

A Comparative Analysis of Humoral Immune Responses Raised by Suppressed and Impaired Mouse Cytomegalovirus Mutants

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Alan Herdman

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Supervisor: Prof. Dr. rer. nat. Mirko Trilling

The experiments underlying the present work were conducted at the Institute for Virology
at the University Hospital Essen, University of Duisburg-Essen.

1. Examiner: Prof. Dr. Mirko Trilling

2. Examiner: Prof. Dr. Astrid Westendorf

Chair of the Board of Examiners: Prof. Dr. Katharina Fleischhauer

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1. Summary

Human cytomegalovirus (HCMV) is a broadly distributed, species-specific betaherpesvirus that frequently causes morbidity and mortality in individuals with impaired, senescent, and immature immunity. If HCMV replicates in pregnant women, virus transmission and congenital infections frequently cause mental defects, deafness, microcephaly, or even death of infants. Despite an urgent need, no approved HCMV vaccine is available. Mice infected with mouse cytomegalovirus (MCMV) serve as a model to investigate general principles of cytomegalovirus (CMV)-specific immune responses.

In principle, viruses can be attenuated through the deletion of genes that are either required for efficient virus production or for immune evasion, and we refer to these two types of alteration as impairing and suppressing attenuation, respectively.

A prototype for a suppressed virus mutant is an MCMV mutant lacking the *M27* gene ($\Delta M27$ -MCMV) that causes an exaggerated interferon (IFN) susceptibility. Accordingly, the replication of $\Delta M27$ -MCMV proceeds wt-like in the absence of IFN signalling *in vitro*, but is drastically suppressed by IFNs *in vitro* and *in vivo*.

A mutant lacking *M34* ($\Delta M34$ -MCMV), which replicates approximately 100-fold impaired in cell culture irrespective of the presence or absence of IFNs and was highly attenuated *in vivo*, is our prototype for an impaired mutant. A recent publication reported a comparable impairment of $\Delta UL34$ -HCMV (Turner et al., 2022). $\Delta M34$ -MCMV infection induced MCMV-specific ELISA-reactive IgG responses. Furthermore, the immune serum of $\Delta M34$ -MCMV-infected mice activated the fragment crystallizable (Fc) receptors CD16, CD32, CD64, and Fc γ RIV in antibody-dependent cellular cytotoxicity (ADCC) surrogate assays. Ultimately, infection with $\Delta M34$ -MCMV protects mice against subsequent challenge infections.

In this project we compare the immune responses of mice infected with $\Delta M27$ -MCMV & $\Delta M34$ -MCMV and show that despite differing in the type of attenuation and susceptibility to innate immunity between these two MCMV mutants, both raise similar Fc γ receptor and MCMV-specific IgG responses. Moreover, we determine that $\Delta M34$ -MCMV is a viable vaccine candidate with the implication that investigation into a $\Delta UL34$ -HCMV live-attenuated vaccine may yield promising results.

Das Humane Cytomegalovirus (HCMV) ist ein weit verbreitetes, artspezifisches Betaherpesvirus, das bei Personen mit geschwächtem, alterndem oder unreifem Immunsystem häufig zu Morbidität und Mortalität führt. In schwangeren Frauen kann die Replikation von HCMV zur Virusübertragung und eine angeborene Infektion häufig zu geistigen Behinderungen, Taubheit, Mikrozephalie oder sogar zum Tod von Säuglingen führen. Trotz des dringenden Bedarfs gibt es keinen zugelassenen HCMV-Impfstoff. Mäuse, die mit dem Maus-Cytomegalovirus (MCMV) infiziert sind, dienen als Modell zur Untersuchung allgemeiner Prinzipien der cytomegalovirus-spezifischen Immunantworten.

Prinzipiell können Viren durch die Deletion von Genen, die entweder für die effiziente Virusproduktion oder für die Umgehung des Immunsystems erforderlich sind, attenuiert werden. Diese beiden Arten von Veränderungen bezeichnen wir als Beeinträchtigung und unterdrückende Attenuierung.

Ein Prototyp eines attenuierten Virusmutanten ist ein MCMV-Mutant ohne das M27-Gen (Δ M27-MCMV), welches eine übersteigerte Empfindlichkeit gegenüber Interferon (IFN) verursacht. Entsprechend verläuft die Replikation von Δ M27-MCMV *in vitro* ohne IFN-Signalübertragung ähnlich wie der Wildtyp, wird jedoch durch IFNs *in vitro* und *in vivo* drastisch unterdrückt.

Ein Mutant ohne M34 (Δ M34-MCMV), der sich in Zellkultur unabhängig von der Anwesenheit oder Abwesenheit von IFNs etwa 100-fach schlechter repliziert und *in vivo* stark abgeschwächt war, ist unser Prototyp eines beeinträchtigten Mutanten. Eine kürzlich veröffentlichte Studie berichtete von einer vergleichbaren Beeinträchtigung des Δ UL34-HCMV (Turner et al., 2022). Die Infektion mit Δ M34-MCMV induzierte MCMV-spezifische ELISA-reaktive IgG-Antworten. Darüber hinaus aktivierte das Immuneserum von Δ M34-MCMV-infizierten Mäusen die Fragment-kristallisierenden (Fc) Rezeptoren CD16, CD32, CD64 und Fc γ RIV in Surrogat-Assays für antikörperabhängige zelluläre Zytotoxizität (ADCC). Letztendlich schützt die Infektion mit Δ M34-MCMV Mäuse vor erneuten Infektionen.

In diesem Projekt vergleichen wir die Immunantworten von Mäusen, die mit Δ M27-MCMV und Δ M34-MCMV infiziert wurden, und zeigen, dass beide, trotz der Unterschiede in der Art der Attenuierung und der Anfälligkeit für die angeborene Immunität dieser beiden MCMV-Mutanten, ähnliche Fc γ -Rezeptor- und MCMV-spezifische IgG-Antworten hervorrufen. Außerdem stellen wir fest, dass Δ M34-MCMV ein potenzieller Impfstoffkandidat ist, was

darauf hindeutet, dass die Untersuchung eines Δ UL34-HCMV-Lebendimpfstoffs vielversprechende Ergebnisse liefern könnte.

2. Introduction

2.1 Cytomegalovirus

Cytomegalovirus (CMV) is a double stranded DNA virus of the herpesviridae family, with the viral genome contained within a capsid. CMVs have the largest genome of any herpesvirus at ~230kb in length (Landolfo et al., 2003). This large genome allows the virus to code for more than 750 translation products (Lodha et al., 2023), including many proteins with immune evasion functions (Patro, 2019).

The virus' capsid is surrounded by a tegument layer, which contains proteins which assist in infection gene expression and immune evasion (Kalejta, 2008) and in the case of pUL48 in Human Cytomegalovirus (HCMV), viral entry (Smith et al., 2014).

The outermost layer is a lipid envelope embedded with proteins essential to CMVs entry into host cells via cell membrane fusion. Here, Glycoprotein B (gB), and a Glycoprotein H (gH)/Glycoprotein L (gL) dimer carry out the first step of CMVs replication cycle by fusing the viral envelope to the host cells membrane and allowing release of the tegument proteins and nucleocapsid into the cytoplasm (Isaacson & Compton, 2009; Vanarsdall et al., 2016; Vanarsdall & Johnson, 2012).

CMV is capable of infecting a broad range of cell types, such as fibroblasts, leukocytes, dendritic cells, epithelial cells, and endothelial cells (Sinzger et al., 2008). A typical infection involves an initial infection of the olfactory epithelium, where the virus will then spread to the rest of the system via infected dendritic cells (Bruce et al., 2022). Early replication can be observed in organs such as the spleen, liver and, lungs, with the virus subsequently spreading to other organs such as the salivary glands and kidney at later stages (Jackson & Sparer, 2018).

Like other viruses, CMV is in constant competition with the host's immune response, and as a result, has developed a high degree of species specificity due to this adaptive 'arms-race'. Notably, species such as Human Cytomegalovirus, Mouse Cytomegalovirus (MCMV) and Rhesus Cytomegalovirus (RhCMV) are distinct and generally incapable of cross-infection *in natura* (Le-Trilling & Trilling, 2017; Mozzi et al., 2020).

This species-specificity is an important consideration in future vaccine development, as a successful vaccine against MCMV in mouse models may not be directly transferrable to HCMV

in a human context (However as I shall discuss, that is not the case with the attenuated MCMVs included in this thesis). As well as this, MCMV vaccine experiments are also invaluable for inferring correlates of protection which are valuable in understanding how to develop a successful HCMV vaccine.

2.2 Clinical Relevance of CMV

HCMV is broadly distributed in the human population. Under circumstances of an uncompromised immune system, the virus is controlled and persists after primary infection in a latent state for example in myeloid cells (Poole et al., 2011). The estimated seroprevalence of HCMV worldwide is approximately 83%, with a seroprevalence of up to 96% in Turkey (Zuhair et al., 2019).

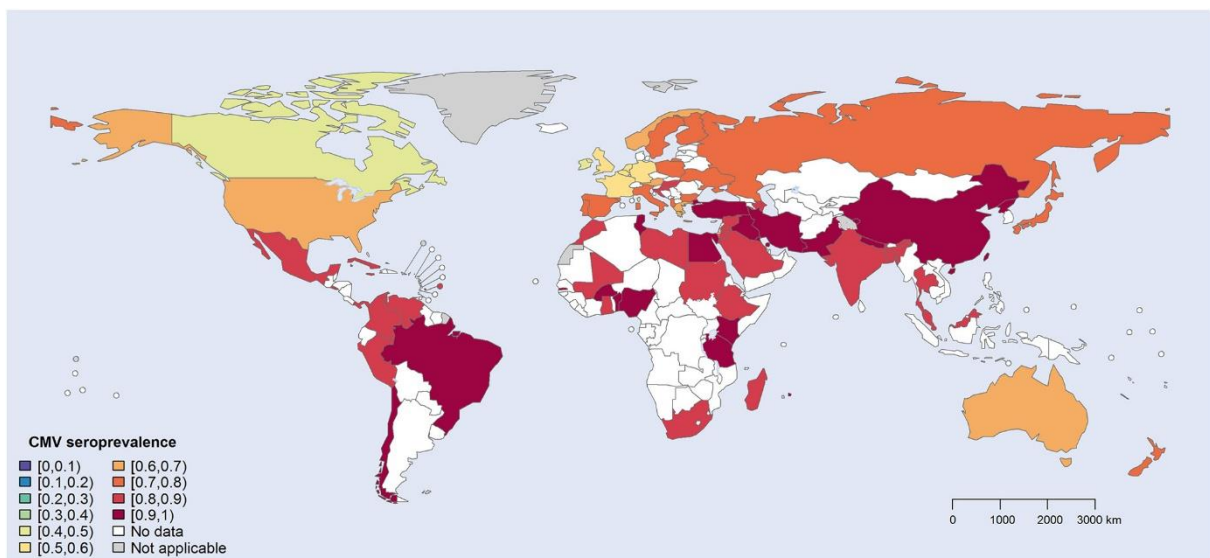


Figure 2.1: HCMV seroprevalence worldwide. Map showing estimated HCMV seroprevalence among women of childbearing age, from a meta-analysis conducted in 2019. Source: (Zuhair et al., 2019)

Complications, however, can occur in individuals with insufficient immunity, meaning that 83% of the worldwide population could be at risk in cases of developed immunodeficiency. Examples of this include transplant patients (Roller et al., 2010), or in Human Immunodeficiency Virus (HIV) patients who can develop HCMV retinitis, the cause of approximately 90% of HIV-related blindness (Munro et al., 2019).

Another significant group at risk from infections are developing foetuses and newborns (Britt, 2018). Approximately 0.5% of newborns in the United States are HCMV positive, with ~10%

of these going on to develop sequelae (Boppana et al., 2011; Kenneson & Cannon, 2007). These congenital infections can be spread from an infected mother either through the placenta during pregnancy, or postnatal via breastmilk (Gaytant et al., 2002; Schleiss, 2006). These congenital HCMV infections frequently cause issues in neurological development, as well as deafness, microcephaly or even death (Boppana et al., 2013; Fowler & Boppana, 2018; Korver et al., 2017).

Antivirals such as letermovir and ganciclovir are available, however, these also frequently cause side effects such as gastroenteritis, nasopharyngitis, dyspnea, and hepatotoxicity (del Rosal et al., 2012; Melendez & Razonable, 2015). As well as this, a long-standing concern about the development of drug-resistant HCMV is an issue to be taken into consideration (Komatsu et al., 2014; Lurain & Chou, 2010).

HCMV has been labelled a high priority disease-causing virus in terms of vaccine-development necessity, yet despite this, to date all attempts to develop a vaccine have been so far unsuccessful (Anderholm et al., 2016; Scarpini et al., 2021).

2.3 Vaccine Development for HCMV

As mentioned, there has been a coordinated effort to develop an HCMV vaccine. There are many ongoing trials, utilising a range of strategies to protect society from this virus (Schleiss, Permar, et al., 2017).

Examples of past attempts at an HCMV vaccine include the live-attenuated Towne strain of HCMV which was shown to reduce the severity of disease in renal transplant patients (Plotkin et al., 1984). However, it was found to be insufficient to protect women of childbearing age (Adler et al., 1995).

Subunit vaccines, which utilise CMV antigens to stimulate a host's immune response, currently show a lot of promise. One example, an adjuvanted gB subunit vaccine named gB/MF59 (glycoprotein B antigen with 'microfluidized adjuvant 59'), has completed its phase 2 trials (Pass et al., 1999) and has been found to boost the humoral response in seropositive trial patients, as well as reduce the number of congenital HCMV infections among vaccinated patients (Pass et al., 2009). A follow-up paper published on the topic has revealed a potential new correlate of protection. In terms of gB, a natural infection raises a humoral immune response against 5 traditional antigenic domains (AD-1-5). Trial patients vaccinated with

gB/MF59 however, did not raise many antibodies against these domains, but rather antibodies against a 6th antigenic domain of gB (AD-6). It has been observed in rabbits that antibodies directed against this AD-6 do not prevent circulating CMV from binding to cell surfaces or infecting cells. However, cell to cell spread is prevented. Furthermore, this observation was backed up in observations of sera from trial vaccine patients where cell to cell spread was prevented (Gomes et al., 2023).

Another current vaccine in development worth noting is mRNA-1647 (Fierro et al., 2024). The company Moderna is currently at work on an mRNA-based vaccine which has been found to raise a humoral immune response in phase 1 trials (Wu et al., 2024).

One further vaccine method is based on vectored vaccines, whereby a modified virus is used to transport and introduce an HCMV surface protein to cells to encourage antigen presentation. An example of this is lymphocytic choriomeningitis virus (LCMV) containing the CMV genes for gB and 65 kDa Phosphoprotein (pp65) antigens (Schleiss, Berka, et al., 2017). Interestingly, HCMV's potential as a vaccine vector is currently being studied (Mendez et al., 2019; Zeng et al., 2023).

HCMV's superinfection ability (Chou, 1986) could allow HCMV vectors to be used in multiple subsequent vaccinations as well as in HCMV-seropositive patients, while infection with even a replication-incompetent HCMV has been shown to raise protection against an implanted gene (Mohr et al., 2010).

Over the years, more than 20 vaccines have reached clinical trials only to be discontinued as candidates for various reasons. As of 2017, there are 8 vaccines in preclinical development (Schleiss, Permar, et al., 2017). These numbers exhibit the need for continuous vaccine development, especially when considering previous efforts towards developing an HCMV vaccine have shown the success of promising vaccines is not guaranteed (Pronker et al., 2013; Starr et al., 1981).

Table 2.1: HCMV vaccines in preclinical development. Source: (Schleiss, Permar, et al., 2017)

Dense body vaccines	Noninfectious
	Humoral and cellular immune response in preclinical testing
	Contain gB, pp65, other envelope and tegument proteins
RNA vaccines	Self-amplifying mRNA vaccines
	Strong antibody and cell-mediated immune responses
	Platform currently under development by GSK Vaccines and Moderna Vaccines
Electroporated DNA vaccines	DNA plasmid vaccines coadministered with electrical stimulation (SynCon platform)
	Excellent immunogenicity in preclinical testing
	Platform under development by Inovio Vaccines
RedBiotech gB/pp65 VLP vaccine	Engineered using recombinant baculovirus
	Generation of virus-like replicon particles (VRPs)
	Phase I clinical trial recently initiated
	Virus-neutralizing antibody and cell-mediated immune responses
	Currently being developed by Pfizer Vaccines
Soluble PC vaccine	Soluble adjuvanted pentameric complex vaccine
	Purified from CHO cells
	Potent, sustained neutralizing antibody responses in mice
	Developed by Humabs Biomed
MVA-vectored PC vaccine	Based on CMV PC
	Induces ELISA and neutralizing antibodies in mice
	Antibodies capable of blocking CMV infection of foetal placental macrophages (Hofbauer cells)
MVA-vectored pp65/IE1 fusion protein	Designated MVA-syn65_IE1
	Expands pp65- and IE1-specific T cells derived from CMV-seropositive donors
	Induces CMV pp65- and IE1 epitope-specific T cells in HLA-transgenic mice
Adenovirus-vectored gB/polyepitope (Ad-gBCMV polyvaccine)	Based on a replication-deficient adenovirus
	Encodes a truncated form of CMV-encoded gB antigen and multiple CMV T-cell epitopes from eight different CMV antigens as a single fusion protein
	Immunogenic in preclinical studies in HLA-A2 transgenic mice

With all these attempted strategies in mind, this project shall be focusing on the potential of live-attenuated vaccines utilising HCMV gene-knockout mutants. Live-attenuated vaccines function by exposing the immune system to a less virulent form of a pathogen, allowing the adaptive immune system to recognise a pathogen in subsequent infections and respond more effectively. In this work, I shall be discussing two attenuated virus mutants, one which is lacking a gene helpful in immune evasion (resulting in a suppressed virus), and one which is lacking a gene which attenuates the virus independent of the immune system (creating an impaired virus).

2.4 Innate and Adaptive Immune Response

Immune responses can be broadly categorised into innate or adaptive response. The innate immune system can be considered the ‘first line of defence against any infection and can be characterised as a broad, fast-acting and non-specialised initial defence. Meanwhile, the adaptive immune system takes longer to develop, but is highly specialised and specific to infections, and can maintain a memory of pathogens to provide a more effective protection against future contact with previously encountered pathogens (La Rosa & Diamond, 2012).

Importantly, these two immune strategies do not function in isolation, but instead overlap with each other through various signalling pathways.

2.4.1 Innate Immune System

The innate immune system is composed of several components, including physical barriers such as skin, tears, and mucous membranes. For this project, however, it is more important to consider the cellular responses.

Fig. 2.2 illustrates the various components of the innate immune response and the various pathways they are involved in.

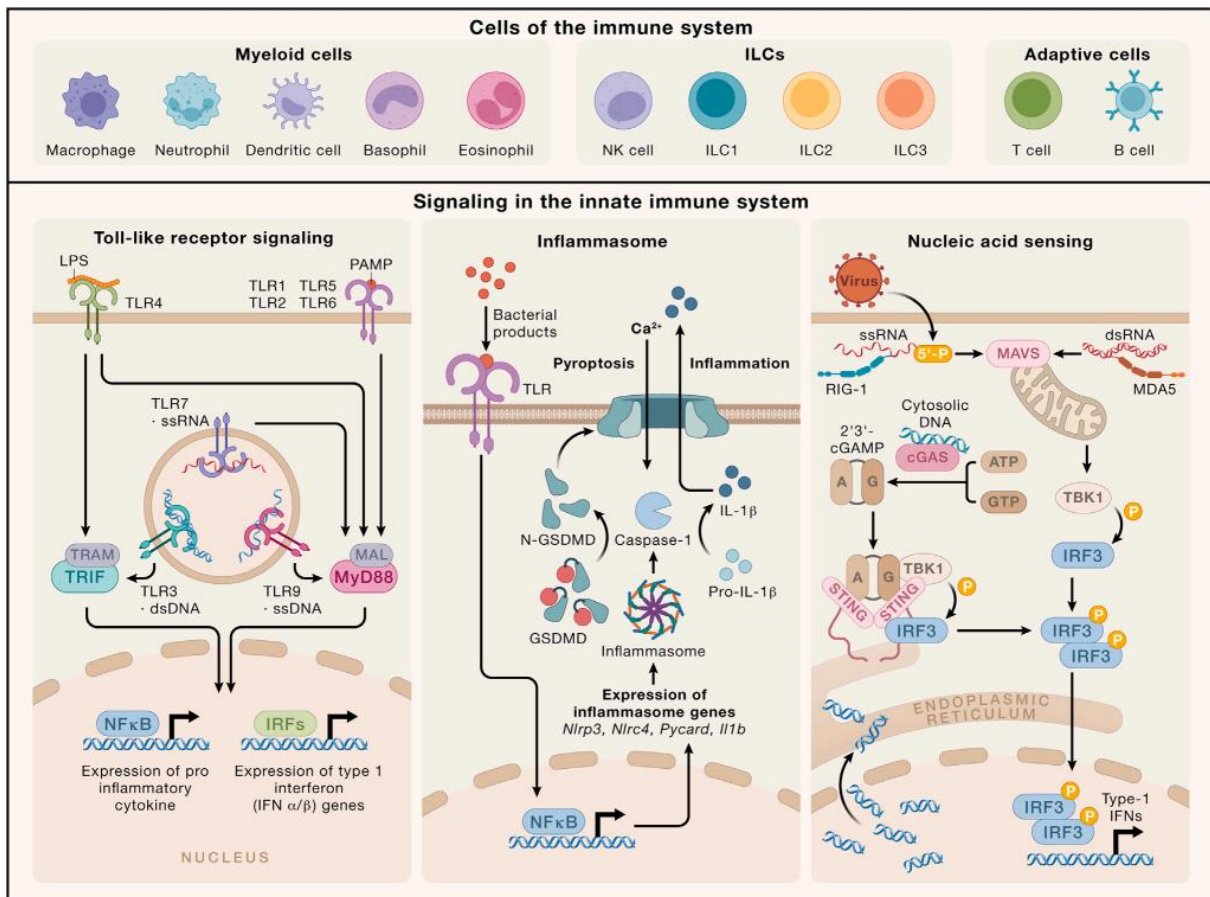


Figure 2.2: Innate immune signalling. Overview of cell types and signalling pathways involved in innate immunity. Source: (Carpenter & O'Neill, 2024)

Many cell types (e.g., neutrophils, dendritic cells, macrophages, and Natural Killer [NK] cells) have Pattern Recognition Receptors (PRR) (Akira et al., 2006). These PRR are crucial to innate immunity as they allow recognition of specific Pathogen-Associated Molecular Patterns (PAMP) present in pathogens but not in host cells (Kumagai & Akira, 2010). As a result, no prior exposure to a pathogen is required to initiate an immune response to foreign material.

An example of this is the Toll-Like Receptor (TLR) 2, which can recognise the gB and gH surface proteins of HCMV and induce Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Boehme et al., 2006) which then induces activation of pro-inflammatory genes (Liu et al., 2017).

Upon detection of PRRs, dendritic cells produce pro-inflammatory cytokines such as IFN-α and β (Inaba et al., 1990). As well as this, they play a crucial role in the interplay between the innate and adaptive immune system as Antigen Presenting Cells (APCs) (Iwasaki & Medzhitov, 2004).

Similarly, macrophages are also APCs. They phagocytose viruses then degrade their viral antigens into small peptide fragments. These are then presented on the cell surface in the context of Major Histocompatibility Complex (MHC) molecules for T cells to recognise (Hennecke & Wiley, 2001).

Immune cell types such as NK cells have been found to play a crucial role in the early control of CMV infections. NK cells function by detecting increased or decreased expression of cell-surface proteins (Kim et al., 2005). For example, healthy cells express MHC-1 molecules on their cell surface (which MCMV mimics through an MHC-like protein encoded by the *m157* gene (Arase et al., 2002)). Meanwhile, viral infection causes the upregulation of stress-induced ligands such as UL16-Binding Proteins (ULBP) (Sutherland et al., 2001). NK cells recognise cells with abnormalities in the number of presented proteins and induce apoptosis in these abnormal cells (Ljunggren & Karre, 1990).

Circulating NK cells may be CD56^{dim} or CD56^{bright}, at about a 9:1 ratio, respectively. CD56^{dim} cells secrete large amounts of cytotoxic granules such as perforin and granzyme (Osinska et al., 2014) to induce cell death, while CD56^{bright} cells produce lower amounts of cytotoxic granules, but produce high levels of cytokines such as IFN-gamma (IFN- γ) (Vivier et al., 2008) which can activate the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway and put cells in an anti-viral state (Paolini et al., 2015; Philips et al., 2022). I shall discuss interferons further in Chapter 2.5.

In terms of MCMV infection, protection against the initial replication in the spleen and liver during the first 8 hours is primarily effected by type 1 IFN and NK cells (Schneider et al., 2008; Verma & Benedict, 2011; Ynga-Durand et al., 2019).

2.4.2 Adaptive Immune System

The adaptive immune system is effected by T cells and B cells.

T lymphocytes originate as hematopoietic stem cells in the bone marrow before migrating to the thymus where they mature and are ‘trained’ through exposure to the body’s proteins to ensure self-tolerance through selection (Andersen et al., 2006; Ashby & Hogquist, 2024). They have specific T Cell Receptors (TCR), which if bound to a recognised antigen presented in an MHC complex activates the cell depending on its type.

Active cytotoxic Cluster of Differentiation 8 (CD8+) T cells release two proteins, perforin and granzymes. These function together, as the perforin forms pores in cellular membranes, which in turn allows entry of granzymes which induce apoptosis (Chowdhury & Lieberman, 2008).

Cluster of Differentiation 4 (CD4+) helper T cells protect against a wide range of pathogens, and may differentiate to have different functions. For example, Th1 cells are important for responses against intracellular pathogens and produce IFN- γ , while Th2 cells are important in defence against extracellular parasites (Luckheeram et al., 2012; Zhu & Paul, 2008).

Notably, pp65-specific CD4+ T cell responses have been shown to play a role in the prevention of cCMV transmission. Younger children with a lower amount of CMV-specific CD4+ T cells were found to have a high rate of viral shedding (Nelson et al., 2020; Stowell et al., 2014). As well as this, CD4+ T-cell depleted rhesus dams display higher viral loads of CMV in amniotic fluid and a lower survival rate (Bialas & Permar, 2016).

There are also regulatory T cells which are vital in the prevention of autoimmunity and chronic inflammation for example through secretion of inhibitory cytokines (Goschl et al., 2019; Vignali et al., 2008).

In HCMV-infected patients, it has been observed that HCMV-specific CD4+ and CD8+ T cells dominate the memory compartments, with as much as 10% of host T cells being dedicated to HCMV in healthy patients (Sylwester et al., 2005). These cells are vital in preventing a reestablished infection (Jonjic et al., 1994). Furthermore, CMV infection leads to a memory inflation whereby the amount of CMV-specific CD8+ T cells increases over time following primary infection, likely as a result of repeated exposure to antigens expressed by latently infected cells (Cicin-Sain, 2019). CD8+ T cells also play a role in reducing viral shedding in breast milk from mothers (Moylan et al., 2017).

Interestingly, HCMV-positive patients also exhibit a stronger response to the influenza vaccine, with increased levels of IFN- γ , increased CD8+ T-cell sensitivity, and heightened antibody responses observed when compared to HCMV-negative individuals (Furman et al., 2015).

B lymphocytes are responsible for the humoral immune response through the production of antibodies. They also originate in the bone marrow as hematopoietic stem cells, where they mature with the development of a unique B-cell receptor (BCR) before migration to the spleen and lymph nodes (Pieper et al., 2013). From here, B cells circulate until they encounter an antigen matching their BCR, whereupon they undergo rapid clonal expansion. Importantly, some of these B cells produced become memory B cells, which are long-living cells which

ensure future protection against previously encountered pathogens long after an infection has been cleared (Kurosaki et al., 2015; Weisel & Shlomchik, 2017).

The newly created B cells produce large numbers of a single type of antibody which specifically binds to its target antigen (Hoffman et al., 2016). Antibodies are Y-shaped proteins and are made up of two identical heavy and two identical light chains connected by disulphide bonds. Each antibody has two domains: a variable antigen-binding domain which is highly specific for target antigens, and a conserved Fc region (Wang et al., 2007) which is recognised and bound by specific Fc receptors found on the surface of cells such as DCs and NK cells (Ravetch & Bolland, 2001).

Fc receptors can be classified based on the type of antibody that they recognise, namely Fc γ R, Fc ϵ RI, Fc μ R, Fc α RI, and FcRn. Fc γ Rs are further categorised into Fc γ RI (CD64), Fc γ RII (CD32), and Fc γ RIII (CD16) (Jenks et al., 2019; Junker et al., 2020). These different Fc γ R families have differing affinities for antibodies and are expressed on different cell types. For example, Fc γ RI is strongly induced by IFN- γ , and is expressed on cells such as DCs, monocytes and macrophages (Corrales-Aguilar et al., 2014; Pan et al., 1990; Ravetch, 1997). As well as this, there are also CMV-encoded Fc γ Rs, which serve to compete with the host's own Fc γ Rs, which reinforces the significant role played by these receptors in the host's immune response (Corrales-Aguilar et al., 2014).

Through the release of antibodies by B cells, the targeted pathogen can have its cell-binding sites neutralised. Alternatively, coated cells are 'marked' for effector cells such as NK cells, macrophages or neutrophils. These effector cells can recognise and attach to the Fc region of these antibodies via the specific Fc receptors on their cell surface and activate ADCC to lyse the cell, as well as cause the release of cytokines to further amplify the immune response (Corrales-Aguilar et al., 2014; Nimmerjahn & Ravetch, 2008).

Notably, work conducted by this research group concluded that antibodies passed from a mother are sufficient to protect offspring against challenge infections with MCMV (Le-Trilling, Jagnjic, et al., 2023). This antibody data is backed up by another study which found anti CMV-IgG was sufficient to prevent placental transmission in a rhesus macaque model (Nelson et al., 2017).

2.5 Interferons

Recognition of PAMPs triggers secretion of interferons by several cell types such as dendritic cells, macrophages, fibroblasts, CD4+, and CD8+ T cells. These interferons can belong to one of three classes:

Type 1 (IFN- α , IFN- β): Produced by most cell types, conferring resistance to viral infections (Swiecki & Colonna, 2011).

Type 2 (IFN- γ): Secreted by T cells and NK cells, stimulating antigen presentation by MHC molecules (Boehm et al., 1997).

Type 3 (IFN- λ): Expressed in response to viral infections, similar to Type 1, but differing in being mainly produced and detected by epithelial cells (Dellgren et al., 2009).

Interferons play a key role in the innate immune response and are capable of putting cells in an antiviral state through activation of the JAK-STAT pathway (Au-Yeung et al., 2013). Through this, cells can make transcriptional changes in response to extracellular stimuli (Schindler et al., 2007).

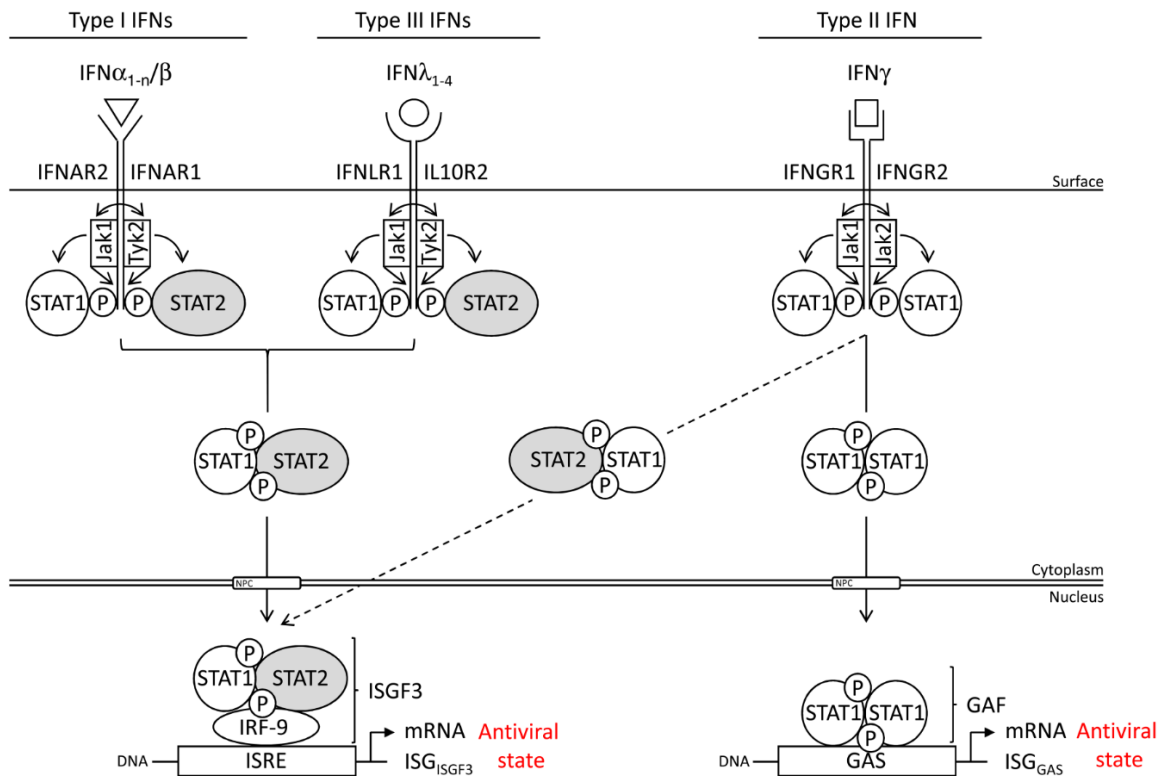


Figure 2.3: JAK-STAT pathway. Interferon binding interferon receptors activates the JAK-STAT pathway, resulting in the transcription of interferon-stimulated genes. Source: (Le-Trilling et al., 2018)

The JAK-STAT pathway begins with transmembrane cytokine receptors. Upon binding of a corresponding interferon to a surface receptor, a conformational change brings JAK proteins into close proximity, whereupon they transphosphorylate each other on key tyrosine residues. This in turn attracts inactive STAT proteins in the cytosol to the phosphorylated JAKs, which then phosphorylate the STAT proteins which dimerize and become activate (Schindler, 2002; Xin et al., 2020).

Once dimerized, these STAT protein complexes translocate into the nucleus. At this point, the STAT complex may bind to Interferon Stimulated Response Elements, which are specific promotor regions of the DNA. Once these regions are bound, transcription of Interferon-Stimulated Genes (ISG) is stimulated, and this puts the cell into an antiviral state (Schneider et al., 2014; Schoggins, 2019).

Worth noting, there is also a group of Interferon-Repressed Genes (IRepG) which are downregulated in the presence of IFN- γ (Megger et al., 2017) presumably to assist further in protecting the cell (Trilling et al., 2013).

For one of my experiments, shown in Fig 5.3, it is also worth noting that several cancer drug strategies such as that of Ruxolitinib involve selectively inhibiting this pathway by blocking the kinase activity of JAK1 and JAK2 and preventing activation of the JAK-STAT pathway (Elli et al., 2019; Mesa et al., 2012).

2.6 Mice Infected with MCMV as a Model for HCMV Infections in Humans

Mice have been an enduring and popular model for the development of vaccines and medical knowledge. They have many advantages as an animal model.

Their quick life-cycle and high rate of replication allows for experiments to be conducted in a relatively short period of time.

They are far cheaper and more ‘space-efficient’ when compared with other models such as *Macaca mulatta*, yet they have similar genetics and physiology to humans unlike *Danio* and *Drosophila* models.

Unlike in human studies, all conditions can be carefully monitored and controlled to ensure the exclusion of external factors affecting results.

As a result of extensive research conducted with mice, their genetics are very well understood. As well as this, specific genetic variants such as STAT2 knockout mice (Park et al., 2000) allow a deeper study into the effect of specific genes.

As discussed in Chapter 2.3, knowledge of the mechanisms involved in virus infection, growth, and immune invasion can be used in vaccine development. Crippling its abilities in any of these regards can develop an attenuated version of that virus which may have much milder side effects while still providing adequate defence against subsequent exposures to more severe wildtype forms of the virus (Minor, 2015).

For this, utilising MCMV in a mouse model is a widely popular strategy in CMV research for developing this knowledge, as well as knowledge of the correlates of protection (Reddehase & Lemmermann, 2018). MCMV and HCMV have many homologous and analogous genes whose functions can be elucidated upon in a mouse model (Schleiss, 2013).

The mice used in this project are BALB/c mice, which have been in use for over a century (Potter, 1985). Adult BALB/c mice are genetically more susceptible to MCMV infection when compared to other strains as it mainly uses adaptive CD8⁺ T cells to control infection, while other strains of mice also have high NK-cell activity in addition to this (Brizic et al., 2018).

It is through work with mice that an attenuated Δ M27-MCMV virus was developed by our research group (Le-Trilling et al., 2018).

2.7 Δ M27-MCMV

In MCMV, the *M27* gene encodes the M27 protein, a 79kDa protein with early-late expression. This protein is beneficial to successful MCMV infection as it antagonises STAT2 and prevents successful implementation of the JAK-STAT pathway (Hu et al., 2021; Zimmermann et al., 2005). pM27 achieves this by exploiting DNA Damage Binding Protein 1 (DDB1) (Landsberg et al., 2018) and utilising the Cullin RocA ubiquitin ligases to poly-ubiquitinate STAT2 (Becker et al., 2019). These poly-ubiquitinated STAT2 are subsequently degraded by the proteasome (Flores-Martinez et al., 2021; Trilling et al., 2011).

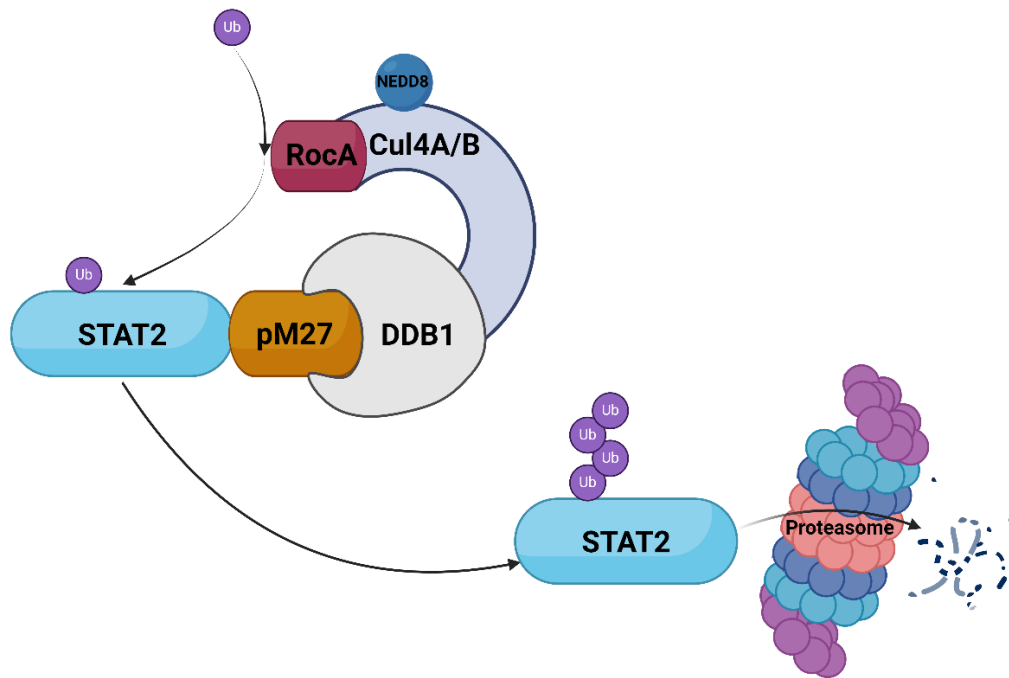


Figure 2.4: pM27 interacts with DDB1 to effect proteasomal degradation of STAT2. The M27 protein interrupts the JAK-STAT pathway by means of degrading STAT2. As a result, no complex can be formed to translocate to the nucleus to activate transcription and the cell does not enter an antiviral state. Figure adapted from (Trilling et al., 2011). Created with BioRender.

As a result of this, STAT2 proteins cannot form a complex for entry into the cell nucleus, thus removing its ability to activate the cells transcription of antiviral factors. This STAT2 downregulation is also effected by the Rat Cytomegalovirus (RCMV) E27 protein (Le-Trilling, Banchenko, et al., 2023) but notably the homologous pUL27 of HCMV does not induce STAT2 degradation and hardly binds DDB1 (Landsberg et al., 2018; Le et al., 2008). Instead, pUL27 induces the degradation of the acetyltransferase Tip60 (Reitsma et al., 2011).

It is HCMV's *UL145* gene that is analogous in terms of function to the MCMV's *M27* gene (Le-Trilling et al., 2020; Le-Trilling & Trilling, 2020). This gene codes for two pUL145 isoforms which are also responsible for STAT2 downregulation, the early-infection expressed pUL145-Short and the late-infection expressed pUL145-Long. The N-terminus of these proteins has been found to have residues which closely resemble the DDB1-binding interface of DDB1-cullin-associated-factors (DCAF) which are involved in the recruitment of DDB1. These are conserved across several species of CMV and indicate this process of DDB1/CRL utilisation plays a valuable role in CMV immune evasion and infection across multiple species (Le-Trilling et al., 2020).

It is important to note also that this research group has previously shown that a mutant MCMV lacking the *M27* gene is attenuated *in vitro* in the presence of IFN and *in vivo*, and notably its replication is restored *in vivo* in mice lacking STAT2 (Le-Trilling et al., 2018). Δ M27-MCMV is an example of a suppressed live attenuated virus, whereby its replication is attenuated due to lacking a gene helpful in evading the host's immune response.

As mentioned in Chapter 2.4.2, Δ M27-MCMV is also capable of raising an antibody response in mother mice which can be passed to offspring and protect them against exposure to MCMV. JHT mice cannot produce antibodies when 'vaccinated' and subsequently offspring succumb to infection when challenged with MCMV (Le-Trilling, Jagnjic, et al., 2023).

2.8 Δ M34-MCMV & Δ UL34-HCMV

The *M34* gene of MCMV encodes the pM34 protein. Previously, *M34* had been reported to be essential to MCMV replication (Baluchova et al., 2008), but this was disproven by Dr Mareike Eilbrecht who successfully developed a replication competent Δ M34-MCMV mutant with 10-100 fold reduced replication efficacy (Eilbrecht et al., 2020). Using Hemagglutinin (HA)-tagged pM34, the research group also showed that the pM34 protein is expressed with early-late kinetics, and is localised in the nucleus (Eilbrecht et al., 2020).

Through a clustal alignment, it was shown that pM34 is homologous to the HCMV protein pUL34 (34% identity, 55% similarity) (Eilbrecht et al., 2020). pUL34 is encoded by HCMV's *UL34* gene, which was also previously believed to be an essential gene (Dunn et al., 2003; Rana & Biegelke, 2014), but recent findings have also proven otherwise (Turner et al., 2022).

pUL34 had previously been found to bind to multiple sites in the HCMV genome and downregulate expression of the *US3* and *US9* genes (Liu & Biegelke, 2013). These genes, perhaps counterintuitively, are both involved in immune evasion as one would expect increased expression of immune evasion genes would be beneficial to successful HCMV replication.

US3 functions to inhibit peptide-loaded MHC1 molecules transferring to the membrane from the endoplasmic reticulum (ER) (Biegelke et al., 2004; Hewitt, 2003; Jones et al., 1996). pUL34 prevents this by binding to the transcriptional repressive element (TRE) of the *US3* gene (Biegelke, 1998; LaPierre & Biegelke, 2001). pUS9 on the other hand inhibits IFN β responses. It achieves this by targeting both the Stimulator of Interferon Genes (STING) and the Mitochondrial AntiViral Signalling protein (MAVS) (Choi et al., 2018).

In addition to this, pUL34 is theorised to act as a guide protein, interacting with viral proteins essential to viral DNA replication such as pIE2, pUL44, and pUL84 and then binding to regions in proximity of the origin of lytic replication (oriLyt) (Slayton et al., 2018). This function may explain pM34s 'non-essential but attenuated' characteristic, as if this is the case, the protein would be acting as a catalyst for essential interactions needed for DNA replication. Without the protein, this process is still carried out but at a much lower rate.

With this in mind, Δ M34-MCMV can be seen as an example of an impaired live-attenuated virus mutant. It is lacking a gene required for efficient replication, but as I show in Fig. 5.3, the virus remains attenuated through factors independent of the host's immune response.

3. Aims and Scope of the Work

Given the lack of viable vaccine candidates for human cytomegalovirus, the current work aims to develop an understanding of the correlates of protection in order to determine the required characteristics of a successful vaccine.

The aims laid out in this thesis are as follows:

- Characterise the immune response raised by $\Delta M34$ -MCMV.
- Compare the immune responses raised by this $\Delta M34$ -MCMV and $\Delta M27$ -MCMV.
- Determine the replication competence of $\Delta M34$ -MCMV as a live-attenuated vaccine.

4. Materials and Methods

4.1 Materials

4.1.1 Reagents

3, 3', 5, 5' Tetramethylbenzidine 1-step Ultra TMB-ELISA	ThermoScientific
Agarose	Biozym
Ammonium Persulphate (APS)	Roth
Beetle Juice	PJK
Ciprofloxacin Hydrochloride	MP Biomedicals
Complete Protease Inhibitors Cocktail EDTA Free	Roche
DMEM	Gibco
DMSO	Roth
DNA Stain Clear G	Serva
ECL Reagent	SignalFire ECL Reagent, Cell Signaling Technology
Ethanol, Absolute	Roth
FBS Superior	Capricorn Scientific
Gel Loading Dye, Purple (6X)	New England Biolabs
GeneRuler DNA Ladder Mix	Thermofisher
Glycerol	Roth
Hydrochloric Acid (HCl)	Roth
Isopropanol	Roth
MEM	Sigma
Methylcellulose	Sigma Aldrich
Milk Powder	Sucotin
NaHCO₃	Roth
PBS	Gibco
PBS Tablets	Applichem
PenStrep	Gibco
Polyacrylamide 30	Roth
Ponceau S	Roth

Q (L-Glutamine)	Gibco
Reblot Solution	Millipore
RNAase Cleaner	Molecular BioProducts
RPMI	Gibco
Ruxolitinib	Cell Guidance Systems
Saccharose	Roth
Sodium Dodecyl Sulphate (SDS)	Roth
Sodium Pyruvate	Gibco
Streptavidin POD	Dianova
TEMED	PanReac Applichem
TRIS	Roth
Triton	Roth
Trypan Blue (0.4%)	Gibco
Trypsin	Gibco
Tween-20	Roth
β-mercaptoethanol	Gibco

4.1.2 Cell Lines

CIM	Crisis-immortalised MEF from C57BL/6 embryos (Rattay et al., 2015)
MNC	Primary mouse embryonic newborn cells isolated from BALB/c or 129S1 mice (Le-Trilling & Trilling, 2017)
MEF	Primary mouse embryonic fibroblasts, isolated from BALB/c (Le-Trilling & Trilling, 2017)
BWmCD16	BW5417 thymoma cells expressing only CD16 surface receptor (Corrales-Aguilar et al., 2013)
BWmCD32	BW5417 thymoma cells expressing only CD32 surface receptor (Corrales-Aguilar et al., 2013)
BWmCD64	BW5417 thymoma cells expressing only CD64 surface receptor (Corrales-Aguilar et al., 2013)
BWFcRIV	BW5417 thymoma cells expressing only Fc surface receptor IV (Corrales-Aguilar et al., 2013)

4.1.3 Mouse Lines

BALB/c Bagg albino inbred mouse strain

129S1 129 inbred mouse strain

4.1.4 Virus Stocks

wt-MCMV Reconstituted from wt-MCMV-BAC

Provided by Dr. Khanh Le-Trilling

ΔM27-MCMV Reconstituted from ΔM27-MCMV-BAC

Provided by Dr. Khanh Le-Trilling (Le-Trilling et al., 2018)

ΔM34-MCMV Reconstituted from ΔM34-MCMV-BAC

Provided by Dr. Mareike Eilbrecht (Eilbrecht et al., 2020)

MCMV:eGFP (RVG102) (Henry S.C *et al.*, 2000)

4.1.5 Antibodies

Table 4.1: List of antibodies

Antibody	Species	Producer	Product No.	Dilution
Anti-GAPDH	Mouse	Santa Cruz	Sc-25778	1:10,000
Anti-gB	Mouse	Stipan Jonjić	15A12-H9	1:1,000
Anti-IE1	Mouse	Stipan Jonjić	CROMA101	1:100
Anti-Rabbit POD	Goat	Sigma	A6154	1:10,000
Goat Anti-Mouse POD	Goat	Dianova	115-035-062	1:10,000
Rat-anti-mouse IL-2 (Without Biotin)	Rat	BD Biosciences	554424	1:500
Rat-anti-mouse IL-2 (With Biotin)	Rat	BD Biosciences	554426	1:1,250

4.1.6 Oligonucleotides

ΔM34-Ctrl-1 1:10 Primer	5'-GCTGTCGAACCGCATCTATC-3'
ΔM34-Ctrl-2 1:10 Primer	5'-GCTTGCCAGTTATGCATTGC-3'
KL-MCMV IE2-1 1:10 Primer	5'-ATGGAGCGTGTTCGGGGAGC-3'
KL-MCMV IE2-2 1:10 Primer	5'-CTGAATCTTCTTCCTGACGGTC-3'

4.1.7 Buffers

Bradford Reagent	8.5% (v/v) Phosphoric Acid 4.75% (v/v) Ethanol 100 mg/L Coomassie Blue G-250
5% Blocking Buffer	5% (w/v) Milk Powder 1X TBST
ELISA Binding Buffer	0.1 M Na ₂ HPO ₄ , pH 9
ELISA Blocking Buffer	10% (v/v) FBS in PBS
ELISA Sample Buffer	10% (v/v) FBS in PBS-T
10X Laemmli	250 mM Tris
Electrophoresis Buffer	1.92 M Glycine 1% (w/v) SDS
Methylcellulose Complete	Methylcellulose 8.8g Millipore H ₂ O 360 mL Minimum Eagle's Medium (MEM) 40 mL FBS 20 mL NaHCO ₃ (55 g/L, autoclaved) 20 mL PenStrep 5 mL L-Glutamine 5 mL
PBS-T	0.1% (v/v) Tween 20 in PBS
RIPA Buffer	0.5 M TRIS/HCl pH 7.5 5 M NaCl 20% (v/v) SDS 100 mM DTT

	100 mM PMSF
	0.1 mg/mL Pepstatin
	0.2 mg/mL Leupeptin
	1 M Sodium Fluoride
4X SDS Sample Loading Buffer	Tris-HCl: 0.2 M DTT: 0.4 M SDS: 277 mM, 8.0% (w/v) Bromophenol Blue: 6 mM Glycerol: 4.3 M
10X TBST	100 mM Tris/HCl 1.5 M NaCl 10 mL Tween
10X Western Blot Blotting Buffer	TRIS:Glycine (2:1 (v/v))
4X Western Blot Sample Buffer	0.25 M Tris/HCl, pH 6.8 40% (v/v) Glycerol 8% (w/v) SDS 20% (v/v) β -Mercaptoethanol 6 mM Bromophenol Blue

4.1.8 Equipment

Aspirator Pump	N881 Laboport, KNF
Cell Culture Flasks	Cellstar TC Sterile Filter Cap Cell Culture Flask, Greiner Bio-One
Cell Culture Hood	HERAsafe, Thermo Heraeus HERAsafe KS, Thermo Scientific
Cell Incubator	BINDER
Cell Strainer	MACS SmartStrainer (70 μ m), Miltenyi Biotec
Cellulose Papers	3MM Chr Cellulose Chromatography Paper, Cytiva Life Sciences
Centrifuge	ROTANA 460 R, Hettich 5415R, Eppendorf

	5417R, Eppendorf
Clingfilm	Pro, Sarogold
Cryotubes	Cryo.s PP 2 mL Sterile, Greiner Bio-One
DNA Extraction Kit	INSTANT Virus RNA/DNA Kit, Analytik Jena
DNA Ladder	New England Biolabs, Color Protein Standard Broad Range, P7719S
Electronic Balance	572, Kern Sartorius Research
Electrophoresis Chamber	PerfectBlue Horizontal Gelsystem, Peqlab
Eppendorf Tubes (1.5 mL & 2 mL)	Eppendorf
Falcon Tubes	Cellstar PP Conical Bottom Sterile Tube 15 & 50 mL, Greiner Bio-One
Freezer -20°C	MediLine Lab Freezer, Liebherr
Freezer -80°C	MDF-DU702VH-PE VIP ECO ULT Freezer, PHCbi
Freezer Boxes	Cryobox, GLW
Freezing Container	CoolCell, Biocision
Fridge	Liebherr
Gel Chamber	VWR
Gel Documentation	Intas Science Imaging
Glassware	Rasotherm, Roth
Heating Block	Thermomixer Comfort, Eppendorf
Hemocytometer	Fuchs-Rosenthal 0.2 mm, Marienfeld Superior
Homogeniser	Dounce Tissue Grinder, Wheaton
Ice Machine	AF80, Scotsman
Magnetic Stirrer	Heating and Magnetic Stirrer MH15, Roth JK IKAmag REO, IKA
Microscopes	Primovert Inverted Cell Culture Microscope, Zeiss CKX41, Olympus
Multichannel Pipette	Research Plus Multichannel Pipette, Eppendorf Pipet-Lite XLS, Rainin
Multimode Reader	Mithras2 LB 943, Berthold Technologies
Multipipette	Multipipette Plus, Eppendorf

Multiwell Cell Culture Plate	96-well Clear Flat Bottom TC-treated Culture Microplate, with Lid, Individually Wrapped, Sterile, Falcon Cell Culture Multiwell Plate, PS, Greiner Bio-One U-Well Plate, Greiner Bio-One
Plate Rocker	GFL
Neutralisation Plate	Microplate 96 well, PS, V-bottom, Clear, Greiner Bio-One
PCR Cycler	Professional TRIO Thermocycler, Biometra
pH Meter	Lab 850, Schott Instruments
Photometer	BioPhotometer Plus, Eppendorf
Pipette	ErgoOne Single-Channel Pipettes, Starlab
Pipette Tips	TipOne pipette tips, Starlab RC LTS 300 µL Pipette Tips, Rainin
Pipetboy	Pipetboy 2, Integra
Power Supply	EV202, Peqlab PS300T Mini Power Pack, Biometra
Serological Pipettes	CELLSTAR Serological Pipettes, Greiner Bio-One
Spectrophotometer	NanoDrop 2000c Spectrophotometer, Thermo Scientific
Stirring Bar	PTFE Cylindrical Stirrer Bar, VWR
Syringes	Discardit II, BD
Syringe Needles	Eclipse Needle, BD
Ultracentrifuge	Optima L80XP Ultracentrifuge, Beckman Coulter
Ultrapure Water System	Milli Q, Millipore
UV Microscope	EL6000, Leica
Vacuum Filter	Vacuumfiltration 500 'Rapid' Filtermax 0.22 µm poresize, TPP
Vortexer	Vortex 3, IKA
Water Sonicator	Sonorex, Bandolin
Water Bath	GFL
Western Blot Developer	Cawomat 2000IR, CAWO
Western Blot Membrane	0.45 µm NC Nitrocellulose Blotting Membrane Nucleic Acid & Protein Application, Amersham Protran
Wipes	Prestige, Satino
	Lysis Buffer High Q AllIn Sample Direct Kit
	Polymerase Buffer (5X AllIn HIFI Buffer)

4.1.9 Software

BioRender

Endnote 20

GraphPad PRISM 8

ImageJ

Microsoft Office (Word, Excel, Powerpoint)

Mithras MikroWin 2010

Nanodrop2000

4.2 Cell Culture

4.2.1 Preparation of Cell Growth Medium (Standard)

Dulbecco's Modified Eagle Media (DMEM) (Gibco) was supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Capricorn Scientific) and penicillin-streptomycin (PenStrep) stock (Gibco) (100U/mL Pen, 100 µg/mL Strep [w/v]). This complete medium was stored at 4°C.

4.2.2 Preparation of Cell Growth Medium (BW Cell Line)

Roswell Park Memorial Institute (RPMI) Medium (Gibco) was supplemented with 10% (v/v) FBS and 1% (v/v) Sodium Pyruvate (Gibco). Before use on the cell line, this solution was supplemented further with 0.05 mM beta-mercaptoethanol (β -ME) (Gibco). This complete medium was stored at 4°C.

4.2.3 Cell Thawing

A vial of previously frozen cells was removed from liquid nitrogen and allowed to thaw. The cells were then transferred to a 15 mL tube containing 5 mL of pre-warmed complete growth medium and centrifuged at 330G for 3 minutes. The supernatant was removed, and the cell pellet resuspended in fresh complete medium before being transferred to the appropriately sized cell culture flask and stored in a 37°C incubator.

4.2.4 Passaging Adherent Cell Lines

Old medium was removed from the flask. The flask was then rinsed with Phosphate Buffered Saline (PBS) (Gibco) before the addition of a 2X solution of Trypsin (Gibco) diluted in PBS. This trypsin was spread evenly across the cells by rotation of the cell culture flask before being removed. The flask was then placed in a 37°C incubator for approximately 5 minutes (varies depending on cell line) and the flask was gently bumped to assist in the detachment of cells. A predetermined dilution of the cells was then placed in a fresh cell culture flask of desired size, and the cells stored in a 37°C incubator.

4.2.5 Passaging Suspension Cell Lines

RPMI complete cell culture medium was added to a fresh cell culture flask. The suspension cells were mixed to ensure homogeneity before a predetermined dilution of the medium was transferred to the fresh flask containing medium which was then stored in a 37°C incubator.

4.2.6 Resuscitation of MNCs for Virus Titration

Mouse Newborn Cells (MNC) (Le-Trilling & Trilling, 2017) were resuscitated and split for 3 days before resuspending cells in 24 mL media and transferring to 48 well plates with 500 µL per well.

4.2.7 Cryopreservation

Cryopreservation medium was made up using 90% (v/v) FBS and 10% (v/v) Dimethyl Sulfoxide (DMSO) (Roth). Cells were suspended in media before being centrifuged at 330G for 3 minutes. The cell pellet was then resuspended in 1 mL of cryopreservation medium and placed in a cryovial. This cryovial was then placed in a freezing container which was placed in a -80°C freezer. The cryovial was then transferred for storage in liquid nitrogen 24 hours later.

4.2.8 Preparation of Cell Lysates for Western Blot

CIM cells were allowed to become 90% confluent in a 6-well cell culture plate before infection (as described in 4.3.1) with chosen virus for a desired length of time. Following this, cells were resuspended in wells via physical means (pipetting force), and 1 mL at a time, added to a labelled 1.5 mL Eppendorf tube on ice. Cells were centrifuged at 7000 RPM for 1 minute at

4°C. Supernatant was disposed of and a further 1 mL of cell suspension was added and centrifuged. This process was repeated until all media was removed from the wells. Wells were then rinsed with 1 mL PBS, which was also centrifuged in the tubes. Tubes were centrifuged one further time with 1 mL fresh PBS which was discarded before the pellet was suspended in 100 µL RIPA buffer before freezing and storing in a -20°C freezer.

4.2.9 Determination of Protein Concentration

Lysates were allowed to thaw on ice before being centrifuged at 13,500 RPM for 5 minutes at 4°C. 1.5 mL Eppendorf tubes were labelled, and the supernatant of each lysate was transferred. 995 µL of Bradford Reagent was placed into an additional 1.5 mL Eppendorf tube along with 5 µL of lysate to be measured. This mix was then measured against a blank at an absorbance wavelength of 595 nm.

4.2.10 Preparation of Protein Lysates for Enzyme-Linked Immunosorbent Assay (ELISA)

Infected cells were prepared as described in 4.3.1 as far as the washing with PBS. Cells were infected for 72 hours and an MOI of 5. After the washing step, the cell pellet was resuspended in 1 mL + 30 µL of complete protease inhibitors cocktail (Roche) per tube and centrifuged at 13,000 RPM for 1 minute at 4°C.

The cell pellet was then resuspended in the supernatant and sonicated (Sonorex) 3 times for 10 seconds before freezing lysates in 200 µL aliquots at -20°C (approximately 1×10^6 cells per 100 µL).

4.3 Virus Infections

4.3.1 *In vitro* Infection of Cells

For all *in vitro* infections, plates were centrifuged at 931G for 15 minutes at 24°C. Plates were then rotated 180 degrees and centrifuged again at 931G for 15 minutes at 24°C. Plates were then stored in an incubator at 37°C to allow viral replication.

4.3.2 Generation of Δ M34-MCMV Stock

A 6-well cell-culture plate with CIM cells at 90% confluency was infected with Δ M34-MCMV. 24 hours after infection, the supernatant of these plates was added to DMEM containing 100U/mL Pen, 100 μ g/mL Strep (w/v) and 10 μ g/mL ciprofloxacin hydrochloride (w/v) (MP Biomedicals). The plate was then washed with PBS before cells were trypsinised and suspended in this DMEM. The DMEM was then split between 16 prepared T175 cell-culture flasks containing CIM cells at 90% confluency.

CIM cells were stored in an incubator at 37°C and checked regularly. Five mL of DMEM containing 5% FBS (v/v), 100U/mL Pen, 100 μ g/mL Strep (w/v), and 10 μ g/mL ciprofloxacin hydrochloride (w/v) was added if media was turning orange.

When cells were fully infected, they were scraped from all T175s using a sterile cell scraper and resuspended in their media. Media from all flasks was combined and mixed before being transferred to centrifuge tubes which were centrifuged at 6000 RPM for 5 minutes at 10°C. All but 10 mL supernatant was placed in fresh 250 mL centrifuge tubes, with the cell pellet being resuspended in the remaining supernatant and transferred to a Dounce tissue grinder (Wheaton). This was then ground 30 times using a 7/16 mL tissue grinder before being added along with the rest of the supernatant into the used centrifuge tubes and centrifuged at 6000 RPM for 10 minutes at 10°C.

Supernatants were transferred to a new centrifuge tube and centrifuged at 13000 RPM for 4 hours at 10°C. Supernatant was discarded and the cell pellets were gently covered with 15 mL DMEM total, stored on ice, and placed in a 4°C room overnight.

Virus pellets were then resuspended and homogenised and 20 mL of Saccharose/VSF (Roth) solution was added on top of each tube. Tubes were centrifuged at 27,000 RPM for 70 minutes at 10°C and supernatant was discarded. Two mL of Saccharose/VSF solution was added and tubes were incubated overnight at 4°C. The virus pellet was resuspended and homogenised by use of a 1 mL tissue grinder, and aliquots of 20 and 50 μ L were stored at -80°C. Virus titre was calculated by virus titration of a thawed stock as described in Chapter 4.5.2.

4.3.3 Confirmation of *M34* Gene Deletion

129 mouse MNCs were plated and infected with either wt-MCMV or Δ M34-MCMV prepared in Chapter 4.3.2 above. 10 μ L of supernatant from each sample was then combined with 50 μ L of Master Mix Proteinase K Digestion (Table 4.1)

Table 4.2: PCR reagent mix

Component	Volume (μ L)
PCR-grade H ₂ O	35 μ L
5x Lysis Buffer High Q AllIn Sample Direct Kit	10 μ L
10x DPK Protease Buffer High Q AllIn Sample Direct Kit	5 μ L

The mixture was placed on a heating block at 75°C for 5 minutes, being quickly vortexed every 2 minutes. This was then transferred to a heating block at 95°C for 10 minutes before the addition of 450 μ L of PCR-grade H₂O. The mixture was then centrifuged at 6500 RPM for 1 minute at room temperature before transferring the supernatant to a new tube.

One μ L of this template of each sample was then mixed with 14 μ L of PCR Mastermix (Table 4.2).

Table 4.3: PCR mastermix

Component	Volume (μ L)
PCR-grade H ₂ O	7.7
5X Polymerase Buffer	3
Taq Polymerase	0.3
10 μ M Δ M34-CTRL1 1:10 Primer	0.75
10 μ M Δ M34-CTRL-2 1:10 Primer	0.75
10 μ M KL-MCMV-IE2-1 1:10 Primer	0.75
10 μ M KL-MCMV-IE2-2 1:10 Primer	0.75

Samples were then loaded into a Biometra Professional TRIO Thermocycler and run as in Table 4.3.

Table 4.4: PCR thermocycler settings

Repeats	Step	Temperature [°C]	Time (s)
	1	95	Hold
	2	94	120
11x	3	94	20
	4	65	15
	5	68	10
29x	6	94	15
	7	60	15
	8	72	10
	9	72	120
	10	4	Hold

A 1.5% (w/v) agarose gel solution was prepared, heated in a microwave, and mixed before being poured into a gel electrophoresis chamber containing a well-comb and allowed to harden when 4 μ L Serva DNA Stain Clear G was added. The well comb was removed and 3 μ L of 6x Gel Loading Dye (Biolab) was added to 15 μ L of PCR product before loading 14 μ L into the well. 5 μ L Gene Ruler DNA Ladder Mix (ThermoFisher) was loaded alongside samples, and the gel was run at 120 V, 2000 mA for 60 minutes before being visualised using an Intas Science Imaging Gel Imager.

4.4 Mouse Experiments

4.4.1 Infection of Mice

All mice were infected intraperitoneally under sterile conditions using virus diluted in sterile PBS. Mice infected with different viruses were kept separate with no cross-contamination in bedding, food or water.

4.4.2 Sacrificing Mice

Mice were weighed a final time before being sacrificed humanely either by carbon dioxide asphyxiation or cervical dislocation.

4.4.3 Collection of Blood and Organs

Immediately after sacrificing, mice were cut open using sterile scissors and tweezers in the designated animal use hood. Blood was collected via a syringe connected to the heart and placed in a prelabelled 1.5 mL Eppendorf tube on ice. Organs were then removed, placed in prelabelled cryopreservation tubes and immediately placed in liquid nitrogen for transfer to a freezer box and stored in a -80°C freezer.

4.4.4 Isolation of Serum from Blood

Eppendorf tube containing blood was centrifuged at 1500G for 10 minutes at room temperature. Supernatant was transferred to a new tube and centrifuged once more at 1500G for 10 minutes at room temperature. Supernatant was then placed in a fresh, labelled tube and stored at -20°C.

4.5 Organ Titration

4.5.1 Straining of Organ

Frozen organs of choice were thawed on ice. Tubes containing the organ had their weight recorded. Using the sterile plunger of a syringe, organs were homogenised through a fresh MACS SmartStrainer(70µM) (Miltenyi Biotec) along with 5 mL of PBS containing 3% FBS (v/v) (5% FBS [v/v] for the liver) into a 50 mL Falcon tube.

This organ homogenate was then centrifuged at 330G (931G for liver homogenate) for 5 minutes at 4°C. Homogenate was transferred to 15 mL Falcon tubes and centrifuged for a further 5 minutes at 931G (2095G for liver) at 4°C.

4.5.2 Titration

Serial dilutions of the homogenate on the 48 well plates of MNCs were carried out using a multichannel pipette before centrifuging all plates for infection as in Chapter 4.3.1. Cells were then incubated at 37°C for 2 hours (1 hour for liver titrations). Supernatant was then aspirated and 500 µL of methylcellulose complete (Chapter 4.1.7) was added. Cells were then further incubated and viral plaques were recorded after 72 hours. The protocol is the same for regular virus titration.

4.6 Western Blot

4.6.1 Preparation of Western Blot Gel

A Western blot chamber was assembled for gel preparation. Ten percent running gel was prepared (Table 4.4) and the chamber was filled to 2 cm below the bottom of the well-comb. A layer of isopropanol was placed on top to ensure a level gel. The gel was allowed to solidify, and the isopropanol was removed before the addition of the loading gel, at which point the well-comb was placed in the apparatus and removed once the loading gel had solidified.

Table 4.5: 10% running gel components

Component	Running Gel Volume	Loading Gel Volume
PAA-30 (mL)	32	6
2 M TRIS/HCl pH 8.8 (mL)	20	0
20% SDS (μ L)	480	180
H ₂ O (mL)	42.4	16.8
0.5 M Tris/HCl pH 6.8 (mL)	0	4.8
60% Saccharose (mL)	0	8.4
TEMED (μ L)	192	48
APS (μ L)	1000	240

4.6.2 Running Western Blot Gel

Western blot chamber was assembled for gel running and confirmed to have no leakage. Laemmli buffer was added to the chamber and the well-comb was removed. Empty lanes were then flushed with a syringe of Laemmli buffer to ensure they were clear of debris. Chamber mixtures were prepared by diluting protein lysates (As prepared in Chapter 4.2.8) to matching concentrations, adding loading buffer and then incubating at 95°C for 5 minutes before loading into the wells. An appropriate ladder was loaded alongside the proteins.

The gel was then run for 30 minutes initially at room temperature, before being run overnight for 16 hours at 150 V and 14 mA (for two large gels) until the proteins had run sufficiently through the gel.

4.6.3 Blotting

The slides were removed from the apparatus and placed on a paper towel to be prised open using a western blot tool. Excess gel was cut away, and 8 pieces of Whatman paper were cut to size, along with a sheet of polyvinylidene difluoride (PVDF membrane) (Amersham Protran). The electroporation device was opened and 1X western blot buffer was placed in a container. Four sheets of Whatman paper were dipped in this container and placed on the electroporation device before having air bubbles rolled out. The PVDF membrane was then placed in the container before being placed on top of the prepared Whatman paper. Following this, the gel containing the protein bands was placed on the PVDF membrane and air bubbles were removed. Four more sheets of Whatman paper were placed in the buffer and then placed on top. Bubbles and excess buffer were removed before closing the device and running at 12 V, 2000 mA for 75 minutes.

Ponceau red solution (Roth) was used to confirm successful transfer of protein bands, and the PVDF membrane was then rinsed in deionised (DI) water and several times in several times in 1X Tris-buffered saline with 0.1% Tween-20 (v/v) (Roth) (TBST).

4.6.4 Blocking

Blocking solution of 5% (w/v) milk powder in 1X TBST was made up. PVDF membranes were placed in a container, covered with blocking solution, and left on a rocker for minimum 1 hour at room temperature, or overnight at 4°C.

4.6.5 Primary Antibody

The desired dilution of primary antibody was made up in 1X TBST with 0.5% (w/v) milk powder) and placed with the PVDF membrane in a container on a rocker for minimum 1 hour at room temperature, or overnight at 4°C. The primary antibody was then removed and stored at -20°C).

4.6.6 Secondary Antibody

The PVDF membrane was washed with 1X TBST for 10 minutes before being incubated on a rocker with a 1:10,000 dilution of the secondary antibody (Goat-anti-mouse IgG [Dianova]) for 1 hour.

The secondary antibody was removed and the PVDF membrane rinsed with 1X TBST several times for 30 minutes.

4.6.7 Detection of Protein Band

ECL reagent (Cell Signaling Technology) was prepared and manually rolled over the membrane for 2 minutes. Following this, the membrane was blotted dry with Whatman paper before being wrapped in cling film and placed in a developing box with bands facing up. A sheet of developing film (Fujifilm) was placed over the PVDF membrane for the desired exposure time before the developing film was treated and developed using a Cawomat 2000IR.

4.6.8 Reblotting Membrane

Reblotting of the PVDF membrane was carried out by initially rinsing the membrane once in 1X TBST. The membrane was then rinsed 5 times with DI water and incubated for 5 minutes in Reblot solution (Millipore). The membrane was rinsed a further 5 times with 1X TBST followed by 5 rinses with DI water before being incubated in blocking buffer for 1 hour at room temperature. The membrane could then be exposed once again to the primary and secondary antibodies and be detected.

4.7 Measuring MCMV-Specific IgG Response

4.7.1 Coating of Plates with wt-MCMV-Infected Fibroblasts

A solution of 200 μL (2×10^6 cells) of lysate as prepared in Chapter 4.2.10 (72 hours, MOI 5) and 5 mL of ELISA Binding Buffer (0.1 M Na_2HPO_4) was made up per plate. Microtitre plates (MaxiSorp Surface NUNC) were coated with 50 μL /well of this solution and plates were incubated overnight at 4°C.

Lysate was removed and nonspecific binding sites were blocked with 100 μL /well of blocking buffer for 1 hour at room temperature before buffer was also removed and wells were washed two times with 100 μL /well of PBS-T.

4.7.2 Loading Plates with Sera Dilutions

Serum samples were diluted 5-fold in ELISA sample buffer. These dilutions were done from 1/50 to 1/156,250.

Fifty μL /well of each serum dilution was added per well and plates were incubated for 90 minutes at room temperature. Following incubation, sera was removed and plates were washed three times with 100 μL /well of PBS-T.

4.7.3 Detection of MCMV-Specific IgG

A 1:5000 dilution of GAM in ELISA sample buffer was prepared and 50 μL /well was loaded onto plates. Plates were incubated at room temperature for 1 hour before a further 4 washes with 100 μL /well of PBS-T.

50 μL /well of TMB was loaded into each well for 90 seconds, before the colour reaction was stopped with 50 μL /well of 0.5 M HCl. O.D was measured immediately at 450/620 nm using the Multireader and recorded.

4.8 Measurement of ADCC Response

96-well plates of CIM cells were infected with wt-MCMV with an MOI of 7.5 and infected as in Chapter 4.3.1 before being incubated at 37°C overnight. Meanwhile on a separate plate, a solution of 50 μL /well binding buffer (0.1 M Na_2HPO_4) containing 0.2% (v/v) Rat anti-mouse IL-2 without biotin was used to coat Nunc Maxisorp 96 well plates at 4°C overnight. This solution was then removed and replaced with 100 μL /well of blocking buffer (90% [v/v] PBS, 10% [v/v] FBS) for a further overnight incubation at 4°C.

The day after the CIM cells were infected, the medium was replaced with 100 μL /well DMEM and plates were incubated for 2 hours at 37°C. Plates were then washed three times with DMEM before addition of 1:30 dilutions of mouse sera. Plates were incubated again for 2 hours at 37°C. After this incubation, plates were rinsed once with a 3% (v/v) solution of FBS in PBS before the addition of 100 μL cells/well of 1×10^6 cells/mL of a BW cell line. Cells were then incubated for 16 hours at 37°C.

In a fresh 96 u-bottom well plate, 70 μL of Sample Buffer was loaded, and 70 μL of supernatant from the corresponding wells of the BW cell plate was added for a 15-minute incubation at room temperature.

Meanwhile, the prepared Maxisorp plates were washed and beaten out three times with PBS-T before adding 100 μL of samples from the prepared u-well plates. A positive control of 50 μL /well of 1:1250 (v/v) mIL-2 was used. Maxisorp plates were then incubated at 4°C overnight.

On the final day, Maxisorp plates were washed three times with PBS-T before the addition of 50 μL /well of 1:500 (v/v) Rat anti-mouse (with Biotin). Plates were then incubated on a rocker for 2 hours at room temperature before a further three washes with PBS-T. Fifty μL /well of 1:500 (v/v) Strep-HRP in Sample Buffer was added and plates were incubated for 30 minutes at room temperature.

Plates were washed 4 final times with PBS-T before the addition of 100 μL /well of TMB. Plates were observed and timed until the negative control just began to show colour, then the reaction was stopped by the addition of 100 μL /well 0.1 M HCl.

Plates were then measured using the multimode reader (Berthold Technologies).

5. Results

5.1 Vaccination Doses of Δ M27-MCMV Below 10^4 PFU Raise wt-like MCMV-Specific IgG Responses

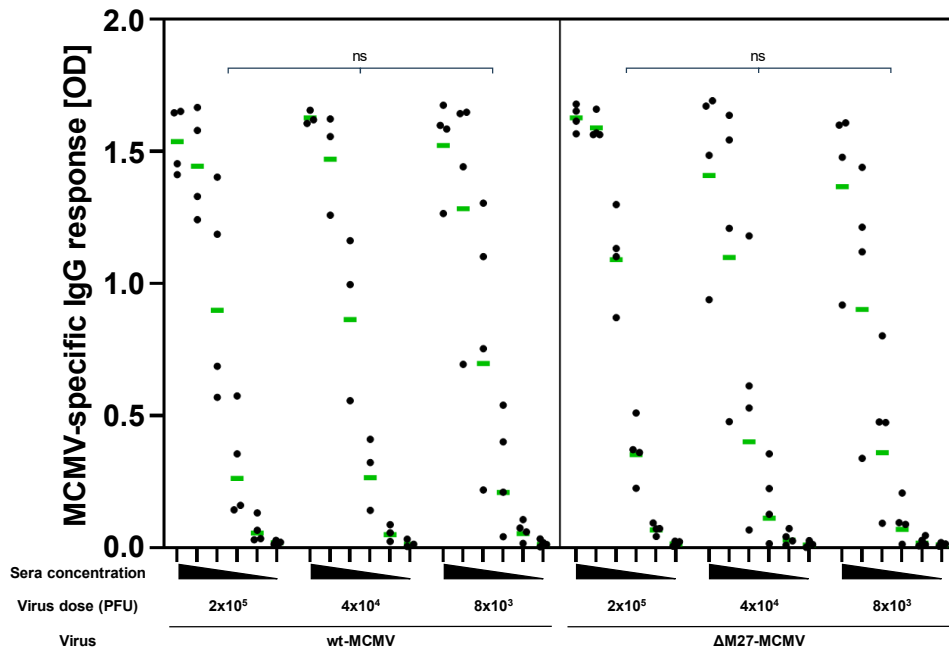


Figure 5.1: MCMV-specific IgG response at 21 dpi in mice vaccinated with differing amounts of virus. BALB/c mice were infected i.p. with 2×10^5 PFU, 4×10^4 PFU or 8×10^3 PFU of wt-MCMV or Δ M27-MCMV. Sera was isolated from collected blood, and MCMV-specific IgG recognising MCMV-infected cell antigens were quantified by ELISA performed in triplicate. Horizontal bars depict geometric mean, while dots indicate individual mice (n=4). Significance calculated by Two-way ANOVA. ns: Not Significant.

Previous work in this research group showed that even vaccination with as low as 8×10^3 PFU of the attenuated Δ M27-MCMV was sufficient to protect against challenge MCMV infections. Analysing mice infected with three different PFUs of wt-MCMV and Δ M27-MCMV, it was found that there was no significant difference in MCMV-specific IgG response between wt-MCMV and Δ M27-MCMV infected mice, confirming previous findings that these viral doses were sufficient to protect mice from challenge infections (Le-Trilling, Jagnjic, et al., 2023).

5.2 Growth of wt-MCMV, Δ M27-MCMV and Δ M34-MCMV in Absence and Presence of Ruxolitinib

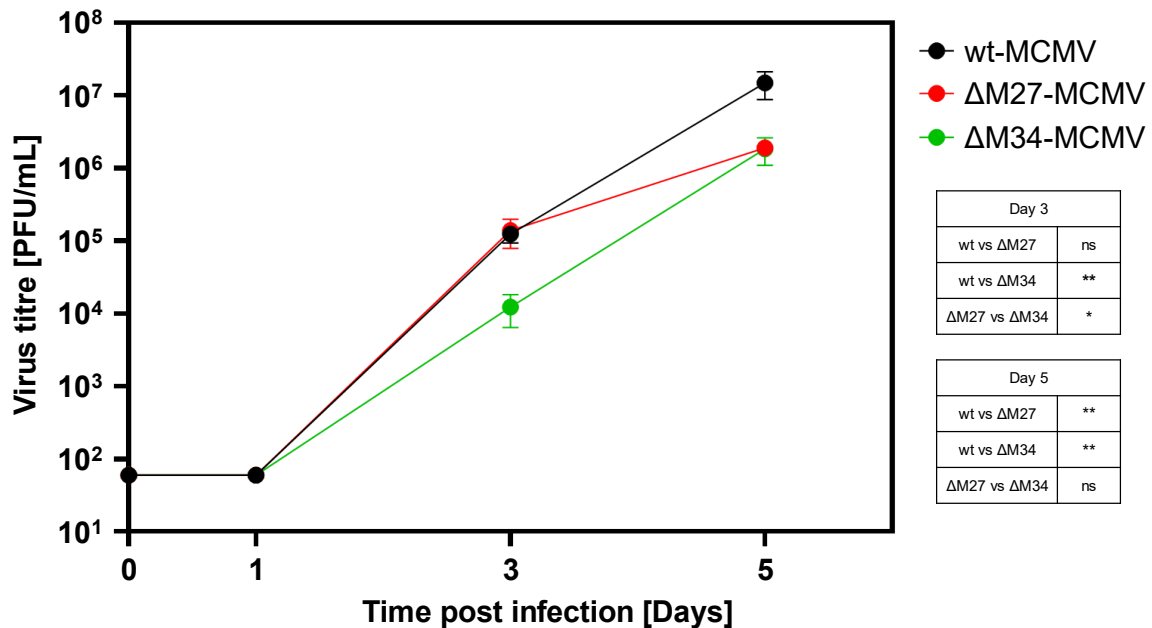


Figure 5.2: Δ M34-MCMV is attenuated *in vitro*. wt-MCMV, Δ M27-MCMV or Δ M34-MCMV with an MOI of 0.001 were used to infect MNCs. Supernatants were collected at 0, 1, 3, and 5 days post infection (dpi), and viral titres were determined by plaque titration assay performed in triplicate to form a growth curve. Significance calculated by Two-way ANOVA. *: $p < 0.05$. **: $p < 0.01$. ns: Not Significant.

Previous work by Dr Mareike Eilbrecht showed that an MCMV mutant lacking the *M34* gene was attenuated but replication competent *in vitro*. To validate this finding, MNCs were infected with wt-MCMV, Δ M27-MCMV or Δ M34-MCMV at an MOI of 0.001 and titrations were carried out on aliquots of supernatant collected at days 0, 1, 3, and 5 post infection. At three dpi, significant attenuation of Δ M34-MCMV was observed compared to wt-MCMV. By five dpi both Δ M27-MCMV and Δ M34-MCMV exhibited significant reduction in growth compared to wt-MCMV: however, both viruses were still replication competent despite their mutations. This supported both Dr Eilbrecht's findings as well as established work with Δ M27-MCMV (Eilbrecht et al., 2020; Le-Trilling et al., 2018) which concluded that both Δ M27-MCMV and Δ M34-MCMV are attenuated relative to wt-MCMV.

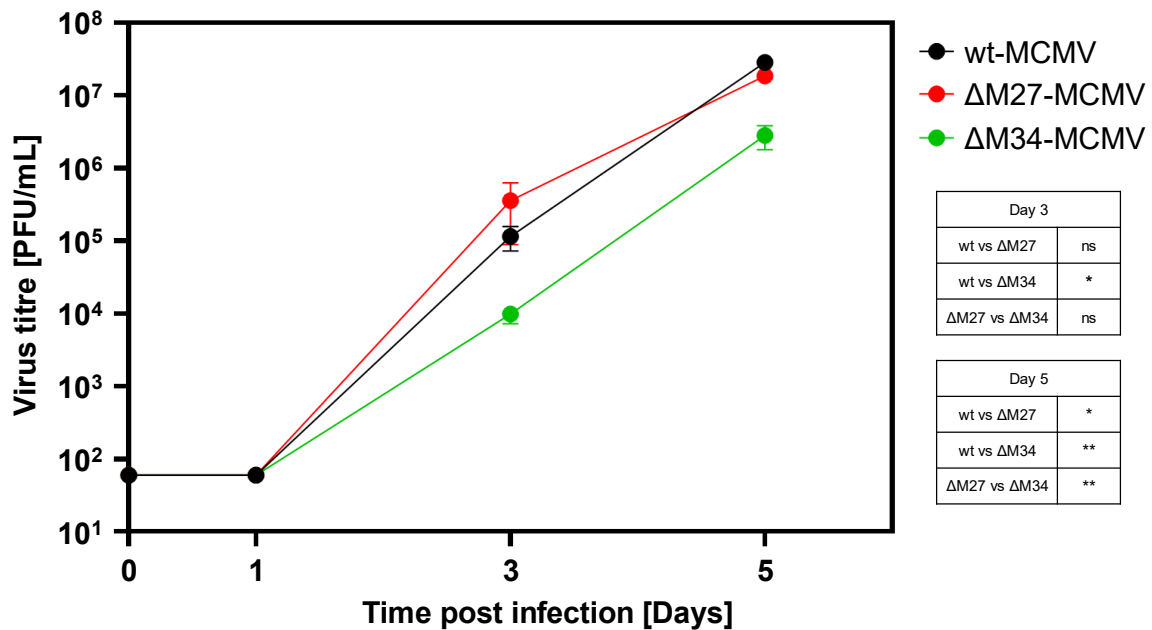


Figure 5.3: Δ M34-MCMV replication is not restored in conditions of inactive Janus Kinases (JAK). Virus growth curves of wt-MCMV, Δ M27-MCMV, and Δ M34-MCMV at days 0, 1, 3, and 5 post infection. wt-MCMV, Δ M27-MCMV or Δ M34-MCMV with an initial dose of infection MOI of 0.001 when grown on MNCs treated with a 4 μ M solution of Ruxolitinib. Viral titre was determined by plaque titration assay performed in triplicate. Significance calculated by Two-way ANOVA. *: $p < 0.05$. **: $p < 0.01$. ns: Not Significant

With this growth curve established, it was important to test whether Δ M34-MCMVs susceptibility to the innate immune system was what was causing its attenuation (as established in Δ M27-MCMV) or whether it was attenuated through different means. To accomplish this, MNCs treated with ruxolitinib were infected with wt-MCMV, Δ M27-MCMV or Δ M34-MCMV. Ruxolotinib, a JAK1/JAK2 inhibitor, prevents phosphorylation and activation of STAT proteins, thus preventing activation of IFN-Stimulated Response Elements (ISREs) (Le-Trilling et al., 2018).

When infecting cells treated with ruxolitinib, Δ M27-MCMV growth was restored to wild-type levels (Fig. 5.3). This is in agreement with previous reports (Le-Trilling et al., 2020), indicating that the M27 protein is beneficial to the virus due to its ability to counteract IFN responses. In clear contrast, however, Δ M34-MCMV remained significantly attenuated (with more than a tenfold reduction in replication compared to wt-MCMV) in these conditions at both 3 dpi and 5 dpi suggesting its attenuation is achieved via different means when compared to Δ M27-MCMV; Δ M27-MCMV being attenuated by virus suppression (Le-Trilling et al., 2018) and Δ M34-MCMV being attenuated by virus impairment (Slayton et al., 2018).

5.3 Δ M34-MCMV Attenuation *in vivo*

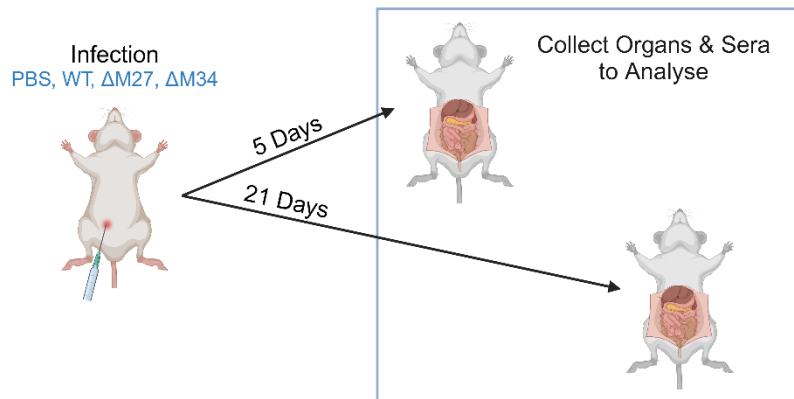


Figure 5.4: Schematic of mouse infection. BALB/c mice were infected i.p. with 2×10^5 PFU of wt-MCMV, Δ M27-MCMV or Δ M34-MCMV as well as a PBS control. Organs and sera were collected and frozen at 5 and 21 dpi.

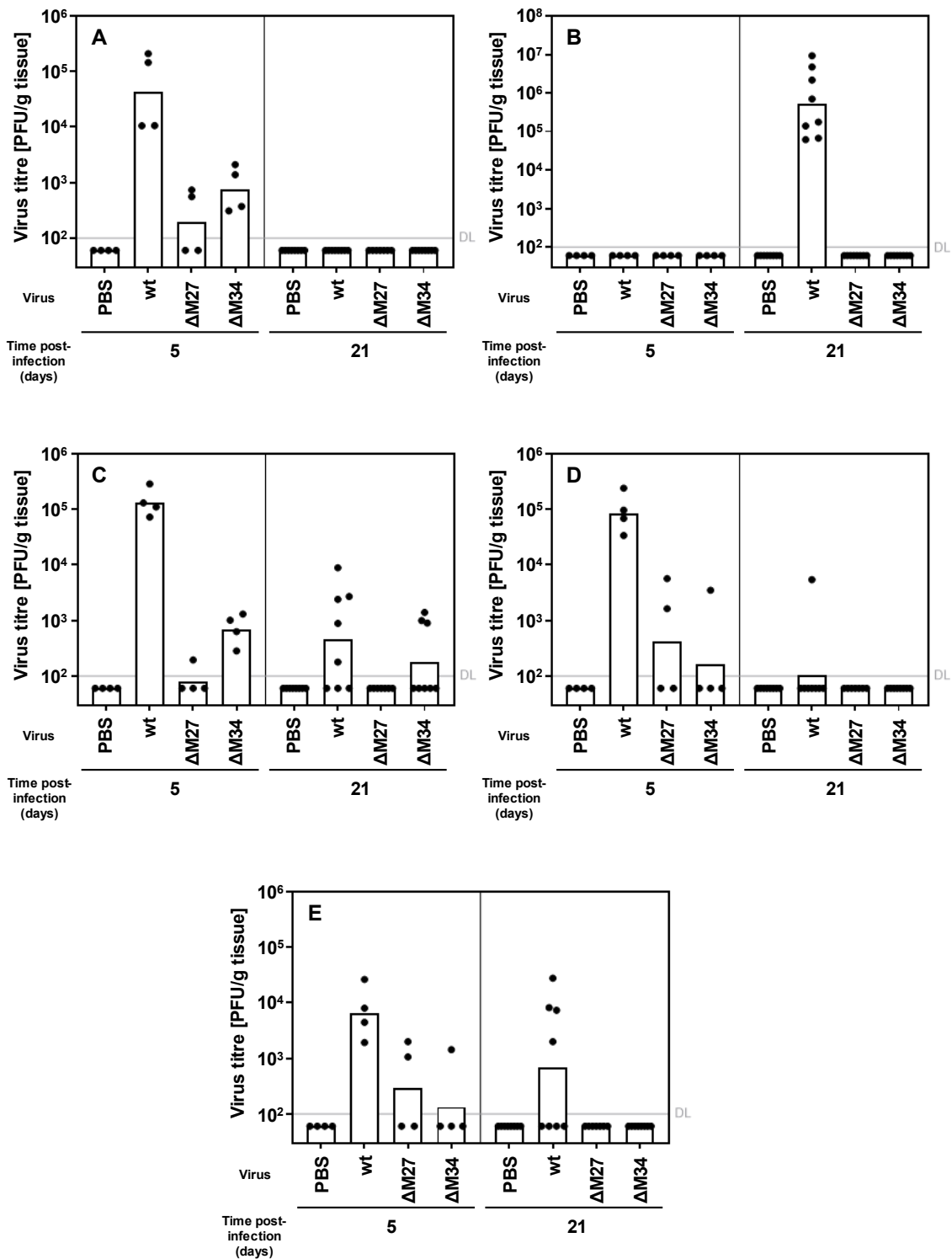


Figure 5.5: *In vivo* growth of wt-MCMV, ΔM27-MCMV, and ΔM34-MCMV. MCMV plaque titrations carried out to determine the amount of virus present in each tissue collected following experimental design laid out in Fig. 5.4. **A)** MCMV titres in spleen, **B)** MCMV titres in salivary gland, **C)** MCMV titres in liver, **D)** MCMV titres in kidney, **E)** MCMV titres in lung. Each dot represents one mouse (5 dpi n=4, 21dpi n=8). Titrations for each sample were performed in quadruplicate. DL: >100PFU/g tissue.

Having shown the attenuation of $\Delta M34$ -MCMV *in vitro*, we next asked if this finding would also be evident in an *in vivo* model. To this end, we used a BALB/c mouse model to see if it was still attenuated and thus a viable vaccine candidate in a living host.

Mice were injected with PBS, wt-MCMV, $\Delta M27$ -MCMV or $\Delta M34$ -MCMV (Fig 5.4). Organs were collected at 5 and 21 dpi and viral titres were calculated. Inhibition of both $\Delta M27$ -MCMV and $\Delta M34$ -MCMV was also evident *in-vivo* in a mouse model. The viral replication was attenuated but the virus was in principle still replication competent as viral growth can be observed in the Spleen at 5 dpi (Fig. 5.3.A) which under regular infection kinetics is an early organ to be infected. This growth is possibly more attenuated in $\Delta M27$ -MCMV than $\Delta M34$ -MCMV. $\Delta M34$ -MCMV replicates more in the liver (Fig 5.3.C) than $\Delta M27$ -MCMV; there is a possibility it is not fully cleared from the liver even at 21 dpi but further experiments would be required to clarify whether this is indeed the case.

The general growth kinetics seen in wt-MCMV-infected mice were as expected based on previous publications (Jackson & Sparer, 2018), with early MCMV growth in the spleen, liver and kidney, and later growth then seen in the salivary glands and lung (Jackson & Sparer, 2018).

As was observed, $\Delta M27$ -MCMV and $\Delta M34$ -MCMV are either cleared from, or do not even reach the salivary gland by 21dpi (Fig. 5.3.B).

5.4 Antibody Responses Raised by Δ M27-MCMV and Δ M34-MCMV Infections

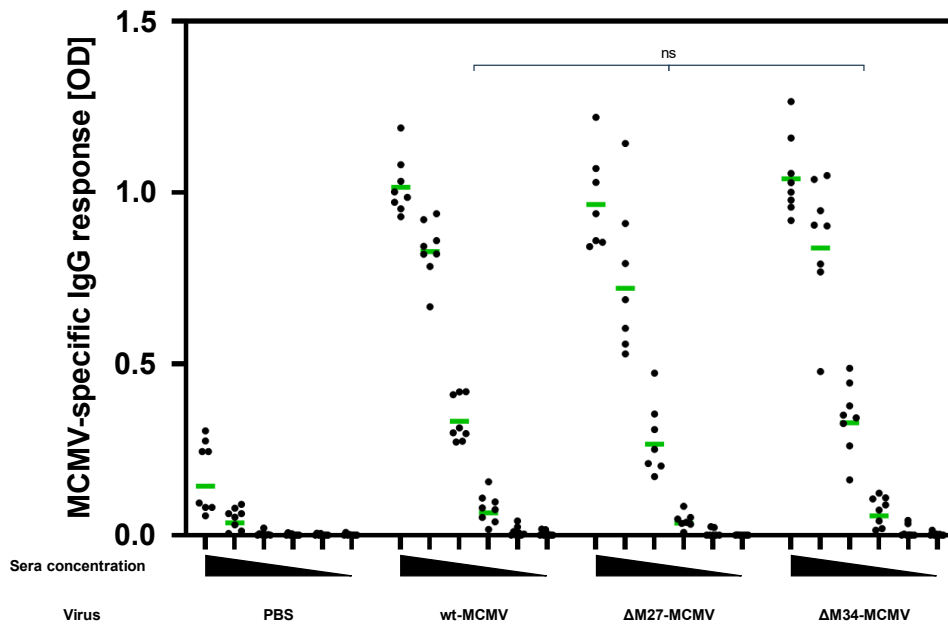


Figure 5.6: MCMV-specific IgG response at 21 dpi is consistent in wt-MCMV, Δ M27-MCMV, and Δ M34-MCMV infected mice. BALB/c mice were infected with wt-MCMV, Δ M27-MCMV or Δ M34-MCMV as depicted in Fig. 5.4. Sera were isolated from collected blood, and MCMV-specific IgG determined by ELISA performed in triplicate. Horizontal bars show geometric mean, dots indicate individual mice (n=8). Significance calculated by Two-way ANOVA. ns: Not Significant.

With proven attenuation of Δ M34-MCMV *in vitro* and *in vivo*, we then asked if it was still capable of raising a sufficient immune response and thus protecting the host from future infections with MCMV. To this end, we determined MCMV-specific IgG responses in mice infected with PBS, wt-MCMV, Δ M27-MCMV or Δ M34-MCMV.

Only sera from mice 21 dpi were analysed as there would be no antibodies at 5 dpi. Δ M27-MCMV and Δ M34-MCMV attenuated viruses were found to elicit a similar level of MCMV-specific IgG response compared with that in a wt-MCMV infection. Similar antibody responses were observed despite the attenuation of the virus as observed in Fig. 5.5.

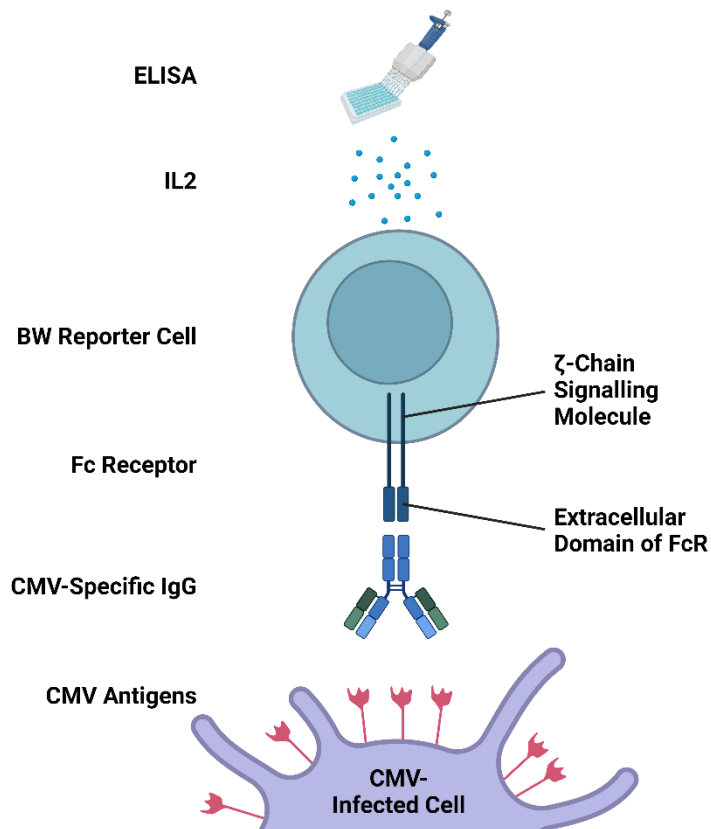


Figure 5.7: Schematic showing a surrogate assay for the activation of individual Fc γ receptors. CIM cells are infected with Δ M138-MCMV to express MCMV antigens. These MCMV-antigen presenting cells are then exposed to sera of wt-MCMV or Δ M34-MCMV infected mice which contain MCMV-specific IgG. The IgG then binds to the Fc receptor on developed BW cells which contain only a single receptor type. Binding of receptors causes secretion of IL-2 which is detectable by ELISA. Adapted from (Corrales-Aguilar et al., 2013). Created with BioRender.

Knowing that Δ M34-MCMV raised similar IgG responses to wt-MCMV infections, the next step was to determine which individual Fc-receptors were activated, and whether there was any notable difference in the levels of activation in a Δ M34-MCMV infection and a wt-MCMV infection.

To this end, we used a previously established surrogate assay for the activation of individual Fc γ -receptors developed by (Corrales-Aguilar et al., 2013) (Schematic in Fig 5.7).

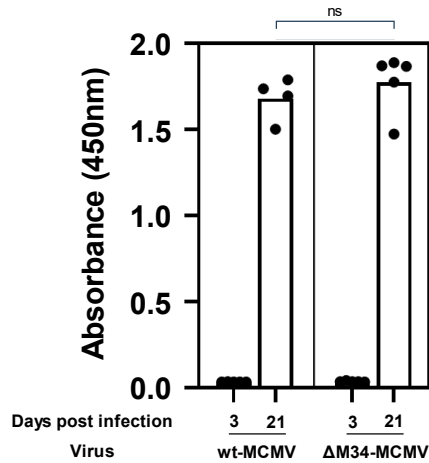


Figure 5.8: Δ M34-MCMV induces a wt-MCMV-like response in terms of CD16 (Fc γ RIII) antibodies. BALB/c mice were infected i.p. with 2×10^5 PFU of wt-MCMV or Δ M34-MCMV and sera collected at 3 and 21 dpi. Dots indicate individual mice (n=5). Significance calculated by Two-way ANOVA. ns: Not Significant.

CD16 (Fc γ RIII) activation was detected at 21 dpi in both wt-MCMV and Δ M34-MCMV infected mice. There was no significant difference in the level of activation. As expected, no activation was detected at 3 dpi due to no prior exposure to the virus (Fig 5.8).

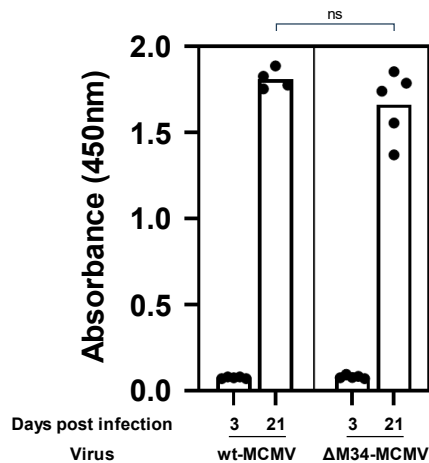


Figure 5.9: Δ M34-MCMV induces a wt-MCMV-like response in terms of CD32 (Fc γ RII) activating antibodies. BALB/c mice were infected with 2×10^5 PFU of wt-MCMV or Δ M34-MCMV and sera collected at 3 and 21 dpi. Dots indicate individual mice (n=5). Significance calculated by Two-way ANOVA. ns: Not Significant.

CD32 (Fc γ RII) activation was detected at 21 dpi in both wt-MCMV and Δ M34-MCMV infected mice. There was no significant difference in the level of activation. As expected, no activation was detected at 3 dpi due to no prior exposure to the virus (Fig 5.9).

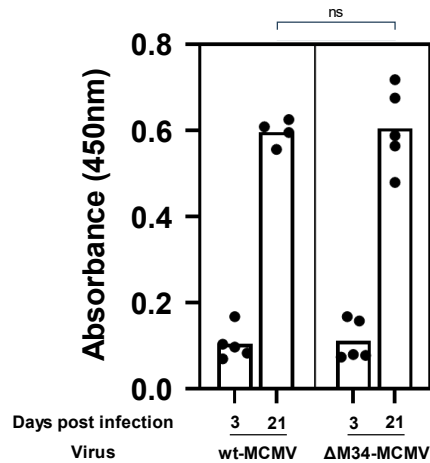


Figure 5.10: Δ M34-MCMV induces a wt-MCMV-like response in terms of CD64 (Fc γ RI) activating antibodies. BALB/c mice were infected with 2×10^5 PFU of wt-MCMV or Δ M34-MCMV and sera collected at 3 and 21 dpi. Dots indicate individual mice (n=5). Significance calculated by Two-way ANOVA. ns: Not Significant.

CD64 (Fc γ RI) activation was detected at 21 dpi in both wt-MCMV and Δ M34-MCMV infected mice. There was no significant difference in the level of activation. As expected, no activation was detected at 3 dpi due to no prior exposure to the virus. Notably, less activation of CD64 (Fc γ RI) by Fc receptor activating antibodies was observed when compared to other Fc receptors by the sera of wt-MCMV and Δ M34-MCMV infected mice.

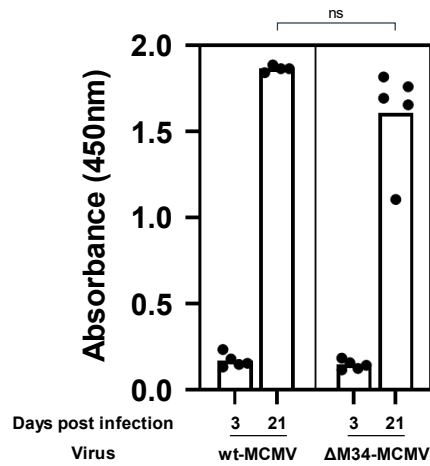


Figure 5.11: Δ M34-MCMV induces a wt-MCMV-like response in terms of Fc γ RIV (Fc γ RIIIA) activating antibodies. BALB/c mice were infected with 2×10^5 PFU of wt-MCMV or Δ M34-MCMV and sera collected at 3 and 21 dpi. Dots indicate individual mice (n=5). Significance calculated by Two-way ANOVA. ns: Not Significant.

Fc γ RIV (Fc γ RIIIA) activation was detected at 21 dpi in both wt-MCMV and Δ M34-MCMV infected mice. There was no significant difference in the level of activation. As expected, no activation was detected at 3 dpi due to no prior exposure to the virus (Fig 5.11).

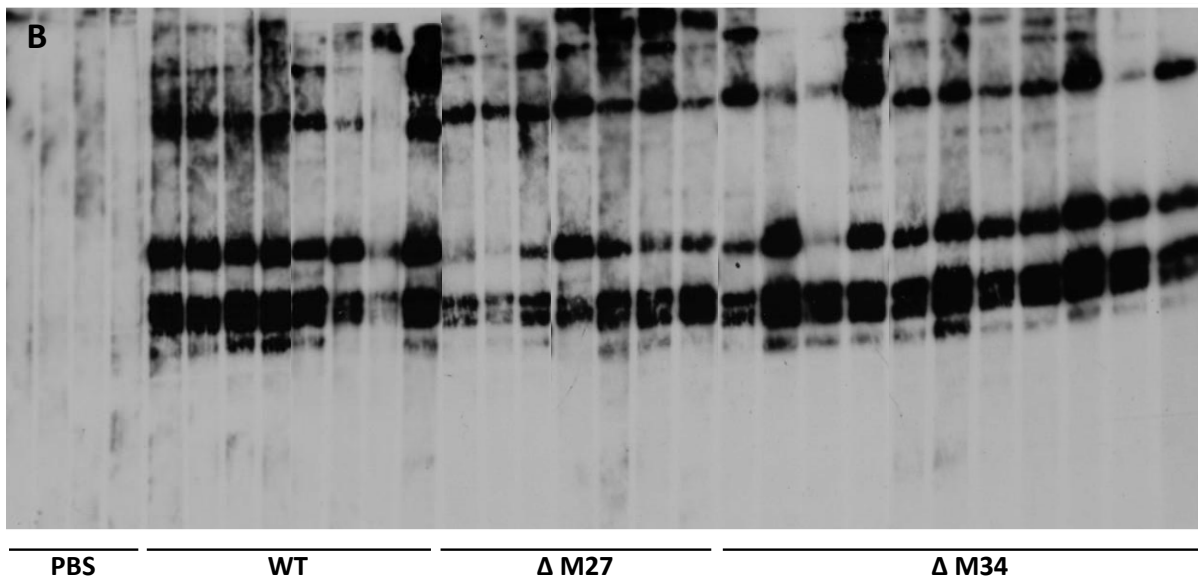
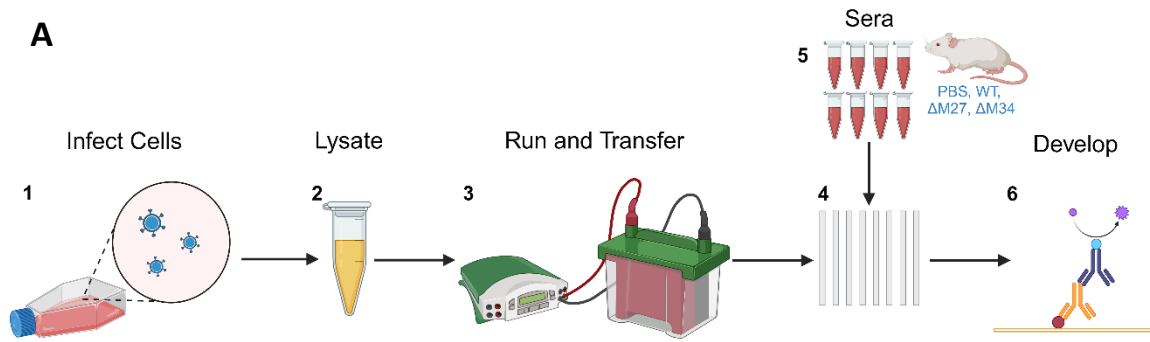


Figure 5.12: Sera originating from mice infected with wt-MCMV, $\Delta M27$ -MCMV or $\Delta M34$ -MCMV recognise similar MCMV antigens. **A)** Schema of experiment. **1)** CIM cells infected with wt-MCMV **2)** Lysate of infected CIMs collected **3)** Lysate run on Western Blot gel and transferred to PVDF membrane **4)** PVDF membrane cut into strips **5)** Sera from individual BALB/c mice infected as described in Fig. 5.4 at 21 dpi was used as a primary antibody on individual membrane strips to allow for detection of recognised antigens **6)** Membranes exposed to secondary antibodies and image developed. Created with BioRender. **B)** Western Blot showing similar antibody recognition by antigens produced during infection in mice with wt-MCMV, $\Delta M27$ -MCMV or $\Delta M34$ -MCMV.

With knowledge of the $\Delta M34$ -MCMV Fc-receptor responses established as being similar to those of wt-MCMV infections, we next asked whether the antibodies raised by wt-MCMV, $\Delta M27$ -MCMV, and $\Delta M34$ -MCMV infections had any unique recognised antigens which could give further clues to vital antibodies in protection. The experimental design is laid out in Fig

5.12.A. CIM cells were infected with wt-MCMV, and their lysates were collected at 3 dpi. These lysates were then run on a Western Blot gel, transferred to PVDF, and the PVDF was cut into strips of equal width. Each strip was then incubated with the sera of an individual mouse which had been infected with either PBS, wt-MCMV, Δ M27-MCMV or Δ M34-MCMV for 21 days. The strips were then rearranged for an image to be developed.

Across all mice which had been infected with wt-MCMV, Δ M27-MCMV or Δ M34-MCMV, there are consistently recognised antigens by the antibodies within the sera. This would suggest that even with the differing cause of attenuation between Δ M27-MCMV, and Δ M34-MCMV, the host immune response raises similar antibodies.

5.5 IgG Responses in Vaccination Experiments

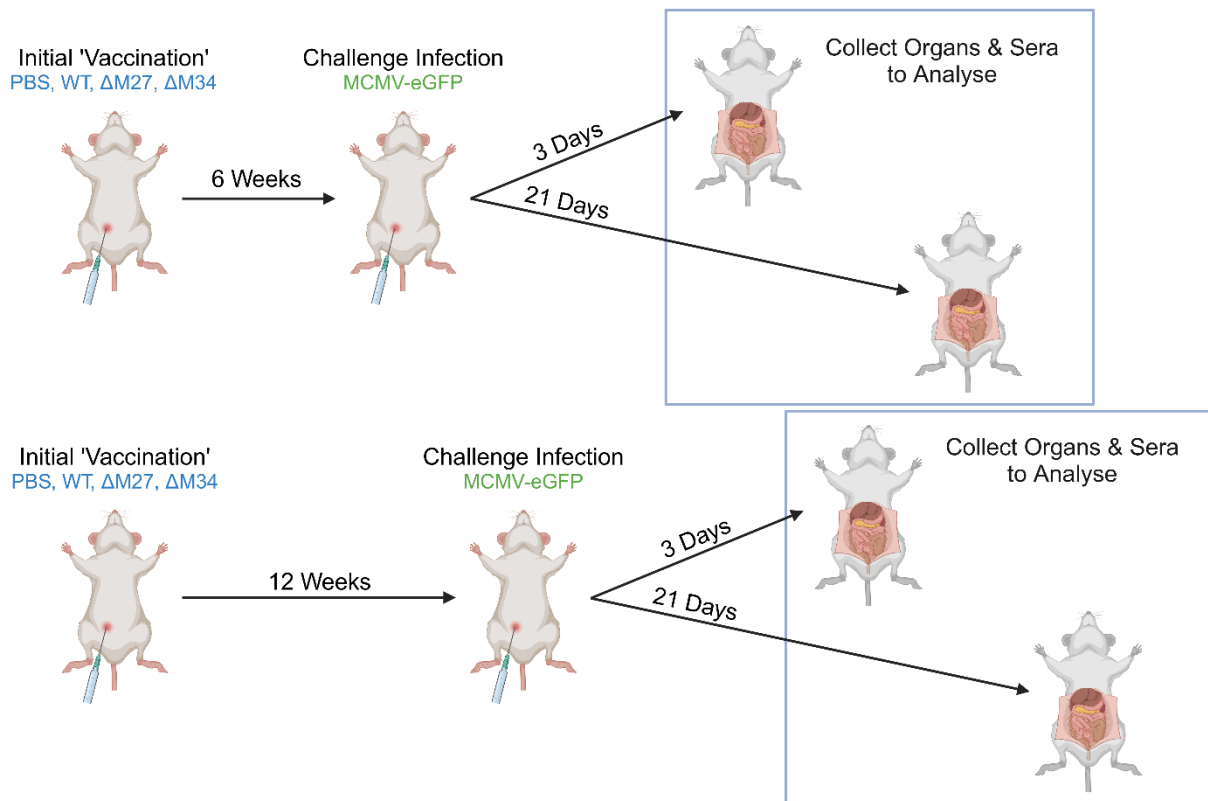


Figure 5.13: Experimental schema of vaccination experiment. BALB/c mice were given initial i.p. vaccinations with 2×10^5 PFU of wt-MCMV, $\Delta M27$ -MCMV or $\Delta M34$ -MCMV. Six or 12 weeks after the vaccination, mice were given a challenge infection with 2×10^5 PFU of MCMV:eGFP. Organs and sera were then collected at either 3 or 21 days post challenge (dpc). Created with BioRender.

With the knowledge that $\Delta M27$ -MCMV and $\Delta M34$ -MCMV are attenuated compared to wt-MCMV but raise similar antibody responses, we next asked if the $\Delta M34$ -MCMV mutant was capable of protecting a host against infection with a challenge MCMV infection. To do this, several cohorts of mice were infected as indicated in Fig. 5.13, and the sera and organs analysed.

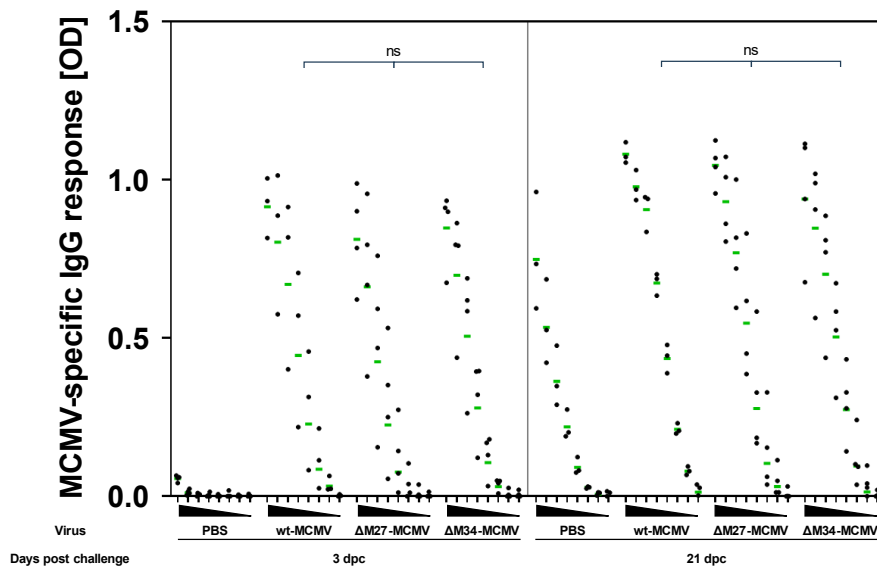


Figure 5.14: MCMV-specific binding IgG response at 3 and 21 dpi in vaccinated mice at 6 weeks post vaccination. Mice were infected as indicated in Fig 5.13. MCMV-specific IgG determined by ELISA performed in triplicates. Lysates from MCMV-infected CIM cells used for antigen. Horizontal bars depict geometric mean, while dots indicate individual mice (n=4). Significance when comparing wt-MCMV, Δ M27-MCMV, and Δ M34-MCMV vaccinated mice calculated by Two-way ANOVA. ns: Not Significant.

Wt-MCMV, Δ M27-MCMV and Δ M34-MCMV all raised similar MCMV-specific IgG responses in combined vaccination and challenge infections (Fig. 5.14).

There is a clear anamnestic antibody response as mice with previous contact with wt-MCMV, Δ M27-MCMV and Δ M34-MCMV showed strong MCMV-specific IgG responses as early as 3 dpc, whereas PBS vaccinated mice show little to no MCMV-specific IgG response at 3 dpc, with an increase by 21 dpc as antibodies are produced in response to the challenge virus, but this response is still below that observed in the sera of mice with previous exposure to wt-MCMV, Δ M27-MCMV and Δ M34-MCMV.

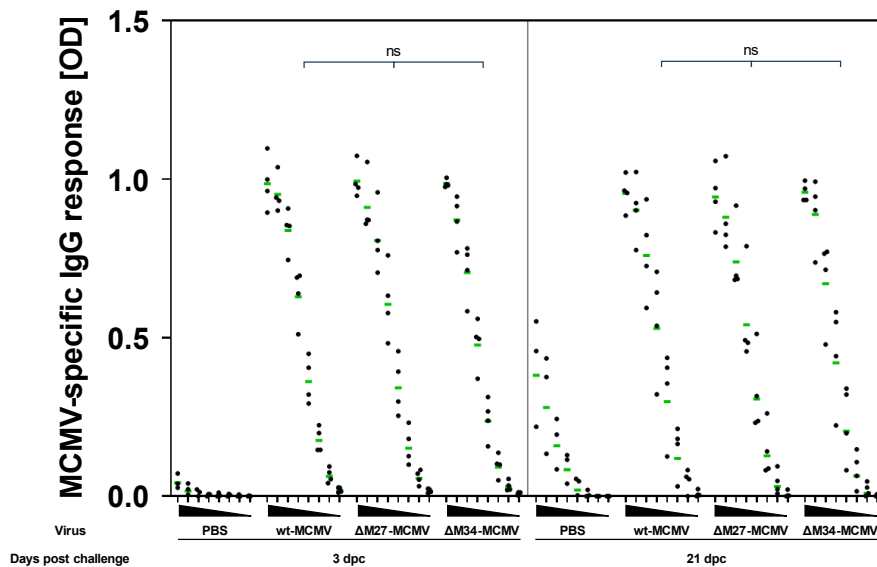


Figure 5.15: MCMV-specific binding IgG response at 3 and 21 dpi in vaccinated mice at 12 weeks post vaccination. Mice infected as indicated in Fig 5.13. MCMV-specific binding IgG determined by ELISA performed in triplicates. Lysates from MCMV-infected CIM cells used for antigen. Horizontal bars depict geometric mean, while dots indicate individual mice (n=4). Significance when comparing wt-MCMV, Δ M27-MCMV and Δ M34-MCMV vaccinated mice calculated by Two-way ANOVA. ns: Not Significant.

This analysis was repeated with the sera of mice given a challenge infection 12 weeks post vaccination. Once again, wt-MCMV, Δ M27-MCMV and Δ M34-MCMV raised similar MCMV-specific binding IgG responses in combined vaccination and challenge infections.

Similar to the experiment carried out with a challenge infection given 6 weeks post vaccination, there is a clear anamnestic antibody response. Once more, mice with previous contact with wt-MCMV, Δ M27-MCMV and Δ M34-MCMV showed strong MCMV-specific IgG responses as early as 3 dpc, whereas PBS vaccinated mice show little to no MCMV-specific IgG response at 3 dpc, with an increase by 21 dpc as antibodies are produced in response to the challenge virus, but this response is still below that observed in the sera of mice with previous exposure to wt-MCMV, Δ M27-MCMV and Δ M34-MCMV.

5.6 Protection Provided by Δ M34-MCMV Vaccination Against Challenge Infections

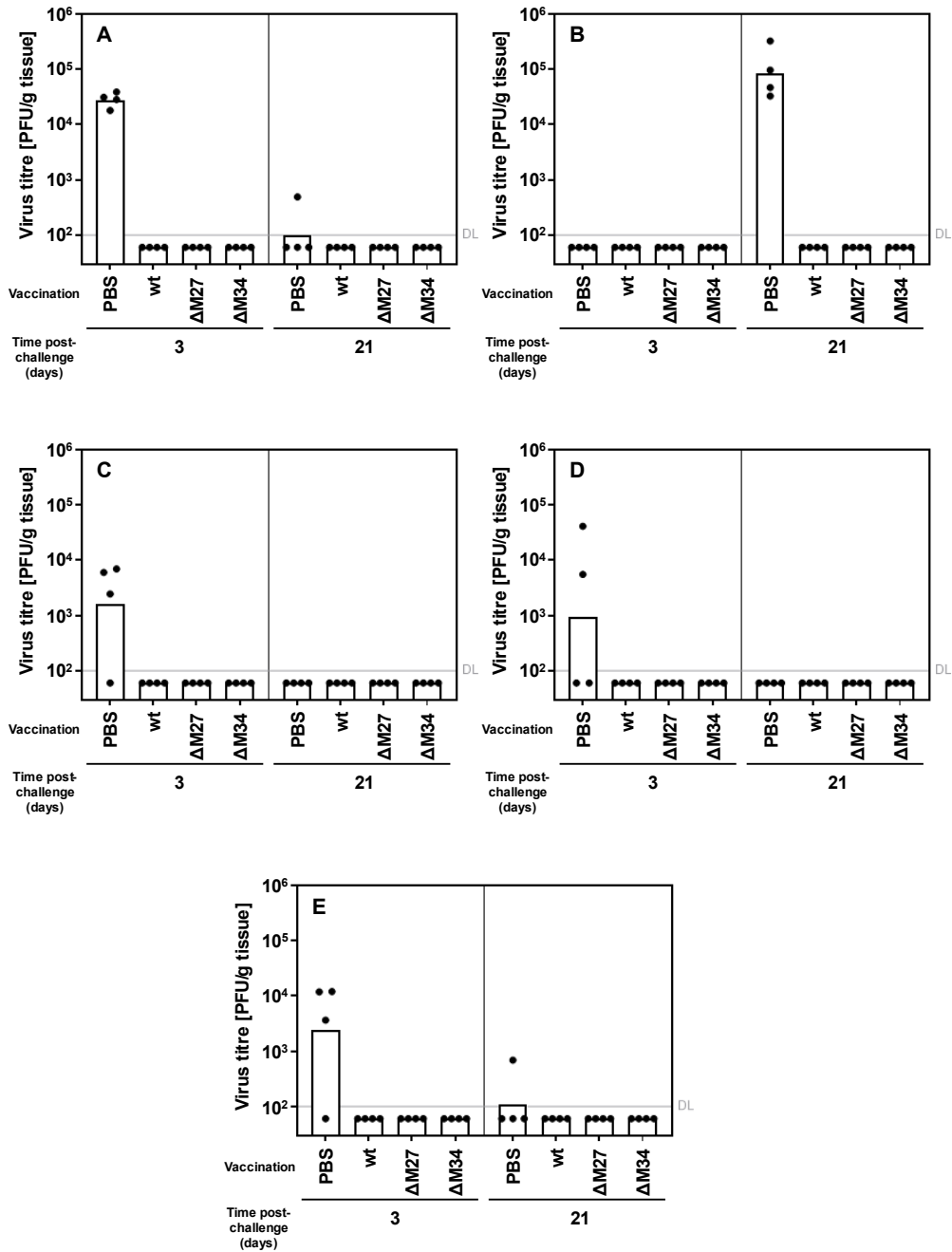


Figure 5.16: *In vivo* growth of a challenge infection at 6 weeks post vaccination in mice vaccinated with wt-MCMV, Δ M27-MCMV or Δ M34-MCMV showing vaccinated mice are protected against a challenge infection. BALB/c mice were infected as described in Fig. 5.13. Titres of virus in indicated organs were determined by plaque titration assay. A) MCMV titres in spleen, B) MCMV titres in salivary gland, C) MCMV titres in liver, D) MCMV titres in kidney, E) MCMV titres in lung. Dots indicate individual mice (n=4). Titrations were performed in quadruplicates. DL: >100PFU/g tissue.

Δ M27-MCMV and Δ M34-MCMV are attenuated *in vivo* and raise similar IgG immune responses to wt-MCMV infections. With this in mind, information regarding the ability of these attenuated mutants to offer protection against a challenge infection was investigated.

Vaccination with wt-MCMV, Δ M27-MCMV or Δ M34-MCMV provides protection against a challenge infection, preventing viral replication in all organs at both 3 and 21 dpc. Thus, despite the attenuation of the viruses through different means, both are viable vaccine candidates.

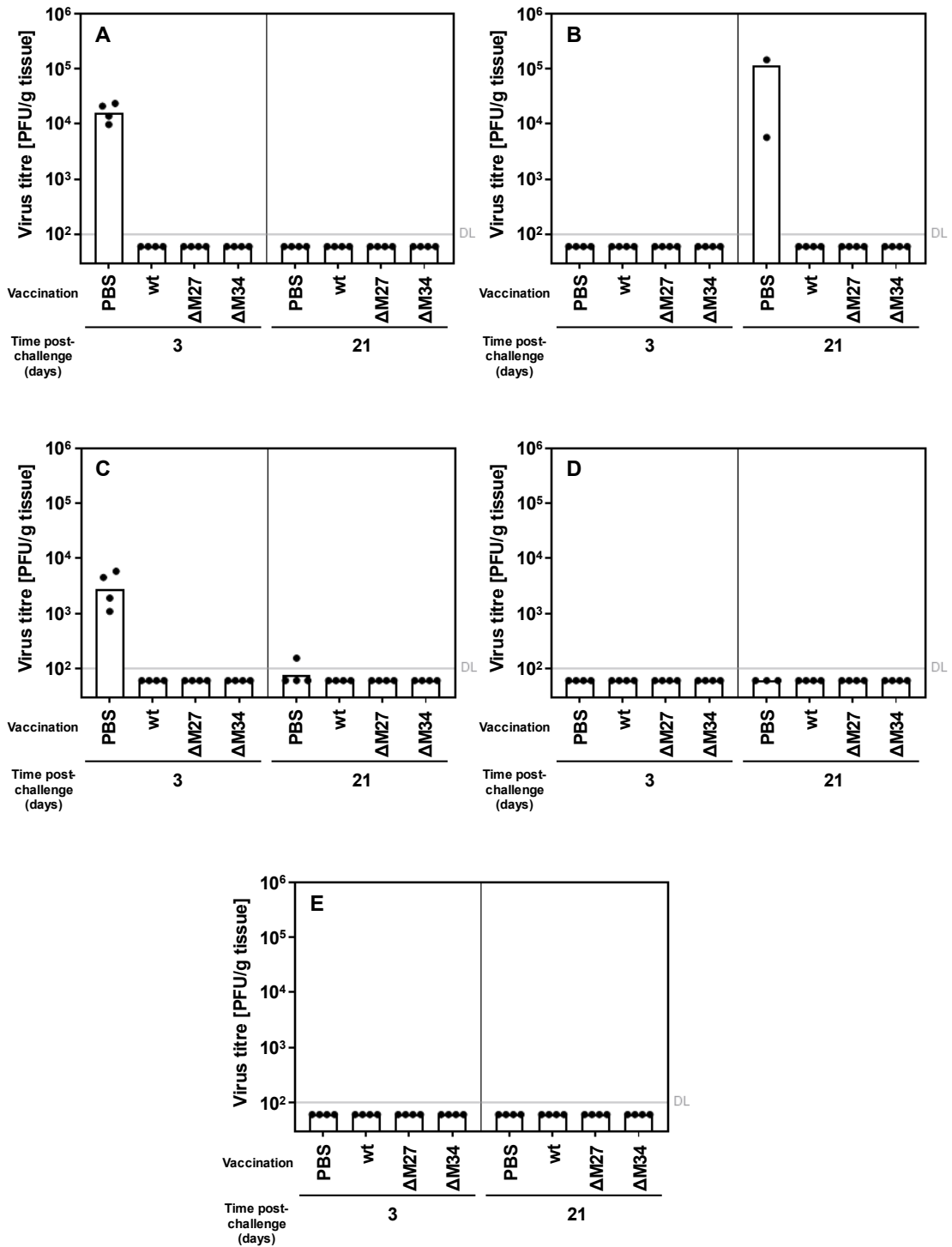


Figure 5.17: *In vivo* growth of a challenge MCMV infection at 12 weeks post vaccination in mice vaccinated with wt-MCMV, ΔM27-MCMV, and ΔM34-MCMV showing vaccinated mice are protected against a challenge infection. BALB/c mice were infected as described in Fig. 5.13. MCMV titres in indicated organ were determined by plaque titration assay. **A)** MCMV titres in spleen, **B)** MCMV titres in salivary gland, **C)** MCMV titres in liver, **D)** MCMV titres in kidney, **E)** MCMV titres in lung. Dots indicate individual mice (n=4). Titrations were performed in quadruplicates. DL: >100PFU/g tissue.

A further vaccination experiment in which mice were infected with a challenge virus 12 weeks after the initial vaccination with wt-MCMV, Δ M27-MCMV or Δ M34-MCMV also proved to prevent spread of the challenge virus to the organs. This would suggest that vaccination with both Δ M27-MCMV and Δ M34-MCMV provides protection for longer periods of time (of at least 12 weeks).

6. Discussion

6.1 Δ M34-MCMV is Attenuated *in vitro* in a Manner Independent of JAK1/2 Activation

The findings presented in Fig. 5.2 confirm previous data showing that Δ M34-MCMV is an attenuated MCMV mutant. As published by Mareike Eilbrecht (Eilbrecht et al., 2020), the virus is replication competent but it is attenuated 10-100 fold.

As shown in Fig. 5.3 that in cells treated with ruxolitinib, Δ M34-MCMV remained significantly attenuated while Δ M27-MCMV virus growth appears to be restored. As ruxolitinib inhibits the ATP-binding catalytic sites of JAKs (Ajayi et al., 2018; Mascarenhas & Hoffman, 2012) this would suggest that Δ M34-MCMV attenuation is achieved via means independent of the JAK-STAT pathway.

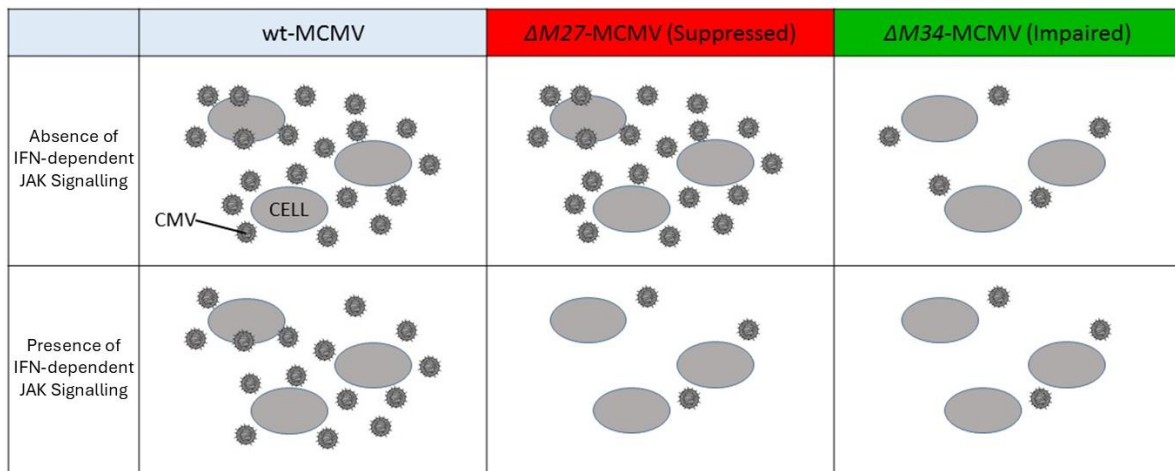


Figure 6.1: Suppressed vs impaired viral growth in presence and absence of IFN-induced JAK1/JAK2 signalling. wt-MCMV replicates efficiently in cells due to its full arsenal of gene products. Meanwhile Δ M27-MCMV is attenuated in the presence of interferon due to missing a gene coding for a protein beneficial to its innate immune evasion, blockade of the cell intrinsic interferon signalling allows Δ M27-MCMV to replicate efficiently once more as it is not suppressed by the immune system. Δ M34-MCMV remains impaired even in the absence of IFN-induced JAK1/JAK2 signalling.

6.2 Δ M34-MCMV is Attenuated *in vivo*

When BALB/c mice were infected with Δ M34-MCMV or Δ M27-MCMV, both viruses were attenuated when compared to a wild-type MCMV infection (Fig. 5.5).

This is especially evident in the salivary gland, where at 21 dpi copies of the virus mutants were not detectable. Even at early timepoints in organs such as the spleen, liver and kidney, where viral replication is evident in early stages of infection, Δ M27-MCMV and Δ M34-MCMV virus titres were considerably lower than with wt-MCMV.

Notably when comparing Δ M34-MCMV to wt-MCMV, viral growth is 10-100 fold more attenuated in the spleen by 5 dpi. Meanwhile, comparing Δ M34-MCMV growth to Δ M27-MCMV across all organs the difference in viral growth observed is far smaller.

By 21 dpi, Δ M27-MCMV and Δ M34-MCMV are cleared from all organs, with the exception of Δ M34-MCMV, which in some cases (3/8 mice) appears to not be fully cleared from the liver (Fig. 5.5.C). Future work would first necessitate looking further into this possibility, followed by observation of the liver of Δ M34-MCMV infected mice at later timepoints than 21 dpi. If the virus is in fact not cleared from the liver, it may pose a risk of hepatitis, however, it is believed that cases of CMV hepatitis in healthy patients requiring hospitalisation is rare (Da Cunha & Wu, 2021) which would suggest this is not the case.

It is important to note, however, in the two cohorts of mice analysed, virus plaques could not be seen in the kidney or lung of one cohort (n=4 for each condition). Visual inspection of these wells revealed dead and dying cells. This is potentially because the organ homogenate was too toxic to the cells. My theory is that due to the high number of enzymes present in the liver, at concentrations of the homogenate where virus plaques would be expected to be observed cells were damaged beyond the point of being observable during these two separate runs of this assay for this dataset.

6.3 Both Δ M27-MCMV and Δ M34-MCMV Raises Wild Type Like IgG Response, Despite Attenuation

With our proof of Δ M34-MCMV's attenuation in a mouse model, we next characterised the MCMV-specific IgG response. This is important as previous work by the research group indicated that antibodies are sufficient to raise protection against a challenge MCMV infection

in a cCMV model (Le-Trilling, Jagnjic, et al., 2023). Furthermore, the characteristic of reduced viral replication is essential in the development of a live-attenuated vaccine, but if the attenuated virus no longer raises an immune response, then it is not a viable vaccine candidate.

An example of this was observed previously in UV-treated MCMV, where a lack of viral replication is hypothesised to have not raised a protective immune response in infected hosts, and thus they were not protected against a further challenge infection. This will be discussed in Chapter 6.8.

As shown by Fig. 5.6, Δ M34-MCMV raised a wt-MCMV-like MCMV-specific IgG response by 21 dpi in BALB/c mice. Δ M27-MCMV also raised a similar response. This data pointed towards Δ M34-MCMV potentially raising similar levels of protection against challenge infections, and justified a vaccination experiment to explore this further.

To address whether these IgG resulted in neutralisation, the next step was to carry out a neutralisation assay to determine the different levels of neutralisation across the sera of mice infected with each virus. However, trial experiments determined that neutralisation could not be clearly determined in sera samples with a dilution greater than 1/20. Thus, an experiment would require a minimum concentration of 1/10 sera to obtain conclusive data. From a practical point of view, the volume of sera required for this would surpass the total material collected from single mice in some cases, rendering this technique not widely applicable to understand the mechanism of protection.

6.4 Wild-Type MCMV and Δ M34-MCMV Infections Elicit Similar Levels of Fc-Receptor Activating Antibodies

With our establishment that infections with wt-MCMV, Δ M27-MCMV, and Δ M34-MCMV raise similar levels of MCMV-specific IgG response in their hosts, we next determined which Fc receptors are activated by these antibodies, and whether it is possible to determine a difference in levels of activation of specific receptors between wt-MCMV and Δ M34-MCMV infections.

To achieve this, an Fc-receptor activation assay as described by (Corrales-Aguilar et al., 2013) was used (explanation in Fig. 5.7).

The results in Figs. 5.8, 5.9, 5.10, and 5.11, together with the previous ELISA result from Fig. 5.6 demonstrated that despite Δ M34-MCMV being attenuated 10-100 fold compared to a wt-

MCMV infection, Δ M34-MCMV raised a similar response across all Fc receptors. This supports the viability of Δ M27-MCMV and Δ M34-MCMV candidate vaccines for MCMV.

6.5 Mouse Sera Obtained from wt-MCMV, Δ M27-MCMV and Δ M34-MCMV Infection Contain Antibodies that Recognise Similar MCMV Antigens

We have shown, despite the differing levels of attenuation between wt-MCMV and the two mutant viruses (Δ M27-MCMV and Δ M34-MCMV), there is a remarkably similar level of antigen recognition the sera of all three mouse. Interestingly, Δ M27-MCMV and Δ M34-MCMV infected mouse sera contained antibodies recognising similar antigens across the board. This is despite the two viruses being attenuated by two different methods, suppression and impairment, respectively.

Indeed, the techniques used here (Western Blot) would not indicate subtle changes in various immune responses. To compare subtle differences in immune responses elicited by these three viruses, a quantitative technique such as proteomics or transcriptomics (transcriptional level changes) would have to be applied. Additionally, it would be of particular interest to carry out an immunoprecipitation with aforementioned sera originating from vaccinations with attenuated MCMV mutants so that the recognised antigens could be identified and further investigated for their significance. Taken together, though we cannot observe gross differences in immune responses as of now, other techniques need to be applied for a more comprehensive overview.

In a recent publication by (Vlahava et al., 2021) HCMV viral antigens that mediated antiviral ADCC were identified, and neutralizing monoclonal antibodies (mAb) were developed. These mAbs were sufficient to raise an ADCC response against fibroblasts expressing their specific antigen and increase NK-mediated cell death. These antigens were HCMV's pUL16 and pUL141, which are not conserved in MCMV. However, by identifying the antigens recognised in Fig. 5.12, potential analogous proteins could be identified in HCMV and selected as targets for development of mAbs.

If further experiments could be conducted to determine antigens presented during viral-latency in HCMV (pUS28 was used as an example by Vlahava, but suitable mAb candidates could not

be generated for this antigen), then potentially an ADCC response could be induced against these latently infected cells in an effort to clear chronic HCMV infections in the future.

6.6 Vaccinated Mice Show Similar MCMV-Specific IgG Responses Across All Tested Viruses

Looking at MCMV-specific IgG responses raised by the combined vaccination and challenge infections, wt-MCMV, Δ M27-MCMV, and Δ M34-MCMV all raised similar responses. Interestingly, when mice had only ever been exposed to one infection there is little to no response observed at 3 dpc (as can be seen in mice vaccinated with PBS). Likewise, the response is still lower at 21 dpc. This suggests an anamnestic response to the vaccination showing that both attenuated viruses would be potential candidates for long-term (12 week at least) protection. Longer-term experiments would be required to strengthen this finding.

6.7 Δ M34-MCMV as a Potentially Viable Vaccine Against MCMV

Taken together our data show that Δ M34-MCMV is an attractive candidate for an MCMV vaccine. The virus was attenuated *in vivo*, being cleared from all organs except potentially the liver by day 21. Notably, infected liver endothelial cells and hepatocytes produce MCMV, but MCMV produced in the liver does not spread to other organs (Sacher et al., 2008). Despite this attenuation, Δ M34-MCMV raised a wt-like IgG response as well as exhibiting similar levels of Fc-receptor activation. Most importantly, vaccination with Δ M34-MCMV protects against reinfection with a challenge MCMV.

Both Δ M27-MCMV and Δ M34-MCMV completely protected against the growth of any detectable level of MCMV upon reexposure at both 3 and 21 dpi in all organs tested (Fig. 5.16). This was also the case with a longer time period between vaccination and challenge infection, suggesting that these attenuated viruses both provide protection over a longer period of at least 12 weeks (Fig. 5.17).

Considering the *M34* gene in MCMV is the homolog of the *UL34* gene in HCMV, this data shows promise for the potential of a Δ UL34-HCMV vaccine candidate in humans.

In a future experiment, it may be worthwhile testing the limit of this protection over even longer time periods, perhaps up to 9 months to determine whether a single vaccination dose could protect potential mothers against a challenge infection for the full term of a human pregnancy. This would have to be planned accordingly, vaccinating mice at a younger age to maximise the probability the results could be obtained during their natural, short lifespan.

Further, to address limitation posed by species specificity, humanised mouse models have been developed to test various vaccines against viruses such as HIV, Hepatitis C virus, and Epstein-Barr Virus (EBV), reviewed in (Kaushik et al., 2024). Indeed, a humanised mouse model has recently been proposed for HCMV vaccine development. Here a mouse with a human immune system is developed through human precursors or differentiated immune cells biodistributed in an immune-deficient mouse model to mimic human immune responses to various stimuli (Koenig et al., 2020).

6.8 MCMV Correlates of Protection

With all of these experiments taken together, several important correlates of protection are evident and the similarities in the results obtained in protection experiments between wt-MCMV, Δ M27-MCMV, and Δ M34-MCMV would suggest all three of these viruses protect against a challenge infection in the same manner.

In the vaccinations depicted in Fig. 5.16 and Fig. 5.17, there was growth of the challenge virus in PBS-“vaccinated” mice, but no challenge virus growth can be seen in mice vaccinated with wt-MCMV, Δ M27-MCMV or Δ M34-MCMV. This early protection is likely mediated by memory B-cells which recognise the MCMV antigens and produce antibodies accordingly.

This is backed up by the data seen in Fig 5.14 and Fig 5.15 where wt-MCMV, Δ M27-MCMV and, Δ M34-MCMV vaccinated mice have MCMV-specific IgG present even at 3 dpc, as they have B cells which were raised prior exposure to MCMV. Meanwhile, PBS exposed mice do not have these MCMV-specific IgG-producing memory B cells.

These produced antibodies would bind their recognised MCMV antigens, at which point NK cells bind the Fc region of these antibodies to then kill infected cells by opsonization.

In Fig. 5.8, Fig. 5.9, Fig 5.10 and, Fig. 5.11, the ADCC assay detected similar levels of Fc-receptor activation between wt-MCMV and Δ M34-MCMV infected mice at 21 dpi, with only

background activation detected at 3 dpi. Most likely, this is because B cells specific to MCMV antigens have not been produced yet.

Due to limitations in sera volume, this experiment could not be repeated in vaccinated mice, but one could hypothesise that if this experiment was to be conducted, high levels of Fc-receptor activation would be observed in MCMV, Δ M27-MCMV, and Δ M34-MCMV vaccinated mice at both 3 dpc and 21 dpc.

This is all further supported in Fig. 5.12, where antibodies raised by MCMV, Δ M27-MCMV, and Δ M34-MCMV infected mice largely recognise similar antibodies as shown by western blot.

And thus, despite being attenuated by two differing methods (impairment and suppression), Δ M27-MCMV and Δ M34-MCMV both show similar correlates of protection.

6.9 Application of a Δ UL34-HCMV Attenuated Virus

As has been demonstrated in this thesis, Δ M34-MCMV is an impaired attenuated MCMV mutant which raises sufficient immunity to protect mice against a challenge infection, thus proving to be an effective vaccine in terms of MCMV. With the knowledge that HCMV lacking the homologous *UL34* gene has also been shown to be attenuated \sim 100-fold (Turner et al., 2022) this points towards a very promising HCMV vaccine candidate for the future in a Δ UL34-HCMV attenuated virus.

This vaccine would have many applications (beyond the obvious advantage of a vaccination program reducing the incidence rate of HCMV infections worldwide).

Importantly, seronegative women of childbearing age would benefit greatly from the introduction of this vaccine. This would reduce the risk of cCMV to the infant, and associated complications and mortality.

As an attenuated virus mutant, it would likely be unsafe for vaccination of immunosuppressed individuals, however a successful Δ UL34-HCMV would be of great interest to seronegative patients anticipating immunosuppressive therapy in the future, for example, patients on a waiting-list to receive an organ transplant as an added layer of security.

6.10 Potential of a ‘Double Knock-Out’ Vaccine

The development of a ‘double knock-out’ variant of these attenuated viruses (i.e., a $\Delta M27$ - $\Delta M34$ -MCMV) could also be of interest in the future. On the one hand, this may be beneficial to immunosuppressed individuals as it has been found that a knockout $\Delta UL145$ -MCMV (the human analogue of $\Delta M27$ -MCMV) may not sufficiently attenuated to be a perfect vaccine candidate by itself. This is because $\Delta M27$ -MCMV has been found to still result in the death of Severe Combined ImmunoDeficient (SCID) mice (Abenes et al., 2001).

On the other hand, the possibility that eliminating a gene important for defence against the host’s immune response ($\Delta M27$ -MCMV) as well as impairing the virus ($\Delta M34$ -MCMV) may prove to be too much, and perhaps attenuate the virus to the point that is no longer replication competent *in vivo*. The virus could still be cultivated *in vitro* however through the use of ruxolitinib as we have demonstrated, as this would eliminate the attenuating effect of lacking the *M27* gene.

This would be a problem that has been seen before in our research group (Le-Trilling, Jagnjic, et al., 2023) where for example UV-exposed MCMV does not confer protection against challenge infections when used as a ‘vaccine’. This may also have been the case in the failed ALVAC-CMV (vCP139) vector vaccine, where virus-neutralising titres were not raised, likely due to insufficient replication stemming from the canarypox vector’s inability to replicate in human cells (Adler et al., 1999). Contradictorily, however, replication incompetent $\Delta M94$ -MCMV was previously found to raise neutralizing antibodies and T-cell responses. Further investigation found that the virus was confined to the initially infected cell (Mohr et al., 2010). Taken together with our UV-MCMV data this may suggest that intercellular replication without viral release is sufficient to raise a protective immune response.

It could certainly be worth considering, however, as if it did successfully protect against challenge infections and was even more attenuated than $\Delta M34$ -MCMV then it may have a lower risk of potential side effects in patients in the future. Though this could perhaps simply be overcome through the use of a lower vaccination dose of $\Delta M34$ -MCMV.

Overall, this work has described two potential MCMV vaccine candidates through their potential to confer protection in mouse models. These are strong candidates for future translation into a human context.

6.11 Potential Use as a Vaccine Vector

As discussed previously, the use of HCMV as a viral vector is being researched. A replication incompetent CMV is favourable in this role due to the reduced risk of side effects, and as discussed in Chapter 6.8 $\Delta M94$ -MCMV is capable of raising protection against an embedded gene despite being replication incompetent.

As also discussed in Chapter 6.10, there is a risk that a $\Delta M27$ - $\Delta M34$ -MCMV double knockout ($\Delta U145$ - $\Delta U134$ -HCMV in the case of HCMV) may be too attenuated to raise an immune response due to no replication. If this was the case, the double knockout could then see use as a vaccine vector.

A problem with this would be that if the virus is replication incompetent, then it could not be produced in bulk for development of the vaccine. However, this limitation could be overcome by using a STAT2KO cell line to 'override' the attenuation provided by the missing *M27* gene as the function of pM27 as a STAT antagonist would be unnecessary. With this, we would have a useful CMV viral vector which would be replication incompetent in its host but be capable of replicating *in vitro* for mass production of the vaccine. It is also worth noting here that an alternative option could also be to cultivate the virus in culture medium containing ruxolitinib.

This 'win-win' situation makes the double-knockout $\Delta M27$ - $\Delta M34$ -MCMV an even more attractive knockout mutant to develop. It will either serve as an even more attenuated and thus safer vaccine, or will be attenuated to the point that it will become a promising vaccine vector candidate.

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8. Supplementary Information

8.1 List of Abbreviations

°C	Temperature in Degrees Celsius
Ab	Antibody
ADCC	Antibody Dependant Cellular Cytotoxicity
APC	Antigen-Presenting Cell
APS	Ammonium Persulphate
APS	Ammonium Persulphate
BALB/c	Bagg Albino Mouse Strain
BCR	B-Cell Receptor
cCMV	Congenital Cytomegalovirus
CD	Cluster of Differentiation
CIM	Crisis-Immortalised MEF
CMV	Cytomegalovirus
DDB1	DNA-Damage Binding Protein 1
DI	Deionised
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dpc	Days Post Challenge
dpi	Days Post Infection
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FBS	Foetal Bovine Serum
Fc	Fragment crystallizable
G	Gravity
GAM	Goat Anti-Mouse
gB	Glycoprotein B

GFP	Green Fluorescent Protein
gH	Glycoprotein H
gL	Glycoprotein L
HCl	Hydrochloric Acid
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency Virus
i.p.	Intraperitoneal
IE1	Immediate-Early 1
IE2	Immediate-Early 2
IFN	Interferon
IgG	Immunoglobulin G
IL-2	Interleukin 2
IRepG	Interferon-Repressed Gene
ISG	Interferon-Stimulated Gene
ISRE	Interferon Stimulated Response Elements
JAK	Janus Kinase
LCMV	Lymphocytic Choriomeningitis Virus
mAb	Monoclonal Antibody
MAVS	Mitochondrial Antiviral Signalling Protein
MCMV	Murine Cytomegalovirus
MEF	Mouse Embryonic Fibroblasts
MEM	Minimum Essential Medium
MF59	Microfluidized Adjuvant 59
MHC	Major Histocompatibility Complex
mL	Millilitre
mM	Millimole
mm	Millimetre
MNC	Mouse Embryonic Newborn Cells
MOI	Multiplicity of Infection
mRNA	Messenger RNA
NaCl	Sodium Chloride
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NK	Natural Killer

nm	Nanometre
OD	Optical Density
oriLyt	Origin of Lytic Replication
PAA	Polyacrylamide
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PBS-T	Phosphate-Buffered Saline with Tween
PCR	Polymerase Chain Reaction
PenStrep	Penicillin/Streptomycin
PFU	Plaque Forming Units
pH	power/potential of Hydrogen
PMSF	Phenylmethylsulphonyl Fluoride
POD	Peroxidase
pp65	65 kDa Phosphoprotein
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene Fluoride
RCMV	Rat Cytomegalovirus
RhCMV	Rhesus Cytomegalovirus
RNA	Ribonucleic Acid
RPM	Rotations Per Minute
RPMI	Roswell Park Memorial Institute (Medium)
SCID	Severe Combined ImmunoDeficient
SDS	Sodium Dodecyl Sulphate
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of Interferon Genes
TBST	Tris-Buffered Saline with Tween
TCR	T-Cell Receptor
TEMED	Tetramethylethylenediamine
TLR	Toll-Like Receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TRE	Transcriptional Repressive Element
ULBP	Unique Long (UL)16-Binding Protein
v/v	Volume/Volume
w/v	Weight/Volume

wt	Wild-Type
β -ME	2-Mercaptoethanol

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9. Acknowledgements

The acknowledgements are not included in the published version.

10. Declarations

Declaration:

In accordance with § 6 (para. 2, clause g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I represent the field to which the topic “A Comparative Analysis of Humoral Immune Responses Raised by Suppressed and Impaired Mouse Cytomegalovirus Mutants” is assigned in research and teaching and that I support the application of Alan Herdman.

Essen, date _____

_____	_____
Name of the scientific supervisor/member of the University of Duisburg-Essen	Signature of the supervisor/member of the University of Duisburg-Essen

Declaration:

In accordance with § 7 (para. 2, clause d and f) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I have written the herewith submitted dissertation independently using only the materials listed, and have cited all sources taken over verbatim or in content as such.

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In accordance with § 7 (para. 2, clause e and g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I have undertaken no previous attempts to attain a doctoral degree, that the current work has not been rejected by any other faculty, and that I am submitting the dissertation only in this procedure.

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Signature of the doctoral candidate

11. Curriculum Vitae

The curriculum vitae is not included in the published version for the purpose of data protection.