An investigation of MCMV-induced suppression of TNF production *in vitro* and *in vivo*

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Declaration

I hereby declare that this thesis is of my own composition, and that 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 acknowledgement is made in the text.

Abstract

The murine cytomegalovirus (MCMV) immediate early 1 (IE1) protein has been described as a trans-activator of viral and host gene expression. However, the precise role that IE1 plays in the viral life cycle, and in particular its effect on the host immune response is not known. This thesis investigates the functional relationship of the IE1 protein and the immune response induced after infection. By using an *ie1*-deletion mutant MCMV (MCMVdie1) it was demonstrated that, early after infection, tumor necrosis factor (*tnf*) gene activation and protein production was significantly induced in infected-primary macrophages (Mθ) to a much greater extent than its wild type counterpart. In addition, preliminary studies on the signalling pathways activated upon infection were carried out in order to gain information about the pathways that might be involved in MCMV-induced modulation of *tnf* activation. Initial observations on the MAPK family members Erk1/2, p38 and JNK did not revealed any differential activation in the absence of IE1. However, due to a number of limitations, it was not possible to draw any firm conclusions from this study.

Investigation of the role of IE1 in the *in vivo* production of TNF were also performed in both susceptible (BALB/c) and resistant (C57Bl/6) mice. These experiments confirmed the attenuated phenotype of MCMVdie1 *in vivo*, whereby the mutant strain grew to much lower titers than wild type. When cytokine production was assessed in relation to PFU levels a significant production of TNF after infection is observed in different organs of both mice strains. This raises the question whether IE1 contributes to MCMV modulation of TNF production in the natural host. Although, because it is still unclear whether the phenotype of MCMVdie1 *in vivo* is due to a defect in the virus or the result of a immune

response, it was not possible to conclude unequivocally that IE1 is responsible for dampening this cytokine response.

This thesis also tested whether the attenuated replication of MCMVdie1 *in vivo* was due to the increased TNF production induced after infection. An initial investigation in *tnf* depleted mice revealed that the MCMVdie1 growth phenotype is not due to TNF response.

Overall, this study has provided insight into a potential immune modulatory function by MCMV associated with IE1 protein and the regulation of TNF *in vivo* and *in vitro*.

Dedication

A los que siempre creyeron en mi.

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List of Acronyms

aa	amino acid
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1
AIDs	Acquired immunodeficiency syndrome
AK2	adenylate kinase 2
ANOVA	Analysis of variance
AP1	Activator protein 1 (ATF-2/c-Jun)
APC	Antigen Presenting Cell
APC	Allophycocyanin
APS	Amonium persulfate
ASFV	African swine fever virus
ATCC	American Type Culture Collection
ATF2	Activating transcription factor 2
BAC	bacterial artificial chromosome
BALB/c	laboratory mouse strain
BM	bone marrow
BMMθ	bone marrow derived macrophages
bp	base pairs
BSA	Bovine serum albumin
°C	Degrees Celcius
C57Bl/6	laboratory mouse strain
CBA	Cytometric bead array
CBP	CREB-binding protein
CCL8	Chemokine ligand 8
cDNA	complementary DNA

CH25H	Cholesterol 25-hydroxylase
ChIP	chromatin immuno-precipitation
CIITA	class II transactivator
cm^2	centimetres square
CMV	cytomegalovirus
CNS	Central nervous system
CO_2	Carbon dioxide
CPE	cytopathogenic effect
CREB	cAMP response element binding protein
CRE	cAMP response element
cRNA	
CS	Calf serum
CTL	Cytotoxic T cell
DAPK1	Death-associated protein kinase 1
Daxx	Death-associated protein 6
DC	dentritic cell
DD	Death domain
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
E1	early protein 1
EBV	Epstein Barr virus
ELISA	enzyme-linked immunosorbent assay
Egr1	Early growth response protein 1
Erk1/2	Extracellular signal-regulated kinases 1/2
FABP3	Fatty acid binding protein 3
FACS	fluorescent-activated cell sorter
FADD	Fas-Associated protein with Death Domain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate

g	gravitational force or grams, depending on context
GAS	IFNγ-activation site elements
gB	glycoprotein B
GOLGA3	Golgi autoantigen, golgin subfamily a, 3
HCV	Hepatitis C virus
HCMV	Human cytomegalovirus
HDC	Histidine decarboxilase
HDCA2	Histone deacetylase 2
HIPK1	Homeodomain interacting protein kinase 1
HIPK3	Homeodomain interacting protein kinase 3
HIST1H1C	histon cluster 1
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
hr	hour
HRP	horseradish peroxidase
h.p.i	hours post infection
HSP27	Heat shock 27kDa protein 1
HSPA9	Heat shock 70kDa protein 9
HSV	Herpes simplex virus
HVEM	Herpesvirus entry mediator
ICP0	Human Herpes Virus (HHV) Infected Cell Polypeptide 0 (ICP0)
IE	Immediate early
IE1	Immediate early 1 protein
IE2	Immediate early 2 protein
IE3	Immediate early 3 protein
IFN	Interferon
IFNAR	Interferon$\alpha\beta$ receptor
IFNGR	IFNγ receptor
ifitm1	Interferon induced transmembrane protein 1
ifitm2	Interferon induced transmembrane protein 2
ifitm6	Interferon induced transmembrane protein 6

IκBα	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α
IL	Interleukin
i.p	intraperitoneal route
IPA	Ingenuity Pathway Analysis
IRF1	Interferon regulatory factor 1
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
IRF9	Interferon regulatory factor 9
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response elements
IVT	*in vitro* transcription
JNK/SAPK	c-Jun amino-terminal kinase/stress-activated protein kinase
Jak1	Janus kinase 1
Jak2	Janus kinase 2
kb	kilo bases
kDa	kilo Dalton
kg	kilogram
L	litre
LIPA	Cholesterol esterase
LPL	Lipoprotein lipase
LPS	lipopolysaccharide
LT	Lymphotoxin
M	Molar
Mθ	Macrophages
MAPK	Mitogen-activated protein kinases
MCMV	Murine cytomegalovirus
MCMVdie1	*ie1*-deletion mutant MCMV
MCMVrev	*ie1*-deletion mutant MCMV revertant
MCP-1	Monocyte CHemoattractant Protein-1
MED21	Mediator complex subunit 21

MEF	Murine embryonic fibroblasts
MFI	Median Fluorescent Intensity
MHC	Major histocompatibility complex
MIEP	Major Immediate Early Promoter
min	minutes
mg	milligram
mL	mililitre
mM	milimolar
MNK	Mitogen- activated protein kinase signal-integrating kinases
MOI	Multiplicity of infection
mRNA	Messenger ribose nucleic acid
mx1	Myxovirus resistance 1, interferon-inducible protein p78
NAP1L1	Nucleosome assemble protein 1
ND10	Nuclear domain 10
NFκB	Nuclear factor κB
nm	nanometre
NIH 3T3	Fibroblast cell line
NK	Natural Killer
NKG2D	NK cell activator receptor
ORF	Open reading frame
OSBP	Oxysterol binding protein
p53	protein p53
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
pg	picograms
PML	Promyelocytic leukemia protein
PODs	PML oncogenic domains
PPAR	peroxisome proliferator-activated receptor
PVDF	Poy vinylidene floride membrane
RIP1	Receptor interacting protein 1

RMA	Robust multi-chip analysis
RNA	Ribose nucleic acid
rpm	Revolutions per minute
r/t	Room temperature
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGV	Salivary gland-derived virus
Sp100	Sp100 nuclear antigen
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TBST	Tris buffered saline plus Tween 20 detergent
TCR	T cell receptor
TEMED	N,N,N,N-tetramethylethylenediamine
TNF	Tumor Necrosis Factor
TNFAIP2	tumor necrosis factor, alpha-induced protein 2
TNIP1	TNFAIP2 interaction protein 1, also known as NAF-1 or ABIN-1
TNFSF12	Tumor necrosis factor (ligand) superfamily, member 12, also known as TWEAK
TNFR	TNF Receptor
TLR	Toll-like receptor
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand, also known as TNFSF10
Tyk2	Tyrosine kinase 2
U	Units
μg	micrograms
μl	microlitre
V	Volts

w/vol	Weight per volume

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CHAPTER 1

Introduction

1.1 Herpesviruses: General overview

Herpesviridae are a large family of viruses highly disseminated in nature which can cause disease in humans. All Herpesviruses share a common structure, they all are DNA viruses and replicate in the nucleus of infected cells. Another common feature of this family is the capacity to establish latency after the initial infection. The cells where the viruses establish latent infection varies from one virus to another as well as the reactivation mechanism of the virus from latency. The exact mechanisms by which this process takes place is still under study.

1.1.1 Structure and Classification of Herpesviruses

Initially, inclusion within the Herpesvirus family was based on the virion structure (see Figure 1.1). The Herpesvirus virion consists of a torus-shape core associated with the viral double-stranded DNA of about 120 to 230 kilobase pairs, depending on the Herpesvirus. The viral DNA is housed in an icosahedral capsid composed by approximately 162 capsomers, or morphological units. Surrounding the capsid is the tegument which also contains virally expressed proteins. These proteins are introduced in the host cell with the aim of modifying the cellular environment for the successful initiation of the infection. Examples of this include the shut down of the cellular protein synthesis, enhancement of viral gene transcription or immune evasion strategies. Finally, the outer layer is called envelope and is composed by altered host membranes and viral glycoproteins. The envelope can also contain external glycoproteins spikes which play a key role during the initial stages of viral infection.

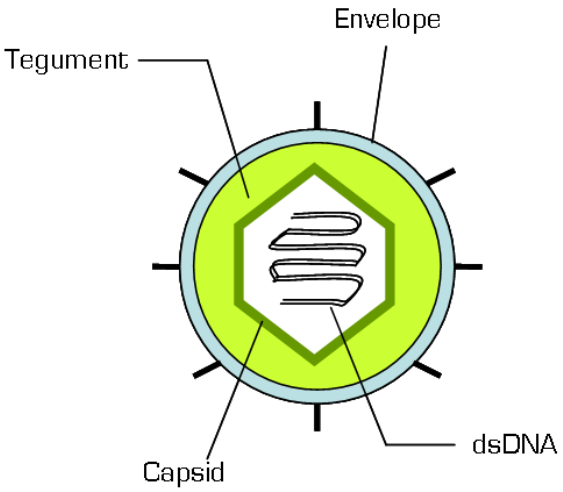

Figure 1.1: Typical Herpesvirus virion

In addition to the virion architecture, there are also four main characteristics shared by all Herpesviruses [Pellett P. E., 2006]:

1. All viruses encode a number of enzymes involved in nucleotide metabolism, DNA replication and protein synthesis.
2. Viral DNA replication and assembly of new virions occur in the nuclear compartment of the cell.
3. Production of new progeny virions during lytic replication generally results in the destruction of the infected cell.
4. All Herpesviruses are able to remain in the host cell in a latent state. After correct stimuli latent virus can be reactivated and cause disease.

Approximately 100 Herpesviruses have been isolated and to date 8 Herpesviruses in this family infect and cause disease in humans. They are subdivided into three subfamilies based on their biological properties (Table 1.1).

Inspite of the common characteristics of the members of the Herpesvirus family, there are also specific features for each virus. For instance, the host cell range is very wide for the *alpha* Herpesviruses whereas it is more restricted for *beta* and *gamma* Herpesvirus. This restriction is related to the distribution of cellular

Table 1.1: Classification of Herpesviruses

Type	Name	Subfamily
HHV-1	Herpes simplex virus-1 (HSV-1)	Alphaherpesvirinae
HHV-2	Herpes simplex virus-2 (HSV-2)	Alphaherpesvirinae
HHV-3	Varicella zoster virus (VZV)	Alphaherpesvirinae
HHV-4	Epstein-Barr virus (EBV)	Gammaherpesvirinae
HHV-5	Cytomegalovirus (CMV)	Betaherpesvirinae
HHV-6	-	Betaherpesvirinae
HHV-7	-	Betaherpesvirinae
HHV-8	Kaposi's sarcoma-associated herpesvirus (KSHV)	Gammaherpesvirinae

receptors for the viral particle, making the cells more or less permissive for infection. There is also variation in the length of the replication cycle, HSV has a short replicative cycle of approximately 18 hours while CMV requires up to 48 hours to complete it.

1.1.2 Herpesvirus Replication Overview

The replication cycle of the Herpesviruses can be divided into several stages (see Figures 1.2 and 1.3): (1) attachment of the virus to the host cell and penetration, (2) transcription and (3) viral replication, both events happening in the nuclear compartment of the host cell, (4) assembly of the new progeny of virions and (5) egress from the cell to the exterior. A general introduction of these phases is described below.

Attachment and entry

The initial step in the Herpesvirus life cycle is the attachment of the viral particle to cellular receptors, followed by the fusion of the viral envelope with the cell plasma membrane. Once the virion has been attached both viral envelope and cell plasma membrane fuse. Glycoproteins found in the virion envelope are key players in the initiation of the cycle. Upon entry the viral nucleocapsid with associated tegument proteins is transported by the cellular cytoskeleton to the nuclear pores. It is believed that the presence of these tegument proteins during these early stages of infection is essential to create the correct environment for efficient replication of the virus. For example, along with HSV capsid the tegumentary proteins VP16 and vhs are also introduced in the host cell. VP16

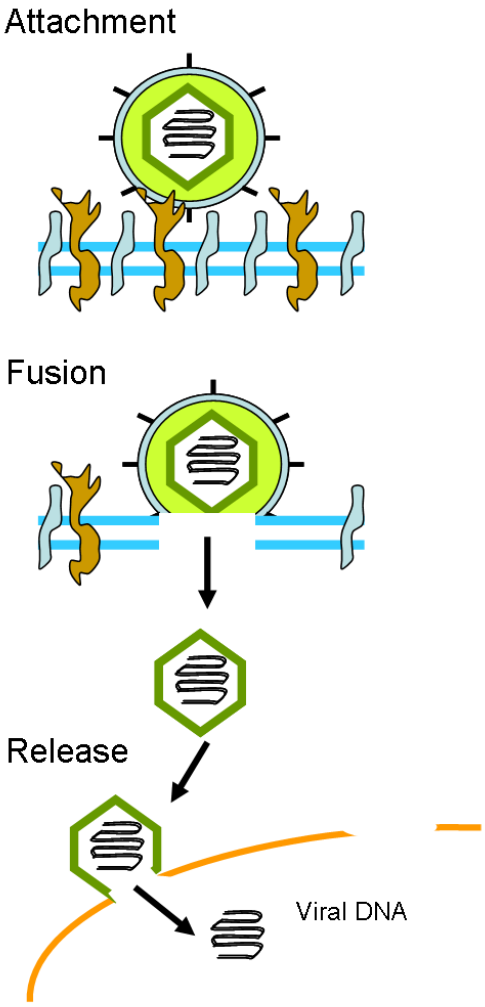

Figure 1.2: Life cycle of Herpesviruses

is transported to the nucleus where acts as an inducer of immediate early gene transcription [Campbell et al., 1984] whereas vhs remains in the cytoplasm where it induces degradation of both cellular and viral mRNAs [Fenwick and Clark, 1982, Fenwick and McMenamin, 1984].

Transcription

Transcription of all Herpesviruses takes place in the nucleus and viral proteins are synthesized in the cellular cytoplasm. In infected cells the cellular RNA polymerase II is responsible for the transcription of viral genes. In addition, this process occurs in a regulated temporal cascade starting with the expression of the α

or immediate early genes, followed by the β or early genes and γ or late genes. The expression of immediate early genes does not require prior viral protein synthesis, however sometimes the virus particle has some proteins which enhance this initial process. Expression of the early genes requires the presence of some immediate early proteins but not the onset of viral DNA replication. The early genes encode for proteins required for viral replication, nucleotide metabolism and expression of the last set of viral genes. The late gene expression is normally increased after viral DNA replication and they encode for virion structure proteins.

Replication

Herpesviruses encode their own DNA-dependent DNA polymerase responsible for viral DNA replication. The replicated DNA is present as concatameric molecules, that is a long DNA molecule containing multiple repeats of the viral genome, which is generate by the rolling-circle method of replication. The concatemers are then cleaved to correct size of DNA to be packaged into new progeny of virions.

Assembly

Assembly of new capsids takes place in the nucleus, therefore new capsid proteins are transported from the cellular cytoplasm to the nucleus. Along with viral scaffolding proteins the assembly of capsids occurs. These scaffolding proteins will be later degraded by a viral protease. Finally, the viral DNA which is in the form of concatemers is cleaved and packaged into the capsid.

Viral envelopment and egress.

Once the capsid has been formed the virion needs to obtain the tegument and envelope. This complex procedure is not completely understood but it is known to start by fusion of the capsid with the nuclear membrane obtaining an initial envelop. There are two different pathway of envelopment: (1) the dual envelopment pathway and (2) the single nuclear envelopment pathway. In the dual envelopment pathway the virus acquires the envelope from the nuclear inner membrane and follows a progressive de-envelopment and re-envelopment as the

virion moves from different cellular compartments [Jeffrey I. Cohen, 2001, Pellett P. E., 2001]. On the other hand, in the single envelopment pathway the virus acquires the envelop from the inner nuclear membrane after which enters a vesicle at the outer nuclear membrane in which the virus is transported to the plasma membrane. Finally, by fusion with the plasma membrane the virus is released from the cell.

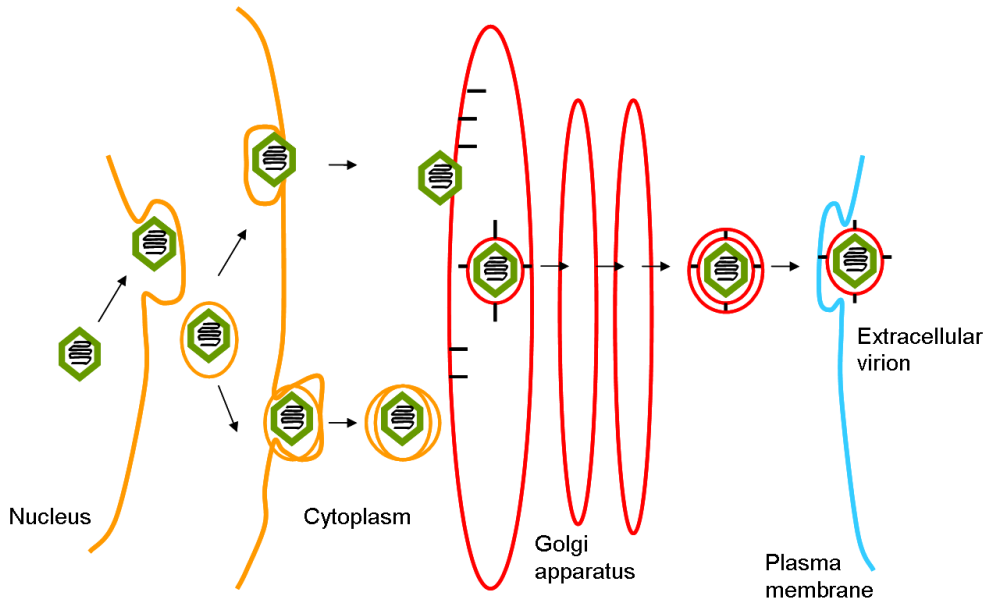

Figure 1.3: Possible egress pathways

The egress of the new virion from the infected cell can follow two pathways. One involves the de-envelopment of the virion when it passes through the outer nuclear membrane and re-envelopment from the trans-Golgi network, being released in secretory vesicles. The second pathway does not involve the re-envelopment sequence and is transported in vesicles to the plasma membrane. Adapted from [Roizman and Knipe, 2001]

1.2 *Alpha* Herpesviruses

The *Alpha* Herpesviruses exhibit a wide range of host cells, and are also characterized by a short replicative cycle and rapid cell-to-cell spread in culture. Infection with *Alpha* Herpesviruses results in the effective destruction of the cells and also the establishment of a latent infection in sensory ganglia. This subfamily includes Herpes Simplex Virus 1 and 2 (HSV1 and HSV2) and Varicella Zoster virus (VZV).

Infection with HSV1 and HSV2 is distributed worldwide. Initial infection with HSV1 appears to be very frequent at early age during childhood. However geographic location and socioeconomic status seem to be factors affecting acquisition of HSV. In fact, several studies have revealed the high seroprevalence of HSV in the population [Roizman B., 2006]. In the case of pregnant women this also increase the risk of reactivation of the virus and transmission of the virus to the newborn. When infection is initiated viral replication takes place at the site of infection. A key step in viral pathogenesis is the ability of the virus to replicate and subsequently be transported to dorsal root ganglia where it can establishment of latency. The recurrence of HSV infection is also normal, resulting in vesicular lesions of the skin in the lips or genitalia.

The capacity of HSV to infect the nervous system is an important viral property for human pathogenesis. Animal models have been very useful to study viral pathogenesis of HSV infection.

VZV infection is initiated by inoculation of the respiratory mucosa or close contact with an infected individual. VZV causes varicella, also known as chicken pox, a highly contagious infection, causing annual outbreaks. Unlike other members of the *alpha* Herpesvirus subfamily, VZV has a tropism for T lymphocytes. VZV can also establish latency after primary infection in cells of the dorsal root ganglia. Reactivation of VZV from latency results in herpes zoster, commonly known as shingles. VZV reactivation can also cause post-herpetic neuralgia.

1.3 *Beta* Herpesviruses

Beta Herpesviruses are characterized by a strict species specificity, relatively slow replication cycle and the induction of the enlargement of infected cells, also known as cytomegalia. *Beta* Herpesviruses can also establish latency in the host as do other Hespesviruses. This subfamily contains Cytomegalovirus and Human Herpesvirus 6 and 7. Although HHV6 and 7 are relatively recently discovered viruses, the murine virus (MCMV) has been extensively used as a model for

studying human virus (HCMV)-induced pathogenesis, thanks to the biological similarities between viruses and to the ease of handling the virus.

Like HCMV, infection with MCMV results normally in an asymptomatic infection and establishment of latency. However, in immunocompromised hosts the outcome of the infection could be fatal. The immune system controls the level of reactivation from latency leading to a life-threatening disease in immunosuppressed patients. AIDs patients and transplant recipients are commonly vulnerable to reactivated viral infection. Congenital infection also occurs and is associated with serious damage of the central nervous system, including maldevelopment and deafness.

HHV6 and HHV7 compose the Roseolovirus within the *beta* Herpesvirus subfamily, sharing some features with CMV. As seen for CMV infection HHV6 and 7 are ubiquitous with more than 90% of the population infected. Two HHV6 variants have been identified, HHV6A and HHV6B. With HHV7, infection of these viruses cause the infant disease exanthem subitum (ES), also referred to as roseola infantum. Infection with the variant B of HHV6 is more common than the A variant, and so far no clear disease has been associated with HHV6A.

1.4 *Gamma* Herpesviruses

Gamma Herpesviruses have a more restricted host and host cell range. They are lymphotropic viruses, specific to either T or B cells, where latency is also established. This subfamily contains two genera: (γ-1) Lymphocryptovirus, which includes EBV, and (γ-2) Rhadinovirus, which includes KSHV, Herpesvirus Saimiri (HVS), Rhesus monkey rhadinovirus (RRV), Equine Herpesvirus 2 and murine gammaherpesvirus 68 (MHV68). An important feature of the *gamma* Herpesviruses is the association with cancer.

The study of *gamma* Herpesviruses has not been easy due to the difficulty of these viruses to replicate *in vitro*, as well as the tendency of establishing latency instead of a lytic infection. On the other hand, ethical restrictions to study human *gamma* Herpesviruses in the natural host have also impeded to have a better

information about the biology of these viruses. However of the interest in veterinary medicine *gamma* Herpesviruses are now very valuable animal models, providing important information regarding the infection in the natural host.

EBV was the first Herpesvirus to be sequenced and cloned [Baer et al., 1984] and it has been extensively studied in contrast to the rest of *gamma* Herpesviruses. There are two variants of EBV, subtype 1 or A and subtype 2 or B and they both present antigenically differences. There are studies suggesting that EBV subtype 1 is more prevalent that subtype 2 but this last one is found prominently in Africa [Gratama and Ernberg, 1995]. EBV is capable of infecting several cell types, T- and B-cells, as well as epithelial cells. Transmission of EBV infection is mainly through the saliva of infected individuals, however EBV has also been detected in genital secretions. EBV is the agent of infectious mononucleosis and Burkitt's lymphoma and it has also been associated with Hodgkin's lymphoma, nasopharangeal carcinoma and some forms of gastric cancer [Rickinson A. B., 2006]. An important characteristic of EBV infection is the different latency programs. In addition, these programs are also characteristic of various EBV-associated malignancies [Rowe et al., 1992]. Interestingly, the viral latent gene expression varies according to the program.

The other member of the *gamma* Herpesvirus subfamily to infect humans is the very recently discovered KSHV or HHV-8. It resembles EBV in its tropimsm for B cells, in the establishment of latent state and difficulty of using the virus *in vitro*. KSHV infection has been associated with Kaposi's sarcoma, a tumour of endothelial cells origin, Primary Effusion Lymphoma and Multicentric Castleman's Disease, both of which are rare B-cell lymphoproliferative diseases.

1.5 Focus on Murine CMV

The ability to study a natural infection in the natural host makes MCMV a virus worth studying. Due to the strict species specificity of CMV, studies on HCMV have been restricted to *in vitro* systems. However the biological similarities to MCMV (virion structure, life cycle, general biology) makes the murine virus an

excellent model to study HCMV biology and pathogenesis. There are also a number of similarities in the immune response to control viral infection. For instance, in both human and murine MCMV infection $CD8^+$ T cells play an important role for viral control [E. S. Mocarski, 1996]. In fact, the most important target for both HCMV and MCMV-specific $CD8^+$ T cells is the immediate early 1 (IE1) protein [Kern et al., 1999, Reddehase et al., 1985]. Nevertheless, information obtained from the MCMV model should be not extrapolated to the HCMV since there are also a number of differences between these viruses [Rawlinson et al., 1996], for instance both viruses have developed immune evasion strategies to overcome host response against infection, however the molecular mechanisms of such strategies are not identical.

1.5.1 Virion structure and Genetics of MCMV

CMVs are the principal members of the *beta* Herpesvirus subfamily. They also share common characteristics with other Herpesviruses like virion structure and establishment of latency. CMV virion structure consists on an icosahedral capsid where the linear double-stranded DNA of 235 kb is housed. CMV has also an envelope and between the capsid and envelope there is the tegument. Both HCMV and MCMV genomes have been sequenced and it has been established that approximately 80 of the 170 ORFs share homology with HCMV [Rawlinson et al., 1996]. In contrast to HCMV genome organization, MCMV presents a single unique sequence flanked by terminal repeats [Rawlinson et al., 1996].

As other Herpesviruses, MCMV gene expression is regulated as a 3-phase temporal cascade: (1) the immediate early phase, independent on any previous viral gene expression, (2) the early phase which relies on the IE proteins and (3) the late phase dependent on viral DNA replication. During the late phase structural proteins are encoded. The genetic regions encoding for these 3 different classes of genes were described by Keil and coworkers [Keil et al., 1984].

The IE genes of the MCMV are organized in a genomic region known as the major immediate early (MIE) locus from where the IE proteins are expressed under the strict control of the MIE enhancer/promoter. This enhancer region

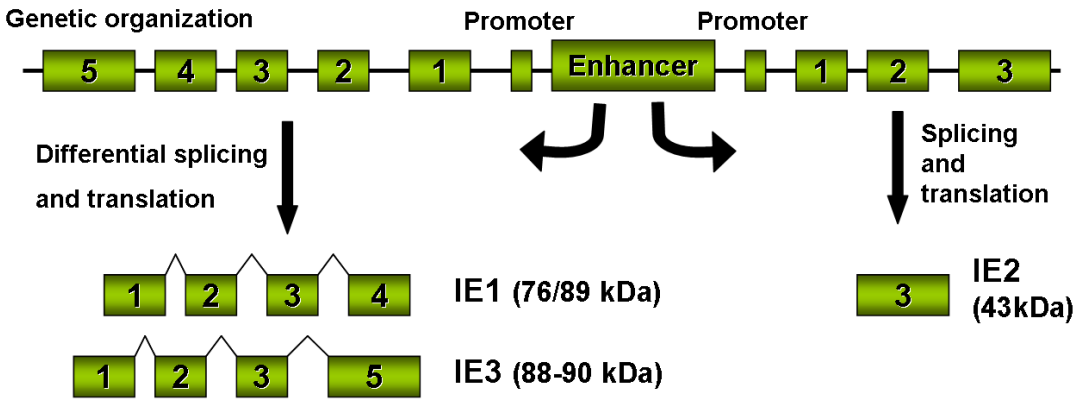

Figure 1.4: Genetic organization of the major immediate early (MIE) region of the Murine Cytomegalovirus

The MIE consists in two transcriptional units: the *ie1/ie3* and the *ie2*, both under the control of specific promoters, at both sides of the enhancer region. The differential splicing from the *ie1/ie3* unit drive to the production of two proteins, the IE1 and IE3. The exon 3 from the *ie2* transcriptional unit is responsible for encoding the IE2 protein. Adapted from [Reddehase, 2002]

is a large sequence containing a number of binding sites for different transcription factors, including NFκB, retinoic acid receptor (RAR), AP1 and CREB. These factors are shared within the different CMVs enhancers [Stinski and Isomura, 2008]. The enhancer region is necessary for viral replication, since it has been shown that a mutant lacking the enhancer is deficient for replication in tissue culture [Angulo et al., 1998]. Moreover, this study also showed that the enhancer is not responsible for the species specificity of the virus since it was possible to restore viral growth when the human virus enhancer region was introduced to the enhancerless virus. In addition, the enhancer region of MCMV is absolutely essential for viral pathogenesis in the natural host [Ghazal et al., 2003].

Two different transcription units can be differentiated at both sides of the enhancer region, the *ie1/ie3* and *ie2*, each one driven by specific promoters (Figure 1.4). Differential splicing of the *ie1/ie3* region results in the production of two proteins: the IE1, encoded by exons 1-4, and IE3, encoded by exons 1-3 and 5. The IE2 protein is encoded by the exon 3 of the *ie2* gene. The structure of this last protein was studied by [Messerle et al., 1991] and different investigations

revealed no actual function for the MCMV IE2 protein, as well as complete unaffected viral growth in the absence of this protein [Manning and Mocarski, 1988]. Therefore, the following sections will be focused on IE1 and IE3 proteins.

Immediate early 1 protein

Sequence and structural organization of the immediate early 1 gene was described by Keil and coworkers [Keil et al., 1987b]. Years later, and thanks to the bacterial artificial chromosome (BAC) technology, which allowed for cloning of the viral genome, studies were designed in order to dissect the role that viral genes might play in the context of a viral infection. Therefore, by using BAC strategy, a MCMV genome carrying a mutation within the *ie1* gene was constructed [Messerle et al., 1997]. The initial observation when studying the function of this gene was that, in the absence of *ie1*, the virus presented slightly lower replication in tissue culture. Ghazal and coworkers generated a second mutant virus where the *ie1* exon 4 was deleted from the viral genome and showed that the IE1 protein was not required for efficient replication *in vitro* [Ghazal et al., 2005]. However, in the natural host the *ie1*-deletion mutant MCMV had an attenuated phenotype, demonstrating a critical role of the viral protein for viral replication *in vivo*. Moreover, the construction of a revertant virus, where the exon 4 was reintroduced, further confirmed that these results were due to IE1, since no differences were found between parental and revertant MCMV.

It has been shown that MCMV IE1 contributes to the transcriptional activation of the early genes, but in combination with the IE3 protein [Messerle et al., 1992]. The IE1-induced activation of gene expression is not completely understood, although the ability of IE1 to interact with chromatin through histones [Munch et al., 1988, Munch et al., 1992] might be responsible for this regulatory function. The ability of the MCMV IE1 protein to activate gene expression has also been demonstrated [Koszinowski et al., 1986], and it was later confirmed by showing that this viral protein induces the transcription of specific genes such as NFκB [Gribaudo et al., 1996] and *c-fos*, both involved in the cellular cycle [Schickedanz et al., 1988], as well as genes involved in thymidylic acid metabolism [Gribaudo et al., 2000], and ribonucleotide reductase [Lembo et al.,

2000], which are required for viral DNA replication and cell cycle regulation. This ability of IE1 to activate gene transcription is also shared with the HCMV homolog IE1 protein [Wade et al., 1992, Hayhurst et al., 1995], although very little homology exist between the nucleic acid and amino acidic sequence of both proteins [Keil et al., 1987b].

Another functional characteristic of the IE1 protein is its interaction with Nuclear domain 10 (ND10), also known as promyelocytic leukemia (PML) oncogenic domains (PODs). ND10s are nuclear structures comprising protein aggregates including PML, Sp100 and Daxx. These aggregates have been shown to be nuclear sites of viral initiation of replication, including herpesvirus and adenovirus [Maul, 1998]. Components of ND10 are known transcriptional repressors, such as PML and Daxx. In addition, they have been associated with antiviral activities since their expression is induced by IFN [Grotzinger et al., 1996, Gongora et al., 2001]. However, the role that these structures play in CMV infection is not completely understood. Infection with HCMV results in the disruption of ND10 nuclear bodies which seems to relieve the transcriptional repression induced by proteins like Daxx. HCMV has been shown to induce degradation of this protein by the tegument protein pp71 [Saffert and Kalejta, 2006], which will subsequently allow expression of IE genes. This would be consistent with the enhancement of viral replication seen in the absence of PML or Daxx [Tavalai et al., 2008]. HCMV IE1 protein has also been involved in ND10 disruption by the modification of PML [Lee et al., 2004]. Like HCMV, MCMV IE1 disperses ND10 bodies soon after infection [Tang and Maul, 2003, Ghazal et al., 2005], an ability which is also shared by other herpesviruses [Everett, 2001], including HSV-1. The HSV-1 ICP0 protein has been shown to disrupt ND10 nuclear bodies by inducing the proteosomal degradation of PML [Everett et al., 1998]. In contrast, MCMV IE1 does not induce the degradation of either PML or Daxx. Instead, it has been suggested that it might induce changes in the phosphorylation status of Daxx, which affects its repression abilities [Maul and Negorev, 2008].

Interestingly, HCMV and HSV-1 mutant viruses, failing to express IE1 and ICP-0, respectively, exhibit a dose-dependent phenotype in cultured cells [Greaves

and Mocarski, 1998, Everett et al., 2004]. This phenotype has been associated with the failure of ND10 disruption, accompanied by the transcriptional repression induced by ND10 components [Gawn and Greaves, 2002, Everett et al., 2008] which limit the expression of IE genes. However, in contrast to these mutants, the MCMV *ie1*-deletion mutant showed comparable replication to wild type in a number of permissive cells [Ghazal et al., 2005]. As expected, disruption of ND10 at early times did not take place in *ie1* mutant-infected cells, indicating that ND10 bodies are not required for MCMV initiation of replication. However, later in MCMV *ie1*-deletion mutant infection, ND10 disruption was seen in an IE1-independent manner, perhaps by other viral products which take over this function for future replication cycles. It is also noteworthy that the ratio particle to PFU in HCMV and HSV-1 IE1 mutants are high [Greaves and Mocarski, 1998, Everett, 1989]. This ratio measures the number of viral particles to infectious particles of virus. However, Ghazal and coworkers showed an indistinguishable growth kinetics of MCMV *ie1*-deletion mutant in a series of permissive cell types and therefore so far it has been assumed that the absence of *ie1* does not result in a defective new virion progeny. In any case, it would be very informative to investigate what the particle to PFU ratio is in this mutant virus.

Immediate early 3 protein

MCMV IE3 is similar to its human homolog IE2, in both structural and functional characteristics [Keil et al., 1987a]. This protein is encoded by exons 1, 2, 3 and 5 from the *ie1/ie3* transcriptional unit. Characterization of the protein resulted in a 88 kDa protein, with similar kinetics as the IE1 protein [Messerle et al., 1992]. The same study demonstrated that the switch from the immediate early phase to the early phase requires IE3 protein which, at the same time, acts as a transcriptional repressor of the *ie1/ie3* promoter.

The role of the *ie3* gene product has been studied by the construction of an *ie3*-deletion MCMV mutant [Angulo et al., 2000]. In order to disrupt the *ie3* gene and prevent the production of the IE3 protein, the exon 5 of the *ie1/ie3* transcriptional unit was deleted. At the same time, and in order to study whether the resulting phenotype was due to solely the *ie3* gene, a revertant virus was also constructed.

In this study, Angulo and coworkers demonstrated the absolute requirement of *ie3* for viral growth. The fact that only *ie1* transcripts were found in infected fibroblasts and neither early nor late proteins were expressed also confirmed the role of the IE3 protein in the activation of viral gene expression. Indeed, IE3 has been shown to negatively regulate the *ie1/ie3* promoter, indicating the regulatory role of this protein in the viral gene expression [Messerle et al., 1992].

Early and Late genes

IE gene products are required for expression of the early genes, which play a role in viral DNA replication. Upon viral replication late genes are then expressed, encoding for structural proteins which make up the viral capsid as well as proteins involved in virion maturation and egress from the cell.

1.5.2 Pathogenesis

CMV is transmitted by bodily secretions (saliva, semen, blood). During pregnancy, transplacental transmission might occur, as well as during breastfeeding, which is considered the most common source of transmission from mother to child. Primary infection of immunocompetent hosts with HCMV is very common and frequently results in asymptomatic disease. However, the virus is never completely cleared by the immune response and the virus establishes latency. Furthermore, when the immune system of the host is undeveloped as in the case of congenital infections, or compromised as in the case of transplant recipients or patients with AIDS, the outcome of the infection could be fatal. Along with the immune status of the host, there are several other factors affecting viral pathogenesis, such as viral replication, evasion of the immune system and dissemination.

Congenital HCMV infection is characterized by damage to the central nervous system (CNS). Maldevelopment of the CNS, deafness and impaired vision are very common in congenitally-infected newborns [Stagno et al., 1986].

HCMV-related disease is very important for transplant recipients, where pneumonitis and gastrointestinal inflammation are very common. The source of viral infection in these patients could be as a result of reactivation of latent virus or transmission from the organ donor. HCMV has also been associated with atherogenesis. The mechanisms by which the disease is initiated are not known, although injury to endothelial cells has been implicated as an important factor. HCMV has tropism for endothelial cells and viral replication induces an inflammatory response. The activation of Mθs, a central player in inflammation, results in the production of cytokines and chemokines which also contribute to the maintainance of inflammation and the evolution of atherogenesis. In fact, there is evidence showing that MCMV infection of Mθs favor the differentiation of these cells into an active pro-inflammatory Mθs, which will contribute to the aggravation of atherosclerosis [Vliegen et al., 2004]. It is also known that HCMV infects monocytes and disseminates throughout the host by using these cells as vehicles. This dissemination process is also important for pathogenesis, as well as cytokine production which has been implicated in reactivation of virus from latency, as seen for TNF [Simon et al., 2005, Hummel et al., 2001]. HCMV infection of intestinal cells has been associated with inflammatory bowel disease by induction of cytokine production [Rahbar et al., 2003].

Viral induction of adhesion molecules in the cell surface could also affect cellular migration and subsequent tumor invasiveness [Soderberg-Naucler, 2006]. HCMV has also been suggested to play a role in immune evasion of tumor cells, since it could help to avoid the immune response.

Because clinical abnormalities of HCMV infection are similar to that observed in corresponding animal models, the use of these systems have helped to understand the pathologies associated with CMV infections. The murine model have been extensively used in this regard. When studying MCMV pathogenesis several factors have to be taken into consideration regarding the outcome of infection. For instance, the route of infection or the origin of the viral inoculum and immune status of the host are key factors which could affect the infection

[Krmpotic et al., 2003]. Infection of immunocompetent mice with salivary gland-derived virus (SGV) results in high viral replication in several organs, including the liver. In this tissue, viral replication induces an inflammatory response, characterized by high levels of TNF [Trgovcich et al., 2000], which have been associated with hepatitis. Cytokine-induced activation of bystander cells could also aggravate damage to the tissue. As in HCMV, infection of immunocompromised hosts could lead to life-threating disease.

1.5.3 Innate immunity against MCMV

Study of the control of MCMV infection has been the focus of many groups in the last decade. This effort has made it possible to understand the specific mechanisms involved with controling virus infection. The studies have identified the key immune cells that play an important role against MCMV infection, as well as their molecular mediators. The establishment of the animal model for studying the human virus has been crucial to determine what immune cells are important in the control of viral infection. Several studies have identified the following cellular populations as the main contributors for the immunity against MCMV as well as targets for the immune evasion strategies developed by the virus.

Natural Killer Cells (NK)

The use of NK cell-depleted mice using either genetically strategies or specific antibodies against this cellular population have demonstrated the key role of NK cells in the innate immune response to MCMV [Orange et al., 1995, Krmpotic et al., 2002]. The response has also been shown to be organ-specific [Tay and Welsh, 1997]. An important feature of these cells is that they allowed the identification of genetic MCMV susceptibility based on the ability of these cells be activated after viral infection. This difference in susceptibility relies on the level of NK cell activation, which is under control of the *Cmv1* locus [Scalzo et al., 1990] which lies within the NK gene complex [Scalzo et al., 1992]. *Cmv1* encodes the activated NK cell receptor Ly49H. NK cells from BALB/c mice lack this locus

and consequently they do not induce the correct signals for NK cell activation after MCMV infection. However, 50% of the NK cells of C57Bl/6 mice bear the *Cmv1* locus and express Ly49H. Therefore, the BALB/c mouse strain is considered as MCMV-susceptible while the C57Bl/6 are MCMV-resistant.

MCMV-infected cells express the viral protein M157, which has been shown to be a strong ligand for Ly49H [Arase et al., 2002]. It is interesting that the virus expresses a protein which will induce activation of NK cells and therefore the innate immune response. However, despite this viral protein, MCMV has also evolved immunevasive mechanisms to escape from NK cells. For instance, Arase and coworkers also showed that M157 binds the inhibitory receptor Ly49I, which association will block NK activation. A second receptor essential for NK cell activation is NKG2D. This receptor is a potent activator of NK cells which has also been shown to be targeted by both HCMV and MCMV (reviewed in [Lenac et al., 2008].

Monocytes/Macrophages (Mθs)

Monocytes are produced in the bone marrow from haematopoietic precursors. They circulate in the blood until they move to the tissues, where they mature into Mθs. Monocytes and Mθs have been shown to be a replication site for MCMV. Moreover, upon infection, MCMV disseminates to other organs aided by blood monocytes, which act as vehicles. It has been reported that the differentiation process defining the maturation of monocytes into Mθs favors viral infection [Hanson et al., 1999]. Mθs also play a role during latency, since it has been found that these cells are a major site of persistence of MCMV DNA in the latent state. Moreover, the bone marrow has been defined as a 'source' of latent MCMV [Pollock et al., 1997, Mitchell et al., 1996].

Besides, Mθs represent the first line of defense along with NK and dendritic cells. Upon activation, Mθs produce a number of mediators which will activate and recruit other immune cells to the site of infection. Mθs also coordinate innate and adaptive immunity. Therefore, it is not surprising that different viruses have developed strategies to avoid Mθs activation or Mθ-induced immune responses.

Indeed, MCMV has been demonstrated to reduce the expression of MHC-class II on the surface of infected macrophages, by inducing the production of IL10 [Redpath et al., 1999].

Because Mθs also play a role in CMV-induced pathogenesis, there are several studies trying to identify the viral genes responsible for Mθs tropism, and find homologues for HCMV.

Dendritic Cells (DC)

DCs are professional antigen presenting cells (APC). Upon activation, DCs migrate to lymphoid tissues where they present antigens to T cells. Several subtypes of DCs have been described. They differ in Toll like receptors (TLRs), costimulatory proteins and cytokines produced. Therefore, the type of immune response induced by DCs depends on the subtype infected or activated. MCMV can infect DCs. Moreover, there is evidence showing that MCMV induces changes in the cytokine profile of the cells as well as their capacity to activate T cells [Andrews et al., 2001].

Direct contact between DCs and NK cells is required for T lymphocyte effector cell expansion. Therefore, interfering with the expression of costimularoty proteins on the surface of DCs might block this expansion and interfere with both innate and adaptive cellular immune response. MCMV has been reported to interfere with the expression of costimulatory proteins in APC. In fact, the *m138* gene product has been reported to downmodulate B7.1 in DCs [Mintern et al., 2006] and *modB7-2* has been identified to downmodulate B7.2 in Mθs [Loewendorf et al., 2004]. These proteins are important T cell stimulators and therefore by modulating their expression the virus also interferes with T cell activation.

Activated DCs produce IL12 which is important for NK activation and subsequent IFNγ production. DCs have been described as major producer of type I IFN after MCMV infection [Dalod et al., 2002].

HCMV has been also reported to target DC function. Chang and coworkers showed that HCMV induces an alteration in DC migration and IL12 production which will then have an effect on NK and T cell activation. Interestingly, these

effects were seen to be mediated by a IL10 viral homolog, known as cmvIL10 [Chang et al., 2004]. In addition to this strategy there are other studies showing the ability of HCMV to manipulate the expression of several chemokines responsible for DC migration to lymphoid organs [Varani et al., 2005].

A recent report has shown that HCMV infection of plasmacytoid DC results in a differential regulation of B and T cells. Activation of pDC by HCMV resulted in the release of several cytokines which favor B cell activation. However, in contrast to this response, the ability of pDC to activate T cells were decreased [Varani et al., 2007]. These studies highlight the importance of cross-talk amongst cells of the immune response against viral infection.

Cytokines and chemokines

An important part of the immune response against MCMV is played by cytokines and chemokines. These chemical mediators are crucial for recruiting more immune cells to the site of infection and alerting non-infected bystander cells to the presence of the pathogen. In the following section, several cytokines known to mediate a key role during MCMV infection are briefly described, as well as the immune evasion mechanisms known to be used by the virus, to counteract these mediators.

Interferon (IFN) Type I IFN comprising members of the IFN$\alpha\beta$ family induce antiviral responses following binding to the receptors IFNAR1 and IFNAR2. Upon ligand binding, Jak1 and Tyk2 are phosphorylated, leading to the phosphorylation of STAT1 and STAT2. Activation of these proteins drives to the formation of a transcriptional complex known as IFN-stimulated gene factor 3 (ISGF3), which is a trimolecular complex composed of STAT1, STAT2 and the transcription factor IRF9. This transcriptional complex will then bind to IFN-stimulated response elements (ISRE) and induce gene expression.

Studies on IFNβ production have shown that full induction of the IFNβ gene required the contribution of three transcription factors, c-Jun/ATF-2, NF-κB and interferon regulatory factor 3 (IRF3). This first wave of IFN induces a positive

feedback loop dependent on IRF7 which will contribute to a robust IFN$\alpha\beta$ response.

After MCMV infection, IFN$\alpha\beta$ levels can be early detected in spleen and liver of infected mice. Plasmacytoid (pDC) and non-plasmacytoid dentritic cells (DC) are responsible for this first induction of IFN$\alpha\beta$ [Orange and Biron, 1996, Hokeness-Antonelli et al., 2007, Zucchini et al., 2007]. In addition, a very recent report has demonstrated that stromal cells in the spleen of infected mice play a key role in the production of IFN$\alpha\beta$ [Schneider et al., 2008]. B cells-produced lymphotoxin β (LTβ) which activate the splenic stromal cells through the LTβ-LTβR signalling pathway, leading to the production of IFN$\alpha\beta$. In contrast, pDC-induced IFN$\alpha\beta$ which is dependent on the activation of the TLR9/MyD88 signalling pathway in the spleen, but it is TLR9-independent and MyD88-dependent in the liver [Delale et al., 2005, Hokeness-Antonelli et al., 2007]. A second wave of IFN$\alpha\beta$ production has been observed in an IRF8-dependent fashion [Tailor et al., 2007], which ensures the optimal cytokine antiviral response.

The role of IFN$\alpha\beta$ in the control of MCMV infection in the natural host has also been studied. It is now well established that IFN$\alpha\beta$ activates natural killer (NK) and CD8$^+$ T cells which in turn are induced to produce IFNγ. Furthermore, this cascade of events has to be under strict control since high levels of IFN$\alpha\beta$ could lead to autoimmune damage [Hahm et al., 2005]. A recent study has shown that NK cells balance the pDC-induced IFN$\alpha\beta$ by controlling viral replication and avoiding overwhelming activation signals to CD8$^+$ T cells [Robbins et al., 2007].

CMV has evolved with the host by developing evasive strategies: the human CMV IE1 protein prevents binding of the ISGF3 complex to IFN-induced promoters by physically interacting with STAT1 and STAT2 [Paulus et al., 2006]. Unlike HCMV, MCMV IE1 has not been shown to share the same function. Instead, a recent investigation suggests that MCMV IE1 is required for an efficient induction of IFN-induced genes [Busche et al., 2008]. Another recent report has revealed

that MCMV inhibits IFNβ transcription by interfering with the transcription factor involved in the formation of the enhanceosome [Le et al., 2008]. Although this report showed that MCMV gene expression is required for this immune evasive strategy, the exact mechanisms of this process remain undetermined. Tyk2 has been shown to be crucial for the antiviral response against MCMV infection both *in vitro* and *in vivo* [Strobl et al., 2005], however since the IFN$\alpha\beta$-induced response was not impaired in the absence of Tyk2, the mechanisms by which this kinase exerts its antiviral effects still remains unidentified.

Type II IFN refers to IFNγ. Following activation of NK and $CD8^+$ T cells, IFNγ is produced and binds to the IFNγ receptor (IFNGR), which is composed of two subunits: IFNGR1 and IFNGR2, both of which are ubiquitously expressed. IFNγ helps to activate bystander cells and is known by the induction of the antiviral state by which the cells prepare to control viral infection. However, the mechanism of IFNγ-induced inhibition of viral replication is not entirely understood. Ligand binding to the receptor induces the phosphorylation of Jak1 and Jak2. Activated Jak1 then phosphorylates STAT1 which associates with other phosphorylated STAT1 to form homodimers. These complexes translocate to the nucleus and bind to IFNγ-activation site elements (GAS) which will induce the activation of IFNγ-induced gene expression. Although IFNγ-induced signalling pathways are STAT1-dependent, there are several studies showing that also STAT1-independent pathways also exist which are important for antiviral activity [Ramana et al., 2002].

The role of this cytokine in the control of MCMV infection has been extensively studied both *in vivo* [Pomeroy et al., 1998, Heise and Virgin, 1995] and *in vitro* [Lucin et al., 1994]. Considering the role that IFNγ plays in antiviral immunity it is not surprising that MCMV has evolved strategies to evade IFNγ-induced antiviral activity. Indeed, it has been demonstrated that MCMV inhibits IFNγ-induced MHC class II expression on the cell surface by affecting expression of the MHC II components [Heise et al., 1998] and by inducing cellular IL10, which is a known anti-inflammatory cytokine [Redpath et al., 1999]. It has been

also reported strategies by which the virus affects both type I and II IFN pathways by targeting STAT2. In fact, the MCMV viral protein pM27 specifically binds to STAT2 and leads to its degradation [Zimmermann et al., 2005]. This mechanism would affect the crosstalk between both pathways during the antiviral activity. This mechanism allows MCMV to escape from the activation of $CD4^+$ T cells. However, and despite the antiviral effect of IFNγ, its production is maintained in spleen cells even after the resolution of the acute infection, suggesting a role in the activation of latent virus [Shanley et al., 2001]. IFNγ is also important for viral clearance from the salivary glands [Lucin et al., 1992].

Interleukins Different interleukins have been shown to play a role in the control of MCMV infection. In this section some of them will be described in more detail according to the relevance of their function in the context of MCMV infection.

IL10 is produced by a variety of immune cells including Mθs, DCs, and T cells. The anti-inflammatory function of this cytokine is well established, its role in limiting the production of proinflammatory cytokines, such as tumor necrosis factor (TNF), IL6 and IL12 has been throughly characterized. IL10 has also been reported to have a role in MCMV infection in primary Mθs. Redpath and coworkers demonstrated that MCMV-induced down-regulation of MHC class II in infected cells was driven by the induction of IL10 in these cells [Redpath et al., 1999]. Very recently IL10 has been described as an important regulator of the immune response against infection, since it can control both pathogen invasion and pathology [Couper et al., 2008]. This would be consistent with a recent study showing that the depletion of IL10 resulted in more severe symptoms of MCMV infection in the natural host, due to the uncontrolled production of inflammatory cytokines such as IFNγ, IL6 and TNF [Oakley et al., 2008]. IL10 is also crucial for the control of MCMV infection in salivary glands, where the virus can still replicate even after clearance of the acute infection. Humphreys and coworkers suggest that MCMV persists in this gland by manipulation of IL10-induced blockade of IFNγ production [Humphreys et al., 2007]. Human CMV, but not the murine virus, encodes a viral homolog of IL10 known as cmvIL10 [Kotenko et al.,

2000]. This viral cytokine is also capable of engaging the IL10 receptor as well as activating signalling molecules such as STAT3 [Spencer, 2007]. Other viruses, such as Epstein Barr (EBV) and Orf virus, have also been reported to encode viral homologues of IL10 (reviewed in [Alcami, 2003]).

The IL6 receptor is a heterotrimeric complex consisting of an α chain and a signal transducing chain, also known as gp130. After ligand binding the consequent association between the α chain and gp130 drives the activation of Jak1, Jak2 and Tyk2 which leads to phosphorylation of STAT1 and STAT3. IL6 is produced by Mθs, T and B cells, endothelial cells and fibroblasts. It is induced by a variety of stimuli, including TNF, IL1 and LPS. It was initially identified as a factor required for B cell differentiation into plasma cells. In addition, IL6 has been shown to be involved in T cell activation. IL6 also has a role in the inflammatory response by inducing the production of MCP1, which recruits other immune cells [Rahbar et al., 2003]. However, there is evidence demonstrating that IL6 might also act as an anti-inflammatory cytokine. For instance, IL6 induces the release of glucocorticoids, which negatively regulate immune functions [Barton, 1997]. MCMV infection results in the production of IL6 [Ruzek et al., 1997]. In addition, IL6 production has been associated with MCMV-induced atherogenesis [Rott et al., 2003]. In HCMV-infected patients IL6-induced inflammatory response might have consequences in patients with inflammatory bowel disease [Rahbar et al., 2003]. The human virus induces a transcriptional regulation of IL6 mRNA in Mθs by a mechanism involving the IE1 protein [Geist and Dai, 1996], maybe through the activation of NFκB. NFκB has also been shown to be essential for *il6* expression [Libermann and Baltimore, 1990].

IL12 is also produced very early after infection and is required for the optimal activation of NK cells and subsequent production of IFNγ in spleen and liver after acute infection. The strength of IL12 signalling is related to the activation of NK cells [Orange et al., 1995]. In addition, IL12 production is also under control since it has been reported that IFN$\alpha\beta$ inhibits IL12 production, which in turn

affects the consequent NK-induced IFNγ. It has also been shown that MCMV infection of DCs results in a significant reduction in IL12 production after 4 days post infection [Andrews et al., 2001], resulting in a decrease in the activation of NK and T cells. However, the mechanisms by which this reduction is regulated have not been elucidated.

IL1 has also been shown to play a role in the control of CMV infection. This pro-inflammatory cytokine is mainly produced by monocytes and Mθs, and it has been reported that it induces the blockade of viral spread in different cell types [Randolph-Habecker et al., 2002]. It is suggested in this study that IL1 might limit viral spread by induction of IFNβ production.

Tumor necrosis factor (TNF) and Lymphotoxin (LT) TNF, also known as tumor necrosis factor-α, is mainly produced by Mθs, and also by NK, DC and activated T cells. TNF exerts its biological activities via signalling through two receptors: TNR receptor 1 (TNFR1, CD120a or p55) and TNR receptor 2 (TNFR2, CD120b or p75), both of which belong to the TNF receptor superfamily. Members of this family can be subdivided into different groups depending on their cytosolic signalling domains: death domain (DD)-containing, also known as death receptors, and TNFR-associated factor (TRAF) binding receptors [Benedict et al., 2003]. TNFR1 is a DD-containing receptor along with others such as Fas, TNF-related apoptosis inducing ligand receptor 1 (TRAIL-R1) and TRAIL-R2. Engagement of these receptors leads to the activation of apoptosis, via recruitment of signalling molecules which induce caspase activation. The second group of receptors signal through the recruitment of TRAF molecules which results in the activation of gene expression by activation of NF-κB as well as members of the mitogen activated protein kinases (MAPK). Despite these two opposite responses, cell death or survival, these signalling cascades are also characterized by crosstalks which ultimately will decide the final outcome of the TNF-induced response. Therefore, it is understandable that TNF has been considered to be a 'double-edged sword' in the immune response, since it can induce processes both positive and negative for the cell [Aggarwal, 2003].

Although both receptors can be found in a wide range of cell types, TNFR2 appears to be restricted to immune cells. It has been proposed that TNFR1 mediates the majority of TNF-induced responses. TNF is important for lymphoid tissue and hematopoietic proliferation and protection against pathogen invasion but uncontrolled TNF production also leads to tissue destruction, as in rheumatoid arthritis and other diseases.

One of the main activities of TNF is to provide protection against pathogen invasion. Therefore, and considering the role of this cytokine in the fate of cells, TNF might be considered as the perfect target for pathogens, since both cell death and gene expression could be modulated by controlling TNF-induced signalling pathways. Indeed, there are a number of pathogens which have developed several mechanisms to inhibit or modulate different stages of the TNF response, from the blockage of TNF binding to the receptor to inhibition of specific TNF-induced responses, such as gene expression or caspase activation [Herbein and O'Brien, 2000, Benedict and Ware, 2001, Rahman and McFadden, 2006]. To date both human and murine CMV have been reported to block TNF-induced gene expression by interfering with TNF receptor expression [Popkin and Virgin, 2003, Baillie et al., 2003, Montag et al., 2006]. Others have shown that HCMV inhibits TNF-induced caspase-dependent apoptosis by encoding viral inhibitors [Skaletskaya et al., 2001]. Very recently murine MCMV has been shown to block caspase-independent apoptosis by direct binding and degradation of receptor-interacting protein RIP1 by the viral protein M45 [Mack et al., 2008]. RIP1 degradation occurs upstream of activation of NF-κB and MAPK during the TNF-induced signalling pathway.

Despite the large amount of information detailing viral strategies to modulate TNF-induced responses, there are only a few examples in the literature of viral proteins which interfere with *tnf* gene expression or protein production. One of these examples is the African Swine fever virus (ASFV). It has been reported that ASFV infection of Mθs results in the inhibition of TNF expression by interfering with the activity of CBP/p300 [Granja et al., 2006]. Epstein Barr (EBV) has also been shown to inhibit TNF production in cell culture [Gosselin

et al., 1992], however the molecular mechanism of this regulation is not clearly understood. By contrast, another study has shown that EBV gp350 induces TNF [D'Addario et al., 2000]. In contrast to the TNF evasion measures that CMV has evolved, work done by Geist and coworkers have indicated that the IE1 protein of HCMV is involved in the induction of *tnf* gene expression in Mθs, probably by interfering with the transcription factors involved in the activation of the promoter [Geist et al., 1994, Geist et al., 1997]. However, the exact mechanism of this regulation is not completely understood. So far there is no evidence showing the same regulation by the murine virus.

In MCMV infection, there have been several studies trying to elucidate the role of this cytokine in the control of viral infection in the natural host. However, results provide contradictory evidence. Shanley and colleagues showed that after both lethal and sublethal infection of BALB/c mice TNF levels were undetectable in serum. However, levels of this cytokine in serum were found in MCMV-infected BALB/c [Trgovcich et al., 2000] and C57BL/6 [Lenzo et al., 2001] and in all organs examined. It also appears that TNF was produced in a viral dose-dependent manner. In addition, studies by Trgovcich showed that the *tnf* gene was expressed during sublethal infection in spleen, liver and lung. Tang-Feldman reported on the levels of expression of different cytokines after MCMV infection in BALB/c mice, showing an induction in TNF expression at day 5 p.i in spleen, liver, lungs and salivary glands [Tang-Feldman et al., 2006]. Thus, the production of TNF early after infection suggests a role in the control of *in vivo* infection of different strains of mice in serum, spleen, liver and heart.

Although the use of specific antibodies to neutralize TNF activity *in vivo* did not result in a significant increase of viral titers [Shanley et al., 1994, Heise and Virgin, 1995], Lucin and coworkers demonstrated that TNF in combination with IFNγ has a more effective anti-MCMV effect [Lucin et al., 1994]. Also TNF has an autocrine/paracrine action. By its paracrine action it can help to activate bystander cells to respond to viral infection. In fact, TNF blockade has been shown to affect Mθs activation and infiltration during MCMV and HSV infection [Heise and Virgin, 1995].

Within the TNF superfamily, another ligand has to be taken into consideration in the context of MCMV infection, namely Lymphotoxin (LT). As with TNF, LT also has a role in the development of lymphoid tissues and immune cells. LTα binds to the same receptors as TNF (TNFR1 and TNFR2). LTα can also form with LTβ the heterotrimer LT$\alpha\beta$ which binds to the LTβ receptor (LTβR). This receptor also binds a second ligand known as LIGHT, which also binds to the herpesvirus entry mediator or HVEM [Aggarwal, 2003]. The role of the LT$\alpha\beta$-LTβR system in the control of MCMV infection has been studied. Different investigations have demonstrated that the LTβ signalling pathway plays an important role in human CMV infection by inducing IFNβ production. However, this response relies on the presence of HCMV [Benedict et al., 2001]. They have also reported that LT$\alpha\beta$-LTβR is pivotal for MCMV infection *in vivo*, since genetic depletion of LTα resulted in a high susceptibility to MCMV infection. LTβ is also produced after infection and LTβ-LTβR signalling is crucial for the production of IFN$\alpha\beta$ by splenic stromal cells. Thus, it is not surprising that blocking LTβ- induced signalling results in high viral burden. The crosstalk between LTβ-LTβR signalling and IFNβ was demonstrated when the antiviral activity was restored by administrating IFNβ [Banks et al., 2005].

Chemokines Chemokines are a small family of chemoattractant cytokines whose main function is to induce trafficking and recruitment of other cells. Chemokines are divided into different groups depending on the molecular structure. There are four main groups: CC chemokines (or β-chemokines), CXC chemokines (or α-chemokines), C chemokines (or γ-chemokines) and CX_3C chemokine (or δ-chemokines). Amongst the functions of these cytokines are: trafficking of leukocytes and regulation and differentiation of cells of hematopoietic lineage and inflammation [Schall and Bacon, 1994]. There are also several studies showing the production of chemokines and their effect on viral infection (reviewed in [Melchjorsen et al., 2003]). Moreover, the fact that an important number of viruses encode their own chemokines and chemokine receptors reveals the key

role that these mediators play during the immune response against viral infection. Both human and murine CMV encode viral chemokines. For instance, HCMV UL146 product vCXC-1 has been reported to function as an agonist of human IL18. It induces neutrophil chemotaxis to the site of infection favoring viral dissemination [Penfold et al., 1999]. MCMV chemokines MCK-1 and MCK-2 also control viral dissemination in the natural host by recruiting leukocytes to the site of infection [Saederup et al., 1999]. HCMV encodes 4 chemokine receptors, UL33, UL78, US27 and US28 [Chee et al., 1990]. US28 has been reported to bind and sequester chemokines from the extracellular environment, which will inhibit lymphocyte recruitment and activation [Bodaghi et al., 1998]. In addition, the ability of HCMV US28 to induce migration of smooth muscle cells has implicated this viral chemokine receptor in the development of atherosclerosis [Streblow et al., 1999]. The murine homolog of HCMV UL33, known as M33, has been reported to be important for viral replication *in vivo*, particularly in the salivary glands [Davis-Poynter et al., 1997].

In addition to several other herpesviruses which have been reported to encode viral chemokines, there are other viruses too which have evolved this evasion strategy (reviewed in [Alcami, 2003], demonstrating the key role that these proteins play in the regulation of the immune response.

1.5.4 Adaptive immunity

The adaptive immune response follows the initial innate response, and is characterized by the activation of T and B cells. The protective role of T cell subsets has been studied. $CD8^+$ T cells become activated by the recognition of antigens presented by MHC class I molecules, as well as the presence of several coestimulatory proteins. Even though it is known that $CD8^+$ T cells play an important role in MCMV protection, different studies revealed that MCMV was also cleared when this subset was absent [Jonjic et al., 1990]. Surprisingly, viral clearance appeared to rely on $CD4^+$ T cells. These results are also important since they revealed the flexibility of the immune response to adapt to specific conditions and

induce compensatory mechanisms keeping the organism protected. In fact, this central role of $CD4^+$ T cells is only apparent in the absence of $CD8^+$ T cells.

Naive $CD4^+$ T cells are activated following interaction between the T-cell receptor (TCR) and antigens bound to MHC class II molecules, expressed on the surface of APC. Activation of $CD4^+$ T cells results in the expression of a wide range of cytokines which will orchestrate the immune response. Jonjic and coworkers also studied the role of $CD4^+$ T cells during MCMV infection. *In vivo* studies showed that these cells are key players in control of MCMV infection in the salivary glands [Jonjic et al., 1989], where they mediate immunity through the production of TNF and IFNγ. In the absence of $CD4^+$ T cells, $CD8^+$ T cells are still central in MCMV clearance, although with slower kinetics.

In addition to viral evasion strategies for escaping the innate immune response, there are also studies showing viral immunomodulatory strategies to counteract adaptive immunity. These are generally based on the disruption of antigen presenting pathways. These evasion strategies are also shared by other viruses, including HSV, EBV and HIV, revealing the importance of this pathway in the immune response (reviewed in [Alcami et al., 2005]). Both human and murine CMV encode proteins which reduce the expression of MHC class I molecules, either by inducing their degradation, like HCMV US2 and US11 proteins, or MCMV gp34, gp48 and gp40 (reviewed in [Hengel et al., 1998]). Disruption of $CD4^+$ T cell activation due to HCMV and MCMV has also been reported by interference with expression of MHC class II molecules. HCMV infection reduces the expression of IFNγ-induced MHC class II by either interfering with the IFNγ signalling pathway or by inducing the production of HCMV cmvIL10, an inhibitor of MHC class II expression [Spencer et al., 2002]. MCMV however induces cellular IL10 in order to inhibit IFNγ-induced MHC II expression in peritoneal Mθs [Redpath et al., 1999]. Also in Mθs it was seen that type I IFN might also be involved in the inhibition of the expression of MHC class II [Heise et al., 1998].

Unlike T cells, the role of B cells in MCMV infection is not well defined. On the one hand, it is very difficult to completely delete B cells, since antibody depletion strategies do not inhibit plasma cells. On the other hand, the interaction between B and T cells has to be taken into consideration when drawing any conclusions, since B cells are important for T cell expansion. However, it has been shown that B cell-induced immunity is not required for viral clearance during primary infection [Jonjic et al., 1994]. Later, by using an immunocompromised mouse model, protection against MCMV after adoptive transfer of memory B cells was demonstrated [Klenovsek et al., 2007]. This study also showed that the antiviral protection was antibody-dependent. Understanding the role of these cells in the immune response is crucial for bone marrow transplantation.

1.5.5 Latency

One of the hallmarks of herpesviruses is the ability to establish a long term infection within the host, which might last for life, known as a latent infection. The study of HCMV latency has been problematic due to the fact that, unlike other viruses in which the establishment of latency is restricted to specific tissues, HCMV can be found in a latent state in several tissues, including lungs, kidney, liver and bone marrow [Koffron et al., 1997, Minton et al., 1994] and the same situation has been described for MCMV as well [Koffron et al., 1998]. Although the molecular mechanisms controlling establishment and maintenance of CMV latency are not known, the role of the immune system has been extensively studied. For instance, after primary infection cell-mediated immunity does not completely clear the virus, even though viral replication is inhibited primarily by NK and CD8 T cells. Instead, the virus persists for longer periods by the establishment of latency. The initiation of this process has also been suggested to be part of the viral immune evasion strategy. Therefore, by shutting down viral gene expression the virus limits the number of antigens which might be recognized by CTLs.

The role of Mθs in CMV latency has been extensively reported. Thus, it is now known that DNA for human and murine CMV can be found in Mθs during latent infection [Jarvis and Nelson, 2002, Mitchell et al., 1996, Pollock et al., 1997]. Moreover, bone marrow cells have also been shown to harbor latent DNA, suggesting that it might act as a source of latent virus. Mθs have also been implicated as vehicles for dissemination not only during acute infection [Stoddart et al., 1994] but also during latency [Pollock et al., 1997].

Although in some of the aforementioned studies no viral transcripts were found, it is generally accepted that latency might involve a low basal transcriptional activation in latently-infected cells. For instance, Kurz and coworkers showed that during latency, the MCMV *ie1/ie3* transcriptional unit remains active in lungs [Kurz et al., 1999]. This transcriptional activity is restricted to *ie1* but is not sufficient to induce reactivation of the virus. Since IE3 is responsible for activation of the early phase of the viral cycle, the lack of IE3 explains why, even though IE1 transcripts are detected, no reactivation can be seen. In fact, CD8 memory T cells specific for IE1 are key players in this stage of latency, since there is a continuous control for viral antigens and inhibit any attempt of reactivation [Simon et al., 2006]. This study suggest an important role of the MIE locus in dictating MCMV reactivation in this tissue.

Viral reactivation from latency has been suggested to occur though several mechanisms, including inflammatory signals, cellular differentiation and allogeneic stimulation. Notably, the role of the immune system is common to all mechanisms proposed. Under immunosuppressive conditions the monitoring of viral antigens performed by memory T cells might be absent. Therefore, the basal transcriptional activity which has been described in latent CMV infection might continue towards the expression of viral genes leading to a replicative cycle and complete reactivation. On the other hand, during transplantation and cellular differentiation, cytokine production might occur. Within the cytokine pool, TNF has been shown to play a role in viral reactivation. This proinflammatory cytokine is known to induce activation of several transcription factors which bind to the MCMV MIEP enhancer region, including NF-κB and AP1 [Hummel et al.,

2001]. However, it has also been shown that this TNF-dependent viral gene expression reactivation might be organ-specific [Hummel et al., 2001, Hummel and Abecassis, 2002] and would not explain the reactivation process in other organs.

In any case, it seems that the immune system is crucial during the whole latency period, as described by Redpath and coworkers in their model for viral latency [Redpath et al., 2001]. Latency might be described as a state of balance between the virus and the immune system. This model suggests that the maintenance of latency depends on the activation threshold of immune cells, which changes with the immune status of the host. In this model the associated risk to both virus and host due to reactivation is also considered; injury to the host due to virus-associated disease and inhibition of the virus due to immune control. Importantly, this model is not only restricted to CMV but also to other viruses which are capable of establishing latency.

1.5.6 Human Herpesvirus 6 and Human Herpesvirus 7

HHV6 and HHV7 also belongs to the *beta* Herpesvirus subfamily, and they compose the *Roseolovirus* genus. They all share characteristics with the more extensively studied CMV. These viruses are T lymphotropic but they also infect other types of cells. HHV6 infects T and B cells, macrophages and NK cells. In contrast, HHV7 has a more restricted cell tropism based on the finding that it utilises CD4 as a receptor [Lusso et al., 1994], infecting T cells. *In vivo* epithelial cells from the salivary gland have also been identified as targets for HHV7. Because Roseoloviruses have been recently discovered the kinetics of gene expression have not been studied in extent, however it seems to follow the typical temporal cascade characteristic of other Herpesviruses. During the latent infection viral gene expression is limited to those genes involved in maintenance of latency. HHV6 latent infection can be detected in monocytes-macrophages as well as in salivary glands. A site for HHV7 latency is CD4 T cells, epithelial cells from the salivary glands among others. Roseoloviruses share with VZV the re-envelopment pathway for virion egress as previously explain in Section 1.1.2.

After HHV6 and HHV7 infection cellular immunity seems to have a key role in the control of viral infection, particularly T cells response [Yakushijin et al., 1991, Yakushijin et al., 1992, Yasukawa et al., 1993]. Clinical data also indicate the role for IFNα and NK cells [Takahashi et al., 1992]. Other cytokines like TNF, IFNγ and IL1β have also been shown to be produced after HHV6 infection [Flamand et al., 1991].

HHV6 and HHV7 infection occurs during infancy. Although the transmission mechanism is not clear yet it seems that saliva is the most common route of infection. HHV6 has also been detected in the cervix of pregnant women, suggesting a possible transmission to newborns. Breast-feeding has also been suggested to help the transmission of HHV7. Other routes of infections have also been suggested such as bone marrow or solid organ transplantation in the case of HHV6.

As introduced before, HHV6 and HHV7 infection causes ES in infants characterized by transient rash after following by sudden fever. In rare cases febrile convulsion could happen as a result of the sudden high fever. In most cases ES does not have any clinical complication. In adults infection could cause mononucleosis-like disease. HHV6 can persist in the central nervous system and it might be associated with cases of HHV6-induce encephalitis. Like CMV infection, infection of immunocompromised hosts could be fatal, as for those transplant recipients or AIDS patients. Reactivation of HHV6 has been reported after bone marrow or solid organ transplantation, as well as potential association with HCMV.

1.6 This Thesis: Rationale and Central Hypothesis

As has been discussed, the IE1 protein of MCMV plays a role in the regulation of viral and host gene expression. In the virus, it *trans*-activates the expression of early genes, in coordination with the IE3 protein, and in the host it seems that the IE1-induced gene expression prepares the cellular environment to favor viral replication. By using BAC technology an *ie1*-deletion mutant of MCMV, (referred to as MCMVdie1 hereafter), was constructed in order to determine the exact role

that this viral protein plays in the MCMV infection. In addition, the revertant of this mutant, named MCMVrev, was also constructed in order to rule out any second site mutations. So far, it has been demonstrated that, unlike the IE3 protein, IE1 is not required for efficient replication in different cell types. However, in immunecompetent mice the mutant virus presents an attenuated phenotype. Moreover, in SCID mice lacking B and T cells and relying on innate immunity to control the infection, the mutant virus also showed a growth defect. These observations have led to the hypothesis that IE1 is involved in the regulation of the host immune response. This would be consistent with the growth defect seen for MCMVdie1 *in vivo*, most likely because the host immune response is not under the control of the viral protein, leading to a stronger response which control viral infection sooner. In other words, IE1 might act as a immunomodulator. To investigate this hypothesis the following questions were prompted and define the different Chapters of this thesis:

1. **How does the absence of IE1 affect viral replication in BMMθs?**
   BMMθs were used as a experimental system in this project, since they play a crucial role not only for viral infection but also in the immune response against MCMV. Moreover, MCMVdie1 replication has not been studied in this cellular population. Therefore it was of interest to study MCMVdie1 infection in a cell type involved in the control of viral infection. In addition, IE1 protein has been described as a *trans*-activator of cell and viral gene expression. Therefore it was also of interest to study the effect that the absence of this viral protein might have in the host gene expression. In order to have an initial observation of the effect of MCMVdie1 infection in BMMθs a pilot study was carried out between MCMVdie1 and MCMVrev using microarray technology. The following objectives were set:
   - To determine the effect of the absence of IE1 in viral replication in BMMθs
   - To determine to what extent host gene expression were affected by the absence this viral protein

- To make an initial observation of the biological processes and pathways most affected after infection with MCMVdie1 by performing analysis based on Pathway biology.

2. **What effect does MCMVdie1 infection have on cytokine production induced by BMMθs?**

   Considering the key role that BMMθs play in the control of MCMV infection, the next step in this investigation was to study in more detail the cytokine production in the context of MCMVdie1 infection. The following objectives were set up:

   - To study the effect of MCMVdie1 infection in the cytokine production in BMMθ
   - To study the transcript level of those cytokines of interest

3. **What are the molecular mechanisms for MCMVdie1-induced cytokine modulation?**

   As it has been introduced, TNF is a key player in the control of MCMV infection. Several viruses have been shown to target different steps of the TNF biology however only few have been reported to target TNF production. Results also revealed a significant regulation of TNF response by MCMV infection. Therefore the next objective was to study in more detail the molecular mechanisms of TNF response in the context of MCMV infection in the presence and absence of *ie1*. To fulfill this aim two different approaches were taken:

   - A literature review was performed in order to understand the mechanisms of TNF production and create a model of the different transcription factors that might be involved in the induction of *tnf* expression after MCMV infection.
   - Based on the review, a extensive study on the activation of signalling molecules was performed in infected-BMMθ. This study was done in collaboration with Upstate (Millipore, UK). Furthermore, in order

to validate this study, conventional Western Blot approach was also taken.

4. **Is TNF response to MCMVdie1 infection also seen *in vivo*?**
   Experimental findings in tissue culture led to the final stage of this investigation, where it was of interest to determine whether MCMVdie1-induced TNF production was also seen *in vivo*. The essential role of IE1 for an efficient replication of the virus in BALB/c and SCID mice has been reported [Ghazal et al., 2005]. Therefore the following objectives were set:
   - Study the role of the IE1 protein in the viral replication in MCMV-resistant C57Bl/6 mice and confirm the attenuated phenotype in MCMV-susceptible BALB/c.
   - To establish whether MCMVdie1 infection induces a significant TNF production in both BALB/c and C57Bl/6 mice.

5. **Is TNF responsible for the growth defect of the *ie1*-deletion mutant MCMV?**
   The final question of this investigation was to study whether the replication defect seen for the mutant virus was due to the regulation of TNF response. In the first place the effect of TNF was studied in the viral replication in BMMθs and later was expanded to an *in vivo* system where genetically depleted TNF C57Bl/6 mice were used.

CHAPTER 2

Materials and Methods

2.1 General Chemicals and Solutions

For general solution recipes refer to Appendix I

2.2 General Methods in Tissue Culture

2.2.1 Culturing NIH 3T3

The murine fibroblast cell line NIH-3T3 cells (ATCC CRL1658) was obtained from American Type Culture Collection (Manassas, VA.). Cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% calf serum (CS), 2 mM glutamine (Invitrogen, UK) and 100 U of penicillin/streptomycin (Invitrogen, UK) per ml. Cells were incubated at 37°C and 5% CO_2 in tissue culture flasks (Corning, UK), until they reached 70-80% confluency. Cells were expanded as described in Section 2.2.3.

2.2.2 Culturing Primary Murine Embryonic Fibroblasts (MEFs)

MEFs were prepared from embryos of pregnant BALB/c mice on day 16 of gestation. Cells were grown in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine (Invitrogen, UK) and 100 U of penicillin/streptomycin (Invitrogen, UK) per ml. Cells were incubated at 37°C and 5% CO_2 in tissue culture flasks (Corning, UK), until reached confluency, but never left to 100% confluency to avoid overgrowth of the cells. Cells were expanded as described in Section 2.2.3.

2.2.3 *Handling cells for passaging*

Cells were expanded when they reached 70-80% confluency. Medium was discarded and cells were washed with PBS (Sigma, UK). PBS was discarded and cells were detached from the flask by using Trypsin. The flask was gently rocked for a couple of min and most of the Trypsin solution was discarded. Cells were transferred to a 15 ml Falcon tube and spun at 1100 rpm for 5 min at room temperature in 4 ml of DMEM supplemented with the specific serum. The cell pellet was resuspended in culture media and transferred to a new tissue culture flask.

Table 2.1: Handling cells for passaging

Tissue Culture Flask	PBS (ml)	Trypsin (ml)
25 cm^2	5	3
75 cm^2	10	4
175 cm^2	20	5

2.2.4 *Freezing cells for long term storage*

Cells were trypsinised as described previously in Section 2.2.3. Cells were transferred to a 15 ml Falcon tube and centrifuged at 1100 rpm for 5 min at room temperature. The cell pellet was resuspended in 3 ml of freezing medium. 1 ml aliquots were transferred to 1.8 ml cryovials (Corning, UK). The cryovials were labelled and stored in liquid nitrogen until use.

2.2.5 *Thawing cells from liquid nitrogen storage*

Cryovials containing frozen cells were removed from liquid nitrogen and thawed slowly at 37°C. Cells were then tranferred to a 15 ml Falcon tube and 5 ml of culture medium was added to wash out the DMSO contained in the freezing medium. Cells were centrifuged at 1100 rpm for 5 min at room temperature. Supernantant was discarded and cell pellet was resuspended in 1 ml of culturing medium and transferred to a 25 cm^2 tissue culture flask and grown as previously described (Sections 2.2.1 and 2.2.2)

2.2.6 *Bone Marrow Derived Macrophages (BMMθs)*

1. Preparation and culture of BMMθs

BMMθs were prepared from 10-12 week old male BALB/c mice. Animals were killed by cervical dislocation. Fur was cleaned with ethanol, an incision was made from the abdomen and the femurs exposed by removing muscles surrounding the bone. The hip and knee joints were cut and any remaining tissue was removed using desinfectant wipes. The femur was held with sterile forceps and the marrow cavitiy was flushed with DMEM:F12 supplemented with 10% FCS and 10% L929 conditioned medium. Cells were recovered in a 50 ml Falcon tube, centrifuged at 1100 rpm at room temperature for 5 min and resuspended in DMEM:F12 medium. Cell number was determined by microscopy using a haemocytometer (described in Section 2.3). Cells were plated into either 24- or 6-well plates and cultured for 7 days in DMEM:F12 containing 10% FCS and 10% L929 conditioned medium to stimulate the differentiation of macrophages. Medium was replaced every 2 days.

2. Characterization of BMMθs

   (i) Cytospin followed by Diff-quik staining.

   Morphology and maturation of BMMθs was examined on Cytospins stained with Diff-quik, a commercial stain commonly used to differentiate a variety of smears. 100-200 μl from a BMMθs suspension (approximately 1 x 10^6 cells/ml) was added to the well of a 'cytospin holder' and the cells were cytocentrifuged at 300 g for 3 min in a Shandon Cytospin II (Shandon, UK). The cells were then fixed with 100% methanol for 2 min, followed by staining in the Diff-quik Red solution for 1 min and Diff-quik Blue solution (both from Gamidor Ltd, UK) for 1 min. All steps were performed at room temperature. The slides were rinsed with distilled water, left to air-dry overnight and finally coverslipped and mounted (Figure 2.1).

   (ii) Flow Cytometry.

   Maturation of BMMθs was also tested by flow-cytometric analysis of surface markers. Thus, an analysis was performed for expression of murine macrophage cell surface proteins F4/80 and CD11b. Double

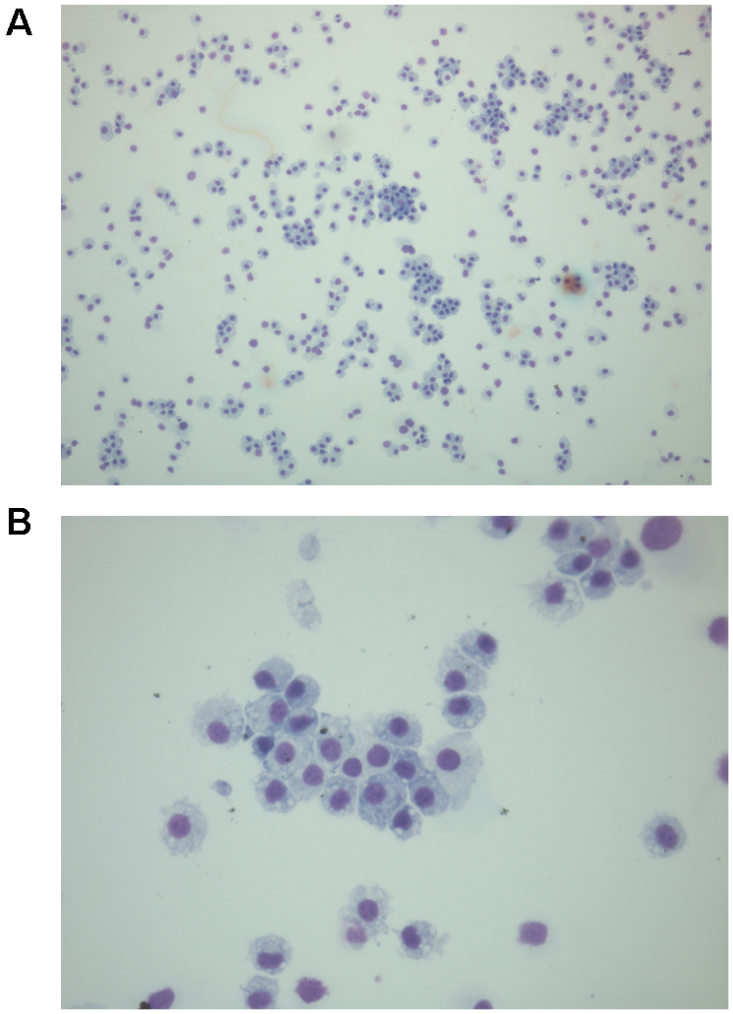

Figure 2.1: Phenotype of BMMθs after 7 days of culture

Maturation of day 7 BMMθs was determined by cytopsin followed by Diff Quik staining. **A.** x100 magnification of mature BMMθs, without any contaminating cells and **B.** x1000 magnification indicating the features of BMMθs.

labelling allows the detection of mature BMMθs compared to monocytes, which only express CD11b. At day 7, BMMθs were detached from the culture plates using a cell scraper, transferred to polystyrene FACS tubes (BD Falcon, UK) and centrifuged at 300 g for 5 min at 4°C. The supernatant was discarded and the cells were washed by adding 2 ml of cold PBS, followed by centrifugation at 300 g for 5 min at 4°C. Cells were resuspended (2x10^5 cells/ml per FACS tube) in 100 μl blocking solution (PBS containing 10% mouse serum) and incubated for 30 min on ice. Allophycocyanin (APC) conjugated monoclonal

rat-anti-mouse F4/80 (IgG2aκ, Caltag Laboratories, UK) and fluorescein isothiocyanate (FITC) conjugated rat-anti-mouse CD11b (IgG2bκ, eBiosciences, UK), both diluted 1/100 in blocking solution, or their respective isotype controls were then added. Cells were mixed gently and incubated on ice for 30 min in the dark. Before undergoing flow cytometric analysis, the cells were washed with cold PBS and resuspended in 200-300 μl of PBS. Analyses were performed using a FACScan instrument (Becton Dickinson, UK) and the results were analysed with Cell Quest software (Becton Dickinson, UK) or FlowJo software (Treestar, USA). A typical flow cytometric analysis of day 7 BMMθs after staining for F4/80 and CD11b is shown in Figure 2.2.

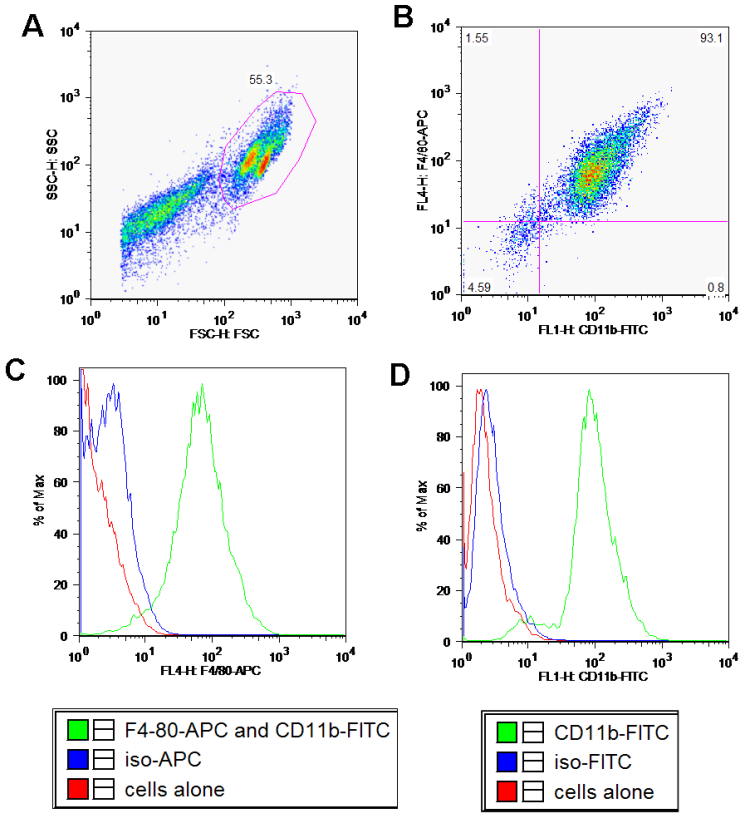

Figure 2.2: Phenotype of BMMθs after 7 days of culture by Flow Cytometry Analysis

Maturation of day 7 BMMθs assessed by staining for the specific expression of murine Mθs cell surface proteins F4/80 and CD11b. The level of expression was analysed by flow cytometry. **A.** FACS dot blot showing the gating in forward scatter (FSC) and side scatter (SSC). **B.** A pure Mθs population (93.1%) expressing both F4/80 and CD11b is seen in the upper right quadrant of the FACS dot blot. Histograms for **C.** F4/80 and **D.** CD11b staining.

2.2.7 TNF treatment of BMMθs

Recombinant mouse tumor necrosis factor α (Biosource International, USA) was prepared in DMEM:F12 supplemented with 10% FCS and 10% L929 conditioned medium as 1U/mL or 10U/mL (10 μg/mL corresponds to 10^5 U/mL). Cytokine was added to the cells 24h prior infection. After infection, cytokine was added to the medium.

2.2.8 Cell viability assay

To determine the number of viable cells after TNF treatment CellTiter-Blue Cell Viability Assay (Promega, UK) was performed following manufacturer's instructions. Briefly, BMMθs were plated out in a 96-well plate at a seeding density of 4 x 10^4 cells/well. Cells were either treated for 24h with 1U/mL or 10U/mL of TNF or mocked treated by adding medium. All treatments were done in triplicates. After the specified time 5μL of the reagent were added to each well leaving the plate for 4h incubation at 37°C. Viabiliy of cells was measured according to a fluorescent signal, correponsing to the metabolic activity of viable cells. Fluorescence was measured in a POLARstar OPTIMA Multifunction Microplate Reader (BMG LabTech, UK).

2.2.9 Testing cell culture for presence of Mycoplasma

Tests for the presence of the mycoplasma were carried out every 4 months by Minerva Biolabs GmbH (Berlin, Germany), all found negative.

2.2.10 Testing cell culture for Lymphotoxin contamination

Tests for LPS contamination were carried out regularly by SNBTS (Edinburgh, UK), all found negative.

2.3 Cell counting

Cell counts were determined by using a haemocytometer. Cells were harvested and resuspended in 2 ml of the appropiate medium. The haemocytometer slide and coverslip were washed with 70% ethanol prior to use. Carefully both chambers of the haemocytometer were filled with an aliquot of the cell suspension by capillary action using a pipette. Using the microscope viable cells were counted

only in the centre square of the 9 squares on the main grid. Counting was repeated for the grid on the other side of the haemocytometer. To calculate the number of viable cells per ml in the original cell suspension the following equation was used:

Cell/ml = Average count x 10^4 x Dilution Factor

2.4 General Methods in Virology

2.4.1 Preparation of viral stocks

All viruses used during this thesis were propagated in NIH 3T3 cells. Cells were cultured as described in Section 2.2.1. and infected at a MOI 0.001 with the desired virus. The viral inoculum was prepared in DMEM supplemented with 3% FCS, 2mM Glutamine and 100 U of penicilin/streptomycin per ml. 10 days after infection, viral stock was harvested. Cells were detached from the flask and spun at room temperature at 1100 rpm for 5 min. Supernantant was then aliquoted and kept at -70°C until use.

2.4.2 Titration of viral stock: plaque assay

Viral titers were quantitated by standard plaque assay. Briefly, MEFs were grown (Section 2.2.2) and plated out in a 24- well plate at a seeding density of 10^5 cells/well and left at 37°C and 5% CO_2 for 24 hours. Plates were gently agitated vertically and horizontally to evenly distribute the cells. On the day of the assay, a serial dilution of virus was made in DMEM supplemented with 3% FCS in a 96-well plate. MEFs were then infected with 250 μl of the specific viral dilution, and left for 1 hour adsorption gently shaking the plate every 10 min. After 1 hour the viral inoculum was carefully aspirated and monolayer were washed with PBS. Meanwhile DMEM supplemented with 3% FCS was mixed with melted 2.5% agarose (1:10) and 1.5 ml were added to each well. Plates were placed at 37°C and 5% CO_2. Lysis plaques were counted in each well after 3-4 days of culture and viral titer was determined as plaque forming units per ml (PFU/ml).

2.4.3 *Viral infection of BMMθs*

BMMθs were prepared (Section 2.2.6) and plated out at a seeding density of 5x10^5 cell/well for a 24-well plate or 8x10^5 cell/well for a 6- well plate, unless specified. After 7 days culture cells were infected with virus at a MOI of 1. As described for NIH 3T3 and MEFs, the viral inoculum was prepared in DMEM:F12 supplemented with 3% FCS and left for 1 hour adsorption with gently shaking of the plate every 10 min. BMMθs were washed in PBS and fresh media was added. Plates were placed at 37°C and 5% CO_2.

2.5 General Methods for RNA

2.5.1 *Trizol/RNA extraction*

RNA extraction was done using Invitrogen reagents and protocols. Briefly, 200 μl of chloroform were added per 1 ml of Trizol. The mixture was shaken vigorously by hand and incubated at room temperature for 15-30 min. After incubation, samples were centrifuged at 12,000 xg for 15 min at 4°C. The upper phase was carefully removed and transferred to a fresh microcentrifuge tube. 500 μl of isopropanol alcohol were added per 1 ml of Trizol and incubated for 10 min at room temperature. After incubation samples were centrifuged once again at 12,000 xg for 10 min at 4°C. Supernatant was then removed and the pellet was washed with 75% ethanol, vortexed and centrifuged at 7,500 xg for 5 min at 4°C. The pellet was air dried and dissolved in RNAse free water. Extracted RNA was kept at -80°C.

2.5.2 *Quantification of RNA concentration.*

RNA concentration was determined by using a NanoDrop ND-1000 spectophotometer (NanoDrop Technologies, USA).

2.5.3 *qRT-PCR*

For each sample, 2x Taqman® PCR mix (Applied Biosystems, USA) was mixed with 40 u of Superscript III (Invitrogen, USA). 4 μL total RNA was then added

and each sample split into two reactions. A Taqman® primer/probe set (Applied Biosystems, USA) for *tnf* was then added to one reaction at the recommended concentration while a Taqman® primer/probe set for *gapdh* was added to the other reaction. Samples were then run on a MX1000P quantitative PCR thermal cycler (Stratagene, USA). Samples were first heated to 50°C for 30 min then heated to 95°C for 10 min. Samples were then subject to 40 cycles under Taqman® standard conditions. Data was analysed by using Stratagene MXPro software.

2.5.4 Microarray

RNA samples from MCMVdie1 or MCMVrev infeced-BMMθs were processed using the Affymetrix Genechip Mouse 430A 2.0 protocol and the One Cycle cDNA synthesis method with a starting amount of 2.5 μg of RNA. This was followed by a cDNA clean-up step and an *in vitro* transcription step to synthesise biotin-labelled cRNA. Biotin labelled cRNA was then processed through a clean-up step prior to quantification (260/280nm) using a Nanodrop spectrophotometer.

Samples were then run on an Agilent 2100 Bioanalyser to check the quality of the cRNA produced. All samples produced the expected profile at this stage and were therefore put forward for the fragmentation procedure. Following fragmentation, samples were again run on the Agilent Bioanalyser to check for the correct fragmentation of the cRNA. Only high quality samples (MCMVdie1 n=4, MCMVrev n=3) were put forward for the array hybridisations.

All samples passed the basic Affymetrix QC report file tests based mostly on the spiked in Poly-A controls.

2.5.5 Data analysis

Data from the microarray were analyzed by using the Ingenuity Pathway Analysis (IPA) application (Ingenuity Systems® www.ingenuity.com).

2.6 Protein Methods

2.6.1 Whole cell extract preparation.

Whole lysate was extracted from BMMθs using Beadlyte® Cell Signaling Lysis Buffer (Millipore, UK), following manufacter's instructions. BMMθs were cultured for 7 days (Section 2.2.6) in 6- well plates at a seeding density of 10^6 cells/well. After treatment or infection cells were washed with ice-cold TBS and 10 ml of 1X lysis buffer prepared. At this stage 1 tablet of Complete Mini-proteinase inhibitor (Roche, UK) and 100 μl of Phosphatase Inhibitor cocktail I and II (both from Sigma, UK) were also added to the lysis buffer. 100 μl of lysis buffer was added to the cells. Cells were then scraped from the plate using a plastic cell scraper and transferred to a 1.5 ml Eppendorf tube. The suspension was then gently rocked for 15 min at 4°C to lyse the cells. The lysate was clarified by centrifugation at 14,000 g for 15 min at 4°C. Supernatant was transferred to a new 1.5 ml Eppendorf and the lysate was kept at -70°C until use.

2.6.2 Measure of protein concentration

Total protein concentration was determined using the Micro BCA protein assay (Pierce, UK), following manufacturer's instructions. A set of protein standards was prepared by diluting an Bovine Serum Albumin standard (BSA) ampule (2000μg/ml) as follows:

Table 2.2: Preparation of Micro BCA protein assay standard

Vial	Final Concentration	Volume (Diluent + Stock)
A	2000 μg/ml	300μl of stock
B	1,500 μg/ml	375μl of stock
C	1,000 μg/ml	325μl of stock
D	750 μg/ml	175μl of vial B + 125 μl of diluent
E	500 μg/ml	325 μl of vial C + 325μl of diluent
F	250 μg/ml	325 μl of vial E + 325μl of diluent
G	125 μg/ml	325 μl of vial F + 325μl of diluent
H	25 μg/ml	100 μl of vial G
I	0 μg/ml	400μl of diluent

Along with the standards, 20 μl of the sample was loaded onto a 96- well plate. The samples were analysed in duplicates. Working reagents were prepared in a 50:1 (Reagent A:B) ratio adding 200 μl of the mixture per well. The plate was incubated for 30 min at 37°C and protein concentration was determined from the measurement of the absorbance at 562 nm on a POLARstar OPTIMA Multifunction Microplate Reader (BMG LabTech, UK).

2.6.3 PathwayProfiler^{TM}

Pathwayprofiler^{TM}is used to detect changes in the phosphorylated status of a number of proteins in cell lysates by using the Luminex^{TM}x-MAP^{TM}system. This assay has been developed as an alternative to Western Blotting and immunoprecipitation procedures. Cell lysates from infected- and non-infected-BMMθs was extracted at the specified times and protein concentration was determined as described in Sections 2.6.1 and 2.6.2, respectively. Lysates were then analysed in the Pathwayprofiler^{TM}by the Upstate team based in Dundee, UK. Analysis of the assay was carried out by comparing the median fluorescent value (MFI) provided by Upstate (Dundee, UK).

2.6.4 SDS-PAGE and Western Blotting

Equal amounts of proteins were mixed with 2X Laemmli Sample Buffer, containing 10% of DTT and loaded onto 10% SDS-PAGE gels, along with 6 μl of molecular weight markers (Prestained Protein Marker Broad Range, New England Bioloabs, UK). The gel was run for 45 min approximately at 200 V constant on a BioRad PowerPac 200 Power Supply. Filter papers (BioRad) were soaked in transfer buffer along with fiber pads. Polyvinylidene difluoride (PVDF) membranes were cut to the dimension of the gel (maximum size 7.5 cm x 10 cm) and soaked in methanol for 10 seconds, washed in distilled water for 5 seconds and soaked in transfer buffer for a couple of minutes. Once the electrophoresis was finished the gel cassette was dissasembled and foot and wells were cut. With the help of a filter paper, the gel was separated from the glass and placed on the transfer sandwich, on the top of the PVDF membrane. The gel sandwich was placed in the TransBlot module along with the cooling unit filled with cold

water. The tank was then filled with transfer buffer and run for 1 hour at 100 V/constant. When transfer was finished, the cassette was carefully dissasembled and the PVDF membranes were blocked with 20 ml of blocking buffer at room temperature with gentle shaking on a Belly Dancer (Stovall Life Sciences, USA). The membrane was then washed 3 times for 15 min with washing buffer. The primary antibody was diluted into the primary antibody dilution buffer. The membrane was incubated with the primary antibody overnight at 4°C with gentle rocking. After primary antibody incubation membranes were again washed in washing buffer and then incubated with the secondary antibody, diluted in blocking solution for 1 hour at room temperature. Membrane was washed again and overlaid onto mix of detection reagents (ECL Plus Western Blotting Detection Reagents, Amersham Biosciences, UK) for 5 min. The membrane was then exposed to the VersaDoc imaging system 4000. Densitometric analysis of the blots was performed by Quantity One software 4.5.0.

Table 2.3: Primary Antibodies

Antibody	Species	Dilution	Supplier
Phospho-Erk1/2	Mouse	1:1000	Sigma
Erk1/2	Rabbit	1:1000	Cell Signalling
Phospho-p38	Mouse	1:1000	Cell Signalling
p38	Rabbit	1:1000	Cell Signalling
Phospho-SAPK/JNK	Rabbit	1:1000	Cell Signalling
SAPK/JNK	Rabbit	1:1000	Cell Signalling
β-Actin	Rabbit	1:1000	Cell Signalling
CROMA101	Mouse	1:1000	*
CROMA103	Mouse	1:1000	*

* CROMA101 and CROMA103 antibodies were kindly donated by Prof. Stipan Jonjinc (Riejka University, Croatia)

Table 2.4: Secondary Antibodies

Conjugate	Antibody	Species	Dilution	Supplier
HRP-linked	mouse IgG	Horse	1:2000	Cell Signalling
HRP-linked	rabbit IgG	Goat	1:2000	Cell Signalling

2.7 Cytokine measurement

2.7.1 Flow cytometry

Cytokine levels in cell culture supernantants were detected by Cytometric Bead Array mouse inflammation kit (BD Biosciences, UK), and flow cytometry analysis. This kit allows quantification of Interleukin-6 (IL-6), Interleukin-10 (IL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Interferon-γ (IFNγ), Tumor Necrosis Factor (TNF), and Interleukin-12p70 (IL-12p70) protein levels in a single sample. Cytokines can be detected in a range of 20-5000 pg/ml. The procedure was performed following manufacter's instruction. Briefly, protein standards were first prepared by serial dilution. 50μl of Capture Beads, standards and samples were mixed into assay tubes along with the detection reagents. After 2 h incubation at room temperature and avoiding direct light samples were washed in the washing solution and centrifuged at 200 g for 5 min. Analysis was performed using BD CBA Software.

2.7.2 ELISA

Murine TNF levels were also determined from cell culture supernatants using a mouse TNF-α/TNFSF1A DuoSet ELISA Development kit (R&D Systems Europe LTd.) designed to detect TNF in the range of 32.5-2000 pg/ml. ELISA was performed following manufacturer's instruction. Briefly, an ELISA 96-well plate was coated with the capture antibody and left overnight at room temperatuere. The antibody was then washed and the plate was blocked for 1 h at room temperature. A series of standards was prepared and after blocking 100 μl of each standard along with 100 μl of sample was added to the plate. Standards and samples were left for 1 h incubation at room temperature, after which the plate was washed 3 times followed by 1 h incubation at room temperature with the detection antibody. The washing step was repeated and Streptavidin- horseradish peroxidase (HRP)-conjugated solution was added at the recommended working concentration. The plate was incubated in the dark at room temperature for 20 min, washed and 100 μl of substrate solution was added. The plate was then incubated in the dark for 20 min at room temperature. To stop the reaction, stop

solution was added (50 μl). TNF concentration was determined by reading the absorbance at 450 nm in a POLARstar OPTIMA Multifunction Microplate Reader (BMG LabTech, UK).

2.8 General Methods for *in vivo* experiments

2.8.1 Mice and Infection

BALB/c (Charles Rivers, France), C57Bl/6 (B&K, UK) and TNF-/- C57Bl/6 (B&K, UK) mice were housed in the animal facilities at the University of Barcelona or the University of Edinburgh under specific-pathogen-free conditions. All experiments were carried out under appropiate personal and project licenses in accordance with the Home Office and the University of Barcelona and University of Edinburgh regulations. Mice were inoculated with the specified PFU by the intraperitoneal route (i.p). During the course of the different experiments, mice were monitored daily. At designated times mice were killed by cervical dislocation.

2.8.2 Sample processing

Dissection instruments were sterilized before use and between mice. Fur was cleaned with ethanol and an incision was made in the abdomen. Spleen, liver, kidneys, lungs and hearts were removed and placed in 500 μl of PBS. Organs were then weighed and processed as a 10% (wt/vol) tissue homogenate. Homogenates were sonicated (Fisher Scientific) for 10 seconds on ice . A small volume of the homogenate was not sonicated in order to measure cytokine levels. Both samples were washed by centrifugation and kept at -70°C until use. Viral titers were determined by standard plaque assays (Section 2.4.2) and cytokine levels by ELISA (Section 2.7.2).

CHAPTER 3

MCMVdie1 replication in BMMθ

3.1 Introduction

The β-herpesvirus human cytomegalovirus (HCMV) can establish both acute and latent infection, remaining in the host for life. As a human pathogen, it is highly relevant with more than 60% of the population worldwide infected. Clinically HCMV infection varies according to the status of the patient's immune system; infection of immunocompetent individuals is usually asymptomatic whereas a variety of severe pathologies are presented in immunocompromised patients such as those with AIDS or organ transplant recipients. Also, CMV infection in newborn children can result in birth defects such as nervous system maldevelopment, deafness and mortality [Jarvis and Nelson, 2002, Stagno et al., 1986].

The murine cytomegalovirus (MCMV) model provides a useful tool for studying the human virus and its pathogenesis thanks to the ease of handling the virus *in vivo* and its genotypic and biological similarities to HCMV. Similar to HCMV, the murine virus has a dsDNA genome that is sequentially expressed in three phases, immediate early (IE), early (E) and late (L) genes [Keil et al., 1984]. The IE phase is independent on newly synthesized proteins, while the expression of early genes requires control by IE proteins. The last phase of the transcription cascade which starts after viral replication encodes late proteins responsible for the structure of the viral particle. The MCMV IE1 protein has been described as a

trans-activator for cellular and viral gene expression [Gribaudo et al., 1996, Gribaudo et al., 2000, Koszinowski et al., 1986]. However, the role of IE1 in the regulation of gene expression and immune response has not been described. So far the requirement of IE1 for efficient replication of the virus in the natural host has been reported [Ghazal et al., 2005].

Macrophages (Mθs) play an important role in CMV infection as a major site for viral replication and establishment of latency, as well as providing a vehicle for viral dissemination throughout the host [Hanson et al., 1999, Stoddart et al., 1994]. In addition, Mθs constitute one of the principal effectors of innate immunity. Upon activation, Mθs produce a number of cytokines such as TNF, IL1, IL6, IFN$\alpha\beta$, and IL12, which have been reported to be key mediators of the response against MCMV [Orange and Biron, 1996, Orange et al., 1995, Randolph-Habecker et al., 2002]. This cytokine response will recruit other immune cells, such as NK, very well known to mount an antiviral activity by inducing IFNγ [Orange et al., 1995]. These two faces that Mθs seem to have in MCMV infection has been considered as 'paradoxical' [Hanson et al., 1999], since the same cellular population is equally important for viral replication and for viral clearance. However and regardless of this duality, it is clear that Mθs are crucial for MCMV infection.

3.2 Aim of the chapter

MCMV *ie1*-deletion mutant (MCMVdie1) replication *in vitro* in fibroblasts and peritoneal Mθs is similar to the parental and revertant virus [Ghazal et al., 2005], demonstrating that IE1 is dispensable for MCMV replication in these cells. However, the replication of MCMVdie1 in BMMθs has not been described. Considering the importance of Mθs in the immune response against viral infection, the aim of this chapter was to study MCMVdie1's growth kinetics in this cellular population, as well as gain an insight in what the effect of the absence of IE1 would be on BMMθ gene expression upon infection.

3.3 Results

3.3.1 Replication in bone marrow derived macrophages

BMMθs from 10-12 week old male BALB/c mice were cultured for 7 days as described in Section 2.2.6. After one week and prior to infection, maturation of the Mθs was characterized by flow cytometry (Section 2.2.6). Cells were then infected at a multiplicity of infection (MOI) of 1 and 0.2. Previous experience in the laboratory showed that infection of BMMθs with MOIs higher than 1 resulted in high cell death. Therefore at MOI of 1 was used in all experiments of this thesis unless specified. Intracellular and extracellular infectious infectious virus were determined by standard plaque assays as described in Section 2.4.2. Figure 3.1 shows MCMV, MCMVdie1 and MCMVrev growth kinetics after infection at a MOI of 1 and MOI of 0.2 of two independent experiments.

From the graph it can be seen that infection at a MOI of 1 did not result in notable differences in the replication of MCMVdie1 when compared to the parental and revertant virus. However, a slight decrease, not statistically significant, in MCMVdie1 yields was seen at late times of infection. Similar results were found when infections were done at a lower MOI. All together, these results indicate

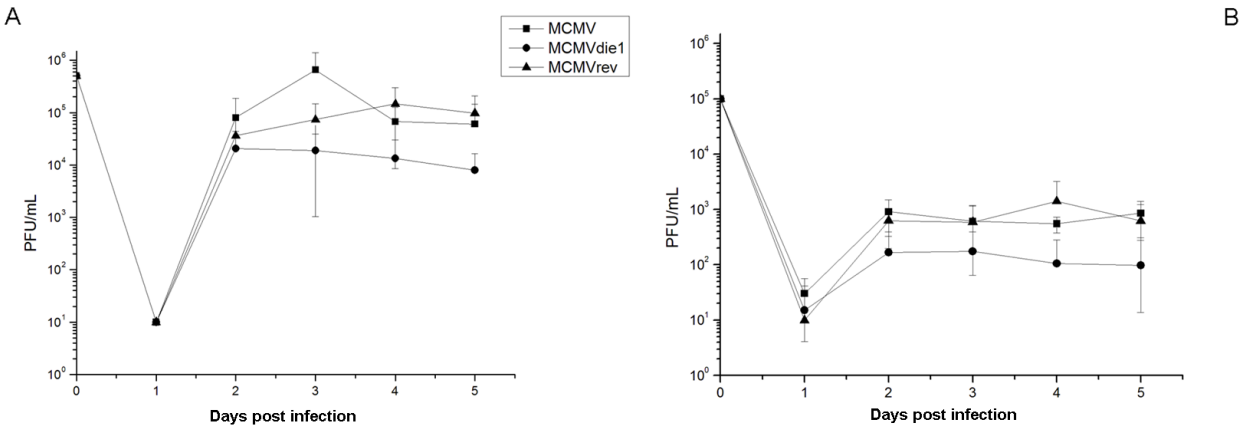

Figure 3.1: Growth curves in infected-bone marrow derived macrophages

Cells were infected with the different viruses at a (A) MOI 1 or (B) MOI 0.2. At indicated times intracellular and extracellular virus were harvested and viral titers were determined by standard plaque assay in MEFs. Each data point represents the average and SD of results of different samples from 2 independent experiments (n=6).

that MCMVdie1 establishes an infection in BMMθs and although the mutant virus replication tended towards lower yields at later times of infection these differences were not significant (t-test, p=0.15 and p=0.52 when comparing MCMV and MCMVrev against MCMVdie1, respectively).

3.3.2 Microarray and data anlysis

Study of viral growth kinetics in BMMθs showed that MCMVdie1 replication tends to be slightly lower than parental or revertant MCMV. Work done by Ghazal and coworkers revealed that the IE1 protein is essential for viral growth in the natural host [Ghazal et al., 2005]. The same study revealed that the absence of IE1 also resulted in an attenuation of MCMVdie1 replication in SCID mice, lacking T and B cells and relying on the innate immune response to control the infection. The fact that the trend towards lower viral yields is not seen in fibroblasts but in BMMθs raised the possibility of that the absence of IE1 might have an effect on cellular mechanisms involved in viral control, as seen *in vivo*. Therefore, to gain an insight in the effect of MCMVdie1 infection of BMMθs a comparative study on MCMVdie1- and MCMVrev-induced host gene expression was performed using microarray technology. It is known that MCMV induces changes in host gene expression when compared to mock-infected samples, therefore this pilot study was only focused on MCMVdie1 and MCMVrev infection at early times post infection, in order to test the effect of the absence of *ie1*. Considering the limitations of the study, as well as the small numer of samples, the observations from this experiment were not considered as conclusive. Instead they have been used as a guide of what might be happening after infection, as well as to provide an example of how microarray data could be analysed.

BMMθs from male BALB/c mice were infected with either MCMVdie1 (n=4) or MCMVrev (n=3) at a MOI of 1 for 10 h. After the indicated time, total RNA was isolated as described in Chapter 2. As indicated in Section 8.1.2, RNA quality was confirmed on RNA 6000 Nanochips in the Agilent 2100 Bioanalyzer. Only high quality RNA preparations, indicated by the RNA integrity number (RIN>7.5), were considered for microarray screening in the the Affymetrix Mouse 430v2

array. This work was done in collaboration with S. Burgess and P. Lacaze (Edinburgh University).

A total number of 22690 probesets from the Affymetrix Mouse 430v2 array were imported into Partek® Genomic Suite™and normalized using the RMA and quantile methods. An expression level filter was applied to the data for probesets with absolute expression values of above 100 in two or more of the seven arrays. 1455 filtered probesets were obtained and analyzed for differential expression between the MCMVdie1- and MCMVrev-infected samples using ANOVA. 532 probesets were found to be differentially expressed to a statistically significant level using a p value (<0.05) and fold change (>1.2) cut off. In general, significant p values are more important than the magnitude of the effect. However, in practice, a combination of statistically significant p value and fold change are commonly used. Although this cut off is not very stringent it was used in order to obtain a reasonable number of genes to work with in the analysis. In any case, the results obtained from microarray always require independent validation. The differentially expressed probesets were then used to identify genes whose expression was likely to be altered after MCMVdie1 infection. The complete list of these genes can be found in Appendix B.

From the data analysis, it was seen that, although the vast majority of the genes were not affected in the absence of *ie1*, approximately 550 genes showed differential expression. Strikingly, according to gene ontology analysis performed with DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp), nearly 33% were relevant for the immune response triggered upon infection (see Figure 3.2). In order to gain a better understanding of the biological processes affected by these genes, a more extensive study was performed by pathway analysis.

3.3.3 Pathway Analysis

One of the obstacles of the microarray technology is the difficulty of analysing the amount of data generated. However, there are several tools which help to understand microarray data. In this thesis, Ingenuity Pathway Analysis (IPA) (Ingenuity Systems®, www.ingenuity.com) tool was used in order to identify the

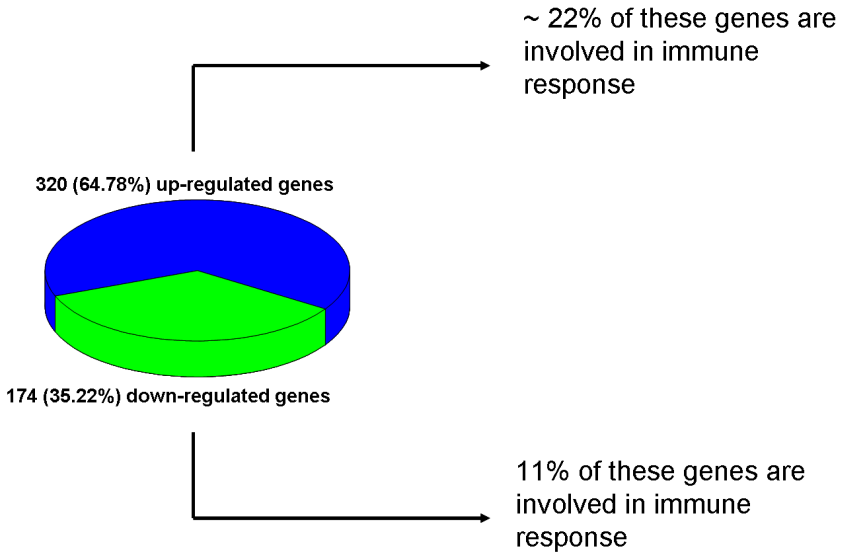

Figure 3.2: General description of microarray data analysis

BMMθs were infected with the MCMVdie1 and MCMVrev at a MOI 1 (n=4 and n=3, respectively) for 10 hours. RNA was extracted and microarray was performed. Approximately 550 genes were differentially expressed, ~65% of which were upregulated and 35% were downregulated. An important percentage of those genes were involved in the immune response.

pathways and biological processes which were most affected after MCMVdie1 infection. Therefore, genes accession numbers from the 532 genes with differential expression along with the fold change were imported to IPA. This application has also an extense manual-curated database, known as Ingenuity Pathways Knowledge Base, derived from an important number of journals and text books, which enables the identification of those genes of interest. The analysis of the gene list was based on (1) the most relevant genetic networks identified and, (2) the canonical pathways affected after infection.

Networks of gene interaction after viral infection

Those identified genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base and interconnected algorithmically by using the Network Generation Algorithm [Calvano et al., 2005]. Networks are also scored based on the number of molecules included. Thus, the higher the score of a network the lower probability of finding molecules by chance. This score is calculated with the right-tailed Fisher's Exact test. Furthermore, IPA also carries out a functional analysis of a network,

identifying the biological functions and/or diseases that were most significant to the genes in the network. Fisher's exact test was also used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

The top 5 genetic networks identified by the Ingenuity Pathways Knowledge Base are summarized in Table 3.1. The first 3 networks will be described in more detail in this section, since they showed the highest scores and they have been associated with functions relevant for viral infections, including cell death. It has to be noticed that several genes appeared in more than one network which indicates that they are associated with several biological processes.

CELL DEATH. As can be seen in Figure 3.3, this first genetic network represents genes affected after MCMVdie1 infection and mainly involved in the regulation of gene transcription and induction of apoptosis. Regarding the regulation of DNA transcription different genes were found, including histone cluster 1 (HIST1H1C) and nucleosome assemble protein 1 (NAP1L1). The expression of these genes was affected after MCMVdie1 infection, as well as MED21, which is involved in the regulation of the RNA polymerase II, HSPA9, Daxx, known transcriptional repressor, and p53. This last one seems so have a central role in this network, since the vast majority of the genes interact with p53. Other genes involved in the aminoacid metabolism are also represented in this network such as histidine decarboxylase (HDC). All together, it can be seen that the expression of genes involved in transcription are affected after MCMVdie1 infection. Indeed, as has been already introduced IE1 protein acts as a *trans*-activation of cell gene expression, so it is not surprising that these genes appear altered in the absence of IE1.

Kinases like Death-associated protein kinase 1 (DAPK1) and Homeodomain interacting protein kinase 3 (HIPK3) were also affected after MCMVdie1 infection. DAPK1 has also been reported to mediate IFNγ-induced apoptosis [Inbal et al., 2000], whereas HIPK3 has been shown to phosphorylate the adaptor protein FADD [Rochat-Steiner et al., 2000], modulating an alternative apoptosis pathway involving Fas ligand-induced JNK activation. These results might

Table 3.1: Genetic networks affected by MCMVdie1 infection

Network	Genes in Ingenuity network	Associated Pathways	Score†
1	**AKAP12**, Calpain, **CARHSP1**, **CBX4**,**CSDE1**, Ctbp, **DAPK1**, **DAXX**, E2f, **ENG**, **GLRX**, Glutathione peroxidase, **HDC**, **HIPK3**, **HIST1H1C**, **HSPA9**, **MAFB**, **MED21**, **MED31**, **MT2A**, **MTDH**, **NAP1L1**, **P4HA1**, **PHC2**, **PRNP**, **PTPN12**, **RBL1**, **RFC3**, **SARS**, **TGFBI**, Top2, **TP53**, **TPX2**, **UBE2I**, **ZYX**	Cell Death, Neurological Disease, Cardiovascular System Development and Function	49
2	**ABCA1**, **ALDH1B1**, **ANXA6**, **CASP2**, **CCL8**, **CD36**, **CDKN2D**, **CFB**, **CST7**, **ETV6**, **FABP3**, **GLRX3**, **GMFB**, **GOLGA3**, Itgal-Itgb2, LDL, **LIPA**, **LIPE**, **LIPG**, **LPL**, **LRRC8C**, **MTSS1**, N-cor, NCOR-LXR-Oxysterol-RXR-9 cis RA, NFκB, PPARα-RXRα, **RHOH**, **RIPK3**, Rxr, **SDC4**, **SLC12A7**, **STK10**, **STK40**, **TNFSF13**, **USP18**	Lipid Metabolism, Small Molecule Biochemistry, Cardiovascular Disease	44
3	**AK2**, **ALCAM**, ALT, **AMPD3**, Angiotensin II receptor type 1, **BTG3**, **BTRC**, Caspase 3/7, **CCRN4L**, **CD38**, **CH25H**, **CNP**, **EHD1**, **EIF2AK2**, **HIPK1**, Iκb, IKK, IL1/IL6/TNF, **IL21R**, NF-κB, **NFκB2**, NfκB-RelA, **NFκBIE**, **OASL**, **POU3F1**, **QKI**, **RELB**, **RGS14**, **RHOV**, **TNF**, **TNFAIP2**, **TNFSF10**, **TNIP1**, **TPST1**, Ube3	Viral Function, Cell Death, Connective Tissue Disorders	39
4	**ARHGEF3**, C8, **CHAC1**, **CXCL9**, **CXCL10**, Cyclooxygenase, **FPRL1**, GOT, **ICAM1**, Ifnγ, IL1, **IL18**, IL-1R, **IL1B**, **IL1RN**, JINK1/2, **MX1**, **NKIRAS1**, Nos, Notch, **PDE4B**, **PELI1**, **PSCD1**, **PSCDBP**, **PYCARD**, Rar, **RHOB**, Saa, **SAA2**, Secretaseγ, **SOCS3**, Sod, **SOD2**, **TLR3**, **UGCG**	Connective Tissue Disorders, Inflammatory Disease, Skeletal and Muscular Disorders	31
5	**ADAM17**, Cbp/p300, **CCL3**, **CSF1**, **CSF3R**, Fcgr3, **FCGR3A**, **HLX**, **IFITM1**, Ige, **IL6**, **IL10**, **IL15RA**, **IL4R**, **IRF1**, JAK, MHC Class II, Mmp, Pdgf, **PDGFC**, Pdgfra-Pdgfrb, PLC γ, SOCS, **SOCS1**, **SOCS6**, STAT, **STAT2**, STAT5a/b, **STAT5B**, SYK/ZAP, **TDG**, **TIAM1**, Timp, **TIMP1**, **TLR2**	Cellular Development, Cellular Growth and Proliferation	29

Genes in bold are those included in the microarray analysis list. Other genes were not in the list but included in the network for known interaction. † Score represents the probability of finding molecules in the network only by chance. The higher the score the lower probability.

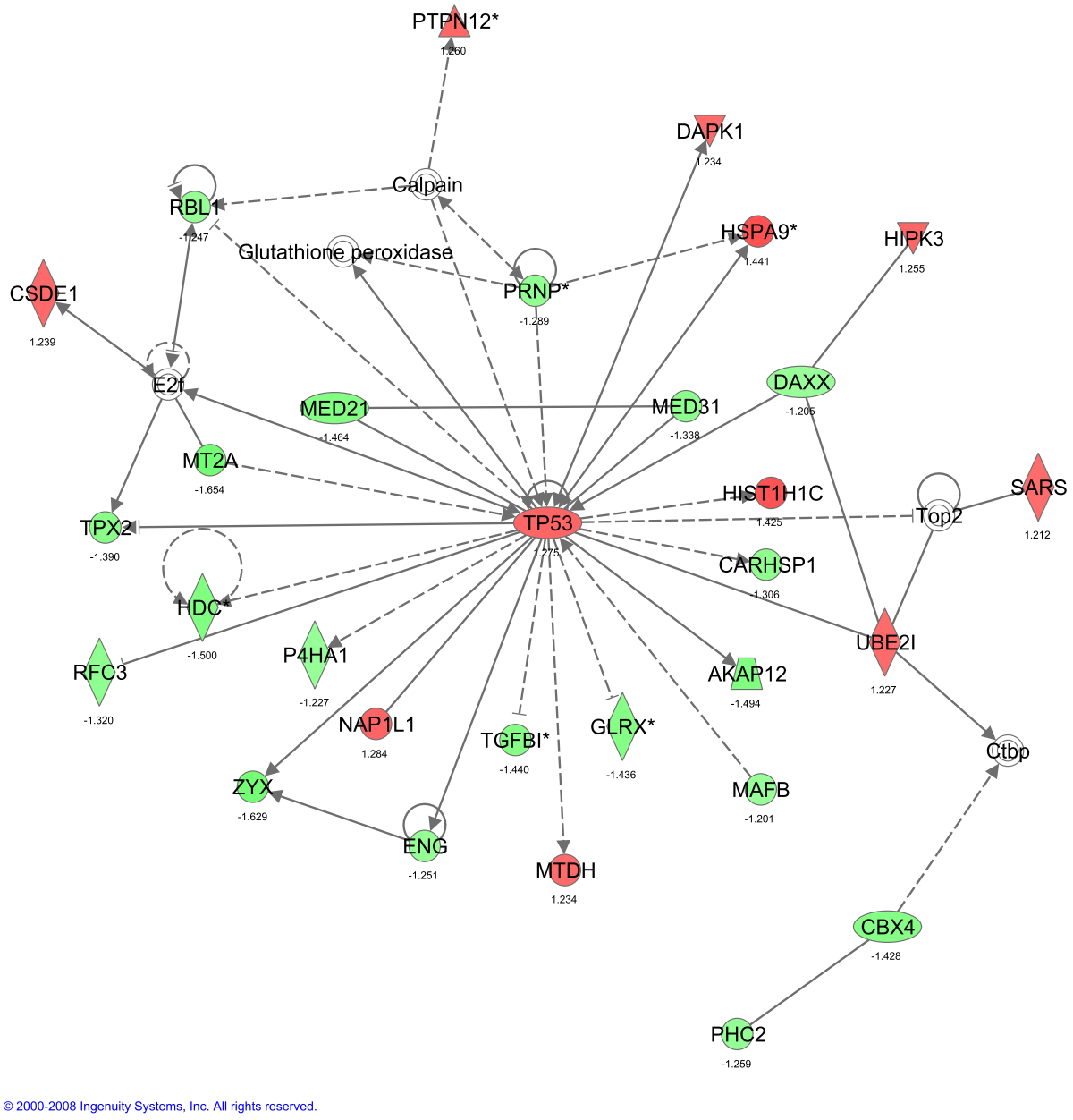

Figure 3.3: Genetic Network associated with Cell Death.

point to a regulation of the activation of apoptosis after MCMV infection, however qRT-PCR validation would be required to further investigate this potential mechanism.

LIPID METABOLISM. Little is known about the role of lipids in infection of MCMV, however there are some reports showing that Hepatitis C virus (HCV) interacts with lipid aggregates known as 'lipid rafts' in order to start the viral

replication cycle [Aizaki et al., 2004]. Human CMV replication in endothelial cells has also been shown to require the activity of Hydroxymethyl-Glutaryl Coenzyme A (HGM-CoA) reductase, known to be part of the metabolic pathway that produces cholesterol. When this enzyme was blocked by cholesterol lowering drugs it was seen that the expression of viral immediate early and late antigens was inhibited [Potena et al., 2004]. Interestingly, microarray analysis pointed to an alteration of the expression of several genes involved in the cellular lipid metabolism after MCMVdie1 infection. These genes have been mapped as shown in Figure 3.4. Within the genes differentially expressed it could be found lipoprotein lipase (LPL), esterases (LIPA), binding proteins (FABP3) and lipid transporters (ABCA1).

Intermediate compounds originated from the lipid metabolism are also known to have an integral role in signalling pathways, such as Ras family of small GTPases. Indeed, members of this family, including Rho and Ras, are localized in the plasma membrane thanks to posttranslational lipid modification of the proteins. Alteration of this lipid portion of the protein would result in a change in cellular localization as well as biological activity [Wennerberg et al., 2005]. In addition, these molecules are involved in the activation of the immune response by leading to the activation of gene expression [Monick et al., 2003]. Moreover, evidences on the anti-inflammatory role of LPL has been reported [Ziouzenkova et al., 2003].

There are also experimental results showing a link between lipids and regulation of apoptosis. It has been reported that LPL can induce apoptosis in human cells by promoting caspase activation [Napoli et al., 2000]. Indeed, this genetic network revealed an interaction between LDL and caspase-2. GOLGA3, also known as Golgin 160, is a caspase-2 substrate which transduces pro-apoptotic signals [Mancini et al., 2000].

Overall, this genetic network raises the possibility that MCMV targets lipid metabolism by regulating the expression of several enzymes. The alteration of this metabolism might also affect other cellular responses, including the immune response. Therefore, it would be of interest to investigate further the interaction

between MCMV and the lipid metabolism, and how that would affect either viral infection and cellular responses to infection.

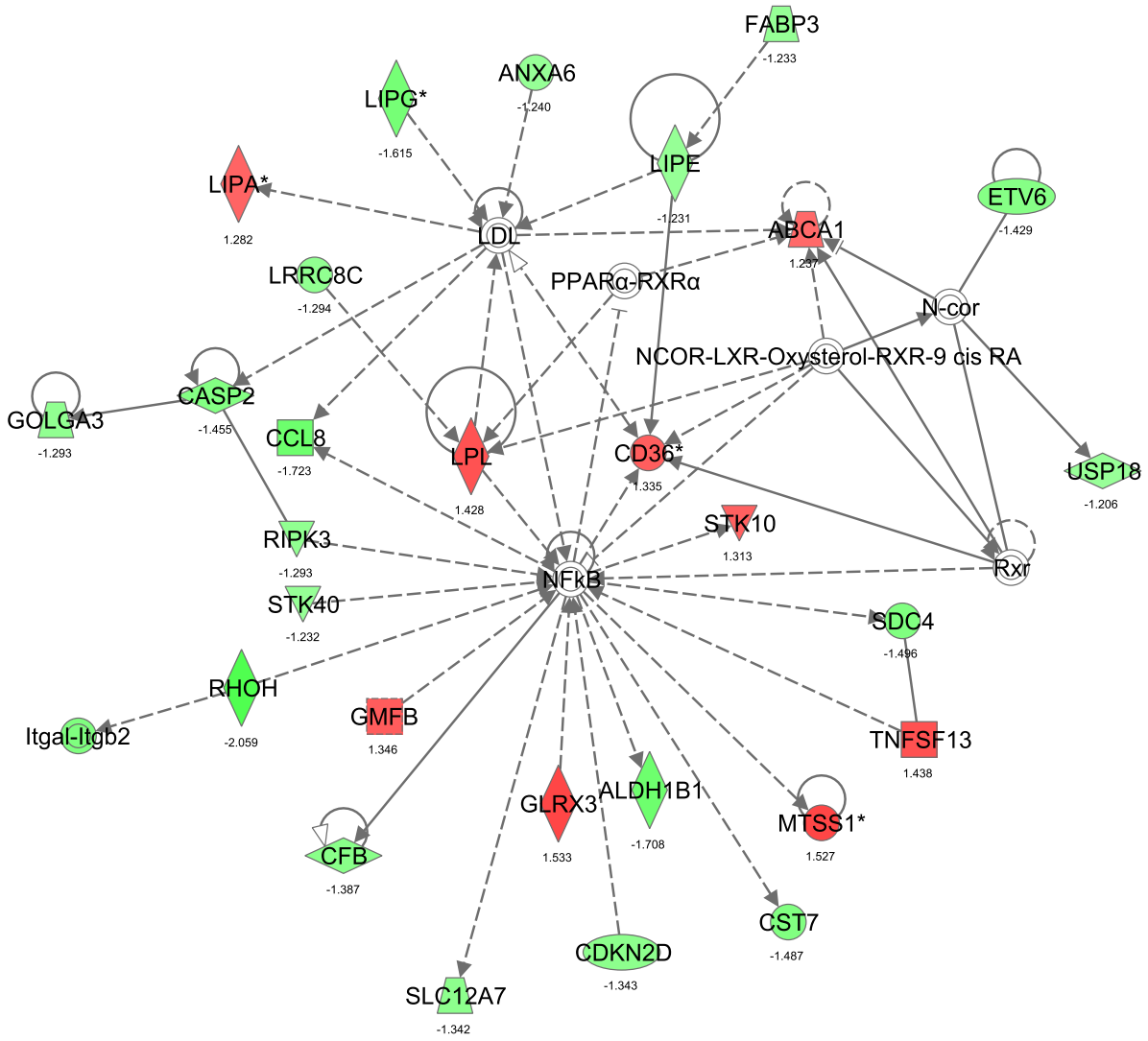

Figure 3.4: Genetic Network associated with Lipid metabolism.

VIRAL FUNCTION. This genetic network includes those genes which are involved in biological process happening after viral infection, in particular with viral replication and apoptosis. TNF appears to have a central role in this specific network (see Figure 3.5), as well as members of the canonical and alternative NFκB pathway, involved in the innate immune response and development of lymphoid organs. Both human and murine CMV induce activation of

NFκB. MCMV IE1 has been shown to induce the expression of NFκB subunits p65 and p50/p105 [Gribaudo et al., 1996] and human IE1 induces expression of *relb* [Wang and Sonenshein, 2005]. Another study has shown that human IE1 leads the activation of the MIE region by *trans*-activating NFκB sites in human fibroblasts [Sambucetti et al., 1989]. However, work done by different groups have also demonstrated that this factor is dispensable for HCMV and MCMV replication [Benedict et al., 2004, Gustems et al., 2006]. In addition, inhibition of NFκB activation in human cell cultures reduced the antiviral effect induced by TNF and IFNγ [Eickhoff and Cotten, 2005], and very likely many other responses mediated by this factor. However, microarray data could indicate a differential expression of both *relb* and *p100* in the absence of IE1. Results indicated that TNFAIP3 interaction protein 1 (TNIP1), also known as NAF-1 or ABIN-1, is also altered after MCMVdie1 infection. NAF-1 is induced by TNF in human cells [Gallagher et al., 2003] and it negatively regulates NFκB-dependent gene expression, by interfering with IKK complex [Mauro et al., 2006]. Other groups have revealed that this protein can also act as a attenuator of the Erk1/2 signalling pathway [Zhang et al., 2002]. Therefore, it might be that NAF-1 could be acting as a negative regulator of TNF-induced signalling, and the absence of IE1 affects NAF-1 expression via TNF.

Several TNF family members are also part of this network, such as TNFSF10 (also known as TRAIL) and TNFAIP2, both of which induce apoptosis [Aggarwal, 2003]. The expression of the mitochondrial adenylate kinase 2 (AK2) also appeared altered. This kinase initiates the activation of the FADD/caspase-10 pathway [Lee et al., 2007] and, consequently, the activation of caspase- 9 and -3. These data may suggest that the absence of IE1 affects the expression of AK2, raising a possible strategy to block the AK2/FADD/caspase-10 induced apoptosis.

Strikingly, in the genetic networks described above, expression of several other genes has been shown to be affected in the absence of *ie1*, and these also

play a role in the regulation of apoptosis, such as DAPK1 and HIPK3. Therefore, this analysis might indicate that apoptosis is a key cellular response for the control of MCMV infection which might involve the IE1 protein.

In this regard it is noteworthy that the presence of the homeodomain interacting protein kinase 1 (HIPK1). TNF has been shown to induce translocation of HIPK1 [Li et al., 2005]. When it is in the nucleus, HIPK1 interacts with Daxx and regulates transcription. HIPK1 relocalized Daxx from ND10 to chromatin, where Daxx acts as a repressor. However, HIPK1-induced phosphorylation of Daxx also regulates Daxx repressor capacity [Ecsedy et al., 2003]. As has been introduced, Daxx is part of the ND10 aggregates, along with PML and Sp100. The precise function of these bodies in MCMV infection has not been totally elucidated, however it is known that both human and murine CMV disrupt these protein complexes [Tang and Maul, 2003, Lee et al., 2004]. Unlike MCMV, HCMV induces the degradation of Daxx [Saffert and Kalejta, 2006]. Therefore, it might be possible that MCMV has evolved other mechanisms to counteract Daxx-induced repression by interfering with its localization and activity, rather than inducing its degradation. Although *ie1*-deletion mutant did not disrupt ND10 bodies early after infection it did not affect viral replication, since MCMVdie1 replicates as wild type in tissue culture [Ghazal et al., 2005]. However, this function is not shared by other viruses such as HCMV and HSV because they show defective replication in the absence of their respective IE1 proteins [Greaves and Mocarski, 1998, Everett et al., 2004]. Nevertheless, this possible function of IE1 where it might regulate Daxx function by interfering with its phosphorylation status might be the alternative to the degradation seen for human CMV. It has been suggested the role of these molecules in antiviral defense, since they are induced by IFN [Gongora et al., 2001, Grotzinger et al., 1996]. IFN-induced Daxx is also known to act as a transcriptional repressor. Microarray analysis pointed to the differential expression of Daxx and HIPK1. However, why these proteins might be targeted by MCMV requires further investigation.

The interaction between TNF and cholesterol 25-hydroxylase (CH25H) is also of great interest. Microarray data might indicate that TNF expression is altered

in the absence of IE1. The previous section pointed to the alteration of several genes involved in the regulation of the lipid metabolism. This enzyme is known to transform cholesterol into 25-hydroxycholesterol, which has been reported to negatively regulate the LPS-induced TNF production in Mθs [Englund et al., 2001]. Moreover, it has been shown that cholesterol plays a role in the regulation of TNF production. The mitogen activated protein kinase Erk1/2 plays a role in the induction of *tnf* gene expression in Mθs [Shi et al., 2002]. As a protein kinase, Erk1/2 activity is regulated by a specific kinase and phosphatase. It has been described that cholesterol is essential for the negative regulation of the phosphatase complex [Wang et al., 2005], which ultimately might affect the activation of gene expression, including *tnf*. Microarray data might indicate that TNF expression is regulated after MCMVdie1 infection. One possible explanation might be that the apparent regulation of *tnf* expression might be partially cholesterol-dependent. However, further validation has to be done in order to investigate these speculations.

Canonical pathways affected by MCMVdie1 infection

In the previous section it has been described how the genes differentially expressed after MCMVdie1 infection interact with each other in different genetic networks, giving an initial idea of the biological processes which might be targeted. The Ingenuity Pathway Analysis also includes the study of the different canonical pathways which have been altered after MCMVdie1 infection. As for the genetic networks, these pathways have also been curated and drawn from information found in the literature. The significance of the association between the data set and the canonical pathway was measured by Fischer's exact test, giving a p-value which determines the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. Below, a number of pathways are described not only for their relevance on the control of infection, but also for the significance with which they were scored in the analysis.

IL10 AND IL6 SIGNALLING PATHWAYS. The production of IL10 after MCMV infection of primary Mθs has been reported. This anti-inflammatory cytokine

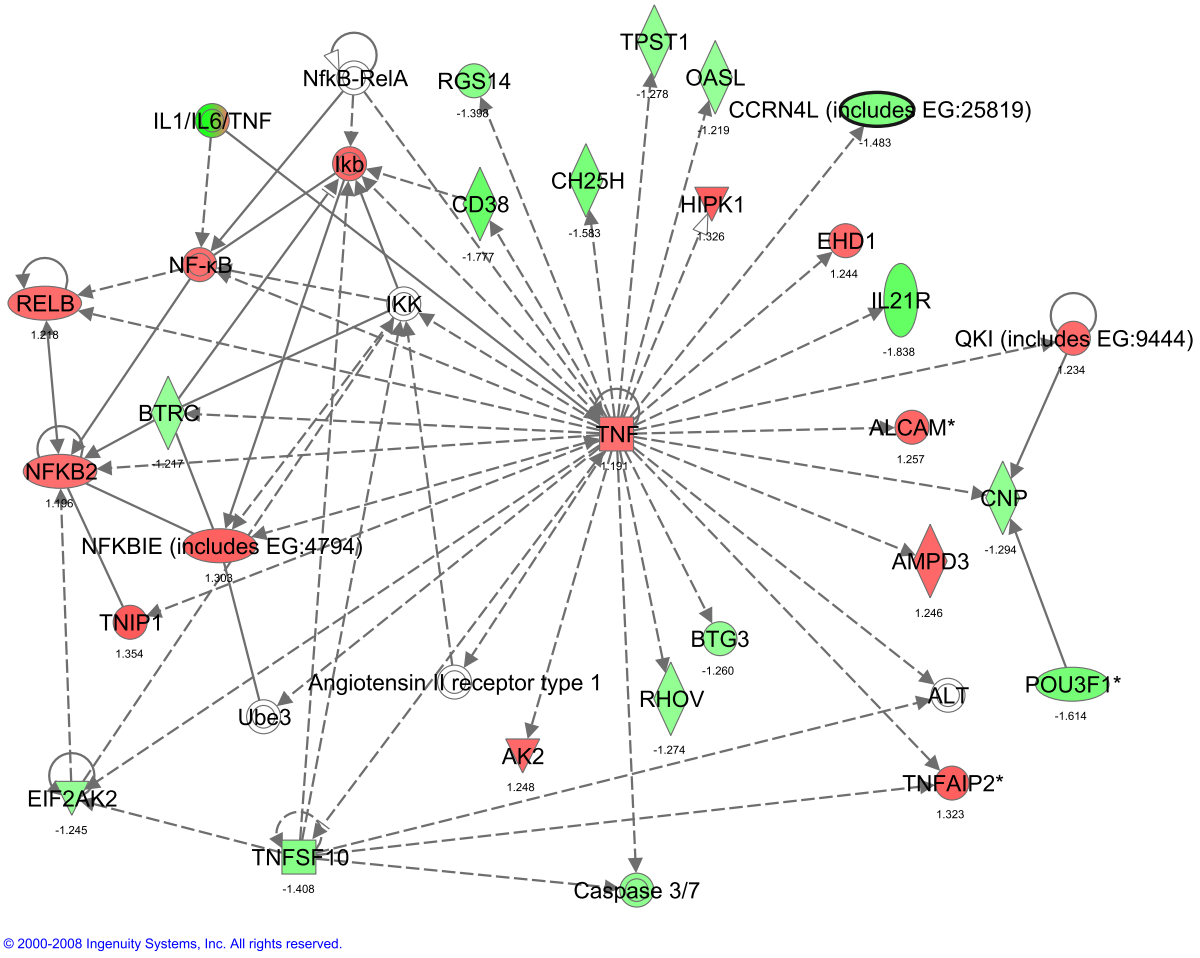

Figure 3.5: Genetic Network associated with Viral function.

appeared to be involved in the MCMV-induced inhibition of the MHC class II molecules in infected cells [Redpath et al., 1999]. Moreover, it has been shown that IL10 is responsible for the control of MCMV in the salivary glands, suggesting a mechanism involving the balance between IL10 and IFNγ production [Humphreys et al., 2007]. However, very recently it has also been demonstrated that IL10 has a pivotal role during MCMV infection, since MCMV-infected mice with severe disease were found in the absence of IL10 because of the abundant and persistent production of other cytokines including TNF, IFNγ and IL6 [Oakley et al., 2008]. Pathway analysis from the microarray data might indicate that the absence of IE1 has a strong effect on IL10 expression early after infection

of BMMθs, as well as other molecules involved in the IL10 signalling pathway (Figure 3.6).

Another signalling pathway altered in the absence of IE1 was IL6 (Figure 3.7). After MCMV infection IL6 levels can be detected *in vitro* and *in vivo*. Moreover, Ruzek and coworkers also reported the association between IL6 and the production of glucocorticoids in the natural host [Ruzek et al., 1997], which has been shown to inhibit immune response [Kunicka et al., 1993]. IL6 can also induce other immune cells by inducing MCP1 [Rott et al., 2003] as well as the activation and expansion of B cells [Barton, 1997]. There is evidence demonstrating that human CMV induces *il6* expression by its IE1 [Geist and Dai, 1996], but this analysis pointed to a possible regulation of IL6 by MCMV. Figure 3.7 shows that by interfering with the expression of *il6* the signalling cascade induced by this cytokine might also be altered, as well as the induced biological activities.

IFN SIGNALLING PATHWAYS. The role that IFN plays in the control of MCMV infection has been extensively studied, as has been already introduced in this thesis. Pathway analysis identified this as one of the most affected by MCMVdie1 infection. Microarray data showed that IFN-induced genes *ifitm1, irf1* and *mx1*, were up-regulated by the IE1 protein (see Figure 3.8). Besides, interferon induced transmembrane protein 2 and 6 (*ifitm2*, *ifitm6*) expression also was altered by MCMVdie1 infection (see complete gene list from Appendix B). Moreover, these results are consistent with the work recently published by Busche and coworkers, where they show that the induction of IFN response elements were decreased in the absence of IE1 [Busche et al., 2008].

It has been reported that human IE1 protein targets type I IFN-induced gene expression by interfering with the complex STAT1-STAT2-IRF9, preventing the activation of transcription [Paulus et al., 2006]. However, this microarray study along with the recently published work might indicate that, unlike the human virus, MCMV IE1 induces this specific response rather than blocking it.

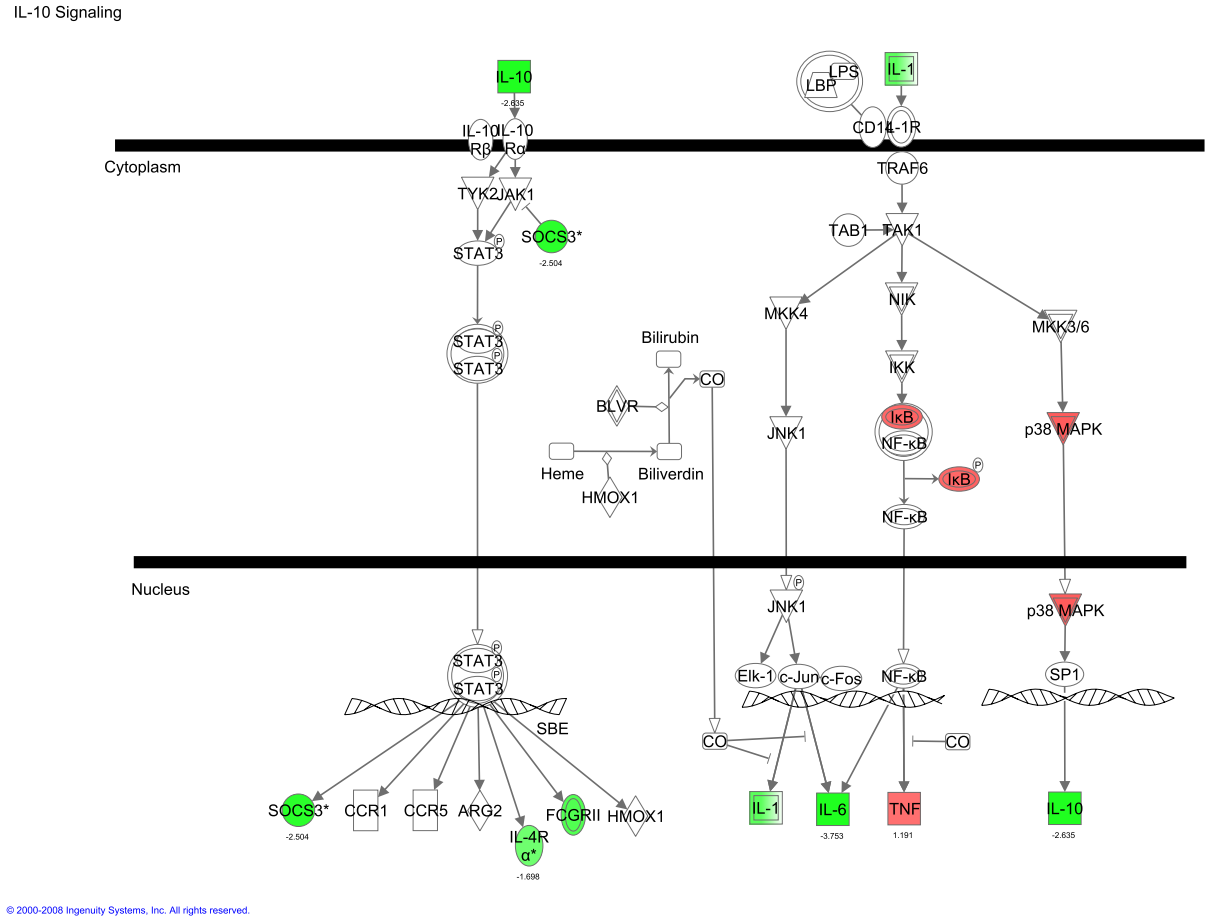

Figure 3.6: IL10 signalling pathway

Analysis of the IL10 signalling pathway by IPA showed genes differentially expressed from the microarray data. Green and red color denotes genes which expression is down- and up-regulated, respectively.

3.4 Conclusions

MCMV IE1 has been shown to be dispensable for viral replication in different types of cells *in vitro*, however, the role of this viral protein in viral replication in primary Mθs has not been studied. Mθs are key players in both immune response against viruses and other pathogens and MCMV infection. Therefore, it was of interest to study viral infection in this particular cellular population. BMMθs were infected at different MOIs with MCMVdie1, parental MCMV and a revertant. Viral growth kinetics revealed that, there was a marginal decrease in MCMV replication was seen in the absence of *ie1*, although not statistically

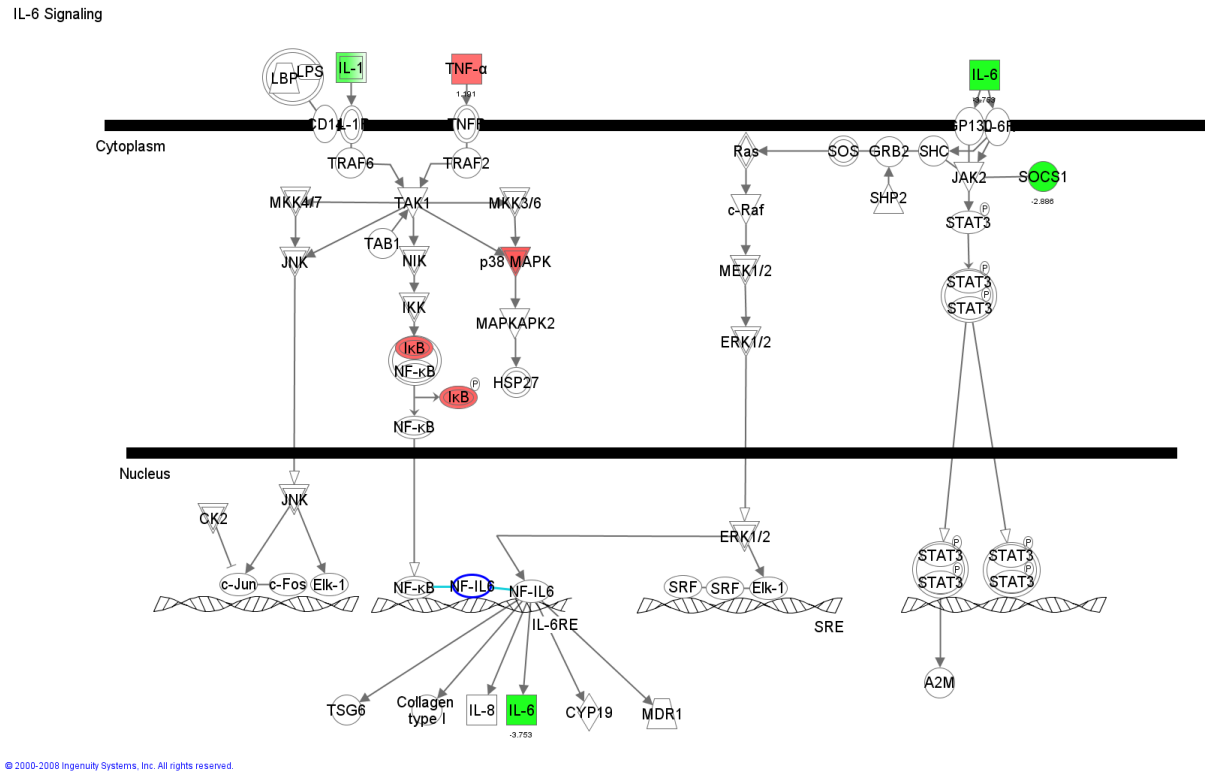

Figure 3.7: IL6 signalling pathway

Analysis of the IL6 signalling pathway by IPA showed genes differentially expressed from the microarray data. Green and red color denotes genes which expression is down- and up-regulated, respectively.

significant. The reason of trend towards lower replication is not known, however there are several factors which could explain this difference. For instance, the virus could have adapted to replicate in fibroblasts and does not take into consideration factors to which the virus could be exposed in BMMθs. It is also known that Mθs are semipermisive cells which could also affect viral replication. Considering that the phenotype of MCMVdie1 seen *in vivo* it might be that BMMθs simulate the closest environment to in vivo conditions, including factors involved in the immune response which are not present in fibroblasts, and all together could explain the trend towards lower viral yields.

When gene expression was studied after infection of BMMθs it was seen that, although the vast majority of genes had a similar expression after Mθs infection with MCMVdie1 and MCMVrev, approximately 550 genes were differentially

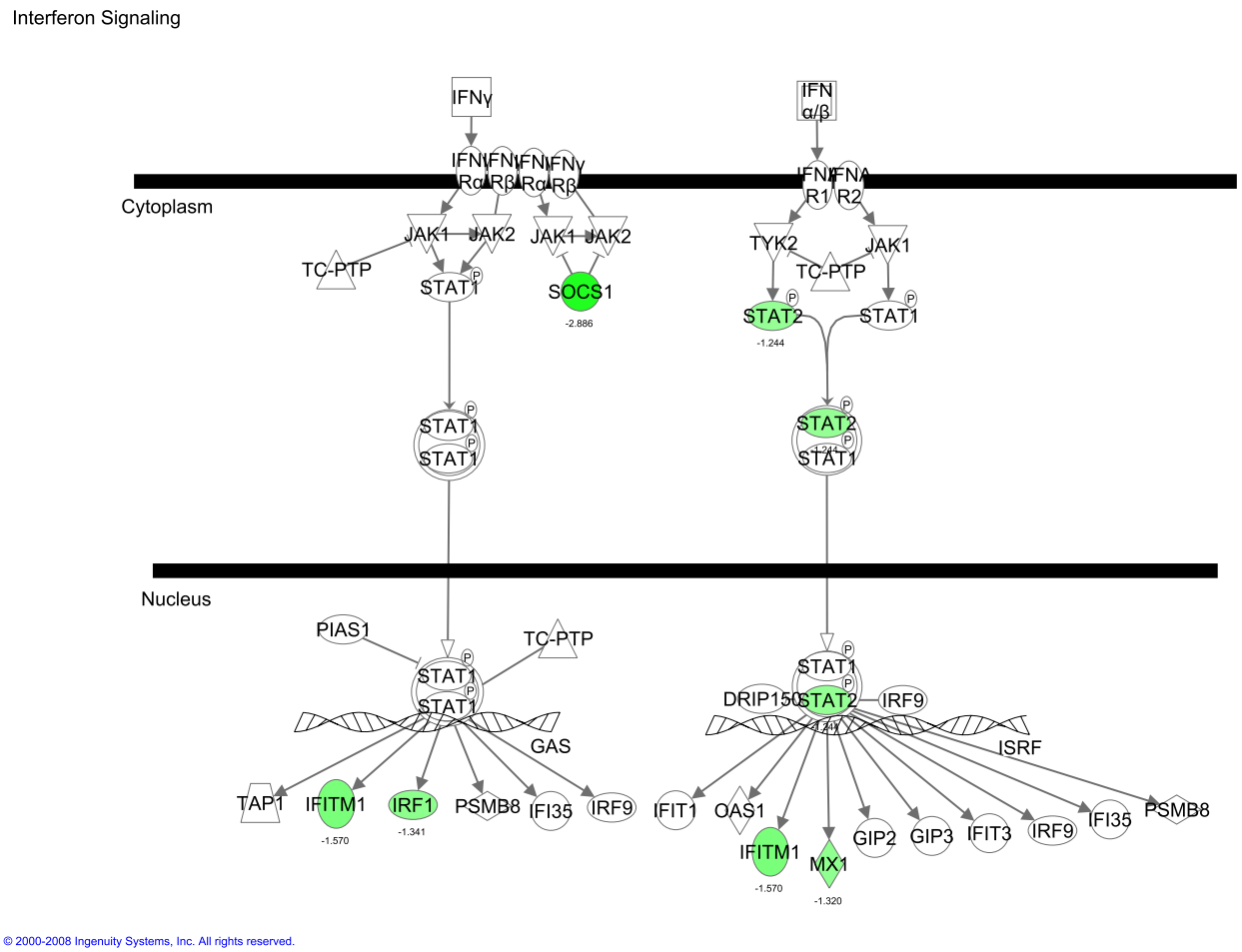

Figure 3.8: IFN signalling pathway

Analysis of the IFN signalling pathway by IPA showed genes differentially expressed from the microarray data. Green and red color denotes genes which expression is down- and up-regulated, respectively.

expressed in the absence of IE1. Of these, ∼33% are genes involved in the regulation of the immune response, including cytokines. In order to gain an insight of the biological processes in which those genes are involved, the Ingenuity Pathway Analysis application was used. It has to be noticed that this tool is used with the goal of obtaining a broad idea of the effect of the viral infection on cell host expression and validation of the data is always required in order to make firm conclusions. The use of the *ie1*-deletion mutant has shown that IE1 might to be involved not only in the expression of genes required for viral replication, but

also in the regulation of the immune response triggered by BMMθs upon infection. This study has helped to point to potential targets for viral regulation, and further validation and work has to be done to completely elucidate the role that IE1 might have in the regulation of immune pathways in the cell host.

IL10, IL6, TNF, IFN and NFκB signalling pathways are affected after viral replication, and the absence of IE1 resulted in the differential expression of several members of these pathways. Because all these signalling cascades interact with each other, viral interference with a single gene might have an effect on many others. For instance, by regulating the expression of IκBα, not only the activation of the NFκB might be affected, but also the biological activity induced by other cytokines which depend on the activation of this transcription factor. This includes IL1, TNF and other members of the TNF superfamiliy, such as TWEAK and TRAIL. IL1 has been shown to play a role in the control of CMV infection, by blocking viral spread in different cell types [Randolph-Habecker et al., 2002]. By targeting this cytokine, or IL1-induced responses, MCMV might control its own dissemination.

TNF superfamily members TWEAK and TRAIL also appeared to be differentially expressed after MCMVdie1 infection. Both proteins have been shown to activate caspase 8-induced apoptosis [Aggarwal, 2003]. Modulation of the expression of these ligands suggests that MCMV might also regulate the induction of apoptosis. Very recently, it has been reported that MCMV also targets other members of the TNF signalling pathway in order to counteract the immune response induced after receptor engagement [Mack et al., 2008]. Human CMV has been reported to encode a viral protein that blocks caspase 8 activation [Skaletskaya et al., 2001]. In addition, the human virus has also been shown to induce the activation of the caspase-inhibitor c-FLIP by its immediate early 2 protein (correspondent to the IE3 MCMV) [Chiou et al., 2006]. The activation of c-FLIP is NFκB-dependent. Microarray data pointed to the regulation of the IκBα gene expression in the absence of IE1, suggesting that MCMV might also block caspase 8/10-induced apoptosis. Moreover, it was seen that the AK2/FADD/caspase-10

signalling pathway was also affected by MCMVdie1 infection, as well as DAPK1 and HIPK3. Considering the importance of apoptosis in the immune response against viral infection, it is of interest to study further the interaction between IE1 and the regulation of apoptosis.

Microarray analysis might also indicate that IE1 may be required for the activation of IFN-induced genes, including *irf1*, *ifitm1* and *mx1*. In fact, this result would be consistent with another report demonstrating that IE1 is required for induction of IFN response elements [Busche et al., 2008]. However, another study has shown that MCMV blocks IFNβ expression, although the molecular mechanism is not known [Le et al., 2008]. Human CMV has been shown to prevent binding of STAT1-STAT2-IRF9 to specific DNA sites in order to block IFN$\alpha\beta$-induced gene expression.

The Ingenuity Pathway Analysis tool is able to represent the interaction of different genes, giving a number of genetic networks which might reveal new interactions. Table 3.1 summarizes the top 5 genetic networks found in this analysis, along with the different genes involved. As can be seen, cell death, lipid metabolism and biological processes involved with viral infections are included. From these genetic networks it was seen that IE1 protein might also be involved in regulation of lipid metabolism. The enzyme CH25H appeared to be differentially expressed. This enzyme has a role in regulation of the cholesterol metabolism. Moreover, it has been shown that cholesterol plays an important role in activation of Erk1/2 phosphatase, by supporting the OSBP and specific enzymes in an active complex.

Overall, microarray study and pathway analysis are useful tools to obtain information about the functional relationship between BMMθs and MCMV in the abscense of IE1. Despite the need for further validation of the microarray data this is the first study done trying to understand the impact of this viral protein in the host cell. The results presented in this chapter gives an overview on how MCMVdie1 might affect host cell gene expression. IE1 has been suggested to

be a *trans*-activator of viral and host gene expression to favor viral replication, however Pathway Analysis has pointed to a possible regulation of expression of genes involved in the immune response in the absence of *ie1*. Moreover, microarray analysis could be very useful to identify potential targets for viral infections. For instance, in the context of MCMVdie1 infection: (1) lipid metabolism which has not been described until now to be crucial for MCMV infection, but might be important in the regulation of the immune response and (2) apoptosis which is known to be part of the cellular response against viral infection and is also exploited for some viruses to their own convenience. This study might indicate a role for IE1 in the regulation of such a response. Cytokine production also appeared to be regulated in the absence of IE1, as seen for TNF, IL10 and IL6. Considering the relevance of these biological processes and molecules in the control of cell survival and immune response further work has to be done in order to validate the microarray data as well as make firm conclusions about these possible immune evasion strategies.

CHAPTER 4

Modulation of TNF response by MCMV

4.1 Introduction

In the previous chapter the growth kinetics of the MCMVdie1 were described, showing a trend towards a lower replication in BMMθ. Although this result was not statistically significant, the difference in viral replication, which is not seen in other cell types such as fibroblasts, might be due to the production of factors involved in the control of viral replication such as cytokines. Moreover, cytokines have been shown to play a crucial role in the control of MCMV infection *in vivo* and *in vitro*, so it is not be surprising that MCMV has evolved mechanisms to suppress or evade cytokine-induced responses in order to persist within the host.

IL10 has been reported to have a role in MCMV infection in primary Mθs. Redpath and coworkers demonstrated that MCMV-induced down-regulation of MHC class II in infected cells was driven by the induction of IL10 in these cells [Redpath et al., 1999]. Very recently IL10 has been described as an important regulator of the immune response against infection, since it can control both pathogen invasion and pathology [Couper et al., 2008]. This would be consistent with a recent study showing that depletion of IL10 resulted in dramatic symptoms of MCMV infection in the natural host, related to uncontrolled production of other pro-inflammatory cytokines such as IFNγ, IL6 and TNF [Oakley et al., 2008]. Unlike MCMV, the human virus encodes a viral homolog of IL10, cmvIL10, which is capable of engaging the IL10 receptor as well as activating signalling molecules such as STAT3 [Spencer, 2007].

IL6 has also been shown to be induced upon MCMV infection [Ruzek et al., 1997]. Moreover, the human virus has been reported to induce a transcriptional regulation of IL6 mRNA in Mθs by a mechanism involving the IE1 protein [Geist and Dai, 1996].

Several viruses have been shown to induce TNF production [D'Addario et al., 2000, Ameglio et al., 1994, Paludan and Mogensen, 2001], however very few examples can be found in the literature regarding viral regulation of TNF production. One of these examples is the African swine fever virus (ASFV). It has been reported that ASFV infection of Mθs results in inhibition of TNF expression by interfering with the activity of CBP/p300 [Granja et al., 2006]. Epstein Barr virus (EBV) has also been shown to inhibit TNF production in cell culture [Gosselin et al., 1992], however the molecular mechanism of this regulation is not clearly understood. Besides, another study has shown that EBV gp350 induces TNF production [D'Addario et al., 2000], contrary to the previous report. To date both human and murine CMV have been reported to block TNF-induced gene expression by interfering with TNF receptors expression [Popkin and Virgin, 2003, Baillie et al., 2003, Montag et al., 2006], TNF-induced caspase-dependent apoptosis [Skaletskaya et al., 2001] and very recently caspase-independent apoptosis by direct binding and degradation of RIP1 by the viral protein M45 [Mack et al., 2008]. Other viruses, such as Dengue Virus and Hepatitis C virus,have been also shown to overcome the TNF-induced response [Saito et al., 2006, Choi et al., 2006, Wati et al., 2007].

4.2 Aim of the chapter

Mθs are essential for MCMV infection as well as the cytokine response triggered upon infection. However, little is known about the role of IE1 protein in the infection of primary Mθs. There is evidence showing that IE1 activates transcription of cellular genes in order to create the optimal environment for viral replication, but there is not much information about the role that IE1 could be playing in regulation of the immune response induced upon viral infection. Therefore, in order to characterize the role of IE1 protein in regulation of the immune response

against MCMV, the aim of this chapter was to study the effect of this viral protein in BMMθs-induced cytokine production during early hours of infection.

4.3 Results

4.3.1 Cytokine production after infection

To investigate the role of IE1 protein in modulation of the immune response in infected-BMMθs, cytokine secretion at early times after infection was studied. Thus BMMθs were prepared as described before and after 7 days of culture were either mock infected or infected with MCMVdie1 or MCMV at a MOI 1 (n=3). Supernatants were harvested at 10 and 24 hpi and flow cytometry-based Cytometric Bead Array (CBA) was performed for IL6, IL10, IFNγ, TNF and IL12p70.

Figure 4.1 shows that MCMV infection resulted in the induction of IL6 (75 pg/mL) at 10 hpi when compared to the mock-infected cells, which levels were under limit of detection ($p<0.05$). At 24 hpi IL6 levels were reduced, just above the limit of detection (55 pg/mL). MCMV is known to induce production of IL10 in macrophages [Redpath et al., 1999]. In this experimental system, MCMV also induced IL10 production after 10 hours (118 pg/mL, $p=0.03$, when compared to mock infection), with a moderate decrease by 24 hpi (58 pg/mL). IFNγ levels were under limit of detection, as expected, but also the levels of IL12p70 at both time points. The most significant response detected after MCMV infection was TNF production, which reached 206 pg/mL ($p=0.006$) and 160 pg/mL at 10 and 24 hpi, respectively, although TNF levels found in the supernatants of mock-infected cells were above the base line at 24 hpi.

When cytokine production was studied after MCMVdie1 infection it was seen that, except for IFNγ and IL12p70, all cytokines were also found in the cellular supernatant early after infection. Moreover, the TNF response was markedly higher, when compared to the levels seen for wild type infection.

Considering the role that TNF plays in the control of viral infections, and the significant production of the cytokine after infection with MCMVdie1, it was of

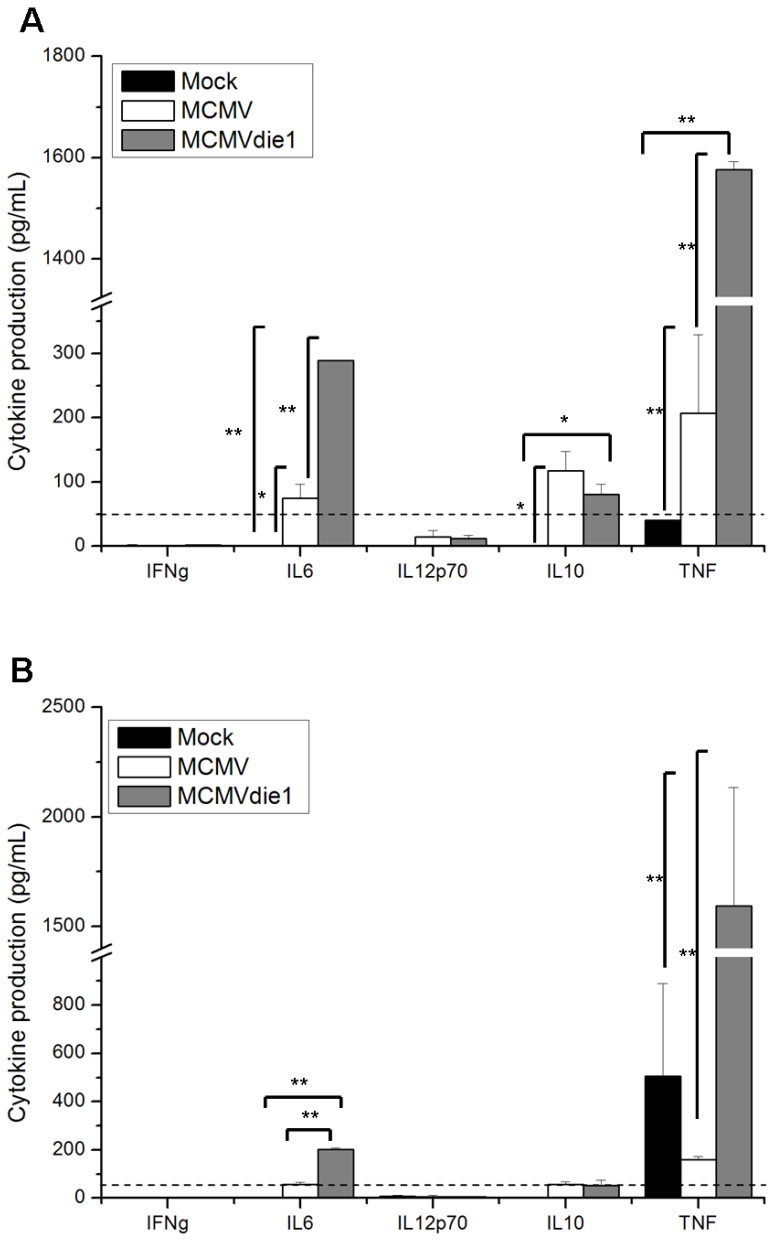

Figure 4.1: Cytokine production after 10 and 24 hours post infection

Cytokine production was determined from supernatant of infected-BMMθs after A. 10 and B. 24 hours post infection (n=3). Mean values and SD are shown. Dash lines represent limit of detection. Significance of the cytokine production is given by Student's T-test p values (* $p<0.05$, ** $p<0.01$).

interest to confirm the TNF production in the absence of IE1. Therefore, a second experiment was performed where BMMθs were infected with either MCMV or MCMVdie1, or mock-infected and cytokine levels were measured by ELISA. As a positive control for macrophage activation, cells were also treated with LPS

and TNF levels were measured after 6 hours post stimulation. Figure 4.2 clearly shows that MCMV infection induced TNF production. After 10 hpi MCMV induced 50 pg/mL of TNF. When compared to the mutant virus however, the absence of IE1 protein resulted in a significant production of the cytokine, 3-fold higher amount of TNF. At 24 hpi both MCMV and MCMVdie1-induced TNF production slightly decreased, but there was still a significant difference between viral infections. It is of note that there was a difference in the cytokine concentration between the flow cytometry based-CBA and ELISA. The higher sensitivity of the first assay is well known [Jimenez et al., 2005], in comparison to conventional ELISA, which would explain the difference in protein concentration. However and despite the sensitivity difference of the technique used, these independent experiments showed a significant production of TNF in the absence of IE1. Therefore, these data suggest that MCMV modulates the production of TNF in BMMθs at early times post infection, in a mechanism that involved the presence of the IE1 protein.

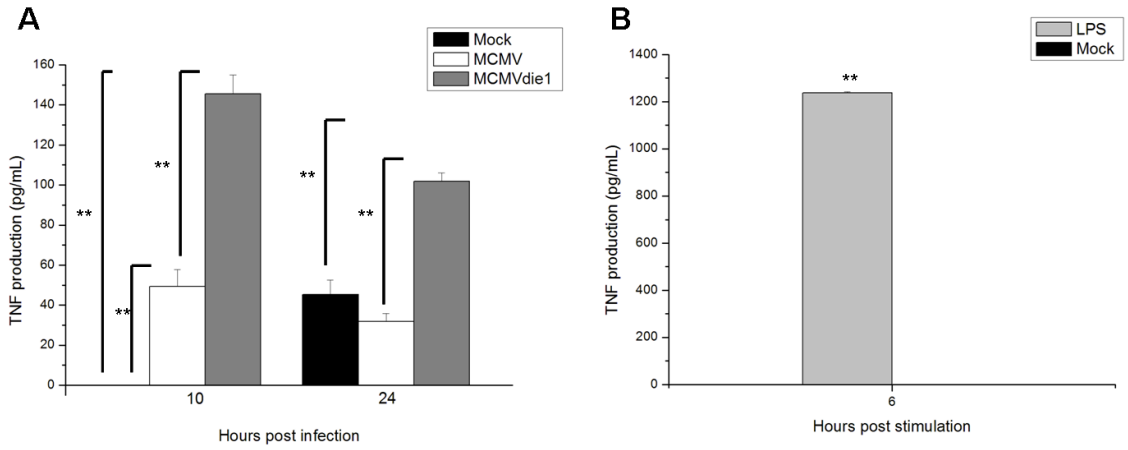

Figure 4.2: Confirmation of TNF production in BMMθs by ELISA.

Mθs were either infected with MCMV or MCMVdie1 or mock-infected (n=3). Cytokine production was determined from the supernatant of infected-BMMθs after A. 10 and B. 24 hours post infection. Mean values and SE are shown. Significance of the cytokine production is given by Student's T-test p values (* $p<0.05$, ** $p<0.01$).

4.3.2 Role of MCMVdie1 in tnf expression in BMMθs.

To further investigate regulation of TNF production after infection by MCMV, the activation of *tnf* gene expression in BMMθs was studied. Cells were infected

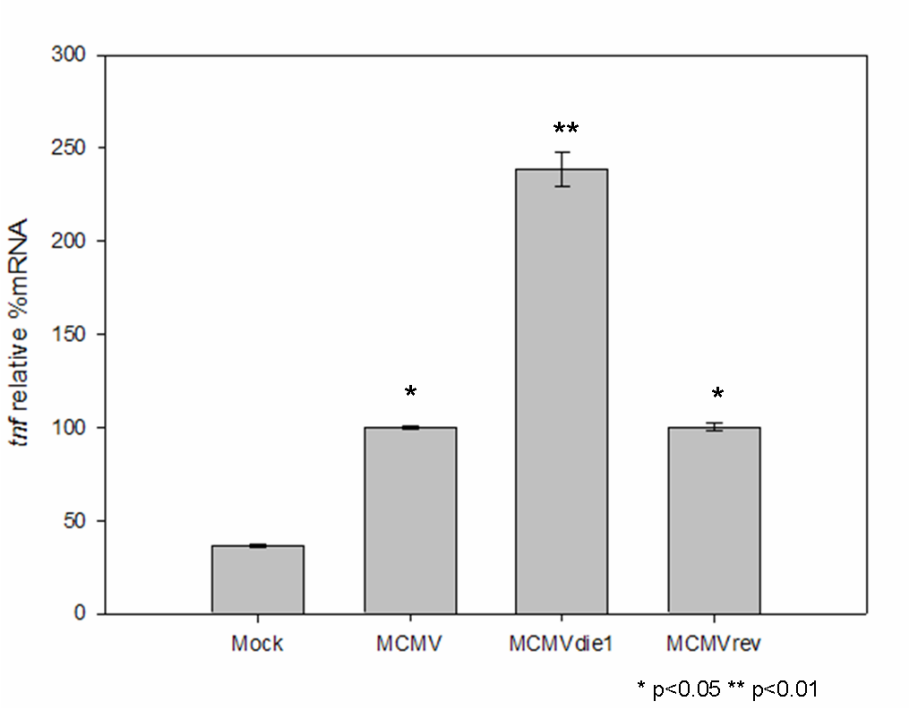

Figure 4.3: Relative expression of *tnf* after infection

Relative fold expression of *tnf* gene in infected-BMMθs. Total RNA was extracted at 10 hours post infection from BMMθs. Quantitative RT-PCR was performed for relative expression levels of *tnf* in these cells. Shown are relative levels of gene expression which have been normalized against *gapdh* gene and calibrated against MCMV induced-gene expression. SE bars also shown. Significance of mRNA levels is given by Student's T-test p values.

with MCMVdie1, MCMVrev or parental MCMV or mock infected (n=3) and total RNA was extracted after 10 hpi. This time point was chosen since TNF production was found to be higher (Figure 4.2). Quantitative (q)RT-PCR was performed to detect *tnf* mRNA levels. Data was normalized against expression of *gapdh* and calibrated against MCMV infection. Figure 4.3 shows relative levels of *tnf* transcripts. When compared to the mock-infected samples, MCMV infection induced expression of *tnf* ($p<0.05$). However, TNF mRNA levels in MCMVdie1-infected BMMθs were 2.5- fold higher than those seen for the parental and revertant MCMVs ($p<0.01$). MCMVrev induction of *tnf* expression was similar to that induced by wild type virus. It has been reported that MCMV infection inhibits expression of TNF receptors in macrophages [Popkin and Virgin, 2003], altering the signalling cascade of cellular activation. However, this is the first time that *tnf* gene expression has been shown to be regulated by MCMV. Therefore, the data

suggest that MCMV modulates both *tnf* transcript levels and protein production in BMMθs.

4.4 Conclusion

Mθs are a key cellular population for MCMV infection. MCMV disseminates throughout the host by using blood monocytes derived from bone marrow progenitors as vehicles [Hanson et al., 1999]. Differentiation from monocytes to Mθs has been shown to favor the cellular environment for viral replication. Mθs also play a role during latency, since it has been found that these cells are a major site of persistence of MCMV DNA in latent state. Moreover, the bone marrow has been defined as a 'source' of latent MCMV [Pollock et al., 1997, Mitchell et al., 1996]. Mθs are as important for MCMV infection as for the immune response against MCMV. Mθs are part of the first line of defense, becoming active and secreting cytokines, such as TNF, IL12, IL1 and IL6 and chemokines to alert and recruit other cells to the site of infection. Together, cells in collaboration with cytokines control pathogen invasion and kill infected cells to avoid further spread of the virus. However, and despite all the efforts made by the immune cell 'army' to control MCMV infection, infectious virus can still be found in salivary glands after resolution of acute infection. That indicates that MCMV has evolved mechanisms to sabotage Mθs function. It is reported here the modulation of TNF production by MCMV in the absence of IE1. TNF has a central role in control of MCMV infection. In synergy with IFNγ MCMV replication is reduced in MEFs [Lucin et al., 1994], confirming the antiviral activity of this cytokine. TNF-induced immune effects in the cells are mediated through a signalling cascade which involves both TNF receptors. For that reason, it is not surprising that one of the CMV's immune evasion strategies is to block the activation of such a response. Indeed, it has been shown that both human and murine CMV targets expression of TNF receptor 1 (TNFR1) on the surface of Mθs [Baillie et al., 2003, Popkin and Virgin, 2003], however the exact viral protein responsible for this regulation is not known.

Experiments described in this chapter show the modulation of BMMθs-induced TNF production by MCMV in the absence of the *ie1* gene. It is shown that MCMV suppresses the *tnf* gene expression and protein production. By using an *ie1*-deletion mutant MCMV it was seen that the induction of the cytokine early after infection was significantly higher when compared to the cytokine production induced by the wild type MCMV. Moreover, when *tnf* mRNA levels were quantified it was seen that MCMVdie1-induced activation of *tnf* expression was 2.5-fold higher. Work done by Geist and coworkers showed that HCMV IE1 protein induced expression of the *tnf* gene in cultured cells [Geist et al., 1994, Geist et al., 1997]. However, in contrast to the work described in this chapter which was done in the context of viral infection, these studies were performed by using transient transfection assays.

It could be argued that the dramatic TNF production is not due to a viral-induced regulation but the result of a defect of the mutant virus instead. However, this investigation has been focused on early times post infection, prior to viral replication. In addition, previous observations show that MCMVdie1 is able to replicate as wild type in other cell types such as fibroblasts. Since the trend towards lower yields was only found in BMMO it was hypothesized that IE1 is involved in the regulation of the immune response, since this cellular population is known to have a role in the control of MCMV infection. Data presented in this chapter show independent experiments where the modulation of TNF is shown in the absence of IE1, suggesting that MCMV is regulating this cytokine response. The mechanism by which this modulation takes place is not known but it is clear that involves the presence of IE1. In any case, a comparative study of the genome copies found in infected cells could be very informative to confirm that cultures were equally infected. Therefore, the observations described here were made under the assumption of that equal number of cells being infected (see Figure 5.3 for controls). Hence, it can be concluded that MCMV regulates the production of TNF in BMMθs at early times post infection, in a mechanism that involved the presence of the IE1 protein.

IL6 protein production presented a similar profile to TNF, although not as significant as TNF. A possible explanation for this induction of IL6 after MCMVdie1 infection could be that IL6 is produced as secondary response to the dramatic levels of TNF found in the cellular supernatant, since IL6 production is induced by TNF.

Overall, data presented in the above sections indicates that MCMV modulates TNF at both RNA transcripts and protein levels. The mechanisms of such modulation are not known however data also indicates that IE1 plays a role. This raises a potential strategy of evasion of the immune response by MCMV, particularly the escape from the known TNF-induced response. These data, along with previous work regarding the inhibition of TNF-induced signalling pathway by blocking expression of TNF receptors, reinforce the key role that TNF plays in control of MCMV infection.

CHAPTER 5

Molecular study of MCMV-induced TNF production

5.1 Introduction

Nearly every step of TNF biosynthesis and the TNF-induced signalling pathway has been shown to be targeted by different pathogens. For instance, members of the Poxvirus family have been reported to directly interact with TNF by a viral TNF receptor homolog [McFadden et al., 1997], avoiding TNF binding to the receptor and induction of the immune response. Blocking of caspase activation is another common mechanism used by pathogens. That is the case for Hepatitis C virus (HCV) which suppresses TNF-induced caspase 8-dependent apoptosis by sustaining expression of the caspase-8 inhibitor c-FLIP [Saito et al., 2006].

HCV also inhibits TNF-induced NFκB activation by interfering with TRAF2 and the inhibitory complex IKKα by the viral proteins NS5A and NS5B, respectively [Choi et al., 2006]. It is more common for viruses to target either TNF superfamliy ligands or TNF-induced signalling rather than production of the protein. However, different bacteria and parasites such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Plasmodium falciparum*, *Toxoplasma gondii* among others, have been reported to interfere with the expression of the *tnf* gene, mainly by targeting NFκB and members of the MAPK [Rahman and McFadden, 2006], known to be crucial for *tnf* expression and which will be described in more detail in different sections of this Chapter.

HCMV has also been reported to block TNF-induced NFκB-dependent responses by targeting the TNFR1 [Baillie et al., 2003]. A similar mechanism has been described by MCMV which inhibits TNF-induced ICAM-1 expression by altering the levels of both TNFR1 and TNFR2 [Popkin and Virgin, 2003].

5.2 Aim of the Chapter.

Work presented in previous Chapters has shown that the absence of *ie1* resulted in a significant production of TNF at both transcript and protein levels in infected-BMMθs. The aim of this Chapter was to explore the molecular mechanism of this potential modulation of TNF production in primary Mθs.

5.3 Bibliographic characterization of *tnf* promoter activation.

In order to study at the molecular level the MCMVdie1-induced production of TNF it was of interest to firstly understand the induction of this response. From the literature it can be seen that *tnf* activation occurs by different mechanisms depending on the stimuli, as well as the species under study, that is between human and murine gene expression. Therefore, a literature review was performed in order to characterize the molecular mechanisms by which murine *tnf* promoter is activated, and to identify common transcription factors which might also be involved in the activation of the gene, as well as the signalling pathways leading to the activation of these molecules.

The first part of this study involved an extensive literature review in order to characterize which factors are crucial for *tnf* activation. This study was done in colaboration with Y. Naiyachit, as part of an MSc project done during the time of this thesis. As it has been previously introduced, TNF is mainly produced by macrophages, however other cells like T cells, B cells and NK cells also produce TNF. The cytokine response normally occurs after cellular activation by a wide variety of stimuli, such as bacterial peptidoglycan, lypopolysacharide, viral and bacterial infection, among others. Interestingly, all these stimuli induce the activation of *tnf* expression by the recruitment and activation of different transcription factors. Moreover, this response has also been reported to be cell type- and stimuli-dependent [Falvo et al., 2000].

LPS-induced TNF production has been extensively studied in both human and murine monocytic cell line. For instance, comparative studies have shown similarities in both promoters, but there are also several differences regarding the contribution of transcription factor NFκB in the induction of *tnf* promoter [Kuprash et al., 1999]. In contrast to this study, others have shown that NFκB is not required for a full induction of the promoter, and the recruitment of transcription factors such as Sp1, Egr-1, Ets and ATF2/Jun is sufficient for an optimal activation of *tnf* expression in LPS-activated Mθs [Tsai et al., 2000]. However, a recent study has shown that blocking NFκB activation resulted in a reduction in the activation of *tnf* by LPS, suggesting that although NFκB might not be essential for the initial activation of the gene expression it is required for the stability of the mRNA [Tsytsykova et al., 2007]. The coactivator proteins CREB binding protein (CBP) and p300 seem to function to maintain the correct alignment of the factors for the optimal induction of the promoter [Barthel et al., 2003].

The molecular mechanisms of TNF production by Mycobacteria has also been studied in macrophages, showing a number of similaties with LPS [Barthel et al., 2003] regarding the induction of the enhanceosome formation. This study also compared the assembly of the enhanceosome in virus-infected cells or in response to ionophore. The results are shown in Figure 5.1, where it can be seen that, even though cell type and and stimili play a role in the formation of the initiation complex, there are a number of factors which are equally essential in all three scenarios. They are ATF2/Jun, Sp1, Ets, Egr-1 and coactivators CBP/p300.

Taking all the information together, figure 5.2 shows the transcription factors which have been shown to be involved in the activation of the murine *tnf* promoter. The identification of these transcription factors along with the upstream signals driving their activation might give the basis of the molecular mechanisms underlying MCMV IE1-induced suppression of *tnf* expression in Mθs.

5.4 Investigation of Mitogen Activated Protein Kinases.

Once the transcription factors involved in *tnf* promoter activation were determined, the second step in this investigation was to identify which signalling pathways would drive the recruitment and activation of these factors, which also might be targeted by MCMV IE1. It has been shown that the engagement of different TLRs results in activation of pro-inflammatory cytokines, including TNF. In fact, *M. tuberculosis* and LPS activate TLR2- and TLR4- signalling pathways, respectively. It is also known that viral infection is normally sensed by TLR3 or TLR9, or both, inducing the signalling cascade. These receptors induce the activation of NFκB and mitogen activated protein kinases (MAPK), which cultimate in the activation of an pro-inflammatory response.

MAPK is a family of signalling proteins expressed in all eukaryotic cells and involved in many different cellular responses and functions, from cellular growth and differentiation to cytokine responses. Three important groups of MAP kinases have been characterized in mammalian cells; the extracellular signal regulated protein kinase 1 and 2 (Erk1/2), p38 MAPK, and c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK). All of these have been shown to be activated in different macrophage cell lines [Rao, 2001]. Their effects on the induction of TNF gene expression and protein release have been extensively documented, as well as their involvement in the activation of cytokine responses against different pathogens [Pazdrak et al., 2002, Nakamichi et al., 2004, Lee et al., 2005, Zhang et al., 2005, Zhu et al., 2005], and immune evasion strategies [Prive and Descoteaux, 2000]. Moreover, it has been shown that activation of at least Erk1/2 and p38 is required for successful induction of the TNF gene [Rutault et al., 2001]. This suggests a cooperative effect between the different MAP kinase signalling pathways, which would regulate different stages of TNF production [Zhu et al., 2000].

The activation of Erk1/2 has also been reported in LPS-stimulated bone marrow derived macrophages [Valledor et al., 2000], as well as its role in TNF production. However, the precise role that these molecules, and the subsequent

cascades triggered upon kinase activation, play in the processes responsible for TNF production remains unclear. Both Elk-1 and Egr-1 are involved in Erk1/2 signalling pathway in different Mθs cell lines. It is known that there are binding sites for both transcription factors in the *tnf* promoter (see Figure 5.2), and that Elk-1 can activate Egr-1 expression [Shi et al., 2002, Xu et al., 2001]. MAPK also phosphorylate CREB, shown to be involved in activation of the transcription of *tnf* gene [Roach et al., 2005]. Very recently it has been reported that MNK kinases, downstream Erk1/2 and p38, also play an essential role in regulation of *tnf* expression, as well as balance the activation of a pro-inflammatory response against anti-inflammatoy response [Rowlett et al., 2008].

TLR3 and TLR9 [Tabeta et al., 2004], and very recently TLR7 [Zucchini et al., 2008], have been shown to play a role in innate immunity against MCMV infection. These TLRs are also known to activate MAPK, however this has not been described in the context of MCMV infection. Moreover, data presented in Chapter 3 might indicate that MCMV regulates lipid metabolism of the infected cell, including cholesterol. A recent study has shown that cholesterol is also important for the regulation of Erk1/2 kinase activity, by being an integral part of the Erk1/2 specific phosphatase complex [Wang et al., 2003, Wang et al., 2005]. Nevertheless, since members of the MAPK family and NFκB are common targets for different pathogens in the inhibition of TNF, it was of interest to investigate whether MCMVdie1 infection resulted in a differential activation of any of these molecules which could give an insight into the molecular mechanisms of TNF production.

Hence, BMMθs from 10-12 week old male mice were cultured as described before and made quiescent by serum starvation for 24 hours prior infection. Infection was carried out at a MOI 1 with MCMVdie1, wild type MCMV or MCMVrev for 10 hours. The length of infection used was determined according to the peak of TNF shown in the results described in Chapter 4. This study was done in collaboration with Upstate (Millipore, UK), using the PathwayProfiler^{TM}service. This multiple bead-based array was used as an alternative to Western Blot for

Table 5.1: Panel of target

Analyte	Phophosyte	Total protein	Validation
Active β-Catenin		$\surd$	
Akt/PKBα	Phospho-Ser473	$\surd$	
c-Jun	Phospho-Ser73	$\surd$	$\surd$
c-Kit	Phospho-Tyr	$\surd$	
CREB	Phospho-Ser133	$\surd$	$\surd$
EGF-Receptor	Phospho-Tyr	$\surd$	
Erk1/2	Phospho-Thr185/Tyr187	$\surd$	$\surd$
HSP27	Phospho-Ser78	$\surd$	
IκBα	Phospho-Ser32	$\surd$	$\surd$
IRS1	Phospho-Tyr	$\surd$	
JNK/SAPK1	Phospho-Thr183/Tyr185	$\surd$	$\surd$
p38/SAPK	Phospho-Thr180/Tyr182	$\surd$	$\surd$
STAT3	Phospho-Tyr705	$\surd$	
STAT3	Phospho-Ser727	$\surd$	
STAT5 A/B	Phospho-Tyr694/699		
STAT6	Phospho-Tyr641	$\surd$	$\surd$

Analytes that are targeted by the assay. Table shows the list of targets along with the specific antibody, the total protein analysis and validation by Western Blot.

the study of the activation of members of the MAPK family, along with other signalling molecules.

In order to validate this technology, Western Blot was also used on the proteins indicated on the last columm of Table 5.1. Therefore, two of the biological replicates were analysed using PathwayProfiler^{TM}, and the third replicate was used for validation in Western Blot. At the same time, a second experiment was performed and activation of signalling molecules was analyzed by Western Blot. After infection, the whole cellular lysate was extracted as described in Section 2.6.1 and analysed by the PathwayProfiler^{TM}assay and by Western blot for the activated forms of the MAPK family members and several other signalling molecules. In order to establish the intensity of activation, data was normalized against β-Actin protein, followed by normalization against the mock infected signal, and total amount of protein when applicable, showing fold activation values. Values above 1 indicates that viral infection induces stronger activation of the kinase than non-infected samples.

As a control of infection, Western Blot was performed against IE1 and early 1 (E1) viral proteins. As it can be seen from the first panel in Figure 5.3 IE1 protein was absent in both non-infected and MCMVdie1-infected samples. Comparable levels of E1 protein were seen in all infected samples, but not in mock-infected. The slight background found in the mock-infected line could be the result of several washes and stripping of the blot. In any case, the analysis was done on the basis of equal amounts of protein, as seen in the rest of the blots. Panel B shows the positive control for BMMθs activation. Cells were stimulated with LPS for 6 h and Western Blot was performed against IκBα. Degradation of the protein after LPS stimulation demonstrated normal activation of the cells.

5.4.1 MAPK family

Figure 5.4 shows the activation of members of the MAPK family (A-C). The top panels shows the PathwayProfiler^{TM}analysis and with the Western Blot validation along with the densitometric analysis of each blot below. Panel A.1 depicts Erk1/2 activation and from the graph it can be seen that after infection with MCMV kinase phosphorylation was similar to the mock-infected BMMθs. However, when infection was carried out with the revertant virus a 3-fold activation was detected. The phosphorylation of Erk1/2 after MCMVdie1 was 4-fold higher than with mock-infection ($p<0.05$). Figures 5.4 A.2 and A.3 show Western Blot and correspodent densitometric analysis, respectively. In contrast to what was seen in A.1, MCMV infection resulted in a significant activation of Erk1 ($p=0.03$) after 10 hours of infection, whereas Erk2 activation was only modest when compared to mock-infected samples ($p=0.5$). A similar response was seen after infection with revertant virus, however both proteins were significantly activated ($p<0.05$), with 4.8 and 3.6-fold higher phoshorylation of Erk1 and 2, respectively. Similar to what was seen from the Luminex analysis, Erk1/2 activation after MCMVdie1 was 5.5 and 4.2-fold higher than mock-infected samples ($p=0.01$).

Other members of the MAPK family, p38 and JNK, were also analyzed. Luminex based PathwayProfiler™analysis shows in panel B1 that p38 was activated after infection, however median fluorescent intensity (MFI) values from MCMVrev-infected cells were below base line. MCMVdie1-induced p38 phosphorylation seemed to be slightly higher than MCMV-induced activation, however Western Blot analysis did not reveal any difference between viruses. Analysis of JNK was not included since MFI values were under limit of detection. However, Western Blot analysis showed (panel C.2) that MCMVdie1 infection resulted in activation of the kinase, but no differences were found when activation was compared to MCMV and MCMVrev infection.

In summary, this analysis showed that PathwayProfiler™was not consistent with the results obtained by Western Blot, indicating that it is not a reliable technology for the molecules tested. On the other hand, based on Western Bot results, no differences were found in the activation of Erk1/2, p38 or JNK between the different viral infections. Therefore, the significant production of TNF seen after MCMVdie1 infection does not seem to be due to a stronger activation of these pathways.

5.4.2 IκBα, CREB and STAT6 activation

Next, the activation of other molecules which have been shown to play a role in the activation of *tnf* gene expression was studied. It is well documented that the transcription factor NFκB participates in the activation of *tnf* gene expression in macrophages, although there is some controversy regarding the contribution of this factor in the initial steps of the promoter activation. The role of NFκB has also been studied in MCMV replication. Although there are several binding sites of this transcription factor in both human and murine CMV enhancer region [Stinski and Isomura, 2008], NFκB is not required for either HCMV or MCMV replication *in vitro* [Benedict et al., 2004, Gustems et al., 2006]. Moreover, mutation of the NFκB sites did not have any effect on the expression of the *ie1* gene in both viruses. Human IE1 has been shown to induce the expression of members of the NFκB, such as RelB [Wang et al., 2005] or the NFκB p105/p50

promoter [Gribaudo et al., 1996]. The dispensable role of NFκB in HCMV replication has also been demonstrated by Eickhoff and coworkers. However, the same study also showed that blockade at NFκB activation decreases both TNF and IFNγ-induced inhibition of HCMV replication [Eickhoff and Cotten, 2005]. In contrast, Sambucetti and coworkers showed that the IE1 protein was leading the activation of the MIE region by *trans*-activating NFκB sites [Sambucetti et al., 1989]. Taken all together, it can be seen from the literature that the role that NFκB plays in CMV infection is not completely understood, but it is clear that this factor acts as a mediator in the immune response. In fact, several NFκB sites have been localized in the *tnf* promoter (Figure 5.2), therefore activation of NFκB was also included to test the activation of TNF after MCMV infection.

In resting cells, NFκB is sequestered in the cytoplasm by association with IκBα. Cell activation leads to the phosphorylation and ubiquitination of the NFκB inhibitor IκBα. Once free from IκBα, the active form of NFκB translocates to the nucleus and activates gene expression. The activation of NFκB was studied in two different ways. Figure 5.5 D.1 shows the phosphorylation of IκBα, which precedes its degradation. Below is Western Blot for IκBα the degradation of the protein and therefore the activation of NFκB. Densitometric analysis of the blot are also shown in Figure 5.5 D.3. Analysis from the PathwayProfiler^{TM}shows that IκBα phosphorylation was induced after MCMVdie1 infection, however both MCMV-induced inhibitor phosphorylation was not as strong as in the mutant virus. MCMVrev infection did not seem to activate phosphorylation. Western Blot and densitometric analysis show the degradation of IκBα. Results did not reveal any significant degradation between viral infections, and IκBα levels in MCMVrev-infected samples were similar to mock-infected samples. From this data it cannot be concluded that MCMV infection induced activation of NFκB at the time point under study, and that IE1 did not seem to play a role in this activation.

Along with IκBα, the activation of the transcription factor CREB was also studied. As it has been introduced before, CREB is phosphorylated by members

of the MAPK family, and takes part in the enhanceosome complex during *tnf* induction after cellular activation with different stimuli. Once phosphorylated, the active form of CREB binds to the cyclic AMP response elements (CRE) localized in the *tnf* promoter as part of the transcriptional machinery responsible for activation of gene expression. The phosphorylation analysis can be seen in Figure 5.5 E.1-E.3. Luminex-based PathwayProfiler™revealed that after 10hpi, MCMV infection induced a modest phosphorylation of CREB, however both MCMVdie1 and MCMVrev-induced phosphorylation was significantly higher than the mock-infected sample ($p<0.001$ in both cases). As seen for Erk1/2 activation in the previous section, there were differences between MCMV and MCMVrev, and CREB activation was higher after MCMVrev infection. However, MCMVdie1 infection results in a stronger phosphorylation of CREB when compared to both parental and revertant virus ($p<0.001$ in both cases). In contrast to the PathwayProfiler™, Western Blot showed strong phosphorylation of CREB after viral infection and no difference was found in the phosphorylation in the absence of IE1.

Finally, the activation of STAT6 was studied. STAT6 is reported to be involved in the negative regulation of cytokines [Ohmori and Hamilton, 2000]. Analysis can be seen in Figure 5.5 F.1-F.3. PathwayProfiler™analysis showed that STAT6 activation was similar to the levels of mock-infected samples. Western blot showed similar results, and no differences were found between either mock-infection or between viruses.

Taking all the data together, the Luminex-based PathwayProfiler™was not found to be a reliable technique for the purpose of this study since there was no consistency with the data obtained by Western Blot. Nevertheless, initial observations might indicate that MCMVdie1 infection does not apparenlty result in a stronger activation of members of the MAPK family Erk1/2, p38 and JNK, when compared to MCMV and MCMVrev. Very similar results were found in the activation of NFκB where, by looking at the phosphorylation and degradation of its

inhibitor IκB, Western blot analysis did not reveal differences between MCMV and the mutant virus.

The rest of the proteins included in this study (see Table 5.1) were not validated by Western Blot because an important variability were found between samples. Nevertheless, results from PathwayProfiler^{TM}analysis can be found in Appendix C.

5.5 Conclusion

It was of interest to explore the potential regulation of *tnf* activation after MCMV infection. Hence, a study on the activation of the *tnf* promoter was performed, followed by the identification of different signalling molecules which might be involved. An extensive literature review identified a number of transcription factors which are commonly recruited to the *tnf* promoter after cellular activation with different stimuli such as LPS and *Mycobacterium tuberculosis*. These transcription factors included ATF2/Jun, CREB, Ets, Elk, Egr-1 and Sp1, as well as different κB sites in distal sequences of the promoter. It is known that after BMMθs infection, a number of signalling molecules are activated to start a cascade of events which drive the activation of gene expression. Within this cascade, and in the context of the activation of *tnf* expression, the MAPK family has been shown to play a role. It has been reported that several stimuli lead to phosphorylation of Erk1/2, p38 and JNK, after which they translocate to the nucleus and activate different transcription factors and gene expression. The use of specific inhibitors for Erk1/2 and p38 has shown that LPS-induced *tnf* gene expression can be completely abrogated [Rutault et al., 2001]. Together with the MAPK family, NFκB is also important for the activation of a number of immune genes, including *tnf*. There are several IκB sites within the *tnf* promoter, making NFκB another candidate for MCMV. This study was done as part of a collaboration with Upstate (Millipore, UK) in order to validate the Luminex-based PathwayProfiler^{TM}. This protein array is presented as an alternative to Western Blot, since it allows testing of a number of different molecules simultaneusly.

The validation by Western Blot was not consistent with the analysis and therefore no conclusions were drawn from this study. Initial observations indicate that MCMVdie1-induced TNF production does not involve the differential activation of Erk1/2, p38, JNK and NFκB. However, further work has to be done to elucidate the mechanisms of the TNF response.

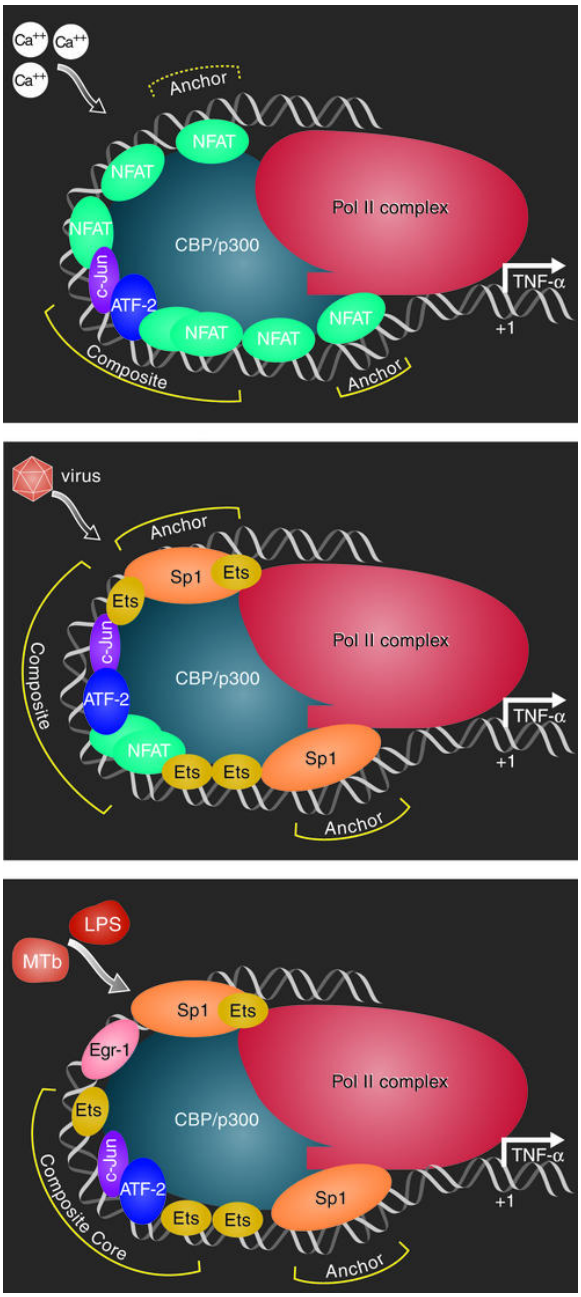

Figure 5.1: Proposed model for TNF promoter activation after different stimuli in T cells and monocytes.

Schematic diagrams representing models for transcription factor recruitment and enhanceosome formation on the *tnf* promoter in T cells (top and middle panel) and monocytes (bottom panel) after different stimuli. Illustration taken from [Barthel et al., 2003] with the permission of the American Society of Microbiology.

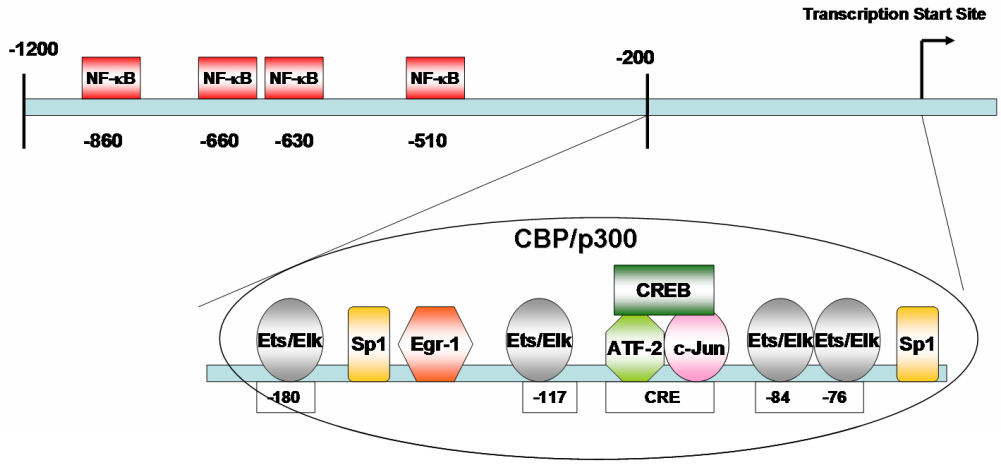

Figure 5.2: Murine TNF promoter topography (adapted from Lee and Schorey 2005).

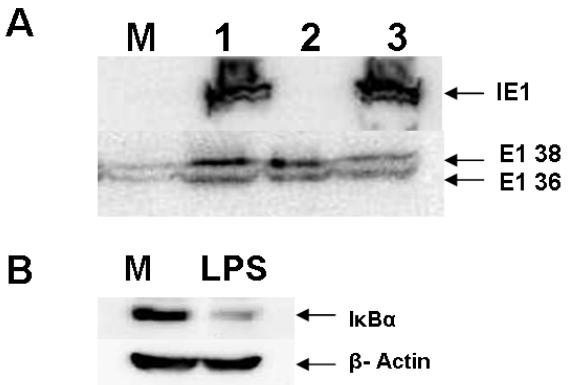

Figure 5.3: Western Blot for IE1 and E1 proteins and LPS positive control

BMMθs were infected at a MOI of 1 with MCMV, MCMVdie1 or MCMVrev or mock infected as a negative control (n=2). Western blot was perfomed as a control of infection using anti-IE1 and anti-E1 specific monoclonal antibodies. A. Viral proteins IE1 and E1 after M. mock infection or infection with 1. MCMV, 2. MCMVdie1, 3. MCMVrev. B. Cells were also M. mock-stimulated or treated for 6 h with LPS as a positive control. Western blot shows LPS-induced degradation of IκBα.

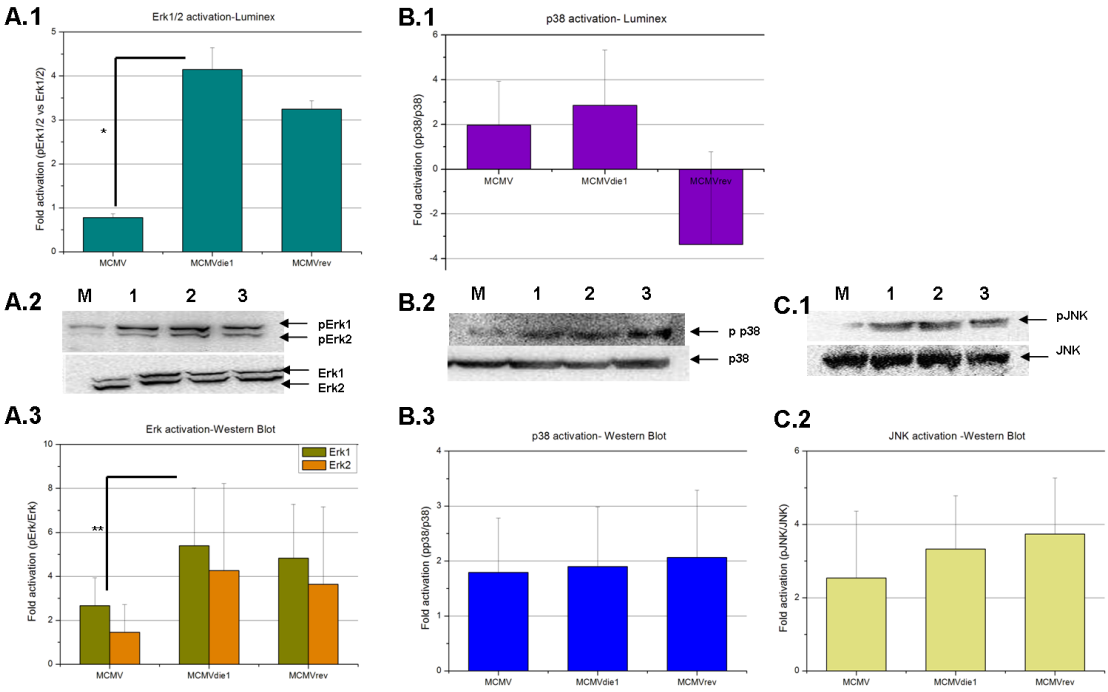

Figure 5.4: Activation of MAPK after infection of BMMθs

BMMθs were infected at a MOI of 1 with MCMV, MCMVdie1 or MCMVrev or mock infected as a negative control. Samples were analyzed as n=4 using the PathwayProfiler^{TM}service by Upstate (Millipore, UK) or Western Blot (n=3). **1.** Fold activation of the kinases are shown. MFI data have been normalized against total amount of protein and mock-infected samples. Blank values were also extrated from the final values. Bars show media values and SD. **2.** Western blot for the activated forms of the kinases and total amount of protein (M mock-infected, 1 MCMV, 2 MCMVdie1, 3 MCMVrev). **3.** Densitometric analysis of the blot. Fold activation of the kinases are shown. Data have been normalized against β-Actin, mock-infected and total amount of protein. Bars show media values and SD.

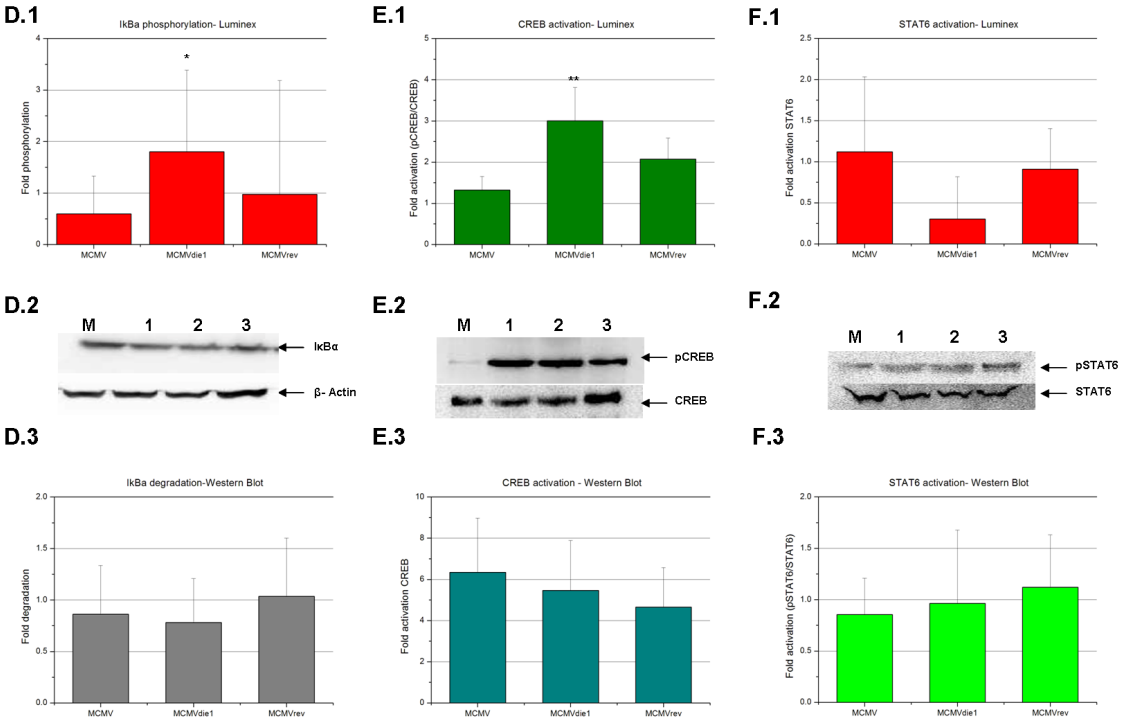

Figure 5.5: Activation of IκBα, CREB and STAT6 after infection of BMMθs

BMMθs were infected at a MOI of 1 with MCMV, MCMVdie1 or MCMVrev or mock infected as a negative control (n=3). Samples were analyzed in duplicate as n=2 using the PathwayProfiler^{TM}service by Upstate (Millipore, UK) or Western Blot (n=2). **1.** Fold activation of the kinases are shown. MFI data have been normalized against total amount of protein and mock-infected samples. Blank values were also extrated from the final values. Bars show media values and SD. **2.** Western blot for the activated forms of the kinases and total amount of protein (M mock-infected, 1 MCMV, 2 MCMVdie1, 3 MCMVrev). **3.** Densitometric analysis of the blot. Fold activation of the kinases are shown. Data have been normalized against β-Actin, mock-infected and total amount of protein. Bars show media values and SD.

CHAPTER 6

Investigation of MCMVdie1 regulation of TNF levels *in vivo*

6.1 Introduction

In vitro systems allow scientists to easily examine the behavior of individual cellular populations. However, this misses one of the most important factors for the optimal activation of the cellular immune response upon infection, and that is cell-to-cell interaction, within the context of intact multicellular tissues. To date, this can only be modeled in intact organisms so that it is essential to have animal models to study.

Studying MCMV infection *in vivo* is a well established model. Moreover, because of the strict species specificity of MCMV infection the mouse model is a useful tool to study viral-induced pathogenesis in the natural host. The outcome of any infection is both host and virus-dependent and there are a number of factors which could affect the outcome of experimental infection, including the origin of the viral preparation. Whether MCMV has been prepared *in vivo* from salivary glands (SVG) or tissue culture is an important factor since SVG-derived MCMV has been shown to be more virulent than MCMV generated in tissue culture. This observation has already been documented [Krmpotic et al., 2003] and it might be due to factors in the salivary glands but the reasons remain unknown. In addition, the passage of MCMV in culture results in a loss of viral infectivity, maybe as a result of an adaptive response of the viral strain to tissue culture. The route of inoculation has also to be taken into consideration. Although saliva is

considered to be the most natural route of infection, intraperitoneal injection is one of the common routes used in *in vivo* studies, after which spleen and liver become infected. But other routes such as intravenous, intraglandular or nasal aspiration are also used. The immune status of the mouse at the time of infection is crucial for *in vivo* studies, since infection of immunocompromised mice results in high mortality, pneumonitis and myocarditis [Krmpotic et al., 2003]. Infection of neonatal mice differs from immunocompetent adult mice in the tissue tropism seen. Neonatal infection is characterized by an early infection of the Central Nervous System (CNS) which results in high mortality whereas adult mice have asymptomatic infection which is normally controlled within days. The genetic background of the mouse to be infected is also important. Infection of various mouse strains has been reported but the MCMV-susceptible BALB/c and MCMV-resistant C57Bl/6 are the most commonly used. This difference in susceptibility relies on the level of NK cell activation, which is under control of the *Cmv1* locus [Scalzo et al., 1990] within the NK gene complex [Scalzo et al., 1992]. *Cmv1* encodes for the activated NK cell receptor Ly49H. NK cells from BALB/c mice lack this locus and as a consequence they do not induce the correct signals for NK cell activation after MCMV infection. However, 50% of the NK cells of the C57Bl/6 mice bear the *Cmv1* locus and express Ly49H. MCMV-infected cells express the viral protein M157 which is a strong ligand for Ly49H. The interaction between Ly49H/M157 induces effective activation of NK cells. This results in secretion of IFNγ, which is a very potent cytokine involved in the control of viral infection.

Infection with MCMV results in a rapid cytokine and chemokine response to recruit and activate immune cells to the site of infection and control viral replication and dissemination to other organs. Within this pool of cytokines, special mention has to be made of type I and II IFN, IL12, LTβ and TNF. The interplay between these cytokines and immune cell population determines the outcome of the infection. However, the fact that MCMV successfully establishes latency in its

host means that MCMV has evolved several mechanisms to evade the immune response against the infection.

As early as 2 days post infection, production of IFN$\alpha\beta$ is detectable in spleen and liver of infected mice. This first wave of IFN$\alpha\beta$ production comes from plasmacytoid (pDC) and non-plasmacytoid dentritic cells (DC) [Orange and Biron, 1996, Hokeness-Antonelli et al., 2007, Zucchini et al., 2007]. pDCs have been considered to be a major source for this cytokine. However, the exclusivity of pDC in the production of IFN$\alpha\beta$ has been ousted by a very recent report showing that stromal cells in the spleen of infected mice are also key in the production of IFN$\alpha\beta$ [Schneider et al., 2008]. Interestingly, both pDC and splenic stromal cells induce IFN$\alpha\beta$ response using different signalling pathways. Thus, spleen stromal cells are activated through the crosstalk with B cells which produce lymphotoxin β (LTβ). After production, LTβ binds to LTβ receptor (LTβR) on the surface of the stromal cell, and LTβ-LTβR signalling pathway leads to the production of IFN$\alpha\beta$. It has been also shown that pathways leading to IFN$\alpha\beta$ production depends on the tissue, for instance TLR9 and MyD88 are activated in the spleen however IFN$\alpha\beta$ production is TLR9-independent in the liver [Delale et al., 2005, Hokeness-Antonelli et al., 2007]. The production of IFN$\alpha\beta$ is also under control; as documented by [Tailor et al., 2007] where IRF8 is involved in the second wave of cytokine production, ensuring the optimal antiviral response.

Independently of the source and molecular mechanism by which IFN$\alpha\beta$ is produced, its role in the control of MCMV infection in the natural host has been extensively studied. Activation of NK and CD8$^+$ T cells depends in part to IFN$\alpha\beta$. Upon activation, these cells produce IFNγ. However, the uncontrolled production of this cytokine could cause damage on the host and therefore, IFN$\alpha\beta$ production requires to be under control [Hahm et al., 2005]. One example of this cytokine control has been recently documented by [Robbins et al., 2007] where it is shown that overactivation of CD8$^+$ T cells could be avoided by the control of pDC-induced IFN$\alpha\beta$.

IFNγ is produced by activated-NK and CD8$^+$ T cells. It has been well documented the effect of this cytokine in MCMV infection both *in vivo* [Pomeroy et al., 1998] and *in vitro* [Lucin et al., 1994]. IFNγ is responsible for the establishment of the antiviral state by inducing the transcription of a huge number of genes. The IFNγ receptor is ubiquitously expressed, which helps to activate bystander cells and prepare them to control viral infection. Although how IFNγ blocks viral replication remains unknown. Nevertheless, MCMV has developed strategies to circumvent the antiviral activity induced by IFNγ. One examples is the degradation of STAT2 by the viral protein pM27 [Zimmermann et al., 2005]. By blocking STAT2 activation both type I and II IFN responses are affected, suggesting that both pathways cross-talk during the antiviral activity. Another strategy to escape from IFNγ-induced effects has been shown by [Heise et al., 1998]. In this study it is shown how MCMV has evolved to inhibit the expression of MHC class II on the cell surface induced by IFNγ. With this strategy MCMV also circumvents the activation of CD4$^+$ T cells.

IL12 is also produced very early after infection and is required for the optimal activation of NK cells and subsequent production of IFNγ in spleen and liver after acute infection. The strength of IL12 signalling is related with the activation of NK cells [Orange et al., 1995]. In addition, IL12 production is also under control since it has been reported that IFN$\alpha\beta$ inhibits IL12 production, which in turn affects the consequent NK-induced IFNγ. It has been shown that MCMV infection of DC results in significant reduction in IL12 production after 4 days post infection [Andrews et al., 2001], resulting in a decrease in activation signals to NK and T cells. However, the mechanisms by which this reduction is regulated have not been elucidated.

Along with type I and II IFN and IL12, TNF is also produced. Macrophages and activated NK and CD8$^+$ T cells are the main producers of this cytokine. TNF is involved with cellular differentiation, proliferation and apoptosis. Although

the presence of TNF is sufficient to reduce viral replication in infected cells, it has been shown that IFNγ in combination with TNF has a more effective antiviral effect [Lucin et al., 1994]. Also TNF has a paracrine action, which will induce the activation of bystander cells which will respond to viral infection. Considering the important role that TNF plays in the control of viral infection, there have been several studies trying to elucidate its role in natural settings. However, there is a lot of controversy in the results. Shanley et. al. showed that after both lethal and sublethal infection of BALB/c mice TNF levels were undetectable in serum. However, levels of this cytokine in serum were found in MCMV-infected BALB/c [Trgovcich et al., 2000] and C57BL/6 [Lenzo et al., 2001] and in all organs examined. It also appears that TNF was produced in a viral dose-dependent manner. Trgovcich et. al. showed as well that the *tnf* gene was expressed during sublethal infection in spleen, liver and lung. Tang-Feldman reported on the levels of expression of different cytokines after MCMV infection in BALB/c mice, showing an induction in TNF expression at day 5 p.i in spleen, liver, lungs and salivary glands [Tang-Feldman et al., 2006]. Thus, the production of TNF early after infection suggest a role in the control of *in vivo* infection of different strains of mice in serum, spleen, liver and heart.

6.2 Aim of the chapter

Because *in vitro* conditions may not correspond to those of the natural host, it is essential to study viral infection *in vivo*, where an important number of interactions and crosstalk between cells take place, which may influence the outcome of the infection. Work presented in Chapter 4 of this thesis suggests that MCMV regulates the production of BMMθ-induced TNF production. Therefore, the next question to be addressed was whether the modulation of TNF production by MCMVdie1 also occurs *in vivo*. Two different mouse strains were used. An attenuated phenotype for the *ie1*-deletion mutant MCMV was previously described in immunocompetent MCMV-susceptible BALB/c mice [Ghazal et al., 2005]. The work presented in the following sections confirms these results and

describes viral growth in the MCMV-resistant C57Bl/6 mice. The production of TNF is described for the first time in MCMVdie1-infected mice *in vivo*.

6.3 Results

6.3.1 MCMVdie1 induces higher levels of TNF in competent BALB/c mice.

In order to characterize the induction of TNF in MCMVdie1 infected mice, the following experiment was designed. Groups of 8-weeks old, male BALB/c mice were inoculated i.p. with 1.6 x 10^6 PFU of tissue culture-derived MCMVdie1 or the revertant virus or mocked infected. At 4 and 7 days post infection, 4 mice from the different infected-groups and 2 from the mock-infected group were killed and selected organs were harvested to determine viral titers and TNF induction. Wild type MCMV was not included in this experiment since there were no significant differences between the replication of MCMV and MCMVrev in infected BALB/c mice, as had been described in previous publications [Ghazal et al., 2005]

Confirmation of the attenuated growth phenotype of MCMVdie1 in vivo.

Spleen, liver, kidney, lung and heart were removed from mice at specified time points. As described in Section 2.8.2 from Material and Methods, samples were weighed and homogenized as in 10% w/v. Tissue homogenate was then sonicated and after clearing by centrifugation, viral titers were determined by standard plaque assay (Section 2.4.2). Figure 6.1 depicts viral titers per gram of tissue in selected organs. From the graph it can be seen that, as expected, MCMVdie1 shows an attenuation in its growth phenotype, when compared to MCMVrev replication. At day 4 pi MCMVrev viral load was high in spleen and liver, as normally occurs after an acute infection [Krmpotic et al., 2003], and 6-, and 12-fold higher than MCMVdie1 replication, respectively (t-test, $p<0.05$). A very dramatic difference in viral titers was also seen in kidneys where MCMVdie1 titers were more than 100-fold less (t-test, $p=0.0094$). MCMVdie1 replication in heart and lung was 21- and 35-fold lower when compared to MCMVrev, respectively (t-test, $p<0.05$).

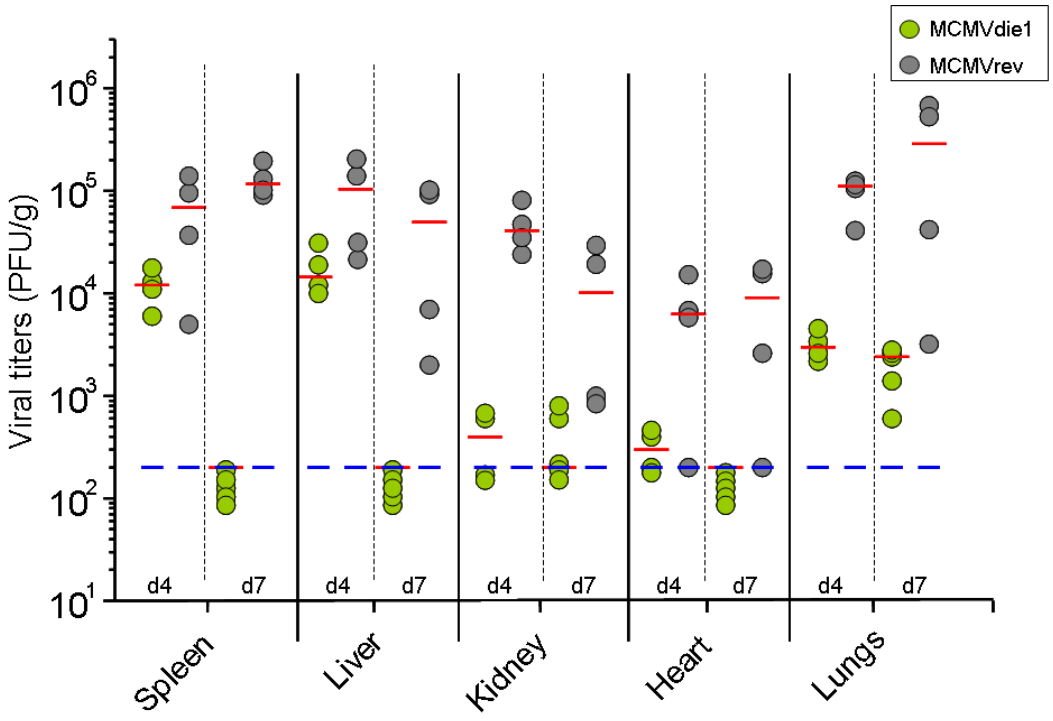

Figure 6.1: Growth of MCMVdie1 and MCMVrev in different organs from infected BALB/c mice at day 4 and 7 post infection.

Groups of BALB/c mice (n=4 mice per group, except MCMVdie1-infected mice at 7 days was n=5) were inoculated with 1.6 x 10^6 PFU of tissue-derived MCMVdie1 or MCMVrev. On day 4 and 7 post infection mice were sacrificed and the indicated organs were harvested, weighted, and sonicated as a 10% (wt/vol) tissue homogenate in DMEM. Viral titers were determined by standard plaque assay on MEFs. Red lines show median values and blue lines show limit of detection. All titers were significantly different between MCMVdie1 and MCMVrev (t-test, $p<0.05$).

At day 7 pi MCMVdie1 could not be detected in spleen, liver or heart. In order to calculate median values of viral titers in these particular organs the limit of detection was set for 2 x 10^2 PFU/g, which corresponds to one single PFU. However, MCMVrev burden in these organs remained elevated (600-, 250- and 45-fold higher in spleen, liver and heart, respectively). MCMVdie1 titers in kidneys were 2-fold lower than at day 4 p.i. No changes in titers were found in lungs of MCMVdie1-infected mice, whereas after 7 days of infection with MCMVrev there was a 2.5-fold higher titers in this organ, suggesting that MCMVrev had spread to the lungs in the period of a week of infection.

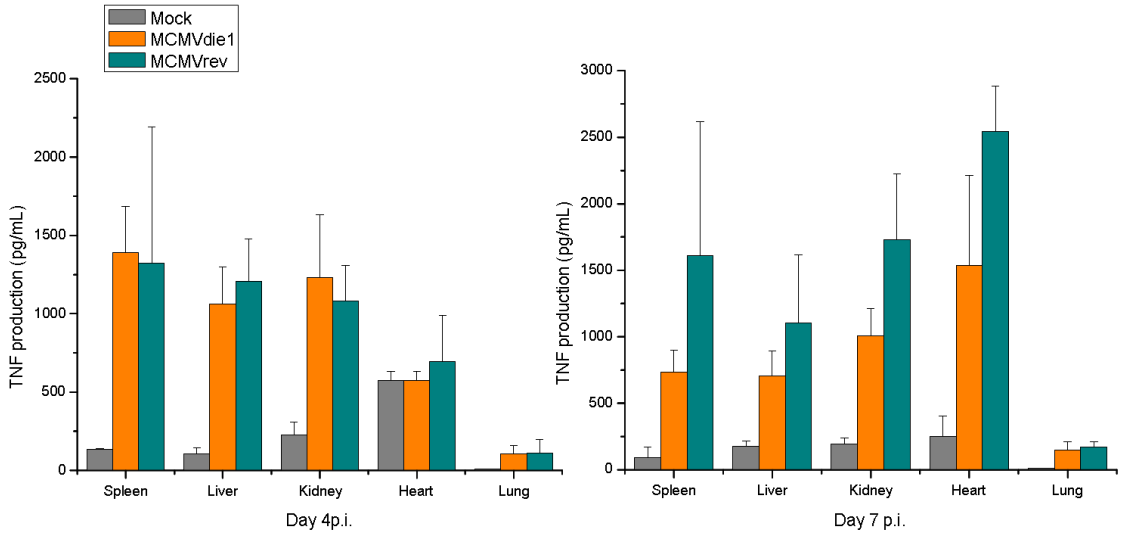

Figure 6.2: TNF production in selected organs from BALB/c mice at day 4 and 7 post infection.

TNF levels were detected in tissue homogenate by ELISA at indicated times. Compared to mock-infected mice, both MCMVdie1 and MCMVrev induced significant levels of TNF. No differences were found in TNF levels induced by MCMVrev and MCMVdie1.

TNF production in infected-BALB/c mice.

In order to asses induction of TNF after MCMV infection, cytokine concentration was measured in selected organs by ELISA as described in Material and Methods (Section 2.7.2). It has been already shown that MCMV infection of immunocompetent mice induced TNF production at early times after infection [Trgovcich et al., 2000, Lenzo et al., 2003, Tang-Feldman et al., 2006]. When compared to the mock-infected mice, MCMVrev induced significant levels of TNF in all organs after 4 days p.i. (Figure 6.2). Spleen and liver of infected-mice showed 10-fold higher levels of TNF when compared to uninfected-mice and a difference of 5-fold was found in kidneys. Although the levels of TNF found in heart and lung were not statistically significant, it appears that viral infection also results in induction of this cytokine in these organs. However, what was more surprising about these results was that after infection with MCMVdie1 no significant differences were seen in the levels of TNF produced when compared to those seen for the revertant virus (t-test, $p>0.05$) on day 4.

After day 7 p.i (Figure 6.2) TNF was also detectable after both *ie1*-deletion mutant and revertant MCMV infection. Data showed (Figure 6.2, right pannel) that after a week of infection, MCMVrev-induced TNF levels remained elevated in spleen and kidneys, whereas a slight drop in hepatic levels was seen when compared to levels detected after 4 days of infection. A significant 3.5-fold increase was detected in the heart at this time point. There was no difference in TNF levels in the lungs. When TNF induction was compared between viral infections there were no differences in splenic, hepatic or lung levels. However, MCMVdie1-induced levels in kidneys and heart were significantly lower compared to MCMVrev-induced TNF. These differences might be due to lower replication rate of the mutant virus in these organs. In order to establish a relation between viral replication and cytokine response a correlation study was performed, which is been described in the following section.

Correlation between PFU and TNF levels in infected-BABL/c mice

It has been reported that levels of cytokine expression correlates with viral replication in different organs [Tang-Feldman et al., 2006]. An earlier study showed contrary results regarding the expression of TNF after infection [Trgovcich et al., 2000]. However, these differences might be explained due to the higher sensitivity of the qRT-PCR used in the latter report. Nevertheless, in order to better understand the results of the experiment described above, a correlation study was carried out to test whether TNF levels corresponded to viral loads in the different organs. Figure 6.3 shows a significant positive correlation between TNF levels and viral load in kidneys and heart after 4 days p.i. (Pearson correlation coefficient, R^2=0.89 and R^2=0.85 in kidney and heart, respectively).

Since these two organs showed a significant correlation between viral titers and TNF levels, it was assumed that the relation also existed in the rest of the organs. The relation between PFU and TNF can be seen in Figure 6.4. The graph shows that at day 4 p.i., MCMVdie1 induced similar levels of TNF in spleen and liver than those detected for MCMVrev. Higher levels of TNF were found in kidneys, heart and lungs, even though viral yields were more than 10-fold less in these organs. At 7 days pi (Figure 6.4) the difference between MCMVdie1 and

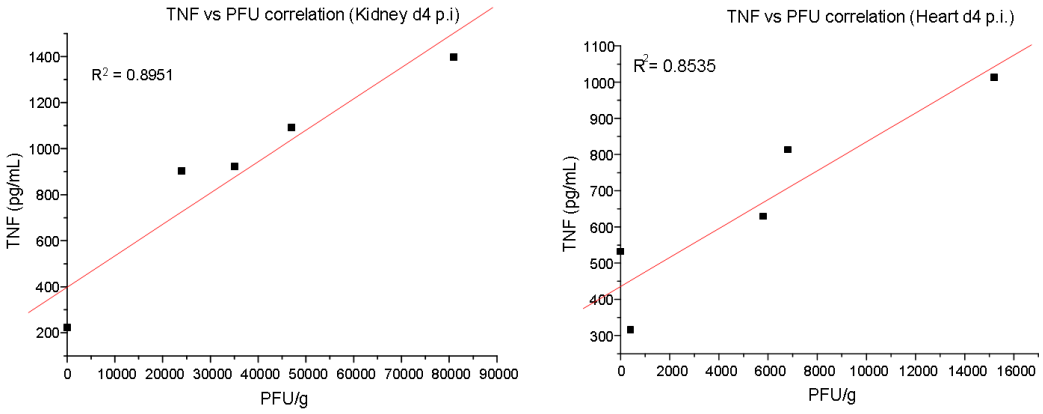

Figure 6.3: Correlation in heart and kidney after 4 days post infection.

Pearson's correlation coefficient showed a significant relation between TNF levels and viral load in kidneys and heart from MCMVrev-infected BABL/c mice after 4 days p.i.

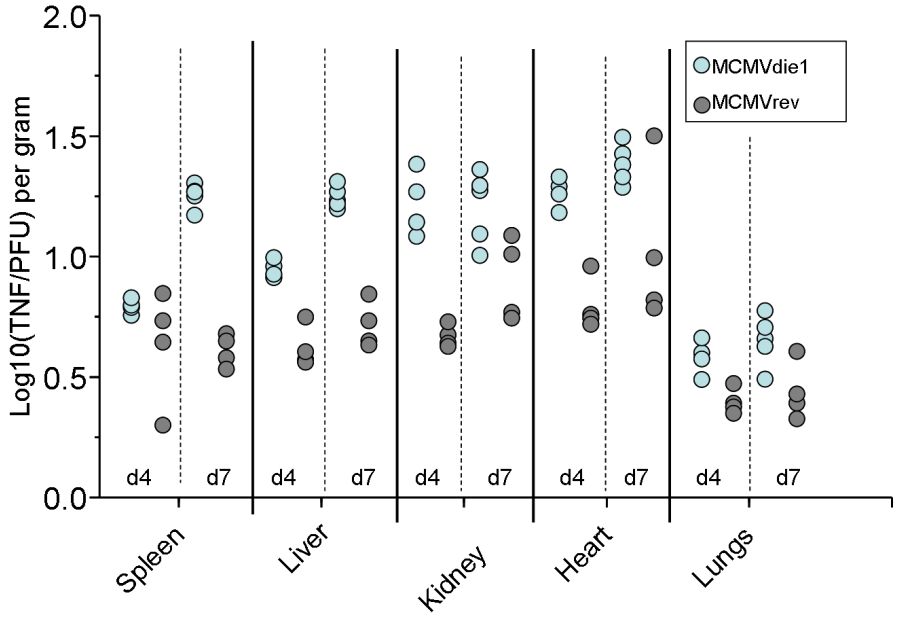

Figure 6.4: Correlation between TNF levels and PFU per gram of tissue

Correlation between TNF levels and PFU in different organs at day 4 and 7 p.i.

MCMVrev induction of TNF was even more dramatic since the mutant viral load was 25-1000-fold lower than the revertant virus.

Because of the different titers found in selected organs after MCMVdie1 and MCMVrev infection, BALB/c mice were also infected with a dose of MCMV

which resultant titers were similar to those seen for MCMVdie1. Thus, mice were infected with 3 x 10^5 PFU of MCMV, and titers and TNF production were also determined and compared to both mock-infected mice and MCMVdie1. Figure 6.5 shows viral titers (right panel) and TNF production (left panel) in selected organs after 4 and 7 dpi. Viral titers show that MCMV and MCMVdie1 replication was comparable in all organs examined, except in the kidney, where replication of MCMV in the kidneys after 4 dpi was significantly higher than titers found after MCMVdie1 infection ($p=0.02$), but in the rest of the organs at both times post infection, it was shown comparable PFU per gram of tissue.

When TNF levels were determined by ELISA it was seen that MCMV infection induced the cytokine production after 4 dpi. Only in the heart TNF levels were similar to those seen after mock-infection. Interestingly, when compared to MCMVdie1 infection, the mutant virus-induced TNF production was significantly higher in all organs, except in the heart. After 7 days, TNF levels induced by MCMV dropped to similar levels as mock-infected mice. However, MCMVdie1-induced TNF production was significantly higher in all the organs examined. It could be argued that the levels of TNF found after MCMVdie1 infection were due to the initial dose of virus, but it has to be noted that the interpretation of the data has been based on the output levels of infection.

6.3.2 MCMVdie1 attenuation is not strain-dependent.

A second study was designed in a different mouse strain. In this way, the strain-dependency on the cytokine response would also be ruled out. Thus, the murine strain C57Bl/6 was used for the second part of these *in vivo* studies. Groups of 4, 8-week old, male C57Bl/6 mice were infected by the intraperitoneal route with 2x10^6 PFU. A higher dose of virus was used in this experiment since C57Bl/6 strain is considered as a MCMV-resistant strain, based on the optimal activation of NK cell by the MCMV protein M157-Ly49H interaction [Scalzo et al., 1992]. In this way, the viral dose would be high enough to induce a clinical infection in C57Bl/6 and MCMVdie1 titers could be detectable.

From Figure 6.6 it can be seen that no statistical difference was found between MCMV and MCMVrev replication (t-test, $p>0.05$ in lungs and $p>0.5$ in the rest

of the organs), whereas MCMVdie1 titers were lower in all organs examined. In more detail, a 20- and 12-fold reduction was seen in spleen and liver, respectively when compared to the parental and revertant viral load (t-test, $p<0.03$). Replication in the heart was 11-fold lower (t-test, $p=0.005$) and 6-fold in lungs (t-test, $p=0.016$). The attenuation was even more dramatic in kidneys with a difference in viral load of 150-fold (t-test, $p=0.0015$). Therefore, it can be concluded that MCMVdie1 attenuation *in vivo* and operates in both susceptible and resistant strains.

TNF production in infected-C57Bl/6 mice

Again ELISA was performed to measure TNF levels in selected organs after 4 days of infection. In agreement with previous studies [Orange and Biron, 1996, Lenzo et al., 2003], MCMV infected-C57Bl/6 mice induced TNF production. As seen in BALB/c (Figure 6.2), there were no significant differences in the levels of TNF produced after MCMV, MCMVdie1 or MCMVrev infection of C57Bl/6 mice. The highest cytokine production was found in spleen, where 2.5-fold more levels were detected when compared to the uninfected-mice (t-test, $p=0.03$), which showed a basal TNF production. Just a modest induction of TNF was found in the liver, and 1.5-fold higher level in kidneys (t-test, $p=0.013$) and lung (t-test, $p<0.01$) when compared to control samples. Although higher levels were also seen in heart of infected mice, it was not statistically significant (Figure 6.7).

Correlation between PFU and TNF levels in infected-C57Bl/6 mice.

Following the same procedure as in BALB/c and assuming the correlation between TNF and PFU, Figure 6.8 represents the relation between these two factors. From the graph it can be seen that the levels of TNF induced by MCMVdie1 are significantly higher, considering equal input of PFU in all groups. MCMV and MCMVrev however, showed a normal profile where TNF levels are high when viral titers are also high.

6.4 Conclusion

After MCMV infection, a number of cells are activated to control viral replication and spread throughout the host. This immune response involves production of cytokines or even direct lysis of infected cells. *tnf* gene expression has been demonstrated to be activated upon infection in different organs [Tang-Feldman et al., 2006] as well as protein production, which is detected early after infection in the spleen, liver [Orange and Biron, 1996] and heart [Lenzo et al., 2003] of infected mice. MCMV has evolved with its natural host in a way to escape immunosurveillance and endure in a latent state.

Data presented in Chapter 4 suggest a potential modulation of TNF production in infected-BMMθs, involving IE1. The experiments described in the present chapter were undertaken in order to assess whether MCMV infection had the same effect on cytokine production in the natural host. Thus, two different mouse strains were used and infection was carried out with parental MCMV and *ie1*-deletion mutant MCMV. A revertant virus was also included in the experiment (MCMVrev).

In summary, the results presented in this study showed that MCMV IE1 protein is necessary for the growth of the virus in the natural host, since the *ie1*-MCMV mutant failed to replicate as the parental and revertant virus. Moreover, the attenuation seen for MCMVdie1 is not strain-dependent, since in both BALB/c (which has been confirmed with these experiments) and C57Bl/6 mice MCMVdie1 showed the attenuated phenotype. When TNF levels were studied it was found that cytokine levels in all organs examined were similar after infection with either the mutant or parental and revertant virus. Overall, these results showed that MCMVdie1 induced TNF production in the natural host but does not demonstrate that IE1 is responsible for TNF regulation. In fact, it could be argued that the levels of TNF after MCMVdie1 infection might not be due to a modulatory mechanism but due to a defect on MCMVdie1 instead, resulting in lower yields and, therefore, the correlation led to high levels of TNF. Perhaps an investigation in the number of infected cells would be helpful to determine whether MCMVdie1-infected cells is similar to those infected by the parental and

revertant virus. Finally, it would also be helpful to determine the source of TNF to know whether bystander cells are also responsible for such cytokine response. Therefore, the role of IE1 in the regulation of TNF remains open for further investigation.

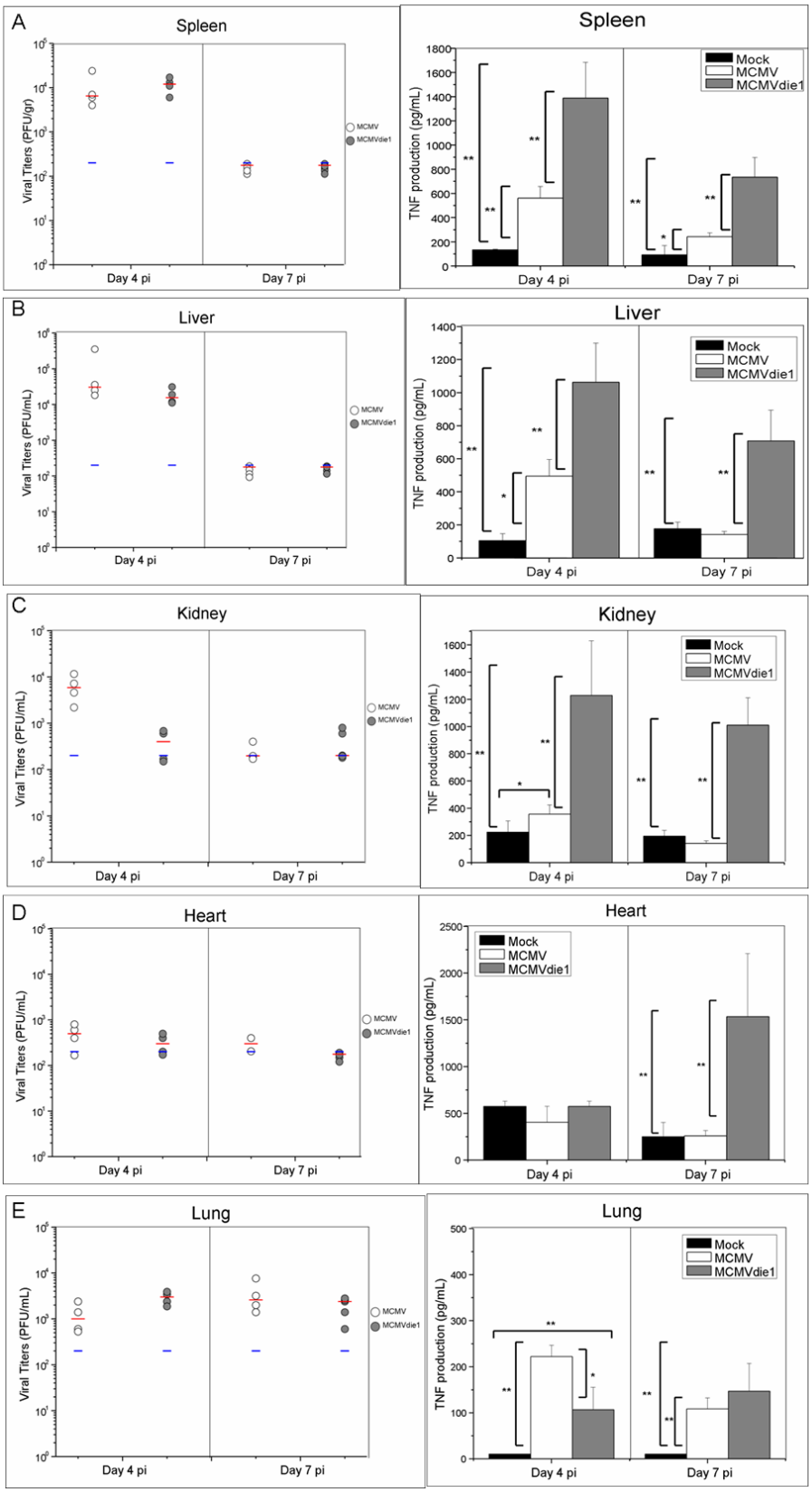

Figure 6.5: Titers and TNF production by MCMV.

Groups of BALB/c mice (n=4 mice per group, except MCMVdie1-infected mice at 7 days was n=5) were inoculated with 3 x 10^5 PFU of tissue-derived MCMV and compared to MCMVdie1. On day 4 and 7 post infection mice were killed and indicated organs were harvested, weighted, and sonicated as a 10% (wt/vol) tissue homogenate in DMEM. Viral titers were determined by standard plaque assay on MEFs (left panels). Red lines show median values and blue lines show limit of detection. Right panels show TNF levels detected from tissue homogenate by ELISA at indicated times. Significance in TNF levels is shown as $p<0.05$ (*) and $p<0.01$ (**).

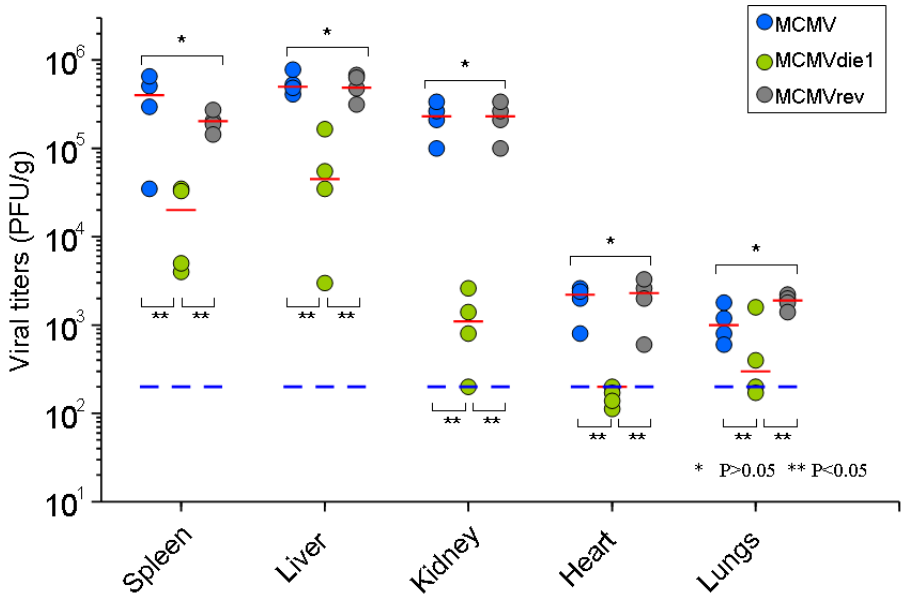

Figure 6.6: Growth of MCMV, MCMVdie1 and MCMVrev in different organs of infected-C57Bl/6 mice at day 4 post infection.

Groups of C57Bl/6 mice (4 mice per group) were inoculated with 2 x 10^6 PFU of tissue-derived parental MCMV, MCMVdie1 or MCMVrev. On day 4 post infection mice were sacrificed and the indicated organs were harvested, weighted, and sonicated as a 10% (wt/vol) tissue homogenate in DMEM. Viral titers were determined by standard plaque assay on MEFs cells. Red lines show median values and blue lines show limit of detection. There was no significant differences in the growth of MCMV and MCMVrev (t-test, $p>0.05$), but MCMVdie1 replication was significantly lower when compared to the parental and revertant MCMV (t-test, $p<0.05$).

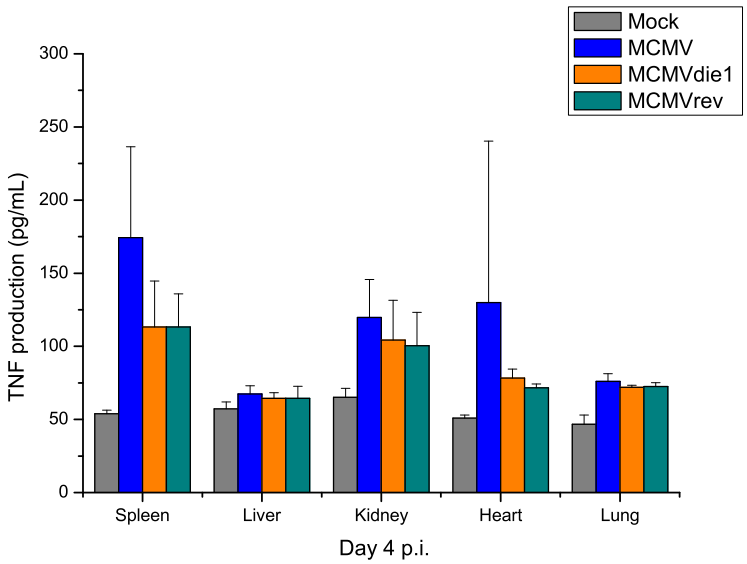

Figure 6.7: TNF levels in C57Bl/6 mice at day 4 post infection.

Levels of TNF were determined by ELISA after 4 days post infection. All three infections induced TNF productions in organs examined. No significant differences were found in the cytokine levels induced by MCMVdie1 and parental or revertant virus.

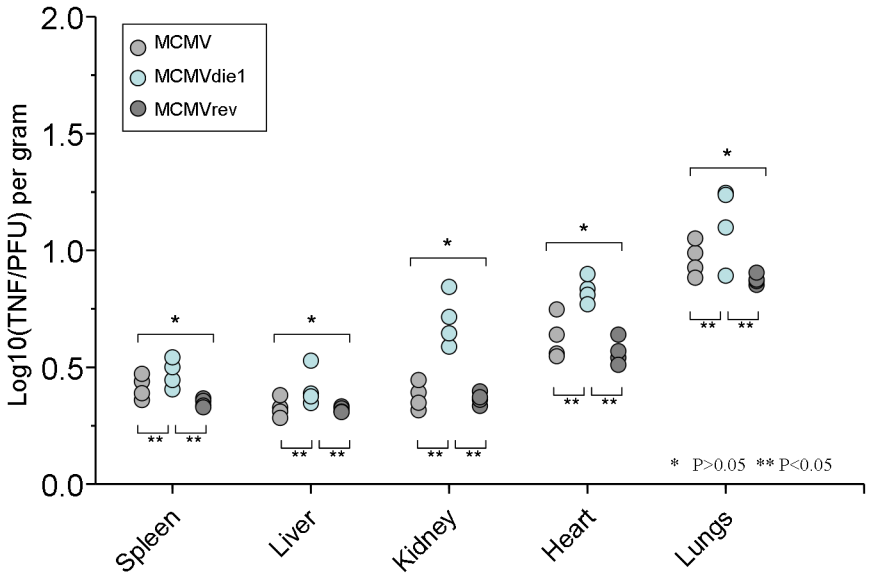

Figure 6.8: TNF levels versus PFU in C57Bl/6 mice at day 4 post infection

Levels of TNF against PFU in different organs of infected C57Bl/6 mice. At indicated times organs were harvested, weighted and homogenate as a 10% (wt/v). TNF levels were determined by ELISA from the homogenate.

CHAPTER 7

Role of TNF in MCMV infection *in vivo*

7.1 Introduction

So far it has been shown that IE1 MCMV protein is necessary for optimal viral replication *in vivo*, since in both immunocompetent BALB/c and C57Bl/6, MCMVdie1 presented an attenuated phenotype, incapable of reaching viral loads as MCMV or the revertant virus did. When TNF levels were studied after infection, it was seen, despite of the growth defect seen with the mutant virus, it was able to induce similar levels of TNF to those seen for the parental and revertant virus. These results gave a hint towards a potential modulatory strategy where MCMV by its IE1 protein reduces TNF levels not only *in vitro*, as seen from Chapter 4, but also *in vivo*.

The use of neutralizing antibodies, as well as genetically modified mice, has helped to evaluate in more depth the role that cytokines and immune cells play in the immune response against viral infections. Thus, blocking IL12 activity showed how important this cytokine is for an optimal activation of NK cells and the subsequent production of IFNγ after MCMV infection [Orange et al., 1995]. Moreover, the effect of depleting IL12 is specific for MCMV, since the blockade of IL12 activity does not have any effect on NK-induced IFNγ production after LCMV infection [Cousens et al., 1997]. MCMV infection of mice depleted of NK cells by neutralizing antibodies resulted in an increased MCMV burden in spleen, liver [Orange and Biron, 1996] and lungs [Krmpotic et al., 2002]. These results established the relevance of this cellular subset for the early control of

viral infection. Furthermore, it appeared that NK-induced antiviral activity to MCMV infection is organ-specific, since neutralizing IFNγ in MCMV-infected mice showed higher viral replication in the liver, but not in the spleen. The results shown in this study led to the conclusion that control of MCMV infection was NK-induced IFNγ-dependent in the liver whereas in the spleen the antiviral mechanism was IFN-independent but perforin-dependent [Tay and Welsh, 1997].

In agreement with the findings from Tay et. al., and regarding the role of IFNγ, neutralizing IFNγ by specific antibodies [Orange et al., 1995] or genetic depletion [Pomeroy et al., 1998] resulted in high susceptibility to MCMV infection and increased viral replication. In addition, $CD4^+$ T cell-induced IFNγ has been demonstrated to be critical for MCMV clearance from the salivary glands [Lucin et al., 1992], whereas $CD8^+$ T cells are necessary for the control of infection in the rest of the organs [Pavic et al., 1993]. Interestingly, there is evidence showing that in the absence of the $CD8^+$ T cells subset, $CD4^+$ T cells alone are capable of controlling viral infection in tissues other than the salivary glands [Jonjic et al., 1990]. This fact showed how flexible the immune system is in adapting the response in specific situations. Besides the protective effects that IFNγ has in control of the MCMV acute infection, IFNγ is significantly produced after a second infection [Shanley et al., 2001], suggesting a role in the control of viral replication in a second exposure to the pathogen, and also in control of reactivation of virus from latency.

Blocking IFN$\alpha\beta$ by administration of specific antibodies considerably increased MCMV titers in the liver of infected-mice [Orange and Biron, 1996]. The depletion of IFN$\alpha\beta$ has been shown to have other effects in the control of MCMV replication. For instance, enhanced IL12 production and NK-induced IFNγ production [Cousens et al., 1997], demonstrating that type I IFN also regulates the production of these cytokines, maintaining the balance in the cytokines response. $CD8^+$ T cell expansion and activation are also impaired. Overall, by blocking the

activity of a single cytokine such as IFN$\alpha\beta$ the activation of the innate response to control MCMV infection is affected. This demonstrates the key role that IFN$\alpha\beta$ plays in orchestrating the antiviral activity at early times upon infection. Nevertheless, Krug et. al showed that *in vivo* depletion of cells responsible for interferon production by neutralizing antibodies did not result in higher MCMV titers in spleen. Moreover, it appeared that infected mice were able to counterbalance the reduction of IFN$\alpha\beta$ by increasing the production of other cytokines, such as IL12 and IFNγ [Krug et al., 2004]. This example of compensatory mechanism is very important when interpreting *in vivo* data.

Lymphotoxin has also been shown to be critical for controlling viral replication. In previous sections of this thesis it has been quoted that LTβ is produced after infection and that LTβ-LTβR signalling is crucial for the production of IFN$\alpha\beta$ by splenic stromal cells. Thus, it is not surprising that blocking LTβ-induced signalling results in high viral burden. The crosstalk between LTβ-LTβR signalling and IFNβ was demonstrated when the antiviral activity was restored by administrating IFNβ [Banks et al., 2005]. Moreover, it has been reported that LTβ signalling activates the production of IFNβ, but requires the presence of MCMV [Benedict et al., 2001]. Hence, type I IFN is not only crucial for activation of NK and $CD8^+$ T cells, and subsequent cellular responses, but also affects LTβ-induced antiviral activity which ultimately results in inhibition of viral replication.

7.2 Aim of the chapter

The aim of this Chapter is to further analyse the role of TNF in the antiviral response against MCMV infection *in vivo*. From the data presented in previous Chapters it was suggested that MCMV IE1 protein modulates the TNF response induced after infection, since the *ie1*-deletion mutant MCMV led to a significant cytokine production, in both primary Mθ and in the natural host. These results

led to study in more detail the role of TNF in the control of viral infection. Therefore, work presented in this Chapter was designed to answer the following questions: (1) what would be the effect of TNF on MCMVdie1 replication in BMMθs, considering the significant cytokine production registered after infection and (2) is TNF responsible for the attenuated phenotype of MCMVdie1 *in vivo*. Taking into account the levels of TNF produced in the different organs analyzed it was expected that the growth defect seen for MCMVdie1 would be restored to normal levels in the absence of TNF response.

7.3 Results

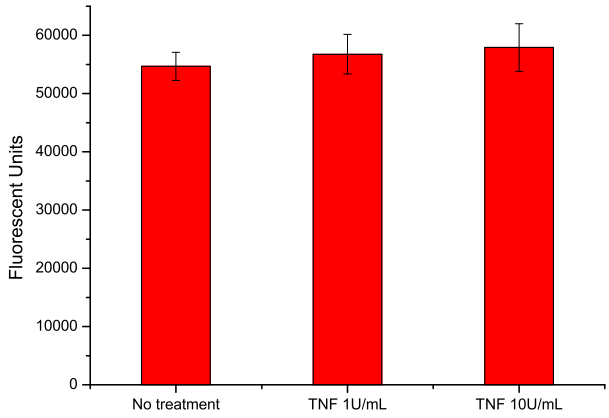

Figure 7.1: CellTiter-Blue^R Cell Viability Assay

Viable cells were determined by fluorescent-based cell viability assay after treatment for 24 hours of BMMθs with different concentrations of TNF (1U and 10U/mL) along with a non-treated control. Fluorescent units are shown along with standard deviation bars. No differences were seen between treatments and non-treated cells.

7.3.1 Effect of TNF on viral replication.

Previous sections in this thesis have demonstrated that MCMVdie1 infection results in a significant production of TNF in BMMθs. It has been reported that TNF pre-treatment of MEFs results in a reduction of MCMV replication [Lucin et al., 1994]. So it was hypothesized that TNF-treated BMMθs control MCMVdie1 replication by cooperative antiviral effect exerted by both exogenous and endogenous TNF. In order to ascertain if this hypothesis was true, BMMθs were cultured and incubated with 1U/mL or 10U/mL of TNF 24 h, after which cells were infected

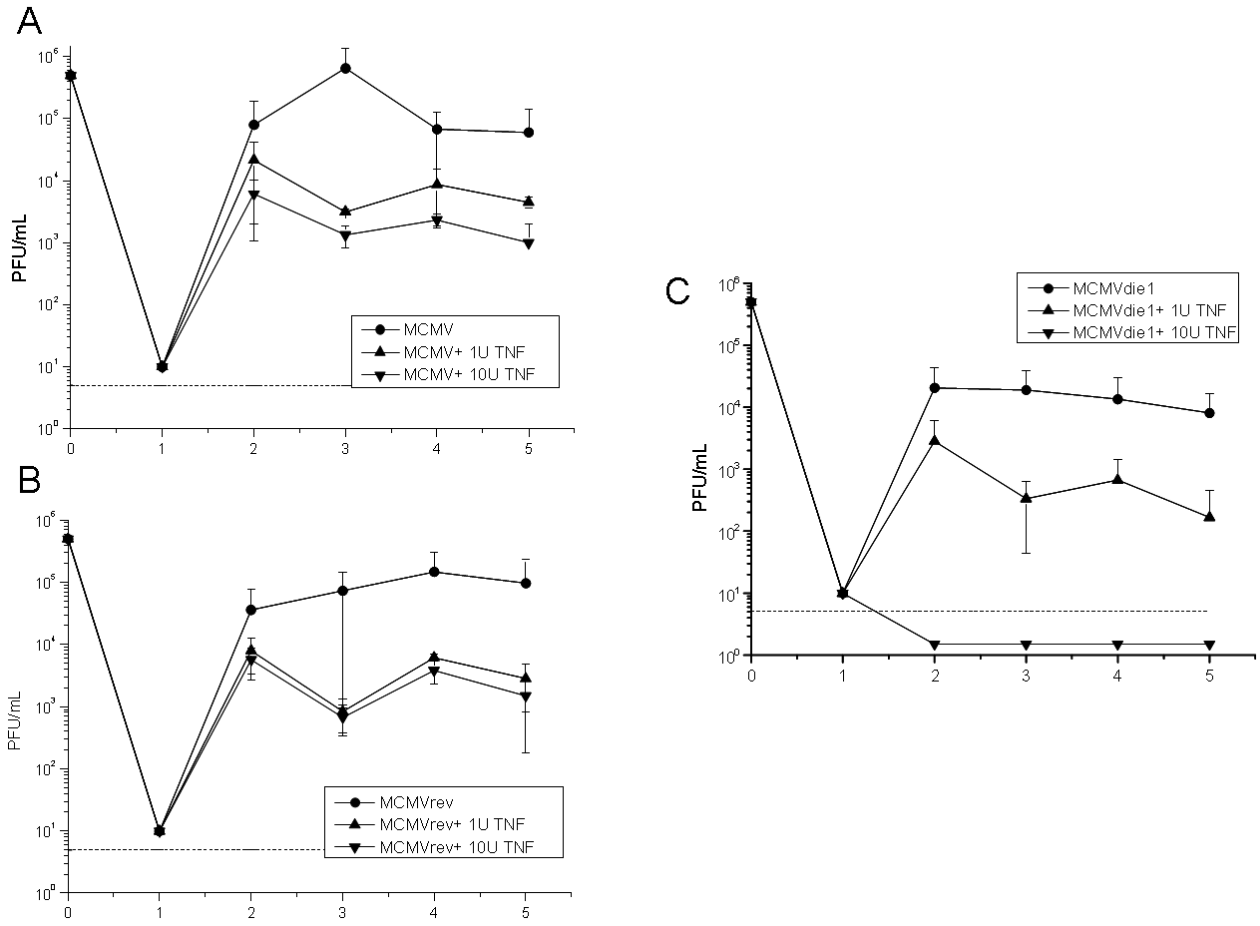

Figure 7.2: TNF effect on viral replication in BMMθs

Cells were treated with either 1 or 10U of TNF for 24h prior to infection. After 24h cells were infected with the different viruses at MOI 1. Intracellular and extracellular titers were determined by standard plaque assay. A. MCMV, B. MCMVdie1 and C. MCMVrev. Each data point represents the average and standard deviation of results of three different samples. Dash line represents limit of detection.

at a MOI 1 with MCMV, MCMVdie1 and correspondent revertant (n=3) and viral replication was compared to non- TNF treated and infected BMMθs. Prior to infection, cell viability was tested to rule out that the dose of TNF was toxic or induced apoptosis of the cells. Figure 7.1 shows that a 24 h treatment of the cells with either 1U or 10U of TNF did not have any effect on the viability of the cells. Cells were then pre-treated and infected and the antiviral effect of TNF was determined by standard plaque assay.

As described in MEFs, TNF has a weak but clear antiviral effect on MCMV (Figure 7.2.A) since a reduction of 23% and 37% of MCMV titers was seen after treatment with 1U and 10U of TNF, respectively. Replication of MCMVrev was also impaired by TNF in a very similar way as observed for the wild type MCMV, with a reduction of viral replication of 30% after 1U TNF treatment and 38% after 10U TNF (Figure 7.2.B). A reduction of 45% in MCMVdie1 titers was found in 1 U/mL pre-treated BMMθs. Moreover, the complete inhibition of MCMVdie1 replication after 10U TNF treatment was seen (Figure 7.2.C). These results are consistent with previous findings demonstrating the antiviral effect exerted by TNF [Lucin et al., 1994]. The antiviral effect exerted by TNF alone in MEFs was weaker than the effect reported here. This difference might be explain by the fact that fibroblast do not produce TNF, and cytokine levels induced after infection would synergyze with exogenous TNF resulting in a higher reduction of viral titers. Considering the high levels of TNF induced by MCMVdie1 it is not surprising that the higher reduction were seen in the mutant titers. It has to be noted that the levels of TNF used for BMMθs treatment did not exceed from virus-induced TNF levels.

It could be argued that suppression in MCMVdie1 replication might be due to a defect in the virus, however MCMVdie1 does not have a phenotype in tissue culture as shown by [Ghazal et al., 2005]. Results presented on Chapter 3 showed that in BMMθs MCMVdie1 tended towards lower yields, although no statistical difference was found. This observation might be explained by the fact that the cytokine repertoire induced by fibroblasts do not include TNF which could be the reason of such a high sensitivity of MCMVdie1. In addition, sensitivity of MCMVdie1 to TNF could also be explained by the result of the engagement with other cytokines that, when combined, successfully control viral replication. For instance, IL6 is produced after infection (see Chapter 3) and the antiviral effect of this cytokine on MCMV replication has been documented, therefore it is possible that together, along with chemokines, suppress viral growth.

7.3.2 *Role of TNF in viral replication in vivo.*

Work presented in Chapter 6 pointed to a potential role of IE1 in the modulation of TNF production in the natural host. This result along with the attenuated phenotype that the *ie1*-deletion mutant MCMV showed *in vivo*, and the high sensitivity that MCMVdie1 presented to TNF treatment, led to pose the question of whether TNF was responsible for such attenuation *in vivo*. To test this hypothesis, genetically modified C57Bl/6 mice lacking *tnf* gene ($TNF^{-/-}$ C57Bl/6) were infected with MCMVdie1 and MCMVrev in order to establish the role of TNF in the immune response against MCMV infection *in vivo*. Wild type MCMV was not included in these experiments since there were no significant differences in the replication between MCMV and MCMVrev in infected C57Bl/6 mice. Therefore, groups of 8 week old, male wild type C57Bl/6 and $TNF^{-/-}$ C57Bl/6 mice were infected by the i.p. route with 2 x 10^6 PFU. After 4 days of infection mice were killed and spleen, liver, kidneys, heart and lung were harvested and processed as described before (section 2.8.2). The significance of the data presented in this chapter was determined by Student's T-test, after the normality of the population was established, according to Lilliefors' hypothesis test.

When MCMVrev replication was studied in $TNF^{-/-}$ C57Bl/6 mice, and in agreement with previous data [Pavic et al., 1993], no significant differences were seen in viral burden in the spleen when compared to infected-wild type C57Bl/6 mice. Viral replication in the kidneys of TNF-depleted and MCMVrev-infected mice was not higher either in comparison to wild type mice. In the liver a slight increase in viral replication was found, and in heart and lung the highest viral load was observed, being statistically significant only in the heart (t-test, $p<0.05$). Similar results have been already seen in the lungs [Pavic et al., 1993] and liver of TNF-depleted and MCMV-infected mice [Orange and Biron, 1996]. However no significant differences were observed in MCMVrev replication in these organs. Surprisingly, when replication of the *ie1*-deletion mutant was studied in $TNF^{-/-}$ mice it was seen that viral growth was not restored. Contrary to what was expected, there was just a slight improvement in the growth of MCMVdie1 in the absence of TNF, similar to that seen for the revertant virus. Only in the heart of

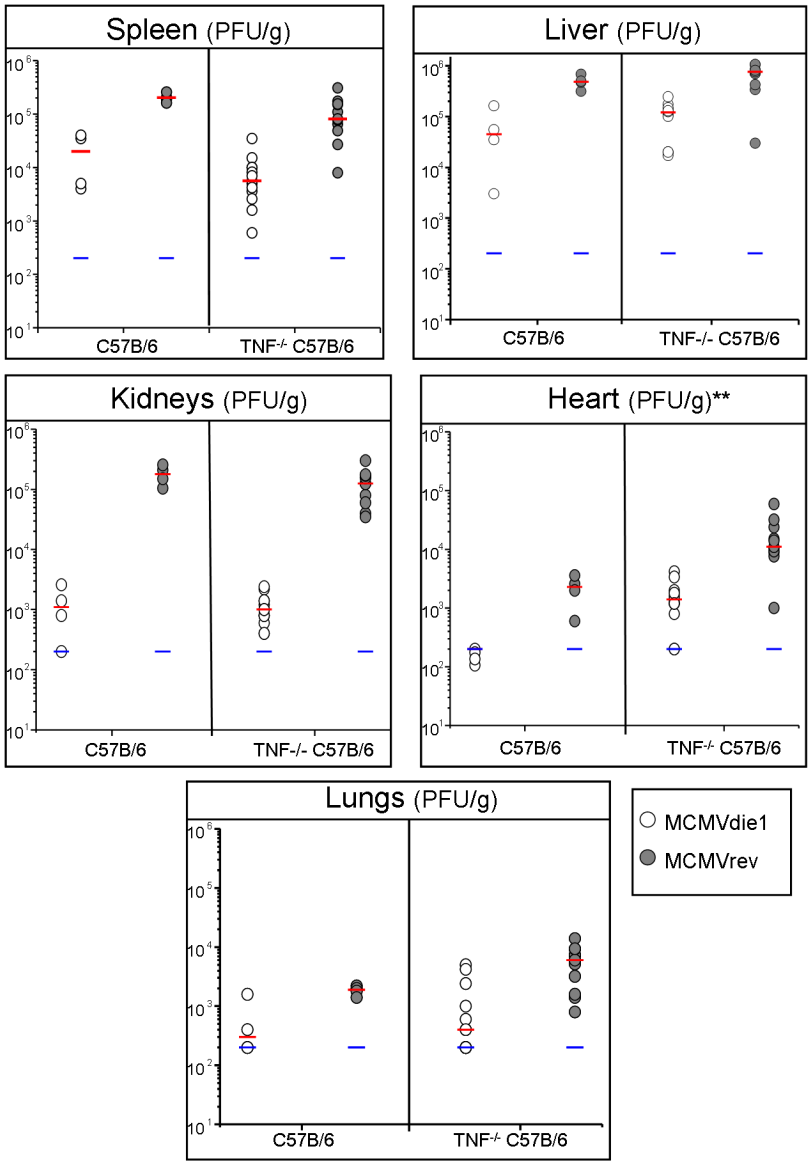

Figure 7.3: Growth of MCMVrev and MCMVdie1 in different organs in wild type and TNF$^{-/-}$ C57Bl/6 mice after 4 days of infection.

Groups of 13 TNF$^{-/-}$ C57Bl/6 mice were inoculated i.p. with 2 x 10^6 PFU of tissue culture-derived MCMVrev or MCMVdie1. On day 4 post infection mice were killed and selected organs were removed, weighted and sonicated as a 10%w/v. Viral titers were determined by standard plaque assays in MEFs. Viral growth in TNF$^{-/-}$ C57Bl/6 is compared to titers found in wild type C57Bl/6 mice. Only viral replication in the heart was significantly different for both MCMVdie1 and MCMVrev (t-test, $*$ $p<0.05$). Red lines show median values and blue lines limit of detection.

infected mice the replication of MCMVdie1 was significantly higher when compared to titers of virus in immunocompetent infected-mice (Figure 7.3). As in MCMVrev-infected TNF$^{-/-}$ mice, viral titers in liver, kidneys and lungs were

modestly higher than those seen in infected-wild type C57Bl/6, but not statistically significant. Finally, the absence of TNF did not have any effect in viral replication in the spleen.

From the data presented in this chapter it can be concluded that TNF is not responsible for the attenuation of MCMVdie1 *in vivo*, since no significant changes were seen in the replication of MCMVdie1 in the absence of TNF.

7.4 Conclusions

Infection with MCMV results in the activation of a network of cells and cytokines which control viral replication. Within this pool of cytokines, TNF is produced in spleen, liver, kidneys, lung and heart after viral challenge of both immunocompentent BALB/c and C57Bl/6 mice, as demonstrated in Chapter 6 of this thesis. *In vitro* work has suggests that MCMV modulates the production of TNF by its IE1 protein. MCMVdie1 replication in BMMθs cells resulted in a slight decrease on viral yields at late time of the course of infection. When TNF effect was studied on viral replication it was seen that, as seen previously in other cell type, TNF exerted an antiviral activity and viral replication was reduced after TNF treatments in a dose dependent manner. However, TNF-induced antiviral effect was more dramatic in MCMVdie1-infected cells, resulting in the complete inhibition of viral replication after 10U/mL TNF treatment. It can be concluded that TNF antiviral effect in BMMθs is more effective than in MEFs probably because of the lack of synergy between exogenous and endogenous TNF, and that MCMVdie1 presents high sensitivity to TNF since it was not able to overcome the TNF-induced antiviral effect *in vitro*. It has to be taken in consideration the activation of other pathways and cytokines leading to the control of the viral infection.

Moreover, using *ie1*-deletion mutant MCMV it was seen that the absence of this immediate early protein resulted in a growth defect *in vivo*. When TNF production was studied after infection with MCMVdie1 it was seen that MCMV might also modulates TNF induction in the natural host, since cytokine levels induced by MCMVdie1 were similar to those seen for the parental and revertant

virus. Considering the growth defect presented by MCMVdie1 and the significant cytokine production, it was hypothesized that the attenuated phenotype of MCMVdie1 was due, at least partially, to the strong TNF response induced in the absence of IE1 protein. The results presented in this Chapter also showed that, even though MCMV modulates the production of the proinflammatory cytokine TNF *in vitro* (Chapter 4) and *in vivo* (Chapter 6), TNF is not responsible for the attenuated phenotype that the *ie1*-deletion mutant MCMV presents in the natural host. However, the slight increase in viral yields found in most organs analyzed do not exclude TNF as a player in the control of MCMV infection. Moreover, it cannot be ruled out that the presence of another cytokine such as IFNγ, which has been shown to be essential for the complete control of MCMV infection *in vitro*, blocks viral replication but requires the presence of TNF for the complete viral clearance *in vivo*.

The initial hypothesis was based on the attenuated phenotype that MCMVdie1 presents in immunocompetent BALB/c mice, as well as in SCID mice, lacking T and B cells [Ghazal et al., 2005]. These results led to question whether the attenuation was due to a high susceptibility to the innate immune response. Since data might indicate that MCMV suppresses TNF production throught an unkown mechanism which involves IE1 *in vitro* and *in vivo*, it was hypothezised that TNF was responsible for the attenuation of the *ie1*-deletion mutant MCMV *in vivo*. However, the results showed in the above sections of this Chapter showed that TNF is not responsible for the attenuated phenotype of the mutant virus.

It has been reported that neutralization of TNF *in vivo* does not result in a significant increase in viral titers [Shanley et al., 1994]. The lack of improvement in viral replication might be explained by the fact that MCMV is not producing the optimal amount of TNF, since it is expressing IE1, which is suppressing the cytokine production. However, and assuming that this hypothesis was true, the depletion of TNF would have an effect in the mutant virus replication. Contrary to what was expected, the results presented in this Chapter showed that TNF alone is not required for the control of viral infection *in vivo*.

On the other hand, the fact that all mice survived MCMV infection, led to the question whether the genetic depletion of the *tnf* gene could have induced the development of compensatory mechanisms to counterbalance the lack of TNF. For instance, overactivation of immune cells or overproduction of other cytokines such as type I or II IFN has been previously described [Krug et al., 2004]. Such compensation would help, at least, to control the infection and forestall the death of the host. Thus, the fact that an organism is able to counteract the lack of a specific cytokine or cellular subset does not mean that it is not important for the control of infection. Perhaps, by forcing the overproduction of other cytokines or overwhelming different cells with activation signals the success in viral clearance is transient and may not be successful in a second exposure.

CHAPTER 8

Final Conclusions

8.1 Summary of results and discussion

The IE1 protein of murine CMV has been shown to be involved in the activation of the viral gene transcription in collaboration with the IE3 protein, and in driving the switch from the immediate early to the early phase of the MCMV transcription cascade [Angulo et al., 2000]. In addition, IE1 induces the activation of several cellular genes in order to create the optimal environment for viral replication [Koszinowski et al., 1986, Gribaudo et al., 1996, Schickedanz et al., 1988, Gribaudo et al., 2000, Lembo et al., 2000]. It has also been reported the dispensable role of IE1 in viral replication in tissue culture [Ghazal et al., 2005] since an *ie1*-deletion mutant MCMV (MCMVdie1), lacking the entire exon 4 of the *ie1* gene, replicated as wild type in several cell lines. In contrast, IE1 is required for optimal replication in the natural host [Ghazal et al., 2005]. The fact that the *ie1* mutant virus also presented a growth phenotype in SCID mice, which exclusively relies on the innate immune response to control the infection raise the possibility of an immunomodulatory role for IE1 protein. Hence, the initial hypothesis of this investigation was that the IE1 protein is involved in the regulation of the immune response triggered upon infection.

8.1.1 Effect of IE1 protein in viral replication in BMMθs.

It is now well established that after MCMV infection, innate immunity plays a critical role in the initial control of the virus. This first defense is characterized by the activation of macrophages, NK and dendritic cells. Upon activation a cascade of cytokines and chemokines production is initiated in order to alert bystander

cells of the invasion and recruit immune cells to the site of infection. This initial response is triggered to inhibit viral replication and dissemination throughout the host. However, as other viruses, MCMV has also developed strategies to escape from immunosurveillance. The success of such an evolution can be seen from the fact that MCMV can be found in organs such as the salivary glands after resolving the acute infection.

Within the cells activated early after infection, macrophages have been described as having 'two-faces' in the context of MCMV infection. On one hand, Mθs are essential for viral replication, dissemination and pathogenesis, and on the other hand they are crucial for the control of viral infection. Therefore, it was of interest to study the role of the IE1 protein in the control of the immune response in this cellular population. In addition, it was possible to further understand the interplay between MCMV and Mθs. Therefore, the first step in this investigation was to characterize the role of the IE1 protein in viral replication in BMMθs. Contrary to other viruses, it has been shown that IE1 is not essential for viral replication in fibroblasts, endothelial cells and peritoneal macrophages. However, replication in BMMθs has not been described. Results from independent experiments revealed that the *ie1*-deletion mutant MCMV also showed comparable replication in BMMθs (see Figure 3.1), however, a trend towards lower viral yields is observed at late times of the infection at both MOIs studied. These differences were not found to be statistically significant, reflecting high level of variability between samples and the overall sensitivity of the plaque assay. In addition, the fact that this cellular population is considered as semi permissive for viral infection might also explain that titers were not statistically significant, which might indicate a similar replication of the mutant in this cellular population. It could also be argued that the trend towards a lower replication of MCMVdie1 could be due to a critical defect on the viral replication. Time constraints prevented to explore this possibility and therefore this investigation was carried out under the assumption of the MCMVdie1 is not defective. Nevertheless, it would be very informative to investigate the PFU/viral genome copies ratio, since it would elucidate whether or not the absence of IE1 results

in a defective virus. In addition, in order to rule out any second site mutations and ensure that the effect seen was solely due to IE1 protein a revertant virus (MCMVrev) was also included in this investigation. As expected there was no differences between replication of wild type and revertant virus. The trend toward lower titers of MCMVdie1 is not completely understood. The effect of the absence of the IE1 protein on viral gene expression has not been elucidated yet. However, since MCMVdie1 does not present a phenotype in culture cells this response might be due to factors produced by macrophages which might have an effect on viral replication, such as cytokines.

8.1.2 Role of IE1 protein in the regulation of host cell gene expression after early hours post infection.

As part of this project, an initial study was carried out in order to obtain information about how IE1 might affect host cell gene expression. Due to the expensive cost of microarrays, experiments were only performed for MCMVdie1 and MCMVrev-infected BMMθs. While unequivocally conclusions cannot be drawn from this experiment, the data has been used for informative purposes only, as well as an example of how microarray data could be analysed. Considering the role of the IE1 in the expression of cellular genes an investigation of the host gene expression by the presence of IE1 was conducted, with particular interest in immune genes. Hence, a comparative study between MCMVdie1 and MCMVrev-induced gene expression was performed (see Section 8.1.2). Microarray technology was used in this investigation since it gives an important amount of information regarding the genes which expression is affected after viral infection. From the microarray it was seen that, after 10 hpi, approximately 550 genes were differentially expressed. Surprisingly nearly 30% of these genes were involved in the immune response, including surface markers, receptors, cytokines and transcription factors. To further understand how these genes interact and relate to each other and what biological processes might be associated with the Ingenuity Pathway Analysis (IPA) application was used. The extensive database of IPA enabled the generation of genetic networks showing the interaction between those selected genes. Along with the interaction networks, IPA also identified

the canonical pathways which were the most affected. Surprisingly, both networks and pathways also recognized processes such as apoptosis and cytokine signalling pathways as relevantly affected by the viral protein. Therefore, this initial study raises the possibility of a role of IE1 protein in the regulation of the host cell gene expression. Moreover, IE1 might also be specifically targeting the immune response triggered upon infection.

The use of IPA as a tool for analysing microarray data has been shown to be useful regarding the identification of potential new candidates for regulation. In this investigation, it was interesting the fact that the lipid metabolism was one of the biological processes to appear as targeted after infection. There is no much information in the literature regarding the interaction between viral infection and lipids. On the other hand, there are experimental data showing a link between lipids and inflammatory response. One of this examples could also be highlighted from the data. The enzyme CH25H appeared to be differentially expressed. This enzyme has a role in the regulation of the cholesterol metabolism. Interestingly, it has been reported that cholesterol plays an important role in the activation of the Erk1/2 phosphatase [Wang et al., 2003], by supporting the OSBP and the specific enzymes in an active complex [Wang et al., 2005]. Furthermore, this kinase has been involved in the regulation of the expression of proinflammatory cytokines, such as TNF, which also plays a role in the control of MCMV replication. Therefore, pathway analysis could be useful to raise new hypothesis such as IE1-induced modulation of the immune response throught the regulation of the lipid metabolism.

All together, microarray and pathway analysis are useful tools to give an insight into potential regulatory functions.

8.1.3 IE1 and modulation of TNF production in infected-BMMθs at the transcript and protein level.

To further characterize the role of IE1 protein in infected-BMMθs, the cytokine production was studied after early hours of infection. These experiments were focused on the cytokine response based on the important role that they play in the immune response against MCMV infection. Therefore, it was of interest to

study the effect of IE1 protein in this response. Independent experiments were carried out (see Section 4.3.1) and, markedly, when cytokine levels were measured from the supernatants of infected-BMMθs it was seen that MCMVdie1 induced a very significant production of TNF but not other cyokine except IL6, when compared to MCMV and MCMVrev. The IL6 response may be explained by the fact that IL6 production has been shown to be activated by TNF and therefore, the concentration of cytokine after MCMVdie1 infection is just the result of the significant TNF production.

Next, it was of interest to study whether the cytokine response was also regulated at the transcript level (see Section 4.3.2). Thus, *tnf* gene expression was studied by determining the relative levels of *tnf* mRNA. Quantitative RT-PCR revealed a similar regulation after infection with the mutant virus. MCMVdie1-induced *tnf* expression was 2.5-fold higher than MCMV and the revertant virus. Therefore, results pointed to a regulation of TNF involving the IE1 protein in BMMθs.

One of the responses in which macrophages have a role is cytokine and chemokine production. MCMV has also developed strategies to counteract cytokine production and/or cytokine-induced responses. Within the pool of MCMV's strategies to modulate cytokine responses, TNF has been targeted in virtually every step of its biology, although the regulation of TNF production is the less studied. Different groups have shown that both human and murine CMV regulate TNF-induced immune response by blocking the expression of TNF receptors [Popkin and Virgin, 2003, Baillie et al., 2003, Montag et al., 2006]. Interfering with TNF receptors is also shared with other viruses, such as Poxviruses which were the first example of a virus encoding a TNF receptor homolog [McFadden et al., 1997]. Along with the receptor, other molecules involved in the activation of TNF-induced responses have been shown to be on the list of targets. For instance, the viral protein M45 has been reported to block RIP1, in order to inhibit the proinflammatory response induced by TNF [Mack et al., 2008]. However, results from this investigation could indicate that MCMV modulates the production of TNF by mechanisms which involve IE1 protein. The direct or indirect

effect of this viral protein or whether other viral proteins are involved is yet to be discovered.

It has been shown, by using transient expression assays, that HCMV IE1 activates *tnf* expression [Geist et al., 1994] by modulation of transcription factors involved in the regulation of TNF [Geist et al., 1997]. However, these studies were based on expression plasmid experiments and outside the context of an infection. Experiments described in this investigation were based on complete viral genomes and respective deletion mutant revertants. Therefore, it is of interest to study how TNF production is affected under similar experimental conditions, considering the significance of HCMV as a human pathogen and TNF as cytokine.

8.1.4 Signalling studies to unmask a potential mechanism for TNF modulation

The next step in this investigation was to explore a potential transcriptional regulation of TNF production. An extensive literature review was done regarding the regulatory mechanisms of TNF production in different cells and in the context of several pathogens. From the literature, it was seen that several transcription factors were commonly involved in the activation of *tnf* by different stimuli, such as viral and bacterial infection. Within the transcription factors identified it was seen that some of them like Elk, CREB, are activated by members of the MAPK family through a signalling cascade. Therefore, this study also included the characterization of the activation of signalling molecules after viral infection in order to study the effect of *ie1* defective virus in the activation of these molecules, as well as the downstream effect in the induction of the TNF promoter. This study was carried out by two approaches. The first approach was a Luminex-based highthroughput technology analyzing a number of signalling molecules simultaneously. This experiment was done as part of a collaboration with Upstate (Millipore, UK). The second approach and in order to validate the first technology, conventional Western Blot was also performed against activated forms of the molecules of interest. Unfortunately, Luminex-based analysis was not consistent and therefore was not reliable for this study. On the other hand, it was also difficult to draw conclusions from the Western blot analysis since there was

a large variability between the samples. Nevertheless, initial observations might indicate that member of the MAPK family such as Erk1/2, p38 and JNK are not differentially activated in the absence of IE1 and therefore the regulation of TNF does not depend on the activation of these pathways. Clearly, further work has to be done in order to clarify the molecular mechanism by which IE1 targets TNF production.

8.1.5 TNF production after infection of the natural host.

It is possible that the observed *in vitro* phenotype of MCMVdie1 and TNF production may be restricted to tissue culture conditions. Hence, the next step of this investigation was to assess whether the absence of IE1 also resulted in the modulation of TNF production in the natural host. Several studies have already shown the activation of TNF production after MCMV infection *in vivo*. For instance, *tnf* gene expression in different organs has been demonstrated [Tang-Feldman et al., 2006] as well as protein production, which is detected early after infection in the spleen, liver [Orange and Biron, 1996] and heart [Lenzo et al., 2003] of infected mice.

The role of the IE1 protein in the production of TNF was studied by using two different mouse strains and cytokine production was measured after 4 and 7 (only in BALB/c) dpi. First viral growth was studied in order to (1) confirm attenuation of MCMVdie1 in BALB/c mice and (2) study viral growth in C57Bl/6 (see Section 6.3.2). Results confirmed that MCMV IE1 protein is necessary for viral replication *in vivo*. As expected, the *ie1*-MCMV mutant failed to grow as the parental and revertant virus in both competent BALB/c and C57Bl/6. TNF production was also determined in spleen, liver, kidney, heart and lung of infected mice, and compared to the cytokine production in non-infected control. Remarkably, similar levels of cytokine between viral infections were found in all organs examined. Since TNF production upon MCMV infection is positively correlated with the output of infectious virus (PFU/g tissue), these results indicate that IE1 also regulates the production of TNF *in vivo*. MCMVdie1-induced TNF production reached similar levels to those seen after MCMV and MCMVrev infection, regardless of the low viral titers detected. Taking into consideration

the difference in viral titers and in order to rule out that the results were affected by the non-equivalency of viral synthesis, a second study was carried out where BALB/c mice were infected with a different dose of parental MCMV and MCMVdie1 to obtain similar PFU and measure cytokine production under this condition (see Figure 6.5). ELISA showed that TNF induction after MCMVdie1 infection was significantly higher than the levels observed after MCMV infection. It could be argued that the levels of cytokine found in the different tissues is the result of the higher dose of MCMVdie1 used for the infection. However TNF levels were based on the output levels of infection it is also important to account for the input. Perhaps an investigation on the actual number of cells infected and the identification of the cells responsible for the cytokine response would help to resolve this issue. Therefore further studies couild be need to determine the contribution of input virus in the TNF response.

8.1.6 TNF is not responsible for the attenuated phenotype of MCMVdie1 in the natural host.

The *in vivo* studies raised the final question of whether the significant TNF production seen after MCMVdie1 infection was responsible, at least in part, for the growth phenotype that the mutant virus presents in the natural host. It has been demonstrated the antiviral role of TNF in tissue culture [Lucin et al., 1994], in synergy with IFNγ. However, the effect of the cytokine in MCMVdie1 replication has not been described. Therefore, TNF effect was studied in infected-BMMθs. As expected, infection of TNF-primed BMMθs resulted in a reduction of viral replication in a dose dependent fashion (see Figure 7.2). Moreover, TNF-induced antiviral effect was more dramatic in MCMVdie1-infected cells, resulting in the complete inhibition of viral replication after 10U/mL TNF treatment. This result might be explained by the synergy between exogenous and endogenous TNF in MCMVdie1-infected cells, however the activation of alternative pathways as well as the production of other factors involved in the control of the virus have also need to be taken into considerantion. On the other hand, as it has been stated before, it is also possible that the susceptibility of MCMVdie1 could be due to the fact that this is not a competent virus, which could explain these results.

To go further in this investigation, the role of TNF in the attenuation of MCMVdie1 replication *in vivo* was also tested (see Section 7.3.2). Thus, genetically depleted mice lacking *tnf* gene were infected with either MCMV or MCMVdie1 and viral replication was compared in different organs. If the significant TNF response seen after MCMVdie1 infection was responsible for the growth defect of the mutant virus, the absence of TNF production should result in an increase in MCMVdie1 burden. However, the results showed that TNF is not responsible for the attenuated phenotype that the *ie1*-deletion mutant MCMV presents in the natural host. Only a slight increase in viral yields found in most organs analyzed was seen but it does not exclude TNF as a player in the control of MCMV infection. Moreover, it cannot be ruled out that the presence of another cytokine such as IFNγ, which has been shown to be essential for the complete control of MCMV infection *in vitro*, blocks viral replication but requires the presence of TNF for the complete viral clearance *in vivo*. However, the results of this experiment clearly showed that TNF is not responsible for the attenuation of MCMVdie1 in the natural host.

On the other hand, the fact that all mice survived MCMV infection, lead to the question whether the genetic depletion of the *tnf* gene could have induced the development of compensatory mechanisms to counterbalance the lack of TNF. For instance, overactivation of immune cells or overproduction of other cytokines such as type I or II IFN has been previously described [Krug et al., 2004]. Such compensation would help, at least, to control the infection and forestall the death of the host.

8.2 Concluding remarks

This thesis has investigated the role of the MCMV IE1 protein in the regulation of the immune response triggered upon infection in BMMθs and in the natural host (BALB/c and C57Bl/6 mice). Previous studies have demonstrated that IE1 protein is required for viral replication *in vivo*, but not *in vitro*, where a mutant virus lacking the entire exon 4 of the *ie1* gene replicated as the parental virus. Since the *ie1* mutant virus showed an attenuated phenotype in competent BALB/c mice

and SCID mice, it was hypothezied that IE1 protein acts as a immuno modulator. The central hypothesis of the thesis was addressed by the following questions:

1. Does IE1 protein play a role in the gene expression of BMMθs?
2. Is it involved in the modulation of the cytokine response in BMMθs?
3. What are the molecular mechanisms underlying the cytokine production regulation?
4. Does MCMV regulate the cytokine response in the natural host as *in vitro*?
5. Is TNF responsible for the attenuated phenotype of MCMVdie1 *in vivo*?

During the course of this investigation, a number of important limitations were found and made not possible to draw any conclusions regarding the immunomodulatory function of IE1. Several issues should be addressed in order to clarify and better explain the results presented in previous chapters. Taking all the data together, it is not clear why MCMVdie1 does not show a replication defect in cells like fibroblasts but shows a decrease, although not statistically significant, in BMMθs. Initially it was assumed the competence of MCMVdie1 and therefore the hypothesis of the immunomodulatory function of IE1 was investigated. However, after reviewing the data it is clear that a comparative study on the MCMVdie1 genome copies could have been very useful to the interpretation of the results. On the other hand, the study of the activation of signalling molecules was unsuccessful to identify a possible pathway by which this modulation might be taking place. Same limitations were identified in studies done in the natural host where it was difficult to descifrer whether the production of TNF in different tissues were due to a regulatory function of the virus or just as a result of a defective virus.Finally, TNF depleted mice experiment did not reveal any significant change on MCMVdie1 replication *in vivo*, which means that this cytokine is not responsible for the attenuation of the virus *in vivo* in the absence of IE1.

Overall, work presented in this thesis has not proved the initial hypothesis of that IE1 is an immunomodulator targeting TNF *in vitro* and *in vivo*. Instead it has highlighted a number of limitations which have to be taken into account

for future work and that will definetly clarify most of the problems presented in previous chapters.

8.3 Future work

This thesis has demonstrated a novel function for the IE1 protein of MCMV and therefore has expanded the existing knowledge regarding the role of this viral protein in the host-pathogen interaction. However, the different findings obtained from this investigation also has raised new questions:

Investigation on the PFU/genome copies ratio. This study will clarify MCMVdie1 phenotype being possible to determine whether the lower replication of the virus is due to the immune control induced in the absence of IE1 or because the mutation resulted in a defective virus.

Molecular mechanism of TNF production. The results presented in this study has given an insight of a possible regulation of TNF production. Since it is known the role that TNF plays in the control of viral infection, and the fact that several pathogens have been shown to developed strategies to suppress this cytokine production, it could be very interesting to investigate the molecular mechanisms involved in this response in the context of MCMV infection.

Role of cytokines in the control of viral infection *in vivo*. Following findings presented in previous sections of this thesis it was seen that regardless the significant TNF production *in vivo* after infection with MCMVdie1, wild type phenotype was not recovered when TNF was genetically depleted. However, there are a number of other cytokines involved in the control of viral infections *in vivo*, such as type I and II IFN. To study how MCMV infection is affected by these factors could be also be very informative.

Production of TNF after HCMV infection. Human CMV is an important pathogen for immunocompromised patients. HCMV-induced pathogenesis has

been associated in part with the immune response triggered upon infection, therefore it would be of a great interest to determine whether the human virus has also evolved a mechanism for TNF production modulation. So far, it has been shown that the IE1 protein induces the activation of the TNF promoter, but no study has been done in the context of the life virus.

Role of MCMV in the lipid metabolism and how this regulation affect TNF production. Very few examples can be found in the literature regarding the role of lipids in viral infection. Microarray analysis pointed out that several enzymes involved in the metabolism of cholesterol were altered after viral infection. Hence, it would be interesting to further study to what extent viral infection, and in particular the IE1 protein, affects this metabolism and how the lipids affect viral and cellular response against infection.

APPENDIX A

General solutions and buffers

A.1 General solutions

- **10% Ammonium persulphate (APS)**
  1 g of APS (Sigma, UK) in 10 ml of distilled water. Solution was stored at 4C covered in foil.
- **10% sodium dodecyl sulfate (SDS)** (Invitrogen, UK)
- **TEMED** (Biorad, UK) Stored at 4°C.
- **Glycerol** (BDH Ltd., UK)
- **2x Laemmli sample loading buffer**
  0.125M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.01% bromophenol blue and 100 mM DTT (Sigma, UK) (just added prior to use).
- **TBS**
  6.05 g Tris base (Sigma, UK), 8.76 g NaCl (Sigma, UK). Add distillied water up to 800 mL and adjust pH to 7.5 with HCl. Make up to 1 L with distilled water.
- **1 M Tris buffer (pH 6.8)**
  12.1 g of Tris base (Sigma, UK) was dissolved in 80 ml of distilled water. pH was adjusted with HCl and volume was adjusted to 100 ml. Solutions was kept at RT after sterilization by autoclave.
- **1 M NaCl**
  5.84 g NaCl (Sigma, UK) into 80 mL distilled water Dissolved and made up to 100 mL with distilled water
- **Complete mini-Protease Inhibitor Solution**

1 tablet of the inhibitor (Roche) was added to 10 ml of lysis buffer. Stored at 4°C.

- **Phosphatase inhibitor Cocktail I**
  100 μL of phosphatase inhibitor cocktail (Sigma, UK) was added to lysis buffer just prior to use.
- **Phosphatase inhibitor Cocktail II**
  100 μL of phosphatase inhibitor cocktail (Sigma, UK) was added to lysis buffer just prior to use.
- **Beadlyte Cell Signaling Lysis Buffer** (Millipore, UK)
- **PBS** (Sigma, UK) Dissolve 1 tablet in 200mL.
- **10% Acrylamide gel for SDS-PAGE**
  For 10 mL, 4 mL of distilled water, 3.3 mL of 30% Acrylamide (Sigma,UK), 2.5 mL of 1.5 M Tris (pH 8.8), 0.1 mL of 10% SDS (Invitrogen, UK), 0.1 mL of 10% APS (Invitrogen, UK), 0.004 mL TEMED (Biorad, UK)
- **Stacking gel for SDS-PAGE**
  For 5 mL, 3.4 mL of distilled water, 0.83 mL of 30% Acrylamide (Sigma,UK), 0.63 mL of 1 M Tris (pH 6.8), 0.05 mL of 10% SDS (Invitrogen, UK), 0.05 mL of 10% APS (Invitrogen, UK), 0.005 mL TEMED (Biorad, UK)

A.2 Solutions for Tissue Culture

- **Calf Serum (CS)** (Lonza, UK)
  500 mL heat inactivate CS (56°CC for 35 min) were filtered in a StericupR vacuum filtration unit (Millipore, UK). The filtered serum was then aliquoted into 50 mL lots and store at - 20°C until use.
- **Fetal Calf Serum (FCS)** (Lonza, UK)
  500 mL heat inactivate FCS (56°C for 35 min) were filtered in a StericupR vacuum filtration unit (Millipore, UK). The filtered serum was then aliquoted into 50 ml lots and store at - 20°C until use.
- **L929 conditioned medium**
  Grow cells in a 165 cm^2 flask in DMEM:F12 growth medium. When confluent, trypsinise and re-suspend in 30 ml growth medium. Add 1 ml cells to

49 mL growth medium and seed into several 165 cm^2 flasks. Culture for 5 days, harvest medium, centrifuge at 1500 rpm for 10 min, filter, aliquot and freeze at -20°C.

- **Freezing medium**
  30 mL DMEM, 1.5 mL FCS (Lonza, UK), 5 mL DMSO (Sigma, UK). Aliquoted into 8 x 6 mL and stored at -20°C.
- **Trypsin/Versene** (Lonza, UK)
  200 mg/L Versene and 170000 U Trypsin/L

A.3 Solutions for SDS-polyacrylamide-gel electrophoresis (SDS-PAGE)

- **10X SDS running buffer**
  303 g of Tris base (Sigma, UK), 1442 g of glycine (Sigma, UK), SDS were dissolved in 8 L of distilled water. pH was adjusted to pH 8.3 and volume was adjusted to 10 L. Solution was kept at room temperature and was used as in 1X solution diluted in distilled water.
- **Solutions for chemiluminescent blotting**
  ECL Plus Western Blotting Detection reagents (Amersham Biosciences, UK)
- **Transfer buffer**
  25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5). Buffer was prepared fresh for every transfer.
- **10X Tris Buffered Saline (TBS)**
  To prepare 1 L of 10X TBS: 24.2 g Tris base, 80 g NaCl; pH to 7.5.
- **Washing buffer**
  1X TBS, 0.1% Tween-20 (TBS/T)
- **Blocking buffer**
  1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk
- **Primary Antibody Dilution Buffer**
  1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
- **Secondary Antibody Dilution Buffer**
  1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.

APPENDIX B

Microarray Data

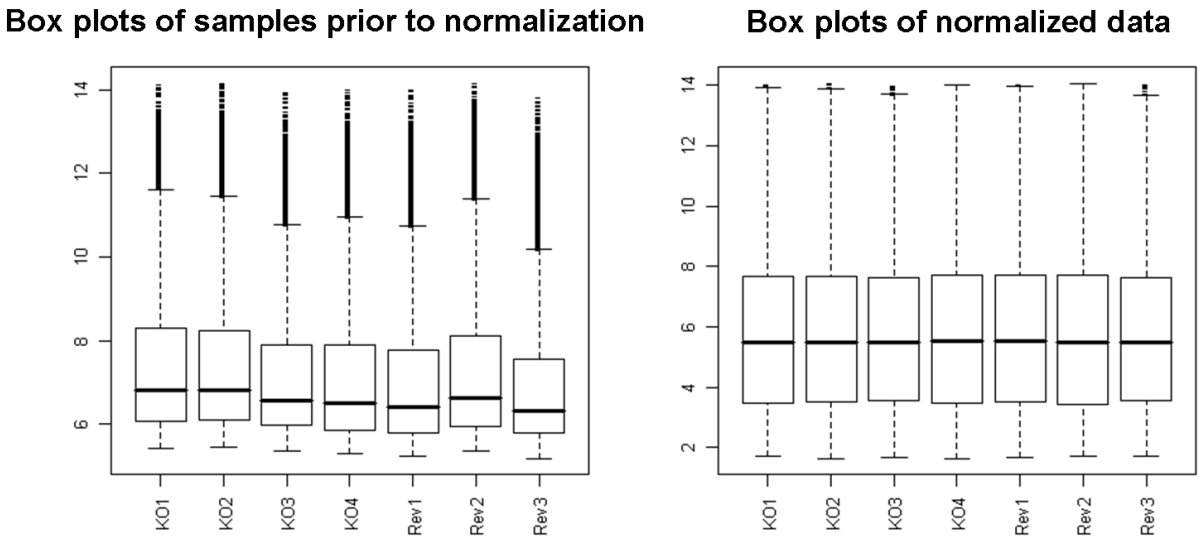

Figure B.1: Box plots before and after normalization

Box plot showing log2 intensity values from Affymetrix Mouse 430v2 array individual .CEL files. The bottom and top of the box represents the 25^{th} and 75^{th} percentile, and the band in the middle of the box represents the median. The horizontal lines, also known as the "whiskers", represent observed data below the first quartile and beyond the third quartile. In other words, the whiskers represent all observed value outside the box. The left pannel shows spread of the data before RMA and quantile normalization and right pannel shows data after normalization. Little variation was observed prior normalization, which was then reduced to minimal after normalization.

Figure B.2: List of genes down-regulated after MCMV infection in the absence of IE1 protein

Affymetrix ID	Gene Name	Gene symbol	Fold Change	p-value
1450297_at	interleukin 6	Il6	-3.75	0.00000089
1450446_a_at	suppressor of cytokine signaling 1	Socs1	-2.89	0.00015700
1450330_at	interleukin 10	Il10	-2.63	0.00021432
1416576_at	suppressor of cytokine signaling 3	Socs3	-2.5	0.00001817
1455899_x_at	suppressor of cytokine signaling 3	Socs3	-2.45	0.00052384
1450808_at	formyl peptide receptor 1	Fpr1	-2.29	0.00000488
1419123_a_at	platelet-derived growth factor, C polypeptide	Pdgfc	-2.19	0.00016388
1456212_x_at	suppressor of cytokine signaling 3	Socs3	-2.13	0.00243144
1455393_at	ceruloplasmin	Cp	-2.12	0.00013138
1429319_at	ras homolog gene family, member H	Rhoh	-2.06	0.00015354
1435477_s_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	-2.05	0.00002547
1420393_at	nitric oxide synthase 2, inducible, macrophage	Nos2	-2.04	0.00110776
1451941_a_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	-2.01	0.00009659
1460227_at	tissue inhibitor of metalloproteinase 1	Timp1	-1.97	0.00080724
1449399_a_at	interleukin 1 beta	Il1b	-1.94	0.00013698
1428503_a_at	NFKB inhibitor interacting Ras-like protein 1	Nkiras1	-1.93	0.00048040
1416572_at	matrix metallopeptidase 14 (membrane-inserted)	Mmp14	-1.93	0.00023859
1450826_a_at	serum amyloid A 3	Saa3	-1.93	0.00009183
1450456_at	interleukin 21 receptor	Il21r	-1.84	0.00018292
1426889_at	RIKEN cDNA 4930566A11 gene	4930566A11Rik	-1.82	0.00007381
1425002_at	secreted and transmembrane 1A	Sectm1a	-1.82	0.00004092
1423511_at	ASF1 anti-silencing function 1 homolog A (S. cerevisiae)	Asf1a	-1.81	0.00002534
1435476_a_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	-1.81	0.00006121
1417926_at	non-SMC condensin II complex, subunit G2	Ncapg2	-1.8	0.00204277
1449450_at	prostaglandin E synthase	Ptges	-1.78	0.00008038
1433741_at	CD38 antigen	Cd38	-1.78	0.00063060
1419654_at	transducin-like enhancer of split 3, homolog of Drosophila E(spl)	Tle3	-1.77	0.00010207
1453851_a_at	growth arrest and DNA-damage-inducible 45 gamma	Gadd45g	-1.75	0.00000423
1455332_x_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	-1.74	0.00002368
1418776_at	RIKEN cDNA 5830443L24 gene	5830443L24Rik	-1.72	0.00477355
1419684_at	chemokine (C-C motif) ligand 8	Ccl8	-1.72	0.00055764
1451260_at	aldehyde dehydrogenase 1 family, member B1	Aldh1b1	-1.71	0.00003397
1449453_at	bone marrow stromal cell antigen 1	Bst1	-1.7	0.00001382
1421034_a_at	interleukin 4 receptor, alpha	Il4ra	-1.7	0.00249656
1427257_at	versican	Vcan	-1.69	0.00057706
1449454_at	bone marrow stromal cell antigen 1	Bst1	-1.69	0.00004936
1448383_at	matrix metallopeptidase 14 (membrane-inserted)	Mmp14	-1.69	0.00012346
1428942_at	metallothionein 2	Mt2	-1.65	0.00065479
1422397_a_at	interleukin 15 receptor, alpha chain	Il15ra	-1.65	0.00052709
1423996_a_at	interleukin 4 receptor, alpha	Il4ra	-1.64	0.00466906
1448681_at	interleukin 15 receptor, alpha chain	Il15ra	-1.64	0.00001923
1418932_at	nuclear factor, interleukin 3, regulated	Nfil3	-1.63	0.00258196
1418317_at	LIM homeobox protein 2	Lhx2	-1.63	0.00003835
1417240_at	zyxin	Zyx	-1.63	0.00000390
1422869_at	c-mer proto-oncogene tyrosine kinase	Mertk	-1.62	0.00139034
1421262_at	lipase, endothelial	Lipg	-1.61	0.00003592
1424518_at	RIKEN cDNA 2310016F22 gene /// cDNA sequence BC020489	BC020489	-1.61	0.00011617
1422068_at	POU domain, class 3, transcription factor 1	Pou3f1	-1.61	0.00038425

Figure B.3: List of genes down-regulated after MCMV infection in the absence of IE1 protein (continuation)

Affymetrix ID	Gene Name	Gene symbol	Fold Change	p-value
1419655_at	transducin-like enhancer of split 3, homolog of Drosophila E(spl)	Tle3	-1.61	0.00042575
1424895_at	G-protein signalling modulator 2 (AGS3-like, C. elegans)	Gpsm2	-1.61	0.00001980
1451458_at	transmembrane protein 2	Tmem2	-1.58	0.00099228
1449227_at	cholesterol 25-hydroxylase	Ch25h	-1.58	0.00000638
1450188_s_at	lipase, endothelial	Lipg	-1.58	0.00008990
1434306_at	RAB3A interacting protein	Rab3ip	-1.58	0.00030730
1427256_at	versican	Vcan	-1.58	0.00011555
1448786_at	RIKEN cDNA 1100001H23 gene	1100001H23Rik	-1.58	0.00446137
1424254_at	interferon induced transmembrane protein 1	Ifitm1	-1.57	0.00027050
1424250_a_at	Rho guanine nucleotide exchange factor (GEF) 3	Arhgef3	-1.57	0.00072325
1451340_at	AT rich interactive domain 5A (Mrf1 like)	Arid5a	-1.54	0.00003834
1419322_at	FYVE, RhoGEF and PH domain containing 6	Fgd6	-1.54	0.00294707
1416432_at	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Pfkfb3	-1.53	0.00155409
1450449_a_at	RIKEN cDNA 2900002H16 gene	2900002H16Rik	-1.53	0.00005984
1452519_a_at	zinc finger protein 36	Zfp36	-1.52	0.00013127
1417694_at	growth factor receptor bound protein 2-associated protein 1	Gab1	-1.52	0.00188825
1435697_a_at	pleckstrin homology, Sec7 and coiled-coil domains, binding protein	Pscdbp	-1.52	0.00111701
1426288_at	low density lipoprotein receptor-related protein 4	Lrp4	-1.52	0.00032291
1425367_at	integrin alpha L /// similar to Integrin alpha-L precursor	Itgal	-1.51	0.00048273
1451796_s_at	histidine decarboxylase	Hdc	-1.5	0.00111309
1421031_a_at	RIKEN cDNA 2310016C08 gene	2310016C08Rik	-1.5	0.00281873
1448793_a_at	syndecan 4	Sdc4	-1.5	0.00181643
1419706_a_at	A kinase (PRKA) anchor protein (gravin) 12	Akap12	-1.49	0.00023966
1424711_at	transmembrane protein 2	Tmem2	-1.49	0.00017608
1419202_at	cystatin F (leukocystatin)	Cst7	-1.49	0.00029119
1419315_at	SLAM family member 9	Slamf9	-1.49	0.00009043
1425837_a_at	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	Ccrn4l	-1.48	0.00005129
1421267_a_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain,	Cited2	-1.48	0.00028525
1431429_a_at	ADP-ribosylation factor-like 4A	Arl4a	-1.48	0.00010894
1421694_a_at	versican	Vcan	-1.47	0.00002136
1460038_at	POU domain, class 3, transcription factor 1	Pou3f1	-1.47	0.00177589
1426721_s_at	TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	-1.47	0.00001141
1419282_at	chemokine (C-C motif) ligand 12	Ccl12	-1.46	0.00039744
1425806_a_at	SRB7 (suppressor of RNA polymerase B) homolog (S. cerevisiae)	Surb7	-1.46	0.00080890
1452291_at	centaurin, delta 1	Centd1	-1.46	0.00149215
1416885_at	RIKEN cDNA 1110038F14 gene	1110038F14Rik	-1.46	0.00048801
1448165_at	caspase 2	Casp2	-1.45	0.00001126
1424268_at	spermine oxidase	Smox	-1.45	0.00040168
1448377_at	secretory leukocyte peptidase inhibitor	Slpi	-1.44	0.00383944
1448123_s_at	transforming growth factor, beta induced	Tgfbi	-1.44	0.00143191
1452203_at	oligonucleotide/oligosaccharide-binding fold containing 2A	Obfc2a	-1.44	0.00004120
1417394_at	Kruppel-like factor 4 (gut)	Klf4	-1.44	0.00559870
1416593_at	glutaredoxin	Glrx	-1.44	0.00504811
1434537_at	solute carrier organic anion transporter family, member 3a1	Slco3a1	-1.43	0.00017273
1418856_a_at	Fanconi anemia, complementation group A	Fanca	-1.43	0.00013250
1449124_at	ral guanine nucleotide dissociation stimulator,-like 1	Rgl1	-1.43	0.00044054
1421578_at	chemokine (C-C motif) ligand 4	Ccl4	-1.43	0.00026534
1448918_at	solute carrier organic anion transporter family, member 3a1	Slco3a1	-1.43	0.00115420
1423401_at	ets variant gene 6 (TEL oncogene)	Etv6	-1.43	0.00120534
1419583_at	chromobox homolog 4 (Drosophila Pc class)	Cbx4	-1.43	0.00000052
1418045_at	inositol polyphosphate-1-phosphatase	Inpp1	-1.43	0.00116967
1451574_at	B-cell CLL/lymphoma 9	Bcl9	-1.43	0.00032995
1426013_s_at	pleckstrin homology domain containing, family A	Plekha4	-1.42	0.00044463
1452204_at	ankyrin repeat and SAM domain containing 1	Anks1	-1.42	0.00167573
1425225_at	Fc receptor, IgG, low affinity IV	Fcgr4	-1.42	0.00139661
1422557_s_at	metallothionein 1	Mt1	-1.42	0.00134090
1418030_at	solute carrier organic anion transporter family, member 3a1	Slco3a1	-1.42	0.00122024

Figure B.4: List of genes down-regulated after MCMV infection in the absence of IE1 protein (continuation)

Affymetrix ID	Gene Name	Gene symbol	Fold Change	p-value
1453819_x_at	syntaxin 18	Stx18	-1.41	0.00096494
1452160_at	TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	-1.41	0.00183200
1421077_at	SERTA domain containing 3	Sertad3	-1.41	0.00114139
1420412_at	tumor necrosis factor (ligand) superfamily, member 10	Tnfsf10	-1.41	0.00110176
1425011_x_at	syntaxin 18	Stx18	-1.41	0.00036777
1455372_at	cytoplasmic polyadenylation element binding protein 3	Cpeb3	-1.4	0.00087571
1417587_at	timeless homolog (Drosophila)	Timeless	-1.4	0.00118615
1418535_at	ral guanine nucleotide dissociation stimulator,-like 1	Rgl1	-1.4	0.00215539
1415871_at	transforming growth factor, beta induced	Tgfbi	-1.4	0.00018035
1452207_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain	Cited2	-1.4	0.00314403
1417583_a_at	charged amino acid rich leucine zipper 1	Crlz1	-1.4	0.00375198
1418077_at	tripartite motif protein 21	Trim21	-1.4	0.00388866
1419221_a_at	regulator of G-protein signaling 14	Rgs14	-1.4	0.00000157
1422953_at	formyl peptide receptor, related sequence 2	Fpr-rs2	-1.4	0.00002889
1416097_at	leucine rich repeat containing 4	Lrrc4	-1.39	0.00224665
1460302_at	thrombospondin 1 /// similar to thrombospondin 1	Thbs1	-1.39	0.00173514
1428104_at	TPX2, microtubule-associated protein homolog (Xenopus laevis)	Tpx2	-1.39	0.00047183
1419561_at	chemokine (C-C motif) ligand 3	Ccl3	-1.39	0.00025763
1417406_at	SERTA domain containing 1	Sertad1	-1.39	0.00010184
1417371_at	pellino 1	Peli1	-1.39	0.00177194
1417314_at	complement factor B	Cfb	-1.39	0.00032017
1418736_at	UDP-GalNAc:betaGlcNAc beta 1,3-galactosaminyltransferase, polypeptide 1	B3galnt1	-1.38	0.00092940
1430005_a_at	basic leucine zipper transcription factor, ATF-like 2	Batf2	-1.38	0.00008525
1448940_at	tripartite motif protein 21	Trim21	-1.38	0.00004993
1427072_at	START domain containing 8	Stard8	-1.38	0.00012906
1424733_at	purinergic receptor P2Y, G-protein coupled, 14	P2ry14	-1.38	0.00046943
1418103_at	solute carrier family 12 (potassium/chloride transporters), member 9	Slc12a9	-1.38	0.00021957
1452161_at	TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	-1.38	0.00083369
1416086_at	protein-tyrosine sulfotransferase 2	Tpst2	-1.38	0.00015343
1418346_at	insulin-like 6	Insl6	-1.37	0.00205372
1418483_a_at	glycoprotein galactosyltransferase alpha 1, 3	Ggta1	-1.37	0.00329474
1451253_at	PX domain containing serine/threonine kinase	Pxk	-1.37	0.00021084
1450949_at	katanin p60 (ATPase-containing) subunit A1	Katna1	-1.37	0.00052060
1452036_a_at	thymopoietin	Tmpo	-1.36	0.00015567
1449110_at	ras homolog gene family, member B	Rhob	-1.36	0.00096999
1427573_at	cysteine-rich hydrophobic domain 1	Chic1	-1.36	0.00167216
1433750_at	solute carrier family 31, member 1	Slc31a1	-1.36	0.00013231
1416592_at	glutaredoxin	Glrx	-1.36	0.00059485
1421922_at	SH3-domain binding protein 5 (BTK-associated)	Sh3bp5	-1.36	0.00002852
1439008_at	zinc finger protein 319	Zfp319	-1.36	0.00003960
1452515_a_at	xylosyltransferase II	Xylt2	-1.36	0.00244217
1439121_at	histocompatibility 2, T region locus 22	H2-T22	-1.36	0.00177651
1428701_at	poliovirus receptor-related 4	Pvrl4	-1.35	0.00032741
1454713_s_at	histidine decarboxylase	Hdc	-1.35	0.00157672
1423837_at	RIKEN cDNA 2400003C14 gene	2400003C14Rik	-1.35	0.00007537
1422046_at	integrin alpha M	Itgam	-1.35	0.00039080
1435133_at	UDP-glucose ceramide glucosyltransferase	Ugcg	-1.34	0.00537301
1416253_at	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	Cdkn2d	-1.34	0.00109065
1418257_at	solute carrier family 12, member 7	Slc12a7	-1.34	0.00521241
1420796_at	aryl-hydrocarbon receptor repressor	Ahrr	-1.34	0.00027771
1448436_a_at	interferon regulatory factor 1	Irf1	-1.34	0.00045445
1421923_at	SH3-domain binding protein 5 (BTK-associated)	Sh3bp5	-1.34	0.00053679
1449354_at	zinc finger (CCCH type), RNA binding motif and serine/arginine rich 1	Zrsr1	-1.34	0.00175803
1424444_a_at	RIKEN cDNA 1600014C10 gene	1600014C10Rik	-1.34	0.00003920
1417387_at	mediator of RNA polymerase II transcription, subunit 31 homolog (yeast)	Med31	-1.34	0.00175980
1422141_s_at	component of Sp100-rs	Csprs	-1.34	0.00312966
1450377_at	similar to thrombospondin 1	LOC640441	-1.33	0.00477582

Figure B.5: List of genes down-regulated after MCMV infection in the absence of IE1 protein (continuation)

Affymetrix ID	Gene Name	Gene symbol	Fold Change	p-value
1426488_at	bifunctional apoptosis regulator	Bfar	-1.33	0.00000707
1417586_at	timeless homolog (Drosophila)	Timeless	-1.33	0.00101107
1418806_at	colony stimulating factor 3 receptor (granulocyte)	Csf3r	-1.33	0.00438316
1450967_at	protein tyrosine phosphatase-like A domain containing 2	Ptplad2	-1.33	0.00529272
1454018_at	tousled-like kinase 2 (Arabidopsis)	Tlk2	-1.33	0.00149645
1416997_a_at	huntingtin-associated protein 1	Hap1	-1.33	0.00294550
1417460_at	interferon induced transmembrane protein 2	Ifitm2	-1.33	0.00055555
1426165_a_at	caspase 3	Casp3	-1.32	0.01975910
1432538_a_at	replication factor C (activator 1) 3	Rfc3	-1.32	0.00084961
1416600_a_at	Down syndrome critical region homolog 1 (human)	Dscr1	-1.32	0.00419999
1419676_at	myxovirus (influenza virus) resistance 2	Mx2	-1.32	0.00077796
1424089_a_at	transcription factor 4	Tcf4	-1.32	0.00107708
1460430_at	RAP2C, member of RAS oncogene family	Rap2c	-1.32	0.00244045
1423762_at	aarF domain containing kinase 1	Adck1	-1.31	0.00444928
1439030_at	GDP-mannose pyrophosphorylase B	Gmppb	-1.31	0.00417320
1418057_at	T-cell lymphoma invasion and metastasis 1	Tiam1	-1.31	0.00312945
1422591_at	transcription elongation factor B (SIII), polypeptide 3	Tceb3	-1.31	0.00043865
1424625_a_at	DENN/MADD domain containing 1A	Dennd1a	-1.31	0.00350274
1422517_a_at	zinc ribbon domain containing, 1	Znrd1	-1.31	0.00032187
1454169_a_at	epithelial stromal interaction 1 (breast)	Epsti1	-1.31	0.00297829
1449862_a_at	phosphatidylinositol 4-kinase type 2 beta	Pi4k2b	-1.31	0.00203920
1423838_s_at	RIKEN cDNA 2400003C14 gene	2400003C14Rik	-1.31	0.00025689
1423878_at	glycophorin C	Gypc	-1.31	0.00111052
1431724_a_at	purinergic receptor P2Y, G-protein coupled 12	P2ry12	-1.31	0.00216432
1415975_at	calcium regulated heat stable protein 1	Carhsp1	-1.31	0.00013699
1440865_at	interferon induced transmembrane protein 6	Ifitm6	-1.31	0.00310897
1441986_at	zinc finger, CCHC domain containing 6	Zcchc6	-1.3	0.00142320
1452087_at	epithelial stromal interaction 1 (breast)	Epsti1	-1.3	0.00028595
1434348_at	fasciculation and elongation protein zeta 2 (zygin II)	Fez2	-1.29	0.00009107
1426004_a_at	transglutaminase 2, C polypeptide	Tgm2	-1.29	0.00240036
1423614_at	leucine rich repeat containing 8 family, member C	Lrrc8c	-1.29	0.00031432
1418980_a_at	cyclic nucleotide phosphodiesterase 1	Cnp1	-1.29	0.00016674
1419159_at	golgi autoantigen, golgin subfamily a, 3	Golga3	-1.29	0.00042395
1448449_at	receptor-interacting serine-threonine kinase 3	Ripk3	-1.29	0.00511559
1417346_at	PYD and CARD domain containing	Pycard	-1.29	0.00101472
1427405_s_at	RAB11 family interacting protein 5 (class I)	Rab11fip5	-1.29	0.00283516
1449379_at	kinase insert domain protein receptor	Kdr	-1.29	0.00008056
1416130_at	prion protein	Prnp	-1.29	0.00138828
1421303_at	IKAROS family zinc finger 1	Ikzf1	-1.29	0.00387121
1424309_a_at	molybdenum cofactor synthesis 2	Mocs2	-1.29	0.00033495
1416724_x_at	transcription factor 4	Tcf4	-1.29	0.00347022
1434117_at	transcription elongation factor B (SIII), polypeptide 3	Tceb3	-1.29	0.00069904
1436899_at	RIKEN cDNA 2700019D07 gene	2700019D07Rik	-1.28	0.00275383
1424383_at	transmembrane protein 51	Tmem51	-1.28	0.00011579
1427994_at	CD300 antigen like family member F	Cd300lf	-1.28	0.00040397
1418703_at	RNA binding motif, single stranded interacting protein 1	Rbms1	-1.28	0.00381226
1421733_a_at	protein-tyrosine sulfotransferase 1	Tpst1	-1.28	0.00124119
1448757_at	promyelocytic leukemia	Pml	-1.28	0.00034430
1416958_at	nuclear receptor subfamily 1, group D, member 2	Nr1d2	-1.28	0.00152577
1424976_at	ras homolog gene family, member V	Rhov	-1.27	0.00339879
1452669_at	RIKEN cDNA 2810012G03 gene	2810012G03Rik	-1.27	0.00211431
1455731_at	solute carrier family 29 (nucleoside transporters), member 3	Slc29a3	-1.27	0.00177890
1418135_at	AF4/FMR2 family, member 1	Aff1	-1.27	0.00001433
1424552_at	caspase 8	Casp8	-1.27	0.00038898
1418154_at	cDNA sequence BC004022	BC004022	-1.27	0.00281327
1448233_at	prion protein	Prnp	-1.27	0.00003771
1423609_a_at	mannoside acetylglucosaminyltransferase 1	Mgat1	-1.27	0.00028424

Figure B.6: List of genes down-regulated after MCMV infection in the absence of IE1 protein (continuation)

Affymetrix ID	Gene Name	Gene symbol	Fold Change	p-value
1435792_at	component of Sp100-rs /// predicted gene, EG665338	Csprs	-1.27	0.00088532
1449575_a_at	glutathione S-transferase, pi 1	Gstp1	-1.27	0.00140652
1419043_a_at	interferon inducible GTPase 1	Iigp1	-1.27	0.00269335
1426450_at	phospholipase C-like 2	Plcl2	-1.27	0.00210808
1437102_at	YTH domain family 1	Ythdf1	-1.27	0.00204589
1452178_at	plectin 1 /// similar to poly (ADP-ribose) polymerase family, member 10	Plec1	-1.27	0.00037874
1417193_at	superoxide dismutase 2, mitochondrial	Sod2	-1.26	0.00002585
1431293_a_at	claudin domain containing 1	Cldnd1	-1.26	0.00045954
1422766_at	staufen (RNA binding protein) homolog 1 (Drosophila)	Stau1	-1.26	0.00201203
1426489_s_at	bifunctional apoptosis regulator	Bfar	-1.26	0.00004689
1425663_at	interleukin 1 receptor antagonist	Il1rn	-1.26	0.00255054
1416489_at	phosphatidylinositol 4-kinase type 2 beta	Pi4k2b	-1.26	0.00063041
1418634_at	Notch gene homolog 1 (Drosophila)	Notch1	-1.26	0.00375819
1449007_at	B-cell translocation gene 3 /// predicted gene, EG654432	Btg3	-1.26	0.00014683
1420498_a_at	disabled homolog 2 (Drosophila)	Dab2	-1.26	0.00248606
1416048_at	polyhomeotic-like 2 (Drosophila)	Phc2	-1.26	0.00186996
1422471_at	peroxisomal biogenesis factor 13	Pex13	-1.26	0.00089390
1416440_at	CD164 antigen	Cd164	-1.26	0.00162671
1417793_at	interferon inducible GTPase 2	Iigp2	-1.26	0.00068871
1417166_at	PC4 and SFRS1 interacting protein 1	Psip1	-1.26	0.00382879
1436026_at	zinc finger protein 703	Zfp703	-1.26	0.00424244
1421720_a_at	deltex 2 homolog (Drosophila)	Dtx2	-1.26	0.00436782
1421307_at	carbonic anhydrase 13	Car13	-1.25	0.00444812
1453915_a_at	solute carrier family 37 (glycerol-3-phosphate transporter), member 3	Slc37a3	-1.25	0.00332598
1424348_at	RIKEN cDNA 1110007A13 gene	1110007A13Rik	-1.25	0.00021678
1450987_a_at	RIKEN cDNA 2310004I24 gene	2310004I24Rik	-1.25	0.00034040
1449839_at	---	---	-1.25	0.00049383
1417271_a_at	endoglin	Eng	-1.25	0.00505614
1452653_at	solute carrier family 25 (mitochondrial carrier, glutamate), member 22	Slc25a22	-1.25	0.00034441
1418126_at	chemokine (C-C motif) ligand 5	Ccl5	-1.25	0.00015523
1450129_a_at	suppressor of cytokine signaling 6	Socs6	-1.25	0.00387058
1450868_at	heparan-alpha-glucosaminide N-acetyltransferase	Hgsnat	-1.25	0.00077373
1450676_at	transcription elongation factor B (SIII), polypeptide 3	Tceb3	-1.25	0.00473534
1424156_at	retinoblastoma-like 1 (p107)	Rbl1	-1.25	0.00032594
1423780_at	3-hydroxyisobutyrate dehydrogenase	Hibadh	-1.25	0.00229421
1426861_at	aftiphilin	Aftph	-1.25	0.00108801
1422005_at	eukaryotic translation initiation factor 2-alpha kinase 2	Eif2ak2	-1.25	0.00092475
1433645_at	solute carrier family 44, member 1	Slc44a1	-1.24	0.00045060
1421911_at	signal transducer and activator of transcription 2	Stat2	-1.24	0.00119735
1418183_a_at	pleckstrin homology, Sec7 and coiled-coil domains 1	Pscd1	-1.24	0.00298974
1417141_at	interferon gamma induced GTPase	Igtp	-1.24	0.00003679
1453299_a_at	purine-nucleoside phosphorylase /// similar to purine-nucleoside phosphorylase	Pnp	-1.24	0.00268105
1417932_at	interleukin 18	Il18	-1.24	0.00198252
1448610_a_at	superoxide dismutase 2, mitochondrial	Sod2	-1.24	0.00005630
1426008_a_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	Slc7a2	-1.24	0.00339461
1415818_at	annexin A6	Anxa6	-1.24	0.00554551
1418939_at	H2.0-like homeo box 1 (Drosophila)	Hlx1	-1.24	0.00509226
1427273_at	ring finger protein 214	Rnf214	-1.24	0.00036520
1452045_at	zinc finger protein 281	Zfp281	-1.24	0.00381847
1452813_a_at	RIKEN cDNA 5033428A16 gene	5033428A16Rik	-1.24	0.00105100
1448391_at	RAB9, member RAS oncogene family	Rab9	-1.24	0.00235601
1428196_a_at	RIKEN cDNA 1200015F23 gene	1200015F23Rik	-1.24	0.00010276
1417500_a_at	transglutaminase 2, C polypeptide	Tgm2	-1.24	0.00233509
1426762_s_at	amine oxidase (flavin containing) domain 2	Aof2	-1.24	0.00091126
1448613_at	extracellular matrix protein 1	Ecm1	-1.24	0.00492877
1451321_a_at	RNA binding motif protein 43	Rbm43	-1.24	0.00010753
1454605_a_at	phosphatidylinositol 4-kinase type 2 alpha	Pi4k2a	-1.23	0.00128900

Figure B.7: List of genes down-regulated after MCMV infection in the absence of IE1 protein (continuation)

Affymetrix ID	Gene Name	Gene symbol	Fold Change	p-value
1416023_at	fatty acid binding protein 3, muscle and heart	Fabp3	-1.23	0.00413146
1429650_at	serine/threonine kinase 40	Stk40	-1.23	0.00292370
1416856_at	RIKEN cDNA 3230401D17 gene	3230401D17Rik	-1.23	0.00018710
1422820_at	lipase, hormone sensitive	Lipe	-1.23	0.00493090
1424575_at	RAB, member of RAS oncogene family-like 5	Rabl5	-1.23	0.00325354
1435967_s_at	3-hydroxyisobutyrate dehydrogenase	Hibadh	-1.23	0.00036717
1434372_at	expressed sequence AW112010	AW112010	-1.23	0.00024091
1423394_at	prenylcysteine oxidase 1	Pcyox1	-1.23	0.00055507
1452094_at	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha	P4ha1	-1.23	0.00073663
1460319_at	fucosyltransferase 8	Fut8	-1.23	0.00089635
1422628_at	RIKEN cDNA 4632417K18 gene	4632417K18Rik	-1.23	0.00105265
1426798_a_at	protein phosphatase 1, regulatory (inhibitor) subunit 15b	Ppp1r15b	-1.23	0.00043704
1426750_at	filamin, beta	Flnb	-1.22	0.00424420
1419768_at	CD22 antigen	Cd22	-1.22	0.00009935
1425639_at	centaurin, alpha 2	Centa2	-1.22	0.00162632
1448914_a_at	colony stimulating factor 1 (macrophage)	Csf1	-1.22	0.00060729
1429884_at	SLIT-ROBO Rho GTPase activating protein 2	Srgap2	-1.22	0.00399191
1423976_at	RIKEN cDNA 4930453N24 gene	4930453N24Rik	-1.22	0.00337067
1418323_at	feminization 1 homolog b (C. elegans)	Fem1b	-1.22	0.00303417
1424354_at	transmembrane protein 140	Tmem140	-1.22	0.00278023
1448583_at	expressed sequence AA960436	AA960436	-1.22	0.00236017
1415852_at	inosine 5'-phosphate dehydrogenase 2	Impdh2	-1.22	0.00205551
1416009_at	tetraspanin 3	Tspan3	-1.22	0.00111720
1415767_at	YTH domain family 1	Ythdf1	-1.22	0.00034812
1424339_at	2'-5' oligoadenylate synthetase-like 1	Oasl1	-1.22	0.00059962
1425680_a_at	beta-transducin repeat containing protein	Btrc	-1.22	0.00056375
1415710_at	COX18 cytochrome c oxidase assembly homolog (S. cerevisiae)	Cox18	-1.22	0.00010358
1426761_at	amine oxidase (flavin containing) domain 2	Aof2	-1.21	0.00073657
1424929_a_at	tripartite motif protein 26	Trim26	-1.21	0.00029537
1422782_s_at	toll-like receptor 3	Tlr3	-1.21	0.00552468
1451330_a_at	inositol polyphosphate-5-phosphatase B	Inpp5b	-1.21	0.00239502
1449981_a_at	N-acetyltransferase 2 (arylamine N-acetyltransferase)	Nat2	-1.21	0.00455912
1420196_s_at	TBC1 domain family, member 14	Tbc1d14	-1.21	0.00129054
1422103_a_at	signal transducer and activator of transcription 5B	Stat5b	-1.21	0.00051735
1423613_at	---	---	-1.21	0.00031488
1418191_at	ubiquitin specific peptidase 18	Usp18	-1.21	0.00008454
1434005_at	RNA binding motif, single stranded interacting protein 1	Rbms1	-1.21	0.00072644
1419026_at	Fas death domain-associated protein	Daxx	-1.21	0.00209802
1460243_at	serine palmitoyltransferase, long chain base subunit 2	Sptlc2	-1.2	0.00221371
1423502_at	bromodomain containing 2	Brd2	-1.2	0.00033342
1424286_at	protein kinase, X-linked	Prkx	-1.2	0.00218137
1418930_at	chemokine (C-X-C motif) ligand 10	Cxcl10	-1.2	0.00114448
1451716_at	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	Mafb	-1.2	0.00469316
1452316_at	integrator complex subunit 12	Ints12	-1.2	0.00120106

Figure B.8: List of genes up-regulated after MCMV infection in the absence of IE1 protein (continuation)

Affymetrix ID	Gene Name	Gene symbol	Fold Change	p-value
1433966_x_at	asparagine synthetase	Asns	2.08	0.00047071
1418937_at	deiodinase, iodothyronine, type II	Dio2	1.74	0.00113433
1422851_at	high mobility group AT-hook 2	Hmga2	1.69	0.00007279
1418652_at	chemokine (C-X-C motif) ligand 9	Cxcl9	1.68	0.00025863
1420895_at	transforming growth factor, beta receptor I	Tgfbr1	1.66	0.00004121
1454831_at	forkhead box N2	Foxn2	1.63	0.00213758
1450780_s_at	high mobility group AT-hook 2	Hmga2	1.62	0.00081930
1450781_at	high mobility group AT-hook 2	Hmga2	1.62	0.00149479
1451095_at	asparagine synthetase	Asns	1.58	0.00052654
1421098_at	expressed sequence AI586015	AI586015	1.57	0.00182980
1456244_x_at	thioredoxin-like 2	Txnl2	1.53	0.00380612
1434036_at	metastasis suppressor 1	Mtss1	1.53	0.00015387
1424826_s_at	metastasis suppressor 1	Mtss1	1.52	0.00002695
1422474_at	phosphodiesterase 4B, cAMP specific	Pde4b	1.5	0.00028998
1418503_at	heat shock protein 9	Hspa9	1.44	0.00238737
1418345_at	tumor necrosis factor (ligand) superfamily, member 13	Tnfsf13	1.44	0.00247101
1449036_at	ring finger protein 128	Rnf128	1.43	0.00324102
1415904_at	lipoprotein lipase	Lpl	1.43	0.00249769
1436994_a_at	histone cluster 1, H1c	Hist1h1c	1.43	0.00207864
1449677_s_at	transmembrane protein 38B	Tmem38b	1.42	0.00023576
1417377_at	cell adhesion molecule 1	Cadm1	1.42	0.00011859
1433935_at	expressed sequence AU020206	AU020206	1.41	0.00239700
1430447_a_at	leukocyte-associated Ig-like receptor 1	Lair1	1.41	0.00039189
1426705_s_at	isoleucine-tRNA synthetase	Iars	1.4	0.00037005
1421859_at	a disintegrin and metallopeptidase domain 17	Adam17	1.4	0.00006810
1451980_at	CAS1 domain containing 1	Casd1	1.4	0.00118325
1449383_at	adenylosuccinate synthetase like 1	Adssl1	1.39	0.00194532
1419253_at	methylenetetrahydrofolate dehydrogenase (NAD+ dependent)	Mthfd2	1.39	0.00079503
1435903_at	CD300A antigen	Cd300a	1.39	0.00196346
1425264_s_at	myelin basic protein	Mbp	1.39	0.00023670
1417376_a_at	cell adhesion molecule 1	Cadm1	1.39	0.00003872
1418318_at	ring finger protein 128	Rnf128	1.39	0.00033517
1432164_a_at	glycine cleavage system protein H (aminomethyl carrier)	Gcsh	1.39	0.00173792
1451382_at	ChaC, cation transport regulator-like 1 (E. coli)	Chac1	1.38	0.00424049
1453634_a_at	endoplasmic reticulum protein 29	Erp29	1.38	0.00054931
1419254_at	methylenetetrahydrofolate dehydrogenase (NAD+ dependent)	Mthfd2	1.38	0.00542940
1421857_at	a disintegrin and metallopeptidase domain 17	Adam17	1.38	0.00243387
1436549_a_at	heterogeneous nuclear ribonucleoprotein A1	Hnrpa1	1.38	0.00165318
1449670_x_at	G protein-coupled receptor 137B	Gpr137b	1.38	0.00347638
1423233_at	CCAAT/enhancer binding protein (C/EBP), delta	Cebpd	1.37	0.00287888
1422473_at	phosphodiesterase 4B, cAMP specific	Pde4b	1.37	0.00081722
1439255_s_at	G protein-coupled receptor 137B	Gpr137b	1.37	0.00092279
1424683_at	RIKEN cDNA 1810015C04 gene	1810015C04Rik	1.37	0.00209257
1423721_at	tropomyosin 1, alpha	Tpm1	1.37	0.00456739
1417378_at	cell adhesion molecule 1	Cadm1	1.37	0.00086569
1437111_at	zinc finger CCCH type containing 12C	Zc3h12c	1.36	0.00008587
1455493_at	synaptic nuclear envelope 1	Syne1	1.36	0.00478662
1449175_at	G-protein coupled receptor 65	Gpr65	1.36	0.00155384
1417133_at	peripheral myelin protein	Pmp22	1.36	0.00003357
1420643_at	lunatic fringe gene homolog (Drosophila)	Lfng	1.36	0.00102303
1450849_at	heterogeneous nuclear ribonucleoprotein U	Hnrpu	1.35	0.00196938
1427689_a_at	TNFAIP3 interacting protein 1	Tnip1	1.35	0.00003227
1450881_s_at	G protein-coupled receptor 137B	Gpr137b	1.35	0.00334133
1448570_at	glia maturation factor, beta	Gmfb	1.35	0.00200706
1416703_at	mitogen activated protein kinase 14	Mapk14	1.34	0.00082113
1421038_a_at	potassium intermediate/small conductance calcium-activated channel, subfamily N,	Kcnn4	1.34	0.00583759
1424720_at	mannoside acetylglucosaminyltransferase 4, isoenzyme B	Mgat4b	1.34	0.00013130
1423166_at	CD36 antigen	Cd36	1.34	0.00423232
1421858_at	a disintegrin and metallopeptidase domain 17	Adam17	1.33	0.00017392

Figure B.9: List of genes up-regulated after MCMV infection in the absence of IE1 protein (continuation)

Affymetrix ID	Gene Name	Gene symbol	Fold Change	p-value
1424954_a_at	---	---	1.33	0.00047749
1424700_at	transmembrane protein 38B	Tmem38b	1.33	0.00464081
1452093_at	transmembrane protein 185B	Tmem185b	1.33	0.00195337
1424540_at	homeodomain interacting protein kinase 1	Hipk1	1.33	0.00099212
1438855_x_at	tumor necrosis factor, alpha-induced protein 2	Tnfaip2	1.32	0.00037282
1428097_at	RIKEN cDNA 2510009E07 gene	2510009E07Rik	1.32	0.00429351
1451475_at	plexin D1	Plxnd1	1.32	0.00206257
1448406_at	EP300 interacting inhibitor of differentiation 1	Eid1	1.32	0.00192661
1448185_at	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like d	Herpud1	1.32	0.00439800
1437401_at	---	---	1.32	0.00148258
1426722_at	solute carrier family 38, member 2	Slc38a2	1.31	0.00244054
1417751_at	serine/threonine kinase 10	Stk10	1.31	0.00316380
1450883_a_at	CD36 antigen	Cd36	1.31	0.00278738
1434472_at	dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related)	Dusp3	1.3	0.00133090
1431804_a_at	trans-acting transcription factor 3	Sp3	1.3	0.00506080
1431843_a_at	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, ep	Nfkbie	1.3	0.00173087
1424290_at	oxidative stress induced growth inhibitor family member 2	Osgin2	1.3	0.00251020
1423602_at	Tnf receptor-associated factor 1	Traf1	1.3	0.00183476
1426371_at	male sterility domain containing 2	Mlstd2	1.3	0.00013336
1424293_s_at	transmembrane protein 55A	Tmem55a	1.29	0.00104896
1452440_at	tumor necrosis factor (ligand) superfamily, member 12r	Tnfsf12	1.29	0.00578527
1435602_at	Transcribed locus	---	1.29	0.00156900
1453722_s_at	splicing factor, arginine/serine-rich 1 (ASF/SF2)	Sfrs1	1.28	0.00179096
1423213_at	plexin C1	Plxnc1	1.28	0.00206204
1427504_s_at	splicing factor, arginine/serine-rich 2 (SC-35)	Sfrs2	1.28	0.00563507
1420478_at	nucleosome assembly protein 1-like 1	Nap1l1	1.28	0.00487405
1449151_at	PCTAIRE-motif protein kinase 3	Pctk3	1.28	0.00357163
1423989_at	RIKEN cDNA 2210010N04 gene	2210010N04Rik	1.28	0.00504376
1423141_at	Lysosomal acid lipase A	Lipa	1.28	0.00147340
1424915_s_at	---	---	1.28	0.00031982
1424289_at	oxidative stress induced growth inhibitor family member 2	Osgin2	1.28	0.00515075
1434940_x_at	regulator of G-protein signaling 19	Rgs19	1.28	0.00560472
1422638_s_at	Ras association (RalGDS/AF-6) domain family 5	Rassf5	1.28	0.00435798
1456133_x_at	integrin beta 5	Itgb5	1.28	0.00203492
1423346_at	degenerative spermatocyte homolog 1 (Drosophila)	Degs1	1.28	0.00221071
1418397_at	zinc finger protein 275	Zfp275	1.28	0.00034486
1427739_a_at	transformation related protein 53	Trp53	1.28	0.00190265
1416543_at	nuclear factor, erythroid derived 2, like 2	Nfe2l2	1.27	0.00053767
1450882_s_at	G protein-coupled receptor 137B	Gpr137b	1.27	0.00058691
1451311_a_at	adiponectin receptor 1	Adipor1	1.27	0.00537793
1422455_s_at	N-ethylmaleimide sensitive fusion protein	Nsf	1.27	0.00241264
1434468_at	OTU domain containing 4	Otud4	1.26	0.00431614
1423195_at	hippocampus abundant gene transcript 1	Hiat1	1.26	0.00043663
1422045_a_at	protein tyrosine phosphatase, non-receptor type 12	Ptpn12	1.26	0.00071284
1423642_at	tubulin, beta 2c	Tubb2c	1.26	0.00069583
1426301_at	activated leukocyte cell adhesion molecule	Alcam	1.26	0.00004513
1426819_at	homeodomain interacting protein kinase 3	Hipk3	1.25	0.00553995
1426708_at	anthrax toxin receptor 2	Antxr2	1.25	0.00005320
1418192_at	max binding protein	Mnt	1.25	0.00405395
1448306_at	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Nfkbia	1.25	0.00265420
1426826_at	RNA binding motif protein 16	Rbm16	1.25	0.00210766
1448450_at	adenylate kinase 2	Ak2	1.25	0.00306736
1448124_at	glucuronidase, beta	Gusb	1.25	0.00200344
1422573_at	AMP deaminase 3	Ampd3	1.25	0.00023806
1455105_at	protein tyrosine phosphatase, non-receptor type 12	Ptpn12	1.25	0.00444371
1425486_s_at	myotubularin related protein 6	Mtmr6	1.24	0.00367132
1426884_at	required for meiotic nuclear division 5 homolog A (S. cerevisiae)	Rmnd5a	1.24	0.00347978
1417786_a_at	regulator of G-protein signaling 19	Rgs19	1.24	0.00092135
1416012_at	EH-domain containing 1	Ehd1	1.24	0.00086002
1454705_at	DNA segment, Chr 15, ERATO Doi 621, expressed	D15Ertd621e	1.24	0.00249646

Figure B.10: List of genes up-regulated after MCMV infection in the absence of IE1 protein (continuation)

Affymetrix ID	Gene Name	Gene symbol	Fold Change	p-value
1432543_a_at	Kruppel-like factor 13	Klf13	1.24	0.00101369
1427396_a_at	cold shock domain containing E1, RNA binding	Csde1	1.24	0.00010103
1448425_at	eukaryotic translation initiation factor 3, subunit 10 (theta)	Eif3s10	1.24	0.00226775
1426877_a_at	polybromo 1	Pbrm1	1.24	0.00268838
1421840_at	ATP-binding cassette, sub-family A (ABC1), member 1	Abca1	1.24	0.00295929
1419589_at	CD93 antigen	Cd93	1.24	0.00157855
1451097_at	vasodilator-stimulated phosphoprotein	Vasp	1.24	0.00257674
1422569_at	YY1 transcription factor	Yy1	1.24	0.00137615
1418504_at	heat shock protein 9	Hspa9	1.23	0.00282261
1426402_at	synaptotagmin binding, cytoplasmic RNA interacting protein	Syncrip	1.23	0.00279531
1427358_a_at	death associated protein kinase 1	Dapk1	1.23	0.00539571
1417073_a_at	quaking	Qk	1.23	0.00577329
1455129_at	Metadherin	Mtdh	1.23	0.00017485
1416660_at	eukaryotic translation initiation factor 3, subunit 10 (theta)	Eif3s10	1.23	0.00045766
1429296_at	RAB10, member RAS oncogene family	Rab10	1.23	0.00525101
1423134_at	cDNA sequence BC003324	BC003324	1.23	0.00379312
1429775_a_at	G protein-coupled receptor 137B	Gpr137b	1.23	0.00366350
1422714_at	ubiquitin-conjugating enzyme E2I /// predicted gene, EG546265	Ube2i	1.23	0.00053976
1438376_s_at	tripartite motif protein 27	Trim27	1.23	0.00009239
1439389_s_at	myeloid-associated differentiation marker	Myadm	1.23	0.00028686
1438853_x_at	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54	Ddx54	1.23	0.00507530
1418539_a_at	protein tyrosine phosphatase, receptor type, E	Ptpre	1.22	0.00241935
1416273_at	tumor necrosis factor, alpha-induced protein 2	Tnfaip2	1.22	0.00164121
1448385_at	solute carrier family 15, member 4	Slc15a4	1.22	0.00292972
1426300_at	activated leukocyte cell adhesion molecule	Alcam	1.22	0.00065617
1419645_at	cleavage stimulation factor, 3' pre-RNA subunit 2	Cstf2	1.22	0.00227370
1416359_at	sorting nexin associated golgi protein 1	Snag1	1.22	0.00370816
1417856_at	avian reticuloendotheliosis viral (v-rel) oncogene related B	Relb	1.22	0.00145977
1422566_at	transcription factor EB	Tcfeb	1.22	0.00591783
1419132_at	toll-like receptor 2	Tlr2	1.22	0.00186883
1416406_at	phosphoprotein enriched in astrocytes 15A	Pea15a	1.22	0.00543658
1448454_at	splicing factor, arginine/serine-rich 6	Sfrs6	1.21	0.00104728
1426908_at	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferas	Galnt7	1.21	0.00046160
1434888_a_at	matrin 3	Matr3	1.21	0.00441652
1438673_at	solute carrier family 4, sodium bicarbonate cotransporter, member 7	Slc4a7	1.21	0.00372263
1450479_x_at	protein tyrosine phosphatase, non-receptor type 12	Ptpn12	1.21	0.00464498
1433737_at	similar to U2AF homology motif (UHM) kinase 1	LOC677213	1.21	0.00453984
1426257_a_at	seryl-aminoacyl-tRNA synthetase	Sars	1.21	0.00393678
1415839_a_at	nucleophosmin 1	Npm1	1.21	0.00555968
1423955_a_at	longevity assurance homolog 2 (S. cerevisiae)	Lass2	1.21	0.00125622
1450905_at	plexin C1	Plxnc1	1.21	0.00333071
1428666_at	asparaginyl-tRNA synthetase	Nars	1.21	0.00145426
1417339_a_at	dynein light chain LC8-type 1 /// predicted gene, EG627788 /// similar to dynein	Dynll1	1.21	0.00001899
1418302_at	palmitoyl-protein thioesterase 2	Ppt2	1.21	0.00587620
1450872_s_at	lysosomal acid lipase A	Lipa	1.2	0.00059120
1435715_x_at	thymine DNA glycosylase /// predicted gene, EG545124 /// similar to G/T mismatch	Tdg	1.2	0.00044671
1422456_at	N-ethylmaleimide sensitive fusion protein	Nsf	1.2	0.00208491
1417542_at	ribosomal protein S6 kinase, polypeptide 2	Rps6ka2	1.2	0.00422023
1428262_s_at	heterogeneous nuclear ribonucleoprotein A3 /// similar to heterogeneous nuclear	Hnrpa3	1.2	0.00322749
1424637_s_at	RIKEN cDNA 2610204L23 gene	2610204L23Rik	1.2	0.00346695
1425902_a_at	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	Nfkb2	1.2	0.00069331
1437457_a_at	myotrophin	Mtpn	1.2	0.00320139
1434080_at	AE binding protein 2	Aebp2	1.19	0.00326944
1424067_at	intercellular adhesion molecule	Icam1	1.19	0.00100407
1419607_at	tumor necrosis factor	Tnf	1.19	0.00503352

APPENDIX C

Luminex-based PathwayProfiler^{TM}analysis

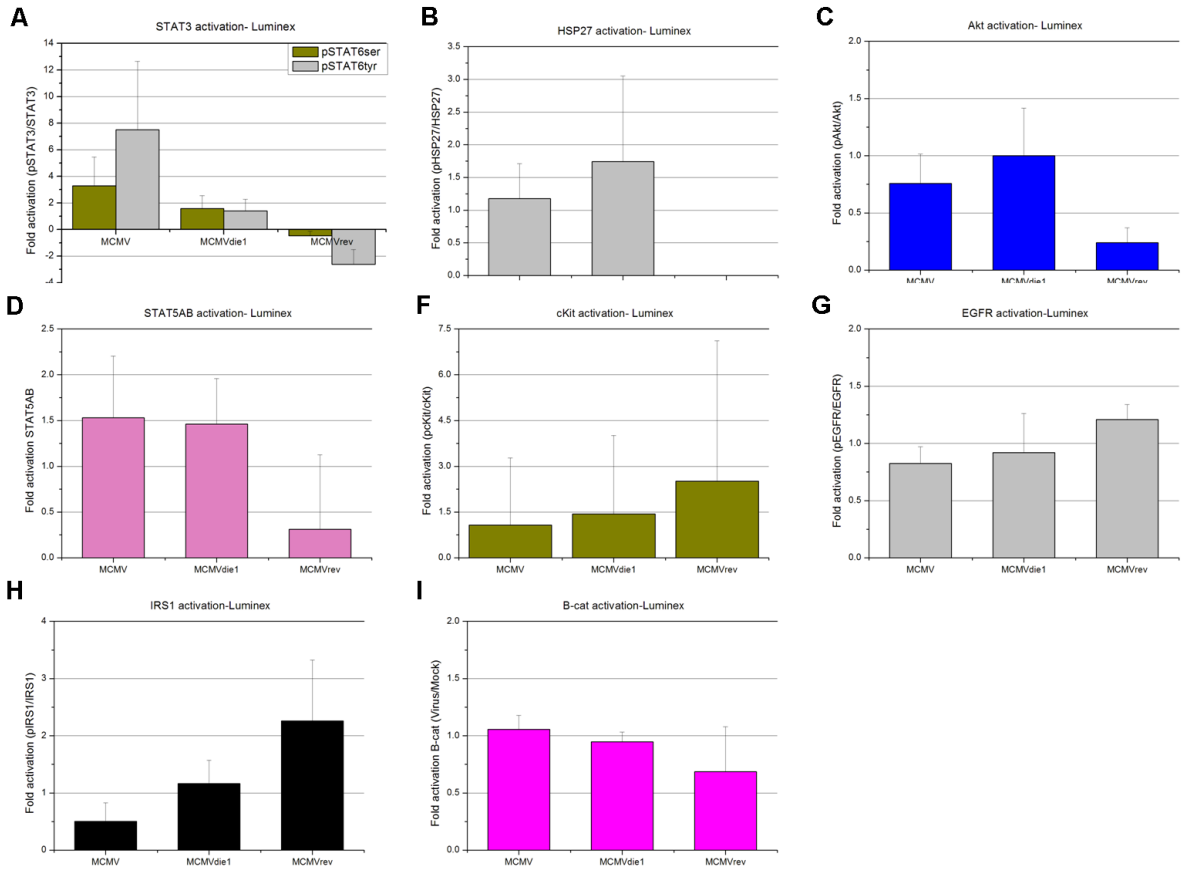

Figure C.1: Analysis of the rest of the signalling proteins

BMMθ were infected at a MOI of 1 with MCMV, MCMVdie1 or MCMVrev or mock infected as a negative control. Samples were analyzed as n=4 using the PathwayProfiler^{TM}service by Upstate (Millipore, UK). Fold activation of the kinases are shown. MFI data have been normalized against total amount of protein and mock-infected samples. Blank values were also extrated from the final values. Bars show media values and SD.

References

[Aggarwal, 2003] Aggarwal, B. B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol*, 3(9):745–56.

[Aizaki et al., 2004] Aizaki, H., Lee, K. J., Sung, V. M., Ishiko, H., and Lai, M. M. (2004). Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology*, 324(2):450–61.

[Alcami, 2003] Alcami, A. (2003). Viral mimicry of cytokines, chemokines and their receptors. *Nat Rev Immunol*, 3(1):36–50.

[Alcami et al., 2005] Alcami, A., Hill, A. B., and Koszinowski, U. H. (2005). *Viral interference with the host immune response*. Hodder Arnold.

[Ameglio et al., 1994] Ameglio, F., Capobianchi, M. R., Castilletti, C., Cordiali Fei, P., Fais, S., Trento, E., and Dianzani, F. (1994). Recombinant gp120 induces IL-10 in resting peripheral blood mononuclear cells; correlation with the induction of other cytokines. *Clin Exp Immunol*, 95(3):455–8.

[Andrews et al., 2001] Andrews, D. M., Andoniou, C. E., Granucci, F., Ricciardi-Castagnoli, P., and Degli-Esposti, M. A. (2001). Infection of dendritic cells by murine Cytomegalovirus induces functional paralysis. *Nat Immunol*, 2(11):1077–84.

[Angulo et al., 2000] Angulo, A., Ghazal, P., and Messerle, M. (2000). The major immediate-early gene ie3 of mouse Cytomegalovirus is essential for viral growth. *J Virol*, 74(23):11129–36.

[Angulo et al., 1998] Angulo, A., Messerle, M., Koszinowski, U. H., and Ghazal, P. (1998). Enhancer requirement for murine cytomegalovirus growth and genetic complementation by the human cytomegalovirus enhancer. *J Virol*, 72(11):8502–9.

[Arase et al., 2002] Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B., and Lanier, L. L. (2002). Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science*, 296(5571):1323–6.

[Baer et al., 1984] Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, C., and et al. (1984). Dna sequence and expression of the b95-8 epstein-barr virus genome. *Nature*, 310(5974):207–11.

[Baillie et al., 2003] Baillie, J., Sahlender, D. A., and Sinclair, J. H. (2003). Human cytomegalovirus infection inhibits tumor necrosis factor alpha (TNF-alpha) signaling by targeting the 55-kilodalton TNF-alpha receptor. *J Virol*, 77(12):7007–16.

[Banks et al., 2005] Banks, T. A., Rickert, S., Benedict, C. A., Ma, L., Ko, M., Meier, J., Ha, W., Schneider, K., Granger, S. W., Turovskaya, O., Elewaut, D., Otero, D., French, A. R., Henry, S. C., Hamilton, J. D., Scheu, S., Pfeffer, K., and Ware, C. F. (2005). A lymphotoxin-IFN-beta axis essential for lymphocyte survival revealed during cytomegalovirus infection. *J Immunol*, 174(11):7217–25.

[Barthel et al., 2003] Barthel, R., Tsytsykova, A. V., Barczak, A. K., Tsai, E. Y., Dascher, C. C., Brenner, M. B., and Goldfeld, A. E. (2003). Regulation of tumor necrosis factor alpha gene expression by mycobacteria involves the assembly of a unique enhanceosome dependent on the coactivator proteins CBP/p300. *Mol Cell Biol*, 23(2):526–33.

[Barton, 1997] Barton, B. E. (1997). IL-6: insights into novel biological activities. *Clin Immunol Immunopathol*, 85(1):16–20.

[Benedict et al., 2004] Benedict, C. A., Angulo, A., Patterson, G., Ha, S., Huang, H., Messerle, M., Ware, C. F., and Ghazal, P. (2004). Neutrality of the canonical NF-kappaB-dependent pathway for human and murine cytomegalovirus transcription and replication in vitro. *J Virol*, 78(2):741–50.

[Benedict et al., 2001] Benedict, C. A., Banks, T. A., Senderowicz, L., Ko, M., Britt, W. J., Angulo, A., Ghazal, P., and Ware, C. F. (2001). Lymphotoxins and cytomegalovirus cooperatively induce interferon-beta, establishing host-virus detente. *Immunity*, 15(4):617–26.

[Benedict et al., 2003] Benedict, C. A., Banks, T. A., and Ware, C. F. (2003). Death and survival: viral regulation of TNF signaling pathways. *Curr Opin Immunol*, 15(1):59–65.

[Benedict and Ware, 2001] Benedict, C. A. and Ware, C. F. (2001). Virus targeting of the tumor necrosis factor superfamily. *Virology*, 289(1):1–5.

[Bodaghi et al., 1998] Bodaghi, B., Jones, T. R., Zipeto, D., Vita, C., Sun, L., Laurent, L., Arenzana-Seisdedos, F., Virelizier, J. L., and Michelson, S. (1998). Chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of chemokines from the environment of cytomegalovirus-infected cells. *J Exp Med*, 188(5):855–66.

[Busche et al., 2008] Busche, A., Angulo, A., Kay-Jackson, P., Ghazal, P., and Messerle, M. (2008). Phenotypes of major immediate-early gene mutants of mouse cytomegalovirus. *Med Microbiol Immunol*.

[Calvano et al., 2005] Calvano, S. E., Xiao, W., Richards, D. R., Felciano, R. M., Baker, H. V., Cho, R. J., Chen, R. O., Brownstein, B. H., Cobb, J. P., Tschoeke, S. K., Miller-Graziano, C., Moldawer, L. L., Mindrinos, M. N., Davis, R. W., Tompkins, R. G., and Lowry, S. F. (2005). A network-based analysis of systemic inflammation in humans. *Nature*, 437(7061):1032–7.

[Campbell et al., 1984] Campbell, M. E., Palfreyman, J. W., and Preston, C. M. (1984). Identification of herpes simplex virus dna sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. *J Mol Biol*, 180(1):1–19.

[Chang et al., 2004] Chang, W. L., Baumgarth, N., Yu, D., and Barry, P. A. (2004). Human cytomegalovirus-encoded interleukin-10 homolog inhibits maturation of dendritic cells and alters their functionality. *J Virol*, 78(16):8720–31.

[Chee et al., 1990] Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, C. A., r., Kouzarides, T., Martignetti, J. A., and et al. (1990). Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr Top Microbiol Immunol*, 154:125–69.

[Chiou et al., 2006] Chiou, S. H., Yang, Y. P., Lin, J. C., Hsu, C. H., Jhang, H. C., Yang, Y. T., Lee, C. H., Ho, L. L., Hsu, W. M., Ku, H. H., Chen, S. J., Chen, S. S., Chang, M. D., Wu, C. W., and Juan, L. J. (2006). The immediate early 2 protein of human cytomegalovirus (HCMV) mediates the apoptotic control in HCMV retinitis through up-regulation of the cellular FLICE-inhibitory protein expression. *J Immunol*, 177(9):6199–206.

[Choi et al., 2006] Choi, S. H., Park, K. J., Ahn, B. Y., Jung, G., Lai, M. M., and Hwang, S. B. (2006). Hepatitis C virus nonstructural 5b protein regulates tumor necrosis factor alpha signaling through effects on cellular IkappaB kinase. *Mol Cell Biol*, 26(8):3048–59.

[Couper et al., 2008] Couper, K. N., Blount, D. G., and Riley, E. M. (2008). IL-10: The master regulator of immunity to infection. *J Immunol*, 180(9):5771–7.

[Cousens et al., 1997] Cousens, L. P., Orange, J. S., Su, H. C., and Biron, C. A. (1997). Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection. *Proc Natl Acad Sci U S A*, 94(2):634–9.

[D'Addario et al., 2000] D'Addario, M., Ahmad, A., Morgan, A., and Menezes, J. (2000). Binding of the Epstein-Barr virus major envelope glycoprotein gp350 results in the upregulation of the TNF-alpha gene expression in monocytic cells via NF-kappaB involving PKC, PI3-K and tyrosine kinases. *J Mol Biol*, 298(5):765–78.

[Dalod et al., 2002] Dalod, M., Salazar-Mather, T. P., Malmgaard, L., Lewis, C., Asselin-Paturel, C., Briere, F., Trinchieri, G., and Biron, C. A. (2002). Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. *J Exp Med*, 195(4):517–28.

[Davis-Poynter et al., 1997] Davis-Poynter, N. J., Lynch, D. M., Vally, H., Shellam, G. R., Rawlinson, W. D., Barrell, B. G., and Farrell, H. E. (1997). Identification and characterization of a G protein-coupled receptor homolog encoded by murine cytomegalovirus. *J Virol*, 71(2):1521–9.

[Delale et al., 2005] Delale, T., Paquin, A., Asselin-Paturel, C., Dalod, M., Brizard, G., Bates, E. E., Kastner, P., Chan, S., Akira, S., Vicari, A., Biron, C. A.,

Trinchieri, G., and Briere, F. (2005). MyD88-dependent and -independent murine cytomegalovirus sensing for IFN-alpha release and initiation of immune responses in vivo. *J Immunol*, 175(10):6723–32.

[E. S. Mocarski, 1996] E. S. Mocarski, J. (1996). *Cytomegaloviruses and their replication*. Philadelphia : Lippincott Williams and Wilkins.

[Ecsedy et al., 2003] Ecsedy, J. A., Michaelson, J. S., and Leder, P. (2003). Homeodomain-interacting protein kinase 1 modulates Daxx localization, phosphorylation, and transcriptional activity. *Mol Cell Biol*, 23(3):950–60.

[Eickhoff and Cotten, 2005] Eickhoff, J. E. and Cotten, M. (2005). NF-kappaB activation can mediate inhibition of human cytomegalovirus replication. *J Gen Virol*, 86(Pt 2):285–95.

[Englund et al., 2001] Englund, M. C., Karlsson, A. L., Wiklund, O., Bondjers, G., and Ohlsson, B. G. (2001). 25-hydroxycholesterol induces lipopolysaccharide-tolerance and decreases a lipopolysaccharide-induced TNF-alpha secretion in macrophages. *Atherosclerosis*, 158(1):61–71.

[Everett, 1989] Everett, R. D. (1989). Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. *J Gen Virol*, 70 (Pt 5):1185–202.

[Everett, 2001] Everett, R. D. (2001). DNA viruses and viral proteins that interact with PML nuclear bodies. *Oncogene*, 20(49):7266–73.

[Everett et al., 2004] Everett, R. D., Boutell, C., and Orr, A. (2004). Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0. *J Virol*, 78(4):1763–74.

[Everett et al., 1998] Everett, R. D., Freemont, P., Saitoh, H., Dasso, M., Orr, A., Kathoria, M., and Parkinson, J. (1998). The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *J Virol*, 72(8):6581–91.

[Everett et al., 2008] Everett, R. D., Parada, C., Gripon, P., Sirma, H., and Orr, A. (2008). Replication of ICP0-null mutant herpes simplex virus type 1 is restricted by both PML and Sp100. *J Virol*, 82(6):2661–72.

[Falvo et al., 2000] Falvo, J. V., Uglialoro, A. M., Brinkman, B. M., Merika, M., Parekh, B. S., Tsai, E. Y., King, H. C., Morielli, A. D., Peralta, E. G., Maniatis, T., Thanos, D., and Goldfeld, A. E. (2000). Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter. *Mol Cell Biol*, 20(6):2239–47.

[Fenwick and Clark, 1982] Fenwick, M. L. and Clark, J. (1982). Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. *J Gen Virol*, 61 (Pt l):121–5.

[Fenwick and McMenamin, 1984] Fenwick, M. L. and McMenamin, M. M. (1984). Early virion-associated suppression of cellular protein synthesis by herpes simplex virus is accompanied by inactivation of mrna. *J Gen Virol*, 65 (Pt 7):1225–8.

[Flamand et al., 1991] Flamand, L., Gosselin, J., D'Addario, M., Hiscott, J., Ablashi, D. V., Gallo, R. C., and Menezes, J. (1991). Human herpesvirus 6 induces interleukin-1 beta and tumor necrosis factor alpha, but not interleukin-6, in peripheral blood mononuclear cell cultures. *J Virol*, 65(9):5105–10.

[Gallagher et al., 2003] Gallagher, J., Howlin, J., McCarthy, C., Murphy, E. P., Bresnihan, B., FitzGerald, O., Godson, C., Brady, H. R., and Martin, F. (2003). Identification of Naf1/ABIN-1 among TNF-alpha-induced expressed genes in human synoviocytes using oligonucleotide microarrays. *FEBS Lett*, 551(1-3):8–12.

[Gawn and Greaves, 2002] Gawn, J. M. and Greaves, R. F. (2002). Absence of IE1 p72 protein function during low-multiplicity infection by human cytomegalovirus results in a broad block to viral delayed-early gene expression. *J Virol*, 76(9):4441–55.

[Geist and Dai, 1996] Geist, L. J. and Dai, L. Y. (1996). Cytomegalovirus modulates interleukin-6 gene expression. *Transplantation*, 62(5):653–8.

[Geist et al., 1997] Geist, L. J., Hopkins, H. A., Dai, L. Y., He, B., Monick, M. M., and Hunninghake, G. W. (1997). Cytomegalovirus modulates transcription factors necessary for the activation of the tumor necrosis factor-alpha promoter. *Am J Respir Cell Mol Biol*, 16(1):31–7.

[Geist et al., 1994] Geist, L. J., Monick, M. M., Stinski, M. F., and Hunninghake, G. W. (1994). The immediate early genes of human cytomegalovirus upregulate tumor necrosis factor-alpha gene expression. *J Clin Invest*, 93(2):474–8.

[Ghazal et al., 2003] Ghazal, P., Messerle, M., Osborn, K., and Angulo, A. (2003). An essential role of the enhancer for murine cytomegalovirus in vivo growth and pathogenesis. *J Virol*, 77(5):3217–28.

[Ghazal et al., 2005] Ghazal, P., Visser, A. E., Gustems, M., Garcia, R., Borst, E. M., Sullivan, K., Messerle, M., and Angulo, A. (2005). Elimination of ie1 significantly attenuates murine cytomegalovirus virulence but does not alter replicative capacity in cell culture. *J Virol*, 79(11):7182–94.

[Gongora et al., 2001] Gongora, R., Stephan, R. P., Zhang, Z., and Cooper, M. D. (2001). An essential role for Daxx in the inhibition of B lymphopoiesis by type I interferons. *Immunity*, 14(6):727–37.

[Gosselin et al., 1992] Gosselin, J., Flamand, L., D'Addario, M., Hiscott, J., Stefanescu, I., Ablashi, D. V., Gallo, R. C., and Menezes, J. (1992). Modulatory effects of Epstein-Barr, herpes simplex, and human herpes-6 viral infections and coinfections on cytokine synthesis. a comparative study. *J Immunol*, 149(1):181–7.

[Granja et al., 2006] Granja, A. G., Nogal, M. L., Hurtado, C., Del Aguila, C., Carrascosa, A. L., Salas, M. L., Fresno, M., and Revilla, Y. (2006). The viral protein A238L inhibits TNF-alpha expression through a CBP/p300 transcriptional coactivators pathway. *J Immunol*, 176(1):451–62.

[Gratama and Ernberg, 1995] Gratama, J. W. and Ernberg, I. (1995). Molecular epidemiology of epstein-barr virus infection. *Adv Cancer Res*, 67:197–255.

[Greaves and Mocarski, 1998] Greaves, R. F. and Mocarski, E. S. (1998). Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus ie1 mutant. *J Virol*, 72(1):366–79.

[Gribaudo et al., 1996] Gribaudo, G., Ravaglia, S., Guandalini, L., Cavallo, R., Gariglio, M., and Landolfo, S. (1996). The murine cytomegalovirus immediate-early 1 protein stimulates NF-kappa B activity by transactivating the NF-kappa B p105/p50 promoter. *Virus Res*, 45(1):15–27.

[Gribaudo et al., 2000] Gribaudo, G., Riera, L., Lembo, D., De Andrea, M., Gariglio, M., Rudge, T. L., Johnson, L. F., and Landolfo, S. (2000). Murine cytomegalovirus stimulates cellular thymidylate synthase gene expression in quiescent cells and requires the enzyme for replication. *J Virol*, 74(11):4979–87.

[Grotzinger et al., 1996] Grotzinger, T., Sternsdorf, T., Jensen, K., and Will, H. (1996). Interferon-modulated expression of genes encoding the nuclear-dot-associated proteins Sp100 and promyelocytic leukemia protein (pml). *Eur J Biochem*, 238(2):554–60.

[Gustems et al., 2006] Gustems, M., Borst, E., Benedict, C. A., Perez, C., Messerle, M., Ghazal, P., and Angulo, A. (2006). Regulation of the transcription and replication cycle of human cytomegalovirus is insensitive to genetic elimination of the cognate NF-kappaB binding sites in the enhancer. *J Virol*, 80(19):9899–904.

[Hahm et al., 2005] Hahm, B., Trifilo, M. J., Zuniga, E. I., and Oldstone, M. B. (2005). Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. *Immunity*, 22(2):247–57.

[Hanson et al., 1999] Hanson, L. K., Slater, J. S., Karabekian, Z., Virgin, H. W. t., Biron, C. A., Ruzek, M. C., van Rooijen, N., Ciavarra, R. P., Stenberg, R. M., and Campbell, A. E. (1999). Replication of murine cytomegalovirus in differentiated macrophages as a determinant of viral pathogenesis. *J Virol*, 73(7):5970–80.

[Hayhurst et al., 1995] Hayhurst, G. P., Bryant, L. A., Caswell, R. C., Walker, S. M., and Sinclair, J. H. (1995). Ccaat box-dependent activation of the tataless human dna polymerase alpha promoter by the human cytomegalovirus 72-kilodalton major immediate-early protein. *J Virol*, 69(1):182–8.

[Heise et al., 1998] Heise, M. T., Pollock, J. L., O'Guin, A., Barkon, M. L., Bormley, S., and Virgin, H. W. t. (1998). Murine cytomegalovirus infection inhibits IFN gamma-induced MHC class II expression on macrophages: the role of type I interferon. *Virology*, 241(2):331–44.

[Heise and Virgin, 1995] Heise, M. T. and Virgin, H. W. t. (1995). The T-cell-independent role of gamma interferon and tumor necrosis factor alpha in macrophage activation during murine cytomegalovirus and herpes simplex virus infections. *J Virol*, 69(2):904–9.

[Hengel et al., 1998] Hengel, H., Brune, W., and Koszinowski, U. H. (1998). Immune evasion by cytomegalovirus–survival strategies of a highly adapted opportunist. *Trends Microbiol*, 6(5):190–7.

[Herbein and O'Brien, 2000] Herbein, G. and O'Brien, W. A. (2000). Tumor necrosis factor (TNF)-alpha and TNF receptors in viral pathogenesis. *Proc Soc Exp Biol Med*, 223(3):241–57.

[Hokeness-Antonelli et al., 2007] Hokeness-Antonelli, K. L., Crane, M. J., Dragoi, A. M., Chu, W. M., and Salazar-Mather, T. P. (2007). IFN-alphabeta-mediated inflammatory responses and antiviral defense in liver is TLR9-independent but MyD88-dependent during murine cytomegalovirus infection. *J Immunol*, 179(9):6176–83.

[Hummel and Abecassis, 2002] Hummel, M. and Abecassis, M. M. (2002). A model for reactivation of CMV from latency. *J Clin Virol*, 25 Suppl 2:S123–36.

[Hummel et al., 2001] Hummel, M., Zhang, Z., Yan, S., DePlaen, I., Golia, P., Varghese, T., Thomas, G., and Abecassis, M. I. (2001). Allogeneic transplantation induces expression of cytomegalovirus immediate-early genes in vivo: a model for reactivation from latency. *J Virol*, 75(10):4814–22.

[Humphreys et al., 2007] Humphreys, I. R., de Trez, C., Kinkade, A., Benedict, C. A., Croft, M., and Ware, C. F. (2007). Cytomegalovirus exploits IL-10-mediated immune regulation in the salivary glands. *J Exp Med*, 204(5):1217–25.

[Inbal et al., 2000] Inbal, B., Shani, G., Cohen, O., Kissil, J. L., and Kimchi, A. (2000). Death-associated protein kinase-related protein 1, a novel serine/threonine kinase involved in apoptosis. *Mol Cell Biol*, 20(3):1044–54.

[Jarvis and Nelson, 2002] Jarvis, M. A. and Nelson, J. A. (2002). Mechanisms of human cytomegalovirus persistence and latency. *Front Biosci*, 7:d1575–82.

[Jeffrey I. Cohen, 2001] Jeffrey I. Cohen, S. E. S. (2001). *Varicella-Zoster Virus and Its Replication*. Philadelphia : Lippincott Williams and Wilkins.

[Jimenez et al., 2005] Jimenez, R., Ramirez, R., Carracedo, J., Aguera, M., Navarro, D., Santamaria, R., Perez, R., Del Castillo, D., and Aljama, P. (2005). Cytometric bead array (CBA) for the measurement of cytokines in urine and plasma of patients undergoing renal rejection. *Cytokine*, 32(1):45–50.

[Jonjic et al., 1989] Jonjic, S., Mutter, W., Weiland, F., Reddehase, M. J., and Koszinowski, U. H. (1989). Site-restricted persistent cytomegalovirus infection after selective long-term depletion of CD4 T lymphocytes. *J Exp Med*, 169(4):1199–212.

[Jonjic et al., 1990] Jonjic, S., Pavic, I., Lucin, P., Rukavina, D., and Koszinowski, U. H. (1990). Efficacious control of cytomegalovirus infection after long-term depletion of CD8 T lymphocytes. *J Virol*, 64(11):5457–64.

[Jonjic et al., 1994] Jonjic, S., Pavic, I., Polic, B., Crnkovic, I., Lucin, P., and Koszinowski, U. H. (1994). Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. *J Exp Med*, 179(5):1713–7.

[Keil et al., 1984] Keil, G. M., Ebeling-Keil, A., and Koszinowski, U. H. (1984). Temporal regulation of murine cytomegalovirus transcription and mapping of viral RNA synthesized at immediate early times after infection. *J Virol*, 50(3):784–95.

[Keil et al., 1987a] Keil, G. M., Ebeling-Keil, A., and Koszinowski, U. H. (1987a). Immediate-early genes of murine cytomegalovirus: location, transcripts, and translation products. *J Virol*, 61(2):526–33.

[Keil et al., 1987b] Keil, G. M., Ebeling-Keil, A., and Koszinowski, U. H. (1987b). Sequence and structural organization of murine cytomegalovirus immediate-early gene 1. *J Virol*, 61(6):1901–8.

[Kern et al., 1999] Kern, F., Surel, I. P., Faulhaber, N., Frommel, C., Schneider-Mergener, J., Schonemann, C., Reinke, P., and Volk, H. D. (1999). Target

structures of the cd8(+)-t-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. *J Virol*, 73(10):8179–84.

[Klenovsek et al., 2007] Klenovsek, K., Weisel, F., Schneider, A., Appelt, U., Jonjic, S., Messerle, M., Bradel-Tretheway, B., Winkler, T. H., and Mach, M. (2007). Protection from CMV infection in immunodeficient hosts by adoptive transfer of memory B cells. *Blood*, 110(9):3472–9.

[Koffron et al., 1998] Koffron, A. J., Hummel, M., Patterson, B. K., Yan, S., Kaufman, D. B., Fryer, J. P., Stuart, F. P., and Abecassis, M. I. (1998). Cellular localization of latent murine cytomegalovirus. *J Virol*, 72(1):95–103.

[Koffron et al., 1997] Koffron, A. J., Patterson, B. K., Yan, S., Kaufman, D. B., Fryer, J. P., Stuart, F. P., and Abecassis, M. I. (1997). Latent human cytomegalovirus: a functional study. *Transplant Proc*, 29(1-2):793–5.

[Koszinowski et al., 1986] Koszinowski, U. H., Keil, G. M., Volkmer, H., Fibi, M. R., Ebeling-Keil, A., and Munch, K. (1986). The 89,000-Mr murine cytomegalovirus immediate-early protein activates gene transcription. *J Virol*, 58(1):59–66.

[Kotenko et al., 2000] Kotenko, S. V., Saccani, S., Izotova, L. S., Mirochnitchenko, O. V., and Pestka, S. (2000). Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). *Proc Natl Acad Sci U S A*, 97(4):1695–700.

[Krmpotic et al., 2003] Krmpotic, A., Bubic, I., Polic, B., Lucin, P., and Jonjic, S. (2003). Pathogenesis of murine cytomegalovirus infection. *Microbes Infect*, 5(13):1263–77.

[Krmpotic et al., 2002] Krmpotic, A., Busch, D. H., Bubic, I., Gebhardt, F., Hengel, H., Hasan, M., Scalzo, A. A., Koszinowski, U. H., and Jonjic, S. (2002). MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells in vivo. *Nat Immunol*, 3(6):529–35.

[Krug et al., 2004] Krug, A., French, A. R., Barchet, W., Fischer, J. A., Dzionek, A., Pingel, J. T., Orihuela, M. M., Akira, S., Yokoyama, W. M., and Colonna, M. (2004). TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity*, 21(1):107–19.

[Kunicka et al., 1993] Kunicka, J. E., Talle, M. A., Denhardt, G. H., Brown, M., Prince, L. A., and Goldstein, G. (1993). Immunosuppression by glucocorticoids: inhibition of production of multiple lymphokines by in vivo administration of dexamethasone. *Cell Immunol*, 149(1):39–49.

[Kuprash et al., 1999] Kuprash, D. V., Udalova, I. A., Turetskaya, R. L., Kwiatkowski, D., Rice, N. R., and Nedospasov, S. A. (1999). Similarities and differences between human and murine TNF promoters in their response to lipopolysaccharide. *J Immunol*, 162(7):4045–52.

[Kurz et al., 1999] Kurz, S. K., Rapp, M., Steffens, H. P., Grzimek, N. K., Schmalz, S., and Reddehase, M. J. (1999). Focal transcriptional activity of murine cytomegalovirus during latency in the lungs. *J Virol*, 73(1):482–94.

[Le et al., 2008] Le, V. T., Trilling, M., Zimmermann, A., and Hengel, H. (2008). Mouse cytomegalovirus inhibits beta interferon (IFN-beta) gene expression and controls activation pathways of the IFN-beta enhanceosome. *J Gen Virol*, 89(Pt 5):1131–41.

[Lee et al., 2005] Lee, C., Tomkowicz, B., Freedman, B. D., and Collman, R. G. (2005). HIV-1 gp120-induced TNF-alpha production by primary human macrophages is mediated by phosphatidylinositol-3 (PI-3) kinase and mitogen-activated protein (MAP) kinase pathways. *J Leukoc Biol*, 78(4):1016–23.

[Lee et al., 2007] Lee, H. J., Pyo, J. O., Oh, Y., Kim, H. J., Hong, S. H., Jeon, Y. J., Kim, H., Cho, D. H., Woo, H. N., Song, S., Nam, J. H., Kim, H. J., Kim, K. S., and Jung, Y. K. (2007). AK2 activates a novel apoptotic pathway through formation of a complex with FADD and caspase-10. *Nat Cell Biol*, 9(11):1303–10.

[Lee et al., 2004] Lee, H. R., Kim, D. J., Lee, J. M., Choi, C. Y., Ahn, B. Y., Hayward, G. S., and Ahn, J. H. (2004). Ability of the human cytomegalovirus IE1 protein to modulate sumoylation of PML correlates with its functional activities in transcriptional regulation and infectivity in cultured fibroblast cells. *J Virol*, 78(12):6527–42.

[Lembo et al., 2000] Lembo, D., Gribaudo, G., Hofer, A., Riera, L., Cornaglia, M., Mondo, A., Angeretti, A., Gariglio, M., Thelander, L., and Landolfo, S.

(2000). Expression of an altered ribonucleotide reductase activity associated with the replication of murine cytomegalovirus in quiescent fibroblasts. *J Virol*, 74(24):11557–65.

[Lenac et al., 2008] Lenac, T., Arapovic, J., Traven, L., Krmpotic, A., and Jonjic, S. (2008). Murine cytomegalovirus regulation of NKG2D ligands. *Med Microbiol Immunol*, 197(2):159–66.

[Lenzo et al., 2001] Lenzo, J. C., Fairweather, D., Shellam, G. R., and Lawson, C. M. (2001). Immunomodulation of murine cytomegalovirus-induced myocarditis in mice treated with lipopolysaccharide and tumor necrosis factor. *Cell Immunol*, 213(1):52–61.

[Lenzo et al., 2003] Lenzo, J. C., Mansfield, J. P., Sivamoorthy, S., Cull, V. S., and James, C. M. (2003). Cytokine expression in murine cytomegalovirus-induced myocarditis: modulation with interferon-alpha therapy. *Cell Immunol*, 223(1):77–86.

[Li et al., 2005] Li, X., Zhang, R., Luo, D., Park, S. J., Wang, Q., Kim, Y., and Min, W. (2005). Tumor necrosis factor alpha-induced desumoylation and cytoplasmic translocation of homeodomain-interacting protein kinase 1 are critical for apoptosis signal-regulating kinase 1-JNK/p38 activation. *J Biol Chem*, 280(15):15061–70.

[Libermann and Baltimore, 1990] Libermann, T. A. and Baltimore, D. (1990). Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol Cell Biol*, 10(5):2327–34.

[Loewendorf et al., 2004] Loewendorf, A., Kruger, C., Borst, E. M., Wagner, M., Just, U., and Messerle, M. (2004). Identification of a mouse cytomegalovirus gene selectively targeting CD86 expression on antigen-presenting cells. *J Virol*, 78(23):13062–71.

[Lucin et al., 1994] Lucin, P., Jonjic, S., Messerle, M., Polic, B., Hengel, H., and Koszinowski, U. H. (1994). Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon-gamma and tumour necrosis factor. *J Gen Virol*, 75 (Pt 1):101–10.

[Lucin et al., 1992] Lucin, P., Pavic, I., Polic, B., Jonjic, S., and Koszinowski, U. H. (1992). Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. *J Virol*, 66(4):1977–84.

[Lusso et al., 1994] Lusso, P., Secchiero, P., Crowley, R. W., Garzino-Demo, A., Berneman, Z. N., and Gallo, R. C. (1994). Cd4 is a critical component of the receptor for human herpesvirus 7: interference with human immunodeficiency virus. *Proc Natl Acad Sci U S A*, 91(9):3872–6.

[Mack et al., 2008] Mack, C., Sickmann, A., Lembo, D., and Brune, W. (2008). Inhibition of proinflammatory and innate immune signaling pathways by a cytomegalovirus RIP1-interacting protein. *Proc Natl Acad Sci U S A*.

[Mancini et al., 2000] Mancini, M., Machamer, C. E., Roy, S., Nicholson, D. W., Thornberry, N. A., Casciola-Rosen, L. A., and Rosen, A. (2000). Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis. *J Cell Biol*, 149(3):603–12.

[Manning and Mocarski, 1988] Manning, W. C. and Mocarski, E. S. (1988). Insertional mutagenesis of the murine cytomegalovirus genome: one prominent alpha gene (ie2) is dispensable for growth. *Virology*, 167(2):477–84.

[Maul, 1998] Maul, G. G. (1998). Nuclear domain 10, the site of DNA virus transcription and replication. *Bioessays*, 20(8):660–7.

[Maul and Negorev, 2008] Maul, G. G. and Negorev, D. (2008). Differences between mouse and human cytomegalovirus interactions with their respective hosts at immediate early times of the replication cycle. *Med Microbiol Immunol*.

[Mauro et al., 2006] Mauro, C., Pacifico, F., Lavorgna, A., Mellone, S., Iannetti, A., Acquaviva, R., Formisano, S., Vito, P., and Leonardi, A. (2006). ABIN-1 binds to NEMO/IKKgamma and co-operates with A20 in inhibiting NF-kappaB. *J Biol Chem*, 281(27):18482–8.

[McFadden et al., 1997] McFadden, G., Schreiber, M., and Sedger, L. (1997). Myxoma T2 protein as a model for poxvirus TNF receptor homologs. *J Neuroimmunol*, 72(2):119–26.

[Melchjorsen et al., 2003] Melchjorsen, J., Sorensen, L. N., and Paludan, S. R. (2003). Expression and function of chemokines during viral infections: from molecular mechanisms to in vivo function. *J Leukoc Biol*, 74(3):331–43.

[Messerle et al., 1992] Messerle, M., Buhler, B., Keil, G. M., and Koszinowski, U. H. (1992). Structural organization, expression, and functional characterization of the murine cytomegalovirus immediate-early gene 3. *J Virol*, 66(1):27–36.

[Messerle et al., 1997] Messerle, M., Crnkovic, I., Hammerschmidt, W., Ziegler, H., and Koszinowski, U. H. (1997). Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. *Proc Natl Acad Sci U S A*, 94(26):14759–63.

[Messerle et al., 1991] Messerle, M., Keil, G. M., and Koszinowski, U. H. (1991). Structure and expression of murine cytomegalovirus immediate-early gene 2. *J Virol*, 65(3):1638–43.

[Mintern et al., 2006] Mintern, J. D., Klemm, E. J., Wagner, M., Paquet, M. E., Napier, M. D., Kim, Y. M., Koszinowski, U. H., and Ploegh, H. L. (2006). Viral interference with B7-1 costimulation: a new role for murine cytomegalovirus fc receptor-1. *J Immunol*, 177(12):8422–31.

[Minton et al., 1994] Minton, E. J., Tysoe, C., Sinclair, J. H., and Sissons, J. G. (1994). Human cytomegalovirus infection of the monocyte/macrophage lineage in bone marrow. *J Virol*, 68(6):4017–21.

[Mitchell et al., 1996] Mitchell, B. M., Leung, A., and Stevens, J. G. (1996). Murine cytomegalovirus DNA in peripheral blood of latently infected mice is detectable only in monocytes and polymorphonuclear leukocytes. *Virology*, 223(1):198–207.

[Monick et al., 2003] Monick, M. M., Powers, L. S., Butler, N. S., and Hunninghake, G. W. (2003). Inhibition of Rho family GTPases results in increased TNF-alpha production after lipopolysaccharide exposure. *J Immunol*, 171(5):2625–30.

[Montag et al., 2006] Montag, C., Wagner, J., Gruska, I., and Hagemeier, C. (2006). Human cytomegalovirus blocks tumor necrosis factor alpha- and interleukin-1beta-mediated NF-kappaB signaling. *J Virol*, 80(23):11686–98.

[Munch et al., 1988] Munch, K., Keil, G. M., Messerle, M., and Koszinowski, U. H. (1988). Interaction of the 89K murine cytomegalovirus immediate-early protein with core histones. *Virology*, 163(2):405–12.

[Munch et al., 1992] Munch, K., Messerle, M., Plachter, B., and Koszinowski, U. H. (1992). An acidic region of the 89K murine cytomegalovirus immediate early protein interacts with DNA. *J Gen Virol*, 73 (Pt 3):499–506.

[Nakamichi et al., 2004] Nakamichi, K., Inoue, S., Takasaki, T., Morimoto, K., and Kurane, I. (2004). Rabies virus stimulates nitric oxide production and CXC chemokine ligand 10 expression in macrophages through activation of extracellular signal-regulated kinases 1 and 2. *J Virol*, 78(17):9376–88.

[Napoli et al., 2000] Napoli, C., Quehenberger, O., De Nigris, F., Abete, P., Glass, C. K., and Palinski, W. (2000). Mildly oxidized low density lipoprotein activates multiple apoptotic signaling pathways in human coronary cells. *Faseb J*, 14(13):1996–2007.

[Oakley et al., 2008] Oakley, O. R., Garvy, B. A., Humphreys, S., Qureshi, M. H., and Pomeroy, C. (2008). Increased weight loss with reduced viral replication in interleukin-10 knock-out mice infected with murine cytomegalovirus. *Clin Exp Immunol*, 151(1):155–64.

[Ohmori and Hamilton, 2000] Ohmori, Y. and Hamilton, T. A. (2000). Interleukin-4/stat6 represses stat1 and nf-kappa b-dependent transcription through distinct mechanisms. *J Biol Chem*, 275(48):38095–103.

[Orange and Biron, 1996] Orange, J. S. and Biron, C. A. (1996). Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *J Immunol*, 156(12):4746–56.

[Orange et al., 1995] Orange, J. S., Wang, B., Terhorst, C., and Biron, C. A. (1995). Requirement for natural killer cell-produced interferon gamma in defense

against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J Exp Med*, 182(4):1045–56.

[Paludan and Mogensen, 2001] Paludan, S. R. and Mogensen, S. C. (2001). Virus-cell interactions regulating induction of tumor necrosis factor alpha production in macrophages infected with herpes simplex virus. *J Virol*, 75(21):10170–8.

[Paulus et al., 2006] Paulus, C., Krauss, S., and Nevels, M. (2006). A human cytomegalovirus antagonist of type I IFN-dependent signal transducer and activator of transcription signaling. *Proc Natl Acad Sci U S A*, 103(10):3840–5.

[Pavic et al., 1993] Pavic, I., Polic, B., Crnkovic, I., Lucin, P., Jonjic, S., and Koszinowski, U. H. (1993). Participation of endogenous tumour necrosis factor alpha in host resistance to cytomegalovirus infection. *J Gen Virol*, 74 (Pt 10):2215–23.

[Pazdrak et al., 2002] Pazdrak, K., Olszewska-Pazdrak, B., Liu, T., Takizawa, R., Brasier, A. R., Garofalo, R. P., and Casola, A. (2002). MAPK activation is involved in posttranscriptional regulation of RSV-induced RANTES gene expression. *Am J Physiol Lung Cell Mol Physiol*, 283(2):L364–72.

[Pellett P. E., 2001] Pellett P. E., D. G. (2001). *Human Herpesviruses 6A, 6B, and 7 and Their Replication*. Philadelphia : Lippincott Williams and Wilkins.

[Pellett P. E., 2006] Pellett P. E., R. B. (2006). *The Family Herpesviridae: A Brief Introduction*. Philadelphia : Lippincott Williams and Wilkins.

[Penfold et al., 1999] Penfold, M. E., Dairaghi, D. J., Duke, G. M., Saederup, N., Mocarski, E. S., Kemble, G. W., and Schall, T. J. (1999). Cytomegalovirus encodes a potent alpha chemokine. *Proc Natl Acad Sci U S A*, 96(17):9839–44.

[Pollock et al., 1997] Pollock, J. L., Presti, R. M., Paetzold, S., and Virgin, H. W. t. (1997). Latent murine cytomegalovirus infection in macrophages. *Virology*, 227(1):168–79.

[Pomeroy et al., 1998] Pomeroy, C., Delong, D., Clabots, C., Riciputi, P., and Filice, G. A. (1998). Role of interferon-gamma in murine cytomegalovirus infection. *J Lab Clin Med*, 132(2):124–33.

[Popkin and Virgin, 2003] Popkin, D. L. and Virgin, H. W. t. (2003). Murine cytomegalovirus infection inhibits tumor necrosis factor alpha responses in primary macrophages. *J Virol*, 77(18):10125–30.

[Potena et al., 2004] Potena, L., Frascaroli, G., Grigioni, F., Lazzarotto, T., Magnani, G., Tomasi, L., Coccolo, F., Gabrielli, L., Magelli, C., Landini, M. P., and Branzi, A. (2004). Hydroxymethyl-glutaryl coenzyme a reductase inhibition limits cytomegalovirus infection in human endothelial cells. *Circulation*, 109(4):532–6.

[Prive and Descoteaux, 2000] Prive, C. and Descoteaux, A. (2000). Leishmania donovani promastigotes evade the activation of mitogen-activated protein kinases p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase-1/2 during infection of naive macrophages. *Eur J Immunol*, 30(8):2235–44.

[Rahbar et al., 2003] Rahbar, A., Bostrom, L., Lagerstedt, U., Magnusson, I., Soderberg-Naucler, C., and Sundqvist, V. A. (2003). Evidence of active cytomegalovirus infection and increased production of IL-6 in tissue specimens obtained from patients with inflammatory bowel diseases. *Inflamm Bowel Dis*, 9(3):154–61.

[Rahman and McFadden, 2006] Rahman, M. M. and McFadden, G. (2006). Modulation of tumor necrosis factor by microbial pathogens. *PLoS Pathog*, 2(2):e4.

[Ramana et al., 2002] Ramana, C. V., Gil, M. P., Schreiber, R. D., and Stark, G. R. (2002). Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. *Trends Immunol*, 23(2):96–101.

[Randolph-Habecker et al., 2002] Randolph-Habecker, J., Iwata, M., Geballe, A. P., Jarrahian, S., and Torok-Storb, B. (2002). Interleukin-1-mediated inhibition of cytomegalovirus replication is due to increased IFN-beta production. *J Interferon Cytokine Res*, 22(7):765–72.

[Rao, 2001] Rao, K. M. (2001). MAP kinase activation in macrophages. *J Leukoc Biol*, 69(1):3–10.

[Rawlinson et al., 1996] Rawlinson, W. D., Farrell, H. E., and Barrell, B. G. (1996). Analysis of the complete dna sequence of murine cytomegalovirus. *J Virol*, 70(12):8833–49.

[Reddehase, 2002] Reddehase, M. J. (2002). Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. *Nat Rev Immunol*, 2(11):831–44.

[Reddehase et al., 1985] Reddehase, M. J., Weiland, F., Munch, K., Jonjic, S., Luske, A., and Koszinowski, U. H. (1985). Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. *J Virol*, 55(2):264–73.

[Redpath et al., 1999] Redpath, S., Angulo, A., Gascoigne, N. R., and Ghazal, P. (1999). Murine cytomegalovirus infection down-regulates MHC class II expression on macrophages by induction of IL-10. *J Immunol*, 162(11):6701–7.

[Redpath et al., 2001] Redpath, S., Angulo, A., Gascoigne, N. R., and Ghazal, P. (2001). Immune checkpoints in viral latency. *Annu Rev Microbiol*, 55:531–60.

[Rickinson A. B., 2006] Rickinson A. B., K. E. (2006). *Epstein-Barr Virus*. Philadelphia : Lippincott Williams and Wilkins.

[Roach et al., 2005] Roach, S. K., Lee, S. B., and Schorey, J. S. (2005). Differential activation of the transcription factor cyclic AMP response element binding protein (CREB) in macrophages following infection with pathogenic and nonpathogenic mycobacteria and role for CREB in tumor necrosis factor alpha production. *Infect Immun*, 73(1):514–22.

[Robbins et al., 2007] Robbins, S. H., Bessou, G., Cornillon, A., Zucchini, N., Rupp, B., Ruzsics, Z., Sacher, T., Tomasello, E., Vivier, E., Koszinowski, U. H., and Dalod, M. (2007). Natural killer cells promote early CD8 T cell responses against cytomegalovirus. *PLoS Pathog*, 3(8):e123.

[Rochat-Steiner et al., 2000] Rochat-Steiner, V., Becker, K., Micheau, O., Schneider, P., Burns, K., and Tschopp, J. (2000). FIST/HIPK3: a Fas/FADD-interacting serine/threonine kinase that induces FADD phosphorylation and inhibits Fas-mediated Jun NH(2)-terminal kinase activation. *J Exp Med*, 192(8):1165–74.

[Roizman and Knipe, 2001] Roizman, B. and Knipe, D. M. (2001). *Herpes Simplex Viruses and Their Replication*. Philadelphia : Lippincott Williams and Wilkins.

[Roizman B., 2006] Roizman B., Knipe D. M., W. R. J. (2006). *Herpes Simplex Viruses*. Philadelphia : Lippincott Williams and Wilkins.

[Rott et al., 2003] Rott, D., Zhu, J., Zhou, Y. F., Burnett, M. S., Zalles-Ganley, A., and Epstein, S. E. (2003). IL-6 is produced by splenocytes derived from CMV-infected mice in response to CMV antigens, and induces MCP-1 production by endothelial cells: a new mechanistic paradigm for infection-induced atherogenesis. *Atherosclerosis*, 170(2):223–8.

[Rowe et al., 1992] Rowe, M., Lear, A. L., Croom-Carter, D., Davies, A. H., and Rickinson, A. B. (1992). Three pathways of epstein-barr virus gene activation from ebna1-positive latency in b lymphocytes. *J Virol*, 66(1):122–31.

[Rowlett et al., 2008] Rowlett, R. M., Chrestensen, C. A., Nyce, M., Harp, M. G., Pelo, J. W., Cominelli, F., Ernst, P. B., Pizarro, T. T., Sturgill, T. W., and Worthington, M. T. (2008). MNK kinases regulate multiple TLR pathways and innate proinflammatory cytokines in macrophages. *Am J Physiol Gastrointest Liver Physiol*, 294(2):G452–9.

[Rutault et al., 2001] Rutault, K., Hazzalin, C. A., and Mahadevan, L. C. (2001). Combinations of ERK and p38 MAPK inhibitors ablate tumor necrosis factor-alpha (TNF-alpha) mRNA induction. evidence for selective destabilization of TNF-alpha transcripts. *J Biol Chem*, 276(9):6666–74.

[Ruzek et al., 1997] Ruzek, M. C., Miller, A. H., Opal, S. M., Pearce, B. D., and Biron, C. A. (1997). Characterization of early cytokine responses and an interleukin (IL)-6-dependent pathway of endogenous glucocorticoid induction during murine cytomegalovirus infection. *J Exp Med*, 185(7):1185–92.

[Saederup et al., 1999] Saederup, N., Lin, Y. C., Dairaghi, D. J., Schall, T. J., and Mocarski, E. S. (1999). Cytomegalovirus-encoded beta chemokine promotes monocyte-associated viremia in the host. *Proc Natl Acad Sci U S A*, 96(19):10881–6.

[Saffert and Kalejta, 2006] Saffert, R. T. and Kalejta, R. F. (2006). Inactivating a cellular intrinsic immune defense mediated by daxx is the mechanism through which the human cytomegalovirus pp71 protein stimulates viral immediate-early gene expression. *J Virol*, 80(8):3863–71.

[Saito et al., 2006] Saito, K., Meyer, K., Warner, R., Basu, A., Ray, R. B., and Ray, R. (2006). Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein. *J Virol*, 80(9):4372–9.

[Sambucetti et al., 1989] Sambucetti, L. C., Cherrington, J. M., Wilkinson, G. W., and Mocarski, E. S. (1989). NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. *Embo J*, 8(13):4251–8.

[Scalzo et al., 1990] Scalzo, A. A., Fitzgerald, N. A., Simmons, A., La Vista, A. B., and Shellam, G. R. (1990). Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. *J Exp Med*, 171(5):1469–83.

[Scalzo et al., 1992] Scalzo, A. A., Fitzgerald, N. A., Wallace, C. R., Gibbons, A. E., Smart, Y. C., Burton, R. C., and Shellam, G. R. (1992). The effect of the Cmv-1 resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. *J Immunol*, 149(2):581–9.

[Schall and Bacon, 1994] Schall, T. J. and Bacon, K. B. (1994). Chemokines, leukocyte trafficking, and inflammation. *Curr Opin Immunol*, 6(6):865–73.

[Schickedanz et al., 1988] Schickedanz, J., Philipson, L., Ansorge, W., Pepperkok, R., Klein, R., and Koszinowski, U. H. (1988). The 89,000-Mr murine cytomegalovirus immediate-early protein stimulates c-fos expression and cellular DNA synthesis. *J Virol*, 62(9):3341–7.

[Schneider et al., 2008] Schneider, K., Loewendorf, A., De Trez, C., Fulton, J., Rhode, A., Shumway, H., Ha, S., Patterson, G., Pfeffer, K., Nedospasov, S. A., Ware, C. F., and Benedict, C. A. (2008). Lymphotoxin-mediated crosstalk between B cells and splenic stroma promotes the initial type I interferon response to cytomegalovirus. *Cell Host Microbe*, 3(2):67–76.

[Shanley et al., 1994] Shanley, J. D., Goff, E., Debs, R. J., and Forman, S. J. (1994). The role of tumor necrosis factor-alpha in acute murine cytomegalovirus infection in BALB/c mice. *J Infect Dis*, 169(5):1088–91.

[Shanley et al., 2001] Shanley, J. D., Shanley, J. A., Albert, G., and Biegel, D. (2001). Characterization of virus-induced interferon-gamma responses in mice previously infected with murine cytomegalovirus. *J Infect Dis*, 183(5):697–706.

[Shi et al., 2002] Shi, L., Kishore, R., McMullen, M. R., and Nagy, L. E. (2002). Lipopolysaccharide stimulation of ERK1/2 increases TNF-alpha production via Egr-1. *Am J Physiol Cell Physiol*, 282(6):C1205–11.

[Simon et al., 2006] Simon, C. O., Holtappels, R., Tervo, H. M., Bohm, V., Daubner, T., Oehrlein-Karpi, S. A., Kuhnapfel, B., Renzaho, A., Strand, D., Podlech, J., Reddehase, M. J., and Grzimek, N. K. (2006). CD8 T cells control cytomegalovirus latency by epitope-specific sensing of transcriptional reactivation. *J Virol*, 80(21):10436–56.

[Simon et al., 2005] Simon, C. O., Seckert, C. K., Dreis, D., Reddehase, M. J., and Grzimek, N. K. (2005). Role for tumor necrosis factor alpha in murine cytomegalovirus transcriptional reactivation in latently infected lungs. *J Virol*, 79(1):326–40.

[Skaletskaya et al., 2001] Skaletskaya, A., Bartle, L. M., Chittenden, T., McCormick, A. L., Mocarski, E. S., and Goldmacher, V. S. (2001). A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. *Proc Natl Acad Sci U S A*, 98(14):7829–34.

[Soderberg-Naucler, 2006] Soderberg-Naucler, C. (2006). Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *J Intern Med*, 259(3):219–46.

[Spencer, 2007] Spencer, J. V. (2007). The cytomegalovirus homolog of interleukin-10 requires phosphatidylinositol 3-kinase activity for inhibition of cytokine synthesis in monocytes. *J Virol*, 81(4):2083–6.

[Spencer et al., 2002] Spencer, J. V., Lockridge, K. M., Barry, P. A., Lin, G., Tsang, M., Penfold, M. E., and Schall, T. J. (2002). Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. *J Virol*, 76(3):1285–92.

[Stagno et al., 1986] Stagno, S., Pass, R. F., Cloud, G., Britt, W. J., Henderson, R. E., Walton, P. D., Veren, D. A., Page, F., and Alford, C. A. (1986). Primary

cytomegalovirus infection in pregnancy. incidence, transmission to fetus, and clinical outcome. *Jama*, 256(14):1904–8.

[Stinski and Isomura, 2008] Stinski, M. F. and Isomura, H. (2008). Role of the cytomegalovirus major immediate early enhancer in acute infection and reactivation from latency. *Med Microbiol Immunol*, 197(2):223–31.

[Stoddart et al., 1994] Stoddart, C. A., Cardin, R. D., Boname, J. M., Manning, W. C., Abenes, G. B., and Mocarski, E. S. (1994). Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. *J Virol*, 68(10):6243–53.

[Streblow et al., 1999] Streblow, D. N., Soderberg-Naucler, C., Vieira, J., Smith, P., Wakabayashi, E., Ruchti, F., Mattison, K., Altschuler, Y., and Nelson, J. A. (1999). The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell*, 99(5):511–20.

[Strobl et al., 2005] Strobl, B., Bubic, I., Bruns, U., Steinborn, R., Lajko, R., Kolbe, T., Karaghiosoff, M., Kalinke, U., Jonjic, S., and Muller, M. (2005). Novel functions of tyrosine kinase 2 in the antiviral defense against murine cytomegalovirus. *J Immunol*, 175(6):4000–8.

[Tabeta et al., 2004] Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J., Alexopoulou, L., Flavell, R. A., and Beutler, B. (2004). Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci U S A*, 101(10):3516–21.

[Tailor et al., 2007] Tailor, P., Tamura, T., Kong, H. J., Kubota, T., Kubota, M., Borghi, P., Gabriele, L., and Ozato, K. (2007). The feedback phase of type I interferon induction in dendritic cells requires interferon regulatory factor 8. *Immunity*, 27(2):228–39.

[Takahashi et al., 1992] Takahashi, K., Segal, E., Kondo, T., Mukai, T., Moriyama, M., Takahashi, M., and Yamanishi, K. (1992). Interferon and natural killer cell activity in patients with exanthem subitum. *Pediatr Infect Dis J*, 11(5):369–73.

[Tang and Maul, 2003] Tang, Q. and Maul, G. G. (2003). Mouse cytomegalovirus immediate-early protein 1 binds with host cell repressors to relieve suppressive effects on viral transcription and replication during lytic infection. *J Virol*, 77(2):1357–67.

[Tang-Feldman et al., 2006] Tang-Feldman, Y. J., Wojtowicz, A., Lochhead, G. R., Hale, M. A., Li, Y., and Pomeroy, C. (2006). Use of quantitative real-time PCR (qRT-PCR) to measure cytokine transcription and viral load in murine cytomegalovirus infection. *J Virol Methods*, 131(2):122–9.

[Tavalai et al., 2008] Tavalai, N., Papior, P., Rechter, S., and Stamminger, T. (2008). Nuclear domain 10 components promyelocytic leukemia protein and hDaxx independently contribute to an intrinsic antiviral defense against human cytomegalovirus infection. *J Virol*, 82(1):126–37.

[Tay and Welsh, 1997] Tay, C. H. and Welsh, R. M. (1997). Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. *J Virol*, 71(1):267–75.

[Trgovcich et al., 2000] Trgovcich, J., Stimac, D., Polic, B., Krmpotic, A., Pernjak-Pugel, E., Tomac, J., Hasan, M., Wraber, B., and Jonjic, S. (2000). Immune responses and cytokine induction in the development of severe hepatitis during acute infections with murine cytomegalovirus. *Arch Virol*, 145(12):2601–18.

[Tsai et al., 2000] Tsai, E. Y., Falvo, J. V., Tsytsykova, A. V., Barczak, A. K., Reimold, A. M., Glimcher, L. H., Fenton, M. J., Gordon, D. C., Dunn, I. F., and Goldfeld, A. E. (2000). A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo. *Mol Cell Biol*, 20(16):6084–94.

[Tsytsykova et al., 2007] Tsytsykova, A. V., Falvo, J. V., Schmidt-Supprian, M., Courtois, G., Thanos, D., and Goldfeld, A. E. (2007). Post-induction, stimulus-specific regulation of tumor necrosis factor mRNA expression. *J Biol Chem*, 282(16):11629–38.

[Valledor et al., 2000] Valledor, A. F., Comalada, M., Xaus, J., and Celada, A. (2000). The differential time-course of extracellular-regulated kinase activity

correlates with the macrophage response toward proliferation or activation. *J Biol Chem*, 275(10):7403–9.

[Varani et al., 2007] Varani, S., Cederarv, M., Feld, S., Tammik, C., Frascaroli, G., Landini, M. P., and Soderberg-Naucler, C. (2007). Human cytomegalovirus differentially controls B cell and T cell responses through effects on plasmacytoid dendritic cells. *J Immunol*, 179(11):7767–76.

[Varani et al., 2005] Varani, S., Frascaroli, G., Homman-Loudiyi, M., Feld, S., Landini, M. P., and Soderberg-Naucler, C. (2005). Human cytomegalovirus inhibits the migration of immature dendritic cells by down-regulating cell-surface CCR1 and CCR5. *J Leukoc Biol*, 77(2):219–28.

[Vliegen et al., 2004] Vliegen, I., Duijvestijn, A., Stassen, F., and Bruggeman, C. (2004). Murine cytomegalovirus infection directs macrophage differentiation into a pro-inflammatory immune phenotype: implications for atherogenesis. *Microbes Infect*, 6(12):1056–62.

[Wade et al., 1992] Wade, M., Kowalik, T. F., Mudryj, M., Huang, E. S., and Azizkhan, J. C. (1992). E2f mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. *Mol Cell Biol*, 12(10):4364–74.

[Wang et al., 2003] Wang, P. Y., Liu, P., Weng, J., Sontag, E., and Anderson, R. G. (2003). A cholesterol-regulated PP2A/HePTP complex with dual specificity ERK1/2 phosphatase activity. *Embo J*, 22(11):2658–67.

[Wang et al., 2005] Wang, P. Y., Weng, J., and Anderson, R. G. (2005). OSBP is a cholesterol-regulated scaffolding protein in control of ERK 1/2 activation. *Science*, 307(5714):1472–6.

[Wang and Sonenshein, 2005] Wang, X. and Sonenshein, G. E. (2005). Induction of the relB NF-kappaB subunit by the cytomegalovirus IE1 protein is mediated via Jun kinase and c-Jun/Fra-2 AP-1 complexes. *J Virol*, 79(1):95–105.

[Wati et al., 2007] Wati, S., Li, P., Burrell, C. J., and Carr, J. M. (2007). Dengue virus (DV) replication in monocyte-derived macrophages is not affected by tumor necrosis factor alpha (TNF-alpha), and DV infection induces altered responsiveness to TNF-alpha stimulation. *J Virol*, 81(18):10161–71.

[Wennerberg et al., 2005] Wennerberg, K., Rossman, K. L., and Der, C. J. (2005). The Ras superfamily at a glance. *J Cell Sci*, 118(Pt 5):843–6.

[Xu et al., 2001] Xu, Z., Dziarski, R., Wang, Q., Swartz, K., Sakamoto, K. M., and Gupta, D. (2001). Bacterial peptidoglycan-induced tnf-alpha transcription is mediated through the transcription factors Egr-1, Elk-1, and NF-kappaB. *J Immunol*, 167(12):6975–82.

[Yakushijin et al., 1991] Yakushijin, Y., Yasukawa, M., and Kobayashi, Y. (1991). T-cell immune response to human herpesvirus-6 in healthy adults. *Microbiol Immunol*, 35(8):655–60.

[Yakushijin et al., 1992] Yakushijin, Y., Yasukawa, M., and Kobayashi, Y. (1992). Establishment and functional characterization of human herpesvirus 6-specific cd4+ human t-cell clones. *J Virol*, 66(5):2773–9.

[Yasukawa et al., 1993] Yasukawa, M., Yakushijin, Y., Furukawa, M., and Fujita, S. (1993). Specificity analysis of human cd4+ t-cell clones directed against human herpesvirus 6 (hhv-6), hhv-7, and human cytomegalovirus. *J Virol*, 67(10):6259–64.

[Zhang et al., 2005] Zhang, P., Martin, M., Michalek, S. M., and Katz, J. (2005). Role of mitogen-activated protein kinases and NF-kappaB in the regulation of proinflammatory and anti-inflammatory cytokines by Porphyromonas gingivalis hemagglutinin B. *Infect Immun*, 73(7):3990–8.

[Zhang et al., 2002] Zhang, S., Fukushi, M., Hashimoto, S., Gao, C., Huang, L., Fukuyo, Y., Nakajima, T., Amagasa, T., Enomoto, S., Koike, K., Miura, O., Yamamoto, N., and Tsuchida, N. (2002). A new ERK2 binding protein, Naf1, attenuates the EGF/ERK2 nuclear signaling. *Biochem Biophys Res Commun*, 297(1):17–23.

[Zhu et al., 2005] Zhu, J., Krishnegowda, G., and Gowda, D. C. (2005). Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of plasmodium falciparum: the requirement of extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase and NF-kappaB pathways for the expression of proinflammatory cytokines and nitric oxide. *J Biol Chem*, 280(9):8617–27.

[Zhu et al., 2000] Zhu, W., Downey, J. S., Gu, J., Di Padova, F., Gram, H., and Han, J. (2000). Regulation of TNF expression by multiple mitogen-activated protein kinase pathways. *J Immunol*, 164(12):6349–58.

[Zimmermann et al., 2005] Zimmermann, A., Trilling, M., Wagner, M., Wilborn, M., Bubic, I., Jonjic, S., Koszinowski, U., and Hengel, H. (2005). A cytomegaloviral protein reveals a dual role for STAT2 in IFN-gamma signaling and antiviral responses. *J Exp Med*, 201(10):1543–53.

[Ziouzenkova et al., 2003] Ziouzenkova, O., Perrey, S., Asatryan, L., Hwang, J., MacNaul, K. L., Moller, D. E., Rader, D. J., Sevanian, A., Zechner, R., Hoefler, G., and Plutzky, J. (2003). Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an antiinflammatory role for lipoprotein lipase. *Proc Natl Acad Sci U S A*, 100(5):2730–5.

[Zucchini et al., 2007] Zucchini, N., Bessou, G., Robbins, S. H., Chasson, L., Raper, A., Crocker, P. R., and Dalod, M. (2007). Individual plasmacytoid dendritic cells are major contributors to the production of multiple innate cytokines in an organ-specific manner during viral infection. *Int Immunol*.

[Zucchini et al., 2008] Zucchini, N., Bessou, G., Traub, S., Robbins, S. H., Uematsu, S., Akira, S., Alexopoulou, L., and Dalod, M. (2008). Cutting edge: Overlapping functions of TLR7 and TLR9 for innate defense against a herpesvirus infection. *J Immunol*, 180(9):5799–5803.