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Gene products of the embedded m41/m41.1 locus of murine cytomegalovirus differentially influence replication and pathogenesis

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**Article info**

**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.

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**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 beta herpesviruses, 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; Jurák 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; Karbowský 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.

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 (Fig. 1A), employing a strategy of selection/counterselection and an intermediate Δm41/m41.1-BAC with a Kan<sup>®</sup>/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 (Fig. 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 threonyne to prevent translation initiation (Fig. 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<sup>®</sup>/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 (Fig. 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 (Fig. 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<sup>®</sup>/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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 2D). This phenotype also extended to primary bone marrow-derived macrophages (BMDM) where mutant viral titers were more than 10-fold lower than controls (Fig. 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 (Fig. 2C) and J774A.1 (Fig. 2D) macrophage lines as well as primary macrophages (Fig. 2E). The slight difference in m41-mutant viral titers in RAW264.7 cells at 7 and 9 DPI (Fig. 2C) was not observed in either J774A.1 or
Fig. 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) and (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).
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 (Fig. 2C–E). As expected from previous work (Cam et al., 2010), m41.1-encoded vIBO sustained viability of infected macrophages (Fig. 3A–C). That is, cell viability of RAW264.7 (Fig. 3A), J774A.1 (Fig. 3B) and primary BMDM (Fig. 3C) was reduced during m41.1-mutant viral infection compared to controls. Furthermore, when infected with this mutant virus, Bak−/− BMDMs showed restored viability (Fig. 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 (Fig. 3E and F). This demonstrates that 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 4C) and salivary glands (data not shown), but not
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 (Fig. 5A) or lung (Fig. 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. viB0-deficient virus exhibited wild-type levels of replication in the lungs of BALB/c mice at 5 DPI (Fig. 6A); however, at 10 DPI viB0-mutant virus titers were 10-fold lower in the lungs than the other viruses (Fig. 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, viB0-mutant virus replication was modestly compromised in the salivary glands, where levels were up to 10-fold lower than control viruses at 10 DPI (Fig. 6C) or 14 DPI (Fig. 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 Fig. 2B and E). Despite these elevated titers of the parental control, viB0-mutant viral titers remained significantly reduced in the salivary glands in comparison to all controls \( p < 0.05 \).

The role of viB0 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 viB0 during dissemination, viremia was analyzed at
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 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, 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 outside of lymphoid organs, 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 Bak 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 fibroblasts to determine the presence of reactivated virus by detected CPE.

Fig. 5. Evaluation of the role of the m41 during reactivation in BALB/c mice. 6-week-old BALB/c mice were inoculated with 10⁶ 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.

Discussion

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 (Fig. 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 × 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 (Fig. 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.
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 Bcl-2 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. While it is formally possible that m41 is non-functional in the K181-BAC strain, we favor the view that m41 may encode 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 KanR/SacB cassette, is more attenuated than either independent mutant. However, because of the potential for this large insertion to alter gene

![Diagram](image-url)
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.

**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% CO₂ at least 18 h before infection. DMEM containing 4.5 g/mL glucose, 1 mM sodium pyruvate, 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen). Bone marrow-derived macrophages were harvested from B6 x 129P2 backcrossed to C57BL/6 mice for three generations (referred to as C57BL/6) and maintained in house as well as B6 x 129-Bax⁻/⁻ Bak1⁻/⁻ mice (referred to as Bak⁻/⁻) were provided by Douglas Green (St. Jude Children's Research Hospital) with permission from Jackson Laboratories (stock number 006329). 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 milk. Salivary glands were disrupted by sonication and centrifuged at 2300 × g for 10 min 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 titrated 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 h at 37 °C. Following adsorption, the viral inoculum was replaced with complete DMEM. Samples were harvested at the indicated times and titrated by plaque assay.

**Cell viability assay**

Cells (20,000 cells/well) were seeded into 96-well plate in quadruplicate. Approximately 18 h 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 manufacturer's protocol.

**Mice, infections, and organ harvests**

BALB/c mice were obtained from Jackson Laboratory. B6 x 129-Bax⁻/⁻ Bak1⁻/⁻ mice in combination with B6129SF2/J mice (Jackson Laboratory) were used for in vivo experiments. Five to eleven-week old mice were inoculated in one hind footpad or IP with either 10⁴ PFU of salivary gland-derived virus or 10⁵ 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 titrated by plaque assay.

Peripheral blood leukocytes (PBL) were isolated from blood collected via heart puncture in syringes loaded with 0.5 M EDTA (Cellogro). Blood was diluted in PBS, layered onto Histopaque-1199 (Sigma) density medium, and centrifuged (30 min, 700 × 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 NH₄Cl, 0.1 M NaHCO₃, and 0.01 M Na₂-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 10⁶ 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.

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