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CMV Chemokines and Co-infection: A Dissemination Plot that Peptides Can Foil

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To the Graduate Council:

I am submitting herewith a dissertation written by Pranay Dogra entitled "CMV Chemokines and Co-infection: A Dissemination Plot that Peptides Can Foil." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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CMV Chemokines and Co-infection : A Dissemination Plot that Peptides Can Foil

**A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

Pranay Dogra

August 2015

DEDICATION

I dedicate this dissertation to my grandparents. Your words of encouragement and your love have always been an inspiration for me to achieve all I can.

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ABSTRACT

Human Cytomegalovirus (HCMV) is the leading cause of both non-hereditary mental retardation and hearing loss, and CMV infection/reactivation causes serious complications in transplant and immune compromised patients. Due to these issues, development of a CMV vaccine and/or therapeutics is required. To achieve this goal, it is necessary to gain a better understanding of CMV pathogenesis. Because of its coevolution with humans, HCMV has evolved genes with homology to human immune modulatory genes. Several of these genes help CMV establish a successful and lifelong infection within the host. An example is the viral CXC chemokine homolog *UL146* gene (vCXCL-1). *UL146* varies between clinical isolates and has been associated with clinical outcomes of HCMV infection. In this dissertation we characterized the vCXCL-1 protein from different clinical isolates *in vitro* (Chapter 1). We hypothesized that, **variability in vCXCL-1 leads to differential activation of neutrophils**, which in turn leads to the observed differences in HCMV pathogenesis. In this study we identified the similarities and differences in the functional activity of vCXCL-1s from different HCMV isolates and suggest how the variability can affect neutrophil function and CMV pathogenesis. In order to understand the contribution of vCXCL-1 in the pathogenesis of CMV infection *in vivo* (Chapter 2), we tested the hypothesis that **vCXCL-1 from chimpanzee CMV (vCXCL-1_{CCMV}) is a functional CXC chemokine and contributes to viral dissemination**, similar to the MCMV CC chemokine. However, contrary to this hypothesis, we found that overexpression of the chemokine is detrimental to the dissemination of MCMV by recruiting more inflammatory monocytes and NK cells to the site of infection. In an effort to develop a novel anti-CMV treatment, we tested the hypothesis that **heparan sulfate binding peptides can act as potential antivirals** (Chapter 3). Peptides of different lengths and net charge were generated and tested for their ability to prevent MCMV infections. Of those tested, the cationic peptides reduced MCMV infection *in vitro* by ~ 90%. In summary my research suggests that over expression of chemokines can attenuate CMV dissemination making it a potential vaccine candidate and that a peptide that binds to heparan sulfate can be a potential CMV therapeutic.

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INTRODUCTION

PART I: What We Have Learned From Animal Models of HCMV

This part (Part I) is a publication by the same title published in the book titled *Human Cytomegaloviruses: Methods and Protocols* in 2014 authored by Pranay Dogra and Tim E. Sparer and has been reproduced here with permission from the publisher (Appendix 5)

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My primary contributions to this paper include (1) researching the topic and, (2) writing of this review article.

1. Abstract

Although human cytomegalovirus (HCMV) primary infection is generally asymptomatic, in immune-compromised patients HCMV increases morbidity and mortality. As a member of the betaherpesvirus family, *in vivo* studies of HCMV are limited due to its species specificity. CMVs from other species are often used as surrogates to express HCMV genes/proteins or used as models for inferring HCMV protein function in humans. Using innovative experiments, these animal models have answered important questions about CMV's life cycle, dissemination, pathogenesis, immune evasion, and host immune response. This chapter provides CMV biologists with an overview of the insights gained using these animal models. Subsequent chapters will provide details of the specifics of the experimental methods developed for each of the animal models discussed here.

2. Introduction

One of the hallmarks of β herpesviruses is their species specificity. This means that human CMV (HCMV) does not productively infect mouse cells and vice versa. The species barrier is not due to attachment or entry, but a combination of factors including blocks in immediate early gene expression and specificity of anti-apoptotic proteins (3-8). Without the ability to use

HCMV in animal models, it necessitates the development and utilization of animal models of HCMV infection. Despite these limitations, animal models have been useful for studying pathogenesis, immune control, immune evasion, dissemination within the host, latency and reactivation, and vaccine/drug development (9-14). In this chapter we will discuss the four main animal models used to study HCMV. After a discussion of the advantages and disadvantages of each model, the proceeding sections will focus on the characteristics of HCMV infection that have been investigated in the different animal models. The main animal models are:

1. The mouse CMV (MCMV) model.
2. The rat CMV (RCMV) model.
3. The guinea pig CMV (GPCMV) model.
4. The rhesus CMV (RhCMV) model.

3. Advantages and Disadvantages of the Animal Models

The complexity of host-viral interaction makes an exact mimic of HCMV infection of humans difficult. Nonetheless we have gained tremendous insight into the pathogenesis of HCMV with these animal models. However it must be kept in mind that these models have their limitations for studying HCMV pathogenesis. Below are some of the major advantages and disadvantages of the different animal models discussed in this chapter.

3.1. Mouse model

Advantages

- Characteristics of MCMV infection in mice are similar to that of HCMV infection in humans (15-18).
- MCMV contains homologues and/or at least functional homologues of many HCMV genes and gene products. The MCMV genome can be easily manipulated to either delete or exchange genes between HCMV and MCMV (19, 20).

- The mouse has a well-characterized immune system, short gestational periods, and large litter sizes. There are numerous immunologic reagents available including transgenic and knockout mice (20, 21).

Disadvantages

- A major disadvantage of the mouse model is that the placental barrier is refractory to CMV transmission (22), except in a severe combined immune deficient (SCID) mice (23). This is most likely due to the three-cell-thick trophoblast layer that separates maternal and fetal circulations (20).

3.2. Rat model

Advantages

- Similarity between HCMV and RCMV pathogenesis (14)
- HCMV genetic counterparts in the RCMV genome and the availability of viral mutants (20, 24)
- Availability of immunologic reagents and transgenic animals (20).
- Larger size makes it better suited to surgical manipulation

Disadvantages

- No clear disease phenotype in pups for modeling congenital infection.
- Congenital and placental infections have only recently been described (20).

3.3. Guinea pig model (Reviewed in (9, 25))

Advantage

- GPCMV can cross the guinea pig placenta, causing infection *in utero*. This is probably due to single trophoblast layer separating maternal and fetal circulations. This makes the guinea pig well suited for the study of vaccines designed to interrupt transplacental transmission of infection (10, 20).
- The presence of HCMV counterpart genes in the GPCMV genome (26).

Disadvantage

- The lack of immunologic reagents for guinea pig studies.
- Lengthy guinea pig gestational periods with relatively small litter size slows down animal studies (20).

3.4. Rhesus model (Reviewed in (11))

Advantage

- High similarity between the pathogenesis of infection of HCMV and RhCMV (11, 27, 28).
- Relatedness of the genomes of RhCMV and HCMV and the availability of viral mutants (20, 29).

Disadvantage

- The high cost of the animal maintenance.
- The paucity of RhCMV-seronegative animals because RhCMV infection is ubiquitous in most colonies (20).

4. Pathogenesis

Before discussing the animal models used for studying HCMV pathogenesis, it is important to discuss what is known about HCMV disease in humans. HCMV infection causes severe disease in immunocompromised patients including individuals with AIDS, organ transplant patients, cancer (30) and newborns (31). Infection in these patients can sometimes cause clinical disease including mononucleosis-like syndrome, interstitial pneumonia, gastroenteritis, retinitis, or transplant rejection. Acute rejection and cardiac allograft vascular disease is reduced with suppression of subclinical cytomegalovirus infection (32-34). HCMV is the leading viral cause of congenital birth defects following infection *in utero*. Worldwide between 0.5 to 2% of newborns are infected. The majority of newborns are asymptomatic at birth but some exhibit outward signs of infection including microcephaly, jaundice, and hepatosplenomegaly (35, 36). About 10% of the asymptomatic newborns develop neurological dysfunction, most prominently sensorineural hearing loss (SNHL) that appears when they are older (20, 37, 38). Animal models provide some insights into these different aspects of HCMV disease.

In immunocompetent individuals HCMV infection is generally asymptomatic. However clinical studies point to HCMV's contribution to cardiovascular (39-42) and inflammatory bowel diseases (43-45). HCMV infection is associated with many types of cancers (46-50), however there is no strong evidence of transformation of normal human cells after HCMV infection (48, 49). In cardiovascular disease there is an association between HCMV infection and the

thickening of the arteriole walls during heart transplantation rejection (51), vasculopathy (52), and arteriolar dysfunction (53).

Some aspects of these HCMV-related diseases can be recapitulated in different animal models. This, in turn, allows the assessment of CMV's contribution to the disease and exploration of potential treatment. Although not all aspects parallel human infection and disease, these animal models are an excellent resource for dissecting particular CMV disease models. Discussed below are the main observations gleaned from animal models of HCMV pathogenesis.

4.1 Congenital infection

Animal model systems that mimic HCMV-induced developmental abnormalities have been used to study the pathological outcomes of CMV congenital disease (9-11, 54-57). GPCMV and RhCMV, due to their natural ability to cross the placental barrier and cause *in utero* infection have been the models of choice for congenital infections (10, 57-60). Although MCMV and RCMV are very inefficient at crossing the placenta due to the unique features of the trophoblastic layer, MCMV in the SCID model and a new strain of RCMV are capable of crossing the placenta and cause symptoms similar to HCMV congenital disease (12, 23). Because this is an inefficient process, direct injection of the virus into the central nervous system (CNS) of the fetus, uterus, placenta, brain or peritoneum of neonatal animals is most commonly used to recapitulate HCMV induced congenital disease (21, 54-57, 61-65). One of the drawbacks of these models is assessing whether the infection has led to SNHL or other developmental defects.

Discoveries using these models have led to a better understanding of how HCMV infection leads to developmental defects. Mouse studies have shown that the susceptibility to CMV infection is dependent on gestational age/developmental stage of the embryo. Although embryonic stem cells are resistant to CMV infection initially, as they differentiate they become permissive to MCMV replication (61, 66). Once the pup is born there is a reduction in the susceptibility of the brain during development from neonate to adult. This may be due to a decrease in the number of susceptible cells in the developing brain (67) or increased immune responses, providing protection against infection (68). CMV targets neural stem cells and the auditory nerve spiral ganglion in the developing brain (28, 57, 69-71). Several factors that contribute to the development of SNHL have been identified using the murine model of CMV

congenital infection, including ultrastructural lesions of the neurons, reduction in the number of spiral ganglion neurons (21) and cytopathic effects of viral replication in the inner ear (cochlea) (20, 28, 57, 70, 72). The virus spreads to the inner ear most likely via the perilymphatic routes (20) where the inflammation induced by CMV viral chemokines may contribute towards pathogenesis (72, 73).

Similarly, the intrauterine model of rhesus CMV infection identified that CMV infection of neuronal stem/progenitor cells before neuronal migration, differentiation, and organization, results in more severe outcomes (57). Infection after these developmental processes are completed results in less severe disease suggesting the timing of CMV infection during fetal development is one factor determining disease severity. CMV infection is not limited to the developing CNS. Systemic CMV infection can cause non-CNS diseases like intra uterine growth restriction, renal and hepatic damage to the fetus (28, 57).

Animal models of neonatal CMV infection have provided tremendous insight into CMV neuropathogenesis and the role of immune responses in controlling infection (66, 71, 74-76). The neonatal mouse model of CMV infection in the presence of maternal antibodies has contributed to current vaccination strategies. The fact that experimental intrauterine infection of rhesus monkeys does not always lead to adverse outcomes implies that other factors limit CMV disease (57).

4.2 Vasculopathy and Graft Rejection

The rat and mouse models have been used to investigate the role of CMV infection in cardiovascular disease (CVD). In humans, circumstantial evidence points to the contribution of HCMV infection to the development of arterial restenosis following angioplasty, atherosclerosis, and solid organ transplant vascular sclerosis (TVS) (77, 78). In the mouse model, MCMV infection accelerates atherosclerosis development in mice with high cholesterol (79-83). CMV infection has a proinflammatory influence on the microvasculature that increases its susceptibility to both proinflammatory and thrombotic responses caused by hypercholesterolemia (84). MCMV and RCMV chemokine receptors M33 and R33 respectively, which are functional homologs of HCMV US28, are required for smooth muscle cell migration to the site of vascular injury. Their accumulation in the vessel intima leads to vessel narrowing and development of CVD (85, 86).

In rat organ transplantation models of chronic rejection (CR), active CMV replication (87, 88), contributes to accelerated graft rejection and increased vasculopathy in allograft vessels (89-93). Several tissue specific RCMV genes involved in host modification of inflammatory and tissue repair processes are upregulated in allograft recipients and may contribute to CR (94). RCMV chemokine receptor R33 also plays an important role in acceleration of CR (86). Although prophylactic treatment with ganciclovir in humans delays the time to allograft rejection (95, 96), the rat model shows that recipients of latently infected donor hearts treated with ganciclovir does not prevent CR or TVS. One explanation for this discrepancy is that RCMV induces tertiary lymphoid structure formation and alteration of donor tissue T cell profiles prior to transplantation (97). Both the mouse and rat models provide unique tools to dissecting the role that CMV plays in CVD and graft rejection.

4.3 Retinitis

The mouse model of CMV retinitis is a common model for HCMV retinitis. MCMV readily infects ocular tissue (98-101) and establishes latency in the eye (102). During the acute phase of the response to CMV infection, there is a rapid expansion and infiltration of CD8⁺ T cells into the infected retina. This is followed by a contraction phase where viral antigen presentation and CD8⁺ T cell activation occurs in the spleen and the draining lymph nodes but not in the retina or iris (103). Using this model, TNF α was shown to induce apoptosis of retinal neurons and that bystander cells contribute to the pathogenesis of CMV retinitis (104, 105), while not being T-cell dependent (105). The mouse model also highlights the protective role of CD8⁺ T cells (106, 107) and NK cells (108) against MCMV-induced necrotizing retinitis. The mouse model, although not perfect, provides a system for exploring not only the mechanism of CMV retinitis but also potential treatment options.

4.4 CMV Infection of an Immunodeficient Host

HCMV is one of the opportunistic infections in late-stage AIDS patients, leading to pneumonitis, gastroenteritis and/or retinitis. The animal models that best recapitulate this scenario are mouse retrovirus-induced immunodeficiency syndrome (MAIDS), and either spontaneous simian AIDS (SAIDS) or experimental infection rhesus monkeys with SIV (109, 110). In the rhesus model, CMV infection is similar to human infections of the gastrointestinal tract, hepatobiliary system,

lungs, and testicles (111, 112). Much like in humans, reactivation of CMV and the development of disseminated CMV disease are the result of diminished CMV-specific CD4⁺ T cell and CD8⁺ T cell immune responses (113, 114). Interestingly in this model, RhCMV and SIV co-infection suggested that concurrent primary infection with CMV could augment the development of AIDS (11, 115). In the mouse model, although T cell subsets play role in MAIDS/MCMV pathology, Dix and colleagues suggested that the type of T cell response (i.e., perforin-mediated cytotoxicity) contributes to the severity of MCMV-retinitis (116).

Without an HIV equivalent in the guinea pig model, cyclophosphamide treatment is used as an immune suppressant. The suppression of T and B cell immunity following CMV infection leads to lethal CMV infection in these animals (117). This once again illustrates the importance of these immune cells for maintaining control of CMV infection.

5. Immune Control

In the mouse and rhesus models of CMV infection CD8⁺ T cells, CD4⁺ T cells, and NK cells are the major cell types responsible for immune control of replication, latency and reactivation (113, 118-120). In the rhesus model, the target antigens for cytotoxic T lymphocytes (CTL) responses are the immediate-early proteins 1 and 2, and pp65-2, the homolog of HCMV pp65 (121, 122). With the identification of the major target antigens, it is now possible to explore vaccination strategies using the major CTL target as antigens.

Using these animal models, virus-specific antibodies have also been shown to play a crucial role in preventing CMV induced pathology (123, 124). The neutralizing antibodies target mainly glycoprotein B (gB). However in the recently completed human vaccine trials, gB vaccination generated a strong antibody response and with a vaccine efficacy that exceeded predictions albeit with less than 50% efficacy (125). In order to test whether inclusion of additional antigens could increase vaccine efficacy, a DNA vaccination/vaccinia virus prime-boost regimen was used to vaccinate rhesus monkeys. They were subsequently challenged with RhCMV and the amount of viral shedding was measured. Even with these additional antigens, the monkeys were not protected completely from infection and still shed virus (126). It will be interesting to see if this level of protection is sufficient to protect the developing fetus in RhCMV (57).

For understanding immune control the mouse model has allowed an in depth analysis of the immune responses for controlling MCMV infection (127-130). Besides showing that CTLs and NK cells are important in controlling MCMV infection (131-133), this model system also showed that different immune cells control MCMV infection in different organs. For example, CD4⁺ T cells expressing IFN γ control MCMV in the liver and IL10 expressing CD4⁺ T cells are important for clearance in the salivary gland (134-136).

6. Immune Evasion

The previous section discussed how animal hosts control CMV infection, but CMV has evolved mechanisms for evading many of these responses. Using animal models of CMV infection, many factors involved in virus immune evasion and their role in CMV survival and damage in their host have been identified. These CMV proteins not only allow the virus to avoid the immune system, but can also activate it to the virus' advantage (reviewed in (137, 138)). In the mouse model the MCMV chemokine homolog MCK-2 (m131-m129) has a potent pro inflammatory property and plays a crucial role in dissemination and immune evasion (139-141). The function of the HCMV viral chemokines, vCXCL-1 and vCXCL-2, *in vivo* has been inferred from this data, even though they are from a different subclass of chemokines (142, 143). The constitutively active CMV chemokine receptors utilize the signaling from the chemokine receptors for its advantage. In HCMV there are four chemokine-like receptors: US27, US28, UL33, UL78 (reviewed in (144)). The rodent homologues, M33 and M78 in MCMV and R33 in RCMV, are the counterparts of HCMV UL33 and have a crucial role in immune evasion and/or dissemination (145-147). Recently the mouse model was used as a surrogate for replacing the function of M33 with the HCMV G-Protein coupled receptor (GPCR) homologues US28 and UL33 (148). The RhCMV genome also encodes six CXC chemokines and five viral GPCRs (29, 149-152), and although they are dispensable for virus growth *in vitro*, the function of most of these proteins *in vivo* is unknown (153).

HCMV also encodes proteins with cytokine homology. HCMV encodes a homolog to host IL10 (154-159). Endogenous IL10 is an immune suppressive cytokine that down regulates T cell activation. RhCMV also encodes a homolog of rhesus IL-10, which possesses potent anti-inflammatory activity that weakens the antibody and cellular immune responses *in vivo* (160,

161). MCMV lacks an equivalent IL10 homologue, which reduces its usefulness as a model of HCMV IL10 *in vivo*.

The MHC class I homologs MCMV M144 and RCMV R144 are the counterparts of UL18 in HCMV. Because of their MHC class I homology, it was speculated that UL18 and its counterparts would therefore be important for preventing NK cell lysis. Using knockout viruses in their respective models, these proteins contribute to virus survival and dissemination (162, 163) (reviewed in (164)). *In vivo*, the MCMV protein m04/gp34, which escort class I proteins to the cell surface, was shown to prevent NK cell activation (165). Taken together the mouse system has been valuable for mapping which immune evasion proteins are important for resistance to NK cell lysis.

In human, mouse, and rhesus CMVs, there are several proteins that alter class I expression/antigen processing and presentation *in vitro*. These proteins are “functional” homologues of proteins in HCMV (i.e., limited sequence homology but similar functions). The HCMV encoded proteins, gpUS2, gpUS3, gpUS6, and gpUS11, interfere with MHC class I surface expression and antigen presentation (146, 166). The RhCMV homologues of HCMV gpUS2 and gpUS11 are the functionally related gpRh182 and gpRh189 proteins, while gpRh185 also has many of the functional features of gpUS2, gpUS3, and gpUS11 (167). It was initially hypothesized that these proteins would diminish CD8⁺ T cell detection but *in vivo* evidence following infection with recombinant viruses lacking some or all of these proteins leads to a similar immune response and equivalent viral titers. Recent evidence from animal models points to a role for these MHC homologs in superinfection (168). Superinfection not only explains how humans can be infected multiple times with the same strain of CMV but also presents a problem for vaccinologists. The data from the rhesus experiment points to the difficulties of effective vaccine development, which will require more than this partially protective CD8⁺ T cell response (169). Paradoxically, the MCMV equivalents of the class I immune modulating proteins (m152/gp40, m04/gp34, and m06/gp48) contribute to an increase in processed and presented antigens leading to a *greater* CD8⁺ T cell response. This casts into doubt the working hypotheses about these proteins dampening CD8⁺ T cell responses (170). Perhaps this is the host’s countermeasure for the viral counter measure!

CMV infection also induces inflammatory mediators, which seem to have an important role in viral replication. Cyclooxygenase-2 (COX-2), for example, is an enzyme that leads to the

generation of inflammatory lipid-derived compounds such as prostaglandin E(2). Although HCMV does not have a COX-2 homolog, it up regulates cellular COX-2 protein expression upon infection. COX-2 and the production of prostaglandin E(2) are necessary for HCMV infection. In fact, COX-2 inhibitors prevent normal HCMV replication (171). Unlike HCMV, RhCMV encodes a COX-2 homolog, which is critical for viral growth in endothelial cells (172). Thus, controlling inflammation is not only important for immune responses but also contributes to efficient viral replication. The role of the CMV induced or encoded inflammatory mediators *in vivo* have yet to be determined.

HCMV inhibits apoptotic cellular defenses (Reviewed in (173)). Using knockout mice and viral deletion mutants, Upton *et al* showed the importance of the viral inhibitor of RIP (vIRA), encoded from the M45 locus of MCMV (174). vIRA inhibits RIP3 activation of necrosis (174). Although there is no equivalent gene in HCMV, it encodes other inhibitors of apoptosis, which may serve a similar function (175, 176). Perhaps HCMV must only counteract apoptotic pathways instead of RIP3/necrosis pathways (reviewed in (177, 178))

7. Dissemination within the Host

Clinical studies have revealed the routes of HCMV person to person spread. Vertical transmission of HCMV occurs via transplacental transfer of virus (179-181), intrapartum transmission (182) and via breastfeeding from infected mother to child (183-187). Horizontal transmission includes organ transplantation from an infected donor, exposure to infected secretions (i.e., saliva), contact with infected urine during childhood (188), and sexual activity in adulthood (189). Inside the host the infection spreads mainly via leukocytes (190).

MCMV is an excellent experimental model for studying the interaction of CMV with different tissues and cells following infection (191-195). CMVs can productively infect many different cell types. These include epithelial cells of the salivary glands, kidneys, lung, liver, and intestines (196). MCMV can infect endothelial cells lining the spleen (197), myocytes, brown fat adipocytes, connective tissue fibrocytes, bone marrow stromal cells, dendritic cells, monocytes and tissue macrophages, but the B- and T-cell compartments of lymphoid organs, including the thymus are not infected (198). The mouse model has demonstrated that circulating leukocytes, predominantly mononuclear cells, disseminate MCMV and that cell-associated viremia is biphasic. First, primary dissemination of MCMV leads to infection of

reticuloendothelial organs the liver and spleen. This is followed by viral amplification and a more intense secondary viremia to organs such as the salivary gland (199). This system also allowed the identification of the genes that are necessary for virus replication in the different tissues (200). Recently an MCMV conditional gene expression system was used to quantify viral productivity in specific cell types and determine the role that each one plays in viral dissemination *in vivo* (201, 202). Viral factors including chemokines, GPCRs, anti-apoptotic genes, and tegument proteins play a role in viral dissemination and full pathogenicity in the mouse (139-141, 146-148, 175, 176, 203).

8. Latency and Reactivation

In humans HCMV remains latent in endothelial cells and cells of the myeloid lineage (reviewed in (204)). Animal models of latency and reactivation have played a role in our somewhat limited understanding of the maintenance of CMV latency and the signals necessary for reactivation (reviewed in (198, 205)). Studies with mouse and guinea pig models of CMV confirmed the role of myeloid lineage cells in virus persistence and the specificity of the CD8⁺ T cell responses during latent infection (102, 206-210). Viral and host factors, including novel “unfit” NK cells, have been identified that contribute to persistence and latency in different organs (145, 175, 176, 211). Also in the murine system, the helper function of CD4⁺ T cells (212) and antigen presentation on non-hematopoietic and hematopoietic cells (213) play important roles in memory inflation in latently infected hosts.

CMV reactivation in the mouse model includes a kidney transplantation model (214). Several factors inducing reactivation have been identified (198, 215, 216). However immune suppression and cytokine mediated activation of the productive viral cycle appears to be the most common inducers of recurrence (118, 198, 217). Transcription of IE1 and the differentiation state of the cell may not be sufficient for virus reactivation (218, 219). However allogeneic organ transplantation, tissue implantation, or cell transfer (220) are important factors for inducing the reactivation of MCMV and RCMV (219, 221, 222). The mouse system was also used to understand the source of the reactivated virus in models of organ transplantation. Using a kidney transplantation model in the mouse, Klotman *et al.* showed that the source of reactivated MCMV in an uninfected recipient comes from the transplanted organ, but if the recipient is latently infected, the majority of the time the reactivated virus comes from the recipient (223). The

rodent models have been very useful for modeling transplantation reactivation and for dissecting mechanisms of reactivation.

9. Vaccine and Drug Development and Testing

The animal models for CMV infection have been used to test new candidate vaccines and the potency of existing ones. The gB vaccine that has shown some promise in clinical trials was initially tested in animal models of both congenital infection and immunosuppression. (123-125). All of the models except for the rat have provided some clues to vaccine design. In the guinea pig model, systemic immunity to GPCMV has been shown to protect against hearing loss following congenital infection (76, 224) (reviewed in (25)). Antibodies against gB have been shown to be protective against congenital disease, which provided hope that this would also work in humans (225, 226). Vaccination with gB DNA subunit has shown some capacity to provide protection against congenital CMV infection (227, 228). More recent vaccination studies with GPCMV matrix protein GP83, a homolog of HCMV pp65, generated protective T cell-mediated immune responses against congenital GPCMV infection and disease (229). This highlights other possible avenues for protective vaccination. In the rhesus model, DNA vaccination with plasmids encoding gB, pp65-2 and IE-1 has shown promise. The gB-pp65 combined vaccine significantly reduces RhCMV copy numbers in plasma and oral shedding of the virus and has proven to be much better than vaccines directed against only gB (126, 230).

Because of its affordability and the availability of reagents for dissecting the immune response, the mouse has been particularly useful for testing potential vaccine strains such as attenuated viruses or viruses that overexpress potential immune stimulators (i.e., for NK cells) (231-236). Although these animal models of vaccination have not yielded an efficacious vaccine in humans, they have provided the foundational baseline of which proteins, routes, and attenuation genes to target.

The susceptibility of animal CMVs to various antiviral drugs makes them ideal for the identification of potential antiviral compounds (reviewed in (237)). MCMV is susceptible to ganciclovir (GCV) (238). More recently, a number of nucleoside analogues with Z- or E-methylenecyclopropane structures have been evaluated in the mouse model and possess better activity than GCV (239, 240). GPCMV is resistant to GCV (241). However, it is susceptible to cidofovir and cyclic cidofovir (242). The guinea pig model has been used to demonstrate the

efficacy and safety of cidofovir and cyclic cidofovir (243, 244). The administration of these drugs during pregnancy prevents GPCMV mortality in pups (245). Other novel non-nucleoside analogues have also been tested in guinea pigs using the immunosuppressive model of CMV infection (246).

RhCMV has comparable susceptibility to GCV, foscarnet, and benzimidazole nucleosides (247). The highly conserved sequence of the drug target proteins in HCMV and RhCMV make this model ideal for testing the efficacy and safety of novel anti-HCMV drugs both in immunocompetent and immunocompromised animals. (29, 149, 247)

10. Animal Models for HCMV Studies *in vivo*

True animal models of HCMV infection are extremely difficult to develop due to its strict species-specificity. However, several attempts have been made to develop models of HCMV infection in animals that recapitulate one or more phases of the viral replication cycle. Human cells/tissue fragments implanted into mouse (248) and rats (249) have been shown to support viral infection *in vivo*, but only within the implanted cells.

In SCID mice human fetal thymus and liver implants were placed under the kidney capsule (SCID-hu mice) (250) or fragments of human fetal retina were placed in the anterior chamber of the eye and supported HCMV growth (240, 251-254). Using the SCID-hu mouse model Wang *et al* demonstrated that the ULb' region, encoding 19 open reading frames, present in all virulent strains but deleted from attenuated strains is essential for HCMV replication *in vivo* (255). An *in vivo* model of HCMV retinal infection in athymic rats has also been developed using the same approach (256).

SCID-hu mice do not support systemic infection nor do these mice develop viral latency (250, 254). In order to study systemic and latent HCMV infection, reactivation, and viral spread within myeloid progenitors, monocytes, and macrophages, a model system in which huCD34⁺ hematopoietic stem cells (HSCs) are engrafted into NOD/SCID mice has been developed. In this model, granulocyte-colony stimulating factor (G-CSF) leads to reactivation of latent HCMV in monocytes/macrophages that have migrated into organ tissues. The results from this study also suggest that G-CSF mobilized blood products from seropositive donors pose an elevated risk for HCMV transmission to recipients (257). These implant-based animal models for HCMV

infection have also proved to be valuable for drug testing and vaccine development against HCMV (237).

11. Summary

This chapter has provided an overview of CMV infections in the different animal models. Each one has provided important insights into the lifecycle of HCMV and each has its promoters and detractors as models for HCMV infection. In the following chapters, experts in the field will provide details of how to use both animal and cellular systems to address important questions in CMV biology. Importantly, the animal models described in this chapter will ultimately provide the conduit by which discoveries in fundamental virological processes can be examined and extended to an *in vivo* setting.

PART II: Literature Review

1. Chemokines

Chemokines are important in the regulation of a number of biological processes.. They are small proteins (approximately 9-30 amino acids or 8-12 kDa in size) produced by leukocytes that mediate their signaling through G-protein coupled receptors (258-260). Chemokine gradients provide directed movement of cells through blood or tissues. They are classified based on the number and spacing of the two cysteine residues on the N-terminus of the chemokine. Following this criteria, chemokines are classified as C, CC, CXC, and CX3C chemokines. For example, the CXC chemokine (α subfamily) chemokines have two cysteines separated by an amino acid residue while the CC chemokines (β subfamily) have two juxtaposed cysteine residues. The C chemokines (γ subfamily) have only one cysteine, and the CX3C chemokines (δ subfamily) have two cysteines separated by three amino acids (261).

CXC chemokines are further subdivided into ELR or non-ELR CXC chemokines depending on the presence of a glutamate-leucine-arginine (ELR) motif directly preceding the CXC motif of the chemokine (262). The ELR CXC chemokines bind to CXCR1 and/or CXCR2 and are responsible for the activation and migration of neutrophils (262, 263). Single amino acid substitutions in the ELR motif showed all three residues, especially arginine, are sensitive to

modification and are critically important for CXCL8 (IL-8) function (264, 265). The non ELR chemokines attract cells other than neutrophils (e.g., CXCL10 (IP-10) attracts monocytes, T lymphocytes, and natural killer (NK) cells (266, 267), CXCL9 (MIG) attracts tumor-infiltrating T lymphocytes (268), CXCL12 (SDF-1) stimulates the proliferation of B cells (269)). The ELR motif is critical to the specificity of chemokine function as illustrated by the experiments wherein the addition of ELR to the N-terminal domain of the non-ELR CXC chemokine, CXCL4 (PF4), transforms it into a neutrophil chemoattractant (264). This addition, however, was not sufficient for IP-10, which also requires additional Gly31 and Pro32 changes to become a fully functional on human neutrophils (270). ELR CXC chemokines have also been shown to possess angiogenic properties. For example, CXCL8 (IL-8), CXCL7 (NAP-2), CXCL1 (GRO- α), CXCL2 (GRO- β), CXCL3 (GRO- γ), and CXCL5 (ENA-78) have proangiogenic properties because of their ability to recruit endothelial cells (271, 272). ELR CXC chemokines like CXCL1 (GRO- α), CXCL2 (GRO- β), and CXCL3 (GRO- γ) have also been reported to possess angiostatic properties at higher concentrations (>~1000 fold higher) (273-275). Unlike the ELR CXC chemokines, many non-ELR CXC chemokines only have angiostatic properties (262, 274).

The **CC chemokines** form the largest family of chemokines with 28 members and attract a variety of cell types including monocytes, basophils, eosinophils, and dendritic cells (DCs) (262, 276). The first CC chemokine to be characterized, CCL2 (monocyte chemoattractant protein-1: MCP-1), attracts monocytes but not neutrophils (277). MCPs not only attract monocytes (277-280), but also CD4⁺ and CD8⁺ T lymphocytes (281-283) and basophils (284-287). CCL4 (macrophage inflammatory protein-1 β : MIP-1 β), which is produced mainly by macrophages, has been shown to attract NK cells and monocytes (288). The CCL5 (RANTES) (289) and CCL11 (eotaxin) (290) have also been shown to be potent eosinophils attractants.

CX3CL1 (fractalkine) is the only member of the **CX3C chemokine** family. Fractalkine is produced as a long protein (373 amino acids) with an extended mucin-like stalk and a chemokine domain on top (291). CX3CL1 is expressed on neurons, lung epithelial cells, kidney, and in the intestine (292-294). CX3CL1 binds to CX3CR1 on cells and to induce chemotaxis, cellular adhesion and increases cell survival during hemostasis and inflammation (292). The mucin-like stalk anchors CX3CL1 to the surface of endothelial cells and promotes strong adhesion of leukocytes to the endothelium (295, 296) and may play a role in promoting atherosclerosis (297). The membrane-bound form can also be enzymatically cleaved to become a soluble chemokine

(85 or 95 kDa) that can potentially attract monocytes and T lymphocytes (291, 295, 296). CX3CL1 has been speculated to be involved with low-grade inflammation in adipose tissue and function as an adipokine positively upregulated in obesity and diabetes (298, 299). Recent studies show that CX3CL1 plays a direct role in type 2 diabetes by controlling insulin production (300) and enhancing pancreatic β cells survival (301).

The members of the **C chemokine** family lack two of the four cysteine residues that are characteristic of chemokines (302). This family is represented by two chemokines, XCL1 (lymphotactin α) and XCL2 (lymphotactin β) (302-305). Lymphotactin (Lptn) has also been reported to be chemotactic for B lymphocytes, T lymphocytes and neutrophils through CXCR1 receptor (306). Therefore, Lptn could potentially play an important role in the regulating T and B lymphocyte and neutrophil trafficking during inflammatory and immunological responses (306).

Secreted chemokines, being cationic, can bind negatively charged glycosaminoglycans (GAGs) such as heparan sulfate (HS) on the surface of cells or within the extracellular matrix (259, 307, 308). The GAGs provide a solid support for gradient formation allowing for the directed movement of cells under conditions of blood flow at the endothelial surface as well as receptor interactions (259, 307, 309, 310). For example CXCL8 (IL-8) binds to GAG through its C-terminal α -helix and shows enhanced chemotactic activity towards neutrophils when bound to HS (311). In studies using extracellular matrix (ECM)-coated culture dishes, CCL4 (MIP-1 β) and CCL5 (RANTES) mediated leukocyte adhesion and chemotaxis that was GAG-dependent (312). *Proudfoot et al.* demonstrated the importance of chemokine-GAG interactions *in vivo* using mutated chemokines that do not bind GAGs efficiently and showed that these chemokines induced chemotaxis *in vitro* but were severely limited in the recruitment of cells compared to wild type chemokines when injected into the peritoneum of mice (313).

Microbial products and inflammatory cytokines like TNF induce the expression of chemokines and their receptors to initiate the inflammatory response (314). For example, MIP-1 produced by macrophages in response to bacterial endotoxins leads to the activation and influx of neutrophils to the site of infection (308, 315). Chemokines are also necessary for efficient homing of leukocytes during homeostasis. For example, CCL19 and CCL21 induce the migration of T cells and CXCL12 the migration of B cells into secondary lymphoid tissues (316). Chemokines also control tissue retention by controlling lymphocyte egress via the afferent lymph. For example, CCR7 expression on CD4 $^{+}$ and CD8 $^{+}$ T cells is essential for the egress of

these cells from the lung during an inflammatory response (317-319). Chemokines induce chemotaxis by regulating the expression of leukocyte adhesion molecules such CD11a, b, and c. These adhesion molecules are the α component of the β_2 -integrin heterodimeric receptor. For example, CXCL8 upregulates the expression of β_2 -integrins on neutrophils (320). Conformational changes to the β_2 -integrins in response to chemokine leads to a strong adhesion and arrest of the leukocytes to the endothelium facilitating their transmigration into tissues (321, 322).

In addition to chemotaxis, chemokines also participate in inducing effector functions of immune cells. Activation of granulocytes in response to chemokines leads to their degranulation contributing to the inflammatory response (323). For example, neutrophils activated in response to chemokines release antimicrobial proteins such as defensins and lysozyme as well as proteases capable of degrading proteins of the extracellular matrix and basement membrane (324). Chemokines are also capable of triggering respiratory burst in immune cells leading to the formation of reactive oxygen species, oxygen metabolites with potent antimicrobial activities (325). Chemokines can also provide important survival signals for CD4+ T cells in peripheral tissue. For example, CX3CR1 was found to be important for the development of allergic airway inflammation in mice (326). Chemokines also play a critical role in the development of memory cell populations by determining their migration and surveillance properties (327).

2. Chemokine Receptors

2.1 Structure, Ligand Binding, and Activation

Chemokine signaling is mediated through their cognate G protein coupled receptors (GPCRs). GPCR activation requires interaction between specific domains of the receptor and several motifs on the chemokine. Approximately 20 signaling chemokine receptors have been reported as well as three non-signaling scavenger receptors that dampen the immune response by binding, internalizing, and degrading chemokines (328-330). Chemokine receptors are GPCR seven-transmembrane (7TM) helical regions connected by extra-cellular loops (ECLs) (Figure II.1A). Until 2007, none of chemokine receptors had been crystallized. The models were based on bovine rhodopsin, the only 7TM receptor for which three-dimensional structures had been solved at the time (331-334). From this structure, the N terminus and three of ECLs are extracellular, whereas the C terminus and three intracellular loops (ICLs) face the cytoplasm.

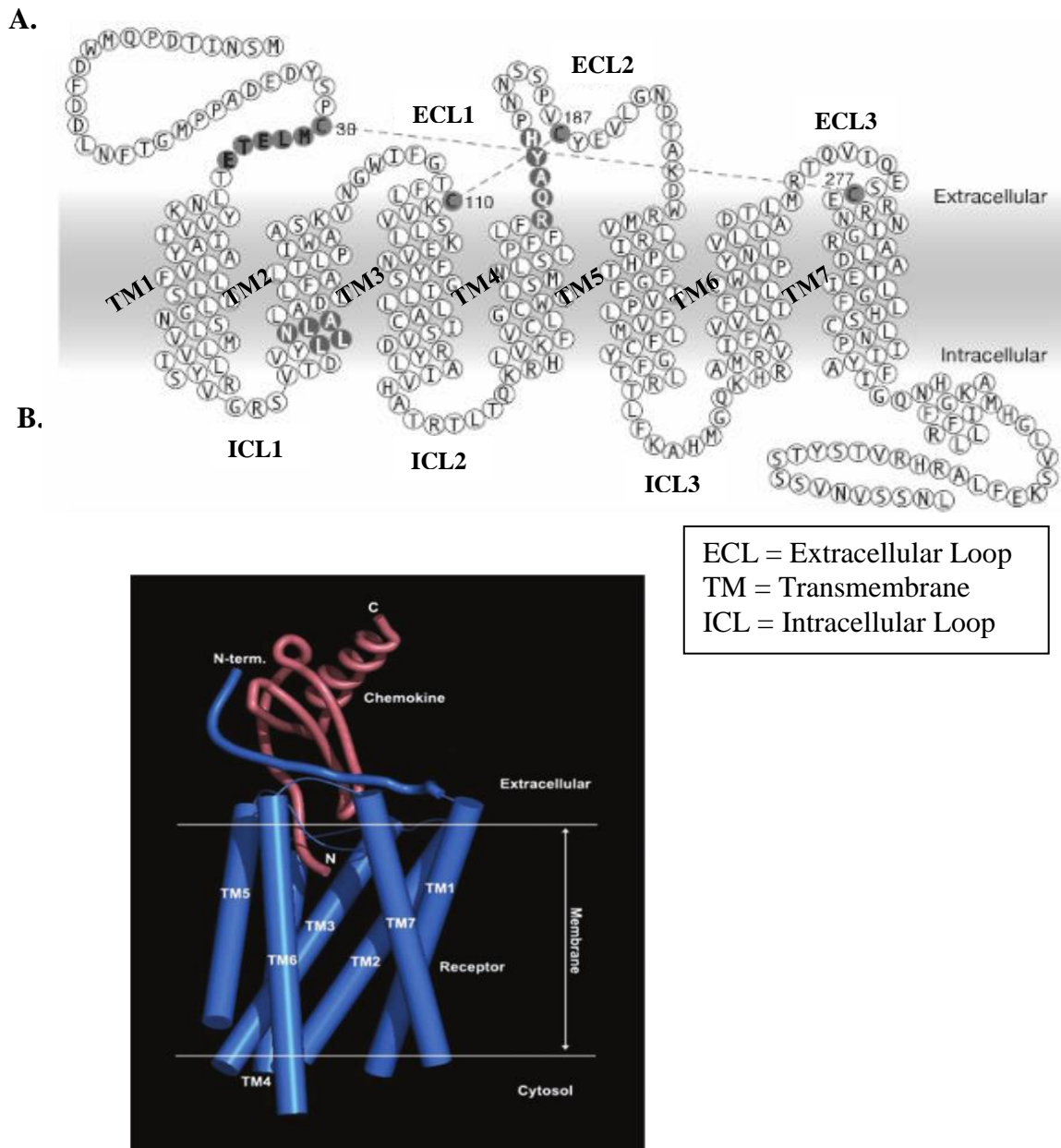


Figure I1.1. Receptor structure and receptor-ligand interaction. (A) Structure of CXCR1 showing the 7TM regions, ECL and ICLs. The two disulphide bonds are shown as dotted lines. (B) Hypothetical model of the interaction of a chemokine (pink) with its receptor (blue). The model illustrates the interaction between the N-terminal domain of the ligand with the receptor helical bundle, and the interaction of the core domain of the ligand with the ECLs of the receptor (as observed for CXCR4-vMIPII, CCR1-CCL3). Figures adapted from *Park et.al, 2012, Nature* (336) and *Allen et al., 2007, Annu. Rev. Immunol* (340).

The structures of CXCR1 (335) and CXCR4 (336) have recently been described and show significant similarities. Both the structures show a kink that changes the direction of TM helix TM2 (335, 336). The extracellular portion of TM7, just after ECL3, is tilted towards the central axis of the receptor in CXCR1 (335). The TM7 helix is also about one turn longer in CXCR1 at the intracellular end compared to CXCR4. The H8 helix present in CXCR1 immediately preceding the mobile C terminus and absent in CXCR4, has a distinctly amphipathic amino acid sequence that interacts with the phospholipid bilayer to stabilize the conformation of CXCR1 (335).

Two disulphide bonds, one connecting the N terminus to the extracellular part of TM7 and the other connecting the extracellular end of TM3 to ECL2 have been identified in the structures of the chemokine receptors (335, 336). These Cys pairs are conserved in the sequences of other chemokine receptors as well. They play an important role in shaping the extracellular structure of the receptor, ligand binding, and provide restraints for receptor structure determination (335). Charged residues are located near the membrane-water interface in both CXCR1 and CXCR4, where they interact with basic residues on the ligand (337). In addition, four charged residues, contributed by helices TM2, TM3 and TM7, form a polar cluster in the core of the helical bundle of CXCR1, and may be involved in ligand binding and signal transduction (335). Residues in the ICL2 (i.e., the aspartic acid, arginine, tyrosine (DRY) motif (338)) and in the ICL3 between Thr 228 to Gln 236 connecting helices TM5 and TM6 are important for CXCR1 coupling to G proteins. These residues are critical for the ability of the chemokine receptor to induce calcium mobilization, chemokine mediated migration, and cell adhesion (335, 336, 338). The importance of these residues in chemokine receptor signaling is apparent in scavenger receptors that lack the DRY motif, and thus are unable to induce a downstream signal (339).

The use of chimeric proteins and mutagenesis studies identified the N terminus of several receptors (CCR2, CCR3, CCR5, and CXCR1) as important for ligand binding (337, 340, 341). For example, it has been shown that the chemokine interacts via its globular core with the receptor N terminus chemokine recognition site 1 (CRS1) and via its N terminus with the CRS2 (Figure II.1B) (337, 339, 342). *Blanpain et al.* (343) showed that the globular body of the chemokine contacts the ECL2 in addition to the N terminus orienting the chemokine as suggested by *Skelton et al* (337). By generating chemokine receptor elements on a soluble scaffold (i.e., CROSSes), *Stone et al.* also demonstrated that the N termini and ECL3 were needed for proper binding of the ligand to CCR3 and CCL11 (344, 345). Many of the chemokine receptors (CCR2, CCR5, CCR8, CXCR4, CX3CR1) are also glycosylated and/or tyrosine sulfated on their N termini (346-352). The tyrosine sulfation increases the affinity of the receptors for their ligands, but the function, if any, of glycosylation is unknown.

GPCRs are thought to exist as homo or hetero dimers and/or higher-order oligomers (353-361), with hetero- and homodimers activating distinct signaling pathways (357). Dimerization might influence receptor function by regulating the trafficking of receptors to the cell surface and other locations within the cell (356), receptor specificity, and signaling (357, 362). Residues in TM1 and TM4 (Ile52 and Val150) have been shown to be involved in the ligand dependent dimerization of CCR5 (363). Dimerization of CXCR2 occurs shortly after synthesis and is therefore likely to be ligand-independent (360). However, other reports describe agonist-dependent oligomerization that could lead to complex signaling (364). Receptors can also undergo heteromerization (e.g., CCR2 can heteromerize with CCR5 when co-expressed in a cell. In this case, ligands of CCR5 can block signaling through CCR2 and vice versa (365). The activation of the receptors will also depend on the ligands. Chemokines can bind to the receptor dimer as a monomer (366) or dimer (357, 361). Thus, receptor and chemokine dimerization contributes to the complexity of chemokine responses including ligand recognition, signaling specificity and receptor trafficking.

Amino acid residues at the N-terminus of both CC and CXC chemokines preceding the first cysteine are considered critical for receptor binding, activation, and specificity (259, 367-371). In studies with N-terminus truncation mutants of CXCL12 (SDF-1), the residues 1-8 preceding first cysteine in are essential for receptor binding (372). Additionally the deletion of the first two residues generates a receptor antagonist (372). For CCL2 (MCP-1), all of the 10 amino acid residues preceding the first cysteine are required for full activity (367, 368). Altering even a single amino acid residue at the N-terminus of CCL7 (MCP-3) and CCL5 (RANTES) produces potent antagonists (370, 371). In addition to that, the ELR CXC chemokines require the ELR motif and the N-loop region following the second cysteine for receptor recognition, activation, and specificity (259, 265, 270, 337, 369, 373, 374). *Skelton et al.* showed that the CXCR1 N-terminus fragment interacts along the ELR and N-loop motifs of CXCL8 (IL-8) (337). Moreover, swapping the N loops of CXCL8 and CXCL1 caused an interchange in their receptor binding and specificity (369). Synthetic peptides comprising the N-terminus and the N-loop have reduced activities compared to the full-length chemokines suggesting that the other regions within the chemokine also contribute to receptor interactions and activation (262).

With a better understanding of the structure-function relationship of chemokines and their receptors we can now begin to understand how differences in the sequence and structure of the virally encoded chemokines and receptors (as discussed in section 3 below) can induce different

signaling cascades that benefit the virus. This difference in signaling of the viral chemokines may play a role in viral pathogenesis by inducing differential migration and effector functions of immune cells leading to increased dissemination, inflammation, or increased viral replication.

2.2 Signaling

The G proteins that interact with the GPCRs are heterotrimeric and composed of α , β , and γ subunits (375, 376). To date, 17 $G\alpha$, 5 $G\beta$, and 12 $G\gamma$ proteins have been described (376-378). The binding of a chemokine to the GPCR induces conformational changes in the receptor, which activates its guanine-nucleotide exchange factor (GEF) function. The activated GPCR exchanges GDP for GTP on the $G\alpha$ subunit that causes the dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ dimer. Both the dissociated $G\alpha$ and $G\beta\gamma$ subunits in turn activate several downstream effectors (379-381). The $G\alpha$ subunit remains in the activated GTP-bound state until the hydrolysis of GTP to GDP, which leads to re-association of the heterotrimer and termination of the signal by the regulator of G-protein signaling protein signaling protein (382)

There are four subfamilies of the α subunit of the G proteins: $G\alpha_s$, $G\alpha_i/o$, $G\alpha_q/11$, and $G\alpha_{12/13}$ and each of the $G\alpha$ protein signals several downstream effectors. Signaling via $G\alpha_s$ activates adenylyl cyclase (AC), which increases the levels of cyclic AMP (cAMP) subsequently activating mitogen-activated protein kinases (MAPK) and protein kinase A signaling cascades (383). On the other hand, signaling via $G\alpha_i/o$ inhibits AC, reducing the levels of cAMP, which in turn activates the G-protein-coupled potassium channels leading to the hyperpolarization of the cell membrane (384). $G\alpha_q/11$ protein activates phospholipase C β (PLC β), and $G\alpha_{12/13}$ subunit activates Rho guanine nucleotide exchange factors (385). 8 of the 17 $G\alpha$ proteins are widely distributed, but the remaining 9 are expressed in selective cell types (377), which leads to the specific type of signaling pathway induced in response to receptor engagement in a particular cell type (377).

The dissociated $G\beta\gamma$ subunits also regulate signaling pathways leading to the phosphorylation of PLC β (386, 387). Activated PLC β cleaves phosphatidylinositol 4,5-bisphosphate to inositol triphosphate (IP $_3$) and diacylglycerol (DAG). IP $_3$ in turn elicits calcium mobilization from the endoplasmic reticulum into the cytoplasm while DAG activates protein kinase C (PKC) (388). PKC activation is required to upregulate β_2 integrins, such as CD11b/CD18, on neutrophils and eosinophils (389, 390). In addition, $G\beta\gamma$ also stimulates

serine-threonine kinase, Akt, through phosphatidylinositol 3- kinase (PI3K) (378). Akt activation, as well as ERK pathway, is responsible for chemotaxis of HL60 cells or CXCR2 transfected HEK293 cells (391, 392). The diversity of the subunits that make up G proteins and overlapping signaling cascades through $G\alpha$ and $G\beta\gamma$ is responsible for the complex and multiple downstream signaling events in response to the activation of GPCRs.

After initial signaling, the G protein receptor kinases (GRKs) and β -arrestins shut off signaling through GPCRs (393). GRKs phosphorylate serine and threonine residues at the C-terminus of the GPCR, providing a binding site for β -arrestin. β -arrestin binding to the GPCR sterically interferes with the association of the G protein with the GPCR leading to the desensitization of the receptor (394). The GRK- β -arrestin system also induces clathrin-mediated internalization of the inactivated GPCR into the endosomal compartments for receptor recycling (395, 396). β -arrestins can also act as adaptors to mediate signaling through GPCRs independently of G proteins leading to the activation of MAPK, SRC, nuclear factor- κ B (NF- κ B) and phosphoinositide 3-kinase (394, 397, 398). To conclude, the coupling of a GPCR with multiple combinations of G proteins and β -arrestins can generate numerous complex signaling pathways and cellular effects even from similar ligands.

3. Cytomegalovirus Encoded Homologs of Chemokines, Cytokines, and their Receptors

In vitro experiments have shown that only 41 CMV ORFs are part of the core set of genes essential for HCMV replication in culture (399). There are about 88 genes that are non-essential for replication of the virus *in vitro* but may play a role in the survival/replication of the virus *in vivo* (399). Some of these genes encode immune modulatory chemokine (400) and cytokine (161, 401) homologs and receptors (402, 403). The following section discusses the details about the diversity and function of these CMV proteins.

3.1 CXC Chemokine Homologs in CMV

The HCMV genome carries two open reading frames (ORFs), *UL146* and *UL147*, that encode CXC chemokine homologs: viral CXCL-1 and 2 (vCXCL-1 and vCXCL-2) respectively (400). Similar to HCMV, chimpanzee CMV (CCMV) also encodes homologs of *UL146* and *UL147* in addition to *UL146*-related genes *UL146a* and *UL157* that are not present in other CMVs (404).

The rhesus CMV (RhCMV) also carries a homolog of the HCMV *UL147*, *rh158* (29). The virulent HCMV Toledo strain contains at least 19 ORFs in the 15 kb UL/b' region including the viral chemokine genes (*UL146* and *UL147*) which are missing in the lab adapted and attenuated AD169 strain (400). The absence of these genes does not affect the growth of AD169 *in vitro*, leading to the speculation that these genes may have a role in HCMV pathogenesis (400, 405, 406). The *UL146*-encoded protein, vCXCL-1, is a functional CXC chemokine (407). It can bind both hCXCR2 and hCXCR1 (407, 408) and is capable of inducing chemotaxis and calcium flux in isolated human neutrophils similar to CXCL8 (407, 409). The evidence for the role of viral CXC chemokines in HCMV dissemination comes from experiments with HCMV *UL146-UL147* deletion recombinants. In these experiments, viral passage to neutrophils was impaired while retaining its tropism for other cell types (410). No formal proof exists for the secretion of vCXCL-2 and there is no functional data for vCXCL-2 (138, 411). All data to date focuses on vCXCL-1 and not vCXCL-2.

Data from sequencing studies shows that the *UL146* gene is one of the most variable genes in the entire HCMV genome (405, 406, 412-418). *UL146* from clinical isolates shows 4% identity (5 residues) and 5% similarity (6 residues) to each other (416). It has been hypothesized that this variability may correlate to the severity of CMV disease (414, 415, 419). A recent study conducted by *Paradowska et al.* provided for evidence in support of this hypothesis by concluding that there is a correlation between HCMV vCXCL genotypes and the clinical sequela observed (405). However, other similar studies could not find such a correlation between the *UL146* genotype and CMV pathogenesis, which was attributed to the small sample size in these studies (412, 413, 415).

3.2 CC Chemokine Homologs in CMV

The HCMV *UL128* ORF encodes a protein with limited homology to CC chemokines (420). The encoded protein promotes the migration of human peripheral blood mononuclear cells (PBMC) and induces expression of pro-inflammatory cytokines TNF- α and IL-6 similar to that reported for MIP-1 α (421, 422). The HCMV *UL128* protein however, is non-functional in clinical HCMV strains (i.e., Toledo and Merlin) due to a partial inversion or frame shift mutation (420). CCMV and RhCMV also have a *UL128* ORF homolog (143, 149). The HCMV *UL130*

ORF encodes a protein containing a putative C chemokine fold (423), although functional data is lacking.

In addition to being a potent chemokine, the *UL128* locus (*UL128*, *UL130* and *UL131A*) of HCMV are essential for growth in endothelial cells, DC, epithelial cells, and for transmission to leukocytes (410, 424, 425). The UL128/130/131 proteins form a complex with glycoprotein H and L (gH/gL) on the virion surface to form the pentameric complex (426). The pentameric complex mediates entry of HCMV into endothelial and epithelial cells whereas the gH/gL/gO complexes mediate virus entry into fibroblasts (425, 426). Therefore, the genes at the UL128 locus play a dual role, as a potent chemokine and tropism determinant.

Splicing of the *m131* and *m129* genes of murine CMV (MCMV) produces a CC chemokine homolog MCK-2 (427, 428). Initially, the protein product of *m131* (MCK-1) alone was characterized as a functional chemokine (429). However, MCK-1 is a portion of a CC chemokine including a long carboxy-terminal domain, which includes the entire *m129* ORF. Together, MCK-1 with this carboxy domain form MCK-2 (427, 428). *In vivo* studies using MCMV recombinants deleted in MCK-2 demonstrated the role of MCK-2 in viral dissemination *in vivo* (139, 140). The recombinants are defective in their dissemination to the salivary gland and demonstrate lesser inflammation at the site of inoculation in the foot pad (139, 140). MCK-2 has been shown to recruit myeloid progenitor cells (141, 430) and monocytes (429). These cell types participate in the viral dissemination to the salivary gland (141, 430) and also provide a reservoir for establishing latency (206, 431-433). MCK-2 can also attract cells similar to the myeloid-derived suppressor cells (MDSCs) to the site of infection and impair proliferation and the differentiation of CMV-specific CD8⁺ T cells (434). *Wagner et al.* recently showed that the complex formed between MCK-2 and gH is incorporated into the virion envelope and plays a role in promoting MCMV infection monocytes/macrophages *in vitro* and *in vivo* (435). Therefore like the HCMV CC chemokine homolog, MCK2 also has dual role as a chemokine as a determinant of MCMV cell tropism.

Rat CMV (RCMV) genes *r129* and *r131* have limited homology to MCMV genes *m129* and *m131* genes (24). Deleting the *r131* ORF was shown to encode a functional CC chemokine that is a functional homolog of the MCMV MCK-2 protein. The *r131* deleted recombinants develop less swelling at the site of infection in the paw and demonstrate impaired viral

dissemination to the salivary gland as well as lower viral titers in the salivary gland compared to wild type virus (436) paralleling the MCK-2 phenotype.

Guinea pig CMV (GPCMV) encodes a CC chemokine with homology to CCL3 (MIP-1 α) and CCL14 (hemofiltrate CC-chemokine 1, HCC-1) not encoded by other animal CMVs (437, 438). GPCMV-MIP signals via hCCR1 and induces Ca⁺² flux similar to CCL3 (438, 439). Both CCL3 and CCL14 are known to enhance the proliferation of myeloid progenitor cells. As such, GPCMV-MIP production aids in viral dissemination and latency *in vivo* (440). Intracochlear inoculation of guinea pigs with the GPCMV-MIP deletion viruses resulted in reduced hearing loss compared to wild type virus, demonstrating its role in CMV pathogenesis (441).

3.3 Chemokine Receptor Homologs in CMV

The HCMV genome encodes for four chemokine receptors: US27, US28, UL33 and UL78 (402, 403). US28 has pleiotropic roles as an immune modulator and oncogene (144, 442, 443). US28 displays 30% homology with the β -chemokine receptor CCR1 and to lesser extent to CCR2 and CCR5 (444). The protein, pUS28, is the only viral chemokine receptor shown to bind CC as well CX3C chemokines (444-446). pUS28 is expressed on infected cells and can signal upon ligand binding to induce cellular responses similar to host β -chemokine receptors (444, 447-450). pUS28 also displays constitutive signaling activity, which leads to the activation of phospholipase C causing the accumulation of IP3 and DAG, as well as promoting NF- κ B activation (451-455). Signaling through these pathways induces smooth muscle cell migration (SMC)(448), which may contribute to the development of HCMV associated atherosclerosis (456). Using integrated analysis of sequence, structure, and simulations of pUS28, *Burg et al.* discovered that pUS28 has a distinctive structure near the cytoplasmic end of TM3. This results in a destabilization of the receptor's inactive state and is responsible for the observed ligand-independent constitutive activity (457). US28 is also transcribed during HCMV latency (437, 458, 459). However, its role in this setting is yet to be determined.

The ability of pUS28 to bind, sequester, and internalize chemokines allows it to act as an immune modulator by disrupting the chemokine gradient (460, 461). Experiments with HCMV US28 deletion mutants demonstrated that the chemotaxis of monocytes towards CCL5 and CCL2, which are expressed in response to infection, could be inhibited (462). pUS28, by virtue of its constitutive signaling activity, can also promote tumorigenesis by up-regulating signaling

via the STAT3/IL-6 axis to induce the proliferation of cells (463-465). Transgenic mice expressing pUS28 under the control of the villin promoter developed intestinal neoplasia that could be further enhanced by co-expression of the US28 ligand CCL2 (466). Transcripts of US28 and pUS28 have also been detected in primary cultures of glioblastomas and paraffin embedded glioblastoma tissue samples in nearly 60% of cases analyzed (465, 467), suggesting the involvement for pUS28 in tumorigenesis in humans.

Very little is known about the functions of other members of the HCMV chemokine receptor family. They are considered orphan receptors with no known ligands. pUS27 does not possess constitutive signaling activity (468). The protein localizes mainly to intracellular multivesicular bodies, but can also be detected at the cell surface and is incorporated into the virion envelope. The envelope-localized pUS27 appears to play a role in the extracellular spread of the virus in both fibroblasts and endothelial cells (468-470). pUS27 can colocalize and heteromerize with pUS28 without inhibiting its constitutive signaling activity (471). The role of this complex in CMV pathogenesis, however, is unknown.

The UL33 gene is conserved in all β -herpesviruses, including HCMV (UL33), RCMV (R33), and MCMV (M33). The UL33 protein and its rodent homologs also show constitutive activity, although they differentially activate specific signaling pathways (452, 455, 472, 473). The pR33 and pM33 are not essential for viral replication *in vitro*, nor does pM33 play a role in hematogenous dissemination *in vivo* (474). However, these proteins are important for viral replication and cell to cell spread in the salivary gland (147, 474-476) and reactivation from spleen and lung explants (148, 475). The constitutive activity of pM33 is required for efficient dissemination and replication MCMV *in vivo* (477). pM33 can be activated upon binding CCL5 and both pM33 and pR33 induce SMC migration similar to pUS28 (85, 86). Moreover, replacing M33 with US28 from HCMV, partially complements the activity of MCMV M33 deletion recombinant (148). This data suggests that pM33 is a functional homolog of the HCMV US28 (85) and that GPCRs of the UL33 family might modulate cellular trafficking contributing to viral pathogenesis.

UL78 is the newest member of the HCMV chemokine receptor family and is not required for the replication of the virus *in vitro* (478). The genomic location of both UL33 and UL78 is conserved among all known β -herpesviruses, suggesting a role for these proteins in the viral life cycle *in vivo* (479). A recent study has shown that pUL33 and pUL78 can form a complex with

pUS28 without affecting its constitutive activity. However, this might interfere with the ability of pUS28 to induce NF- κ B activation, suggesting a potential regulatory role for this complex in infected cells (471, 480). Both pUL78 and pUL33 can also heteromerize with the host chemokine receptors CCR5 and CXCR4 in THP-1 cells. This interaction inhibits HIV-1 infection of infected cells (481) and may function to modulate the host immune response to chemokines that bind to CCR5 and CXCR4 (481).

3.4 Cytokine Homologs in CMV

During productive infection the HCMV gene *UL111A* encodes a homolog of human IL-10 (hIL-10) called cmvIL-10 (161, 401). Alternative splicing of the *UL111A* gene leads to the expression of another viral IL-10 homolog during latency termed latency associated cmvIL-10 (LAcmvIL-10)(482). The cmvIL-10 transcript encodes a 175 amino acid protein, whereas LAcmvIL-10 has 139 amino acid residues. Both cmvIL-10 and LAcmvIL-10 share 27% amino acid identity with hIL-10. Deletion of the *UL111A* gene from HCMV neither affects virus replication (483) nor the establishment and maintenance of HCMV latency *in vitro* (484). Although divergent in its sequence from hIL-10, cmvIL-10 can still bind and signal through the hIL-10 receptor (161, 401, 485) and has immunomodulatory functions similar to those of hIL-10. For example, similar to hIL-10, cmvIL-10 can inhibit the production of pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 from lipopolysaccharide (LPS) stimulated PBMCs and monocytes. They also decrease the monocytic expression of major histocompatibility complex (MHC) class I and MHC class II (159, 486, 487) and inhibit the maturation and effector function of monocyte derived DCs (156, 157, 488), plasmacytoid DCs (154) and microglial macrophage, which results in a limited chemotaxis of activated T lymphocytes towards these cells (489). cmvIL-10 can also stimulate proliferation and differentiation of B lymphocytes (158) and monocyte differentiation towards a more macrophage like phenotype rather than a dendritic cell phenotype. This allows the virus to replicate in these cells and not be presented to T cells in the lymph node (490). During latency, viral IL-10 down-regulates the cytokines associated with DC differentiation (491) and the surface expression of MHC class II on latently infected cells leading to an impaired CD4⁺ T cell recognition of latently infected cells *in vitro* (484).

While both cmvIL-10 and LAcmvIL-10 can suppress MHC class II on primary human myeloid progenitor cells and monocytes (159, 492), LAcmvIL-10 does not impair DC

maturation, nor does it inhibit the expression of proinflammatory cytokines and costimulatory molecules CD40, CD80, and CD86 expressed on stimulated DCs, which cmvIL-10 and hIL-10 do (492). Furthermore, it also lacks the ability to stimulate B cells and phagocytic macrophages (158, 490). Structural differences between cmvIL-10 and LAcmvIL-10 might account for this difference in the function range of cmv IL-10 and LAcmvIL-10. Studies using hIL-10 receptor blocking antibodies have shown that LAcmvIL-10 may either not bind and signal through the hIL-10 receptor or it may do so differently than cmvIL-10 and hIL-10 (492), which might result in a restricted range of functions.

RhCMV encodes a functional IL-10 homolog (RhcmvIL-10) and has been used to evaluate the role of cmvIL-10 *in vivo* (160, 161, 493). In studies using RhcmvIL-10 deleted mutants, rhesus macaques infected with the mutants displayed enhanced inflammatory response and diminished macrophage infiltration (160). At the same time, these infected macaques demonstrated a significantly higher DC numbers, a stronger CD4⁺ T cell proliferative response and higher IgG titers (160). cmvIL-10 can also enhance congenital HCMV disease by impairing cytotrophoblast remodeling of the uterine vasculature to limit fetal growth (494). The combination of cmvIL-10's ability to suppress pro-inflammatory mediators accompanied by stimulation of B cells, works to skew the host immune response such that it is less than effective at controlling virus replication. In addition the ability of viral IL-10 to alter the differentiation of latently infected myeloid progenitors, inhibition of the formation of mature DCs could be another mechanism by which HCMV restricts immune clearance of latently infected cells. It has also been hypothesized that viral IL-10 expressed during HCMV infection induces an anti-inflammatory macrophage phenotype (M2 macrophage) to enhance virus pathogenesis *in vivo*.

3.5 Cytokine Receptor Homologs in CMV

The *UL144* gene of HCMV encodes a type I transmembrane glycoprotein homologous to tumor necrosis factor receptor (TNFR) superfamily members (495). *UL144* is also one of the most variable genes in the HCMV genome (416, 419, 496). The protein, pUL144, however, does not function through binding of TNF ligand family members (495, 497). A pUL144 fusion protein was shown to bind to B- and T-lymphocyte attenuator (BTLA), an inhibitory receptor found on T-cells (498, 499). This binding inhibits the proliferation of CD4⁺ T cells induced by anti-CD3

and anti-CD28 stimulation (498). HCMV might employ a similar strategy to attenuate the anti-viral T-cell response *in vivo*.

pUL144 also interacts with TNFR associated factor (TRAF6) and the cellular tripartite motif 23 protein (TRIM23) as a co-factor to activate NF- κ B signaling (497, 500). This signaling induces the chemokine CCL22 (497, 501), which attracts regulatory T-cells (502) suggesting the role of pUL144 as an immunoevasin for HCMV (497).

3.6 Secreted Chemokine Binding Protein Homologs in CMV

pUL21.5 is a small secreted chemokine binding glycoprotein (CBP) encoded by HCMV. The CBP shows no homology to known proteins and is not conserved across viruses (503). pUL21.5 is capable of binding CCL5 (RANTES) but not CCL3, CCL2 or CXCL8 (504). This suggests that it can act as a sink to sequester free CCL5 to compete for CCL5 binding to its receptor (504). The mRNA for UL21.5 is incorporated into the mature virion and is available for translation immediately following infection (505). CCL5 is a potent chemoattractant of immune effector cells such as monocytes, T-cells, macrophages, and dendritic cells (506). HCMV has multiple strategies to modulate CCL5 expression and function, suggesting an important role of CCL5 in the anti-HCMV immune response. However, there is currently no data evaluating the function of p21.5 to interfere with CCL5 function and its effect on viral survival *in vivo*.

4. Current status of vaccines and anti-virals against HCMV

4.1 Current status of CMV vaccines

As discussed in chapter 1, animal models have been used to evaluate strategies to develop a CMV vaccine. Some of these products are currently in human clinical trials (507). Initial studies in renal transplant recipients using the Towne vaccine provided evidence of reduced severity of end organ disease (EOD) (508). Several chimeric live virus vaccines, including ones with a Towne backbone and segments of the attenuated Toledo and others have also been evaluated in phase I trials (509-511). Sanofi Pasteur is testing a subunit vaccine containing the soluble glycoprotein B (gB) in phase II double-blind randomized placebo-controlled trials (125, 512). In this study, the vaccine protected ~ 50% of seronegative women from acquiring primary infection and reduced viral load parameters post kidney or liver (125, 512). GlaxoSmithKline

has also evaluated a gB subunit vaccine in a phase I clinical trial (513, 514). Astellas is currently using a DNA vaccine expressing gB and pp65, in which they noted reduced reactivation of CMV in hematopoietic stem cell recipients (515, 516). Currently, Novartis is currently exploring a novel alphavirus recombinant vaccine expressing gB, pp65, and the major immediate-early antigen (517). Recently it has been shown that entry into epithelial and endothelial cells is prevented by antibodies to gB or the pentameric complex (426, 518). Newer targets of cytotoxic T-lymphocytes have also been identified and are currently under investigation (519). Although it has been demonstrated that vaccination can boost immune responses in seropositive women (520), it is currently unknown if this is sufficient to prevent congenital infection.

4.2 Current status of CMV anti-virals

In the absence of any licensed CMV vaccine, antiviral therapy has been critical. The current clinical protocol results in dramatically improved outcomes for immunocompromised patients. Currently licensed drugs for the treatment of CMV infections include the nucleoside analogs ganciclovir (GCV), valganciclovir (VGCV), foscarnet (FOS), and cidofovir (CDV) (521-523). These nucleoside analogs have been highly successful due to the potential for chemical diversity within the class and the differentiation of target viral DNA polymerases from host enzymes (521). Ganciclovir has become the gold standard for management of CMV diseases in the majority of patient settings (521). These drugs have been used to prevent CMV infection and disease, primarily in solid organ and hematopoietic stem cell transplants (521-523). The anti-sense RNA, fomivirsen is licensed for intravitreal administration to treat CMV retinitis and EOD in AIDS patients (521-524). The potential use of these drugs is being explored for the treatment of congenital CMV infection (525, 526). Continuous efforts in the industry and academia have led to the development of newer candidates with enhanced antiviral efficacy and apparently minimal side effects (e.g. Maribavir, Cidofovir ester, etc.) (521, 523). Recently, the phospholipid specific antibody baviximab, that targets phosphatidylserine in the outer leaflet of the virion envelope has been shown to be effective against MCMV by preventing cellular attachment and entry (527). These compounds are still in the early stages of clinical development and have yet to be approved.

At the same time, the clinical utility of most of these agents is limited by poor oral bioavailability, associated toxicities, and the potential for development of resistance with

extended use (521-524). Therefore, novel therapeutic agents with a different mechanism of action than the currently used drugs and low toxicity are needed to address these limitations.

4.3 Challenges in developing an effective CMV vaccine

Natural immunity against CMV is imperfect, in part due to the ability of CMV to evade the host immune response (138, 411). Vaccines could improve upon natural immunity by stimulating a protective immune response. However, a major barrier in the development of a CMV vaccine is the ability of CMV to co-infect, re-infect and reactivate even in the face of a strong immune response. Recent studies have shown that high titers of anti-CMV antibodies are unable to prevent congenital infection (528). Moreover, immune competent individuals generate a very strong immune response against CMV, with up to 10% of CD4 and CD8+ T cells being CMV specific and yet still unable to prevent CMV re-infection (411, 519). These observations suggest that there are still gaps in our knowledge regarding CMV transmission and epidemiology that need to be closed in order to develop a successful CMV vaccine.

5. Conclusion and Statement of Research Aims

Due to high morbidity and mortality in HCMV infected newborns and immunocompromised individuals, the development of an HCMV vaccine or other effective therapeutic treatment is necessary. However, as discussed above, no vaccine against CMV is currently available despite over three decades of research. This in part, is due to the limited understanding of HCMV pathogenesis including viral dissemination.

As described above CMV encodes homologs of several host proteins with the ability to exploit existing cellular signaling pathways and evade the anti-viral immune response. These proteins facilitate viral infection, dissemination, and survival and complicate the understanding of the virus even further. CMV disseminates within the host via cell-associated viremia in cells of hematopoietic origin including monocytes and neutrophils. Although the virally encoded homologs share some activities with their host counterparts, they also demonstrate specific differences in function (e.g., constitutive signaling of chemokine receptor homologs, which is attributed to specific structural aspects of the viral receptor not found in the human receptor). The CMV-encoded chemokine homologs can alter the response of leukocytes during viral

infection and could have an effect on viral pathogenesis. For example, the CC chemokine of MCMV and RCMV, MCK2 and pR131 respectively, induce inflammatory responses that enhance virus dissemination to the salivary gland *in vivo*, while GPCMV-MIP induces an inflammatory response leading to hearing loss. HCMV encoded vCXCL-1 demonstrates hypervariability and the vCXCL-1 genotype could be a determinant of HCMV pathogenesis. Further study is required to characterize the vCXCL-1 gene from different HCMV isolates *in vitro* and evaluate to its role in the context of viral infection in an appropriate animal model *in vivo*.

Although effective, the current antivirals have several shortcomings including toxic side effects and the emergence of drug resistant CMV strains. Therefore, research that would contribute to closing these gaps in our knowledge or that lead to the development of new therapeutics is needed.

We address these issues using recombinant proteins, viral recombinants, and synthetic cationic peptides and in the mouse model of CMV to test the following hypotheses in this dissertation:

1. Polymorphisms in vCXCL-1, lead to differences in cellular activation that contribute to HCMV pathogenesis.
2. vCXCL-1 is a functional CXC chemokine that contributes to viral dissemination.
3. Heparan sulfate binding peptides prevent CMV infection of the cells.

We hope that these studies will broaden our understanding of HCMV immune evasion strategies and provide evidence to support the development of novel HCMV vaccines/therapeutics.

**CHAPTER 1: NOVEL HUMAN CYTOMEGALOVIRUS VIRAL CHEMOKINES,
vCXCL1-1s, 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).
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My use of “we” in this chapter refers to my coauthors and myself. My primary contributions to this paper include (1) researching the topic, (2) generating and purifying the chemokines (used to generate figures 1.2 and 1.5) (3) performing experiments to generate data for figure 1.2 and for the rebuttal letter from the reviewers, (4) writing up the results and discussion of the figures generated for the main manuscript and rebuttal letter.

1. 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.

2. Introduction

Human cytomegaloviruses (HCMVs) are ubiquitous pathogens that are well adapted to modulate host immune responses (138, 529). HCMV contains genes for immune evasion that function to increase viral survival, dissemination, and may contribute to pathogenesis (411). There are a large number of open reading frames (~82) in HCMV that are non-essential for virus replication *in vitro* but may have a role in immune evasion *in vivo* (168, 400). In one of these regions, the UL/b' region, the open reading frames (ORFs), *UL146* and *UL147*, have limited homology to host CXC chemokines (400). Yet, the *UL146* protein from the Toledo strain of HCMV, vCXCL-1_{Toledo}, acts as a functional CXC chemokine (407) that binds to CXCR1 and CXCR2, induces neutrophil chemotaxis and calcium mobilization (408). This gene is one of the most variable in the entire HCMV genome (412, 413, 415, 417, 418). 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 (259, 369). Some strains even alter the Glu-Leu-Arg (ELR) prior to the CXC motif, which is a critical for receptor recognition and activation (373, 374). 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 (416). In these groups the N-loop region was highly variable. In addition one isolate, vCXCL-1_{TX15}, encoded a non-ELR CXC chemokine. Although the genetic variability of vCXCL-1 does not definitively correlate 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. In order to address functional variability of the vCXCL-1s, recombinant vCXCL-1s from each clade were generated. Competition binding, signaling, and neutrophil activation assays were utilized to assess the effect of vCXCL1 variability on chemokine function.

3. Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were obtained from Hyclone Laboratories (Logan, Utah). Fetal bovine serum was purchased from Mediatech (Manassas, Virginia). DMEM containing 25 mM HEPES and L-glutamine, OPTI-MEM, Hygromycin-B and Geneticin were obtained from Invitrogen (Paisley, United Kingdom). Bovine Serum Albumin Fraction V (BSA) was purchased from Roche (Mannheim, Germany). Polyethylenimine (PEI) was obtained from Polysciences (Warrington, PA, USA). ^{125}I -CXCL8 and ^{35}S -GTP γ S was obtained from PerkinElmer Life Sciences (Boston, MA, USA).

Clinical isolates used for cloning of the vCXCL-1 open reading frames 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 in (416).

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.

PathHunterTM HEK293-CXCR2 cells (DiscoverRx, Fremont, USA), were grown at 5% CO₂ 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% CO₂ 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 -GTP γ S experiments, HEK293T cells were grown at 5% CO₂ 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 2.5 µg 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 (530).

Neutrophil isolation

PBNs were isolated from EDTA-treated blood from healthy human volunteers using dextran sedimentation and density gradient centrifugation as previously described (531). 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 5×10^6 cells/ml were loaded with 4 μ M 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 1×10^6 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.

β 2 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 μ l 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 μ m 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 μ l 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 (407). 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 -GTP γ S 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 1500g for 10 min at 4°C. The pellet was resuspended in ice-cold PBS and centrifuged. Cells were resuspended in ice-cold membrane buffer (15 mM Tris, 1 mM EGTA, 0.3 mM EDTA, and 2 mM MgCl₂, pH 7.5), followed by homogenization using a Teflon-glass homogenizer and rotor. The membranes were subjected to two freeze-thaw cycles using liquid nitrogen, followed by centrifugation at 40,000g 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 μ g/well) were incubated in 96-well plates in assay buffer (50 mM Hepes, 10 mM MgCl₂, 100 mM NaCl, pH 7.2 with 5 μ g saponin/well, 3 μ M GDP and approximately 500 pM of ^{35}S -GTP γ S added) and the indicated concentrations of CXCL8 or vCXCL-1 to a final volume of 100 μ l. 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 MgCl₂, pH 7.4). ^{35}S -GTP γ S 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

PathHunterTM HEK293-CXCR2 cells were plated out overnight at 1×10^4 cells/well (384-wells format) in 20 μ l OPTI-MEM. A pre-incubation with vehicle (PBS + 0.1 % BSA) of 30 min at 37°C and 5% CO₂, was followed by 90 min with CXCL8 or vCXCL-1 stimulation at 37°C and 5% CO₂. 12 μ l 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 Victor² 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 μ l 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 $\Delta CT_{\text{target}}$ or reference = threshold cycle (CT) of the control gene (ACT1) – CT of the target gene (CCL22), and $\Delta\Delta CT = \Delta CT_{\text{reference}} - \Delta CT_{\text{target}}$ (532). 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.

4. Results

Amino acid sequences alignment

Previously, we sequenced the *ULI46* gene from 51 clinical isolates and showed that it comprised 11 genetic clades (416). Representative isolates from the 11 clades were aligned with vCXCL-1 from the Toledo strain (vCXCL-1_{Toledo}) (Figure 1.1). 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 (270), C-terminus (533, 534), and even in the ELR motif (506), led us to evaluate differences in chemokine receptor binding and functional responses (535).

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 (407) 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 (143, 407). CXCL8 and CXCL1 were shown to induce calcium flux at similar concentrations via CXCR2 (536). 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 1.2. However they differ in their ability to induce a calcium flux at other concentrations tested (Figure 1.2). 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.

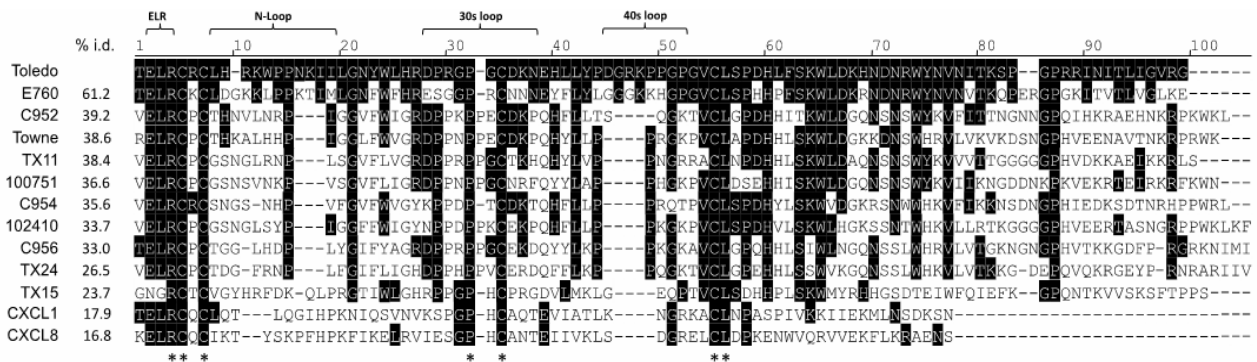


Figure 1.1 Amino-acid alignment of the mature forms of recombinant vCXCL-1s and the host chemokines CXCL1 and CXCL8 with vCXCL-1Toledo. Seven amino acid residues that are 100% conserved are indicated with an *. The important ELR, N-loop region, and 30s and 40s loops are indicated at the top.

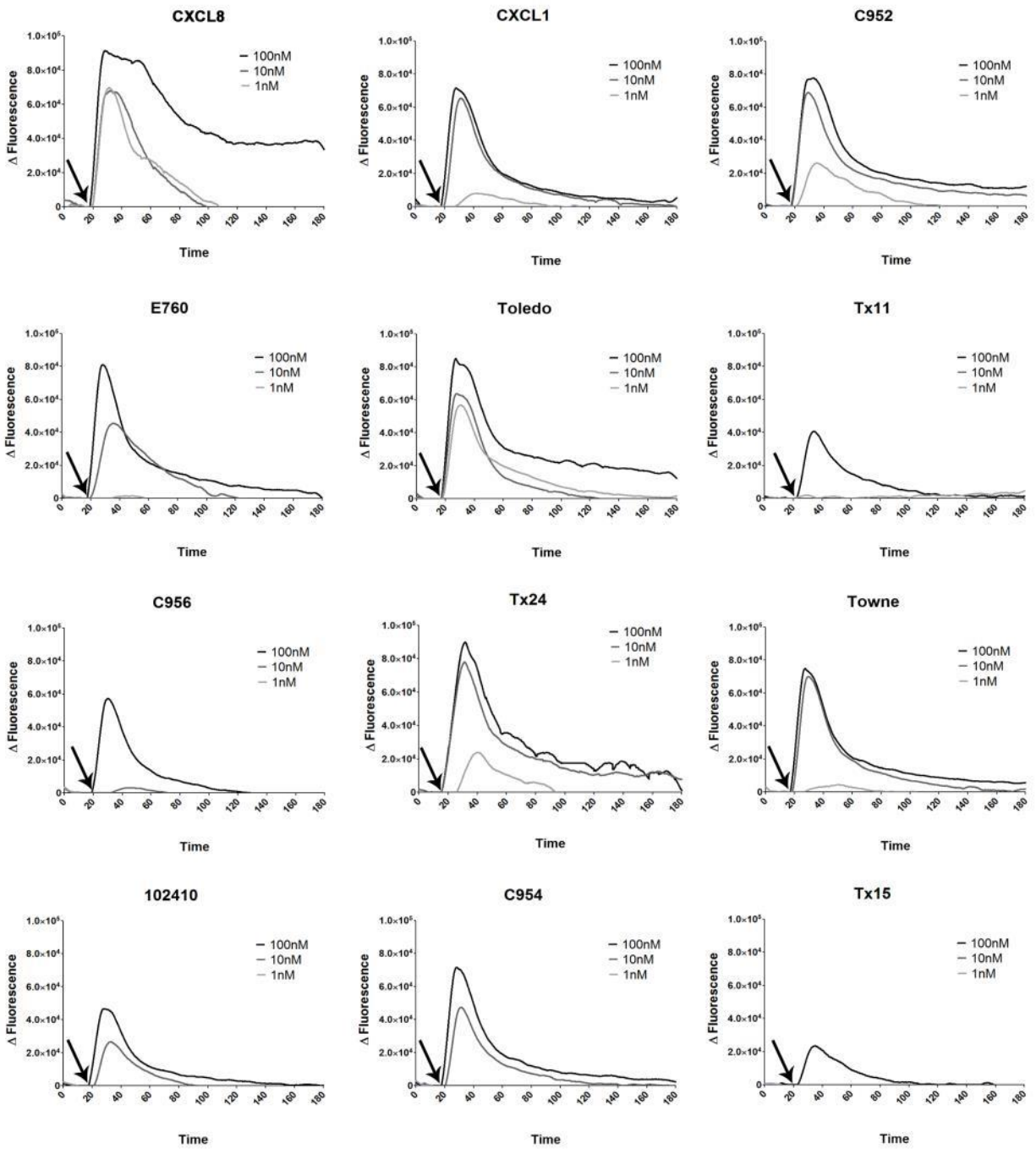


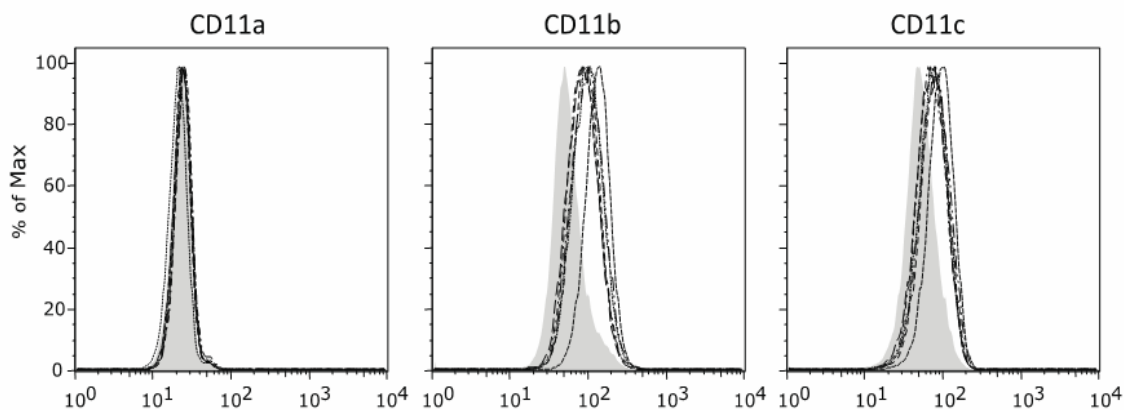
Figure 1.2 CXCL8, CXCL1, and the different vCXCL-1s induce intracellular calcium mobilization on human PBNs. Changes in fluorescence were measured over time after exposure to different concentrations of chemokines (after 20 seconds at baseline as indicated with an arrow). Data shown are representative figures of three independent experiments.

vCXCL-1s upregulate CD11b and CD11c

β 2 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 (537). Host chemokine, CXCL8, upregulates CD11b and CD11c expression (538, 539). Moreover, vCXCL-1_{Toledo} and the vCXCL-1 from chimpanzee CMV also increases integrins on PBNs (143). In this study, we tested the ability of vCXCL-1s to alter the surface expression of these receptors on PBNs (Figure 1.3). 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 β 2 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 (373, 407). 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 1.4). 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-1₁₀₀₇₅₁, 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.



| 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 1.3 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.

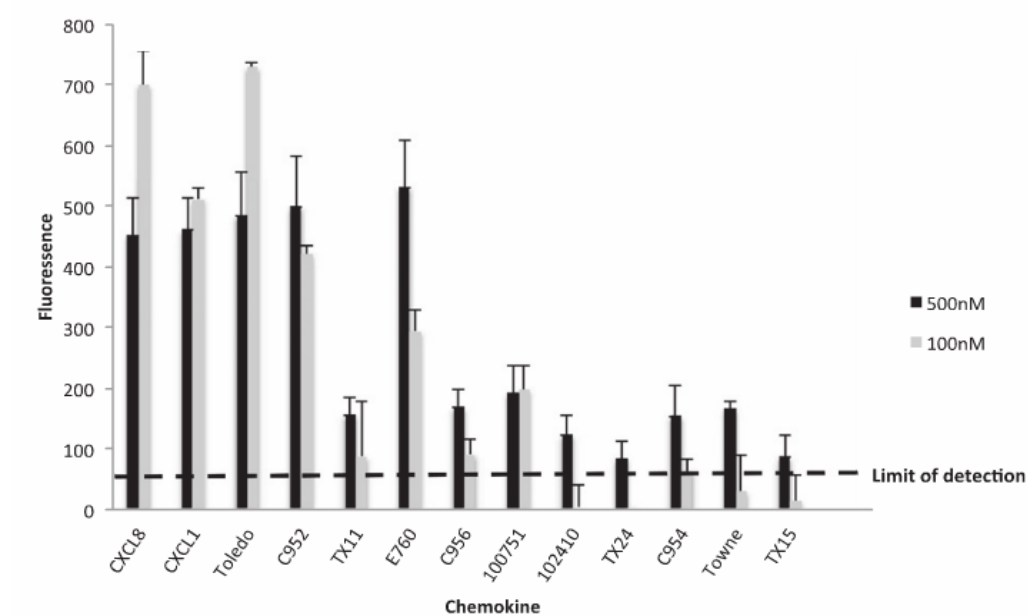


Figure 1.4 Differential chemotaxis of PBNs to vCXCL-1s partially correlates with affinity. Chemotactic response of human PBNs to 500 nM and 100nM of CXCL8, CXCL1, or vCXCL-1s. The chemotactic response was measured as fluorescence intensity of migrated PBN labeled with CalceinAM. Background chemotaxis was subtracted from all samples. Data shown are representative data of three independent experiments performed in triplicate.

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 (540-542), 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 1.5A). 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-1₁₀₀₇₅₁ and medium-low members are vCXCL-1₁₀₂₄₁₀, 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 1.5B). 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-1₁₀₀₇₅₁ and vCXCL-1_{C956}) could induce migration (Figure 1.4).

This data implies that affinity for CXCR2 (i.e., high affinity equals high migration) and/or CXCR1 usage are potential factors in PBN migration (541, 542). Because it is not strictly correlated with affinity, differential agonist activation signals could also contribute to PBN migration as well.

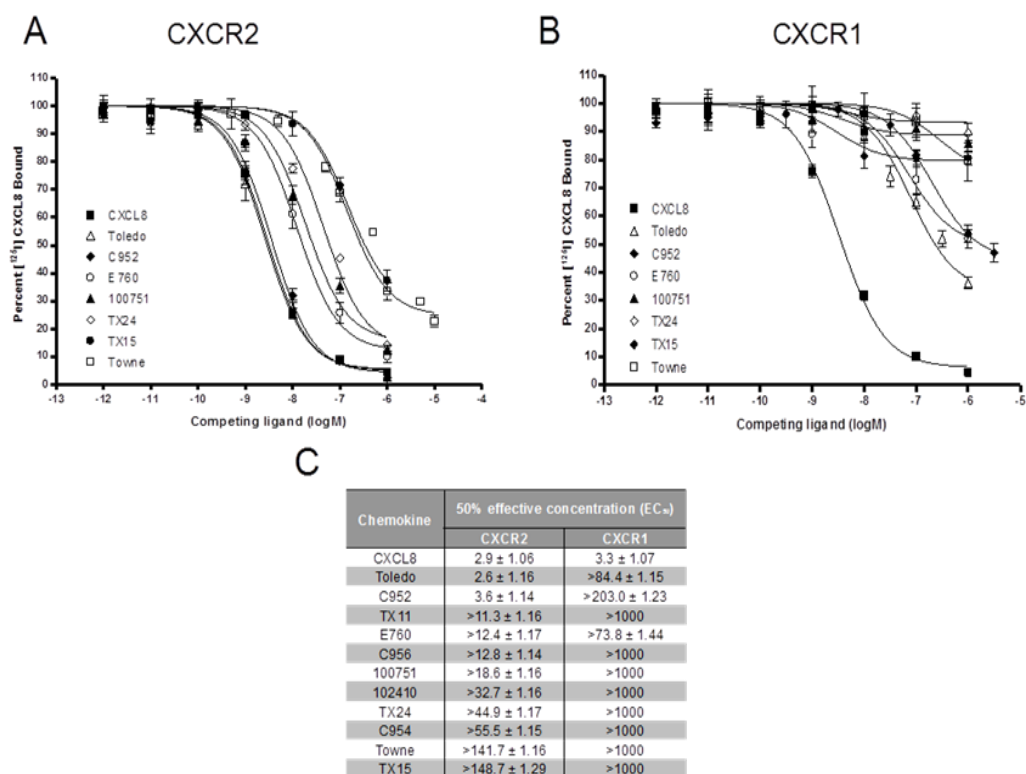


Figure 1.5 vCXCL-1s have different binding affinities for human CXCR1 or CXCR2. Displacement of ¹²⁵I-CXCL8 binding to HEK293 cells stably expressing human CXCR2 (A) or CXCR1 (B). Cells were incubated with indicated concentration of vCXCL-1s and 200 pM ¹²⁵I-CXCL8 for one hour at room temperature. For simplicity, curves for TX11, C956, 102410, and C954 are not shown. (C) The average IC₅₀ +/- 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 IC₅₀. Those chemokines with no competition at all concentrations tested are listed as >1000 IC₅₀.

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

Chemotactic responses can be mediated via G-protein dependent and/or G-protein independent signaling. Berger *et al* (539) demonstrated that CXCL8-induced β2 integrin CD11b upregulation and migration of neutrophils is Gα_i dependent. Chemokine-induced calcium flux involves Gα_i proteins as well (543, 544). Based on these studies and observation of differences in migration and binding, we investigated whether vCXCL-1s display differences in G-protein dependent and independent signaling that could explain the differences in migration. ³⁵S-GTPγS binding experiments were performed on HEK293T membranes expressing human CXCR2 (Figure 1.6A-B). The pEC₅₀ 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 1.6) and uses G proteins (Figure 1.6A). 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-1₁₀₀₇₅₁, vCXCL-1_{C956}, vCXCL-1₁₀₂₄₁₀, 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 (394, 545). The involvement of β-arrestins in chemokine-induced chemotaxis was first described for the CXCR4/CXCL12 axis (546) and involves the p38 MAPK pathway (547). Following from these studies, a role for β-arrestin2 in CXCR2 directed chemotaxis was shown (534, 548, 549). β-arrestin2 induced chemotaxis could explain the differences seen with the different chemokines (Figure 1.6). 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 (550). The pEC₅₀ value of CXCL8 in this assay is 9.1.

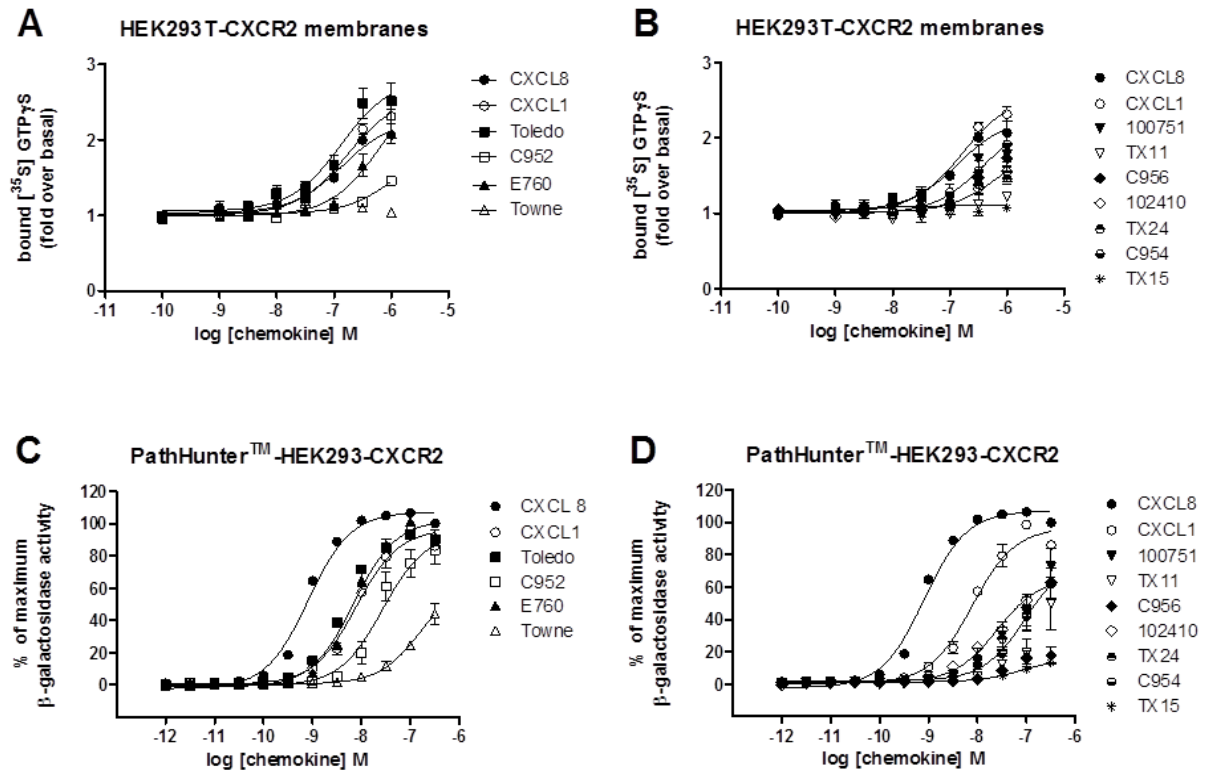


Figure 1.6 G protein activation and β -arrestin2 signaling correlates with CXCR2 affinity. vCXCL-1 chemokine induction of ^{35}S -GTP γ S binding to HEK293T membranes expressing CXCR2 (A-B). Data are corrected for basal ^{35}S -GTP γ S binding (n=3-4). vCXCL-1 β -arrestin2 recruitment in PathHunterTM 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 1.6C-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 (pEC₅₀ = 9.1 nM) [high affinity for CXCR2] \geq CXCL1 (pEC₅₀ = 8.3 nM) [high affinity for CXCR2] \sim Toledo (pEC₅₀ = 8.4 nM) [high affinity for CXCR2] \sim E760 (pEC₅₀ = 8.1 nM) [med-high affinity for CXCR2] \geq C952 (7.5 nM) [high affinity for CXCR2] \geq C956 [med-high affinity for CXCR2] \sim 102410 [med-low affinity for CXCR2] \sim 100751 [med-high affinity for CXCR2] \sim C954 [med-low affinity for CXCR2] \sim TX24 [med-low affinity for CXCR2] \geq C952 [med-low affinity for CXCR2] \geq TX15 [low affinity for CXCR2] \sim TX11 [med-high affinity for CXCR2] \sim 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 (551).

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 Th₂ subset of T cells (552). 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 (553, 554). In fact, another UL/b' protein, UL144, upregulates CCL22 and has been implicated in immune modulation (i.e., recruitment and activation of Th₂ and T_{reg}) (497). 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 1.7). 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-1₁₀₀₇₅₁, induce CCL22. In contrast, all the members in the medium-low (i.e., vCXCL-1₁₀₂₄₁₀ 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.

5. 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 (555-557). 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 1.1). 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* (437, 438, 558). Furthermore, this virally induced inflammation contributes to cytomegalovirus-related inner ear injury (i.e., auditory pathology) (441). 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*.

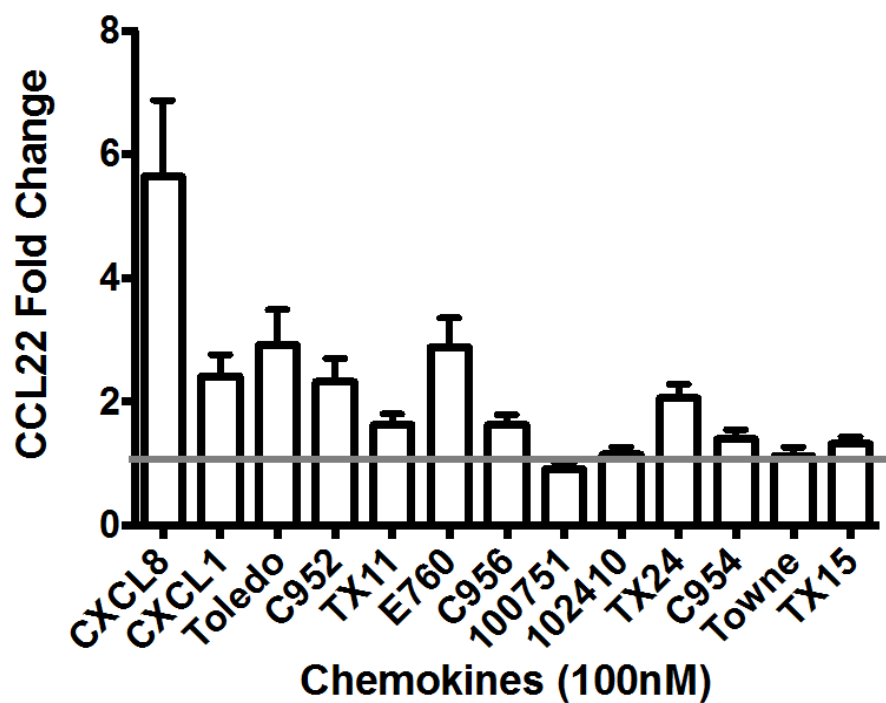


Figure 1.7 vCXCL-1s differentially induce CCL22 expression via CXCR2. Neutrophil-like HL60 T2 cells were incubated in the presence of 100 nM of the indicated vCXCL-1s or host chemokine. Each bar represents the average from three separate experiments of the fold change in CCL22 mRNA expression levels (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.

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 (559). PBNs pick up HCMV during neutrophil transendothelial migration and subsequently transmit infectious virus to fibroblasts (560, 561). We analyzed PBN induction of calcium flux and adhesion molecules upon vCXCL-1 treatment as indicators of neutrophil activation (Figure 1.2, 1.3), which could affect subsequent cell-mediated viral dissemination (562). 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 (563).

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 1.5), all vCXCL-1s induce intracellular calcium mobilization in PBNs, albeit to different degrees at the concentrations tested (Figure 1.2) and upregulate β 2 integrins on the surface of PBNs (Figure 1.3) 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 1.5C). 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-1₁₀₀₇₅₁). 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 (265, 564) and elastase production (270). Others have observed a complex relationship between binding and activation similar to our observations with our medium affinity vCXCL-1s (565). 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 (566). 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 (567-570) while PBN infections are nonproductive (561). 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 (568). 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 1.7). 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-1₁₀₀₀₇₅₁, vCXCL-1₁₀₂₄₁₀, 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 (416). 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 (551). 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* (571) 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 1.6). 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 1.1). 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 (572). 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 (573). 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.

6. Acknowledgments

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Table 1.1 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 | +/- | + | - | - | - | - |

**CHAPTER 2: A LITTLE COOPERATION HELPS MCMV GO A LONG WAY – THE
INTERACTION BETWEEN DIFFERENT MCMVs RESCUES A DISSEMINATION
DEFECT WITHIN HOST**

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Abstract

Evaluation of the role of vCXCL-1 in the context of HCMV infection is limited to *in vitro* and *in silico* analysis due to the species specificity of the CMV. In this study, we used the murine CMV (MCMV) mouse model to evaluate the function of this chemokine *in vivo*. The primary dissemination to the popliteal lymph node, spleen, and lung of the recombinant MCMVs expressing vCXCL-1_{CCMV} and mCXCL1 was similar to the parental MCMV, which does not express any chemokine (RM4511). However, neither of the recombinants was recovered from the salivary gland (SG) at any time point. The absence of the recombinants from the SG of SCID mice suggested that the adaptive immune system is not responsible for viral clearance of the recombinants. Neutrophil depletion was unable to rescue the dissemination of the recombinants to the SG, even though the recombinants overexpress proteins that attract neutrophils. The dissemination was restored upon immune ablation using cyclophosphamide and in NSG mice, which lack T, B lymphocytes, and NK cells. These results suggest that the aberrant expression of the chemokine induces cells of the innate immune system to curtail the dissemination of the recombinants to the SG. To better understand the mechanism of this dissemination defect, a series of co-infections between parental and recombinant MCMVs were carried out to address whether dissemination defect was dominant. Similar to the experiments with immune ablation, dissemination of the recombinants to SG was restored in immune sufficient mice during co-infection of parental and overexpressing recombinants. We show that co-infection of the same cells was necessary in order to overcome the dissemination defect. We also found that co-infection *in vitro* reduced chemokine levels, without affecting the virus titers. During the co-infection *in vivo*, we measured reduced numbers of NK cells and inflammatory

monocytes recruited to the site of infection, compared to a recombinant alone infection during co-infection. Therefore, from this study we concluded that vCXCL-1 is a functional chemokine *in vivo*. However, continuous expression of the chemokine is detrimental to the dissemination of MCMV by recruiting more inflammatory monocytes and NK cells to the site of infection. These cells types are absent or reduced in NSG mice, cyclophosphamide treated animals, and during co-infection. During the co-infection, there is an intracellular resource competition that reduces the chemokine levels produced from the recombinants that also reduces the inflammatory cells (i.e., NK and inflammatory monocytes) recruited to the site of infection, restoring 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 (509, 574). It infects between 50% and 90% of the population resulting in largely asymptomatic infections (574). However, HCMV can cause serious complications in immunocompromised individuals and newborns (574, 575). Primary or reactivated HCMV is a frequent cause of retinitis in AIDS patients (576) and increases the incidence of organ rejection and graft versus host disease in transplant recipients (577, 578). Central nervous system damage due to congenital HCMV infection affects between 5000 and 8000 newborns in the U.S. each year (579). As a result, HCMV is the leading cause of infectious hearing loss and non-hereditary mental retardation (574). Understanding HCMV pathogenesis is important for the development of an effective vaccine or potential therapeutics. Due to the long-term morbidity associated with CMV congenital infections, the Institute of Medicine lists the development of a CMV vaccine as a top priority (509).

CMVs encode numerous proteins that modulate the host immune system. CMVs can inhibit apoptosis of infected cells, impair antigen processing, and alter the inflammatory response including the chemokine network (580). Chemokines are small, chemotactic cytokines that are important for leukocyte trafficking and activation. Chemokines are divided into four groups (CC, CXC, CX3C, C) based on the spacing of their amino-terminal cysteines (261). Most chemokines are members of either the CC or CXC class. These chemokines attract and activate a variety of immune cells responsible for the viral clearance and contribute to CMV pathogenesis (581, 582).

HCMV infection alters the expression of host chemokines (583) and many CMVs also encode viral homologs of chemokines and their receptors (138, 411, 584, 585). The virulent lab strain of HCMV, Toledo (557), 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) (407, 408). In our previous studies, we have shown that the viral chemokine from chimpanzee cytomegalovirus (vCXCL-1_{CCMV}) is a homolog of vCXCL-1_{Tol}, and triggers calcium release and chemotaxis of PBNs (143). Both viral chemokines were also shown to upregulate the expression of adhesion molecule on PBNs and downregulated neutrophil apoptosis, albeit with different potencies (143). These findings provide circumstantial evidence for a role of vCXCL-1 in activating and recruiting neutrophils for CMV dissemination. Clinical evidence, including the recovery of HCMV from neutrophils of immunocompromised patients (586-588) and the presence of neutrophilic infiltrates in CMV associated retinitis (589), suggests neutrophils, as well as monocytes (590-592) are important in HCMV dissemination. While the direct evaluation of vCXCL-1 in HCMV dissemination is limited by the species specificity of CMVs, the function of vCXCL-1_{CCMV} may correlate with its HCMV homolog.

MCMV is a well-established animal model of CMV infection and has similar cellular tropism and disease manifestations as HCMV (194). MCMV expresses a chemokine homolog, MCK2, which has been shown to increase the inflammatory response in mice and enhance dissemination of MCMV to the salivary gland (139, 140). Therefore, MCMV was chosen to characterize the role of vCXCL-1_{CCMV} *in vivo*. To test this in the mouse system, we generated a recombinant MCMV expressing vCXCL-1_{CCMV} and infected mice. Results from our experiments show that the aberrant expression of host or viral chemokine is detrimental to the dissemination of the recombinants to the salivary gland (SG) during secondary viremia. The NK cells and inflammatory monocytes play a role in an innate immune system-mediated blockade of dissemination. Co-infection with a non-chemokine-expressing strain of MCMV restored the dissemination of the recombinant to the SG by tipping the immune response to make conditions more favorable for SG dissemination of the recombinants. Although our data shows that the aberrant expression of vCXCL-1_{CCMV} is antagonizing viral dissemination, in reality CMV has many checks and balances regulating the expression of the viral chemokine. It is therefore possible that normal expression of this chemokine may aid the dissemination of CMV *in vivo*.

Materials and Methods

Cells and Viruses

Murine NIH3T3 and M210B4 cells (ATCC) were propagated in DMEM supplemented with 10% Fetalclone III (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 FCIII to a final concentration of 10%, P/S and L-Gln to a final concentration of 1%. The parental MCMV strain used in this study was MCMV RM4511 (593) 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 2.1 A). This virus was obtained from Dr. Edward Mocarski, Emory University. For UV inactivation of the virus, the stock virus was divided into 50µl drops in a petri dish and exposed to UV light in a UV Crosslinker (Stratagene Stratalinker) at a setting of 1200 for 8 minutes. Complete inactivation of the virus was tested in a plaque formation assay.

Mice

Initially, mice that over express human CXCR2 (hCXCR2) or have replace murine CXCR2 (mCXCR2) were used as there was concern that vCXCL-1_{CCMV} might not stimulate the mCXCR2 as seen with vCXCL-1_{Tol} (142). The hCXCR2 transgenic BALB/c mice express hCXCR2 under the control of the neutrophil-specific, human myeloid related protein-8 promoter (142). In the co-infection experiments, mice that have the hCXCR2 gene replacing the murine CXCR2 were used (594). These mice have normal expression levels of hCXCR2 and expression on all the appropriate cell types. We have subsequently have 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 purchased from Jackson Laboratory (Bar Harbor, ME) and SCID/NCr were purchased from Taconic Labs. All mice were housed under specific pathogen free conditions in the WLS LAF.

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

***In Vitro* Protein Expression**

Aliquots from the single-step growth assay were removed and used to verify chemokine expression. vCXCL-1_{CCMV} and mCXCL1 were isolated from 100 µg of total protein by incubation with Ni-NTA agarose beads. 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.

***In Vivo* Growth of Parental and Recombinant Viruses**

10⁶ PFUs of parental or recombinant viruses were inoculated in the footpad of hCXCR2 transgenic, Balb/c, 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, sonicated in growth medium and centrifuged at 400 x g to pellet debris. Supernatants were stored at -80°C until titered.

Plaque formation assay

Viral titers in the organs were determined by plaque formation assay as per lab protocol on mouse embryonic fibroblast (MEF) 10.1 cells. Briefly MEF 10.1 cells were plated in a 6 well dish. Organs were harvested and homogenized. The homogenate was diluted and added the MEF 10.1 cells and incubated for 1 hr. After incubation the diluted virus was removed from the plates and cells were overlaid with carboxy methyl cellulose media and incubated for 7 days. At the end of the incubation period, CMC was removed and plates were stained and plaques counted using a dissection microscope.

Depletion of Cellular Subsets

In vivo depletion of cellular subsets was performed by 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.

Results

Construction of the chemokine expressing MCMVs

The strict species specificity of the CMVs precludes the use of CCMV directly in the mouse model. Therefore, we created a recombinant MCMV expressing vCXCL-1_{CCMV} (RMvCXCL-1_{CCMV}) to study the role of this viral chemokine in virus dissemination *in vivo*. mCXCL1 (KC) is the murine equivalent of CXCL-1 (Gro- α) with a high affinity for CXCR2. Therefore, we also generated MCMV expressing KC as a control to evaluate specific effects of the viral chemokine on dissemination. MCMV RM4511 was the parental strain used for construction of these recombinants. RM4511 lacks the expression of the 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 endogenous MCMV encoded chemokine (schematic diagram in figure 2.1 A).

The recombinant plasmid, L120.1, was constructed to contain either the vCXCL-1_{CCMV} or mCXCL1 coding sequence under the control of the HCMV IE promoter (figure 2.1B). This promoter has been used to drive expression of other genes inserted in the MCMV genome and was chosen to ensure adequate chemokine expression. We chose to insert the chemokine cassette into the *ie2* region as the *ie2* has been shown to be dispensable for growth of MCMV *in vivo*. The cassette displaces the puromycin-GFP segment present in RM4511. Loss of GFP expression allowed visual selection of recombinant viruses.

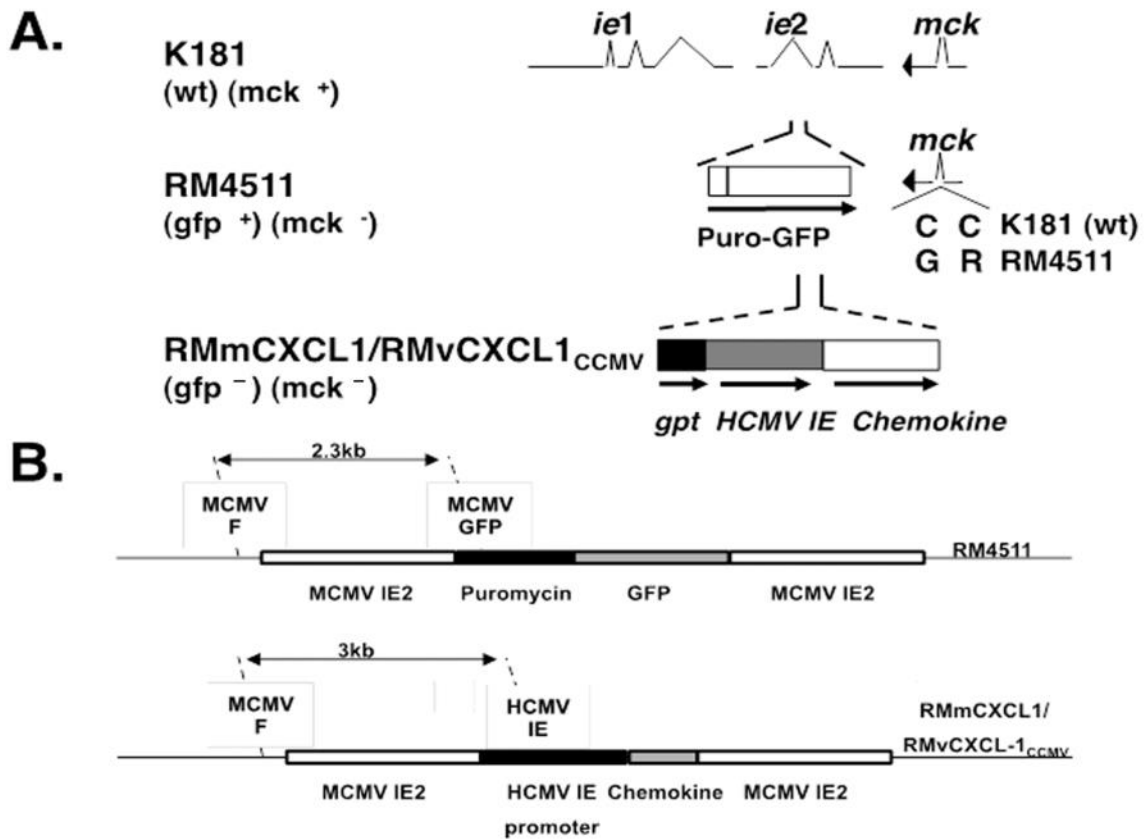


Figure 2.1 Schematic Representation of the wild type (WT) MCMV, parental MCMV (RM4511), RMmCXCL1, and RMvCXCL1_{CCMV} (A) The RM4511 recombinant MCMV contains a double point mutation resulting in a missense mutation that converts amino acids CC to GR in MCK2. This in effect creates an MCK2 negative strain. RM4511 also has a puromycin/green fluorescent protein expression cassette (*puro-GFP*) cassette in the *ie2* locus that is replaced in the chemokine expressing recombinants (RMmCXCL1, and RMvCXCL1_{CCMV}) was inserted of the MCMV genome in RM4511, replacing the Puro. The mCXCL1 or vCXCL1_{CCMV} expression cassette containing a *gpt* selectable marker that was used for selection (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.

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 virus expressing *gpt*. The loss of GFP expression identified recombinant viruses and each virus was plaque purified three times. PCR was used to confirm the recombinants and correct insertion of the promoter chemokine cassette. No product was detected when the parental virus was used as a template (supplemental figure S2.1).

***In Vitro* growth kinetics and chemokine expression of the recombinant MCMVs**

The growth kinetics of RMvCXCL-1_{CCMV} and RMmCXCL1 were evaluated to determine whether insertion of the vCXCL-1_{CCMV} or mCXCL1 cassette affected growth of the recombinant viruses in cell culture. We performed both single (MOI=5) and multi (MOI=0.05) step growth curves to identify any growth defects in replication or spread of the recombinant viruses relative to the parental strain, RM4511. RMvCXCL-1_{CCMV} and RMmCXCL1 replicated as well as RM4511 in both assays figure 2.2A, indicating the absence of any deleterious effects of the insertion on growth of virus in cell culture.

Before evaluating the role of RMvCXCL-1_{CCMV} and RMmCXCL1 *in vivo*, temporal expression of the chemokines in the supernatants of virally infected cell was confirmed (figure 2.2B). Immunoblotting of Ni-NTA-concentrated supernatants from each time point of the single step growth curve detected RMvCXCL-1_{CCMV} and RMmCXCL1 proteins. Beginning on the second day post infection and continuing for the duration of the experiment, both chemokines were detected in the supernatants. This illustrates both host and viral chemokines are secreted in detectable quantities beginning at day 2 post infection (p.i).

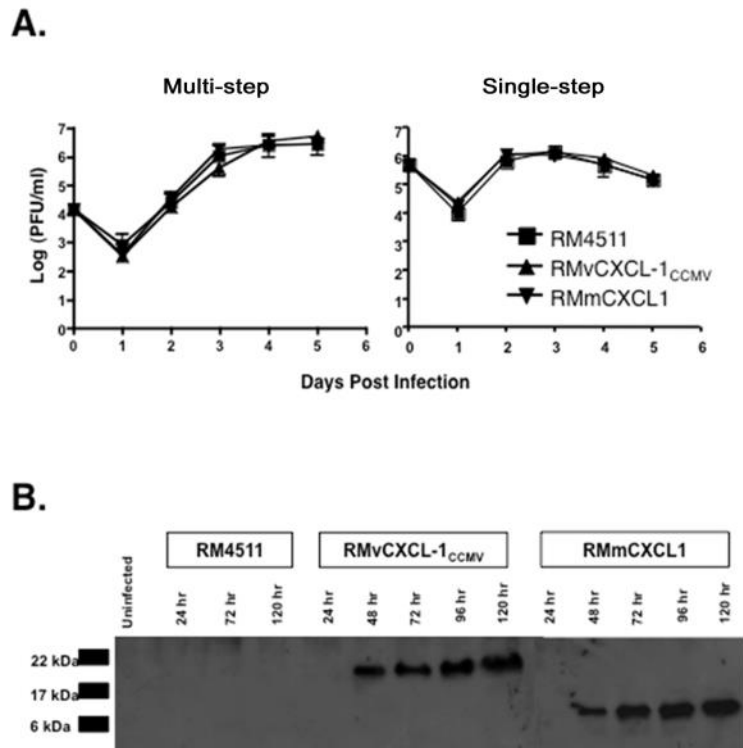


Figure 2.2 Recombinant viruses have equivalent growth rates and overexpress host and viral chemokines in vitro. (A) NIH3T3 cells were infected in triplicate with RM4511, RMmCXCL1 or RMvCXCL1_{CCMV} at a MOI of 5 (single-step) or 0.05 (multi-step). Supernatants were harvested daily for five days p.i. Plaque formation assays were performed on M210B4 cells. (B) Western blot analysis was used to analyze the expression of mCXCL1 and vCXCL1_{CCMV}. NIH3T3 cells were infected with RM4511, RMmCXCL1, or RMvCXCL1_{CCMV} at a MOI of 5. Supernatants were harvested at the indicated times post infection. Recombinant proteins were enriched using Ni-NTA agarose beads from 100 mg of total protein. The eluted protein samples were subjected to Western blot analysis using anti-6-His antibody to detect the 6-His tagged chemokine.

Dissemination of virus *in vivo*

To evaluate the contribution of vCXCL1 in the replication, dissemination of the recombinant viruses *in vivo*, we infected mice with 1×10^6 PFU of RM4511, RMmCXCL1, and RMvCXCL1_{CCMV} in the foot pad and measured the viral titers in the organs by plaque formation assay. The recombinants reached similar titers as the parental virus at the site of inoculation in the footpad (FP), and in the organs of primary dissemination, the lymph node, lung, and the spleen (figure 2.3). However, we could not detect the recombinant virus in the salivary gland (SG) at day 7 and 14 p.i, when the parental virus reaches peak titers in the organ (figure 2.3). To exclude the possibility that the dissemination of the recombinant virus to the salivary gland was not delayed, viral load in the SG was also measured at day 21 p.i. No recombinant viruses were detected even at this later time point (data not shown). The route of infection also did not alter the dissemination outcome, as even when infected i.p with 1×10^6 PFU per mouse, no recombinant viruses were detected in the SG (data not shown).

Next we investigated if the defective secondary dissemination of the recombinants to the SG was a result of defective viremia. For this, mice were infected intra peritoneally (i.p.) with 1×10^6 PFU of each virus and an infectious centers assay was performed on isolated peripheral blood leukocytes (PBLs) at day 4 p.i. As seen in figure 2.4 E left panel, viremia was observed only for 4511 and not for the recombinants. Taken together these data suggest the absence of recombinants in the SG is due to defective dissemination during secondary dissemination of the virus.

Immune cell depletion rescues the dissemination of the recombinants

Both vCXCL-1_{CCMV} and mCXCL1 bind and activate neutrophils via CXCR2 (143, 595), and it is possible that the overexpression of these chemokines recruits and activates neutrophils, triggering an inflammatory response capable of clearing the recombinant viruses (596). To evaluate the contribution of the neutrophils in the clearance and/or dissemination, mice were depleted of neutrophils using anti-Ly6G antibody before infection. Viral load in the SG was measured at day 14 p.i. Neutrophil depletion did not restore the dissemination of the recombinants to the SG (figure 2.4 A). Moreover in un-depleted mice, we did not see any difference in the neutrophil frequency in the salivary gland of mice infected with either the recombinants or RM4511 at day 14 p.i. (data not shown). These results show that neutrophils are not responsible for increased clearance of the recombinants in the SG or the dissemination defect observed *in vivo*.

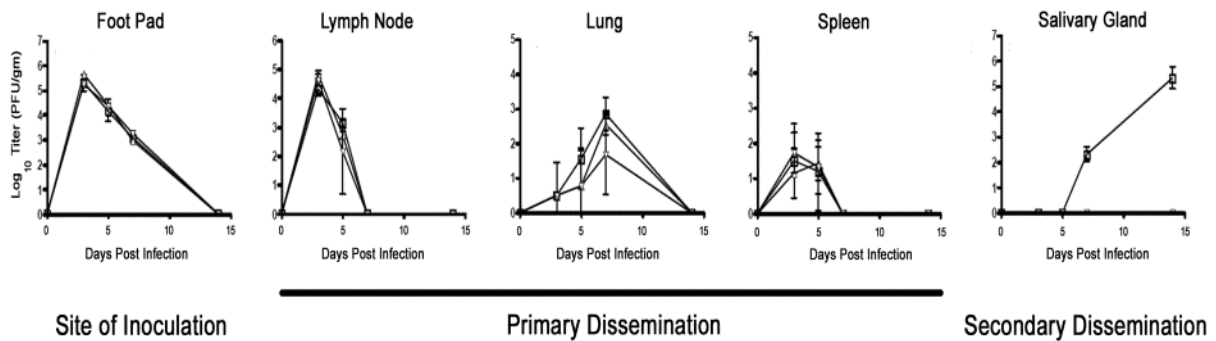


Figure 2.3 Overexpression of host or viral chemokine prevents MCMV dissemination to the salivary gland. hCXCR2 transgenic mice were inoculated in the footpad with 10^6 PFUs of either RMmCXCL1 (\blacktriangledown), RMvCXCL-1_{CCMV} (\blacktriangle), RM4511 (\square). Organs were harvested at indicated day p.i. and virus titers were determined via plaque assay. Each symbol represents the mean virus titer of 5 mice (\pm standard error). This data is a representative of 2 experiments.

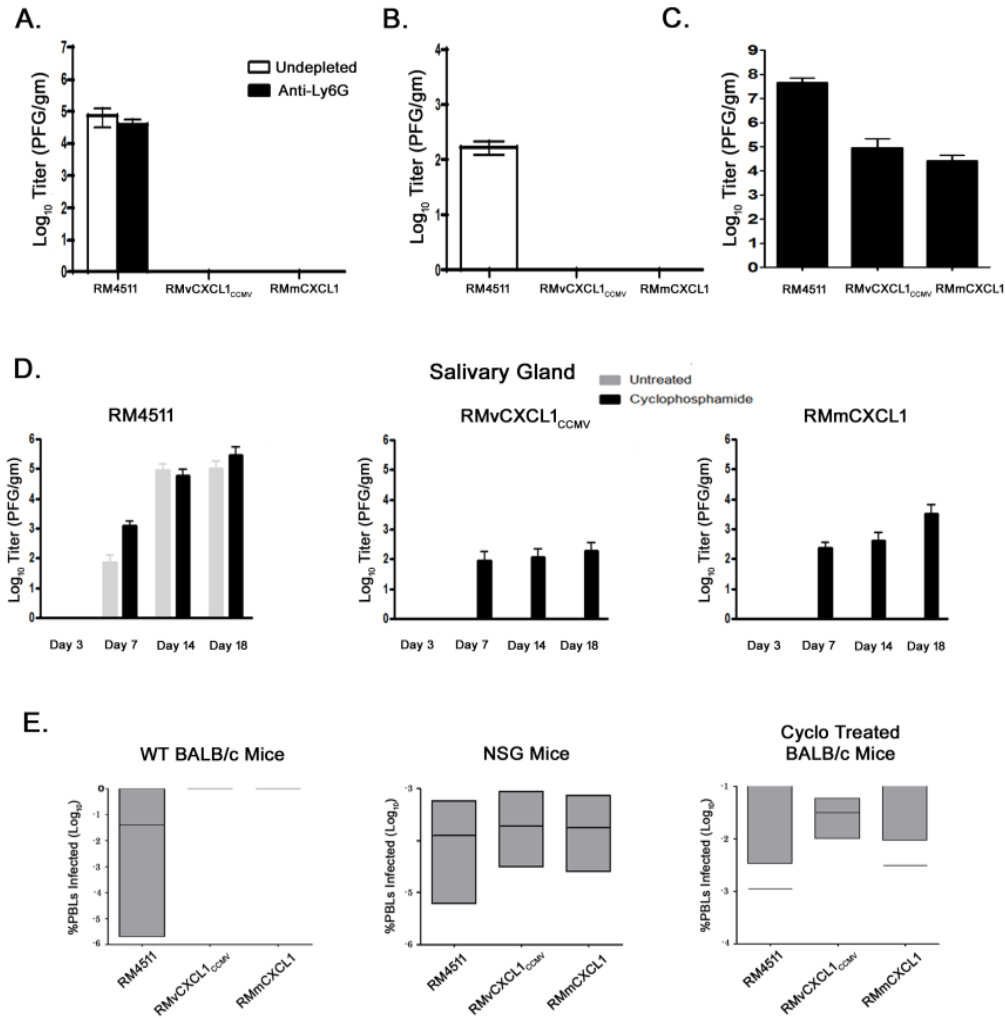


Figure 2.4 Evaluation of MCMV dissemination to the SG in the absence of cellular subsets. BALB/c mice depleted of neutrophils using anti-Ly6G antibody (A) or SCID mice (B) were infected by FP inoculation (B). NSG mice (C), which lack NK cells in addition to T and B lymphocytes, were infected i.p. and SG were harvested at day 14 p.i and viral titers measured by plaque assay. Mice were treated with cyclophosphamide to cause systemic immune ablation prior to infection with the viruses. Viral titer in the SG was measured at days 3, 7, 14 and 18 p.i. (D). Results are from 5-10 mice per infection and are representative of 2 or more experiments. Bars represent the mean virus titer (+/- standard deviation) (E) Viremia on PBLs isolated from WT BALB/c mice on day 4 p.i.,(left panel), NSG mice at day 14p.i. (center panel), or cyclophosphamide-treated BALB/c mice at day4 p.i (right panel).. Data is represented as Log₁₀ % infected PBLs and the line represents mean from at least 3 mice for each experiment.

T cells have also been shown to play an important role in controlling CMV infection. While CD8⁺ T cells effectively clear MCMV from many organs in the periphery (597), viral clearance from the SG is dependent on CD4⁺ T cells (136, 598). To determine the role of the adaptive immunity in clearing RMvCXCL-1_{CCMV} and RMmCXCL1 from the SG, SCID mice were infected with 1×10^6 PFU of recombinant or parental MCMV in the FP. SGs were harvested at 14 day p.i. and viral load measured. No virus was recovered from SG of SCID mice infected with recombinant viruses, while parental virus disseminated to the SG (figure 2.4 B). This data suggests that the absence of RMvCXCL-1_{CCMV} and RMmCXCL1 from the SG is not due to an amplified adaptive immune response against the recombinants.

NK cells form an important arm of the innate immune response to MCMV infection (131, 211, 599). To address their role in our model, we utilized the NSG mouse model which is severely immune deficient (600, 601). These mice not only lack T and B lymphocytes but have a defective IL2 receptor that leads to absence of NK cells from these mice (600, 601). NSG mice were infected i.p. with the different viruses, the SG was harvested at day 14 p.i. and viral load was determined. Unlike the previous experiments, we were able to recover the recombinant viruses from the SG of NSG mice, albeit much lesser than the parental virus (figure 2.4 C). Also, as seen in figure 2.4 E middle panel, in NSG mice, we observed viremia for the recombinants as well as the parental virus. Data from this experiment suggests that NK cells play a role in preventing the dissemination of the recombinant virus to the SG.

To evaluate the full extent the innate immune system in preventing the dissemination of the recombinants to the SG, mice were administered cyclophosphamide (cyclo) to deplete immune cells (602-604). Cyclo is an antimitotic that depletes neutrophils within one week. Mice become completely immune suppressed with continued cyclo treatment. At different times p.i. viral load in the organs was determined. We did not observe any difference in the primary dissemination between the recombinants and the parental virus in cyclo treated mice (supplemental figure S2.2). However, the recombinant were recovered from the SG of cyclo treated mice (figure 2.4 D), with detectable viremia measured at day 4 p.i (figure 2.4 E right panel). Thus, the data from these experiments suggests that cells of the innate immune system of the hematopoietic lineage are responsible for the lack of dissemination of the recombinant viruses to the SG.

Co-infection rescues the dissemination of recombinant virus in immune competent mice

The chemokine gene in the recombinants is under the control of the strong and constitutively expressed HCMV MIEP promoter (605). This leads to an aberrant expression of the chemokine from virally infected cells. This has an adverse effect on the dissemination of the recombinants to the SG during secondary dissemination. Therefore, we hypothesized that in a setting of co-infection (parental + recombinant) that the dissemination defect would dominate. This would lead to an inhibition or reduction in the dissemination of the parental virus to the SG. To test this hypothesis, mice were infected in the same FP with a mixed inoculum (1:1 ratio parental:recombinant). The SGs from these mice were harvested 14 days p.i. and the virus load determined. To our surprise, and contradictory to our hypothesis, we were able to recover the recombinant virus from the SG of mice infected with the mixed inoculum (figure 2.5 A and B). This rescue in dissemination was not due to higher replication of the virus in the FP, spleen, or lung post co-infection (supplemental figure S2.3). The rescue in dissemination was also not due to higher virus at the site of infection as even twice as much RMmCXCL1 inoculum did not rescue the dissemination of the recombinant to the SG (data not shown). This rescue of dissemination was also observed when mice were infected i.p. (figure 2.5 C). However we did not observe the rescue of dissemination of the recombinants when the infection with the two viruses was spaced apart by 2 or 7 days (supplemental figure S2.4).

Our data shows that the rescue of the dissemination of the recombinants to the SG is independent of MCMV endogenous CC chemokine (MCK2) as both the CC expressing RM4503 and non-expressing RM4511 rescue the dissemination to the SG. It also demonstrates that the co-infection is temporally restricted and that the viruses interact early during infection. In addition the data also shows that the interaction between the two viruses skews the immune response such that it allows the dissemination of the recombinants to the SG. Thus with co-infection, we identified an immune competent mouse model where the recombinant virus can successfully disseminate to the SG. This model can therefore be used to investigate how the recombinants stimulate the immune system to prevent their dissemination to the SG.

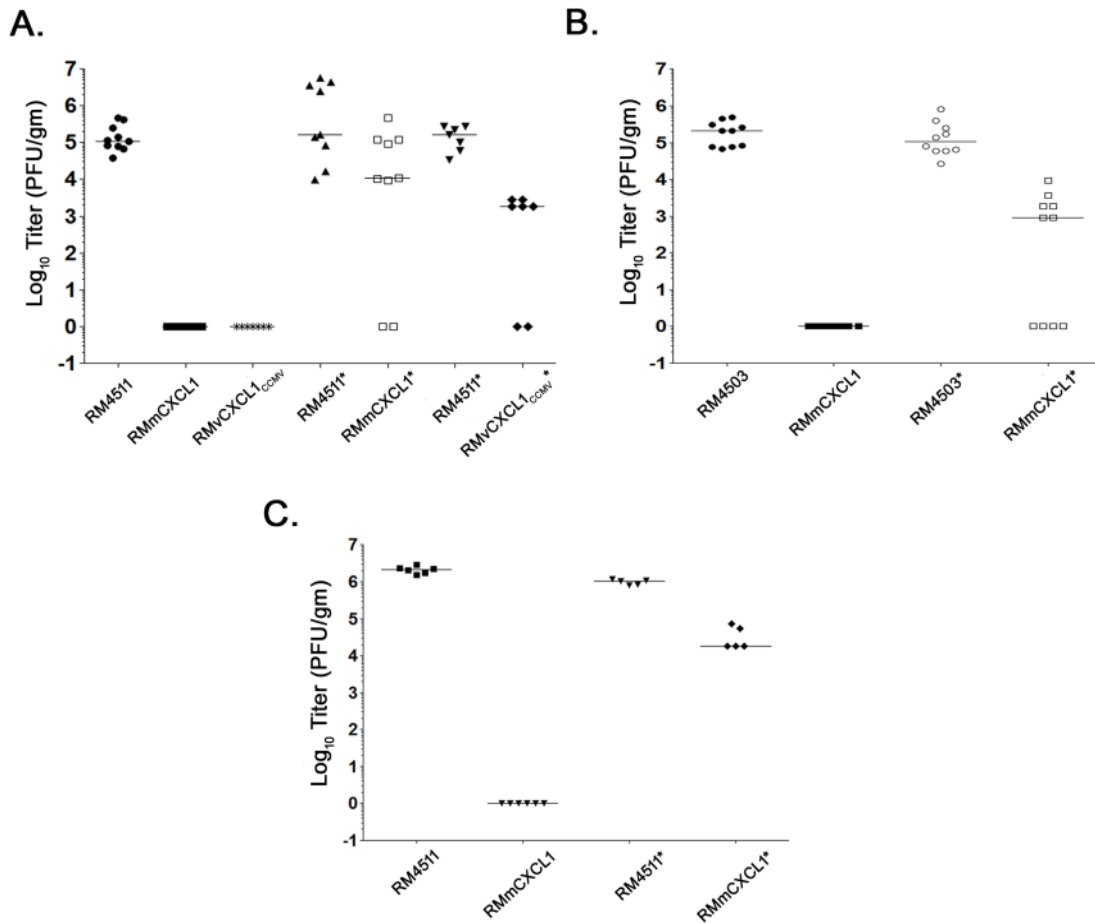


Figure 2.5 Co-infection with parental and recombinant viruses rescues the dissemination of the recombinant to the SG. (A and B) Mice were infected in the foot pad with either 1×10^5 PFU of parental (RM4511 or RM4503) alone, recombinant (RMmCXCL1 or RMvCXCL1_{CCMV}) alone or a mixed inoculum containing 1:1 mix of 1×10^5 PFU of each of parental and recombinant viruses. Viral titer was measured in the SG at day 14 post infection. (C) Mice were infected i.p with either the parental virus alone, recombinant virus alone, or a mixed inoculum containing 1:1 mix of 1×10^5 PFU each parental and recombinant. Virus titer was measured in the SG at day 14 p.i. The symbols indicate titer from each individual mouse. The * indicates viral titers in the SG from mice infected with a mixed inoculum. The horizontal line is the median titer for each infection group.

Co-infection at the site of infection is necessary for SG recombinant dissemination

To characterize how coinfection allows recombinant viruses to disseminate, we wanted to know whether spatial localization rescues the dissemination of the recombinants. Mice were infected with the two viruses in separate foot pads on the same day. This would eliminate the possibility of the viruses interacting at the site of infection, but will still allow the viruses to interact at the sites of primary dissemination. As seen in figure 2.6 A, we did not recover recombinant viruses from the SG of these mice at 14 days p.i. The viruses grew to similar levels at the site of infection and primary dissemination sites even after separate foot pad infections (supplemental figure 2.3).

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 mixed inoculum containing 100 PFU each of RM4511 and RMmCXCL1, effectively reducing the probability of co-infection by 10,000 fold. As seen in figure 2.6 B, the infection with a low PFU inoculum reduces the frequency of dissemination rescue. 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. (schematically described in figure 2.6 C). During the amplification step, we were able to detect GFP positive and negative plaques from ~ 49% of the purified GFP plaques (figure 2.6 C). Purified GFP negative plaques yielded all GFP negative plaques during the amplification step in all instances. Therefore, these results indicate that in this model system, the two viruses need to be at the same site of infection, where they interact by co-infecting the same cell.

Live virus is required to rescue the dissemination of recombinant virus

We show above that the rescue of the dissemination of the recombinant to the SG in mice infected with the mixed inoculum is due to the viruses co-infecting a cell at the site of infection. One possible explanation is that the presence of the virus particles itself could stimulate an immune response that overcomes the blockade of dissemination. We infected mice in the same FP with a mixed inoculum containing a 1:1 mixture of viable RMmCXCL1 or RM4511 and UV inactivated RM4511 or RMmCXCL1 respectively. As seen in figure 2.7 A, UV inactivated RMmCXCL1 did not affect the dissemination of RM4511 to the SG. However, UV inactivation of RM4511 completely abolished the rescue of the dissemination of RMmCXCL1 to the SG (figure 2.7 A). This proves that live replicating parental virus is necessary to mediate the rescue of the recombinant to the SG.

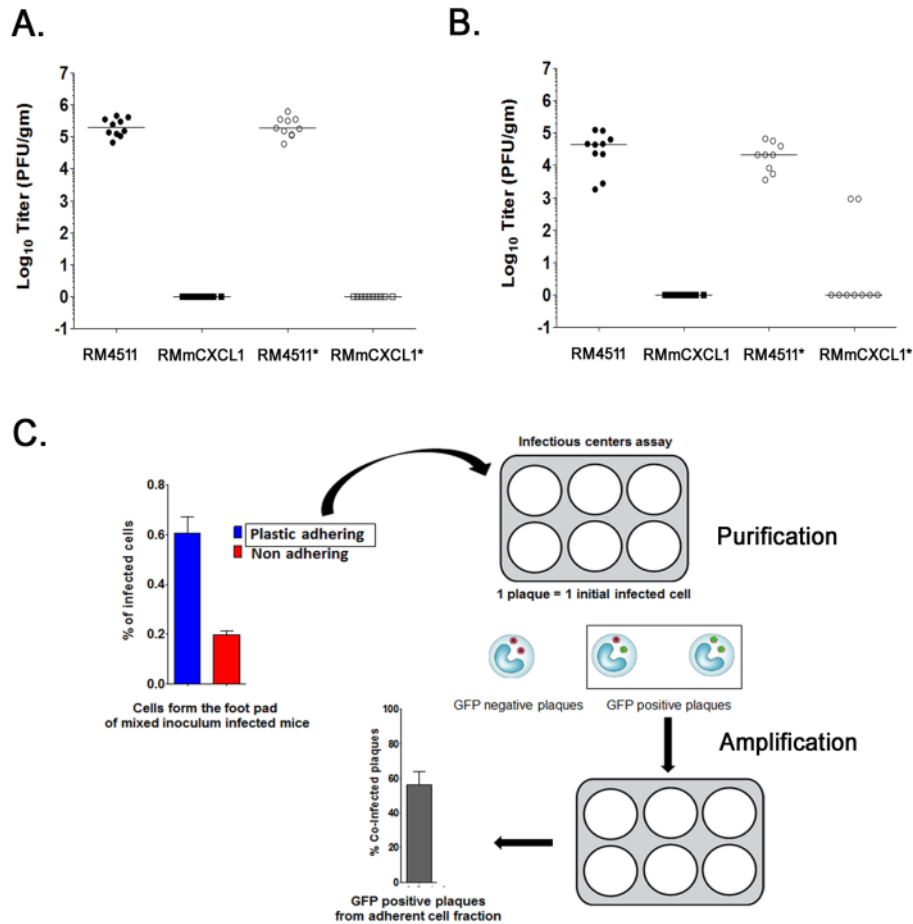


Figure 2.6 Co-infecting viruses interact with each other at the site of infection. (A) Mice were infected in different foot pads with 1×10^5 PFU each of parental (RM4511) and the recombinant (RMmCXCL1) virus. Viral titers in the SG were measured at day 14 p.i. (B) Mice were infected in same FP with a mixed inoculum containing a 1:1 mix of 1×10^2 PFU each of parental and the recombinant virus. Viral titers in the SG were 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. (C) Plastic adherent leukocytes were isolated from FP of mice infected with a mixed inoculum of a 1:1 mix of 1×10^5 PFU each of parental and the recombinant virus at day 3 p.i. GFP positive plaques were purified from an infectious centers assay. These plaques underwent a round of amplification to ascertain the presence of GFP negative virus within the purified plaques, represented as % co-infected plaques. Bars represent the average from 9 mice + SD.

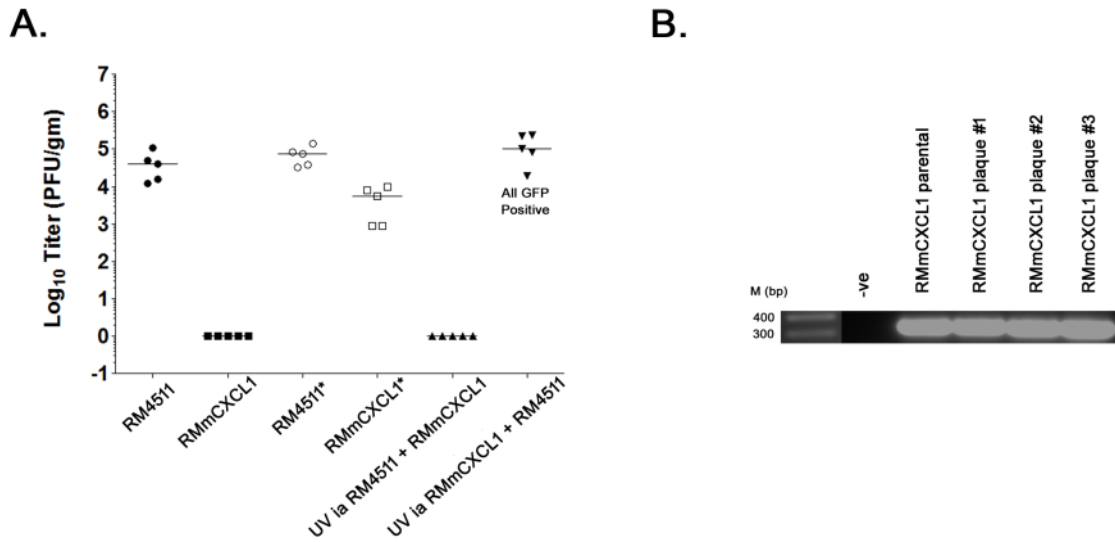


Figure 2.7 Live parental virus required for the rescue the dissemination to the SG and no recombination occurs during rescue. (A) Mice were infected in the FP with 1×10^5 PFU of either parental (RM4511) alone, recombinant (RMmCXCL1) alone, or a mixed inoculum containing 1:1 mix of 1×10^5 PFU of UV inactivated (UV ia) RMmCXCL1 and RM4511 and live RM4511 and RMmCXCL1. Viral titer was measured in the SG at day 14 p.i. The symbols indicate titer from each individual mouse. The * indicates viral titers in the SG from mice infected with a mixed inoculum. The horizontal line is the median titer from the experiment. (B) Viral DNA was isolated by phenol chloroform method and used to set up a diagnostic PCR using mCXCL1 specific forward primer and 6His reverse primer to detect the presence of the chemokine gene within the recombinants isolated from the SG of mice at day 14 p.i infected with a mixed inoculum. -ve is the negative control for the PCR experiment.

One possibility that we needed to address is whether RMmCXCL1 undergoes homologous or illegitimate DNA recombination with RM4511. This could lead to the loss of the chemokine gene from the recombinant and explain the subsequent dissemination. To ascertain the integrity of the chemokine gene in the rescued recombinants, GFP negative plaques were purified from the SG of co-infected mice and DNA isolated. As seen in figure 2.7 B, these isolated recombinants still carried the chemokine gene. There is also the possibility that chemokine gene was mutated such that the chemokine is no longer secreted. However, sequencing of the chemokine gene from the isolates did not show any mutations in the resulting protein, and we were also able to detect the His-tagged chemokine in the culture supernatant of the isolates (supplemental figure S2.5 A and B). We also performed RFLP analysis of these isolates, and no evidence of overt recombination was found (supplemental figure S2.5 C).

Co-infection *in vitro* reduces KC production

Another possible explanation for the rescue of dissemination defect is that the parental virus could affect the production of the chemokine from the recombinant during co-infection. To test this possibility, we carried out an *in vitro* co-infection assay. Cells were infected with the recombinant and parental virus alone at an MOI of 5, or with a mixed inoculum which provides an MOI of 5 for both the viruses (MOI 10 total). Supernatants were harvested every 24 hrs. for 5 days and the amount of chemokine was measured. As seen in figure 2.8 A, the relative expression of the chemokine is reduced during mixed infection compared to recombinant alone. There was also a reduction in chemokine production when the cells were infected with the recombinant alone at an MOI of 10 (data not shown). At the same time, there was no difference in the viral titers at these time points for either of these infections (figure 2.8 B). This data suggests that it is possible that, during *in vivo* co-infection, there may be less chemokine produced without affecting the amount of virus produced. This reduction may be sufficient to relieve the negative effects of the over expressed chemokine to allow for dissemination of the recombinant.

Co-infection modulates the immune response to rescue dissemination

We have already shown that the adaptive immune system does not interfere with the dissemination of the recombinants to the SG (figure 2.4 B). Focusing on the innate immune system, as expected, we did see a slight increase in the number of neutrophils recruited to the site of inoculation at day 3 p.i. in mice infected with the recombinants compared to parental (figure 2.9 A). However, as neutrophils do not contribute to the dissemination defect (figure 2.4 A),

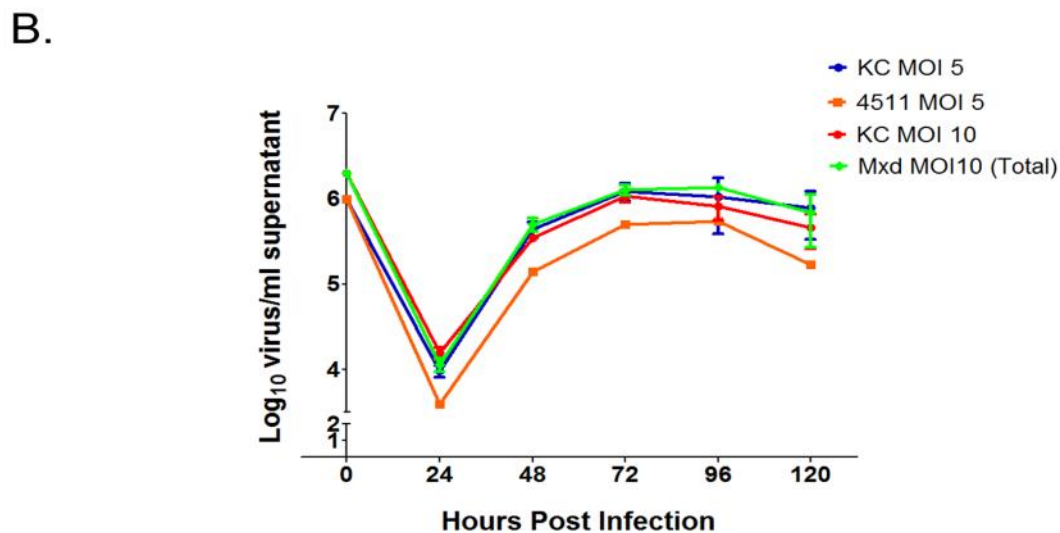
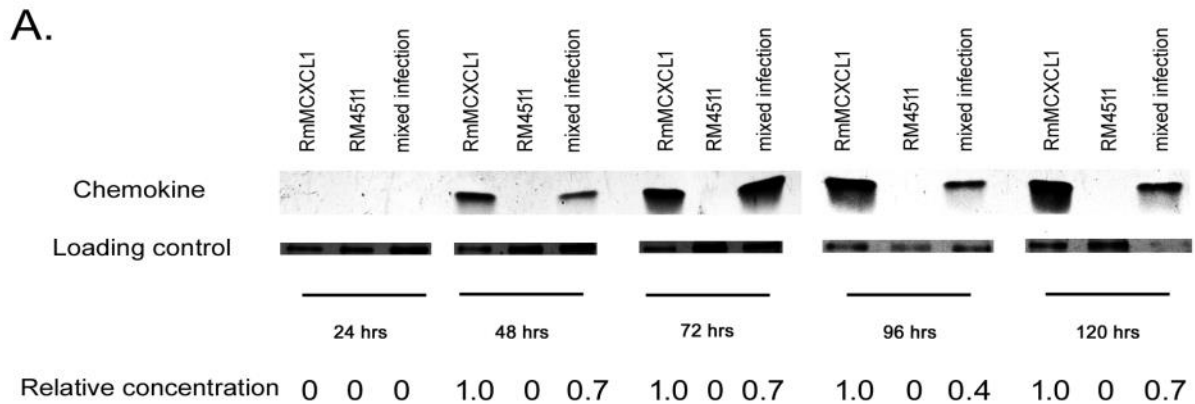


Figure 2.8 Co-infection *in vitro* reduces the recombinant MCMV chemokine levels without affecting viral growth. (A) *In vitro* co-infection assay was performed on MEF 10.1 cells by infecting them at an MOI of 5 with RMmCXCL1, RM4511, or a 1:1 mixed inoculum of RMmCXCL1 and RM4511 at a MOI of 5 for each of the viruses. The supernatant from the infection was collected at the given time points. The 6xHis-tagged chemokine was enriched using Ni-NTA agarose beads and analyzed on a silver stained SDS PAGE gel. Silver stained image is representative of 2 experiments and the relative concentration is the average from those experiments. (B) Viral titers were measured in the supernatant collected from the *in vitro* co-infection assays. Mxd MOI 10 = (MOI5 4511 + MOI5 KC). The data is representative from 3 experiments. Symbols represent the average titer +/- SD.

we did not pursue these cell types further. Experiments with the NOD-NSG mice suggest a role of the NK cells in mediating the dissemination defect. Indeed, we observed more NK cells recruited to the spleen and lung in mice infected with the recombinants compared to the parental (figure 2.9 B). We also observed greater numbers of inflammatory monocytes, at the site of infection in recombinant infected mice recombinants compared to the RM4511 infected mice (figure 2.9 C).

We then used the immune-sufficient co-infection model, to evaluate the contribution of these cell types in preventing the normal secondary dissemination of the recombinants. For this mice were infected i.p with RM4511 alone, RMmCXCL1, or a mixed inoculum of RM4511 + RMmCXCL1. We observed a reduction in the NK cells recruited to spleen and lung in mice infected with the mixed inoculum, compared to RMmCXCL1 alone infected mice (figure 2.10 A). At the same time we also observed a reduction in the number of inflammatory monocytes in the lungs of mixed inoculum infected mice compared to RMmCXCL1 alone infected mice (figure 2.10 B). The data from these experiments suggests that infection with the recombinant viruses leads to a higher infiltration of NK cells and inflammatory monocytes compared to the parental virus to the site of infection. These cell types might interfere with the normal secondary dissemination of the recombinants. Co-infection reduces the number of recruited NK cells and shifts the scale in favor of patrolling monocytes that help the recombinant disseminate to the SG along with the parental virus.

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} (143). Due to the species specificity of CMV, the *in vivo* function of vCXCL-1_{CCMV} is unknown. We have gained significant knowledge about various aspects of HCMV dissemination using the MCMV model (574, 585). MCMV has similar tropism to HCMV (574, 585), and both the viruses demonstrate a cell-associated viremia in cells of the myelomonocytic lineage such as neutrophils, monocytes, and their precursors (430, 574, 585, 586, 606, 607). Although the mechanism and relative contribution of each of these cell type to viral dissemination *in vivo* has been studied in some detail, the role of host and viral chemokines on this dissemination remains to be discovered (141, 430, 574, 585).

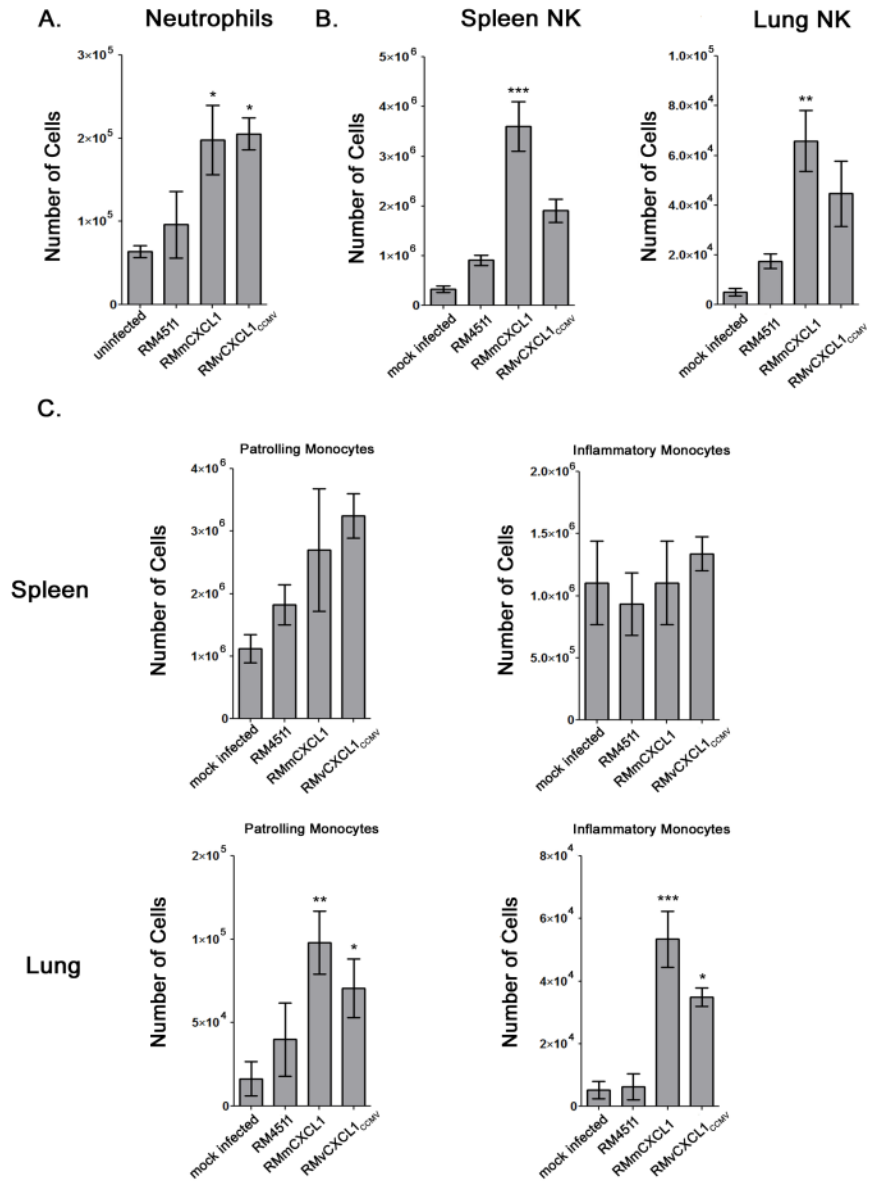


Figure 2.9 Cellular infiltrate is altered at the sites of infection after parental and recombinant virus infection. Mice were infected in the foot pad with RM4511, RMmCXCL1 or RMvCXCL1. Flow cytometry was used to analyze number and types of cellular infiltrate into the different locations. (A) The number of neutrophils infiltrating the FP at day 3 p.i. (B) Mice infected i.p. with RM4511, RMmCXCL1, and RMvCXCL1. The number of NK cells (B) and patrolling and inflammatory monocytes (C) recruited into the spleen and the lung at day 4 p.i. was measured. Bars represent the average of the data from at least 5-6 mice per experiment \pm SEM. One Way ANOVA was used to compare the data. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$

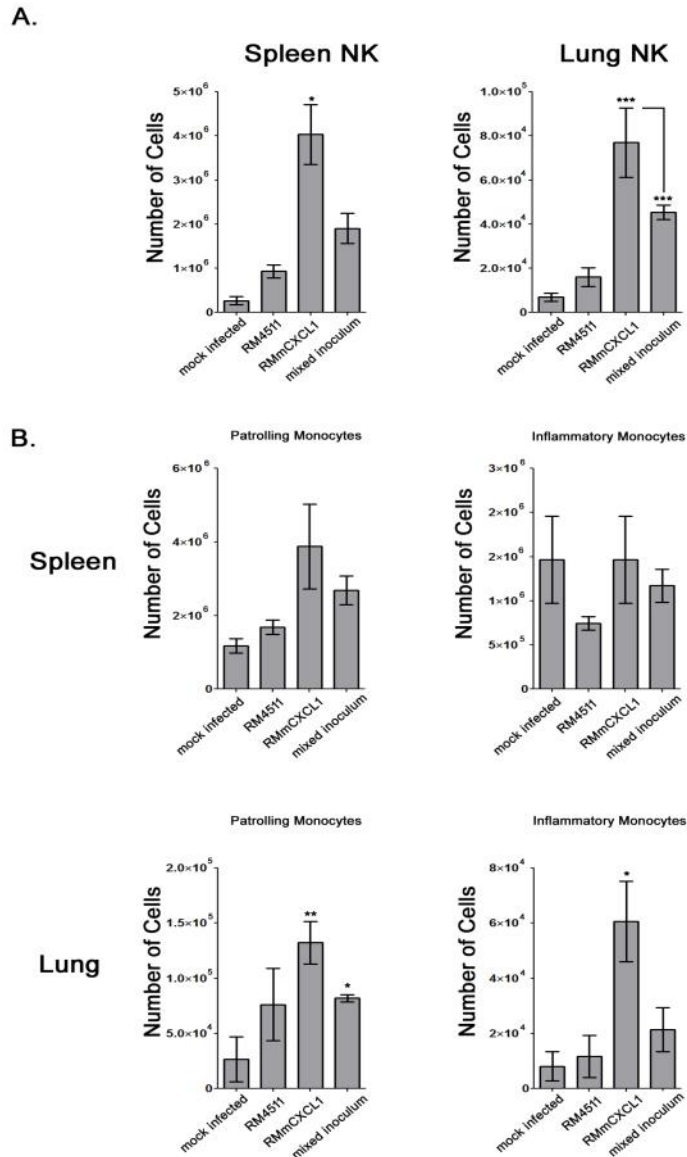


Figure 2.10 Co-infection alters the cellular infiltrate at the sites of infection to favor dissemination. Mice were infected i.p. with RM4511, RMmCXCL1 or a mixed inoculum of 1:1 mix of 1×10^6 PFU RM4511:RMmCXCL1. Flow cytometry was used to analyze NK cells (A), patrolling and inflammatory monocytes (B) within the spleen and the lung recruited into the spleen and the lung at day 4 p.i. Bars represent the average of the data from at least 5-6 mice per experiment +/- SEM. One Way ANOVA was used to compare the data. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$

The MCMV CC chemokine, MCK2, contributes to the dissemination of MCMV to the salivary gland while spread to other organs is MCK2-independent (140). In this study, we used MCMV RM4511, which does not express functional MCK2, to generate recombinant MCMV expressing viral and host CXC chemokines 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 2.3). However, the recombinant viruses were not recovered from the SG (figure 2.3). The absence of the recombinants from the SG could not be explained due to impaired viral growth at other sites of infection, as we did not see any difference in the viral growth of the recombinant compared to the parental virus at the site of inoculation in the foot pad, or the primary dissemination organs: the popliteal lymph node, spleen, and the lung (figure 2.3). It is possible that our recombinants are unable to replicate in the SG and carry a mutation in the *sggI* gene, which has been shown for other MCMV recombinants with a salivary gland growth defect (608, 609). However, this is not likely 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 NSG mice, cyclo treatment or co-infected mice, they are able to replicate in the SG (figure 2.4 C and D). However, we did observe defective viremia for the recombinants (figure 2.4 E). Therefore, the recombinants are sufficiently capable of SG replication, but 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 (136, 598). However, the absence of recombinants from the SG of SCID mice, lacking B and T lymphocytes, argues against this possibility (figure 2.4 B). However, we recovered the recombinants from the salivary gland in NSG mice and after systemic immune ablation with cyclo treatment, implicating some aspects of the innate immune system are responsible for the SG dissemination defect (figure 2.4 C and D). Interestingly, restored viremia for the recombinants in both the cases paralleled SG dissemination (figure 2.4 E). Thus, although the recombinants are not susceptible to adaptive immune mediated clearance in the SG, they show an innate immune-mediated defect in dissemination to the SG.

While working with the co-infection model (i.e., parental + recombinant virus), we made the serendipitous discovery that the recombinants were able to disseminate to the SG even in

immune-competent mice (figure 2.5). This rescue of dissemination required the two viruses to infect simultaneously and at the same site, as separating the infection of the two viruses spatially or temporally does not support the rescue of dissemination of the recombinants to the SG (figure 2.6 A and supplemental figure S2.4). This localization of the infection is required for the two viruses to infect the same cell, most likely a monocyte/macrophage population at the site of infection (figure 2.6 C). Our experiments also show that live replicating virus was required to rescue the dissemination of the recombinants to the SG, not just viral particle interference with recombinant dissemination (figure 2.7 A). This gives rise to the possibility that, the recombinant might undergo recombination, mutation or the deletion of the chemokine gene while replicating inside the parentally infected cell. We show that the recombinants that disseminate to the salivary gland still carry a fully intact chemokine gene (figure 2.7 B and supplemental figure S2.5). There is the additional possibility that during co-infection, the recombinants might grow to higher titers, which would help seed the SG better. This does not seem likely as, we did not observe an increased viral growth in the organs of primary dissemination during co-infection (supplemental figure 2.3). On the other hand 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 the viral growth, however we did observe a modest reduction in the chemokine levels produced (figure 2.8). Therefore, the reduced chemokine level seen during mixed infection is not due to lesser virus produced, but probably due to intracellular resource competition (610-616) or some direct parental alteration within the infected cell leading to reduced chemokine expression. Even though we observed no effect on virus growth in the primary dissemination organs during co-infection (supplemental figure S2.3), we were unable to show a concomitant reduction in the chemokine levels *in vivo* due to experimental limitations of chemokine 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* (610-616). From these experiments, it is clear that immune suppression/ablation is not an absolute requirement to restore the dissemination of the recombinants to the SG, and lends support to the idea that overproduction of the chemokine by the recombinants is the culprit. The over produced chemokines may be recruiting or activating cells of innate immune system that are detrimental for the dissemination of the recombinants to the SG. This data also brings up the interesting observation that the

immune system mediated blockade of the dissemination of the 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 proper dissemination.

Neutrophil depletion did not restore dissemination to the salivary gland (figure 2.4 A). Therefore another innate cell type that expresses CXCR2 and is not depleted with the anti-Ly6G antibody could be playing a role in preventing the dissemination of the recombinants. For example, dendritic cells, a subset of monocytes, and NK cells can be induced to express CXCR2 and CXCR1 (617-620) and may be involved in this process. Although an exhaustive analysis of vCXCL1_{CCMV} receptor usage is lacking, mCXCL1 receptor usage is well-characterized (621), and as both have the same phenotype in our experiments, this does not seem to be exclusively a vCXCL1_{CCMV} phenomenon. We used the co-infection model to evaluate the role the innate immune cell types in dissemination blockade of the recombinants. NK cells play a major role in the antiviral response against MCMV (131, 211, 599). BALB/c mice which are unable to induce NK cell activation via the m157-Ly49H axis are susceptible to MCMV infection and show much higher viral titers in peripheral organs (622, 623). In our experiments we observed higher number of NK cells recruited to the site of infection when mice were infected with the recombinants (figure 2.9 B), which might be compensating for proper activation of the NK cells and prevent the dissemination of the recombinants. Evidence in support of this premise comes from the following observations all of which have restored SG dissemination (i) Co-infection is associated with a reduction of NK cells recruited to spleen and lung (figure 2.10 A), (ii) There was a significant reduction in the NK cells in mice treated with cyclo (supplemental figure 2.6), and (iii) NSG mice, which lack NK cells. The reduction or absence of NK cells correlates with a reduced inflammation at the site of infection or around the foci of during primary organ infection, which could potentially be beneficial for virus growth and escape from the organ.

This model is also useful to evaluate the role of inflammatory monocytes in the dissemination of MCMV, given the experimental limitations of selectively depleting either inflammatory or patrolling monocytes. While patrolling monocytes have been shown to play a role in viral dissemination (430), inflammatory monocytes extravasate into the tissue during inflammation and differentiate into M1 macrophages that contribute to the antiviral response (434, 624). In addition, the infected macrophages can support productive infection of MCMV (198, 625-627). Interestingly, this rescue of dissemination was independent of MCK2 (figure 2.5

A). The dissemination of MCMV to the SG is aided with MCK2 expression; viral dissemination to the other organs is independent of MCK2. This most likely is because the first round of early infiltrating patrolling monocytes that carry the virus are recruited independent of MCK2 expression (430). Indeed we can detect virus in the popliteal lymph node of mice infected in the foot pad as soon as 30 min post infection (unpublished data). Mechanisms contributing to this early exit of the virus from the site of inoculation are currently under investigation. In mice infected with the recombinant, we observed higher number of inflammatory monocytes in the lungs and spleens compared to mice infected with the parental virus (figure 2.9 C). Co-infection reduced the number of inflammatory monocytes in the lung, while not affecting the number of patrolling monocytes (figure 2.10 B). This data supports our hypothesis that the monocytes at the site of infection or around the foci of infection might be the wrong type (i.e., 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 with adoptively transferring leukocytes isolated at day 3 p.i. from the FP of mice infected with parental, recombinant, or a mixed inoculum show that, while we could recover the virus from the SG of mice receiving cells from mice infected with parental or a mixed inoculum, no virus was recovered from the SG of mice receiving cells infected with the recombinant (supplemental figure S2.7).

Therefore we propose a model where the virus is carried out of the FP to the organs of primary dissemination initially in the absence of viral gene expression by patrolling monocytes. During infection with the recombinant alone, the over expression of the chemokine in the organs of primary dissemination (LN, SP, LU) 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 around the foci of infection may also interfere with ability of patrolling monocytes to gain access to virus infected cells. During co-infection, there is reduction in chemokine levels without affecting viral titers. The reduced chemokine leads to a reduction in the number of NK cell at the site of infection, reduction in the inflammatory environment granting patrolling monocyte access to the foci of infection, which then allows for the dissemination of recombinants to the salivary gland. This model has been summarized in figure 2.11.

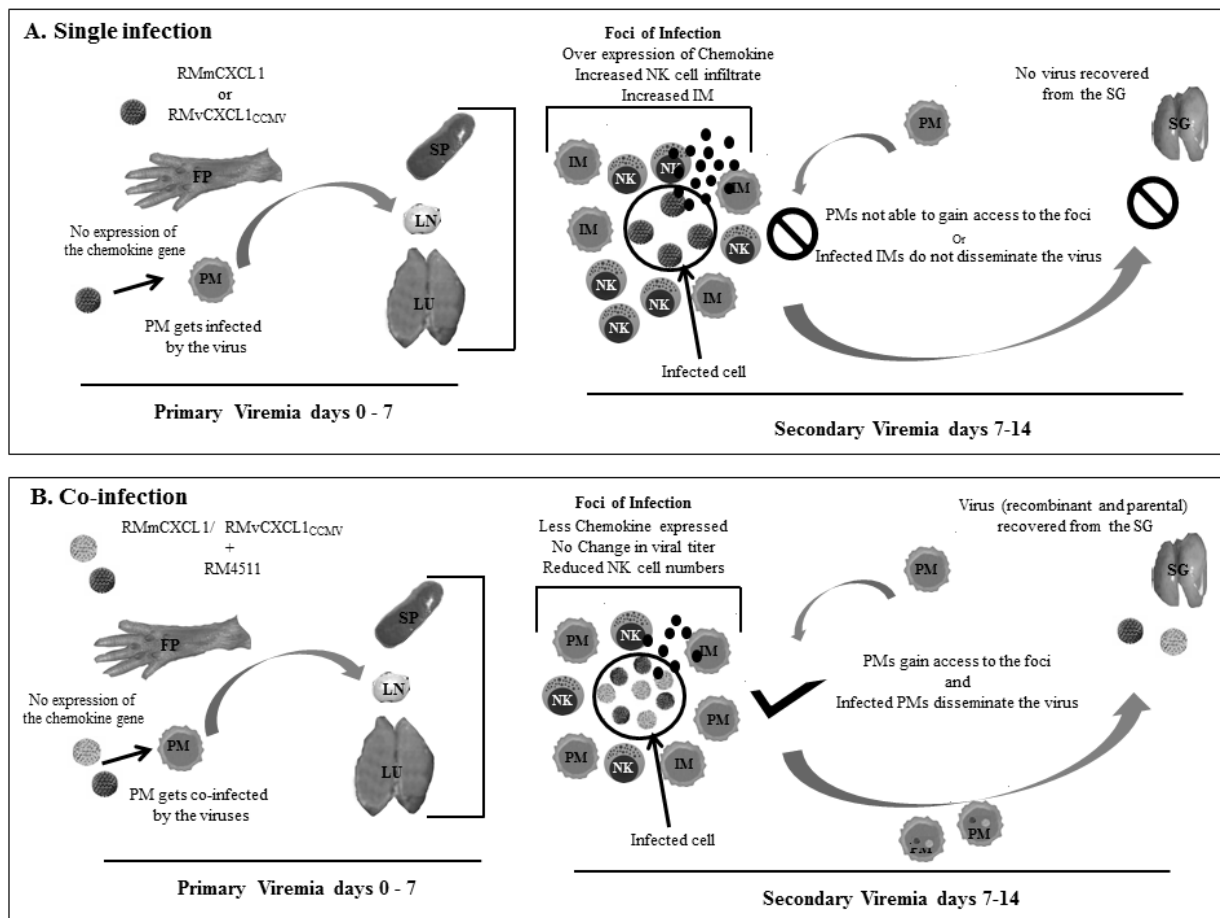


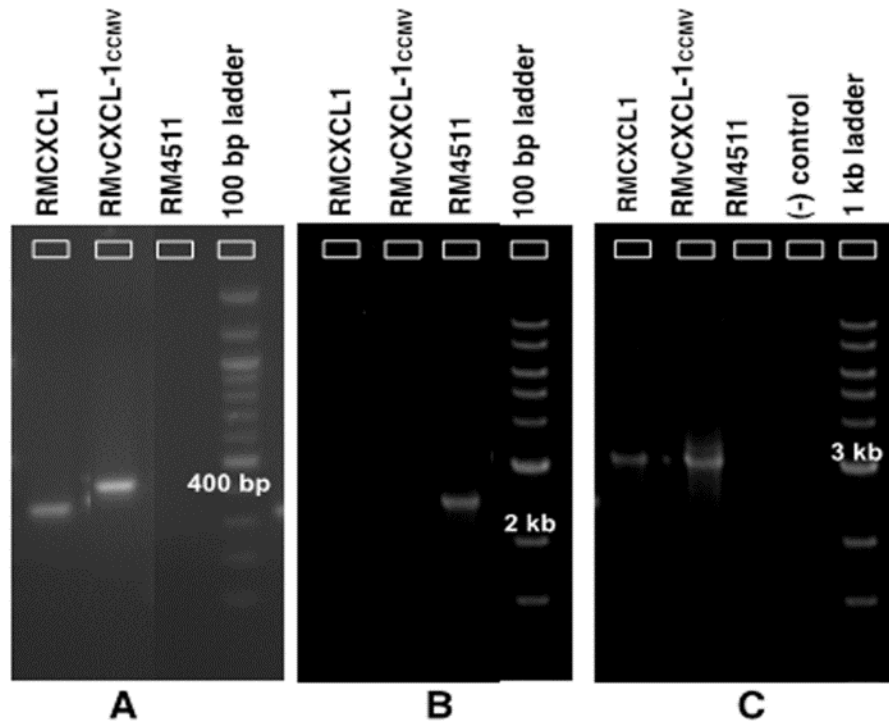
Figure 2.11 Model of dissemination defect and rescue of dissemination of the recombinants during co-infection. (A) During single infection, the overproduction of the chemokine at the site of infection in the primary organs of dissemination recruits inflammatory cells (NK and IM) to the site and interferes with normal virus dissemination during secondary viremia. (B) During co-infection, the reduction in the chemokine levels at the site of infection in the primary organs of dissemination 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 are able to disseminate the virus to the SG during secondary viremia.

Abbreviations used: 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, ● = RMmCXCL1/RMvCXCL1_{CCMV}, ● = RM4511

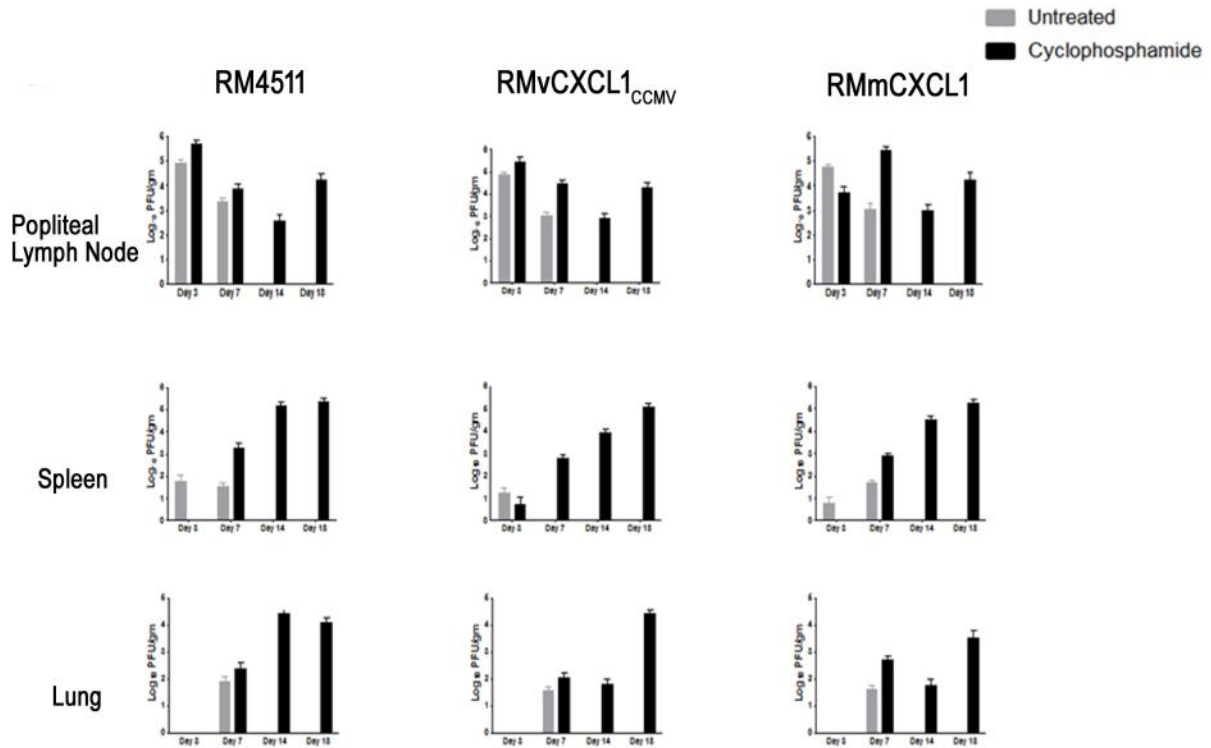
The recombinants that we have generated are attenuated, in the sense that they do not disseminate to the SG. Therefore, they cannot be shed and transmitted horizontally through mouse biting. However, as shown with the co-infection model, the parental and the recombinant virus collaborate to allow the otherwise dissemination defective virus to reach the SG. This aspect of our study raises an important concern about using attenuated strains for CMV vaccination purposes (507). The infection of humans with multiple strains of HCMV is very common (628-636). Therefore, it is possible that in the presence of a previously existing infection, or a newly acquired HCMV infection, the vaccine strain can regain some of its infectious potential depending on the type of attenuation. This finding thus warrants the careful testing of attenuated viral vaccine strains in the context of co-infections during the development phase.

MCMV has several genes regulating the spread and replication of the virus in the salivary gland highlights the importance of this organ to MCMV biology, as it is both a site of viral persistence and of transmission from host to host (199, 598, 607). For example, *sgg1* gene is important for MCMV replication in the SG (608, 609), and the MCMV G protein-coupled receptor homolog, M33, is also needed for efficient salivary gland replication based on mutagenesis studies (145, 473-475, 477). The virally encoded chemokine, MCK2, enhances MCMV dissemination to the salivary (139-141). It also suggests MCMV may require unique mechanisms to facilitate dissemination to and growth in the SG. Although our results may seem to contradict our original hypothesis (i.e., that the expression of HCMV CXC chemokines helps in its dissemination), we have to consider the caveat that our recombinants over express the chemokines. In reality, the expression of the vCXCL1 gene in HCMV is tightly regulated and it is expressed with late expression kinetics (407, 417), and not constitutively, as in the case of our recombinants. Studies have shown that CXC chemokines are capable of making the monocytes adhere to and halt on the endothelium (637-639), which could allow them to be infected more efficiently. Not only can the monocytes be infected by CMV, but the infection increases their life span (640), allows them to re-circulate (569) and promotes their differentiation into a macrophage (590-592). This differentiation then allows for productive infection of the virus in these cell types (641-643). Because monocytes play an important role in HCMV dissemination *in vivo* (568, 569, 640), it is conceivable that, HCMV has evolved to express vCXCL at the stage when the virus is budding from the infected cell. The 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 carrying the virus to a distal site spreading infection within the host.

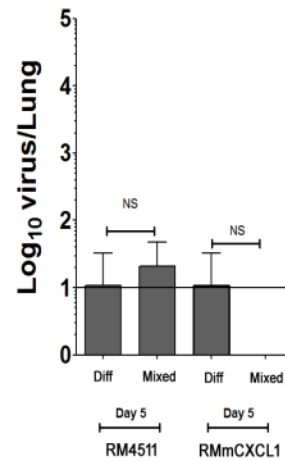
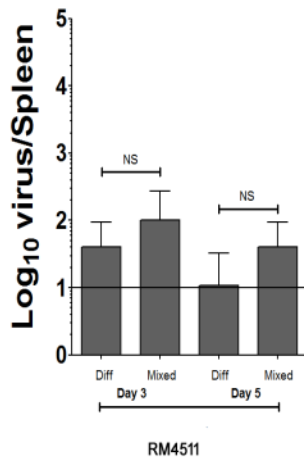
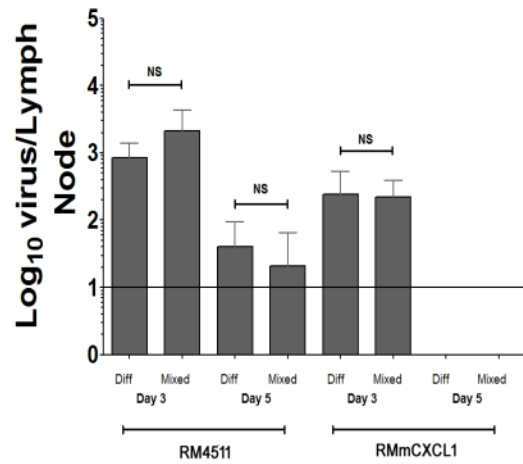
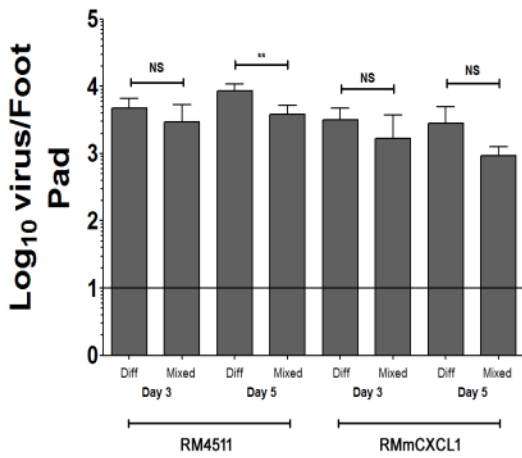
SUPPLEMENTAL FIGURES



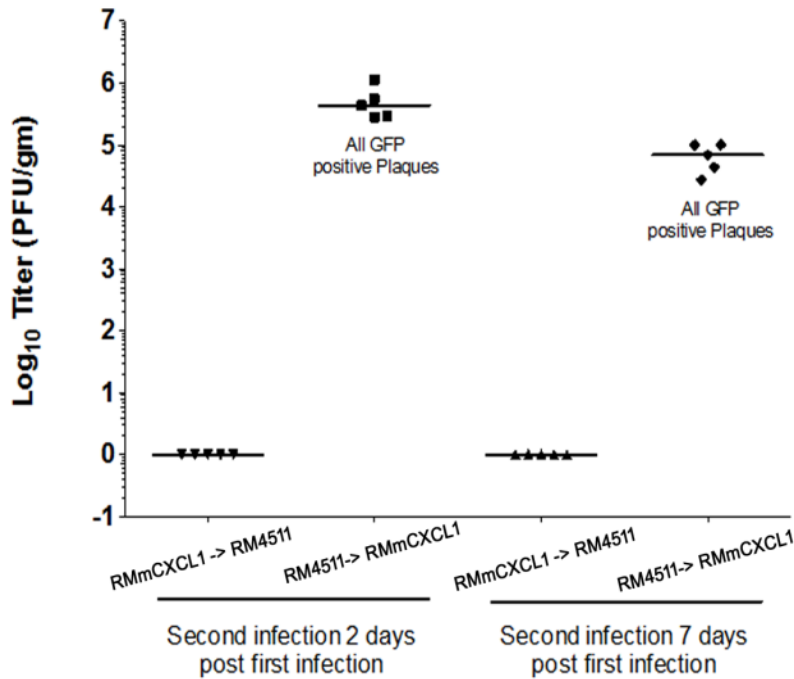
Supplemental figure S2.1 Confirmation of recombination and correct insertion of the expression cassette. Three PCR reactions were performed using viral DNA as template. A) Amplification of either the mCXCL1 or vCXCL-1CCMV gene using HCMV IE and 6 His primers. This generates a ~325 bp product (mCXCL1) and a ~425bp (vCXCL-1CCMV). B) 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. C) 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.



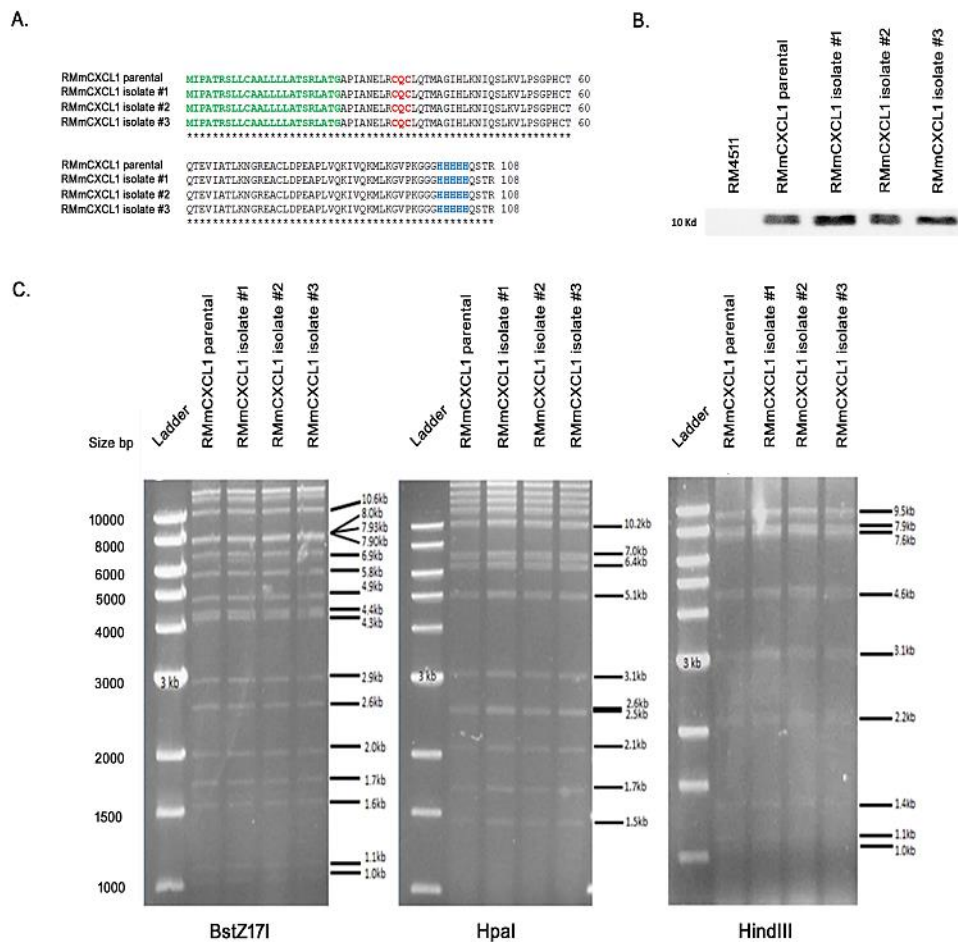
Supplemental figure S2.2 Primary dissemination of virus in cyclophosphamide treated mice. Mice were treated with cyclophosphamide to deplete the immune cells prior to infection with the viruses. Un-treated mice were use as control. Organs (popliteal lymph node, spleen and lung) were harvested at days 3, 7, 14, 18 post infection, homogenized and viral titer measured by performing a plaque formation assay as described in materials and methods. Bars represent average virus titer from at least 5 mice per time point + SD.



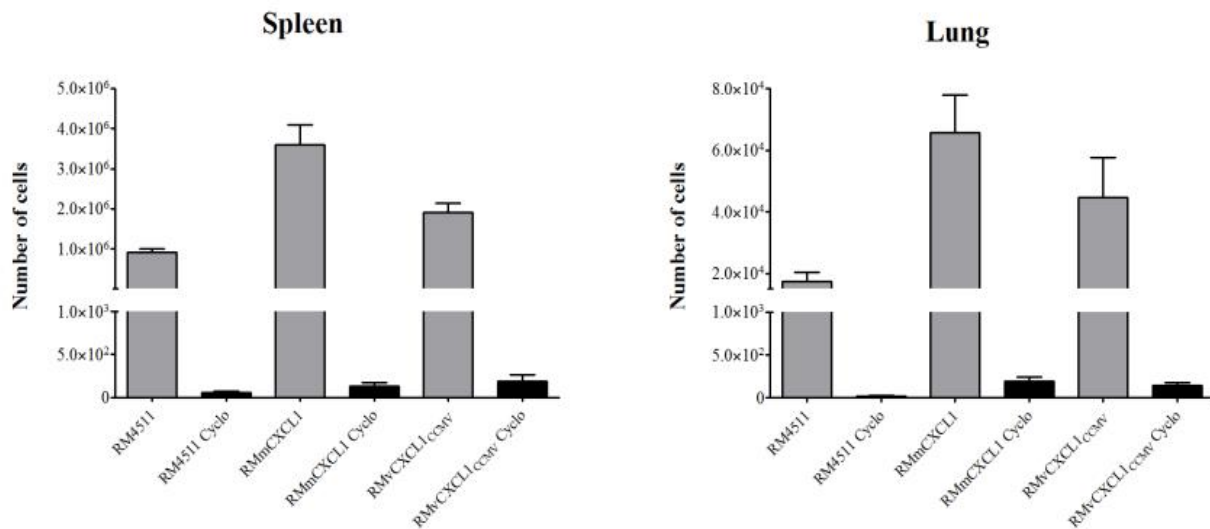
Supplemental figure S2.3 No difference in the titer of recombinant virus when co-infected with the parental virus either in the same or different foot pad. Mice were infected in the same foot pad with a mixed inoculum consisting of a 1:1 mix 1×10^5 PFU each of RM4511 and RMmCXCL1 (Mixed) or in the different foot pad with 1×10^5 PFU each of RM4511 and RMmCXCL1. Organs (Foot pad, Popliteal lymph node, Spleen and Lung) were harvested at days 3 and 5 p.i, homogenized and viral titer measured by plaque formation assay. Bars represent average virus titer from 5 mice per group + SD. The horizontal line represents the limit of detection in the experiment.



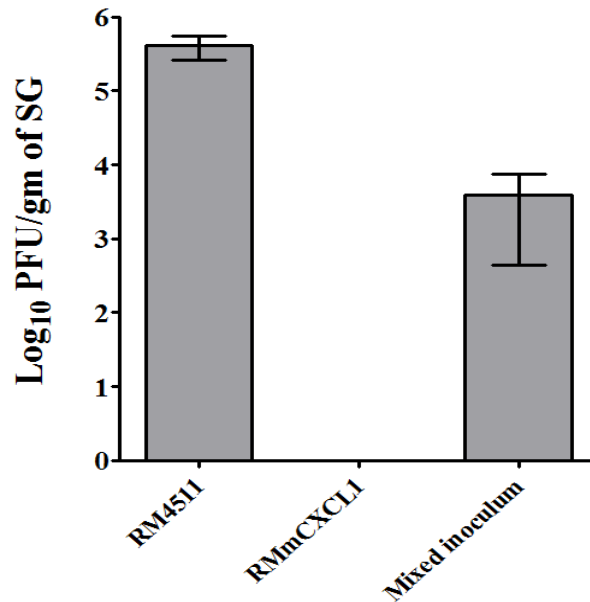
Supplemental figure S2.4 The dissemination of the recombinant is not rescued when the infection with the two viruses is temporally separated. Mice were infected with 1×10^6 PFU of RM4511 and RMmCXCL1 by spacing the infections 2 or 7 days apart. The salivary glands from these mice were harvested at 14 days post the second infection, homogenized and viral titer measured by performing plaque formation assay as described in materials and methods. Symbols represent viral titer from each individual mouse, line is the median titer from the experiment. RMmCXCL1->RM4511 = first infection RMmCXCL1 and second infection with RM4511, RM4511->RMmCXCL1 = first infection RM4511 and second infection with RMmCXCL1.



Supplemental figure S2.5 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) Viral DNA was isolated by phenol chloroform method and used for sequencing using HCMV IE and 6His primers described in materials and methods. Protein sequence was 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) Results from western blot using anti-His antibody to detect the presence of the chemokine in the supernatant from large scale virus cultures (C) RFLP analysis was performed on viral DNA isolated from the RMmCXCL1 virus used for inoculation (RMmCXCL1 parental) and the isolates from the SG using BstZ171, HpaI and HindIII restriction endonuclease.



Supplemental figure S2.6 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 prepared and stained for analysis by flow cytometry. The graph shows the number of NK cells in the spleen and lung of infected untreated and cyclo treated mice. Bars represent average NK cell numbers from 5 mice per treatment group + SEM.



Supplemental figure S2.7 Dissemination of virus after adoptive transfer of infected leukocytes. Mice were infected with 1×10^5 PFU of RM4511/RMmCXCL1 or a mixed inoculum consisting of a 1:1 mix 1×10^5 PFU each of RM4511 and RMmCXCL1. Leukocytes were isolated from the foot pad at day 3 p.i and adoptively transferred to mice via tail vein injection. The salivary glands from these mice were harvested at day 14 post transfer, homogenized and viral titer measured by plaque formation assay. Bars represent average virus titer from 3 mice per group \pm SEM.

**CHAPTER 3: NOVEL HEPARAN SULFATE-BINDING PEPTIDES FOR BLOCKING
HERPESVIRUS ENTRY**

This chapter is a publication by the same title published in PLOS One in 2015 authored by Pranay Dogra, Emily B. Martin, Angela Williams, Raphael L. Richardson, James S. Foster, Nicole Hackenback, Stephen J. Kennel, Tim E. Sparer, and Jonathan S. Wall

Dogra P, Martin EB, Williams A, Richardson RL, Foster JS, et al. (2015) Novel Heparan Sulfate-Binding Peptides for Blocking Herpesvirus Entry. PLoS ONE 10(5): e0126239. doi: 10.1371/journal.pone.0126239

My use of “we” in this chapter refers to my coauthors and myself. My primary contributions to this paper include (1) researching the topic, (2) performing lab work, (3) data analysis and, (4) writing the paper.

1. Abstract

Human cytomegalovirus (HCMV) infection can lead to congenital hearing loss and mental retardation. Upon immune suppression, reactivation of latent HCMV or primary infection increases morbidity in cancer, transplantation, and late stage AIDS patients. Current treatments include nucleoside analogues, which have significant toxicities limiting their usefulness. In this study we screened a panel of synthetic heparin-binding peptides for their ability to prevent CMV infection *in vitro*. A peptide designated, p5+14 exhibited ~ 90% reduction in murine CMV (MCMV) infection. Because negatively charged, cell-surface heparan sulfate proteoglycans (HSPGs), serve as the attachment receptor during the adsorption phase of the CMV infection cycle, we hypothesized that p5+14 effectively competes for CMV adsorption to the cell surface resulting in the reduction in infection. Positively charged Lys residues were required for peptide binding to cell-surface HSPGs and reducing viral infection. We show that this inhibition was not due to a direct neutralizing effect on the virus itself and that the peptide blocked adsorption of the virus. The peptide also inhibited infection of other herpesviruses: HCMV and herpes simplex virus 1 and 2 *in vitro*, demonstrating it has broad-spectrum antiviral activity. Therefore, this peptide may offer an adjunct therapy for the treatment of herpes viral infections and other viruses that use HSPGs for entry.

2. Introduction

Human cytomegalovirus (HCMV) is a beta-herpesvirus with nearly 90% prevalence in the adult human population in developing countries (644). Initial viral infection is generally asymptomatic in immune competent individuals. However, severe CMV disease occurs in individuals with a deficient immune system (*e.g.*, transplant patients suppressed to avoid graft rejection, late stage AIDS patients, and the developing fetus). In immune deficient adults, HCMV can cause pneumonitis, multi-organ disease, and death (644-646). Retinitis and blindness are also common in HCMV-infected, late-stage AIDS patients in the absence of highly active antiretroviral therapies (646). *In utero* infection can cause neurological sequela in infants, including sensorineuronal hearing loss (SNHL) and mental retardation (644, 647).

Attempts to develop a vaccine for CMV infection are ongoing but have met with limited success (507, 511). Current regimens to treat HCMV infection (*i.e.*, ganciclovir, foscarnet, and cidofovir) target viral DNA synthesis (648) but can have detrimental side effects (649). Furthermore the increased use of these drugs has led to HCMV drug-resistance to these therapies (650-653). Due to these limitations, it is clinically important to develop new therapeutics against HCMV that are selective, less toxic, and circumvent resistance. One avenue for drug development is to target other aspects of the HCMV life cycle besides genome replication.

One of these potential targets is virus attachment to the cell. HCMV uses heparan sulfate (HS) for entry into cells and to initiate viral replication (654, 655). Virtually all cells express HS glycosaminoglycans as long un-branched chains associated with protein cores in the form of cell surface heparan sulfate proteoglycans (HSPGs) (656). Heparan sulfate and heparin are both linear glycosaminoglycans (GAGs) composed of alternating glucosamine and uronic acids that can be *N*-acetylated and *N*-sulfated (656-658). Although both HS and heparin are highly sulfated, HS has fewer modifications, making heparin more electronegative than HS GAGs (657, 658). This is an important distinction as heparin is often used as a surrogate for HS GAGs in spite of these differences.

HSPGs act as docking sites for growth factors (657, 659), parasites such as the malarial sporozoite (660), pathologic amyloid-related proteins (661), and many human and non-human pathogenic viruses including HCMV (654) and herpes simplex virus (HSV) (662). The HCMV envelope glycoproteins glycoprotein B (gB) and the glycoprotein M/N (gM/gN) heterodimer

complex are involved in virus adsorption via interaction with HSPG expressed on the cell surface (654). The ability of HS to act as a binding site for numerous distinct viruses can be attributed to its diverse structure and variable negative-charge density (656, 663, 664). Despite the critical role that HS has in HCMV infection, therapeutics targeting HS to treat CMV infections are lacking. This is likely due to its ubiquitous expression on mammalian cells and its important role in facilitating the biological activity of growth factors.

Recently, a panel of heparin reactive peptides has been shown to preferentially bind the HSPG GAGs associated with pathologic deposits containing amyloid fibrils, *in vitro* and *in vivo* (665, 666). Of these peptides, a synthetic, 31 amino-acid, polybasic peptide with a +8 net positive charge, designated p5, was shown to bind amyloid in visceral organs, including the liver, spleen, heart, and kidneys (667). Notably, this peptide does not bind to HS-related GAGs expressed in healthy (*i.e.*, amyloid-free) organs and tissues. Specific reactivity with amyloid-associated HSPGs and not healthy tissues is likely due to the fact that the amyloid-associated tissues are hypersulfated and electrochemically similar to heparin (668, 669). Based on these properties, we hypothesized that these peptides could block CMV entry.

In this study we screened a panel of synthetic, heparan sulfate reactive, p5-related peptides to identify novel inhibitors of CMV HS-mediated adsorption and subsequent infection. We explored the mechanism of action of the peptide and whether it could prevent other viruses that use HS for entry.

3. Materials and Methods

Peptide Synthesis and Purification

Peptides were purchased from Keck Laboratories as semi-pure preparations. Routine purification was performed by HPLC (1100 series; Agilent) using elution from a reverse-phase C3 matrix in a linear gradient of 0–50% acetonitrile in water with 0.05% trifluoroacetic acid. Peptide peaks were eluted from the column using a flow rate of 1 mL/min; 1 mL fractions were collected, peak fractions were pooled, and the mass was determined by mass spectrometry (MS) using a single quadrupole MS (Applied Biosystems). If multiple peaks were observed, peptides were further purified by RP-HPLC and the mass of each confirmed by MS. In all cases, the purified peptides used in these studies appeared as single peaks during HPLC purification and as single bands

following electrophoresis by using SDS-polyacrylamide gel electrophoresis. The purified peptides were lyophilized as 5 mg aliquots and re-suspended in phosphate-buffered saline (150 mM NaCl, pH7.2; PBS) before use. The re-suspended peptides were stored at 4°C until use.

Cells and virus

Low passage-number cells (< 20) were used for all the experiments. Mouse embryonic fibroblast 10.1 (MEF 10.1 (670)) were cultured in DMEM (Lonza, Rockland, ME) supplemented with Fetal Clone III serum (FCIII) to a final concentration of 10% (Hyclone, Logan, UT), Pen/Strep (P/S) to a final concentration of 100 U/ml and L-glutamine (L-Gln) to a final concentration of 2 mM. Human foreskin fibroblast cells (HFF; obtained from ATCC) were cultured in DMEM (Lonza, Rockland, ME) supplemented with Fetal Bovine Serum (FBS) to a final concentration of 10% (Hyclone, Logan, UT), L-Gln to a final concentration of 2 mM, and sodium pyruvate to a final concentration of 1 mM. Human Aortic Endothelial Cells (HAEC) were cultured in EGM-2 Bullet Kit (Lonza, Rockland, ME) supplemented with FBS to a final concentration of 6%. Human retinal pigment epithelia (ARPE-19) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM):F12 medium (Lonza, Rockland, ME) supplemented with FBS to a final concentration of 10%. Human normal lung fibroblast (MRC-5) cells cultured in Minimal Essential Medium (MEM) (Lonza, Rockland, ME) supplemented FBS to a final concentration of 10% and L-Gln to a final concentration of 2 mM. These lines were a kind gift from Dr. Mike McVoy, VCU. African green monkey kidney epithelial (VERO; ATCC) cells were cultured in DMEM media supplemented with FBS to a final concentration of 10%, sodium pyruvate to a final concentration of 1 mM, HEPES buffer to a final concentration of 10 mM and P/S to a final concentration of 100 U/ml.

MCMV RM4503 (140), was cultured *in vitro* in MEF 10.1 cells. The virus stock was titered using plaque assay (described below) and stored at -80°C. Bacterial artificial chromosome generated HCMV TB40/E-mCherry (470, 671) and TB40/E-pp150-GFP (672, 673) were cultured *in vitro* on HFF cells. The virus stock was titered using a plaque assay and stored at -80°C. Low passage number HCMV (passaged 2-3 times) was used for all experiments. Herpes Simplex Virus (HSV-1 KOS and HSV-2 186 Syn⁺) were cultured *in vitro* on VERO cells. The virus stock was titered using a plaque assay and stored at -80°C.

Plaque reduction assay

Peptides were screened for their ability to reduce viral infection using a plaque reduction assay. Cells were cultured in 12-well (VERO) or 24-well culture plates (MEF 10.1 and HFF). When cells reached ~80 % confluence the media was removed and washed once with PBS before addition of peptide. As a control, cells were incubated with PBS alone. After a 30 min incubation with peptide in PBS, virus (~100 PFU/well for MCMV and ~30-40 PFU/well for HCMV and HSV) was added and incubated for another 90 min (HSV and HCMV) or 60 min (MCMV). Following virus incubation the peptide/virus mixture was removed and replaced with 0.75% carboxymethyl cellulose (Sigma Aldrich, St. Louis, MO) (CMC) + complete media (DMEM + P/S + L-Gln) for MCMV and HSV experiments or 0.5% agarose (Lonza, Rockland, ME) in complete media for HCMV experiments. The plates were incubated at 37° C in 5% CO₂ for 4 days and when plaques began to develop, plates were stained with Coomassie stain (AMRESCO, Solon, Ohio). Due to the inability of HCMV to form distinct plaques on HAEC and ARPE-19 cells, infection in these cell types was measured by counting mCherry positive foci 14 days post infection. Plaques were counted manually using a dissection microscope. Data was analyzed using Prism 5.0 (GraphPad Software, La Jolla, CA). Data were expressed as percent infection (100 x (number of plaques after treatment/ the number of plaques in the PBS-treated wells)).

Flow cytometric analysis of attached virus

HFF cells were grown in a 24 well dish and allowed to reach ~80% confluency. The cells were cooled to 4°C to prevent virus internalization before addition of peptide (100µM) and incubated for ½ h. Following the incubation, HCMV TB40/E pp150-GFP was added (MOI 10) at 4°C and incubated for 1h. Following the incubation, cells were removed from the wells using non-enzymatic cell stripper solution (Corning), fixed (with paraformaldehyde) and the data acquired using a BD FACS Calibur flowcytometer (BD Biosciences). The data was analyzed using FlowJo software (TreeStar).

Heparin blockade of peptide-mediated plaque reduction

Peptide p5+14 (100 µM) was pre-incubated with heparin sodium salt (Acros Organics, NJ) at different concentrations for 1 h at 37°C. This heparin/peptide mix was added to the cells and

incubated for 30 min at 37°C. Following the incubation, supernatant was aspirated and cells washed once with PBS to remove unbound/excess heparin or peptide. The cells were subsequently infected with ~100 PFU/well of MCMV. To test whether heparin treatment of cells interferes with virus infection, MEF 10.1 cells in a 24 well dish were pre-incubated with different concentrations of heparin for 1 hour and washed as described above. Following this pre-incubation, infection was initiated as described above. Finally to test the effect of heparin treatment on the infectivity of virus, MCMV was incubated with different concentrations of heparin for 1h before infecting cells. For all treatments, virus was removed 1h post infection and cells were overlaid with CMC. Plates were incubated for 4 days before staining and counting the plaques.

Enzymatic treatment of cells

Heparinase I, Heparinase II, Heparinase III, and Chondroitinase ABC were purchased from Sigma Aldrich (St. Louis, MO). MEF 10.1 cells in culture were treated with heparinase in heparinase buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM CaCl₂, and 0.01% bovine serum albumin (BSA)) at a concentration of 1U/ml or chondroitinase re-suspended in chondroitinase buffer (50 mM Tris, pH 8.0, 60 mM sodium acetate and 0.02% BSA) at a concentration of 1 U/ml for 1 h at 37°C. As a control, cells were treated with enzyme buffer alone. Following incubation, the enzyme solution was removed and cells were washed with PBS to remove excess enzyme. Subsequently the cells were treated with peptide and infected with virus. Data was collected and analyzed as described above.

Visualization of bound peptide

Coverslips with fixed MEF cells were prepared, washed in PBS, and blocked with 1% BSA/PBS for 5 min. Following a PBS wash, the nuclei were stained using Hoechst (Life Technologies Molecular Probes, Grand Island, NY) 1:100 in H₂O for 30 min at 37°C. Cells were then blocked using a casein block solution (Scytek) for 5 min, AVIDIN/Biotin blocks (VECTOR) for 20 min each at room temperature (RT) followed by a 5 min PBS wash. Biotinylated p5+14 or CGGY-p5G (control) at 1.6 µg/mL in PBS was added and incubated overnight at 4° C. Following a PBS wash Alexa Fluor 594-conjugated streptavidin (Molecular Probes) was added at a 1:200 dilution in PBS for 1h at RT. Cells were then permeabilized with 0.2% Triton X-100 (Sigma) in PBS for

10 min at RT and washed with a solution of 1% BSA in PBS for 30 min. The cells were then stained with Alexa Fluor 488-conjugated phalloidin (Molecular Probes) at a 1:100 dilution of stock in 1% BSA/PBS, for 45 min at RT to visualize actin filaments. Slides were cover-slipped using a fluorescent mounting medium (Dako) to minimize photobleaching.

Measuring bound peptide

MEF 10.1 Cells were grown in 24-well cell culture plates as described above. Each well was probed with 100 μ L of biotinylated peptides at 1 μ g/mL in cold DMEM/F12 with 0.1% BSA and incubated for one hour at 4°C. Following the incubation, cells were washed twice with ice cold PBS and fixed with 1.25% glutaraldehyde. Fixed samples were washed twice and stored in PBS for 24 hours. The samples were then blocked with 1% BSA in PBS and probed with 100 μ L of Europium-conjugated streptavidin (Perkin-Elmer, Waltham, MA) in PBS/0.1% BSA for 30 min at RT. The plate was washed three times with PBS and enhancement solution added. The fluorescence counts of the control peptide (*i.e.*, background), P5+14 treated cells, and P5+14 treated cells with added enzymes were measured using time resolved fluorescence on the Wallac Victor 3 (Perkin-Elmer) plate reader. Background counts were subtracted from all treatments. The percent reduction in bound peptide was calculated as $100\% - (\text{enzyme treated fluorescence counts} / \text{no enzyme treated counts} \times 100)$.

Statistical analysis

The data presented are pooled results from three or more experiments performed independently (*i.e.*, repeats), with at least three replicates in each experiment. Error bars represent the standard deviation (SD). Statistical significance was calculated using one tailed student's *t* test or 1 way ANOVA followed by Tukey's Multiple Comparison Test in GraphPad Prism following the recommendations of Vaux et al (674, 675). Significance was determined for each separate run for each of the repeats. A *p* value < 0.05 was considered statistically significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, NS = non-significant reduction in infection. In the case of experiments with only three samples, statistical significance should be interpreted with caution. The small sample size could be susceptible to type II error.

4. Results

Screening of peptides

Seven synthetic peptides based on the structure of peptide p5 were screened for their ability to reduce MCMV infection *in vitro* (Table 3.1). In the initial screening assays all peptides were tested at a single concentration (500 $\mu\text{g/ml}$) using a plaque-reduction assay, in which mouse embryonic fibroblasts were incubated with the peptides for 30 min prior to the addition of virus. The polybasic peptides exhibited a range of viral inhibition up to >90% inhibition for peptide p5+14 (Fig. 3.1A). In contrast, the poly anionic, uncharged, and hydrophobic p5 variant peptides, CGGY-p5E, CGGY-p5G, and CGGY-p5L, respectively, did not reduce MCMV infection (Fig. 5.1A). The presence of an N-terminal Cys residue, which was originally generated to facilitate incorporation of the radionuclide $^{99\text{m}}\text{Tc}$ in peptide CGGYp5, did not alter the efficacy of GGGY N-terminal variant (p5) (Fig. 3.1A). However, the CGGYp5 was prone to self-aggregation (data not shown) and was therefore not further considered in this study.

Following the initial screen, peptide p5+14 was selected for further analysis because it induced the greatest reduction in infection. Serial dilution of p5+14 peptide resulted in significant reduction in infection at concentrations > 5 $\mu\text{g/mL}$ (Fig. 3.1B) with an IC_{50} of 5.2 μM .

Structural aspects and insights into the mechanism of action

ITASSER software (676, 677) predicted the secondary structure of peptide p5+14 to be α -helical with the majority of the Lys residues aligned along one face of the peptide due to the heptad repeat in the protein sequence (678) (Fig. 3.2A). To test our hypothesis that peptide p5+14 prevents MCMV infection by competing effectively for negatively charged cell surface HSPG, biotinylated p5+14 was incubated with fibroblasts in culture. Biotinylated peptide CGGY-p5G, which replaces Lys with Gly throughout the peptide, served as a negative control. The p5+14 bound mouse fibroblasts in culture as evidenced by the red (Alexa 540) fluorescence stain associated with the cells (Fig. 3.2B left). In contrast, the electro-neutral peptide CGGY-p5G did not bind (Fig. 3.2B right), suggesting that the binding of the peptide to fibroblasts was dependent upon the presence of basic (Lys) residues.

Table 3.1 Primary sequence of peptides

| Peptide | Sequence | Net Charge | Plaque Reduction (Average from Fig.1) |
|----------------|---|-------------------|--|
| CGGY-p5 | CGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK Q | +8 | ~61 % |
| CGGY-p5E | CGGYS EAQEA QAEQA EQAQE AQEAQ AEQAE Q | -8 | 0 |
| CGGY-p5L | CGGYS LAQLA QALQA LQAQL AQLAQ ALQAL Q | 0 | 0 |
| CGGY-p5G | CGGYS GAQGA QAGQA GQAQG AQGAQ AGQAG Q | 0 | 0 |
| p5 | GGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK Q | +8 | ~53 % |
| p5R | GGGYS RAQRA QARQA RQAQR AQRAQ ARQAR Q | +8 | ~75 % |
| p5+14 | GGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK QAQKA QKAQA KQAKQ | +12 | ~90 % |
| G2 | MPRRR RIRRR QK | +8 | |

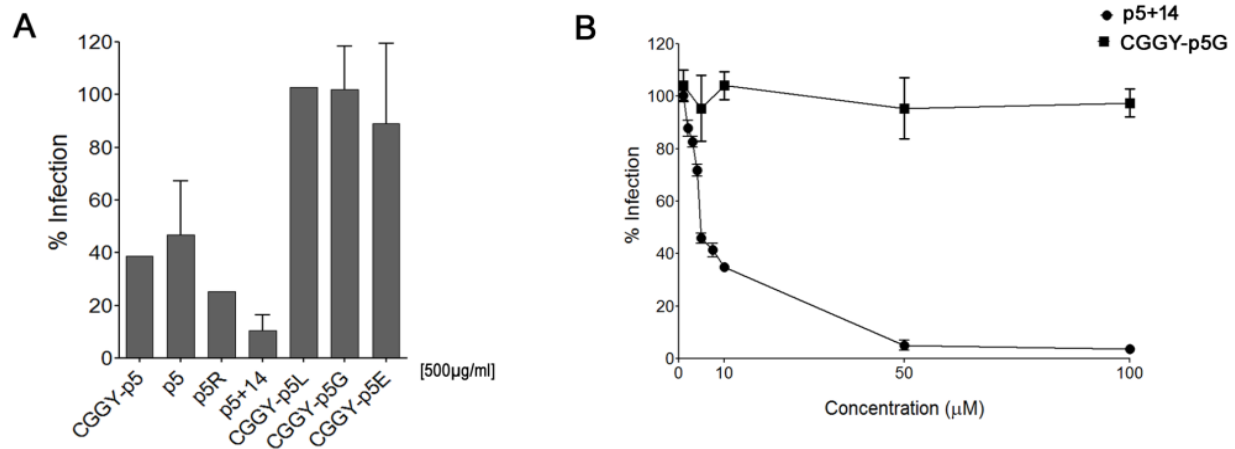


Figure 3.1 Heparin-reactive peptides reduce MCMV infection *in vitro*. (A) Peptides (500 µg/ml) with different net charges and lengths were incubated with cells 30 min prior to addition of MCMV (~100 PFU/well). Bars represent the average of the percent reduction in infection compared to PBS-treated control from three independent experiments with at least three replicates in each + SD. (B) p5+14 and CGGY-p5G (control peptide) were serially diluted and assayed in a plaque reduction assay as described in materials and methods.

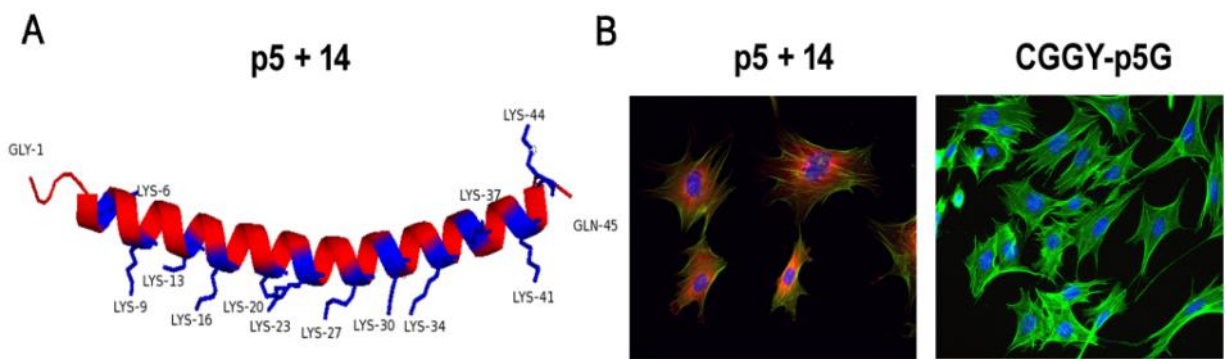


Figure 3.2 p5+14 binding to cells is charge dependent. (A) Predicted α -helix structure of peptide p5+14 based on ITASSER modeling. (B) Biotinylated peptide p5+14 (left panel) or CGGY-p5G (right panel) was added to MEF 10.1 cells followed by addition of Alexa Fluor 594-conjugated streptavidin (red). Nuclei are stained blue with Hoechst and F-actin stained green with Alexa Fluor 488-conjugated phalloidin.

Peptide-mediated reduction of MCMV infection through cell surface HS binding

If p5+14 binds to negatively charged HS moieties on the cell surface, pre-incubation of the peptide with heparin, which has similar charge and structural properties to HS, should interfere with peptide-mediated reduction of infection. To test this, we incubated p5+14 with various concentrations of heparin. Pre-incubation of peptide with heparin before addition to the cells reduced its ability to inhibit MCMV infection in a dose-dependent manner (Fig. 3.3A). It should be noted that the ~50% reduction in infection with peptide and no heparin (*i.e.*, 0ug/ml heparin concentration in Fig. 3.3A) is different than the ~90% reduction in Fig. 3.1. We ruled out degradation of the peptide during the pre-incubation step as an explanation for this discrepancy (data not shown). This disparity could however be due to the additional wash step after incubation of peptide + heparin. This additional wash could remove cell-surface bound peptide decreasing peptide interference with infection. This step is necessary to avoid any free heparin neutralizing the virus so it could not be eliminated from the protocol. In contrast, pre-incubation of the cells with negatively charged heparin prior to virus addition did not alter MCMV infection (Fig. 3.3B). However, when heparin was pre-incubated with MCMV (without peptide), infection was reduced > 80% at all heparin concentrations $\geq 2 \mu\text{g/mL}$ (Fig. 3.3C). This data supports our hypothesis that p5 is binding the negatively charged GAGs on the cell surface which can be counteracted by incubation with the negatively charged heparin (679).

Because p5+14 can bind both HS and CS GAGs, the reduction in infection could be mediated by direct competition for virus adsorption sites via HS on the cell surface, steric hindrance mediated by peptide bound to CS on the cell surface, or both. To distinguish between these possibilities, cells were treated with heparinase or chondroitinase enzymes to cleave the different GAGs from the cell surface. Treatment of cells with heparinase caused a ~40% reduction in the amount of bound peptide, whereas chondroitinase treatment resulted in a ~11% reduction (Fig. 3.4A). Treatment of cells with heparinase (1U/ml) led to a ~60% reduction in MCMV infectivity as expected, which was enhanced further by the addition of p5+14 leading to an ~80% reduction (Fig. 3.4B). There was no significant difference between peptide alone and peptide in conjunction with heparinase treatment. In contrast, treatment of cells with chondroitinase (1U/ml) did not reduce MCMV infectivity nor did it have any effect on the activity of the peptide (Fig. 3.4C). Pre-treatment of MCMV itself with heparinase or chondroitinase prior to addition to MEFs did not alter its infectivity (data not shown). These results indicate that p5+14 blocks MCMV infectivity via heparan sulfate and not steric hindrance after binding to chondroitin sulfate.

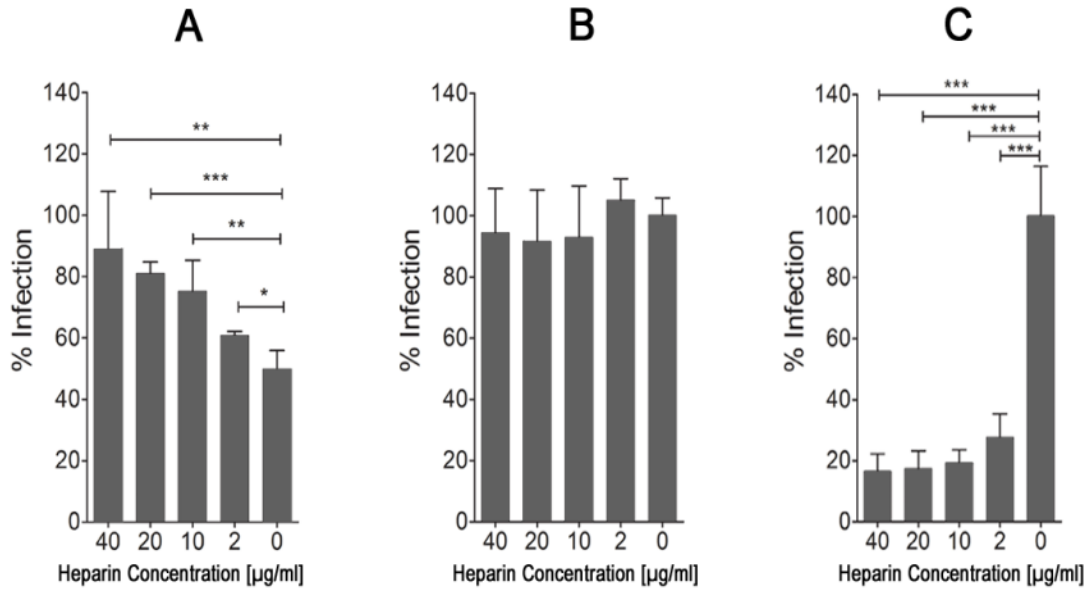


Figure 3.3 Soluble heparin interferes with peptide inhibition of virus infection. The effect of heparin on the activity of peptide and MCMV viral infectivity *in vitro* when (A) pre-incubated with the peptide (100 µM), (B) incubated with the cells before adding virus, and (C) pre-incubated with virus alone in a plaque reduction assay as described in materials and methods. Bars represent the average of the percent reduction in infection compared to PBS-treated control from three independent experiments with at least three replicates in each + SD. Statistical significance is indicated as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

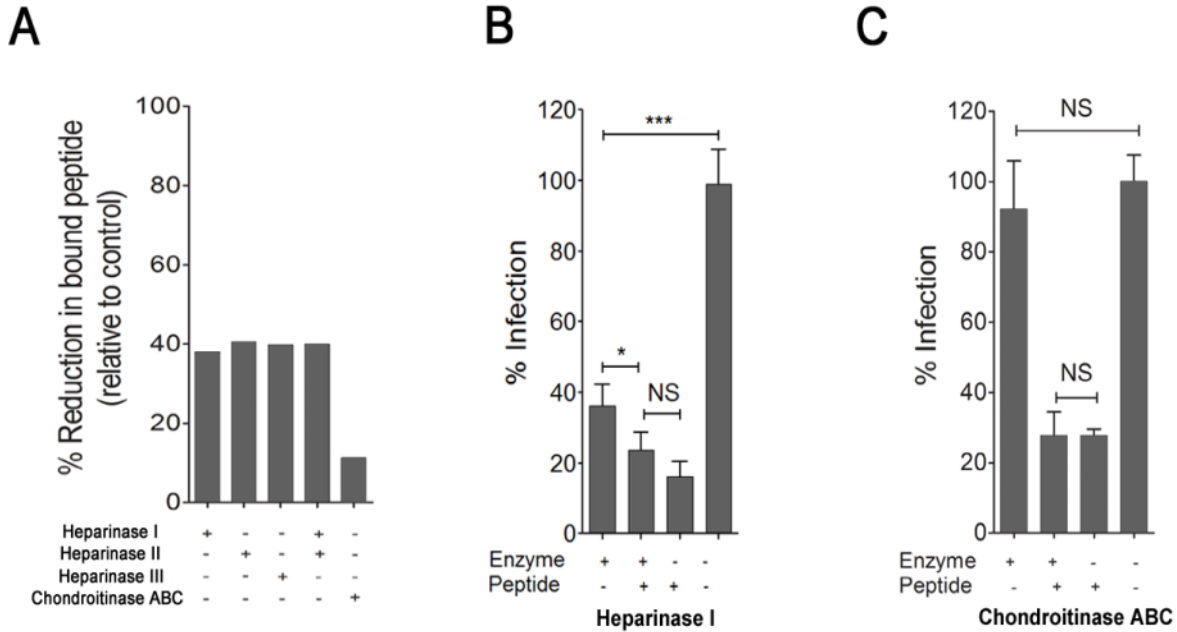


Figure 3.4 Peptide interacts with cell surface heparan sulfate but not chondroitin sulfate to mediate anti-viral activities. (A) MEF 10.1 cells were treated with heparinase I, II, III or chondroitinase ABC and the amount of bound peptide was assessed as described in materials and methods. (B) Cells were treated with heparinase I (1U/ml) or (C) chondroitinase ABC (1U/ml) and peptide p5+14 was added. The amount of plaque reduction of MCMV infection in each treatment was measured in a plaque reduction assay as described in materials and methods. Bars represent the average of the percent reduction in infection compared to PBS-treated control from three independent experiments with at least three replicates in each + SD. Statistical significance is indicated as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, NS = non-significant difference in the reduction of infection.

Peptide competes for virus adsorption to the cell surface

In the infectivity assays described above, peptide and virus were co-incubated with the cells. In this experimental setup, the peptide could bind to the virus, to cells, or both and reduce infection. Therefore to ensure that the peptide was not directly inactivating the virus, MCMV was co-incubated with 100 μ M (~20x the IC₅₀) peptide at 37°C for 1 h, diluted to an *ineffective* peptide concentration (1 μ M) and infection of fibroblasts measured. There was no reduction in MCMV infection under these conditions, whereas addition of peptide and virus simultaneously to the cells showed significant reduction in infection (Fig. 3.5A).

To determine at which stage of the MCMV entry cycle the peptide interferes, four different peptide treatment protocols were tested: 1) 30 min prior to infection (pre-adsorption) 2) simultaneously with virus (during adsorption) 3) after letting the virus adsorb to the cells at 4°C for 1h (post adsorption, then shifted to 37°C to induce membrane fusion) or 4) after allowing the virus to fuse with the cellular membrane at 37°C for 1h (post fusion) (Fig. 3.5B). Addition of the p5+14 peptide before or in conjunction with virus addition to the cells resulted in >80% reduction in infection. However, when peptide was added after the adsorption or fusion phase of viral entry, no significant reduction in plaque formation was observed (Fig. 3.5B).

To specifically show that p5+14 prevents adsorption of HCMV to cells, HCMV expressing a tegument protein-green fluorescent fusion protein, pp150-GFP, was incubated with HFF cells at 4°C in the presence or absence of the different peptides. The fluorescence of cell-associated virus was measured via flow cytometry (Fig. 3.5C). Incubation of the cells with p5+14 reduced the amount of fluorescent virus attached to the cell surface, whereas there was no reduction in fluorescence when cells were incubated with peptide CGGY-p5G compared to PBS treated cells.

Comparison of p5+14 to other inhibitory peptides

The efficacy of p5+14 to reduce infection was compared to the recently reported inhibitor peptide, G2, which was also inhibits infection of herpes viruses (HSV and MCMV) (680) (Table 1). Both peptides effectively inhibited MCMV infection at 100 μ M, but p5+14 leads to a >80% reduction in infection at 10 μ M at which concentration peptide G2 was ineffective (Fig. 3.6).

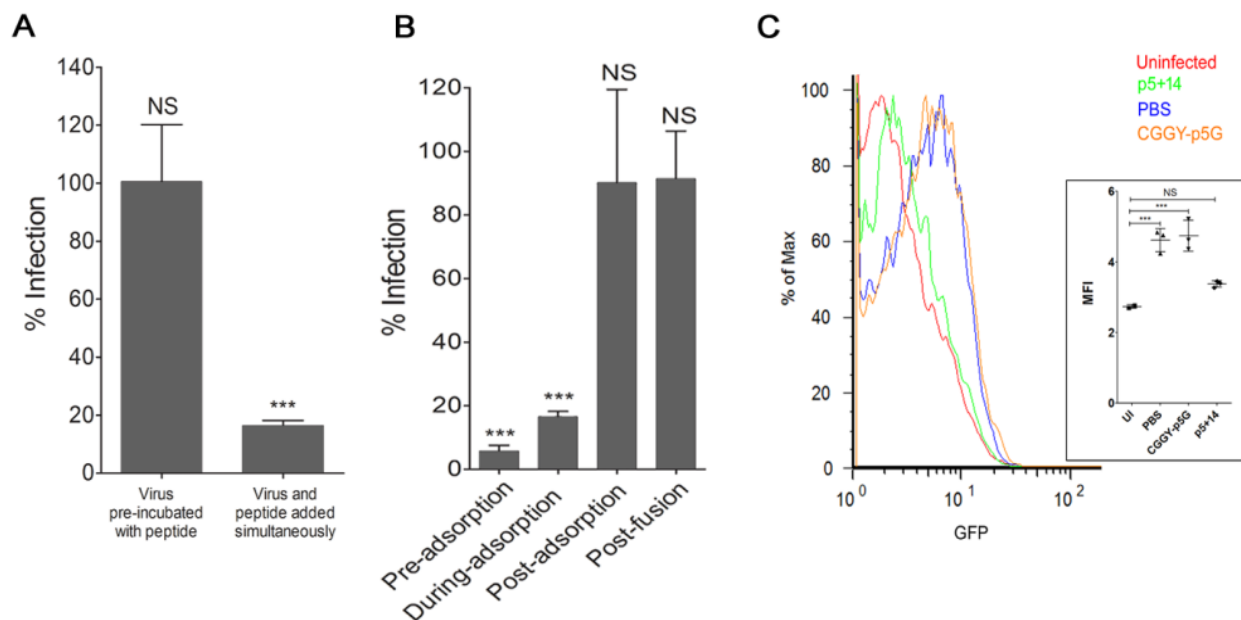


Figure 3.5 Peptide p5+14 blocks adsorption of MCMV. (A) MCMV was pre-incubated with p5+14 peptide (100 μ M) before diluting the virus/peptide to an ineffective peptide concentration (1 μ M) and assayed in the plaque reduction assay as described in materials and methods. As a control, virus and peptide (100 μ M) were added to cells simultaneously. (B) Cells were incubated with p5+14 peptide either prior to virus adsorption, during virus adsorption, after virus adsorption (at 4°C) but prior to fusion, or after fusion (at 37°C). Plaque reduction was measured in a plaque reduction assay as described in materials and methods. Bars represent the average of the percent reduction in infection compared to PBS-treated control from three independent experiments with at least three replicates in each + SD. Statistical significance is indicated as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, NS = non-significant difference in the reduction of infection compared to PBS treated control wells. (C) Adsorption of HCMV TB40/E-pp150-GFP (MOI 10) fusion protein expressing HCMV was measured via flow cytometry in the presence of p5+14 (green), control peptide CGGY-p5G (orange) and PBS (blue). Red line represents uninfected cells. Inset is a scatter plot of the mean fluorescence intensity (MFI) for GFP with the line representing average of 3 replicates +/- SD for the different treatments.

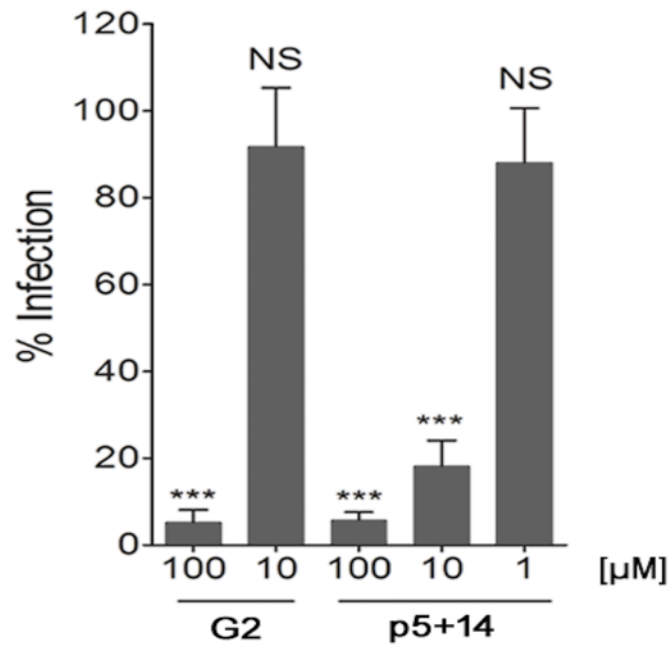


Figure 3.6 Comparison of the efficacy of p5+14 and peptide G2 to reduce MCMV infection in vitro. Peptides G2 and p5+14 were added at different concentrations (100, 10, 1 µM) in a plaque reduction assay as described in materials and methods. Bars represent the average of the percent reduction in infection compared to PBS-treated control from three independent experiments with at least three replicates in each + SD. Statistical significance is indicated as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, NS = non-significant difference in the reduction of infection compared to PBS treated control wells.

p5+14 inhibition of other herpesvirus infection

Because most herpesviruses use HS for their initial attachment to cells and can infect different cell types, we evaluated the efficacy of the peptide to block infection of other human herpesviruses infecting different cells types. Addition of peptide p5+14 at a concentration of 100 μ M 30 min prior HCMV infection resulted in a reduction of ~70% on HFF, ~50% on HAEC, ~90% on ARPE-19 and ~ 60% on MRC-5 cells (Fig. 3.7). An ~80% reduction in infection was observed with herpes simplex virus (HSV) 1. However, reduction of HSV 2 infection was less remarkable (~40% reduction).

5. Discussion

Cytomegalovirus infection is a significant clinical problem in infants and immunodeficient populations. There are two major problems with current anti-CMV treatments. First, current anti-CMV therapies have significant organ toxicity. Secondly, resistance to current therapies is increasing. In this study we examined a panel of synthetic peptides that bind hypersulfated GAGs for their ability to inhibit herpesvirus infection, using MCMV as a model system. Of the seven peptides evaluated in this study, peptide p5+14 demonstrated effective inhibition of MCMV infection and reduced infection of both HCMV and HSV (HSV-1 and 2) *in vitro* (Figs. 3.1A and 3.7). This suggests a broader applicability of GAG-binding synthetic peptides for inhibiting virus-cell interactions. We established that the peptide effectively competed for adsorption of CMV to susceptible cells, thereby reducing infection. We also demonstrated that the peptide does not have a direct neutralizing effect on the virus itself.

The p5-related peptides are synthetic polybasic reagents with a predicted α -helical secondary structure. The heptad amino acid repeat -KAQKAQA- positions the Lys residues along one face of the helix. This structural feature was engineered and intended to facilitate an interaction with linear sulfated GAG molecules, notably heparin (678, 681). Due to their ability to preferentially bind hypersulfated GAGs these peptides have been used to effectively target and image tissue amyloid deposits (666, 668), which contain hypersulfated HS and possibly CS proteoglycans (682). Remarkably, when radiolabeled the p5 and p5+14 peptides were injected in disease-free mice, peptide did not bind to GAGs expressed in healthy organs or tissues (666). This lead us to hypothesize that the linear positive charge on peptide p5+14 facilitates binding to negatively charged PGs on the cell surface, which mediates antiviral activity. This is supported

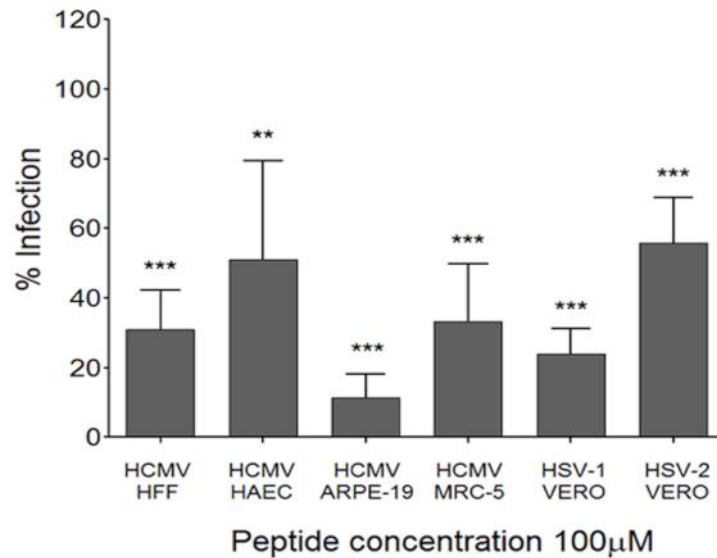


Figure 3.7 Peptide p5+14 inhibits HCMV and HSV infections in vitro. Peptide p5+14 (100 μ M) was added in a plaque reduction assay as described in materials and methods using HCMV (TB40/E) on different cell types (HFF, HAEC, and ARPE-19) and HSV-1 or HSV-2 on VERO cells. Bars represent the average of the percent reduction in infection compared to PBS-treated control from three independent experiments with at least three replicates in each + SD. Statistical significance is indicated as: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

by the fact that peptides with the same net positive charge exhibit differential anti-viral effects that are consistent with the peptide affinity for the GAGs and subsequently amyloid. Thus, peptide p5R (+8 charge), which has a higher affinity for heparin (678) and amyloid (683) as compared to peptide p5 (+8 charge), blocks viral infection 2-fold better (Fig. 3.1A). These data suggest that the secondary structure of the peptides, as well as the overall net charge, affects binding to specific GAGs on the cell surface and their subsequent anti-viral activity. Based on the known restricted reactivity of peptides p5 (666) and p5+14 *in vivo*, our data using p5+14 suggests that CMV may preferentially bind hypersulfated GAGs, such as 6-O-sulfated GAGs (679) on the cell surface of cultured fibroblasts. This may differ from the ubiquitously expressed GAGs found in tissue HSPG and CSPG proteins *in vivo*. This is similar to the proposed mechanism for HSV that uses multiple different interactions for entry including 3-O sulfated GAGs, which differ between cells grown *in vitro* and *in vivo* (684).

An alternative mode of action for these peptides may involve internalization of the peptide along with the GAG ligands that the virus uses for entry. For example, peptides that are rich in Arg or Lys are known to bind HS on the cell surface resulting in internalization of the peptide/HS complex (685, 686). Because of this mechanism, these peptides are being considered for drug delivery or diagnostic/therapeutic nanoparticles (687-689). It remains to be evaluated whether peptide p5+14 binding to the HSPG ligands results in internalization of the peptide-ligand complex, resulting in less HS for virus to bind and enter. These studies are underway. If this is the case, it would provide an alternative explanation for HS binding peptides' inhibition of CMV infection and suggest that p5+14 could also be used as a reagent for delivery of intracellularly active payloads.

Tiwari *et al.* (680) and Borst *et al.*'s (679) recent work identified HS-reactive anti-viral peptides G2 and CYVIP from a phage library screen and human hemofiltrate, respectively. In concordance with our findings, the positive charge of these peptides was critical for their anti-viral activity. Notably, our peptide p5+14 was more effective at lower concentrations in inhibiting MCMV infection of mouse fibroblasts *in vitro* when compared with peptide G2 (Fig. 3.6). Although these peptides have similar modes of action, there are significant differences in their size and charge distribution. The length and spatial arrangement of charged amino acids affect binding to heparin (678), HS-laden amyloid (690), and cell surface HS (685). Although we used the L form of the peptide G2 in the current study, recently the D form of the G2 peptide

was shown to be 4 times as efficacious as the L form *in vitro* (691). This form has the additional advantage of being proteolytically stable. Thus the authors propose that D form could be important for *in vivo* treatments because it would be more stable in serum. A systematic evaluation of the physical, electrochemical, and structural characteristics that contribute to anti-viral activity of all these peptides will aid in the design of next generation antivirals.

In this study we show that peptide p5+14 exhibited significant anti-viral activity against HCMV, HSV-1 and 2 (Fig. 3.7). It is interesting that the antiviral effects were more robust on the HSV-1 than on HSV-2. Even though we propose a similar mode of action against each virus (*i.e.*, blocking of viral adsorption to cell surface HS) the difference in peptide p5+14 efficacies is intriguing. Differences in the viral gB glycoproteins could lead to preferential use of specific GAGs to adsorb to the cell surface (692) that lead to differences in the efficacy of the peptide against the two HSV serotypes. Indeed, the fine structure and distribution of HS GAGs can be different on different cell types. This can explain differences in the efficiency of peptide blockade on different strains and cell types (693, 694). It is possible, indeed likely, that the p5+14 peptide and similar reagents exhibit preferential binding to GAGs that could lead to differences in cell-surface binding and antiviral efficacy. Notably, circular dichroism measurements showed that peptide p5 preferentially binds heparin and adopts an α -helical configuration compared to HS, CS, dermatan sulfate, and hyaluronic acid (681).

Using SPECT imaging and micro-autoradiography, we have previously shown that the “ligand” bound by peptides p5 (666) and p5+14 (unpublished data) has a restricted distribution *in vivo*. The peptides do not bind cellular GAGs or those in the extra-cellular matrix of healthy tissues (666). This observation, taken together with the fact that these peptides compete with herpesviruses for binding to cells in culture suggests that viruses may preferentially bind to a subset of HS *in vivo* that is characterized by a high sulfation pattern, (*i.e.*, electrochemically more reminiscent of heparin). This pattern has been observed with HSV (663, 692). This remains to be established *in vivo*.

CMV and other herpes viruses establish latency within the host, which is dependent upon virus entry and infection of host cells. Preventing viral entry using competitive peptides could potentially reduce the ability of virus to establish latency. Even though HS on the cell surface is an attractive target for developing antivirals, reports targeting this pathway during viral infections *in vivo* are scarce (680). This is likely due to the fact that HS is ubiquitous and

involved in numerous critical cell-signaling pathways. Thus, peptides such as p5+14 that specifically targeted heparin-like HS may provide selective viral competition *in vivo* without detrimentally affecting biological processes through a more common HS.

6. Acknowledgements

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CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The Human Cytomegalovirus (HCMV) genome carries several genes that have immune modulatory properties (138, 411). An example is the *UL146* gene which encodes for a functional viral CXC chemokine (vCXCL-1), which exhibits significant variability between the clinical isolates and has been demonstrated to be associated with clinical outcome of HCMV infection (405, 416). *Gerna et. al* showed viral transmission from HCMV infected endothelial cells to peripheral blood neutrophils (PBNs) *in vitro* after as little as 1 hour of co-culture (560, 695). Even though HCMV infection of PBN is abortive, vCXCL-1's recruitment of PBN could be beneficial for HCMV, as it may use them as a means of transportation (695). HCMV transmission requires contact between the endothelial cells and PBNs, during which transitory microfusion events allow the PBNs to pick up the virus and disseminate HCMV through the bloodstream (695). This study characterized the vCXCL-1 protein from different clinical isolates *in vitro* (chapter 1) and evaluated the contribution of vCXCL-1 in the pathogenesis of CMV infection *in vivo* (chapter 2). In addition, we also tested the efficacy of heparan sulfate binding peptides as potential antivirals (chapter 3).

Because the hypervariability observed in the *UL146* gene from the different clinical isolates occurs within receptor binding motifs, such as ELR and N-loop (416), we hypothesized that, **variability in vCXCL-1 leads to differential activation of neutrophils, which leads to the observed difference in HCMV pathogenesis.** This hypothesis was addressed in chapter 1 by synthesizing vCXCL-1 proteins using a baculovirus protein expression system. All the vCXCL-1s bound to CXCR2 with different binding affinities, whereas only three of them bound to CXCR1. All vCXCL-1s were capable of inducing intracellular calcium mobilization in human peripheral blood neutrophils (PBNs). The chemotaxis of PBNs was dependent on the affinity of vCXCL-1 for CXCR2, and vCXCL-1s also differentially upregulate CD11b and CD11c on the surface of PBNs. β 2-integrins are important in the adhesion and extravasation of PBNs across the endothelium and this might lead to difference in the infectivity of these cell types during CMV infection. In addition the induction of secondary chemokine CCL22 (macrophage-derived chemokine (MDC)), which can attract other leukocytes including monocytes, dendritic cells, and

the Th2 cells (552) was also dependent on the affinity of the CXCL-1 for CXCR-2. Monocytes and dendritic cells support productive infection of the virus upon activation (430, 561, 696) and a Th2 responses also can help HCMV pathogenesis by diminishing CTL responses (553, 554). Taken together, in this study we identified the similarities and differences in the functional activity of vCXCL-1 from different HCMV isolates and suggest how the variability can affect neutrophil function and CMV pathogenesis.

In chapter 2 we evaluated of the role of vCXCL-1 *in vivo* using the murine model of CMV infection. We generated recombinant murine CMV (MCMV) expressing the vCXCL-1_{CCMV} from chimpanzee CMV. We tested the hypothesis that **vCXCL-1_{CCMV} (vCXCL1_{CCMV}) is a functional CXC chemokine that contributes to viral dissemination**, similar to MCMV CC chemokine. The primary dissemination of recombinant MCMV expressing vCXCL-1_{CCMV}, and mCXCL-1_{KC} was similar to RM4511 (parental MCMV not expressing any chemokine). However, neither of the recombinants was recovered from the SG at any time point. The absence of the recombinants from the SG of SCID mice suggested that the virus was not cleared in the organ by the adaptive immune system. Neutrophil depletion in mice was unable to rescue the dissemination of the recombinants to the SG. This suggested that neutrophils too are not involved in blocking the dissemination of the recombinants to the SG. The dissemination of the recombinants to the SG was restored upon cyclophosphamide induced immuneablation and in NSG mice, which lack T, B lymphocytes and NK cells. These results suggest that the over expression of the chemokine induced cells of the innate immune system which curtail the dissemination of the recombinants to the SG. We also observed restoration of the dissemination of the recombinants to SG in an immune competent co-infection system (RM4511 + recombinant). The two viruses co-infecting the cell at the site of infection is critical for restoration of the dissemination to the SG. In an *in vitro* system of co-infection we observed reduced the chemokine levels, without affecting the virus titer compared to single infection. The co-infection also reduced the number of NK cells and inflammatory monocytes at the site of infection, compared to a recombinant alone infection. Therefore, from this study we concluded that vCXCL-1 is a functional chemokine *in vivo*. However, overexpression of the chemokine is detrimental to the dissemination of MCMV because recruitment of more inflammatory

monocytes and NK cells to the site of infection. In case of co-infection, there occurs a resource competition that reduces the chemokine levels and allows SG dissemination.

Like other herpes viruses, once infected with CMV, the host is infected for life. Latent virus reactivates and can cause serious complications in immune-compromised individuals. Antivirals are the only recourse in this situation, but their use is limited due to toxic side effects. Moreover HCMV strains are becoming resistant with prolonged treatment with these drugs (697-700). CMV binds to negatively charged heparan sulfate on the cell surface during the attachment step of viral entry into the cell, making it a potential target for anti-CMV drug development (654, 701). We targeted this step of the CMV infection lifecycle to develop novel therapeutics. These drugs would attack a different step in the viral life cycle than the currently used drugs. In chapter 3 we tested the hypothesis that **heparan sulfate binding peptides will block CMV infection of the cells**, by preventing the attachment and subsequent entry of the virus into the cells. Peptides of different lengths and net charge were generated. Of them, the cationic peptides reduced MCMV infection *in vitro* by ~ 90%. The peptides bound to the cells in a charge dependent manner. Pre-incubation of the peptides with soluble heparin reduced the activity of the peptide and viral infectivity of the cells suggesting that the peptide and the virus interact with a negatively charged moiety on the cell surface. Treating the cells with GAG cleaving enzymes, demonstrated the peptide preferably binds HS and mediates reduction in infection via blocking the attachment of virus to the cells, which is HS dependent. The peptide did not neutralize the virus itself and was more efficacious than similar antiviral peptide G2 (680). Finally the peptide was shown to be effective against HCMV and herpes simplex 1 and 2 virus on a wide variety of cells. Thus we characterized a novel antiviral peptide, which is effective against herpesviruses that use HS to attach to cells and has a mechanism of action different than the existing antiviral drugs. This peptide could therefore be used to treat HCMV disease in the conjunction with current therapies and in the treatment of drug resistant strains of HCMV.

Future Directions

Chapter 1:

In this chapter we characterized some of the functional differences in the vCXCL-1 chemokines from different clinical isolates. Future work along the following lines will help to better understand vCXCL-1 and 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 (702). Therefore, investigating the signal transduction pathways 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 difference in the leukocyte response (703). Therefore it would be interesting to investigate the contribution of the each of these receptors individually in functional assays (calcium flux, chemotaxis, adhesion molecule upregulation, CCL22 induction etc.). This can be done by using receptor blocking antibodies while performing these assays.
3. In our previous studies we had observed significant variability in and around the ELR motif and the N-loop regions of the vCXCL1s (416). Both of these regions have been shown to be involved in the chemokine-receptor interactions (259, 265, 270, 337, 369, 373, 374). Therefore, we should perform domain swap studies to understand the key elements in vCXCL1s that are responsible for the functional differences of the vCXCL1 from the different isolates.
4. Variability in the sequence of protein isoforms could translate into structural differences, which could trigger different downstream signaling cascades from the receptor (457, 485) or ligand binding affinities e.g. for Hemoglobin isoforms. Therefore, it would be worthwhile to carry out structure homology modelling of the vCXCL1s from different isolates, using other previously crystalized chemokines and receptors as templates. These can then be used to carry out molecular docking (protein-protein) studies to visualize how the different vCXCL1s interact with the chemokine receptors. This might help us

understand why and how these different vCXCL1s elicit different downstream signaling responses.

5. Previous studies have shown that treating fibroblasts with CXCL8 (IL-8) leads to increased viral replication (704, 705). It would therefore 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.
6. We have shown that the viral chemokines upregulate the expression of adhesion molecules on neutrophils and propose that this aids in virus transfer to the neutrophils. However, it is also possible that vCXCL1s upregulate adhesion molecules (e.g. selectins, and integrins) on endothelial cells. This too might allow for better interaction of leukocytes with the infected endothelial cells also allowing for better transfer of the virus to the leukocytes from the infected cells.
7. Neutrophils can transmigrate through a monolayer of endothelial cells and pick up virus from the infected cells while doing so (560). It will be valuable in understanding how the variability in the vCXCL1s effects the transmigration and the ability of neutrophils to pick up the virus from infected endothelial cells by carrying out endothelial transmigration assays in the presence of vCXCL1.
8. As an alternate to the experiment mentioned above, we could also perform co-culture experiments with infected endothelial cells and neutrophils (560, 695). During these experiments we can treat the cell types in isolation or together with the different viral chemokines and evaluate the transfer to virus to the neutrophils from the infected endothelial cells.

Chapter 2:

In this chapter we evaluated the *in vivo* functionality of vCXCL1 from CCMV (vCXCL-1CCMV). 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 (416). These recombinants could be used to investigate the role viral CXCL1 from

HCMV in CMV pathogenesis and dissemination. In addition, this model will also allow us to investigate how the variability in the sequence of the chemokine affects CMV dissemination and pathogenesis *in vivo*.

2. The vCXCL1 gene is expressed with late expression kinetics (407, 417). It has been shown that the amount and timing of the chemokine can lead to an aberrant immune response (706, 707). Our model may not be mimicking the real life scenario, as the recombinants overexpress the chemokine constitutively. Therefore, 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 for chemokine expression and usage *in vivo*.
3. Viral titers and persistence, both of which are controlled by the immune system, determine the ability of MCMV to establish latency and subsequent reactivation at the site of infection (430). Because the recombinant viruses alter the immune response, it will also be important to assess how the expression of vCXCL1 affects the establishment of latency and the ability of MCMV to reactivate.
4. The BALB/c mice are susceptible to MCMV infection because they cannot induce an effective NK response (622). From our experiments it seems that when infected with the recombinants, they recruiting more NK cells to the site of infection. Using the NSG mouse model we have shown that NK cells mediate the dissemination blockade of the recombinants to the salivary gland (SG). However there are several other components of the immune system are defective/lacking in this mouse model including dendritic cells, macrophages, T and B lymphocytes etc. (600, 601). Therefore, I would also suggest carrying out *in vivo* dissemination experiments in WT BALB/c mice following NK cell depletion (708, 709) to demonstrate the role of these cells types in an otherwise immune sufficient mouse.
5. It is also possible that the overproduction of the chemokine leads to chemokine receptor desensitization (706, 707, 710-712). This may lead to poor recruitment to the site of infection or impaired re-circulation of cells, which might contribute to the blockade of dissemination of the recombinant to the SG. To test this possibility we should sort the cells from the sites of infection from mice infected with either the parental or the

recombinants. These isolated cells would then be tested in *in vitro* functional assays for responsiveness to chemokine stimuli.

6. Infection of the host with multiple strains of HCMV is a very common phenomenon (628-636), which happens even in the face of pre-existing immunity to CMV (519). The co-infection model can be used to investigate the host pathogen-pathogen interaction further. For example we have shown that recombinants generate an abnormal immune response, and the co-infection skews the immune response to favor the dissemination of the recombinant. In the future we can carry out experiments with other combinations of recombinants and parental virus to evaluate how mixed infection or sequential infection affects the development of T and B cell responses to CMV infection.

Chapter 3:

In this chapter we tested heparan sulfate (HS) binding peptides as potential anti virals. 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 Jon Wall). It is necessary to define the HS subtype being targeted by the peptide is the same one that is also 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 by using HS arrays available at the University of Georgia, Complex Carbohydrate Research Center. This data will help in revealing the specificity of the peptides for HS subtypes, which will help us to improve the peptide even further and to avoid 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*.
2. In the future, we should also carry out a systematic analysis to identify the sequence and structural features of the peptide that contribute to its function. This will help in the development and optimization of the peptides. For example, from our experiments it seems that peptides with Arg function better than peptides with Lys, even if they have the same charge. Future experiments to test the validity of this observation can be carried out

by using Arg and Lys containing peptides of different lengths. In addition, we show that the greater the positive charge on a peptide the better it functions. Experiments could also be performed to identify the optimum charge:length ratio that would offer the best protection against infection. Structural features of the peptide may also contribute to the function of the peptides. The peptide we tested forms an α -helix. However some other peptides that we tested initially and the ones published elsewhere do not. Because the structure might contribute to the turnover and ability of the peptide to bind to the cell surface, it would be interesting to investigate the relative contribution of the secondary structure of the peptide to the overall function of the peptide.

3. Cationic peptides have the tendency to be internalized after being bound to cell surface HS. Therefore, they have been used to transport small molecules into the cells (687). It has also been shown in other studies that the internalized peptide can interfere with viral replication (713-715). It would therefore be interesting to test if our peptides also get internalized. In addition to being a new use for these peptides (i.e., small molecule transporter), if the peptides interfere with virus replication after entering the cell, it will increase the usability of these peptides to treat other viral infections too.
4. Although we did not observe any cytotoxicity for the peptide *in vitro*, in order to develop the peptides for *in vivo* use, we need also need to perform toxicity assays in animals. In addition we also need to address the issue of bioavailability and stability of the peptides *in vivo*. Peptides with modifications to increase their stability and bioavailability e.g. acylation and PEGylation (716) need to be tested *in vitro* before proceeding to *in vivo* testing. One of the factors that might reduce the stability of the peptide is the proteolytic environment *in vivo*. To address this issue we have developed proteolytically stable peptides. These peptides are made up of D amino acids rather than L amino acids. The use of the D amino acids makes these peptides resistant to proteolytic cleavage (manuscript in preparation) (691). In addition, it has also been proposed that this lack of cellular proteolytic processing of peptides with D amino acids, they are less likely to induce an immune response (717-719).
5. Because CMV can infect different cell types in various organs, for a peptide to be used successfully as a therapeutic *in vivo*, we need to know the bio distribution of the peptide. This can be done using radiolabeled peptide with SPECT/CT scans in Dr. Wall's lab. At

the same time we can also assess the bioavailability of the peptide in different organs and tissue.

6. Finally after carefully evaluating the peptides for toxicity, stability, and distribution we should proceed to *in vivo* experiments. We need to evaluate the utility of peptides for *in vivo* use by comparing the route of administration (i.e., intravenous, local, oral etc.). We can also test the efficacy of the peptide in a mouse model of re-activation vs. primary infection. These experiments will help us to determine the best route where these peptides will be most effective.
7. We show that the peptide functions by blocking viral attachment to the cells. This mechanism is different from the currently used nucleoside analog antivirals (e.g. ganciclovir, valganciclovir, foscarnet etc., that inhibit viral DNA polymerase (521-523)). This suggests the interesting possibility that the peptides could still function against drug resistant HCMV strains, which should be tested in future experiments. In addition, the currently used drugs are also associated with toxic side effects (521-523). Therefore, we should also experiment with treating cells with peptide and the anti-CMV drugs simultaneously with the goal of reducing the IC_{50} of the anti-CMV drug being tested. A reduction in the IC_{50} of drug could translate into less toxicity when used *in vivo*.
8. We show that the peptides do not target the virus directly and hypothesize that there is lesser possibility that the virus develops resistance against these peptides. To test the robustness of these peptides as antivirals, 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 several times with increasing concentration of the peptides. The escape variants could then be sequenced to examine what changes lead to their resistance to the peptide. If our observation is true that the peptide does not exert any direct selective pressure on the virus, the virus should not be able to develop resistance to the peptide by switching to use a different HS subtype during infection.
9. In other experiments we observed that SG isolated MCMV was less susceptible to peptide mediated reduction of infection compared to the tissue culture grown virus. This susceptibility however could be restored upon one round of passaging the virus in fibroblasts *in vitro*. This observation in addition to the observations that i) SG isolated

virus is less capable of infecting macrophages (430, 625) and ii) HCMV shed in the urine is not neutralized by anti CMV antibodies and susceptibility could be restored by one round or passaging the virus in fibroblasts (720) suggest that virus grown *in vivo* is different in its attachment and/or entry process. This might be because the cell type from which the virus is derived affects the subsequent infectivity of CMV (721, 722). The peptides could be used to elucidate the mechanistic difference in the infection process between the viruses from the two sources. This in turn can lead to the identification of targets/features that can be exploited to develop newer therapeutics in future.

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APPENDICES

APPENDIX 1: MATHEMATICAL MODELLING OF MCMV DISSEMINATION *IN VIVO*

Abstract

Human cytomegalovirus (CMV) infects with seroprevalence reaching 100% in some countries. It causes life threatening disease in immune compromised patients and congenital defects if infection occurs *in utero*. Mouse CMV (MCMV) is used as model to understand the spread of virus in the host. Following foot pad infection of mice with MCMV, the viral load is followed over time in organs including foot pad (FP), spleen (SP), popliteal lymph nodes (LN), and salivary glands (SG). Our experiments show a decrease of virus in the FP, accumulation then decline of the virus in LN and SP, and delayed appearance of virus in SG. In the presented here, we developed mathematical models to understand viral dynamics during the first 2 weeks of MCMV infection of mice. From our final model, we conclude that, the virus diffuses out of the FP without any growth at site of inoculation, both the LN and the SP contribute equally to the seeding of virus to the SG and the net growth rate for virus was positive only in the SG as in other organs the death rate (natural/immune system mediated) cancelled out the growth rate. However, data from recent experiments challenges this model and necessitates a reevaluation of our model.

Introduction

Human Cytomegalovirus (HCMV) infects people throughout the world with adult seroprevalence approaching 100% in many countries (723). It causes severe disease in immunocompromised patients including individuals with AIDS, organ transplant patients, cancer (30) and newborns (31). Infection in these patients can lead to clinical disease including mononucleosis-like syndrome, interstitial pneumonia, gastroenteritis, retinitis, or transplant rejection. HCMV is the leading viral cause of congenital birth defects following infection *in utero*. Worldwide between 0.5 to 2% of newborns are infected. The majority of newborns are asymptomatic at birth but some exhibit outward signs of infection including microcephaly, jaundice, and hepatosplenomegaly (35, 36). About 10% of the asymptomatic newborns develop neurological dysfunction, most prominently sensorineural hearing loss (SNHL) (20, 37, 38). In

immunocompetent individuals HCMV infection is generally asymptomatic. However, clinical studies implicate HCMV's contribution to cardiovascular (39-42) and inflammatory bowel diseases (43-45). HCMV pathogenesis is diverse but with an inflammatory component to each.

In order to develop a CMV vaccine or effective treatments, an understanding of CMV pathogenesis and viral dissemination are required. Due to the strict species specificity of HCMV, murine CMV (MCMV) is the preferred model to study CMV infection. The dissemination of MCMV within the mouse is cell associated. The cells of hematopoietic origin including monocytes, neutrophils, and late monocyte progenitors have been implicated in this process (141, 429, 430, 434). However, the exact role that these different cell types play in viral dissemination is not clearly defined. It has been shown that the MCMV CC chemokine, MCK2 plays a role in the dissemination of the virus *in vivo* and also contributes to virus-cell tropism (139-141, 429, 435). In the footpad model of MCMV infection, the virus disseminates within the host with biphasic kinetics. In the first phase (primary dissemination) the virus disseminates from the site of infection, the foot pad (FP) to the popliteal lymph node (LN), spleen (SP), lung (LU) and liver (days 3-5). During the second phase (secondary dissemination) the virus can be isolated in the salivary gland (SG) (days 7-18). MCMV spreads in the mouse population by saliva transferred to an uninfected mouse during biting. Hence the ability of the virus to disseminate to the salivary gland is used as readout for the characterization and successful dissemination of MCMV *in vivo*.

Mathematical modelling studies to understand the aspects of pathogen dissemination and or colonization *in vivo* e.g. for HIV, salmonella etc., have helped in defining several pathogen associated attributes such as preferred route of entry, anatomical localization, replication, migration, and death rates *in vivo* (724-726). We do not full understand the contribution of the intermediate organs, cell types, chemokines etc. in the dissemination of MCMV to the SG. Recent studies looking at the dissemination of MCMV *in vivo* have identified the cell types infected and viral reservoirs *in vivo* (141, 429, 430, 599, 727); however, currently there is no quantitative model for the *in vivo* dissemination of MCMV. A well-defined model of MCMV dissemination *in vivo* could help identify key events during MCMV infection life cycle that would be more suitable for therapeutic intervention e.g. replication or migration. In this study we developed a mathematical model to determine the rate of spread of MCMV after foot pad infection of mice. From our study we concluded that the virus diffuses out of the FP and does not

undergo any replication there. However viral replication was required in the SG to explain the high titers observed. Our model also suggests that both the SP and the LN might contribute equally to seeding the SG with disseminated MCMV.

Materials and methods

Cells and Viruses

MEF 10.1 cells (ATCC) were propagated in DMEM supplemented with FCIII to a final concentration of 10%, P/S and L-Gln to a final concentration of 1%. MCMV RM4511 strain was used to infect mice. This virus 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 (593). This was obtained from Dr. Ed Mocarski, Emory University.

Mice

hCXCR2 transgenic BALB/c mice expressing hCXCR2 under the control of the neutrophil-specific, human myeloid related protein-8 promoter were used for the experiments. For infection the mice were infected with 1×10^6 PFU of RM4511 in the foot pad. All mice were housed under specific pathogen free conditions in WLS LAF.

Plaque formation assay

Viral titers in the organs were determined by plaque formation assay as per lab protocol on mouse embryonic fibroblast (MEF) 10.1 cells. Briefly MEF 10.1 cells were plated in a 6 well dish. Organs were harvested at selected time points post infection (p.i) and homogenized. The homogenate was diluted and added the MEF 10.1 cells and incubated for 1 hr. After incubation the diluted virus was removed from the plates and cells were overlaid with carboxy methyl cellulose media and incubated for 7 days. At the end of the incubation period, CMC was removed, plates were stained and plaques counted using a dissection microscope.

Mathematical model and statistical analysis

Wolfram Mathematica, the mathematical computational software was used to generate the models and to carry out statistical analysis. Bootstrap was done to determine the bias of the parameters and the correlation matrix for the parameters. Confidence interval estimates were obtained from 1000 resampling events during bootstrapping. The quality of the fits was assessed with a χ^2 goodness of fit test.

Results and discussion

Dissemination of RM4511 *in vivo*

The dissemination of RM4511 *in vivo* following a foot pad infection was determined by measuring viral load in the FP, LN, SP and the SG on days 3, 5, 7, and 14 post infections by performing plaque formation assay as described in materials and methods (Figure A1.1). For our analysis we used total virus as it provides a better estimate of the viral load in each organ. Virus per gram of tissue on the other hand gives ambiguous results for smaller organs like LN which are very small in size and can have higher values.

Mathematical modelling for of spread of MCMV after foot pad infection

Based on the experimental data for MCMV dissemination after foot pad infection (Figure A1.1), we developed a simple model, defined by a set of ordinary differential equations (ODEs) that describe the replication, clearance and migration of the virus from the FP to the LN, SP and subsequently to the SG. The initial “full model” assumes that the virus replicates in all the organs and there is viral death in all the organs. It also assumes that MCMV dissemination *in vivo* is unidirectional (Figure A1.2). Subsequently, we reduced the number of parameters in the full model to generate sub models and fitted the curves to the data (Figure A1.3). From this analysis we identified that any model that does not consider viral replication in the SG could not explain the observed data sub model 1.1 and figure A1.3 B. Following this reductionist approach, we reached the final “minimal model” defined by the sub models 1.4a and 1.4b, figure A1.3C and graphically depicted in figure A1.4 A and B. The parameter values for these models were estimated by fitting the models to the *in vivo* dissemination data, and the confidence interval determined by bootstrapping with 1000 resampling events (Table A1.1).

In conclusion, the minimal model that could explain the spread kinetics suggests that (i) the virus migrates from FP to LN and SP without death or replication in the FP (ii) the virus half-life $T_{1/2} = 23\text{hrs}$ in the LN and $T_{1/2} = 2.3\text{hrs}$ in SP suggesting an immune system mediated clearance of virus in these organs (iii) the virus migrates to SG from either LN or SP; however a definite answer cannot be provided with available data (iv) they delayed appearance of virus in SG is due to extremely low migration rates of virus into SG, and (v) increased viral titer in SG is due to viral replication at the rate of 0.9/day, which is very close to that predicted for MCMV .

Alternate models were also tested that could explain the MCMV spread kinetics. These include models where it is assumed that (i) multiple organs serve as reservoirs for virus before it reaches SG, (ii) there time dependent migration of virus from LN to SG or S to SG: using a piece

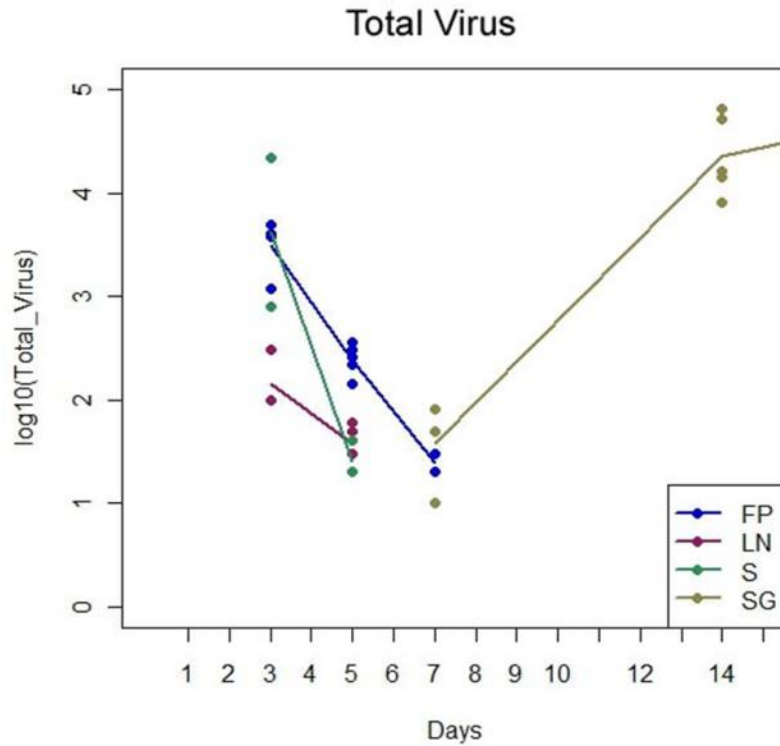
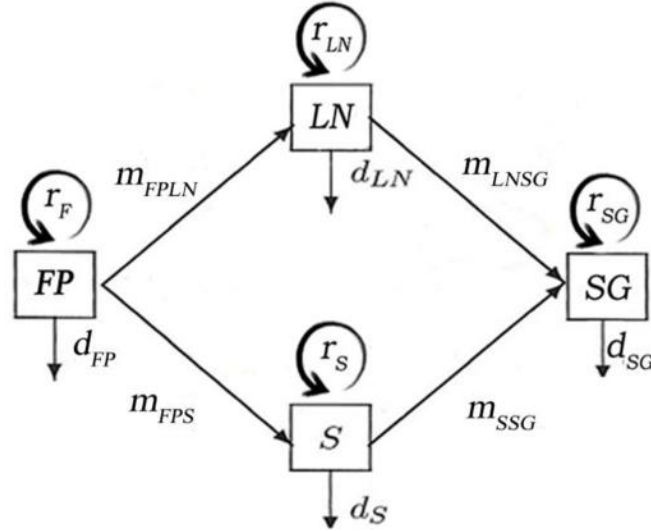


Figure A1.1 Dissemination of RM4511 in mouse following foot pad infection. Mice were infected in the foot pad with RM4511 as described in materials. Organs were harvested at days 3, 5, 7 and 14 p.i, and viral titer in each organ was determined by plaque formation assay. Dots represent viral titer for individual mouse, and the lines connect the average values. FP = Foot pad, LN = Popliteal lymph node, S = Spleen and SG = Salivary gland.

Full Model



$$\begin{aligned} \frac{dv_{FP}(t)}{dt} &= -(m_{FPLN} + m_{FPS} + d_{FP})V_{FP}(t) + r_{FP}V_{FP}(t) \\ \frac{dv_{LN}(t)}{dt} &= m_{FPLN}V_{FP}(t) - (m_{LNSG} + d_{LN})V_{LN}(t) + r_{LN}V_{LN}(t) \\ \frac{dv_S(t)}{dt} &= m_{FPS}V_{FP}(t) - (m_{SSG} + d_S)V_S(t) + r_SV_S(t) \\ \frac{dv_{SG}(t)}{dt} &= r_{SG}V_{SG}(t) + m_{LNSG}V_{LN}(t) + m_{SSG}V_S(t) - d_{SG}V_{SG}(t) \end{aligned}$$

Figure A1.2 The full model. The full model assumes that MCMV replication and death in all organs. It also assumes that the dissemination of MCMV is unidirectional indicated by the direction of the arrows. The following parameters are used to define the model above:
 $V_X(t)$, where X can be Foot Pad (FP), Lymph Node (LN), Spleen (S), Salivary Gland (SG) = Number of viruses in organ X.

m_{XY} , where X,Y could be FP, LN, S, SG = Migration rate from organ X to organ Y.

d_X , where X could be FP, LN, S, SG = Death rate in organ X.

r_X , where X could be FP, LN, S, SG = Replication rate in each organ.

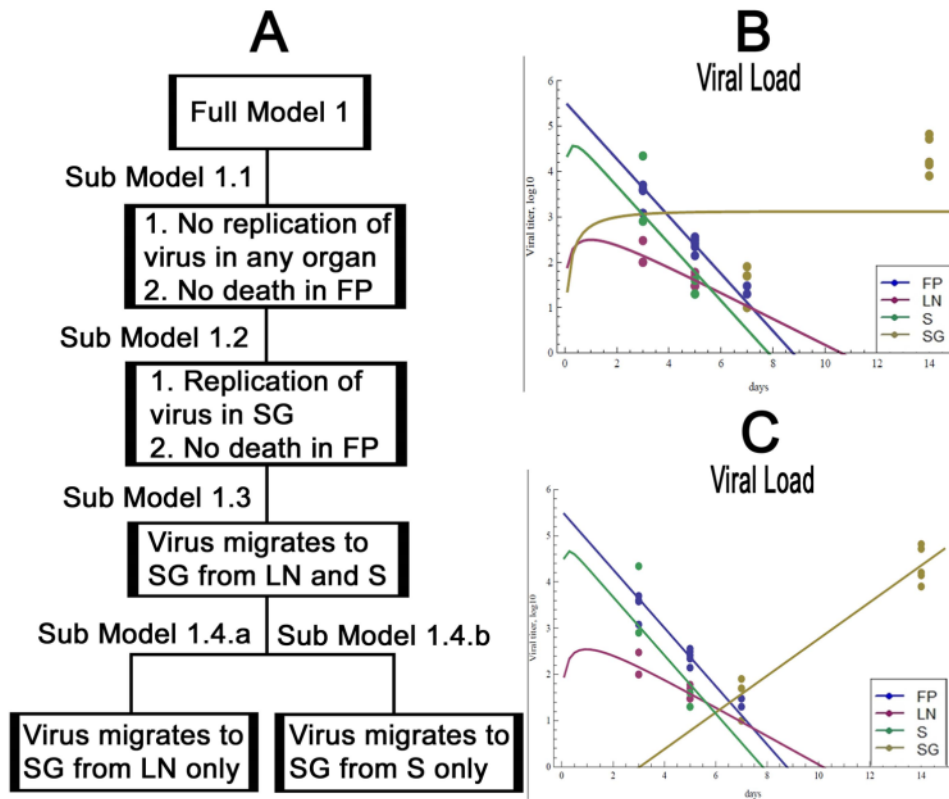


Figure A1.3. Various sub models. (A) Various sub models with salient features of each model (B) Plot for best fit for Sub Model 1.1, dots represent experimental data points (C) Representative plot for best fit for Sub Models 1.3, 1.4.a, 1.4.b, dots represent experimental data points. Sub Model 1.1 was discarded as it could not explain the increase of virus in SG. Sub Models 1.3, 1.4.a, 1.4.b could explain the data equally well.

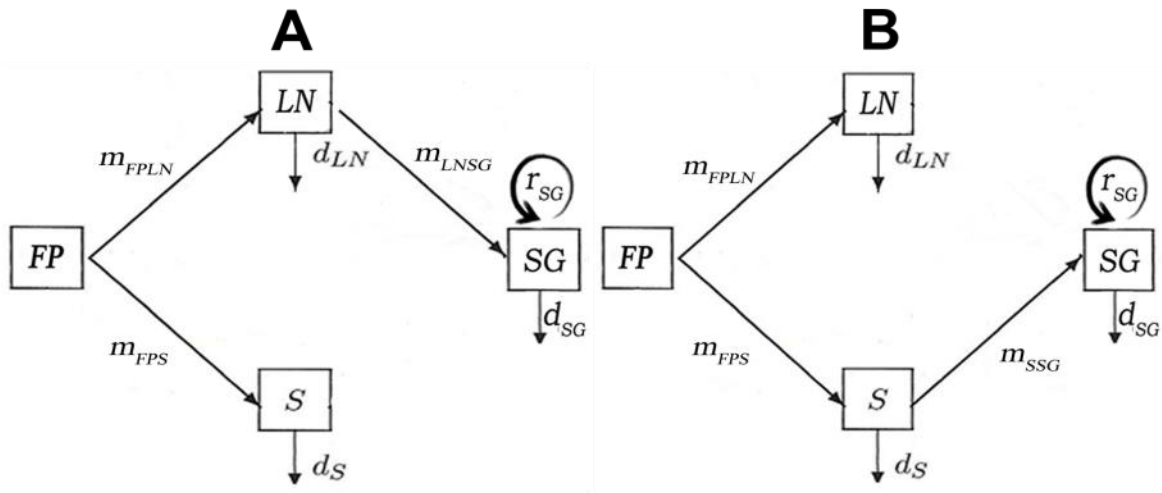


Figure A1.4 The final minimal models. (A) No death in FP, Death in LN and S, replication only in SG and migration of virus from only from LN to SG (B) No death in FP, Death in LN and S, replication only in SG and migration of virus only from S to SG (parameter abbreviations same as those in figure A1.2)

Table A1.1 Parameter values from model fitting and Confidence Interval (CI) from bootstrapping (parameter abbreviations same as in figure A1.2.)

$(r_X - d_X)$: Net viral growth rate in organ (X = FP, LN, S, SG)

| Parameter | Sub Model 1.4.a | | Sub Model 1.4.b | |
|---------------------------------------|----------------------|--|----------------------|--|
| | Fitted Values | CI | Fitted Values | CI |
| $V_{FP}(t)$ pfu | 3.4×10^5 | $(7.0 \times 10^4, 1.5 \times 10^6)$ | 3.4×10^5 | $(7.0 \times 10^4, 1.5 \times 10^6)$ |
| m_{FPLN} day ⁻¹ | 2.9×10^{-3} | $(4.5 \times 10^{-4}, 2.6 \times 10^{-2})$ | 2.9×10^{-3} | $(5.0 \times 10^{-4}, 2.1 \times 10^{-2})$ |
| m_{FPS} day ⁻¹ | 1.45 | (1.1, 1.8) | 1.45 | (1.1, 1.8) |
| m_{LNSG} day ⁻¹ | 2.4×10^{-4} | $(2.0 \times 10^{-5}, 1.3 \times 10^{-3})$ | | |
| m_{SSG} day ⁻¹ | | | 2.4×10^{-6} | $(1.8 \times 10^{-7}, 3.5 \times 10^{-5})$ |
| $(r_S - d_S)$ day ⁻¹ | -7.1 | (-16.7, -2.7) | -7.1 | (-17.5, -2.9) |
| $(r_{LN} - d_{LN})$ day ⁻¹ | -0.7 | (-1.2, -0.3) | -0.7 | (-1.2, -0.4) |
| $(r_{SG} - d_{SG})$ day ⁻¹ | 0.9 | (0.8, 1.0) | 0.9 | (0.8, 1.0) |

wise function to represent $m_{\text{LNSG}}(t)$ and $m_{\text{SSG}}(t)$, and (iii) the viral load dependent migration of virus from LN to SG and S to SG: using piece wise function to represent $m_{\text{LNSG}}(V_{\text{LN}}(t))$ and $m_{\text{SSG}}(V_{\text{S}}(t))$. Further experimentation is required allow for better understanding how MCMV disseminates, to exclude the alternate models.

Future Directions

It has been shown that the early events following viral infection such as, induction of cytokines and chemokines or cell types recruited, are critical in determining the fate and outcome of the infection (728-730). Even for CMV, the cells recruited to the site of infection early on after infection play a role in dissemination (430). Therefore, it would be interesting to expand our existing model to include the early time points after infection. However due to lack of data, we could not model the MCMV spread kinetics at early time points after infection during our initial analysis. To address this issue, we performed experiments measuring the dissemination of MCMV at very early time points (30 min, 60 min, 2hrs, 1 day and 2 day p.i). The results from these experiments make us question some of the conclusions drawn from our previous model. In particular, in these recent experiments we noted that the virus disappears from the foot pad and becomes un-detectable by day 1 p.i, and then re-appears at day 2 p.i. (figure A1.5). This suggests that the virus might be replicating at the site of inoculation in the foot pad. It is still possible that MCMV diffuses out of the foot pad into the LN early on during infection and the virus undergoes the “latent phase” of the infection/replication cycle, and then reappears at day 2 p.i. Therefore, in light of these recent observations, it is required that in future we update our existing model to more accurately represent the *in vivo* growth/dissemination kinetics of MCMV.

Acknowledgements

I would like to thank Dr. Yiding Yang for her assistance in developing the model and carrying out the statistical analysis. I would also like to thank Dr. Mindy Miller-Kittrell for generating the data used for modelling in this study.

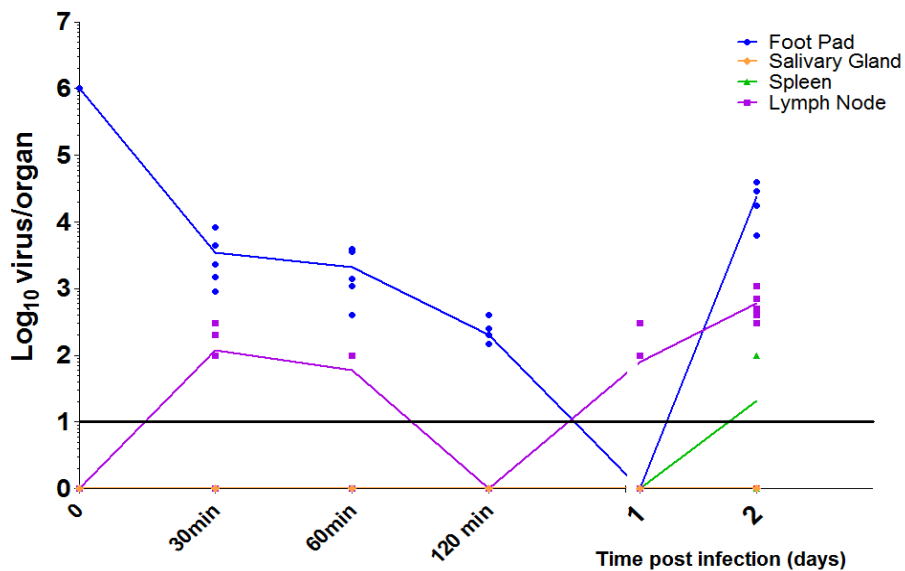


Figure A1.5 Dissemination of RM4511 in mouse at early time points following foot pad infection. Mice were infected in the foot pad with RM4511 as described in materials. Organs were harvested at 30, 60, 120 min p.i, and on days 1 and 2 p.i, and viral titer in each organ was determined by plaque formation assay. Dots represent viral titer for individual mouse, and the lines connect the average values.

APPENDIX 2: THE ROLE OF mir-155 IN CMV PATHOGENESIS

Abstract

Human CMV (HCMV) is widespread β -herpes virus that is prevalent in upwards of 90% of the population in developing countries and 30-60% of women of childbearing age in developed nations. Primary infection in healthy adults is controlled by the cells of the adaptive system after which it establishes latency. Suppression of the immune system can lead to re-activation as seen in transplantation patients. In this study we investigated the role of micro RNA-155 (miR-155) in immune response to MCMV infection. miR-155 knock out (miR-155 KO) mice generate a weaker CD4⁺T cell response as expected. However, there was lower virus titer in the salivary gland (SG) of the knock out mice. Upon further investigation we discovered a higher percentage of macrophages in the SG of miR-155 KO. We also observed higher numbers of IFN- β transcripts and reduced levels of IL-10 transcripts in the SG of miR-155 KO mice. Therefore we suggest a mechanism where an increase in activated macrophages coupled with lower IL-10 levels contribute to the control of MCMV infection in the SG. This highlights a role of macrophages in an organ where control of infection was believed to be exclusively under the control of CD4⁺ T cells.

Introduction

Human Cytomegalovirus (HCMV) is a ubiquitous β -herpesvirus. It is estimated that its prevalence is between 90-100% in developing nations (723). Even in the USA HCMV infects 30-60% of women of child bearing age (731). HCMV is the leading cause of birth defects and congenital infection causes neurodevelopmental defects (38, 574). In immune compromised individuals like AIDS and transplant patients, CMV infection can lead to serious complications, which can be fatal in some cases (574). In healthy adults however, HCMV infection is usually asymptomatic, with mononucleosis-like symptoms (574). After clearance of the primary infection, HCMV establishes lifelong latency in an immune competent individual (574). Cells of both the innate and adaptive immune system contribute to controlling CMV infection. Animal models have contributed significantly to our understanding of the contribution of the immune system components in the control of CMV infection (127-130). Studies using the mouse model of HCMV infection have demonstrated that NK cells, neutrophils, and CD8⁺ T cells play a critical role in the control of infection in the primary organs of dissemination i.e. spleen, lung,

and liver (131-133). However, MCMV growth in the salivary gland is controlled only by CD4+ T cells (134-136). In humans, ~10% of the total CD4+ and CD8+ T cells are HCMV specific and prevent virus reactivation (519). Indeed, in immune suppressed individuals HCMV reactivation is observed (574). Therefore it is important to understand the mechanisms that control the immune response to viral infections.

Micro RNAs (miRNA) are 19-24 nucleotides non-coding RNAs that bind to the 3' UTR of their target genes and suppress or up-regulate the expression of the gene (732-738). They regulate the expression of their target genes 1.2 to 4 folds fine tuning the response of a cell (734, 736, 738) and play a critical role in immune cell homeostasis and function (734-737, 739-741). miR155, miR21, miR146a etc. are regulated in response to inflammatory cues, disease states, and stages of cell differentiation (734-737, 741-743). They directly or indirectly regulate the expression of genes of the inflammatory response pathways (734-737). Fredman *et.al* demonstrated that different miRNAs expressed between acute and chronic/delayed inflammation causes impaired homeostasis (744). Studies in mice suggest that miR-155 influences inflammatory disease by both promoting the expansion of proinflammatory Th1 and Th17 cells and amplifying inflammatory gene expression in macrophages and T cells (745, 746). Studies evaluating the role of miR-155 in HSV infection show that mice lacking mir-155 show high virus titers and are more susceptible to encephalitis due to impaired CD4+ and CD8+ T cells response (747-749). Previous studies have shown that several miRNAs are expressed in the mouse salivary gland in steady state (750). A presentation at the AAI meeting 2012 implicated a role for mir-155 in CMV infection. In this study Sun *et.al* showed that that knocking out miR-155 from mice lead to an impaired NK and CD8+ T cell response (751).

In this study we investigated the role of mir-155 during MCMV infection in mice. The primary target cells for virus replication in salivary glands are the sub mandibular salivary gland acinar cells (SMSG-acinar cells). To our surprise, contrary to previous studies where knocking out miR-155 leads to higher virus titers, we observed lower titers of MCMV in the salivary gland (SG) of mir-155 knock out mice (miR-155 KO). This difference could be traced to an increased infiltration of macrophages in the SG of miR-155 KO mice. We also observed higher levels of IFN- β and lower levels of IL-10 transcripts in the SG of miR-155 KO mice compared to WT mice. Therefore, even though the number of CD4+ T cells recruited to the SG of miR-155 is less, this study shows reduced MCMV titers in the SG of miR-155 KO mice that could be due to the recruitment of highly activated macrophages to the SG. These macrophages create an environment unsuitable for virus growth in the SG. This study sheds new light on the role of

macrophages for controlling MCMV infection in the SG, an organ in which virus growth was thought to be exclusively controlled by CD4+ T cells.

Materials and Methods

Mouse Infection

Breeder pairs miRNA-155 knock out mice on B6 background and WT B6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Subsequent mouse colonies were housed in WLS LAF. The mice used in this study were a generous gift of Dr. Barry T. Rouse. For the experiments, mice were infected i.p with 1×10^6 PFU MCMV K181 per mouse.

QPCR

Total mRNA and miRNA was isolated from salivary gland using mirVana miRNA Isolation Kit (Ambion). For RNA, cDNA was made with 500 ng RNA using oligo (dT) primer and ImProm-II™ Reverse Transcription System (Promega). For miRNA, cDNA was made with 5ng of miRNA using the TaqMan microRNA reverse transcription kit (Applied Biosystems) and primers for miR -155 and small nucleolar RNA 202 (SnoRNA202). QPCR was set up using the cDNA samples from above. Primer for SYBR Green PCR (IFN- β , IFN- γ , IL-10, MCP-1, KC and beta actin) and TaqMan probes (for miR-155 and SnoRNA202) were purchased from Applied Biosystems and were used to quantify microRNAs and mRNAs using a 7500 Fast Real-Time PCR System (Applied Biosystems). The expression levels of the target genes were normalized to β -actin for mRNA and SnoRNA202 for miRNA with the Δ CT method, and relative quantification between control and infected mice was performed using the $(2^{-\Delta\Delta CT}) * 1000$ formula.

Plaque formation assay

Viral titers in the organs were determined by plaque formation assay as per lab protocol on mouse embryonic fibroblast (MEF) 10.1 cells. Briefly MEF 10.1 cells were plated in a 6 well dish. Organs were harvested and homogenized. The homogenate was diluted and added the MEF 10.1 cells and incubated for 1 hr. After incubation the diluted virus was removed from the plates and cells were overlaid with carboxy methyl cellulose media and incubated for 7 days. At the end of the incubation period, CMC was removed and plates were stained and plaques counted using a dissection microscope.

Flow Cytometry

Single cell suspensions were prepared from SG days 10 post infection (p.i.) and surface staining was performed. To enumerate the number of IFN- γ and IL-17 producing CD4⁺ T cells, cells were stimulated with PMA at 100ng/ml and Ionomycin at 1 μ g/ml for 6 hrs. at 37°C in 5% CO₂. Brefeldin A (5 mg/ml) was added for the duration of the culture period to facilitate intracellular cytokine accumulation. After incubation, cell-surface staining was performed, followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD Pharmingen) to enumerate the number of IFN- γ - and TNF- α -producing CD8 T cells as previously described (22). Finally, the cells were washed three times and resuspended in 1% paraformaldehyde. The stained samples were acquired with BD LSR II (BD Biosciences) and the data were analyzed using the FlowJo software.

Statistical analysis

The statistical significance between two groups was determined using unpaired two-tailed Student t test. For our analysis *** = $p < 0.001$, ** = $p < 0.01$, and * = $p < 0.05$ were considered significant. All statistical analysis was done using, GraphPad Prism software.

Results and Discussion

miR-155 expression in the SG of B6 mice

To measure the expression level of miR-155 following infection with MCMV, salivary glands were collected at day 10 p.i. and miRNA levels were quantified by QPCR. As seen in figure A2.1, miR-155 is upregulated ~12 fold p.i compared to the uninfected control group. Because miR-155 is upregulated in response to infection and a sign of activated and functional CD4⁺ T cells, change in expression levels of this miRNA might affect viral titers in the organ.

Effect of miR-155 on virus growth

To investigate the role of miR-155 on virus growth *in vivo*, we infected miR-155 KO and WT B6 mice with MCMV following intraperitoneal (i.p.) infection. The lungs and salivary glands were isolated at day 10 p.i. and viral titers were measured by viral plaque assay. No virus was detected in the lungs of both WT and miR-155KO mice at this time point (data not shown). However, we observed 10 fold lower viral titers in the SG of miR-155 KO mice at day 10 p.i compared to WT mice on the same day (figure A2.2). This observation is contrary to previously published reports where knocking out miR-155 leads to higher virus titers following HSV infection.

Immune cell infiltration in response to MCMV infection in SG of miR-155 KO mice

We evaluated the infiltration of immune cells in the SG of infected WT and miR-155 KO mice by flow cytometry. As expected, KO mice demonstrate a severely diminished infiltration of the cells of the adaptive immune system in the SG at day 10 p.i. (Figure A2.3B). We did not

**Expression of miR-155 in Salivary Gland
Day 10 post infection**

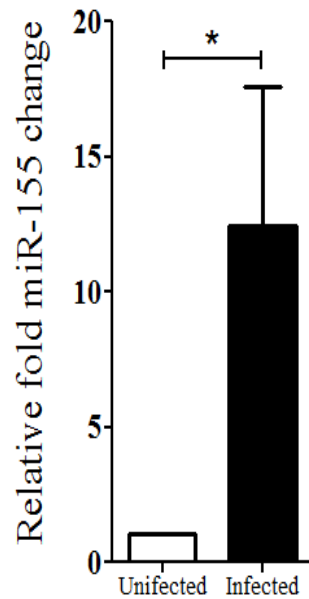


Figure A2.1 Change in miR-155 level in salivary gland post infection. WT B6 mice were infected i.p. with 1×10^6 PFU of MCMV K181. Salivary glands were isolated at day 10 post infection. miRNA was isolated and quantified as described in materials and methods. Data represents fold change in the expression of miR-155 over uninfected control WT B6 mouse. Bars represent the average fold change + standard deviation from 3 mice in each group. All mRNA samples were run in triplicates. * = P value <0.05

**Salivary Gland Titer Day 10
Post Infection**

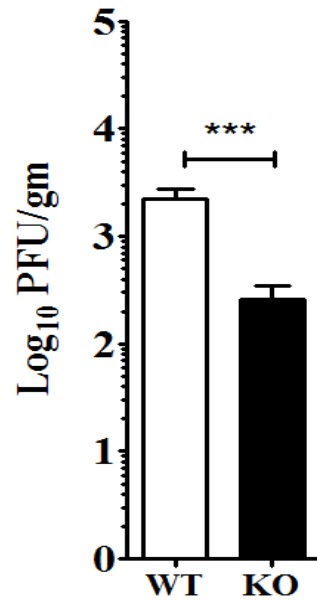


Figure A2.2 Decreased virus in the SG of KO mice. WT B6 and miR-155 knockout (KO) mice were infected i.p. with 1×10^6 PFU of MCMV K181. Salivary glands were isolated at day 10 p.i., homogenized and viral titer was determined by plaque formation assay described in materials and methods. Bars represent average from 12 mice in each group + SEM. *** = P value < 0.001.

observe any difference in the number of infiltrating neutrophils in the SG, but observed a reduction in the infiltration of NK cells in miR-155 KO mice. However, at the same time, we observed a higher frequency of macrophages in the SG. (Figure A2.3A). Due to a poor CD4⁺ T cell response that controls virus growth in SG in miR-155 KO mice, it was expected that the virus growth will be enhanced in the SG. However as seen in figure A2.2, we observed lower virus titers. This could be explained by the increase in macrophages in the SG.

Effector function of infiltrating cells in response to infection in miR-155 KO mice

In order to understand how the weaker CD4⁺ T cell response results in lower virus titers and the contribution of infiltrating macrophages in this phenomenon, we measured the transcript levels of several effector molecules in the SG of WT and miR-155 KO mice. Using QPCR we observed reduced levels of CXC chemokine KC, IFN- γ and IL-10 in miR-155 KO mice (Figure A2.4). However no difference was observed for MCP-1. We did observe an increase in the transcript levels of IFN- β in the SG of miR-155 KO mice. Previous studies had shown that CD4⁺ T cells are the primary source of IL-10 in the SG (598, 752), which could explain the reduced levels of IL-10 in the SG of miR-155 KO mice. It has also been shown that blocking IL-10 signaling reduces viral titers in the SG (134). IFN- β is synthesized by several cell types including macrophages (753, 754). It is possible that the increased level of IFN- β is a consequence of an increased macrophage presence in the SG of miR-155 KO mice.

Conclusion and Future Directions

Taken together, we speculate that two mechanisms are at work here which are responsible for the reduced viral titers in the miR-155 knock out mice, 1) reduction in the level of IL-10 as a result of diminished CD4⁺ T cell infiltration, which supports viral clearance and 2) highly activated macrophages in the SG are producing greater amounts of IFN- β , which creates an environment not suitable for virus growth. Future experiments are required to evaluate the activation and function of the macrophages recruited into the SG of miR-155 KO mice. Additionally, experiments with depletion of macrophages (using clodronate) in miR-155 KO mice could also be performed to confirm the role of these cell types in the observed phenotype.

Acknowledgements

I would like to thank Dr. Sachin Mulik and Dr. Siddheshvar Bhela for sharing the reagents and for their technical assistance while carrying out this study.

**Cell infiltrate in Salivary Gland at Day 10
Post Infection**

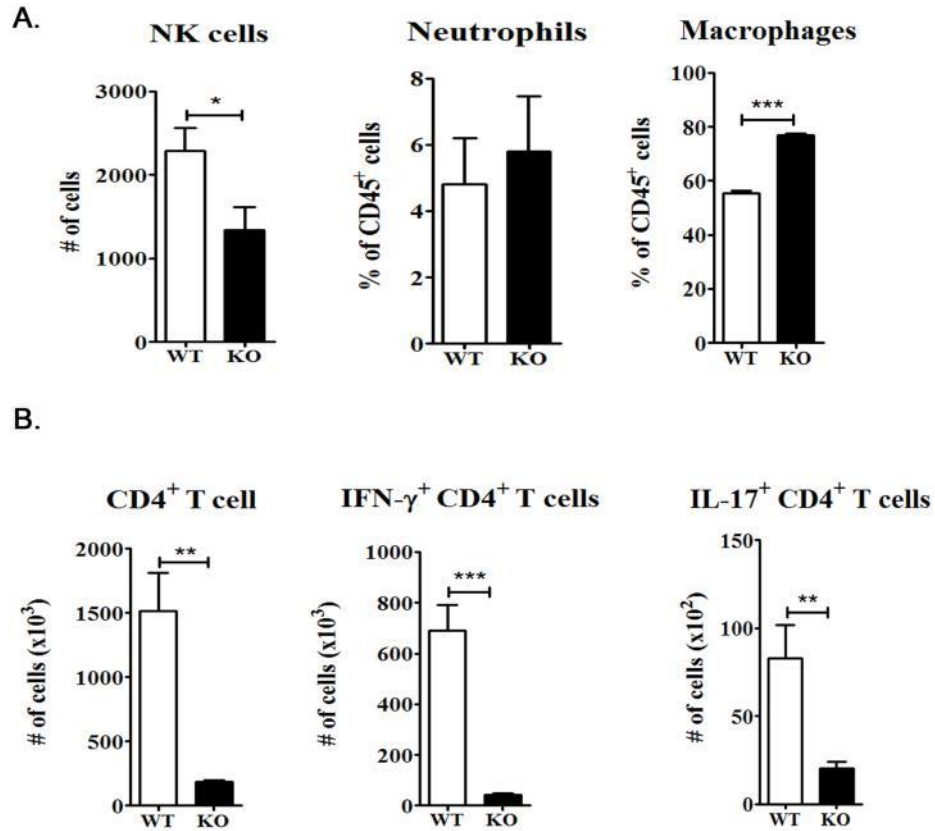


Figure A2.3 Alteration in the cellular infiltrate in the SG in could be responsible for difference in SG viral titers. WT B6 and miR-155 knockout (KO) mice were infected i.p. with 1×10^6 PFU of MCMV K181. Salivary glands were isolated at day 10 p.i. Cells were stained for flow cytometry and analyzed. Bars represent the average from 3 mice in each group + SEM. * = P value < 0.05, ** = P value < 0.01, *** = P value < 0.001

**RNA Levels of Effector Molecules
in Salivary Gland at Day 10 Post Infection**

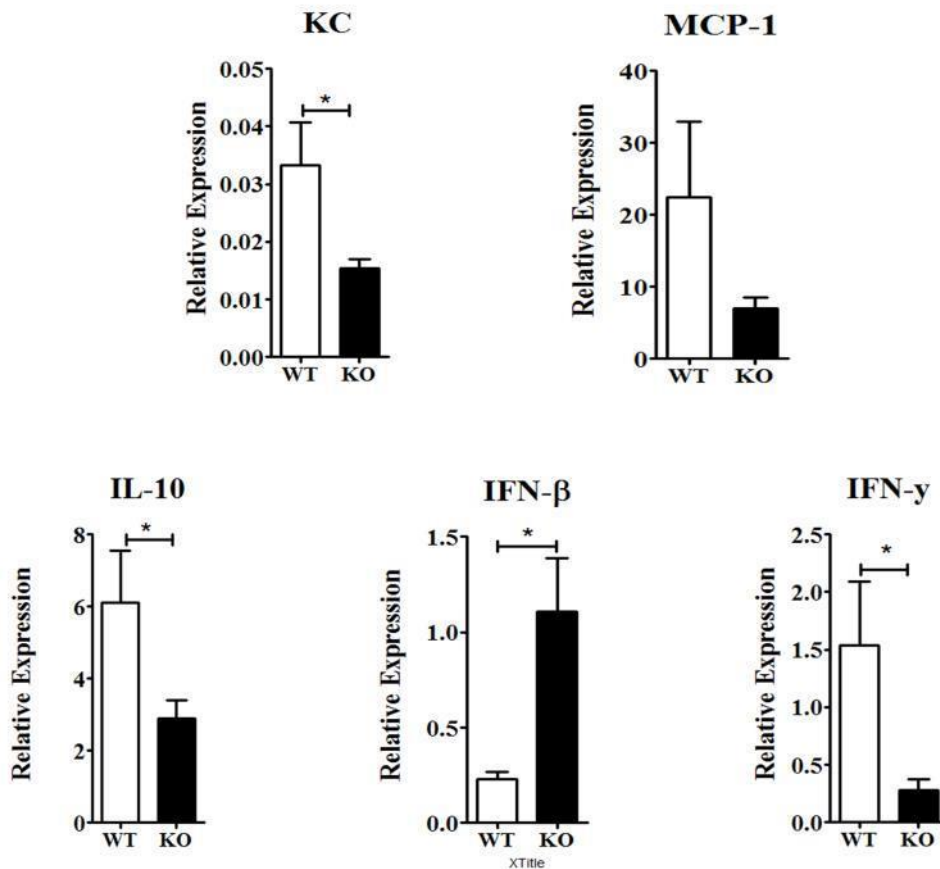


Figure A2.4 miR155KO mice have increased IFN β and decreased IL10, KC, MCP-1, and IFN γ mRNA. WT B6 and miR-155 knockout (KO) mice were infected i.p. with 1×10^6 PFU of MCMV K181. SG were isolated at day 10 p.i. and mRNA was assayed via qPCR. Bars represent the average from 3 mice in each group + SEM. * = P value < 0.05.

APPENDIX 3: THE ROLE OF PD-L1 EXPRESSING NEUTROPHILS IN CMV PATHOGENESIS

Abstract

Human CMV (HCMV) is widespread β -herpes virus that is prevalent in upwards of 90% of the population in developing countries and 30-60% of women of childbearing age in developed nations. Primary infection in healthy adults is controlled by the cells of the adaptive immune system after which it establishes latency. Suppression of the immune system can lead to re-activation as seen in transplantation patients. The *UL146* gene of CMV encodes a potent CXC chemokine, vCXCL1 which induces migration and calcium flux in peripheral blood neutrophils (PBNs). It has been suggested that vCXCL1 attracts neutrophils that help CMV disseminate within the host. In this study we show that neutrophils recruited to the site of CMV infection express PD-L1, which could suppress CD4+ and CD8+ T cell immune responses. This might be an additional immune evasive strategy used by the virus to buy some extra time to replicate at an initial site and then disseminate successfully to another site.

Introduction

Human Cytomegalovirus (HCMV) is a ubiquitous β -herpesvirus. Reports have estimated its prevalence being between 90-100% in developing nations (723). Even in the USA HCMV infection is prevalent in between 30-60% of women of child bearing age (731). It is the leading cause of child birth defects and congenital infection with HCMV causes neurodevelopmental defects (38, 574). In immune compromised individuals like AIDS and transplantation patients, CMV infection can lead to serious complications, which can be fatal in some cases (574). In healthy adults however, HCMV infection is usually asymptomatic with mononucleosis-like symptoms in some cases (574). After clearance of the primary infection, HCMV establishes lifelong latency in an immune competent individual (574). Cells of both the innate and adaptive immune system contribute to controlling CMV infection. Animal models have contributed significantly in understanding the contribution of the immune system components in the control of CMV infection (127-130). Studies using the mouse model of CMV infection have

demonstrated that NK cells, neutrophils and CD8⁺ T cells play a critical role in the control of infection in the primary organs of dissemination (i.e., spleen, lung, and liver (131-133)). However, MCMV growth in the salivary gland (SG) is controlled only by CD4⁺ T cells (134-136). In humans, ~10% of the total CD4⁺ and CD8⁺ T cells are HCMV specific and prevent the virus from reactivation (519). Indeed, in immune suppressed individuals e.g., transplant patients, HCMV reactivation is observed (574). Therefore it is important to understand the mechanisms that control the immune response to viral infections.

In the setting of a chronic infection, signaling through PD-1 expressed on the surface of T cells leads to a suppression of the functionality of these cells and exhaustion of these cells. Blocking of signaling through this receptor can lead to reversal of exhaustion and restoration of the effector function of these cells (755-759). Interestingly, in a recent study it was shown that neutrophils recruited to the site of HIV infection express PD-L1, the ligand for PD-1 and suppress the response of T cells against virus; this allows the virus to persist in the host (760). In this study we investigated the expression of PD-L1 on neutrophils recruited to the site of CMV infection in the footpad. PD-L1 expressing neutrophils might suppress the T cell response against CMV, creating a more favorable environment for the virus to replicate and establish latency. Because the CMV genome encodes for a functional CXC chemokine with the ability to induce migration of neutrophils, we propose that the viral chemokine-mediated recruitment of PDL-1 expressing neutrophils to the site of infection might be an additional immune evasion mechanism employed by the virus.

Materials and Methods

Mice an Infection

WT BALB/c mice were bred in WLS animal facility and were used at the age of 6-8 weeks for all the experiments. Mice were infected in the foot pad with 1×10^6 PFU of MCMV RMmCXCL1 per mouse.

Flow Cytometry

Single cell suspensions were prepared from the foot pads mice at day 3 post infection. Feet were minced and digesting with in collagenase D. After this, cell-surface staining was performed to

stain for neutrophils expressing PDL-1 (CD45+ Cd11b+ Ly6G+ PD-L1+). Finally, the cells were washed three times and resuspended in 1% paraformaldehyde. The stained samples were acquired with a BD LSR II (BD Biosciences), and the data were analyzed using the FlowJo software.

Results and Discussion

Neutrophils recruited to site of CMV infection express PD-L1

Neutrophils are the first cell types recruited to the site of infection and form an important part of the host anti-viral defense (761). As seen in figure A3.1 the neutrophils recruited to the site express PD-L1. We did not observe any difference in the level of PD-L1 expression on the neutrophils between mice infected with RM4511 and RMmCXCL1, a recombinant MCVm that expresses the host chemokine mCXCL1. It is possible that CMV has evolved to carry *UL146* gene to recruit PD-L1 expressing neutrophils to the site of infection, which leads to a suppressed immune response. This might provide the virus enough time to replicate at the site of infection before being controlled by the adaptive immune system. It has also been shown that CMV specific CD4+ and CD8+ T cells express PD-1, and blocking the (PD-1)-(PD-L1) signaling axis leads to better control of viral infections post transplantation (762). It is therefore conceivable that the neutrophils at the site of infection are the source of PD-L1 and help the virus evade T-cell mediated control. Further experimentation is required to fully evaluate the contribution of this observation in the CMV pathogenesis including its role in replication, latency, reactivation, and superinfection.

Future Directions

In order to investigate the role of PD-L1 expressing neutrophils in CMV immune evasion, future experiments need to be done to show the ability of PD-L1 expressing neutrophils to suppress CMV specific T cell function *in vitro* and whether the depletion of neutrophils leads to a better CMV specific T cell response *in vivo*. In their study, Bowers *et al.* show that the infection of neutrophils by HIV was sufficient to upregulate PD-L1 on their surface (760). Because CMV can infect neutrophils, we should also evaluate whether CMV infection of neutrophils can upregulate PD-L1 on their surface. In addition, it would also be interesting to evaluate whether the different viral chemokines can induce a differential expression of PDL-1 on the surface of neutrophils. Experiments should also be carried out with isolated human PBNs to show the relevance of this mechanism in the setting of HCMV infection of humans.

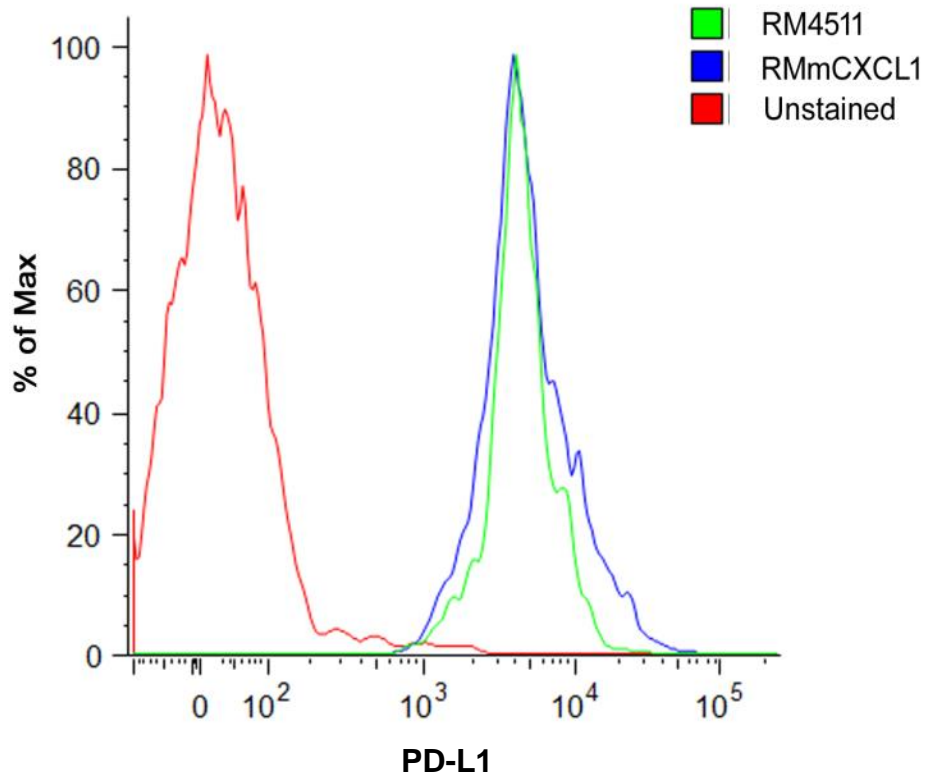


Figure A3.1 PD-L1 is expressed on neutrophils at day 3 post infection. Mice were infected in the footpad with either RM4511 (green) or RMmCXCL1 (blue). Cells of the footpad were stained for PD-L1 expression on neutrophils and analyzed by flow cytometry. The histogram represents the expression of PDL-1 on neutrophils or the unstained control (red). Data is representative of 3 mice for each infection group.

APPENDIX 4: THE ROLE OF VIRAL IL-10 IN CMV PATHOGENESIS

Abstract

Human cytomegalovirus (HCMV) infects with seroprevalence reaching 100% in some countries. It causes life threatening disease in immune compromised patients and congenital defects if infection occurs *in utero*. The HCMV genome encodes for potent immunomodulatory proteins such as the human interleukin (IL-10) homolog, cmvIL-10 that aids in viral dissemination and survival within the host. The cmvIL-10 is structurally different from human IL-10 (hIL-10) and has been suggested to have functional differences from its human counterpart. Due to CMV's host specificity, murine CMV (MCMV) has been used as model for *in vivo* studies. MCMV lacks a homolog of cmvIL-10 and it has no effect on mouse cells. The biological basis of this non-functionality is unknown, thus a mouse model to investigate the role of cmvIL-10 *in vivo* does not exist. The present study was undertaken to develop a recombinant MCMV expressing a mouse-adapted cmvIL-10 (MAcmvIL-10), which would be functional in mice and may subsequently lead to the development of a mouse model to test this viral cytokine.

Introduction

Human cytomegalovirus (CMV) infects people throughout the world with adult seroprevalence approaching 100% in many countries (723). CMV infections cause life-threatening diseases in immunocompromised individuals and congenital birth defects if infection occurs *in utero*. In healthy individuals it has been associated with vascular disease (39-42, 51-53). The CMV genome encodes interleukins (e.g., cmvIL-10) and chemokines (e.g., vCXCL-1) (401, 407). Cytokines are small proteins responsible for regulation of cell proliferation, differentiation, production of other cytokines, leukocyte trafficking, inflammation etc. Interleukins are a class of cytokines secreted by leucocytes, having effects on other leukocytes. Interleukin 10 (IL-10) is the major anti-inflammatory and immunosuppressive cytokine with effects on a wide range of immune cells including T cells, B cells, NK cells, monocytes, and macrophages (763). cmvIL-10 sequence shares only 25 to 27% identity with hIL-10 at amino acid level (401). This suggests that cmvIL-10 may not only function similarly to hIL-10, but it could also have different or

additional properties. Based on this knowledge, we hypothesize that these viral factors have a global effect on host immune response and is involved in viral dissemination and survival.

The goal of this study is to assess the *in vivo* role of cmv-IL10 in virus dissemination and persistence. *In vivo* studies with cmvIL10 have been impeded because it is ineffective on mouse cells (156). In order to identify the molecular mechanisms of the lack of cmvIL-10 functionality on mouse cells, we synthesized hIL-10 and cmvIL-10 using the *Pichia* expression system. These cytokines will be used in *in vitro* functional assays in the future. In addition, using bioinformatics we identified key amino acids in cmvIL-10 for subsequent mutation and generation of a mouse adapted cmvIL-10 (MAcmvIL-10) that would function on mouse cells. Recombinant Mouse CMV (MCMV) expressing MAcmvIL-10 will be used to carry out *in vivo* studies to assess whether this viral cytokine functions to manipulate the host immune response and the survivability of the virus.

Materials and methods

Genes

The genes for hIL-10 and cmvIL-10 were codon optimized for protein production in yeast and artificially synthesized (Gene Script, NJ). The proteins were FLAG tagged at the N-terminus to facilitate identification and purification of the induced protein.

Cloning and selection

The hIL-10 and cmvIL-10 genes were cloned in to *pPICZ A* Expression Vector (Life Technologies, NY) under the control of the methanol inducible *S. cerevisiae* AOX promoter. Transformation of *Pichia pastoris* (strain X-33) was achieved by electroporation. Successful transformants were selected by plating on Yeast Extract Peptone Dextrose (YEED) agar plates with 100µg/ml zeocin. Clones with multiple integrations of the expression cassette were selected on 1mg/ml zeocin plates.

Protein induction and purification

Selected clones were grown as 2.5 ml mini- induction cultures and supplemented with methanol for 4 days to induce protein production. For large scale protein production, clones expressing high levels of hIL-10 and cmvIL-10 were grown to high density in 1L cultures overnight. The

cells from the overnight cultures were transferred to 250ml induction medium and supplemented with methanol for 4 days to induce protein production. Medium from the large-scale induction experiment was dialyzed in a 3kDa cutoff dialysis membrane against FPLC buffer. The pH and conductivity of the dialyzed medium was adjusted to match the FPLC buffer. The processed medium was run over a Q (anion exchange) column at pH6.5 with a NaCl gradient of 0 – 1 M to purify the protein. The fractions from the Q column-FPLC run containing the protein were pooled and run over a Sepharose DEAE column at pH9.0 and a NaCl gradient of 0 – 0.5 M.

Screening for protein production

Western blotting using the anti-FLAG antibody was performed on supernatants to screen for protein expressing clones. The fractions obtained from the FPLC of the large scale induction cultures were also silver stained and blotted to determine the fraction(s) containing the protein.

Results

Production of host and viral chemokine in yeast

To determine the reason for the lack of functionality of cmvIL-10 in mice, we produced and purified cmvIL-10 and hIL-10 from the yeast, *Pichia pastoris* expression system (Figure A4.1). These proteins will be tested on mouse cells for receptor binding and downstream signaling defects that may render the cmvIL-10 functionless on mouse cells.

Structure based sequence alignment

It is possible that cmvIL-10 is unable to bind the mouse IL-10 receptor which leads to its lack of functionality on mouse cells. Structural studies of cmvIL-10 bound to sIL-10R1 identified several residues that are involved in the receptor ligand interaction (485, 764). Building upon these structural studies we carried out an *in silico* analysis of the amino acid sequences of hIL-10, mouseIL-10 (mIL-10) and cmvIL-10 to identify residues within the receptor binding domains (485, 764), which are depicted by the circles in figure A4.2. The amount of the buried surface area for these cytokine residues in the receptor binding pocket is denoted by the different numbers of circles with 1 > 5 Å², 2 > 10 Å² < 35 Å², 3 > 35 Å² < 60 Å², 4 > 60 Å² < 85 Å², and 5 > 85 Å². As observed in figure A4.2, mIL-10 and cmvIL-10 share only 30% identity in the receptor binding domain I and only 27% identify in the binding domain II. Changing the residues of cmvIL-10 to match the residues at the same position in mIL-10, would result in 100% identity of the interacting residues in the receptor binding domains.

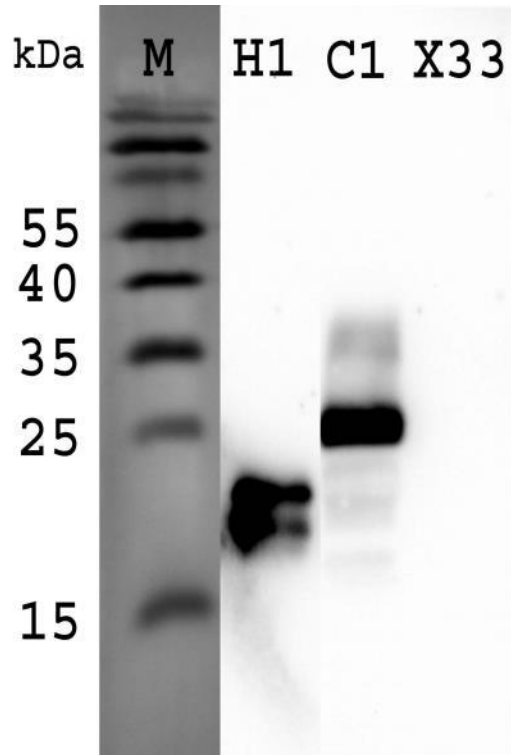


Figure A4.1 Detection of the expressed cytokine. The gene for hIL-10 and cmvIL-10 was codon optimized and cloned into yeast and the expression of the cytokine was induced by adding methanol as described in materials and methods. Cytokine expression was detected by western blotting using anti-FLAG antibody. M is the molecular weight marker. H1 is the yeast clone expressing hIL-10, C1 is the yeast clone expressing cmvIL-10, and X33 negative control.

This would be designated as mouse adapted cmvIL-10 (MAcmvIL-10) (figure A4.2). Because the residues in the receptor binding domain of MAcmvIL-10 match that of mIL-10, we hypothesize that MAcmvIL-10 will bind and signal via the mouse IL-10R and gain functionality in the mouse model. This hypothesis needs to be tested with future experiments.



Figure A4.2 Structure based sequence alignment of hIL-10, cmvIL-10, mIL-10 and MAcmvIL-10. The cmvIL-10 or hIL-10 residues that are buried into surface into sIL-10R1 are marked with circles. The amount of buried surface area for the cytokine residues is denoted by different numbers of circles with 1 > 5 Å², 2 > 10 Å² < 35 Å², 3 > 35 Å² < 60 Å², 4 > 60 Å² < 85 Å², and 5 > 85 Å². Figure adapted from *Jones et al, 2002, PNAS*

APPENDIX 5:

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Pranay Dogra was born on March 17th, 1986 in India. He attended Hislop College, University of Nagpur, India where he received his Bachelors of Science degree with triple majors in Botany, Chemistry and Biotechnology in 2007 and his Masters in Biotechnology from the Nagpur University, India in 2009. He started his doctoral program at the University of Tennessee Knoxville in August 2010 in the lab of Dr. Tim Sparer. His work included investigating the role of CMV encoded chemokines in the pathogenesis of virus and to develop antiviral peptides in collaboration with Dr. John Wall at the University of Tennessee Health Science Center. He completed his studies and graduated with a Doctor of Philosophy in Microbiology in August 2015.