LATENT HERPES VIRUS REACTIVATION IN SPACE AND THE EFFECTS OF SIMULATED MICROGRAVITY ON CMV INFECTION

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A Dissertation

Presented to

The Faculty of the Department

of Health and Human Performance

University of Houston

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In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy

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By

Bridgette V. Rooney

August 2020

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Abstract

Cytomegalovirus (CMV) is a common beta-herpes-virus that is oftentimes shed in the body fluids of astronauts during short space shuttle (10-16 days) and longer International Space Station (≥180 days) missions. The presence of viral DNA in body fluids indicates reactivation from latency, as well as replication, and can be observed as early as 10 days before launch. The viral load, or copies of viral DNA, continue to rise throughout the spaceflight endeavor. This is problematic for astronauts tasked for extended missions such as to the Moon, Mars and beyond during future exploration-class missions. Current anti-viral treatments are only moderately effective and have significant toxicities further compounding the problem, and there is no treatment for latent CMV. Uncontrolled CMV infections can lead to inflammation of major organs, as well as major cognitive and functional disabilities, as has been shown in immunocompromised bone marrow and solid organ transplant recipients. CMV reactivation in astronauts is further complicated by a spaceflight-induced dysregulation of the immune system where control of viral replication and expansion is impaired. NK-cells of the innate arm and T-cells of the adaptive arm of the immune system suffer cytotoxic deficits that render them incapable of suppressing and/or eliminating free virus or virally infected cells. Though many factors have been implicated in terrestrial reactivation of CMV, including stress hormones and inflammatory cytokines, the specific factors responsible for the nearly 8-fold increases in CMV viral titer observed during spaceflight have not been elucidated. The implication is that factors unique to the spaceflight environment are responsible, and this makes microgravity a prime candidate for investigation. This dissertation,

therefore, sought to determine the history and prevalence of CMV reactivation during spaceflight, as well as the unique contribution of simulated microgravity (SMG), using rotating wall vessel (RWV) technology, on the *in vitro* CMV infection of, and replication amid, immortalized myeloid progenitor cells. Further, we sought to determine the effect of SMG on CMV virulence or its magnitude of expansion measured by the percentage of lysed fibroblast cells due to CMV infection. The outcome of this study highlights microgravity's influence on CMV infection and reactivation.

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List of Abbreviations

AP-1- Activating Protein-1

AUCg- Area Under the Curve relative to the ground

BAC- Bacterial Artificial Chromosome

cAMP- cyclic Adenosine Monophosphate

CMV- Cytomegalovirus

CCMV- Chimpanzee Cytomegalovirus

CD(x)- Cluster of Differentiation

DHEA- Dehydroepiandrosterone

DNA- Deoxyribonucleic Acid

EGFR- Epidermal Growth Factor Receptor

HCMV- Human Cytomegalovirus

HLA-E- Human Leukocyte Antigen class I histocompatibility antigen, alpha chain E

HPA- Hypothalamus-Pituitary-Adrenal

IE- Immediate early

Ig(x)- Immunoglobulin

IκB- NFκB Inhibitor

IL- Interleukin

JSC- Johnson Space Center

L-(x)- Number of days before launch

LIR-1- Leukocyte Immunoglobulin-like receptor 1

MCMV-Murine Cytomegalovirus

MIEP- Major Immediate Early Enhancer/promoter

NASA- National Aeronautics and Space Administration

NFkB- Nuclear-Factor-kappa-light-chain-enhancer of activated B cells

NK(Cell)- Natural Killer

ORF- Open Reading Frame

PKA- Protein Kinase A

R+(x)- Number of days after return

RCMV- Rat Cytomegalovirus

RhCMV- Rhesus Cytomegaolvirus

RWV- Rotating Wall Vessel

SAM- Sympathetic-Adrenal-Medullary

SMG- Simulated Microgravity

T(Cell)- Thymus-derived-lymphocytes

TGF-β- Tumor Growth Factor

TNF- Tumor Necrosis Factor

UL- Unique Long

ULBP- UL16 Binding Protein

US- Unique Short

Chapter 1

Introduction

Cytomegalovirus (CMV) reactivation during space flight has been evident since the early 2000's and poses a significant health risk to the astronaut population, especially when considering that the magnitude of reactivation increases with more time spent in space [1]. The early CMV studies evaluated astronauts assigned to space shuttle missions (10-16 days) where only pre- and post-flight sampling was possible. In these studies, scientists were able to show that 27% of the astronauts shed CMV DNA in either pre- or post-flight urine samples, and that their plasma anti-CMV IgG antibody titers continued to increase through each time point, all of which were significantly different from their baseline values [2]. These results have been corroborated but also extended when active flight samples from astronauts assigned to long duration International Space Station (ISS) missions (≥ 180 days) were evaluated. In these longer duration studies, 61% of astronauts shed CMV DNA in their urine during and after spaceflight in complete contrast to their negative L-180 urine samples. In total for all space shuttle missions, the incidences of CMV reactivation were 47%. This increases to 61% for total ISS missions evaluated to date [1,3]. This 30% increase in frequency, along with time point dependent increases in CMV viral load, underscores the imperative nature of fully understanding and curtailing this phenomenon.

Terrestrial reactivation of herpes viruses is not unusual. In fact, it is common during stressful events incurred through daily living [4], as well as events such as the taking of major exams [5]. However, in these cases, as well as during terrestrial space analog studies, which are designed to emulate aspects of the spaceflight experience, the magnitude of the reactivation is less than the response seen in space [1,3,6–8]. The difference is likely due to factors unique to the spaceflight environment and which are impossible to replicate on Earth such as true microgravity. Whether CMV reactivates in response to microgravity, is yet unknown. Additionally, the spaceflight

environment induces physiological changes that result in immune system dysregulation, which is responsible for the impaired control of latent virus reactivation.

The stress and/or environment of space stimulate the secretion of glucocorticoids and catecholamines in chronically high concentrations and this negatively affects the regulation of the immune system and its individual components [9]. The spaceflight induced changes in these hormones and the cytokine profiles of the astronauts results in decreased cell-mediated immunity, which promotes opportunistic latent viral reactivation [3,10,11].

Lymphocytes such as T-cells and NK-cells play a major role in maintaining viral latency. Spaceflight samples of these lymphocytes indicate dramatic deficits in their function. This is likely due to the pro-inflammatory and TH1-TH2 cytokine changes that occur during spaceflight [9,12]. Major increases in the TH2 cytokine IL-4 (21-fold) and the pro-inflammatory cytokine IL-6 (33-fold) are evident for those astronauts who shed viral DNA. Regardless, T-cells and NK-cells are markedly incapacitated during spaceflight as documented in recent flight studies and these deficits can persist up to several months post flight [11,13]. In the absence of effective lymphocytes, the immune system simply cannot maintain control of opportunistic viral reactivation.

The absence of immune control and existence of a progressive CMV reactivation pose tremendous risk to the health and wellbeing of astronauts as their mission durations extend beyond 6 months. Uncontrolled CMV infections can result in diseases such as chorioretinitis, encephalitis, gastroenteritis, and pneumonia [14]. Additionally, active CMV infection further exacerbates immune dysfunction with its own characteristic immunosuppressive infection of immune cells such as monocytes, macrophages, dendritic cells, and hematopoietic stem cells [15–17].

Immune changes do exist in terrestrial space analogs, but as stated earlier, CMV reactivation and replication during space flight results in a viral load that far exceeds that seen in these analog studies. Again, this is probably due to spaceflight specific factors like microgravity. True microgravity would be the optimal setting for CMV reactivation studies using canonical tissue culture techniques. However, this is

a logistically difficult enterprise. For this study, microgravity was be simulated using a validated analog called the rotating wall vessel (RWV) system [18,19]. This apparatus was engineered by the National Aeronautics and Space Administration (NASA) Johnson Space Center (JSC) Biotechnology Group as an archetypal system for low-shear, low-turbulence conditions mirroring circumstances predicted for space [19]. Cells suspended in medium are contained within a continually rotated disc about an axis perpendicular to the gravitational force vector. This induces a free fall column of fluid where individual cells, maintained in suspension within the RWV, experience very little gravitational force. In fact, the time-averaged gravitational vector on these cells is a residual $10^{-3}g$ force that approximates SMG [19]. Experiments using this technology have illustrated gene expression changes [19] and increases in virulence for pathogens such as *S. Typhimurium* commensurate to results seen in actual space-flight studies [20]. Increases in virulence for both spaceflight and RWV studies, have indicated that the removal of gravitational forces amplifies virulence for a number of pathogens [21,22]. Altogether, this underscores the importance of the use of RWV technology in evaluating space specific objectives and hypotheses. Importantly for this dissertation, this analog facilitated the evaluation of the influence of microgravity on CMV infection, replication and virulence independently of the immune system.

The use of primary cells is optimal over immortalized cells for functional immune studies because it avoids alloreactivity. However, the use of primary cells is not trivial. The CD34+ hematopoietic cell yield, magnetically separated from either of its sources bone marrow or cord blood, is generally small, and within this small population, the number of cells that support infection is even smaller. For that reason, immortalized Kasumi-3 myeloid progenitor cells were used to evaluate SMG effects on CMV's capacity to infect and to replicate, as well as for the initial part of the virulence experiments. Kasumi-3 cells originated from the bone-marrow-blast-cells of a 57 year old Japanese man with minimally differentiated acute myeloblastic leukemia [23]. They are CMV negative, they express cell surface markers indicative of myeloid progenitors (CD13, CD33 and CD34), and they retain their capacity to differentiate down the myeloid pathway. The phorbol-ester, 12-o-tetradecanoylphorbol-13-acetate

(TPA, but aka PMA) specifically directs these cells toward monocyte formation. Most importantly, they are well suited for CMV infection studies [17,24–26].

CMV infection is highly cell associated, especially in the blood [14], with very little free virus present in circulation. Characterization of the capacity of CMV to infect (bind to and enter) Kasumi-3 cells during *in vitro* experiments is possible by evaluating the extent to which the virus is cell associated. Cell association is evaluated by separating samples into cell pellet and supernatant fractions, via high-speed centrifugation, DNA extraction and then polymerase-chain-reaction (PCR). Additionally, the expression of lytic CMV transcripts such as IE-1 and IE-2 within the cell pellet fraction indicate infection. Replication of the virus while in culture is represented by changes in the viral load (copy number per µL), quantified via the amplification of the viral DNA present in the sample. While all of the above analysis is critical to understanding the changes in CMV behavior due to SMG at the genetic level, it is imperative to understand how these changes materialize at the whole virus level. DNA evidence only highlights the presence of CMV activity but does not fully illustrate the viability or infectiousness of the live virus. For that reason, virulence of CMV, or its capacity to generate disease or death in the host, will be evaluated at the whole virus level. Virulence can be extrapolated from a plaque-forming-unit (PFU) assay accomplished in fibroblast cells. The timing from infection of fibroblasts, generally at 30 minutes post infection [27], to their ultimate demise (time to cell death) along with the number of PFU would demonstrate the capacity of the virus to generate disease. For example, a 5.0 $x10^5$ pfu/mL would be less virulent than 7.0 $x10^6$ pfu/mL and would therefore cause less disease.

The primary aim of this study was to determine the history and prevalence of CMV reactivation during spaceflight, as well as determine the effect of simulated microgravity (SMG) on CMV infection and replication. The overarching hypothesis was that space flight would promote latent herpes viral reactivation, and that SMG would enhance CMV infection and replication in Kasumi-3 cells, as well as increase CMV's virulence (speed and capacity to cause disease).

Literature Review

Discovering Cytomegalovirus

The earliest observations of CMV occurred in 1881 by Hugo Ribbert, a German pathologist, who noticed enlarged cells with enlarged nuclei in renal-epithelium tissue samples taken from stillborn fetuses. In 1921, pathologists Goodpasture and Talbot, having observed the same morphological changes in a variety of postmortem epithelial cells, coined the term “cytomegalia” [28]. This term is derived from the Greek *cyto*- meaning cell, and *megalo*- meaning large. In both observations, the enlarged epithelial cells are due to the characteristic inclusion cysts, or owl’s eyes inclusion cysts, which are indicative of latent CMV infection [29] . The modern age of CMV discussion and determined research was ushered in during the late 1960’s and early 1970’s with the first allografts of human organs. Accompanying these transplant procedures was the administration of immunosuppressive drugs which resulted in devastating multisystem CMV disease [28,30,31]. Forty years later, even with all the advances in medicine, both procedurally and therapeutically, CMV remains a leading cause of morbidity and mortality in these immunosuppressed transplant populations [32,33].

Cytomegalovirus classification and species tropism

CMV is a β-member of the herpesvirus family and, as such, has linear double-stranded DNA packaged in an icosahedral capsid. The capsid is surrounded by a tegument protein layer which is encapsulated by a glycoprotein lipid bilayer. The lipid bilayer is coated with more than 25 glycoproteins among which are the six vitally important ones: gB, gH, gL, gM, gN, and gO. Glycoproteins B, H, and L are conserved across all herpes viruses, but only CMV has glycoprotein O which forms a trimerized structure with glycoproteins H and L [34]. The glycoprotein complex gH/gL/(gO)-UL128-131 plays an integral role in viral entry into target cells, especially myeloid progenitors and monocytes, through interactions with the target cell epidermal-growth-factor receptors (EGFR) and β1-β3 integrins [35].

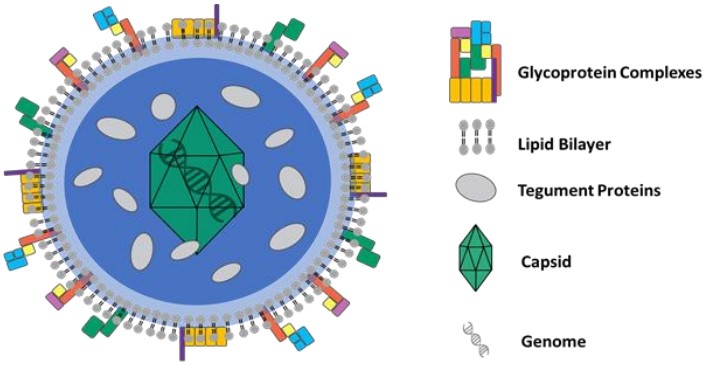

Figure 1.1 Cartoon schematic of the CMV virion. The CMV virion consists of a ~235kb double-stranded DNA genome contained within an icosahedral protein capsid. The tegument layer (blue region) consists of various phosphoproteins and lies between the capsid and the outer membrane. The lipid bilayer surface membrane contains multiple glycoprotein complexes.

There are three major criteria for the categorization of CMV as a β-herpesvirimae subfamily member. The first is CMV's large genome. It has the largest genome in comparison to its β-herpesvirimae counterparts at 235 kbp. The genome contains several open reading frames (ORFs) that include both unique long (UL) and

unique short (US) regions. Major portions of the genetic material are dedicated to immune evasion and modulation rather than merely replication [36]. Secondly, it has an extensive replication cycle and remains significantly cell associated. Lastly, it does not normally cross species barriers. In fact, CMV is highly species restrictive only infecting humans (HCMV), non-human primates (chimpanzees (CCMV) and rhesus (RhCMV)) and rodents (mice (MCMV) and rats (RCMV)) [37]. This strict host specificity restricts the opportunities to study active HCMV infection and relegates analysis to either diagnostic patient samples or autopsy material.

Strains

CMV strains are characterized as either laboratory or clinical strains. The laboratory strains originate from clinical sources that were selected as candidates for vaccine therapy. Clinical isolates were serially passaged on fibroblasts to attenuate the viruses, however this resulted in each of the candidate isolates altering their biological and genomic properties. They lost their capacity for broader tropism, meaning they could no longer efficiently enter and replicate within a variety of cell types including, but not limited to, epithelial cells, endothelial cells, smooth muscle cells and macrophages. Mutations in the ORF that encodes elements of the glycoprotein complex gH/gL-UL128-UL131 were the cause of this cellular entry barrier. However, these isolates which grow more rapidly, release more free virus and are passaged to much higher yields in fibroblasts, could now be studied more extensively than their parent viruses. Subsequently, these strains, AD169 and Towne, have been designated laboratory strains [37,38].

There are six known clinical strains of CMV, all of which retain their capability for broad cellular tropism. FIX, TR, PH, Toledo, and TB40/E were passaged to a limited extent on fibroblasts before they were cloned as bacterial artificial chromosomes (BAC's) and then sequenced. BAC's are beneficial because they allow for phenotype homogeneity making all genetic analyses comparable. The Merlin strain remains uncloned but has been sequenced. FIX [39], a derivative of VR1814 [40], was isolated from a pregnant female with primary CMV infection. TR is a ganciclovir-resistant isolate from the retina of an AIDS patient with CMV induced retinitis [41]. PH was isolated from a bone marrow transplant patient [42], and Toledo [43] and Merlin [44] were isolated from the urine of a congenitally infected child. Lastly, we have TB40/E which was isolated by bronchoaveolar lavage of an allogeneic bone marrow transplant patient with CMV [45]. The E simply indicates that this strain is primarily passaged in endothelial cells. This strain, like the others, retains its ability to infect a broad range of cells, but it has proven especially effective in infection of hematopoietic stem cells and myeloid progenitor cells [17].

Cellular Tropism and hematogenous dissemination of CMV

The pathogenesis of CMV is highly dependent upon the range of target cells it can infect, or its broad cellular tropism. In fact, CMV can infect almost all cells of the body with only a few exceptions. Lymphocytes and granulocytes are among the cells that do not support infection or replication, however they might help distribute the virus because they pick up viral particles and express immediate early (IE) antigens [37]. Systemic dissemination of CMV to target organs is predominantly due to CMV's preferential infection of hematopoietic stem cells, myeloid progenitors and monocytes

[16,35,46]. Consequently during acute infection, CMV replication can be detected in almost every organ of the body such as liver, gastrointestinal tract, lungs, retina and the brain [37].

Cytomegalovirus transmission, infection and disease

CMV is generally acquired during childhood and persists lifelong as a latent infection, all the while, remaining asymptomatic in immunocompetent individuals because of constitutive immune surveillance and control. However, CMV does replicate at subclinical levels, or periodically reactivate from latency, during ordinary physiological processes such as differentiation of monocytes into macrophages or lactation [47–50]. These events are well controlled by the immune system but likely facilitate transmission of the virus. It is transmitted via body fluids and has a worldwide prevalence ranging between 75-90%. Primary infection, especially in the immunocompetent, may result in mild symptoms similar to mononucleosis infection which is accompanied by a low-level viremia [17]. CMV related disease, however, is predominantly due to viral reactivation. Uncontrolled or unrestricted CMV replication is directly responsible for CMV viral syndrome and invasive end-organ diseases such as nephritis, hepatitis, myocarditis, pancreatitis, colitis, retinitis and encephalitis [14,29]. Indirectly, CMV seropositivity has been linked to a number of metabolic disorders that contribute to metabolic syndrome and cardiovascular disease [51–53]. It is also positively associated with cognitive and behavioral disorders including dementia and Alzheimer's [54,55], chronic autoimmune and inflammatory conditions, HIV pathogenicity [56], and finally early immune senescence [57,58].

CMV treatments

There is no treatment for latent CMV, but active CMV can be treated with antivirals with fairly positive results. The antivirals Ganciclovir and Valganciclovir seem to be the most effective against the virus, though neither of them “cure” CMV and their use may result in bone marrow suppression, neutropenia and other organ toxicities. Additionally, because of myelosuppression resulting in leukopenias, there is an increased risk for fungal and/or bacterial infections [59–61]. Upon completion of antiviral treatment, there is a risk for reoccurrence which may be worse than the original reactivation because antiviral drugs often delay virus-specific immune recovery [62]. As a result, patients with continued viral complications may require multiple treatments, which is costly and facilitates drug resistance. Approximately 14.5% of CMV strains are already resistant to antiviral therapies [63].

Other treatment options could include CMV hyper-immune globulin (Cytogam) and viral specific T-cell immunotherapy. Cytogam is used to treat mothers with active CMV infection in order to try to prevent congenital infection of the fetus. T-cell immunotherapy has been used in clinical populations, mostly immune-ablated transplant patients, with reactivated CMV disease. It is a relatively new treatment option but it has shown impressive efficacy in clinical trials [64,65].

Universal vaccinations against CMV are actively being researched and pursued, but as of yet none have made it past clinical trial phases or have proven universally efficacious. This is due in large part to the complications due to multiple strains of CMV and its inherent capacity to mutate [66,67].

Characteristics of lytic and latent infection and immune evasion

CMV has developed intricate strategies for latency and immune evasion over millennia of co-evolution with its host. Ultimately, these strategies ensure preservation of the viral genome, as well as, promote the life-long persistence of the virus. Though many of the host-virus interactions governing the initiation and maintenance of latency, or even the cues that stimulate reaction from latency, have not been fully delineated, it is clear that many factors are involved. Unraveling, or separating out these factors, has been likened to the impossible untying of the Gordian Knot [50]. This complicated orchestration of simultaneous events manipulates host cell physiology and signaling, viral epigenetic remodeling, transcription of viral miRNA, and the innate and adaptive host immune responses.

It begins with the infection of cell types permissive to infection and to the CMV life cycle and is furthered by viral promotion of host cell survival. For example, CMV infection of monocytes promotes their extended survival through the manipulation of Bcl-2 family proteins [35]. Additionally, a series of viral proteins affect changes in the host cell expression of surface receptors. Viral protein UL138 (pUL138), encoded by a gene from the unique long (UL) ORF UL138, is a constitutively active protein which promotes the preservation of the epidermal-growth-factor receptor (EGFR) at the host cell surface. Viral protein UL135, by contrast, targets the EGFR for internalization and degradation by overcoming the action of UL138. Preservation of the EGFR at the cell surface promotes CMV latency, while internalization of the EGFR supports reactivation [68]. EGFR and its downstream signaling pathways are important homeostatic regulators of cell survival, differentiation and proliferation. It has been

suggested that high levels of EGFR activity might impede post entry mechanisms of viral replication, and by extension support viral latency [69,70]. Viral protein US28 is a G-coupled cell surface protein expressed during lytic and latent CMV phases. It binds host chemokines, causing their internalization, and thereby alters the local concentrations of inflammatory and chemotactic cytokines [71].

CMV also affects changes in the host cell secretome. The CMV protein UL111A produces a latency-associated splice product that encodes a viral (v)IL-10 homologue. IL-10, both viral and cellular, is a powerful inhibitor of Th1 cytokines such as IFN-γ and IL-2, as well as inflammatory cytokines from monocytes and macrophages. vIL-10 secretion results in the downregulation of MHC Class II cell surface expression. This reduction in MHC Class II expression interferes with CD4+ T-cell recognition of infected cells [72]. Likewise, there are a number of lytic CMV genes that interfere with both MHC Class I and II restricted antigen processing and presentation, ultimately resulting in dramatic reductions in CD4+ and CD8+ T-cell recognition [73,74], as well as costimulatory T-cell signaling [75]. Latent infection of CD34+ cells yields high concentrations of immunosuppressive cytokines such as cellular (c)IL-10 and TGF-β. These cytokines inhibit Th1-antiviral CD4+ cytokine and cytotoxicity cell effector functions. Additionally, the neighboring, uninfected, CD34+ cells and antigen-specific CD4+ regulatory T-cells are induced to secrete their own cIL-10 and TGF-β, further enhancing the immunosuppressive microenvironment [71,76]. All of which protects or prevents clearance of latently infected cells.

CMV expresses a substantial number of proteins targeted towards various NK-cell recognition pathways. In this way, CMV has tremendous potential for moderating

NK-cell recognition and response to infected cells. Some of these proteins prevent infected cells from expressing cell surface ligands such as MIC A/B and the ULBP (UL16 binding protein) family that would otherwise engage the NK-cell activating receptor NKG2D [77]. In other cases, the virus promotes the expression of ligands such as the UL18 molecule that binds the inhibitory LIR-1 (leukocyte immunoglobulin-like receptor) cell surface receptor [78]. Perhaps most importantly, there is a viral peptide processed by CMV viral protein UL40 that promotes HLA-E (HLA class I histocompatibility antigen, alpha chain E) cell surface expression. HLA-E binds the NK-cell heterodimeric receptor CD94/NKG2A/B/C producing an inhibitory effect on the cytotoxic activity of the NK cell. However, binding of HLA-E to CD94/NKG2C can also result in NK cell activation, which may trigger expansion of NK cell subsets during antiviral responses [79].

CMV Reactivation: terrestrial models, mechanisms and cues

CMV reactivation is the result of epigenetic changes in its chromatin structure, by means of histone acetylation, that open up the major-immediate-enhancer/promoter (MIEP) regions of the CMV DNA. This allows for the expression of immediate early (IE) genes and IE protein synthesis [80]. The expression of these viral proteins is a remarkably controlled cascade of events divided into three temporal classes: immediate-early, early (delayed-early or early-late) and late. Each class of proteins regulates specific aspects of the infectious cycle.

The prevailing terrestrial models explaining the causes of CMV reactivation include immunosuppression, cellular differentiation, inflammation and stress. Immunosuppression, generally chemically or radiologically induced, results in high

levels of cell death and the subsequent release of pro-inflammatory cytokines. These cytokines are likely to play more of a role in reactivation than is immunosuppression itself. Cellular differentiation like the transition of monocytes-to-macrophages, for example, may also be a localized contributor to CMV reactivation but would not explain a full body or systemic reactivation event. However, inflammation [81–83], and stress [4,5,84], significantly influence systemic CMV reactivation.

The specific cues for inflammation-induced reactivation have not been fully elucidated but significant evidence exists to suggest that pro-inflammatory cytokines interleukin-1 (IL-1) and tumor-necrosis-factor-α (TNF-α) play key roles. Both IL-1 and TNF-α facilitate the translocation of active NFκB subunits, important for CMV reactivation, to the nucleus through phosphorylation and ubiquitin-mediated degradation of IκB [81]. However, TNF-α is thought to be the greater contributor to this process [26]. Additionally, TNF-α induces the activation of activator protein-1 (AP-1). NFκB and AP-1 transcription factors have been shown to be important regulators of CMV MIEP activity, as evidenced by the multiple independent, as well as tandem binding sites within the MIEP [80,85].

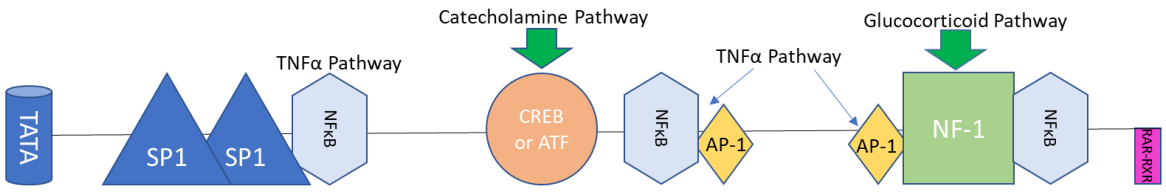

Figure 1.2 Truncated cartoon depiction of Human Cytomegalovirus MIEP with stress and inflammatory response elements

The sympathetic system response to physical and/or perceived (psychological) stress results in the secretion of both glucocorticoids and

catecholamines. It has been shown that each of these hormone classes has its own response element at the CMV MIEP, and therefore can themselves be responsible for CMV reactivation. Cortisol, a glucocorticoid steroid, initiates reactivation via the NF-1 element on the MIEP [86], while the catecholamine epinephrine acts through β_2-adrenergic receptors and the PKA/cAMP cell-signaling pathway stimulating the cAMP response element on the CMV MIEP [84].

Activation of the CMV MIEP, regardless of the cell-signaling pathway, would first result in the lytic or active expression of IE genes: IE1 (UL123) and IE2 (UL122). These genes serve to influence the cellular microenvironment making it more conducive to viral replication, as well as to transactivate other early genes that are all expressed independently of viral DNA synthesis. These events would then be followed by the expression of early and late genes, whose expression is characterized by their increasing independence on viral genome synthesis [50].

CMV Reactivation: Space Flight

CMV reactivation has occurred in more than half of the astronauts deployed to space over the last 2.5 decades, regardless of their mission duration. In fact, 47% of shuttle crew and 61% of ISS crew, to date, have been positive for CMV reactivation [87]. Reactivation can occur as early as ten days before launch and is indicated by the shedding of CMV DNA in astronaut urine samples, and by increases in their plasma anti-CMV IgG antibody titers. Though both of these can also be observed in ground analog studies concerning stress [5], and perhaps in response to cortisol, the magnitude of the space response is unique. The anti-CMV IgG titers in space can rise up to eight fold beyond their negative baseline values [88], potentially implicating

a space-specific factor such as microgravity. Additionally, the viral load and titers steadily increase with more time spent in space. Moreover, though CMV reactivation can occur in isolation, it happens more often in concert with other herpes viruses. This further exacerbates the risks to crewmembers regarding adverse health effects.

Collective herpes reactivation in space

Only four of the eight herpes viruses that parasitize humans have been shown to reactivate in astronauts during shuttle, Russian Soyuz and International Space Station missions [1,3,89,90]. Other than CMV, the viruses that reactivate are Epstein-Barr (EBV), Varicella Zoster (VZV), and Herpes Simplex-1 (HSV-1). EBV preferentially infects B-lymphocytes, is responsible for infectious mononucleosis, and is associated with several malignancies [91–93]. EBV reactivates in 90% of astronauts. VZV infects neurons in ganglia and is responsible for both chicken pox (primary infection) and shingles (secondary infection). It reactivates in 60% of astronauts from combined shuttle and ISS missions. Most VZV reactivations are subclinical, though there have been 4-5 clinical cases of rash associated with its reactivation. HSV-1 also infects neurons in ganglia, and is generally referred to as oral herpes, though lesions/rash may appear anywhere on the body. HSV-1 reactivation statistics are very low with only 8% of astronaut saliva samples testing positive for viral DNA. Recently, however, an astronaut with persistent dermatitis during a long duration spaceflight, >180 day, had positive HSV-1 saliva and lesion samples. The impaired control of all of these latent viruses is symptomatic of space-induced dysregulation of the immune system, and microgravity/stress induced

decreases in cell-mediated immunity. Again, whether latent viruses themselves are also responding to microgravity remains unanswered.

Impaired Immune Function in space

Spaceflight induced immunological problems have been evident since the first Apollo missions, where more than half of the astronauts suffered from bacterial or viral infections [94–96]. The literature, subsequently amassed over the last four decades of spaceflight investigations, has clearly shown that spaceflight negatively affects the immune system [97–99]. Among the changes are altered distributions of circulating leukocytes, altered production of cytokines, decreased number and activity of lymphocytes and monocytes, increased numbers of granulocytes with diminished function, and altered levels of circulating immunoglobulins [100]. Additionally, significant reductions in the stimulatory responses and effector functions of T-cells has been highlighted along with significantly altered neuroendocrine responses.

Astronaut stressors in space

Astronauts endure both physical and psychological stressors in the execution of their extra-terrestrial duties. The physical stressors include acceleration to escape velocities reaching 7 miles per second (25,000 miles per hour), exposure to both cosmic radiation and microgravity, acceleration due to gravity upon atmospheric reentry, and deceleration upon landing. Psychologically they are confronted with social separation from friends and family, confinement, isolation at 254 miles above the Earth’s surface, sleep deprivation, circadian rhythm disruption and anxiety. Altogether, these stressors have been shown to correlate and/or contribute to the

dysregulation of the immune and endocrine systems in space [101,102]. The dysregulation of these systems is predominantly due to the consistently elevated concentrations of salivary, plasma and urinary stress hormones that commonly accompany spaceflight [103].

Catecholamine, glucocorticoid and cytokine changes in space

Neuroendocrine mediation of the stress response via the hypothalamus-pituitary-adrenal (HPA) and sympathetic-adrenal-medullary (SAM) axes results in the secretion of catecholamines and glucocorticoids [104–106]. Acute (transient) responses to stress are positive while chronic (long lasting) responses, where circulating concentrations of hormones remain high, are harmful to the immune system and its individual components [9]. The phenotypical and functional changes that occur in immune cells as a result of chronic catecholamine/glucocorticoid exposure culminate in decreased cell-mediated immunity [13,107].

Catecholamines, epinephrine and norepinephrine, are secreted by the adrenal medulla in response to stress. Their circulating concentrations in astronauts are subject to considerable variation dependent upon gender, mission tasks such as extra-vehicular activities (EVA), and the exposure and/or coping history of individual astronauts [108]. Epinephrine changes very little from baseline, for both men and women, during preflight and flight time points. However, it increases 2-3 fold around the time of landing [103,109]. Norepinephrine secretion patterns seem to exhibit gender dependent differences, where levels of norepinephrine do not change for females but are substantially decreased in males [110].

Glucocorticoids, such as cortisol and dehydroepiandrosterone (DHEA), on the other hand, remain consistently altered through all phases of flight. Both cortisol and DHEA are steroid hormones released by the adrenal cortex in response to stress. Cortisol is anti-inflammatory and immunosuppressive, but DHEA antagonizes the action of cortisol. This relationship, often represented as a molar ratio [C]/[D], can be an important indicator of immune regulation [111]. Salivary cortisol circulates in significantly higher concentrations before and during flight, while DHEA waking concentrations are significantly lower during flight in comparison to samples taken before and after flight. Altogether, diurnal patterns of salivary cortisol are significantly higher during flight while DHEA is significantly lower. Consequently, the [C]/[D] molar ratio is increased during spaceflight indicating potential immune challenge and exerting negative influences on the intrinsic cytokine profile. Glucocorticoids inhibit IL-12 production and increase IL-10 production by monocytes driving the cytokine profile towards TH2 (IL-4, IL-5 and IL-10) and away from TH1 (Il-2, IL-12 and lFγ). These changes in the inflammatory cytokine response and the TH2 shift have been observed in many spaceflight studies [9,112].

Cytokines, small cell-signaling proteins, are largely responsible for directing the human immune response. They modulate both pro- and anti-inflammatory immune states, and are broadly categorized as either inflammatory cytokines (IL-1α, IL-1β, TNFα, lL-6, IL-8), lymphoid growth factors (IL-2, IL-7, IL-15), Th1/17 cytokines (IFNγ, lL-12, IL-17), Th2 cytokines (IL-4, IL-5, IL-10, IL-13), myeloid growth factors (G-CSF, GM-CSF), and chemokines (eotaxin, MCP-1, M1P1α, lP-10). Many pro-inflammatory plasma cytokines are significantly increased from baseline a week

before launch including IL-1α, IL-6, IL-8, IFNγ, IL-4, eotaxin, and IP-10 [8,9,12]. These same cytokines are also significantly elevated in samples taken immediately upon return to Earth (R+0). IL-4 is the most responsive Th2 cytokine with 35-fold and 21-fold increases from baseline values at L-10 and R+0, respectively.

Astronauts who shed virus exhibit significantly elevated levels of cytokines including, but not limited to, IL-1α, IL-6, IL-8, IFNγ, IL-4, and IL-10, with the largest plasma cytokine increases in IL-4 (21-fold increase) and IL- 6 (33-fold increase) [112]. This accentuates a dynamic shift away from the antiviral Th1 immune state towards an antibacterial/antifungal Th2 immune state. The ratio of IFNγ: IL-4 is another mechanism for determining the Th1-Th2 balance. This ratio decreases significantly for shedders compared to astronauts who did not shed any viruses during their space flights.

Impaired control of latent viruses: lymphocyte function

As referred to before, in order for the body to maintain control of viral latency, the immune system must be both stalwart and sentinel. That means that the agents of its primary and secondary defenses must be both primed and proficient, this includes cytotoxic T-cells and NK-cells. Any changes in immune status, lending to the decreased effectiveness of these cells, tend to promote viral reactivation. This is evident in both terrestrial space-analog studies [8,113,114] and spaceflight studies [1,3,6,115]. The alterations in immune status for terrestrial analog studies are relatively mild, though not trivial, and coincide with low-level viral reactivation. Spaceflight studies illustrate significant immune dysregulation accompanied by functional changes that result in large-scale viral reactivation. Substantial changes in

cell-mediated immunity exist for most the astronauts who reactivated a single or multiple herpes viruses [3,11].

Altered function of viral specific T-cells and NK-cells in space

Cytokine profile changes, acting either independently or in conjunction with microgravity, critically alter the fate of many important leukocyte populations. Eventually this results in significant changes to the numbers, proportions, and functions of leukocytes. Monocyte [116], granulocyte [117] and lymphocyte functions [11,13] are diminished, consequently resulting in a reduction in their effectiveness against pathogens. This includes their capacity to prevent and/or control viral reactivation and expansion. T-cells and NK-cells are lymphocytes designed to seek out and destroy viruses and virally infected cells. However, their capacity to do that declines substantially during spaceflight, as evidenced by flight studies concentrating on the phenotypes and effector functions of these lymphocyte subpopulations. CD4+ and CD8+ T-cells taken from astronauts in space respond ineffectually against common stimuli. Under normal circumstances, as evidenced by responses in control subjects, these same stimuli elicit profound responses by the T-cells. This T-cell malaise can last the entirety of the flight phase [11]. NK-cells sampled during spaceflight exhibit decrements in cytotoxicity against leukemic cell lines due to decreased production of the enzymes perforin and granzyme B [13]. These enzymes are the key armaments for NK-cells and their absence render the NK-cell ineffective against target cells/pathogens. This impairment may last up to 60 days post flight. In both cases, reductions in T-cell and NK-cell function handicap the capacity of the immune system to control and/or conquer opportunistic viral reactivation.

Microgravity induced changes in anatomy and physiology

Microgravity exerts unique and profound influences on the anatomy and physiology of human beings in space. It is directly responsible for morphological changes of major organs such as the eye, the brain and the heart. Flattening of the globe of the eye, changes to the retina, optic disc and optic nerve are all due to the microgravity environment and affect the visual acuity of astronauts [118]. In the absence of downward gravitational force, the brain shifts upwards, but more critically there is narrowing of the central sulcus and cerebral spinal fluid spaces [119]. Cardiovascular changes that occur in microgravity include the heart becoming 9.4% more spherical due to the elimination of the normal cephalic-caudal hydrostatic gradient. The loss of this gradient lessens the demand on arterial blood pressure regulatory mechanisms culminating in a reduced workload for the heart [120]. Though many of these changes are temporary and resolve quickly upon return to normal gravity, deficits in visual acuity resulting from the microgravity, can last up to 5 years post flight [121,122].

The absence of gravitational loading on muscles and bones precipitates detrimental structural changes in both. Muscles lose mass and strength during space flight. Individual muscle fibers begin to shrink almost immediately upon exposure to microgravity resulting in 20-30% reductions in overall mass. Intuitively, the reduction in mass is accompanied by reductions in strength, sometimes up to 50% [123,124]. Exercise as a countermeasure has helped attenuate the deleterious effects of microgravity, but it does not fully prevent muscle and bone loss [100]. Gravitational unloading on the bone induces bone density loss at a rate of 1-2% a month. Bone

demineralization begins immediately upon exposure to microgravity with early mission urine samples revealing 60-70% increases in calcium content, in addition to increases in bone resorption markers [125,126].

Microgravity induced changes affecting immunity/immune control

The internal anatomy of the bone also changes with microgravity or mechanical unloading whereby the gross composition of the bone marrow irrevocably converts from red (hemopoietic) to yellow (fatty). Bone marrow, which is contained within a protective calcified cortex, is a primary hematopoietic organ shown to be important in the active function and trafficking of immune cells including regulatory T-cells, T-cells, B-Cells, dendritic cells and NK-T-cells [127]. The marrow conversion adversely affects the populations and functions of cells contained within it, and this could negatively affect the control, or fine tuning, of immunity [127].

The marrow houses mesenchymal and hematopoietic stem cells (HSC). The mesenchymal stem cells can differentiate into osteoprogenitors, which regulate the balance between bone anabolism and catabolism. However in response to mechanical unloading, these primitive cells lose their commitment to osteogenic lineage and commit instead to adipogenic lineage with devastating effects on bone density and HSC signaling and function [128–130]. Further, in response to microgravity, fully differentiated bone forming osteoblasts fail to proliferate, they exhibit blunted metabolism and they increase secretion of chemical factors enhancing osteoclast recruitment [131]. HSC, which differentiate into cells of both lymphoid and myeloid origins, generally position and home themselves in close proximity to cells of mesenchymal origin. This highlights the intrinsic regulatory signaling between these

cells types, which is hugely important for the healthy generation and function of many immune cells. In fact, osteoblasts have been shown to support HSC activity in vivo [132], and they are important mediators of B-Cell differentiation [133]. B-Cells are key mediating components of the humoral immune response, and though data from rodents flown in space has illustrated significant reductions in mature B-Cells [134], a recent study of human crew members has shown no effect of spaceflight on numbers and proportions of B-Cell subsets [135], which may be a positive result of current exercise countermeasures.

Microgravity induced changes in immune cells

Cells of the immune system are exceptionally sensitive to microgravity, in that their effector function is highly dependent upon well-defined spatial orientation and contact and/or exchange with other cells. T-cells, for example, require a distinct alignment of membrane and adhesion molecules ICAM1 and LFA-1 when consorting with antigen-presenting cells (APC) [136]. Incidentally, long-term spaceflight alters the expression of ICAM-1 [137], potentially disrupting the interaction between T-cells and APC. Microgravity induced spatial ambiguity, therefore, probably influences the immunological synapse, an interface not only between APC and T-cells, but also between target cells and NK-cells.

Many of the decisions cells make regarding their proliferation, migration, commitment, matrix synthesis and maintenance is dependent upon omnipresent mechanical forces. In the absence of these forces, divergences from the normal patterns of all these cellular functions has been illustrated for various immune cell types after spaceflight. Spacelab investigations showed that human lymphocytes

failed to proliferate after several days in microgravity [138]. Additionally, general alterations in human-mononuclear-leukocyte populations and their weakened responses to mitogen stimulation have been noted during spaceflight studies [139]. Microgravity induced disruptions in specific cell-signaling pathways and cytoskeletal development have been discovered for lymphocytes [140,141] and monocytes [116,142,143]. All of which significantly impede the effective response of the immune system to endogenous or exogenous pathogens.

Pathogen responses to microgravity

Bacterial strains, including *Salmonella enterica* and *Escherichia coli*, benefit from microgravity [20,144] . Spaceflight also seems to promote higher genetic mutation accumulation resulting in increased antibiotic resistances [145,146]. In fact, in a study designed to characterize the total and viable bacterial and fungal communities aboard the ISS [147], multiple drug resistant bacteria have been discovered. Additionally, two fungal isolates of *Fusarium oxysporum* were detected which are genomically different from any of the 65 known terrestrial strains [148]. This fungus can cause infections in humans ranging from superficial dermatological issues in immunocompetent individuals, to invasive and systemic blood and deep tissue involvement in immunocompromised patients [149,150]. Further, many of the organisms discovered aboard the ISS are known to form bacterial and fungal biofilms, which also promote antibiotic resistance [151]. If astronauts become infected with these biofilm forming organisms, this could pose significant problems, especially with an already challenged immune system. Further, significant proportions of the bacterial and fungal populations represented opportunistic pathogens such as *S.*

aureus, Staphylococcus hominis, Staphylococcus haemolyticus, P. conspicua, Acinetobacter pittii, Klebsiella quasipneumoniae, and *A. fumigatus*, further exacerbating risks to the astronauts [147].

Viral pathogen dynamics in the spaceflight environment are largely unknown. We do know that viruses reactivate at increased rates and with greater magnitude during spaceflight. We also know that viral shedding in saliva is infectious as evaluated by culturing saliva samples with fibroblast cells [152]. What remains to be elucidated is whether the virus itself responds to changes in gravitational load, and whether viral reactivation during spaceflight is a communicable risk to seronegative astronauts. For instance, the Flu virus can be disseminated via direct aerosolized body fluid and/or via fomite transmission from objects in communal spaces. A current study regarding the microbial tracking of viruses aboard ISS seeks to answer whether herpes viruses are found outside of the human body. So far, just EBV, VZV and HSV-1 have been detected. EBV was found in a navel body swab sample, and VZV was found in a body swab and several surface samples, while HSV-1 has been detected in a surface sample [153]. Clearly, a communicable risk to seronegative crew does exist, though no study to date has been able to quantify infectious risk or virulence changes of viruses in space.

Aims and Hypotheses

Specific Aims: The overarching aim of this study was to determine the history and prevalence of latent CMV infection during spaceflight and then to investigate the effect of simulated microgravity (SMG) on CMV infection and replication. The overarching hypothesis was that spaceflight would promote significant latent herpes virus reactivation, and that SMG would enhance CMV infection and replication in Kasumi-3 cells, as well as increase CMV's virulence.

Aim 1: Review the scientific literature to determine the history and frequency of latent herpes viral reactivation during spaceflight, with specific attention paid to CMV.

Hypothesis: Spaceflight promotes latent herpes reactivation in large numbers of space flight crewmembers.

Questions:

1. What is the history of latent herpes viral reactivation during spaceflight missions of variable duration?
2. What is the frequency of latent herpes viral reactivation among otherwise healthy spaceflight crewmembers?
3. What are the spaceflight related factors that contribute to, or are associated with, latent herpes viral reactivation?

Aim 2: Investigate the effects of SMG, using RWV technology, on the capacity of CMV to infect Kasumi-3 myeloid progenitor cells (cell association and IE-

1/IE-2 expression), as well as evaluate SMG influence on CMV's replication kinetics (changes in viral load-viral DNA).

Hypothesis: SMG will enhance *in vitro* CMV infection (binding to and entry) of Kasumi-3 myeloid progenitor cells, as well as increase replication (viral load) frequency.

Questions:

1. Are there differences in viral load between infected cells cultured in SMG versus 1G culture?
2. Is there an effect of SMG timing on CMV viral load in Kasumi-3 Cells?
3. Are there differences in the onset or concentrations of IE-1, IE-2, US28 and UL138 transcription between SMG culture and traditional 1G culture?

Aim 3: At the whole virus level, investigate the effects of SMG on CMV's virulence (capacity to generate disease) and continued capacity to infect (bind to and enter) fibroblasts.

Hypothesis: SMG will increase CMV virulence resulting in an increased PFU after SMG culture, as well as promote retention of its capacity to infect fibroblasts.

Questions:

1. Will the viral titer (PFU) change with exposure to SMG versus control cultures maintained in 1G?
2. Will exposure to SMG alter the infection timing and capacity of CMV to infect fibroblasts?

Chapter 2

Materials and Methods

Aim 1: Review the scientific literature to determine the history and prevalence of latent herpes viral reactivation during spaceflight, with specific attention paid to CMV.

We performed a systematic review of publications related to herpes virus reactivation during spaceflight and the factors that might influence this phenomenon, using “Virus reactivation in space” search parameters in PubMed.

Aim 2: Investigated the effects of SMG, using RWV technology, on the capacity of CMV to infect Kasumi-3 myeloid progenitor cells (IE-1 expression/IE-2 expression), as well as evaluated any SMG influences on CMV’s replication kinetics (changes in viral load).

Viral propagation and harvest

Using a single vial of a low fibroblast passaged *BAC*-derived TB40/E strain of CMV (7.8×10^7 pfu/mL) obtained from Dr. Ritesh Tandon’s lab, we passaged and propagated our own viral stock by infecting MRC5 (ATCC CCL- 171) human embryonic lung fibroblast cells maintained in 37°C/95% humidity/5% CO_2 incubators. We infected the MRC5 cells at a multiplicity of infection (MOI) of 2 calculated by MOI (x) = [(x) x number of cells]/pfu/mL. MRC5 cells were incubated 1-2 hours in 37°C/95% humidity/5% CO_2 incubators and then harvested and centrifuged at 14,000 rpm for 60 minutes. 1mL of media was removed and replaced with autoclaved milk.

Each aliquot was sonicated on ice at a power of 2 for 10 seconds, then 1mL aliquots was accomplished and frozen at -80°C.

Determination of viral infectivity (PFU) & validation of MRC5 infection protocol

The “virus concentration or infectious dose” was determined by accomplishing a plaque-forming unit (PFU) assay using whole virus aliquots. Serially diluted CMV virus was added atop MRC5 cells cultured in 6 well plates. Infection culture was agitated and incubated for 30 minutes in 37°C/95% humidity/5% CO_2 incubators. Infection media was removed and replaced with 1-2mL’s of fresh 37°C complete RPMI containing IgG at a concentration of 1:500. Culture media was removed, and then cells were washed with phosphate buffered saline (PBS) and then fixed with formalin for 15-20 minutes. The plates were washed/rinsed with PBS, and then stained with crystal violet for 30 minutes. The cells were washed a final time with PBS. The PFU and success of infection were determined by visualization of stained cells TB40/E or fluorescent cells GFP-Towne-*BAC*. PFU/mL= number of plaques/ (dilution factor $_x$ volume of diluted virus).

Validation of Kasumi-3 in SMG

Kasumi-3 cells (ATCC CRL-2725, Manassas, VA, USA) were brought up and maintained in RPMI 1640 (ATCC 30-2001, Manassas, VA, USA) supplemented with 20% fetal bovine serum (FBS) (ATCC 30-2020, Manassas, VA, USA) and 1.4mL of Penicillin-Streptomycin-Glutamine (25 U/mL penicillin, 25 µg/mL Streptomycin, and 2 mM L-Glutamine Gibco®, Carlsbad, CA, USA). Cultures were sustained in 37°C/95% humidity/5% CO_2 incubators. Cells were collected, combined and divided among 4

culture vessels, two standard T-75cm^2 canted neck flasks and two 50 mL rotating wall vessels (RWV) (Synthecon Inc, Houston, TX, USA) rotated at 10 rpm, so that the plating density/concentration will be 25e6 cells per vessel. Aliquots from each RWV or FLASK culture vessel were taken on days 1, 4, 8 and 12.

Cell Count, Size and Viability Assessment

Each experimental aliquot was assessed for cell count (concentration per mL), cell size (diameter) and percentage viability using Cellometer Auto 2000 (Nexcelom, Lawrence, MA, USA). In short, a homogenized sample was taken from each experimental condition; 20uL was removed and mixed with 20uL of arginine-orange/propidium-iodine (AOPI), this mixture was added to a Nexcelom counting chamber and then placed into the Nexcelom Cellometer.

Kasumi-3 Cell infection and validation of infection

For validation of our Kasumi-3 infection protocol, we infected the Kasumi-3 cells from the SMG validation study above; from both conditions, in 12 well plates, at a MOI of 1, via high-speed centrifugation and then plated and maintained them in T-75cm^2 FLASKS with a plating density of 25e6 cells per vessel. Non-infected Kasumi-3 cells were maintained for use as our negative control, also plated in the FLASK condition. We took aliquots from each FLASK on days 1, 4, 8, 12, and 16 to analyze cell count, cell size and cell viability, and to confirm infection. Infection of cells was confirmed by RT-PCR, confocal microscopy, direct single-color antibody staining using mouse anti-human mab810X-AlexaFluor-488 (IE-1) (EMD Millipore, Temecula,

CA, USA) via flow cytometry, and reverse transcriptional analysis of viral lytic protein UL123 (IE-1) and UL122 (IE-2).

Kasumi-3 1G Infection and then culture in SMG versus FLASK

We brought up fresh Kasumi-3 cells and maintained them in ATCC RPMI 1640 supplemented with 20% fetal bovine serum (FBS) and 1.4mL of Penicillin-Streptomycin-Glutamine. These cultures were sustained cultures in 37°C/95% humidity/5% CO_2 incubators. They were maintained until cell concentrations conducive to large-scale infection were accomplished. Then cells were then collected, combined and then further divided (25e6) among SMG and FLASK conditions, as well as the non-infected controls for each gravitational group. Cells fated for infection were infected in 50mL conical tubes at a MOI of 1 and then plated on 12 well plates. Non-infected cells were plated on separate 12 well plates, and then all plates will be centrifuged at high speed. Cells collected from the 12 well plates were then plated in their respective vessels for their designated condition with a plating density of 25e6 cells per vessel. Non-infected Kasumi-3 cells were maintained as our negative control in both SMG and FLASK conditions. SMG was accomplished in 50mL RWV rotating at 10 rpm. Aliquots were taken from each SMG or FLASK culture vessel on days 1, 4, 8 and 12. Cell count, concentration, size, and percentage viability for all aliquots were accomplished using Cellometer Auto 2000 (Nexcelom, Lawrence, MA, USA).

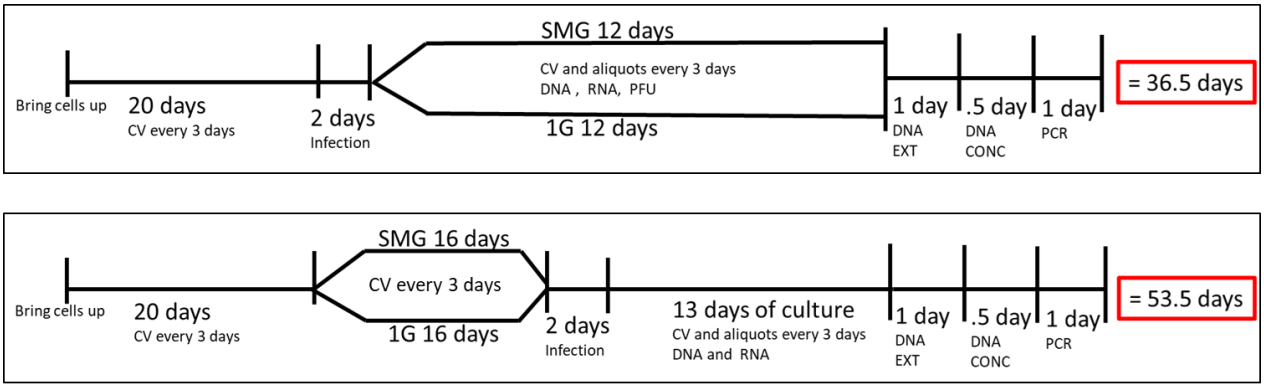

Figure 2.1. Kasumi-3 myeloid progenitor cell project timelines. Top panel outlines the experimental procedure followed for infecting the cells before separating them into gravitational conditions, either simulated microgravity (SMG) or normal gravity (1G). The bottom panel outlines the experimental procedure for cells pre-treated to SMG for 16 days before they are infected and maintained in 1G for 13 days.

DNA extraction

One million CMV-infected Kasumi-3 cells were separated from the supernatant by centrifugation at 14,000 rpm for 10 minutes. DNA was extracted from both cells and supernatant using an ArchivePure DNA Tissue kit (5 Prime, Gaithersburg, MD, USA-Qiagen). In short, contaminants, such as proteins, were removed by salt precipitation while the genomic DNA was recovered by precipitation with alcohol and dissolved in DNA hydration solution.

DNA Concentration for normalization

For each sample, viral copy numbers from duplicate readings were averaged, and then normalized by the DNA concentration to produce the main outcome measure of viral load: copies of DNA per nanogram of DNA. DNA concentration was determined using a Qubit 2.0 Fluorometer and Invitrogen™ Quant-iT™ Qubit™ dsDNA HS Assay Kits (Invitrogen, Carlsbad, CA). The Qubit Fluorometer converts

the fluorescent signal into DNA concentration (ng/μL) using DNA standards of known concentration.

DNA qPCR

Real-time Polymerase Chain Reaction (PCR)- CMV PCR assays were performed in 20-μL volumes containing 13.4μL 2× TaqMan Universal PCR Master Mix (Perkin–Elmer, Norwalk, CT) and 6.6μL of extracted DNA. Standard curves were generated with diluted viral DNA (1–10^5 copies) extracted from virus-infected cells. Each sample was analyzed in duplicate. Viral DNA was quantified by Real Time PCR (qPCR) using a TaqMan™ 7900HT Fast Real-time PCR System (Applied Biosystems, Grand Island, NY, USA). The fluorogenic probe and primers used, shown below, were synthesized from Integrated DNA Technologies ((IDT) Coralville, IA, USA). The viral primer and probe sequences are outlined below:

CMV	IE-EX4 Forward	TCC CGC TTA TCC TCR GGT ACA
	IE-EX4 Reverse	TGA GCC TTT CGA GGA SAT GAA
	IE-EX4 Probe	TCT CAT ACA TGC TCT GCA TAG TTA GCC CAA TAC A
	gB F	TGG GCG AGG ACA ACG AA
	gB R	TGA GGC TGG GAA GCT GAC AT
	gB Probe	TGG GCA ACC ACC GCA CTG AGG

RNA isolation and qPCR

RNA was isolated from 5e5-1e6 infected kasumi-3 cells after fresh culture samples were centrifuged at 14,000 rpm for 2 minutes, decanted and then resuspended in 350μL of lysis buffer. RNA was isolated from each sample using an AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany). RNA

concentration was measured using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using the miScript II RT Kit (Qiagen, Hilden, Germany), and conversion to cDNA was performed in the DNA Engine® Thermal Cycler (BioRad, Hercules, CA, USA). cDNA was diluted in 40µL RNase-free water. The PCR reagent consisted of 12.5µL 2xQuantiTect SYBR Green PCR Master Mix, 7.5µL RNase-free water, 2.5µL 10x Primer Assay from the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany), and 2.5 µL Template cDNA. PCR was performed in a CFX96TM Thermal Cycler (BioRad, Hercules, CA, USA).

The primer sequences for viral transcription analysis will be as follows:

UL123	Forward Primer	GCC TTC CCT AAG ACC ACC AAT
	Reverse Primer	ATT TTC TGG GCA TAA GCC ATA ATC
UL122	Forward Primer	ATG GTT TTG CAG GCT TTG ATG
	Reverse Primer	ACC TGC CCT TCA CGA TTC C
US28	Forward Primer	CCA GAA TCG TTG CGG TGT CTC AGT
	Reverse Primer	CGT GTC CAC AAA CAG CGT CAG GT
UL138	Forward Primer	GGT TCA TCG TCT TCG TCG TC
	Reverse Primer	CAC GGG TTT CAA CAG ATC G

Microscopy

GFP-Towne-*BAC* infected Kasumi-3 cells were removed from culture, fixed in 3% Ultra-pure formaldehyde+2mM EGTA, and then stained with the membrane stain wheat germ agglutinin (WGA) and the nuclear DAPI stain. Imaging was accomplished on LICA Confocal SP8 microscope. Wavelength stimulation for GFP, WGA and DAPI was at 488, 568 and 405, respectively. The images were collected in 50 nm Z-stacks

for optimal signal detection where pixel overlap indicated the true signal. The images were post-processed using point spread functioning of individual images, as well as deconvolution for clarity.

Aim 3: Investigated the effects of SMG on CMV's virulence, and capacity to infect fibroblasts at the whole virus level.

Assessing Kasumi-3 virulence between SMG and Flask cultures

We infected Kasumi-3 cells in the same way as accomplished in Aim1, 25e6 per culture vessel. We took sample aliquots at time points similar to those above with the inclusion of an infection day sample at Day 0. At the Day 4 time point, the time latency was confirmed, we harvested the cultures, pooling by SMG or Flask condition into 50 mL conical tubes. We then centrifuged each sample and then removed the excess media. We resuspended the cell pellets in the remaining volume, and then added 250uL of TPA to each conical tube for a final working concentration of 20nM. We incubated the cells in 37°C/5%CO_2 for 30 minutes, and then harvested the cells from their respective conical tubes and returned them to their original culture condition, SMG or Flask. We took subsequent sample aliquots at days 7, 10 and day 13. Changes in virulence were analyzed by reverse transcriptional analysis of UL123 and UL122, as well as by culturing the whole virus aliquots atop naïve (uninfected) fibroblasts (Dr. Ritesh Tandon Lab). At the same time, culturing atop naïve fibroblast cells illustrated how SMG altered the capacity to infect, or the timing of infection in these cells. Generally, CMV infects fibroblasts within 3 hours.

Power and Statistical Analysis

AIM2: An *a priori* power analysis was computed using the G*Power computing software for CMV infection and replication (expansion). AIM1 includes both longitudinal measures and two experimental conditions: SMG and 1G. The significance level was set at $\alpha = 0.05$ and the preliminary CMV data effect size was approximately 0.85. The power analysis projected that for an effect between cell culture conditions across time required a sample population of 14 (7 each experimental condition) to have 83.1% power. AIM2: Differences in viral load is response to the different gravitational conditions was evaluated using paired sample t-test analysis. To determine if the viral load was different between gravitational conditions, or with-in each condition across the different time points, a univariate ANOVA analysis was performed. Prior to analysis the data were screened to ensure all assumptions were met. Normality was confirmed using histograms and Fisher's skewness and Kurtosis coefficients, as well as descriptive statistics and normal Q-Q plots. Bonferroni corrected post-hoc comparisons were used to determine which days of culture were significantly different from one another. SPSS version 22 (IBM; Armonk, NY, USA) was used for statistical analyses with significance indicated as ($p<.05$).

AIM2 RNA differences within and between SMG and FLASK conditions was analyzed by mixed-effects REML regression using ΔΔCt values. Ct values for the target gene were subtracted from the averaged Ct of the control gene (GAPDH). The normalized value is the result of the ΔCt. The difference between the normalized

values in the control (FLASK) group and the experimental group (SMG) equals the ΔΔCt values.

AIM3: A two-tailed unpaired *t* test with Welch's correction (unequal variance assumption) was used for statistical analysis of differences between two samples at same time point. Sample comparisons were between two groups: SMG and 1G; with aliquots from these two conditions cultured atop fibroblast cells to assess differences in virulence across condition and time point, as well as after TPA stimulation of Kasumi-3 cell differentiation and CMV reactivation in SMG.

Chapter 3

Herpes virus reactivation in astronauts during spaceflight and its application on Earth

Co-authored by Brian Crucian, Duane Pierson, Mark Laudenslager and Satish Mehta

Abstract

Latent herpes virus reactivation has been demonstrated in astronauts during shuttle (10-16 days) and International Space Station (≥ 180 days) flights. Following reactivation, viruses are shed in the body fluids of astronauts. Typically, shedding of viral DNA is asymptomatic in astronauts regardless of mission duration; however, in some cases, live/infectious virus was recovered by tissue culture that was associated with atopic-dermatitis or skin lesions during and after spaceflight. Hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal-medullary (SAM) axes activation during spaceflight occurs as indicated by increased levels of stress hormones including cortisol, dehydroepiandrosterone, epinephrine, and norepinephrine. These changes, along with a decreased cell mediated immunity, contribute to the reactivation of latent herpes viruses in astronauts. Currently, 47/89 (53%) astronauts from shuttle-flights and 14/23 (61%) astronauts from ISS missions shed one or more herpes viruses in saliva/urine samples. Astronauts shed Epstein-Barr virus (EBV), varicella-zoster virus (VZV), and herpes-simplex-1 (HSV-1) in saliva and cytomegalovirus (CMV) in urine. Larger quantities and increased frequencies for these viruses were found during spaceflight as compared to before or after flight samples and their matched healthy controls. The shedding did not abate during the longer ISS missions, but rather increased in frequency and amplitude. These findings coincided with the immune system dysregulation observed in astronauts from shuttle and ISS missions. VZV shedding increased from 41% in space shuttle to 65% in ISS missions, EBV increased 82% to 96%, and CMV increased 47% to 61%. In addition, VZV/CMV shed ≤ 30 days after ISS in contrast to shuttle where VZV/CMV shed up to 5 and 3 days after flight respectively. Continued shedding of infectious-virus post flight may pose a potential risk for crew who may encounter newborn infants, sero-negative

adults or any immunocompromised individuals on Earth. Therefore, developing spaceflight countermeasures to prevent viral reactivation is essential. Our spaceflight-developed technologies for saliva collection/rapid viral detection have been extended to include clinical applications including zoster patients, chicken pox, postherpetic neuralgia (PHN), multiple sclerosis, and various neurological disorders. These protocols are employed in various clinics and hospitals including the CDC and Colombia University in New York, as well as overseas in Switzerland and Israel.

Introduction

1. Herpes virus

Herpes viruses have co-evolved with humans for millennia and subsequently employ sophisticated strategies to evade the host immune response. Consequently, after primary infection, they persist lifelong in a latent or dormant phase, and are generally asymptomatic in immunocompetent individuals. However, they may reactivate during periods of increased stress, isolation, and during times of immune challenge. Eight major herpes viruses parasitize humans with worldwide infection rates of 70-95%. Four of the eight are shed in the body fluids of NASA astronauts during both short and long duration spaceflight. Though viral load (virus detected in the body fluids) can be high, these astronauts often have no clinical symptoms associated with reactivation [154]. Post reactivation, replication of the virus may also be enhanced which could account for the significant increase in viral shedding during spaceflight. Yet, there have been a few cases where the reactivation culminated in commensurate atopic dermatitis and/or viral lesions [155].

2. Astronaut stress/exposures

Exposure of astronauts, during both short and long duration spaceflight, to non-terrestrial hazards such as variable gravitational forces including acceleration/deceleration, cosmic radiation, and microgravity result in a unique set of stressors that contribute to the dysregulation of the immune and endocrine systems [101,156]. In addition, they also endure some common stressors including but not limited to social separation, confinement, sleep deprivation, circadian rhythm

disruption and anxiety. There is increasing evidence to suggest that these spaceflight-associated stressors chronically amplify the release of stress hormones, which negatively affects the immune system, especially the adaptive immune system facilitating latent herpes virus reactivation during and after spaceflight. Increased levels of salivary, plasma and urinary stress hormones such as cortisol and catecholamines commonly accompany spaceflight [103].

3. Altered immunity

Maintenance of viral latency requires a vigorous and vigilant immune system, highly dependent upon competent cytotoxic T-cells, and any changes in immune status tend to promote viral reactivation. This is evident in both terrestrial space-analog studies [8] and spaceflight [1,6,7,157]. The alterations in immune status for terrestrial analog studies are minor and coincide with mild viral reactivation. Spaceflight studies illustrate major immune dysregulation and functional changes in conjunction with significant viral reactivation, regardless of mission duration. In fact, substantial changes in cell-mediated immunity exist in most astronauts that reactivated one or more herpes viruses [11,157]. This was also highlighted by Glaser, [10], who previously showed an association of EBV reactivation and diminished cell-mediated immunity.

The hypothalamus-pituitary-adrenal (HPA) axis along with the sympathetic-adrenal-medullary (SAM) axis partially mediate the stress response where glucocorticoids and catecholamines are secreted in proportionate concentrations relative to the stress stimulus **(Figure 3.1)**, [104–106]. Though acute responses to stress can be positive, long duration or chronically high levels of stress hormones can negatively affect the regulation of the immune system and its individual components [9]. Changes in a variety of immune cells, both in form (phenotype) and function (killing capacity), result in decreased cell-mediated immunity, which facilitates opportunistic reactivation of latent viruses, [11,13].

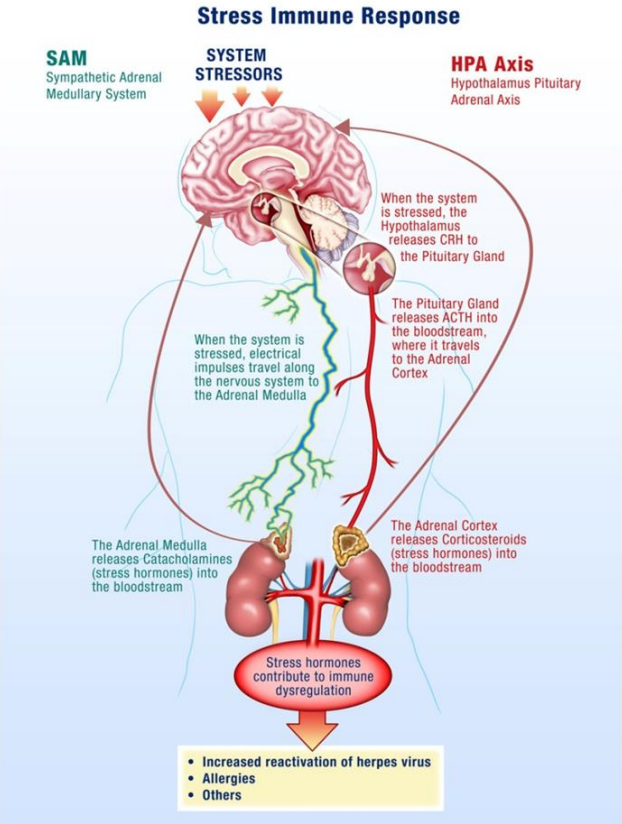

Figure 3.1. Spaceflight is a stressful environment with various stressors acting through the hypothalamus-pituitary-adrenal (HPA)-axis and the sympathetic-adrenal-medullary (SAM)-axis. Increases in stress hormones, such as cortisol from the adrenal glands, result in reductions in cellular immunity which facilitates opportunistic viral reactivation.

3.1 Stress hormones/cytokines

Cortisol and dehydroepiandrosterone (DHEA) are glucocorticoid steroid hormones released by the adrenal glands in response to stress. Cortisol is anti-inflammatory and immunosuppressive, but DHEA is an important antagonist to cortisol. For that reason, the molar ratio of cortisol to DHEA [C]/[D] is an important indicator of immune regulation. In recent flight studies, the regular diurnal release of these hormones was tracked in saliva samples to evaluate any changes/trends occurring through the various phases of flight; launch/pre-flight, flight, and return [157]. Salivary cortisol was present in significantly higher concentrations in samples taken before and during flight. Salivary DHEA followed its normal daily decline kinetics in the samples taken before, during and after flight, but has been found to have significantly lower waking concentrations during the flight phase in comparison

to samples taken before and after flight. Altogether, diurnal patterns of salivary cortisol were significantly higher during flight while DHEA was significantly lower. The cortisol area under the curve relative to ground (AUCg) did not change significantly during flight relative to baseline whereas DHEA AUCg significantly declined during flight relative to baseline. Ultimately, this results in an increased [C]/[D] molar ratio during spaceflight **(Figure 3.2)** which potentially indicates immune challenge, and has been linked to immune modulation [111], including the increased inflammatory cytokine response and the TH2 shift observed in earlier spaceflight studies [9,12].

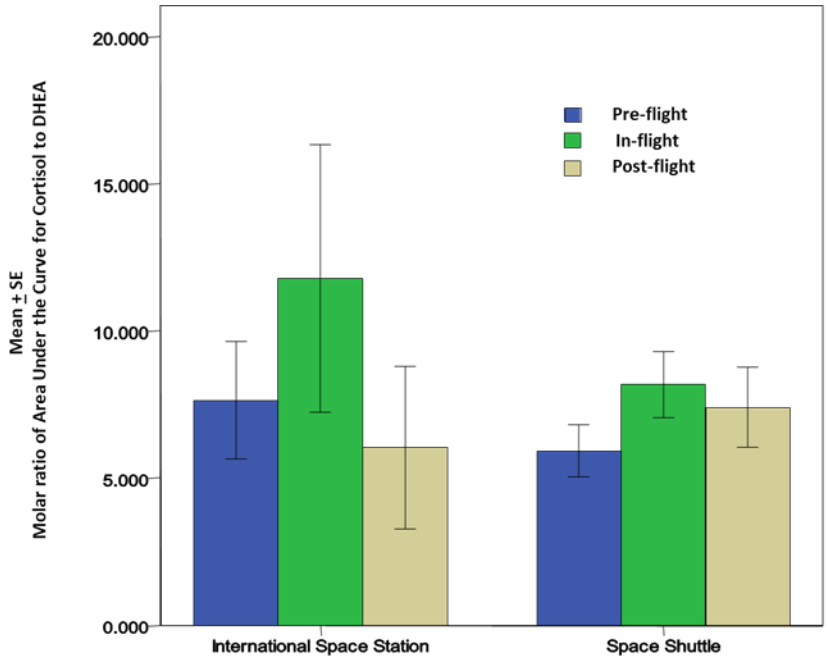

Figure 3.2. Cortisol and DHEA were analyzed in saliva from astronauts before, during and after the space flights using a commercially available ELISA assays (Salimetrics, LLC, State College, PA). There was a significant increase in the molar ratio of cortisol to DHEA during the flight phase for both Space Shuttle (N= 17) or ISS (N=10). The increase in this ratio may be associated with lower cellular immunity and innate immunity; potentially contributing to greater inflammatory cytokines that may affect bone remodeling and bone growth. * indicates significance when comparing flight against pre-flight and post-flight, $p<0.01$

Cytokines are small cell-signaling proteins that play a crucial role in the modulation of the human immune response. They can facilitate both pro- and anti-inflammatory immune states and are generally analyzed in the categories of inflammatory cytokines (IL-1α, IL-1β, TNFα, IL-6, IL-8), lymphoid growth factors (IL-2, IL-7, IL-15), Th1/17 cytokines (IFNγ, IL-12, IL-17), Th2 cytokines (IL-4, IL-5, IL-10, IL-13), myeloid growth factors (G-CSF, GM-CSF), and chemokines (eotaxin, MCP-1, M1P1α, IP-10). Recent flight studies [9,11,12] have shown that astronauts displayed

significant increases in the pro-inflammatory plasma cytokines IL-1α, IL-6, IL-8, IFNγ, IL-4, eotaxin, and IP-10 in samples taken 10 days before launch (L-10), in comparison to their baseline samples taken 180 days before launch (L-180). The increase of IL-6, IL-8, IL-4, eotaxin, and IP-10 is also evident immediately upon return to Earth at landing, designated as R+0. The Th2 cytokine IL-4 was the most sensitive/responsive to the phases of flight with 35-fold and 21-fold increases from baseline values at L-10 and R+0, respectively.

When analyzing plasma cytokine levels in the context of virus shedding, there seems to be a connection between astronauts who shed virus and significantly elevated levels of cytokines (IL-1α, IL-6, IL-8, IFNγ, IL-12p70, IL-4, IL-10, IL-13, eotaxin, and IP-10), [12]. Lymphoid and myeloid growth factors are also elevated in virus shedding astronauts, by about two-fold. As mentioned earlier, the Th2 cytokine IL-4 shows the largest fold increases through launch and return flight phases, and this is evident again when restricting the analysis to only viral-shedding astronauts at the return time point R+0. For these astronauts, the single largest plasma cytokine increases were IL-4 (21-fold increase) and IL- 6 (33-fold increase). This indicates a dynamic shift from a Th1 antiviral immune state to a Th2 antibacterial/antifungal immune state. Further emphasizing the Th1-Th2 shift is an analysis of the ratio of IFNγ: IL-4. The results from some of the most recent flight studies suggest a significant decrease in the IFNγ: IL-4 ratio for shedders compared to astronauts who did not shed any viruses during their duty rotation [9,12].

3.2 Viral specific T-Cell and NK-Cell function

Alterations in the aforementioned cytokines play a critical role in the fate of many important leukocyte populations. The cytokine profile changes, acting either independently or in conjunction with microgravity, generate a variety of immune vulnerabilities by significantly changing the numbers, proportions, and functions of leukocytes. Monocyte [116], granulocyte [117] and lymphocyte functions [11,13] are diminished, critically reducing the effectiveness of the immune response to pathogens, as well as its capacity to prevent viral reactivation. T-Cells and NK-Cells in particular, which function to attack and destroy viruses/virally infected cells, are

substantially debilitated during spaceflight. Flight studies focusing on T-Cell function have elucidated that both CD4+ and CD8+ T-Cells taken from astronauts during the flight phase respond ineffectively against a variety of stimuli. Under normal circumstances, these same stimuli would have elicited a more profound response by the T-Cells. The weakened response can last the duration of the flight phase, [11]. Additional flight studies focusing on the function of NK-Cells have shown decrements in cytotoxicity due to decreased production of the enzymes perforin and granzyme B [158]. Without these enzymes, NK-Cells are rendered ineffective against the target cell/pathogen and this impairment may last up to 60 days post flight. In both cases, reductions in T-Cell and NK-Cell function lead to the inability of the immune system to suppress/sequester/eliminate opportunistic viral reactivation.

4. Viral latency

As stated earlier, herpes viruses share a long-term co-evolutionary history with humankind. This promotes a relatively benign life-long persistence of the virus within the host. In healthy individuals with robust immune surveillance, viral activity can occur in the absence of clinical symptoms [159]. Injury to the host is antithetical to viral survival. Viral persistence in the host is aided by viral strategies for latency. Latency is a well-orchestrated series of concomitant events that allow for viral genome maintenance, while actively repressing lytic (replicative) gene expression and promoting latent gene expression. A hallmark of viral latency is that infectious viral progeny are not produced, so the surrounding cells remain uninfected or naïve. Viral latency is the culmination of a handful of factors; infection of cell types permissive to latency, viral promotion of infected cell survival, and the general evasion of the host immune response. Cellular tropism is dictated by cell surface receptor expression, as well as intracellular conditions permissive to viral activity, and is very specific to the individual herpes viruses. HSV and VZV infect neurons/nerve ganglia, while EBV and CMV preferentially infect the cells of the immune system, B cells and myeloid progenitor cells, respectively. Other cell types can be infected by the viruses, but the aforementioned cell types serve as the greater viral latent reservoirs. The promotion of infected cell survival is the product of viral manipulation of host cell machinery. For example, the manipulation of Bcl-2 family proteins promotes survival

of CMV infected monocytes [35]. Not only can herpes viruses manipulate the cells they infect, but they can also affect the host immune response. Interestingly, latent viruses are still very genetically active even in the absence of replication. There is emerging evidence of significant miRNA activity during latency that can act to override lytic transcription, as well as to alter the cell secretome. Though the role of miRNA are yet to be fully teased out, they seem to facilitate the transcription of proteins that mimic host cytokines and chemokines which ultimately inhibit host anti-viral activity. For the many nuances of viral latency, specific to each virus, the reader is directed to the following review articles: [71,160–162].

5. Viral reactivation

Reactivation and shedding of latent herpes viruses has been reported in astronauts during space shuttle, Russian Soyuz and International Space Station missions [1,89,90,157,163]. Virus reactivation has also been observed in ground-based models of spaceflight including Antarctica, undersea habitat, artificial gravity and bed rest studies, though not to the extent seen during spaceflight studies. So far, 47 out of 89 (53%) astronauts from short duration space shuttle flights, and 14 out of 23 (61%) from long duration ISS spaceflight missions shed at least one or more herpes viruses in their saliva or urine samples. Significant reactivations of EBV, CMV and VZV occurred during flight phase and the magnitude and frequency of viral shedding during spaceflight directly correlates with duration of spaceflight. VZV shedding increased from 41% in space shuttle to 65% in ISS missions, EBV increased from 82% to 96%, and CMV increased from 47% to 61%. In addition, VZV and CMV shed up to a month post long duration flight. Percent distribution of these viruses during shuttle and ISS missions is depicted in **Figure 3.3**. These viruses often reactivate in concert with one another, but they may also reactivate independently of the other viruses. Reactivation of latent viruses during long-duration spaceflight could increase risk for adverse medical events during exploration-class deep-space missions [101]. Taken altogether, and to our knowledge, there have been six incidences of astronauts with complaint of symptoms related to herpes viral reactivation [155]. VZV is an important health risk to crewmembers (several have

experienced shingles during flight). Furthermore, CMV can be immuno-suppressive and may play a role in the well-documented immune dysfunction observed in crewmembers.

EBV: Epstein-Barr virus is responsible for infectious mononucleosis and is associated with several malignancies [91–93]. It is a highly infectious DNA virus transmitted by aerosolized micro-droplets and by direct contact with saliva. It has a 95% infection rate among adults worldwide, which makes it an ideal target for investigation among a limited and unique astronaut population. EBV preferentially infects B-lymphocytes and these cells serve as a latent virus reservoir. Early flight studies from the shuttle missions were the first to demonstrate that EBV DNA was shed in astronaut saliva samples taken before, during, and after space flight, [6,163,164]. These studies highlighted a 10-fold increase in viral load during the flight phase in comparison to samples taken before or after flight. Additionally, EBV copies shed during space flight seemed to increase as a function of time in space, and as a result of diminished cell immunity [165]. These early findings have been repeatedly corroborated in longer duration ISS missions [1,115]. Altogether, flight studies have illustrated that approximately 90% of astronauts, regardless of mission duration, shed EBV during spaceflight.

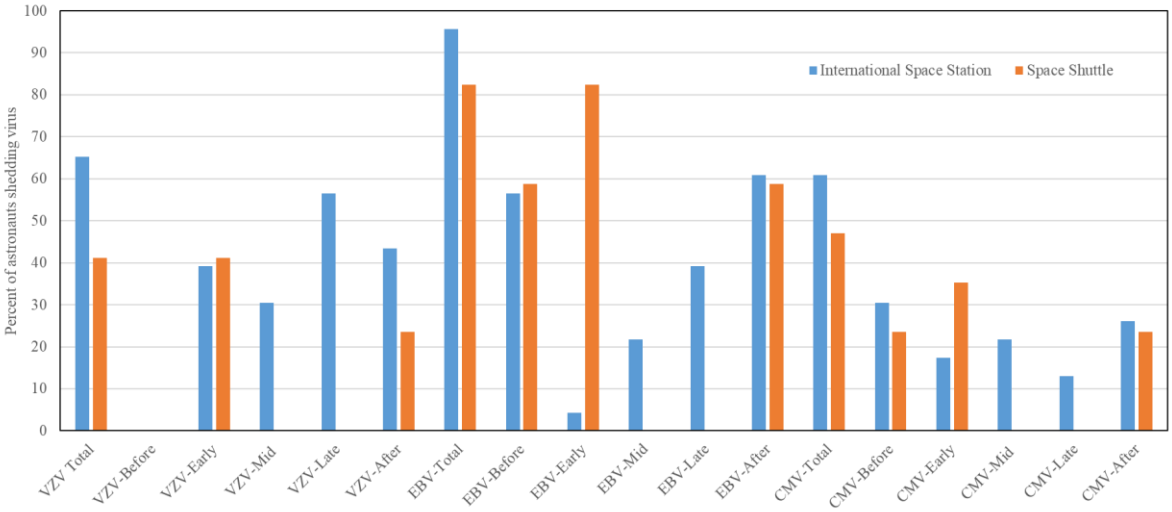

Figure 3.3. Percent distribution of astronauts shedding VZV, EBV and CMV before, during the mission at time point early-mission, mid-mission, late-mission and after either short and long duration space flights. Saliva and urine samples were collected from 112 astronauts (89 short duration and 23 long duration) before, during, and after the spaceflight. Saliva was analyzed for Epstein-Barr virus (EBV) and varicella-zoster virus (VZV), and urine was analyzed for Cytomegalovirus CMV by real time PCR assay using Taqman 7900 (Thermofisher Inc). The shedding of EBV, VZV and CMV DNA in body fluids is significantly higher during spaceflight as compared to pre-flight, post-flight, and the control $p<0.01$ (Mehta et al , 2014 and 2017). However, when comparing these shedding patterns between space shuttle and ISS missions, the differences were not significant.

VZV: Varicella Zoster virus is highly communicable and responsible for both chicken pox (primary infection) and shingles (secondary infection). The virus is transmitted via saliva and can be aerosolized by sneezing and coughing. After primary infection, VZV becomes latent in various nerve ganglia [166] (cranial, dorsal root, autonomic) along the entire length of the neuroaxis, and reactivation often results in characteristic skin lesions that range from aggravating to painful. Though reactivation of VZV is evident from flight studies where viral DNA was found in saliva of astronauts from both shuttle and ISS missions, astronauts do not often develop symptoms or rash [154,167]. Additionally, we have found that saliva samples taken 2-6 days following landing were infectious by culturing that saliva with human fetal lung (HFL) cells. Infectious VZV was present and confirmed by visual inspection of the culture where viral plaques were obvious, as well as by antibody staining and real-time PCR DNA analysis. This poses a risk to the welfare of both astronauts and their seronegative contacts back on Earth, as VZV viral load also increases with time in space and is present in saliva of about 60% of astronauts from combined shuttle and ISS missions.

HSV-1: Herpes Simplex virus-1 is highly prevalent and communicable and persists as a latent virus lifelong. Generally referred to as oral herpes, reactivation can be either asymptomatic or lead to lesions/rash anywhere on the body. Incidences of HSV-1 reactivation are very low with only 8% of astronaut saliva samples test positive for viral DNA, though recently, an astronaut suffering persistent dermatitis during a long duration spaceflight, >180 day, was positive for HSV-1 viral DNA in saliva and lesion samples. The saliva containing virus was infectious, as evidenced by a culture of the saliva atop HFL cells, where visual disruption of HFL cells was apparent at 3 days post infection with saliva, with viral load verified/quantified by real-time PCR.

CMV: Cytomegalovirus is the only beta-herpes virus known to reactivate in astronauts. It is typically acquired asymptomatically during childhood and has a worldwide prevalence of 75-90%. Though it remains asymptomatic in immunocompetent people, it may reactivate in individuals whose immune systems are either immature or immunocompromised causing multiple diseases such as encephalitis, gastroenteritis, pneumonia, and chorioretinitis [14]. Moreover, several

studies have suggested that CMV infection is immunosuppressive because it directly infects leukocytes as well as hematopoietic cells [15–17]. Additionally, CMV has been uniquely linked to early immune senescence [57,58]. However, a study was able to illustrate a benefit of CMV infection specifically to young adults (20-30 years old) regarding increased antibody response to the influenza vaccine [168]. Spaceflight studies have shown that 27% of the astronauts from short-term space missions shed CMV DNA in either pre- or post-flight urine samples, and that anti-CMV IgG antibody titers increased significantly for all shedders from each time point compared to their baseline values [2]. In long duration spaceflight, 61% of astronauts shed CMV DNA in their urine during and after spaceflight in stark contrast to the absence of CMV DNA in urine samples taken 180 days before flight. These findings demonstrate that CMV reactivation occurs in astronauts regardless of mission duration, and this may pose additional threats to the health of crewmembers during longer-duration missions. [1,3].

6. Rapid detection of virus for application in patient populations

The most obvious signs of VZV reactivation are the vesicular rash and the pain associated with zoster, however even in the absence of rash, the virus is active and can spread to the retina causing blindness, to the spinal cord causing paralysis and incontinence, and to cerebral arteries resulting in stroke [169,170]. Associating VZV with a disease asymptomatically can be challenging. For example, when stroke occurs in the elderly, especially many months following zoster, the association with VZV reactivation requires cerebral spinal fluid (CSF) analysis for VZV antibodies [171]. Likewise, detection of asymptomatic VZV reactivation, which often is only seen as an increase in antibody titer against VZV (but may also result in virus transmission), is difficult to detect. In such instances, virological verification of VZV disease has relied on the detection of VZV DNA or anti-VZV IgG antibodies in CSF or, less often, the presence of VZV DNA in blood mononuclear cells or anti-VZV IgM antibodies in serum.

However, VZV DNA has been detected in the saliva samples from patients with acute zoster [172], zoster sine herpete [173], chickenpox [174] and postherpetic

neuralgia (PHN) [175] even before the rash appears, which now makes diagnosis less invasive and less time consuming. In fact, a rapid and sensitive virus detection method has been developed and used to detect virus in saliva samples taken from asymptomatic patients with neurologic and other VZV related disease [174]. **Figure 3.4** illustrates VZV copy numbers in saliva from shingles patients before anti-viral treatment. For this method, the saliva is collected by passive drool or by way of a synthetic swab and then processed for DNA within an hour from sample collection. The results from a few studies using this technique have shown that VZV DNA is present in 100% of patients tested before antiviral treatment and is exclusively in the cell pelleted fraction of saliva. These studies further showed that VZV, isolated from zoster patient saliva, was primarily associated with the epithelial cell membrane but could also be inside the cell. Epithelial cells with VZV continued to be present in the saliva of a single zoster patient up to 10 months after recovery. These kinds of studies are ongoing and our spaceflight-developed technology for rapid viral detection continues to be used locally and around the world for patients with zoster [174], chicken pox [172], PHN [175], multiple sclerosis [176], and various other neurological disorders [177,178].

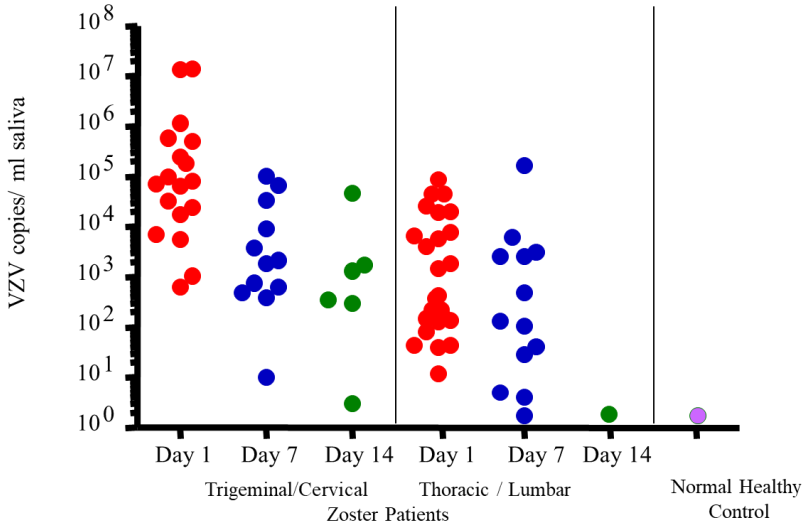

Figure 3.4. VZV copy number per mL of saliva in Fifty-four zoster patients treated with valacyclovir and 14 healthy subjects.. On treatment day 1, 7- and 14-days later, pain was scored (data not given here) and saliva examined for VZV-DNA by real time PCR. Patients were divided into two groups based upon the infected dermatome, Trigeminal/Cervical and Thoracic / Lumbar. VZV-DNA was found in every patient the day treatment was started and disappeared in 82% with the treatment. Analysis of human saliva has potential usefulness in diagnosing neurological disease produced by VZV without rash. When comparing patient shedding against normal healthy controls, it was significantly higher, $p<0.01$, (Mehta et al, 2008).

7. Conclusion

Reactivation of latent viruses is a powerful biomarker of immune status for astronauts deployed to space. There are multiple factors that influence reactivation including increases in glucocorticoid/catecholamine secretion, cytokine profile shifts, and decreased function in the major leukocyte and lymphocyte subsets designed to suppress and eliminate viruses/virally infected cells. Viral reactivation is evident through the shedding of viral DNA in the body fluids of astronauts, and the viral load only increases with more time in space. Additionally, more than one virus generally reactivates at a time, potentially compounding the physiological ramifications of uncontrolled viral reactivation to not only rashes, but also severe target organ failures, and permanent vision and hearing loss. The occupational hazards for astronauts are profound, but research into the causes and mechanics of viral reactivation not only benefit the astronaut but also the general patient population. As our understanding of viral reactivation widens, we are better able to develop and implement effective countermeasures for our astronaut professionals, as well as targeted treatment regimens for immunocompromised individuals suffering the consequences of viral reactivation. As a result, this research has tremendous clinical relevance.

Ultimately, the information gleaned from these space studies will shape the way we prepare for and design exploration-class missions, beyond the moon and mars, where reactivation of latent viruses could result in increased risk for wide-ranging adverse medical events. Partial-gravity environments, e.g. on Mars, might be sufficient to curtail serious viral reactivation, but this needs to be addressed in future research. In the interim, because astronaut saliva contains increasingly significant viral DNA, during and after spaceflight that can be infectious, we recommend prophylactics (vaccines), where available, to the astronauts before they go in space.

Chapter 4

SMG alters CMV viral load and lytic transcription

Abstract

Cytomegalovirus (CMV) is a highly prevalent and communicable beta herpes virus that persists lifelong and requires an incessant commitment of immune resources to maintain asymptomatic latency. Reactivation of CMV in astronauts is evident by shedding of its viral DNA in urine samples taken before, during and after missions of variable durations. Though many factors have been implicated in terrestrial reactivation of CMV, including stress hormones and inflammatory cytokines, the specific factors responsible for the 8-fold increases in CMV viral load observed during spaceflight have not been elucidated. Our laboratory, therefore, sought to understand the unique contribution of microgravity to this phenomenon. We established a positive infection model for Kasumi-3 myeloid progenitor cells using a low fibroblast passaged, BAC-derived TB40/E clinical strain of CMV, and high-speed centrifugation. First, we show that Kasumi-3 myeloid progenitor cells, infected with the CMV virus and then plated for 12 days in simulated microgravity (SMG), using the rotating wall vessel (RWV), have significantly lower viral loads ($p<.007$) than their 2D/1g controls through all 5 time points: days 0, 1, 4, 8 and 12. We also show that, though viral load is lower in SMG, lytic transcription of UL122 is higher in SMG. This increase in lytic gene transcription does not translate to increases in whole virus lytic behavior within the 12-day assay period. Second, we cultured Kasumi-3 cells for 16 days in SMG before infecting them with the BAC-derived TB40/E strain of CMV. We infected at multiplicity

of infection (MOI) of 1 via high-speed centrifugation and then plated these cells in T-75 flasks. We used the Kasumi-3 cells cultured in normal gravity, pretreated to 1G for 16 days and then infected with CMV, as our experimental controls. We observed these cultures over a 13-day period, with specific aliquots taken from both SMG pretreated and 1G pretreated (control) vessels on days 0 (12-14 hours post infection), 1 (24 hours post infection), 4, 7, 10 and 13. Cell count, cell size and cell viability were obtained for all aliquots, and then viral load was determined for both cell pellet and supernatant fractions by real time-PCR. Cell concentration and viability remain very similar between SMG and 1G pretreated cultures for all time points. However, there was a 25% decrease in the viral load for SMG pre-treated cells at days 0 and 1, in comparison to the 1G condition. (D0 flask 7992 ± SE 1486 copies/ng/ SMG-pre-treated 5987 ± SE 1193 copies/ng, D1 flask 3870 ± SE 632 copies/ng/SMG pre-treated 2908 ± SE 236 copies/ng.) This reduction in viral load for the pre-treated condition is statistically different from the 1G pre-treated control at D0 (t-stat 6.707, $p \leq .05$), and begins to lose significance in D1 samples with (t-stat 1.425, $p \leq .09$). This trend was replicated over three experimental trials with multiple replicates, SMG pre-treated (n=7) and 1G pre-treated (n=7). This phenomenon may be due to microgravity-induced changes in epidermal growth receptor (EGFR) expression or spatial misalignment at the cell/virus synapse, but this requires further investigation.

Introduction

The ubiquitous beta herpesvirus CMV is a significant worldwide pathogen infecting between 75-90% of the world's population. It is asymptomatic in immune competent people but can have severe consequences for people who are immune immature or challenged [60]. CMV is immunotropic, meaning that it preferentially infects cells of the immune system, specifically those of hematopoietic stem cell lineage. In fact, it is universally accepted that CMV's latent reservoir resides within hematopoietic stem cells of the bone marrow, predominantly in undifferentiated cells of the myeloid lineage and monocytes [179,180]. This facilitates its systemic dissemination during active infection, as well as allows it to evade a robust immune response during latency, ultimately promoting its lifelong presence in the host [181,182].

CMV reactivation has occurred in more than half of the astronauts deployed to space over the last 2.5 decades, regardless of their mission duration. In fact, 47% of shuttle crew and 61% of ISS crew, to date, have been positive for CMV reactivation [87]. Reactivation can occur as early as ten days before launch and is indicated by the shedding of CMV DNA in astronaut urine samples, and by increases in their plasma anti-CMV IgG antibody titers. Though both of these can also be observed in ground analog studies concerning stress [5], and perhaps in response to cortisol, the magnitude of the space response is unique. The anti-CMV IgG titers in space can rise to eight-fold beyond their negative baseline values [88], and both the viral load and titers steadily increase with more time spent in space. This likely implicates a space-specific factor such as microgravity.

For that reason, in the current study we sought to determine the unique contribution of simulated microgravity (SMG), using rotating wall vessel (RWV) technology, on the *in vitro* CMV infection of, and replication amid, immortalized Kasumi-3 myeloid progenitor cells. Further, we sought to determine the effect of SMG on CMV virulence or its magnitude of expansion measured by the percentage of lysed fibroblast cells due to CMV infection. We hypothesized that SMG would enhance CMV infection and replication in Kasumi-3 cells, as well as increase CMV's virulence (speed and capacity to cause disease).

Methods

Cells and Virus

We used Kasumi-3 myeloid progenitor cells purchased from ATCC (ATCC CRL-2725, Manassas, VA, USA) and a single vial of a low fibroblast passaged *BAC*-derived TB40/E strain of CMV (7.8×10^7 pfu/mL) obtained from Dr. Ritesh Tandon's lab.

Kasumi-3 Cell infection, infection validation and plating in SMG/Flask

Kasumi-3 cells are infected in 12 well plates at a MOI of 1 via high-speed centrifugation and then plated and maintained in culture vessels (RWV or Flask) at a plating density of 25e6 cells per vessel and maintained 37°C/95% humidity/5% CO_2 incubators. Non-infected Kasumi-3 cells serve as the negative control. SMG was accomplished in 50mL RWV rotating at 10 rpm. Aliquots were taken from each vessel on days 1, 4, 8, 12, and 16 to analyze cell count, cell size and cell viability, and to confirm infection. Cell count, concentration, size, and percentage viability for all

aliquots were accomplished using Cellometer Auto 2000 (Nexcelom, Lawrence, MA, USA). Infection of cells was confirmed by q-PCR, confocal microscopy, direct single-color antibody staining using mouse anti-human mab810X-AlexaFluor-488 (IE-1) (EMD Millipore, Temecula, CA, USA) via flow cytometry, and reverse transcriptional analysis of viral lytic protein UL123 (IE-1) and UL122 (IE-2).

DNA extraction

One million CMV-infected Kasumi-3 cells were separated from the supernatant by centrifugation at 14,000 rpm for 10 minutes. DNA was extracted from both cells and supernatant using an ArchivePure DNA Tissue kit (5 Prime, Gaithersburg, MD, USA-Qiagen). In short, contaminants, such as proteins, were removed by salt precipitation while the genomic DNA was recovered by precipitation with alcohol and dissolved in DNA hydration solution.

DNA Concentration for normalization

For each sample, viral copy numbers from duplicate readings were averaged, and then normalized by the DNA concentration to produce the main outcome measure of viral load: copies of DNA per nanogram of DNA. DNA concentration was determined using a Qubit 2.0 Fluorometer and Invitrogen™ Quant-iT™ Qubit™ dsDNA HS Assay Kits (Invitrogen, Carlsbad, CA). The Qubit Fluorometer converts the fluorescent signal into DNA concentration (ng/μL) using DNA standards of known concentration.

DNA qPCR

Real-time Polymerase Chain Reaction (PCR) - CMV PCR assays were performed in 20-µL volumes containing 13.4µL 2× TaqMan Universal PCR Master Mix (Perkin–Elmer, Norwalk, CT) and 6.6µL of extracted DNA. Standard curves were generated with diluted viral DNA (1–10^5 copies) extracted from virus-infected cells. Each sample was analyzed in duplicate. Viral DNA was quantified by Real Time PCR (qPCR) using a TaqMan™ 7900HT Fast Real-time PCR System (Applied Biosystems, Grand Island, NY, USA). The fluorogenic probe and primers used, shown below, were synthesized from Integrated DNA Technologies ((IDT) Coralville, IA, USA). The viral primer and probe sequences are outlined below:

CMV	IE-EX4 Forward	TCC CGC TTA TCC TCR GGT ACA
	IE-EX4 Reverse	TGA GCC TTT CGA GGA SAT GAA
	IE-EX4 Probe	TCT CAT ACA TGC TCT GCA TAG TTA GCC CAA TAC A
	gB F	TGG GCG AGG ACA ACG AA
	gB R	TGA GGC TGG GAA GCT GAC AT
	gB Probe	TGG GCA ACC ACC GCA CTG AGG

RNA isolation and qPCR

RNA was isolated from 5e5-1e6 infected kasumi-3 cells after fresh culture samples were centrifuged at 14,000 rpm for 2 minutes, decanted and then resuspended in 350µL of lysis buffer. RNA was isolated from each sample using an AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany). RNA concentration was measured using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using the

miScript II RT Kit (Qiagen, Hilden, Germany), and conversion to cDNA was performed in the DNA Engine® Thermal Cycler (BioRad, Hercules, CA, USA). cDNA was diluted in 40µL RNase-free water. The PCR reagent consisted of 12.5µL 2xQuantiTect SYBR Green PCR Master Mix, 7.5µL RNase-free water, 2.5µL 10x Primer Assay from the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany), and 2.5 µL Template cDNA. PCR was performed in a CFX96TM Thermal Cycler (BioRad, Hercules, CA, USA).

The primer sequences for viral transcription analysis will be as follows:

UL123	Forward Primer	GCC TTC CCT AAG ACC ACC AAT
	Reverse Primer	ATT TTC TGG GCA TAA GCC ATA ATC
UL122	Forward Primer	ATG GTT TTG CAG GCT TTG ATG
	Reverse Primer	ACC TGC CCT TCA CGA TTC C
US28	Forward Primer	CCA GAA TCG TTG CGG TGT CTC AGT
	Reverse Primer	CGT GTC CAC AAA CAG CGT CAG GT
UL138	Forward Primer	GGT TCA TCG TCT TCG TCG TC
	Reverse Primer	CAC GGG TTT CAA CAG ATC G

Power and Statistical Analysis

An *a priori* power analysis was computed using the G*Power computing software for CMV infection and replication (expansion). Our experiments included both longitudinal measures and two experimental conditions: SMG and 1G. The significance level was set at $\alpha = 0.05$ and the preliminary CMV data effect size was approximately 0.85. The power analysis projected that for an effect between cell culture conditions across time required a sample population of 14 (7 each experimental condition) to have 83.1% power. Differences in viral load is response to

the different gravitational conditions was evaluated using paired sample t-test analysis. To determine if the viral load was different between gravitational conditions, or with-in each condition across the different time points, a univariate ANOVA analysis was performed. Prior to analysis the data were screened to ensure all assumptions were met. Normality was confirmed using histograms and Fisher's skewness and Kurtosis coefficients, as well as descriptive statistics and normal Q-Q plots. Bonferroni corrected post-hoc comparisons were used to determine which days of culture were significantly different from one another. SPSS version 22 (IBM; Armonk, NY, USA) was used for statistical analyses with significance indicated as ($p<.05$).

RNA differences within and between SMG and FLASK conditions was analyzed by mixed-effects REML regression using ΔΔCt values. Ct values for the target gene were subtracted from the averaged Ct of the control gene (GAPDH). The normalized value is the result of the ΔCt. The difference between the normalized values in the control (FLASK) group and the experimental group (SMG) equals the ΔΔCt values.

A two-tailed unpaired t test with Welch's correction (unequal variance assumption) was used for statistical analysis of differences between two samples at same time point. Sample comparisons were between two groups: SMG and 1G; with aliquots from these two conditions cultured atop fibroblast cells to assess differences in virulence across condition and time point, as well as after TPA stimulation of Kasumi-3 cell differentiation and CMV reactivation in SMG.

Results

Kasumi-3 myeloid progenitor cells remain viable in SMG

Uninfected Kasumi-3 cells brought up and maintained in ATCC RPMI 1640 supplemented with 20% fetal bovine serum (FBS), 1.4 mL of Penicillin-Streptomycin-Glutamine, and sustained in humidified 37°C/5%CO_2 incubators, remained viable in SMG with numbers ranging between 90-95% for all time points: Days 1, 4, 8 and 12.

MRC5 and Kasumi-3 cells are susceptible to infection using our protocol

Using single vials of the TB40/E and GFP-Towne-BAC CMV virus, we passaged, propagated and determined virus concentration (infectious dose) of 5.4e7 pfu/mL in MRC5 fibroblast cells. Infection of these cells was confirmed using crystal violet staining and fluorescent imaging, (figure 3).

Infection of the Kasumi-3 cells from our SMG validation study, done above, was confirmed by RT-PCR, confocal microscopy and direct single-color antibody staining using mouse anti-human mab810X-AlexaFluor-488, (figure 3). Cells taken from the SMG condition for this infection study exhibited lower viral load at time points up to 24 hours. This was confirmed in subsequent studies shown later in this section, **(Figure 4.1)**.

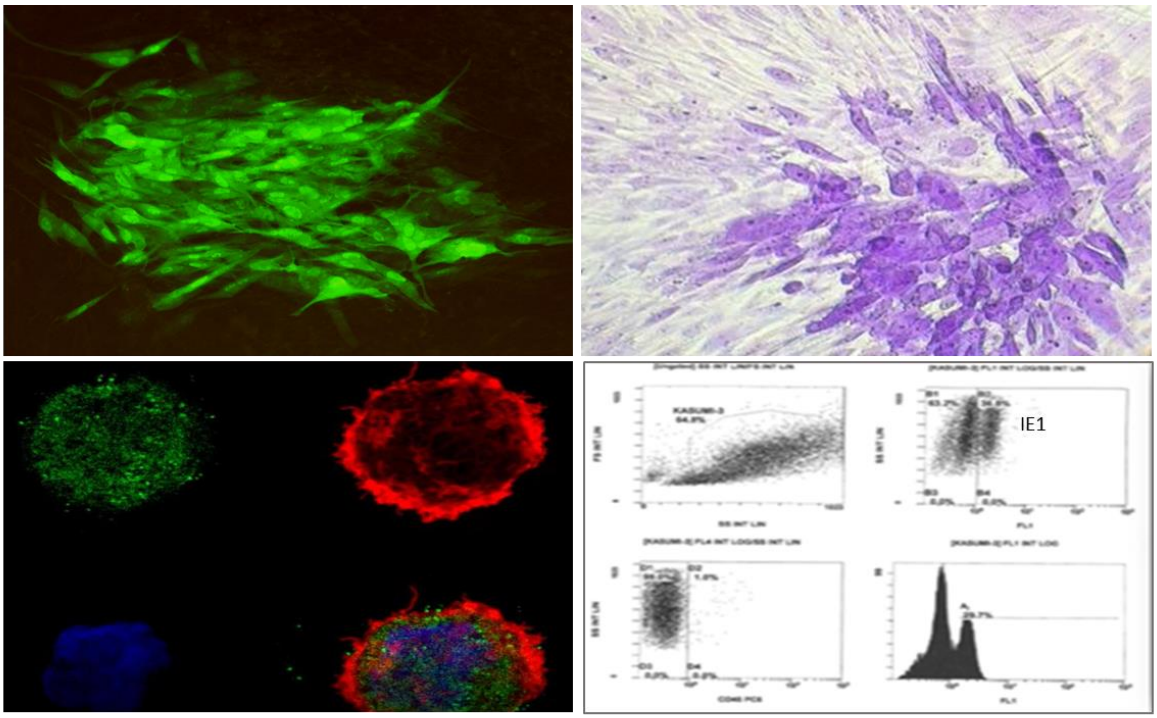

Figure 4.1. MRC5 fibroblast cells infected with GFP-Towne-BAC (top left) or a BAC- derived TB40/E (top right) CMV strain, imaged with fluorescent imaging microscope or by crystal violet staining, respectively. Kasumi-3 myeloid progenitor cells infected with GFP-Towne-BAC (bottom left) or a BAC-derived TB40/E (bottom right) CMV strain, imaged using confocal microscopy or direct single-color antibody staining using mouse anti-human mab810X-AlexaFluor-488. Bottom left image: green is GFP-labeled CMV, red is wheat germ agglutinin (WGA) stained cell membrane, and blue is DAPI stained nuclei.

SMG impedes *in vitro* CMV viral expansion in infected Kasumi-3 cells

Aliquots taken from each SMG or Flask culture vessel on days 1, 4, 8 and 12, revealed that cell pellet (CP) fractions yield vastly higher viral load (ranged between 3 and 255, mean 94.78 ± SE 10.13) than supernatant (SN) fractions (predominantly less than 20 copies/ng of total DNA). We further determined by paired sample t-test analyses that the CP viral loads from the Flask/1G (M=116.05, SD=71) conditions were significantly higher than the RWV (M=42.8, SD=44) condition (t_{55}=-7.39, *p*<.007), **(Figure 4.2)**. Results of the univariate ANOVA indicate a significant main effect of time ($F_{7,48}$=10.11, *p*<.001), where all times points are significantly different from Day 1, (*p*<.001), and Day 8 and 12 are significantly different from Day 4, (*p*<.05), but not from each other.

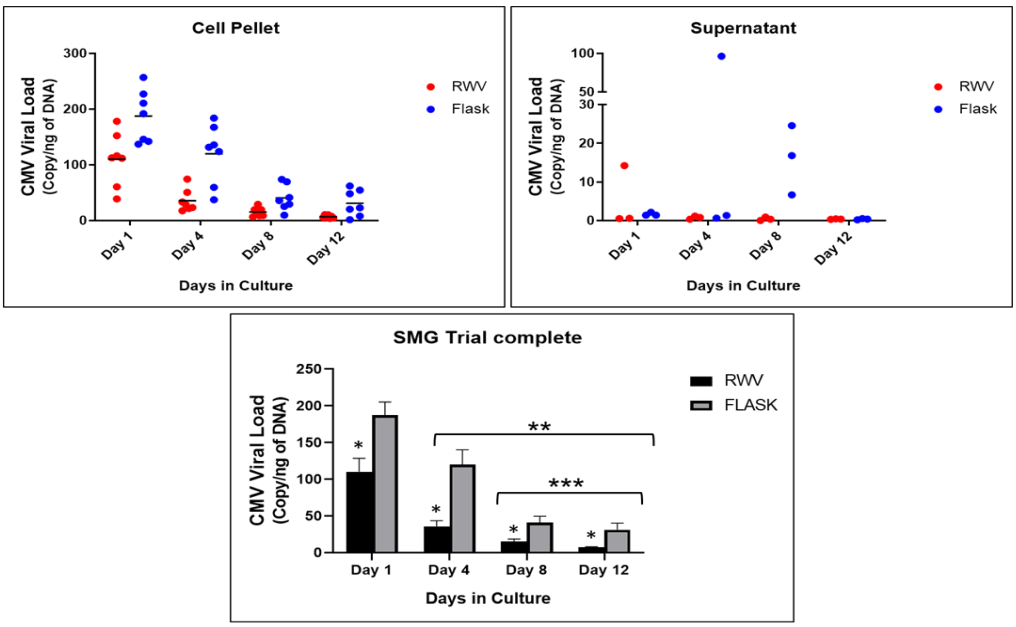

Figure 4.2. 25e6 Kasumi-3 cells per predesignated culture vessel were infected with a BAC-derived TB40/E CMV strain in 1G by high speed centrifugation at an MOI of 1. 12-14 hours post infection (hpi), cells were then separated and plated at 25e6 cells per culture vessel in either simulated microgravity (SMG) using the rotating wall vessel (RWV n=7) or remained in 1G in T-75cm^2 (n=7) flasks. Post infection aliquots were taken Day 1 (24hpi), Day 4, Day 8, and Day 12. 1e6 cells from each aliquot were removed, separated into cell pellet (top left) and supernatant fractions (top right) for DNA extraction, and then viral load was quantified using real-time-PCR. Values were then normalized to total DNA concentration in the sample aliquot, (copy per ng of DNA). Bottom graph depicts the average viral copy number per ng of DNA in positively infected Kasumi-3 cells. Values are mean ± SE. *indicates significant difference from the flask condition, p<.05. ** indicates significant difference from Day 1, p<.001, *** indicates significant difference from Day 4, p<.05

16 days of SMG delays primary CMV infection *in vitro*

25e6 Kasumi-3 cells pre-treated to 16 days of SMG before infection in 50 mL conical tubes at a MOI of 1, revealed 25% decreases in viral load in comparison to their 1G controls at Day 0 (12-14hpi) and Day 1 (24hpi). Further, using a paired sample t-test analysis, we found a significant difference between CP viral load in pre-treated RWV conditions in comparison to their 1G controls, $p<.05$, (**Figure 4.3, Table 1**). Results of the univariate ANOVA indicate a significant main effect of time ($F_{4,60}=69.19$, $p<.001$), where all times points are significantly different from Day 1, ($p<.001$), and Day 10 and 13 are significantly different from Day 4, ($p<.05$), but not from Day 7 or each other. Concentrations and viability shown in figure 4.4.

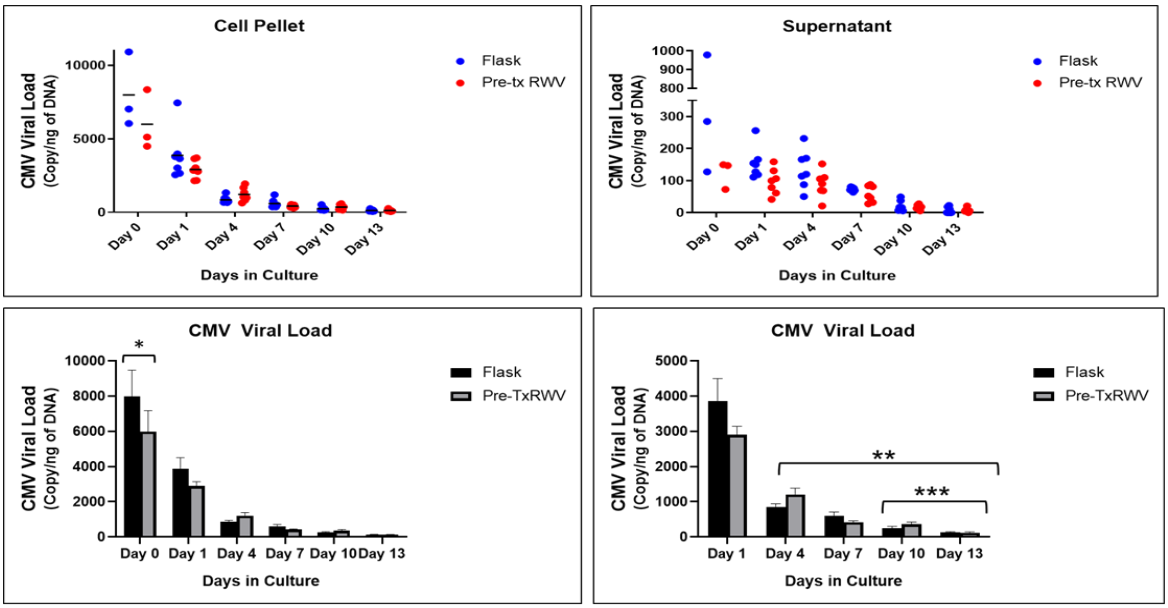

Figure 4.3. Graphs in top panels illustrate individual data points from the following experiment: 25e6 Kasumi-3 cells per culture vessel were pre-treated to simulated microgravity (SMG n=7) for 16 days prior to 1G infection with a BAC-derived TB40/E CMV strain at an MOI of 1. 25e6 cells plated in T-75cm^2 flasks (n=7) served as the pre-treatment control. Post infection aliquots were taken at Day 0 (12-14hpi), Day 1 (24hpi), Day 4, Day 7, Day 10 and Day 13. 1e6 cells from each aliquot were removed, and then separated into cell pellet (top left panel) and supernatant fractions (top right panel) for DNA extraction, and then viral load was quantified using real-time-PCR. Values were then normalized to total DNA concentration in the sample aliquot, (copy per ng of DNA). Graphs in bottom panels depict the average viral copy number per ng of DNA in positively infected Kasumi-3 cells. Left panel includes Day 0 time point, right panel does not include Day 0 for better visualization of Days 4-13 values. Values are mean ± SE. *indicates significance between flask and pre-treated RWV conditions, p<.05. Though not significant, the difference between flask and pre-treated RWV at Day 1 is p<.09. ** indicates significant difference from Day 1, p<.001, *** indicates significant difference from Day 4, p<.05

Table 1. CMV viral copies per ng of DNA in cells either pre-treated to 16 days SMG or 1G, with results from paired sample t-test.

	Pre-Tx SMG	Flask	t-stat	p value
Day 0 (12-14hpi)	5987 ± SE 1193 copies/ng	7992 ± SE 1486 copies/ng	6.707	≤ .05
Day 1 (24hpi)	2908 ± SE 236 copies/ng	3870 ± SE 632 copies/ng	1.425	≤ .09

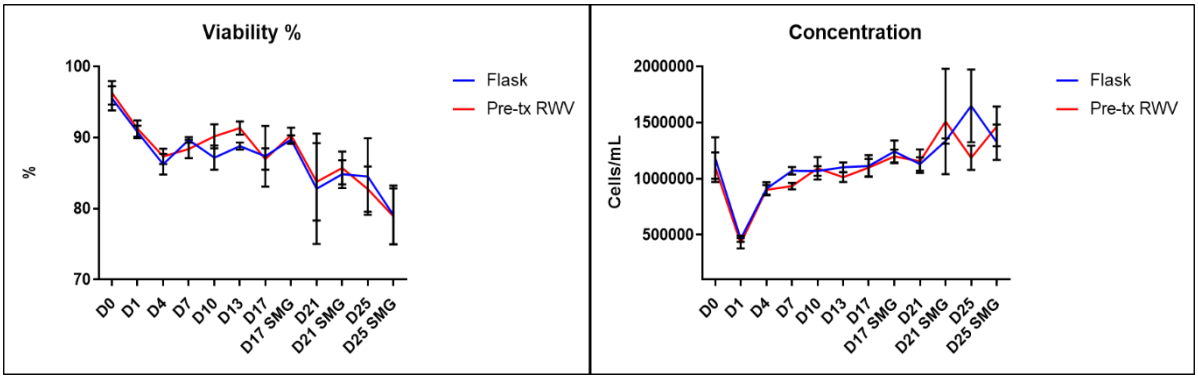

Figure 4.4. Graphs above depicting Kasumi-3 viability% (left panel) and cell concentration in cells/mL (right panel) are compiled averages for three identical trials. Cells pre-treated to simulated microgravity (SMG) in rotating wall vessels (RWV) for 16 days, are then infected at MOI of 1 via high speed centrifugation, and then plated in 75cm^2 flasks for 25 days of observation. Aliquots are taken on days 0 (12-14hpi), day 1 (24hpi), day 4, day 7, day 10, day 13, day 17, day 21 and day 25. Error bars represent SEM.

SMG alters CMV lytic gene transcription

Transcriptional analysis was accomplished using 1mL aliquots from 25e6 Kasumi-3 cells that were infected at MOI of 1 and then separated into SMG or Flask conditions. These aliquots were taken from each SMG or Flask culture vessel on days 1, 4, 7 and 10. Each sample was probed for GAPDH, UL123, UL122, US28 and UL138. Lytic transcript UL123 was not detected in any of the aliquots, but the lytic transcript UL122 was detected and its normalized gene expression was significantly different between SMG (M=11.14, SD=.54) and Flask (M=10.8, SD=.65) conditions, t_{11}=-2.23, p<.05, **(Figure 4.5, left)**. Further, the relative fluorescent unit (RFU) fold change from the normal gravity Day 1 control was significantly different between SMG (M=1.203, SD=.079) and Flask (M=1.02, SD=.14) conditions, t_3=-4.615, p<.05, **(Figure 4.5, right)**. Samples with higher quantities of amplified DNA, have higher corresponding RFU. The expression of latent transcripts US28 and UL138 were

highly variable in both conditions with no discernable patterns or changes in response to SMG.

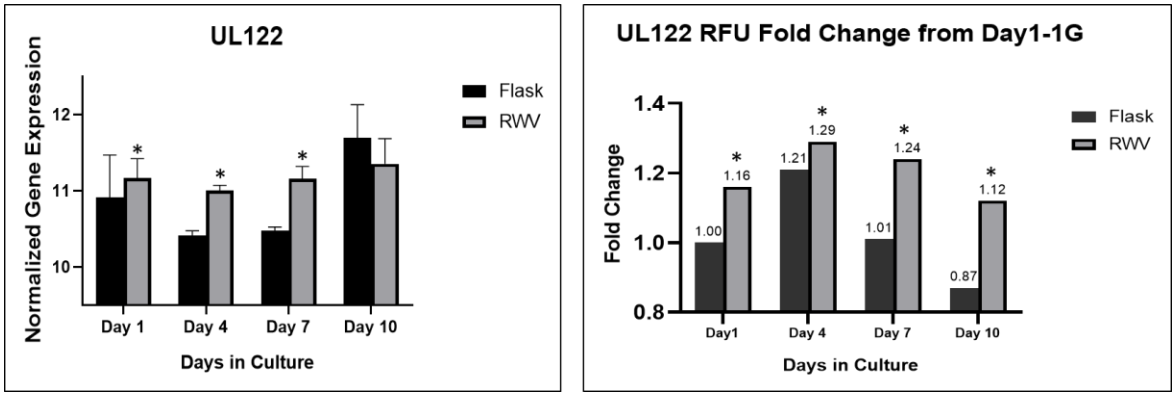

Figure 4.5- **(Left)** Normalized UL122 lytic gene expression between normal gravity (n=7) and simulated microgravity (SMG) (n=7) conditions observed over 10 days. RWV represents SMG. Cycle threshold (Ct) values were used to analyze the gene expression. The Ct value represents the **cycle** number at which the fluorescence generated within a reaction crosses the fluorescence **threshold**, and amplifies above the background fluorescence. The Ct values for GAPDH were averaged for the replicates of a single time point and then subtracted from the individual Ct values of UL122 for the same time point. UL122 expression is significantly higher in the rotating wall vessel (RWV) condition, p≤.05. **(Right)** Relative fluorescent unit (RFU) fold change from normal gravity Day 1 control observed over 10 days between normal gravity and SMG cultures. Samples which contain higher quantities of amplified DNA have higher corresponding RFU values. UL122 amplification is significantly higher in the rotating wall vessel (RWV) condition, p≤.05. This amplification remains high even in RWV samples from later time points, Day 7 and Day 10. Values are mean ± SE. *indicates significant difference from the flask condition, p<.05.

Whole virus lytic behavior not different between SMG and Flask

Viral titer was accomplished using 500uL aliquots from 25e6 Kasumi-3 cells that were infected at MOI of 1 and then separated into SMG or Flask conditions. Aliquots were taken from each SMG or Flask culture vessel on days 1, 4, 7 and 10, **(Figure 4.6)**. Viral titer appears to decrease in both conditions overtime indicating latency.

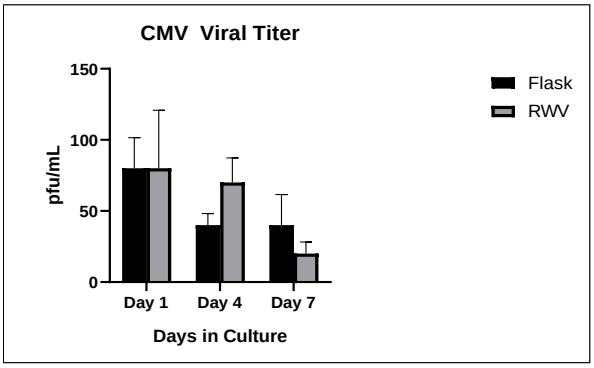

Figure 4.6 – Viral titers assessed for Kasumi-3 cells infected with Bac derived TB40/E CMV and then cultured in normal gravity (n=4) or simulated microgravity (SMG) (n=4) conditions. Aliquots were taken on days 1, 4 , 7, and 10. RWV represents SMG. Aliquot volumes were then cultured atop fibroblast cells to assess viral titer. Viral titers decrease in both conditions overtime indicating the emergence of latency. Values are mean ± SE.

Discussion

The results of our current study highlight that simulated microgravity (SMG) impedes CMV binding and entry into Kasumi-3 myeloid progenitor cells, as evidenced by significantly lower viral load in the SMG fractions of our experiments. Further, when Kasumi-3 cells are pre-treated to SMG for 16 days before being infected and cultured in 1G, SMG offers them significant protection against infection for up to 24 hours. This is especially interesting when considering that SMG promotes lytic transcription of UL122 for those cells that are capable of being infected during SMG exposure. The first two revelations were unexpected considering our hypothesis was SMG would enhance CMV infection and replication to mirror observations during space flight. The third observation was not surprising as we expected to see significant increases in lytic gene expression in response to SMG. However, it was interesting to see that the lytic expression of UL122, which remained significantly higher throughout the experimental time course, followed a characteristic parabola where expression peaked at day four [26] and then declined in later time points. We believe that many of the above observations are likely related to the expression and/or action of epidermal-growth-factor receptors (EGFR) on the surface of the Kasumi-3 cells.

CMV requires activation of the host cell EGFR to enter during infection [183,184], and binding of the CMV glycoprotein complex gH/gL/(gO)-UL128-131 to target cell EGFR and β1-β3 integrins is key to infection of myeloid progenitors and monocytes [35]. EGFR and its downstream signaling pathways are important homeostatic regulators of cell survival, differentiation and proliferation. Once infected, preservation of the EGFR at the host cell surface promotes CMV latency by impeding

post entry mechanisms of viral replication [69,70], while internalization of the EGFR supports reactivation [68]. Interestingly, viral proteins affect changes in the host cell expression of EGFR. Viral protein UL138 is a constitutively active protein which promotes latency by preserving EGFR expression at the host cell surface. Viral protein UL135 must overcome the replication-suppressive activity of UL138 to target the EGFR for internalization and degradation. Though we measured UL138 transcription for our samples cultured in either SMG or flasks, we saw no discernable differences in its expression between the two gravity conditions. Further, SMG itself has been shown to affect EGFR expression and activation [185,186]. Unfortunately, we did not measure the expression or activation of EGFR. However, it might also be possible, especially for the first observation where SMG impedes viral expansion, that SMG simply mechanically interferes with the cell-to-cell contact necessary for CMV transmission. In the flask condition, cells form a sedimentary layer along the bottom of the flask and remain close for the duration of the experiments. Though we recognize this possibility, we contend that there is a unique effect of SMG which is highlighted by the protection of SMG pre-treated Kasumi-3 cells from infection, and the increase in UL122 expression in response to SMG.

Regardless, viral load, UL122 transcription and viral titer decrease over time in all our experiments. This indicates a dedicated fate towards latency and demonstrates that SMG alone does not promote or sustain increases in CMV infection and replication, at least not in our Kasumi-3 model. This is further exemplified by experiments where, once latency was assumed by low viral load yields, cultures were returned to SMG with no increases in viral load (data not shown).

Though our experimental design is unique, our observations support previous findings in Kasumi-3 cells where lytic gene expression is activated at early times post infection and then precipitously decreases in later time points during the establishment of latency [17,26,187–189].

Kasumi-3 cells are myeloid progenitor cells that originated from the bone-marrow-blast-cells of a 57 year old Japanese man with minimally differentiated acute myeloblastic leukemia [23]. They were selected for this study because they are CMV negative, they express cell surface markers indicative of myeloid progenitors (CD13, CD33 and CD34), they represent the cells preferentially infected by CMV during initial infection, and most importantly, because they are well suited for CMV infection studies [17,24–26]. Interestingly, studies have shown that though Kasumi-3 cells support the full cycle of CMV lytic replication, they are also capable of shutting down viral replication in order to survive [26], and therefore are prone to supporting latent infection [17]. In retrospect, another cell line or a primary cell source might have been optimal for investigating CMV lytic differences between cells cultured in SMG versus 1G. However, the use of primary cells is not trivial. The CD34+ hematopoietic cell yield, magnetically separated from either of its sources bone marrow or cord blood, is generally small, and within this small population, the number of cells that support infection is even smaller. For that reason, many select to use these cells only when recapitulating their salient findings.

SMG did not seem to promote increased CMV infection or replication on its own in our Kasumi-3 infection model. However, there are other space related elements that could exert influence on CMV reactivation, such as stress and galactic

cosmic reactivation (GCR). Both radiation [190,191] and stress hormones [4,84] have been shown to reactivate viruses on Earth, and future studies should focus on investigating the interaction of these elements with viral reactivation in space. Travel beyond the Van Allen Radiation belt will potentially expose crews to higher concentrations of radiation via GCR, as well as contribute to higher circulating concentrations of stress hormones such as glucocorticoids and catecholamines.

Conclusion

Simulated microgravity (SMG) impedes CMV binding and entry into Kasumi-3 myeloid progenitor cells and pre-treatment of Kasumi-3 cells in SMG for 16 days before infection and maintenance in 1G, results in significant protection against infection for up to 24 hours. SMG also promotes lytic transcription of UL122 in cells successfully infected during SMG. However, viral load, UL122 transcription and viral titer all decrease over time and this indicates a commitment towards latency. Altogether, this seems to demonstrate that SMG alone does not promote or sustain increases in CMV infection and replication, at least not in our Kasumi-3 model of infection.

Chapter 5

Discussion

Here for the first time, we consolidate the significant history and prevalence of CMV reactivation during space flight, as well as present the unique observations from our CMV infection studies done in SMG. CMV reactivates during space flight and this is interesting because it is not a virus that commonly reactivates in immune competent people. This is especially evident in terrestrial analog studies that elicit responses from other herpes viruses, but not CMV. It is possible that CMV has a higher activation threshold, and this may be due to its considerable capacity to generate disease, as devastating the host is antithetical to viral survival. Moreover, the immune system dedicates substantial resources to controlling and/or suppressing the virus. Latent CMV infection preferentially promotes clonal expansion (almost to the point of functional exhaustion) of highly differentiated CMV-specific NK cell and T cell subsets [192,193]. However, in the absence of immune control, CMV infections often lead to inflammation of major organs, as well as major cognitive and functional disabilities. This has been consistently observed in immunocompromised bone marrow and solid organ transplant recipients [14,28]. Unfortunately, space flight negatively affects the immune system, and this poses a significant risk to the health and wellbeing of astronauts, especially as it relates to the inability to control/suppress CMV reactivation [13,101,194]. This is particularly concerning for crew members selected for future exploration class missions that will take them farther from Earth and away from access to advanced clinical care. For this reason, we endeavored to explore

what is known about CMV reactivation in space and what space related factors might be involved in its amplified reactivation.

From our review of the literature, we concluded that, even though astronauts are relatively healthy people whose fitness and wellbeing are intensely scrutinized, over half of them reactivate one or more of the herpes viruses while deployed to space. This is regardless of their mission duration. In fact, 53% astronauts from shuttle-flights and 61% astronauts from ISS missions shed at least one herpes virus in their saliva/urine samples. The viruses that appear to reactivate most often are Epstein-Barr virus (EBV), varicella-zoster virus (VZV), herpes-simplex-1 (HSV-1) and cytomegalovirus (CMV). Not only do most of these viruses reactivate to a greater extent in space than in their comparative analog studies, but the magnitude of their reactivation, indicated by increased viral load, continues to expand with more time spent in space. The frequency of reactivation also increases, for example, VZV shedding increased from 41% of crewmembers from space shuttle missions to 65% of crewmembers from ISS missions, EBV increased 82% to 96%, and CMV increased 47% to 61% [195].

CMV shedding in response to space flight has been evident since the early 2000's [2], where mission samples revealed that CMV DNA is present in the urine of astronauts as early as ten days before launch and continues to increase throughout the space mission. The reactivation of CMV can also be assessed by looking at levels of plasma anti-CMV IgG antibody titers. These anti-CMV IgG titers can rise to eight-fold beyond negative baseline values in space [88]. Seemingly, a feature unique to the space environment is responsible for these heightened responses. With many

potential space specific factors from which to choose, we singled out maybe the most obvious element (microgravity) to assess its contribution.

Simulated microgravity (SMG) was accomplished using the rotating wall vessel (RWV), which continually rotated at 10 rpm about an axis perpendicular to the force vector. This allowed the cells to freefall and to effectively experience a residual 10^{-3} *g*-force. We found that viral load in our SMG treated cultures was significantly lower than in our 1G controls. This indicates that SMG impedes viral expansion or CMV binding and entry into Kasumi-3 myeloid progenitor cells. Further, pre-treatment of Kasumi-3 cells to SMG for 16 days before infecting and maintaining them in 1G, offered them significant protection against infection for up to 24 hours. Both observations potentially indicate that SMG induces a change in Kasumi-3 cell morphology or cell membrane-receptor composition. Epidermal-growth-factor receptors (EGFR), for example, are critical for CMV infection of naïve or uninfected cells [35,183,184], and EGFR expression and activity are sensitive to microgravity [185,186]. Further evidence for reduced EGFR expression might be provided by the observation that lytic transcription of UL122 (IE-2) was higher in infected cells from the SMG condition. It has been shown that internalization and/or degradation of the EGFR supports increased lytic CMV behavior [68].

The EGFR is a transmembrane protein whose activation results in intracellular tyrosine kinase activity. It is essential for homeostatic regulation of cell survival, differentiation and proliferation. However, it has also been shown to play a critical role in modulating both CMV viral replication and latency in CD34+ progenitor cells (PC), with activation favoring long-term latency and inhibition favoring viral reactivation and

replication [68]. CMV viral determinants act opposingly on the EGFR with discrete effects on its endocytic trafficking and activity. UL138 promotes latent infection by sequestering EGFR at the cell surface and preserving its signaling activity, while UL135 promotes reactivation and replication by downregulating EGFR expression and activity [68,187,196]. Though we measured UL138 transcription in both SMG and flasks cultures, we saw no discernable differences in its expression between the two gravity conditions. Altogether, CMV and the EGFR have a complex interaction, whereby CMV manipulates the EGFR, not only to achieve lifelong persistence, but also to be able to sense and respond to changes in the cellular microenvironment. Understanding this relationship has provided researchers with opportunities to investigate novel therapeutics to target this receptor. This was the case in a recent study where researchers used a heparin sulfate mimetic called sulfated pentagalloylglucoside (SPGG) to bind EGFR and subsequently block CMV entry into cells [197].

Nevertheless, in our microgravity experiments, we found that viral load, UL122 transcription and viral titer decrease over time. This indicates a dedicated fate towards latency and seemingly demonstrates that SMG alone does not promote or sustain increases in CMV infection and replication, at least not in our Kasumi-3 infection model. This is supported by experiments where, once latency was assumed by low viral load yields, we reintroduced the cultures to SMG. We found no increases in viral load (data not shown). Though our experimental design is unique because of the SMG component, our observations are supported by previous findings in Kasumi-3 cells where lytic gene expression was activated at early post-infection time points

and then precipitously decreased in later time points during the establishment of latency [17,26,187–189].

Kasumi-3 cells can support the full cycle of CMV lytic replication, but they are also capable of shutting down viral replication in order to survive [26] and are therefore prone to supporting latent infection [17]. They were selected for this study because they are CMV negative, they are CD33 and CD34 positive, they represent the cells preferentially infected by CMV during initial infection, and most importantly, because they are well suited for CMV infection studies [17,24–26]. However, they may not be well suited for longer term lytic infection studies. In future studies, the use of a cell line or primary cell source that is less permissive to latency might be more effective. It would be interesting to see if UL122 expression, and by extension viral titer, would continue to increase in the SMG condition under those circumstances. Unfortunately, primary cells such as monocytes, which are preferentially infected by CMV for dissemination and persistence, are difficult to maintain in long-term culture. This is due to their propensity to differentiate into macrophages and dendritic cells [198]. Spontaneous differentiation in response to their culture environment increases the unwanted probability of highly heterogenous cultures comprised of cells at various stages of differentiation [199,200]. Obviously, this makes experimental results difficult to quantify and highly unreproducible. Further, CMV infection of monocytes promotes their differentiation into macrophages, further complicating the effort for a homogenous culture environment [46,182].

Countermeasures and Future Directions

Though the concern for future exploration class missions persists, two recent International Space Station (ISS) studies have highlighted that pre-flight fitness and current countermeasures might be positively related to decreased incidences of latent herpes reactivation. Agha et al. report that crewmembers with higher pre-flight cardiorespiratory fitness had 29% lower rates of overall herpes virus reactivation [201]. Further, they found that higher preflight upper body muscular endurance was associated with a 39% reduction in EBV and VZV viral reactivation. This included an increasingly delayed viral reactivation and lowered overall magnitude of reactivation. Unfortunately, they were unable to find significant differences between preflight fitness and reduced incidences of CMV reactivation, potentially because only half of their sample population was seropositive for CMV versus almost 100% for other viruses such as EBV and VZV. However, Crucian et al. presented the findings of several years of ISS space flight observations, and conclude that improvements in latent herpes virus reactivation, including CMV, are likely countermeasure-based [202]. They report the results from three separate space flight investigations with performance periods spanning 14 years: Integrated Immune (2006-2012), Salivary Markers (2013-2016) and Functional Immune (2016-present). The cumulative data from these investigations indicate a reduced DNA shedding (reactivation) rate of CMV for the Salivary Markers performance period in comparison to the earlier Integrated Immune period. There have been no observations of CMV shedding in any of the Functional Immune samples. Samples taken for Salivary Markers and Functional Immune studies indicate that VZV shedding is absent, EBV reactivation has trended

downward and HSV-1 seems to be on the rise. Though not stated in their review, the likely reason for the absence of VZV in samples from the latest studies is that crewmembers are now vaccinated against VZV. Further, the emergence of HSV-1 could be directly related to the suppression of VZV. These two viruses are related alpha-herpes viruses that share a latent reservoir in the neuronal nuclei of sensory ganglia, but they are different enough to have adopted unique modes of reactivation and shedding. Perhaps, HSV-1 is now taking full advantage of the absence of its more dominant family member.

Irrespective, Crucian et al. postulate that the evolution of the ISS as a vehicle is responsible for these positive changes, specifically as it relates to the countermeasures implemented overtime. This includes major improvements in exercise equipment, such as the advanced resistance exercise device (ARED), and advances in nutrition, especially the capacity to grow fresh vegetables on ISS. Of course, the current vehicles designed for exploration beyond low Earth orbit will not have the same amenities or support improvements as the ISS. They are considerably smaller with little to no living space, they will have limited communication capacity and finally, less, if any, direct contact with resupply vessels. These conditions mirror the early ISS habitat and are likely to induce similar latent herpes virus reactivation profiles. A condition sure to be exaggerated by exposure to the dangerous galactic cosmic radiation (GCR) beyond the protective shield of the Van Allen radiation belt.

GCR is comprised of different types of charged particles, such as protons (87% hydrogen) and high energy heavy ions, that are emitted by the Sun and galactic sources. While our planet's magnetosphere and the Van Allen radiation belt generally

provide significant protection for astronauts in low Earth orbit (LEO), travelers beyond these natural shields will be at increased risk for adverse health events. GCR particles move at nearly the speed of light and easily penetrate shuttle and space station walls, as well as human skin, cells, and DNA. This ionizing radiation can cause changes in the DNA integrity complicating the cells ability to repair and reproduce itself [203,204]. The cumulative effect of this damage to cells has been associated with health problems including cataracts, cancer, and damage to the central nervous system [205–209]. Further, ionizing radiation has been shown to reactivate viruses both *in vivo* (Hepatitis B and EBV) and *in vitro* (EBV) [190,191,210]. No study, to our knowledge, has observed CMV reactivation in response to radiation. It is therefore imperative that we investigate the impact of GCR (real or synthesized) on CMV latency, reactivation and lytic replication, specifically at the genome/transcriptome level.

Future studies must be concerned with strategies for reducing radiation exposure, or the effects of irradiation, in space, especially as it may relate to prevention of latent viral reactivation. Radioprotective foods and supplements have been recommended by several research teams [207,211]. This includes diets rich in fruits and vegetables, specifically those high in retinoids and vitamins A, C, and E - which proved to be effective for atomic-bomb survivors in Japan [212]. Hormones such as melatonin and glutathione, phytochemicals from plant extracts (including green tea and cruciferous vegetables), and metals (especially selenium, zinc, and copper salts) are also under study as dietary supplements for individuals overexposed to radiation, including astronauts [213]. One commentary actually

advocated for radiation pre-dosing with low doses of ionizing radiation which would reduce oxidative damage in normal tissues [214]. In this commentary, the authors introduce work that showed that low dose radiation stimulates antioxidant production which protects the organism from oxidative damage [215]. The time is now for these exposures, risks and countermeasures to take center stage. In our very near future, we will again seek to trek beyond low Earth orbit, back to the Moon, to Mars and then "to boldly go where no one has gone before!"

Strengths

The strength of our study lies in both its design and duration. We employed a randomized longitudinal experimental design with uninfected and infected 1G controls. Each experiment was carried out multiple times with internal replicates over a three-year time period, so we are confident with our findings. Additionally, many of the results obtained using RWV technology have been confirmed in real microgravity [216–218]. This makes the RWV cell culture system a highly physiologically relevant cell culture model.

Limitations

We also recognize a few limitations to our study. First, the expression or activation of EGFR was not measured for any of our cultures. EGFR was not part of our original design or hypothesis, so our speculation concerning potential EGFR involvement was retrospective. Regardless, measuring its expression would have been helpful in determining whether the SMG related impediment to CMV expansion or the protective effect of 16 days of SMG before infection was related to receptor

density. This should be considered for similar studies done in the future. Second, our lab does not have a FACS-laser cell sorter which is used in many CMV infection studies for the selection and quantification of positively infected cells in culture. That means that we were not able to identify the number or percentages of positively infected cells in either SMG or 1G culture vessels. This potentially complicates the interpretation of our results where amplification is normalized to GAPDH. Lastly, the RWV system might not be optimal for viral transmission studies where cell-to-cell contact is imperative. It is possible that the divergence between our SMG impediment observations and CMV viral expansion in space is due to the diminished contact between cells in our RWV freefall model. In a freefall model of microgravity, especially with a controlled number of cells (infected cells), contact between cells is probably lower than under normal anatomical conditions where cells are less likely to escape contact with each other.

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