# Human Cytomegalovirus US28: A Functionally Selective Chemokine Receptor

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## Abbreviations

7-TM: 7-transmembrane Domain

aa: amino acid

AIDS: Acquired Immune Deficiency Syndrome

AngII: Angiotensin II

BAC: Bacterial Artificial Chromosome

bFGF: basic Fibroblast Growth Factor

BSA: Bovine Serum Albumin

BRET: Bioluminescence Resonance Energy Transfer

CCL: Chemokine Ligand

CCMV: Chimpanzee Cytomegalovirus

CCR: Chemokine Receptor

CK2: casein kinase 2

CMV: Cytomegalovirus

CNS: Central Nervous System

Co-IP: Co-immunoprecipitation

CR: Chronic Rejection

CRP: C-reactive Protein

CTL: Cytotoxic Lymphocyte

DB: Dense Bodies

DC: Dendritic Cells

DMEM: Dulbecco's Modified Eagle's Medium

EBV: Epstein-Barr Virus

EC: Endothelial Cells

ECL: Extracellular Loop

ECM: Extracellular Matrix

ER: Endoplasmic Reticulum

ERM: ezrin-radixinmoesin

ERGIC: ER-Golgi Intermediate Compartment

FACS: Fluorescence Activated Cell Sorting

FAK: Focal Adhesion Kinase

FAT: Focal Adhesion Targeting

FCS: Fetal Calf Serum

FERM: erythrocyte band 4.1-ezrin-radixin-moesin

FPLC: Flow Pressure Liquid Chromatography

FRET: Fluorescence Resonance Energy Transfer

GAG: Glycosaminoglycan

GAP: GTPase-activating Protein

GBM: Glioblastoma Multiforme

GFAP: Glial Fibrillary Acidic Protein

GPCR: G-Protein Coupled Receptor

GRK: G-protein Coupled Receptor Kinase

gX: Glycoprotein X (i.e. gB, gH, gL)

HCMV: Human Cytomegalovirus

HDAC: Histone Deacetylase

HHV: Human Herpesvirus

HIV: Human Immunodeficiency Virus

HPV: Human Papilloma Virus

HRP: Horseradish Peroxidase

HSV: Herpes Simplex Virus

IBD: Irritable Bowl Disorder

IBE: Inclusion Body Extract

ICL: Intracellular Loop

IE: Immediate-early

IFN: Interferon

IL: Interlukin

InsP: Inositol Phosphate

KS: Kaposi's Sarcoma

KSHV: Kaposi's Sarcoma Herpes Virus

LDL: Low-Density Lipoprotein

LPA: Lysophosphatidic Acid

MCMV: Murine Cytomegalovirus

mCBP: Minor Capsid Binding Protein

MCP: Major Capsid Protein

mCP: Minor Capsid Protein

MCV: Molluscum Contagiosum Virus

mDC: Monocyte-derived DC

MHC: Major Histocompatibility Complex

MIEP: Major Immediate-Early Promoter

MOI: Multiplicity of Infection

NIEP: Non-infectious Enveloped Particle

NIH: Neointimal Hyperplasia

NK Cells: Natural Killer Cells

NLS: Nuclear Localization Signal

ORF: Open Reading Frame

pp (prefix): phosphoprotein

pAP: Assembly Protien Precursor

PBS: Phosphate Buffered Saline

pDC: Plasmacytoid DC

PDGF: Platelet-Derived Growth Factor

PFA: Paraformaldehyde

PLC: Phospholipase C

PML: Polymorphonuclear Leukocytes

PML-NB: Promyelocytic Leukemia Nuclear Bodies

PORT: Portal Protein

pPR: Protease Precursor

PSG: penicillin-streptomycin-

L-glutamine

PTK: Protein Tyrosine Kinase

PTX: Pertussis Toxin

RA: Rheumatoid Arthritis

RBC: Red Blood Cells

RBD: RhoA Binding Domain

RCMV: Rat Cytomegalovirus

RGS: Regulators of G-protein Signaling

RSMC: Rat Smooth Muscle Cells

ROCK: RhoA Effector Associated Kinase

SCP: Smallest Capsid Protein

SLE: Systemic Lupus Erythematosus

SMC: Smooth Muscle Cells

SS: Sjogren's Syndrome

SV40: Simian Virus 40

TFA: Trifluoroacetic Acid

TLO: Tertiary Lymphoid Organ

TLR-X: Toll-like Receptor (i.e. TLR-1, TLR-2)

TM: Transmembrane Domain

TM: Tropomyosin

Tmod: Tropomodulin

TNF: Tumor Necrosis Factor

TVS: Transplant Vascular Sclerosis

VCAM: Vascular Endothelial Adhesion Molecule

VEGF: Vascular Endothelial Growth Factor

WT: Wild-Type

## Abstract

Human Cytomegalovirus (HCMV) is a ubiquitous human pathogen that is associated with the development of numerous inflammatory diseases including vascular disease in solid organ allografts and certain forms of cancer. HCMV establishes life-long persistent/latent infections via nuanced manipulation of the host immune response. As such, HCMV encodes both chemokines and chemokine receptor homologs and is able to subvert the host chemokine-signaling network in infected cells and tissues. The pathological consequences of CMV chemokine mimicry are only beginning to be understood. In this dissertation, we investigate signaling from the HCMV-encoded chemokine receptor US28 in multiple HCMV susceptible cell types and identify a novel CMV-encoded chemokine. In Chapter 2, we demonstrate that US28 is a functionally selective chemokine receptor. Binding of CC-chemokines is pro-migratory when US28 is expressed in SMC and Fractalkine is an anti-migratory stimulus to SMC. Conversely, Fractalkine stimulus is chemotactic to US28-expressing macrophages but CC-chemokines block US28-mediated migration of macrophages. We determine that US28 functional selectivity occurs at the level of G-protein coupling to the ligand-bound receptor. In Chapter 3, we identify the non-receptor protein tyrosine kinase Pyk2 as a critical cellular mediator of US28 signaling in SMC. We demonstrate that Pyk2 autophosphorylation is required for US28 pro-migratory signaling via activation of the GTPase RhoA. Moreover, we identify US28 signaling to Pyk2 as a potential mechanistic link between HCMV biology and the pathogenesis of malignant glioblastoma. We determine US28-specific Pyk2 binding partners in both SMC and U373 glioma cells. Importantly, Pyk2 binding partners are cell type-specific, providing further evidence that the functional consequences of US28 signaling are highly context-specific. The results presented herein significantly refine and extend our understanding of US28 as a functionally selective chemokine receptor and provide new mechanisms for the

participation of HCMV in the pathogenesis of inflammatory diseases.

In Chapter 4, I identify and characterize the RCMV gene product r129 as a functional CC-chemokine. I map the functional domains of r129 with respect to its chemotactic activity and determine that r129 targets immature CD4+ T cells *in vitro*. Moreover, I determine that r129 is expressed with late kinetics in RCMV infection and that r129 is incorporated into progeny virions. I construct and perform initial characterization of two recombinant RCMV strains containing critical mutations in the r129 ORF. These mutants will be important for future studies of RCMV r129 in the pathogenesis of inflammatory diseases and transplant vascular sclerosis.

# I

# Chapter 1 - Introduction

## 1.1 Overview

The family *herpesviridae* is comprised of large double-stranded DNA viruses with coding capacity for a large array of proteins in addition to virion structural components. Herpesviruses characteristically persist in their natural host and can establish a latent replication cycle in which the virus genome is maintained in the absence of large scale protein synthesis or the production of progeny virus (Roizman 1996). Family *herpesviridae* is broken down into three sub-families designated α- β- and γ-herpesviruses based on both genomic characteristics and distinct biological properties (McGeoch, Dolan et al. 2000). There are a total of eight human herpesviruses (HHV). Herpes Simplex Viruses 1 and 2 (HSV-1 and -2) are α- herpesviruses distinguished by their relatively short and highly lytic replication cycle as well as their preference for establishing latent infection in sensory ganglia. The β-herpesvirus subfamily is made up of the cytomegaloviruses discussed in detail herein. Subfamily γ-*herpesvirinae* includes the human pathogens Epstein-Barr Virus (EBV, HHV-4) and Kaposi's Sarcoma Herpes Virus (KSHV, HHV-8) and is characterized by a restricted host range and a preference for latent infection of T or B lymphocytes.

Human cytomegalovirus (HCMV) is member of the β-herpesvirus subfamily of family *herpesviridae*. The viruses replicate in the nucleus of the infected cell. Spread in tissues is primarily via direct cell-to-cell contact, and in most cases HCMV replication results in lysis of the host cell (Roizman 1996; Mocarski 2007). Members of the β-herpesvirus subfamily are distinguished from other herpesvirus family members based on several characteristics: (1) viruses are highly species specific, (2) they exhibit limited tissue tropism *in vivo*, and (3) infection proceeds slowly in tissue culture. β-herpesviruses display high genetic and evolutionary divergence compared to other herpesviruses and, as such, many members of the subfamily remain unclassified (Mocarski 2007).

### *1.1.1 HCMV Epidemiology*

HCMV infection is endemic in the human population. Seroepidemiologic studies have revealed high rates of infection in every population studied. Due to the serious consequences of congenital HCMV infection, the majority of epidemiologic data is acquired from women of childbearing age (Britt and Alford 1996; Mocarski 2007). Infection with HCMV generally occurs early in life. In the United States 0.2-2.2% of newborns acquire HCMV *in utero* (Gaytant, Steegers et al. 2002) and 30-40% of individuals acquire the virus from breast milk in their first year of life or shortly thereafter from other infected children (Onorato, Morens et al. 1985; Grillner and Strangert 1986). Thereafter, HCMV can be acquired via close contact with infected children or individuals (Pass, Little et al. 1987) or by sexual contact (Handsfield, Chandler et al. 1985). The age of HCMV acquisition varies according to socioeconomic status and economic development, with infections occurring later in life in developed countries and in wealthier populations within those countries (Mocarski 2007). A recent population-based study in the United States demonstrated that women (63.5%) have a slightly higher infection rate than men (54.1%), and that HCMV seroprevalence was higher overall and acquired earlier in life in non-hispanic black and hispanic minority populations (Staras, Dollard et al. 2006).

### *1.1.2 Morbidity and Mortality*

Primary HCMV infection in individuals with normal immune function is generally subclinical but may result in symptoms related to general viremia, including low-grade fever and fatigue infrequently coupled with mild liver damage and transient bone marrow suppression (Cohen and Corey 1985; Britt 2008). Additionally, there is evidence that the chronic inflammation associated with persistent HCMV infection can spur the development of autoimmune diseases, vascular diseases and cancer (discussed in detail in section 1.3).

HCMV infection of the human fetus *in utero* is the most common cause of birth defects in the United States affecting up to 8000 children per year. Congenital HCMV infection causes enlargement of the fetal spleen and liver as well as severe damage to the central nervous system often resulting in deafness, vision loss and cognitive impairment (Alford, Stagno et al. 1990). Central Nervous System (CNS) complications of congenital CMV infection are more frequent and more severe in cases of primary CMV infection during pregnancy than in children of persistently infected mothers (Fowler, Stagno et al. 1992).

In adults, severe CMV disease is primarily associated with alterations in immune function. In the absence of specific antiviral chemotherapy, transplant recipients undergoing immunosuppressive therapy experience significant morbidity and mortality from HCMV infection. These patients can experience reactivation of existing CMV infection or primary infection from the allograft with the latter case resulting in more severe disease even when post-transplant antiviral prophylaxis is used (Bonatti, Tabarelli et al. 2004; Murray and Subramaniam 2004). CMV viremia in transplant patients can result in acute rejection of the transplanted organ, as well as pneumonia and a generalized syndrome associated with CMV replication that negatively affects liver function (Britt 2008). Importantly, there is significant evidence that CMV infection can cause vascular pathologies in transplanted solid organs, ultimately resulting in chronic rejection (CR) of the allograft (discussed in detail in section 1.3.1).

Patients with Acquired Immune Deficiency Syndrome (AIDS) as a result of Human Immunodeficiency Virus (HIV) infection are another population that experiences significant morbidity and mortality as a result of HCMV infection. The incidence of CMV disease correlates with decreases in CD4+ T cell counts. One study of AIDS patients undergoing zidovudine treatment showed that while 21.4% of patients with initial CD4 counts under 100 cells/$mm^3$ showed appreciable CMV-associated disease only 10.3% of patients with

CD4 counts above 100 cells/mm$^3$ were similarly affected (Gallant, Moore et al. 1992). Interestingly, CMV pathology in AIDS patients is markedly dissimilar to that seen in other susceptible populations. In particular, CMV-associated organ disease of the digestive system and retina are unique to AIDS patients and occur only rarely in transplant patients undergoing chemotherapeutic immunosuppression (Britt 2008). In the aforementioned study of CMV disease in AIDS patients, retinitis was the most common CMV-associated pathology in the affected patients while colitis, and esophagitis were less common (Gallant, Moore et al. 1992). Infrequently, AIDS patients experience CMV-associated pathologies of the nervous system as well as pneumonia and hepatitis (Mocarski 2007).

## 1.2 Cytomegalovirus Biology

### *1.2.1 Virion Structure*

Cytomegalovirus particles are comprised of a lipid bilayer containing glycoproteins and G-protein coupled receptors (GPCRs) surrounding a complex layer of protein known as tegument and a 130nm icosahedral nucleocapsid which contains the double-stranded DNA genome (Mocarski 2001) (Figure 1.1A). Detailed analysis of the HCMV virion proteome revealed proteins from 71 HCMV open reading frames ORFs and over 70 host cellular genes represented in the virus particle (Varnum, Streblow et al. 2004) (Table 1.1). In addition to infectious virions and non-infectious enveloped particles (NIEPs) which lack only viral DNA, HCMV-infected fibroblasts produce large numbers of particles known as dense bodies (DB) which differ significantly in both protein content and structure compared to infectious HCMV virions and lack the viral genome as well (Table 1.1, Figure 1.1B). Although the *in vivo* function of DB is unknown, they have been shown to be highly immunogenic suggesting that HCMV may utilize DB to skew the antiviral immune response (Pepperl, Munster et al. 2000).

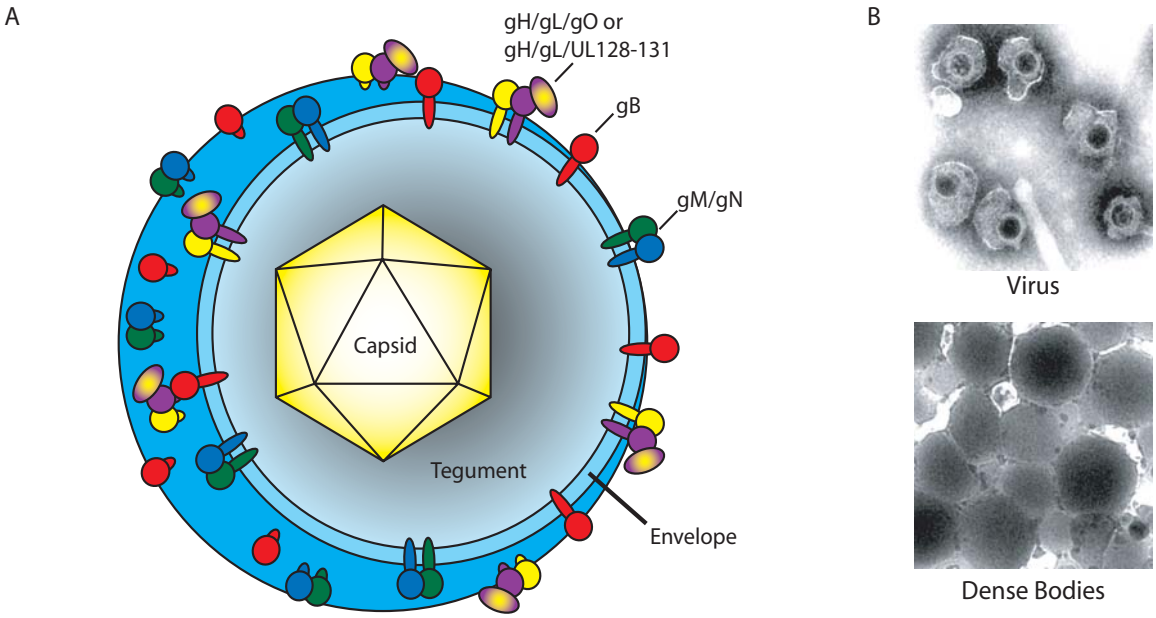



**Figure 1.1 - Structure of the HCMV Virion**
**(A) Illustration of the HCMV virion structure (B) Electron microscopy of negatively stained HCMV virion and dense body preparations. Magnification, x8,400. (Varnum et. al., 2004. used with permission of D. Streblow)**

### i. Viral Envelope

The lipid bilayer enveloping HCMV particles originates from the endoplasmic reticulum (ER) and ER-Golgi intermediate compartment (ERGIC) membranes (Mocarski 2007). Mass spectrometric analysis of the HCMV virion has shown that 19 viral glycoproteins reside in the viral membrane (Varnum, Streblow et al. 2004) (Table 1.1). Three glycoprotein complexes have been characterized as critical mediators of viral entry: (1) glycoprotein B (gB) encoded by UL55, (2) gH:gL encoded by UL75 and UL115, respectively and (3) gM:gN encoded by UL100 and UL73, respectively (Figure 1.1A) (Mocarski 2007). gM is the most prevalent glycoprotein component of the virion comprising 10% of the total protein content, far in excess of its binding partner gN which makes up only 0.1% of the virion protein (Varnum, Streblow et al. 2004). gH:gL complexes have been observed containing gO (pUL74) (Huber and Compton 1998; Theiler and Compton 2001) as well as pUL128/UL130/UL131 (Ryckman, Rainish et al. 2008) and these complexes have been shown to

**A. Virion proteins**

| Viral Protein Group | HCMV ORF | LCQ MS/MS | | FTICR |
|---|---|---|---|---|
| | | # Different Peptides | Max XCorr | # Different Peptides |
| Capsid | UL46 | 20 | 5.30 | 14 |
| | UL48-49 | 8 | 6.52 | 5 |
| | UL80 | 37 | 6.36 | 30 |
| | UL85 | 21 | 6.73 | 22 |
| | UL86 | 149 | 3.97 | 123 |
| Tegument | UL24 | 8 | 5.06 | 9 |
| | UL25 | 60 | 7.04 | 59 |
| | UL26 | 9 | 4.77 | 10 |
| | UL32 | 135 | 3.01 | 100 |
| | UL43 | 7 | 5.50 | 10 |
| | UL47 | 53 | 6.10 | 64 |
| | UL48 | 111 | 4.29 | 109 |
| | UL82 | 70 | 6.39 | 47 |
| | UL83 | 123 | 5.44 | 86 |
| | UL94 | 10 | 5.08 | 12 |
| | UL99 | 8 | 5.87 | 9 |
| | US22 | 2 | 3.16 | 2 |
| | US23 | 1 | 2.61 | 1 |
| | US24 | 1 | 4.83 | 2 |
| Glycoprotein | RL10 | 5 | 2.36 | 4 |
| | TRL14 | * | | 1 |
| | UL5 | * | | 1 |
| | UL22A | 1 | 5.04 | 1 |
| | UL33 | 4 | 6.11 | 4 |
| | UL38 | * | | 1 |
| | UL41A | 2 | 5.72 | 2 |
| | UL50 | 1 | 2.82 | 4 |
| | UL55 | 21 | 6.16 | 23 |
| | UL73 | 2 | 3.47 | 2 |
| | UL74 | 4 | 5.07 | 4 |
| | UL75 | 21 | 6.15 | 22 |
| | UL77 | 14 | 5.65 | 12 |
| | UL93 | 15 | 5.35 | 14 |
| | UL100 | 13 | 5.24 | 7 |
| | UL115 | 11 | 4.73 | 9 |
| | UL119 | 2 | 2.23 | 1 |
| | UL132 | 8 | 5.89 | 8 |
| | US27 | 4 | 4.25 | 2 |
| Transcription / Replication Machinery | IRS1 | 15 | 6.01 | 17 |
| | TRS1 | 10 | 6.92 | 23 |
| | UL44 | 1 | 4.32 | 9 |
| | UL45 | 43 | 5.85 | 52 |
| | UL54 | * | | 1 |
| | UL57 | * | | 1 |
| | UL69 | 6 | 4.17 | 7 |
| | UL72 | * | | 1 |
| | UL84 | 1 | 2.50 | 3 |
| | UL89 | * | | 1 |
| | UL97 | 13 | 5.95 | 9 |
| | UL122 | 2 | 4.26 | 4 |
| Un-characterized | UL35 | 42 | 6.27 | 40 |
| | UL51 | * | | 1 |
| | UL71 | 12 | 6.32 | 11 |
| | UL79 | * | | 1 |
| | UL88 | 14 | 6.8 | 17 |
| | UL96 | 1 | 4.46 | 1 |
| | UL103 | 8 | 5.18 | 8 |
| | UL104 | 9 | 4.68 | 9 |
| | UL112 | 1 | 3.30 | 4 |

**B. Dense body proteins**

| Viral Protein Group | HCMV ORF | LCQ-MS/MS | | FTICR |
|---|---|---|---|---|
| | | # Different Peptides | Max Xcorr | # Different Peptides |
| Capsid | UL46 | 1 | 3.6 | 6 |
| | UL48-49 | 2 | 5.8 | 1 |
| | UL80 | 1 | 6.1 | 2 |
| | UL85 | 4 | 5.0 | 4 |
| | UL86 | 22 | 5.0 | 19 |
| Tegument | UL25 | 17 | 6.3 | 13 |
| | UL26 | 3 | 3.6 | 3 |
| | UL32 | 11 | 5.4 | 15 |
| | UL35 | 5 | 5.6 | 9 |
| | UL47 | 2 | 4.3 | 6 |
| | UL48 | 7 | 5.4 | 12 |
| | UL82 | 9 | 5.1 | 6 |
| | UL83 | 40 | 6.3 | 14 |
| | UL75 | 4 | 5.6 | 2 |
| Transcription/ Replication Machinery | UL45 | 2 | 4.3 | 6 |
| | IRS1 | 3 | 5.6 | 2 |
| | TRS1 | 1 | 4.7 | 5 |

*Indicates proteins whose LC-MS/MS SEQUEST scores were lower than our cutoff values, however the proteins were identified with the more sensitive FTICR.

**Table 1.1 - Proteins Components of HCMV Virions and Dense Bodies**

**Proteomics analysis of the protein components of (A) HCMV Virions and (B) Dense Bodies by tandem mass spectrometry and FTICR analysis (Varnum et. al., 2004, used with permission of DN Streblow)**

mediate specific interactions with different CMV-susceptible cell types (discussed in detail in section 1.2.3.i.). The viral GPCR homologs (UL33, UL78, US27 and US28) have also been detected in the viral envelope but their role in the biology of the virion remains unclear (Mocarski 2007).

**ii. Tegument**

The tegument portion of the CMV virion is an amorphous protein layer between the envelope and the capsid, and is defined as any protein constituent of the virion not directly associated with either the capsid or the envelope (Figure 1.1A). As such, the majority of the virion-incorporated proteins, both viral and cellular, and approximately 40% of the overall mass of the virion are associated with the tegument (Varnum, Streblow et al. 2004; Mocarski 2007). Less than half of the identified tegument proteins have defined roles in CMV biology. Some tegument components are essential for HCMV replication, others contribute to the efficient replication or spread of the virus and still others are dispensable for HCMV replication *in vitro* but may still have undefined functions *in vivo*. Generally, tegument proteins act very early in the infectious cycle to modulate host cellular functions (ppUL69), immunity (pp65, pIRS1, pTRS1) and initiate viral transcription (pp71) prior to the *de novo* production of viral gene products, or very late in infection where they participate in the assembly, maturation and egress (pp150, pp28) of infectious virions. The mechanisms by which HCMV incorporates specific proteins into the viral tegument while excluding others are unknown. The observation that most tegument proteins are phosphorylated has produced speculation that phosphorylation may play a role in the virion incorporation of some tegument proteins. However, the functional significance of phosphorylation in CMV tegument is largely unknown (Kalejta 2008). Interestingly, viral and cellular RNA is incorporated into the virion tegument in quantities proportional to the overall abundance of each RNA in the cell (Terhune, Schroer et al. 2004). The incorporation of RNA into the

tegument implies that proteins could potentially be translated early in infection prior to *de novo* viral transcription, however the functional implications of tegument-associated RNA remain a mystery (Kalejta 2008).

#### iii. Capsid

HCMV genomic DNA is enclosed in an icosahedral nucleocapsid with T=16 symmetry, approximately 130nm in diameter (Figure 1.1A). The capsid shell is comprised of four viral proteins. The 150 triangular faces of the icosahedron are comprised of hexameric complexes of major capsid protein (MCP, encoded by UL86) with a single copy of the smallest capsid protein (SCP, encoded by UL49a) at the tip of each hexon. The icosahedral vertices are pentameric structures of MCP except for a single vertex comprised instead of 12 copies of the portal protein (PORT, encoded by UL104), through which the DNA is inserted into the assembled capsid. Triplexes of minor capsid binding protein (mCBP, pUL46) and minor capsid protein (mCP, pUL85) connect the MCP capsomeres. Assembly of HCMV capsids is scaffolded by the assembly protein precursor (pAP) and the protease precursor (pPR), both encoded by the UL80 open reading frame (Mocarski 2007; Gibson 2008). The assembly scaffold proteins were originally thought to be completely eliminated from mature infectious capsids, however mass spectrometry analysis of infectious HCMV particles revealed large quantities of AP, suggesting that this protein may play a role in the biology of the mature capsid (Varnum, Streblow et al. 2004). .

### *1.2.2 Genome structure*

The HCMV genome is approximately 250,000 bp of double stranded DNA. The genome can isomerize into four forms depending upon recombination between repeat elements flanking the two major domains (long and short) and, as such is classified as a E-type genome within the herpesvirus family. Each domain is comprised of a unique region ($U_L$ and $U_S$) and is

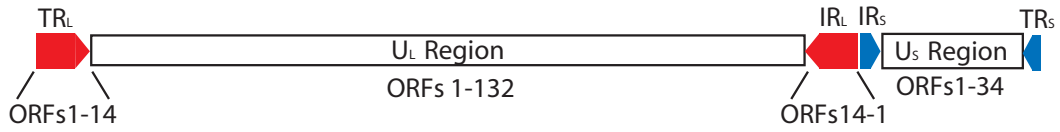



**Figure 1.2 - HCMV Genomic Structure**
**Schematic representation of consensus genome structure of HCMV Laboratory Isolates.**

flanked by terminal and internal repeat regions ($TR_L$, $IR_L$, $IR_S$, $TR_S$) (Figure 1.2) (Murphy and Shenk 2008). HCMV DNA isolated from infectious particles is linear, with single base 3' overhangs at either end of the genome. This 3' overhang facilitates circularization of the genome in the infected cell (Tamashiro and Spector 1986). The protein coding capacity of the HCMV genome has been a subject of much debate. Comparison of multiple HCMV strains revealed 252 potential protein-coding ORFs present within four separate clinical isolates (Murphy, Yu et al. 2003). A more conservative estimate of 164 to 167 protein coding ORFs was made by comparing the sequences of two HCMV laboratory strains to chimpanzee CMV (CCMV) (Davison, Dolan et al. 2003). This estimate is likely less than the actual number of ORFs. Comparison of the HCMV coding capacity to that of CCMV suggests that each of the ORFs identified has a high likelihood of being functional.

### *1.2.3 Life cycle*

#### i. Viral Entry

The glycoprotein complexes of the HCMV viral envelope work in concert to mediate viral attachment and entry into host target cells (Figure 1.3). The gM:gN complex (Mach, Kropff et al. 2000) interacts with heparin sulfate proteoglycans and is thought to mediate the initial, non-specific adsorption of virions onto the cell surface (Compton, Nowlin et al. 1993; Mocarski 2007). The gB homodimer has been implicated in multiple steps of HCMV entry including attachment to heparin sulfate (Compton, Nowlin et al. 1993) and other cell surface receptors (Mocarski 2001) as well as viral membrane fusion (Navarro,

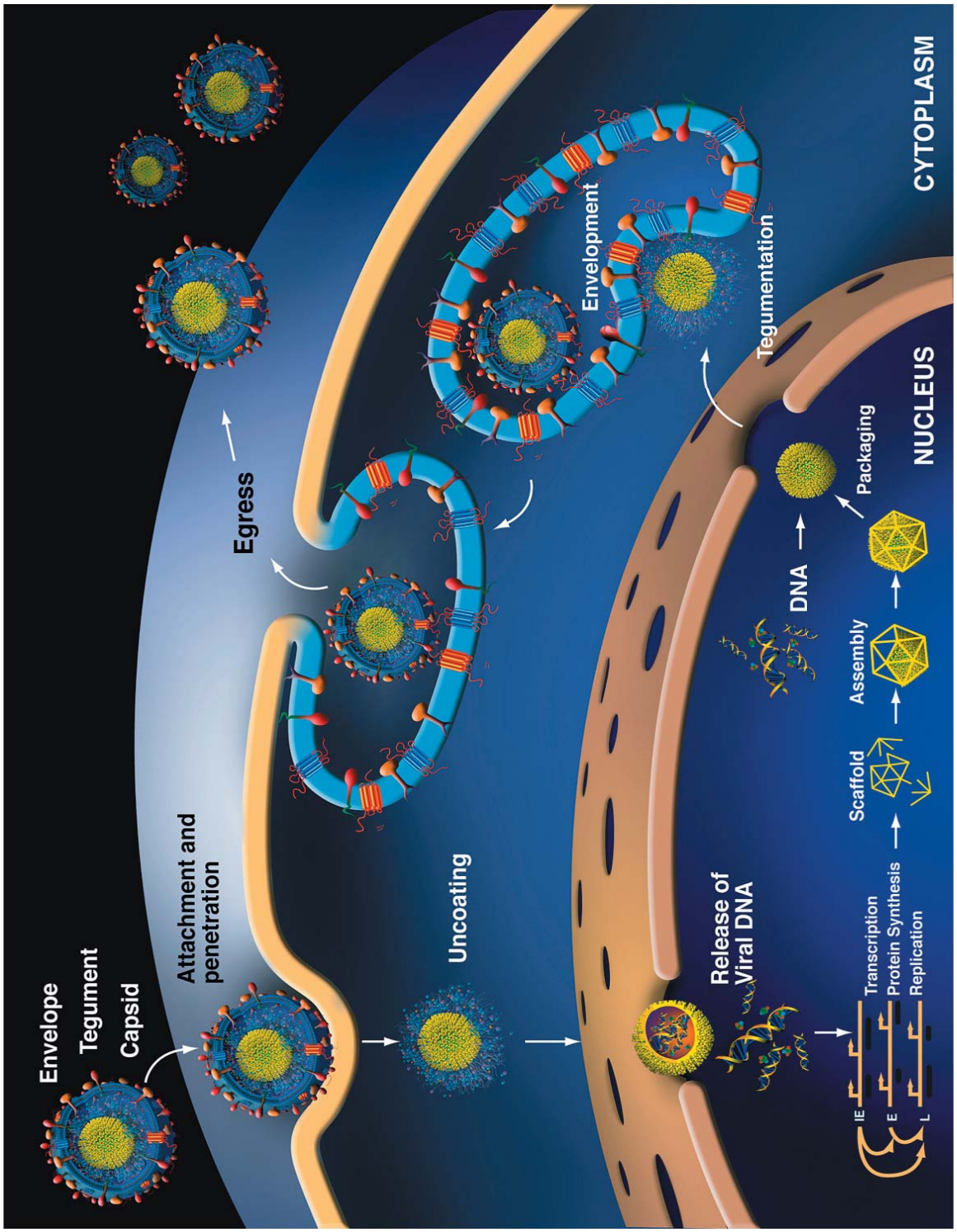
Paz et al. 1993). Soluble forms of gB interact with several cell surface molecules including β1-integrins (Feire, Koss et al. 2004), epidermal growth factor receptor (EGFR) (Wang, Huong et al. 2003), and Toll-like receptor-2 (TLR-2) (Boehme, Guerrero et al. 2006). gH has been demonstrated to interact with both TLR-2 (Boehme, Guerrero et al. 2006) and αvβ3 integrins (Wang, Huang et al. 2005). However, requirements of specific receptor interactions for virus entry remain unclear. The complex of gH:gL is common to all human herpesviruses (HHV) and is essential for virus entry. gL acts as a chaperone for the localization of gH, which is the active fusogenic molecule of the complex (Mocarski 2007). Compelling evidence suggests that the cellular tropism of HCMV is mediated by differential association of gO or UL128, UL130 and UL131 with gH:gL complexes. In studies using the laboratory-adapted HCMV strain AD169, gO can be observed in complex with gH:gL on infected cell membranes and in virion envelopes and this tripartite complex of gH:gL:gO has long been thought to specifically mediate entry into fibroblasts via pH-independent fusion at the cell surface (Huber and Compton 1998; Theiler and Compton 2001; Ryckman, Jarvis et al. 2006). Clinical strains of HCMV incorporate a 5-member complex of gH:gL with UL128, UL130 and UL131 (gH:gL:UL128-131), and this complex is critical for entry into epithelial and endothelial cells via endocytosis and a low pH-mediated fusion process (Wang and Shenk 2005; Ryckman, Jarvis et al. 2006). The accumulation of mutations in UL128-131 accounts for the restricted cellular tropism of AD169 (Wang and Shenk 2005). Ultimately, AD169 has proved to be a poor model for understanding the complex nature gH:gL entry complexes. More recent studies utilizing the HCMV clinical isolate TR reveal that gO is not stably associated with gH:gL but instead transiently associates with gH:gL complexes in the ER in order to facilitate export of gH:gL complexes to the cell surface (Ryckman, Chase et al. 2010). Importantly, an HCMV TR-based strain lacking gO was severely defective in infection of fibroblast strains due to a defect in the incorporation of

**Figure 1.3 - HCMV Life Cycle (opposite)**
**HCMV virions attach via uncharacterized receptor-mediated events and enter cells at the plasma membrane or via endocytosis and low pH-mediated fusion (not pictured). Viral capsids are released into the cytoplasm and transit to the nucleus via microtubules. Virus genomic DNA is released into the nucleus and viral transcription proceeds temporally via intermediate early (IE), early (E) and late (L) genes. Genomic DNA is replicated and packaged into nascent capsids. Mature capsids exit the nucleus via envelopment and de-envelopment at the nuclear membrane (not pictured). In the cytoplasm, capsids acquire tegument proteins and bud into ER or ERGIC compartments to acquire the viral envelope. Egress of particles proceeds via the cellular secretory pathway.**

gH:gL into virions (Wille, Knoche et al. 2010). Furthermore, the association of gO with gH:gL in the ER competes with UL128-131 binding to gH:gL complexes, resulting in a mixture of gH:gL and gH:gL:UL129-131 in the mature virion envelope for clinical strains (Ryckman, Chase et al. 2010).

### ii. Early Events and Transcription Initiation

Following fusion of the viral envelope with cellular membranes, tegument proteins and intact HCMV capsids are found in the cytoplasm of infected cells. Some tegument proteins (i.e. pp71 and pp65) are translocated to the nucleus independently of the nucleocapsid (Hensel, Meyer et al. 1996). Other tegument proteins (pp150, pUL47 and pUL48) remain associated with the capsid and mediate the microtubule-dependent migration of the capsid to the cell nucleus and the subsequent injection of the viral DNA through the nuclear pore complex (NPC) (Bechtel and Shenk 2002; Ogawa-Goto, Tanaka et al. 2003). Viral DNA is rapidly associated with histones and chromatin remodeling proteins upon entry into the nucleus, and this chromatin structure represses viral transcription from the major immediate early promoter (MIEP) via the actions of Daxx histone deacetylase proteins (HDAC) and other PML nuclear body (PML-NB) proteins (Woodhall, Groves et al. 2006; Maul 2008). The tegument protein pp71 (pUL82) interacts with Daxx and mediates its proteasomal degradation thereby relieving the cellular repression of transcription from the MIEP (Saffert and Kalejta 2006). Nascent IE1 (pUL123) proteins further modulate repressive chromatin structures surrounding the MIEP systematically deconstructing PML-


Envelope
Tegument
Capsid
Attachment and penetration
Egress
Uncoating
Release of Viral DNA
Envelopment
Tegumentation
CYTOPLASM
NUCLEUS
DNA
Packaging
Assembly
Scaffold
Transcription
Protein Synthesis
Replication
IE
E
L

NB and allowing viral transcription to proceed (Korioth, Maul et al. 1996; Maul 2008). The major IE2 gene product, IE86 is the master regulator of viral and cellular gene synthesis and is essential for transcription of both early and late genes from the HCMV genome (Marchini, Liu et al. 2001). IE86 mediates the transcription of cellular messages necessary to induce S-phase transition in HCMV infected cells via activation of E2F responsive genes (Song and Stinski 2002) while simultaneously inhibiting cell division via stabilization and upregulation of p53 and p21 tumor suppressor proteins (Song and Stinski 2005). By these mechanisms HCMV acquires a stable pool of the cellular enzymes necessary for viral genome synthesis.

### iii. Genome Replication

Replication of the circularized genome proceeds via a rolling circle replication method originating from the large cis-acting origin of lytic replication (*oriLyt*) (Anders, Kacica et al. 1992). Lytic DNA replication from the *oriLyt* requires cooperative transactivation by both IE86 and pUL84 (Xu, Cei et al. 2004) in addition to the core essential replication components pUL54 (polymerase), pUL44 (polymerase accessory), pUL105 (helicase), pUL70 (primase), pUL102 (primase accessory), pUL57 (ssDNA binding), IRS1 (RNA binding, transactivation), UL112/113 (transactivation) and UL36-38 (anti-apoptotic) (Pari and Anders 1993; Pari, Kacica et al. 1993). Replication of HCMV results in multiple concatenated genomes that are subsequently cleaved into single linear genomic units upon packaging into mature capsids (McVoy and Adler 1994).

### iv. Capsid Assembly and Maturation

The assembly of nascent HCMV capsids begins in the cytoplasm with interactions between newly synthesized structural proteins. pAP, pPR and MCP proteins form protocapsomers and the nuclear localization signals (NLS) present in pAP mediate nuclear translocation

of the capsid precursor complexes (Wood, Baxter et al. 1997). Triplexes of mCP dimers and a single mCBP also assemble in the cytoplasm and are imported into the nucleus via the mCBP NLS (Gibson 2008). Subsequent nuclear assembly of capsid precursors (procapsids) is poorly understood for HCMV. However, the assembly of these unstable structures containing the capsid proteins, portal protein and scaffolding proteins but lacking viral DNA has been studied extensively in the context of HSV (Newcomb, Homa et al. 1999; Newcomb, Trus et al. 2000), and HCMV procapsid assembly is thought to proceed via a similar mechanism.

Maturation of procapsids requires removal of the scaffolding proteins and incorporation of the viral genomic DNA. Following assembly of the immature procapsid, the protease activity of pPR mediates both autoproteolysis and proteolytic degradation of pAP driving maturation of the capsid (Brignole and Gibson 2007). Concurrently, the HCMV terminase proteins (pUL56 and pUL89) and PORT cooperate in the ATP-dependent cleavage and insertion of a single HCMV genome into the maturing capsid (Scheffczik, Savva et al. 2002; Dittmer and Bogner 2005; Dittmer, Drach et al. 2005).

### v. Viral Egress

The nuclear egress of mature capsids and acquisition of tegument proteins and viral envelope is believed to be common among HHV strains, and many viral proteins involved in egress are conserved throughout *herpesviridae*. Egress proceeds via a process of envelopment and de-envelopment of the capsid at the nuclear membrane followed by tegumentation in the cytoplasm and re-envelopment via budding into the ER or ERGIC compartment (Mettenleiter 2002; Sampaio, Cavignac et al. 2005; Gibson 2008). Primary envelopment of HCMV capsids at the inner nuclear leaflet requires a complex interaction between viral and cellular proteins. The nuclear lamin structure is locally disrupted at sites of HCMV envelopment (Buser, Walther et al. 2007). This disruption of the nuclear lamin is mediated

by both the cellular kinase PKC (Milbradt, Auerochs et al. 2007) and the viral kinase pUL97 and is required for HCMV egress (Prichard, Britt et al. 2005; Prichard 2009). Recruitment of these kinases and other cellular and viral proteins necessary for primary envelopment is mediated by interactions of pUL50 and pUL53 (Dal Monte, Pignatelli et al. 2002; Bjerke, Cowan et al. 2003; Milbradt, Auerochs et al. 2007; Camozzi, Pignatelli et al. 2008). De-envelopment then occurs at the outer leaflet of the nuclear membrane releasing naked capsids into the cytoplasm. The exact mechanisms of capsid tegumentation are poorly understood. Tegument proteins are largely acquired in the cytoplasm, however some tegumentation could occur during transit across the nuclear membrane (Gibson 2008). The budding of tegumented capsids through ER/ERGIC membranes requires pp28 (pUL99) (Silva, Yu et al. 2003). Following secondary envelopment, mature virions are released from the cell via normal secretory pathways (Figure 1.3).

### *1.2.4 HCMV Tissue Tropism, Susceptible Cell Types and Pathogenesis*

HCMV productively infects cells in nearly every tissue of the human body. As an obligate intracellular pathogen, this broad tissue tropism determines the spread, maintenance and pathogenesis of HCMV infection. Endothelial cells, epithelial cells, smooth muscle cells and firoblasts are considered the primary targets for HCMV infection *in vivo* (Sinzger, Grefte et al. 1995). HCMV can also infect connective tissue and parenchymal cells in a variety of organs as well as several hematopoietic cell types including tissue macrophages (Sinzger, Digel et al. 2008). Lymphocytes (Sinzger, Grefte et al. 1995) and polymorphonuclear leukocytes (PML) (Grefte, Harmsen et al. 1994; Sinzger, Plachter et al. 1996) are among the few cell types that are not permissive for HCMV replication. In immunocompromized hosts, HCMV replication causes clinical symptoms in retina and brain as well as the gut, lung and liver (Plachter, Sinzger et al. 1996) (see section 1.1.2).

Most natural HCMV infections are thought to occur via infection of epithelial cells in the

mucous membranes or the gastrointestinal tract (Sinzger, Digel et al. 2008). However, infection of dendritic cells (DCs) may also play a role in early infection and dissemination of HCMV. DCs may take up virions at mucosal surfaces and carry the virus to secondary lymphoid organs where infection can spread to other cells. HCMV infected DCs are defective in immune activation (discussed in detail in section 1.2.6), and this dysfunction may play a role in modulating and suppressing the immune response to HCMV infection (Raftery, Schwab et al. 2001). Hematogenous spread of HCMV is generally accepted as the most probable method for dissemination of the virus to multiple organ systems (Britt 2008; Sinzger, Digel et al. 2008). Primary monocytes isolated from peripheral blood can be infected *in vitro* via contact with infected endothelial cells and then be used to transfer infectious virus to secondary endothelial cell cultures, (Waldman, Knight et al. 1995). Importantly, upon infection with HCMV, monocytes are induced to migrate across endothelial cell barriers and differentiate into tissue macrophages where they produce progeny virus (Smith, Bentz et al. 2004). Taken together, these studies provide a compelling picture of the capacity of monocytes to disseminate HCMV *in vivo*.

The ability of cultured fibroblasts to produce high titers of HCMV *in vitro* suggests that the infection of connective tissue *in vivo* may contribute to proliferation during acute infection and reactivation. Productive infection of smooth muscle cells (SMC), endothelial cells (EC) and organ parenchymal cells (i.e hepatocytes) *in vivo* may also contribute to viremia and viral maintenance during persistent infection (Sinzger, Digel et al. 2008). Lytic replication of HCMV in these cell types is the cause of organ dysfunction and cellular damage in HCMV infection as well as the inflammatory processes that have been implicated in HCMV-associated chronic disease (discussed in detail in section 1.3) (Britt 2008).

HCMV transmission primarily occurs via contact with virus shed in body fluids. Infection of epithelial cells in the salivary gland, mammary gland, kidney and intestinal mucosa can

be detected in late stages of acute productive HCMV infection and is primarily responsible for the presence of virus in saliva, breast milk, urine and stool, respectively.

### *1.2.5 Latency and Persistence*

HCMV is able to establish a life-long infection in the host, however the mechanisms of HCMV persistence are poorly understood. For most other human herpesviruses, long-term carriage of viral genomes in the host is connected to the ability of the virus to establish latent infection in certain cell types. Latency is defined as the persistence and maintenance of the viral genome in the absence of significant viral transcription and production of progeny virions. Identification of a truly latent reservoir of HCMV genomes *in vivo* has been difficult (Reeves and Sinclair 2008). A significant body of data suggests that bone marrow resident CD34+ hematopoietic progenitor cells committed to the myeloid lineage can be latently infected with HCMV *in vivo* (Mendelson, Monard et al. 1996; Sindre, Tjonnfjord et al. 1996; Sinclair and Sissons 2006), and that viral reactivation in these cells is driven by differentiation of the infected progenitors into tissue macrophages or DC (Hahn, Jores et al. 1998; Reeves, Lehner et al. 2005). The molecular mechanism of latency and reactivation in these cell types remain poorly understood, but appears to be at least partially controlled by epigenetic silencing of the MIEP via the establishment and maintenance of higher-order chromatin structures in a differentiation-dependent manner (Murphy, Fischle et al. 2002; Reeves, Lehner et al. 2005; Reeves, MacAry et al. 2005). Transcriptional analysis of CMV infection via microarray studies has revealed that transcription from the CMV genome is highly regulated by the cell type infected. In HCMV infected hematopoietic progenitors, an initial burst of viral gene transcription is followed by a period of quiescence, and co-culture of quiescent cells with permissive fibroblasts results in reactivation of viral transcription (Goodrum, Jordan et al. 2002). Additionally, different transcriptional profiles are seen depending upon the cell type and differentiation of the progenitor cells infected (Goodrum,

Jordan et al. 2004). Further evidence for tissue-specific transcription of CMV genes was established in our rat model using RCMV infection. When transcription from the RCMV genome was analyzed in a variety of tissue culture cells approximately 80% of the viral gene products were transcribed by 16 hours post-infection and transcription profile was very similar between cell types. However, when the same microarray analysis was performed on cDNA from infected rat tissues the transcription profiles were highly restricted and varied significantly between tissue types (Streblow, van Cleef et al. 2007). Taken together, these results suggest that CMV gene expression is tightly regulated and highly tissue-specific *in vivo* and this regulation is achieved via unknown factors that are not present in tissue culture. Tissue specific transcriptional programs undoubtedly have significant implications for mechanisms of CMV persistence and latency and warrant further study.

A latent origin of replication for HCMV has yet to be identified, raising significant questions regarding the maintenance of HCMV genomes in latently infected cells. HCMV genomes may not replicate in CD34+ progenitor cells. Progenitors may acquire the virus from persistently infected bone marrow stromal cells and the viral genome could be maintained in an inactive state until the progenitors have migrated to peripheral sites and differentiated into macrophages and dendritic cells. This hypothesis remains to be verified experimentally, but would argue against the establishment of true latency in HCMV infection (Reeves and Sinclair 2008).

### *1.2.6 CMV and the Immune System*

The ability of CMV to establish a life-long persistent/latent infection in its natural host requires a nuanced relationship with the host immune system. Indeed, a significant component of the cytomegalovirus genome encodes immunomodulatory factors (Loewendorf and Benedict 2010). Importantly, an estimated 3% of the mouse genome is specifically evolved to resist murine cytomegalovirus (MCMV) infection (Beutler, Georgel

et al. 2005). Taken together, the evidence suggests that the persistence of CMV infection requires a delicate and highly evolved balance between CMV immune evasion and host immune surveillance of CMV infection.

Initial immune responses to CMV infection rely heavily on the actions of type I interferons and natural killer (NK) T cells (Biron, Byron et al. 1989; DeFilippis 2007). CMV employs several known mechanisms to evade early immune detection. The HCMV immediate early-2 (IE2) gene product IE86 specifically blocks the transcription of IFN-β, and several other pro-inflammatory cytokines (Taylor and Bresnahan 2006) by interfering with the ability of NFκB to bind to promoters and induce transcription of these genes (Taylor and Bresnahan 2006). Later in infection, HCMV inhibits the host cell ability to respond to TNF-α and IL1β pro-inflammatory signals by blocking receptor-proximal signaling events (Montag, Wagner et al. 2006).

Although DCs are variably permissive for CMV infection depending upon the DC subset studied and the CMV strain applied (Riegler, Hebart et al. 2000; Varani, Cederarv et al. 2007; Schneider, Meyer-Koenig et al. 2008), the effects of CMV infection on DC function are varied and profound. Monocyte-derived DC (mDC) are permissive for lytic CMV infection (Riegler, Hebart et al. 2000) and CMV infection inhibits mDC maturation, MHC class I (MHC-I) expression and CD8 T cell priming (Moutaftsi, Mehl et al. 2002; Beck, Meyer-Konig et al. 2003). In contrast, plasmacytoid DCs (pDC) undergo partial maturation upon CMV infection (Varani, Cederarv et al. 2007). CMV-infected pDCs secrete inflammatory cytokines and induce B cell proliferation but are defective in T cell priming (Varani, Cederarv et al. 2007; Schneider, Meyer-Koenig et al. 2008). In a RCMV model of bone marrow-derived DC infection, MHC Class II is significantly downregulated in virus-infected cells via lysosomal degradation of MHC II molecules providing a secondary mechanism for CMV to evade recognition by CD4 T cells (Baca Jones, Kreklywich et al.

2009).

NK cell activation is mediated by inflammatory cytokines as well as direct interaction with DCs (Borg, Jalil et al. 2004; Ferlazzo, Pack et al. 2004; Gerosa, Gobbi et al. 2005). Significantly, CMV-infected pDC are able to cause NK cell migration and stimulate the production of TNFα and IFNγ, however NK cell cytotoxicity is impaired (Cederarv, Soderberg-Naucler et al. 2009). Additionally, HCMV encodes six separate genes (UL16, UL18, UL40, UL83, UL141, and UL142) and a microRNA (miR-UL112) that modulate the expression of NK cell activating ligands, significantly modulating the ability of NK cells to recognize and induce apoptosis in CMV-infected cells (Wilkinson, Tomasec et al. 2008).

The maintenance and expansion of T cell memory in CMV infection has been the subject of intense study (Loewendorf and Benedict 2010). However, few studies have effectively examined the generation of an adaptive immune response to primary CMV infection. What data exists suggests that a large, diverse CD8+ cytotoxic T cell repertoire is established shortly after infection and that these CD8+ effectors are rapidly selected and focused into memory subsets. Notably, there is an unusual prevalence of CD8+, CD45RA+, CD27+/-, CD28- cells (TEMRA cells) in the early stages of CMV memory selection, the implications of which are poorly understood (Waller, Day et al. 2008). In HCMV seropositive adults nearly 10% of the circulating CD4+ and CD8+ memory T cells are specific for HCMV antigens, underscoring the large immunological burden of persistent HCMV infection (Sylwester, Mitchell et al. 2005). The expansion of CMV-specific memory cells continues as infection persists such that in some elderly individuals up to 25% of their cytotoxic T cell (CTL) repertoire is specific for HCMV (Khan, Shariff et al. 2002). This oligoclonal expansion of CMV-specific CTL is thought to contribute to immune dysfunction seen in the elderly (Looney, Falsey et al. 1999; Khan, Shariff et al. 2002).

## 1.3 Cytomegalovirus Pathogenesis Associated with Chronic Inflammation

Although the persistence of HCMV infection is generally asymptomatic in immunocompetent individuals, long-term persistent HCMV replication results in chronic inflammation. These inflammatory processes are thought to contribute to the progression of inflammatory diseases including autoimmune disorders, vascular disease (discussed in detail in section 1.3.1) and some cancers (Soderberg-Naucler 2006). In particular, HCMV has been implicated in the exacerbation of autoimmune disorders such as rheumatoid arthritis (RA) (Hamerman, Gresser et al. 1982; Murayama, Tsuchiya et al. 1994), Sjögren's Syndrome (SS) (Thorn, Oxholm et al. 1988), systemic lupus erythematosus (SLE) (Hayashi, Lee et al. 1998; Nawata, Seta et al. 2001), psoriasis (Asadullah, Prosch et al. 1999), and irritable bowl disorders (IBD) including Crohn's disease and ucerative colitis (Rahbar, Bostrom et al. 2003). Congenital HCMV infection has been linked to epilepsy in infants and children (Suzuki, Toribe et al. 2008) and anti-CMV chemotherapy as been efficacious in preventing seizures in affected children (Dunin-Wasowicz, Kasprzyk-Obara et al. 2007). Additionally, HCMV infection has been linked to several types of cancer including colon cancer (Harkins, Volk et al. 2002), prostate cancer (Samanta, Harkins et al. 2003), EBV-negative Hogkins Lymphoma (Huang, Yan et al. 2002), and glioblastoma multiforme (GBM) (discussed in detail in section 1.3.2). The evidence that CMV infection is associated with these diseases is quite clear from the literature, however debate still remains as to the significance of these findings. In some cases, an established mechanism of HCMV host-cell manipulation can be linked to the molecular pathogenesis of a disease associated with the virus. For example, HCMV infected cells secrete a wide array of soluble factors (known as the HCMV secretome) which includes pro-inflammatory molecules known to play a role in the progression of autoimmune diseases and vascular disease as well as angiogenic factors that

could promote the growth of blood vessels into tumor microenvironments where HCMV infected cells are also present (Dumortier, Streblow et al. 2008). HCMV infection could also be an epiphenomenon in these disorders such that the disease process promotes an environment ideal for HCMV replication rather than the converse. However, as mechanisms of HCMV host-cell modulation continue to be discovered, the body of evidence suggests that HCMV infection can exacerbate inflammatory conditions and drive the pathogenesis of cancer, vascular disease and autoimmune disorders. The data presented herein specifically focuses on the role of the HCMV-encoded chemokine receptor US28 in the pathogenesis of vascular disease (Chapters 2, 3&4) and also presents evidence that US28 signaling activates a pathway known to participate in GBM tumor formation (Chapter 4).

### *1.3.1 Vascular Disease and Chronic Rejection of Solid Organ Allografts*

#### i. Formation of Atherosclerotic Plaques

Atherosclerosis is a progressive disease process primarily driven by chronic inflammation. Atherosclerotic plaques are characterized morphologically by a core of necrotic, lipid-rich cellular debris and a fibrotic cap comprised of SMC and extracellular matrix (ECM) components. Advanced atherosclerotic lesions can be partially vascularized and undergo varying degrees of calcification, contributing to plaque instability. Clinical manifestations of atherosclerosis are usually due to plaques growing sufficiently large to occlude blood flow or partial rupture of the plaque resulting in thrombotic vessel occlusion (Lusis 2000).

Atherosclerotic lesions are initiated by an insult to the vascular endothelium resulting in a pro-inflammatory response. These insults may take many forms including the infiltration of low-density lipoproteins (LDL) into the vessel wall (Watson, Leitinger et al. 1997), vascular wounds or by an infectious process. The activated endothelium expresses a variety of pro-inflammatory adhesion molecules and cytokines resulting in the recruitment of monocytes

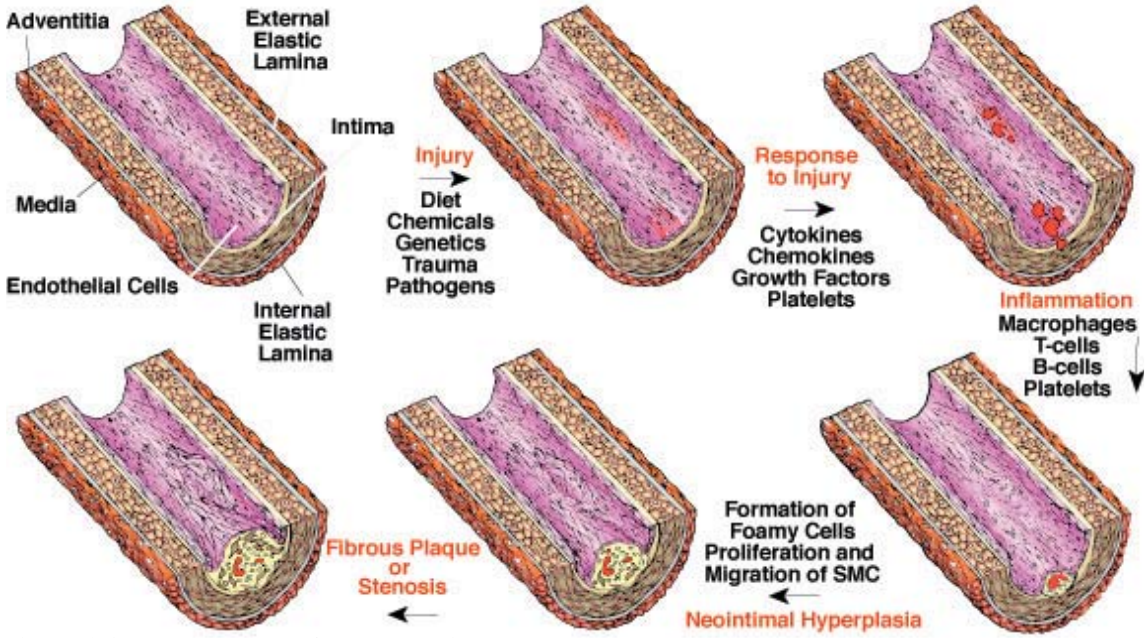



**Figure 1.4 - Development of Vascular Disease**

**The pathogenic process is initiated by an injury to the vascular endothelium which can be due to a variety of factors. The immune response to the injury causes the accumultaion of soluble inflammatory mediators which recruit immune cells which infiltrate the vessel wall and reinforce the pro-inflammatory environment. Recruited macrophages absorb excess LDL in the vessel wall and become foamy cells. SMC migrate from the vessel periphery into the intima where they proliferate and secrete ECM components contributing to fibrosis of the developing plaque and vessel stenosis. (Streblow et. al., 2001, reprinted via limited license from the Journal of Nutrition, see Appendix 2)**

and T cells into the vessel wall. The recruitment of leukocytes initiates a positive feedback loop, further amplifying the inflammatory process. Excess LDL in the vessel wall is modified into highly-oxidized LDL species by a variety of reactive oxygen species and lipase enzymes. The recruited monocytes are induced to differentiate into macrophages, which take up highly-oxidized LDL and become lipid-rich foam cells. Vascular SMC are stimulated to migrate into the vessel lumen via the action of pro-inflammatory cytokines and growth factors. They proliferate in the plaque forming the SMC-rich fibrous cap characteristic of advanced atherosclerotic lesions. The formation of the atherosclerotic plaque continues as the mislocalized SMC produce ECM components resulting in fibrosis of the vessel wall (Lusis 2000) (Figure 1.4).

### ii. Participation of Inflammatory Mediators in Atherosclerosis

Atherogenesis is a multifactoral process involving a complex interplay of chemokines, cytokines, growth factors and their responding cells (Lusis 2000; Libby 2002). Recent advances in mouse genetics have enabled researchers to dissect risk factors and critical mediators in mouse models of vascular disease (Smithies and Maeda 1995). In the initial stages of plaque formation, adhesion of monocyte/macrophages and T cells to activated EC is mediated by vascular endothelial adhesion molecule-1 (VCAM-1) (Cybulsky, Iiyama et al. 2001) as well as P- and E- selectin (Dong, Chapman et al. 1998). Monocyte chemotaxis into the vessel wall is driven by the chemokine MCP-1 (CCL2) interactions with CCR2 (Boring, Gosling et al. 1998; Gu, Okada et al. 1998). Additionally, the membrane-anchored chemokine $CX_3CL1$ (Fractalkine) also plays a role in atherosclerosis by acting as an adhesion molecule and a chemotactic signal to macrophages during atherogenesis (Combadiere, Potteaux et al. 2003; Lesnik, Haskell et al. 2003). Following recruitment to inflammatory sites in the vessel wall, maturation of monocyte/macrophages inside the vessel wall is mediated by M-CSF (Smith, Trogan et al. 1995). Mature macrophages and T cells secrete growth factors and inflammatory mediators including cytokines such as interferon-ɣ (IFN-γ) and chemokines. Chemokines mediate the migration of SMC into the atherosclerotic plaque where growth factors and cytokines initiate SMC proliferation and secretion of ECM components, driving fibrotic development of the plaque. The transition of macrophages to foam cells is mediated by a variety of factors including IFN-γ. The development of the lipid-rich necrotic core of the atherosclerotic plaque is ultimately a result of apoptotic cell death of foam cells (Lusis 2000).

### iii. Association of HCMV Infection and Atherosclerosis

HCMV infection has been linked to the development of atherosclerosis, arterial restenosis following angioplasty, and solid organ TVS (Melnick, Petrie et al. 1983; Speir, Modali et

al. 1994; Melnick, Adam et al. 1998). Epidemiological data suggests HCMV infection can increase an individual's risk for the development of atherosclerosis particularly when combined with other genetic and environmental risk factors. In particular, a study of patients with diagnosed coronary artery disease showed that CMV seropositive patients had significantly increased mortality, and that this effect was more pronounced in patients with both HCMV infection and increased levels of the inflammatory marker C-reactive protein (CRP) (Muhlestein, Horne et al. 2000). Importantly, analysis of human atherosclerotic lesions reveals the presence of HCMV DNA and antigens in a high proportion of diseased vessels (Melnick, Petrie et al. 1983; Hendrix, Dormans et al. 1989; Hendrix, Salimans et al. 1990; Pampou, Gnedoy et al. 2000). Detection of CMV DNA and protein is histologically localized to SMC and EC both in the vessel wall of both normal and lesional vessels as well as SMC localized in the vessel intima in early stage lesions (Pampou, Gnedoy et al. 2000).

Animal models of vascular pathologies provide further evidence that CMV can accelerate the process of atherosclerosis. ApoE -/- mice, which spontaneously develop atherosclerosis due to high levels of LDL, are particularly valuable in analyzing the contribution of pathogens to disease development and plaque formation (Nakashima, Plump et al. 1994). MCMV infection accelerates the development of atherosclerosis in ApoE -/- mice, even in the absence of excess fat in the diet (Burnett, Gaydos et al. 2001; Streblow, Orloff et al. 2001). The contribution of MCMV infection to atherogenesis is thought to be due to an induction of both local and systemic pro-inflammatory mediators including TNF$\alpha$ and IFN$\gamma$ as well as increased recruitment of T cells to atherosclerotic lesions (Vliegen, Stassen et al. 2002; Vliegen, Duijvestijn et al. 2004).

### iv. Development of Transplant Vascular Sclerosis

Although significant advances have been made in solid organ transplantation techniques,

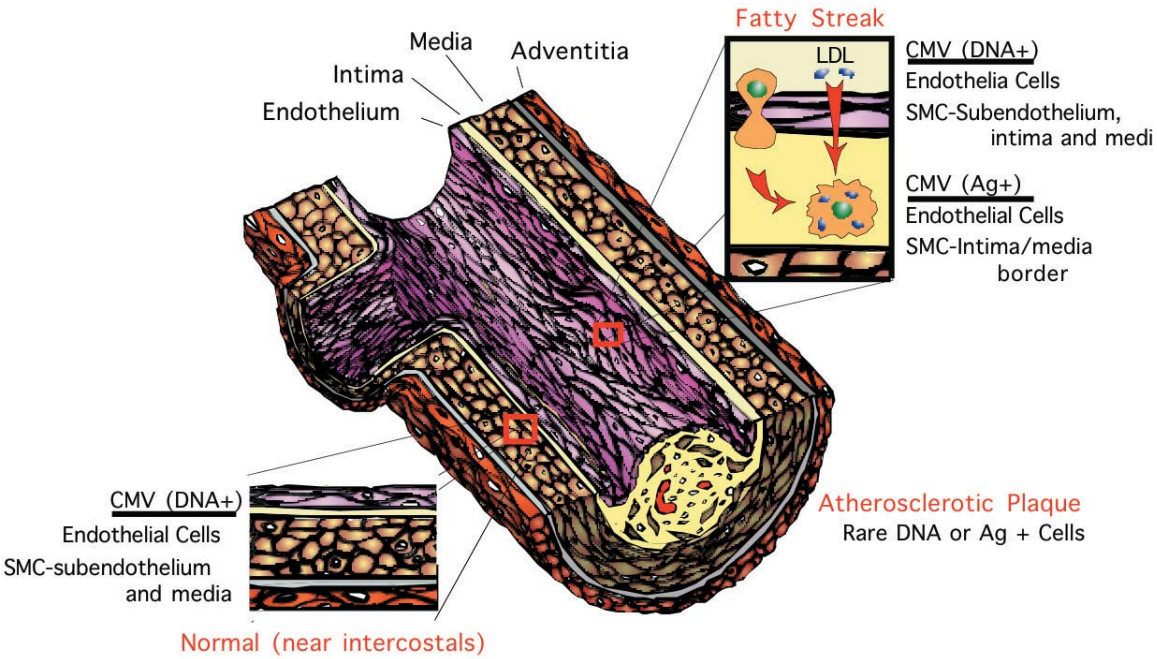



**Figure 1.5 - Detection of HCMV DNA in Atherosclerotic Vessels**
**HCMV DNA has been detected in pre-atherosclerotic fatty streaks as well as endothelium and SMC in both normal vessels those undergoing the early stages of atherogenesis. HCMV DNA + cells are rarely detected in advanced atherosclerotic lesions (Streblow et. al., 2001, reprinted via limited license from the Journal of Nutrition, see Appendix 2)**

chronic rejection (CR) remains a significant source of morbidity and mortality in transplant patients. CR pathology is characterized by an accelerated form of arteriosclerosis referred to as transplant vascular sclerosis (TVS). Approximately 10-20% of solid organ allograft recipients per year experience TVS, and at 5 years post-transplantation TVS prevalence reaches 60% of surviving allograft recipients (Hosenpud, Shipley et al. 1992; Miller 1992). There are several risk factors for CR: donor age, acute rejection, HLA mismatches, hypertension, hypercholesterolemia and HCMV infection. However, the mechanisms linking viruses to vascular disease are unclear (Eich, Thompson et al. 1991; Almond, Matas et al. 1993; Isoniemi, Nurminen et al. 1994; Kobashigawa and Kasiske 1997). The only effective treatment for CR is retransplantation and, therefore, effective treatments to prevent the development of TVS are urgently needed.

Early TVS lesions show the accumulation of macrophages, T Cells, NK cells and B

cells in the vessel intima. TVS pathology progresses to a diffuse concentric intimal proliferation primarily consisting of SMC and macrophages that ultimately occludes the vessel resulting in graft failure (Billingham 1992; Cramer, Wu et al. 1992). Although the pathogenic mechanism of TVS is unknown, several lines of evidence suggest that the disease is primarily immune-mediated: (1) TVS is specific to allograft vessels, (2) low-grade inflammatory processes have been characterized in the sub-endothelial layer of TVS vessels, (3) the lesion distribution is diffuse and concentric (Libby, Salomon et al. 1989; Billingham 1992; Hosenpud, Shipley et al. 1992).

### v. Role of HCMV in the Development of TVS

HCMV infection doubles the rate of liver graft failure at 3 years post-transplantation (Deotero, Gavalda et al. 1998; Rubin 1999) and nearly doubles rejection of cardiac allografts at 5 years post-transplantation (Grattan, Moreno-Cabral et al. 1989). Graft failure in both instances is linked to TVS pathology. Significantly, CMV DNA is frequently detected in the coronary arteries of heart transplant patients with accelerated atherosclerotic pathology, and the hybridization of CMV DNA in diseased vessels suggests the presence of infected SMC, macrophages and endothelial cells (Wu, Hruban et al. 1992) (Figure 1.5).

Importantly, studies using the CMV chemotherapeutic agent gancyclovir in transplant patients provide significant evidence that CMV replication has an effect on the development of TVS. In one controlled study, heart transplant recipients who were either CMV positive at the time of transplant or were receiving CMV positive organs were treated with gancyclovir for 28 days post-transplant and followed for an additional 120 days to determine the onset of CMV disease. Gancyclovir treatment significantly decreased the incidence of primary CMV disease in these patients immediately following transplant (Merigan, Renlund et al. 1992). Significantly, the same patients were followed for 4-6 years post-transplant and the gancyclovir-treated group had a reduced incidence of TVS over this time period compared

to patients who did not receive anti-CMV chemotherapy (Valantine, Gao et al. 1999).

Determining the mechanisms involved in HCMV-associated TVS has been difficult because the high prevalence of HCMV results in a lack of negative controls. Furthermore, the multifactoral etiology of TVS, coupled with the lifelong nature of HCMV infection confounds efforts to establish a clear mechanistic link between virus infection and TVS. Therefore, much of the data characterizing the association between CMV infection and TVS have utilized animal models of solid organ transplantation.

In several rat models of transplantation, rat cytomegalovirus (RCMV) accelerates graft failure via a TVS process with similar histology to that seen in human samples (Lemstrom, Bruning et al. 1993; Bruning, Persoons et al. 1994; Lemstrom, Koskinen et al. 1995; Orloff 1999; Orloff, Streblow et al. 2002). In our rat model of heart transplantation, acute infection with RCMV increases the severity of TVS in graft vessels and dramatically decreases the mean time to develop TVS and graft failure (Orloff, Yin et al. 1999; Orloff, Yin et al. 2000). Importantly, the effects of CMV on TVS acceleration are not organ specific but occur in a broad range of solid organ transplants including heart, kidney, lung, and small bowel (Orloff 1999; Orloff, Yin et al. 1999; Orloff, Yin et al. 2000; Soule, Streblow et al. 2006). These observations verify the link between CMV and TVS, and establish this as a model system to study the viral mechanisms involved in the acceleration of CR.

Our rat model of RCMV-accelerated TVS has provided important mechanistic information into the development of vascular pathologies in solid organ transplants. Syngeneic transplants and transplants in which the recipient has been tolerized to the graft organ via bone marrow transplantation do not develop TVS. These results suggest that the progression of TVS requires an interplay between CMV replication and the alloreactive response of the recipient's immune system to the host organ (Orloff 1999; Orloff, Streblow et al. 2002). Additionally, in RCMV-infected recipients of heart transplants, a dramatic

increase in expression of chemokines is temporally associated with the increased infiltration of T cells and macrophages in the vessels of the allograft compared to uninfected allograft recipients (Streblow, Kreklywich et al. 2003). A similar association of RCMV infection with increased chemokine expression and chronic allograft nephropathy was observed in a rat model of kidney transplantation (Soule, Streblow et al. 2006).

Several CMV genes have been implicated in the development of vascular disease, including IE and early genes. These genes are attractive candidates for the acceleration of vascular pathologies since latently infected cells can exhibit partial viral gene expression without producing virus. Viral genes such as GPCRs that may be expressed in nonproductively infected cells are potential mediators of long-term diseases including vascular disease (Speir, Modali et al. 1994). The RCMV-encoded chemokine receptor R33 is necessary for RCMV-mediated migration of SMC *in vitro*. Significantly, infection of rat heart transplant recipients with RCMV lacking R33 resulted in increased graft survival and decreased TVS disease compared to wild-type RCMV-infected transplant recipients (Streblow, Kreklywich et al. 2005). These data provide strong evidence that cellular chemotaxis resulting from CMV-encoded chemokine receptors plays a role in the inflammatory processes that lead to TVS. Similarly, the HCMV-encoded chemokine receptor US28 is necessary and sufficient for HCMV-mediated SMC migration *in vitro* (Streblow, Söderberg-Nauclér et al. 1999). Therefore, US28-expression is one mechanism by which CMV could control the migration of cellular infiltrates into atherosclerotic plaques (see Chapters 2 and 3 for detailed discussion and data).

### *1.3.2 CMV and Cancer*

Although HCMV DNA, gene products and antigens have been detected in a variety of tumor tissues (see section 1.3 for primary references), a consensus has not been reached as to the role of HCMV biology in the origin or development of malignant neoplasm.

Other DNA viruses including EBV (Henle, Diehl et al. 1967; Pope, Achong et al. 1968), human papilloma virus (HPV) (Schlegel, Phelps et al. 1988; Storey, Pim et al. 1988), and Simian Virus 40 (SV40) (Vilchez, Kozinetz et al. 2003; Ahuja, Saenz-Robles et al. 2005) are directly linked to the transformation of normal cells in tissue culture. However, no such data exists for HCMV. Furthermore, the high prevalence of HCMV infection in the human population confounds epidemiological studies. The hypothesis most consistent with the current evidence is that HCMV infection is oncomodulatory rather than directly oncogenic. This oncomodulation hypothesis suggests a symbiotic feedback between the transformed cell and HCMV biology in which the profound cellular changes that take place in transformed cells produce an environment ideal for the robust replication of HCMV. HCMV gene products further modulate the tumor cell to increase survival, limit immune surveillance and ultimately modify the cellular microenvironment to promote the formation and invasion of tumors (Cinatl, Scholz et al. 2004).

Glioblastoma multiforme (GBM; also called WHO grade IV astrocytoma) is a malignant brain tumor of primarily astrocytic origin (Louis, Ohgaki et al. 2007). These tumors are highly aggressive and lack effective treatments, resulting in 97% mortality within 5 years of diagnosis (Miller 2009). There has been significant controversy surrounding the association of HCMV with GBM tumors in the last decade. The first report of GBM tumor tissue containing HCMV proteins and mRNA was published by Cobbs et. al. in 2002 (Cobbs, Harkins et al. 2002) but was not independently confirmed until 2008 (Mitchell, Xie et al. 2008; Scheurer, Bondy et al. 2008). Although it is now widely accepted that a large number of GBM tumors are HCMV positive, current data are not sufficient to determine whether the HCMV infection of glioma cells is etiological, exacerbatory or a non-specific epiphenomenon in the development of GBM tumors. To date, several studies have demonstrated compelling mechanistic connections between HCMV biology and

GBM pathogenesis.

The increased pathological grade in astrocytomas correlates with a decrease in expression of glial fibrillary acidic protein (GFAP), and several studies have shown that GFAP deficient glial cells have greater proliferative and invasive potential than astrocytes with normal GFAP expression (Rutka, Hubbard et al. 1994; Toda, Miura et al. 1999). HCMV infection or expression of the IE1 protein can decrease the expression of GFAP in the U373MG glioma cell line (Lee, Jeon et al. 2005; Koh, Lee et al. 2009). Furthermore, using the same model cell line Lee et. al. discovered that IE1 expression downregulated the p53 tumor suppressor and thrombospondin-1 (Lee, Jeon et al. 2005), both of which participate in the vascularization of glial tumors (Harada, Nakagawa et al. 2003; Gaiser, Becker et al. 2009). Another study examining the effect of IE1 expression in glioma cell lines revealed differential phenotypes for cellular growth and activation depending upon the cell line examined (Cobbs, Soroceanu et al. 2008). Although these results confirm that IE1 can modulate the tumor cell biology, the data also suggest that the background of genomic mutations present in the tumor can significantly alter secondary effects of HCMV on the tumor. HCMV IE proteins are implicated in the activation of telomerase activity in both normal fibroblasts and malignant glioma cell lines, providing another link between HCMV and tumorigenesis (Straat, Liu et al. 2009).

The HCMV-encoded chemokine receptor US28 is a potential mediator of HCMV oncomodulation. US28 expression has been linked to malignant transformation of NIH-3T3 cells and is implicated in glioma vascularization via transactivation of vascular endothelial growth factor (VEGF) in HCMV infected U373 cells (Maussang, Verzijl et al. 2006). Another recent study showed that HCMV infection specifically modulates cellular migration of glioma cells via activation of focal adhesion kinase (FAK) (Cobbs, Soroceanu et al. 2007). We have previously demonstrated that US28 signaling activates FAK in

fibroblasts and SMC and that this activation is required for US28-mediated migration of SMC (Streblow, Vomaske et al. 2003). In this volume, I further demonstrate that US28 signals to Pyk2, a critical mediator of glioma cell invasiveness (Chapter 3).

Taken together, the current data provide compelling suggestions that HCMV can participate in the proliferation, migration and invasion of glioma tumor cells. Whether antiviral treatments may be efficacious in extending the life of GBM patients remains to be determined. Certainly, further study is warranted in order to characterize the complex interactions of HCMV biology and GBM pathogenesis.

## 1.4 The Chemokine Signaling Network

Chemokines and their receptors participate in a wide variety of essential biological processes including development, inflammation and immunity via initiating directed cellular migration (Baggiolini 1998; Moser and Willimann 2004). Dysregulation of the chemokine signaling network is implicated in the pathogenesis of inflammatory diseases and the development of cancers (Gerard and Rollins 2001; O'Hayre, Salanga et al. 2008). There are 20 known chemokine receptors (CCRs) and 50 chemokines expressed in humans (Salanga, O'Hayre et al. 2009). Depending upon their expression profiles and funtions, chemokines can be subdivided into inflammatory (induced by inflammatory stimulus) and homeostatic (constitutively expressed). Inflammatory chemokines are involved in orchestrating active immune responses while homeostatic chemokines function in embryonic development, leukocyte development and trafficking, and immune surveillance. CCRs are 7-transmembrane (7-TM) spanning GPCR proteins, which bind a variety of chemokine ligands and induce cellular migration via G-protein mediated signaling. CCRs are named for the class of chemokine ligand they bind (i.e. CXCR- or CCR-). CCRs lacking known ligands are named as orphans. In general, the chemokine signaling network displays significant redundancy within chemokine-receptor classes. Chemokines generally

bind multiple receptors within the same receptor class with different affinities. As such, the spatial and temporal expression of chemokines and their receptors contributes significantly to the function and regulation of chemokine signaling (Colobran, Pujol-Borrell et al. 2007; Salanga, O'Hayre et al. 2009).

### *1.4.1 Chemokine Structure and Function*

Chemokines are a large family of small (8-15 kDa) cytokines. They are subdivided into four chemokine classes (CC-, CXC-, CX3C-, and C-) based upon the N-terminal motif containing two structural cysteines or a single cysteine in the case of the C-chemokine lymphotactin. Despite significant sequence divergence, chemokines share a common structural fold. There is a short, unstructured N-terminus preceding the first structural cysteine motif. Disulfide bonds between the N-terminal dicysteine and a third and fourth structural cysteine form the basis of two loops connecting three anti-parallel beta strands followed by a C-terminal alpha helix (Figure 1.6) (Baggiolini 1998; Colobran, Pujol-Borrell et al. 2007).

#### i. Receptor Binding and Activation

Numerous structure/activity studies have elucidated the sequence determinants of chemokine receptor binding and activation. Although some of these requirements are chemokine and receptor-specific, in general primary receptor interaction (docking) is determined by sequences in the first loop between the second and third structural cysteine and receptor activation sequences are found in the N-terminus preceding the first structural cysteine (Loetscher and Clark-Lewis 2001). The CXC-chemokine IL-8 contains an ELR motif in the N-terminus that is necessary but not sufficient for receptor activation (Hebert, Vitangcol et al. 1991; Clark-Lewis, Dewald et al. 1993). The non-ELR CXC-chemokine SDF-1 requires its first two amino acids for receptor activation, and mutations

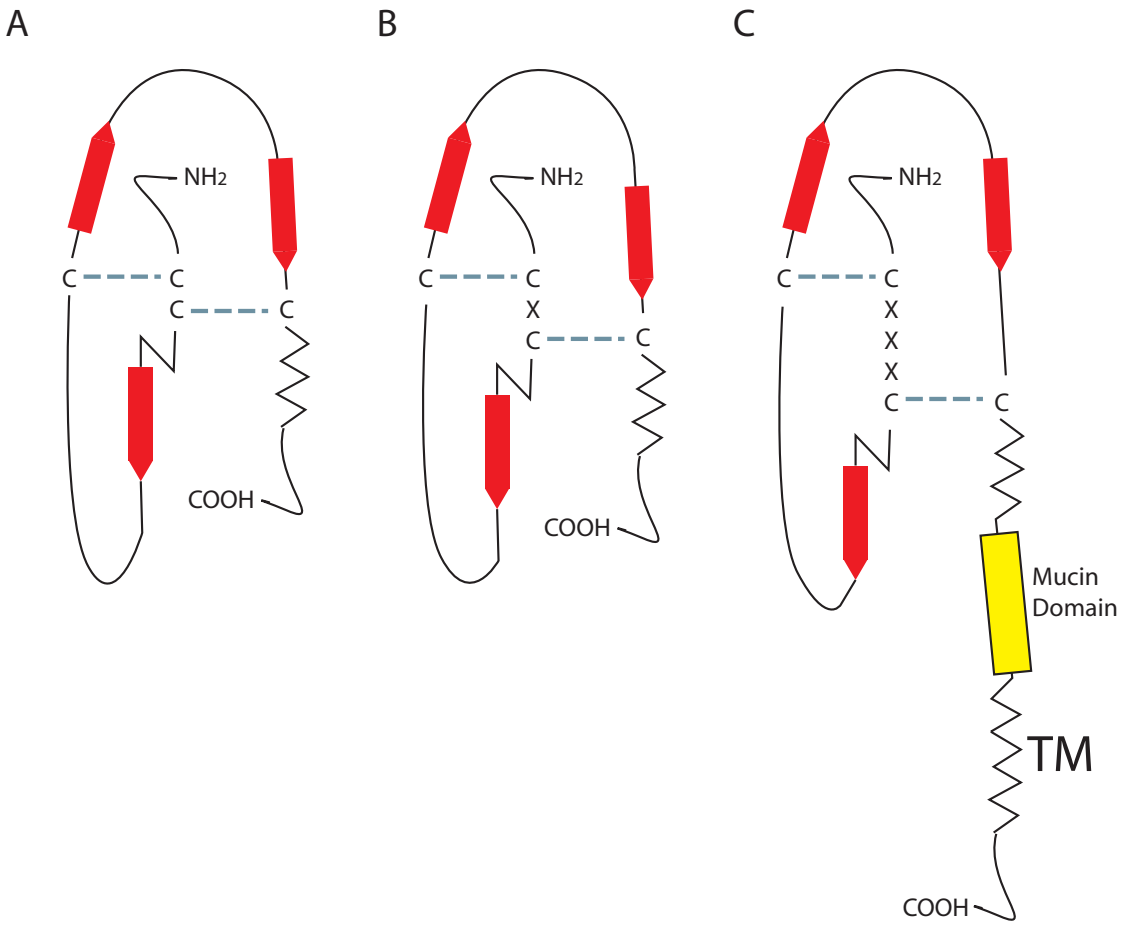



**Figure 1.6 - Chemokine Structure**
**Schematic of the tertiary structures of (A) CC-chemokines, (B) CXC-chemokines and (C) the CX3C-chemokine Fractalkine. Red arrows indicate beta-strands and jagged lines indicate alpha-helical structure.**

in this region produce chemokines that are potent receptor antagonists (Crump, Gong et al. 1997). Interestingly, peptides corresponding to the SDF-1 N-terminal eight amino acids are chemotactic but display very low receptor affinity compared to the complete SDF-1 sequence. Addition of the REFESH motif found in the first inter-cysteine loop to these N-terminal peptides increases receptor affinity significantly, underscoring the role of this region in chemokine-receptor binding (Loetscher, Gong et al. 1998). Mutagenesis studies of the CC-chemokine MCP-1 demonstrate similar roles for the N-terminus in receptor activation and charged residues in the first loop region for receptor interaction. However,

there are stricter requirements for the intact N-terminus for CC-chemokines and peptides corresponding to these sequences are not independently chemotactic (Zhang, Rutledge et al. 1994).

### ii. Potential Role of Oligomerization

Multiple chemokines are known to form dimers higher order oligomers in solution. However the role of oligomerization in the biology of chemokines remains a subject of debate (Allen, Crown et al. 2007). Chemokine oligomers form at much higher concentrations than the optimal chemotactic concentration *in vitro*, raising questions as to whether local chemokine concentrations *in vivo* are ever sufficient for the formation of higher order structures (Czaplewski, McKeating et al. 1999). Moreover, mutant chemokines that are deficient in the formation of oligomers retain full chemotactic function *in vitro* (Rajarathnam, Sykes et al. 1994; Rajarathnam, Clark-Lewis et al. 1995; Paavola, Hemmerich et al. 1998; Laurence, Blanpain et al. 2000). However, studies comparing monomeric mutants to wild-type oligomeric forms of RANTES (CCL5), MCP1 (CCL2) and MIP1β (CCL4) revealed that monomeric forms of these chemokines are significantly deficient in chemotactic function *in vivo* (Proudfoot, Handel et al. 2003). The authors speculate that oligomerization may play a role in the function of chemokines under flow conditions by modulating chemokine interactions with glycosaminoglycans (GAGs) thereby regulating the establishment of an effective chemotactic gradient. There is also evidence for hetero-oligomerization between different chemokines but the physiological relevance of these interactions remains unclear (Allen, Crown et al. 2007).

### ii. Fractalkine and Adhesion

Fractalkine (CX3CL1) is the only known CX3C-chemokine and is unique among chemokines in that it functions as both an inducible inflammatory chemokine and an adhesion factor.

Fractalkine has a unique structure wherein there are three amino acids between the first two structural cysteines and the C-terminal end of the chemokine fold is extended into a mucin stalk followed by a transmembrane domain and a short (37 amino acid) cytoplasmic domain (Figure 1.6C) (Umehara, Bloom et al. 2004). Fractalkine is also found in a soluble form produced by proteolytic cleavage of the full-length molecule by TNFα-converting enzyme ADAM17 (Garton, Gough et al. 2001; Tsou, Haskell et al. 2001). As such, the function of Fractalkine in lymphocyte homing to sites of inflammation is regulated by factors influencing expression of Fractalkine itself as well as factors controlling the expression of ADAM17. EC-expressed membrane-bound Fractalkine binds its receptor CX3CR1 and mediates the secure adhesion of receptor-bearing cells to EC monolayers under flow conditions (Haskell, Cleary et al. 1999; Goda, Imai et al. 2000). Dysregulation of Fractalkine expression has been associated with a variety of inflammatory disorders including vascular disease, acute rejection of allografts, renal disease and RA (Umehara, Bloom et al. 2004).

### *1.4.2 Chemokine Receptor Structure and Function*

CCRs are 7-transmembrane domain (7-TM) GPCRs of the Class A (Rhodopsin-like) superfamily of GPCRs. Critical features of CCR structure include: (1) bonded cysteines in each extracellular loop regulating the barrel-shape structure of the CCR; (2) acidic residues and sites for tyrosine sulfation in the N-terminus determining initial interactions with chemokine ligands; (3) a highly-conserved D/ERY (DRY) motif in the second intracellular loop and (4) a basic motif in the fourth intracellular loop (Figure 1.7) (Murphy, Baggiolini et al. 2000). CCR activation is mediated by chemokine interactions with the extracellular face of the CCR, which stabilizes a conformational change in the TM helices. This ligand-bound activated CCR conformation exposes intracellular domains that mediate GDP-GTP exchange and activation of heterotrimeric G-proteins, thereby initiating intracellular signal

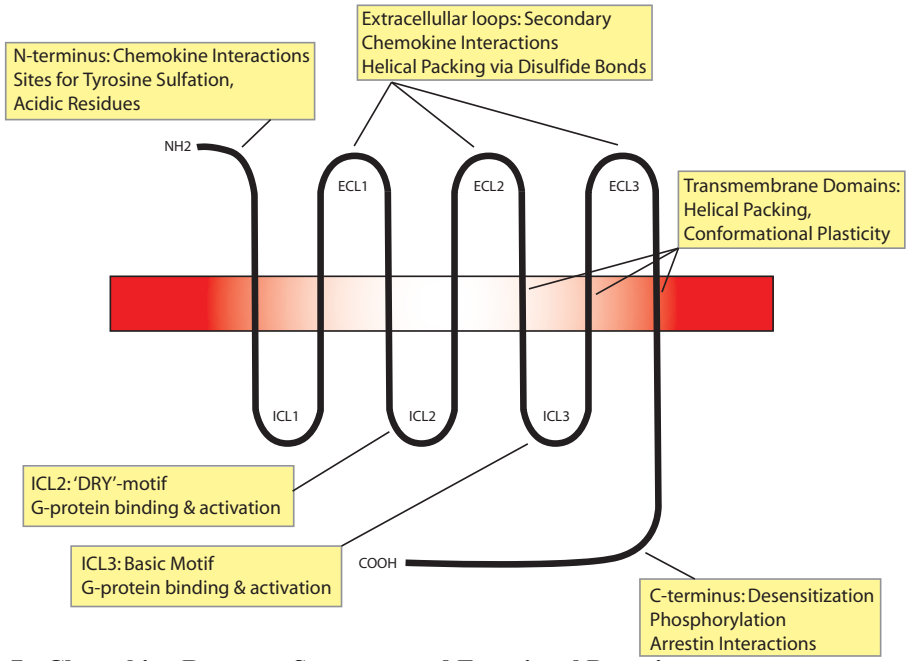



**Figure 1.7 - Chemokine Receptor Structure and Functional Domains**
**Schematic representation of a 7-TM chemokine receptor with critical functional domains.**

transduction. Following G-protein activation, the intracellular C-terminus of the CCR is phosphorylated by G-protein coupled receptor kinases (GRKs) and other cellular kinases. Phosphorylation mediates the recruitment of arrestin proteins that function to desensitize the receptor via internalization (Figure 1.8) (Murphy, Baggiolini et al. 2000; Schwartz, Frimurer et al. 2006; Allen, Crown et al. 2007; DeWire, Ahn et al. 2007). In some cases arrestin recruitment initiates a second wave of intracellular signal transduction (DeWire, Ahn et al. 2007).

### i. Ligand Binding

The specific structural determinants of chemokine binding to CCRs remains poorly understood, and accumulating evidence suggests that each CCR ligand may have slightly different binding determinants (Blanpain, Doranz et al. 2003; Allen, Crown et al. 2007). In many cases, the CCR N-terminus is required for high-affinity chemokine binding (Ahuja, Lee et al. 1996; Monteclaro and Charo 1996) and tyrosine sulfation of CCR N-terminal

sequences increases the affinity of ligand-receptor interactions (Veldkamp, Seibert et al. 2006). Secondary interactions of chemokine ligands with the extracellular loops (ECL) of CCRs are also important for effective receptor activation (Allen, Crown et al. 2007). One study showed via extensive mutagenesis that residues in ECL2 and ECL3 of CCR5 were important for functional MIP1α interactions but had no effect on RANTES binding to the receptor (Blanpain, Doranz et al. 2003). Another elegant study used soluable CCR analogs of CCR2 and CCR3 to determine that the receptor N-terminus and ECL3 sequences are sufficient for selective and high affinity binding of chemokines (Datta-Mannan and Stone 2004).

### ii. G-protein Binding and Activation

The DRY-motif found in the TM3-proximal end of ICL2 on most CCRs is believed to be a critical determinant of heterotrimeric G-protein binding and activation and CCRs lacking this motif are generally classified as non-signaling scavenger receptors (Allen, Crown et al. 2007). However, mutagenesis of the DRY motif does not result in a single signaling-inactive phenotype (Rovati, Capra et al. 2007). Mutations in the conserved arginine residue of the DRY-motif have also been studied in the context of CCR3 and CX3CR1 and result in decreased signaling in response to ligand stimulation (Haskell, Cleary et al. 1999; Auger, Pease et al. 2002). This phenotype is most likely a result of decreased G-protein binding to the ligand-bound active receptor conformation. CXCR2 mutants in the D/E residue of the DRY-motif display an increase in agonist-independent constitutive activity, suggesting that this mutation destabilizes the inactive receptor conformation (Burger, Burger et al. 1999). However, when similar mutations are introduced into CX3CR1 and CCR3 no increase in ligand-independent activation is observed (Haskell, Cleary et al. 1999; Auger, Pease et al. 2002).

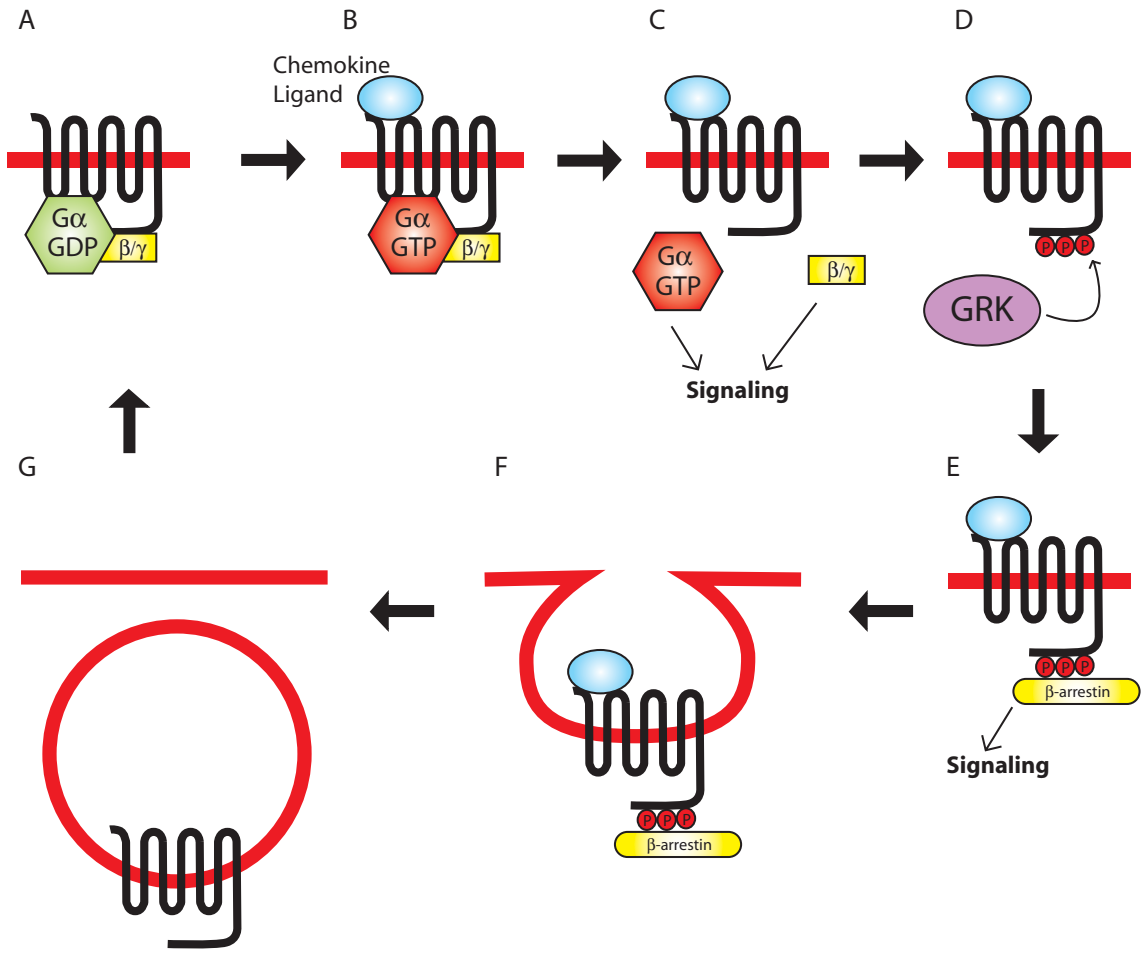



**Figure 1.8 - Chemokine Receptor Signaling**
**(A) Chemokine receptors interact with inactive (GDP-bound) heterotrimeric G-proteins. (B) Ligand binding to the N-terminus transduces a conformational change catalyzing the activation of the bound G-protein. (C) G-protien subunits initiate intracellular signaling. (D) GRK phosphorylate the activated receptor. (E) The phosphorylated receptor binds β-arrestin proteins which signal in some cases. (F) β-arrestin mediates the internalization of the activated receptor. (G) Internalized receptors are either degraded or recycled back to the cell surface in inactive form.**

### iii. Regulation and Desensitization

Following chemokine binding and the initial wave of G-protein mediated signaling, the C-terminal domain of an activated CCR is phosphorylated by GRKs. This phosphorylation mediates recruitment of β-arrestin proteins to the receptor C-terminus. Arrestins sterically inhibit further interaction between the receptor and G-proteins thereby terminating G-protein mediated signaling. In some cases, the β-arrestin molecule may initiate a second

wave of intracellular signaling (Rajagopal, Rajagopal et al. 2010). β-arrestin recruitment initiates the clathrin-mediated internalization of the CCR resulting in desensitization. Following internalization, the receptor is either recycled back to the cell surface in an inactive conformation targeted for lysosomal degradation. It is clear that the expression of GRK and β-arrestin proteins has an effect on CCR function. Decreases in GRK expression result in increased chemotactic responses from CXCR2, CXCR4 and CCR5 in hematopoietic cells (Fan and Malik 2003; Vroon, Heijnen et al. 2004; Vroon, Heijnen et al. 2004). Importantly, the expression of GRK molecules is both tissue specific and inducible in various pro-inflammatory contexts, allowing for marked differences in GPCR regulation via phosphorylation depending on the cell-type and the signaling context (Tobin, Butcher et al. 2008). Moreover, ligand-specific differences in the phosphorylation of GPCRs has been observed, suggesting that different agonists initiate different programs of GPCR regulation and desensitization (Kelly, Bailey et al. 2008). Although ligand-specific phosphorylation has been observed for CCR5 the functional implications of these differential phosphorylation events remains to be determined (Pollok-Kopp, Schwarze et al. 2003). Interestingly, β-arrestin-deficient T-cells are defective in CXCR4-mediated chemotaxis although marked GTPase activity is associated with the receptor in these cells (Fong, Premont et al. 2002). These data may indicate that a lack of coordinated receptor desensitization is anathema to directed cellular migration. Alternatively, β-arrestin signaling to ERK1/2 and/or p38 MAPK may be important for CXCR4-mediated chemotaxis (Rajagopal, Rajagopal et al. 2010).

## 1.5 Herpesvirus Chemokine Receptor Mimicry

Human herpesviruses have been evolving with their natural hosts for millions of years (McGeoch, Dolan et al. 2000). Over the course of this co-evolution the virus has acquired and modified a number of cellular genes in order to more effectively manipulate the host

cell metabolism and modulate cellular defenses. In the β- and γ-herpesvirus subfamilies, these hijacked genes include, chemokines, chemokine binding proteins and chemokine receptors (CCRs). HHV-encoded chemokine receptors (vCCRs) are known to signal both constitutively and in response to cellular and viral chemokine ligands (Maussang, Vischer et al. 2009) (Table 1.2). However the implications of vCCR expression in the pathogenesis of herpesvirus infections is still under investigation.

### *1.5.1 HHV-encoded Chemokine Receptors*

The γ-herpesviruses Epstein-Barr Virus (EBV) and Kaposi's Sarcoma Associated Herpesvirus (KSHV) each encode one chemokine receptor homolog. The EBV-encoded CCR, BILF is unusual among HHV CCRs in that the functions attributed to BILF are primarily related to EBV immune evasion. BILF remains an orphan chemokine receptor and all functions attributed to BILF are thought to be either constitutive or independent of receptor signaling events. Ligand-independent BILF signaling inhibits the phosphorylation of PKR, thereby preventing host cell translational shutoff and ensuring the efficient translation of EBV lytic genes (Beisser, Verzijl et al. 2005). More recently, BILF was shown to interact with MHC Class I molecules at the surface of infected cells and mediate their internalization and lysosomal degradation, thereby hiding EBV-infected cells from CD8 T-cell surveillance (Zuo, Currin et al. 2009).

The KSHV-encoded chemokine receptor ORF74 performs a myriad of functions in KSHV-infected cells. ORF74 resembles CCRs and binds multiple cellular chemokine ligands as well as the KSHV-encoded CXC chemokine (vCXCL2) (Figure 1.6). However, many of the functions of ORF74 are attributed to ligand-independent constitutive activity (Maussang, Vischer et al. 2009; Jham and Montaner 2010). ORF74 is expressed in KS lesions and KS-associated lymphoma tissues (Cesarman, Nador et al. 1996) and has been implicated in tumorigenesis via induction of multiple pro-inflammatory signaling pathways. Importantly, ORF74 was the

**Table 1.2 - HHV-Encoded Chemokine Receptors and their known Ligands**

| Virus | Receptor | Ligands |
|---|---|---|
| HHV-6 | U12 | CCL2, CCL4, CCL5, CCL3 |
| | U51 | CCL2, CCL5, CCL7, CCL11, CCL13, CCL19, CCL22, XCL1, CX3CL1 |
| HHV-7 | U12 | CCL17, CCL19, CCL21, CCL22 |
| | U51 | CCL17, CCL19, CCL21, CCL22 |
| KSHV(HHV-8) | ORF74 | CCL1, CCL5, vCCL2, CXCL1, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL10 |
| EBV | BILF1 | Unknown |
| HCMV | US27 | Unknown |
| | US28 | CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CCL13, CCL26, CCL28, CX3CL1 |
| | UL33 | Unknown |
| | UL78 | Unknown |

only putative KSHV oncogene able to produce a transformed angioproliferative sarcoma in a mouse model of KSHV tumorigenesis (Montaner, Sodhi et al. 2003). ORF74 is implicated in the progression and maintenance of angioproliferative tumors by inducing the cellular secretion of a number of growth factors and pro-inflammatory factors including VEGF, basic fibroblast growth factor (bFGF), TNFα, MIP-1 and IL-1β, -2, -4, -6 and -8 (Jham and Montaner 2010). This extensive paracrine signaling induces a hyperproliferative, pro-inflammatory and pro-angiogenic phenotype in both KSHV-infected and uninfected bystander cells in KS lesions. Elimination of ORF74 expression in murine KS lesions results in tumor regression (Jensen, Manfra et al. 2005) underscoring the importance of ORF74 signaling in KS pathogenesis.

The β-herpesviruses HHV-6 and HHV-7 encode analogous CCRs, U12 and U51. In both HHV-6 and HHV-7 these receptors bind a number of cellular chemokine ligands (Figure 1.6). HHV-7 U12 induces ligand-dependent chemotaxis in Jurkatt cells. In contrast, HHV-7 U51 lacks chemotactic function (Tadagaki, Nakano et al. 2005). Interestingly, both HHV-7 U12 and U51 can modify the function of cellular CCRs via heterodimerization (Tadagaki, Yamanishi et al. 2007). HHV-6 U51 is an effective chemokine sink for CCL5/RANTES

in mutiple systems (Milne, Mattick et al. 2000; Catusse, Spinks et al. 2008). Additionally, HHV-6 U51-expressing cells respond to chemotactic gradients, and U51 has been implicated in immune evasion via downregulation of the hematopoietic transcriptional repressor FOG-2 (Catusse, Spinks et al. 2008). The significance of U12 and U51 signaling is difficult to determine since the pathogenesis of HHV-6 and HHV-7 are poorly understood.

### *1.5.2 CMV-encoded Chemokine receptors*

Similarly, CMV-mediated manipulation of the host chemokine signaling network via the actions of virus-encoded chemokines and CCRs could play an important role in the pathogenesis of CMV-associated inflammatory diseases. HCMV encodes four CCR homologs, pUL33, pUL78, pUS27 and pUS28 (Murphy, Yu et al. 2003). pUS28 signaling is the subject of this volume and is summarized extensively in Section 1.6. pUL33 remains an orphan receptor, but can cause the phospholipase C-dependent (PLC) accumulation of inositol phosphates (InsP) and the accumulation of cAMP in cells in a ligand-independent manner (Casarosa, Gruijthuijsen et al. 2003). Deletion of UL33 homologs in MCMV (M33) or RCMV (R33) results in virus with restricted dissemination and replication within the salivary glands of infected animals (Davis-Poynter, Lynch et al. 1997; Beisser, Vink et al. 1998). M33 and R33 have both been shown to be necessary for the CMV-mediated migration of mouse and rat SMC (Melnychuk, Smith et al. 2005; Streblow, Kreklywich et al. 2005), respectively. Importantly, deletion of R33 from the RCMV genome results in a virus (RCMV-ΔR33) that fails to replicate in the salivary gland (Kaptein, Beisser et al. 2003) and the development of TVS following heart transplant is significantly decreased in RCMV-ΔR33 infected rats (Streblow, Kreklywich et al. 2005). The ligand specificity and signaling potential of pUL78 and pUS27 are unknown. However, deletion of the MCMV (M78) and RCMV (R78) homologs of these proteins resulted in decreased viral fitness *in vivo.* M78 mutant MCMV displays decreased viral replication in all organs of infected mice (Oliveira and Shenk 2001), while deletion

of R78 inhibited RCMV replication specifically in the spleen of infected rats (Kaptein, Beisser et al. 2003). Interestingly, heterodimers of two separately encoded CCRs can have modulated function compared to each CCR individually (Mellado, Rodriguez-Frade et al. 2001; Tadagaki, Yamanishi et al. 2007). This observation suggests that orphan viral CCRs that lack apparent intrinsic activity may function via dimerization and modulation of signaling from cellular CCRs. This possibility has yet to be explored experimentally.

## 1.6 HCMV US28

The HCMV-encoded chemokine receptor US28 is the most well-characterized of the four HCMV CCRs. US28 biology is extensively studied in models of HCMV-mediated vascular disease (Streblow, Orloff et al. 2001). Moreover, US28 has recently been implicated in HCMV-associated malignancy (Maussang, Verzijl et al. 2006). US28 binds numerous CC-chemokines (RANTES, MCP-1, MCP-3 and MIP1a) and the CX3C-chemokine Fractalkine (Kledal, Rosenkilde et al. 1998). This property of binding chemokines from multiple classes makes US28 highly unusual among CCRs. Importantly, the functional consequences of US28 ligand promiscuity remain to be determined.

### *1.6.1 US28 Functions in HCMV Infection*

#### i. Chemotaxis

US28 is a functional receptor for CC-chemokines and is necessary and sufficient for chemotaxis of HCMV-infected SMC *in vitro* (Streblow, Soderberg-Naucler et al. 1999). These findings have significant implications for the participation of HCMV in the development of TVS. Our group has proposed a model by which US28-mediated chemotaxis recruits SMC into atherosclerotic lesions, thereby accelerating the formation of an SMC-rich atherosclerotic plaque (Streblow, Orloff et al. 2001). Detailed examination of signaling initiated by US28 binding to CC-chemokines has revealed critical cellular

signaling mediators involved in US28-mediated chemotaxis. In particular, the activation of focal adhesion kinase (FAK), Src, and the small GTPase RhoA are absolutely required for pro-migratory signaling from US28 (Streblow, Vomaske et al. 2003; Melnychuk, Streblow et al. 2004). Moreover, US28 signaling activates the MAP kinase ERK1/2 downstream of FAK (Streblow, Vomaske et al. 2003). The identification of additional cellular signaling pathways activated by US28 pro-migratory signaling will provide potential targets for inhibition of US28-mediated chemotaxis of SMC. Such treatments may prove important in the treatment of HCMV-associated vascular diseases. In this dissertation, we examine US28 signaling to one such cellular target, the non-receptor tyrosine kinase Pyk2. Moreover, the chemotactic potential of Fractalkine binding to US28 remains to be determined, and is the subject of Chapter 2 of this dissertation.

### ii. Chemokine Scavenging

Several bodies of data attribute a chemokine scavenging function to US28. One study showed that the supernatants of fibroblast cultures infected with a US28-expressing HCMV are deficient in CC-chemokines but cultures infected with a US28-deficient HCMV contain higher levels of CC-chemokines in their supernatants (Bodaghi, Jones et al. 1998). The authors attribute this chemokine deficiency to US28-mediated binding and internalization of supernatant CC-chemokines. Moreover, other studies have shown that US28 is constitutively internalized and recycled when expressed in immortalized cell lines (Mokros, Rehm et al. 2002; Waldhoer, Casarosa et al. 2003). However, the *in vivo* relevance of these observations has been challenged by others who have shown normal recruitment and adhesion of monocytes to US28-expressing EC (Boomker, de Jong et al. 2006). These data suggest that physiological concentrations of chemokine are too high to be effectively scavenged by US28.

#### iii. Cellular Activation

Early studies of US28 signaling characterized US28 as a generic 'cellular activator'. In COS-7 cells, US28 signals to phospholipase C and NF-kB (Casarosa, Bakker et al. 2001). This signaling is thought to play a role in the NF-kB mediated activation of the MIEP in HCMV infected cells (DeMeritt, Milford et al. 2004; Boomker, The et al. 2006). Other studies show that US28 signaling can initiate caspase-dependent apoptosis in a variety of cell lines (Pleskoff, Casarosa et al. 2005). However the pathophysiological implications of this signaling remain to be examined in detail. Many of these studies suggest that US28 is constitutively active. US28 constitutive activity is usually observed in immortalized cell lines in which profound cellular dysregulation may effect US28 function. Moreover, most studies do not specifically test for the presence or absence of US28 ligands. Importantly, the *in vivo* relevance of US28 'constitutive activity' has not been determined.

### *1.6.2 US28 Structure and Function*

#### i. Ligand Binding

The N-terminus of US28 contains a conserved hexapeptide sequence that is required for high-affinity chemokine interactions (Casarosa, Waldhoer et al. 2005). This domain is also critical for chemokine binding to the cellular chemokine receptors CCR1 and CCR2 (Preobrazhensky, Dragan et al. 2000). Interestingly, mutagenesis of the US28 N-terminus reveals that US28 interactions with CC- and CX3C-chemokines have distinct but overlapping sequence requirements. Specifically, Phe-14 is a determinant of CC-chemokine binding but has little effect on the affinity of Fractalkine for US28. However, mutation of Tyr-16 inhibits binding of both chemokine classes but is more profoundly deleterious to US28-Fractalkine interactions (Casarosa, Waldhoer et al. 2005). Potential secondary interactions of chemokine ligands with US28 extracellular loop domains have

not been explored experimentally.

### ii. G-protein Binding and Activation

US28 is promiscuous in its G-protein interactions. This G-protein promiscuity has potential functional implications for US28 functions in different cell types and signaling contexts, however this hypothesis remains to be rigorously tested. To date, functional consequences of US28 binding to Gaq/11, Gai1, Ga16 and Ga12 have been demonstrated experimentally. The earliest studies of US28 signaling demonstrated that US28 stimulation with RANTES can activate ERK1/2 via Gai1 and Ga16-mediated mechanisms (Billstrom, Johnson et al. 1998). US28 activation of Gaq/11 is responsible for the 'constitutive' activation of PLC and NFkB (Casarosa, Bakker et al. 2001; Waldhoer, Kledal et al. 2002). This Gaq/11-mediated signaling can be inhibited by the addition of Fractalkine (Casarosa, Bakker et al. 2001), suggesting that Fractalkine binding can co-opt US28-Gaq/11 signaling for activation of a secondary, as yet uncharacterized, signaling pathway. Our group has characterized the Ga12/13-mediated activation of FAK, ERK and RhoA in US28-expressing cells (Streblow, Soderberg-Naucler et al. 1999; Melnychuk, Streblow et al. 2004). Mutagenesis of the highly conserved DRY-motif of US28 has produced conflicting results. An intact DRY-motif is required for US28 signaling to PLC (Waldhoer, Casarosa et al. 2003). However, US28-mediated SMC migration in response to CC-chemokine stimulus is unaffected by mutation of this motif (Figure A.2, this volume). These data suggest that US28 may have different sequence requirements for binding and activation of different G-proteins and that the DRY-motif is not absolutely required for G-protein mediated signaling from certain US28 active conformations.

### iii. Regulation and Desensitization

Regulation of US28 signaling proceeds via phosphorylation of C-terminal serine residues followed by recruitment of β-arrestin-2. A variety of cellular kinases interact with the US28

C-terminus including PKC, casein kinase 2 (CK2), GRK2 and GRK5 (Mokros, Rehm et al. 2002; Miller, Houtz et al. 2003; Sherrill and Miller 2006). US28 is constitutively phosphorylated in transformed cell lines. However, US28 ligands can modulate levels of US28 phosphorylation, suggesting that the regulation of US28 is not entirely ligand-independent (Mokros, Rehm et al. 2002; Miller, Houtz et al. 2003). Moreover, these data demonstrate that phosphorylation by cellular kinases differs for specific US28 active conformations. Although US28 interacts with β-arrestin-2 in a phosphorylation-dependent manner (Miller, Houtz et al. 2003), expression of US28 in b-arrestin deficient cells has no effect on the endocytosis and recycling of US28 (Fraile-Ramos, Kohout et al. 2003). Mutation or deletion of US28 C-terminal phosphorylation sites generally results in increased surface expression and increased constitutive signaling to PLC and NFkB from US28 (Mokros, Rehm et al. 2002; Casarosa, Menge et al. 2003; Miller, Houtz et al. 2003). However, the effect of phosphorylation and β-arrestin binding on ligand-dependent US28 signaling remains to be determined.

## 1.7 Herpesvirus Chemokine Mimicry

Viral subversion of host immunity can also be affected via manipulation of host CCR and viral CCR signaling through expression of viral chemokine homologs. Viral chemokine homologs have been discovered for several human herpesviruses and one poxvirus (molluscum contagiosum virus (MCV)) (Luttichau 2008) (Table 1.3). Viral chemokines can be receptor agonists or competitive receptor antagonists. The *in vivo* implications of viral chemokine mimicry in viral pathogenesis and immune evasion are only beginning to be understood.

### *1.7.1 HHV Chemokine Homologs*

The U83 ORF of HHV6 encodes a functional CC-chemokine termed vCCL4. vCCL4

**Table 1.3 - Chemokine Homologs of Human Pathogens and their known Functions**

| Virus | Chemokine | Receptor | Functional Outcomes |
|---|---|---|---|
| HHV-6 | vCCL4 | CCR2 | Calcium flux, Chemotaxis |
| KSHV(HHV-8) | vMIP-I | CCR8 | Calcium flux, Chemotaxis |
| | vMIP-II | CCR3 | Chemotaxis of Eosinophils |
| | | CCR1, 2 &5; XCR1; CX3CR1; CXCR4 | Antagonism of Endogenous Ligand Binding |
| | vMIP-III | XCR1 | Calcium flux, Chemotaxis |
| HCMV | vCXCL1 | CXCR2 | Calcium flux, Chemotaxis |
| | vCXCL2 | Unknown | Unknown |
| | UL128 | Unknown | Unknown |
| | UL130 | Unknown | Unknown |
| Molluscum Contagiosum (MCV) | MCC | CCR8 | Antagonism of Endogenous Ligand Binding |

binds human CCR2 with high affinity and can mediate the chemotaxis of CCR2-expressing cells *in vitro* (Luttichau, Clark-Lewis et al. 2003). HHV6 infected cells may express vCCL4 in order to attract susceptible monocyte and/or lymphocyte populations in which the virus can establish latency. KSHV/HHV8 encodes three separate viral chemokine homologs termed vMIP-I (vCCL1), vMIP-II (vCCL2) and vMIP-III (vCCL3). vMIP-II is well-studied as a broad-spectrum chemokine receptor antagonist, which interacts with a number of cellular CCRs and blocks their activation by endogenous chemokine ligands. Thus, vMIP-II is a powerful immune evasion molecule that is able to efficiently prevent the recruitment of immune cells to sites of KSHV infection (Kledal, Rosenkilde et al. 1997). Moreover, vMIP-II can mediate the *in vitro* chemotaxis of eosinophils via activation of CCR3 (Boshoff, Endo et al. 1997). KSHV vMIP-I is a specific CCR8 agonist and can mediate calcium flux and chemotaxis of CCR8-expressing T cells *in vitro* (Dairaghi, Fan et al. 1999; Endres, Garlisi et al. 1999). As such, vMIP-I is thought to skew KSHV immune responses towards a Th2 phenotype thereby dampening the generation of robust cellular immunity. vMIP-III, the most

recently discovered KSHV-encoded chemokine, binds the lymphotactin receptor XCR1 with high affinity and can mediate the *in vitro* chemotaxis of XCR1-expressing neutrophils (Luttichau, Johnsen et al. 2007). Intriguingly, two of the KSHV-encoded chemokines interact with XCR1. vMIP-I is a potent XCR1 antagonist and vMIP-III is a XCR1 agonist. These data suggest that XCR1 signaling is of particular importance to KSHV biology.

### *1.7.2 CMV-encoded Chemokines*

The HCMV genome encodes two CXC-chemokines (UL146 and UL147) and two putative CC-chemokines (UL128 and UL130) (Penfold, Dairaghi et al. 1999). Of these four molecules only the UL146 gene product vCXCL1 has been characterized as a functional chemotactic cytokine. vCXCL1 is a potent agonist for the cellular CCR CXCR2 and can mediate calcium flux and chemotaxis of CXCR2-expressing cells *in vitro* (Penfold, Dairaghi et al. 1999). Moreover, in a transgenic mouse model, vCXCL1 is highly specific for human CXCR2-expressing neutrophils, providing a model in which to study the *in vivo* relevance of vCXCL1-mediate chemotaxis (Sparer, Gosling et al. 2004). Although there is no data characterizing the chemotactic function of the UL128 gene product, the MCMV homolog of UL128, known as MCK-2 (M129/131) is well characterized as a functional CC-chemokine (MacDonald, Burney et al. 1999; Saederup, Aguirre et al. 2001). MCK-2 recruits susceptible monocyte populations to sites of MCMV inoculation and participates in early monocyte-associated viremia and dissemination of MCMV within the infected host (Saederup, Lin et al. 1999; Saederup, Aguirre et al. 2001). Moreover, one study demonstrated that MCK-2 specifically recruits immature myelomonocytic leukocytes from the bone marrow independent of MCMV infection (Noda, Aguirre et al. 2006). Taken together these data suggest that MCK-2 participates in primary viral dissemination in MCMV infection and that the principal vehicles of MCMV spread within the host are myeloid progenitor cells. Importantly, the analogous region in the RCMV genome encodes two separate ORFs

with homology to CC-chemokines, r129 and r131. Studies have demonstrated that r131 is the functional homolog of MCK-2 in RCMV (Kaptein, van Cleef et al. 2004). However, the chemotactic potential of r129 remains to be explored.

## 1.8 Overview and Aims

In this dissertation I examine signaling from the HCMV-encoded chemokine receptor US28 in order to further characterize the contribution of HCMV chemokine receptor mimicry to the development of chronic inflammatory diseases. I hypothesize that the capacity of US28 to bind multiple classes of chemokine ligand has differential effects on the biology of HCMV and its participation in the pathogenesis of inflammatory diseases. I further hypothesize that the intracellular signaling from US28 will be both ligand-specific and cell type-specific such that identification of US28-specific cellular signaling intermediates will provide novel targets for blocking specific effects of US28 signaling in HCMV infected cells.

In Chapter 2, I demonstrate that signaling from US28 is both ligand and cell-type specific and determine that the two classes of US28 chemokine ligands have differential effects on migration in SMC and macrophages. Although US28 binding of CC-chemokines initiates pro-migratory signaling in SMC, binding of the CX3C-chemokine Fractalkine (CX3CL1) to US28-expressing SMC is actively anti-migratory and can competitively inhibit a CC-chemokine mediated pro-migratory stimulus. Importantly, I observe the opposite phenotype when US28 is expressed in macrophages. Wherein Fractalkine is able to initiate pro-migratory signaling and CC-chemokines are inhibitory to Fractalkine-mediated US28 migration. I present evidence that the observed US28 functional selectivity is controlled at the level of heterotrimeric G-protein coupling to the ligand activated US28 receptor. These findings refine our understanding of the participation of US28 in the chemotaxis of HCMV-infected SMC and macrophages *in vivo* and the role both of these cell types to play in the pathogenesis of vascular disease.

Chapter 3 focuses on the contribution of the cellular non-receptor tyrosine kinase Pyk2 to US28-mediated pro-migratory signaling. Pyk2 is expressed with limited tissue distribution *in vivo*, primarily in HCMV permissive cell types, making Pyk2 an attractive target for inhibition of US28 signaling. I establish that Pyk2 signaling is necessary for US28-mediated migration in SMC and that the autophosphorylation of Pyk2 is required for US28 pro-migratory signaling via the activation of RhoA. These results suggest that Pyk2 plays a necessary role as a signaling scaffold in these signaling events and provides a specific Pyk2 function that could be a target for chemotherapeutic intervention. I determine that in addition to SMC, US28 signals to Pyk2 in fibroblasts and U373 glioblastoma cells. Importantly, Pyk2 plays a significant role in the invasion of glial tumors. These results provide a potential mechanistic link between HCMV biology and the progression of glioblastoma. In order to further characterize the participation of Pyk2 in US28 signaling I identify US28-specific and cell type-specific binding partners for Pyk2 in both SMC and U373 further demonstrating that the consequences of US28 are highly dependent upon the cellular environment.

In Chapter 4, I demonstrate that the RCMV ORF r129 encodes a functional CC-chemokine. I utilized site-directed mutagenesis to characterize the domains of r129 required for chemotactic function and determine that r129 specifically recruits immature CD4+ T cells *in vitro*. Moreover, I present the initial characterization of two recombinant RCMV strains containing r129 mutants.

The findings presented herein refine and extend our understanding of cytomegalovirus subversion of the host chemokine signaling network. I characterize HCMV US28 as a functionally selective chemokine binding receptor and demonstrate that US28 functional selectivity has differential consequences for the virus and the biology of the host cell depending upon the intracellular signaling environment and the extracellular inflammatory

environment with significant implications for the participation of HCMV and US28 in the development of inflammatory diseases. Furthermore, I extend our understanding of US28 pro-migratory signaling via characterization of Pyk2 as a critical signaling intermediate linking US28 to the activation of Rho. Moreover, I provide new insight into the function of Pyk2 as a pro-migratory protein tyrosine kinase by identifying novel Pyk2 binding partners in both SMC and glioblastoma cells. I identify a novel pro-inflammatory chemokine encoded by RCMV r129 and determine that pr129 may participate in the recruitment of immature CD4+ T cells to sites of infection. These results have significant implications for RCMV biology and the pathogenesis of chronic rejection in CMV-infected solid organ allografts.

# II

# Chapter 2 - Differential Ligand Binding to a Human Cytomegalovirus Chemokine Receptor Determines Cell Type-Specific Motility

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## 2.1 Abstract

While most chemokine receptors fail to cross the chemokine class boundary with respect to the ligands that they bind, the human cytomegalovirus (HCMV)-encoded chemokine receptor US28 binds multiple CC-chemokines, and the $CX_3C$-chemokine Fractalkine. US28 binding to CC-chemokines is both necessary and sufficient to induce vascular smooth muscle cell (SMC) migration in response to HCMV infection. However, the function of Fractalkine binding to US28 is unknown. In this report, we demonstrate that Fractalkine binding to US28 not only induces migration of macrophages but also acts to inhibit RANTES mediated SMC migration. Similarly, RANTES inhibited Fractalkine-mediated US28 migration in macrophages. While US28 binding of both RANTES and Fractalkine activate FAK and ERK-1/2, RANTES signals through Gα12 and Fractalkine through Gαq. These findings represent the first example of differential chemotactic signaling via a multiple chemokine family binding receptor that results in migration of two different cell types. Additionally, the demonstration that US28-mediated chemotaxis is both ligand-specific and cell type-specific has important implications in the role of US28 in HCMV pathogenesis.

## 2.2 Author Summary

Chemokines are small cytokines that are critical for recruiting and activating cells of the immune system during viral infections. Many viruses including the large herpes virus human cytomegalovirus (HCMV) encode mechanisms to impede the effects of chemokines or have gained the ability to use these molecules to its own advantage. HCMV encodes multiple chemokine receptors including US28, which binds two different classes of chemokines namely the CC and CX3C families. In this report, we demonstrate that US28 binding to CC chemokines or the CX3C chemokine, Fractalkine, elicit different responses. RANTES

(CC chemokine) binding to US28 mediates smooth muscle cell migration but Fractalkine blocks this process in a dose-dependent manner. However, Fractalkine binding to US28 can specifically mediate the migration of macrophages, another important cell type during viral pathogenesis. We explored the intracellular signaling pathways responsible for each migration event and determine that they differ in the G-proteins that couple to US28 following addition of ligand and that this occurs in a cell type-specific manner. These results provide a new mechanism for HCMV acceleration of vascular disease via the specific migration of macrophages and provide the first example of cell type-specific migration via multiple chemokines binding to a single receptor.

## 2.3 Introduction

All β and γ-herpesviruses encode molecules with the potential to modulate the host immune response, including chemokines and/or chemokine receptor homologs. The β-herpesvirus human cytomegalovirus (HCMV) encodes a CXC-chemokine (UL146), a potential CC-chemokine (UL128), and four potential chemokine receptors (US27, US28, UL33 and UL78) with the most characterized being US28 (Chee, Bankier et al. 1990; Chee, Satchwell et al. 1990; Penfold, Dairaghi et al. 1999; Akter, Cunningham et al. 2003). Chemokines are small, inducible cytokines that have critical roles in the induction and promotion of cellular migration and activation upon binding 7-transmembrane spanning G-protein coupled receptors (GPCRs). There are four major chemokine subfamilies that are categorized according to the spacing of the first two conserved amino-terminal cysteine residues: CC-, CXC-, $CX_3C$- and XC-. Most chemokine receptors bind a limited subset of ligands belonging to a single subfamily. The ability to bind multiple ligands from different chemokine subfamilies is unique to a select few receptors including the Duffy antigen/receptor for chemokine (DARC-receptor) and the HHV-8-encoded chemokine receptor Orf74. These receptors have been reported to bind to both CC- and CXC-chemokines

(Szabo, Soo et al. 1995; Rosenkilde, Kledal et al. 1999; Lentsch 2002). US28 also binds multiple ligands from different subfamilies. US28 contains homology to CC-chemokine receptors, with greatest homology to CCR1 (Gao and Murphy 1994) and binds to a broad spectrum of CC-chemokines with high affinity including: RANTES, MCP-1, MIP-1α and MIP-1β (Kuhn 1995). Interestingly, US28 also binds the $CX_3C$-chemokine Fractalkine and with greater affinity than CC-chemokines. Although the N-terminal 22 amino acids of US28 are required for binding of both chemokine classes (Casarosa, Menge et al. 2003), binding is not competed with saturating quantities of selected CC-chemokines (Kledal, Rosenkilde et al. 1998). Therefore, Fractalkine is predicted to bind unique regions of US28 compared to the CC-chemokines. Indeed, recent mutagenesis studies of the US28 N-terminus revealed that the phenylalanine residue at position 14 of US28 is important for binding of CC chemokines but is dispensable for Fractalkine binding, while mutation of tyrosine 16 negatively effects binding of both classes of chemokines (Casarosa, Waldhoer et al. 2005).

Binding of chemokines to their respective receptors stimulates the cell type-dependent activation of a plethora of cellular signaling pathways specific to the chemokine/receptor pair. The CC-chemokines are known to be potent stimulators of cellular activation through US28. For example, in 293 cells, RANTES binding to US28 activates ERK-1/2 pathways through the G-proteins Gαi1 and Gα16 (Billstrom, Johnson et al. 1998). We have previously demonstrated that US28-mediated SMC migration is ligand-dependent requiring either exogenously added RANTES or endogenously expressed MCP-1 (Streblow, Söderberg-Nauclér et al. 1999). This migratory process is not blocked by treatment with pertussis toxin (PTX), a Gαi/o G-protein inhibitor, suggesting that other G-proteins are involved in this event (Streblow, Söderberg-Nauclér et al. 1999). Subsequent studies revealed that US28 couples with Gα12/13, promoting SMC migration and ligand-dependent signaling

through the small G-protein RhoA (Melnychuk, Streblow et al. 2004). US28 mediated SMC migration is also sensitive to treatment with protein tyrosine kinase (PTK) inhibitors, and the PTKs focal adhesion kinase (FAK) and Src are activated in US28 expressing cells upon RANTES binding (Streblow, Vomaske et al. 2003). Dominant negative inhibitory FAK molecules blocked US28 induced SMC migration suggesting that FAK activation is critical for US28 mediated SMC motility (Streblow, Vomaske et al. 2003).

Although US28 binding to CC-chemokines leads to the activation of a multitude of cellular signaling pathways, the only activities associated with US28 binding to Fractalkine involve the modulation of constitutive signaling activity (Casarosa, Bakker et al. 2001; Fraile-Ramos, Kledal et al. 2001; Mokros, Rehm et al. 2002). Treatment of US28 expressing cells with Fractalkine or the US28 synthetic inverse agonist VUF2274 leads to substantial decreases in the ability of US28 to promote the Gαq/11 dependent constitutive activation of phospholipase-C (PLC) and NF-κB, whereas MCP-1 and RANTES have only negligible effects on constitutive signaling levels (Casarosa, Bakker et al. 2001; Casarosa, Menge et al. 2003). Additionally, Fractalkine treatment of US28 expressing HEK293A cells reduces constitutive US28 phosphorylation (Mokros, Rehm et al. 2002) and steady state levels of surface US28, but has little influence on the rapid endocytosis observed in HeLa cells (Fraile-Ramos, Kledal et al. 2001). The ability of US28 to efficiently bind ligands from multiple chemokine subfamilies coupled with the vastly different signaling responses elicited by divergent ligands is intriguing and suggests that US28 signaling is not only ligand and cell-type dependent, but also ligand-specific.

In the current study, we investigate the signaling potential of US28 upon stimulation with CC-chemokines compared to the $CX_3C$-chemokine Fractalkine. We demonstrate that Fractalkine binding to US28 inhibits the ability of CC-chemokines to induce SMC migration. RANTES, MCP-1, and Fractalkine binding to US28 induced similar levels

of FAK activation in fibroblasts. Overexpression studies indicate that RANTES-mediated stimulation of FAK occurs via a Gα12-dependent mechanism while Fractalkine utilizes Gαq. In contrast to SMC, when US28 is expressed in macrophages, Fractalkine stimulation produces robust migration These results suggest that US28-signaling is ligand-specific and cell type-specific, and that RANTES and Fractalkine promote differential G-protein coupling leading to the activation of alternative signaling pathways depending on the cell-type and the complement of endogenously expressed G-proteins.

## 2.4 Results

### *2.4.1 Ligand-Specific US28 Mediated Smooth Muscle Cell Migration*

The unique ability of US28 to bind both CC- and CX3C-chemokine ligands raises the question of whether US28 signaling is not only ligand-dependent, but also ligand-specific (Schall, Stein et al. 1994; Kuhn 1995; Billstrom, Johnson et al. 1998; Bodaghi, Jones et al. 1998). To determine whether US28 signaling and SMC migration are ligand-specific, we performed SMC migration and signaling assays on US28 adenovirus expressing primary rat SMC in the presence of RANTES or Fractalkine. In this assay, RANTES readily induced US28-mediated SMC migration, however, increasing concentrations of Fractalkine failed to stimulate cellular motility above Ad-tet-transactivator (Trans) infected and RANTES stimulated controls, indicating that not all US28 ligands evoke the same functional response (Figure 2.1A). Visual analysis of the cells prior to and following the migration assay indicated that the lack of migration was not due to overt cell death mediated by US28 expression and subsequent treatment with Fractalkine (data not shown). A competition assay was performed to determine whether Fractalkine inhibits the ability of RANTES to induce SMC migration. In these experiments, RANTES alone promoted SMC migration, as expected. However, Fractalkine, at concentrations as low as 10 ng/ml, was sufficient to

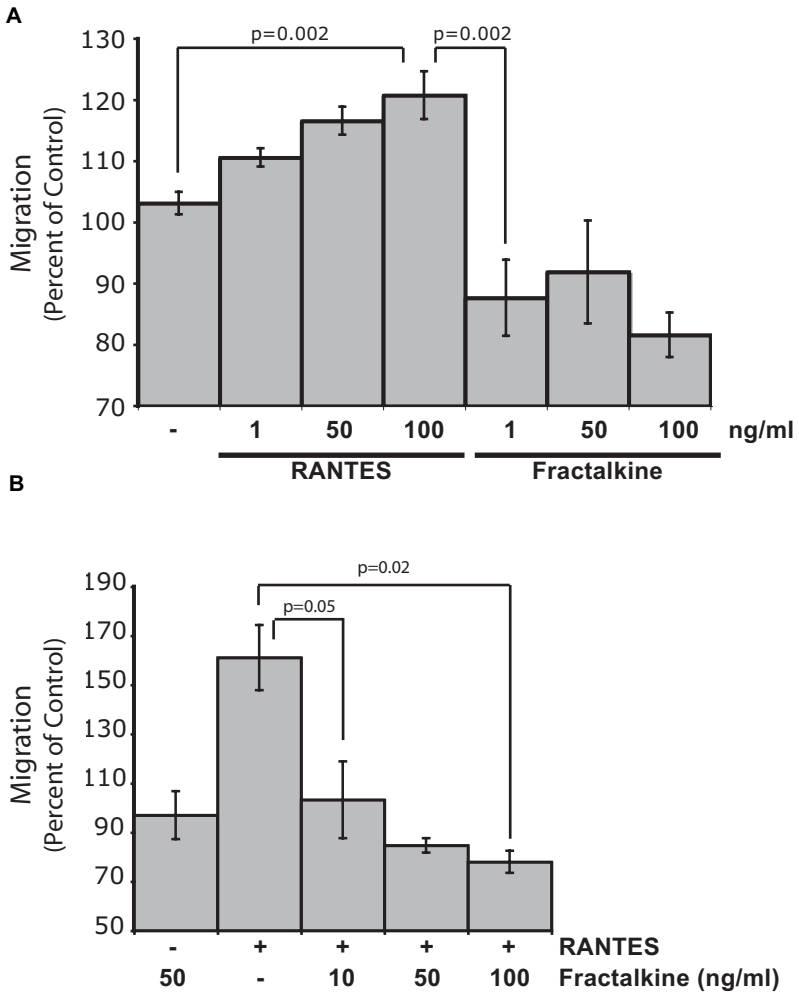


**Figure 2.1 - Fractalkine Inhibits US28-Mediated SMC migration induced by RANTES.**
**(A)SMC migration assays were performed on cells infected with adenovirus expressing US28-HA treated with either RANTES or Fractalkine at the indicated concentrations. Data are represented as a percentage of unstimulated cells infected with control adenovirus transactivator only. For all conditions, n>6 from two independent experiments. (B) SMC migration assays were performed on US28-expressing cells treated with RANTES, Fractalkine or 40ng/ml of RANTES and the indicated concentrations of Fractalkine as a competing ligand.**

block RANTES-mediated SMC migration (Figure 2.1B), suggesting that Fractalkine is a competitive inhibitor to CC-chemokine induced SMC migration.

Since RANTES but not Fractalkine caused the migration of US28 expressing SMC, and since Fractalkine blocks this migration event, we hypothesized that the difference in the ability to promote motility occurred at the level of signaling. To determine whether there exists a gross difference in the ability of these chemokine receptors/ligands to modulate intracellular signaling cascades, host transcriptional profiles were examined using DNA microarrays. Interestingly, the cellular gene expression profile of US28-expressing SMC

stimulated with RANTES substantially differs from the profile obtained upon stimulation with Fractalkine. In fact, Fractalkine down-regulated most of the genes that were up-regulated upon RANTES stimulation. Specifically, RANTES binding to US28 induced expression of a number of cellular genes involved in cellular migration, while Fractalkine down-regulated many of these same genes (data not shown). These findings indicate that there are ligand-specific differences in US28 signaling that parallel the ability of either RANTES or Fractalkine to promote SMC migration.

### *2.4.2 Ligand-Specific Signaling Mediated By US28*

To determine if the different phenotypic outcomes of RANTES or Fractalkine binding to US28 is reflected in differences at the level of signal transduction, we examined the ability each class of chemokine ligand to activate FAK through binding to US28. We have previously demonstrated that RANTES binding to US28 stimulates the activation of FAK, promoting a specific association between phosphorylated FAK and the adaptor protein Grb2. FAK is a critical mediator of focal adhesion turnover and plays important roles in cellular adhesion and motility. As such, it displays high basal activity levels in most cell types. For these experiments we developed a clean inducible signaling assay using FAK knockout mouse fibroblasts (FAK -/-) that have been reconstituted with an adenovirus vector expressing wild-type FAK concurrent with the addition of Ad-US28 (Streblow, Vomaske et al. 2003). To determine the ability of CC-chemokines and the CX3C-chemokine Fractalkine to promote US28 mediated activation of FAK and formation of active Grb2/FAK complexes, FAK -/- cells expressing US28 alone or in combination with FAK were stimulated with RANTES, MCP-1 or Fractalkine (40 ng/ml) for 0 (unstimulated), 5, 10, 15 or 30 minutes. Grb2 was immunoprecipitated and active FAK associated with Grb2 visualized by western blotting for Phospho-Tyr (Streblow, Vomaske et al. 2003). RANTES, MCP-1 and Fractalkine all promoted US28-mediated FAK activation and formation of

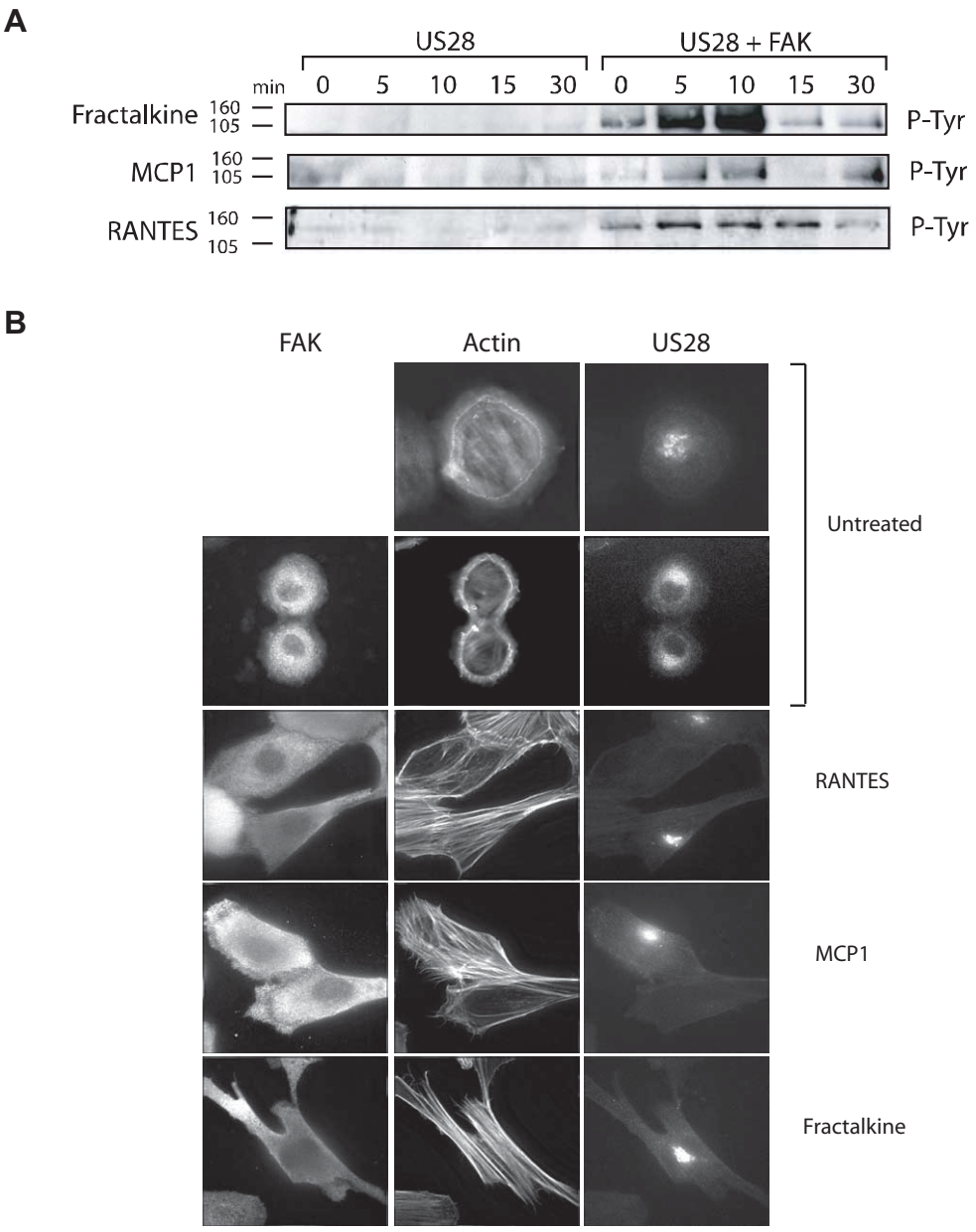


**Figure 2.2 - All US28 ligands are capable of activating FAK and inducing Actin Stress Fiber Formation in reconstituted FAK -/- cells.**

**(A)FAK activation was determined by Grb2/FAK co-immunoprecipitation reactions on Ad-FAK reconstituted FAK -/- cells infected with Ad-US28 that were treated with RANTES, Fractalkine, MCP-1. Cells were harvested in modified RIPA buffer at 0 (unstimulated), 5, 10, 15, and 30 minutes post addition of ligand. Active FAK associated with Grb2 was visualized by western blotting for phospho-FAK. (B) FAK null cells infected with Ad-US28 were reconstituted with WT FAK via adenovirus transduction. RANTES, MCP-1, or Fractalkine treated cells were fixed two hours post addition of ligand. Cells were stained for actin with phalloidin (actin) and FAK using antibodies directed against the FAK-N'terminal HA-tag, and US28 using antibodies directed against the N-terminal Flag epitope present on US28. All images are 60X magnification.**

Grb2/FAK complexes with similar kinetics but slightly different magnitudes of activation (Figure 2.2A).

RANTES (CCL5)-induced signaling through US28 also promotes pronounced actin-cytoskeletal changes in multiple cell types (Streblow, Söderberg-Nauclér et al. 1999; Streblow, Vomaske et al. 2003; Melnychuk, Streblow et al. 2004). Therefore, we also examined the ability of RANTES, MCP-1, or Fractalkine to promote actin cytoskeletal re-arrangements through US28 in FAK -/- fibroblasts. FAK -/- cells infected with adenoviruses expressing US28 and FAK were stimulated with RANTES, MCP-1, or Fractalkine (40ng/ml). Two hours post-ligand stimulation, fixed and permeabilized cells were incubated with

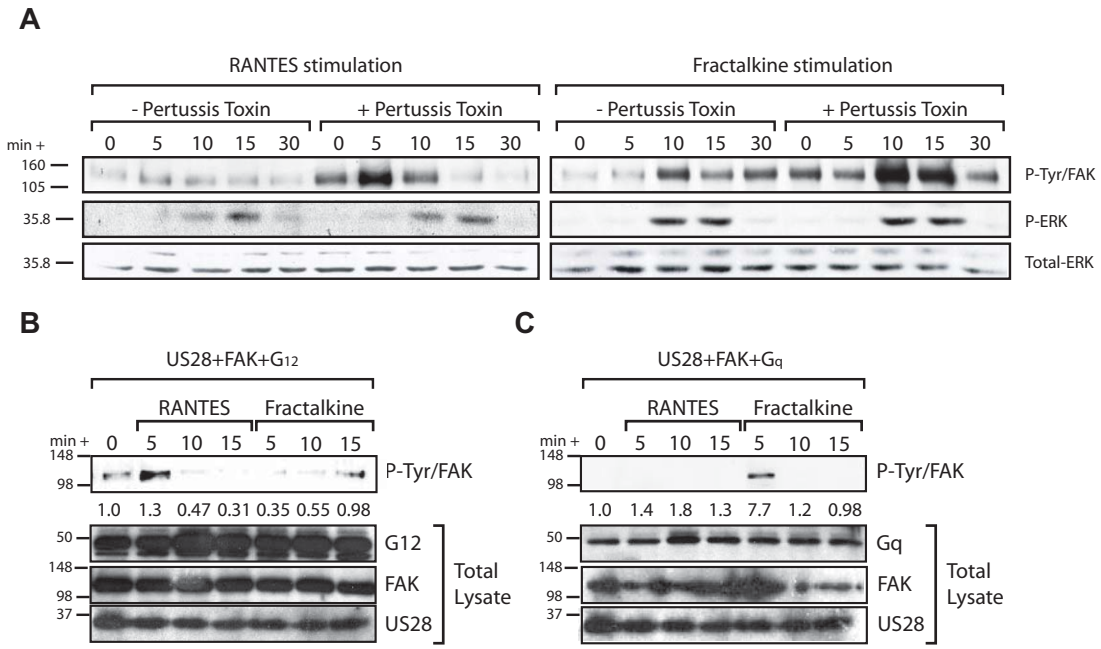


**Figure 2.3 - RANTES and Fractalkine activation of FAK is dependent on different G-proteins**
**(A) FAK activity in FAK -/- cells expressing both US28 and wt-FAK in response to either Fractalkine or RANTES and in the presence or absence of pertussis toxin was assessed by Grb2/FAK co-immunoprecipitation reactions. Cells were harvested in modified RIPA buffer at 0 (unstimulated), 5, 10, 15, and 30 minutes post addition of ligand. Active FAK associated with Grb2 was visualized by western blotting for phospho-tyrosine. (B)The ability of Gα12 and Gαq to enhance or abrogate RANTES and Fractalkine mediated activation of FAK through US28 was assessed by overexpressing Gα12 or Gαq in FAK -/- cells. FAK -/- cells infected with adenovirus expressing US28, wt-FAK and Gα12 or Gαq were stimulated with either RANTES or Fractalkine. As in (A), FAK activity was assessed by Grb2/FAK co-immunoprecipitation reactions and active FAK associated with Grb2 was visualized by western blotting for phospho-tyrosine. Western blots were quantified by densitomitry and fold FAK activation compared to unstimulated control is indicated below each lane.**

antibodies directed against the Flag (US28) and HA (FAK) epitopes, and actin visualized by staining with Phalloidin. While RANTES, MCP-1, and Fractalkine failed to stimulate morphological changes in the absence of US28 (data not shown) each of the three ligands readily promoted actin cytoskeletal re-arrangements in US28 expressing cells (Figure 2.2B). Although RANTES, MCP-1 and Fractalkine differ with respect to their ability to promote SMC migration through US28, all are capable of promoting FAK activation and formation of active Grb2-FAK complexes, as well as re-organization of the actin-cytoskeleton in fibroblasts.

### *2.4.3 Fractalkine and RANTES induced FAK activation through US28 require different G-proteins.*

Our data indicate that although CC- and $CX_3C$-chemokine stimulation of US28-expressing SMC produces different migratory phenotypes, both classes of ligands are capable of activating common pro-migratory signaling cascades in US28-expressing fibroblasts. We hypothesized that the disparate phenotypes seen in US28-expressing cell types is a result of differential coupling of G-proteins to US28. To identify the G-proteins involved in RANTES and Fractalkine stimulated FAK activation through US28, Grb2-FAK co-immunoprecipitation reactions were performed on lysates from reconstituted FAK-/- cells expressing US28. Cells were pre-treated with the Gαi/o inhibitor PTX or were left untreated and then stimulated with either RANTES or Fractalkine (40 ng/ml) and Grb2/FAK co-immunoprecipitations were visualized by western blotting. Pre-treatment with PTX significantly enhanced both Fractalkine and RANTES mediated activation of FAK through US28, suggesting that both ligands promote coupling to G-proteins other than Gαi/o family G-proteins to induce FAK activation (Figure 2.3A). Interestingly, stimulation of US28 expressing cells with either RANTES or Fractalkine led to the PTX resistant activation of ERK-1/2. Unlike US28 mediated FAK activation, which was enhanced by PTX, ERK-1/2

activation was not affected by PTX pre-treatment. Therefore, US28 mediated activation of ERK-1/2 in reconstituted FAK-/- cells is independent of Gαi/o family G-proteins, differing from PTX-sensitive MCP-1 and RANTES induced ERK-2 activation by US28 observed in 293 cells (Billstrom, Johnson et al. 1998).

We have previously determined that US28-mediated SMC migration requires the Gα12/13-dependent activation of RhoA (Melnychuk, Streblow et al. 2004). Additionally, Fractalkine stimulation of US28 has been used as an inhibitor of Gαq/11-mediated constitutive activation of phospholipase-C (PLC) and NF-κB (Casarosa, Menge et al. 2003). Since RANTES- and Fractalkine-induced activation of FAK through US28 is independent of Gαi/o family G-proteins, and US28 is known to signal through Gα12 to promote cellular migration in SMC, we assessed the role of Gα12 in promoting RANTES and Fractalkine mediated activation of FAK. Reconstituted FAK-/- cells infected with adenoviruses expressing US28 and wild-type Gα12 were stimulated with either RANTES or Fractalkine. FAK activation was determined using Grb2-FAK co-immunoprecipitation reactions as described above. Introduction of high levels of Gα12 had little effect on the kinetics of FAK activation by RANTES, but significantly delayed and reduced FAK activation by Fractalkine (Figure 2.3B). In similar assays, over-expression of Gαq abrogated RANTES-mediated FAK activation while Fractalkine mediated FAK activation was unaffected by expression of this G-protein (Figure 2.3C). These data are consistent with the observation that Fractalkine binding to US28 specifically decreases the constitutive activation of PLC and NF-κB via a Gαq/11 dependent mechanism. This study, combined with our previous findings, shows that US28 G-protein coupling occurs in a ligand-specific manner wherein RANTES promotes US28 coupling to Gαi/o, Gα16 and Gα12/13, while Fractalkine promotes US28 coupling to Gαq (Melnychuk, Streblow et al. 2004).

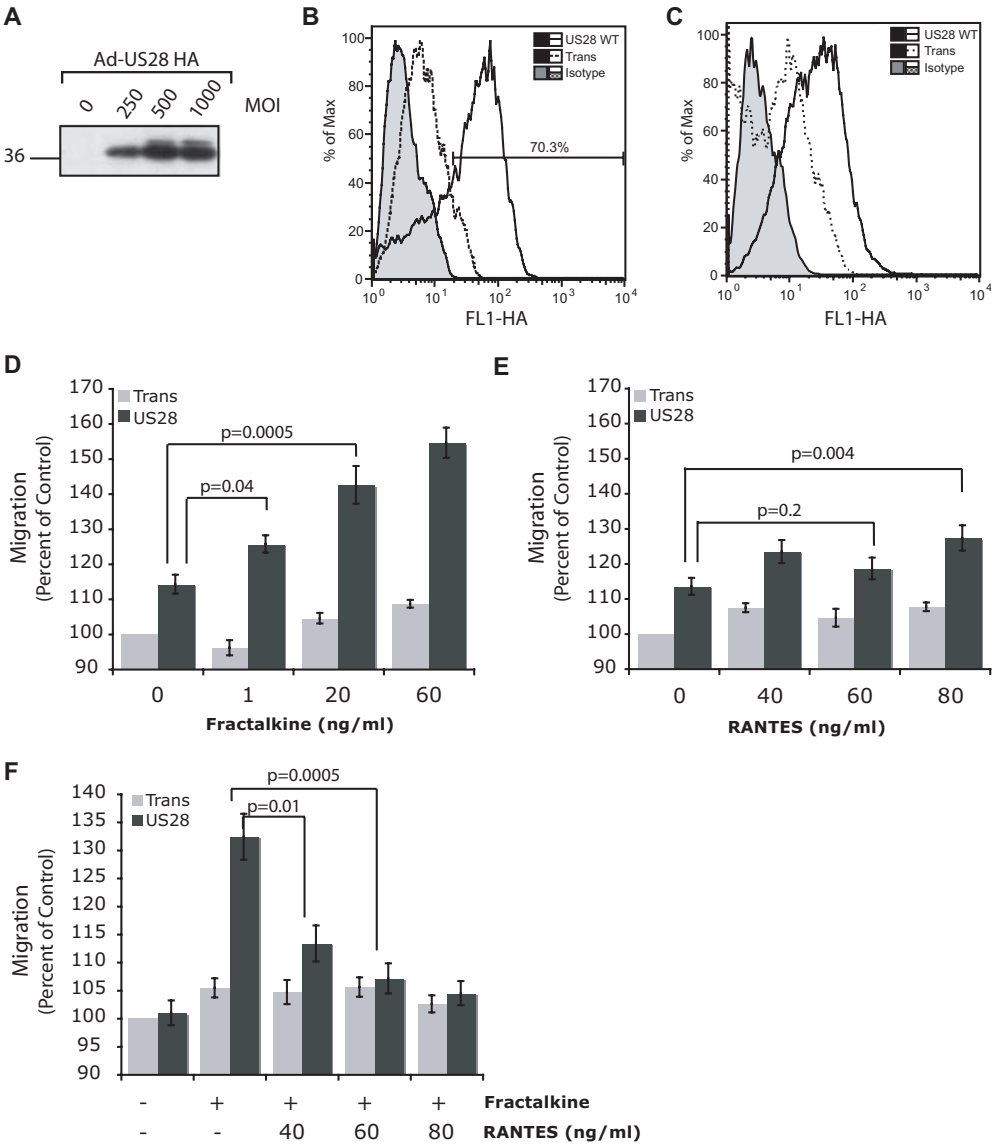


**Figure 2.4 - Figure 4. Fractalkine Induces US28-Mediated Migration of Macrophages**

**(A) Expression of US28 was determined via western blot analysis of total cellular lysate for the HA epitope tag. 2x105 rat macrophages were infected for 72 hours with US28-HA adenovirus vector at the indicated MOI. (B) The efficiency of adenovirus transduction was determined by FACS analysis of permeablized rat macrophages infected for 72 hours at MOI 250 with US28-HA adenovirus vector. (C) Surface expression of US28 was confirmed via FACS analysis of non-permeablized rat macrophages infected for 72 hours at MOI 250 with US28-HA adenovirus vector. In vitro migration assays were performed on 1x105 Ad-US28 and/or Ad-Trans infected rat macrophages treated with the indicated concentrations of (D) Fractalkine or (E) RANTES. For all conditions, n≥12 from four independent experiments. Percentages are calculated relative to unstimulated macrophages infected with adenovirus transactivator (Trans). (F) Similar migration assays were performed on Ad-US28 expressing macrophages treated with 40ng/ml of Fractalkine and the indicated concentrations of RANTES as a competing ligand. For all conditions, n≥12 from two independent experiments. Percentages are calculated relative to unstimulated macrophages infected with Ad-Trans.**

### *2.4.4 Ligand-Specific US28 Mediated Macrophage Migration*

Although Fractalkine binding to US28 fails to promote migration in SMC, we have demonstrated that Fractalkine stimulation causes cytoskeletal rearrangements and activates pro-migratory signaling pathways in fibroblasts via Gαq. Given that the endogenous complement of G-proteins differs between cell types, we hypothesized that Fractalkine binding to US28 may mediate migration of a second HCMV-susceptible cell type. Fractalkine (CX3CL1), is the only known CX3C chemokine and is unique among chemokines in that it has both membrane bound and soluble forms. Fractalkine is both a chemotactic signal for monocytes and sufficient for monocyte activation and adhesion under flow conditions (Umehara, Bloom et al. 2004). HCMV infection of monocyte/macrophages is an important dissemination vehicle in vivo (Waldman, Knight et al. 1995; Gerna, Percivalle et al. 2000). We hypothesized that the capacity of US28 to bind Fractalkine with high affinity, in addition to CC-chemokine ligands, may play a role in HCMV infection of monocytes and that, in contrast to SMC; Fractalkine stimulus may be pro-migratory in US28-expressing monocytes. We attempted these experiments in human monocytes in the context of HCMV infection. However, the presence of endogenous chemokine receptors (including RANTES-binding CCR1 and CCR5 as well as the human Fractalkine receptor CX3CR1) and endogenous chemokine ligands in these cells made the experimental results difficult to interpret. To compensate for technical difficulties, US28 was expressed from an adenoviral vector in the context of a rat macrophage cell line. We reasoned that compared to ligands produced in human monocytes fewer endogenous rat chemokines would functionally interact with US28 and, similarly, fewer endogenously expressed rat chemokine receptors would signal productively in response to stimulation with recombinant human chemokines.

Using a low temperature, low volume infection protocol, rat macrophages were infected with adenovirus expressing US28 at various MOI (Figure 2.4A). Flow cytometry analysis

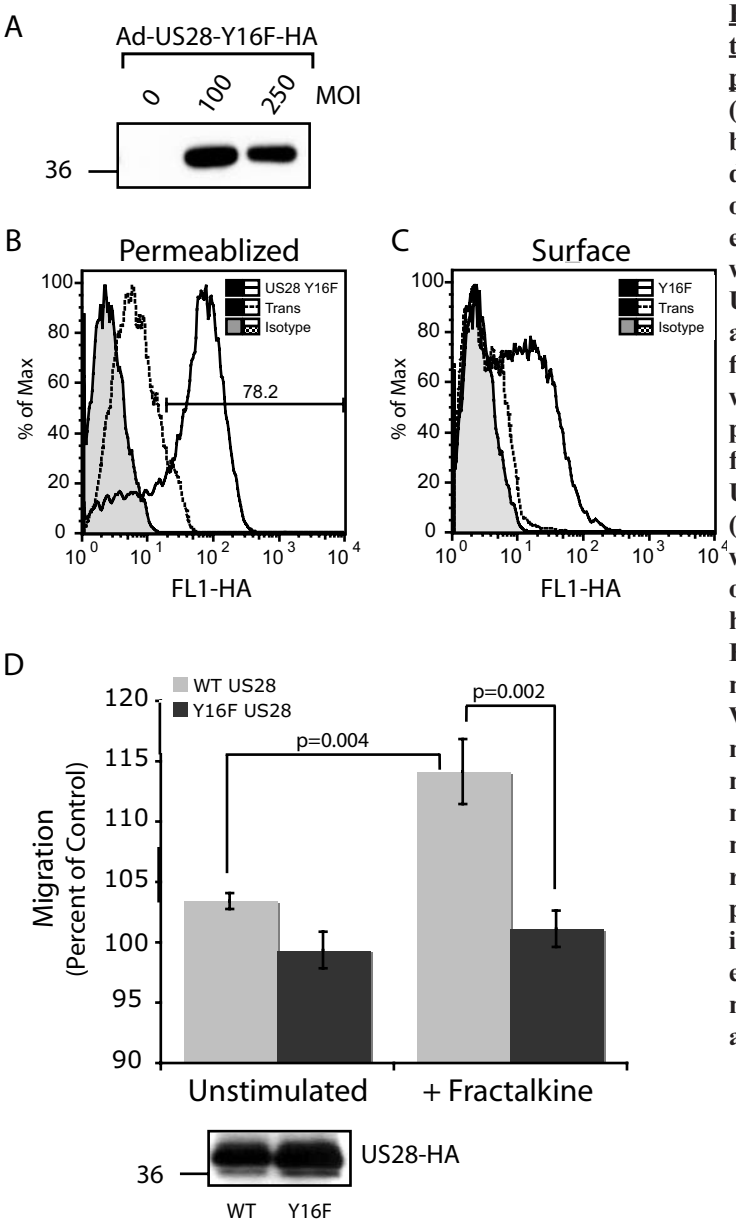


**Figure 2.5 - US28-Mediated Migration of Macrophages is Ligand-Dependent**

**(A) Expression of a chemokine binding mutant US28-Y16F-HA was determined via western blot analysis of total cellular lysate for the HA epitope tag. 2(10)5 rat macrophages were infected for 72 hours with US28-Y16F-HA adenovirus vector at the indicated MOI. (B) The efficiency of adenovirus transduction was determined by FACS analysis of permeablized rat macrophages infected for 72 hours at MOI 250 with US28-Y16F-HA adenovirus vector. (C) Surface expression of US28-Y16F was confirmed via FACS analysis of rat macrophages infected for 72 hours at MOI 100 with US28-Y16F-HA adenovirus vector. (D) In vitro migration assays were performed on WT US28 or US28-Y16F infected rat macrophages with or without 10ng/ml Fractalkine. For all conditions, n=8 from two independent experiments. Percentages are calculated relative to unstimulated macrophages infected with Ad-Trans. Inset is the western blot showing equal expression of US28-WT and Y16F in macrophages used for this migration assay.**

was used to demonstrate US28 expression as approximately 70% of permeablized macrophages stained for the HA tag (Figure 2.4B). US28 was expressed on the cell surface of adenovirus-infected macrophages. (Figure 2.4C). The response of US28-expressing macrophages to treatment with recombinant human RANTES and Fractalkine was assessed using a quantitative in vitro migration assay. In these assays, Fractalkine induced robust migration of US28-expressing macrophages (Figure 2.4D). Statistically significant

migration was seen at very low (1 ng/ml) concentrations of chemokine but not in control cells expressing only Trans. In contrast, RANTES caused weak migration of macrophages presumably due to low levels of Gα12 expressed in these cells. Only the highest dose (80 ng/ml) of RANTES achieved statistical significance and this response was not titratable with increasing chemokine as seen with Fractalkine stimulation (Figure 2.4D&E). These results suggest that Fractalkine is the predominant chemotactic signal in US28-expressing macrophages. To test this possibility, we performed chemokine competition experiments similar to those performed in SMC (Figure 2.1B) to determine whether RANTES and Fractalkine have any synergistic effect on US28-mediated macrophage migration. Fractalkine-dependent macrophage migration was inhibited in a dose-dependent manner by increasing concentrations of RANTES as a competing ligand (Figure 2.4F). These results show that, in direct contrast to results seen in SMC, RANTES is a competitive inhibitor of Fractalkine-mediated macrophage migration.

To demonstrate that the US28-induced macrophage migration specifically required US28-Fractalkine interaction, we expressed the US28 mutant (Y16F), which is deficient in RANTES and Fractalkine binding (Casarosa, Waldhoer et al. 2005). US28-Y16F is efficiently expressed in adenovirus-infected macrophages (Figure 2.5A) and is present on the cell surface (Figure 2.5B). Macrophages expressing Y16F mutant did not migrate in response to Fractalkine (Figure 2.5C). Taken together these results demonstrate that US28-expressing macrophages respond to stimulus with recombinant human chemokine in a ligand-specific manner. Furthermore, in contrast to the CC-chemokine mediated migration phenotype in SMC, Fractalkine binding to US28 produces robust migration in macrophages. These are the first data to demonstrate a specific cellular phenotype mediated by US28 binding to Fractalkine and the first example of ligand-specific chemotaxis mediated by a multiple chemokine family binding receptor.

## 2.5 Discussion

In the current report, by examining the functional responses, signaling characteristics, and transcriptional profiles induced by US28 upon binding a diversity of ligands, we demonstrate that not only is US28-signaling ligand- and cell-type dependent but also ligand and cell type-specific. While RANTES stimulation of US28 causes robust SMC migration, Fractalkine provides an anti-migratory signal in these cells. Similarly, RANTES but not Fractalkine increases transcription of genes involved in SMC migration. In contrast, Fractalkine but not RANTES provides a strong chemotactic stimulus for US28-expressing macrophages, and RANTES competitively inhibits Fractalkine-mediated macrophage migration. Interestingly, while these ligands display differential signaling characteristics with respect to cellular migration, they both are capable of activating FAK and producing actin cytoskeletal rearrangements in fibroblasts. Importantly, we demonstrate that these phenotypic differences can be attributed to RANTES and Fractalkine causing differential G-protein coupling to US28. Fractalkine induced-US28 signaling occurs in a Gαq-dependent manner and is abrogated in the presence of Gα12 but not by PTX. However, RANTES induced migration and signal transduction occurs in a Gα12 dependent manner and is blocked by over expression of Gαq. Ultimately, our findings indicate that US28 binding to RANTES or Fractalkine results in differential G-protein coupling/activation leading to unique functional consequences.

While most chemokine receptors bind a limited subset of chemokines from a single chemokine subfamily, there are three examples of chemokine receptors that bind chemokines from multiple subfamilies: the DARC-receptor, Orf74 of HHV-8, and US28 (Szabo, Soo et al. 1995; Kledal, Rosenkilde et al. 1998; Rosenkilde, Kledal et al. 1999; Lentsch 2002). To date DARC, which binds both CC- and CXC-chemokines (CCL2, CCL5, CXCL1, and CXCL8), is the only true chemokine sink because this receptor binds and internalizes these

ligands without inducing signaling events. Orf74 has also been demonstrated to bind both CC- and CXC-chemokines; however, there is a significant difference in the affinity of individual ligands for this receptor. Despite being referred to as an IL-8 receptor, Orf74 has greater affinity for GRO peptides (α, β, γ) than for IL-8 (Rosenkilde, Kledal et al. 1999). In competition binding assays with IL-8, Orf74 binding to the CC-chemokines MIP-1α, MIP-1β, MCP-1, and RANTES was virtually undetectable, while MCP-3 and aminooxypentane (AOP)-RANTES display affinities in the 200 nm range. Interestingly, the structurally distinct, CXC-chemokines lacking the ELR motif (non-ELR CXC chemokines) IP-10 and SDF-1α can displace IL-8 binding, and function as efficient inverse agonists of Orf74 signaling at nanomolar concentrations (Rosenkilde, Kledal et al. 1999). Although Orf74 binds to chemokines from multiple chemokine subfamilies, Orf74 signaling only occurs in the presence of ELR, and pro-inflammatory/angiogenic chemokines, whereas the angiostatic non-ELR CXC-chemokines function as efficient inverse agonists. Unlike Orf74, US28 binds multiple ligands from different chemokine subfamilies with near equal affinity (Kuhn 1995; Kledal, Rosenkilde et al. 1998), and as we demonstrate in the current report, these distinct ligands promote cellular activation upon binding US28. Therefore, to date, US28 is the only chemokine receptor capable of signaling upon binding ligands from multiple chemokine subfamilies.

We have demonstrated that both MCP-1 and RANTES promote US28-mediated SMC migration (Streblow, Söderberg-Nauclér et al. 1999). While Fractalkine is a known modulator of US28-induced constitutive signaling activity (Casarosa, Bakker et al. 2001; Mokros, Rehm et al. 2002), we have shown that Fractalkine does not promote US28-mediated SMC migration and actually inhibited RANTES mediated SMC migration. In accordance with these ligand-specific functional responses, microarray analysis of US28-expressing SMC stimulated with either RANTES or Fractalkine revealed profound differences at the

level of gene induction. In the context of CMV-infection of SMC, the ability of US28 to adhere to mobilized Fractalkine, coupled with our finding that this chemokine reverses the transcriptional activation required for cellular migration in SMC, suggests that Fractalkine may arrest US28-induced SMC migration and promote the subsequent adhesion of US28 expressing SMC to the vascular endothelium. The migration of HCMV infected and US28 expressing SMC from the vessel media to inflammatory sites in the vessel intima and the subsequent adhesion and accumulation of SMC in the vessel intima may have important implications in the dissemination and *in vivo* pathogenesis of HCMV, as well as in the exacerbation of vascular disease.

In this study, we also demonstrate that Fractalkine causes robust migration of US28-expressing macrophages, which is the first known cellular phenotype associated with Fractalkine binding to US28. This finding indicates that, in addition to being ligand-dependent and ligand-specific, the function of US28 signaling is also cell type-specific. Our finding that Fractalkine causes migration of US28-expressing macrophages suggests a further role for US28 in the development of vascular disease. US28 has been shown to be expressed in HCMV-infected peripheral blood mononuclear cells (Patterson, Landay et al. 1998). Foam cells found in atherosclerotic lesions originate as circulating monocytes and chemokines play an important role in the deposition of monocytes in lesions (Glass and Witztum 2001). In particular, Fractalkine expression is known to be important for the development of atherosclerosis in mouse models of heart disease via recruitment of macrophages into atherosclerotic plaques (Combadiere, Potteaux et al. 2003; Lesnik, Haskell et al. 2003). Expression of membrane-bound Fractalkine can be induced on endothelial cells by numerous cytokines including IFN-$\gamma$, TNF-$\alpha$ and IL-1, resulting in the recruitment of inflammatory cells and contributing to chronic inflammatory vascular diseases such as atherosclerosis, restenosis following angioplasty and transplant vascular sclerosis (Bazan,

Bacon et al. 1997). Unlike other chemokines which are secreted as soluble molecules that must associate with proteoglycans and other components of the extracellular matrix to establish chemokine gradients (Tanaka, Adams et al. 1993), Fractalkine is generated as a membrane-bound ligand with the chemokine domain presented at the top of the cell-bound mucin-like stalk (Bazan, Bacon et al. 1997; Pan, Lloyd et al. 1997). In many instances this ligand is more effective than other ligands in promoting leukocyte activation and migration. Our current findings suggest a secondary mechanism for US28 in CMV-mediated vascular pathology by which circulating CMV-positive monocytes infiltrate atherosclerotic plaques mediated by Fractalkine binding to US28.

We demonstrate for the first time that Fractalkine is a potent agonist capable of inducing cellular migration in macrophages and activation of signaling pathways upon binding US28. Prior to this study, Fractalkine had been employed as a modulator of US28-mediated constitutive signaling activity. Some of the signaling pathways activated by Fractalkine were similar to those activated by the CC-chemokines. For example, RANTES, MCP-1, and Fractalkine all display similar abilities to induce ERK-1/2, actin cytoskeletal rearrangements and formation FAK-Grb2 complexes in fibroblasts. Pre-treatment with PTX enhanced Fractalkine mediated FAK activation through US28, which indicated that Fractalkine promoted US28 coupling to G-proteins other than Gai/o. Expression of Gα12 delayed and reduced FAK activity via Fractalkine signaling through US28 but had no effect on RANTES/US28 activation of FAK. Importantly, overexpression of Gαq blocked RANTES signaling to FAK but had no effect on Fractalkine-mediated FAK activation. In a number of different activation scenarios FAK is a known point of signaling convergence and has been demonstrated to be phosphorylated in response to Gαq/11, Gαi/o, and Gα12/13 coupled receptors in various cell types and signaling environments (Chikumi, Fukuhara et al. 2002; Deo, Bazan et al. 2004; Bian, Mahanivong et al. 2006; Morishita, Ueda et al.

2007). In one study, lysophosphatidic acid (LPA) signaling stimulated both membrane association and autophosphorylation of FAK but these two effects were separable and mediated by different G-alpha subunits (Gαi1 and Gα12/13, respectively) presumably via signaling from two different LPA receptors (Bian, Mahanivong et al. 2006). Importantly, in a receptor-decoupled system of constitutively active G-alpha subunits, significant FAK phosphorylation can be observed via signaling through Gαq, Gα12 and Gα13 (Chikumi, Fukuhara et al. 2002). These results are consistent with our observations that both RANTES and Fractalkine binding to US28 can activate FAK via different signaling cascades mediated by different G-proteins. Our results suggest that overexpression of off-target G-proteins inhibit signaling from a particular ligand via competition with the G-proteins that would normally promote signaling from the ligand-bound activated receptor. Therefore, in these experiments overexpression of Gα12 may act as a dominant inhibitory molecule that prevents Gαq-receptor interactions, which would normally activate FAK following Fractalkine coupling to US28. Overexpression of Gαq prevents Gα12 coupling to the RANTES-bound activated form of US28 thereby abrogating the downstream signaling to FAK. Therefore, RANTES stimulates varying signaling pathways through different G-proteins in SMC (Gα12-dependent) and fibroblasts (Gαi/o independent). Fractalkine signals from US28 via coupling of Gαq in fibroblasts, SMC and macrophages. Together these findings demonstrate that not only is US28 signaling ligand-dependent and ligand-specific, it utilizes differential G-protein coupling to produce cell-type specific signaling and differential phenotypic responses.

In this report, we demonstrate that similar to RANTES and MCP-1, Fractalkine is a potent US28 agonist that promotes migration in macrophages, robust signaling through FAK and ERK1/2 and induces actin cytoskeletal rearrangements in fibroblasts. Unlike RANTES and MCP-1, Fractalkine fails to induce SMC migration, or increase expression of cellular

genes involved in motility and signaling in SMC, thus demonstrating that US28 signaling is ligand-specific and cell type-specific. In addition, the US28 ligand-specific and cell-type dependent activation of differential signaling pathways suggest that this chemokine receptor has the capacity to couple to different G-proteins depending upon the ligand bound and the cellular G-protein environment. Therefore, US28 binds to a diversity of chemokines, which promote US28 coupling to multiple G-proteins, eliciting functional signaling through these various G-proteins. HCMV encounters and infects a multitude of distinct cell types *in vivo* including fibroblasts, monocyte/macrophages, endothelial cells and SMC. These cell types differ substantially with respect to the G-proteins that they express. The ability of US28 to respond to multiple signaling environments and couple to multiple G-proteins may have important implications in the persistence and pathogenesis of HCMV in these different cell-types.

## 2.6 Materials and Methods

*Cell Lines.* The life-extended human pulmonary artery smooth muscle cell line, PAT1 (Melnychuk, Streblow et al. 2004) were maintained in Medium 199 supplemented with 20% fetal calf serum (FCS) and penicillin-streptomycin-L-glutamine (PSG; Gibco). For migration and microarray experiments, PAT1 cells were utilized between passage 5 and 30 post-telomerization. Primary F344 rat smooth muscle cells (RSMC) were maintained in Dulbecco's modified Eagle's Medium (DMEM) with 10% FCS and PSG. RSMC were used between passage 5 and 20. NR8383 rat alveolar macrophages were maintained in RPMI with 10% FCS and PSG. Mouse FAK-/- fibroblasts were maintained on gelatin coated culture dishes in DMEM supplemented with 10% FCS, PSG, non-essential amino acids (Cellgro), and G418 (Sigma; 500μg/ml) as previously described (Ilic, Furuta et al. 1995; Sieg, Ilic et al. 1998). FAK-/- cells used in experiments were between passage 5 and 15.

*Reagents.* Recombinant human RANTES, MCP-1, and Fractalkine were purchased from R&D Systems. Anti-Grb2 (C-7), anti-phosphotyrosine (PY99), anti-Gα12 (S-20), anti-Gαq (E-17) and anti-HA (F-7) antibodies were purchased from Santa Cruz Biotechnology. Phospho-specific ERK-1/2 (Thr202/Tyr204) and total ERK-1/2 antibodies were from Cell Signaling Technologies. Anti-M2-Flag antibody (F-3165) was purchased from Sigma. Secondary anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (NA934V and NA931V) were purchased from Amersham.

*Adenovirus Construction.* Adenoviruses expressing Gα12 Gαq, WT-FAK, US28-Flag, and US28-HA were previously described (Streblow, Söderberg-Nauclér et al. 1999; Streblow, Vomaske et al. 2003; Melnychuk, Streblow et al. 2004). Adenovirus vectors expressing US28-Y16F-HA were constructed by mutagenesis of the US28-HA construct in pAdTet7. This vector contains the tet-responsive enhancer within a minimal CMV promoter followed by the SV40 late poly(A) cassette, adenovirus E1A, and a single loxP site to increase recombination frequency. Complementary 30bp primers containing coding sequence for amino-acids 2-25 of US28-HA and including a phenylalanine codon in place of the tyrosine at position 16 (5'-ACGACGGAGTTTGACTTCGACGATGAAGCG-3' and 5'-CGCTT CATCGTCGAAGTCAAACTCCGTCGT-3') were used to PCR amplify mutated vector using Pfu Turbo DNA Polymerase (Stratagene). Non-mutated methylated parental DNA was digested using *DpnI* and mutated plasmid was propagated in DH5α. Recombinant adenoviruses were produced by pAdTet7 US28-Y16F-HA construct co-transfection of 293 cells expressing the Cre-recombinase with adenovirus DNA (Ad5-ψ5) that contains an E1A/E3-deleted adenovirus genome (Hsia, Mitra et al. 2003). Recombinant adenoviruses were expanded on 293-Cre cells and the bulk stocks were titered on 293 cells by limiting dilution. Gene expression was driven by co-infection with Ad-Trans expressing the Tet-off transactivator as previously described (Streblow, Söderberg-Nauclér et al. 1999).

*Flow Cytometry.* To monitor surface expression of recombinant proteins and total adenovirus transduction, adenovirus-infected cells were fixed in 2% PFA for 15min, washed 2X with PBS, blocked for 15min on ice in Fc Block (PBS+20%Normal goat serum (NGS)+0.1% sodium azide). To determine the rate of adenovirus transduction, cells were permeablized with PBS containing 0.2% Saponin and 0.02%NGS for 15min on ice. For both cell surface and intracellular staining assays the cells were incubated for 30min with either mouse IgG2b isotype control or primary αHA antibody diluted 1:200 in FACS wash buffer (PBS+1% NGS+0.01% sodium azide +/- 0.2% saponin as appropriate) on ice and washed 2X with FACS wash buffer. Primary antibody staining was detected with anti-mouse Alexa-Fluor597 antibody diluted 1:1000 in FACS wash. After 20 min incubation with secondary antibody on ice cells were washed as above and surface expression was quantified using flow cytometry (FACS Calibur, BD Biosystems). Data analysis was performed using FlowJo software v8.8 (Treestar Inc.).

*Immunocytochemistry.* FAK**-/-** fibroblasts were grown in 0.1% gelatin coated 4-well chamber slides (Nalge-Nunc). US28 and/or FAK was expressed using the adenovirus vectors described above and were left untreated or were treated with MCP-1, RANTES or Fractalkine (20ng/ml) for 2 hrs. The cells were washed in PBS and fixed in phosphate buffered 2% paraformaldehyde (PFA) for 15 minutes at r.t., then permeabilized and blocked with 0.2% Saponin + 0.02% BSA in PBS for 15min at r.t. Thereafter, the cells were incubated with antibodies against US28-Flag epitope or FAK-HA epitope in a 1:200 dilution for 1 hr at room temperature. Cells were washed three times in blocking buffer and binding of the primary antibody was detected with a fluorescein isothiocyanate-tetramethyl (FITC) conjugated goat anti-mouse or rhodamine conjugated goat anti-rabbit antibody for 1 hr at room temperature. At this time the cells were also stained for actin using Phalloidin (Molecular Probes, Eugene, OR) to monitor alterations in cellular actin cytoskeleton

induced by US28 and FAK. Fluorescence positive cells were visualized on an inverted Applied Precision Deltavision™ deconvolution microscope.

*Immunoprecipitation Reactions.* FAK-/- cells were plated in 10cm culture dishes and serum starved for 6 hrs upon achieving 50% confluence. The cells were co-infected with Ad-Trans and/or Ad-US28 and/or Ad-FAK WT at MOI 50. After 16 hrs the cells were stimulated with RANTES (40ng/ml), Fractalkine (40ng/ml), or MCP-1 (40ng/ml) and then harvested at times 0 (unstimulated), 5, 10, 15, and 30 minutes post ligand addition. Cells were lysed in modified RIPA buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS and total Grb2 was immunoprecipitated and samples analyzed by western blotting using antibodies directed against phospho-Tyr (Streblow, Vomaske et al. 2003). Co-precipitation of FAK-HA was demonstrated by stripping the blots in buffer containing 0.1M Tris pH 6.8, 1% SDS, and 1% 2-mercaptoethanol and staining using antibodies directed against HA. Prior to immune-complex reactions, a total of 50µl of cellular lysate was assayed by SDS-PAGE/western blotting for the presence of input US28 and FAK using antibodies directed against the HA-epitope present on both recombinant proteins.

*SMC migration assay.* SMC migration assays were performed as previously described (Streblow, Söderberg-Nauclér et al. 1999). Briefly, 4x104 primary rat SMCs were added to each upper well of a transwell (12 mm diameter, 3.0 µm pore size, Costar Corning, Cambridge, MA). Cells were serum starved for 16 hrs, and then infected with Ad-Trans only or Ad-Trans and Ad-US28-HA at MOI 200. After 4 hrs, the inserts were washed and transferred to fresh 12-well plates with chemotactic stimulus. Cells migrating to the lower chamber were quantified at 48-72 hrs p.i. via fluorescence using CyQuant (Invitrogen) and read on a Molecular Devices Flexstation® II fluorescence plate reader. Migration was determined from 4-6 independent wells per assay per condition. Mean and standard deviation were calculated. Percent of control values were generated by comparing chemokine

stimulated US28-expressing cells to unstimulated control cells (Trans-only) and compared using Student's t test. P values <0.05 were considered statistically significant.

*Macrophage Migration Assay.* NR8383 macrophages were co-infected with Ad-Trans and Ad-US28 WT or Ad-US28-Y16F at MOI 100. Macrophages were incubated with adenovirus in 200µl total volume for 30min at room temperature, diluted into 10ml complete RPMI and incubated at 37°C. At 72 hrs post-infection, 1x105 infected macrophages were added to the top well of a chemotaxis chamber (96-well Millipore Multiscreen, 3.0µm pore size) with Fractalkine and/or human RANTES in the bottom chamber. Chemotaxis was allowed to proceed for 1 hr at 37°C. Top chambers were discarded and migrated cells in the bottom chamber were quantified via fluorescence using CyQuant (Invitrogen) and read on a Molecular Devices Flexstation® II fluorescence plate reader. Migration was determined from 4-6 independent wells per assay per condition. Mean and standard deviation were calculated. Percent of control values were generated by comparing chemokine stimulated US28-expressing cells to unstimulated control cells (Trans-only) and compared using Student's t test. P values <0.05 were considered statistically significant. Recombinant protein levels were monitored by western blotting and flow cytometry staining for total and surface expression and equalized by adjusting the adenoviral vector MOI accordingly.

# III

# Chapter 3 - HCMV pUS28 Initiates Pro-migratory Signaling via Activation of Pyk2 Kinase

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## 3.1 Abstract


### *3.1.1 Background*

Human Cytomegalovirus (HCMV) has been implicated in the acceleration of vascular disease and chronic allograft rejection. Recently, the virus has been associated with glioblastoma and other tumors. We previously showed that the HCMV-encoded chemokine receptor pUS28 mediates smooth muscle cell (SMC) and macrophage motility and this activity has been implicated in the acceleration of vascular disease. pUS28 induced SMC migration involves the activation of the protein tyrosine kinases (PTKs) Src and Focal adhesion kinase as well as the small GTPase RhoA. The PTK Pyk2 has been shown to play a role in cellular migration and formation of cancer, especially glioblastoma. The role of Pyk2 in pUS28 signaling and migration are unknown.

### *3.1.2 Methods*

In the current study, we examined the involvement of the PTK Pyk2 in pUS28-induced cellular motility. We utilized in vitro migration of SMC to determine the requirements for Pyk2 in pUS28 pro-migratory signaling. We performed biochemical analysis of Pyk2 signaling in response to pUS28 activation to determine the mechanisms involved in pUS28 migration. We performed mass spectrometric analysis of Pyk2 complexes to identify novel Pyk2 binding partners.

### *3.1.3 Results*

Expression of a mutant form of Pyk2 lacking the autophosphorylation site (Tyr-402) blocks pUS28-mediated SMC migration in response to CCL5, while the kinase-inactive Pyk2 mutant failed to elicit the same negative effect on migration. pUS28 stimulation with CCL5 results in ligand-dependent and calcium-dependent phosphorylation of Pyk2 Tyr-

402 and induced the formation of an active Pyk2 kinase complex containing several novel Pyk2 binding proteins. Expression of the autophosphorylation null mutant Pyk2 F402Y did not abrogate the formation of an active Pyk2 kinase complex, but instead prevented pUS28-mediated activation of RhoA. Additionally, pUS28 activated RhoA via Pyk2 in the U373 glioblastoma cells. Interestingly, the Pyk2 kinase complex in U373 contained several proteins known to participate in glioma tumorigenesis.

### *3.1.4 Conclusions*

These findings represent the first demonstration that pUS28 signals through Pyk2 and that this PTK participates in pUS28-mediated cellular motility via activation of RhoA. Furthermore, these results provide a potential mechanistic link between HCMV-pUS28 and glioblastoma cell activation.

## 3.2 Background

Human cytomegalovirus (HCMV) is a β-herpesvirus able to establish a life-long persistent infection after primary infection has been cleared. Although infection is ubiquitous in the human population, persistent HCMV infection is commonly asymptomatic in healthy, immunocompetent individuals. However, HCMV causes significant disease in immunosuppressed patients and, despite effective antiviral therapies, HCMV infection is still a significant problem in congenital disease and in bone marrow transplant recipients. Additionally, HCMV has been associated with long-term diseases including the vascular diseases atherosclerosis, restenosis following angioplasty, and chronic allograft rejection following solid organ transplantation (Almond, Matas et al. 1993; Speir, Modali et al. 1994; Melnick, Adam et al. 1998; Cobbs, Harkins et al. 2002). Recently, HCMV was detected in human glioblastomas, and data suggest that HCMV exacterbates the progression of this disease (Scheurer, Bondy et al. 2008). Chemokines and their receptors have been

identified as key mediators in chronic inflammatory processes that attend the development of vascular diseases and play a role in tumor development. Herpesviruses manipulate the host chemokine system by regulating the expression of host chemokines and chemokine receptors as well as by encoding chemokine and chemokine receptor homologues. Indeed, HCMV encodes a CXC chemokine (UL146), a putative CC chemokine (UL128), and four potential chemokine receptors (US27, US28, UL33 and UL78). pUS28 is one of the best characterized chemokine receptors and has been implicated in the development of long-term pathologies associated with HCMV infection, including vascular disease and malignancies (Chee, Bankier et al. 1990; Chee, Satchwell et al. 1990; Penfold, Dairaghi et al. 1999; Akter, Cunningham et al. 2003; Maussang, Verzijl et al. 2006).

pUS28 contains homology to the CC-chemokine receptors (Gao and Murphy 1994) and binds to a broad spectrum of CC-chemokine ligands, including RANTES/CCL5, MCP-1/CCL2, MCP-3/CCL7, and MIP-1β/CCL4. pUS28 is unusual in that it is able to bind the $CX_3C$-chemokine Fractalkine/CX3CL1 with high affinity in addition to binding a variety of CC-chemokines (Kuhn 1995; Kledal, Rosenkilde et al. 1998). Although the N-terminal 22 amino acids of pUS28 have been shown to be essential for binding of both chemokine classes, CC-chemokines fail to compete with CX3CL1 for receptor occupation. Mutagenesis studies of pUS28 indicate that different N-terminal residues are critical for binding to each chemokine class (Kledal, Rosenkilde et al. 1998; Casarosa, Waldhoer et al. 2005). Chemokine binding to pUS28 initiates a variety of cellular signaling pathways (Billstrom, Johnson et al. 1998; Streblow, Vomaske et al. 2003; Melnychuk, Streblow et al. 2004; Vomaske, Melnychuk et al. 2009). Additionally, pUS28 has been shown to signal constitutively in the absence of ligand binding in several transformed cell lines (Casarosa, Bakker et al. 2001). pUS28 mediated migration of arterial SMC requires binding of the CC-chemokines CCL5 or CCL2 (Streblow, Söderberg-Nauclér et al. 1999), while CX3CL1

inhibits pUS28-mediated SMC migration via the CC-chemokines (Vomaske, Melnychuk et al. 2009). However, CX3CL1 actively promotes the robust migration of pUS28 expressing macrophages suggesting that pUS28 has a dual function dependent upon the cell type in which it is expressed and the local chemokine environment of that cell (Vomaske, Melnychuk et al. 2009). The binding of each chemokine ligand class (CC- vs. $CX_3C$-) results in differential signaling at the level of G-protein coupling to pUS28 (Vomaske, Melnychuk et al. 2009). In fact, pUS28 binding to the CC-chemokines promotes SMC migration through the activation of Gα12/13 and the small G-protein RhoA. pUS28-mediated SMC migration can be blocked by expression of a dominant negative RhoA or RhoA effector associated kinase-1 (ROCK-1) (Melnychuk, Streblow et al. 2004).

pUS28 signals through the non-receptor tyrosine kinases focal adhesion kinase (FAK) and Src in a ligand-dependent manner, and this activity is required for induction of pUS28-mediated SMC migration. (Streblow, Vomaske et al. 2003). FAK is comprised of a central kinase domain flanked on one side by an N-terminal FERM (erythrocyte band 4.1-ezrin-radixin-moesin) domain, which is involved in linking FAK to integrins and growth factor receptors and provides regulation of the Tyr-397 autophosphorylation site (Sieg, Hauck et al. 2000; Jacamo and Rozengurt 2005). Additionally, the FERM domain regulates FAK kinase activity via direct interaction with the kinase domain thereby blocking the access of substrates to the catalytic cleft (Lietha, Cai et al. 2007). The F.A.T. (focal adhesion targeting) domain is located C-terminal of the central kinase domain and is comprised of multiple protein-protein interaction motifs. FAK tyrosine phosphorylation following cellular stimulation is enhanced by its association with Src-family PTKs at Tyr-397. pUS28-mediated SMC migration is sensitive to the Src inhibitor PP2 and mutation of the FAK autophosphorylation site at Tyr-397 blocks SMC migration in a dominant-negative fashion. Conversely, a kinase-negative FAK mutant (FAK R454K) had no effect on

pUS28-mediated SMC migration (Streblow, Vomaske et al. 2003) suggesting that FAK acts as a protein scaffold rather than an active kinase in the signaling cascade leading to SMC migration.

Proline-rich Tyrosine kinase 2 (Pyk2), also a non-receptor tyrosine kinase is highly related to FAK, and plays an important role in cell motility (Gelman 2003). Recently, Pyk2 has been shown to be involved in the CCR5-mediated chemotaxis of dendritic cells via binding of HIV-1 gp120 (Anand, Prasad et al. 2009). In addition, Pyk2 is critical for Angiotensin II (AngII)-mediated migration of vascular SMC (VSMC), which is mediated activation of RhoA and its effector kinase ROCK via phosphorylation of PDZ-Rho-GEF (Ohtsu, Mifune et al. 2005; Ying, Giachini et al. 2009).

FAK and Pyk2 share an overall 45% nucleotide sequence homology with 60% amino acid identity within the catalytic domain and also have analogous sites for tyrosine phosphorylation and Src binding (Avraham, Park et al. 2000). Despite their striking sequence similarity, it is becoming increasingly clear that these molecules play very different roles in signaling cascades leading to cellular migration. Pyk2 is expressed in cells of the brain, hematopoetic cells, osteoblasts, some types of epithelium, SMC and fibroblasts (Gelman 2003). Unlike FAK, Pyk2 activation has a significant dependence on intracellular calcium levels (Li and Earp 1997; Avraham, Park et al. 2000). Calcium-mediated regulation of Pyk2 activity proceeds via binding of Ca2+/calmodulin to the Pyk2 FERM domain, allowing homodimerization and autophosphorylation at Tyr-402 (Kohno, Matsuda et al. 2008). Autophosphorylation of Pyk2 is believed to be a critical initial step in the activation of Pyk2 via the recruitment of Src to the phosphorylated Tyr-402 site, resulting in further tyrosine phosphorylation of Pyk2 (Dikic, Tokiwa et al. 1996; Park, Avraham et al. 2004). However, Pyk2 activation can proceed by both Src-dependent (Dikic, Tokiwa et al. 1996; Sieg, Ilic et al. 1998; Sorokin, Kozlowski et al. 2001; Cheng, Chao et al. 2002) and Src-independent

(Le Bouf, Houle et al. 2006; Wu, Jacamo et al. 2006) mechanisms. Additionally, Pyk2-mediated phosphorylation of paxillin is independent of Tyr-402 autophosphorylation in SYF cells (Park, Avraham et al. 2004). Collectively these data demonstrate that Tyr-402 autophosphorylation is not absolutely required for Pyk2 activity. While Pyk2 can partially compensate for the lack of FAK in fibroblasts derived from FAK-/- mouse embryos, Pyk2 alone is not sufficient to reconstitute a migratory phenotype in these cells (Sieg, Ilic et al. 1998). Pyk2 and FAK interact in signaling events leading to cellular migration (Du, Ren et al. 2001; Avraham, Lee et al. 2003; Lipinski, Tran et al. 2003; Jiang, Jacamo et al. 2006), but the relationship between these two proteins remains poorly defined and appears to be highly signal- and cell context-specific. Additionally, phosphorylation kinetics differ between the two proteins (Wu, Jacamo et al. 2006).

Here, we examined the involvement of Pyk2 in pUS28-induced signaling and SMC migration. Pyk2 is activated in SMC expressing pUS28 following the addition of ligand in a calcium-dependent manner. Expression of an autophosphorylation point mutant of Pyk2 (F402Y) blocks SMC migration in a dominant-negative fashion by preventing RhoA activation, while a kinase-inactive mutant has no effect on migration. Pyk2 forms an active kinase complex containing several novel Pyk2 binding proteins in CCL5 stimulated SMC expressing pUS28. Suprisingly, the autophosphorylation null mutant Pyk2 F402Y did not abrogate the formation of an active Pyk2 kinase complex but did prevent the pUS28-mediated activation of RhoA. Lastly, we demonstrate the pUS28-mediated activation of RhoA via Pyk2 also occurs in U373 glioblastoma cells. The Pyk2 kinase complex in U373 is distinct from the complex observed in RSMC and contains a number of proteins known to participate in glioma tumorigenesis. These results provide a potential mechanistic link between pUS28 signaling and glioma pathogenesis.

## 3.3 Methods

*Cell Lines.* The life-extended telomerized pulmonary artery SMC line, PAT1 (Melnychuk, Streblow et al. 2004) were maintained in Medium 199 supplemented with 20% fetal calf serum (FCS) and penicillin-streptomycin-L-glutamine (PSG; Gibco). For migration experiments described below, PAT1 cells were utilized between passage 5 and 30 post-telomerization. Mouse FAK-/- fibroblasts were maintained on gelatin coated culture dishes in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FCS, PSG, non-essential amino acids (Cellgro), and G418 (Sigma; 500µg/ml) as previously described (Ilic, Furuta et al. 1995; Sieg, Ilic et al. 1998). FAK-/- cells used in experiments were between passage 5 and 15. Primary F344 rat SMC (RSMC) were maintained in Dulbecco's modified Eagle's Medium (DMEM) with 10% FCS and PSG. RSMC were used between passage 5 and 20. U373 glioblastoma cells were maintained in DMEM supplemented with 10% FCS and PSG. U373 were used between passage 5 and 20.

*Reagents.* Recombinant human CCL5 was purchased from R&D Systems and BAPTA-AM was from Sigma-Aldrich (A1076). Anti-Pyk2 (N-19), anti-phospho-402-Pyk2, anti-myc (9E10), anti-RhoA and anti-HA (F-7) antibodies were purchased from Santa Cruz Biotechnology. Secondary anti-mouse, anti-goat and anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies were purchased from Amersham. Goat anti-mouse Alexa Fluor-488 was purchased from Molecular Probes (A11017).

*Adenovirus Construction.* The adenovirus expressing pUS28-HA was previously described (Streblow, Söderberg-Nauclér et al. 1999; Streblow, Vomaske et al. 2003; Melnychuk, Streblow et al. 2004). Myc-tagged Pyk2 WT, Pyk2 A457K (kinase inactive mutant) or Pyk2 F402Y (autophosphorylation mutant) (Sieg, Ilic et al. 1998) were constructed by cloning of the cDNA into pAdTet7, which contains the tet-responsive enhancer within a minimal CMV promoter followed by the SV40 late poly(A) cassette. Recombinant adenoviruses were

produced by co-transfection of 293 cells expressing the Cre-recombinase with pAdTet7 constructs and adenovirus DNA (Ad5-Ψ5, an E1A/E3-deleted adenovirus genome) (Hsia, Mitra et al. 2003). Recombinant adenoviruses were expanded on 293-Cre cells and the bulk stocks were titered on 293 cells by limiting dilution. Gene expression was driven by co-infection with an adenovirus expressing the Tet-off transactivator (Ad-Trans) (Streblow, Söderberg-Nauclér et al. 1999).

*SMC migration assay.* Cell migration assays were performed as previously described (Streblow, Söderberg-Nauclér et al. 1999). PAT1 cells were infected with HCMV (MOI 10) for 2 hrs followed by co-infection with Ad-Trans and Ad-Pyk2 (WT, A457, F402Y) at MOI of 50 for an additional 2 hrs. Subsequently, the transwells were transferred to new 12-well plates containing fresh medium with CCL5 (50ng/ml) added to the lower chamber. Cells migrating into the lower chamber were counted at 48-72 hours post-infection (hpi) using a Nikon TE300 microscope at magnification 10X. Experiments were performed in at least triplicate wells and ten random fields were read in each well. The average number of cells per well was determined by multiplying the average number of cells per 10X field by the number of fields per well. Mean and standard deviation were calculated. Pyk2 recombinant protein levels were monitored by western blotting and equalized by adjusting the adenoviral vector MOI accordingly.

*Immunofluorescence.* Subcellular localization of adenovirus-expressed proteins was visualized by fixing adenovirus-infected SMC in 4-well chamber slides (Nunc Lab-Tek) at 24 hpi in 2% paraformaldehyde for 15 min. Cells were then permeablized with PBS containing 0.2% Saponin and 0.02% BSA (sapPBS) for 15 min. Anti-myc primary antibody was diluted 1:500 in sapPBS and cells were incubated for 1 hr at room temperature (r.t.), washed twice with sapPBS, and incubated with goat anti-mouse Alexa Fluor-488 diluted 1:1000 in sapPBS for 30 min at r.t. After two more washes in sapPBS cells were visualized

on a Deltavision deconvolution fluorescence microscope (Applied Precision).

*Pyk2 Y402 Phosphorylation Assay.* FAK-/- or U373 cells plated onto 6-well culture dishes at 50% confluence were serum-starved for 6 hrs. The cells were infected with Ad-Trans and Ad-pUS28 at MOI 150 with Ad-Pyk2 or Ad-Pyk2 F402Y at MOI 50 and placed in serum free medium. For calcium chelation assays, cells were pre-treated with 50μM BAPTA-AM for 30 min prior to stimulation. At 16 hpi, the cells were stimulated with CCL5 (40ng/ml) and then scraped in 2X Laemmeli's Sample Buffer. Unboiled samples were loaded on 10% SDS-PAGE. The gels were transferred to Immobilon-P membranes then blocked in PBS containing 3% milk + 0.1% Tween 20 for 15 min at r.t. The primary anti-Phospho-Y402 Pyk2 rabbit polyclonal antibody was added at 1:1000 dilution in blocking buffer for 1 hr at r.t. The blots were washed with TBS-Tween buffer (10mM Tris pH 7.2, 100mM NaCl, 0.2% Tween-20). The secondary antibody (goat anti-rabbit conjugated to HRP) was added at 1:20,000 dilution in blocking buffer for 30 min at r.t. After washing 3 times with TBS-Tween buffer and incubation with ECL reagents, the blots were visualized by autoradiography. Blots were dried to inactivate the HRP secondary reagent and then reprobed as above with anti-Pyk2 goat polyclonal antibody at 1:1000 dilution in blocking buffer followed by secondary donkey anti-goat conjugated to HRP at 1:100,000 dilution.

*RhoA Activation Assay*. FAK-/- or U373 cells plated onto 6-well culture dishes at 50% confluence were serum-starved for 6 hrs. The cells were infected with Ad-Trans only or co-infected with Ad-Trans and Ad-pUS28 at MOI 150 with or without Ad-Pyk2 or Ad-Pyk2 F402Y at MOI 50 and placed in serum free medium. After 16 hrs, the cells were stimulated with CCL5 (40ng/ml) and then scraped in equal volumes modified RIPA buffer (10mM Tris pH7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (100mM AEBSF, 80μM aprotinin, 1.5mM E-64, 2mM leupeptin hemisulfate, and 1mM pepstatin A) at 0 (unstimulated), 2.5, 5, or 10

minutes post-stimulation. Cell lysates were homogenized via sonication, then incubated with 20µl Rho Assay Reagent (Rhotekin-RBD-GST Agarose; Upstate) for 1 hr at 4°C, then washed 3 times with 1ml of RIPA. The final bead pellet was resuspended in 40µl of 2x Laemmli's sample buffer, boiled and then run on 10% SDS-PAGE. The gels were transferred to Immobilon-P membranes then blocked in blocking buffer (PBS containing 3% Milk + 0.1% Tween 20) for 15 min at r.t. The primary anti-RhoA mouse monoclonal antibody was added at 1:1000 dilution in blocking buffer for 1 hr at r.t. The blots were washed with TBS-Tween buffer. The secondary antibody (goat anti-mouse conjugated to HRP) was added at 1:40,000 dilution in blocking buffer for 30 min at r.t. After washing 3 times with TBS-Tween buffer and incubation with ECL reagents, the blots were visualized by autoradiography.

*Pyk2* In Vitro *Kinase Assays*. *In vitro* kinase assays were performed on on immunoprecipitated Pyk2 from F344 aortic RSMC. Cells were plated in 10cm culture dishes and serum starved for 24 hrs. The RSMC were co-infected with Ad-Trans and Ad-Pyk2 or Ad-Pyk2 F402Y at an MOI of 50 with or without Ad-pUS28 at an MOI of 150. After 16 hrs, the cells were stimulated with recombinant human CCL5 (40ng/ml) and then harvested at times 0 (unstimulated), 2.5, 5, 10, and 15 minutes post ligand addition. Cells were rinsed in PBS and lysed in modified RIPA containing protease inhibitor cocktail and 200µM sodium orthovanadate. Total Pyk2 was immunoprecipitated using mouse anti-myc tag monoclonal antibody and Protein-A/G conjugated agarose beads (Santa Cruz Biotechnology). Precipitation reactions were washed one time in Triton-only lysis buffer (modified RIPA without sodium deoxycholate and SDS), two times in HNTG buffer (50mM HEPES, 150mM NaCl, 1% Triton, 10% Glycerol, pH 7.4), and two times in kinase buffer (20mM HEPES, 10mM $MgCl_2$, 10mM $MnCl_2$, 150mM NaCl, 10% Glycerol pH 7.4) and then resuspended in 50µl kinase buffer plus 10µCi $^{32}$P-γ-ATP (Perkin-Elmer). The kinase reaction was allowed to proceed for 30

minutes at r.t. then stopped by the addition of 2X Laemmeli's sample buffer. After boiling for 5 minutes, the reactions were run on 10% SDS-PAGE and transferred to Immobilon-P membranes. The incorporation of $^{32}$P-g-ATP was visualized via autoradiography. After allowing radioactivity to decay, the membranes were further analyzed via western blot as above for the presence of Pyk2-myc and pUS28-HA.

*Preparation of Pyk2 Complexes for Proteomics Analysis*. Proteomics analysis was performed on immunoprecipitated Pyk2 from F344 aortic RSMC or U373 glioblastoma cells. Cells were plated in 15cm culture dishes. The RSMC were serum starved for 24 hrs prior to co-infection with Ad-Trans and Ad-Pyk2 at an MOI of 50 with or without Ad-pUS28 at an MOI of 150. After 16 hrs, the cells were stimulated with recombinant human CCL5 (40ng/ml) and then triplicate dishes were harvested at times 0 (unstimulated), 5, 10, 15, 30 and 60 minutes post ligand addition. Cells were rinsed in PBS and lysed in modified RIPA containing protease inhibitor cocktail and 200μM sodium orthovanadate. Total Pyk2 was immunoprecipitated using mouse anti-myc tag monoclonal antibody and Protein-A/G conjugated agarose beads (Santa Cruz Biotechnology). Precipitation reactions were washed twice with RIPA. Pyk2 and associated proteins were eluted with 200μl of Pierce Gentle Ag/Ab Elution buffer. Triplicate eluates were combined and the samples were sequenced by tandem mass spectrometry analysis at the EMSL user facility at Pacific Northwest National Laboratories.

*Tryptic digestion of Pyk2 Complexes.* Proteins from the Pyk2 complexes were desalted and concentrated with Amicon ultra filtration units (Millipore, Billerica, MA). Protein concentration was determined using BCA protein assay (Thermo Scientific) and the concentrated protein was denatured by the addition of trifluoroethanol (TFE) to a final concentration of 50% and heating to 37°C for 60 min. Denatured proteins were reduced with DTT (2mM final concentration) and diluted fivefold with 50mM NH4HCO3. Methylated,

sequencing-grade porcine trypsin (Promega, Madison, WI) was added at a substrate-to-enzyme ratio of 50:1 (mass to mass) and incubated at 37°C for 3 hrs. The peptides were concentrated with a speed vac and stored at 80°C until analysis.

*Tandem mass spectrometric analysis of peptides.* Peptide samples were analyzed using an automated custom built capillary LC system containing a four capillary column system (Livesay et al, Anal Chem 2008, vol 80, page 294). Eluate from the LC was coupled directly to a hybrid linear ion-trap-orbitrap (LTQ_Orbitrap, Thermo Electron Corp.). The reverse-phase capillary column was prepared by slurry-packing 3-micron Jupiter C18 bonded particles (Phenomenex, Torrence, CA) into a 65cm long, 75-micron-inner diameter fused silica capillary (Polymicro Technologies, Phoenix, AZ). After peptide loading onto the column, the mobile phase was held at 100% A (0.05% trifluoroacetic acid (TFA) and 0.2% acetic acid in water) for 20 min, followed by a linear gradient from 0 to 70% buffer B (0.1% TFA in 90% acetonitrile, 10% water) over 80 min with a flow rate of ~500nL/min. Orbitrap spectra (AGC $1 \times 10^6$) were collected from 400–2000 m/z at a resolution of 100 k followed by data-dependent ion trap tandem mass spectra (AGC $1 \times 10^4$) of the six most abundant ions using a collision energy of 35%. The heated capillary was maintained at 200°C, and the ESI voltage was held at 2.2 kV.

*SEQUEST analysis.* The SEQUEST algorithm was run on each of the datasets against the human.fasta from the International Protein Index database (version 3.54, 75,419 entries, released January 2009). MS/MS peaks were generated by "extract_msn.exe," part of the SEQUEST software package. A peptide was considered to be a match by using a conservative criteria set developed previously by Yates and coworkers [45]. Briefly, peptides were retained if they met the following criteria: 1). SEQUEST DelCN value of $\geq$0.1 and 2). SEQUEST correlation score (Xcorr) $\geq$ 1.9 for charge state 1+ and fully tryptic; Xcorr $\geq$ 2.2 for charge state 2+ and fully or partially tryptic; Xcorr $\geq$ 3 for charge state 2+

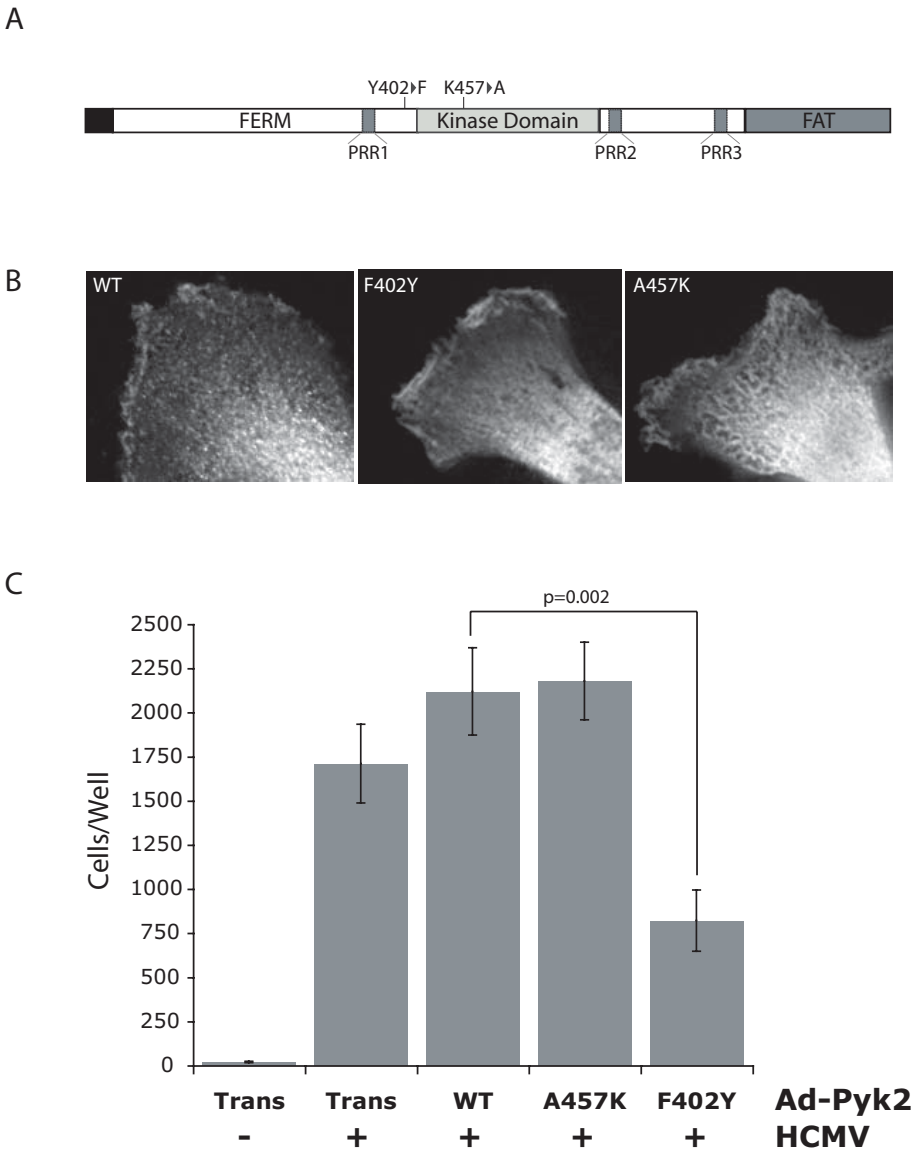


**Figure 3.1 - Pyk2 Autophosphorylation Mutant Inhibits HCMV-mediated SMC**
**(A) Schematic of Pyk2 domain structure with A457K and F402Y mutations annotated. (B) Sub-cellular localization of Myc-tagged adenovirus constructs was determined via indirect immunofluorescence staining for the Myc epitope. From left Ad-Pyk2 WT, Ad-Pyk2-F402Y and Ad-Pyk2-A457K in lamellipodia of adenovirus-infected PAT1 SMC. Gene expression was driven my co-infection with an adenovirus expressing the Tet-off transactivator (Ad-Trans) (C) SMC migration assays were performed on PAT1 SMC co-infected with HCMV at MOI 10 and adenoviruses expressing control Ad-Trans only or Ad-Trans+Pyk2-WT, Pyk2-A457K, or Pyk2-F402Y at MOI 50 in transwell chambers. At 2 hrs post-infection fresh medium containing 50ng/ml CCL5 was added to the bottom chamber and cells were allowed to migrate for 48 hrs. Migrated cells in the lower chamber were counted via light microscopy. For each condition, n=6 from two independent experiments.**

regardless of tryptic state; Xcorr ≥ 3.75 for charge state 3+ for fully or partially tryptic.

## 3.4 Results

### *3.4.1 Pyk2 Autophosphorylation is Required for pUS28-mediated SMC Migration*

In order to establish the involvement of Pyk2 in pUS28-mediated SMC migration, we constructed a panel of adenovirus vector expressing *myc*-tagged versions of wild-type Pyk2 (Pyk2-WT), an autophosphorylation null mutant (Pyk2-F402), and a kinase-inactive mutant (Pyk2-A457) (Figure 3.1A) (Sieg, Ilic et al. 1998). These Pyk2 mutant adenoviruses were expressed in human pulmonary artery smooth muscle (PAT1) cells. Gene expression was driven by co-infection with the Tet-off transactivator adenovirus (Ad-Trans) and Ad-Trans infection alone is used to control for any non-specific effects of adenovirus infection. All Pyk2 constructs displayed similar cellular distribution patterns in the cytoplasm and at lamellipodia of growing cells (Figure 3.1B). As previously described, human SMC cells

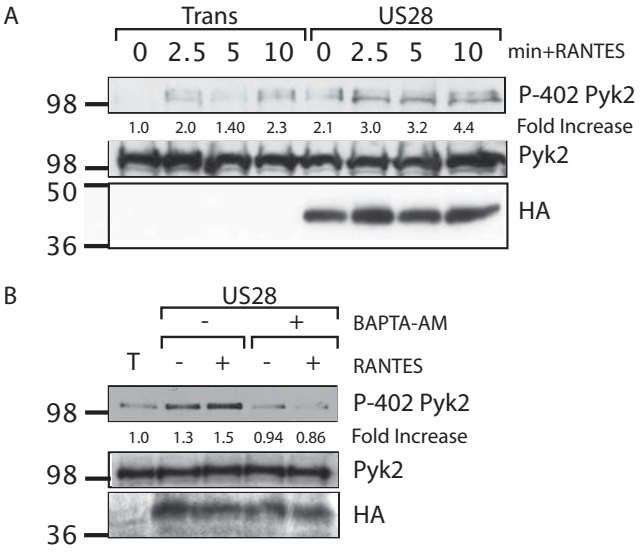


**Figure 3.2 - pUS28 Signaling Causes Calcium-Dependent Phosphorylation of Pyk2 at Y402**

**(A) FAK-/- fibroblasts were infected with Ad-Trans only or Ad-Trans+Ad-pUS28 for 18 hrs. Cells were stimulated with 40ng/ml CCL5 for the indicated times and analyzed via western blot with a phospho-specific Pyk2-Y402 antibody or total Pyk2 antibody. (B) Adenovirus-infected FAK-/- were pre-treated for 30 min with BAPTA-AM to chelate intracellular calcium and then stimulated with 40ng/ml CCL5 for 5 min. Total cell lysates were analyzed via western blot for phospho-Y402 Pyk2 and total Pyk2. For both experiments, blots were stripped and reprobed to verify pUS28-HA expression. Phospho-specific blots were quantified via densitometry using ImageJ software and are expressed as fold change compared to unstimulated, Ad-Trans infected control.**

**Figure 3.3 - pUS28 Signaling Causes Formation of an Active Kinase Complex Involving Pyk2**
**SMC were infected with Ad-Trans and Ad-Pyk2 +/- Ad-pUS28 and stimulated with 20% serum or 40ng/ml CCL5 for indicated times. Pyk2 was immunoprecipitated using myc antibodies. In vitro kinase reactions were performed on immunoprecipitated material and reactions were loaded on SDS-PAGE, transferred to immobilon-P membranes and visualized via autoradiography. (A) Autoradiogram of in vitro kinase reactions. Total Pyk2 and pUS28 expression was determined by western blot for myc and HA tags, respectively. (B) Densitometric lane plots of results shown in panel A, generated using ImageJ software. The darker curve (overlay) is unstimulated and the lighter curve (background) is 5 min post-stimulation for Pyk2 only stimulated with serum (top) or Pyk2+pUS28 stimulated with CCL5 (bottom). Black arrows indicate bands present only in stimulated samples. Molecular weight markers are shown on the density plot to facilitate comparison to the autoradiogram in panel A. (C) Overall optical density quantification of each lane in Pyk2 in vitro kinase reactions shown in panel A. Values are displayed as percentages compared to unstimulated samples infected with Ad-Trans+Ad-Pyk2.**

infected with HCMV migrated in response to CCL5 (Streblow, Söderberg-Nauclér et al. 1999; Streblow, Orloff et al. 2001) (Figure 3.1C). Expression of Pyk2-WT produced similar levels of migration compared to Ad-Trans only controls. Surprisingly, co-infection with adenovirus producing kinase-inactive Pyk2 (Pyk2-A457) did not abrogate HCMV-mediated SMC. In contrast, expression of a mutant Pyk2 that lacks the Tyr-402 autophosphorylation site (Pyk2-F402) acted as a dominant negative inhibitor of HCMV-mediated SMC migration, causing a 60% reduction in SMC migration compared to HCMV-infected SMC expressing Pyk2-WT (Figure 3.1C). These results demonstrate that Pyk2 kinase activity is dispensable for pUS28-mediated SMC migration and suggest that the primary role of Pyk2 in the pro-migratory signaling cascade in these cells is to act as a signaling scaffold for the activation of Src kinase (Dikic, Tokiwa et al. 1996).

### *3.4.2 pUS28 Signaling leads to Phosporylation of Pyk2 at Y402*

In order to further characterize the role of Pyk2 in pUS28-mediated cellular migration, we examined phosphorylation of Pyk2 at Y402 in Ad-pUS28 infected mouse fibroblasts lacking FAK ($FAK^{-/-}$). $FAK^{-/-}$ cells are known to have elevated levels of endogenous Pyk2, partially compensating for the lack of FAK (Sieg, Ilic et al. 1998; Owen, Ruest et al. 1999). We have utilized $FAK^{-/-}$ fibroblasts for pUS28 signaling assays in previous studies

A
250
144
98
64
50
36
22
144
36
0 5 5 0 2.5 5 10 15 min
Serum RANTES Stimulus
Pyk2 US28 + Pyk2 Adenovirus
WB: myc
WB: HA
B
Pyk2 (Serum)
US28+Pyk2 (RANTES)
Optical Density
250 144 98 64 50 36 22
C
% of Control
195.00 185.00 175.00 165.00 155.00 145.00 135.00 125.00 115.00 105.00 95.00
0 5 5 0 2.5 5 10 15 min
Serum RANTES Stimulus
Pyk2 US28 + Pyk2 Adenovirus

(Streblow, Vomaske et al. 2003; Vomaske, Melnychuk et al. 2009). FAK$^{-/-}$ cells exhibit low baseline activation of pro-migratory signaling molecules and are therefore a highly inducible system in which to study these signaling events. Furthermore, using this system we can study pUS28 signaling to Pyk2 independent of FAK.

pUS28-expressing FAK$^{-/-}$ cells stimulated with CCL5 showed sustained phosphorylation of Pyk2 at Y402. Levels of Pyk2 phosphorlyation were ~2-fold higher in the presence of pUS28 than control Ad-Trans infected cells similarly stimulated. CCL5 stimulation had a small but quantifiable effect on Pyk2 phosphorylation in FAK$^{-/-}$ cells infected with Trans only, we believe this activation is attributable to the effects of calcium gradients produced by the addition of media upon stimulation (Figure 3.2A). Pyk2 kinase activity has been shown to be dependent on intracellular calcium levels (Li and Earp 1997; Avraham, Park et al. 2000; Kohno, Matsuda et al. 2008). Therefore, we treated pUS28-expressing FAK$^{-/-}$ fibroblasts with BAPTA-AM to chelate intracellular calcium prior to performing signaling assays. The cells were then stimulated with CCL5 and examined for Pyk2 Y402 phosphorylation via western blot. As expected, chelation of intracellular calcium abrogated phosphorylation of Pyk2 in response to pUS28 signaling (Figure 3.2B) and prevented Pyk2 activation in Ad-Trans controls (data not shown). We observed a low, but measurable, effect of US28 expression on Pyk2 activity in the absence of ligand (Figure 3.2A&B), which could be attributed to either low-level constitutive signaling or minor effects of endogenously expressed ligands.

### *3.4.3 pUS28 Signaling Causes formation of a Pyk2-Associated Active Kinase Complex*

We have shown that phosphorylation of Pyk2 at Y402 is required for SMC migration (Figure 3.1C) and that pUS28 signaling causes phosphorylation at this critical site independent of any pUS28 activation of FAK (Figure 3.3A). Although activation at the autophosphorylation site

is important for Pyk2 function (Park, Avraham et al. 2004), we wanted to further characterize the effect of pUS28 signaling on the activation and function of Pyk2. To accomplish this, we performed *in vitro* kinase assays on Pyk2 immunoprecipitation reactions. For these experiments we returned to examining Pyk2 signaling in the context of SMC using our well-characterized primary rat SMC cultures (RSMC) (Vomaske, Melnychuk et al. 2009), Cells were serum starved and co-infected with adenovirus vectors expressing Pyk2-WT with or without pUS28-HA. The cells were then stimulated with either 20% serum, as a positive control for Pyk2 activation, or 40ng/ml CCL5 and harvested at 0, 2.5, 5, 10 or 15min post-stimulation. Pyk2 was then immunoprecipitated from total cell lysates using a monoclonal antibody directed against the myc tag and immunoprecipitated proteins were

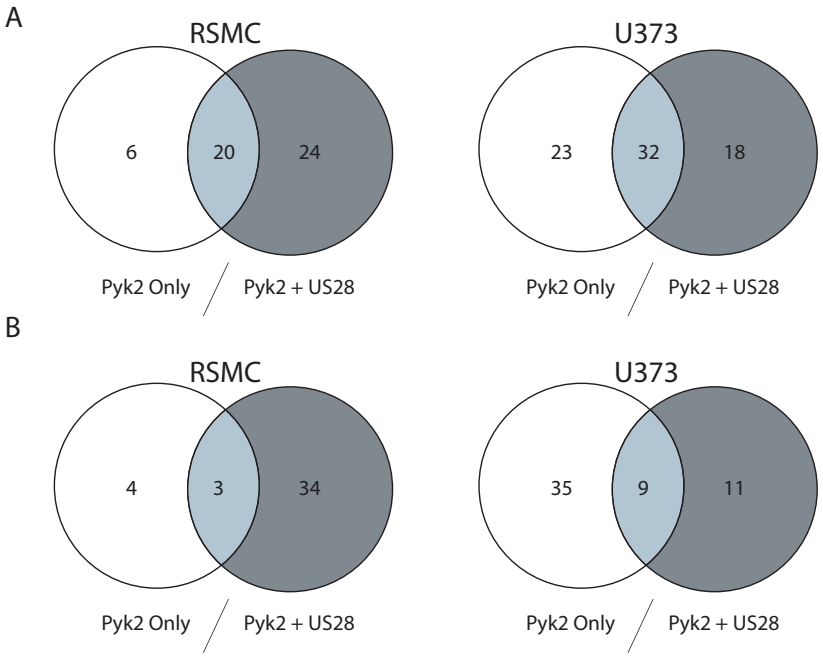


**Figure 3.4 – Pyk2-Associated Proteins are Cell-Type and Signal-Specific**
**SMC or U373 were infected with Ad-Trans and Ad-Pyk2 +/- Ad-pUS28 and stimulated with 20% serum or 40ng/ml CCL5 for 0, 5, 10, 15, 30 or 60 min. Pyk2 was immunoprecipitated using Myc antibodies. Pyk2 complexes were analyzed via tandem mass spectrometry. Results shown are proteins identified with >3 spectral hits for all peptides. (A) Total hits for Pyk2-associated proteins in RSMC and U373. (B) Proteins for which Pyk2-association was induced over the timecourse of stimulation in RSMC and U373. For data analysis, a protein was considered induced if the average spectral hits for all peptides for timepoints 5, 10, 15, 30 and 60 min was greater than twice the spectral hits for all peptides in unstimulated cells.**

subjected to *in vitro* kinase assay. The Pyk2 kinase activity was analyzed via SDS-PAGE and autoradiography. pUS28 signaling promoted the formation of a large kinase complex involving Pyk2 and a number of other interacting phosphoproteins. This protein complex begins to form by 2.5 min after the addition of CCL5 peaking at 10 min post ligand treatment (Figure 3.3A & C). Plotting the optical density of unstimulated versus 5 min stimulated timepoints using ImageJ software revealed several pUS28-specific proteins associated with Pyk2 (Figure 3.3B). When a parallel experiment was performed in the presence of Pyk2-F402Y, a similar kinase complex was observed (data not shown), indicating that Tyr-402 is not critical for formation or maintenance of Pyk2 in an active kinase complex but rather mediates the binding of critical signaling elements necessary for the Pyk2 scaffold

**Table 3.1 - Selected Pyk2 binding partners found in pUS28-expressing RSMC**

| CATEGORY | PROTEIN(S) IDENTIFIED | SELECTED REFERENCES |
|---|---|---|
| Cytoskeleton Structure and Dynamics | **β-actin, α1-actin** | |
| | F-actin capping proteins-α1, -α2*, -β | |
| | Gelsolin, | (Wang, Xie et al. 2003; Spinardi and Witke 2007; Bucki, Levental et al. 2008; Gunning, O'Neill et al. 2008) |
| | **Myosin-9**, -10, -11 | |
| | **Myosin light chain-6**, | |
| | Tropomodulin-3 | (Gunning, O'Neill et al. 2008) |
| | **Tropomyosin-α1**, α3, α4 -β | (Gunning, O'Neill et al. 2008; Lindberg, Schutt et al. 2008; Bach, Creed et al. 2009) |
| | Tubulin-β-2C | |
| | Vimentin | (Ivaska, Pallari et al. 2007) |
| Signal Transduction | Calmodulin | (Kohno, Matsuda et al. 2008; Xie, Allen et al. 2008) |
| | G-protein subunit β5 | (Dupre, Robitaille et al. 2009) |
| | KSR-2* | (Costanzo-Garvey, Pfluger et al. 2009; Dougherty, Ritt et al. 2009) |
| | Rhophilin-2* | (Fujisawa, Madaule et al. 1998) |
| | Trio* | (Rossman, Der et al. 2005; Rojas, Yohe et al. 2007) |
| | **TFG** | (Miranda, Roccato et al. 2006) |
| Inflammation and Immunity | **Immunoglobulin lambda light chain** | (Bliznyukov, Kozmin et al. 2005) |
| | **NFκB p105** | |
| Cellular Proliferation | **AF-17** | (Lin, Ono et al. 2001) |
| | **RTEL-1*** | (Barber, Youds et al. 2008) |

**Boldface font** indicates proteins identified as Pyk2-associated in multiple cell types

* indicates proteins identified with low abundance (< 3 spectral hits on a single peptide).

to participate in pro-migratory signaling.

### 3.4.4 Identification of Pyk2 Binding Partners

We performed proteomics analysis on Pyk2 kinase complexes in order to determine whether pUS28 signaling promotes the association of Pyk2 with novel binding partners. We isolated Pyk2 complexes via immunoprecipitation as described above from RSMC expressing pUS28-HA and Pyk2 at 0, 5, 10, 15, 30 or 60 min post-addition of CCL5. As a control for the pUS28-specificity of Pyk2 interactions, we performed identical immunoprecipitation reactions in RSMC expressing Pyk2 only stimulated with 20% serum. Bound proteins were analyzed via tandem mass spectrometry. We identified a total of 50 Pyk2 binding partners in RSMC and 24 of these associated with Pyk2 specifically in response to pUS28 expression. Only 6 proteins were specific for Pyk2 in the absence of pUS28 (Figure 3.4A, Table 3.1). When we examined the proteins whose Pyk2 association was specifically induced over the signaling timecourse, we determined that 34 proteins associated with Pyk2 in response to pUS28 stimulation by CCL5. In contrast, only 4 proteins were specifically activated by serum stimulation of RMSC expressing Pyk2 only (Figure 3.4B). As expected, we identified a number of cytoskeleton structural proteins in our proteomics screen confirming that Pyk2 is directly associated with the cytoskeleton when activated (Ohanian, Gatfield et al. 2005) (Table 3.1). We identified several proteins known to interact with Pyk2 in other systems including calmodulin (Xie, Allen et al. 2008) and gelsolin (Wang, Xie et al. 2003). In support of our hypothesis that Pyk2 serves as a scaffold for pUS28 signaling in RSMC, we found several signaling intermediates associating with Pyk2 over the timecourse. These included a RhoGEF (Trio) known to link G-protein signaling to RhoA activation (Rojas, Yohe et al. 2007), a regulator of MAPK signaling (KSR-2) (Dougherty, Ritt et al. 2009) as well as a regulator of the SHP-1 tyrosine phosphatase (TFG) (Miranda, Roccato et al. 2006). Interestingly, we observed a pUS28 inducible association of Pyk2 with the NFκB

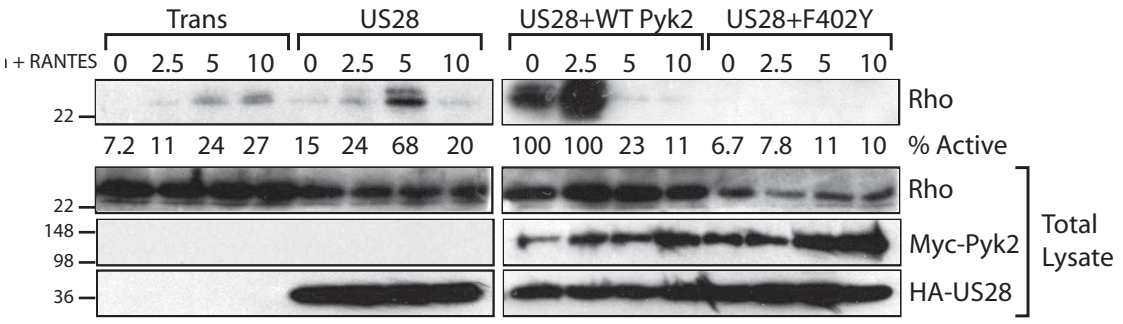


**Figure 3.5 – Pyk2-F402Y Blocks pUS28 Signaling to RhoA**

**FAK-/- fibroblasts were infected with Ad-Trans and Ad-pUS28 +/- Pyk2-WT or -F402Y for 18 hrs. Cells were stimulated with 40ng/ml CCL5 for the indicated times. Lysates were immunoprecipitated with Rhotekin-RBD-GST Agarose and analyzed by western blot for RhoA. Input lysates were analyzed for total RhoA and to confirm adenovirus infection efficiency. The percent active RhoA was quantified via ImageJ densitometry of both IP and total lysate western blots.**

precursor p105. Although Pyk2 signaling has been implicated in NFkB activation (Shi and Kehrl 2001; Xi, Xiong et al. 2010), we believe that our data showing NFκB p105 present in an activated Pyk2 complex is novel. This interaction may provide a secondary, ligand-dependent mechanism for pUS28-mediated activation of NFκB.

### *3.4.5 Pyk2 F402Y is Defective in RhoA Activation*

We have previously demonstrated that activation of RhoA is necessary for pUS28-mediated SMC migration (Melnychuk, Streblow et al. 2004). Therefore, we hypothesized that the dominant negative effect of the Pyk2 F402Y mutant is due to a defect in RhoA activation. To test this hypothesis, we performed a RhoA activation assay in cells expressing pUS28 that were stimulated with CCL5. For these experiments, Rhotekin-RhoA Binding Domain (RBD) conjugated beads were used to specifically pull-down active RhoA from FAK-/- cells expressing Trans only, or Trans + pUS28 with or without Pyk2-WT or Pyk2-F402Y. Percent of RhoA activated was calculated by comparing immunoprecipitated RhoA with total RhoA in the same sample. As expected, within 5 min, CCL5 stimulation of pUS28-expressing FAK$^{-/-}$ cells results in activation of 68% of total RhoA compared to a maximum of 27% activation in control Trans-infected cells treated with CCL5 (Figure

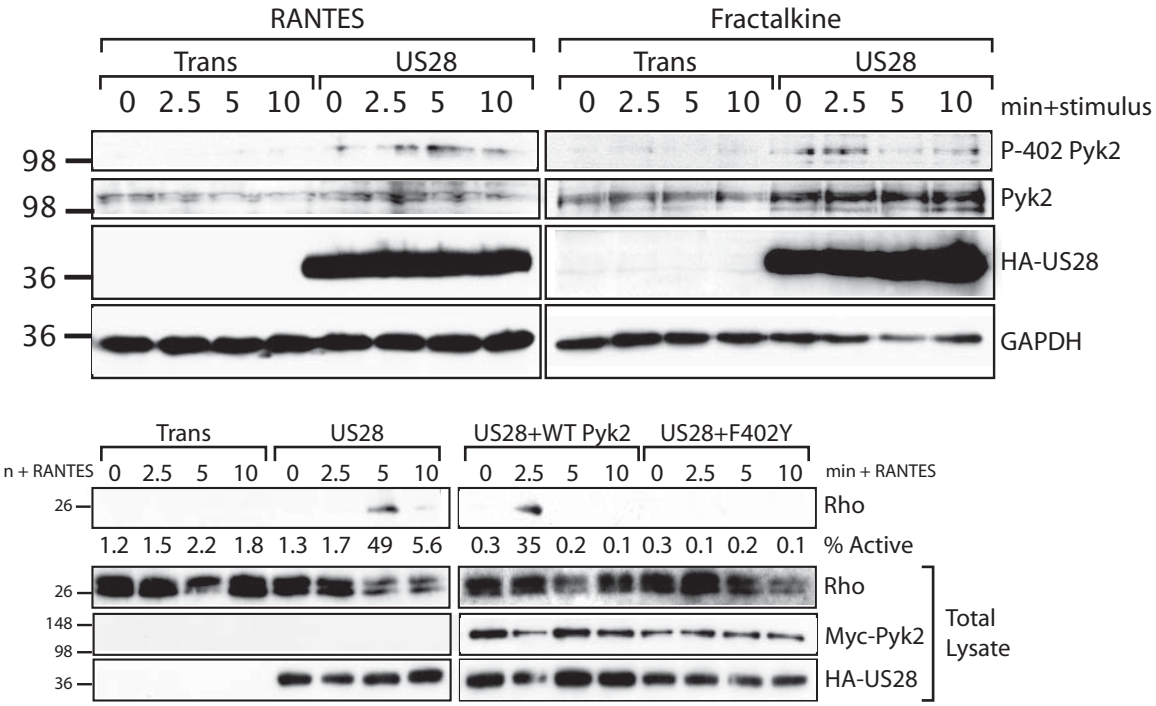


**Figure 3.6 – pUS28 Activates Pyk2 and RhoA in U373 Glioblastoma Cells**

**(A) U373 cells were infected with Ad-Trans or Ad-pUS28 for 18 hrs. Cells were stimulated with 40ng/ml CCL5 (left) or 40ng/ml CX3CL1 (right) for the indicated times and analyzed via western blot with a phospho-specific Pyk2-Y402 antibody or total Pyk2 antibody. (B) U373 were infected with Ad-Trans, Ad-pUS28 or Ad-pUS28 + Pyk2 WT or F402Y for 18 hrs. Cells were stimulated with 40ng/ml CCL5 for the indicated times. Lysates were immunoprecipitated with Rhotekin-RBD-GST Agarose and analyzed by western blot for RhoA. Input lysates were analyzed for total RhoA and to confirm adenovirus infection efficiency. The percent active RhoA was quantified via ImageJ densitometry of both IP and total lysate western blots.**

3.5). Overexpression of Pyk2-WT markedly raised baseline activation of RhoA in pUS28 expressing cells and resulted in the activation of 100% of RhoA at 2.5 min post addition of CCL5. The phenomenon of increased baseline activation of Pyk2 and downstream effectors in the context of overexpression has been observed by others, and appears to be due to the $Ca^{2+}$-mediated regulation of Pyk2 activity (Kohno, Matsuda et al. 2008). Conversely, overexpression of Pyk2-F402Y abrogated pUS28-mediated activation of RhoA, resulting in a maximum of 10% activation over the timecourse of stimulation (Figure 3.5). We observed some cellular toxicity in samples overexpressing Pyk2 F402Y which resulted in slightly lower total RhoA levels in these samples, but as each sample was internally

controlled we believe this has no effect on the data as shown. These results demonstrate that activation of Pyk2 at Y402 is necessary for Pyk2-mediated signaling to RhoA and subsequent signaling leading to SMC migration.

### *3.4.6 pUS28 Activates Pyk2 in Glioblastoma Cells*

Having established a functional link between pUS28, Pyk2 and migration in SMC, we hypothesized that pUS28 mediates pro-migratory signaling to Pyk2 in other CMV-susceptible cell types. Although HCMV infection has been associated with poor clinical outcome in glioblastoma multiforme (GBM) patients, a clear mechanistic link between HCMV and GBM tumorigenesis has not been established (Streblow, Orloff et al. 2001; Cobbs, Soroceanu et al. 2007; Scheurer, Bondy et al. 2008). However, many studies have

**Table 3.2 - Selected Pyk2 binding partners found in pUS28-expressing U373 cells**

| CATEGORY | PROTEIN(S) IDENTIFIED | SELECTED REFERENCES |
|---|---|---|
| Cytoskeleton Structure and Dynamics | **β-actin**, **α1-actin**, α2-actin | |
| | Nectin-3 | (Nakanishi and Takai 2004) |
| | **Myosin-9**, **Myosin light chain-6**, **Tropomyosin-α1** | (Gunning, O'Neill et al. 2008; Lindberg, Schutt et al. 2008; Bach, Creed et al. 2009) |
| Signal Transduction | USP6 oncogene* | (Dechamps, Bach et al. 2006) |
| | NIM1 kinase* | (Jaleel, McBride et al. 2005) |
| | Copine-5* | (Tomsig and Creutz 2002) |
| | Abl-interactor-2* | (Grove, Demyanenko et al. 2004) |
| | **TFG** | (Miranda, Roccato et al. 2006) |
| Inflammation and Immunity | **Immunoglobulin lambda light chain** | (Bliznyukov, Kozmin et al. 2005) |
| | **NFκB p105** | |
| Cellular Proliferation | TCF-4 | (Xie, Allen et al. 2008; Pu, Zhang et al. 2009) |
| | **AF-17** | (Lin, Ono et al. 2001) |
| | **RTEL-1*** | (Barber, Youds et al. 2008; Shete, Hosking et al. 2009; Wrensch, Jenkins et al. 2009) |
| | N-acetyltransferase-10 | (Shen, Zheng et al. 2009) |
| | Integrator complex subunit 6 | (Filleur, Hirsch et al. 2009) |
| Stress Response | BiP | (Pyrko, Schonthal et al. 2007; Le Mercier, Mathieu et al. 2008) |
| | Mortalin | (Takano, Wadhwa et al. 1997; Yi, Luk et al. 2008) |
| | Hsc70 | (Zeise, Kuhl et al. 1998; Vila-Carriles, Zhou et al. 2007) |

**Boldface font** indicates proteins identified as Pyk2 associated in multiple cell types

* indicates proteins identified with low abundance (< 3 spectral hits on a single peptide).

associated aberrant activation of Pyk2 with increased invasiveness in GBM (Lipinski, Tran et al. 2003; Lipinski, Tran et al. 2005; Lipinski, Tran et al. 2006; Lipinski, Tran et al. 2008). To test whether pUS28 can signal to Pyk2 in a glioma model, we examined phosphorylation of Pyk2 at the Y402 site in pUS28 adenovirus-infected U373 glioblastoma cells. Interestingly, we observed pUS28-specific phosphorylation of Pyk2 in U373 cells in response to both CCL5 and CX3CL1 stimulation (Figure 3.6A). Interestingly, Pyk2 phosphorylation in response to CX3CL1 stimulation is biphasic, with two separate peaks of phosphorlyation, the first at 2.5 and second at 10 min post-ligand addition. This biphasic pattern of phosphorylation at the Y402 site has been observed in other systems (Wu, Jacamo et al. 2006).

We tested the participation of Pyk2 in the pUS28-mediated activation of RhoA in this system. Similar to results obtained in FAK-/- fibroblasts, pUS28-specific activation of RhoA is readily observed in U373 cells. pUS28-expressing U373 cells showed 49% activation of RhoA at 5 min post addition of CCL5. Adenovirus expression of WT Pyk2 resulted in similar activation of RhoA, but altered the timing of RhoA activation such that the peak of RhoA activation was seen at 2.5 min post addition of CCL5. Overexpression of the Pyk2 F402Y mutant prevents pUS28-mediated activation of RhoA resulting in less than 1% activation of RhoA throughout the timecourse of stimulation (Figure 3.6B).

In order to compare pUS28-mediated activation of Pyk2 in U373 cells to that seen in RSMC, we performed proteomics analysis of Pyk2 complexes in U373 cells (Figure 3.4, Table 3.2). The conditions for this experiment were the same as the previous experiment in RSMC. Overall, Pyk2 seems to have more binding partners in U373 cells, underscoring the importance of Pyk2 in glioma cell biology. A total of 73 proteins were found associated with Pyk2 in this system, however only 18 of these were found specifically in pUS28-expressing samples (Figure 3.4A). In contrast to results seen in RSMC, nearly all of the

proteins associated with Pyk2 in U373 were induced by signaling (Figure 3.4B), revealing low baseline activity of Pyk2 in these cells. Many of the same proteins binding to Pyk2 in response to pUS28 signaling were detected in samples from both cell types (Tables 1 & 2, boldface text). However, there were a number of proteins that were unique to pUS28 signaling in U373. Importantly, several heat shock proteins of the Hsp70 family (BiP, mortalin and Hsc70) known to be involved in glioma tumorigenesis (Takano, Wadhwa et al. 1997; Zeise, Kuhl et al. 1998; Pyrko, Schonthal et al. 2007; Vila-Carriles, Zhou et al. 2007) were found to complex with Pyk2 in response to pUS28 signaling. Additionally, several regulators of the Wnt/beta-catenin/Tcf signaling pathway, also implicated in glioma pathogenesis (Pu, Zhang et al. 2009; Sareddy, Challa et al. 2009) are associated with pUS28-activated Pyk2 complexes (Table 2).

## 3.5 Discussion

In this paper, we examined the ability of the HCMV-encoded chemokine receptor pUS28 to activate Pyk2 and determined the role of Pyk2 in pUS28 mediated SMC migration. Pyk2 expression is limited to a subset of cell types *in vivo* including brain, hematopoetic cells, endothelial cells, SMC and fibroblasts. Interestingly, many of these cell types are capable of undergoing migration events in response to various external stimuli including integrin, growth factor, hormone and chemokine-mediated signals. There is very little consensus in the literature regarding the participation of Pyk2 in such migration events. Indeed, requirements for Pyk2 signaling, and downstream effectors of Pyk2 activation are highly cell-type and signal type-specific (Avraham, Park et al. 2000).

### *3.5.1 Pyk2 and migration in vascular smooth muscle*

Migration of vascular SMC is induced by a variety of stimuli including growth factors, cytokines, extracellular matrix (ECM) components and peptide hormones. Pro-migratory

signaling in SMC is best understood in the context of platelet-derived growth factor (PDGF) binding through receptor tyrosine kinases PDGFR-α and β. PDGF-mediated migration in SMC is characterized by Src-dependent activation of FAK, RhoA mediated activation of ROCK and subsequent phosphorylation and inhibition of myosin light chain phosphatase as well as activation of various other effector molecules via ERK, p38MAPK and JNK MAPK families (Gerthoffer 2007). Angiotensin II (Ang II) is another well-studied pro-migratory signal in SMC. Ang II binding to the GPCR angiotensin receptor type 1 (AT-1) initiates the only pro-migratory signaling cascade in SMC previously reported to involve Pyk2 (Yin, Yan et al. 2003). In this signaling context, Pyk2 is activated by PKCδ (Frank, Saito et al. 2002) and signals to Rho/ROCK via association with PDZ-RhoGEF (Ohtsu, Mifune et al. 2005). Additionally, Pyk2 has been identified as an essential signaling scaffold linking GPCR signaling to MAP kinase activation via Src activation upon binding to Tyr-402 autophosphorylated Pyk2 (Dikic, Tokiwa et al. 1996). Activation of JNK and ERK MAP kinase pathways are necessary for Ang II-mediated SMC migration (Ohtsu, Mifune et al. 2005).

The results presented herein represent the first example of a chemokine-mediated pro-migratory signaling cascade in VSMC involving Pyk2. We previously demonstrated that chemokine binding to the viral chemokine receptor pUS28 causes SMC migration via a Src-mediated activation of FAK and ERK (Streblow, Vomaske et al. 2003), and the Gα12-mediated activation of RhoA and ROCK (Melnychuk, Streblow et al. 2004). These results refine our understanding of the pUS28 receptor-proximal events leading to the activation of RhoA via the ligand-dependent phosphorylation of Pyk2 at Tyr-402. Pyk2 kinase activity is dispensable for pUS28-mediated migration in SMC, suggesting that Pyk2 participates primarily as a signaling scaffold in pUS28 pro-migratory signaling. We previously showed pUS28-induced SMC migration is sensitive to the Src inhibitor PP-2, and that

FAK functionally interacts with Src in pUS28-expressing cells stimulated with CCL5. We further demonstrated that Src activation is upstream of FAK in this pathway (Streblow, Vomaske et al. 2003). Similarly, pUS28 can signal to Pyk2 in FAK-/- fibroblasts suggesting that Pyk2 activation is upstream or independent of FAK in this signaling cascade. Our proteomics analysis of Pyk2 binding partners in the context of pUS28 signaling revealed several novel proteins that shed new insight into the function of Pyk2 in pro-migratory signaling. In particular, we show that Pyk2 interacts with several tropomyosin (TM) isoforms and the TM interacting protein tropomodulin isoform 3 (Tmod3) in response to pUS28 signaling. These proteins are well-characterized regulators of actin polymerization and cytoskeletal dynamics (Kostyukova 2008). Tmod3 is broadly expressed in cells with dynamic actin filament structures and is unique among tropomodulin proteins in that, in addition to regulating the capping of actin filament pointed ends, it binds free actin monomers and may regulate the nucleation of new actin filaments (Fischer, Yarmola et al. 2006). Our discovery of an association of Pyk2 with TM isoforms is novel and particularly intriguing. TM proteins regulate the structure and specific properties of actin filaments but the mechanisms of TM regulation remain obscure (Gunning, O'Neill et al. 2008). One possible mechanism of TM regulation is phosphorylation. Indeed, the phosphorlyation of TM1 in response to ERK signaling in endothelial cells increases the formation of both actin stress fibers and focal adhesions contributing to increased cellular contractility (Houle, Rousseau et al. 2003). Our results suggest that the Pyk2 signaling complex participates in cytoskeleton remodeling via modification of Tmod and TM proteins. Taken together, these results suggest that Pyk2 acts together with Src, FAK and other adaptor molecules to promote pro-migratory signaling and that Pyk2 phosphorylation at Tyr-402 is required for the downstream activation of RhoA. Our finding that the scaffolding activity of Pyk2 is separable from its kinase activity is novel. In the context of AngII-mediated SMC migration

as well as migration of endothelial cells, Pyk2 kinase activity is an essential component of pro-migratory signaling (Kuwabara, Nakaoka et al. 2004; Ohtsu, Mifune et al. 2005).

### *3.5.2 pUS28 signaling and CMV-associated malignancy*

In this study, we explore the contribution of pUS28 signaling to Pyk2 in migration processes associated with HCMV-mediated vascular disease. However, HCMV has been implicated in other disease processes in which the induction of cellular migration may play a role. In particular, HCMV infection has been associated with several human malignancies including prostate and colon cancers as well as glioblastoma multiforme (GBM) (Soderberg-Naucler 2006). GBM is the most common and most malignant type of brain cancer and currently has no effective treatment (Louis, Ohgaki et al. 2007). HCMV DNA is present in over 90% of human malignant gliomas (Cobbs, Harkins et al. 2002; Mitchell, Xie et al. 2008), suggesting a role for HCMV in glioma oncogenesis. The connection between HCMV and GBM was recently strengthened by a study showing that vaccination against the HCMV tegument protein pp65 can improve survival in GBM patients (Mitchell, Archer et al. 2008). However, the molecular mechanisms of HCMV participation in glioma development remain unclear.

pUS28 signaling reportedly produces a transformed phenotype in transfected NIH-3T3 cells that is characterized by increased proliferative capacity and increased expression of the pro-angiogenic marker VEGF. In addition, the study demonstrated that WT HCMV infection of a human glioblastoma cell line (U373) induces VEGF expression while a pUS28 deletion mutant of HCMV fails to induce VEGF upregulation in glioblastoma cells (Maussang, Verzijl et al. 2006) implicating pUS28 signaling in glioma pathogenesis. However, this study did not explore the contribution of pUS28 ligand binding to the observed phenotypes.

### *3.5.3 Pyk2 and glioma tumorigenesis*

In addition to its role in vascular remodelling, Pyk2 has been identified as a pro-metastatic and pro-angiogenic molecule in several cancer models including small cell lung cancer (Roelle, Grosse et al. 2008; Zhang, Qiu et al. 2008), prostate cancer (Stanzione, Picascia et al. 2001; Picascia, Stanzione et al. 2002; Iiizumi, Bandyopadhyay et al. 2008) and glioblastoma (Lipinski, Tran et al. 2003; Lipinski, Tran et al. 2005; Lipinski, Tran et al. 2006; Lipinski, Tran et al. 2008). A number of elegant studies by Lipinski et. al. have established roles for Pyk2 and FAK in glioma tumorigenesis both *in vitro* and *in vivo*. In these studies, Pyk2 is demonstrated to promote migration and invasion of glioma cells while FAK controls their proliferation. The authors demonstrate that the migratory capacity of individual glioma cell lines can be correlated to the relative levels of Pyk2 expression and activation. Perhaps the most compelling evidence for the role of Pyk2 and FAK in glioma tumor progression utilized an *in vivo* xenograft model of glioblastoma in mice. In this study, Pyk2 deficient cells were observed to invade only locally and survival was significantly increased when mice were engrafted with Pyk2 or FAK deficient glioma cells compared to mice engrafted with wild-type glioma cells (Lipinski, Tran et al. 2008). Our current study and previous work characterizing the pUS28-mediated activation of FAK suggest a mechanism for acceleration of glioma tumor formation by HCMV via pUS28-mediated signaling through the critical mediators of glioma tumor progression, Pyk2 and FAK. Our results are consistent with existing literature in which the Pyk2 FERM domain is established as a target for reducing glioma cell migration (Lipinski, Tran et al. 2006; Loftus, Yang et al. 2009). Pyk2 undergoes dimerization and autophosphorylation upon FERM domain interaction with Ca2+/calmodulin (Kohno, Matsuda et al. 2008). Therefore inhibition of the Pyk2 FERM domain by mutation or antibody targeting may prevent dimerization and phosphorylation of Pyk2 at Tyr-402 thereby preventing the activation of

RhoA.

In U373 glioblastoma cells, we observed the formation of a cell type-specific Pyk2 complex in response to pUS28 signaling. This complex included several proteins previously implicated in glioma tumorigenesis, but never before identified as Pyk2 binding partners. Our data suggests at least two novel mechanisms for the participation of Pyk2 and pUS28 in glioma tumorigenesis. First, we identified TCF-4 and AF-17 binding to Pyk2 in response to pUS28 signaling. Both proteins are members of the Wnt/β-catenin signaling pathway that has recently been linked to glioma progression (Pu, Zhang et al. 2009; Sareddy, Challa et al. 2009). Indeed, siRNA mediated inhibition of this pathway decreases glioma proliferation and invasion capacity (Pu, Zhang et al. 2009). Interestingly, Pyk2 has been shown to phosphorylate β-catenin in endothelial cells (van Buul, Anthony et al. 2005) suggesting that Pyk2 may be involved in the activation of canonical β-catenin signaling in multiple cell types. Second, we discovered that pUS28 signaling caused Pyk2 to associate with three members of the Hsp70 family of molecular chaperone proteins. Interestingly, all three of these proteins are known to be specifically upregulated in glioma tissue and play various roles in the progression of glioma tumorigenesis (Takano, Wadhwa et al. 1997; Pyrko, Schonthal et al. 2007; Vila-Carriles, Zhou et al. 2007) as well as other cancer models (Daugaard, Rohde et al. 2007). Mortalin, in particular, is functionally regulated via tyrosine phosphorylation (Mizukoshi, Suzuki et al. 2001). Our results suggest that Pyk2, or kinases associated with Pyk2 may play a role in regulating the function of multiple Hsp70-family chaperones, thereby contributing to the proliferation and survival of tumor cells.

These findings refine our understanding of the pro-migratory signaling pathways activated by pUS28 in SMC and establish Pyk2 as an attractive target for the treatment of CMV-mediated vascular disease because it acts early in the pUS28 pro-migratory signaling

cascade and its expression is restricted to a few cell types, reducing the opportunities for off-target effects. Further, these results suggest a role for HCMV pUS28 in pathologies associated with aberrant activation of Pyk2 including some malignancies.

## 3.6 Conclusions

In this study, we determined the involvement of Pyk2 in pUS28 signaling and cellular migration. We found that Pyk2 autophosphorylation activity, but not kinase activity, is necessary for HCMV-mediated SMC migration. Furthermore, pUS28 signaling causes ligand-dependent phosphorylation of Pyk2 at Tyr-402 independent of pUS28 signaling to FAK. Pyk2 is incorporated into an active kinase complex in CCL5 stimulated SMC expressing pUS28. Pyk2 associates with a number of other proteins in response to pUS28 signaling including kinases, signaling molecules, cytoskeletal proteins, and molecular chaperones. We determined that although the Pyk2 autophosphorylation mutant (F402Y) does not prevent the formation of an activated kinase complex, it exerts a dominant negative effect on SMC migration by preventing the pUS28-mediated activation of the small G-protein RhoA. Importantly, the effect of pUS28 activating Pyk2 is not cell-type specific as we observed comparable activation of Pyk2 and RhoA via pUS28 signaling in U373 glioblastoma cells. This finding suggests that pUS28 signaling to Pyk2 may be important in glioma cell motility. In addition to establishing Pyk2 as a potential target in the treatment of HCMV-mediated vascular disease, and establishing a number of novel Pyk2-associated proteins, these results provide a potential link between pUS28 and CMV-associated malignancy.

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# IV

# Chapter 4 - RCMV r129 is a Chemotactic Cytokine

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## 4.1 Introduction

In rat solid organ transplantation, infection with RCMV accelerates TVS (Lemstrom, Bruning et al. 1993; Bruning, Persoons et al. 1994; Lemstrom, Koskinen et al. 1995; Orloff 1999; Orloff, Streblow et al. 2002) leading to graft failure. In a rat heart transplant model, acute infection with RCMV dramatically decreases the mean time to develop TVS and graft failure, and also increases the severity of TVS in graft vessels (Orloff, Yin et al. 1999; Orloff, Yin et al. 2000). Importantly, the effects of CMV on TVS acceleration are not organ specific but occur in a broad range of solid organ transplants including heart, kidney, lung, and small bowel (Orloff 1999; Orloff, Yin et al. 1999; Orloff, Yin et al. 2000). Although these observations demonstrate a link between CMV and TVS, the viral mechanisms involved in the acceleration of disease need further clarification. Understanding these viral mechanisms will also provide us with a tool to understand the development of disease in the absence of viral infection.

Chemokines play a major role in the development of vascular disease. Chemokines are a group of inducible cytokines that promote cellular migration and activation through binding to their respective GPCRs (Rollins 1997). The four classes of chemokines are the CC chemokines, which include MCP-1, MIP-1$\alpha$, MIP-1$\beta$ and RANTES, the CXC chemokines, which include IL-8, IP-10, and SDF-1$\alpha$, the C chemokine, lymphotactin, and the $CX_3C$ chemokine, Fractalkine. Chemokines are present in vascularized grafts at all stages post-transplantation, including during ischemia/reperfusion injury, acute rejection, chronic rejection, and during the healing processes (Melter, McMahon et al. 1999). On the contrary, long-term graft acceptance has been attributed to the absence of chemokines, thus substantiating a major role for chemokines in allogeneic graft rejection and TVS (Russel, Hancock et al. 1995). CC and CXC chemokines have been detected in rejecting human allografts and in experimental animal models of graft rejection (Melter, McMahon

et al. 1999). MCP-1, MIP-1α and β, and RANTES are the major chemokines detected during chronic graft rejection (Nadeau, Azuma et al. 1995; Adams, Hubscher et al. 1996; Fairchild, VanBuskirk et al. 1997; Grandaliano, Gesualdo et al. 1997; Czismadia, Sevillano et al. 1998; Vapnek, Hobart et al. 1998). The most definitive evidence that these molecules are important factors in graft vasculopathies derives from genetically altered mice. For example, deletion of the chemokine receptor CCR1 or CCR5 in a mouse heart transplant model of CR resulted in significant prolongation of graft survival (Gao, Topham et al. 2000). Similarly, wild-type (WT) mouse heart allograft recipients treated with antibodies directed against CCR5 had delayed acute graft rejection (Gao, Faia et al. 2001). These data suggest that CC-chemokines play a major role in acute rejection, TVS, and CR. Accordingly, in our rat heart transplant model we have found that, compared to uninfected allograft recipients, RCMV infected allografts contain immune infiltrates in the grafts at times paralleling the initial acceleration of TVS (Streblow, Kreklywich et al. 2003). This immune cell infiltration is a result of increased expression of chemokines including RANTES, MCP-1, and MIP1-α, IP-10, fractalkine, and lymphotactin. These data suggest that the link between CMV and TVS involves a complex and dynamic interplay between the virus and host inflammatory response mediated by an increase of chemokines.

Herpesvirus subversion of the chemokine network plays an important role in host immune evasion (Vink, Smit et al. 2001; Boomker, de Leij et al. 2005). Many herpes viruses, including KSHV and HCMV, encode soluble chemokine binding proteins, which bind to cellular chemokines, thereby reducing chemotaxis of lymphocytes to sites of virus infection (Wang, Bresnahan et al. 2004; Boomker, de Leij et al. 2005). In addition, many herpes viruses encode chemokine homologues. Generally, the characterized CMV-encoded chemokine homologues are pro-inflammatory, suggesting a role in viral dissemination via recruitment of permissive leukocyte or progenitor cell populations to sites of infection

(Fleming, Davis-Poynter et al. 1999; Penfold, Dairaghi et al. 1999; Saederup, Lin et al. 1999; Saederup, Aguirre et al. 2001; Kaptein, van Cleef et al. 2004; Noda, Aguirre et al. 2006). HCMV encodes two CXC chemokine homologues, UL146 and UL147 and one putative CC chemokine homologue UL128 (Cha, Tom et al. 1996). Only the gene product of UL146 (denoted vCXC-1) has thus far been shown to be functional. (Penfold, Dairaghi et al. 1999). The most well characterized CMV chemokine homologue, MCK-2 is the MCMV-CC chemokine m131/129. MCK-2, is a product of the spliced fusion of m131 and m129, and encodes a functional chemokine with chemotactic activity *in vivo*. MCK-2 mutant viruses show no significant defect in virus replication *in vitro*, at initial sites of infection, nor dissemination to many secondary sites, but have been associated with a significant decrease in virus dissemination to the salivary glands (a crucial site for secondary CMV transmission) (Fleming, Davis-Poynter et al. 1999). MCK-2 was shown to specifically recruit immature myelomonocytic leukocytes from bone marrow to initial sites of MCMV infection (Noda, Aguirre et al. 2006). It has been suggested that MCK-2 functions to recruit a mobile leukocyte population to early sites of CMV infection in order to efficiently disseminate virus to important secondary sites such as the salivary glands. The analogous region in the RCMV genome also contains ORFs collinear to those in MCMV, designated r131 and r129 (Figure 4.1). The gene product of r131 is a homologue of MCK-2. Similar to MCK-2 knockout MCMV viruses, recombinant RCMV strains lacking r131 show defects in dissemination to the spleen and salivary glands (Kaptein, van Cleef et al. 2004). Sequence alignment shows that the r131 gene product shares 12% homology to the rat CC chemokine MIP-1β, a monocyte chemotactic protein. Unlike m129, which merely donates the sequence for the c-terminus of MCK-2 and has no intrinsic CC chemokine characteristics, the putative r129 gene product contains N-terminal CC chemokine consensus sequences suggesting RCMV encodes two separate CC chemokine homologues in this

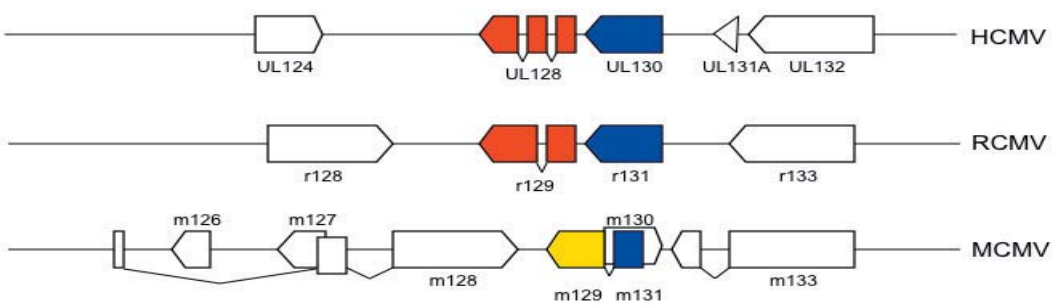


**Figure 4.1 - Genomic Region of Cytomegalovirus-encoded CC-chemokines**
**Schematic representation of the genomic region for HCMV, RCMV and MCMV CC-chemokine homologs.**

region. Interestingly, r129 has significant homology (19.4%) to HCMV UL128 which has been identified in clinical strains and is predicted to encode a CC chemokine (Akter, Cunningham et al. 2003). Detailed characterization of specific chemotactic activities of any of the r131 and r129 gene products or their homologues in HCMV remains to be established.

In the current study, we demonstrate that RCMV r129 encodes a functional CC-chemokine. Recombinant r129 is chemotactic to primary rat lymphocytes and specifically recruits immature CD4+ T cells. We further identify functional domains of r129 critical for migration via site-directed mutagenesis. We determine that r129 is expressed with true late kinetics of RCMV infection and that r129 is a component of the virion envelope. Importantly, we have produced an RCMV containing the r129-ΔNT mutation via BAC mutagenesis. RCMV-GFP-r129ΔNT has normal plaque morphology but spreads slowly within cultures. Western blot analysis of infected cell lysates reveals that r129 protein is expressed from RCMV-GFP-r129ΔNT and accumulates to high levels. However, cell-free virus made from RCMV-ΔNT fails to infect new rat fibroblast cultures, but transfer of infected cells is sufficient to propagate RCMV-ΔNT infection. These results suggest that the N-terminal 10 amino acids of r129 are required for entry of cell-free RCMV particles into fibroblasts but not required for cell-to-cell spread of RCMV within fibroblast monolayers.

## 4.2 Materials and Methods

*Cells and Viruses.* NR8383 rat alveolar macrophages were maintained in RPMI with 10% fetal calf serum (FCS) and penicillin-streptomycin-L-glutamine (PSG, Gibco). RFL6 rat fibroblasts were maintained in DMEM with 10% FCS and PSG. Primary rat PBMC were isolated by overlaying 6ml whole blood of F344 adult male rats over 5ml Ficoll-Paque (GE Healthcare), centrifuging 45 min at 1500 rpm and isolating the buffy coat layer. Lymphocytes were washed with PBS and resuspended in RPMI without supplements. Primary rat bone marrow lymphocytes were isolated by flushing bone marrow from rat femurs and tibias and straining through a 70μm filter. Bone marrow cells were washed in PBS and resuspended in RPMI without supplements. Primary rat splenocytes were isolated via masceration of spleen tissue though a polystyrene mesh followed by lysis of red blood cells (RBC) in 0.84% ammonium chloride for 5 minutes. RBC lysis was stopped by addition of an equal volume of RPMI. Splenocytes were then strained through a 70μm filter and resuspended in RPMI without supplements. RCMV-GFP was constructed as previously described (Baca Jones, Kreklywich et al. 2009).

*Reagents.* Recombinant rat MCP-1 was purchased from R&D Systems (3144-JE). Donkey anti-mouse HRP-conjugated secondary antibody was from GE Healthcare Amersham (NA934V). APC-conjugated mouse anti-rat CD3 antibody (1F4), APC-conjugated mouse anti-rat CD4 (OX-35), Biotin-conjugated mouse anti-rat CD8a (OX-8), PE-conjugated hamster anti-rat CD62L (HRL1) primary antibodies and streptavidin-FITC secondary antibodies were from BD Pharmingen. FITC-conjugated mouse anti-rat CD45R (HIS24) was from eBioscience.

*Cloning and purification of r129-HIS recombinant proteins.* The r129 open reading frame was cloned by making total RNA from RCMV-GFP infected RFL6 cells at 48 hours post-infection (hpi) via Quiagen RNeasy kit. cDNA was made from total RNA using Invitrogen

Superscript III First-strand synthesis kit (18080-051). For bacterial expression and purification r129 was amplified using primers designed to eliminate the r129 signal sequence (predicted using SignalP 3.0 Server) and add a C-terminal 6XHIS tag. Truncation mutants were amplified similarly using primers to truncate the N-terminal 10 amino acids following the signal sequence cleavage site or the C-terminal 121 amino acids. PCR products were cloned into pGEMT-easy (Promega) and subcloned into pRSETB expression vector using NdeI and HindIII restriction sites. Point mutations (r129-C31A and -F43A) were made using 20mer complementary primers including the mutated sequence. Mutagenesis PCR was performed via standard protocol using Pfu Turbo (Stratagene) and transformation into TOP-10 competent cells (Invitrogen). pRSETB clones were confirmed via sequencing and transformed into Rosetta 2 competent cells (EMD4Biosciences 71405). For large-scale purification of r129-HIS proteins, Rosetta 2 cells were grown to $A_{600}$~0.5 in 10L of 2X YT broth and induced with 0.5M IPTG overnight at 30˚C. Induced cells were pelleted at 7000 rpm for 10 minutes and lysed in 3ml/gm lysis buffer (300mM NaCL, 50mM $NaPO_4$, 20mM Tris-HCl, 0.1mM PMSF, 3mM 2-mercaptoethanol (2-ME), pH8.0). Lysosyme (1mg/ml ) and DNase (5µg/ml) were added and incubated for 10 min at room temperature (r.t.) followed by 30 min on ice. Cells were sonicated for three times for 30 seconds each at 84W. The inclusion bodies were pelleted from the cell sonicate at 10,000 rpm for 1 hr at 4˚C. Inclusion body extract (IBE) was made by resuspending pelleted inclusion bodies in binding buffer (300mM NaCL, 50mM $NaPO_4$, 20mM Tris-HCl, 8M Urea, 3mM 2-ME, pH8.0) and incubating 30 min at 60˚C. IBE was applied to 1ml equilibrated TALON® metal affinity resin (Clontech, 63502) in a chromatography column. Resin was washed with 10 column volumes of wash buffer (300mM NaCL, 50mM $NaPO_4$, 20mM Tris-HCl, 8M Urea, 3mM 2-ME, 10% Glycerol, pH 7.5). Bound HIS proteins were eluted sequentially with one column volume each 0.1M, 0.5M and 1M imidazole in wash buffer. Eluted proteins were

analyzed via SDS-PAGE, Coomassie stain and quantified by spectrophotometry. S100 gel filtration column was equilibrated with gel filtration buffer (100mM Tris-HCl, 8M Urea, 3mM 2-ME, pH 8.0) using an Akta FPLC. Approximately, 8-15mg of purified HIS-tagged protein was loaded onto the column and 0.5 ml fractions were collected and analyzed via SDS-PAGE Coomassie stain. Peak protein fractions were pooled and dialyzed sequentially against 6M, 4M, 2M, 1M, 0.5M and 0.1M Urea in 20mM Tris-HCl with a final dialysis into 20mM Tris-HCl. Insoluable or misfolded proteins were removed via centrifugation at 10,000 rpm for 1 hr. Soluble protein was quantified via spectrophotometry and dialyzed into 1% acetic acid and finally into 0.1% TFA. Aliquots of r129 (20µg each) were lyophilized and stored at -80˚C. For chemotaxis assays, the HIS tagged proteins were reconstituted in PBS and stored at 10µg/ml working concentration.

*In vitro migration of lymphocytes.* NR8383 macrophages or primary lymphocytes (derived from peripheral blood, bone marrow or spleen) were suspended in RPMI without supplements at $2x10^6$ cells/ml. 100µl ($2x10^5$ cells) was added to the top well of a chemotaxis chamber (96-well Millipore Multiscreen, 3.0µm pore size). r129-HIS protein or control recombinant protein in RPMI without supplements or media alone was added to the bottom chamber as chemotactic stimulus. Chemotaxis was allowed to proceed for 1 hr at 37°C. Top chambers were discarded and migrated cells in the bottom chamber were quantified via fluorescence using CyQuant (Invitrogen) and read on a Molecular Devices Flexstation® II fluorescence plate reader. Migration was determined from 4-6 independent wells per assay per condition. Mean and standard deviation were calculated. Percent of control values were generated by comparing chemokine stimulated cells to unstimulated media-only control cells and the results were analyzed using Student's t test. P values $<0.05$ were considered statistically significant.

*Fluorescence Accelerated Cell Sorting.* Splenocytes were isolated as described and

resuspended at $1(10)^8$ cells/ml in 10ml of PBS + 1% FBS. Antibodies were added and incubated at 4˚C for 40 minutes. Cells were washed 2X with PBS + 1% FBS and the appropriate secondary antibody (if necessary) was applied at $1\mu g/1(10)^8$ cells for 30 min at 4˚C followed by washing as before. Stained cells were resuspended at $5(10)^7$ cells/ml in PBS + 1% FBS and sorted on a BD FACSAria II cell sorter. Sorted populations were centrifuged for 5 min at 2000 rpm and resuspended at $2(10)^6$/ml in RPMI without supplements. Chemotaxis assays were performed and analyzed as described.

*Generation of RCMV r129-ΔNT virus.* We have constructed a BAC containing the RCMV-Maastricht strain. The BAC vector cassette was inserted into the RCMV genome in r144-146 of the RCMV genome. The BAC cassette contains the chloramphenicol resistance marker and eGFP under control of the HCMV-major immediate early promotor, and is flanked with LoxP sites in direct orientation. We are using a modification of the standard two-step linear recombination protocol utilizing galactokinase (*galK*) for positive and negative selection (Warming, Costantino et al. 2005) coupled with kanamycin resistance (*Kan*$^r$) negative selection to produce a seamless recombination scheme that will not interfere with the r129 ORF. The dual selection cassette containing both the galactokinase (*galK*) gene and a kanamycin resistance gene (*Kan*$^r$) was generously provided by Dr. Dong Yu (Washington University, St. Louis, MO). In the first recombination step, recombinant bacteria (SW105) containing the RCMV BAC were transformed with a PCR product comprised of *galK-Kan*$^r$ flanked by sequences homologous to regions flanking the r129 ORF in the RCMV genome. Recombinants were selected on LB Agar containing kanamycin and verified via PCR of the r129 genomic region and sequencing for the presence of the *galK-Kan*$^r$ cassette. In the second recombination step, the *galK-Kan*$^r$ cassette was replaced with a PCR product with sequence homology to the recombination site containing the r129 ORF with the coding sequence for the first 10 amino acids deleted. Recombinants were selected

on plates containing 2-deoxy-galactose, a substrate toxic in the presence of *galK*, resulting in counter-selection for BAC clones that have lost the *galK* marker and contain the desired mutation. Recombinants were verified via PCR of the r129 genomic region and sequencing for the mutation. Virus was reconstituted from RCMV BAC by electroporation of purified BAC DNA (Epicenter BaxMax Kit) into RCMV permissive fibroblast cells.

*Generation of mouse polyclonal anti-r129 antibody.* Balb/c female mice were injected twice, 21 days apart, ip with 20ug of r129-HIS emulsified in Sigma oil in water emulsion (Sigma catalog # S6322-1VL). Two weeks after the second injection, mice were bled and the titer of anti-r129 was determined in ELISA. Terminal bleeds were then prepared from the best responding mice.

*Western Blot Analysis.* RCMV-infected cells were lysed in 2X Laemmeli's and 20µl was loaded onto NuPAGE 4-12% Bis-Tris gradient gels (Invitrogen) and run in MES buffer (Invitrogen). Proteins were transferred to Immobilon-P blotting membrane and dried overnight to block. Membranes were rehydrated and secondarily blocked in 2% Milk + 0.02% Tween-20 (Block) for 15 min at r.t. Primary mouse polyclonal α-r129 antibody was added at 1:1000 dilution in Block for 1 hour at r.t., and membranes were washed 10 min in TBS + 0.2% Tween (TBST). Secondary goat anti-mouse-HRP conjugate was added at 1:40,000 in Block for 20 min at r.t. and membranes were washed three times for 10 min each in TBST. Membranes were exposed to ECL Advance Lumigen-TNA (GE Healthcare) for 1 min and exposed to Biomax Light Film (Kodak).

## 4.3 Results

### *4.3.1 Recombinant r129-HIS is Chemotactic*

To determine whether the r129 open reading frame encodes a functional CC-chemokine, we expressed r129 as a 6X HIS tagged recombinant protein. r129-HIS was expressed

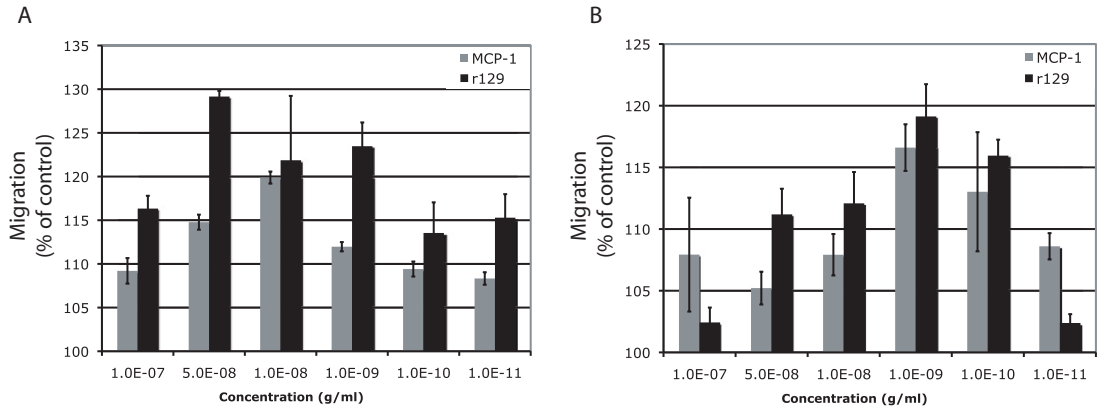


**Figure 4.2 - r129-HIS is chemotactic**
**In vitro migration of (A) NR8383 rat alveolar macrophages or (B) primary rat PBMC in response to varying concentrations of recombinant monocyte chemotactic protein-1 (rMCP-1)or r129-HIS. Shown is percent increase in migration compared to cells treated with media only.**

in bacterial cells and HIS tagged proteins were purified using Ni/Co resin. FPLC was utilized to remove impurities and peak fractions from FPLC were refolded via stepwise dialysis. We performed *in vitro* migration assays with varying concentrations of r129-HIS as the chemotactic stimulus for the migration of the NR8383 rat macrophage cell line or primary rat PBMC. As a positive control for chemotaxis in these experiments we used a commercial recombinant MCP-1. We found that r129-HIS is chemotactic to both PBMC and NR8383 macrophages at the same working concentration range as recombinant MCP-1 (Figure 4.2).

### *4.3.2 Functional Domains of r129*

Having established that r129-HIS is a functional chemokine, we wanted to identify r129 domains that contribute to chemotactic signaling. We constructed four mutants based on structure/function studies of other CC-chemokines (Zhang, Rutledge et al. 1994; Kaptein, van Cleef et al. 2004). Schematic representations of each mutant r129 construct are shown in Figure 4.3. Recombinant proteins were expressed in bacteria and purified as described for r129-HIS. We tested each mutant construct for chemotactic activity using NR8383 rat macrophage cells as well as primary rat lymphocytes isolated from PBMC, bone marrow and

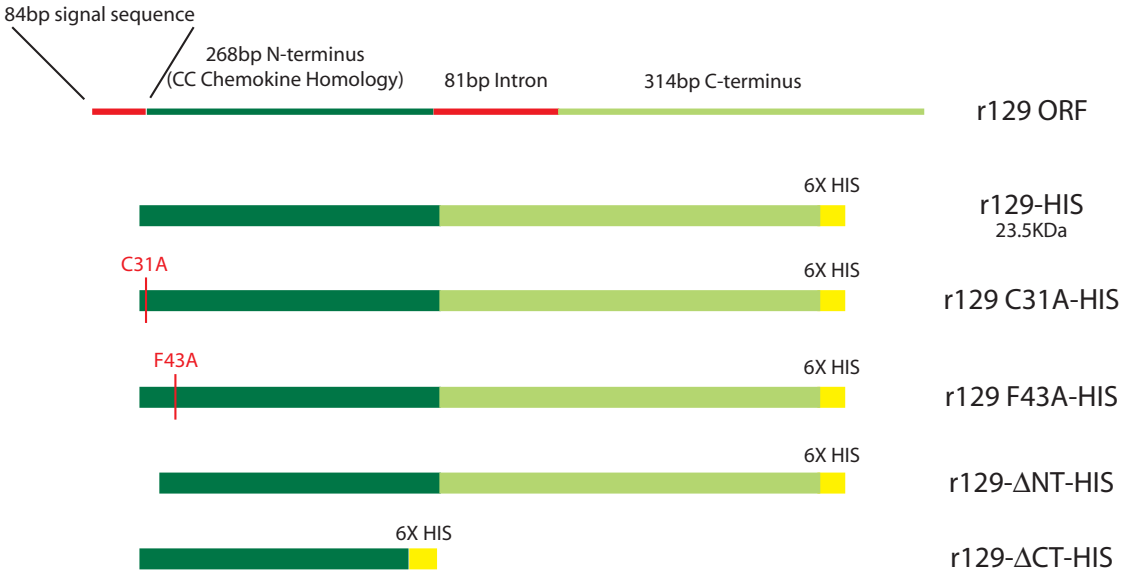


**Figure 4.3 - Schematics of r129 mutants**
**Schematic representations of the r129 gene (top) and recombinant r129-HIS protein and mutants.**

spleen (Figure 4.4). As expected, deletion of the N-terminal 10 amino acids in r129-ΔNT completely abrogated the chemotactic activity of the molecule. Similarly, mutation C31, which comprises the first of the two sequential cysteines in the CC-motif, resulted in a non-functional r129 molecule. Deletion of the C-terminus of r129 had no effect on chemotactic activity. The fact that the C-terminal tail is dispensible for r129 chemotaxis suggests that this molecule may play multiple roles in RCMV biology, however the significance of this finding is still unclear. Interestingly, the r129-F43A mutant displayed a differential migration phenotype depending on the source of the lymphocytes used in the assay. r129-F43A was not chemotactic to NR8383 rat macrophages, but caused migration of PBMC similar to that observed with r129-WT treated cells (Figure 4.4A&4.4B). Lymphocytes isolated from rat bone marrow and rat spleen displayed an intermediate phenotype when stimulated with r129-F43A (Figure 4.4C&4.4D). These results suggest that mutation of F43 results in altered receptor binding but whether this is due to a decrease in receptor affinity or a receptor type switch remains to be determined.

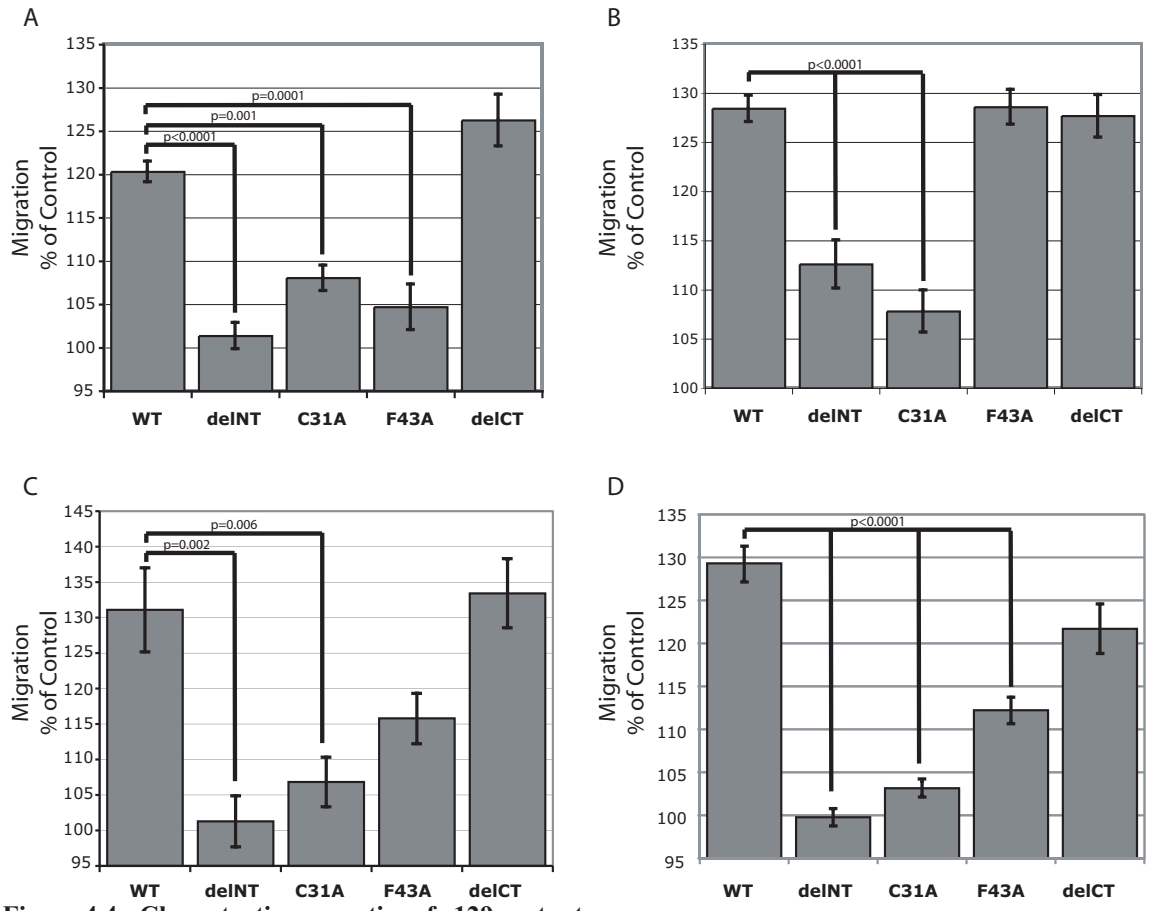


**Figure 4.4 - Chemotactic properties of r129 mutants**

**In vitro migration of (A) NR8383 rat alveolar macrophage cells stimulated with 1ng/ml (B) primary rat PBMC (C) primary rat splenocytes or (D) primary rat bone marrow lymphocytes stimulated with 0.1ng/ml r129-HIS WT, delNT, C31A, F43A, or delCT mutant constructs. For (A-B) n>14 from three independent experiments. For (C-D) n>10 from one experiment.**

To determine whether the non-functional r129 mutants inhibit the migratory action of r129-WT, we performed competition assays using NR8383 rat macrophages in which cells were simultaneously stimulated with r129-WT and varying concentrations of the r129 mutants. Interestingly, r129-ΔNT, r129-C31A and r129-F43A were able to competitively inhibit the migration of NR8383 stimulated with r129-WT (Figure 4.5). The mutant r129-C31A was the most potent inhibitor as 1ng/ml was sufficient to competitively inhibit the pro-migratory signaling of a 10ng/ml r129-WT stimulus (Figure 4.5B). The r129-ΔNT and r129-F43A proteins were inhibitory at concentrations equal to or higher than the amount of r129-WT

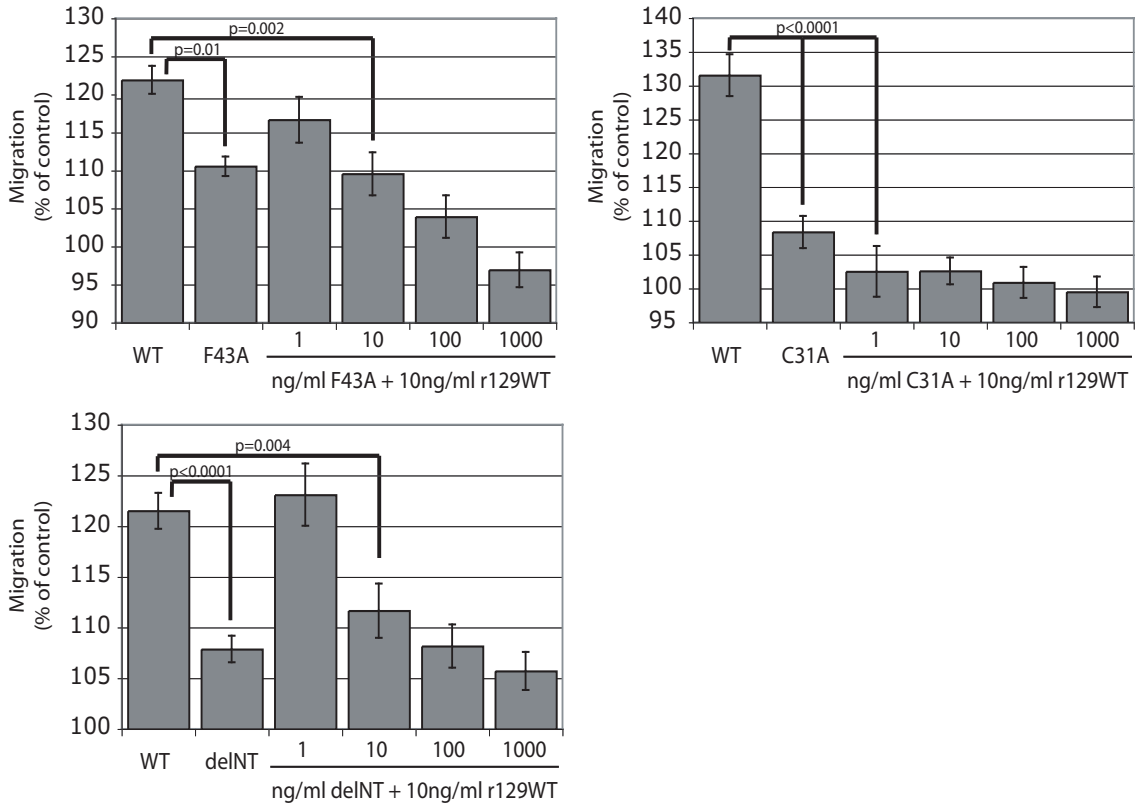


**Figure 4.5 - r129 Mutants Compete with r129-WT Chemotactic Stimulus**
**In vitro migration of NR8383 rat alveolar macrophage cells stimulated with 10ng/ml WT r129-HIS, 10ng/ml mutant r129-HIS or 10ng/ml WT r129-HIS and the indicated concentrations of (A) r129 F43A-HIS, (B) r129 C31A-HIS, or (C) r129-delNT-HIS. as a competing ligand. n>15 from three independent experiments**

used in the assay (Figure 4.5A&4.5C).

### *4.3.3 r129 recruits immature CD4+ T cells*

To determine which cell types are targeted by r129, we initially utilized fluorescence activated cell sorting (FACS) to isolate lymphocyte sub-populations and performed *in vitro* migration assays on the sorted populations. For all sorting experiments, we isolated lymphocytes from spleen via maceration and lysis of red blood cells (RBC). In the first experiment, the isolated splenocytes were stained with antibodies specific for CD3 (T cells) and CD45R (B cells) and the cells were sorted into three populations for *in vitro* migration assays: (1) CD3+ CD45R- (T cells), (2) CD3- CD45R+ (B cells) and (3) CD3- CD45R-

(Non-B/Non-T cells). We found that the T cell subset preferentially migrated in response to r129 (Figure 4.6A) as the B cell population and Non-B/NonT cell populations failed to migrate above background levels. To determine which T cell subset responds to r129, we performed a similar experiment in which splenocytes were stained for CD4, CD8, and for the maturation marker CD62L. CD62L is highly expressed on naïve (Na) and central memory (CM) T cells and is less prevalent on effector memory (EM) T cells. In this experiment we sorted four populations for *in vitro* migration: (1) CD4+ $CD62L^{Hi}$ (CD4 Na/CM), (2) CD4+ $CD62L^{Lo}$ (CD4 EM), (3) CD8+ $CD62L^{Hi}$ (CD8 Na/CM), (2) CD8+ $CD62L^{Lo}$ (CD8 EM). We found that only the CD4 Na/CM population retained the capacity to migrate in response to an r129 chemotactic gradient (Figure 4.6B). Taken together, these results indicate that r129 is able to recruit immature CD4+ T cells to sites of infection. This is consistent with data obtained from our rat heart transplant model of RCMV-accelerated chronic rejection in which we see infiltration of large numbers of CD4+ T cells into latently infected organs prior to transplantation (Streblow et. al., in press).

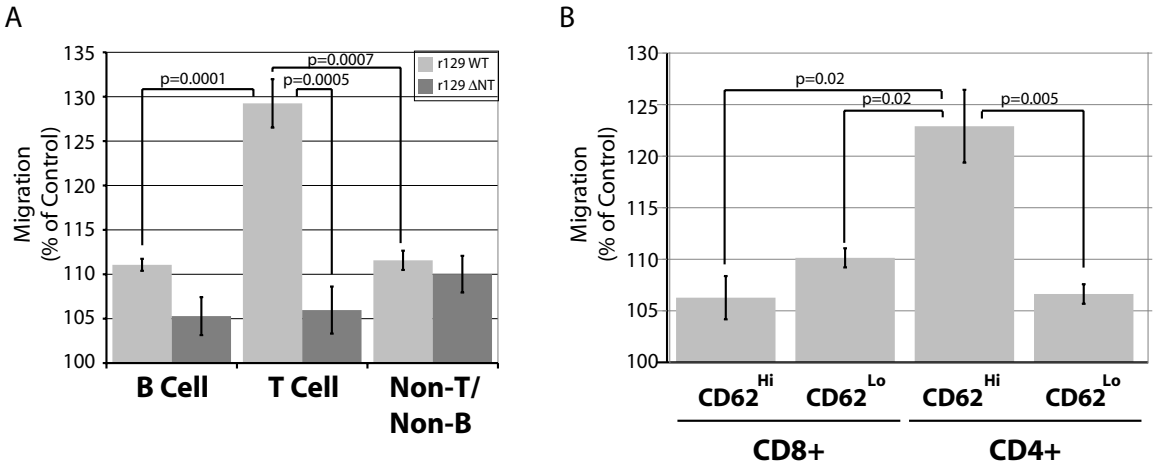


**Figure 4.6 - r129 Recruits Immature CD4+ T cells**

**(A) In vitro migration of primary splenocytes sorted for expression of CD45R (B Cell) and CD3 (T Cell) or neither marker (Non-T/Non-B) and stimulated with 1ng/ml r129-WT or r129ΔNT. n<15 from two independent experiments. (B) In vitro migration of primary splenocytes sorted for expression of CD8 or CD4 and subpopulations expressing the maturation marker CD62L and stimulated with 1ng/ml r129-WT. n<10 from one experiment.**

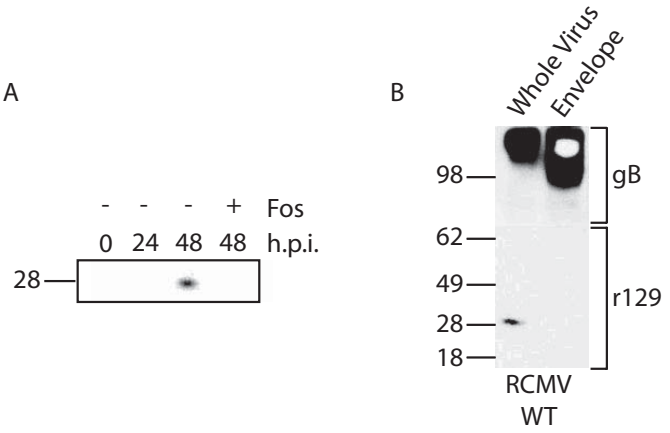


**Figure 4.7 - r129 is Expressed with Late Kinetics in RCMV Infection and is Present in the Virion**
**(A) Western blot of total cell lysates from RCMV-infected RFL6 harvested at 0, 24 or 48 hpi +/- 0.5mM Foscarnet and probed with anti-r129 polyclonal antibody. (B) Western blot of whole RCMV virions or NP40 RCMV envelope preparations with anti-gB (top) or anti-r129 (bottom)**

### *4.3.4 r129 is expressed with late kinetics in RCMV infection*

In order to gain further insight into the function of r129 during RCMV infection, we utilized our recombinant r129-WT-HIS to produce a mouse polyclonal serum directed against r129. Four mice were immunized with 20μg of r129-WT-HIS and boosted with a second 20μg of protein at three weeks post-immunization. After an additional three weeks, a serum sample was tested by ELISA and western blot for reactivity against r129-WT-HIS. Two of four animals had good r129 reactive antibody responses (data not shown). We then tested the ability of the polyclonal mouse serum to detect r129 expression in the context of WT-RCMV infection. Rat fibroblast (RFL6) cells were infected with RCMV at MOI 1 and lysates were harvested at 8 hpi (IE viral kinetics) and 48 hpi (late viral kinetics). An additional infected sample was treated with 0.5mM Foscarnet to block late gene expression and harvested at 48 hpi (early viral kinetics). Lysates were run on SDS-PAGE and western blotted with α-r129 polyclonal mouse serum (Figure 4.7A). We detect r129 expression at 48 hpi, and expression of r129 is sensitive to Foscarnet suggesting that r129 is expressed with late viral kinetics. Additionally, we performed western blot analysis on purified cell-free virions. We detected r129 in whole virions, and r129 was absent in purified envelope fractions (Figure 4.7B). These results indicate that r129 is expressed with true late kinetics in RCMV infection and is incorporated into the virion but is not associated with the viral

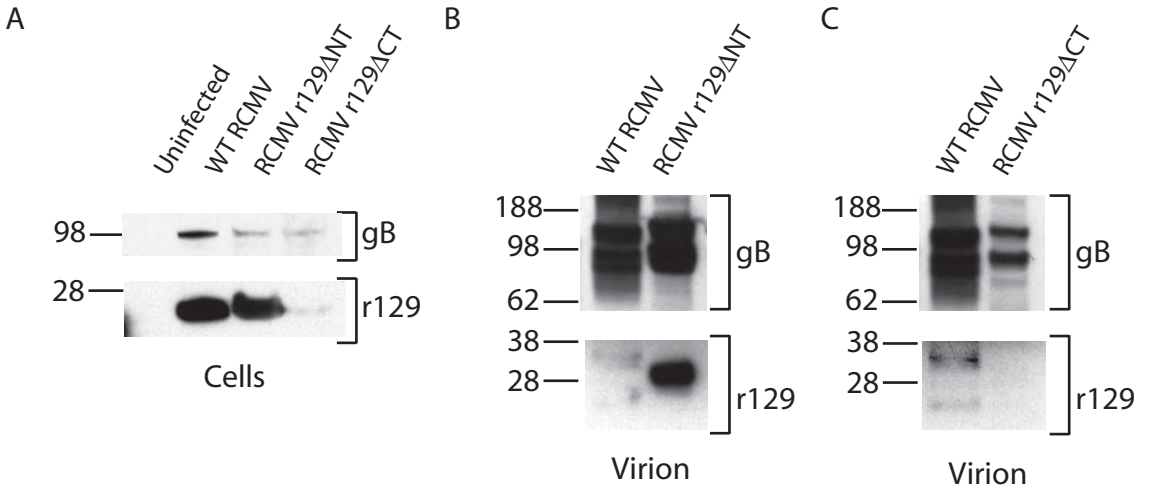


**Figure 4.8 - Preliminary Characterization of RCMV-r129ΔNT**
**(A) Western blot of total cellular lysates from RFL6 fibroblasts infected with RCMV WT or transfected with RCMV-r129ΔNT or RCMV-r129ΔCT and probed with anti-gB (top) or anti-r129 (bottom). Western blot of virion preparations from RCMV WT infection or (B) RCMV-r129ΔNT BAC-transfections or (C) RCMV-r129ΔCT transfections probed with anti-gB (top) or anti-r129 (bottom).**

envelope.

### *4.3.5 RCMV r129 mutants are differentially incorporated into the virion*

To determine the role of r129 in RCMV replication and during pathogenesis, we constructed a RCMV recombinant viruses that expresses the non-migrating r129 mutant-ΔNT or the truncated chemotactic r129-ΔCT mutant instead of the wild type gene. We utilized a two-step seamless linear recombination method to generate BAC-derived RCMV with the mutant r129 sequence introduced into the r129 ORF (RCMV-GFP-r129ΔNT or RCMV-GFP-r129ΔCT) . To reconstitute virus we transfected purified BAC DNA containing the mutations into RFL6 rat fibroblast cells. The spread of infection was monitored by GFP fluorescence microscopy. Both RCMV-GFP-r129ΔNT and -r129ΔCT spread in culture and formed plaques with normal morphology. Western blot analysis of transfected cells revealed that r129 protein is expressed from the mutant RCMV genome in both cases. However, taking into account levels of gB protein, expression of r129ΔCT is significantly

lower than r129-WT and r129ΔNT and r129ΔNT is overexpressed relative to r129-WT (Figure 4.8A). To initially characterize these two mutant RCMV, cell-free virus was isolated via ultracentrifugation through a 10% sorbitol cushion and resulting virions were analyzed for incorporation of r129 protein. We observed that, relative to gB protein levels, RCMV-r129ΔNT incorporated higher levels of r129 protein into particles than WT RCMV (Figure 4.8B). Conversely, r129 is absent or undetectable in RCMV-GFP-r129ΔCT virions (Figure 4.8C). Taken together, these results suggest that r129 mutants are differentially expressed in BAC transfected cells and are differentially incorporated into virus particles. Moreover, the C-terminal domain may contain important determinants for r129 virion incorporation.

## 4.4 Discussion and Future Directions

### *4.4.1 r129-mediated chemotaxis in vascular disease and TVS*

Our observation that r129 is a functional chemotactic cytokine has significant implications for the RCMV-mediated development of TVS. Mechanisms of vascular disease and chronic rejection in solid organ transplants have long been thought to be a result of chronic inflammation. We have recently shown significant recruitment of T cells into RCMV-infected allograft hearts in our rat model of chronic rejection. Importantly, when donor animals are latently infected with RCMV, the chronic rejection phenotype is insensitive to gancyclovir treatment, suggesting that RCMV infection primes the inflammatory process in these organs and active CMV replication is not necessary to drive CR post-transplantation (Streblow et. al., in press). We hypothesize that r129-mediated recruitment of immature T cells into the heart prior to transplantation may contribute to the pro-inflammatory conditioning of the organ. Additionally, we have shown the presence of tertiary lymphoid organ (TLO) structures in the heart tissue of RCMV infected rats (Streblow et. al., in

press) and r129 may play a role in the formation of these structures via recruitment of naïve T cells into infected organ tissues. RCMV strains incorporating r129 mutations will be instrumental in determining the contribution of r129 to T cell recruitment in infected rats. Moreover, our observation that r129-ΔCT is also a functional chemokine suggests that r129 has multiple roles in the biology of RCMV. It will be interesting to determine the phenotype of RCMV-GFP-r129-ΔCT *in vitro* and *in vivo* in order to characterize the possible functions of the r129 C-terminal domain.

### *4.4.2 Characterization of RCMV r129ΔNT and RCMV r129ΔCT*

Our initial characterization of RCMV r129ΔNT and RCMV r129ΔCT suggest that r129 mutants are differentially incorporated in to virions. As such, these mutant RCMV provide an interesting opportunity to study the contribution of r129 incorporation in the biology of the virion. Critical future experiments will characterize the growth characteristics of RCMV r129ΔNT and RCMV r129ΔCT *in vitro* and *in vivo* and determine whether r129 contributes to the pathogenesis of TVS in our rat heart transplant model. We are currently constructing mutant RCMV strains incorporating the other r129 mutations shown in Figure 4.3 as well as an r129 knockout virus and an r129 repair virus control. All RCMV r129 mutants will be characterized for virion incorporation of r129 as well as growth defects *in vitro* and *in vivo* and will be used to determine any contributions of r129 to the pathogenesis of TVS in our rat model.

The HCMV homologue of RCMV r129, UL128, is a critical mediator of HCMV entry into endothelial and epithelial cells but dispensable for entry into fibroblasts (Ryckman, Jarvis et al. 2006; Ryckman, Rainish et al. 2008). The domains of UL128 necessary for its function in entry have not been mapped. It would be interesting to determine whether truncation mutants of UL128 produce a similar phenotype in HCMV virions and whether recombinant UL128 is chemotactic *in vitro*.

### *4.4.3 Identification of the r129 receptor(s)*

Critical future experiments will characterize the cellular receptor(s) for r129. To this end, we have cloned rat CCR1, 2, 3, 4, 5, 6, 7, and 8 into adenovirus vectors for receptor binding studies. To date, functional adenoviruses have been obtained for rat CCR 1, 2, 3, 4, 5 and 7. We are currently optimizing ligand binding assays based upon FACS detection of bound r129-HIS protein as well as calcium flux and arrestin translocation assays.

# V

# Chapter 5 - Conclusions and Directions

## 5.1 Conclusions

The goal of the research presented in this dissertation was to examine CMV subversion of the host chemokine signaling network. I examined signaling from the HCMV-encoded chemokine receptor US28 in order to establish potential mechanisms for the contribution of HCMV to the development of chronic inflammatory conditions. Further, I characterized a novel CMV-encoded chemokine. Three major conclusions can be drawn from the data presented herein. First, the broad ligand specificity of US28 dictates functionally selective signaling. US28 functional selectivity results in ligand and cell-type specific differences in US28 pro-migratory signaling with significant implications for the contribution of HCMV to the pathogenesis of vascular disease. Second, the non-receptor tyrosine kinase Pyk2 is critical for transduction of US28 pro-migratory signaling in multiple HCMV susceptible cell types. Signaling to Pyk2 participates in vascular remodeling associated with disease states including atherosclerosis as well as the migration and invasion of glioblastoma cells. Therefore, US28-mediated activation of Pyk2 is a potential mechanism by which HCMV infection promotes inflammatory diseases. Finally, the RCMV genome contains a functional CC-chemokine encoded by the r129 ORF that is chemotactic to immature CD4+ T cells *in vitro*. These data lay the foundation for characterization of RCMV r129 and its homolog HCMV UL128 as potential factors in the CMV-mediated acceleration of vascular disease and chronic rejection in solid organ allografts.

### *5.1.1 US28 Signaling Displays Functional Selectivity*

I demonstrate in Chapter 2 that US28 pro-migratory signaling is functionally selective and the outcome of US28 interactions with chemokine ligands is cell type specific. In US28-expressing SMC, RANTES (CCL5) initiates pro-migratory signaling, and Fractalkine ($CX_3CL1$) binding to US28 is anti-migratory. However, my data show that in SMC Fractalkine competes out CC-

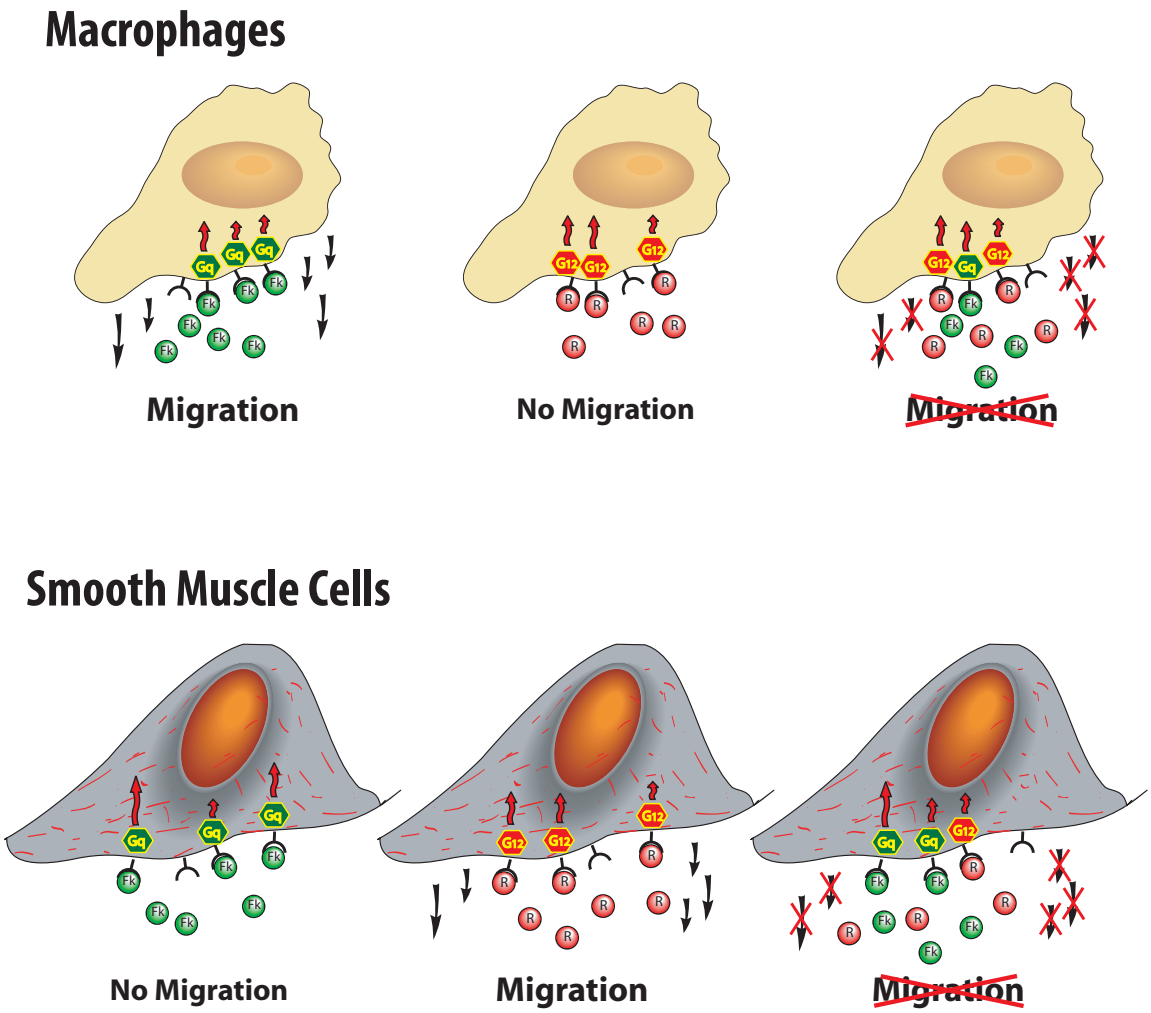


**Figure 5.1 - Model for US28 Ligand and Cell Type Functional Selectivity**
**US28-expressing Macrophages migrate in response to Fractalkine stimulation (green circles) via Gαq signaling. RANTES (red circles) stimulation signals through Gα12 and produces no migration. The RANTES-mediated anti-migratory phenotype is dominant when both ligands are present. US28-expressing SMC migrate in response to RANTES stimulation via Gα12. Fractalkine stimulation signals through Gαq and produces no migration. The Fractalkine-mediated anti-migratory phenotype is dominant when both ligands are present.**

chemokine mediated pro-migratory stimulus. When US28 is expressed in macrophages, the opposite phenotype is observed. Fractalkine binding to US28 produces pro-migratory signaling while RANTES stimluation is actively anti-migratory in US28-expressing macrophages, and RANTES can prevent Fractalkine-mediated migration of macrophages

(Figure 5.1).

Importantly, US28 is the first chemokine receptor to display this level of functional selectivity by mediating the differential chemotaxis of varying cell types in response to two separate classes of chemokine ligands. These intriguing observations suggest that the same US28 receptor activated state (RANTES-bound or Fractalkine-bound) can produce differential signaling depending upon the cellular context in which US28 is expressed. My experiments in Chapter 2 suggest that this phenomenon may occur at the level of G-protein binding to US28 and cell type specific differences in G-protein expression may account for the activation of differential signaling pathways (Figure 3.3). However, the generation of ligand and cell type specific signaling from US28 may involve additional mechanisms, which certainly warrant further study. Moreover, understanding the cellular mechanisms underlying US28 functional selectivity will provide information critical to understanding differential signaling from endogenous chemokine receptors, providing new insight into inflammation and immune function.

In addition to providing essential information about chemokine receptor function, these results provide a compelling mechanism for the participation of HCMV-infected cell types in the formation of atherosclerotic lesions via US28-mediated cell type-specific chemotaxis of SMC and macrophages. My observations suggest two mechanisms by which US28-mediated cellular migration could contribute to vascular disease: (1) CC-chemokine stimulus from infiltrating immune cells mediates migration of SMC into the vessel intima. Concurrently, the pro-inflammatory environment activates EC lining the vessel and drives expression of membrane-bound Fractalkine on the EC luminal membrane. Migrating SMC reach the vessel intima and SMC-expressed US28 is bound by EC-expressed Fractalkine. The US28-Fractalkine interaction overcomes the CC-chemokine mediated pro-migratory signaling cascade and SMC are fixed in the intima by this interaction. The subsequent proliferation

and secretion of ECM components by SMC fixed in the vessel lumen contributes to vessel narrowing and the formation of fibrous plaques. (2) The second mechanism involves the recruitment of HCMV-infected monocytes to sites of inflammation in the vessel via soluble Fractalkine signaling and adherence to the vessel wall in response to EC-expressed membrane-bound Fractalkine. Once inside the vessel wall, CC-chemokine stimulus stops migration. Recruited monocytes differentiate into macrophages, which ultimately become foam cells, contributing to the deposition of lipids and necrotic material in the atherosclerotic plaque.

### *5.1.2 Pyk2 is a Critical Mediator of US28 Pro-Migratory Signaling*

I have established compelling mechanisms by which HCMV could modulate the progression of atherosclerosis and TVS via US28-mediated migration of cells into early atherosclerotic lesions. Effective therapeutic intervention to prevent this process requires a more thorough understanding of both US28 biology and the cellular signaling pathways involved in US28-mediated cellular migration. As discussed in Appendix 1, the functional selectivity of US28 signaling makes pharmacological targeting of US28 difficult. Potential antagonists could prove effective at blocking specific US28 signaling pathways, but could prove to be agonists for other pathways. For example, I observed that US28 expressing cells treated with VUF2334, an inhibitor of constitutive activity, signals through FAK. As such, the identification of potential cellular targets to block US28-mediated cellular migration will be important in the development of therapies to prevent the development of HCMV-associated TVS and other inflammatory diseases. In Chapter 3 I identify one such target, the non-receptor protein tyrosine kinase, Pyk2. Pyk2 is an attractive target for pharmacological intervention because it is expressed with limited tissue distribution primarily found in HCMV susceptible cell types. Therefore, inhibition of Pyk2 would have fewer non-specific effects than targeting ubiquitous kinases involved in US28-mediated

migration such as FAK. Moreover, in Chapter 3 I determine that Pyk2 kinase activity is dispensable for US28-mediated migration. The participation of Pyk2 in these pathways is as a signaling scaffold mediated by autophosphorylation at Y-402, which is critical for promoting activation of the downstream GTPase RhoA. Identification of Y-402 as the critical site for this pathway provides a specific Pyk2 function that could be targeted for more precise inhibition of US28 signaling leaving other cellular Pyk2 functions intact. These results refine our understanding of the cellular mediators of US28 pro-migratory signaling (summarized in Figure 5.2). Importantly, mass spectrometric analysis on US28-specific Pyk2 complexes in both SMC and U373 glioblastoma cells revealed that some Pyk2 associated proteins were cell type-specific. These data could provide further insight into the etiology of cell type-specific functions of US28, and provide potential candidates for mediators of oncogenic signaling from US28 in glioma cells particularly.

### *5.1.3 RCMV ORF r129 Encodes a Functional CC-chemokine*

Our group has established an animal model for the participation of CMV in the development of CR/TVS following solid organ transplantation. RCMV infection of rats exacerbates transplant vasculopathy and has a significant effect on graft survival (ref). This model, together with our ability to construct genomic mutations in RCMV using BAC technology, will allow us to examine the contribution of specific RCMV genes in the development of TVS. Genes of particular interest include the viral chemokine receptors and the putative chemokines encoded by RCMV. In Chapter 4 of this dissertation, I demonstrate that the r129 ORF of RCMV encodes a functional chemokine homolog. I map the functional domains of r129 and determine that r129 shares many receptor binding and activation determinants with its cellular CC-chemokine counterparts. Moreover, I determine that r129 specifically targets immature CD4+ T cells *in vitro*, an observation that has significant implications for the participation of r129 in CR/TVS. I discover that r129 is expressed with late kinetics in RCMV

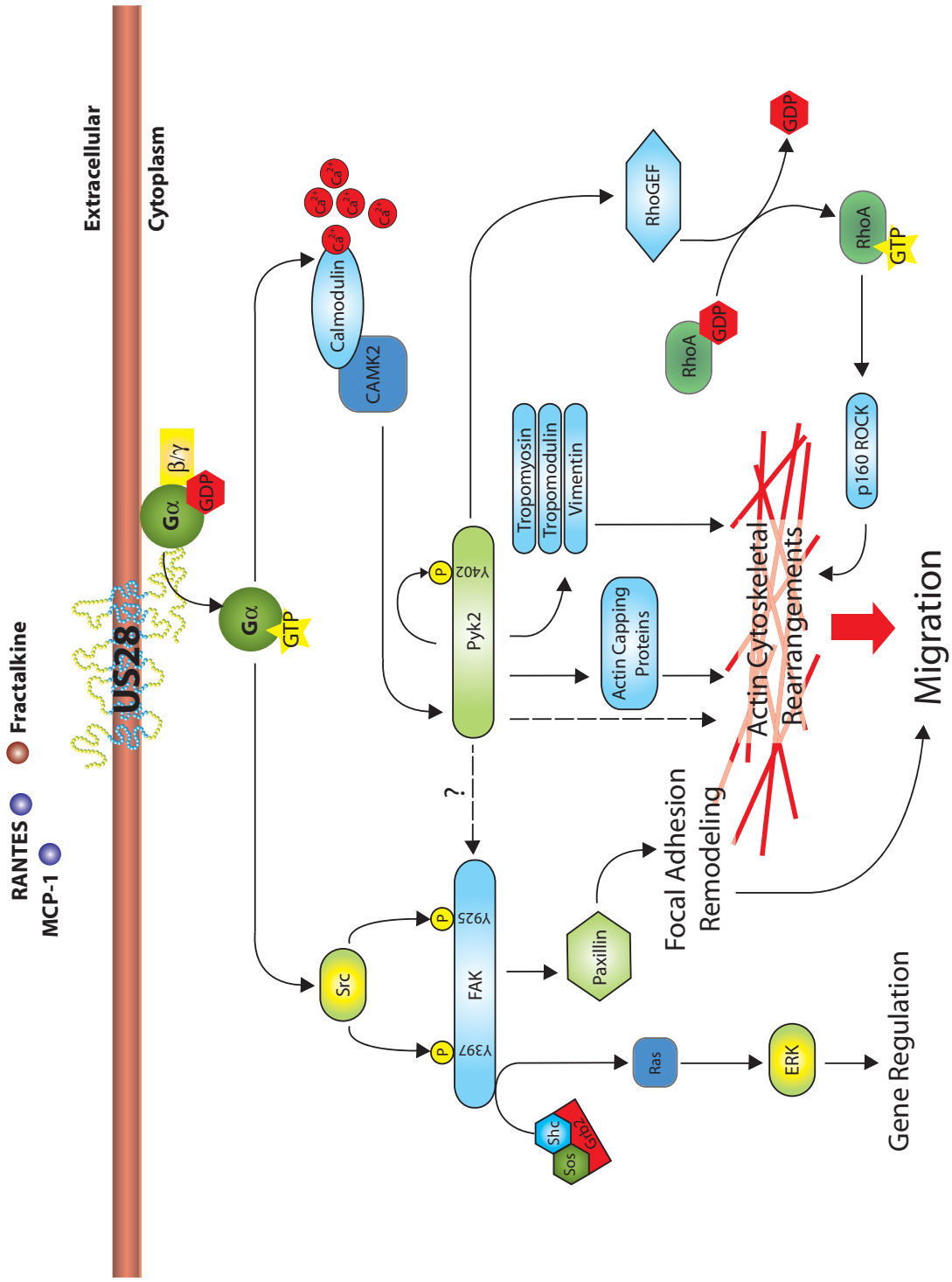


**Figure 5.2 - Model for US28-mediated Pro-migratory Signaling**

infection and that the protein is incorporated into virions. Finally, I describe the construction and initial characterization of two recombinant RCMV that contain specific mutations in the r129 ORF.

## 5.2 Discussion and Future Directions

### *5.2.1 Functions of US28 in Macrophage Biology*

My data demonstrating that US28 can affect the chemotaxis of macrophages via Fractalkine stimulus has further implications for the participation of HCMV in other inflammatory diseases. Macrophages are critical mediators of inflammation and participate in all stages of an inflammatory response (Fujiwara and Kobayashi 2005). The dysregulation of macrophage recruitment and function by US28 and other HCMV-encoded factors could have significant impacts on the development, maintenance and cessation of inflammation and could contribute to the development of a variety of chronic inflammatory conditions. For my data characterizing US28-mediated chemotaxis of macrophages I utilized a rat macrophage system in which I could dissect the effects of US28 response to human chemokine ligands in the absence of background from endogenous human chemokines and CCRs. However, this system prevents us from studying US28 in the context of HCMV infection. Examining US28 signaling in HCMV-infected macrophages will be critical to understanding these phenomena relative to the biology of the virus, however I believe that these experiments will prove to be challenging given that macrophages express a variety of endogenous chemokines and CCRs that are necessary for their normal function. Previous studies have suggested that HCMV infection of macrophages *in vitro* significantly affects macrophage motility via alterations in the expression of endogenous CCRs, particularly CCR1 and CCR5 (King, Baillie et al. 2006; Frascaroli, Varani et al. 2009). However, the viral proteins involved in this process are undefined. Dissecting the contribution of US28 as an additional viral CCR in such a

complex system will require tightly controlled experiments. Additionally, US28 could modify the function of endogenous chemokine receptors in a macrophage system via heterodimerization (see section 5.2.1.i), an intriguing possibility that will be difficult to characterize experimentally but may have significant implications for HCMV biology in macrophages. Utilizing bacterial artificial chromosome (BAC) technology to introduce specific US28 mutants into the HCMV genome and comparing functional outcomes in WT or mutant US28-expressing HCMV infected macrophages will be an important tool in dissecting the significance of US28 signaling the biology of HCMV infected macrophages. The two chemokine binding-deficient mutants Y16F and F14A would be particularly interesting choices for these experiments.

### *5.2.2 Mechanisms of US28 Functional Selectivity*

The most broadly significant conclusion presented in this dissertation is the functional selectivity of the US28 receptor. Ligand-specific interactions with US28 dictate differential migration in two separate HCMV-susceptible cell types. Although my data presents evidence that differential G-protein binding is responsible for ligand-specific responses, a number of other factors could contribute to US28 functional selectivity and cell type-specific signaling that remain to be examined. Three major areas of regulation remain to be explored: (1) The prospect of US28 interaction with cellular chemokine receptors, particularly in the macrophage system. Such oligomerization interactions could modulate both the functions of US28 and the functions of the cellular interacting partners. (2) The cell type specific expression of both G-proteins and Regulators of G-protein Signaling (RGS) molecules, which can be altered by the cellular environment and inflammatory factors. Alterations in the levels of these proteins can have significant cell type-specific and context-specific effects on GPCRs and may affect US28 signaling. (3) The cell type-specific expression of G-protein coupled Receptor Kinase (GRK) molecules and their modulation based on

inflammatory signaling could have significant effects on US28 signaling, desensitization and downregulation in a variety of cell types. Although multiple mechanisms likely interact to produce functionally selective signaling from US28, I will discuss the potential contributions of each of these mechanisms to US28 functional selectivity individually.

**i. Receptor oligomerization**

Historically, signaling through CCRs has been studied in the context of single receptor-ligand interactions activating a single cellular response pathway. In order to simplify and provide the best-controlled experiments, CCRs have often been studied in isolation in heterologous systems (i.e. HEK 293 cells). More recently, data have suggested that the function of a number of CCRs can be modulated by oligomerization with other CCRs in the same cell (Salanga, O'Hayre et al. 2009). Such interactions are particularly important in the context of hematopoietic cells, which generally express a dynamic variety of CCRs depending upon their developmental state and the inflammatory environment.

CCR2/CCR5 heterodimers have been characterized in heterologous systems as well as primary hematopoietic cells. However, the functional significance of these interactions remains obscure. In one study CCR2/CCR5 interactions were ligand-dependent and result in cooperative signaling, lowering the threshold concentration for calcium flux from each ligand and resulting in differential G-protein coupling to the heterodimer compared to CCR2 and CCR5 homodimers (Mellado, Rodriguez-Frade et al. 2001). A second study showed that CCR2/CCR5 heterodimers formed in a ligand-independent manner and displayed cross-inhibition with respect to ligand binding (El-Asmar, Springael et al. 2005). Two fundamental differences between these studies could account for the differing results: (1) each study utilized different cell types for expression of CCRs (HEK 293 cells and CHO cells, respectively) and (2) different analysis methods were used for the detection of receptor dimerization (Co-immunoprecipitation (co-IP) and bioluminescence resonance energy transfer

(BRET), respectively). Importantly, the study by Mellado et. al. verified CCR2 and CCR5 cooperative signaling in primary human PBMC from normal and CCR5-deficient donors. The possibility that US28 forms functional oligomers has not been explored. US28 could homodimerize or associate with other CMV-encoded or cellular chemokine receptors. Interactions with cellular CCRs could modify the constitutive recycling of US28 seen in tissue culture (Fraile-Ramos, Kledal et al. 2001) and potentially stabilize cell surface expression of US28. Heterodimerization of US28 with cellular CCRs has the potential to modify the ligand binding, G-protein recruitment, desensitization and signaling capacity of both receptors, which could result in significant modulation of chemotaxis and inflammatory signaling in HCMV-infected macrophages in particular.

Much of the current data examining the oligomerization of CCRs has been generated via co-IP studies. Immunoprecipitation has a number of significant drawbacks when the target proteins have multiple transmembrane domains as is the case with CCRs. Incomplete solubilization of membrane fractions and non-specific aggregation of CCRs after detergent extraction can be responsible for artifacts that confound co-IP data. Mixing of detergent solubilized membrane fractions from separate cultures containing target CCRs has been proposed as an alternative strategy (Milligan and Bouvier 2005), However, mixed co-IP reactions preclude the possibility of determining functional interactions of CCRs within the same cell and relies on the assumption that CCR dimerization domains remain intact following membrane solubilization. Fluorescence resonance energy transfer (FRET)- or BRET-based assays for CCR oligomerization have the potential for being more physiologically relevant as they can be performed in live cells and are sensitive enough to detect ligand-dependent conformational changes in receptors (Vilardaga, Bunemann et al. 2003), but these techniques are underutilized due to their high degree of technical difficulty (Milligan and Bouvier 2005). One significant limitation of traditional FRET/BRET is

that these techniques necessitate the transient expression of CCR fusion constructs. The addition of bulky bioluminescent or fluorescent tags to CCR proteins could negatively affect their ligand binding, oligomerization, signaling and desensitization. Moreover, the control of transient expression systems to prevent overexpression artifacts could prove challenging. Therefore, antibody-based FRET techniques, which negate the necessity of fused tags and allow the analysis of endogenously expressed CCRs, would be the most specific and relevant way of evaluating interactions between US28 and cellular chemokine receptors, both in the context of HCMV infection of monocyte/macrophages and in US28 expression studies in multiple cell types.

### ii. G-proteins and Regulators of G-protein Signaling (RGS)

The G-protein promiscuity of US28 and some of the known sequence requirements for G-protein binding are discussed in Chapter 1.5.2.ii. I show in Chapter 2 that US28 functional selectivity occurs at the level of G-protein binding in fibroblasts. Fractalkine binding to US28 produces Gαq-dependent activation of FAK while RANTES binding produces Gα12-dependent activation of FAK. These data provide a compelling picture of one mechanism of ligand-specific US28 functional selectivity. However, I believe that multiple cellular factors conspire to produce the cell type specific selectivity presented in Chapter 2. Certainly, determining whether G-protein selectivity plays a role in functionally selective US28 signaling in SMC and macrophages will be critical as cell type specific differences in G-protein expression could partially account for the observed phenotypes.

However, if I adopt the current model that differential ligand binding results in specific receptor active conformations and that these conformations have specific G-protein binding specificities, then the cell type specific differences in US28 signaling between SMC and macrophages cannot be fully accounted for by differences in expression of specific G-proteins. My data indicate that in each migrating cell type (SMC or macrophages) opposite classes of

US28 ligand (Fractalkine or RANTES) are either pro-migratory or anti-migratory. It is important to distinguish between an anti-migratory phenotype which is an active signal producing the effect of cellular fixation as opposed to a non-migratory phenotype that could be attributed to the lack of functional pro-migratory signal. The fact that (1) RANTES can compete out Fractalkine-mediated US28 migration in macrophages and (2) Fractalkine can compete out RANTES-mediated US28 migration in SMC and (3) both ligands can signal to FAK and the cytoskeleton in fibroblasts suggests that, rather than a lack of signaling from one class of ligand due to cellular deficiency in the correct G-protein expression, functional signaling is produced from both Fractalkine and RANTES-bound US28 active states in both cell types with opposite effects on the observed cellular motility. Based on my data, the signaling cascade initiated by each receptor active conformation is cell type specific but, G-proteins bind and initiate signaling in both cases. This argument leads to the more complex hypothesis that cell type-specific responses to different US28 ligands are mediated at the level of regulation of G-protein signaling the differential activation of signaling second messengers.

Regulators of G-protein Signaling are a large family of GTPase-activating proteins (GAPs), which regulate and modulate signaling from G-proteins by several mechanisms. They promote the turnover of GTP for GDP on activated Gα-subunits, thereby providing negative regulation of specific G-proteins. However, RGS proteins are multifunctional cellular regulators and can directly regulate Gβγ subunits, signaling second messengers such as adenalyl cyclase, other non-G-protein coupled receptor signaling events including TGFβ signaling, and even protein translation (Sethakorn, Yau et al. 2010). Importantly, increased G-protein activation and inflammatory signals can alter the expression of specific RGS proteins (Cho, Harrison et al. 2003; Hu, Li et al. 2009; Tsang, Woo et al. 2010). Such alterations in expression could result in both increased regulation of GPCR signaling

and secondarily produces feedback onto the myriad non-G-protein functions of RGS. Such alterations in RGS2 expression have been linked to the development of cardiovascular disease by multiple mechanisms (Tsang, Woo et al. 2010).

Differential expression levels of RGS proteins have been shown to affect chemotaxis and adhesion via CXCR1 and CCR2 in lymphoid cell lines. In this system, overexpression of RGS1, 3 or 4 were able to inhibit migration of lymphoid cells in response to chemotactic stimulus (Bowman, Campbell et al. 1998). The participation of RGS proteins in the promotion of US28-mediated pro-migratory signaling have not been explored, and analysis of the complex cell type specific regulation of G-protein signaling from US28 could provide significant insight into US28 functional selectivity.

### iii. GRK and Arrestin Regulation and Signaling

Following G-protein activation and the initiation of signal transduction, chemokine receptors are phosphorylated at their C-terminus by G-protein coupled Receptor Kinases (GRKs) and other cellular kinases to initiate receptor internalization and desensitization. The potential contribution of GRK phosphorylation to functional selectivity in chemokine receptor biology is discussed in Chapter 1.4.2.iii and A.3.4. Deficiencies in GRK molecules are associated with enhanced chemotaxis in a number of lymphocyte systems (Vroon, Heijnen et al. 2004; Vroon, Heijnen et al. 2004), underscoring the importance of GRK regulation for CCR signaling and cellular migration. In HEK 293 cells, US28 is phosphorylated by GRK2 and GRK5 (Miller, Houtz et al. 2003) and the MCMV-encoded CCR M33 is regulated by GRK2 in mouse cells (Sherrill and Miller 2006). However, the importance of GRK phosphorylation of US28 with respect to cellular migration and functional selectivity remains to be determined. Significantly, ligand-specific GRK recruitment has been shown for CCR7. siRNA knockdown of specific GRK molecules in CCR7-expressing cells has differential effects on CCR7 phosphorylation and arrestin recruitment depending upon the stimulating agonist (Zidar, Violin et al. 2009).

Similar experiments could be performed in US28-expressing cells to determine whether specific US28 active states preferentially recruit specific GRK.

Importantly, GRK2, a molecule with both RGS and GRK activity, is an active transducer of pro-migratory signaling via the Gαq-dependent phosphorylation of ezrin-radixinmoesin (ERM) (Cant and Pitcher 2005). This data is particularly compelling given that two known downstream effectors of US28 pro-migratory signaling, FAK and Pyk2, contain FERM domains that could potentially be targeted by GRK2. It would be particularly interesting to determine whether GRK2 plays a role in US28-mediated pro-migratory signaling either as a signal transducing effector or a regulator. The RGS and kinase activities of GRK2 are separable (Cant and Pitcher 2005), and domain specific mutants could be used to dissect potential functions of GRK2 in US28 signaling. Moreover, mutation of phosphorylation sites in the C-terminal domain of US28 could further elucidate any ligand-specific GRK-mediated regulation of US28 signaling and desensitization.

In the classical model of GPCR desensitization, the C-terminal phosphorylation of GPCR is followed by the recruitment of arrestin molecules to activated receptors. Arrestins bind activated receptors, preventing G-protein binding and thereafter receptor internalization mediates desensitization (Ferguson 2001). More recently, compelling evidence has accumulated that arrestin molecules are capable of scaffolding multiple signaling second messengers and producing functional, G-protein-independent signaling from activated GPCR (DeWire, Ahn et al. 2007). Moreover, arrestin-mediated signaling has been established as a mechanism for agonist selective activation of GPCR (Rajagopal, Rajagopal et al. 2010). Phosphorylated US28 has been shown to bind βarrestin-2 in HEK-293 cells independent of ligand stimulation. The recruitment of βarrestin molecules to ligand-bound US28 active states has not been determined and could be both ligand and cell type-specific. Moreover, the potential β–arrestin-mediated signaling from US28 has not been explored

and could have significant implications for US28-mediated cellular migration.

The potential β–arrestin-mediated activation of the MAP kinase ERK would be particularly intriguing to study in the context of US28 pro-migratory signaling. I have shown that ERK is activated by US28 in a FAK-dependent manner upon stimulation with either Fractalkine or RANTES in $FAK^{-/-}$ fibroblasts (Figure 3.3). Additionally, AT-1 receptor signaling can activate ERK via both G-protein and β–arrestin-dependent signaling. ERK activation from G-protein or β–arrestin-mediated pathways is ligand-specific as well as temporally and spatially distinct (Ahn, Shenoy et al. 2004). Significantly, another study showed ligand-specific AT-1 receptor mediated chemotaxis from a β–arrestin-mediated signaling cascade independent of G-protein activation (Hunton, Barnes et al. 2005). β–arrestin-mediated pro-migratory signaling could be a potential mechanism underlying US28 functional selectivity and should be the subject of future studies.

Importantly, β–arrestin expression has significant impact on the development of neointimal hyperplasia (NIH) following experimental endothelial denudation in a mouse model of atherosclerosis. This study showed that β–arrestin-2 knockout mice had significantly decreased NIH compared to WT mice at 2-4 weeks post injury and that arrestin-1 knockout mice had significantly increased NIH compared to WT mice in the same system. The effects of β–arrestin in this system were linked to the migration and proliferation of SMC into the vessel intima. This study provides compelling evidence suggesting that β–arrestin-2 plays a significant role in the development of atherosclerosis following vessel injury (Kim, Zhang et al. 2008). Future studies to determine whether this effect is due to β–arrestin signaling or β–arrestin-mediated regulation of GPCR signaling will be critical. Moreover, it would be fascinating to determine the phenotypes of β–arrestin transgenic mice in MCMV-accelerated atherogenesis.

### *5.2.3 RCMV r129 as a virulence factor in CR/TVS*

In Chapter 4, I determine that RCMV r129 is a functional CC-chemokine able to recruit immature CD4+ T cells *in vitro*. In order to examine the potential role of this viral CC-chemokine in RCMV pathogenesis, I constructed two recombinant RCMV with specific genomic mutations in the r129 gene. RCMV-r129ΔNT contains an r129 gene that is non-functional for chemotaxis *in vitro*. Preliminary data demonstrate that RCMV r129ΔNT produces abnormally high levels of r129 in infected cells and effectively incorporates r129ΔNT protein into progeny virions (Figure 4.8). Critical future experiments will determine whether RCMV-r129ΔNT is attenuated *in vivo* and whether the chemotactic function of r129 has an effect on the progression of CR/TVS in our rat heart transplant model.

RCMV r129ΔCT contains an r129 gene that is chemotactic but lacks the large C-terminal domain. Many viral chemokines are distinguished from cellular chemokines by a C-terminal domain lacking homology to any known protein. The functional significance of this domain remains unclear. Recombinant r129ΔCT is chemotactic to the same level as r129-WT and targets the same primary cell types as r129-WT (Figure 4.4). Preliminary experiments with RCMV r129ΔCT show that the r129ΔCT mutant is expressed at much lower levels in RCMV infection compared to r129-WT and r129ΔNT, suggesting that this protein may be unstable. Moreover, r129ΔCT is undetectable in virions (Figure 4.8), providing an opportunity to study the significance of r129 virion incorporation *in vitro* and *in vivo*. It will be interesting to determine whether RCMV r129ΔCT is attenuated *in vivo* and whether this virus can accelerate the development of CR/TVS. Based on these results, in would be interesting to determine whether the HCMV UL128 gene product also encodes a functional CC-chemokine.

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# A

# Appendix - Human Cytomegalovirus US28: A Functionally Selective Chemokine Binding Receptor

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## A.1 Abstract

The Human Cytomegalovirus (HCMV)-encoded chemokine receptor US28 is the most well-characterized of the four chemokine receptor-like molecules found in the HCMV genome. US28 been studied as an important virulence factor for HCMV-mediated vascular disease and, more recently, in models of HCMV-associated malignancy. US28 is a rare multi-chemokine family binding receptor with the ability to bind ligands from two distinct chemokine classes. Ligand binding to US28 activates cell-type and ligand-specific signaling pathways leading to cellular migration, an example receptor functional selectivity. Additionally, US28 constitutively activates PLC and NFkB. Understanding the structure/function relationships between US28, its ligands and intracellular signaling molecules will provide essential clues for effective pharmacological targeting this multifunctional chemokine receptor.

## A.2 Introduction

The ubiquitous β-herpesvirus, HCMV establishes a life-long persistent/latent infection in the immunocompetent host. Although HCMV infections are largely asymptomatic in individuals with normal immune function, HCMV has been implicated in the development of vascular diseases including transplant vascular sclerosis associated with chronic rejection of transplanted solid organs, restenosis following angioplasty and atherosclerosis (Melnick, Petrie et al. 1983; Hendrix, Dormans et al. 1989; McDonald, Rector et al. 1989; Speir, Modali et al. 1994; Zhou, Leon et al. 1996; Melnick, Adam et al. 1998; Muhlestein, Horne et al. 2000). HCMV infection is also associated with malignancies (Cobbs, Harkins et al. 2002; Harkins, Volk et al. 2002; Soderberg-Naucler 2006; Scheurer, Bondy et al. 2008), but the mechanisms of cytomegalovirus contribution to cancer remains poorly understood (Soderberg-Naucler 2006) and is more likely oncomodulatory rather than oncogenic in

nature (Cinatl, Vogel et al. 2004).

HCMV encodes four chemokine receptor homologues, namely UL33, US27, US28 and UL78 (Chee, Bankier et al. 1990). Of these, US28 is the most highly characterized. US28 is a key mediator of HCMV-associated vascular disease (Streblow, Orloff et al. 2001) and has recently been implicated in models of HCMV-associated glioblastoma (Maussang, Verzijl et al. 2006). US28 has been extensively studied as a consitutive activator of phospholipase C and NF-kB (Casarosa, Bakker et al. 2001). However, a number of ligand-dependent US28 activities have been characterized including the initiation of calcium flux (Gao and Murphy 1994; Kuhn 1995), the activation of MAP kinase signaling pathways (Billstrom, Johnson et al. 1998), and directed chemotaxis of vascular smooth muscle cells and macrophages (Streblow, Soderberg-Naucler et al. 1999).

Characterized US28 ligands include a number of human CC-chemokines (RANTES, MCP-1, MCP-3 and MIP-1α) as well as the CX3C-chemokine Fractalkine. US28 is unusual among chemokine receptors in its ability to bind chemokines of distinct chemokine families. The functional implications of this unique property of US28 are just beginning to be understood, and indicate that US28 displays "functional selectivity" defined as the ability of a single receptor to activate different signaling pathways in a ligand-specific manner (Urban, Clarke et al. 2007). This extended understanding of US28 signaling has significant implications for rational drug design to target the various functions of US28 in the context of HCMV pathogenesis and, as such, is the major focus of this review.

## A.3 Chemokine Receptor Structure and Function

### *A.3.1 General*

Chemokine receptors (CCRs) comprise a subfamily of the large Class A (Rhodopsin-like) 7-transmembrane domain (7-TM) G protein-coupled receptors (GPCRs). CCRs share

many characteristics with other GPCRs but contain several common characteristics by which they can be distinguished. CCRs are generally 340-370 amino acids (aa) in length and contain both acidic residues and sites for tyrosine sulfation in their N-termini. A cysteine is often found in each of the four extracellular domains. There is a conserved DRY-motif found in the second intracellular loop and the third intracellular loop contains basic residues (Murphy, Baggiolini et al. 2000) (Figure A.1). Although no crystal structure exists for any CCR, their structure has been inferred based upon sequence similarity to rhodopsin (Baldwin 1993; Unger, Hargrave et al. 1997; Lomize, Pogozheva et al. 1999). However, the synthesis of numerous functional and mutagenesis studies have provided a toggle-switch model for GPCR activation in which TM-3, TM-6 and TM-7 move towards each other on the extracellular face and away from each other on the intracellular face, utilizing proline residues as pivot points. The spreading of these three helices on the intracellular face of the receptor allows for the binding of signaling proteins such as G-proteins and subsequent intracellular signal transduction. This model attempts to reconcile the structural similarity of GPCRs and the commonality of their intracellular effector molecules (i.e. G-proteins and arrestins) with the vast diversity of GPCR agonists, which range from metal ions to small molecules to large glycoprotiens. The model is based upon the assumption that agonists function to stabilize, rather than induce, the activated receptor conformation. Therefore, diverse ligands can interact with different residues in distinct portions of the receptor to affect this stabilization. Interestingly, this model also provides for the possibility of spontaneous adoption of the active conformation in the absence of a stabilizing agonist, which may explain the constitutive activity observed with a number of GPCRs (Schwartz, Frimurer et al. 2006).

### *A.3.2 Ligand Binding*

Unlike small molecule agonists that generally bind inside the hydrophilic pocket formed

by the TM helices, chemokines and other peptide ligands are thought to stabilize activated receptor conformations via multiple low affinity interactions in several regions of the extracellular and TM domains. The N-terminus of chemokine receptors is often (but not always) indespensible for high affinity chemokine binding (Ahuja, Lee et al. 1996; Monteclaro and Charo 1996; Lau, Allen et al. 2004).

### *A.3.3 G-protein Binding*

The highly conserved D/ERY or 'DRY' motif at the intracellular end of TM-3 of all GPCRs has received significant attention as a potential G-protein binding and receptor activation determinant. Unfortunately, phenotypes observed with mutations in this region vary widely from receptor to receptor, even within the Class A family of GPCRs (Rovati, Capra et al. 2007). Mutation of the D/E residue often results in an increase in constitutive activity concurrent with an increase in agonist affinity. This phenotype has been observed with CXCR2 mutants (Burger, Burger et al. 1999) and would suggest a destabilization of the inactive receptor conformation. Similar mutations in CCR3 and CX3CR1 did not result in an increase in constitutive activity (Haskell, Cleary et al. 1999; Auger, Pease et al. 2002). There is a similar lack of a consensus phenotype with mutations of the conserved arginine residue (Rovati, Capra et al. 2007). The two chemokine receptors for which these mutations have been studied (CCR3 and CX3CR1) display a decrease in agonist-induced activity, most likely due to a defect in G-protein binding (Haskell, Cleary et al. 1999; Auger, Pease et al. 2002). These results suggest that the conserved arginine plays a significant role in G-protein binding, however the current data are not conclusive in this regard.

Recent evidence suggests that different ligands can stabilize receptor active conformations that have a G-protein affinity bias. This agonist-specific activation of signaling via distinct G-proteins has been demonstrated for the mu-opioid receptor (Saidak, Blake-Palmer et al. 2006), the beta-2 adrenergic receptor (Woo, Wang et al. 2008) and the thromboxane A2

receptor (Zhang, Brass et al. 2008). Taken together, these data indicate that the cellular G-protein environment as well as the agonist environment can influence the signaling capabilities and cellular functions of GPCRs.

### *A.3.4 Desensitization and Regulation*

Regulation of chemokine receptor signaling proceeds via the generally accepted model for GPCR regulation in which the ligand-stabilized, activated receptor transduces signal via G-protein coupling and is subsequently phosphorylated on the intracellular face by one or more G-protein coupled receptor kinases (GRKs). Phosphorylation mediates the coupling of β-arrestin proteins, which prevent further G-protein coupling and facilitate internalization of the desensitized receptor. The receptor is then either recycled to the cell surface in a process of resensitization or targeted for degradation (Krupnick and Benovic 1998; Pitcher, Freedman et al. 1998). This "classical" model for GPCR regulation has been revised and extended recently to account for the tissue-specific (Tobin, Butcher et al. 2008) and ligand-specific (Kelly, Bailey et al. 2008) control of GPCR signaling.

In addition to the seven known human GRKs, second messenger-dependent protein kinases such as PKA and PKC can phosphorylate GPCRs to facilitate desensitization (Benovic, Pike et al. 1985). Interestingly, CCR5 has been shown to be phosphorylated by both PKC and GRK 2 and/or 3 at different sites with different kinetics in a ligand-specific manner (Pollok-Kopp, Schwarze et al. 2003). However, the impact of these differential phosphorylation events on receptor function remains to be elucidated. These regulatory kinases differ in their tissue distribution, providing a mechanism for cell-type specific regulation of GPCR activity (Tobin, Butcher et al. 2008). Furthermore, the expression of GRKs can be modulated in a specific cell type in response to pro-inflammatory stimuli. This is of particular importance in the regulation of chemokine receptor activity in hematopoietic cells and the development of various inflammatory diseases (Vroon, Heijnen et al. 2006). In particular, decreased

GRK2 and GRK6 expression in immune cells is observed in experimental models of arthritis (Lombardi, Kavelaars et al. 2001). Further, TLR-4 mediated signaling via LPS stimulation decreases expression of GRK2 and GRK5 in neutrophils (Fan and Malik 2003). Such inflammatory modulation of GRK expression could result in increased signaling from chemokine receptors normally regulated by these kinases and provide a mechanism for increased recruitment of immune cells to sites of chronic inflammation. Indeed, decreases in GRK expression have been observed to increase chemotactic responses in T-cells via CCR5 (Vroon, Heijnen et al. 2004), and neutrophils via CXCR4 and CXCR2 (Fan and Malik 2003; Vroon, Heijnen et al. 2004).

One might expect that decreased β-arrestin expression might increase cellular chemotaxis via a decrease in receptor desensitization and a concurrent increase in pro-migratory signaling. However, β-arrestin-2-deficient T and B cells display decreased chemotactic activity despite increases in GTPase activity associated with CXCR4 (Fong, Premont et al. 2002). This data could be attributed to the β-arrestin-mediated activation of pro-migratory signaling molecules including ERK1/2 (Ge, Ly et al. 2003), and p38 MAPK (Sun, Cheng et al. 2002). Although very little is known about the specificity of β-arrestin recruitment to phosphorylated receptors, there is clearly the potential for crosstalk between GRK and/or second-messenger dependent kinase-mediated phosphorylation of receptors mediating differential binding of β-arrestin proteins (Violin, Ren et al. 2006) and potentially modulating the signaling capacity of receptor-β-arrestin complexes (Kelly, Bailey et al. 2008; Tobin, Butcher et al. 2008).

## A.4 Functions of US28 and their Pathophysiological Consequences

### *A.4.1 Cellular Activation*

US28 was initially characterized as being able to cause ligand-dependent calcium flux

and Gα16-mediated signaling to ERK1/2 in 293 cells (Billstrom, Johnson et al. 1998). Further signaling functions were elucidated in COS-7 cells where US28 is a constitutive 'cellular activator'. In this system, US28 signals in a ligand-independent manner to both phospholipase C and NF-kB (Casarosa, Bakker et al. 2001). These pathways certainly conspire to produce a cellular environment optimal for HCMV replication and NF-kB has been shown to directly transactivate the HCMV major immediate-early promoter (DeMeritt, Milford et al. 2004; Boomker, The et al. 2006). Constitutive activation of these cellular signaling pathways may contribute to CMV-mediated inflammatory diseases and possibly CMV-mediated oncogenesis (Soderberg-Naucler 2006; Vischer, Leurs et al. 2006). In addition, US28 expression has been shown to activate caspase-dependent apoptosis in a number of cell lines (Pleskoff, Casarosa et al. 2005).

### *A.4.2 Chemokine Scavenging*

The supernatants of HCMV-infected fibroblast cultures are deficient in a number of CC-chemokines. This has been attributed to a chemokine scavenging function of US28 in which these chemokines are actively removed from the supernatant via the constitutive recycling of US28 protein (Bodaghi, Jones et al. 1998). US28 expression in HCMV-infected fibroblasts is sufficient to appreciably decrease the monocyte chemotactic activity of the infected cell supernatants compared to supernatants from fibroblasts infected with a US28 knockout virus (Randolph-Habecker, Rahill et al. 2002). However, this role of US28 as a 'chemokine sink' has recently been challenged by others. US28 expression in endothelial cells is insufficient to prevent static monocyte adhesion, suggesting that physiological concentrations of chemokine are too high to be effectively scavenged by US28 (Boomker, de Jong et al. 2006).

### *A.4.3 Cellular Chemotaxis*

Importantly, US28 plays a role in motility of HCMV-infected cells. US28-mediated cellular migration has important implications for both HCMV dissemination and mechanisms for the development of HCMV-associated vascular disease. US28 binding to CC-chemokines causes migration of HCMV infected SMC (Streblow, Soderberg-Naucler et al. 1999). Conversely, stimulation of US28-expressing SMC with Fractalkine can antagonize CC-chemokine mediated SMC migration. Detailed examination of the signaling cascades involved in US28-mediated SMC migration indicate that CC-chemokine binding to US28 induces the G12-dependent activation of a variety of pro-migratory factors including FAK, Src, ERK1/2, Pyk2 and RhoA (Streblow, Soderberg-Naucler et al. 1999; Streblow, Vomaske et al. 2003; Melnychuk, Streblow et al. 2004). Interestingly, Fractalkine binding to US28 activates many of the same cellular second messengers seen with CC-chemokine binding, including FAK and ERK1/2. Although both chemokine classes activate FAK via US28, they do so via different G-proteins. CC-chemokine activation of FAK is G12-mediated while Fractalkine binding to US28 activates FAK via a Gq-dependent mechanism (Vomaske, Melnychuk et al. 2009). We have recently determined that Fractalkine but not CC-chemokines can induce migration in US28-expressing macrophages (Vomaske, Melnychuk et al. 2009). Therefore, US28 utilizes both the extracellular chemokine environment and the intracellular complement of G-proteins to produce ligand and cell-type specific migration of HCMV-infected cells. Taken together, these observations suggest a dual role for US28 in the acceleration of vascular disease via (1) CC-chemokine mediated migration of SMC into the vessel intima followed by Fractalkine-mediated fixation of SMC and subsequent proliferation leading to vessel narrowing and (2) the Fractalkine-mediated deposition of US28-expressing monocytes into atherosclerotic lesions leading to the formation of foam cells.

## A.5 US28 Structure and Function

### *A.5.1 Chemokine Binding Site*

Although chemokine ligands interact with multiple sites on the extracellular face of chemokine receptors, the N-terminus often dictates high-affinity ligand binding (Monteclaro and Charo 1996). A hexapeptide sequence in the N-terminus of US28 is critical for high-affinity binding of chemokine ligands to US28 (Casarosa, Waldhoer et al. 2005) This region is conserved between US28 and the endogenous human chemokine receptors CCR1 and CCR2 and is known to be a determinant for MCP-1 binding to CCR2 (Preobrazhensky, Dragan et al. 2000). Casarosa et. al. performed extensive mutagenesis studies of this region of US28 and discovered that CC-chemokine binding and fractalkine binding require different residues within this hexapeptide region for high affinity binding. Specifically, mutation of phenylalanine at position 14 negatively affects only CC chemokine binding to US28 but retains high affinity binding of Fractalkine. In contrast, mutation of the tyrosine at position 16 of the US28 N-terminus negatively affects high affinity binding of both classes of chemokine. The authors speculate that sulfation of Y16 may play a role in chemokine binding to US28. Additionally, an aromatic amino acid seems to be required at this position for surface expression of US28 suggesting a role in folding or trafficking of the receptor (Casarosa, Waldhoer et al. 2005).

### *A.5.2 Heterotrimeric G-protein Binding and Activation*

US28 couples with a variety of G-proteins alpha subunits. This G-protein promiscuity appears to provide a level of control and selectivity to US28 signaling depending upon ligand and cell-type context. The coupling of Gq/11 family members to US28 mediates the activation of phospholipase C (PLC) and NFkB in a ligand-independent manner (Casarosa, Bakker et al. 2001; Waldhoer, Kledal et al. 2002). In this system, Fractalkine

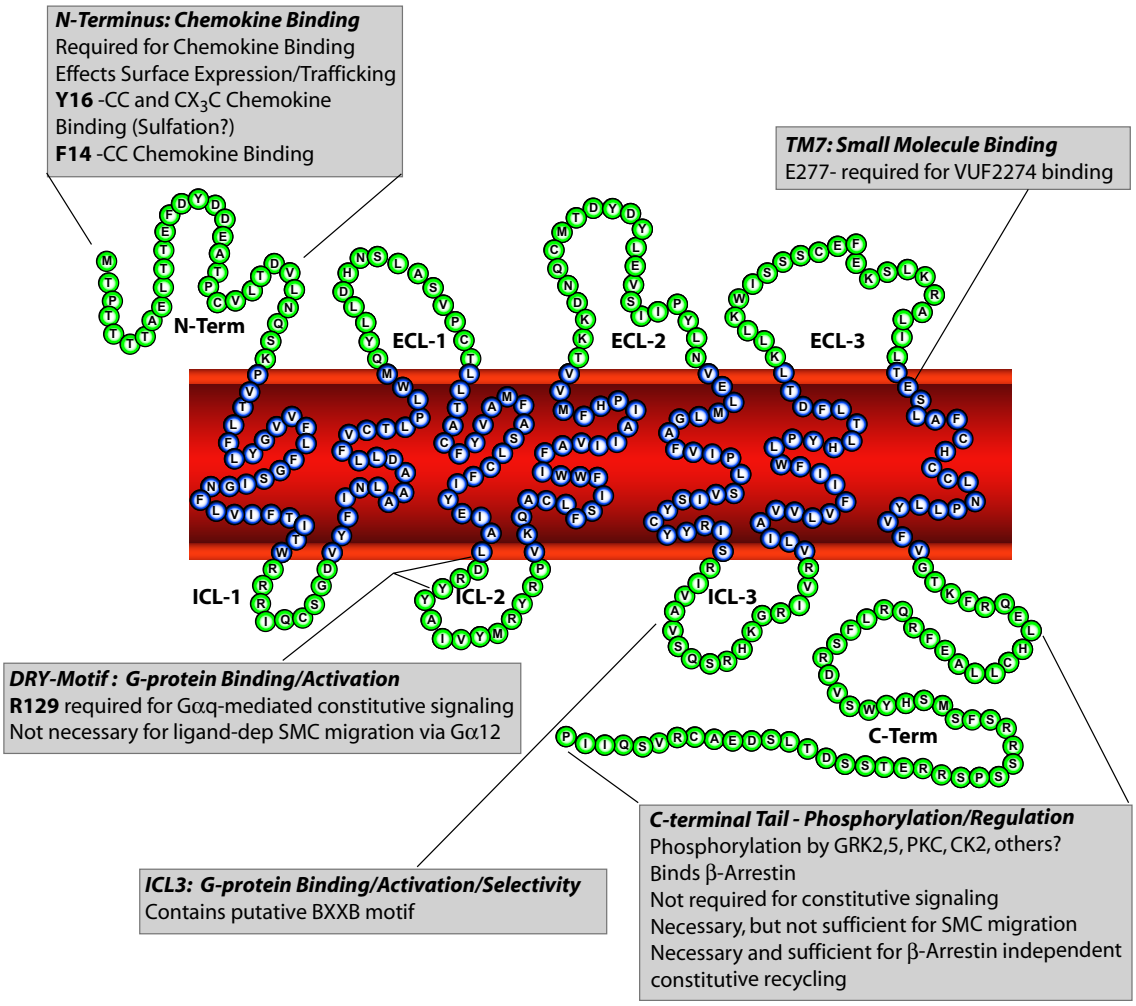


**Figure A.1 - Ribbon structure model for US28.**
**Regions of US28 with known or predicted functional significance are indicated by call outs.Non-italic boldface type indicates specific residues of US28.**

binding to US28 abrogates constitutive activation of PLC (Casarosa, Bakker et al. 2001). Further, Fractalkine binding to US28 in fibroblasts causes Gq/11-dependent signaling to focal adhesion kinase (FAK) (Vomaske, Melnychuk et al. 2009) Taken together, these findings indicate that US28 activates Gq/11 signaling to PLC in the absence of ligand, but Fractalkine binding to US28 co-opts the receptor and utilizes a different Gq/11-dependent pathway to activate FAK. In contrast, CC-chemokine dependent US28 migration of SMC proceeds via G12/13-mediated signaling to FAK, ERK, RhoA and the actin cytoskeleton

(Streblow, Soderberg-Naucler et al. 1999; Melnychuk, Streblow et al. 2004). In 293 cells, RANTES binding to US28 activates ERK1/2 pathways through the G-proteins Gαi1 and Gα16 (Billstrom, Johnson et al. 1998). The highly conserved 'DRY' motif at the end of TM-3 is critical for US28 signaling to PLC (Waldhoer, Casarosa et al. 2003). In contrast, mutagenesis of this motif has no effect on US28-mediated SMC migration (Figure A.2). Experiments with the OT-1 receptor showed that mutation of the DRY motif could simultaneously decouple the receptor from Gi proteins while augmenting signaling via Gq, indicating that different G-proteins may have different sequence requirement at this position and that not all G-protein coupling requires a functional DRY (Favre, Fanelli et al. 2005). Taken together, these results indicate that different activated forms of US28 (i.e. CC- vs. CX3C-chemokine bound or unliganded constitutively active) may utilize slightly different sites for G-protein interaction and activation, contributing to G-protien and ligand binding specificity.

### *A.5.3 Regulation and Desensitization*

US28 is heavily phosphorylated in a ligand-independent manner on several serine residues within the C-terminal 30 amino acids. This phosphorylation can be reduced by pharmacological inhibition of PKC and casein kinase 2 (CK2) and is enhanced by overexpression of GRK2 or GRK5 (Mokros, Rehm et al. 2002; Miller, Houtz et al. 2003; Sherrill and Miller 2006). Although US28 phosphorylation in cell culture systems appears to be ligand-independent, phosphorylation levels can be modulated by US28 ligands. In HEK293 cells, Fractalkine can decrease this basal phosphorylation levels (Mokros, Rehm et al. 2002). The same effect was observed with RANTES treatment in COS7 cells (Miller, Houtz et al. 2003). Taken together, these results indicate that regulatory kinases and phosphatases interact in a dynamic manner with different activated states of US28. Phosphorylated forms of US28 are known to recruit β-arrestin-2 and this association of

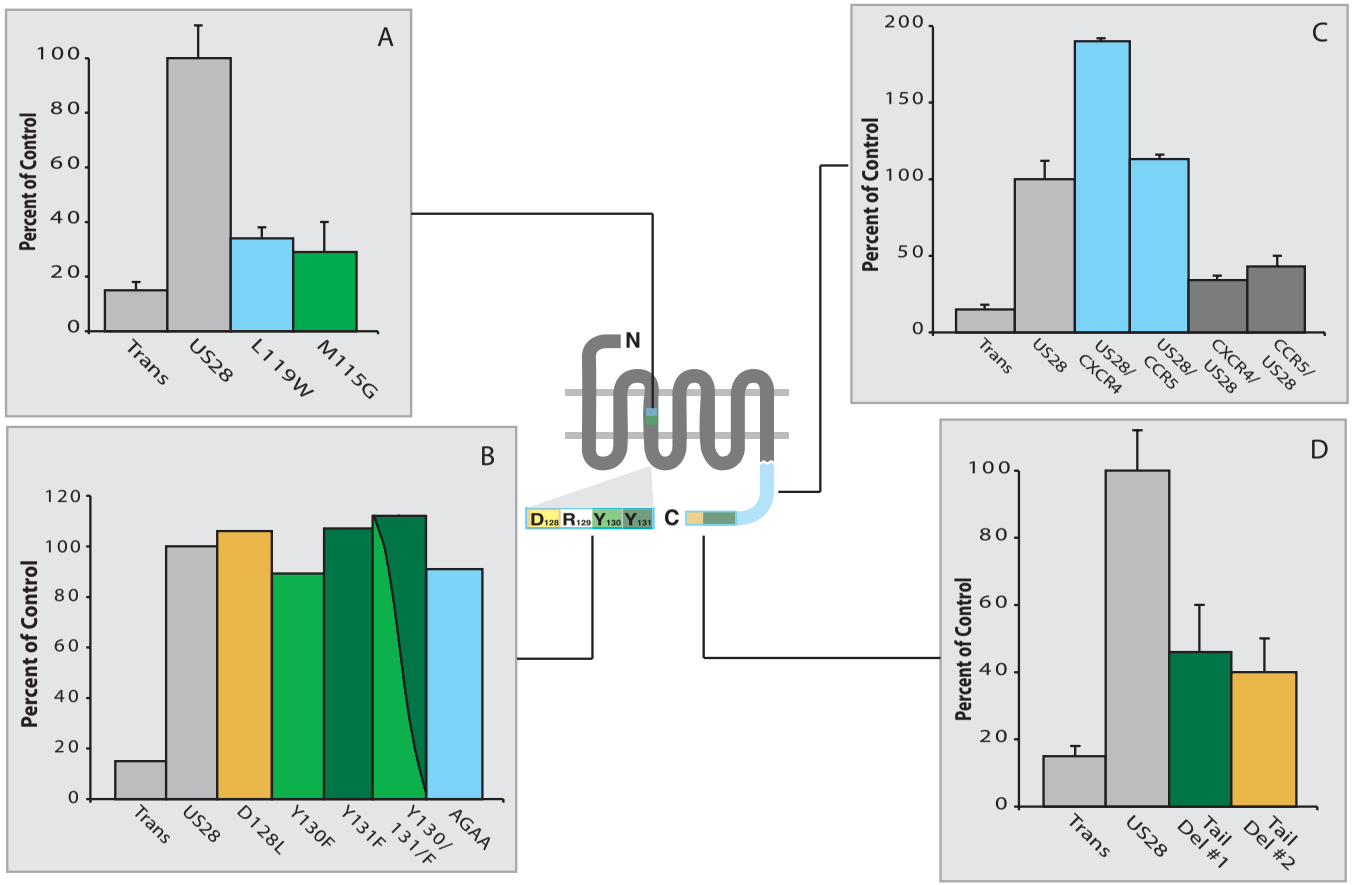


**Figure A.2 - Smooth muscle cell (SMC) migration data for several US28 functional domains.**
**All US28 mutants were expressed via tet transactivator inducible adenovirus vector system. 'Trans' controls indicate cultures infected with Ad-Trans transactivator adenovirus only. (A) US28 mutations affecting intrahelical packing of US28 abrogate SMC migration. (B) Mutations in the 'DRY' motif, a putative active domain of US28 have no effect on CC-chemokine mediated SMC migration. In the US28-AGAA mutant the entire DRYY motif was replaced with the indicated AGAA sequence (C) Chimeric proteins in which the C-terminal tail domain of US28 is replaced with the tail domains of CXCR4 and CCR5 can still mediate SMC migration. Chimeric proteins of CXCR4 and CCR5 containing the US28 C-terminal tail are not able to cause SMC migration. (D) Deletion of the C-terminal 10 (Tail Del #2) or 18 (Tail Del #1) amino acids of US28 significantly abrogates SMC migration.**

β-arrestin with US28 can be augmented by overexpression of GRK2 (Miller, Houtz et al. 2003). However, the constitutive endocytosis and recycling of US28 is not effected by expression in β-arrestin deficient cells (Fraile-Ramos, Kohout et al. 2003). Truncation mutants of US28 that remove the C-terminal phosphorylation sites are generally more prevalent at the cell surface and display increased signaling to PLC, NFkB and CREB (Mokros, Rehm et al. 2002; Miller, Houtz et al. 2003; Waldhoer, Casarosa et al. 2003). However, one study which mutated the C-terminal serine residues rather than truncating the protein showed that serine phosphorylation has no effect on US28 signaling to NFkB (Mokros, Rehm et al. 2002). Interestingly, while Fractalkine treatment generally decreases US28 consitutive signaling, C-terminal truncation mutants display increased accumulation of inositol phosphages and increased transcription from a CREB-responsive element when treated with Fractalkine. The authors attribute this phenotype to increased surface expression of the truncated US28 construct leading to increased binding of the ligand (Waldhoer, Casarosa et al. 2003). Experiments with the MCMV-encoded GPCR M33 suggest that the constitutive activity of this functional homolog of US28 is regulated both by phosphorylation by GRK2 and GRK2-mediated sequestration of Gq proteins (Sherrill and Miller 2006). The effect of ligand binding on US28 phosphorylation via various regulatory kinases has not been determined. Furthermore, the effect of US28 phosphorylation and arrestin binding on ligand dependent US28 signaling pathways such as pro-migratory signaling to FAK and ERK remains to be elucidated. Certainly, the dynamic regulation of US28 signaling in different signaling and cell type contexts deserves further study.

## A.6 Pharmacological Targeting of US28

To date, there is only one characterized pharmacological means for inhibiting US28. Casarosa et. al. identified the nonpetidergic CCR1 antagonist, VUF2274, as an inverse agonist for US28. Their study demonstrates that VUF2274 interaction with US28 is able

to inhibit the constitutive activation of PLC signaling pathways. Mutagenesis of the receptor indicates that VUF2274 does not interact with the chemokine binding domain, but instead binds to residues in the hydrophilic pocket formed by the 7-TM helices (Casarosa, Menge et al. 2003). However, VUF2274 appears to have no inhibitory effect on the chemokine-dependent signaling of US28 to FAK, or the production of actin cytoskeletal rearrangements in fibroblasts (Streblow, unpublished observations). Subsequently, Hulshof et. al. performed detailed pharmacological characterization of VUF2274 and related compounds, producing several drugs with increased selectivity for US28 that are capable of inhibiting the constitutive activation of PLC (Hulshof, Casarosa et al. 2005; Hulshof, Vischer et al. 2006). The efficacy of these drugs for inhibition of US28 ligand-dependent signaling remains to be determined.

## A.7 Summary and Conclusions

Recent research has revealed a startling complexity associated with signaling from the HCMV-encoded chemokine receptor US28. US28 responds to both the extracellular chemokine environment and the intracellular complement of G-proteins, signaling molecules and regulatory molecules to produce a wide variety of signaling and cell motility responses. This highly context-specific functionality requires particular attention with respect to pharmacological targeting of US28. It is apparent that a any potential US28 antagonists must be tested for efficacy against a number of US28-dependent signaling pathways as well as in a number of HCMV-susceptible cell types. Furthermore, characterization of the regulatory proteins interacting with US28 in different cell types may provide targets for cell-type specific inhibition of US28 functions. US28 provides an interesting example of a GPCR able to exert ligand- and cell-type specific signaling. Further study of the mechanisms behind this functional selectivity may serve to elucidate more general aspects of GPCR biology.

# Appendix 2 - Permissions

# A2.1 Limited Use License for Figure 1.4 and 1.5