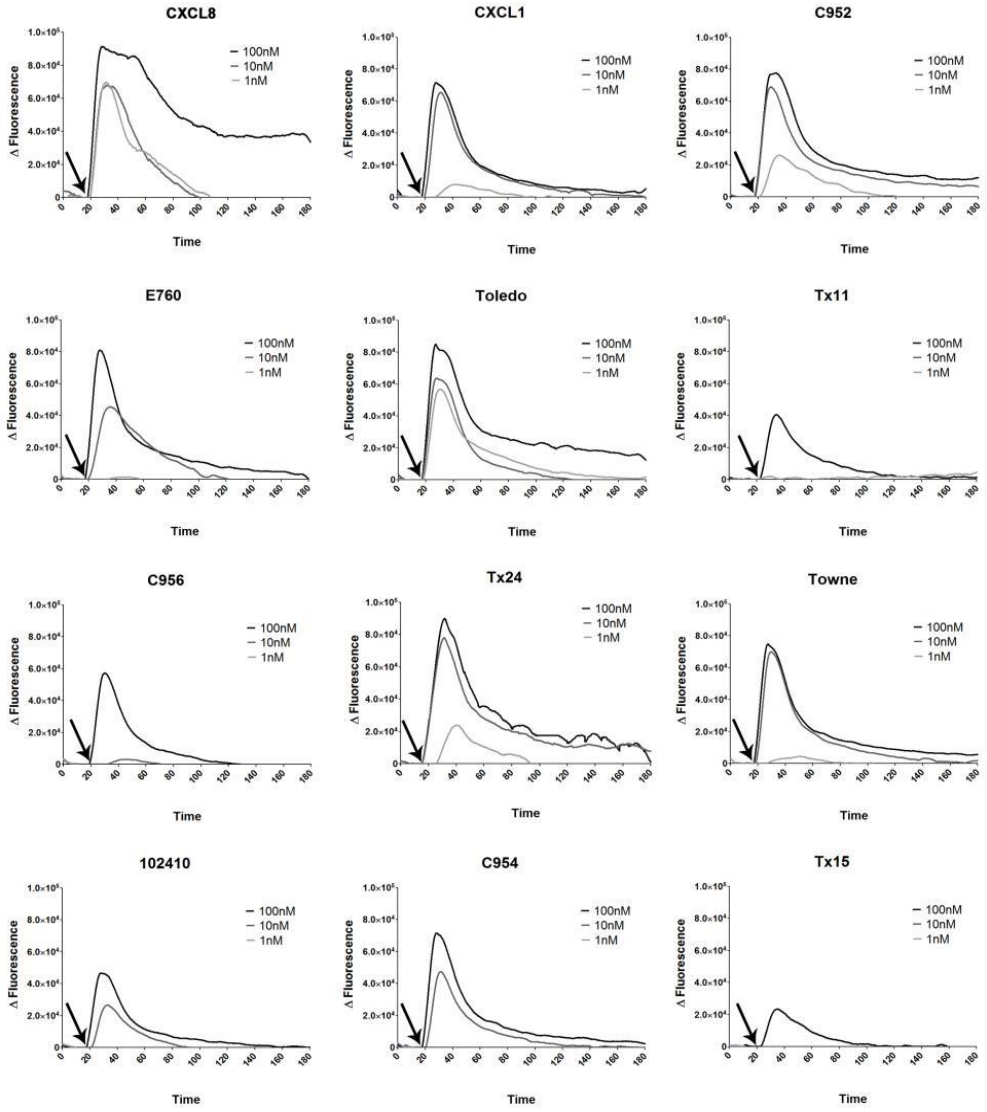
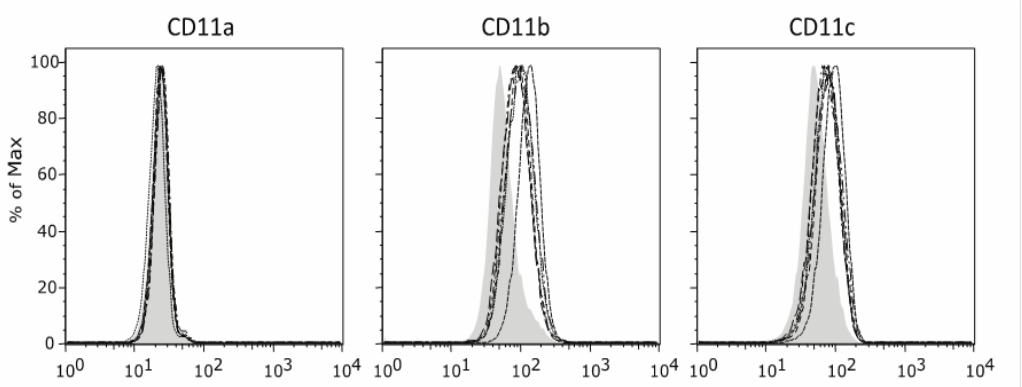


Figure 3.2 CXCL8, CXCL1, and the different vCXCL-1s induce intracellular calcium mobilization on human PBNs. Changes in fluorescence were measured over time after exposure to different concentrations of chemokines (after 20 seconds at baseline as indicated with an arrow). Data shown are representative figures of three independent experiments.



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

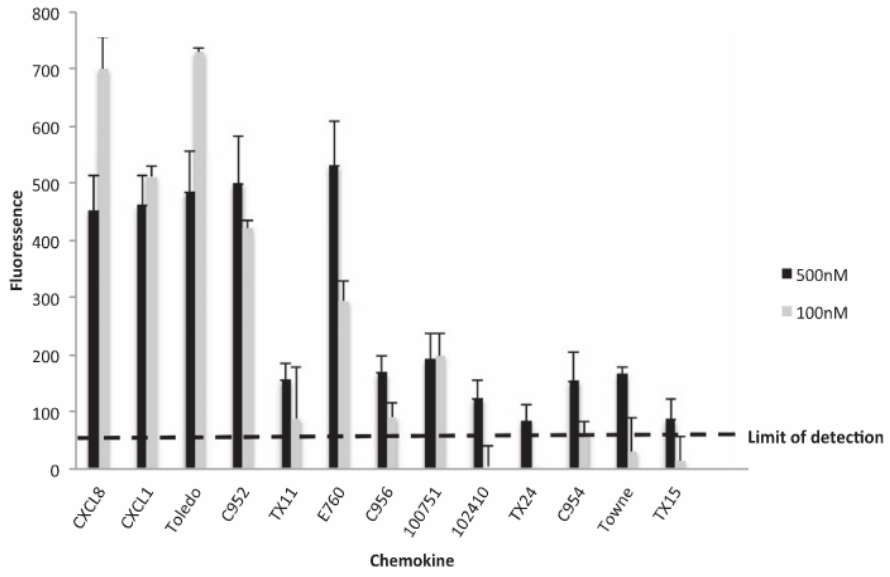
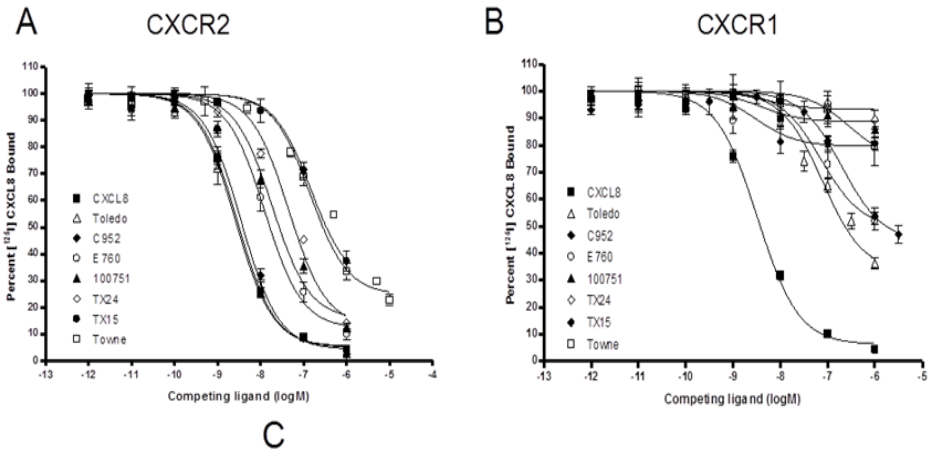


Figure 3.4 Differential chemotaxis of PBNs to vCXCL-1s partially correlates with affinity. Chemotactic response of human PBNs to 500 nM and 100nM of CXCL8, CXCL1, or vCXCL-1s. The chemotactic response was measured as fluorescence intensity of migrated PBN labeled with CalceinAM. Background chemotaxis was subtracted from all samples. Data shown are representative data of three independent experiments performed in triplicate**.**



C

Chemokine	50% effective concentration (EC_{50})	
	CXCR2	CXCR1
CXCL8	2.9 ± 1.06	3.3 ± 1.07
Toledo	2.6 ± 1.16	>84.4 ± 1.15
C952	3.6 ± 1.14	>203.0 ± 1.23
TX11	>11.3 ± 1.16	>1000
E760	>12.4 ± 1.17	>73.8 ± 1.44
C956	>12.8 ± 1.14	>1000
100751	>18.6 ± 1.16	>1000
102410	>32.7 ± 1.16	>1000
TX24	>44.9 ± 1.17	>1000
C954	>55.5 ± 1.15	>1000
Towne	>141.7 ± 1.16	>1000
TX15	>148.7 ± 1.29	>1000

Figure 3.5 vCXCL-1s have different binding affinities for human CXCR1 or CXCR2. Displacement of ^{125}I-CXCL8 binding to HEK293 cells stably expressing human CXCR2 (A) or CXCR1 (B). Cells were incubated with indicated concentration of vCXCL-1s and 200 pM ^{125}I-CXCL8 for one hour at room temperature. For simplicity, curves for TX11, C956, 102410, and C954 are not shown. (C) The average IC_{50} +/- standard error for all vCXCL-1s for either CXCR2 or CXCR1 (n=3-12). Those with incomplete competition curves are indicated with a > of a predicted IC_{50}. Those chemokines with no competition at all concentrations tested are listed as >1000 IC_{50}.

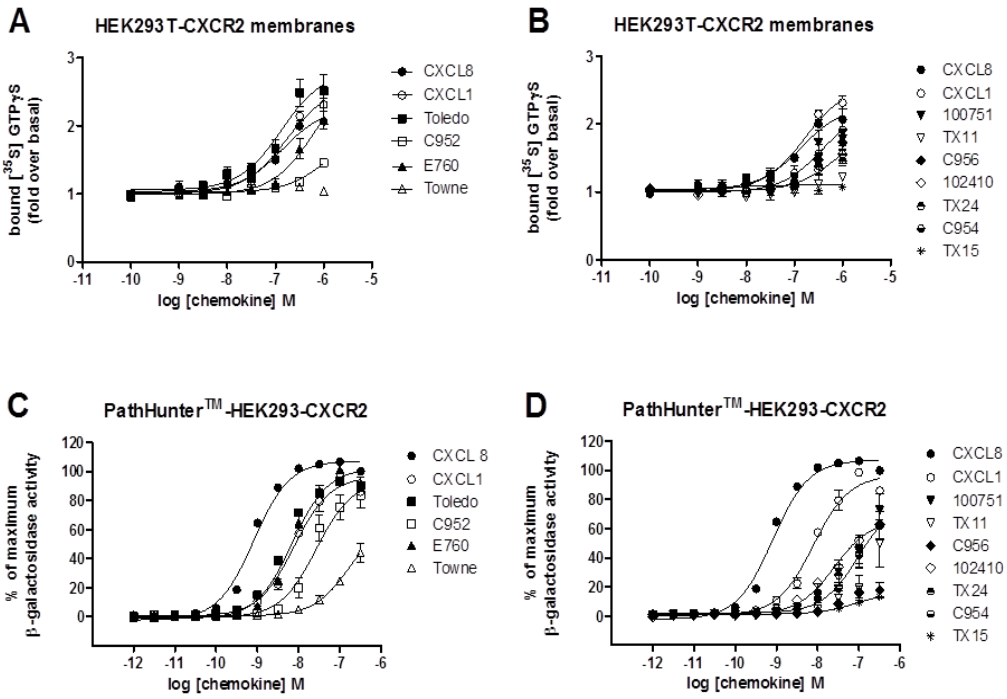


Figure 3.6 G protein activation and β-arrestin2 signaling correlates with CXCR2 affinity. vCXCL-1 chemokine induction of ^{35}S-GTPγS binding to HEK293T membranes expressing CXCR2 (A-B). Data are corrected for basal ^{35}S-GTPyS binding (n=3-4). vCXCL-1 β-arrestin2 recruitment in PathHunter™ indicator cells (C-D). Data are expressed as percentage of β-galactosidase activity**,** in which the response to 1 μM CXCL8 is set to 100% (n=3-4).

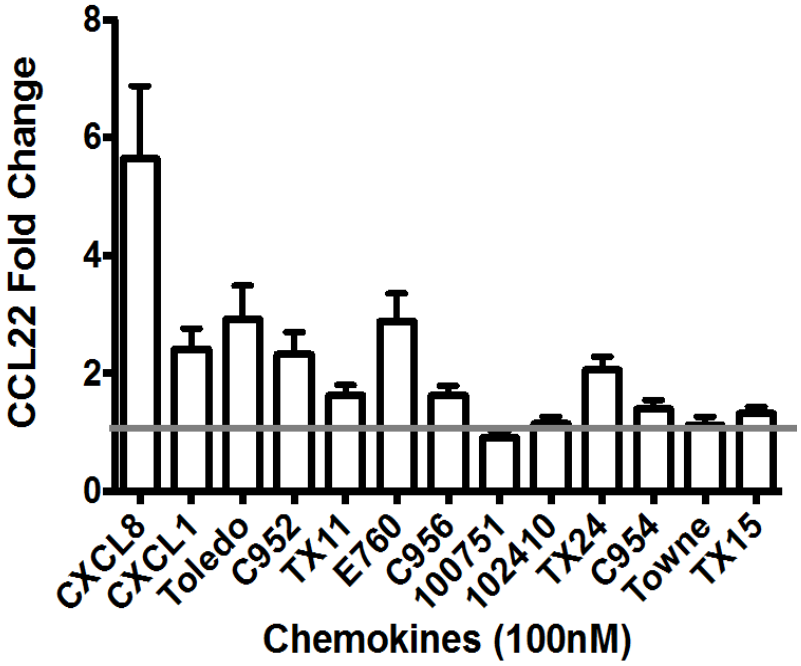


Figure 3.7 vCXCL-1s differentially induce CCL22 expression via CXCR2. Neutrophil-like HL60 T2 cells were incubated in the presence of 100 nM of the indicated vCXCL-1s or host chemokine. Each bar represents the average from three separate experiments of the fold change in CCL22 mRNA expression levels (stimulated /unstimulated cells +/- SEM). All data are normalized to β-actin mRNA expression levels. A ratio of 1, indicated with a gray line, represents no change in expression compared to unstimulated cells.

Table 3.S1 Summary of vCXCL-1 functional outcomes

Key: +++ highest activation; ++ high activation; + activation; +/- weak activation; - no

Chemokine:		Ca++ Flux	Integrin Expression	Migration	G Protein Binding	β Arrestin 2	CCL22 Expression
High affinity	CXCL8	++	++	++	++	++	+++
	Toledo	++	+	++	++	++	++
	C952	++	+	++	++	++	++
Medium-High affinity	TX11	+/-	+	+/-	-	+/-	+
	E760	+	+	+	+	++	++
	C956	+/-	+	+/-	+	-	+
	100751	+	+	+	+	+	-
Medium-Low affinity	102410	+	+	+/-	+	+	-
	TX24	++	+	-	+	+	+
	C954	+	+	+/-	+	+	-
Low affinity	Towne	+/-	+	+/-	-	-	-
	TX15	+/-	+	-	-	-	-

CONCLUSION

Research conclusion and summary

Lifelong infection is a characteristic of herpesvirus infections. In the case of human cytomegalovirus (HCMV) healthy adults will experience lifelong latency. However reactivation or primary infection causes increased morbidity and mortality in immune compromised hosts and congenitally infected newborns [1]. HCMV is treated with nucleoside analogs that inhibit viral DNA replication [2]. Current treatments exhibit varying degrees of toxicity [3]. An added concern is the increasing prevalence of HCMV strains that are resistant to the current antivirals. New therapeutics that target other steps in CMV infection should be effective against the aforementioned resistant strains. CMV binds to negatively charged heparan sulfate on the cell surface during the attachment step of viral entry into the cell [4, 5], making it a potential target for anti-CMV drug development. We targeted this step of the CMV infection lifecycle to develop novel therapeutics. Chapter 1 investigates the efficacy of a protease resistant D-peptide against antiviral resistant HCMVs *in vitro* and against MCMV *in vivo*. The D-peptide was shown to resist protease action and persist in serum compared to its L-peptide counterpart. The D-peptide reduces HCMV infection by greater than 80% in all strains tested. Furthermore our D-peptide reduces MCMV infection of peritoneal exudate cells when administered prior to infection. When the D-peptide is combined with a currently used antiviral treatment it reduces the EC_{50} of that antiviral. This data suggest that our D-peptide may serve as an efficacious anti-CMV therapeutic that can be used to treat GCV resistant HCMV or in conjunction with current antivirals to reduce CMV infection.

HCMV *UL146* gene encodes a CXC chemokine that shows variation among different isolates which may contribute to their varying clinical outcomes [6]. We evaluated the role of HCMV *UL146* encoded chemokine, vCXCL-1, *in vivo* using the murine model of CMV infection (Chapter 2). Recombinant murine CMV (MCMV) expressing the chimpanzee CMV (CCMV) vCXCL-1 (vCXCL-1_{CCMV}) and murine CXCL-1 (KC), mCXCL-1 recombinants were generated to test whether viral or host chemokines contribute to viral dissemination. We hypothesized that vCXCL-1s are functional CXC chemokines that contribute to viral dissemination similar to the MCMV CC chemokine (MCK-2). Primary dissemination is

comparable between the parental RM4511, which does not express a chemokine, and the recombinant MCMVs. However neither MCMV vCXCL-1_{CCMV} nor MCMV mCXCL-1 reached the salivary glands. SCID mice and neutrophil depletions in mice were used to determine whether the adaptive immune system or neutrophils were playing a role in preventing dissemination. Recombinant viruses still did not disseminate in the SCID or neutrophil depleted mice. Only when mice are immune ablated using cyclophosphamide are the recombinant viruses able to disseminate to the SG. To test whether other innate cells are involved in the dissemination defect, NOD-NSG mice lacking T lymphocytes, B lymphocytes, and NK cells were infected with recombinant viruses. In these mice, recombinant viruses disseminate. These results suggest that over expression of CXC chemokines induces cells of the innate immune system preventing viral dissemination to the SGs. SG dissemination is rescued when recombinants are co-infected with the parental RM4511. Co-infection of the same cell at the site of infection is crucial for rescue of dissemination of the recombinants. We hypothesize that co-infection creates a resource competition resulting in less chemokine being produced. This subsequently allows for dissemination to the SG.

The HCMV genome codes for immune modulatory proteins. One specific example is the *UL146* gene which encodes a viral CXC chemokine, vCXCL-1. vCXCL-1 varies greatly between clinical isolates and is associated with varying clinical outcomes [6,7]. vCXCL-1 facilitates viral transmission from endothelial cells to peripheral blood neutrophils (PBNs) *in vitro* during co-culture [8, 9]. HCMV transmission requires contact between the endothelial cells and PBNs during which a transitory microfusion event allows the virus to infect PBNs and disseminate through the bloodstream. It is suggested that HCMV encoded vCXCL-1 is important for recruitment of PBNs for dissemination rather than productive infection in these cells. This study characterized the vCXCL-1 protein from different clinical isolates *in vitro* (Chapter 3). Because the hypervariability observed in the *UL146* gene occurs within the receptor binding motifs, we hypothesized that variability in vCXCL-1 leads to differential activation of neutrophils, leading to the observed difference in HCMV pathogenesis. vCXCL-1 proteins were synthesized and isolated using a baculovirus protein expression system. Our data show that all vCXCL-1s bound to CXCR2 with different affinities, whereas only three bound to CXCR1. All vCXCL-1s induced intracellular calcium mobilization in PBNs. The chemotaxis of PBNs varies

based upon the binding affinities of vCXCL-1s to CXCR2. Furthermore vCXCL-1s differentially upregulate CD11b and CD11c on the surface of PBNs. The induction of CCL22 is dependent on the affinity of the CXCL-1 for CXCR2. CCL22 can attract monocytes, dendritic cells, and Th2 cells [10]. Once activated monocytes and dendritic cells support productive infection of the virus [11-13]. Additionally a Th2 response can promote viral pathogenesis by diminishing cytotoxic T lymphocyte responses [14, 15]. These data show that variation in vCXCL-1 among different isolates affects neutrophil activation which may contribute to CMV pathogenesis.

Future Directions

Chapter I:

In this chapter we tested the D-form of a heparan sulfate (HS) binding peptide as a potential anti-CMV therapeutic. However there are a few questions still remaining that need to be addressed to evaluate the full potential of these peptides as therapeutics.

1. We propose that our peptide interacts with cell surface HS and that this binding is not indiscriminate (un-published data from Dr. Wall). It is necessary to define the HS subtype that the peptide binds to. It is also important to know if the same subtype is used by the virus to attach to the cell during infection. This can be done by either performing peptide-HS pulldown assays using biotinylated peptide or using HS arrays available at the University of Georgia, Complex Carbohydrate Research Center. This data will help reveal the specificity of the peptides for HS subtypes. By narrowing down which subtype the virus uses for attachment we can improve the peptide composition to diminish any side effects associated with non-specific binding. In addition, this knowledge will be of great interest to CMV biologists, as it might help in understanding CMV's cell tropism *in vivo*. We could test this further by performing RT qPCR to determine what HS subtypes are expressed by cells infected with CMV.
2. In the future, we would like to identify the sequence and structural features of the peptides that contribute to their function. This will help in the development and optimization of the peptides. For example, from our experiments and previous study [16], peptides with Arg function better than peptides with Lys, even if they have the same

charge. In addition, the greater the positive charge on a peptide the better it functions. Future experiments to test the importance of certain amino acids, charge and length can be carried out by using Arg and Lys containing peptides of different lengths and charges to identify the optimum inhibition of CMV infection. Structural features of the peptide may also contribute to the peptide functionality. Secondary structure might contribute to the turnover and ability of the peptide to bind to the cell surface. Unpublished work in our lab suggests that a non-conforming secondary structure with a positive 14 charge reduces HCMV infection in fibroblasts when added at a less than 1μM. As a comparison p5R is added at 200 μM to reach the same reduction.

3. Cationic peptides and the HS are internalized after binding [17]. It would be interesting to test if our peptides also get internalized and to determine when the peptide is internalized in order to indicate how long the peptide is efficacious. By determining the turnover rate for the peptides and HS we would be able to determine the timing of peptide administration to inhibit viral infection.
4. Although we did not observe any cytotoxicity for the peptide *in vitro*, in order to develop the peptides for *in vivo* use, toxicity assays in animals should be carried out. Mice will be treated intravenously or potentially intraperitoneally with increasing concentrations of peptides. Weight will be monitored in treated mice. Finally fixed and stained tissue analysis would be used to determine any overt toxicity, inflammation, or necrosis potentially caused by peptide treatment. Peptides with modifications to increase their stability and bioavailability e.g. acylation and PEGylation [18] need to be tested *in vitro* before proceeding to *in vivo* testing. *In vitro* testing would suggest whether these modifications alter function of our peptide. We would treat cells with the modified peptides prior to infection of virus to determine if these peptides inhibit viral entry.
5. We show that the peptides do not target the virus directly and hypothesize that there is less of a possibility that the virus develops resistance against these peptides. To test this possibility we can carry out experimental evolution studies. During these experiments we would infect cells with the virus in the presence of the peptides. Then isolate the virus that infects even in the presence of the peptide. This infection-isolation cycle will be repeated numerous times with increasing concentration of the peptides. The peptide

resistant variants could then be sequenced to examine what changes lead to their resistance to the peptide.

6. We observed that SG MCMV was less susceptible to peptide treatment compared to the TC passaged MCMV. This suggests that virus grown *in vivo* has a different entry process. This specificity could be due to the cell type from which the virus is derived affecting the subsequent infectivity of CMV [19, 20]. The peptides could be used to elucidate the mechanistic difference in the infection process between viruses from the two sources. We can also identify which glycoprotein complexes are expressed by each virus through immunoblot assays. This in turn can lead to the identification of SG isolated MCMV entry pathways and can be exploited to understand viral pathogenesis and develop new therapeutics.

Chapter II:

In this chapter, we evaluated the *in vivo* functionality of vCXCL1 from CCMV (vCXCL-1_{CCMV}) and murine host (mCXCL-1). The following experiments would help to better define the role of vCXCL1 *in vivo* and explain the results obtained from the co-infection model.

1. Generate recombinant MCMVs expressing vCXCL1s from HCMV clinical isolates. These recombinants could be used to investigate the role viral CXCL-1 from HCMV in CMV pathogenesis and dissemination. In addition, this model will also allow us to investigate how the variability the chemokine affects CMV dissemination and pathogenesis *in vivo*.
2. The vCXCL1 gene is constitutively overexpressed in our model. Therefore, it may not mimic the real life scenario. We should generate MCMV recombinants that express the chemokine with late gene kinetics (i.e., the *mck2* promoter). This will provide us with a model more closely matching HCMV chemokine expression *in vivo*.
3. It is also possible that the overproduction of the chemokine leads to chemokine receptor desensitization [21, 22, 23-25]. This may lead to poor recruitment of cells important for dissemination. To test this possibility, sorted cells from the sites of infection from mice

infected with either the parental or the recombinants could be tested in *in vitro* functional assays for responsiveness to chemokine stimuli.

4. Infection of the host with multiple strains of HCMV is a very common phenomenon [26-34]. The co-infection model can be used to investigate the co-infection phenomena better. Our data shows that recombinants generate an abnormal immune response, and that co-infection skews the immune response to favor the dissemination of the recombinants. If this is indicative of true HCMV infections then it would allow us to understand HCMV pathogenesis. Generation of different recombinants that more closely resemble clinical strains can be used in co-infection assays to explore this further.

Chapter III:

In this chapter we characterized some of the functional differences in the vCXCL-1 chemokines from different clinical isolates. Future work focused on vCXCL-1 will help us understand its role in HCMV pathogenesis.

1. Even when bound to the same receptor, subtle differences in the chemokine can lead to different signaling and cellular responses [35]. Therefore, investigating the signal transduction pathway more thoroughly that are induced by the different VCXCL-1 variants will help clarify the polymorphisms at the molecular level.
2. Our experiments show that all vCXCL1s can bind to CXCR2 and some can also bind CXCR1. It is also known that these receptors induce a different downstream signaling cascade leading to differences in the leukocyte response [36]. Therefore it would be interesting to investigate the contribution of each of these receptors individually in functional assays (i.e., calcium flux, chemotaxis, adhesion molecule upregulation, CCL22 induction etc.). This can be done by using receptor blocking antibodies while performing these assays. Or we can exchange residues to map what is essential for the different signaling pathways. This would allow us to determine how differences in vCXCL1 alter signaling and thus functional selectivity.

3. Previous studies have shown that treating fibroblasts with CXCL8 (IL-8) leads to increased viral replication [35, 37]. It would be interesting to investigate whether treating fibroblasts with the different vCXCL1s would also increase viral titers. This might be another mechanism by which viral chemokines contribute to the pathogenesis of CMV.
4. Neutrophils can transmigrate through a monolayer of endothelial cells and pick up virus from the infected cells [38]. Understanding how the variability in the vCXCL1s affects the transmigration of neutrophils and the ability of neutrophils to pick up the virus from infected endothelial cells will help us understand CMV pathogenesis. We are currently carrying out similar experiments in the lab by using endothelial transmigration assays in the presence of chemokines (data not published).
5. We have shown that the viral chemokines upregulate the expression of adhesion molecules on neutrophils and propose that this aids in viral transfer to the neutrophils. However, it is also possible that vCXCL1s upregulate adhesion molecules (e.g. selectins, and integrins) on endothelial cells. This might allow for better interaction of leukocytes and infected endothelial cells allowing for better transfer of the virus.

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APPENDIX I
MCMV ISOLATED FROM SALIVARY GLANDS BYPASSES A HEPARAN SULFATE BLOCKADE FOR ENTRY

Abstract

Human cytomegalovirus (CMV) has a seroprevalence close 100% in developing countries. Infection or reactivation causes life-threatening disease in immune compromised patients and congenital defects following *in utero* infection. MCMV is used as a model to understand the spread of CMV in the host. MCMV eventually disseminates from the site of infection to the salivary glands (SGs). SGs represent a crucial site for MCMV replication and transmission *in vivo*. MCMV isolated from SGs is more virulent than tissue-culture passaged virus. We speculated that there are differences in cellular tropism that are related to CMV entry. CMV glycoproteins gB, gM, and gN initiate entry. gB and gM/gN are responsible for binding heparan sulfate proteoglycans, which consist of heparan sulfate glycosaminoglycans attached to a protein core. A previously characterized heparan sulfate binding peptide, p5+14, is effective at reducing tissue culture (TC) passaged MCMV infection *in vitro.* In order to investigate differences in TC and SG isolated MCMV, we tested the peptide's ability to reduce MCMV infection *in vitro.* SG isolated MCMV infection in fibroblasts is only reduced by ~40% compared to the reduction of TC MCMV. This suggests that SG isolated MCMV may use a different mechanism of entry that is not fully reliant on heparan sulfate. One possibility is that SG MCMV could be using endocytosis to initiate infection. In support of this possibility we show that endocytosis inhibitors reduces SG MCMV infection of epithelial cells but not TC MCMV. Together this data points to a unique entry mechanisms for SG vs. TC passaged MCMV that could relate to the observed differences in pathogenesis.

Introduction

Human Cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus with a seroprevalence reaching over 90% in developing countries [1]. HCMV is the leading cause of birth defects and congenital infections [2]. HCMV infection *in utero* can lead to neurodevelopmental defects, including sensory neural hearing loss (SNHL) [3]. In immune compromised individuals like AIDS and transplantation patients, CMV infection can lead to serious complications with increased mortality [2]. In healthy adults however, HCMV infection is usually asymptomatic. HCMV establishes lifelong latency in an immune competent individual and reactivates during

immune suppression [2]. HCMV infects a wide range of cell types *in vitro* [4]. *In vivo* studies with HCMV are limited due to species specificity. Therefore, infection of mice with murine cytomegalovirus (MCMV) is used and accepted as an animal model for CMV studies *in vivo*.

Salivary glands (SGs) represent a crucial site for MCMV replication and transmission *in vivo* [5]. MCMV isolated from SGs is more virulent than tissue-culture passaged virus [6-8]. However a single passage of the SG isolated MCMV on epithelial cells or fibroblasts attenuates this virulence [8]. There are several differences between tissue-culture passaged MCMV (TC MCMV) and SG isolated MCMV (SG MCMV). There are ultrastructural differences between SG MCMV and TC MCMV. Electron microscopy shows that TC MCMV release progeny that consist of multi-capsid virions [9], while SG MCMV is characterized by one capsid per virion [10]. One study suggested that the virulence attributed to SG MCMV is that this virus is not susceptible to neutralizing antibody, yet still remains susceptible to complement [11]. Tropism differences are also seen in HCMV. For example, clinically isolated HCMV displays different tropism than TC passaged virus [12-14]. This also holds true for MCMV. MCMV isolated from SGs and other organs appears to have different tropism also [15].

A possible explanation for these differences come from HCMV entry mechanisms. Expression of different glycoprotein complexes dictates infection of host cells [16]. Both CMVs require expression of glycoprotein B (gB) and glycoproteins H and L (gH/gL) to infect cells [17,18]. HCMV and MCMV can initially enter fibroblasts using the gH/gL, and glycoprotein O (gH/gL/gO) trimeric complex [19,20]. Entry into endothelial and epithelial cells is mediated by the gH/gL/ pUL128,130,131A pentameric complex in HCMV or the gH/gL/MCK-2 trimeric complex in MCMV [15, 21-26]. HCMV entry into epithelial cells is accomplished using one of two distinct pathways: cell membrane fusion or pH-dependent endocytosis [27]. The gH/gL/pUL128,130,131A complex is dispensable for viral endocytosis implying that the glycoprotein complexes on the virion determine tropism [28-30]. Recent studies have shown that both gH/gL/gO and gH/gL/MCK-2 complexes are important for MCMV spread *in vivo* [15] and that CMV tropism varies depending on the cell types from which its progeny are released [16, 31]. A previously characterized heparan sulfate binding peptide, p5+14, was shown to reduce TC MCMV and HCMV infection of fibroblasts [32]. CMV gB and gM/gN [33] gB and gM/gN are responsible for binding heparan sulfate proteoglycans, which is the initial step in

entry [33]. In this step gB and gM/gN are responsible for binding heparan sulfate proteoglycans, [34]. In this study we tested the hypothesis that despite differences between TC MCMV, and SG MCMV tropism, p5+14 blocks infection of both viruses because the initial step of binding HSPGs is mediated by gB and gM/gN rather than gH/gL complexes.

Materials and Methods

Cells and virus

Mouse embryonic fibroblast (MEF) 10.1 cells and mouse mammary gland epithelial (NMuMG) cells (ATCC) were propagated in DMEM supplemented with 10% Fetal Clone III (FCIII), Penicillin/Streptomycin and L-Glutamine to a final concentration of 1%. Tissue culture passaged MCMV RM4503 [37] (TC MCMV) was cultured *in vitro* in MEF 10.1 cells. SG isolated MCMV RM4503 (SG MCMV) was harvested from BALB/c mouse SGs at 14 days post infection after intraperitoneal injection ($1x10^6$ pfu). SGs were homogenized then centrifuged to remove tissue debris. The remaining homogenate was passed through a 70μM cell strainer (Falcon). The viral titers were determined via plaque assay. All viruses were stored at -80°C until use.

Plaque reduction assay

Peptides were screened for their ability to reduce infection of TC MCMV and SG MCMV on MEF 10.1 cells. Cells were seeded in a 24-well polystyrene cell culture plate and allowed to reach ~85% confluency. MEF 10.1 and NMuMG cells were treated with peptide or PBS for 30 minutes prior to the addition of virus. After one hour of incubation with the virus and peptide, the mixture was removed and replaced fresh 2% carboxymethyl cellulose (CMC; Sigma Aldrich) and complete DMEM (CMC: DMEM). Cells were incubated at 37°C and 5.0% CO_2 for 5 days. Plaques were manually counted using a dissection microscope after staining with Coomassie blue. Data were expressed as percent infection of PBS-treated wells. All data were normalized to 100% using the peptide untreated and infected positive control data. Peptide untreated and uninfected negative controls were subtracted as background. The data are combined from one or more independent experiments with at least three replicates in each. Error bars represent the standard deviation (SD).

Virus purification

TC and SG MCMV was separated from cells and debris using centrifugation at 400xg for 10 minutes then passed over a 70μM cell strainer. Crude purified TC and SG MCMV were further purified using a sorbitol gradient as previously described [38,39].

DNase and RNase treatment

Crude purified SG MCMV was treated with an RNase cocktail (10U/ml A, 400U/ml TI) and DNase (2μg/ml) for 2 hours at 37°C then diluted in PBS. MEF 10.1 cells were treated with 10% FBS/PBS (control) or 100μM of p5+14 for 30 minutes prior to infection with ~50 pfu of RNase and DNase treated SG MCMV.

Heparin treatment of TC and SG MCMV

SG MCMV was treated with PBS, 20 μg/ml or 40 μg/ml of heparin sodium salt (Acros Organics) for 1 hour at 37°C then diluted in PBS. MEF 10.1 cells were infected with ~50 pfu of treated SG MCMV for 1 hour. Virus was replaced with fresh media.

Inhibition of viral endocytosis

In order block pH- dependent changes in the endosome 25μM of chloroquine in PBS stock was pre-incubated with MEF 10.1 and NMuMG cells for an hour followed by infection with ~50pfu of SG MCMV for 1 hour. After absorption media was replaced.

Statistical Analyses

Data were expressed as percent infection of PBS-treated wells. All data were normalized to 100% using the untreated and infected positive control data. The data are the combined data from two or more independent experiments with at least three replicates in each. Error bars represent the standard deviation (SD). Statistical significance was calculated using a Mann-Whitney test or 2 way ANOVA followed by Bonferroni posttests. All analyses were performed using Prism 5.0 (GraphPad). A p-value less than 0.05 was considered statistically significant. Significant values are labeled as *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, NS=non-significant reduction in infection.

Results and Discussion

p5+14 is less effective against SG isolated MCMV

MEF 10.1 cells were treated with p5+14 prior to infection with SG MCMV or TC MCMV. p5+14 reduced infection of TC MCMV >80%, which is in agreement with our previous study [32]. However, p5+14 reduced SG MCMV infection by only 40% (Figure A1.1). It is unclear from this result whether the SG MCMV virions or the homogenate environment contribute to this phenotype. We hypothesized that the homogenate environment may contribute to the lack of efficacy of p5+14.

SG homogenate does not alter p5+14 efficacy

In order to determine if the SG homogenate contributes to decreased efficacy of p5+14 against SG MCMV infection, we treated MEF 10.1 with uninfected SG homogenate for 30 minutes prior to the infection of TC MCMV. There is no observable difference between PBS or homogenate treatments (Figure A1.2). In order to remove potential p5+14 inhibitors from the SG MCMV prep, SG MCMV was purified over a sorbitol gradient. There is no observable difference in p5+14 inhibition of the SG MCMV purified viruses and the "crude" SG MCMV (Figure A1.3). p5+14 is a positively charged peptide [32]. Therefore negatively charged molecules like DNA and RNA in the SG homogenate may neutralize the peptide. Although the sorbitol purification process separates many potential contaminants from the virions, there could still be DNA and RNA in the virion preps. The negative charges of these molecules could neutralize peptide function. In order to address this possibility, we incubated SG MCMV with DNase and RNase prior to infection. Again there is no observable difference between DNase and RNase treated and untreated SG MCMV (Figure A1.4). These data suggest that the SG homogenate is not the reason for the lack of p5+14 efficacy against SG MCMV. We propose that the virion itself and potentially the mechanism of entry differ between TC and SG MCMV.

Heparin treatment neutralizes TC MCMV but not SG MCMV

Heparin and heparan sulfate are chemically related GAGs [38]. Heparin has been shown to reduce infection of HCMV and MCMV [39]. In order to test whether SG MCMV or TC MCMV use HS for entry, we incubated TC and SG MCMV with heparin sodium salt for 1 hour. TC MCMV infection was reduced by ~50% (Figure A1.5A). However, SG MCMV infection

was not reduced after treatment with heparin sodium salt (Figure A1.5B). These data suggest that either SG MCMV is not using HSPGs for entry or SG MCMV may use a HSPG subtype with a different chemical makeup from heparin that p5+14 preferentially binds.

SG MCMV undergoes endocytosis to enter epithelial cells

As previously reported, CMV can enter epithelial cells by membrane fusion or pH-dependent endocytosis [27]. CMV enters fibroblasts by membrane fusion [4,19,20], but there have been no reports of CMV entering fibroblasts via pH-dependent endocytosis. Our data suggest that SG MCMV may not be utilizing HSPGs because heparin sodium salt or the HS binding, p5+14, does not reduce infection. We chose to test whether SG MCMV has the ability to enter fibroblasts, MEF 10.1 cells, and epithelial cells, NMuMG cells, through pH-dependent endocytosis. Cells were treated with 25μM chloroquine solution for 1 hour prior to infection with SG MCMV. Chloroquine reduces SG MCMV infection by >40% in epithelial (Figure A1.6). There is no observable difference in TC or SG MCMV entry in chloroquine treated fibroblasts (Figure 1A.6B). These data suggest SG MCMV entry is partly mediated by a pH-dependent endocytosis mechanism.

Conclusions and Future Directions

SG MCMV infection is reduced by only 40% when cells are pretreated with the heparan sulfate binding, p5+14 peptide. It appears that this phenotype is attributed to the SG MCMV entry mechanism rather than the homogenate environment. A previous study indicated that MCMV isolated from SG MCMV uses different glycosidic linkages on the cell surface glycoconjugates than TC MCMV during entry [40]. Treating cells with N-acetlyglucosaminidase or virus with N-acetylglucosamine neutralized TC MCMV infection. This suggests that TC MCMV binds N-acetylglucosamine. N-acetlyglucosamine can be found at varying amounts in different HSPGs subtypes [41]. Meanwhile SG MCMV infection was inhibited by treating cells with sialidase and N-acetlyglucosaminidase [41]. From this study it appears SG MCMV binds to different carbohydrate moieties than its TC MCMV counterpart. In future studies we would like to test whether TC or SG MCMV bind different HSPG subtypes. This information will map the specificity of p5+14. Determining the specific HSPG subtypes targeted by p5+14 and other p5 related peptides [42, 43] will aid in our understanding which molecules are important for CMV entry *in vivo*.

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Appendix: Figures

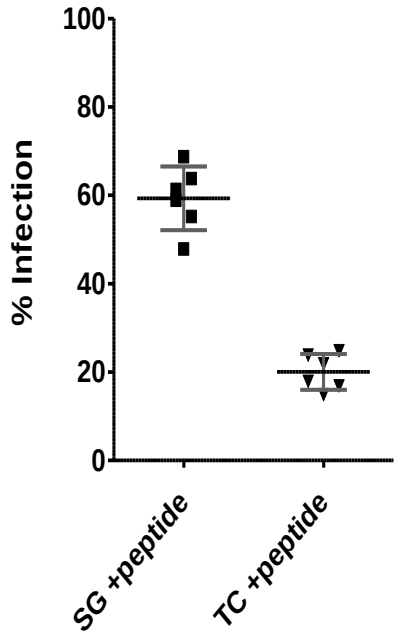

Figure 1A.1. p5+14 is less effective on SG MCMV compared with TC MCMV. MEF 10.1 cells were treated with PBS (control) or 100μM of p5+14 for 30 minutes prior to infection with either SG MCMV or TC MCMV. Horizontal bars ±SD represent the average of the percent reduction compared to PBS-treated control for three separate experiments with three replicates in each.

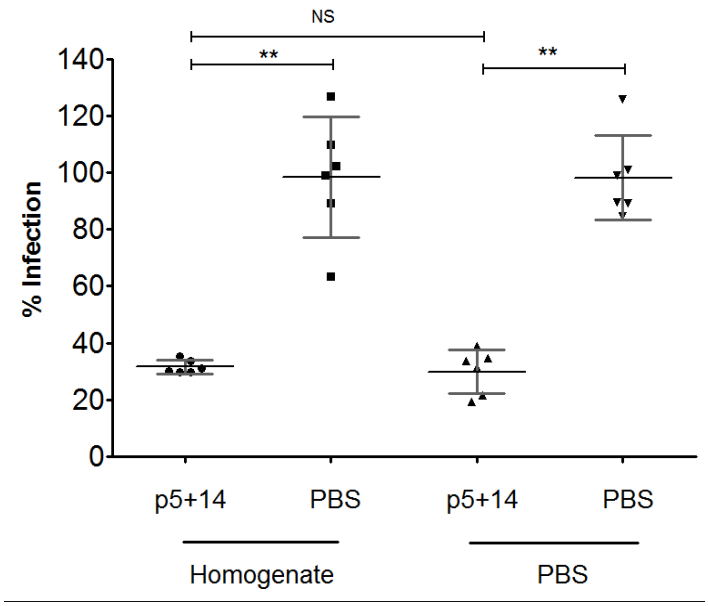

Figure 1A.2. SG homogenate does not reduce p5+14 efficiency. p5+14 was incubated with homogenate from uninfected mouse SG for 1 hour. MEF 10.1 cells were treated with PBS (control) or 100μM of p5+14 + homogenate for 30 minutes prior to infection with TC MCMV. Horizontal bars ±SD represent the average of the percent reduction compared to PBS-treated control for two separate experiments with three replicates in each. Statistical significance was determined using a Kruskal-Wallis One-Way ANOVA test: * p-value<0.05, ** p-value<0.01, *** p-value<0.001, NS=not significant

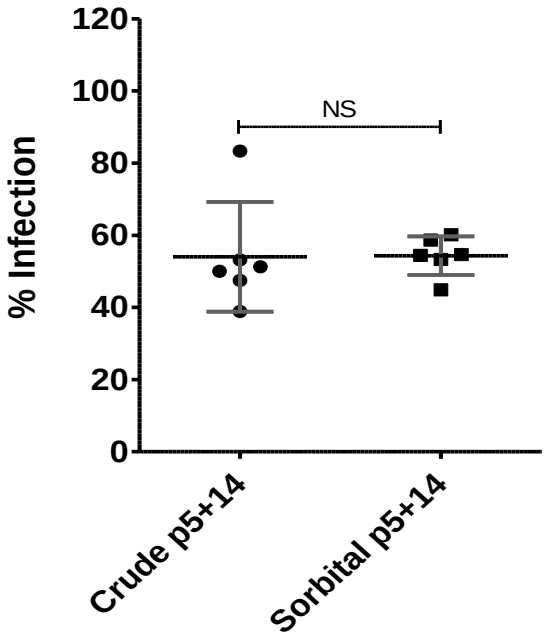

Figure 1A.3. Sorbitol virion purification does not alter p5+14 efficiency. MEF 10.1 cells were treated with PBS (control) or 100μM of p5+14 for 30 minutes prior to infection with either SG MCMV isolated from a crude preparation or SG MCMV following a sorbitol gradient purification. Horizontal bars ±SD represent the average of the percent reduction compared to PBS-treated control for two separate experiments with three replicates in each. Statistical significance was determined using a Mann-Whitney test: NS= not significant

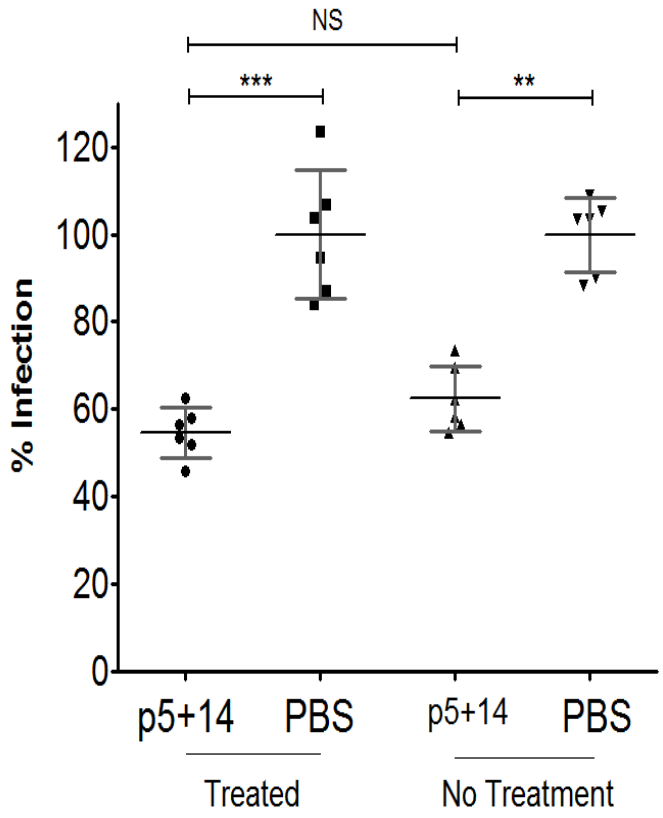

Figure 1A.4. DNase and RNase treatment of SG MCMV does not alter p5+14 efficiency. SG MCMV was treated with RNase cocktail (10U/ml A, 400U/ml TI) and DNase (2µg/ml) for 2 hours at 37°C. MEF 10.1 cells were treated with PBS (control) or 100µM of p5+14 for 30 minutes prior to infection with treated or untreated SG MCMV. Horizontal bars ±SD represent the average of the percent reduction compared to PBS-treated control for one experiment with three replicates in each. Statistical significance was determined using a Kruskal-Wallis One-Way ANOVA test: * p-value<0.05, ** p-value<0.01, *** p-value<0.001, NS= not significant

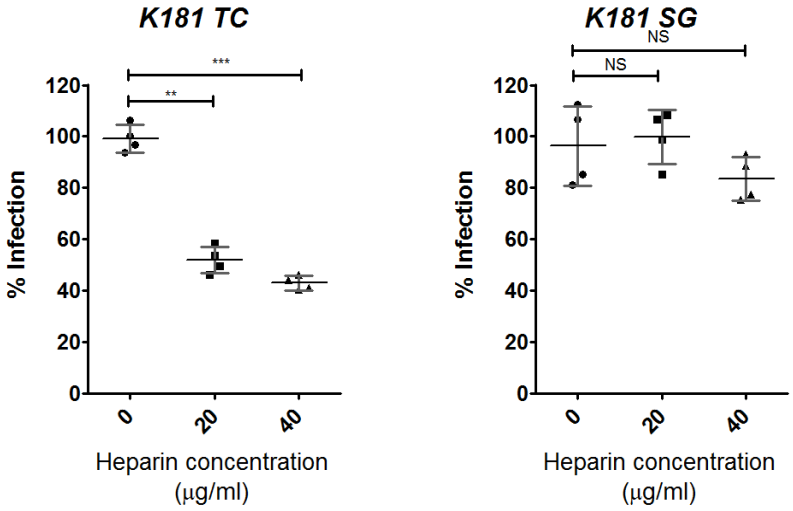

Figure 1A.5. Heparin does not neutralize SG MCMV. SG MCMV was treated with either PBS or 20 µg/ml or 40 µg/ml of heparin sodium salt for 1 hour at 37°C. MEF 10.1 cells were infected with treated SG MCMV for 1 hour. Horizontal bars ±SD represent the average of the percent reduction compared to PBS-treated control for two separate experiments with three replicates in each. Statistical significance was determined by a Kruskal-Wallis One-Way ANOVA: * p-value<0.05, ** p-value<0.01, *** p-value<0.001, NS =not significant.

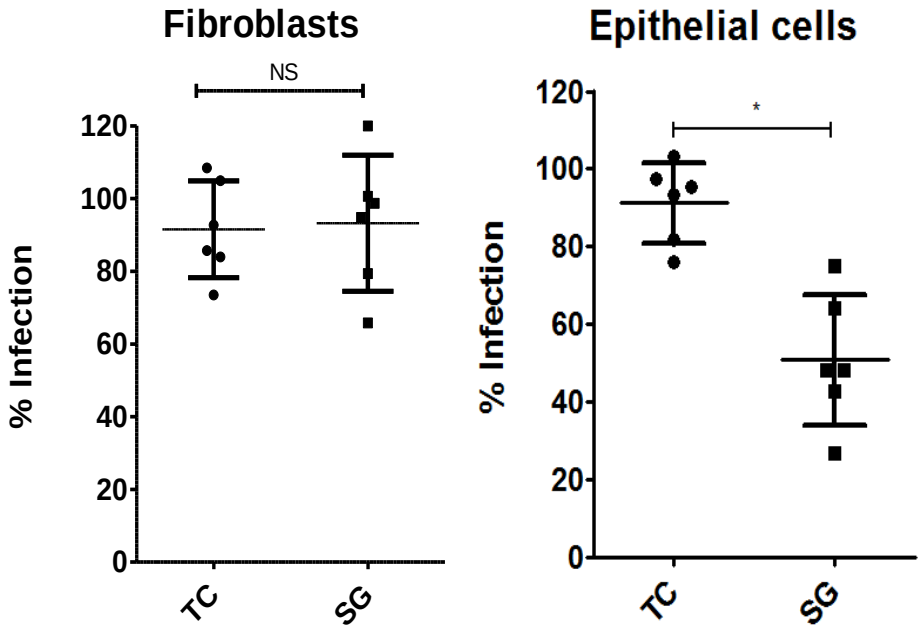

Figure 1A.6. Chloroquine treatment inhibits SG MCMV infection of epithelial cells. MEF 10.1 (fibroblast) and NMuMG (epithelial) cells were treated with 25μM of chloroquine for 1 hour. Cells were washed once with PBS to remove any unbound virus. Cells were infected with either TC MCMV or SG MCMV. Horizontal bars ±SD represent the average of the percent reduction compared to PBS-treated control from two separate experiments with three replicates in each. Statistical significance was determined using a Mann-Whitney t-test: * p-value<0.05, ** p-value<0.01, *** p-value<0.001, NS = not significant.

VITA

Elisabeth Anne Pitt was born January 7, 1992. She received a Bachelor's of Science degree in Microbiology from the College of Arts and Sciences at the University of Tennessee in May 2014. She began her Master's program in the Department of Microbiology at the University of Tennessee Knoxville in August 2014 Dr. Tim Sparer's lab. Her work consisted of investigating the role of CMV encoded chemokines and CMV pathogenesis. She also investigated the efficacy of antiviral peptides in collaboration with Dr. John Wall at the University of Tennessee Health Science Center. She will complete her Master of Science degree in August 2016.