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The Mitochondrial Short Circuit:

Regulation of Bax- and Bak-Mediated Cell Death By Murine Cytomegalovirus

By

Lynsey N. Crosby
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Microbiology and Molecular Genetics

_____
Edward S. Mocarski, Ph.D.
Advisor

_____
Samuel H. Speck, Ph.D.
Committee Member

_____
Victor Faundez, M.D., Ph.D.
Committee Member

_____
David S. Weiss, Ph.D.
Committee Member

_____
Anice C. Lowen, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

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By

Lynsey N. Crosby
B.S., Nebraska Wesleyan University, 2008

Advisor: Edward S. Mocarski, Ph.D.

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Abstract

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Cell death is a host defense strategy, limiting viral pathogenesis. Activation of cell death can be initiated extrinsically at the plasma membrane or from intrinsic stress signals, leading to apoptosis or necrosis. Cytomegaloviruses (CMV), members of *Herpesviridae*, efficiently subvert both death pathways, facilitating lifelong persistence within the host. In particular, CMVs encode conserved suppressors that localize to the mitochondria and subvert cell death signals transmitted through this organelle. Stress signals are relayed to the mitochondrial membrane via members of the B cell lymphoma 2 (Bcl-2) family of proteins. Effector Bcl-2 proteins, Bax and Bak, simultaneously permeabilize the mitochondrial membrane upon activation, allowing for the release of proapoptotic proteins that drive apoptosis. Human CMV (HCMV), an opportunistic pathogen and the leading infectious cause of congenital disease, and murine CMV (MCMV), an important model for HCMV, subvert mitochondrial cell death by regulating Bax and Bak. HCMV encodes a single inhibitor, the viral mitochondrial inhibitor of apoptosis (vMIA), from UL37 exon 1 that blocks activation of both Bax and Bak. MCMV, on the other hand, encodes separate inhibitors of Bax and Bak from m38.5, encoding vMIA, and m41.1, encoding the viral inhibitor of Bak oligomerization (vIBO), respectively. Individually, vIBO promotes efficient replication and dissemination in the host, similar to m38.5 (vMIA); however, the combined impact of these two proteins is greater than their individual contributions. Mutant viruses lacking the ability to suppress both Bax and Bak were largely attenuated in terms of replication in macrophages as well as during replication and dissemination in the host, revealing an essential contribution of mitochondrial cell death in limiting pathogenesis. Individual disruption of upstream Bim, Bid, or PUMA did not reveal a single direct activator of Bax and Bak during infection of macrophages; however, elimination of Bid-dependent pathways throughout the host did provide a modest benefit to viral replication. Thus, Bid may activate Bax and Bak in certain cell types infected throughout the host. All together, these results demonstrate that mitochondrial cell death is a potent antiviral host defense mechanism and viral proteins subverting Bax and Bak, such as HCMV vMIA, are crucial to replication and pathogenesis.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 4:

Figures:

CHAPTER 1

Introduction

1.I. Cytomegalovirus Subversion of Mitochondrial Cell Death

1.II. Figures, Tables, and Legends

Figures:

Tables:

1.I. Cytomegalovirus Subversion of Mitochondrial Cell Death

1.I.i. General Mechanisms of Host Defense and the Intersection with Cytomegaloviruses

Viruses are a group of diverse pathogens that carry the basic building blocks for propagation of progeny and maintenance within a host population (Flint et al., 2008). It is this simplicity that makes them dependent upon the host cell for almost the entirety of their lifecycle, encompassing stages such as attachment and entry, uncoating, nucleic acid replication, protein production, assembly and egress, and dissemination in the host. The virus hijacks the host cellular machinery to support these stages of viral replication and dissemination. However, this takeover is met with resistance by the host. The host is equipped with various defense mechanisms designed to limit the stages of viral replication and promote clearance. These mechanisms fall into three broad categories of host defense: (1) cell intrinsic responses that promote pathogen sensing and cell death; (2) innate immune attributes such as cytokine and chemokine functions, natural killer cell activity, and other antiviral mediators; and (3) adaptive immune functions that involve humoral and cell-mediated viral clearance (Daley-Bauer and Mocarski, 2012). The virus must counter these host defense strategies in order to facilitate replication and persistence within a population. Cytomegaloviruses (CMV) are well characterized for their ability to subvert the various aspects of host defense (McCormick and Mocarski, 2012; Wilkinson et al., 2012). In this document we will discuss how human CMV (HCMV) and the mouse model, murine CMV (MCMV), inhibit cell intrinsic responses, particularly mitochondrial cell death, in order promote viral replication and pathogenesis.

1.I.ii. Human Cytomegalovirus, A Member of *Herpesviridae*

HCMV is a member of the evolutionarily ancient family *Herpesviridae* (Davison, 2007), a family of viruses well known for their hallmark features, such as genome size and makeup, virion morphology, and a replication cycle with two phases (i.e. lytic replication vs latency). Members of this family have large double-stranded DNA genomes contained within a protein capsid that is wrapped in a bilayer lipid envelope. The envelope and the capsid are cushioned by a zone of tegument proteins that function at various phases of viral lifecycle, initiating upon attachment and entry into a host cell. Attachment and entry trigger the start of the lytic replication cycle during which time the virus actively generates progeny virions that infect neighboring cells and disseminate throughout the host (Mocarski et al., 2013). During the lytic replication cycle, herpesviruses replicate their DNA genomes in the nucleus as well as assemble the capsid at this same site (Figure 1.1). The replicated genome is encapsidated and undergoes nuclear egress back out into the cytosol. In the cytosol, the viral capsid acquires a lipid envelope and is eventually released out into the extracellular environment. Lytic replication eventually results in termination of the host cell and can stimulate antiviral host responses. Therefore, herpesviruses utilize an alternative phase in their lifecycle known as latency in order to lower the stress on the host cell and host responses. Latency is a period of limited viral gene expression and protein production. The virus does not actively generate progeny during this phase of the lifecycle but merely maintains the DNA genome within the host cell. This period of limited gene expression allows the virus

to efficiently persist for the life of the host, a characteristic of all members in *Herpesviridae*.

Members of *Herpesviridae* infecting humans are divided into three subfamilies, alpha, beta, and gamma, based on cellular tropism as well as other characteristics, such as sequence homology (Davison, 2007). Herpes simplex virus-1 and -2 (HSV-1 and -2) as well as varicella zoster virus (VZV) comprise the alphaherpevirus subfamily, exhibiting a tropism for neuronal cells. Gammaherpesvirus members, on the other hand, establish latency in cells of lymphoid origin and include Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). HCMV is the prototypic member of the betaherpesvirus subfamily that also includes MCMV, the mouse model for HCMV, as well as Human Herpesvirus-6 and -7 (HHV-6 and -7), all exhibiting broad cellular tropism throughout the host.

1.I.iii. Clinical Impact of Human Cytomegalovirus

HCMV is a ubiquitously distributed worldwide, often transmitting via direct contact with bodily secretions, such as saliva, breast milk, urine, and genital secretions (Mocarski et al., 2013). The virus is shed in these bodily fluids for the life of the host during productive phases of lytic replication, facilitating efficient horizontal transmission. Transmission is most frequent in childhood during which time the virus can be shed for months to years, thus allowing children remain a potent source for a prolonged period of time, transmitting HCMV to parents and childcare workers.

Despite this high rate of transmission, HCMV infection is often asymptomatic in immunocompetent individuals (Mocarski et al., 2013). It is in the face of a compromised

or developing immune repertoire that HCMV is clinically detrimental. HCMV is an important opportunist within the immunocompromised populations and is the leading infectious cause of congenital disease. Congenital complications arise due to sensoneuronal damage occurring after vertical transmission of the virus via the transplacental route during pregnancy. These complications include hearing loss, vision impairment, and/or decreased learning capacity. Sensoneuronal damage impacts roughly 12% to 25% of infected newborns (Dollard et al., 2007), with half of the cases leading to disease development in the first few years of life. The transplacental route of transmission is a rare route for other herpesviruses; however, occurs for HCMV in 1% of pregnancies regardless of serostatus (Mocarski et al., 2013). Primary infection during pregnancy is associated with the highest risk of transplacental transmission, occurring in 33% of cases involving HCMV naïve women (Kenneson and Cannon, 2007). Whereas, seropositive women transmit the virus to the developing fetus in only 1% of cases following infection with a new HCMV variant or reemergence of a latent virus. Transplacental transmission as well as the consequences for development underscores the clinical importance of HCMV.

Immunocompromised populations are also at risk of HCMV-induced disease (Mocarski et al., 2013). These populations include solid organ transplant recipients, individuals undergoing immunosuppressive therapies, and genetic or acquired immunodeficient individuals. The currently licensed antiviral therapies used to treat clinically evident disease include ganciclovir/valganciclovir as well as foscarnet and cidofovir, utilized as back-ups. Despite the availability of these therapies, they remain inadequate in many settings involving immunocompromised individuals as well as

congenital disease. Therefore, there is a demand for newly available drugs that can be utilized to treat HCMV-induced disease. In the interim, education about HCMV may promote practices that reduce horizontal transmission as overall awareness of disease burden remains low. Good hygiene practices, such as hand washing, may be one practice used to curb transmission rates and reduce the overall seroprevalance within the population because the virus requires direct contact with bodily fluids to infect a new host.

All in all, it is these clinical implications as well as the tight association between virus and host that make HCMV a pertinent pathogen to investigate. Studies on HCMV lead to new ideas on how to alleviate the disease burden induced by the virus as well as uncover new insights into the host biology.

1.I.iii. Murine Cytomegalovirus as a Model for Human Cytomegalovirus

HCMV has co-evolved with the host for million of years, similar to other herpesviruses, leading to strict species specificity (McGeoch et al., 2000). This species barrier precludes investigations of HCMV pathogenesis in an animal model, thus restricting HCMV investigations to the confines of *in vitro* tissue culture studies. However, questions regarding viral subversion of host defense strategies cannot be addressed in a tissue culture setting. Therefore, seven animal models have been established for HCMV, including chimpanzee, rhesus macaque, tupaia, pig, rat, mouse, and guinea pig (Britt et al., 2012). Among these different models systems, the murine model, MCMV, is the most extensively studied.

MCMV has been studied since the mid-1950's when Margaret Smith pursued investigations of MCMV isolated from the salivary glands of mice (Smith, 1954). These studies complemented the discovery of human salivary gland virus isolated from the urine of newborns experiencing congential HCMV disease (Mocarski et al., 2013). Margaret Smith named the strain she identified after herself as MCMV strain Smith. A second strain, K181, was later isolated from the salivary glands by June Osborn following serial passage of Smith *in vivo* (Hudson et al., 1988; Misra and Hudson, 1980). K181 and Smith have long been utilized as the predominant laboratory strains of MCMV modeling HCMV infection. There are several advantages to utilizing MCMV as a model for HCMV, including a well-defined genome and proteome, the availability of viral mutants for studies in the host, and the availability of different strains and genetically modified mice. However, the most important advantage is the conserved features between the two viruses. HCMV and MCMV share evolutionarily conserved features, such as pathogenesis in the host and modulation of antiviral host responses, despite their divergence millions of years ago. These conserved features will be highlighted through out this document.

1.I.iv. Murine Cytomegalovirus Replication and Pathogenesis

As stated above, horizontal transmission of HCMV occurs following direct contact with the virus shed in the saliva, breast milk, or genital secretions (Mocarski et al., 2013). Following direct contact with these bodily fluids, the virus must first cross the mucosal surfaces of the upper respiratory tract, upper digestive tract, or genital tract (Britt, 2007). Subsequently, the virus undergoes local amplification either at the mucosal

surface or in a cell type nearby. During this replication at the initial site, the virus mobilizes and activates leukocyte populations through a virally-encoded chemokine, vCXCL-1 (Penfold et al., 1999), presumably to recruit the cell type of dissemination. Dissemination allows the virus to traffic from the initial site of infection to tissues throughout the body. A wide range of cell types are susceptible and permissive to HCMV infection due to the wide spread expression of putative cell surface receptors, such as integrins, proteoglycans, and epidermal growth factor receptor (Britt, 2007). Thus, once the virus reaches these tissues it can replicate in a wide range of cell types, excluding B and T cells.

Studies on MCMV have revealed a similar pattern of viral replication within the host as well as lead the way in pointing to important steps in viral pathogenesis and host control. Following infection MCMV replicates at the initial site, reaching peak titers approximately three days post infection (DPI) (Figure 1.2). During this local amplification, MCMV recruits leukocyte populations via a virally-encoded chemokine, MCK-2 (Fleming et al., 1999; Saederup et al., 2001; Saederup et al., 1999), including two monocytic cells populations, patrolling and inflammatory monocytes (Crane et al., 2009; Daley-Bauer and Mocarski, 2012; Hokeness et al., 2005). Inflammatory monocytes modulate the quality of the CD8 T cell response during MCMV infection in order to delay viral clearance (Daley-Bauer et al., 2012), similar to their role in suppressing CD8 T cell function in tumor immunity (Movahedi et al., 2008). Patrolling monocytes, on the other hand, serve as ferries for dissemination to other organs via the bloodstream (Daley-Bauer and Mocarski, 2012; Stoddart et al., 1994). Before these organs can become infected, it is hypothesized that monocytic cell populations trafficking the virus must first

undergo a macrophage/dendritic cell differentiation program in order to become permissive to viral replication. Subsequent sites targeted for viral replication include the spleen, liver, and lungs (Saederup et al., 2001). Peak viral titers can be detected in these organs approximately 6 to 7 DPI (Figure 1.2). Finally, the virus reaches the final site of dissemination, the salivary glands, at 14 DPI. The window between the time of infection to peak titers in the salivary glands is known as acute MCMV replication. Within the salivary glands, viral replication persists for a prolonged period and represents a potent source of transmission. Following clearance from the salivary glands, the virus establishes latency within the host, with myeloid-lineage cells likely serving as a latent reservoir (Koffron et al., 1998; Pollock et al., 1997).

All in all, MCMV, as well as HCMV, are believed to be dependent upon the different host cell types, particularly monocytic cell lineages, for the various phases of the viral lifecycle. The virus depends upon these host cells for replication and release of progeny, dissemination and trafficking throughout the host, maintenance of latency, and, finally, viral shedding and transmission. Therefore, the viability of the host cell is of central importance to the virus. Intrinsic and extrinsic antiviral response pathways are initiated during CMV infection to drive death of the host cell with the goal of terminating viral replication (McCormick and Mocarski, 2012). Premature termination of viral replication via apoptotic or necrotic death pathways reduces viral load throughout the host, collaborating with other arms of immunity to clear viral infection.

1.I.v. Cytomegalovirus Suppression of Apoptosis and Necrosis

CMV encodes several suppressors to subvert both apoptotic and necrotic death pathways (McCormick and Mocarski, 2012). Both modes of death involve a complex circuitry of biochemical pathways that cleave protein substrates to efficiently dismantle the cell (Lamkanfi and Dixit, 2010). In the case of apoptosis, this dismantling morphologically results in cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation. Alternatively, necrosis drives a morphologically distinct breakdown of the cell in which the plasma and nuclear membranes swell, rupture, and release intracellular contents out into the extracellular space.

Apoptosis is dependent upon a family of cysteine proteases known as caspases, (Bao and Shi, 2007), activated following intrinsic stress signals received from the nucleus, endoplasmic reticulum, and other organelles as well as following extrinsic stress initiated at the plasma membrane (Figure 1.3) (Ferri and Kroemer, 2001; Lamkanfi and Dixit, 2010). The extrinsic stress pathway is activated following ligand (i.e. Fas ligand) engagement of a death receptor (i.e. Fas receptor), leading to recruitment and cleavage of initiator caspases, such as caspase-8. Caspase-8 is a potent activator of apoptosis. It certain cell types, caspase-8 can directly cleave effector caspases (i.e. caspase-3) that orchestrate dismantling of the cell, while in other settings, caspase-8 activates the mitochondrial cell death pathways that amplify the apoptotic signal. Both HCMV and MCMV encode an evolutionarily conserved viral suppressor of caspase-8 activation (vICA) from UL36 and M36, respectively (Figure 1.4) (Cicin-Sain et al., 2008; McCormick et al., 2003a; Skaletskaya et al., 2001). vICA interacts with capase-8 and prevents its cleavage and activation, thus subverting the extrinsic apoptotic pathway (Figure 1.3). However, caspase-8 is necessary to suppress RIP3-dependent necrosis

(Kaiser et al., 2011; Oberst et al., 2011). Thus, inhibition of caspase-8 by vICA may drive RIP3-mediated necrosis. To subvert aberrant necrosis, MCMV encodes an additional suppressor known as the viral inhibitor of RIP activation (vIRA) from the M45 gene (Figure 1.4) (Upton et al., 2010). vIRA interacts with RIP3 via a RIP homotypic interaction motif (RHIM) and suppresses RIP3-mediated necrosis activated during infection (Figure 1.3). An HCMV gene product having a function akin to vIRA in MCMV is yet to be identified (Figure 1.4).

Viral suppression of extrinsic apoptosis as well as necrosis is essential to replication and pathogenesis. HCMV as well as MCMV mutant viruses lacking vICA are largely replication deficient in macrophages, a monocyte-derived cell population (Table 1.1) (McCormick et al., 2010; Menard et al., 2003). As stated above, monocytic cell populations disseminate virus via the bloodstream to other sites of infection (Daley-Bauer and Mocarski, 2012; Stoddart et al., 1994). In correlation to this data, MCMV lacking vICA is impaired in terms of replication and dissemination in the host (Cicin-Sain et al., 2008), indicating that caspase-8 inhibition is crucial to maintaining the monocytic cell disseminating the virus. Similarly, vIRA is also central to the MCMV lifecycle, facilitating replication in a variety of different cell types, such as macrophages, fibroblasts, and endothelial cells as well as during replication and dissemination in the host (Brune et al., 2001; Brune et al., 2003; Upton et al., 2010). In fact, MCMV lacking vIRA does not replicate at the initial site of infection, as indicated by the lack of a local inflammatory response (Upton et al., 2010). Thus, vIRA likely supports viral replication in the initial cell type infected, before monocytic populations can be recruited for dissemination.

In addition to subverting extrinsic apoptosis and necrosis, CMV regulates mitochondrial death that amplifies extrinsic stress signals as well as responds to intrinsic stress. The virus regulates this death pathway via virally encoded suppressors (i.e. vMIA and vIBO discussed below) that maintain the integrity of the mitochondrial membrane (Figure 1.3) (McCormick and Mocarski, 2012). Mitochondria are dynamic organelles, controlling both cellular metabolism and ATP generation as well as cell death (Andersen and Kornbluth, 2013). These two functions initially seem contradictory; however, with them the mitochondria act as a central point of control, supporting energy production until transmission of a danger signal, either extrinsically or intrinsically. Upon receipt of a danger signal, the mitochondria efficiently initiate cell death utilizing factors that are common between the two pathways, such as cytochrome *c*. The mitochondrial cell death pathway is mediated via permeabilization of the outer mitochondrial membrane (OMM) (Lamkanfi and Dixit, 2010). This permeabilization allows soluble factors, such as cytochrome *c*, to be released from the intermembrane space (IMM) out into the cytosol (Figure 1.3). Cytosolic cytochrome *c* interacts with apoptotic protease activating factor 1 (APAF1) to activate caspase-dependent apoptosis. Other factors released from the mitochondria, such as Smac/DIABLO and HtrA2/Omi, promote apoptosis by antagonizing inhibitors of this death pathway as well as drive caspase-independent cell death (Lamkanfi and Dixit, 2010; McCormick et al., 2008). Therefore, the virus must maintain the OMM integrity as stress signals converge at this point in the cell.

Evidence to date suggests that suppression of mitochondrial cell death only modestly promotes replication and pathogenesis (Manzur et al., 2009), particularly in comparison to suppression of extrinsic apoptosis and necrosis. However, stress signals are conveyed to the mitochondrial membrane via a complex circuit of proteins that provide two major avenues for activation of the pathway (i.e. Bax- and Bak-mediated cell death discussed below). Studies have only focused on how suppression one pathway promotes the viral lifecycle; therefore, a lot remains to be revealed as to how this death pathway contributes to host defense.

1.I.vi. Communicating Death Signals to the Mitochondria

Stress signals converging on the OMM are relayed in large part through a complex network of B cell lymphoma 2 (Bcl-2) family members (Chipuk et al., 2010). Membership into the Bcl-2 family is based upon the presence of Bcl-2 homology (BH) domains (Figure 1.5) (Chipuk et al., 2010). Bcl-2 family members have at least one BH domain and up to a maximum of four domains. These protein domains provide the basis for the Bcl-2 family members' localization and interactions. Interactions amongst the different members are how the Bcl-2 family regulates the integrity of the OMM and release of apoptotic factors that initiate caspase activation. The Bcl-2 family members ensure that proapoptotic proteins are retained within the mitochondria in a normal, healthy cell and only released upon transmission of a stress signal.

To carry out this responsibility, the Bcl-2 family members are divided as proapoptotic and antiapoptotic (Chipuk et al., 2010). The antiapoptotic members contain four BH domains (BH1-4) (Figure 1.5A) and are located at the OMM, endoplasmic

reticulum, and in the cytosol. The major antiapoptotic members include Bcl-2, Bcl-w, Bcl-2 related gene, long isoform (Bcl-xL), Bcl-2 related gene A1 (A1), and myeloid cell leukemia 1 (MCL-1) (Chipuk et al., 2010). The antiapoptotic proteins preserve the mitochondrial membrane by interacting with proapoptotic members and antagonizing their functions.

The proapoptotic proteins are further subdivided into three groups, effector proteins, direct activators, and sensitizers/de-repressors (Figure 1.5B) (Chipuk et al., 2010). The effector proteins are responsible for driving mitochondrial membrane permeabilization in the event of a stress signal by homo-oligomerization and pore formation within the membrane, allowing the release of proteins, such as cytochrome *c* (Figure 1.3). Effector protein members include Bcl-2 antagonist killer 1 (Bak) and Bcl-2 associated x protein (Bax) (Figure 1.5). A third potential effector protein is known as Bcl-2 related ovarian killer (Bok); however, it is unclear whether Bok has a function akin to Bax and Bak or functions upstream of these two effector proteins (Echeverry et al., 2013). Direct activators and sensitizers/de-repressors only contain the BH3 domain and are hence referred to as the BH3 only members (Chipuk et al., 2010). The BH3 only members are responsible for driving activation of Bax and Bak as well as relieving the antiapoptotic suppression. However, the BH3 only members function in distinct and different manners from one another and are therefore further subdivided based on their interactions with antiapoptotic Bcl-2 members and effector proteins Bax and Bak. BH3 only members that bind to only the antiapoptotic members are known as sensitizers or de-repressors. Bad, Noxa, Bmf, and Bik function in a manner akin to sensitizers/de-repressors, competing for interactions with antiapoptotic Bcl-2 proteins and thus lowering

the antiapoptotic threshold and/or freeing the direct activators. Direct activator BH3 only proteins simultaneously activate Bax and Bak through direct contact as well as interact with antiapoptotic members.

The only direct activators characterized to date include Bim, Bid, and PUMA (Ren et al., 2010). Bim, Bid, and PUMA are highly regulated proteins as their expression and/or activation represents a cell's commitment to mitochondrial-driven apoptosis (Figure 1.6). PUMA is transcriptionally regulated in a p53-dependent or –independent manner (Hikisz and Kilianska, 2012). Stimuli that drive PUMA up-regulation and activation include inducers of the DNA damage response, such as radiation exposure, replication errors, chemical exposure, and oxidative stress. Bim is also transcriptionally up-regulated (Chipuk et al., 2010). The three isoforms of Bim (i.e. Bim-S, Bim-L, and Bim-EL) produced by alternative splicing can be positively regulated in response to cytokine deprivation and ER stress following activation of transcription factors FOXO3A and CHOP, respectively. The Bim isoforms can also be negatively regulated following phosphorylation by ERK1/2 and JNK, leading to ubiquitination and degradation (Ley et al., 2005). Finally, Bid is activated via post-translational cleavage by proteins such as caspase-8 in response to extrinsic cell death (Chipuk et al., 2010), caspase-2 in response to ER stress (Upton et al., 2008), and granzyme B produced by cytotoxic T cells and natural killer cells (Chipuk et al., 2010).

1.I.vii. Viral Suppression of Mitochondrial Cell Death

Viruses naturally stimulate these stress response mechanisms that drive Bim, Bid, and PUMA activation (Castanier and Arnoult, 2011; He, 2006; Hikisz and Kilianska,

2012). Upon activation of Bim, Bid, and PUMA the mitochondrial death pathway can proceed, limiting viral replication. To subvert this death pathway, viruses encode mimics of the antiapoptotic Bcl-2 members often targeting Bax and Bak, the bottleneck for activating apoptosis through the mitochondria (Table 1.2). These viral Bcl-2 (vBcl-2) proteins are encoded by a wide range of viruses, particularly members of large double-stranded DNA virus families, such as *Adenoviridae*, *Poxviridae*, and *Herpesviridae*. Adenovirus E1B-19K (Chiou et al., 1994; Tarodi et al., 1993), murine gammaherpesvirus 68 viral mitochondrial anti-apoptotic protein (vMAP) (Feng et al., 2007), vaccinia virus F1L (Wasilenko et al., 2005), and myxoma virus M11L (Reboredo et al., 2004) are among the best characterized vBcl-2 proteins, contributing to viral replication in a variety of cell types.

The vBcl-2 proteins have historically been identified by homology to at least one of the BH domains, most often the BH3 domain, showing similarity in sequence and/or structure (Castanier and Arnoult, 2011). Further characterization into the mechanisms of the vBcl-2 proteins has relied heavily on biochemistry, showing direct interactions between vBcl-2 proteins and the cellular Bcl-2 family members. While this characterization has identified prime cellular targets, such as Bax and Bak, further questions in to whether viral replication can be restored upon elimination of cellular Bcl-2 pathways remain unanswered. In fact, the contribution of most of these vBcl-2 proteins to the viral lifecycle, particularly in the natural host, remains unknown. Contribution to the viral lifecycle is best characterized for the mitochondrial inhibitors encoded by HCMV and its mouse model, MCMV.

1.I.viii. Cytomegalovirus Suppression of Mitochondrial Cell Death

HCMV and MCMV encode mitochondrial inhibitors that interact with Bax and Bak, subverting OMM permeabilization (Figure 1.3) (McCormick and Mocarski, 2012). HCMV encodes a single Bax/Bak inhibitor, the viral mitochondrial inhibitor of apoptosis (vMIA) from UL37 exon 1 (Figure 1.4) (Arnoult et al., 2004; Karbowski et al., 2006; Poncet et al., 2004). While MCMV has split Bax and Bak regulation into two individual inhibitors; vMIA encoded by m38.5 (Arnoult et al., 2008; Jurak et al., 2008; Norris and Youle, 2008) and the viral inhibitor of Bak oligomerization (vIBO) encoded by m41.1, respectively (Cam et al., 2010).

HCMV vMIA was originally identified from a screen of a cDNA library for viral genes capable of suppressing extrinsic apoptosis (Goldmacher et al., 1999). Since its discovery, UL37 exon 1 (vMIA) has been characterized as the most broadly antiapoptotic CMV cell death suppressor, capable of inhibiting both extrinsic and intrinsic stress stimuli (McCormick and Mocarski, 2012). The function of vMIA is dependent upon its amino-terminal mitochondrial localization signal (amino acids 5-34) and the carboxy-terminal antiapoptotic domain (AAD) (amino acids 118-147) (Figure 1.7) (Hayajneh et al., 2001). The MLS, comprised of the amino-terminal hydrophobic region and adjacent basic residues, directs vMIA trafficking from the endoplasmic reticulum to the OMM (Bozidis et al., 2008; Mavinakere, 2004; Mavinakere and Colberg-Poley, 2004; Mavinakere et al., 2006). After transfer to the OMM, the MLS spans the mitochondrial membrane and the AAD is exposed to the cytoplasmic space (Mavinakere et al., 2006). The AAD does not contain a sequence homolog to one of the known BH domains but folds into an alpha helical structure (Smith and Mocarski, 2005). This alpha helical

structure allows vMIA to interact with Bcl-2 family members such as Bcl-xL (Smith and Mocarski, 2005), Bak (Karbowski et al., 2006), and Bax (Arnoult et al., 2004). Of these, interactions with Bax are the best characterized. vMIA binds Bax at a unique site and sequesters Bax to the mitochondrial membrane (Arnoult et al., 2004; Ma et al., 2012; Poncet et al., 2004). This sequestration prevents Bax insertion into the OMM and subsequent activation. Evidence suggests that an additional protein, growth arrest and DNA damage 45 (GADD45), interacts with vMIA and facilitates inhibition of Bax (Smith and Mocarski, 2005). Additionally, GADD45 enhances the antiapoptotic function of vMIA binding partner Bcl-xL. Thus, vMIA may inhibit Bax activation by forming an antiapoptotic complex with GADD45 and Bcl-xL at the OMM. It is likely that vMIA inhibits Bak in a similar fashion.

vMIA is necessary for efficient viral replication during infection of human fibroblast cells (Table 1.1); however, the degree to which vMIA supports replication varies based on viral strain. vMIA mutations engineered into HCMV strain AD169 leads to severe replication defects (Reboredo et al., 2004; Sharon-Friling et al., 2006; Yu et al., 2003). These replication defects are rescued when caspases are inhibited, indicating that vMIA regulation of Bax and Bak blocks downstream apoptosis during AD169 infection (Reboredo et al., 2004). In contrast, UL37 exon 1 mutations in strain Towne-BAC produce nearly wild type levels of replication, with only a modest defect observed late in the replication cycle (McCormick et al., 2005). The late impact on Towne-BAC replication is due to premature initiation of an HtrA2/Omi-dependent cell death pathway (McCormick et al., 2008) and not caspase-dependent apoptosis (McCormick et al., 2005). Insight into why these different downstream pathways regulated by vMIA vary based on

the context of the strain of HCMV remains unknown. However, more importantly investigations of vMIA functions during viral infection have been confined to fibroblasts. The pathways regulated by vMIA during infection of other permissive and/or susceptible cell types critical to the viral lifecycle have not been analyzed as well as how these pathways influence replication in the absence of vMIA.

The murine model provides some insight into the role of Bax and Bak suppression during infection of other cell types as well as in the host. m38.5 (vMIA) facilitates MCMV replication in fibroblasts and endothelial cells and to a much larger extent in macrophages by suppressing downstream caspase-dependent apoptosis (Table 1.1) (Jurak et al., 2008; Manzur et al., 2009). As discussed above, macrophages and their monocyte precursors play an important role during infection in the host, disseminating virus from the local site of infection to target organs (Figure 1.2) as well as serving as a source of latent virus (Daley-Bauer and Mocarski, 2012). In conjunction, m38.5 (vMIA) facilitates efficient viral replication and dissemination in the host (Manzur et al., 2009). In the absence of m38.5 (vMIA), viral replication is reduced by up to 100-fold in the salivary glands, the final site of dissemination. Together this data suggests that m38.5 (vMIA) supports efficient dissemination by inhibiting apoptosis, particularly in monocytic cells trafficking the virus.

Similar to vMIA, m41.1 (vIBO) is important for viral replication during infection of macrophages (Table 1.1), presumably inhibiting premature apoptosis by blocking Bak oligomerization at the mitochondrial membrane (Cam et al., 2010). vIBO is encoded from a complex locus, comprised of two overlapping reading frames, m41 and m41.1 (Figure 1.8) (Brocchieri et al., 2005). The m41.1 reading frame is entirely embedded

within the larger alternate m41 reading frame and the start codons of the two protein products are separated by a mere seven nucleotides. m41 was annotated in the original analysis of the MCMV genome; however, computational decisions limited the smallest reading frame size to 100 nucleotides, excluding m41.1 (Rawlinson et al., 1996). In the interim, Golgi-localized m41 was described as a cell death suppressor following insertion of a large transposon disrupting the m41 locus (Brune et al., 2003). Transposon mutagenesis of the region produced fragmentation and cellular blebbing as well as increased DNA fragmentation and reduced cell viability during infection, all indicators of premature apoptosis. Upon re-annotation of the MCMV genome (Brocchieri et al., 2005), it was realized that the transposon also disrupted the embedded m41.1 reading frame. Functional re-analysis of the genetic locus, demonstrated that m41.1 interacts with Bak to suppress mitochondrial-mediated apoptosis and to facilitate viral replication, largely re-assigning the cell death suppressor functions to m41.1 (Cam et al., 2010). However, m41 was necessary to sustain viability of macrophages during infection, indicating that the m41 gene product also functions as a cell death suppressor. This role of maintaining cell viability, however, does not impact viral replication in macrophages or fibroblasts. Therefore, it remains unknown whether or not Golgi-localized m41 truly contributes to cell death suppression during infection.

Studies focused on m38.5 (vMIA) and the m41/m41.1 (vIBO) locus have provided a strong base for understanding the role of mitochondrial cell death suppression during viral replication. Our knowledge, however, as to how regulation of this pathway contributes to the viral lifecycle in the host remains very limited, despite the large array of viruses that encode mitochondrial cell death suppressors. To advance this area of

research, we initially address questions regarding how m41.1 (vIBO) individually contributes to viral replication in the host and if its role in supporting viral replication is dependent upon Bak inhibition (Chapter 2). We hypothesize that inhibition of Bak-mediated cell death is necessary for efficient replication and dissemination in the host, similar to m38.5 (vMIA). Furthermore, we tease apart the role of Golgi-localized m41, encoded from the overlapping reading frame, in cell death suppression. We hypothesize that Golgi-localized m41 does not contribute to maintaining host cell viability, as it does not impact viral replication in any cell types analyzed to date (Cam et al., 2010). Therefore, we predict that m41.1 (vIBO) is the only cell death suppressor encoded from the m41/m41.1 locus. Furthermore, we hypothesize that vIBO works in conjunction with m38.5 (vMIA) to play a central role in the viral lifecycle. Individual analysis of vMIA revealed that inhibition of Bax-mediated cell death was necessary to fully support replication and dissemination (Manzur et al., 2009); however, this impact was modest, especially as compared to M36 (vICA) and M45 (vIRA) (Cicin-Sain et al., 2008; Upton et al., 2010), suppressing extrinsic apoptosis and necrosis, respectively (Table 1.1). Nonetheless, the combined contribution of subverting both Bax and Bak has not been evaluated during viral infection. In Chapter 3, we address the combined contribution of vMIA and vIBO to viral replication and determine if suppression of Bax- and Bak-mediated cell death is central to the viral lifecycle. Furthermore, we extend this analysis to determine which direct activator, Bid, Bim, or PUMA, drive activation of Bax and Bak during infection. Bid, Bim, and PUMA are the only three known direct activators of Bax and Bak and we hypothesize that at least one of these activators stimulates the mitochondrial death pathway during infection.

Overall, in this document we hypothesize that mitochondrial cell death is a potent antiviral host defense mechanism and that viral proteins subverting this cell death pathway are central to the viral lifecycle. We believe that results discussed below will not only extend our understanding as to how suppression of this pathway promotes the MCMV lifecycle but also the lifecycle of HCMV as well as other viruses encoding mitochondrial cell death suppressors.

1.II. Figures, Tables, and Legends

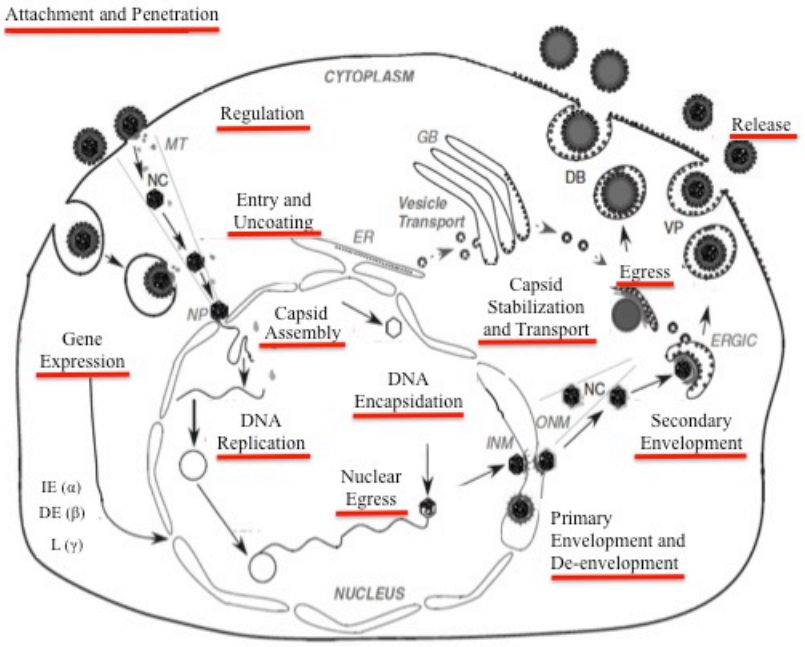

Figure 1.1. The Herpesvirus Replication Cycle. The viral replication cycle initiates with attachment and penetration at the plasma membrane of the host cell. The virus then enters the cell and uncoates, delivering the viral double-stranded DNA genome to the nucleus. In the nucleus the virus expresses immediate early (IE), delayed early (DE), and late (L) genes. These gene products provide functions such as genome replication and capsid assembly. Following DNA replication, the genome is encapsidated and the capsid undergoes nuclear egress. Out in the cytosol, the capsid containing the genome is enveloped and finally released out into the extracellular environment. This image was developed for betaherpesviruses (*adapted from Mocarski et al., 2013*); however, the basic steps described are conserved within *Herpesviridae*.

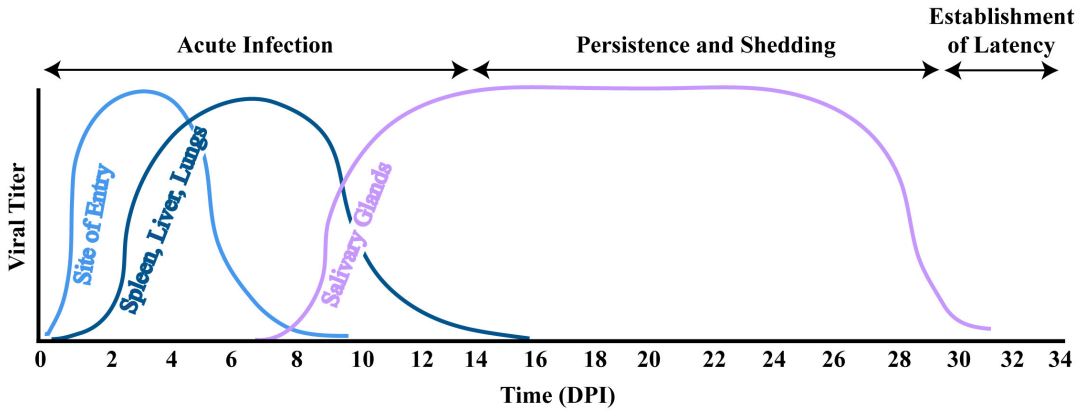

Figure 1.2. MCMV replication kinetics in the host. Acute replication is defined as the window of time following inoculation to peak viral titers in the salivary glands 14 DPI. During the acute phase of infection, the virus replicates at the site of entry/infection and disseminates to the spleen, liver, lungs, and salivary glands. Peak viral titers are observed at each site in accordance to the timing of dissemination. The salivary glands are the final site of dissemination as well as the site in which replication persists for a prolonged period of time. This persistent viral shedding in the salivary glands serves as a potent source of transmission. Following viral clearance from the salivary glands, the virus establishes latency. (*Adapted from E. S. Mocarski*).

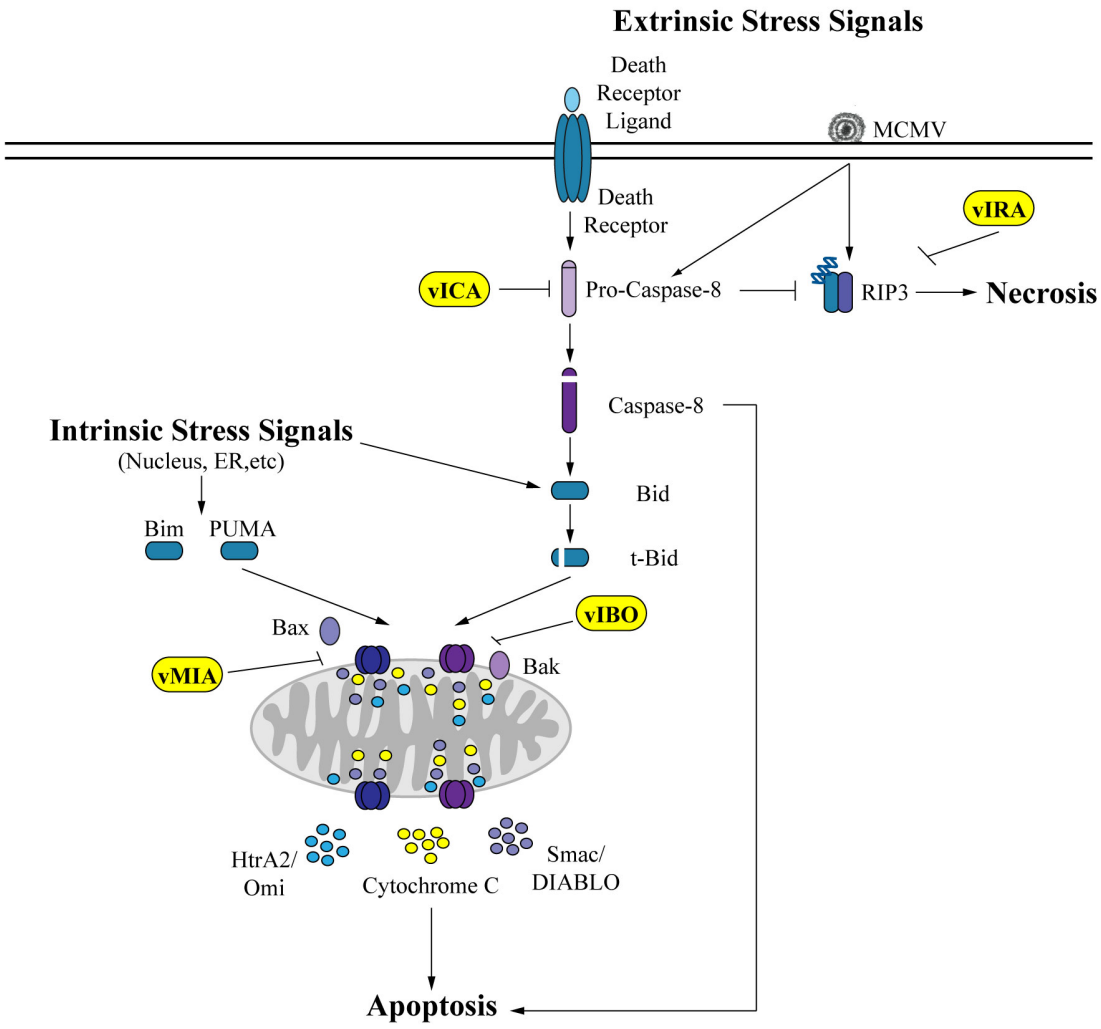

Figure 1.3. Modulation of cell death during MCMV infection. Apoptotic and necrotic cell death pathways are initiated via extrinsic and intrinsic stress signals. MCMV encodes several cell death suppressors to subvert these death pathways. The viral inhibitor of caspase-8 activation (vICA) blocks caspase-8 processing leading to activation of apoptosis, while the viral inhibitor of RIP activation (vIRA) subverts RIP3-mediated necrosis. Two mitochondrial inhibitors, the viral mitochondrial inhibitor of apoptosis (vMIA) and the viral inhibitor of Bak oligomerization (vIBO), suppress Bax and Bak activation, respectively, preventing further amplification and activation of apoptosis.

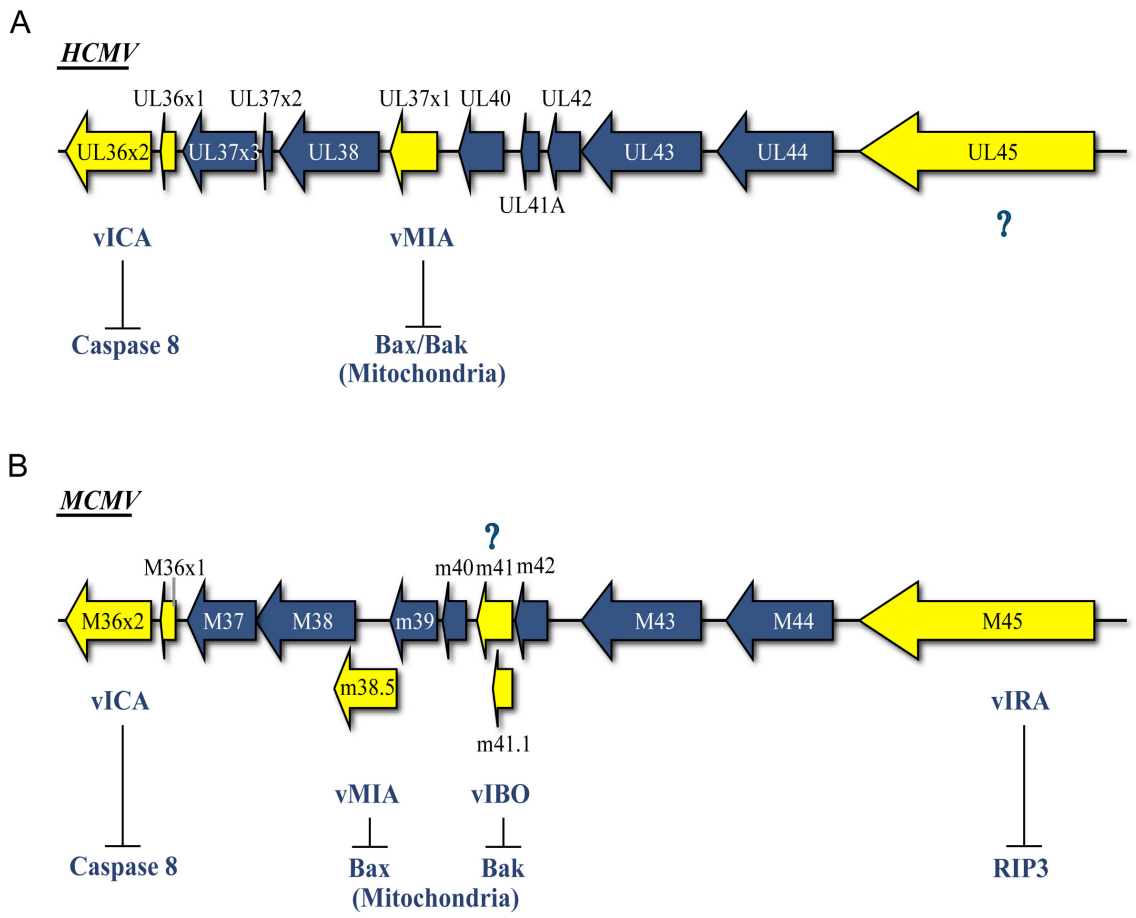

Figure 1.4. Conservation of cell death suppressors encoded by HCMV and MCMV. (A) HCMV and (B) MCMV encode several cell death suppressors that are positionally and functionally conserved. Caspase-8 activation is suppressed by vICA, encoded by HCMV UL36 and MCMV M36. Mitochondrial cell death is suppressed during HCMV infection by vMIA, which suppresses both Bax and Bak and is encoded by UL37 exon 1. MCMV suppresses Bax and Bak through two separate inhibitors, vMIA and vIBO, respectively. vMIA is encoded by m38.5, while vIBO is encoded by m41.1. The m41.1 reading frame is contained within a second reading frame, m41. m41 was originally described as a cell death suppressor; however, its mechanism of cell death suppression is unknown. RIP3-mediated necrosis is subverted during MCMV infection by vIRA,

encoded by M45. It is unknown whether or not HCMV UL45 encodes a protein with vIRA function. (*Adapted from E. S. Mocarski*).

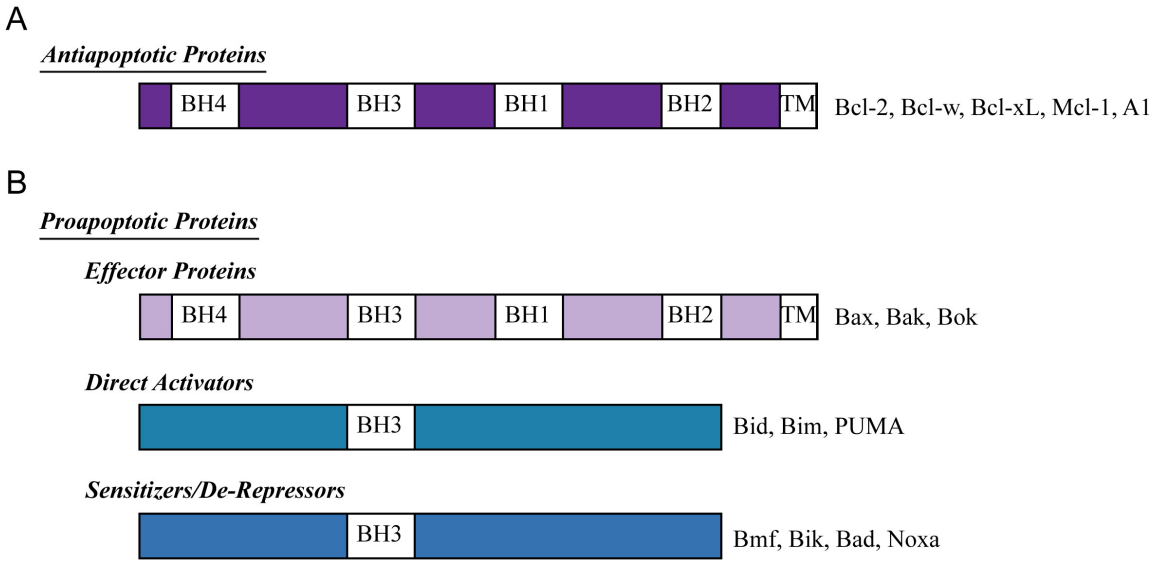

Figure 1.5. The Bcl-2 Family. Membership into the Bcl-2 family is based on the presence of at least one Bcl-2 homology (BH) domain. The members are divided into (A) antiapoptotic, containing BH 1-4 domains and a transmembrane (TM) domain, and (B) proapoptotic. The proapoptotic members are further subdivided into three groups. Effector proteins, Bax, Bak, and Bok, containing BH 1-4 domains and a TM domain, permeabilize the mitochondrial membrane. Director activators, Bid, Bim, and PUMA, can directly interact with the effector proteins and are a BH3 only member. Similarly, the sensitizers/de-repressors, Bmf, Bik, Bad, and Noxa, only contain the BH3 domain and lower the antiapoptotic threshold by interacting with the members in (A). *(Adapted from Martinou and Youle, 2011).*

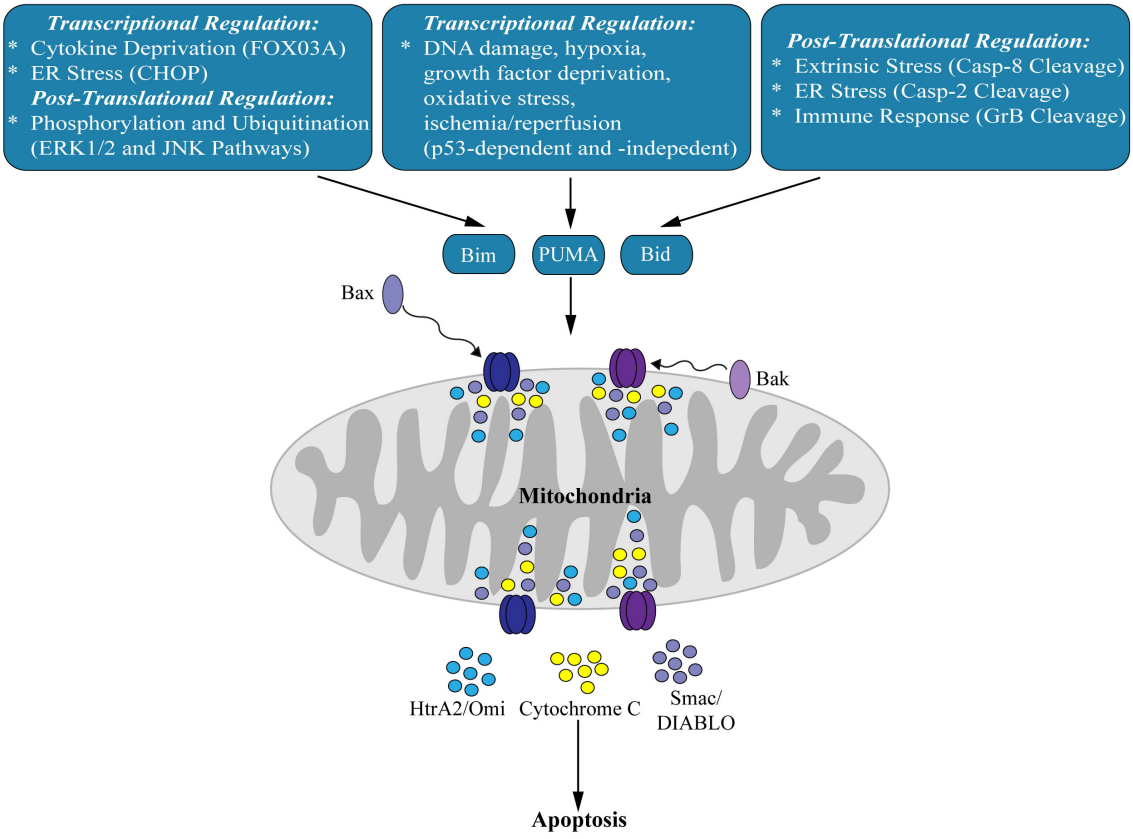

Figure 1.6. Regulation of the Direct Activators, Bim, Bid, and PUMA. The direct activators of the Bcl-2 family are responsible for directly interacting with and activating Bax and Bak and therefore driving mitochondrial cell death. The expression and activation of these proteins is highly regulated both at the transcriptional level and at the post-translational level. Bim expression is up-regulated following cytokine deprivation and ER stress following activation of transcription factors such as FOXO3A and CHOP. Bim expression can also be down-regulated following phosphorylation and ubiquitination. PUMA is up-regulated in response to a wide variety of stimuli, such as DNA damage, in a p53-dependent or –independent manner. Finally, Bid is activated following post-translational cleavage by caspase-8, caspase-2, or granzyme B.

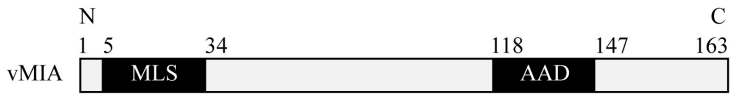

Figure 1.7. Protein Domains of HCMV UL37 exon 1 (vMIA). vMIA is a small protein of 163 amino acids in length. The protein contains two domains required for its localization and function. The mitochondrial localization signal (MLS) (5-34) at the amino terminus is required for proper localization. This domain inserts into the membrane of the mitochondria, exposing the anti-apoptotic domain (AAD) (118-147) out into the cytosol. The AAD forms an alpha helical structure that prevents apoptosis. Inhibition of apoptosis requires recruitment and sequestration of Bax at the mitochondrial membrane. vMIA may also have a similar mechanism of Bak inhibition. (*Adapted from Hayajneh et al., 2001*).

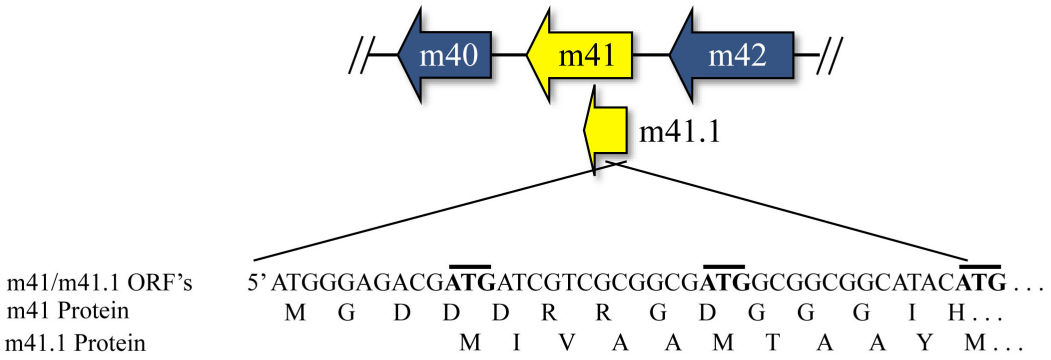

Figure 1.8. Genetic organization of the m41/m41.1 locus. m41.1, encoding vIBO, is embedded within the m41 reading frame, encoding a Golgi-localized protein implicated in cell death suppression. vIBO and Golgi-localized m41 are encoded from alternate reading frames for which the start codons are separate by a mere seven nucleotides. The m41.1 gene contains three potential start codons located towards the 5' end of the reading frame (ATG, bold and over-lined), with protein translation annotated to initiate from the most 5' start codon. However, all three ATG sequences were considered potential start codons for m41.1, particularly during mutagenesis.

Viral Suppressor	Cellular Target	Cell Type(s) with Replication Impact	Impact on Pathogenesis
		HCMV	
UL36 (vICA)	Caspase-8	Macrophages	n/a
UL37x1 (vMIA)	Bax/Bak	Fibroblasts	n/a
		MCMV	
M36 (vICA)	Caspase-8	Macrophages	Severe impact; very limited replication and dissemination
M45 (vIRA)	RIP3	Macrophages, fibroblasts, endothelial cells	Severe impact; very limited replication and dissemination
m38.5 (vMIA)	Bax	Macrophages, fibroblasts, endothelial cells	Modest impact on replication and dissemination
m41.1 (vIBO)	Bak	Macrophages	n/a

Table. 1.1. Impact of cell death suppression on CMV replication and pathogenesis.

Abbreviations: vICA, viral inhibitor of caspase-8; vMIA, viral mitochondrial inhibitor of apoptosis; vIRA, viral inhibitor of RIP activation; vIBO, viral inhibitor of Bak oligomerization; n/a, not available.

Virus	Inhibitor	Cellular Target	Reference
HCMV	UL37x1 (vMIA)	Interacts with Bax and Bak; Inhibits extrinsic and intrinsic cell death	(Arnoult et al., 2004; Karbowski et al., 2006; Poncet et al., 2004)
MCMV	m38.5 (vMIA)	Interacts with Bax to inhibit mitochondrial membrane permeabilization and cell death	(Arnoult et al., 2008; Jurak et al., 2008; Manzur et al., 2009)
	m41.1 (vIBO)	Interacts with Bak to inhibit mitochondrial membrane permeabilization and cell death	(Cam et al., 2010)
γHV-68	vMAP	Interacts with VDAC1 and Bcl-2	(Feng et al., 2007)
	M11	Sequence similarity to BH1 of Bcl-2; inhibits Bax/Bak toxicity in yeast	(Juhasova et al., 2011; Roy et al., 2000)
KSHV	KS-Bcl-2	Suppresses apoptosis similar to Bcl-2 and Bcl-xL	(Cheng et al., 1997)
EBV	BHRF1	Sequentially and structurally similar to Bcl-2 family members; Inhibits apoptosis by extrinsic, *c-myc*, DNA damage, granzyme B, infection, radiation and chemotherapy	(Davis et al., 2000; Fanidi et al., 1998; Huang et al., 1997; Huang et al., 2003; Kawanishi, 1997; Kawanishi et al., 2002; McCarthy et al., 1996)
	BALF1	Interacts with Bax and Bak	(Marshall et al., 1999)
HVS	ORF16	Interacts with Bax and Bak	(Nava et al., 1997)
ADV	E1B-19K	Inhibits apoptosis by TNF; Functionally complemented by Bcl-2	(Chiou et al., 1994; Gooding et al., 1991)
MXV	M11L	Structurally similar to Bcl-2; Interacts with Bax and Bak	(Kvansakul et al., 2007; Su et al., 2006; Wang et al., 2004)
VACV	F1L	Interacts with Bak, Bax, and Bim	(Fischer et al., 2006; Wasilenko et al., 2005)
	N1L	Interacts with Bcl-2, Bid, Bad, Bax	(Aoyagi et al., 2007; Cooray et al., 2007)
FPV	FPV039	Interacts with Bax and Bak	(Banadyga et al., 2007; Banadyga et al., 2009)
PPVO	ORFV125	Sequentially similar to Bcl-2; Interacts with Bik, PUMA, Noxa, Bim, and Bax	(Westphal et al., 2007; Westphal et al., 2009)
ASFV	A179L	Sequentially similar to Bcl-2; Interacts with Bim, Bad, Bmf, Bik	(Galindo et al., 2008; Neilan et al., 1993)

Table 1.2. Viral Bcl-2 (vBcl-2) proteins inhibiting mitochondrial-mediated cell death. Abbreviations: HCMV, human cytomegalovirus; MCMV, murine cytomegalovirus; γHV-68, murine gammaherpesvirus-68; KSHV, Kaposi's sarcoma-associated herpesvirus; EBV, Epstein-Barr virus; HVS, herpesvirus saimiri; ADV, adenovirus; MXV, myxoma virus; VACV, vaccinia virus; FPV, fowl poxvirus; PPVO, parapoxvirus; ASFV, African swine fever virus; vMIA, viral mitochondrial inhibitor of apoptosis; vIBO, viral inhibitor of Bak oligomerization; vMAP, viral mitochondrial anti-apoptotic protein.

CHAPTER 2

Gene Products of the Embedded m41/m41.1 Locus of Murine Cytomegalovirus Differentially Influence Replication and Pathogenesis

Lynsey N. Crosby, A. Louise McCormick, and Edward S. Mocarski

Department of Microbiology and Immunology, Emory Vaccine Center, Emory University School of Medicine, 1462 Clifton Rd NE, Atlanta, GA 30322, USA.

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2.I. Abstract

Cytomegaloviruses utilize overlapping and embedded reading frames as a way to efficiently package and express all genes necessary to carry out a complex lifecycle. Murine cytomegalovirus encodes a mitochondrial-localized inhibitor of Bak oligomerization (vIBO) from m41.1, a reading frame that is embedded within the m41 gene. The m41.1-encoded mitochondrial protein and m41-encoded Golgi-localized protein have both been implicated in cell death suppression; however, their contribution to viral infection within the host has not been investigated. Here, we report that mitochondrial-localized m41.1 (vIBO) is required for optimal viral replication in macrophages and has a modest impact on dissemination in infected mice. In contrast, Golgi-localized m41 protein is dispensable during acute infection and dissemination as well as for latency. All together, these data indicate that the primary evolutionary focus of this locus is to maintain mitochondrial function through inhibition of Bak-mediated death pathways in support of viral pathogenesis.

2.II. Introduction

Cytomegaloviruses (CMVs) are members of *Herpesviridae*, an evolutionarily ancient family of double-stranded DNA viruses. Human CMV (HCMV), a major infectious cause of birth defects and an important cause of opportunistic disease, and murine CMV (MCMV), a natural murine pathogen and important model for HCMV, have complex genomes, encoding over 167 gene products (Mocarski, 2007). As a part of this large genomic landscape, CMV employs overlapping and embedded reading frames to express functions necessary to carry out a complex lifecycle. From a single genetic locus, MCMV encodes mitochondrial and Golgi-localized proteins, m41.1 and m41, respectively (Brocchieri et al., 2005; Brune et al., 2003; Cam et al., 2010). The m41/m41.1 locus is comprised of the m41.1 open reading frame (ORF) embedded entirely within the larger, m41 ORF. Functionally, both proteins have been implicated in suppression of cell death during viral infection (Brune et al., 2003; Cam et al., 2010).

Programmed cell death is an ancient cell-intrinsic host defense strategy to limit pathogen replication and spread (Muñoz-Pinedo, 2012). Viruses evolved to encode suppressors of death pathways to sustain cells and facilitate infection in the host (Mocarski et al., 2011). Large DNA viruses, such as CMVs, are well equipped to modulate these defense pathways and encode inhibitors of caspase 8 and other components of the death pathways to prevent signal transduction to downstream effectors. MCMV-encoded cell death suppressors, in particular, have revealed the importance of apoptotic (Cicin-Sain et al., 2008; Manzur et al., 2009) as well as necrotic (Upton et al., 2010, 2012) cell death pathways in host defense. Due in large part to the evolutionary conservation of cell death suppressors encoded by betaherpesviruses, these

studies have facilitated understanding the contribution of cell death suppression in HCMV pathogenesis (McCormick and Mocarski, 2012).

Viral proteins regulating mitochondrial-mediated cell death are among the conserved modulatory functions encoded by HCMV and MCMV (Arnoult et al., 2004; Arnoult et al., 2008; Cam et al., 2010; Jurak et al., 2008; Norris and Youle, 2008; Poncet et al., 2004). Stress signals, largely relayed through Bcl-2 family member proteins, converge upon the mitochondrial membrane via two pro-apoptotic Bcl-2 family member proteins, Bax and Bak (Danial and Korsmeyer, 2004). Bax and Bak collaborate to drive membrane permeabilization, resulting in the release of cytochrome *c* and other pro-apoptotic factors that activate executioner caspases, driving apoptosis. HCMV encodes the viral mitochondrial inhibitor of apoptosis (vMIA) from the UL37x1 gene. vMIA regulates Bax- and Bak-mediated apoptosis (Arnoult et al., 2004; Karbowski et al., 2006; McCormick and Mocarski, 2012) as well as an HtrA2 serine protease-dependent death pathway (McCormick et al., 2008). MCMV encodes two separate inhibitors, one targeting Bax and one targeting Bak. The m41.1 gene product, encoded from the embedded m41/m41.1 locus introduced above, is a mitochondrial-localized protein that functions as a dedicated viral inhibitor of Bak oligomerization (vIBO) (Cam et al., 2010), working in parallel with m38.5-encoded vMIA, a specific inhibitor of Bax (Arnoult et al., 2008; Jurak et al., 2008; Manzur et al., 2009; Norris and Youle, 2008). These MCMV-encoded inhibitors of Bax and Bak promote viral replication by suppressing cell death (Cam et al., 2010; Jurak et al., 2008; Manzur et al., 2009; McCormick et al., 2005). Inhibition of Bak-mediated cell death by vIBO influences the levels of viral replication in macrophage cell lines but is completely dispensable in fibroblasts (Cam et al., 2010). The

Bax inhibitor, m38.5 (vMIA) has been studied extensively in tissue culture for its role in facilitating viral replication in macrophage, fibroblast, and endothelial cells (Jurak et al., 2008; Manzur et al., 2009; McCormick et al., 2005). Furthermore, Bax-mediated cell death suppression influences virus levels in infected leukocytes during infection of the natural mouse host (Manzur et al., 2009). These studies highlight the importance of m41.1-encoded vIBO as well as m38.5-encoded vMIA in the infection of particular cell types, but also point to the absolute need to study infection in the natural host.

In contrast to m41.1, the function of the underlying m41 gene product is unclear. The m41 reading frame encodes a putative Type II transmembrane protein that localizes to the Golgi apparatus (Brune et al., 2003) and continued to be implicated in cell death suppression during infection of macrophages even after the recognition of m41.1 function (Cam et al., 2010). Death pathways may be initiated extrinsically at the plasma membrane or intrinsically through stress signals sensed by cellular organelles, including the mitochondria as well as the Golgi apparatus (Ferri and Kroemer, 2001; Hicks and Machamer, 2005). Despite growing knowledge of death pathways and how different organelles sense and respond to these pathways, the mechanism of m41 cell death suppression at the Golgi apparatus during infection remains unknown because the gene is dispensable for replication in permissive macrophage as well as fibroblast cell lines (Cam et al., 2010). However, the role of this gene product has not been evaluated in infected mice where the full spectrum of replication, dissemination, and latency places an increased demand for viral genes that exhibit a modest impact in cell culture.

Cell culture analysis has revealed mechanistic insights into the m41/m41.1 locus, particularly related to the function of m41.1-encoded vIBO. However, the contribution of

this protein to the overall viral lifecycle during natural infection has not been assessed. Furthermore, the overlapping m41 ORF, also implicated in cell death suppression (Brune et al., 2003), may also contribute to MCMV pathogenesis and should be evaluated separately. Here, we show that m41 function, originally implicated as a cell death suppressor (Brune et al., 2003), is dispensable for cell death suppression, replication, dissemination, and latency. In contrast, m41.1-encoded vIBO contributes to both viral replication and dissemination during acute infection in mice, affirming the contribution of Bak-mediated cell death to host defense and extending the evaluation to naturally infected mice.

2.III. Results

m41.1-encoded vIBO is necessary for viral replication in macrophages.

Individual mutations were engineered into m41 and m41.1 to evaluate the function of these gene products individually (Figure 2.1A), employing a strategy of selection/counterselection and an intermediate Δm41/m41.1-BAC with a Kan^R/SacB cassette in place of the entire m41/m41.1 locus. Counterselection replaced this cassette with point mutations intended to disrupt the expression of either Golgi-localized m41 protein or m41.1-encoded vIBO. To generate the m41 mutant virus, an in-frame stop codon and the insertion of two nucleotides to frame shift and generate a novel AfeI restriction site were introduced within m41, without affecting the m41.1 (vIBO) ORF. The resultant mutant virus (m41.StopFS-BAC) retains the m41 reading frame with potential to produce a truncated, 66 amino acid protein (Figure 2.1A). The truncated m41 protein product lacks the transmembrane domain required for localization to the Golgi apparatus (Brune et al., 2003; Cam et al., 2010). To eliminate the expression of the embedded m41.1 ORF without disrupting m41 expression, point mutations were inserted into the three potential start codons, changing the m41.1 coding sequence from methionine to a threonine to prevent translation initiation (Figure 2.1A). This mutant virus was called m41.1.ΔStart-BAC. The introduced point mutations did not modify the underlying m41 protein sequence but created an additional PvuI restriction site that was utilized for screening purposes. MR-BAC, used together with K181-BAC as control virus in our studies, was generated from m41.StopFS-BAC by replacing the mutations with a wild-type locus subsequent to re-inserting the Kan^R/SacB cassette. The genomic integrity of all mutant and control BAC clones was assessed by restriction fragment length

polymorphism (RFLP) with five restriction enzymes and found to have the correct structure (Figure 2.1B and data not shown). Furthermore, the insertion of the intended mutations was confirmed by digestion of PCR products amplified from the m41/m41.1 locus with AfeI, the restriction site introduced into the m41 mutant, and PvuI, introduced into the m41.1 mutant virus (Figure 2.1C). Digestion with AfeI produced two products of the expected sizes, 0.8 and 0.2 Kbps, from the m41 mutant virus while the other viruses lacked this restriction site. Similarly, digestion with PvuI produced two products from the m41.1 mutant virus of the expected sizes, 0.6 and 0.4 Kbps, which were not observed with the other viruses. Note that the Δm41/m41.1-BAC PCR product is increased in size due to the insertion of the large Kan^R/SacB cassette. Taken together, the viral BAC clones were found to have the intended structure and mutations.

The replication properties of the m41- and m41.1-deficient viruses were compared to the parental K181-BAC and derived MR-BAC as controls. Cells were infected at a low multiplicity of infection (MOI), 0.05 for fibroblasts, 0.1 for endothelial cells, and 0.5 for macrophages, to yield a similar proportion of cells initially infected, and total viral yields were determined on different days post infection (DPI) by plaque assay. As expected (Brune et al., 2003), these mutant viruses exhibited replication kinetics similar to control viruses in NIH3T3 fibroblasts (Figure 2.2A), although there was a slight delay in the replication of the vIBO-deficient virus. Similarly, both mutant viruses demonstrated replication comparable to control viruses in SVEC4-10 endothelial cells (Figure 2.2B). Together, these data demonstrate that m41 and m41.1 are individually dispensable for viral replication in both fibroblast and endothelial cells.

m41 and m41.1 gene products had a differential impact on viral replication levels and kinetics in both primary and established macrophages (Figure 2.2C-E). During infection of the RAW264.7 macrophage cell line, the vIBO-deficient virus produced titers that were 10- to 50-fold lower than controls (Figure 2.2C), implicating inhibition of Bak-mediated death in efficient viral replication. This phenotype was even more dramatic in J774A.1 macrophages, where mutant viral titers were 50- to 1000-fold lower than K181-BAC or MR-BAC at late times of infection (Figure 2.2D). This phenotype also extended to primary bone marrow-derived macrophages (BMDM) where mutant viral titers were more than 10-fold lower than controls (Figure 2.2E). In contrast, the Golgi-localized m41 protein appeared to be dispensable for replication in macrophages. Mutant virus replication was similar to control viruses in RAW264.7 (Figure 2.2C) and J774A.1 (Figure 2.2D) macrophage lines as well as primary macrophages (Figure 2.2E). The slight difference in m41-mutant viral titers in RAW264.7 cells at 7 and 9 DPI (Figure 2.2C) was not observed in either J774A.1 or primary macrophages, so was not investigated further. Taken together, these data demonstrate that m41.1-encoded vIBO is necessary for efficient viral replication in macrophages while Golgi-localized m41 did not have any impact at all, consistent with an earlier report (Cam et al., 2010).

Inhibition of Bak-mediated death is necessary for maintenance of cell viability and viral replication

m41.1-encoded vIBO was previously identified as critical to viral replication in macrophages and, separately, shown to control infected cell viability (Cam et al., 2010). While instructive, this work left open the question of Bak-dependent control of MCMV

replication in macrophages, an important question, given the apparent specificity of vIBO and the relatively large deficit in replication of m41.1.ΔStart (Figure 2.2C-E). As expected from previous work (Cam et al., 2010), m41.1-encoded vIBO sustained viability of infected macrophages (Fig. 2.3A-C). That is, cell viability of RAW264.7 (Figure 2.3A), J774A.1 (Figure 2.3B) and primary BMDM (Figure 2.3C) was reduced during m41.1-mutant viral infection compared to controls. Furthermore, when infected with this mutant virus, $Bak^{-/-}$ BMDMs showed restored viability (Figure 2.3D), consistent with the Bak-specificity of vIBO when used to block apoptosis in MCMV-infected fibroblasts treated with staurosporine (Cam et al., 2010). In addition to eliminating a need for vIBO in suppressing cell death and extending our current understanding, $Bak^{-/-}$ BMDMs also normalized the replication of vIBO-deficient virus (Figure 2.3E-F). Thus demonstrating that the m41.1 (vIBO) specifically counteracts Bak-dependent death in macrophages in order to sustain viral replication. This is the first report directly linking inhibition of Bak-mediated cell death to efficient viral replication and therefore provides insight into the role of m41.1 during infection.

Finally, Golgi-localized m41 protein, which had been implicated in sustaining macrophage viability when this region was first studied (Brune et al., 2003), does not contribute to viability of either established macrophage cell lines or primary BMDMs in our hands (Figure 2.3A-C). Therefore, m41 is dispensable for viral replication and maintenance of cell viability.

Analysis of viral mutants that modulate host cell or host immune functions may be fleshed out only after analysis in the natural host animal. While cultured cells provide a useful platform for evaluation of intrinsic host defense pathways, including those that

promote cell survival in the host, the analysis is by definition limited to the cell types chosen. In contrast, the viral lifecycle within the host animal involves replication in additional cell types as well as cell differentiation states that are not reflected in cultured cells. Because cell culture may not accurately reflect nuances required for virus dissemination and persistence/latency in the natural host, the studies thus far may not have been sufficient to uncover all contributions of either gene product. Therefore, to more fully dissect the contributions of m41 and m41.1 gene products in viral pathogenesis, mutant viruses were studied further in infected mice.

m41 is dispensable for acute infection in mice.

To assess the contribution of Golgi-localized m41 protein to replication and dissemination in the host, footpad inoculation was used to initiate infection in BALB/c mice. Footpad inoculation introduces virus at a site distal to target organs in contrast to other routes, such as intraperitoneal (IP), intravenous, or intranasal inoculation. Replication at the site of inoculation as well as in the spleen, liver, lungs and salivary glands was assessed over the first two weeks of infection. This time frame is required for control virus to reach peak titers in the salivary glands, a key organ in evaluating MCMV pathogenesis as it is the final site of viral dissemination and source of transmission. Following infection, the footpad of mice infected with m41-mutant virus showed patterns of swelling, a well-characterized aspect of footpad inoculation indicating a robust viral chemokine-dependent inflammatory response (Saederup et al., 2001), as well as replication that was indistinguishable from control virus (Figure 2.4A and data not shown). Viral levels above the limit of detection for either the control or mutant were not

detected in spleen or liver (data not shown). However, mutant virus disseminated to salivary glands and produced titers that increased between 5 and 14 DPI, reaching the same high levels as control (Figure 2.4B).

Salivary gland-derived stocks exhibit significantly increased virulence over tissue culture-derived virus, particularly in terms of the levels of viral replication in target organs such as the lungs. This is a well-recognized feature of MCMV (Osborn and Walker, 1971; Selgrade et al., 1981). We utilized salivary gland-derived stocks in combination with IP inoculation, thereby inducing a systemic infection, to evaluate the impact of m41 in an organ other than the salivary gland. Salivary gland-propagated viral stocks of m41-mutant virus established a productive infection in the lungs (Figure 2.4C) but not in other organs (data not shown), similar to control virus. In contrast, mutant virus levels in the lungs were not sustained to the level of control virus after IP inoculation (Figure 2.4C), an observation that was also made following footpad inoculation with cell culture stock virus (Figure 2.4D). It is important to emphasize that this difference was only observed at late times of infection, approximately 14 DPI or later, and suggested a consistent influence of m41 on clearance of virus from lungs, independent of virus stock or route of inoculation. Despite this behavior, mutant virus reached the final site of dissemination, the salivary glands, and replicated at levels similar to the control (Figure 2.4E). Replication within the salivary glands appears to be independent of host strain as mutant virus was detected in this organ at levels similar to the control in mice on a C57BL/6 background (data not shown), a mouse strain that controls MCMV through a predominate NK cell response contrasting with CD8 T cell control in BALB/c mice (Arase et al., 2002; Brown et al., 2001; Koszinowski et al., 1990; Lee et al., 2001; Smith

et al., 2002). Thus, m41 appears to be dispensable for acute viral replication and dissemination in the intact host, independent of mouse strain.

m41 is dispensable for reactivation from latency.

Following primary infection, herpesviruses establish latency for the life of the host. Replication can recur following reactivation from latency and subsequent progeny can be transmitted to new hosts. Although MCMV gene products controlling cell fate may impact this aspect of viral lifecycle, little experimental analysis has been undertaken in this area. To determine whether m41 contributes to latency, BALB/c mice were inoculated via the IP route, and, after 45 days, the spleen and lungs were harvested and subjected to an explant reactivation assay. Supernatant from the explant cultures was collected over time to detect reactivated virus by plaque assay. The m41-mutant virus reactivated almost as frequently as control virus from explanted spleen (Figure 2.5A) or lung (Figure 2.5B), with only one animal failing to reactivate mutant virus. Importantly, actively replicating virus was not detected in the spleen, lung or salivary glands when a portion was titered at the time of explant (data not shown), affirming that virus harvested from the explant cultures represented reactivated virus. Thus, m41 is dispensable for latency.

Suppression of Bak-dependent death contributes to acute viral replication in the host.

To investigate suppression of Bak-dependent death on viral replication and dissemination, BALB/c mice were inoculated IP with m41.1-mutant virus, and virus in salivary glands and lungs was titered on 5, 10 or 14 DPI. vIBO-deficient virus exhibited

wild-type levels of replication in the lungs of BALB/c mice at 5 DPI (Figure 2.6A); however, at 10 DPI vIBO-mutant virus titers were 10-fold lower in the lungs than the other viruses (Figure 2.6B). This attenuation was significant ($p < 0.01$) in comparison to the K181-BAC at this time. These results indicate that the Bak inhibitor modestly influences maintenance of viral replication in the lungs. In correlation to the lungs, vIBO-mutant virus replication was modestly compromised in the salivary glands, where levels were up to 10-fold lower than control viruses at 10 DPI (Figure 2.6C) or 14 DPI (Figure 2.6D). Parental K181-BAC titers were higher than the other viruses at 14 DPI in this experiment, although such variation was not observed in other experiments (see Figure 2.2B and E). Despite these elevated titers of the parental control, vIBO-mutant viral titers remained significantly reduced in the salivary glands in comparison to all controls ($p < 0.05$).

The role of vIBO in dissemination was also analyzed as MCMV traffics through the blood to the salivary glands and lungs in a subset of monocytes that are recruited during infection by a virally-encoded chemokine (Fleming et al., 1999; Saederup et al., 2001; Saederup et al., 1999; Stoddart et al., 1994). To investigate the role of vIBO during dissemination, viremia was analyzed at early times of infection. vIBO-deficient virus was reduced in the blood at 5 DPI, the period of peak viremia (Saederup et al., 1999), as compared to parental K181-BAC (Figure 2.6E). Four animals infected with vIBO-mutant virus had levels of viremia at or below the limit of detection, while all K181-BAC infected animals had detectable virus in the blood at this time. Overall, the absence of vIBO compromised dissemination within the host, consistent with reduced titers in the lungs and salivary glands.

To determine if the contribution to viral replication in the host is due to the interaction of vIBO with Bak, we next characterized the behavior of vIBO-deficient virus in Bak-deficient mice generated on a C57BL/6 x 129 mixed lineage. Studies in $Bak^{-/-}$ BMDM led us to expect a normalization of the modest defects observed in BALB/c mice. However, following IP inoculation of B6129SF2/J WT strain, there was less than a 10-fold difference in mean titer between m41.1-mutant and control virus in the salivary glands at 14 DPI, a modest difference that remained the same in Bak-deficient mice (Figure 2.6F). It must be noted that this mixed background creates a setting where the antiviral immune response is more complicated than reflected in either purebred C57BL/6 or 129 strains as well as in the susceptible BALB/c strain. The pressures upon MCMV replication in the different host strains may alter the necessity of vIBO and its interaction with Bak. Despite these results, the analysis of m41.1/vIBO in BALB/c mice revealed a role in acute viral replication, thereby promoting MCMV pathogenesis.

2.IV. Discussion

In this study, we examined the impact of two different gene products encoded from alternative ORFs within the m41/m41.1 locus: one (m41) expressing a Golgi localized protein of unknown function and the other (m41.1) encoding vIBO, a suppressor of Bak-dependent death. vIBO supports efficient viral replication in macrophages by suppressing Bak-dependent cell death; however, this activity provides only a modest benefit in enhancing peak viral levels in lungs and salivary glands in the infected mouse. The Golgi-localized m41 protein has no impact on replication or cell death suppression in macrophages and is dispensable for replication, dissemination, and latency in the host.

Previous investigations (Cam et al., 2010) of Bak suppression by m41.1 have focused on infection of fibroblasts, a setting in which m41.1 is dispensable for viral replication. Replication in macrophages was shown to be dependent upon m41.1 in these studies; however, the mechanism in which this gene product supported viral replication remained to be uncovered. Here we demonstrated that inhibition of Bak-mediated cell death by m41.1 (vIBO) is necessary for viral replication in primary macrophages. These results were generated independent of agents, such as staurosporine, that induce exogenous apoptosis and therefore represent a Bak-dependent pathway triggered by natural viral infection. Taken together with previous observations (Cam et al., 2010), Bak-triggered cell death appears to be an independent antiviral host defense mechanism active in macrophages that must be suppressed by vIBO for virus infection to continue at full efficiency. Although the trigger of death during infection remains to be established, recent identification of the DNA sensor DAI in virus-induced programmed necrosis

(Upton et al., 2012) leaves open a role for pathogen sensors in the induction of cell death pathways. Furthermore, an additional note that can be made is given that RAW264.7 macrophage cells are deficient of apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC), a crucial component of inflammasome signaling (Pelegrin et al., 2008), our data also indicates that ASC plays no role in the pathway impacted by vIBO.

Rather, the Bak-dependent impact of vIBO in sustaining viability during infection of cultured macrophages, combined with modest impact of vIBO on acute viral titers in salivary glands, lungs, and blood seems consistent with long-recognized roles for monocyte-derived macrophages in the pathogenesis of viral infection (Fleming et al., 1999; Saederup et al., 2001; Saederup et al., 1999; Shanley and Pesanti, 1980; Stoddart et al., 1994). Monocyte-derived macrophages are responsible for dissemination to the salivary glands, a site of persistent viral replication and source of transmission, and also serve as a reservoir for latent CMV (Hanson and Campbell, 2006). Latent CMV can be found in several organs throughout the host, including the salivary glands and lungs. Upon differentiation to macrophages, monocyte precursors transition from being a vehicle of viral maintenance and dissemination to actively producing progeny virions that infect neighboring cells. Therefore, an impact in macrophages may alter patterns of replication, dissemination, and latency. Thus regulation of Bak-mediated death in a subset of permissive cells, particularly macrophages, contributes to the overall maintenance of MCMV pathogenesis.

Bak inhibition through vIBO has less of an impact than Bax inhibition by vMIA, encoded by m38.5 (Arnoult et al., 2008; Jurak et al., 2008; Manzur et al., 2009; Norris

and Youle, 2008), and neither of these mitochondrial inhibitors impacts viral pathogenesis as profoundly as the viral caspase 8 inhibitor, vICA (Cicin-Sain et al., 2008) or the viral RIP inhibitor, vIRA (Upton et al., 2010, 2012). Given the fact that m38.5 mutants, like the m41.1 mutant virus described here, influence viability of infected macrophages but no other cell type, it seems likely that vMIA and vIBO, respectively, have overlapping roles in cell death suppression even though they evolved independently in MCMV. HCMV vMIA, the UL37x1 gene product, while recognized as an inhibitor of Bax-mediated cell death (Arnoult et al., 2004; Poncet et al., 2004), also interacts with Bak (Karbowski et al., 2006), further supporting an overlap in regulation of Bax- and Bak-mediated death pathways. A full understanding of the contribution that Bax and Bak, as well as inhibitors that are specific for these pro-apoptotic Bcl2 family members, will likely emerge from further studies comparing the behavior of MCMV mutant viruses with individual mutations and the m38.5/m41.1 double mutant.

The role of m41 in MCMV pathogenesis remains an enigma. Originally, Golgi-localized m41 was implicated in cell death suppression, particularly in macrophages (Brune et al., 2003), but this assignment occurred prior to the recognition of embedded m41.1-encoded vIBO (Cam et al., 2010). Here we show that m41 does not contribute to cell death suppression in cultured fibroblasts, endothelial cells, or macrophages, and that m41 mutant viral infection in the host is largely indistinguishable from infection with control viruses. Of course, it is possible that m41 has a function that is linked to m41.1, which would only emerge in a study of m41/m41.1 double mutant virus. Preliminary data suggests that the intermediate insertion mutant, Δm41/m41.1-BAC, carrying a Kan^R/SacB cassette, is more attenuated than either independent mutant. However,

because of the potential for this large insertion to alter gene expression outside of the m41 locus, we believe it is best to continue to evaluate this separate issue of a combined influence of these viral genes with more precisely designed mutations. Nevertheless, it is possible that m41 localizes to Golgi bodies to modulate the activity of vIBO at the mitochondria. Therefore, ongoing research is focused on determining whether m41 and m41.1 collaborate in any way.

In summary, we have demonstrated that m41.1-encoded vIBO influences viral dissemination and replication specifically in lungs and salivary glands, sites important in viral pathogenesis and transmission. A number of issues remain to be resolved, including (i) the cell type(s) sensitive to Bak-mediated cell death within the host, (ii) the combined contribution of mitochondrial cell death suppression by m41.1 (vIBO) and m38.5 (vMIA) to MCMV pathogenesis, and (iii) the possible interaction between m41 and m41.1 (vIBO) functions.

2.V. Materials and Methods

Cells

NIH3T3 fibroblasts (ATCC CRL-1658), 3T3-SA fibroblasts (ATCC CCL-92), RAW264.7 macrophages (ATCC TIB-71), and J774A.1 (ATCC TIB-67) were maintained at 37°C with 5% CO_2 in DMEM containing 4.5 g/mL glucose, 1 mM sodium pyruvate, 10% fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen). Bone marrow-derived macrophages were harvested from B6 × 129P2 backcrossed to C57BL/6 mice for three generations (referred to as C57BL/6) and maintained in house as well as B6 × 129-Bax^{tm2Sjk} $Bak1^{tm1Thsn/J}$ mice (referred to as $Bak^{-/-}$) provided by Douglas Green (St. Jude Children's Research Hospital). Pooled bone marrow cells were flushed from tibias and femurs and differentiated for 7 days in DMEM containing 20% fetal bovine serum and 10% filtered L929 medium containing macrophage colony-stimulating factor (M-CSF). Cells were harvested by scraping, seeded into 48-well plate at a density of 8.5×10^4 cells per well, and then cultured at 37°C with 5% CO_2 at least 18 hours before infection.

BAC Mutagenesis and Recombinant Viruses

Plasmid pSIM6 encoding the λ red recombination functions (Datta et al., 2006) was introduced into *Escherichia coli* strain DH10B harboring the MCMV strain K181 genome (Redwood et al., 2005). Mutations were introduced in to the m41/m41.1 locus of K181-BAC via recombineering protocols as previously described (Tandon and Mocarski, 2008). The following primers were used to insert the selection/counterselection Kan/SacB cassette: Kan/SacB-F 5'

aatagtcatccgatgatcgtgtcgccgcccgaccgccctcctcccccaattaggcccgtagtctgcaaatcc; Kan/SacB-R 5’ cgccgtttcctcacattccgttgtcgtgcgcaggttcctccgaacctttggaaataccgcacagatgcgtaagg. The Kan/SacB cassette was subsequently replaced with the m41/m41.1 locus containing point mutations in m41 (primers: m41.StopFS-F 5’ ctccgacgaagatgacagcgcttcctaggaaccgttgtctcc; m41.StopFS-R 5’ ttaggagacaacggttcctaggaagcgctgtcatcttcgtcggag; homology region-F 5’ tagtcatccgatgatcgtgtcgccgcccgaccgccctcctcccccaattcatctgtcaatgatcacga; homology region-R 5’ cgccgtttcctcacattccgttgtcgtgcgcaggttcctccgaacctttgatgggagacgatgatcgt) or m41.1 (primers: m41.1.ΔStart-R atgggagacgacgatcgtcgcggcgacggcggcggcatacacggctctggctctgcc; homology region-F 5’ tagtcatccgatgatcgtgtcgccgcccgaccgccctcctcccccaattcatctgtcaatgatcacga; homology region-R 5’ tttcctcacattccgttgtcgtgcgcaggttcctccgaacctttgatgggagacgacgatcgtcgcggcg). All BAC-derived mutant and parental clones were diagnostically screened by PCR over the m41/m41.1 locus (primers: m41/m41.1-F 5’ agtcatccgatgatcgtgtcgccgcccgac; m41/m41.1-R 5’ ccgaaccggagcaccgtttgcctac) and restriction fragment length polymorphism (RFLP) analysis by five restriction enzymes. Following proper genetic characterization of the BAC-derived clones, mutant and parental viruses were reconstituted by transfection of NIH3T3 fibroblasts. Cell-associated and supernatant virus were collected and subjected to three rounds of plaque purification. Viral isolates were screened after the first round of plaque purification for the loss of GFP signal and repair of m06-m07 following excision of the BAC insert (Redwood et al., 2005) by PCR (primers: BAC-F 5’ ctcaagcgatacggcaagcggctc; BAC-R 5’ gcgagtgacgggtcgcatccgttag). Following plaque purification, mutant and parental viruses were propagated on NIH3T3

fibroblasts, clarified, concentrated, and titered by plaque assay on 3T3-SA fibroblasts. Salivary gland-derived virus was generated by IP inoculating BALB/c mice with plaque purified, tissue culture-derived K181-BAC or m41.StopFS-BAC virus. Salivary glands were removed 14 DPI following CO_2 asphyxiation and placed in 1:1 mixture of DMEM and sterile milk. Salivary glands were disrupted by sonication and centrifuged at 2300 × g for 10 minutes at 4°C to clarify the salivary gland/DMEM portion from milk mixture. Following clarification, viral stocks were stored in DMEM at -80°C and titered by plaque assay. Growth curves and viral yields were performed by infecting cells in 24-well plates at the indicated multiplicity of infection (MOI) in 0.25 mL for 1 hr at 37°C. Following adsorption, the viral inoculum was replaced with complete DMEM. Samples were harvested at the indicated times and titered by plaque assay.

Cell Viability Assay

Cells (20,000 cells/well) were seeded into 96-well plate in quadruplicate. Approximately 18 hours post seeding, medium was replaced with 50 µl of virus inoculum containing 10 PFU/cell (MOI 10). At the indicated times, cell viability was determined by measuring the ATP levels using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) according to manufacture's protocol.

Mice, Infections, and Organ Harvests

BALB/c mice were obtained from Jackson Laboratory. B6 x 129-Bax^{tm2Sjk} Bak1$^{tm1Thsn/J}$ mice were provided by Douglas Green (St. Jude Children's Research Hospital) with permission from Jackson Laboratory and used in combination with

B6129SF2/J mice (Jackson Laboratory) for *in vivo* experiments. Five to eleven-week old mice were inoculated in one hind footpad or IP with either 10^4 PFU of salivary gland-derived virus or 10^6 PFU of tissue culture-derived virus as previously described (Saederup et al., 2001). The inoculum titers were confirmed after thaw at the onset of animal experiments. At the time of sacrifice, organs were placed in 1 mL of complete DMEM and stored at -80°C until thawed, disrupted by sonication, and titered by plaque assay.

Peripheral blood leukocytes (PBL) were isolated from blood collected via heart puncture in syringes loaded with 0.5 M EDTA (Cellgro). Blood was diluted in PBS, layered onto Histopaque-119 (Sigma) density medium, and centrifuged (30 min, 700 x g, 25°C). Cells within the band at the plasma-Histopaque interface were removed and washed in PBS. Erythrocytes were lysed in a solution containing 1.5 M NH_4Cl_2, 0.1 M $NaHCO_3$, and 0.01 M Na_2-EDTA. Viable cells were counted on a hemacytometer using trypan blue exclusion and evenly divided into three tubes contained 1 mL DMEM. PBL/DMEM suspensions were overlaid onto 3T3-SA fibroblasts to perform an infectious center assay.

Latent Infection and Explant Reactivation

For latent infections and explant reactivation, BALB/c mice were inoculated with 10^6 PFU by IP injection. At the time of sacrifice, one lung and half the spleen were collected for evaluation by plaque assay in order to detect actively replicating virus. The other lung and half of spleen were used to establish explant cultures in duplicate as

previously described (Stoddart et al., 1994). Virus titers were assessed at weekly intervals in the culture supernatants by plaque assay.

2.VI. Figures and Figure Legends

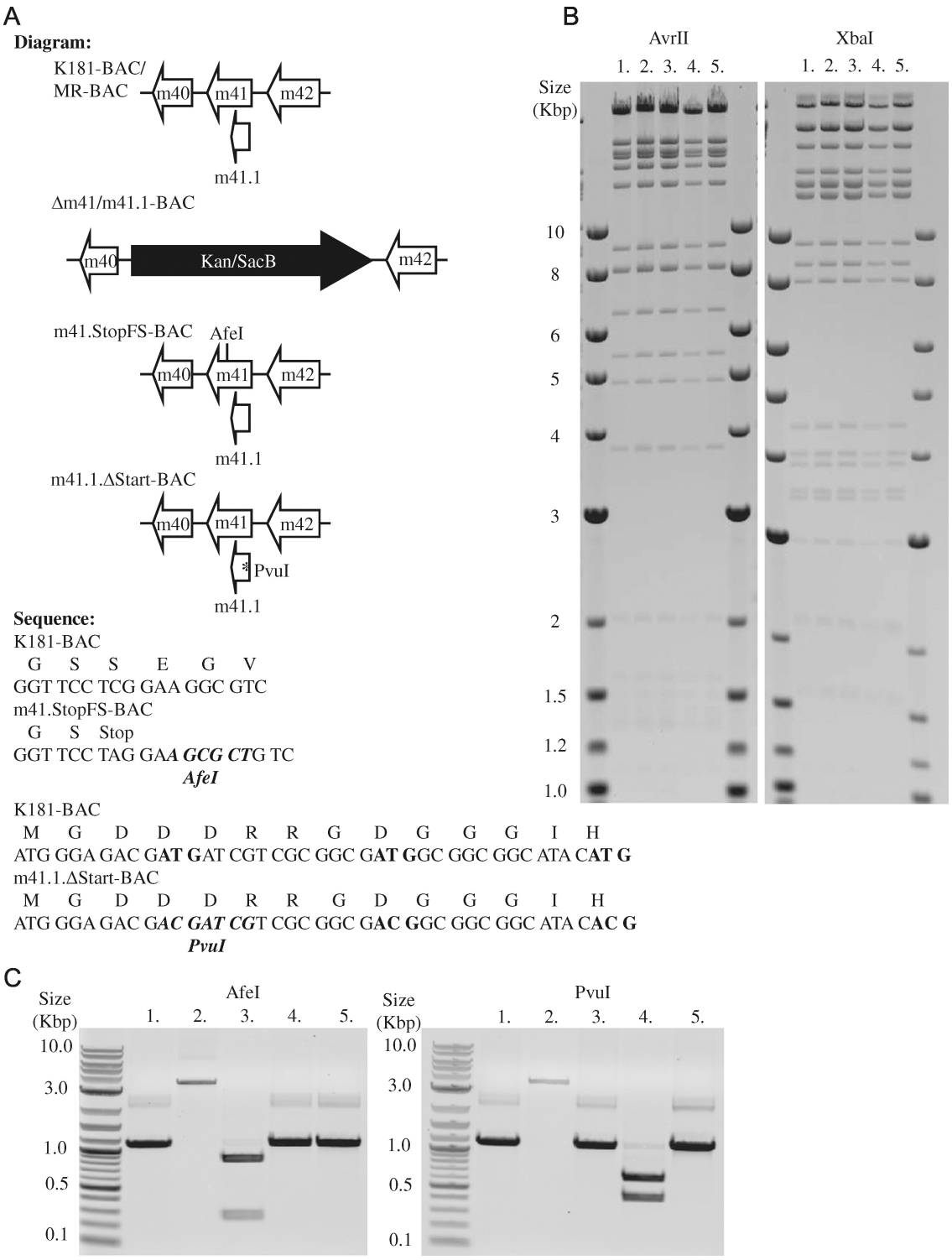

Figure 2.1. Mutagenesis of the m41/m41.1 locus. (A) Representation of the m41/m41.1 mutations introduced into K181-BAC. The m41/m41.1 locus was replaced with a KanR-

SacB cassette (Δm41/m41.1-BAC) that was used to derive m41.StopFS-BAC and m41.1.ΔStart-BAC. The marker rescue (MR-BAC) contains a wild-type m41/m41.1 locus, similar to K181-BAC. Sequence alignment shows the nucleic acid changes made to insert a stop codon and a diagnostic AfeI restriction site in m41, creating m41.StopFS-BAC. To mutate m41.1, point mutations were made in the three potential start codons to encode a threonine. Additionally, a diagnostic PvuI restriction site was inserted. These are silent mutations in m41. (B) Electrophoretic separation of AvrII- and XbaI-digested K181-BAC DNAs on 0.6% agarose as a diagnostic for integrity of the genomes. (C) Electrophoretic separation of AfeI- and PvuI-digested PCR products generated by amplification of the m41/m41.1 locus in each of the derived viruses as a diagnostic for the intended mutations in m41 or m41.1. (B-C) K181-BAC (lane 1), Δm41/m41.1-BAC (lane 2), m41.StopFS-BAC (lane 3), m41.1.ΔStart-BAC (lane 4), and MR-BAC (lane 5).

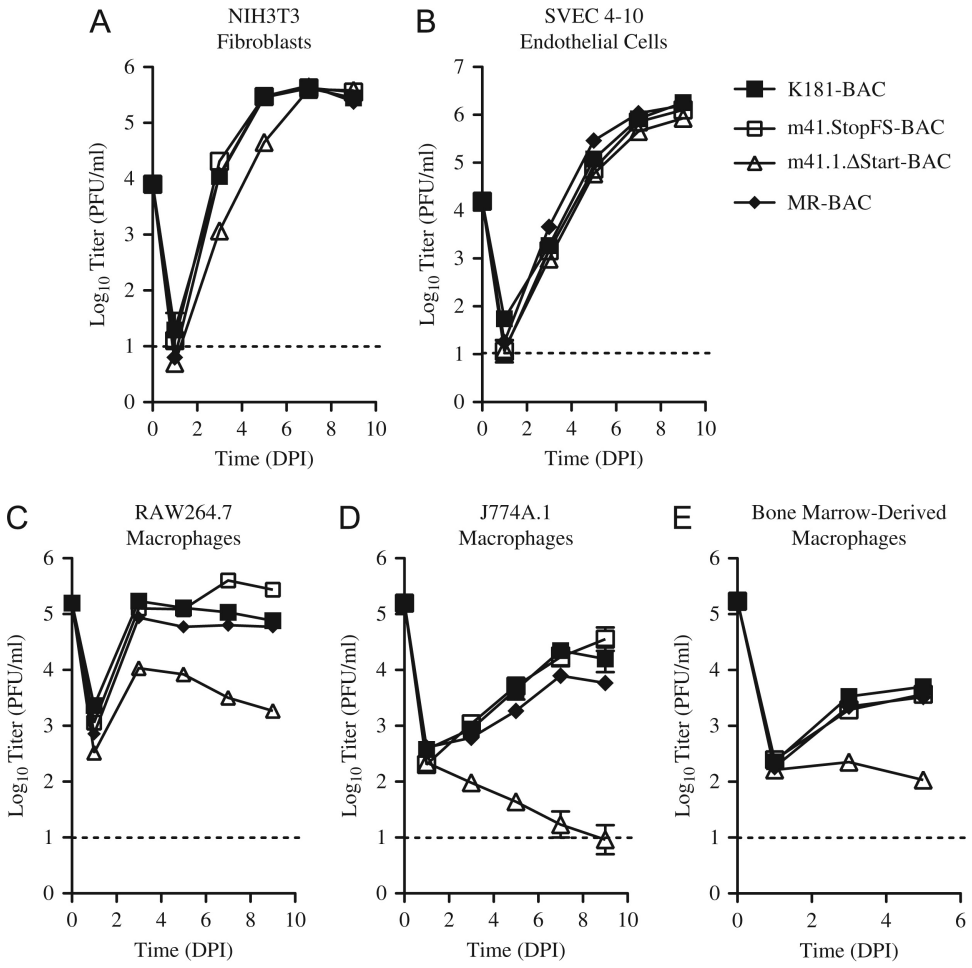

Figure 2.2. Replication kinetics in murine fibroblast and macrophage cell lines. (A) NIH3T3 fibroblast, (B) SVEC 4-10 endothelial cells, (C) RAW264.7 macrophages, (D) J774 macrophages and (E) bone marrow-derived macrophages. NIH3T3 fibroblasts and SVEC 4-10 endothelial cells were infected at an MOI of 0.05 and 0.1, respectively, while macrophages were infected at an MOI of 0.5. Total virus was collected at subsequent times post infection. Titers of cell sonicates were determined by plaque assay on 3T3-SA fibroblasts. Each symbol represents the average of three replicate samples. The dotted line indicates the limit of detection.

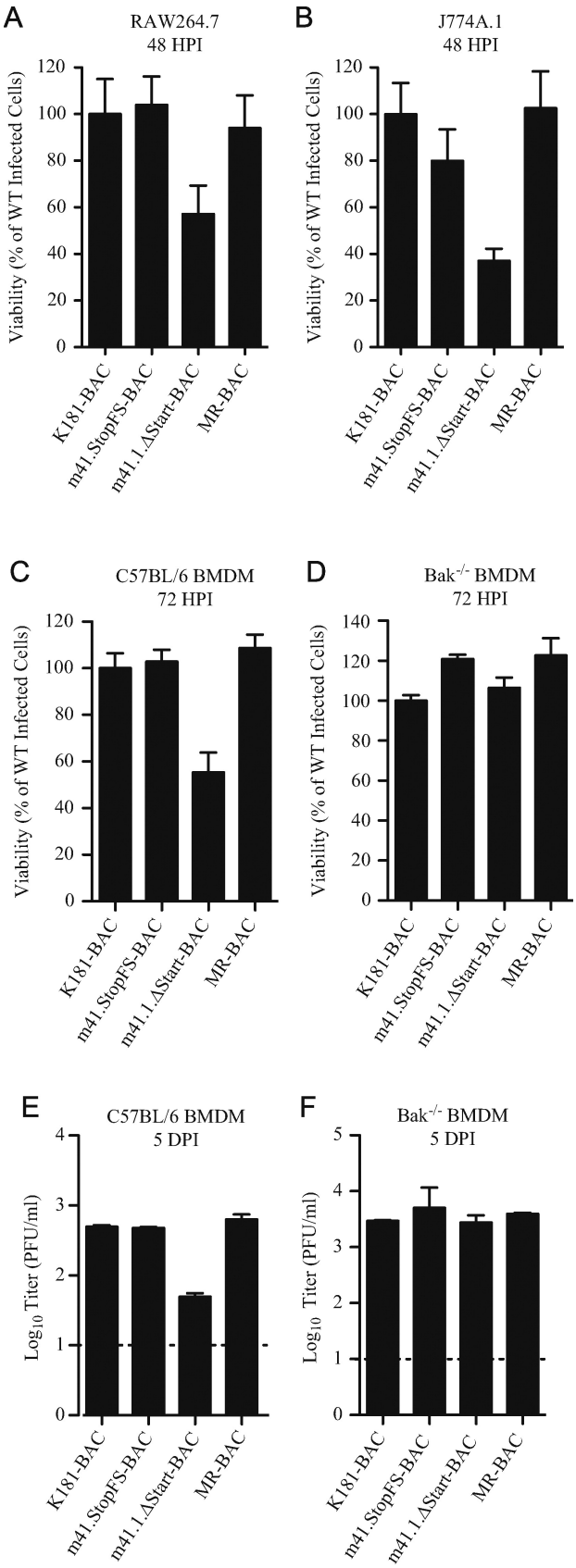
A
RAW264.7
48 HPI
Viability (% of WT Infected Cells)
120
100
80
60
40
20
0
K181-BAC
m41.StopFS-BAC
m41.1.ΔStart-BAC
MR-BAC
B
J774A.1
48 HPI
Viability (% of WT Infected Cells)
120
100
80
60
40
20
0
K181-BAC
m41.StopFS-BAC
m41.1.ΔStart-BAC
MR-BAC
C
C57BL/6 BMDM
72 HPI
Viability (% of WT Infected Cells)
120
100
80
60
40
20
0
K181-BAC
m41.StopFS-BAC
m41.1.ΔStart-BAC
MR-BAC
D
Bak-/- BMDM
72 HPI
Viability (% of WT Infected Cells)
140
120
100
80
60
40
20
0
K181-BAC
m41.StopFS-BAC
m41.1.ΔStart-BAC
MR-BAC
E
C57BL/6 BMDM
5 DPI
Log10 Titer (PFU/ml)
4
3
2
1
0
K181-BAC
m41.StopFS-BAC
m41.1.ΔStart-BAC
MR-BAC
F
Bak-/- BMDM
5 DPI
Log10 Titer (PFU/ml)
5
4
3
2
1
0
K181-BAC
m41.StopFS-BAC
m41.1.ΔStart-BAC
MR-BAC

Figure 2.3. Analysis of viability and viral replication during infection of macrophages. (A) RAW264.7 and (B) J774A.1 macrophage cell lines as well as (C) C57BL/6 and (D) $Bak^{-/-}$ BMDMs were infected at an MOI 10. Cell viability was assessed at the indicated times. Viability was measured as a percent of WT, K181-BAC, infected cells. Each bar represents the average viability obtained from four replicates. (E) C57BL/6 and (F) Bak-/- BMDMs were infected at an MOI 0.5. Total virus was collected 5 DPI and titers of cell sonicates were determined by plaque assay on 3T3-SA fibroblasts. Each bar represents the average titer obtained from three replicates. The dotted line indicates the limit of detection of virus.

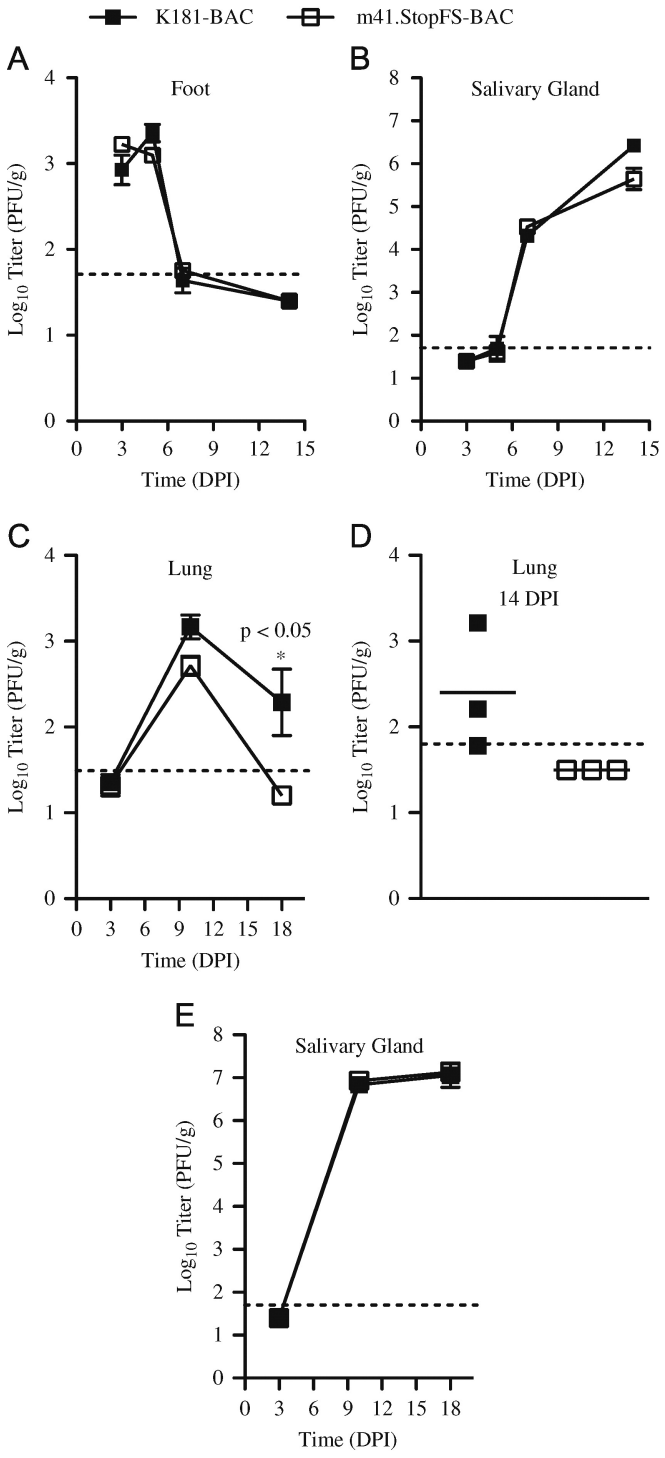

Figure 2.4. Replication pattern for m41.StopFS-BAC in the host. (A, B, D) Footpads of 6-week-old BALB/c mice were inoculated with 10^6 PFU of cell culture stock virus. Each symbol represents the average titer obtained from three mice in panels A and B. In

panel D each symbol represents the titer from an individual mouse. (C, E) 6-week-old BALB/c mice and were inoculated IP with 10^4 PFU of salivary gland-derived virus. Each symbol represents the average titer obtained from five mice. For all experiments, organs were collected over time and titers in organ sonicates were determined by plaque assay on 3T3-SA fibroblasts. The dotted line indicates the limit of detection of virus.

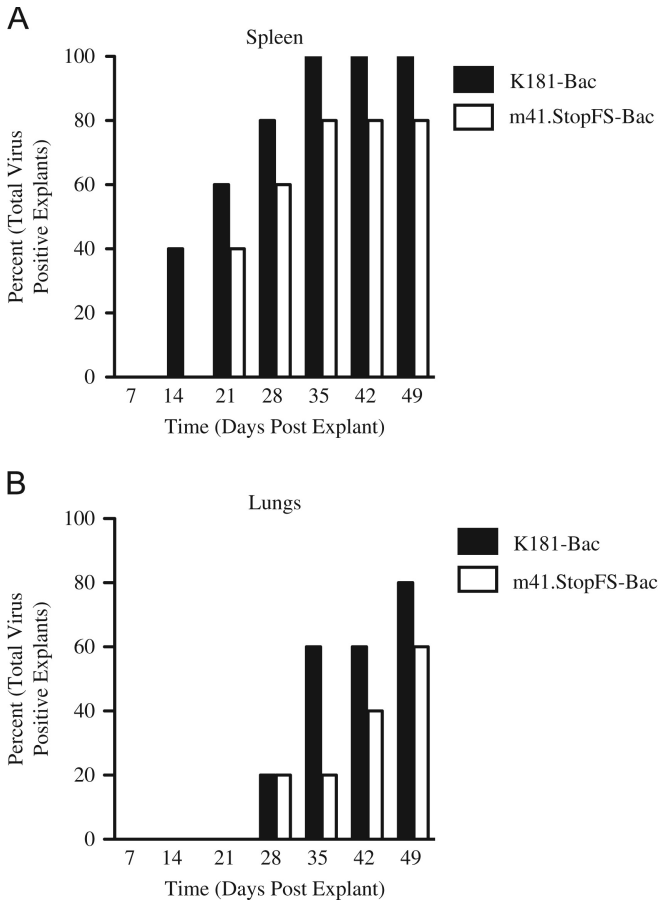

Figure 2.5. Evaluation of the role of the m41 during reactivation in BALB/c mice. 6-week-old BALB/c mice were inoculated with 10^6 PFU via the IP route. Viral reactivation in the spleen and lung was assessed at 45 DPI. Half of the (A) spleen and (B) one lung was harvested and further divided in half to create duplicates. These portions were further diced into smaller pieces and placed in culture. Supernatents were removed periodically

post explant and overlaid onto 3T3-SA fibroblasts to determine the presence of reactivated virus by detected CPE.

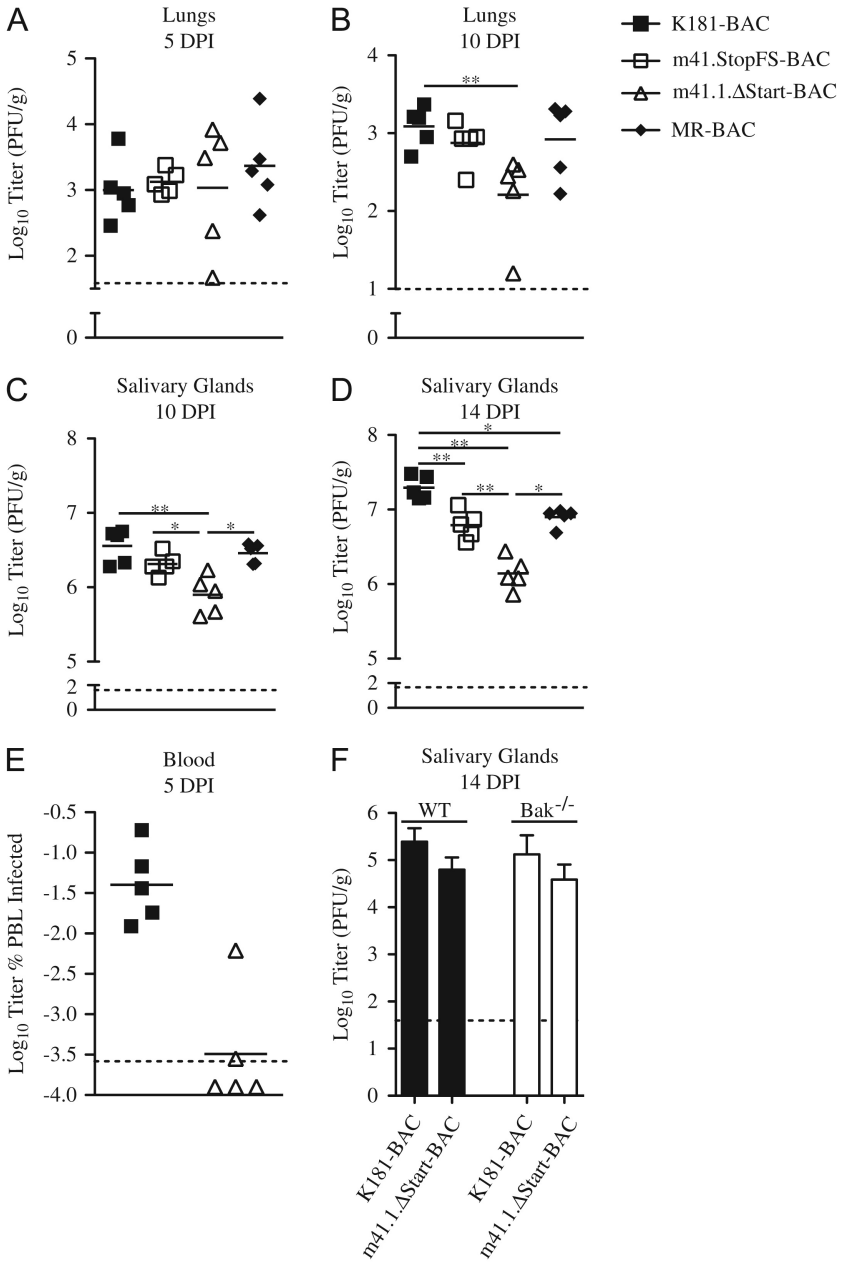

Figure 2.6. Examination of the role of m41.1 (vIBO) during acute replication and dissemination. 6-week-old BALB/c mice were inoculated with 10^6 PFU of tissue culture-derived virus via the IP route. Lungs were collected (A) 5 DPI and (B) 10 DPI. Salivary glands were collected (C) 10 DPI and (D) 14 DPI. Each symbol represents an

individual mouse. (E) 6-week-old BALB/c mice were inoculated as described in (A-D) and PBLs were harvested by heart puncture at 5 days post infection. Infection rates are expressed as a percentage of PBLs that scored positive in an infectious center assay on 3T3-SA fibroblasts. (F) 5-11-week old C57BL/6x129 (WT) or Bak-/- mice were inoculated as described in (A-D). Each bar represents the average titer obtained from 3-4 mice. Titers in organ sonicates were determined by plaque assay on 3T3-SA fibroblasts. The dotted line indicates the limit of detection of virus. *, $p < 0.05$; **, $p < 0.01$.

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CHAPTER 3

Inhibition of Bax- and Bak-Mediated Cell Death by Murine Cytomegalovirus is Crucial to Viral Replication and Pathogenesis

Lynsey N. Crosby[a], Chris Dillon[b], Douglas R. Green[b] and Edward S. Mocarski[a]

[a] Department of Microbiology and Immunology, Emory Vaccine Center, Emory University School of Medicine, 1462 Clifton Rd NE, Atlanta, GA 30322, USA.

[b] Department of Immunology, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105, USA.

3.I. Abstract

Cytomegaloviruses encode evolutionarily conserved cell death suppressors targeting Bax- and Bak-activation that maintain viability of host cells during infection. Human cytomegalovirus relies on a single viral mitochondrial inhibitor of apoptosis (vMIA) inhibiting both Bax and Bak; whereas, murine cytomegalovirus encodes one cell death suppressor targeting Bax (m38.5-encoded vMIA) plus m41.1-encoded viral inhibitor of Bak oligomerization (vIBO). Individually, vMIA and vIBO provide modest support during dissemination in the host, likely by maintaining a monocytic population trafficking the virus. Here we demonstrate, however, that elimination of both mitochondrial death suppressors exacerbates attenuated levels of viral replication in macrophages as well as in the host, revealing an essential contribution of Bax- and Bak-mediated cell death in limiting pathogenesis. Disruption of upstream Bid, Bim and PUMA proapoptotic Bcl-2 family members did not reveal a single activator of Bax and Bak during infection of macrophages; however, elimination of Bid-dependent pathways throughout the host did provide a modest benefit to viral replication in the absence of vMIA and vIBO. Thus, Bid may drive activation of mitochondrial death pathways in certain cell types infected throughout the host. All together, these data indicate that virally encoded suppressors of mitochondrial death pathways have a profound impact on pathogenesis, suppressing the various stress signals initiated during infection of monocyte lineage cells that disseminate virus in the host.

3.II. Introduction

Programmed cell death is an evolutionarily conserved response to infection triggered by cell-intrinsic and cell-extrinsic stress signals that proceeds through caspase-dependent as well as caspase–independent pathways (Muñoz-Pinedo, 2012). The mitochondria play an important role in transmitting intrinsic and extrinsic stress signals, resulting in activation of a caspase-dependent apoptotic pathway (Tait and Green, 2012). Stress signals transmitted through the mitochondria can originate from a variety of sources including, DNA damage, ER stress, tumor necrosis factor-α, interferon signaling, and viral infection. Viruses encode a number of inhibitors to subvert mitochondrial-mediated apoptosis to maintain viability of the host cell and sustain a period of viral replication (Castanier and Arnoult, 2011; McCormick and Mocarski, 2012).

These virally encoded inhibitors mimic the function as well as the sequence and/or structure of the Bcl-2 family (Castanier and Arnoult, 2011). The Bcl-2 family relays and balances stress signals converging on the mitochondrial membrane via proapoptotic and antiapoptotic members (Chipuk et al., 2010). Following initiation of cell death, any of the three proapoptotic proteins, Bid, Bim, or PUMA, directly activate effector proteins Bax and Bak, initiating formation of pores in the mitochondrial membrane and release of proteins such as cytochrome *c* that activate executioner caspases (Chipuk et al., 2010; Ren et al., 2010). Bax and Bak activation is inhibited by antiapoptotic proteins, such as Bcl-2, Bcl-X_L, and Mcl-1, as well as by viral Bcl-2 (vBcl-2) mimics encoded by large double-stranded DNA viruses. Adenovirus E1B-19K (Chiou et al., 1994; Tarodi et al., 1993), vaccinia virus F1L (Wasilenko et al., 2003), and myxoma virus M11L (Everett et al., 2000; Macen et al., 1996) represent only a subset of

identified mitochondrial cell death suppressors. Despite the vast array of vBcl-2 proteins, little is currently known about how mitochondrial cell death contributes to the innate host defense responses limiting viral replication and how suppression of this pathway by the virus may promote pathogenesis.

Cytomegaloviruses (CMV) encode evolutionarily conserved mitochondrial-localized proteins that have provided some insight into how suppression of apoptosis sustains viral replication and pathogenesis. Human CMV (HCMV), a major infectious cause of birth defects and an opportunistic pathogen, encodes a single, viral mitochondrial-localized inhibitor of apoptosis (vMIA) (Goldmacher et al., 1999) that inhibits both Bax and Bak membrane permeabilization (Arnoult et al., 2004; Karbowski et al., 2006; Poncet et al., 2004). During infection of fibroblasts, HCMV vMIA supports viral replication, by making infected cells resistant to stress and prolonging the replication cycle (Goldmacher et al., 1999; McCormick et al., 2005; McCormick et al., 2008; McCormick et al., 2013; Reboredo et al., 2004). Investigations, however, of HCMV vMIA have been confined to fibroblasts so little is known about how suppression of this death pathway supports replication in other permissive cell types. Murine CMV (MCMV), a natural mouse pathogen and important model for HCMV, extends this knowledge base. MCMV regulates Bax and Bak through two individual gene products, m38.5 (vMIA) (Arnoult et al., 2008; Jurak et al., 2008; Manzur et al., 2009; McCormick et al., 2003a; Norris and Youle, 2008) and m41.1, the viral inhibitor of Bak oligomerization (vIBO) (Cam et al., 2010; Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013), respectively. Individually, vMIA supports viral replication in a variety of different cell types, including fibroblasts and endothelial cells; however,

inhibition of Bax or Bak has the greatest impact during replication in macrophages (Cam et al., 2010; Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013; Jurak et al., 2008; Manzur et al., 2009). Macrophages and their monocyte precursors play a central role during CMV pathogenesis (Fleming et al., 1999; Saederup et al., 2001; Saederup et al., 1999; Stoddart et al., 1994). Infected monocyte precursors control dissemination by trafficking through the bloodstream to target organs (Noda et al., 2006). Differentiation from these precursors cells to subsequent lineages, such as macrophages, allows for viral replication and seeding of target organs. In correlation to their role in supporting replication of macrophages, vMIA or vIBO functions are important for efficient MCMV dissemination within the host (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013; Manzur et al., 2009). The parallel role these proteins have in supporting replication in macrophages and during dissemination in the host indicates that these viral proteins are central players to sustain the cell type of dissemination.

However, despite these implications the impacts observed with the individual vMIA or vIBO disruptions are modest in comparison to the effects seen upon disruption of the other cell death suppressors encoded by CMV. Extrinsic apoptosis, dependent upon the self-activation of cysteine protease caspase-8, is subverted during viral infection by a conserved viral inhibitor of caspase-8 activation (vICA) encoded by HCMV UL36 and MCMV M36 (Cicin-Sain et al., 2008; McCormick et al., 2003a; Skaletskaya et al., 2001). Inhibition of extrinsic apoptosis by vICA facilitates viral replication in macrophages (McCormick et al., 2010; Menard et al., 2003), and, in MCMV, is important for sustained infection of the host (Cicin-Sain et al., 2008; Ebermann et al., 2012). However, inhibition of caspase 8 drives caspase-independent necrotic death pathways

due to the necessity of caspase-8 inhibition of RIP3, the central mediator of necrosis (Kaiser et al., 2011; Oberst et al., 2011). To subvert aberrant necrosis induced during viral infection, MCMV encodes the viral inhibitor of RIP activation (vIRA) from M45 that blocks RIP3 activation by the DNA-dependent activator of interferon regulatory factors (DAI) (Upton et al., 2010, 2012). Similar to vICA, vIRA is necessary for efficient viral replication (Brune et al., 2001; Upton et al., 2010). Loss of either vICA or vIRA functions largely eliminates MCMV replication and dissemination within the host (Cicin-Sain et al., 2008; Ebermann et al., 2012; Upton et al., 2010), thereby highlighting the crucial role these proteins play in the viral lifecycle.

Altogether these data suggest that suppression of extrinsic apoptosis and virus-induced necrosis are more central to viral replication and pathogenesis as compared to suppression of mitochondrial death pathways. However, double vMIA and vIBO mutant MCMV viruses lacking all suppression of mitochondrial cell death have not been evaluated. To address the role of mitochondrial cell death suppression, we generated mutations in MCMV that eliminate expression of m38.5 (vMIA) and m41.1 (vIBO) and evaluated viral replication and pathogenesis. Here we demonstrate that, together, vMIA and vIBO provide crucial functions to the MCMV lifecycle. Loss of both vMIA and vIBO resulted in little viral replication during infection of macrophages and during infection of the host due to death occurring prior to replication. These impacts on viral replication were dependent upon the ability of vMIA and vIBO to suppress both Bax- and Bak-mediated cell death. Investigation into upstream pathways did not reveal a single Bim, Bid, and PUMA activator of Bax and Bak during infection of macrophages, however, Bid-dependent pathways modestly contributed to cell death induced in the host.

These results demonstrate that suppression of mitochondrial cell death has a profound impact on viral replication and is central to the viral lifecycle in the host.

3.III. Results

m38.5-encoded vMIA and m41.1-encoded vIBO are necessary for viral replication in macrophages

To characterize the combined contributions of vMIA and vIBO functions to replication and pathogenesis, mutations were engineered into m38.5 and m41.1 (Figure 3.1A). Double mutant viruses lacking both gene products were called Δm38.5/m41.1-BAC, signifying the presence of large insertion disrupting m38.5 and a point mutation in m41.1, and m38.5.StopFS/m41.1.ΔStart-BAC, indicating that a point mutation was present in both reading frames. Single mutant viruses were also utilized at various times throughout the study in comparison to double mutant viruses. These single mutant viruses were called m41.1.ΔStart-BAC and m38.5.StopFS-BAC. The genomic integrity of all mutant and control viruses was analyzed by restriction fragment length polymorphism (RFLP) (Figure 3.1B) and all were found to be intact and have the predicted RFLP pattern.

The replication properties of the double vMIA/vIBO mutant viruses were compared to parental K181-BAC. Cells were infected at a low multiplicity of infection (MOI), 0.3 for 3T3-SA fibroblasts and SVEC4-10 endothelial cells and 0.5 for bone marrow derived macrophages (BMDM), and total virus was collected at different days post infection (DPI) when viral yields were determined by plaque assay. A mutant virus lacking vMIA and vIBO exhibited replication kinetics almost identical to the parental K181-BAC in SVEC4-10 endothelial cells (Figure 3.2A). The second double mutant virus, m38.5.StopFS/m41.1.ΔStart-BAC, confirmed the results produced 5 DPI (Figure

3.2B). Together these results indicate that suppression of Bax- and Bak-mediated cell death is dispensable for viral replication in endothelial cells.

In contrast, the Δm38.5/m41.1-BAC virus exhibited delayed replication kinetics in 3T3-SA fibroblasts (Figure 3.2C), with viral titers reduced approximately 60- and 20-fold lower than the parental virus at 1 and 3 DPI, respectively. Peak viral yield was also observed at a later timepoint (5 DPI) as compared to the parental virus (3 DPI) and was approximately 5-fold lower than peak parental titers. The defect at 3 DPI, however, was not reproduced with either double mutant virus (Figure 3.2D). A possible explanation for the differing results relies on the fibroblast passage number. Higher passage (>20 passages) fibroblasts were infected in Figure 3.2C as compared to low passage (<20 passages) cells (Figure 3.2D). Therefore, a defect in viral replication may be revealed upon continuous passage of fibroblasts in the absence of vMIA and vIBO. Despite this, the results demonstrate that vMIA and vIBO functions are largely dispensable during infection of fibroblasts.

Previous investigations into the individual role of m38.5 (vMIA) during replication in fibroblasts and endothelial cells demonstrated that inhibition of Bax was necessary to support viral replication (Jurak et al., 2008; Manzur et al., 2009). The largest impacts were observed during multi-step replication analyses (MOI ≤ 0.02) (Jurak et al., 2008; Manzur et al., 2009); whereas, single-step analyses (MOI 5) only revealed modest impacts in fibroblasts and only at very late times in the replication cycle (Jurak et al., 2008). Here we did not reveal a role for mitochondrial cell death suppression during multiple cycles of replication in fibroblasts or endothelial cells; however, cells were infected at a higher MOI (MOI 0.3) as compared to the experiment described above

(MOI ≤ 0.02). Therefore, mitochondrial cell death suppression may become of increasing importance and the number of replication cycles increases in these cell types.

In contrast, vMIA and vIBO play an unambiguous and central role during infection of macrophages. Viral titers rapidly declined upon infection of primary bone marrow-derived macrophages (BMDM) with the Δm38.5/m41.1-BAC, resulting in an approximately 1,200-fold reduction as compared to parental K181-BAC by 9 DPI (Figure 3.2E). The second double mutant virus confirmed this large replication defect producing titers similar to Δm38.5/m41.1-BAC at 5 DPI (Figure 3.2F). Overall, these doubly deficient viruses failed to replicate, indicating that suppression of both Bax- and Bak-mediated cell death is crucial to replication in macrophages.

m38.5 (vMIA) and m41.1 (vIBO) are necessary to maintain viability of macrophages during infection

Individually, m38.5 and m41.1 are necessary to maintain viability of the host cell during infection (Arnoult et al., 2008; Cam et al., 2010; Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013; Jurak et al., 2008; Manzur et al., 2009). To determine if their combined function similarly sustained the host cell, we analyzed viability, markers of cell death, and cell morphology during infection. Upon infection with the double vMIA and vIBO mutant virus, Δm38.5/m41.1-BAC, a loss is BMDM cell viability was observed by 24 HPI and was more dramatically reduced (approximately 60%) by 48 HPI as compared to the WT K181-BAC (Figure 3.3A). This loss in viability correlated to the presence of cell death markers (Figure 3.3B). Δm38.5/m41.1-BAC infected BMDMs showed an increase in the proportion of infected cells (IE1 positive) that were positive by

TUNEL assay at 24 and 48 HPI relative to WT infected cells. This demonstrates that cells infected with a double vMIA/vIBO mutant virus have fragmented DNA early in infection, a hallmark of apoptosis. Apoptosis is morphologically characterized by cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation. BMDMs infected with a vMIA/vIBO double mutant virus share these morphological characteristics (Figure 3.3C). Δm38.5/m41.1-BAC infected BMDMs show signs of membrane blebbing and fragmentation. The infected cells are also reduced in size and do not retain the elongated appearance of the mock infected cells. Furthermore, the Δm38.5/m41.1-BAC infected BMDMs do not attain the cytomegalic appearance that is a characteristic of CMV infection as shown in the K181-BAC infected culture. These data indicate that infection of macrophages with a vMIA/vIBO mutant virus is terminated by the onset of cell death. All in all, together vMIA and vIBO are necessary to maintain the viability of macrophages during infection, facilitating viral replication.

vMIA and vIBO facilitate viral replication in a Bax/Bak-dependent manner and are necessary for replication and dissemination in the host

m41.1 (vIBO) individually supports viral replication by targeting Bak (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013). Similarly, m38.5 (vMIA) targets Bax to maintain viability of the host cell (Jurak et al., 2008; Manzur et al., 2009) as well as integrity of mitochondrial membranes (Arnoult et al., 2008; Norris and Youle, 2008). To determine if m41.1 (vIBO) and m38.5 (vMIA) together support viral replication by suppressing Bax- and Bak-mediated cell death, viral replication was analyzed in BMDMs in which Bak expression had been eliminated and the Bax coding region was floxed

(Figure 3.4A). *Bak*$^{-/-}$*Bax*$^{fl/fl}$ as well as WT *Bak*$^{+/+}$*Bax*$^{+/+}$ BMDMs were transduced with a lentivirus expressing Cre recombinase (LV-Cre) or control GFP (LV-GFP) to knockdown expression of Bax. Following transduction, protein levels of Bak and Bax were analyzed. Bax levels were reduced upon transduction of *Bak*$^{-/-}$*Bax*$^{fl/fl}$ BMDMs with LV-Cre as compared to *Bak*$^{-/-}$*Bax*$^{fl/fl}$ and WT *Bak*$^{+/+}$*Bax*$^{+/+}$ BMDMs transduced with LV-GFP. Bak levels, on the other hand, were only detected in WT *Bak*$^{+/+}$*Bax*$^{+/+}$ BMDMs transduced with LV-GFP as predicted.

Viral replication of vMIA/vIBO double mutant viruses increased in these cells as the levels of both Bak and Bax were reduced (Figure 3.4A). Δm38.5/m41.1-BAC replication was significantly increased ($p < 0.05$) more than 8-fold in Bak$^{-/-}$Bax$^{fl/fl}$ BMDMs transduced with LV-Cre as compared to WT Bak/Bax sufficient cells. No increase in replication of Δm38.5/m41.1-BAC occurred when only Bak expression was eliminated (i.e. *Bak*$^{-/-}$*Bax*$^{fl/fl}$ BMDMs transduced with LV-GFP). Together these results indicate that both Bak and Bax expression must be completely eliminated in order to restore replication of a vMIA/vIBO double mutant virus. The precise vMIA/vIBO double mutant confirmed these results. m38.5.StopFS/m41.1.ΔStart-BAC significantly increased ($p < 0.05$) in replication by more than 8-fold when Bak expression was eliminated and Bax levels were reduced (i.e. *Bak*$^{-/-}$*Bax*$^{fl/fl}$ BMDMs transduced with LV-Cre). A subtle decrease in replication was noted when only Bak expression was eliminated; however, this decrease was not significant ($p > 0.05$). The WT K181-BAC also experienced an increase in replication ($p < 0.05$) upon knockout of Bak and knockdown of Bax; however, this difference was less than a 2-fold increase as compared to replication in WT Bak/Bax BMDMs. These results suggest that the WT K181-BAC receives a subtle boost in

replication in the absence of Bax- and Bak-mediated cell death; however, the increase in replication was much greater for the vMIA/vIBO double mutant viruses. Therefore, these results demonstrate that together vMIA and vIBO support viral replication by inhibiting Bax- and Bak-mediated cell death at the mitochondrial membrane.

To determine how suppression of mitochondrial cell death impacts replication and dissemination in the host, viral replication in the salivary glands was analyzed following inoculation of susceptible BALB/c mice. Salivary glands are a key target organ during MCMV infection of the host as they are the final site of dissemination and viral replication persists at this site for a prolonged period of time, creating a potent source of transmission. BALB/c mice were inoculated via the intraperitoneal (IP) route and replication in the salivary glands was evaluated 14 DPI (Figure 3.4B), a time at which peak levels of replication were observed. Δm38.5/m41.1-BAC and m38.5.StopFS/m41.1.ΔStart-BAC viruses were reduced in the salivary glands at 14 DPI by more than 1,900-fold and 700-fold, respectively, as compared to K181-BAC. This decrease in replication and dissemination of the double mutant viruses was greater than the impact observed for individual m38.5 (vMIA) or m41.1 (vIBO) mutant viruses. m38.5.StopFS-BAC levels were reduced by more 50-fold while m41.1.ΔStart-BAC was reduced by 17-fold as compared to K181-BAC. All together, these results demonstrate that suppression of both Bax- and Bak-mediated cell death is crucial to replication and dissemination in the host and that the full impact of mitochondrial cell death suppression is revealed only when both viral suppressors of Bax and Bak are removed.

Investigation of pro-apoptotic pathways upstream of Bax and Bak in MCMV infection

Bax and Bak are activated by three members of the Bcl-2 family of proteins, Bid, Bim, and PUMA, following transduction of a stress signal (Kim et al., 2006; Kuwana et al., 2005; Kuwana et al., 2002; Letai et al., 2002; Ren et al., 2010; Wei et al., 2000). These three Bcl-2 family members are the only recognized direct activators of Bax and Bak amongst the BH3 only proapoptotic proteins. Therefore, stress signals converge upon Bid, Bim, and PUMA, which directly interact with Bax and Bak to drive their activation. To determine which upstream direct activators are responsible for driving Bax and Bak activation during infection, viral replication was analyzed in BMDMs in which upstream Bid-, Bim-, or PUMA-dependent pathways had been eliminated (Figure 3.5). Bid is activated following cleavage in large part by caspase 8 (Chipuk et al., 2010). To assess the contribution of Bid activation during infection, caspase 8 expression was eliminated. However, caspase 8 is necessary to suppress RIP3 (Kaiser et al., 2011; Oberst et al., 2011), the major activator of necrosis. Therefore, elimination of caspase 8 leads to aberrant RIP3-mediated necrosis. To avoid inducing necrotic death, RIP3 expression was also eliminated and viral replication was assessed in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDMs (Figure 3.5A-B). Δm38.5/m41.1-BAC was largely replication attenuated in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDMs as compared to K181-BAC (Figure 3.5A). However, an increase in viral titers of this double mutant virus was noted in this genetic background, increasing nearly 30-fold by 9 DPI. However, this level of replication was not significantly increased over replication in WT BMDMs for both Δm38.5/m41.1-BAC and m38.5.StopFS/m41.1.ΔStart-BAC (Figure 3.5B). These results indicate that stress signals transmitted are likely blocked at caspase 8 by vICA, preventing Bid activation and subsequent mitochondrial-mediated cell death. A second genetic knockout, *Bid*$^{-/-}$*Rip3*$^{-/-}$,

eliminating both Bid-dependent apoptotic and necrotic death pathways confirmed results produced in *Casp8*$^{-/-}$*Rip3*$^{-/-}$ BMDMs (Figure 3.5C). vMIA and vIBO double mutant viruses replicated to similar levels in *Bid*$^{-/-}$*Rip3*$^{-/-}$ BMDMs as compared to WT cells. Finally, individual elimination of Bid was also unable to rescue replication of vMIA/vIBO mutant viruses. A slight increase in replication (approximately 3-fold) in *Bid*$^{-/-}$ BMDMs was noted for m38.5.StopFS/m41.1.ΔStart-BAC; however, this subtle difference was not observed with Δm38.5/m41.1-BAC. Thus, these results demonstrate that Bid is unlikely to be responsible for activation of Bax and Bak during infection of macrophages. Pathways that lead to Bid activation, such as extrinsic apoptosis via caspase 8 or intrinsic stress via caspase 2 (Upton et al., 2008), do not appear to be stimulated or are subverted by other upstream cell death suppressors, such as vICA.

Bim and PUMA may also directly interact with Bax and Bak, leading to mitochondrial-mediated cell death (Chipuk et al., 2010; Ren et al., 2010). To determine whether Bim and PUMA activate Bax and Bak during infection, primary macrophages lacking either Bim or PUMA were infected (Figure 3.5D). Elimination of neither Bim nor PUMA expression was able to restore replication of vMIA/vIBO mutant viruses to K181-BAC levels.

Taken together, this data suggests that neither Bid, Bim, or PUMA individually drive Bax and Bak activation during infection of macrophages. However, MCMV infects a wide range of cells throughout the host beyond macrophages and their monocyte precursors. Infection of *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice modestly restored viral replication in the salivary glands of a mutant virus lacking vMIA and vIBO, suggesting that at least Bid is active in some cell types during infection of the host (Figure 3.5E). Δm38.5/m41.1-BAC

levels were dramatically reduced in the salivary glands of control *Casp*$^{+/-}$*Rip3*$^{-/-}$ mice at 14 DPI as compared to K181-BAC levels. Viral titers of Δm38.5/m41.1-BAC were detected in only one animal whereas high titers were detected in all animals infected with K181-BAC. However, when caspase 8 expression was completely eliminated Δm38.5/m41.1-BAC was detected in the salivary glands of four out of five animals. Additionally, viral titers in two of the Δm38.5/m41.1-BAC infected animals were comparable to levels observed in some K181-BAC infected mice. Thus, this data indicates that vMIA and vIBO are necessary to suppress caspase 8 activation of Bid in some cell types within the host.

3.IV. Discussion

The importance of suppressing Bax as well as Bak at the mitochondrial membrane emerges from our study. We examined the combined impact of Bax and Bak regulation at the mitochondrial membrane by two virally encoded suppressors; m38.5 (vMIA) regulating Bax-mediated cell death and m41.1 (vIBO) suppressing Bak. Together these mitochondrial suppressors support replication in macrophages by maintaining the viability of the host cell through suppression of mitochondrial-mediated cell death. This activity provides a crucial benefit to replication and dissemination in the host, enhancing levels of viral replication at the final site of dissemination, the salivary glands.

Previous investigations of m38.5 (vMIA) (Jurak et al., 2008; Manzur et al., 2009) and m41.1 (vIBO) (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013) revealed that individually these suppressors were crucial for replication in macrophages and to a lesser extent in fibroblasts and endothelial cells for m38.5; however, this activity only provided a modest benefit to replication and dissemination in the host. Replication in both the macrophages and in the host was demonstrated for m41.1 (vIBO) to be dependent upon its ability to suppress Bak (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013). Direct evidence demonstrating that removal of Bax-mediated cell death alleviates replication defects of an m38.5 mutant virus remains unavailable. However, m38.5 suppresses mitochondrial membrane permeabilization and host cell death in a Bax-dependent manner (Arnoult et al., 2008; Jurak et al., 2008; Manzur et al., 2009). Here we demonstrate that the combined contributions vMIA and vIBO not only provides a more dramatic benefit to viral replication in macrophages but also are also central to the viral lifecycle in the host. The combined support of these two viral proteins

is dependent upon their individual abilities to suppress Bax- and Bak-mediated cell death. Therefore, the true impact of suppression of mitochondrial-mediated cell death is only revealed when the two suppressors of Bax and Bak are removed.

Regulation of mitochondrial-mediated cell death is conserved in HCMV. UL37 exon 1 encodes a single inhibitor, vMIA, which localizes to the mitochondrial membrane and is potent suppressor of cell death. Mitochondrial cell death is inhibited during HCMV infection presumably due to vMIA directly interacting with both Bax and Bak; however, vMIA's interactions with these two effector proteins remain controversial. Previous reports extensively characterize vMIA's physical and functional interactions with Bax (Arnoult et al., 2004; Karbowski et al., 2006; Ma et al., 2012; Norris and Youle, 2008; Pauleau et al., 2007; Poncet et al., 2004; Poncet et al., 2006; Smith and Mocarski, 2005; Zhang et al., 2013), solidifying vMIA as a Bax-specific inhibitor. However, some reports indicate that vMIA's specificity stops at Bax and that the viral suppressor does not inhibit Bak-mediated death (Arnoult et al., 2004; Poncet et al., 2006). Strong evidence in support of vMIA as a Bax and Bak suppressor demonstrates that vMIA regulates mitochondrial morphology and death in a Bax/Bak-dependent manner as well as physically interacts with Bak (Karbowski et al., 2006; McCormick et al., 2005). While it seems likely that HCMV vMIA functions as a dual suppressor of Bax and Bak, this question has not been addressed directly. Information present here in combination with previous reports regarding individual roles of m38.5 (vMIA) and m41.1 (vIBO) (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013; Manzur et al., 2009) present an opportunity to directly determine if HCMV vMIA functions as a dual Bax/Bak inhibitor, restoring replication of a double m38.5/m41.1 mutant virus in both macrophages and in the host.

Functional inhibition of both effector proteins would indicate that HCMV vMIA alone is an evolutionary homolog of both m38.5 and m41.1, implicating it a central mediator of viral replication and dissemination.

Activation of Bax and Bak during MCMV infection remains an enigma. Results produced in caspase 8/RIP3 double knockout mice suggest a role for Bid activation during infection; however, this impact was modest. A single clear direct activator of Bax and Bak during infection was not identified possibly because either (i) Bim, Bid, and PUMA pathways are simultaneously active, thus, any relief of mitochondrial cell death that may occur when one pathway is eliminated is obscured by other two active pathways; or (ii) Bim, Bid, and PUMA are not active during infection and another stimulus or pathway is driving Bax and Bak activation during infection. The Bcl-2 pathway regulating the mitochondrial permeabilization is further complicated by the individual identities of Bax and Bak. Apoptosis is a crucial element for normal development (Chipuk et al., 2010). Mice lacking the ability to drive apoptosis throughout all tissues experience developmental impairments, such as an abnormal immune repertoire, autoimmunity complications, and a shortened lifespan. Mice lacking either Bax or Bak are surprisingly viable, with Bax knockout mice experiencing a few more complications, such as an increase in immune cell numbers (Chipuk et al., 2010; Knudson et al., 1995). However, when Bax and Bak expression are simultaneously eliminated mice experience severe developmental impairments, such as pre- and peri-natal lethality, interdigital webbing, and behavioral complications (Lindsten et al., 2000). This data has created an enigma in the field as to the relationship between these two proteins. Bax and Bak appear to either be (i) redundant effector proteins able to

compensate for each other when one protein is suppressed or eliminated or (ii) individual proteins responding to different upstream activators and stress stimuli. The MCMV viral suppressors of Bax and Bak further support this enigma in that elimination of one suppressor, vMIA or vIBO, creates a modest impact (Cam et al., 2010; Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013; Manzur et al., 2009); however, elimination of both suppressors reveals a much stronger attenuation in viral replication and pathogenesis. MCMV vMIA and vIBO present an opportunity to tease apart the Bax and Bak pathways and to determine if there is overlap in the upstream and downstream pathways, indicating if they are individual or redundant.

In summary, we have demonstrated that together m38.5 (vMIA) and m41.1 (vIBO) are central to replication in macrophages and dissemination in the host. A number of issues remain to be resolved, such as (i) other aspects of MCMV pathogenesis influenced by vMIA and vIBO, (ii) the impact of HCMV vMIA on replication in various cell types, such as macrophages, (iii) the ability of HCMV vMIA to substitute for MCMV vMIA and vIBO, and (iv) the upstream pathway(s) driving Bax and Bak activation during infection.

3.V. Materials and Methods

Cells

3T3-SA fibroblasts (ATCC CCL-92), SVEC4-10 endothelial cells (ATCC CRL-2181), and 293-T cells were maintained at 37°C with 5% CO_2 in DMEM containing 4.5 g/mL glucose, 1 mM sodium pyruvate, 10% fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen). Bone marrow-derived macrophages were harvested from C57Bl/6 (referred to as WT), *Bak*$^{-/-}$*Bax*$^{fl/fl}$ (also referred to as *Bax*^{tm2Sjk} *Bak1*$^{tm1Thsn/J}$) (Crosby et al., 2013), *Bim*$^{-/-}$ (Jackson Laboratory), *PUMA*$^{-/-}$, *Bid*$^{-/-}$, *Bid*$^{-/-}$*RIP3*$^{-/-}$ and *Caspase-8*$^{-/-}$ *RIP3*$^{-/-}$ mice (Kaiser et al., 2011). Bone marrow-derived macrophages were harvested and cultured as previously described (Crosby et al., 2013).

BAC Mutagenesis and Recombinant Viruses

Escherichia coli strain DH10B harboring plasmid pSIM6 encoding the λ red recombineering functions (Datta et al., 2006) and MCMV strain K181-BAC (Redwood et al., 2005) was utilized to introduce insertion mutations in the viral genome. m41.1.ΔStart-BAC was generated as previously described (Crosby et al., 2013). This m41.1 mutant was utilized to generate Δm38.5/m41.1-BAC by introducing a selection/counterselection cassette, Kan/SacB, into the m38.5 reading frame. The following primers were used to insert the Kan/SacB cassette: m38.5 Kan/SacB-F 5’ ccaagcgccccagaggcgaagagcagcgctggtcgttcgcttacaaacccaattcgagctcggtacccgg; m38.5 Kan/SacB-R 5’ ggttgtagttgtggaggggacagcgatggagagtgtgcgccgacccttctatcccgggaaaagtgccacc. To introduce

point mutations into the viral genome the more efficient *En Passant* mutagenesis system (Tischer et al., 2010; Tischer et al., 2006) was employed. K181-BAC as well as m41.1.ΔStart-BAC were introduced into *E. coli* strain GS1783. A point mutation in m38.5 that created a stop codon, frame shift, and an XbaI restriction site was inserted into K181-BAC, generating m38.5.StopFS, and m41.1.ΔStart-BAC, generating m38.5.StopFS/m41.1.ΔStart-BAC. The following primers were used to insert this point mutation: m38.5.StopFS-F 5' gcgccccagaggcgaagagcagcgctggtcgttcgcttacaaaccctctagaagggtcggcgcacaggatgacgacgataa gtaggg; m38.5.StopFS-R 5' gtagttgtggaggggacagcgatggagagtgtgcgccgacccttctagagggtttgtaagcgaaccaaccaattaaccaattct gattag. All BAC-derived clones were screened by PCR over the m41/m41.1 locus as previously described (Crosby et al., 2013) and over the m38.5 locus. The following primers were used to amplify and screen the m38.5 locus: m38.5-F 5' gcagaagtcacgtcggatccag; m38.5-R 5' ggctgctacgagaacgtgac. Furthermore, BAC-derived clones were analyzed by restriction fragment length polymorphism (RFLP) with five restriction enzymes to ensure the integrity of the viral genome. Following proper genetic characterization, mutant and parental viruses were reconstituted as previously described (Crosby et al., 2013). Growth curves and viral yields were performed by infecting cells in a 48-well plate in 0.25 ml of viral inoculum at the indicated multiplicity of infection (MOI) for 1 hour at 37°C with 5% CO_2. Following adsorption, the inoculum was replaced with complete DMEM. Samples were harvested at the indicated times, sonicated, and titered by plaque assay on 3T3-SA fibroblasts.

Mice, Infections, and Organ Harvests

BALB/c and B6 × 129P2 mice were obtained from Jackson Laboratory. B6 x 129-*Bax*^{tm2Sjk} *Bak1*$^{tm1Thsn/J}$ (also referred to as *Bak*$^{-/-}$*Bax*$^{fl/fl}$) were obtained and used as previously described (Crosby et al., 2013). *Caspase 8*$^{-/-}$ *RIP3*$^{-/-}$ were bred and maintained as previously described (Kaiser et al., 2011). 6-week old BALB/c mice were infected via the intraperitoneal (IP) route of inoculation with 2 x 10^5 PFU of tissue culture-derived virus. Other strains of mice (6-18 weeks old) were infected via the IP route with 1 x 10^6 PFU. At the time of sacrifice, organs were placed in 1 ml of complete DMEM, disrupted by sonication, and titered by plaque assay. Emory University Division of Animal Resources maintained mice and the Emory University Institutional Animal Care and Use Committee approved all procedures.

Knockdown of Bax

Bax knockdown in *Bak*$^{-/-}$*Bax*$^{fl/fl}$ BMDM cells was achieved by infection with lentivirus expressing Cre recombinase. Lentiviral stocks were generated by transfecting 293-T cells with LV-Cre (Addgene) or control LV-GFP (Addgene) along with packaging plasmids psPAX2 and VSV-G. Supernatant containing the lentiviral particles was collected 24, 48, and 72 hours post transfection, filtered through a 0.45 micron filter, subjected to a flash-freeze, and stored at -80°C until use. WT and *Bak*$^{-/-}$*Bax*$^{fl/fl}$ BMDM cells were thawed and cultured in the lentiviral supernatant collected at 24 hours post transfection for 8 hours. Medium was replaced and the BMDM cells were allowed to recover overnight. This process was repeated twice using supernatant collected at 48 and 72 hours post

transfection. Following infection with lentiviral particles, BMDM cells were allowed to rest for two days before seeding.

Cell Viability Assay

Cells (50,000 cells/well) were seeded into 96-well plate in triplicate. Approximately 18 hours post seeding, medium was replaced with 100 µl of virus inoculum containing 10 PFU/cell (MOI 10). At the indicated times, cell viability was determined by measuring the ATP levels using the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) according to manufacture's protocol.

Cell Death Assay

Cells (60,000/well) were seeded into 48-well plate in triplicate. Approximately 18 hours post seeding, cells were infected at an MOI of 10. At 24 HPI, DNA fragmentation was visualized by the DeadEnd Fluorometric TUNEL System (Promega). The number of cells in which fragmented DNA could be detected were expressed as a percent of cells expressing IE1. Cell morphology 24 HPI was visualized by phase-contrast microscopy.

Immunoblot Analysis

Cells were lysed in 50 mM Tris (pH 7.0), 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 2.75% sucrose. Cell lysates were boiled for 5 minutes, separated by SDS-polyacrylamide gel electrophoresis (15% gel), transferred to Immobilon-P PVDF membranes (Millipore), and subjected to immunoblot analysis. Antibodies used to detect

various proteins included anti-Bax (BioLegend, clone 5B7), anti-Bak (Cell Signaling), and anti-β-actin (Sigma, clone AC-74).

3.VI. Figures and Figure Legends

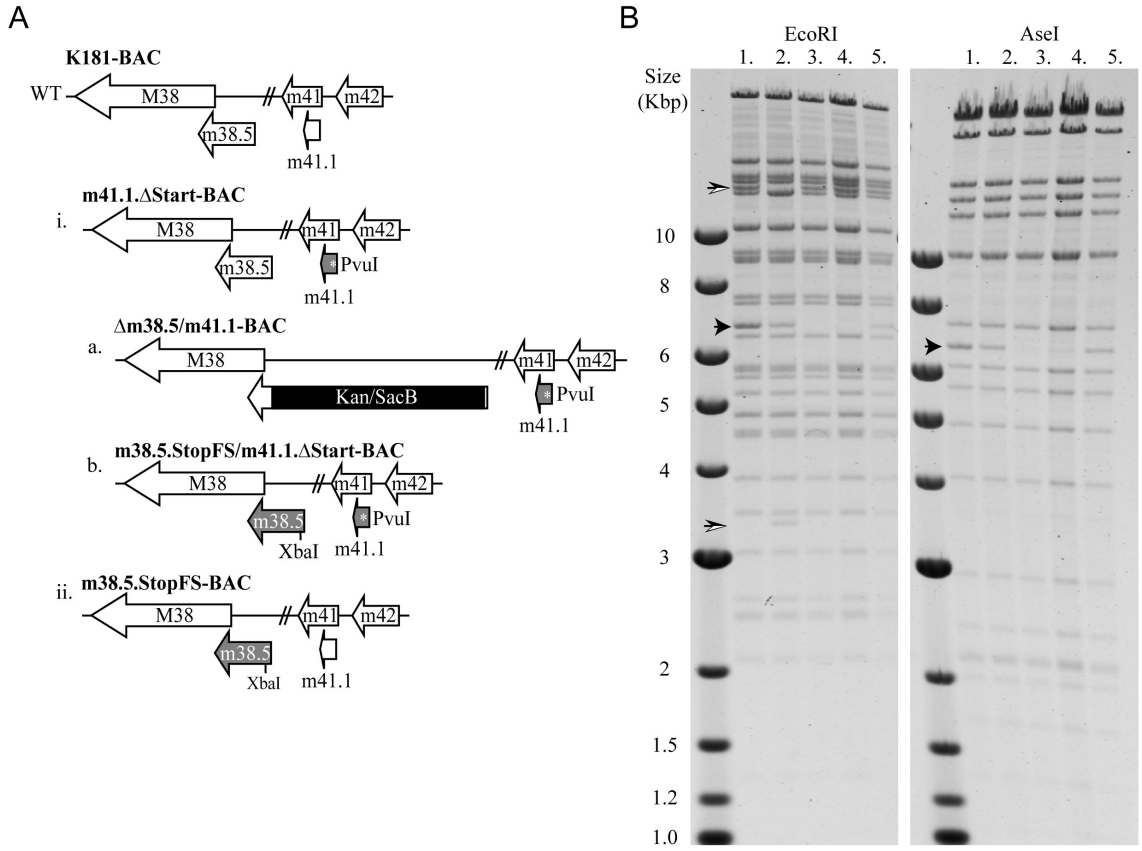

Figure 3.1. Mutagenesis of m38.5 and m41.1. (A) Representation of the mutations inserted into m38.5 and m41.1. The m38.5 reading frame, upstream of M38, was replaced with a Kan/SacB cassette in the m41.1.ΔStart-BAC, generated as previously described (Crosby et al., 2013). This created a double m38.5/m41.1 mutant virus, referred to as Δm38.5/m41.1-BAC. A second double mutant virus was generated using the m41.1.ΔStart-BAC in which a stop codon and frame shift mutation were inserted into m38.5. These point mutations created an XbaI restriction site utilized to screen for m38.5.StopFS/m41.1.ΔStart-BAC. A single m38.5 mutant was generated from the WT K181-BAC. The same stop codon and frame shift mutations described above were inserted into m38.5, creating m38.5.StopFS-BAC. (B) The genomic integrity of the WT

and mutant viruses was analyzed by electrophoretic separation of EcoRI- and AseI-digested BAC DNA on 0.6% agarose.

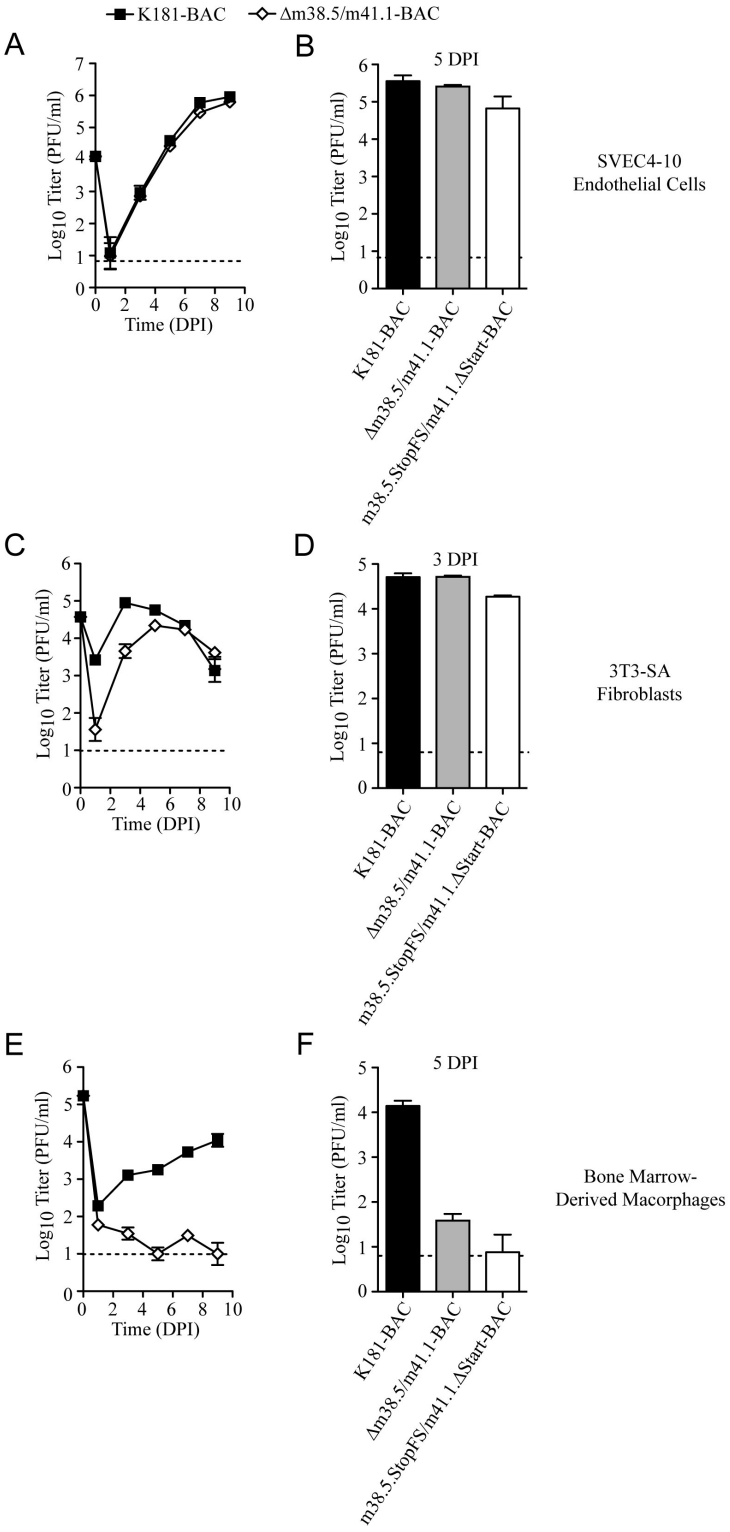

Figure 3.2. Replication kinetics in murine endothelial cells, fibroblasts, and macrophages. (A-B) SVEC4-10 endothelial cells and (C-D) 3T3-SA fibroblasts were infected at an MOI of 0.3. (E-F) Primary bone marrow-derived macrophages were infected at an MOI of 0.5. Total virus was collected at the indicated times post infection. Titers of cell sonicates were determined by plaque assay on 3T3-SA fibroblasts. Each symbol and bar represents the average of three replicate samples. The dotted line indicates the limit of detection.

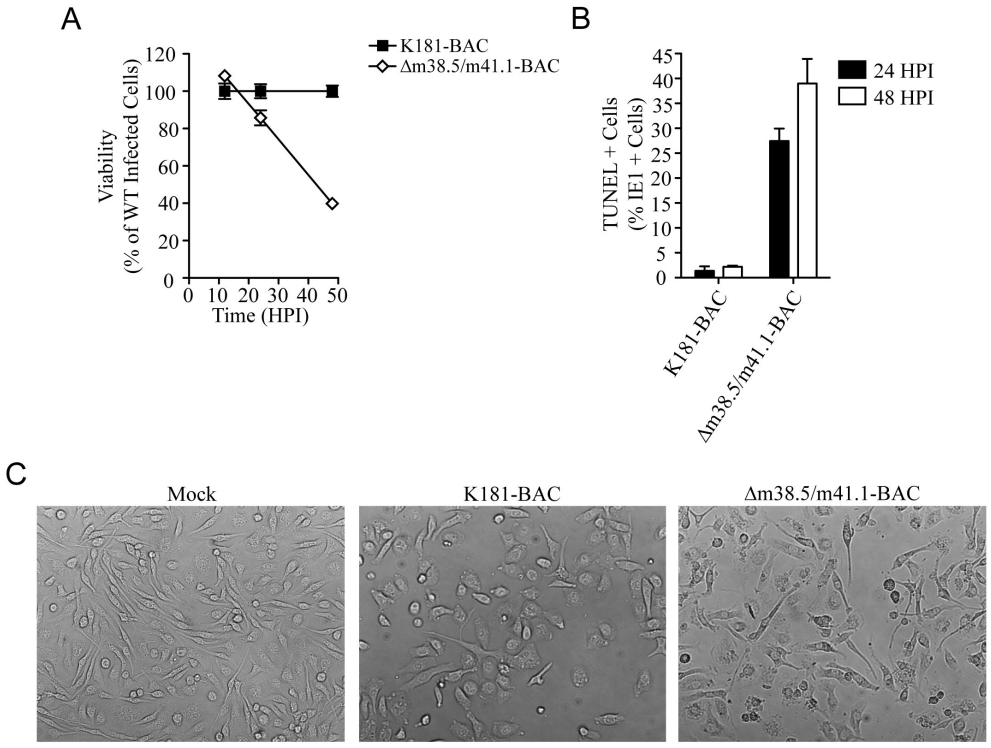

Figure 3.3. m38.5 (vMIA) and m41.1 (vIBO) are necessary to maintain viability of macrophages during infection. (A) BMDM were infected at an MOI of 10 and cell viability was analyzed at the indicated times post infection. Viability was measured as a percent of WT (K181-BAC) infected cells. Each symbol represent the average viability obtained from three replicates. (B) Nuclear DNA fragmentation was assessed in infected BMDM by TUNEL assay. BMDM were infected at an MOI of 10 and TUNEL positive

cells were enumerated as a percentage of infected, IE1 positive cells at the indicated time post infection. Each bar represents the average percentage of TUNEL positive cells obtained from three replicates. (C) Cell morphology during infection was visualized by phase-contrast microscopy. BMDM were infected at MOI of 20 and representative images were taken 24 HPI.

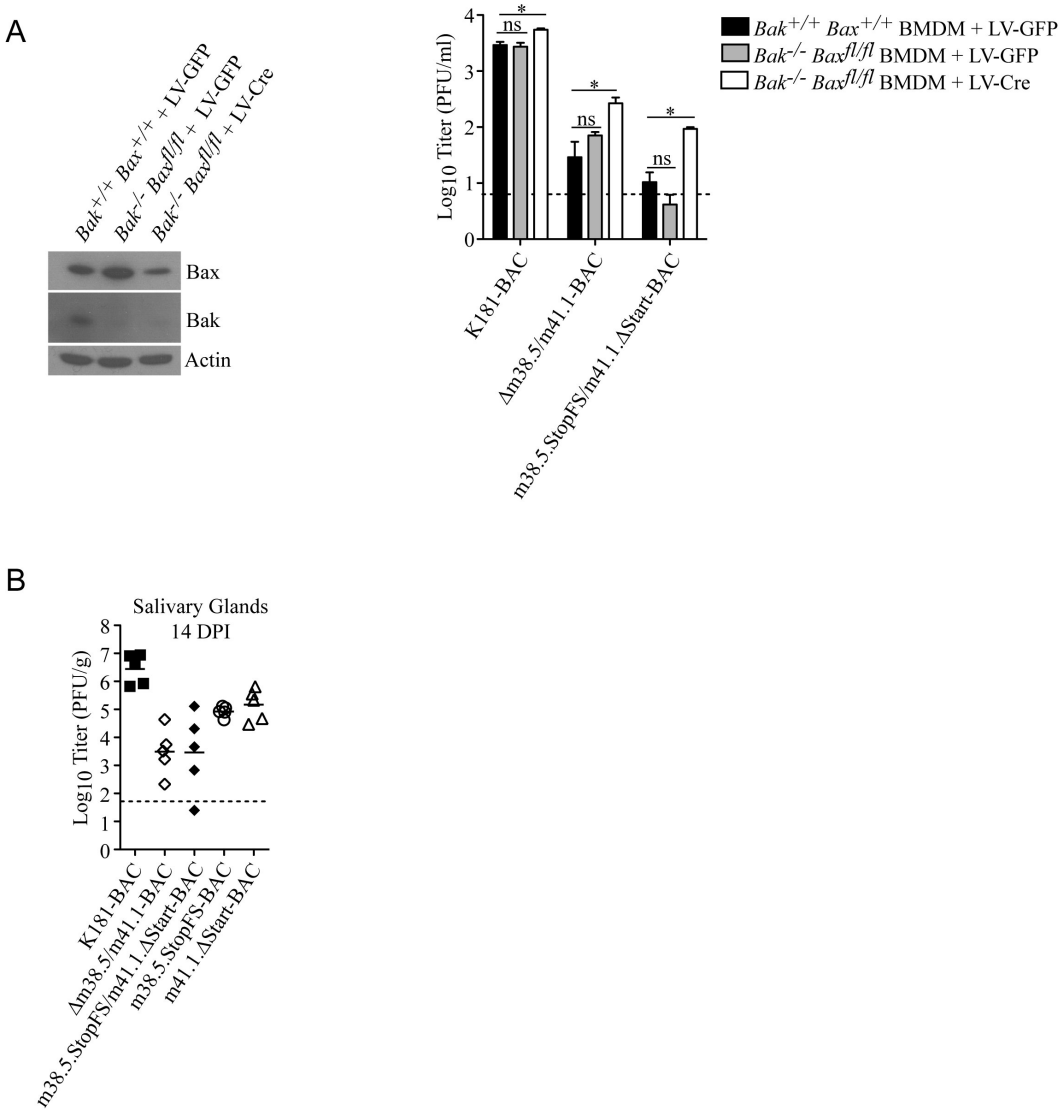

Figure 3.4. m38.5 (vMIA) and m41.1 (vIBO) facilitate viral replication in macrophages in a Bax/Bak-dependent manner and facilitate dissemination in the host. (A) *Bak*$^{+/+}$*Bax*$^{+/+}$ and *Bak*$^{-/-}$*Bax*$^{fl/fl}$ BMDM were transduced with a lentivirus

expressing GFP (LV-GFP) or Cre recombinase (LV-Cre). Bak and Bax expression was assessed by Western blot analysis at the time of infection with K181-BAC-derived viruses (6 DPI). BMDM macrophages were infected with BAC-derived viruses at an MOI of 0.5 and total virus was collected 5 DPI. Each bar represents the average of three replicate samples. (B) 6-week-old BALB/c mice were inoculated IP with $2x10^5$ PFU of tissue culture-derived virus. Salivary glands were collected 14 DPI and disrupted by sonication. Each symbol represents the titer obtained from an individual mouse. Viral titers in cell and organ sonicates were determined by plaque assay on 3T3-SA fibroblasts. The dotted line indicates the limit of detection. *, $p < 0.05$.

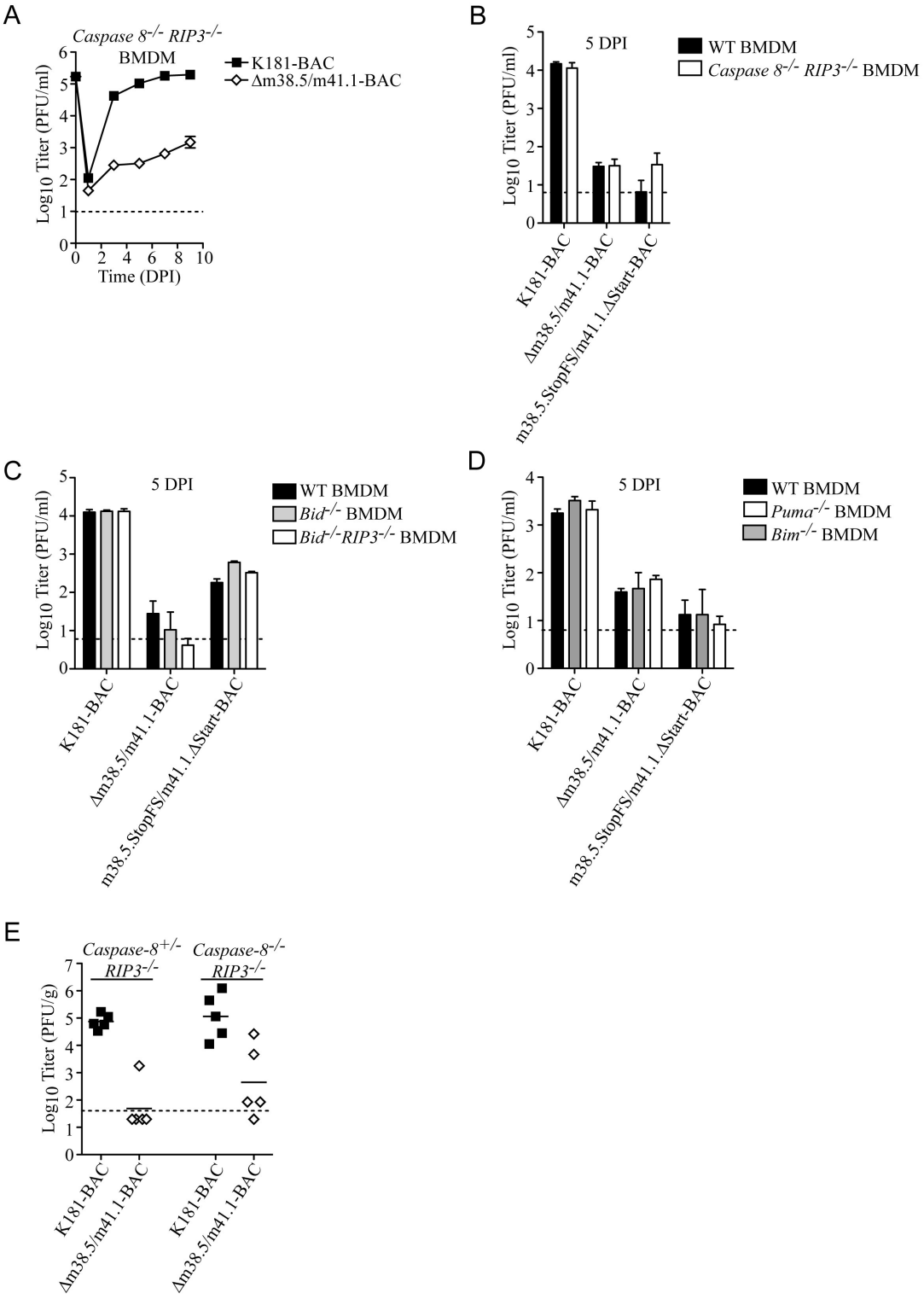

Figure 3.5. Characterization of the upstream pathways that drive Bax and Bak activation during MCMV infection. (A-B) *Caspase8*$^{-/-}$*RIP3*$^{-/-}$, (C) *Bid*$^{-/-}$, *Bid*$^{-/-}$*RIP3*$^{-/-}$,

(D) *PUMA*$^{-/-}$, and *Bim*$^{-/-}$ BMDM were infected along with WT control BMDM at an MOI of 0.5. Total virus was collected at the indicated times. Each symbol and bar represent the average viral titer obtained from three replicates. (E) *Caspase8*$^{-/-}$*RIP3*$^{-/-}$ and control *caspase 8*$^{+/-}$*RIP3*$^{-/-}$ C57Bl/6 mice were infected IP with 10^6 PFU. Salivary glands were collected 14 DPI and disrupted by sonication. Viral titers in cell and organ sonicates were titered on 3T3-SA fibroblasts. The dotted line indicates the limit of detection.

3.VII. References

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CHAPTER 4

Conclusions and Future Directions

4.I. Cytomegalovirus Subversion of Mitochondrial Cell Death

4.I.i. General Overview

Modulation of cell death provides an opportunity for the virus to establish infection within an organism and maintain the environment necessary for efficient replication. However, the host can initiate cell death through several different pathways that the virus must subvert, including via pathways requiring the mitochondria. Mitochondrial cell death pathways efficiently limit the replicative capacity of a virus, limiting production of progeny, pathogenesis, and transmission. MCMV as well as HCMV must overcome this death pathway in order to complete the replication cycle.

MCMV subverts Bax-mediated cell death by vMIA, encoded by m38.5, and Bak by vIBO, encoded from the m41.1 reading frame contained within the m41/m41.1 locus. The m41/m41.1 locus encodes an additional product, localized to the Golgi apparatus and implicated in cell death suppression (Brune et al., 2003; Cam et al., 2010). In Chapter 2, we demonstrated that this larger Golgi-localized gene product was individually dispensable for replication, dissemination, and latency in the host. Furthermore, it did not act as a cell death suppressor during infection (Crosby et al., 2013). In contrast, m41.1 (vIBO) was central to the viral lifecycle (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013). In Chapter 3, we demonstrated that together vMIA and vIBO subverted the mitochondrial death pathway, supporting viral replication and pathogenesis. Their individual impacts were modest (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013; Manzur et al., 2009), initially indicating a minor role for the mitochondria in host defense; however, the full impact of the mitochondrial death pathway was revealed when both vMIA and vIBO were absent during infection (Chapter

3). In the absence of mitochondrial cell death suppression, viral replication was essentially eliminated in macrophages, a monocyte-derived cell population. Monocytic cell populations are central to viral infection, disseminating virus throughout the host via the bloodstream (Daley-Bauer and Mocarski, 2012). In correlation to impacting replication in this cell lineage, viral replication and dissemination was greatly attenuated in the host in the absence of vMIA and vIBO, implicating mitochondrial cell death suppression as central to supporting the cell type of dissemination during infection.

Overall, these results highlight the efficiency of the mitochondrial death pathways in host defense and provide insight and future directions in several areas. These areas include (1) the role of mitochondrial cell death suppression in monocytic cell populations disseminating virus in the host; (2) the functional interactions between Bax and Bak during infection; (3) the contribution of HCMV vMIA to replication and pathogenesis; and (4) the function of Golgi-localized m41 during infection. These insights and future directions are discussed below.

4.I.ii. Cell Death Suppression in Monocytic Cell Populations

Mitochondrial cell death suppression is an important feature of CMV biology. Regulation of these pathways supports pathogenesis as well as replication in a variety of cell types, particularly cells of a monocytic cell lineage, such as macrophages (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013; Manzur et al., 2009). Monocytic cell populations are central to CMV infection of the host, disseminating virus via the bloodstream to other target organs and tissues as well as serving as a source of latent virus (Daley-Bauer and Mocarski, 2012). Patrolling monocytes are the specific

monocytic cell population responsible for disseminating MCMV following local infection. Mutant viruses lacking the ability to suppress mitochondrial cell death exhibit signs of impaired dissemination as low levels of replication are observed in the salivary glands, the final site of dissemination (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013; Manzur et al., 2009). These low levels of replication in the salivary glands paired with limited replication in macrophages in tissue culture suggests the viral suppressors vMIA and vIBO play a central role in maintaining the monocytic cell population trafficking the virus throughout the host.

This hypothesis is supported by two key pieces of evidence. First, a study published at the same time as Chapter 2 also analyzed the impact of vIBO on MCMV pathogenesis and revealed a distinct pattern of dissemination to the salivary glands over time (Fleming et al., 2013). Viral titers in the salivary glands of a vIBO mutant virus started to rise at 7 DPI, similar to wild-type control. These titers continued to increase over time; however, the rate declined to levels below wild-type, leading to decreased vIBO mutant virus at 10 DPI. These results suggest that vIBO mutant virus is attenuated in terms of seeding the salivary glands, as the rate of increase was not sustained to control levels over the course of infection. Subsequently, mutant virus eventually reached the same level of replication as K181-BAC, indicating that once the vIBO mutant infected the acinar epithelial cells of the salivary glands normal rates of replication were restored. Thus, these data indicate that vIBO supports dissemination to the salivary glands likely by maintaining the viability of a myeloid lineage cell.

A second key piece of evidence is that M36 (vICA) and M45 (vIRA) also exhibit common impacts on replication as vMIA and vIBO (Brune et al., 2001; Cicin-Sain et al.,

2008; Menard et al., 2003; Upton et al., 2010). Specifically, vICA and vIRA individually have a profound impact on replication in macrophages. This defect in macrophages also correlates to limited replication and dissemination in the host. Thus it appears that suppression of apoptosis and necrosis is central to viral dissemination. This is modeled in Figure 4.1 in which dissemination is initiated upon infection of the host. At the time of infection the virus must undergo a period of local amplification, expressing the viral suppressors that facilitate viral replication. At the time of local amplification, the cell type of dissemination is recruited and infected by progeny virus. These susceptible cells traffic via the bloodstream to other sites of infection and eventually differentiate into a permissive macrophage or dendritic cell. In these permissive differentiated cells, the viral lifecycle is initiated and the suppressors are also expressed during this second round of replication, supporting production of progeny and infection of neighboring cells. vIRA appears to be important very early during local amplification as indicators of a local inflammatory response and infiltration of the cell type of dissemination are not observed in the absence of this gene product (Upton et al., 2010). Thus it appears that replication is terminated immediately by induction of necrosis. This corresponds to the role vIRA plays in supporting replication in other cell types, such as fibroblasts and endothelial cells expressing high levels of RIP3 (Table 1.1) (Brune et al., 2001; Brune et al., 2003; Upton et al., 2010).

Overall, suppression of apoptosis and necrosis is likely central to the viral lifecycle during dissemination, particularly within monocytic cell populations, such as patrolling monocytes. To further characterize the relationship between monocytic cell populations and cell death suppression future directions include (1) analyzing viral

replication of the different viral suppressors mutants in specific monocyte-derived populations, such as macrophages and dendritic cells derived from patrolling monocytes, (2) characterization of the death pathways (i.e. levels of RIP3, Bax, Bak, etc.) in patrolling monocyte derived cells, (3) characterizing how elimination of these death pathways in patrolling monocytes influences pathogenesis of the mutant viruses lacking the different viral suppressors, and finally (4) characterize the impacts of viral cell death suppression on establishment, maintenance, and reactivation from latency.

4.I.iii. Bax and Bak Activation During Murine Cytomegalovirus Infection

Bax and Bak are the central bottleneck to mitochondrial cell death. Stress signals are conveyed through these two effector proteins, leading to termination of the host cell (Chipuk et al., 2010). During this process, Bax and Bak functions are almost identical, oligomerizing upon activation in order to create pores in the mitochondrial membrane. Upstream stress signals converge on Bax and Bak via the direct activators, Bim, Bid, and PUMA (Chipuk et al., 2010; Ren et al., 2010). These proapoptotic, direct activators induce simultaneous activation of Bax and Bak, initiating mitochondrial permeabilization.

Investigations into vMIA and vIBO during replication clearly demonstrated that Bax and Bak are activated during infection (Crosby et al., 2013; Fleming et al., 2013; Handke et al., 2013; Manzur et al., 2009) (Chapter 3); however, characterization of the upstream pathway driving this activation failed to identify a primary activator. Rather, results demonstrated that replication of a mutant virus lacking vMIA and vIBO could not be restored by individual elimination of Bim, Bid, or PUMA, or pathways dependent

upon their activation (i.e. caspase-8). While these results were initially perplexing, two models explain the relationship between the direct activators and Bax and Bak during infection (Figure 4.2). First, viral infection may stimulate so many different stress signals that all three direct activators, Bid, Bim, and PUMA, are simultaneously active (Figure 4.2A). Therefore, when one pathway (i.e. Bim) is eliminated the other two pathways (i.e. Bid and PUMA) are present and active, driving Bax and Bak oligomerization. Second, a fourth direct activator, yet to be identified, may be initiating the mitochondrial death pathway (Figure 4.2B). The later model (Figure 4.2B) is supported by evidence describing the role that effector and direct activator Bcl-2 proteins play during development.

Individually, Bax and Bak have a minor role during development. Elimination of Bax in mice lead to modest impacts, such as B and T cell hyperplasia, abnormal morphology of gonads and germ cells, prolonged ovarian lifespan, and reduced central and peripheral nervous system cell death (Knudson et al., 1995). These impacts were not significant enough to terminate the lifespan of the developing embryo, leading to viable *Bax*$^{-/-}$ mice. A similar story was revealed upon Bak elimination in the whole animal with even fewer biological impacts in the viable mouse (Hutcheson et al., 2005; Lindsten et al., 2000). However, upon simultaneous elimination of Bax and Bak the developmental process was significantly altered (Lindsten et al., 2000). Mice lacking both effector proteins were often pre- and peri-natal lethal as well as exhibited signs of interdigital webbing and immunological, nervous system, and reproductive defects. Therefore, this evidence demonstrated that together Bax and Bak play a significant role in the developmental process.

Investigation into the upstream activators of Bax and Bak, revealed that together Bid, Bim, and PUMA also play a key role during development (Ren et al., 2010). However, simultaneous elimination of all three known upstream direct activators did not lead to a full phenocopy of mice lacking both Bax and Bak (Lindsten et al., 2000; Ren et al., 2010). *Bim*$^{-/-}$*Bid*$^{-/-}$*PUMA*$^{-/-}$ mice did display developmental defects, such as interdigital webbing and imperforated vaginas, observed in *Bax*$^{-/-}$*Bak*$^{-/-}$ mice. But these triple knockout, direct activator mice were often viable and therefore had a less severe phenotype in terms of embryonic lethality as compared to *Bax*$^{-/-}$*Bak*$^{-/-}$ mice. Thus, this data indicates that alternative direct activators exist that drive Bax and Bak oligomerization.

To tease apart these two models of Bax and Bak activation during infection future experiments should initially focus on elimination of more than one direct activator. If Bim, Bid, and PUMA are simultaneously active during infection, then elimination of all three pathways should restore replication of a virus lacking vMIA and vIBO. Elimination of at least two of the pathways may even partially restore replication of a double vMIA/vIBO mutant virus. However, if viral replication cannot be restored by elimination of these pathways then the vMIA/vIBO mutant virus can be utilized to screen for alternative direct activators. These studies may advance knowledge as to how Bax and Bak are activated and how then contribute to host defense.

4.I.iv. Relating Murine Cytomegalovirus vMIA/vIBO Back to Human Cytomegalovirus vMIA

Mitochondrial cell death suppression is conserved in HCMV, encoding a single suppressor, vMIA, from UL37 exon 1 (Goldmacher et al., 1999). HCMV vMIA localizes to the mitochondria and can suppress both intrinsic and extrinsic death signals in human cells (Arnoult et al., 2004; Goldmacher et al., 1999; McCormick et al., 2005; Poncet et al., 2004). Similar to MCMV m38.5 (vMIA), HCMV vMIA's functions are necessary for efficient viral replication in fibroblasts (McCormick et al., 2005; Reboredo et al., 2004); however, the extent to which the virus depends on this gene product for replication varies based on laboratory strain. HCMV vMIA is more essential to AD169 replication as compared to Towne-BAC (McCormick et al., 2005; Reboredo et al., 2004). Replication defects observed with Towne-BAC infection in the absence of vMIA are attributed to premature termination of replication due to early initiation of a death pathway dependent upon serine protease HtrA2/Omi and independent of caspases (McCormick et al., 2008). The pathway downstream of the mitochondria has not been characterized during AD169 infection. This pathway may be similarly dependent upon HtrA2/Omi or dependent upon caspases, a defining feature of apoptosis.

Activation of either of these pathways is likely subsequent to activation of Bax and Bak at the mitochondrial membrane, as vMIA has been shown to interact with both effector proteins (Karbowski et al., 2006). Despite this evidence, vMIA's interactions with Bak remain controversial. vMIA has been very well characterized as a Bax-inhibitor, binding to the effector protein and re-localizing it from the cytosol to the mitochondrial membrane (Arnoult et al., 2004; Poncet et al., 2004). This re-localization is typically observed following activation of Bax in which the protein undergoes conformation changes that allow oligomerization and insertion into the membrane

(Chipuk et al., 2010). vMIA appears to stabilize key elements in Bax needed for oligomerization and insertion, thus preventing this process and subsequent death (Ma et al., 2012). Studies focused on vMIA have not eloquently teased apart similar interactions with Bak. In fact, only one biochemical investigation to date has been able to detect a direct interaction between these two proteins (Karbowski et al., 2006). Other functional data, however, focused on mitochondrial morphology supports vMIA as a Bak inhibitor.

Expression of vMIA, either in the context of viral infection or overexpression, results in disruption of mitochondrial networks (Karbowski et al., 2006; McCormick et al., 2003b). Mitochondria are dynamic organelles, undergoing a constant process of fission and fusion that morphologically alters their appearance (Youle and van der Bliek, 2012). Fusion results in elongated mitochondrial chains that appear filamentous when visualized by microscopy. Fission, on the other hand, results in the fragmentation of these networks, resulting in punctate mitochondria. Together, Bax and Bak promote fusion of the mitochondria, resulting in an elongated network (Karbowski et al., 2006). vMIA's disruptions of this network is dependent upon its interactions with both Bax and Bak (Karbowski et al., 2006; McCormick et al., 2003b), supporting the model in which it acts as an inhibitor of both proteins.

In contrast, studies arguing in favor of vMIA acting solely as an inhibitor of Bax failed to detect direct interactions with Bak (Arnoult et al., 2004; Poncet et al., 2004). These studies also demonstrated that vMIA was only able to protect mitochondrial membrane permeabilization in the presence of Bax when cells were treated with staurosporine but not in the presence of Bak. Furthermore, studies in murine cells demonstrated that vMIA is able to maintain mitochondrial membrane integrity only in the

presence of Bax (Arnoult et al., 2008) and that MCMV vIBO, the Bak inhibitor, is necessary in conjunction with HCMV vMIA to suppress extrinsic apoptosis (Cam et al., 2010).

Therefore, it remains unclear whether HCMV vMIA truly inhibits both effector proteins (Figure 4.3A) or whether it is solely an inhibitor of Bax (Figure 4.3B). Alternatively, some have suggested that cells can be divided into two categories, (1) Bax dominant cells or (2) cells in which both Bax and Bak pathways are co-dominant (Arnoult et al., 2004). Human cells may represent Bax dominant cells, hence vMIA is sufficient to inhibit mitochondrial cell death (Figure 4.3C), whereas murine cells are co-dominant (Figure 4.3D).

The Bax/Bak conundrum of HCMV vMIA can be sorted out by studies focused on complementation of HCMV and MCMV mutant viruses lacking the mitochondrial cell death suppressors (Figure 4.4). The model shown in Figure 4.3A, in which HCMV vMIA functions as a suppressor of both Bax and Bak, can be tested by utilizing an MCMV mutant lacking both m38.5 and m41.1. If both Bax and Bak are suppressed by HCMV vMIA then this viral protein would be able to maintain viability of macrophages during infection with the MCMV mutant lacking m38.5 and m41.1 and therefore restore viral replication (Figure 4.4A). Furthermore, HCMV vMIA would complement the replication and dissemination defects observed in the host with this MCMV double mutant virus. However, if HCMV vMIA functions solely as an inhibitor of Bax (Figure 4.3B) then this viral protein would only be able to completely restore the impacts of a single MCMV m38.5 (vMIA) mutant virus in terms of viability and replication in macrophages, fibroblasts, and endothelial cells as well as replication and dissemination in the host

(Figure 4.4B). A partial restoration of the double m38.5 (vMIA)/m41.1 (vIBO) mutant virus would also be achieved in this setting by HCMV vMIA. Finally, to determine if human cells behave in a Bax-dominant manner (Figure 4.3C) m38.5 (vMIA) can be utilized to complement HCMV lacking vMIA (Figure 4.4C). In this setting, m38.5 (vMIA) should be able to restore replication defects observed with AD169 and Towne-BAC lacking vMIA. However, current data does not support this latter model shown in Figure 4.3C (McCormick et al., 2005). Overexpression of m38.5 (vMIA) in human fibroblasts is not sufficient to suppress extrinsic apoptosis whereas UL37 exon 1 (vMIA) is able to block this stress signal. This indicates that human fibroblasts do not function in a Bax-dominant manner, favoring models shown in Figure 4.3A-B.

A second line of investigation that will clarify vMIA's role during HCMV infection revolves around manipulation of the host cellular pathways. Studies have demonstrated that vMIA is necessary for efficient replication in fibroblasts, particularly for strain AD169, while separately demonstrating that this viral protein subverts Bax- and/or Bak-dependent pathways. Direct evidence demonstrating that Bax and/or Bak functions limit viral replication in the absence of vMIA is not available. Experiments focused on viral replication of vMIA mutant viruses in the absence of Bax and/or Bak would provide evidence directly linking the viral lifecycle to the mitochondrial pathways. Similar lines of investigation in which host cellular processes have not been directly tied to viral replication have lead to a growing list of vMIA functions. vMIA has been demonstrated to function in collaboration with viperin, GADD45, Bax, and Bak as well as influence mitochondrial respiration and control downstream caspase-dependent and –independent pathways (Arnoult et al., 2004; Kaarbo et al., 2011; Karbowski et al., 2006;

McCormick et al., 2008; Poncet et al., 2004; Seo et al., 2011; Smith and Mocarski, 2005). This is a long list of functions for a viral protein of a mere 163 amino acids containing a small number of identified interaction domains (Figure 1.7). This long list of functions and interacting partners is not unique to vMIA. Other vBcl-2 proteins, such as Vaccinia virus encoded F1L, also have a growing list of functions. Vaccinia virus F1L was originally described as a mitochondrial-localized viral anti-apoptotic protein, mimicking the antiapoptotic members of the Bcl-2 family (Wasilenko et al., 2003). Since its discovery F1L interacting partners include Bcl-2 family members, Bax, Bak, and Bim, as well as players involved in the inflammasome formation, such as NLRP1 (Campbell et al., 2010; Gerlic et al., 2013; Taylor et al., 2006; Wasilenko et al., 2005). How these host players impact viral replication remains unknown; however, the Bcl-2 family members do impact the host cell as elimination of various mitochondrial death pathways restores viability of the cell during infection with a mutant virus lacking F1L (Eitz Ferrer et al., 2011).

Ultimately, the vBcl-2 proteins must be studied in a cell type in which their function is necessary for viral replication. The sequence characterization of the BH domains as well as the biochemical interactions provides a good base for functional predictions during infection. But the natural host cell type in which the viral suppressor contributes to replication must first be identified, followed by rescue of this defect by eliminating candidate host pathways. Investigations of HCMV vMIA have been confined to infection of fibroblasts. While vMIA is necessary for efficient replication in this setting, studies presented here focused on MCMV predict that HCMV vMIA has a more central role in myeloid lineage cells, such as macrophages. Future investigations will

focus on (1) the impact of vMIA on HCMV replication in various cell types, such as macrophages and endothelial cells; (2) elimination or knockdown of Bax and/or Bak to restore replication defects identified; and (3) characterization as to how HCMV vMIA can influence MCMV replication and vice versa.

These studies will determine the contribution of vMIA to HCMV replication as well as the mechanism of function during infection. As stated earlier, evidence favors a model in which HCMV vMIA suppresses both Bax- and Bak-mediated apoptosis, indicating it is an evolutionary homolog of MCMV vMIA and vIBO combined. In this scenario, these studies predict that mitochondrial cell death suppression by UL37 exon 1 (vMIA) has a profound impact on HCMV infection of the host. Elimination of vMIA will likely lead to large reductions in levels HCMV replication and dissemination due to increased apoptosis. Studies indicate that apoptosis as well as necrosis influence the immune response (Ferguson et al., 2011). Apoptotic and necrotic cells can drive an immune response that in some cases is tolerogenic and in other cases is immunogenic. Increased immunogenicity is a desired parameter for vaccine candidates. Initial studies indicate that elimination of viral mitochondrial cell death suppressors, such as F1L, enhance immune responses during infection (Perdiguero et al., 2012). These early studies illustrate a need to evaluate the immune responses that develop in the absence of mitochondrial cell death suppression as well as in the absence of suppression of other apoptotic and necrotic death pathways. MCMV is a perfect candidate for these studies as it carries several apoptotic and necrotic suppressors that are in most cases evolutionarily conserved in the human virus and it drives a well-characterized acute immune response that promotes clearance (Snyder, 2011). Therefore, future studies will also focus on

characterization of these immune responses in the presence of increased apoptosis or necrosis. These studies may impact vaccine design for HCMV in order to increase immunogenicity.

4.I.v. Golgi-Localized m41: A Possible Role in Conjunction with m41.1 (vIBO)

The tight genetic intricacies between m41 and m41.1 confounded early investigations into the function of this locus during infection. Coarse mutations, such as large transposon insertions and whole locus replacements, identified the presence of a cell death suppressor but failed to appreciate the smaller, un-annotated m41.1 reading frame (Brune et al., 2003). Therefore, Golgi-localized m41 gained the title of a virally encoded cell death suppressor. Re-annotation of the MCMV genome lead to the characterization of m41.1 functioning in cell death suppression (Brocchieri et al., 2005; Cam et al., 2010). Despite the re-assignment of a majority of these functions, Golgi-localized m41 has remained a cell death suppressor in the literature (Brune, 2011) due to a result demonstrating that infection of macrophages with an m41 mutant virus lead to decreased cell viability as compared to infection with wild-type virus (Cam et al., 2010). The decrease in cell viability, however, did not correspond to an increase in cell death markers, such as DNA fragmentation, effector caspase cleavage, and oligomerization of Bak. Whereas, infection of macrophages with an m41.1 (vIBO) mutant virus did lead to an obvious increase in these cell death markers. Therefore, cell death suppression by Golgi-localized m41 has remained ambiguous due to limited data indicating a role in blocking these pathways and due to the difficulty in modeling suppression of cell death from the Golgi apparatus as there is a low abundance of stress pathways intersecting with

this organelle (Ferri and Kroemer, 2001). In Chapter 2, we removed the title of cell death suppressor from m41 as this viral gene product does not impact cell viability during infection (Crosby et al., 2013). In fact, m41 does not impact replication in permissive cell types assayed *in vitro* as well as in the host. A virus lacking m41 is able to establish acute infection in the host, with only a modest impact on viral clearance from the lungs, as well as reactivate from latency. All together, this data indicates that m41 is completely dispensable during infection of the host.

At the time of publication of these results, another paper identified an upstream exon between m42 and M43 spliced onto the amino terminus of m41 (Fleming et al., 2013). This splicing event produced two gene products during infection, m41L and the original m41. m41L utilizes the entire m41 reading frame during translation in addition to the short, spliced exon. Mutations engineered into m41 during our studies were contained within the original m41 reading frame, just downstream of m41.1 (Crosby et al., 2013), and therefore disrupted both m41 and m41L gene products. This indicates that both m41L and m41 are dispensable during infection in the host. The paper identifying the two gene products supported this conclusion by demonstrating that m41 and m41L mutant viruses replicate to similar levels as the parental virus, with only a modest impact on viral clearance from the lungs (Fleming et al., 2013).

All together, m41 gene products appear to be completely dispensable during infection. The identification of a dispensable gene is a rare event as viruses have a limited coding capacity that must be utilized to produce progeny and subvert the antiviral host tactics. Only one other dispensable gene, IE2, has been identified amongst CMVs (Cardin et al., 1995). However, it remains possible that an aspect of biology yet to be discovered

obscures the function of m41 and/or that m41 functions in conjunction with another protein, such as m41.1. Preliminary characterization of a virus in which the m41/m41.1 locus had been replaced with a large selection/counterselection cassette, Δm41/m41.1-BAC (Figure 2.1), indicated a potential combined role for these two gene products. The replication kinetics of the Δm41/m41.1-BAC was analyzed during infection of BALB/c mice. Viral titers in the salivary glands at 14 DPI were greatly reduced for the Δm41/m41.1-BAC in comparison to WT and m41 mutant levels (Figure 4.5A). This decreased level of replication at 14 DPI in the salivary glands was greater than that observed for a single m41.1 mutant virus (data not shown). Despite these lower levels of replication, Δm41/m41.1-BAC replication persisted at 45 DPI, a time period in which control K181-BAC and MR-BAC as well as m41 and m41.1 mutant viruses are cleared (Figure 4.5B-C). CD4 T cells clear viral infection in the salivary glands through an unknown mechanism that is independent of CD8 T cells and humoral responses (Jonjic et al., 1989; Jonjic et al., 1990; Jonjic et al., 1994; Lucin et al., 1992). Preliminary evaluation of the CD4 T response in the draining cervical lymph nodes revealed a lower percentage of cells expressing IL2 and TNF-α upon infection with Δm41/m41.1-BAC as compared to K181-BAC and the m41 mutant virus (Figure 4.6). Thus indicating an insufficiently primed CD4 T cell response in the salivary glands during infection with Δm41/m41.1-BAC. To determine if increased antigen levels lead an increased priming of the CD4 T cells response and thus viral clearance at 45 DPI, Δm41/m41.1-BAC was co-infected with K181-BAC. Upon co-infection, viral titers were elevated to WT levels at 14 DPI, promoting clearance by 45 DPI (Figure 4.7). This result demonstrates that Δm41/m41.1-BAC insufficiently primed the immune response that clears the salivary

glands. All together, a combined deletion of m41 and m41.1 revealed a greater impact on pathogenesis as compared to single mutants, indicating that these two gene products may cooperate during infection. If this hypothesis holds true, the role of m41 will only be revealed if it is studied in conjunction with m41.1. Refined point mutations eliminating both gene products are necessary to tease apart a cooperative function. Results presented here are preliminary as the large selection/counterselection cassette inserted in to the locus may impact neighboring genes, confounding the results. Therefore, future directions include (1) the insertion of point mutations disrupting both m41 and m41.1 (vIBO) and (2) analysis of the impacts on replication and pathogenesis of this double mutant virus in comparison to individual m41 and m41.1 mutant viruses.

4.II. Figures, Tables, and Legends

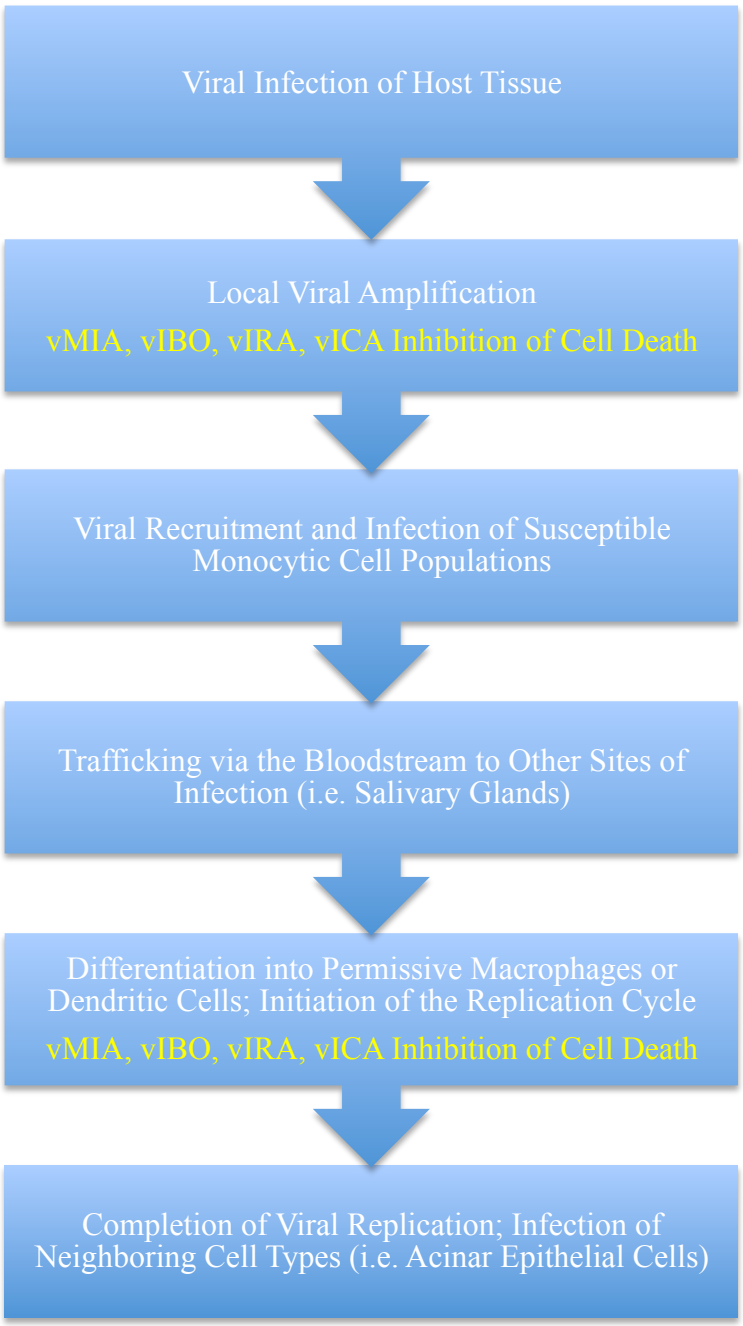

Figure 4.1. Model of the Impact of Cell Death Suppression During Dissemination in the Host. Following infection, the virus undergoes a round of viral replication (i.e. local amplification), recruiting the cell type of dissemination at the same time. During local

amplification, viral cell death suppressors (i.e. vMIA, vIBO, vICA, vIRA) are expressed, supporting replication by inhibiting death of the host cell. Progeny virus infects recruited monocytic cell populations that are susceptible but not permissive to viral replication. These cells traffic to other tissues throughout the host (i.e. salivary glands) via the bloodstream and eventually differentiate in to permissive macrophages or dendritic cells. Following differentiation, viral replication is initiated and the viral cell death suppressors are expressed for a second time. These cell death suppressors inhibit cell death that is initiated upon exposure to intrinsic and extrinsic stress signals thus supporting viral replication, production of progeny, and infection of neighboring cells (i.e. acinar epithelial cells).

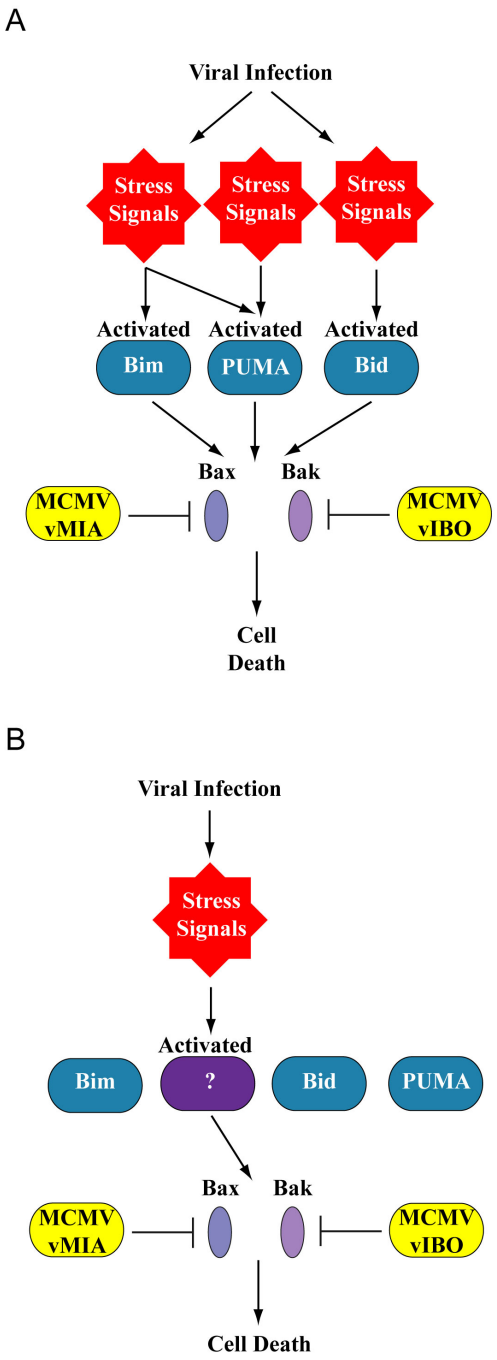

Figure 4.2. Model of Upstream Activation of Bax and Bak During MCMV Infection. MCMV vMIA and vIBO suppress Bax and Bak-mediated cell death during infection. Characterization of MCMV replication in the absence of these two viral inhibitors as well as the upstream pathway driving mitochondrial cell death reveals different models of Bax

and Bak activation. (A) MCMV infection may induce stress signals that drive several pathways, leading to activation of all three direct activators. Therefore, Bim, Bid, and PUMA may simultaneously activate redundant Bax and Bak. (B) An additional direct activator that is yet to be identified may activate redundant Bax and Bak during infection.

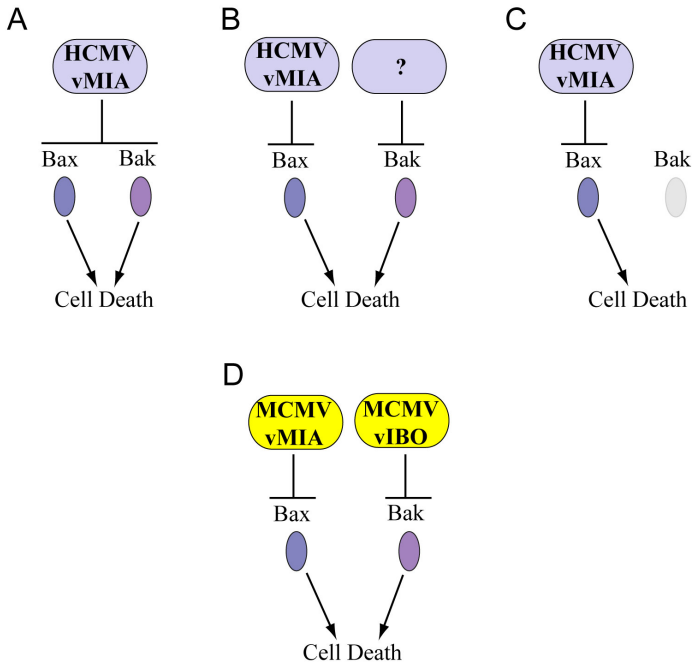

Figure 4.3. Model of HCMV vMIA Function as Compared to MCMV Mitochondrial Cell Death Suppression. Studies investigating the role of HCMV vMIA in suppression of mitochondrial death pathways have developed models in which the viral protein inhibits (A) both Bax- and Bak-mediated cell death, (B) solely inhibits Bax-mediated cell death and the viral protein suppressing Bak remains to be identified, or (C) dominant Bax-dependent pathways (Bak-dependent pathways are not sufficient to drive apoptosis. (D) Murine cells are able to drive both Bax- and Bak-dependent pathways, therefore, MCMV encodes two inhibitors, vMIA and vIBO, to suppress apoptosis.

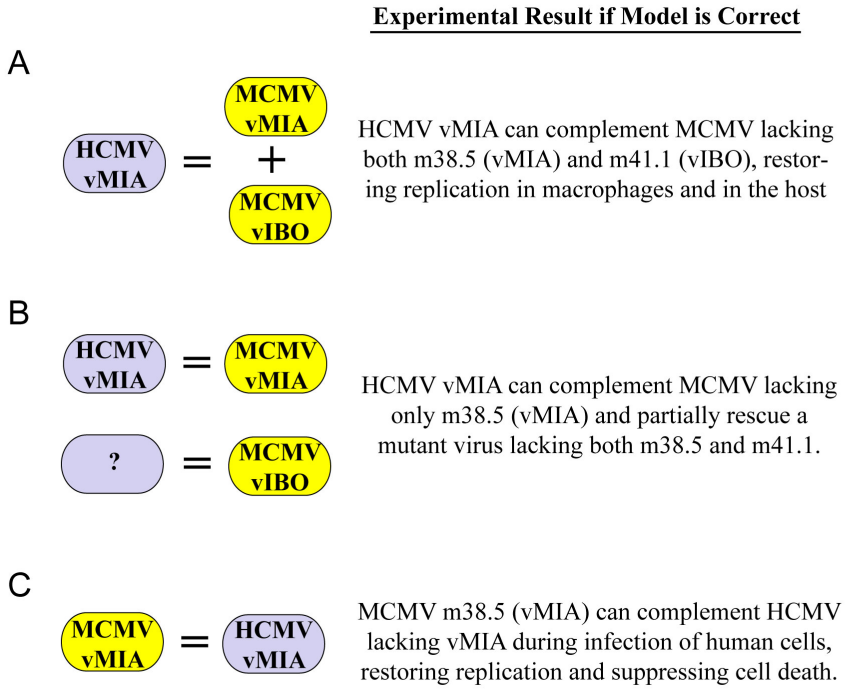

Figure 4.4. Experimental Designs to Determine if HCMV Functions as an Inhibitor of Both Bax and Bak or solely Bax. (A) If HCMV vMIA functions as an inhibitor of both effector proteins (modeled in Figure 4.4A) then this viral protein can complement the defects observed with a double MCMV mutant lacking m38.5 (vMIA) and m41.1 (vIBO). (B) If HCMV vMIA functions only as an inhibitor of Bax then only the replication defects observed with a single m38.5 (vMIA) mutant virus can be completely complemented. Another unidentified inhibitor of Bak mimics the function of vIBO. (C) If human cells exhibit a Bax-dominant phenotype then m38.5 (vMIA) can complement the defects observed with HCMV strains lacking vMIA.

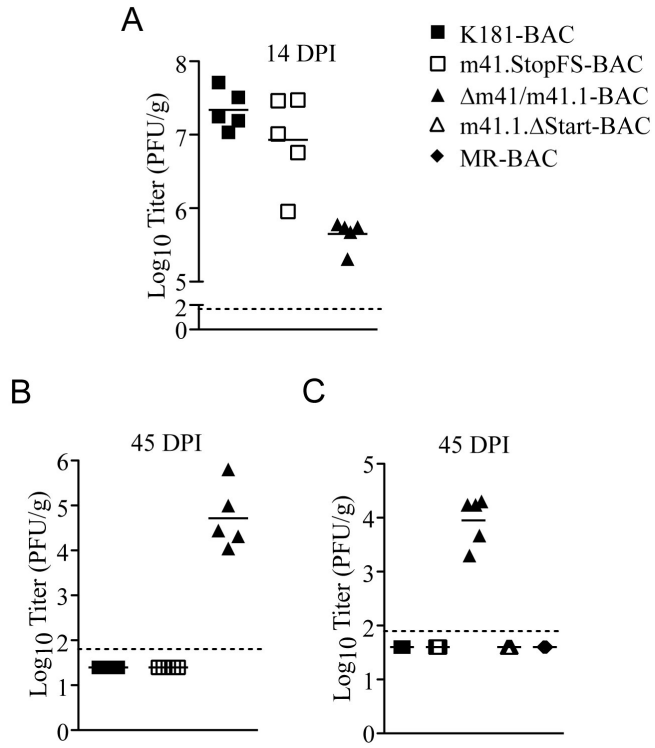

Figure 4.5. Evaluation of the combined role of m41 and m41.1 (vIBO) during replication and dissemination in the host. (A-C) 6-week-old BALB/c mice were inoculated with 10^6 PFU via the IP route. Salivary glands were harvested at the times indicated and disrupted via sonication. Titers in organ sonicates were determined by plaque assay on 3T3-SA fibroblasts. Each symbol represents the titer obtained from a single mouse. The dotted line indicates the limit of detection of virus.

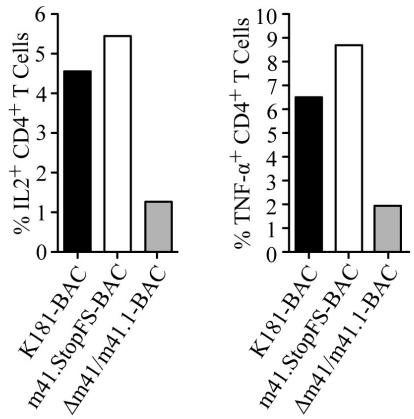

Figure 4.6. Decreased CD4 T Cell Response in the Cervical Lymph Nodes During Δm41/m41.1-BAC Infection. 6-week old BALB/c mice were infected with 10^6 PFU of K181-BAC or derived mutant viruses. The cervical lymph nodes draining the salivary glands were harvested at 45 DPI. Frequencies of CD4 T cells expressing IL2 and TNF-α are shown.

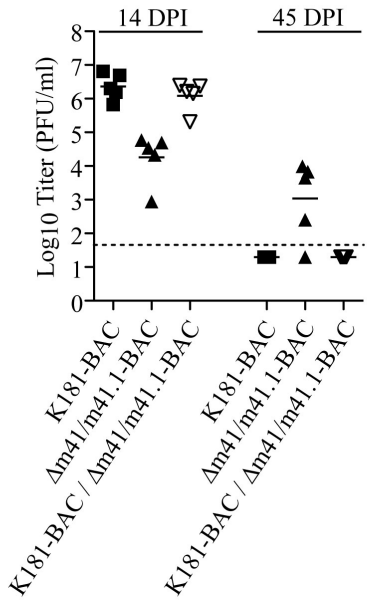

Figure 4.7. Increased Levels of Replication in the Salivary Glands Promotes Clearance of Δm41/m41.1-BAC. 6-week-old BALB/c mice were inoculated with 10^6 PFU of K181-BAC, Δm41/m41.1-BAC, or a combination of K181-BAC and Δm41/m41.1-BAC (total 2 x 10^6 PFU). Salivary glands were harvested at the times indicated and disrupted via sonication. Titers in organ sonicates were determined by plaque assay on 3T3-SA fibroblasts. Each symbol represents the titer obtained from a single mouse. The dotted line indicates the limit of detection of virus.

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