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Metabolic programming in murine cytomegalovirus infected macrophages

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Declaration

I hereby declare that this thesis is of my own composition, and it contains no material previously submitted for the award of any other degree. The work reported in this thesis has been executed by myself, except where due acknowledgment is made in the text.

Konstantinos Kotzamanis

Abstract

Immunity and metabolism have been viewed as separate fields, however recent evidence show that these two systems are intimately integrated, share resources and cross-regulate each other. Activated immune cells have to alter their metabolism in order to support effector functions. On the other hand, viruses are obligatory parasites that counter and exploit host pathways, including metabolism, to effectively propagate. Like immune cells, viruses have to alter the metabolic profile of infected cells in order to propagate.

The regulation of metabolism in immune cells or virally infected cells has been well studied. However, the precise metabolic regulation that ensues when both immune system and viral infection in immune cells interact and compete for the limited resources and metabolic pathways available is not clear. In this thesis, I have sought to investigate the integrative process by studying the metabolic programming of macrophages infected with murine cytomegalovirus (MCMV)

The central hypothesis of this thesis is that productive infection of macrophages by MCMV takes advantage of the early inflammatory metabolomic reprogramming of activated macrophages to establish infection, and modulates metabolism at late stages of infection towards fatty acid (FA) production to promote viral progeny.

To study this interaction, I have analysed the temporal profile of the transcriptome and metabolome of bone marrow derived macrophages (BMDM) infected with productive (WT) and non-productive (attenuated) (MCMV) strains. This aimed to unravel the host-directed versus virus-driven metabolic alterations.

I show evidence indicating that during early times of productive and non-productive MCMV infection glycolysis is, in infected BMDM, markedly increased. Furthermore, pharmacological and siRNA mediated inhibition of glycolysis resulted in attenuation of viral growth demonstrating the dependency of MCMV on this pathway. Additionally, using interferon receptor A (IFNAR) and interferon receptor A (IFNB) deficient BMDM showed that type-I interferon (IFN) signalling is essential for the early upregulation of glycolysis that was observed. In addition to the changes in

glycolysis, MCMV infection alters the tricarboxylic acid (TCA) cycle in infected BMDM. Metabolomic and transcriptomic data revealed a shift from catabolic to anabolic function for the TCA to promote production of TCA intermediates. Finally, the urea cycle is also altered both on transcriptional and metabolomic level, consistent with the support of Nitric oxide (NO) production which is a hallmark metabolite in classically activated macrophages.

These changes observed in the TCA cycle and glycolysis are consistent with supporting the FA elongation pathway during late time points of productive infection. Only productive MCMV infection upregulates this pathway. At the same time, pharmacological and siRNA mediated inhibition of FA elongation pathway greatly attenuates viral growth. This indicates that MCMV growth is dependenton FA elongation. The effect was very specific for the elongation and not the *de novo* synthesis pathway indicating that MCMV remodels FA that already in the cells. It is argued, that in agreement to known literature, MCMV uses these FA for the formation of its lipid membrane.

To further investigate the dependency of MCMV on FA elongation pathways I studied additional lipids pathway associated with the former. I found that MCMV infection also upregulates the triacylglycerol formation and membrane remodelling pathways, which are dependent on FA biosynthesis and elongation. The inhibition of triacylglycerol formation and membrane remodelling pathway also attenuated MCMV growth. This indicates that apart from the formation of its lipid membrane MCMV requires FA to remodel the cellular environment.

I have also explored the effects of infection on regulating lipid mediators, in particular eicosanoids. Eicosanoids are lipid signalling molecules that can act as potent inflammation modulators. Here I demonstrated that productive MCMV infection specifically increases PGE_2 production in infected BMDM. Moreover, addition of PGE_2 increased viral replication in infected fibroblasts in comparison to non-treated cells, while pharmacological blocking of EP4 (PGE_2 receptor) rescued the phenotype. These studies reveal how MCMV advantageously use inflammatory lipid pathways to promote growth

In conclusion, the data presented in this thesis support my hypothesis and provide an insight in the role of metabolism during viral infection. Evidence is provided to show that MCMV co-ops the early alterations that metabolic pathways undergo in activated macrophage, including but not limited to glycolysis, TCA cycle and urea cycle. These early changes in metabolism appear to be coupled with upregulation of FA elongation pathways and remodeling of lipids in infected cells. Finally, MCMV co-ops the function of regulatory lipids, in particular PGE_2, to promote viral growth. It is further argued that MCMV productive infection dictates these fatty acid metabolism alterations in order to remodel the host cell's environment, regulate the immune system response and provide resources for its lipid membrane.

Layman Abstract

The immune system protects the host from infections. Recent advances in the field of immunology have shown that when immune cells are activated they alter their metabolomic profile/metabolic state. This has been shown to be crucial to support their immune effector functions. The alterations of metabolism allow immune cells to accumulate more energy and nutrients that allow them, among others, to replicate and generate cytokines, a class of proteins necessary for immune cells to communicate with each other. On the other hand, viruses are obligate parasites that need to use the host's cellular machinery to replicate. They have evolved multiple ways to co-op, supress and evade the immune response of the host. Multiple studies have shown that ~~for~~ various viruses alter the metabolism of infected cells to support their own growth.

In this thesis, I attempt to study the interaction between host immune system-viral infection-and metabolism. By infecting immune cells with virus, I uncovered a link between the metabolism of immune cells and the metabolism of virally infected cells immune cells metabolism and viral infected cells metabolism. Murine cytomegalovirus, the virus that I use as a model throughout my thesis, was shown to co-opt the metabolism of activated macrophages to establish infection in the cells.

Moreover, it is revealed that fatty acid (FA) biosynthesis is crucial for generation of viral progeny. Inhibition of FA biosynthesis and/or other enzymes that are involved in lipid remodelling in cells, block viral growth. Although it is not clear, there are some indications of how FA biosynthesis facilitates viral growth. It is hypothesised that alterations of lipid membrane content might support the assembly of virions in cells. Additionally, some of the FA synthesised might be crucial components of viral lipid membranes.

The results presented in this thesis provide insight into how metabolism is crucial in determining infection outcome. This set a paradigm for a new approach in therapeutics for infectious diseases.

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Common Abbreviations

2-DG	2-Deoxyglucose
BMDM	Bone marrow derived macrophage
DC	Dendritic Cell
ELOVL	Elongation of very long chain fatty acids
FA	Fatty acids
FCS	Fetal calf serum
GFP	Green Fluorescence Protein
HCMV	Human cytomegalovirus
HFF	Human Foreskin fibroblasts
HIF-1α	hypoxia-inducible factor 1
hpi	Hours post infection
IE	Immediate early
IFN	interferon
IRF3	IFN-regulatory factor 3
IRF7	IFN-regulatory factor 7
JAK	Janus kinase
LD	Lipid droplets
LPS	Lipopolysaccharides

MBOAT	Membrane bound O-acyl transferase
MCMV	Murine cytomegalovirus
MIEP	Viral Major Immediate Early Promoter
MOI	Multiplicity of infection
МΦ	Macrophage
NF-kB	Nuclear Factor-κB
NO	Nitric Oxide
PCA	Principal Components analysis
PFU	Plaque forming unit
PG	Prostaglandin
PGE_2	Prostaglandin E2
PPP	Pentose phosphate pathway
PRR	Pattern recognition receptor
TCA cycle	Tricarboxylic acid cycle
TF	Transcriptional Factor
TLR	Toll-like receptor
VLCFA	Very long chain fatty acids

1 Chapter 1 Introduction

1.1 Characteristics of Cytomegalovirus

Cytomegalovirus (CMV) is a double-stranded enveloped DNA virus, which belongs to the Herpesviridae family. Its name is derived from two Greek words; cyto which means cell and megalo which means big. The name is a reference to the characteristic morphology that CMV infected cells exhibit, which become enlarged and rounded. Human CMV (HCMV) is the human adapted strain and it is ubiquitous in human population with infection rates ranging from 40-100% depending on socio-economic background, race/ethnicity, geographic location, age (Cannon, Schmid, and Hyde 2010).

Infection with HCMV usually goes unnoticed in healthy individuals, but it has been related to long term health risks (Reinhardt et al. 2006). After infection, HCMV establishes a lifelong infection that is not life-threatening to immunocompetent individuals. However, the virus can cause life threatening infections in immunocompromised individuals, such as HIV infected patients and transplant recipients (Crough and Khanna 2009; Griffiths and Grundy 1987). HCMV also poses a serious threat to neonates and foetuses. Congenital infection with HCMV can result in death, or serious defects, including hearing loss and microcephaly (Gerna, Baldanti, and Revello 2004; Burny et al. 2004).

1.2 Murine cytomegalovirus as a model for HCMV

One of the characteristics of HCMV is its high species specificity, which prevents *in vivo* studies of HCMV using animal models. For this reason, several other CMV strains have been used in animal models, with murine cytomegalovirus (MCMV) being the most established model. Despite their genetic differences, HCMV and MCMV share a lot of biological and pathological similarities (Schleiss 2013; M. J. Reddehase et al. 2008). The two viruses have about 70% genomic sequence homology, they have similar virion structure, viral life cycle, cell tropism and interact with the host in a

similar way (Krmpotic et al. 2003; Matthias J Reddehase, Podlech, and Grzimek 2002).

As stated above HCMV is a clinically important pathogen from immunocompromised individuals, to neonates and foetuses. Recipients of organs and HIV infected people are also in danger because the latent virus can opportunistically reactivate under unspecified conditions. Additionally, it has been observed that secondary reactivation of the virus during pregnancy leads to less severe effects to the foetus than congenital infection does (Arvin et al. 2004; Stratton et al. 1999). Finally, there are a lot indications linking HCMV infection to heart disease (Nieto et al. 1999; Lenzo et al. 2002). For these reasons, a great deal of research is dedicated to studying the mechanisms through which the immune system pushes the virus into latency and how it reactivates, as well as for the development of a vaccine in an attempt to curb HCMV.

One of the characteristics of HCMV, and other cytomegalovirus species, is the high host species specificity. This readily reduces our ability to perform *in vivo* studies with HCMV and thus some of the most important aspects of the viral life cycle cannot be studied extensively. Studying HCMV can only be done using human immortalised cell lines that are permissive to the virus or recruiting human volunteers that donate primary cells. This latter procedure is very time consuming due to the ethical issues that arise and the difficulty in finding suitable donors. Moreover, host genetics that are important to viral infections and cannot be standardised as easily when human donors are recruited(Nolan, Gaudieri, and Mallal 2006). To circumvent the difficulties in studying HCMV infection, the murine model system and MCMV infection has been extensively used. In its favour, an extensive number of knockdown mice models exists, allowing studies to better understand the role of different host factors in MCMV infection. Finally, the development of human vaccines is to date practically impossible and any effects of CMV immunisations should ideally, be studied in animal models first (Schleiss 2006).

For these reasons, several other CMV strains have been used in animal models, with murine cytomegalovirus (MCMV) being the most established model to study HCMV infection. Despite their genetic differences, HCMV and MCMV share a lot of biological and pathological similarities (Schleiss 2013; M. J. Reddehase et al. 2008).

The two viruses have about 70% genomic sequence homology, a similar virion structure, viral life cycle, cell tropism and they interact with the host in a similar way (Krmpotic et al. 2003; Matthias J Reddehase, Podlech, and Grzimek 2002). Moreover, the immune system of the mouse is one of the most well-defined, making the murine model of the infection very attractive for host-virus studies. For these reasons, the use of an animal model in the study of HCMV infection is not just useful, but necessary. MCMV is the most extensively studied cytomegalovirus and the mouse is the most extensively studied animal models making the use of this system to study HCMV infection one of the most suitable.

1.3 MCMV and HCMV infection cycle

1.3.1 Viral entry

MCMV and HCMV share similar host cell entry mechanisms. The entry of HCMV is facilitated through the interaction of the viral glycoprotein complexes gB, gM, gL, gO and gN, on the surface of the virion with cellular receptors (Compton, Nepomuceno, and Nowlin 1992; Xin Wang et al. 2005; Marisa K Isaacson and Compton 2009). HCMV entry in fibroblasts occurs through direct fusion, or endocytosis into epithelial and endothelial cells (Ryckman et al. 2008; Compton, Nepomuceno, and Nowlin 1992). MCMV infects fibroblasts in a similar manner as HCMV, by using the m74 encoded protein, which is homologous to the gO of HCMV (Scrivano et al. 2010).

After the virus enters the cell, the capsid is internalized and migrates to the nucleus with the help of tegument proteins associated with microtubules (Kalejta 2008; Li, Chen, and Cristea 2013; Cristea et al. 2010; Bresnahan and Shenk 2000).

1.3.2 Viral gene expression

In the nucleus, the viral gene transcription begins. Viral transcription is a tightly regulated process and the genes involved can be organised into 3 categories based on the time of their transcription; immediate early, early and late genes (Keil, Ebeling-Keil, and Koszinowski 1987). Genes that do not require *de novo* viral protein synthesis are defined as immediate early genes. Genes that require *de novo* viral protein

synthesis, but not viral genome replication are categorised as early genes, and finally genes that require both *de novo* protein synthesis and viral gene replication are late genes.

The immediate early genes IE1 and IE2 do not require *de novo* protein synthesis and begin transcription immediately (Greaves and Mocarski 1998; Iskenderian et al. 1996; Meier and Pruessner 2000). Their functions include acting as master transcriptional regulators of viral gene expression, promoting viral DNA replication, while also altering the host intercellular environment making it suitable for viral replication by arresting cell cycle, inhibiting apoptosis and the host immune response (Bresnahan et al. 1996; Castillo, Yurochko, and Kowalik 2000)

Following the replication of viral DNA and the transcription of late viral proteins, the DNA is packed in capsids. The capsids get an initial envelopment as they pass through the nuclear envelope to the cytoplasm. In the cytoplasm mature virions with the addition of tegument proteins are formed followed by secondary envelopment (Das, Vasanji, and Pellett 2007; AuCoin et al. 2006; Sanchez et al. 1998, 2000). Finally, the infectious virions are released from the cell.

1.3.3 Latency

Herpesviruses establish latency in their host, during which, viral DNA exist in the host cells, but it does not produce infectious virions (M. J. Reddehase et al. 2008; Redpath et al. 2001). MCMV and HCMV can establish latency in multiple sites. Latent MCMV infections have been shown to occur in lungs, heart, kidneys salivary glands and other body organs (Kurz et al. 1997; Matsuzawa et al. 1995; Koffron et al. 1998; M J Reddehase et al. 1994), but for both pathogens the myeloid cells are considered an important site of latency and reactivation in these tissues (Pollock et al. 1997; Sinclair 2008; Mendelson et al. 1996). Reactivation of CMV can occur after latency and represents a serious threat in immune compromised patients.

The precise reactivation mechanisms for MCMV and HCMV are not yet known, but is generally believed to involve the host immune system and multiple signalling pathways (Sinclair and Sissons 2006; Poole, Wills, and Sinclair 2014). Interestingly,

there appears to be a requirement for latently myeloid cell to reach terminal differentiation to support productive replication(Lucin et al. 1992; Sinclair and Sissons 2006).

1.4 Immune response to cytomegalovirus infection

1.4.1 Pattern recognition receptors and CMV detection by the immune system

The innate immune system is the non-specific defence system of the host that provides broad non-specific protection against pathogens. Evolutionarily, it is the first form of immunity that appeared and it can be found in plants, fungi, and insects. Until recently it was considered that unlike the adaptive immunity, which is pathogen specific with immunological memory, the innate immunity does not confer long lasting host protection against the same pathogens. This dogma has started sifting with the discovery of trained innate immunity (Saeed et al. 2014).

In mammals, the innate immune system includes, chemicals barriers, physical barriers (such as skin), innate immune cells (e.g. macrophages) and components of humoral immunity, such as the complement system. After the innate immune system is activated it drives the adaptive system response to clear an infection.

Cells of the innate immune system can recognise pathogens through pattern recognition receptors (PRRs). These are receptors, which have evolved to specifically bind to pathogen-derived molecules that are not found in the host. The existence of PRRs was proposed by Charles Janeway in 1989, in the Infectious non-self-model (Janeway 1989). Today numerous PRRs that recognise different pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) have been discovered (Thompson et al. 2011).

Lipopolysaccharide (LPS) is a lipoglycan produced specifically by gram-negative bacteria, and it is detected by Toll-Like receptor-4 (TLR4) (Akira and Takeda 2004; Poltorak et al. 1998). As LPS is only found on the membranes of gram-negative bacteria and not eukaryotic cells, the detection of LPS by TLR4 indicates the presence of bacteria, and subsequently the immune is activated to mount a response.

1.4.2 Toll-like receptors (TLR)

Toll-like receptors (TLRs) are the most well studied and evolutionarily conserved PRR protein family and were first discovered in *Drosophila melanogaster* (Kluding et al. 1985). TLRs are named after the Toll receptor that confer similar function in *Drosophila* (Hansson and Edfeldt 2005). In mice, there are 13 TLRs, with TLR 10 having lost its function due to a retrovirus insertion (Kawai and Akira 2010). In humans, only 10 TLRs exist, with TLRs 1-9 being conserved in both mouse and humans (Kawai and Akira 2010). All TLRs are transmembrane proteins with leucine rich motifs that act as binding sites for PAMPs and an intracellular Toll–interleukin 1 (IL-1) receptor (TIR) that transduces the signal (Kawai and Akira 2010; L. A. J. O'Neill, Golenbock, and Bowie 2013).

After the engagement of TLRs by PAMPs the signal is transduced using two main pathways; the Myeloid differentiation primary response gene 88 (MyD-88) pathway and the TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway. The MyD-88 dependent pathway is used by all TLRs, except TLR3, and the TRIF pathway is used by TLR3 and TLR4. The MyD-88 pathway results in the activation of nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) pathways, which induces the production of proinflammatory cytokines and eventually type-I IFN. The TRIF pathway activates IRF3 and/or IRF7 depending on the signalling TLR and leads to the production of type-I Interferon (IFN), which then activate the transcription of numerous inflammatory genes (L. A. J. O'Neill, Golenbock, and Bowie 2013; Akinori Takaoka and Yanai 2006; Akira and Takeda 2004; Kawai and Akira 2010).

HCMV can be detected at the surface of the cell by TLR2 (Boehme, Guerrero, and Compton 2006; Compton et al. 2003). TLR2 detects hydrophobic PAMPs such as lipoteichoic acid, but it is not clear which component of the virus it detects (Botos, Segal, and Davies 2011). Antibodies specific for gB and gH can block this interaction. Thus, it is assumed that TLR2 detects hydrophobic peptides in these proteins (Boehme, Guerrero, and Compton 2006). TLR2 has been shown to be important in MCMV immunity too, with mice lacking TLR2 showing elevated levels of the virus (Szomolanyi-Tsuda et al. 2006).

After entry of CMV in the cell, the virus can be detected by TLR9, which is located in endosomes. TLR9 detects DNA containing unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs, which are abundant in viral and bacterial DNA, but not vertebrate (Hemmi et al. 2000). TLR9 activates IRF7, which leads to rapid induction of type-I IFN expression, both in humans and mice. Studies have provided evidence that both human and mouse CMV are detected by TLR9 (Varani et al. 2007; Tabeta et al. 2004). Unlike mice though, TLR9 expression in humans is restricted to B cells and plasmacytoid DCs (pDCs) (Takeuchi and Akira 2010). The latter are renowned for their ability to produce huge amounts of type-I and contribute significantly to the antiviral response during CMV infection (Zucchini, Bessou, Robbins, et al. 2008). TLR9 deficient mice infected with MCMV exhibited reduced production of IFN-γ, type-I IFN, higher viral loads and were more susceptible to MCMV infection than what WT mice were (Delale et al. 2005).

CMV is also detected by TLR3 and TLR7. TLR3 is located in endosomes and can detect dsRNA, as challenge with poly(I:C) has demonstrated (Alexopoulou et al. 2001). Nahum *et al.* has demonstrated that TLR3 plays an important role in HCMV immunity by studying a the TLR3 receptor variant, L412F (Nahum et al. 2012). At the same time MCMV infected TLR3 deficient mice have been shown to have greatly augmented viral load in their spleen (Tabeta et al. 2004). TLR7 can detect ssRNA and it is, like TLR3, located in endosomes (Nahum et al. 2012). Zucchini *et al.* demonstrated that TLR7 and TLR9 have many overlapping functions during MCMV infection (Zucchini, Bessou, Traub, et al. 2008). In this study it was demonstrated that when TLR7 or TLR9 deficient mice were challenged with MCMV the level of multiple cytokines in serum were similar for both mice strains(Zucchini, Bessou, Traub, et al. 2008).

In summary the human and mouse CMV have been shown to be detected by TLR2,3,7, and 9. Despite the fact that the signalling patterns in these molecules might appear overlapping and redundant, the differences in cell localisation and pathways through which they signal might contribute to slight adaptations of immune signalling allowing the immune system to respond to the plethora of microbes (Takeuchi and Akira 2010).

1.4.3 RIG-I-like receptors

After the discovery of TLRs it became clear that they are not the only PPRs in cells. TLR3 deficient cells for example are capable of detecting dsRNA, indicating the existence of more PRRs (Yamamoto 2003; Hoebe et al. 2003). In addition to TLRs there are other PPRs in cells, such as NOD-like receptors (NLR), C-type lectin receptors (CLR), and RIG-I-Like receptors (RLR). RLR are a family of receptors which consist of three members; retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (Wilkins and Gale 2010) .

The members of this family have a DExD/H- box RNA helicase domain, which is used to bind to the RNA. Additionally, RIG-I and MDA-5 have a Caspase activation and recruitment domain (CARD) that is involved in the downstream signalling after the activation (Yoneyama et al. 2005; Takahasi et al. 2008). RLRs reside in the cytoplasm and their activations results in the activation of the Interferon regulatory factor-3 (IRF3), NF-κB and the downstream activation of type-I IFN (Wilkins and Gale 2010; Yoneyama et al. 2004). Further studies have demonstrated that MDA-5 preferentially detects dsRNA while RIG-I recognizes 5-triphosphorylated, uncapped ssRNA (Kato et al. 2008; Sachidanandam 2011). It is not clear if MCMV or HCMV are recognised by RLRs, but Scott et al. has demonstrated that RIG-I is targeted for proteosomal degradation by HCMV(Scott 2009).

1.4.4 Absent in melanoma 2 receptor

Given that cytomegaloviruses are DNA viruses it is expected that they would mainly be detected by DNA sensors. Absent in melanoma 2 (AIM-2) is a dsDNA cytosolic PRR that can recognise host and pathogen DNA in the cytosol through an interaction with its HIN domain. Its activation stimulates the formation of caspase-1 inflammasome in an apoptosis-associated speck-like protein (ASC) depend manner(Hornung et al. 2009).

The formation of the inflammasome is critical for the maturation or pro-IL-1β and pro-18 during MCMV infection *in vitro*. The expression of both cytokines is induced by

TLR activation, but caspase-1 inflammasome activation is critical for their maturation (Rathinam et al. 2010). It is tempting to infer that *in vivo* AIM-2 activation is also required for inflammasome activation and maturation of IL-1β because of the findings *in vitro*. However, the mechanism through which viral DNA reaches AIM-2 and activates it not yet defined(Marc; Dalod 2013). Critically, absence of AIM-2 in MCMV infected mice reduces the number of IFN-γ producing NK cells which is critical for driving the virus in latency and controlling the infection(Rathinam et al. 2010).

1.4.5 Dependent activator of interferon regulatory factor

DNA Dependent activator of interferon regulatory factor (DAI) is a cytosolic DNA sensor that upon activation interacts with TANK-binding kinase 1 (TBK1) which leads to type-I IFN activation in cells (Takaoka et al. 2007). Defillips *et al.* reported that HCMV is detected by cytoplasmatic dsDNA sensors, and more specifically the ZBP1 (also known as DAI, DNA-Dependent activator of interferon regulatory factor) (DeFilippis, Sali, et al. 2010; DeFilippis, Alvarado, et al. 2010).

1.4.6 Type I, II & III IFN antiviral response

1.4.6.1 Type-I interferons

Interferons (IFN) are secreted cytokines that can function in a paracrine and autocrine manner, inducing the transcription of numerous genes as part of the immune system response. There are three interferon families. The family of type-I interferon family in humans consists of 13 homologous IFN-α proteins, one IFN-β protein and IFN-δ, IFN-ζ, IFN-ε, IFN-κ, IFN-τ and IFN-ω, which are poorly characterised and understood. Type-II interferon has only one member which is IFN-γ and finally the type-III IFN family comprises IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4 (Pestka, Krause, and Walter 2004; Platanias 2005).

Upon detection of a pathogen by various PRRs, type-I IFNs are produced *in vivo* and *in vitro*, resulting in the induction of the transcription of multiple genes termed interferon stimulated genes (ISGs) that have potent antiviral, immunomodulatory and anti-

proliferative properties (Platanias 2005). Type-I interferons are produced and recognised by almost all cell types (Piehler et al. 2012).

Secreted type-I IFNs are detected by a common cell surface receptor cell surface receptor called IFN-α receptor (IFNAR), which consists of two subunits, IFNAR1 and IFNAR2. Engagement of the IFNAR leads to the activation of the receptor associated tyrosine kinases, Janus kinase 1 (JAK1) and TYK2. These kinases phosphorylate the signal transducer and activator of transcription (STAT) proteins, STAT1 and STAT2. Phosphorylated STAT1 and STAT2 dimerise and associate with IRF9 to form a complex called the ISG factor 3 (ISGF3) (Stark and Darnell 2012). The ISGF3 complex binds to the IFN-stimulated response elements (ISREs) on the promoter regions and promotes the transcription of interferon-stimulated genes (ISGs). ISGs have a plethora of antiviral, immunomodulation and anti-proliferative properties that shape the immune response to various pathogens (Stark and Darnell 2012). This order of events is known as the canonical type -I IFN signalling pathway. More recently, however, it was found that type-I interferons can signal through different pathways, which could explain the broad effects of IFN-I (McNab et al. 2015). For example, type-I interferon detection by cells can also lead to the formation of STAT1 homodimers, which are usually associated with IFN-γ signalling.

The regulation of this type of immune response is governed by multiple feedback loops and it has been observed that infection by different viruses activates different gene sets (M. S. Diamond and Schoggins 2013). Additionally, the ability of type-I IFNs to activate immune cells and to induce different ISGs depends on the type of IFN, their binding affinity and concentration, adding to the complexity of the system (Levin et al. 2014; Lavoie et al. 2011; Levin, Harari, and Schreiber 2011).

After the MCMV has been detected, type-I interferons are secreted, which activates ISGs leading to the expression of more inflammatory cytokines. The lymphotoxin β receptor (LTβR) has been shown to contribute to this initial burst of IFNα/β production (Schneider et al. 2008). The first wave of the bi-phasal type-I IFN expression peaks at 8 hours and then declines, but it is crucial for restricting viral replication through various mechanisms including the enhancement of NK cells cytotoxicity (Loewendorf and Benedict 2010; Orr, Murphy, and Lanier 2010; Schneider et al. 2008). The

accumulation of type-I IFN increases again at 36 hours post infection and plasmacytoid dendritic cells (pDCs) appear to be its main source, as stated above(Marc Dalod et al. 2003; Zucchini, Bessou, Robbins, et al. 2008). Interestingly unlike other conventional DC (cDC) types (CD8+ cDCs and CD11b+ cDCs and MΦ), pDCs are not permissive to viral infection (Marc Dalod et al. 2003; Andrews et al. 2010; Marc; Dalod 2013).

The importance of type-I interferon in the control of CMV infection has been demonstrated by Strobl *et al*. In their study they show that defects in the JAK-STAT signalling pathway renders cells more susceptible to infection by MCMV (Strobl et al. 2005). Furthermore blocking IFN-α/β, or IFN-γ during MCMV infection *in vivo* increases viral titres

(Heise and Virgin 1995).

1.4.6.2 Type-II interferon

The only member of this protein family is IFN-γ, and it is structurally distinct from type-I IFNs. It binds to the interferon gamma receptor (INFGR), which is a transmembrane dimer of IFNGR1 and IFNGR2 (Pestka, Krause, and Walter 2004). IFNGR binds JAK1 and JAK2 at its intracellular domain, which upon ligation to IFNGR phosphorylate STAT1. STAT1 forms homodimers that translocate to the nucleus, where they bind at the Gamma interferon activation site (GAS) promoting the transcription of genes. There is some overlap between the genes induced by IFN-γ and IFN-I, but this is not always the case(Der et al. 1998).

IFN-γ can act on a wide range of cells that have the IFNGR. Two of the most important cell types affected by IFN-γ are T cells and macrophage (MΦ) (Schroder et al. 2004). In the case of macrophages, IFN-γ is critical for their activation due to its ability to induce transcription of microbicide encoding genes (J. D. MacMicking 2012). This has been illustrated in the case of *Listeria* infection, where IFN-γ is essential for the clearance of the parasite once it has been phagocytosed by MΦ (Soudja et al. 2014; S. H. Lee et al. 2013).

During CMV infection, IFN-γ appears to be one of the most important cytokines to control the infection. IFN-γ deficient mice are unable to control the infection and drive

the virus into latency (Presti et al. 1998). Despite its crucial role, IFN-γ on its own is not sufficient to control MCMV infection (Lucin et al. 1992). Studies in MCMV infected MΦ have demonstrated that IFN-γ induces a primed state on the type-I IFN network of the cells, which enables the establishment of an antiviral state capable of suppressing major immediate early promoter (MIEP) activity (K. a Kropp et al. 2011).

1.4.6.3 Type III Interferon

In mice, there are two type interferons IFNλ2/IL-28A and IFNλ3/IL-28B. In humans there are four members IFNλ1/IL-29, IFNλ2/IL-28A, IFNλ3/IL-28B, IFNλ4 (Kotenko et al. 2003). This last variant of IFNλ in humans, INFλ4 is frameshift variant of IL28B and it is associated with impaired HCV clearance (Prokunina-Olsson et al. 2013).

Type-I INFs and type-III IFN share a lot of common characteristics. Like type-I INFs type-IFN is induced by a number of pathogens including RNA and DNA viruses (Ank et al. 2006; Sheppard et al. 2003). Type-III Interferons signal through a unique receptor INFλR which is compromised or two chains IL10Rβ and IL28Rα (Sheppard et al. 2003). Finally, like the other IFN types type-III IFN are able to activate a large number of ISG which have a lot of potent anti-microbial properties.

The first main difference observed between type-I and type-III interferons is their expression pattern. While virtually all cell types produce type-I interferons and respond to them only a small subset of cells express and respond to type-III IFN. There is a compartmentalisation to type-III IFN response and it is increasingly believed that this interferon regulates immune responces specifically at mucosal areas (Kotenko and Durbin 2017; Odendall and Kagan 2015) .

1.5 Metabolism in infection and Immunity

1.5.1 Introduction

Immunity and metabolism have been viewed as separate fields for a long period of time. The association between the two fields was studied in the past, but only by a small number of groups, which showed metabolic adaptations in immune cells during activation (Newsholme et al. 1986). However, research in the field of

immunometabolism has over the last 15 years research exploded with studies clearly demonstrating a tight integration, sharing of resources and cross-regulation between the two systems (Pearce et al. 2013). In particular macrophages, among other immune cells, reprogram and tightly regulate their metabolism upon activation from IFN and/or TLRs (Van den Bossche, O'Neill, and Menon 2017). It has also become obvious that these changes are essential to support their effector functions.

On the other hand, viruses are obligate parasites that both exploit and counter selective host pathways, including metabolism, to effectively propagate. Numerous studies have demonstrated that viruses depend on alterations of metabolism in infected cells to propagate (Munger et al. 2006; Koyuncu et al. 2013; J.-Y. Seo, Yaneva, and Cresswell 2011; Munger et al. 2008; Vastag et al. 2011; D. L. Diamond et al. 2010). Moreover, recent publications from our group and others have revealed a central connection between infection and metabolism in regulating the host response (Blanc et al. 2013, 2011; Kotzamanis, Angulo, and Ghazal 2015).

Under the light of the accumulating evidence in immunometabolism and virology it is becoming apparent that metabolism, as a source of energy and metabolic products, is critical for the outcome of infection and/or other diseases. Here, I provide a summary of how HCMV infection modifies metabolism of the host cell; how specific immune cells, like MΦ, regulate their metabolism during activation; and how metabolic intermediates can regulate all three processes: infection, metabolism and the immune response. Throughout my thesis I attempt to place metabolism at the centre of an infection, metabolic and immune axis, which can determine the outcome of infection by intergrading signals both from host and pathogen.

A crucial and novel aspect of my work is employing an infectious agent in its natural host to study metabolism in immunity. Most of the studies presented below study immunometabolism in innate immune cells, usually, Dendritic cells (DCs), or Macrophages (MΦ), in the content of TLR and/or other cytokine activation. While this might be a sufficient model to describe the state of metabolism in immune cells during, perhaps some autoimmune diseases, it is not sufficient to describe it during viral infections. During viral infection innate immune cells have to intergrade signals that stem from multiple PRRs and cytokines at the site of inflammation. It has already been

stated that different viruses activate different set of immune response genes (M. S. Diamond and Schoggins 2013). It is, thus, not far-fetched to assume that based on that different immune cells, apart from their phenotype, might also alter their metabolism in subtle ways.

At the same time, most of these studies employ the use of LPS in combination with other cytokines to study immunometabolism. LPS is detected on the surface of gram-negative bacteria and engages TLR4. This TLR ligand is not a suitable for the emulation of viral infections, but rather only specific bacterial ones. Furthermore, even if another TLR ligand were employed to emulate viral infection it would not be sufficient. Some viruses can infect immune cells and affect their metabolism directly. Even if the viral infection of the cell does not result in productive infection the initial transcription of any genes and/or tegument proteins could affect the phenotype/metabolism of the cells.

In similar fashion, the studies of alterations in the metabolism of virally infected cells takes place usually outside of the context of an immune response. For this reason, these studies are not sufficient for describing the full range of events during infection. Viruses normally grow in the niche/environment/context of an immune response from the host. Thus, they have evolved mechanisms to counteract and subvert pathways including metabolic and immune signalling pathways. They have even evolved methods to counteract and exploit immune signalling (Bowie and Unterholzner 2008; Netterwald et al. 2005). Thus, studying the metabolism of infected cells outside of the context of an immune response might produce artefacts not encountered in a real infection.

1.5.2 Central carbon metabolism in cells

1.5.2.1 Glycolysis (glycolytic pathways)

Cellular metabolism is the set of sustaining biochemical reactions that take place within a cell. Through them a cell takes up and exports the metabolites that it uses to generate energy and to produce the chemicals that are essential for its survival. Central carbon metabolism is an integral part of metabolism and it is responsible for processing

carbon atoms derived from sugars (Noor et al. 2010). Figure 1.1 shows an overview of the Central carbon metabolism in a quiescent cell.

Although there are other pathways for the metabolism of sugars, the glycolytic pathway, which is responsible for the metabolism of glucose, is traditionally considered the most important and most common pathway that the description of most metabolic networks start with. The glycolytic pathway in cells starts with the import of glucose from the extracellular environment and its processing in the cytosol of the cell.

In a quiescent cell, glycolysis catabolises glucose to pyruvate through 10 metabolic steps. The generated pyruvate can then enter the Tricarboxylic acid (TCA) cycle, where it is further oxidised and used to produce energy through oxidative phosphorylation. For each glucose molecule that runs through the glycolytic pathway, two ATP molecules are "invested" by the cell to process glucose and four ATP molecules are generated as end product. This provides a net generation of two new ATP molecules, plus the regeneration of NADH from NAD^+. NADH is an important coenzyme in many biochemical pathways in cell, it is predominately used as a reductive agent and as a provider of protons for the electron transport chain (ETC) in the mitochondria.

Under normoxic conditions most of the glucose is turned into pyruvate. However, when cells are deprived of oxygen they cannot generate enough NAD^+. The inability to generate NAD^+ is detrimental to the cell as NAD^+ is an essential cofactor for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the enzyme that catalyses the sixth reaction in the glycolytic pathway. Thus, the lack of NAD^+ can inhibit glycolysis and deprive cells of essential molecules. To overcome this, cells reduce pyruvate to lactate in a reaction that oxidises NADH to NAD^+, which can then be used to keep glycolysis running. **Figure 1.1** demonstrates the Central carbon metabolism.

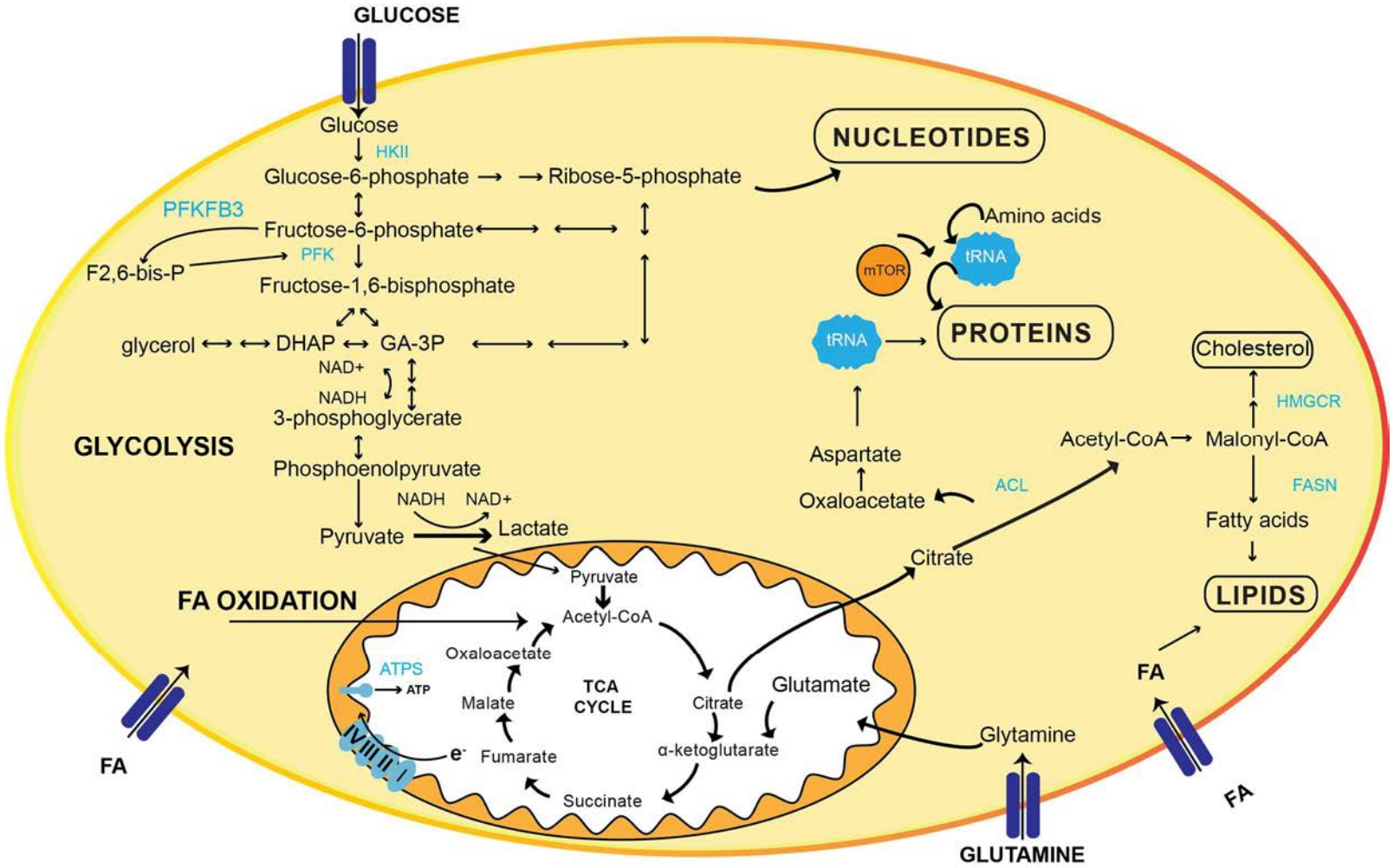


Figure 1.1 Overview of Cell Central Carbon metabolism. Glucose is imported into the cell and is oxidised via glycolysis or

the PPP. Glucose molecules oxidised via the PPP can either re-enter the glycolytic pathway or feed into other pathways to produce nucleotides. Glucose molecules oxidised via glycolysis are converted to pyruvate, which can either be reduced to lactate and generate NAD+ or get transported into the mitochondria and be oxidised via the TCA cycle. Pyruvate enters the TCA cycle to either be catabolised to produce energy via OXPHOS or can be utilised by other pathways anabolically. The TCA cycle can be replenished by glutamate if needed or via FA that can be oxidised in the mitochondrion to produce acetyl-CoA. Carbon atoms from pyruvate can be shuttled outside the mitochondrion via citrate to synthesise lipids like FA and cholesterol. Enzymes are indicated with blue colour. F2,6Bis-P: Fructose-2,6-Bisphosphatase; HK: Hexokinase; PFKFB3: 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase 3; ATPS: ATP synthase; ACL: ATP citrate lyase; HMGCR: 3-Hydroxy-3-Methylglutaryl-CoA Reductase; FASN: Fatty acid synthase; FA: Fatty Acid. Adopted and Modified from (Finley et al. 2013).

1.5.2.2 The Pentose Phosphate Pathway

The pentose phosphate pathway (PPP) (**Fig.1.2**) connects to the glucose pathway in two steps. Connection with the glucose pathway occurs at the first step, where D-glucose is converted to D-glucose-6P, and at the fifth step, where D-glyceraldehyde is produced. The pathway acts as an alternative pathway for glucose oxidation without generating ATP. Instead, the PPP accomplishes two other equally important functions:

1. It generates NADPH, which is crucial for the generation of fatty acids and the maintenance of the redox balance in the cytosol of cells through reduction of glutathione.
2. It generates D-ribose-5P, an important precursor for nucleotides synthesis.

The PPP pathway is composed of two branches. The first branch is the oxidative branch, which leads to the generation of NADPH and D-ribulose-5P from D-glucose-6P through three irreversible reactions. The second branch is the non-oxidative branch, which leads to the generation of D-ribose-5P. Because of the branching of the pathway and the fact that the reactions in the non-oxidative branch are reversible, the flux in the PPP can be modulated to better suit the needs of the cells (**Fig. 1.2**).

Depending on the needs of the cells, the PPP can support the generation of NADPH and ATP, by allowing carbon atoms to re-enter glycolysis through its non-oxidative branch. Alternatively, it can support the generation of nucleotides without producing extra NADPH by operating only its non-oxidative branch. This flexibility is important for the pathway to support the cells functions.

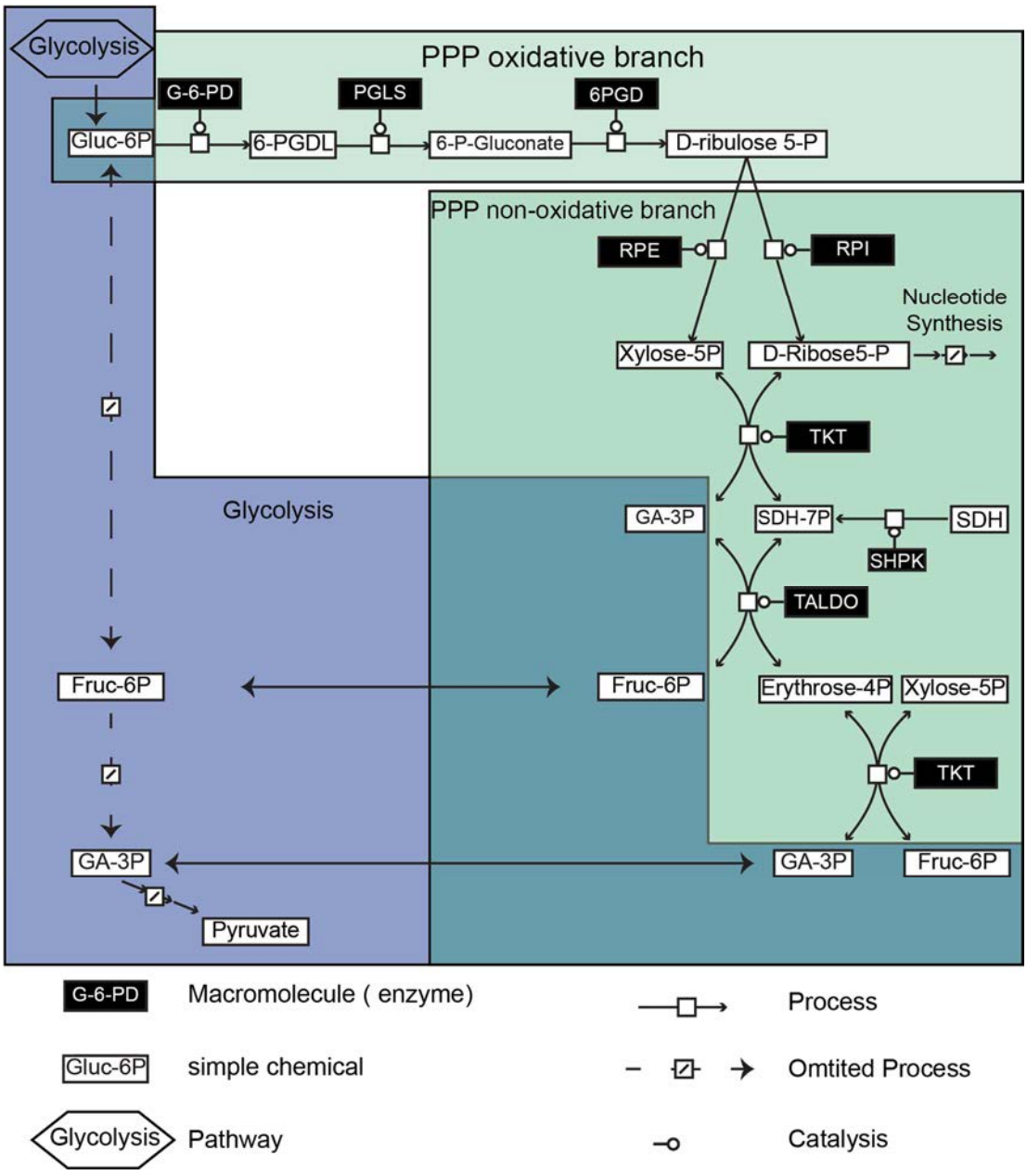


Figure 1.2 The Pentose Phosphate Pathway. The Pentose Phosphate pathway with both its branches and its interaction with the glycolytic pathway. G-6-PD: Glucose 6-P dehydrogenase; PGLS: 6-phosphogluconolactonase: 6PGD: 6-Phosphogluconate dehydrogenase; RPI: Ribulose 5-Phosphate Isomerase; RPE: Ribulose 5-Phosphate 3-Epimerase; TKT: Transketolase; TALDO: Transaldolase; SHPK: Sedoheptulose: GA-3P: glyceraldehyde 3-phosphate

1.5.2.3 The tricarboxylic acid cycle or Krebs cycle

The tricarboxylic acid cycle (TCA), or the Krebs cycle, is a series of metabolic reactions that takes place in the mitochondria of eukaryotic cells. It oxidises Acetyl-

CoA coming from carbohydrates, amino acids and fatty acids. The pathway acts as a hub where multiple pathways converge and through which carbon atoms can flow.

In most quiescent cells this pathway is the major generator of ATP. The oxidation of Acetyl-CoA does not generate large amounts of energy itself, but it is important for the generation of NADH and $FADH_2$, which can be utilised for the generation of ATP through oxidative phosphorylation (OXPHOS). Electrons from NADH and $FADH_2$ can enter the electron transport chain and generate ATP through chemiosmosis created in the inner membrane of mitochondria.

The TCA cycle and OXPHOS coupling is the most efficient way of producing energy, but it is not the only function of the TCA cycle. Due to its integral part in the heart of carbon metabolism it can be utilised to generate precursors for other pathways when it is required. Two notable examples of this are 1) citric acid that can be used for the generation of fatty acids through conversion into Malonyl-CoA, while 2) α-ketoglutarate (α-KG), which can be reversibly converted to glutamate for the generation of glutamine, proline and arginine.

1.5.2.4 Glutaminolysis

Glutamine is the most abundant circulating amino acid in blood and it is critical in a number of cell function including, generation of antioxidants, synthesis of other amino acids, purine and pyrimidine synthesis and mitochondrial metabolism. Interestingly glutamine metabolism is a hallmark of cancer metabolism (DeBerardinis et al. 2007).

The process by which glutamine is converted to a number of different TCA cycle metabolites is called glutaminolysis. Glutamine is taken up by cells and then converted to glutamate via glutaminase. Subsequently it can be converted to α-KG via two distinct pathways. The first one is transamination from glutamate–oxaloacetate transaminase (GOT), or glutamate–pyruvate transaminase (GPT), and the second is via glutamate dehydrogenase (GLUD) (L. Yang, Venneti, and Nagrath 2017).

1.5.2.5 Fatty acids biosynthesis and elongation

Fatty acids (FA) are carboxylic acids with aliphatic tails. Based on the length of the aliphatic tail the solubility of FA in water changes and as a result only some short chain FA are slightly soluble in water due to the polar carboxylic moiety. The acyl- group of the FA can be 4-36 carbon atoms long, and is usually an even number due to the way that FA are synthesized by cells.

FAs have three primary functions, 1) They are used to store energy. For this purpose, FAs are stored in cells, in the form of triglycerides, in lipid droplets. Triglycerides are formed when three FAs are attached to a glycerol molecule through the formation of ester bond between the carboxylic group of the FA and the alcohol moiety of the glycerol. 2) FAs are essential components of cellular membranes where they form an amphipathic lipid bilayer. 3) As signalling molecules (Iwasaki et al. 2015; Furuhashi and Hotamisligil 2008; Nakamura, Yudell, and Loor 2013). In general, two lipid classes form membranes in mammalian cells:

1) Glycerophospholipids: consist of a glycerol molecule that has a hydrophilic polar group at the sn3 position and two FA attached at the other two positions. The hydrophilic group is a phosphoric acid that has another polar moiety attached to its phosphate group.
2) Sphingolipids: which are composed of a sphingosine, a FA attached to it, serving as hydrophobic group, and a polar group joined by a glycosidic or a phosphodiester bond.

The nature of the FAs attached to the lipids (length of tails, saturation degree etc.) defines the physiochemical properties of the lipids. This allows for different cells and cellular compartments to have membranes with properties tuned to their needs.

Most FA can be obtained from diet or synthesized *de novo.* Some specific species of FA though cannot be synthesized by humans and other animals and, thus, must be obtained by diet. The *de novo* synthesis of straight-chain FA requires citrate from the TCA cycle. Citrate is exported to the cytosol where it is converted to Acetyl-CoA, which acetyl-CoA carboxylase (ACC) converts to malonyl-CoA, the building block

for FA. The conversion of acetyl-CoA to malonyl-CoA is a commitment step for the formation of FA.

The generation of Malonyl-CoA is followed by the synthesis of FA by Fatty acid synthase (FASN). The mammalian FASN is a huge polymer protein comprising 7 different proteins that synthesize and elongate FA up to 16 carbon atoms long (Wakil 1989). Fatty acids can by further elongated by the elongation system in the Endoplasmic reticulum (ER). Elongase Of Very Long chain fatty acid (ELOVL), in combination with other enzymes, are used in the ER to further elongate FA (Cinti et al. 1992). The FA can also be further processed by desaturases, which results in unsaturated FA. Because of its importance, the FA biosynthesis pathway is tightly regulated by mTOR and sterol regulatory element-binding proteins (SREBP) amongst others (Peterson et al. 2011; Porstmann et al. 2008).

More recently, a novel function for FA in immunity has been described. It has been shown that FA can act as signalling molecules, exhibit anti-inflammatory properties and contribute to the regulation of metabolism (Nakamura, Yudell, and Loor 2013; Oishi et al. 2016).

1.5.3 Central carbon metabolism in HCMV infected cell

Viruses are obligate parasites that are dependent on the host cells biological machinery and biosynthetic pathways to replicate. Homeostatic regulation of these pathways and the limited resources associated with them enables the cell to maintain a dynamic equilibrium and respond to wide range of external stimuli, which is crucial for survival in an ever-changing environment. Given the interplay of pathways, the extreme coordination and integration that cells have to use to control them, in addition to the cost associated, both in resources and energy, with proliferation it is not a surprise that both viruses and the immune system have developed ways to tightly regulate them. A large number of viruses, including, but not limited to, HSV-1, Dengue Virus and HCMV have been shown to exploit host metabolic pathways to proliferate (Koyuncu et al. 2013; Moser, Schieffer, and Cherry 2012; Zheng et al. 2003; Vastag et al. 2011).

Fibroblasts infected with HCMV have been shown to dramatically increased glycolysis and produce lactate in the presence of O_2. This can be perceived as

unexpected given the fact that the glycolytic pathway is not very efficient in producing ATP in comparison to the TCA cycle (Munger et al. 2006, 2008; Vastag et al. 2011), however, ^{13}C labelling experiments in HCMV infected cell have demonstrated that glycolysis transfers an increased number of carbon atoms to the TCA cycle.

Pyruvate generated from glycolysis is converted through decarboxylation to Acetyl-CoA by pyruvate dehydrogenase (PDH), which is followed by citrate synthase (CS) incorporating Acetyl-CoA to oxaloacetate and generating citrate. Citrate molecules are shuttled outside the cell at an increased rate to synthesise FA and are also used to run through the TCA (**Fig. 1.3**). At the same time uptake of glutamine is hugely increased to support anaplerotically (replenishment) the TCA cycle, to compensate for the increased efflux of citrate atoms to the cytosol. This is more evident due to the fact that while glutamine is not required for fibroblasts, it is for HCMV infected fibroblast an essential nutrient (Chambers, Maguire, and Alwine 2010; Yu, Clippinger, and Alwine 2011)

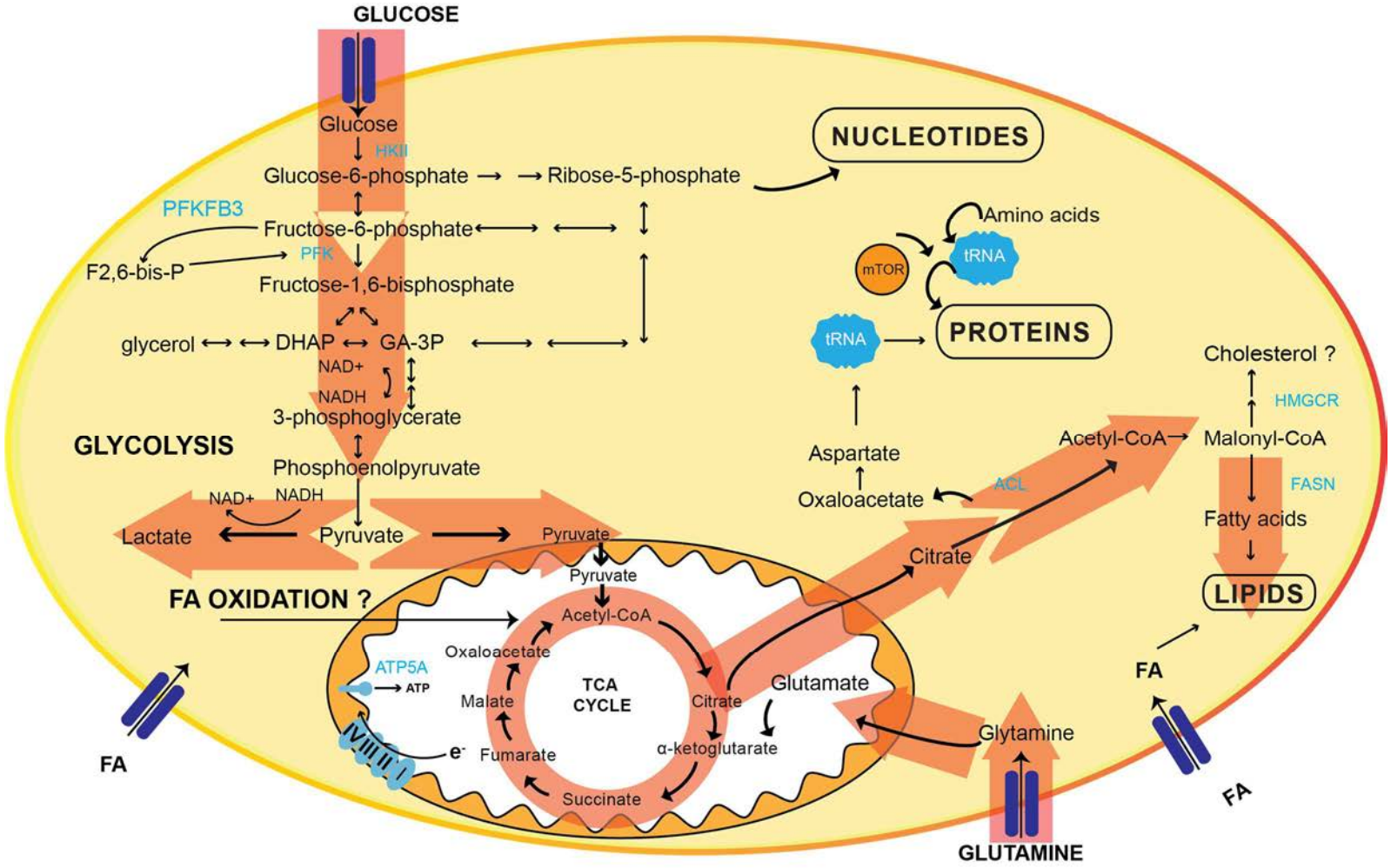


Figure 1.3 Overview of Cell Central Carbon metabolism in HCMV infected fibroblasts. HCMV greatly increases import of

glucose into the infected cell. At the same time lactate and pyruvate production is increased. The TCA cycle shunt is greatly enchanced and carbon atoms are both utilised for energy production via OXHPOS and shuttled via citrate from the mitochondrion to the cytosol where they are utilised to synthesise FA. Glutaminolysis is also greatly increased to replenish the TCA cycle despite the fact that it is not required in uninfected fibroblasts. The main pathways upregulated by HCMV are indicated by orange arrows. The question mark near some pathways indicated lack of information regarding its contribution to the metabolism of the infected cell.Abreviations same as Fig.1.1

Due to its central position in cell metabolism, the TCA cycle is, in an HCMV infected cell, turned into a major biosynthetic hub that synthesise precursor molecules for multiple pathways. HCMV manages this by increasing almost globally the metabolic fluxes. Given this, it should not come as a surprise that HCMV infected cells utilize glycolysis for ATP generation since the TCA cycle's main function is shifted.

To support the energy demands of viral replication, the virus employs multiple methods to subvert and co-op metabolic pathways within the cell. By increasing the expression of glucose transporter 4 (*Glut4*), HCMV can increase glucose uptake greatly in infected fibroblasts (Yu, Maguire, and Alwine 2011). However, increased glucose import alone is not enough, the glucose needs to be oxidised and shuttled to other pathways where it contributes to viral progeny. To manage this, HCMV increases the expression of multiple transcriptional factors while also actively interfering with growth, nutrient and energy sensors within the cell. The expression of carbohydrate response element-binding protein (ChREBP) is greatly induced and its inhibition can attenuate HCMV growth in infected fibroblasts (Yu, Maguire, and Alwine 2014). ChREBP can translocate to the nucleus and induce genes related to glycolysis, protein synthesis and FA synthesis (Postic et al. 2007; Denechaud et al. 2013). The ability of ChREBP to induce, along with glycolysis, protein and FA synthesis makes it is critical for HCMV metabolomic reprogramming of cells.

Interestingly, HCMV appears to also induce sterol regulatory binding element protein (SREBP), another transcription factor associated with FA biosynthesis. There are two SREBP genes that express SREBP1 and SREBP2, respectively. Moreover, the SREBP1 protein exist as two isoforms SREBP1a and SREBP1c (Jeon and Osborne 2012). Published data indicate that the two SREBP1 variants are associated with regulating FA metabolism and that all the SREBPs are associated with sterol

metabolism(Sato 2010). The studies reviewed here focus on the SREBP1 variants, as they are associated with FA synthesis (Yu, Maguire, and Alwine 2012). In the context of HCMV infection, it has been shown HCMV induces SREBP1 translocation from the ER, where it is bound and inactive, to the nucleus where it promotes the expression of FA synthesis genes (Yu, Maguire, and Alwine 2012; Spencer et al. 2011). Moreover, while high sterol levels normally inhibit cleavage of SREBP1, HCMV can still induce cleavage of SREBP1 despite of high sterol levels (Yu, Maguire, and Alwine 2012). HCMV has been shown to induce the translocation of inactive ER-bound SREBP1, to the nucleus where it induces the expression of FA biosynthesis genes (Yu, Maguire, and Alwine 2012; Spencer et al. 2011). Moreover, while high sterol levels normally inhibit cleavage of SREBP1 the virus can still induce it (Yu, Maguire, and Alwine 2012). Yu *et al.* suggest that one of the mechanism, through which the virus induces SREBP1 activation, is the induction of PKR-like endoplasmic reticulum kinase (PERK) (Yu et al. 2013).

HCMV, is furthermore, known to interfere with multiple cellular sensors, such AMP-activated protein kinase (AMPK), which it activates. AMPK is an energy level sensor that is activated by high levels of AMP, an indicator that ATP reserves are low in the cells. Upon activation, AMPK induces pathways associated with energy production, such as glycolysis and FA oxidation, while it inhibits energy consuming pathways, e.g. FA synthesis, and stops cell growth (Hardie and Sakamoto 2006; H. M. O'Neill, Holloway, and Steinberg 2013). Interestingly, HCMV counteracts some of the adverse effects that AMPK would have for its replication by regulating AMPK signalling. For example, while, protein synthesis is inhibited by AMPK, the HCMV protein UL38 supresses that AMPK function, resulting in continued protein synthesis. AMPK prevents inhibition of mTOR. When mTOR is deactivated, protein synthesis is restricted (McArdle, Moorman, and Munger 2012; Moorman et al. 2008; Mattijssen and Pruijn 2012), thus, by inhibiting the inhibition of mTOR, HCMV ensures continued protein synthesis for its own benefit.

Seo and Creswell suggested that this cascade of events might also be regulated by HCMV's interference with Viperin (J.-Y. Seo and Cresswell 2013). Viperin is an type-I IFN, and to a lesser extent type-II IFN, induced antiviral protein (Mattijssen and

Pruijn 2012). Upon expression, Viperin localises to the ER and lipid droplets, where it has divergent antiviral roles (Hinson and Cresswell 2009b, 2009a). These include induction of IFN-I production in plasmacytoid DCs (pDCs), inhibition of influenza A virion release and inhibiting HCV replication, most likely by inhibiting HCV's interaction with lipid droplets (Saitoh et al. 2011; Xiuyan Wang, Hinson, and Cresswell 2007; Mattijssen and Pruijn 2012).

HCMV induces Viperin independently of IFN, which then associates with a viral protein called mitochondrial inhibitor of apoptosis (vMIA). Upon interaction with vMIA, Viperin translocate to the mitochondria where it disrupts ATP production from β-oxidation of FA (J.-Y. Seo et al. 2011; J.-Y. Seo, Yaneva, and Cresswell 2011; Mattijssen and Pruijn 2012). The decreased ATP levels disrupt cytoskeletons formation, which has been shown to enhance HCMV replication (Poncet et al. 2006; Xin Wang et al. 2005). Additionally, it is proposed that this interaction results in the activation of AMPK, which results in increased glucose levels in infected cell that activates ChREBP (J.-Y. Seo and Cresswell 2013). Taken together, these indicate that HCMV is a master manipulator of cellular metabolism.

These alterations appear to converge to one metabolic pathway, FA synthesis. ^{13}C labelling studies have shown that carbon atoms are shuttled from glucose to citrate and then to FA synthesis (Munger et al. 2008). Liu et al. showed that inhibition of HCMV alters the lipidome of infected cells so much that fibroblasts produce vesicles that have lipid patterns similar to synaptic vesicles (Liu et al. 2011). Inhibition of FA synthesis enzymes, ACC and FASN, or FA elongation enzymes results in the inhibition of viral replication (Koyuncu et al. 2013). Further examination of the data indicated that the virus does not engage *de novo* synthesis of FA, but preferentially induces FA elongation and remodelling of existing FA (Koyuncu et al. 2013). Additionally, HCMV pushes FA elongation towards the production of very long saturated FAs (VLSFAs). HCMV manages this by upregulating specific elongases, particularly *Elovl7*, which has been shown, albeit *in vitro*, to facilitate the elongation of saturated FA (Purdy, Shenk, and Rabinowitz 2015; Naganuma et al. 2011; Ohno et al. 2010; Ofman et al. 2010; Leonard et al. 2002; Tamura et al. 2009; Harkewicz et al. 2012).

The advantages of these complicated manipulations of cellular metabolic process by HCMV are not clearly understood. However, examination of viral membranes indicated that they are enriched with saturated FA and it is known that saturated FA are favourable for the curvature of membranes (Munger et al. 2008; Koyuncu et al. 2013). It is, thus, assumed that HCMV utilises the VLSFAs synthesised in the cells to form its viral membrane with the physiochemical properties required for successful infection. This hypothesis is in agreement with data that have shown that inhibition of FA does not reduce greatly the number of virions, but that it greatly impairs their infectivity, indicating that they are not able to properly form and engage with cells to infect them(Yu, Maguire, and Alwine 2014). Additionally, Influenza A replication is also inhibited by restricting FA synthesis. Treatment of Influenza A infected cells with TOFA, a FA synthesis inhibitor, resulted in a reduced number of virions (Munger et al. 2008). Since Influenza A has almost no common elements with HCMV apart from the fact that it also has a lipid envelope, these results further support the notion that FA synthesis is critical for enveloped viruses' life cycle (Munger et al. 2008).

On the other hand, HSV-1, another herpes virus, does not induce the same metabolic programming. Studies have shown that the metabolic programming of cells infected with HSV-1 appears to be the opposite of that in cells infected with HCMV, indicating a clear divergence in replication strategies of the two viruses (Vastag et al. 2011). HSV-1 still uses FA from host cells to form its membrane, it just does not focus on FA synthesis as much as HCMV. It is difficult to understand what lead HCMV to choose to adopt such a complicated metabolomic program. Nevertheless, FA synthesis for incorporation of FA in its membrane appears to be one reason behind it.

1.5.4 Metabolism during immunity

Historically, immunity and metabolism have been viewed as separate fields, however recent evidence indicate that these two systems are intimately integrated, share resources and cross-regulate each other. Immune cells pre-programme and tightly regulate their metabolism upon activation by IFN and/or TLRs. These changes are essential to support their effector functions (L. A. J. O'Neill, Kishton, and Rathmell 2016). During inflammation, different immune cell types adopt different phenotypes depending on the inflammatory stimulus. New studies reveal that each phenotype also

adopts and depends on a unique metabolomic profile (O'Sullivan and Pearce 2015; Cham et al. 2008; Cao, Rathmell, and Macintyre 2014; C.-H. Chang et al. 2013; Chiba et al. 2017; Rafael Montenegro-Burke et al. 2016; El Kasmi et al. 2008; Biswas and Mantovani 2012; Mills et al. 2016; EL Kasmi and Stenmark 2015; Jha et al. 2015).

The integration of the two systems appears to be tight and important that even slight changes to the metabolism of certain immune cells, e.g. macrophages, can induce a change in the phenotype. For example, induction of GLUT1 expression in macrophages drive the cells to adopt an M1 (or classically activated) like phenotype (Freemerman et al. 2014). Alterations in the metabolism of an immune cell can affect the phenotype for the cell. This opens new possibilities for drug intervention on immune cell level, as potentially, the immune system could be regulated by inhibiting specific metabolic pathways without affecting cell viability (L. A. J. O'Neill, Kishton, and Rathmell 2016; Kotzamanis, Angulo, and Ghazal 2015).

1.5.5 Metabolism in macrophage and dendritic cells

While macrophages (MΦ) and dendritic cells (DCs) exhibit differences regarding phenotype and function in disease, the two cell types share quite a few similarities. Some similarities appear to be in the metabolomic profile that both cell types adopt when they are activated by TLR receptors in classical manner. This similarity might be explained by the fact that these cells types during infection have similar energy and metabolites requirements during infection and may explain similar metabolic profiles.

Both MΦ and DC needs to phagocyte and process antigens. The process and functional outcome behind phagocytosis might be different in each cell type, but the function is similar and should have similar metabolic requirements. Moreover, both cell types need to produce huge amounts of cytokines to coordinate the immune response, to external stimuli both by the host and the pathogens and finally resolve inflammation. It has recently become obvious that for all these processes, even resolution of inflammation, metabolism plays a pivotal role.

Differences between the two cell types are expected to exist but it is not clear yet if, and which, subtle differences should be attributed to differences between the cell types

and/or differences in treatment. Macrophages and DCs are extremely plastic cells and slight differences in treatments might produce different results. Macrophages for example have been distinguished into distinct phenotypes for a long time. M1 macrophages are macrophages treated with LPS or LPS+IFN-γ. These macrophages are associated with inflammation and are mobilised during viral, bacteria and/or fungal infections. M2 macrophages are macrophages treated with IL-4 and they have anti-inflammatory properties and promote wound healing. They are associated with Th2 immune response to helminths and other parasites. Macrophages though are highly plastic cells that respond to a plethora of stimuli and recent studies though have indicated that there is a big spectrum of activated macrophages rather than just these two extreme examples(Mosser and Edwards 2008).

Conventional dendritic cells (cDCs) and Macrophages (MΦ) engage aerobic glycolysis when they are activated under certain conditions. cDCs dramatically increase their glucose uptake and lactate production upon TLR stimulation (Everts et al. 2014) and MΦ adopt, in a similar manner, a highly glycolytic metabolism in the presence of oxygen (Jha et al. 2015). This adaptation might appear unexpected because of the inefficiency glycolysis has in generating energy, but it has been shown to be important for these cell types. Both macrophages and dendritic cells have been shown to produce nitric oxide (NO) during inflammation by inducing expression of inducible Nitric oxide synthase (iNOS). NO has bacteriostatic effect, but also inhibits OXPHOS by inhibiting complexes I, II and IV of the electron transport chain (Beltrán et al. 2000; Everts et al. 2012). Thus, the switch to glycolysis is necessary for the cells to survive as it has been shown for DCs, that inhibition of glycolysis by 2 Deoxyglucose (2-DG) prevented the cells from becoming activated (Everts et al. 2012).

The mechanisms that drive these changes appear to be complex and work on the transcriptional and post transcriptional level. TLR stimulation greatly induces expression of several genes encoding enzymes associated with glycolysis in macrophages, such as pyruvate kinase isoenzyme M2 (PKM2), GLUT1 and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3). PKM2 catalyses the last step in glycolysis, GLUT1 facilitates import of glucose in the cells and finally PFKFB3 produces fructose-2,6-biphosphatase. PFKFB3 is an allosteric activator of

phosphofructokinase (PFK) that catalyses one of the rate limiting step of glycolysis(De Bock et al. 2013). In DCs it has been shown that TBK1-IKKε, through Protein kinase B (PKB or AKT), promotes glycolysis by translocating hexokinase 2 (HK2) to the mitochondrion where it presumably has access to increased levels of ATP which increases turnover time(Everts et al. 2014).

In addition of the glycolytic pathway, the glucose shunt through the Pentose Phosphate Pathway (PPP), is also greatly increased in proinflammatory macrophages and DCs. Shunting glucose through PPP offers an alternative way of oxidising glucose, which re-enters the glycolytic pathway at a later stage, while at the same time allow for the generation of NADPH (Wamelink, Struys, and Jakobs 2008). NADPH is vital for M1 macrophages and DCs as it is used for the generation of reactive oxygen species (ROS) (Singh et al. 2017). It is also used to reduce glutathione and prevent damage caused by oxidative stress and generated ROS.

Sedoheptulose kinase (SDK or CARKL) has been demonstrated to play a critical role in regulation of PPP and glucose by providing Sedoheptulose-7P (S7P) independently of glucose (Nagy and Haschemi 2013). High S7P levels negatively regulate hexose phosphate isomerase and block fructose phosphorylation by phosphoglucose isomerase (Nagy and Haschemi 2013). LPS signalling supress SDK, which in turn has a positive effect on the PPP and glycolytic pathways. Contrary to M1 macrophages, M2 macrophages upregulate CARLK and, thus, limit the shunt in both pathways (Haschemi et al. 2012).

Inflammation and metabolism are tightly interlinked. Pro-inflammatory cytokines and signals promote glycolysis while anti-inflammatory cytokines, such as IL-10, limit glycolysis in DCs. IL-4 activated macrophages (alternatively activated MΦ or M2 MΦ) exhibit intact OXPHOS and limited glycolysis (Jha et al. 2015; Ganeshan and Chawla 2014). Additionally, not only proinflammatory signals and cytokines drive a specific type of metabolism, but metabolism can also dictate the phenotype of the cell. For example, macrophages overexpressing GLUT1 adopt a phenotype that is pushed towards the M1 spectrum(Freemerman et al. 2014). Arguably, the study was performed in RAW264.7 cells, which would have their own metabolic adaptations since they are a stable cell line and not primary cells.

There are on the other hand other metabolites, prostaglandins, that are known to act as inflammatory signals and that have been studied more extensively. Prostaglandins are the most well studied metabolites that, amongst others, during inflammation function as paracrine and endocrine signalling lipid molecules. Prostaglandins are a subset of prostanoids, a class of molecules that are derived from arachidonic acid (AA). All prostaglandins are oxygenated 20 carbon atoms long fatty acids that contain a cyclopentane ring(Bos et al. 2004).

When it is required, AA is released from phospholipids by lipases. The released AA is converted to prostaglandin G2 (PGG_2), the precursor molecule of all prostaglandins. This conversion is catalysed by cyclooxygenase (COX), which exists in two forms. COX-1 is constitutively expressed and COX-2, is not, but is greatly induced by stimuli such as LPS(Funk et al. 1991; Herschman 1996; Aderem et al. 1986). There are 4 different prostaglandins, prostaglandin E2 (PGE_2), PGD_2, $PGF_2$and PGI_2. The produced prostaglandin profile differs depending on cell type. Macrophages produce mainly prostaglandin E2 (PGE_2), while mast cells mainly produce PGD_2 (Tilley, Coffman, and Koller 2001). After their production, prostaglandins act as signals that can regulate the function of immune cells, including DCs and macrophages. Prostaglandins can both promote or attenuate inflammation depending on the setting and cell type making (Ricciotti and FitzGerald 2011).

Succinate has also been shown to act as a proinflammatory signal through different mechanisms in MΦ and DC. In MΦ, succinate accumulation inhibits propyl hydroxylases (PHD) from hydroxylating their target proteins, including hypoxia-inducible factor 1 (HIF-1α) (EL Kasmi and Stenmark 2015) . This results in HIF-1α stabilisation and increasing expression of IL-1β and glycolytic enzymes, among others (EL Kasmi and Stenmark 2015; Tannahill et al. 2013). Accumulated succinate also increases production of reactive oxygen species (ROS), by the mitochondria, which also can inhibit PHDs and further stabilise HIF-1α (Brunelle et al. 2005; Dröse 2013; Infantino et al. 2011). TLR signalling greatly induces ROS, enhancing the bactericidal activity of MΦ (West et al. 2011) and further promotes HIF-1α stabilisation and signalling.

These described changes might be useful as MΦ need to mount a robust immune response in open wounds and other environments where oxygen is low (Palazon et al. 2014; Nizet and Johnson 2009). More recently, studies have found that high succinate levels on their own are not sufficient to act as proinflammatory signal, but that succinate dehydrogenase (SDH) function and high mitochondria membrane potential is also required (Mills et al. 2016). Under these conditions it has been shown that SDH contributes to the generation of ROS through reverse electron transport (RET) although whether this is the mechanism that contributes to the effects of succinate remains unresolved (Mills et al. 2016; Votyakova and Reynolds 2001; Lambert and Brand 2004).

These metabolic alterations have profound effects on the TCA cycle of MΦ and DCs. M2 macrophages have an intact and functioning TCA along with OXPHOS. It is believed that one of the functions that this serves in M2 macrophage, is the glycosylation of receptors associated with M2 function (Jha et al. 2015; Palsson-McDermott et al. 2015). On the other hand, in M1 MΦ, the TCA cycle breaks down at the succinate, as described above, and at citrate (Andreyev et al. 2010). Citrate is one of the major carbon sources for FA synthesis (Hatzivassiliou et al. 2005). Numerous studies have demonstrated that both macrophage and DC shuttle carbon atoms from glucose to FAS through citrate (Feingold et al. 2012; Jha et al. 2015; Andreyev et al. 2010; Everts et al. 2014). In activated DCs, the newly produced FAs have been shown to be utilised to expand the ER and Golgi to support the increased requirement for protein synthesis and secretion (Everts et al. 2014). Similarly, fatty acids were found to be important for supporting the phagocytic function in macrophages (Ecker et al. 2010).Thus, these changes are essential for DCs and macrophages to transform themselves from small quiescent cells into potent phagocytes that secrete a vast number of cytokines and present antigens.

A recent study has indicated that FA synthesis might play a crucial role in regulating inflammation. It was demonstrated that SREBP1 contributes to resolution of inflammation by altering FA metabolism (Oishi et al. 2016). In the resolution phase of inflammation, SREBP1 is activated and promotes the transcription of FAS enzymes

that generate FA with anti-inflammatory properties such as Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) (Oishi et al. 2016).

The TCA cycle is also linked to NO production through the arginosuccinate shunt (J. MacMicking, Xie, and Nathan 1997). NO is critical for clearance of bacteria by macrophages (Qualls et al. 2012; Morris 2004; El Kasmi et al. 2008). The arginosuccinate shunt has been shown to be upregulated in M1 macrophages (Jha et al. 2015) and citrulline is produced at high levels in M1 MΦ during NO synthesis (Qualls et al. 2012). This excess citrulline along with aspartate from the malate aspartate shunt can be utilised by Argino-succinate synthase (ASS) to synthesise arginosuccinate. Arginosuccinate in turn can be broken down to arginine and fumarate by arginosuccinate lyase (ASL).

The remodelling of the aspartate-malate shuttle and arginosuccinate shunt allows the macrophage to be autotrophic - support its requirements without external replenishment - for arginine, which is essential for NO synthesis by iNOS (J. MacMicking, Xie, and Nathan 1997). Additionally, the produced fumarate can replenish the TCA cycle. It could possibly also reinforce the generation of succinate by reverse function of SDH, although this has not yet been demonstrated in macrophages (Chouchani et al. 2014). Nevertheless, the ability to generate NO, regardless of the availability of arginine in the environment, is undisputed for M1 macrophages.

There is an interesting association between the fate of arginine in macrophages and their cell phenotype. Arginine can either be utilised by iNOS to produce NO or converted by Arginase (ARG) to ornithine (Rath et al. 2014). The first reaction is associated with proinflammatory response while the second is associated with the arise of a tolerogenic and wound healing phenotype in macrophages (Albina et al. 1988; Campbell et al. 2013).

Finally, fatty acid oxidation (FAO) is another pathway that is altered during activation of macrophages. FAO takes place in the mitochondria and peroxisomes depending on the length of the FA. It is believed that FA longer than 22 carbon atoms are first oxidised in peroxisomes, to the size of medium chain acyl-CoAs, and then transported

to the mitochondria where they are further oxidised(Wanders, Waterham, and Ferdinandusse 2016). Peroxisomes are also able to oxidise odd-number and unsaturated fatty acids. FAO starts with the transfer of FA by carnitine palmitoyl transferase I (CPT1) and carnitine palmitoyl transferase II (CTP2) in mitochondria. In peroxisomes, the same function is performed by ATP-binding cassette (ABC) transporter ABCD1 and ABCD2.

In immunity, increased FAO is associated with an anti-inflammatory phenotype(L. A. J. O'Neill, Kishton, and Rathmell 2016). Early studies indicated that M2 macrophages rely on FAO, which is perpetuated through PPARγ-co-activator 1β (PGC1β) and Signal transducer and activator of transcription 6 (STAT6) (Vats et al. 2006). This was further substantiated by a later study in murine macrophages (S. C.-C. Huang et al. 2014). Interestingly, a study in human macrophages indicated that FAO was not essential for human macrophages (Namgaladze and Brüne 2014). A later short report indicated that mice CTP2 deficient BMDM were able to fully acquire a M2 phenotype (Nomura et al. 2016). Figure 1.4 synopsises the differences between the two MΦ phenotypes.

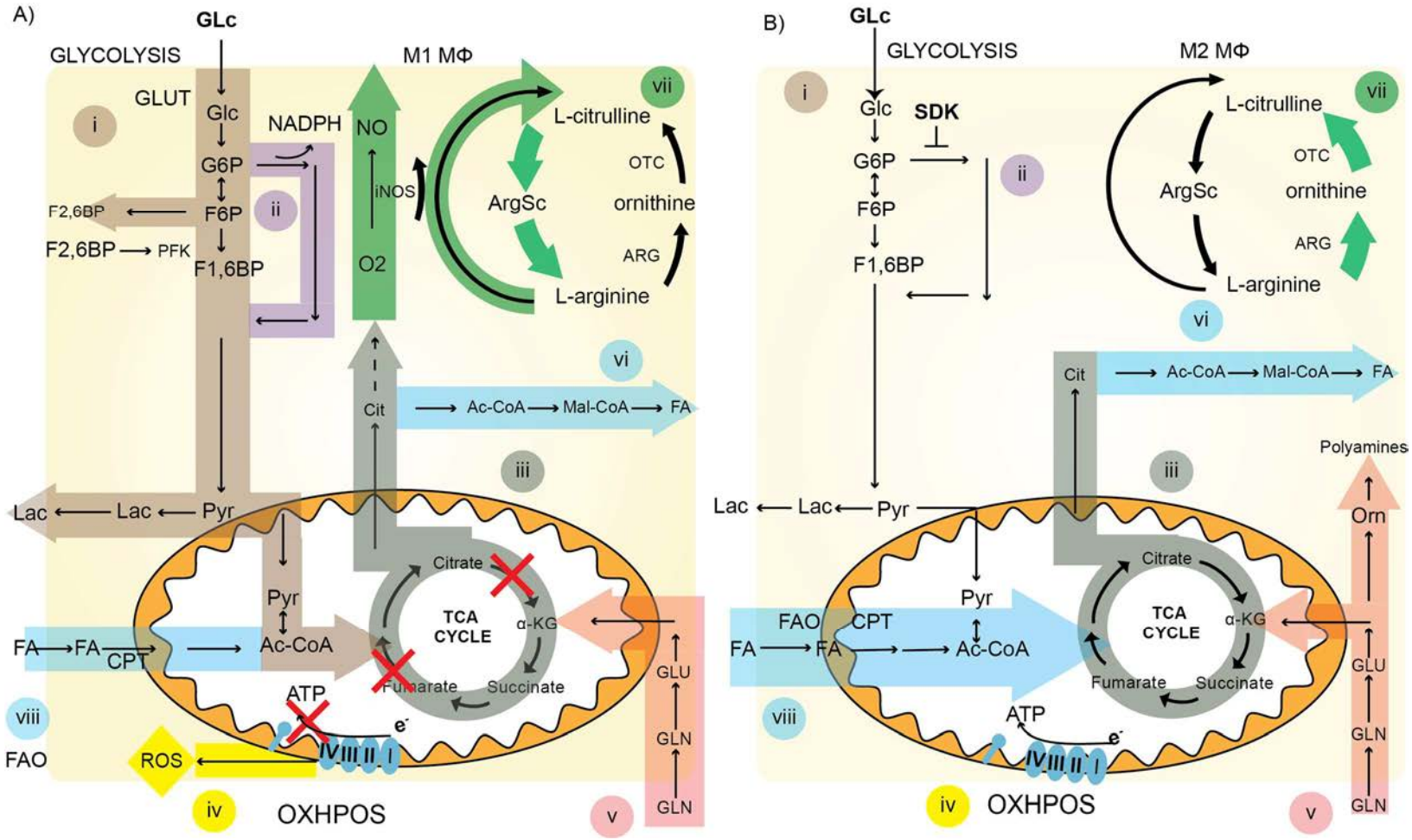


Figure 1.4 Macrophage metabolism during immunity. A) M1 MΦ are characterised by increased **i)** glycolytic shunt via increased glucose import and upregulation of the glycolytic pathway on the transcriptional and posttranscriptional level **ii)**

increased oxidation of glucose via the PPP and production of NADPH. **iii)** The TCA cycle is broken down and citrate is shuttled out of the mitochondrion to provide carbon atoms for **vi)** FA synthesis. **iv)** OXPHOS does not operate and the electron transport chain (ETC) is utilised to produce ROS. **v)** Glutamine is imported into the cell to replenish the TCA cycle and help maintain the mitochondria. Finally, **vii)** the arginosuccinate shunt is utilised to produce NO, which has important bacteriocidic properties and **viii)** FAO is present on a basal level. **B)** M2 MΦ are characterised by **i)** low glycolysis and low **ii)** PPP shunt. Upregulation of SDK inhibits the PPP and glycolysis. Instead of glycolysis, M2 MΦ rely on **viii)** FAO for energy generation**. iii)** The TCA cycle and **iv)** are functional and intact, and glutamine is utilised to produce Polyamines or to replenish the TCA cycle. Finally, **vii)** arginine is catabolised to ornithine by arginase and not utilised for the generation of NO Glc: Glucose; G6P: Glucose-6-Phosphate; F6P: Fructose-6-Posphate: F1-6BP: Fructoce-1,6-Phosphate; Pyr: Pyruvate; Lac: Lactate; FAO: Fatty acid oxidation; CTP: Carnitine Palmitoyltransferase; Ac-CoA: Acyl-CoA; GLN: glutamine; GLU: Glutamate Cit: citrate; NO nitric oxide; ArgSc: ArgininoSuccinate; Arg: Arginase; OTC: ornithine transcarbamylase; Orn: Ornithine. Adopted from (Ghesquière et al. 2014) and altered.

Another piece of evidence linking decreased FAO with increased inflammation comes from a study on X-linked adrenoleukodystrophy (X-ALD). In X-ALD patients, mutations in the *ABCD1* gene cause neurodegeneration and inflammatory cerebral demyelination (Engelen et al. 2012). The microglia and bone marrow derived macrophages appear to be the main drivers of the disease progression in humans.

In mice, however, X-ALD does not exhibit the same strong phenotype as in humans. Previous studies have demonstrated that murine *Abcd1* deficient macrophages exhibited a highly pro-inflammatory phenotype because of accumulation of very long chain saturated fatty acids (VLCSFA) (Yanagisawa et al. 2008). Using *Abcd1* and *Abcd2* single and double deficient mice, Muneer *et al.* demonstrated that ABCD2 compensates partially for ABCD1 function. In mice deficient for both both *Abcd1* and *Abcd2*, VLCFA accumulated at an increased rate in comparison to single *Abcd1* knockouts (KO) (Muneer et al. 2014). Based on their observations, and the fact that human BMDM and microglia do not express ABCD2, they hypothesised that this is the driver behind the metabolic phenotype in humans. Thus, BMDM and microglia in humans with X-ALD rapidly accumulate VLCFA and become more proinflammatory exacerbating the progression of the disease. These differences between mice and human macrophages reveal the complexity of immunometabolism and how small differences at the site of inflammation and the genetic background of the cells can lead to great differences in the outcome of an infection, and or other underlying pathology.

Interestingly, there is a great deal of studies in other human pathologies that reveal a correlation between accumulation of FA and lipoproteins and inflammation(Lusis 2000; Shoelson, Lee, and Goldfine 2006). Additionally, proinflammatory macrophages exhibit only basal levels of FAO (Ghesquière et al. 2014). Finally, in conjunction with the above, overexpression of *CPT1A* in human macrophages increased FAO and reduced the production of inflammatory cytokines (Malandrino et al. 2015).

Despite the huge advances in the field of immunometabolism, little has been done to study metabolism and immunity in the context of infection. Most of the studies presented above are examining metabolism during immunity using either MΦ or DC that have been activated by TLRs. Usually the TLR ligand used is LPS, which is prototypical of a response to bacterial infection, specifically by gram negative bacteria. During an immune response, immune cells are going to be engaged by numerous PRRs and cytokines, which should further tune metabolism and immune response. Furthermore, bacterial infections are different from viral ones, especially when the pathogen can infect immune cells, like MCMV infects MΦ. In this case, MΦ integrate signals from immune signalling, metabolism and the pathogen itself.

1.5.5.1 Metabolism in T cells

The changes observed in the metabolism of macrophages and DCs upon activation are not only limited to cells of the innate immune system. Cells of the adaptive immune system exhibit similar alterations to their metabolism.

The adaptive immune system is comprised of T lymphocytes and B lymphocytes. T lymphocytes are key elements of the immune system and they include a wide subset of cells, including CD4+ (helper) and CD8+ T (cytotoxic) cells. These cells are important in the process of eliminating pathogens and regulating the progression of non-infectious diseases such as cancer and diabetes 2 (Iwasaki et al. 2015; Gajewski, Schreiber, and Fu 2014; Eheim, Medrikova, and Herzig 2014). There are three stages that T cells undergo when mounting an immune response: priming, expansion and contraction. Each stage in the life of a T cell has different metabolic demands, which need to be met to ensure that the cell can realise its function.

Similar to quiescent cells, naïve T cells have low metabolic demands and, thus, a similar metabolic phenotype. Upon priming by an antigen, T cells are activated and must undergo great metabolic changes to support their need to rapidly proliferate and generate large amounts of cytokines. After expansion and clearance of the pathogens, a small population of antigen-specific long-lived memory T cells (T memory cells, T_M) is left behind (Pearce et al. 2013).

All effector T cell subtypes, including CD8+ T cells, T helper cells1 (T_H1), T helper cells17 (T_H17) and T helper cells2 (T_H2) have been shown to rely heavily on glycolysis(Shi et al. 2011; Gubser et al. 2013; Michalek et al. 2011; Buck, O'Sullivan, and Pearce 2015). Despite this, and unlike DCs and MΦ, effector T cells have functional TCA and OXPHOS, and can function without glycolysis, but with impaired effector functions (C.-H. Chang et al. 2013; Sena et al. 2013; Cham et al. 2008). T effector cells have increased FAS while at the same time reduced FAO (Robichaud et al. 2013; Metallo et al. 2011). On the other hand, T regulatory cells (Treg) rely heavily on FAO and not glycolysis (Michalek et al. 2011). Similar to myeloid cells, changes in the metabolism of T cells can influence their phenotype; inhibition of FA synthesis in T_H17 was shown by *Berod et al*. to result in the acquisition of a Treg phenotype (Berod et al. 2014).

T_H17 cells are important cells during inflammation, but some can become "pathogenic" by inducing tissue inflammation and promoting responses to self-antigens. A study demonstrated that the expression of CD5 antigen-like (CD5L) in T_H17 promotes a non-pathogenic phenotype by regulating lipid metabolism in T_H17 (C. Wang et al. 2015). CD5L appears to bind fatty acid synthase and promote the generation of polyunsaturated fatty acids (PUFAs). It is hypothesised that PUFAs modulate cholesterol metabolites, which act as agonists for RAR-related orphan receptor gamma thymus (RORγt). This prevents RORγt from upregulating the expression of *Il-23* and *Il-17* synthesis, while at the same expression of *Il-10* is increased (C. Wang et al. 2015).

Oishi *et al.* (presented above) has demonstrated that during resolution of inflammation, MΦ alter their FA metabolism to attenuate inflammation (Oishi et al. 2016). Together, these two studies demonstrate clearly the ability of metabolism, and

lipid metabolism, to influence the outcome of inflammation during pathogen evasion or other pathology. This is now an area of intense research.

1.6 Rationale and experimental design

Accumulated evidence in the last 15 years support the notion that metabolism and the immune system interact and regulate each other in ways that were not though of previously. Immune cells have been demonstrated to drastically alter the metabolism upon immune activation in a clear and defined manner. These metabolic adaptations are important for the cells to acquire their effector functions and complete their function.

On the other hand, viruses are extreme parasites that exploit and counter pathways, including metabolism, to effectively propagate. HCMV alters the metabolism of infected host cells in a way that resembles the metabolism that proinflammatory macrophages acquire when they become activated in a classical manner. Moreover, macrophages play a critical role in the dissemination and infection strategy of this clinically important pathogen.

Viruses are known to exploit and counter-act signalling of the immune system. The parallels between the metabolism of HCMV infected cells and classically activated macrophages indicate that, metabolism might be a novel mechanism which viruses can exploit to subvert the immune response. Thus, I will attempt to investigate this integrative process by studying the metabolic programming of macrophages infected with MCMV.

The main hypothesis of the thesis is that productive infection of macrophages by MCMV takes advantage of the early metabolomic reprogramming of activated macrophages, and modulates metabolism at late stages of infection towards fatty acid production to promote viral progeny.

In this thesis, I will try to answer the following question to refute or support the hypothesis:

i. Does MCMV upon infection, similar to HCMV, alter the metabolomic programming of macrophages towards fatty acid synthesis?
ii. Is metabolism of macrophages during early times of productive and non-productive MCMV infection similar at the transcriptional and metabolomic level?
iii. Is metabolism of macrophages during late times of productive and non-productive MCMV infection different?
iv. Does productive MCMV infection alter the metabolism of infected macrophages and promote FA synthesis?
v. Does MCMV infection take advantage of prostaglandin metabolism, a key component of FA metabolism, to evade the immune system response?

Taking all these considerations into mind in the thesis presented here I attempt to study immunometabolism by employing a more suitable model. A novel and crucial aspect of my work is employing a viral infectious agent to study metabolism in immunity. To achieve this I, utilise a series of transcriptomics and metabolomics studies in combination with pharmacological/siRNA inhibition of metabolic pathways in an *in vitro* model for host pathogen interaction. In my model system, to study metabolism, MCMV is used as the model for viral infection and Bone Marrow Derived Macrophages (BMDM) are utilised as a model for the immune system. Crucially, MCMV is a natural infectious agent for murine BMDM and, thus, the generation of artefacts is reduced.

In my model system, I mainly utilise MCMV-pSM3fr and MCMV-pSM3frdie3 strains (from now on MCMV-WT and MCMV-KO). MCMV-WT can infect and produce virally progeny (productive infection), while MCMV-KO can infect cells, but it does not produce infectious virions. BMDM were infected with both strains and from the analysis of the data between productive and non-productive infection the host-directed versus, virus-driven metabolic alterations could be unravelled.

2 Chapter 2 Materials and Methods

2.1 Cell cultures

2.1.1 Cell lines

All cell lines used in this thesis were grown at 37°C and 5% CO_2 in tissue culture flasks or dishes (Corning, UK), and were handled in a class 2 tissue culture hood. The NIH-3T3 immortalized cell line of embryonic mouse fibroblasts was obtained by American Type Culture Collection (ATCC) (Manassas, VA, USA). They were grown in Dulbecco's modified Eagle medium (DMEM) (Lonza, UK), supplemented with 5% calf serum (CS) (Invitrogen, UK), 2mM glutamine (Lonza, UK) and 100U/ml of penicillin/streptomycin (Lonza, UK). NIH-3T3-Bam25 cells were derived by co-transfection of the pBam-25 and pPUR plasmid in NIH-3T3 cells, as previously described(Angulo, Ghazal, and Messerle 2000). The same growth conditions were used as for NIH-3T3 cells. p-53-/- Murine Embryonic Fibroblasts (MEF p53-/-) were purchased from ATCC. They were grown in DMEM, supplemented with 5% fetal calf serum (FCS) (Invitrogen, UK), 2mM glutamine and 100U/ml of penicillin/streptomycin. RAW264.7 murine macrophages from blood were grown in DMEM supplemented with 10% FCS, 2mM glutamine and 100U/ml of penicillin/streptomycin.

2.1.2 Bone Marrow Derived Macrophages (BMDM)

C57BL/6 JCR male mice, aged between 7 and 14 weeks were obtained and culled from Chancellor's Building animal unit. Femur and Tibia bones, extracted from the hind legs of the mouse, were flushed in a 50ml flacon tube that contained DMEM/F-12 (Ham 1:1) and L-glutaMax (LONZA, UK). The supernatant with the cells was passed through a 50 μm cell strainer and then centrifuged at 244 x g for 5 minutes at room temperature (RT). The supernatant was removed and the pellet was resuspended in DMEM/F-12 (Ham 1:1) and L-glutaMax, supplemented with 10% FCS, 10% L929 and 100U/ml of penicillin/streptomycin. The cells were counted and seeded in 6 well (1×10^6 cells/ml) or 24 well (2.5×10^5) plates for 7 days. The medium was replenished on days 3 and 5. On day 7 the wells cells were ready for treatment.

2.1.3 Primary Murine Embryonic Fibroblasts(pMEFS)

Embryos, aged 7-14 weeks, were obtained from C57BL/6 JCR mice. From the embryos the brain, intestines and eyes were removed and subsequently placed in 10 cm dishes that were placed on ice. The embryos were homogenized manually using razor blades. The homogenized tissue was then suspended and stirred, for 1 hour at 37°C, in a Falcon tube containing 30 ml 1x trypsin and glass pellets. An additional 30 ml of 1x trypsin was added every 30 minutes. The cells were then filtered centrifuged at 244 x g for 5 minutes and washed 3 times with PBS, followed by collection and re-suspension in EMEM (Lonza, UK) supplemented with 10% FCS, 2mM glutamine and 100U/ml of penicillin/streptomycin. The cells were counted using haemocytometer and seeded in 175cm^2 flasks (1 x 10^7 cells/flask). The following day, cells were washed once with 1x PBS to remove erythrocytes. The culture was then left to reach a confluency of 80%. Upon reaching that confluency, cells were harvested and then either frozen for later use or passaged for use.

2.1.4 Preparation of L929 conditioned media for BMDM differentiation

L929 cells were obtained from Sigma, UK. L929 cells are subclones of the parental strain that is derived from subcutaneous areolar and adipose tissues of the C3H/An male mouse strain. L929 cells produce Macrophage Colony Stimulating Factor 1 (CSF-1), a cytokine essential for the survival, proliferation and differentiation of hematopoietic stem bone marrow cells into macrophages. L929 were grown in DMEM/F-12 (Ham 1:1) and L-glutaMax supplemented with 10%FCS and 100U/ml of penicillin/streptomycin for 14 days. The medium was then collected, sterile filtered and checked for the presence of endotoxin and mycoplasma. Each new batched of L929 media was tested for its ability to induce BMDM differentiation.

2.1.5 Characterisation of BMDM by Flow cytometry

Macrophages express on their surface a variety of markers than can be used to assessed the efficiency of differentiation by Flow cytometry. To ensure the maturation stage of BMDM after 7 days of maturation, cells were stained for F4/80 and CD11 markers.

On Day 7 of maturation, cells were washed twice with PBS and scraped off the bottom of the cell plates. The cells were then transferred to polystyrene FACS tubes (BD Falcon, UK). The tubes were centrifuged at 1,000 g for 5 minutes at 4°C to isolate the cells. The cells were washed twice with cold PBS (4°C) and suspended in blocking solution (Flow cytometry buffer +0.5µg/ml of Mouse BD Fc block™ (BD Biosciences, UK:cat.no.553142) + 10% mouse serum (Invitrogen, UK:cat.no.10410)) for 10 minutes on ice to avoid non-specific antibody binding. The cells were then incubated, for 1 hour at RT, with Rat anti-mouse F4/80-PE (Invitrogen, UK, cat.no.MF48004) and with Rat anti-mouse Cd11b-FITC (Caltag, UK, cat. no. RM2801), which were both diluted 1:100 in flow cytometry buffer. Then the cells were washed three times with flow cytometry buffer and the pellet was suspended in 300 µl flow cytometry buffer. The cells were then transferred to ice for flow cytometry. The staining experiments were performed in collaboration with Dr. Muhamad Fairus Noor Hassim or Alan Ross. A BD FACScalibur (BD, UK) instrument was used to analyse the samples and FlowJO software (Treestar, USA) was used to analyse the data.

2.1.6 Cell freezing protocol

Cell were removed from their flask/dish with the use of trypsin, transferred to a 50 ml Flacon tube and centrifuged at 244 x g for 5 minutes. The pellet was then re-suspended in media containing 90% FCS or CS depending on the cell type and 10% DMSO. The media was then aliquoted into 1.8ml cryovials, which were placed in a cryopreservation chamber and kept overnight at -80°C before transfer to Liquid nitrogen for long-time storage.

2.1.7 Thawing of Frozen cells

Cryovials containing frozen cells were quickly thawed in a water bath at 37°C. The thawed cells were then transferred to a 50 ml Falcon tube that contained appropriate media, followed by centrifugation at 244 x g for 5 minutes. The pellet was re-suspended in appropriate media and transferred to a T75 flask (T25 for (pMEFS)). The cells were washed the following day to remove dead cells and incubated until they reached confluency.

2.1.8 Cell passaging

Cells were expanded by passaging them 1:10 – 1:2 depending on the cell type. Cultured cells were allowed to reach 70-80% confluency and were subsequently incubated with trypsin/EDTA for a few minutes at 37˚C to allow cells to detach from the flask surface. After the cells were detached from the flask, the cells were collected in 50 ml falcon tubes and mixed with an equal volume of fresh media. The cells were then pelleted by centrifuged at 244 x g for 5 minutes and re-suspended in an appropriate cell media volume and re-seeded in new tissue flasks. The maximum passage number was 25 times.

2.1.9 Cell counting and seeding

In order to seed an appropriate number of cells in well plates, cell concentrations were determined by cell counting. To count cells, 10 µl of suspended cells were diluted in the appropriate volume of 0.4% Trypan Blue stain, (Invitrogen, UK). 10 µl of the solution was injected into a haemocytometer chamber and the number of alive cells per square was calculated using the formula.

$$\frac{Cells}{ml} = N * df * 10^4$$

- N = number of cells counted
- df= dilution factor

2.2 General methods of virology

2.2.1 Viruses

MCMV-C3X (referred to as MCMV-WT in this thesis) was obtained from the recombinant bacterial artificial chromosome clone pSM3fr, which itself was derived from MW97.01(Messerle et al. 2000). Construction of the pSM3frdie3 mutant (referred to as MCMV-KO in this thesis) is described by Angulo *et al.* (Angulo, Ghazal, and Messerle 2000). MCMV-KO lacks the immediate Early (IE) 3 gene and is unable to replicate. From this mutant, a GFP-encoding revertant was constructed

originally named pSM3fr-rev (termed MCMV-GFP in this thesis). Figure2.1 shows a representation of the MCMV genome and its mutants.

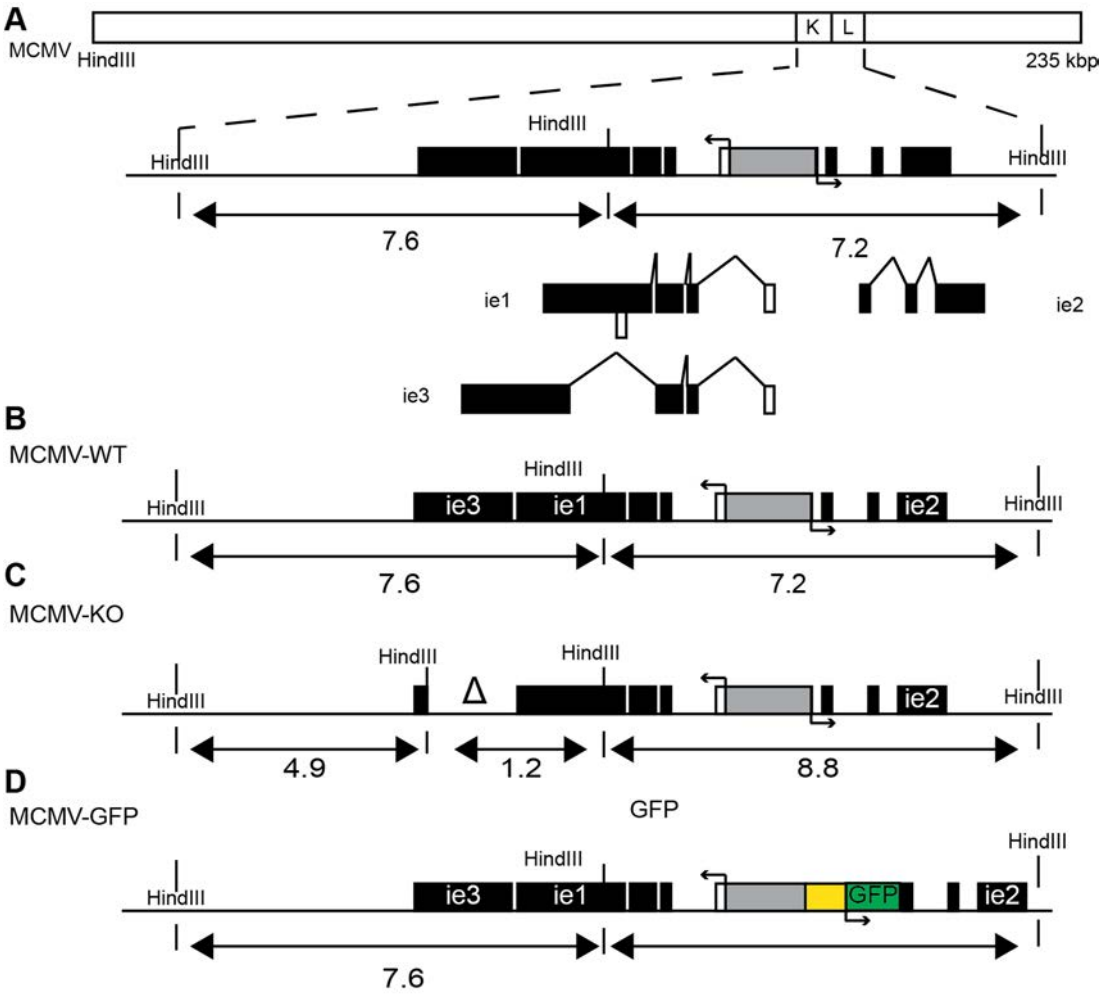


Figure 2.1 Schematic representation of the MCMV genome and its mutants. (A) The MCMV the region containing immediate early (IE) genes and the MCMV enhancer is expanded to reveal the structure of the *ie1*, *ie2* and *ie3* genes. Coding exons are shown in black and noncoding exons are shown as white rectangles. The arrows indicate the direction of transcription. **(B)** The parental MCMV strain derived from bacterial artificial chromosome clone pSM3fr. **(C)** MCMVdie3 strain **(D)** The MCMVdie3rev strain, which contains a GFP reporter gene (green box) and a HCMV enhancer (yellow) to promote GFP transcription. Adopted from (Angulo, Ghazal, and Messerle 2000)

While this thesis was conducted, it was uncovered that the MCMV pSM3fr strain used to derive all the previously mentioned strains had a single base pair deletion positioned in the m129 ORF. This deletion affects the MCMV-encoded chemokine 2 (MCK2) resulting in a non-functional product(Jordan et al. 2011). The MCK2 protein has been

used to demonstrate increased viral titre in salivary glands of MCMV infected adults BALB/c mice(Fleming et al. 1999). Additionally, as described in the introduction, it has been proposed that MCK2 attracts myeloid cells helping the promotion of monocyte-associated dissemination within the host (Saederup et al. 1999).

2.2.2 Preparation of virus stock

Cells were seeded in flasks until they reached 70-80% confluency. The cells were subsequently infected with virus at a Multiplicity of Infection (MOI) of 0.001 plaque forming units per cell (PFU/cell). After 5-7 days, depending on the state of the culture, the cells with the media was harvested and stored at -80°C.

2.2.3 Concentration and purification of viral stock

The harvested virus media (stock) is not useful in the form that it is collected (see section 2-2-2) because of the low viral titre. In order to increase the viral titre and purify prepared stocks, the harvested virus was concentrated using high-speed centrifugation. The harvested media containing the virus was centrifuged at 7,500 rcf for 20 minutes at 4°C. The Supernatant (SN1) was then kept on ice and the pellet was re-suspended in a total of 10 ml media and homogenized using a homogenizer. The mixture containing the disrupted cells was then centrifuged at 3,600 rcf for 20 minutes. Following centrifugation, the produced supernatant (SN2) was pooled with SN1, centrifuged at 30,000 rcf for 3 hours 4°C.

Following ultracentrifugation, the SN was discarded and the pellets are collected and re-suspended in maximum 4 ml and disrupted by homogenision in a homogenizer. A 20% sorbitol solution was added to 30 ml centrifuge tubes, into which the homogenized viral concentrate was then slowly pipetted (on top of the sorbitol). The tubes were then centrifuged at 30,000 rcf for 3 hours. The pellet was collected, re-suspended in a total volume of 3 ml 20% sorbitol solution, aliquoted into Eppendorf tubes and frozen at -80°C.

2.2.4 Viral infection assays.

Cells were seeded, one day prior to infection, into plates at the appropriate cell density. Based on the desired MOI and the cell number, the right viral PFU was calculated. Subsequently, the appropriate amount of virus was mixed with infection media and added to the plates. The virus was allowed to adsorb to the cells for 1 hour, after which the virus-containing media was removed. The cells were washed two times with 1x PBS and growth media was added. In the case of NIH-3T3, cells were not washed following adsorption due to the fragile nature of this cell strain.

2.2.5 Viral titration (Plaque assay)

The plaque assay is an assay to determine the viral titre and it is considered the gold standard. P53-/- MEF cell were seeded the day before in 24 well plates at a density of 5x 10^4 cells/well. Serial dilutions of the samples that were to be titred were prepared and then used to infect the P53-/- MEF cells prepared earlier. After the adsorption period the cells were covered with growth media mixed with 2.5% agarose for a final concentration of 0.36%. After 3-4 days, the number of plaques formed on the monolayer of the cells was counted and used to calculate the PFU/ml using the following equation.

$$\frac{PFU}{ml} = \frac{Numer\ or\ Plaques * dialution\ of\ viral\ stock}{Infection\ volume}$$

Of note, the MCMV-KO strain cannot be titred in p53-/- MEF cells as it is unable to replicate. For this strain the Bam-25 cell line was used that expresses the IE3 gene, thus, allowing the virus to replicate.

2.3 Genomics techniques

2.3.1 qRT-PCR for quantitation of mRNA transcripts

qRT-PCR is employed to quantify the relative abundance of mRNA transcripts. The mRNA isolated from harvested cells is transcribed into cDNA by reverse trancriptase. The cDNA transcripts are then amplified by PCR and the relative quantity is

determined by first normalizing the expression relative to the internal control values. based on comparative analysis of their Ct values.: The normalized data is then used to quantify the relative concentration of a given mRNA in accordance with the comparative cycle threshold ($2^{-\Delta\Delta CT}$) analysis(Livak and Schmittgen 2001; Lyman and Enquist 2009)

2.3.2 One Step qRT-PCR

Automated isolation of RNA from harvested cells was performed using Qiagen RNAeasy® Plus Mini Kit (cat. no. 74136) and QIAcube (Qiagen).

All one step qRT-PCRs performed for this thesis had RT negative controls to ensure that the sample did not contain DNA contamination and non-template controls to ensure there was no RNA contamination. Quanta 1-Step RT-qPCR ToughMix (95123) was used to perform all the qRT-PCR assays. The assay was performed in 96 well plates supplied by ThermoScientific (AB-0600/W) on a MXPRO3000P cycler (Stratagene, CA, USA). All the probes/primes were obtained by Life Technologies and their details can be reviewed in the appendix. All reactions were performed in 10 µl volumes using 25-75 ng of total RNA.

The reaction started with a 5 minutes cDNA synthesis step at 50°C, followed by an initial denaturation at 95°C for 1 minute. Finally, the samples are cycled from 95°C for 3 seconds to 60°C for 30 seconds (data collection step) for 40 cycles.

Table 2.1 Probes Information

Probe target	Order number
Actb	Mm02619580_g1
Hk3	Mm0131942_m1
Pfkfb3	Mm00504650_m1
Acly	Mm01302282_m1

Elovl4	LU-010269-00-0002
Elovl6	Mm00851223_s1
Elovl7	Mm00512434_m1
Mboat1	Mm00463555_m1
Mboat2	Mm00509052_m1
Dgat2	Mm00499536_m1
Scd1	Mm00772290_m1

2.4 Transfection and siRNA knockdown (KD) assays

2.4.1 siRNA knockdown

To transfect cells with siRNA two methods were employed. During reverse transfection, the transfection reagents are prepared and added to the well the same day that the cells are added. On the other hand, during the standard transfection method the cells are seeded into the wells and left overnight. The transfection reagents are added the next day. Reverse transfection has the advantage that it reduces the handling time and increases the absorption of siRNA by the cells. Forward transfection was utilized for some of siRNA targets, because it was found that it reduced the cell toxicity, while maintaining an acceptable KD level.

Transfection were performed in a 96-well plate with a final siRNA concentration of 25 nM/well. All siRNAs (ON-TARGET plus SMARTpool) were obtained by Dharmacon at a concentration of 2nmol and diluted to 2μM using siRNA buffer (5x siRNA buffer # B-002000-UB-100). On the day of transfection 1.25 μl of siRNA SMARTpool was mixed with 8.75 μl Optimem reagent (Invitrogen). 0.4 μl of Dharmafect1 (Dharmafect) was mixed with 9.6 μl Optimem. Following incubation for 5 min at room temperature the Dharmafect mix was added to the siRNA mix and

incubated for 30 minutes at room temperature. Subsequently, the siRNA mixture was added to the wells and $1.5x10^4$ cells/well (pMEFs or NIH-3T3), in a volume of 80 µl, was added to the wells and incubated for 48 hours. During transfection cells were grown in their respective growth media without the presence of antibiotics. The volumes described here was calculated for one well, but was scaled up appropriately for the assay.

After 48 hours the cells were treated depending on the experiment requirements. For all KD, three controls were used. A RISC free siRNA, which cannot be incorporated to the RISC complex, and a series of non-treated wells were used as negative controls. For viral infections M54 targeting siRNA was used as positive control. M54 targets specifically the MCMV polymerase and it was a custom made order from Dharmacon. Its 5'-3' sense strand sequence is AGAAAGACGACCTGAGCTA.

Table 2.2 SiRNAs Information

siRNA target	SiRNA name	Order number
Risc Free	SiGenome™ Control	0-001220-01-05
M54	-	-
Hk3	TARGETplus SMARTpool - Hk3 siRNA	L-045823-00-0005
Pfkfb3	ON-TARGETplus SMARTpool - Pfkfb3 siRNA	L-050027-00-0005
Elovl4	ON-TARGETplus ELOVL4 siRNA	LU-010269-00-0002
Elovl6	ON-TARGETplus ELOVL4 siRNA	LU-040378-00-0002
Elovl7	ON-TARGETplus SMARTpool - Elov17 siRNA	L-059708-01-0005
Mboat1	ON-TARGETplus MBOAT1 siRNA	LU-054725-01-0002
Mboat2	ON-TARGETplus MBOAT2 siRNA	LU-063482-01-0002

Dgatt2	ON-TARGETplus MBOAT2 siRNA	LU-060914-00-0002
Scd1	ON-TARGETplus SCD1 siRNA	LU-005061-00-0002

2.4.2 siRNA KD efficiency

In order to monitor the transfection efficiency for siRNA, qRT-PCR was used to measure the reduction in the relative abundance for each targeted gene transcript. Cells were transfected as described in section 2.4.1 followed by harvesting, RNA isolation and qRT-PCR (performed as in section 2.3.2). Some of the genes investigated in this thesis have very low expression levels in mock-treated cells making it difficult to determine accurate KD efficiency values. These genes are, however, highly upregulated during late time points of viral infection. For these genes, a different assay was devised to calculate the KD efficiency. Cells were first transfected for 24 hours and subsequently infected with MCMV-GFP (MOI 1). After 24 hours of infection, RNA was harvested and the abundance of each transcript was assayed by qRT-PCR.

2.5 Microarray gene expression analysis

2.5.1 Microarrays designs and experiments

Two microarrays datasets are used for this thesis, Macrophage Infection/Immunity Time-Course Hours 12 (MITCH12), and Macrophage Infection/Immunity Time-Course Hours 24 Knockout (MITCHKO24).

MITCH12 was performed for the thesis of Mathieu Blanc and MITCHKO24 for the thesis of Paul Lacaze(Blanc 2011; P. (University O. E. Lacaze 2011). Both experiments can be reviewed in detail in the thesis or the respective publications associated with them. Briefly, for MITCH12 BMDM from Balb/c mice were mock treated, or treated with MCMV-WT (MOI 1) or treated with 10 Units of IFN-γ. For each treatment, RNA was isolated every 30 minutes and used to perform the microarray experiment on a Mouse Agilent V2 array platform (G4121A, 20868 annotated probes)

In MITCHKO24, BMDMs from WT and IFNAR or IFNB deficient C57/BL6 mice were used. These were mock treated or treated with MCMV-WT (MOI 1), or MCMV-KO (MOI 1), or poly(I:C). The RNA from each treatment and cell type was isolated and high quality RNA was hybridised Affymetrix Mouse Gene 1.0ST microarray to profile the transcriptional response at 0, 2, 4, 6, 8, 10, 12 and 24 hours post treatment.

2.5.2 Microarray analysis

The MITCHKO24 raw data were imported into Partek Genomics Data 6.5 (Bilkei-Gorzo et al. 2017). The data were normalized using the quantile RMA method. This was followed by a QC check during which sample number 49 (IFNAR-/- BMDM, mock 0 hpi) did not pass the test and was subsequently excluded from all analysis. Finally, a per gene normalization step was followed.

The MITCH12 dataset was given to me, already normalized using Bioconductor R statistical package, by Dr. Thorsten Forster based at the Department of Infection and Pathway Medicine. The data were then smoothened using a lowess smoothing with span parameter of (f)=20% by Dr. Mizanur Khondoker to facilitate study and visualization. Further processing and analysis was performed by me using Partek Genomics Data 6.5 and Microsoft Excel.

2.6 Metabolomics

2.6.1 BMDM metabolome during productive vs. non-productive infection.

To study the metabolome of BMDM during infection, a metabolomics study was devised. This was a collaborative study between our Lab and the lab of Dr. David Watson at Strathclyde University.

BMDM from C57BL/6 JCR male mice were isolated and seeded in 6 well plates. After 7 days of maturation they were mock treated or treated with MCMV-WT (MOI 1) or MCMV-KO (MOI 1). Metabolites were isolated from the cells at 4 hpi and 24 hpi and shipped for quantification at Strathclyde University. To isolate metabolites, the cells were quickly washed twice with 3 ml 37°C of phosphate-buffered saline (PBS). Quenching of the cell's metabolism was performed by placing the culture plates on ice

and then adding 500 µl of ice cold extraction solution (methanol: acetonitrile: water – all HPLC grade – 50:30:20; pre-cooled by keeping on dry ice/methanol mixture). Subsequently the cells were scrapped off the plates, transferred to Eppendorf tubes and incubated on a rocker for 15 minutes at 4°C. The samples were spun down at 4°C (15,000 rpm) for 15 minutes and stored at -80°C, before being shipped to Dr. David Watson's lab for LC-MS analysis. The data were obtained on a Dionex 3000 HPLC (Thermo Fisher Scientific) coupled to an Exactive Orbitrap (Thermo Fisher Scientific).

LC-MS data were acquired on an Dionex 3000 HPLC (Thermo Fisher Scientific) coupled to an Exactive Orbitrap (Thermo Fisher Scientific) in both positive and negative mode set at 50,000 resolution (controlled by Xcalibur version 2.1.0; Thermo Fisher Corporation). The mass scanning range was m/z 75–1200, the capillary temperature was 320 °C and the sheath and auxiliary gas flow rates were 50 and 17 arbitrary units, respectively. The separation was performed on a ZIC-pHILIC column (150 × 4.6 mm, 5 µm from HiChrom, Reading UK) in binary gradient mode. The mobile phase used was 20 mM ammonium carbonate buffer (pH 9.2) and pure ACN; the flow rate was 300 $\mu L \cdot min^{-1}$. The gradient was programmed as follows: 0 min 20% A 80% B to time 30 min 80% A 20% B. The injection volume was 10 µL and the sample tray temperature was controlled at 12 °C during the measurement.

The Raw LC-MS files were processed by using m/z Mine 2.14 and searched against an in-house metabolite database. Graphical representations, tabular features and statistical analysis (*p*-value generation) were performed in Excel (Microsoft Office 2013).

SIMCA-P version 14.0 (Umetrics, Sweden) was used for PCA analysis which included PCA. The data were centred, and Pareto scaled.

2.6.2 Detection of eicosanoids in BMDM.

This study was a collaborative project with Dr Vallerie O'Donnel based at the University of Cardiff. BMDM from C57BL/6 JCR male mice were seeded in 6 well plates and mock infected or infected with MCMV-WT (MOI=1) or MCMV-KO (MOI=1) or MCMV-MCK2+ GFP. At 0, 12 and 24 hpi the cells and the supernatant

were collected and flash frozen in Liquid nitrogen. Subsequently they were stored at -80 0C before being shipped to Cardiff.

At Cardiff the samples were analysed for eicosanoids with LC/MS/MS and they were quantified using a 4000 Q-Trap and in comparison to deuterated internal standards. Standards were added to the 1ml sample (5ng 15-HETE-d8, 13-HODE-d4 and PGE2-d4 per sample) and the lipids were extracted by adding a solvent mixture of 1M acetic acid/isopropanol/hexane (2:20:30, v/v/v) to the sample at a ratio of 2.5ml solvent mixture:1ml sample. The new mixture was vortexed and 2.5 ml or hexane was added. Subsequently the sample was vortexed again and then centrifuged at 1500 rpm, 4oC for 5 min. The lipids were recover from the upper hexane layer and a new extraction of lipids from the samples took place as described above. The hexane layers were combined and dried under N_2 flow, or in vacuum (program1, vacuum 200, temp 30oC, Speed 35). The extracted lipids were stored at-70 ^{o}C.

Eicosanoids were quantified using LC/MS/MS: Samples were separated on a C18 Spherisorb ODS2, 5 μm, 150 x 4.6-mm column (Waters, Hertfordshire, UK) using a gradient of 50–90% B over 10 min (A, water:acetonitrile:acetic acid, 75:25:0.1; B, methanol:acetonitrile:acetic acid, 60:40:0.1) with a flow rate of 1 ml/min. Products were quantitated by LC/MS/MS electrospray ionization on an Applied Biosystems 4000 Q-Trap using parent-to- daughter transitions of m/z 319.2 (HETE, [M-H]-) to m/z 179.1 (12-HETE), 219.1 (15-HETE), 167.1 (11-HETE), 115.1 (5-HETE), m/z 351.2 (PGE, [M-H]-) to m/z 189.1 (PGD2), 271.1 (PGE2), and m/z 327.2 to 226.2 for 15-HETE-d8, and m/z 355.2 to 275.1 for PGE2-d4 with collision energies of -20 to -30 V. Products were identified and quantified by mass spectrometry using standard curves generated with the equivalent HETEs and PGs against 15-HETE-d8 and PGE2-d4 run in parallel under the same conditions.

2.6.3 Study of metabolism using Seahorse XF Extracellular Flux Analyzer

The Seahorse XF Extracellular Flux Analyzer can be used to directly measure the oxygen consumption rate (OCR) of cells and to indirectly measure the glycolytic

activity of cells by measuring the extracellular acidification rate (ECAR). I employed this instrument to compare the OCR and ECAR between mock treated and treated macrophage.

Macrophage were grown for grown for 7 days as described in 2.1.1, in Teflon pots instead of culture flasks or plates. Cells were seeded in Seahorse XF24 V7 PS Cell Culture Microplates one day before the experiment. The following morning, cells were treated or mock treated, for the specified time required in each experiment. Subsequently they were washed 3 times with Seahorse XF base medium. Next the plate was placed at 37°C for 1 hour to equilibrated CO_2 with the atmosphere, and then returned to the Seahorse XF Extracellular Flux Analyzer to perform the glycolysis rate assay. The seahorse experiment took place with the help of Dr. Roderick Carter.

2.6.4 Drugs used

The following inhibitors were used in a number of experiments during the thesis.

5-(Tetradecyloxy)-2-furoic acid (TOFA) Sigma Aldrich (T6575-5MG)

4-Methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75) Sigma Aldrich (C5490-5MG)

2-Deoxy-D-Glucose (2-DG) Sigma Aldrich (D6134-5G)

3 Chapter 3 Characterisation of productive and non-productive MCMV infection

3.1 Introduction

The first aim in this chapter is to characterise the growth characteristics of the different viral strains; MCMV, MCMV-pSM3fr, MCMVdie3 and MCMVdie3rev (from now on MCMV-WT, MCMV-KO and MCMV-GFP) that were used as a model for infection throughout this thesis and are reviewed in (Angulo, Ghazal, and Messerle 2000). The growth characteristics of the virus were studied in BMDM and NIH/3T3 permissive cells that constitute the main cell systems used throughout this thesis.

As a model for non-productive infection, MCMV-KO was used. MCMV-KO lacks the immediate early (IE) 3 gene, making it impossible for this viral strain to replicate outside of the complementing cell line Bam-25, which express the IE3 gene. For my thesis I had to corroborate that MCMV-KO can be used as a model for non-productive infection and does not replicate in NIH/3T3 or BMDM (Angulo, Ghazal, and Messerle 2000). MCMV-KO has all the components of the MCMV-WT strain but lacks the IE3 gene, which is essential for viral growth (Angulo, Ghazal, and Messerle 2000). Thus, MCMV-KO can, like MCMV-WT, enter cells, elicit an immune response, but unlike MCMV-WT cannot transcribe any gene apart from the IE1 gene (P. Lacaze et al. 2011). By comparing the effects that MCMV-WT and MCMV-KO infection have on cells, it is possible to pinpoint which alterations of metabolic pathways are due to viral manipulation or part of the immune response.

Regulation of metabolism is emerging as an important factor in infection. Multiple studies have demonstrated a tight integration of metabolism and immunity. The ability and need for both host and pathogen to alter the metabolome appears to be a key checkpoint that can determine the outcome of infection (Kotzamanis, Angulo, and Ghazal 2015). At the same time multiple viruses, including HCMV, have been shown to alter the metabolome of infected cells. HCMV, the human strain of MCMV, has been shown to manipulate lipid metabolism and FA biosynthesis extensively during

infection (Munger et al. 2006; Koyuncu et al. 2013; J.-Y. Seo, Yaneva, and Cresswell 2011; Munger et al. 2008; Vastag et al. 2011).

While similar studies have not been conducted for MCMV infection, it has been demonstrated that metabolic pathways are altered by MCMV infection. More specifically, the cholesterol metabolic pathway is downregulated on transcriptional level by the immune system (Blanc et al. 2011, 2013). Taking all the above into consideration I hypothesised that MCMV manipulates metabolic pathways associated with lipid and FA metabolism to promote viral progeny.

To tackle this problem, I analysed a dataset consisting of the temporal transcriptomic response of macrophages during MCMV productive (MCMV-WT), or non-productive (MCMV-KO) infection. By studying the differences in the transcriptome between productively infected and non-productively infected macrophages I could set apart key transcriptional events that are initiated by the virus or the MΦ in response to the viral infection. The genes identified were then clustered according to function, revealing that MCMV induces the expression of genes that belong to metabolic pathways associated with FA elongation and membrane remodelling during late stages of infection.

3.2 MCMV growth characteristics.

BMDM have been shown to be less permissive to MCMV infection than fibroblasts (KKropp et al. 2011). Kropp et al. 2011 demonstrated that BMDMs have a primed anti-viral state that can interfere with the MIEP of MCMV. BMDM-STAT1-/- were fully permissive to MCMV infection as they fail to induce IFN-I signalling, which has been to shown to inhibit MCMV growth in BMDM and fibroblasts (Gribaudo et al. 1993).

To corroborate these results and characterise my model virus, plaque assays were performed to study the growth kinetics of the MCMV-WT, MCMV-GFP and MCMV-KO used in this study. In our lab, a live cell replication assay was developed that was sensitive enough to measure the effects of drug- and siRNA-treatments in fibroblasts infected with MCMV-GFP. After MCMV-GPF strain infects cells it produces GFP

and thus the viral infection level can be assessed by detecting GFP fluorescence. The assay does not require the destruction of the cell cultures and thus the kinetics of the infection can be monitored more often, faster and at a lower cost than by using a plaque assay. BMDM infection could not be assessed using this assay, because BMDM have high auto-fluorescence making it impossible to get good signal-to-noise-ratio and obtain meaningful data.

According to this assay, NIH/3T3 were infected with a range of MOIs for 1 hour in a 96 well plate. Subsequently, an OPTIMA Polarstar plate reader was used to monitor the increase of viral growth by measuring GFP signal. As shown in (**Fig.3.1A)**, there is a correlation between the MOI with which the cells were initially infected and the GFP expression level over time.

BMDM or NIH/3T3 fibroblasts were infected with MCMV-WT, MCMV-GFP or MCMV-KO at a multiplicity of infection (MOI) of 1. Subsequently, supernatant was collected every day for 5 days and a plaque assays was performed to determine viral titres. The plaque assay was performed on p-53-/- fibroblasts for the MCMV-WT and MCMV-GFP strains and in BAM-25 cells for the MCMV-KO strain.

The results demonstrate that in each cell type MCMV-WT and MCMV-GFP have similar growth kinetics in either cell type used (**Fig.3.1B, C**). Additionally, it is shown that MCMV-KO growth was not detected in BMDM or NIH/3T3 beyond the initial day of infection revealing that it cannot replicate in the cells. MCMV-KO replication is detected in the media at day 0 because even after rigorous washing of the cells, from the infection media, it is impossible to remove all viral particles. It should be noted here that because NIH/3T3 cells are very fragile they were not washed after infection resulting in increased virus detection on day 1 post infection.

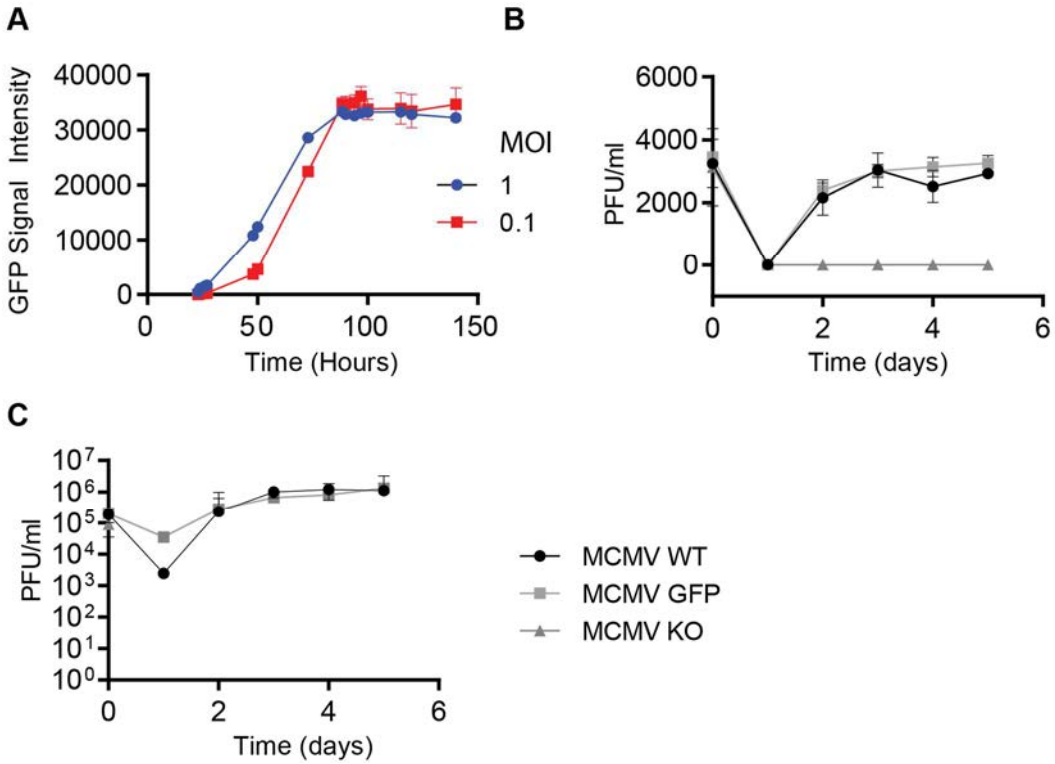


Figure 3.1 Growth kinetics of MCMV-WT, MCMV-GFP and MCMV-KO in BMDM and NIH/3T3. (A) NIH/3T3 cells infected at different MOIs with MCMV-GFP in 96 well plates. The GFP signal was measured using an OPTIMA Polarstar plate reader over 5 days. Three independent experiments mean SEM ± with 6 biological replicates (n=6). **(B)** BMDM were infected at an MOI of 1 with MCMV-WT, MCMV-GFP or MCMV-KO. At the indicated time points, samples were taken and the viral titres were determined using plaque assay. Data are mean SEM ± for three independent experiments with 3 biological replicates (n=3) **(C)** NIH/3T3 cells treated as in (B) The data are logarithmic and because of that data only data with values greater than 0 can be shown. Therefore MCMV-KO PFU/ml is only shown for day it the rest of the days 0 plaques were formed. Data are mean SD ± for three independent experiments with 3 biological replicates in each.

3.3 Analysis of transcriptomics data of bone marrow derived macrophages (BMDM) following productive infection (MCMV-WT), or non-productive infection (MCMV-KO) or poly(I:C) treatment.

Previous microarray studies conducted in our lab have generated a data resource studying the temporal transcriptomic profiling of BMDMs treated with MCMV-WT, MCMV-KO, or poly(I:C). The experiment called MITCH24KO (**Methods 2.4.12),** was conducted for the needs of Paul Lacaze's thesis and it can be reviewed in greater detail there (P. (University O. E. Lacaze 2011). I acquired the raw data from this resource and reanalysed it to study the differential effects that productive and non-productive infection would have over time in BMDM. The first aim of this analysis was to establish whether productive and non-productive infection program BMDM similarly at the transcriptional levels during early times of infection. The second was to identify whether MCMV infection alters metabolic pathways in infected BMDM and if this is related to FA metabolism.

MITCH24KO data set was generated by Paul Lacaze. Briefly, WT BMDM from C56BL/6 mice were treated with MCMV-WT (MOI=1) or MCMV-KO (MOI=1) or poly(I:C) over a 24-hour period. At 0, 2, 4, 6, 8, 10 and 24 hours post infection (hpi), RNA was isolated and used for the microarray analysis. In this experiment, poly(I:C) was used as a control for the non-productive infection. Poly(I:C) is a TLR-3 ligand which is a pattern recognition receptor that detects dsRNA and that has been shown to contribute to MCMV detection (Tabeta et al. 2004).

The raw dataset was retrieved and imported them into Partek Genomic Suite (MO) for analysis. The raw data was normalized using the RMA algorithm followed by a per-gene normalisation step. The normalised expression data was visualized for all the probes using a Principal Components Analysis (PCA) (**Fig.3.2A**). PCA is a statistical analysis that is useful in identifying patterns in complex data. PCA uses the covariance of these data to calculate the principle components (PC) with the first PC always having the bigger variance.

The 3D PCA graph of all probes in the dataset reveals how the transcriptomic profiles of the differently treated BMDM evolve spatially over time. At time point 0, the PC of MCMV-WT, MCMV-KO and poly(I:C) treatments are similar, indicated by their close clustering in the graph. This is expected as the genomic profile of all BMDM should be similar at time point 0, since the MCMV infections and poly(I:C) treatment have not had time to affect the cells' transcriptome yet. Over time the clusters for each treatment deviate reflecting the divergent gene expression of the BMDM due to the different treatments. It is interesting that all treatments are clustered together until 4 hours post treatment. After that time point, productive infection by MCMV-WT begins to increasingly deviate from the other treatments resulting in a different trajectory from the other treatments. This could be explained by the fact that by 4-6 hpi the virus would have initiated the expression of its Immediately Early (IE) genes allowing the virus to start taking control of the host cells and affecting its gene expression.

The results presented in **Fig 3.2A** included all the probes present on the microarray, even those that represented genes with low expression levels or whose expression was steady during all three treatments. Genes with expression levels below the detection threshold were subsequently removed as part of the initial processing of the dataset. A filtering step was performed in which probes showing no difference in their expression levels during at least one treatment and/or were expressed at a very low level were filtered out. This reduced the dataset to 6377 probes. On this probe set a second PCA was performed, shown in (**Fig.3.2B)**.

The new dataset shows the same trend as indicated previously, namely that the productive infection diverges from the other two treatments over time (**Fig.3.2B** compared to **Fig.3.2A**). In this instance, though, the divergence is faster, possibly due to the filtering that was applied. The removal of very low expressed probes and/or non-variant ones increases the variance the data set, as shown in the PCA used to calculate the principal components. The file of the 6383 probes can be found in the appendix file name [**6383_probes**].

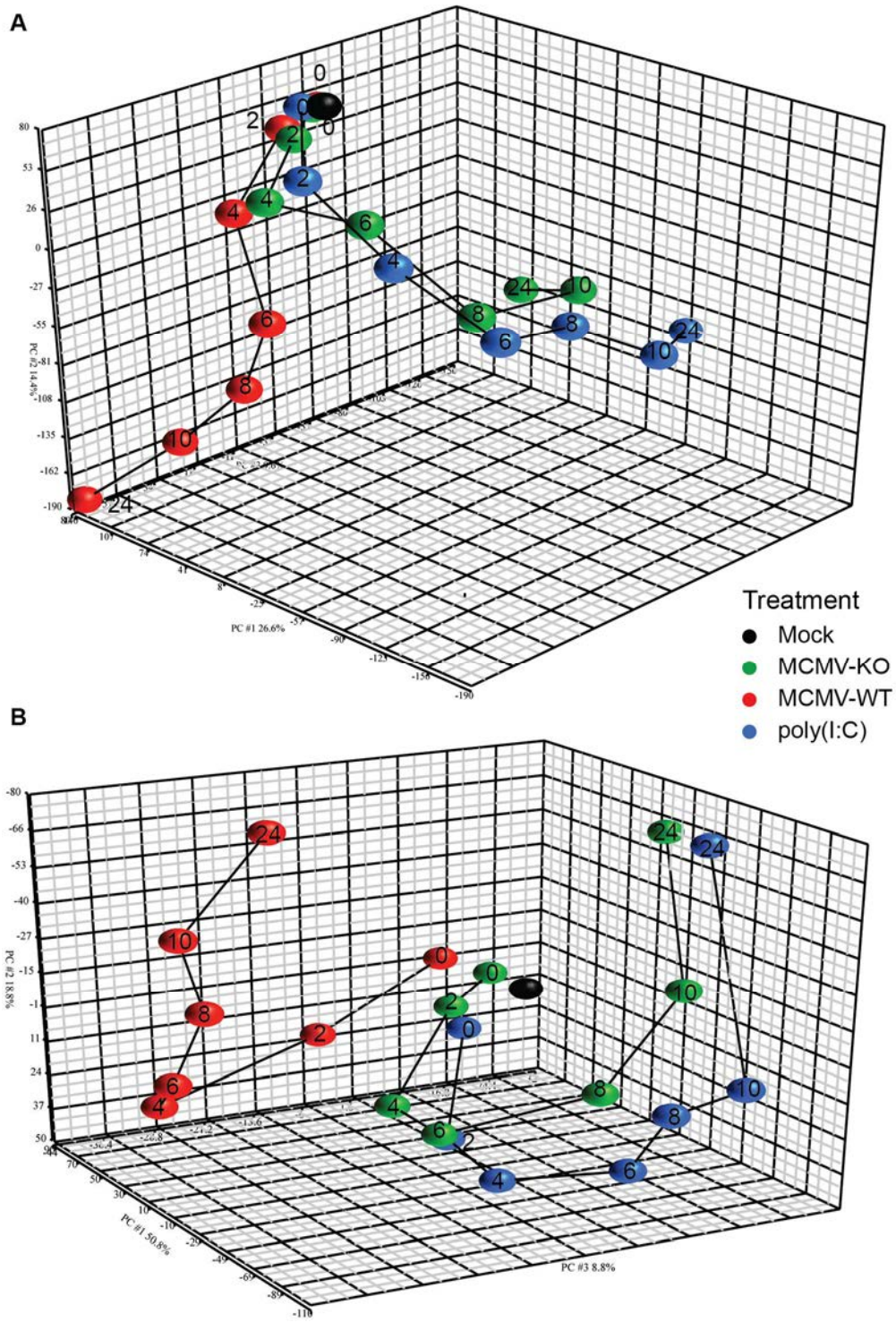


Figure 3.2 The transcriptomic profile of BMDM during infection (A) Genome wide PCA analysis of BMDM following productive infection (MCMV-WT), non-productive infection (MCMV-KO) and poly(I:C) treatment. Each treatment is represented by different colour shown in the legend. Each ball represents a time point after treatment in hours. **(B)** PCA analysis of 6377 probes filtered from the previous dataset shown in A.

3.4 Bioinformatics analysis of the transcriptome of infected BMDM's identifies fatty acid metabolism genes upregulated specifically during productive MCMV infection

3.4.1 Genes upregulated specifically during MCMV infection

Based on the results obtained above, it was hypothesized that there could be cellular genes that are critical for MCMV replication. Some of the genes that are pro-viral should therefore be expected to be upregulated, specifically during productive infection.

As shown in **Fig.3.2 A&B** the differences in gene expression between productive infection, non-productive infection and poly(I:C) treatment are more pronounced at 10 and 24 hpi. By focusing on these two-time points and filtering only the gene probes with a fold change of ≥1.5 in productively infected cells, I identified a subgroup of genes that represent a transcriptional response specifically associated with MCMV-WT infection.

Out of these of these gene probes, *Elongation of very long chain fatty acid 7* (*Elovl7*) was identified. *Elovl7* encodes for ELOVL7, an elongase that is involved in the elongation of very long chain fatty acids(Tamura et al. 2009). Since HCMV has been shown to induce synthesis of very long chain fatty acids and to investigate the hypothesis in this chapter I utilized the expression profile of this gene to identify other genes with similar expression profile (Koyuncu et al. 2013).

Elovl7 was also a good candidate because it shows a high magnitude of expression (fold change of 22) at 24 hpi, in comparison to 0 hpi. Furthermore, in mock, MCMV-KO or poly(I:C) treated cells *Elovl7* has a very low expression, close or even below the detection limit. This is also true for MCMV-WT treatment, up to 10 hpi. At 10 hpi or later *Elovl7* expression is greatly induced further indicating that this is a pro-viral gene with important function for MCMV. This suggests that the gene is not normally expressed in MΦ, but it is greatly induced specifically by MCMV-WT infection.

To identify genes with similar expression profile to *Elovl7,* the Pearson Dissimilarity algorithm in Partek Genomics Suite was used to rank the list of 6838 probes previously obtained. The top 250 gene probes with expression profiles most similar to *Elovl7* were filtered. After removing the duplicate probes 243 probes remained, corresponding to 243 individual genes. A heatmap of the genes was created to show their change in expression over time (**Fig.3.3A**). As shown in the heatmap (**Fig.3.3A**) a significant increase in the expression level for all genes was observed only in the productively infected samples after 8 hpi. Some of the genes, including *Elovl7*, have up to 22-fold change increase in their expression levels compared to their mean value. All the genes can be reviewed in the attached appendix file [**250_proves_Elovl7**].

Viruses have been known to exploit the host's cell-machinery including the mechanisms used to regulate it (Bolinger and Boris-Lawrie 2009). Thus, it was hypothesised that MCMV could be hijacking several transcription factors (TFs). These TFs should be, in such case, induced specifically by MCMV productive infection in early stages of infection.

Following the same workflow that was used for *Elovl7, Foxo1,* a well-known TF, was identified as being upregulated specifically during productive infection before 6 hpi. *Foxo1* was selected because it was upregulated specifically at early stages of productive infection, (fold change >4), a similar expression profile with *Elovl7* and it has been associated with regulating metabolism including FA. Using the Pearson Dissimilarity algorithm, the 6838 probes of differentially expressed genes were ranked based on their similarity to *Foxo1*. The transcription profiles of the top 250 most similar gene probes were selected. From these, 58 probes were common with the gene list generated using *Elovl7* as seed list leaving 192 unique probes. These 192 probes were attributed to 192 genes and a heatmap of these genes was created and can be viewed in (**Fig.3.3B**). The file of the of the probes can be seen in the appendix file name [**250_probes_foxo1**]

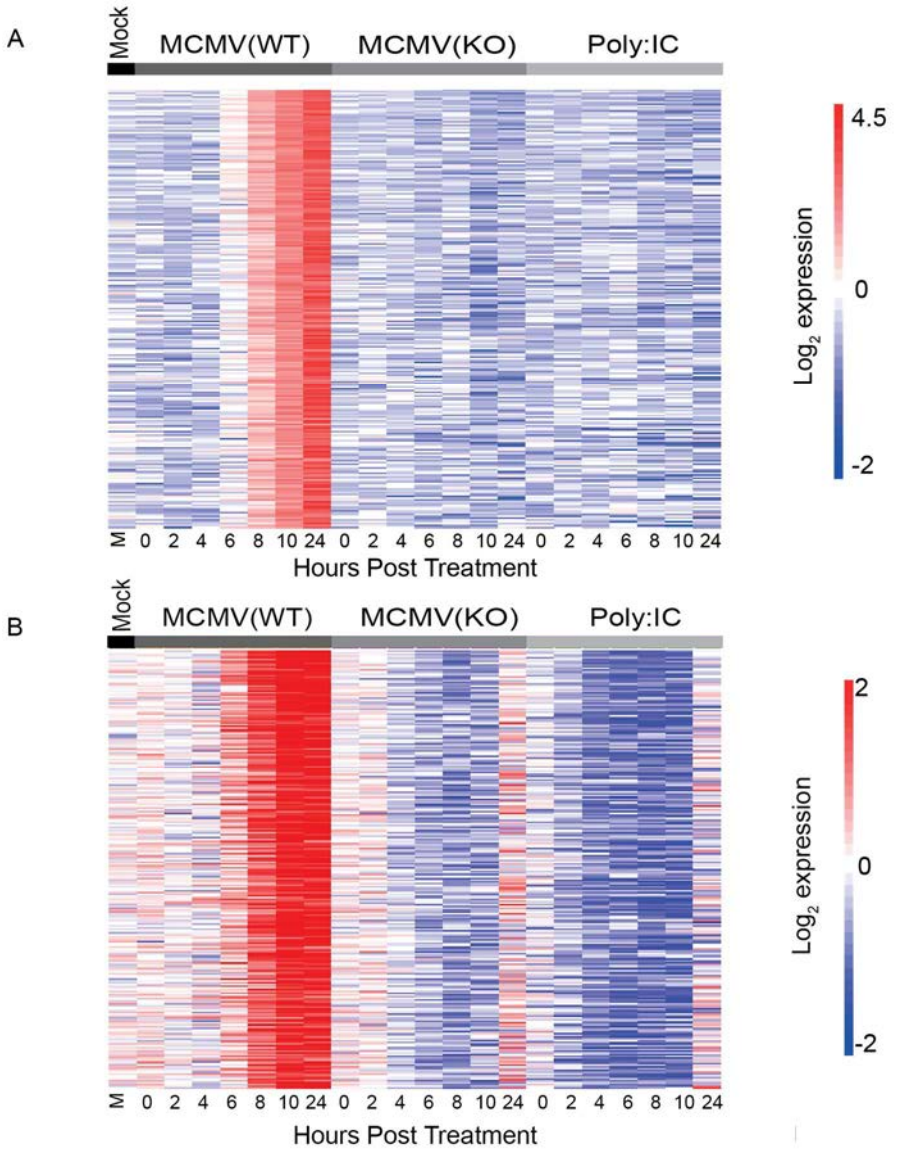


Figure 3.3 Subsets of macrophages genes based on the expression profile of *Elovl7* and *Foxo1.* (A) Heatmap of the 243 most correlated genes to *Elovl7*'s expression profile. Pearson correlation was used to identify genes with expression profiles highly correlated with *Elovl7*. Each gene is represented in a row and has been normalised to its mean value to show relative expression. Blue indicates a decrease relative to the mean and red an increase. **(B)** As is (A) for 192 most correlated genes to *Foxo1*.

3.4.2 Functional characterisation of genes identified to be upregulated specifically during MCMV infection

The genes identified above was in overrepresentation analysis to determine enriched metabolic pathways. The KEGG pathway database was queried using DAVID (**D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery) (D. W. Huang,

Sherman, and Lempicki 2008, 2009), which at the time this thesis was written could be found at https://david.ncifcrf.gov/.

Out of the 243 genes in my list, 34.9% (85) were identified as being part of 21 KEGG metabolic pathways (**Table 3.1**).

3.1 Significant metabolic pathways identified in the list of 243 genes

KEGG pathway	Count	*p*-Value
mmu04015:Rap1 signalling pathway	13	2.95E-06
mmu04390:Hippo signalling pathway	9	2.21E-04
mmu05200:Pathways in cancer	14	3.22E-04
mmu04360:Axon guidance	8	4.81E-04
mmu04810:Regulation of actin cytoskeleton	10	4.95E-04
mmu04530:Tight junction	8	7.53E-04
mmu04014:Ras signalling pathway	10	8.08E-04
mmu04520:Adherens junction	6	0.001069
mmu05205:Proteoglycans in cancer	9	0.001566
mmu05217:Basal cell carcinoma	5	0.002918
mmu04960:Aldosterone-regulated sodium reabsorption	4	0.009189
mmu04151:PI3K-Akt signalling pathway	10	0.013634
mmu04550:Signaling pathways regulating pluripotency of stem cells	6	0.016978
mmu04310:Wnt signalling pathway	6	0.018465
mmu05213:Endometrial cancer	4	0.018728
mmu04961:Endocrine and other factor-regulated calcium reabsorption	4	0.018728
mmu04010:MAPK signalling pathway	8	0.019853
mmu04916:Melanogenesis	5	0.022485
mmu00062:Fatty acid elongation	3	0.032112
mmu05216:Thyroid cancer	3	0.039286
mmu04918:Thyroid hormone synthesis	4	0.040389
mmu04971:Gastric acid secretion	4	0.043336

DAVID applies a Fisher Exact test to determine the *p*-value for the overrepresentation analysis. A *p*-value <0.05 was selected to filter out pathways.

In my second list of 192 genes, 14.7% (28) of them, were identified as being part of 6 KEGG metabolic pathways (**Table 3.2**.) using again a *p*-value <0.05 as significant.

Table 3.2 Significant metabolic pathways identified in the list of 191 genes

KEGG pathway	Count	*p*-value
mmu01100:Metabolic pathways	17	0.009665
mmu03020:RNA polymerase	3	0.017793
mmu04710:Circadian rhythm	3	0.018937
mmu04146:Peroxisome	4	0.019086
mmu00240:Pyrimidine metabolism	4	0.033398
mmu00230:Purine metabolism	5	0.033912

My hypothesis suggests that genes involved in lipid metabolism would be important for MCMV growth. With this in mind I compared the list of genes I had generated with a list of lipid genes that was generated in our lab based on the LIPIDMAPS database (Fahy et al. 2007). This comparison identified 22 lipids (5%) out of 442 genes that I had previously identified as related to lipid metabolism. Additionally, I was attempting to identify host factors that MCMV exploits to target and alter metabolism in infected cells. Thus I also compared the gene lists that I had generated with a list of genes that are either transcriptional factors (TF) or predicted to have TF function, according to Vaquerizas *et al.* (Vaquerizas et al. 2009). This last comparison indicated that 70 (16.1%) out of the 442 identified genes were TF or predicted to be TF.

3.4.3 Functional characterisation of genes using Ingenuity pathway analysis

To corroborate my results, I used the Ingenuity pathway analysis (IPA) software to perform a pathway analysis. IPA uses the gene lists you provide to creates functional networks with other genes with known functions. The genes from my lists were imported to the software and a twin tailed Fisher's exact test was performed using, this time, a more stringent *p*-value of <0.01. The results of the analysis can be viewed in the **Table 3.3**.

Table 3.3 IPA analysis of the gene lists

Clustered Genes	Networked genes
ABCB1,AMIGO2,ARFGAP1,ARMCX2,CECR2,CHFR,CHP1,COPB1, CREB3L4,DOCK9,ELOVL4,EPB41L5,FAM101B,GEMIN4,IFIH1,IGS F3,KDELR1,KDELR2,KDELR3,LAPTM4B,LRPPRC,MORC4,PCGF1, PNKD,RAC2,RARRES3,RBPMS2,RNF125,SLC9A2,SPTA1,SRSF5, TLCD1,UBC,UBE2E2,UBE2L6	Infectious Disease, Embryonic Development, Organismal Development
CACNB3,CLCN5,COBLL1,DACT1,FAM129A,FAM50A,FBXL20,FOS, GON4L,HDAC1,HVCN1,KCNIP3,MID2,NBPF15 (includes others),NT5C,NT5E,OCLN,P2RY8,PRCP,PXK,RAB37,RIMS1,SATB 1,SATB2,SBF2,SEPT5,SIPA1L2,SLC9A3R2,TET1,TPMT,TSPYL4,U BC,UNC13B,VDR,YPEL5	Cellular Assembly and Organization, Cancer, Tumour Morphology
AQP11, BOC,ELAVL1,ELOVL7, ELOLV4 F2R,Gpcr,GPR33,GPR61,GPR84,GPR111, GPR114,GPR144,GPR152,GPR162,GPR171,GPR173,GPR174, GPRC6A,HAC4,HEY1,LPAR4,MCAM,MYB,NDRG2,PDLIM1, PPARGC1A,PRKAA,SLC17A7,SREBF1,STAT3,STAT5A,TAAR8, TMEM56,TP53,VN1R2	Cell Death and Survival, Lipid Metabolism, Molecular Transport
ACVR1C,AGPAT1,ASCL2,BCL11A,BDH1,BMP2/4,BRCA1,C8orf4,CI TED1,CLYBL,CNTFR,DAB2,DSCR3,DUSP9,EPS8L3,ESR1,FGFR3, FGFR6,FGFRL1,FKBPL,GPANK1,GRB2,growth factor receptor,HIST1H2AD,HNF4A,LRP2,MBOAT2,PANX2,PEX16,PGGT 1B,RIOK2,RRAS2,RSPH3,SMAD3,TGFBR3	Developmental Disorder, Skeletal and Muscular Disorders, Cellular Development
ARMC7,B3GNT5,C2orf88,CDC25C,DNMT3B,EFNA1,EFNA2,EFNA3 ,EFNA4,EFNA5,Efna,ELL2,EPHA1,EPHA2,EPHA3,EPHA4,EPHA5,E PHA6,EPHA7,EPHA8,EPHA,EPHB1,EPHB2,EPHB4,HOXA13,MKI6 7,MYF6,MYOD1,NUPR1,RAB39B,RECK,RPP25,SAFB,SLC2A1,ZNF 238	Cell Morphology, Cellular Compromise, Cellular Assembly and Organization
GCNT1,GCNT3,GCNT4,N-acetyllactosaminide beta-1,6-N-acetylglucosaminyl-transferase	Embryonic Development, Organ Development, Organ Morphology

Most of the networks shown involve embryonic development or cellular assembly and organization. MCMV and HCMV are known to reorganise the cytoskeleton and affect

the morphology of infected cells (Lyman and Enquist 2009; J.-Y. Seo et al. 2011; Le et al. 2008).

3.5 Discussion

Metabolism is emerging as an important factor during immunity and viral infection (L. A. J. O'Neill, Kishton, and Rathmell 2016; Vastag et al. 2011). In this chapter I employed analysis of temporal transcriptomic data in MCMV infected BMDM to identify metabolomics pathways specifically induced by MCMV-WT.

Following an unbiased initial analysis of the data I identified temporal expression profile of host genes in BMDM treated with MCMV-WT, MCMV-KO and poly(I:C). This indicates that at early time of infection, the immune system response triggered by MCMV or poly(I:C) is driving the cells programming. After 4 hours, MCMV has initiated its transcriptional program and, thus, starts interfering with the host cell before taking over infected cells. MCMV-WT infection begins diverging from the MCMV-KO and poly(I:C) treatments at 4 hpi, and at 24 hpi it has diverged the most. MCMV-KO and poly(I:C) treatments evolve differently, but they are very close spatially and they appear to follow similar paths. This is an indication that BMDM respond similarly to these two treatments, which is expected as both poly(I:C) and MCMV-KO initiate an immune response, but after the initial signalling there is no more interaction. The differences in the response could be explained by the fact that MCMV-KO is an inactive virus, but that it still has several tegument proteins. Some of tegument proteins are known to interact and subvert immune signalling during CMV infection (Chevillotte et al. 2009). Additionally, MCMV-KO is recognized by a wider range of PRR than poly(I:C).

After the initial analysis, I wanted to focus on metabolic genes that were specifically induced by MCMV-WT infection during late time of infection. HCMV has been reported to upregulate genes related to FA synthesis strongly after 24 hours in its 96 hours replication cycle *in vitro* (Purdy, Shenk, and Rabinowitz 2015; Spencer et al. 2011). The initial induction of *Elovl* genes expression starts at 24 hours and FA biosynthesis inhibition is able to block HCMV replication at even 48 hours post infection (Spencer et al. 2011; Purdy, Shenk, and Rabinowitz 2015).

Genes that were upregulated specifically during MCMV-WT infection, with a fold change > 1.5, were filtered out from my gene list. *Elovl4&7* genes were in this list of genes and because of the relevance of FA metabolism to HCMV, I chose to use their transcriptomic profile to identify genes with a similar strong induction, specifically during late stages of infection (Purdy, Shenk, and Rabinowitz 2015). This indicates that MCMV like HCMV is dependent on similar pathways.

I chose to use the *Elovl7* gene over *Elovl4* for the generation of my list, because ELOVL7 has been associated with the generation of very long saturated fatty acids, the same kind of FA that HCMV induces during infection (Tamura et al. 2009; Vastag et al. 2011; Jakobsson, Westerberg, and Jacobsson 2006; Koyuncu et al. 2013). ELOVL4 is associated with the elongation of polyunsaturated fatty acids (Ohno et al. 2010; Tamura et al. 2009)

The use of *Elovl7* on a genome wide analysis in MCMV infected BMDMs at late and early time of infection identified 243 and 192 genes specifically upregulated by MCMV productive infection. Out of these, 70 were predicted to be TF based on a list published by (Vaquerizas et al. 2009).The bioinformatics analysis indicated that MCMV might have a strong dependency on fatty acid and lipid metabolism, identifying 13 genes of the former and 9 genes of the latter, that are specifically upregulated during productive infection.

Functional characterisation of the 243 genes using DAVID associated them with, amongst others, Rab & Ras signalling, regulation of actin cytoskeleton, PI3K-AKT signalling, Wnt signalling, fatty acid elongation pathway and pathways associated with cancer. As discussed earlier Rab & Ras proteins have been found to be crucial for HCMV and MCMV infection. Additionally, HCMV has been shown to remodel the cytoskeleton of infected cells (Lyman and Enquist 2009; J.-Y. Seo et al. 2011; Le et al. 2008). Furthermore, given the huge morphological changes that HCMV and MCMV infected cells undergo it is expected that both viruses would interact with cell development and cell organisation pathways, such as the Wnt and the cytoskeleton organisation pathways (Jones, Lewis, and Kilpatrick 1986; Angelova et al. 2012; Melnick, Deluca, and Jaskoll 2014). Finally, Munger et. al have shown that FA synthesis is upregulated upon HCMV infection in Human Foreskin Fibroblasts (HFF)

(Munger et al. 2008). This pathway also came up in my functional analysis further supporting its role in CMV infection. IPA analysis also reinforces all of the above by revealing the presence of networks associated with developmental, cellular morphology, molecular transport and lipid pathways.

The association of cancer-related pathways with MCMV is captivating. This association could be expected as the energy metabolism of HCMV infected cells and tumour cells is similar (Munger et al. 2008, 2006). The PI3K-AKT signalling pathway that was identified in my analysis has been shown to be crucial signalling pathway for survival and cell cycle control (L. Yang, Venneti, and Nagrath 2017). Among other functions it regulates proteins translation and energy metabolism. HCMV and MCMV have been shown to directly interact with it in numerous ways to alter metabolism and prevent the apoptosis of infected cells (Yatim and Albert 2011; Moorman et al. 2008; Poglitsch et al. 2012). It is interesting that cancer cells also manipulate the PI3K-AKT pathway for similar reasons, to promote survival and also alter their metabolism and support their need to propagate (Raja Singh et al. 2017; Tserga et al. 2016).

Furthermore, based on several studies and data obtained by another member of our lab, Dr. Wein Hsiesh, the third network in **Table 3.3**, which includes many G-proteins, lipid metabolism and molecular transport associated genes, is of particular interest (Das and Pellett 2007; Das, Vasanji, and Pellett 2007; Liu et al. 2011; Indran and Britt 2011). The vesicular transport system, in which Rab proteins play an important role, has been shown to be crucial for HCMV replication (Das, Vasanji, and Pellett 2007; Liu et al. 2011; Indran and Britt 2011). Wein Hsiesh, has identified the prenylation pathway, which is directly linked to the vesicular transport system and is part of the mevalonate pathway, to be crucial for viral progeny during MCMV infection (Hsiesh 2015).

Additionally, the same network includes proteins involved in lipid metabolism and FA elongation. In eukaryotes, very long chain fatty acids (VLCFA) are formed form the elongation of fatty acids that are either produced by fatty acid synthase (FASN) or obtained by diet (Cinti et al. 1992). Despite their small presence in mammalian tissues VLCFA play an important role in number of biological processes and are essential for the synthesis of phospholipids and sphingolipids(Poulos 1995). Furthermore, FA

elongases, two of which are included in this network, have been shown to be important for HCMV replication (Purdy, Shenk, and Rabinowitz 2015).

In conclusion, the data presented here support the notion that productive MCMV infection affects a plethora of genes in infected BMDM. The functional characterisation of these genes revealed an extensive manipulation of metabolic and cell developmental/organisational pathways by MCMV infection. This appears to be consistent with the known effects that MCMV and HCMV infection has in infected cells. Although the analysis alone in not sufficient to reach final conclusions it appears that productive MCMV infection has a dependency on a number of pathways associated with lipid metabolism. These pathways are related to FA elongation, and/or vesicular transporting supporting the notion that MCMV extensively manipulates the host cell's molecular transport and resources.

4 Chapter 4 Co-option of BMDM metabolism by MCMV productive infection during early time of infection

4.1 Introduction

Homeostasis is the ability of a system to maintain equilibrium by actively regulating itself and other systems that cross it. To maintain a dynamic equilibrium, a system must be able to respond to a wide range of external stimuli and tightly coordinate and integrate numerous diverse processes. It is a key characteristic of living organisms and underpins the regulation of metabolism and the immune system.

Traditionally metabolism and immunity have been studied by different research communities and out of the context of each other. However, recently a number of advances in both fields revealed that they are tightly integrated(L. A. J. O'Neill, Kishton, and Rathmell 2016; Pearce et al. 2013). Immune cells alter their metabolism upon activation, a critical step for them to support their effector functions. This becomes evident in the case of T cells for example that upon activation have to proliferate extensively and rapidly. For T cells to proliferate and mature they need to switch from catabolic to anabolic metabolism. To support these increased bioenergetic needs, naive T cells increase their oxidative phosphorylation along with inducing aerobic glycolysis(Palmer et al. 2015; Shi et al. 2011; Gubser et al. 2013; Michalek et al. 2011; Buck, O'Sullivan, and Pearce 2015). Inhibition of glycolysis doesn't inhibit the ability of effector T cells to undergo clonal expansion, but prevents them from fully acquiring their effector functions(C.-H. Chang et al. 2013; Palmer et al. 2015; Cham et al. 2008).

Similarly, classically activated DCs and MΦ upregulate their glycolytic pathway and alter their Tricarboxylic acid (TCA) cycle. In contrast to T-cells, MΦ and DCs exhibit a broken-down TCA cycle at two points, at citrate and succinate. Citrate is used by the cells to support FA and itaconate production, while succinate accumulation has been show to promote the secretion of certain cytokines. Because of their uncoupled TCA cycle these cells rely on glycolysis for ATP generation. It is not very well understood

why these cells change to this more inefficient way of producing energy. Several studies have indicated that Nitric oxide (NO), an important antimicrobial metabolite that is produced by classically activated DC and MΦ, inhibits the electron transport(Beltrán et al. 2000; Everts et al. 2012).

Control of homeostasis is very important for viruses too, as upon infection viruses have to regulate metabolism to meet their own metabolic needs. Viruses are obligatory parasites that are dependent on their host's cell machinery to replicate. They need to exploit and counter host pathways, including metabolism, to effectively propagate. A number of viruses have been shown to do that, including but not limited to, HCV, HIV, rift valley virus, and human cytomegalovirus (HCMV) (Vastag et al. 2011; Moser, Schieffer, and Cherry 2012; Zheng et al. 2003; M.-L. Chang 2016).

The metabolome of HCMV infected human foreskin fibroblasts (HFF) has been studied extensively(Vastag et al. 2011; Munger et al. 2008; Koyuncu et al. 2013; Purdy, Shenk, and Rabinowitz 2015; Terry et al. 2012; Munger et al. 2006; Yu, Maguire, and Alwine 2011; Chambers, Maguire, and Alwine 2010). HFF infected by HCMV have increased glucose uptake and FA synthesis. Glucose is converted to lactate to a greater extent in HCMV infected HFF than in mock infected HFF(Vastag et al. 2011). The TCA cycle flux is, thus, increased enabling it to support energy production through oxidative phosphorylation and anabolic demands by providing important intermediates. (Munger et al. 2008; Purdy, Shenk, and Rabinowitz 2015) Through the TCA cycle, carbon atoms from the glycolytic pathway are shuttled to FA biosynthesis. The FA biosynthesis pathway is crucial for HCMV proliferation and inhibiting it blocks viral progeny (Koyuncu et al. 2013). In this context, the metabolic alteration that MCMV infected cells has not been studied yet.

It becomes apparent that activated immune cells, and especially macrophages have similar metabolism to HCMV infected Human foreskin fibroblasts (HFF) (Vastag et al. 2011; Biswas and Mantovani 2012; Kotzamanis, Angulo, and Ghazal 2015). Interestingly the increased glycolysis rate and alteration in the TCA cycle appear to play a critical role both for the outcome of infection and the effector function of MΦ. According, to the central hypothesis that MCMV coops the metabolic programming of activated BMDM during early times of infection in order establish infection, I

propose that the glycolytic pathway and the TCA cycle should be primary candidate pathways for co-option during early times of infections. This raises a few questions that I will attempt to answer in this chapter.

1) Does MCMV upregulate glycolysis during infection in macrophages, and is its growth dependent on this.
2) Does MCMV infection affect the TCA cycle and/or other linked pathways early during infection.
3) Are these alterations in the metabolic programming of BMDM driven by host or pathogen events?

4.2 Early temporal activation of the glycolytic capacity and activity upon macrophage infection

To investigate glycolysis in the context of viral infection, transcriptomic and metabolomics studies were performed on MCMV infected Bone Marrow Derived Macrophages (BMDM). I analysed MITCH12 raw data, a data resource available at our lab, which was performed by Mathieu Blanc. MITCH12 is a time series analysis of gene expression of MCMV infected Bone Marrow Derived Macrophages (BMDM) (**Fig.4.1A-B**). Briefly, BMDM were infected with MCMV and RNA was harvested every 30 minutes up to 12 hours post infection (hpi) and hybridised on an Agilent microarray chip.

The expression of genes in the glycolytic pathways normalised at time point 0 of infection was plotted in a heatmap diagram. A rapid induction of key enzymatic steps in the glycolytic pathway was observed over the course of the infection (**Fig.4.1A-B**). The infection affects enzymes involved in at least three, of the four, key regulatory steps of

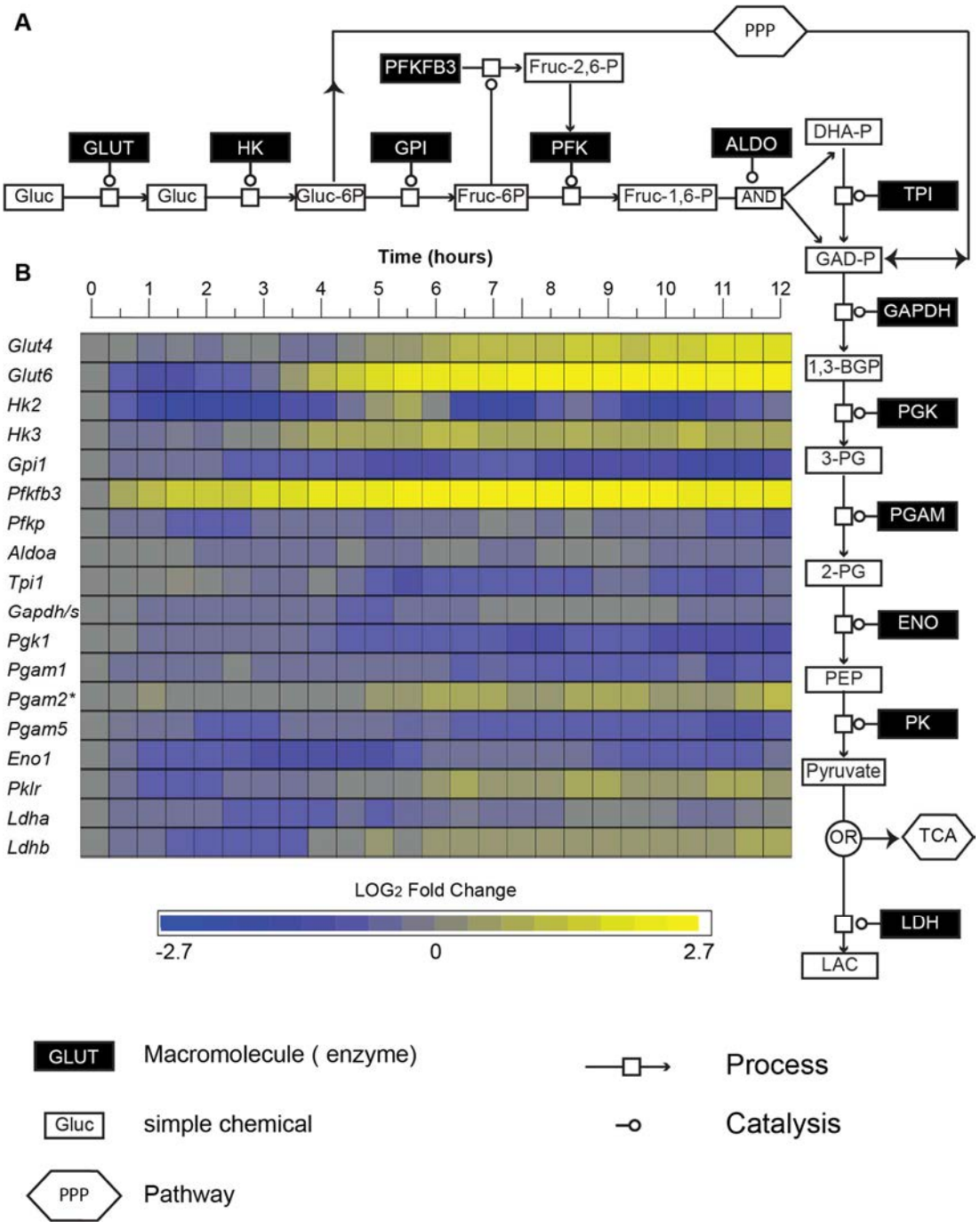


Figure 4.1 The glycolytic pathway is upregulated at the transcriptional level in MCMV-WT infected macrophages. (A) The glycolytic pathway. **(B)** Heatmap of the glycolytic pathway temporal gene's expression over 12 hours in BMDM infected with MCMV-WT (MOI 1). Each time point corresponds to one independent biological sample, and columns indicate time in hours. Fold changes of expression levels are represented on a Log_2 scale compared to time point zero. The * near a gene represents genes close to the level of detection. **(A)** Gluc: Glucose; Gluc-6p: Clucose-6P; Fruc-6P: Fructore-6P; Fruc-1,6-P; Fructose-1,6P; Fruc-2,6-P; Fructose-2,6P; DHA-P: Dihydroxyacetone-P; GAD-P Glyceraldehyde-3P; 1,3BGP: 1,3-Bisphosphoglyceric acid; 3-PG: 3-Phosphoglyceric acid; 2-PG: 2-Phosphoglyceric acid; PEP; Phosphoenolpyruvic acid; LAC: lactate; **(B)** *Glut4*: Glucose transport 4:

Glut6: Glucose transporter 6; *Hk2*: hexokinase 2; *Hk3*: hexokinase 3; *Gpi1*: glucose phosphate isomerase 1; *Pfkfb3*: 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; *Pfkp*: phosphofructokinase, platelet; *Aldoa*: aldolase 1, A isoform; *Tpi1*: triosephosphate isomerase 1; *Gapdh/s*: glyceraldehyde-3-phosphate dehydrogenase /spermatogenic; *Pgk1*: phosphoglycerate kinase 1; *Pgam1*: phosphoglycerate mutase1; *Pgam2*: phosphoglycerate mutase 2: *Pgam5*: phosphoglycerate mutase family member 5; *Eno1*: enolase 1, alpha non-neuron; *Pklr*: pyruvate kinase liver and red blood cell; *Ldha*: lactate dehydrogenase A; *Ldhb*: lactate dehydrogenase B

glycolytic pathway(**Fig.4.1A**). The first regulatory step of glycolysis is the import of glucose in a cell. The import of glucose is regulated by proteins that belong to the Solute Carrier Family 2 (*Sl27a*), commonly named Glucose Transporters (*Glut*). The expression of *Glut4* and *Glut 6* is rapidly induced in MCMV infected BMDM as early as 3.5 hpi and remains high until 12 hpi (**Fig.4.1B**). This is similar to previous work shown in HCMV infected fibroblasts were *Glut4* expression was also increased (Yu, Maguire, and Alwine 2011; J.-Y. Seo and Cresswell 2013).

Following import, cytosolic glucose is converted to glucose-6-phosphate (glucose-6P) by hexokinase (HK). This step is kept under tight regulation in cells because it requires ATP and it could deplete the cell's ATP reserves very quickly upon high accumulation of glucose. Microarray analysis indicates that *Hk3* expression is induced by MCMV infection early in infection at 3.5 hpi.

The conversion of fructose-6P to fructose-1,6-P, by phosphofructokinase (PFK), is the most prominent step in regulating the mammalian glycolytic pathway. This conversion has a high negative ΔG^{o} making it practically irreversible and it has the lowest kinetic rate in the glycolytic pathway. Moreover, this is the first commitment step for glucose to enter the glycolytic pathway. Alternatively, glucose-6P generated by HK can be utilised by other pathways such as the pentose phosphate pathway (PPP) or glycogen generation (Daemen, Kutmon, and Evelo 2013).

Notably, MCMV infection appears not to directly induce *Pkf*, but instead induces the expression of *6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3* (*Pfkfb3*) as early as within 30 minutes of infection. PFKFB3 catalyses the conversion of fructose-6P to fructose-2,6-P (fruc-2,6-P), which is a strong allosteric activator of the PFK. High levels of ATP allosterically inhibit PFK, while AMP reverses that effect. Fruc-

2,6-P is a very strong allosteric activator of PFK and it can reverse the effects of ATP. Furthermore, PFKFB3 silencing using siRNA, has been shown to reduce glycolysis by up to 40% in some cell types(De Bock et al. 2013). *Pfkfb3* upregulation has been shown to be crucial in proinflammatory macrophages (Jiang et al. 2016).

To examine if these changes were reflected at the metabolomic level, a metabolomics analysis of intercellular metabolites from BMDM was conducted. BMDM were infected or mock infected with MCMV-WT; intracellular metabolites were isolated at 4 hpi and analysed using LC-MS. Three metabolites were detected from the glycolytic pathway: Glucose-6P, Glyceldehyde-3P and Glycerate-3P (**Fig.4.2A-C**). The depletion of Glucose-6P and Glycerate-3P in combination with the increased levels of Glyceraldehyde-3P suggests increased levels of glycolysis in the infected BMDM.

To investigate functional requirement of the glycolytic activity for MCMV infection, the glycolytic activity of MCMV infected or mock-infected BMDM was examined using Seahorse XF Extracellular Flux Analyzer. Seahorse XF Extracellular Flux Analyzer instrumentation can directly measure the oxygen consumption rate (OCR) and glycolytic activity indirectly of cells by measuring the extracellular acidification rate (ECAR) (C.-H. Chang et al. 2013; Wu et al. 2016; Berod et al. 2014) . BMDM were infected, or mock infected, with MCMV-WT and 1.5 hpi their OCR and ECAR were measured. The mock infected BMDM appear to have increased OCR levels in comparison to MCMV-WT infected MΦ before the addition of glucose in the media (**Fig.4.2D**). The high OCR levels of mock-infected cells were reduced after the addition of glucose, indicating that in the absence of glucose, these cells use oxidative phosphorylation to produce ATP. On the other hand, the MCMV-WT BMDM have a steady level of OCR through the duration of the experiment, indicating that despite the glucose starvation they do not engage oxidative

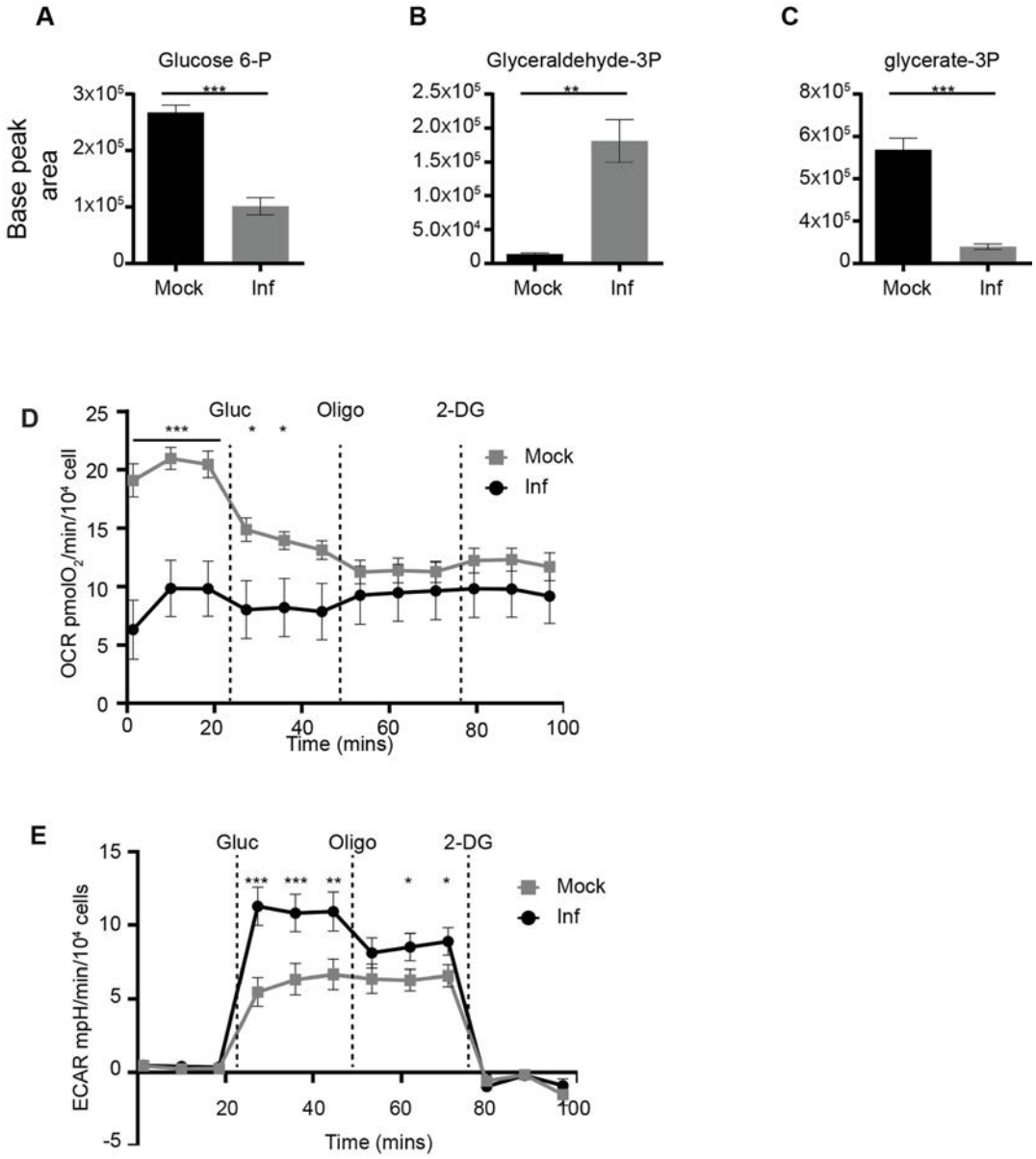


Figure 4.2 Upregulation of glycolysis on the transcriptional level is reflected on the metabolomic level. (A-C) Levels of glycolysis metabolites level of infected versus mock infected BMDM 4 hpi. Each bar point represents a mean ± SEM of one experiment with 6 biological replicates *p<0.05, **p<0.01, ***p<0.001 with an unpaired Student's t test (**D-E**) Real-time changes of OCR and ECAR of BMDM 1.5 hours after they have been mock infected or infected with MCMV-GFP and subsequently treated with Glucose (Glug), Oligomycin (Oligo) and 2-Deoxyglucose (2-DG). Each data point represents a mean ± SEM from 2 independent experiments with 5 biological replicates each.

phosphorylation. This is in agreement with the previously published studies that indicate that DC cells do not utilise OXPHOS for energy generation when they are

engaged by the TLR ligand LPS, which is reflected by lower consumption of O_2 (Everts et al. 2014).

The ECAR levels for both mock infected and MCMV-WT infected MΦ are close to 0 at the start of the experiment, which is expected since there is no glucose in the media (**Fig.4.2E**). After the addition of glucose to the media both the mock and MCMV-WT infected cells increase their glycolytic activity significantly. The MCMV-WT infected MΦ have greater glycolytic activity in comparison to the mock infected cells, which corroborate the transcriptomics and metabolomics data presented in **Fig.4.1B** and **Fig.4.2A-C**. The data are also in accordance with previously published studies that demonstrate that under certain circumstances some immune cells rely mainly on glycolysis for energy production to support their effector functions (Krawczyk et al. 2010; Pearce et al. 2013; Everts et al. 2014; Everts and Pearce 2014).

Taken together these data demonstrate that MCMV-WT infected MΦ have increased glycolytic activity in comparison to mock-infected cells. The effects at the transcriptional and metabolomic level appear to take effect very early during infection. More specifically the effect on the mRNA levels of *Hk3* and *Glut6* are evident at 3.5 hpi while *Pfkfb3* expression is induced earlier, 30 minutes post infection (**Fig.4.1B**). The effects on the metabolome are evident as early as 1.5 hpi. This is an indication that the induction of the glycolytic pathway is triggered by the immune system and not by viral manipulation of the infected cells as MCMV could not have initiate its transcriptional program so early during infection.

4.3 Enhanced glycolytic activity of MΦ is required for viral growth

To investigate the importance of glucose metabolism for viral replication I next blocked the pathway pharmacologically as well as by knocking down (KD) the expression of two glycolytic enzymes *Hk3* and *Pfkfb3* using RNA interference (RNAi). NIH/3T3 and primary murine embryonic fibroblasts (pMEFs) were infected with MCMV-GFP and treated with increased doses of 2-Deoxyglucose (2-DG). The viral growth rate was then monitored over time by monitoring the increase in fluoresce. The

Inhibitory Concertation for the drugs was determined to quantify the IC_{50} levels of the drug on the viral growth. IC_{50} is the concentration of an inhibitor that reduces the activity of an enzyme or an effect by half.

2-DG treatment inhibited viral growth in a dose dependent manner in both cells types (**Fig.4.3A&B)**. Furthermore, the IC_{50} concentration for 2-DG was lower than the concentrations at which 2-DG exhibited cytotoxic effects (**Fig.4.3C**). In addition to its inhibitory effects on viral growth in NIH/3T3 cells, 2-DG was able, in a dose dependent manner, to inhibit viral growth in BMDM infected MCMV-GFP (**Fig.4.3D**).

To further corroborate these results, I used RNA interference (RNAi) to KD *Hk3* and *Pfkfb3* in NIH/3T3 cells. I used ON-TARGET Plus™ siRNA from GE Dharmacon that reduces the chance of off targets effects by using 4 different siRNAs to target the same gene. The KD efficiencies for *Hk3* was below the 50% reduction threshold commonly used as a cut off for a successful KD (**Fig.4.3E**) and thus I cannot exclude that any effects observed are not due to side targets of the siRNAs. Despite the KD of either gene product, adverse effects on the cell viability of the treated cells was not observed (**Fig.4.3F**). The M54 siRNA targets M54, which is the viral DNA polymerase and acts as positive control for the treatment.

Despite the reduced KD efficiency of *Hk3*, both treatments inhibited the viral growth in the treated cells (**Fig.4.3G**), further supporting the role of glycolysis in promoting viral replication.

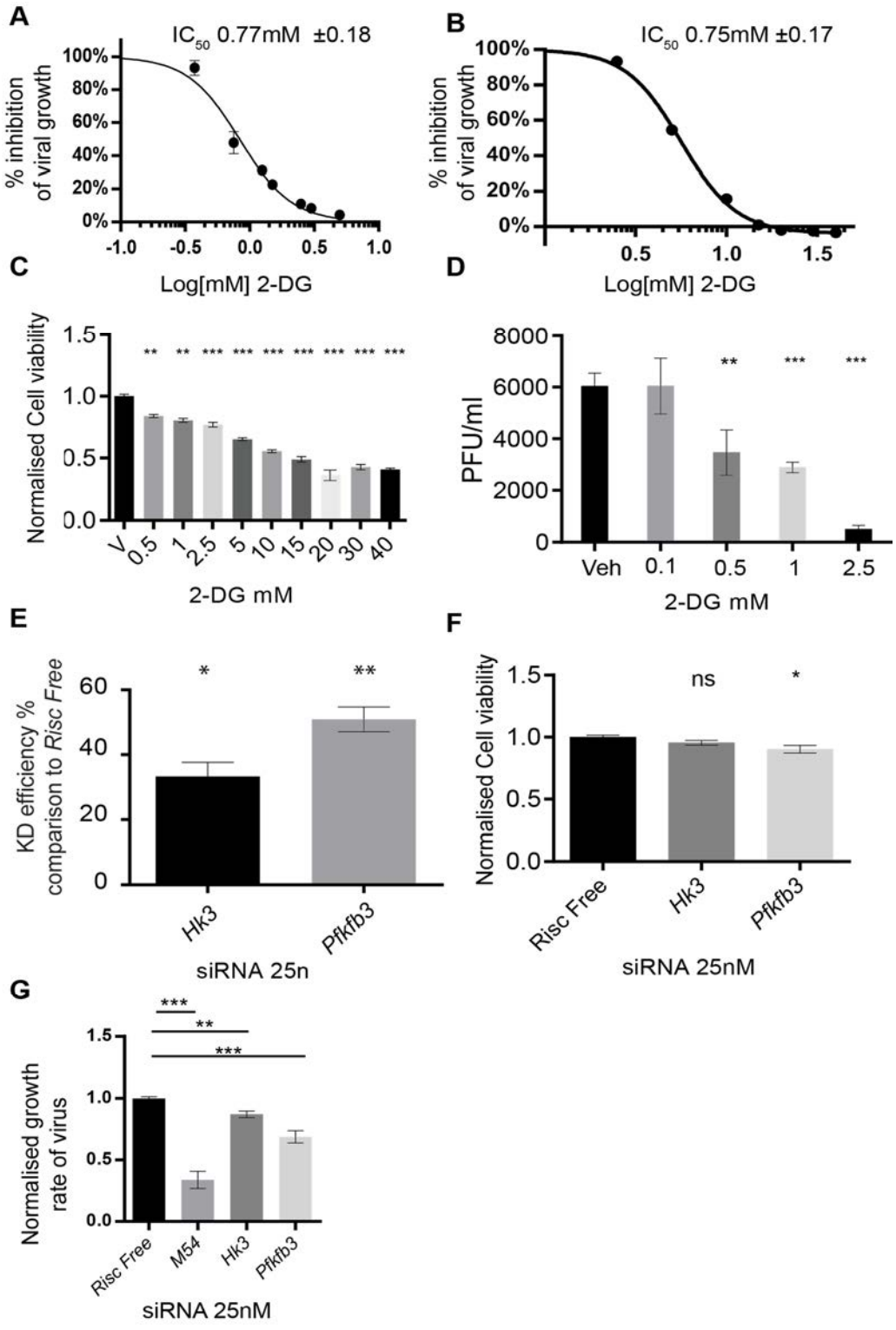


Figure 4.3 Inhibition of the glycolytic pathway Inhibits MCMV growth. (A) NIH/3T3 cells were infected with MCMV-GFP (MOI 0.1) and subsequently treated with varying concentrations of 2-DG. GFP was measured to measure the level of infection over time. **(B)** Similar to A for p-MEFs data points represent a mean ± SEM of three independent experiments with n = 6 for each experiment. **(C)** NIH/3T3 cells were treated with varying concentrations of 2-DG. 48 hours post treatment the cell viability

was asserted using Celltiter-Blue®. Data points represent a mean ± SEM of three independent experiments with n = 6. **(D)** BMDM were infected with MCMV-GFP (MOI 1) and subsequently treated with varying concentrations of 2-DG. Data points represent a mean ± SEM of three independent experiments with n = 3 for each experiment. **(E)** NIH/3T3 cells were transfected with 25 nM of siRNA hour ON-TARGET Plus™ siRNA smart pool for 48 hours, and then infected with MCMV-GFP (MOI of 0.1). The graph represents growth rate of viral replication normalised to RISC free treated cells. Data points represent a mean ± SEM of four independent experiments with n = 6 for each experiment. **(F)** NIH/3T3 cells were transfected with 25 nM ON-TARGET Plus™ siRNA smart pool for 48 hours, subsequently the cell viability was asserted using Celltiter-Blue®. Data points represent a mean ± SEM of three independent experiments with n = 4. **(G)** NIH/3T3 cells were transfected with 25 nM ON-TARGET Plus™ siRNA smart pool for 48 hours. Subsequently the KD efficiency for each siRNA was asserted with qRT-PCR in comparison to RISC free treated cells. M54 is a control siRNA treatment that targets the viral DNA polymerase. C and D* an unpaired Student's t test. E,F,G One way ANOVA with Dunnett's correction for multiple comparisons $p<0.05$, $**p<0.01$, $***p<0.001$

The reduced KD efficiency could explain the minimal effect that the treatment Hk3 KD had on viral growth. Nevertheless, the inhibition of viral growth was statistically significant when either *Hk3* or *Pfkfb3* was knocked down. This, in in combination with the clear inhibitory effect of 2-DG on MCMV growth, presented above, demonstrate the dependency MCMV growth on the glycolytic pathway. Inhibition of glycolysis uptake also blocks HCMV growth (Yu, Maguire, and Alwine 2011).

4.4 Enhanced glycolytic activity coincides with metabolic reprogramming and adaptation of the TCA cycle breakpoints in infected macrophages

After glucose is converted to pyruvate it can either be broken down to lactate by lactate dehydrogenase (LDH), or it can enter the TCA cycle (**Fig.4.4A**) where it is oxidised and the protons generated during the process generate ATP. In quiescent cells the TCA cycle and the oxidative phosphorylation (OXPHOS) are the main pathways for generating energy, but this is not the same for all cell types (De Bock et al. 2013; Groschner et al. 2012).

After the glycolytic pathway, I investigated the TCA cycle of MCMV-WT infected macrophages at the transcriptomic and metabolomic level. The transcriptional profile of all the genes that encode enzymes for the TCA cycle was generated using the

MITCH12 microarray dataset (**Fig.4.4B**). Although glutamate dehydrogenase 1 (*Glud1*) is not part of the TCA cycle, the temporal transcriptional profile of the gene was added here because of the importance that glutaminolysis plays during CMV infection(Chambers, Maguire, and Alwine 2010; Yu, Clippinger, and Alwine 2011). It should also be noted here that a lot of the TCA cycle enzymes are composed of subunits that are encoded by distinct genes, and this is illustrated with a rounded rectangle around these genes (**Fig.4.4A**).

The temporal transcriptional profile of genes in BMDM infected by MCMV-WT demonstrates that there is a change in the transcription of genes encoding TCA cycle enzymes, albeit not as big as for the glycolysis genes. Nevertheless, even small increases in cellular enzyme concentration can have immense impact on the substrate turnover time, especially in a pathway like TCA. Additionally, in the TCA a lot of the enzymes are regulated allosterically, which is essential for the TCA cycle, as it is the linking point between multiple metabolic pathways and need to respond and adapt fast to stimuli.

The TCA cycle in the infected BMDM experimentally appears to be broken at two points on the transcriptional level. The enzymes leading up to isocitrate dehydrogenase (IDH) are downregulated, and the rest up to malate dehydrogenase MDH upregulated. Thus, two mini cycles appear to be formed. Fumarate Hydratase (FH) appears to be downregulated too, but the microarray platform employed here does not distinguish between the cytosolic and the mitochondrial isoforms of FH and, thus, does not allow for clear picture.

The division of TCA cycle in two minicycles was first proposed after studies conducted in the 1990s. It was observed that the flux in the TCA cycle appears to be conscripted at two steps due to the limited availability of oxaloacetate and α-Ketoglutarate (α-KG). These two products are in constant balance with their transamination products, aspartate and glutamate. Thus, a model was proposed that allows the TCA cycle to be broken into two small minicycles between these two substrates, (McCammon 2003).

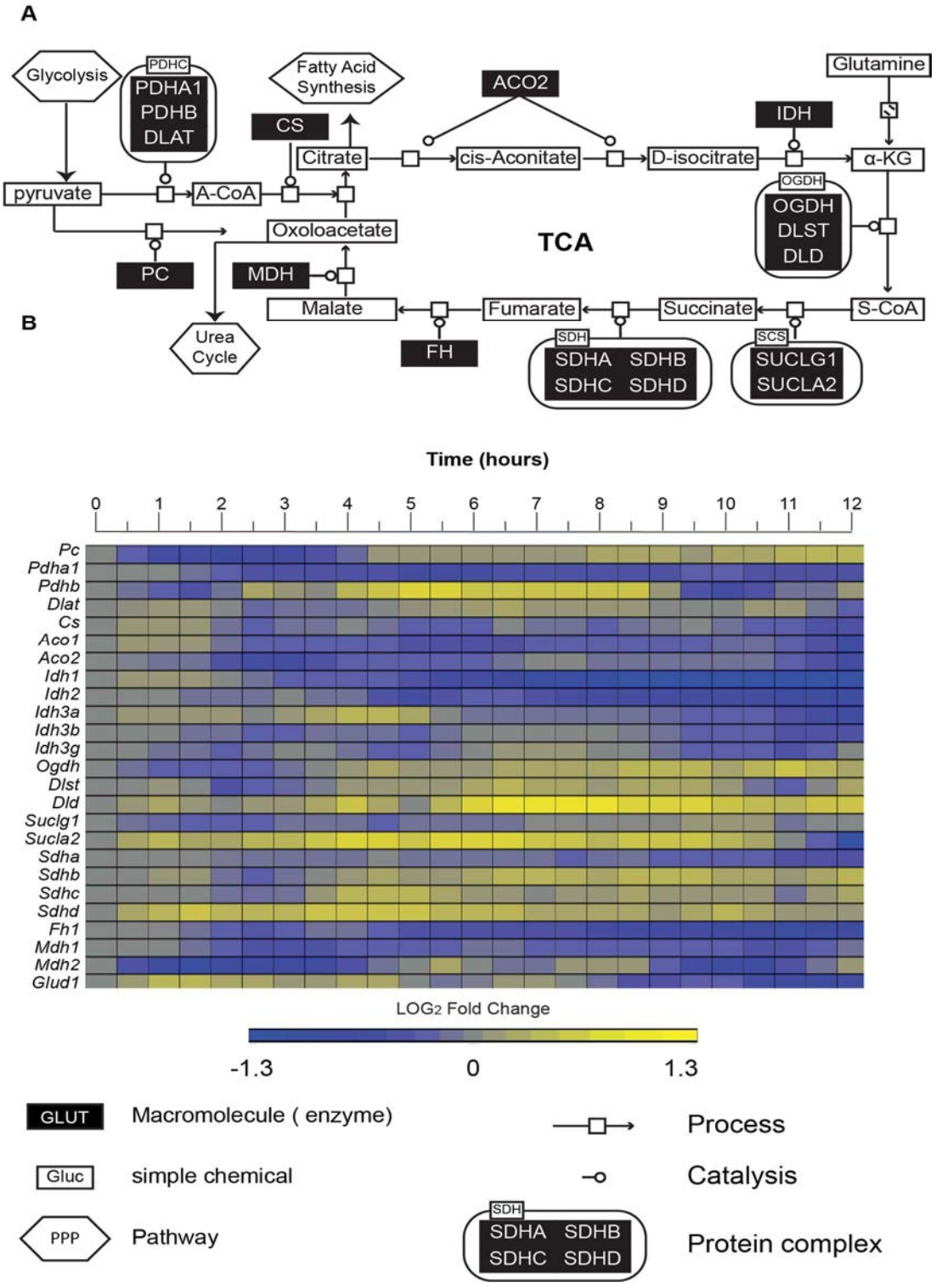


Figure 4.4 TCA cycle transcriptomics during MCMV-WT infection of BMDM indicate uncoupling of TCA cycle. (**A**) Schematic of the TCA cycle **(B)** Temporal gene's expression over 12 hours in BMDM infected with MCMV-WT (MOI 1). Each time point corresponds to one independent biological sample, and columns indicate time in hours. Fold changes of expression levels are represented on a Log_2 scale normalised to time point zero. *Pc*:pyruvate carboxylase; *Pdha1*:pyruvate dehydrogenase E1 alpha 1; *Pdhb*: pyruvate dehydrogenase (lipoamide) beta; *Dlat*:

dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex); *Dld*: dihydrolipoamide dehydrogenase; *Cs*: citrate synthase; *Aco2*: aconitase 2, mitochondrial; *Idh3a*: isocitrate dehydrogenase 3 (NAD+) alpha; *Idh3b*: isocitrate dehydrogenase 3 (NAD+) beta; *Idh3g*: isocitrate dehydrogenase 3 (NAD+), gamma; *Ogdh*; oxoglutarate dehydrogenase; *Dlst*: dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex); *Dld*: dihydrolipoamide dehydrogenase; *Suclg1*: succinate-CoA ligase, GDP-forming, alpha subunit; *Sucla2*: succinate-Coenzyme A ligase, ADP-forming, beta subunit; *Sdha*: succinate dehydrogenase complex, subunit A, flavoprotein (Fp); *Sdhd*:succinate dehydrogenase complex, subunit D, integral membrane protein; *Sdhb*: succinate dehydrogenase complex, subunit B, iron sulphur (Ip); *Mdh1*: malate dehydrogenase 2, NAD; *Mdh2*: malate dehydrogenase 2, NAD (mitochondrial); *Glud1*: glutamate dehydrogenase 1

In order to corroborate the transcriptomics data of the infected BMDM I analysed the set of untargeted metabolomics data from infected BMDM that I had previously generated, focusing on metabolites of the TCA cycle. In agreement with the transcriptomics data and the model described above I was able to capture, the break-up of the TCA cycle in the two minicycles (**Fig.4.5A-H**). The metabolites of the first three enzymatic steps in the TCA cycle are depleted in the infected cells, while succinate and fumarate are not changed and malate accumulates.

This suggests that citrate is either being consumed by another process in the cells or that there is a reduced generation of citrate, which limits the flux to the other metabolites. The decreased concentration of α-KG, the increase in *Glud1* expression and the steady concentrations of succinate and fumarate suggest that citrate is probably being consumed by other biochemical pathways while α-KG replenishes the TCA cycle. The reactions from malate to succinate are all reversible and this could indicate the reversal of TCA cycle, but this is not likely due to α-KG concentration being reduced. Moreover, at the same time *Glud1* expression is increased, indicating increased production and consumption of α-KG. Oxaloacetate was not captured in this experiment, but this could be anticipated as oxaloacetate has usually very low concentrations in cells.

When combined, the transcriptomic and metabolomic data indicate that citrate is being shuttled outside of the mitochondrion while the rest of the TCA cycle works in an anaplerotic mode to replenish other intermediates and to maintain the mitochondrion membrane potential. This is crucial because without the mitochondria membrane

potential, the mitochondrial membrane could break up, which activates caspases and apoptosis (Henry-Mowatt et al. 2004).

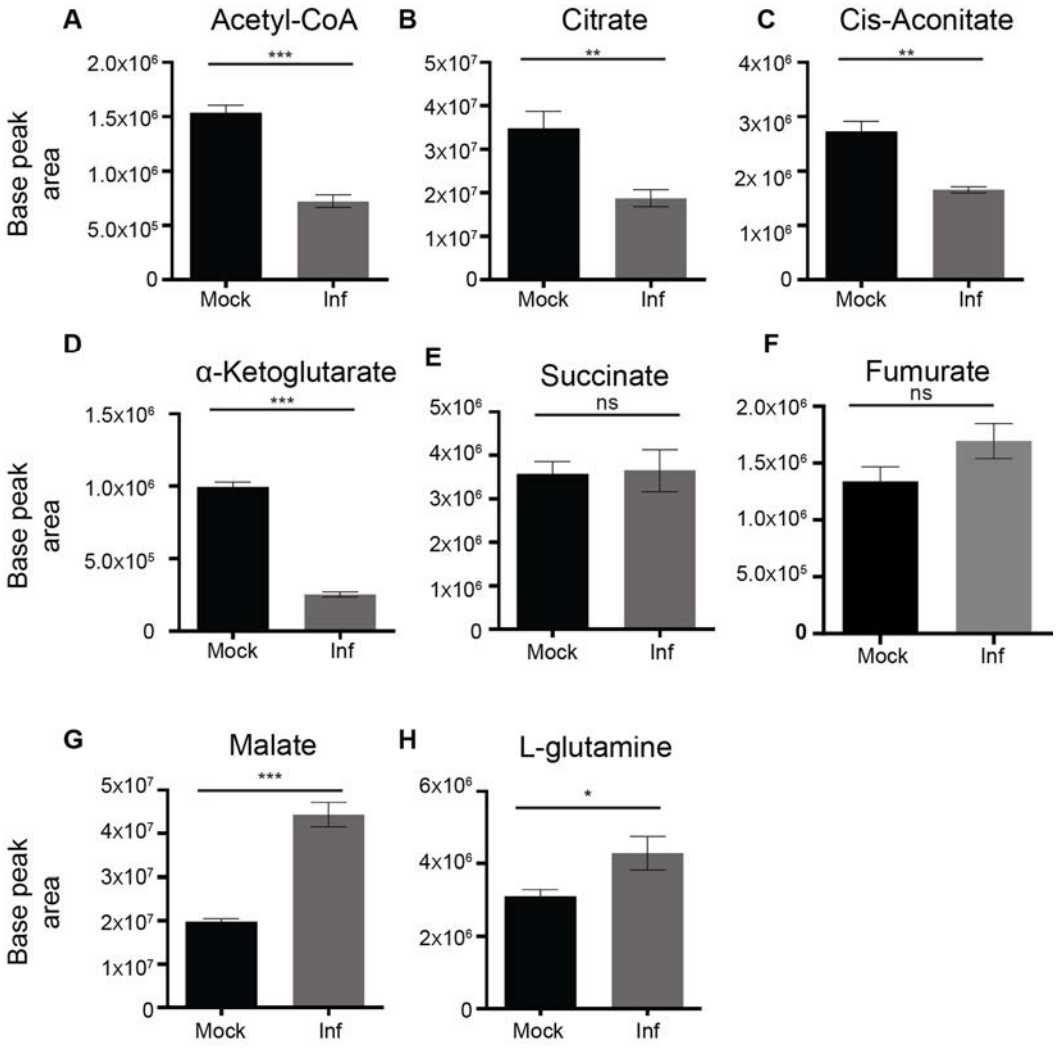


Figure 4.5 TCA metabolomics of MCMV infected BMDM. **(A-H)** TCA cycle metabolites. BMDM were infected with MCMV-WT (MOI 1). At 4 hpi the metabolism was quenched and metabolites were extracted from the cells, followed by metabolite quantification using LC-MS. Each bar point represents mean ± SEM of one experiment with 6 biological replicates. *p<0.05, **p<0.01, ***p<0.001 with an unpaired Student's t test

4.5 Enhanced arginosuccinate and citrulline in Urea/NO cycle in infected MΦ is concurrent with the TCA cycle reprogramming.

One of the many metabolic cycles that is connected to the TCA cycle and crucial for proinflammatory macrophages, is the Urea cycle. The Urea cycle plays a central role

in the removal of nitrogen from cells by converting NH_4^+ to the less toxic urea, which is produced during the conversion of arginine to ornithine (**Fig.4.6A**) (Crombez and Cederbaum 2005). Arginine from this process can be used to produce nitric oxide (NO), which plays an important role in the effector functions of proinflammatory macrophages (Qualls et al. 2012; Morris 2004; El Kasmi et al. 2008; Jha et al. 2015). This comes without its own harmful consequences, as NO can inhibit cytochrome C oxidase, a key enzyme in the electron transport chain, and therefore blocks the production of ATP through oxidative phosphorylation(Beltrán et al. 2000; Everts et al. 2012). As mentioned in the introduction the fate of arginine is entwined with the phenotype of the macrophage. With the M1 phenotype promoting NO production while M2 macrophages promote degradation of arginine to ornithine (Qualls et al. 2012; Jha et al. 2015; Campbell et al. 2013; El Kasmi et al. 2008).

The transcriptomic profile for the enzymes in the urea cycle reveal an increased shunt towards NO production, with increased expression of argininosuccicanate synthase (ASS) (**Fig.4.6B**). iNOS expression was below the detection threshold in MITCH12 dataset, while the MITCH24KO dataset indicate a strong induction (**Fig.S7.1**). In MITCH12, BALB/c mice were used while in the MITCH24KO dataset, BMDM from C57BL/6J were used. The difference could be attributed to differences in mouse strain. Nevertheless, the metabolomics data indicate that iNOS is induced too. The metabolomics data are in agreement with the transcriptional profile of these enzymes, revealing that L-aspartate and N(L-Arginino)succinate accumulate, probably due to the upregulation of arginosuccinate synthase, and not that of Argininosuccinate lyase (*Asl*) (**Fig.46C-G**). At the same time L-arginine remains steady, while L-citrulline levels are increased indicating that the pathway

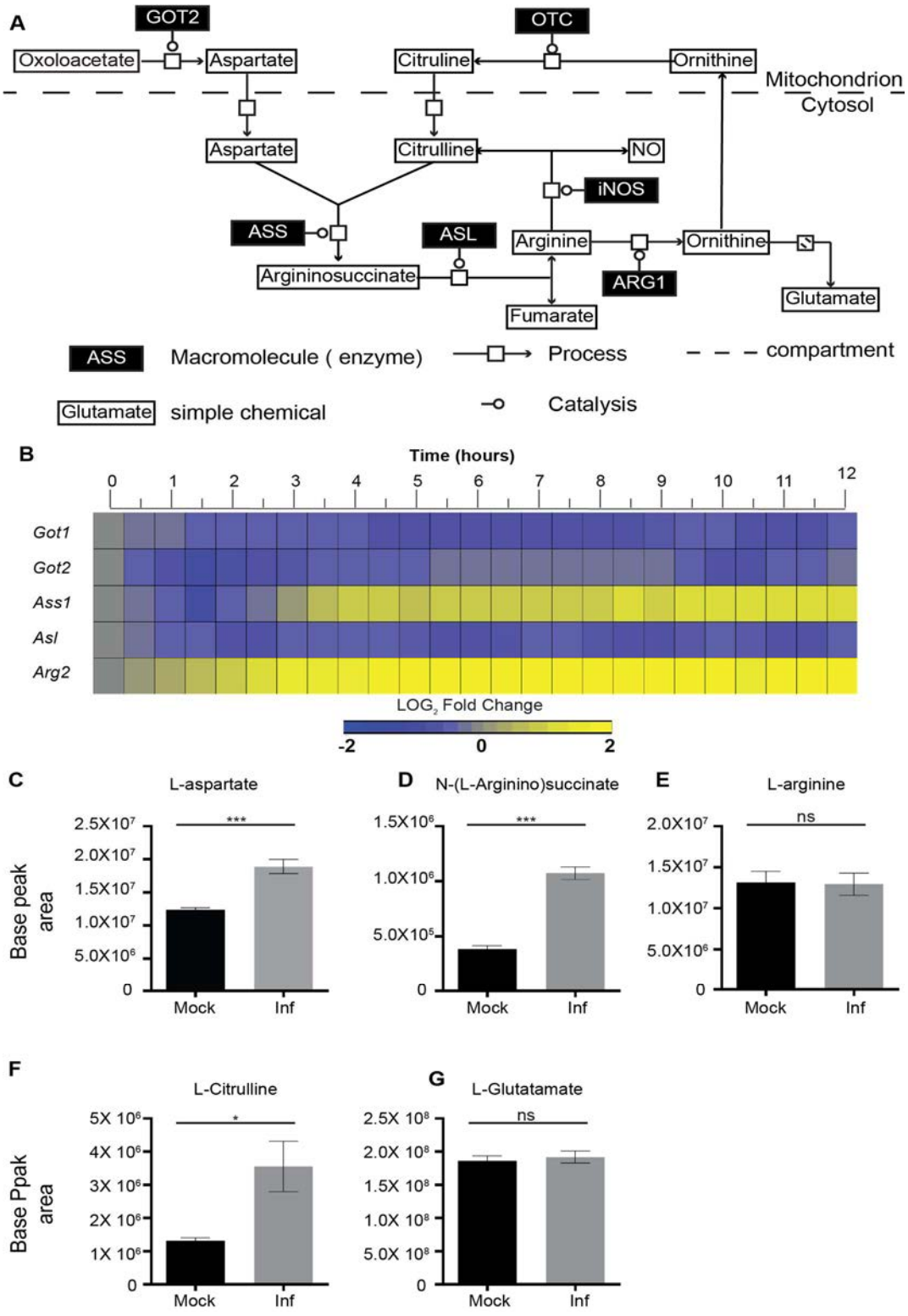


Figure 4.6 Increased NO production form Urea cycle. (A) the Urea cycle. **(B)** Temporal gene's expression over 12 hours in BMDM infected with MCMV-WT (MOI 1). Each time point corresponds to one independent biological sample, and columns indicate time in hours. Fold changes of expression levels are represented on a Log_2 scale normalised to time point zero. **(C-G)** Urea cycle metabolites. BMDM were

infected with MCMV-WT (MOI 1). At 4 hpi the metabolism was quenched and metabolites were extracted from the cells and the metabolites were quantified using LC-MS. Each bar point represents mean ± SEM of one experiment with 6 biological replicates. *p<0.05, **p<0.01, ***p<0.001 with an unpaired Student's t test. *Got1*; glutamate oxaloacetate transaminase 1, soluble; *Got2*: glutamate oxaloacetate transaminase 2, mitochondrial; *Ass1*: arginosuccinate synthetase 1; *Asl*: argininosuccinate lyase; *Arg2*: arginase type II; *Nos2*: nitric oxide synthase 2, inducible

is driven towards NO production. This is expected as the macrophages studied here are of the proinflammatory type that would favour NO production.

Arginase 2 (*Arg2*) expression is also induced early during infection (1 hpi). This might, in the first instance appear controversial as arginase activity is associated with M2 macrophage phenotype and not a proinflammatory one(Campbell et al. 2013; EL Kasmi and Stenmark 2015). However, while this is true for ARG1, the effects of ARG2 on MΦ phenotype have not been studied extensively(Z. Yang and Ming 2014). There are, however, studies that have associated ARG2 activity specifically to proinflammatory macrophages and thus the association between ARG2 activity and M2 phenotype can be dismissed (Hardbower et al. 2016; Ming et al. 2012).

4.6 Type-I IFN signalling is essential for the early upregulation of glycolysis for productive and non-productive infection of macrophages

The changes observed above, on the transcriptional and metabolomics level, appear very early during infection (3-4 hpi) with some starting as early as 30 minutes post infection. Transcription of viral genes have not initiated, apart from maybe some IE genes, suggesting that these early metabolomic events might be a host driven response to the infection and not an event driven by *de novo* viral synthesised viral products.

To examine this further, I sought to investigate the effect that different types of treatment had on the glycolytic pathway of infected BMDM. BMDM were mock treated or treated with MCMV-WT, or MCMV-GFP or poly(I:C) and the glycolytic activity was tested by Seahorse XF Extracellular Flux Analyzer at 1.5 hours post treatment (**Fig.4.7A**). The data reveal that at 1.5 hours, all three treatments

significantly increased the glycolytic capacity of the cells in comparison to the mock treated cells. This is in agreement with previously published data in DCs and MΦ, where, in comparison to untreated cells, cells treated with TLR ligands significantly upregulate their glycolytic flux early after treatment (Jha et al. 2015; Everts et al. 2014, 2012).

The ability of poly(I:C) treatment to increase the glycolytic activity of treated cells in MΦ indicates that this is a host driven event. The virus might not be the master regulator of some of the metabolomics changes that were observed during infection, but instead the immune system is. To further substantiate these results the experiment was repeated using MCMV-GFP, MCMV-KO and poly(I:C). The results clearly demonstrate that all the treatments induce the glycolytic activity of BMDM (**Fig.4.7B**) revealing a common regulator behind the induced glycolytic efflux. Since the cell type (MΦ) is the only thing all treatments have in common, it is deduced that the response of the macrophage to the stimuli must be triggering the metabolic changes observed. Since all treatments have in common only the MΦ it can be deduced that the response of the macrophage to the stimuli must be triggering the metabolic changes observed.

To further investigate the role of the immune response in regulating the transcriptomic changes of metabolic genes the whole MITCH24 dataset was used. The MITCH24 data presented in the previous chapter included the transcriptome of WT BMDM treated with MCMV-WT, or MCMV-KO, or poly(I:C). The entire MITCH24 data includes also the same data for BMDM that are IFNB1, or IFNAR1, deficient. I analysed the temporal expression profile of the key glycolytic genes, (*Glut6, Hk3* and *Pfkfb3*) identified earlier (**Fig.4.1A**), in all conditions.

The data demonstrate that *Glut6, Hk3* and *Pfkfb3* are all upregulated early upon treatment of the WT macrophages with either MCMV-WT, or MCMV-KO or poly(I:C) (**Fig.4.7C-E**). This suggests that the upregulation of the genes is an event associated with the immune response reprogramming of the macrophage upon engagement of specific TLRs, including

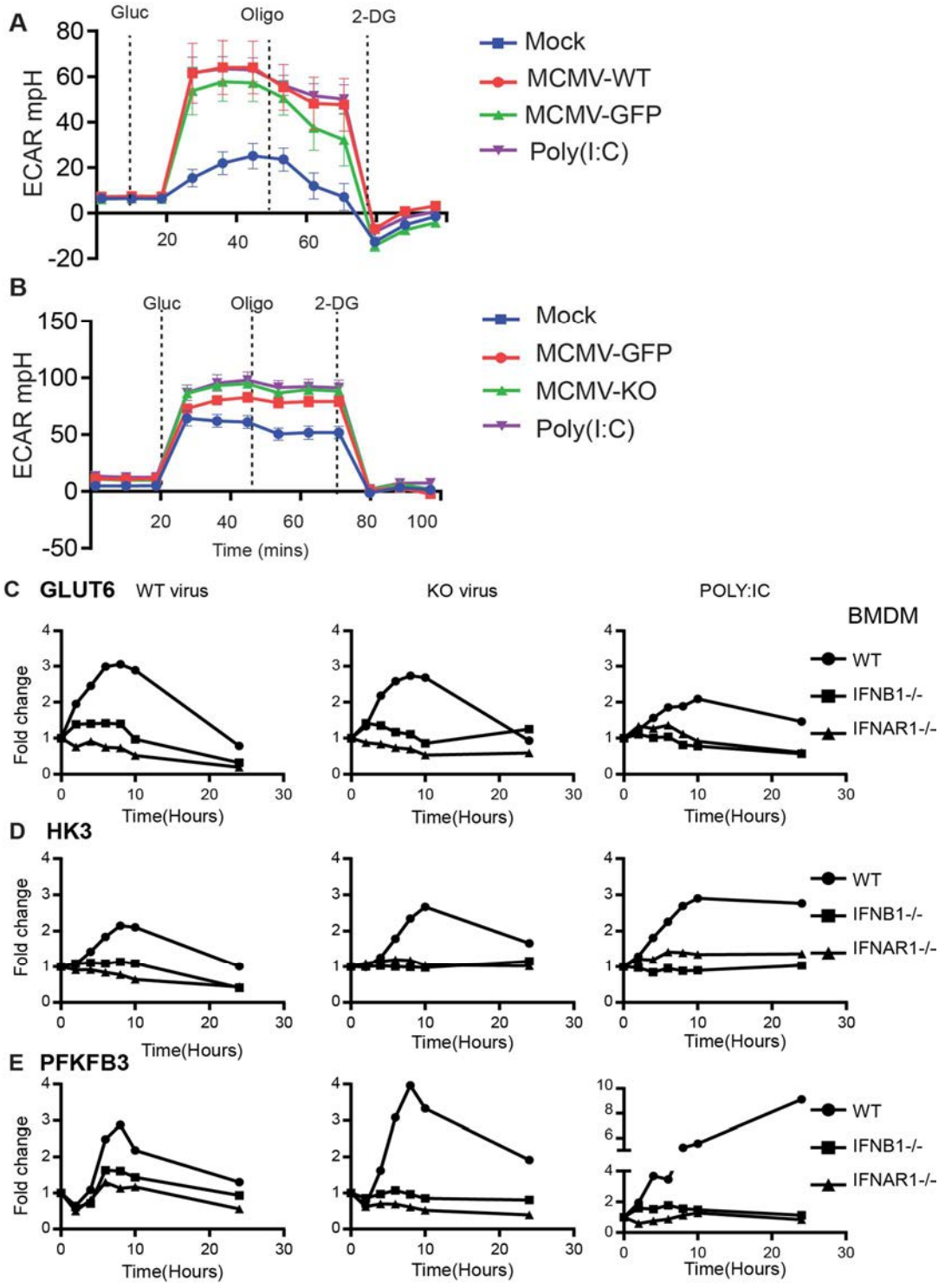


Figure 4.7 Type-I IFN signalling is essential for the upregulation of glycolysis. (A) BMDM were mock treated, or treated with MCMV-WT (MOI 1), or MCMV-GFP (MOI 1) or poly(I:C). 1.5 Hours post treatment ECAR was measured using Seahorse XF Extracellular Flux Analyzer and were treated with Glucose (Gluc), Oligomycin (Oligo) and 2-Deoxyglucose (2-DG)**.** Each data point represents a mean ± SEM from one experiment with 5 biological replicates each. Poly(I:C) treatment results are obscured by MCMV-WT**(B)** As in (A) for BMDM, mock treated or treated with MCMV-

WT (MOI 1), or MCMV-KO (MOI 1), or poly(I:C). **(C-E)** MITCH24 data. Fold change of expression of glycolysis genes (*Glut6*, *Hk3* and *Pfkfb3*) over 24 hours in WT, or IFNB1-/-, or IFNAR1-/- macrophages that they have been infected with MCMV-WT (MOI 1), or MCMV IE3KO-KO (MOI 1) or treated with poly(I:C).

TLR3 and/or TLR9 which are known to be engaged by MCMV-WT (Tabeta et al. 2004), and other PRRs.

The upregulation of *Glut6* and *Hk3* is almost completely ablated in the IFNB1-/- and IFNAR1-/- BMDM. This suggests that the increased expression of these genes is dependent on type-I IFN signalling under these conditions. The *Pfkfb3* has slightly increase expression during WT infection in the deficient BMDM, suggesting that MCMV interferes with the expression of this gene, but further experiments are required to reach this conclusion as the current data are insufficient (**Fig.4.7E**). Nevertheless, *Pfkfb3* expression is higher in the WT BMDM and its expression does not alter in the deficient BMDM in the other two treatments, suggesting that type-I IFN signalling is also required for the upregulation of the gene expression.

Taken together, these results demonstrate that, during the early time of infection, type I IFN is crucial in affecting glycolysis and the TCA cycle, two pathways important for virus replication.

4.7 Profiling IFN-γ stimulation of macrophages shows correlated and non-correlated glycolytic, TCA and Urea cycle

Based on the beneficial role that IFN-I appears to have during early time of MCMV infection we decided to investigate the effects of INF-γ, a key cytokine for macrophages, on the previously investigated pathways.

I analysed the MITCH12 dataset to examine whether the effects of IFN-γ treatment on BMDM was sufficient to induce gene expression of metabolic pathways. Similarly, to the experiment previously described, mature BMDM were treated with IFN-γ and the RNA was harvested every 30 minutes for up to 12 hours post infection (hpi) for microarray analysis. The first pathway investigated was glycolysis (**Fig.4.8A**). The heatmap reveals that INF-γ treatment has a profound effect on the transcriptomic

profiles of glycolytic genes. More specifically, all three glycolytic enzymes previously identified (*Glut6, Hk3, Pfkfb3*) exhibited increased expression levels similar the ones they had during MCMV-WT infection.

There are key differences though; the expression of *Glut4* and *Ldhb* was not upregulated from IFN-γ treatment (**Fig.4.8A**). This suggests that the expression induction observed for these genes in the MCMV infected MΦ could be a virus driven event or due to differences in signalling. Importantly, at six hours post infection, a distinct expression profile of almost all genes is observed (**Fig.4.8A**). IFN-γ ubiquitously upregulated the expression of almost all glycolytic enzymes especially at 5 hours post treatment. In contrast MCMV-WT infection suppress some of these genes post 5 hours indicating differences in both treatments. It is interesting that earlier studies in type-IFN and their relationship to MCMV have indicated that it takes 6 hours for MCMV to block IFN-β signalling.

The next pathway investigated was the TCA cycle (**Fig.4.8B**). Similar to MCMV-WT infection, IFN-γ appears to not have an effect on the TCA cycle, at least not on the transcriptomic level. The expression of the genes involved in the TCA cycle does not change as dramatically as in the glycolytic genes do upon with IFN-g treatment, making it difficult to draw clear conclusions. Further experiments are required to support the transcriptomics data.

Finally, the transcription of the genes encoding enzymes in the urea cycle was studied (**Fig.4.8C**). There are similarities here between the two treatments and some differences as well. *Ass* is upregulated along with *Arg2* indicating that NO production is as expected favoured in proinflammatory macrophages. On the other hand, in contrast to what was observed during productive infection, were the genes had a slight reduction in their

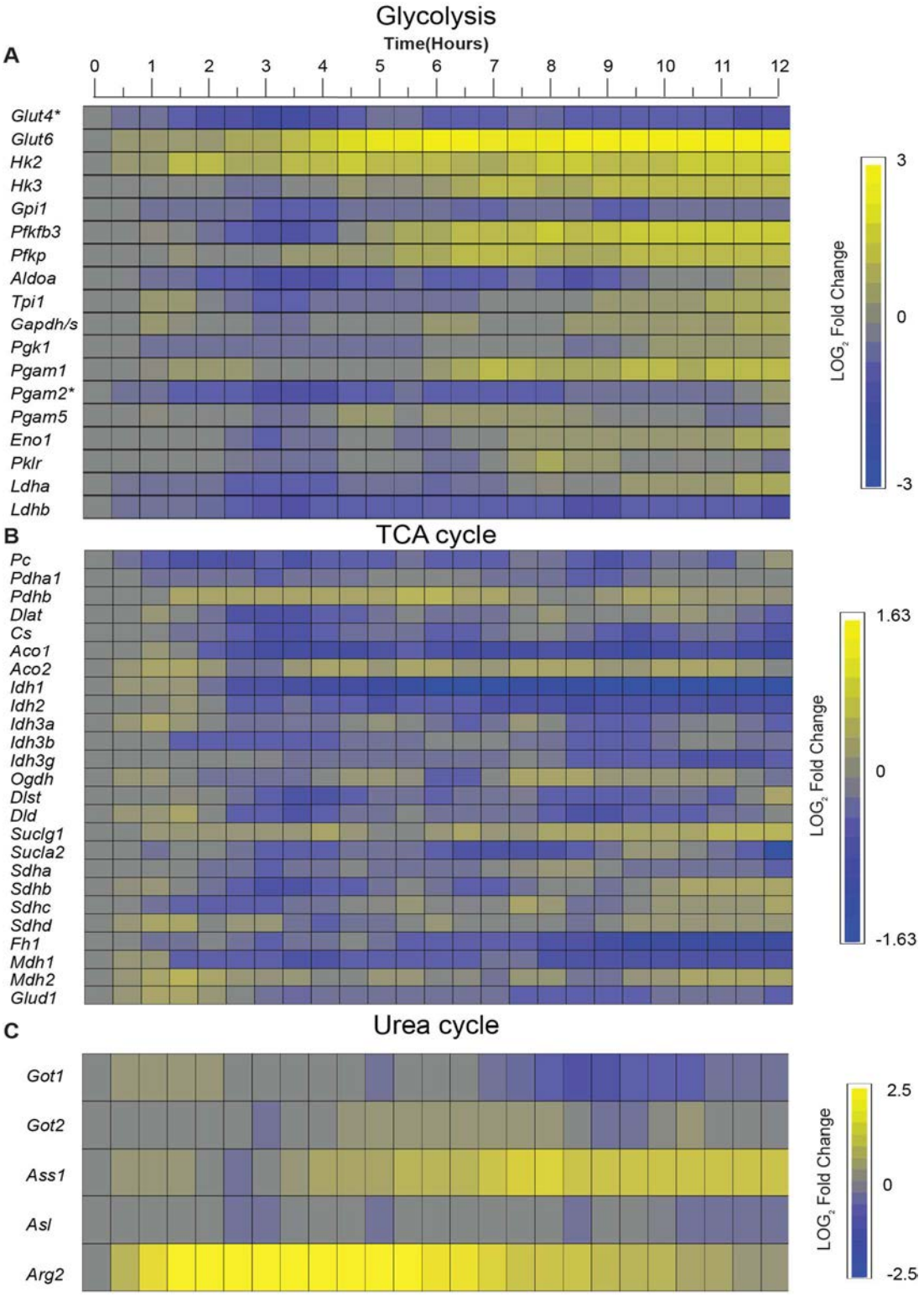


Figure 4.8 Effects of IFN-γ on BMDM metabolome. (A-C) MITCH12 data for glycolysis. BMDM were treated with 10 Units/ml of IFN-γ and subsequently RNA was harvested every 30 minutes for 12 hours and hybridized on an Illumina array. **(A)** Temporal gene's expression over 12 hours of the glycolytic pathway. Each time point corresponds to one independent biological sample, and columns indicate time in

hours. Fold changes of expression levels are represented on a Log_2 scale normalised to time point zero **(B)** as in (A) for TCA cycle **(C)** as in (A) for the Urea cycle.

expression levels, the rest of the genes in the cycle appear to have no change in their transcription levels.

Taken together, these results suggest that INF-γ alters the metabolism of macrophages. Additionally, during early times of IFN-γ treatment, glycolysis and the urea cycle have a similar transcriptional profile with the productive infection, in a similar manner to that of the early phase of MCMV infection. This further reinforces the concept that alterations of the metabolism from the immune system might be beneficial to the viral infection especially early after proinflammatory signals have been received, prior to the cells acquiring the full anti-viral state.

4.8 Productive infection differentially reprograms infected macrophage at late time of infection, associated with enhanced depletion of energy reserves in infected macrophages

I hypothesized, that part of the responses observed above are a result of increased energy demands associated with infection and/or activation of macrophages, for supporting viral progeny, and the effector function of the macrophages respectively.

To test this hypothesis BMDM were mock infected, or infected with WT MCMV-WT, or MCMV-KO. After four hours and 24 hours of infection their intracellular metabolites were isolated and analysed using LC-MS. ATP levels are decreased in MCMV-WT infected macrophages, while in both MCMV-WT and MCMV-KO infected macrophages an increase of ADP levels was observed at four hours post infection (**Fig.4.9A**). Another source of ATP that is readily available to cells is Phosphocreatine(P-C). During rest, cells can store energy in the form of P-C that can quickly, and without

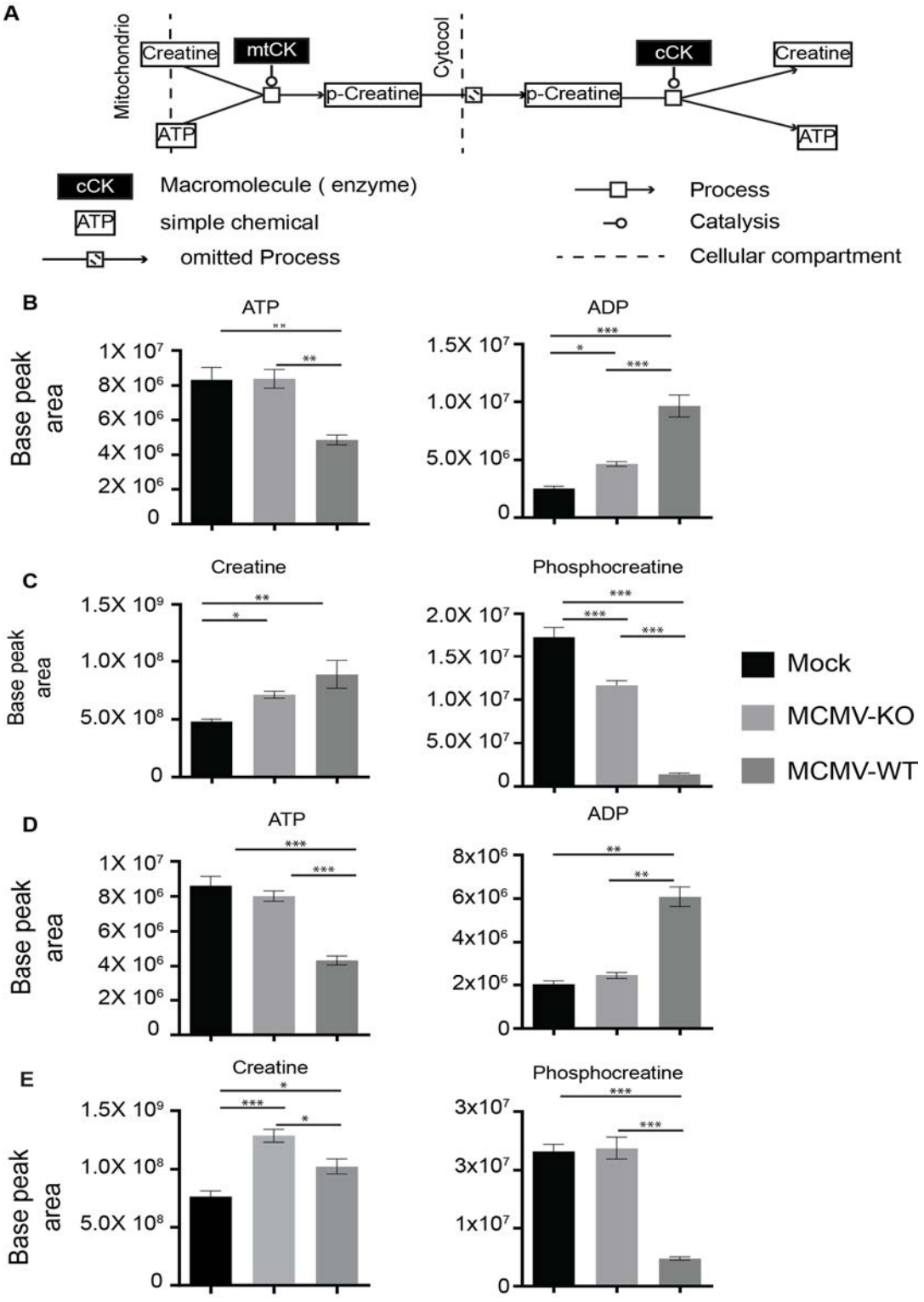


Figure 4.9 Energy state of BMDM cells. (A) The creatine-phosphocreatine energy shuttle. **(B-C)** ATP-ADP and P-Cr levels in BMDM infected with MCMV-WT (MOI 1). At 4 hpi the metabolism was quenched and metabolites were extracted from the cells and the metabolites were quantified using LC-MS. Each bar point represents mean ± SEM of one experiment with 6 biological replicates. **(D-E)** AS in **(B-E)** at 24 hpi. One

way ANOVA with turkey's correction for multiple corrections *p<0.05, **p<0.01, ***p<0.001 Non-significant results are not shown.

the use of oxygen, be used to replenish the ATP pool of a cell (**Fig.4.9A**). Infection with both MCMV strains depleted Phosphocreatine and increased creatine levels. However, productive infection appears to be more energy demanding than non-productive infection at 4 hours post infection. Creatine levels increased more in MCMV-WT than in MCMV-KO infected cells and at the same time MCMV-WT infection almost completely depleted the cells of Phosphocreatine (**Fig4.9B-C)**.

At 24 hours the MCMV-WT infected macrophages continued to exhibit depleted energy levels, indicated by low ATP levels and high ADP levels, in comparison with mock and MCMV-KO infected cells (**Fig.4.9D**). Interestingly the levels of these two metabolites in MCMV-KO infected macrophages have returned to levels similar to mock infected cells. Phosphocreatine levels are depleted for MCMV-WT infected BMDM, but similar for mock and MCMV-KO infected cells (**Fig.4.9E**).

Another indication that the productive infection is more energy demanding is the increased expression of lactate dehydrogenase b (*Ldhd)* presented in MCMV-WT BMDM. *Ldhd* is responsible for converting pyruvate to lactate converting NADH to NAD^+ during the process in the absence of oxygen. It is important to keep glycolysis active, as NAD^+ is required at an earlier part of glycolysis and without its regeneration it would seize to function. This also indicates that BMDM are pushed toward anaerobic glycolysis. *Ldhd* appears to be targeted by MCMV specifically since it has its expression upregulated in all three macrophage types, while at the same time, infection with MCMV-KO virus, or treatment with poly(I:C) does not alter the expression levels of the enzyme (**Fig.4.10A**).

Taken together these experiments indicate that infection of macrophages by MCMV-WT or MCMV-KO adds an energy burden on the cells, expected by the numerous new process that an activated macrophage has to acquire. This demand for increased energy appear to be ablated in the MCMV-KO treated cells at 24 hours in contrast to the MCMV-WT infected ones. The upregulation of *Ldhb* that was also observed at the MICTH12 dataset (F**ig4.1B**) started at later time points, at 6 hpi and peaked at 24 hpi.

This demonstrates clearly that MCMV starts taking over the host cell and supports the notion that type-I interferon signalling at early time points of infection may positively contribute to establish infection in BMDM.

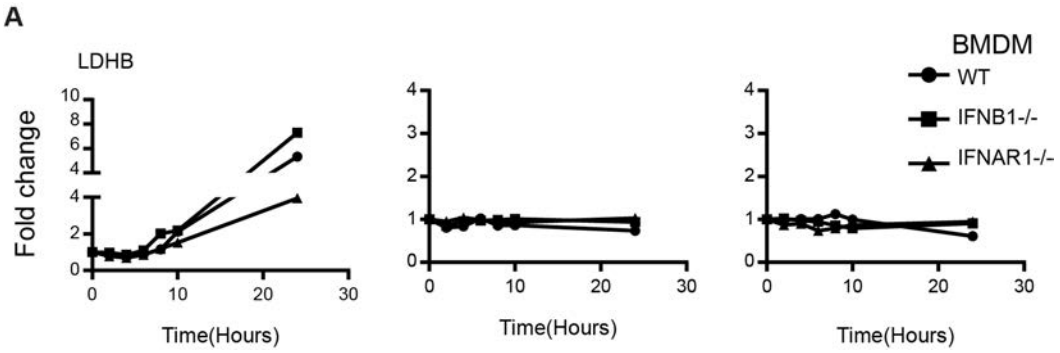


Figure 4.10 *Ldhb* expression in infected BMDM. (A) MICTH24 dataset. Fold change of expression of glycolysis genes (*Glut6*, *Hk3* and *Pfkfb3*) over 24 hours in WT, or IFNB1-/-, or IFNAR1-/- macrophages that they have been infected with MCMV-WT (MOI 1), or MCMV IE3KO-KO (MOI 1) or treated with poly(I:C).

4.9 Discussion

The data presented in this chapter demonstrate that infection of MCMV alters the metabolome of infected BMDM significantly. More specifically, the expression of *Glut6*, *Hk3* and *Pfkfb3* was induced. These genes are involved in key regulatory steps of theglycolytic pathway. Functional assays using Seahorse XF Extracellular Flux Analyzer in agreement indicate an increase of lactate production in MCMV infected BMDM in comparison to mock-infected cells. The metabolomics data in combination with the transcriptomic data also reflect this change, indicating an increase in the glycolytic flux very early during infection. Multiple studies have been published by other groups demonstrating that DCs or macrophages treated with TLR ligands demonstrate an increase in their glycolytic fluxes, associated with transcriptional and post transcriptional changes in their metabolism(Everts et al. 2014; Krawczyk et al. 2010; Jantsch et al. 2008; Rodríguez-Prados et al. 2010; Haschemi et al. 2012).

The TCA cycle was also significantly altered. The expression of enzymes up to OGDH was down regulated. At the same time the expression of *Ogdh*, *Scs*, *Sdh*, was upregulated. The fact that the changes in mRNA level are small and that a lot of these enzymes are composed of multiple components do not allow for a clear picture.

However, combined with metabolomics data, these changes indicate that the TCA cycle is uncoupled at two points, at the enzymatic reaction catalysed CS and OGDH, Acetyl-CoA, Citrate and cis-Aconitate are all consumed this in conjunction with the transcriptomics data of the TCA cycle indicating that citrate is being consumed by other pathways outside of the mitochondria. At the same time α-KG levels are diminished while fumarate and succinate levels are steadily increasing, indicating a rapid conversion of α-KG to these two molecules. Malate appears to be accumulated, which could be attributed to increased synthesis of malate either by fumarate or oxaloacetate. Malate is used in the malate-aspartate shuttle to transfer NADH equivalent outside of the mitochondrion or oxaloacetate because both molecules cannot leave the mitochondrion (Jespersen et al. 2017).

The data presented here support the notion that citrate is being shuttled outside the mitochondria, probably to support FA production in the cytosol, while the rest of the TCA cycle operates anaplerotically. Many studies havedemonstrated that OXPHOS is abolished and the TCA cycle is uncoupled in DCs and MΦ treated with TLR ligands (EL Kasmi and Stenmark 2015; Mills et al. 2016; Everts et al. 2014; Jha et al. 2015; McGettrick and O'Neill 2013). It has been shown that under these conditions MΦ accumulate succinate that can promote ROS generation and inflammation through HIF-1a. I did not sought to observe these effects in my thesis, but it should be noted that the vast majority of these studies have focused on researching the effects of a single TLR molecule on the metabolism of these cells, which is usually LPS.

In my thesis, I study a more complicated and realistic model of infection. MCMV is a natural infectious virus for murine MCMV, and MCMV engages at the same time multiple PRR, as reviewed in the introduction. The multiple signalling inputs should tune the infected macrophage differently than the engagement of a single TLR. Finally, the virus will initiate its own transcriptional programming in the infected cells further modifying amongst others the metabolism of infected cells.

The Urea cycle was also heavily altered to support NO production in MCMV infected BMDM. The transcriptomic data reveal a rapid induction of Ass1 and Arg2. The metabolomics data indicate that NO production is heavily favoured with accumulation of citrulline in the infected cells, indicating that the pathway is altered to support NO

production. The production of NO from proinflammatory macrophages is well established in the literature and has a protective role especially during bacterial infections (Qualls et al. 2012; Morris 2004; El Kasmi et al. 2008; Jha et al. 2015). Arginase activity though is usually associated with M2 macrophage and not proinflammatory ones. However, while this is true for ARG1, the association of ARG2 activity and its effects on macrophage phenotype have not been studied so extensively (Z. Yang and Ming 2014)

The amino acids homology of both enzymes is 50% and rises to 100% for amino acids at their catalytic centres. Moreover, both enzymes can hydrolyse arginine. Because of this it is usually extrapolated that induced expression of *Arg2* would have the same effects as *Arg1* in macrophages. While ARG1 localises in the cytoplasm and ARG2 localises in the mitochondrion, and not enough is known about the consequences that their respective localisation has on the function of these enzymes (Crombez and Cederbaum 2005). Ming et al. have demonstrated induction of *Arg2* expression in macrophage after LPS stimulation (Ming et al. 2012). They also note that the expression is induced in parallel with iNOS. Khallou-Laschet *et al.* have demonstrated that M1 BMDM express preferably *Arg2* while M2 BMDM preferably express *Arg1* (Khallou-Laschet et al. 2010). There is no conclusive answer yet as of the role of ARG2 in macrophages, despite that these studies indicate that the induced expression of *Arg2* in my dataset may not be an oddity.

My experiments indicate that upregulation of glycolysis is critical for MCMV growth, as pharmacological inhibition of the pathway hampered viral growth. KD of *Hk3* and *Pfkfb3* by siRNA inhibited viral growth and 2-DG inhibition of the glycolytic pathway also blocked MCMV growth. The idea that inhibition of glycolysis will inhibit viral infection is not new, as Radsak and Weder used, in 1981, 2-DG to inhibit HCMV replication (Radsak and Weder 1981).

Interestingly, while the virus appears to be dependent on the glycolytic pathway, so are macrophages during inflammation. Although I have not conducted any studies to support this, it is well established by literature that proinflammatory DCs and macrophages require increased glycolytic activity to support their effectors functions. The inhibitory effects of NO in OXPHOS make glycolysis the only alternative

pathway in energy production. Thus, its upregulation promotes DC survival and allows the cell to acquire its full phenotype by upregulating receptor CD40, interleukin 6 (IL-6), and TNF-α among others(Everts et al. 2014; Krawczyk et al. 2010; Everts et al. 2012). Similarly, upregulation of glycolysis supports MΦ proinflammatory function by a number of ways, such as promotion of IL1-β production, efferocytosis of infected cells, cell migration, and NPLR-3 inflammasome (Tannahill et al. 2013; Jiang et al. 2016; Palsson-McDermott et al. 2015; Xie et al. 2016; Semba et al. 2016).

These observations by myself and others lead to the hypothesis that MCMV might be co-opting proinflammatory signals to promote a metabolic profile in infected MΦ that suitable to sustain viral progeny. My experiments indicate that the upregulation of glycolysis, crucial for both MΦ function and MCMV growth, is dependent on type-I IFN signalling. The induction of the *Glut6*, *Hk3* and *Pfkfb3* enzymes is evident predominately in WT macrophages, while BMDM deficient in type-I IFN signalling exhibited none or very low induction. This is in agreement with previously published data by Pantel *et al.* and Jiang *et al*. (Pantel et al. 2014; Jiang et al. 2016). Pantel et al. clearly demonstrated that the shift to glycolysis in poly(I:C) treated DCs was depended on type-I IFN and not MD5A/TLR signalling. Jiang *et al.* showed that IFN-β induces *Pfkfb3* mRNA in treated BMDM. Moreover, the induction of the three enzymes takes place very early during infection (*Pfkfb3* at 30 min post infection, *Glut6* and *Hk3* at 3.5 hpi). At this time, it is unlikely for MCMV to have taken control of infected cells and it is more likely that immune signalling is directing the metabolic changes observed in the glycolytic pathway. Moreover, it has already been established that it takes more than 6 hours for MCMV to block IFN-β signalling in MΦ(Le et al. 2008). Thus, any effects that IFN-I on MΦ metabolism will be unhindered so early during infection.

MCMV is, however, not a bystander, at later stages of infection the virus takes over the cell and alters its metabolome, as evident by the data presented in Chapter 3 and here by the temporal transcription of *Ldhb*. The induction of *Ldhb* transcription is driven mainly by the MCMV-WT infection as both non-productive infection and poly(I:C) treatment do not induce it. Furthermore, this induction is evident only at later time points in both MITCH12 and MITCH24 data sets at 8 hpi for both. The

upregulation of LDH is crucial as glycolysis to be active even when oxidative phosphorylation (OXPHOS) is not functional or when enough NAD^+ cannot be generated. In the absence of OXPHOS or when not enough NAD^+ is regenerated by the TCA cycle, e.g. when under exercise, LDH replenishes NAD^+, which is a crucial coenzyme for GAPDH to function.

Given the evidence provided, my hypothesis that MCMV coops the metabolic programming of activated BMDM during early times of infection is supported. I have, however, not generated any data to demonstrate that this adaptation of MCMV allows the virus to establish infection in the host *in vivo* during the first few hours of infection.

However, MCMV has already been described to co-opt immune system signalling to establish infection in the host. Several studies have demonstrated that MCMV can co-op the immune signalling by promoting its viral enhancer activity (K. A. Kropp et al. 2015; Benedict et al. 2004). Kropp et al. have demonstrated that there is a temporal opening early during infection when TLR activity promotes viral growth, after which TLR activity inhibitory because of the establishment of an antiviral state in the cells(K. A. Kropp et al. 2015). Similar evidence has been published for HCMV. LPS treatment of CD14+ monocytes promotes their permissiveness to MCMV (Kew, Wills, and Reeves 2017). Furthermore, it has been shown that APC-1 and NF-κB and AP-1 activity promotes viral IE gene expression (Caposio et al. 2007; Isern et al. 2011; M. K. Isaacson, Juckem, and Compton 2008). Additionally, HCMV MIEP contains Gamma interferon activation sites (GAS) elements and, which can make the IE gene expression inducible by IFN-γ(Netterwald et al. 2005). In fact multiple immune genes are hijacked by viruses are seen in this review (Santoro, Rossi, and Amici 2003).

It is, thus, not difficult to hypothesise that since MCMV and HCMV have already evolved ways to co-op immune signalling, co-opting the metabolic programming in cells should not be that far-fetched. After all viruses grow in the niche environment of an immune response, so there should be an evolutionary pressure onto them to co-op, or counteract the environment they meet. During the first few hours of infection when few cells have been infected, the virus has not yet been detected by the immune system, but some cells included MΦ are infected and an immune response is triggered in them. Co-opting the cellular environment of host cells might be a good strategy to establish

infection during this time. Typically, in *in vitro* and *in vivo* MCMV infections, high MOIs are used, which can overwhelm an *in vitro* cellular system or an *in vivo* system. This is not the case in normal infections were very few cells are infected by the virus. Thus, while this strategy might not be important in artificial systems that are usually studied, it might be important for natural infections. Crucially, the deletion of the GAS elements from the HCMV MIEP appears to have an effect on viral growth only at low MOIs and not high ones, which is closer a natural infection scenario (Netterwald et al. 2005).

Regarding the functional role of glycolysis during MCMV infection some data are presented in this chapter. The data support the idea that it glycolysis is upregulated to sustain the increased energy demands by the activated macrophages early and viral replication later. Both productive and non-productive infection deplete macrophages of energy at early time points indicating, as expected that the activation of the MΦ and acquisition of their pro-inflammatory phenotype requires energy. At 24 hpi however non-productively infected BMDM appear to have replenished their phosphocreatine levels and have similar ones with mock-infected cells and only MCMV-WT infected BMDM remain depleted in energy reserves. The increased energy demands of MCMV-WT infected macrophages could be supported by the induction of *Ldhb*, while the non-productively.

Increased energy demands are not the only reason for increased glycolysis and the alterations observed in the TCA cycle. Increased flux through glycolysis allows for the generation of extra energy and the shuttling of carbon molecules in the TCA cycle, where they can be oxidised up for more energy generation and/or to support biosynthesis of other molecules. Several studies have demonstrated that HCMV increases the FA synthesis pathway (Purdy, Shenk, and Rabinowitz 2015; Koyuncu et al. 2013; Munger et al. 2008; Vastag et al. 2011). Briefly, the authors of these studies demonstrate using ^{13}C labelling that citrate is being shuttled outside the mitochondria to support FA elongation in infected human fibroblasts. MCMV might be pushing metabolism of infected cells in the same direction, which will be studied in the next chapter.

5 Chapter 5 Fatty acid and signalling lipid metabolism during MCMV infection in BMDM

5.1 Introduction

This chapter will be separated in two smaller chapters that study different aspect of lipid metabolism. In the first section I will address the effect of MCMV infection in BMDM on FA biosynthesis. In the second smaller chapter I will extend this analysis on the study of signalling lipids and more precisely eicosanoids.

5.2 Fatty acid metabolism during MCMV infection in BMDM

5.2.1 Introduction

In chapter 4, evidence is presented to suggest that MCMV infection opportunistically co-opts early pro-inflammatory changes that take place in infected MΦ (chapter 4). One of the pathways affected was the glycolytic pathway that was rapidly induced during infection. This response is under type-I IFN control, since type-I IFN deficient BMDM infected with MCMV did not exhibit the same induced expression in glycolysis enzymes. MCMV is dependent on the increased glycolytic flux and its inhibition blocks and appears to be co-opting the early response of the immune system.

I did not study the effects of increased glycolysis on proinflammatory macrophages, but there are numerous studies that have demonstrated its importance and the ways it is beneficial, which was reviewed earlier(Jiang et al. 2016; Jha et al. 2015; Everts et al. 2012; Galván-Peña and O'Neill 2014) The dependency of MCMV growth on the glycolytic pathway, which is also required by innate immune cells had not yet been studied. I provided evidence demonstrating that the induction of the glycolytic pathway could support the increased energy demands of MCMV during productive infection. MCMV-WT infected BMDM have depleted energy reserves in comparison to mock and infected cells at 4 and 24 hpi.

This is not conclusive though as the glycolytic pathway, apart from providing energy, also shuttles carbon atoms to the TCA cycle through its end product pyruvate. In the

TCA cycle pyruvate, can be further oxidised to generate energy or serve as precursor for other molecules in pathways that intersect with the TCA cycle. The increased carbon atoms flux though could be utilised by other biosynthetic pathways one of the most notables being FA.

HCMV, the human homolog of MCMV, has been shown to dramatically alter the cell metabolome in a manner that resembles the metabolism of highly proliferating cancer cells and induce FA synthesis(Yu, Clippinger, and Alwine 2011; Spencer et al. 2011; Swinnen, Brusselmans, and Verhoeven 2006; D. L. Diamond et al. 2010). Munger *et al.* demonstrated, using ^{13}C labelling, that HCMV infection dramatically increases the uptake of glucose and alters the TCA cycle(Munger et al. 2008). During HCMV infection of Human foreskin fibroblasts (HFF) the TCA cycle was not used only catabolically to generate ATP, but also anabolically to facilitate the fatty acid (FA) production(Munger et al. 2008). In the TCA cycle, pyruvate is converted to citrate, which should be shuttled out of the mitochondrion to be converted in Malonyl-CoA, the building blocks for FA biosynthesis and elongation.

By inhibiting two key FA biosynthesis enzymes; Acetyl-CoA carboxylase (ACAC) and FASN, Spencer *et al.* demonstrated that HCMV growth was also inhibited (Spencer et al. 2011). HCMV does not simply upregulate FA biosynthesis, but rather requires specifically the biosynthesis of saturated Very Long Chain FAs (VLCFA) to propagate effectively(Koyuncu et al. 2013). Additionally, in this study it was shown that HCMV specifically upregulates *Elovls*, with *Elovl7*, *3*, and *4* being the most important.

Given the data presented in the previous chapter and the studies reviewed here it was hypothesized that: MCMV modulates metabolism in infected MΦ at late stages of infection towards FA elongation and lipid membrane remodelling to promote viral progeny: This raises a series of questions to be answered in this chapter.

1) Does MCMV-WT infection specifically upregulate FA elongation pathway?

2) Is this an effect triggered by the WT infection or the immune response?

3) Does inhibition of FA biosynthesis block MCMV replication?

5.2.2 Temporal transcriptomics and metabolomics analysis of Fatty acid metabolism during MCMV infection in BMDM

5.2.2.1 MCMV-WT specifically upregulates genes related to FA and lipid biosynthesis

De novo FA biosynthesis starts in the cytosol by the conversion of citrate to Acetyl-CoA via ATP Citrate Lyase (ACLY). Two Acetyl-CoA molecules are then converted by Acetyl-CoA Carboxylase Alpha (ACACA) to Malonyl-CoA which can then be used either by FASN (Wakil, Stoops, and Joshi 1983) to synthesise palmitate or by the elongation system in the ER to elongate FAs (**Fig.5.1A**). To investigate FA biosynthesis in the context of viral infection I performed (as described in Methods) a temporal analysis of the gene expression of infected BMDM cells. For this purpose, the microarray dataset MITCH24KO, a resource available in to our lab, was used. Briefly for MITCH24KO, BMDM from WT, or IFNAR, or IFNB1 deficient C57BL/6J mice were used. These were mock treated, or treated with MCMV-WT (MOI=1), or MCMV-KO (MOI=1), or poly(I:C). The RNA from each treatment and cell type was isolated at 0, 2, 4, 6, 8, 10, 12 and 24 hours post treatment and high-quality RNA was hybridised to an Affymetrix Mouse Gene 1.0ST microarray.

An analysis of this data shows that MCMV infection of BMDM results in the induction of key enzymes that participate in the elongation of FAs (**Fig.5.1B**). The expression of *Elovl3, 4* and *7* exhibit a substantial increase specifically during productive infection. *Elovls* catalyse the first and rate limiting step in FA elongation (Jakobsson, Westerberg, and Jacobsson 2006). ELOVL7 has been identified by Purdy et al. as the most important enzyme for the catalysis of the lipidome remodelling in HCMV infected cells (Purdy, Shenk, and Rabinowitz 2015).

Acyl-CoA Synthetase Long-Chains (*ACSLs*) are essential proteins for the *de novo* lipid synthesis, FA metabolism, and membrane remodelling. FA are chemically inactive because of the -COOH moiety. In order for the cells to use FA, they convert FA to their active thioester form. This reaction is being catalysed by *ACLSs* (Soupene et al. 2012). In the treated BMDM (**Fig.5.1B**), *Acsl1* was the only *Acsl* gene that exhibited changed temporal expression. This change was observed in all three treatments,

indicating it might be linked to alterations in the lipidome of activated macrophages, which has also been observed in LPS treated macrophage(Y. L. Huang et al. 2014).

Membrane Bound O-Acyltransferases (*MBOATs*) do not contribute directly to FA biosynthesis, but are important for lipids processing (C. C. Y. Chang, Sun, and Chang 2011). *Mboat*2 was significantly upregulated specifically during productive infection indicating that MCMV-WT induces the transcription of the gene. Additionally, *Mboat1* also appears to be under viral control since the gene was initially downregulated, but returned to its previous transcription levels only during productive infection. Furthermore, the data from *Ifnar1-/-* and *Ifnb1-/-* BMDM indicate that the gene is not under type-I interferon control (**Fig.5.2A-B**), and the MITCH12 data reveal that *Mboat1* is downregulated after IFN-γ treatment (**Fig.S7.2**).

Mboats are divided in 3 subgroups; both *Mboat1* and *Mboat2* belong to the third subgroup named Lysophospholipid acyltransferases (LPATs). These proteins are essential in membrane phospholipid remodelling. Phospholipases can cleave the FA that occupies position two in a phospholipid and then a LPAT re-acylates the phospholipid (C. C. Y. Chang, Sun, and Chang 2011; Naganuma et al. 2011; Tamura et al. 2009).

Diacylglycerol O-Acyltransferase 2 (DGAT2) expression is also specifically induced during MCMV-WT infection. Diacylglycerol O-Acyltransferases (DGATs) are proteins involved in the generation of triacylglycerols and catalyse the final and committing step in triacylglycerol synthesis (Stone et al. 2009). Triacylglycerols form Lipid Droplets (LD) in a cell were fatty acids and other lipids like, cholesterol, are stored for later use.

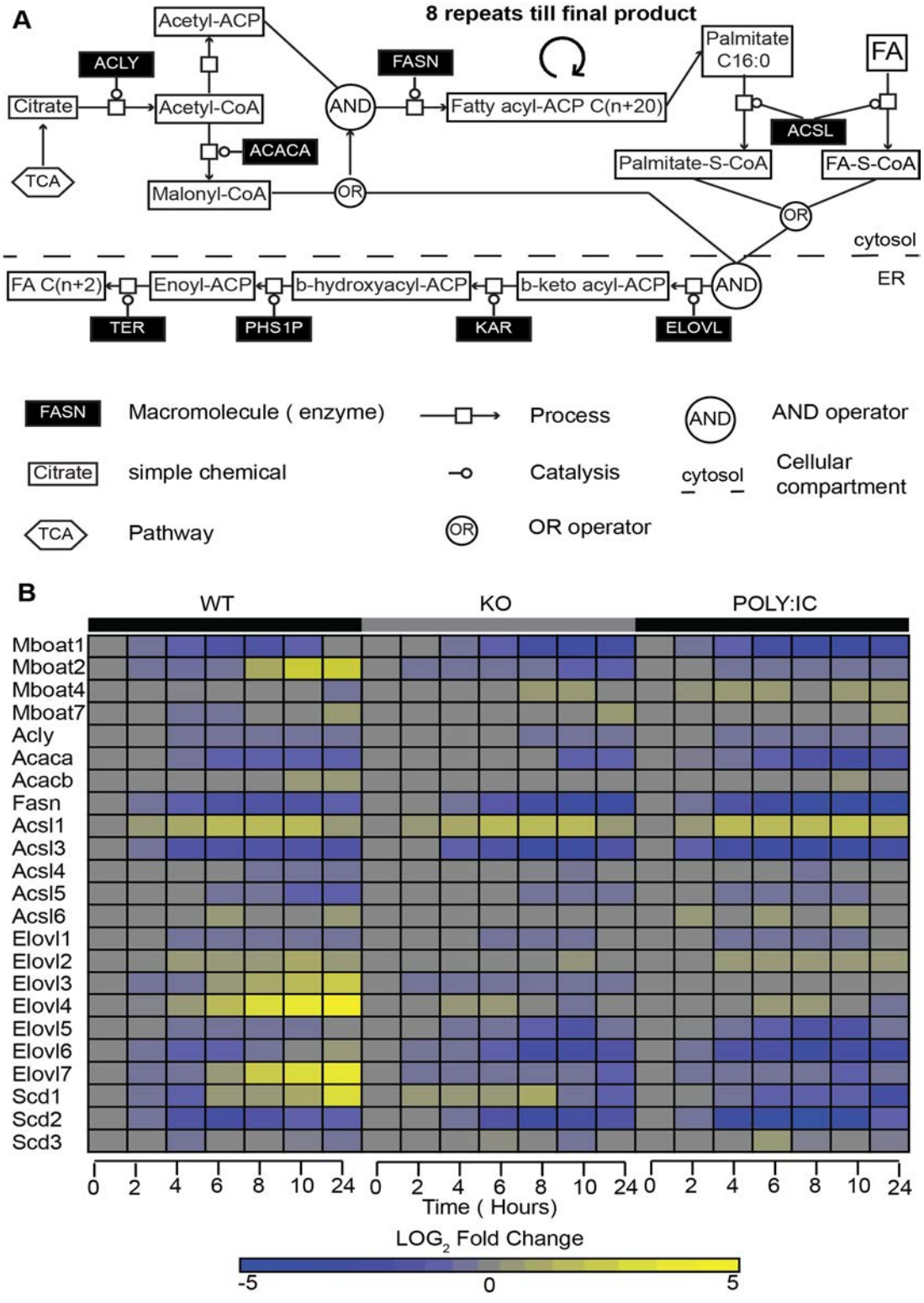


Figure 5.1 Temporal transcriptomics analysis of FA biosynthesis in WT macrophages. (A) The FA biosynthesis and elongation pathway. **(B)** Heatmap of the FA biosynthesis temporal gene's expression over a 24 hour period in BMDM treated with MCMV-WT (MOI 1), or MCMV-KO (MOI 1) or POLY(I:C). Each gene is represented in a row and has been normalised to time point 0. *Mboat1*: membrane

bound O-acyltransferase domain containing 1; *Mboat2*: membrane bound O-acyltransferase domain containing 2; *Mboat3*: membrane bound O-acyltransferase domain containing 3; *Mboat4*: membrane bound O-acyltransferase domain containing 4; *Acly*: ATP citrate lyase; *Acaca*: acetyl-Coenzyme A carboxylase alpha; *Acacb*: acetyl-Coenzyme A carboxylase beta; *Fasn*: fatty acid synthase; *Acsl1*: acyl-CoA synthetase long-chain family member 1; *Acsl3*: acyl-CoA synthetase long-chain family member 3; *Acsl4*: acyl-CoA synthetase long-chain family member 4; *Acsl5*: acyl-CoA synthetase long-chain family member 5; *Acsl6*: acyl-CoA synthetase long-chain family member 6; *Elovl1*: elongase of very long chain fatty acids 1; *Elovl2*: elongase of very long chain fatty acids 2; *Elovl3*: elongase of very long chain fatty acids 3; *Elovl4*: elongase of very long chain fatty acids 4; *Elovl5*: elongase of very long chain fatty acids 5; *Elovl6*: elongase of very long chain fatty acids 6; *Elovl7*: elongase of very long chain fatty acids 7; *Scd1*: stearoyl-Coenzyme A desaturase 1; *Scd2*: stearoyl-Coenzyme A desaturase 2; *Scd3*: stearoyl-Coenzyme A desaturase 3; *Scd4*: stearoyl-Coenzyme A desaturase 4; *Dgat1*: diacylglycerol O-acyltransferase 1; *Dgat2*: diacylglycerol O-acyltransferase 2.

Finally, Stearoyl-CoA Desaturases (SCDs) are essential for the desaturation of FAs. There are four isomers in mouse, and SCD1 is a Δ^9 desaturase that converts 12-18 carbon saturated FA, to monounsaturated FA by inserting a cis double bond at the 9^{th} carbon atom(Sampath and Ntambi 2014).

To confirm that the changes observed above were not induced by type-I IFN signalling, the temporal gene expression of the same genes was also generated in *Infb1-/-* and *Ifnar1-/-* BMDM. Heatmaps for the same genes were generated, as above, for both macrophage types deficient in type-I IFN (**Fig.5.2A-B).** The expression of *Elovl3, 4* and *7*, *Scd1*, *Mboat1* and *Mboat2* increased specifically during productive infection even in these type-I deficient BMDM, suggesting that the genes are upregulated selectively by MCMV taking control of the host cells. Interestingly *Scd1* appears to be upregulated in the poly(I:C) treated *Ifnar1-/-* MΦ. This could be either a false signal, or it could be the result of the differences in signalling among the different cell lines and treatments. Poly(I:C) treatment engages TLR3, but cells productive or non-productive infection engage multiple PRR resulting in differences in the outcome. *Scd1* might be under immune system control, but not type-I interferon. *Acsl1*, on the other hand, has an increased expression across all treatments and all BMDM types reinforcing the idea that this gene might be part of the immune system response, but again not solely under the control of type-I IFN.

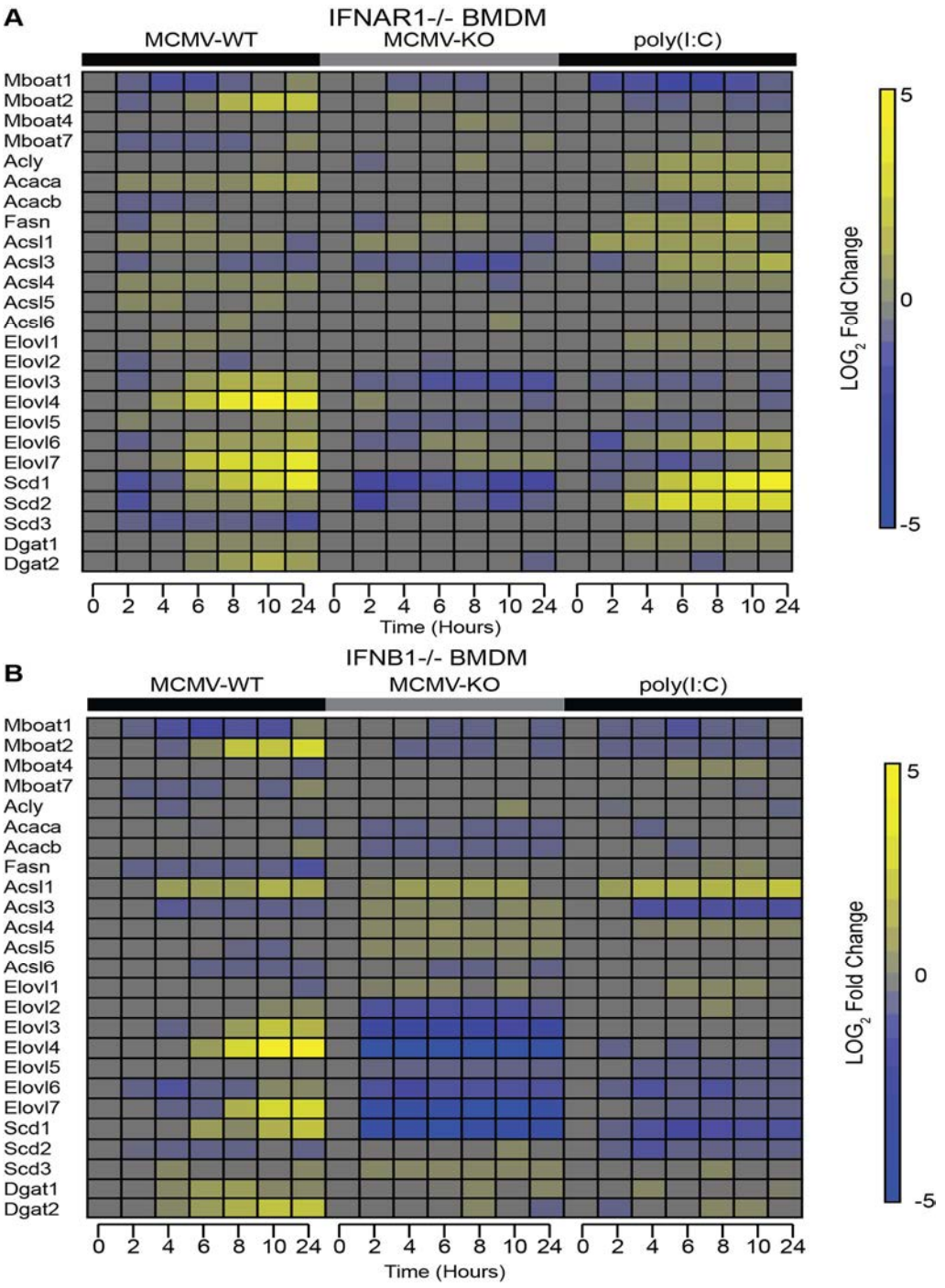


Figure 5.2 Temporal transcriptomics analysis of FA biosynthesis in WT macrophages. (A) Heatmap of the FA biosynthesis temporal gene's expression over a 24-hour period in IFNAR-/- BMDM treated with MCMV-WT (MOI 1), or MCMV-KO (MOI 1) or poly(I:C). Each gene is represented in a row and has been normalised to time point 0. **(B)** Like A for IFNB1-/- BMDM.

To validate the results obtained by microarray, the expression level of some of the genes was confirmed using qRT-PCR, I chose at least one gene from each of the

protein categories listed above, *Elovls, Dagts, MBoats* and *Scds*, and genes that exhibited a fold change >1.5 in combination with high expression levels late in productive infection. It should be noted that *Elovl3, 5* and *7, Scd1, Mboat1, Mboat2 and Dgat2* expression is very low in uninfected cell or early during infection (6 < hpi) and it was a challenge to accurately measure them, even by qRT-PCR.

The expression levels for all genes, except *Acly*, and *Scd1*, was significantly upregulated specifically during productive infection (**Fig.5.3A-F**). *Scd1* expression is downregulated in both treatments (MCMV-WT and MCMV-KO) before the expression was induced specifically during productive infection. The down regulation of *Scd1* during inflammation might be important and not an artefact. Another study has demonstrated that genes involved in the production of unsaturated FA are downregulated early in BMDM treated with Kdo2 LIPID A (KLA), while their expression is induced later to help resolve inflammation through the mediation of unsaturated FAs (Oishi et al. 2016).

As mentioned above the expression levels of *Elovl4, Elovl7, Mboat2, and Scd1* are very low in uninfected cells making it very challenging to detect them. Their expression is, however, markedly increased during MCMV-WT infection allowing for reliable readout.

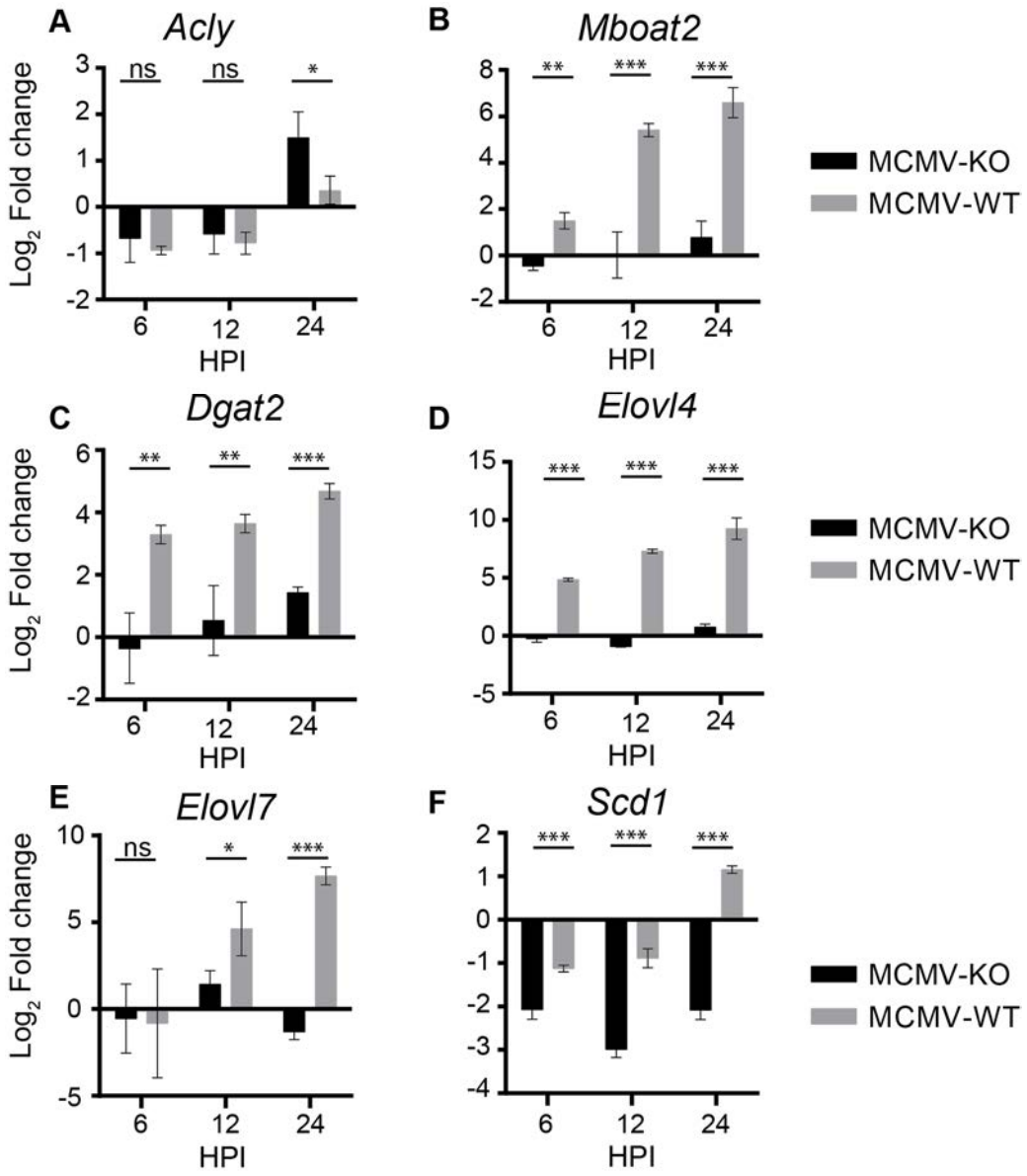


Figure 5.3 Fatty acid metabolism genes are induced specifically during productive infection. (A-F) BMDM were infected with MCMV-WT (MOI 1) or MCMV-KO (MOI 1) and total RNA was harvested at 6, 12 and 24 hpi. The transcript levels of *Acly*, *Mboat2*, *Dgat2*, *Elov4&7* and *Scd1* were calculated in comparison to mock infected cell at 0 hpi. 3 Independent experiments with (n=3) mean ± SEM. T-test with Welch's correction *p<0.05, **p<0.01, ***p<0.001.

The two genes with the highest induction (*Elovl4* and *Elovl7*) in the productively infected cells versus non-productively infected cells were chosen for further study. The

expression levels of *Elovl4* and *Elovl7* was also investigated using different MCMV strains (MCMV-GFP and MCMV-(MCK2+ GFP)) and measured using qRT-PCR. MCMV-(MCK2+ GFP) is discussed in more detail in the methods section. Briefly the MCMV-WT strain that I used during my thesis work does not express the MCK2 protein. MCK2 increases infectivity of MCMV during *in vivo* infection via multiple methods, one of which is by attracting MΦ at the site of infection (Saederup et al. 1999; Stahl et al. 2014). Based on my experiments *in vitro*, infection of BMDM or NIH/3T3 cells by MCMV-(MCK2+ GFP) resulted in similar infection levels as that observed upon infection with MCMV-WT.

For these experiments, BMDM were mock infected or infected with MCMV-KO, or MCMV-GFP, or MCMV-(MCK2+ GFP) for 24 hours. *Elovl4* and *Elovl7* both exhibit remarkably increased expression compared to non-productively infected cells or mock infected cells (**Fig.5.4A**). Taken together these data confirm that the productive infection, and not the immune system, specifically upregulates the expression of the *Elovl4* and *Elovl7*.

To determine if the upregulation of the *Elovl* genes was dependent on viral DNA replication, an experiment was devised using Ganciclovir. Ganciclovir, an antiviral drug, inhibits viral DNA replication (McGavin and Goa 2001) by targeting MCMV DNA polymerase and preventing DNA elongation. BMDM were infected with MCMV-KO or MCMV-WT, subsequently cells infected with MCMV-WT were treated with vehicle or with 10μM Ganciclovir. BMDM treated with Ganciclovir showed a similar expression profile for *Elovl4* and *Elovl7* as BMDM infected with MCMV-WT (**Fig.5.4B**).

In herpes virology genes are classified as IE genes, Early genes and Late genes based on the timing of their transcription. IE early genes require no *de novo* viral protein synthesis to begin transcription, while Early genes required *de novo* viral protein synthesis and Late genes require both *de novo* protein and viral genome replication. Since Ganciclovir blocks late gene transcription, it can be inferred that the induction of *Elovl4* and *7* is regulated by an IE or Early gene product.

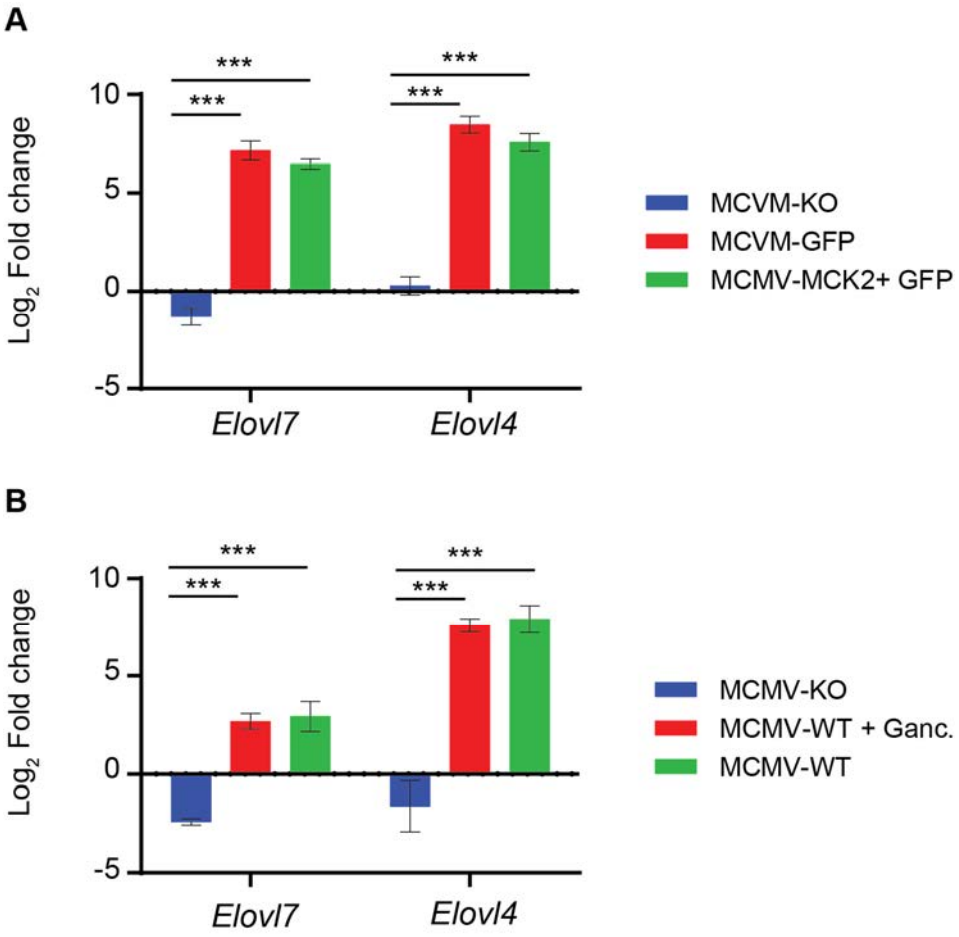


Figure 5.4 Fatty acid elongation genes induction occurs in the presence of Ganciclovir. (A) BMDM were mock infected or infected with MCMV-KO (MOI 1), or MCMV-GFP (MOI 1), or MCMV (MCK2+ GFP+) (MOI 1). At 24 hpi RNA was isolated and the relative expression of the transcripts was determined with qRT-PCR **(B)** BMDM were mock infected or infected with MCMV-KO (MOI 1), or MCMV-WT (MOI 1). After 1 hour of absorption period cells infected with MCMV-WT were treated or not treated with 10μM Ganciclovir. 2 Way ANOVA with Sidak's multiple correction method was used*p<0.05 **p<0.01, ***p<0.001.

5.2.2.2 MCMV-WT greatly increases the presence of long chain FA in infected macrophages

In productively infected cells, the increased gene expression of *Elovls* results in an altered metabolomics profile, as productively infected macrophages have increased levels of free FA during late stages of infection in comparison to non-productively infected cells (**Fig.5.5A-I**). All the long chain FAs identified in the macrophages, apart from docosahexaenoic (DHA), are more abundant in the productively infected

macrophages. At the same time the increase in biotin levels, an important FA biosynthesis coenzyme, and the depletion of Acetyl-CoA (**Fig.5.5J-K**) also support the notion that there is a switch to FA anabolism, as shown by the transcriptomics data presented earlier.

In comparison to non-productively infected macrophages, DHA is the only fatty acid that is not detected as more abundant in the productively infected macrophages. DHA is an omega-3 fatty acid that has 22 Carbons and 6 double bonds with the first double bond at position 4 and the rest at 7, 10, 13, 16 and 19. For many years it was assumed that the synthesis of this fatty acid required the presence of a Δ^4 desaturase (Sprecher 2000). Contrary to this Howard Sprecher demonstrated that some polyunsaturated long FA, like DHA, are synthesised through the Sprecher shunt. More specifically, he concluded that polyunsaturated long FA, like DHA, are first elongated to 24 carbon atoms, they then get unsaturated and finally they are cleaved to the appropriate length (Voss et al. 1991; Leonard et al. 2002; Sprecher 2000). This might explain why DHA levels appear to be similar in BMDM infected with MCMV-WT and MCMV-KO. Since the production of DHA requires, in addition to the FA elongation pathway, the Sprecher shunt, which does not appear to be engaged according to my data at the transcriptional level.

The data presented here complement the transcriptomic analysis that I undertook in section 5.2.2.1, and support the notion that there is an increase in the FA synthesis in infected macrophages with productive infection showing a greater induction in FA biosynthesis.

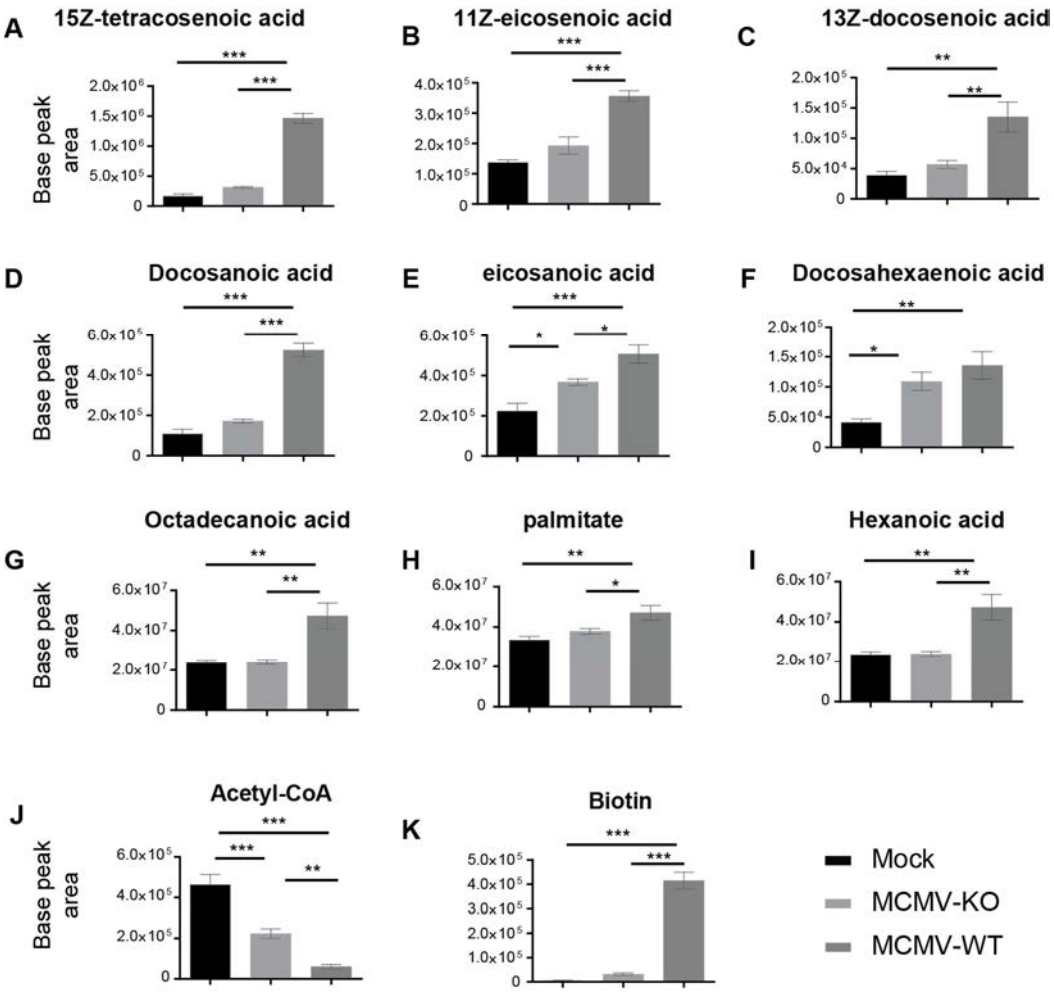


Figure 5.5 Fatty acids levels in productively or non-productively infected macrophages. (A-K) BMDM were grown for 7 days. Then they were mock infected or infected with MCMV-WT or MCMV-KO. At 24 hpi intercellular metabolites were isolated by the cells and were measured with LS-MS. Mean ± SEM (n=6). One way ANOVA with Turkey's correction correction *p<0.05, **p<0.01, ***p<0.001. Non-significant results are not shown.

5.2.3 Inhibition of fatty acid biosynthesis attenuates MCMV growth

5.2.3.1 Pharmacological Inhibition of fatty acid biosynthesis attenuates MCMV growth

I hypothesised that productive infection of MCMV specifically requires fatty acid biosynthesis. To examine this hypothesis, I pharmacologically inhibited fatty acid biosynthesis using C75, a FASN inhibitor, and 5-(Tetradecyloxy)-2-furoic Acid (TOFA), an ACACA inhibitor (Jin et al. 2010; Kariya and Wille 1978). It is important to note that TOFA inhibits both fatty acid elongation and the *de novo* fatty acid biosynthesis, because it inhibits malonyl-CoA production, whereas C75 inhibits only the *de novo* fatty acid biosynthesis.

To assess the level of cytotoxicity in NIH/3T3 cells, NIH/3T3 cells were treated with increased doses of C75 or TOFA and their cell viability was assessed 48 hours post treatment using CellTiter-Blue assay. Both drugs did not affect the cells' viability at the concentrations used (**Fig.5.6A-B**).

NIH/3T3 cells were then infected with MCMV-GFP and subsequently treated with increased doses of C75 or TOFA to determine the effect of both drugs on viral growth. The viral growth was assessed by measuring the GFP signal. The generated IC_{50} values for C75 and TOFA demonstrate that both drugs were able to inhibit viral growth at concentrations below any cytotoxicity effects(**Fig.5.6C-D**). Both drugs exhibited the same inhibitory effects on viral growth, as assessed using plaque assay, when they were tested in BMDM or RAW264.7 cells that were infected with MCMV-GFP (**Fig.5.6E-F**).

Cells grown in lipid-deficient media would have increased need for fatty acid biosynthesis, thus, it is likely that any effects fatty acid inhibitors have will elicit a different response under these conditions. To test this, NIH/3T3 cells were grown in media supplemented with delipidated serum (DLPS). Subsequently, cells were infected with MCMV-GFP and treated with C75. The results demonstrate that in the presence of DLPS, MCMV-WT is more sensitive to inhibition of the FA biosynthesis when cells are grown in media not containing full serum (**Fig.5.6C**).

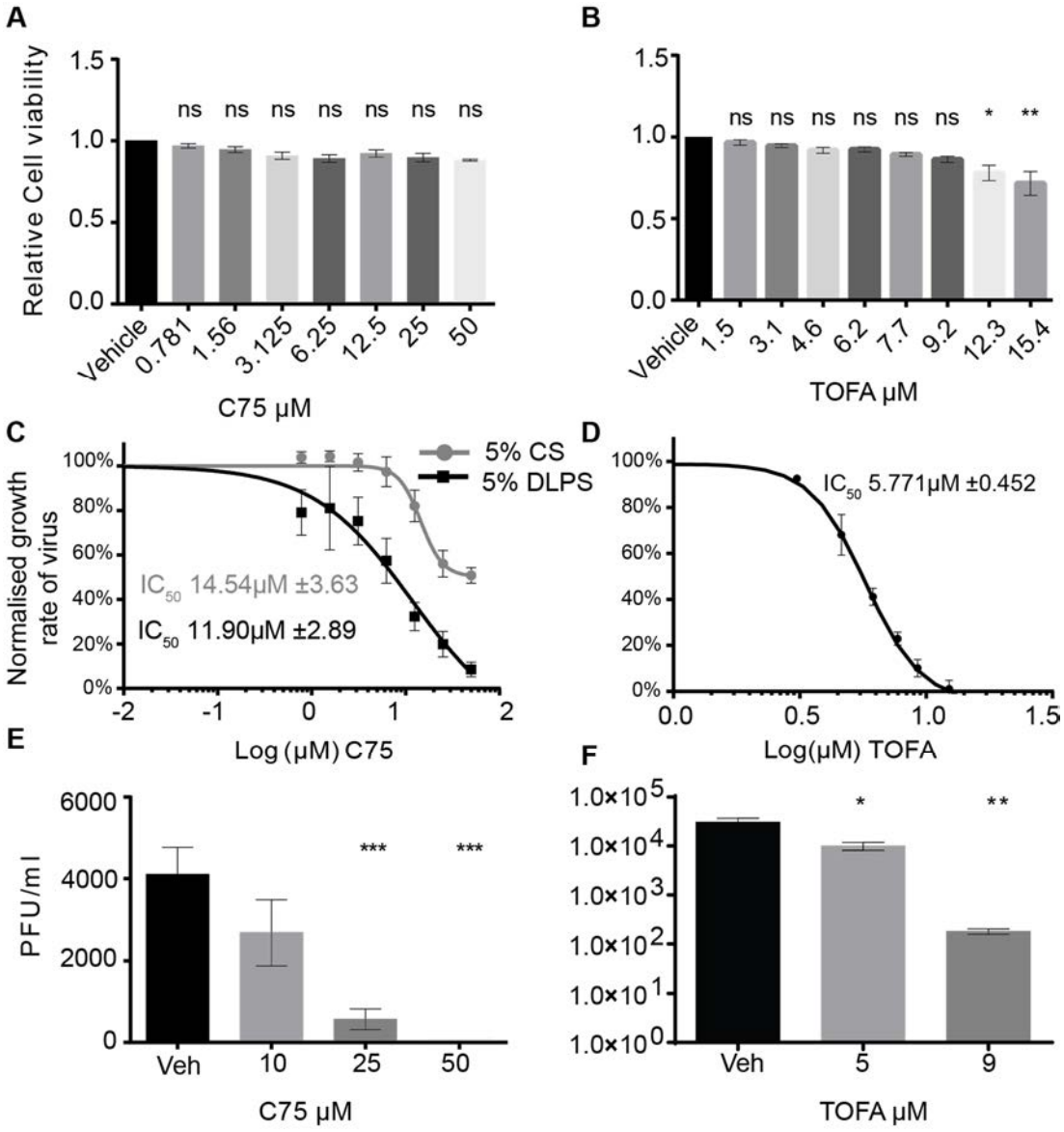


Figure 5.6 Inhibition of fatty acid biosynthesis hampers MCMV growth. (A) NIH/3T3 fibroblasts were seeded in 96 well plates and treated with increasing dosage of C75 the following day. After 48 hours, their viability was assessed using the CellTiter-Blue Assay. Mean ± SEM of four independent experiments (n=6) **(B)** Same as B for TOFA. **(C)** NIH/3T3 fibroblasts were seeded in 96 well plates. Cells were infected with MCMV-GFP the following day (MOI 0.1) and subsequently incubated with media supplemented with C75 at increasing concentrations. The level of infection was determined by measuring the GFP signal. Mean ± SEM of four independent experiments (n=6). **(D)** Same as C for TOFA. **(E)** BMDM from C57BL/6J mice were seeded in 24 well plates. They were subsequently infected with MCMV-GFP (MOI 1) and incubated in media supplemented with C75. On day 4, post infection, the media was collected and the viral titre was assayed by plaque assays. Mean ± SEM of three independent experiments (n=3). **(F)** Same as E with RAW264.7 macrophages treated with TOFA. Mean ± SEM of three independent experiments (n=3). *p<0.05, **p<0.01, ***p<0.001.

Taken together these results reveal the importance of FA biosynthesis pathways for MCMV in multiple cell types including the FA elongation pathway. The evidence provided here is not conclusive regarding the effect of inhibition of FA elongation on MCMV growth. TOFA inhibits both pathways by restraining the production of Malonyl-CoA, which is the building block for FAs, while on the other hand C75 inhibits only *de novo* fatty acid biosynthesis. Since both drugs exhibited antiviral effects it is impossible to characterise the effects that FA elongation has on viral growth. I can only conclude that *de novo* fatty acid biosynthesis inhibition attenuates MCMV growth.

5.2.3.2 siRNA knockdown of fatty acids elongation and membrane remodelling pathways inhibits MCMV growth

Previously, I demonstrated that pharmacological inhibition of FA biosynthesis attenuates viral growth in different cell types. However, the drug inhibitors to my disposal affected either *de novo* FA biosynthesis only, or affected both *de novo* FA synthesis and elongation pathways. Additionally, drugs can have off-target effects especially at higher concentrations.

To independently corroborate the studies presented above and further investigate the effects of the FA elongation and lipid remodelling pathway, I utilised a siRNA knockdown (KD) assay. Several genes of the elongation pathway (*Elovl4, 6 & 7*), two genes of the *Mboat* family (Mboat*1&2*) and D*gat2,* were chosen for knockdown in the knockdown assay in NIH/3T3 cells that were infected with MCMV. Prior to the infection experiments, the knockdown efficiency (KD) and the toxicity of the siRNAs were tested. To assess cell toxicity, NIH/3T3 were transfected for 48 h and subsequently cell viability was assayed using the CellTiter-Blue Assay. **Figure 5.7A** demonstrates that KD of *Mboat2, Dgat*2 and *Scd1* was toxic to the cells. This was also apparent by visual examination of the wells, as the monolayer was severely damaged. These three siRNAs were excluded from the subsequent study.

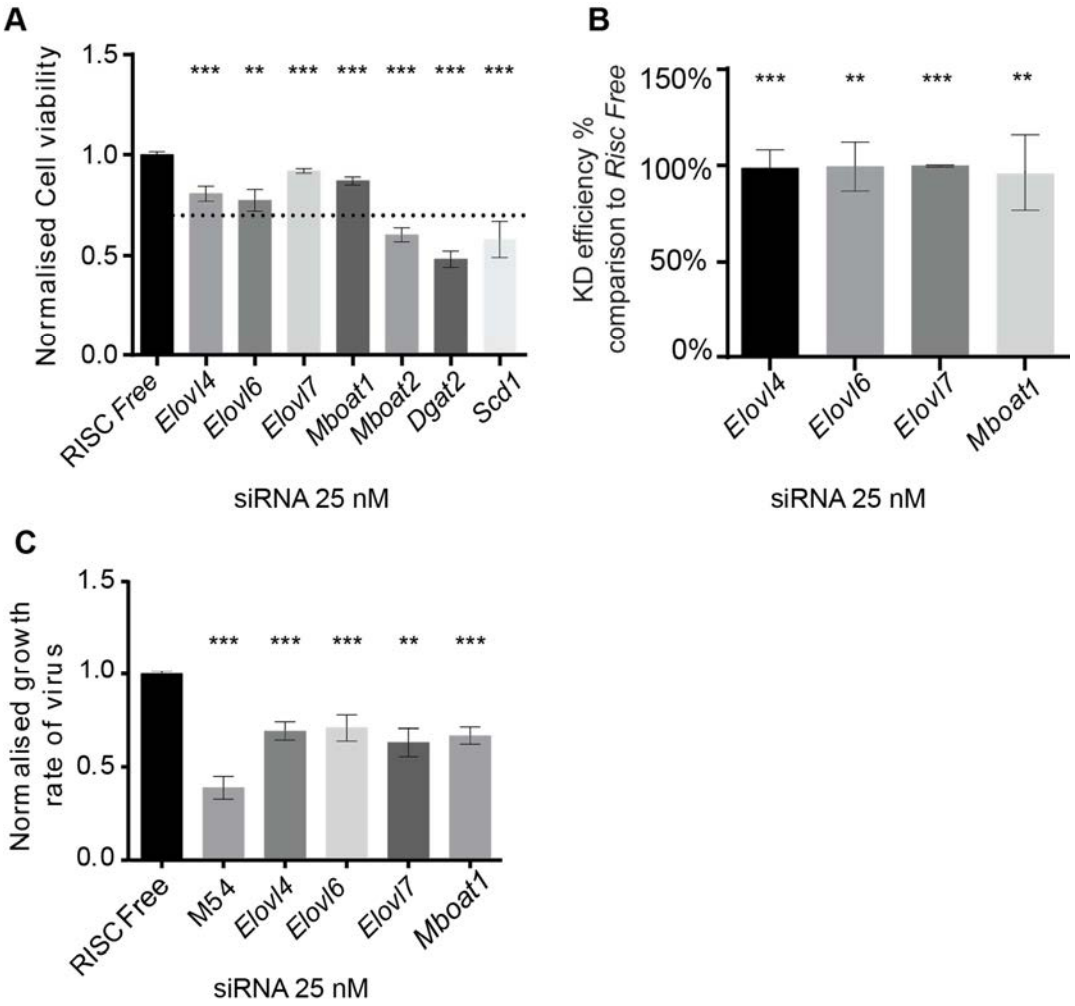


Figure 5.7 siRNA knockdown of fatty acids elongation and membrane remodelling pathways inhibits MCMV growth. (A) NIH/3T3 fibroblasts were transfected in 96 well plates with 25 nM siRNA for 48 hours. Subsequently, the cell viability for each treatment was assessed using the CellTiter-Blue Assay Mean ± SEM of three independent experiments (n=6) **(B)** NIH/3T3 fibroblasts were transfected in 48 well plates with 25 nM siRNA for 24 hours. Subsequently, the cells were infected for 24 with MCMV-GFP (MOI 1), and their RNA was isolated and the transcript level of each gene was determined using qRT-PCR. Mean ± SEM of two independent experiments (n=4). Comparison to *Risc Free* treated cells **(C)** NIH/3T3 fibroblasts were transfected in 96 well plates with 25 nM siRNA for 48 hours. Subsequently, they were infected for 24 with MCMV-GFP (MOI 0.1) and the level of infection was determined by measuring the GFP signal. Mean ± SEM of four independent experiments (n=6). One way ANOVA with Dunnett's correction for multiple comparisons *p<0.05, **p<0.01, ***p<0.001.

To determine the KD efficiency, NIH/3T3 were transfected and after 48 hours RNA was harvested and the abundance of each transcript was assayed by qRT-PCR.

Because of the very low expression values of these genes in cells, it was difficult to determine accurate KD values. To overcome this problem, an alternative method was employed described in Methods 2.4.2. Briefly, in order to induce the expression of the genes, cells were first transfected for 24 hours followed by infection with MCMV-GFP (MOI 1). After 24 hours of infection (48h after transfection), RNA was harvested and the abundance of each transcript was assayed by qRT-QPCR. KD efficiencies greater than 90% was achieved for the genes assayed (**Fig.5.7B**).

Knocking down the expression of the *Elovl4,6, &7* and *Mboat1* genes reduces viral growth in infected NIH/3T3 cells (**Fig5.7C**), demonstrating the importance of the FA elongation and membrane remodelling-pathways for MCMV replication. The importance of ELOVLs, and especially ELOVL7, in the growth of HCMV has already been demonstrated(Purdy, Shenk, and Rabinowitz 2015). MBOATs have not been implicated or shown to have an effect. This is the first time that one of these proteins has been shown to affect MCMV growth.

To further study this interaction, a plaque number and size assay was also performed. The difference with previous plaques assays described earlier is that the diameter of the plaques was also measured in addition to their number. Additionally, for this assay, NIH/3T3 cells were transfected using the forward transfection method instead of reverse transfection (see methods 2.4.1). This transfection method reduced the toxicity of multiple siRNAs and no siRNA treatment appears to affect cell viability (**Fig.5.8A**).

Cells were forward transfected for 48 hours and were subsequently infected with MCMV-GFP at a low MOI of 0.025 to allow the observation of plaque formation on the monolayer. After 48 hours of infection, the cells were fixed and the plaque number and diameter were determined using Celligo Cell Counter. Celligo is a cell counter that can count the number of GFP infected cells and the size of the plaques.

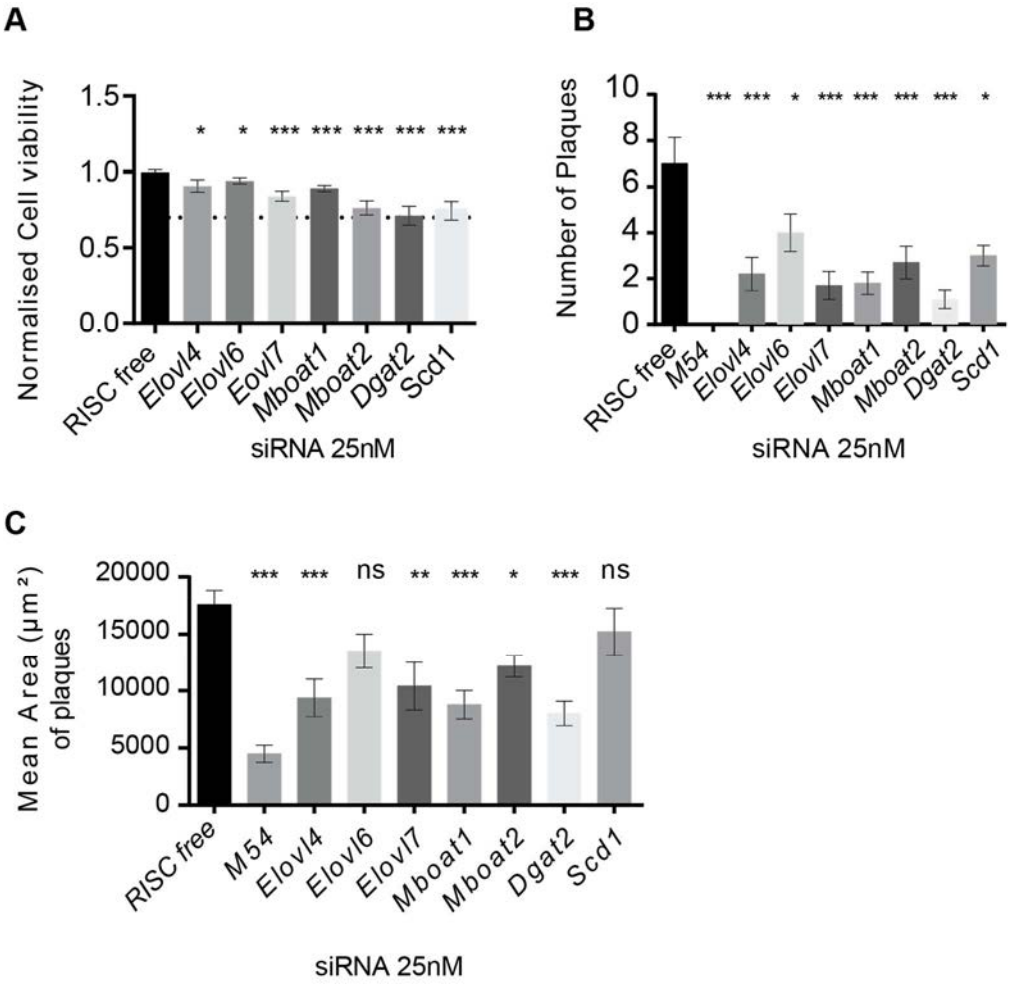


Figure 5.8 siRNA KD of FA elongation and lipid remodelling genes reduces the number and the size of the plaques in infected cells. (A) NIH/3T3 fibroblasts were transfected in 96 well plates with 25 nM siRNA for 48 hours. Subsequently, the cell viability for each treatment was assessed using the CellTiter-Blue Assay Mean ± SEM of three independent experiments (n=5) **(B)** NIH/3T3 cells were forward transfected with 25 nM siRNA for 48 hours. Subsequently, they were infected with MCMV-GFP (MOI 0.025). After 48 hours, the cells were fixed and the number of plaques was determined using photographs of the plates taken by Celligo cell counter. **(C)** Same as B, the mean area of plaques for each treatment was determined by the Celligo cell counter. One way ANOVA with Dunnett's correction for multiple comparisons *p<0.05, **p<0.01, ***p<0.001.

All siRNAs treatments inhibited viral growth, in comparison to RISC-free siRNA treated cells, when plaques were counted (**Fig.5.8B**). It should be noted here that despite the results of the cell viability assay some wells, specifically for *Scd1* KD, showed reduced growth and not complete formation of the monolayer. Taking this into consideration along with the fact that *Scd1* KD viability was close to the cut off value of 70%, the data for these the gene should be viewed with some scepticism. Another form of cell viability assay, such as caspase activation, to test apoptosis would be useful to determine the effect of the KD.

The cell counter used can also count the mean area of the plaques formed on the monolayer during the infection as it can detect the GFP signal produced by the virally infected cells (**Fig.5.8C**). The results demonstrate that all treatments apart from Scd1 KD inhibit MCMV growth. The results presented here complement my previous siRNA study, by demonstrating that KD of *Elovl4,6&7* and *Mboat1* is effective at inhibiting viral growth when the formation and size plaques is monitored on top of GFP signal induction. Furthermore, knockdown of *Mboat2* appears to have no detrimental effect on the cell viability, when forward transfection is used, while at the same time successfully inhibit MCMV growth. This is also true for *Dgat2* KD. *Scd1* KD has a smaller impact on cell viability, but because the results obtained are very close to the cut off set for this study and of the monolayer status in some treated wells, the antiviral effects exhibited should reviewed more critically.

5.2.4 Discussion

MΦ are known to engage their lipid metabolism when they are classically or alternatively activated. Although the function of lipid metabolism during inflammation is not clear yet, it is considered that FA oxidation is associated with tolerogenic events while FA synthesis is associated with inflammatory process(L. A. J. O'Neill, Kishton, and Rathmell 2016). Moreover, proinflammatory signals, like cytokines and LPS have been repeatedly demonstrated to induce FA synthesis in MΦ (Posokhova et al. 2008; Feingold et al. 2012). Additionally, SREBP1, a major FA metabolism regulator, has been found to promote inflammation by regulating genes associated with Il-1β (Im et al. 2011).

In this chapter I analysed the metabolism of FA biosynthesis and lipid remodelling genes during MCMV infection in BMDM. Analysis of temporal transcriptomics analysis of FA biosynthesis and membrane remodelling pathways indicated that MCMV-WT infection specifically upregulates enzymes in these pathways. In accordance to what has been published for HCMV, MCMV also upregulates the FA elongation pathway. *Elovl4 & 7* are upregulated specifically during productive infection (Munger et al. 2008). MΦ from mice deficient in type-I IFN also have induced expression of *Elovl4 & 7* indicating that this is an event associated with productive infection. These genes have a very low abundance in mock or non-productively infected macrophages and are hard to detect even with qRT-PCR, which reinforces the idea that the genes are upregulated specifically by MCMV infection.

Numerous studies have tried to characterise the enzymatic properties of ELOVLs. According to them, ELOVL 1,3,4,and 7 can elongate saturated FA, while ELOVL 2, 4, and 5 elongate monounsaturated FAs (Ohno et al. 2010; Tamura et al. 2009; Ofman et al. 2010; Harkewicz et al. 2012; Leonard et al. 2002; Kitazawa et al. 2009). However, complete characterisation of these proteins and their functions remains difficult due to their nature (big membrane bound proteins) and the nature of their substrates. I cannot, for this reason, reach any conclusions regarding the specific FAs that *Elovl4&7* produce in my system. Despite this, the metabolomics data presented here indicate that there is a markedly increase in FA synthesis in agreement with the transcriptomic data.

Dgat2 was also upregulated specifically during productive infection. *Dgat2* catalyses the last step in the formation of triglycerides (TAG). Macrophages challenged with LPS or poly(I:C) have been shown to have increased TAG formation and expression of *Dgat2* (Feingold et al. 2012; Y. L. Huang et al. 2014). In my analysis, I do not find upregulation of *Dgat2* in macrophages infected with MCMV-KO or treated with poly(I:C). This could be explained by the fact that these studies used peritoneal and/or RAW264.7 and J774 cell lines, which are different phenotypically from BMDM. Especially RAW264.7 and J774 cells are immortalised cell lines, which is bound to have a different metabolic profile than primary cells. Increased TAG synthesis leads

to accumulation of LDs, which certain viruses, including HCMV, have been shown to induce in infected cells (J.-Y. Seo and Cresswell 2013).

Mboat1,2 and *Scd1* are also upregulated specifically during productive infection. Interestingly, *Scd1* has been shown recently to be implicated in the regulation of the inflammatory response in TLR4 activated macrophages(Oishi et al. 2016). At early time points after LPS stimulation macrophages downregulate FA genes, including *Scd1*. Later during the resolution phase of inflammation SREBP1 is upregulated, inducing the expression of FA synthesis genes, which produce FA that help resolve inflammation (Oishi et al. 2016). This is a prime example of FA acting as signalling molecules in addition to their structural and energy storage functions, which has been well characterised.

These data indicate that MCMV infection manipulates FA metabolism in infected cells. FA metabolism is essential is cells for a lot of processes including the synthesis of phospholipids and sphingolipids(Poulos 1995). Apart from the FA elongation MCMV manipulates *Mboat1&2* along with *Dgat2*, which contribute to the growth and maintenance of cell membranes and other organelles, such as lipid droplets, something crucial for several cellular functions (C. C. Y. Chang, Sun, and Chang 2011; Helle et al. 2013; Stone et al. 2009).

Drug inhibition of *de novo* FA synthesis and FA elongation demonstrated the dependency of MCMV-WT on these pathways in multiple cell types. This dependency is increased when cells are grown in lipid-depleted media. The absence of lipids and FA forces the cell to *de novo* synthesise its own lipids as the cell now has limited supply of them. This in turn increases the effects that FA synthesis inhibitors have on viral growth. C75 does not directly affect the FA elongation pathways and TOFA inhibits both *de novo* synthesis and FA elongation, therefore I could not reach a conclusion regarding the importance of elongation versus *de novo* FA biosynthesis. At the same time, I was conducting these experiments there were no readily available drugs that could specifically inhibit FA elongation that would allow me to distinguish between the two pathways. Additionally, my temporal transcriptomics analysis indicated that more genes associated with lipid and membrane remodelling were specifically upregulated during MCMV-WT infection.

In order to overcome this problem, the genes were knock-downed using siRNA. This study revealed that productive infection is inhibited if FA elongases and *Mboat1* are inhibited. *Mboat2*, *Dgat2* and *Scd1* KDs were toxic for the cells when the reverse transfection method was used. There was an improvement in cell viability when the forward transfection method was used, especially for *Mboat2*. The data obtained from *Dagt2* KD also demonstrated inhibition of the virus while the cell viability is not affected. The results for *Scd1* KD, on the other hand, could be dismissed because their toxicity was, even with this method, near the cut-off value of 70% viability.

I hypothesised that the reason for why the forward transfection method works better for these genes might have to do with cell growth. According to the reverse transfection method, the transfection reagents are added on the same day as the cells are seeded in the wells of the plate. The cells are allowed to grow over 48 hours to form a monolayer. Cells that are growing need to process and divide their membranes. To do so *Mboats* and other lipid remodelling genes and processing genes are crucial. MBOATS participate in membrane remodelling by altering the FA composition phospholipid of membranes. Cell membranes are dynamic structures and alterations in their composition greatly affect their rigidity and their physicochemical properties. Disruption of the phospholipid and/or cholesterol content of membranes can have adverse effects on cell viability. *Scd1* also has great effect on the composition of lipid membranes as demonstrated by the abnormal lipid metabolism that *Scd1* KO mice have (Dobrzyn et al. 2005; Attie et al. 2002; Sampath and Ntambi 2014). Knocking down the expression of these genes, when there is increased demand for the enzymes they encode, prevents the cells from multiplying physiologically. This could also be observed under the microscope. The monolayer of the cells with these genes KD was not properly formed and the cells were growing abnormally. In the forward transfection protocol, cells are seeded 24 h in the plates, prior to the transfection, allowing them to grow and form a monolayer.

Taken together, these results demonstrate that MCMV manipulates FA elongation system and remodelling of membranes upon infection. The upregulation in the glycolytic flux discussed in the previous chapter could be feeding into FA synthesis through the citrate shuttle. However, I did not perform any ^{13}C labelling experiments

to trace the flux of carbon atoms and, thus, this conclusion cannot be derived by the data presented here. Additionally, the contribution of other metabolomics pathways that can provide carbon atoms to FA synthesis cannot be excluded for the same reason.

According to the bibliography HCMV increases the production of saturated VLCFA, which it incorporates in its membrane(Koyuncu et al. 2013; Munger et al. 2008). One of the hypotheses is that these FA favour the membrane curvature. I did not analyse the MCMV viral composition and did not performed ^{13}C experiments that would allow me to analyse which FA species are incorporated in the MCMV membrane. Additionally, my metabolomics data were not designed to target specifically FA and lipid metabolites, preventing me from make any claims regarding which FA species are being favoured by the productive infection. They clearly indicate, though, that there is increased production of long chain FA. However, my transcriptomic data in conjunction indicate that, at least in the case of MCMV, the virus engages with the lipid metabolism of infected cells extensively and does not only upregulate the FA elongation pathway. It is quite interesting that MCMV upregulates *Dgat2*. Dgats forms TAGs which decreases the availability of FA in the cell, something that would be contradictory if the virus needed FA.

There must be an additional role for long chain fatty acids, apart from mere structural components for the viral membrane, indicated by the complex way that MCMV engages the pathway. As mentioned before, cell membranes are a dynamic structure and their physicochemical properties greatly affect cellular process like the formation of lipid rafts. Disruption of the phospholipid and/or cholesterol content of membranes has been shown to affect the growth and/or the entry of various pathogens in host cells (Makino and Jenkin 1975; Ohol et al. 2015; Orynbayeva et al. 2007; Haque et al. 2005; Vitiello et al. 2011). The formation of MCMV virions is a very complicated process that requires an initial envelopment at nucleus and a second one at the cytoplasm. Some FA, like C26:0 have been shown to affect the packing of phospholipids and promote the formation of lipid rafts. These studies were conducted in model membranes, but C26:0 has been shown to affect the localisation of an ATPse in yeast, independently of the phospholipid head group that it is attached to(Ho et al. 1995; Hannich, Umebayashi, and Riezman 2011). Additionally, Lipid droplets LDs are not

as passive organelles as it was believed. They actively regulate cellular lipids and different RAB proteins, and have been shown to specifically associate with them, presumably to facilitate lipid molecule exchange with other membranes(Martin and Parton 2006). This last procedure especially is very tightly regulated and allows for different organelles to have different membranes composition, something vital to maintain their functions (Mesmin et al. 2013; Helle et al. 2013).

All these indicate that MCMV might be manipulating lipid metabolism extensively to facilitate virion assembly in addition to using FA as structural components. In fact, MCMV must be under selective pressure to regulate lipid metabolism closely rather than just upregulate FA elongation as the slightest abnormality in lipid metabolism could have adverse effects for the cellular processes. To this complicated picture I must add the function of FA as signalling molecules that regulate numerous processes like metabolism and immunity by interacting with G coupled receptors (Nakamura, Yudell, and Loor 2013; Alvarez-Curto and Milligan 2016; Miyamoto et al. 2016). The signalling properties of some FA like prostaglandins are already known and have been extensively studied, however, it is becoming more and more apparent that more FA can act as signalling molecules (Alvarez-Curto and Milligan 2016). Especially polyunsaturated FA, like DHA appear to be vital in maintaining homeostasis in the host and have extensive anti-inflammatory properties (Kuda et al. 2016).

It should be noted here that even though MCMV appears to be dictating the metabolic programming in infected BMDM late during infection, I cannot be sure that this is not a host driven effect, even partly. The transcriptomic data clearly demonstrate that non-productive infection does not induce upregulation of *Elovls*, *Mboats* or *Dgat2*. However, early studies in vaccines have clearly demonstrated that vaccines with live bacteria are more effective in generating antibodies. Sander *et al.* demonstrated that MΦ can distinguish between live or dead bacteria through the detection or prokaryotic mRNA (Sander et al. 2012). Thus, it cannot be excluded that MΦ might possess a mechanism to distinguish between a replicating virus and a non-replicating one.

Despite that the data presented here demonstrate that FA and lipid metabolism manipulation is a very complicated process that could allow MCMV to manipulate and alter the host cell environment to facilitate growth. Based on this work my hypothesis

cannot be refuted. Namely, that MCMV modulates metabolism in infected MΦ at late stages of infection towards FA elongation to promote viral progeny. The exact mechanisms through which this is beneficial to the virus have, however, not been studied here.

5.3 Study of Prostaglandins synthesis during MCMV infection in BMDM

5.3.1 Introduction

The first eicosanoids were discovered by Ulf von Euler in 1936, who called them prostaglandins because they appeared to be produced in the prostate gland of animals(von Euler 1936). What Ulf von Euler had discovered was a subclass of eicosanoids termed prostanoids. All prostanoids are fatty acids, composed of 20 Carbon atoms, that have been are oxygenated and contain a cyclic ring.

Prostaglandins (PG), which will be the focus of this chapter, have a cyclopentane ring while thromboxanes (TBX) contain a cyclo-hexane ring in their structure. There are four main naturally occurring prostaglandins termed prostaglandins D, E, F and I. The last letter D, E, F, or I is followed by a number, which indicated the number of double bonds that the side carbon chains have attached to the cyclopentane ring. The most commonly enzymatically synthesised prostaglandins are PGD_2, PGE_2, PGF_2 and PGI_2(Bos et al. 2004).

Prostaglandin biosynthesis starts in the cytosol by the cleavage of Arachidonic acid (AA) from the phospholipids in the outer cellular membrane. PTGS11 and PTGS2 (alternatively (COX1 and COX2) then synthesise PGH_2, which is a precursor prostaglandin for thromboxane2 and all PG (**Fig.5.9**). COX1 is expressed constitutively in in most cells while COX2 is induced by inflammation, growth factors and hormones(Dubois et al. 1998). Each COX isomer associates preferably, but not exclusively, with specific prostaglandin synthases(Smyth et al. 2009). The profile of the produced prostaglandins differs depending on the prostaglandins synthetic enzymes present in each cell(Ricciotti and FitzGerald 2011). For example

macrophages produce mainly PGE_2 and TXA_2, while mast cells predominately synthesise PGD_2 (Tilley, Coffman, and Koller 2001).

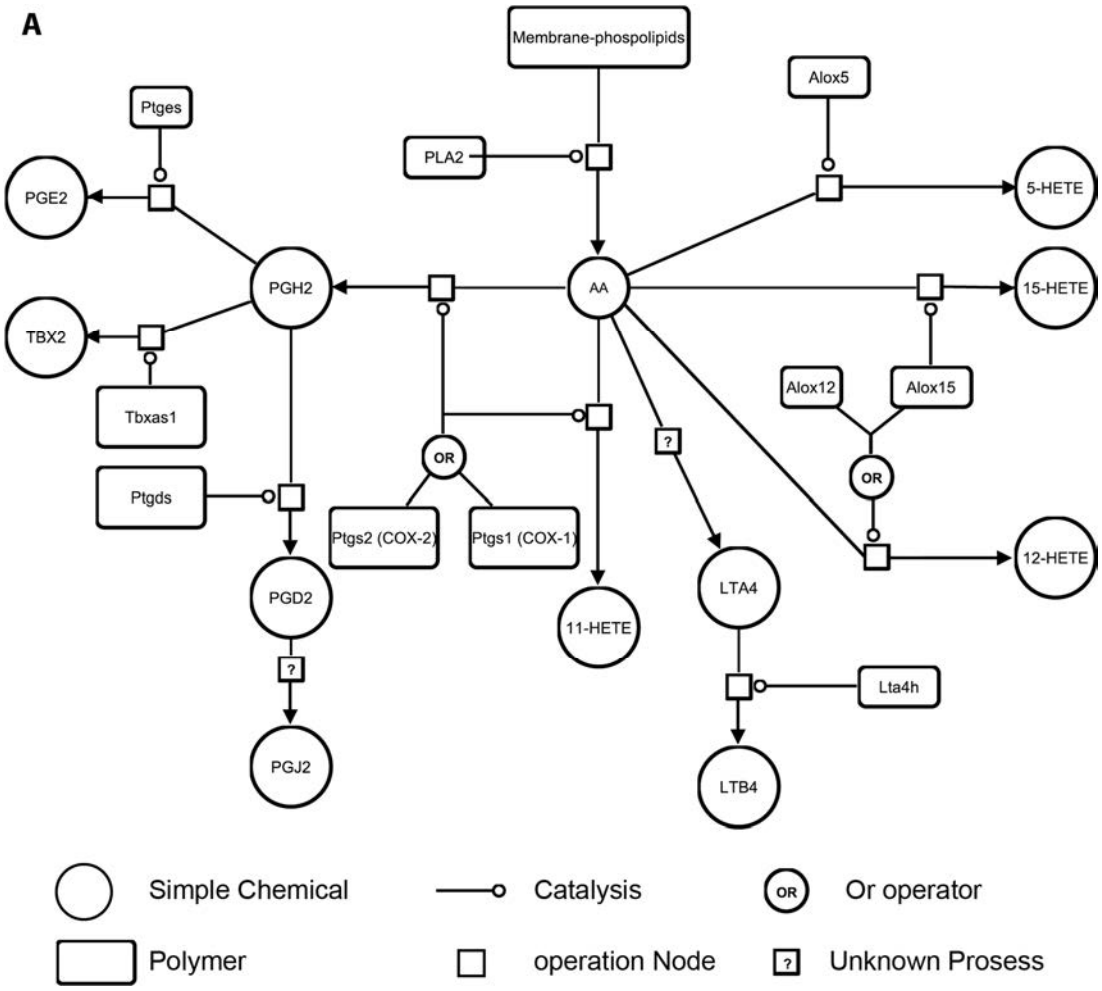


Figure 5.9: The Eicosanoids pathway.

PG act through rhodopsin-like 7-transmembrane-spanning G protein-coupled receptors. There is one PG receptor for each prostaglandin apart from PGE_2, which has 4 E proteinoids receptors (EP1 to EP4) (Ricciotti and FitzGerald 2011).

PGD_2 can spontaneously dehydrate and give rise to another class of molecules, 15-deoxy-Δ12,14-PGJ2 (15d-PGJ_2)(Bell-Parikh et al. 2003). 15d-PGJ_2 has been reported to have anti-inflammatory properties and pro-resolving properties (Surh et al. 2011). These appear to be exerted by a number of mechanisms including inhibition of NF-κB, and suppression of JAK-STAT pathway(Bernardo, Levi, and Minghetti 2000; Castrillo et al. 2000; S.-K. Seo et al. 2014). Despite these findings, very little is known

about the pharmacokinetics and/or the biosynthesis of this molecule *in vivo* making it difficult to assess any potential effects it has in an *in vivo* setting.

Lipidomics studies both in MΦ and DC have shown that PG production is increased upon TLR4 activation (Dennis et al. 2010). Depending on a number of factors including, but not limited to, cell type, site and length of exposure, PG have different effects, proinflammatory and anti-inflammatory(Biswas and Mantovani 2012; Hirata and Narumiya 2012; Tilley, Coffman, and Koller 2001). For example PGE_2 has been shown to inhibit inflammation in DC and MΦ by blocking TNF-α production in MΦ, stimulate IL-10 production and suppress MHC class II (Vassiliou, Jing, and Ganea 2003; Harizi, Grosset, and Gualde 2003; Nataraj et al. 2001). At the same time PGE_2 can stimulate DCs by inducing IL-23 production (Yao et al. 2009).

Due to the central role that PG can play during inflammation there is several viruses that appear to exploit their signalling properties. Coulombe *et al.* in 2014 demonstrated that PGE_2 can inhibit type-I IFN signalling (Coulombe et al. 2014). HCMV has also been shown to upregulate the production of PG upon infection of fibroblasts(Browne et al. 2001; Simmen et al. 2001). The rhesus cytomegalovirus even encodes a COX2 homolog indicating the importance of these enzymes for some viruses (Schröer and Shenk 2008).

HCMV stimulates the expression of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (COX2) leading to the production of PGH_2 (AbuBakar, Boldogh, and Albrecht 1990). Inhibition of COX2 activity has been demonstrated to block HCMV growth in infected fibroblasts (Zhu et al. 2002). Later, studies by Jörg Schröer and Thomas Shenk revealed that COX2 inhibition blocks cell-to-cell spread of the virus (Schröer and Shenk 2008). Both studies indicated that addition PGE_2 could reverse the effect that COX2 inhibition had on the viral growth. Similarly herpes simplex virus type 1 and pseudorabies virus infections of rat embryonic fibroblasts can be blocked using COX1/2 inhibitors, and PGE_2 was able to reverse the effect (Ray, Bisher, and Enquist 2004).

An earlier study on the effects of synthetic prostanoids on MCMV growth, demonstrated that prostanoids can affect MCMV growth depending on their chemical

structure (K. J. Lee et al. 1999). Together, these studies reveal that the regulatory functions of PG during the immune response can have profound effects on the growth of MCMV and determine the outcome of the infection.

Thus, in this chapter I will attempt to examine the effects that MCMV infection has on A key regulatory lipid pathway the PG pathway, and conversely determine the role that the PG pathway has on the viral growth. Based on the studies above I hypothesise that MCMV regulates the PG pathway to produce specific PG that promote viral growth by inhibiting type-I IFN responses.

5.3.2 Temporal transcriptomics and metabolomics analysis of prostaglandins biosynthesis during MCMV infection

5.3.2.1 Transcriptomics analysis of Prostaglandin Pathway

To investigate Prostaglandin (PG) biosynthesis in the context of viral infection I performed (as described in Methods) a temporal analysis of the gene expression of infected BMDM cells. For this purpose, I analysed a data from a resource available in the lab MITCH24KO (University O. E. Lacaze 2011). Briefly, for MITCH24KO, BMDM from WT, or IFNAR, or IFNB1 deficient C57BL/6J mice were used. These were mock treated, or treated with MCMV-WT (MOI 1), or MCMV-KO (MOI 1), or poly(I:C) (10μg/ml). The RNA from each treatment and cell type was isolated at 0, 2, 4, 6, 8, 10, 12 and 24 hours post treatment and high-quality RNA was hybridised to an Affymetrix Mouse Gene 1.0ST microarray.

All treatments of BMDM resulted in the induction of COX2 at 4 hours post treatment. However, MCMV-WT infection and poly(I:C) treatment resulted in a higher expression of COX2 in treated BMDM compared to mock, or MCMV-KO treatments (**Fig.5.10A**). The increased levels of COX2 were expected to result in increased production of PGH_2, the precursor molecule for all PG and Thromboxane 2 (TBX2).

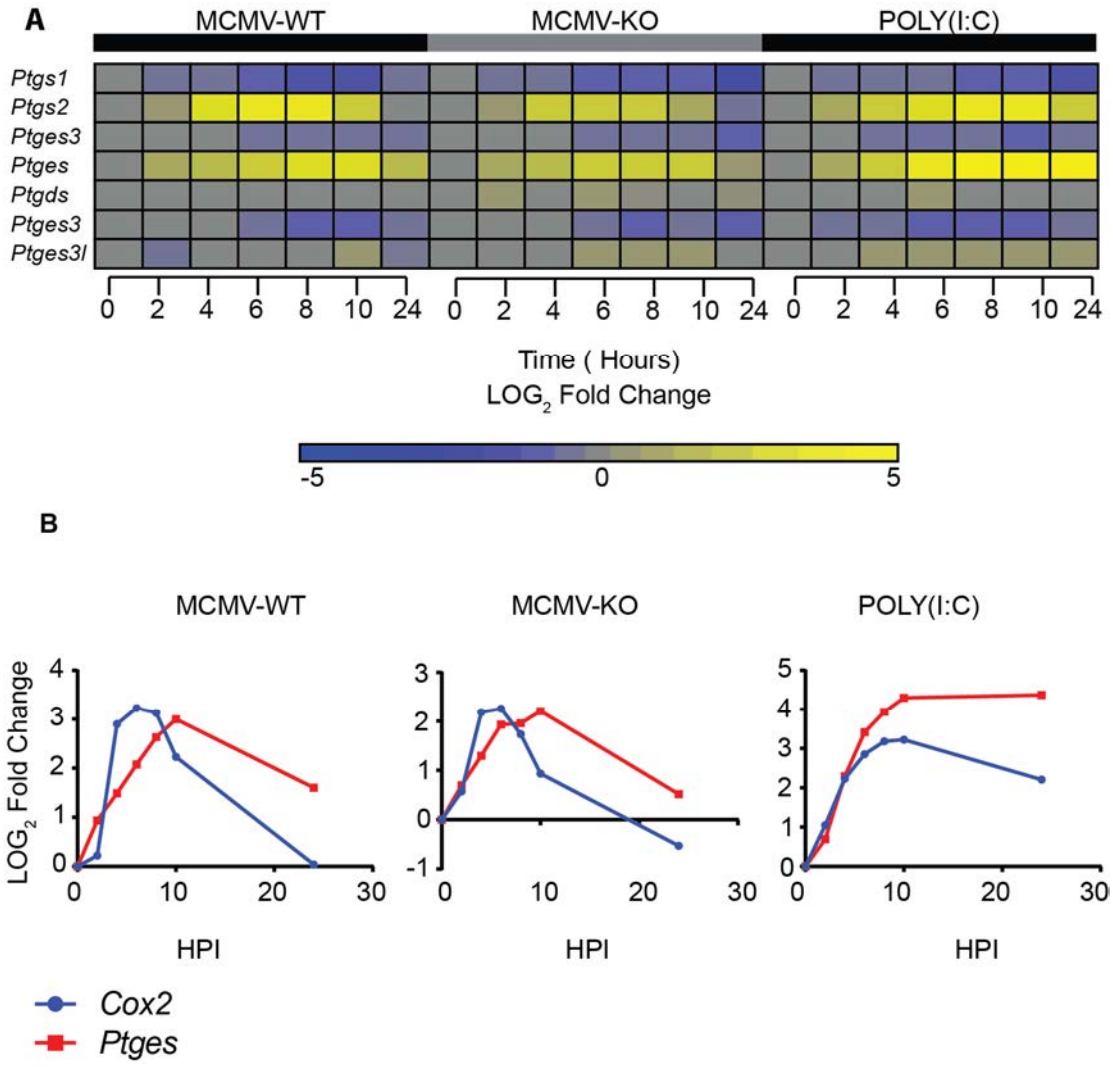


Figure 5.10 Temporal transcriptomics of the prostaglandin pathway in WT macrophage (**A**) Heatmap of the temporal gene expression of prostaglandin synthases (prostagladin pathway) over a 24 hour period in BMDM treated with MCMV-WT (MOI 1), or MCMV-KO (MOI 1) or POLY(I:C). Each row represents the expression of a single gene in the presence of different treatments, and has been normalised to time point 0. **(B)** Temporal gene expression of *Cox2* (blue) and *Ptges* (red) over a 24 hour period in BMDM treated with MCMV-WT (MOI 1), MCMV-KO (MOI 1) or POLY(I:C).

The expression of prostaglandin E synthase (*Ptges*) was induced in a similar manner that almost mirrored the expression of COX2 (**Fig.5.10B**). PTGES catalyses the production of PGE_2 from PGH_2. The increased expression of both enzymes (*Cox2* and *Ptges*) at similar time points and similar levels indicates that the pathway is tuned towards PGE_2 production. The rest of the enzymes, part of the pathway, detected by the microarray were also plotted, but they are not discussed further in this chapter. The

data can be found in the appendix (**Fig.S7.3)**. The data indicate that the productive infection specifically induces the expression of arachidonate lipoxygenases (*Alox*) -8, -12 and -15 at 10 hpi till 24 hpi **FigureS7.3**.

5.3.2.2 Metabolomics analysis of the prostaglandin pathway

While the infection induced transcriptional changes in the COX2 and PGE_2 genes, this is only indicative of an effect/change in the metabolome of prostaglandins. Therefore, to further examine the effects of MCMV infection on the eicosanoids pathway in BMDM a metabolomics analysis of PG was performed.

BMDM were mock treated, or treated with MCMV-KO, MCMV-WT or MCMV-(MCK2+ GFP). The cells were then scraped in the growth media and flash frozen using liquid nitrogen at 0, 12 and 24 hpi. The samples were shipped to Professor Valerie O'Donnell's lab in Cardiff where they were analysed for eicosanoids using LS-MS.

The only PG detected in the samples were PGE_2 and PGD_2. AA was also identified along with some Hydroxyicosatetraenoic acids **(**HETES). The data for HETES can be found in the appendix (**FigS.7.4**). Similar high concentration of AA was detected at 0 hpi in all samples regardless of treatment (**Fig.5.11A**). At later time points the concentration of AA was reduced in all conditions, but it remained higher in samples infected with productive virus. The two productive infections also had similar levels of AA between them. Moreover, the non-productive infection with MCMV-KO, had the same concentration of AA at 12 and 24 hpi as the non-infected samples did.

The data reveal a similar trend for the two detected prostaglandins, PGD_2 and PGE_2 (**Fig.5.11B-C**). PGD_2 concentrations is similar for all treatments at 0 hpi, apart from MCMV-WT infection where it is slightly decreased (**Fig.5.11B**). At 12 and 24 hpi the concentration of PGD_2 is markedly increased in MCMV-WT and MCMV-(MCK2+ GFP), in comparison to mock or MCMV-KO treated cells.

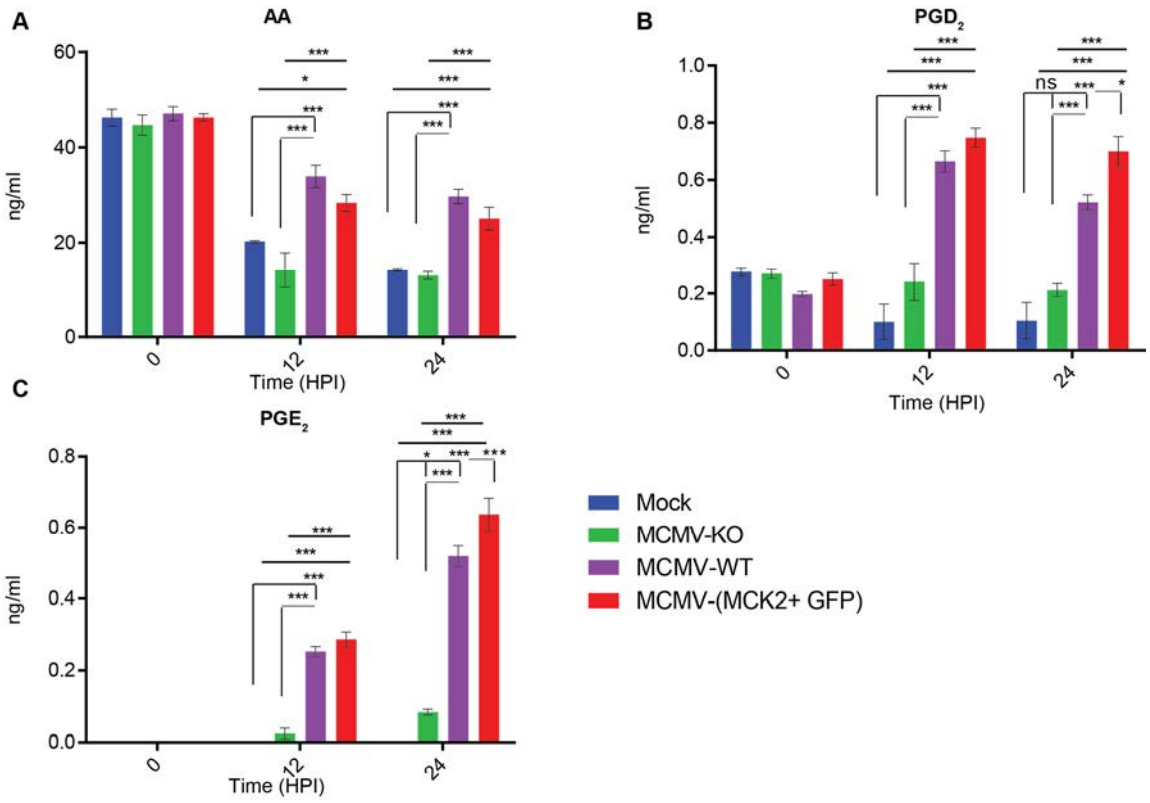


Figure 5.11 Levels of Arachidonic acid and Prostaglandins in productively or non-productively infected macrophages. (A-C) BMDM were grown for 7 days, followed by mock infection or infection with MCMV-KO, MCMV-WT, or MCMV-(MCK2+ GFP). At 0, 12 and 24 hpi cells were scraped in the growth medium, flash frozen in liquid nitrogen and shipped to Cardiff for PG analysis using LS-MS. 1 experiment with Mean ± SEM (n=6). 2 way ANOVA with Turkey correction for multiple comparisons *p<0.05, **p<0.01, ***p<0.001. Non-significant results are not shown.

PGE_2 is not detected in any sample at 0 hpi and remained undetected in mock treated cells at all time points (**Fig.5.11C**). At 12 hpi, the concentration increased significantly to the same level in MCMV-WT and MCMV-(MCK+GFP) cells. While MCMV-KO infected cells appeared to produce a small amount, it was not statistically significant in comparison to mock treated cells.

Taken together, the data indicate that productive infection of MCMV specifically induces PG synthesis in BMDM. PGE_2 and PGD_2 are synthesised at increased levels in comparison to mock, or MCMV-KO treated BMDM at later time points of infection and, thus, the data presented here complement the transcriptional data of the eicosanoids pathway discussed earlier.

5.3.3 PGE_2 promotes MCMV growth in fibroblasts

Inhibition of COX2 has been shown to block HCMV replication and subsequent treatment with PGE_2 can abrogate this effect(Zhu et al. 2002). Additionally, Coulombe *et al.* have demonstrated that PGE_2 can inhibit type-I IFN signalling in macrophages (Coulombe et al. 2014). It was, thus, hypothesised that MCMV induces PGE_2 production in BMDM to block type-I IFN signalling during infection.

To assess the effects of PGE_2 on MCMV growth, NIH/3T3 were infected with MCMV and subsequently treated with PGE_2 and/or EP4 (PF-04418948) antagonist. Addition of PGE_2 to MCMV infected NIH/3T3 promoted viral growth in a dose dependent manner (**Fig.5.12A**). The EP4 antagonist did not affect viral growth on its own, but could reverse the effects of added PGE_2. None of the treatments affected the viability of the cells (**Fig.5.12B**).

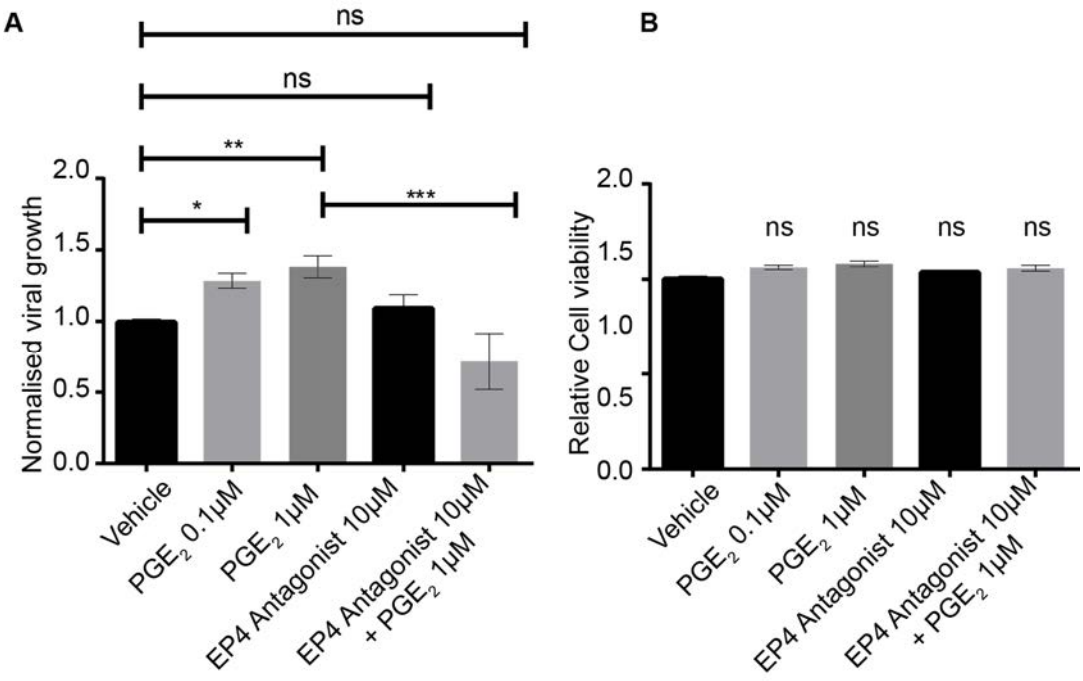


Figure 5.12 Effects of PGE_2 on MCMV growth (A) NIH/3T3 fibroblasts were seeded in 96 well plates. They were infected the following day with MCMV-GFP (MOI 0.1) and subsequently incubated with media supplemented with DMSO (vehicle) PGE_2, EP4 antagonist or with a combination of the two. Mean ± SEM of three independent experiments (n=6). One way ANOVA with Dunnett's correction for multiple comparisons *p<0.05, **p<0.01, ***p<0.001.

These results demonstrate that PGE_2 promotes MCMV growth in infected fibroblasts. The effect is elicited at least in part by EP4 since blockage of the receptor block the signal.

5.3.4 Discussion

In this chapter I studied the effect of MCMV infection on prostaglandin metabolism. Evidence was presented that revealed a correlation between productive MCMV infection and increased biosynthesis of two prostaglandins, PGE_2 and PGD_2, in infected macrophages. PGE_2 was shown to promote MCMV infection in infected fibroblasts through at least EP4.

The effect of MCMV infection on PG biosynthesis was specific on the transcriptional and post-transcriptional level, with an observed coordinated induction of both *Cox2* and *Ptges* over time, which showed a remarkable correlation between the two enzymes.

On the post-transcriptional level, at 0 hpi, AA was detected in all samples at a similar level. This could be attributed to the treatment of the cells before being harvested. In accordance to the infection protocol that we follow in our group, cell growth media was removed, followed by a 1 hour adsorption period in virus-containing media and a PBS wash before the addition of new growth media and cell harvesting. This handling of the cells could lead to AA production induced by the stress caused on the cells. The levels of AA are reduced over time, but they remain higher at the productively infected samples.

At the same time, PGE_2 synthesis was increased in a transient manner. PGE_2 was not detected at 0 hpi in any sample, but was detected at 12 and 24 hpi, mainly in the productively infected cells. Similarly, PGD_2 levels were increased in a transient manner from 0 to 24 hpi with the increase being predominant in the productively infected MΦ despite *Ptgds* not being upregulated. The data presented above are in agreement with a published study by *Kihara et al.* on the eicosanoid fluxes in BMDM(Kihara et al. 2014).

In their study, *Kihara et al.* provided evidence for a functional coupling of COX2 and PTGES in BMDM treated with Kdo2-Lipid A (KLA) and then stimulated with the ATP. The metabolomics and transcriptomics data of the pathway presented in this study are very similar with the PG data presented in this chapter.

Treatment of PGE_2 in MCMV in infected fibroblasts induced viral growth. *Zhu et al*. and Schröer *et al.* have published that COX2 inhibition blocks HCMV replication and that treatment with PGE_2 could reverse the effect (Zhu et al. 2002; Schröer and Shenk 2008). Although the inhibition of COX2 blocks the biosynthesis of multiple PG, neither study reported on the effects of other PG apart from PGE_2, nor did they monitor the concentration of them over the course of infection. Additionally, Schröer *et al.* reported that the effect of COX2 inhibition blocked cell to cell spread of the virus (Schröer and Shenk 2008). Whether PGE_2 treatment affects MCMV in a similar way remains to be seen. In contrast to what has been reported for HCMV, *Clemente et al.* reported that post-infection with PGE_2 blocked HIV cell to cell spread (Clemente et al. 2014). HIV is a completely different virus from HCMV and has no similarities HCMV, but this finding adds to the complicated picture of PG effects during inflammation.

More recently, other studies have demonstrated that PGE_2 could inhibit proinflammatory signalling and, thus, promote viral growth(Coulombe et al. 2014; Alfajaro et al. 2017). *Coulombe et al.* demonstrated that PGE_2 inhibits IFN-I signalling and apoptosis in influenza A virus (IAV) infected alveolar MΦ via EP2 and EP4 receptors (Coulombe et al. 2014). Interestingly, in their study infection with UV-inactivated IAV (non-productive infection) did not induce production of PGE_2 in alveolar MΦ, however, analysis was done only at 4 hours post treatment. In the study presented here, non-productive infection induced PGE_2 production by only a small amount and at 24 hours post infection.

Another more recent study demonstrated the effects of PGE_2 during porcine sapovirus (PSaV) infection in LLC-PK cells (Alfajaro et al. 2017). PGE_2 was shown to promote viral growth in the cells through inhibition of NO production. Since NO induction is downstream of type-I IFN the effects observed in this study could be attributed to inhibition of type-I IFN signalling by PGE_2 like *Coulombe et al.* demonstrated. Both

studies suggest that PGE_2 has anti-inflammatory properties that allows viruses to evade the immune system response by inhibiting signalling, but both studies routinely use 10 μM of PGE_2,a concentration much higher than the physiological levels at which PGE_2 is present *in vivo*.

My results clearly demonstrated that productive MCMV infection of BMDM induces PG metabolism on the transcriptional and metabolomic levels. It was also demonstrated that PGE_2 promoted MCMV growth in fibroblasts and that this occurs through an EP4 dependent manner, but other EP receptors might contribute too. The lack of an effect by the EP4 antagonist on its own might indicate that NIH/3T3 cells do not produce PGE_2 or produce it at very low quantities. Thus, blocking signalling would not have an effect since the receptor would not be engaged by PGE_2.

The data presented here cannot support my full hypothesis. Productive infection of MCMV does promote the biosynthesis of specific PG, PGE_2 and PGD_2 in infected BMDM on the transcriptional and metabolomic levels. However, further experimentation is required to support or refute my hypothesis, namely that PG attenuates type-I IFN signalling during MCMV infection and, thus, promote viral growth.

It is possible to study these effects on the post-transcriptional (mRNA levels of type-I IFN ISGs) and protein level of type-I IFN signalling. Using RT-qPCR in mock or MCMV infected BMDM that haven been treated with PGE2 and or EP4 antagonist, the mRNA levels of *Stat1*, *Ifnb1*, *Irf3* and 25-hydroxycholesterol (*25-hc*) could be detected and compared between each treatment to assess any effect that PGE_2 might have on them. Using the same experimental setup, the phosphorylation levels of STAT and IRF3 (activation) could be studied to detect, on protein level, the effects that PGE_2 might have on type-I IFN signalling.

6 Discussion

In this thesis, evidence has been presented demonstrating the importance of metabolism for MCMV growth in infected macrophages. More specifically, transcriptomic and metabolomic data of MCMV infected BMDM reveal the upregulation of key regulatory steps in the glycolytic pathway early during infection. Critically, MCMV growth is blocked by inhibition of the glycolytic pathway. Additionally, when glycolysis is inhibited, activated DC fail to acquire a fully activated phenotype(Everts and Pearce 2014).

These same data indicate that these changes in glycolysis are concurrent with altered TCA cycle function. The TCA cycle is broken at isocitrate formation and at α-KG generation during MCMV infection according to my data. It is in agreement with work by other groups that have already demonstrated similar alterations in both pathways, in proinflammatory DCs and MΦ(Everts et al. 2014; EL Kasmi and Stenmark 2015). The transcriptomics and metabolomics data further indicate that MCMV infection alters the Urea cycle, a pathway intersecting the TCA cycle, to support NO production. The production of NO from MΦ has long being considered a hallmark of proinflammatory phenotype because of the bacteriostatic effects of NO (EL Kasmi and Stenmark 2015)

The data presented here, with regard to MCMV effect on MΦ , show similarities in the metabolism of HCMV infected and MCMV infected cells. There are differences, which could be attributed to the cell type utilised in the studies and different viruses. In HCMV studies, the metabolism was studied in infected HFF while I utilised BMDM for my experiments. HCMV infected cells and activated macrophages use glycolysis for the generation of energy (Jha et al. 2015; Munger et al. 2008). HCMV infected HFF, among others, upregulate glycolysis and the TCA cycle during infection. The glycolytic pathway provides energy and extra carbon molecules that are shuttled to generate FA. HCMV increases the flux in glycolysis, the TCA cycle and FA synthesis to synthesise the precursor molecules required to replicate (Munger et al. 2008). It should be noted here that OXPHOS is still functioning in HCMV infected HFF unlike in proinflammatory DCs and MΦ(L. A. J. O'Neill, Kishton, and Rathmell

2016).However, a lot of carbon atoms are shuttled outside of the TCA cycle in HCMV infected HFF to support FA synthesis (Koyuncu et al. 2013).

Functional assays using Seahorse Extracellular Analyser offered evidence that early during infection the glycolytic flux is greatly increased in BMDM infected with MCMV-WT, or MCMV-KO, or treated with poly(I:C). Thus, the programming for the induction of glycolysis has an immune driven component and appears not to require *de novo* viral protein synthesis. The temporal transcriptomic data of glycolytic enzymes (*Glut6*, *Hk3* and *Pfkfb3*) expression further supported the hypothesis that the reprogramming of the glycolytic pathway and its subsequent induction is dependent on type-I IFN. Type-IFN deficient BMDM were not able to induce these glycolytic genes, or at least not as efficiently in the case of *Pfkfb3*. This is consistent with previously published data in DCs. Pantel *et al.* demonstrated using IFNAR-/- DCs that type-IFN is required for the glycolytic pathway to be engaged, while at the same time TLR3/MDA5 signalling on its own was not sufficient to initiate the same metabolic programming (Pantel et al. 2014).

Thus, in this thesis, I present the first paradigm, to my knowledge, suggesting that a virus co-opts the metabolic programming induced in immune cells during their activation to establish infection in host cells. However, there are gaps to be filled in my studies such as whether this co-option takes place *in vivo* and if it confers any actual advantage to MCMV allowing it to establish infection in a host as discussed in chapter 4. The evidence provided in this thesis along with numerous publications suggest that MCMV co-opts the metabolomic environment in recently immune-activated MΦ. More experiments are required to thoroughly test this part of my hypothesis.

On the translational side of my research, even though glycolysis inhibition blocks viral growth it is not a recommended strategy. Numerous studies have indicated that the glycolytic pathway plays a key role in facilitating innate and adaptive immune cells to fulfil their effector functions (Pearce et al. 2013; L. A. J. O'Neill, Kishton, and Rathmell 2016; Jiang et al. 2016). Inhibiting it would be more detrimental, in the long run, to the host rather than the virus. MCMV infection appears to opportunistically utilise the metabolomic programming of early-activated macrophages, but this is

probably a temporal advantage much like the capture of TLR signalling as discussed in chapter 4. After some time, cells have an established antiviral state and the immune system is activated, which tilts the scales in favour of the host.

It appears though that there are other pathways that could be inhibited to block viral growth without affecting the host. HCMV has been demonstrated to induce the generation of saturated Very Long Chain FA (VLCFA). *Elovl3&7* have been shown to be specifically induced by HCMV infection, with *Elovl7* being the most important elongase for viral growth in infected HFF (Purdy, Shenk, and Rabinowitz 2015). ELOVL7 has been implicated to support the generation of saturated VLCFA.

In this thesis, I provided evidence that MCMV productive infection, similarly to HCMV, targets FA biosynthesis. Temporal transcriptomic data indicate that productive infection specifically upregulates the expression of *Elovl3, 4 & 7* enzymes at later times of infection. The upregulation is specific to productive infection and driven by the virus and not type-I IFN signalling. In agreement, my metabolomics data reveal induction of long chain FA biosynthesis. Moreover, pharmacological inhibition of FA biosynthesis blocks viral growth or siRNA KD of *Elovl4,6 & 7* also blocks viral growth.

Thus, both HCMV and MCMV in a similar manner appear to be inducing FA elongation. Furthermore, evidence is provided demonstrating that MCMV manipulates lipid metabolism in a broad context. Members of the *Mboat (Mboat1&2)*, and *Dgat (Dgat2)* protein families are also specifically upregulated during productive infection. siRNA KD of these genes inhibited blocked viral growth. Additionally, MCMV extensively interferes with eicosanoid metabolism. My data indicate that MCMV promotes synthesis of PGE_2. PGE_2 treatments of MCMV infected cell promotes viral growth. Although, more experiments are required to unravel the mechanism through which PGE_2 acts beneficially to the virus, my initial data and previous studies by other labs suggest that PGE_2 can block type-I IFN signalling (Coulombe et al. 2014).

Together these data suggest, as discussed in chapter 5, that MCMV manipulates lipid metabolism extensively to generate the lipid species required while also maintaining lipid homeostasis is the cell. These lipid species might support the generation of

structural components for virion assembly and/or create a cellular environment that facilitates their complicated assembly while also suppressing interference by the host immune system. More studies are required to determine what are functional requirements for these genes by MCMV. Nevertheless, it is becoming apparent that there are similarities in the metabolism of MCMV infected BMDM and HCMV infected HFF. Thus, this thesis has also provided evidence that demonstrate the suitability of studying metabolism induced by MCMV infection as model for HCMV infection.

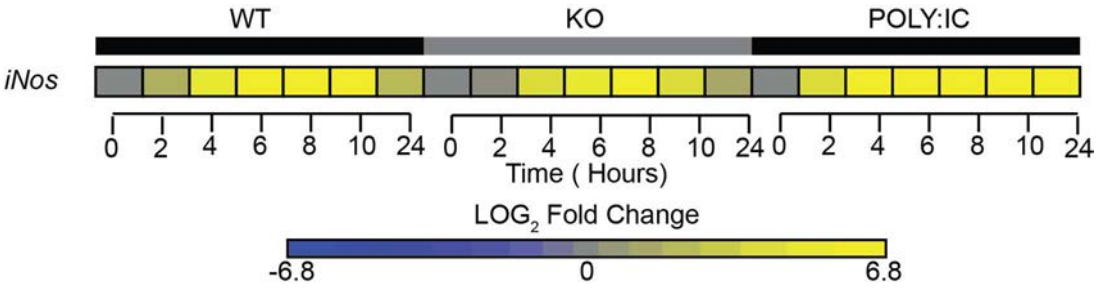
To summarise, my data indicate the MCMV co-ops the metabolism of recently activated immune cells to establish infection and then tunes host cell metabolism towards FA biosynthesis and lipid remodelling. It appears that MCMV is dependent on extensively manipulating metabolism and especially lipid metabolism. It is possible that one of the reasons behind this is latency. MCMV, like HCMV remains latent in cells of the myeloid lineage, but it reactivates only when they terminally differentiate. We still do not clearly understand which signals induce viral reactivation, but metabolism could be one of these. Metabolism has already been established to signal and regulate the immune response. Monocytes appear to exhibit the metabolism of a quiescent cell, but a dramatic upregulation to FA metabolism is observed upon differentiation (Munger et al. 2008; Ecker et al. 2010). Moreover, newly differentiated macrophages that are activated in a site of infection, and have not established an anti-microbial state yet, will exhibit the high glycolytic metabolism and environment that MCMV is known to co-op to replicate. Thus, metabolic signals in conjunction with other ones might promote MCMV and/or HCMV reactivation.

In conclusion, the evidence provided in this thesis do not refute my hypothesis, namely that: productive infection of macrophages by MCMV takes advantage of the early inflammatory metabolomic reprogramming of activated macrophages to establish infection, and modulates metabolism at late stages of infection towards fatty acid (FA) production to promote viral progeny. Part of my hypothesis is the notion that MCMV co-ops metabolic programming to establish infection in activated macrophages. Concrete evidence to support this notion has, however, not been provided in this thesis. Further experiments are required to prove that the co-option of metabolism by MCMV

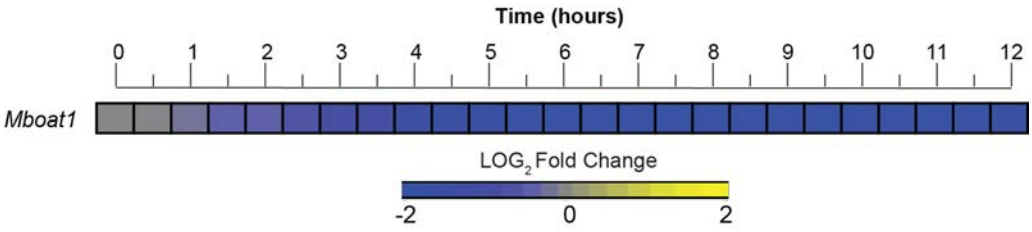
in activated macrophages assists in the establishment of infection and to determine the role of lipid metabolism for MCMV infection.

The thesis presented here is filling a niche in the fields of immunometabolism and virology by studying the manipulation of metabolism in the context a host-pathogen interaction. This increases our understanding about the importance of metabolism in determining the outcome of an infection. The study has arguably presented a new paradigm of viral manipulation of the immune response. MCMV co-opts the metabolic environment of host MΦ cells to establish infection. It also further establishes the suitability of MCMV as a model for HCMV infection in the context of metabolism as the two viruses appear to share several similarities in this aspect too.

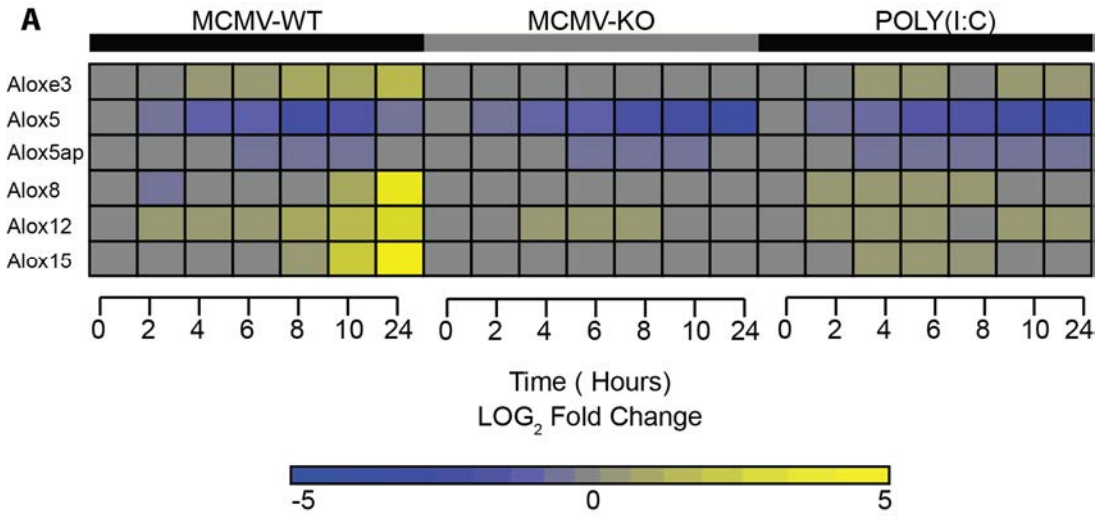
7 Appendix



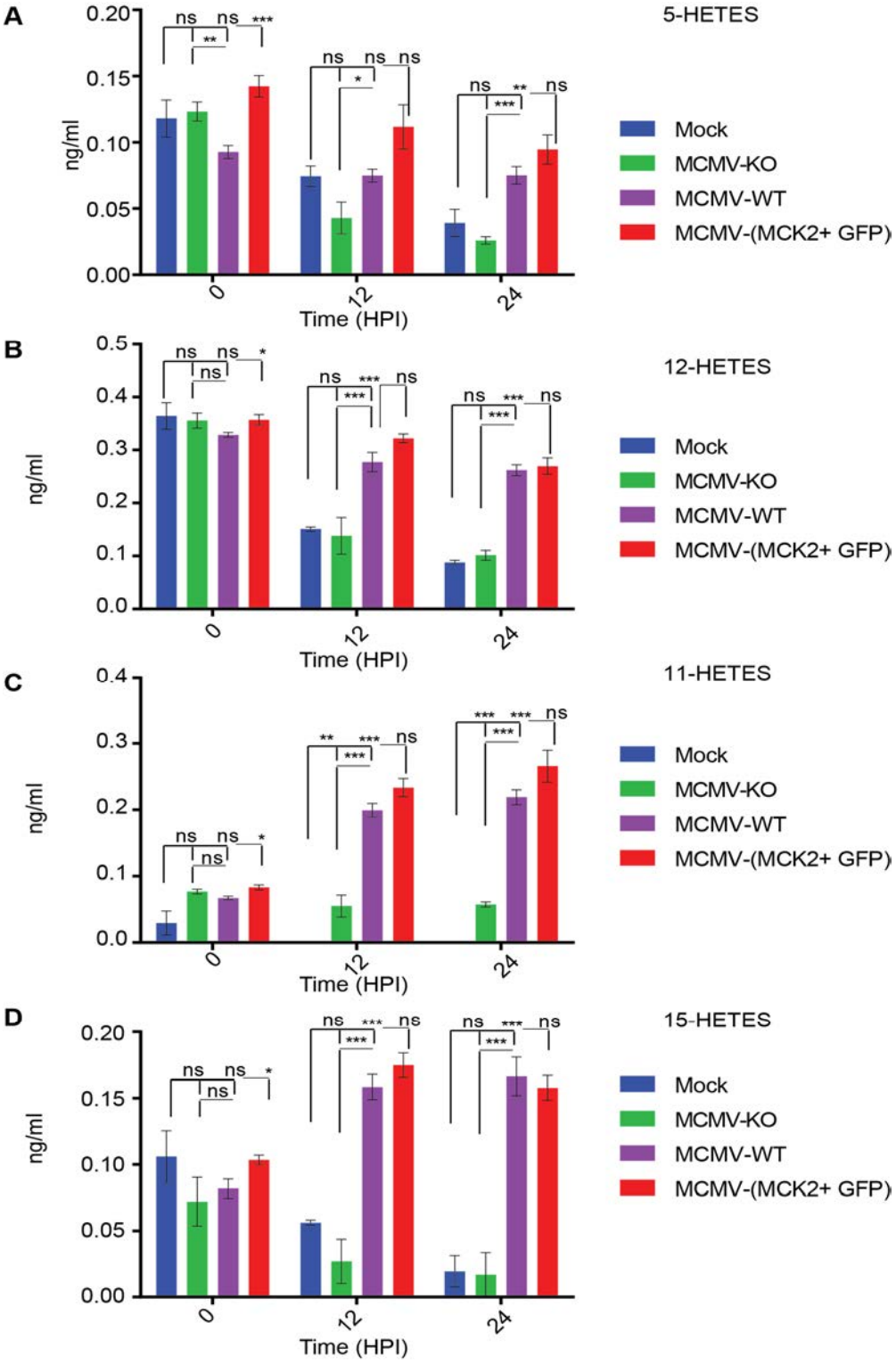
FigureS7.1 Increased iNOS expression during infection. Heatmap of the iNOs temporal expression over a 24 hour period in BMDM treated with 1 MOI MCMV-WT, or 1 MOI MCMV-KO or POLY(I:C). The gene is represented in a row and has been normalised to time point 0.



FigureS7.2 IFN-γ supress expression of *Mboat1*.Temporal expression of Mboat1 in BMDM treated with FN-γ over 12 hours.



FigureS7.3 Figure 7.4 Temporal transcriptomics of the prostaglandin pathway in WT macrophage (A) Heatmap of the temporal gene expression of Arachidonate Lipoxygenases (Aloxs) over a 24 hour period in BMDM treated with MCMV-WT (MOI 1), or MCMV-KO (MOI 1) or POLY(I:C). Each row represents the expression of a single gene in the presence of different treatments, and has been normalised to time point 0.



FigureS7.5 Levels HETES in productively or non-productively infected macrophages. (A-D) BMDM were grown for 7 days, followed by mock infection or infection with MCMV-KO, MCMV-WT, or MCMV-(MCK2+ GFP). At 0, 12 and 24 hpi cells were scraped in the growth medium, flash frozen in liquid nitrogen and shipped to Cardiff for eicosanoids analysis using LS-MS. 1 experiment with Mean ± SEM (n=6). T-test with Welch's correction *p<0.05, **p<0.01, ***p<0.001.

Publications

Abstract: Homeostasis underpins at a systems level the regulatory control of immunity and metabolism. While physiologically these systems are often viewed as independent, there is increasing evidence showing a tight coupling between immune and metabolic functions. Critically upon infection, the homeostatic regulation for both immune and metabolic pathways is altered yet these changes are often investigated in isolation. Here, we summarise our current understanding of these processes in the context of a clinically relevant pathogen, cytomegalovirus. We synthesise from the literature an integrative view of a coupled immune–metabolic infection process, centred on sugar and lipid metabolism. We put forward the notion that under- standing immune control of key metabolic enzymatic steps in infection will promote the future development of novel therapeutic modalities based on metabolic modifiers that either enhance protection or inhibit infection.

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REVIEW

Infection homeostasis: implications for therapeutic and immune programming of metabolism in controlling infection

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Abstract Homeostasis underpins at a systems level the regulatory control of immunity and metabolism. While physiologically these systems are often viewed as independent, there is increasing evidence showing a tight coupling between immune and metabolic functions. Critically upon infection, the homeostatic regulation for both immune and metabolic pathways is altered yet these changes are often investigated in isolation. Here, we summarise our current understanding of these processes in the context of a clinically relevant pathogen, cytomegalovirus. We synthesise from the literature an integrative view of a coupled immune–metabolic infection process, centred on sugar and lipid metabolism. We put forward the notion that understanding immune control of key metabolic enzymatic steps in infection will promote the future development of novel therapeutic modalities based on metabolic modifiers that either enhance protection or inhibit infection.

Keywords Infection · Glycolysis · CMV · Fatty acid synthesis · Metabolism · Inflammation

This article is part of the Special Issue on Cytomegalovirus.

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Introduction

Viruses are extreme obligate parasites that are strictly dependent on the host's energy and biosynthetic pathways for their replication and morphogenesis, and therefore, these pathways, in particular, afford the host critical immune and non-immune checkpoints to control infection [1]. Notably, the homeostatic regulation of these pathways not only acts to maintain a dynamic equilibrium but also plays a vital role in responding to a wide range of external stimuli, such as infection, which at a systems level must integrate and coordinate multiple diverse processes ranging from the homeostasis of membranes to energy and immunity [2–4]. Using this line of reasoning, we previously put forward the concept for the 'homeostatic regulation of infection' which is highly relevant to a wide range of pathogens including cytomegalovirus (CMV) and other members of the herpes virus family that are clinically important and well known to establish lifelong infections [3]. Homeostasis therefore underpins many of the regulatory principles discussed in this review.

Historically, the immune system and metabolism have been intensively investigated at different times and by separated research communities (immunologist and biochemists) leading to an unintended misperception that these act as independent systems. However, there is now increasing evidence to show that these two systems are intimately integrated, share resources and cross-regulate each other [5–10]. While we will highlight some of these recent studies in relation to cytomegalovirus infection, we refer readers to a number of excellent recent reviews that cover the elucidation of the molecular mechanisms linking some of these systems [6–10]. At the cellular level, viral infection adds considerable burden to the cell with the increased demand for energy and metabolic building blocks that are commandeered for the production of new viral progeny. A

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number of different viruses have been demonstrated to alter the metabolism of infected cells, including, but not limited to hepatitis C virus, rift valley virus, human immunodeficiency virus and human CMV (HCMV) [11–14]. These changes may be pro, or antiviral, or simply bystander effects. In the case of cytomegalovirus infection of macrophages, some of these metabolic alterations, in particular lipid biosynthesis, have been mechanistically linked to the interferon (IFN) response [15, 16].

Here, we provide a discussion of how cytomegalovirus infection modifies metabolism of the host cell, how immune signalling regulates metabolism and how intermediate metabolites can regulate all three processes: infection, the immune response to infection and metabolism itself. Finally, as a perspective for grappling with this new and emerging understanding, we place metabolism at the centre of an immune–metabolic–infection axis that integrates signals from both the host and pathogen. We propose that metabolism provides a highly tunable switch used by both viruses and the immune system to regulate infection. Hence, the lessons learnt from how the immune system regulates metabolism will inform new therapeutic strategies targeting metabolic enzymes to control acute and latent infections.

Sugar requirement and immune regulation

In normal resting cells, glucose provides the main carbon energy source and is actively imported into cells and rapidly converted to pyruvate in order to enter the tricarboxylic acid cycle (TCA), where it is catabolised to CO_2 through oxidative phosphorylation and which leads to the production of ATP [17, 18]. These processes of using glucose require oxygen. However, under anaerobic conditions, cells can switch to a more inefficient way of using glucose that converts it to lactate. This leads to a smaller number of ATP production per glucose molecule (two ATP molecules) in comparison with oxidative phosphorylation (36 ATP molecules) [19]. Importantly, this now allows the sugar carbons to be used as building blocks for anabolic processes rather than eliminating through the generation of CO_2.

Indeed, it was Otto Warburg in 1924 that made the key fundamental observation that cancer cells, despite the fact that they have adequate supply of oxygen, utilise anaerobic metabolism [20]. He posited that in order for a cell to divide it needs to increase its biomass by reprogramming the TCA cycle for anabolic purposes [21, 22]. In this scenario, pyruvate (a product of the glycolytic pathway) in the TCA cycle can get converted to citrate that can be shuttled to the cytosol instead of completing the whole TCA cycle (Fig. 1). There it can be converted to acetyl-CoA and be used for the biosynthesis of fatty acids which could be employed to form cell organelles and support division. In fact, a number of studies have shown that as cancer progresses, there is an increased production in fatty acid biosynthesis [23]. A similar process in reprogramming the TCA cycle also occurs during immune stimulation and upon infection of cells.

Glycolytic and glutaminolysis pathways

Virus infection

A series of systematic metabolomics studies documented that infection of fibroblasts with HCMV dramatically alters the cell metabolome towards fatty acid biosynthesis [14, 24, 25]. A comparison of Figs. 1 and 2 will aid in following the discussion of the changes that occur upon infection. Studies investigating the flux of ^{13}C labelling in infected fibroblasts revealed that during infection, uptake of glucose is greatly enhanced and through increased glycolytic activity, carbon atoms are shuttled from glucose to citrate and then to fatty acid biosynthesis [14, 25, 26]. There are multiple regulatory steps involved in this process: according to *McArdle* and co-workers, HCMV activates AMP-activated protein kinase (AMPK), an energy sensor responding to low ATP/ADP ratios in cells, which in turn up-regulates glycolysis [27]; PKR-like endoplasmic reticulum kinase (PERK), an ER-associated stress sensor protein [28] is induced; the sterol regulatory protein 1 (SREBP-1), a transcription factor for regulating fatty acid biosynthesis is up-regulated at late times of infection [29]; and expression of Glut-4, a glucose transporter (GLUT), and carbohydrate-response element-binding protein (ChREBP), a transcriptional factor that regulates glucose metabolism and lipogenesis [30, 31] are induced during HCMV infection. All these alterations lead collectively to higher glucose uptake and increased lipogenesis [29–31]. Along with changes in glycolysis, the virus also increases glutaminolysis, and glutamine gets converted to α-ketoglutarate and through anaplerosis allows the TCA cycle to function [32]. Thus, while in normal diploid fibroblasts glutaminolysis is not required, it is essential for infected cells, and blockade of glutaminolysis significantly impacts on the viability of HCMV-infected cells [32]. Finally, Seo and Creswell [33] suggest that this cascade may also be regulated by viperin. Viperin is an IFN-stimulated multifunctional antiviral protein that gets hijacked by viral mitochondrial inhibitor of apoptosis (vMIA), a HCMV protein, resulting in alteration of its function [33–35]. HCMV appears to up-regulate these pathways and inhibition of them and/or of fatty acids synthesis leads to reduction in viral progeny [11, 25, 36]. In fact, the concept of inhibiting metabolic pathways to block CMV infection it is not so new. As early as 1981, 2-deoxy-D-glucose (2-DG), a glycolysis inhibitor, was shown to potently inhibit viral replication [37]. A schematic of these alterations is shown in Fig. 2.

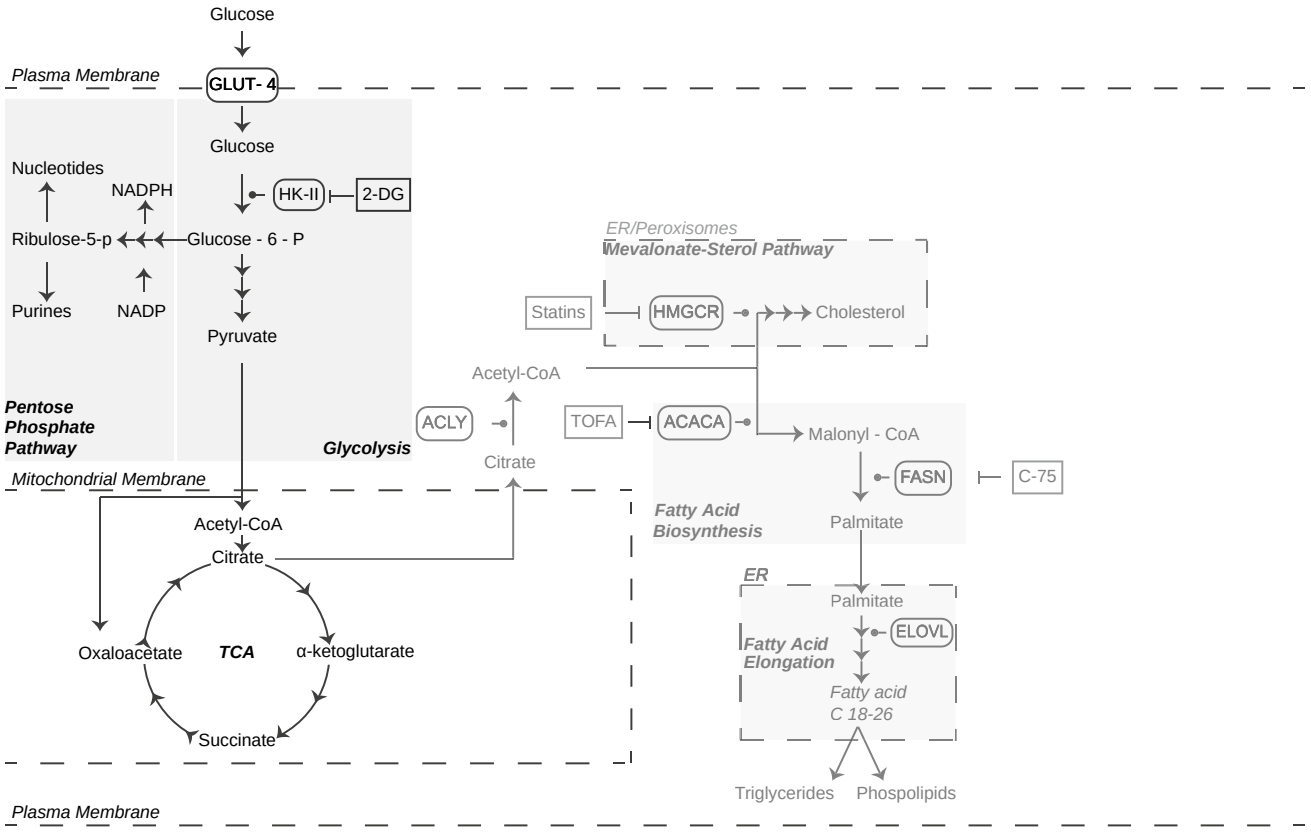

Fig. 1 Metabolism of a resting uninfected cell. In a resting cell, glucose is actively imported and used to fuel oxidative phosphorylation. The glycolytic pathway in the cytosol converts glucose to pyruvate. Pyruvate is imported into the mitochondria where it is used to generate NADH and FADH through the TCA cycle. NADH and FADH can be oxidised by the electron transport chain to generate ATP. Citrate generated in the mitochondria can be shuttled out to generate cholesterol and fatty acids when this is required. *Repression arrows* indicate specific drug inhibitors. Glucose-6-P can also be shuttled to the pentose phosphate pathway where it can be used for generation of NADPH, nucleotides and purines. *GLUT* glucose transporter, *TCA cycle* tricarboxylic acid cycle

Immune activation

The question arises as to whether these changes upon infection are in isolation of immune activation. A comparison of Fig. 3 with 1 will aid in following the discussion of the changes that occur upon immune activation. In this regard, it is now emerging that immune cell activation also leads to marked detectable metabolic changes in cells [38]. Upon activation, lymphocytes, macrophages and dendritic cells have all been shown to alter dynamically their metabolism [39–42]. The precise metabolic changes that occur differ not only from cell type to cell type, but also between different phenotypes of the same cell type [43]. For the purpose of this review, we will focus on discussing macrophages and dendritic cells (DCs) that are permissive to CMV and play a key role in viral dissemination and latency. A schematic summary of these changes is shown in Fig. 3.

Immune-activated dendritic cells and macrophages show enhanced glycolysis and are further compromised in their ability to sustain oxidative phosphorylation in the presence of immune-activated nitric oxide (NO) production, as this molecule potently inhibits oxidative phosphorylation in the mitochondria [44]. Furthermore, in the case of immune-activated macrophages, the reduced mitochondrial respiration is also highly effective at augmenting the generation of anti-infective reactive oxygen species (ROS) [45]. In the case of resting DCs, oxidative phosphorylation is mainly used for energy purposes. However, when treated with Toll-like receptor agonists, these cells adopt a Warburg style metabolism and after 12 h of activation, mitochondrial oxygen consumption ceases and glucose uptake is increased even though it is not using the TCA cycle for ATP production [44, 46]. As described above, this change allows DCs to survive the production of NO and when DCs are treated with 2DG at early stages of activation (before 6 h), the development of the activated phenotype is inhibited [44, 46]. More recent studies reveal that this Toll-like receptors (TLR) activation supports anabolic demands of DCs which include biosynthesis of fatty acids through the shuttling of carbon atoms from glucose to TCA cycle and then de novo

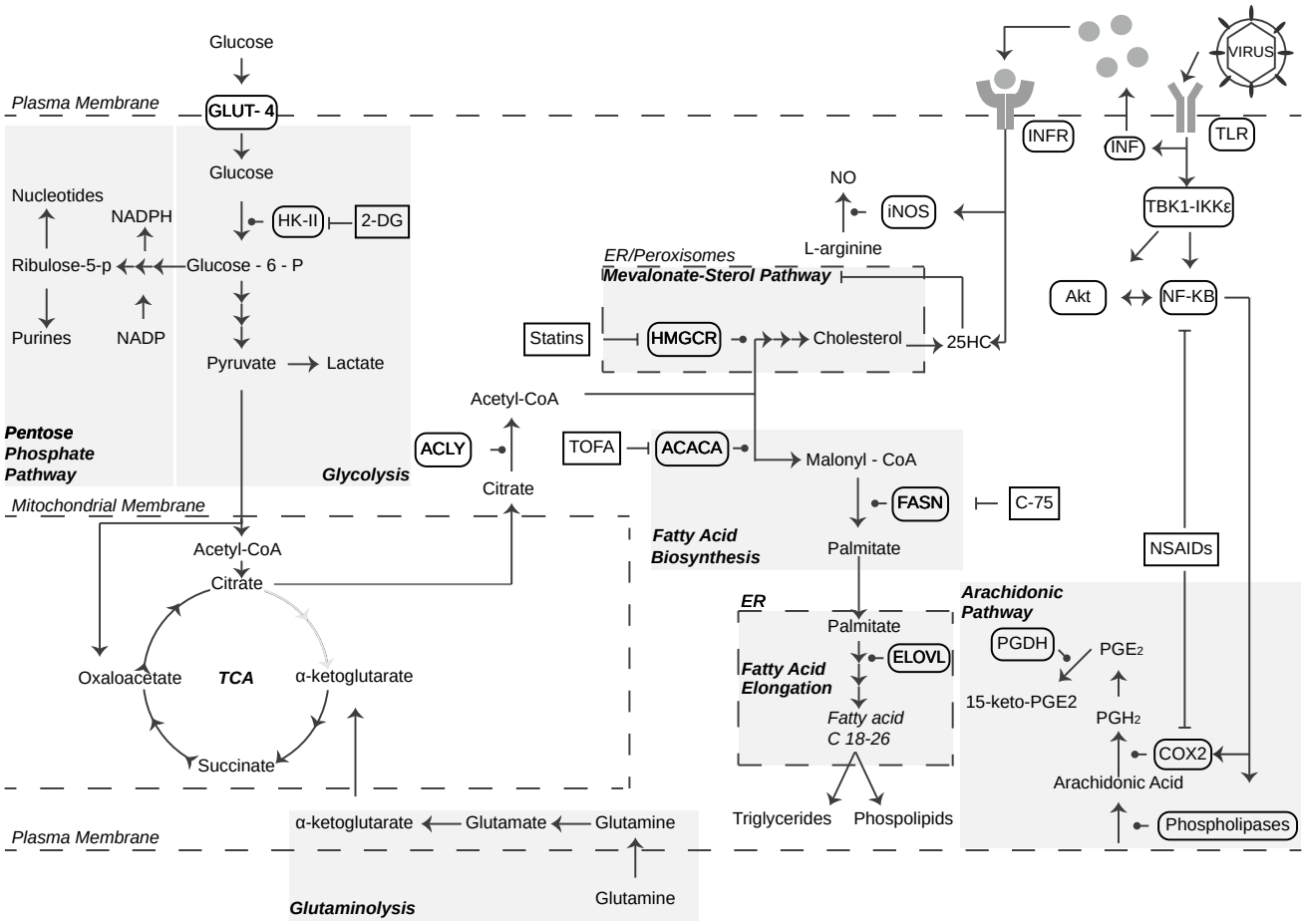

Fig. 2 Metabolism of a cell infected by HCMV. A cell infected by HCMV has its metabolism heavily modified. Glucose importation is up-regulated due to up-regulation in the expression of GLUT-4. Glucose imported is converted to pyruvate, which can be oxidised to lactate for the generation of two molecules of ATP. Some of the pyruvate can be imported in the mitochondria and converted to citrate and instead of being used in the TCA is rapidly shuttled out where it is employed for fatty acid biosynthesis. Glutamine importation is required in order to support the TCA cycle through anaplerosis, which is vital to maintain the mitochondrial membrane potential. If the mitochondrial membrane potential is lost, cytochromes get released in the cytosol and caspases are activated leading to apoptosis. The arachidonic pathway is also up-regulated, and there is increased release of arachidonic acid from membranes by lipases, which COX-2 converts to PGH_2 a precursor for prostanoids. *Repression arrows* indicate specific drug inhibitors. *TLR* Toll-like receptor, *IFN* interferon, *GLUT* glucose transporter, *TCA cycle* tricarboxylic acid cycle, *HK-II* hexokinase-II, *Akt* protein kinase B, *NO* nitric oxide, *NSAIDS* non-steroidal anti-inflammatory inhibitory drugs, *PPP* pentose phosphate pathway

synthesis of fatty acids and prostaglandins [47]. Similarly, IFN-activated macrophages are highly glycolytic and also adopt a Warburg style metabolism [42]. Much like DCs, macrophages shuttle glucose carbon to fatty acids and lipids synthesis, and at the same time, their pentose phosphate pathway (PPP) flux is increased [42]. These changes are required for meeting the demands for enhanced production of membranes in the ER and golgi to accommodate the stimulated protein production as part of the immune-activated functions, including proteins for antigen presentation and cytokine production.

A critical part of this metabolic reprogramming is the very rapid (within minutes) accumulation and secretion of succinate, a metabolic intermediate of the TCA cycle, that has been shown to act as a signalling molecule for the activation of the alpha subunit of hypoxia-inducible factor-1 (HIF-1a) and the induction of HIF-1a-dependent genes [48, 49]. A further interesting aspect of the crosstalk that occurs between metabolism and immunity is the availability of substrates that may also alter the phenotype of macrophages. For instance, overexpression of GLUT-1 leads to increases in glycolytic flux that drives a pro-inflammatory M1-like phenotype by macrophages without any other signalling [50].

Altogether, broadly comparing the isolated infection and immune activation studies, it appears that the alterations in energy and central carbon metabolism show metabolic cooperation, despite different cell type-based

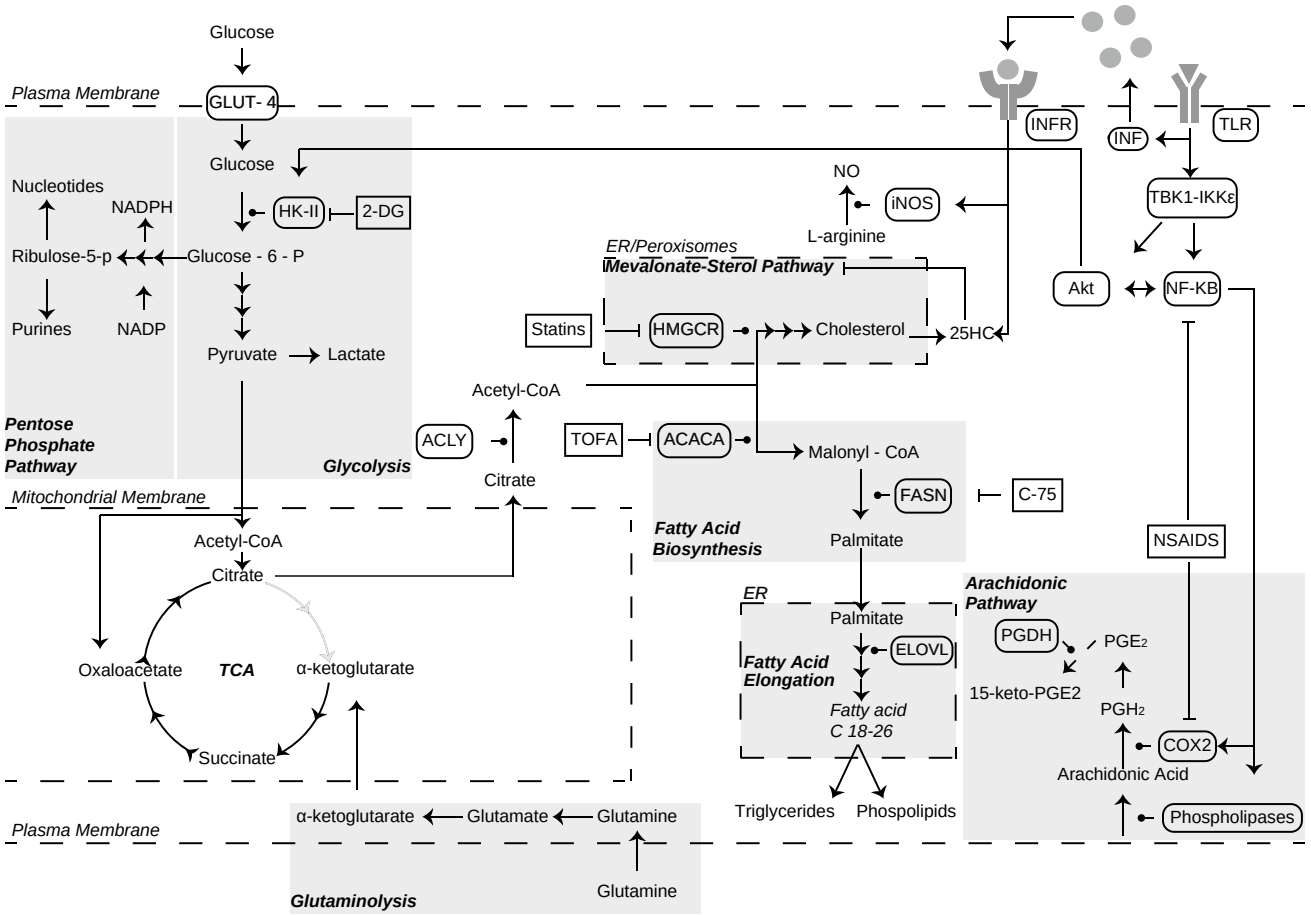

Fig. 3 Metabolism of a macrophage activated by immune signals. Upon activation of TLRs, the cell releases downstream IFN and at the same time, TBK1-IKKε kinases are activated leading to iNOS, Akt and NF-KB activation. This leads to increased glycolysis through activation of HK-II. NO production is also up-regulated from iNOS and blocks generation of ATP by mitochondria, and this also leads in up-regulation of glycolysis in order to produce ATP from pyruvate fermentation. Part of the pyruvate produced by glycolysis is imported to mitochondria where it gets converted to citrate and shuttled to the cytosol and used for fatty acids synthesis required by the cell for its activation. At the same time due to direct signalling by IFN, the mevalonate–sterol pathway is down-regulated. In the arachidonic pathway, COX2 is activated by NF-KB and prostanoids produced by are used as signals in an autocrine and paracrine way to regulate the immune response. *Repression arrows* indicate specific drug inhibitors. The key differences to note in comparison Fig. 2 are TLR activation alone involving HK-II activation by Akt and that TLR agonist is the activator and not virus. *TLR* Toll-like receptor, *IFN* interferon, *GLUT* glucose transporter, *TCA cycle* tricarboxylic acid cycle, *HK-II* hexokinase-II, *Akt* protein kinase B, *NO* nitric oxide, *NSAIDS* non-steroidal anti-inflammatory inhibitory drugs, *PPP* pentose phosphate pathway

investigations. The apparent parallel trajectories of infected and immune-activated cells (compare Figs. 2, 3) strongly suggest that rather than the virus driving these changes, it has the option for co-opting at early times of infection the TLR-mediated signalling of metabolism. It will be important in the future to determine whether CMV-infected macrophages also exhibit a similar alteration in flux and metabolic functions.

Lipogenic requirement and immune regulation

Lipids are a diverse family of chemically distinct molecules that can be hydrophobic or amphipathic and serve multiple roles in a cell. They are used to store energy, signal and maintain cellular structure and have a key building role for all membranes and organelles [51] including for the growth of viruses. For instance, an important class of non-steroidal lipids that act as signalling molecules in immunity are retinoids, which are also known to directly and indirectly control HCMV and MCMV gene expression and viral replication [52–54].

Figure 4 shows the results of very early experiments that we conducted to directly test a potential lipogenic requirement for CMV growth in cells. For these studies, fibroblasts were incubated in medium supplemented with charcoal-treated serum (delipidised media), in which biologically active lipid factors had been removed, and were

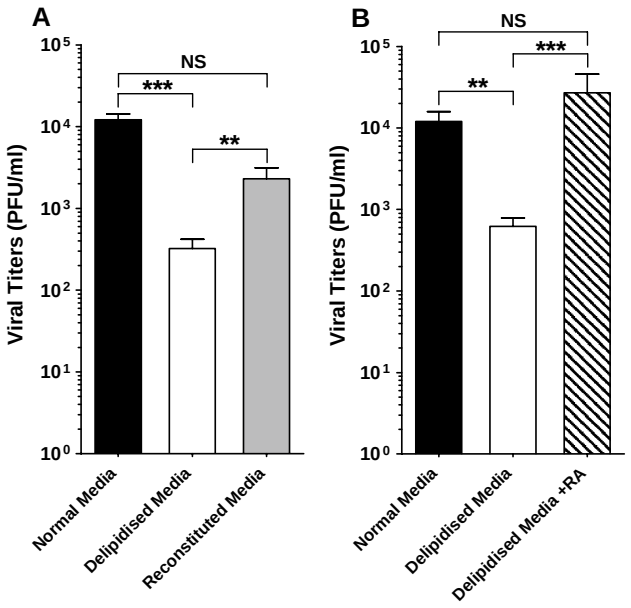

Fig. 4 Lipogenic factor requirement for MCMV growth. **a** NIH 3T3 cells are pre-treated with normal (DMEM 3 % CS), delipidised (DMEM 3 % charcoal resin-treated CS) or reconstituted (DMEM 3 % charcoal resin-treated CS containing chloroform–methanol serum extracted lipids) medium for 4 h and then infected with MCMV at a multiplicity of infection (MOI) of 0.02. After infection, cells received the corresponding medium (normal, delipidised or reconstituted). Viral titers are determined by plaque assay at day 7 after infection from culture supernatants. $n = 4$, data are mean ± SD. **b** NIH 3T3 cells are pre-treated with normal, delipidised or 10 μM 9-*cis* RA in delipidised medium for 4 h and then infected with MCMV at an MOI of 0.01. After infection, cells received the corresponding medium (normal, delipidised without or with 10^{-9} M 9-*cis* RA. Viral titres are determined as in **a**. $n = 3$, data are mean ± SD. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$, determined with an unpaired Student's *t* test

subsequently infected with murine CMV (MCMV) and analysed for viral production. As shown in Fig. 4, the growth of CMV was statistically strongly attenuated under these lipid-depleted conditions. It is worth noting that cell viability was not significantly affected by the removal of lipids in the culture medium in the conditions of the assay. We next asked whether a pro-viral lipogenic component in the serum was possibly removed. To test this notion, a chloroform–methanol method for removing lipids was used [55] whereby neutral lipids (triglycerides, waxes and pigments) are extracted by chloroform and phospholipids; glycoproteins and cholesterol are removed by methanol. These extracted lipids were subsequently used to reconstitute the delipidised serum. Notably, we found that addition of the reconstituted lipids to the delipidised media substantially reversed the viral growth defect exhibited after lipid depletion (Fig. 4a). It is possible that the delipidised media conditions simply impacts the cell by reducing an essential 'lipid' resource for the production of viral membranes, or alternatively but not mutually exclusive, is the possibility for a pro-viral regulatory lipogenic factor. In order to distinguish between these possibilities, we sought to test whether the capacity of infected cells to generate infectious particles under delipidised media conditions is due to limited membrane lipid resources. In order to assess the maximal capacity for additional virus production under delipidised culture conditions, we employed a well-established and potent regulatory lipid for enhancing CMV, 9-*cis* retinoic acid (RA) [54]. Treatment of cultures in delipidised medium with 9-*cis* RA led to a notable increase in MCMV growth that reached levels comparable or even higher than those obtained in cultures containing the normal medium, indicating that resources are not limiting the capacity of the cell to produce virus (Fig. 4b). Thus, these investigations reveal a requirement of lipogenic factors for optimal MCMV infection and indicate that this dependency is not only due to a deficit of membrane resources for viral growth in the cells. Please note that we do not infer from these experiments that retinoic acid is the regulatory limiting lipogenic component although this possibility cannot be excluded. Most importantly, these experiments underscore that careful consideration should be given to interpreting experiments that block or deprive cells of lipids and assessing the impact on viral growth. For this reason, the view that a pro-viral host lipid represents a depleted resource may not necessarily be the case and should be distinguished from possible effector or regulatory signalling functions. In the next section, we discuss the functional roles and alterations that occur for lipids upon infection and immune activation.

Long chain fatty acid pathway

Virus infection

As described above, HCMV infection shuttles carbon atoms to fatty acid synthesis (compare Figs. 1, 2). Fatty acids are carboxylic acids with long aliphatic tails. Fatty acid synthesis begins in the cytosol with the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACACA), the committing step in fatty acid biosynthesis (Fig. 1). Fatty acid synthase (FASN) then uses malonyl-CoA to add two more carbon atoms to the aliphatic chain of a fatty acid. Fatty acids are elongated up to 16 carbons from FAS, and then, they can be elongated further in the ER by the elongation system in which fatty acid elongases (ELOVLs) play a vital role [56] (Fig. 1).

Drug inhibition of ACC with TOFA and of FASN with C-75 or other inhibitors have shown marked inhibition of HCMV replication [25, 36]. HCMV has also been shown to up-regulate ACC expression along with a number of other enzymes important for fatty acid biosynthesis. More recent studies have demonstrated that the virus requires very long

chain fatty acids (VLCFAs) to replicate effectively [11]. Inhibition of specific ELOVLs attenuated the replication of the virus, and the fact that the envelope of the virus in untreated fibroblasts was strikingly enriched for those specific fatty acids, suggesting that indeed the requirement for this pathway may act at the viral membrane level. In further support of this, it was also noted that inhibition of the pro-viral ELOVLs does not significantly reduce the number of virions, but instead greatly reduces infectivity. This was attributed to the ability of those VLCFAs to produce an envelope with correct curvature or other physicochemical properties necessary to bud virions from cells [11]. In this connection, phospholipids are essential membrane structural lipids consisting of a hydrophobic fatty acid tail. For HCMV, the glycerophospholipid composition of the virus membrane has been found to be very similar to synaptic vesicles suggesting a re-purposing or modelling of the host lipid membranes for its morphogenesis [57]. HCMV in general dramatically remodels the structure of a cell, leading to the gross enlargement of infected cells (cytomegalic) and the formation of a new compartment termed the virion assembly compartment (VAC) [58]. VAC is a kidney-shaped structure adjacent to the nucleus that is crucial for HCMV replication and is formed by extensive remodelling of the secretory membrane apparatus [59–61].

Immune activation

The immune activation of cells also impacts on lipid and fatty acid metabolism [62–64], see Fig. 3 for a schematic summary of pathways. As described above, macrophages and DCs of specific phenotypes promote de novo fatty acid synthesis upon activation [47, 65]. Although still not well understood, these changes appear to be important for the activated function of the cell; for instance, inhibiting fatty acid synthesis can impair phagocytosis [65]. An activated macrophage leads to significant changes in phospholipids and fatty acids that promote the formation of filopodia and the biogenesis of new organelles such as mitochondria, lysosomal compartments, and expanded ER and golgi [47, 65, 66]. These metabolic changes are therefore essential for activated macrophages and dendritic cells to be transformed, from small quiescent cells to cells that are actively phagocytic, present antigens and secrete a huge number of cytokine and immune regulatory molecules.

Altogether, these immune-driven changes appear to have similarity to those observed in infected cells despite the studies being conducted in different cell types and point to a more unified response (compare Figs. 2, 3). This raises again the question of whether immune activation initiates the metabolic changes that upon infection also opportunistically favour viral growth. In this scenario, such immune-initiated re-programming of metabolism may be required and co-opted by the virus but alone may not be sufficient. Future studies should address whether it is likely that additional metabolic state changes are further needed to encourage viral growth, and we speculate that viral gene products may have a contributory role in governing metabolic programming in the infected cell.

Prostanoid biosynthesis pathway

Virus infection

As mentioned at the start of the lipid section, not all lipids will necessarily have a structural role as membrane lipids but may also contribute regulatory functions or signals that promote or inhibit viral growth. In this regard, an interesting class of signalling lipids that HCMV appears to depend on are eicosanoids. These are produced by oxidation of 20 carbon fatty acids and play critical regulatory signalling roles in immunity and inflammation. Upon infection of fibroblasts, HCMV up-regulates the expression of genes related to the eicosanoids pathway [67, 68]. Among them are cytosolic phospholipase A2 (cPLA2) and cyclooxygenase-2 (COX-2) that are responsible for the generation of prostaglandin H2 (PGH2) acid from cell membranes and its conversion to prostaglandin H2 (PGH_2) [69–71]. The inhibition of COX-2 has been shown to reduce HCMV progeny while the introduction of prostaglandin E2 (PGE_2) rescues the drug blockade [71]. While the antiviral mechanism for inhibition is not clear, PGE_2 has been reported to stimulate the activity of the virus major immediate early promoter [72]. A later report from Schröer and Shenk [71] demonstrated that inhibition of COX-2 also blocks cell-to-cell viral spread although the precise mechanism has not been defined.

In addition to the studies indicating that blocking prostaglandin synthesis inhibits viral growth, we have previously reported through evaluation of a highly selective and chemically diverse synthetic prostanoid library that the replication of MCMV can be enhanced or strongly curtailed depending on the specific prostanoid chemical structure [73]. Thus, altogether these studies suggest key regulatory functions for prostanoid signalling in determining the outcome of a viral infection. In this context, we have been successful, as a proof-of-principle study, to identify a potential therapeutic prostanoid that had potent anti-CMV activity [73].

Immune activation

The inflammatory induction of prostaglandins and other related lipids by macrophages and DCs have a critical secondary autocrine and paracrine signalling role in immunity [70, 74, 75]. Notably, lipidomic studies of the macrophages

have revealed that upon activation with a selective TLR4 agonist, a rapid increase in eicosanoid synthesis takes place [76]. Depending on factors such as the nature of the signalling or length of exposure to it, different outcomes on lipid alterations have been reported in macrophages and DCs [77, 78]. Moreover, differential gene regulation of enzymes that participate in the eicosanoid biosynthesis pathway has been documented in pro-inflammatory (M1) and anti-inflammatory (M2) polarised macrophages [79]. Thus, IFN and LPS activation of M1 macrophages potently induced COX-2, and down-regulated COX-1 and other pathway-related enzymes, such as leukotriene A4 hydrolase. In contrast, M2 macrophages activated with IL-4 exhibited an up-regulation of COX-1. TLR treatment of macrophages induced the microsomal isoform of PGE synthase (mPGES). On the other hand, Th2-associated cytokines IL-4 and IL-13 down-regulated the expression of mPGEs in macrophages [80].

In turn, prostanoids released from macrophages and DCs participate in autocrine loops to regulate COX-2 expression. In macrophage cells, PGE2 has been reported to induce COX-2 and mPGES-1 [81]. In addition, released prostanoids are able to affect, either in a positive or in a negative manner, a number of macrophage and DC functions [78]. As an example, PGE2 has been shown to usually exert inhibitory actions on macrophages and DCs, such as suppressing MHC class II expression and TNF-α production and inducing IL-10 expression [82–84]. However, PGE2 has also been documented to exert stimulatory effects on these two cell types, including the induction of IL-23 in DCs through cooperation of PGE2 with CD40-mediated signalling [85]. Most significantly, PGE2 release from myeloid cells has been shown to restrain T cell activation, and clinical trials have revealed that blockade of PGE2 synthesis exacerbates severe systemic inflammation. In this connection, it has also been suggested that PGE2 released from HCMV-infected macrophages may contribute to the immunosuppressive activity of HCMV [86]. Future studies elucidating the regulatory role of PGE2 in immunity will significantly advance our understanding of lipid host protection pathways modulated in cytomegalovirus disease.

Mevalonate–sterol pathway for cholesterol biosynthesis

Virus infection

Cholesterol is known to have a causal link to inflammatory diseases and its oxidised metabolites (oxysterols) alter adaptive and innate immunity, and innate immune signalling has been shown to modulate the dynamics of cholesterol homeostasis [62–64, 87]. In this connection, over the last century, natural and experimental infection studies have reported for a wide range of pathogens a state of hypocholesterolemia during the acute phase (2–4 days) of infection that is often followed by a rebound period of hypercholesterolemia [88–96]. Currently in the critical care of sepsis, monitoring of cholesterol levels is used as a biomarker for prognosis. Although blood cholesterol levels for CMV infection have not been measured, acute hepatic infection by MCMV has been shown to increase total cholesterol levels in the liver [97]. Hence, the biosynthesis and metabolism of cholesterol takes centre stage in infection biology. Work from Alwine et al. [29] suggest that HCMV induces adipocyte-like lipogenesis and overrides normal sterol feedback controls in order to maintain high levels of constitutive lipid synthesis during infection.

Cholesterol biosynthesis starts with acetyl-CoA whereby the reductase, HMGCR, represents a critical regulated step in the mevalonate arm of the sterol biosynthesis pathway (Fig. 1). The pathway is responsible not only for the production of cholesterol but also for generating immune and sex steroid hormones while the upstream mevalonate arm of the pathway produces metabolites used in a diverse array of biological processes including the synthesis of ubiquinone for mitochondrial aerobic respiration with functions in the electron transport chain, and other isoprenoids required for the N-gylcosylation and lipidation of proteins (farnesylation and geranylation of proteins). Thus, the inhibition of HMGCR by statins not only reduces cholesterol but also has the ability to affect multiple products of the mevalonate side branch. Perhaps not surprisingly statins are potent inhibitors for human and murine CMV [15, 98–100], and we were the first to show that the antiviral activity of statin on CMV can be metabolically reversed by mevalonate but not cholesterol treatment [15]. Recent studies have since independently confirmed the inhibition of HCMV by statins and metabolic rescue of antiviral action by using mevalonate [98]. The metabolic rescue experiments unequivocally demonstrate the absolute requirement for mevalonate–sterol biosynthesis pathway for optimal CMV growth. In a series of systematic loss of function and drug inhibition assays, we have provided further evidence implicating mevalonate-derived products such as isoprenoids involved in the lipidation of proteins for CMV growth [16, 101]. Inhibition of the mevalonate–sterol arm does not affect the binding or entry of CMV but evidence to date points to the suppression of the major immediate early gene expression as well as blocking cell-to-cell spread [16].

It is important to note that while these studies indicate that cholesterol alone is not the primary metabolite for statin antiviral action, they do not rule out an important biological requirement for cholesterol in the CMV life cycle. In this regard, studies evaluating the proteomes of fibroblasts cells before and after HCMV infection by mass spectroscopy identified LRP1, an LDL receptor-related protein involved in cholesterol uptake, as a transiently increased protein at 24 h after infection. In human fibroblasts,

knockdown of LRP1 was found to enhance HCMV infectivity and also lead to increased cholesterol in released virions. Importantly, the cholesterol content of the virus envelope was shown to be critical for HCMV fusion with the host cell. These results indicate that increases in cellular and virion cholesterol content lead to more efficient fusion of the virion envelope with the plasma membrane and therefore increased virion infectivity [102].

Upon infection of macrophages with MCMV, we found by metabolic and transcriptional profiling studies that one of the most statistically significant pathways suppressed upon infection was mevalonate–sterol metabolism. The reasons for this reduction are complex and may not be identical or equivalent in all cell types and media conditions as this pathway is directly regulated by the immune response and by extracellular cholesterol. For instance in macrophages but not fibroblasts, we find a highly specific and dramatic increase in the production of an oxidised cholesterol metabolite generated by the hydroxylase CH25H. The regulation of CH25H appears to be highly cell type and tissue specific and is mainly restricted to immune (myeloid and lymphoid) and to certain epithelial cell types. We, and others, identified and showed that the metabolic product of CH25H enzyme, 25-hydroxycholesterol (25HC), a well-characterised inhibitor of the mevalonate–sterol biosynthesis pathway, acts as a potent antiviral regulatory factor by principally suppressing the isoprenoid branch of the pathway and through affecting cellular membranes [16, 66, 103]. It remains an open question if CMV encodes viral proteins that counter these inhibitory effects. Previous studies from Alwine et al. [29] have suggested that HCMV infection of fibroblast can override normal sterol feedback control in order to maintain high levels of constitutive lipid synthesis during infection.

Overall, these studies underscore that the interplay between immune activation, infection and metabolic changes cannot be considered in isolation.

Immune activation

Similar to the early infection studies examining blood cholesterol, in completely unrelated early investigational studies of recombinant IFN treatment in humans, for a range of different disease indications, a transient drop in blood cholesterol levels has also been repeatedly documented. Radiolabelled metabolic studies have further indicated that this particular side effect of IFN treatment is due to a suppression of endogenous sterol synthesis [94]. Strikingly, mechanistic and functional physiological linkages between the observational based infection and IFN studies have not, until recently, been investigated or considered. IFN and TLR activation are now known to coordinate the reduction in cholesterol biosynthesis for human and murine macrophages [10, 15, 66, 76, 104]. We demonstrated that IFN-induced inhibition of sterol biosynthesis serves as an integral component of the very early cellular response to virus infection [15]. Metabolic profiling [15] and molecular screening [103] studies led ourselves and others to identify IFN-elicited 25HC as an important effector of the IFN-induced antiviral response in macrophages [15, 16]. This lipid has emerged to have broad-spectrum antiviral and immune modulatory functions [10].

We demonstrated that CMV infection leads to a TLR-induced type I IFN-mediated down-regulation of the master transcription factor for sterol biosynthesis, SREBP2 [15]. More recently, we have also shown that IFN signalling specifically targets in a SREBP-independent manner the statin-targeted reductase, HMGCR, for rapid proteosomal degradation that is strictly coupled to the endogenous synthesis of 25HC [105]. Our studies of immune-activated macrophages identified STAT1 transcriptional regulation of the cholesterol hydroxylase, CH25H, as a direct mechanistic link between IFN-mediated antiviral activity and the regulation of sterol metabolism [16]. Investigations of SREBP2-dependent IFN effects have implicated the sterol biosynthesis pathway involved in both antiviral and anti-inflammatory pathways [16, 106]. Further studies are required to more fully understand the role of SREBP2 and the mevalonate–sterol pathway in immunity and infection.

Therapeutic perspective

Here we have discussed how cytomegaloviruses are critically dependent on and co-opt many aspects of cellular sugar and lipid metabolism to facilitate their life cycles across species and how the inhibition of these processes can curtail virus replication. Overall, we find a remarkable level of metabolic cooperation between infection and immunity. Defining how immunity and metabolism are integrated, share resources and cross-regulate one another during infection is therefore an essential yet incompletely understood area of infection biology. Notably, unbiased systems biology approaches are providing valuable new insights into this integrative complexity and computational studies based on predictive modelling will be essential in the future functional design and control of an immune–metabolic–infection axis [107].

In this regard and to advance this future perspective, Fig. 5 summarises and shows as a schematic representation of the inputs and outputs for this axis, with metabolism positioned in the middle connecting in an integrative manner the various distinct biological processes. Conceptually, this diagram extends our previous ‘cellular clockwork model of infection’ [1] that underscored the dependency of

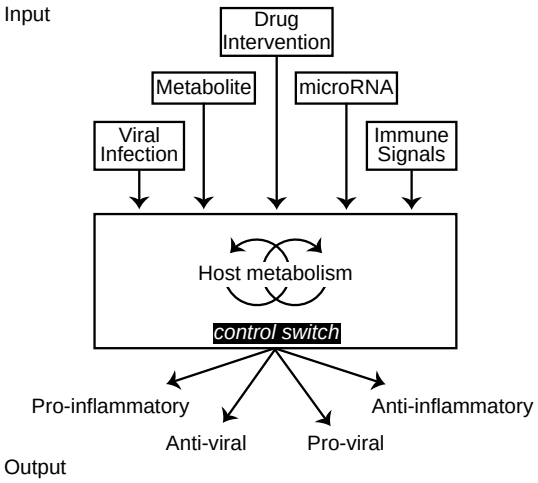

Fig. 5 Metabolism acts as a tunable switch device that integrates multiple input signals to direct a range of biological outputs

pathogens on cellular pathways as a means for therapeutic drugs or the immune system to control infections. Here we extend the previous model to emphasise, at a systems control level, an important design principle suggesting that cellular metabolism behaves as a 'tunable device' and that the control of metabolic flux in these pathways is necessary for driving the range and type of biological response (Fig. 5).

While the concept of metabolism acting as a 'tunable device' for governing cellular functions is new, it remains speculative yet testable for the future. Thus, we believe that further understanding and identifying the host molecular circuitry for new and recently discovered immune-regulated metabolites and enzyme pathway systems will aid in the development of new therapeutic innovations to prevent infection (as an anti-infective) or enhance immunity (as adjuvant-based metabolic modifiers).

Although we have focussed our discussions on sugar and lipid pathways, these are not the only examples of an immune–metabolic–infection axis known to control infections. In particular, the immune-activated metabolism of amino acids has been long established. Stimulation of macrophages with IFN results in the rapid induction of the tryptophan-catabolising enzyme indoleamine-2,3-dioxygenase resulting in broad-spectrum antimicrobial and immunosuppressive effector functions. Other examples include the immune-regulated activities of the arginine-hydrolysing enzymes nitric oxide synthase 2 (iNOS) and arginase 1 (Arg1). In the case of these enzyme systems, NO production by iNOS acts as an important pro-inflammatory and antiviral mediator, while Arg1-expressing macrophages contribute to the resolution of inflammation and wound repair. In the context of viral infections, expression of these enzymes can result in a variety of outcomes for the host [108]. Interestingly, HCMV infection of fibroblast is capable of effectively impairing this response [109] and might suggest a similar mode of action for blocking the IFN-induced suppression of sterol biosynthesis.

Strikingly, the glycolytic and fatty acid metabolism changes that favour CMV growth in fibroblasts, parallels the metabolic profile of the pro-inflammatory immune-activated macrophages, but not the anti-inflammatory macrophages. In future, it will be important to determine whether the metabolic cooperation in glycolytic and fatty acid pathways also occurs upon CMV infection of macrophages. Since CMV remains latent in monocytes and reactivates upon immune activation and differentiation, it is plausible that CMV may also exploit these different metabolic stages of the macrophage phenotypes to remain latent and reactivate when conditions favour replication. If so, then therapeutic targeting ('tuning') of metabolism in latently infected monocytes may provide a means for curing or eliminating latently infected cells.

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