The roles of human cytomegalovirus US27 gene product during virus infection

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The roles of human cytomegalovirus US27 gene product during virus infection

by

Carolyn Tu

Thesis
Submitted in partial Fulfillment of the Requirements
For the degree of

Master of Science

In Biology

In the
College of Arts and Sciences
University of San Francisco
San Francisco, California

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Approved: [Signature] 8.20.2015
Dean, College of Arts and Sciences
Human cytomegalovirus (HCMV) is a widespread pathogen that causes lifelong latent infection. Successful persistence of HCMV in healthy individuals involves extensive manipulation of host cellular functions and immune responses. HCMV encodes four genes for putative G protein-coupled receptors (GPCRs): US27, US28, UL33, and UL78. The US28 gene product is a functional chemokine receptor that exhibits a variety of signaling outcomes. In contrast, the US27 gene product is an orphan with no known chemokine ligands. We have found that US27 triggers several biological effects: increased cellular proliferation, changes in gene expression, and enhanced signaling of cellular chemokine receptor CXCR4. Enhanced cell survival and proliferation were mediated by the DRY box and C-terminal domain (CTD) of US27. PCR array analysis revealed that expression of US27 led to up-regulation of pro-survival factor Bcl-x, AP-1 transcription factor components jun and fos, and down-regulation of cell cycle regulators CDKN1A (cyclin dependent kinase inhibitor 1A) and SESN2 (Sestrin2 or Hi95). These results indicate that increased cell proliferation due to US27 may be linked to suppression of negative growth regulators. In addition, US27 was shown to increase calcium mobilization of CXCR4 in response to its natural ligand CXCL12. cmvIL-10, an ortholog of human IL-10, was also found to enhance CXCR4 signaling and act synergistically with US27. In both HCMV-infected and US27-transfected cells, exposure to cmvIL-10 increased calcium and migration responses to CXCR4 ligand CXCL12. This cooperative effect may enable HCMV to manipulate CXCR4 to alter host immune responses and modify cell trafficking patterns. Overall, US27 has a wide range of functions that may alter activities of HCMV-infected cells.
Acknowledgements

I’d like to first express my sincerest gratitude to my thesis advisor, Dr. Juliet V. Spencer. I thank her for her patience, guidance, enthusiasm, and mentorship throughout my time as a graduate student. Because of her, I’ve had the chance to travel to many national and international conferences. I’ve been able to give oral presentations to renowned scientists in the virology field and interact with many of her colleagues. Words cannot express enough my utmost respect and appreciation for her tireless efforts in securing grants and allowing me the opportunity to learn and utilize many cutting edge techniques and instruments. I’d like to thank Dr. James Sikes for being a fantastic and caring graduate director. I will never forget all the conversations we’ve had from science to everyday life. I’d like to thank Dr. John Sullivan and Dr. Christina Tzagarakis-Foster for being on my committee and their time in giving advice and reviewing my thesis. I sincerely appreciate their amazing support throughout my time at USF. I’d like to thank professor Leslie King for letting me TA throughout my time as a grad student and learn what it takes to ensure a great lab for my students. I’d like to give a special thanks to Dr. Mary Jane Niles for being one of the best professors I’ve had the privilege of knowing and learning from. I’d like to thank Jeff Oda for being the best lab manager and instrument specialist I’ve had the pleasure of knowing and working with, as well as infusing me with hours of laughter. And of course, I’d like to thank the entire biology department for their support, including their generous travel grants. As importantly, I’d like to thank my mom and brother for their love and support. And lastly, I’d like to take a moment to thank my father. He was the hardest working human being I know, and I hope I can carry on his unwavering grace, poise, and courage as I embark on my new professional journey.
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<tbody>
<tr>
<td>ADRB1</td>
<td>beta-1 adrenergic receptor</td>
</tr>
<tr>
<td>CALCRL</td>
<td>Calcitonin receptor-like</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif ligand 2, systematic nomenclature for MCP-1 (Monocyte chemoattractant protein-1)</td>
</tr>
<tr>
<td>CCL3</td>
<td>chemokine (C-C motif) ligand 3, systematic nomenclature for MIP-1α (macrophage inflammatory protein 1 alpha)</td>
</tr>
<tr>
<td>CCL5</td>
<td>C-C motif chemokine ligand 5, systematic nomenclature for Rantes (regulated on activation, normal T cell expressed and secreted)</td>
</tr>
<tr>
<td>CCR2</td>
<td>C-C chemokine receptor 2</td>
</tr>
<tr>
<td>CD4+</td>
<td>cluster of differentiation 4</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
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<tr>
<td>cmvIL-10</td>
<td>cytomegalovirus interleukin-10</td>
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<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>C-X3-C motif chemokine ligand 1</td>
</tr>
<tr>
<td>CXCL12</td>
<td>C-X-C motif ligand 12, systematic nomenclature for SDF-1α</td>
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<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor 4</td>
</tr>
<tr>
<td>CXCR7</td>
<td>C-X-C chemokine receptor 7</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>cytochrome P450 19A1</td>
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<tr>
<td>DAY</td>
<td>aspartic acid, alanine, tyrosine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRY</td>
<td>aspartic acid, arginine, tyrosine</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FCGR1A</td>
<td>Fc fragment of IgG, high affinity Ia, receptor</td>
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<tr>
<td>Fluo-4 AM</td>
<td>fluorescent-4 acetoxymethyl ester</td>
</tr>
<tr>
<td>GABARAP</td>
<td>gamma-aminobutyric acid type A receptor-associated protein</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HCMV</td>
<td>human cytomegalovirus</td>
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<tr>
<td>hIL-10</td>
<td>human interleukin-10</td>
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<tr>
<td>hIL-10R</td>
<td>human interleukin-10 receptor</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>M33</td>
<td>Mouse cytomegalovirus 33</td>
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<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
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<td>OPRK1</td>
<td>human opioid receptor gene</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PI3CG</td>
<td>phosphoinositol-3-kinase, catalytic, gamma polypeptide</td>
</tr>
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<td>PI3K/AKT</td>
<td>phosphatidylinositol 3-kinase/protein kinase B (PKB)</td>
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<tr>
<td>PTGS2</td>
<td>prostaglandin G/H synthase and cyclooxygenase</td>
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<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SOCS2</td>
<td>suppressor of cytokine signaling 2</td>
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<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TYK2</td>
<td>tyrosine kinase 2</td>
</tr>
<tr>
<td>UL111A</td>
<td>unique long 111 A gene</td>
</tr>
<tr>
<td>UL33</td>
<td>unique long gene 33</td>
</tr>
<tr>
<td>UL78</td>
<td>unique long gene 78</td>
</tr>
<tr>
<td>US27</td>
<td>unique short gene 27</td>
</tr>
<tr>
<td>US28</td>
<td>unique short gene 28</td>
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Introduction

Human cytomegalovirus (HCMV), also known as human herpesvirus-5, is a widespread pathogen that can lay dormant in healthy individuals and establish lifelong latent infection [1]. Seroprevalence ranges from 45-100% worldwide [2] and while infection is generally asymptomatic in healthy individuals, it can be life-threatening in immune-compromised hosts [3, 4]. If symptoms do occur upon infection, they are typically flu-like [3, 4] or a mononucleosis-like fever [5]. Transplant recipients and AIDS patients are more prone to becoming infected with HCMV due to reactivation of latent virus, resulting in serious conditions like retinitis and pneumonitis [4]. HCMV also remains the most common viral cause of birth defects and childhood disabilities in the United States [6].

The HCMV virion is composed of a genome of 230 kb linear double-stranded DNA that is enclosed by an icosahedral capsid, which itself is surrounded by a proteinaceous layer termed the tegument [7] (Figure 1). The outermost layer is the viral envelope, which consists of a lipid membrane containing glycoproteins for virus attachment onto host cells. HCMV has the largest genome of all herpesviruses, encoding over 200 open reading frames (ORF) [8]. Replication of HCMV is relatively slow and requires at least 24 hours to produce infectious progeny virions, which are generally not released until 72-96 hours later [9]. The viral genome is transcribed slowly in a sequential and highly regulated order, and transcription is divided into three major phases: immediate-early (IE), early (E), and late (L). The immediate-early proteins are first synthesized and act as transcription factors to activate early genes required for the
Figure 1. Schematic image HCMV virus particle. The virion of HCMV contains a linear double-stranded DNA genome packaged in an icosahedral capsid. The capsid is surrounded by a tegument and an enveloped composed of a lipid bilayer membrane.
replication process itself. The late genes are involved in encoding structural virion components and are transcribed only after viral DNA replication has begun [9, 10].

Upon infection with HCMV, the virus establishes a lifetime coexistence with the host through chronic virus shedding and latency [11]. Throughout the chronic infection, there is minimal yet constant shedding of the virus in bodily fluids such as urine, blood, and saliva. Latent infection is characterized by a reversibly quiescent state, which enables the virus to reactivate and periodically produce viral progeny [12]. HCMV is able to maintain a successful coexistence with its host by evading and manipulating the immune system. One strategy involves interference with the major histocompatibility complex (MHC). MHC class I molecules are cell surface glycoproteins that play a role in the immune surveillance against viruses by displaying antigens on infected cells, whereby the immune cells can recognize and ultimately destroy them [13]. HCMV has been shown to down-regulate surface expression of MHC class I, thus preventing antigen presentation during the IE phase of infection [14, 15]. HCMV also encodes a viral ortholog of MHC class I, which may function as an immune modulator and disrupt the presentation of antigens to cytotoxic T cells while also preventing recognition by natural killer cells [13, 16]. Mimicking and inhibiting the MHC complex helps contribute to viral evasion and protection from the host immune system. In addition, successful coexistence of HCMV with a healthy host is also mediated by the production of viral proteins that are similar to normal immune modulators like cytokines, chemokines, and chemokine receptors [17].

One such mimic is the HCMV US27 gene product that has seven transmembrane domains and similarity to the human chemokine receptor family of G-protein coupled
receptors (GPCRs), including conserved cysteines in the extracellular loops and extensive glycosylation of the extracellular domain [18]. US27 also contains a DRY (aspartic acid, arginine, tyrosine) motif in the second intracellular loop that is critical in other GPCRs for activation of G proteins following ligand binding [19], as well as a di-leucine motif in the carboxy-terminal domain that mediates receptor endocytosis [20] (Figure 2). Despite having these characteristics, US27 is considered an orphan since no human chemokine ligands have been shown to engage the receptor [20]. US27 is found in the envelope of the virus particle [18], but in virus-infected cells, the majority of the US27 protein is found in endosomes, the Golgi apparatus, and perinuclear compartments [21]. The US27 gene is non-essential for virus replication, and mutants that lack US27 are replication competent [22]. However, US27 deletion mutant viruses are incapable of spreading via the extracellular route in endothelial cells [23], suggesting that US27 might play a role in virion assembly or egress.

Interestingly, HCMV encodes three other genes that give rise to proteins having similarity to human chemokine receptors [24, 25]. One of these, US28, has been shown to elicit intracellular signaling both constitutively and in response to several human chemokines, including CCL3/MIP-1α, CCL5/Rantes, and CX3CL1/Fractalkine [26-28]. UL33 also has constitutive signaling ability [29], and rodent homologs of both UL33 and UL78 have been shown to play a role in virus dissemination in vivo [30, 31]. The M33 gene of murine cytomegalovirus, a homolog of HCMV UL33, was found to be required for salivary gland tropism and efficient reactivation from latency, and the HCMV US28 gene was able to complement and partially rescue those deficiencies [32, 33].
Figure 2. Schematic image of the HCMV US27 gene product. US27 contains conserved cysteines in the extracellular loops and contains a DRY (aspartic acid, arginine, tyrosine) motif in the second intracellular loop.
Recent evidence suggests that US28 forms heteromeric complexes with US27, UL33 and UL78 [34]. While no functional changes were observed with the US28:US27 heteromer, the US28:UL33 heteromer and the US28:UL78 heteromer both ablated activation of NF-κB transcriptional activity by US28. This suggests a complex level of regulation in which these viral receptors may interact in particular combinations to either promote or block signaling through specific pathways in particular cell types or at specific times during the course of virus infection. US27, US28, UL33, and UL78 are all likely to play important roles in immune modulation and viral persistence, and the presence of multiple receptors in the viral genome could be due to the need to control cellular activity in a large variety of cell types infected by HCMV, which include monocytes, lymphocytes, epithelial cells, endothelial cells, and fibroblasts [35]. Considering that GPCRs constitute a major target in pharmaceutical development, discerning the function of viral GPCRs during HCMV infection could be highly beneficial in the quest for novel anti-viral therapeutics.

Although no chemokine ligands for US27 have been identified, US27 has been shown to enhance cell proliferation, survival [36, 37] and signaling activity of the human chemokine receptor CXCR4 [38]. Chemokines, or chemotactic cytokines, and their receptors play crucial roles in inflammation by directing immune cells, such as leukocytes, to sites of injury or infection [39]. CXCR4 exhibits many physiological roles, including chemotaxis, embryogenesis, hematopoiesis, vascularization, and the immune response [40]. CXCR4 is a human G-protein-coupled receptor (GPCR) and binds to ligands CXCL12/SDF-1α and ubiquitin [41, 42]. CXCR4 signaling plays key roles in hematopoiesis and immune homeostasis. The effects of CXCL12 are mediated
through G protein signaling, causing activation of downstream pathways including mitogen activated protein kinase (MAPK) family members and PI3K/Akt cascades [43, 44]. Early studies found CXCR4 to function as a co-receptor for entry of HIV into CD4+ T cells [45]. CXCR4 is also found to be up-regulated in many different types of cancer cells [46-48], and linked to enhanced tumor growth and metastases [44].

While CXCL12-CXCR4 signaling has been found to be down-regulated by both viral and cellular GPCRs, such as CCR2 [49], CXCR7 [50], EBV BILF1 [51], and HCMV UL33 and UL78 [52], HCMV US27 was shown to potentiate CXCR4 activity [38]. In stably-transfected HEK293 cells expressing both US27 and CXCR4, a greater calcium mobilization was seen in response to CXCL12 than control HEK293 cells. CXCR4 signaling can also be enhanced by other proteins, such as T-cell receptor [53] and human IL-10 [54]. Human IL-10 has been shown to potentiate the chemotactic effect of CXCL12 in B-lymphocytes, while other cytokines such as IL-5, IL-6, and IL-9 do not [54]. Furthermore, IL-10 also increased the effect of CXCL12 on the proliferation and survival of B-lymphocytes [54]. In this thesis, we investigated whether cmvIL-10, a viral homolog of human IL-10, can also alter CXCR4 activity.

cmvIL-10, encoded by the UL111A gene, shares only a 27% amino acid sequence identity to human IL-10 [55]. Despite this low conservation, cmvIL-10 exhibits the same immunosuppressive properties as human IL-10, such as inhibition of pro-inflammatory cytokines and T cell proliferation, and down regulation of the MHC expression [56]. Like human IL-10, cmvIL-10 can also inhibit nuclear factor κB (NF-κB) transcriptional activity in human monocytes and suppress pro-inflammatory cytokines TNFα and IL-6 [57].
cmvIL-10 binds and signals through the human IL-10 receptor (hIL-10R) [55]. Once the hIL-10R is engaged by its ligand, the receptor dimerizes and activates JAK (Janus kinases), which is followed by STAT (signal transducer and activator of transcription) recruitment and phosphorylation (Figure 3). After STAT dimerizes, it will translocate into the nucleus and activate genes responsive to STAT. STATs are key integrators of cytokine and growth factor receptor signaling required for cell growth, survival, differentiation, and motility [58, 59]. Thus, the virus has the ability to hijack the cellular JAK-STAT signaling pathway and manipulate the host immune response.

In this thesis project, we show that HCMV US27 enhances cell proliferation (chapter 1), protects cells from apoptosis and cause changes in host gene expression (chapter 2), and affects CXCR4 synergistically with cmvIL-10 (chapter 3). These findings are the first reported biological activities associated with US27 and help shed light on the function of this protein during infection.
Figure 3. Schematic image of cmvIL-10 binding to IL-10 receptor. All three receptors are in close proximity to each other, thereby increasing intracellular signaling.
Chapter 1 – The human cytomegalovirus US27 gene product enhances cell proliferation and alters cellular gene expression

(Lares, A.P., Tu C.C., and J.V. Spencer. 2013 Virus Research 176: 312-320)
Chapter 1  Introduction to published manuscript

In this study, we found that US27 significantly increased both cell proliferation and DNA synthesis compared to control cells. PCR array analysis revealed that expression of US27 led to changes in a limited number of cellular genes, but genes that were up-regulated included the pro-survival factor Bcl-x, AP-1 transcription factor components jun and fos, and the IL-6 family cytokine oncostatin M. These results demonstrate that US27 can impact host cell physiology and may shed light on the function of this orphan viral GPCR.

A former graduate student, Angela P. Lares, started this project and it was a privilege for me to carry it on. My role included confirming expression of US27 and control receptors in both western blotting and immunofluorescence staining. I carried out the cell count data and calculated the mean doubling time as another way to confirm that measurement of growth was not due to an effect of US27 on ATP levels (per cell) or prevention of cell death from transfection. I also repeated the qPCR arrays to include three biological replicates and ensured all plates were set to the same baseline threshold for accurate reading of quality controls and comparisons between US27 and controls. And lastly, I confirmed that US27 causes up-regulation of AP-1 complex genes jun and fos not only on the RNA level, but also the protein level through western blotting.
Short communication

The human cytomegalovirus US27 gene product enhances cell proliferation and alters cellular gene expression

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A R T I C L E   I N F O
Article history:
Received 24 March 2013
Received in revised form 2 July 2013
Accepted 3 July 2013
Available online 11 July 2013

Keywords:
HCMV
Cytomegalovirus
GPCR
Chemokine receptor

A B S T R A C T

Human cytomegalovirus (HCMV) is a prevalent pathogen worldwide. Although generally harmless in healthy individuals, HCMV can pose a serious threat to immune compromised individuals and developing fetuses in utero. HCMV encodes four genes predicted to give rise to G protein–coupled receptors (GPCRs): US27, US28, UL33, and UL78. The US28 gene product is a functional chemokine receptor that enhances cell growth in some cell types but induces apoptosis in others. In contrast, the US27 gene product has not been demonstrated to signal either constitutively or in a ligand-induced manner. In this study, US27 was expressed in transfected cells, and both cell proliferation and DNA synthesis were significantly increased compared to control cells. PCR array analysis revealed that expression of US27 led to changes in a limited number of cellular genes, but genes that were up-regulated included the pro-survival factor Bcl-x, AP-1 transcription factor components jun and fos, and the IL-6 family cytokine oncostatin M. These results demonstrate that US27 can impact host cell physiology and may shed light on the function of this orphan viral GPCR.

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Human cytomegalovirus (HCMV) is a member of the *Herpesviridae* family, and infection is widespread in the general population (Staras et al., 2006). While most healthy adults exhibit only mild or no symptoms of infection, serious disease can occur in immunocompromised individuals, especially AIDS patients, transplant recipients, and newborn infants (Kesson and Kakakios, 2007). HCMV is the leading infectious cause of birth defects, and congenital HCMV infection continues to be problematic due to deficiencies in public awareness, diagnostic procedures, and treatment options (Revello et al., 2008).

Like all other herpesviruses, the HCMV virion consists of a large DNA genome enclosed in an icosahedral capsid surrounded by a dense tegument layer, all encircled in a lipid envelope containing numerous viral glycoproteins. HCMV has the largest genome of all the human herpesviruses at 230 kb and encoding at least 167 genes (Mocarski et al., 2006). More than half of these genes are not required for virus replication in vitro (Yu et al., 2003) but instead play roles in vivo in the manipulation of host immune responses and the establishment of latency (Jackson et al., 2011). The US27 gene, which encodes a putative G protein–coupled receptor (GPCR) found in the viral envelope, is one of these non-essential genes (Chee et al., 1990; Margulies and Gibson, 2007). Virus mutants lacking US27 are replication competent (Bodaghi et al., 1998), although a single log reduction in virus titers produced from both infected fibroblasts and endothelial cells was observed (O’Connor and Shenk, 2011). The US27 deletion mutant virus also exhibited a defect in extracellular spreading, but the virus was still able to infect neighboring cells, presumably via the cell–cell route (O’Connor and Shenk, 2011). The US27 gene is expressed late during infection, and the gene product is found mainly in the endosomes, the Golgi apparatus, and perinuclear compartments of infected cells (Fraile-Ramos et al., 2002).

The US27 gene product has many conserved features of the chemokine receptor subset of the GPCR superfamily, such as seven transmembrane domains, a DRY (aspartic acid–arginine–tyrosine) motif in the second intracellular loop, conserved cysteines (C104 and C176) in the second and third extracellular loops, and extensive glycosylation of the extracellular domains (Margulies and Gibson, 2007). Despite having these characteristics, US27 is considered an orphan since no human chemokine ligands have been shown to engage the receptor (Stapleton et al., 2012). Interestingly, HCMV encodes three other genes that give rise to proteins having similarity to human chemokine receptors (Beisser et al., 2002; Chee et al., 1990). One of these, US28, has been shown to elicit intracellular signaling both constitutively and in response to several human chemokines, including CCL3/MIP-1α, CCL5/Rantes, and CX3CL1/Fractalkine (Gao and Murphy, 1994; Neote et al., 1993; Stropes et al., 2009). UL33 also has constitutive signaling ability (Casarosa et al., 2003), and rodent homologs of both UL33...
and UL78 have been shown to play a role in virus dissemination in vivo (Beisser et al., 1999, 1998). The M33 gene of murine cytomegalovirus, a homolog of HCMV UL33, was found to be required for salivary gland tropism and efficient reactivation from latency, and the HCMV US28 gene was able to complement and partially rescue those deficiencies (Cardin et al., 2009; Farrell et al., 2011).

Recent evidence suggests that US28 forms heteromeric complexes with US27, UL33 and UL78 (Tschische et al., 2011). While no functional changes were observed with the US28:US27 heteromer, the US28:UL33 heteromer and the US28:UL78 heteromer both ablated activation of NF-kB transcriptional activity by US28. This suggests a complex level of regulation in which these viral receptors may interact in particular combinations to either promote or block signaling through specific pathways in particular cell types or at specific times during the course of virus infection. US27, US28, UL33, and UL78 are all likely to play important roles in immune modulation and viral persistence, and the presence of multiple receptors in the viral genome could be due to the need to control cellular activity in the large variety of cell types infected by HCMV, which include monocytes, lymphocytes, epithelial cells, endothelial cells, and fibroblasts (Mocarski et al., 2006). Considering that GPCRs constitute a major target in pharmaceutical development, discerning the function of viral GPCRs during HCMV infection could be highly beneficial in the quest for novel anti-viral therapeutics.

To study the function of US27, the gene from HCMV strain AD169 was cloned into the p3XFLAG expression vector and transiently transfected into HEK293 cells, as described (Stapleton et al., 2012). The cells were seeded into 96-well plates at a density of 1 x 10⁴ cells per well, and cell proliferation was monitored using the CellTiter-Glo Assay (Promega, Madison, WI). Briefly, a luciferin substrate that is converted to oxyluciferin in the presence of O₂ and ATP was added to each well. The resulting luminescence is proportional to the amount of ATP present, reflecting the number of viable cells in the well. Cells expressing US27 had greater luminescence, suggesting that they had an enhanced growth rate compared to controls, as shown in Fig. 1A. The control cells included mock transfected cells treated with transfection reagent only, cells transfected with the empty p3XFLAG vector, and cells transfected with the p3XFLAG vector expressing HCMV US28 or human chemokine receptor CXCR3, which was cloned from human peripheral blood mononuclear cells as previously described (Stapleton et al., 2012). US27-expressing cultures consistently exhibited 14–26% greater luminescence over the course of the experiment than most of the other cell lines, which is indicative of higher cell numbers and a faster growth rate. Each of the other control cell lines had comparable growth rates except for the US28-transfected cells, which exhibited reduced luminescence, indicating fewer viable cells. The transfection efficiency was comparable among the cell lines at 60–70%, as indicated by staining with an anti-FLAG antibody (Sigma–Aldrich, St. Louis, MO), followed by FITC-conjugated secondary antibody and flow cytometry (Fig. 1B).

To rule out any possibility that the FLAG tag might be affecting US27 functional activity, the US27, US28 and CXCR3 genes were also cloned into a pEGFP vector (Clontech, Mountain View, CA) and expressed as fusion proteins linked to the C-terminal domain of EGFP. As shown in Fig. 1C, HEK293 cells transiently transfected with pEGFP-US28 also exhibited greater cell proliferation than corresponding control cells. The increase in growth rate for these cells was more modest, with an increase of 9–16% over each of the control cell lines over the course of the experiment. The growth of the pEGFP–US27 transfected cells was 26–30% higher than pEGFP–US28 transfected cells. Transfection efficiency with the pEGFP plasmids was comparable among the cell lines, but lower than the p3XFLAG plasmids, at 46–52% (Fig. 1D). Overall, the US27–expressing HEK293 cultures grew faster while US28 cells grew at a reduced rate compared to the control cell lines. These results suggested that the US27 gene product might enhance cell growth.

Previous studies have shown that the US28 gene product may have different effects on cell growth and survival depending on cell type. Transient transfection of GFP-US28 induced apoptosis in 293T, HeLa, and Cos cells (Pleskoff et al., 2005), whereas US28 was found to enhance cell growth and cell cycle progression in stably transfected NIH-3T3 cells (Maussang et al., 2006). Since we found increased growth rates associated with US27 and decreased growth rates with US28 in HEK293 cells, we next transfected and evaluated two additional cell types, HeLa and Cos cells (Fig. 1E and F). The results indicate that these cell types also exhibited greater proliferation when expressing HCMV US27 than control cells. Cells expressing US28 were found to have the slowest growth rate, possibly due to the loss of some cells undergoing apoptosis, a result that would be consistent with previous studies (Pleskoff et al., 2005). Although we also attempted to examine the effects of US27 in NIH-3T3 cells, the rate of transfection was extremely low (1%) and prevented their inclusion in this study.

In order to confirm that the differences in growth rates that we observed were specifically due to the presence of US27 and not the result of well-to-well differences in plasmid purity, transfection efficiency, or transgene expression, stable HEK293 cell lines were created. Following transfection with p3XFLAG-US27, cells were cultivated in the presence of 1 mg/ml genetin to eliminate untransfected cells, and clonal cell lines were created using limiting dilution. As shown in Fig. 2A, comparable levels of FLAG-tagged protein were expressed in each of the cell lines. CXCR3 was detected as a 42 kDa band, US28 as a 44 kDa band, and US27, which is extensively glycosylated, was detected as a 45–55 kDa smear. Immunofluorescence microscopy also indicated that protein expression levels were comparable among the cell lines (Fig. 2B). When the growth rate of the stable cell lines was examined via cell counting, the US27 cultures were found to have significantly higher cell numbers after 72 h (Fig. 2C). Although one representative clonal cell line is shown here, three individual 293-US27 clonal cell lines were examined and all had comparable expression levels and growth rates (data not shown), suggesting that the integration of the expression cassette did not account for the enhanced proliferation of cells expressing US27. The mean doubling time was 12.1 h for the 293-US27 cultures, compared to 13.9 h for empty vector transfected cells, 14.4 h for 293-CXCR3 cells and 17.5 h for 293-US28 cells (Fig. 2D). When cell proliferation was examined using the Cell Titre-Glo assay, 293-US27 cells still exhibited significantly greater proliferation (14–25% higher) than control cells (Fig. 2E). In addition, DNA synthesis was examined using BrdU incorporation, and the rate of DNA synthesis was found to be significantly higher (26–49%) in cells expressing US27 compared to the control cell lines (Fig. 2F). These results clearly demonstrate that the HCMV US27 gene product stimulates cell proliferation and increases the rate of DNA synthesis.

In order to identify cellular genes that might be affected by the US27 gene product and contribute to the enhanced proliferative effect observed here, PCR array analysis was performed on the stable cell lines. RNA was extracted from each cell type (HEK293, 293-US27, 293-US28, and 293-CXCR3) and expression levels of 84 genes involved in the JAK-STAT signaling pathway were compared using RT2 Profiler Arrays (SA Biosciences, Valencia, CA). For each of the stable cell lines, three biological replicates were assayed, averaged, and compared to control HEK293 cells, as shown in Fig. 3. Some genes were found to be up-regulated in a non-specific manner, for example, JUNB expression was increased by more than 2-fold in all three cell lines compared to control HEK293 cells. Other genes that were up-regulated by all three receptors included MYC, CEBP, and JAK1, suggesting that overexpression of any GPCR might yield the induction of a certain subset of inflammatory genes. In
order to eliminate these non-specific effects, the fold change values for 293-US27 and 293-US28 were then calculated compared to the 293-CXCR3 values in order to identify changes specific to the viral receptors. A select subset of the results for US27 is shown in Fig. 4, with a line marking changes of 1.8-fold or higher compared to controls. A fold change cutoff of 2.0 is recommended by the assay manufacturer, but other sources indicate that fold change values of 1.5 or higher should not be ignored, especially when they indicate genes that are physiologically relevant (Dalman et al., 2012). We selected a mid-range cut-off of 1.8-fold change and have identified seven JAK-STAT pathway genes whose expression level is significantly increased in the presence of US27: BCL2L1, FCGR1A, JUN, OSM, SOCS2, SOCS5, and TYK2. Table 1 includes a brief description of each cellular gene significantly impacted by either US27 or US28, as well as the fold change value for each cell type compared to cells expressing CXCR3. Six of the seven genes that exhibited up-regulation by US27 were also up-regulated in the presence of US28, suggesting that the two viral receptors may work together...
to enhance expression of certain cellular genes that foster a favorable environment for virus infection. The seventh gene, BCL2L1, was up-regulated by US27 but down-regulated in cells expressing US28.

In addition to the JAK-STAT array, genes involved in GPCR signaling were also evaluated via the RT2 Profiler Array System. Expression profiles for each cell type compared to HEK293 cells are shown in Fig. 5. Again, certain genes were up-regulated by the expression of all three GPCR, including ACGT1, CALCR, and CASR, suggesting that these effects are non-specific and might be induced by the overexpression of any GPCR. When the fold change values for 293-US27 and 293-US28 were compared to the 293-CXCR3 values, only three cellular genes were found to be significantly up-regulated by HCMV US27: FOS, JUN and CYP19A1, as shown in Table 2. A greater number of cellular genes were impacted by US28, which also caused upregulation of CYP19A1, as well as CALCR, CCL2/MCP-1, OPRK1, PI3CG, and PTGS2. Notably, expression of BCL2L1 and ADRB1, the β-adrenergic receptor, were significantly down-regulated by US28 compared to controls. There were two genes that were represented on both the JAK/STAT array and the GPCR array: BCL2L1 and JUN. JUN was found to be significantly up-regulated in the presence of US27 on both arrays, while the BCL2L1
Fig. 3. JAK-STAT pathway gene expression levels in cells expressing US27, US28, or CXCR3. RNA from stable cell line was extracted using RNEasy Midi Kit (Qiagen, Valencia, CA), cDNA was prepared using the RT2 First Strand Kit (Qiagen), and then diluted cDNA was mixed with the RT2 SYBR green master mix (Qiagen) according to the manufacturer’s instructions and loaded into the Human Jak-Stat RT2-PCR Profiler Array (SABiosciences, Valencia, CA). Real Time PCR was performed using the CFX96 (BioRad, Hercules, CA) by heating to 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data was analyzed using the ΔΔCt method according to the SABiosciences web portal (www.SABiosciences.com/pcrarray.dataanalysis.php) and further recalculated manually. The same threshold value was used across all plates in the same data analysis to ensure accurate reading of quality controls. The data were normalized across all plates to the following housekeeping genes: beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L13a (RPL13A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and beta actin (ACTB). Controls for genomic DNA contamination RNA quality, and PCR performance were all in the recommended ranges. Data are represented as fold change compared to expression levels in HEK293 cells. The results are the average of three biological replicates.

results were slightly more variable, with a fold change of 1.97 when analyzed on the JAK-STAT array but only 1.45 on the GPCR array. For two genes, JUN and FOS, protein expression was also examined by Western blot, and a modest increase in jun and fos protein levels was detected in both the 293-US27 and 293-US28 cells (Fig. 6), which further supports findings of the PCR array.

During infection, HCMV can have a striking impact on cell gene expression and other cellular functions. We have focused on one
viral gene, US27, and its effect on cell physiology when expressed in isolation in human cells. We found that the presence of the US27 gene product leads to enhanced cell proliferation and DNA synthesis compared to controls. This suggests that like US28 (Mausang et al., 2006, 2009; Slinger et al., 2010) and ORF74 of KSHV (Kaposis's sarcoma associated herpesvirus) (Bais et al., 1998), under certain conditions, US27 can stimulate cell division and promote enhanced growth. Evaluation of cellular gene expression using PCR profiler arrays led to the identification of several host genes that may play a role in the enhanced growth rate observed in cells expressing HCMV US27. The JAK-STAT PCR array was utilized in this study because US28 has been previously shown to stimulate cell proliferation through induction of IL-6, with downstream signaling by JAK1 and STAT3 (Slinger et al., 2010). Here, we report that the gene encoding oncostatin M, a cytokine that belongs to the IL-6 family with roles in hematopoiesis and inflammation, is significantly up-regulated by both US27 and US28. This result is consistent with transcriptome analyses that found upregulation of oncostatin M gene expression in HCMV-infected monocytes (Chan et al., 2008a, 2008b). Oncostatin M was also found to be one of the most abundant proteins present in the supernatants of virus-infected cells (Dumontier et al., 2008) and has been identified as a major growth factor for Kaposi's sarcoma spindle cells (Nair et al., 1992).

In addition to oncostatin M, increased expression of the JUN and FOS genes was observed in cells expressing US27. The jun and fos proteins typically form homo- or heterodimers to comprise the transcription factor AP-1, which plays a critical role in the regulation of multiple genes controlling cell proliferation and apoptosis (Angel and Karin, 1991). Induction of AP-1 upon infection with HCMV has been well-documented (Boldogh et al., 1990.

![Figure 4. JAK-STAT pathway gene expression levels in 293-US27 cells. The fold change values from three biological replicates of the PCR array were analyzed in comparison to 293-CXCR3 cells as a baseline control. Error bars indicate standard error; * indicates genes with a 1.8-fold change or higher.](image)

### Table 1

<table>
<thead>
<tr>
<th>UniGene</th>
<th>Refseq</th>
<th>Symbol</th>
<th>Description</th>
<th>Fold change&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>HS:516866</td>
<td>NM_138578</td>
<td>BCL2L1</td>
<td>BCL2-like 1</td>
<td>1.97 US27, 1.43 US28</td>
</tr>
<tr>
<td>HS:709456</td>
<td>NM_000567</td>
<td>CRP</td>
<td>C-reactive protein, pentraxin-related</td>
<td>1.36 US27, 3.11 US28</td>
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<tr>
<td>HS:654394</td>
<td>NM_005211</td>
<td>CSF1R</td>
<td>Colony stimulating factor receptor 1</td>
<td>1.63 US27, 4.09 US28</td>
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<tr>
<td>HS:592192</td>
<td>NM_000395</td>
<td>CSF2RB</td>
<td>Colony stimulating factor receptor 2, beta, low-affinity (granulocyte-macrophage)</td>
<td>1.36 US27, 3.11 US28</td>
</tr>
<tr>
<td>HS:77367</td>
<td>NM_004216</td>
<td>CXCL9</td>
<td>Chemokine (C-X-C motif) ligand 9</td>
<td>1.36 US27, 3.11 US28</td>
</tr>
<tr>
<td>HS:77424</td>
<td>NM_000566</td>
<td>FGR1A</td>
<td>Fc fragment of IgG, high affinity Ia, receptor (CD64)</td>
<td>1.84 US27, 2.61 US28</td>
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<tr>
<td>HS:519805</td>
<td>NM_001311</td>
<td>HMGAI</td>
<td>High mobility group AT-hook 1</td>
<td>1.43 US27, 2.49 US28</td>
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<tr>
<td>HS:529400</td>
<td>NM_000629</td>
<td>IFNAR1</td>
<td>Interferon (alpha, beta and omega) receptor 1</td>
<td>1.45 US27, 2.04 US28</td>
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<td>HS:504035</td>
<td>NM_001558</td>
<td>IL10RA</td>
<td>Interleukin 10 receptor, alpha</td>
<td>1.73 US27, 2.64 US28</td>
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<tr>
<td>HS:272373</td>
<td>NM_0018724</td>
<td>IL20</td>
<td>Interleukin 20</td>
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<tr>
<td>HS:73917</td>
<td>NM_00089</td>
<td>IL4</td>
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<td>HS:532082</td>
<td>NM_002184</td>
<td>IL6ST</td>
<td>Interleukin 6 signal transducer (gp130, oncostatin M receptor)</td>
<td>1.27 US27, 1.97 US28</td>
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<td>HS:436061</td>
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<td>Interferon regulatory factor 1</td>
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<tr>
<td>HS:458485</td>
<td>NM_003101</td>
<td>ISG15</td>
<td>ISG15 ubiquitin-like modifier</td>
<td>1.60 US27, 2.23 US28</td>
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<tr>
<td>HS:714791</td>
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<td>Jun proto-oncogene</td>
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<td>HS:379129</td>
<td>NM_00422</td>
<td>MMP3</td>
<td>Matrix metalloproteinase 3 (stromelysin 1, progelatinase)</td>
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<tr>
<td>HS:524769</td>
<td>NM_002534</td>
<td>OAS1</td>
<td>2′-5′-Oligoadenylate synthetase 1, 40/46 kDa</td>
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<td>NM_0020530</td>
<td>OSM</td>
<td>Oncostatin M</td>
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<tr>
<td>HS:74615</td>
<td>NM_0020626</td>
<td>PDGFRα</td>
<td>Platelet-derived growth factor receptor, alpha polypeptide</td>
<td>1.05 US27, 3.45 US28</td>
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<tr>
<td>HS:654514</td>
<td>NM_002388</td>
<td>PTPRC</td>
<td>Protein tyrosine phosphatase, receptor type, C</td>
<td>1.36 US27, 3.11 US28</td>
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<td>HS:15744</td>
<td>NM_0015503</td>
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<td>SH2B adaptor protein 1</td>
<td>1.40 US27, 2.06 US28</td>
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<td>HS:713578</td>
<td>NM_175077</td>
<td>SLA2</td>
<td>Src-like-adaptor 2</td>
<td>1.20 US27, 2.71 US28</td>
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<tr>
<td>HS:485572</td>
<td>NM_003877</td>
<td>SOCS2</td>
<td>Suppressor of cytokine signaling 2</td>
<td>1.94 US27, 1.45 US28</td>
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<tr>
<td>HS:527973</td>
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<td>Suppressor of cytokine signaling 3</td>
<td>1.55 US27, 2.05 US28</td>
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<tr>
<td>HS:468426</td>
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<td>SOCS5</td>
<td>Suppressor of cytokine signaling 5</td>
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<tr>
<td>HS:75516</td>
<td>NM_001331</td>
<td>TYK2</td>
<td>Tyrosine kinase 2</td>
<td>1.82 US27, 2.07 US28</td>
</tr>
</tbody>
</table>

<sup>a</sup> The complete list of genes analyzed on this array is shown in Fig. 3 and includes: A2M, SH2B2, BCL2L1, CCND1, COX11A, CEBPB, CRK, CRP, CSF1R, CSF2RB, CXCL9, EGFR, EPOR, F2, FMR1, FCER1A, FCGR1A, ISG15, GATA3, GBP1, GHR, HMG1, HEF1, A2M, AFNG1, IL10RA, IL20, IL2RA, IL12B, IL14, IL18, IL6ST, INS, IRE1F, ISG35, JAK1, JAK2, JAK3, JUN, JUNB, JUNF, MEF3, MPL, MYC, NFKB1, NOS2A, NR3C1, OAS1, OSM, PDGFRα, PIA1, PIA2, PIP2, PIP3, PIP4, PIK3CA, SHB1, SIT1, SLA2, SMAD1, SMAD2, SMAD3, SMAD4, SMAD5, SOC1, SOC2, SOCS3, SOCS4, SOCS5, SP1, SP11, SRC, STAM, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6, STUB1, TYK2, USF1, YY1.

<sup>b</sup> Gene expression was analyzed using Human JAK-STAT RT2 Profiler PCR Arrays (SABiosciences, Valencia, CA) in triplicate as described in the legend for Fig. 3. Fold change was calculated via the comparative threshold cycle method using values for 293-CXCR3 cells as the baseline.
Fig. 5. GPCR pathway gene expression levels in cells expressing US27, US28, or CXCR3. RNA from each stable cell line was extracted using RNEasy Midi Kit (Qiagen, Valencia, CA), cDNA was prepared using the RT2 First Strand Kit (Qiagen), and then diluted cDNA was mixed with the RT2 SYBR green master mix (Qiagen) according to the manufacturer’s instructions and loaded into the Human GPCR RT2-PCR Profiler Array (SABiosciences, Valencia, CA). Data analysis was performed as described for the JAK-STAT array in the legend for Fig. 3 except that the GPCR results are the average of two biological replicates.

1991), and stimulation of AP-1 activity has previously been linked to at least two specific viral proteins: IE1 (Kim et al., 1999; Wang and Sonenshein, 2005) and the tegument protein pp71 (Liu and Stinski, 1992). We show not only an increase in gene expression for JUN and FOS, but also an increase in jun and fos protein levels, strongly supporting the notion that US27 may induce changes in cellular gene expression through the action of the AP-1 transcription factor.

Cells expressing US27 also exhibited an increase in expression of the BCL2L1 gene. While there was some variability in the fold-change of up-regulation detected on the JAK-STAT array compared to the GPCR array, the overall trend indicated increased BCL2L1 gene expression. The protein encoded by this gene is also known as Bcl-x, a member of the Bcl-2 protein family that plays critical roles in regulation of cell survival (Boise et al., 1993). Bcl-x is expressed as two isomeric forms, Bcl-xL and Bcl-xS, which are typically present in the cytosol in association with the mitochondrial membrane. Bcl-xL forms heterodimers with various proteins, including Bax, Bak and Bcl-2, and prevents cell death by blocking...
the formation of channels in the mitochondrial membrane and inhibiting activation of the caspase cascade (Straten and Andersen, 2010). Overexpression of Bcl-2 family pro-survival genes is a common mechanism for preventing apoptosis, and cells infected with HCMV have previously been found to contain elevated levels of Bcl-2 protein (Cinat et al., 1998). Although levels of Bcl-x protein in HCMV-infected cells have not been reported to date, microarray analysis of infected monocytes revealed a 4.9-fold increase in the level of BCL2A1 (Bcl-2 related protein A1) mRNA (Chan et al., 2008a). In fact, Chan et al. found that infection of monocytes led to the induction of many genes associated with the differentiation into a macrophage phenotype, with 583 genes significantly up-regulated and 621 genes significantly downregulated (Chan et al., 2008a). One of the genes found to be up-regulated in that study was SOCS3, a suppressor of cytokine signaling. In our study with HCMV US27 expressed in isolation, no significant changes in SOCS3 expression were noted, but both SOCS2 and SOCS5 were found to be up-regulated. It is not surprising that HCMV might have multiple mechanisms for activating SOCS proteins and suppressing inflammatory cytokines, since this would likely aid the virus in avoiding immune clearance.

Another global analysis of host cell gene expression during virus infection utilized the AD169 strain of HCMV compared to a US28-deletion mutant (Hertel and Mocarski, 2004). The results showed that while numerous changes occur upon infection, only a small subset of genes involved in cell cycle progression could be specifically attributed to the presence of US28, and none of those were the same genes identified as being impacted by US28 expression in this study. Future work will focus on the protein products of the genes affected by US27, both in transfected and virus-infected cells, in order to confirm that activity is increased and thus contributing to US27-mediated enhancement of cell proliferation. These studies will help clarify the role of US27 in virus infection and could provide a potential target for novel anti-viral therapeutics.

Acknowledgements

This work was supported by NIH Grant AI074029 (to JVS), the Lily Drake Cancer Fund, and USF Faculty Development funds. The authors thank Lauren Hart, Nandini Chitale, and Kathleen Arnold for technical assistance and helpful discussions.

References


Chapter 2 - The DRY box and C-terminal domain of the human cytomegalovirus US27 gene product play a role in promoting cell growth and survival

Chapter 2  Introduction to published manuscript

HCMV US27 is a viral receptor with similarities to chemokine receptors. While US27 is an orphan receptor, we found that US27 expression results in increased cellular proliferation and survival in HEK293 cells. In this study, we examined the involvement of two protein domains, the DRY (aspartic acid, arginine, tyrosine) box and the C-terminal intracellular domain (CTD) of US27, in mediating both cell proliferation and survival. While both domains were required for a proliferative effect, loss of either domain only moderately impacted cell survival, suggesting that US27 may interact with cell survival pathways through protein regions other than the DRY box and CTD. Cells expressing US27 had the lowest percentage of apoptotic cells when compared to control cells. Quantitative RT-PCR arrays were used to profile changes in cellular gene expression in the HEK293-US27 cell line, and down-regulation of cell cycle regulators CDKN1A/p21/CIP1 (cyclin dependent kinase inhibitor 1A) and SESN (Sestrin2 or Hi95) was observed. These results indicate that increased cell proliferation due to US27 may be linked to suppression of negative growth regulators, and that these interactions require the DRY box and CTD.
The DRY Box and C-Terminal Domain of the Human Cytomegalovirus US27 Gene Product Play a Role in Promoting Cell Growth and Survival

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Abstract

Human cytomegalovirus (HCMV) is a widespread pathogen that can lay dormant in healthy individuals and establish lifelong latent infection. This successful co-existence is facilitated by a number of viral gene products that manipulate host cellular functions and immune responses. Among these immunomodulatory genes are four G-protein coupled receptors (GPCRs) encoded by HCMV, designated US27, US28, UL33, and UL78. Studies have shown the US28 gene product to be a functional chemokine receptor that signals both constitutively and in a ligand-dependent manner, resulting in a wide range of cellular effects. In previous work, we have found that US27 expression results in at least two biological effects: enhanced CXCR4 signaling and increased in cellular proliferation in HEK293 cells. Here, we examined the involvement of two protein domains, the DRY box and the C-terminal intracellular domain (CTD) of US27, in mediating both cell proliferation and survival. While both domains were required for a proliferative effect, loss of either domain only moderately impacted cell survival, suggesting that US27 may interact with cell survival pathways through protein regions other than the DRY box and CTD. Quantitative RT-PCR arrays were used to profile changes in cellular gene expression in the HEK293-US27 cell line, and down-regulation of cell cycle regulators CDKN1A/p21/CIP1 (cyclin dependent kinase inhibitor 1A) and SESN (Sestrin2 or Hi95) was observed. These results indicate that increased cell proliferation due to US27 may be linked to suppression of negative growth regulators, and that these interactions require the DRY box and CTD.

Introduction

Human cytomegalovirus (HCMV) is a member of the Herpesviridae family that manipulates the host immune system and establishes life-long latent infection [1]. HCMV is widespread in the general population, but infection is typically asymptomatic in immune competent individuals [2]. Transplant recipients and HIV patients, on the other hand, can suffer debilitating disease upon virus reactivation [3,4]. In addition, during pregnancy, HCMV may be transmitted in utero to the developing fetus, resulting in severe congenital defects like deafness, blindness, mental retardation, and impaired motor function [5,6]. Successful co-existence of HCMV with a healthy host is mediated to some extent by the production of viral proteins that mimic normal immune modulators like cytokines, chemokines, and chemokine receptors [7]. The US27 gene encodes a protein with seven transmembrane domains and similarity to the human chemokine receptor family of G-protein coupled receptors (GPCRs), including conserved cysteines in the extracellular loops and extensive glycosylation of the extracellular domain [8]. US27 also contains a DRY (aspartic acid, arginine, tyrosine) motif in the second intracellular loop that is critical in other GPCRs for activation of G proteins following ligand binding [9], as well as a di-leucine motif in the carboxy-terminal domain that mediates receptor endocytosis [10]. US27 is found in the envelope of the virus particle [6], but in virus-infected cells, the majority of the US27 protein is found in endosomes, the Golgi apparatus, and perinuclear compartments [11]. The US27 gene is non-essential for virus replication and mutants that lack US27 are replication competent [12]. However, US27 deletion mutant viruses are incapable of spreading via the extracellular route in endothelial cells [13], suggesting that US27 might play a role in virion assembly or egress. Although no chemokine ligands for US27 have been identified, signaling activity of the human chemokine receptor CXCR4 is increased in the presence of US27 [14]. In addition, cells expressing US27 demonstrate increased rates of cell proliferation and DNA synthesis [15]. Here, we further investigated the mechanism for this proliferative effect by examining the requirement for the DRY box motif and the carboxy-terminal domain (CTD) of US27 in cell growth and resistance to apoptotic stimuli.
Materials and Methods

Cells
Human embryonic kidney (HEK) 293 cells were grown in Eagle’s minimal essential media (MEM) with 10% fetal bovine serum (Cellgro, Herndon, VA) in a humidified incubator at 37°C and 5% CO₂ atmosphere. Stable HEK293 cell lines expressing 3XFLAG-tagged HCMV US27, US28, or human CXCR3 were generated previously and cultured under the same conditions [10]. In addition, stable cell lines expressing 3XFLAG-tagged US27-DAY (R128A) and US27/XR3CT were also cultured as above [14]. HEK293 or HeLa cells were maintained as above, then seeded in white 96-well plates at a density of 1 x 10⁵ cells per well and transiently transfected 24 hours later with the indicated pEGFP expression vectors using Fugene transfection reagent (Roche Biosciences, Basel, Switzerland) at a ratio of 3:1 (µl Fugene: µg plasmid DNA per manufacturer’s instructions). Transfection efficiency was 40-50% as determined via flow cytometry.

Western Blotting
Cells were seeded into 100 mm dishes at a density of 8 x 10⁵ cells and lysed in the dish after 48 hours via the addition of lysis buffer (150 mM NaCl, 20 mM HEPES, 0.5% Cymal-5, 1 mM NaVO₄, 1 mM EDTA, 0.1% NaN₃, and 4 M urea). The lysate was freeze-thawed three times, clarified via centrifugation at 14,000 rpm for 15 minutes at 4°C, followed by addition of loading dye. Samples were separated using SDS-PAGE, and then proteins were transferred to a nitrocellulose filter then blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). The membranes were incubated with either anti-p21 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-FLAG M2 monoclonal antibody (Sigma Aldrich, St. Louis, MO) at a ratio of 3:1 (µg antibody: µl TBS-T) for one hour. The membranes were incubated with either anti-p21 or anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) at a 1:1000 dilution for one hour at 37°C and then washed. Following washes, the coverslips were incubated with Alexa Fluor 514 Goat Anti-Mouse (Life Technologies) at a 1:500 dilution for one hour at 37°C, washed again, and then mounted on a glass slide using Prolong Gold anti-fade reagent with DAPI (Invitrogen, Carlsbad, CA). For cell surface receptor staining only, the protocol was followed as described above but cells were not permeabilized. All images were acquired using a Zeiss LSM710 laser scanning confocal fluorescence microscope and Zen Black software (Carl Zeiss, Inc., Oberkochen, Germany).

Proliferation Assays
Cells were seeded in white 96-well dishes at a density of 5 x 10⁵ cells per well. Cell growth was measured at indicated time points via the addition of CellTiter-Glo reagent (Promega) followed by luminometry according to manufacturer’s instructions. Bromodeoxyuridine (BrdU) incorporation was assayed on cells seeded as above using the BrdU ELISA kit (Roche Bioscience, South San Francisco, CA). For both assays, each condition was performed in triplicate within the assay, and three independent replicates of each experiment were performed. For cell counts, cells were seeded into 6-well dishes at 2 x 10⁶ cells per well, harvested via trypsinization at the indicated times, and then 3 x 10⁶ µl aliquots were analyzed from each well using the BD Accuri C6 Flow Cytometer (Becton Dickinson, San Jose, CA). Three independent experiments assessing cell counting were performed.

Apoptosis Assays
Cells were seeded into 6-well dishes and treated with 10 µM etoposide (Sigma-Aldrich) for 48 hours, then harvested and stained with Annexin V and propidium iodide using the TACS Annexin V-FITC Staining Kit ( Trevigen, Gaithersburg, MD) before analysis by flow cytometry. In addition, cells were seeded into white 96 well plates at a density of 5 x 10⁵ cells per well in the presence of 10 µM etoposide, and cell viability determined at indicated time points via the addition of CellTiter-Glo reagent as above. For transiently transfected HEK293 and HeLa cells, 10 µM etoposide was added six hours post-transfection. In all cases, each condition was performed in triplicate within the assay, and three independent experiments were performed.

PCR
RNA from HEK293 or stable cell lines that were 70-80% confluent in a T75 flask was extracted using RNeasy Midi Kit (Qiagen, Valencia, CA), cDNA was prepared using the RT2 First Strand Kit (Qiagen), and then diluted cDNA was mixed with the RT2 SYBR green master mix (Qiagen, Valencia, CA) and aliquoted using the BRAINER Microplate RT2-PCR Profiler Array (SA Biosciences, Valencia, CA). Real-Time PCR was performed using the CFX96 (BioRad, Hercules, CA) by heating to 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were analyzed using the ΔΔCt method according to the SA Biosciences web portal (www.SABiosciences.com/ pcrarray.dataanalysis.php) as described previously [15] and are provided as Supplementary Data. The same threshold value was used across all plates in the same data analysis to ensure accurate reading of quality controls. The data were normalized across all plates to the following housekeeping genes: beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L13a (RPL13A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and beta actin (ACTB). Controls for genomic DNA contamination, RNA quality, and PCR performance were all in the manufacturer’s recommended ranges. The fold change values from three biological replicates of the PCR array were analyzed in comparison to either HEK293 or 293-
Results and Discussion

The US27 gene product has been shown to promote an increase in cell proliferation when compared to cells expressing US28 or other cellular chemokine receptors like CXCR3 [15]. Here, we investigated whether specific domains of US27 are necessary to mediate this proliferative effect by making use of two mutants available in our laboratory. First, we examined the requirement for the DRY box motif that is known to play a role in signaling for many GPCRs [9] by using a stable HEK293 cell line expressing US27-DAY, which contains a single amino acid substitution of arginine 128 to alanine (R128A). We also explored the requirement for the intracellular carboxy-terminal domain (CTD) using both a stable HEK293 cell line expressing US27/XR3CT, a chimeric receptor that has the CTD of CXCR3 in place of the CTD from US27, as described previously [10], and a mutant lacking the CTD, US27ACT. As shown in Figure 1, wild-type US27 and the US27 mutants were expressed at comparable levels. Cell lysates were immunoblotted with antibody directed against the US27 gene product, which has heavily glycosylated, was detected (Fig. 1A). Comparable bands were noted for both the US27-DAY and US27/XR3CT receptors, demonstrating that these changes do not adversely affect protein expression. US28 and CXCR3, an HCMV and a human chemokine receptor, respectively, serve as controls in several subsequent experiments and were also detected at the expected sizes of 44 kD and 42 kD. No 3XFLAG labeled proteins were detected in the parent HEK293 cell line. Each receptor was also viewed by immunofluorescence microscopy, and the expression level for the US27-DAY and US27/XR3CT proteins was similar to that of wild-type US27, US28, and CXCR3 (Fig. 1B). To confirm that the mutations in US27 did not alter receptor trafficking to the surface, cells were fixed but not permeabilized, then incubated with anti-FLAG antibody. As shown in Fig. 1C, the level of staining was comparable between US27 and the mutants, indicating that these receptors are present on the cell surface and that the mutations in US27 have no effect on receptor recycling.

In order to examine the impact of these mutations on the ability of US27 to enhance cell proliferation, growth of the stable cell lines was monitored over time. Cell viability was evaluated using a substrate for total cellular ATP, reflecting the number of metabolically active cells in the well (Fig. 2A). Cells expressing wild-type US27 exhibited ATP levels that were higher than any of the other cell lines, which included the parent HEK293 cells, cells expressing US28, CXCR3, or the US27 mutants. Cells expressing US27-DAY and US27/XR3CT were highly and exhibited growth, but the ATP levels were more similar to control cells than US27-expressing cells. There was no evidence that either mutation resulted in a protein that facilitated the same growth advantage as wild-type US27.

Cells expressing US27 mutants were further investigated using BrdU incorporation to determine whether there was any effect on the rate of DNA synthesis (Fig. 2B). While cells expressing wild-type US27 exhibited a marked increase in the rate of DNA synthesis relative to control cells, the US27-DAY and US27/XR3CT cell lines had a rate of DNA synthesis that was on par with the control cells. In addition, standard cell counts confirmed that only US27-expressing cells, and not the US27 mutant-expressing cell lines, contained higher cell numbers than controls when counted at the indicated times (Fig. 2C). While we have previously shown that US27 conveys increased proliferative properties in a variety of cell types [15], we wanted to examine the US27 mutants in a different cell type. Both HEK293 and HeLa cells were transiently transfected using EFGP-tagged US27, US27-DAY, or US27ACT constructs, and cell growth monitored (Fig. 2D, E). In both cell types, US27 cells grew at a significantly higher rate than cells expressing either mutant receptor or the controls. These results suggest that both the DRY box and the CTD of US27 may be required for stimulating cell proliferation.

Next we investigated whether US27 could protect cells from apoptosis. Cells were treated with etoposide to block topoisomerase function, thereby impeding DNA replication and leading to induction of the apoptotic cascade, which was detected via flow cytometry with Annexin V and propidium iodide staining. As shown in Figure 3A, cells expressing US27 had the lowest levels of apoptosis, with only 4.9% of the population staining double positive (DP) for Annexin V and propidium iodide after 48 hours. In contrast there were 15.3% DP cells in the US28 cell cultures, which is not surprising since this receptor has been reported to induce apoptosis when expressed in a variety of cell types [16]. Cultures expressing US27-DAY had 6.2% DP, which was comparable to the control HEK293 cell line (6.9%), and the US27/XR3CT cultures had 7.3% DP cells. Overall, US27-expressing cells had the lowest rate of etoposide-induced apoptosis (Fig. 3B), while cells expressing the US27 mutants responded more like the control cells, indicating that the DRY box and CTD may help mediate cell survival.

When cell viability was monitored over time, US27-expressing cells exhibited significantly greater viability than the other cell types (Fig. 3C). Surprisingly, US27/XR3CT cells also had greater viability than most other cultures from 48 to 96 hours. The viability of cells expressing US27/XR3CT was significantly higher than US28 or control HEK293 cells, but significantly lower than US27-expressing cells, suggesting that this mutant may still be able to either partially block apoptosis or stimulate survival pathways, conferring a moderately protective effect. While US28 cells exhibited the highest levels of apoptosis at 48 hrs (Fig. 3A), the death rate in these cultures at later time points was significantly lower than in control HEK293 or CXCR3-expressing cells (Fig. 3B). The overall viability in the US27-DAY cultures was almost identical to that of US28 and also significantly lower than in control cells, indicating that this mutant might also retain some ability to protect cells from programmed cell death. Thus, unlike the proliferative ability of US27, which was completely abrogated by mutation of the DRY box and CTD, the ability to protect from etoposide-induced death was only partially diminished by these mutations. Given that the protection was less evident at the earliest time points, it may be that mutations delayed the activation of survival pathways by US27, but ultimately the US27-DAY and...
Figure 1. Stable cell lines express HCMV US27 or US27 mutants. A) Cell lysates were separated by SDS-PAGE and then immunoblotted using anti-FLAG M2 antibody or anti-MAPK as a control. (B) Immunofluorescence staining of fixed, permeabilized cells with anti-FLAG M2 antibody followed by Alexa Fluor 514-conjugated mouse secondary. Optical sectioning was performed and one slice of the Z-stack is shown. (C) Cell surface staining with anti-FLAG M2 antibody on non-permeabilized cells, followed by Alexa Fluor 514 secondary antibody.

doi:10.1371/journal.pone.0113427.g001
Figure 2. Expression of US27 conveys a proliferative advantage. A) Cells were seeded into 96-well plates at a density of \(5 \times 10^3\) cells per well and cell number was monitored via the addition of CellTiter-Glo reagent at the indicated time points. B) The rate of DNA synthesis for each cell line was measured using bromodeoxyuridine (BrdU) incorporation and luminometry. C) Cells were seeded in 6-well dishes at \(2 \times 10^5\) cells per well, then harvested with trypsin and counted via flow cytometry at the indicated time points. D) HEK293 and (E) HeLa cells were seeded into 96-well plates at a density of \(1 \times 10^4\) cells per well and transfected 24 hours later with the indicated pEGFP expression vector at a ratio of 3:1 (μl Fugene:μg plasmid DNA), cell number was monitored via the addition of CellTiter-Glo reagent at the indicated time points. Error bars represent standard error of three triplicate data points within one experiment. These results are representative of three independent experiments. ** indicates \(p < 0.001\), * indicates \(p < 0.05\) by Student t-test.

doi:10.1371/journal.pone.0113427.g002
Figure 3. HEK293 cells expressing wild type US27 are more resistant to apoptosis. Cells were treated with 10 μM etoposide to induce apoptosis. A) After 48 hours, cells were stained with propidium iodide and Annexin V then analyzed by flow cytometry. B) The average percent positive cells for Annexin V, propidium iodide (PI) or both (double positive, DP) from three independent experiments, as indicated in A above. * indicates p<0.01 by Student t-test compared to US27-cells. Overall cell viability was measured at the indicated time points via the addition of CellTiter-Glo reagent and total luminescence quantified in C) stable HEK293 cell lines, and in transiently transfected D) HEK293 cells and E) HeLa cells following etoposide treatment. Error bars represent standard error of three triplicate data points within one experiment. These results are representative of three independent experiments. ** indicates p<0.001, * indicates p<0.05 by Student t-test. In C, pairwise comparisons were performed between US27/XR3CT and US27, US28, and HEK293 and between US27-DAY and US27 and HEK293.

doi:10.1371/journal.pone.0113427.g003
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1The complete list of genes analyzed using the Human p53 Signaling Pathway RT2 Profiler PCR Array (SABiosciences, Valencia, CA) includes: APAF1, ATM, ATR, BAK1, BAX, BCL2, BCL2A, BID, BIRC3, BRCA1, BRCA2, BTG2, CASP2, CASP9, CEBP, CCNE2, CCNE4, CDK1, CDK2, CDK2A, CDK3, CDK4, CDKN1A, CDKN1B, CDKN2A, CDKN2B, CHEK1, CHEK2, CME1, DHH, EZF2, ESR1, FADD, FASLG, FOXO3, GADD45A, GMI, HDAC1, HMC, IFN1, IFN2R1, IGF1, IGF1R, IGFR, JUN, KARS, KDR, MCL1, MDM2, MDM4, MLH1, MSH2, MYC, MYOD1, NF1, NRAS, TP53, TP53AIP1, KAT2B, PCBP4, PCNA, PPM1D, PRC1, PRKCA, Pten, PTG1, RB1, RELA, RRM1, SESN1, SESN2, SIAH1, SIRT1, STAT1, TADA3, TNF, TNFRSF10B, TNFRSF10, TP53, TP53B, TP73, TP63, TRAF2, TSC1, TUT1, XIKC.
US27/XR3CT proteins were still able to convey anti-apoptotic effects to the cell.

In order to further explore the effect of the US27, US27-DAY, and US27/XR3CT on cell survival, quantitative PCR array analysis was performed on the stable cell lines. RNA was extracted from each cell type and expression levels of 84 genes involved in the p53 signaling pathway were compared to the parent HEK293 cells. As shown in Table 1, only a small subset of genes exhibited a fold change of 1.5 or higher that was statistically significant. Some genes were found to be down-regulated in all five cell lines stably expressing viral or human GPCRs, like CDKN1A, or p21, the cyclin-dependent kinase inhibitor. Although cells expressing US27 had the greatest fold change decrease (~2.41), the overall trend was that CXCR3, US28, and the US27 mutants all caused down-regulation of CDKN1A, suggesting that overexpression of any GPCRs might affect the p21 gene. Other genes, like BTG2 and BAI1, were specifically down-regulated in cells expressing the viral GPCRs but not in cells expressing CXCR3. For BTG2, (also known as NGF-inducible anti-proliferative protein PC3 or NGF-inducible protein TIS21), which is a p53-induced transcriptional co-regulator of cell cycle progression [17], cells expressing US27 exhibited a −2.03-fold change compared to the control HEK293 cells, while cells with the US27-DAY mutant had only a −1.47-fold decrease in expression. This suggests that US27 impacts the expression of BTG2 and that this effect is mediated to some extent by the DRY box and to an even greater extent by the CTD of US27, which may interact with intracellular signaling proteins. For BAI1, brain-specific angiogenesis inhibitor 1, in the adhesion GPCR family that is regulated by p53 [18], the opposite effect was observed. Cells expressing US27 were found to have a ~3.47 fold decrease in expression, compared to ~1.02 for US27-DAY and ~2.03 for US27/XR3CT, suggesting that the DRY box is critical for down-regulation of BAI1 by US27, while the CTD may play a less significant role in mediating that effect.

For some genes, the changes in gene expression conveyed by any of the viral GPCRs were quite distinct from that of the human GPCR CXCR3. Thus, the fold changes were also calculated using the values from 293-CXCR3 cells as a baseline (Table 2). As shown in Figure 4A, both EGR1 and SESN2 were significantly down-regulated for all four viral GPCR. For SESN2, this pattern was also evident at a range of time points of cell cultivation (24 hours, Fig 4B), and at the protein level. Western blotting revealed decreased levels of Sestrin-2 (also known as HI95) in cell lysates expressing US27 and US27-DAY compared to parent HEK293 or CXCR3-expressing cells (Fig. 4C), although for US27/XR3CT, the changes in RNA level did not correlate as clearly with protein level. Sestrin-2 has been identified as a stress sensor that functions in a p53-independent manner, possibly through the PI3K/Akt signaling pathway [19,20]. Although Sestrin-2 is generally considered a pro-survival factor, our results suggest that the protein is down-regulated by US27, indicating that the signaling pathways involved may be complex and multi-functional.

Figure 4. HCMV US27 correlates with decreased expression of p21 and Sestrin-2. A) Fold changes in gene expression compared to levels in cells expressing CXCR3. B) RNA was harvested from each stable cell line, reverse transcribed, then gene specific primers were used to amplify either Sestrin-2 or β-actin. C) Cell lysates were separated by SDS-PAGE and immunoblotted with antibodies directed against p21, sestrin-2, or MAPK as indicated.
doi:10.1371/journal.pone.0113427.g004
Table 2. p53 Pathway Gene Expression Analysis II.

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The complete list of genes analyzed using the Human p53 Signaling Pathway RT2 Profiler PCR Array (SABiosciences, Valencia, CA) includes: APAF1, ATM, ATR, BAI1, BAX, BCL2, BCL2A1, BID, BIRC5, BRCA1, BRCA2, BTG2, CASP2, CASP9, CCNB2, CCNE2, CCNG2, CCNH, CDK1, CDC25A, CDC25C, CDK4, CDKN1A, CDKN2A, CHEK1, CHEK2, CRADD, DNMT1, E2F1, E2F3, EGR1, EI24, ESR1, FADD, FASLG, FOXO3, GADD45A, GML, HDAC1, HK2, IFNB1, IGF1R, IL6, JUN, KRAS, PIDD, MCL1, MDM2, MDM4, MLH1, MSH2, MYC, MYOD1, NF1, NFKB1, TP53AIP1, KAT2B, PCBP4, PCNA, PPM1D, PRC1, PRKCA, PTEN, PTTG1, RB1, RELA, RPRM, SESN1, SESN2, SIAH1, SIRT1, STAT1, TADA3, TNF, TNFRSF10B, TNFRSF10, TP53, TP53BP2, TP73, TP63, TRAF2, TSC1, WT1, XRCC5.

doi:10.1371/journal.pone.0113427.t002
For CDKN1A, a slightly different picture emerged when using CXCR3 expressing cells as a baseline, compared to the parent HEK293 cells. While all cell lines showed some level of down-regulation compared to HEK293 cells, US28 was virtually identical to CXCR3 (1.04-fold change). In contrast, US27 cells exhibited a nearly 2-fold down-regulation of CDKN1A, which was nearly ablated in cells expressing either US27-DAY or US27/XR3CT. This decrease in gene expression correlated to a modest reduction in p21 protein levels compared to HEK293, US28, or CXCR3 cells (Fig. 4C). Since p21 is a negative regulator of cell cycle progression [21,22], down-regulation of this protein by US27 supports our observations that the US27 protein conveys a proliferative and survival advantage. Further analysis of the possible interaction between US27 and the p21 signaling pathway may provide valuable insights into the mechanistic basis for this proliferative effect.

Some changes were notable because of a significant impact by only one of the GPCR examined. Cells expressing US28 had a dramatic increase in TNF (tumor necrosis factor) expression (4.41 fold) compared to the other cell lines. IFNβ (interferon-β) was found to be up-regulated by 2-fold in cells expressing the US27/XR3CT mutant, but not by US27 or US27-DAY. However, these changes could not be confirmed at the protein level, and no TNF or IFNβ was detected in the supernatants of any of the cell lines by ELISA (data not shown).

Another interesting change was the significant increase in expression of ESRI, the estrogen receptor, in cells expressing either the US27-DAY (3.02-fold change) or US27/XR3CT mutant (5.18 fold change), whereas expression was down-regulated in cells expressing either US27, US28, or CXCR3. In contrast, the WT1 (Wilms tumor) gene, a tumor suppressor implicated in childhood nephroblastoma [23,24], was significantly down-regulated in cells with US27-DAY (~4.49-fold) with little impact from wild type US27 or US27/XR3CT, suggesting that there may be negative regulation of WT1 that is blocked by US27 and mediated through signaling involving the DRY box. Although US28 has previously been implicated in tumorigenesis [25,26], future studies will be necessary to determine whether the US27 gene might play a role in tumor formation in either breast, kidney, or other forms of cancer.

In summary, we have shown that expression of HCMV US27 can promote cell proliferation, and that this effect requires both the DRY box motif and the carboxy terminal intracellular domain (CTD). While US27 has not yet been associated with any constitutive signaling pathway [27], our findings suggest that the viral receptor does likely associate with intracellular signaling proteins, such as p21 or Sestrin-2, and that the DRY box and CTD are important for these interactions. The requirement for the DRY box and CTD was less clear in protection from apoptotic stimuli, where the protective effect of US27 was diminished, but not completely abrogated, by the loss of either domain. Additional work is needed to fully understand the functions and signaling capabilities of this viral receptor, including whether these proliferative and pro-survival effects might play a possible role in tumorigenesis.

**Supporting Information**

**Table S1** Gene expression analysis of US27 compared to HEK293. RNA was extracted and expression levels of 84 genes involved in the p53 signaling pathway were analyzed using the p53 RT2 Profiler PCR Array. Raw data and analysis for three biological replicates of 293-US27 cells compared to values for the parent HEK293 cells is shown in this supplementary table. (XLS)

**Table S2** Gene expression analysis of US27-DAY compared to HEK293. RNA was extracted and expression levels of 84 genes involved in the p53 signaling pathway were analyzed using the p53 RT2 Profiler PCR Array. Raw data and analysis for three biological replicates of 293-US27-DAY cells compared to values for the parent HEK293 cells is shown in this supplementary table. (XLS)

**Table S3** Gene expression analysis of US27/XR3CT compared to HEK293. RNA was extracted and expression levels of 84 genes involved in the p53 signaling pathway were analyzed using the p53 RT2 Profiler PCR Array. Raw data and analysis for three biological replicates of 293-US27/XR3CT cells compared to values for the parent HEK293 cells is shown in this supplementary table. (XLS)

**Table S4** Gene expression analysis of US28 compared to HEK293. RNA was extracted and expression levels of 84 genes involved in the p53 signaling pathway were analyzed using the p53 RT2 Profiler PCR Array. Raw data and analysis for three biological replicates of 293-US28 cells compared to values for the parent HEK293 cells is shown in this supplementary table. (XLS)

**Table S5** Gene expression analysis of CXCR3 compared to HEK293. RNA was extracted and expression levels of 84 genes involved in the p53 signaling pathway were analyzed using the p53 RT2 Profiler PCR Array. Raw data and analysis for three biological replicates of 293-US28 cells compared to values for the parent HEK293 cells is shown in this supplementary table. (XLS)

**Table S6** Gene expression analysis of US27 compared to CXCR3. RNA was extracted and expression levels of 84 genes involved in the p53 signaling pathway were analyzed using the p53 RT2 Profiler PCR Array. Raw data and analysis for three biological replicates of 293-US27 cells compared to values for the 293-CXCR3 cells is shown in this supplementary table. (XLS)

**Table S7** Gene expression analysis of US27-DAY compared to CXCR3. RNA was extracted and expression levels of 84 genes involved in the p53 signaling pathway were analyzed using the p53 RT2 Profiler PCR Array. Raw data and analysis for three biological replicates of 293-US27-DAY cells compared to values for the 293-CXCR3 cells is shown in this supplementary table. (XLS)

**Table S8** Gene expression analysis of US28 compared to CXCR3. RNA was extracted and expression levels of 84 genes involved in the p53 signaling pathway were analyzed using the p53 RT2 Profiler PCR Array. Raw data and analysis for three biological replicates of 293-US28 cells compared to values for the 293-CXCR3 cells is shown in this supplementary table. (XLS)

**Table S9** Gene expression analysis of US27/XR3CT compared to CXCR3. RNA was extracted and expression levels of 84 genes involved in the p53 signaling pathway were analyzed using the p53 RT2 Profiler PCR Array. Raw data and analysis for three biological replicates of 293-US27/XR3CT cells compared to values for the 293-CXCR3 cells is shown in this supplementary table. (XLS)
Author Contributions

Conceived and designed the experiments: CCT JVS. Performed the experiments: CCT. Analyzed the data: CCT JVS. Contributed reagents/materials/analysis tools: CCT JVS. Wrote the paper: CCT JVS.

References

Chapter 3 - Human Cytomegalovirus UL111A and US27 gene products synergize to enhance signaling of host chemokine receptor CXCR4

(Tu, C.C. and J.V. Spencer. 2015. In preparation for manuscript submission)
While no chemokine ligands for US27 have been identified, US27 has been shown to be the first viral receptor to enhance the signaling activity of the human chemokine receptor CXCR4 [38]. Here, we found that US27 can synergize with cmvIL-10, an ortholog of human IL-10, to enhance CXCR4 calcium signaling in response to CXCL12 more than either viral protein alone. In HCMV-infected and US27-transfected HEK293 cells, exposure to cmvIL-10 significantly increased calcium and migration responses to CXCL12, suggesting that the two viral proteins work synergistically to enhance CXCR4 signaling. This cooperative effect may enable HCMV to manipulate CXCR4 to alter host immune responses and modify cell trafficking patterns.

Furthermore, we also confirmed that the IL-10 receptor and STAT3 activation are required for the cmvIL-10-mediated enhancement of CXCR4 activity. These results suggest that HCMV exhibits two distinct mechanisms in potentiating CXCR4 signaling. US27 and CXCR4 can be possibly forming heterodimers, while there also might be some receptor crosstalk through the IL-10R involving STAT3. Among many of the roles CXCR4 exhibits, one major role is that it can direct HCMV-infected cells to the bone marrow, which is a primary site of HCMV latency. Consequently, a new round of infection can be initiated and expand the reservoir of latently infected cells.
Human Cytomegalovirus UL111A and US27 gene products synergize to enhance signaling of host chemokine receptor CXCR4

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One sentence summary: HCMV manipulates cell trafficking by enhancing CXCL12-CXCR4 signaling through two distinct mechanisms that have additive effects.

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Abstract

Human cytomegalovirus (HCMV) is a herpesvirus that establishes lifelong latent infection. Successful persistence of HCMV in healthy individuals is characterized by extensive manipulation of host cellular functions and immune responses. The UL111A gene encodes cmvIL-10, a homolog of human interleukin-10 (hIL-10), a multi-functional cytokine with potent immunosuppressive properties. Despite having only 27% sequence identity to hIL-10, cmvIL-10 retains most functions of hIL-10 and acts by binding and signaling through the hIL-10 receptor complex (IL-10R). Recently hIL-10 was found to potentiate the signaling activity of CXCR4, a human chemokine receptor that promotes migration of immune cells toward sites of injury or infection and also plays key roles in hematopoiesis and immune homeostasis. We found that cmvIL-10 can also enhance signaling of CXCR4 in response to its natural ligand, CXCL12. HEK293 cells endogenously express both CXCR4 and IL-10R, and these cells exhibited significantly increased calcium mobilization and migration in response to CXCL12 in the presence of cmvIL-10. Treatment with a STAT3 inhibitor or siRNA blocked the enhanced calcium flux, indicating that STAT3 activation is required for potentiation of CXCR4 by cmvIL-10. These findings demonstrate that cmvIL-10 triggers events that augment the signaling activity of a cellular chemokine receptor, most likely through receptor crosstalk. We have previously reported that HCMV US27, an orphan chemokine receptor, is the first viral receptor known to enhance CXCR4 signaling. Here, we found that cmvIL-10 and US27 together enhanced CXCR4 calcium signaling in response to CXCL12 more than either viral protein alone. In US27-transfected HEK293 cells and in virus infected cells, exposure to cmvIL-10 was still able to increase calcium and migration responses to
CXCL12, suggesting that the two viral proteins work synergistically to enhance CXCR4 signaling. This cooperative effect may enable HCMV to manipulate CXCR4 to alter host immune responses and modify cell trafficking patterns.
Introduction

Human cytomegalovirus (HCMV) is a member of the herpesvirus family that is widespread in the general population [60]. Seroprevalence ranges from 45-100% worldwide [2], and while infection is generally asymptomatic, life-threatening disease can occur in immune-compromised hosts, particularly transplant recipients [3] [4]. HCMV is the most common infectious cause of congenital defects in the United States, including deafness, blindness, and debilitating neurological conditions [6].

Following primary infection, HCMV establishes lifelong latency [11]. Latent infection is characterized by a quiescent state in which no virus particles are produced, punctuated by periods of reactivation and virus replication. Transmission occurs due to shedding of infectious virus in body fluids such as urine, blood, and saliva [12]. HCMV is highly adapted for successful coexistence with humans through an arsenal of mechanisms for manipulation of host immune responses, particularly host cytokine and chemokine signaling pathways. HCMV encodes one functional cytokine (UL111A), one functional chemokine (UL146), one receptor with similarity to TNF family receptors (UL144), and four putative chemokine receptors (US27, US28, UL33, and UL78) [61].

The UL111A gene encodes a viral ortholog of human interleukin 10 (hIL-10). The viral cytokine, known as cmvIL-10, has only 27% sequence identity to hIL-10, but binds with high affinity to the cellular IL-10 receptor (IL-10R) [55, 62]. Engagement of IL-10R by cmvIL-10 dimers results in activation of the Jak/Stat3 signaling cascade to produce
immune suppressive effects, including inhibition of inflammatory cytokine synthesis, down-regulation of MHC class I and class II, and impaired dendritic cell maturation [56, 63, 64]. The UL111A gene is expressed during both lytic and latent infection, and these immune suppressive effects likely play a critical role in facilitating virus persistence. However, not all functions of cmvIL-10 are immune suppressive. While Th1 type immune responses are inhibited, Th2 type responses are promoted by both hIL-10 and cmvIL-10, including B cell survival and proliferation [65-67]. Maintenance of the peritoneal B cell compartment requires hIL-10, which stimulates proliferation and chemotaxis of B lymphocytes by enhancing signaling through CXCR4 [54].

CXCR4 is a human chemokine receptor that plays critical roles in development, hematopoiesis, and immune cell trafficking [40]. A member of the G protein-coupled receptor (GPCR) superfamily, CXCR4 induces calcium mobilization and chemotaxis upon binding of its ligand CXCL12 (also known as stromal derived factor, or SDF-1). CXCR4 activity is regulated in a number of ways, including formation of homodimers and heterodimers with other cellular and viral GPCRs, including CCR2 [49], CXCR7 [50], Epstein-Barr Virus BILF1 [51], and HCMV UL33 and UL78 [52]. While these interactions tend to inhibit CXCL12-CXCR4 signaling, HCMV US27 was found to interact with CXCR4 and enhance CXCL12-induced calcium flux and chemotaxis [38]. CXCR4 signaling can also be increased by other the actions of other cellular proteins, including the T cell receptor [53], and, as described above, hIL-10 [54]. Here, we investigated whether cmvIL-10 could also potentiate CXCR4 signaling. Our results show that cmvIL-10 enhances CXCL12-induced CXCR4 signaling outcomes, and also that
US27 and cmvIL-10 together have additive effects to increase CXCL12-CXCR4 signaling. Thus, HCMV has developed two distinct mechanisms for enhancing CXCR4 signaling, which may enable the virus to alter host cell trafficking patterns.
Results

**cmvIL-10 enhances calcium mobilization by CXCR4 in response to CXCL12**

To determine whether cmvIL-10 could potentiate the signaling activity of CXCR4, HEK293 cells were loaded with a calcium indicator dye, and then stimulated with CXCL12. Chemokine binding induced the release of sequestered calcium ions into the cytosol, causing a rapid and transient increase in fluorescence, as shown in Figure 3.1 A. When cells were treated with CXCL12 in the presence of cmvIL-10, there was increased calcium mobilization, and the peak fluorescence intensity was significantly higher. The viral cytokine alone did not induce calcium mobilization (Figure 1 A, B). When the CXCL12 concentration was fixed at 100 ng/ml, the magnitude of the calcium response increased as the dose of cmvIL-10 increased (Figure 1 B). In addition, cmvIL-10 was effective at increasing calcium flux to range of doses of CXCL12 (Figure 1 C). These results demonstrate that the UL111A gene product, cmvIL-10, amplifies CXCR4-mediated calcium signaling in a dose dependent manner.

**cmvIL-10 augments chemotaxis towards CXCL12**

In order to investigate whether cmvIL-10 could enhance downstream signaling outcomes of CXCR4, transwell migration assays were performed. HEK293 cells were placed in the upper chamber, separated from the lower chamber containing CXCL12 by a 5.0 µm filter. After four and a half hours, cells that traversed the filter were collected and quantified. The classic bell-shaped curve for chemotaxis was observed, with maximal migration toward 0.1 ng/ml CXCL12 and less cell movement at higher and lower doses (Figure 1 D). While the basal movement of cells was comparable in the presence or absence of
cmvIL-10, migration toward CXCL12 was significantly increased in the presence of cmvIL-10. Although the dose of CXCL12 eliciting maximal chemotaxis remained unchanged, the number of migrating cells increased when cmvIL-10 was included in the lower chamber. The data shown here are for experiments conducted with HEK293 cells, which endogenously express both CXCR4 and IL-10R (Supplemental Figure S1), but the same results were also observed with two monocytic cell lines (Supplemental Figure S2). Taken together, these findings indicate that cmvIL-10 specifically enhances chemotaxis toward CXCL12.

**IL-10R and Stat3 activation are required for cmvIL-10 to potentiate CXCR4 signaling.** Since cmvIL-10 is known to bind to IL-10R and activate the JAK-STAT pathway, we investigated whether this pathway was required for cmvIL-10 effects on CXCR4 signaling. Calcium mobilization was examined in HEK293 cells pretreated with an IL-10R neutralizing antibody, and cmvIL-10 was unable to enhance the CXCL12-induced response in these cells (Figure 2 A). There was no effect on the ability of CXCL12 to mobilize calcium, demonstrating that though antibody did not impair CXCR4 function, IL-10R was required for cmvIL-10 to affect CXCR4 signaling activity. This was also confirmed using siRNA treatment to knockdown IL-10R prior to the calcium flux assay (Figure 2 B). A western blot verified that IL-10R expression was decreased (Figure 2 C), and in these cells, cmvIL-10 failed to increase CXCR4 signaling. Next, cells were pretreated with either a chemical inhibitor of STAT3 (Figure 2 D) or siRNA to knockdown STAT3 (Figure 2 E,F) and assayed for calcium mobilization after stimulation with CXCL12 in the presence or absence of cmvIL-10. While the response to CXCL12
was not affected by treatments that inhibited STAT3, cmvIL-10 was unable to augment CXCR4 signaling under these conditions. Treatment with inhibitors of other signaling pathways did not interfere with the ability of cmvIL-10 to enhance calcium flux (Figure 2 G) suggesting that STAT3 is a critical mediator of cmvIL-10 effects on CXCR4. Finally, a transwell migration assay was performed using cells treated with either the Stat3 inhibitor or a p38MAPK inhibitor (Figure 2 H). Untreated cells migrated toward CXCL12, and migration was increased in the presence of cmvIL-10. The enhanced migration effect by cmvIL-10 was completely ablated when cells were treated with the STAT3 inhibitor, whereas the p38MAPK inhibitor had no effect. These results demonstrate that cmvIL-10 acts through the IL-10R/Stat3 signaling pathway to increase calcium signaling and migration by CXCR4.

*cmvIL-10 mediates a greater calcium flux and chemotaxis than hIL-10 in response to CXCL12*

To examine whether cmvIL-10 can act as a more potent chemo-kinetic agent than human IL-10, calcium flux assays were performed as described previously but here hIL-10 was included. Results showed that hIL-10 can enhance CXCR4 calcium flux, but the response was greater in the presence of cmvIL-10 (Figure 3 A). We also wanted to examine the combined effects of hIL-10 and cmvIL-10. Results showed a modest additive effect when both were added compared to cmvIL-10 alone, suggesting that both are competing to bind the IL-10 receptor and inducing a quicker and stronger secondary effect. In addition, we wanted to compare the downstream signaling effects of hIL-10 and cmvIL-10 on chemotaxis and performed the same migration experiment as described...
previously. In HEK293, hIL-10 enhanced CXCR4-directed migration than CXCL12 alone, but chemotaxis was even greater in the presence of cmvIL-10 (Figure 2 B). cmvIL-10 also enhanced CXCR4-directed chemotaxis more than hIL-10 in the monocytic U937 cell line (supplemental figure S2). To confirm that STAT3 is getting phosphorylated, levels of phosphorylated STAT3 levels were immunoblotted and detected through western blotting. Phosphorylated STAT3 levels were detected after 15 minutes of treatment with hIL-10, cmvIL-10, or both at the expected size of 86 kDa, while total STAT3 levels were detected at the expected 91kDa (Figure 2 C). Phosphorylated STAT3 was not detected in untreated or in cells treated with CXCL12 alone.

**cmvIL-10 does not affect CXCR4 gene expression**

One possible explanation for the increased CXCR4 signaling observed in the presence of cmvIL-10 was that CXCR4 gene expression was up-regulated. To evaluate CXCR4 mRNA levels, cmvIL-10 was added to HEK293 cultures for 2 hours or 24 hours. RNA was isolated and both RT-PCR and quantitative PCR performed. There was no change in CXCR4 band intensity (Figure 4 A) at either time point, indicating that cmvIL-10 did not impact CXCR4 gene expression. Likewise, there was no change in total cellular CXCR4 protein levels, as determined by western blotting of cell lysates (Figure 4 B). These results are consistent with a previous report that hIL-10 enhancement of CXCR4 signaling found no evidence of up-regulation of CXCR4.
cmvIL-10 and US27 synergize to enhance CXCR4 calcium flux and migration while US27 effects are Stat3-independent

Previous work in our lab showed that HCMV US27 can enhance CXCR4 activity [38], and here we wanted to see if cmvIL-10 and US27 could synergize to enhance CXCR4 signaling than either viral protein alone. To examine whether cmvIL-10 and US27 can synergize to enhance CXCR4 calcium flux, the same assay was performed as described previously, but here we compared HEK293 and 293-US27 cells. Results showed that 293-US27 cells caused a greater CXCL12-induced calcium flux in the presence of cmvIL-10 than in parent HEK293 cells (Figure 5 A), suggesting that cmvIL-10 and US27 can synergize to potentiate CXCR4 activity. Interestingly, cells pretreated with STAT3 inhibitor did not block US27-mediated potentiation of CXCR4 in the presence of cmvIL-10, suggesting STAT3 is not required for the effects of US27 (Figure 5 A). In addition, migration assays were performed as described previously but here we compared 293-US27 cells to 293-US28 and HEK293 cells. Results showed that cmvIL-10 and US27 synergize to enhance calcium flux (Figure 5 B) and chemotaxis (Figure 5 C) greater than both 293-US28 and HEK293 cells. Basal movement without CXCL12 was comparable in both cell lines in the presence of cmvIL-10.

cmvIL-10 and US27 synergy does not happen in trans

To examine whether the cmvIL-10 and US27 synergy occurs in trans, we labeled cells with Fluo-4 AM dye and performed calcium flux in the presence of unlabeled cells. When we loaded the labeled HEK293 cells with US27 unlabeled cells for calcium flux in the presence of cmvIL-10, the response was not as high compared to US27 labeled cells.
(Figure 6). The US27 labeled cells were still able to induce a significant increase of calcium flux in the presence of cmvIL-10 either in combination with US27 unlabeled or HEK293 unlabeled cells. This suggests that the synergy of cmvIL-10 and US27 on CXCR4 activity is specific to US27 being present on the same cell with both CXCR4 and IL-10R.

**cmvIL-10 enhances CXCR4 activity in infected with AD169 lab strain and TB40/E clinical strain**

To examine the effect of CXCR4 signaling during HCMV infection,

Human foreskin fibroblast (HFF-1) and newborn human foreskin fibroblast (NUFF) cells were infected with either the AD169 strain of HCMV at an MOI of 0.25 for 48 hours or TB40/E clinical strain and US27 deletion mutant at an MOI at 0.25 for 96 hours. Infected cells exhibited a 40-45 % CPE at time of harvest for calcium flux assay. Infected HFF-1 cells exhibited a greater CXCL12-induced calcium response in the presence of cmvIL-10 than CXCL12 alone (Figure 7 A). Interestingly, to further corroborate the cooperation of US27 and cmvIL-10 on CXCR4 activity in the context of virus infection, infected NUFF cells of the TB40/E and US27 deletion mutant were harvested for calcium flux. TB40/E showed a significant increase in calcium flux in the presence of cmvIL-10, while US27 deletion mutant had a lower and comparable response to uninfected cells (Figure 7 B). TB40/E also showed greater chemotaxis in the presence of cmvIL-10 compared to controls (Figure 7 C). HCMV IE1 gene expression served as positive control to ensure the fibroblast cells were infected with the virus (Figure D). IE1 gene expression was detected in infected, but not in mock-infected cells. US27 gene
expression was detected in only TB40/E virus-infected cells, while UL111A gene expression was detected in both TB40/E and TB40/E and US27 deletion mutant infected cells.

**US27, IL-10R, and CXCR4 are in close proximity to each other and form heteromeric complexes**

Proximity ligation assay was used to examine whether US27, IL-10R, and CXCR4 are in close proximity to each other. Primary antibodies were used to detect for native proteins. Oligonucleotide-conjugated secondary antibodies served as plus and minus probes. If the probes are within 15-30 nm of each other, they will ligate and amplify as a fluorescent amplicon. Results showed that in US27 cells, each pairwise receptor exhibited discrete spots inside and around the cell, indicating US27, IL-10R and CXCR4 are all in close proximity to each other and thus possibly form heteromeric complexes and get internalized together (Figure 8). In US27 cells, they exhibited significantly more IL-10R:CXCR4 complexes than HEK293 cells, suggesting all three receptors are forming a large signaling complex allowing for the cmvIL-10 and US27 synergy to take place. Oncostatin M served as a negative control receptor to confirm that CXCR4, US27, and IL-10R do not form complexes with any surface receptor.

**Bone marrow tissue highly expresses both IL-10R and CXCR4**

To confirm the expression of CXCR4 and IL-10R in the bone marrow, we stained paraffin sections with antibodies to both receptors. We visualized the tissue sections under confocal microscopy and found that the bone marrow has high expression of both
CXCR4 and IL-10R on the same cell (Figure 9). After visualizing the sections under confocal microscopy, we de-stained the sections for immunohistochemistry analysis. We stained the sections with hematoxylin and eosin (reagents provided by Dr. John Sullivan, University of San Francisco) to stain the nuclei and cytoplasm, respectively. Red blood cells are stained intensely red. We visualized the sections using SPOT Advanced imaging software under a compound microscope. Our images show that the bone marrow consists of many different types of cells, including macrophages, megakaryocytes, and red blood cells. This suggests that CXCR4 can direct migration of a variety of cell types to the bone marrow to further promote HCMV latency.
Discussion

The successful co-existence of HCMV with its host is intricate and complex. The virus is very widespread in the human population and can cause debilitating disease in the immune-compromised. HCMV has evolved many strategies to manipulate the host immune system and establishes lifelong latency. One way is by encoding cmvIL-10, a viral homolog of human interleukin-10 (IL-10). Despite its low 27% homology to human IL-10, cmvIL-10 can bind and signal through the IL-10 receptor [55]. Moreover, studies have found that cmvIL-10 can exhibit the same immunosuppressive properties as human IL-10 [56].

Studies have shown that human IL-10 can potentiate the signaling activity of cellular chemokine receptor, CXCR4 [54]. CXCR4 exhibits many physiological roles, including chemotaxis, embryogenesis, and the immune response [40]. CXCR4 is also considered to be the most common chemokine receptor found to be present in cancers, such as breast, prostate and lung cancer [68, 69]. Since human IL-10 has been shown to enhance signaling of CXCR4, we investigated whether cmvIL-10 can also augment CXCR4 signaling. Because cmvIL-10 signals via interaction with the human IL-10 receptor, it was first necessary to identify cells that express both CXCR4 and the human IL-10 receptor in order to investigate whether cmvIL-10 has the ability to enhance CXCR4 signaling. Using HEK293, 293-US27 stable, THP-1, and U937 cells, we evaluated the presence of both receptors via flow cytometry. Our results showed both receptors are expressed on the cell surface, confirming that these cell lines are good candidates for exploring the role of cmvIL-10 on CXCR4 signaling (supplemental figure S1). Our
results showed that cmvIL-10, like human IL-10, can enhance the signaling activity of CXCR4. Multiple cell lines stimulated with CXCL12 in the presence of cmvIL-10 exhibited a significant increase in calcium mobilization (Figure 1, S2). These results are noteworthy and may open the doors to some significant implications for HCMV modulation and dissemination. It could be that cmvIL-10 binding to the IL-10 receptor activates a large signaling complex that enhances ligand CXCL12 mediated signaling through CXCR4. Among the many physiological roles of CXCR4 is that it can direct immune cells to the bone marrow [70], a primary site of HCMV latency [71]. We also found that cmvIL-10 can also affect downstream signaling such as chemotaxis, which could shed light on a specific strategy that HCMV employs to target virus-infected cells.

Proximity ligation showed US27, IL-10R, and CXCR4 to form heteromeric complexes and possibly suggest they’re getting endocytosed together and localizing to certain organelles. Recent studies have shown that CXCR4 undergoes rapid CXCL12 mediated internalization onto the early endosomal compartments [72]. Moreover, US27 has been shown to localize to the endosomes and golgi apparatus, further suggesting that US27:CXCR4:IL10R are getting endocytosed together. Studies have also shown that ubiquitin can function as a ligand for CXCR4 [42]. These findings suggest that cmvIL-10 might be causing CXCR4 to be rapidly internalized and being targeted for lysosomal degradation through a pathway involving ubiquitination. Moreover, cmvIL-10 may be playing a role in regulating the endosomal sorting of activated CXCR4 by targeting the receptor to the endosomal sorting complex required for transport pathway.
We also confirmed that the IL-10 receptor and STAT3 activation are required for the cmvIL-10-mediated enhancement of CXCR4 activity (Figure 2). These results suggest that HCMV exhibits two distinct mechanisms in potentiating CXCR4 signaling (Figure 10). US27 and CXCR4 can be possibly forming heterodimers, while there might be some receptor crosstalk through the IL-10R involving STAT3. Moreover, cmvIL-10 caused a greater enhancement of CXCR4 calcium flux and chemotaxis than human IL-10. Recent studies have shown that human IL-10 can generate functional decoy receptors in the chemokine system [73]. In an inflammatory environment, these decoy receptors can act as molecular sinks for chemokines and prevent signal transduction. Specifically, studies found that CCR1, CCR2, and CCR5 were retained on the cell surface of IL-10 treated cells and can trap inflammatory cytokines, removing them from the inflammatory site [73]. These receptors are essentially “frozen” chemokine receptors that are uncoupled and unable to elicit migration. Hence, decoy receptors can inhibit the chemokine receptor switch. Moreover, CXCR4 has been suggested to be involved in certain inflammatory disorders [74, 75]. These findings suggest that in an inflammatory environment, cmvIL-10 can possibly act as a decoy cytokine and disturb the CXCL12 binding to CXCR4. Hence, cmvIL-10 can possibly disrupt normal CXCL12-mediated effects.

cmvIL-10 is a very intriguing viral homolog of HCMV, one that the virus employs to evade the immune system. Despite its low homology to human IL-10, cmvIL-10 has been shown to bind the IL-10 receptor and exhibit the same immunosuppressive properties. Furthermore, cmvIL-10 has been found to activate the same pattern of STAT1 and STAT3 DNA-binding complexes that was characteristic of IL-10 signaling.
Moreover, human IL-10 has been shown to potentiate the chemotactic effect of CXCL12, while other cytokines such as IL-5, IL-6, and IL-9 do not [54]. Since we found that cmvIL-10 and US27 can synergize to enhance CXCR4 signaling (Figure 5), this suggests that cmvIL-10 may be the main viral cytokine acting as a chemokinetic agent, potentiating the chemotactic effect of CXCL12. This synergy was specific to US27 and not US28. The increased signaling of CXCR4 can further promote the directed cell movement of HCMV-infected cells to tissues expressing CXCL12 (Figure 11). These virus-infected cells can be directed to the bone marrow and help facilitate viral latency. Consequently, a new round of infection can be initiated and expand the reservoir of latently infected cells. In addition, CXCL12 is highly expressed in the placenta, thus it can attract virus-infected CXCR4-expressing cells into the maternal circulation and promote virus dissemination during placental development [76, 77].

The wide range of physiological and pathological effects CXCR4 exhibits makes it intriguing to study. Furthermore, examining the role cmvIL-10 may play in HCMV infection can help contribute to the understanding of how the virus can employ strategies to manipulate host cellular functions. Little is known about US27 and since we found that cmvIL-10 and US27 act in synergy to potentiate CXCR4 signaling, it would be exciting to further explore the mechanism between these two viral gene products and other downstream effects on CXCR4 signaling. Data produced from this research can possibly serve as the foundation for future follow-up studies in vivo and help clarify the role of cmvIL-10 in HCMV latency and develop better treatment options for preventative or antiviral therapy.
Materials and Methods

Cell Lines and Tissue Culture

Human embryonic kidney (HEK293, American Type Culture Collection, Manassas, VA) cells and HeLa (human cervical adenocarcinoma, ATCC) cells were grown and maintained in Eagles Minimal Essential Media (MEM) (VWR, Arlington Heights, IL) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA). HEK293 cells stably expressing HCMV US27 and US28 encoded in the p3X-FLAG vector (Sigma-Aldrich, St. Louis, Missouri) were maintained in MEM + 10% FBS with 1mg/ml Geneticin (Gibco, Grand Island, NY). Suspended monocytic cell lines THP-1 (acute monocytic leukemia, ATCC) and U937 (human leukemic monocyte lymphoma, from Dr. Jonathan Jarvik, Carnegie Mellon University) were grown in RPMI media supplemented with 200mM glutamine, 100X glucose, 1M HEPES, 100mM sodium pyruvate, 7.5% sodium bicarbonate, and 10% FBS. All cells will be subcultured twice weekly by washing with 5 ml of Dulbecco Phosphate Buffered Saline (DPBS), followed by 1 ml of 0.25% Trypsin-EDTA (VWR) to detach HEK293 cell lines from flask. All cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

Flow Cytometry

Cells were harvested at 75-80% confluence by using cell stripper (VWR) and re-suspended with MEM + 10% FBS. Cells were then spun at 1000 rpm for 5 minutes at room temperature. Cell pellets were then re-suspended with cold FACS buffer (PBS + 1%BSA + 0.1% NaN₃) and transferred into a clear round-bottom 96-well plate in a total volume of 100 µl per well. Cells were incubated with anti-CXCR4-PE mouse antibody
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(BD Biosciences), IgG_{2B}-PE mouse isotype control antibody (R&D Systems), anti-IL-10Rα-PE goat antibody (R&D Systems), or IgG-PE goat isotype control antibody (R&D Systems) for one hour on ice, protected from light. For double-staining cells with CXCR4 and IL-10R, cells were co-stained with anti-CXCR4-PE mouse antibody and -IL-10R antibodies followed by anti-goat FITC antibody (Santa Cruz Biotechnology).

After an hour, cells were washed with 100 µl cold FACS buffer, and pelleted at 1000 rpm at 4°C for 5 minutes. Cells were washed for a total of three times. Cell pellets were re-suspended in FACS buffer followed by the addition of 2% paraformaldehyde. Surface staining of samples were detected using a BD FACSCalibur flow cytometer (BD Biosciences).

**Calcium Flux Assay**

HEK293 cell lines were washed with PBS and harvested via cell stripper (VWR) and RPMI 1640 (Roswell Park Memorial Institute) medium without L-glutamine and phenol red (VWR) with 25 mM HEPES (VWR). Cells were spun at 1000 rpm for 5 minutes at room temperature, pellets with washed and spun again followed by a re-suspension of 2 ml RPMI + 25 mM of HEPES at a density of 5 x 10^5 cells/ml per sample. For each sample, 2 µl of Fluo-4AM (Invitrogen, Grand Island, NY) were added and immediately covered with foil to protect from light. Samples were incubated at 37°C with an atmosphere of 5% CO₂ for 30 minutes with agitation at 10-minute intervals. Following incubation, samples were spun at 1000 rpm for 5 minutes at room temperature with pellets re-suspended in RPMI + 25 mM HEPES and transferred into eppendorf tubes. Un-stimulated cells were collected for 20 seconds followed by addition of CXCL12
(0.1µg/ml) in the presence or absence of cmvIL-10 (0.1µg/ml). For Stat3, PI3K, and p38MAPK inhibitors, HEK293 cells were pre-incubated with 10µM of each inhibitor followed by calcium flux. DMSO served as a negative control for the inhibitors. For IL-10R neutralization studies, cells were pre-incubated with 30µg/ml neutralizing antibody for 30 minutes followed by calcium flux. For Stat3 and IL-10R knockdown, HEK293 cells were seeded at 1.5 x 10^5 cells per well in a 6-well plate followed by transfection 48 hours later with either 30 pmol control siRNA (Cell Signaling), IL-10R siRNA (Santa Cruz Biotechnology), or Stat3 II siRNA (Cell Signaling) using Lipofectamine RNAiMAX transfection reagent (Invitrogen). After 72 hours, cells were harvested for calcium flux assay with or without cmvIL-10 treatment. Calcium flux was monitored using a BD FACSCalibur and BD Accuri C6 flow cytometer (BD Biosciences). Fluorescence units were analyzed using FlowJo software v9.4.9 (FlowJo, Ashland, Oregon). FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). In all calcium flux assays, ionomycin (VWR) served as the positive control to ensure cells were healthy and able to exhibit a calcium flux, while PBS served as a negative control to ensure that the addition of stimulus did not impact response.

**Cell Migration Assay**

Cells were harvested via cell stripper (VWR) followed by growth media and spun at 1000 rpm for 5 minutes at room temperature. Pellets were washed with PBS to eliminate any residual medium. Cells were re-suspended at a density of 0.5 x 10^5 cells/well with media + 0.5% FBS and 75 µl of cells were placed onto the upper chamber of a 96-well transwell system with 5.0 µm pores (VWR). The bottom chamber contained 235 µl of media +
varying doses of CXCL12 in the presence or absence of hIL-10 (0.1 µg/ml) or cmvIL-10 (0.1 µg/ml) in media + 0.5% FBS, each in triplicate. For Stat3 and p38MAPK inhibitors, 10 µM of inhibitor was added to the chemo-attractant media. DMSO served as a negative control for the inhibitor. Samples were incubated for four hours in a humidified incubator at 37°C with an atmosphere of 5% CO₂. Cells that traversed through the filter were harvested from the bottom chamber by the addition of CellTiter-Glo reagent (Promega, Madison, WI) with a two-minute incubation, shaking at room temperature followed by a 10-minute incubation on bench. Luminescence was detected using a GloMax 96 Microplate Luminometer (Promega).

**RT-PCR**

Cells 75-80 % confluent were treated with CXCL12 (0.1µg/ml) in the presence of absence of cmvIL-10 (0.1 µg/ml). RNA from cells was extracted using RNeasy Midi Kit (Qiagen, Valencia, CA). cDNA was prepared using the iscript cDNA synthesis kit (Bio-Rad). For PCR reactions, each contained cDNA template, primers, dNTP mix, Ex-Taq buffer, and Ex-Taq polymerase (Clontech, Mountain View, California) in a final volume of 30 µl. The gene specific primers for CXCR4 were 5’ –

CCGTGGCAAAACTGGTACTTT – 3’ (forward) and 5’ –
CCCTTGGAGTGTGACAGCTT – 3’ (reverse) and for B-actin specific primers, 5’ –
AAGAGAGGCATCCTCACC – 3’ (forward) and 5’ – TACATGGCTGGGTGTTG – 3’ (reverse). The gene specific primers for IE1 were 5’ -
GGTCACTAGTGACGCTTGTATGATGACCATGTACCGA – 3’ and
5’ – GATAGTCGGGTACAGGGGACTCT – 3’ (reverse) and for US27 5’ –
GACGGACAAGCTTCGATGACCACCTCTACAATAATC – 3’ (forward) and 5’ -
CGACGAGCTGCAGTTACAACAGAAATTCCTCCTC – 3’ (reverse) and for UL111A
5’ – TCCTGAGACAGCCGACTAATCAGGCACGACG – 3’ (forward) and 5’ –
TCTCGAGTCAGACTCTTTGCAGACG – 3’ (reverse). The PCR reaction
underwent the following protocol on a MyCycler Thermal Cycler (Bio-
Rad): 94C, 5 min; 35 cycles: 94C for 30 sec, 58C for 30 sec, 72C for 60 sec; 72C for 5 min; a final hold at 4
°C. The PCR products were visualized on a 2 % agarose gel.

**Western Blotting**

Cells were seeded into 10 cm dishes at a density of 8 x 10^5 cells and treated 48 hours later
with CXCL12 (0.1 µg/ml) in the presence of absence of cmvIL-10 (0.1 µgat varying time
points. Cells were scraped in the dish via the addition of cold RIPA lysis buffer (25 mM
Tris-HCL pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS),
followed by PBS wash, and resuspension of pellet with 100 ul of cold RIPA buffer. Cell
lysates were agitated every 15 minutes for one hour, sonicated, and centrifuged at 14,000
rpm at 4°C for 15 minutes. Clarified cell lysates were then transferred into eppendorf
tubes. After the addition of 4X Laemmli buffer (Bio-Rad) and 10X sample reducing
agent (Life Technologies), cell lysates were heated at 42°C for 10 minutes. Samples were
then separated using SDS-PAGE and transferred onto a nitrocellulose membrane as
described above, followed by overnight incubation at 4°C with anti-CXCR4 goat
polyclonal primary antibody (Santa Cruz Biotechnology) at a 1:100 dilution. For
detection, membrane was incubated with appropriate alkaline phosphatase-conjugated
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secondary antibody. Bands were visualized via the addition of Western Blue Substrate reagent (Promega, Madison, WI).

**Proximity Ligation Assay (PLA)**

Cells were seeded into 16-well chamber slides (Lab-Tek, Fisher-Scientific) at a density of 1.5 x 10^5 cells per well and then treated 24 hours later with CXCL12 (0.1 µg/ml) in the presence of absence of cmvIL-10 (0.1 µg/ml) for 15 minutes in a humidified incubator at 37°C with an atmosphere of 5% CO₂. Cells were then fixed with 4% paraformaledyde for 20 minutes at room temperature followed by a PBS wash for five minutes in a coplin jar with agitation then decant, three washes total. Cells were treated with 0.5% Triton X-100 in PBS for 10 minutes without agitation at RT then washed with 0.05% Tween-20 in TBS (TBS-T) for 3 x 5 min in a Coplin jar with agitation at RT. Cells were blocked with one drop of Duolink blocking solution (1X) (DUOLINK PLA kit, Sigma-Aldrich) per well and incubated for 1 hour at 37°C. After one hour, cells were co-stained with anti-CXCR4 goat polyclonal antibody at 1:100, anti-IL10Rα rabbit polyclonal antibody (Santa Cruz Biotechnology) at 1:100, or anti-FLAG M2 mouse monoclonal antibody (Sigma-Aldrich) at 1:500 at 37°C for one hour. Cells were washed with wash buffer A for 2 x 5 min in a Coplin jar with agitation at RT. Cells were then co-stained with PLA probes PLUS and MINUS at 37°C for one hour. Cells were washed with wash buffer A for 2 x 5 min, and incubated with ligation mix at a final dilution of 1:40 for 30 min at 37°C. After 2 x 2 min washes with wash buffer A, cells were incubated with amplification mix at a final dilution of 1:80 for 30 min at 37°C. Cells were washed for 2 x 10 min each in wash buffer B in Coplin jar with agitation followed by a one dip in 0.1X wash buffer B before
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removing the silicone from the slide. Mounting medium with DAPI of 40 µl was added to the slide and coverslips were placed on top. Slides were stored in the dark for 24 hours. Images were captured using a Zeiss LSM700 laser scanning confocal fluorescence microscope and Zen Black software (Carl Zeiss, Inc., Oberkochen, Germany).

**Bone marrow paraffin-embedded tissue staining**

Five-micron thick tissue sections (Zyagen, San Diego, California) were immersed in xylene 2 x 10 minutes in a coplin jar, shaking, followed by a series of ethanol concentrations: 2 x 10 min washes in 100% ethanol; 5 minutes each in 95%, 70%, and 50% ethanol. The tissue section was then rinsed in dH₂O, then rehydrated with wash buffer (1X PBS) for 10 minutes followed by draining of the excess buffer. Tissue sections were incubated with blocking buffer (1% horse serum in PBS) for 30 minutes at room temperature. Primary antibodies CXCR4 mouse antibody and IL-10R rabbit antibody (1:100, Santa Cruz Biotechnology) were added to the tissue sections and incubated at 4°C overnight. Tissue sections were washed in wash buffer 3x 15 minutes, followed by addition of secondary antibodies Alexa Fluor 514 and Alexa Fluor 594 for one hour at room temperature. Tissue sections were washed in wash buffer 3 x 15 minutes, followed by the addition of DAPI and coverslip. Images were captured on a Zeiss LSM700 confocal microscope. For de-staining tissue sections, they were immersed in 70% ethanol overnight to gently remove coverslip. Tissue sections were immersed in hematoxylin for 5 min then rinsed with tap water for 20 minutes. The tissue sections were immersed in 70% ethanol than 95% ethanol, each for three minutes. Next, slides were immersed in 100% alcohol containing eosin for three minutes, followed by 3 x 3
minute immersions in 100 % alcohol. Then, tissue sections were immersed 2 x 3 minute immersions in xylene, followed by addition of Permount mounting medium and coverslip. Images were captured with oil immersion on a compound microscope using the SPOT Advanced imaging software.
Figure 1. cmvIL-10 enhances CXCL12/CXCR4 calcium mobilization and migration in HEK293 cells. A) HEK293 cells were loaded with Fluo-4 AM calcium indicator dye, then stimulated with 0.1 µg/ml of CXCL12 in the presence or absence of cmvIL-10 (0.1 µg/ml). cmvIL-10 alone served as a negative control. Relative fluorescence intensity (RFI) was measured by flow cytometry. Calcium flux showed cmvIL-10-mediated CXCR4 potentiation is B) dose-dependent on cmvIL-10 and C) dose-dependent on CXCL12. D) HEK293 cells were seeded in a 96-well plate at a density of 5 x 10^4 cells in the upper chamber of an 5.0 µm transwell filter. Varying doses of CXCL12 in the presence or absence of cmvIL-10 (0.1 µg/ml) was placed in the lower chamber where indicated. After 4 ½ hours of incubation, cells that traversed the filter were harvested and quantified by CellTiter-Glo. * indicates p < 0.05 by Student’s t-test.
Figure 2. The IL-10 receptor and Stat3 activation are required for cmvIL-10 mediated potentiation of CXCR4. A) HEK293 cells were pretreated with either A) IL-10R inhibitor (10 µM) or B) IL-10R siRNA (30 pmol). Calcium flux was measured following treatment with 0.1µg/ml CXCL12 in the presence or absence of cmvIL-10 (0.1µg/ml). C) Cells transfected with siRNA were lysed and immunoblotted with IL-10R antibody. MAPK served as a control for total protein levels. HEK293 cells were pretreated with D) Stat3 inhibitor (10 µM) or E) Stat3 siRNA (30 pmol) and calcium flux was measured following treatment with 0.1µg/ml CXCL12 in the presence or absence of cmvIL-10 (0.1µg/ml). F) Western blot confirming Stat3 knockdown. G) HEK293 cells were pretreated with 10 µM inhibitor and calcium flux was performed following treatment with 0.1µg/ml CXCL12 in the presence or absence of cmvIL-10 (0.1µg/ml). H) HEK293 cells were seeded and pretreated with 10 µM inhibitor in the upper chamber of a 5.0 um transwell filter in a 96-well plate. Varying doses of CXCL12 in the presence or absence of cmvIL-10 (0.1 µg/ml) were placed in the lower chamber where indicated. Migrated cells were quantified. Error bars represent standard error, * indicates p < 0.05, ** p < 0.01
Figure 3. cmvIL-10 enhances CXCR4 calcium flux and chemotaxis greater than hIL-10. A) HEK293 cells were loaded with Fluo-4AM calcium indicator dye, then stimulated with CXCL12 (0.1µg/ml) in the presence or absence of cmvIL-10, hIL-10, or both (0.1µg/ml). Ionomycin served as positive control. PBS served as negative control. * indicates p < 0.05; ** indicates p < 0.005 by Student’s t-test. B) HEK293 cells were seeded in a 96-well plate in the upper chamber of a 5.0 µm transwell filter. Varying doses of CXCL12 in the presence or absence of cmvIL-10 or hIL-10 (0.1 µg/ml) was placed in the lower chamber where indicated. Migrated cells were quantified. C) P-Stat3 levels were detected after 15 minutes of treatment with cmvIL-10, hIL-10, or both through western blotting. Total Stat3 served as loading control. * indicates p < 0.05 by Student’s t-test.
Figure 4. cmvIL-10 does not cause changes in CXCR4 gene expression and protein levels but affects receptor levels. HEK293 cells were treated with CXCL12 (0.1 µg/ml) in the presence or absence of cmvIL-10 (0.1 µg/ml), or both at the indicated times. RNA was harvested and reverse transcribed into cDNA, followed by A) RT-PCR using primers against CXCR4. B) Cells were lysed and CXCR4 protein level was detected through western blotting. MAPK served as loading control. C-D) As above, but cells were treated for 24 hours.
**Figure 5.** US27 and cmvIL-10 synergize to enhance CXCR4-directed chemotaxis while US27-CXCR4 calcium flux does not require Stat3 activation.  

A) HEK293 and 293-US27 cells pretreated in the presence or absence of Stat3 inhibitor (10 µM) were harvested and loaded with Fluo-4AM, followed by CXCL12-induced calcium flux performed in the presence or absence of cmvIL-10 (0.1µg/ml).  

B) Cells were harvested and loaded with Fluo-4AM, followed by CXCL12-induced calcium flux performed in the presence or absence of cmvIL-10 (0.1µg/ml).  

C) Cells were seeded in a 96-well plate at a density of 5 x 10⁴ cells in the upper chamber of an 5.0 µm transwell filter. Varying doses of CXCL12 in the presence or absence of cmvIL-10 (0.1 µg/ml) was placed in the lower chamber where indicated. After 4 ½ hours of incubation, cells that traversed the filter were harvested and quantified by CellTiter-Glo. * indicates p < 0.05; ** indicates p < 0.005 by Student’s t-test.
Figure 6. cmvIL-10 and US27 synergy does not happen in trans. HEK293 and US27 cells were harvested for calcium flux as previously described. Cells were labeled with Fluo-4 AM calcium sensitive dye and calcium flux was performed in the presence of unlabeled cells as indicated. Cells were loaded with CXCL12 (0.1µg/ml) in the presence or absence of cmvIL-10 (0.1µg/ml).
Figure 7. cmvIL-10 enhances CXCR4 signaling in infected fibroblasts with strains AD169 and TB40/E. A) Uninfected and HFF-1 infected with AD169 lab strain at an MOI of 0.25 for 48 hours. Uninfected and NUFF infected with clinical isolate TB40/E or US27 deletion mutant at an MOI of 0.25 for 96 hours. Cells were harvested and either B) labeled with Fluo-4AM for calcium flux assay with CXCL12 treatment in the presence or absence of cmvIL-10 (0.1 µg/ml) or C) seeded in a 96-well plate at a density of 5 x 10⁴ cells in the upper chamber of an 5.0 µm transwell filter. Varying doses of CXCL12 in the presence or absence of cmvIL-10 (0.1 µg/ml) was placed in the lower chamber where indicated. After 4 ½ hours of incubation, cells that traversed the filter were harvested and quantified by CellTiter-Glo. D) IE1 mRNA expression confirmed virus infection of each strain, while beta-actin served as a housekeeping gene.

Error bars represent standard error, * indicates p < 0.05, ** p < 0.01.
Figure 8. Proximity Ligation Assay of US27, IL-10R, and CXCR4. Primary antibodies to each native receptor were used, followed by oligonucleotide-conjugated secondary antibodies that act as plus and minus probes. If probes are within 15-30 nm of each other, they will ligate and get amplified, resulting in discrete fluorescent spots when viewed by confocal microscopy. A) Each indicated paired receptor show to be in close proximity to each other and thus form heteromers in 293-US27 stable cells. B) Direct comparison of IL-10R and CXCR4 heteromers in HEK293 and 293-US27 stable cells. Quantification of fluorescent spots shown on the right. C) Oncostatin M was used as a negative control.
HCMV exhibits two distinct mechanisms to enhance CXCR4 signaling.
Figure 10. Schematic model of HCMV-infected cells being directed to tissues expressing CXCL12.

cmvIL-10 + US27 enhance directed movement of HCMV-infected cells to tissues expressing CXCL12, such as the bone marrow and placenta, thereby promoting virus dissemination.
Figure 11. Bone marrow tissue expresses CXCR4 and IL-10R. A) Tissue sections were immersed in xylene, followed by a series of ethanol concentrations; 100%, 95%, 70%, 50%, dH2O, then wash buffer to rehydrate the sections. Slides were incubated with blocking buffer for 30 minutes at room temperature. Primary antibodies CXCR4 mouse antibody and IL-10R rabbit antibody (1:100, Santa Cruz Biotechnology) were added to the tissue sections and incubated at 4°C overnight. Cells were washed in wash buffer followed by addition of secondary antibodies Alexa Fluor 514 and Alexa Fluor 594 for one hour at room temperature. Cells were washed and DAPI were added to stain the nuclei. Images were captured on a Zeiss LSM700 confocal microscope. Images show IL-10R (red), CXCR4 (green), and nuclei (blue). B) Tissue sections were de-stained with hematoxylin for 5 min, washed with tap water, followed by 70% ethanol, then 95% ethanol, each for three minutes. Next, slides were immersed in 100% alcohol containing eosin for three minutes, followed by three 100% alcohol washes and two washes of Xylene. Images were captured with oil immersion on a compound microscope using the SPOT Advanced imaging software.
Supplemental figure 1 (S1). Human cells express both CXCR4 and IL-10R on cell surface. Cells were stained with fluorochrome-conjugated anti-CXCR4 (mouse, FL-2) or anti-IL-10R (rabbit, FL-1) antibodies. Fluorescence intensity was measured by flow cytometry. Double positive cells indicated as percentage. Cell types are as follows: HEK293, epithelial cells; 293-US27 and -US28 stable cell lines; THP-1 and U937, monocytic leukemia cell lines.
Supplemental figure 2 (S2). cmvIL-10 enhances CXCR4 calcium mobilization and migration in THP-1 and U937 cells.  **A)** THP-1 and **B)** U937 cells were loaded with Fluo-4 AM calcium indicator dye, then stimulated with 0.1 µg/ml of CXCL12 in the presence or absence of cmvIL-10 (0.1 µg/ml). cmvIL-10 alone served as a negative control. Relative fluorescence intensity (RFI) was measured by flow cytometry.  **C)** U937 cells were seeded in a 96-well plate in the upper chamber of a 5.0 µm transwell filter. Varying doses of CXCL12 in the presence or absence of cmvIL-10 or hIL-10 (0.1 µg/ml) was placed in the lower chamber where indicated. Migrated cells were quantified. * indicates p < 0.05 by Student’s t-test.  **D)** U937 cells were seeded and pretreated with 10 µM inhibitor in the upper chamber of a 5.0 um transwell filter in a 96-well plate. Varying doses of CXCL12 in the presence or absence of cmvIL-10 (0.1 µg/ml) were placed in the lower chamber where indicated. Migrated cells were quantified.
References


70. Mohle, R., et al., *The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial
Chapter 4 – Other Work
HCMV US27 enhances NFκB signaling
HCMV US27 enhances NFκB signaling

Studies have shown that CXCL12/CXCR4 can activate and promote translocation of NFκB to the nucleus, activating the NFκB signaling pathway [78, 79]. CXCR4 can also activate NFκB signaling and promote cancer cell migration and invasion [78, 79]. We found that NFκB signaling is enhanced in the presence of HCMV US27. We used a NFκB secreted luciferase reporter system to detect for the promoter activity. Our findings show that US27 wild-type and cmvIL-10 can synergize to enhance NFκB signaling (Figure 1). However, CXCL12 and cmvIL-10 also seemed to enhance NFκB signaling in control HEK293 cells.

NFκB activity may cause certain cytokines to be expressed in immune cells, leading to STAT3 activation in both malignant and immune cells [80]. This suggests that NFκB may be activating cmvIL-10, leading to more STAT3 activation and causing more downstream signaling of STAT3. The putative crosstalk between NFκB and STAT3 include its physical interaction between the two and cooperation of the two at specific gene promoter/enhancer regions [80]. Since NFκB signaling can be activated by CXCR4, we propose increased signaling in the presence of US27. More studies need to be done to understand the effects of US27 and cmvIL-10 on NFκB signaling. For example, NFκB and STAT3 can control the regulation of certain anti-apoptotic, pro-proliferative and immune response genes. Some of these genes overlap and need the interaction of the two factors. One future experiment may be looking at certain genes to see if there is a difference in expression in the presence of US27 and cmvIL-10.
Figure 1. HCMV US27 and cmvIL-10 synergize to enhance NFκB signaling. Cells were seeded in a 96-well plate at 1.0 x 10^4 cells/well, followed by next day transfection with pMetLuc2-Control Vector or pNFκB-MetLuc2-Reporter Vector at 3:1 Fugene to DNA. After six hours of post-transfection, cells were treated as indicated. Reporter activity was quantified with the addition of substrate at the indicated times.
The human cytomegalovirus US27 gene product binds to the cellular GABA$_A$-receptor associated protein (GABARAP)
The human cytomegalovirus US27 gene product binds to the cellular GABAA-receptor associated protein (GABARAP)

We employed the yeast two-hybrid system to screen a human bone marrow library and identified GABARAP (gamma-aminobutyric acid type A receptor-associated protein) as a binding partner with the C-terminal domain of US27. The GABARAP protein contains hydrophobic binding pockets that tend to associate with a four amino acid motif, WXXL, where W represents the amino acid tryptophan [81], X represents any amino acid, and L represents leucine. GABARAP is a 14 kDa protein expressed in a many cell types and has roles in membrane trafficking and fusion events, receptor trafficking, autophagy, and apoptosis [82].

Co-immunoprecipitation assays demonstrate the US27-GABARAP interaction in HEK293 cells (Figure 2). In addition, we used fluorescence microscopy to examine the US27-GABARAP interaction in human embryonic kidney cells (HEK293). Using a series of proteins with mutations in this motif, we found that the WXXL motif in the C-terminal domain of US27 is required for both GABARAP binding (Figure 3) and for localization to the endosomes (Figures 4). Mutation of the tryptophan (position 306) and leucine (position 309) residues in the C-tail of US27 disrupts its binding to GABARAP and localization to the endosomes. These results show that US27 functions primarily as an intracellular protein (Figure 5). Our findings suggest that the viral receptor could induce internalization of cellular chemokine receptors and may modulate host chemokine signaling responses. Future work includes identifying additional proteins that are part of the US27-GABARAP complex with the aim of clarifying the role of US27 during virus infection.
Figure 2. Co-immunoprecipitation with HCMV US27 and GABARAP. 293-p3XFLAG-US27 stable cells were seeded in a 6-well dish at 2.0 x 10^5 cells, followed by immunoprecipitation using A) GABARAP antibody (1:100, Santa Cruz Biotechnology) and probing with anti-Flag M2 antibody (1:500, Sigma-Aldrich), or B) anti-FLAG M2 antibody and probing with GABARAP antibody. Western blot of glycosylated p3X-US27 is detected as a smear from 50-98 kDa in both the IP and cell lysate lanes, while GABARAP protein appears at 17 kDa. * We have not been able to detect GABARAP in the co-IP lane with anti-FLAG IP antibody.
Figure 3. **US27 and mutants co-localize with GABARAP.** The US27 gene was cloned into the pEGFP vector to create a US27-EGFP fusion protein (green). The GABARAP gene was cloned into the pDsRed vector to create a GABARP-DsRed fusion protein (red). HEK293 cells were seeded onto coverslips and transfected with both vectors. After 48 hours, cells were fixed and mounted using Prolong Gold with DAPI to stain nuclei blue. In the merged image, areas of yellow represent co-localization (arrow).
US27 localizes to the endosomes; US27 mutants do not

Figure 4. Wild-type US27 localizes to the endosomes. HEK293 cells were seeded onto coverslips and transfected with GFP fusion vectors. After 48 hours, cells were fixed and stained with antibodies to EEA1 (early endosome antigen 1) followed by TRITC-conjugated goat anti-rabbit antibody (red). Blue represents the DAPI-stained nucleus, and green represents US27. In the merged image, areas of yellow represent co-localization (arrow).
Figure 5. Schematic image of the US27 receptor binding to GABARAP and localizing to the endosomes inside a cell.
Discussion

Human cytomegalovirus (HCMV) is a ubiquitous pathogen with seroprevalence that ranges 45-100% worldwide [2]. The virus can lay dormant in healthy individuals but be life threatening in immune-compromised hosts. In addition, successful coexistence of HCMV with a healthy host is mediated by the production of viral proteins that mimic normal immune modulators like cytokines, chemokines, and chemokine receptors [17]. One such mimic is the US27 gene product, which has similar features to human chemokine receptors. In this thesis, we focused on the US27 gene product and discovered that it can trigger several biological effects. In chapter one, we found that cells expressing US27 showed enhanced growth rate compared to controls. We also found US27 increased gene expression for JUN and FOS, indicating that US27 may induce changes in cellular gene expression through the action of the AP-1 transcription factor, thereby controlling cell proliferation and apoptosis [83]. US27 also increased BCL2L1 gene expression, which plays critical roles in regulation of cell survival. These results suggest that like US28, US27 can stimulate cell division and promote enhanced growth during virus infection. In summary, we found that during HCMV infection, US27 can have a striking impact on cell gene expression and other host cellular functions.

Next, we wanted to examine whether certain domains of US27 were required for the enhanced cell proliferation. We found that the DRY motif and C-terminal domain of US27 mediate enhanced cell proliferation and survival. We also found that wild-type US27 down-regulates CDKN1A, a negative regulator of cell proliferation. This result suggests that US27 can drive cell proliferation by causing changes in host genes such as blocking CDKN1A. In addition, cells-expressing US27 showed a down-regulation of
BTG2 and were impacted by modification of its carboxy-terminal domain (CTD), which suggests that US27 may be interacting with other host intracellular signaling proteins. Future work is needed to tease out the functions and signaling capabilities of this viral receptor, including whether these proliferative and pro-survival effects might play a possible role in tumorigenesis.

Although no chemokine ligands for US27 have been identified, we found that US27 can synergize with cmvIL-10 to enhance the signaling activity of the human chemokine receptor CXCR4. We also confirmed that the IL-10 receptor and STAT3 activation are required for the cmvIL-10-mediated enhancement of CXCR4 activity. These results suggest that HCMV exhibits two distinct mechanisms in potentiating CXCR4 signaling. US27 and CXCR4 can be possibly forming heterodimers, while there also might be some receptor crosstalk through the IL-10R involving STAT3. In addition, cmvIL-10 caused a greater enhancement of CXCR4-mediated calcium flux and chemotaxis than human IL-10. Moreover, proximity ligation showed US27, IL-10R, and CXCR4 to form heteromeric complexes and suggests all three receptors are endocytosed together to form one large signaling complex. Furthermore, among the many physiological roles of CXCR4 is that it can direct immune cells to the bone marrow [70], which is a primary site of HCMV latency [71, 84] and thus could expand the reservoir of latently infected cells. Chemotaxis in HCMV-infected cells may be significantly increased in the presence of US27 and cmvIL-10.

Taken together, research detailed in this thesis found that US27 enhances cell proliferation, protect cells from apoptosis, and causes changes in host gene expression levels. We identified that the DRY motif and C-terminal domain to be responsible for
US27-induced cell proliferation. Since US27 can promote cell proliferation and synergize with cmvIL-10 to enhance CXCR4 activity, this suggests US27 can cause more HCMV-infected cells to be directed to the bone marrow and facilitate HCMV latency. HCMV remains the most common viral cause of birth defects and childhood disabilities in the United States [6]. Studies have shown that the decidual trophoblast cells that line the maternal blood vessels secrete CXCL12 [76, 77]. This suggests increased homing of HCMV-infected cells into the placenta, thereby attracting virus-infected CXCR4-expressing cells into the fetal circulation.

We also found that US27 can interact with cellular protein GABARAP, shown through preliminary co-immunoprecipitation and immunofluorescence microscopy. Specifically, we found that the WXXL motif of US27 is required for both GABARAP binding and localization to the endosomes. GABARAP is expressed in a variety of cell types and has roles in membrane trafficking and fusion events, receptor trafficking, autophagy, and apoptosis [82]. These results indicate that US27 functions primarily as an intracellular protein and may alter host trafficking patterns.

CXCL12/CXCR4 has been shown to activate and promote translocation of NFkB to the nucleus, thereby activating the NFkB signaling pathway [78, 79]. Preliminary findings suggest that NFkB signaling is enhanced in the presence of HCMV US27. Since NFkB signaling can be activated by CXCR4, we speculate increased signaling in the presence of US27. Future studies need to be done to understand the effects of US27 and cmvIL-10 on NFkB signaling.

Lastly, a different form of cmvIL-10 has also been identified, denoted latency-associated cmvIL-10 (LAcmvIL-10) [85]. The LAcmvIL-10 homologue results from
alternative splicing and is among the few viral proteins synthesized during the latent phase of viral infection, possibly contributing to host immune evasion. Future work may include examining the role of LAcmvIL-10 on CXCR4 signaling, in the presence or absence of US27.
References

18. Margulies, B.J. and W. Gibson, The chemokine receptor homologue encoded by US27 of human cytomegalovirus is heavily glycosylated and is present in


