THE ROLE OF VIRALLY ENCODED CHEMOKINES AND CHEMOKINE INHIBITORS IN HEALTH AND DISEASE

by

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TABLE OF CONTENTS

4. Viral manipulation of the immune system

iv

LIST OF FIGURES AND TABLES

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ABSTRACT

Chemokines are small, secreted cytokines with the capacity to induce cellular migration, also referred to as chemotaxis. Although primarily known for their ability to mediate leukocyte migration as part of the immune response to injury or pathogenic insult, chemokines also play an integral role in a variety of cellular processes like tissue and organ development, angiogenesis, and hematopoiesis. As such, some viruses have evolved mechanisms to either mimic or subvert normal chemokine function in an attempt to further their own lifecycle. In this work, we will address two such proteins, one, a chemokine homolog encoded by rhesus rhadinovirus, the other, a chemokine inhibitor encoded by monkeypox virus.

Rhesus rhadinovirus (RRV) is the simian homolog of Kaposi sarcoma-associated herpesvirus (KSHV), and like KSHV, it encodes a viral macrophage inflammatory protein homolog (RRV vMIP) that, in many respects, mimics the function of cellular chemokine, rhesus macrophage inflammatory protein-1α (MIP-1α). In the first half of this thesis, we show that recombinant RRV vMIP induces migration of monocytic THP-1 cells *in vitro*. Moreover, RRV vMIP -saturated implants induce the recruitment of CD14$^+$ cells *in vivo*, suggesting RRV vMIP might function as a chemokine during RRV infection. Acute *in vitro* infection of isolated peripheral blood mononuclear cells (PBMC) with RRV shows that CD14$^+$ cells are permissive to RRV infection. Taken together, these data strongly suggest that RRV vMIP encodes a functional chemokine with the ability to recruit permissive CD14$^+$ cells during acute infection, which may provide a mechanism for viral dissemination.

Monkeypox virus (MPV) is an orthopoxvirus with considerable homology to variola major, the etiologic agent of smallpox. Like other orthopoxviruses, MPV encodes a secreted chemokine binding protein, MPV vCCI, that is abundantly expressed and secreted during MPV infection. In the second half of this thesis, we show via Electrophoretic mobility shift assay (EMSA) that MPV vCCI efficiently binds rhesus MIP-1α (rhMIP-1α). Furthermore, *in vitro* chemotaxis experiments demonstrate that MPV vCCI completely inhibits rhMIP-1α mediated chemotaxis, while *in vivo* recruitment assays in rhesus macaques using chemokine-saturated implants show a decrease in the number of $CD14^+$ cells responding to rhMIP-1α when MPV vCCI is present, suggesting MPV vCCI is effectively inhibiting chemokine function both *in vitro* and *in vivo*. More importantly, we demonstrate that MPV vCCI can diminish the severity of the acute phase of infection and completely inhibit the relapsing phase of experimental allergic encephalomyelitis (EAE) disease. These data represent the first *in vitro* and *in vivo* characterization of MPV vCCI emphasizing its function as a potent inhibitor of rhMIP-1α. Furthermore, the ability of MPV vCCI to inhibit relapsing EAE disease represents a novel therapeutic approach for treating chemokine-mediated diseases.

Chapter 1

INTRODUCTION

1. Human Herpesviruses

a. *Classification*

Members of the *Herpesviridae* family are common pathogens that infect a wide range of vertebrate hosts (325). A hallmark of the *Herpesviridae* family is the ability to persist within the infected host for long periods of time without any associated pathologies. Some herpesviruses accomplish this by entering a state of latency, wherein viral replication is halted and viral gene expression is suppressed, while others maintain a persistent infection, wherein viral replication is minimal or abortive. In either case, under certain environmental conditions, viral reactivation may ensue and lead to pathogenesis and/or the production of viral progeny. To date, eight human herpesviruses have been discovered and although during periods of latency or persistence are relatively benign, under conditions of reactivation, this can result in serious health concerns.

The *Herpesviridae* family is divided into three distinct subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. The placement of a herpesvirus into one of these subfamilies is based on a number of biological properties including cellular tropism, host range, and growth characteristics (325). The human *Alphaherpesvirinae* subfamily contains two genera, *Simplexvirus*, which includes herpes simplex virus type 1, also referred to as human herpesvirus-1, or HHV-1, and type 2

(HHV-2), and *Varicellovirus*, which includes varicella zoster virus (HHV-3).

Alphaherpesviruses share a wide host range, have a short replication cycle, often result in

substantial cellular disruption, and establish a lifelong latent infection in sensory ganglia.

Alphaherpesviruses are quite common in the human population with up to 80-95%

seropositivity among the worldwide population (299, 325). The human

Betaherpesvirinae subfamily is also comprised of two genera, *Cytomegalovirus*, which

includes cytomegalovirus (HHV-5), and *Roseolovirus*, which includes HHV-6 (A and B

variants) and -7. Betaherpesviruses exhibit a more restricted host range, long

reproduction cycle, infection progresses slowly in culture, and infected cells frequently

become enlarged, referred to as cytomegalia. Betaherpesviruses can establish a life-long

persistent infection in a variety of tissues including myeloid cells, lymphoreticular cells,

kidneys and secretory glands (325, 403). Prevalence within the human population varies

depending on region and socio-economic status, but ranges from 50-100% (325, 461).

The last subfamily, *Gammaherpesvirinae*, contains two genera as well,

Lymphocryptovirus which includes Epstein Barr virus (EBV or HHV-4) and

Rhadinovirus, which includes Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-

8). Gammaherpesviruses are lymphotropic with infection and latency typically

associated with lymphoid tissues. Furthermore, gammaherpesviruses exhibit the most

restricted host range of the herpesviruses, which is limited to the family or order to which

the natural host belongs. Like the betaherpesviruses, gammaherpesviruses have a long

replication cycle (325). Prevalence of the gammaherpesviruses in the human population

varies greatly between the two genera. Epstein Barr virus (HHV-4) is present in >90% of

the world's population, while Kaposi's sarcoma-associated herpesvirus (HHV-8) varies

between 1% and 60% depending on region (179, 374, 380) .

b. *Virion structure*

A common trait among *herpesviridae* family members is the structure of the

virion. Proteomic analysis of EBV and CMV viral particles has identified up to 71

virally encoded proteins that make up the virion (205, 217). Additionally, a variety of

cellular proteins, as well as viral and cellular encoded RNAs, can also be found within

the mature virion (58). All herpesvirus virions are made up of four conserved regions:

core, capsid, tegument, and envelope (figure 1.1). The herpesvirus virion can vary in size

between 120 and 260 nm depending on the thickness of two of the conserved structures:

the tegument and envelope (325). The core of a mature virion contains the double

stranded DNA genome in the form of a torus, or doughnut, and although the exact

mechanism is unknown, the viral genome is believed to be suspended via a proteinaceous

network that attaches to the underside of the viral capsid (127, 301, 463). The capsid is a

proteinaceous shell that encases the viral genome. The 100 nm diameter of the capsid is

highly conserved among herpesviruses and is made up of 162 capsomers forming an

icosahedron. The tegument is an amorphous protein layer between the capsid and

envelope comprised of viral and host proteins of that are believed to be involved in

establishing the environment within a newly infected cell by blocking cellular protein

synthesis, inhibiting cellular defense mechanisms, and promoting viral gene expression.

The outermost layer of the virion is the envelope. Believed to be formed from altered
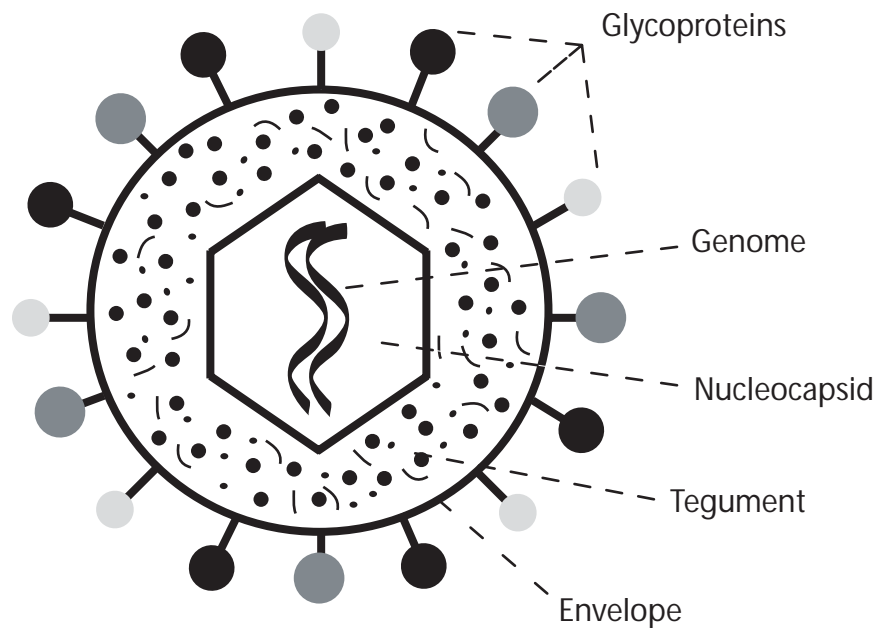
Figure 1.1 General herpesvirus structure. A) Cross-sectional diagram representing the general organization and components of a typical herpesvirus virion.

cellular membranes, the envelope is a lipid bilayer studded with virally encoded

glycoproteins (325).

c. *Herpesvirus life cycle*

The herpesvirus lifecycle begins with a virion binding and entering a permissive

cell, which starts with viral glycoproteins interacting with cellular receptors on the

surface of the host cell. Studies on HSV-1 have shown that binding is primarily mediated

by the interaction between glycoproteins C and B (gC and gB) with cell-surface

glycosaminoglycans (GAG) (181, 395). Following binding, fusion of the viral envelope

with the plasma membrane is mediated by the interaction of gD and different cellular

adhesion molecules, such as herpes virus entry mediator (HVEM) or 3-*O*-sulfated

heparan sulfate (3-OS HS) (150, 396). Although gD is critical for fusion, other

glycoproteins, such as gB, gH, and gL have also been shown to be important for the

fusion process (408). The use of GAG's to facilitate entry is ubiquitous among the

herpesvirus family and like HSV, a similar interaction occurs between heparin sulfate and

the envelope glycoprotein, K8.1, of KSHV (13, 42). Studies using soluble heparin or

enzymatic cleavage of heparin from the target cell surface demonstrate a deceased ability

to infect permissive cells. Heparin sulfate is thought to enhance membrane fusion and

therefore increase the efficiency of infection, however, K8.1 knockout virions retain

infectivity suggesting other receptors are also involved (266). Evidence suggests that

DC-SIGN, integrins, and xCT have the ability to mediate KSHV infection (12, 210, 342).

Akula *et al.* demonstrated that integrin α_3/β_1 co-immunoprecipitated with KSHV gB.

This interaction was mediated by an RGD motif present in gB and conferred

permissibility (12, 442). Moreover, integrin α_v/β_3 has also been shown to mediate KSHV infectivity via a similar mechanism. Recent findings by Kaleeba *et al*. describe the ability of the cystine transporter, xCT, to mediate KSHV infectivity in otherwise non-permissive cells. Interestingly, xCT has been shown to form a complex with CD98, and this heterodimer is known to associate with α_3/β_1 (141). Although xCT and intergrins mediate KSHV infection in various cell types, their lack of expression in B lymphocytes suggests that additional entry mechanisms had yet to be discovered. Along these lines, Rappocciolo *et al*. recently discovered that the surface protein, DC-SIGN, was capable of mediating KSHV infectivity in dendritic cells, macrophages, and more importantly B lymphocytes (341, 342). Once binding and fusion take place, the capsid and tegument proteins are released into the cytoplasm of the now infected cell. The capsid is actively transported along the microtubule network from the site of deenvelopment to the nuclear pore, where the viral DNA is released into the nucleus and takes on a circular form (401). Transcription of viral genes is mediated by cellular RNA polymerase II, while viral DNA replication is mediated by the virally encoded DNA polymerase, utilizing a rolling circle mechanism (357).

Herpesvirus lytic genes follow a transcription pattern that is temporal in nature and is comprised of three distinct classes: immediate early (or α class), early (or β class), and late (or γ class) (figure 1.2). The first class of genes to be transcribed immediately after the viral DNA enters the nucleus, the immediate early genes, do not require viral protein synthesis for their expression and utilize viral proteins (e.g. VP16 for HSV-1 and UL82 for CMV) brought in with the virion tegument to enhance transcription (285, 325).
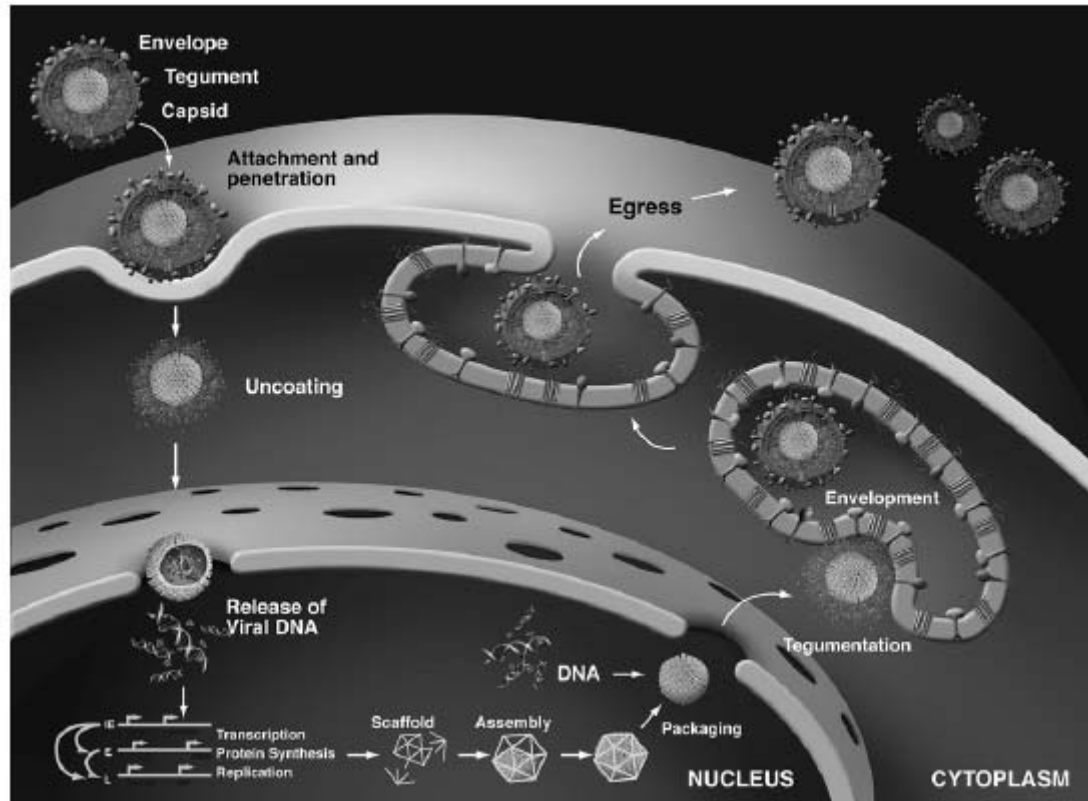
Figure 1.2 General herpesvirus lifecycle. Virus binding and uncoating releases the nucleocapsid and tegument proteins into the cytoplasm. Nucleocapsid is translocated to the nucleus where viral DNA is released. Viral gene transcription occurs via a temporal cascade: immediate early genes → early genes → late genes. Late protein synthesis is typically associated with an accumulation of structural proteins involved in forming progeny virions. Viral DNA is packaged into nascent capsids via a head-full mechanism. Nucleocapsid is translocated out of the nucleus and acquires a tegument layer. The acquisition of the viral envelope occurs by budding into the trans-Golgi-network, which has been loaded with viral glycoproteins. Finally, viral egress. *With permission – Andrew Townsend*

Immediate early genes encode proteins that are involved in promoting further viral gene expression. The second class of genes to be expressed, the early genes, are dependent on immediate early gene expression, are expressed independently of viral DNA synthesis, and the proteins encoded by early genes are generally involved in viral DNA replication. Finally, once viral DNA synthesis has been well established (i.e. early gene expression), the late genes are expressed. Late genes typically encode the structural elements of the virion (357). Although their classification is largely based on time of expression, the use of chemical agents to inhibit DNA replication and protein synthesis provides other criteria for qualifying placement into one of the herpesvirus gene classes. Genes whose expression is not inhibited by cyclohexamide (CHX), a protein synthesis inhibitor, are considered immediate early genes, since their transcription does not require prior viral protein synthesis. Genes whose transcription is not inhibited by phosphonoacetic acid (PAA), an inhibitor of DNA synthesis, are considered immediate early or early genes depending on their sensitivity to CHX. Lastly, because of their dependency on both viral DNA synthesis and viral protein translation, late genes are sensitive to both chemical agents.

After viral proteins are synthesized, constituents of the capsid are translocated to the nucleus and assembly of the progeny virions begins. Viral DNA is packaged into nascent capsids via rolling circle replication. A "head-full" sensing mechanism appears to maintain proper genomic content by triggering cleavage at conserved sequences that define the genomic termini. Following the acquisition of the tegument, capsids are exported out of the nucleus. Three mechanisms for nuclear egress are currently postulated (252, 282, 283). The first involves newly formed capsids transitioning thru the

8

nuclear membrane directly into the rough endoplasmic reticulum on the inside of membrane-bound tubes and vesicles. The next is thought to progress via an envelopment/de-envelopment mechanism, where nascent capsids eventually enter the cytoplasm without an envelope. Lastly, nascent capsids may exit the nucleus thru enlarged nuclear pores, therefore not acquiring an envelope. The latter two pathways would acquire an envelope by budding into cytoplasmic vesicles prior to egress. Irrespective to the pathway used, release of progeny virions from the host cell occurs either by lysis of the infected cells or by budding from the host cell plasma membrane.

Along with lytic replication, a hallmark of the herpesvirus family is the ability to establish a life-long latent infection in infected host cells. During latency, an altered replication program is initiated where very few viral genes are expressed and as a result, the production of progeny virions is halted. To ensure proper maintenance of viral DNA during latency, the viral genome exists as an episome and is replicated and passed onto progeny cells. This process is mediated by both host and viral components to ensure faithful segregation of the viral genome into daughter cells during mitosis. For KSHV, the process is mediated by the latency-associated nuclear antigen (LANA) protein (27). LANA is one of the few KSHV genes expressed during latency and its presence is often used as an indicator of latent KSHV infection. LANA binds the terminal repeat of the viral genome, an interaction that initiates semi-conservative replication via host machinery (145). Following replication, segregation of the viral episomes is achieved by LANA interacting with mitotic chromosomes. Therefore, during chromosomal separation, viral episomes are also separated faithfully into daughter cells (328).

In this quiescent state, the virus can exist indefinitely until a lytic replication cycle is initiated, termed reactivation. Although the exact mechanisms for initiation and reactivation of lytic replication are not well understood, various stimuli have been identified that promote viral reactivation. These include: heat, physical, and emotional stress, immunosuppression, and exposure to UV light (357).

d. *Human herpesviruses and disease*

Alphaherpesviruses share a preference for infection, replication, and eventual latency in neurons. HSV-1 and -2, the causative agents for oral and genital herpes, infect naïve hosts through mucosal epithelium or broken skin. After initial infection, viral spread results in infection of sensory nerve endings close to the initial site of entry. From there, virus is transported to the sensory nerve ganglia where latency is established (414). Although the typical pathology associated with HSV infection is a painful lesion of the epidermis, in rare cases, systemic or ocular infection can occur resulting in life-threatening infections of the central nervous system. Under certain environmental conditions, HSV can reactivate leading to reoccurring outbreaks (412). VZV, the causative agent of chicken pox (varicella), typically infects naïve hosts during childhood. Primary infection occurs through inhalation of aerosolized droplets and infection of respiratory epithelium, where the virus gains access to highly permissive T cells in the tonsillar lymphoid tissue resulting in a systemic disease characterized by wide-spread skin lesions. (233). Similar to HSV, VZV eventually spreads to sensory nerves and traffics to the dorsal root ganglia, the site of latency. Reactivation of VZV may occur later in life, leading to shingles (herpes zoster) (95).

Like alphaherpesviruses, betaherpesvirus exhibit a wide distribution throughout the human population, with up to 95% of certain populations showing seropositivity. Unlike alphaherpesviruses that establish latency in neurons, betaherpesviruses can latently infect a variety of cells types of myeloid and epithelial origin (345, 403). Primary infection by CMV is often asymptomatic, but occasionally can cause acute febrile illness resembling mononucleosis, and in rare cases, CMV mononucleosis can lead to severe complications involving specific organ systems. More importantly, congenital CMV infection is a leading cause of birth defects of the CNS, resulting in brain damage, deafness, and impaired vision (285). Latent CMV infections pose little to no risk to the immunocompetent host, but in individuals with compromised immune systems, CMV may reactivate leading to CMV disease, typified by retinitis, esophagitis, or colitis. Recipients of solid organ transplant are another group susceptible to CMV reactivation. In this case, CMV reactivation may lead to serious complications, such as graft rejection, atherosclerosis, and an increased risk of opportunistic fungal or bacterial infections (87, 285). The other human betaherpesviruses, HHV-6 and -7, are considerably less pathogenic than CMV and have been identified as the etiologic agents for exanthema subitum (roseola infantum), a wide spread childhood disease characterized by a systemic rash and febrile seizures (164, 253, 456). Additionally, HHV-6 has been shown to be neurotropic and, although a direct link has not been established, HHV-6 components have been detected in brain samples from individuals who suffered from multiple sclerosis (80, 93).

A hallmark of the gammaherpesviruses is the propensity to infect and establish latency in B cells. EBV was the first human gammaherpesvirus to be identified and

exhibits a prolific, wide spread distribution throughout the human population (124, 179).

EBV is generally spread asymptomatically among children, however when primary infection occurs later in life, infectious mononucleosis occurs in 50% of cases (270).

EBV has also been associated with several B cell malignancies, including nasopharyngeal carcinoma, Hodgkin's lymphoma, and Burkett's lymphoma (18, 156, 352). As with most herpesviruses, the incidence of these malignancies is greatly increased in individuals with compromised immune systems. The most recently discovered human herpesvirus is the gammaherpesvirus, KSHV (or HHV-8), and was identified as the etiologic agent of Kaposi's sarcoma, as well as the B cell malignancies primary effusion lymphoma (PEL) and multicentric Castlemen's disease (MCD) (77, 83, 141, 406). Epidemiological studies on KSHV have shown that, unlike other herpesviruses, which typically share wide spread distribution, KSHV seropositivity varies greatly based on the region sampled.

Worldwide, 2% to 5% of the human population is seropositive for KSHV, but in Mediterranean regions, the number increases to 15% to 25% (6, 64, 74, 390). In sub-Saharan Africa where KSHV is endemic, seropositivity for KSHV ranges from 50% to 60% (110, 120, 221). In the United States and Western Europe where KSHV is more prevalent among homosexual males, studies have shown that 25 to 60% of HIV-positive men were also seropositive for the KSHV (84, 144, 220).

e. *Kaposi's sarcoma-associated herpesvirus*

i. **Identification and classification of Kaposi's sarcoma-associated herpesvirus**

KSHV/HHV-8 is the most recently discovered human herpesvirus. Although rare in the United States and Western Europe, during the 1980's as the number of HIV cases began to substantially rise, so did the incidence of a rare form of cancer, Kaposi's sarcoma. Referred to as AIDS-KS, this form of KS was found to be much more prevalent among homosexual males, compared to transplant patients, hemophiliacs, and children, who acquired their HIV infection either from grafted tissues, blood products, or paternally. This difference, provided the first observational evidence suggesting a possible sexually transmitted cause (141, 144, 220). In 1994, using representational difference analysis to search for DNA sequences, Chang *et al.* confirmed the presence of viral DNA in AIDS-KS tissue that was absent from normal tissue (83). Upon further sequence analysis, the virus was shown to contain features consistent with those of the *Herpesviridae* family, including 66 conserved herpesvirus genes and the presence of G+C rich terminal repeat sequences (367). The virus was subsequently named human herpesvirus 8 (HHV-8), though commonly referred to as Kaposi's sarcoma-associated herpesvirus (KSHV). Since the discovery of KSHV, sexual transmission has always been thought to be the most common route of infection among immunocompetent populations within low seroprevalence. However, recent work by Casper *et al.* has questioned this long accepted concept. Tracking oropharyngeal shedding of KSHV in 46, HIV-negative, homosexual males, the authors noted 61% of those men experienced oropharyngeal shedding of KSHV over the course of the study (72). This data is supported by work by

13

Kazanji *et al.* who demonstrated that children from low-income, over crowded areas where KS is endemic, readily secrete virus in their saliva. Their findings suggest that within high prevalence areas, children may be an important source for child-to-child, child-to-sibling, and child-to-parent transmission of KSHV (218). Together, these data suggest that oral shedding and saliva may play a larger role in KSHV infection, both in high and low prevalence areas, then previously suggested.

Phylogenetic analysis of the major capsid protein of KSHV revealed that it is a member of the gamma-2 or rhadinovirus genus of the *Gammaherpesvirinae* subfamily and is closely related to Herpesvirus saimiri (HVS), a rhadinovirus that naturally infects squirrel monkeys (*Saimiri sciureus*). Although HVS infection of squirrel monkeys is not associated with disease, when infection occurs in other species of new world monkeys, it is associated with fatal T cell lymphomas. Furthermore, HVS has been shown to possess transforming properties with the ability to immortalize human T cells in vitro (133). Another rhadinovirus with considerable sequence homology to KSHV, rhesus rhadinovirus (RRV), was isolated from rhesus macaques (113, 382). As RRV is a subject of this thesis dissertation, it will be discussed in greater detail in later sections.

ii. KSHV-associated diseases

1) *Kaposi's sarcoma (KS)*

Originally described by Moritz Kaposi in 1872 as "idiopathic multiple pigmented sarcomas of the skin", KS is characterized as inflamed papular nodules that are typically found on the skin, but can also form in the mouth, gastrointestinal tract and respiratory

tract (212). Despite the name, KS is not a classic malignancy, in the sense that, many

cancers generally arise from a single cell type and metastases are often clonal in nature,

while KS lesions are typically comprised of many types of cells. The predominant cell

type of a KS lesion is the spindle cell, which can have either a lymphatic and vascular

endothelial origin (53). While the spindle cell is a large component of a KS lesion, the

rest of the tumor is an amalgam of cell types forming a network of abnormal vasculature

filled with various inflammatory infiltrates. These additional cells may or may not be

infected with KSHV, but in either case are responsible for promoting the characteristic

bruise-like appearance (114, 122, 180). Furthermore, in patients suffering from AID-

related KS, the KS lesion may or may not be clonal, in that, separate lesions may come

from a common progenitor (e.g. clonal origin) or, possibly arise from separate KSHV

infections (e.g. polyclonal origin) (117, 122, 180, 339). Regardless of clonality, KS

lesions produce increased levels of pro-inflammatory cytokines, chemokines, and growth

factors such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin (IL) -

1β, IL-6, regulated upon activation, normal T cell expressed and secreted (RANTES),

macrophage inflammatory protein (MIP) -1α and -1β, monocyte chemotactic protein-1

(MCP-1), IL-8, as well as angiogenic factors like basic fibroblast growth factor (bFGF)

and vascular endothelial growth factor (VEGF) (121). Several *in vitro* models using KS

spindle cells report a survival and/or growth requirement for many of the aforementioned

inflammatory proteins (121, 122). It is this requirement that suggests that KS is in deed a

hyperplasic lesion and not a true neoplastic growth (141).

There are four types of KS, all of which have been shown to be associated with

KSHV infection. Classic KS is characterized as a relatively indolent disease affecting

elderly men from Mediterranean region or those of Eastern European descent, usually

affecting the lower extremities and rarely involving internal organs. Endemic KS is a

much more aggressive form of the disease prevalent in sub-Saharan Africa. Normally

occurring in children and young adults, endemic KS results in skin lesions of lower

extremities (like classic KS), but in a much more aggressive nature, can also affect

visceral and lymphatic organs, which can lead to increase mortality (423). Although rare,

post-transplant patients under immunosuppressive therapy can develop a mild form of

KS, referred to iatrogenic KS. The disease presents with KS lesions on the skin and oral

mucosa and generally resolves once immunosuppression is halted (122). The last form of

KS is associated with late stage HIV disease, or AIDS-KS. Unlike previous forms of KS,

AIDS-KS is much more aggressive. Early lesions typically appear on the face and torso,

and present as reddish to pink macules (no topology, only coloration) or papules

(elevation). In prolonged episodes, these lesions can spread over the entire body and can

coalesce into larger plaques. Furthermore, in extreme cases, the visceral organs and

gastrointestinal tract may also contain KS lesions (151). In numerous cases, appropriate

treatment with antiviral therapies such as protease inhibitors can result in complete

remission of KS (75).

Like other herpesviruses, KSHV infection is relatively asymptomatic in the

immunocompetent host, the result of a proper functioning immune system. However,

when a person becomes immune suppressed, KSHV can exit latency and reactivate into

lytic replication, which can lead to disease. Given that KSHV infection and the onset of

KSHV-associated diseases is much worse in patients with a diminished T cell responses

(e.g. immune suppression and AIDS), this suggests that proper T cell function is critical

for controlling KSHV infection (141). Several studies have demonstrated that Cytotoxic

lymphocytes (CTL) play vital roles in controlling KSHV infection. Lambert *et al.*

demonstrated that patients with AIDS-KS displayed reduced capacity to mount anti-

KSHV CTL responses, as compared to patients with transplant-KS and classic-KS (241).

These differences correlate well with the progression of KS, where patients with larger

and more frequent anti-KSHV responses were able to control the infection and not

progress to KS.   Furthermore, Bihl *et al.* showed that CTL responses exist for both lytic

and latent antigens, underscoring the ability of the healthy immune system to provide

constant pressure against KSHV infection, independent of the viral life cycle (41).

Although representing a relatively small proportion of CTLs (less than 5%), $\gamma\delta$ T cells,

have been shown to react strongly to KSHV *in vitro* and prevent the release of infectious

virus during induction of lytic replication, further underscoring the importance of

immunological control (28). The molecular mechanisms of KSHV that evade CTL-

mediated killing are discussed in greater detail in section 4a. In addition to CTL

responses, antibody titers also correlate with KS disease progression, albeit in an inverse

fashion. Newton *et al.* tracked 189 patients seropositive for KSHV. Their findings show

that patients with high anti-KSHV antibody titers have an increased risk of developing

KS (304). This correlation is independent of HIV status and provides an indicator for

those who may be at a higher risk for developing KS.

KSHV infection may be necessary for the development of KS, but the virus alone

is not sufficient for the onset of KS. As previously stated, approximately 3% of the

population in the United States and Western Europe are seropositive for KSHV, but the

risk of these individuals developing KS is estimated at roughly 1 in 10,000 per year,

suggesting additional factors must be present for advancement of KS disease (149, 170).

In the case of AIDS-KS, two observations clearly suggest that the additional factor is

HIV infection. First, a strong association exists between KS risk and HIV viral load

(275). Second, the significant decrease in incident KS as well as remission of clinical

AIDS-KS in HIV-positive patients undergoing highly active antiretroviral therapy

(HAART) has been noted (147, 333). Although the mechanism by which HIV promotes

a higher risk of KS is unknown, Ensoli *et al.* has suggested that secreted HIV tat protein

might be acting as a growth factor and/or proangiogenic stimuli for KS cells (122, 123).

Furthermore, decreased immune function as a result of HIV-induced or chemically-

induced immunosuppression might result in increased KSHV lytic replication. Much less

is known about the additional stimuli needed to promote classic and endemic KS disease,

although several reports have linked higher risk of classic and endemic KS to increased

environmental iron oxide levels. Interestingly, this might offer a non-hormonal

explanation for the higher prevalence of KS among men, as women tend to have lower

iron reserves (397, 398, 464).

2) *Primary effusion lymphoma (PEL)*

Primary effusion lymphoma, also referred to as body cavity lymphoma (BCL), is

a rare disease predominantly seen in patients with end-stage AIDS. The disease is

characterized by the formation of neoplastic lesions due to unregulated B cell

proliferation within serosal cavities and sometimes in solid organs (141). In contrast to

KS, PEL is a classic malignancy with all of the B cells being clonal and all containing

high levels of KSHV DNA. All PELs are thought to arise from KSHV-infected

postgerminal center B cells, and express CD138, an adhesion molecule expressed by a subset of pre-B cells and by plasma cells (151). Interestingly, approximately 80% of PEL cells are also positive for EBV, but to date, no synergy between KSHV and EBV has been described. Unfortunately, PEL is generally unresponsive to chemotherapeutics and carries a poor prognosis (54).

3) *Multicentric Castlemen's disease (MCD)*

Castleman's disease is a rare, polyclonal B cell malignancy seen in both HIV-positive and HIV-negative patients (115). The disease presents in two forms, a localized form and a systemic form. The localized form is typically seen in HIV-negative patients and is characterized by a hyperplastic lesion isolated into a single node or space. This form of the disease is not associated with KSHV infection and is often treated by excision of the affected area. The systemic form, also known as multicentric Castleman's disease, is a much more aggressive illness characterized by sweats, fever, lymphadenopathy, splenomegaly, and excessive weight loss. Although MCD can be observed in HIV-negative patients, it is more frequent in AIDS patients (406). Furthermore, MCD is generally associated with KSHV infection, and up to 30% of B cells in mantle zones surrounding germinal centers are positive for KSHV DNA (215, 318).

iii. **Models of KSHV pathogenesis**

Researchers studying KSHV pathogenesis are faced with a number of challenges specific to KSHV. Although KSHV has been found to infect many different cell types (e.g. B cells, endothelial cells, fibroblasts, epithelial cells, and macrophages), the virus's

strong drive toward latency means that lytic replication is limited, and in several of the cell types mentioned, although viral infection was confirmed in many cell types, progeny virus was never detected (347). Moreover, in most *in vitro* studies of KSHV infection, a majority of cells become latently infected, while only a small percentage (~1-5%) of cells support lytic replication (32, 292). As a result, KSHV is difficult to propagate to high titers and does not form plaques in traditional plaque assays. This lack of lytic replication poses a significant problem to investigating properties of KSHV during *de novo* infection, such as lytic gene transcription and viral replication. More importantly, without a reliable *in vitro* system of lytic replication, the production of virus stocks or recombinant virus is difficult.

There are several *in vitro* model systems currently used to study KSHV pathogenesis, but to date, no adequate *in vivo* model exists for KSHV. Latently infected PEL cell lines can be induced to undergo lytic replication following treatment with phorbol esters or sodium butyrate, and are most frequently used for the study of lytic replication, and as a source for infectious virus (23, 349). Although induced PEL cells are a valuable resource for many aspects of KSHV pathogenesis, the system is not efficient because only ~20% of the cells enter lytic replication. Furthermore, without induction, ~2% of the PEL cells enter spontaneous lytic replication (284). The presence of a mixed population of lytic/latent cells is not optimum for studying aspects of viral life cycle, such as replication and transcription.

The complex microenvironment of a KS tumor is notoriously difficult to recapitulate *in vitro*. Primary cultures of KS spindle cells require the presence of serum and growth factors to survive *ex vivo* and will not grow in soft agar assays. More

importantly, primary KS cells lose the ability to maintain the KSHV genome over time

(135, 247, 346). Together, these findings have led to the paracrine hypothesis for KS

pathogenesis, where KSHV induces a microenvironment rich in growth factors that help

support the proliferation of KS tumor cells, and suggest that the transforming nature of a

KS tumor is a highly concerted process (372). Several *in vitro* system utilizing

immortalized endothelial are currently being used to mimic the KS microenvironment.

KSHV infection of dermal microvascular endothelial cells (DMVEC) immortalized with

either E6 and E7 from human paplomavirus or human telomerase reverse transcriptase

(h-TeRT) results in a latent infection characterized by the formation of spindle cells that

closely resemble cellular morphology associated with KS (32, 92, 239, 292).

Furthermore, like PEL cells, lytic replication can be induced using phorbol esters, albeit

with fewer cells entering lytic replication (~5%). Unlike primary cultures, these cells

maintain the KSHV genome and represent a valuable tool for studying KSHV

oncogenesis.

Several open reading frames of KSHV (e.g. vGPCR, vIL-6, and vIRF1) have been

shown to possess tumorgenic properties (20, 26, 143). A common technique for studying

in vivo oncogenic potential is the use of severe combined immunodeficient or SCID mice.

Since these mice are deficient in both B and T cells, exogenous cells stably transfected

with genes of interest can be administered without the risk of rejection. Tumorigenesis is

typically evaluated 10-12 weeks following injection and is based on the size of the

resulting tumor, as compared to negative controls.

In 1999, Dittmer and colleagues reported *de novo* KSHV infection in SCID-hu

Thy/Liv mice. These SCID mice were humanized by the coimplantation of fetal thymus

and liver under the kidney capsule of recipient mice. Following recovery (~4 to 7 months), these mice were infected with ~10^7 DNA equivalents of KSHV directly into the grafted tissues, and lytic and latent infection was followed using a variety of techniques. Their findings suggested that limited lytic replication occurred in the implanted tissue followed by a sustained latent infection, and that CD19$^+$ B cells were the site of infection. Furthermore, the infection was limited to the grafted tissue and did not spread to the blood or surrounding tissues. Although limited *de novo* infection was detected by real-time PCR, no virus was isolated or pathology was associated with the model and shows the limited nature of the technique.

Recently, several investigators have described the ability of KSHV to infect and elicit KSHV-like disease in a NOD/SCID mouse model. The first of these was Parsons *et al.*, who demonstrated that they could establish a productive KSHV infection in the NOD/SCID mice (320). Following infection, the authors were able to detect the presence of KSHV DNA, both lytic and latent genes, up to four months post infection by qRT-PCR. Furthermore, they show LANA expression in approximately 0.5% to 1% of isolated splenocytes. Additional characterization showed that the expression of LANA could be seen in murine B cells, NK cells, macrophages, and dendritic cells. Most importantly, using transmission electron microscopy, the authors demonstrate the presence of KSHV virions in a small number of splenocytes preparations.

Mutlu et al. utilized the NOD/SCID model in a slightly different manner. The authors used a KSHV bacterial artificial chromosome (BAC) that encodes GFP under a KSHV promoter. Using this BAC, the author's transfected mouse bone marrow endothelial cells and selected for antibiotic resistance and GFP expression. These cells

were: 1) injected subcutaneously (s.c.) into the flanks of NOD/SCID mice, and 2) injected intravenously. Animals that received s.c injections developed green "vascularized spindle cell sarcomas", histologically reminiscent of human KS tissue (298). Furthermore, mice that received i.v. injections, developed multi-focal invasive spindle cell sarcoma lesions in their lungs. These KS-like tumors were highly angiogenic and expressed high levels of VEGF and podoplanin, many of the same hallmarks as human KS tissue. Therefore, for the first time, a small animal model is capable of supporting KSHV growth and induces disease consistent with KS. The work by Mutlu et al. also used small interfering RNA (siRNA) against vGPCR, a well characterized KSHV oncogene, which led to a block in VEGF expression and tumorigenesis. The ability to target single genes and measure a clear effect on tumor growth, demonstrates the utility and value of this model.

Although these models are valuable and provide considerable information, they do not take into account the dynamic host/pathogen interactions that take place during a natural infection and inevitably lead to disease. An alternative approach is to develop an animal model that naturally harbors a viral pathogen that is closely related to KSHV. Rhesus macaques are an excellent model for studying human pathogens because of their related physiology and the observation that many human pathogens have rhesus homologues or are themselves rhesus pathogens. Rhesus macaques harbor a virus referred to as rhesus rhadinovirus (RRV) that is closely related to KSHV (113, 382) and will be discussed in the next section.

f. *Rhesus rhadinovirus as a model for studying KSHV pathogenesis*

i. General overview of RRV

Rhesus rhadinovirus strain 17577 (RRV) was isolated from a simian immunodeficiency virus (SIV)-infected rhesus macaque (*Macacca mulatta*) that had developed B cell hyperplasia. Upon further genomic and phylogenetic analysis, RRV was found to be a γ-herpesvirus that closely resembled KSHV (figure 1.3) (14, 54, 113, 453). The RRV and KSHV genomes exhibit a high degree of co-linearity of, with 67 of 79 ORFs sharing some level of homology (*reviewed in(382)*. Furthermore, RRV appears to have homologues for many of the genes involved KSHV pathogenesis (e.g. vGPCR, vIL-6, vCD200, vMIP, LANA) (382). Importantly, rhesus macaques experimentally co-infected with SIV and RRV develop pathology similar to humans infected with HIV and KSHV, such as chronic lymphadenopathy, B cell hyperplasia, as well as other lymphoproliferative disorders. However, these animals do not develop a KS-like disease. Based on genomic and pathogenic evidence, RRV is the rhesus macaque homologue of KSHV.

ii. RRV-associated diseases

Following experimental coinfection of RRV and SIV, macaques develop disease that is consistent with the disease progression and lymphoproliferative disorders observed in KSHV/HIV patients. Initial onset of disease in the SIV/RRV coinfected monkeys generally presents as lymphadenopathy, splenomegaly, and hepatomegaly, but more importantly, B cell hyperplasia is detected in the peripheral blood, lymph node, and
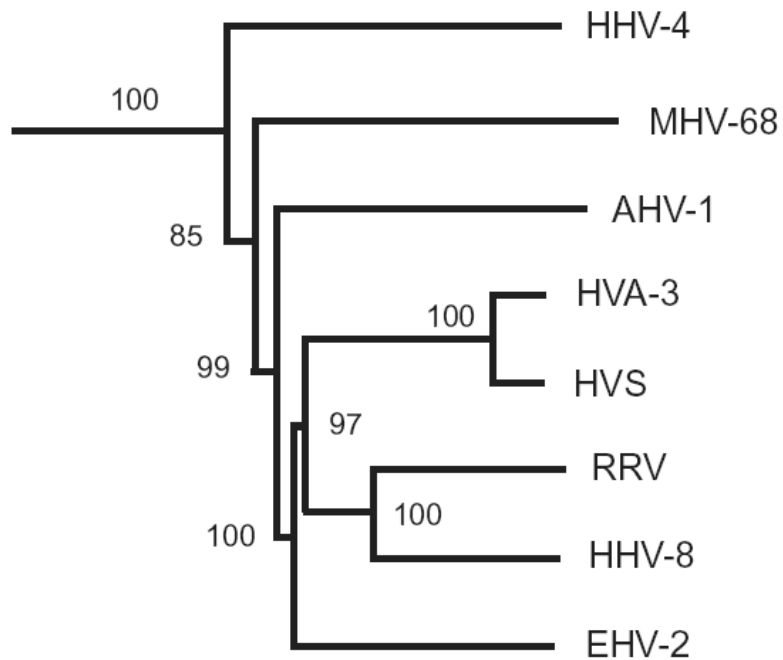
Figure 1.3 Phylogenetic analyses of the *Gammaherpesvirinae* subfamily. The diagram represents the consensus tree constructed from phylogenetic analysis of conserved regions in 26 herpesvirus open reading frames. The numbers represent the number of Protpars trees out of 100 that contained the same sequence to the right of the branch point as in the consensus tree. HHV-4 = human herpesvirus 4 or Epstein-Barr virus, MHV-68 = murine herpesvirus 68, AHV-1 = alcelaphine herpesvirus, HVA-3 = ateline herpesvirus 3, HVS = Herpesvirus saimiri, RRV = rhesus rhadinovirus, HHV-8 = human herpesvirus 8 or Kaposi's sarcoma-associated herpesvirus. *Adapted from reference (14) with permission.*

spleen. Additionally, hypergammaglobulinemia, a condition often associated with B cell hyperplasia and characterized by elevated serum IgG levels (> 2000 mg/dl), is often observed in the infected macaques (242, 276, 319). The majority of rhesus macaques coinfected with SIV and RRV develop persistent multicentric angiofollicular lymphadenopathy resembling the multicentric plasma cell from of MCD. Furthermore, 20% of SIV/RRV coinfected animals go on to develop non-Hodgkin's B cell lymphoma (453). RRV DNA can be detected in all lymphoid tissues associated with disease in the infected macaques. Furthermore, RRV is able to establish a lifelong persistent infection in CD20$^+$ B cells of naturally infected macaques (37). Although RRV is highly prevalent among colony-raised rhesus macaques, disease is not observed in immunocompetent animals, indicating that coinfection with SIV and the subsequent immunosuppression may be required for the onset of RRV pathogenesis.

iii. **Utility of RRV in studying KSHV pathogenesis**

Rhesus Rhadinovirus offers a reliable and reproducible model system for studying KSHV pathogenesis both *in vitro* and *in vivo*. Unlike KSHV, RRV is not hindered by the same *in vitro* limitations, and given that RRV grows well in culture and to high titers, many aspects of viral biology that require *de novo* lytic infection, such as lytic viral replication and gene transcription can now be studied. With a better understanding of RRV pathogenesis and viral biology, we can draw parallels between the two viruses and answer questions regarding KSHV pathogenesis that would otherwise go unanswered. Furthermore, the ability of RRV to infect and cause disease in the SIV-infected macaque represents an ideal *in vivo* system for investigating many aspects of KSHV pathogenesis

that cannot be answered by *in vitro* studies alone. Specifically, the SIV/RRV system is the only *in vivo* model for studying lymphoproliferative disorders associated with KSHV.

Although RRV represents the most attractive model for studying KSHV pathogenesis, other viral models do exist. Murine gammaherpesvirus-68 (MHV-68) is often used as a murine model for studying KSHV pathogenesis. While useful for studying vasculitis, a disease that presents with abnormal vasculature similar to that of KS, mice infected with MHV-68 do not develop the more serious lymphoproliferative disorders (e.g. PEL, MCD, and other B cell lymphomas) associated with KSHV infection. Furthermore, phylogenetic analysis shows that MHV-68 is more distantly related to KSHV as compared to RRV.

Another strain of RRV, RRV_{H26-95} has been utilized as a model of KSHV pathogenesis. Like RRV_{17577}, RRV_{H26-95} has considerable sequence homology to KSHV and is capable of lytic infection *in vitro*. Unlike RRV_{17577}, RRV_{H26-95} does not cause disease in SIV-infected macaques. The reasons for this difference are unknown, but recent sequence analysis has revealed genomic differences which may play a role in RRV pathogenesis. Although point mutations exist throughout the RRV_{H26-95} genome (as compared to RRV_{17577}), one gene in particular appears to be significantly different. While most genes encoded by RRV_{H26-95} and RRV_{17577} share considerable amino acid similarity (~98%), the compliment control protein (RCP) encoded by ORF 4 is considerably less homologous between the two strains (~60%). In addition, Mark *et al.* has shown that RCP exhibits alternative splicing in RRV_{17577}, producing three separate transcripts, all of which are expressed and secreted, while RRV_{H26-95} only expresses one.

Whether this difference confers disease *in vivo* is unclear, but the lack of *in vivo* pathology associated with RRV$_{H26-95}$ highlights the limited nature of its use.

Rose and colleagues have described viral sequences found in retroperitoneal fibromatosis (RF), a rare mesenchymal lesion with similar cellular morphology to KS found in rhesus macaques. Sequence analyses on 4.3 kilobases of DNA found in RF tissue, suggest that they originate from a gammaherpesvirus with homology to KSHV, which they named rhesus fibromatosis herpesvirus (162, 363, 416). Utilizing a lambda phage library prepared from RF tissue, they have been able to reconstruct the majority of the RFHV genome, yet to date; the authors have not been successful in isolating virus from RF tissue or from a macaque, so the contribution of this virus to furthering KSHV pathogenesis is quite limited (368).

2. Human Poxviruses

a. *Classification*

The *Poxviridae* family is a unique group of large, complex DNA viruses that replicate exclusively in the cytoplasm. With the exception of *Molluscipoxvirus* genus, all poxviruses possess a lifecycle that is highly lytic, often resulting in significant pathology, and in some cases death. The *Poxviridae* family is divided into two subfamilies: *Chordopoxvirinae*, composed of eight genera of vertebrate poxviruses, and *Entomopoxvirinae*, containing three genera of insect poxviruses. Within each subfamily, the genera are segregated based on genomic similarity (size and G+C content), virion morphology, mode of transmission, and host range. The genera of *Chordopoxvirinae* are

Orthopoxvirus, *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*,

Molluscipoxvirus, *and Yatapoxvirus*. Several of these genera have been shown to contain

viruses capable of infecting humans and will be discussed later in this section. The

genera of *Entomopoxvirinae* are *Alphaentomopoxvirus*, *Betaentomopoxvirus*, and

Gammaentomopoxvirus, but these viruses will not be covered in this body of work.

Table 1.1 details the genera of the *Chordopoxvirinae* subfamily and provides

characteristic features of each genus and the species contained within (294).

b. *Virion structure*

Compared to other viruses, poxviruses are exceptionally large and, with the

typical size of a poxvirus being roughly 360x 270x250 nm, represent the only virus

visible using light microscopy. Poxviruses can take on several morphological forms, but

the fundamental infectious unit is the mature virion (MV). High resolution images on

vaccinia virus (VACV) have shown that MVs are comprised of several key structures: the

core, lateral bodies, and envelope (104). Although slight structural variations are present

between the different genera, these key structural elements are consistent among all

poxviruses.

The inner most structure is the viral core, which is comprised of a dense inner

layer, housing the viral genome, RNA polymerase, transcription factors, capping enzyme,

poly(A) polymerase, and a variety of non-enzymatic proteins, all of which are encased by

a core wall. The core wall itself is also made of two different structures, an inner layer

and out layer. With the exception of several channels, the inner layer is a continuous

lipid membrane. The outer layer forms a palisade structure with T-shaped spikes

Table 1.1 Members of the *Chordopoxvirnae* Subfamily

Genus	Virus Species	Features
Orthopoxvirus	*camelpox*, *cowpox*, *ectromelia*, *monkeypox, raccoonpox*, *skunkpox*, Uasin Gishu*, vaccinia, variola,* volepox	DNA ~200kbp, G+C ~36%, wide to narrow host range
Parapoxvirus	Auzduk disease, *bovine papular stomatitis*, chamois contagious ecthyma*, orf,* pseudocowpox*, parapox of red deer*, sealpox, squirrelpox	DNA ~140kbp, G+C ~64%,
Avipoxvirus	*canarypox*, crowpox*, fowlpox, juncopox, mynahpox, pigeonpox, psittacinepox, quailpox*, peacockpox, peguinpox*, sparrowpox, starlingpox, turkeypox*	DNA ~260kbp, G+C ~35%, birds, arthropod vector
Capripoxvirus	*goatpox, lumpy skin disease, sheeppox*	DNA ~150kbp, ungulates, arthropod vector
Leporipoxvirus	*hare fibroma, myxoma, rabbit fibroma, squirrel fibroma*	DNA ~160kbp, G+C ~40%, leporids and squirrels
Suipoxvirus	swinepox	DNA ~170kbp, narrow host range
Molluscipoxvirus	*molluscum contagiosum*	DNA ~180kbp, G+C ~60%, human host, localized tumors, contact spread
Yatapoxvirus	*tanapox, yaba monkey tumor*	DNA ~145kbp, G+C ~33%, primates and possibly rodents

anchored into the inner membrane (182). Detailed structural analysis of the "dumb-bell"

shaped core shows two prominent features, a dense layer just under the core wall with a

fiber-like morphology consistent with nucleoprotein, and cylindrical elements that form

S-like structures that co-localize with the core wall channels (196, 327).

Poxviruses have a linear double-stranded DNA genome that can vary

considerably in length between viral species.   All poxvirus genomes have inverted

terminal repeats (ITRs) consisting of an A+T rich, incompletely base-paired, hairpin loop

that links the two DNA strands (30, 146). Furthermore, ITRs also contain a small (less

than 100bp), highly conserved region required for resolution of replicating concatemeric

DNA (111).   The viral DNA exists in a complex with a variety of viral nucleoproteins in

a structure consistent with cellular nucleosomes (294).

Almost 100 genes are conserved among all chordopoxvirues. Genes that are more

conserved (e.g. involved in replication processes) tend to be located near the center of the

genome, while less conserved genes (e.g. involved in host interaction) tend to be located

toward the ends of the genome (435). The arrangement of the genes is so similar, that a

convention for naming was established. Digestion of VACV Copenhagen (COP) strain

with the endonuclease Hind III generates 15 fragments, lettered A through O. Within

each fragment, ORFs are numbered from left to right, and finally, an L or R designation

is used to describe the direction of the ORF. Additionally, a numbering scheme was also

developed using VACV Western Reserve (WR) strain, where ORFs were numbered

sequentially from one end of the genome to the other. For the purposes of consistency,

the Hind III convention will be used when describing VACV genes (294).

Zartouw *et al.* determined the chemical composition of VACV and estimated that roughly 90% of the total mass of the MV is protein. Proteomics on non-core associated fractions have shown the presence of 30 prominent protein bands on a reduced one-dimensional SDS-PAGE gel. This number increases to 75 when samples are resolved in two dimensions (202). Roughly 25 proteins have been localized at or near the virion surface. These proteins have been divided structurally into groups based on the presence or absence of a transmembrane domain and functionally into those required for morphogenesis and those with a role in viral entry (294). Roughly 50 proteins are associated with the viral core. Of these, 30 possess some enzymatic function and half of these proteins are involved in RNA synthesis. The remaining proteins are postulated to be involved in morphogenesis or play a structural role (118).

As mentioned, poxviruses possess four distinctly different morphological forms: mature virion (MV), enveloped virion (EV), cell-associated enveloped mature virion (CEV), as well as an intermediate form that possesses three membranes, termed wrapped virion (WV) (Figure 1.4). Previous nomenclature took viral location within the infected cell into account and referred to MVs as intracellular mature virion (IMV) and EVs as extracellular enveloped mature virion (EEV). The intermediate form, WV, was referred to as intracellular enveloped virion (IEV). For the purposes of consistency, the current nomenclature of MV, EV, CEV, and WV will be used in this work. Much of the information presented has been based on MVs, because EV and CEV particles contain an additional membrane, they possess unique features not present in MV particles. The primary difference between MV and envelope particles is the additional membrane, containing eight virally encoded glycoproteins. Studies suggest that differences between
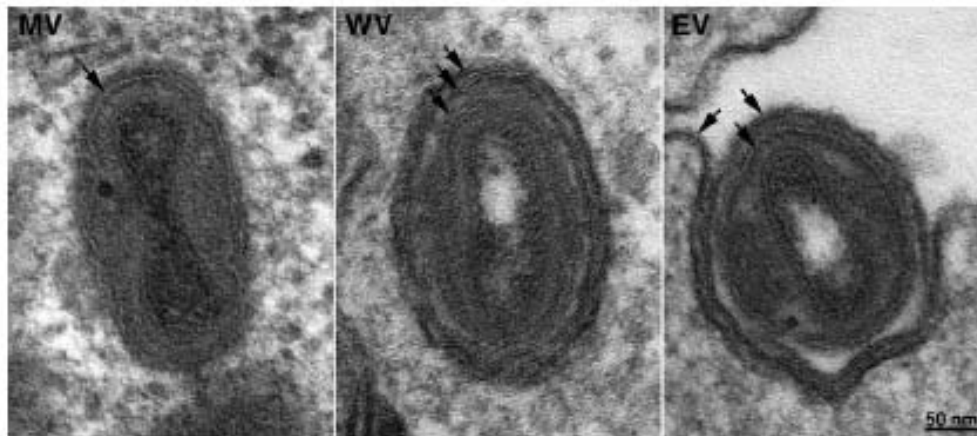
Figure 1.4 Morphological forms of poxviruses. (Left) Mature virion (MV) with the characteristic dumbbell shaped core flanked by lateral bodies, and surrounded by a single membrane (arrow). (Middle) The intermediate form, wrapped virion (WV), containing two additional membranes (arrows). (Right) Extracellular virion (EV) budding from the infected host cell, the third membrane present of the WV has fused with the plasma membrane of the cell (left arrow), thereby releasing the EV particle. *With permission (295).*

MV and EV surface proteins might play a role in mediating different mechanisms of viral entry (82, 437). In general, enveloped virions tend to mediate viral spread within the infected host, either by dissemination throughout the body (EV or CEV) or through cell-to-cell spread (CEV) (21, 47, 102, 323). However, studies investigating VARV and MPV infection of non-human primates suggest that a major part of these infections is mediated by monocyte-associated (CEV) viremia, and that levels of EV in the serum of infected animals are undetectable (199, 460). Given that the presence of an additional membrane reduces the overall stability of EV and CEV particles, host-to-host spread is thought to be mediated by the more stable MV particles (294).

The last conserved structural elements found within poxviruses are the lateral bodies. These are regions of aggregated, heterogeneous material that typically occur in the concave regions of the core. Specific functions have not been assigned to the lateral bodies or its constituents.

c. *Poxvirus lifecycle*

i. **Fusion and entry**

The poxvirus lifecycle begins with the virus gaining entry into a permissive cell. Although a specific cellular receptor utilized by poxviruses has not been identified, studies have shown that poxviruses can enter permissive cells through a variety of different mechanisms (295). At neutral pH, VACV attachment proteins on the surface of MV particles can directly interact with glycosaminoglycans on the surface of a permissive cell, resulting in fusion of the viral envelope with the plasma membrane,

thereby releasing the viral core into the cytoplasm. This effect can be inhibited by depleting plasma membrane cholesterol, suggesting that lipid rafts may be involved (90). Further evidence suggests that the attachment protein D8L binds chondroitin sulfate, while two other viral proteins, A27L and H3L, bind heparin sulfate (89, 190, 191, 259). The use of genetic knockout viruses in attempts to isolate specific attachment proteins responsible for entry has been unsuccessful, with all of the attachment proteins classified as "non-essential". This suggests redundant systems may be in place (69).

Through the use of conditional mutants, a number of viral proteins have been identified as having key roles in viral entry. The VACV proteins A16L, A21L, A28L, G3L, G9R, H2R, J5L, and L5R have been suggested as a putative entry complex (384). Six of the eight proteins are required for VACV entry, but not assembly of MVs or EVs (309, 385, 386). All eight proteins of the entry complex are highly conserved among all poxviruses, suggesting a conserved function. Two additional proteins, F9L and I2L, are not part of the entry complex, but are required for entry and fusion (295).

Prior to high resolution imagery, evidence for other morphological forms containing additional membranes initially came from failed inactivation assays, where neutralizing antibodies specific for MV failed to protect against viral challenge. Once high resolution imagery was available, images showed the presence of MV particles within cytoplasmic vesicles of VACV-infected cells. This coupled with evidence showing inhibition of endosomal acidification or inhibition by agents that block actin-mediated internalization profoundly inhibits VACV entry, suggests that poxviruses may also utilize a mechanism involving endocytosis of the viral particle, followed by endosomal acidification, and finally fusion of the MV envelope and the membrane of the

endosome resulting in the release of the viral core into the cytoplasm (106, 295, 324, 436). Research suggests that this latter mechanism is believed to mediate CEV and EV entry, where the additional outer membrane is lost during the endocytosis process and fusion and entry proceed via the conserved MV entry complex (295). Regardless of which pathway is utilized, once fusion has occurred, the poxvirus lifecycle proceeds in the same manner.

ii. **Viral transcription and gene expression.**

Like the herpesviruses, poxvirus transcription follows a strict cascading mechanism to ensure proper timing and expression of viral proteins. The three poxvirus gene classes are early, intermediate, and late. Shortly after entry, the viral core is translocated on microtubules to the site of replication within the cytoplasm of the newly infected cell. The viral core contains all the necessary machinery for early gene transcription, which begins roughly 20 minutes after the viral core is released into the cytoplasm. Early genes encode enzymes and cofactors required for the synthesis of viral DNA and the transcription of intermediate genes. These include, DNA-dependant RNA polymerase, RNA-associated protein 94 (RAP94), topoisomerase, VACV early transcription factor (VETF), nucleotide phosphohydrolase I (NPH1), as well as enzymes responsible for polyadenylation, methylation, and capping of viral mRNA. Nearly half of the VACV genome is transcribed prior to DNA replication, and in addition to the transcriptional machinery listed above, includes proteins involved in host interaction and DNA synthesis (50, 70). Additionally, all poxvirus genes appear to encode a single

protein and there is no evidence of RNA splicing. Early gene transcription ends with the disruption of the viral core, a process referred to as uncoating (61, 294).

Intermediate gene transcription is dependant on viral DNA synthesis, and begins following the disassociation of the viral core, roughly 60-90 minutes post-infection. Intermediate gene products are largely comprised of enzymes and transcription factors required by the late gene class. Three key intermediate gene products are the viral RNA polymerase and viral intermediate transcription factor 1 and 3 (VITF 1 and 3). VITF-1 is believed to be a viral homologue of cellular transcription factor IIS (TFIIS), and both VITF-1 and -3, although lacking ATPase activity, exhibit sequence-specific DNA binding (360, 361). There is evidence for an *in vivo* requirement for the cellular protein VITF-2, a transcription factor normally found in the nucleus of uninfected cells, but present in the cytoplasm of infected cells. One possible role for VITF-2 in the viral lifecycle is to act as a check point between pre- and post-replicative phases of viral replication, signaling when a newly infected cell has reached a point to allow optimum viral replication (295).

The late gene class is a large class of transcriptional and structural genes, the products of which are involved in the final stages of viral transcription and producing the structural and non-structural elements for the formation of progeny virions. Late gene transcription begins when sufficient quantities of the prerequisite factors have been made through intermediate transcription, roughly 120 minutes to 48 hours, and continues until the death of the host cell (295). The four viral late transcription factors, VLTF-1, -2, -3, and -4, mediate the transcription of late genes, and although described in more detail in the following section, the structural and non-structural elements produced during late

gene transcription will make up the immature virion (IV), begin the process of packaging viral DNA and the transcription machinery, and are incorporated into nascent virions (219, 230, 279).

The rapid decline in mRNA levels for each of the three gene classes following transition into the next phase of transcription, suggests a mechanism for rapid turnover is in place. This adds an additional mechanism to insure the faithful timing of poxvirus gene expression (295).

iii. **Assembly and egress.**

Assembly of nascent poxvirus virions begins approximately 6 hours post infection, and starts with the accumulation of crescent-shaped structures within the cytoplasm, specifically within the electron dense, granular area referred to as the viral factory or viroplasm. These crescents will eventually make up the immature virion (IV) envelope. The exact origin of the crescents is controversial, but there are two hypotheses currently being tested. The lack of biochemical evidence linking crescents to any known cellular organelle suggests that the crescents are formed *de novo* (187). Alternatively, the crescents may be formed as flattened cisterna (i.e. flattened membrane) derived from a cellular organelle. The evidence for the latter comes from high resolution images that seem to support two closely apposed membranes, consistent with a flattened organelle. Neither model has been ruled out and further research is needed (163). Immature virions (IV) are formed when components of the core and viral particle come together in the presence of the D13L scaffold protein.

Although many aspects of poxvirus assembly are largely unknown, through the use of conditional VACV mutants, a number of proteins have been identified as essential for IV formation. Two IV membrane proteins, A14L and A17L, are essential for proper IV formation. Repression of either protein results in the accumulation of small vesicles and little to no crescent formation (355, 356, 429). Seven core proteins, A15L, A30L, D2L, D3R, F10L, G7L, and J1R, have been identified as required for association of crescents with viral factories (82, 422). The current hypothesis is that the proteins complex with one or more, membrane proteins present in the crescent that have not been identified. The function of one of these core-associated proteins, F10L, has been well documented as a Src-family kinase. Moreover, phosphorylation of A14L and A17L by the F10L kinase is required for proper IV formation (40, 138). Furthermore, repression of the major core protein, A10L, results in a similar phenotype as the core protein mutants, crescents unable to associate with the viral factories (294). Three other non-core proteins, H5R, G5R, and A1L, are also required for IV formation (105, 112, 350, 428, 443).

Immature viral DNA concatemers are processed into mature DNA genomes and accumulate in the cytoplasm as large crystalline structures (165). Following the formation of the IV, packaging of viral genomic DNA takes place using a head-full mechanism (294). Three viral proteins have been shown to be essential for proper DNA packaging and subsequent morphogenesis. The protein encoded by A32L is predicted to possess a role in DNA packaging because of sequence homology to gene I of filamentous single-strand DNA bacteriophages, as well as IVa2 of adenovirus, both of which are ATPases involved in DNA packaging (227). Confirmation of this hypothesized function

was demonstrated as repression of A32L inhibits VACV genome packaging (73). Repression of the telomere binding protein, I6L, or the viral membrane protein, A13L, also results in a defect in DNA packaging, through an unidentified mechanism (168, 433).

As mentioned earlier, viral cores possess all the components required for early transcription. Therefore, along with the packaging of the viral genome, proteins of the early transcription machinery are also incorporated into the IV. The exact mechanism of how these proteins are faithfully incorporated into the IV is unknown. However, when expression of the RNA polymerase, RAP94, is repressed, non-infectious virus particles are formed. These particles are morphologically normal and contain the early transcription factor VETF, but lack the RNA polymerase, as well as the enzymes poly(A) polymerase, capping enzyme, topoisomerase, NPH I, and NPH II. The repression of one protein leading to a deficiency of five others, suggests that RAP94 is part of a larger tertiary structure and without RAP94, this structure is, in some fashion, disrupted (286, 294).

Once the genomic components and transcription machinery are incorporated into the IV, the particle undergoes a series of changes which promote the shift from IV to MV. The gross morphology of the particle changes from spherical to barrel-shape and although the exact mechanism behind the morphological change is unknown, the shift to the barrel-shaped MV is accompanied by the loss of the D13L scaffold protein, incorporation of additional membrane proteins, proteolytic processing, and reorganization of core proteins (176, 287, 402).

The processing and maturation of some of the proteins within the MV highlight the unique mechanisms poxviruses have adapted to their cytoplasmic environment. Several of the membrane proteins in the MV contain intramolecular disulfide bonds. Typically, these would be formed within the reducing environment of the endoplasmic reticulum (ER). However, proteins encoded by poxviruses do not pass through the ER, suggesting that poxviruses encode their own redox mechanism. Interestingly, three poxvirus proteins, E10R, A2.5L, and G4L have been shown to be part of a unique cytoplasmic redox mechanism (386-388). At least nine viral membrane proteins have disulfide bonds formed through this cytoplasmic redox system. In addition to disulfide bond formation, one membrane protein (A17L) and several core proteins (A3L, A10L, A12L, L4R, and G7L) require proteolytic processing in order for morphogenesis to occur. The cleavage point for all of the proteins is the same and occurs at the consensus sequence AG$\downarrow$X (438). Evidence suggests that the viral gene responsible for the cleavage is I7L (211) and repression of I7L, or another putative viral protease, G1L, effectively blocks morphogenesis (19, 176).

Following morphogenesis, the final stage of the poxvirus lifecycle is egress. Poxviruses utilize two methods of egress: 1) passive egress, where virus accumulates within the cell and is released upon lysis of the infected cell, and 2) active egress, where the virus is actively transported to the cell surface and released. During passive egress, the MVs of certain poxviruses can form dense, inclusion bodies as a means of storage prior to release via cell disruption. These inclusions are referred to as A-type inclusions to differentiate them from the viral factories, termed B-type inclusions (216). The formation of extracellular virions requires active egress, where MVs are trafficked within

41

the infected cell and either: 1) wrapped in a double membrane derived from modified trans-Golgi and endosomal cisternae and shuttled to the cell surface or 2) directly budded from the infected cell via exocytosis. In either case, the result is an EV with an additional membrane (186, 376). Several poxvirus proteins have been shown to be involved in the active egress pathway. The VACV protein A36R has been shown to interact with Kinesin, a motor protein, and therefore may help MVs to tether to the microtubule transport machinery (444). Although as many as ten viral proteins are incorporated into the wrapping membranes and, to varying degrees, are required for proper envelopment, conditional mutants of A27L, F13L, and B5R exhibit the most severe phenotypes. Two of these, B5R and F13L, are functionally linked, with F13L being required for Golgi localization of the B5R glycoprotein (195). Additionally, F13L contains a putative phospholipase domain that is required for proper wrapping and the formation of post-Golgi vesicles (194). Once the outer membrane of the wrapped virion (WV) fuses with the plasma membrane, EVs are released from the infected cell. Although some EVs are released into the medium and can promote long-range dissemination, the majority of EV particles remain associated with the cell, termed cell-associated enveloped virions (CEV). These virions can later be released when the host cell disintegrates or they can mediate cell-to-cell spread through their association with actin containing microvilli, also referred to as "comet tails" because of their distinct morphology. During the latter, a CEV-loaded microvilli rapidly thrusts into an adjacent cell when actin is rapidly polymerized (102, 186). Three viral proteins, A33R, A34R, and A36R, are vital to this actin-mediated process. Repression of any of these genes results in the loss of actin-containing microvilli formation and as a result, a small plaque phenotype is observed (359, 373).

42

Studies have shown that phosphorylation of A36R by Src-family kinases is required for an interaction between A36R and the adaptor protein Nck, which promotes the association of the actin assembly (138, 375).

d. *Poxviruses and disease*

Of the eight poxvirus genera, four have been shown to contain species with the ability to infect humans: *Parapoxvirus*, *Yatapoxvirus*, *Molluscipoxvirus*, and *Orthopoxvirus*. Most of these human infections are zoonotic in nature and occur with relatively low frequency, while two viruses are obligate human pathogens.

The genus, *Parapoxvirus*, has four species that have been shown to infect humans: Bovine popular stomatitis virus (BPSV), orf virus (ORFV), pseudocowpox virus (PCPV), and seal parapoxvirus. ORFV is a common pathogen in sheep and goats, while PCPV and BPSV are seen in cattle. All represent common occupational hazards for those in contact with infected livestock, and transmission to humans has been documented since the 18th century (Figure1.5a) (311, 354). In 1987, the first case of seal parapoxvirus infection of a human was reported when a seal handler contracted the disease (185). The clinical features of Parapoxvirus infection in humans are seen one to two weeks post infection, and often present as a single, painless, nodular lesion (107).

The genus, *Yatapoxvirus*, has two species capable of infecting humans, tanapox virus and yabapox virus, both indigenous to monkeys. First described in 1957, yatapox virus infection of human appears to localized to Kenya and is transmitted from monkeys to humans, where the disease progression is swift with a febrile response observed within

two to four days post-infection, followed by the development of skin lesions on the lower extremities, typically less than ten (Figure 1.5b) (203).

The genus, *Molluscipoxvirus*, contains only one species, molluscum contagiosum (MCV), and is pathologically unique among poxviruses. Unlike other poxviruses, MCV presents with benign, self-limiting skin tumors or papular eruptions (Figure 1.5c). There is no febrile response and little to no inflammatory infiltrates associated with MCV nodules. Transmission of the virus occurs through contact with broken skin, and although lesions can occur anywhere on the body, however, in adults genital lesions are often spread via sexual contact, leading to MCV's designation as a sexually transmitted disease (45).

The final genus, *Orthopoxvirus*, is the most well studied of all poxvirus genera, and contains four viruses capable of infecting humans: variola virus (VARV), cowpox virus (CPV), monkeypox virus (MPV), and vaccinia virus (VACV). Clinical manifestations of naturally occurring VARV infection begin 7 to 17 days post infection, typically with a high fever, followed by a systemic rash that starts out macular, but quickly progresses into a papular stage (Figure 1.5d). Lesions are more prominent on the face, oral mucosa, and extremities, but can also form on the torso. Mortality rates for VARV range from 10-30%, and death most often occurs between days 10 and 16 (107). Death is usually the result of pneumonia (both viral and/or bacterial), encephalitis, heart failure, and/or pulmonary edema (130). Prior to its successful eradication in 1977, VARV killed an estimated 300-500 million people worldwide in the 20th century (228).

Cowpox virus is a naturally occurring pathogen present in a wide host range in Europe and Asia. Although maintained in rodents, CPV is able to establish a fast

spreading infection in livestock. The most common source of zoonotic infection in

human is the domestic cat. CPV infection of humans results in single or multiple lesions

on the face and/or hands and "flu-like" symptoms that can last for several weeks. CPV is

most known for being the agent used by Edward Jenner to induce immunity to smallpox

(VARV) (107).

Monkeypox virus is a naturally occurring pathogen in African rodents.

Transmission to humans is thought to occur as the result of respiratory, cutaneous, or

mucosal exposure to fluids from MPV-infected animals. The disease progression is

nearly indistinguishable from that of VARV; with patients exhibiting high fevers and

systemic papular rashes roughly 7 to 17 days post infection (Figure 1.5e) (204).

Although less lethal as VARV, the Centers for Disease Control and Prevention lists the

mortality rate of human MPV infection at 1-10% (4).

The final orthopoxvirus capable of infecting humans is vaccinia virus (VACV).

The origin and natural host of vaccinia virus are unknown, but two hypotheses are that: 1)

it is the result of years of *in vitro* passaging of CPV, and 2) phylogenetically, VACV is

most similar to, and possibly derived from, horsepox virus. Regardless of its origin,

VACV has been extensively studied for many years and was the first virus to be seen,

grown, titered, purified, and chemically analyzed (294). Currently used as a vaccine

against smallpox (VARV), VACV infection of humans typically results in a mild

inflammatory response at the site of inoculation, referred to as a "take", but can give rise

to a variety of complicating symptoms, such as systemic vaccinia infection, eczema

vaccinatum, vaccinia necrosum, and postvaccinal encephalitis (Figure 1.5f) (107).

Figure 1.5 Poxvirus infections in humans. (A) Characteristic "milker's nodule" associated with human psuedocowpox virus infection (B) Day 10 of a Tanapox virus infection in Congolese youth (C) Benign, papular skin lesions of molluscum contagiosum (D) Systemic, papular rash associated with variola major virus (E) Day 8 of a monkeypox virus infection of 8 year old Congolese girl (F) Complications associated with vaccinia virus inoculation: left panel –eczema vaccinatum from a secondary inoculation from a vaccinated sibling; right panel – vaccinia necrosum in an immune compromised child. *Images modified from http://www.cdc.gov – public domain*
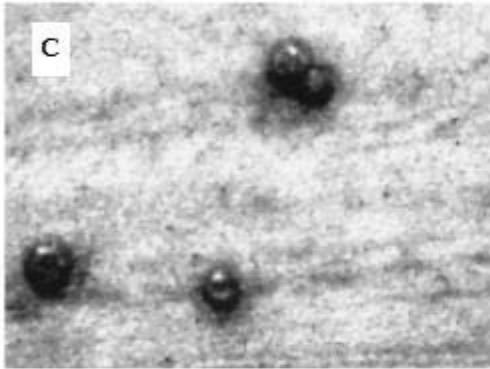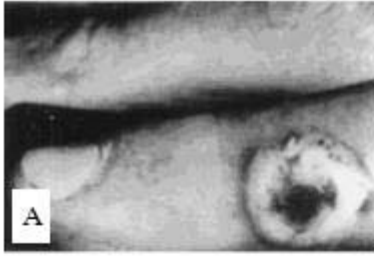
e. *Monkeypox virus and its use as a model for variola*

Smallpox is one of the most deadly human pathogens in recorded history, and despite mummified remains of Egyptian pharaohs showing the telltale signs of a smallpox infection, the earliest records of smallpox infection come from China in the fourth century AD, where the disease was described as endemic and initially introduced from the west in 48 AD (131). In 1798, Edward Jenner published his findings that experimental inoculation with CPV provided protection against VARV infection. Over the next 179 years, CPV and later VACV were used to provide immunological protection against VARV infection, a process term "vaccination". On January 1, 1967, the World Health Organization (WHO) launched the Smallpox Eradication Programme, where through mass vaccination and quarantine, the number of smallpox cases began to wane. Following the last case of naturally occurring smallpox, in 1977 the WHO declared smallpox eradicated (108, 130). Although eradicated, smallpox is not extinct; cryopreserved samples of the virus still exist at the Centers for Disease Control and Prevention, as well as in laboratories in Russia.

Prior to 1970, MPV was classified as an endemic pathogen of West Africa and the Congo River basin. Although infection was described in rodents, the natural reservoir has not been definitively identified. The first human case of MPV infection occurred in Congo (now the Democratic Republic of the Congo, DRC) in 1970, and over the next 25 years, 418 cases of human MPV infection would be confirmed (272). By 1999, the number of human MPV infections reported in the DRC was nearing 1000, with several hundred cases reported in 1999 alone, however many of these cases have yet to be

48

verified, and in some cases, MPV infection was mistakenly diagnosed for varicella zoster virus infection, or chickenpox (192, 204).

In 2003, an outbreak of MPV occurred in the United States with 37 confirmed cases (257). Although the route of infection and severity varied from cases to case, all cases stemmed from close contact with infected prairie dogs. These prairie dogs contracted the virus while being group housed with infected rodents imported from West Africa. No deaths were associated with the U.S. MPV outbreaks and more importantly, there were no reported cases involving human-to-human transmission. Furthermore, initial observations suggested that the disease described during the U.S. MPV outbreak was milder than previously described DRC outbreaks. Furthermore, concurrent with the 2003 U.S. outbreak, 10 cases of human MPV were reported from the DRC, and mortality and human-to-human transmission were reported (246).

Based on pathogenesis, transmission characteristics, and genomic sequencing, two different clades of MPV appear to exist. One clade, Congo Basin, is much more pathogenic and has the ability (roughly 30%) to spread through person-to-person contact. Infections by Congo basin MPV account for roughly 85% of human MPV infections. The other clade, West African/USA, is seen less frequently, appears to be less pathogenic, and does not show the ability to spread from person-to-person (257). Genomic analysis between MPV_{Zaire} and MPV_{USA} show the two viruses share 95% nucleotide identity, however five open reading frames are notably different, these include the IL-1β receptor homologue, SPI-1 homologue, C7L host range factor, MT4-like apoptosis factor, and the compliment control protein (CCP). All of these proteins are involved in immune evasion or host range determination. In the West Africa/USA clade,

these genes are either missing or truncated. Additional studies are underway to determine the effect each of these proteins has on MPV pathogenesis.

Several poxviruses are currently used as models of VARV pathogenesis. Because of their use of small animal models, ectromelia virus and VACV infection of mice, as well as myxoma virus and rabbitpox virus infection of rabbits are the most common models used today. Although each model has provided valuable information into areas of poxvirus replication, morphogenesis, and pathogenesis, it is difficult to directly infer that these models predict the mechanisms of VARV infection of humans. Therefore, a model system in primates would provide the most accurate understanding about smallpox pathogenesis in humans.

MPV infection of rhesus macaques represents an excellent model for studying VARV pathogenesis in humans. The genomes of VARV and MPV share 85% similarity at the genomic level. More importantly, MPV infection of both humans and macaques results in a disease progression that is clinically indistinguishable from VARV infection (see figure 1.5d and e). Monkeys experimentally infected with MPV$_{Zaire}$ develop a febrile rash, pulmonary distress, and systemic papular lesions. Additionally, histopathology on macaque tissues following MPV infection are consistent with VARV infected human samples, namely the papular lesions and increased number of immunological infiltrates in the lung (unpublished results).

3. Chemokines and their role in immunity

a. *Overview of the immune system*

The role of the immune system is to protect the host against disease by identifying

and removing pathogens, damaged tissue, and tumors. Examples of the most basic

immune mechanisms from ancient eukaryotes can be seen in their modern descendants,

such as plants, fish, reptiles, and insects. These conserved mechanisms include

antimicrobial peptides (e.g. defensins), phagocytosis, as well as the complement system.

Eventually, more sophisticated mechanisms evolved in mammals consisting of

specialized cells, receptors, secreted proteins, organs, and tissues that work together in a

concerted effort to protect us from the constant exposure to pathogens (33, 263).

i. Innate versus adaptive immunity

Mounting a successful defense against pathogenic challenge relies on both the

innate and adaptive immune responses. The innate immune response is the first line of

defense and is triggered by germline-encoded receptors, called pattern recognition

receptors (PRR) that recognize common molecular patterns found on or within most

pathogens (e.g. lipopolysaccharide – LPS, peptidoglycan – PGN, and unmethylated DNA

containing a CpG motif – CpG-DNA) (200). Triggering of PRRs leads to the activation

of innate immune cells (macrophages, dendritic cells) and the production of cytokines,

chemokines, complement proteins, and antimicrobial peptides (125).   These responses

are often tailored for the specific class of infectious agent. For example, the production

of interferons α and β (IFN-α and -β) appears to be unique to viral infections (136). The

innate immune response plays a critical role in the outcome of the overall immune response by promoting the development of the antigen-specific adaptive immune response (43, 44).

In contrast to the innate immune response, which occurs shortly after infection through the recognition of PRRs, the adaptive immune response requires days to develop and is mediated by the recognition of specific pathogens. The adaptive immune response provides long lasting immunity to pathogens by functioning through two different lymphocyte subsets: cell-mediated immunity is mediated by antigen-specific T cells while humoral immunity is mediated by B cells (200). The primary effector cells of the adaptive immune response are B cells and T cells. These lymphocytes arise from a common progenitor within the bone marrow, and although B cells remain in the bone marrow to develop, T cells migrate to and develop within the thymus. In these tissues, developing lymphocytes generate a wide range of antigen binding receptors with diverse repertoires, referred to as T cell receptor (TCR) and B cell receptor (BCR), through a process called gene rearrangement (54). Additionally, these receptors are subject to positive and negative selection, which helps to remove cells with receptors that possess an affinity for self-antigens, as well as those with poor affinity to a specific foreign antigen. The end result of gene rearrangement and the selection process is the production of naïve lymphocytes, which then make their way to the lymph nodes, where they wait for activation and further maturation (201). A more detailed description of the innate and adaptive immune responses, as well as the role of chemokines within those responses, will be discussed in more detail later in this section.

ii. **Cytokines**

Cytokines are a family of secreted signaling proteins that modulate the function of white blood cells, influence tissue remodeling, mediate cell survival and death, as well as regulate the expression of other effector molecules. Ranging in size from 8 to 30kDa, these proteins were originally referred to as lymphokines and monokines to reflect the cell type from which they were secreted, lymphocytes and monocytes, respectively. However, it became apparent that this nomenclature was an over-simplification since it was discovered that some lymphokines and monokines were in fact produced by a variety of cell types including endothelial cells, leading to the current term, cytokine. Interleukins (IL) refers to cytokines made by leukocytes that directly act on other leukocytes (251).

In general, cytokines have short half-lives and act over short distances at very low concentrations. Cytokines function by binding to specific membrane receptors, which then signal through the cell via second messengers, often receptor-bound tyrosine kinases. Cytokines have both autocrine and paracrine signaling abilities and represent a primary mode of communication within the immune system promoting cellular activation and differentiation, gene expression and silencing, as well as apoptosis and survival (251).

Cytokines can be broadly classified as either pro-inflammatory or anti-inflammatory based on whether they result in the potentiation or dampening of an immune response. Pro-inflammatory cytokines include IL-2, tumor necrosis factor-α, IL-6 and Interferon-γ. The most well studied of the inflammatory cytokines is IL-2, which is produced by activated T cells and acts in both an autocrine and paracrine fashion to drive

proliferation and differentiation of T cells into armed effector cells. In addition to the activation of T cells, IL-2 also stimulates the growth of NK cells, boosting the innate immune response (201). IFN-γ, produced primarily by activated macrophages and T_H1 CD4 T cells, induces a pro-inflammatory environment by: 1) inhibiting T_H2 T cell growth and cytokine production, 2) activating macrophages and increasing surface expression of MHC class I and II, 3) activation of NK cells, and 4) promoting differentiation of, and the production of antibodies from B cells (171, 377). TNF-α is a potent pro-inflammatory molecule produced primarily by activated phagocytes which alters the permeability of endothelium of capillary vessels allowing for increased movement of inflammatory cells into and out of the affected tissue. Additionally, TNF-α is a vital component of the febrile response and induces the production of nitric oxide, a potent antimicrobial compound secreted by macrophages (22, 201).

Anti-inflammatory cytokines include IL-10, IL-13, and IL-4. Upon binding the IL-4 receptor (IL-4R), IL-4 stimulates B cells to proliferate, express MHC class II, and produce IgG1 and IgE. Also, IL-4 expression induces T cell and mast cell proliferation and survival. However, in addition to the stimulatory effects, IL-4 expression inhibits macrophage activation, thereby promoting a humoral response over a cell-mediated response (193, 201). The expression of IL-10 stimulates the growth of mast cells, NK cells, and some B cell subsets, as well as promotes the production of MHC class II on the surface of B cells. However, IL-10 is best known for inhibiting DC maturation as well as suppressing pro-inflammatory cytokine expression from DCs and macrophages. Some key pro-inflammatory cytokines inhibited by IL-10 include TNF-α, IL-1α, IL-1β, and IL-

54

6. Furthermore, IL-10 expression can inhibit monocyte-dependent T cell proliferation and can induce anergy among antigen-specific T cells (167, 251, 458).

In 1987, a small cytokine called neutrophil-activating factor (NAF) was discovered and found to possess a unique property. The expression and secretion of NAF by monocytes could induce the migration of neutrophils, a process referred to as chemotaxis (379, 439, 459). In the years that followed this initial finding, many previously discovered cytokines were also shown to possess the ability to induce chemotaxis. These proteins, called chemotactic cytokines or chemokines, are a large part of this thesis work and will be discussed in greater detail in the sections that follow.

b. *Overview of chemokines*

When the body incurs an injury or an infection, cells of the immune system, primarily leukocytes, must respond to help heal the injury or battle the infection. Since this can happen anywhere throughout the body, these cells must constantly circulate through the blood, waiting for signals to enter tissues, a process referred to as extravasation (figure 1.6). The process of cells migrating to the affected area is referred to as chemotaxis and the chemotactic signals that most leukocytes respond to, come from resident leukocytes as well as other cell types within affected tissues. Upon activation as a result of trauma or infection, these resident cells begin to secrete chemokines.

Chemokines belong to a superfamily of small (8 – 14 kDa) proteins that possess similar structural and functional properties (297). The chemokine family is further subdivided based on the position of conserved cysteines located in the N- terminus of the protein, these are: C, CC, CXC, and CX_3C. Most of the known chemokines (~94%) are
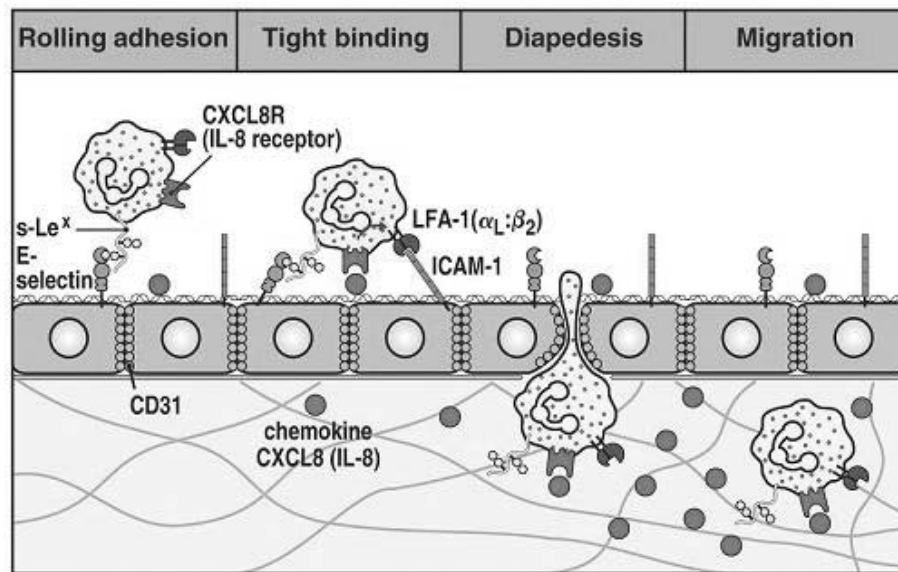
Figure 2-44 part 3 of 3 Immunobiology, 6/e. (© Garland Science 2005)

Figure 1.6 Example of leukocyte extravasation. Chemokine-dependent upregulation of adhesion proteins (e.g. E-selectin and ICAM-1) expressed on the surface of cells in the affected area contact their binding partners on a migrating cell, as a result, the cell slows and begins to roll along the vasculature. Additional adhesion contacts are made, and chemokine (e.g. IL-8) binds to its specific receptors (e.g. CXCR1 and 2), all of which effectively halt the migrating cell. Finally, the leukocyte migrates out of the blood stream, through tight junctions, and into the affected tissue where it migrates up the chemokine gradient to the site of infection or injury. *With permission (200)*

part of the CXC or CC subtypes and although primarily known for their ability to mediate

recruitment of effector leukocytes and lymphocytes during injury or infection,

chemokines are also involved in a variety of cellular processes, such as development,

apoptosis, angiogenesis, and hematopoiesis (297, 358). Chemokines function by binding

to seven transmembrane G-protein-coupled receptors (GPCRs), which initiates

downstream signaling events, that lead to adhesion, contraction, and actin

polymerization, all of which are required for cell motility (297). Additionally,

chemokine binding to glycosaminoglycans (GAGs) on the surface of some cell types may

also enhance chemokine-mediated chemotaxis (446). The roles of GPCRs and GAGs, as

they pertain to chemokine function, will be discussed in a later section.

i. Chemokine nomenclature

Chemokines are named in a manner that reflects their function (e.g. macrophage

inflammatory protein - MIP, monocyte chemotactic protein - MCP, and regulated upon

activation, normal T-cell expressed, and secreted - RANTES). Several chemokines have

multiple forms that are designated by a numeral following the name (e.g MIP-1 through

MIP-4), as well as multiple isoforms, which are designated by Roman letters (e.g MIP-1α

and MIP-1β). Different forms (e.g. MIP-1 versus MIP-3) tend to have different

biological characteristics; whereas different isoforms (e.g. MIP-1α versus MIP-1β) may

or may not (297). As more and more chemokines were discovered, naming the novel

proteins was a challenge and, in some cases, two different chemokines discovered within

a short time were given the same common name. In addition, many chemokines have

several common names which can lead to a discontinuity of the literature. In 2000,

Zlotnik *et al*. proposed a new nomenclature system for chemokines based on their subfamily and the order in which they were discovered. For example, the first chemokine with a "CC" motif to be discovered, I-309, was given the designation CC ligand 1, or CCL1. Similarly, the first chemokine with a "CXC" motif to be discovered, growth-regulated oncogene-α (GROα), was given the designation CXC ligand 1, or CXCL1 (466). Table 1.2 provides a detailed, up-to-date list of the chemokine subfamilies, their constituents, and receptor usage. In order to maintain consistency and provide a more descriptive characterization, common names will be used throughout this work.

ii. **Chemokine structure**

At the amino acid level, chemokines are quite conserved, sharing roughly 25 to 50% similarity. Although small insertions and deletions can be present from chemokine to chemokine, most of the variation is often located in the N- and C-termini of the proteins (132).   Despite differences in the termini, structural comparison of representatives from all four chemokine subfamilies and one viral chemokine have revealed a conserved tertiary structure consisting of three antiparallel β-sheets forming a "Greek key" formation (91, 100). These sheets, preceded by the N-loop, themselves are connected by short loops, referred to as 30's loop and 40's loop respectively, and are packed by hydrophobic interactions with an amphipathic C-terminal α-helix (307). The overall chemokine structure is referred to as a "chemokine fold" (297).

Chemokines are very stable proteins that require chaotropic salts, reducing agents, and elevated temperatures for complete denaturation to occur. The stability of the structure is due, in part, to disulphide bonds formed between the conserved cysteines

Table 1.2 Chemokines and Chemokine receptors *Adapted from ref (200)*

Systemic Name	Common Name(s)	Receptor (s)
CCL1	I-309, TCA-3	CCR8
CCL2	MCP-1	CCR2
CCL3	MIP-1α	CCR1, 5
CCL4	MIP-1β	CCR1, 5
CCL5	RANTES	CCR1, 3, 5
CCL6	C10, MRP-2	CCR1
CCL7	MARC, MCP-3	CCR2
CCL8	MCP-2	CCR2
CCL9/CCL10	MRP-2, CCF18, MIP-1γ	unknown
CCL11	Eotaxin	CCR3
CCL12	MCP-5	CCR2
CCL13	MCP-4, NCC-1, Ckβ10	CCR2, 3
CCL14	HCC-1, MCIF, Ckβ1, NCC-2, CCL	CCR1
CCL15	Leukotactin-1, MIP-5, HCC-2, NCC-3	CCR1, 3
CCL16	LEC, NCC-4, LMC, Ckβ12	CCR1
CCL17	TARC, dendrokine, ABCD-2	CCR4
CCL18	PARC, DC-CK1, AMAC-1, Ckβ7, MIP-4	unknown
CCL19	ELC, Exodus-3, Ckβ11	CCR7
CCL20	LARC, Exodus-1, Ckβ4	CCR6
CCL21	SLC, 6Ckine, Exodus-2, Ckβ9, TCA-4	CCR7
CCL22	MDC, DC/β-CK	CCR4
CCL23	MPIF-1, Ckβ8, MIP-3, MPIF-1	unknown
CCL24	Eotaxin-2, MPIF-2, Ckβ6	CCR3
CCL25	TECK, Ckβ15	CCR9
CCL26	Eotaxin-3, MIP-4α, IMAC, TSC-1	CCR3
CCL27	CTACK, ILC, Eskine, PESKY, skinkine	CCR10
CCL28	MEC	CCR10, 3
CXCL1	Gro-α, GRO1, NAP-3	CXCR2>>1
CXCL2	Gro-β, GRO2, MIP-2α	CXCR 2
CXCL3	Gro-γ, GRO3, MIP-2β	CXCR 2
CXCL4	PF-4	unknown
CXCL5	ENA-78	CXCR 2
CXCL6	GCP-2	CXCR 2
CXCL7	NAP-2, CTAPIII, β-Ta, PEP	CXCR 2
CXCL8	IL-8, NAP-1, MDNCF, GCP-1	CXCR 1, 2
CXCL9	MIG, CRG-10	CXCR 3
CXCL10	IP-10, CRG-2	CXCR 3
CXCL11	I-TAC, β-R1, IP-9	CXCR 3
CXCL12	SDF-1, PBSF	CXCR 4, 7
CXCL13	BCA-1, BLC	CXCR 5
CXCL14	BRAK, bolekine	unknown
CXCL15	Lungkine, WECHE	unknown
CXCL16	SRPSOX	CXCR 6
CXCL17	DMC, VCC-1	unknown
CX_3CL	Fractalkine, Neurotactin, ABCD-3	CX_3CR1
XCL1	Lymphotactin α, SCM-1α, ATAC	XCR1
XCL2	Lymphotactin β, SCM-1β	XCR1

within each chemokine. For CC and CXC chemokines, these occur between Cys-1 and -3 and Cys-2 and -4 (94). Interestingly, the C chemokine, lymphotactin contains a single disulfide bond between the only cysteines in the protein, Cys-1 and -2. However, these cysteines are in a conserved location, which would be labeled Cys-2 and -4 in CC and CXC chemokines. More importantly, the single disulphide bond formed in XCL1 provides sufficient stability to the protein, indicating that this conserved location provides a large portion of the structural stability (237).

Most chemokines likely function as monomeric proteins at physiological concentrations. However, at elevated concentration, some can form large multimeric structures. Monomers, dimers, and in some cases, tetramers have been observed via NMR and crystallographic techniques and are all biologically active (63, 316). The physiological relevance of this is unclear, but Witt *et al*. has suggested CXC chemokine dimer formation creates a structure similar to the peptide binding groove of major histocompatibility complex (MHC) molecules, and that this topology creates a more favorable surface for binding to cell surface GAGs (451). The dimer interface of CC chemokines is different than CXC chemokines, but the end result is quite similar, an extended molecule with an exposed binding surface, possibly for GAG binding (264). The possible roll of GAG binding will be discussed in more detail in the next section.

iii. Chemokine receptors

Chemokines bind to cell surface receptors to elicit downstream signaling events. Chemokine receptors are expressed on a variety of cell types, including leukocytes, endothelial cells, and neurons (297). Chemokine receptors are G protein-couple

receptors (GPCRs) and are further classified into four classes based on the types of

ligands they bind. The structure of these 7-transmembrane (7-TM) receptors is unknown,

but through comparison to bovine rhodopsin, one of the only GPCRs to be crystallized,

the structure of chemokine receptors can be inferred (434). Chemokine receptors are

comprised of seven membrane spanning domains connected by 3 extracellular loops

(ECL) and 3 intracellular loops (ICL), an extracellular N-terminus, and an intracellular C-

terminus. Other conserved features include an acidic N-terminal section, the "dry-box"

sequence, DRYLAIVHA, present in ICL2, a basic ICL3, and a cysteine present in each of

the four extracellular domains. Although, these conserved features can be present in

other GPCRs, their presence as a group is uncommon among other types of GPCRs

(297). To date, eighteen human receptors have been isolated: eleven CC receptors

(CCRs), five CXC receptors (CXCRs), one C receptor (XCR), and one CX_3C receptor

(CX_3CR).

Chemokines bind GPCRs using two distinct binding sites. The first site binds the

N-loop domain on the chemokine, with high affinity, while the second accepts the N-

terminus once the first site is occupied and induces signaling (38, 177). Prior to

chemokine binding, the ICLs on the receptor form a large docking complex for the

heterotrimeric G proteins, which are comprised of α, β, and γ subunits. Once a

chemokine has successfully bound to the GPCR, a conformational change within the

receptor induces a guanine nucleotide exchange factor (GEF) that catalyzes the exchange

from GDP to GTP on the α subunit of the G protein. This releases the G protein

heterotrimer from the receptor and the α subunit dissociates from the βγ subunits, which

remain associated as a membrane-bound dimer. Signals are transduced via interactions

of the α and/or βγ subunits with downstream effectors (302). Upon GTP hydrolysis, the α subunit re-associates with the βγ subunits, and G protein signaling is thus terminated. Although many chemokines, typically those of the CXC subfamily, bind a single receptor, some chemokines, primarily those within the CC subfamily, have the ability to bind and function through multiple receptors (see Table 1.2).

One of the more recent developments within the chemokine field is the ability of glycosaminoglycans, or GAGs, to bind and promote dimerization of certain chemokines *in vivo* (338). Glycosaminoglycans are long unbranched polysaccharides expressed on the surface of various cell types. The most prevalent GAG, Chondroitin sulfate, is a major component of extracellular matrix, and is important in maintaining the structural integrity of tissues within which it is expressed. Another physiologically important GAG is heparin sulfate, which is a ubiquitously expressed anticoagulant present on the surface of most cells that make up connective and vascular tissues (46, 109).

The mechanism by which GAGs affect chemokine function *in vivo* is not completely understood, but characterization of GAG binding sites on chemokines has shown that GAG binding may or may not interfere with receptor binding depending on the chemokine, indicating that in some cases the GAG binding site is spatially distinct from the GPCR binding site (s). However, for other chemokines, GAG binding reduces GPCR binding suggesting some overlap is occurring (338). Therefore, GAG binding may competitively inhibit some chemokines from binding their respective GPCRs. An alternative hypothesis suggests that GAG binding may augment GPCR binding. This hypothesis is supported by *in vivo* data showing reduced numbers of peritoneal exudate cells (PECs) when mice are injected with chemokines with mutated GAG binding sites.

Proudfoot *et al.* suggests that the binding of a dimerized chemokine to GAG may induce a conformational change within the dimer, possibly exposing GPCR binding sites or even promoting the release of monomeric chemokine (338). Johnson *et al.* argues that binding to GAGs may present a relatively high concentration of immobilized chemokine at or near the site of injury or infection, providing a fixed signal for recruited cells to stop, and enter the affected area (206). Although the *in vivo* role of GAG binding remains unclear, the hypothesis that dimerization promotes binding to GAGs thereby inhibiting or possibly enhancing normal function, may prove to be a critical process *in vivo*.

iv. Chemokine and chemokine receptor antagonists

Chemokines and chemokine receptors have been targeted by the pharmaceutical industry since the early 1990's. Unlike many cytokines, which typically require a large number of protein-protein interactions to initiate function (e.g. IL-1 requires seven contact points with IL-1R to elicit function), functional binding of a chemokine to a chemokine receptor requires relatively few contacts (i.e. a small, discrete binding site) (238). The inhibition of the larger cytokines usually requires biological agents, such as antibodies or soluble protein antagonists, whereas the smaller chemokines offer potential targets for small molecule inhibitors, as well as the use of biologicals (336).

Three different classes of chemokine/chemokine receptor inhibitors: small molecule inhibitors, neutralizing antibodies, and modified chemokines have been tested with varying degrees of success. The use of small molecule inhibitors is the favored approach by pharmaceutical companies because of advances in high throughput chemistry that have made discovery and screening less time consuming. Two FDA-

approved chemokine receptor inhibitors, TAK779 and SCH-C, both non-peptide, CCR5

antagonists, have been shown to block all three CCR5 ligands (e.g. RANTES, MIP-1α,

and MIP-1β) and possess potent anti-HIV activity (25). While both inhibitors show

significant anti-HIV activity, phase II trails show they were most efficient when used in

conjunction with other HIV therapies, and in patients who were treatment-experienced

(i.e. HIV-positive patients who have been on a long-term anti-HIV regimen) (366).

Another small molecule inhibitor, DF-2162, is an allosteric inhibitor of CXCR1/2 and

shows promise for the treatment of neutrophil-mediated inflammatory diseases, such as

rheumatoid arthritis (103). The production of neutralizing, monoclonal antibodies

directed toward chemokines and their receptors has shown some promise in small animal

studies (189, 213, 214). However, targeting GPCRs with monoclonal antibodies can be

challenging given that very little of the receptor extrudes from the plasma membrane and

is highly conformation specific. Lastly, modified chemokines have been used in several

small animal models. A broad-spectrum RANTES antagonist, Met-RANTES (RANTES

with its start methionine still present) has been effective in murine models for treating

inflammatory diseases such as asthma (airway hyperactivity), chronic colitis, arthritis,

and transplant rejection (11, 159, 166, 330). Likewise, a truncated version of MCP-1 has

also proven effective against arthritis in a murine model (157).

A complicating factor for inhibiting chemokines is redundancy within the certain

sub-families, namely the β-chemokines. For example, MIP-1α, MCP-1, and RANTES

have the ability to bind their cognate receptor(s) and elicit similar physiological

outcomes. Therefore, inhibiting one chemokine via a small molecule inhibitor or

antibody may not result in a significant phenotypic change because of the overlapping

functions of other chemokines. For this reason, a broad-spectrum chemokine inhibitor

that selectively targets inflammatory chemokines without affecting those involved in

housekeeping functions would represent the best case scenario for chemokine inhibition.

Along these lines, the viral chemokine binding protein M-T7 has been shown to be

effective in reducing inflammatory side effects of angioplasty injury, leading Proudfoot *et

al.* to comment, "viruses may be able to teach us many lesions on how to evade the

immune resonse" (240, 336).

v. **Chemokine regulation**

Chemokines are regulated by a complex series of positive and negative stimuli.

Exposure to oxidative stress, pathogens, pro-inflammatory cytokines, and cell adhesion

proteins can promote the expression of proinflammatory chemokines, such as MIP-1α,

RANTES, and IL-8, while immunoregulatory cytokines promote the expression of both

pro-inflammatory (e.g. MIG, IP-10, and I-TAC) and homeostatic (e.g. eotaxin)

chemokines. Furthermore, expression of the homeostatic chemokine, eotaxin, suppresses

IL-8 expression, suggesting that chemokines also play a role in regulating the pro-

inflammatory response (207). Expression of glucocorticoids and anti-inflammatory

cytokines, such as IL-10 and IL-4, effectively inhibit the expression of most chemokines.

Some of these regulatory mechanisms act directly on chemokine expression, while others

act through indirect mechanisms (297). Montecucco *et al*. recently showed that TNF-α

treatment of neutrophils increased MIP-1α-mediated migration by upregulating

expression of the adhesion molecule Mac-1, which in turn promoted CCR5 expression, a

known MIP-1α receptor. Therefore, by upregulating Mac-1, TNF-α indirectly promotes

MIP-1α migration (289).

Many chemokines are regulated at the site of inflammation by being subjected to

extracellular enzymatic processing within the affected area. Several enzymes including

neutrophil elastase, chymotrypsin, matrix metalloproteases, and cathepsins are capable of

modifying chemokines at the site of inflammation. Another example of enzymatic

regulation is Dipeptidyl peptidase IV (CD26), where this enzyme is capable of cleaving

the N-terminus of the chemokines. In the case of eotaxin, CD26-mediated cleavage

creates a truncated version of the chemokine capable of binding CCR3, but unable to

initiate signaling (365, 419).

Chemokine function can also be regulated at the receptor level through several

processes. When a GPCR has initiated a signaling event, G protein-independent kinases

are recruited to the receptor, where they phosphorylate serines and theonines in the C-

terminus of the receptor. These phosphorylated residues recruit β-arrestin, which

prevents additional G proteins from coupling with the receptor. Furthermore, β-arrestin

interacts with the adaptor molecules, AP-2 and dynamin-I, which promotes the

internalization of the receptor via clathrin-coated pits. The combination of no associated

G proteins and receptor internalization means the receptor is unavailable for further

chemokine signaling, a process termed "receptor desensitization" (129).

Along with the known chemokine receptors, two chemokine decoy receptors or

"interceptors" (referring to their function as chemokine *inter*nalizing (pseudo)re*ceptors*)

have also been described. Duffy antigen, also referred to as Duffy antigen receptor of

chemokines or DARC (human), and D6 (mice) are 7-TM proteins, but lack the crucial

"DRY-box" region required for proper G protein docking (172). The exact physiological role of DARC and D6 are unknown. They are expressed on erythrocytes, endothelial cells, and Purkinje cells. Without the ability to transduce signals, these decoys could either positively or negatively regulate inflammation depending on whether they sequester and remove chemokine from the blood or bind and present chemokine in a similar fashion to GAGs.

Certain chemokines, when present in high concentration, form large, high molecular weight aggregates. Apparently as a result of their large structure, these aggregates exhibit reduced chemokine function, therefore raising the possibility that chemokines may possess some form of concentration dependent auto-regulation (321, 399).

c. *Functional role of chemokines during inflammation*

Functionally, chemokines can be classified as inducible or "inflammatory" chemokines and constitutive or "homeostatic" chemokines, and as described below, these classes of chemokines have been shown to play an intrinsic role in both the innate and adaptive arms of the immune response (figure 1.7) (125).

i. **The role of chemokines during the innate immune response**

Inflammatory chemokines are thought to play a key role during innate immunity by mediating the recruitment of effector cell types, such as neutrophils, monocytes and macrophages, natural killer cells, and eosinophils (200). These chemokines, typically of

Figure within image (labels): Pathogen, LPS, 'Immature' dendritic cell, MIP-3α CCR6, Activated 'mature' dendritic cell, Afferent lymph, CCR1 CCR5, CCR7, SLC, TLR, MIP-1α MIP-1β, Tissue macrophage, IP-10, Endothelium lymphatics, MIP-1α MIP-1β, IL-8, Lymph node, B-cell follicle, HEV, Capillary endothelium, CXCR1/2, CCR5, CXCR3, CXCR5, BLC, B, DC-CK1 ELC, SLC, ELC, T, CCR7, Blood, Neutrophil, NK cell, Activated T cell, CXCR5, T, B, CXCR5, Blood, T-cell zone, CCR7, SLC, T, CXCR3, Activated T cell, Efferent lymph, Current Opinion in Immunology

Figure 1.7 Chemokine-mediated trafficking of leukocytes during an immune response. In response to infection, tissue resident macrophages begin to secrete pro-inflammatory chemokines, while immature dendritic cells begin to mature as they migrate to the draining lymph node. Once there, now mature dendritic cells present antigen to naïve lymphocytes stimulating their activation. Activated lymphocytes are now responsive to the pro-inflammatory chemokines expressed by tissue resident macrophages and along with NK cells and neutrophils respond to the site of infection. *With permission (267)*

the CC subfamily, generally bind more than one receptor, however, they are not considered redundant because they often differ in their biological properties (465).

The first step in initiating an innate immune response is the detection of pathogen-specific motifs via PRRs on the surface of surveilling cells, such as the epithelial cells lining the gastrointestinal tract and tissue resident dendritic cells and macrophages. Detection by PRRs produces an activated phenotype for the host cell, resulting in the secretion of proinflammatory cytokines and chemokines (229). One of the key functions of these proinflammatory molecules is the upregulation of cell adhesion proteins within the vasculature. These adhesion molecules are members of the selectin family (e.g. P- and S- selectin), integrin family (e.g. LFA-1 and VLA-5), and immunoglobulin superfamily (e.g. ICAMs, VCAM-1, and PECAM). Upregulation of adhesion molecules, as well as chemokine-bound GAG, promotes the extravasation of leukocytes into the affected areas (figure 1.6). Once in the affected tissue, responding cells follow the chemokine gradient to the site of infection.

In general, neutrophils are the most abundant leukocytes in the peripheral blood and the first cells to respond to microbial infection. Neutrophils are phagocytic granulocytes, capable of ingesting microorganisms and debris. Pathogens are internalized into a compartment, referred to as a phagosome, where reactive oxygen species and hydrolytic enzymes are secreted and effectively kill most pathogens. Neutrophils also release several effector molecules by degranulation; these proteins include a variety of degradation enzymes and antimicrobial polypeptides, and can be targeted to specific cells or diffuse into the microenvironment (200). Neutrophils expressing CXCR1, CXCR2, and CCR1 are the first cells of the innate immune system

that respond to the infected area, typically in response to the CXC chemokines: IL-8, GROα, and ENA-78, and the CC chemokines: MIP-1α and MIP-1β (figure 1.7) (249, 365).

Monocytes and other mononuclear cells, including basophils and eosinophils, that express CCR1, CCR3, and CCR5 appear shortly after neutrophils in response to the CC chemokines: MCP-1, MIP-1α, and RANTES (365). Monocytes and macrophages play a vital role in both the innate and adaptive immune responses by performing a variety of antimicrobial functions, such as phagocytosis and degranulation like that described for neutrophils, as well as antigen presentation, which is vital for initiating the adaptive immune response (160, 200). Basophils and eosinophils are the least common leukocytes and require additional activation by T_H2 cytokines or activation through their Fc receptors (FcR). When the FcR on basophils or eosinophils contacts an infected cell decorated by host antibodies (e.g. IgG, IgA, and IgE), this causes the cells to degranulate and release histamine, proteoglycans, as well as proteolytic enzymes which effectively kill the targeted cell in a process referred to as antibody-dependant cellular cytotoxicity (ADCC). In addition, eosinophils also play a role in controlling viral infections by secreting RNAses contained within their granules (200).

Natural killer (NK) cells are important for early protection against viral infection. These cells are considered cytotoxic lymphocytes, but since they lack a diverse repertoire of antigen receptors, NK cells are considered part of the innate immune response. NK cells attack infected cells through ADCC as well as recognition of "missing self" (200). The latter is accomplished by recognizing two types of surface receptors: activating and inhibitory receptors. When a cell is infected with an intracellular pathogen, peptides

70

from the infecting agent are processed via the proteosome and loaded onto major histocompatibility complex class I (MHC I) molecules, which are presented on the cell surface for cytotoxic T cells to detect and initiate their cytolytic function. However, many pathogens have evolved mechanisms to avoid cytotoxic T cells, by simply down regulating MHC I expression and this will be discussed in greater detail in section four. NK cells recognize MHC I molecules as "self", and therefore MHC I expression provides an inhibitory stimulus, which explains why NK cells kill cells possessing low levels of MHC class I molecules, or "missing self" (343). NK cells kill their targets by degranulation, releasing small cytoplasmic granules containing proteins such as perforin and granzyme. These proteins disrupt membrane permeability causing the target cell to die by apoptosis (200).

Resting NK cells lack CCR7 expression, which is important for homing to secondary lymphoid tissue (271). Therefore, NK cells are thought to migrate to peripheral non-lymphoid tissue through some undefined mechanism, where they await activation. Chemokine receptor expression on NK cells is somewhat controversial, but resting NK cells appear to express CXCR3, CXCR4, XCR1, and CX_3CR1, and upon activation, CCR2, CCR4, CCR7, and CCR8 are also upregulated. This would indicate that resting NK cells are responsive to the CXC chemokines: MIG, IP-10, I-TAC, the XCR chemokine lymphotactin, and the CX_3C chemokine fractalkine, while activated NK cells respond to the CC chemokines: I-309, TARC, MDC, as well as members of the MIP and MCP families (figure 1.7) (125).

***ii.* The role of chemokines during the adaptive immune response**

While the innate immune response is tailored toward recognizing common patterns present on pathogens, the adaptive immune response is tailored toward recognizing specific pathogens. It is this specificity and the generation of long lasting memory T and B cells which provides long term immunity to pathogens. The development of a successful adaptive immune response has strict requirements for proper architecture within the lymphoid tissues, and the presentation of antigen, all of which are modulated by chemokines (201). However, unlike the innate immune response, which is primarily driven by inflammatory chemokines, the adaptive immune response requires a concerted effort by both inflammatory and homeostatic chemokines (125).

Homeostatic chemokines like MIP-3β and secondary lymphoid-tissue chemokine (SLC) play an important role mediating the migration of naïve lymphocytes, as well as mature dendritic cells (mDC) and certain subsets of NK cells, to the T cell zone of a lymph node, while B cells eventually localize to the follicle of the lymph node, a process mediated by the homeostatic chemokine B lymphocyte chemoattractant (BCA-1) (312). Migration of cells into the T cell zone is largely mediated by CCR7 expression and its ligands MIP-3β and SLC by stromal cells and lymphatic endothelium (137) (figure 1.7). Studies have shown that CCR7 knockout mice do not form normal T cell zones and as a result have seriously impaired primary immune responses (137). Therefore, this concerted movement of cells within the lymph node is a requirement for proper lymph node architecture and demonstrates the important role that homeostatic chemokines play in this process (296).

Another important aspect of the adaptive immune response is antigen

presentation. There are two ways antigens can be presented, bound to MHC class I or

MHC class II molecules. As briefly described in the previous section, cells infected with

an intracellular pathogen process pathogenic proteins via the proteosome and load these

antigenic peptides onto MHC I molecules. The surface presentation of antigen bound to

MHC class I molecules triggers circulating $CD8^+$ T cells to kill the infected cell.

Phagocytic cells can engulf pathogenic material (microbes, microbial proteins, etc),

which gets degraded in the lysosomal compartment, and these antigenic peptides are then

loaded onto MHC class II molecules. These cells, along with B cells, are referred to as

professional APCs because along with presenting antigen in the context of MHC class II,

these cells also express other activating signals which help to differentiate $CD4^+$ T cells

into effector T_H1 or T_H2 T cells, .

Trafficking of APCs to sites of inflammation where they capture antigens and

then back to the secondary lymphoid tissues, where they present processed antigen to

naïve lymphocytes is critical to antigen presentation. Inflammatory chemokines, such as

IL-8, MIP-1α, MIP-1β, MIP-3α, and RANTES are responsible for recruiting APCs, like

immature DC (iDC), monocytes, and macrophages to the site of inflammation (76). Once

these cells have become activated by pathogen, they upregulate CCR7 and become

responsive to MIP-3β and SLC, which mediate homing back to the lymph node (figure

1.7) (312). Within the lymph node, and to a lesser extent in the peripheral blood, APCs

present MHC-bound peptides to the TCR on the naïve T cells. This signal alone is not

enough to activate the naïve cells, which require an additional co-stimulatory signal for

activation. Dendritic cells and macrophages are referred to as professional APCs because they express co-stimulatory molecules on their surface (201).

d. *Chemokines and their role in disease*

Chemokines play an important role during inflammation by mediating the recruitment of inflammatory cells into and out of the affected area, and as a result, are often involved during inflammatory diseases. Although chemokines are typically not directly responsible for pathology, their expression can promote an environment primed for an immune response, thereby resulting in pathology.

i. Inflammatory diseases

Inflammation is a vital component of a healthy immune response and involves the increased movement of plasma and leukocytes out of the blood and into injured or infected tissues, resulting in redness, swelling, pain, and heat. Inflammation and the associated symptoms typically persist until the injury or infection has been resolved. Although vital in the short term, inflammation occurring over a long period of time, referred to as chronic inflammation, which can lead to a number of diseases, such as asthma, atherosclerosis, and allograft rejection, represents a serious health risk for millions of people (1, 2, 5).

1) *Atherosclerosis*

Coronary heart disease caused by atherosclerosis, or the narrowing of the vasculature of the heart, is the leading cause of death in the United States with roughly

500,000 deaths annually (2). Atherosclerotic plaques form when oxidized low density

lipoproteins (ox-LDL) are deposited on the inner wall of blood vessels. In response to

the presence of ox-LDL, endothelial cells lining the vessel wall within the affected area

begin to secrete numerous chemokines, as well as upregulate surface expression of

adhesion molecules (140). Although many cell types have been described with an

atherosclerotic plaque, the expression of IL-8, MCP-1, and RANTES promote the influx

of neutrophils, macrophages and T cells, which represent the most abundant

inflammatory cells present within the plaque (153). As more and more ox-LDL is

deposited on the plaque, the immune system responds in kind, resulting in increased

inflammation. Eventually, the blood vessel becomes too occluded and blood flow is

blocked resulting in a heart attack.

2) *Asthma*

Asthma affects 1 in 15 Americans resulting in roughly 4000 deaths annually (1).

The disease is characterized as a restriction of the passages of the lung caused by

bronchial spasm and inflammation. The bronchial spasms associated with asthma can be

induced by a number of environmental triggers, but the most common is the inhalation of

allergens. Asthma-associated inflammation is largely mediated by two responses, a

humoral response and a cell-mediated response. The first is a humoral response caused

by plasma cells releasing antibodies specific for the inhaled antigen. The antibody-bound

antigen then triggers phagocytes to engulf the antigen in an attempt to remove the antigen

(236). The cell-mediated response is largely mediated by monocytes and macrophages

responding to MCP-1, followed by T cells and eosinophils in response to RANTES and

eotaxin, respectively. The cytokines released by these cells result in a large portion of the

inflammation that occurs during an asthma attack (158).

3) *Allograft rejection*

In 2007, 28,353 solid organ transplants were performed in the United States (5).

In nearly every case, patients are treated with immunosuppressive drugs, such as

cyclosporin and rapamycin to diminish the immune response to the allograft and improve

chances of success. However, transplant recipients can develop acute rejection months to

years after transplantation (5).   Transplant rejection is largely mediated by the T_H1

immune response (173). Transplant studies using knock out mice have shown that the

rejection process is largely mediated by ligands for CXCR3 (e.g. IP-10) and CCR5 (e.g.

MIP-1a, MIP-1b, and RANTES) and to a lesser extent CX3CR1 (e.g. fractalkine) and

CCR2 (e.g. MCP family) (297). As a result, inflammatory cells begin to accumulate

within the grafted tissue almost immediately following transplant. The ensuing

inflammatory response can cause scarring and, if severe enough, loss of the grafted

organ.

ii. **Autoimmune diseases**

The immune system has the important task of differentiating between healthy and

infected or injured cells. This process relies on the ability of T and B cells to distinguish

between self and non-self antigens through a rigorous selection process of negative and

positive selection whereby potentially auto-reactive lymphocytes are either eliminated or

induced to become hypo-responsive. If this process is disrupted, the immune system can

attack healthy tissue, a disorder referred to as autoimmunity. The autoimmune response is a complicated process that involves both the innate and adaptive arms of the immune response. The simplified view of autoimmunity is that memory T cells cross-recognize self antigens on healthy tissues which initiates an aberrant immune response. Alternatively, healthy tissue may become targeted by autoreactive antibodies. The presence of these antibodies on the surface of healthy tissues leads to their destruction through the complement system (201). Autoimmune disorders are a subset of inflammatory diseases which include various ailments such as rheumatoid arthritis, multiple sclerosis, Grave's disease, inflammatory bowel disorders including Crohn's disease and ulcerative colitis, and type I diabetes. More importantly, autoimmune disorders represent a considerable health concern with approximately 1 in 31 people in the United States affected by an autoimmune disorder every year (362).

1) *Rheumatoid arthritis (RA)*

Rheumatoid arthritis is a degenerative autoimmune disease characterized by the destruction of the synovial membranes, connective tissue, and bone within many of the joints of the human body. Although the exact trigger is not known, recent evidence suggests that autoreactive B cells are activated possibly through TLR9 (245), and differentiate into antibody producing plasma cells, as well as stimulate T cells to differentiate into autoimmune effector T cells. The presence of autoreactive antibodies further activates the immune system through Fc and complement receptors. The result is the accumulation of inflammatory infiltrates including T and B cells, macrophages, and NK cells (278). The role chemokines play during RA is not fully understood, but the

77

expression of inflammatory chemokines and tissue infiltration by autoreactive lymphocytes and monocytes expressing the corresponding inflammatory chemokine receptors has been documented (152, 155). Interestingly, several studies have demonstrated that MCP-1 and MIP-1α protein levels are significantly elevated in RA patient serum, synovial fluid, and eroded tissues (225, 226). The logical deduction from these findings is that chemokines are responsible for the recruitment of mononuclear cells into the affected tissues where they contribute to the chronic inflammation.

2) *Type 1 diabetes (T1D)*

Type 1 diabetes is a degenerative autoimmune disease wherein the insulin-producing cells of the pancreas, the beta cells, are slowly destroyed (197). The result of this is an absolute dependence on the administration of insulin in order to maintain metabolic balance and ultimately survival. Like RA, the exact trigger(s) that induce autoimmunity is unclear; however, the destruction of beta cells is mediated by a variety of inflammatory cells (e.g. T cells, B cells, macrophages, NK cells, and dendritic cells) (24). Recent evidence suggests that autoreactive T_H1 and T_H17 subsets of $CD4^+$ T cells, as well as B cells producing autoreactive antibodies are the primary suspects for the induction of the autoimmune response (7, 139, 175). As with previous examples, chemokine expression promotes the influx of the inflammatory cells that, in the end, cause the damage. Serum samples taken from T1D patients indicate that the inflammatory chemokines, such as MCP-1, MIP-1α, MIP-1β, and RANTES are present at elevated levels (265). This observation is consistent with data from murine models where, along with the chemokines listed above, MCP-3, MCP-5, and IP-10 are also

present in the serum of experimentally-induced diabetic mice (57, 65, 85). Interestingly, one of the chemokines listed, IP-10, specifically mediates the recruitment of activated T_H1 T cells, one of the cell types implicated in the establishment of autoimmunity.

3) *Multiple sclerosis (MS)*

Multiple sclerosis (MS) is a degenerative condition caused by the progressive destruction of the myelin sheath surrounding the nervous tissue of the brain and spinal cord. The purpose of the myelin sheath is to insulate the nerve from the surrounding tissues and produce a conduit for nerve impulses to travel down. When the myelin sheath is compromised, normal nerve signaling is lost leading to impaired physical and cognitive functions (418). Studying histopathology in humans with MS is difficult because of a lack of samples prior to death. Most of our understanding about MS in humans comes from post-mortem sampling. These data show the presence of many types of cellular infiltrates, but the disease is believed to be the result of autoreactive T_H1 CD4+ T cells and their proinflammatory products (413). Experimental autoimmune encephalomyelitis (EAE) is an experimentally induced condition that resembles MS in humans. The administration of myelin basic protein peptides induces an autoreactive response against the protective myelin sheath, resulting in a disease progression that mimics MS in humans. As with MS in humans, there are varying degrees of EAE in mice: 1) acute EAE – acute paralysis, followed by full recovery, 2) chronic EAE – acute paralysis that partially improves, however the animal remains in a chronic state of partial paralysis, and 3) relapsing and remitting EAE – acute paralysis followed by relapsing episodes of paralysis. Chemokines and their role in recruiting inflammatory cells have always been

implicated in MS. Kennedy *et al*. demonstrated that the administration of neutralizing antibodies for MCP-1 and MIP-1α, reduced the severity and duration of EAE (214).

Chemokines represent a vital component of the immune system. Without the ability to mediate and guide the migration of effector cells, the immune system would be rendered ineffective against pathogenic challenge. Along those lines, a proper understanding of how chemokines function in health and in disease is the first step towards the development of potential therapeutics for diseases that involve any kind of inflammation.

4. Viral manipulation of the immune system.

For a virus to productively infect an immunocompetent host, it must gain entry to the host, infect a permissive cell, and produce progeny. Importantly, the virus must do so in the face of the immunological machinery described in the preceding section.   Through millions of years of co-evolution and co-existence, viruses have developed mechanisms which help the virus cope with this hostile environment, a process commonly referred to as "immune evasion".   Although the term can be applied to a single viral gene, the process is typically a concerted effort by multiple genes, often with redundant functions. In many cases, these genes represent pirated versions of cellular homologs, encoding proteins that may or may not retain a similar function as the cellular version. The nomenclature for these types of proteins is generally the same as that of their cellular counterparts, with a "v" preceding the gene name (e.g. vIL-10).

With their large genome size and ability to express numerous accessory genes, poxviruses and herpesviruses harbor numerous mechanisms to manipulate the

intracellular environment, as well as the host immune system. For this purpose, and for the focus of this work, this section will explore the following mechanisms encoded by poxviruses and herpesviruses: a) downregulation of cellular proteins and receptors, b) virally-encoded receptors and receptor inhibitors, and c) expression and secretion of viral effectors and inhibitors.

a. Downregulation of cellular proteins and receptors*

Upon infection, the presence of virus particles and viral gene products can trigger the host cell to initiate a variety of self defense mechanisms in an attempt to thwart the viral life cycle, as well as to alert the immune system to the infection within. For instance, virally infected cells express pro-inflammatory surface markers (e.g. MHC class I and TNF-αR) and cytokines (e.g. IFN-α/β) to increase the likelihood that the infected cell will be targeted and killed by an immunological effector cell. Another common self defense mechanism in response to viral infection is programmed cell death, or apoptosis of the infected cell. Both of these mechanisms attempt to kill the targeted cell without lysis, at which time the remnants are targeted by phagocytic cells and removed. This non-lytic mechanism is vital for reducing viral spread, since lysis could result in the release of intracellular progeny virions (201). As a result, some viruses possess mechanisms to reduce the expression of these host defense proteins, either through a physical, transcriptional, or translational blockade.

i. Major histocompatibility complex (MHC)

Major histocompatibility complex class I and II are responsible for surface presentation of antigenic peptides derived from both intracellular and extracellular pathogens, respectively. Major histocompatibility complex is a heterotrimeric protein consisting of a heavy chain (HC) and β2 microglobin (β2m). During synthesis, MHC constituents are synthesized into the lumen of the endoplasmic reticulum (ER) where chaperone proteins stabilize the transient complex. Antigenic peptides generated by the proteosome are translocated into the ER lumen by the peptide transporter TAP, loaded onto the transient complex, and eventually presented to the extracellular environment (201). As outlined in the previous section, surface presentation of antigenic peptides, in conjunction with other stimulatory molecules, results in the targeting and eventual death of the infected cell. For this reason, many viruses encode proteins that target both MHC classes.

1) *HCMV US2 through US11*

Human cytomegalovirus (HCMV) infects and efficiently establishes a life-long persistent infection within the host. The ability to maintain a persistent infection while under constant immune surveillance highlights the ability of HCMV to evade the immune system. Critical to this delicate game of "hide and seek", HCMV contains several well studied genes that mediate the downregulation of MHC. The genes responsible are located in a unique short (US) region between open reading frames *US2* and *US11* and include *US2*, *US3*, *US6*, and *US11*. These gene products act through different mechanisms to reduce MHC levels on the surface of infected cells. US2 and US11

remove newly synthesized MHC molecules from the lumen of the ER by targeting them

to the proteosome, where they are degraded (449, 450). Although the exact mechanism

of how US2 binds MHC is unknown, US11 does not bind to MHC directly, instead

binding the MHC chaperone protein, derlin 1 (29, 258, 457). US6 inhibits peptide

translocation into the lumen of the ER membrane by blocking the peptide transporter

TAP. This effectively prevents the loading of antigenic peptides onto nascent MHC I

molecules (10, 178, 183, 250). Lastly, US3 interferes with the MHC chaperone tapasin,

an ER protein that controls the loading of antigenic peptide. As a result, mature MHC

does form and therefore is retained in the ER (9, 169, 208, 248).

2) *K3 family*

The γ2-herpesviruses and some poxviruses, demonstrate yet another mechanism

for viral downregulation of surface glycoproteins like MHC. Kaposi sarcoma-associated

herpesvirus (KSHV), murine gamma herpesvirus-68 (MHV-68), and some poxviruses

encode proteins containing RING-CH domains known as the K3 family. Important for

their function, the RING-CH domain belongs to the larger zinc-finger motif and functions

as an E3 ubiquitin ligase. Several reports have shown that binding of K3 and K5

(KSHV), K3 (MHV-68), and p28 (vaccinia virus – WR) promotes ubiquitination of, and

the ultimate degradation of MHC class I. This process effectively reduces surface

expression of MHC class I (31). Furthermore, KSHV-K5 has been shown to

downregulate other immunologically important glycoproteins such as the co-stimulatory

molecules, B7.2 and ICAM-1 (98).

3) *MHC homologs*

As outlined in the previous section, the homeostatic expression of MHC on the surface of cells is a vital process in immune surveillance, by providing inhibitory signals to passing NK cells. Therefore, a lack of MHC on the cell surface would result in a loss of inhibition, thereby leading to NK cell-mediated killing (343). In addition to the loss of inhibitory signals, viral infection can result in increased stimulatory signals. These signals typically are in the form of the ligands for the activation receptor, NKG2D, such as MHC class I-related chains A and B (MICA and MICB). Together, this combination of NK signals promotes the targeting of the infected cell by NK cells. Along with mechanisms to reduce MHC surface expression, HCMV has also evolved mechanisms to combat the loss of MHC surface expression thereby resulting in NK cell-mediated killing. The first of these is the expression of the MHC-like molecule, UL18 (34). This virally encoded MHC class I homolog is capable of binding β2m and presenting endogenous peptide at the cell surface. Another HCMV protein, UL40, is capable of binding and stabilizing HLA-E, a form of MHC class I, resulting in increased expression on the surface of HCMV infected cells. HLA-E binds the heterodimeric inhibitory receptor CD94/NKG2A expressed on NK cells. Through these two proteins, HCMV is capable of providing its own inhibitory signals to patrolling NK cells (99, 426, 441). In order to suppress virally induced stimulatory signals, HCMV encodes two proteins that bind and sequester these stimulatory receptors. UL16 is a membrane bound glycoprotein that can bind several well known NK cell stimulatory receptors, MICB, ULBP1, and ULBP2. The interaction between UL16 and these receptors causes them to be retained in the ER (116). Furthermore, UL16 expression appears to stabilize the plasma membrane

integrity of HCMV infected cells making them resistant to granzyme B secretion (116, 308). Finally, HCMV actively downregulates surface levels of the stimulatory receptor MICA. This is accomplished by the viral protein, UL142, which blocks the surface expression of MICA. The exact mechanism is unknown, but like other proteins, MICA may be retained in an intracellular compartment or possibly even degraded (81).

ii. Tumor necrosis factor-α receptor 1 (TNF-αR-1)

While many cytokines may elicit a response from a small set of target cells, some cytokines, like tumor necrosis factor-α (TNF-α), are generalized effectors promoting a pro-inflammatory environment. Although primarily made by macrophages in response to infection, TNF- α can be made by a variety of cells, such as endothelial cells, lymphocytes, adipocytes, fibroblasts, and neuronal tissues. The expression of TNF-α is associated with increased permeability of capillary beds, increased cellular activation, increased recruitment of neutrophils, apoptosis, and other antiviral properties (256, 452). Additionally, TNF-α synergizes with IL-1 and IL-6 on the hypothalamus to mediate the host febrile response (96). Like many cytokines, TNF-α works best over short distances within the inflamed microenvironment. When large concentrations of TNF-α are secreted, they can induce a deadly shock syndrome (8).

The binding of TNF- α to the TNF-α receptor (TNFαR) results in several possible outcomes, all of which are mediated by the complex interaction of the TNFRSF1A-associated via death domain adaptor protein (TRADD) on the cytoplasmic tail of the TNFαR and various other signaling proteins. The first is the activation of the NF-κB signaling pathway. This is accomplished by the interaction of the signaling proteins,

TRAF2 and RIP, with the TRADD protein. TRAF2 recruits the signaling protein, IKK, to the receptor and RIP phosphorylates IKK. Phosphorylated IKK, in turn, phosphorylates the inhibitory protein IκB causing its release from NF-κB. Free NF-κB is then capable of translocating to the nucleus and initiating transcription of a number of pro-inflammatory and pro-survival genes (340). The second outcome is the activation of the MAP kinase pathway. Like NF-κB, the process starts with TRAF2 recruiting the signaling protein MEKK1 to the TRADD protein. Once there, MEKK1 is phosphorylated by the kinase ASK1, which initiates a series of signaling events resulting in the activation of a number of transcription factors involved in cell differentiation and proliferation (351). Finally, the last process mediated by TNF-α is apoptosis. This is accomplished by the recruitment of the death domain FADD to the TRADD protein. FADD, in turn, associates with procaspase 8 and when enough procaspase 8 has accumulated, an autolytic reaction occurs, producing caspase 8. Caspase 8 can activate several downstream enzymes resulting in cell death (39).

1) *Epstein-Barr virus gene Lmp-1*

Epstein-Barr virus (EBV) is a γ-herpesvirus that has the ability to infect lymphocytes and epithelial cells. Like most herpesviruses, EBV encodes a variety of defense mechanisms to adapt to the hostile environment within the host and augment its own pathogenesis. One such mechanism is the downregulation of TNF-αR-1 by the EBV gene *latent membrane protein-1* (*LMP-1*). Expression of LMP-1 causes T cells to up-regulate TNF-α through TRAF2 and TRAF5 and the NF-κB pathway. Interestingly, these LMP-1 expressing T cells became resistant to TNF-α-induced apoptosis. This

resistance is conferred by the considerable reduction in TNF-αR-1 expression.

Furthermore, the TRADD protein is constitutively recruited by LMP-1, and the apoptotic activities of caspases 3, 8, and 9 are also suppressed (88). The result of this is a latently infected T cell that possesses a diminished capacity for apoptosis. This has considerable pathogenic implications considering EBV reactivation can lead to certain types of cancer. Additional mechanisms for inhibiting TNF-αR signaling will be discussed below.

iii. Viral inhibition of host cell transcription and translation

1) HCMV immediate early proteins 1 and 2

Another common mechanism used by viruses to inhibit the expression of cellular proteins is through a transcriptional blockade. There are many examples of this throughout the herpesvirus family. One example of this type of mechanism comes from the 72 kDa immediate early-1 protein (IE1-72kDa) of HCMV. Recently, work by Paulus *et al.* demonstrated that IE1-72kDa inhibits the expression of interferon-stimulated genes (ISG). IE1-72kDa blocks the signaling proteins STAT1 and 2 from interacting with interferon regulatory factor 9 (IRF9) thereby blocking the transcription of ISGs and conferring a partial resistance to IFNs (322). The next HCMV protein with a profound inhibitory effect on cellular transcription is the 86kDa immediate early 2 product (IE2p86). In the context of HCMV infection, IE2p86 imposes an inhibitory effect over a number of host genes, blocking the expression of several pro-inflammatory molecules including IFN-β, RANTES, MIG, MIP-1α, MCP-2, IL-6 and IL-8 (148, 424, 425). Although the exact mechanisms are unclear, the inhibitory effects of IE2p86 are possibly

the result of impaired NF-κB function, since the inhibited proteins share the NF-κB pathway as a common link (60). It is important to note that both IE1 and IE2 are expressed very early following viral infection and therefore represent two of the first mechanisms HCMV uses to evade the host immune response.

2) *KSHV K9, vIRF homolog*

Like other herpesviruses, Kaposi sarcoma-associated herpesvirus (KSHV) encodes a variety of mechanisms to evade detection by the immune system. Pertinent to this section, the KSHV gene *K9*, which encodes a viral homolog of interferon regulatory factor 1 (vIRF1), downregulates both interferon and IRF-mediated transcription of interferon stimulated genes (ISG) (260). Additionally, vIRF1 also inhibits transforming growth factor-β (TGF-β), presumably to avoid TGF-β-induced apoptosis. The inhibition of TGF-β is accomplished by vIRF1 directly interacting with and inhibiting SMAD proteins. These proteins are recruited to the cytoplasmic tail of the TGF-β receptor and transduce signals via the MAPK8 pathway to the nucleus, resulting in apoptosis (389).

3) *HSV UL41, viral-host shutoff (vhs) protein*

The disruption of host cell translation provides viruses with yet another means to prevent infected cells from signaling to the immune system, and one of the best examples of this phenomenon is the *UL41* gene of herpes simplex 1 and 2 (HSV 1 and 2). Both HSV 1 and 2 are capable of suppressing host cell protein synthesis even in the absence of viral gene expression, a process termed virion-associated host shutoff (126). *UL41* encodes a 58 kDa phosphoprotein that is present in the tegument layer of the infecting

virion. As a component of the tegument, UL41 is capable of imposing function immediately after uncoating, thus making this effect independent of viral gene expression (315). Although the exact function of UL41 is unclear, evidence suggests that UL41 functions as either a ribonuclease or as a subunit of a ribonuclease, causing the disruption of polysomes and increasing mRNA degradation. Association of UL41 with the alpha-transinducing factor (α-TIF) down regulates UL41 activity during lytic infection. Therefore, immediately after infection, UL41 suppresses host cell protein synthesis in order to establish an environment within the cell favorable for HSV replication. Once immediate early gene transcription begins and adequate levels of α-TIF are present, UL41 activity is effectively silenced (277).

b. *Virally-encoded receptors and receptor inhibitors*

Another mechanism that viruses employ to influence their environment is the expression of virally encoded surface receptors and receptor inhibitors. Although these receptors are often pirated homologs of cellular receptors, they may or may not retain functional similarity. Virally encoded proteins that inhibit normal cellular receptor function often share enough homology to cellular ligands or their protein domains to allow for receptor binding. However, most contain amino acid substitutions possibly conferring the inhibitory phenotype.

i. **Inducible and constitutive viral surface receptors**

The HCMV US28 and KSHV vGPCR are two well characterized viral surface receptors that provide excellent examples of functional viral receptors that augment viral

pathogenesis. Despite US28 and vGPCR sharing the same general topology (e.g. 7-TM GPCR) they differ greatly in the roles that they play within their respective viral life cycles.

1) *HCMV US28*

HCMV encodes four chemokine receptors: UL33, UL78, US27, and US28, all of which share the conserved 7-TM topology of a GPCR. US28 is the most studied of these viral GPCRs, possessing structural similarities to CCR1, but with a unique ability to bind chemokines from different chemokine families, such as RANTES, MIP-1α, MCP-1, and Fractalkine (223, 235, 303). Gao *et al.* demonstrated that US28 not only bound RANTES, MIP-1α, and surprisingly, MCP-1, a ligand of CCR2, but also induced the release of intracellular Ca^{++}, which is an indicator of functional agonistic binding (142). Importantly, US28 can mediate the migration of smooth muscle cells (SMC) in response to RANTES and MCP-1 expression (417). This is an interesting finding given that HCMV has been implicated in a number of vascular pathologies, all of which involve an influx of SMCs (36, 409). However, along with chemokine-dependent signaling, US28 has also been shown to be constitutively active, inducing phospholipase C and NF-κB signaling in a ligand-independent manner (71). Interestingly, Kledal *et al.* demonstrated that the membrane-bound CX_3C chemokine, fractalkine, could also bind US28 with very high affinity. The authors hypothesized that the interaction between US28 expressed on virions and infected cells with fractalkine expressed on target cells may enhance infection or cell-to-cell fusion. Therefore, not only is US28 capable of promiscuously binding various chemokines, inducing both ligand-dependent and ligand-independent downstream

90

signaling events, it is also capable of mediating the recruitment of SMCs, ultimately

resulting in vascular pathology.

2) *KSHV vGPCR*

Like HCMV, KSHV also encodes a viral chemokine receptor, vGPCR. A

homolog of the cellular chemokine receptor CXCR2, vGPCR exhibits promiscuous

binding of chemokines like RANTES, I-309, and IL-8, and like US28, vGPCR is

constitutively active, capable of transducing signals in the absence of ligand (23, 66).

This constitutive signaling is mediated through a number of signaling pathways including

protein kinase C (PKC), MAPK, and Phosphoinositide 3-kinase (PI3K)/Akt, resulting in

the activation of numerous transcription factors including activator protein-1 (AP-1),

cAMP response element-binding (CREB), NF-κB, and nuclear factor of activated T cells

(NFAT) (67, 288). Expression of vGPCR in KSHV infected cells is associated with

several potentially pathogenic consequences. Transformation assays show that vGPCRs

cause cellular transformation *in vitro* and lead to KS-like tumors in transgenic mouse

models. This effect appears to be the result of vGPCRs ability to stimulate the

production of vascular endothelial cell growth factor (VEGF), which is associated with

angiogenesis (26, 51). Moreover, vGPCR expression is associated with increased

expression of a number of pro-inflammatory cytokines such as the KSHV encoded viral

IL-6, cellular IL-6, IL-8, and GRO-α (66, 288). The ability of vGPCR and its aberrant

signaling to influence the cellular microenvironment by promoting transformation,

angiogenesis, and inflammation via the secretion of pro-inflammatory cytokines and

chemokines explains why vGPCR is considered a key pathogenic determinant in sarcomagenesis.

ii. Receptor inhibitors

The process of competitive inhibition is a fundamental component of the biological regulatory machinery within the cell. Along these lines, viruses have adapted strategies that utilize this mechanism to establish, adjust, and protect the intracellular environment within an infected cell. By inhibiting specific responses produced by secreted effector proteins like cytokines, chemokines, and growth factors, viral receptor inhibitors can influence overall phenotypic changes, such as cell activation and deactivation, growth, motility, and apoptosis.

1) *Viral tumor necrosis factor-α receptor 1 homologs (vTNF-αR-1)*

Given the general pro-inflammatory nature of TNF-α, some viruses have evolved mechanisms to help mitigate its effects. Myxoma virus (MV) is a leporipoxvirus that naturally infects rabbits, resulting in a highly lytic and often fatal infection. MV expresses a soluble and secreted protein similar to cellular TNF-αR. Specifically, the gene *M-T2* contains a region with significant homology to the ligand binding domain of cellular TNF-αR. Evidence suggests that M-T2 is expressed and secreted from infected cells, where it specifically binds and inhibits the cytolytic activity of rabbit TNF-α. Therefore, M-T2 acts as a soluble competitive inhibitor of TNF-α, and as a result, is an important virulence factor during MV infection (378).

While MV mitigates TNF-α signaling by secreting a soluble form of the TNF-αR, HCMV utilizes the TNF-αR in a different fashion. The unique long b' (UL/b') region of clinical HCMV isolates contains a number of open reading frames that are not present in the extensively passaged laboratory strains. Although these open reading frames may be dispensable for growth *in vitro*, their conserved nature among the clinical strains and their ability to infect humanized SCID mice, while the attenuated laboratory strains cannot, suggest that these genes play an important role *in vivo* (79). One of these genes, *UL144*, encodes a TNF-αR super family member, but unlike the poxvirus protein MT-2 that only possess the ligand binding domain, UL144 is a membrane-anchored glycoprotein with a transmembrane domain and a short cytoplasmic tail. Furthermore, while poxviruses secrete their TNF-α binding protein, UL144 is retained within the cell. The purpose of this is not completely understood, since UL144 has not been shown to bind any known TNF-αR superfamily ligands (35). However, UL144 has been shown to possess two critical functions that could be advantageous for the virus. The first, expression of UL144 promotes the association of TRAF6, a signaling protein with similar functions to TRAF2, with the TRADD domain, thereby stimulating NF-κB activity. Along with pro-survival signals, NF-κB activity stimulates the production of the chemokine, macrophage-derived chemokine (MDC). This chemokine has been implicated in the recruitment of T_H2 lymphocytes which may diminish the host T_H1 immune responses (331, 332). Secondly, the extracellular domain of UL144 has been shown to interact with B and T lymphocyte attenuator (BTLA or CD272), a surface glycoprotein belonging to the Ig superfamily that, when complexed with UL144, can inhibit T cell proliferation *in vitro* (86). Therefore, the function of UL144 may be to promote a cellular/host

environment with a diminished antiviral response.

2) *Poxvirus encoded receptor homologs and inhibitors*

Poxviruses encode a number of receptor homologs and inhibitors. While most of the experimental work has been carried out using vaccinia virus, many of these genes are conserved among the poxvirus family. The proteins encoded by the vaccinia genes *A46R* and *A52R* are intracellular receptor inhibitors. Both of these proteins contain a TIR (toll-IL1 receptor) domain, which is a conserved signaling motif found in Toll receptors, the IL-1 receptor, as well as the five known downstream adaptor proteins for these receptors: MyD88, MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif containing protein (SARM) (306). Following receptor activation, adaptor proteins are recruited to the Toll/IL-1 receptor and form a signaling complex. The association of specific adaptor proteins with the receptors is mediated by TIR domains and confers various physiological responses. With regard to viral infection, TRIF, SARM, and MyD88 are the most important of the adaptor molecules as they transduce signals that ultimately lead to activation of IRF3, AP-1, and NF-κB (306). Expression of A46R and A52R inhibits IL-1R, TLR4 and IL-18 signal transduction, presumably by interfering with the assembly of adaptor molecules, effectively reducing their pro-inflammatory signals (55).

Another vaccinia receptor homolog is encoded by the gene *B15R* which encodes a homolog for the IL-1 receptor (IL-1R). B15R is a 50-60kDa secreted glycoprotein with considerable homology to cellular IL-1R. Interestingly, this viral receptor inhibitor

exhibits a novel specificity for only IL-1β and not IL-1α or natural IL-1 receptor antagonists. Studies utilizing a *B15R* deletion virus demonstrated that infected mice exhibited accelerated symptoms of disease and mortality. Therefore, expression of B15R may diminish the systemic acute phase response to infection and modulate the severity of the disease (15).

The vaccinia receptor analog encoded by the gene *B8R*, produces a 43 kDa glycoprotein that is secreted shortly after infection. B8R possesses significant amino acid homology to he extracellular domain of cellular IFN-γ receptor (IFN-γR). Similar to cellular IFN-γR, B8R is expressed as a homodimer and binds and competitively inhibits IFN-γ away from cellular IFN-γR (421). With its rapid expression and inhibitory function, B8R typifies pox virus immune evasion.

c. *Expression of viral effectors and inhibitors*

The last group of viral proteins to be discussed in this section includes those that are expressed and secreted from virally infected cells. These include viral cytokines and chemokines, as well as virally encoded inhibitors of cellular processes.

i. **Virally encoded effectors**

We have thus far presented several viral mechanisms that influence both the intracellular and extracellular environments in an effort to evade the immune system. The focus of this section is to expand on this and highlight virally encoded effector proteins that, in many cases, mimic cellular cytokines, chemokines, and other effectors and inhibitors.

1) *vIL-6*

Cellular IL-6 is a powerful cytokine possessing both pro- and anti-inflammatory properties. IL-6 functions by binding to the heterodimeric complex of IL-6R and gp130, with IL-6R responsible for ligand binding and gp130 responsible for signal transduction. As a pro-inflammatory cytokine, IL-6 is a key mediator of the acute phase and febrile responses. The anti-inflammatory nature of IL-6 comes from its ability to promote IL-10 expression, which promotes a shift toward T_H2 response, resulting in reduced expression of TNF-α and IL-1 (201). The ability to inhibit pro-inflammatory cytokines while promoting B cell proliferation represents a combination of factors that make IL-6 an attractive cytokine for members of the γ-herpesviruses subfamily that depend upon B cells for latency. As such, KSHV encodes a viral IL-6 homolog (vIL-6) that functions in a similar manner to cellular IL-6. vIL-6 is capable of supporting growth of IL-6 dependant B cell lines, but vIL-6 is unique in its ability to bind and signal in the absence of IL-6R. Binding of vIL-6 to gp130 induces a signaling cascade through both the JAK/STAT and Ras-MAPK pathways, triggering the same responses as cellular IL-6. As a result, vIL-6 is capable of sustained IL-6 signaling, promoting cellular proliferation and anti-apoptotic signals, while being resistant to IL-6R downregulation (314).

2) *Viral IL-10 homologs*

The Epstein-Barr virus gene *BCRF-1* encodes a homolog of IL-10 (vIL-10) that is 84% identical to cellular IL-10. vIL-10 possesses the same immunosuppressive properties as cellular IL-10, including the ability to downregulate the expression of MHC class I, ICAM-1, B7, IFN-γ, IL-12, IL-18, TNF-α, as well as many pro-inflammatory

chemokines (290, 371). More importantly, vIL-10 lacks IL-10's immunostimulatory abilities to induce MHC class II expression and increase B cell proliferation and survival (154). Therefore, expression of vIL-10 potentially impairs antigen presentation and pro-inflammatory cytokine and chemokine expression, producing an environment favorable for viral replication.

HCMV also encodes an IL-10 homolog (UL111a/cmvIL-10). However, unlike vIL-10, which is highly homologous to cellular IL-10 (e.g. 84% identical), cmvIL-10 shares only 27% amino acid sequence identity with cellular IL-10. Like vIL-10 of EBV, cmvIL-10 maintains many of the immunosuppressive properties of cellular IL-10. However, a significant difference between vIL-10 and cmvIL-10 is the ability of cmvIL-10 to enhance B cell proliferation, suggesting that cmvIL-10 is functionally more homologous to cellular IL-10 than vIL-10. Interestingly, treatment with cmvIL-10 stimulates the autocrine production of cellular IL-10 from B cells, thereby further promoting an anti-inflammatory environment (410, 411).

3) *Viral macrophage inflammatory protein (vMIP) homologs*

Macrophage inflammatory protein-1α (MIP-1α) is a potent β-chemokine capable of inducing the migration of a number of cell types, including monocytes, macrophages, T cells, B cells, neutrophils, eosinophils, NK cells, and dendritic cells (297). KSHV and the related rhesus rhadinovirus (RRV) encode MIP-1α homologs that are referred to as vMIPs. While KSHV encodes three vMIPs (e.g. vMIP-1, -II, and III), RRV only encodes a single homolog (RRV vMIP). Much of what is known about vMIPs comes from the study of the KSHV homologs. Therefore, this section will focus on the vMIPs of KSHV

97

and their wide range of effects. RRV vMIP is one of the main focuses of this thesis work and will be discussed at length in chapter 2.

The vMIPs encoded by KSHV have been shown to possess a number of biological functions, each fulfilling a specific role within the KSHV lifecycle. These functions include: functional chemokine, receptor antagonist/chemokine inhibitor, and angiogenic factor. Given that vMIPs have a variety of functions; the role of vMIPs as effector molecules will be covered in this section, and in section c. *ii* the inhibitory nature of vMIP-II will be discussed.

The first of the vMIPs to be discovered, vMIP-I, is encoded by the KSHV gene *K6* and is only expressed during lytic infection (291). Functional studies have shown that vMIP-I is a specific agonist for CCR8, capable of mediating the migration of endothelial cells and T_H2 T cells, which has significant ramifications regarding the KSHV life cycle (174). First, endothelial cells are a major component of Kaposi sarcoma lesions, and the ability of vMIP-I to induce their migration may promote either further infection of endothelial cells or the spread of KS. Secondly, the ability of vMIP-I to selectively recruit cells of the T_H2 lineage suggests a virally mediated shift away from the antiviral T_H1 response to a more viral friendly T_H2 response (119).

The KSHV gene *K4* encodes the second vMIP to be discovered, vMIP-II. The most studied of the vMIPs, vMIP-II exhibits the widest range of biological functions of all viral chemokines. As a chemokine, vMIP-II is capable of mediating the recruitment of many cell types, including monocytes (300), eosinophils (52), T_H2 T cells (407, 447), and endothelial cells (174). Although specific receptor usage is somewhat controversial, evidence suggests that vMIP-II-mediated recruitment occurs through agonistic binding of

CCR3 and CCR8 (52, 174, 222). Similar to vMIP-I, vMIP-II appears to influence the

immune response by inhibiting the expression of T_H1 cytokines, while promoting the

recruitment of T_H2 T cells. Specifically, vMIP-II expression inhibits chemokine-induced

migration of CD8$^+$ T cells (261). Moreover, immunohistochemical analysis of KS

lesions shows vMIP-II expression and mononuclear cell infiltration, represented largely

by CCR3$^+$ T_H2 T cells, therefore suggesting that vMIP-II can drive a T_H2-type immune

response *in vivo* (447). Finally, a unique functional property of both vMIP-I and -II is the

ability to induce angiogenesis. Abnormal vasculature is a hallmark of KS lesions and

angiogenesis is a key component of both KS and KSHV-related malignancies, yet the

cause of KSHV-mediated angiogenesis remains only loosely defined. Using chicken egg

chorioallantoic membrane (CAM) assays, Boshoff *et al.* demonstrated that the presence

of vMIP-I and -II induced "a clear angiogenic response", suggesting a potential role for

vMIP-I and -II in angiogenesis (52).

     The last of the KSHV vMIPs, vMIP-III encoded by the gene *K4.1,* was initially

thought to be the result of a duplication of the *K4* gene that encoded a non-functional

product. However, in 2000, Stine *et al.* published their findings showing that vMIP-III

was actually an agonist for CCR4, a receptor commonly expressed on T_H2 T cells.

Consistent with this finding, vMIP-III preferentially recruits T_H2 T cells in chemotaxis

assays (415).

4) *UL146, a CMV encoded chemokine*

     Several chemokine homologs have been identified among the various species-

specific CMVs. HCMV encodes two chemokine homologs, UL146 and UL147,

designated vCXC-1 and vCXC-2 because of their limited sequence homology to α-chemokines. Despite their sequence homology, only UL146 has been shown to possess any chemokine-like properties. *UL146* encodes a small glycoprotein with late gene kinetics that is secreted from HCMV infected cells. Recombinant vCXC-1 possesses many of the functional properties of an α-chemokine, including high affinity binding to CXCR2, induction of calcium mobilization, chemotaxis, and degranulation of neutrophils (326). The role of vCXC-1 during CMV infection, albeit hypothetical, is believed to promote the recruitment of neutrophils to sites of CMV infection for the purposes of viral dissemination (305, 326).

ii. **Virally encoded inhibitors**

Virally encoded inhibitors of cellular processes, particularly those involved in immunity, are the topic of this section. Many of these inhibitors are secreted from infected cells such that they can function in both an autocrine and paracrine fashion. However, some of these inhibitors remain cell-associated and help to augment the viral lifecycle by affecting the microenvironment directly surrounding the infected cell.

1) *Complement control proteins*

The complement system is an aspect of innate immunity that consists of a number of blood proteins that lead to the removal of pathogens from circulation. The complement proteins circulate through the blood as inactive enzymatic precursors (e.g. C1, C2, and C4 thru C9), and under certain conditions, such as opsonified antibody on the surface of an infected cell (classical pathway) or the presence of pathogen itself

(alternative pathway), when enough of the precursors are present, they are cleaved via an autolytic process resulting in the assembly of a functional protease (C3 convertase) with the capability to cleave other complement proteins. The end result of this protease cascade is the formation of the C5b protein, which has the ability to insert in the plasma membrane and associate with C6 through C9, creating a pore forming unit called a membrane attack complex (MAC). These MACs produce holes in the plasma membrane of the affected cell, bacteria, or virus, resulting in their death (201).

To combat the complement system, some members of the herpesvirus and poxvirus families encode complement control proteins that interfere with the formation of the larger, biologically active complexes. One such example comes from KSHV open reading frame 4, which encodes the KSHV complement control protein (KCP). KCP is expressed as a membrane bound protein on the surface of KSHV infected cells. KCP accelerates the decay of classical C3 convertase by the cellular control protein decay accelerating factor (DAF). This induces the degradation of activated complement factors C4b and C3b by a cellular serine protease, factor I, thereby effectively blocking the formation of MAC (273, 274). The association of KCP with C3, C4b and C3b, demonstrates the ability of KCP to inhibit both the classical and alternative complement pathways.

The complement control proteins encoded by poxviruses work in a similar fashion to the mechanism described for KSHV. Vaccinia virus produces the 35 kDa vaccinia virus complement control protein (VCP) that binds to the C3 convertase, thus inhibiting both the classical and alternative complement pathways. Unlike KSHV, VCP is produced in both a secreted form and a membrane bound form (364). Therefore VCP has

the ability to prevent the complement-dependent lysis of the infected cell or adjacent

cells. Variola virus also encodes a soluble complement regulator called smallpox

inhibitor of complement enzymes (SPICE). Although they differ only by 11 amino acids,

SPICE has been shown to be a much more potent in inactivator of the complement

cascade than VCP. This increased potency is due to a higher affinity for the C3

convertase (455).

2) *Viral macrophage inflammatory protein-II (vMIP-II)*

As mentioned in the previous section, the KSHV-encoded vMIPs all possess

functional chemokine abilities. However, a unique property of vMIP-II is the ability to

competitively inhibit endogenous chemokines. Kledal *et al.* demonstrated through

radiolabeled receptor-binding and internal Ca++ mobilization assays that vMIP-II,

although capable of high affinity binding to β-chemokine receptors CCR1, CCR5, and

CCR2, was unable to mobilize internal Ca^{++} stores. Taken together, these results suggest

that vMIP-II can act as a competitive inhibitor by binding to chemokine receptors,

thereby blocking other chemokines from functionally binding to their cognate receptors.

Interestingly, vMIP-II can also bind and inhibit the α-chemokine receptor CXCR4, as

well as the viral receptor US28 (222). Therefore, through its promiscuous binding and

varied functional outcomes, vMIP-II can influence both the immediate and distant

environments.

3) *Viral chemokine binding proteins (vCKBP)*

While vMIP-II functions as a chemokine inhibitor by antagonizing chemokine

receptors, the viral chemokine binding proteins (vCKBP), encoded by numerous

poxviruses and some herpesviruses, are soluble proteins secreted from infected cells that

act directly on chemokines themselves. These vCKBPs function by binding to

chemokines and sequestering them away from their specific receptors, effectively

reducing inflammation. The vCKBP family includes three proteins encoded by some

herpesviruses and many poxviruses. vCKBP-I is encoded by myxoma virus and has

homology to cellular IFN-γR. A unique property of vCKBP-I is the ability to scavenge

many different types of chemokines. Moreover, vCKBP-I can bind directly to

chemokines from three of the four chemokine families (e.g C, CC, CXC), in addition to

IFN-γ (297). vCKBP-II is encoded by multiple poxviruses (e.g. cowpox, variola,

monkeypox, myxoma, and vaccinia) and is much more specific, binding only β- or CC

chemokines. Throughout the literature, this protein is known by several other names:

"T1" in leporipoxviruses, "35kD" in orthopoxviruses, and commonly referred to as viral

CC chemokine inhibitor (vCCI). Chapter 3 will discuss in greater detail the vCKBP-II

protein of monkeypox virus (referred to as MPV vCCI). vCKBP-III is encoded by

murine herpesvirus-68 (MHV-68) and scavenges chemokines from all chemokine

families (e.g. C, CC, CXC, and CX$_3$C). Like the previous vCKBPs, vCKBP-III also

functions to reduce the host inflammatory response. However, unlike vCKBP-I and -II,

vCKBP-III is required for the establishment of latency in B cells, in part by blocking

chemokine activation of CD8+ T cells (59). Human cytomegalovirus encodes another

vCKBP, the secreted glycoprotein UL21.5, which specifically binds RANTES with very

high affinity and effectively inhibiting its contact with cognate receptors (440). Together, expression of vCKBPs during viral infection marks an attempt by the viruses to diminish the chemokine-dependent inflammatory response, underscoring the fundamental importance of proper chemokine function for the immune system to combat viral infection.

5. Overview of thesis project

As powerful mediators of cellular recruitment, chemokines play an important role in both health and disease. Viruses have therefore evolved mechanisms to either mimic or subvert normal chemokine function in an attempt to augment their own pathogenesis. The basis for this thesis project is the biological characterization of two such viral proteins. The first is rhesus rhadinovirus viral macrophage inflammatory protein (RRV vMIP), which, based on homology with β-chemokines as well as the MIP homologs encoded by KSHV, is predicted to possess chemokine-like function. The second protein is the monkeypox virus viral chemokine inhibitor (MPV vCCI). This common poxvirus protein has been partially characterized *in vitro* for CPV, VV, and RPV, and to date only RPV vCCI has been studied *in vivo*. However, MPV vCCI has never been characterized. The work presented in chapter 2 describes our preliminary characterization of RRV vMIP and its role as a functional chemokine homolog. Chapter 3 provides a detailed description of the biological functions of MPV vCCI and its role as a potent chemokine inhibitor.

a. *Significance of work*

Kaposi sarcoma (KS) is the number one AIDS-related malignancy worldwide. Unfortunately, Kaposi sarcoma-associated herpesvirus (KSHV), the etiological agent of KS, presents several obstacles to researchers seeking insights into KSHV pathogenesis. These include poor growth *in vitro*, the lack of an adequate animal model that supports KSHV growth, and a rapid progression to latency. The closely related RRV overcomes each of these obstacles, and therefore holds great promise as an experimental model for understanding KSHV biology. However, the analysis of individual RRV open reading frames remains an important task for validating RRV as a model for studying KSHV pathogenesis. The studies described in this work, present novel findings with regard to our understanding of the role of vMIPs in γ-herpesvirus pathogenesis.

Variola virus (VARV), the causative agent of smallpox, was eradicated in 1976, and although smallpox is no longer considered a human health concern, emerging zoonotic infections like MPV pose a significant risk, not only during zoonosis, but also in the unlikely event that MPV is utilized as a weapon of bioterrorism. MPV infection of humans results in a disease progression nearly indistinguishable from that of VARV. Both viruses encode chemokine binding proteins, which have been implicated as major pathogenic determinants in the development of disease. As such, our studies provide the first *in vitro* and *in vivo* characterization of MPV vCCI. More importantly, our findings suggest that MPV vCCI could be used as a novel therapeutic for the treatment of chemokine-driven diseases.

b. *Authors contribution*

All of the work presented in chapter 2 was performed by the author of this thesis, with the exception of: 1) figure 6, in which surgical implantations and explantation were performed by Drs. John Fanton and Anne Lewis (acknowledged in the text), and 2) figure 7, which was conducted in collaboration with Dr. Ilhem Messauodi. All of the work in chapter 3 was performed by the author, with the exception of figure 6, which was performed in collaboration with Dr. Ilhem Messauodi. All other authors in Chapters 2 and 3 were included for their scientific input, expertise, and guidance.

Chapter 2

Rhesus Rhadinovirus expresses a functional chemokine homolog

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ABSTRACT

Rhesus rhadinovirus strain 17577 (RRV_{17577}), a simian γ2-herpesvirus, shares considerable sequence homology with Kaposi's sarcoma-associated herpesvirus/ human herpesvirus 8 (KSHV/HHV-8). Furthermore, RRV infection of immune-deficient rhesus macaques results in the development of B cell disorders similarly to what has been described for AIDS patients co-infected with KSHV. Both KSHV and RRV encode viral macrophage inflammatory protein homologues (vMIPs), but while KSHV encodes three vMIPs (ORF-K6, -K4, and -K4.1), RRV encodes one from ORF-R3. On average, the amino acid similarity of RRV vMIP and the KSHV vMIPs is roughly 37%, with KSHV vMIP-II exhibiting the highest homology of 38.5%, while RRV vMIP is 37.6% similar to rhMIP-1α. Both RRV and KSHV vMIPs are expressed and secreted from infected cells. However, despite the significant homology with cellular MIPs, the role of these viral homologues in the pathogenesis of RRV and KSHV remains poorly understood. In this study, we show that recombinant RRV vMIP induces migration of monocytic THP-1 cells *in vitro*. Moreover, RRV vMIP -saturated implants induce the recruitment of $CD14^+$ cells *in vivo*, suggesting RRV vMIP might function as a chemokine during lytic RRV infection. Acute *in vitro* infection of isolated peripheral blood mononuclear cells (PBMC) with RRV shows that $CD14^+$ cells are permissive to RRV infection. Taken together, these data strongly suggest that RRV vMIP encodes a functional chemokine with the ability to recruit permissive $CD14^+$ cells during acute infection, which may provide a mechanism for viral dissemination.

INTRODUCTION

Members of the *herpesviridae* family have co-evolved with their specific hosts, managing a delicate balance between the productions of progeny virions through lytic infection, and the establishment of a persistent, life-long infection. To achieve this complex interaction within the host, many herpesviruses possess mechanisms to interact with and subvert various cellular processes. These interactions are in part mediated by viral genes with considerable homology to host cellular genes involved in cell cycle control, apoptosis, interferon regulation, cytokines and chemokines expression, and cell surface receptors (134). The acquisition and adaptation of these cellular processes suggests the viruses have pirated these cellular genes to, in some manner, augment their own existence.

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), also referred to as, human herpesvirus 8 (HHV-8), is a γ2-herpesvirus and the etiologic agent of KS, a highly vascularized tumor of endothelial origin, and the most common AIDS- related malignancy. KSHV DNA has also been shown to be present in both AIDS and non-AIDS related B cell disorders, such as primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (78, 83, 405). Studying KSHV lytic replication *in vitro* is difficult, due to the virus's ability to quickly establish a latent infection. To date, the current method for KSHV viral preparation involves using phorbol esters to induce latently infected B cells (e.g. BCBL-1) to produce virus (348, 349). Furthermore, Renne *et al.* tested numerous cell lines for permissivity to KSHV and found that eleven of thirty-eight tested cell lines supported KSHV infection, but in every case, infection

proceeded in an inefficient manner, and was often characterized as "semi-productive" (347). Furthermore, studies on KSHV pathogenesis are hampered by the fact that infection of laboratory animals with KSHV fails to reproduce clinical signs of disease.

The infection of nonhuman primates with homologous viruses offers an alternative model where host-pathogen interactions can be studied in a natural setting. Rhesus Rhadinovirus strain 17577 (RRV_{17577}) is a γ2-herpesvirus with significant similarities to KSHV. Sequence analysis shows that the genomes of KSHV and RRV_{17577} are essentially co-linear with 67 of 79 open reading frames sharing some level of homology (113, 367, 382). Furthermore, rhesus macaques co-infected with SIV and RRV_{17577} develop several B cell disorders consistent with those seen in KSHV/HIV co-infected humans (453). Importantly, RRV can establish a robust lytic infection in primary rhesus fibroblasts which greatly facilitates *in vitro* functional studies (382). Based on the genomic and pathological similarities, RRV_{17577} provides a suitable animal model to investigate KSHV-like diseases in an AIDS-like setting.

Interestingly, both KSHV and RRV encode homologues of cellular chemokines. Chemokines belong to a superfamily of small (8 – 14 kDa) proteins that possess similar structural and functional properties (297). The chemokine family is further divided into the following subtypes: C, CC, CXC, and CX_3C, based on the position of conserved cysteines located in the N-terminus of the protein. Although primarily known for their ability to induce chemotaxis as a result of injury or pathogenic insult, chemokines are also involved in a variety of cellular processes, such as development, angiogenesis, and hematopoiesis (297, 358). Virally encoded chemokines have been described for a number of herpesviruses (e.g. HCMV, MCMV, and KSHV) and have been shown to

possess a variety of biological properties such as: chemotaxis, cellular activation and differentiation, angiogenesis, anti-apoptosis, and competitive inhibition of cellular receptors (52, 188, 222, 326, 369).

The viral macrophage inflammatory protein homologues (vMIPs) of KSHV and RRV possess the classic β-chemokine "CC" motif and are ~ 25-40% homologous to cellular MIP-1α (222). While KSHV encodes 3 vMIPs (vMIP-I, -II, and -III), RRV encodes a single MIP homolog (RRV vMIP) from ORF-R3. Several biochemical studies have shown that KSHV vMIPs bind to an array of chemokine receptors to induce a variety of biological functions. Work by Haque *et al.* showed that KSHV vMIP-I binds to CCR-8 causing endothelial cell migration (174), while Boshoff *et al.* showed that KSHV vMIP-II is a potent chemoattractant for eosinophils via CCR3, and both KSHV vMIP-I and -II possess angiogenic properties (52). A unique characteristic of KSHV vMIP-II is the ability to function as a powerful, broad spectrum antagonist of several chemokine receptors: CCR1, 2, 4, 5, 8, and 10, CXCR3 and 4, XCR1 and CX_3CR1 (268). Along these lines, work by Lindow *et al.* suggests that vMIP-II interferes with migration of T_H1 T cells, thereby disrupting the anti-viral immune response (261). Despite having relatively low sequence homology and possessing a wide range of biological functions, crystallographic studies have shown that KSHV vMIP-I and -II share the same three dimensional (3-D) structure, the typical chemokine tertiary fold, as Eotaxin-1, RANTES, and MCP-3. Furthermore, except for some variation in the N-terminus, the 3-D structures of vMIP-I, vMIP-II, RANTES, MCP-3, and Eotaxin are essentially superimposable (132, 269). KSHV vMIP-III binds CCR4 and XCR1, promoting angiogenesis (268, 415). However, the functions of KSHV encoded vMIPs have not

111

been validated *in vivo.* The infection of rhesus macaques with RRV is a powerful model of KSHV pathogenesis. Furthermore, RRV encodes a viral homologue of MIP, ORF R3.

The goal of this study is to characterize RRV vMIP and its role in RRV pathogenesis in an effort to further our understanding of the role of vMIP in KSHV pathogenesis. Here, we provide the first evidence that RRV vMIP is expressed and secreted during lytic RRV infection. Furthermore, RRV vMIP acts as a functional chemokine by inducing chemotaxis of activated THP-1 cells *in vitro*, and more importantly, CD14$^+$ cells *in vivo*. During acute *in vitro* infections, we show for the first time that CD14$^+$ cells are a major target of RRV. These data suggest a model where RRV secretes vMIP during lytic infection thereby recruiting permissive CD14$^+$ cells which can then participate in viral dissemination.

MATERIALS AND METHODS

Protein alignments. Protein alignments were performed using ClustalW from

MacVector version 9.0 software (Accelrys, Inc., Madison, WI). A Blosum scoring

matrix was used in pairwise alignment of each sequence, with a gap introduction penalty

of 10 and a gap extension of 0.1.

Cell Lines, cell culture, and RRV specific antibodies. Primary rhesus fibroblasts were

maintained as previously described (420). THP-1 cells are maintained in RPMI plus 10%

heat-inactivated fetal bovine serum (HyClone, Logan, UT). For chemotaxis assays, THP-

1 cells are activated prior to use by culturing in the presence of 80 nM Vitamin D_3 for 48

hours. All RRV specific antibodies were made on-site by the monoclonal antibody core

at the Vaccine and Gene Therapy Institute (VGTI, Beaverton, OR). For RRV vMIP, the

recombinant vMIP described below was utilized as antigen for immunization. Gradient

purified RRV was utilized as antigen for the glycoprotein B specific monoclonal

antibody.

RRV infections and RNA isolation. Approximately $1x10^6$ primary rhesus fibroblasts

were infected with RRV_{17577} at an M.O.I = 5, in the presence of 75 µg/ml cyclohexamide

(CHX; Sigma, St. Louis, MO), 300 µM phosphonoacetic acid (PAA; Sigma, St. Louis,

MO), or left untreated. RNA was isolated from uninfected and infected cells using TRI

REAGENT™ (Sigma, St. Louis, MO).

Generation of RRV_{gfp} virus. To facilitate *in vitro* tropism studies of RRV, a
recombinant RRV expressing the enhanced green fluorescent protein (eGFP) under the
transcriptional control of the elongation factor 1-α (EF-1α) promoter was created to serve
as a marker of RRV infection. The recombinant was generated by co-transfecting
construct pGEM7-9-1ES-GFP (containing the eGFP cassette inserted into the Hind III
site present in the intergenic region between ORFs 57 and R6) and infectious RRV DNA
(derived from a plaque purified isolate of RRV$_{strain\ 17577}$) into primary rhesus fibroblasts.
Recombinant viruses expressing eGFP were purified from parental wild type virus by
three rounds of single cell sorting on the basis of fluorescence of infected cells, which
were subsequently plated onto primary rhesus fibroblasts. Southern blot analysis was
utilized to confirm correct recombination in the RRV genome and 5' and 3' RACE
analysis was utilized to verify that expression of ORF57 and R6 were un-affected.

Cloning and expression of recombinant RRV vMIP and RhMIP1α. Based on the
amino acid sequence and SignalP predicition, the first 20 amino acids of RRV vMIP
represent a secretion signal, which is cleaved from the mature protein. The coding
sequence for mature RRV-R3 was isolated from RRV$_{17577}$ genomic DNA via PCR using
primers specific for RRV-R3 which also contained a 6x-histidine tag (underlined region)
and restriction sites for NdeI (5-CATATGGCCTTTCCTATGGGCTCC-3') and XhoI
(5'-
CTCGAGTCATCA<u>GTGGTGGTGGTGGTGGTG</u>CACAATCCCGCTGCCAAGGCC-
3'). A 6x-histidine tagged RhMIP1α was isolated in a similar manner from another
expression plasmid (graciously provided by Dr. Todd Reinhart, Univ. of Pittsburg) also

using NdeI (5'-CATATGGCTGACACCCCGACCTC-3') and XhoI (5'-

CTCGAGTCATCAGTGGTGGTGGTGGTGGTGCACGGCACTCAGCTCTAGGTC-

3'). The resulting products were cloned into pRSETb (invitrogen, Carlsbad, CA) for

expression. Rosetta 2® DE3 cells (Novagen, Madison, WI) were transformed with the

pRSETb expression plasmids. Expression cultures were set up by diluting overnight

cultures 1:20 into 1 L of LB media without antibiotic and incubated for 3 hours at 37°C

with agitation. At 3 hours, the temperature of the cultures was reduced to 25°C and

protein expression was induced with 0.5 μM isopropyl-β-D-thiogalactoside (IPTG -

Fisher, Fair Lawn, NJ) with continued agitation for 6 hours. Cells pellets were harvested

by centrifugation (5,000xg for 12 minutes) and stored at -80°C until use.

Purification of recombinant RRV vMIP and RhMIP1α. Purification of recombinant

proteins from bacteria was based on previously described methods (337). Harvested

bacteria are resuspended in lysis buffer (300 mM NaCl, 50 mM NaPO4, 20 mM Tris-

HCl, 0.1 mM PMSF, 3 mM βME, pH 8.0) and lysed by 2 freeze/thaw cycles, incubation

with 1 mg/ml lysozyme, 5 μg/ml DNAase, and 5 μg/ml RNAase for 30 minutes on ice,

and then sonicated 30 seconds (3x). Lysates were separated into soluble and insoluble

fractions by centrifugation at 20,000xg for 60 minutes at 4°C. Proteins were purified via

inclusion bodies by resuspending the insoluble fraction in 20 ml binding buffer (300 mM

NaCl, 50 mM NaPO4, 8 M urea, 3 mM βME, pH 8.0) and stirring at 60°C for 30

minutes. The solubilized protein was cooled to room temperature and applied to pre-

equilibrated BD Talon® metal affinity resin (Clontech Laboratories Inc, Mountain View,

CA) (1 ml resin per 2 L culture), where it was incubated on a rotator at RT for 1 hour.

Protein-bound resin was pelleted and washed (2x) with 20 ml wash buffer (300 mM

NaCl, 50 mM NaPO4, 8 M urea, 10% glycerol, 3 mM βME, pH 7.5). Protein was eluted

from the resin by adding 3 ml elution buffer (300 mM NaCl, 50 mM NaPO4, 8 M urea, 3

mM βME, pH 3.0) and incubated on a rotator at room temperature for 5 minutes (3x).

Eluted protein is 0.22 μm filtered and run over a HiPrep 16/60 Sephacryl S-100 HR

column (GE Healthcare, Piscataway, NJ) equilibrated in denaturing buffer (100 mM Tris-

HCl, 8 M urea, 3 mM βME, pH 8.0). Protein purity and size were determined on 4-12%

Bis-Tris NuPAGE® gels and the purest fractions were pooled together and re-natured by

10-fold "dropwise" dilution into refolding buffer (20 mM Tris-HCl, 0.01 mM glutathione

(oxidized - GSSG), 0.1 mM glutathione (reduced - GSH), pH 8.0) overnight at 4°C with

stirring. Aggregates were pelleted (10,000xg for 30 minutes) and the pH of the

supernatant was adjusted to 4.5 with acetic acid. Diluted protein solution was further

purified and concentrated by binding to a HiTrap SP FF column and eluting with a 0-2 M

NaCl gradient over 20 ml. Protein purity and size were determined on 4-12% Bis-Tris

NuPAGE® gels and the purest fractions were pooled together and dialyzed into 1%

acetic acid for 4 hours at 4°C (2x) and 0.1% trifluoroacetic acid for 4 hours at 4°C.

Protein concentration was determined by absorbance spectroscopy. Purified proteins

were lyophilized and stored at -80°C, while reconstituted protein was kept at -20°C.

Northern Blot Analysis. A 1% formaldehyde agarose gel was loaded with 10 μg of each

RNA sample and following resolution was transferred overnight to a nitrocellulose

membrane using capillary action. Membranes were UV cross-linked and baked at 80°C

for 1 hour. Radiolabeled double-stranded DNA probe specific for RRV ORF-R3 was

made using an R3-specific PCR product and a Prime-It® II random primer labeling kit (Stratagene, La Jolla, CA). Membranes were rehydrated in pre-hybridization buffer (PHB; 2X SSC – 900 µM NaCl and 90 µM sodium citrate, 6X Denhardt's solution, 0.1% SDS, 50% formamide, 100 µg/ml tRNA) and then incubated with radiolabeled probed overnight at 37°C. Membranes were washed twice with 2X SSC + 0.1% at RT, once with 2X SSC + 0.1% at 42°C for 30 minutes, once with 2X SSC + 0.1% at 60°C for 20 minutes, and twice with 0.2X SSC + 0.1% at 60°C from 15 minutes. Membranes were air dried and exposed to film.

Flow cytometry of RRV infected peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from the blood of five rhesus macaques via centrifugation over Histopaque®-1077 (Sigma, St. Louis, MO). Complete blood cell counts were obtained using an AcT 5 diff cell counter (Beckman Coulter, Fullerton, CA). Isolated PBMCs were infected with RRV_{17577} expressing green fluorescent protein (RRV-GFP) at an M.O.I. = 3. 72 hours post infection, cells were washed and stained using a panel of conjugated antibodies directed against: CD3 (PECy7 - Pharmingen, San Diego, CA), CD14 (Alexa 700 - Biolegend, San Diego, CA), CD20 (ECD - Beckman Coulter, Fullerton, CA), HLA-DR (PerCP Cy5.5 - Pharmingen, San Diego, CA), and CD16 (Pacific Blue - Pharmingen, San Diego, CA). Samples were acquired using an LSR-II flow cytometer (Becton Dickinson Co., Franklin Lakes, NJ) and the data was analyzed using FlowJo (Treestar, Ashland, OR).

Immunoprecipitation and Western blot analysis. RRV vMIP was immunoprecipitated

from infected cell supernatants using the following protocol. Supernatants were clarified

and concentrated 10-fold via 5,000 MWCO Amicon® Ultra centrifugal filtration device

(Millipore, Bedford, MA). An RRV vMIP specific mouse monoclonal antibody (clone

#10D12) was added to the concentrated supernatants at 12.5 µg/mL and incubated for 1

hour at 4°C with agitation. 100 µL Protein A/G-plus agarose (Santa Cruz Biotechnology

Inc., Santa Cruz, CA) was added to the mixture and allowed to incubate for 1 hour at 4°C

with agitation. Protein bound agarose was pelleted and washed twice with cold PBS.

Bound proteins were denatured by adding 2x NuPAGE® LDS sample buffer (Invitrogen,

Carlsbad, CA) and heating to 70°C for 10 minutes. Proteins (15 µL load) were resolved

on 4-12% NuPAGE® Bis-Tris polyacrylamide gels and wet transferred to PVDF

membranes at 30V for 1 hour. Protein blots were probed using our 10D12 antibody

(1:1000) followed by a horseradish peroxidase-conjugated secondary antibody (1:5000)

(Sigma, St. Louis, MO). Bands were visualized using chemiluminescence.

Immunofluorescence analysis. Approximately 0.8×10^5 Primary rhesus fibroblast were

seeded onto 12mm glass cover slips (Fisher Scientific, Pittsburg, PA). After 48 hours,

cells were infected with RRV$_{17577}$ at an M.O.I.= 1. Infection levels were monitored using

parallel infections with RRV$_{GFP}$. At 48 hours post-infection, cells were fixed with 4%

paraformaldehyde in PBS at 25°C for 20 minutes. Fixed cells were then permeabilized

with 0.2% triton-x 100 in PBS. Staining for RRV vMIP and RRV gB was performed

using mouse monoclonal antibodies (RRV vMIP – clone #10D12 and RRV gB – clone

#10B5.2), followed by a biotinylated horse anti-mouse secondary antibody (Dako,

Cuppertino, CA). Visualization was performed using streptavidin conjugated to Alexa-488 (RRV gB) and Alexa-594 (RRV vMIP) (Invitrogen, Carlsbad, CA) followed by a nuclear counterstain with a Hoechst dye (Sigma, St. Louis, MO). All antibodies used were at 1:100 dilution.

***In vitro* chemotaxis assay.** Migration of THP-1 cells was carried out using Transwell® plates (6.5 mm x 3.0 μm pore, Corning, New York, NY) equilibrated in assay media (RPMI 1640 supplemented with 0.5% heat-inactivated fetal bovine serum) for 1 hour prior to assay. Various concentrations of RRV vMIP, 10^{-9} M RhMIP-1α, or buffer were added to 600 μl assay media in the lower chamber. Approximately 5×10^5 activated THP-1 cells suspended in 100 μl were added to the upper chamber of the transwell and incubated for 4 hours at 37°C with 5% CO_2. Migrated cells were counted using CyQuant cell proliferation assay kit (Invitrogen, Carlsbad, CA).

***In vivo* chemotaxis assay.** *In vivo* recruitment assay was adapted from a previously published angiogenesis assay (128). Gelfoam® plugs (Pharmacia & Upjohn Company, Kalamazoo, MI) were cut 5 mm^3 and rehydrated overnight in PBS at 4°C. On the day of implantation, plugs were briefly dried between two pieces of filter paper and 500 ng of chemokine (or PBS) mixed with 0.4% agarose warmed to 42°C was absorbed into the plugs and placed at 4°C until implantation. For implantation, rhesus macaques are anesthetized with ketamine (15-20 mg/kg i.m.), placed in oblique ventral recumbency, and the hair clipped from the mid-scapular region to the shoulder. Skin prep was performed in routine fashion with betadine scrub and solution, followed by placement of

a medium fenestrated drape. A 5-10 mm skin incision was made in the lateral mid-

scapular region, the skin is undermined with a Kelly forceps for a distance of

approximately 2-3 cm from the incision, and the Gelfoam® implants were inserted in the

undermined space. The skin was then closed with several simple interrupted sutures.

Spacing between implants was maximized to avoid potential functional overlap. The

implants remained in the animal for 7 days, at which time, the Gelfoam® plugs and

surrounding tissue were excised and cryopreserved in tissue freezing media (Triangle

Biomedical Sciences, Durham, NC) and stored at -80°C for later sectioning.

Immunohistochemistry. Sections of the cryopreserved samples 10 µm thick were cut

and mounted onto Superfrost®/Plus slides (Fisher Scientific, Pittsburg, PA) at RT

overnight. Slides were fixed with ice cold acetone for 10 minutes and then washed three

times with tris-buffered saline (pH 7.4) + 0.1% tween-20 (TBST) to remove freezing

media. Slides were blocked with PBS + 1% BSA and 10% donkey serum at RT for 1

hour, followed by PBS + 0.3% H_2O_2. A CD14-specific mouse monoclonal primary

antibody (BD Pharmingen, San Diego, CA) diluted in PBS + 1% BSA was incubated on

the sections overnight at RT. Following TBST washes, sections were incubated with

horse anti-mouse secondary antibody conjugated to horse radish peroxidase for 1 hour at

RT. CD14 specific staining was visualized using a DAB substrate kit (Dako, Cuppertino,

CA) and counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA).

RRV vMIP bears significant homology to KSHV vMIPs, rhesus (rh) MIP-1α, rhMIP-1β, and rhRANTES. The amino acid sequence of RRV vMIP was aligned with those of rhMIP-1α, rhMIP-1β, and rhRANTES to determine the level of amino acid sequence homology. Figure 2.1 shows the protein alignments for all four proteins and confirms the presence of the four conserved cysteines and the classic β-chemokine "CC" motif at their N-termini, as well as other conserved chemokine residues. Interestingly, the C-terminus RRV vMIP possesses an additional 20 amino acids that do not align with the other listed rhesus chemokines. On average, RRV vMIP shares approximately 38% similarity with the above listed chemokines (Table 2.1). These similarities suggest that RRV vMIP shares similar sequence and structural elements as β-chemokines, and may possess chemotactic properties.

Transcriptional analysis of RRV ORF-R3 (RRV vMIP). Based on the published RRV sequence, ORF-R3 is located in the 5' region (nt21753 to nt21406) of the RRV genome flanked by open reading frames R2 (vIL-6) and 16 (vBcl-2) (Fig. 2.2a) (382). To examine expression of the RRV ORF-R3 transcript during RRV infection, northern blot analysis was performed on RNA isolated from RRV infected primary rhesus fibroblasts either treated with cyclohexamide (CHX) for 24 hours, phosphonoacetic acid (PAA) for 48 hours, or left untreated for 72 hours. These time points represent the immediate early, early, and late lytic gene classes of herpesvirus transcription, respectively. A single R3-specific band was detected only in the untreated (72 hour) sample suggesting that R3 is

transcribed as a single transcript with late gene kinetics (Fig. 2.2b). Furthermore, the size

of the transcript, 500 bp, is consistent with the size of the R3 open reading frame.

RRV vMIP is expressed and secreted during lytic RRV infection. We next

determined when RRV vMIP protein is expressed during RRV infection. To do so, we

performed an immunofluorescence assay on RRV infected rhesus fibroblasts using an

RRV vMIP specific mouse monoclonal antibody (10D12) (Fig. 2.3a). To correlate RRV

vMIP expression with RRV infection, cells were co-stained using a mouse monoclonal

antibody specific for an RRV late gene, glycoprotein B (clone #10B5.2) (Fig. 2.3b). As

shown in figure 2.3d, cells that stain positive for RRV gB, also stain positive for RRV

vMIP, clearly indicating RRV vMIP expression in RRV infected cells.

The presence of a secretion signal peptide, suggested that RRV vMIP would be

secreted during infection. To test this possibility, we performed immunoprecipitations on

clarified supernatants from rhesus fibroblasts infected with RRV for 24, 48, or 72 hours.

Immunoprecipitation with RRV vMIP-specific antibody (10D12), followed by SDS-

PAGE and western blot analysis, shows the presence of a RRV vMIP specific band at the

predicted molecular weight of 11.2kDa in the 48 and 72 hour RRV-infected samples (Fig.

2.3e). Furthermore, the RRV vMIP band was PAA sensitive, indicating that viral DNA

synthesis is required for RRV vMIP expression and secretion. These data, coupled with

our transcriptional analysis, demonstrate that RRV vMIP is expressed and secreted during

lytic RRV infection.

Expression and purification of recombinant RRV vMIP and rhMIP-1α. The DNA

sequences encoding mature RRV vMIP and mature rhMIP-1α were amplified by PCR

and a 6x-histidine tag was placed in frame at the C-terminus of each protein for purification purposes. After a multi-step purification protocol, SDS-PAGE on fractions from size exclusion chromatography shows purified recombinant RRV vMIP and rhMIP-1α running at the predicted molecular weights of 11.2 kDa and 8.3 kDa, respectively (Fig. 2.4a and b). At high protein concentrations, the presence of RRV vMIP dimers, a common trait among several chemokines, can be observed (Fig. 2.4b). Limulus amebocyte lysate (LAL) assays were conducted on purified proteins to determine endotoxin levels. In all samples tested, the endotoxin levels were well below the 5.0 EU/kg limit set by the Food and Drug Administration for *in vivo* studies in humans and small animals (data not shown).

RRV vMIP induces chemotaxis in vitamin D$_3$ activated THP-1 cells. In order to assess the chemotactic abilities of RRV vMIP, we first utilized an *in vitro* transwell assay using vitamin D$_3$ activated THP-1 cells (231, 317, 353). Vitamin D$_3$ was used as our activating agent based on its ability to upregulate many chemokine receptors without significantly changing the morphology of the THP-1 cells, unlike phorbol-12-myristate-13-acetate (PMA), which significantly alters morphology and adherence of the THP-1 cells (John Jones and Scott Wong, unpublished data). Rhesus MIP-1α at 10^{-9} M and PBS were used as positive and negative controls, respectively. RRV vMIP induces chemotaxis of activated THP-1 cells exhibiting a classic chemokine curve with a sharp increase in mobility at an optimum concentration of 10^{-6} M, followed by a gradual decrease at higher concentrations (Fig. 2.5). These data strongly suggest that RRV vMIP possesses chemotactic activity.

KSHV vMIPs have been shown to inhibit cellular chemokine function by antagonistically binding chemokine receptors. To assess if RRV vMIP can inhibit rhMIP-1α function, we designed an inhibition assay using non-activated THP-1 cells, which are chemotactic to rhMIP-1α at an optimum concentration of 10^{-9} M, where as RRV vMIP requires vitamin D_3 activation to induce chemotaxis in THP-1 cells. *In vitro* chemotaxis assays were performed using increasing concentrations of RRV vMIP titrated against 10^{-9} M rhMIP-1α. However, at all concentrations tested, RRV vMIP was unable to inhibit rhMIP-1α-mediated migration (data not shown).

RRV vMIP induces chemotaxis of CD14$^+$ cells *in vivo*. To better understand the *in vivo* function of RRV vMIP and identify responding cell types, we designed an *in vivo* recruitment assay using chemokine-saturated Gelfoam® implants (see methods). Following surgical implantation, incubation, and subsequent removal of the chemokine-saturated implants, frozen sections of the Gelfoam® implants and surrounding tissue were analyzed by immunohistochemistry using antibodies directed against T and B cells (CD3 and CD20, respectively) as well as monocytes (CD14). In Figures 2.6a-d, the Gelfoam® implants can be differentiated from surrounding tissue by its intense H and E (dark blue/purple) staining pattern. Our data suggests that RRV vMIP induces recruitment of CD14$^+$ cells, as indicated by increased DAB positive (dark grey/brown) staining in and around the Gelfoam implant (Fig. 2.6a). The CD14$^+$ staining pattern for RRV vMIP is similar to that seen with the positive control, rhMIP-1α (Fig. 2.6b). CD3$^+$ or CD20$^+$ cells were not detected in the sections analyzed (data not shown). In an effort to quantify the levels of CD14$^+$ staining, the number of DAB positive pixels for each image was

normalized to the PBS control and graphed as a migration index. Figure 2.6e shows

significantly higher levels of CD14$^+$ staining are observed in implants containing rhMIP-

1α and RRV vMIP, as compared to PBS. Furthermore, the levels of CD14$^+$ infiltration

are similar between rhMIP-1α and RRV vMIP. Taken together, our *in vitro* and *in vivo*

data strongly suggest that RRV vMIP is a functional chemokine.

RRV preferentially infects CD14$^+$ cells during *in vitro* infection. Given that

recombinant RRV vMIP recruits CD14$^+$ cells *in vivo*, we sought to determine whether

CD14+ cells were susceptible to infection with RRV. To that end, PBMCs isolated from

RRV negative animals were either infected with a green fluorescent protein expressing

RRV (RRV$_{GFP}$) or left untreated for 72 hrs, stained using a panel of antibodies directed

against CD3, CD14, CD20, and HLA-DR and then analyzed by flow cytometry. A

representative example of the data is shown in figure 7. Infected cells were identified

based on GFP expression. We measured the prevalence of CD14$^+$DR$^+$ cells in infected

(GFP$^+$, Fig. 2.7e) and uninfected cells (GFP-, Fig. 2.7c). This data shows that a

significantly higher percentage of the GFP$^+$ cells are CD14$^+$ cells as compared to GFP- or

uninfected cells. Further analysis shows that the remainder of the GFP$^+$ cells are CD20$^+$ B

cells, CD3$^+$ T cells, and CD3- CD20- CD14- DR$^+$ cells which are mostly dendritic cells

(data not shown). Additionally, we characterized GFP expression within the CD3$^+$ (Fig.

2.7h), CD14$^+$ (Fig. 2.7g), and CD20$^+$ (Fig. 2.7i) subsets from the live gate (Fig. 2.7f),

which further demonstrates that a higher percentage of CD14$^+$ monocytes are GFP$^+$

compared to CD3$^+$ T cells and CD20$^+$ B cellls. These data further confirmed the

apparent preference of RRV to infect CD14+ cells. The combined data set for all five

animals is summarized in Table 2.2 where we calculated the ratio of CD14$^+$ cells within

infected versus uninfected cells. These data indicate that CD14$^+$ cells exhibit a higher

percentage of infectivity and might be preferentially infected by RRV during acute *in*

vitro infection.

DISCUSSION

In this report, we show that RRV, a simian homologue of KSHV, encodes a functional homologue to the chemokine MIP1α, RRV vMIP, which is expressed and secreted during the late stage of lytic infection. RRV vMIP mediates chemotaxis in both *in vitro* and *in vivo*. Furthermore, we have shown that RRV preferentially infects CD14+ cells during *in vitro* infection of rhesus PBMCs.

Like its KSHV counterparts, RRV vMIP possesses key β-chemokine features, namely the N-terminal C-C motif, as well as sequence similarities which correspond to conserved structural elements (e.g. N-loop, 30's loop, and 40's loop). At the amino acid level, full length RRV vMIP contains 40% similarity to rhMIP-1α and although this is considerably less than the homology of KSHV vMIPs to human MIP-1α, when the unique C-terminal tail of RRV vMIP is excluded from the homology calculation, the homology of RRV vMIP to rhMIP -1α goes up substantially to 38% identical and 64% similar (data not shown).

The strength of the RRV model is the ability to observe lytic gene expression without the use of inducing agents. We used an RRV vMIP antibody to monitor RRV vMIP protein expression and secretion during lytic RRV infection. Although our northern blot analysis classifies RRV vMIP as a late lytic gene, the presence of RRV vMIP in infected cells at 48 hours post infection clearly demonstrates that although RNA levels are below the limits of detect by northern blot, enough transcript is present for efficient protein expression. Additionally, our immunofluorescence assay shows a localization shift in RRV vMIP. In RRV vMIP+/gB- cells, RRV vMIP exhibits punctuate

127

staining consistent with localization within the trans-Golgi network. However, in RRV vMIP$^+$/gB$^+$ cells, RRV vMIP appears to stain in a more diffuse pattern. The cause for this is unknown, but gB expression correlates with increasing virus production, and the shift in RRV vMIP localization may be the result of increasing viral disruption of cellular compartments. Consistent with our immunofluorescence data, significant levels of RRV vMIP can be detected in infected supernatants at 48 hours post infection. Taken together, these data show for the first time vMIP expression and secretion during lytic infection.

Our results also show that recombinant RRV vMIP possesses chemotactic properties and has the ability to recruit cells *in vitro* and *in vivo*. Despite inducing migration, the observed activity of RRV vMIP during *in vitro* assays (optimum at 10^{-6}M) was two to three logs less than cellular MIP1α. Decreased biological activity is a common feature of viral homologs of cellular proteins encoded by RRV (e.g. vIL-6 and vCD200) (209, 243). Although rhMIP-1α activity was not affected, the decreased activity of RRV vMIP might be due to its poor interaction with human chemokine receptors expressed by THP-1 cells used in the chemotaxis assays. This possible explanation is supported by the observation that RRV vMIP induces migration of CD14$^+$ cells during *in vivo* recruitment assays to similar levels as the rhMIP-1α. Our findings represent the first time that a vMIP has been shown to directly mediate chemotaxis *in vivo*.

RRV, like KSHV and EBV, persists in B cells. Although the role that CD14$^+$ cells play during RRV pathogenesis has not been well characterized, many viruses, such as CMV, HIV, Dengue, HSV, and EBV, utilize CD14$^+$ cells to aid in dissemination throughout the infected host (293, 329, 370, 391, 432). Our observation that RRV

successfully infects CD14$^+$ *in vitro*, coupled with the knowledge that RRV vMIP

possesses chemotactic properties and can recruit CD14$^+$ cells, suggests RRV might

secrete vMIP during acute infection in order to actively recruit CD14$^+$ cells which are

highly permissive to RRV infection. Furthermore, our laboratory has shown that RRV

encodes a soluble CD200 homologue capable of reducing TNF-α production in

monocyte-derived macrophages, thereby reducing their anti-viral response (243). These

data are consistent with several recent studies showing that KSHV can infect monocytes

and macrophages *in vivo*, as well as monocyte-derived macrophages *in vitro* (48, 122,

342).

In this study, we characterized the function of RRV vMIP *in vivo* and *in vitro* and

have demonstrated for the first time that RRV vMIP is expressed and secreted by infected

cells. More importantly, our work has shown that secreted RRV vMIP is a functional

chemokine mediating recruitment of monocytic cells *in vitro*, as well as, *in vivo*.

Furthermore, we have showed for the first time that RRV preferentially infects CD14$^+$

cells during acute *in vitro* infection. By looking at other viruses and the role monocytes

play in viral dissemination, specifically Epstein-Barr virus [reviewed in (431), we

hypothesize a situation where RRV vMIP expression from RRV infected cells is

recruiting permissive CD14$^+$ cells for the purpose of trafficking progeny virus to other

areas of the infected host, perhaps increasing contacts with B cells thereby contributing to

the establishment of persistence.

ACKNOWLEDGEMENTS

Table 2.1 Homology of RRV vMIP to KSHV vMIPs and other rhesus β-chemokines

Chemokine	% Identical to RRV vMIP	% Similar to RRV vMIP
KSHV vMIP-I	22.2	37.6
KSHV vMIP-II	25.6	38.5
KSHV vMIP-III	18.5	34.7
rhMIP1α	19.7	37.6
rhMIP1β	21.4	36.8
rhRANTES	17.1	35.9

Table 2.2 Percentage of CD14+ cells infected with RRV$_{GFP}$

Animal	%GFP+	% CD14+ DR+ in GFP+	% CD14+ DR+ in GFP-	Ratio GFP+/GFP-
1	10	4.72	1.66	2.84
2	22.8	29.50	6.24	4.73
3	16.1	22.60	12.30	1.84
4	19.9	21.40	11.90	1.80
5	7.57	57.00	9.60	5.94
AVE	15.27	27.04	8.34	3.43
SEM	2.88	8.52	1.99	0.82
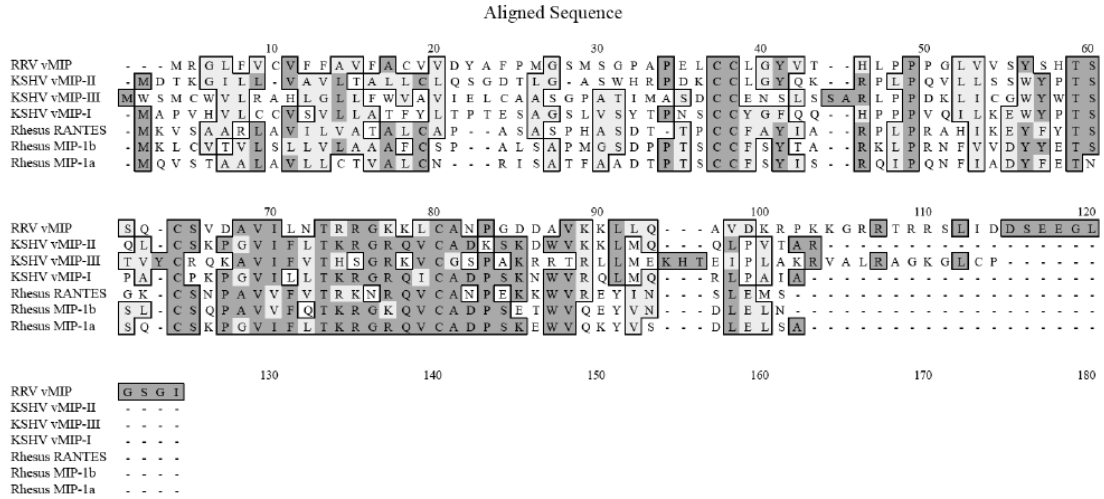
Figure 2.1 Amino acid comparison of RRV vMIP to KSHV vMIPs and other rhesus β-chemokines. Alignments were performed with ClustalW using Blosum scoring matrix. Dark shaded boxes indicate either: 1) identical residues, or 2) unique residues to RRV vMIP. Lightly shaded boxes represent similar residues to RRV vMIP.

Figure 2.2 Northern blot analysis of RRV ORF-R3 (RRV vMIP). A) Schematic

representation of RRV-R3 and surrounding open reading frames. B) RNA from RRV

infected rhesus fibroblasts treated with CHX or PAA or left untreated, was collected at

24, 48, and 72hrs, respectively. 10μg of total RNA was resolved on denaturing agarose

gels and transferred to nitrocellulose for hybridization to a radio-labeled double-stranded

DNA probe specific for R3. To insure equal loading, northern blots were stripped and re-

probed using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific probe.

A.

B.
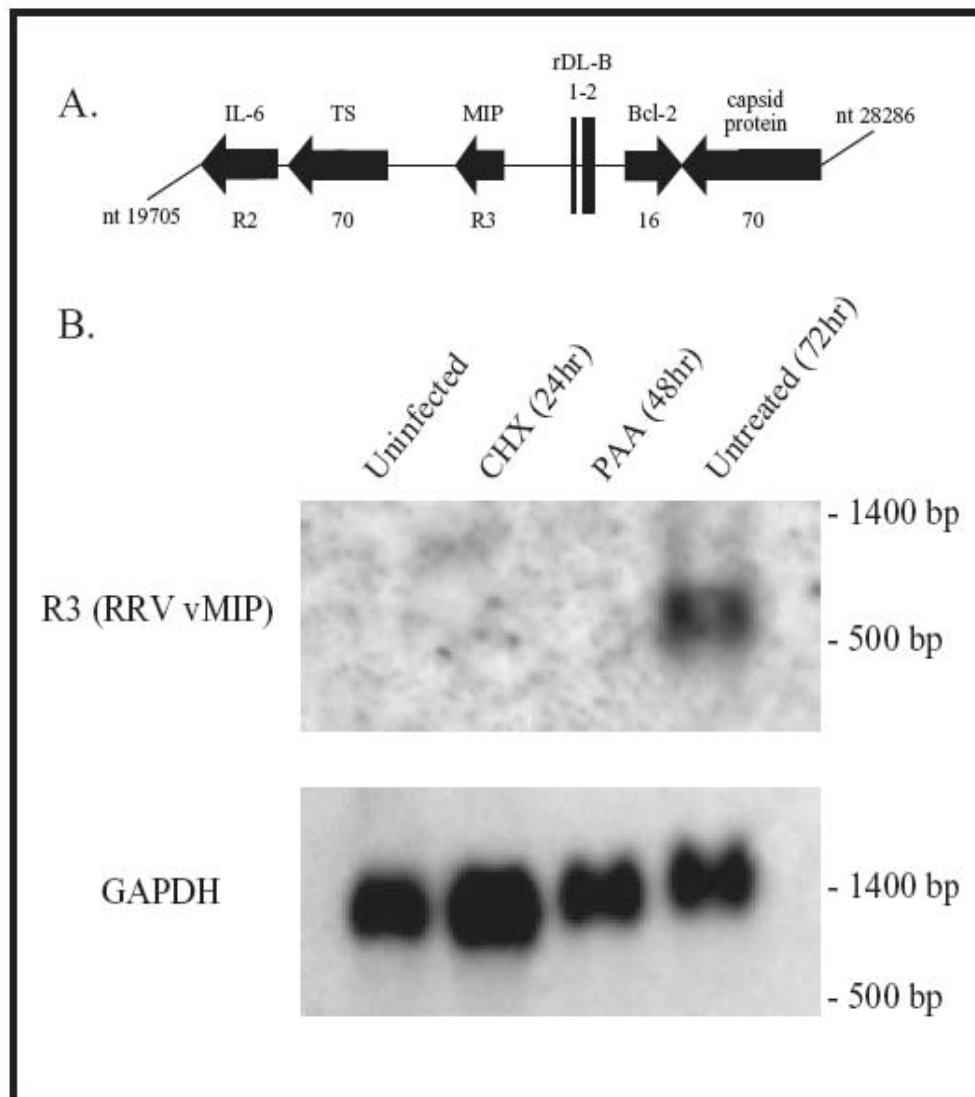
Figure 2.3 RRV vMIP is expressed and secreted from RRV infected cells. Primary rhesus fibroblast were infected with RRV$_{17577}$ at an M.O.I. = 1. 48 hours post-infection, cells were fixed, permeabilized, and stained for RRV vMIP (A), RRV gB (B), or a Hoechst nuclear stain (C). Figure 4D represents the merged image from all three channels.   Secretion of RRV vMIP from RRV infected cells was detected by immunoprecipitation from RRV infected supernatants at 24, 48, and 72 hours post infection. Western blot analysis was performed on the infected or mock samples and a clear band of the predicted size (11.2kDa) is visible in the 48 and 72 hours samples (E). Heavy (HC) and light (LC) chain from the mouse antibody use in the immunoprecipitation can also be seen.

A. RRV vMIP
B. RRV gB
C. DAPI
D. Merge

hours post infection

vMIP MW 24   48   72
      - + - + - + PAA
16
6

E.
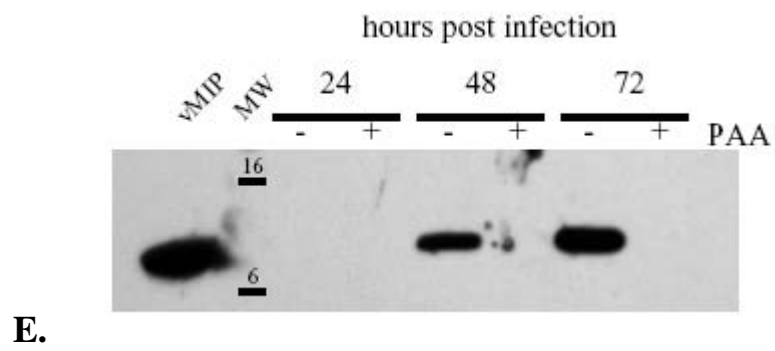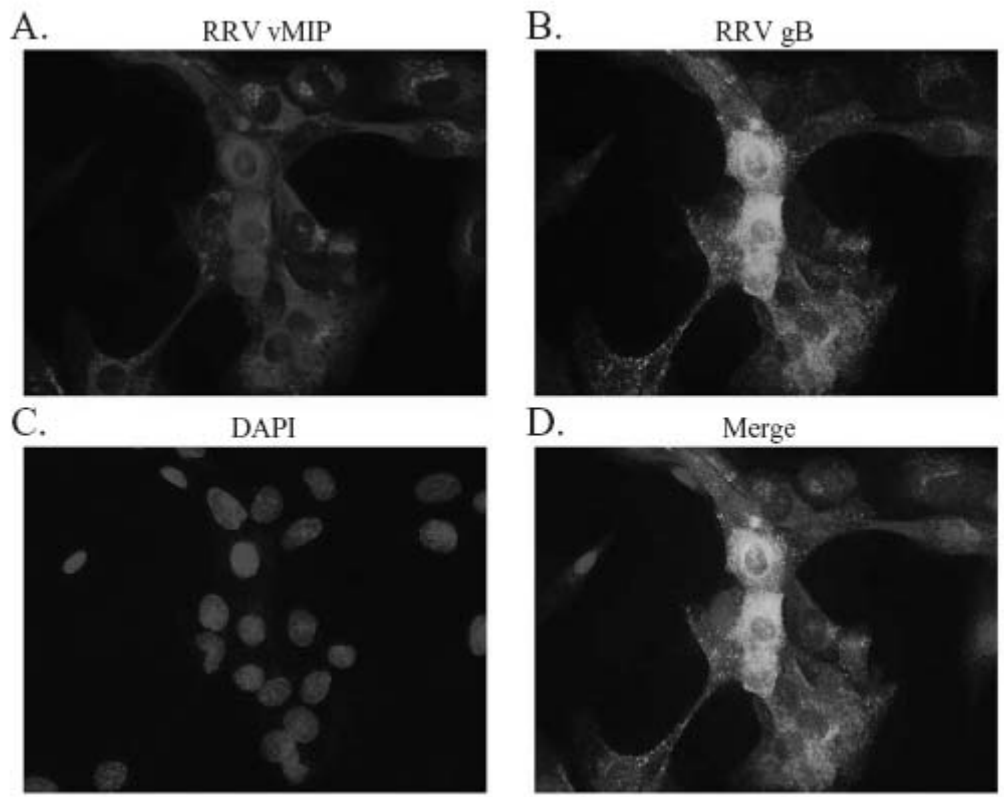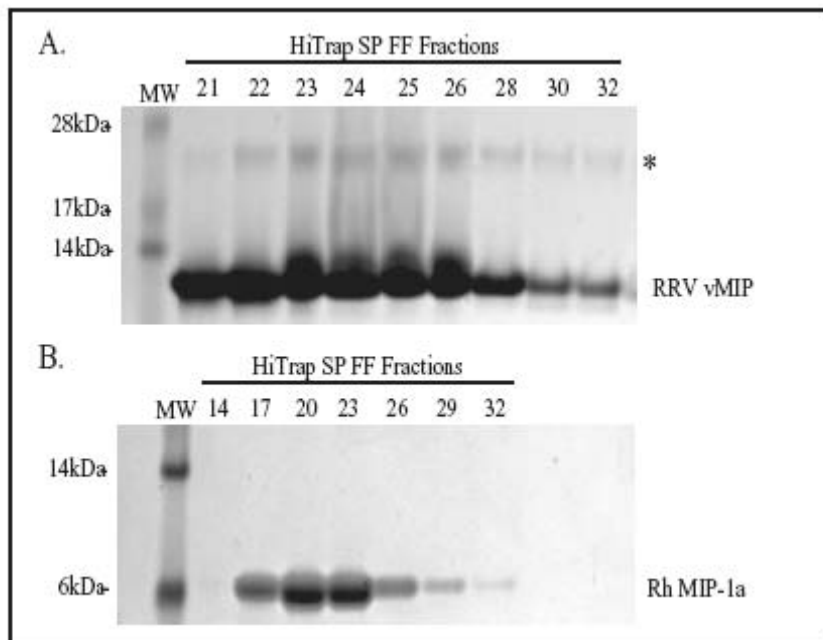
Figure 2.4 SPS-PAGE of expressed and purified chemokines. Purified rhMIP-1α (A) and RRV vMIP (B) were eluted from HiTrap SP columns and purity of fractions was assessed on 4-12% Bis-Tris NuPAGE® gels. The observed bands correspond to the predicted molecular weights of rhMIP-1α and RRV vMIP, 8.3 kDa and 11.2 kDa, respectively. At higher concentrations, the presence of RRV vMIP dimers (*) can is observed.
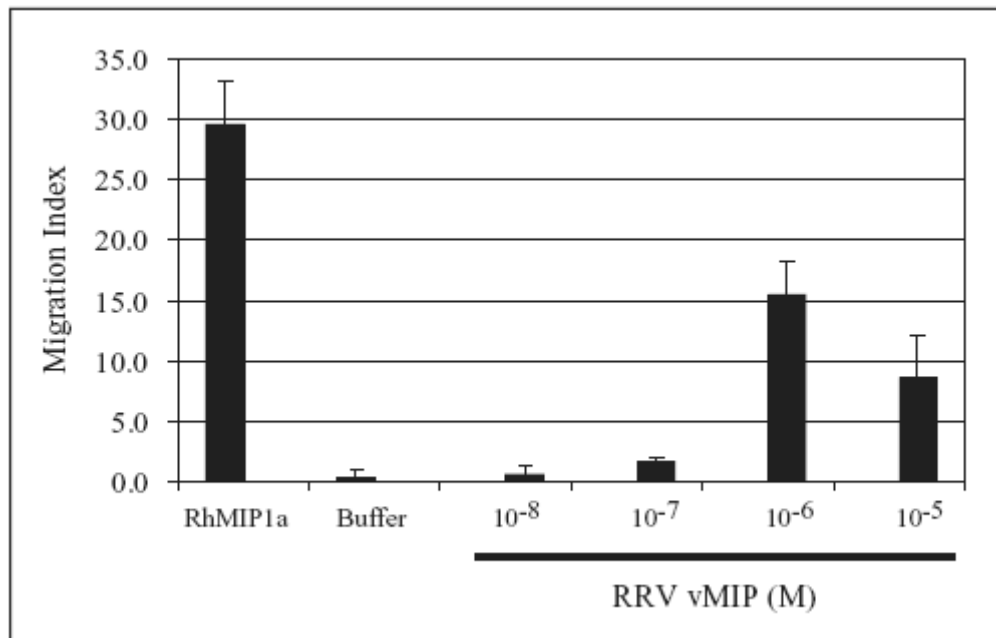
Figure 2.5 In vitro migration of vitamin D_3 activated Human THP-1 cells. 5×10^5 THP-1 cells suspended in 100μL of assay media (RPMI 1640 + 0.5% fetal bovine serum) were placed in 3μm pore size transwell inserts and placed in 24-well culture plates containing 600μL assay media with various concentrations of RRV vMIP, 10^{-9} M rhMIP1α, or a PBS control. Following a 4hr incubation at 37°C (5% CO_2), THP-1 cells migrating through the transwell were counted using a CyQuant[®] cell proliferation assay kit (Molecular Probes, Eugene, OR). Represented data are the average of 3 wells, normalized to the PBS control, and expressed as a migration index ± SEM.

Figure 2.6 In vivo migration assay. Gelfoam® sponges containing agarose-embedded (A) RRV vMIP or (B) rhMIP1α (500ng each) or (C) PBS were implanted s.c. in the back of a rhesus macaque (≥8 cm apart), where they remained for 7 days before being harvested, sectioned, and stained. CD14 staining shows more CD14$^+$ infiltrates (presence of dark grey/brown coloration) in the Gelfoam® sponges containing either RRV vMIP or rhMIP1α, as compared to PBS and (D) IgG Isotype controls, neither of which yielded significant CD14$^+$ staining (C and D respectively). Quantification of CD14$^+$ infiltrates (E) was performed by comparing the number of DAB$^+$ pixels in each image and normalizing to PBS. All images (A-D) were taken using a 20x objective and are the same size, 345000 pixels.

A. RRV vMIP
B. rhMIP-1α
C. PBS
D. Isotype Control
E.
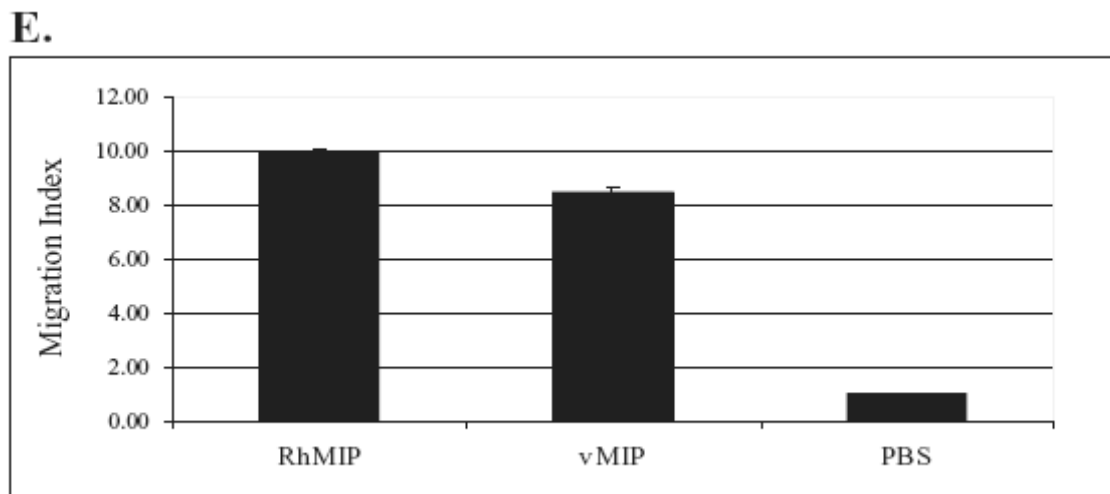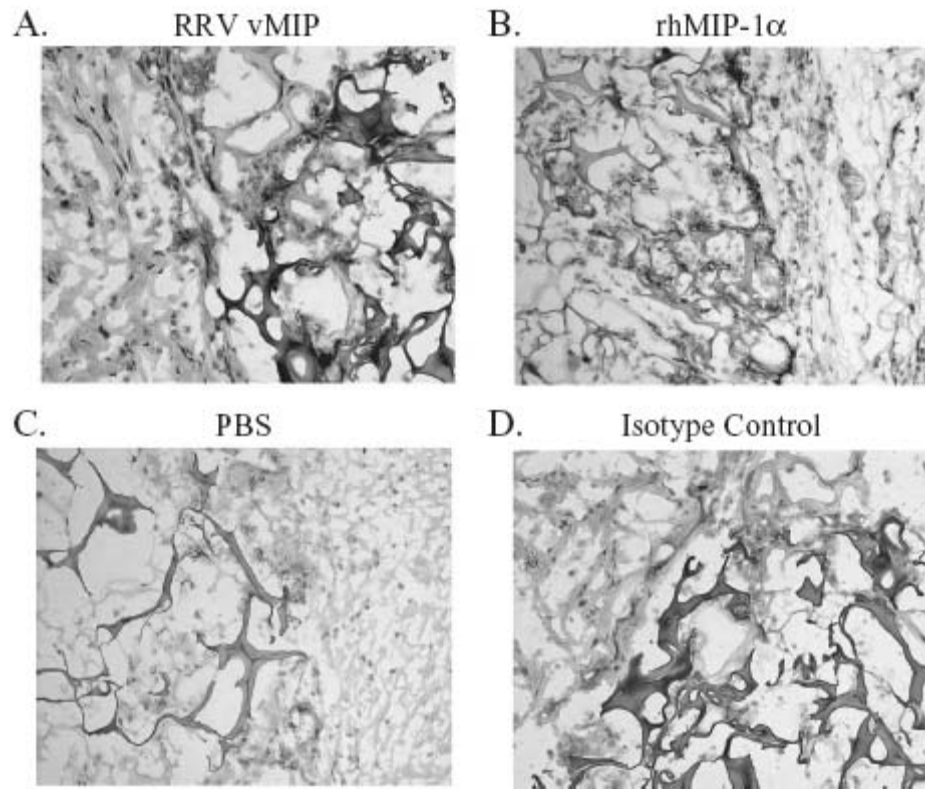
Figure 2.7 Monocytes are highly susceptible to RRV infection in vitro. Rhesus

peripheral blood mononuclear cells (PBMC) from five separate animals were either

infected with RRV_{GFP} at an M.O.I = 3.0 or left untreated for 72 hrs. The cells were then

washed with PBS and stained with surface antibodies specific for CD3, CD14, CD20, and

HLA-DR. Samples were run on an LSR-II flow cytometer (Beckton Dickinson, San

Jose, CA) and analyzed using FlowJo software (TreeStar, Ashland, OR). Panel A shows

non-infected PBMC side scatter versus GFP. Panel B displays the percentage of HLA-

DR+CD14+ (monocytes) CD14-DR+ (B cells and dendritic cells), and CD14-DR- (T,

NK cells) present in non-infected PBMC. In non-infected cells, monocytes are a minor

population of PBMC. Panels C-E are a representative example of immune cell subset

distribution within infected (GFP^+ - panel E) versus uninfected (GFP^- - panel C) PBMC,

with panel D showing the total infected population (GFP^+ and GFP-). In contrast to

uninfected cells, the infected cells show a clear enrichment for CD14+DR+ cells (9.6%

versus 59.3%). Panel F shows our live gate on RRVgfp infected cells, with panels G-I

show the percentages of RRVgfp infected cells witin the $CD14^+$, $CD3^+$, and $CD20^+$

populations.

A. Day 3 non-infected

B. Day 3 non-infected
9.6%

C. GFP-negative
6.7%

D. Day 3 infected

E. GFP-positive
57.1%

F. Live Gate
59.3

G. CD14 vs. GFP
3.9%

H. CD3 vs. GFP
0.56%

I. CD20 vs. GFP
0.38%
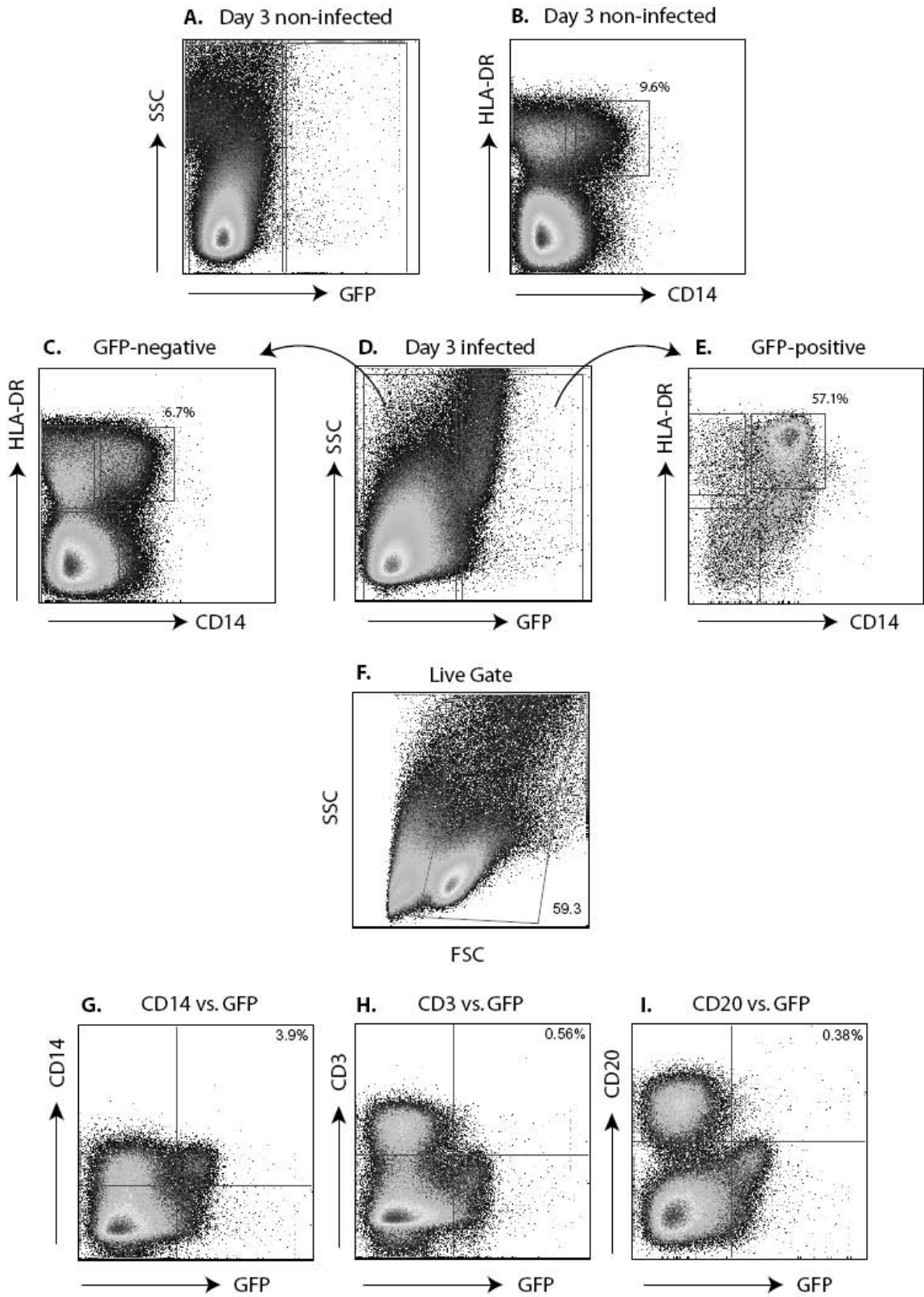
Chapter 3

Monkeypox Viral Chemokine Inhibitor (MPV vCCI), a Potent Inhibitor of Rhesus Macrophage Inflammatory Protein – 1

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ABSTRACT

Monkeypox virus (MPV) is an orthopoxvirus with considerable homology to variola major, the etiologic agent of smallpox. Although smallpox was eradicated in 1976, the outbreak of MPV in the U.S. highlights the health hazards associated with zoonotic infections. Like other orthopoxviruses, MPV encodes a secreted chemokine binding protein, MPV vCCI that is abundantly expressed and secreted from MPV infected cells. EMSA data shows MPV vCCI efficiently binds rhesus MIP-1α (rhMIP-1α) at near one-to-one stoichiometry. *In vitro* chemotaxis experiments demonstrate that MPV vCCI completely inhibits rhMIP-1α mediated chemotaxis, while *in vivo* recruitment assays in rhesus macaques using chemokine-saturated implants show a decrease in the number of $CD14^+$ cells responding to rhMIP-1α when MPV vCCI is present, suggesting MPV vCCI is effectively inhibiting chemokine function both *in vitro* and *in vivo*. More importantly, we demonstrate that MPV vCCI can diminish the severity of the acute phase and completely inhibit the relapsing phase of experimental allergic encephalomyelitis (EAE) disease. These data represent the first *in vitro* and *in vivo* characterization of MPV vCCI emphasizing its function as a potent inhibitor of rhMIP-1α. Furthermore, the ability of MPV vCCI to inhibit relapsing EAE disease represents a novel therapeutic approach for treating chemokine-mediated diseases.

INTRODUCTION

Monkeypox virus (MPV) is a member of the genus Orthopoxvirus, which includes

variola major, the etiologic agent of smallpox (392-394). Monkeypox virus and variola

major share considerable homology, approximately 85% at the genomic level, and cause

similar disease manifestations in infected humans. Although variola major is no longer a

worldwide threat, MPV is a virus that naturally infects rodents and primates in sub-

Saharan Africa, and since its discovery, thousands of cases of human MPV infection have

been reported. The disease is primarily transmitted from animals to humans, either

through animal bites or through direct contact with animal body fluids. Person-to-person

transmission is rare, and is acquired through close contact and exposure to aerosol

droplets or contaminated body fluids (97, 392). More importantly, MPV infection of

humans is clinically indistinguishable from smallpox, sharing similar pathology and

disease progression, and without proper medical attention, a 1-10% mortality rate (335).

Further complicating diagnosis, the early stages of human MPV infection are often

misdiagnosed as chickenpox, caused by varicella-zoster virus. Although smallpox was

officially eradicated in 1976 by world-wide vaccination, recent cases of MPV in the

United States indicates that MPV should be considered as an emerging zoonotic infection

that poses a threat to the millions of non-vaccinated individuals.

The *poxviridae* family is characterized as large, DNA viruses that cause disease in

a wide variety of organisms. Many poxviruses encode proteins that inhibit normal

chemokine function, collectively, these proteins are referred to as viral chemokine

binding proteins (vCBPs) (17, 49, 188, 224). Members of the *orthopoxvirus* and

leporipoxvirus genera express a secreted, 35 kDa protein, commonly referred to as vCCI, vCBP-I, or 35 kDa, that binds to human and rodent CC and CXC chemokines with high affinity, competitively inhibiting their normal interaction with cellular chemokine receptors (400). Members of the myxomavirus genus also encode a secreted CC chemokine inhibitor (referred to as T7 or vCBP-II), additionally, these proteins have also been shown to effectively scavenge γ-IFN (280). As a result of their inhibitory nature, all of these secreted proteins function as anti-inflammatory proteins during viral infection. All vCBPs represent a structurally unique family that does not share homology to any known cellular chemokine receptors, or any other mammalian or eukaryotic proteins (16, 68, 161, 383). To date, two animal models have been used to investigate the effect vCCI has on poxvirus pathogenesis. Expression of vCCI during experimental vaccinia infection in mice has shown to greatly reduce the number of infiltrating cells in the lungs of vaccinia infected mice (344). Additionally, skin lesions from rabbits infected with rabbitpox show reduced infiltrates, compared to a vCCI knockout virus (161).

Chemokines belong to a superfamily of small (8 – 14 kDa) proteins that possess similar structural and functional properties (297). The chemokine family is further divided into the following subtypes: C, CC, CXC, and CX_3C, based on the position of conserved cysteines located in the N-terminus of the protein. Most of the known chemokines (~94%) belong to the CXC or CC subtypes. Chemokines impose function by binding to seven transmembrane G-protein-coupled receptors (GPCRs) and glycosaminoglycans (GAGs), initiating downstream signaling events leading to adhesion, contraction, and actin polymerization (297, 446). Although primarily known for their ability to mediate recruitment of effector leukocytes and lymphocytes during injury or

146

pathogenic insult, chemokines are also critically involved in a variety of cellular processes, such as the development of secondary lymphoid tissue, organogenesis, angiogenesis, and hematopoiesis (297, 358). As a fundamental component of both the innate and adaptive immune responses, chemokines have been targeted by many viruses who have obtained the ability to modulate and mimic chemokine function.

Along with their role in mediating inflammation due to injury or pathogens, some chemokines can play key roles in the progression of many auto-immune and neurodegenerative diseases, such as rheumatoid arthritis, Grave's disease, multiple sclerosis, Alzheimer's disease, human immunodeficiency virus-associated dementia, Type 1 diabetes, and Parkinson's disease (152). Most auto-immune diseases involve autoreactive lymphocytes that can express chemokines, such as IL-8, MCP-1, MIP-1α, MIP-1β, and RANTES, which promote the recruitment of inflammatory cells. It is this influx of inflammatory cells and their secreted products which mediate the auto-immune destruction of host cells and tissue, thus promoting disease. Current therapies for treating chemokine-mediated diseases generally involve suppression of the host immune system, but as with any immunosuppressive regime, there is substantial risk for secondary infection. Initially developed as possible blocking agents for HIV infection, small molecule antagonists for chemokine receptors are currently being evaluated in both animal models and clinical trials for effectiveness in treating chemokine-mediated diseases, but to date, no therapies exist that specifically target the chemokine protein itself (313).

Experimental autoimmune encephalomyelitis (EAE) is a murine disease intiated by the introduction of myelin-derived peptides (262, 310). Following peptide injection,

mice develop a scoreable, tail-to-head paralysis, which is reminiscent of multiple sclerosis in humans. The cellular mechanisms behind this paralysis have been well characterized, and largely involve autoreactive $CD4^+$ T_H1 and T_H17 T cells (101, 244, 381). The introduction of myelin-derived peptides emulsified in Freund's complete adjuvent generates these activated, auto-reactive, myelin-specific $CD4^+$ T cells that have broken immune tolerance. These activated T cells can cross the blood-brain barrier and gain access to neuronal tissues, where they are re-activated by antigen-presenting cells expressing MHC class II loaded with myelin peptide (56, 184, 281, 427). The ensuing inflammatory response involves the expression of pro-inflammatory cytokines (e.g T_H1 - TNF-α and IL-1, and T_H17 - IL-1, IL-18, and IL-17), as well as inflammatory chemokines, that primarily recruit macrophages and neutrophils to the site of inflammation (101, 234, 445). Furthermore, evidence suggests that inflammatory chemokines are important for the initiation and maintainence of EAE, since neutralizing antibodies to MIP-1α and MCP-1 reduce the effects of EAE and CCR2 -/- mice are resistant the EAE induction (198, 213). The primary histopathology associated with EAE is the presence of demylinated plaques within the nervous tissues. These plaques are the result of the inflammatory response generated by the auto-reactive $CD4^+$ T cells and activated macrophages (404).

Infection of rhesus macaques with MPV represents an excellent non-human primate model for variola and the goal of this study was to biologically characterize MPV encoded vCCI and how it may contribute to MPV pathogenesis. As such, these data represent a foundation for further studies into the biological significance of MPV vCCI in a non-human primate model. Here we provide the first evidence that MPV vCCI is

expressed and secreted during MPV infection and that MPV vCCI interacts with rhesus MIP-1α (rhMIP-1α) in vitro and in vivo inhibiting normal chemokine function. Additionally, we tested the utility of MPV vCCI in treating chemokine-mediated disease and show that MPV vCCI can inhibit relapsing EAE in mice, which represents a novel therapeutic approach for treating disease mediated by chemokine function.

MATERIALS AND METHODS

Protein alignments. Protein alignments were performed using ClustalW from MacVector version 9.0 software (Accelrys, Inc., Madison, WI). A Blosum scoring matrix was used in pairwise alignment of each sequence, with a gap introduction penalty of 10 and a gap extension of 0.1.

Virus, cell culture, and MPV vCCI specific antibodies. Human monkeypox virus (MPX V79-I-005, herein referred to MPV) was provided by Dr. Inger Damon (Center for Disease Control and Prevention, Atlanta, GA) and propagated in BSC_{40} cells (African green monkey kidney cells – American Type Culture Collection (ATCC), Manassas, VA) cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 1% penicillin, streptomycin, and L-glutamine (Invitogen, Carlsbad, CA). Viral titers were determined by plaque assay. HeLa cells and primary rhesus fibroblasts were maintained in DMEM and human THP-1 cells were maintained in RPMI 1640 (Mediatech, Herndon, VA), both supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, streptomycin, L-glutamine. RPMI 1640 was further supplemented with HEPES, and sodium pyruvate, 2% sodium bicarbonate (Invitogen, Carlsbad, CA). MPV vCCI specific monoclonal antibodies were made on-site in the monoclonal antibody core at the Vaccine and Gene Therapy Institute (Beaverton, OR) using purified recombinant MPV vCCI (see below) as antigen.

Immunofluorescence analysis. Approximately 0.8×10^5 BSC$_{40}$ cells were seeded onto 12mm glass cover slips (Fisher Scientific, Pittsburg, PA). The following day, cells were either infected with MPV at a multiplicity of infection (MOI) = 1 or mock and at 24 hours post-infection, cells were fixed with 4% paraformaldehyde in PBS at 25°C for 20 minutes. Fixed cells were then permeabilized with 0.2% triton-x 100 in PBS. Staining for MPV vCCI was performed using mouse monoclonal antibodies (Clone #11A3.4.2), followed by a biotinylated horse anti-mouse secondary antibody (Dako, Cuppertino, CA). The 11A3.4.2 clone was used specifically for immunofluorescence because of its low background in this application. Visualization was performed using streptavidin conjugated to Alexa-488 (Invitrogen, Carlsbad, CA) followed by a nuclear counterstain with a Hoechst dye (Sigma, St. Louis, MO).

Immunoprecipitation and Western blot analysis. Approximately 2.5×10^6 BSC$_{40}$ cells were infected with MPV at MOI = 10. Following 24 hours of incubation, supernatants were clarified and concentrated 10-fold via 5 000 MWCO Amicon® Ultra centrifugal filtration device (Millipore, Bedford, MA), while infected cells were washed with PBS and lysed in ice cold RIPA buffer (PBS, 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate). An MPV vCCI-specific mouse monoclonal antibody (clone #3D1) was added to the concentrated supernatants at 12.5 µg/mL and incubated for 1 hour at 4°C with agitation. 100 µL Protein A/G-plus agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was added to the mixture and allowed to incubate for 1 hour at 4°C with agitation. Protein bound agarose was pelleted and washed twice with cold PBS. Bound proteins were denatured by adding 2x NuPAGE® LDS sample buffer (Invitrogen,

Carlsbad, CA) and heating to 70°C for 10 minutes.   Proteins (15 µL load) were resolved

on 4-12% NuPAGE® Bis-Tris polyacrylamide gels and wet transferred to PVDF

membranes at 30 V for 1 hour. Protein blots were probed using our anti-MPV vCCI

mouse monoclonal antibody (clone #3D1) followed by a horseradish peroxidase-

conjugated goat anti-mouse secondary antibody (1:2 000) (Santa Cruz Biotechnology

Inc., Santa Cruz, CA). Bands were visualized using chemiluminescence. For the co-

immunoprecipitation of rhMIP-1α with MPV vCCI, 6 ug of recombinant MPV vCCI was

mixed with increasing amounts of recombinant rhMIP-1α (from 0.1 ug to 2 ug).

Following 10 minute room temperature incubation, 10ug of our anti-MPV vCCI mouse

monoclonal antibody (clone #3D1) was added to the reaction and immunoprecipitation

was carried out as described above. Western blot analysis for rhMIP-1α was conducted

in a similar fashion as described for MPV vCCI using a cross-reactive human MIP-1α

polyclonal antibody (# BAF270 – R & D Systems, Minneapolis, MN)

Cloning and expression of recombinant MPV vCCI. The coding sequence for MPV-

J1L was isolated from MPV genomic DNA via PCR using primers specific for MPV-J1L

which also contained a 6x-histidine tag (underlined region) and restriction sites for NdeI

(5'-CATATGATCCCTACCAGTCTTCAGCA-3') and XhoI (5'-

CTCGAGTCATCA<u>GTGGTGGTGGTGGTGGTG</u>GACACATGCTTTGAGTTTTGT-

3'). A non-sense mutation (in quotation marks) was introduced into an internal NdeI site

via site directed mutagenesis using the following primers: 5'-

AACAAACATCA"C"ATGGGAATCG - 3' and 5'-

CGATTCCCAT"G"TGATGTTTGTT - 3'. A 6x-histidine tagged rhMIP1α was isolated

in a similar manner from another expression plasmid (graciously provided by Dr. Todd

Reinhart, Univ. of Pittsburg) also using NdeI (5'-CATATGGCTGACACCCCGACCTC-

3') and XhoI (5'-

CTCGAGTCATCA<u>GTGGTGGTGGTGGTGGTG</u>CACGGCACTCAGCTCTAGGTC-

3'). The resulting products were cloned into pRSETb (invitrogen, Carlsbad, CA) for

expression. Rosetta 2® DE3 cells (Novagen, Madison, WI) were transformed with the

pRSETb expression plasmids. Expression cultures were set up by diluting overnight

cultures 1:20 into 1 L of LB media without antibiotic and incubated for 3 hours at 37°C

with agitation. At 3 hours, the temperature of the cultures was reduced to 25°C and

protein expression was induced with 0.5 μM isopropyl-β-D-thiogalactoside (IPTG -

Fisher, Fair Lawn, NJ) with continued agitation for 6 hours. Cells pellets were harvested

by centrifugation (5,000xg for 12 minutes) and stored at -80°C until use.

Purification of recombinant MPV vCCI. Induced cell pellets were resuspended in

lysis buffer (300 mM NaCl, 50 mM NaPO4, 20 mM Tris-HCl, 0.1 mM PMSF, 3 mM

βME, pH 8.0) and lysed by 2 freeze/thaw cycles, incubation with 1 mg/ml lysozyme, 5

μg/ml DNAase, and 5 μg/ml RNAase for 30 minutes on ice, and then sonicated 30

seconds (3x). Lysates were separated into soluble and insoluble fractions by

centrifugation at 20,000xg for 60 minutes at 4°C. Proteins were purified via immobilized

metal affinity chromatography (IMAC) by applying the soluble fraction to pre-

equilibrated BD Talon® metal affinity resin (Clontech Laboratories Inc, Mountain View,

CA) (1 ml resin per 2 L culture), where it was incubated on a rotator at RT for 1 hour.

Protein-bound resin was pelleted and washed (2x) with 20 ml wash buffer (300 mM

NaCl, 50 mM NaPO4, 20 mM Tris-HCl, 10% glycerol, 3 mM βME, pH 7.5). Protein

was eluted from the resin by adding 3 ml elution buffer (300 mM NaCl, 50 mM NaPO4,

20 mM Tris-HCl, 250 mM imidazole, 3 mM βME, pH 7.0) and incubated on a rotator at

room temperature for 5 minutes (3x). Eluted protein was 0.22 μm filtered and run over a

HiPrep 16/60 Sephacryl S-100 HR column (GE Healthcare, Piscataway, NJ) pre-

equilibrated in running buffer (20 mM NaPO4, 150 mM NaCl, 3 mM βME, pH 7.0).

Pooled fractions were further purified and concentrated by binding to a HiTrap Q FF

column and eluted with a 0-1 M NaCl gradient over 20 ml. Protein purity and size were

determined on 4-12% Bis-Tris NuPAGE® gels and the purest fractions were pooled

together. Endotoxin levels were assessed using a limulus amebocyte lysate (LAL) assay

(Cambrex, Walkersville, MD), followed by endotoxin removal using AffintyPak™

Detoxi-Gel™ endotoxin removal gel (Pierce, Rockford, IL). Protein concentration was

determined by absorbance spectroscopy. Purified proteins were lyophilized and stored at

-80°C, while reconstituted protein was kept at -20°C.

Electrophoretic mobility shift assays. Purified recombinant MPV vCCI was incubated

with rhMIP-1α, murine MIP-1α, murine MCP-1, or murine RANTES at room

temperature for 10 minutes. Samples were resolved on a non-denaturing, non-reducing

12% polyacrylamide gel at 30 mA. Bands were visualized using SimplyBlue® SafeStain

(Invitrogen, Carlsbad, CA).

In vitro chemotaxis inhibition assay. Inhibition of THP-1 cell migration was carried

out using Transwell® plates (6.5 mm x 3.0 μm pore, Corning, New York, NY)

equilibrated in assay media (RPMI 1640 supplemented with 0.5% heat-inactivated fetal

bovine serum) for 1 hour prior to assay. Ten minutes prior to beginning the assay, 10^{-9} M

rhMIP-1α was mixed with increasing amounts of MPV vCCI and incubated at 25°C.

The protein mixture was then added to 600 μL of assay media in the lower chamber.

Approximately $5x10^5$ THP-1 cells suspended in 100 μl were added to the upper chamber

of the transwell and incubated for 4 hours at 37°C with 5% CO_2. Migrated cells were

counted using CyQuant cell proliferation assay kit (Invitrogen, Carlsbad, CA).

In vivo chemotaxis assay. In vivo recruitment assay was adapted from a previously

published angiogenesis assay (128). Gelfoam® plugs (Pharmacia & Upjohn Company,

Kalamazoo, MI) were cut 5 mm^3 and rehydrated overnight in PBS at 4°C. On the day of

implantation, plugs were briefly dried between two pieces of filter paper and soaked with

a) 500 ng of rhMIP-1α , b) 500 ng of rhMIP-1α plus 1.5 ug MPV vCCI (1:1 molar ratio),

or c) PBS mixed with 0.4% agarose warmed to 42°C. The soaked implants were stored

at 4°C until implantation. For implantation, rhesus macaques are anesthetized with

ketamine (15-20 mg/kg i.m.), placed in oblique ventral recumbency, and the hair clipped

from the mid-scapular region to the shoulder. Skin prep was performed in routine

fashion with betadine scrub and solution, followed by placement of a medium fenestrated

drape. A 5-10 mm skin incision was made in the lateral mid-scapular region, the skin is

undermined with a Kelly forceps for a distance of approximately 2-3 cm from the

incision, and the Gelfoam® implants were inserted in the undermined space. The skin

was then closed with several simple interrupted sutures. Spacing between implants was

maximized to avoid potential functional overlap. The implants remained in the animal

for 7 days, at which time, the Gelfoam® plugs and surrounding tissue were excised and cryopreserved in tissue freezing media (Triangle Biomedical Sciences, Durham, NC) and stored at -80°C for later sectioning. All aspects of the experimental implantation studies were performed according to institutional guidelines for animal care and use at the OHSU, West Campus.

Immunohistochemistry. Sections of the cryopreserved samples 10 µm thick were cut and mounted onto Superfrost®/Plus slides (Fisher Scientific, Pittsburg, PA) at RT overnight. Slides were fixed with ice cold acetone for 10 minutes and then washed three times with tris-buffered saline (pH 7.4) + 0.1% tween-20 (TBST) to remove freezing media. Slides were blocked with PBS + 1% BSA and 10% donkey serum at RT for 1 hour, followed by PBS + 0.3% H_2O_2. A CD14-specific mouse monoclonal primary antibody (Clone# M5E2 – BD Pharmingen, San Diego, CA) diluted in PBS + 1% BSA was incubated on the sections overnight at RT. Following TBST washes, sections were incubated with horse anti-mouse secondary antibody conjugated to horse radish peroxidase for 1 hour at RT. CD14 specific staining was visualized using a DAB substrate kit (Dako, Cuppertino, CA) and counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, CA).

Experimental allergic encephalomyelitis (EAE) model. Our EAE model follows the published protocol of Stromnes and Goverman (418) and was performed according to institutional guidelines for animal care and use at the OHSU, West Campus. Briefly, on day zero, 8 week old, female SJL/J mice (Jackson Labs, Bar Harbor, MA) were injected

subcutaneously (s.c.) with 200 ug of myelin proteolipid peptide residues 139-151

(PLP$_{139-151}$); Peptides Intl., Louisville, KY) emulsified in complete Freud's adjuvant

(Sigma, St. Louis, MO), and 100 ng of pertussis toxin (List Biological Laboratories, Inc.,

Campbell, CA) was given intraperitoneally (i.p.), these mice serve as positive controls.

Each mouse in the experimental group received an additional 25 ug of MPV vCCI i.p..

Mice receiving 25 ug MPV vCCI alone or buffer alone serve as negative controls. On

day 3, an additional boost of 100 ng of pertussis and 25 ug of MPV vCCI were given to

the appropriate groups. Mice were monitored daily and disease was scored using the

following scale: 0 – Normal, 0.5 – Partially limp tail, 1.0 – Paralyzed tail, 2.0 – Hind

limb paresis, 2.5 – One hind limb paralyzed, 3.0 – both hind limbs paralyzed, 3.5 – Hind

limbs paralyzed; fore limbs weak, 4.0 – Fore limbs paralyzed, 5.0 – Moribund.

Additional care was given to mice exhibiting disease, such as, soaked chow and the

administration of s.c. fluids to mice exhibiting a 25% reduction in weight.

RESULTS

Protein alignment of various poxvirus vCCI sequences. The predicted product of the MPV ORF-J1L is a 27.6kDa protein, MPV vCCI. The amino acid sequence of MPV vCCI was aligned with other vCCI sequences encoded by variola virus (VARV), cowpox virus (CPV), rabbitpox virus (RPV), and vaccinia stain Copenhagen (VV COP) to determine the level of amino acid sequence homology. Figure 1 shows the protein alignments for all five proteins and confirms conserved homology between them. On average, MPV vCCI shares approximately 85.8% similarity and 82.5% identity with the other chemokine inhibitors (Table 1). Although highly homologous, there is one area of divergence from amino acid 72 to 94, where the vCCIs of MPV and CPV differ from the other viral vCCIs. Based on previously published studies, these similarities suggest that MPV vCCI likely functions in a similar manner as the vCCIs of CPV and VV, and will inhibit CC chemokine function in an infected host.

Expression and purification of recombinant MPV vCCI. The DNA sequence encoding MPV vCCI was amplified by PCR and a 6x-histidine tag was placed in frame at the C-terminus for purification purposes. After a multi-step purification protocol, SDS-PAGE on fractions from anion exchange chromatography shows purified recombinant MPV vCCI. Despite having a predicted molecular weight of 27.6 kDa, MPV vCCI migrates roughly 5-6kDa higher on SDS-PAGE, which is consistent with other vCCI species, like VARV, CPV, and VV COP, and is more than likely the result of charged residues in the primary sequence (data not shown).

MPV vCCI is expressed and secreted during MPV infection. To determine if MPV vCCI protein is expressed during MPV infection, an immunofluorescence assay was performed on MPV infected BSC_{40} cells using an MPV vCCI specific mouse monoclonal antibody (11A3.4.2). As shown in Fig 2a, MPV infected cells begin to stain positive for MPV vCCI, as early as 24 hours post infection. Positive cells show an intense cytoplasmic staining as compared to mock infected cells.

Next, to determine if MPV vCCI is secreted from MPV-infected cells, western blot analysis was performed on clarified/concentrated supernatants and cellular lysates from BSC40 cells infected with MPV for 24 hours. Western blot analysis shows the presence of a MPV vCCI specific band at, or near the apparent molecular weight of ~35kDa in supernatant from infected samples, but not in supernatants from mock samples (Fig. 2b). Recombinant MPV vCCI was loaded as a positive control. Taken together, these data clearly demonstrate MPV vCCI is expressed and secreted during MPV infection, either via active transport or during cell lysis.

MPV vCCI interacts with various β-chemokines. To assess the ability of MPV vCCI to bind various β-chemokines, we utilized a modified electrophoretic mobility shift assays (EMSA) to visualize differences in MPV vCCI mobility with and without rhMIP-1α present (Fig. 3a). Because of its small size and amino acid content, rhMIP-1α does not stain at the concentrations used (lanes 2 and 5). Therefore, if MPV vCCI is forming a complex with rhMIP-1α, we should see an increase in the apparent molecular weight (MW_{app}) of MPV vCCI. Compared to free MPV vCCI (lane 4), MPV vCCI runs at a higher MW_{app} when incubated with rhMIP-1α (lane 3). Moreover, to address MPV vCCI

aggregation as a possible explanation for the shift in molecular weight, twice the amount

MPV vCCI was loaded (lane 1), and although some "smearing" is observed, the higher

molecular weight band is not observed. To confirm the presence of both MPV vCCI and

rhMIP-1α, the shifted band (lane 3) was excised and in-gel trypsin digest was performed,

followed by mass spectrophotometry. Following analysis of unique peptide hits, the

presence of two species, MPV vCCI and rhMIP-1α, was confirmed (data not shown).

To further demonstrate the formation of the MPV vCCI: rhMIP-1α complex, we

set up a titration assay where increasing amounts of MPV vCCI were incubated against a

fixed amount of rhMIP-1α. Figure 3b shows that with limiting amounts of MPV vCCI,

the only species present is the higher MW_{app} species (lanes 1 and 2). As MPV vCCI

begins to be in excess, the presence of the free MPV vCCI begins to be seen (lanes 4 and

5). As seen in Figure 3a, 2x MPV vCCI was loaded to verify that aggregation was not

the reason for the shifted band (lane 8).

In order to confer specificity, a co-immunoprecipitation assay was performed on a

mixture MPV vCCI and rhMIP-1α using an anti-MPV vCCI monoclonal. As shown in

figure 3c, as increasing amounts of rhMIP-1α were added to the incubation mixture, more

rhMIP-1α is co-immunoprecipitated with MPV vCCI (lanes 4-8). This effect is

dependent on MPV vCCI, as rhMIP-1α alone does not immunoprecipitate with the MPV

vCCI antibody (lane 3). Taken together, these data show that MPV vCCI binds and

forms a complex with rhMIP-1α.

Studies on CPV vCCI have demonstrated an ability to bind murine MIP-1α and

MCP-1 (62). To verify future MPV vCCI studies within a murine model, we performed

shift assays using several murine β-chemokines. Figure 3d shows the ability of MPV

vCCI to bind murine MIP-1α and MCP-1, as indicated by the shift in vCCI MW_{app}. Interestingly, MPV vCCI was also able to weakly interact with mRANTES, as indicated by a partial shift in vCCI MW_{app}. This is in contrast to CPV vCCI, which was able to bind mRANTES.

MPV vCCI inhibits rhesus MIP-1α mediated chemotaxis of THP-1 cells. In order to assess the inhibitory properties of MPV vCCI, we first utilized an in vitro transwell assay using human THP-1 cells, a premonocytic cell line. We used THP-1 cells for their consistency, as opposed to isolating cells from different rhesus macaques and dealing with animal to animal variability. Furthermore, we have previously determined that THP-1 cells are responsive to rhMIP-1α with maximum chemotaxis occurring at 10^{-9} M (data not shown). Figure 4 shows that with increasing concentrations of MPV vCCI, rhMIP-1α mediated chemotaxis is reduced to levels similar to PBS controls. The use of heat inactivated MPV vCCI restores rhMIP-1α mediated migration confirming that the observed effect is mediated by MPV vCCI. These findings clearly show that MPV vCCI is binding to rhMIP-1α and effectively inhibiting chemotaxis.

MPV vCCI inhibits rhMIP-1α mediated chemotaxis of CD14^{+} cells during in vivo recruitment assays. To better understand the in vivo function of MPV vCCI, we designed an in vivo assay to observe whether or not MPV vCCI could effectively inhibit rhMIP-1α mediated recruitment. To introduce our samples into a macaque in a controlled setting, we modified a previously published angiogenesis protocol by Fan *et al.* (128). Gelfoam is an inert, sponge-like material used as a hemastatic material during

161

surgery. When a soluble agent, such as a chemokine, is absorbed into Gelfoam in the

presence of 0.4% agarose, it can be handled as a solid and once implanted is slowly

released into the external environment over time. Based on previous work in our

laboratory that showed rhMIP-1α mediates recruitment of $CD14^+$ cells during in vivo

recruitment assays, rhMIP-1α was incubated with MPV vCCI at a 1:1 molar ratio prior to

absorption into Gelfoam® plugs. Following surgical implantation, incubation, and

subsequent removal of the protein-saturated implants, frozen sections of the Gelfoam®

implants and surrounding tissue were analyzed by immunohistochemistry using a CD14

specific antibody. In Figures 5a-d, the Gelfoam® implants can be differentiated from

surrounding tissue by its intense H and E (dark blue/purple) staining pattern. As

compared to rhMIP-1α alone, our data suggests that MPV vCCI inhibits rhMIP-1α

mediated recruitment of $CD14^+$ cells, as indicated by a decrease in DAB positive (dark

grey/brown) staining in and around the Gelfoam implant (Fig. 5b).   In an effort to

quantify the levels of inhibition, the number of DAB positive pixels for each image was

normalized to the PBS control and graphed as a migration index. Figure 5e shows

significant inhibition of rhMIP-1α mediated recruitment. These findings are consistent

with our in vitro data and clearly indicated the MPV vCCI is a potent inhibitor of rhMIP-

1α, both in vitro and in vivo.

MPV vCCI inhibits relapsing experimental allergic encephalomyelitis (EAE). In

order to assess the ability of MPV vCCI to treat a chemokine-mediated disease, we

utilized the well described EAE mouse model. Four groups of mice were used for our

experiment: Group 1) Positive controls – mice that received $PLP_{139-151}$ only; Group 2)

Experimental group – mice that received recombinant MPV vCCI and PLP$_{139-151}$; Group 3) MPV vCCI alone – mice receive MPV vCCI alone; and Group 4) Buffer alone – mice receive buffer alone. Groups 3 and 4 serve as negative controls. Figure 6 shows that on day 12, both group 1 and 2 began to exhibit early signs of acute EAE and by day 16 the disease had peaked and both groups began to resolve the disease with complete recovery occurring by day 20. Interestingly, although administration of MPV vCCI did not stop or delay the onset of EAE, animals that received MPV vCCI showed a slight reduction in severity during the acute phase of disease. On day 24, animals of group 1 began to show signs of EAE relapse, lasting approximately 6 days. While the majority of animals fully recovered from EAE relapse, one animal developed chronic EAE, thus the consistent score from day 30 on. More importantly, none of the animals that received MPV vCCI showed any signs of relapse, which was confirmed in a second cohort of animals. Animals receiving MPV vCCI alone or buffer alone, showed no clinical signs of EAE or other pathologies. These data suggest that administration of recombinant MPV vCCI is capable of reducing, and possibly inhibiting, chemokine-mediated disease.

DISCUSSION

Chemokines play an important role in mediating the recruitment of leukocytes to sites of infection, and ultimately establishing effective innate and adaptive immune responses. As a result, many viruses encode proteins which subvert normal chemokine function. In this report, we sought to biologically characterize MPV vCCI both in vitro and in vivo. We show that MPV vCCI is expressed and secreted during MPV infection and that MPV vCCI efficiently inhibits rhMIP-1α mediated chemotaxis in both in vitro and in vivo assays. Furthermore, we have shown that MPV vCCI has the ability to halt relapsing EAE in mice, suggesting that MPV vCCI might represent a novel therapeutic for the treatment of chemokine-mediated disease.

The MPV vCCI staining pattern seen during MPV infection is consistent with the cytoplasmic replication of poxviruses and represents the first time that any vCCI has been visualized using immunofluorescence. Furthermore, immunoprecipitation from infected supernatants and cell lysates clearly shows the presence of MPV vCCI. Interestingly, the presence of a single band in the lysate and a broadened band in the supernatant suggests that the secreted form of MPV vCCI may undergo some post-translational modification.

A commonality among all vCCI research from both the *leporipoxvirus* and *orthopoxvirus* genera is their ability to bind α- and β-chemokines. Structural analysis has determined that binding and subsequent inhibition is much stronger with β-chemokines. In fact, vCCI binding to α-chemokines occurs with such a low affinity that many question whether it is physiologically relevant (16, 400). Our work on MPV vCCI is consistent with these previous results, in that MPV vCCI forms a complex with rhMIP-1α resulting

164

in a significant shift in MPV vCCI MW_{app}. Although there is some debate as to the exact stoichiometry, stoichiometric analysis suggests that vCCI binds MCP-1 at nearly 1:1 ratio (16, 383). Our data with rhMIP-1α is supported by these findings, in that at a 1:1 ratio, all of the MPV vCCI is migrating at the higher MW_{app}, only when in excess does MPV vCCI migrate at the lower MW_{app}.

Structural analysis of rabbitpox virus vCCI (RPV vCCI) complexed with human MIP-1β has provided significant insight as to a possible mechanism behind vCCI-mediated inhibition. Zhang *et al*. reported that RPV vCCI possess a number of important contacts with MIP-1β. In particular, the highly conserved vCCI resides Ser-182 to Thr-187 make "extensive contacts" with the chemokine (462). This region is critical for receptor binding, therefore high affinity association with vCCI appears to inhibit cc-chemokine receptor interactions. Along these lines, our in vitro inhibition assay clearly demonstrates the inhibitory power of MPV vCCI as it completely blocks rhMIP-1α-mediated chemotaxis in a dose-dependent manner. This effect is dependent on MPV vCCI function, since heat inactivation of MPV vCCI restores rhMIP-1α-mediated migration to near rhMIP-1α alone levels. Although we have shown that MPV vCCI interacts with rhMIP-1α at approximately 1:1 stoichiometry (fig 3b and 5), our in vitro inhibition assay requires 100 fold excess MPV vCCI for complete inhibition of rhMIP-1α mediated migration (fig. 4). More than likely, this discrepancy is the result of using the human THP-1 cells in our in vitro inhibition assay. Subtle differences between human and rhesus GPCRs may explain the requirement for excess vCCI to be present in order to achieve complete inhibition in THP-1 cells.

Our *in vivo* studies on vCCI further confirm that the inhibitory potential is not limited to the in vitro setting. Although several reports have studied VV vCCI in mice and guinea pigs showing that vCCI can inhibit leukocyte recruitment in these animals, these are not natural host for VV, therefore slight differences may exist in the natural host (16, 344, 400). Graham *et al*. investigated the inhibitory potential of RPV vCCI in rabbits showing a marked increase in leukocyte infiltrates when rabbits were infected with a vCCI knockout virus (161). For our in vivo studies, we performed two tests, both of which utilized purified recombinant MPV vCCI. The first involved an in vivo inhibition assay in rhesus macaques using protein saturated Gelfoam® plugs. Although rhMIP1-α alone induces significant recruitment of $CD14^+$ cells, when complexed with MPV vCCI, $CD14^+$ recruitment was drastically reduced. Secondly, we tested the ability of MPV vCCI to mitigate a chemokine-mediated disease. Experimental allergic encephalomyelitis (EAE) is an induced disease in mice that closely mimics multiple sclerosis in humans. Previous work by Karpus e*t al.* has shown that administration of neutralizing antibodies for MIP-1α and MCP-1 causes a significant reduction in EAE disease, and therefore MIP-1α and MCP-1 must play an integral part in the establishment and progression of EAE (214). Prior to initiating the EAE study, we confirmed that MPV vCCI interacts with several mouse chemokines, namely MIP-1α and MCP-1, via shift assay (data not shown). These results were consistent with work by Smith *el al.*, who showed that CPV vCCI bound with high affinity to mouse MCP-1, MCP-5, MIP-1α, MIP-1β, C10, and Eotaxin (400). As our EAE study progressed into the acute phase we observed that mice receiving MPV vCCI exhibited reduced severity of disease compared to mice that did not receive MPV vCCI. More importantly, as mice from our positive

166

control group began to exhibit signs of relapsing-remitting EAE (around day 24); the mice that received MPV vCCI did not and remained free of relapse until the end of the study.

This represents the first time that any vCCI has been shown to inhibit or mitigate a chemokine-mediated disease. The implications of our in vitro and in vivo characterization are significant, laying the ground work for additional studies to investigate the effectiveness of MPV vCCI in treating chemokine-mediated disease in non-human primates. Furthermore, we feel that MPV vCCI represents a legitimate therapeutic candidate for the treatment of chemokine-mediated disease.

ACKNOWLEDGEMENTS

Table 3.1 Homology of MPV vCCI to CPV vCCI, RPV vCCI, VARV vCCI, and VV COP vCCI.

vCCI	% Identical to MPV vCCI	% Similar to MPV vCCI
CPV	79	84
RPV	85	89
VARV	83	87
VV COP	83	83
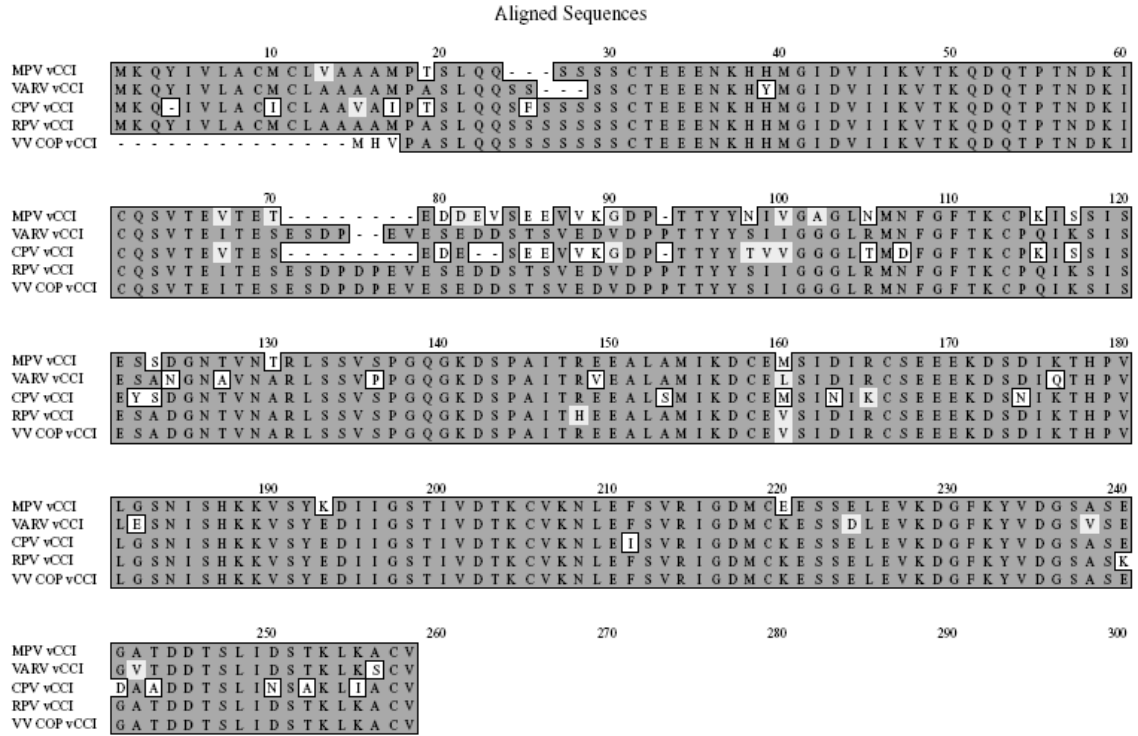
Figure 3.1 Amino acid comparison of MPV vCCI to vCCIs encoded by variola virus (VARV), cowpox virus (CPV), rabbitpox virus (RPV), and vaccinia Copenhagen strain (VV COP). Alignments were performed with ClustalW using Blosum scoring matrix. Dark shaded boxes indicate either: 1) identical residues, or 2) unique residues to MPV vCCI. Lightly shaded boxes represent similar residues to MPV vCCI.

MPV infected
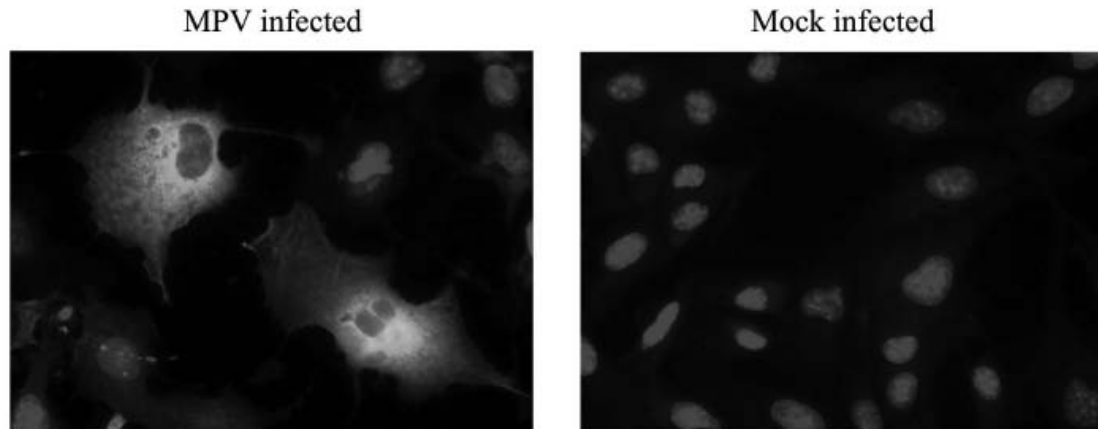
Mock infected

Figure 3.2 Expression of MPV vCCI during MPV infection. (A) Immunofluorescence

analysis on MPV-infected (panel A) or mock-infected (panel B) BSC_{40} cells fixed at 24

h.p.i. Cells were stained with a mouse anti-vCCI monoclonal antibody, followed by a

biotinylated horse anti-mouse secondary antibody, and visualized using an alexa-488

conjugated to streptavidin. Nuclear staining was performed using Hoescht stain. All

images were taken with 20x objective. (B) Secretion of MPV vCCI during MPV

infection. Samples of supernatants and lysates from MPV (lanes 1 and 3) and Mock

(lanes 2 and 4) infected BSC_{40} cells were resolved on 4-12% Bis-Tris NuPAGE® gels and

transferred to PVDF. Western blot analysis was performed using a mouse anti-vCCI

monoclonal antibody (3D1) and an HRP-conjugated goat anti-mouse secondary antibody.

Purified MPV vCCI was used as a positive control (lane 5).

Figure 3.3 MPV vCCI binds β-chemokines. (A) Purified MPV vCCI and rhMIP-1α

were mixed together at a 1:1 molar ratio and incubated for ten minutes at room

temperature. Purified MPV vCCI alone and rhMIP-1α alone were used as controls.

Reactions were resolved on a 12% native PAGE gel and stained with SimplyBlue™ Safe

Stain. (B) MPV vCCI was titrated from limiting to excess, into a reaction mixture with a

fixed amount of rhMIP-1α. MPV vCCI alone and rhMIP-1α alone were used as controls.

(C) Co-immunoprecipitation of rhMIP-1α with MPV vCCI. Increasing amounts of

rhMIP-1α (0.1ug to 2.0ug – lanes 4 to 8) were incubated with a fixed amount of MPV

vCCI (6ug), as a result more rhMIP-1α co-elutes with immunoprecipitated MPV vCCI.

3ug of rhMIP-1α (lane 2) and 6ug of MPV vCCI (lane 1) were used as positive controls.

As a negative control, MPV vCCI immunoprecipitation was performed on 3ug of rhMIP-

1α alone (lane 3). Proteins were resolved on 4-12% Bis-Tris NuPAGE® gels. (D) MPV

vCCI binds the murine chemokines: MIP-1α, MCP-1, and RANTES. (D) In similar

assays described in panel A, shift assays using MPV vCCI and mMIP-1α and mMCP-1,

mRANTES were also conducted.

A.

	2x MPV vCCI	2x rhMIP-1α	MPV vCCI + rhMIP-1α	MPV vCCI	rhMIP-1α	
Std	1	2	3	4	5	Lane

20kDa -

B.

Ratio of MPV vCCI/rhMIP-1α

	0.25/1	0.5/1	1/1	1.25/1	1.5/1			2x MPV vCCI	
Std	1	2	3	4	5	6	7	8	Lane

← MPV vCCI:rhMIP-1α

20kDa -

← free MPV vCCI

C.

IP: MPV vCCI

MPV vCCI	6.0	–	–	6.0	6.0	6.0	6.0	6.0	μg protein
rhMIP-1α	–	3.0	3.0	0.1	0.5	1.0	1.5	2.0	
	1	2	3	4	5	6	7	8	Lane

35kDa -

WB: MPV vCCI

10kDa -

WB: rhMIP-1α

D.

174
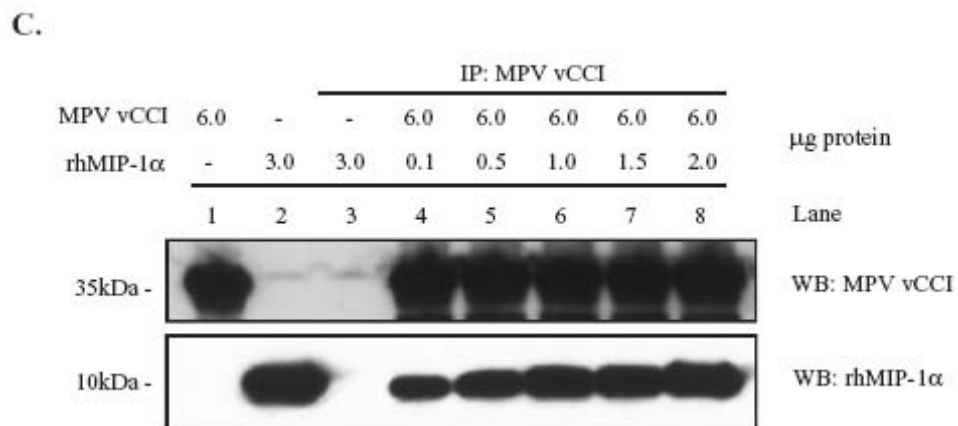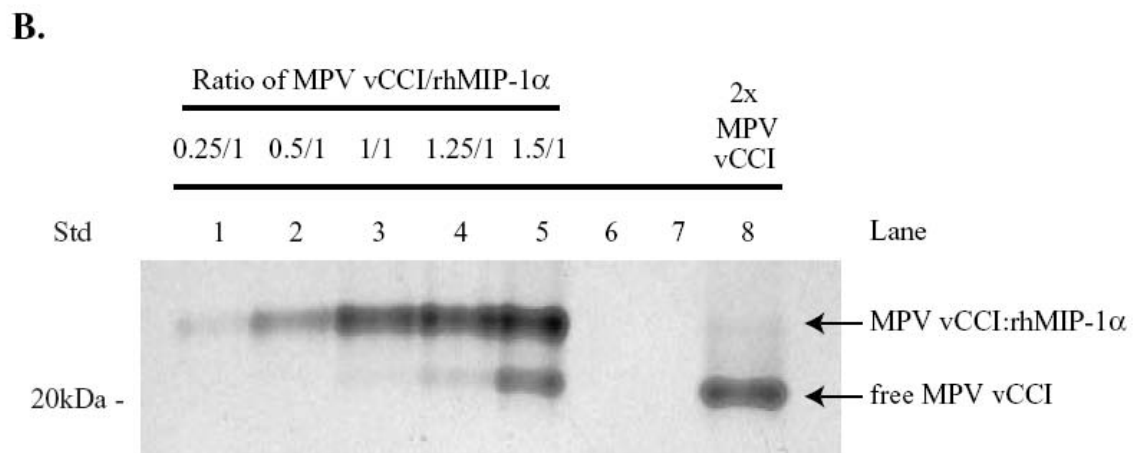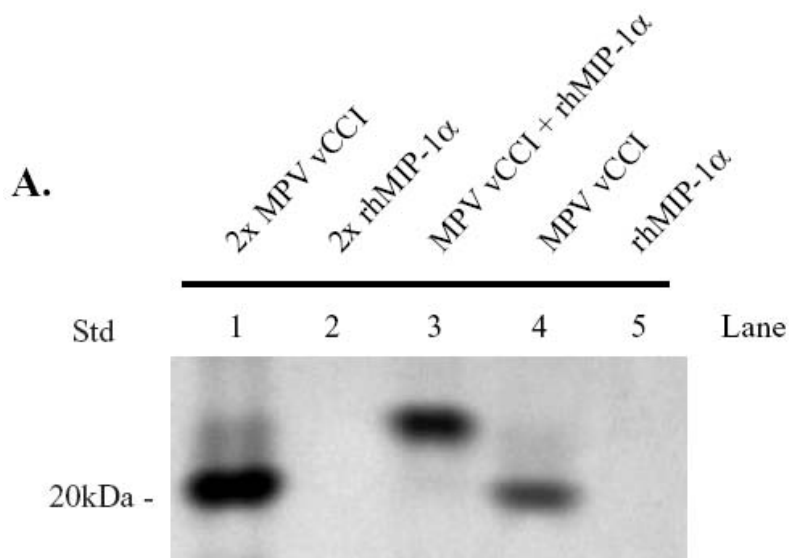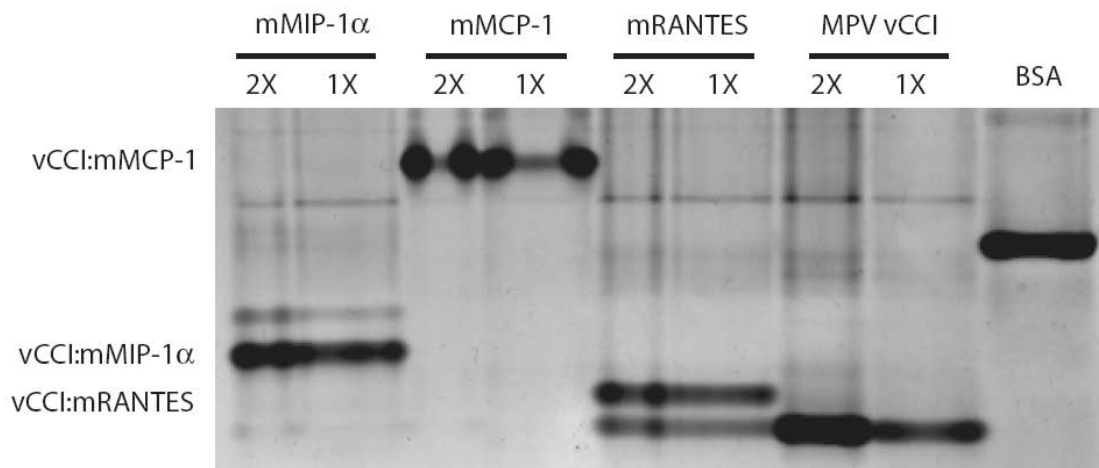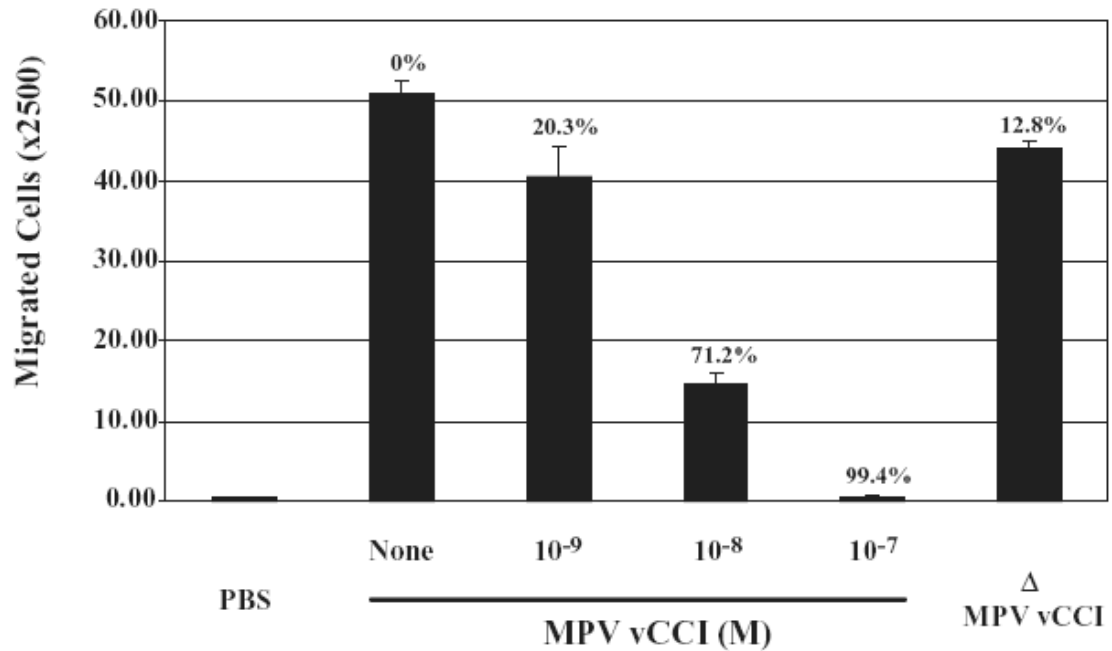
Figure 3.4 Inhibition of rhesus MIP-1α mediated migration of Human THP-1 cells.

$5x10^5$ THP-1 cells suspended in 100μL of assay media (RPMI 1640 + 0.5% fetal bovine

serum) were placed in 3μm pore size transwell inserts and placed in 24-well culture

plates containing 600μL assay media with 10^{-9} M rhMIP-1α plus increasing

concentrations of MPV vCCI or 10^{-7} M heat inactivated MPV vCCI (Δ MPV vCCI).

PBS was used as a negative control. Following a 4hr incubation at 37°C (5% CO_2),

THP-1 cells migrating through the transwell were counted using a CyQuant® cell

proliferation assay kit (Molecular Probes, Eugene, OR). Represented data are the

average number of migrated cells of 3 wells (x2500) ± SEM.

Figure 3.5 In vivo inhibition of rhesus MIP-1α mediated chemotaxis. Gelfoam®
sponges containing agarose-embedded (A) rhMIP1α or (B) rhMIP1α + MPV vCCI or
(C) PBS were implanted s.c. in the back of a rhesus macaque (≥8 cm apart), where they
remained for 7 days before being harvested, sectioned, and stained. CD14 staining shows
a clear reduction in CD14$^+$ infiltrates (lack of dark grey/brown coloration) in the
Gelfoam® sponges containing rhMIP1α + MPV vCCI, as compared to rhMIP1α. An
isotopically matched primary antibody was used on a section of rhMIP1α-containing
Gelfoam® as an antibody control (D). Quantification of CD14$^+$ infiltrates (E) was
performed by comparing the number of DAB$^+$ pixels in each image and normalizing to
PBS and represented as a migration index ± SEM. All images (A-D) were taken using a
20x objective and are the same size, 345000 pixels.

A. rhMIP-1α

B. rhMIP-1α + MPV vCCI

C. PBS

D. IgG Isotype

E.
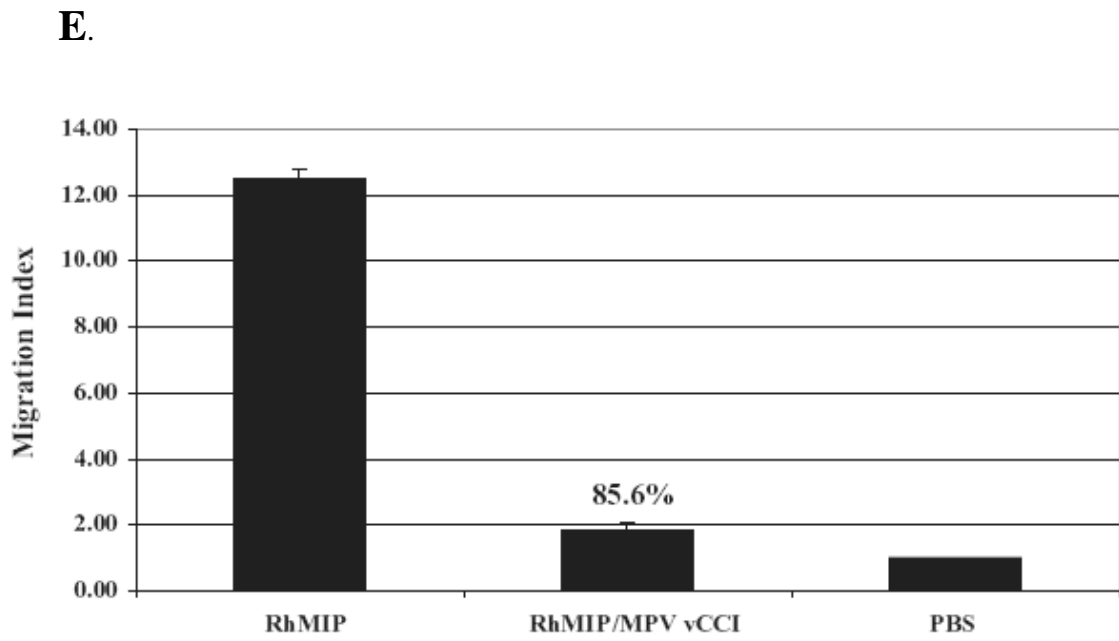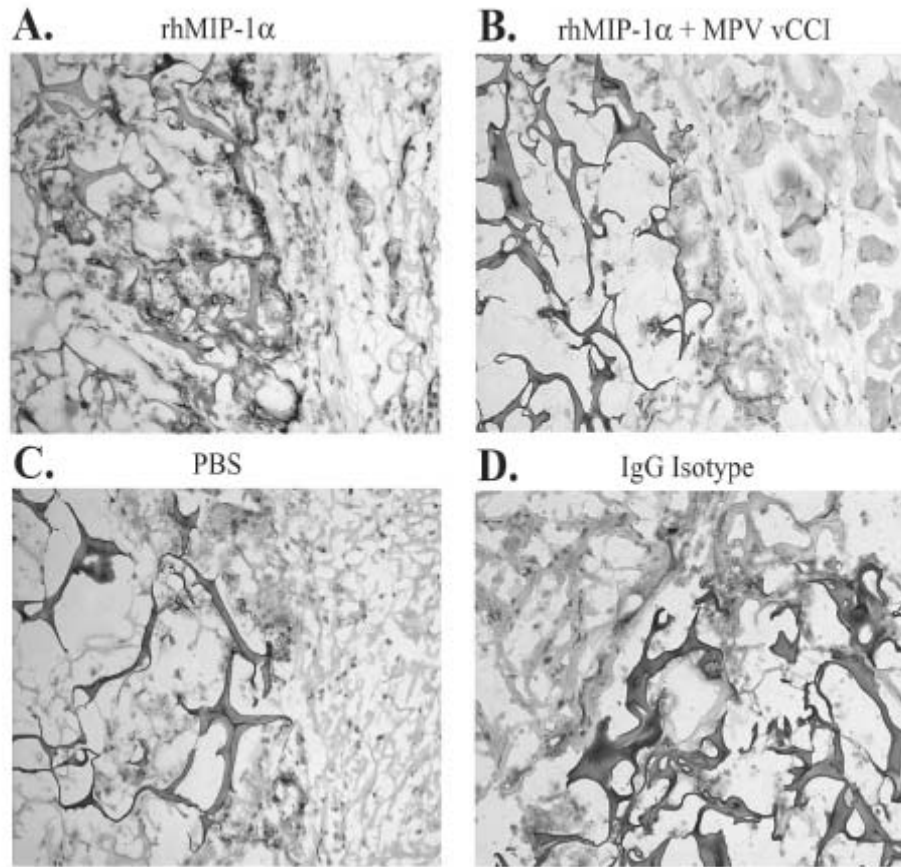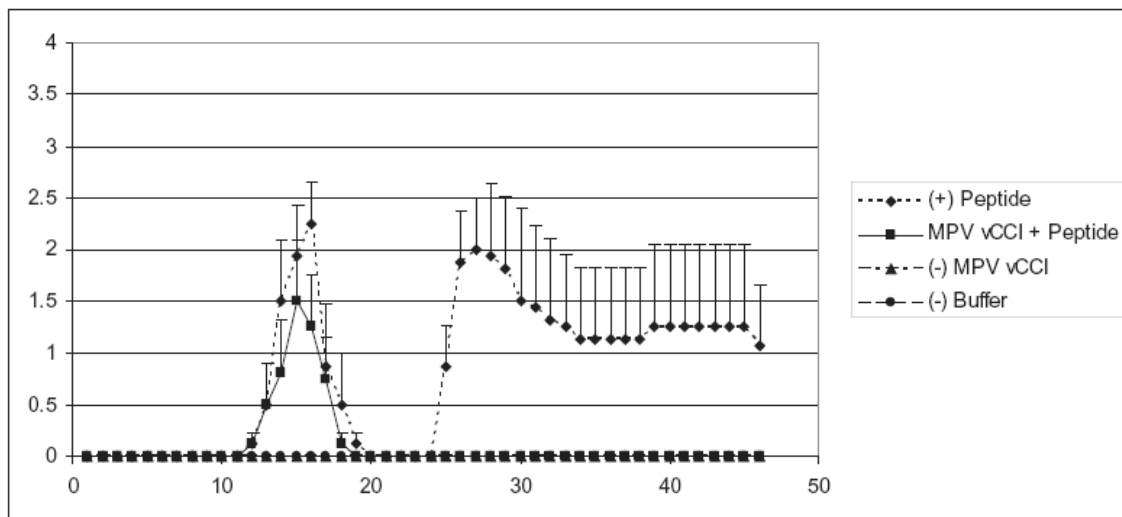
Figure 3.6 MPV vCCI inhibits relapsing EAE. Following induction of EAE by administration of $PLP_{139-151}$ peptide $\pm$ MPV vCCI, mice (n=4) were observed on a daily basis and scored for disease using the following scale: 0 – Normal, 0.5 – Partially limp tail, 1.0 – Paralyzed tail, 2.0 – Hind limb paresis, 2.5 – One hind limb paralyzed, 3.0 – both hind limbs paralyzed, 3.5 – Hind limbs paralyzed; fore limbs weak, 4.0 – Fore limbs paralyzed, 5.0 – Moribund. Represented values are the average scores for all mice within each group. Symbols represent the four groups: ♦ - $PLP_{139-151}$ peptide; ■ - $PLP_{139-151}$ peptide + MPV vCCI; ▲ - MPV vCCI alone control; ● - buffer alone control.

Chapter 4

SUMMARY AND CONCLUSIONS

1. RRV ORF-R3 encodes a vMIP homolog similar to those encoded by KSHV

a. *Functional characterization of RRV vMIP*

In chapter 2 of this thesis, we describe the functional characterization of RRV vMIP. Amino acid sequence analysis suggested that the product of RRV ORF- R3 is a secreted protein containing β-chemokine features. Our work establishes that RRV ORF-R3 is transcribed during lytic RRV infection, displaying late viral gene kinetics. This is somewhat different than what has been described for KSHV vMIPs, which are described in the literature as primary and secondary lytic genes. However, this may be an artifact from the use of phorbol esters to induce KSHV lytic replication, which could result in a more rapid transcription pattern for the KSHV vMIPs. We went on to show that the product of ORF-R3, RRV vMIP, is expressed and secreted from RRV-infected cells, and that, RRV vMIP is capable of inducing chemotaxis both *in vitro* and *in vivo.* These findings are consistent with some of the published reports regarding KSHV vMIPs, specifically the ability of all of the KSHV vMIPs to induce chemotaxis in various cell types. More importantly, we demonstrate via *in vivo* chemotaxis assays that $CD14^+$ monocytes, but not $CD3^+$ T cells or $CD20^+$ B cells, respond to the presence of RRV vMIP and that this response is nearly equivalent to that seen with rhMIP-1α. Finally, this work demonstrates for the first time that RRV selectively infects $CD14^+$ monocytic cells over $CD3^+$ T cells and $CD20^+$ B cells during acute *in vitro* infection of rhesus PBMCs.

b. *The role of RRV vMIP in RRV infection and pathogenesis*

The ability of RRV vMIP to promote recruitment of CD14$^+$ monocytes during RRV infection, at first glance, may seem counterintuitive. As we have described in previous sections, herpesviruses and poxviruses encode many proteins that promote immune evasion, yet RRV vMIP appears to be pro-inflammatory. The ability of RRV vMIP to function as a chemokine coupled with data from our *in vitro* infection experiments suggest that RRV vMIP may play an active role in RRV pathogenesis by promoting the recruitment of permissive cells. Figure 4.1 outlines the possible roles of RRV vMIP during RRV infection. In model #1, roughly 48hr following RRV infection (Figure 4.1.1), RRV vMIP is expressed and secreted from the infected cell (Figure 4.1.2). CD14$^+$monocytes responding to the RRV vMIP gradient migrate into the area surrounding the infected cell (Figure 4.1.3). These permissive monocytes enter a microenvironment primed with newly released RRV virions that exhibit an infectivity preference for the recruited cells. The newly infected monocytes (Figure 4.1.4) can then either harbor further RRV replication and virus production or act as vehicles for the dissemination of RRV throughout the host (Figure 4.1.5).

Our understanding about primary RRV infection and transmission is not completely understood. Infection normally occurs prior to adolescence and evidence suggests that RRV is present in both saliva and blood (448). This suggests that vertical transmission from mother to offspring may be the most likely route for RRV infection. As such, the probable site of primary infection is the epithelial layers of the respiratory tract, but tissue resident macrophages within the lung may also prove to be important for the initial phase of RRV infection. Our data suggests that CD14$^+$ monocytes, responding

to the initial infection, become infected with RRV. Given that CD20$^+$ B cells have already been identified as the site of RRV latency, it is unlikely that monocytes are acting as long-term RRV reservoirs, like members of the β-herpesvirus subfamily (e.g. cytomegalovirus). Therefore, the role of CD14$^+$ monocytes in RRV pathogenesis is likely that of a disseminator, providing a vehicle for the trafficking of virus to other areas within the host, possibly increasing the likelihood of B cell infection, thereby increasing the chances of establishing latency. Further studies are required to fully understand the role CD14$^+$ monocytes play in the RRV lifecycle.

Additional RRV genes may be involved in the proposed RRV vMIP model, specifically, vCD200, which encodes a functional homolog of the cellular protein CD200 (formerly known as OX-2). CD200 is a transmembrane glycoprotein present on many myeloid cells. The interaction between CD200 and CD200R provides an inhibitory signal for myeloid cells, thereby influencing their immunological function (454). The vCD200 homolog of RRV differs from that of cellular or KSHV vCD200, in that, differential splicing results in two forms of the protein being expressed, one membrane bound and the other secreted. Moreover, work in our laboratory has shown that vCD200 reduces TNF-α expression from rhesus macrophages (243). With regards to the RRV vMIP model, we believe that vCD200 is expressed on the surface of RRV infected cells, as well as secreted into the surrounding microenvironment. Therefore, infiltrating CD14$^+$ cells responding to the RRV vMIP gradient are entering an environment rich in vCD200, thereby reducing their pro-inflammatory response and increasing the possibility that they become infected by RRV.

Figure 4.1 Possible roles of vMIP in RRV pathogenesis Model #1 shows the agonistic role of RRV vMIP as a functional chemokine, while model #2 demonstrates how RRV vMIP may act an a receptor antagonist.

Although RRV vMIP can function as a chemokine, as shown for KSHV vMIP-II, it is possible that RRV vMIP may possess antagonistic properties as well. It is this functional duality that may suggest a hypothesis in which RRV vMIP promotes the recruitment of permissive CD14$^+$ monocytes through agonistic GPCR binding, and at the same time, inhibiting the recruitment of other pro-inflammatory cells by antagonistically binding other receptors (Figure 4.1 – model #2). Therefore, the combined functions of three KSHV vMIPs may be consolidated into one multifunctional RRV vMIP. Further ligand-binding studies are required to elucidate additional properties of RRV vMIP and test the plausibility that RRV vMIP possesses antagonistic properties.

c. *Future directions*

Future studies on RRV vMIP will focus on a more biochemical characterization of the protein. Specifically, we hope to identify the chemokine receptor(s) that is targeted by RRV vMIP. Although RRV vMIP has been shown to function as a chemokine, this does not exclude the possibility that RRV vMIP may antagonize other receptors, which is consistent with the published work on KSHV vMIPs. Therefore, ligand-binding studies coupled with Ca^{++} mobilization would help to elucidate the potential role of RRV vMIP as a receptor antagonist. Along with testing the ability of RRV vMIP to bind various rhesus chemokine receptors, the RRV vGPCR could also be evaluated. Although none of the KSHV vMIPs have been shown to bind the KSHV vGPCR, this does not exclude the possibility that RRV vMIP may interact with RRV vGPCR. Further biological studies will hopefully use cells, or perhaps an immortalized cell line, isolated from rhesus macaques. We have tried several isolation techniques, but as of yet, have been unable to

isolate enough cells from rhesus blood to conduct a statistically relevant study. The

presence of human cell lines has provided invaluable in chemokine research and

hopefully in the future rhesus cell lines we become available. Finally, KSHV vMIP-I and

-II have been shown to be highly angiogenic. Although RRV-induced neoplasia are not

usually associated with the abnormal vasculature observed in KS tumors, this does not

preclude the possibility that RRV vMIP may possess angiogenic potential.

2. MPV ORF-J1L encodes a potent inhibitor of rhMIP-1α, MPV vCCI

a. *Functional characterization of MPV vCCI*

In chapter 3 of this thesis, the functional characterization of MPV vCCI is

described. The MPV ORF-J1L encodes a chemokine binding protein that is highly

conserved among orthopoxvirus and some leporipoxviruses. This work provides the first

demonstration of the expression and secretion of MPV vCCI from MPV infected cells.

Furthermore, the immunofluorescence data presented here is the first reported

visualization of vCCI in an infected cell. Functionally, *in vitro* binding assays show that

MPV vCCI is capable of binding rhMIP-1α at a near 1:1 stoichiometry, which is

consistent with previous published reports for CPV vCCI. More importantly, MPV vCCI

efficiently inhibits rhMIP-1α mediated chemotaxis, both *in vitro* and *in vivo*. Although

vCCIs are present in many species of poxvirus, including vaccinia, only one other species

of vCCI has been characterized *in vivo*. Graham *et al.* demonstrated that intradermal

infection of a vCCI knockout rabbitpox virus (RPV) exhibited less macrophage

recruitment as compared to the wild type virus (161). Our system removes the somewhat

complicating aspects of viral infection and instead focuses on the ability of MPV vCCI to bind and inhibit rhMIP-1α. Although the two systems are different, the results are consistent with one another and highlight the important immune evasion role that vCCIs play during poxvirus infection. Finally, we investigated the possible therapeutic potential of MPV vCCI by testing its ability to mitigate the chemokine-driven disease known as experimental autoimmune encephalomyelitis (EAE). Although MPV vCCI was unable to block the onset of acute EAE, the disease score was slightly less for the MPV vCCI + peptide group, as compared to the peptide alone group. More importantly, MPV vCCI was capable of blocking relapsing EAE in the entire MPV vCCI + peptide group.

b. *The role of MPV vCCI in MPV infection and pathogenesis*

As described in earlier sections, chemokines play a vital role in the immune response to viral infection. Therefore, adapting mechanisms to subvert the pro-inflammatory nature of chemokines is advantageous for viruses. Unlike the multifunctional role of KSHV vMIPs and pro-inflammatory role of RRV vMIP, MPV vCCI represents the consummate viral immune evasion protein by efficiently inhibiting rhMIP-1α mediated chemotaxis (Figure 4.2 – top). Collaborative work between our laboratory and the laboratory of Dr. Richard Smith at Pacific Northwest National Laboratories (PNNL) has demonstrated several ways in which MPV vCCI may augment MPV pathogenesis. Interestingly, a proteomic analysis of purified MPV particles shows that MPV vCCI is present in preparations of nascent virions (R. Estep and S. Wong – unpublished work). Whether this is a deliberate event or the result of non-specific incorporation/binding remains to be determined.

Figure 4.2 Roles of MPV vCCI in MPV pathogenesis and EAE. The top panel

illustrates how MPV vCCI inhibits chemokine function and promotes immune evasion.

The bottom panel describes the possible mechanisms of how MPV vCCI is able to inhibit

relapsing EAE

The Role of MPV vCCI in MPV Pathogenesis

MPV vCCI   Chemokine

$+$

Chemokine
sequestration

No chemokine
mediated
chemotaxis

Target Cell
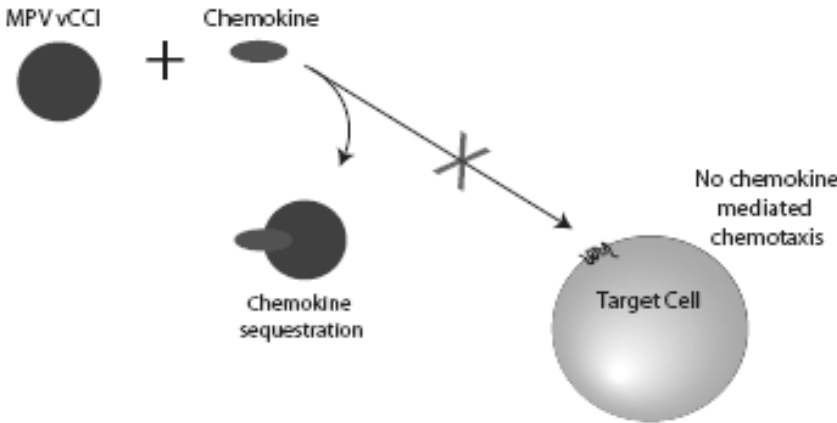
The Role of MPV vCCI in Inhibiting EAE

MPV vCCI

1. Sequestering
of chemokine

3. Anti-idiotypic
response

2. Insufficient memory
population

4. Autoreactive
chemokine
antibodies

Memory T cell
Population

Chemokines

Relapsing EAE
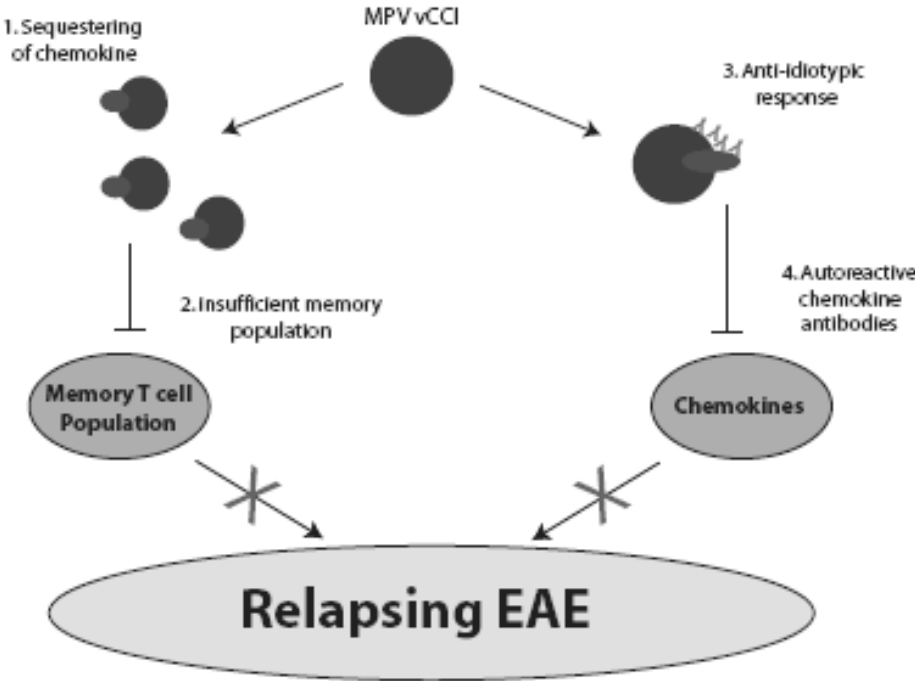
Curiously, vCCIs do not contain a canonical secretion signal peptide, yet are efficiently secreted into the extracellular environment. Therefore, if MPV vCCI was specifically packaged into viral particles or possibly membrane associated, this would provide the virus with an immune evasion mechanism that is independent of viral gene expression and function immediately following viral entry and uncoating. A second proteomics analysis was performed on bronchial alveolar lavage (BAL) fluid taken from MPV infected rhesus macaques. These monkeys were infected intrabronchially and, following the initial wave of viremia, BAL fluid was collected from day 7 to day 32. The results of these analyses demonstrated a continual rise in the levels of MPV vCCI between days 7 and 14 (Figure 4.3). Additional studies are underway to determine the extent of immunological infiltration within the infected lung tissue. When completed, this will provide a better understanding of how MPV vCCI functions in vivo.

In our functional study of MPV vCCI, we investigated the ability of MPV vCCI to sequester rhMIP-1α. This does not exclude the possibility that other chemokines may also be inhibited by MPV vCCI. In fact, studies of CPV vCCI have shown that they can bind a number of chemokines from multiple host species (62). We performed preliminary studies to determine if MPV vCCI could bind several murine chemokines, as well as the RRV vMIP homolog. The results demonstrated that MPV vCCI can interact with mMIP-1α, mMCP-1, and mRANTES *in vitro*. The ability to bind mMIP-1α and mMCP-1 is in agreement with previously published data (213). However, the ability of MPV vCCI to bind mRANTES is contrary to the published CPV vCCI data, indicating that despite their high amino acid identity subtle changes have conferred functional differences.

Figure 4.3 Relative abundance of MPV-specific peptides in BAL fluid of MPV infected monkeys. Proteomic analysis on BAL fluid taken from rhesus macaques pre- and post-infection shows the relative abundance of specific MPV genes. Starting at day 7, both animals have significant levels of MPV vCCI in their BAL fluid.

Macaque1, 3 month pre-infection
Macaque2, 3 month pre-infection
Macaque1, 13 day pre-infection
Macaque2, 13 day pre-infection
Macaque1, 7 day post-infection
Macaque2, 7 day post-infection
Macaque1, 12 day post-infection
Macaque2, 12 day post-infection
Macaque1, 14 day post-infection
Macaque2, 14 day post-infection
Macaque1, 18 day post-infection
Macaque2, 18 day post-infection
Macaque1, 21 day post-infection
Macaque2, 21 day post-infection
Macaque1, 32 day post-infection
Macaque2, 32 day post-infection

-3.0   1:1   3.0

B9R (AJ404603) interferon-gamma receptor [Monkeypox virus]_gnl|poxpep|MPXV-ZRE_170
H5R I5R [Variola virus] -gi|418239|sp|P30062|AG35_VARV ENVELOPE PROTEIN (PROTEIN H5) -gi|420481|pir||S33_gnl|poxpep|MPXV-ZRE_095
J1L (Y11842) D1L [Cowpox virus] -gi|3087052|emb|CAA75307.1| (Y150035) H5R [Cowpox virus]_gnl|poxpep|MPXV-ZRE_001
C22L F16L putative [Vaccinia virus] -gi|137854|sp|P21021|VF16_VACCC PROTEIN F16 -gi|93409|pir||C42508 F1_gnl|poxpep|MPXV-ZRE_048
H4L H4L putative [Vaccinia virus] -gi|133313|sp|P07241|RP94_VACCV RNA-POLYMERASE-ASSOCIATED TRANSCRIPT1_gnl|poxpep|MPXV-ZRE_094
A10L A9L putative [Vaccinia virus] -gi|137347|sp|P20987|VA09_VACCV PROTEIN A9 -gi|93292|pir||B42518 A9L_gnl|poxpep|MPXV-ZRE_120
C19L MAJOR ENVELOPE PROTEIN (37 KD PROTEIN)(P37K) -gi|74269|pir||WMVVZ37 major envelope antigen - vaccini_gnl|poxpep|MPXV-ZRE_045
A25R DNA-DIRECTED RNA POLYMERASE 132 KDA POLYPEPTIDE -gi|66989|pir||RNVZCP DNA-directed RNA polymerase (E_gnl|poxpep|MPXV-ZRE_135
D6L (AJ271165) interleukin 18 binding protein [Ectromelia virus]_gnl|poxpep|MPXV-ZRE_009
I1L TI1L [Vaccinia virus (strain Tian Tan)] -gi|9790992|ref|NP_063719.1| I1L putative [Vaccinia virus]_gnl|poxpep|MPXV-ZRE_082
M1R M1R [Variola virus] -gi|418259|sp|P33040|VL01_VARV PROTEIN L1 -gi|420491|pir||S33087 L1R protein - v_gnl|poxpep|MPXV-ZRE_080

190

Additionally, previous binding studies have shown that CPV vCCI can bind KSHV

vMIP–I and –II, but not –III. Therefore, we tested the ability of MPV vCCI to bind RRV

vMIP. However, at all concentrations tested, no binding was observed. To more fully

understand the degree to which MPV vCCI is limiting the host's chemokine-driven

responses, we will need to evaluate MPV vCCI's ability to bind a much broader panel of

rhesus chemokines.

c. *The possible role of MPV vCCI in treating chemokine-driven diseases*

Since MPV vCCI has demonstrated an ability to bind and inhibit chemokine

function, we sought to determine its usefulness as a possible therapeutic agent. As

described in chapter 1, chemokines have roles in a number of inflammatory diseases.

Current therapies for these conditions involve the use of anti-inflammatory and

immunosuppressive drugs. However, these treatments come with potentially serious side

effects, such as an increased chance of infection. Targeting chemokines may therefore

provide a more specific treatment for inflammatory diseases, potentially resulting in

fewer side effects.

As shown in chapter 3, we demonstrated that the administration of MPV vCCI

can successfully inhibit relapsing experimental autoimmune encephalomyelitis (EAE).

The exact mechanism of this inhibition is unclear, but given the timeframe, it is highly

unlikely that the inhibition is related to ongoing chemokine inhibition by MPV vCCI.

Therefore, treatment with MPV vCCI is capable of priming a long lasting effect. We

believe that the inhibition of relapsing EAE may be the result of two possible

mechanisms (Figure 4.2 – bottom). In the first, MPV vCCI sequesters pro-inflammatory

chemokines (Figure 4.2.1), resulting in treated mice that lack a sufficient memory T cell population and therefore do not develop relapsing EAE (Figure 4.2.2). This model is supported by preliminary data from splenocytes isolated from animals at the completion of the experiment. These splenocytes were pulsed with the $PLP_{131-159}$ peptide or OVA peptide, and their IFN-γ and IL-2 responses were measured by intracellular cytokine staining (ICCS). T cells from mice receiving only the $PLP_{131-159}$ peptide had elevated IL-2 responses, while T cells from mice receiving $PLP_{131-159}$ peptide + MPV vCCI did not (J. Jones, I. Messaoudi, and S. Wong – unpublished results). Since these splenocytes were taken at the completion of the study, one conclusion that can be drawn is that these mice failed to establish a sufficient memory T cell population as a result of MPV vCCI function. The second potential mechanism of MPV vCCI inhibition of relapsing EAE involves the production of anti-idiotypic antibodies. MPV vCCI is highly immunogenic in mice, and following MPV vCCI treatment, anti-MPV vCCI antibodies can easily be detected by immunoblot. It is therefore conceivable that during the generation of anti-MPV vCCI antibodies, the association of chemokines with MPV vCCI results in their targeting by the immune system (Figure 4.2.3). As a result of this chemokine-specific, anti-idiotypic antibody response, the targeted chemokines would be effectively neutralized in the animal (Figure 4.2.4). Given that MIP-1α and MCP-1 have been shown to be important for the acute and relapsing phases of EAE, their neutralization would result in reduced disease.

Despite their fundamental role in inflammation, chemokines have yet to be fully exploited for their therapeutic value in the treatment of inflammatory diseases. Current treatments for inflammatory diseases typically involve the use of corticosteroids and

calcineurin inhibitors. However, the primary concern regarding these treatments is the associated side effects, such as increased opportunistic infections, hypertension, dyslipidemia, hyperglycemia, peptic ulcers, as well as liver and kidney injury (3, 232, 430). As outline in section 3, several approaches for the inhibition of chemokines and their receptors, and the potential role these agents may play in treating inflammation, are being studied. Most of these agents attempt to inhibit inflammatory disease by blocking the recruitment of cells responsible for the inflammatory response, thereby preventing the ensuing inflammation-mediated pathology. The primary benefit to this type of approach is the potential for reduced inflammation without global, non-specific reduction in cellular activation, and therefore potentially fewer of the physiological side effects and opportunitic infections. Moreover, targeting chemokines directly may offer a less toxic option for immune suppression, given that chemokine inhibitors currently studied are typically well tolerated (336).

As with any therapeutic agent, treating inflammation with novel chemokine and chemokine receptor inhibitors is not without potential side effects or disadvantages. The first concern relates to the apparent redundancy within α- and β-chemokine subfamilies. The concept that many chemokine receptors bind multiple chemokines and many chemokines bind to several chemokines receptors, coupled with the ability of some chemokines to function through the same receptors to elitcit the same function (e.g. functional binding of MIP-1α and RANTES to CCR5 and eliciting monocyte chemotaxis), has complicated the potential therapeutic outlook of chemokine and chemokine receptor antagonists. However, encouraging evidence is emerging regarding specific roles for various chemokine receptors within some disease models, suggesting

that the targeting of certain chemokines may be possible (334). The introduction of any

therapeutic agent has the potential to initiate an immune response directed against the

agent. In most cases, the humoral immune response will generate antibodies directed

toward the agent effectively neutralize its therapeutic activity. However, in the worst

case scenario, repeated treatments could potentially result in a much more serious shock-

like syndrome. Another potential problem with this type of approach is the abiltity of the

therapeutic to work in different species. For example, several CCR1 inhibitors have

showed significant promise in blocking CCR1 function in humans and rabbits, but are not

effective in inhibiting murine CCR1 (254, 255). This underscores the importance of

selecting a proper model system which best represents the final target species.

The administration of MPV vCCI, which directly targets multiple inflammatory

chemokines, may provide an alternative to conventional methodologies for the treatment

of inflammatory diseases. Furthermore, because MPV vCCI can efficiently bind multiple

chemokines, specifically those inducible chemokines involved in inflammation, the effect

of chemokine redundancy would be reduced when treating with MPV vCCI.

d. *Future directions*

Further research on MPV vCCI will involve a combination of biochemical and *in*

vivo approaches. Additional binding studies are needed to determine which of the known

rhesus chemokines MPV vCCI is capable of binding. The results of such studies will not

only further our understanding of MPV pathogenesis, but they will also identify

additional MPV vCCI therapeutic targets. A major focus of future experiments will

involve the characterization of the role of that MPV vCCI plays during MPV infection of

194

rhesus macaques. To do so, an MPV vCCI knockout virus will be constructed. Rhesus

macaques will be infected with either the ΔMPV vCCI or the wild type virus, and

differences between the two will be assessed via co-culture, serology, and histology. We

expect that in the absence of MPV vCCI, infected animals will show an increased

immune infiltrates coupled with a reduced viral load. Finally, we wish to transition our

EAE model into primates. To do so, we will utilize a small percentage of colony-kept

Japanese macaques that develop a disease that closely mimics multiple sclerosis (MS).

These animals may represent the closest non-human primate model for the study of MS.

As such, we plan on testing the efficacy of MPV vCCI to mitigate the symptoms

associated with this condition in the Japanese macaque. If successful in the primate

model, one might envision the use of MPV vCCI, as a novel treatment in humans, not

only for MS, but potentially any for other chemokine-driven disease.

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