Defining the molecular pathways governing antiviral Natural Killer Cell Immunity

A thesis submitted in candidature for the degree of

Doctor of Philosophy (PhD)

by

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Summary

Natural Killer (NK) cells are important effector cells for control of cytomegalovirus (CMV) infection. Understanding the mechanisms which contribute to antiviral control by NK cells may inform on the development of safe and effective treatments for viral infection such as NK cell-based therapies. The first aim of this thesis was to identify genes required for control of cytomegalovirus replication using an *in vivo* MCMV infection screen. Several genes of interest which affected acute viral replication control were discovered from the *in vivo* screen including *Fam111a, Fam114a2, Far1, Heatr9, Serpinb9b* and *Sytl3*. The second aim was to examine how gene(s) of interest regulate NK cell functions. The novel gene Synaptotagmin-like 3 (*Sytl3*) was chosen for further investigation as it was almost exclusively expressed by mouse (and human) NK cells and *Sytl3* deficiency led to a significant impairment in MCMV replication control. Members of the sytl family have been implicated in vesicular trafficking events due to their ability to bind Rab27 proteins which are important in secretory pathways (Taruho S. Kuroda *et al.*, 2002). Interestingly, *Sytl3* deficient NK cells had reduced degranulation in response to a variety of stimuli and my studies revealed that sytl3 may drive microtubule organising centre (MTOC) polarisation towards the immunological synapse, which is a key step for efficient degranulation. Therefore, this work provides novel insight into NK cell effector function whereby optimal degranulation in response to MCMV (and possibly other stimuli) contributes to the effective control of acute viral infection.

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AIM2	Absent in melanoma 2
AP-1	Activator protein-1
AP3	Adaptor protein 3
APCs	Antigen presenting cells
ART	Antiretroviral therapy
ASC	Apoptosis-associated speck-like protein containing a CARD
BAC	Bacterial artificial chromosome
BSA	Bovine serum albumin
C-type	Carboxyl terminal type
cAMP	Cyclic AMP
CAR	Chimeric antigen receptor
Cas9	CRISPR-associated protein 9
CCL3 (or MIP-1α)	C-C motif chemokine ligand 3
cDCs	Classical DCs
cGAS	Cyclic GMP-AMP synthase
CHS	Chediak-Higashi syndrome
CIP4	Cdc42-interacting protein-4
CLPs	Common lymphoid progenitors
CMP	Common myeloid progenitors
CREB	cAMP response element binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
cSMAC	Central supramolecular activation cluster
CTLs	C-type lectins
CXCR5	C-X-C chemokine receptor 5
DAP(10/12)	DNAX-activating protein of 10KDa/12KDa
DCs	Dendritic cells
DDX3	DEAD box protein 3
DE	Delayed early phase of viral gene transcription
DNA	Deoxyribonucleic acid
DNAM	DNAX accessory molecule
ds	Double stranded
E	Early phase of viral gene transcription
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
F-actin	Filamentous actin
Fam111a	Family with Sequence Similarity 111 Member A
Fam114a2	Family with Sequence Similarity 114 Member A2
Far1	Fatty acyl CoA reductase 1

FHL	Familial hemophagocytic lymphohistiocytosis
FSC	Forward scatter
gB	Glycoprotein B
gB/MF59	MF59-adjuvanted gB protein subunit vaccine
GTP	Guanosine-5'-triphosphate
HCMV	Human Cytomegalovirus
Heatr9	HEAT Repeat Containing 9
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSCs	Haematopoietic stem cells
IE	Immediate early phase of viral gene transcription
IFI16	Interferon gamma inducible protein 16
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILCs	Innate lymphoid cells
Immgen	Immunological Genome Project
IR	Internal region
IRF	Interferon-regulatory factors
ITAMs	Immunoreceptor tyrosine-based activation motifs
JAK	Jansus kinase
JNK	c Jun N terminal kinase
KCS	Kenny-Caffey Syndrome
KIRs	Killer cell immunoglobulin-like receptors
KLRG1	Killer cell lectin-like receptor subfamily G member 1
L	Late phase of viral gene transcription
LAK cells	lymphokine activated killer cells
LAMP-1 (or CD107a)	lysosomal associated membrane protein-1
LIMP	lysosomal integral membrane protein
LIR-1	Leukocyte immunoglobulin-like receptor
LIRs	Leukocyte immunoglobulin-like receptors
LMPP	Lymphoid-primed multipotent progenitors
LPS	Lipopolysaccharide
LYST	Lysosomal trafficking regulator
MAPK	Mitogen activated protein kinase
MCMV	Mouse cytomegalovirus
MHC	Major histocompatibility complex
MIC	MHC class I related molecule
MTOC	Microtubule organising centre
mTOR	Mammalian/mechanistic target of Rapamycin
MULT	UL16-binding protein like transcript
MyD88	Myeloid differentiation primary response 88

NETs	Neutrophil extracellular traps
NK cell	Natural Killer cell
NKD	Natural killer receptor domain
NKP	NK cell progenitor
NLRs	NOD-like receptors
OCS	Osteocraniostenosis
OR14I1	Olfactory Receptor Family 14 Subfamily I Member 1
p.i.	Post-infection
PAMPs	Pathogen-associated molecular patterns
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
pDCs	Plasmacytoid DCs
PDGFRα	Platelet-derived growth factor receptor α
PFU	Plaque forming units
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PM	Plasma membrane
PMA-Iono	Phorbol-Myristate-Acetate and Ionomycin
PRRs	Pattern recognition receptors
pSMAC	peripheral supramolecular activation cluster
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Serpinb9b	Serine (or cysteine) peptidase inhibitor, clade B, member 9b
SHD	*Sytl* homology domain
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SOCE	Store operated calcium entry
SOT	Solid organ transplant patients
SSC	Side scatter
STAT	Signal transducer and activator of transcription
STX11	Syntaxin 11
SV40	Simian Virus 40
Syt	Synaptotagmin
Sytl	Synaptotagmin-like-gene
Sytl3	Synaptotagmin-like-3
TCRs	T-cell receptors
TGF	Transforming growth factor
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TR	Terminal region
TRAIL	TNF-related apoptosis inducing ligand
Treg	T-regulatory

TRIF	TIR-domain-containing adapter inducing interferon-β
U_L	Unique long
U_S	Unique short
VAMP	Vesicle-associated membrane protein
VSV	Vesicular stomatitis virus
VSV-OVA	Vesicular stomatitis virus-ovalbumin peptide infection
VZV	Varicella-zoster virus
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein
WTSI	Wellcome Trust Sanger Institute
ZBP1	Z-DNA binding protein 1
Zfp53	Zinc finger protein 53
Δm157	m157 knockout MCMV

Chapter 1 – Introduction

1.1 Cytomegalovirus

1.1.1 Herpesviridae

Human cytomegalovirus (HCMV) belongs to the herpesvirus family which is a large family of dsDNA viruses with a distinctive virion architecture due to its unique four-layered structure including the DNA-containing core enclosed by the isocapentahedral capsid and the surrounding tegument encased by a glycoprotein envelope. Herpesviruses in most cases persist for the lifetime of the host and can be divided into three groups according to their sequence, cellular host range and biological characteristics: alpha-, beta-, and gamma-herpesviruses (Whitley RJ.; Chapter 68., 1996; Weir, 1998; McGeoch, Rixon and Davison, 2006; Davison *et al.*, 2009; Forte *et al.*, 2020). Alpha-herpesviruses have an extremely short reproductive cycle and replicate in a wide variety of host tissues. Alpha-herpesviruses include herpes simplex type 1 and 2 and the varicella-zoster virus (VZV), which causes chickenpox (Whitley RJ.; Chapter 68., 1996; Weir, 1998; Sloutskin *et al.*, 2014). Beta-herpesviruses include HCMV and human herpesviruses 6 and 7 which have a long replicative cycle and form characteristic enlarged cells upon infection (Whitley RJ.; Chapter 68., 1996; Weir, 1998; Nishimura and Mori, 2019). Gamma-herpesviruses, which have the most limited host range, have cellular tropism for lymphocytes and epithelial cells and commonly induce lymphoproliferation and cancers. Gamma-herpesviruses include Epstein-Barr virus and human herpesvirus 8 (Longnecker and Neipel, 2007; Münz, 2018). This thesis will focus on the beta-herpesvirus HCMV.

1.1.2 HCMV structure and replication cycle

1.1.2.1 HCMV genomic and virion structure

HCMV is the largest herpesvirus with a genome of ~235kb ± 1.9 kbp which encodes at least 165 canonical genes (Davison *et al.*, 2003; Dolan *et al.*, 2004; Görzer *et al.*, 2015; Sijmons *et al.*, 2015). The genome is composed of unique long (U_L) and unique short (U_S) regions flanked by terminal (TR_L and TR_S), and internal (IR_L and IR_S) repeated regions (Van Damme and Van Loock, 2014) shown in Figure 1.1. Recombination between repetitive regions, at the junction between U_L and U_S, yields four genomic isomers which

are found at equimolar proportion in any infective virus population (Kilpatrick, Huang and Pagano, 1976; Weststrate, Geelen and van der Noordaa, 1980). A huge repertoire of coding and non-coding transcripts adds further complexity to regulate HCMV replication and life-long persistence (Murphy and Shenk, 2008; Van Damme and Van Loock, 2014).

Figure 1. 1 Structure of the HCMV genome.
The HCMV genome is organised with unique long (U_L) and unique short (U_S) regions flanked by terminal (TR_L and TR_S), and internal (IR_L and IR_S) repeated regions.

The linear genome is packaged within an icosahedral nucleocapsid enveloped by a proteinaceous matrix known as the tegument (Chen *et al.*, 1999). This is all bound by the outermost element, a lipid bilayer which contains host and viral glycoproteins (Mocarski Jr., 2007; Murphy and Shenk, 2008). These components all make up the structure of the virion illustrated in Figure 1.2.

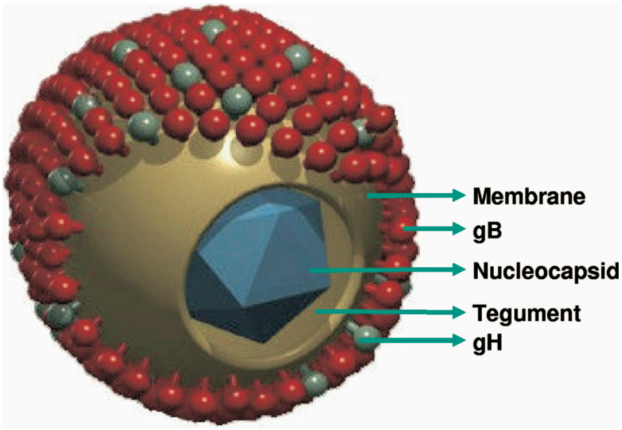

Figure 1. 2 A virtual 3D model of HCMV showing the virion structure.
The viral DNA is encased within the nucleocapsid which is encased by the tegument layer. The outermost layer of HCMV, which contains viral and host glycoproteins (gB and gH), forms the viral envelope. Source: Crough and Khanna, 2009.

1.1.2.2 HCMV entry and replication

To replicate, HCMV virions may enter different cell types according to distinct pathways which require different glycoprotein complexes. HCMV enters fibroblasts through direct membrane fusion with the plasma membrane and entry into epithelial and endothelial cells occurs via endocytosis (Ryckman *et al.*, 2006; Wang *et al.*, 2007; Vanarsdall and Johnson, 2012). HCMV attaches to and enters cells using its virion envelope glycoproteins (Frascaroli *et al.*, 2006, 2018; Weekes *et al.*, 2014). These glycoproteins form complexes which are required for entering different host cell types. The gHgLgO (trimer) complex, required for infection of all cell types, binds platelet-derived growth factor receptor α (PDGFRα) on fibroblasts (Kabanova *et al.*, 2016; Wu *et al.*, 2017) and binds to a wider variety of cell types through transforming growth factor (TGF)βR3 (Kschonsak *et al.*, 2021). The gHgLpUL128pUL130pUL131A (pentamer) complex, which binds to Neuropilin-2 and Olfactory Receptor Family 14 Subfamily I Member 1 (OR14I1), is required for HCMV entry in epithelial cells and endothelial cells (Ryckman *et al.*, 2008; Ciferri, Chandramouli, Donnarumma, *et al.*, 2015; Chandramouli *et al.*, 2017; Martinez-Martin *et al.*, 2018; Xiaofei *et al.*, 2019). Membrane fusion between HCMV virions and the host cell surface is catalysed by glycoprotein B (gB) which forms a homotrimer (Burke and Heldwein, 2015; Chandramouli *et al.*, 2015). Once the viral envelope fuses with the host cell membrane, the nucleocapsid is released into the cytoplasm. The tegument proteins facilitate its transport through the cytoplasm along microtubules towards the nuclear envelope (Kalejta, 2008). Uncoating of the virus occurs at the nuclear envelope, where viral DNA is released before initiating the expression of immediate early genes and other important proteins. HCMV regulates host gene expression through a relatively long replication cycle (>72 hr) to facilitate viral replication while evading immune defences (Stanton *et al.*, 2007).

HCMV gene expression during productive viral infection is conventionally divided into (IE) (0-2hr), delayed-early (DE) (<24hr) and late stages (L) (>24hr) which can be further subdivided (Stinski, 1978; Mocarski and Courcelle, 2001). According to the phases, IE gene expression is defined as those transcribed in the absence of *de novo* virus-encoded protein synthesis, early gene expression induces initiation of viral DNA replication, DE genes are transcribed at low levels until the onset of viral replication and L genes encode proteins needed during the assembly of HCMV virions (Mocarski *et al.*, 2013). However,

the understanding of functional proteins produced during these phases was superficial until analysis of HCMV viral proteins at several timepoints during infection was performed by Weekes *et al.*, 2014. From this analysis at least 5 distinct temporal protein profiles were revealed which had similar grouping and timepoints to the recognised functional gene expression cascades IE/E/DE/L (Weekes *et al.*, 2014). For example, the known L proteins UL99, UL94, UL75, UL115 and UL32 (Omoto and Mocarski, 2014) were classified within the fifth temporal protein profile and it was suggested that the late category of transcripts could be substantially expanded (Weekes *et al.*, 2014).

The most abundant and important products from the IE gene, IE1 (UL123) and IE2 (UL122), regulate transcription and are critical for counteracting innate host cell defence (Paulus and Nevels, 2009). The predominant product of the IE1 transcript (IE72), is essential under most conditions as IE72 deficient viruses replicate in fibroblasts only at a high multiplicity of infection (MOI) (Mocarski *et al.*, 1996; Greaves and Mocarski, 1998). On the other hand, the predominant product of the IE2 transcript (IE86) is required for productive viral replication in all conditions (Marchini, Liu and Zhu, 2001; Heider, Bresnahan and Shenk, 2002). Other IE gene products manipulate the host cell cycle such as the UL82 gene product pp71 which induces DNA synthesis in quiescent cells by degrading hypophosphorylated Rb tumour suppressors and also distinctly accelerates G1 phase progression (Kalejta and Shenk, 2003). Accelerating G1 and then eventual arrest at the G1/S border enables the virus to use available DNA precursors which are not being consumed by the synthesis of the host genome (Kalejta and Shenk, 2002). Late genes encode proteins for other regulatory functions such as UL75 and UL115 (which encode gH/gL viral glycoproteins) (Leatham, Witte and Stinski, 1991; McWatters, Stenberg and Kerry, 2002; Wang, Duh and Wu, 2004), and UL32 (which encodes the abundant tegument protein pp150) (Sanchez *et al.*, 2000; Perng *et al.*, 2011) for virus assembly.

After uncoating, the viral genome is circularized which generates multiple linked copies in tandem before it is cleaved, linearised and packaged into nucleocapsids (Murphy and Shenk, 2008). The nucleocapsids exit the nucleus by traversing the double nuclear membrane and assembly of the viral products is completed in the cytoplasm at the endoplasmic reticulum (ER)-Golgi intermediate compartment. The final stage involves a complex process of envelopment and egress that leads to virion release by exocytosis at the

plasma membrane (Mocarski Jr., 2007). The HCMV replication cycle is visualised in Figure 1.3.

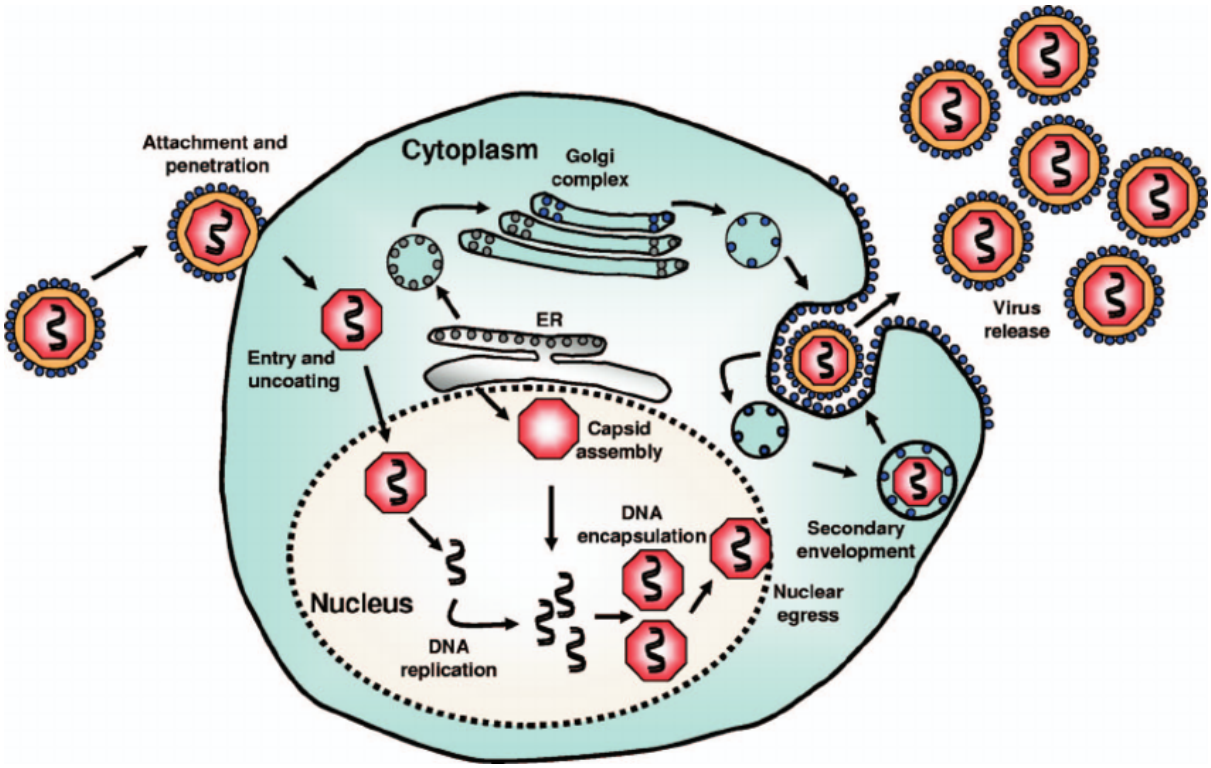

Figure 1.3 The HCMV replication cycle.
First HCMV virions attach to the cell through receptors and enter the cell by fusing with the plasma membrane or via the endocytic pathway. Then the HCMV capsid crosses into the cytoplasm before it can enter the nuclear envelope where viral DNA is delivered and circularised. Tegument proteins regulate host cell responses and initiate the temporal cascade of viral gene expression from immediate early (IE) genes, followed by delayed early (DE) genes which initiate viral replication and late (L) genes. Transcription of viral DNA occurs using host RNA polymerases. The viral genome is replicated, and late gene expression initiates capsid assembly in the nucleus followed by nuclear egress to the cytosol. Capsids associate with tegument proteins in the cytosol and are trafficked to the endoplasmic reticulum (ER), Golgi complex, and endosomal machinery where the capsids acquire tegument and viral envelope by budding into intracellular vesicles. Finally enveloped HCMV virions are released by exocytosis. Source: Crough and Khanna, 2009.

1.1.3 HCMV Pathogenesis

HCMV is among the most prevalent viruses worldwide, infecting 45-100% of individuals depending on socioeconomic and geographical factors (Cannon, Schmid and Hyde, 2010). After the initial lytic HCMV infection the virus establishes latency in the host, which can be defined as the maintenance of the viral genome without production of infectious virions but with the ability to reactivate upon specific conditions (Sinclair, 2008). Reactivation is likely to occur frequently in healthy individuals who can control the virus with their host immune response (Sinclair, 2008). Upon infection, HCMV disseminates through direct contact with fluids from infected individuals who can shed the virus for months or even years post-infection. Examples of bodily fluids include; saliva, urine, stool, breast milk and semen (Stagno *et al.*, 1980; Dworsky *et al.*, 1983; Meyers, 1984; Handsfield *et al.*, 1985; van den Berg *et al.*, 1990; Adler, 1991; Hamprecht *et al.*, 2001). HCMV may be transmitted during organ transplantation, blood transfusions, congenital transmission, through sexual contact and often in childcare settings (Drew, 1993; van der Meer *et al.*, 1996; de Jong *et al.*, 1998; Joseph *et al.*, 2006; Malani, 2010; Ziemann and Hennig, 2014; Azevedo *et al.*, 2015). Usually, healthy individuals with HCMV infection are asymptomatic excluding ~10% of adults who may experience mononucleosis syndrome with fever, malaise, atypical lymphocytosis and pharyngitis (Crough and Khanna, 2009). HCMV infection causes severe disease and mortality in the foetus, neonate, and immunocompromised individuals (Emery, 2001; Crough and Khanna, 2009).

1.1.3.1 Congenital HCMV

The burden of congenital HCMV infection is substantial. Congential HCMV can cause hepatosplenomegaly, retinitis, rash and contributes to neurodevelopmental abnormalities as well as hearing loss in children (Ross and Boppana, 2005; Fowler and Boppana, 2006). The neurodevelopmental outcomes of congenital infection can be severe and include: microcephaly, seizures, lethargy, neuroimaging findings, intellectual disability, balance disturbances and associations with autism, attention deficit hyperactivity disorder and cerebral palsy (Karltorp *et al.*, 2014; Pinninti *et al.*, 2016; Lanzieri *et al.*, 2017). Congenital HCMV infection is the leading infectious cause of deafness in children (Ross and Boppana, 2005; Fowler and Boppana, 2006). A systematic review of deafness in children found 1 in 3 with symptomatic congenital HCMV and 1 in 10 with asymptomatic infection experience hearing loss (Goderis *et al.*, 2014). Studies have shown that antiviral treatments can

improve hearing outcomes in children who have symptomatic disease and neurodevelopment scores but data on preterm infants is lacking (Kimberlin *et al.*, 2003, 2008, 2015).

The risk and severity of HCMV congenital infection is greatest if primary infection occurs in a seronegative female during the first trimester of pregnancy (Pass *et al.*, 2006). For example, 35% of pregnancies where maternal primary infection occurs, congenital transmission also occurs (Stagno *et al.*, 1982). It is also possible for congenital transmission to occur following non-primary infection during pregnancy where HCMV reactivates or where infection occurs with a different HCMV strain (Leruez-Ville *et al.*, 2017; Britt, 2018). HCMV transmission may occur before birth or during the birthing process where cervicovaginal shedding occurs (Reynolds *et al.*, 1973).

1.1.3.2 HCMV infection in immunocompromised groups: transplant recipients and AIDS individuals

Transplant recipients are the main immunocompromised patient group which suffers from HCMV-associated disease. Acquired immunodeficiency syndrome (AIDS) patients can also suffer from HCMV disease. A summary of the different pathological manifestations in these patient groups is shown in Table 1.1.

Symptoms	Definition	Solid organ recipients	Bone marrow recipients	AIDS patients
Fever	High temperature	✓	✓	✓
Hepatitis	Abnormal liver function tests coupled with histological changes and CMV detection in a liver biopsy by culture, histology, or DNA hybridisation	✓✓	✓	
Gastrointestinal		✓	✓	✓
Retinitis	Typical ophthalmological lesions without virological proof	✓	✓	✓✓
Pneumonitis	Different clinical manifestations in transplant recipients and HIV infected patients. Transplant patients: Radiographic changes and/or hypoxia. CMV detected in BAL or lung biopsy. HIV infected patients: Symptoms of pneumonia with hypoxaemia. CMV detected in the lung. Absence of other pathogens	✓	✓✓	
Encephalopathy				✓
Polyradiculopathy				✓
Myelosuppression			✓✓	
Immunosuppression		✓		
Rejection		✓		
Addisonian			✓	

Table 1.1 Pathological manifestation of HCMV in different immunocompromised host.
The relative incidence is indicated by the number of symbols (✓). Source: Emery, 2001.

For HCMV infected AIDS patients the most common sequalae is HCMV retinitis which is a severe vision-threatening disease. Within the eye, HCMV infects vascular endothelial cells followed by retinal pigment epithelial cells which can lead to necrosis and eye damage (Carmichael, 2012). However, incidence of HCMV retinitis has reduced dramatically with the onset of antiretroviral therapy (ART) for AIDS patients therefore CMV disease in this patient group is now generally well managed where ART is available (Munro *et al.*, 2020).

In bone marrow transplant recipients, HCMV pneumonitis is a major disease (Emery, 2001). HCMV pneumonitis has unspecific symptoms including dry cough, breathlessness and fevers, which can be difficult to differentiate from other causes, and can be life threatening (Rafailidis *et al.*, 2008; Piñana *et al.*, 2019; Restrepo-Gualteros *et al.*, 2019). Bone marrow transplant recipients also suffer from virus-induced myelosuppression which enables efficient HCMV latency and causes significant morbidity and mortality (Hancock *et al.*, 2020).

For solid organ transplant (SOT) patients undergoing immunosuppressive therapy, HCMV infection is the most significant threat to patient and graft health and predisposes SOT patients to opportunistic infections and malignancies (Pereyra and Rubin, 2004; Fishman *et al.*, 2007; Levitsky *et al.*, 2008). Approximately 75% of all organ transplants without antiviral prophylaxis have some evidence of active HCMV infection within the first year following transplantation (Pereyra and Rubin, 2004; Yadav *et al.*, 2017). Furthermore, HCMV infection is associated with lower survival rates in both high and low risk transplant patients (Broers *et al.*, 2000; Sagedal, Rollag and Hartmann, 2007; Yadav *et al.*, 2017). The biggest risk factor for HCMV infection in liver transplant patients is the recipient's CMV seronegative status with an incidence of 78-88% without antiviral prophylaxis following transplantation of a CMV-containing organ. This percentage decreases to only 13% in seronegative recipients of seronegative donor organs (Paya *et al.*, 1993; Lautenschlager *et al.*, 2006; Manuel *et al.*, 2013). Other risk factors for CMV disease include: donor and recipient advanced age, human leukocyte antigen (HLA) mismatch, immediate graft rejection by itself, impaired humoral immunity and coinfection with other herpesviruses (HHV 6 and 7) (Singh *et al.*, 2005; Singh and Wagener, 2006; Razonable, 2008). In liver transplant patients a high frequency of HCMV hepatitis is observed which can be self-limiting with moderate changes to liver function or can cause severe viremia, prolonged fever and other organ involvement associated with severe morbidity (Paya *et al.*, 1989).

The risk of CMV disease in liver transplant recipients is higher than in kidney recipients and lower than lung, small intestine and simultaneous heart-lung recipients (Humar *et al.*, 1999; Ambrose *et al.*, 2016; Beam *et al.*, 2016; Nagai *et al.*, 2016).

1.1.4 Antiviral therapies in HCMV infection

1.1.4.1 Pharmacological anti-HCMV therapies

HCMV antivirals are available for severe disease and improve survival in high-risk patients. Examples of available treatments include: 1) the nucleosides Ganciclovir and Acyclovir which are phosphorylated in the host to competitively inhibit the HCMV-encoded DNA polymerase; 2) Cidofovir, a nucleotide drug structurally equivalent to nucleoside monophosphate but without charge therefore once used in the formation of new virions, it prevents the virus from crossing the plasma membrane; 3) Foscarnet which inhibits the HCMV DNA polymerase by binding to its enzymatic site (Griffiths and Boeckh, 2007; Krishna, Wills and Sinclair, 2019). However, these therapies are associated with significant side effects such as neutropenia, anaemia, diarrhoea, and graft rejection in transplant recipients and therapeutic effectiveness is often compromised by drug-resistant strains limiting usage to patients most at risk (Griffiths and Boeckh, 2007; Komatsu *et al.*, 2014; Krishna, Wills and Sinclair, 2019). For example, HCMV resistance to ganciclovir commonly results from mutations in the viral protein kinase UL97 gene responsible for the monophosphorylation of ganciclovir, and mutations in UL54 confer multidrug resistance (Baldanti *et al.*, 2002; Hantz *et al.*, 2005; Chou, 2010; Hakki and Chou, 2011; Chou, Ercolani and Lanier, 2016). In some cases Valganciclovir, a pro-drug of ganciclovir, has been shown at high doses to successfully treat post-transplant CMV infection in the presence of the UL97 mutation (Iwasenko *et al.*, 2009).

A recently approved novel class of HCMV prophylactic drug, Letermovir, may be useful for patients with HCMV where prior treatments have failed or they are unable to tolerate other anti-HCMV compounds (Krishna, Wills and Sinclair, 2019). Letermovir is particularly effective in bone marrow transplant patients where other anti-CMV agents are avoided due to the possibility of myelosuppression (Winston *et al.*, 1993; Boeckh and Ljungman, 2009; Humar *et al.*, 2010). Letermovir inhibits the HCMV DNA terminase complex which leads to compromised late stage viral replication by preventing formation

of viral genomes at the required length, leading to accumulation of immature viral DNA (Goldner *et al.*, 2011; Melendez and Razonable, 2015; Shigle, Handy and Chemaly, 2020). Letermovir is generally well tolerated and it is not expected to generate mechanism-based adverse events since the HCMV target does not have a human equivalent (Kim, 2018; Krishna, Wills and Sinclair, 2019). As a result, Letermovir has generally become a routine pre-emptive therapy in bone marrow transplant recipients. However, cases of HCMV resistance have been noted and the drug has been associated with increased risk of late onset HCMV disease upon cessation of Letermovir treatment (James, 2020; Hofmann *et al.*, 2021). In addition, there is evidence to suggest that minimising antiviral therapy in patients receiving a bone marrow transplant for the treatment of leukaemia may reduce the risk of cancer relapse due to the associated host immune response (Yoon *et al.*, 2016). In SOT recipients, Letermovir is not recommended for treatment of HCMV disease due to drug interactions with immunosuppressants used to prevent graft rejection, including tacrolimus and cyclosporin, leading to toxicity (McCrea *et al.*, 2019; Winstead *et al.*, 2021). New therapies are therefore required to address the shortfalls in currently available treatments including HCMV resistance and adverse drug effects.

1.1.4.2 Cell-based anti-HCMV therapies

Due to the adverse effects and resistance issues associated with anti-HCMV pharmacological therapies, cell-based immunotherapies for HCMV disease in transplant recipients are undergoing extensive research. Several different cell-based immunotherapy approaches exist including; donor derived cells in transplant settings, HCMV-specific T-cell lines from third party donors, and chimeric antigen receptor (CAR) cells (Watanabe *et al.*, 1992; Walter *et al.*, 1995; Einsele *et al.*, 2002; Peggs *et al.*, 2003, 2011; Cobbold *et al.*, 2005; Feuchtinger *et al.*, 2010; Schmitt *et al.*, 2011; Blyth *et al.*, 2013; Leen *et al.*, 2013; Stemberger *et al.*, 2014; Koehne *et al.*, 2015; O'Reilly *et al.*, 2016; Seif, Einsele and Löffler, 2019; Tzannou *et al.*, 2019; Shafat *et al.*, 2020). HCMV directed cell-based immunotherapy has promise in bone marrow transplant settings where patients receive T-cell depleting therapies to limit graft versus host disease and have increased risk of HCMV infection. In this context, cells derived from the bone marrow transplant donor have in general been more successful compared to third party donors (Neuenhahn *et al.*, 2017). Several other variables also affect the success of cell-based therapies including: the phenotype of the cells selected for transfer, the level of HLA matching/mismatching to the donor, culture techniques used in cell preparation, whether antigen specific T-cells are

directly selected or generated during cell culture and the timing of the infusions. A huge number of HCMV directed cell-based immunotherapy trials have taken place which have shown a high level of HCMV infection resolution (Watanabe *et al.*, 1992; Walter *et al.*, 1995; Einsele *et al.*, 2002; Peggs *et al.*, 2003, 2011; Cobbold *et al.*, 2005; Feuchtinger *et al.*, 2010; Schmitt *et al.*, 2011; Blyth *et al.*, 2013; Leen *et al.*, 2013; Stemberger *et al.*, 2014; Koehne *et al.*, 2015; Shafat *et al.*, 2020).

For CAR-T cells, gB is an attractive target as it is expressed during the early phase of viral replication (Smuda, Bogner and Radsak, 1997) therefore infected cells could be targeted before new virions are produced which would protect against severe lytic infections (Bednar and Ensser, 2021). The first successful approach for targeting HCMV using CAR T-cells was directed against gB (Full *et al.*, 2010) but infected cells showed some resistance to cytotoxic lysis by CAR T-cells, which was likely due to viral effector proteins. A more sophisticated approach using gB directed CARs connected to 41BB costimulatory domains showed improvement in HCMV infection control in humanized mice (Olbrich *et al.*, 2020). Therefore, development of the gB-CAR T-cell approach may improve outcomes for patients with severe HCMV in the future. However, translation of cellular therapy into the clinic is limited by the availability of the technique, lack of appropriate mouse models for CAR T-cell development, limited compatibility of phase III clinical trial designs with cellular therapy and regulatory restrictions (Kaeuferle *et al.*, 2019; Seif, Einsele and Löffler, 2019).

1.1.5 Investigating HCMV in a laboratory setting

1.1.5.1 HCMV laboratory strains

To investigate HCMV infection in a laboratory setting, clinical isolates of HCMV have been separated and passaged *in vitro*. HCMV research was dependent for decades on the high-passage strains AD169 and Towne, which were heavily exploited due to their replicative efficiency in fibroblasts. However, as the genetic integrity of these strains is severely compromised their use must be considered with caution. With increased passage number the risk of accumulating mutations increases thereby altering pathogenicity and cellular tropism (Wilkinson *et al.*, 2015). To mitigate this, the low-passage strain Merlin genome was cloned as a bacterial artificial chromosome (BAC) which enables the strain to

be replicated and expanded without the risk of accruing further mutations (Borst *et al.*, 1999; Stanton *et al.*, 2010). The Merlin strain was then sequentially repaired to match the viral sequence in the original clinical sample from which it was derived (Dargan *et al.*, 2010; Stanton *et al.*, 2010). The Merlin transcriptome and proteome has been characterised in unparalleled detail which enables individual researchers to monitor the sequence of their Merlin stocks over time (Wilkinson *et al.*, 2015). As a result, Merlin is now the strain of choice for HCMV studies at Cardiff University. However, the high level of HCMV inter-strain variation in the circulation, particularly in patients with HCMV disease, cannot be modelled accurately using one strain of choice in the laboratory. Furthermore, the heterogeneity of human immune systems cannot be recapitulated *in vitro,* and finally, HCMV infection cannot be investigated *in vivo* due to its high species specificity (Mozzi *et al.*, 2020). As a result, a combination of approaches including studies of HCMV infection in humans, *in vitro* studies of HCMV and animal models are required.

As CMVs infect in a species-specific manner, the species-specific strain of CMV must be used in animal infection studies. A variety of factors are implicated in CMV host specificity which have developed across hundreds of millions of years of coevolution with their respective host of choice. Within the host, antiviral restriction factors prevent the permissive replication of CMVs which are not specific to the host species (Mozzi *et al.*, 2020). In turn, CMVs inhibit apoptosis within its host of choice which allows for successful replication and subsequent dissemination within host cells. For example, the HCMV UL36 gene controls apoptosis through multiple cell death programs including inhibition of caspase-8, blocking of Fas-induced apoptosis, and prevention of apoptosis triggered by tumour necrosis factor α (TNFα) and TNF-related apoptosis inducing ligand (TRAIL) in human cells (McCormick *et al.*, 2010; Collins-McMillen *et al.*, 2018).

1.1.5.2 Animal models of CMV infection

As the species specificity of HCMV prevents investigation *in vivo*, we are reliant on animal models using the relevant species-specific CMVs. Primate CMV strains are most closely related to HCMV (e.g. chimpanzee and rhesus macaque), however, there are ethical, economic and logistical considerations for using these animals in research. Therefore, rodent CMVs (e.g. mouse, rat and guinea pig) are used as an alternative and the most commonly used infection model is mouse CMV (MCMV). MCMV, like HCMV, is a

dsDNA *betaherpesvirus* with a similar size to HCMV at 230Kbp and is predicted to encode ~170 genes (Rawlinson, Farrell and Barrell, 1996). MCMV has relatively similar biological characteristics to HCMV. Both HCMV and MCMV can cause severe infections in immunocompromised hosts, resulting in similar clinical syndromes (Mutter *et al.*, 1988; Craighead, Martin and Huber, 1992; Webb, Lee and Vidal, 2002; Fisher and Lloyd, 2020). For example, high viral titres in mice are associated with pneumonitis (Shanley, 1984), hepatitis (Trgovcich *et al.*, 2000) and retinitis (Hayashi, Kurihara and Uchida, 1985; Hayashi *et al.*, 1995) and high titres in the spleen or liver may lead to fatal infection (Shanley, Biczak and Forman, 1993). MCMV infection resembles HCMV infection with respect to organ and cell tropism, pathogenesis during acute infection, establishment of latency and reactivation upon immunosuppression (Mocarski and Kemble, 1996; Juceviciene *et al.*, 2002; Krmpotic *et al.*, 2003; Smith *et al.*, 2008). In addition, both CMVs are susceptible to the antiviral agents dihydroxyphenylglycine (DHPG) and ganciclovir (Shanley, Morningstar and Jordan, 1985). Furthermore, both CMVs are transmissible through bodily fluids although, unlike HCMV, transplacental transmission of MCMV does not occur. As a result, mouse models of congenital infection have been developed where investigators have directly injected MCMV into placenta or embryo (Tsutsui, 1995; Li and Tsutsui, 2000), directly infected using intracranial inoculation (Shinmura *et al.*, 1997; Kosugi *et al.*, 2002), infection of neonates using milk from MCMV-infected dams (Wu *et al.*, 2011) or i.p. inoculation (Koontz *et al.*, 2008). Arguably the best model is i.p. infection of newborn mice as it more closely recapitulates the presumed route of HCMV dissemination into the CNS during systemic viremia (Cekinović *et al.*, 2008; Koontz *et al.*, 2008).

1.1.5.3 Use of MCMV as a model of HCMV

Many genetic similarities between HCMV and MCMV exist with multiple gene homologs, including structural and immune-evasion genes (Rawlinson, Farrell and Barrell, 1996; Goodier *et al.*, 2017; Fisher and Lloyd, 2020). For example, the positional homolog of HCMV gO, m74 in MCMV, codes for a glycosylated protein which also forms a complex with gH (M75 in mice). M74 knockout MCMV mutants and gO knockout mutants of HCMV show the same shift in spread phenotype from cell-free to cell-associated spread. Therefore, MCMV expresses alternative structural glycoprotein complexes which govern cell spread (Scrivano *et al.*, 2010). However, only 50% of genes identified in MCMV have homologs in HCMV and vice versa while homologs can also target different pathways

(Rawlinson, Farrell and Barrell, 1996; Smith, Shellam and Redwood, 2006; Powers and Früh, 2008; Fisher and Lloyd, 2020). There are also important differences in the organisation of the genetic information. HCMV has unique long and unique short regions with terminal and internal repeat sequences (Van Damme and Van Loock, 2014) as compared with MCMV, which has a single unique sequence with short terminal direct repeats and several short internal repeats shown in Figure 1.4 (Rawlinson, Farrell and Barrell, 1996). Additionally, these viruses adapt to cell culture differently, as the *in vitro* culture of clinical isolates of HCMV invariably leads to loss of genetic regions (Wilkinson *et al.*, 2015) which does not occur to the same extent with MCMV (Cheng *et al.*, 2010), although there is evidence that deletions occur as MCMV is propagated from salivary glands in culture (Cheng *et al.*, 2010).

Figure 1.4 The MCMV genome structure.
The MCMV genome is comprised of a single unique long (U_L) region flanked by terminal (TR) and internal repeat sequences.

1.1.5.3.1 MCMV laboratory strains

The two MCMV strains most used in research are K181 and Smith strain, both of which are serially passaged laboratory strains. Smith strain MCMV was isolated from the salivary gland of infected laboratory mice (Smith, 1954). The K181 strain of MCMV was isolated from mouse salivary glands after serial passages *in vivo* (Misra and Hudson, 1980). The K181 strain is believed to be more virulent, replicates at higher titres in mouse salivary glands (Misra and Hudson, 1980) and demonstrated higher mortality rates in young mice (Hudson, Walker and Altamirano, 1988). Between the first isolation of Smith strain MCMV and 2007, fewer than 1% of MCMV studies use a virus strain other than Smith or K181 (Smith *et al.*, 2008). Studies examining wild-derived MCMVs have shown genetic similarity to Smith strain virus. However, where variations in gene sequences occur between wild-derived and Smith strain MCMVs, these changes are believed to be responsible for distinct differences in viral replication (Smith *et al.*, 2008). As K181 and Smith strain viruses have been serially passaged for decades (Smith, 1956; Misra and

Hudson, 1980), wild-derived CMVs with high variance may serve as a better model of clinical HCMV (Smith, Shellam and Redwood, 2006). Modelling HCMV with MCMV in the laboratory is further complicated by the method of virus strain maintenance. MCMV is generally maintained as either a salivary gland stock or a tissue culture-derived stock, usually prepared from the infection of embryonic mouse fibroblasts (Brizić *et al.*, 2018). This in turn influences the nature of the *in vivo* infection (Osborn and Walker, 1971). Salivary gland virus is generally composed of single capsid virions, produces acute infection in mice and can be lethal at low doses (Grundy, Mackenzie and Stanley, 1981; Allan and Shellam, 1984). In contrast, virus stocks made from other organs (such as liver and spleen) and tissue culture passaged virus in embryonic mouse fibroblasts are comprised of both single and multi-capsid virions and are well tolerated at low doses (Hudson, Misra and Mosmann, 1976).

1.1.5.3.2 Mouse strains for CMV research

In addition to the availability of well-characterised MCMV strains, mice are considered the most appropriate species for infection studies due to our detailed knowledge of the murine immune system. Many gene-deficient mice are available for investigating the role of host genes in response to MCMV. In the laboratory setting, genetically inbred mouse strains such as C57BL/6 and BALB/c are commonly used. Furthermore, it is understood that specific strains have different immune characteristics which can inform on the choice of host for certain investigations and makes the mouse an excellent model to examine heterogeneous responses seen in the human population (Fisher and Lloyd, 2020). For example, BALB/c mice do not express the activating natural killer (NK) cell receptor Ly49H (see section 1.3.3), which contributes to NK cell-mediated control of MCMV. These mouse strains are therefore more susceptible to MCMV infection as compared to C57BL/6 mice which express Ly49H (Fisher and Lloyd, 2020). MCMV has been useful in modelling broad aspects of HCMV disease in many situations, now greatly expanded with the use of recombinant viruses and genetically modified mouse strains (Cicin-Sain *et al.*, 2005; Fisher and Lloyd, 2020). Important insights into CMV-host interactions have been uncovered using MCMV (Picarda and Benedict, 2018). Whilst some of these are likely to be specific for the particular virus-host adaptations, others have revealed general principles such as immune cell crosstalk, antigen processing and presentation which are valid despite the noted differences between mouse and human immunology (Mestas and Hughes, 2004). For example, crosstalk between dendritic cells (DCs) and NK cells (Andrews *et al.*, 2003;

Andoniou *et al.*, 2005), and between T-cells and NK cells (Andrews *et al.*, 2010), are crucial to effective MCMV and HCMV control. Overall, MCMV research is an invaluable resource for understanding infectious disease in the context of a complete immune system, providing insights into cell signalling and immune modulation as well as preclinical opportunities to test therapeutic strategies to counter HCMV.

1.2 Introduction to the immune system

1.2.1 The immune system response to CMV infection

The immune system is split into two main arms: the innate and the adaptive immune system. The main immune cell subtypes from each of these arms are highlighted in Figure 1.5. The innate immune system provides a non-specific response to pathogen challenge and signals to the adaptive immune system. The adaptive immune response, which is not the focus of this thesis, provides highly specific immunity and is able to generate immunological memory for more rapid and robust protection to subsequent infections. Immunological function between mice, which are integral to immunological research, and humans is largely conserved and to date only approximately 300 genes appear to be unique to one species or the other (Waterston *et al.*, 2002). However, key differences between mouse and human immune system development, activation, and responses to infection have been noted (Fisher and Lloyd, 2020). The similarities and differences between mouse and human immune responses to CMV as well as examples of CMV immune evasion strategies will be highlighted in the following section.

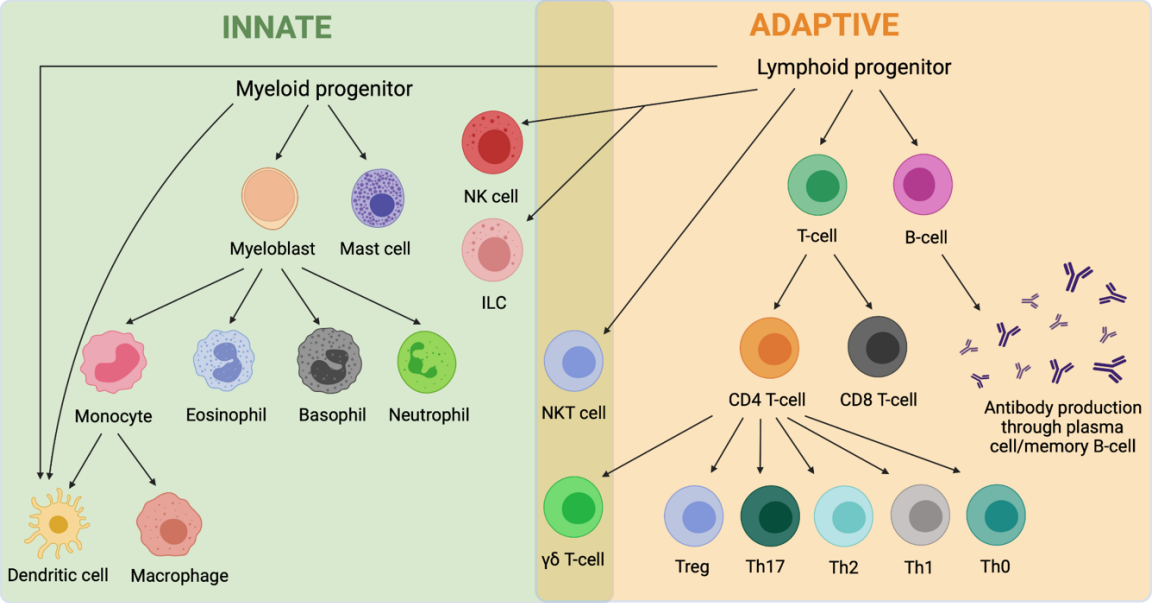

Figure 1. 5 Innate and adaptive immune cell overview.
Innate immune cells are shown in green. Myeloid progenitors give rise to mast cells, myeloblasts and dendritic cells. Myeloblasts give rise to monocytes and granulocytes which are a group of immune cells with large cytoplasmic granules that include eosinophils, basophils, and neutrophils. Monocytes can also give rise to macrophages and dendritic cells. However, dendritic cells can also develop from myeloid and lymphoid precursors. NK cells from a common lymphoid progenitor are classically innate cells. Innate lymphoid cells (ILCs) also derive from a common lymphoid progenitor and are functionally related to NK cells. Adaptive immune cells from the lymphocyte lineage are shown in orange and contain B-cells (which produce antibodies) and T-cells. T-cells can be subdivided into $CD4^+$ helper T-cells and $CD8^+$ cytotoxic T-cells. $CD4^+$ T-cells can also be divided according to the types of responses they induce which include: Treg, Th17, Th2, Th1 and Th0. γδ T-cells and NKT cells are lymphocytes which have innate and adaptive functions.

1.2.2 Innate immunity

1.2.2.1 Barrier immunity

Both the innate and adaptive arms of the immune system contribute to the containment of viral infections such as CMV. The innate system comprises a variety of mechanisms broadly defined as non-specific defences against pathogens and tumour cells they encounter. Mechanisms of non-specific immune defence include physical barriers such as epithelial cell layers which have tight cell-cell contacts, the mucus lining the epithelium in the respiratory, gastrointestinal, and genitourinary tracts and the cilia which sweeps away any mucus which may have been contaminated so it can be constantly refreshed (Nochi and Kiyono, 2006; Gallo and Nizet, 2008). Innate immunity also includes soluble proteins

such as complement, defensins and ficolins (Holmskov, Thiel and Jensenius, 2003; Hiemstra, 2007; Sjöberg, Trouw and Blom, 2009) as well as other bioactive small molecules including cytokines, chemokines, lipid mediators, reactive free radicals and enzymes which contribute to inflammation. Many of these bioactive molecules are released by innate immune cells upon recognition of pathogens through their receptors.

1.2.2.2 Innate sensing through pattern recognition receptors

The host innate immune system can recognise pathogen signatures known as pathogen-associated molecular patterns (PAMPs) such as dsRNA and lipopolysaccharide (LPS) through pattern recognition receptors (PRRs) expressed on or within host cells. PRRs can be broadly classified into the following families: toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), C-type lectins (CTLs), absent in melanoma 2 (AIM2) receptors, and DNA receptors which activate several downstream molecular pathways to control infection (Mogensen, 2009). A summary of the most important PRRs for HCMV and MCMV sensing are highlighted in Table 1.2. Polymorphisms in these PRRs have been associated with increased risk of HCMV disease in humans (Wujcicka, Wilczyński and Nowakowska, 2014; Studzińska *et al.*, 2017; Wujcicka *et al.*, 2017). In addition, mice with defective PRRs have reduced *in vivo* cytokine responses to CMV (Krug *et al.*, 2004; Tabeta *et al.*, 2004; Zucchini *et al.*, 2008).

Pattern recognition receptor	Virus	Site of sensing	Immune response pathway
TLR2	HCMV (Compton *et al.*, 2003) and MCMV (Szomolanyi-Tsuda *et al.*, 2006)	Cell surface	IFNβ via Myd88; IL-8, IL-6, IL-12 via NF-κB
TLR3	HCMV and MCMV (Edelmann *et al.*, 2004)	Endosome	IFNβ via TRIF; IL-8, IL-6, IL-12 via NF-κB
TLR9	HCMV and MCMV (Delale *et al.*, 2005)	Endosome	IFNβ via Myd88; IL-8, IL-6, IL-12 via NF-κB
NOD2	HCMV (Kapoor, Forman and Arav-Boger, 2014)	Cytoplasm	IFNβ via IRF-3; IL-8 via NF-κB
NOD1	HCMV (Fan *et al.*, 2016)	Cytoplasm	IFNβ
NLRC5	HCMV (Kuenzel *et al.*, 2010)	Cytoplasm	IFNα
cGAS	HCMV and MCMV (Lio *et al.*, 2016; Motwani, Pesiridis and Fitzgerald, 2019)	Nucleus, cytoplasm	IFNα, IFNβ via STING
IFI16/p204	HCMV and MCMV (Unterholzner *et al.*, 2010)	Nucleus, cytoplasm	IFNβ via STING
ZBP1	HCMV and MCMV (Maelfait *et al.*, 2017; Sridharan *et al.*, 2017; Jiao *et al.*, 2020)	Cytoplasm	IFNβ via DDX3 and IRF3
AIM-2	HCMV and MCMV (Fernandes-Alnemri *et al.*, 2009; Hornung *et al.*, 2009; Rathinam *et al.*, 2010; Huang *et al.*, 2017; Lu *et al.*, 2019)	Cytoplasm	IFN, IL-1β and IL-18 via ASC inflammasome

Table 1.2 A summary of the proposed HCMV and MCMV sensors.
Pattern recognition receptors which recognise CMV include toll-like receptors (TLRs), nucleotide-binding oligomerisation domain receptors (NOD), NOD-like receptors (NLRs), Cyclic GMP-AMP synthase (cGAS), interferon gamma inducible protein 16 (IFI16), Z-DNA binding protein 1 (ZBP1), and absent in melanoma 2 (AIM2). For each receptor listed, key characteristics are shown including its ability to detect MCMV and/or HCMV, the site of sensing and the downstream immune response pathway. The downstream pathways include nuclear factor-κB (NF-κB), stimulator of interferon genes (STING), DEAD-box helicase family member DDX3X (DDX3X) and apoptosis-associated speck-like protein containing a CARD (ASC) inflammasome which produce interferons (IFN). Adapted from: Biolatti *et al.*, 2018.

The most widely studied family of PRRs are the TLRs which are important for sensing viruses, bacteria, protozoa, and fungi. Thus far, 10 TLRs have been discovered in humans and 12 in mice with TLRs 1-9 conserved in both species (Kumar, Kawai and Akira, 2011). TLRs 1, 2, 4, 5, and 6 are primarily expressed on host cell surfaces and recognise bacterial, fungal and protozoa associated PAMPs. TLR 3, 7, 8 and 9 are exclusively expressed within endocytic compartments and primarily recognise nucleic acid PAMPs including single and double stranded RNA/DNA from viruses and bacteria. The following receptors all sense CMV in humans and mice: TLR3 recognises the dsRNA replicative intermediate during virus replication (Einsele *et al.*, 2004; Tabeta *et al.*, 2004; Yew, Carsten and Harrison, 2010), TLR9 recognises viral dsDNA (Krug *et al.*, 2004; Tabeta *et al.*, 2004; Yew, Carsten and Harrison, 2010), and TLR2 recognises CMV glycoproteins on the cell surface (Compton *et al.*, 2003; Boehme, Guerrero and Compton, 2006; Szomolanyi-Tsuda *et al.*, 2006). Once TLRs recognise PAMPs, they transduce signals through the cytosolic adapter molecule myeloid differentiation primary response 88 (MyD88) (Deguine and Barton, 2014) or, in the case of TLR3, transduce signals through TIR-domain-containing adapter inducing interferon-β (TRIF) (Ullah *et al.*, 2016). These signals lead to activation of transcription factors including nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and cAMP response element binding protein (CREB) (Compton et al. 2003; Boehme et al. 2006) by interferon-regulatory factors (IRF)3/7 and/or mitogen activated protein kinase (MAPK). IRFs and MAPK cooperate to induce cytokine transcription such as interferon (IFN)-α/β for promotion of cellular defences against viral infection (Barnes and Karin, 1997; Akira, Uematsu and Takeuchi, 2006). Accordingly, mice deficient in the IFN I receptor (*Ifnar*$^{-/-}$) succumb to CMV infection (Presti *et al.*, 1998). In addition, HCMV stimulation of peripheral-blood derived monocytes *in vitro* was shown to increase TLR expression alongside CD14, as well as increase adaptor molecules and transcription factors downstream of TLRs (Smith *et al.*, 2014), suggesting active CMV replication induces systemic pro-inflammatory cytokine responses (Clement and Humphreys, 2019).

NLRs can sense a wide range of PAMPs within the cell cytoplasm and have importance for viral recognition. There are 23 NLRs in humans and approximately 34 in mice (Kanneganti, Lamkanfi and Núñez, 2007; Shaw *et al.*, 2008; Franchi *et al.*, 2009; Kumar, Kawai and Akira, 2009, 2011; Takeuchi and Akira, 2010). Upon recognition of pathogen signatures, NLRs induce NF-κB or MAP kinases for cytokine production or the multi-protein complex known as the inflammasome. The inflammasome initiates the

caspase cascade which also leads to inflammatory cytokine production such as IL-1β and IL-18 or initiates cell death cascades. Furthermore, inflammasome-dependent secretion of IL-18 enhances NK cell function during MCMV infection (Madera and Sun, 2015).

Cyclic GMP–AMP synthase (cGAS) senses viral or bacterial cytosolic DNA. Once cGAS is activated by DNA binding it forms a 2′3′-cGAMP product which binds to and activates the stimulator of interferon genes (STING) pathway (Ishikawa and Barber, 2008; Ishikawa, Ma and Barber, 2009; Zhou *et al.*, 2018). STING is an ER resident transmembrane protein which is highly important for triggering cytokine responses to CMV (Ishikawa, Ma and Barber, 2009). cGAS-STING induces downstream activation of IRF3 and NF-κB cytokine transcription pathways (Motwani, Pesiridis and Fitzgerald, 2019).

Interferon gamma inducible protein 16 (IFI16) detects cytoplasmic and nuclear DNA (Unterholzner *et al.*, 2010; Kerur *et al.*, 2011; Orzalli, DeLuca and Knipe, 2012; Horan *et al.*, 2013; Johnson, Chikoti and Chandran, 2013; Li, Chen and Cristea, 2013; Singh *et al.*, 2013) and also directly suppresses the transcriptional activity of HCMV genes required for DNA synthesis (Gariano *et al.*, 2012). IFI16 viral DNA binding also induces STING for production of antiviral cytokines (Jønsson *et al.*, 2017). Interestingly, the HCMV tegument protein pUL83 directly interferes with IFI16 to suppress innate immune sensing (Li, Chen and Cristea, 2013).

Z-DNA binding protein 1 (ZBP1) senses viral nucleic acids (Thapa *et al.*, 2016; Maelfait *et al.*, 2017; Sridharan *et al.*, 2017) which induce DEAD box protein 3 (DDX3) activation and IRF3 activation for production of IFNβ (DeFilippis *et al.*, 2010) and regulated necroptotic death (Sridharan *et al.*, 2017; Kuriakose and Kanneganti, 2018). ZBP-1 has been implicated as the major IRF3 activating PRR in HCMV infection (DeFilippis *et al.*, 2010).

AIM-2 senses cytosolic dsDNA to induce an ASC-dependent (apoptosis-associated speck-like protein containing a CARD) inflammasome leading to downstream activation of caspase-1 and cytokine release (Sepulveda *et al.*, 2015; Wang, Tian and Yin, 2019). A short-lived AIM2 inflammasome was implicated in the reduced control of MCMV infection in BALB/c mice compared to C57BL/6 mice alongside other factors (Lu *et al.*, 2019). During HCMV infection *in vitro*, AIM-2 deficient cells were unable to control

HCMV AD169 strain infection due to increased transcription of HCMV DNA polymerase and a major tegument protein gene as well as limited activation of caspase-1 for inducing cell death in macrophages (Huang *et al.*, 2017). In addition, HCMV was shown to induce immune-evasion through IE gene-directed degradation of the immature cytokine IL-1β during AIM-2 inflammasome activation (Botto *et al.*, 2019).

1.2.2.3 Cellular innate immunity

Upon initiation of transcription pathways which induce cytokine release, innate immune cells are recruited to the site of infection. NK cells, the cell type I will be focussing on in this thesis, are among the first responders and will be discussed alongside innate lymphoid cells (ILCs) in detail in section 1.3. The other key innate immune cell types are discussed alongside their complex host-pathogen relationship with CMV.

1.2.2.3.1 Neutrophils

Neutrophils, which make up 50-70% of immune cells in humans and 10-25% of immune cells in mice (Mestas and Hughes, 2004), are relatively short-lived cells from the granulocyte family (Rosales, 2018). During homeostasis, neutrophils enter the circulation from the bone marrow and migrate to tissues to complete their functions before being eliminated by macrophages (Bratton and Henson, 2011; Rosales, 2018). Their role is to constantly monitor the host for invading pathogens which, once discovered, are destroyed by either phagocytosis, degranulation, or, when the microorganism is too large to be ingested, release of nuclear material in the form of neutrophil extracellular traps (NETs) (Fuchs *et al.*, 2007; Yipp *et al.*, 2012; Rosales, 2018). In addition, neutrophils produce cytokines (Tecchio and Cassatella, 2016), modulate other cells to resolve inflammation (Chen *et al.*, 2014; Greenlee-Wacker, 2016), actively participate in several diseases including cancer (Uribe-Querol and Rosales, 2015; Mishalian, Granot and Fridlender, 2017), and even contribute to innate immune memory-like responses (Netea *et al.*, 2016). In the case of HCMV infection which directly promotes neutrophil survival, neutrophils are believed to aid viral dissemination by inducing pro-inflammatory pathways which recruit and stimulate other immune cells that are permissive to HCMV infection (Taylor-Wiedeman *et al.*, 1991; Pocock *et al.*, 2017). In fact, neutrophils are the major cell type carrying HCMV during acute infection despite their short lifespan (Gerna *et al.*, 1992), but they cannot support viral replication. In comparison to humans where increased neutrophil viability is associated with greater HCMV-induced tissue damage (Rahbar *et al.*, 2003;

Skarman *et al.*, 2006; Pocock *et al.*, 2017), mouse neutrophils are important antivirals which function as TRAIL-dependent effectors (Stacey *et al.*, 2014). During *in vitro* infection, MCMV reduced the chemotactic and killing capacity of neutrophils (Bale Jr., O'Neil and Greiner, 1985). Therefore, the neutrophil response to CMV varies between humans and mice.

1.2.2.3.2 Monocytes and macrophages

Monocytes are circulating mononuclear phagocytes which, during inflammation and possibly less efficiently in the steady state, can differentiate into macrophages, or, under specific environmental cues, DCs (Randolph, Jakubzick and Qu, 2008). Monocytes represent approximately 4% in mice and 10% in humans of all nucleated cells in the blood and they also have pools in the spleen and lungs which allow them to migrate into tissues on demand (van Furth and Sluiter, 1986; Swirski *et al.*, 2009). Migration into tissues and differentiation of monocytes is likely to be determined by the inflammatory milieu and PRR signalling (Serbina *et al.*, 2008). A subset of monocytes with high phagocytic activity known as 'inflammatory monocytes' are preferentially recruited to inflammatory lesions where they can differentiate into macrophages or DCs (Wynn, Chawla and Pollard, 2013). Macrophages reside within lymphoid and non-lymphoid tissues and under steady state are involved in tissue homeostasis through clearance of damaged cells and growth factor secretion. Upon sensing infection (through their PRRs), Macrophages can induce endocytosis, phagocytosis, and cytokine production (Gordon, 2002; Murray *et al.*, 2005; Mercer and Greber, 2013; Hirayama, Iida and Nakase, 2017). Macrophages are also important for the production of chemokines which signal to other immune cells including C-C motif chemokine ligand 3 (CCL3) which recruits NK cells (Salazar-Mather, Orange and Biron, 1998; Salazar-Mather, Lewis and Biron, 2002). Traditionally, macrophages have been broadly categorised into two phenotypes known as M1 and M2. M1 macrophages are 'classical macrophages' which sustain immune responses against pathogens through release of proinflammatory markers, antigen presentation and T-cell stimulation, and are associated with stimulation by IFNγ. M2 macrophages are alternatively activated by IL-4 stimulation and contribute to resolution of inflammation, tissue repair, extracellular matrix remodelling and pathogen scavenging (Gordon, 2003; Mantovani, Sica and Locati, 2005; Martinez *et al.*, 2008; Biswas and Mantovani, 2010, 2012; Sica and Mantovani, 2012). However, new evidence suggests mouse and human macrophages have huge heterogeneity which cannot be accurately interpreted according to the M1/M2 model.

Indeed, macrophages do not form stable subsets and they respond to numerous factors within tissues including growth factors, cytokines and chemokines among others, which demonstrates their heterogeneity (Henson and Hume, 2006; Schultze *et al.*, 2015). In mice, valid *in vivo* markers for M1/M2 macrophages do not exist (Orecchioni *et al.*, 2019; Summers, Bush and Hume, 2020). Several mouse transcriptomic analyses show macrophage populations from different organs are considerably diverse (Stables *et al.*, 2011; Gautier *et al.*, 2012; Summers, Bush and Hume, 2020). A total of 299 transcriptome analyses of human macrophages exposed to 29 individual stimuli showed each stimuli produces distinct outcomes (Schultze *et al.*, 2015). Recent work has demonstrated a broader multi-dimensional model for macrophage classification (Mosser and Edwards, 2008; Hume, 2012; Xue *et al.*, 2014).

Monocytes are the main cell type infected with HCMV in the blood (Taylor-Wiedeman, Sissons and Sinclair, 1994) and the predominate infiltrators into organs (Booss *et al.*, 1989; Pulliam, 1991). HCMV infection directly induces differentiation of infected monocytes, which are short-lived and non-permissive to viral replication (Ibanez *et al.*, 1991; Sinclair and Sissons, 1996; Smith *et al.*, 2004), into macrophages which are long-lived and viral replication permissive (Taylor-Wiedeman *et al.*, 1991; Maciejewski *et al.*, 1993; Taylor-Wiedeman, Sissons and Sinclair, 1994; Mendelson *et al.*, 1996; Smith *et al.*, 2004; Chan *et al.*, 2009; Stevenson *et al.*, 2014). This occurs due to HCMV control of caspase 3 activity, allowing HCMV infected cells to differentiate without being destroyed which enables HCMV dissemination (Chan, Nogalski and Yurochko, 2012). The resulting macrophage phenotype is pro-inflammatory or ‘M1-like’ which may promote recruitment of naïve monocytes to sites of infection to increase the number of infected cells and/or to stimulate movement of infected monocytes from the circulation into peripheral tissue (Chan *et al.*, 2008, 2009; Chan, Nogalski and Yurochko, 2012; Stevenson *et al.*, 2014).

The primary reservoir where HCMV is maintained by CD34+ hematopoietic progenitor cells in the bone marrow where viral DNA is present during latent phases as well as permissive infection (von Laer *et al.*, 1995; Mendelson *et al.*, 1996). Within peripheral blood however, monocytes/macrophages are the primary virus reservoir in HCMV seropositive individuals (Taylor-Wiedeman *et al.*, 1991). As observed during HCMV infection, MCMV infection in mice also induces recruitment of inflammatory and patrolling monocytes which aids viral dissemination. However, monocyte tissue influx can

also improve tissue integrity and induce adaptive immunity which leads to infection control. In addition, inflammatory monocytes are essential for early control of MCMV through a DNAX accessory molecule (DNAM)-1 dependent mechanism (Lenac Rovis *et al.*, 2016). As a result, for both HCMV and MCMV, monocytes and macrophages serve to aid both the virus and the host (Hengel, Koszinowski and Conzelmann, 2005).

1.2.2.3.3 Dendritic cells

DCs are a sparsely distributed, migratory group of bone marrow derived cells specialised for uptake, transport, processing and presentation of antigens to T-cells which contributes to the establishment of immunological memory (Steinman, 1991; Hart, 1997; Banchereau and Steinman, 1998). Human and mouse DCs are relatively similar (Shortman and Liu, 2002). 'Immature' DCs continuously sample the antigenic environment in peripheral tissues. Any encounter with microbial products or tissue damage initiates migration of DCs to lymph nodes. The antigenic sample is processed, and peptides are presented on the DC surface by the major histocompatibility complex (MHC) molecules. The now-mature DCs also upregulate costimulatory molecules required for effective interaction with T-cells. T-cells expressing receptors specific to the foreign peptide MHC complex on the DC surface bind and trigger an immune response (Shortman and Liu, 2002). DCs are a hugely heterogeneous cell population which develop from myeloid precursors, some of which through differentiation into monocytes, and lymphoid precursors. Broad functional groups include classical DCs (cDCs), plasmacytoid DCs (pDCs) and specialist tissue subsets including Langerhans cells in the epidermis of the skin. cDCs are antigen processing and presenting cells. Once mature, cDCs have a high cytokine producing capacity and high phagocytic activity (Banchereau and Steinman, 1998; Mellman and Steinman, 2001). They are continuously replaced by precursors from the bone marrow (Liu *et al.*, 2007). These cells are distinct from tissue resident Langerhans cells (Collin and Bigley, 2018) which are not replaced by blood-borne cells at the steady state (Merad *et al.*, 2002). pDCs are relatively long-lived cells present in the bone marrow and all peripheral organs. pDCs are specialised for responses to viral infection and induce huge type I IFN production but they can also act as antigen presenting cells (APCs) and control T-cell responses (Colonna, Trinchieri and Liu, 2004). It has been suggested that at least some of the pDCs are of lymphoid origin as they express many lymphoid markers and lack myeloid markers (Yasuda *et al.*, 1990; Res *et al.*, 1999).

Upon HCMV infection, DCs generate antiviral immunity by producing chemokines and cytokines including type I IFNs which limit viral spread and have important functions in the activation of adaptive immunity. However, HCMV can also compromise the DC response and infect DCs directly, therefore reducing the effectiveness of the innate and adaptive immune responses coordinated by DCs, to promote HCMV viral spread (Gredmark-Russ and Söderberg-Nauclér, 2012). MCMV infected mouse DCs also have dual functionality. For example, MCMV induces early DC-dependent type I IFN and IL-12 responses which are essential for host resistance (Orange *et al.*, 1995; Orange and Biron, 1996; Presti *et al.*, 1998; Pien *et al.*, 2000; Asselin-Paturel *et al.*, 2001; Dalod *et al.*, 2002, 2003; Salazar-Mather, Lewis and Biron, 2002; Krmpotic *et al.*, 2003). Mature DCs induce MHC and costimulatory signals to activate naïve T-cells from the adaptive system. However, during later times of MCMV infection, DCs downregulate MHC and costimulatory molecules to prevent T-cell activation (Mathys *et al.*, 2003). As DCs are directly infected by CMV, DCs were also shown to serve as sites for virus reactivation. This was shown during latent HCMV infection upon exposure to specific stimuli including IL-6. IL-6 induces extracellular signal regulated kinase (ERK)-MAPK mediated transcriptional induction of HCMV IE genes within DCs (Hargett and Shenk, 2010; Reeves and Compton, 2011). However, DC susceptibility to HCMV infection is dependent on the DC subset and their maturation status. For example, studies suggest monocyte-derived DCs represent a significant portion of infected DCs compared to Langerhans cells and Plasmacytoid DCs which in general have low susceptibility to productive infection (Riegler *et al.*, 2000; Hertel *et al.*, 2003; Kvale *et al.*, 2006). The differences in infection susceptibility due to maturation status was shown *in* vitro where mature Langerhans DCs supported productive infection to a much greater extent compared to immature Langerhans DCs (Hertel *et al.*, 2003). However, the interpretation of these older HCMV studies must be viewed with caution, as many of them use inappropriate HCMV strains and attempt to infect DCs using cell free virus which is technically very challenging. Recent studies have shown cell-cell transfer of HCMV in monocyte-derived DCs and Langerhans cells is more efficient and less vulnerable to disruptive immune functions including IFN, cellular restriction factors and neutralising antibodies than cell free spread (Murrell *et al.*, 2017). As a result, DCs have a huge role in the dissemination of HCMV infection.

1.2.3 Adaptive Immunity

After several days of infection, the adaptive immune system becomes increasingly important in controlling primary CMV infection as innate cell signals, such as IL-12 released by monocytes and DCs, activate adaptive cells (J. Liu *et al.*, 2005). During HCMV latency, adaptive immunity is also vital for maintaining balance between the host and the virus as adaptive immune cells have memory and can rapidly respond when HCMV reactivates. Humoral immunity and cell-mediated immunity are two arms of the adaptive response mediated by antigen specific receptors expressed on the surface of B-cells and T-cells, respectively. The antigen-specific receptors are encoded by genes which are assembled in a process known as somatic recombination to produce immunoglobulin B-cell antigen receptor (Ig) genes and T-cell receptors (TCRs). Millions of different antigen receptors, each with potentially unique specificity for a different antigen, are assembled. The high level of specificity to antigens from invading pathogens is a defining feature of adaptive immunity (Chaplin, 2010).

1.2.3.1 B-cells and antibodies

B-cells constitute approximately 15% of peripheral blood leukocytes and are defined by their production of Ig molecules which identify target cells, viruses and other pathogens for destruction by the immune system (LeBien and Tedder, 2008). B-cells also act as APCs, capturing cognate antigen via their membrane Ig, internalising, and processing it for presentation via MHC class II (Adler *et al.*, 2017). The Ig repertoire during a human hosts life is edited by negative selection against self-antigens, clonal B-cell expansion upon antigen stimulation, activation-induced mutations in Ig genes, and receptor editing. Antibodies can directly neutralise virions and target infected cells by inducing the complement pathway or activating antibody-dependent cellular cytotoxicity (ADCC) (Forthal, 2014). A key function of humoral immunity is to provide immunological memory. After the immune system has encountered a pathogen, a portion of B-cells are retained which are memory B-cells (LeBien and Tedder, 2008). Memory B-cells are available to rapidly induce high levels of high affinity antibody specific to the pathogen upon rechallenge or reactivation of infection from latency. As a result, effective vaccines must induce robust B-cell antibody responses.

In the case of HCMV infection, our knowledge of humoral responses to this pathogen requires further research. Natural HCMV infection in immunocompetent subjects induces robust antibody responses which include neutralising antibody titres and memory B-cell development during latency (Wang *et al.*, 2011; Dauby *et al.*, 2014; Xia *et al.*, 2017). The HCMV viral pentameric complex composed of gH, gL, pUL128, pUL130 and pUL131 (or pUL131a) is an important target for antibody neutralisation against viral infection in epithelial cells (Macagno *et al.*, 2010; Fouts *et al.*, 2012; Ciferri, Chandramouli, Leitner, *et al.*, 2015; Loughney *et al.*, 2015; Gerna *et al.*, 2016). The identification of potently neutralising antibodies against the viral pentameric complex has induced renewed interest in antibody therapies to treat HCMV in transplant settings (Macagno *et al.*, 2010; Lanzavecchia *et al.*, 2016; Ha *et al.*, 2017) and to prevent congenital transmission during pregnancy (Tabata *et al.*, 2019). However, the antigen specificity of neutralising antibodies in seropositive donors and understanding how host neutralising antibodies are shaped by natural HCMV infection requires further investigation (Xia *et al.*, 2017).

In mice, evidence suggests that B-cells and antibodies are not required for primary MCMV infection resolution (Jonjić *et al.*, 1994). A study by Jonjic et al. using B-cell deficient mice infected with MCMV demonstrated that B-cell function is important for limiting viral dissemination during viral reactivation instead (Jonjić *et al.*, 1994). Further evidence in mice also shows the protective role of CMV-specific antibodies (Shanley, Jordan and Stevens, 1981; Farrell and Shellam, 1991; Bootz *et al.*, 2017) and adoptive transfer of B-cells from immune mice reduces viral load and protects against lethal infection (Klenovsek *et al.*, 2007). Furthermore, latent infection of HCMV in a humanised mouse model prompted marked development of B-cells and HCMV-specific antibody responses (Theobald *et al.*, 2018) but evidence of B-cell driven control of latent virus is lacking in humans.

A better understanding of B-cell immunity in response to HCMV will be important for the development of HCMV vaccines which have thus far been slow. The most efficacious vaccine candidate to date, a MF59-adjuvanted gB protein subunit vaccine (gB/MF59), induced 43-50% efficacy in Phase II trials by induing primarily neutralising antibodies (Pass *et al.*, 2009; Griffiths *et al.*, 2011; Rieder and Steininger, 2014). New vaccine candidates continue to be developed and a recent phase I clinical trial showed a live replication defective HCMV vaccine, known as V160, to be safe and induce neutralising

antibodies in phase I clinical trials (Li *et al.*, 2021). A greater understanding of B-cell responses to natural HCMV infections will enable progress towards the first licenced HCMV vaccine.

1.2.3.2 T-cells

Cell-mediated immunity within the adaptive immune system is coordinated by T-cells which are defined by their TCR expression. The TCR recognises peptide antigens presented in a complex with class I or class II MHC proteins. There are two major T-cell subsets known as $CD4^+$ and $CD8^+$ T-cells. $CD4^+$ helper T-cells function to regulate the cellular and humoral response (Gray, Westerhof and MacLeod, 2018; Lim, Jackson and Wills, 2020). Cytotoxic $CD8^+$ T-cells kill infected or transformed cells (Moss and Khan, 2004; Zhang and Bevan, 2011). In the context of CMV infection, both $CD4^+$ T-cells and $CD8^+$ T-cells are important for maintaining virus control.

1.2.3.2.1 $CD4^+$ helper T-cells

$CD4^+$ T-cells recognise specific antigen bound to MHC II molecules found on APCs. As a result of the cytokines in the microenvironment and costimulatory molecules involved in antigen presentation, as well as antigen affinity, $CD4^+$ T-cells will differentiate into distinct populations including T helper 1 (Th1), Th2, Th17 and T regulatory (Treg) cells. Th1 cells, important for the identification and eradication of intracellular pathogens such as viruses and bacteria, produce the effector cytokines IFNγ and TNFα. Th2 cells, which recognise extracellular pathogens including helminths and parasites and activate B-cell antibody responses, produce IL-4, IL-5, IL-10, and IL-13. Th17 cells secrete IL-17A for protection against extracellular pathogens and aberrant Th17 responses have been linked to autoimmune diseases (Harrington *et al.*, 2005; Park *et al.*, 2005; Mirlekar, 2020). Tregs, which are critical for self-tolerance and immune cell homeostasis, secrete immunomodulatory cytokines including IL-10, TGFβ and IL-35 (Sakaguchi *et al.*, 2008; Vignali, Collison and Workman, 2008; Okeke and Uzonna, 2019; Shevyrev and Tereshchenko, 2020). Recently, a new system has been proposed where $CD4^+$ T-cells are divided into central memory and effector memory cells according to CC-chemokine receptor 7 (CCR7) expression which directs lymphocytes to secondary lymphoid organs (Sallusto *et al.*, 1999). Central memory $CD4^+$ T-cells express CCR7 and function within secondary lymphoid organs where they produce IL-2 for priming B-cells and $CD8^+$ T-cells. Effector memory $CD4^+$ T-cells, which reside within peripheral target organs, exert direct

containment of viral infection through production of antimicrobial lymphokines (Jenkins *et al.*, 2001).

During CMV infection $CD4^+$ T-cell types mediate the recruitment, expansion and function of other effector cells as well as directly contributing to viral clearance through cytokine production or cell-mediated cytotoxicity. $CD4^+$ T-cells are primarily known for helping $CD8^+$ T-cells and B-cells during infection. $CD4^+$ T-cells provide help to antiviral $CD8^+$ T cells by maximising their expansion during primary infection (Murali-Krishna *et al.*, 1998; Swain, McKinstry and Strutt, 2012). $CD4^+$ T-cells also facilitate the development of virus-specific memory $CD8^+$ T-cells through downregulation of TRAIL, generation of cytokines such as IL-2, or ligation of CD40 on naïve $CD8^+$ T-cells to CD40L on $CD4^+$ T-cells (Simpson and Gordon, 1977; Husmann and Bevan, 1988; Murali-Krishna *et al.*, 1998; Zajac *et al.*, 1998; Sant and McMichael, 2012; Swain, McKinstry and Strutt, 2012). The importance of $CD4^+$ T-cell help was shown in MCMV infected $CD4^+$ T-cell deficient mice which exhibited delayed $CD8^+$ T cell effector responses, although the mice were still able to control infection (Jonjić *et al.*, 1989). $CD4^+$ T-cells are also involved in the development of antibody responses from B-cells. Antigen engagement on $CD4^+$ T-cells initiates CD40L expression which binds to CD40 on B-cells, leading to B-cell proliferation and differentiation into antibody-producing plasma cells and memory B-cells. A subtype of $CD4^+$ T-cells known as T follicular helper cells, which express the C-X-C chemokine receptor 5 (CXCR5) and the transcriptional repressor Bcl6, produce IL-21 which facilitates the generation of long-term antibody protection (Hale *et al.*, 2013; Hale and Ahmed, 2015). In mice it was shown that the development of neutralising antibodies, which are key to protection, were dependent on the availability of virus-specific helper $CD4^+$ T-cells (Jonjić *et al.*, 1989; Maloy *et al.*, 1999). After CMV infection, there is evidence for the emergence of $CD4^+$ T-cells which are direct antiviral effectors and required for control of CMV across the lifetime of infected hosts (van Leeuwen *et al.*, 2004, 2006; Arens *et al.*, 2011; Pachnio *et al.*, 2016). Most $CD4^+$ T-cells produced in response to primary CMV infection are Th1 which secrete the antiviral cytokine IFNγ (Rentenaar *et al.*, 2000). In mice $CD4^+$ T cells, believed to be Th1, are absolutely required for control of MCMV replication within the salivary gland which is the key site of viral dissemination and where $CD8^+$ T-cells cannot exert control (Lucin *et al.*, 1992; Polić *et al.*, 1998). Furthermore, a key study from Tu et al. showed a lack of IFNγ-producing virus-specific $CD4^+$ T-cells in immunocompetent children was responsible for prolonged viral shedding (Tu *et al.*, 2004). In transplant

recipients, the presence of CMV-specific $CD4^+$ T-cells was associated with a lower risk of developing CMV disease (Avetisyan *et al.*, 2006; Gerna *et al.*, 2006, 2011; Egli *et al.*, 2008; Lilleri *et al.*, 2008; Nebbia *et al.*, 2008; Solano *et al.*, 2008; Pourgheysari *et al.*, 2009; Tormo *et al.*, 2011; Rogers *et al.*, 2020). The presence of $CD4^+$ T-cells is important for CMV control but our understanding of how they orchestrate viral control has thus far been limited.

1.2.3.2.2 $CD8^+$ cytotoxic T-cells

$CD8^+$ cytotoxic T-cells, once primed by $CD4^+$ helper T-cells and directed by antibody binding, function both as direct killers of virally infected cells and as secretors of inflammatory mediators. $CD8^+$ T-cells interact with infected cells upon binding of their TCR to the antigen-presenting MHC I on target cells. Thereafter, $CD8^+$ T-cells release perforin and granzymes to induce direct killing of virally infected cells. $CD8^+$ T-cells also release a number of cytokines including IFNγ, TNFα and IL-2 that recruit other immune cells to the infection site (D'Souza and Lefrançois, 2004; Brehm, Daniels and Welsh, 2005; Lauvau and Goriely, 2016; Bhat *et al.*, 2017).

In CMV-seropositive individuals, CMV specific $CD8^+$ T-cells represent a huge component of the cellular response and make up 4.6% of total T-cells and 10.2% of memory T-cells (Sylwester *et al.*, 2005). During human infection, a high frequency of these CMV-specific $CD8^+$ T-cells are directed towards the viral proteins pp65(UL83) and IE1 (Borysiewicz *et al.*, 1983; McLaughlin-Taylor *et al.*, 1994; Walter *et al.*, 1995; Kern *et al.*, 1999; Khan, Cobbold, *et al.*, 2002) as well as emerging viral proteins of interest (Sylwester *et al.*, 2005; Jackson *et al.*, 2014). CMV-specific $CD8^+$ T-cells are hugely heterogeneous and contain subsets with distinct transcriptional profiles, function, migration patterns and localisation (Holtappels *et al.*, 2000; Podlech *et al.*, 2000; Sierro, Rothkopf and Klenerman, 2005; Munks *et al.*, 2006; Snyder *et al.*, 2008; Hertoghs *et al.*, 2010; Quinn *et al.*, 2015; Smith *et al.*, 2015; Thom *et al.*, 2015). Naïve $CD8^+$ T-cells express the costimulatory proteins CD27 and CD28, are CD45RAhigh which indicates naivety, express the homing molecules CCR7 and CD62L and have low CD11a integrin expression (Hamann *et al.*, 1997; Wills *et al.*, 2002). Upon CMV antigen encounter, all daughter cells permanently upregulate CD11a (Faint *et al.*, 2001) and at least initially upregulate CD45RO (Wills *et al.*, 2002) before they can develop into memory-like phenotypes (Sallusto *et al.*, 1999; Wills *et al.*, 2002). During

chronic HCMV infection, there is a theory suggesting CMV-specific memory $CD8^+$ T-cells remain high (and may even increase with age) (Karrer *et al.*, 2003; O'Hara *et al.*, 2012; Klenerman and Oxenius, 2016) in a process termed memory inflation (Khan, Shariff, *et al.*, 2002; Ouyang *et al.*, 2004; Almanzar *et al.*, 2005; Pourgheysari *et al.*, 2007; Pita-Lopez *et al.*, 2009; Klenerman and Oxenius, 2016). The term 'inflation' was originally defined using longitudinal analysis of T-cells from MCMV-infected mice which had continued expansion of a subset of MCMV-specific $CD8^+$ T-cells and eventual stabilization of a large frequency of memory $CD8^+$ T-cells (Holtappels *et al.*, 2000). In mice the following MCMV proteins have been shown to induce T-cell inflation responses: m38, m139 and IE3 (Munks *et al.*, 2006; Snyder *et al.*, 2008; Turula *et al.*, 2013; Redeker *et al.*, 2017), m164 (Holtappels *et al.*, 2002, 2016), and IE1/pp89 (Holtappels *et al.*, 2000, 2002; Karrer *et al.*, 2003, 2004; Trgovcich *et al.*, 2016). Memory T-cell inflation has also been observed in multiple tissue sites in the mouse including the lung (Holtappels *et al.*, 2000, 2002; Trgovcich *et al.*, 2016; Morabito *et al.*, 2018), spleen (Munks *et al.*, 2006; Snyder *et al.*, 2008) and lymph nodes amongst many sites (Karrer *et al.*, 2003; Arens *et al.*, 2008; Borkner *et al.*, 2017). In humans there is still some debate as to the existence of memory T-cell inflation (Jackson *et al.*, 2019). A number of different approaches have been used to determine whether HCMV induced memory T-cell inflation exists including longitudinal studies and infection during transplantation which has demonstrated evidence for (Gerna *et al.*, 2006; Khan *et al.*, 2007; Suessmuth *et al.*, 2015) and against memory T-cell inflation (Gamadia *et al.*, 2003; Gamadia, Rentenaar, *et al.*, 2004; Gamadia, van Leeuwen, *et al.*, 2004; Hertoghs *et al.*, 2010; Klarenbeek *et al.*, 2012; Jackson *et al.*, 2014, 2017). More studies are required using a range of tissue sites in human subjects to determine the existence of memory $CD8^+$ T-cell inflation. The predominate phenotype of mouse and human CMV-specific memory CD8+ T-cells is defined as effector memory-like and driven by the low-level persistence of CMV (Gamadia *et al.*, 2001; Snyder *et al.*, 2008; Torti *et al.*, 2011; Quinn *et al.*, 2015; Klenerman and Oxenius, 2016). CMV-specific memory CD8+ T-cells are found at high frequencies in tissues as well as in blood (Karrer *et al.*, 2003; Ward *et al.*, 2004; Akulian *et al.*, 2013) and occupy the mature end of T-cell phenotypes with shorter telomeres, re-expression of CD45RA (Wills *et al.*, 1999, 2002; Champagne *et al.*, 2001; Appay *et al.*, 2002; Khan, Shariff, *et al.*, 2002; Weekes *et al.*, 2004), expression of CD57 and killer cell lectin-like receptor subfamily G member 1 (KLRG1) and low expression of the co-stimulating receptor CD28 (Kern *et al.*, 1999; Khan, Shariff, *et al.*, 2002; Gamadia *et al.*, 2003; van Leeuwen *et al.*, 2004; Fletcher *et al.*, 2005). In addition, CMV-specific memory

CD8+ T-cells express a large number of NK cell receptors (van Stijn *et al.*, 2008), and retain high levels of cytokine production (Sierro, Rothkopf and Klenerman, 2005; Munks *et al.*, 2006; Snyder *et al.*, 2008; Hertoghs *et al.*, 2010; Wallace *et al.*, 2011). Further studies are needed to understand the late stage differentiated but highly functional phenotype of CMV-specific $CD8^+$ T-cells.

1.2.3.2.3 γδ T-cells

Approximately 5% of T-cells express the unique γδ TCR that sets them apart from $CD4^+$ and $CD8^+$ T-cells which express αβ TCR. These 'unconventional' T-cells are present in blood and enriched in epithelial and mucosal sites where they function in an innate-like manner within the first line of defence against pathogen challenge. Unlike conventional $CD4^+$ and $CD8^+$ T-cells, the majority of γδ T-cells do not recognise antigens in an MHC dependant manner. The antigens recognised by most γδ T-cells remain unknown but are believed to include metabolites, cell stress signals and MHC-related proteins (Chien, Meyer and Bonneville, 2014; Adams, Gu and Luoma, 2015; Kabelitz and Déchanet-Merville, 2015). γδ T-cells have broad functionality through production of cytokines (including IFNγ, TNFα and IL-17), chemokines, cytolysis, and interaction with other immune cells including monocytes, DCs, neutrophils and B-cells. In the context of HCMV infection, γδ T-cell adaptive responses have also been observed. Healthy and immunocompromised individuals exhibit clonal expansion of a subset of γδ T-cells, known as $V\delta2^-$ γδ T-cells, during HCMV infection (Pitard *et al.*, 2008; Knight *et al.*, 2010; Puig-Pey *et al.*, 2010; Scheper *et al.*, 2013). γδ T-cell expansions in response to MCMV have also been shown in mice within various target organs (Ninomiya *et al.*, 2000; Cavanaugh *et al.*, 2003; Khairallah *et al.*, 2015; Sell *et al.*, 2015). Furthermore, in a study of conventional T-cell deficient mice and another of conventional T-cell deficient mice which also have B-cell deficiency and were infected with a MCMV strain lacking the NK cell activating protein m157, γδ T-cells were shown to protect against MCMV induced organ damage and death (Khairallah *et al.*, 2015; Sell *et al.*, 2015). These studies demonstrate that although γδ T-cells are dispensable in immunocompetent hosts, they are essential in some immunodeficient contexts for controlling infection.

1.3 NK cells

1.3.1 NK cells and their receptors

NK cells are innate lymphocytes (Raulet 2004; Lanier 2005; Sun and Lanier 2009; Sun et al. 2011) that detect aberrant cells in the body and are the first responders among lymphocytes. Infected, transformed, or stressed host cells can stimulate NK cells which respond by killing them or generating cytokines to activate other arms of the immune response. Similar to T-cells and B-cells, NK cells develop through a common lymphocyte progenitor in the bone marrow (Galy *et al.*, 1995; Kondo, Weissman and Akashi, 1997) and require cytokines for their development, homeostasis and survival (Ma, Koka and Burkett, 2006). NK cells from the bone marrow migrate to the blood, tissues and lymphoid organs to exert their function (Castriconi *et al.*, 2018). Patients with NK cell immunodeficiencies suffer from severe recurrent viral infections such as herpesviruses, human papilloma viruses, poxviruses, influenza and HCMV (C. Biron, Byron and Sullivan, 1989; Ballas *et al.*, 1990; Jawahar *et al.*, 1996; Filipovich, 2009; Orange, 2013, 2020).

NK cells express a multitude of activating and inhibitory receptors, the balance of which allows NK cells to coordinate appropriate responses to transformed or infected host cells. A selection of some of the activating and inhibitory receptors expressed on mouse and human NK cells is shown in Figure 1.6. NK cells detect aberrant cells through a multitude of activating receptors which recognise virally derived products, stress-induced host proteins, and cytokines using their surface receptors. However, the intracellular signalling pathways which induce NK cell effector functions are still incompletely understood which has slowed progress on their use as therapeutics (Colucci *et al.*, 2002). As early as the 1980s NK cell-based therapies were investigated and showed promising results but due to severe side effects and the potentiation of regulatory responses, efficacy was not proven (Rosenberg *et al.*, 1985, 1987). Therefore, a better understanding of NK cell signalling pathways could inform on the development of a finely tuned therapy without side effects and regulatory responses. In the following section I will give an overview of the mechanisms and receptors regulating NK cell activation in humans and mice.

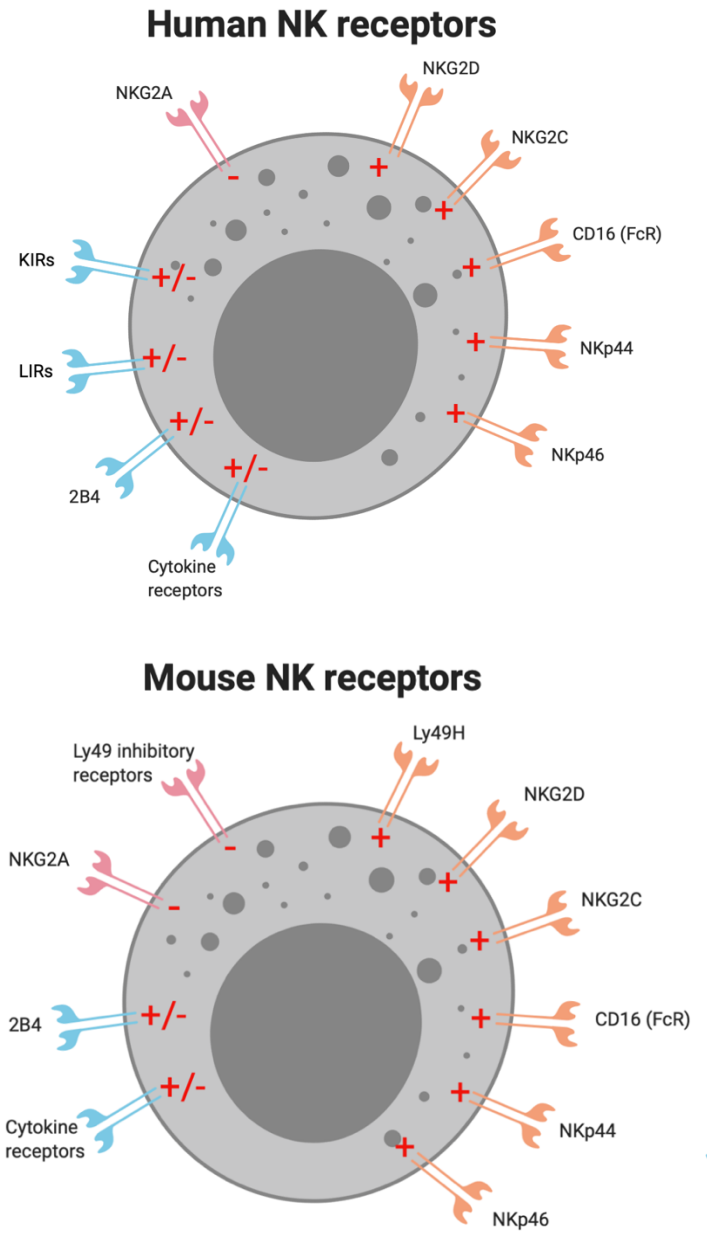

Figure 1. 6 Activating and inhibitory surface NK cell receptors.
Key human and mouse surface receptors important for regulating NK cell activation in response to infection are shown. Inhibitory receptors (pink), activating receptors (orange) and activating and/or inhibitory receptors (blue). Killer immunoglobulin-like receptors (KIRs). Leukocyte immunoglobulin-like receptors (LIRs).

The major factors that induce NK cell activation upon infection are pathogen peptides presented by major histocompatibility complex (MHC), adhesion molecules, stress-induced ligands, immune complexes, and cytokines. The peptides presented by MHC arise from the breakdown of pathogenic proteins through antigen processing. NK cells can become activated upon loss of inhibitory signals usually provided by healthy host cells. Virally infected cells and tumour cells downregulate expression of the MHC class I to avoid T-cell killing (Alcami and Koszinowski, 2000; Garcia-Lora, Algarra and Garrido, 2003; Hewitt, 2003; Revilleza *et al.*, 2011; Koutsakos *et al.*, 2019). MHC class I molecules, ubiquitously expressed by all nucleated cells, interact with inhibitory receptors on NK cells including NKG2A, Ly49 inhibitory receptors in mice and inhibitory killer cell immunoglobulin-like receptors (KIRs) and leukocyte-immunoglobulin-like receptors (LIRs) in humans described in this section. Therefore, loss of MHC class I expression on virus infected cells leads to a loss of NK cell inhibitory signalling and may contribute to subsequent NK cell activation. This 'missing-self' hypothesis is one of the mechanisms for NK cell discrimination between normal cells and aberrant cells, which contributes along with activating signals and may lead to targeting of these aberrant cells for destruction alongside other costimulatory signals (Ljunggren and Kärre, 1990; Lanier, 2005; Waldhauer and Steinle, 2008). A proportion of NK cells do not express self-MHC-I specific inhibitory receptors but are able to maintain self-tolerance by dampening stimulatory signalling which renders them as hyporesponsive (Fernandez *et al.*, 2005; Kim *et al.*, 2005; Anfossi *et al.*, 2006).

NK cells were originally classified as innate cells because they were believed to lack antigen-specific receptors; but recent evidence suggests specific direct interactions between NK cells and aberrant cells. NK cells can directly recognise some pathogen-derived products through their natural cytotoxicity receptors (as well as through the activating Ly49H receptor in mice described in section 1.3.3). The natural cytotoxicity receptors are some of the best described NK cell receptors and include NKp44 and NKp46 (Cantoni *et al.*, 1999; Mandelboim *et al.*, 2001). NKp46 is found on all NK cells and is commonly used to identify NK cell populations. NKp46 recognises the hemagglutinin of influenza virus and the hemagglutinin-neuraminidase of parainfluenza, important for influenza infection control (Mandelboim *et al.*, 2001; Gazit *et al.*, 2006). In HCMV infection studies, NKp46 was shown to be required for driving NK cell responses to infected DCs. However, only infected DCs with HLA class I down-regulation, and until 72 hours post-infection, showed

NKp46 driven responses. The ligand to NKp46 is unknown, but these results suggest NKp46 recognises a ligand of cellular stress which is not specific to viral infection but it cannot be ruled out that the affinity between NKp46 and its ligand increases during the early stage of infection (Magri *et al.*, 2011).

NK cells also recognise stress-induced ligands through their NKG2 receptors which are C-type lectin-like homodimers including NKG2D. Viral infection can induce expression of the host stress proteins MHC class I related (MIC) molecules MICA and MICB, the UL16 binding proteins in humans (Raulet, 2003; González *et al.*, 2008; Jonjić *et al.*, 2008) and murine UL16-binding protein like transcript (MULT)-1, H60 and Retinoic acid induced early transcript (Rae-1) in mice (Lenac *et al.*, 2006). Stress ligands are particularly important for CMV infection control (Andoniou *et al.*, 2005; Slavuljica, Krmpotić and Jonjić, 2011) as well as for vaccinia virus (Martinez, Huang and Yang, 2010) and adenovirus (Zhu, Huang and Yang, 2010). However, in the case of HCMV infection, the virus has evolved mechanisms to bypass the antiviral effects induced by these receptors. These studies show that stress proteins and their receptors critically control NK cell responses to infected target cells.

NK cells also recognise and kill target cells opsonized with antibodies by ADCC (Moretta *et al.*, 2002; Lanier, 2005). ADCC is mediated by IgG antibodies bound to antigen which then signal via NK cell CD16 receptors through their Fc region. In humans, the hIgG3 subclass followed by hIgG1 have the highest affinity for CD16 receptors (FcγRIII). In mice IgG2a and IgG2b have the highest binding affinities for Fcγ receptors (Stewart *et al.*, 2014). The signalling pathways which activate ADCC have been well characterised and resemble those that regulate activation of B-cells and T-cells via their antigen receptors (Lanier, 2008; Wang *et al.*, 2015). NK cell activation induces immunoreceptor tyrosine-based activation motif (ITAM) phosphorylation by Lck, which is a member of the Src family of tyrosine kinases, and thus induces the action of other protein tyrosine kinases leading to cytotoxicity and cytokine production (Smyth *et al.*, 2005; Wang *et al.*, 2015). During CMV infection however, CMV-infected cells can avoid NK cell mediated ADCC as they encode Fc receptors which interfere with ADCC and complement attack (Corrales-Aguilar, Hoffmann and Hengel, 2014).

Cytokines released by other cells, or by NK cells themselves, can activate NK cells and enhance activating receptor-mediated NK cell effector functions (Dokun *et al.*, 2001). Type

I IFNs, IL-12, IL-15, and IL-18, produced by infected cells, macrophages or DCs, are among the most important cytokines for NK cell activation. In general, these cytokines induce the same biological effects in human and mouse NK cells, in part due to the activation of conserved intracellular signalling pathways (Christine A. Biron *et al.*, 1999). Type I IFNs directly induce NK cell mediated immunity (Orange *et al.*, 1995). IL-15 and IL-2 are important for NK cell proliferation and enhancing cytotoxicity. IL-12 and IL-18 derived NK cells have also been associated with greater perforin expression, important for NK cell cytotoxicity (see section 1.3.4) (Lauwerys *et al.*, 2000). IL-12 alone enhances cytotoxicity but has a modest effect on NK cell proliferation (Gately *et al.*, 1998). IL-12 and IL-18 are known to activate NK cell IFNγ production which can limit virus replication and activate other immune cells (Orange *et al.*, 1995). Cytokine receptors on NK cells signal through the janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway and different STAT proteins facilitate the differential effects of each cytokine (Stabile *et al.*, 2018). For example, type I IFNs induce the phosphorylation of STAT1 and STAT2 to drive gene expression which enhances NK cell cytotoxicity (Nguyen *et al.*, 2000). Thus, cytokines are important factors in NK cell driven infection control.

Inhibitory NK cell receptors function to protect healthy host cells from inappropriate NK cell-mediated killing. These include other NKG2 receptors such as NKG2A (Moretta *et al.*, 1994), inhibitory lectin-like Ly49 homodimers in mice (Karlhofer, Ribaudo and Yokoyama, 1992; Schenkel, Kingry and Slayden, 2013) and inhibitory KIRs as well as a related family of receptors known as LIRs in humans (Borges *et al.*, 1997; Raulet, Vance and Mcmahon, 2001). KIRs are a major human NK cell receptor family characterised by extraordinary diversity and recognition of MHC Class I which makes them key to self-tolerance (Trowsdale, 2001; Hsu *et al.*, 2002; Shilling *et al.*, 2002; Jiang *et al.*, 2012; Pyo *et al.*, 2013). Other inhibitory receptors can detect the presence of MHC class I on healthy cells to repress NK cell activation, or they may bind cadherins and collagen to trigger an inhibitory signal (Lanier, 2008). Upon inhibitory receptor ligand binding, the immunoreceptor tyrosine-based inhibition motifs (ITIMs) present in the intracellular domains of inhibitory KIRs, LIRs and Ly49s recruit phosphatases that counterbalance the action of protein tyrosine kinases. A balance of signalling between the receptors ultimately determines cellular function (Long, 2008). If the balance tips towards inhibition, the activation of cytotoxicity and cytokine production is blocked, and NK cell homeostasis is maintained.

NK cell activation is also controlled by cell-extrinsic factors such as cytokines. An important cytokine for NK cell regulation is IL-10 which is known to limit activation induced death of NK cells during acute virus infection (Stacey *et al.*, 2011). B-cells, inflammatory macrophages and splenic DCs are a significant source of IL-10 during acute MCMV infection (Madan *et al.*, 2009; Stacey *et al.*, 2011). IL-10 receptor signalling was shown to promote the accumulation of NK cells, including cytotoxic cells, whilst also suppressing pro-inflammatory cytokine production (Stacey *et al.*, 2011). Pro-inflammatory signalling through signals such as IL-18 is also important to the induction of efficient antiviral responses. NK cell extrinsic IL-18 signalling promotes NK cell activation in response to vaccinia virus through upregulation of NKG2D ligands (Brandstadter, Huang and Yang, 2014). Consequently, multiple immunological factors both cell intrinsic and cell extrinsic are required to regulate the NK cell response to infection.

As a latent virus which has co-evolved with the immune system, CMVs also directly modulate NK cell function upon infection through multiple immune evasion pathways. These strategies for NK cell modulation include regulating MHC I expression to avoid recognition of virus infected cells, decoy molecules encoded by CMVs which preserve inhibitory NK cell receptor expression, and prevention of activating receptor engagement all summarised in Table 1.3. The mechanisms of immune evasion enable life-long persistence of CMV in the host which is balanced by a competent immune response.

Immunoevasin	Target receptor	Action	References
Regulators of MHC I expression			
UL40	NKG2A, NKG2C, KIRs (?)	Provides peptide for loading and stabilization of HLA-E and UL18	Tomasec *et al.*, 2000; Ulbrecht *et al.*, 2000; Wang *et al.*, 2002; Prod'homme *et al.*, 2012
m04 (M)	Inhibitory Ly49s and Ly49P (activating)	Escorts some MHC I to the cell surface, enhances interaction with inhibitory NK cell receptors	Kleijnen *et al.*, 1997; Kavanagh, Koszinowski and Hill, 2001; Kielczewska *et al.*, 2009; Pyzik *et al.*, 2011
Direct binding of virus encoded molecules to block host immune receptor signalling			
UL18	LIR-1, unknown activating receptor	Inhibition of LIR-1+ NK cells and activation of LIR- NK cells (through direct binding of LIR-1 with 1000-fold higher affinity than the standard HLA-I ligand)	Cosman *et al.*, 1997; Willcox, Thomas and Bjorkman, 2003; Prod'homme *et al.*, 2007
m12 (M)	Activating and inhibitory NKR-P1	Activation or inhibition of NK cells, depending on the mouse and viral strain	Carlyle *et al.*, 2004; Aguilar *et al.*, 2015, 2017; Rahim *et al.*, 2016
m144 (M)	Unknown inhibitory receptor	Inhibits NK cell responses	Farrell *et al.*, 1997
m157 (M)	Ly49H (activating), Ly49C, Ly49I (inhibitory)	Ligand for inhibitory Ly49I/C and activating Ly49H receptors. Activation of Ly49H+ cells in C57Bl/6 mouse strains	Arase *et al.*, 2002; Hamish R.C. Smith *et al.*, 2002
Prevention of activating receptor engagement			
US9	NKG2D	Proteasomal degradation of MICA*008	Seidel *et al.*, 2015
UL16		Intracellular retention of MICB, ULBP-1, ULBP-2 and ULBP-6. UL16 with bound ligands is further targeted to lysosomal degradation by US20 family members	Cosman *et al.*, 2001; Dunn *et al.*, 2003; Eagle *et al.*, 2009; Fielding *et al.*, 2017
US18, US20		Lysosomal degradation of full-length MICA	Fielding *et al.*, 2014
US12, US13 and US20		Proteasomal degradation of ULBP-2, MICB and UL16	Fielding *et al.*, 2017
miR-UL112		Inhibition of MICB mRNA translation	Stern-Ginossar *et al.*, 2007
UL142		Downregulation of full-length MICA and ULBP3 from the cell surface	Chalupny *et al.*, 2006; Ashiru *et al.*, 2009
m138 (M)		Downregulation of surface MULT-1, H60a and RAE1-ε	Lenac *et al.*, 2006; Arapović *et al.*, 2009

Table 1. 3A HCMV and MCMV NK cell evasion pathways.
(M) denotes MCMV genes. Original source: Goodier *et al.*, 2017. Edited to include further detail.

Prevention of activating receptor engagement continued			
m145 (M)		Intracellular retention of MULT-1	Krmpotic *et al.*, 2005
m152 (M)		Retention of all Rae isoforms with varying efficacy, H60a and MHC I in the ER.	Lodoen *et al.*, 2003; Arapovic *et al.*, 2009
m155 (M)		Intracellular retention of H60a	Lodoen *et al.*, 2004; Hasan *et al.*, 2005; Lenac *et al.*, 2006; Arapović *et al.*, 2009
m154 (M)	2B4	Lysosomal and proteasomal degradation of CD48	Zarama *et al.*, 2014
US2	DNAM-1, CD96	Degradation of Nectin-2 and 6 α-integrins	Hsu *et al.*, 2015
UL141	DNAM-1, CD96	Downmodulation of surface expression of PVR and Nectin2	Tomasec *et al.*, 2005; Stanietsky and Mandelboim, 2010; Hsu *et al.*, 2015
UL141	TRAIL	Retention of Trail-R1 and Trail-R2 in the ER	Nemčovičová, Benedict and Zajonc, 2013; Smith *et al.*, 2013
m20.1 (M)	DNAM-1	Downmodulation of surface expression of PVR	Lenac Rovis *et al.*, 2016
US18, US20	NKp30	Inhibition of surface expression of B7-H6. Also traffic MICA to the lysosome for degradation which hampers NK cell mediated killing of HCMV infected cells.	Fielding *et al.*, 2017, (Charpak-Amikam *et al.*, 2017)
pp65	NKp30	Dissociation of ζ-chain from NKp30	Arnon *et al.*, 2005
m166 (M)	TRAIL	Surface expression inhibition of TRAILR	Verma *et al.*, 2014
UL148	CD58	Surface expression inhibition of CD58 to reduce ADCC responses	Wang *et al.*, 2018
UL147A	MICA*008	Specific downregulation of MICA*008 which hinders NKG2D-mediated elimination of HCMV infected cells by NK cells	Seidel *et al.*, 2021
UL148A	MICA	Traffic MICA to the lysosome for degradation which hampers NK cell mediated killing of HCMV infected cells. Requires other unknown genes	Dassa *et al.*, 2021
UL135	AB1/AB2	Remodelling the actin cytoskeleton leads to impaired immunological synapse formation and therefore impaired NK cell and T cell degranulation	Takenawa and Suetsugu, 2007; Stanton *et al.*, 2014

Table 1. 3B HCMV and MCMV NK cell evasion pathways. (M) denotes MCMV genes. Original source: Goodier *et al.*, 2017. Edited to include further detail.

1.3.2 NK cell development and maturation in humans and mice

NK cells develop from progenitor cells in specialised bone marrow niches and secondary lymphoid tissues before circulating as mature NK cells in the blood (Scoville, Freud and Caligiuri, 2017). Mouse and human NK cells develop from haematopoietic stem cells (HSCs) which become common lymphoid progenitors (CLPs) before committing to a specific cell lineage. The process of mouse NK cell development in the bone marrow is outlined in Figure 1.7.

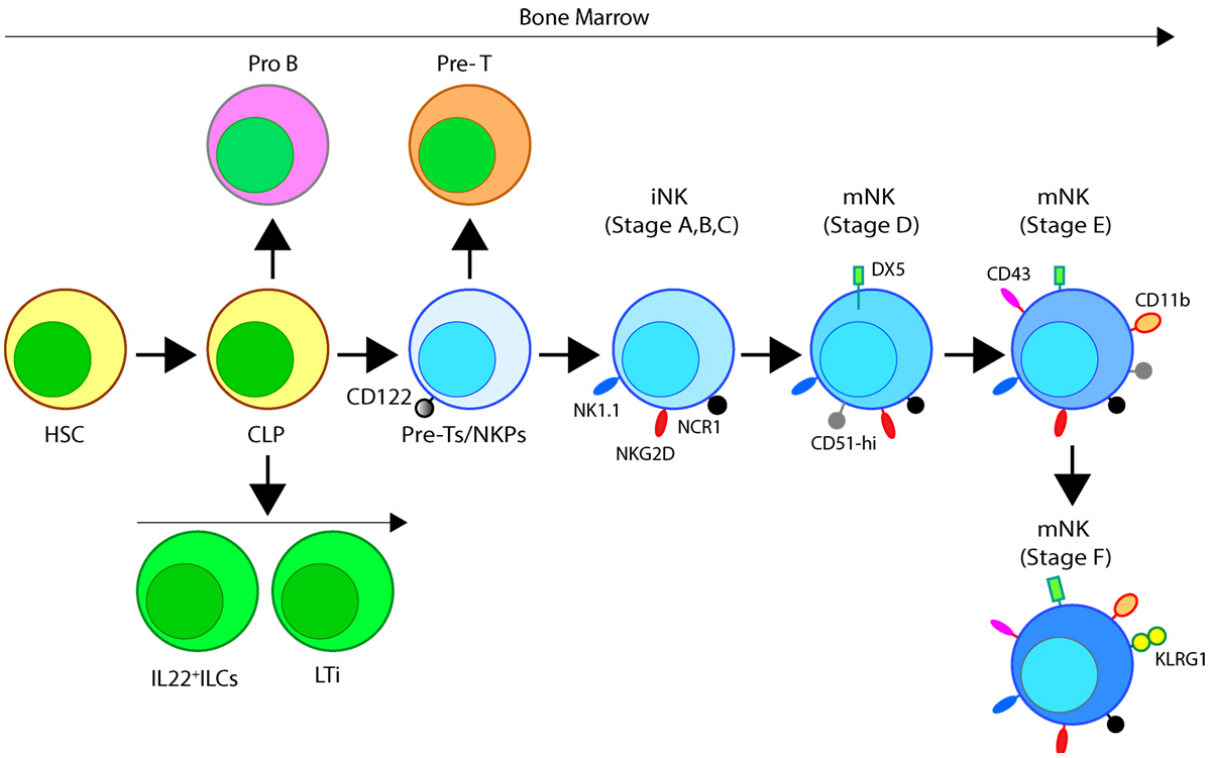

Figure 1.7 Mouse NK cell development from stem cells to mature NK cells.
A subset of multipotent HSCs commits to becoming oligopotent common lymphoid progenitors (CLPs). CLPs give rise to several subsets including Pro-B, Pre-T, innate lymphoid cells (ILCs), lymphoid tissue inducers and CD122+ Pre-T/early NK cell progenitor (NKP) lineages. The earliest transition of NKPs into committed immature NK cells is marked by NKG2D expression on the CD122+ NKPs (stage A). This is followed by NK1.1 and NCR1 expression (stages B and C). The initial stage of mature NK cells is defined by expression of CD51 (Integrin αV) and CD49b (DX5, Integrin VLA-2α) (stage D). Expression of CD43, CD11b and the acquisition of distinct sets of Ly49 receptors define the terminal stage of mature NK cells (stage E). Mouse NK cells migrate into secondary lymphoid organs following the expression of killer cell lectin-like receptor G1 (KLRG1) (stage F). Additional functional classifications of mature NK cells are made using CD27 and CD11b. Source: Abel *et al.*, 2018.

Following the initial stages of mouse NK cell development, the maturation of mouse NK cells may be divided into a 4-stage linear program of functionality (Chiossone *et al.*, 2009). These 4 stages are defined by the expression of CD11b and CD27 and the progressive acquisition of NK cell effector functions (Chiossone *et al.*, 2009) shown in Figure 1.8. It is also possible that the tissue-specific environment of NK cells impacts on their differentiation as NK cell subsets display some tissue preferences, and other immune cell types may also be involved in NK cell maturation. For example, DCs may act as NK cell maturation regulators which influence cellular proliferation and effector function (Lucas *et al.*, 2007; Hochweller *et al.*, 2008).

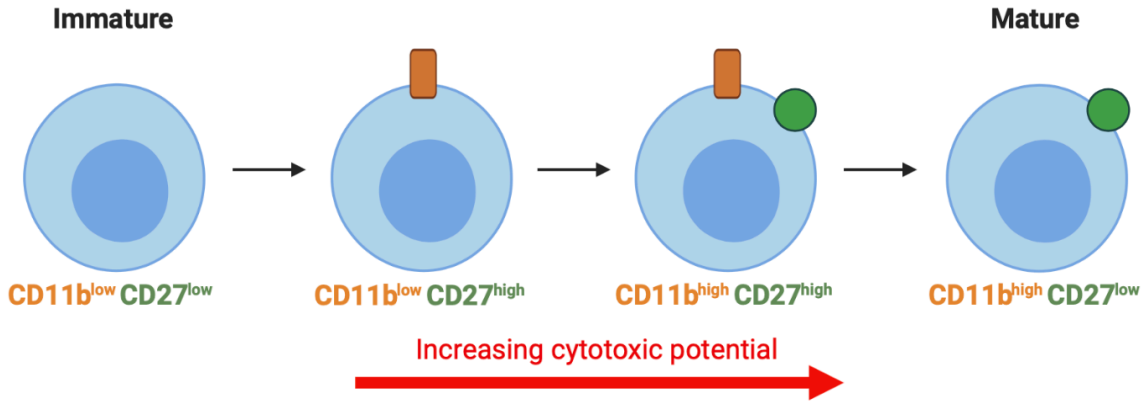

Figure 1.8 The surface density of mouse CD11b and CD27 divides NK cells into developmental stages.
NK cells progress through stages of maturity starting from the most immature ($CD11b^{low}CD27^{low}$) to mature ($CD11b^{high}CD27^{low}$). NK cells gain cytotoxic potential as they progress through the 4 stages of maturation.

Human NK cells follow a similar progression of development from HSCs as mouse NK cells but acquire different receptors at each stage. There are two models for human NK cell development: a linear and a non-linear model shown in Figure 1.9. In the linear model HSCs give rise to progenitors that acquire CD45RA and CD10 before loss of CD10 and the acquisition of CD117. Then progenitor cells are restricted to an NK cell lineage once they have downregulated CD34 and acquired CD117. These immature NK cells then acquire CD94 and represent the $CD56^{bright}$ NK cell subset. Downregulation of CD94 and acquisition of CD16 and killer immunoglobulin-like receptors (KIR) leads to the formation of the $CD56^{dim}$ subset. Human NK cells are broadly divided into immature and mature subsets according to their level of CD56 expression. $CD56^{bright}$ NK cells represent the minor

immature subset found mostly in lymphoid organs (Poli *et al.*, 2009). $CD56^{bright}$ NK cells express few surface KIR molecules, are poorly cytolytic but proliferate vigorously after interacting with activated DCs (Poli *et al.*, 2009). $CD56^{bright}$ NK cells are also believed to differentiate into $CD56^{dim}$ NK cells (Chan *et al.*, 2007; Ouyang *et al.*, 2007; Romagnani *et al.*, 2007). $CD56^{dim}$ NK cells predominate in the blood, have enriched KIR expression and potent killing activity through both natural cytotoxicity and ADCC (Moretta, 2010). The differential expression of chemokine receptors and adhesion molecules on these subsets suggests they have distinct biological roles (Cooper, Fehniger and Caligiuri, 2001). At present, these precise human NK cell subsets have no equivalents in the mouse, although there may be a division of activities between different subsets of murine NK cells (Colucci *et al.*, 2002).

Upon the development of sophisticated analysis strategies, it was found that the human NK cell population is more heterogeneous than previously understood which led to the proposal of a non-linear NK cell development model (Horowitz *et al.*, 2013; Cichocki, Grzywacz and Miller, 2019). However, it remains difficult to ascertain whether less mature populations continually mature and alter their phenotype or whether they are more fixed at their stage of differentiation. Further research is needed to elucidate whether the diversity of the NK cell population is due to the spectrum of maturation states and receptor expression from responses to the environment or whether diversity within the precursor pool dictates NK cell phenotypes (Cichocki, Grzywacz and Miller, 2019).

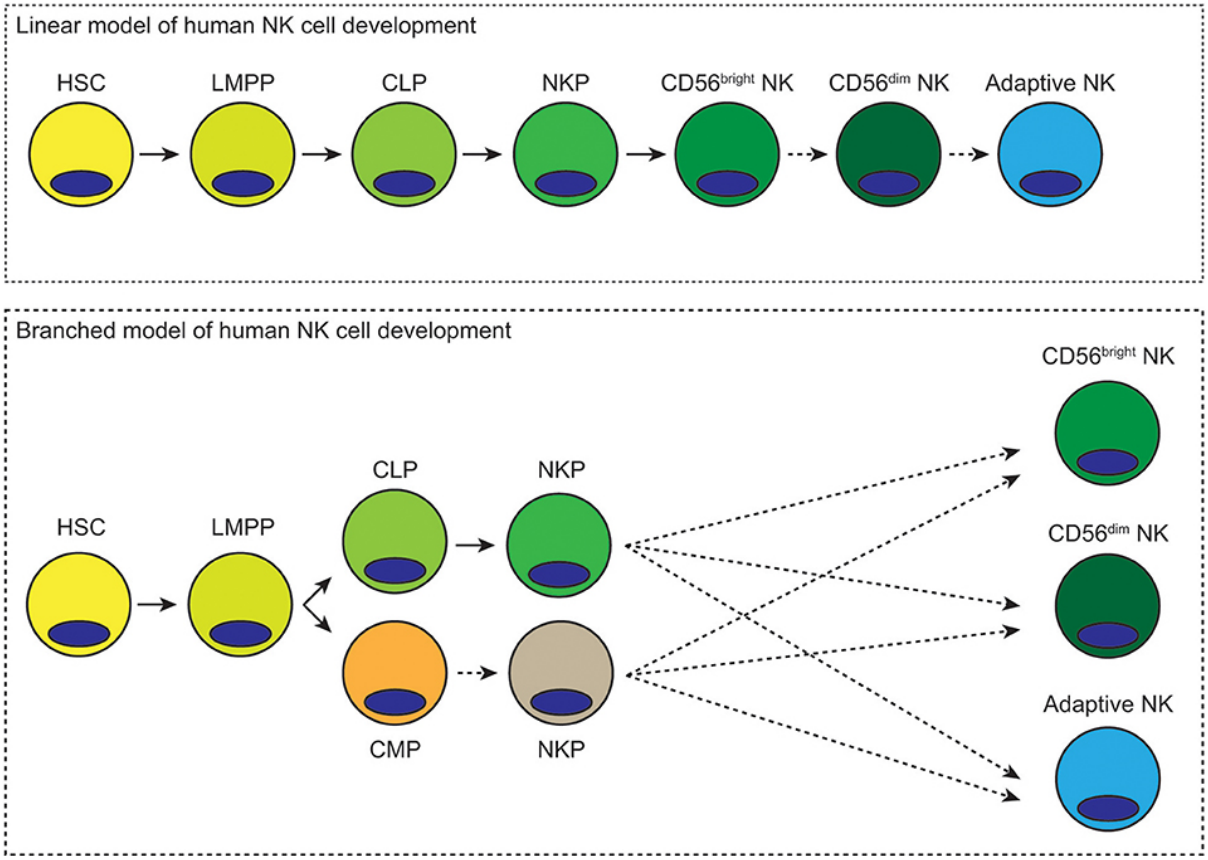

Figure 1.9 Linear and branched models of human NK cell development.
In the linear model of human NK cell development, hematopoietic stem cells (HSC) differentiate into lymphoid-primed multipotent progenitors (LMPP), which then become common lymphocyte progenitors (CLP) before differentiating into NK cell progenitors (NKP). At the precursor stage there is linear commitment to becoming NK cells. These cells first mature into CD56^{bright} NK cells and then CD56^{dim} NK cells. Differentiation into adaptive NK cells (referred to in the text as memory-like NK cells) may subsequently occur in response to viral infection. In the branched model of development, HSCs differentiate into lymphoid primed multipotent progenitors before differentiating into CLPs or common myeloid progenitors (CMP). Either progenitor could give rise to NKPs. The NKPs then differentiate into CD56^{bright}, CD56^{dim}, or adaptive NK cells. Dashed arrows indicate hypothetical routes of development/differentiation. Source: Cichocki, Grzywacz and Miller, 2019.

Human peripheral blood NK cells can be divided into two main subsets: $CD56^{bright}$ and $CD56^{dim}$. Human $CD56^{bright}$ NK cells, the smaller subset in peripheral blood and more abundant subset in tissues (Cooper *et al.*, 2001), have high expression of NKG2A, low expression of CD16 and no KIR expression. $CD56^{bright}$ NK cells respond best to soluble cell-free immunological factors and in response produce high levels of cytokines including IFNγ (Long *et al.*, 2013; Van Acker *et al.*, 2017). Within the $CD56^{bright}$ NK cell subset, there is huge phenotypic and functional heterogeneity due to the existence of tissue-resident $CD56^{bright}$ NK cells in the uterus/maternal decidua, liver, and lymphoid tissues, where these cells exert tissue-specific functions. Uterine $CD56^{bright}$ NK cells express CD49a while liver and lymphoid tissue resident $CD56^{bright}$ NK cells express both CD69 and CXCR6. Increasing evidence indicates that decidual NK cells are vital for preventing miscarriage during pregnancy (Melsen *et al.*, 2016; Guo *et al.*, 2017).

$CD56^{dim}$ NK cells, which predominate in peripheral blood at around 90% of total NK cells, are $CD16^{high}$, express KIRs and/or NKG2A. $CD56^{dim}$ NK cells respond best to receptor binding ligands anchored onto cells and produce high levels of cytokines in response (Fauriat *et al.*, 2010; Long *et al.*, 2013). Increasing evidence suggests that at least a subpopulation of $CD56^{bright}$ NK cells are direct precursors of $CD56^{dim}$ NK cells (as shown in the linear model in Figure 1.9) (Chan *et al.*, 2007; Ouyang *et al.*, 2007; Romagnani *et al.*, 2007). Progressive differentiation of NK cells is associated with phenotypic changes which alter their functional capacity (Goodier, Wolf and Riley, 2020). During differentiation, $CD56^{dim}$ NK cells downregulate cytokine receptors and natural cytotoxicity receptors including NKG2A and express CD16 and KIRs. Highly differentiated NK cells acquire high levels of inhibitory KIRs, inhibitory LIR-1 and a high frequency of NKG2C. In addition, $CD56^{dim}$ NK cells upregulate T-bet and downregulate Eomes transcription factors, which are important for regulating NK cell maturation and function during the last steps of their differentiation. Expression of CD57 on $CD56^{dim}$ NK cells is also associated with a highly differentiated status, poor responsiveness to cytokine-mediated stimulation and greater cytotoxic capacity. Age and infection-related differentiation of intermediately differentiated $CD56^{dim}$ NK cells is believed to induce loss of FcεR1γ adapter protein and the transcriptional regulator proteomyeloid zinc finger molecule expression (Nielsen *et al.*, 2013; Della Chiesa *et al.*, 2016; Bozzano, Marras and De Maria, 2017; Scoville, Freud and Caligiuri, 2017; Goodier, Wolf and Riley, 2020).

Interestingly, CD27 (an important marker for mouse NK cell maturation) appears to be important for delineating NK cell maturation subsets in addition to CD56. It was found that most peripheral blood NK cells were $CD27^{lo}CD56^{dim}$ and the minor $CD56^{bright}$ NK cell subset was $CD27^{hi}$ (Silva *et al.*, 2008). CD11b expression (another important mouse NK cell maturation marker) also appears to be important in identifying developmental subsets in humans. NK cells which express low levels of CD11b have been identified as those at early developmental stages in humans within lymph nodes and tonsils (Freud and Caligiuri, 2006). Thus, the cell-surface density of CD11b and CD27 appears to be relevant in both the mouse and human context for dissecting NK-cell developmental stages. The most functionally mature NK cells are memory-like NK cells which will be discussed in the following section.

1.3.3 The Ly49H receptor and memory-like NK cell responses

1.3.3.1 Ly49 gene family

The Ly49 gene family, encoding C-type lectin Ly49 receptors, contains approximately 20-30 members (Makrigiannis and Anderson, 2000; Lavender and Kane, 2006; Kielczewska *et al.*, 2007; Scarpellino *et al.*, 2007; Belanger *et al.*, 2008; Jonsson *et al.*, 2010; Makrigiannis *et al.*, 2020). Signalling through some Ly49 receptors activates NK cells, invariant NK cells and γδ T-cells, while others induce negative NK cell signalling. All family members have the same basic homodimeric structure, including a stalk and the natural killer receptor domain (NKD) which interacts with ligands. However, the signalling pathways differ between the activating and inhibitory receptors in this family. Inhibitory Ly49 genes have a conserved ITIM domain on their cytoplasmic tails (Belanger *et al.*, 2008). Activating Ly49 genes have a transmembrane arginine residue and associate with the adaptor proteins DAP10 and DAP12 (Tassi *et al.*, 2009). Intracellular signalling is mediated primarily by DAP12 and augmented through DAP10 (Smith *et al.*, 1998; Orr *et al.*, 2009; Tassi *et al.*, 2009). These receptors recognise MHC Class I molecules and other molecules such as viral glycoproteins on the surface of target cells (Yokoyama and Seaman, 1993; Schenkel, Kingry and Slayden, 2013).

1.3.3.2 The Ly49H receptor-m157 interaction

Some Ly49 genes are required for control against viral pathogens. One example is the Ly49H receptor which interacts specifically with MCMV m157 glycoproteins (Arase *et al.*, 2002; H. R. C. Smith *et al.*, 2002; Tripathy *et al.*, 2006; Adams *et al.*, 2007) but is only present in mouse strains resistant to MCMV, such as C57BL/6 mice. The m157 glycoprotein is believed to function as an MHC decoy ligand in other mouse strains which do not contain Ly49H (Arase and Lanier, 2002; Arase *et al.*, 2002). In C57BL/6 mice, Ly49H can recognise m157 presented by infected host cells which initiates intracellular signalling pathways. Studies have demonstrated that the Ly49H-m157 interaction is able to induce NK cell activation, immune targeting and expansion which is highly important to the control of MCMV infection (Gosselin *et al.*, 1999; Daniels *et al.*, 2001; Dokun *et al.*, 2001; Arase *et al.*, 2002; Hamish R.C. Smith *et al.*, 2002). 3 days p.i. a preferential proliferation of Ly49H+ NK cells is evident and by 6 days p.i. the percentage of Ly49H+ NK cells is increased from approximately 50% to 80-90% of the total NK cell expansion (Dokun *et al.*, 2001). The expansion of NK cells in response to MCMV is 'antigen-specific' because infection with MCMV lacking m157 (Δm157) does not drive Ly49H+ NK cell proliferation (Sun, Beilke and Lanier, 2009a). Not only does Ly49H appear to be important for the induction of expansion, but the level of Ly49H expression appears to correspond to the level of expansion individual Ly49H+ NK cells can achieve. This was demonstrated upon tracking Ly49H+ NK cell lineages using heritable fluorescent markers *in vivo* that were adoptively transferred from immunocompetent mice (Grassmann *et al.*, 2019).

During the primary response to MCMV, Ly49H+ NK cells are critical for protective antiviral immunity. Antagonising Ly49H reveals loss of MCMV infection control similar to that observed upon complete loss of NK cells (Brown *et al.*, 2001; Bubić *et al.*, 2004). The Ly49H-m157 interaction has also highlighted important compartment-specific NK cell responses to MCMV. In the liver, NK cells predominantly control MCMV infection through IFNγ secretion (Orange *et al.*, 1995; Tay and Welsh, 1997b). In the spleen, the NK cell-mediated control of MCMV is more dependent on perforin (Tay and Welsh, 1997b). The Cmv-1 resistance gene encodes the Ly49H receptor and maps closely to genes regulating NK cell cytotoxic function (Brown *et al.*, 2001; Daniels *et al.*, 2001; Lee *et al.*, 2001; Scalzo *et al.*, 2005). It has been shown that the Cmv-1 gene locus confers NK cell-dependent resistance to MCMV in the spleen but not the liver (Scalzo *et al.*, 1990). In

addition, Ly49H-dependent MCMV control is specifically mediated through cytotoxicity but not IFNγ production which predominates in the liver (Parikh *et al.*, 2015). The compartmental specificity of Ly49H+ NK cell driven MCMV resistance highlights that NK cell responses to MCMV should be differentially dissected between spleen and liver tissues.

1.3.3.3 Ly49H+ NK cell memory-like responses

The immune system has been traditionally segregated into innate and adaptive arms with immunological memory ascribed only to the adaptive system, mediated by T-cells and B-cells. However, more recent evidence indicates that NK cells have adaptive features of immunological memory. The key determinants of immunological memory are the specific interaction between host and pathogen, the expansion of specific immune cells and their maintenance, allowing them to activate upon a second encounter with their cognate pathogen. The specific interaction between Ly49H on NK cells and m157 glycoproteins (Arase *et al.*, 2002; H. R. C. Smith *et al.*, 2002; Adams *et al.*, 2007), expansion of Ly49H+ NK cells and the antiviral effector function of these cells highlights the existence of memory-like NK cells. Furthermore, evidence suggests that Ly49H+ NK cell pools are maintained long-term and can mount secondary immune responses upon re-challenge. The stages of the Ly49H+ NK cell response to infection is shown in Figure 1.10. At each stage, the gene expression profile of these cells is unique therefore the transcriptional profile differs between resting, effector and memory-like NK cells (Sun *et al.*, 2011).

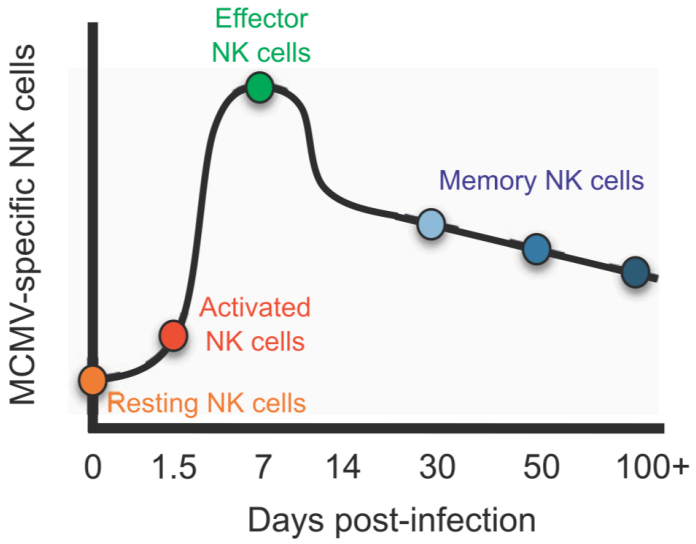

Figure 1.10 NK cell expansion and contraction post-MCMV infection.
During MCMV infection, resting Ly49H+ NK cells become activated and undergo an expansion phase resulting in the generation of more Ly49H+ effector cells. The expansion phase is followed by the contraction of effectors resulting in long-lived memory-like NK cells which remain present months after the initial infection. Source: Sun et al. J. Immunol, 2011.

To understand if memory-like NK cells are maintained long-term, adoptively transferred memory-like NK cells in Ly49H deficient mice post-MCMV infection were observed. The memory-like cells were shown to self-renew but at a slow rate compared to naïve NK cells. The slow rate of renewal may enable greater longevity of memory-like NK cells. Since the rate of renewal is slow, absolute numbers of NK cells steadily decline and in the absence of a secondary infection memory-like NK cells are difficult to detect 6 months post-MCMV infection (Sun et al. 2010). Despite this, 6 months represents a relative life span equivalent to decades in humans (Sun *et al.*, 2011), therefore, memory-like NK cells may also be long lived in humans.

The final key determinant of immunological memory is whether the portion of specific cells retained long-term can re-activate upon a second exposure. The existence of a pool of memory-like NK cells was demonstrated using an adoptive transfer system whereby small numbers of Ly49H+ NK cells proliferated 100-1000-fold following MCMV infection in lymphoid and non-lymphoid tissues. This self-renewing population underwent secondary and tertiary expansions following several rounds of adoptive transfer and virus infection (Sun et al. 2010). *Ex vivo*, NK cells recovered several months after infection and, when

stimulated with plate-bound antibodies and m157-expressing target cells, showed improved virus control compared to NK cells from naïve mice (Sun, Beilke and Lanier, 2009a). These memory-like NK cells produced more IFNγ and exhibited enhanced degranulation upon stimulation. Furthermore, log-fold lower antigen exposed Ly49H+ NK cells were more effective at controlling HCMV compared to naïve NK cells exposed to more antigen (Sun, Beilke and Lanier, 2009a). In addition to the Ly49H-m157 interaction, cytokines can induce NK memory-like responses. Upon *in vitro* exposure to inflammatory cytokines such as IL-12 and IL-18, the pool of resulting NK cells produce more IFNγ than unstimulated NK cells several weeks after adoptive transfer (Cooper *et al.*, 2009). In summary, memory-like NK cells can persist far longer than previously estimated (Koka *et al.*, 2003; Prlic *et al.*, 2003; Jamieson *et al.*, 2004), producing robust responses upon re-encounter, and thus can contribute alongside T-cell and B-cell responses to immunological memory.

1.3.3.4 Human memory-like NK cell responses

There is currently no known direct human counterpart to the Ly49H-m157 interaction. Evidence suggests the possibility of a viral ligand to the human NKG2C receptor. The specificity of this interaction was demonstrated *in vitro* by adding a blocking anti-NKG2C antibody to a coculture containing peripheral blood lymphocytes (PBLs) derived from a HCMV+ donor and Towne HCMV-infected fibroblasts which resulted in loss of NKG2C+ NK cell expansions (Gumá, Budt, *et al.*, 2006). Evidence from HCMV seropositive healthy and HIV-infected adults and children demonstrates that expansions of NKG2C+ NK cells are associated with HCMV infection (Gumá, Cabrera, *et al.*, 2006; Mela, C., Goodier,M,R., 2007; Monsiváis-Urenda *et al.*, 2010; Goodier *et al.*, 2014). There is also evidence suggesting the activation and expansion of NKG2C+ NK cells are altered according to the HCMV UL40 derived peptide introduced into HLA-E therefore NKG2C+ NK cell expansions represent a host adaptation to counteract evasion of the immune system by HCMV (Hammer *et al.*, 2018). NKG2C+ NK cells are retained after acute infection and remain elevated after therapeutic intervention and also in asymptomatic individuals (Guma *et al.*, 2004). Furthermore, a report describing a HCMV-infected immunodeficient infant highlighted the prolific expansion of NKG2C+ NK cells which was a striking 80% of total NK cells (Kuijpers *et al.*, 2008). The expansion observed in this infant is comparable to the 100-fold expansion observed for mouse Ly49H+ NK cells during MCMV infection (Kuijpers *et al.*, 2008). While evidence indicates the importance of the NKG2C interaction,

it may not be crucial to HCMV responses. Approximately 4% of the human population carry a homozygous deletion for the KLRC2 gene which encodes NKG2C. Individuals without NKG2C can still mount a response to HCMV through other activating NK cell receptors (Guma *et al.*, 2004; Liu *et al.*, 2016). However, NKG2C may be required for optimal HCMV responses as NKG2C deletions are associated with reduced NK cell differentiation and maturation (Goodier *et al.*, 2014). Other markers of potential human NK cell memory-like responses exist. A population of NK cells expressing tissue residency markers (CD69, CD62L, CXCR6) exhibit recall responses to varicella-zoster virus (VZV) and appear to be retained long-term (Nikzad *et al.*, 2019). In summary, evidence suggests that the NKG2C receptor on human NK cells may represent a functional human counterpart to the MCMV-specific Ly49H receptor in mice and is used to investigate human NK cell memory.

Activating KIRs in humans, in addition to NKG2C, are also important contributors to NK cell driven CMV infection control and possibly memory-like NK cell responses in humans. Although structurally different, KIR receptors in humans function similarly to Ly49 receptors in mice and they both recognise MHC class Ia (Belanger *et al.*, 2008; Middleton and Gonzelez, 2010; Rahim *et al.*, 2014; Pende *et al.*, 2019). Epidemiological studies have indicated that activating KIRs are associated with reduced risk for HCMV reactivation in an allogeneic stem cell transplant setting (Cook *et al.*, 2006). In support of this, a study of over 200 healthy participants showed that the activating KIRs including KIR2DS4, KIR2DS2 and KIR3DS1 contribute to NK cell expansions upon HCMV infection (Beziat *et al.*, 2013). This evidence suggests activating KIRs in humans are important for developing the initial NK cell response to infection but further links to memory-like NK cell functions have not been elucidated. However, KIR expression is highly variable between individuals due to variable gene content, polymorphisms and transcription regulation (Cichocki, Miller and Anderson, 2011), therefore, the activating KIRs described are not present in all individuals. Consequently, activating KIRs may contribute, but are unlikely to be necessary, for HCMV-specific NK cell memory.

1.3.4 NK cell cytotoxicity

NK cell cytotoxicity is critical for controlling virally infected cells, tumour cells and senescent cells in ageing (Topham and Hewitt, 2009; Parikh *et al.*, 2015). There are two pathways of cytotoxicity which NK cells use to induce target cell death. Firstly, degranulation which is controlled by cell membrane-bound receptors such as NKG2D and DNAM1 or CD16 binding to Fc regions on target cell-bound antibodies (Bryceson *et al.*, 2006; Bryceson, Ljunggren and Long, 2009). Both pathways induce damaging proteins such as perforin and granzymes secreted by exocytosis towards the target cell membrane to initiate cell death processes (Barry and Bleackley 2002; Loh et al. 2005; Chowdhury and Lieberman 2008). Secondly, caspase dependent apoptosis where death receptors such as FasL, CD95 and TRAIL are induced by receptors on NK cells (Kashii *et al.*, 1999; Kayagaki *et al.*, 1999; Takeda *et al.*, 2002). In this section I will focus on NK cell degranulation through granule exocytosis. Human NK cells are armed for cytotoxicity with preformed lytic granules in the resting state which enables rapid deployment of their effector responses. The formation of a functional NK cell lytic synapse is divided into the following three main stages: recognition, effector stages and termination (Orange, 2008). Each of these stages has multiple steps to ensure the complex and tightly controlled process precisely induces cytotoxicity without damaging healthy cells (Orange, 2008; Krzewski and Coligan, 2012). NK cell degranulation is important for destroying virally infected cells and tumour cells as well as other functions including reducing senescent cells in ageing.

1.3.4.1 Degranulation mechanism

1.3.4.1.1 NK cell recognition

The first stage of degranulation involves NK cell recognition of a target cell (Orange, 2008; Krzewski and Coligan, 2012). NK cells establish a close association with the target which may happen by chance or be intentionally induced through chemotactic signals (Grégoire *et al.*, 2007). Early cell association signals may include selectins which lead to NK cell activation (Chen *et al.*, 2005) before firm adhesion receptor-ligand interactions are facilitated by higher affinity interactions including the integrins, such as LFA-1 (CD11a/CD18), on NK cells which cluster rapidly at the NK-cell synapse (Davis *et al.*, 1999; Vyas *et al.*, 2001; Orange *et al.*, 2003). Integrin-induced signalling is essential for the activation and maturation of the synapse but cannot induce degranulation alone (Barber,

Faure and Long, 2004; Bryceson *et al.*, 2005; Kumar, 2018). To induce NK cell degranulation through the formation of an activating immunological synapse, potent NK cell signalling from activating receptors (such as NKG2D, DNAM-1, 2B4, CD2) is required in combination with LFA-1, or, NK cell signalling through CD16 without a requirement for LFA-1 (Bryceson *et al.*, 2006; Kumar, 2018). Alternatively, if inhibitory NK cell receptor signalling overrides activating signals, such as through engagement of inhibitory KIR receptors, then an inhibitory immunological synapse forms (Davis, 2002). An immunological synapse is defined as the intentional arrangement of molecules in an immune cell at the interface with another cell (Davis, 2002; Orange, 2008). NK cells have the unique capability to form many types of immunological synapses such as NK cell activating, inhibitory or regulatory synapses which have specialised functions. These synapses also vary in the spatial organisation between themselves and may differ from immunological synapses formed by other cells of the immune system (Krzewski and Strominger, 2008). If the balance of NK cell receptor signalling enables activation towards a target cell, an activating immunological synapse forms which is the first step towards granule induced cytotoxicity. In contrast, if inhibitory NK cell receptors engage and override the activating signal, an NK cell inhibitory synapse forms to directly interfere with degranulation (Masilamani *et al.*, 2006; Endt *et al.*, 2007).

1.3.4.1.2 Effector stage pSMAC protein accumulation

During the effector stage of degranulation, the NK cell immunological synapse begins to mature. At the initial effector stage, actin filaments accumulate, along with adhesion molecules such as LFA-1 and Mac-1 (CD11b/CD18) and activating receptors such as CD2. These proteins form an actin ring at the peripheral supramolecular activation cluster (pSMAC) highlighted in Figure 1.11 (Vyas *et al.*, 2001; Orange *et al.*, 2003; Roda-Navarro *et al.*, 2004; Liu *et al.*, 2009). At the pSMAC, these proteins mediate the formation of a tight conjugate between the cells and are important for generating activation signals. For example, LFA-1 signalling induces phosphorylation of Src, LAT, SLP76, ZAP70, Vav-1, PKCs, ERK1/2, or JNK signalling molecules (Riteau, Barber and Long, 2003; Perez *et al.*, 2004; Chen *et al.*, 2006) which induce actin reorganisation (Orange *et al.*, 2003; Chen *et al.*, 2007; Krzewski and Strominger, 2008), and are important for lytic granule polarisation to the immunological synapse (Barber, Faure and Long, 2004; Perez *et al.*, 2004; Bryceson *et al.*, 2005). Other molecules that accumulate at the pSMAC and contribute to actin polymerisation include talin, ezrin-radixin-moesin proteins, and Wiskott-Aldrich syndrome

protein (WASP) (Vyas *et al.*, 2001; Orange *et al.*, 2002; Ramoni *et al.*, 2002; McCann *et al.*, 2003). Actin polymerisation and accumulation at the NK cell immunological synapse is indispensable for NK cell cytotoxicity (Orange *et al.*, 2002, 2003; Barber, Faure and Long, 2004).

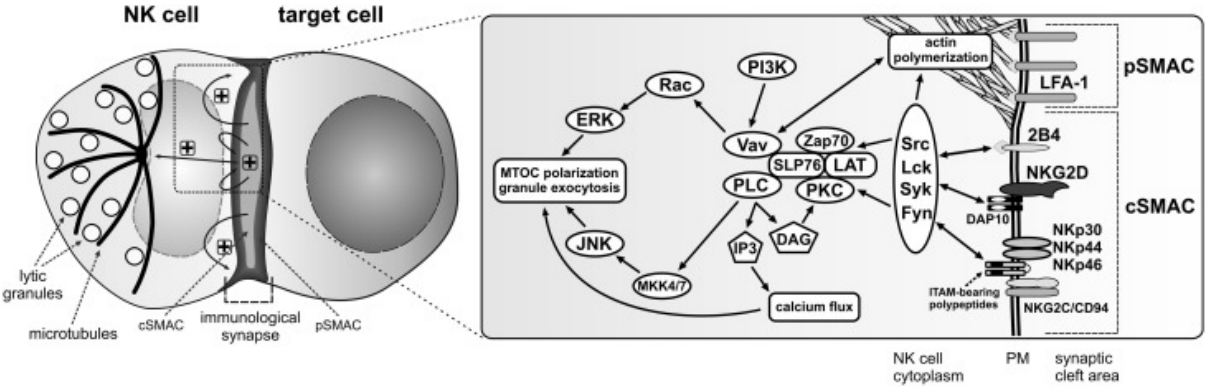

Figure 1.11 Activation signals for lytic granule polarisation in NK cells.
When NK cells encounter a susceptible target cell, they form an activating immunological synapse. Adhesion molecules including LFA-1 move to the peripheral supramolecular activation cluster (pSMAC). NK cell activating receptors move into the central supramolecular activation cluster (cSMAC). NK cell activating receptor engagement to their ligands on the target cell induces phosphorylation of membrane proximal signalling molecules leading to activation of downstream signalling pathways. Positive feedback loops induce robust actin polymerisation at the periphery of the synapse and polarisation of the MTOC and lytic granules to the immunological synapse where granules are released by exocytosis. The diagram shows only selected molecules and the drawings are not to scale. Source: Krzewski and Coligan, 2012.

1.3.4.1.3 Effector stage cSMAC protein accumulation

As adhesion and co-stimulatory signalling molecules are directed to the pSMAC, activating receptors accumulate at the central supramolecular activation cluster (cSMAC) within the immunological synapse (Vyas *et al.*, 2001; Vyas, Maniar and Dupont, 2002). This leads to recruitment of: Src, Lck, Syk, Fyn, ZAP70, and PKC kinases, scaffolding proteins like SLP76, LAT, BLNK, as well as other signalling molecules such as Vav-1, Grb2, PLCγ, PI3K, Pyk2, Rap1, or CrkL at the cSMAC (Sancho *et al.*, 2000; Vyas *et al.*, 2001; Vyas, Maniar and Dupont, 2002; Riteau, Barber and Long, 2003; Upshaw *et al.*, 2006; Segovis *et al.*, 2009). These proteins are integral to the signal transduction of two major pathways involved in cytolytic responses: phosphoinositide 3-kinase (PI3K)-ERK2 (Chen *et al.*,

2006, 2007) and phospholipase C (PLC)γ–c Jun N terminal kinase (JNK) (Li *et al.*, 2008). Signals from these pathways initiate microtubule organising centre (MTOC) polarisation, the first stage of granule exocytosis shown in Figure 1.12.

1.3.4.1.4 Effector stage MTOC polarisation

In the first stage of exocytosis, lytic granules in human NK cells move along microtubules, translocate with the MTOC and move with the MTOC to the cSMAC in a dynein-dynactin complex-dependent manner (Mentlik *et al.*, 2010; Pachlopnik Schmid *et al.*, 2010). MTOC polarisation towards the cSMAC relies on ERK phosphorylation, VAV1 activation and PYK2 (protein tyrosine kinase 2) activity (Sancho *et al.*, 2000; Chen *et al.*, 2006; Graham *et al.*, 2006). Cdc42-interacting protein-4 (CIP4) localisation to the MTOC, which links microtubules and the actin network through its ability to bind microtubules and WASP, is also required to complete MTOC polarisation (Banerjee *et al.*, 2007).

1.3.4.1.5 Effector stage granule transport

The granules then switch from microtubules to the filamentous actin (F-actin) network at the immunological synapse. Although actin accumulation and reorganisation is required for MTOC and lytic granule polarisation, a discrete region of the F-actin network must be disassembled at the cSMAC to create openings for the granules pass through towards the plasma membrane (Vyas *et al.*, 2001; Orange *et al.*, 2003; Roda-Navarro *et al.*, 2004; Andzelm *et al.*, 2007). Granules move through the F-actin mesh, directed by essential activity of the actin motor protein myosin IIA, towards the plasma membrane where they are able to dock (Andzelm *et al.*, 2007; Sanborn *et al.*, 2009, 2011).

1.3.4.1.6 Docking and priming proteins

The secretion of lytic granules also requires docking and priming proteins including Rab27a, Munc13-4, and Munc18-2. In comparison to neural vesicle exocytosis, very few docking and priming proteins have been identified for cytotoxic lymphocyte exocytosis (Sudhof, 2004). Rab27a and Munc13-4 are only present on lytic granule surfaces where there is engagement of different NK cell activating receptors (Wood *et al.*, 2009). Rab27a is a small GTPase required for several functions in lytic granule exocytosis such as retention and cytoskeleton-dependent directional movement of granules towards the plasma membrane as well as recruitment of Munc13-4 to lytic granules (Wood *et al.*, 2009; Liu, Meckel and Long, 2010). Munc13-4 contains two calcium binding domains to respond

to calcium flux, but its function has not been fully elucidated in NK cells. Calcium is essential for lytic granule release but not for granule polarisation. Importantly, granule exocytosis in immune cells is Ca^{2+} dependent; however, the critical Ca^{2+} sensor remains unknown. This is in contrast to the highly established role of synaptotagmin-1 as a Ca^{2+} sensor for neurotransmitter exocytosis in neurons (Geppert *et al.*, 1994; Bin *et al.*, 2018). Calcium release is induced by Inositol-(1,4,5)-triphosphate from intracellular stores such as the ER. Aggregation of the ER calcium sensor STIM1 leads to activation of the ORAI1 calcium channel and calcium influx from the extracellular space in a process known as store operated calcium entry (SOCE) (Feske, 2010; Maul-Pavicic *et al.*, 2011).

1.3.4.1.7 Secretory lysosome release

The last stage of lytic granule release is the fusion of secretory lysosomes with the plasma membrane. Membrane fusion is mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, including Q-SNARE and R-SNARE protein groups, which form a complex at the lytic granule membrane (Jahn and Scheller, 2006). SNARE proteins include syntaxin 11 (Arneson *et al.*, 2007; Bryceson *et al.*, 2007) and VAMP-7 (Marcet-Palacios *et al.*, 2008). The proteins which make up SNARE protein subgroups are not fully defined in NK cells but functional SNARE complexes require one R-SNARE with three (Qa, Qb, and Qc) or two (Qa, Qbc) Q-SNARE proteins (Jahn and Scheller, 2006). Once the SNARE complex is assembled, a fusion pore forms between the lytic granule and the plasma membrane. NK cells can form fully opened, transient and incomplete pores. Complete fusion of lytic granules leads to rapid granule release. Partial fusion pore opening leads to minimal content release and may be important in recycling granule membranes (Liu *et al.*, 2011). It has also been postulated that NK cells only release a subset of the polarised lytic granules following activation (Rak *et al.*, 2011) which may enable killing of several targets (Wiedemann *et al.*, 2006; Bhat and Watzl, 2007). The release of granules is also limited by the available openings in the actin meshwork which allow granules to pass through an estimated 10% of the synapse area (Brown *et al.*, 2012).

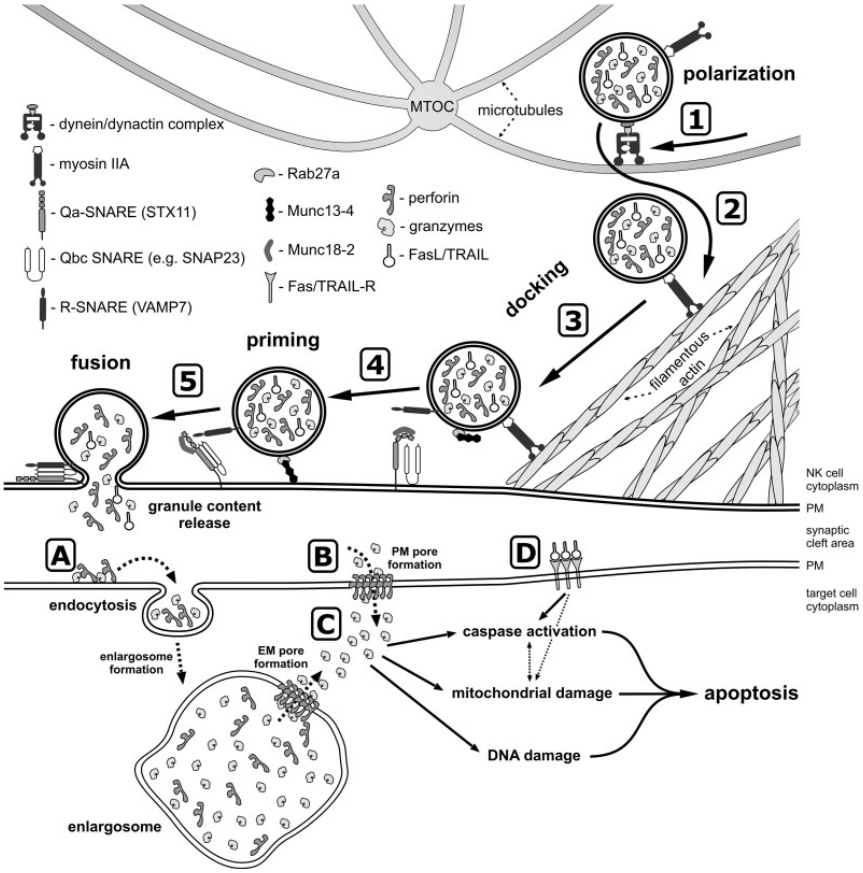

Figure 1.12 A model of lytic granule exocytosis in human NK cells.
After NK cells engage with target cells which induces signalling cascades (not shown), the lytic granules move along microtubules towards the MTOC in a dynein-dynactin dependent manner (1). The MTOC and the granules then polarise towards the immunological synapse, where granules move from microtubules to the filamentous actin (F-actin) network (2) and then navigate through the cortical F-actin meshwork driven by activity of the motor protein myosin IIA (3). Once the lytic granules are near the plasma membrane (PM), they can dock due to activity of Rab27a and Rab27a-mediated recruitment of Munc13-4, as well as through recognition of syntaxin 11 (STX11) and Munc18-2 possibly by R-SNARE proteins at the membrane. Granules which have docked onto the plasma membrane are primed (4) by Munc13-4 in response to an increase in calcium (not shown), likely by the Munc13-4 mediated switch of STX11 to an open conformation (by removal of Munc18-2) and/or by Munc13-4 forming a bridge between the granule membrane and the plasma membrane. Lastly, the granule-associated R-SNARE proteins (such as VAMP7) form a complex with Q-SNARE proteins on the plasma membrane (such as STX11 and SNAP23) (5), enabling the fusion of vesicles with the plasma membrane and granule content release into the synaptic cleft. Perforin and granzyme entry into target cells may occur by (A) endocytosis where perforin mediates pore formation within the endosome-like enlargeosome membrane following internalisation. This enables granzyme release into the cytosol of the target cell. The plasma membrane pore formation model (B) suggests perforin oligomerises in the plasma membrane, disrupting its integrity and thereby allowing granzymes to enter the target cell. Once granzymes have access to the cell cytosol (C), granzymes induce apoptosis through caspase activation, mitochondrial damage, and DNA fragmentation. Also, FasL and TRAIL from lytic granules bind their receptors on the target cell surface (D) which leads to apoptosis. Source: Krzewski and Coligan, 2012.

1.3.4.1.8 Target cell death

Following lytic granule release, in the presence of physiological Ca^{2+} levels and neutral pH, perforin can insert itself into a membrane to generate pores. Perforin forms unstable or stable ring-shaped pores (Praper, A. Sonnen, *et al.*, 2011). Perforin activity is essential for granzyme delivery into target cells (Keefe *et al.*, 2005; Thiery *et al.*, 2011). Granzymes either enter the target cell cytosol through perforin pores (Voskoboinik *et al.*, 2010; Praper, A. Sonnen, *et al.*, 2011) or perforin and granzymes are internalised by endocytosis. Evidence exists for both paradigms (Keefe *et al.*, 2005; Thiery *et al.*, 2010; Praper, A. F.-P. Sonnen, *et al.*, 2011). Nonetheless granzyme delivery to the target cell cytosol results in caspase-dependent and independent mechanisms which generate damaging reactive oxygen species, mitochondrial damage, and DNA fragmentation leading to enforced apoptosis and cell death. For example, caspase dependent mechanisms involve the direct activation of caspases by granzyme B (Barry *et al.*, 2000; Adrain, Murphy and Martin, 2005). Caspase independent mechanisms include granzyme B and granzyme K cleavage of the pro-apoptotic protein Bid which permeabilises the mitochondrial membrane (Adrain, Murphy and Martin, 2005; T Zhao *et al.*, 2007a). Granzymes are also able to induce DNA damage by activating nucleases (Chowdhury *et al.*, 2006; T Zhao *et al.*, 2007b) and cleaving DNA repair enzymes (Lu *et al.*, 2006; Zhu *et al.*, 2006, 2009).

1.3.4.1.9 Termination of the immunological synapse

Compared to initiation and formation of the immune synapse in NK cells, much less is known about signalling events important for the maintenance of the immune synapse and detachment after target cell death. Once the lytic granules are released, a termination stage believed to involve a period of inactivity and downregulation of the activating receptors including NKG2D occurs (Sivori *et al.*, 2000; Sandusky, Messmer and Watzl, 2006). Activating receptors at the SMAC persist at late times after conjugation, suggesting that receptor downregulation at the SMAC occurs relatively late (Orange *et al.*, 2003; Roda-Navarro *et al.*, 2004). Then NK cells detach and recycle their cytolytic capacity. The detachment process may be important for immune regulation as reduced cytotoxic function in patients with immune disorders has been linked to overproduction of inflammatory cytokines leading to inflammatory damage (Jenkins *et al.*, 2015). Detachment may also be important for the regulation of serial killing. The number of target cells an NK cell is able to kill may be up to 16 consecutive targets but this varies between studies according to experimental setup (Bhat and Watzl, 2007; Choi and Mitchison, 2013; Vanherberghen *et*

al., 2013). Of the cells able to complete serial kills, some show killing capacity immediately after detachment (Bhat and Watzl, 2007) and those which undergo downregulation after their first target cell is destroyed need to actively renew their functional capacity before forming new synapses (D. Liu *et al.*, 2005; Li *et al.*, 2011; Vanherberghen *et al.*, 2013). NK cells have also been shown to form more than one activating immunological synapse at the same time, resulting in accelerated detachment of the first synapse (Netter, Anft and Watzl, 2017). The mechanisms for renewing NK cell cytotoxic function are unknown but the ligation of the activating receptors appears to be important. For example, activating NKp30 induces NF-κB (Pandey *et al.*, 2007), which is a transcription factor for perforin (Zhou *et al.*, 2002); and NKG2D and CD16 have been shown to control the amount of perforin secretion, NK cell survival and serial target engagement (Srpan *et al.*, 2018). Further work is needed to fully elucidate the mechanisms involved in detachment.

1.3.4.1.10 Lytic granule contents

Lytic granules have a membrane bilayer which separates their contents from the cell cytoplasm. NK cell lytic granules contain a variety of enzymes typical for lysosomes as well as proteins unique to lytic granules (Bossi and Griffiths, 2005; Krzewski and Coligan, 2012). These are formed though fusion of endosomes with specialised secretory machinery from the trans-Golgi network. As a result, lytic granules have dual functions of secretory and destructive capacity. Adaptor protein 3 (AP3) regulates the addition of lysosomal proteins from the Golgi and lysosomal trafficking regulator (LYST) regulates lysosomal fusion as well as a potential role in protein trafficking (Badolato and Parolini, 2007; Sepulveda *et al.*, 2015). Then, Mannose-6-phosphate dependent and independent mechanisms control the transport of lytic granule contents from the trans-Golgi network (Reczek *et al.*, 2007). Proteins specific to lytic granule contents include: perforin, granzymes, granulysin and antimicrobial peptides (Blott and Griffiths, 2002). Lytic granules in NK cells are heterogeneous which may be due to different stages of granule development (Neighbour, Huberman and Kress, 1982; Krzewski and Coligan, 2012). Lytic granules are also multivesicular in nature and include dense bodies surrounded by layers of vesicles (Neighbour, Huberman and Kress, 1982; Krzewski and Coligan, 2012). The multivesicular bodies contain cathepsins and other lysosomal hydrolases and are mostly devoid of cytotoxic enzymes whereas the dense cores of lytic granules contain lattices of the chondroitin-sulfate proteoglycan serglycin (which gives the cores characteristic electron density), as well as perforin and granzymes (Burkhardt *et al.*, 1990).

Granzymes are a family of structurally homologous serine proteases (which include GrA, GrB, GrH, GrK, GrM in humans and GrA-K, GrM and K in mice) and each one has distinct specificities for proteolytic substrates which enable the initiation of cell death processes (Trapani *et al.*, 2000; Trapani, 2001; Mahrus and Craik, 2005). Within granules, granzymes become active by interacting with cathepsin C and cathepsin H (Smyth, McGuire and Thia, 1995; Meade *et al.*, 2006; D'Angelo *et al.*, 2010), and are packed tightly through complexing with serglycin (Metkar *et al.*, 2002; Raja *et al.*, 2002). Apoptosis induced by granule exocytosis is critically dependent on granzyme B as the main cytotoxic effector molecule (Heusel *et al.*, 1994; Simon *et al.*, 1997; Pardo *et al.*, 2009). It is believed that the cytotoxic activity of granzyme B is induced either directly through activation of executioner caspases (caspase-3 and caspase-7) (Darmon, Nlcholsont and Bleackley, 1995; Thomas *et al.*, 2001; Metkar *et al.*, 2003); or caspase activation is initiated indirectly through mitochondrial permeabilization promoted by granzyme B mediated cleavage of Bid into its active form tBid (Alimonti *et al.*, 2001; Pinkoski *et al.*, 2001; Waterhouse *et al.*, 2005). Granzyme B can induce reactive oxygen species (ROS) production outside the mitochondria through caspase dependent activation of NADPH oxidase(s) (Pardo *et al.*, 2009). Other granzymes show less cytotoxicity or no cytotoxicity at all and therefore have other functions. Human granzyme A can induce human monocytes to secrete IL-1 and TNF in a caspase-1 dependent manner. Other non-cytotoxic functions of granzymes (Froelich, Pardo and Simon, 2009) have been uncovered to control viruses (Knickelbein *et al.*, 2008; Romero and Andrade, 2008) or regulate extracellular matrix dependent processes (Choy *et al.*, 2004, 2005; Buzza *et al.*, 2005). For example, non-cytolytic control of HCMV has been demonstrated through direct granzyme degradation of HCMV IE1 and IE2 proteins (Shan *et al.*, 2020).

The pore-forming protein perforin functions to facilitate granzyme entry into target cells as well as directly lysing cells (Lopez *et al.*, 2013). It is constitutively transcribed in human NK cells (Nakata *et al.*, 1992; Salcedo *et al.*, 1993); however, the immature $CD56^{bright}$ NK cells have lower levels of perforin compared to mature $CD56^{dim}$ NK cells. Once transported into lytic granules, perforin is cleaved by cathepsin L to induce its full activity (Konjar *et al.*, 2010) and the granule acidic environment, as well as interaction with proteins including serglycin, suppresses its activity (Fraser *et al.*, 2000; Metkar *et al.*, 2011). The pro-apoptotic molecules FasL and TRAIL localise to NK cell lytic granules (Monleón *et al.*, 2001; Schmidt *et al.*, 2009; Ghosh *et al.*, 2010).

Granulysin has two isoforms which differ in their function and cellular location. The 15kDa isoform is not cytotoxic but is important for the differentiation and activation of DCs (Tewary *et al.*, 2010; Clayberger *et al.*, 2012). This isoform is present in vesicles negative for perforin or granzymes (Clayberger *et al.*, 2012). Upon cleavage of the 15kDa isoform, the second isoform at 9kDa is generated. The 9kDa isoform has broad cytotoxic function towards bacteria, fungi, parasites and tumours and localises to lytic granules (Krensky and Clayberger, 2009; Clayberger *et al.*, 2012). In addition to the aforementioned functions, both isoforms of granulysin can act as chemoattractants for T-cells, monocytes and NK cells (Deng *et al.*, 2005; Tewary *et al.*, 2010) and induce expression of chemokines (such as CCL2, CCL3, CCL8 and RANTES) and pro-inflammatory cytokines (such as IL-1, IL-6, IL-10 and IFNα) (Deng *et al.*, 2005). Small peptides including peptide LL-37 (cathelicidin) and defensins 1-3 are also present within NK cell lytic granules and contribute to antimicrobial effects (Agerberth *et al.*, 2000; Obata-Onai *et al.*, 2002; Chalifour *et al.*, 2004).

Lysosomal membrane proteins also have roles in controlling signalling processes essential for degranulation (Saftig and Klumperman, 2009). The lysosomal associated membrane protein-1 (CD107a or LAMP-1) is one of the most abundant proteins within the lysosomal membrane (Winchester, 2001) along with LAMP-2 and lysosomal integral membrane protein (LIMP)-2. The functions of CD107a, LAMP-2 and LIMP-2 are largely poorly described. CD107a has a highly glycosylated luminal side and a short tail exposed to the cell cytoplasm (Terasawa *et al.*, 2016). Once the outer membrane of lytic granules merge with the NK cell plasma membrane during degranulation, NK cells express CD107a on their surface in clusters, which may be essential for protecting NK cell membranes from degranulation (Cohnen *et al.*, 2013). As CD107a accumulates at the immunological synapse, CD107a is widely used as a marker of NK cell degranulation (Alter, Malenfant and Altfeld, 2004; Aktas *et al.*, 2009).

1.3.4.2 Dysfunctional lytic granule release and disease

The disruption of several proteins involved in lytic granule release has been implicated in many human diseases associated with impaired infection control. A recessive mutation in LYST, which regulates lysosomal fusion, is the cause of Chediak-Higashi syndrome (CHS)

(Durchfort *et al.*, 2012). CHS patients exhibit enlarged lysosomal compartments, variable defects in T-cell mediated cytotoxicity (Karim *et al.*, 2002; Chiang *et al.*, 2017) and uniformly defective NK cell cytotoxicity (Jessen *et al.*, 2011). Wiskott-Aldrich syndrome (WAS) results from a haematopoietic-cell-specific defect in actin reorganisation and cell signalling due to WASP deficiency. Reduced WASP expression or abnormal WASP leads to decreased cytolytic capacity in NK cells (Orange *et al.*, 2002; Gismondi *et al.*, 2004) as WASP is required for the early effector stage of the NK cell lytic synapse. Therefore, patients with WAS syndrome have increased susceptibility to herpesviruses (Sullivan *et al.*, 1994). Mutations in Munc13-4, Munc18-2, and syntaxin-11 are associated with a fatal immune disorder: familial hemophagocytic lymphohistiocytosis (FHL) type 3 (FHL3), FHL4, and FHL5 respectively (Feldmann *et al.*, 2003; Bryceson *et al.*, 2007; Côte *et al.*, 2009; zur Stadt *et al.*, 2009; Cetica *et al.*, 2010; Meeths *et al.*, 2010). Mutations in these proteins all lead to impaired fusion of the lytic granules with the plasma membrane and therefore impaired granule exocytosis of NK cells and CTLs (Feldmann *et al.*, 2003; Neeft *et al.*, 2005; Marcenaro *et al.*, 2006). Impaired perforin in FHL2 prevents efficient delivery of lytic granule content to target cells. Dysfunction of the small GTPase docking protein, Rab27a, is associated with the immunodeficiency syndrome Griscelli syndrome 2 where granules are unable to dock at the immunological synapse (Mancini, Chan and Paller, 1998; Ménasché *et al.*, 2000; Stinchcombe *et al.*, 2001; Bizario *et al.*, 2004; Gazit *et al.*, 2007). Mutations in the MYH-9 gene, known as May-Hegglin anomaly which leads to defective MyosinIIA, result in impaired granule exocytosis as the granules are unable to penetrate the cortical F-actin to reach the cell membrane at the immune synapse (Althaus and Greinacher, 2009). However, as many of these proteins are expressed across a variety of cell types, the consequences of protein loss for NK cell function is difficult to dissect (Topham and Hewitt, 2009). The loss of cytotoxic function specifically within NK cells is likely to have an important contribution to the pathology of immune disorders involving recurrent infections and tumour development. Further research of cytotoxic pathways, especially within NK cells which are relatively less well understood, is required.

1.3.4.3 Granule exocytosis in mice

It is well known that there are fundamental differences in activating and inhibitory receptors between human and mouse NK cells. Therefore, it is likely that the signalling events leading to granule exocytosis are also different between species. Thus far, our understanding of murine NK cell perforin and granzyme expression is largely derived from

human NK cells, cell lines, cytotoxic T-cells and lymphokine activated killer cells (LAK cells) (Babichuk, Duggan and Chris Bleackley, 1996; Russell and Ley, 2002; Grossman *et al.*, 2003; Fehniger *et al.*, 2007). Studies using murine LAK cells, where splenocytes are cultured in high doses of IL-2, have shown abundant granzyme A, granzyme B and perforin (Pham *et al.*, 1996; Shresta *et al.*, 1999; Revell *et al.*, 2005). However, it is not clear that human NK cells, LAK cells, cell lines or T-cell studies reflect murine NK cell cytotoxicity. It has been shown that unlike human NK cells which require sustained SOCE for cytotoxicity, primary murine NK cells do not. In the absence of SOCE, murine NK cells use alternative DAG-mediated downstream signalling molecules such as protein kinase C (PKC) (Freund-Brown *et al.*, 2017). Furthermore, it has been shown that murine NK cells contain granzyme A but little to no perforin and granzyme B protein. However, abundant mRNAs for all three proteins were found. This suggests that resting murine NK cells are minimally cytotoxic. Upon infection with MCMV *in vivo*, it was shown that granzyme B and perforin protein are induced. Therefore, murine NK cells, unlike human NK cells, require further activation in response to infection for perforin and granzyme mRNA translation (Fehniger *et al.*, 2007). As a result, more work is needed to understand granule exocytosis in mouse NK cells which will inform on the relevance to human NK cell studies.

1.3.5 NK cell cytokine release

The release of antiviral cytokines such as IFNγ and TNFα is another essential NK cell function (Jurisić *et al.*, 2008; Mitrovic *et al.*, 2012). Cytokines activate surrounding inflammatory cells and induce the recruitment and regulation of immune cells from both the innate and adaptive immune systems (Ross and Caligiuri, 1997; C A Biron *et al.*, 1999; Degli-Esposti and Smyth, 2005; Vivier *et al.*, 2008). Uncontrolled cytokine release can lead to destruction of tissue, pain and death therefore these processes must be tightly regulated (Haddad *et al.*, 2001; Stinchcombe *et al.*, 2001; Ishii *et al.*, 2005; van Dommelen *et al.*, 2006; Bradley, 2008). NK cells release a repertoire of pro-inflammatory and regulatory cytokines including IFNγ, TNFα, GM-CSF, IL-10 and TGFβ (C A Biron *et al.*, 1999; Lacy and Stow, 2011). One of the most important cytokines for infection control is IFNγ which is the focus of the following section. IFNγ receptors are present on a large proportion of cell types and tissues, including immune cells (De Weerd and Nguyen, 2012). Upon IFNγ binding to its receptors, the JAK-STAT pathway is initiated which induces interferon stimulated genes (Decker, Kovarik and Meinke, 1997; Lasfar *et al.*, 2014) that regulate

inflammation, innate and acquired cell mediated immunity, apoptosis and the cell cycle (De Weerd and Nguyen, 2012). For example, IFNγ also enables upregulation of MHC Class I to promote NK cell recognition, and activation of adaptive immunity (Schroder *et al.*, 2004).

1.3.5.1 IFNγ production

As with degranulation, multiple signalling steps are required for IFNγ production which include a combination of NK cell activating receptor engagement and cytokine signalling from other innate immune cells. IFN-γ protein expression is regulated at multiple levels, including epigenetically, transcriptionally, and post-transcriptionally. IFNγ regulation within NK cells, has mostly been studied in mice but human studies suggest similarities between species (Savan *et al.*, 2009; Luetke-Eversloh, Cicek, *et al.*, 2014). The binding of cytokines including IL-1, IL-2, IL-12, IL-15, IL-18 and type I IFNs to their receptors can activate STAT proteins and other proteins required for IFNγ transcription (Murphy *et al.*, 2000; Lieberman and Hunter, 2002; Schoenborn and Wilson, 2007; Balasubramani, Mukasa, *et al.*, 2010; Piersma *et al.*, 2019). For optimal IFNγ production, IL-12 is required to activate STAT4. STATs, T-bet, Eomes and NF-κB transcription factors, as well as intronic enhancers and non-coding sequences induce transcription of IFNγ mRNA (Murphy *et al.*, 2000; Lieberman and Hunter, 2002; Schoenborn and Wilson, 2007; Balasubramani, Mukasa, *et al.*, 2010; Balasubramani, Shibata, *et al.*, 2010). Human NK cells have an epigenetically accessible *Ifng* locus and constitutively express the IFNγ transcript. In mature mouse NK cells, the *Ifng* locus is also epigenetically available to the transcription factors T-bet and Eomes (Daniel B. Stetson *et al.*, 2003; Tato *et al.*, 2004; Chang and Aune, 2005). The accessibility of the *Ifng* locus enables NK cells to respond rapidly to infection compared to lymphocytes in the adaptive immune system (Daniel B Stetson *et al.*, 2003; Tato *et al.*, 2004; Chang and Aune, 2005; Balasubramani, Mukasa, *et al.*, 2010). For example, upon HCMV infection, it was found that an expanded pool of NKG2C^{hi} memory-like human NK cells consistently produce IFNγ (Luetke-Eversloh, Hammer, *et al.*, 2014). An enhancer regulating mouse and human IFNγ transcription, known as CSN1, remains in a 'closed state' in naive NK cells but opens in human NKG2C^{hi} memory-like NK cells and mouse memory-like NK cells that were treated with cytokines (IL-12/15/18) (Luetke-Eversloh, Hammer, *et al.*, 2014; Ni *et al.*, 2016). Mouse and human NK cells both have constitutively low levels of IFNγ transcript production without constitutive IFNγ protein

production (Daniel B. Stetson *et al.*, 2003), as observed in mice with perforin and granzyme B production (Fehniger *et al.*, 2007). This mechanism may enable NK cells to respond both rapidly and precisely to infection (Fehniger *et al.*, 2007). Other factors found to regulate IFNγ production include metabolism and micro-RNAs. For example, the metabolic regulator mTOR which is essential to NK cell maturation is also necessary for maximal IFNγ production after IL-15 stimulation (Chi, 2012; Yang and Chi, 2012; Weichhart, Hengstschläger and Linke, 2015). mIR-155 is a positive regulator of IFNγ production in human NK cells through its direct effects on the inositol phosphatase SHIP-1 which can override IL-12 and IL-18 mediated regulation of SHIP-1 (Trotta *et al.*, 2012). Many of the activating NK cell receptors signal through adaptors containing ITAMs, leading to induction of MAPK, PI3K/AKT and PLCγ cascades which contribute to NK cell cytotoxicity or cytokine secretion (Vivier, Nunès and Vély, 2004; Chiesa *et al.*, 2005; Lanier, 2008; Long *et al.*, 2013). NK cell activating receptor engagement is required for IFNγ translation, through stimulation of the TPL2 kinase (Piersma *et al.*, 2019).

1.3.5.2 IFNγ release

Despite the importance of antiviral cytokines, very little is known about their mechanisms of release from NK cells. Multiple pathways of cytokine transport and release have been uncovered in different cell types. Secretory pathways can be grouped into classical secretory pathways including regulated exocytosis (both shown in Figure 1.13), constitutive exocytosis and piecemeal degranulation as well as non-classical secretory pathways including membrane transporter, exosome release, microvesicle shedding and cell lysis (Lacy and Stow, 2011). These pathways all require multiple trafficking machinery molecules as has been demonstrated for degranulation. For example, in macrophages a central organelle for cytokine secretion is the recycling endosome, the site of cytokine regulation and sorting, which can induce the polarised delivery of TNF to phagocytic cups for release (Murray *et al.*, 2005). In addition, helper T-cells can release cytokines using polarised and non-polarised pathways (Huse *et al.*, 2006). Within NK cells, more work is needed to understand cytokine release including the release of the important antiviral cytokine IFNγ.

As NK cells contain pre-formed cytokines, it is likely that NK cells release cytokines through regulated exocytosis. Multiple proteins described in regulated exocytosis of

cytokines, including Cdc42, are required for granule exocytosis in cytotoxicity (Stinchcombe *et al.*, 2001; Stinchcombe, Bossi and Giffiths, 2004; Lacy and Stow, 2011). However, in human NK cells it has been found that the trafficking of IFNγ and TNFα diverges from perforin trafficking at the level of the Golgi complex and is dependent on the recycling endosome. Recycling endosome associated proteins such as Rab11 and vesicle-associated membrane protein 3 (VAMP3) are also essential to cytokine exocytosis (Reefman *et al.*, 2010). Unlike perforin release which is highly directional due to MTOC polarisation, cytokine release can be non-polarised (Reefman *et al.*, 2010) or, in the case of IFNγ, polarised (O'Shea, Ma and Lipsky, 2002; Trapani and Smyth, 2002; Bossi and Griffiths, 2005; Huse *et al.*, 2006). Due to the differences in polarisation, as well as the finding that the localisation of cytokine and granule markers including perforin do not overlap (Reefman *et al.*, 2010), it is believed that cytokine release and granule exocytosis operate with different mechanistic pathways. The separation between these pathways may be important for simultaneous target cell killing and recruitment of other immune cells (Lodoen and Lanier, 2006; Reefman *et al.*, 2010) although it has been found that different populations of NK cells have distinct roles to induce either cytotoxicity or cytokine release (Vahlne *et al.*, 2008).

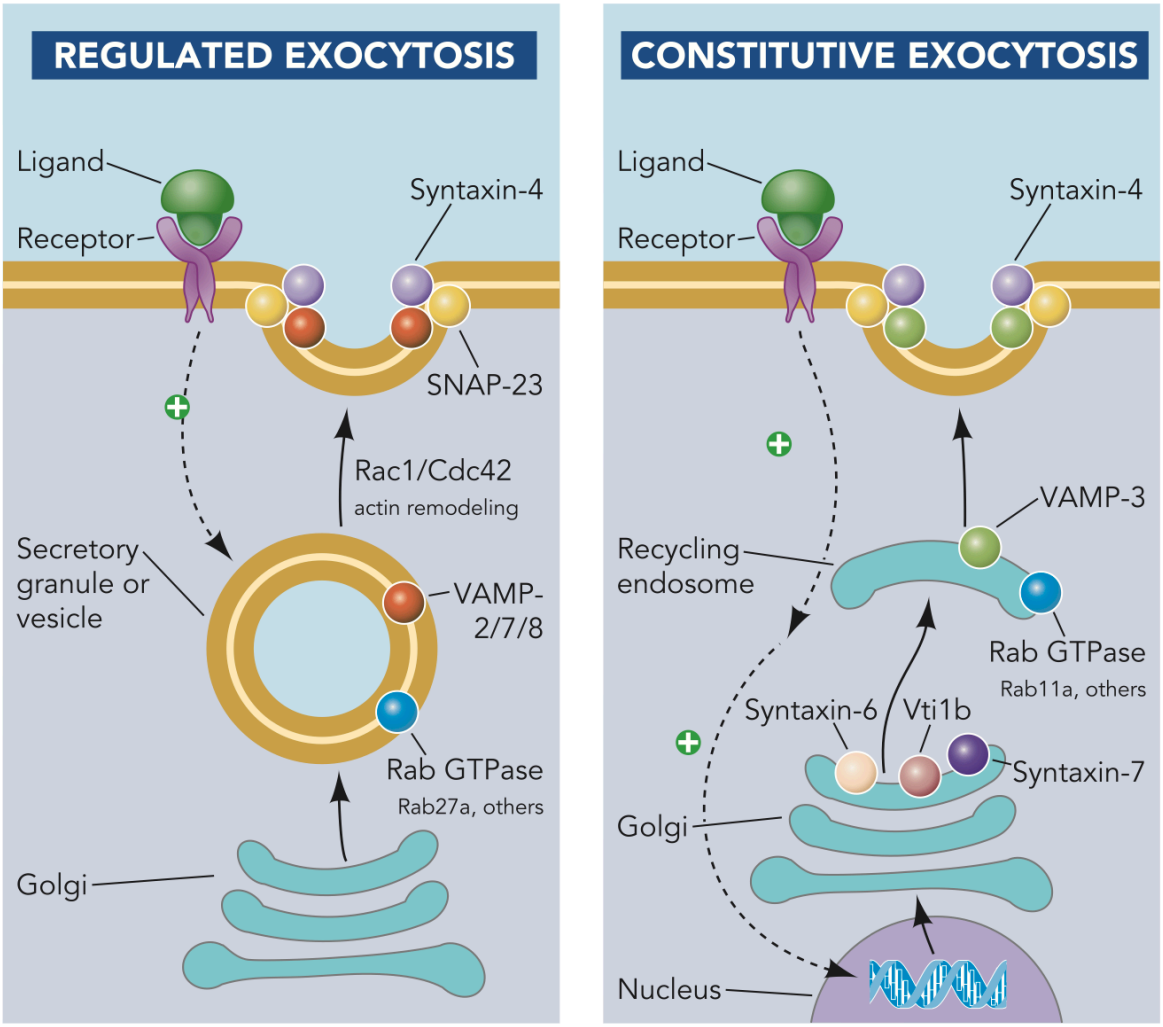

Figure 1.13 The regulated exocytosis and constitutive exocytosis pathways of cytokine release.
Regulated exocytosis involved receptor signalling to secretory granules or vesicles containing cytokines which are mobilised for release. Constitutive exocytosis is dependent on receptor mediated transcription events to enables trafficking of cytokines via the Golgi and recycling endosomes before reaching the cell surface. The pathways shown here are specific to granulocytes in regulated exocytosis and TNF release from macrophages in constitutive exocytosis. The R-SNAREs VAMP-2, -7, and -8 are implicated in regulated secretion, whereas Vti1b and VAMP-3 are essential for constitutive release of recycling endosomes. GTPases including Rab27a and Rab11a are also associated with cytokine release. Source: Stanley and Lacy, 2010.

1.3.6 Innate lymphoid cells

A subset of NK-related cells known as ILCs reside within tissues and contribute to organ homeostasis, metabolism and tissue repair as well as host defence (Artis and Spits, 2015; Diefenbach, Colonna and Romagnani, 2017; Vivier *et al.*, 2018; Rolot and O'Sullivan, 2020). The key hallmark of ILCs are their lack of rearranged antigen receptors, but instead expression of germline encoded activating and inhibitory receptors (Vivier *et al.*, 2018), as well as their residence within tissues. ILCs can be found within lymphoid and non-lymphoid tissues including skin, gastro-intestinal tract, lungs, urogenital tract and are enriched within epithelial barriers (Artis and Spits, 2015; Doisne *et al.*, 2015; Vivier *et al.*, 2018). Within tissues, ILCs rapidly detect signal cytokines and then produce effector cytokines to activate local innate and adaptive effector functions. Mature ILCs are classified into three functional groups: ILC1s and NK cells, ILC2s and ILC3s shown in Figure 1.14. Each group of ILCs has distinct host defence functions which are believed to mirror the activities of T-cell subtypes. ILC1s and NK cells mirror the functions of cytotoxic CD8+ T-cells to destroy bacteria, virally infected cells, and tumour cells. Unlike NK cells, ILC1s are generally non-cytotoxic or weakly cytotoxic but both NK cells and ILC1s produce IFNγ which is a crucial cytokine for viral and intracellular bacteria control (Schoenborn and Wilson, 2007; Vivier *et al.*, 2018). ILC2s mirror T helper 2 cells and respond to large extracellular parasites and allergens. ILC2s produce type 2 cytokines, IL-4, IL-5 and IL-13, which influence B-cell differentiation, antibody production, eosinophil recruitment, and mucus production (Moro *et al.*, 2010; Neill *et al.*, 2010; Price *et al.*, 2010). ILC2s also have an important role in repairing damaged tissues after the infection is resolved (Monticelli *et al.*, 2011, 2015). ILC3s mirror T helper 17 cells which target extracellular microbes including bacteria and fungi (Vivier *et al.*, 2018). In addition, ILC3s are also important for controlling symbiotic bacteria in the intestine and promoting proliferation of intestinal stem cells (Buela, Omenetti and Pizarro, 2015; Ganal-Vonarburg and Duerr, 2020). As most ILCs are tissue-resident (Gasteiger *et al.*, 2015), excluding NK cells which also circulate in the blood, they can adopt slightly different roles according to their local environment which enables them to adopt tissue-specific expression patterns. However, ILC3s are also able to migrate out of the tissue from the intestine into the draining mesenteric lymph node and ILC2s circulate in the bloodstream. Due to ILCs having highly specialised responses according to their environment, 50 distinct ILC clusters have been identified by single-cell RNA sequencing (Gury-BenAri *et al.*, 2016).

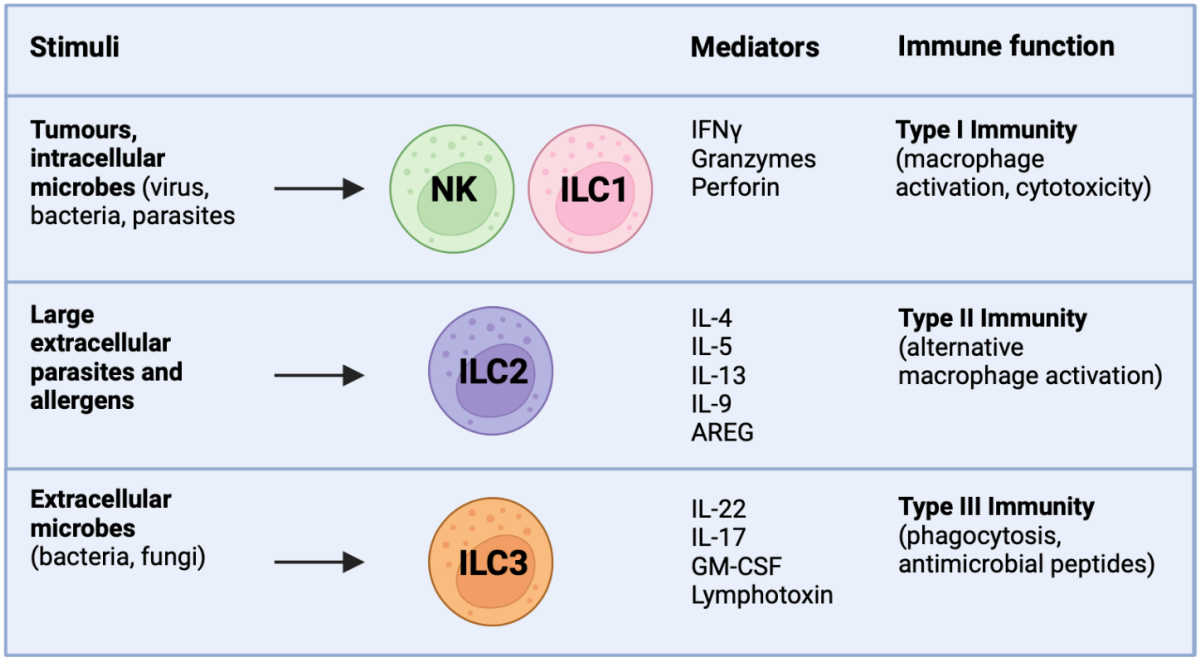

Figure 1.14 A summary of the different types of of Innate Lymphoid cells (ILC) immunity.
Type 1 ILCs (which include NK cells) respond to tumours and intracellular pathogens through release of IFNγ or cytotoxicity. Type 2 ILCs respond to large extracellular pathogens, parasites and allergens and induce alternative macrophage function through release of cytokines. Type 3 ILCs recognise extracellular bacteria and fungi and respond through phagocytosis and release of antimicrobial peptides. Source adapted from: Vivier *et al.*, 2018.

1.4 Thesis hypothesis and objectives

It is well established that NK cells have a vital role in early infection control but the mechanisms underlying their effector function require more research. Efforts have been made to identify novel genes of interest which regulate NK cell function, which will be important for the development of NK cell-based treatment approaches for infection control. In the context of HCMV infection, these are likely to benefit transplant recipients and may overcome the limitations of current treatments. Furthermore, understanding novel genes which regulate NK cells will also improve the diagnosis and treatment of immunocompromised individuals with novel gene mutations.

I hypothesise that the identification of genes required for control of cytomegalovirus replication *in vivo* will help identify novel genes important for NK cell antiviral function.

To test this, I will be using the MCMV infection model to address the following objectives:

1) Assessment of the regulation of antiviral control by 69 novel genes of interest using an *in vivo* viral infection screen of gene-deficient mice
2) Examine how gene(s) of interest identified in the screen regulate NK cell functions

Chapter 2 - Methods

2.1 Buffers, Solutions and Media

All tissue culture reagents were purchased from Gibco, Life Technologies and all chemicals used were purchased from Sigma or Thermo Fisher Scientific unless otherwise stated.

D10:	DMEM containing 4.5g/L L-glucose, 10% (v/v) heat inactivated foetal calf serum (FCS) (Invitrogen), 250units penicillin/streptomycin, 0.26mg/ml L-Glutamine, and 97mg/ml sodium pyruvate.
R10:	RPMI, 10% (v/v) FCS, 250units penicillin/streptomycin, 0.26mg/ml L-Glutamine, and 97mg/ml sodium pyruvate.
Sorbitol layer	20% (w/v) sorbitol (Acros Organics) in PBS, sterile filtered using a 0.22μm filter.
CMC media:	Autoclaved 4g carboxymethyl cellulose (CMC) in 500ml D10.
Crystal violet solution:	0.5% (w/v) crystal violet in ddH_2O.
Red blood cell lysis	150mM NH_4Cl, 10mM $KHCO_3$, 1mM EDTA in ddH_2O.
FACS buffer:	2% (v/v) FCS, 0.05% (w/v) sodium azide in PBS.
Permeabilization buffer	10X solution (Invitrogen) 10% (v/v) in distilled water.
PMA-Ionomycin	PMA 0.2% (v/v) and Ionomycin 0.4% (v/v) in R10.

2.2 Mice for *in vivo* screening

2.2.1 Generation of gene-deficient mice

69 strains of gene-deficient mice were generated by the Wellcome Trust Sanger Institute (WTSI) through use of CRISPR-Cas9 technology using established methods (Doe, Brown and Boroviak, 2018) and in two cases using the EUCOMM/KOMP embryonic stem cell collection (Skarnes *et al.*, 2011) as part of the Sanger Institute Mouse Genetics Project. All mice produced for the screen and WT control mice were on the C57BL6/N genetic background.

2.2.2 MCMV infections of gene-deficient mice at the WTSI

Age and sex-matched mice between 6-12 weeks of age were used for the screen. Mouse MCMV infections were titrated to induce approximately 10% weight loss after 4 days. Virus infections were performed using i.p. injection and mouse weight was monitored daily. All gene-deficient mice were challenged with tissue culture passaged MCMV and in some cases gene-deficient lines were also infected with salivary gland passaged stocks. Mouse spleens and livers were harvested on day 4 post-MCMV infection unless otherwise stated. All animals were treated according to Home Office-approved project licence P653704A5. All animals were cared for according to Home Office regulations and kept in filter top housing at the Home Office designated facility at the WTSI.

2.3 Cell culture

NIH-3T3 cells (ATCC CRL-1658) were grown in D10. P815 cells (ATCC TIB-64) and WEHI3B cells (ECACC; 86013003) were grown in R10. BaF/3 and BaF/3-m157 cells (a kind gift from Professor Wayne Yokoyama, Washington University, USA) were grown in R10 supplemented with 5-10% WEHI3B supernatant as a source of IL-3. BaF/3-m157 cells were also cultured with 1μg/ml puromycin (Gibco) as the m157 construct was under puromycin selection. Puromycin was removed from BaF/3-m157 cell culture 2-3 days before the cells were used in experiments. The cultures were maintained in a humidified incubator at 37°C with 5% CO_2. For sub-culturing NIH-3T3 adherent cells, 70-80% confluent cell monolayers were washed with PBS and incubated with trypsin (Gibco, Life Technologies) for 3-5 minutes at 37°C to detach cells from the flask surface. Subsequently, media was added to inactivate the trypsin. The cells were centrifuged (VWR Mega Star

3.0R) for 5 minutes at 1500rpm (rotor radius at 19.53cm used in all experiments), the supernatant discarded, and the cell pellet resuspended in corresponding media. NIH-3T3 cells were passaged every 2-3 days and split in a ratio of 1:4 to 1:6. Non-adherent cell lines (P815, WEHI3B, BaF/3 and BaF/3-m157) were passaged every 2-3 days and split at a ratio of 1:10. Flasks (150cm^2 or 175cm^2, Thermo Fisher Scientific) containing the cells were prepared and incubated.

2.4 Generation of MCMV stocks

2.4.1 Generation of salivary gland passaged MCMV

To passage salivary gland MCMV, 4-5 week old BALB/c mice were infected i.p. with 5 x 10^3 PFU MCMV Smith strain salivary gland derived virus. The salivary glands were harvested 13 days p.i. before they were put on ice ready for purification.

2.4.2 Generation of tissue culture passaged MCMV

For growing MCMV stocks, virus was put onto a NIH-3T3 cell monolayer at 70-90% confluency. Once the cells showed signs of cytopathic effect, which refers to the structural changes shown in Figure 2.1, media was changed every 3 days and supernatant was collected and stored at -80°C. When almost all the cells in the flask showed signs of cytopathic effect, these were scraped and stored at -80°C. Steps were taken to ensure each batch of virus was as similar as possible. One stock of virus was used to grow each batch and the same process was used to grow and purify the virus batches each time.

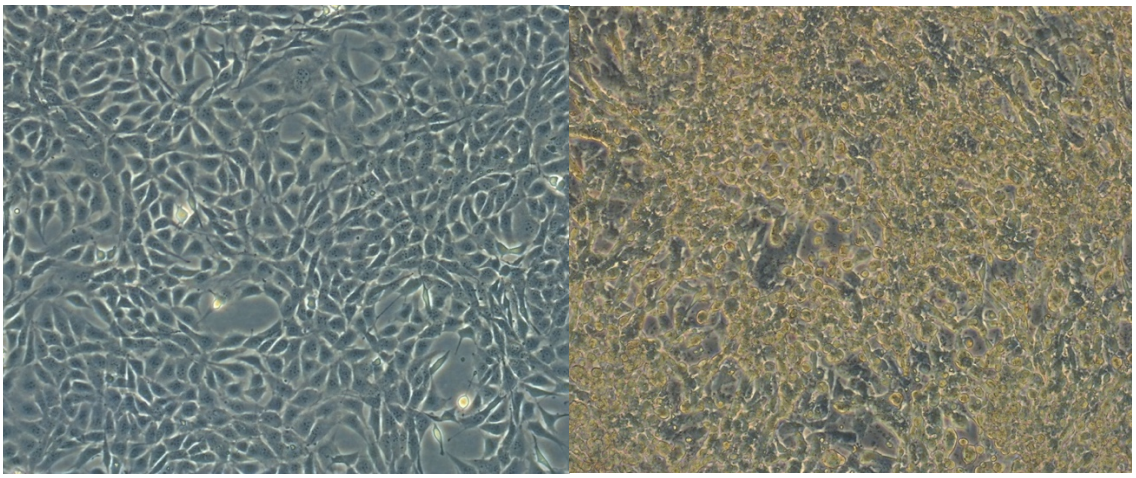

Figure 2.1 Images showing the differences in morphology between uninfected and MCMV infected NIH-3T3 cells.
NIH-3T3 cells without infection (left) and NIH-3T3 cells after 7 days of Smith strain MCMV infection ready for harvesting (right). Magnification 5X.

2.4.3 Salivary gland passaged MCMV purification

The salivary glands harvested from mice were washed with cold PBS, and homogenized. The homogenate was then centrifuged (VWR Mega Star 3.0R) for 10 minutes at 3000rpm at 4°C and the upper fat layer was aspirated. The virus containing supernatant was overlayed onto a sorbitol layer before it was centrifuged (Beckman-Coulter Avanti J-E) at 18,100rpm (rotor radius 108mm) for 75 minutes at 4°C. The supernatant was then removed, and the virus-containing pellet was resuspended in 2ml PBS, sonicated for 5 minutes in an ice water bath, and centrifuged (VWR Mega Star 3.0R) at 2800rpm for 5 minutes 4°C before the virus was aliquoted and frozen at -80°C.

2.4.4 Tissue culture passaged MCMV purification

Virus containing supernatant and cells were thawed and combined before purification. The virus containing mix was centrifuged (VWR Mega Star 3.0R) at 2000rpm for 5 minutes at 4°C. The supernatant containing virus was centrifuged (Beckman-Coulter Avanti J-E) at 10000rpm (rotor radius 98mm) for 90 minutes at 4°C. The cell pellet was resuspended in D10 at 4°C before sonicating for 7 minutes in an ice water bath. The cell pellet was resuspended in D10 media at 4°C and sonicated in an ice water bath for 7 minutes. To remove the cell debris, it was again centrifuged (VWR Mega Star 3.0R) at 3000rpm, 5 minutes, 4°C and the pellet was discarded. All the virus containing sample was combined and centrifuged (VWR Mega Star 3.0R) at 3000rpm, 5 minutes, 4°C to pellet undissolved debris. The sample was placed on top of a sorbitol layer before it was centrifuged (Beckman-Coulter Avanti J-E) at 18,100rpm (rotor radius 108mm) for 100 minutes at 4°C. The cell pellet was resuspended in D10 media, sonicated at 4°C in an ice water bath for 5 minutes before it was centrifuged (VWR Mega Star 3.0R) at 3000rpm, 5 minutes, 4°C to pellet residual debris. The supernatant was aliquoted and stored at -80°C. The virus stocks were titred using a plaque assay 2-3 times to calculate an average titre value. The viral titre from a plaque assay refers to the concentration of infectious viral particles.

2.5 Plaque assay

NIH-3T3 cells were seeded at $1x10^5$ cells per well in a 24 well cell bind plate (VWR) and incubated at 37°C, 5% CO_2 overnight.

2.5.1 MCMV stock titration

Purified virus stocks were serially diluted in DMEM media (Gibco) at 1:10, 1:100 and 1:1000. 200µl of each dilution was added on top of the NIH-3T3 cells in duplicate. The plates were incubated at 37°C, 5% CO_2 for 1 hour. The diluted virus was removed and 1ml of semi-viscous CMC media pre-warmed to 37°C was added to each well. The cell plates were incubated for 6 days at 37°C, 5% CO_2 before the CMC media was removed. The cells were then fixed with 10% v/v formaldehyde in PBS and stained with crystal violet solution for 37°C, 5% CO_2 for 4 hours. The cell fix and stain mix was removed, and the plates were left to dry. The plaques in each well were counted and the virus titre was calculated using the following formula:

Plaque forming unit (PFU) per ml = average number of plaques x dilution factor x 5*

*200µl of the diluted virus was added on top of the cells (i.e. 1/5th of 1ml) therefore multiplying by 5 obtains PFU/ml.

2.5.2 Virus quantification in organs

Mouse organs were homogenised in DMEM media (Gibco) before serial dilutions of the suspension at 1:10, 1:100 and 1:1000 were prepared. The cells were centrifuged (VWR Mega Star 3.0R) at 1000xg for 30 minutes and the cell suspension removed from the cells. 200µl of the suspension was added on top of the prepared NIH-3T3 cells in duplicate. 1ml of semi-viscous CMC media pre-warmed to 37°C was added to each well. The cell plates were incubated for 6 days and then fixed and stained as described for the MCMV stock titration section 2.5.1. The remaining samples were frozen at -80°C for enzyme linked immunosorbent assay (ELISA) analysis. Plaques were counted and calculated using the following formula:

Plaque forming units (PFU) per g organ = average number of plaques x dilution x organ weight x 5*

*200µl of the diluted virus was added on top of the cells (i.e. 1/5th of 1ml) therefore multiplying by 5 obtains PFU/ml.

2.6 Mice and viral infections

Sytl3$^{-/-}$ mice (allele *Sytl3*$^{em1(IMPC)Wtsi}$ and mouse genomics information reference 6153629) were generated via the use of CRISPR-Cas9 using established methods (Doe, Brown and Boroviak, 2018) as part of the WTSI *in vivo* screen and were subsequently shipped to the Heath Park Cardiff University facility. During *in vivo* experiments in Cardiff, in some cases WT mice on the C57BL6/J background were used when WT C57BL6/N mice were not available. Experiments using C57BL6/J mice were initially performed in parallel to experiments using C57BL6/N mice to show there was no difference between the strains. Age- and sex-matched mice 6-12 weeks of age were used for the experiments. Mouse MCMV infections were titrated to induce approximately 10% weight loss after 4 days. Virus infections were performed using i.p. injection and mouse weight was monitored daily. For NK cell depletion, each mouse was injected i.p. with 200µg anti-NK1.1 (clone PK136; Bio X Cell) or PBS control on days −2, 0 and +2 post-infection. For IL-21R blocking, each mouse was injected i.p. 200µg anti-IL-21R (clone 4A9; BioXCell) or PBS control on days −2 and +2 post-infection. Mouse spleens and livers were harvested on 2, 4, 7 or 37 days post-MCMV infection according to the experiment. All animals were treated according to Home Office-approved project licences P7867DADD and P653704A5. All animals were treated according to Home Office regulations and were kept in filter top housing at the Home Office designated facilities at Heath Park Cardiff University or the WTSI.

2.7 Leukocyte isolation

Spleens and livers harvested from mice were passed through a 70µm nylon cell strainer to obtain a single cell suspension in PBS. The single splenocyte cell suspension was centrifuged (VWR Mega Star 3.0R) at 1500rpm for 5 minutes and the cell pellet was resuspended in red blood cell lysis buffer for 5 minutes. The single liver cell suspension was resuspended in 37.5% v/v Percoll solution (GE Heathcare; diluted in PBS) at room temperature before centrifuging (VWR Mega Star 3.0R) at 1800rpm for 6 minutes at room temperature. The supernatant was discarded before RBC lysis as described for splenocytes. Cells were washed in PBS and resuspended in R10 media and counted.

2.8 Blood plasma preparation

At least of 100µl blood/mouse was harvested from the femoral artery and placed into EDTA tubes (SARSTEDT). Then, the blood was spun using a microcentrifuge (Heraeus Biofuge Pico) at 80000rpm (rotor radius at 8.5cm) for 8 minutes at room temperature. The top layer, known as the plasma, was then pipetted off and frozen at -80°C.

2.9 Flow cytometry

2.9.1 Extracellular surface staining

Between $1x10^5$ and $1x10^7$ cells were plated onto V-bottom 96 well plates (Thermo Fisher Scientific). Cells were washed in PBS before staining with a Zombie Aqua Fixable Viability kit at 1:500 (Biolegend; 50µg/ml stock) in PBS for 5 minutes in the dark at room temperature. The cells were washed using PBS and incubated at room temperature with the Fc blocking anti-CD16/32 antibody diluted 1:50 (BioLegend; 0.5mg/ml stock) in FACS buffer for 15 minutes. The cells were washed in FACS buffer before staining with various surface antibodies diluted in FACS buffer at 25µl/well for 20 minutes in the dark at room temperature. The cells were then fixed in 4% paraformaldehyde (Fisher) diluted in PBS for 10 minutes and then resuspended in FACS buffer before analysis or intracellular staining. The surface antibodies are shown in Table 2.1.

Antibody	Conjugate	Clone	Supplier	Dilution	Final concentration
α-NK1.1	APC	PK136	Biolegend	1:100	2μg/ml
α-NK1.1	APCCy7	PK136	Biolegend	1:100	2μg/ml
α-CD3	APCCy7	145-2C11	Biolegend	1:100	2μg/ml
α-CD3	PeCy7	145-2C11	Biolegend	1:100	2μg/ml
α-CD3	PerCP	145-2C11	Biolegend	1:100	2μg/ml
α-NKp46	BV605	29A1.4	Biolegend	1:100	2μg/ml
α-CD11b	PeCy7	M1/70	Biolegend	1:100	2μg/ml
α-CD11b	APCCy7	M1/70	Biolegend	1:100	2μg/ml
α-CD11b	FITC	M1/70	Biolegend	1:100	5μg/ml
α-CD27	PE	LG.7F9	Invitrogen	1:100	2μg/ml
α-CD25	BV605	PC61	Biolegend	1:100	0.8μg/ml
α-KLRG1	PE-Dazzle	2F1/KLRG1	Biolegend	1:100	2μg/ml
α-Ly49H	BV711	3D10	BD Biosciences	1:100	2μg/ml
α-CD107a	FITC	1D4B	Biolegend	1μL/well	20μg/ml
α-CD49a	PerCPCy5.5	HMa1	Biolegend	1:50	4μg/ml
α-CD49b	PeCy7	HMa2	Biolegend	1:100	2μg/ml
α-NKG2D	PeCy7	CX5	eBioscience	1:100	2μg/ml
α-IL-15αR	PE	DNT15Ra	eBioscience	1:100	2μg/ml
α-IL-21R	APC	4A9	Biolegend	1:100	2μg/ml
α-CD11c	PeCy7	N418	Biolegend	1:100	2μg/ml
CD64	PE-Dazzle	X54-5/7.1	Biolegend	1:100	2μg/ml
LY6C	PerCP	HK1.4	Biolegend	1:100	2μg/ml
IAIE	FITC	M5/114.15.2	Biolegend	1:300	1.67μg/ml
F4/80	BV711	BM8	Biolegend	1:100	2μg/ml
Siglec-H	APC	551	Biolegend	1:100	2μg/ml
B220	PB	RA3-6B2	Biolegend	1:100	5μg/ml
LY6G	PE	1A8	Biolegend	1:100	2μg/ml

Table 2.1 Flow cytometry antibodies used to detect cell-surface proteins.

2.9.2 Functional NK cell analysis

2.8.2.1 Ex vivo assessment of degranulation and IFNγ production

For functional NK cell analysis, *ex vivo* whole naïve splenocytes isolated from naïve or MCMV infected mice were stimulated with either unstimulated control (R10 media) or PMA-Ionomycin diluted in R10. Anti-CD107a (Biolegend) was added at the same time as the stimulants for a final volume of 200µl/well. After 1 hour, 25µl/well of Monensin (Biolegend; 1000X solution diluted in R10) was added, and the cells were incubated for a total of 5 hours. Then cells were stained for viability and cell surface proteins as described in section 2.9.1. For IFNγ detection and intracellular markers, flow cytometric antibodies were stained according to section 2.9.3.

2.9.2.2 In vitro assessment of degranulation

For functional analysis of *in vitro* expanded NK cells, $CD3^-$ mouse splenocytes were isolated using a negative selection mouse CD3 isolation kit (Miltenyi) and passed through LS columns twice to maximise purity. The $CD3^-$ cells were then cultured at $4x10^6$ cells/ml with hIL-2 (1000IU; PROLEUKIN®) in R10 for 48 hours in 6 well plates (VWR). Purity was assessed using flow cytometry to ensure >80% $CD3^-$ cells. $1x10^6$ splenocytes per well were then mixed with the following stimulations according to the experimental setup: unstimulated control (R10 media), PMA-Ionomycin, P815 cell targets, P815 cell targets + anti-Ly49H (Biolegend; 10ng/ml), P815 cell targets + anti-MHCI (eBioscience; 10ng/ml), P815 cell targets + anti-NKG2D (Biolegend; 10ng/ml), IL-15 (Biolegend; 30ng/ml) or IL-21 (Biolegend; 60ng/ml). For preparation of the P815 antibody combinations, antibodies were added to the cells in RMPI for 30minutes before centrifuging (VWR Mega Star 3.0R) at 15000rpm, 5 minutes and resuspending in R10. P815 cells were used at an E:T ratio of 2.5:1. Anti-CD107a (Biolegend) was added at the same time as the stimulants. After 1 hour, 25µl/well of Monensin (Biolegend; 1X solution diluted in R10) was added, and the cells were incubated for a total of 4 hours. Then cells were stained for viability and cell surface proteins as described in section 2.9.1.

2.9.2.3 Target cell killing assay

For the BaF/3 control cell line and BaF/3-m157 killing assay, target cells were stained with Cell Trace Violet (Invitrogen; 5mM stock) at 1/1000 for 20 minutes before washing with R10 media. Purified splenic mouse NK cells (>80% purity) were isolated using a negative selection mouse NK cell isolation kit (Miltenyi) and passed through LS columns twice to maximise purity. The NK cells were then cultured at $4x10^6$ cells/ml with human IL-2 (1000IU; PROLEUKIN®) for 72 hours in 12 well plates (VWR). Purity was assessed using flow cytometry to ensure >80% NK cells. The effector cells were plated alongside $2x10^4$ target cells/well at the following E:T ratios: 5:1, 2.5:1, 1.25:1, 0.625:1, 0.3125:1 and 0.15625:1. After incubation at 37°C for 4 hours, the cells were stained using 1µl/well 7AAD (Biolegend) for 5 minutes to quantify viable cells before immediately running the samples on the flow cytometer.

For the CD107a staining of degranulating cells in response to BaF/3 control and BaF/3-m157 effector cells, NK cells and effectors were prepared as described in this section. NK cells and effectors were stained with anti-CD107a (Biolegend) during 4 hours of incubation at 37°C and 25µl/well of Monensin (Biolegend; 1X solution diluted in R10) was added after the first hour. After the incubation, the cell mix was stained with extracellular markers as described in section 2.9.1.

2.9.3 Intracellular staining

Following surface staining, cells were fixed with 4% w/v paraformaldehyde (Thermo Fisher Scientific) for 10 minutes at room temperature and permeabilised with 1X permeabilization buffer (Invitrogen) diluted in distilled water. The cells were stained with intracellular antibodies diluted in permeabilization buffer for 20 minutes in the dark at room temperature. The intracellular antibodies are listed in Table 2.2.

Antibody	Conjugate	Clone	Supplier	Dilution	Final concentration
α-GzB	PB	GB11	Biolegend	1:100	1µg/ml
α-IFNγ	APCCy7	XMG1.2	Biolegend	1:100	2µg/ml

Table 2.2 Flow cytometry antibodies used to detect intracellular proteins.

2.9.4 Flow cytometry analysis

Cells were washed in FACS buffer, and the data was acquired using the Attune NxT Flow Cytometer (Thermo Fischer Scientific). Electronic compensation was performed with Ab capture beads (BD) stained separately with individual antibodies from the experimental panel. A minimum of 20,000 events were acquired and the data was analysed using FlowJo software (Tree Star). Where percentages are displayed on the graph, absolute counts have been checked to ensure they reflect the displayed data. NK cells were defined according to the gating strategy in Figure 2.2. Neutrophils, classical DCs, macrophages, total monocytes and inflammatory monocytes were defined according to the gating strategy shown in Figure 2.3.

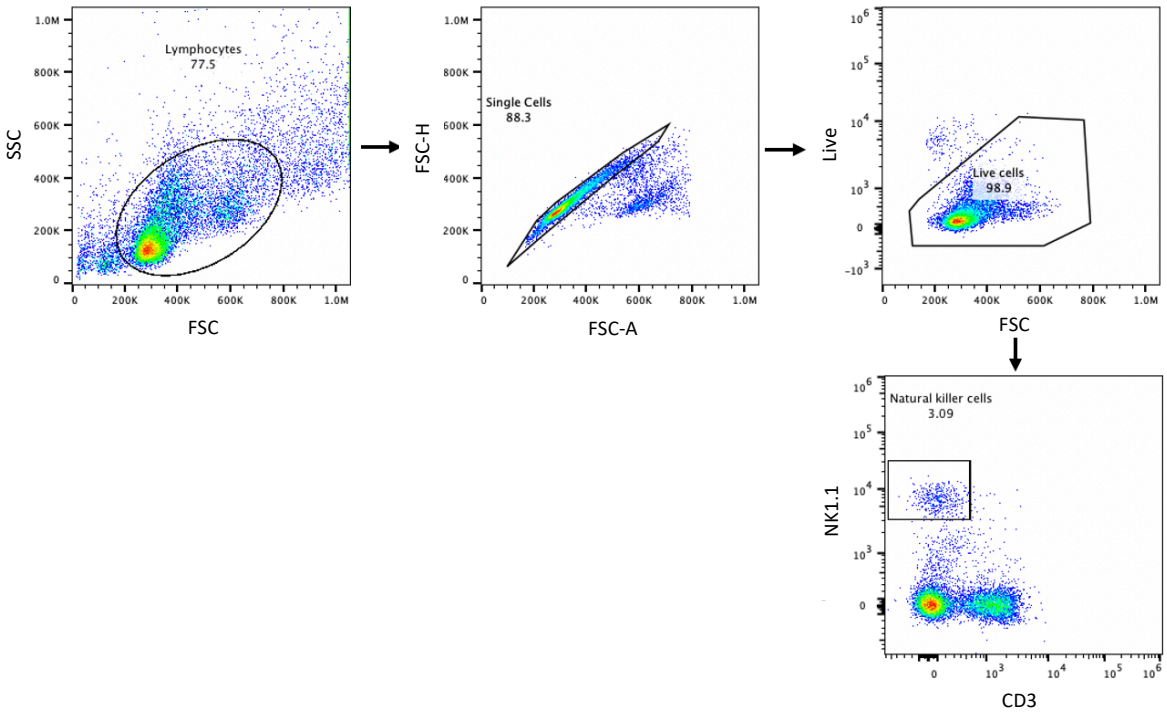

Figure 2.2 Gating strategy for the isolation of NK cells.
Lymphocytes were first gated according to forward scatter (FSC) which was used to detect cell size and side scatter (SSC) which detects cell granularity. Single cells from the lymphocyte population were gated using FSC on both axes. Then using a Live/Dead stain the live cells were gated. Finally, NK cells were defined as $NK1.1^{+}CD3^{-}$.

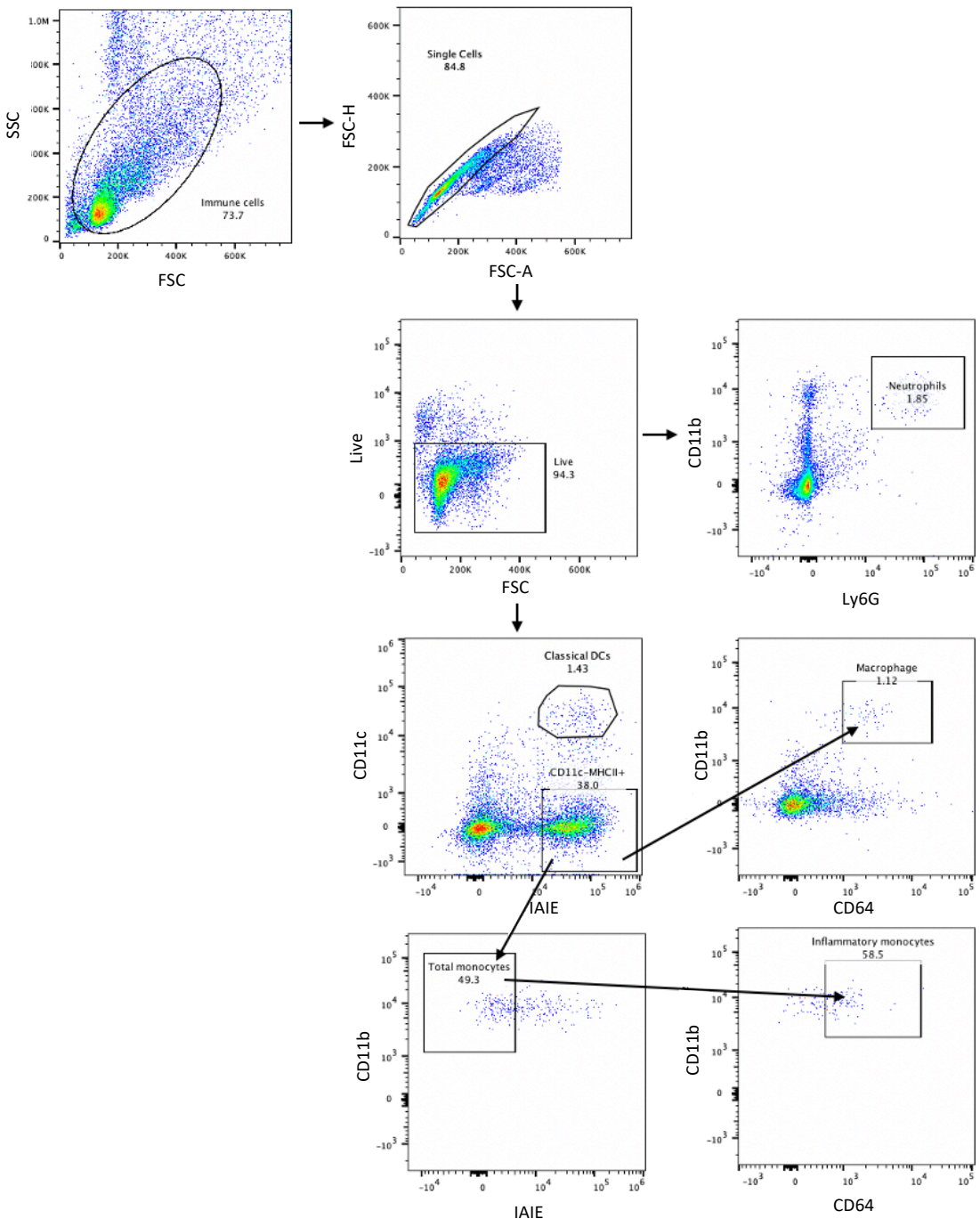

Figure 2.3 Gating strategy for the isolation of neutrophils, classical DCs, macrophages, total monocytes, and inflammatory monocytes.
Immune cells were first gated according to forward scatter (FSC) which was used to detect cell size and side scatter (SSC) which detects cell granularity. Single cells from the immune cell population were gated using FSC on both axes. Then using a Live/Dead stain the live cells were gated. Neutrophils were $CD11b^{+}LY6G^{+}$. Classical DCs were $CD11c^{+}IAIE$ (or $MHCII)^{+}$. $CD11c^{-}IAIE^{+}$ cells were gated and from this population macrophages, and total monocytes were defined. Macrophages were $CD11b^{+}CD64^{+}$. Total monocytes were defined as $CD11b^{+}$. Inflammatory monocytes were defined from the total monocyte population as $CD64^{+}$.

2.10 Cytokine ELISAs

The ELISA plates were coated with IL-21 mouse capture antibody (Biolegend) or IFNγ mouse capture antibody (Biolegend) overnight at 4°C or pre-coated mouse IFNβ ELISA plates were used (PBL assay science). After washing and blocking, thawed cell supernatants were incubated on pre-coated plates alongside standards and a blank control at room temperature for 1 hour. Test samples (splenocyte and liver supernatant or blood plasma) were plated in duplicate and standards and blank controls were plated in triplicate. The plates were washed three times before incubating with biotin-labelled IL-21 antibody (Biolegend), IFNγ antibody (Biolegend) or IFNβ antibody (PBL assay science) for 1 hour according to the kit specifications. HRP-conjugated Streptavidin and TMB substrate (Biolegend and PBL assay science) were sequentially added for detection according to specifications. After stopping the reaction upon addition of a stop solution (1M H_3PO_4 in distilled water), the absorbance was measured at 450nm using a CLARIOstar plate reader (BMG Labtech).

2.11 Confocal imaging

2.11.1 Slide preparation

Mouse NK cells were isolated from total splenocytes using a negative selection mouse NK cell isolation kit (Miltenyi). The isolated NK cells were cultured with hIL-2 (1000IU; PROLEUKIN®) for 48 hours. After 48 hours a sample was taken to determine NK cell purity by flow cytometry (see section 2.9.1) and the rest of the NK cells were combined with target cells. Target cells were pre-stained with CellTrace Violet at 1/1000 (Invitrogen; stock 5mM) in R0 for 20 minutes before washing in R10. $2x10^5$ NK cells were combined with $1.5x10^5$ target cells including P815 cell targets, P815 cell targets + anti-Ly49H (Biolegend; 10ng/ml), P815 cell targets + anti-MHCI (eBioscience; 10ng/ml), P815 cell targets + anti-NKG2D (Biolegend; 10ng/ml) or IL-15 (Biolegend; 30ng/ml). The cells were fixed using 4% w/v paraformaldehyde and permeabilised using 0.1% v/v Triton-X100 (Invitrogen) in PBS before staining in 3% w/v BSA (Invitrogen) and 1% v/v horse serum (Vector) in PBS staining buffer. The cells were stained with Phalloidin-AF488 (1 in 500; Thermo Fisher Scientific), anti-LAMP-AF467 (Santa Cruz Biotechnology; 5μg/mL) and anti-alpha tubulin mouse IgG1 primary antibody (Thermo Fisher Scientific; 1:200) before goat anti-mouse AF568 secondary antibody (Thermo Fisher Scientific; 4μg/mL). The cells

were placed onto slides with coverslips (VWR) and mounted using ProLong Diamond (Thermo Fisher Scientific).

2.11.2 Imaging on confocal microscope

Cells were imaged using a Leica TCS SP8 confocal microscope (Leica Microsystems). A 100X/1.40 NA oil-immersion objective was used with excitation using a 405nm continuous wave laser and a white light pulsed laser set to 488nm, 568nm and 647nm. Fluorescence was detected using Hybrid Detectors using Leica LASX software. Sequential imaging was used to avoid spectral overlap. 10 images were acquired per replicate, with each image showing at least a single NK cell-target cell conjugate.

2.11.3 Image quantification

Images were analysed using QPath to calculate the fold increase in F-actin staining at the synapse relative to the entire NK cell. The MTOC distance was calculated by measuring the distance between the brightest point of the MTOC staining on the NK cell and the nearest point on the immunological synapse.

2.12 Statistical analyses

GraphPad Prism Version 8 was used for all statistical analysis. The results were shown with median values as the most appropriate average measure for data with outliers. The Mann-Whitney U test was used to compare two groups unless otherwise indicated. The statistical tests used for more than two groups are indicated in the relevant graphs. A *P* value of less than 0.05 was considered statistically significant and presented using * in the figures where *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

Chapter 3-An *in vivo* mouse screen to identify genes required for natural killer cell-mediated anti-CMV immunity

3.1. Introduction

3.1.1. *In vivo* mouse screens

The human genome project (completed in 2003) identified over 20,000 protein coding genes as well as many non-coding RNAs. Despite a vastly improved knowledge of human genetic variation, the function of many genes is not understood or is predicted from sequencing analysis alone. To realise the full potential of the information provided in the human genome project, a broader focus is required. Therefore, many studies have attempted to characterise the phenotypes of unknown genes using several organisms including *Escherichia coli* (Qimron *et al.*, 2006), *Saccharomyces cerevisiae* (Giorgini *et al.*, 2005) and *Caenorhabditis elegans* (Savage-Dunn *et al.*, 2003). To gain an improved understanding of these genes in a mammalian context, mutant mouse lines have been increasingly used in a high throughput manner to provide a realistic context for interrogating gene function. Consequently, the Wellcome Trust Sanger Institute (WTSI) alongside the Wellcome Trust Centre for Human Genetics, Omics Laboratory, Harkness Eye Institute, Monash University, Wellcome Trust Centre for Stem Cell Research and the Wolfson Centre for Age-related diseases performed a genome wide mouse screen to identify broad traits and disease features as a step towards the aim of knocking out all genes and screening each line for a broad range of traits (White *et al.*, 2013). The phenotypes of these mouse lines were determined using a wide range of assessments including weight measurements, glucose tolerance and auditory brainstem response measurements across 16 weeks before terminal bleed and necropsy. They found that many novel phenotypes suggest functions for both studied and unknown genes (White *et al.*, 2013). Since then, a number of genome wide mouse screens have been performed to understand the roles of genes in a specific disease context such as cancer (Van Der Weyden, Arends, *et al.*, 2017; Van Der Weyden, Karp, *et al.*, 2017; Abeler-Dörner *et al.*, 2020) and Huntington's (Wertz *et al.*, 2020). The *in vivo* mouse screen described in this chapter specifically focuses on understanding how novel genes regulate NK cell control of CMV infection.

3.1.2. The WTSI NK cell *in vivo* mouse screen

A loss of NK cells or impaired NK cell function renders humans and mice susceptible to infection, particularly herpesviruses, including HCMV and MCMV (Bukowski *et al.*, 1983; C. A. Biron, Byron and Sullivan, 1989). Experimental infection of mice with MCMV is instructive for studying human NK cell responses to viral infection. For example, the MCMV Smith strain has been shown to infect a plethora of different cell types in multiple organs of immunocompromised mice (Podlech *et al.*, 1998; Goodier *et al.*, 2018) closely resembling the broad cell tropism that is characteristic of HCMV in immunocompromised patients (Sinzger, Digel and Jahn, 2008). However, due to the strict host-species specificity of CMVs (Smith 1956; Ostermann et al. 2015; Reddehase 2015), no animal model can be expected to precisely reflect human infection in all aspects.

To provide insight into NK cell biology, I exploited a pool of gene-deficient mice generated by the WTSI that lacked genes hypothesised to be NK cell regulators. Genes were chosen from two mouse NK cell gene expression studies by Chiossone et al. 2009 and Bezman et al. 2012 (with the exception of *Fam114a2* which was chosen due to an internal RNASeq using splenic NK cells index sorted for classification as immature or mature). From the selection of genes shown in these studies, only genes which were novel according to literature searches and where viable selected mouse gene knockouts could be produced were chosen for the *in vivo* screen. Chiossone et al. 2009 sought to address the developmental relationship between NK cell subsets by assessing the kinetics of appearance of each subset during development, gene expression profile, rate of proliferation as well as their potential to give rise to other subsets *in vitro*. Chiossone et al. 2009 focussed on genes which were expressed during different stages of NK cell development which was ascertained using the expression of mouse NK cell maturation markers CD11b and CD27. NK cells can be divided into 4 stages of maturation according to the relative surface expression of CD11b and CD27 (Chiossone *et al.*, 2009) (see section 1.3.2). Table 3.1 shows transcription genes which were upregulated in early and late stages of NK cell maturation.

Category	Differentially expressed transcription factors
Transcription factors up-regulated in CD11b^{low} (immature) NK cells on NK-cell maturation	*Aff3, Apex1, C330003B14Rik, Dmrta1, Dmrta2, Dnajc1, E2f7, Etv3, Hells, Hnrpab, Jmjd1c, Klf4, Maff, Maml2, MGC117846, Myb, Myc, Rel, Rora, Sox6, Ssbp2, Tcf7, 9130211I03Rik*
Transcription factors up-regulated in CD27^{low} (mature) NK cells on NK-cell maturation	*BC066107, C130069I09Rik, D330038O06Rik, Id2, Irf8, Klf12, Klf2, Nfe2, Phf1, Pogk, Rab2, Tbx21, Trappc2, Zbtb39, Zfhx1b, Zfp383, Zfp53, Zfp54, Zfp672, Zfp748, A530094I17Rik, A830058L05Rik*

Table 3.1 Summary of mouse transcription genes which were differentially expressed in the CD11b^{low} (immature) and CD27^{low} (mature) stages of NK cell maturation.
Data from gene expression profiles of mouse NK cell subsets (n=2 for each subset) generated using pan-genomic microarrays. Source: Chiossone et al. 2009.

Bezman et al. 2012 used whole genome microarray datasets to delineate gene expression of NK cells in various states. The transcriptional profile was investigated at several timepoints post-MCMV infection to investigate genes during early effector, late effector, sustained effector, and memory-like NK cell responses, as well as genes upregulated in activated Ly49H+ NK cells. Ly49H binds specifically to m157 glycoproteins expressed on the surface of MCMV infected cells which is important for activating mouse NK cell responses and may have a role in generating memory-like NK cell responses (see section 1.3.3). The largest changes were found during the early stages of infection; 875 genes were upregulated in activated Ly49H+ NK cells including known indicators of inflammation, proliferation, and effector function. Day 7 following MCMV infection, the authors showed that proteins associated with cellular proliferation are elevated in all Ly49H+ NK cells at this timepoint (Bezman *et al.*, 2012), which is consistent with the observation that day 7 is the peak of NK cell expansions (Sun, Beilke and Lanier, 2009a; Schlub *et al.*, 2011; Sun *et al.*, 2011). Following the peak in NK cell expansion, Bezman et al. showed a loss of the survival protein bcl-2 which is consistent with a 'contraction' phase. At day 27 post-infection, a number of genes were specifically upregulated in memory-like NK cells which indicates a distinct population (Bezman *et al.*, 2012). The list of genes upregulated during each stage of infection is shown in Table 3.2.

Category	Significant Genes
Early effector	*1300018I05Rik, 2410002F23Rik, 2610318N02Rik, 2810417H13Rik, 4930412M03Rik, 4930422G04Rik, 4930579G18Rik, 4930588K23Rik, 4932442E05Rik, 4933407C03Rik, 4933437N03Rik, 5930422O12Rik, 6430526N21Rik, 9230105E10Rik, 9330175E14Rik, A230050P20Rik, Aars, Abcb1a, Abcb1b, Abtb2, Actn4, Adap1, Adar, Agfg1, Agpat1, Ahctf1, Ahdc1, Ahr, AI451617, AI848100, Airn, Aldh18a1, Alg8, Alms1, Amfr, Amica1, Amigo3, Ankrd28, Ankrd52, Anp32b, Ap1s3, Apex1, Apobec3, Arf6, Arid1a, Arl5a, Art2b, Asf1b, Asna1, Atad2, Atad5, Atf6, Atf7, Athl1, Atn1, Atp11a, Atp13a1, Atp2a2, Atp2b4, Atr, Atxn2l, Atxn7l1, AU017193, B4galt5, Bak1, Banf1, Bat2, Baz1a, BC016423, BC057170, Bcl2, Bhlhe40, Brca1, Bspry, Bst2, Bysl, C1qtnf6, C530030P08Rik, Calr, Camta2, Canx, Car5b, Carm1, Cars, Casp3, Casp4, Cblb, Cbx5, Ccl3, Ccl4, Ccnd3, Ccne1, Ccnyl1, Ccr5, Cct2, Cd22, Cd38, Cd69, Cdc25a, Cdc2a, Cdc45l, Cdca3, Cdca7, Cdh24, Cdk6, Cdkn1a, Cenpm, Chaf1a, Chaf1b, Chd3, Chd7, Chd7, Chd7, Chek1, Cherp, Chsy1, Chtf18, Cirh1a, Cish, Cit, Ckb, Cks1b, Cldnd2, Clic4, Clpb, Cmpk2, Coro1c, Coro2a, Cpsf7, Csda, Csrnp1, Cxcl10, Cyfip2, Cysltr2, D17Wsu92e, D4Ertd22e, D8Ertd82e, Dazap1, Dcakd, Dctd, Ddx18, Ddx54, Dek, Dennd4a, Dennd4a, Dennd4a, Dennd4a, Dennd4a, Dennd4a, Dennd4a, Dennd4a, Dennd4a, Dennd4b, Dgat1, Dgkh, Dhfr, Dhx58, Dis3, Dkc1, Dnajb11, Dnajc13, Dot1l, Dusp16, Dusp5, E030037K03Rik, E2f1, E2f4, Eftud2, Egr1, Egr2, Ehd4, Eif2ak2, Eif2c2, Eif2s2, Eif3a, Eif3b, Eif4a1, Eif4a1, Eif4enif1, Eif4g1, Eif5a, Eif5a, Elavl1, Elmo2, Entpd1, Eprs, Ern1, Errfi1, Etnk1, Etv6, Ezh2, F2r, F2rl2, F730047E07Rik, Fam120a, Fam188a, Fanca, Fbl, Fen1, Fgr, Fignl1, Fnbp4, Fndc3a, Fosl2, Foxk2, Fubp1, Gadd45g, Gapdh, Gbp4, Gdf11, Gfpt1, Gins2, Glcci1, Gm10521, Gm10537, Gm12070, Gm12250, Gm15470, Gm2173, Gm4955, Gm5069, Gm5145, Gm5523, Gm5785, Gm614, Gm6560, Gm6742, Gm6907, Gm6981, Gm8587, Gm9034, Gnaq, Gnb5, Gns, Got2, Gpd2, Gpr171, Gpr52, Gramd4, Gsk3a, Gspt1, Gsta3, H2-T24, Hdlbp, Hectd1, Hells, Herc5, Hic1, Hif1a, Hist1h3a, Hist1h3f, Hist1h3g, Hist2h3b, Hist2h3c1, Hk2, Hmgn2, Hnrnpl, Hpvc-ps, Hsd3b7, Hsf1, Hsh2d, Hsp90aa1, Hsp90ab1, Hspa5, Hspe1, Hspe1, Huwe1, Hyou1, Iars, Icam1, Ide, Ifih1, Ifitm1, Ifitm3, Ifng, Ifrd2, Ifrg15, Igf2r, Il12rb1, Il12rb2, Il18bp, Il2ra, Il6st, Ints7, Ipo4, Ipo5, Ipo7, Ipo9, Irf1, Irf7, Irf8, Irgm1, Isg20, Jun, Jund, Kat2a, Kpnb1, Krtap10-10, Ksr1, Lag3, Larp1, Larp1, Larp4, Larp4, Larp4, Lig1, Litaf, Lmf2, Lmnb1, LOC100044102, LOC100044416, LOC100047557, LOC433762, LOC641050, LOC670326, Lpp, Lrrc4, Luzp1, Lyar, Map2k4, Map2k7, Map3k8, Mapk6, Maz, Mcm10, Mcm3, Mcm4, Mcm5, Mcm6, Mcm7, Mcph1, Mctp2, Med13l, Med15, Mettl1, Mfsd2, Midn, Mink1, Mlkl, Mllt6, Morc2a, Mov10, Mpa2l, Ms4a6b, Msmp, Mtbp, Mthfd1l, Mthfd2, Muc1, Mx1, Mxd1, Mybl2, Myd88, Myo19, Nacc1, Nap1l1, Ncapg2, Ncl, Nefh, Nek6, Nf1, Nfil3, Nfkbib, Nfkbiz, Nkrf, Nle1, Nlrc5, Noc4l, Nolc1, Nop58, Notch1, Nphp4, Npm1, Nr4a1, Nr4a2, Nr4a3, Nup107, Nup205, Nup98, Oas1a, Oas1g, Oas3, Oasl1, Oasl2, Obfc2a, Obfc2b, Odc1, Odc1, Orc6l, Osm, Otud4, P2ry14, P4hb, Pa2g4, Pabpc4, Pabpc4, Parp12, Parp14, Parp9, Patl1, Paxip1, Pcgf2, Pcna,, Pdia3, Pdia6, Pds5a, Pecam1, Phf11, Picalm, Piga, Pik3ap1, Pim1, Pitpnm2, Pkm2, Pkmyt1, Pla2g6, Plcg2, Plk1, Plk3, Plk4, Plod2, Pmepa1, Pmf1, Pml, Pnet-ps, Pola1, Pole2, Polq, Pols, Pom121, Pop1, Pou5f2, Ppm1h, Ppp1r14b, Ppp1r15b, Ppp1r16b, Ppp1r3b, Pprc1, Prim1, Prp2, Psmd1, Psme1, Psme3, Ptma, Ptpn2, Ptpre, Purb, Pus7, Pvr, Pyhin1, R3hdm1, Rad51, Rad54l, Ranbp1, Rbl1, Rcc1, Rcc2, Rexo1, Rfc3, Rgs16, Riok1, Rnaseh2b, Rnf126, Rnf19b, Rnf213, Rrbp1, Rrm1, Rrm2, Rrp1b, Rsad2, Rtp4, Runx1, Samhd1, Sbno2, Scarb1, Sco1, Sdc3, Sec24c, Sema7a, Senp1, Serpinb9, Serpinb9b, Setbp1, Sf1, Sf3a2, Sfrs2, Sfrs4, Sfrs6, Sh3bp2, Shmt1, Sipa1l1, Sipa1l3, Slamf7, Slc16a6, Slc1a5, Slc25a22,*

Table 3.2A Mouse genes significantly induced post-MCMV infection.
The genes were identified using microarray and clustered according to their expression profile into five groups from early effector responses to memory-like NK cell responses. Data was obtained from timepoints post infection at day 1.5, day 7, and day 27. Source: Bezman et al. 2012.

Category	Significant Genes
Early effector	*Slc29a1, Slc35b1, Slc35f2, Slc41a1, Slc7a6, Slfn8, Smarca4, Smc2, Smchd, Snd1, Snhg1, Snhg1, Snrpa, Snx9, Socs1, Socs3, Solh, Sos1, Spna2, Spred2, Srcap, Srf, Srrm1, Srxn1, Ssr1, Ssrp1, St6galnac4, Stat1, Stat2, Stat3, Stk39, Stk40, Stx11, Sulf2, Sult2b1, Suv39h1, Syncrip, Syvn1, Taf1d, Taf4b, Taf6, Tagln2, Tap1, Tbkbp1, Tbrg4, Tbx21, Tcerg1, Tcf19, Tcof1, Tfrc, Tgfb1, Tgm2, Thada, Thoc4, Thop1, Ticam1, Timeless, Tk1, Tle3, Tmem106a, Tmem184b, Tmem2, Tmprss6, Tnfaip3, Tnks1bp1, Tomm40, Top2a, Topbp1, Tpst1, Tpx2, Trafd1, Trak1, Trak2, Treml2, Trim30, Trip13, Trp53, Ttll4, Tuba1b, Tuba1c, Txn2, Ubap2, Ubap2l, Ube2m, Uck2, Uck2, Uhrf1, Ung, Usp18, Usp2, Usp25, Usp31, Usp37, Vac14, Vasp, Vps37b, Wars, Wbp7, Wdhd1, Wdr43, Wdr62, Wee1, Whsc1, Wiz, Wnk1, Xaf1, Xcl1, Xdh, Xpot, Yrdc, Zbp1, Zbtb32, Zc3h4, Zcchc2, Zcchc7, Zfp335, Zfp367, Zfp384, Znhit1, Zranb3*
Late effector	*1700017B05Rik, 1700066M21Rik, 1700081L11Rik, 2310034G01Rik, 2900026A02Rik, 4931440P22Rik, A530032D15Rik, A630072L19Rik, Arap3, Atxn1, Atxn7l1, Avpr2, BC059842, BC059842, Cbfa2t3, Cdc20, Cercam, Chd7, Ckm, Cnnm2, Dennd1c, Depdc1b, Dnm1, Dock5, Fam117a, Fbrs, Foxk1, Fry, Ggnbp1, Gm10124, Gm10825, Gm10838, Gm2889, Gm5589, Gm8485, Gm8956, Gm8985, Gna15, Grm2, Haao, Hamp2, Hip1, Hmgb2, Inpp4a, Jarid2, Kifc1, Klf11, Klf13, Klrg1, LOC100046894, Lphn1, Lphn1, Maml2, Mef2d, Mlxip, Mnt, Mospd3, Mt1, Myo1e, Nacad, ND4L, Ngfr, Ntng2, Olfr566, Os9, Palm, Pdcd11, Pik3cb, Plekho1, Pltp, Prss27, Ptms, Ptp4a3, Ptprs, Racgap1, Rangrf, Rbm38, Rhbdf2, Rnf43, Setd1b, Sik1, Slain2, Slc36a1, Slc9a3r1, St3gal3, St6galnac6, Tet3, Tmem221, Trp73, Tspan31, Ttyh2, Ube2c, Zdhhc20, Zmiz1*
Sustained effector	*AF362573, Arhgef1, ATP6, Aurkb, B3gnt7, BC059842, BC059842, BC059842, BC059842, BC059842, BC059842, C330019G07Rik, Ccna2, Ccnb2, Cdc6, Cdca2, Chd7, Cyfip2, Dgkz, Diap3, Dock6, Dtl, E2f2, E2f7, E2f8, Eif3c, Eif4g3, Epn1, Foxm1, Fyb, G530012D18Rik, Glcci1, Gm6041, Gopc, Grb7, Grn, Hmgn2, Itpr1, Jak3, Kif11, Kif18b, Kif23, Lrdd, Map3k11, Mark2, Mbd6, Mki67, Mll2, Msl1, Ncapd2, Ncaph, Nlrc5, Pctk1, Prkcz, Psme2, Rnf213, Runx3, Scaf1, Slc43a3, Stom, Thy1, Traf4, Trerf1, Troap, Tyms, Zfhx2*
Infection signature	*AY036118, BC049688, Chd7, F730043M19Rik, Gm12000, Gm7265, Gp49a, Hist1h1b, Hopx, Hsn2, Nlrc5, Trio, Trip11*
Memory	*1700029I01Rik, 2310001H12Rik, 2410080I02Rik, 2610020H08Rik, 3110052M02Rik, 4930444G20Rik, 4930522L14Rik, 4932438A13Rik, 5830415L20Rik, A830080D01Rik, AB010352, Ahsa2, Amotl1, Apoa2, Apold1, Arih2, Armc7, Arx, Atrx, B230307C23Rik, BC094916, Calm1, Camk2b, Casp1, Ccdc137, Ccdc85c, Ccnl1, Cdc20b, Cdkn2b, Cdv3, Cep57, Cetn4, Chadl, Chd7, Clock, Cnih2, Cog3, Dgkz, Dnajb9, Dnajc1, Dusp14, Efemp2, EG547347, ENSMUSG00000072643, Fam111a, Fam178a, Far1, Fasl, Fcho2, Fos, Gcc2, Gm10830, Gm11435, Gm12504, Gm13051, Gm13251, Gm15296, Gm15542, Gm4759, Gm6540, Gm6651, Gm7125, Gm8995, Gm9264, Gm9405, Gsc2, Gvin1, Hbb-b1, Hes1, Hmga1, Hmgb1, Hmox1, Hspa4l, Kif7, Klf12, Klra10, Klra6, Krt8, LOC100043371, LOC100044428, LOC100044517, LOC100045396, LOC638407, LOC640991, LOC676160, Lrrfip1, Ly6c1, Mdfic, Mdga1, Mrpl20, Nktr, OTTMUSG00000010657, Pisd-ps2, Pitx1, Pmaip1, Ppig, Ppp3cc, Ptpn4, Ptprv, Rab11fip4, Rab4a, Rbm25, Rpl17, Rsrc1, Sacs, Sltm, Stxbp3b, Thoc1, Top1, Tpr, Ttc14, Ypel1, Zfp125, Zfp182, Zfp292, Zfp599, Zfp871, Zfp97, Zufsp*

Table 3.2B Mouse genes significantly induced post-MCMV infection.
The genes were identified using microarray and clustered according to their expression profile into five groups from early effector responses to memory-like NK cell responses. Data was obtained from timepoints post-MCMV infection at day 1.5, day 7, and day 27. Source: Bezman et al. 2012.

3.1.3. Aims

I hypothesise that identifying genes which impact NK cell mediated control of viral infection will improve our understanding of the mechanisms regulating NK cell function. My aim is to use an *in vivo* MCMV infection screen to identify and then subsequently validate genes which impact on NK cell-mediated control of virus infection.

3.2 Results

3.2.1. Design of an *in vivo* screen for investigating the role of putative NK cell regulators during MCMV infection

The 69 strains of mice deficient in NK cell-related genes were generated by the WTSI using CRISPR-Cas9 technology and using the EUCOMM/KOMP embryonic stem cell collection (see section 2.2) as part of the Sanger Mouse Genetics Project. Tissue culture passaged MCMV Smith strain (or Δm157 MCMV) used for mouse infections were grown, purified and titred by myself, Morgan Marsden, and Lucy Chapman (see section 2.4 and 2.5). Δm157 MCMV was used to decipher whether loss of the Ly49H-m157 interaction, which is important for NK cell control of MCMV infection, impacted on viral replication in gene-deficient mice. Mice were infected on day 0 before harvesting the spleens and livers on day 4 (Figure 3.1). In some cases, tissue was taken on day 7 to understand whether selected gene deficiency impacted on prolonged control of MCMV replication. Mouse weight was monitored throughout the duration of the experiment as a systemic measure of pathogenesis and to ensure suffering was within boundaries defined by the UK Home Office (set at 20% weight loss by PPL P7867DADD and 25% by PPL P653704A5). These parts of the study were performed at the WTSI by Simon Clare, Cordelia Brandt, and Katherine Harcourt. In the case of *Sytl3*$^{-/-}$ mice all aspects of the *in vivo* experiment were performed by myself in Cardiff. All frozen mouse organ samples from the WTSI were shipped to Cardiff for analysis, which I performed. Plaque assays were used to measure viral replication within spleen and liver snap frozen tissue derived from the gene-deficient mice.

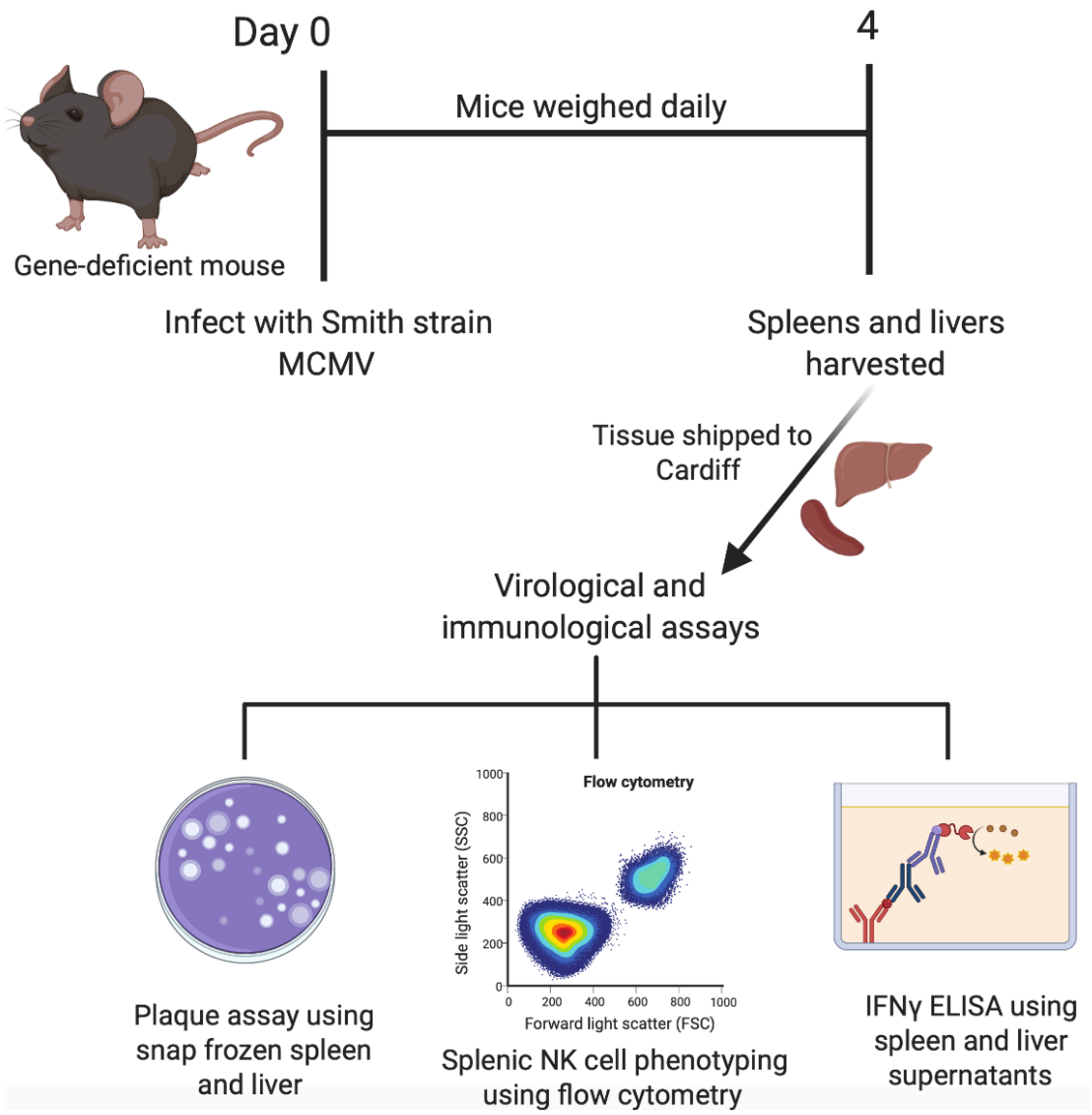

Figure 3.1. Schematic of the *in vivo* MCMV infection screen of gene-deficient mice and tissue analysis.
All mice were tested at a timepoint of day 4 p.i. using Smith strain MCMV before spleens and livers were harvested and frozen for analysis. All snap frozen spleen and liver samples were analysed using a plaque assay to calculate the amount of viral replication. Where plaque assay data was of interest, NK cells from frozen splenocyte samples were phenotyped using flow cytometry and/or tissue supernatants left over from plaque assays were used in an ELISA to investigate the amount of IFNγ protein.

Where differences in viral replication control were observed in the *in vivo* MCMV infection screen, evidence from online gene expression resources was collected to shortlist genes of interest. The gene expression data was used to understand whether the genes were exclusively expressed by NK cells and therefore likely to have importance in NK cell infection control or also expressed by other immune cells. The Immunological Genome Project (Immgen) microarray and ultra-low input RNA-Seq databases which present expression profiles of a selected gene within a chosen group of cell types from male 6-10 week-old C57BL/6J mice analysed by a consortium of laboratories enabled me to identify which immune cell types expressed my genes of interest. Genes which were almost exclusively expressed by NK cells were of most interest. The database from Singhania et al. 2019 was also used to determine genes of interest which quantified gene expression in female 6-18 week-old C57BL/6J mouse whole blood 2 days post-MCMV infection. Genes which were upregulated upon MCMV infection indicated potential importance in virus control. The gene expression data from these online sources is included in this chapter for each gene of interest. This evidence helped me identify six genes of interest to investigate further. The important factors for deciding on these genes of interest are shown Figure 3.2.

Further *in vivo* experiments were completed for each gene-deficient line of interest and this data is presented in the following chapter. This included determining whether changes in virus replication were reproducible in experiments using the same tissue-culture passaged Smith strain MCMV or more pathogenic salivary gland passaged MCMV. In some cases, gene-deficient lines were challenged with Δm157 MCMV to understand whether the Ly49H-m157 interaction influenced the impact of gene deficiency on control of viral replication.

Where changes were observed in weight or viral replication control in the spleen and/or liver between WT and gene-deficient mice in the *in vivo* screen, NK cells from mouse splenocytes were phenotyped using flow cytometry to gain an initial indication if and how NK cell function was affected by gene loss. Mouse splenocytes were prepared from half the spleens in the original screen as the other half was snap frozen and used to detect viral replication in a plaque assay. A portion of mouse liver was also snap frozen for analysis using a plaque assay. I investigated a variety of murine NK cell markers including the cytotoxic marker granzyme B, the activating receptor Ly49H and maturation markers CD11b and CD27. In addition, frozen spleen and liver supernatants left over from plaque

assays were assessed for IFNγ protein using an ELISA. Evidence from all the experiments was used to decide whether to continue investigating each gene and the reasoning is discussed in the following chapter.

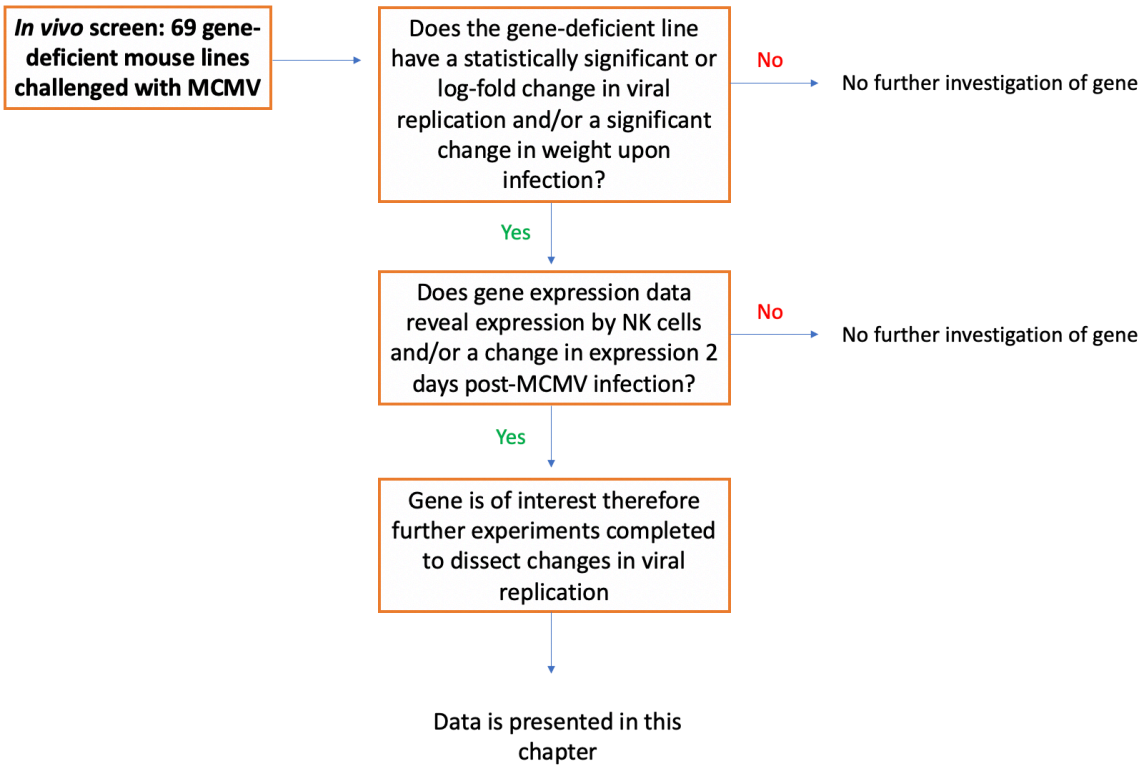

Figure 3.2 Decision map showing how the genes of interest were chosen.
Several factors were considered including the difference in viral replication observed between WT and gene-deficient mice upon infection, difference in weight between WT and gene-deficient mice, gene expression by NK cells and other immune cells and changes to gene expression upon MCMV infection.

3.2.2. Many NK cell-expressed genes do not influence control of MCMV replication *in vivo*

Gene loss in many of the mouse lines investigated had little impact on weight and viral burden in spleen and liver tissue. Table 3.3 is a heatmap summary of the 69 gene-deficient mouse lines and their response to MCMV compared to WT mice. Appendix I contains the original MCMV screen data for each gene in the order presented in summary Table 3.3. In the rest of this chapter, I will now describe the six genes of interest I selected from the screen due to their potential importance controlling viral replication. Mouse gene expression data is shown to support my reasoning for choosing each gene of interest. Then I will describe the *in vivo* screen data, repeat data and other findings for each of the selected genes. The availability of mouse stocks and disruption due to COVID-19 restricted the number of *in vivo* experiments that were possible. I will also explain my reasoning for deciding to continue or not continue investigating each gene of interest.

Gene	Genotype	Weight	Splenic virus replication	Liver viral replication
A830080D01Rik	Hom (X linked)			
Acaa2	Hom			
Apold1	Hom	Decrease		
Brox	Hom			
Ccdc85c	Het			
Ccnl1	Hom	Increase	Decrease	
Cdc20b	Hom			
Cdca2	Hom			
Cetn4	Hom			
Chadl	Hom	Increase		
Chtf8	Het			
Cnpy2	Hom			
Dnajb9	Hom	Decrease	Decrease	Decrease
Dnajc1	Hom			Increase
Dusp14	Hom			
Ergic3	Hom	Increase	Increase	Decrease
Etv3	Hom			
Fam111a	Hom		Decrease (p=0.2984)	Decrease (p=0.2075)
Fam114a2	Hom			Decrease (**p=0.0043)
Fam133b	Het	Decrease		
Fam178a	Het	Decrease		
Fam196b	Hom			
Fam219b	Hom	Increase		
Far1	Hom			Decrease (p=0.3074)
Gcc2	Hom	Increase		
Glcci1	Hom	Increase		
Gzme	Hom			
Gzmf	Hom	Decrease		
Heatr9	Hom	Decrease	Increase (*p=0.0273)	Increase (p=0.1802)
Kbtbd11	Hom	Increase		
Kif23	Het			
Klra6	Hom			
Klrc1	Hom			
Klrc2	Hom		Increase	

Table 3.3A Heatmap summarising the gene-deficient mouse lines and their response to MCMV infection compared to WT mice.
Weight was recorded and viral replication in the spleen and liver was measured using a plaque assay. Genes which impacted MCMV virus replication and/or weight control compared to WT are highlighted in yellow and where significant in red. p values are shown for genes of interest (green) discussed in this chapter.

Klrc3	Hom			
Klrc1to3	Hom			
Ly6f	Hom			
Ly6g	Hom	Increase		
Mdfic	Hom	Increase	Decrease	Decrease
Mrpl20	Het		Decrease	Decrease
Msl1	Het	Increase		Decrease
Ncapd2	Het			
Palm	Hom			
Plekhf1	Het			
Pogk	Hom			
Pou6f1	Hom			
Prr14l	Hom			
2610020H08Rik	Hom	Decrease		
Rsrc1	Hom		Increase	
Scaf1	Hom	Decrease	Increase	Increase
Serpinb9b	Hom		Increase (*p=0.0152)	Increase (p=0.2381)
Stom	Hom			
Sytl3	Hom	Decrease (*p=0.0441)	Increase (p=0.0628)	
Trerf1	Hom			Increase
Troap	Hom			
Ttc14	Het	Increase		
Zfp383	Hom			
Zfp53	Hom		Increase (**p=0.0048)	
Zfp54	Hom			
Zfp58	Hom			
Zfp599	Hom			
Zfp672	Hom			
Zfp748	Hom			
Zfp870	Hom			
Zufsp	Hom	Decrease		
Arpc1b	Hom		Decrease	
Zfp292	Het			
Sidt2	Hom	Increase		
IL6ra	Hom			

Not significant
Significant
Early indication

Table 3.3B Heatmap summarising the gene-deficient mouse lines and their response to MCMV infection compared to WT mice.
Weight was recorded and viral replication in the spleen and liver was measured using a plaque assay. Genes which impacted MCMV virus replication and/or weight control compared to WT are highlighted in yellow and where significant in red. p values are shown for genes of interest (green) discussed in this chapter.

3.2.3 *Family with Sequence Similarity 111 Member A (Fam111a)*

The protein encoded by the gene *Fam111a* is cell cycle regulated and is localised within the nucleus (Tarnita, Wilkie and DeCaprio, 2019). Fam111a is believed to control parathyroid hormone production, calcium homeostasis and skeletal development and growth (Unger *et al.*, 2013). Mutations in *FAM111A* have been linked to Kenny-Caffey Syndrome (KCS) and Osteocraniostenosis (OCS) which are conditions characterised by impaired skeletal development with small and dense bones, short stature, and primary hypoparathyroidism with hypocalcemia (Unger *et al.*, 2013). FAM111A was also found to play a role in Simian Virus 40 (SV40) (Fine *et al.*, 2012) and poxvirus (Panda *et al.*, 2017) host range restriction. The physical interaction of fam111a and the C-terminal region of SV40 is required for efficient viral replication and sustained viral gene expression in restrictive cell types (Fine *et al.*, 2012).

The WTSI selected *Fam111a* for the *in vivo* infection screen as it was significantly induced post-MCMV infection in memory-like NK cells (Bezman *et al.*, 2012). Gene expression data from Immgen highlighted *Fam111a* expression across a wide range of immune cell types including NK cells. However, bone marrow derived neutrophils showed the greatest level of *Fam111a* gene expression and high expression was observed in classical monocytes, and B-cell types including splenic follicular B-cells, splenic germinal centre B-cells, splenic marginal zone B-cells and B-cells from the peritoneal cavity. In comparison, expression of *Fam111a* by NK cell types was relatively low and the NK cell subtype with the greatest *Fam111a* expression was splenic NK cells 1-day post-MCMV infection (Figure 3.3). Prior to memory-like NK cell development at 2 days post-MCMV infection, *Fam111a* gene expression was significantly upregulated within whole blood *in vivo* (*$p<0.0001$, Figure 3.4). Thus, *Fam111a* was identified as an NK cell-expressed gene that may be of possible interest in anti-MCMV immune responses.

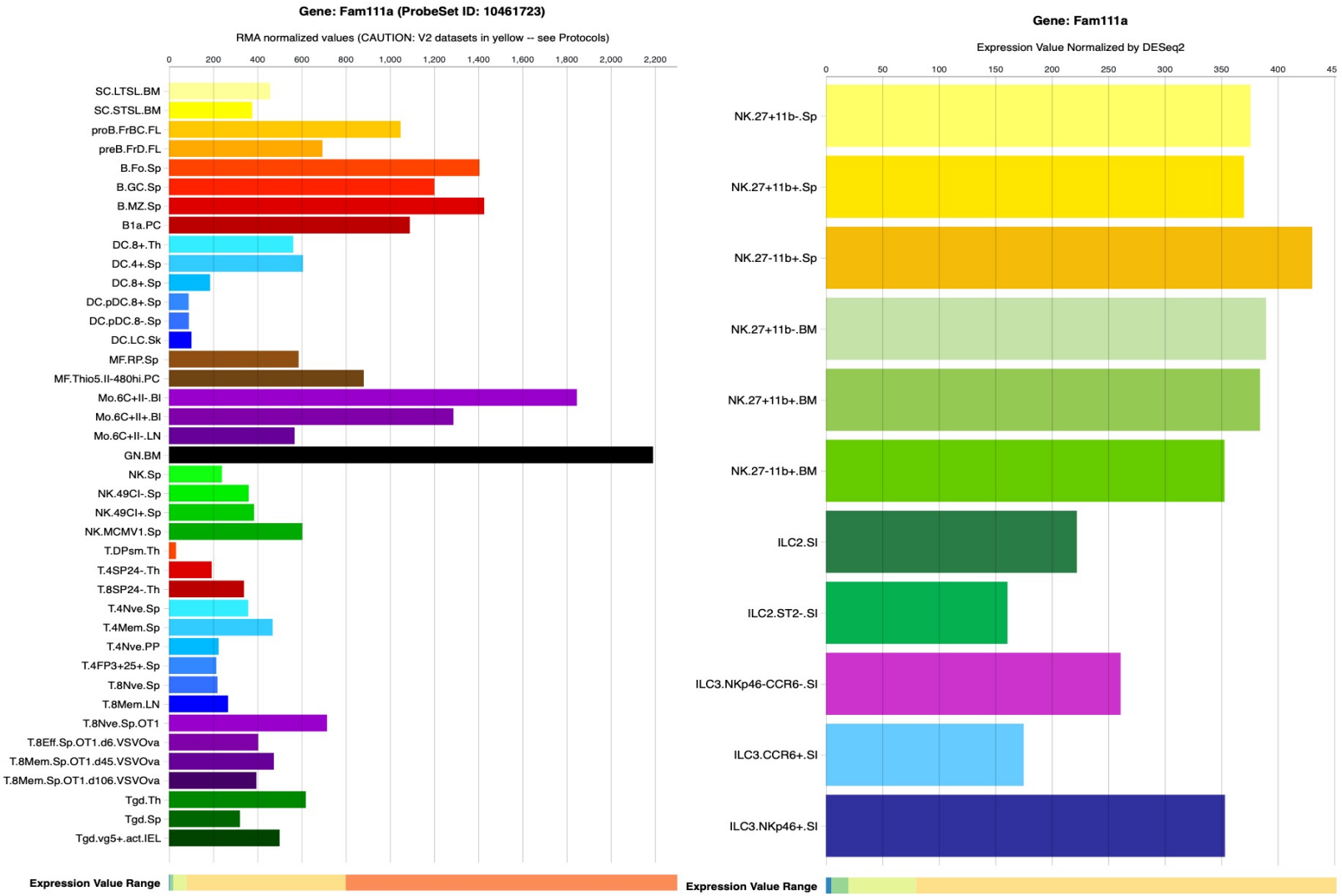

Figure 3.3 *Fam111a* is expressed across a variety of immune cell types.
Mouse *Fam111a* gene expression data from: microarray in various immune cell types (left) and RNASeq in NK cell and ILC types (right). Source: immgen.org.

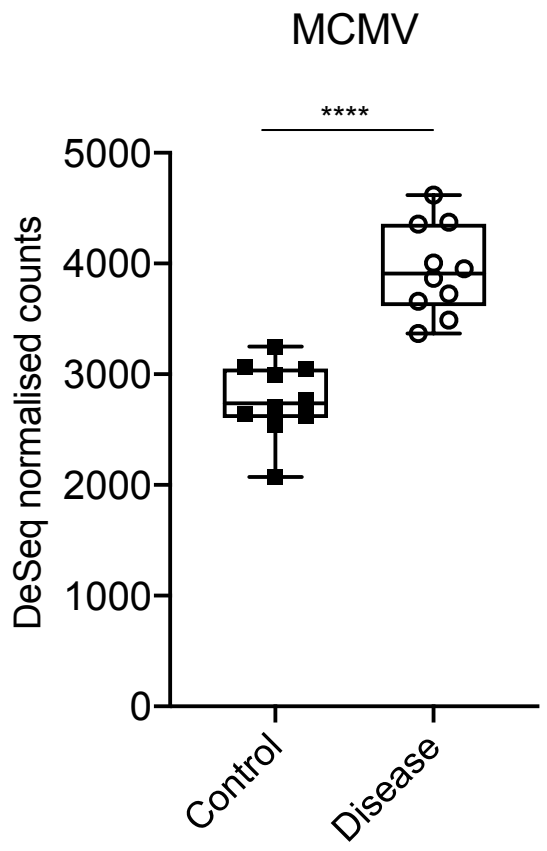

Figure 3.4 *Fam111a* gene expression increases 2 days post-MCMV infection in mouse whole blood.
Female C57BL/6 mice were infected with $3x10^4$ PFU of salivary gland prepared Smith strain MCMV and compared to uninfected control mice. Total blood RNA was extracted, and gene expression was measured using RNA-Seq. Data is expressed as box and whisker diagrams and individual values from each mouse are shown (10 mice per group). ****$p<0.0001$. Source: Singhania et al. 2019.

3.2.3.1 Loss of Fam111a during MCMV infection improves control of viral replication

To examine whether *Fam111a* influences anti-MCMV immune responses, WT and *Fam111a*$^{-/-}$ mice were infected with MCMV, and their weight was measured daily before splenic and liver virus load was measured after 4 days. *Fam111a*$^{-/-}$ mice lost weight in a similar pattern to WT mice (Figure 3.5A). However, *Fam111a*$^{-/-}$ mice exhibited ~1 log-fold reduction in viral replication in the spleen which, although not statistically significant, implied that *Fam111a* impinged upon antiviral immune responses. In the spleen, NK cells predominantly induce their effector function through cytotoxicity (Tay and Welsh, 1997a) and *Fam111a* may therefore impact on this. In the liver, there was no difference in viral replication between groups (Figure 3.5B). As *Fam111a*$^{-/-}$ mice had ~1 log-fold impairment in antiviral control (Figure 3.5) and NK cells expressed *Fam111a* (Figure 3.3), *Fam111a* was selected for further investigation.

Next, to understand whether the change in virus control between *Fam111a*$^{-/-}$ and WT mice was robust, I performed a repeat experiment. Again, infection-induced weight loss was comparable in WT and *Fam111a*$^{-/-}$ mice (Figure 3.6A), although weight loss in both groups was less than previously observed (Figure 3.5A). Furthermore, when virus replication was measured in tissue, there was no difference in MCMV replication in the liver and spleen of *Fam111a*$^{-/-}$ mice compared to WT mice. I hypothesised that with a lower level of infection, any defect in virus control in the absence of *Fam111a* would be less obvious. In both WT and *Fam111a*$^{-/-}$ groups, measurable MCMV replication was tenfold lower than the original data. As there was a low level of infection in the repeat data, I decided not to exclude *Fam111a* from further investigation.

To determine whether *Fam111a* influenced NK cell accumulation, expression of activation markers and NK cell maturation 4 days post-MCMV infection, frozen splenocytes from the *in vivo* screen were analysed using flow cytometry. Of note however, this data was obtained using splenocytes from the repeat experiment where there was a low level of infection and no alteration in viral replication between groups, therefore analysis of NK cell responses from mice which had a greater level of infection is also required. In this initial experiment I observed no difference in NK cell accumulation between WT and *Fam111a*$^{-/-}$ mice (Figure 3.7). There was also no significant difference in the percentage of granzyme B+ NK cells or the MFI of granzyme B, suggesting no impact of *Fam111a* deficiency on cytotoxic content (Figure 3.8A). Furthermore, *Fam111a* did not impact on Ly49H^{+} NK cell

development as the percentage of Ly49H+ NK cells and the MFI of Ly49H on NK cells at 4 days p.i. were comparable in WT and *Fam111a*$^{-/-}$ mice (Figure 3.8B). Next, I assessed whether there were any changes to the percentage of NK cells at each stage of maturation as defined by the level of CD11b and CD27 expression (Chiossone *et al.*, 2009). There was a trend showing a reduced percentage of CD11b^{-}CD27^{-} (immature) NK cells in *Fam111a*$^{-/-}$ mice compared to WT mice (Figure 3.10, p=0.0649). As a result, analysis of splenic NK cells from mice with a greater level of infection may reveal an effect on splenic NK cell maturation.

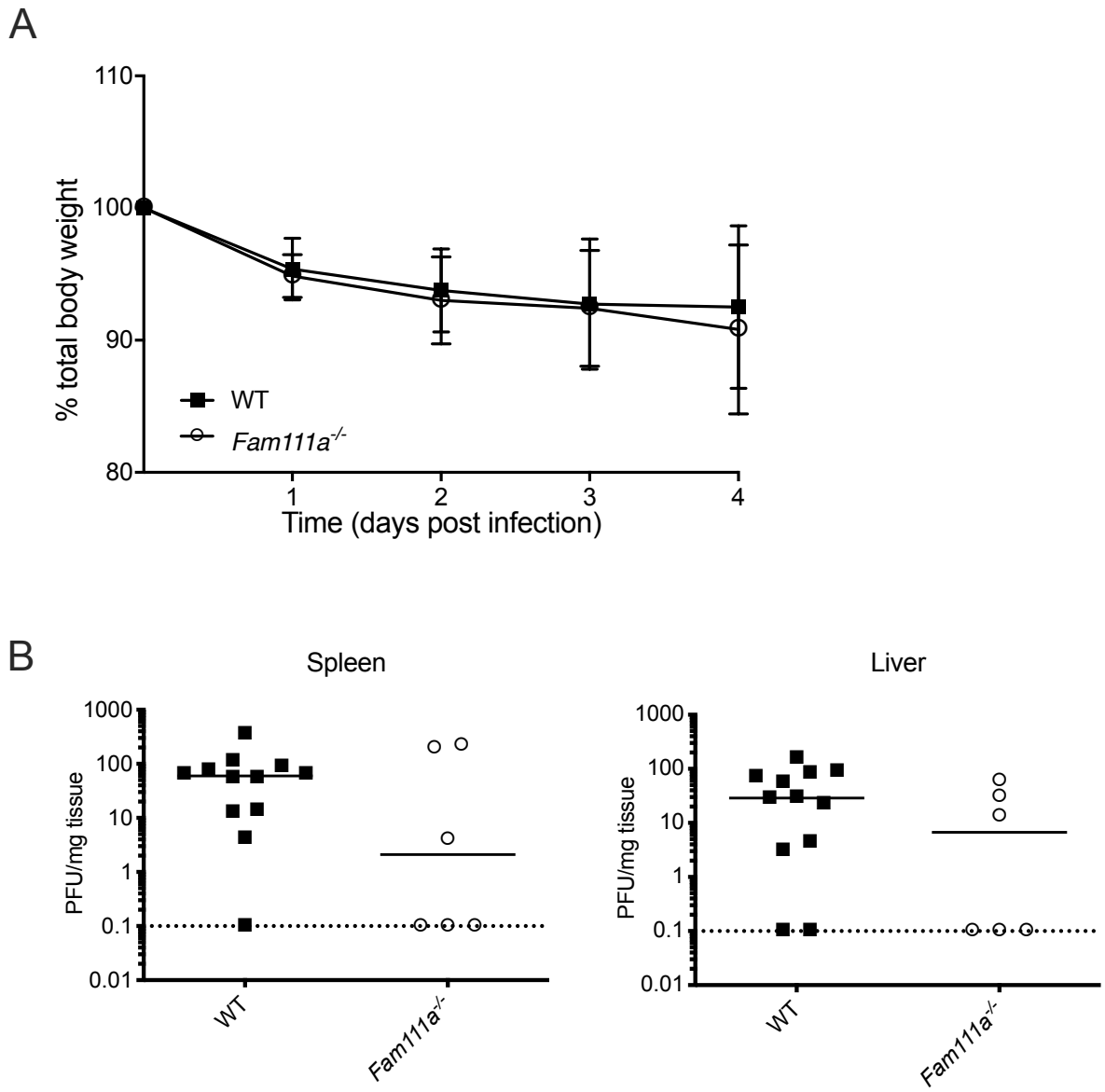

Figure 3.5 *Fam111a*$^{-/-}$ mice have a trend of reduced viral replication in the spleen post-MCMV infection.

(A) Percentage total body weight of *Fam111a*$^{-/-}$ mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Fam111a*$^{-/-}$ mouse spleen (p=0.2984) and liver (p=0.2075). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

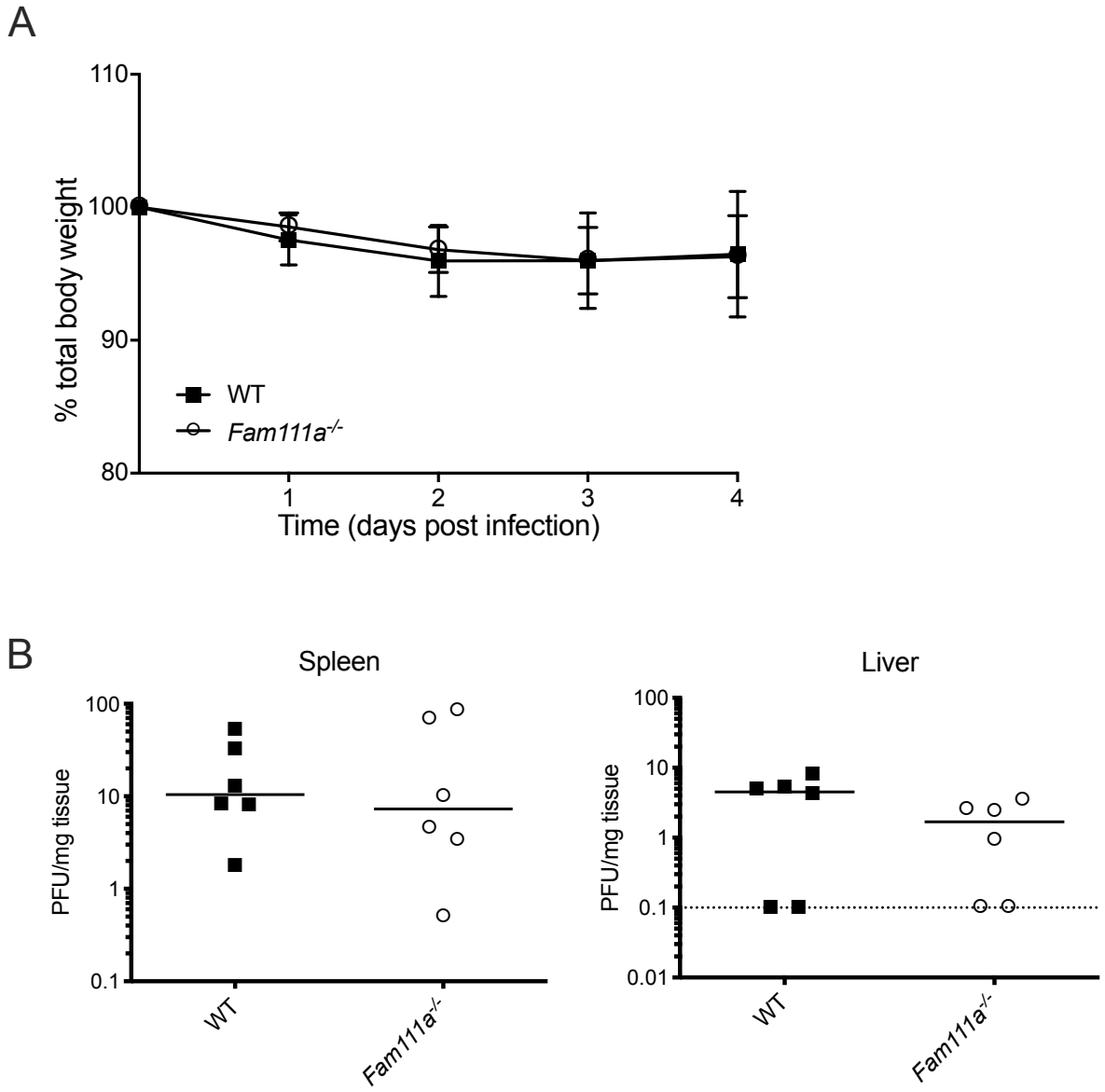

Figure 3.6 Repeat experiment demonstrating no difference in virus replication in *Fam111a*$^{-/-}$ mice.

(A) Percentage total body weight of *Fam111a*$^{-/-}$ mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Fam111a*$^{-/-}$ mouse spleen (p=0.9372) and liver (p=0.2078). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

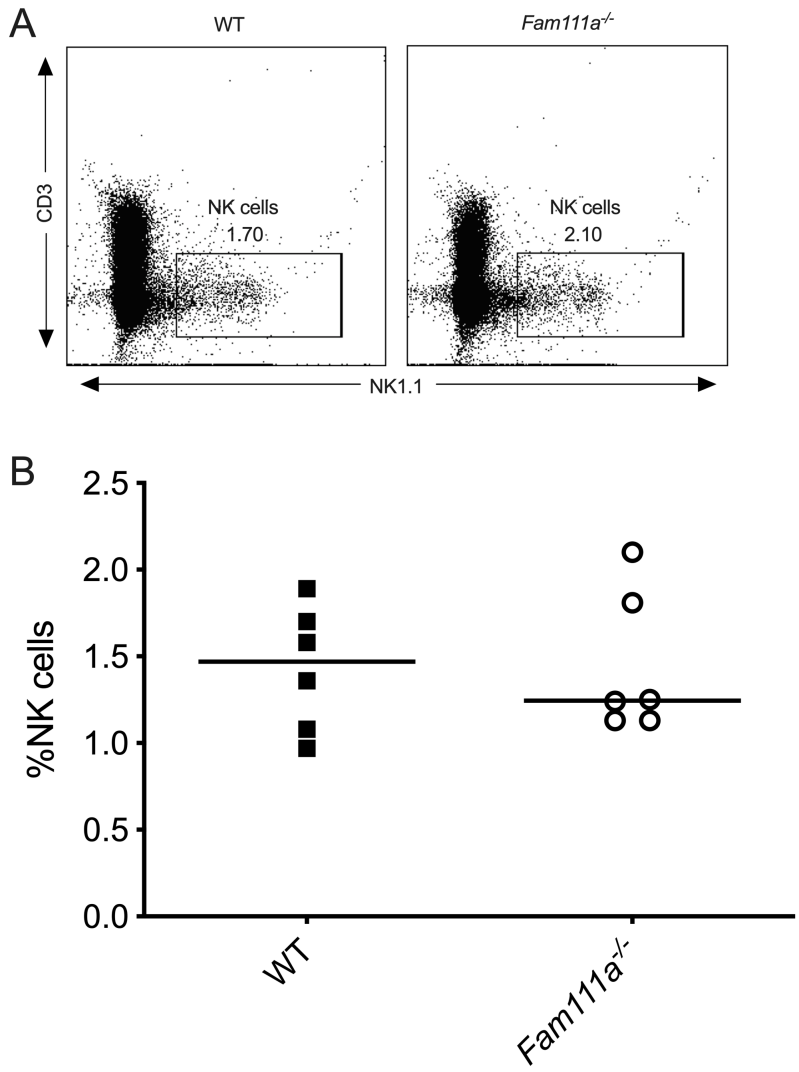

Figure 3.7 The number and proportion of splenic NK cells is unaffected by *Fam111a* deficiency 4 days post-MCMV infection.
(A) Representative flow cytometry plots showing gated NK cells from WT and *Fam111a*$^{-/-}$ mice 4 days post-MCMV which were defined as NK1.1^{+}CD3^{-}. (B) The percentage of NK cells from the total live lymphocyte population in WT and *Fam111a*$^{-/-}$ mice. Individual mice and median are shown (6 mice per group). p=0.9004

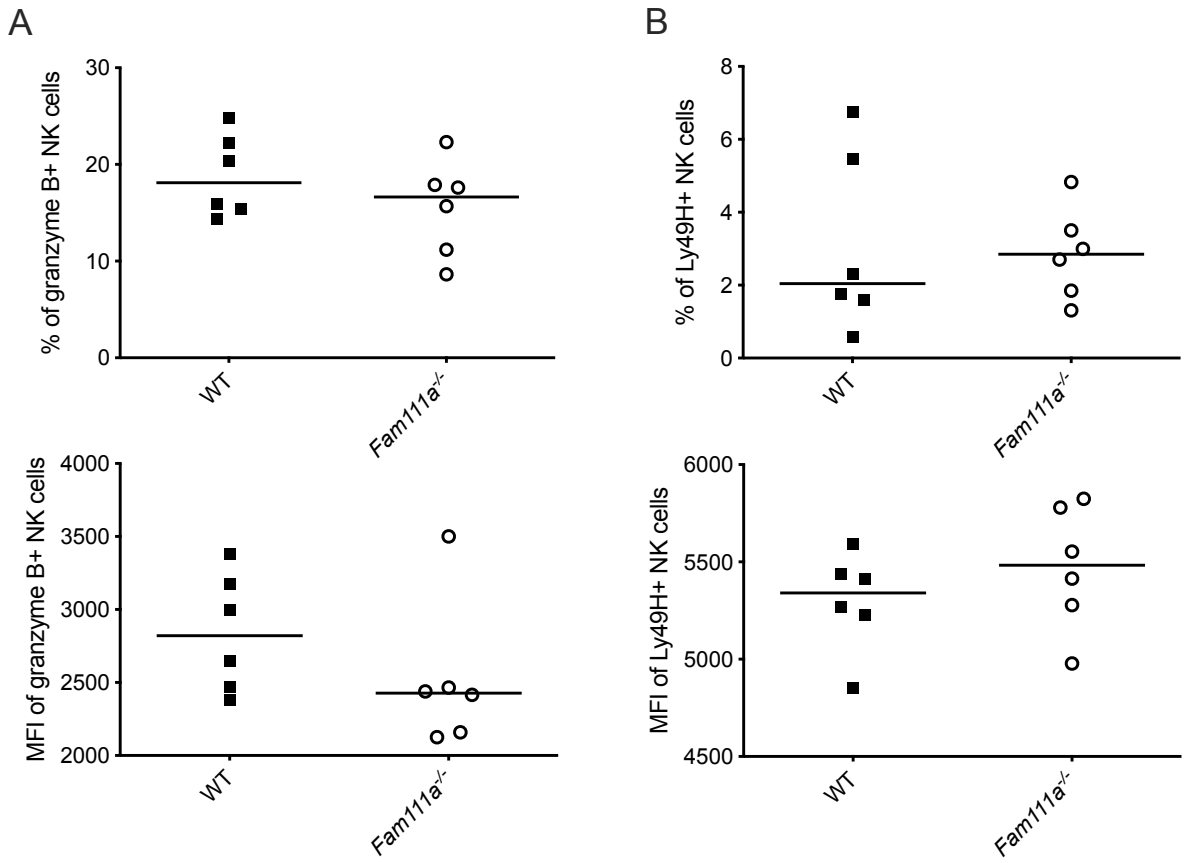

Figure 3.8 *Fam111a* does not impact on granzyme B and Ly49H activating marker expression 4 days after MCMV infection.
(A) The percentage of granzyme B+ NK cells (p=0.4848) and the intensity of expression (MFI) of granzyme B+ NK cells (p=0.4848) from WT and *Fam111a*$^{-/-}$ mice 4 days post-MCMV infection. (B) The percentage of Ly49H+ NK cells (p=0.9372) and the mean fluorescence intensity (MFI) of Ly49H+ NK cells (p=0.3939). Individual mice and median are shown (6 mice per group).

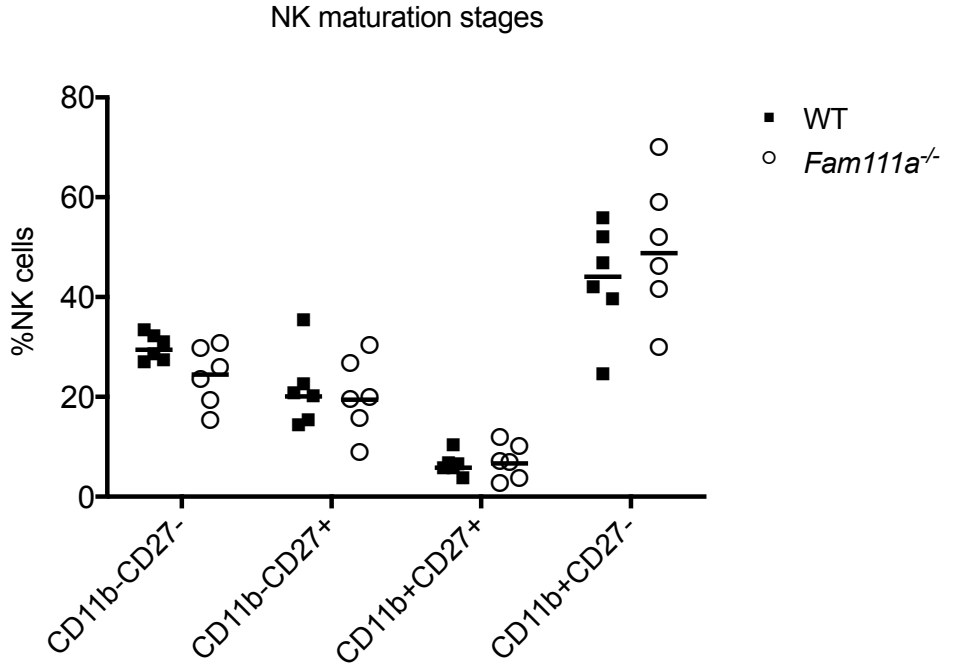

Figure 3.9 The percentage of NK cell maturation subsets does not change between WT and *Fam111a*$^{-/-}$ mice 4 days post-MCMV infection.

The percentage of NK cell maturation populations from immature ($CD11b^{-}CD27^{-}$) to mature ($CD11b^{+}CD27^{-}$) from WT and *Fam111a*$^{-/-}$ mouse splenocytes 4 days post-MCMV infection. Individual mice and median are shown (6 mice per group). $CD11b^{-}CD27^{-}$ p=0.0649, $CD11b^{-}CD27^{+}$ p=0.8182, $CD11b^{+}CD27^{+}$ p=0.5887, $CD11b^{+}CD27^{-}$ p=0.5310.

To understand whether *Fam111a* influenced control of MCMV later during acute infection, responses were studied at 7 days p.i. which is the timepoint when NK cell expansion peaks (Schlub *et al.*, 2011; Sun *et al.*, 2011). Memory-like NK cells were shown to express *Fam111a* (Bezman *et al.*, 2012), therefore, I hypothesised that *Fam111a* may be required during this timepoint when Ly49H+ NK cells are enriched. As previously observed in the *in vivo* infection screen (Figure 3.5 and Figure 3.6), both groups lost weight similarly until day 4 (Figure 3.10A). After day 4, both groups began recovering their weight (Figure 3.10A). By day 7 there was no detectable viral replication in the liver and spleen of both mouse strains (Figure 3.10B). Thus, I concluded that *Fam111a* gene deletion did not influence MCMV replication control at later stages of acute MCMV replication.

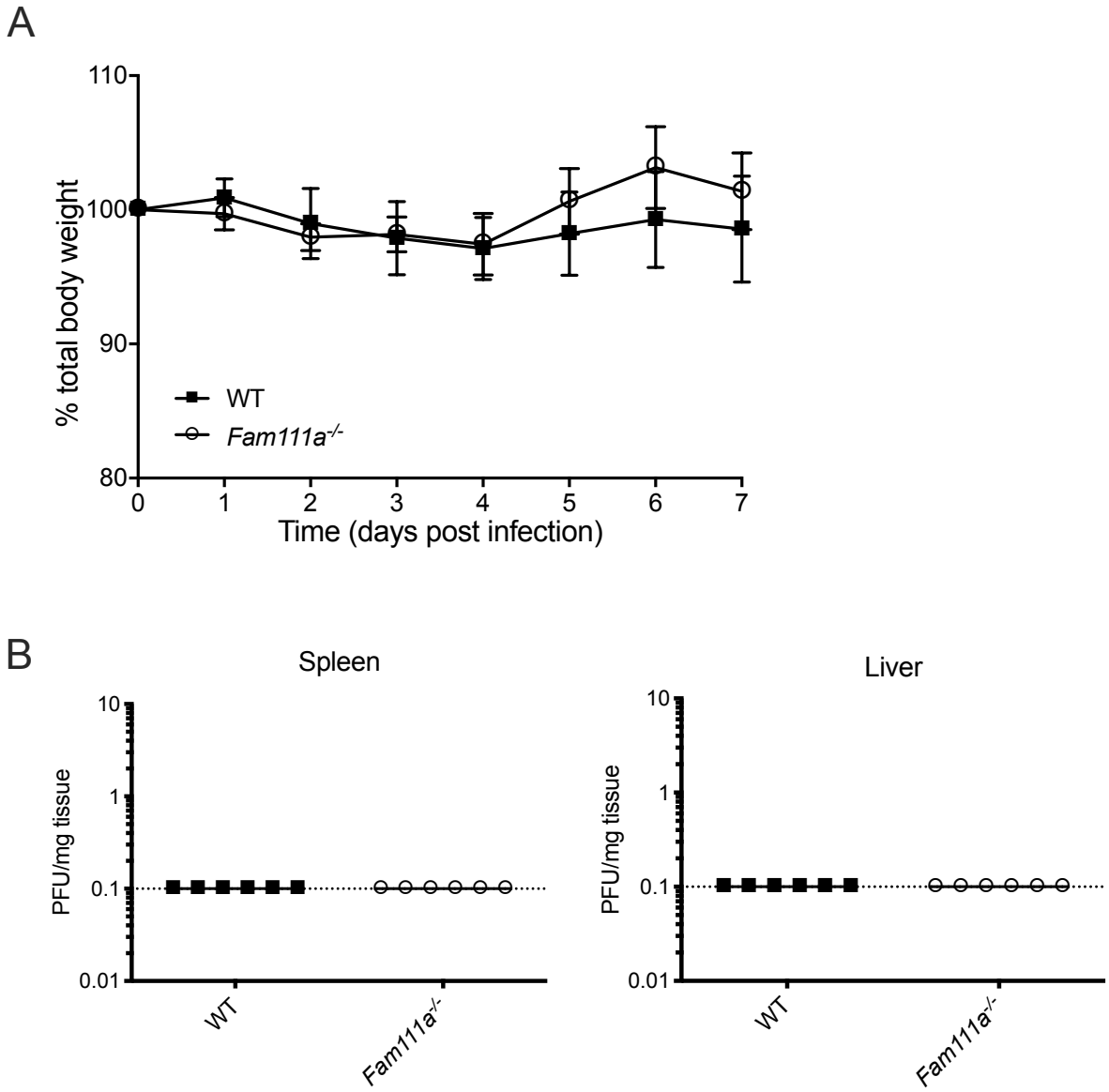

Figure 3.10 *Fam111a* does not impact control of MCMV 7 days after MCMV infection.

(A) The percentage total body weight in *Fam111a*$^{-/-}$ mice compared to WT mice 7 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Fam111a*$^{-/-}$ mouse spleen ($p>0.9999$) and liver ($p>0.9999$). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

Ly49H-mediated recognition of m157 MCMV glycoproteins triggers a strong activation signal in NK cells to induce effector functions (Daniels *et al.*, 2001; Arase *et al.*, 2002; H. R. C. Smith *et al.*, 2002; Parikh *et al.*, 2015). Therefore, more subtle effects of genes on NK cell functionality may be partially or entirely masked in the presence of the m157-Ly49H interaction. As a result, the effect of MCMV infection on *Fam111a*$^{-/-}$ mice in the absence of the m157-Ly49H interaction was investigated. Upon infection of WT and *Fam111a*$^{-/-}$ mice with Δm157 MCMV, a small reduction in weight in both WT and *Fam111a*$^{-/-}$ mice was observed (Figure 3.11A). In the spleen, both groups had viral replication at ~100 PFU/mg tissue suggesting there was a high level of systemic viral replication (Figure 3.11B). In the liver, all *Fam111a*$^{-/-}$ mice infected with Δm157 MCMV had complete loss of viral replication compared to WT where 2 out of the total 6 mice had complete loss of viral replication (*p=0.0152, Figure 3.11B), suggesting *Fam111a* deficiency improves MCMV virus control in the absence of the m157-Ly49H interaction.

I then sought to elucidate whether *Fam111a* gene deficiency influenced splenic NK cell accumulation, expression of activation markers and NK cell maturation 4 days post-Δm157 MCMV infection. No difference in splenic NK cell accumulation between WT and *Fam111a*$^{-/-}$ mice was observed (Figure 3.12) and there was no significant difference in the percentage of granzyme B+ NK cells, the MFI of granzyme B, the percentage of Ly49H+ NK cells and the MFI of Ly49H 4 days post-MCMV infection (Figure 3.13). In addition, there was no difference in the percentage of NK cells at each maturation stage (Figure 3.14). Analysis of liver NK cells is required to inform on phenotypic changes which may contribute to the complete loss of viral replication in the liver upon Δm157 MCMV infection (Figure 3.11B).

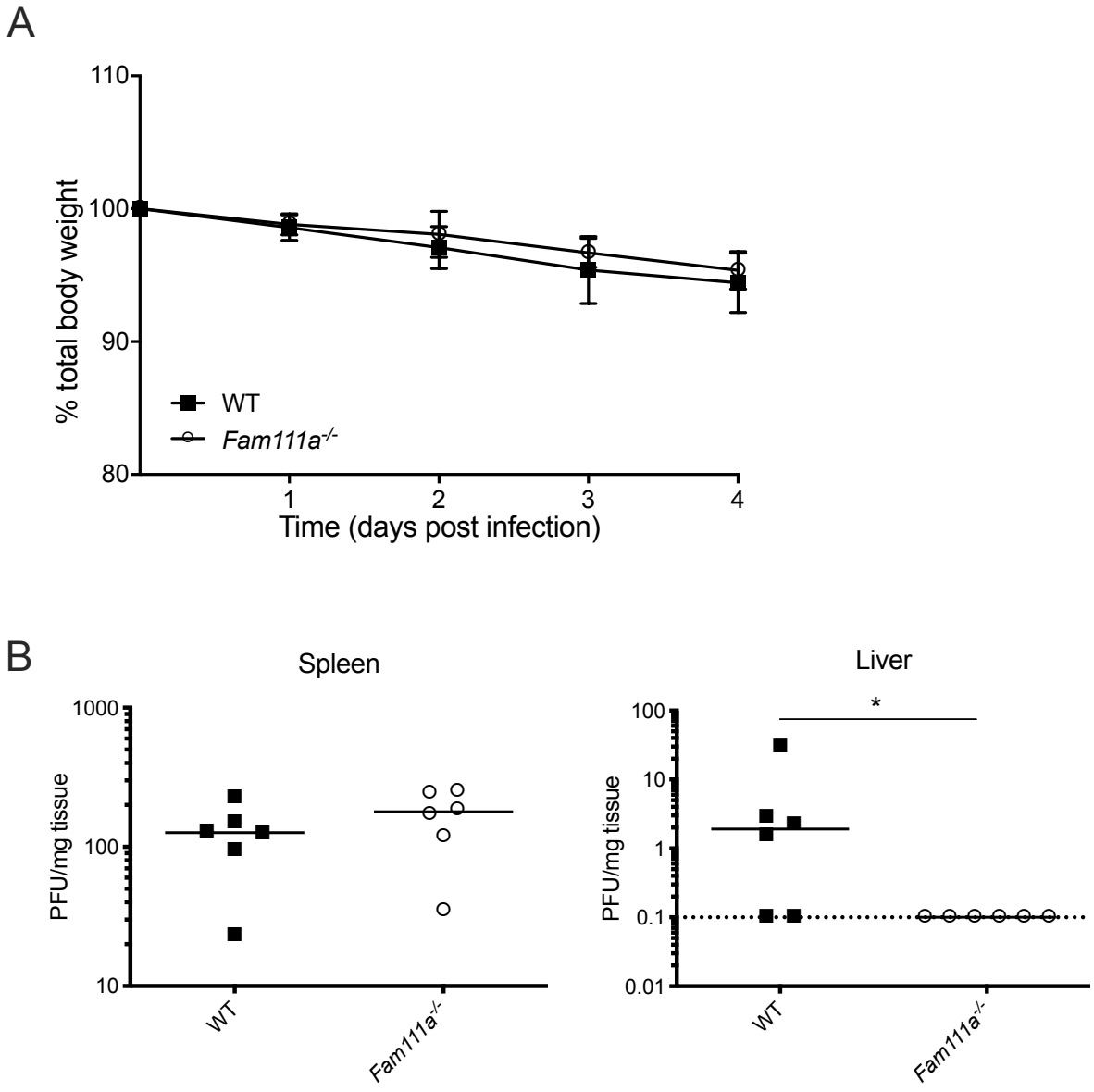

Figure 3.11 *Fam111a*$^{-/-}$ mice exhibit a loss of viral replication in the liver upon Δm157 MCMV infection.

(A) The percentage in total body weight of *Fam111a*$^{-/-}$ mice and WT mice 4 days post-Δm157 MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Fam111a*$^{-/-}$ mouse spleen (p=0.3095) and liver (*p=0.0152). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

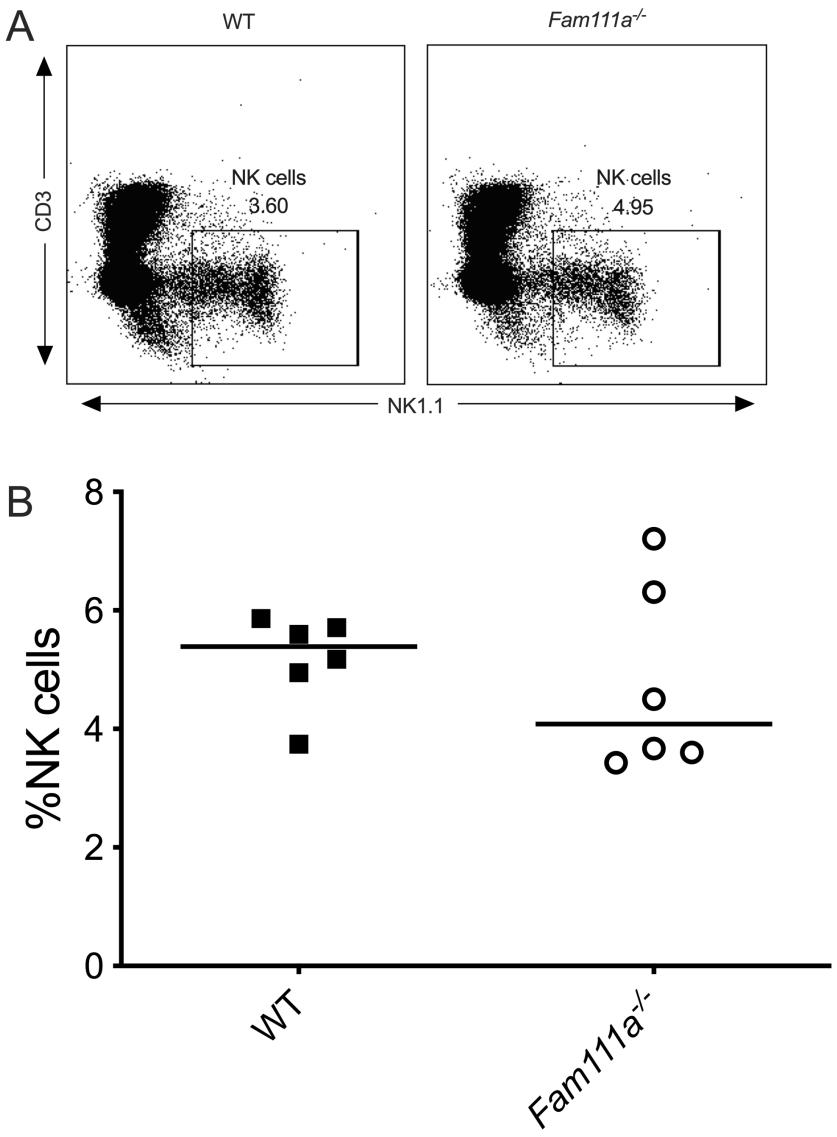

Figure 3.12 The percentage of splenic NK cells is unaffected by *Fam111a* deficiency 4 days post-Δm157 MCMV infection.
(A) Representative flow plots showing gated NK cells from WT and *Fam111a*$^{-/-}$ mice 4 days post-Δm157 MCMV infection which were defined as NK1.1^{+}CD3^{-}. (B) The percentage of NK cells from the total live lymphocyte population in WT and *Fam111a*$^{-/-}$ mice. Individual mice and median are shown (6 mice per group). p=0.4848

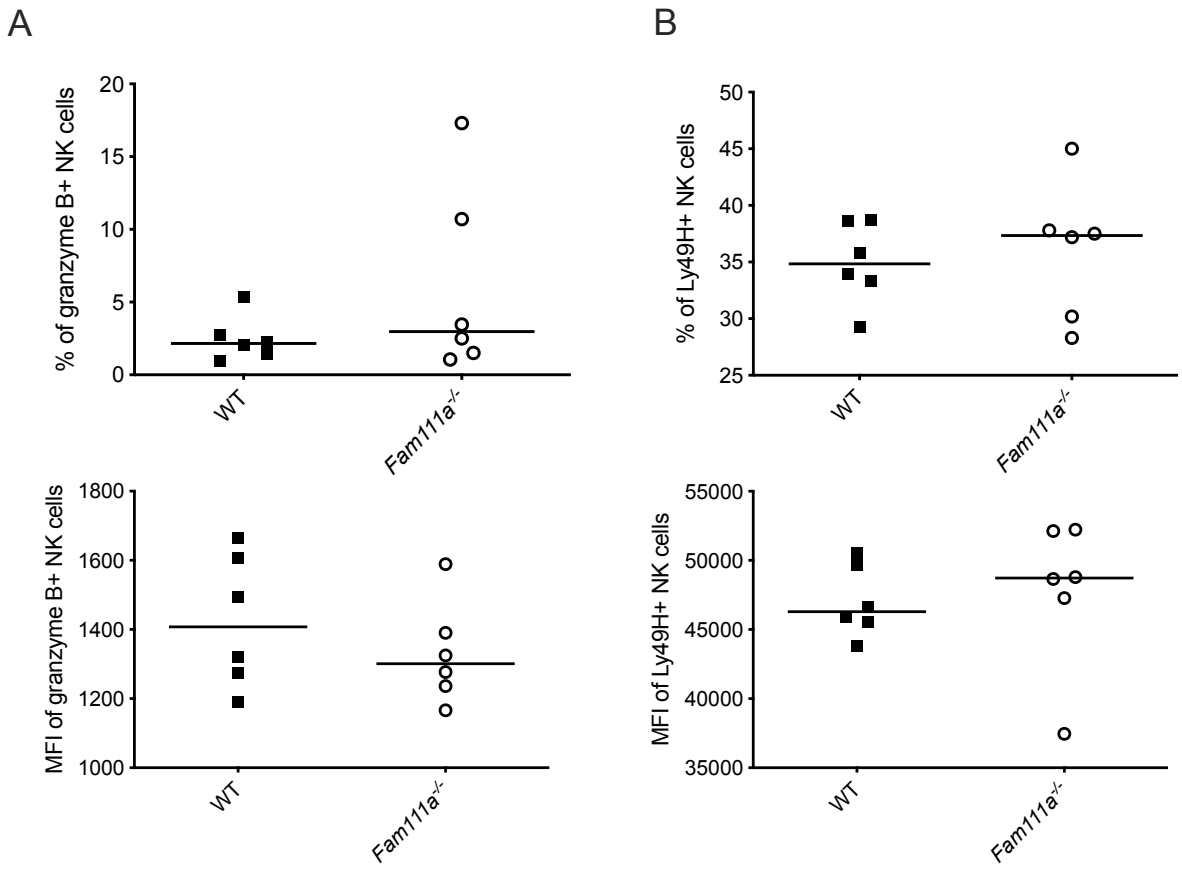

Figure 3.13 Unaltered expression of granzyme B and Ly49H by splenic NK cells from *Fam111a*$^{-/-}$ mice 4 days after Δm157 MCMV infection.
(A) The percentage of granzyme B+ NK cells (p=0.3939) and the mean fluorescence intensity (MFI) of granzyme B+ NK cells (p=0.4848) from WT and *Fam111a*$^{-/-}$ mice 4 days post-Δm157 MCMV infection. (B) The percentage of Ly49H+ NK cells (p=0.9372) and the mean fluorescence intensity (MFI) of Ly49H+ NK cells (p=0.3939). Individual mice and median are shown (6 mice per group).

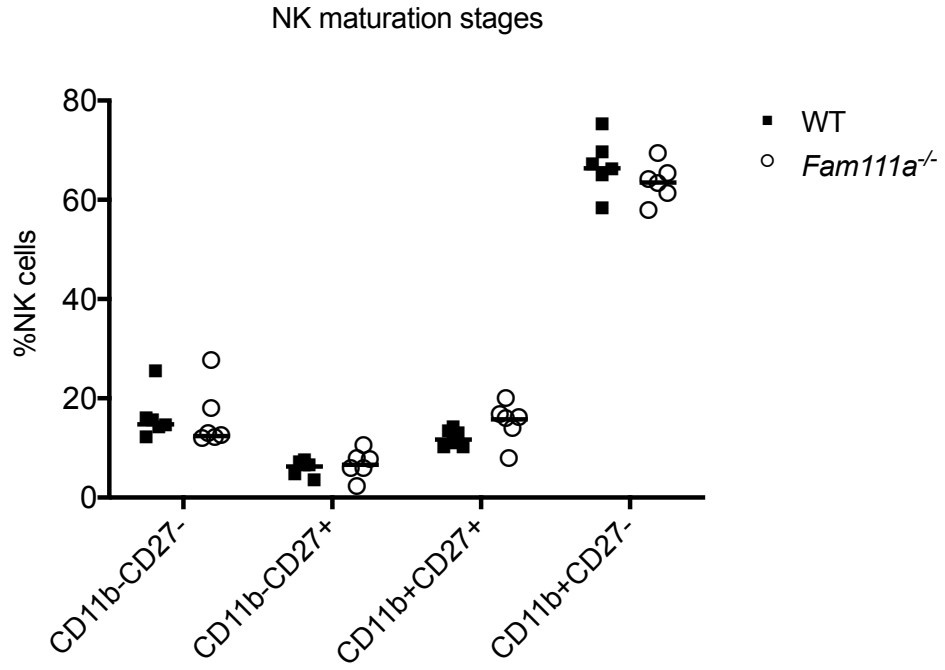

Figure 3.14 NK cell maturation subsets are not impacted by *Fam111a* deficiency during Δm157 MCMV infection.
The percentage of NK cell maturation populations from immature ($CD11b^{-}CD27^{-}$) to mature ($CD11b^{+}CD27^{-}$) from WT and *Fam111a*$^{-/-}$ mouse splenocytes 4 days post Δm157-MCMV infection. Individual mice and median are shown (6 mice per group). $CD11b^{-}CD27^{-}$ p=0.4848, $CD11b^{-}CD27^{+}$ p=0.5887, $CD11b^{+}CD27^{+}$ p=0.0931, $CD11b^{+}CD27^{-}$ p=0.1797.

Finally, I sought to determine whether there were differences in viral replication control in *Fam111a* deficient mice using salivary gland passaged MCMV infection, which has increased pathogenicity compared to tissue culture passaged virus. With increased pathogenicity, differences in virus control should be more obvious. At 4 days post salivary gland passaged MCMV infection, however, no difference in weight loss or control of virus replication was observed between WT and *Fam111a*$^{-/-}$ mice (Figure 3.15). As a result of the salivary gland MCMV infection data which showed no difference between groups, despite a significant difference in virus control without the m157-Ly49H interaction in the liver, I decided to stop investigating this gene.

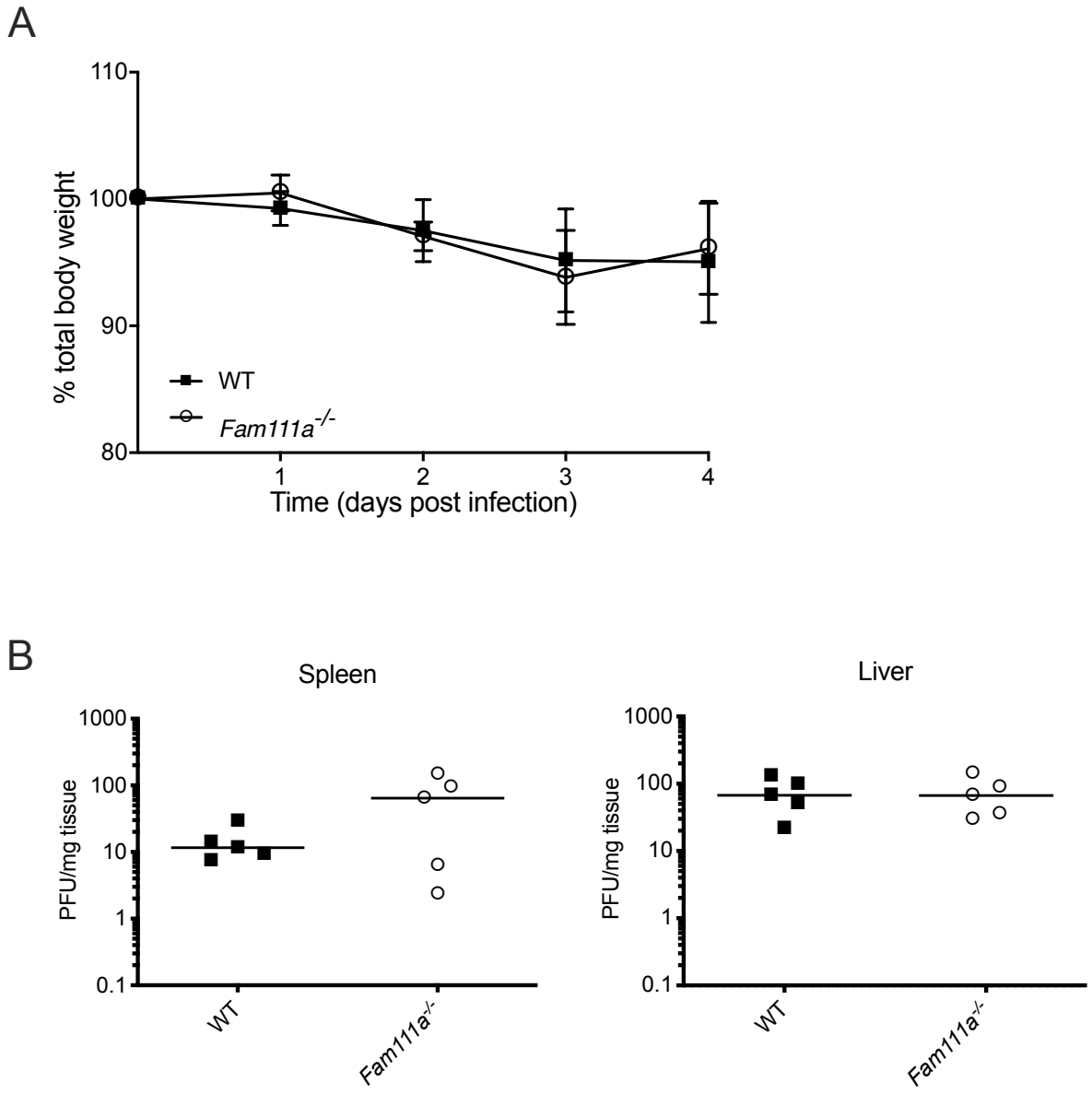

Figure 3.15 *Fam111a*$^{-/-}$ mice exhibit no difference in viral replication upon salivary gland passaged MCMV infection.
(A) The percentage total body weight in *Fam111a*$^{-/-}$ mice and WT mice 4 days post-salivary gland passaged MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Fam111a*$^{-/-}$ mouse spleen ($p=0.6905$) and liver ($p>0.9999$). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (5 mice per group).

3.2.4 *Family with Sequence Similarity 114 Member A2 (Fam114a2)*

Fam114a2 is a highly conserved gene which has a high level of expression in most tissues within the human body (GeneCards, FAM114A2). *FAM111A* has unknown function(s) but has a potential role in cancer (Stransky *et al.*, 2014; Wei *et al.*, 2018) due to association with pathways in oncogenic MAPK signalling (Lu *et al.*, 2017). This gene was chosen by the WTSI for the NK cell screen due to results from an internal RNASeq single cell analysis of mouse splenic NK cells index sorted for classification as immature or mature. Gene expression data from Immgen highlighted significant but not exclusive *Fam114a2* expression by NK cells (Figure 3.16). High levels of *Fam114a2* gene expression were observed across all immune cell types including hematopoietic stem cells, B-cells, DCs, monocytes, neutrophils, and T-cell types. Within mouse whole blood 2 days post-MCMV infection, *Fam114a2* gene expression significantly decreased (*$p<0.0001$, Figure 3.17). Reduced gene expression is of potential interest as the gene may still have important roles in infection control which could highlight novel drug targets. Thus, *Fam114a2* was identified as an NK cell-expressed gene that may be of possible interest in the context of anti-MCMV immune responses.

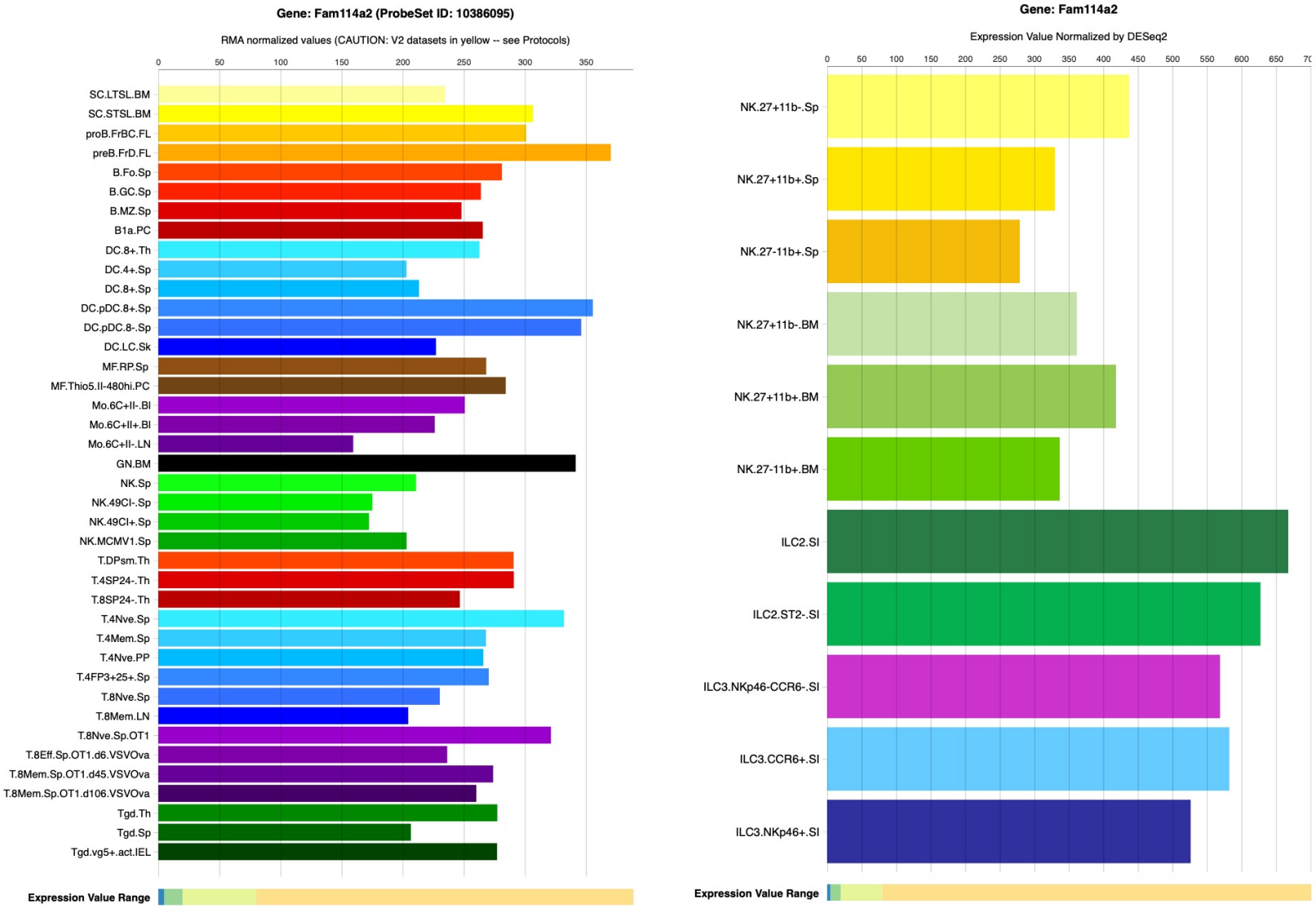

Figure 3.16 *Fam114a2* is expressed across a variety of immune cell types.
Mouse *Fam1114a2* gene expression data from: microarray in various immune cell types (left) and RNASeq in NK cell and ILC types (right). Source: immgen.org.

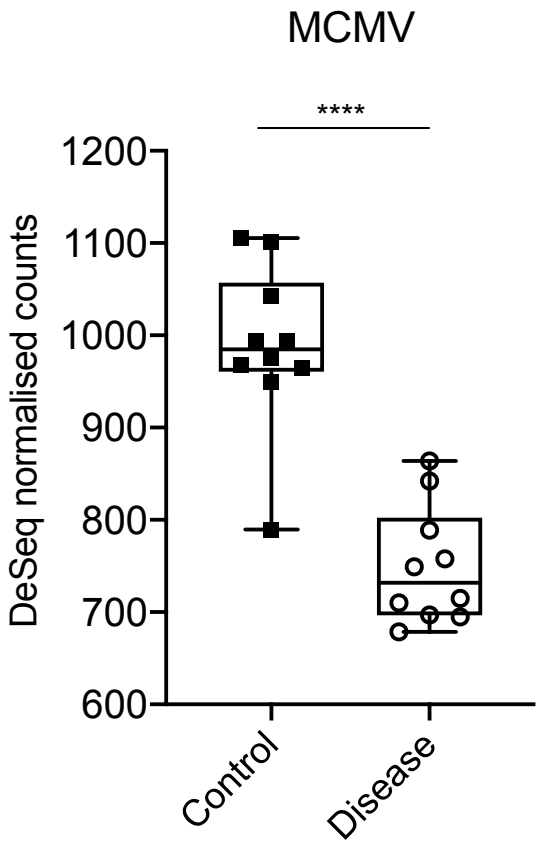

Figure 3.17 *Fam114a2* gene expression decreases 2 days post-MCMV infection in mouse whole blood.
Female C57BL/6 mice were infected with $3x10^4$ PFU of salivary gland prepared Smith strain MCMV and compared to uninfected control mice. Total blood RNA was extracted before assessing gene expression using RNA-Seq. Data are expressed as box and whisker plots and individual values from each mouse are shown (10 mice per group). *p<0.0001. Source: Singhania et al. 2019.

3.2.4.1 Deletion of Fam114a2 during MCMV infection improves control of MCMV replication

To examine whether *Fam114a2* influences MCMV infection control, WT and *Fam114a2*$^{-/-}$ mice were infected with MCMV for 4 days during which their weight was monitored, and viral replication was measured at 4 days. MCMV infections led to a similar weight loss profile in both groups (Figure 3.18A). Although *Fam114a2*$^{-/-}$ mice exhibited no difference in viral replication in the spleen, strikingly, MCMV replication in the liver was significantly reduced (**p=0.0043, Figure 3.18B). Therefore, *Fam114a2* deficiency may be beneficial to viral replication control specifically within the liver.

Next, I repeated the experiment to understand if changes to virus replication control were robust. Upon infection, mice from both groups lost up to 20% of their original weight by day 4 (Figure 3.19A), compared to the original screen data where a maximum of 10% weight loss was observed (Figure 3.18). In addition, *Fam114a2*$^{-/-}$ mice had significantly more weight loss compared to WT on day 1, 2, 3 and 4 (**p=0.022, **p=0.0087, *p=0.0411, *p=0.0152 respectively, Figure 3.19A). Surprisingly, virus replication did not change between groups in the liver (Figure 3.19B). Since the changes in virus replication were not robust, *Fam114a2* was excluded from further investigation.

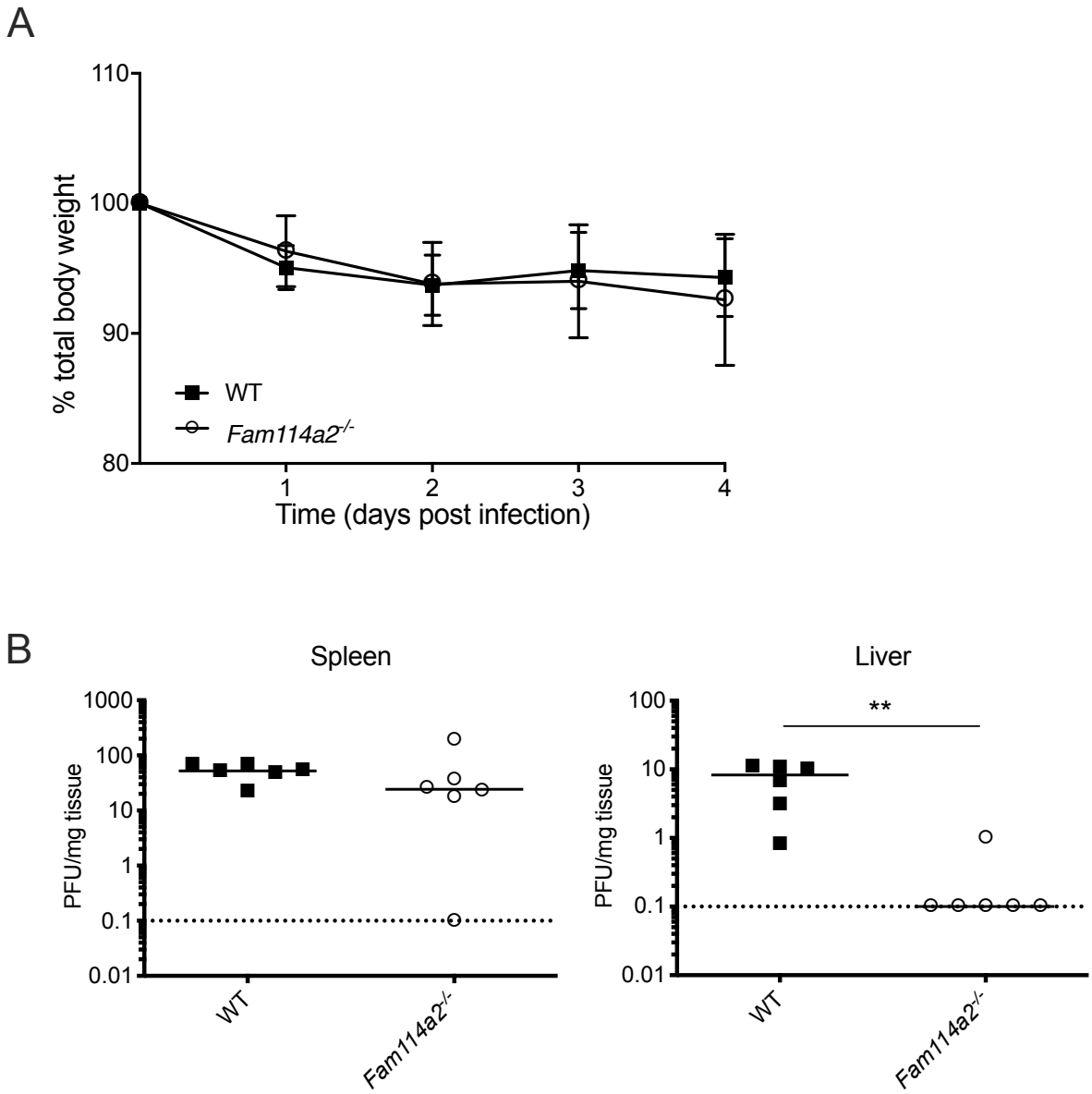

Figure 3.18 *Fam114a2*$^{-/-}$ mice have reduced viral replication in the liver.
(A) Percentage total body weight in *Fam114a2*$^{-/-}$ mice and WT mice 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Fam114a2*$^{-/-}$ mouse spleen (p=0.1797) and liver (**p=0.0043). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

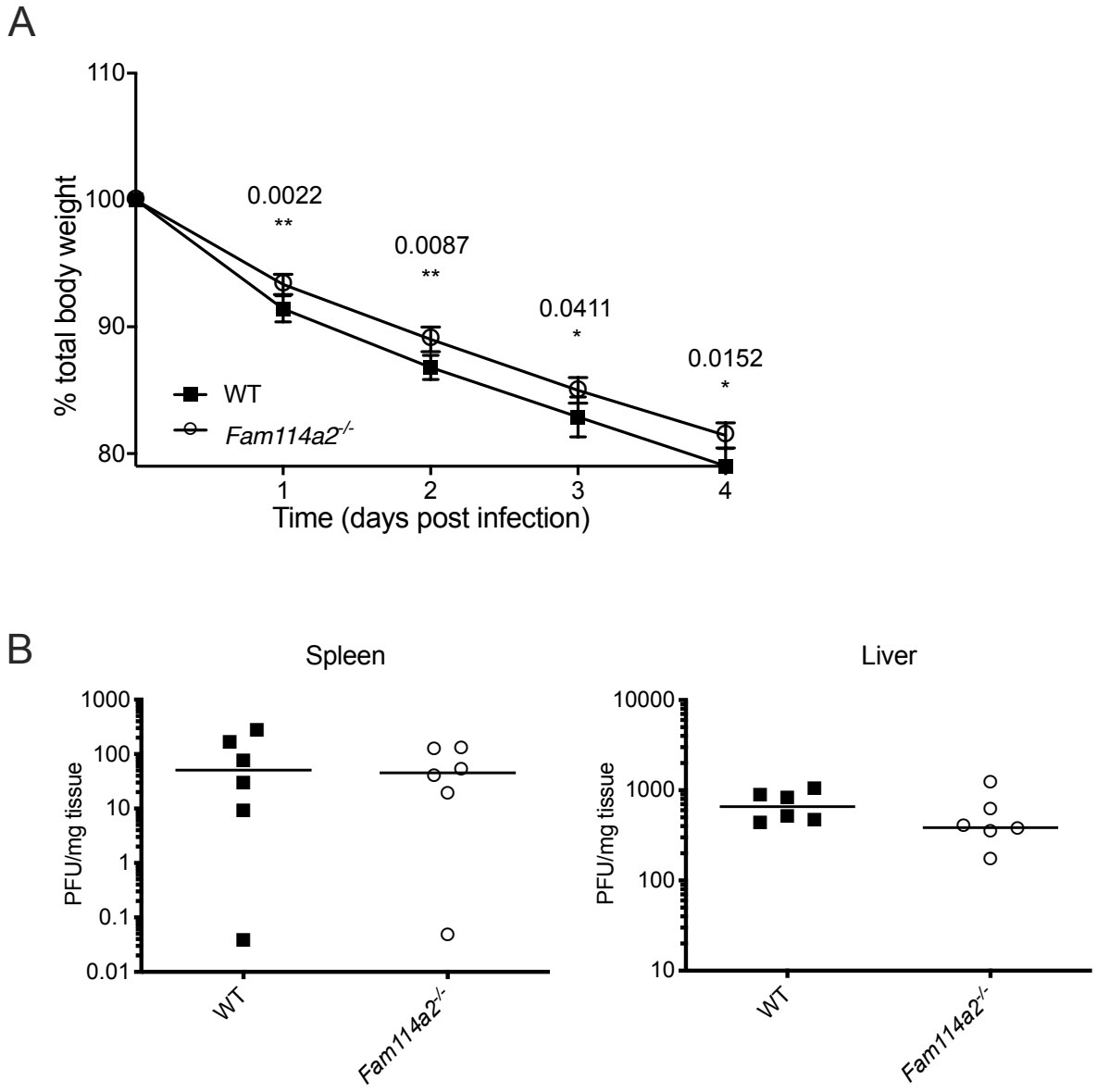

Figure 3.19 Repeat experiment shows less weight loss but no change in viral replication in *Fam114a2*$^{-/-}$ mice.

(A) The percentage total body weight in *Fam114a2*$^{-/-}$ mice and WT mice 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Fam114a2*$^{-/-}$ mouse spleen (p=0.09372) and liver (p=0.1797). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

3.2.5 Fatty acyl CoA reductase 1 (*Far1*)

The *FAR1* gene encodes enzymes which catalyse the reduction of saturated and unsaturated fatty acyl-CoA to fatty alcohols (Jeffrey B Cheng and Russell, 2004) and are localised within peroxisomes (Jeffrey B. Cheng and Russell, 2004). Mouse and human far1 isozymes share ~58% sequence identity and are encoded by *Far1* genes of similar structure, suggesting they arose from a common evolutionary precursor via duplication (Jeffrey B. Cheng and Russell, 2004). *Far1* was chosen for the NK cell screen by the WTSI as it was significantly induced post-MCMV infection in a subset of memory-like NK cells (Bezman *et al.*, 2012). Gene expression data from Immgen highlighted significant but not exclusive *far1* expression by NK cells (Figure 3.20). The greatest level of *Far1* expression was observed in bone marrow-derived neutrophils, although all other immune cell types investigated, including NK cells, showed some level of *Far1* expression. However, at 2 days post-MCMV infection prior to memory-like NK cell development, *Far1* gene expression did not significantly change in whole blood but there was a trend showing increased expression upon acute MCMV infection *in vivo* (Figure 3.21). Thus, as *Far1* was induced post-MCMV in a subset of memory-like NK cells and was expressed by NK cells, *Far1* was identified as a gene which may be of interest in anti-MCMV immune responses.

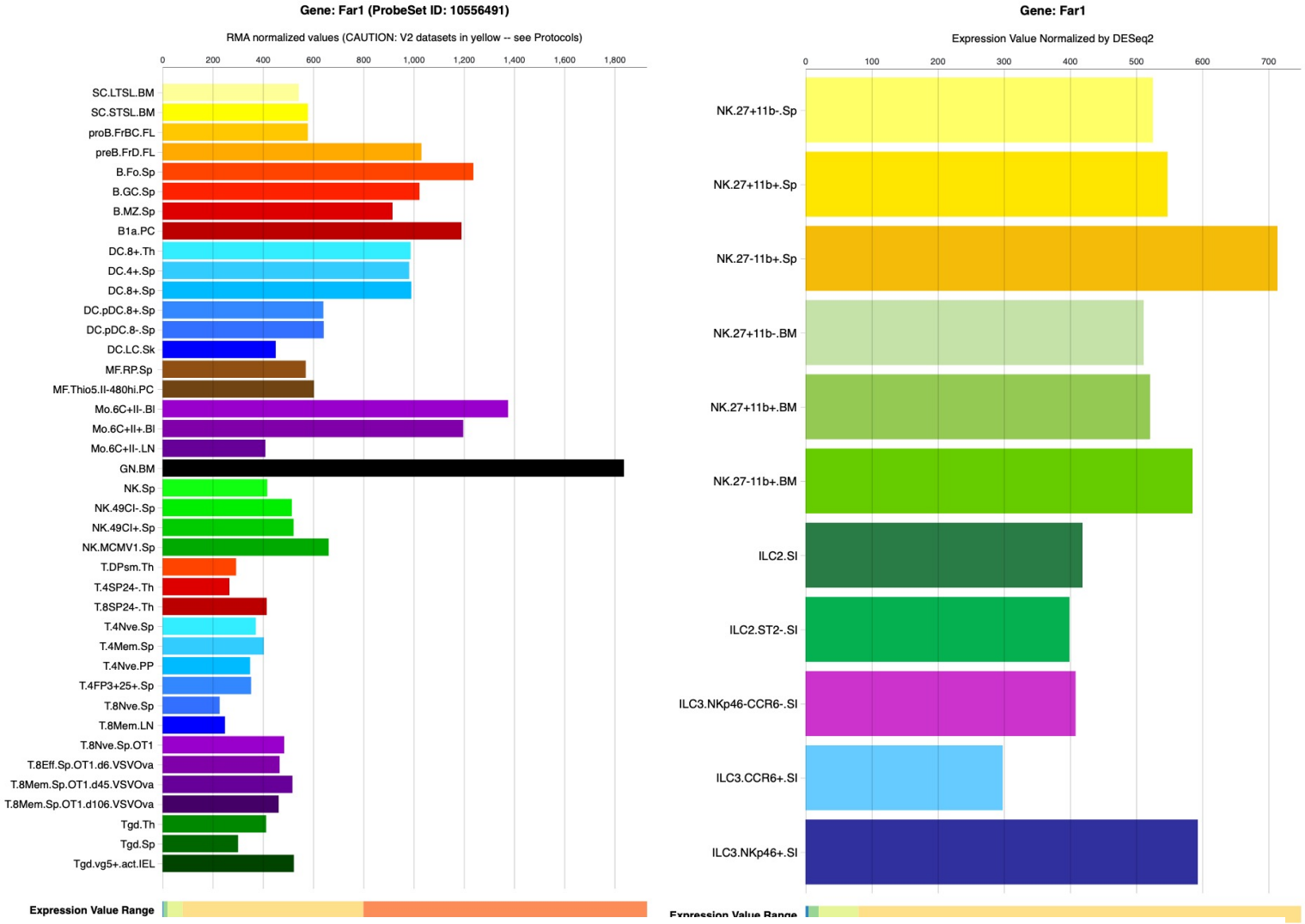

Figure 3.20 *Far1* is expressed across a variety of immune cell types.
Mouse *Far1* gene expression data from: microarray in various immune cell types (left) and RNASeq in NK cell and ILC types (right). Source: immgen.org.

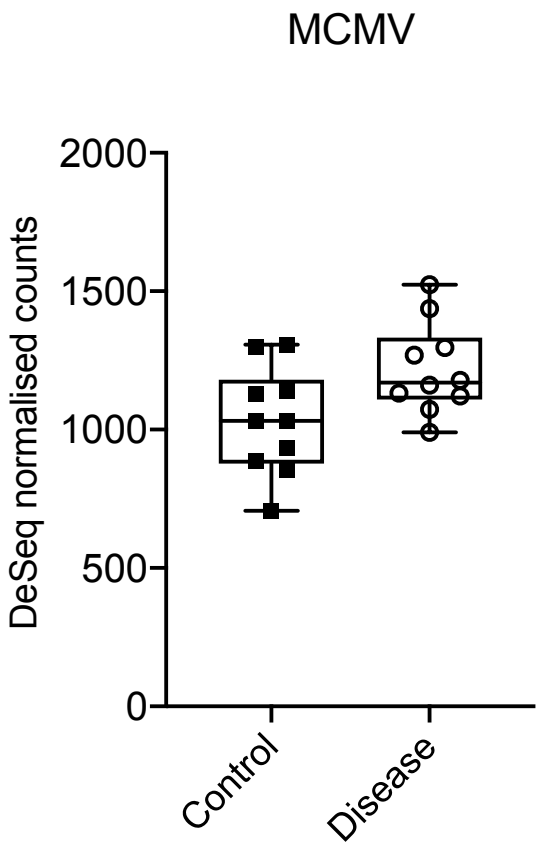

Figure 3.21 The *Far1* gene has a trend of increased expression 2 days post-MCMV infection in mouse whole blood.
Female C57BL/6 mice were infected with $3x10^4$ PFU of salivary gland prepared Smith strain MCMV and compared to uninfected control mice. Total blood RNA was extracted before assessing gene expression using RNA-Seq. Data are expressed as box and whisker plots and individual values from each mouse are shown (10 mice per group). p=0.0630. Source: Singhania et al. 2019.

3.2.5.1 Loss of Far1 during MCMV infection improves MCMV replication control

To elucidate whether *Far1* influences MCMV infection control, WT and *Far1*$^{-/-}$ mice were infected with MCMV and at 4 days spleen and liver viral replication was measured. Of note, *Far1*$^{-/-}$ mice were born smaller in size than WT mice, therefore MCMV infection was postponed until the mice reached a sufficient weight (~25g). The age-matched WT controls at 6 months old weighed ~20g more than *Far1*$^{-/-}$ mice at the time of infection. Upon infection, *Far1*$^{-/-}$ mice had a similar weight loss profile to WT mice (Figure 3.22A) and virus replication did not change between groups in the spleen (Figure 3.22B). Within the liver tissue there was a trend of reduced virus replication in *Far1*$^{-/-}$ mice which was not significant (Figure 3.22B). Notably, virus replication was low in WT and *Far1*$^{-/-}$ groups and I hypothesised that with a lower level of infection any changes to virus control induced by gene deficiency would be more difficult to detect.

I also studied *Far1*$^{+/-}$ responses to MCMV infection as these mice were born a normal size and therefore represented a better comparison to age matched WT controls which were the same weight. *Far1*$^{+/-}$ mice lost significantly less weight than WT mice upon infection at day 3 and 4 (**p=0.0022, **p=0.0022 respectively, Figure 3.23A). No difference in viral replication was observed in the spleen (Figure 3.24). However, *Far1*$^{+/-}$ mice had a trend of improved virus control in the liver compared to WT (Figure 3.23). Of note, the level of infection was greater in the study examining *Far1*$^{+/-}$ mice (Figure 3.23) than the *Far1*$^{-/-}$ mice (Figure 3.24). These data indicated that *Far1* gene deficiency may improve viral replication control.

As the presence of the Ly49H-m157 interaction during MCMV infection may mask subtle effects of gene deficiency on NK cell function, WT and *Far1*$^{+/-}$ mouse infections were repeated with an equivalent dose of Δm157 MCMV. *Far1*$^{+/-}$ mice showed no difference in weight compared to WT mice. Furthermore, in the spleen no difference in viral replication between groups was observed (Figure 3.24). In the liver, *Far1*$^{+/-}$ mice infected with Δm157 MCMV had a trend of improved virus control which was not significant (Figure 3.24), as shown with previous infections with Smith strain MCMV. Therefore, the presence of the m157-Ly49H interaction does not impact *Far1* gene effects.

Overall, my data suggests loss of *Far1* gene function may improve control of MCMV infection, but this was not fully elucidated. However, given the stunted growth of *Far1*$^{-/-}$ mice and the absence of a clear-cut phenotype in heterozygous mice, I decided not to investigate this gene further.

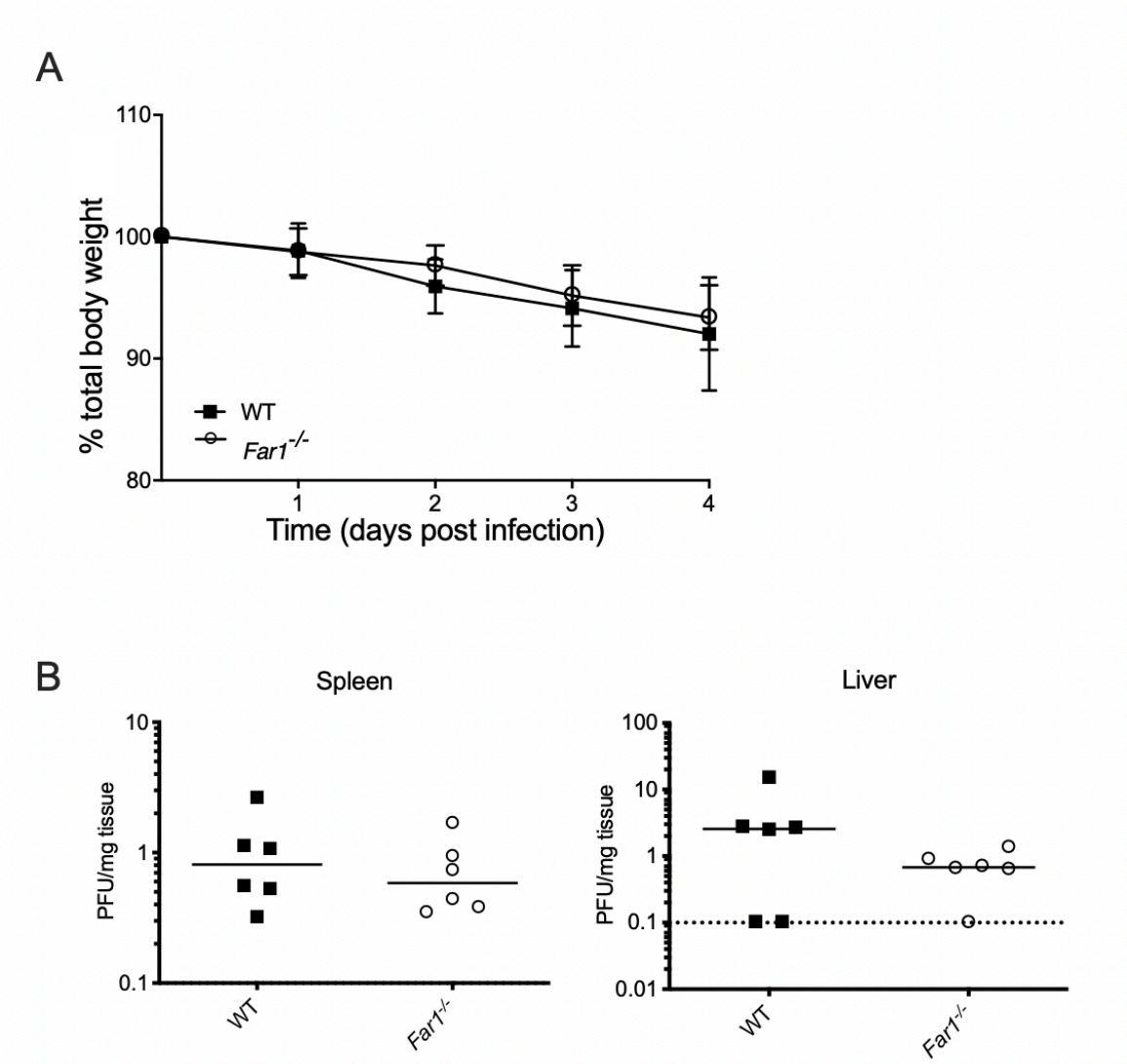

Figure 3.22 *Far1*$^{-/-}$ mice show a trend of reduced virus replication upon MCMV infection.

(A) The percentage total body weight in *Far1*$^{-/-}$ mice and WT mice 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Far1*$^{-/-}$ mouse spleen ($p=0.5887$) and liver ($p=0.3074$). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

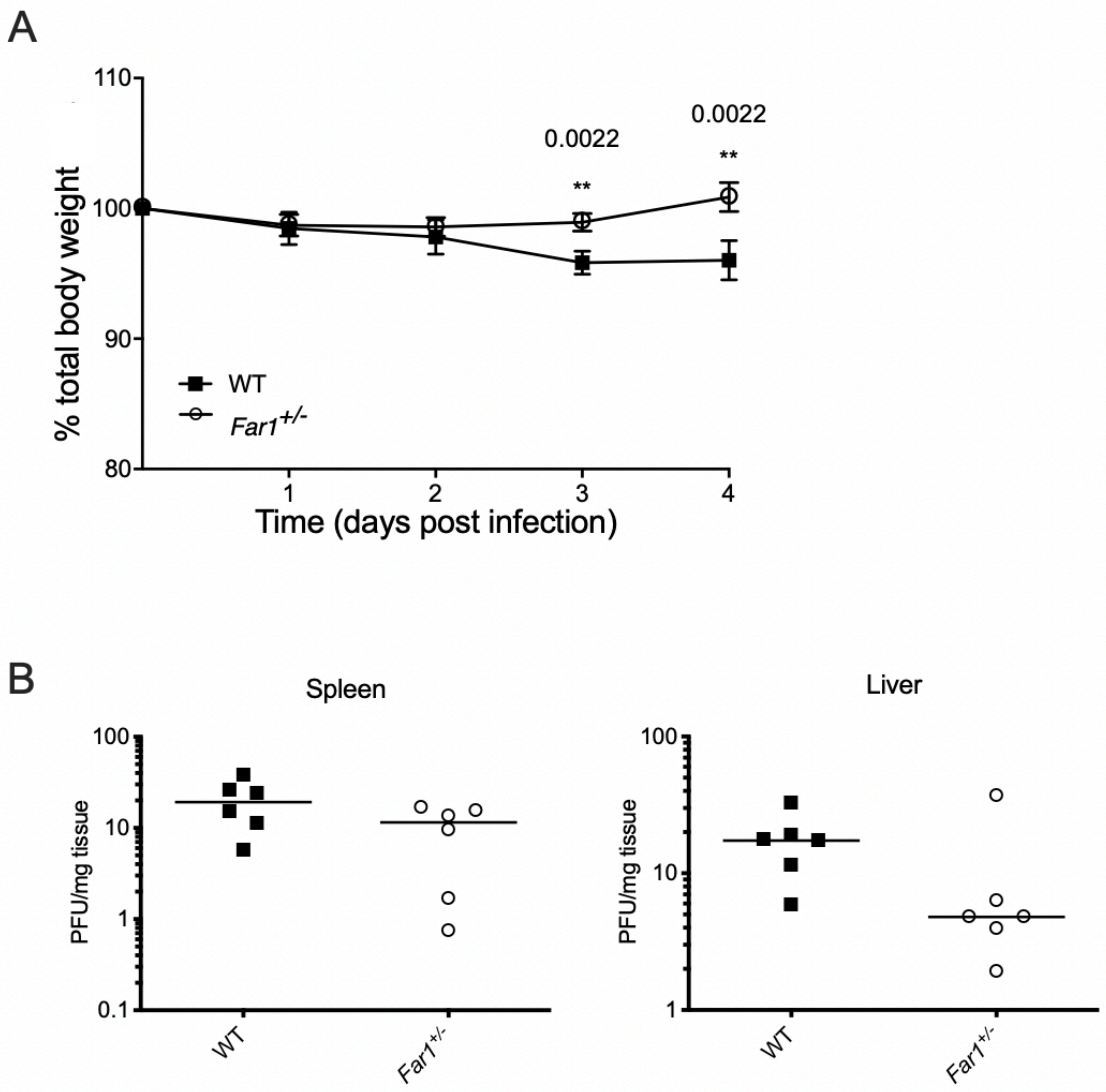

Figure 3.23 *Far*$^{+/-}$ mice demonstrate a trend of reduced virus replication in the liver upon MCMV infection.

(A) The percentage total body weight in *Far*$^{+/-}$ mice and WT mice 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Far1*$^{+/-}$ mouse spleen (p=0.1797) and liver (p=0.0931). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

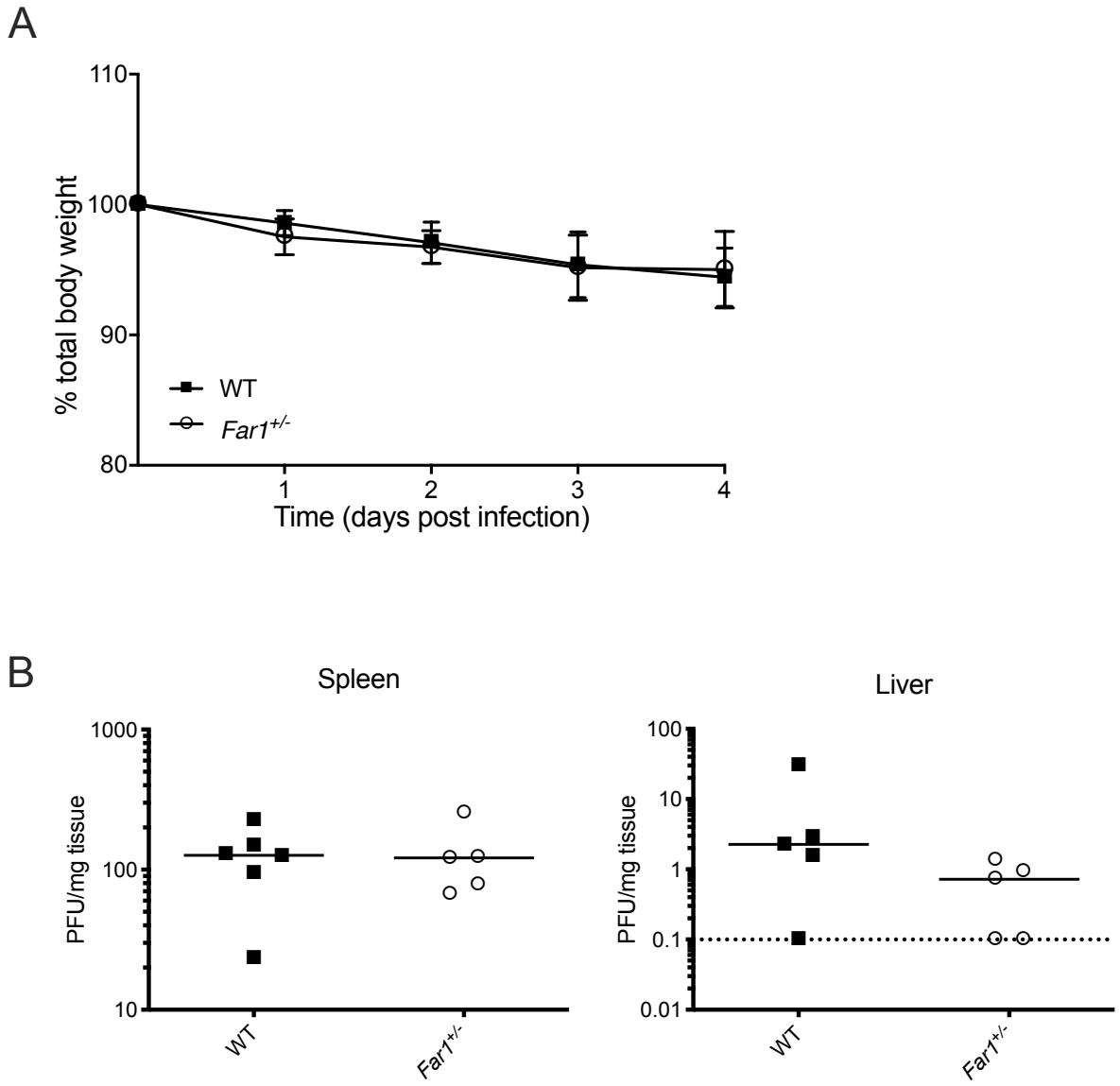

Figure 3.24 Upon Δm157 MCMV infection, *Far1*$^{+/-}$ mice have no change in virus replication.

(A) The percentage total body weight in *Far*$^{+/-}$ mice and WT mice 4 days post-Δm157 MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Far1*$^{+/-}$ mouse spleen (p=0.6623) and liver (p=0.0952). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (5-6 mice per group).

3.2.6 HEAT Repeat Containing 9 *(Heatr9)*

The *Heatr9* gene is conserved in mice and humans with unknown function. Upregulation of *Heatr9* has been observed during viral infections, particularly during influenza infection by lung alveolar epithelial cells (Stairiker *et al.*, 2017, 2018) and has recently been linked to chemokine production (Stairiker *et al.*, 2020). *Heatr9* (gene Gm11435) was chosen by the WTSI for investigation in the NK cell screen as it was significantly upregulated in memory-like NK cells following MCMV infection (Bezman *et al.*, 2012). Gene expression mouse microarray (Figure 3.25) showed *Heatr9* gene expression was almost exclusive to NK cell types including splenic NK cells, Ly49C/I- splenic NK cells, Ly49CI/+ NK cells and splenic NK cells 1-day post-MCMV infection. *Heatr9* was also expressed by effector T-cell and memory T-cell types post-vesicular stomatitis virus (VSV) infection, activated intraepithelial Vγ5+ γδ T-cells, splenic γδ T-cells, and splenic $CD8^+$ DCs. In additon, *Heatr9* gene expression increased significantly in C57BL/6 mouse whole blood 2 days post-MCMV infection (*p=0.0188, Figure 3.26). As this gene was almost exclusively expressed by NK cells and gene expression increased post-MCMV infection, *Heatr9* was of interest.

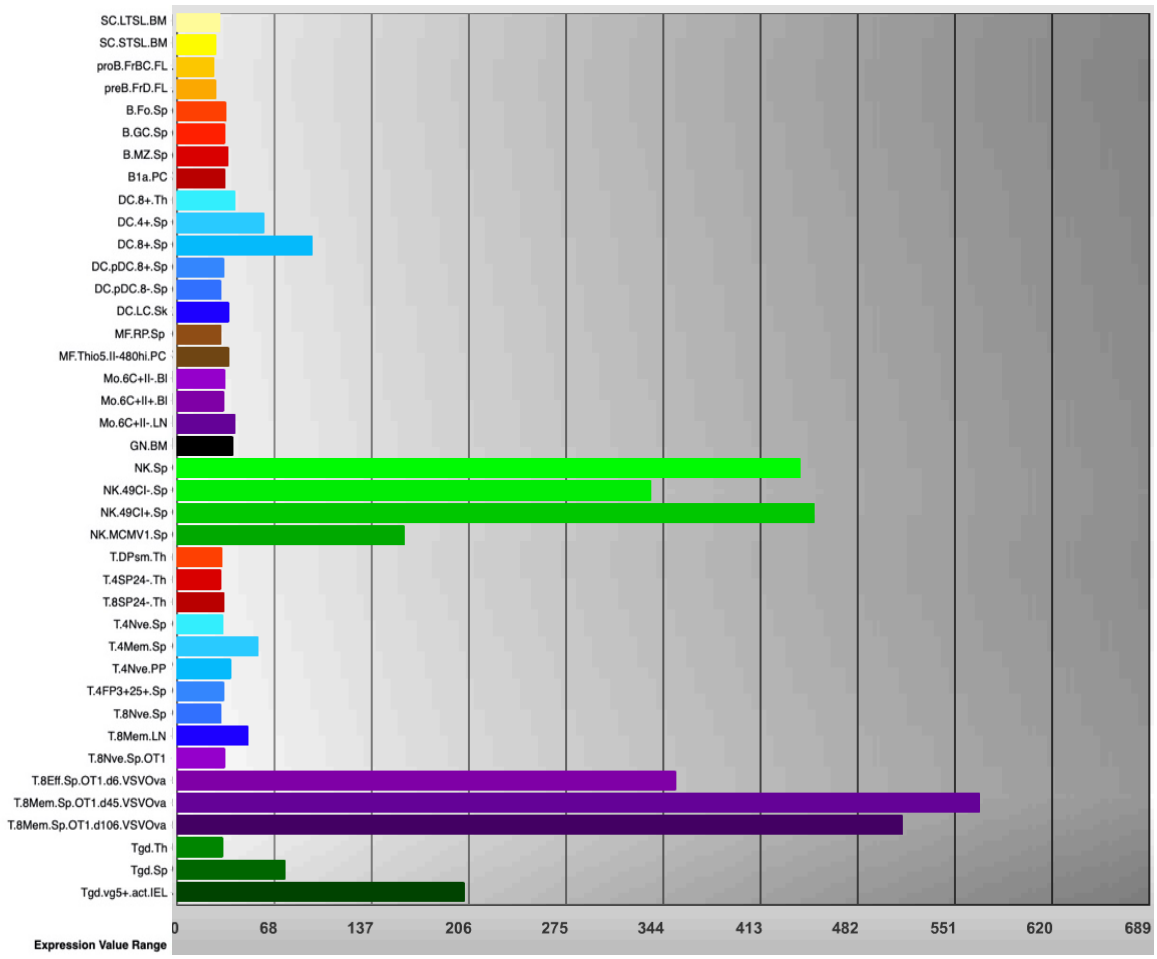

Figure 3.25 Figure 3.26. *Heatr9* has almost exclusive expression by NK cell types as well as OT1 CD8$^+$ effector T-cells and OT1 CD8$^+$ memory T-cells after vesicular stomatitis virus-ovalbumin peptide infection (VSV-OVA).
Mouse *Heatr9* gene expression microarray data. Source: immgen.org.

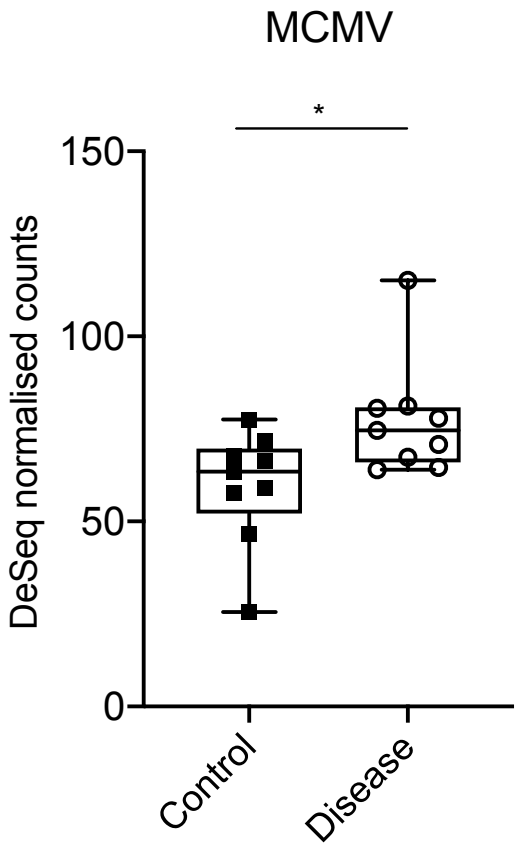

Figure 3.26 *Heatr9* gene expression increases 2 days post-MCMV infection in mouse whole blood.
Female C57BL/6 mice were infected with $3x10^4$ PFU of salivary gland prepared Smith strain MCMV and compared to uninfected C57BL/6 control mice. Total blood RNA was extracted before assessing gene expression using RNA-Seq. Data is expressed as box and whisker plots and individual values from each mouse are shown (10 mice per group). *p=0.0188. Source: Singhania et al. 2019.

3.2.6.1 Deletion of Heatr9 during MCMV infection significantly reduces MCMV replication control

To investigate whether *Heatr9* influenced immune responses against MCMV, WT and *Heatr9*$^{-/-}$ mice were infected with MCMV for 4 days and mouse weight loss and viral replication was measured. The weight loss profile of *Heatr9*$^{-/-}$ mice and WT mice was similar across the 4 days of infection (Figure 3.27A). In the spleen, *Heatr9* deficient mice exhibited significantly elevated viral replication (*p=0.0273) compared to WT mice (Figure 3.27B). Within the liver there was no significant difference in viral replication control upon *Heatr9* deficiency (Figure 3.27B). The difference in viral replication control between tissues is likely due to differences in NK cell effector functions within different tissues. Within the spleen NK cells predominately induce their effector function through cytotoxicity which may be affected by *Heatr9* gene deficiency, whereas liver NK cells predominately induce IFNγ release (Tay and Welsh, 1997a). I also used the spleen and liver samples from *Heatr9*$^{-/-}$ mice and WT controls to assess the amount of IFNγ within each organ and there was no difference between WT and *Heatr9*$^{-/-}$ mice (Figure 3.28). This was expected since no difference in liver viral replication was observed.

To understand whether *Heatr9* influenced MCMV control at later stages of acute infection, I repeated the experiment at a 7-day timepoint. WT mice and *Heatr9*$^{-/-}$ mice showed a similar weight loss and recovery profile across the 7 days (Figure 3.29A). In the spleen and liver, no difference in virus replication was observed between WT and *Heatr9*$^{-/-}$ mice at 7 days and almost all mice had no detectable viral replication (Figure 3.29B). Therefore, any defects in virus replication during the early acute phase upon *Heatr9* deficiency were resolved by 7 days when NK cells are at the peak of their expansion (Schlub *et al.*, 2011; Sun *et al.*, 2011).

As Ly49H recognition of m157 glycoproteins induces strong NK cell activation signals (Daniels *et al.*, 2001; Arase *et al.*, 2002; H. R. C. Smith *et al.*, 2002; Parikh *et al.*, 2015), subtle effects of genes on NK cell functionality may be masked in the presence of this interaction. Therefore, I investigated the effect of MCMV infection on *Heatr9*$^{-/-}$ mice in the absence of the m157-Ly49H interaction using an equivalent dose of Δm157 MCMV. *Heatr9*$^{-/-}$ mice lost significantly more weight on days 2 and 4 post-Δm157 MCMV

infection (*p=0.0260, *p=0.0260 Figure 3.30A). Furthermore, *Heatr9*$^{-/-}$ mice exhibited significantly increased viral replication in the spleen (**p=0.0043) and liver (*p=0.0260) post-Δm157 MCMV (Figure 3.30B). As there was a greater level of viral replication in both tissues upon Δm157 MCMV, unlike infection with MCMV containing m157 where only a difference in the spleen was observed, this indicates that the m157-Ly49H interaction was partially masking the effect of *Heatr9* gene loss. The evidence presented here suggested *Heatr9* was a highly promising gene of interest important for NK cell virus control therefore I planned to continue characterising the effects of this gene. However, due to disruptions caused by the COVID-19 pandemic, I was unable to perform additional studies.

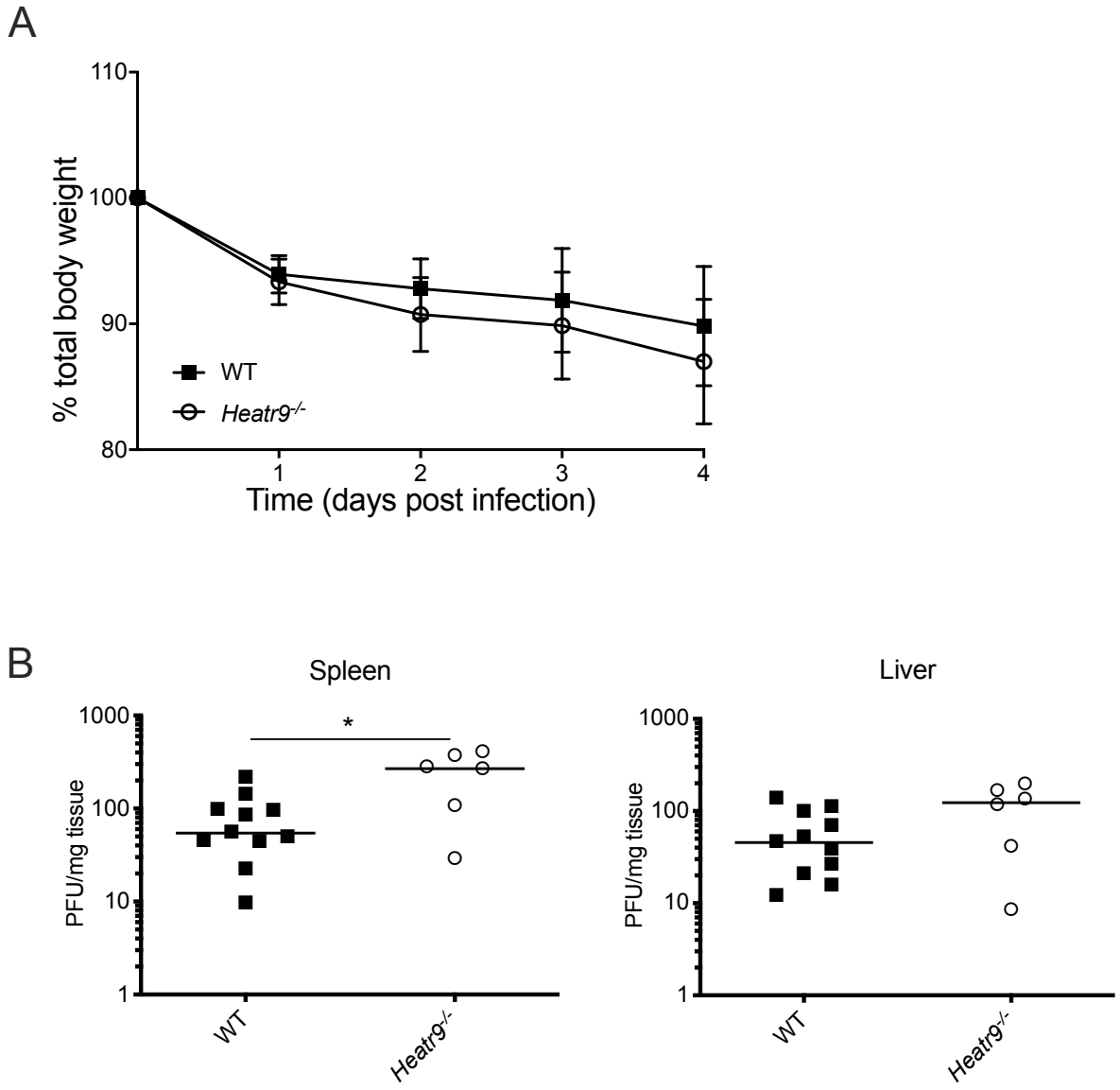

Figure 3.27 *Heatr9*$^{-/-}$ mice show increased viral replication in the spleen upon MCMV infection.

(A) The percentage total body weight in *Heatr9*$^{-/-}$ mice and WT mice 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Heatr9*$^{-/-}$ mouse spleen (*p=0.0273) and liver (p=0.1802). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (at least 6 mice per group).

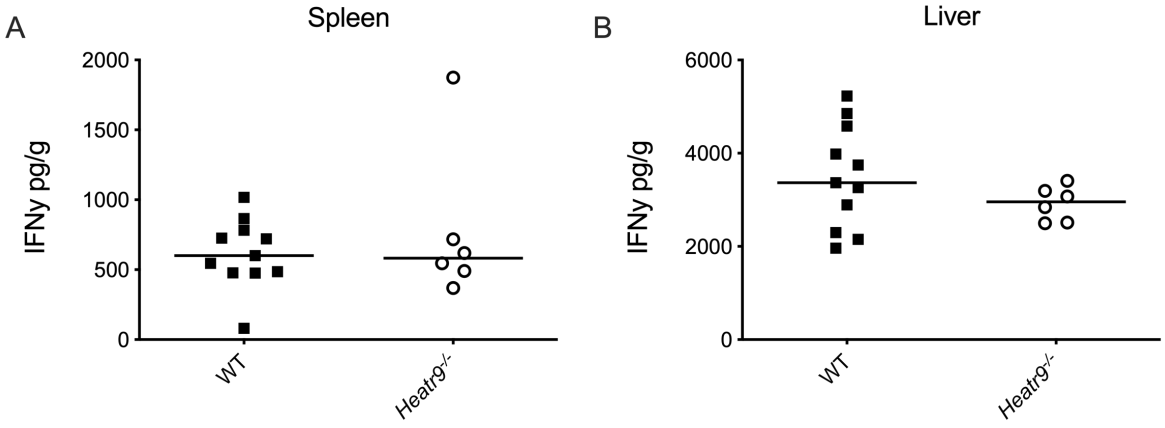

Figure 3.28 *Heatr9* deficiency does not impact on IFNγ protein secretion in the spleen and liver. The amount of IFNγ protein in WT and *Heatr9*$^{-/-}$ mouse (A) spleen ($p>0.9999$) and (B) liver ($p=0.3502$), per g tissue. Individual mice and median are shown (at least 6 mice per group).

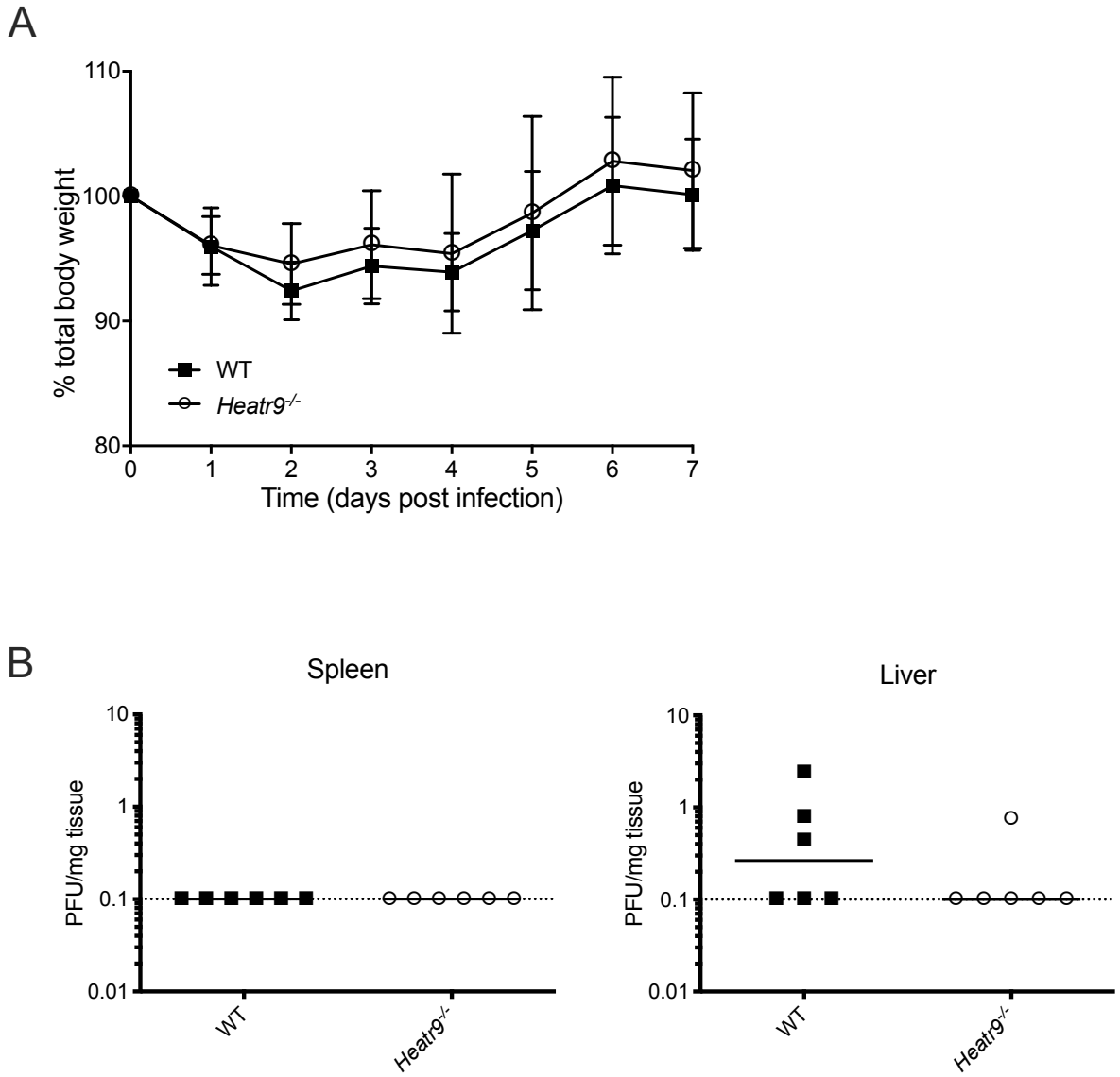

Figure 3.29 *Heatr9*$^{-/-}$ and WT mice have almost no detectable viral replication 7 days post-MCMV infection.

(A) Percentage total body weight in *Heatr9*$^{-/-}$ mice and WT mice 7 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Heatr9*$^{-/-}$ mouse spleen ($p>0.9999$) and liver ($p=0.3030$). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

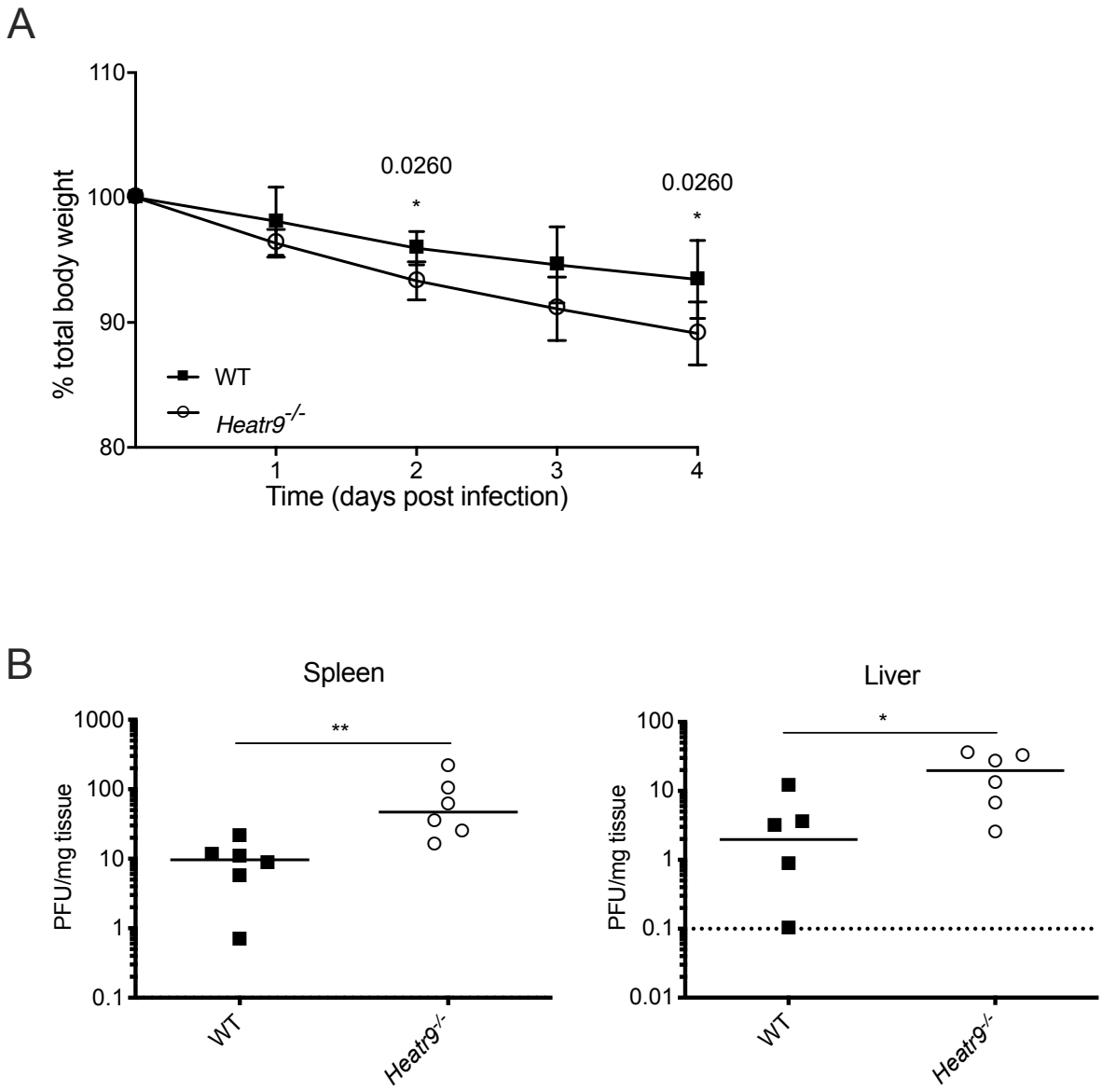

Figure 3.30 *Heatr9*$^{-/-}$ mice have increased viral replication upon Δm157 MCMV infection.

(A) Percentage total body weight in *Heatr9*$^{-/-}$ mice and WT mice 4 days post-Δm157 MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Heatr9*$^{-/-}$ mouse spleen (**p=0.0043) and liver (*p=0.0260). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (5-6 mice per group).

3.2.7 Serine (or cysteine) peptidase inhibitor, clade B, member 9b (*Serpinb9b*)

The SERPINB9 gene is believed to encode a granzyme M inhibitor which may protect cytotoxic lymphocytes including mouse NK cells from being killed by their own cytolytic molecules (Kaiserman and Bird, 2010). However, *Serpinb9b* is completely absent in humans with no known pseudogene although other serpins present in humans have also been reported as granzyme inhibitors (Kaiserman and Bird, 2010). *Serpinb9b* was selected by the WTSI for the NK cell screen as it was found to be induced during the early effector NK cell response to MCMV (Bezman *et al.*, 2012). Gene expression data from Immgen highlighted exclusive *Serpinb9b* expression by NK cells and no expression in other ILC types (Figure 3.31). The NK cell microarray data from Immgen shows high expression of *Serpinb9b* on splenic NK cells, Ly49C/I- splenic NK cells, Ly49C/I+ NK cells and splenic NK cells 1-day post-MCMV infection. Interestingly, there was a significant increase in *Serpinb9b* gene expression in whole blood upon acute MCMV infection *in vivo* (****$p<0.0001$, Figure 3.32). Thus, *Serpinb9b* was identified as an NK cell-expressed gene which may be of interest in anti-MCMV immune responses.

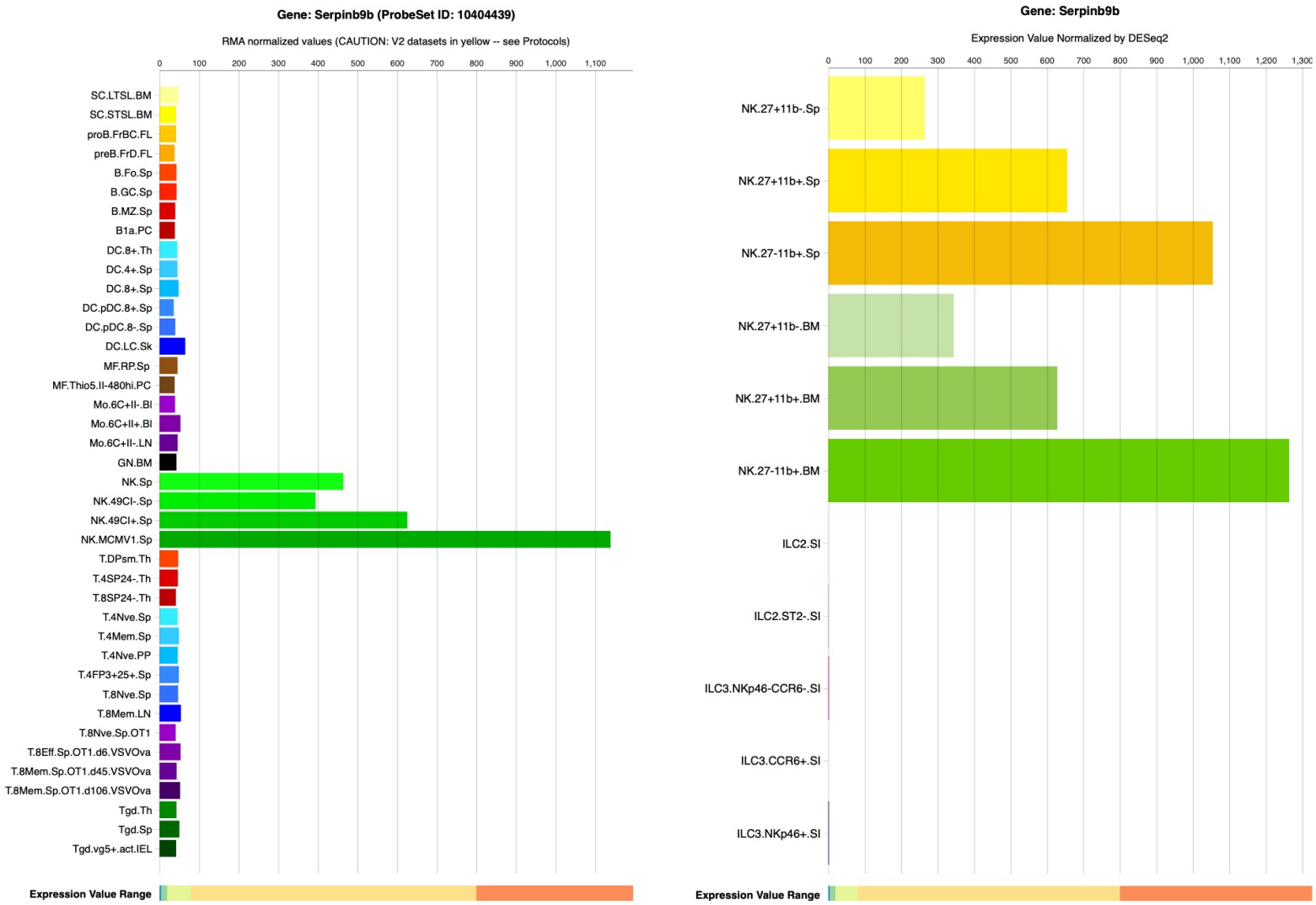

Figure 3.31 *Serpinb9b* is almost exclusively expressed by NK cells.
Mouse *Serpinb9b* gene expression data from: microarray in various immune cell types (left) and RNASeq in NK cell and ILC types (right). Source: immgen.org.

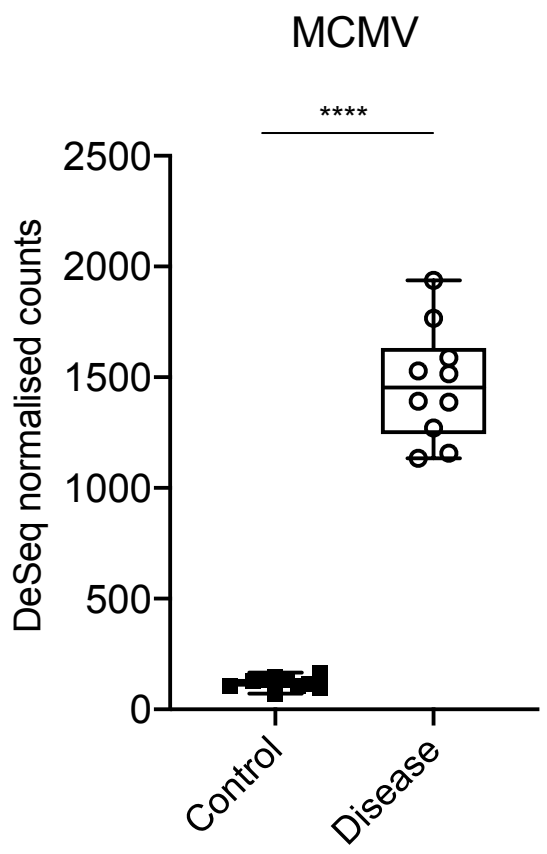

Figure 3.32 *Serpinb9b* gene expression increased 2 days post-MCMV infection in mouse whole blood.
Female C57BL/6 mice were infected with $3x10^4$ PFU of salivary gland prepared Smith strain MCMV and compared to uninfected control mice. Total blood RNA was extracted before assessing gene expression using RNA-Seq. Data is expressed as box and whisker plots and individual values from each mouse are shown (10 mice per group). ****$p<0.0001$. Source: Singhania et al. 2019.

3.2.7.1 Loss of Serpinb9b upon MCMV infection significantly reduces control of MCMV replication

MCMV infections in WT and *Serpinb9b*$^{-/-}$ mice were used to investigate whether *Serpinb9b* influences immune responses upon infection. It was observed that *Serpinb9b*$^{-/-}$ mice and WT mice had a similar weight loss profile, suggesting no impact on viral disease (Figure 3.33A). However, in the spleen, *Serpinb9b*$^{-/-}$ mice had a significant increase in virus replication (*p=0.0152) compared to WT mice (Figure 3.33B). In the liver there was no significant difference in virus replication between groups. The amount of IFNγ in the spleen and liver at 4 days post-MCMV infection was also assessed using an ELISA. In the spleen, there was no difference in the amount of IFNγ between WT and *Serpinb9b*$^{-/-}$ mice (Figure 3.34A). In the liver, there was a trend of increased IFNγ in *Serpinb9b*$^{-/-}$ mice compared to WT which was almost significant (p=0.0649, Figure 3.34B). As there was no reduction in IFNγ and no significant change in virus control within the liver where IFNγ release is more important, a change in another mechanism of virus control such as cytotoxicity may be responsible for the difference observed in the spleen (Tay and Welsh, 1997b).

Next, *Serpinb9b*$^{-/-}$ mouse infections were repeated at a 7-day timepoint to elucidate whether *Serpinb9b* may be involved in MCMV control at later stages of acute infection. *Serpinb9b*$^{-/-}$ mice did not fully recover their weight by 7 days p.i. (Figure 3.35A), while WT mice increased in weight. On days 4, 5 and 6 post-MCMV infection, *Serpinb9b*$^{-/-}$ mice weighed significantly less than WT mice (**p=0.022, *p=0.0260, *p=0.0152, respectively, Figure 3.35). The weight loss profile observed across the first 4 days of the 7-day infection differed from the weight loss profile shown during the 4-day infection where mouse weight was unaffected by gene loss (Figure 3.33A). In the liver, no significant difference in virus replication was observed between *Serpinb9b*$^{-/-}$ and WT mice (Figure 3.35B). This data demonstrated that *Serpinb9b* deficiency only had a negative impact on virus control in the spleen during the early acute phase and by 7 days there was no longer a defect. In addition, the trend showing increased liver IFNγ in *Serpinb9b* mice may have been responsible for the improved virus clearance in the liver at 7 days as liver NK cells predominantly release IFNγ in response to infection (Tay and Welsh, 1997a).

Upon Δm157 MCMV infection, I observed a small reduction in weight with no difference between WT and *Serpinb9b*$^{-/-}$ groups (Figure 3.36A). In the spleen and liver, there was no difference in viral replication between WT and *Serpinb9b*$^{-/-}$ mice, suggesting any impact of *Serpinb9b* deficiency on control of viral infection was likely related to NK cell responses driven by m157-Ly49H mediated activation (Figure 3.36B).

Although *Serpinb9b* is almost exclusively expressed by mouse NK cells and viral replication was significantly elevated in the spleen of *Serpinb9b*$^{-/-}$ mice which are key factors for determining genes to continue investigating, I decided not to pursue *Serpinb9b*. This was due to a lack of homologue in humans which means it was of less relevance to humans compared to other genes of interest.

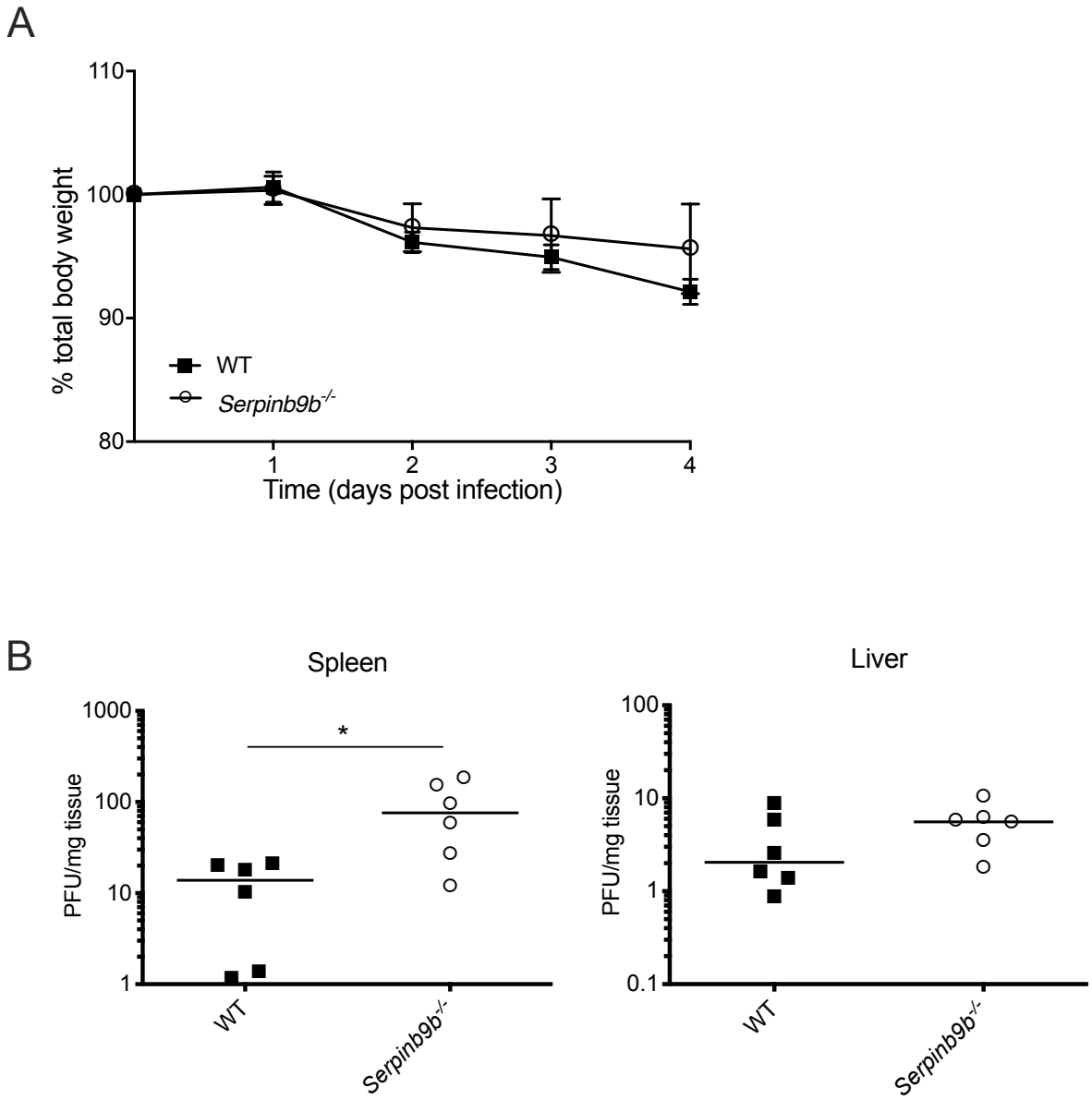

Figure 3.33 *Serpinb9b*$^{-/-}$ mice show increased virus replication in the spleen upon MCMV infection.

(A) Percentage total body weight in *Serpinb9b*$^{-/-}$ mice and WT mice 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Serpinb9b*$^{-/-}$ mouse spleen (*p=0.0152) and liver (p=0.2381). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (5-6 mice per group).

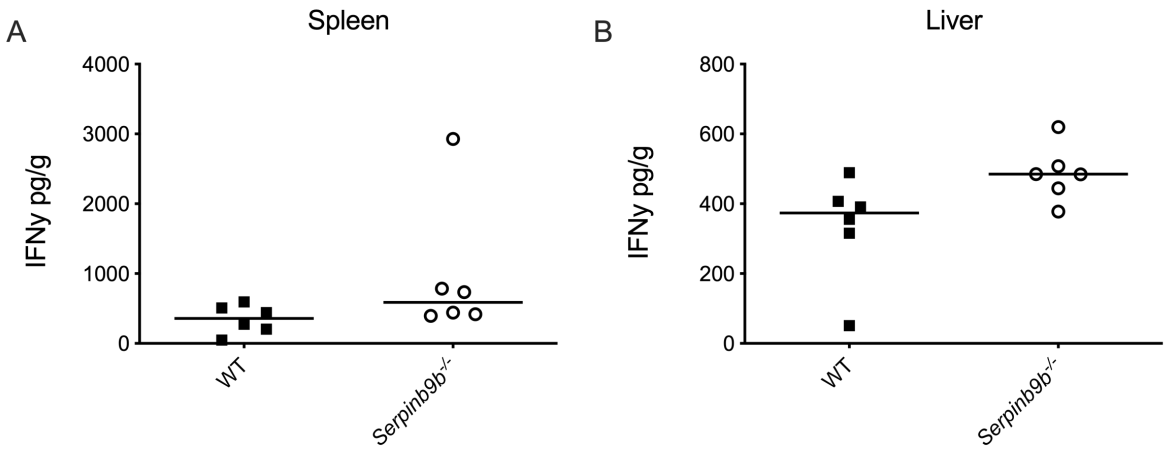

Figure 3.34 *Serpinb9b* gene deficiency does not affect IFNγ in the spleen and liver. The amount of IFNγ protein in WT and *Serpinb9b*$^{-/-}$ mouse (A) spleen (p=0.1320), and (B) liver (p=0.0649) per g tissue. Individual mice and median are shown (6 mice per group).

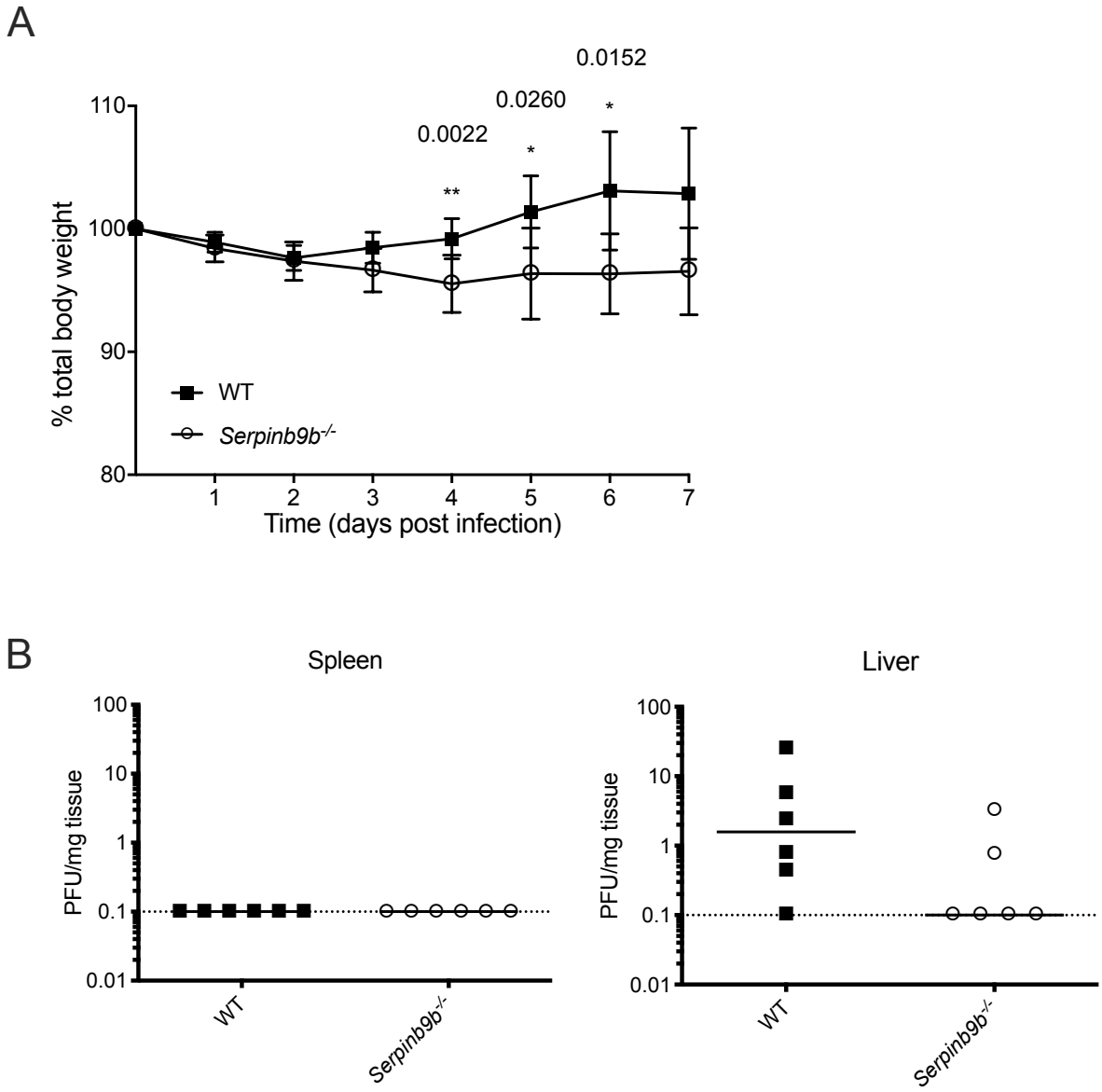

Figure 3.35 *Serpinb9b* deficiency influences weight gain during the latter stages of acute MCMV infection.

(A) Percentage total body weight in *Serpinb9b*$^{-/-}$ mice and WT mice 7-days post-MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Serpinb9b*$^{-/-}$ mouse spleen ($p>0.9999$) and liver ($p=0.1126$). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

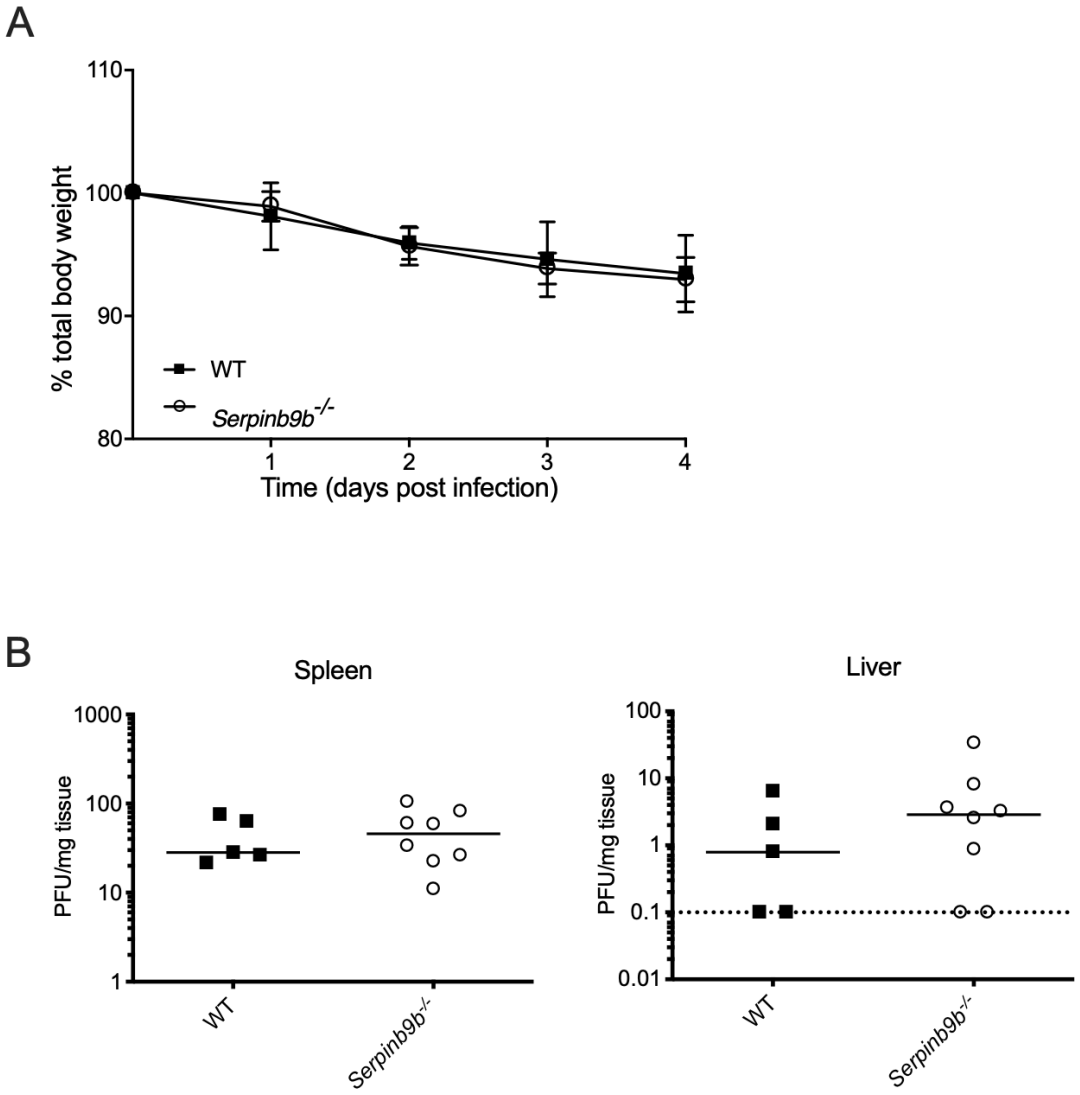

Figure 3.36 Upon Δm157 MCMV infection, *Serpinb9b*$^{-/-}$ mice show no difference in viral replication.

(A) The percentage total body weight in *Serpinb9b*$^{-/-}$ mice and WT mice 4 days post-Δm157 MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Serpinb9b*$^{-/-}$ mouse spleen (p=0.9433) and liver (p=0.3240). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (5-6 mice per group).

3.2.8 Synaptotagmin-like-3 (*Sytl3*)

SYTL3 is a novel gene associated with vesicular trafficking of cytotoxic molecules (Taruho S. Kuroda *et al.*, 2002; Ostrowski *et al.*, 2010) such as perforin secretion via Rab27a (Wood *et al.*, 2009). Members of the structurally related synaptotagmin protein family have been characterised with roles in controlling exocytosis in mast cells (Baram *et al.*, 1998), acrosomes (Hutt *et al.*, 2002) and phagocytosis in macrophages (Duque, Fukuda and Descoteaux, 2013). In addition, dysfunctional synaptotagmin-like protein and Rab27a mediated secretory lysosome release has been implicated in impaired cytotoxic lymphocyte responses in a syndrome characterised by albinism with immunodeficiency known as Griscelli Syndrome (Ménasché *et al.*, 2003, 2008; Stinchcombe, Bossi and Giffiths, 2004). The WTSI selected *Sytl3* for the *in vivo* infection screen as it was differentially expressed across different NK cell maturation subsets (Chiossone *et al.*, 2009). In addition, using data mining tools, *Sytl3* was found to be a significant regulator of the NK cell signature (Bezman *et al.*, 2012). Mouse microarray expression data (Figure 3.37) from Immgen demonstrated almost exclusive *Sytl3* expression by the following NK cell subtypes: splenic NK cells, Ly49C/I- splenic NK cells, Ly49C/I+ NK cells and splenic NK cells 1-day post-MCMV infection. *Sytl3* was also expressed by activated Vγ5+ intraepithelial γδ T-cells, $CD4^{+}CD8^{+}$ small resting helper T-cells and effector T-cell and memory T-cell types post-VSV infection (Figure 3.37). Interestingly, *Sytl3* gene expression was also significantly increased in whole blood during acute MCMV infection *in vivo* (****$p<0.0001$, Figure 3.39). Thus, *Sytl3* was identified as an NK cell-expressed gene that was of possible interest in anti-MCMV immune responses.

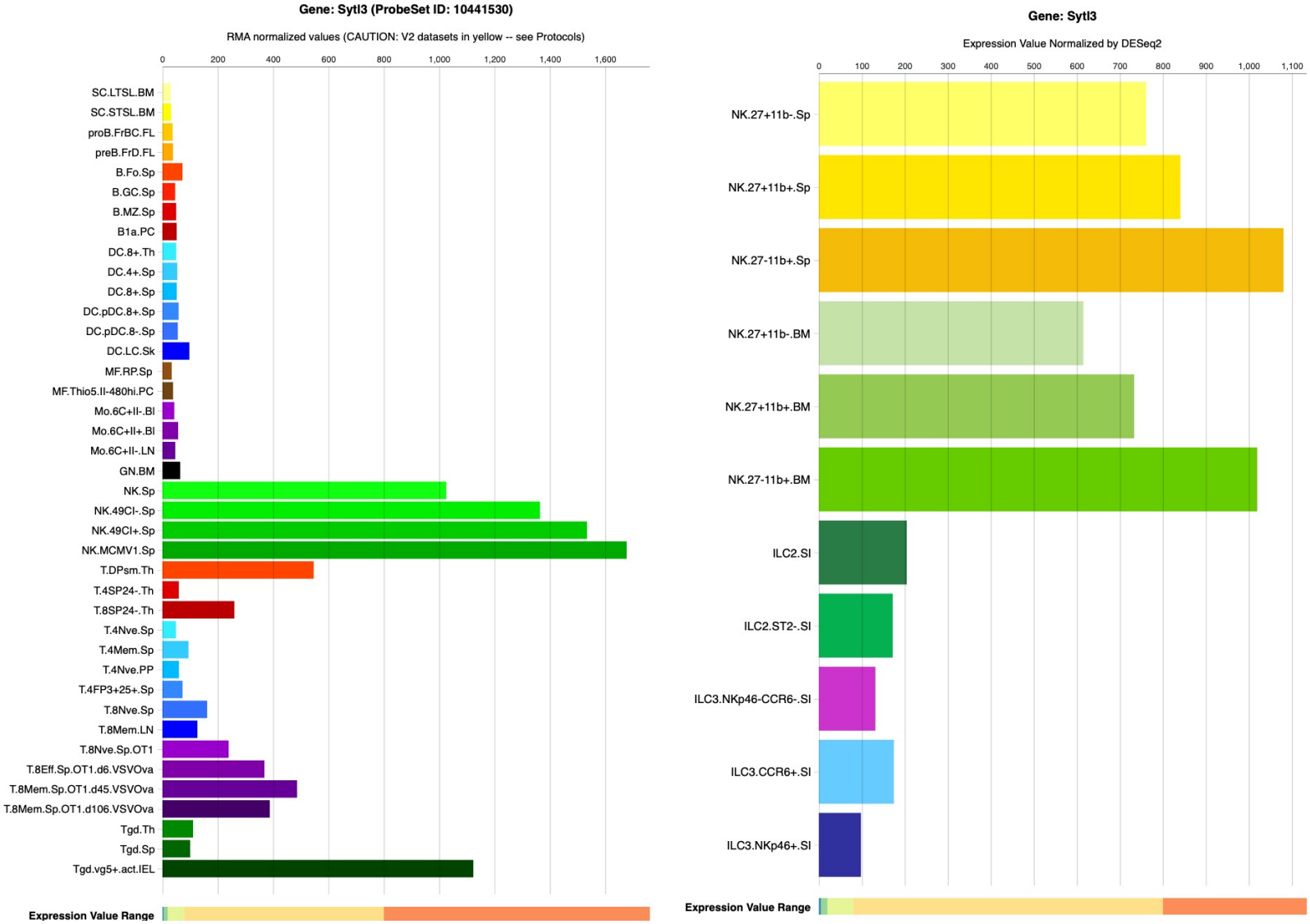

Figure 3.37 *Sytl3* is almost exclusively expressed by NK cells.
Mouse *Sytl3* gene expression data from: microarray in various immune cell types (left) and RNASeq in NK cell and ILC types (right). Source: immgen.org.

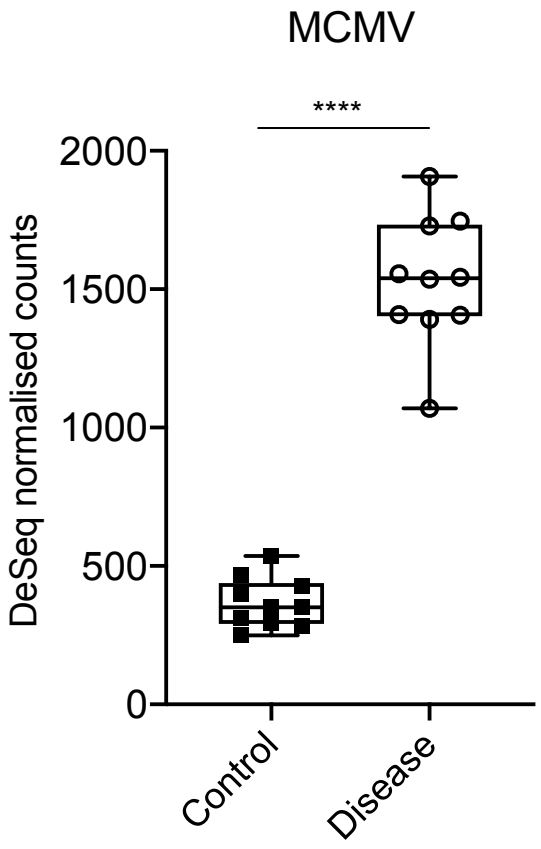

Figure 3.38 *Sytl3* gene expression increased 2 days post-MCMV infection in mouse whole blood.
Female C57BL/6 mice were infected with $3x10^4$ PFU of salivary gland prepared Smith strain MCMV and compared to uninfected control mice. Total blood RNA was extracted before assessing gene expression using RNA-Seq. Data is expressed as box and whisker plots and individual values from each mouse are shown (10 mice per group). ****$p<0.0001$. Source: Singhania et al. 2019.

3.2.8.1 Loss of Sytl3 upon MCMV infection reduces virus replication control

To uncover whether *Sytl3* influences anti-MCMV immune responses, WT and *Sytl3*$^{-/-}$ mice were infected with MCMV for 4 days. Mice from the *Sytl3*$^{-/-}$ group lost more weight than WT mice which was significant at day 4 p.i. (*p=0.0411, Figure 3.39A). There was a log-fold increase in virus replication in the spleen upon *Sytl3* gene deficiency which was not significant (p=0.0628, Figure 3.39B) and there was no difference in the liver (Figure 3.39B). Low viral replication was observed in this experiment, as WT groups in both the spleen and liver had a median viral replication of ~1 PFU/mg, which was lower than most experiments in the *in vivo* infection screen by 10 to 100-fold. Therefore, I repeated the experiment to understand if the change in virus replication within the spleen was robust. In the repeat there was no significant weight difference between groups, but the weight loss profile showed the same trend as the original screen data with *Sytl3*$^{-/-}$ mice losing more weight (Figure 3.40A). In the spleen, there was a significant log-fold increase in virus replication in *Sytl3*$^{-/-}$ mice compared to WT mice (*p=0.0152, Figure 3.40B). As expected, there was no difference in virus replication between *Sytl3*$^{-/-}$ and WT mice in the liver (Figure 3.40B). Therefore *Sytl3*$^{-/-}$ mice have impaired splenic virus control, and as splenic NK cells predominantly induce cytotoxicity during infection (Tay and Welsh, 1997a), this effect may be related to impaired cytotoxic responses.

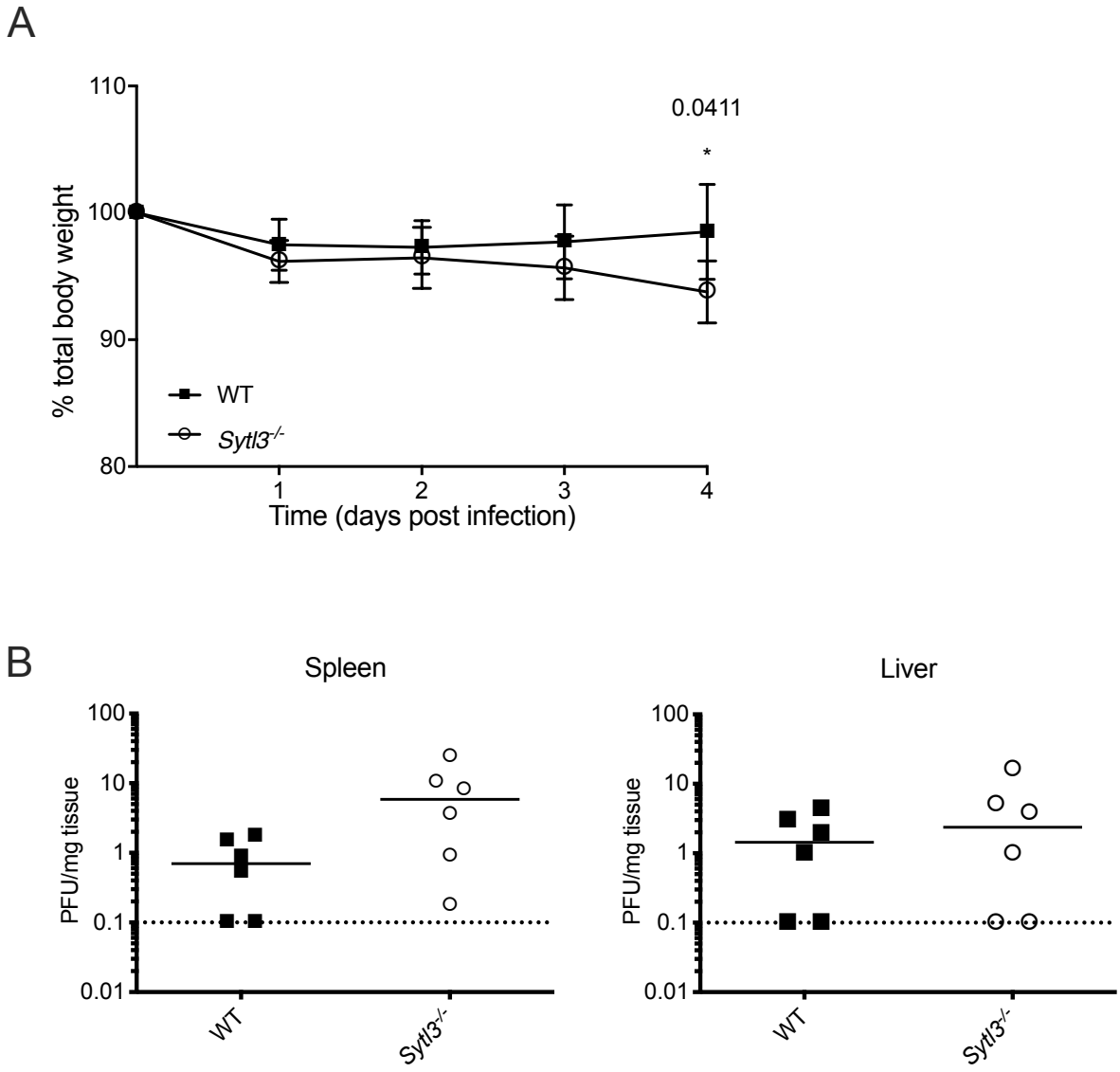

Figure 3.39 *Sytl3*$^{-/-}$ mice have a log-fold increase in virus replication in the spleen upon MCMV infection.

(A) The percentage total body weight in *Sytl3*$^{-/-}$ mice and WT mice 4 days post- MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Sytl3*$^{-/-}$ mouse spleen ($p=0.0628$) and liver ($p=0.6104$). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

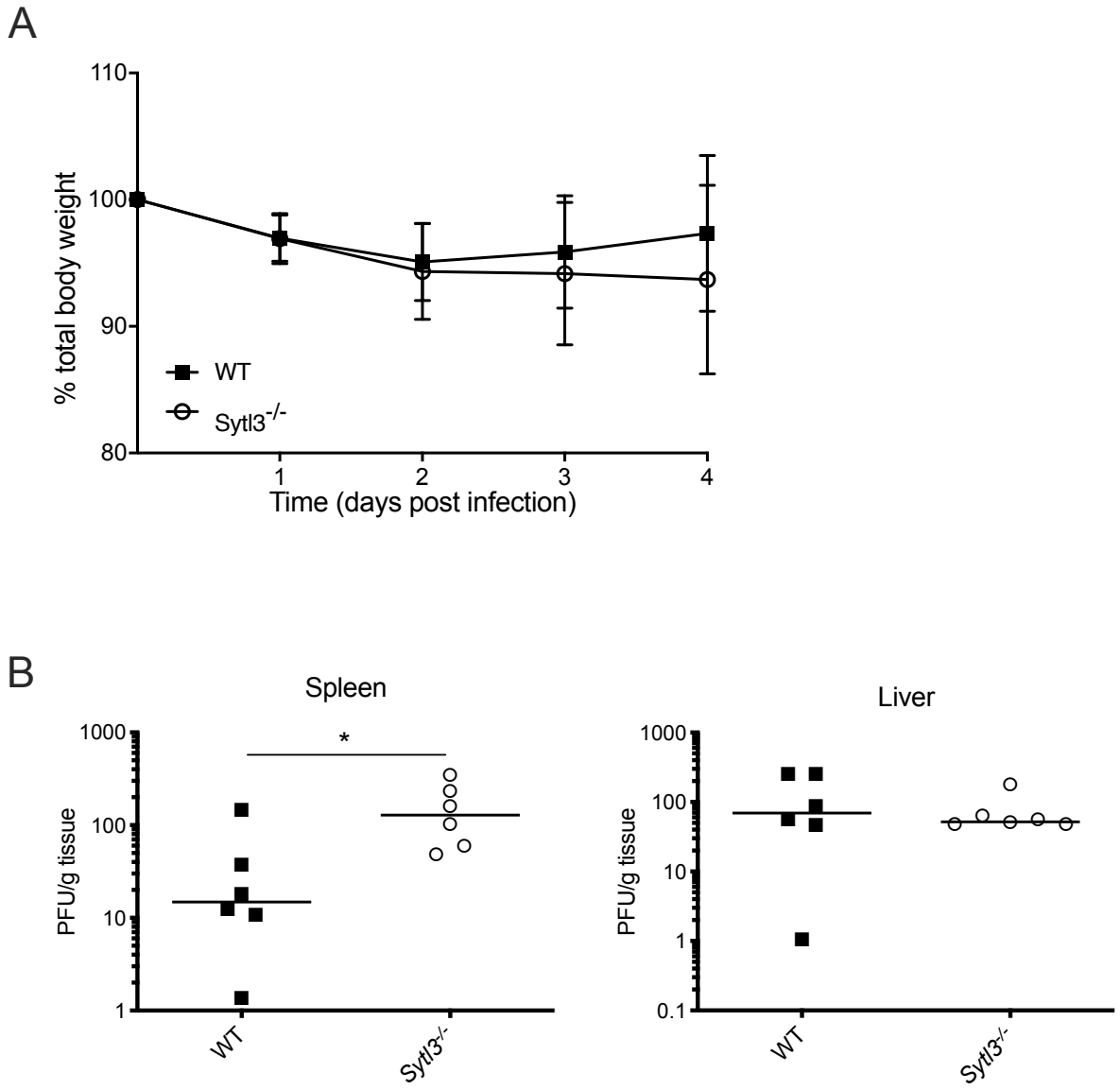

Figure 3.40 Repeat experiment demonstrating increased virus replication in the spleen of *Sytl3*$^{-/-}$ mice.
(A) The percentage total body weight in *Sytl3*$^{-/-}$ mice and WT mice 4 days post- MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Sytl3*$^{-/-}$ mouse spleen (*p=0.0152) and liver (p=0.6691). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6 mice per group).

Finally, I sought to determine whether the observed increase in viral replication in *Sytl3* deficient mice was robust using salivary gland passaged MCMV infection which has increased pathogenicity compared to tissue-culture passaged virus. Viral replication was significantly increased in *Sytl3*$^{-/-}$ mice compared to WT mice in the spleen (*p=0.0357) and liver (*p=0.0357) 4 days post-salivary gland passaged MCMV (Figure 3.41). This indicates that *Sytl3* deficiency could also impact liver NK cell responses such as IFNγ, although to a lesser extent than within the spleen due to tissue culture passaged MCMV infection results. As the trend of increased splenic virus replication was robust and *Sytl3* was almost exclusively expressed by NK cells, I decided to continue investigating *Sytl3* (Chapter 4).

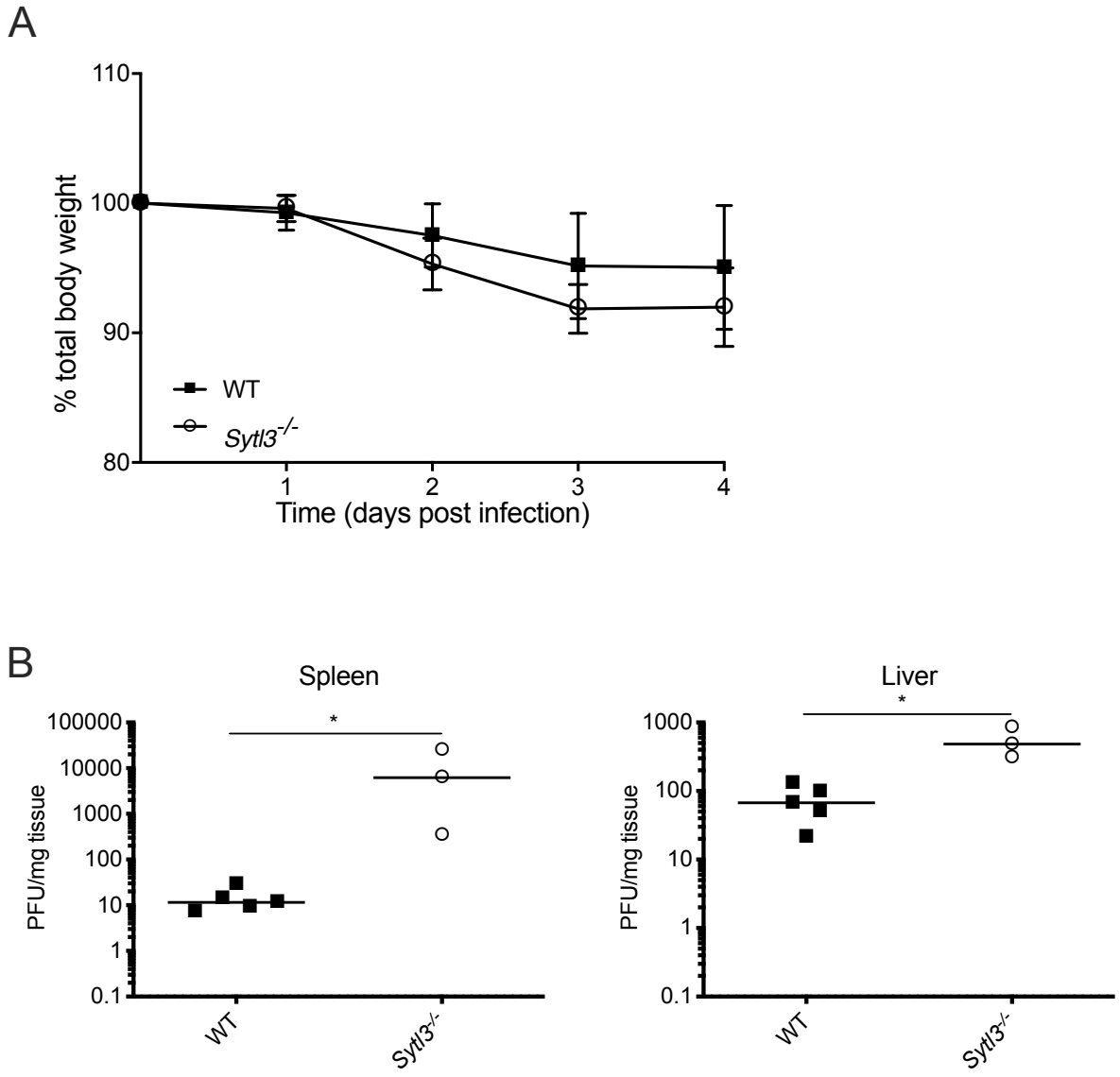

Figure 3.41 *Sytl3*$^{-/-}$ mice have increased viral replication compared to WT mice 4 days post-salivary gland passaged MCMV infection.
(A) The percentage total body weight in *Sytl3*$^{-/-}$ mice and WT mice 4 days post-salivary gland passaged MCMV infection. Lines represent mean ± standard deviation. (B) Viral replication titres from WT and *Sytl3*$^{-/-}$ mouse spleen (*p=0.0357) and liver (*p=0.0357). Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (3-5 mice per group).

3.3 Discussion

3.3.1 *In vivo* mouse screen

In this chapter, the results from an *in vivo* MCMV infection screen of gene-deficient mice were evaluated. Selected genes of interest from the screen were described and additional experiments were performed to assess whether to continue investigating them. These genes of interest described in the chapter included: *Fam111a, Fam114a2, Far1, Heatr9, Serpinb9b, and Sytl3.*

3.3.2 *Fam111a*

Upon tissue-culture passaged MCMV infection *Fam111a* gene deficiency led to improved infection control in the spleen. However, there was some variability in the infection studies due to changes in the level of infection. A low level of infection with some virus stocks was a recurring problem in the infection screen as virus stocks are tissue-culture passaged *in vitro* and can be variable. There was also inherent variability in the mouse stocks due to the mix of sexes and variability in age of the mice used as well as the need to complete experiments in batches at different times.

There was a trend in the splenic phenotypic analysis showing a reduced percentage of the most immature NK cell population. With a reduced percentage of the most immature NK cell population, the percentage of more mature subsets is thus increased and mature NK cells show an improved ability to secrete cytokines and exhibit higher cytolytic function (Fu *et al.*, 2011). Therefore, I hypothesised that the improved splenic MCMV infection control observed upon *Fam111a* gene deficiency may be due to a shift towards a greater percentage of more mature splenic NK cells, with no other NK cell activation changes observed in the phenotypic analysis. This hypothesis supports the findings by Bezman et al. which showed significant *Fam111a* expression post-MCMV infection in memory-like NK cells (Bezman *et al.*, 2012).

However, interpretation of the NK cell phenotyping data must be made with caution as the splenocytes were taken from the MCMV repeat data where there was a low level of infection and therefore no effect between WT and gene-deficient groups was observed.

This data was also derived from frozen cells. Consequently, compared to experiments using fresh splenocytes, a small proportion of cells may not have recovered due to the impact of the freeze thaw process, which in turn can affect results.

By 7 days of MCMV infection, there was a trend showing improved recovery of weight in *Fam111a*$^{-/-}$ mice. As both mouse groups showed no MCMV replication by 7 days, further investigation, perhaps using a 7-day infection of salivary gland passaged MCMV which has greater pathogenicity, is necessary to determine whether *Fam111a* is beneficial for improving virus recovery after the early acute phase.

Responses to Δm157 MCMV, which does not trigger Ly49H-mediated control of MCMV, varied between the spleen and liver in *Fam111a*$^{-/-}$ mice. As there was a reduction in viral replication in the spleens of *Fam111a*$^{-/-}$ mice upon infection with MCMV containing m157 and it was not observed upon infection with Δm157 MCMV, these data suggest that *Fam111a* suppressed a m157-Ly49H driven NK cell response. Within the liver however, virus control improved when there was no m157-Ly49H interaction. As a result of these differential effects in the liver, the m157-Ly49H interaction may have masked the improved infection control within this tissue which could be due to the differential mechanisms of NK cell (or non-NK cell) control within the spleen and liver (Tay and Welsh, 1997b).

Phenotyping of splenic NK cells derived from Δm157 MCMV infections revealed no difference in NK cell maturation, unlike the trend observed upon infection with MCMV containing m157. It is already known that the m157-Ly49H interaction is key to viral recognition required for expansion of functionally mature antiviral NK cells (Sun, Beilke and Lanier, 2009b), but whether the presence of *Fam111a* can enhance development of mature NK cells remains to be investigated. Future assessments of liver NK cell phenotypes are required to understand how differential effects were observed in this tissue when the m157-Ly49H interaction was not present. Due to the complete loss of viral replication within the liver upon infection without the m157-Ly49H interaction, I predict that NK cell phenotyping would show increased liver NK cell function upon *Fam111a* gene loss when the m157-Ly49H interaction is not present. Increased liver NK cell functionality may be demonstrated through a shift towards increased liver NK cell

maturation, as this was indicated in the spleen where reduced viral replication was observed.

Notably, a final determining experiment using the highly pathogenic salivary gland derived MCMV revealed no difference in virus control between *Fam111a*$^{-/-}$ and WT mice. Salivary gland passaged MCMV experiments were reserved for genes of interest which I may want to continue investigating due to promising initial *in vivo* screen data and follow-up experiments. This was due to ethical and economic considerations as mice are required to passage the salivary gland virus *in vivo*. However, in the case of *Fam111a* gene deficiency no difference in salivary gland virus control was observed and as a result I decided to stop investigating this gene.

3.3.3 *Fam114a2*

Fam114a2 deficiency led to an almost complete loss of detectable viral replication in the liver. This indicated that *Fam114a2* gene effects may be dependent on the liver-specific phenotype of NK cells such as IFNγ release (Tay and Welsh, 1997a), or, as *Fam114a2* had wide immune cell expression, on other immune cell types present within the liver. For example, IL-22 induced neutrophils show effector function in the liver but not in the spleen during acute MCMV infection through a mechanism linked to TRAIL (Stacey *et al.*, 2014). However, the *Fam114a2* results were not robust which may have been due to different batches of virus or differences in the mice which were housed at different times, a recurring issue in the *in vivo* screen. As the results were not robust, I decided to stop investigating this gene.

3.3.4 *Far1*

My data suggests that *Far1* deficiency, which was difficult to investigate due to the smaller size of *Far1*$^{-/-}$ mice, may improve infection control. The best evidence of this was shown in heterozygous mice which gained more weight than WT controls and showed a trend of improved infection control within the liver. However, as no significant differences in viral replication in the spleen and liver were observed, further investigation is required to determine whether *Far1* gene deficiency improves virus control. Heterozygous mice challenged with MCMV without the m157-Ly49H interaction showed no notable

difference in virus control compared to WT mice. As *Far1* was induced in a subset of memory-like NK cells post-MCMV infection (Bezman *et al.*, 2012), the lack of change in virus control upon Δm157 MCMV infection may be because *Far1* is involved in memory-like responses which require the m157-Ly49H interaction. Therefore, the benefit of improved virus control during infection with $m157^{+}$ MCMV in $Far1^{-/-}$ mice may be lost when the m157-Ly49H interaction is not present although this improvement in virus control was not fully elucidated. Due to this and the difficulty interpreting results due to size differences in homozygous mice and the absence of evidence for a striking impact on viral replication, I decided not to investigate this gene further.

3.3.5 *Heatr9*

Heatr9 deficiency was detrimental to virus control during acute MCMV infection. As this effect was most obvious in the spleen and *Heatr9* was almost exclusively expressed by NK cells, the effect of increased splenic viral replication may be related to NK cell cytotoxicity which is the predominate effector function of splenic NK cells (Tay and Welsh, 1997a). Upregulation of *Heatr9* occurs not only during early MCMV infection in the blood (Singhania *et al.*, 2019), but also within lung alveolar epithelial cells during influenza infection (Stairiker *et al.*, 2017, 2018), which supports the idea that its function is related to NK cell effector function. However, *Heatr9* was also induced in memory-like NK cells post-MCMV infection so it may also have longer-term effects on NK cell infection control (Bezman *et al.*, 2012).

Alternatively, other unknown splenic NK cell phenotypic effects could be responsible. At the timepoint where NK cells have large expansions in response to MCMV at 7 days (Schlub *et al.*, 2011; Sun *et al.*, 2011), the infections were almost resolved in both groups. Therefore, *Heatr9* deficiency is not detrimental to virus control beyond the early acute phase. During early acute infection with Δm157 MCMV $Heatr9^{-/-}$ mice showed significant virus control defects in the spleen and liver as well as increased weight loss. This indicates that the presence of m157-Ly49H may partially mask the effect of *Heatr9* gene deficiency. *Heatr9* is therefore likely to affect NK cells across all compartments and could be related to NK cell recognition and activation. The effects are less likely to be directed through effector function mechanisms which are differential between organs (Tay and Welsh, 1997a). Furthermore, since there was more severe loss of virus control when the highly

specific interaction between the m157 MCMV glycoprotein and Ly49H on NK cells was not present, *Heatr9* virus control is likely to occur independently to the m157-Ly49H interaction. I planned more experiments before deciding whether to continue investigating this gene as the initial investigations showed *Heatr9* is almost exclusively expressed by mouse NK cells and *Heatr9* may be important for controlling acute MCMV infection, especially during Δm157 MCMV infection. However, due to the impact of the COVID-19 pandemic, I was unable to investigate this gene further.

3.3.6 *Serpinb9b*

Serpinb9b$^{-/-}$ mice had increased viral replication in the spleen 4 days post-MCMV with no difference in the liver. I hypothesised that the trend of increased liver IFNγ was responsible for controlling viral replication in the liver and therefore masked the effect of *Serpinb9b* deficiency in this organ. Increased IFNγ may be caused by increased viral burden in *Serpinb9b*$^{-/-}$ mice. As there was an increase in viral replication in the spleen, where NK cells predominately induce cytotoxicity (Tay and Welsh, 1997a), this data indicates that *Serpinb9b* may be involved in NK cell cytotoxicity. This hypothesis supports the idea that the *Serpinb9b* gene encodes a granzyme M inhibitor for protection of NK cells against their own cytolytic molecules (Kaiserman and Bird, 2010).

In addition, no change in virus replication was observed upon Δm157 MCMV infection, unlike during m157^{+} MCMV infections, which suggests that *Serpinb9b* function is associated with the m157-Ly49H interaction. In addition, as reduced virus control was mainly observed within the spleen, this fits with our understanding of the known role of Ly49H in controlling viral replication through cytotoxicity in this site of MCMV infection (Parikh *et al.*, 2016).

During analysis of *Serpinb9b*$^{-/-}$ mice up to 7 days post-MCMV infection, a timepoint where NK cells are significantly expanded (Schlub *et al.*, 2011; Sun *et al.*, 2011), gene-deficient mice recovered their weight more slowly although no effect on viral replication was observed. As a result, *Serpinb9b* deficiency may affect longer term viral responses, but it is not essential for obtaining virus control.

Although the data indicated a potential role for *Serpinb9b* in controlling the NK cell-driven response to MCMV, I decided not to continue investigating this gene due to a lack of a homologue in humans since my aim was to uncover genes with translational potential.

3.3.8 *Sytl3*

Sytl3 deficiency during early acute MCMV infection led to reduced virus control within the spleen. *Sytl3* gene expression was also almost exclusive to NK cells. Therefore, I hypothesised that the gene of interest has a role in NK cell cytotoxicity which occurs mainly within the spleen (Tay and Welsh, 1997a). This hypothesis fits with associations between the synaptotagmin-like protein family, including sytl3, with vesicular trafficking pathways (Baram *et al.*, 1998; Hutt *et al.*, 2002; Taruho S. Kuroda *et al.*, 2002; Ostrowski *et al.*, 2010; Arango Duque, Fukuda and Descoteaux, 2013). Degranulation is a vesicular trafficking pathway where multivesicular structures containing proteins including perforin and granzymes are trafficked to the NK cell-target cell interface to induce target cell cytotoxicity (Neighbour, Huberman and Kress, 1982; Burkhardt *et al.*, 1990; Krzewski and Coligan, 2012). *Sytl3* also showed expression on another innate immune cell type, activated Vγ5+ activated intraepithelial γδ T-cells, therefore the impact of *Sytl3* deficiency on other mechanisms of innate immune cell control cannot be ruled out. γδ T-cells have been shown to expand, differentiate into effector/memory phenotypes and enhance their antiviral effector function in response to MCMV (Ninomiya *et al.*, 2000; Cavanaugh *et al.*, 2003; Sell *et al.*, 2015). During infection with highly pathogenic salivary gland passaged MCMV, viral replication was not only increased within the spleen, but also in the liver. As a result, *Sytl3* may also have a lesser impact on IFNγ responses. Thus, as the increase in splenic viral replication in *Sytl3* deficient mice was robust across several experiments and the gene was almost exclusively expressed by NK cells, I decided to continue investigating *Sytl3* in Chapter 4.

Chapter 4 - *Sytl3* regulates NK cell degranulation and control of MCMV infection

4.1 Introduction

4.1.1 *Sytl3*

Sytl3, the gene of interest identified in Chapter 3, is a member of the synaptotagmin-like (*Sytl)* gene family. Mouse sytl proteins were originally identified as synaptotagmin-related proteins since both protein families contain a carboxyl terminal type (C-type) tandem C2 protein domain but sytl proteins do not contain the transmembrane domain at the N-terminus (Fukuda and Mikoshiba, 2001). At the N-terminus sytl proteins contain a conserved motif named the sytl homology domain (SHD) although some family members have several splice variants which lack one of the SHD or tandem C2 protein domains (Fukuda, Saegusa and Mikoshiba, 2001). The SHD region of sytl proteins is able to bind all Rab protein isoforms (small GTPases described in section 1.3.4.) and therefore sytl proteins have a putative role as specific effector domains for Rab27a and Rab27b (Taruho S. Kuroda *et al.*, 2002). Synaptotagmins have an established role in vesicle exocytosis including neurotransmitter release (Südhof, 2012; Rizo and Xu, 2015; Zhou *et al.*, 2015; Brunger *et al.*, 2018), mast cell exocytosis (Baram *et al.*, 1998), and macrophage phagocytosis and cytokine secretion (Duque, Fukuda and Descoteaux, 2013). Due to the structural similarity of sytl proteins with synaptotagmins which have established roles in vesicle exocytosis*,* sytl protein*s* may also have a role in vesicular trafficking events. This has been shown in sytl1 proteins which have a putative role in platelet dense granule secretion (Neumüller *et al.*, 2009), sytl2-a in melanosome transport (Taruho S Kuroda *et al.*, 2002b; Kuroda and Fukuda, 2004), and finally sytl4-a proteins in insulin exocytosis (Tomas *et al.*, 2008) and dense core vesicle exocytosis in PC12 neuroendocrine cells (Fukuda *et al.*, 2002).

Sytl1-5 are conserved between humans and mice (Fukuda and Mikoshiba, 2001; Taruho S Kuroda *et al.*, 2002a). As mouse *Sytl* mRNAs are differentially distributed across different tissues and developmental stages, sytl proteins may have tissue or cell specific roles in vesicular exocytosis (Gálvez-Santisteban *et al.*, 2012). In Chapter 3 I identified that mice lacking the NK cell expressed gene *Sytl3* were less able to control MCMV

infection. Sytl3 has two splicing isoforms known as sytl3-a which contains an N-terminal SHD with zinc finger domains and sytl3-b. The C2A domain of sytl3 may function as an atypical Ca^{2+} dependent phospholipid binding domain, with key structural differences to the calcium binding C2A domain of synaptotagmin-1 (Fukuda, 2002). Synaptotagmin-1 has an established role as a calcium sensor and synchroniser of neurotransmitter release (Geppert *et al.*, 1994; Bin *et al.*, 2018) but in lymphocyte granule exocytosis the functional protein with this role is unknown. To date, limited analysis of *Sytl3* in the context of granule exocytosis exists. However, sytl3 proteins have been detected on the endosomal exocytotic vesicles of cytotoxic lymphocytes (Kurowska *et al.*, 2012). In addition, a study in cytotoxic T lymphocytes by Wood and Bryceson in 2012 suggests that sytl3 proteins form a complex with Rab27a and kinesin-1 which may facilitate anterograde transport of lytic granules from the microtubule organising centre (MTOC) towards the immunological synapse at the plasma membrane. The authors suggest further work is needed to understand if mutations in *Sytl3* could be related to immunodeficiencies (Wood and Bryceson, 2012) as seen with Rab27a deficiencies (Mancini, Chan and Paller, 1998; Ménasché *et al.*, 2000; Stinchcombe *et al.*, 2001; Bizario *et al.*, 2004; Gazit *et al.*, 2007). Indeed, overexpression of *Sytl3* in combination with Rab27a and Munc13-4 has been shown to restore granule secretion in cytotoxic lymphocytes from Chediak-Higashi Syndrome (CHS) patients who have serious degranulation defects (Sepulveda *et al.*, 2015). In this chapter I will describe how *Sytl3* gene deficiency leads to dysfunctional granule exocytosis in mice.

4.2 Aims

I hypothesise that Sytl3 deficiency, associated with reduced infection control of MCMV in vivo, is associated with dysfunctional NK cell degranulation. The aim of the work described in this chapter is to understand the mechanisms underlying *Sytl3* function in murine NK cells.

4.3 Results

4.3.1 *Sytl3* gene deficiency is associated with reduced MCMV infection control *in vivo* and impaired NK cell function

As *Sytl3* was identified as a gene of possible interest in anti-MCMV immune responses in Chapter 3, I chose to investigate this gene further. *Sytl3* mRNA has been detected within mouse spleen, lung, kidney and testis (Fukuda, Saegusa and Mikoshiba, 2001). As also shown in Chapter 3, mouse gene expression data from Immgen.org demonstrated that *Sytl3* was almost exclusively expressed by NK cells in mice (Figure 4.1) and humans (not presented here). *Sytl3* was also expressed by a group of activated intraepithelial Vγ5+ γδT cells, T-cell effector and T-cell memory types post-VSV infection and on double-positive small resting helper T-cells (Figure 4.1).

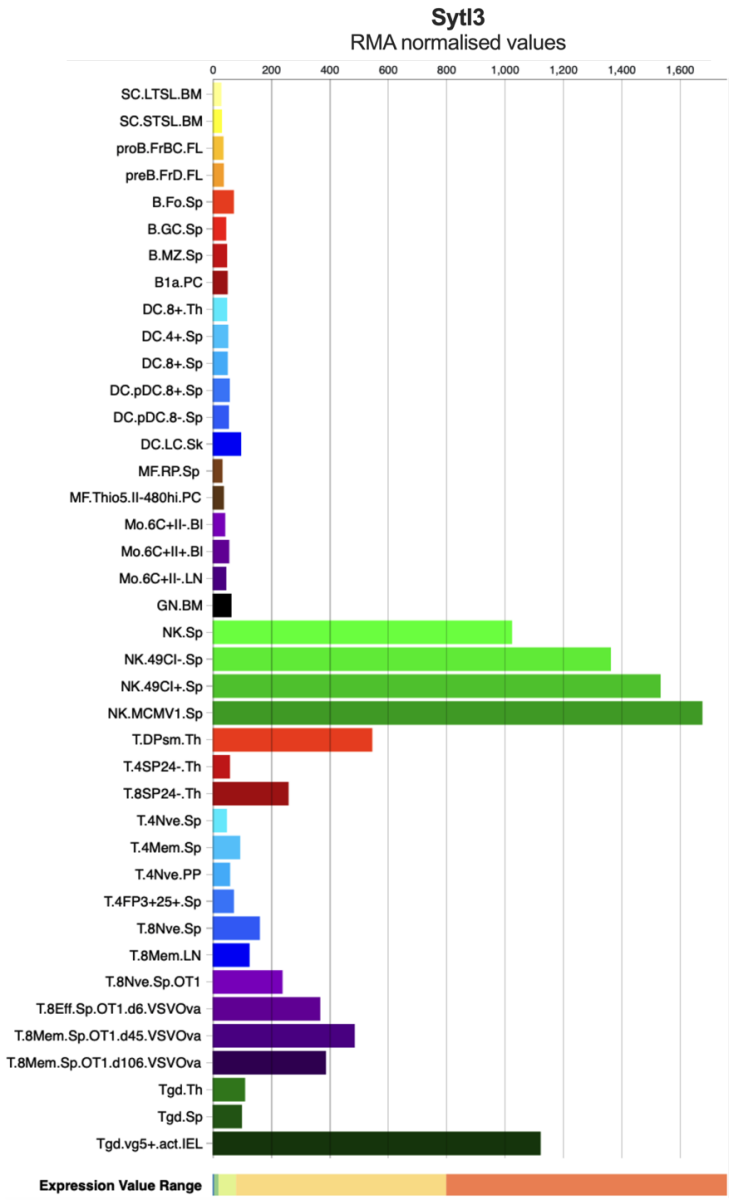

Figure 4.1 *Sytl3* gene expression is almost exclusive to NK cells and is induced upon MCMV infection.
Sytl3 gene expression mouse microarray within different immune cell subsets from Immgen.org.

To uncover when during MCMV infection the *Sytl3* gene influences virus replication, WT and *Sytl3*$^{-/-}$ mice were infected with MCMV and harvested at 2 days, 4 days, and 37 days post-infection (p.i.). MCMV viral replication in spleen, liver and salivary glands was assessed using a plaque assay. The day 4 viral replication data is the same as presented in Chapter 3 (Figure 3.41). No significant difference in viral replication was observed within the spleen and the liver at 2 days p.i. (Figure 4.2A). However, there was a trend showing reduced MCMV infection control in *Sytl3*$^{-/-}$ mice at 2 days p.i. which requires validation. At 4 days p.i. there was a significant increase in viral replication in *Sytl3*$^{-/-}$ mice compared to WT mice within the spleen (Figure 4.2B). No difference in virus replication between groups was observed within the liver at 4 days p.i. (Figure 4.2B). At 37 days p.i., no virus replication was detected in WT and *Sytl3*$^{-/-}$ salivary glands, indicating there was no uncontrolled chronic MCMV infection in *Sytl3*$^{-/-}$ mice. As it was known that NK cells control acute infection, *Sytl3* was almost exclusively expressed by NK cells (Figure 4.1), and there was a significant defect in MCMV control in *Sytl3*$^{-/-}$ mice at 4 days p.i. (Figure 4.2B), these data suggest *Sytl3* may have a role in NK cell-driven MCMV replication control.

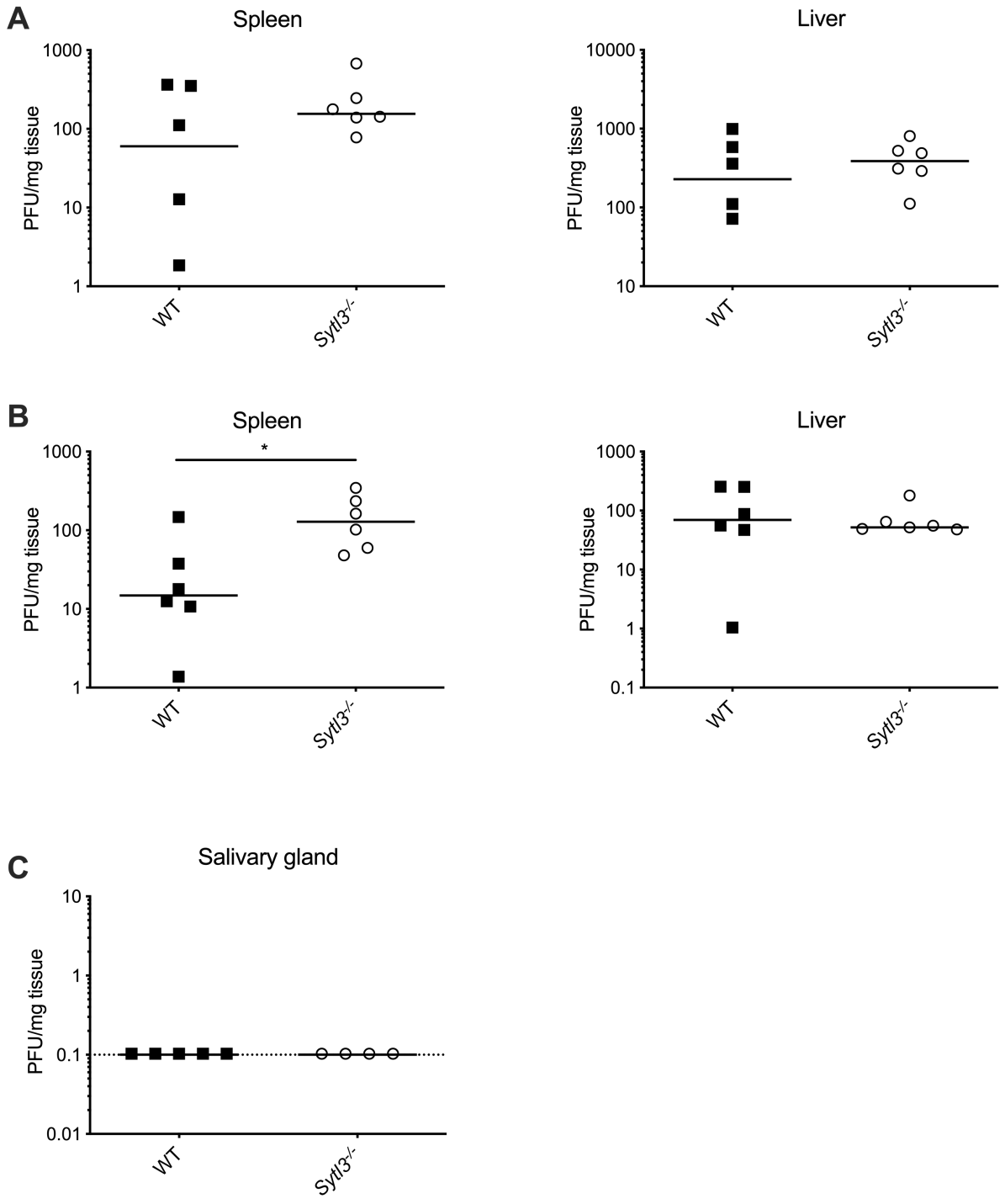

Figure 4.2 *Sytl3*$^{-/-}$ mice have increased viral replication in the spleen 4 days post-MCMV infection.

Spleen and liver viral replication titres from WT and *Sytl3*$^{-/-}$ mice (A) 2 days post-MCMV infection in the spleen and liver (p=0.3095). (B) 4 days post-MCMV infection in the spleen (*p<0.0155) and liver. (C) 37 days post-MCMV infection within the salivary gland. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (4-6 mice per group).

4.3.2 *Sytl3* deficient mice exhibit no defects in the frequencies of innate immune cell types in the spleen and blood

To understand whether the innate immune composition was affected by *Sytl3* deficiency which may impact acute infection control, innate immune cell types from the blood and spleen of WT and *Sytl3*$^{-/-}$ mice were phenotyped using flow cytometry. The percentage of *γδ* T cells, NK cells, DCs and monocytes was calculated as a percentage of the total CD45+ population. The percentage of Ly49H+ NK cells and Killer Cell Lectin Like Receptor 1 (KLRG1)+ NK cells as well as NK cell maturation subsets were calculated as a percentage of total NK cells. Ly49H is an important activating receptor for MCMV responses (Sun *et al.*, 2011) and KLRG1 is an activation marker highly upregulated on NK cells following activation by a variety of stimuli, including viral infection (Robbins *et al.*, 2002, 2004). No significant defects in the percentage of innate immune cell types were observed in *Sytl3*$^{-/-}$ mice, suggesting that the loss of viral replication control (Figure 4.2) was unlikely to be due to a change in percentage of the immune cells shown. In addition, there was an increase in the percentage of total DCs (Figure 4.4), Ly49H+ NK cells (Figure 4.5) and CD11b+CD27- (mature) NK cells (Figure 4.6) within the spleen which indicates increased innate immune function. The following phenotyping data was completed at the WTSI by Anneliese Speak as part of the Sanger Institute Mouse Genetics Project.

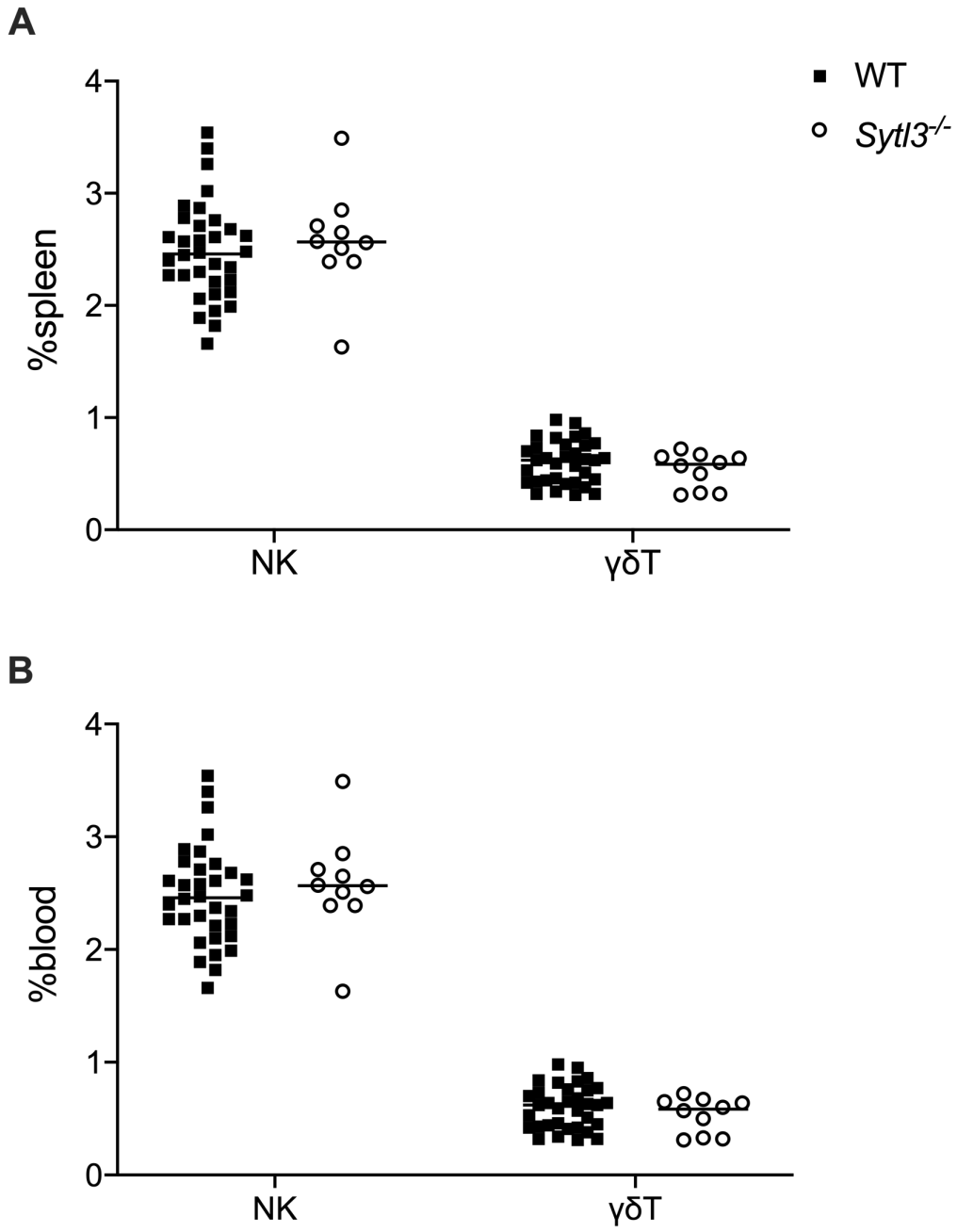

Figure 4.3 *Sytl3*$^{-/-}$ mice exhibit no difference in the percentage of NK cells and γδT cells.

Percentage of total NK cells and γδT cells within the live CD45+ population from naïve WT and *Sytl3*$^{-/-}$ mice (A) within the spleen (B) within the blood. Individual mice and median are shown (10-37 mice per group). NK1.1^{+}CD3^{-} cells were defined as NK cells and αβTCR^{-}CD3^{+} cells were defined as γδT cells. Statistical analysis was performed using Phenstat to account for batch effects as the mice were assayed over several collection days with concurrent controls and the p-values were adjusted using the Benjamini-Hochberg method.

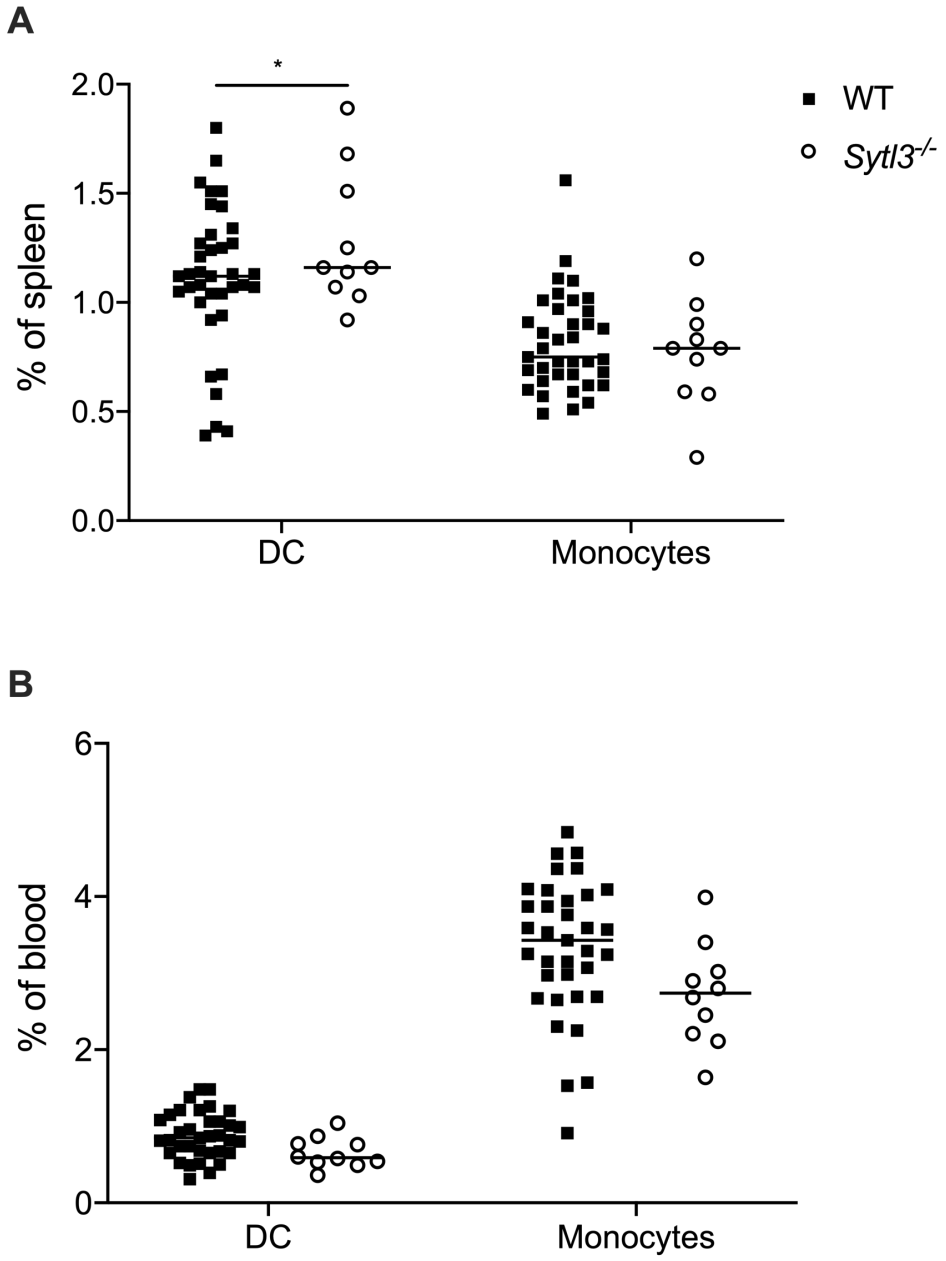

Figure 4.4 *Sytl3*$^{-/-}$ mice exhibit no defect in the percentage of DCs and monocytes. Percentage of total DCs and monocytes within the live CD45+ population from naïve WT and *Sytl3*$^{-/-}$ mice (A) in the spleen (B) in the blood. Individual mice and median are shown (10-37 mice per group). Statistical analysis was performed using Phenstat to account for batch effects as the mice were assayed over several collection days with concurrent controls and the p-values were adjusted using the Banjamini-Hochberg method. *p=0.0111

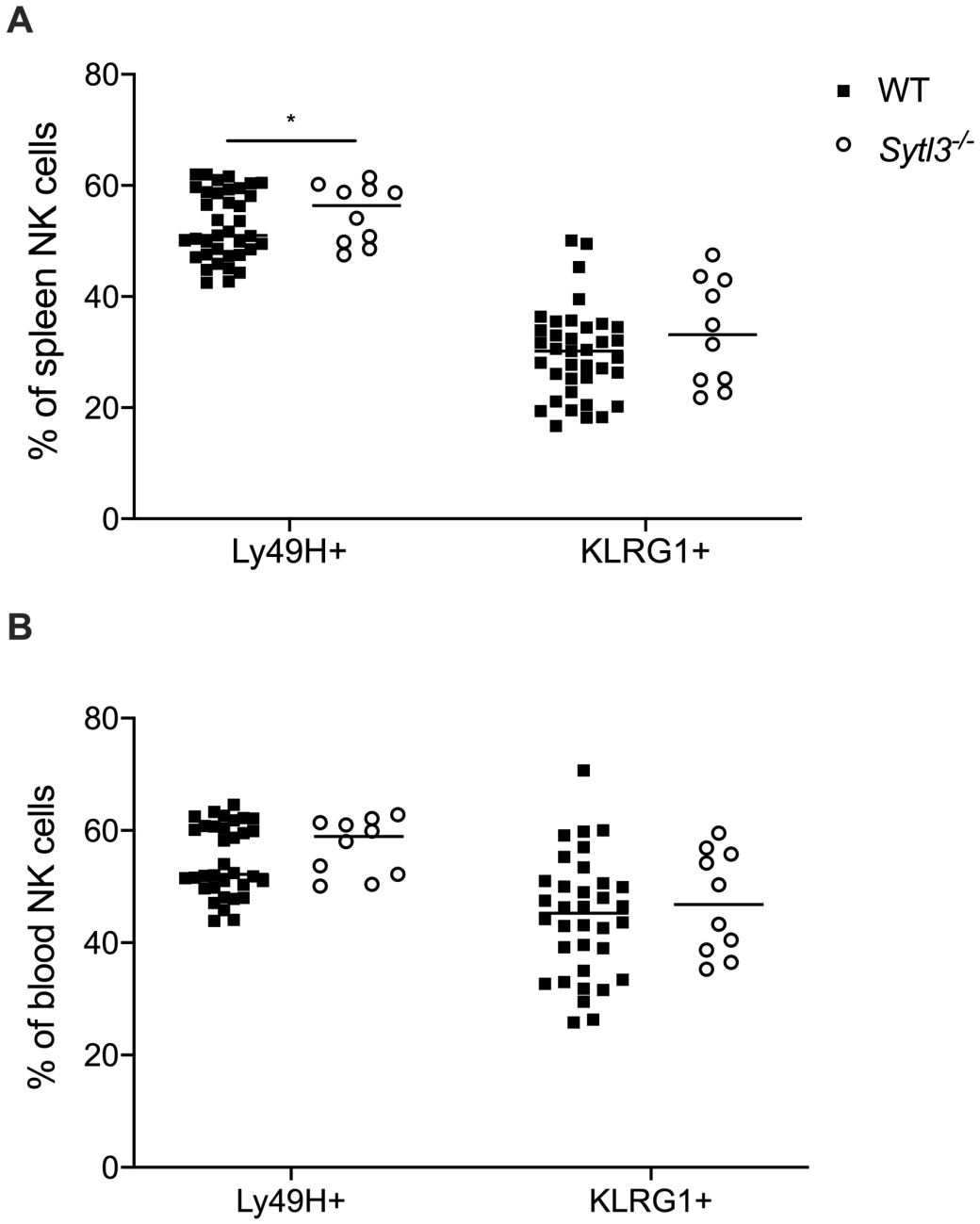

Figure 4.5 *Sytl3*$^{-/-}$ mice exhibit no defects in the percentage of Ly49H+ NK cells and KLRG1+ NK cells.

Percentage of total Ly49H+ NK cells and KLRG1+ NK cells within the total NK cell population from naïve WT and *Sytl3*$^{-/-}$ mice (A) in the spleen (B) in the blood. Individual mice and median are shown (10-37 mice per group). Statistical analysis was performed using Phenstat to account for batch effects as the mice were assayed over several collection days with concurrent controls and the p-values were adjusted using the Banjamini-Hochberg method. *p=0.0447

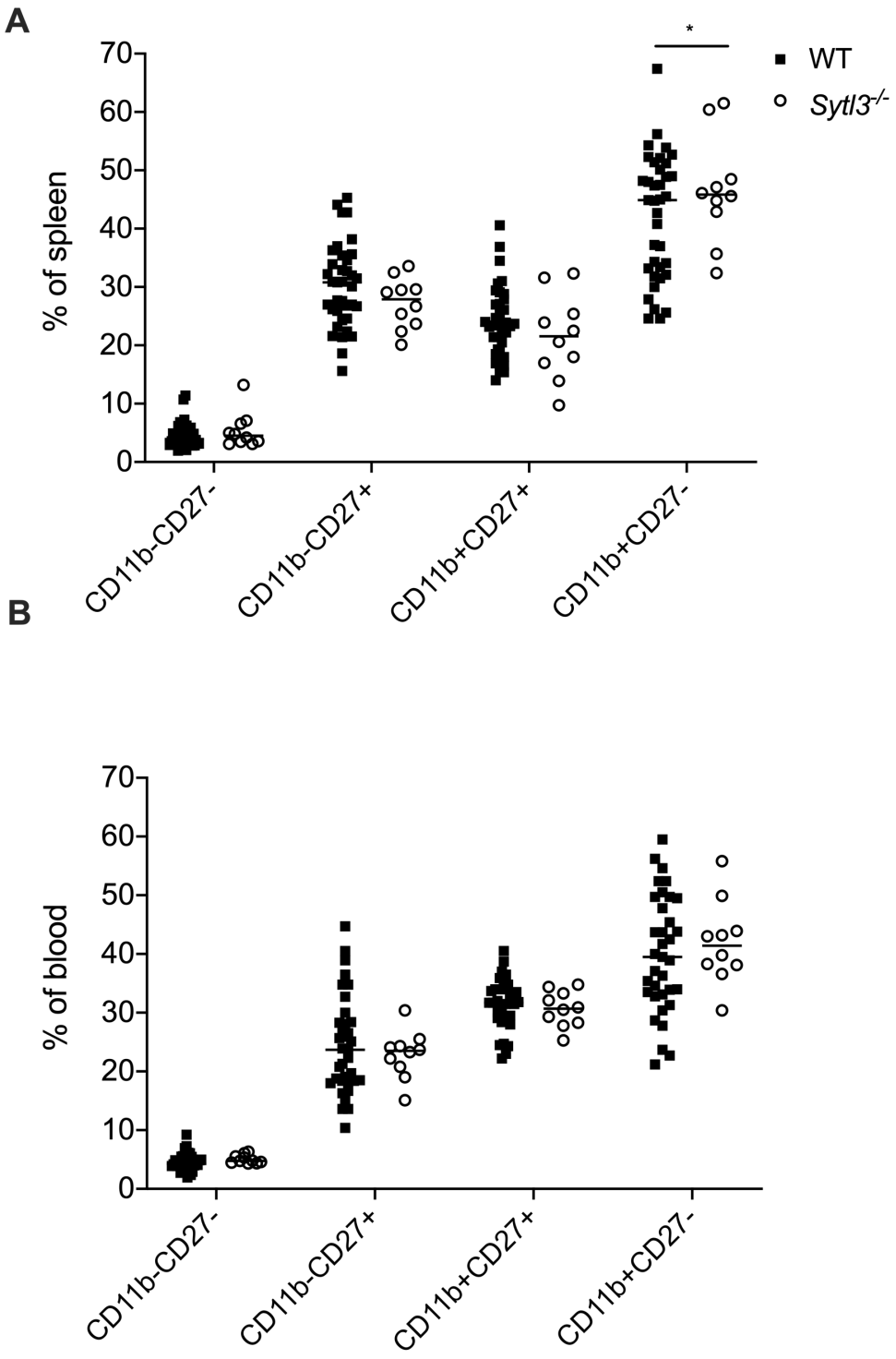

Figure 4.6 *Sytl3*$^{-/-}$ mice exhibit no NK cell maturation defects.
Percentage of CD11b-CD27- (immature), CD11b-CD27+, CD11b+CD27+ and CD11b+CD27- (mature) cells within the whole NK population from naïve WT and *Sytl3*$^{-/-}$ mice (A) within the spleen (B) within the blood. Individual mice and median are shown (10-37 mice per group). Statistical analysis was performed using Phenstat to account for batch effects as the mice were assayed over several collection days with concurrent controls and the p-values were adjusted using the Banjamini-Hochberg method. *p=0.0311

4.3.3 Reduced splenic MCMV infection control in *Sytl3*$^{-/-}$ mice is due to impaired NK cell function

To understand whether *Sytl3* gene deficiency impacts NK cell mediated control of MCMV, mice were dosed with a depleting αNK1.1 antibody during MCMV infection. I administered αNK1.1 antibody or PBS control on days -2, 0 and +2 post-MCMV infection to WT and *Sytl3*$^{-/-}$ mice before harvesting spleens and livers for viral replication analysis and identification of NK cell numbers 4 days post-MCMV infection. αNK1.1 antibody treated groups showed almost complete loss of NK cells (Figure 4.7A). As observed in previous data (Figure 4.2B), the viral replication during *Sytl3* deficiency significantly increased 4 days p.i. compared to WT in the control treated groups (Figure 4.7B). Upon depleting αNK1.1 treatment, WT and *Sytl3*$^{-/-}$ mice demonstrated no difference in the level of viral replication (Figure 4.7B), indicating that the impairment in viral replication control at this timepoint in *Sytl3*$^{-/-}$ mice was due to specific NK cell functional impairment.

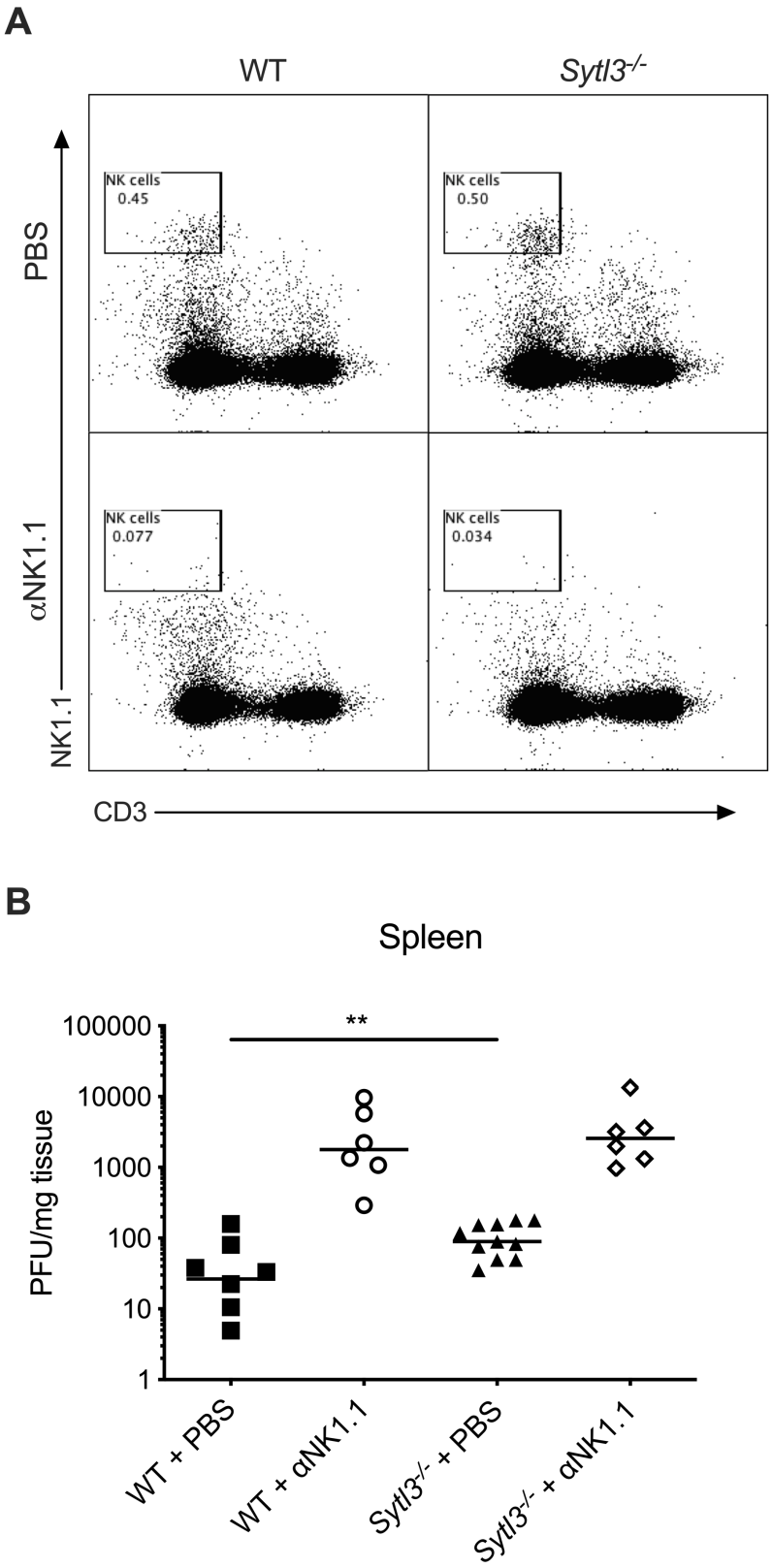

Figure 4.7 Upon NK cell depletion, *Sytl3*$^{-/-}$ mice have the same level of viral replication as WT mice.

WT and *Sytl3*$^{-/-}$ mice treated with PBS control or αNK1.1 depleting antibody 4 days post-MCMV infection. (A) Representative flow cytometry plots of NK cells (defined as NK1.1+CD3-). (B) Splenic viral replication. Individual mice and median are shown from two independent experiments (5-11 mice per group). **p=0.0068

4.3.4 *Sytl3* deficient mice have unaltered NK cell accumulation, NK cell activating receptor expression and NK cell maturation in response to MCMV

I hypothesised that *Sytl3* influenced NK cell functionality therefore I investigated the number of NK cells, NK cell activating receptor expression and maturation. To test this, WT and *Sytl3*$^{-/-}$ splenic NK cells from naïve and MCMV infected mice 4 days p.i. were examined using flow cytometry to determine NK cell accumulation and expression of different NK cell functional markers. At day 4 p.i., *Sytl3* deficiency had no impact on total NK cell number (Figure 4.8A), the percentage of NK cells expressing CD25 (Figure 4.8B), Ly49H (Figure 4.8C), and the maturation markers CD27 and CD11b (Figure 4.8E). There was a significant increase in intracellular granzyme B which may indicate reduced cytotoxic granule release (Figure 4.8D). This suggested the impairment of NK cell function in *Sytl3*$^{-/-}$ mice was not associated with deficiencies in these functional markers, nor was it due to impaired NK cell recruitment, expansion and/or survival *in vivo*.

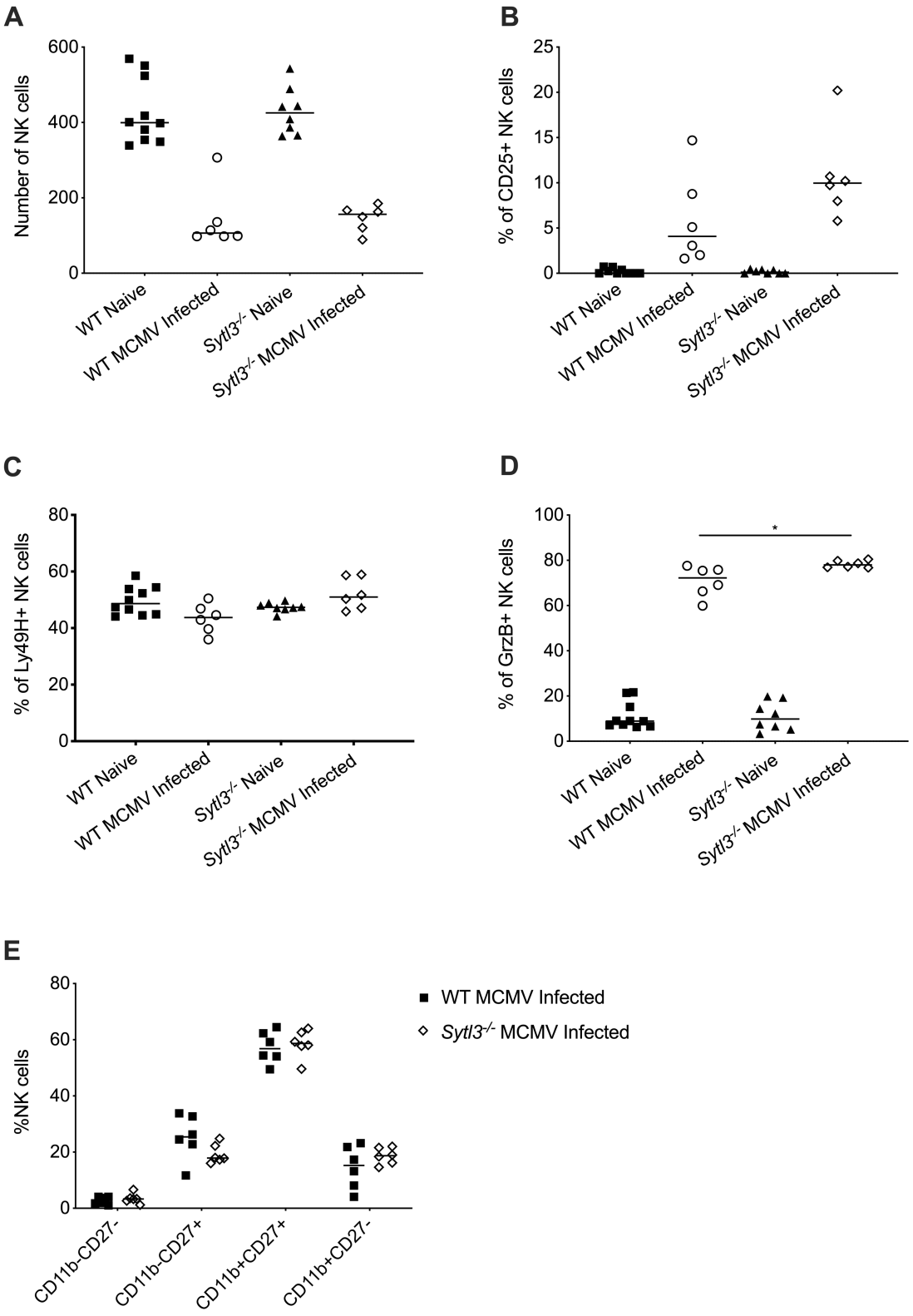

Figure 4.8 *Sytl3* does not impact NK cell accumulation, activating receptor expression and maturation.

WT and *Sytl3*$^{-/-}$ mice either naïve or 4 days post-MCMV infection. (A) The total number of splenic NK cells (defined as NK1.1^{+}CD3^{-}) detected by flow cytometry. The percentage of (B) CD25+ NK cells, (C) Ly49H+ NK cells, (D) granzyme B (GrzB)+ NK cells. (E) The percentage of NK cell maturation populations from immature (CD11b-CD27-) to mature (CD11b+CD27-). Individual mice and median are shown (6-10 mice per group). *p=0.0152

4.3.5 NK cells derived from *Sytl3* deficient mice have impaired degranulation responses and IFNγ production compared to WT mice

To examine whether impaired NK cell control of MCMV was due to deficient degranulation or cytokine production, WT and *Sytl3*$^{-/-}$ mouse splenocytes from naïve and MCMV infected mice 2 days p.i. were stimulated with phorbol 12-myristate 13-acetate (PMA)-Ionomycin and compared to unstimulated controls. PMA activates protein kinase C and ionomycin is a calcium transporter. Both compounds bypass membrane bound receptor complexes and initiate several activating intracellular signalling pathways. As a result, PMA-Ionomycin stimulation is a useful tool for understanding changes to activation signals including degranulation and cytokine release independently of specific NK cell receptor signalling. CD107a expression by NK cells is widely used as a marker of granule secretion during degranulation (Alter, Malenfant and Altfeld, 2004; Aktas *et al.*, 2009). After PMA-Ionomycin stimulation, the percentage and number of NK cells expressing CD107a in *Sytl3*$^{-/-}$ mice compared to WT mice did not change significantly (Figure 4.9A-B). However, there was a trend showing a degranulation defect in *Sytl3*$^{-/-}$ mice which requires further validation. The percentage and number of NK cells producing IFNγ in *Sytl3*$^{-/-}$ mice compared to WT mice also did not significantly change (Figure 4.9C). However, there was also a trend showing defective IFNγ production in *Sytl3*$^{-/-}$ mice. Therefore, at 2 days p.i., there was an early indication of defective *Sytl3*$^{-/-}$degranulation and IFNγ production but validation with larger group sizes is required.

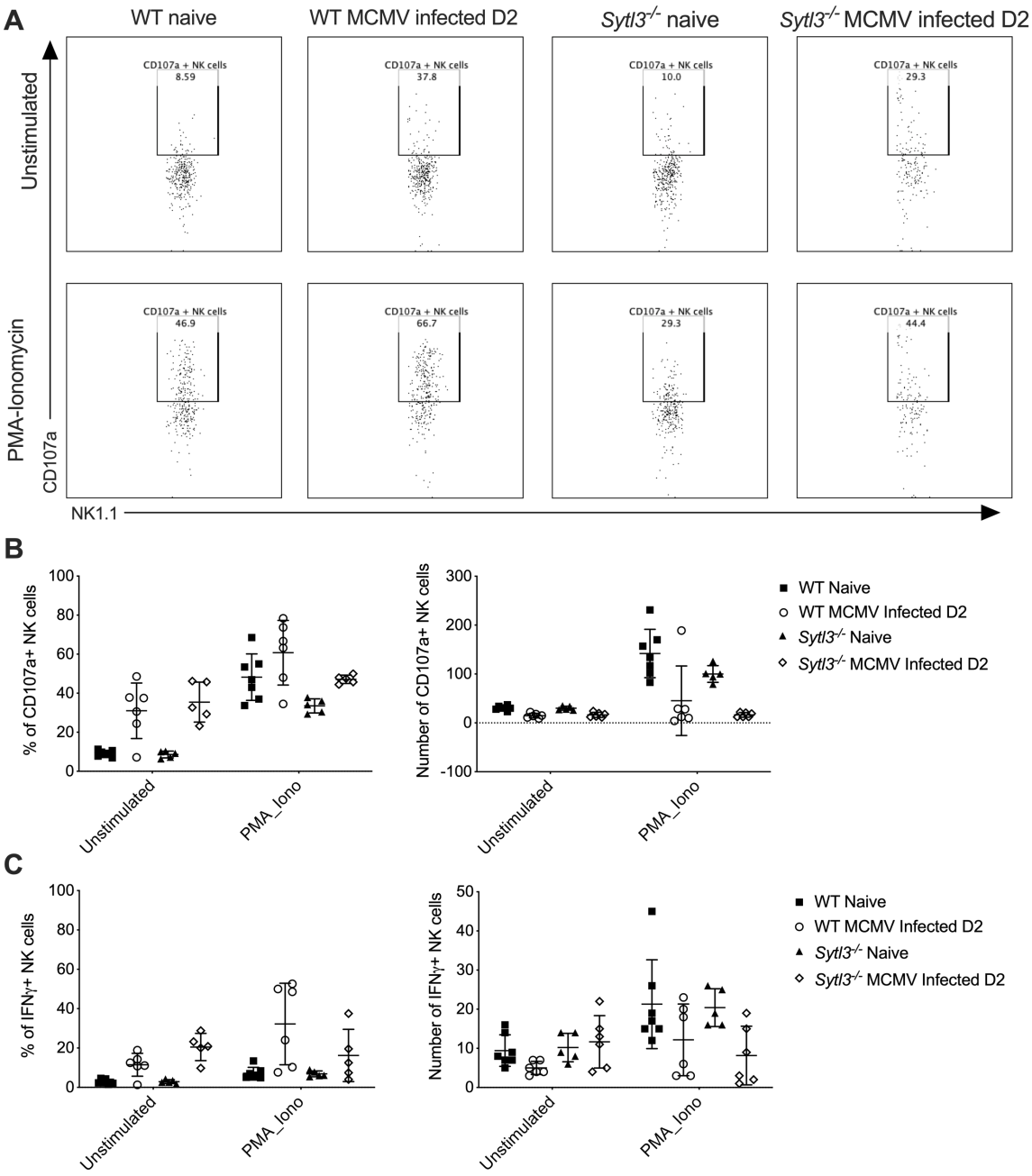

Figure 4.9 At 2 days p.i. *Sytl3* deficient NK cells exhibit an early indication of reduced CD107a expression and IFNγ production.
WT and *Sytl3*$^{-/-}$ naïve mice at 2 days post-MCMV Smith strain infection. (A) Representative FACS plots showing %CD107a+ splenic NK cells (B) The percentage of splenic CD107a+ NK cells and the number of splenic CD107a+ NK cells. (C) The percentage of IFNγ+ splenic NK cells and the number of splenic IFNγ+ NK cells. Individual mice, median and interquartile range are shown (3-6 mice per group).

NK cell degranulation and IFNγ production in response to PMA-Ionomycin stimulation were also investigated at 4 days p.i. which was the timepoint where significant viral replication defects were observed (Figure 4.2B). At 4 days p.i. after PMA-Ionomycin stimulation, I observed a reduction in both the percentage and total number of CD107a+ NK cells indicating a degranulation defect (Figure 4.10A-B). Furthermore, at 4 days p.i. without further stimulation there was a significant increase in granzyme B+ NK cells from *Sytl3*$^{-/-}$ mice compared to WT mice (Figure 4.8D) which may indicate impaired granzyme B release therefore further supporting the hypothesis of defective NK cell degranulation upon *Sytl3* deficiency. Interestingly, I also observed a significant reduction in the total number of IFNγ+ NK cells in *Sytl3*$^{-/-}$ mice (Figure 4.10C), suggesting *Sytl3* deficiency may influence intracellular IFNγ protein levels in addition to degranulation. However, the amount of plasma IFNγ was also assessed in these mice and was unaltered by *Sytl3* deficiency suggesting no significant *in vivo* effect of *Sytl3* on IFNγ responses (Figure 4.11). Therefore, further experiments are required to understand the effects of reduced IFNγ production within *Sytl3* deficient NK cells. As the degranulation defect was more pronounced than the IFNγ production impairment, and due to literature showing the predominant effector function of splenic NK cells is cytotoxicity (Tay and Welsh, 1997b), I decided to investigate the degranulation impairment in more detail.

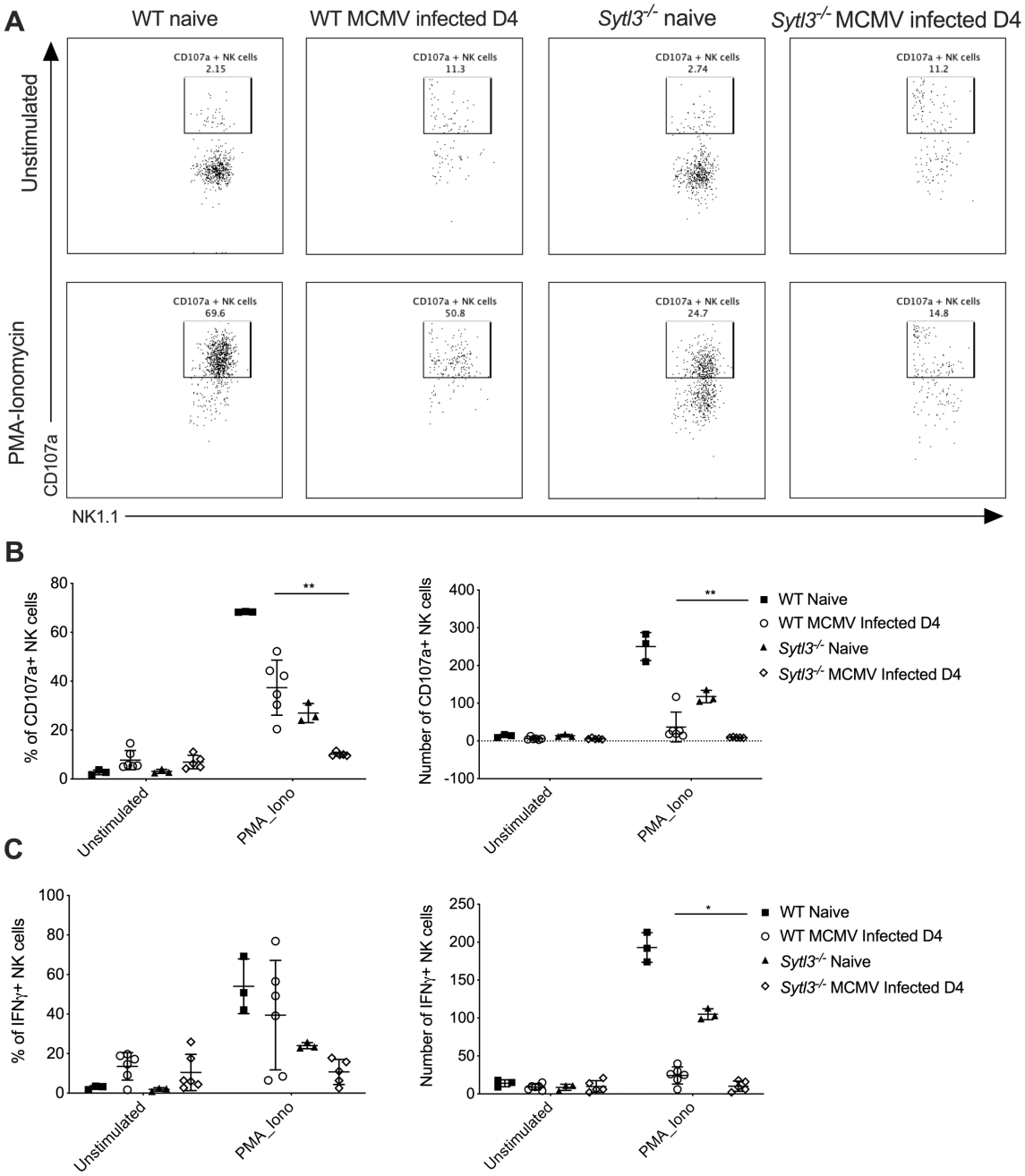

Figure 4.10 *Sytl3*$^{-/-}$ mice 4 days p.i. exhibit dysfunctional degranulation and IFNγ production.

WT and *Sytl3*$^{-/-}$ naïve mice at 4 days post-MCMV Smith strain infection. (A) Representative FACS plots showing %CD107a+ splenic NK cells (B) The percentage of splenic CD107a+ NK cells (**p=0.0016) and the number of splenic CD107a+ NK cells (**p=0.0043). (C) The percentage of IFNγ+ splenic NK cells and the number of IFNγ+ splenic NK cells (*p=0.0325). Individual mice, median and interquartile range are shown (3-6 mice per group).

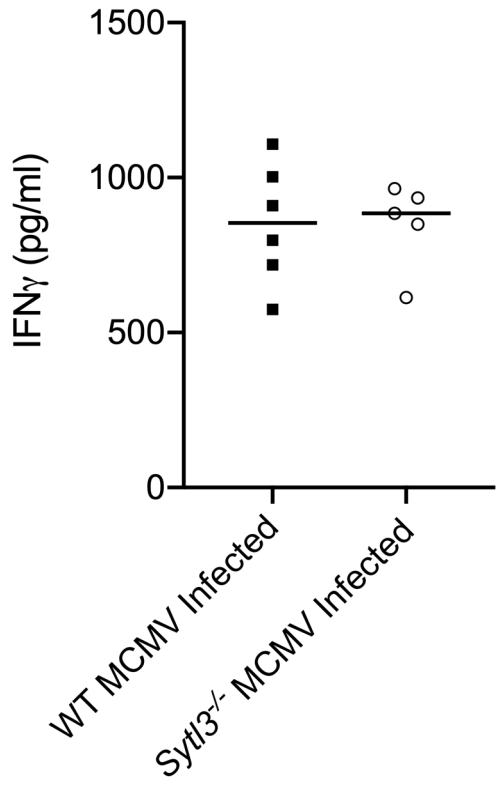

Figure 4.11 *Sytl3* deficiency does not impact blood plasma IFNγ.
The amount of plasma IFNγ protein from WT and *Sytl3*$^{-/-}$ mice 4 days post-MCMV infection. Individual mice and median are shown (6 mice per group).

4.3.6 Characterisation of monocyte and neutrophil innate cell populations in *Sytl3* deficient mice during acute MCMV infection

As MCMV responses were impaired at the acute stage of MCMV infection (Figure 4.2) and to ensure the defective *Sytl3*$^{-/-}$ response was predominantly due to dysfunctional NK cells, I investigated populations of other innate cells important for virus control at 2 days p.i. The percentage of total innate cells including total monocytes (Figure 4.12A), inflammatory monocytes (Figure 4.12B), classical DCs (Figure 4.12D) and neutrophils (Figure 4.12E) did not change between WT and *Sytl3*$^{-/-}$ naïve groups and between WT and *Sytl3*$^{-/-}$ MCMV infected groups. The percentage of macrophages significantly increased in *Sytl3*$^{-/-}$ MCMV infected mice compared to WT MCMV infected mice (Figure 4.12C). Macrophages respond during the early acute phase at 2 days p.i. and have a reduced role in infection control by 4 days p.i. (Hokeness-Antonelli *et al.*, 2007; Crane, Hokeness-Antonelli and Salazar-Mather, 2009). As the change in virus replication at 2 days p.i did not change significantly but did change at 4 days p.i. (Figure 4.2A-B), these data indicate that increased macrophage responses may enhance control of MCMV replication during dysfunctional *Sytl3*$^{-/-}$ NK cell responses in the early stages of acute infection. Since type I IFNs are important for inducing NK cell function during acute infection (Krug *et al.*, 2004; Hokeness-Antonelli *et al.*, 2007; Schneider *et al.*, 2008), I investigated the amount of IFNβ protein in WT and *Sytl3*$^{-/-}$ mice at 2 days p.i alongside naïve controls. There was no significant difference in plasma IFNβ protein levels in *Sytl3*$^{-/-}$ mice and WT controls. However, there was a trend showing increased IFNβ in *Sytl3*$^{-/-}$ mice 2 days post-MCMV infection which may also help control viral replication at this timepoint. Thus, these data suggest that non-NK cell innate pathways may partially control viral replication 2 days p.i. in the absence of *Sytl3*.

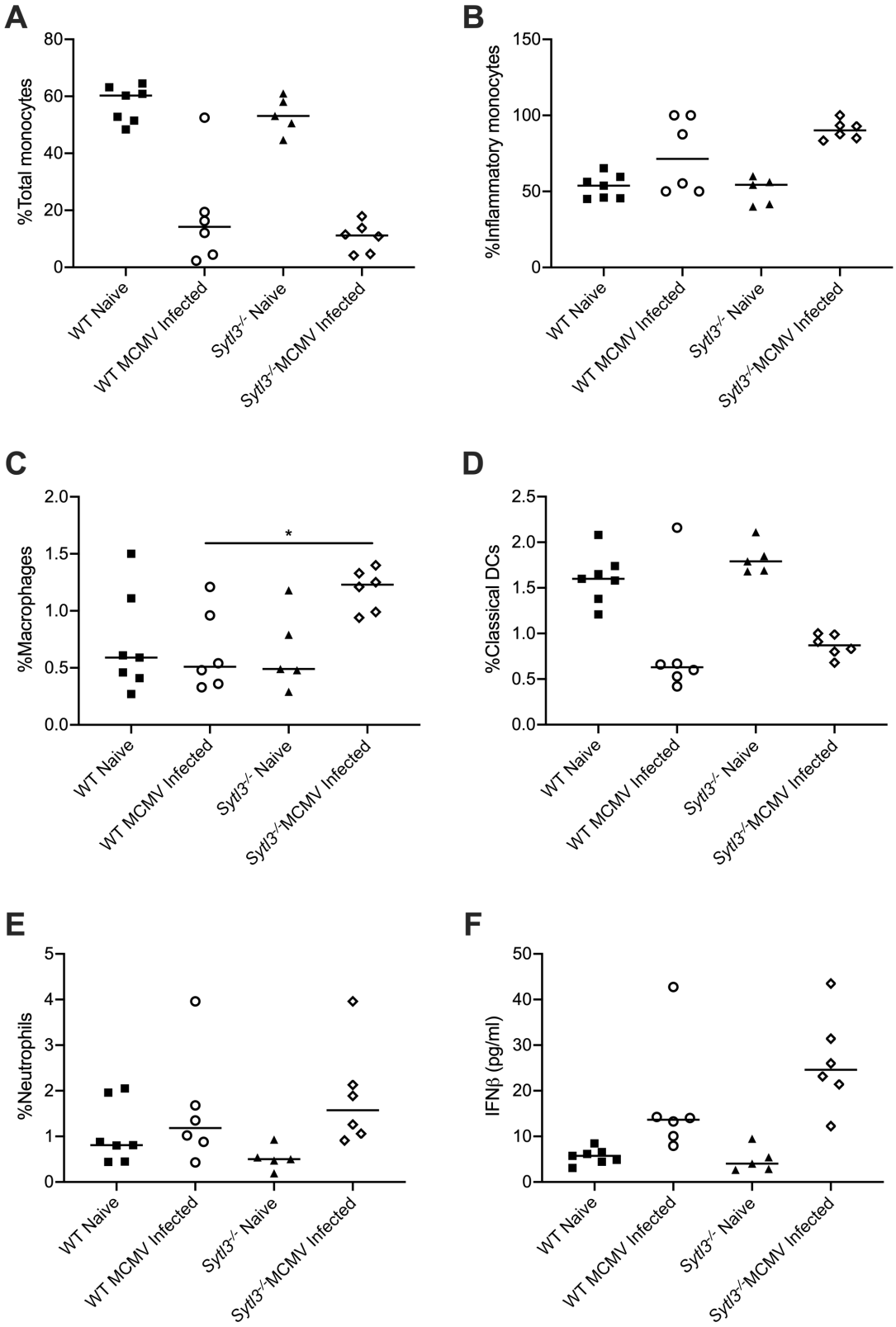

Figure 4.12 *Sytl3* deficient mice have an increased percentage of macrophages 2 days p.i. and show no change to the percentage of monocytes, DCs and neutrophils.
WT and *Sytl3*$^{-/-}$ naïve controls and 2 days post-MCMV infection. (A) The percentage of total monocytes (B) inflammatory monocytes (C), macrophages (*p=0.0173), (D) classical DCs (naïve p=0.0732) and (E) neutrophils within the splenic compartment. (F) The amount of plasma IFNβ protein. Individual mice and median are shown (5-7 mice per group).

4.3.7 NK cells derived from *Sytl3* deficient mice have impaired degranulation in response to a variety of NK cell stimuli.

To specifically investigate the impact of *Sytl3* on NK cell degranulation, I performed an *in vitro* assay using isolated splenocytes from naïve WT and *Sytl3*$^{-/-}$ mice cultured with IL-2 for 48 hours to induce their proliferation before stimulating with different conditions for 4 hours (Figure 4.13A). NK cell CD107a externalisation was examined after stimulation for 4 hours with unstimulated control, PMA-Ionomycin, P815 target cells, P815 target cells with αLy49H antibody, P815 target cells with αMHCI antibody (to model ADCC), P815 target cells with αNKG2D antibody, IL-15, and IL-21. IL-15 and IL-21 cytokines were chosen as they are known to induce NK cell cytotoxic responses (Brady *et al.*, 2004; Skak, Frederiksen and Lundsgaard, 2008; Zhang *et al.*, 2008). The P815 target cells are a mouse mastocytoma cell line which are MHC mismatched to the C57BL6 background of the WT and *Sytl3*$^{-/-}$ mice used in the study. P815 cells do not express any other NK cell activating ligands but it is possible to induce activation of specific NK cell receptors using antibodies. To activate NK cell receptors, the αLy49H and αNKG2D antibodies bind to P815 cells at the Fc portion, leaving the NK cell receptor specific proteins on the Fab region of the antibody free to bind the corresponding NK cell receptors. To mimic ADCC responses, the αMHCI (H-2Kd/H-2Dd) antibody was used which binds at the Fab portion to P815 αMHCI receptors, leaving the Fc portion of the antibody available to activate NK cells. A significant degranulation defect in *Sytl3*$^{-/-}$ NK cells was observed upon stimulation with PMA-Ionomycin, P815 target cells with αLy49H antibody, P815 target cells with αNKG2D antibody and IL-15 cytokine (Figure 4.13B). Consequently, *Sytl3*$^{-/-}$ mouse NK cells showed dysfunctional degranulation responses to a variety of different cell stimuli.

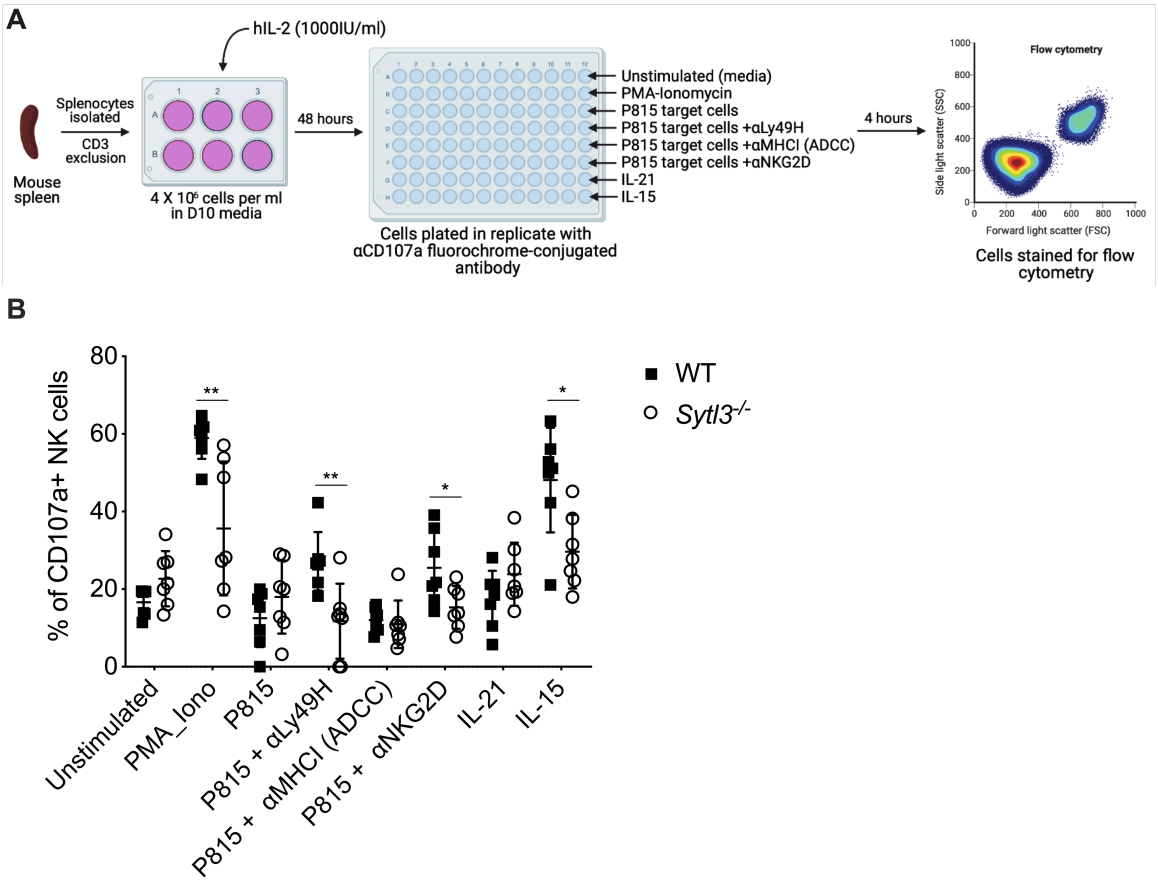

Figure 4.13 NK cells derived from *Sytl3*$^{-/-}$ mouse splenocytes have impaired cytotoxicity in response to a variety of cell stimuli.
(A) Schematic showing splenocyte preparation and stimulations for analysis by flow cytometry. (B) NK cells from naïve WT and *Sytl3*$^{-/-}$ mice were stimulated with PMA-Ionomycin (**p=0.0051), P815 cell targets, P815 targets alongside αLy49H (**p=0.0059), P815+ αMHCI (ADCC) and P815+ αNKG2D (*p=0.0301) antibodies, IL-21, and IL-15 (*p=0.0119). Cells were pooled within groups where lymphocyte numbers were insufficient. Individual mice (or pooled mouse lymphocytes from 2 mice), median and interquartile range are shown (7 mice per group). Statistical analysis was performed using an unpaired t test between WT and *Sytl3*$^{-/-}$ groups.

To confirm that reduced CD107a externalisation in *Sytl3*$^{-/-}$ mice (Figure 4.13B) was due to a defect in degranulation rather than differences in NK cell activating receptor expression, IL-2 cultured NK cells were analysed for expression of receptors corresponding to the stimulations used in Figure 4.13 using flow cytometry. The number of NK cells within the total splenocyte population did not change between WT and *Sytl3*$^{-/-}$ mice *in vitro* (Figure 4.14A). The expression of Ly49H, NKG2D, IL-21R and IL-15αR also did not change between mouse WT and *Sytl3*$^{-/-}$ cells *in vitro* (Figure 4.14B-C). Therefore, I concluded that the reduction in CD107a externalisation by *Sytl3*$^{-/-}$ NK cells (Figure 4.13B) was most likely due to dysfunctional degranulation.

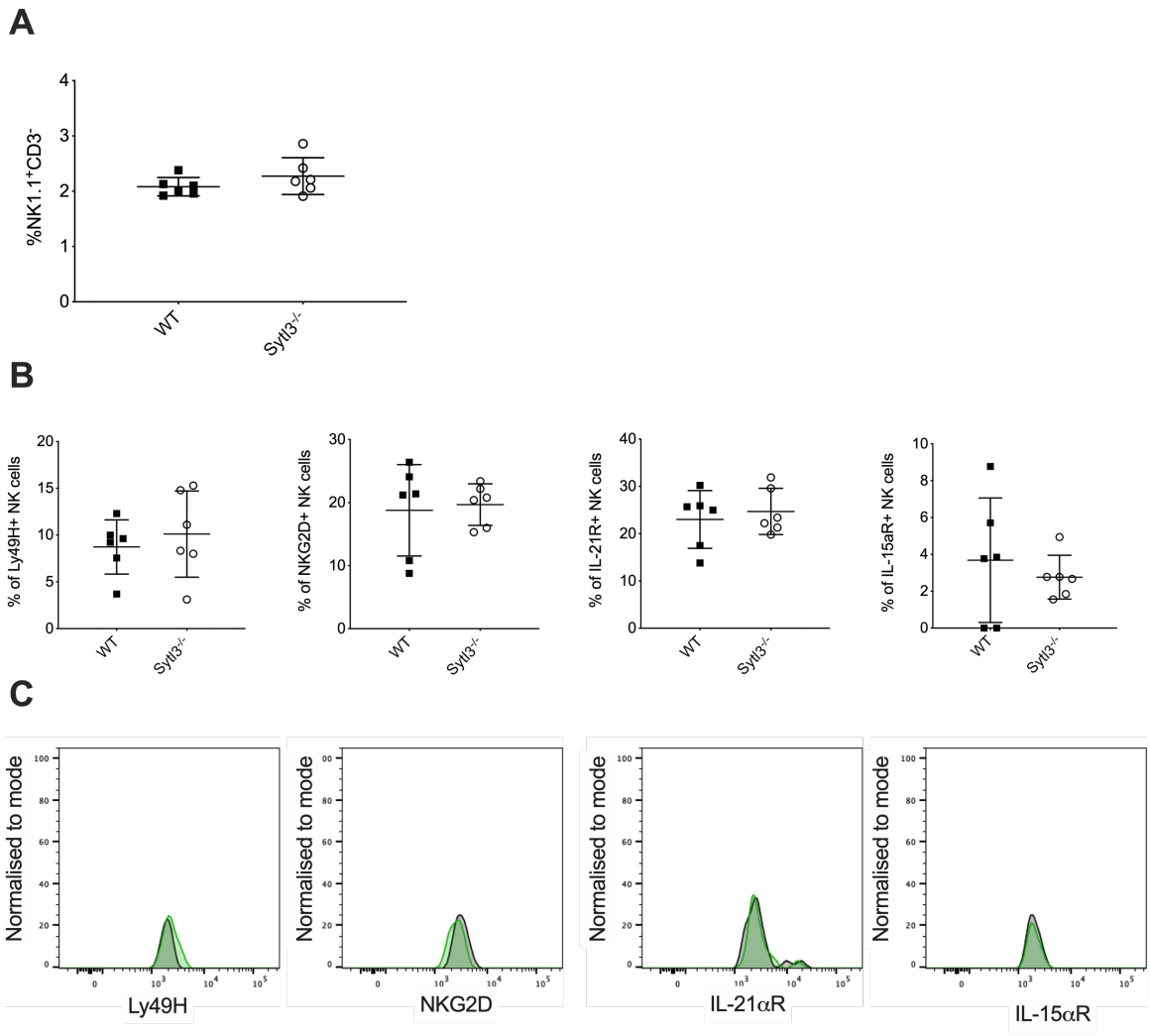

Figure 4.14 *Sytl3* gene deficiency does not impact splenic NK cell degranulation-inducing receptor expression.
Splenocytes from naïve WT and *Sytl3*$^{-/-}$ mice cultured in IL-2. (A) The percentage of NK cells. (B) The percentage of NK cells expressing Ly49H, NKG2D, IL-21R and IL-15αR. (C) Representative histogram overlay plots showing the amount of expression of Ly49H, NKG2D, IL-21R and IL-15aR by NK cells in WT (black) and *Sytl3*$^{-/-}$ (green) mouse NK cells. Cells were pooled within groups where lymphocyte numbers were insufficient. Individual mice (or pooled mouse lymphocytes from 2 mice), median and interquartile range are shown (7 mice per group).

4.3.8. NK cells derived from *Sytl3* deficient mice show no impairment in target cell killing

As a degranulation defect in *Sytl3* deficient NK cells was observed 4 days p.i. and in response to a variety of cell stimuli (Figure 4.10 and Figure 4.13) and these mice exhibited reduced viral replication control (Figure 4.2), I hypothesised that NK cell killing of target cells may also be impaired. Therefore, NK cells derived from WT and *Sytl3* deficient mice were grown in IL-2 (prepared as in Figure 13A but pure NK cells were cultured for 72 hours) before stimulating with BaF/3 control or BaF/3-m157 target cells. BaF/3 cells are a mouse pro-B cell line MHC mismatched to the WT and *Sytl3*$^{-/-}$ mouse C57BL6 background. The BaF/3-m157 cells model MCMV infected cells which also express the m157 glycoprotein that stimulates the Ly49H activating receptor on NK cells. In order to show that *Sytl3* deficient mice exhibit reduced degranulation in response to BaF/3 target cells, NK cells were stained for CD107a. As expected, *Sytl3* deficient mouse NK cells demonstrated reduced degranulation in response to BaF/3 control and BaF/3-m157 cells compared to WT (Figure 15A). To determine the amount of target cell killing, BaF/3 target cells were stained with Cell Trace Violet before combining with NK cells and staining for live/dead markers. Unexpectedly, BaF/3 control and BaF/3-m157 target cell killing was unaffected by *Sytl3* gene deficiency (Figure 15B). This indicates that other compensatory mechanisms may be involved in NK cell killing within this *in vitro* assay. As a result, further work is needed to understand how *Sytl3* deficiency impacts the functional target cell killing NK cell response to MCMV infection.

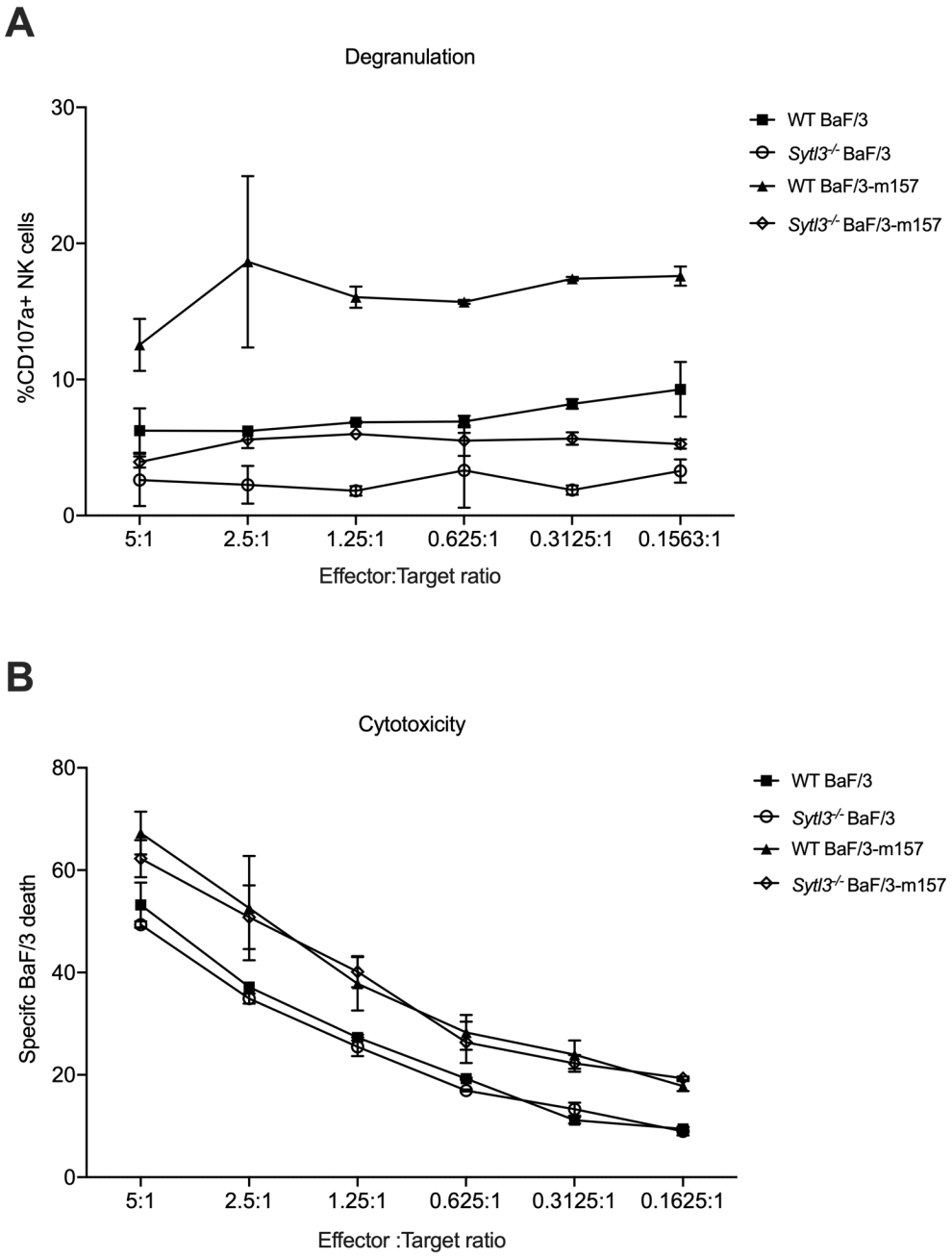

Figure 4.15 *Sytl3* deficient NK cells do not show killing defects in response to BaF/3-m157 cell targets.
NK cells derived from WT and *Sytl3*$^{-/-}$ mouse spleens isolated using negative selection and grown in hIL-2 for 72 hours before stimulating with BaF/3 control and BaF/3-m157 cells across several E:T ratios. (A) The percentage of CD107a+ NK cells. BaF/3 WT vs. BaF/3 *Sytl3*$^{-/-}$ *p=0.04347, BaF/3-m157 WT vs. BaF/3-m157 *Sytl3*$^{-/-}$ *p=0.0437. (B) Specific BaF/3 cell death. The mean of 2 technical replicates is shown (using NK cells pooled from 6 mice per group) ±standard deviation. Statistical analysis was performed using the Dunn's multiple comparisons test.

4.3.9. Imaging NK cells derived from *Sytl3* deficient mice reveal the role for *Sytl3* in granule exocytosis.

To understand how the degranulation pathway was impaired in *Sytl3* deficient mice, splenic NK cells grown in IL-2 for 48 hours were stained for MTOC and F-actin markers. MTOC proteins ensure the cellular machinery required for degranulation, including the lytic granules, are polarised towards the immunological synapse (Mentlik *et al.*, 2010; Pachlopnik Schmid *et al.*, 2010). F-actin is required for delivery of lytic granules from the MTOC to the plasma membrane (Vyas *et al.*, 2001; Orange *et al.*, 2003; Roda-Navarro *et al.*, 2004; Andzelm *et al.*, 2007). The P815-antibody conditions which showed significant differences in degranulation markers between WT and *Sytl3*$^{-/-}$ including αLy49H and αNKG2D (Figure 4.13B), were chosen to combine with NK cells before staining and imaging of NK cell-target cell conjugates using confocal microscopy. F-actin polarisation measured as the fold increase in intensity of F-actin staining at the immunological synapse compared to the whole NK cell and distance between the MTOC and the immunological synapse was calculated using Q-Path. Each immunological synapse image used in the analysis is shown (Figure 4.16-4.19). The F-actin polarisation did not significantly change between WT and *Sytl3*$^{-/-}$ NK cells upon P815 + αLy49H and P815 + αNKG2D stimulation (Figure 4.20A). However, there was a trend showing increased F-actin polarisation in *Sytl3*$^{-/-}$ NK cells stimulated by P815 + αNKG2D cells compared to WT which was not observed upon stimulation with P815 + αLy49H. This may indicate differential effects between Ly49H and NKG2D-induced NK cell responses during *Sytl3* deficiency, but further investigation is required. The MTOC distance was calculated as the length between the centre of the brightest MTOC staining to the closest point on the immunological synapse. The MTOC distance significantly increased upon *Sytl3* deficiency in both P815 + αLy49H and P815 + αNKG2D stimulated NK cells (Figure 4.20B). Therefore, the directionality of the degranulation response is impaired during *Sytl3* loss, which is likely to reduce the efficiency of cell killing by granule exocytosis.

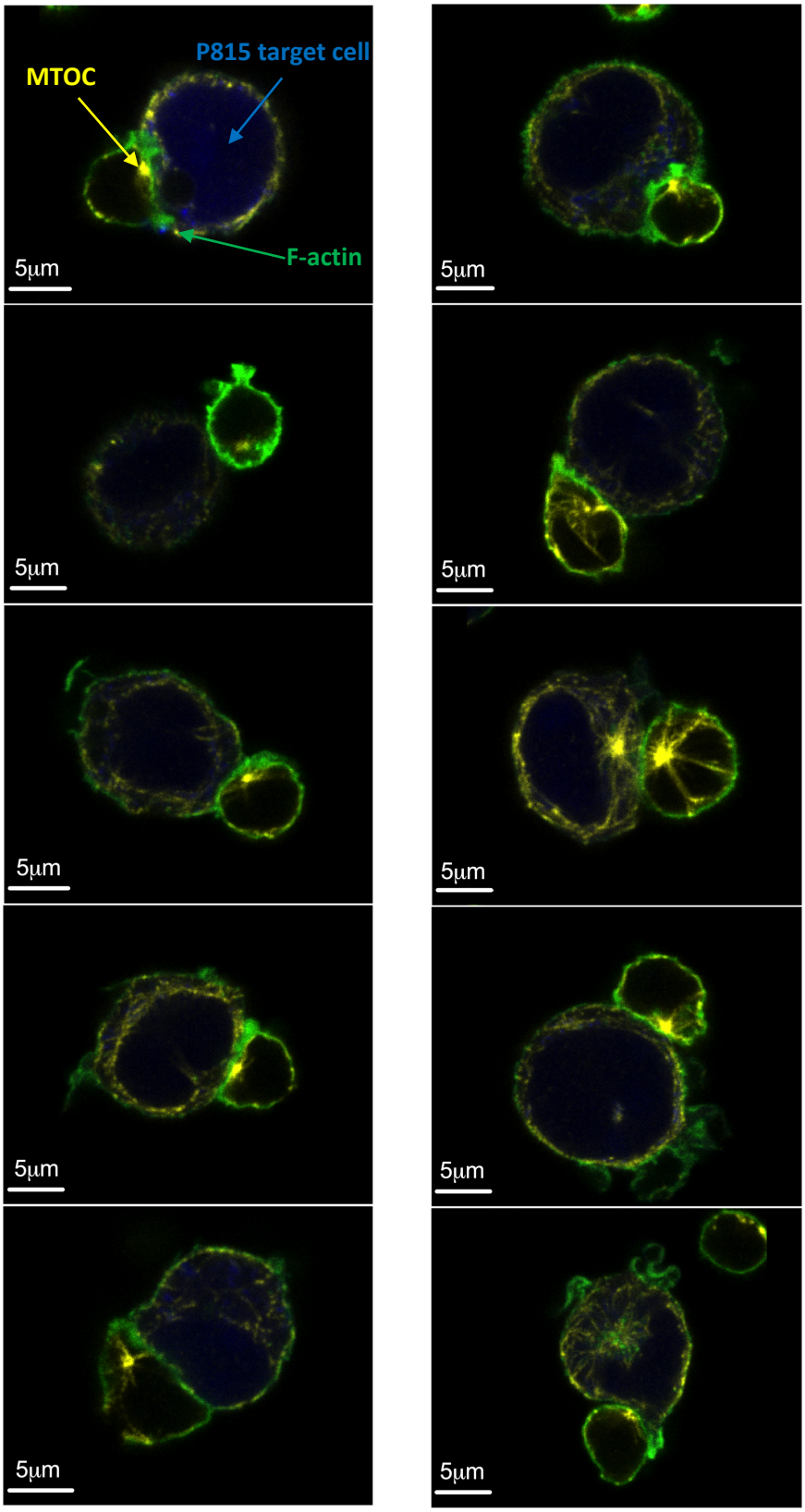

Figure 4.16 WT-derived NK cells show normal immunological synapse formation with P815 + αLy49H target cells.
Confocal imaging of NK cells derived from WT mice grown in hIL-2 for 48 hours and their conjugates with P815 + αLy49H target cells. P815 + αLy49H target cells (blue), MTOC staining (yellow) and F-actin staining (green). These particular images were taken by Roseanna Hare and all other aspects were performed by myself.

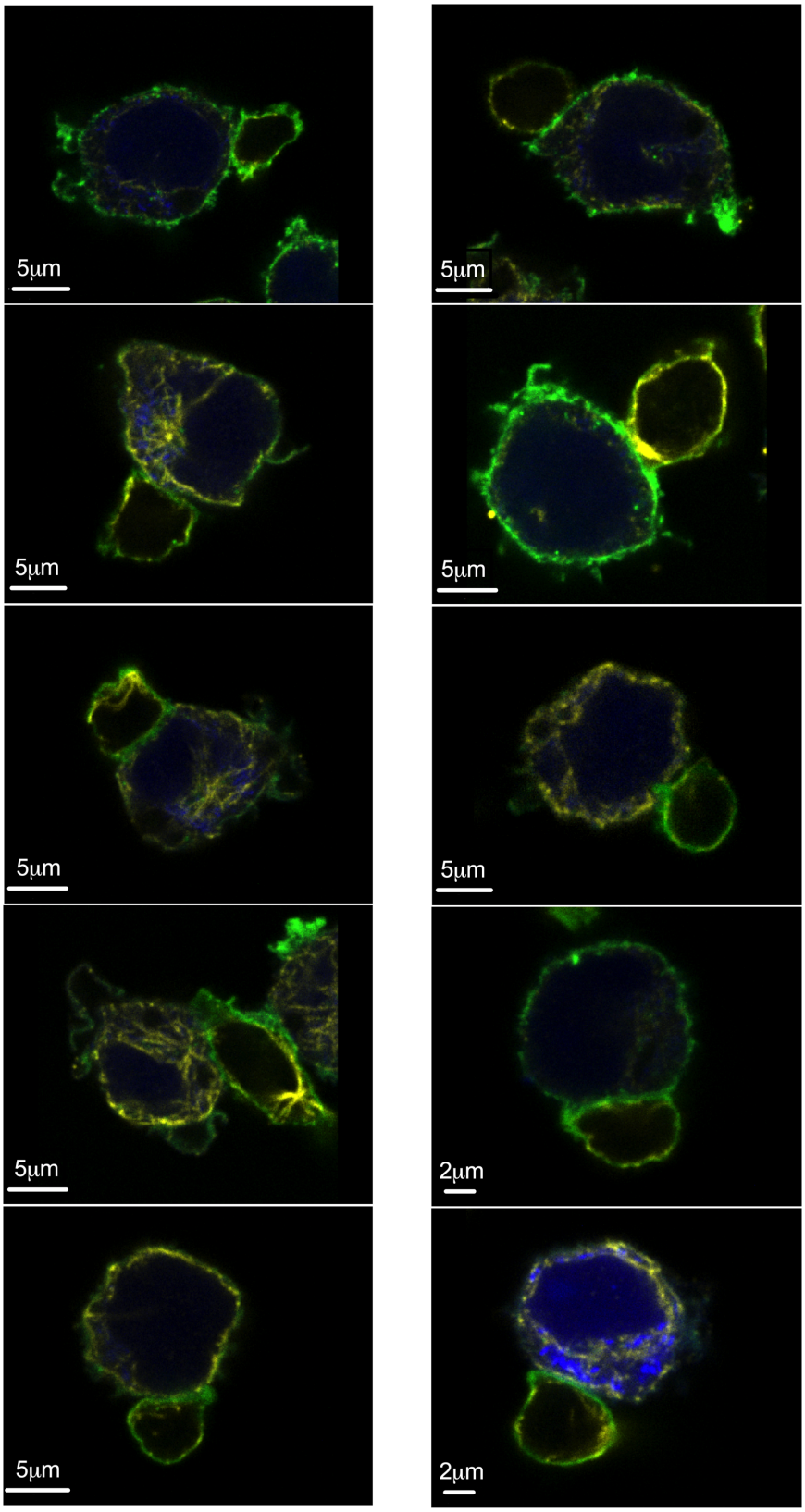

Figure 4.17 *Sytl3*$^{-/-}$ derived NK cells show impaired MTOC directionality upon forming immunological synapses with P815 + αLy49H target cells.
Confocal imaging of NK cells derived from *Sytl3*$^{-/-}$ mice grown in hIL-2 for 48 hours and their conjugates with P815 + αLy49H target cells. P815 + αLy49H target cells (blue), MTOC staining (yellow) and F-actin staining (green). These particular images were taken by Roseanna Hare and all other aspects were performed by myself.

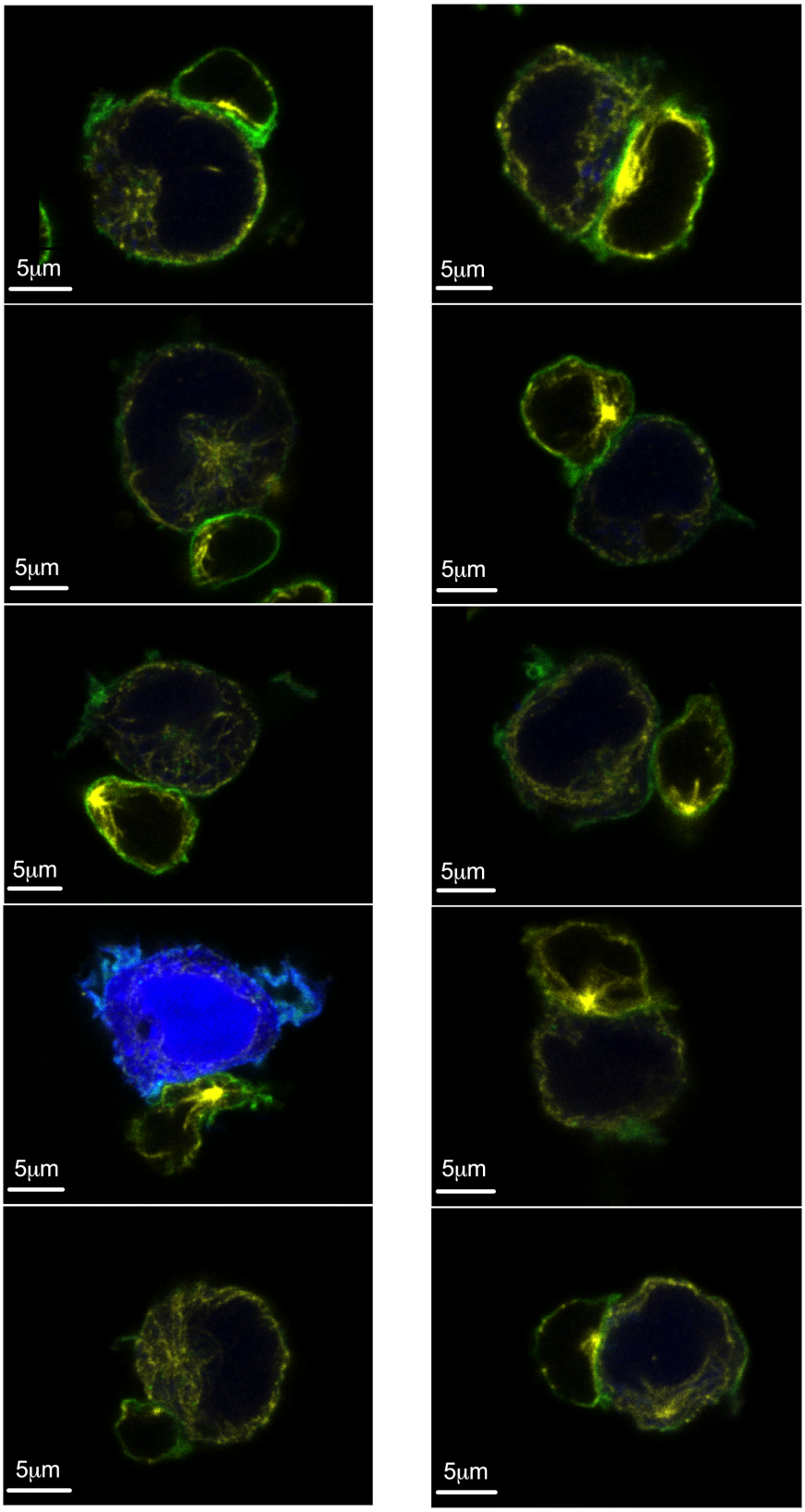

Figure 4.18 WT-derived NK cells show normal immunological synapse formation with P815 + αNKG2D target cells.
Confocal imaging of NK cells derived from WT mice grown in hIL-2 for 48 hours and their conjugates with P815 + αNKG2D target cells. P815 + αNKG2D target cells (blue), MTOC staining (yellow) and F-actin staining (green). These particular images were taken by Roseanna Hare and all other aspects were performed by myself.

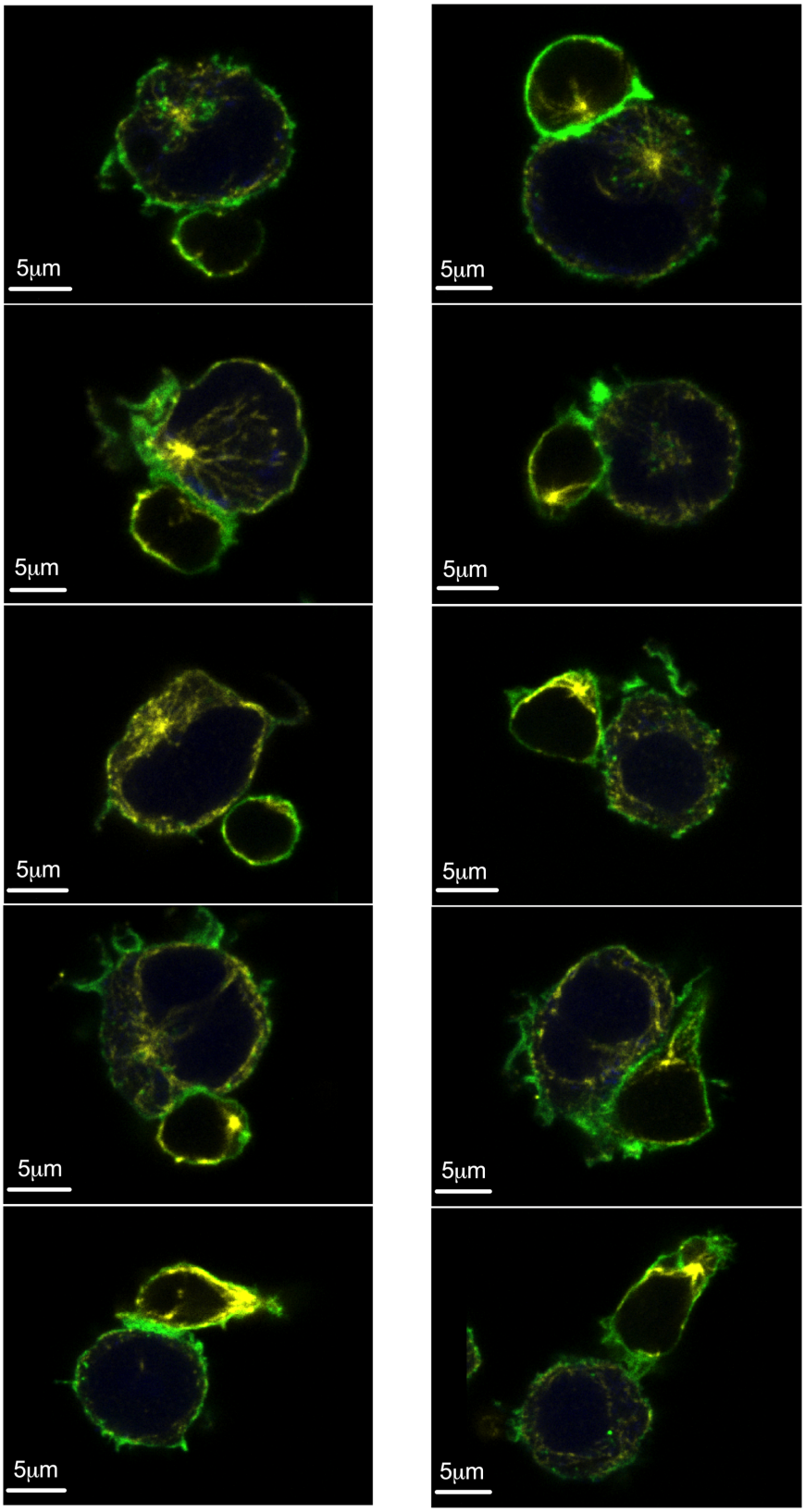

Figure 4.19 *Sytl3*$^{-/-}$ derived NK cells show impaired MTOC directionality upon formation of immunological synapses with P815 + αNKG2D target cells.
Confocal imaging of NK cells derived from *Sytl3*$^{-/-}$ mice grown in hIL-2 for 48 hours and their conjugates with P815 + αNKG2D target cells. P815 + αNKG2D target cells (blue), MTOC staining (yellow) and F-actin staining (green). These particular images were taken by Roseanna Hare and all other aspects were performed by myself.

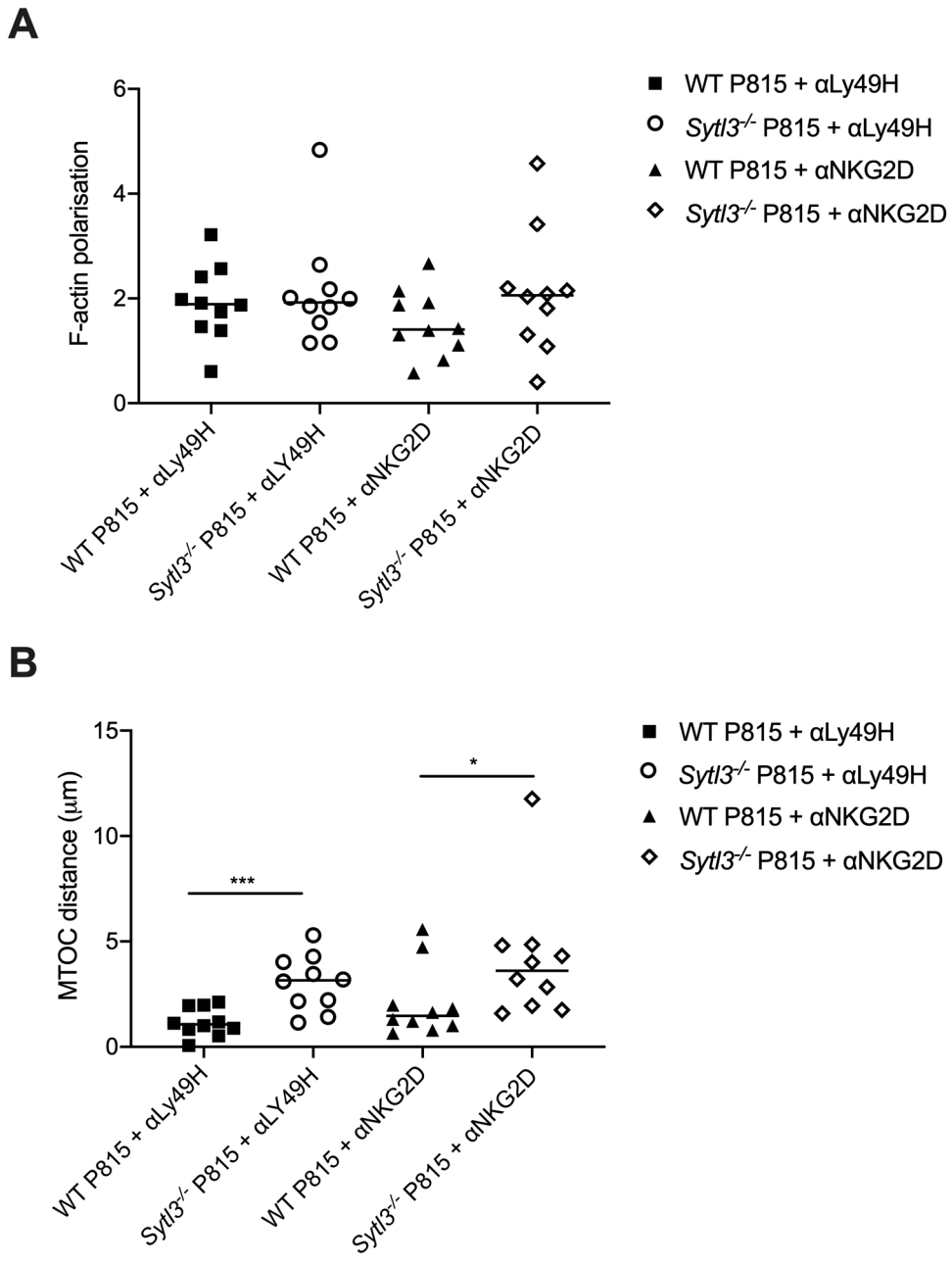

Figure 4.20 *Sytl3* deficient NK cells have reduced MTOC polarisation towards the immunological synapse.
WT and *Sytl3*$^{-/-}$ splenic NK cells were cultured in hIL-2 for 48hours before combining with either stained P815 + αLy49H target cells or stained P815 + αNKG2D target cells and subsequent staining for F-actin and MTOC markers for imaging. (A) F-actin polarisation which was measured as the fold increase in mean F-actin intensity per pixel at the immunological synapse compared to the whole NK cell from each image. (B) The MTOC distance was measured as the distance between the centre of the brightest MTOC stain on the NK cell and the nearest point on the immunological synapse (***p=0.0005, *p=0.0355). The value obtained for each image and the mean of each group is shown (10 conjugate images per group).

4.3.10. IL-21 is important for optimal NK cell cytotoxicity

As *Sytl3*$^{-/-}$ mouse NK cells exhibited some CD107a externalisation upon different stimulations *in vitro* (Figure 4.13B), I decided to investigate *Sytl3* independent degranulation by granule exocytosis. Since there was no significant impairment in CD107a+ NK cells upon IL-21 stimulation *in vitro* (Figure 4.13B), I decided to investigate the importance of IL-21 induced degranulation responses. To do this, I administered an αIL-21R blocking antibody to C57BL/6 mice alongside PBS control treated mice at -2 and +2 days post-MCMV infection before harvesting the splenocytes for analysis of NK cell functional markers at 4 days post-MCMV infection. The total number of NK cells did not change significantly between PBS control treated mice and αIL-21R blocking antibody treated mice. The percentage of NK cells expressing CD107a did not significantly change between control and αIL-21R blocking antibody treated mice (Figure 4.21B). However, there was a trend which showed reduced degranulation upon blockade of IL-21 receptors suggesting IL-21 may be required for optimal degranulation. Interestingly, the percentage of granzyme B+ NK cells was significantly reduced upon αIL-21R blocking antibody administration which indicates that IL-21 may be important for inducing optimal production of essential proteins for degranulation (Figure 4.21F). The percentage of IFNγ+ NK cells (Figure 4.21E), Ly49H+ NK cells (Figure 4.21C) and KLRG1+ NK cells (Figure 4.21D) did not change between control and αIL-21R blocking antibody treated mice. In addition, the percentage of NK cells expressing the maturation markers CD11b and CD27 did not change (Figure 4.21G). The amount of viral replication also did not change upon IL-21R depletion (data not shown) however with a greater number of mice an effect may be observed. To ensure the αIL-21R blocking antibody was effective, the levels of IL-21 cytokine within mouse spleens from PBS treated and αIL-21R blocking antibody treated mice were investigated. The level of IL-21 cytokine upon αIL-21R blocking antibody administration was significantly increased compared to control (Figure 4.21H), therefore indicating that the antibody was effective at blocking IL-21 receptors. The expression analysis of IL-21R on NK cells showed it was also unaffected by *Sytl3* deficiency (Figure 4.14B-C). Overall, these data suggest that IL-21 exerts *Sytl3*-independent effects on NK cell degranulation and NK cell granzyme B expression may to some degree compensate for the absence of *Sytl3* in orchestrating NK cell responses to MCMV.

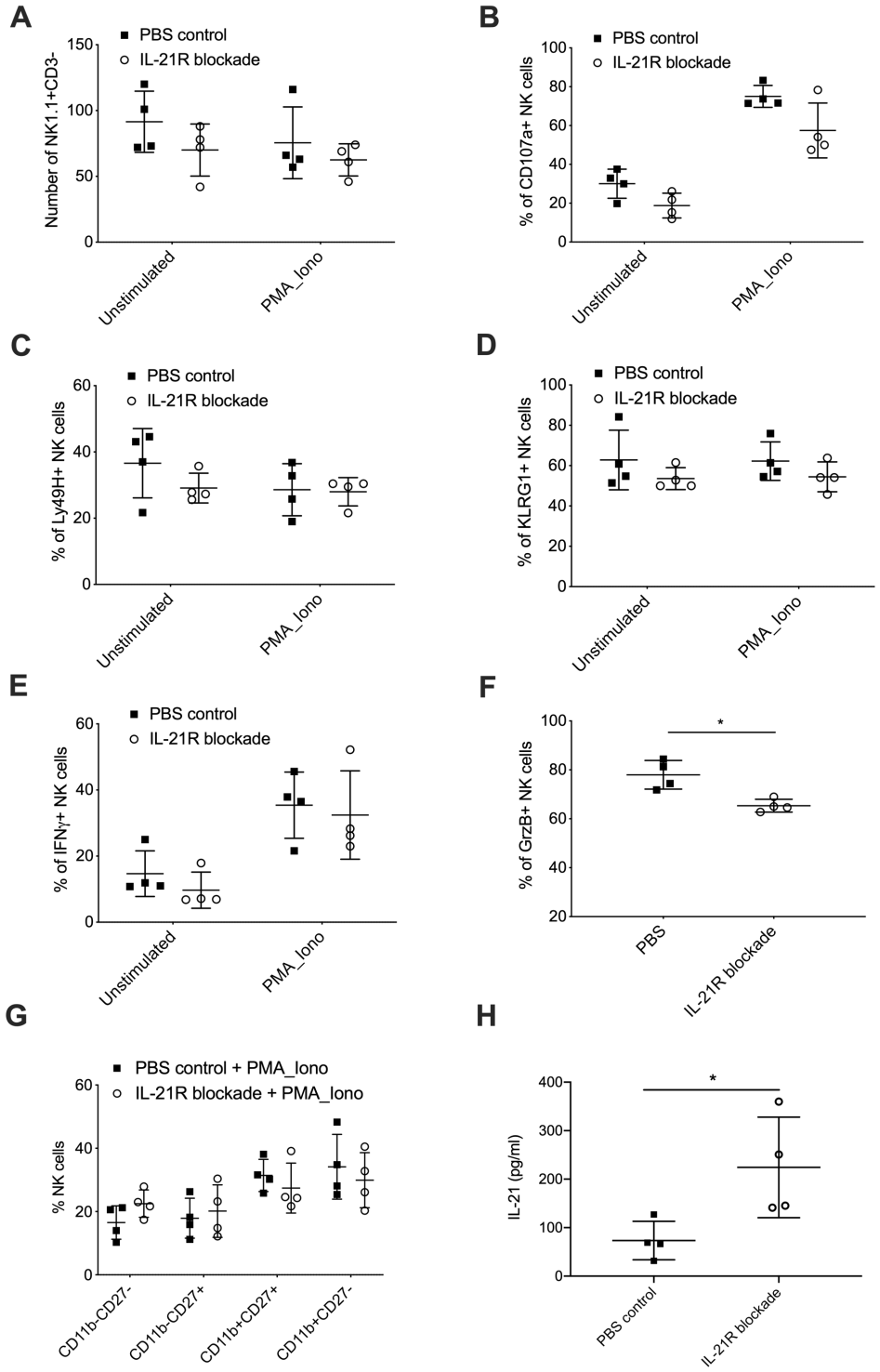

Figure 4.21 IL-21R blockade reduces granzyme B production 4 days post-MCMV infection.

NK cells from PBS control treated and αIL-21R blocking antibody treated C57BL/6 mice unstimulated or stimulated with PMA-Ionomycin. (A) The number of NK cells in total mouse splenocytes. The percentage of (B) CD107a+ NK cells, (C) Ly49H+ NK cells, (D) KLRG1+ NK cells, (E) IFN+ NK cells. (F) The percentage of unstimulated granzyme B+ NK cells (*p=0.0286). (G) The percentage of NK cell maturation populations from immature (CD11b-CD27-) to mature (CD11b+CD27-). (H) IL-21 protein in the spleen (*p=0.0286). Individual values and median ±standard deviation is shown (4 mice per group).

4.4 Discussion

Improving our understanding of NK cell effector function is important for developments in viral infection control. The key pathways for inducing NK cell control of MCMV infection are cytotoxicity and cytokine release. Thus, NK cells from *Sytl3*$^{-/-}$ mice, which demonstrated reduced MCMV replication control in Chapter 3, were assessed for degranulation function and antiviral cytokine production.

My data suggests that *Sytl3* is important for optimal NK cell antiviral function. This is in accordance with mouse gene expression analysis from online data resources showing almost exclusive *Sytl3* expression by NK cells (Immgen.org) and significantly induced gene expression 2 days p.i. (Singhania *et al.*, 2019). In this study, viral replication in *Sytl3*$^{-/-}$ mice was significantly impaired within the spleen 4 days p.i., demonstrating the importance of *Sytl3* during the acute phase when NK cells are vital for infection control (Bukowski, Woda and Welsh, 1984). Since NK cells are induced during the acute phase of infection and *Sytl3* was almost exclusively expressed by NK cells, these results indicated that *Sytl3* impairment is associated with reduced NK cell-driven acute infection control. To test my hypothesis that *Sytl3* has importance specifically within NK cells, WT and *Sytl3* deficient mice were treated with αNK1.1 depleting antibody during infection and the results showed no difference in infection control upon NK cell depletion between WT and *Sytl3*$^{-/-}$ mice. Therefore, during acute infection, only NK cell-directed infection control is likely to be impacted by *Sytl3* gene deficiency.

The phenotyping of NK cells derived from naïve *Sytl3*$^{-/-}$ and WT mice, performed at the WTSI, showed an increased percentage of Ly49H+ NK cells in *Sytl3*$^{-/-}$ mice which was just over the threshold for significance. However, upon assessment of Ly49H expression by NK cells derived from naïve and MCMV infected mice at 4 days p.i. by myself, no difference was observed. However, there was a smaller number of mice tested in my experiment therefore further investigation of Ly49H expression using a greater number of mice may generate the same result as observed by the WTSI. The slight increase in percentage of DCs ($CD11C^{+}MHCII^{+}B220^{-}NK1.1^{-}CD3^{-}$) shown in the naïve mouse phenotyping by the WTSI was reflected in my study using fewer naïve (and infected) mice. In my experiment a trend showing an increased percentage of classical DCs ($CD11C^{+}MHCII^{+}$) in *Sytl3* deficient naïve mice was close to significance. For most parameters tested in both studies, no change to the percentage of different immune cell

types upon *Sytl3* deficiency was shown. Finally, the phenotyping data generated by the WTSI generally reflected what was observed in my studies therefore the phenotyping data was robust.

As NK cell activation markers did not change upon *Sytl3* deficiency, NK cell effector function mechanisms were assessed at 2- and 4-days post-MCMV infection. At 4 days p.i., the timepoint where there was a significant increase in viral replication, *Sytl3*$^{-/-}$ mouse NK cells showed reduced degranulation. This is in accordance with a previous study linking *Sytl3* to cytotoxic pathways within cytotoxic T-cells due to the association of sytl3 proteins with Rab27a and Kinesin-1 which are known components of degranulation machinery (Kurowska *et al.*, 2012). Surprisingly, in addition to the defect in degranulation, these results also showed a reduced number of IFNγ+ NK cells which may implicate *Sytl3* in the activation of more global effector function mechanisms. For example, as the level of IFNγ was measured intracellularly this may indicate reduced IFNγ protein production therefore *Sytl3* may be involved in the activation of transcription pathways. Alternatively, reduced intracellular IFNγ may be due to increased IFNγ release in *Sytl3* deficient NK cells but this is less likely as their degranulation effector response was reduced. Upon analysis of IFNγ release during assessment of protein levels in blood plasma p.i., I observed no change in the absence of *Sytl3*, suggesting minimal *in vivo* impact of this *ex vivo* phenotype on the antiviral immune response. Understanding the mechanism behind reduced intracellular IFNγ during *Sytl3* deficiency therefore requires further study.

I also investigated the impact of *Sytl3* deficiency on non-NK innate cells during MCMV infection. At 2 days p.i. the percentage of macrophages significantly increased in *Sytl3*$^{-/-}$ mice. Therefore, as the change in virus replication at 2 days p.i. did not change significantly but did change at 4 days p.i., the increased macrophage response may enhance control of MCMV replication during dysfunctional *Sytl3*$^{-/-}$ NK cell responses. Another important aspect of NK cell infection control is mediated by type I IFNs produced by macrophages, DCs and non-immune cells such as fibroblasts and epithelial cells (Zhu, Huang and Yang, 2008; Ivashkiv and Donlin, 2014; Madera *et al.*, 2016). Consequently, I also investigated the level of plasma IFNβ which did not change therefore the NK cell impairment was not mediated by a change in activation through IFNβ. Further experiments investigating the level of IFNα, as another important type I IFN also produced by monocytes and DCs, would improve the confidence in the interpretation of the data.

I then performed more detailed analysis of *Sytl3* deficient splenic mouse NK cells *in vitro* to understand which NK cell activating receptor pathways lead to downstream *Sytl3* function. *Sytl3* was induced by a variety of activating pathways including P815 target cells expressing the ligand to the activating Ly49H receptor, which is important for MCMV specific control (Orr *et al.*, 2009), P815 target cells expressing the ligand to the cytotoxic activating receptor NKG2D and cytokine induced cytotoxicity from IL-15 stimulation. Consequently, *Sytl3* is essential to optimal degranulation responses in NK cells upon a variety of cell stimuli.

As degranulation was impaired in *Sytl3* deficient NK cells, I hypothesised that target cell killing may also be impaired. Therefore, BaF/3-m157 cell targets, which were used as a model of MCMV infected cells which express m157, were combined with NK cell effectors. However, the *Sytl3* deficient NK cells exhibited no defect in target cell killing. This may have been due to the purity of the NK cells not being as high as one would like (at 82-90%) and therefore other immune cells may have compensated for impairments in NK cell-directed killing control. Alternatively, since the splenic NK cells were first cultured in IL-2 for 72 hours before use and TRAIL is upregulated upon IL-2 stimulation (Kayagaki *et al.*, 1999), apoptosis induced by death receptors may compensate for deficiencies in degranulation through lytic granule release. This is supported by data showing that Rab27a deficient mouse lymphocytes, the protein binding partner for sytl3, have defective granule exocytosis but normal killing through Fas death receptors (Haddad *et al.*, 2001).

To uncover how *Sytl3* impairment leads to defective degranulation, IL-2 activated NK cells combined with P815 cell targets + αLy49H and P815 cells + αNKG2D were stained for F-actin and MTOC which are essential proteins involved in degranulation. There was a significant increase in the MTOC distance in *Sytl3*-deficient NK cells which indicates reduced MTOC polarisation towards the immunological synapse and therefore the degranulation pathway is likely to be less efficient. Reduced degranulation efficiency links to the observed reduction in CD107a externalisation. This data supports findings by Bryceson et al. 2012 who suggests that a complex of sytl3, Rab27a and kinesin-1 may facilitate anterograde transport of lytic granules from the MTOC towards the immunological synapse at the plasma membrane (Wood and Bryceson, 2012). In addition, a trend showing increased F-actin polarisation in *Sytl3* deficient NK cells compared to WT

during synapse formation with P815 + αNKG2D cells may partly compensate for the impairment in degranulation function. Although this requires further investigation, this hypothesis is supported by the degranulation data which showed a greater defect upon P815 + αLy49H stimulation than P815 + αNKG2D. Therefore, *Sytl3* impairment consistently reduced MTOC polarisation but did not significantly impair F-actin polarisation during degranulation.

As *Sytl3*$^{-/-}$ mouse NK cells retained some cytotoxic response in these data, I decided to investigate *Sytl3*-independent degranulation. In these data I showed no impairment in degranulation in *Sytl3* deficient NK cells upon stimulation with IL-21 *in vitro.* Therefore, I investigated IL-21 induced degranulation by blocking IL-21 receptors during acute MCMV infection. However, I observed no significant difference in the percentage of CD107a+ NK cells. On the other hand, there was a significant reduction in granzyme B+ NK cells. In mice, previous data showed that resting murine NK cells are minimally cytotoxic but as they contain abundant mRNAs for granzyme B they can be rapidly induced upon infection (Fehniger *et al.*, 2007). Taken together, this indicates that IL-21 may be important for the development of granzyme B producing NK cells which is also in accordance with studies linking IL-21 to the frequency of granzyme B producing cells in humans (Lindner *et al.*, 2013).

In conclusion, these data suggest that the novel gene *Sytl3* is required for optimal NK cell degranulation. In addition, *Sytl3* may potentially have a role in the control of antiviral cytokine production. Further work is required to show how the sytl3 protein orchestrates NK cell effector function during viral infections.

Chapter 5 – General discussion

Despite the generation of effective antivirals, additional innovative treatment strategies such as cell-based immunotherapies may be needed due to adverse drug effects and virus resistance. The main patient group which would benefit from improved treatment strategies for HCMV is transplant recipients who are highly susceptible to viral disease which can cause graft rejection and death. NK cells are a key cell subset for defence against HCMV infection, therefore NK cell-based treatment strategies hold a great deal of promise. However, currently there are no approved NK cell-based immunotherapies, partly due to an incomplete understanding of the mechanisms controlling NK cell effector function. However, there are some NK cell-based approaches currently undergoing clinical trials (Liu *et al.*, 2021).

The aims of this thesis were to identify genes with importance in CMV infection control through use of an *in vivo* mouse cytomegalovirus (MCMV) infection screen and to examine how chosen gene(s) of interest regulate NK cell function. Initially I identified: *Fam111a, Fam114a2, Far1, Heatr9, Serpinb9b* and *Sytl3* as genes with potential importance in CMV infection control from the 69 genes investigated. *Sytl3* was chosen to investigate further as it was almost exclusively expressed by mouse (and human) NK cells and showed robust effects on viral replication control in the spleen during acute MCMV infection. Further, I demonstrated that *Sytl3* has importance in generating optimal NK cell degranulation responses during acute MCMV infection and in response to various activating stimuli. Finally, I demonstrated that defective degranulation during *Sytl3* deficiency may be due to reduced polarisation of the MTOC towards the immunological synapse, which has implications in improving our understanding of diseases where there is defective NK cell degranulation and the development of NK-cell based treatments.

5.1 *In vivo* mouse screening for the discovery of novel genes

Most genetic screens are conducted *in vitro* using cell lines or primary cells and are therefore unable to fully recapitulate complex cellular interactions (Shalem, Sanjana and Zhang, 2015; Meyers *et al.*, 2017; Kuhn, Santinha and Platt, 2021). The presence of a full and functioning immune system is crucial for discovery of genes important in immune cell

control as intra-cellular signals and crosstalk between cells in distinct microenvironments are essential to the effective orchestration of the immune response. Although *in vitro* systems such as organoids are being increasingly used to mimic the complexity of living organisms (Shankaran *et al.*, 2021), most *in vitro* infection models still lack complexity of the *in vivo* situation (Kuhn, Santinha and Platt, 2021). Instead, direct *in vivo* screening, which was used in Chapter 3, enables the study of genes within a complex living organism and, in the case of the mouse for which we have a detailed understanding of the immune system, we have the potential to translate findings to humans. However, there are several important considerations for *in vivo* infection screens. These considerations include the dose, strain of virus and how it is cultured, the route of infection which can hugely alter responses and the age, sex, and strain of the mice.

In the *in vivo* infection screen described in Chapter 3, I used tissue culture passaged Smith strain MCMV which generates robust immune responses and is well tolerated by the C57BL/6 mice used in the screen. However, MCMV has several differences to HCMV described in section 1.1.5, and HCMV cannot be used to infect mice since CMVs are highly species-specific. In addition, as MCMV was grown in culture, virus batches can have varied pathogenicity and, despite steps being taken in my experiments to reduce this by using the same batch of virus to grow subsequent batches and by calculating the titre of individual batches using a plaque assay, there was still significant variability in virus load and pathogenicity in the stocks used. The dose of new batches of virus stock was also tested in WT mice to ensure 5-10% weight loss. The mode of infection used was i.p. injection which is technically straightforward and well-tolerated. However, other routes of infection may more closely reflect a natural infection, such as the intranasal route of infection, although these are more difficult to control and are relatively inefficient (Tan, Frederico and Stevenson, 2014; Oduro *et al.*, 2016). In addition, as there were 69 different mouse lines, the screen was split up into several experiments occurring at different times and therefore batch effects impacted the results. A mix of males and females were also used to minimise the number of mice produced for the screen and all mice used in the experiments were 6-12 weeks of age. Therefore, although several steps were taken to reduce variability in the screen, there was still some variability in the virus and mice used for the experiments which may have impacted results. However, we only chose genes where robust changes in weight and/or viral replication were observed.

There are also ethical implications in the use of so many animals for discovery of genes. In my experiments, only six genes of interest were identified in the screen out of the 69 investigated which calls into question whether *in vivo* models should be used for gene discovery. The use of *in vitro* screening may be more appropriate to reduce the number of animals used in experiments. Then, once genes of potential importance have been discovered *in vitro*, murine models could be used to validate results. As my project was focussed on the role of NK cell expressed genes, next time I would reduce the number of mouse lines investigated by identifying which of the 69 genes are highly or exclusively expressed by mouse NK cells using the immgen.org gene expression database and identified genes with relevance to humans before performing *in vivo* experiments. This approach would reduce the number of mice used in experiments and improve the efficiency of gene discovery.

The aim of the MCMV infection screen was to discover genes which may have relevance for the development of NK cell-based therapies. Where genes of interest encode intracellular proteins, this may lead to the identification of signalling pathways which could be altered to improve NK cell-based therapies, or, targeted using sophisticated drug delivery approaches such as receptor-mediated endocytosis. Where genes encode cell surface proteins which can also be manipulated to improve NK cell-based approaches, other approaches such as antibody-directed therapies could also be employed. In addition, since many receptors expressed on the surface of mouse NK cells differ to those in humans, an *in vitro* human infection screen of genes encoding extracellular proteins may have more translational potential.

Of the genes of interest identified in the *in vivo* screen in Chapter 3, some showed promising initial findings which I was unable to continue investigating due to lack of time and resources, particularly due to COVID-19 disruptions. These genes include *Heatr9* which was almost exclusively expressed by NK cells. *Heatr9* deficient mice showed defects in viral replication control which were amplified upon Δm157 MCMV infection. If I had more time, I would first repeat the MCMV and Δm157 MCMV infections to ensure the data was robust before phenotyping the spleen and liver NK cells using flow cytometry. Then, I would analyse the functional response of NK cells to specific NK cell activating receptor ligands expressed on target cells to find the activating receptors responsible for inducing *Heatr9* infection control and the effector function mechanisms impacted by

Heatr9 deficiency. I hypothesised that NK cell cytotoxicity is more severely impaired by *Heatr9* deficiency than IFNγ release as my experiments showed viral replication control was impaired only in the spleen during MCMV infection and in both organs, but more severely in the spleen, during Δm157 MCMV infection. This is due to literature showing NK cell effector function predominately occurs through cytotoxicity in the spleen and IFNγ in the liver (Tay and Welsh, 1997b). As a recent study suggested *Heatr9* is important for chemokine production in response to infection, I would also investigate chemokine production in gene-deficient mice during MCMV infection (Stairiker *et al.*, 2020). In addition, as *Heatr9* was significantly upregulated in memory-like NK cells following MCMV infection (Bezman *et al.*, 2012), I hypothesise that *Heatr9* deficiency may reduce NK cell-driven chronic viral replication control. Therefore, I would also investigate the impact of *Heatr9* deficiency on viral replication control at 30 days p.i., the timepoint when a pool of memory-like NK cells is established post-MCMV (Sun *et al.*, 2011). Further insights into memory-like NK cells could also be generated by investigating the impact of *Heatr9* deficiency on viral replication control upon re-challenge with MCMV after memory-like NK cells have been generated. Heatr9 proteins are predicted to be intracellular (The Human Protein Atlas, Heatr9) therefore these experiments would inform on the development of NK cell-based therapies, drugs using sophisticated targeting approaches to manipulate heatr9, and inform on mechanisms underlying immunodeficiency in humans.

I would also investigate *Fam111a* gene deficiency in the liver as *Fam111a* deficient mice showed complete loss of viral replication in the liver upon Δm157 MCMV infection. I hypothesise that *Fam111a* reduces virus control within the liver which is impacted by Ly49H-m157 activation and may involve reducing IFNγ responses which occur within liver NK cells (Tay and Welsh, 1997b). However, *Fam111a* is expressed highly across a wide range of immune cells. As a result, *Fam111a* may affect other immune cell types with roles in acute viral replication control within the liver such as macrophages and DCs therefore this gene gives potential for learning about non-NK cell antiviral effector responses. Future experiments to investigate *Fam111a* would include NK cell depletion during MCMV infection to determine whether the loss of viral replication within the liver in *Fam111a* deficient mice during Δm157 MCMV infection impacts on NK cell function outside the m157-Ly49H interaction, or, whether it has an impact on other immune cell types at this timepoint. Fam111a proteins are predicted to be intracellular

(The Human Protein Atlas, FAM111A) therefore these experiments may inform on NK cell-based therapy development, sophisticated intracellular targeting approaches to manipulate fam111a, and the potential to discover other drug targets in this signalling pathway which could have relevance in therapeutic development. In addition, mutations in FAM111a have been associated with conditions including Kenn-Caffey Syndrome (KCS) and Osteocraniostenosis (OCS) characterised by impaired skeletal development and hypoparathyroidism with hypocalcaemia as well as immune defects in KCS Type 2 (Sanjad *et al.*, 1991; Unger *et al.*, 2013) therefore, these experiments would also inform on the underlying pathology of these diseases which may improve diagnosis and treatment.

5.2 *Sytl3* as a facilitator of NK cell degranulation

The experiments in Chapter 4 demonstrated that the novel gene *Sytl3*, expressed almost exclusively by NK cells, affects degranulation responses through reduced MTOC polarisation. The sytl protein family is so-called due to its structural similarity to synaptotagmins. Sytl proteins and synaptotagmins both contain C-type tandem C2 protein domains but differ at the N-terminus (Fukuda and Mikoshiba, 2001). At the N-terminus synaptotagmin-like proteins contain a conserved *sytl* homology domain (SHD). The SHD region of sytl proteins is able to bind all Rab protein isoforms (small GTPases involved in degranulation) and therefore sytl proteins have a putative role as specific effector domains for Rab27a and Rab27b (Taruho S. Kuroda *et al.*, 2002). Due to the structural insight into *sytl* proteins and the established role of synaptotagmins in vesicle exocytosis, particularly in the context of neurotransmitter release (Südhof, 2012; Rizo and Xu, 2015; Zhou *et al.*, 2015; Brunger *et al.*, 2018) as well as mast cell exocytosis (Baram *et al.*, 1998) and macrophage exocytosis (Duque, Fukuda and Descoteaux, 2013), the sytl proteins are also believed to have importance in pathways of vesicular exocytosis. This has been demonstrated by sytl1 which has a putative role in controlling dense granule secretion in platelets (Neumüller *et al.*, 2009), sytl2-a in melanosome transport within melanocytes (Taruho S Kuroda *et al.*, 2002b; Kuroda and Fukuda, 2004) and finally sytl4-a in insulin granule exocytosis (Tomas *et al.*, 2008) and dense core vesicle exocytosis in PC12 neuroendocrine cells (Fukuda *et al.*, 2002). A recent study highlighted SYTL3 as an important regulator of presynaptic neurotransmitter release and cortical neuronal migration

in developing brains (Dong *et al.*, 2021). In the context of immune function, one study has shown an association between human SYTL3-encoded proteins with degranulation in cytotoxic T-cells due to evidence of a complex between sytl3, Rab27a and kinesin-1 (Kurowska *et al.*, 2012). Thus far, no links have been made between human or mouse *sytl3* and degranulation specifically within NK cells.

As *Sytl1, Sytl4* and *Sytl5* show wide immune cell expression, these proteins may have importance across several different pathways which may be difficult to dissect. However, alongside *Sytl3*, *Sytl2* also exhibits high NK cell expression compared to other immune cells (Immgen.org). *Sytl2* also shows high expression on intraepithelial Vγ5+ γδ T-cells and, unlike *Sytl3*, also has expression on both $CD4^+$ and $CD8^+$ T-cell effector and memory types. Therefore, future studies investigating *Sytl2* deficiency may enable dissection of the individual roles of different sytl proteins in vesicle exocytosis. Future investigation of this protein family will improve our understanding of a wide spectrum of biological functions all involving vesicle exocytosis including neurotransmitter release, secretion of hormones, enzymes, and antibodies. In the context of immune function, investigation of *Sytl* encoded proteins, in particular sytl2 and sytl3, is likely to have an impact on our understanding of NK cell degranulation and how it differs from the degranulation pathways of other immune cells.

It is important to note that I did not observe reduced NK cell killing of an m157-expressing cell line by *Sytl3* deficient NK cells, therefore further assessments of NK cell effector function are required. As large defects in NK cell degranulation were observed through stimulation of Ly49H and NKG2D receptor signalling using P815 cell targets, downstream signalling through extracellular-signal regulated kinase (ERK) which induces granule exocytosis downstream of these receptors should be assessed. For example, through detection of phosphorylated ERK post-NK cell stimulation with Ly49H and NKG2D receptor activating target cells. This may inform on whether there are defects in NK cell activation given both degranulation and intracellular IFNγ were impacted by *Sytl3* deficiency. A result showing comparable ERK activation in WT and *Sytl3* deficient NK cells, in combination with the data presented in this thesis showing MTOC polarisation was impacted, would indicate a post-activation NK cell defect. In addition, NK cells were cultured with IL-2 before assessment in the killing experiment and IL-2 induces expression of TRAIL death receptors (Kayagaki *et al.*, 1999). Therefore, killing through death

receptors such as TRAIL may compensate for the observed loss of degranulation via granule exocytosis. This hypothesis is also supported by data showing that lymphocytes from Rab27a-deficient mice, the binding partner for sytl3, have defective granule exocytosis but normal killing through Fas death receptors (Haddad *et al.*, 2001). As a result, assessment of death receptor expression after culturing in IL-2 and subsequent target cell priming is required to discover whether death receptors compensate for loss of degranulation in this *in vitro* assay. If this is the case, further assessments of NK cell killing using cell lines which do not express death receptor ligands or using pharmacological inhibitors of death receptors may identify deficiencies in granule exocytosis-induced killing.

In Chapter 4, there was a surprising defect in the number of IFNγ+ NK cells from *Sytl3* deficient mice post-MCMV infection and PMA-Ionomycin stimulation. Therefore, if NK cell activation is not impaired by *Sytl3* deficiency, future assessment to dissect the contribution of *Sytl3* to degranulation and IFNγ production are important. For example, through treatment of *Sytl3*-deficient mice with anti-IFNγ receptor blocking antibodies during infection and subsequent assessment of viral replication and degranulation responses of the NK cells. This may inform on the contribution of *Sytl3* to degranulation-induced virus control. The reduced level of intracellular IFNγ indicates reduced IFNγ protein production. Upon analysis of IFNγ released in blood plasma p.i., I observed no change upon *Sytl3* deficiency, suggesting minimal *in vivo* impact of this phenotype on the antiviral immune response. Understanding the mechanism behind reduced intracellular IFNγ during *Sytl3* deficiency requires further study.

The aim of this research was to uncover translationally relevant genes, therefore future experiments involving CRISPR-Cas9 gene knock-out of SYTL3 in primary human NK cells would be beneficial. The *in vitro* MCMV infection responses of SYTL3 deficient NK cells could therefore be studied. For example, through assessment of viral replication, NK cell activating receptor expression, CD107a and IFNγ expression in response to cell stimuli and imaging to understand whether SYTL3 deficiency in humans reduces MTOC polarisation, as was observed in mice. As Ly49H receptor activation-induced responses showed significant deficiencies in *Sytl3*$^{-/-}$ NK cell degranulation, further assessment of the importance of this signalling pathway may prove useful. However, data from Δm157 MCMV infection showed mixed effects on viral replication control (data not shown), and

since *Sytl3* deficiency impacted degranulation in response to a variety of different cell stimuli, this may not be required.

In addition to NK cells, *Sytl3* was highly expressed by intraepithelial Vγ5+ γδ T-cells. Therefore, *Sytl3* is also likely to impact Vγ5+ γδ T-cell degranulation which should be assessed through staining of degranulation markers in response to cell stimuli. However, as γδ T-cell subsets vary hugely between humans and mice (Pang *et al.*, 2012), and the expression of SYTL3 by γδ T-cells in humans has not been investigated, mouse γδ T-cell research may not be as relevant to humans compared to NK cell research.

5.3 Translational implications for *Sytl3*

As with *Sytl3* in mice, SYTL3 is almost exclusively expressed by human NK cells (Immgen.org). Therefore, these experiments showing reduced NK cell degranulation in *Sytl3*-deficient mice during acute MCMV infection may also inform on mechanisms for controlling other viral infections, bacterial and fungal infections where NK cell control is important, cancer and autoimmune diseases in humans.

The discovery of *Sytl3,* alongside other genes from the *in vivo* screen, is likely to give insight into rare gene defects which cause impaired immune responses to infection. For example, diseases where the underlying genetic cause of recurrent and/or severe infections is not understood such as the cohort selected for whole genome sequencing by Thaventhiran et al. (Thaventhiran *et al.*, 2020). This cohort was a heterogeneous primary immunodeficient group who exhibited a huge range of mutations in different genes (Thaventhiran *et al.*, 2020). Impaired virus control linked specifically to reduced NK cell cytotoxicity has been observed with several different viral infections including: recurrent herpesvirus infections where the common mechanism of NK cell degranulation dysfunction is not fully understood (Murugin *et al.*, 2011), hospitalised patients infected with SARS-CoV2 where the percentage of CD107a+ NK cells is reduced (Zheng *et al.*, 2020) and Epstein-Barr virus+ Hodgkin lymphoma where a reduced frequency of cytotoxic NK cells has been observed (Pánisová *et al.*, 2021). NK cell degranulation is also important for control of bacterial and fungal infections including *Chlamydia psittaci* (Radomski *et al.*, 2019), *Listeria monocytogenes* through maternal decidual NK cells during pregnancy (Crespo *et al.*, 2020) and a number of fungal infections including *Cryptococcus*

neoformans and *Candida albicans* (Ogbomo and Mody, 2016). A greater insight into the underlying mechanisms causing recurrent and severe infections may inform on potential treatment strategies and disease monitoring.

This research also has relevance in cancer as NK cell cytotoxicity is also important for destruction of cancer cells. Alongside the MCMV infection screen, the WTSI performed an *in vivo* cancer screen using the same mouse lines challenged with the B16-F10 tumour model. In the case of *Sytl3*, no significant impacts on cancer growth were observed. However, the B16-F10 model is poorly immunogenic (Wang *et al.*, 1998) therefore, in other tumours which have NK cell tumour infiltration, the discovery of *Sytl3* as an NK cell expressed gene involved in degranulation may be relevant. Assessment of *Sytl3* gene defects in immunogenic tumour models would provide insight into the impact of *Sytl3* on tumour growth.

The discovery of *Sytl3* is also likely to inform on several autoimmune disorders where reduced NK cell cytotoxicity has been observed. For example, hemophagocytic lymphohistiocytosis (HLH) which are a group of heterogeneous life-threatening disorders characterised by uncontrolled immune activation and decreased NK cell cytolytic responses (Risma and Jordan, 2012). Mutations in several different proteins required for degranulation have been observed in HLH including STXBP2, RAB27A, LYST, PRF1, UNC13D and AP3B1 therefore SYTL3 gene effects could also have relevance (Mukda *et al.*, 2017; Miao *et al.*, 2019; Gadoury-Levesque *et al.*, 2020). Despite not showing a killing defect during *Sytl3* deficiency in my experiments, and only showing a loss of degranulation function through testing CD107a expression, these experiments remain highly relevant to HLH. This is due to a study showing CD107a testing is superior to NK cell functional testing for screening patients with genetic HLH (Rubin *et al.*, 2017). The study highlights that NK cell functional testing, usually through chromium release assays, is less sensitive and no more specific than analysing NK cell expression and upregulation of CD107a alongside perforin granule contents. In addition, dysfunctional Rab27a mediated secretory lysosome release has been implicated in the impaired cytotoxic lymphocyte responses of Griscelli Syndrome patients. Griscelli Syndrome is characterised by albinism with immunodeficiency, therefore both melanin transport and degranulation are impacted (Ménasché *et al.*, 2003, 2008; Stinchcombe, Bossi and Giffiths, 2004). Since Rab27a binds to sytl proteins, this research may also have relevance to uncovering the mechanisms underlying Griscelli Syndrome (Taruho S Kuroda *et al.*, 2002b).

Several other disorders have shown reduced NK cell cytolytic function such as: Fanconi anemia, a rare inherited syndrome caused by genomic instability leading to hematopoietic stem cell death; myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (Eaton-Fitch *et al.*, 2019) and juvenile idiopathic arthritis (Cifaldi *et al.*, 2015). However, in the case of juvenile idiopathic arthritis, NK cell granule exocytosis occurs normally but granule contents are reduced. Therefore, as my results showed reduced MTOC polarisation upon *Sytl3* deficiency, with no impairment in granzyme B granule content production, *Sytl3* is likely to have most relevance to disorders which present with impaired granule exocytosis.

5.4 Conclusions

I have shown that the following novel genes *Fam111a, Fam114a2, Far1, Heatr9, Serpinb9b* and *Sytl3* regulate responses to acute MCMV infection. Of these genes, *Sytl3* was shown to facilitate degranulation through granule exocytosis and affect the amount of IFNγ production within NK cells in response to acute MCMV infection and multiple cell stimuli. The mechanism for *Sytl3*-directed degranulation is likely to involve driving MTOC polarisation towards the immunological synapse. However, a killing defect upon *Sytl3* deficiency has not thus far been demonstrated. Although further studies are needed to elucidate whether there is an impact on NK cell induced killing and the mechanism behind this, the overall data presented here suggests that *Sytl3* has importance in NK cell directed virus control, predominantly through granule exocytosis. Finally, this work is likely to have relevance to human NK cell cytotoxic function within infection, cancer, and autoimmunity.

Chapter 6 – References

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Chapter 7 Appendix

7.1 Appendix I

Primary data describing the *in vivo* MCMV infection screen including weight change over time and viral replication 4 days p.i. in WT and gene-deficient mice. Technical assistance for some of these assays was provided by Morgan Marsden and Lucy Chapman.

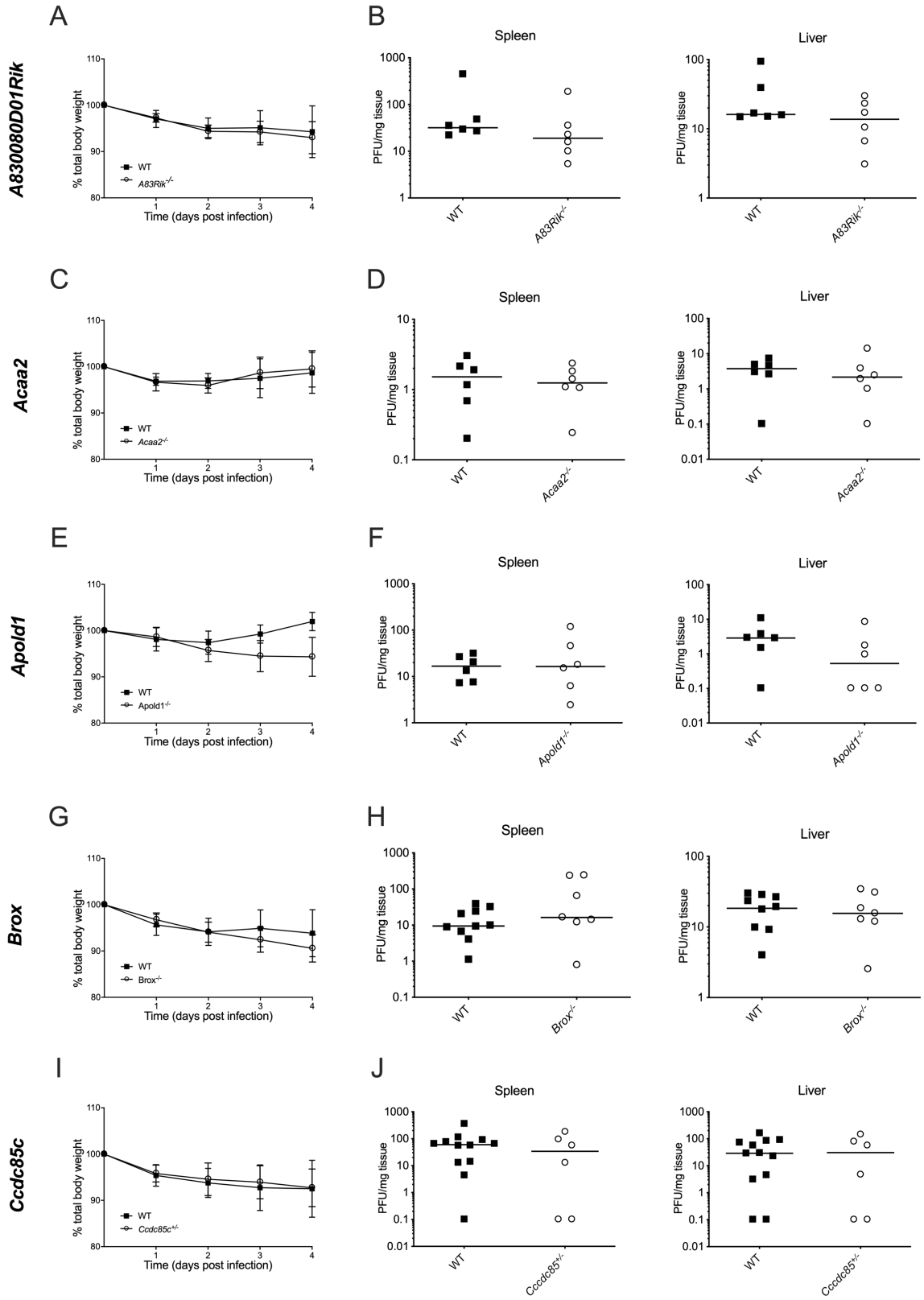

Figure 7. 1 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

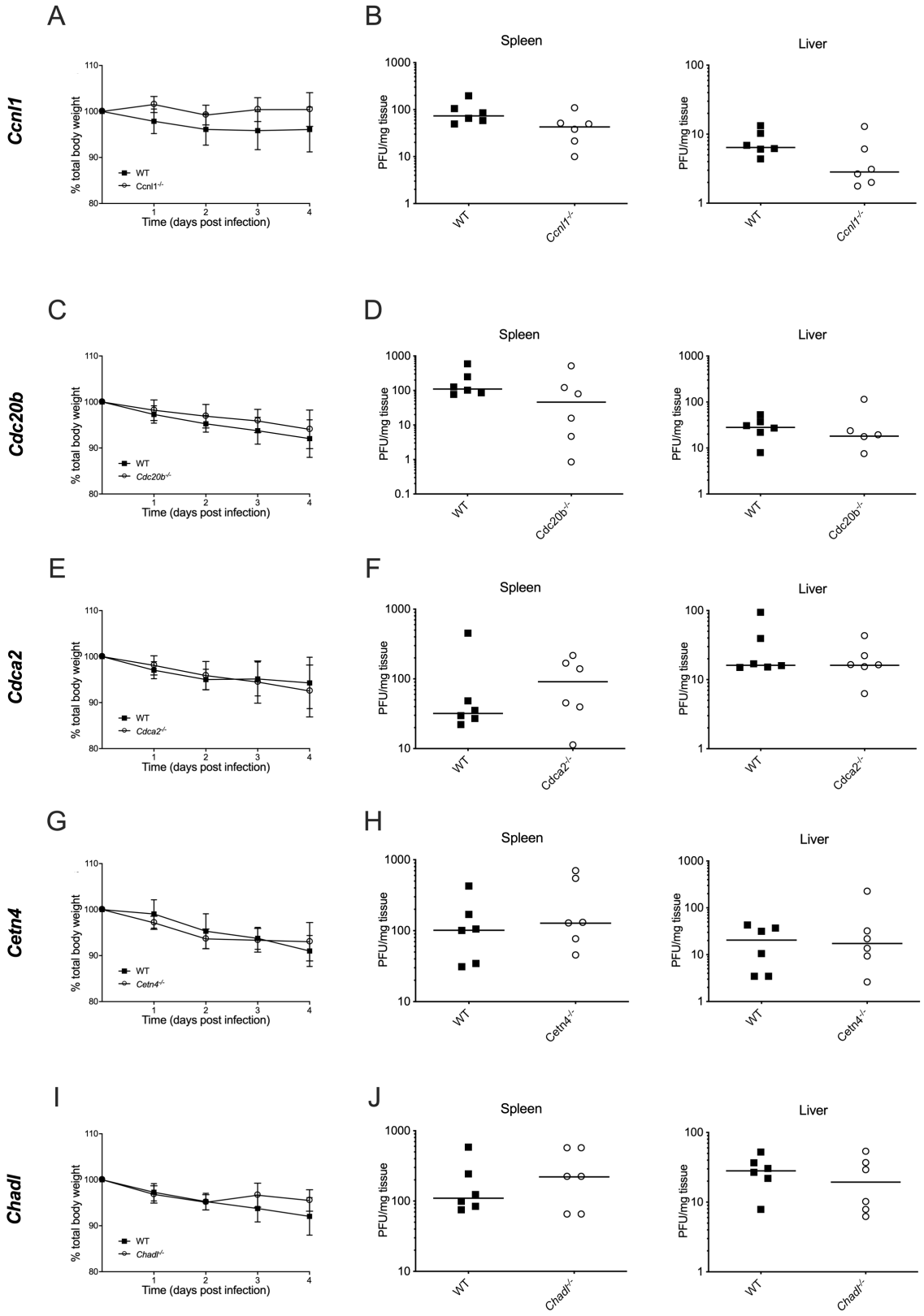

Figure 7. 2 In vivo mouse screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

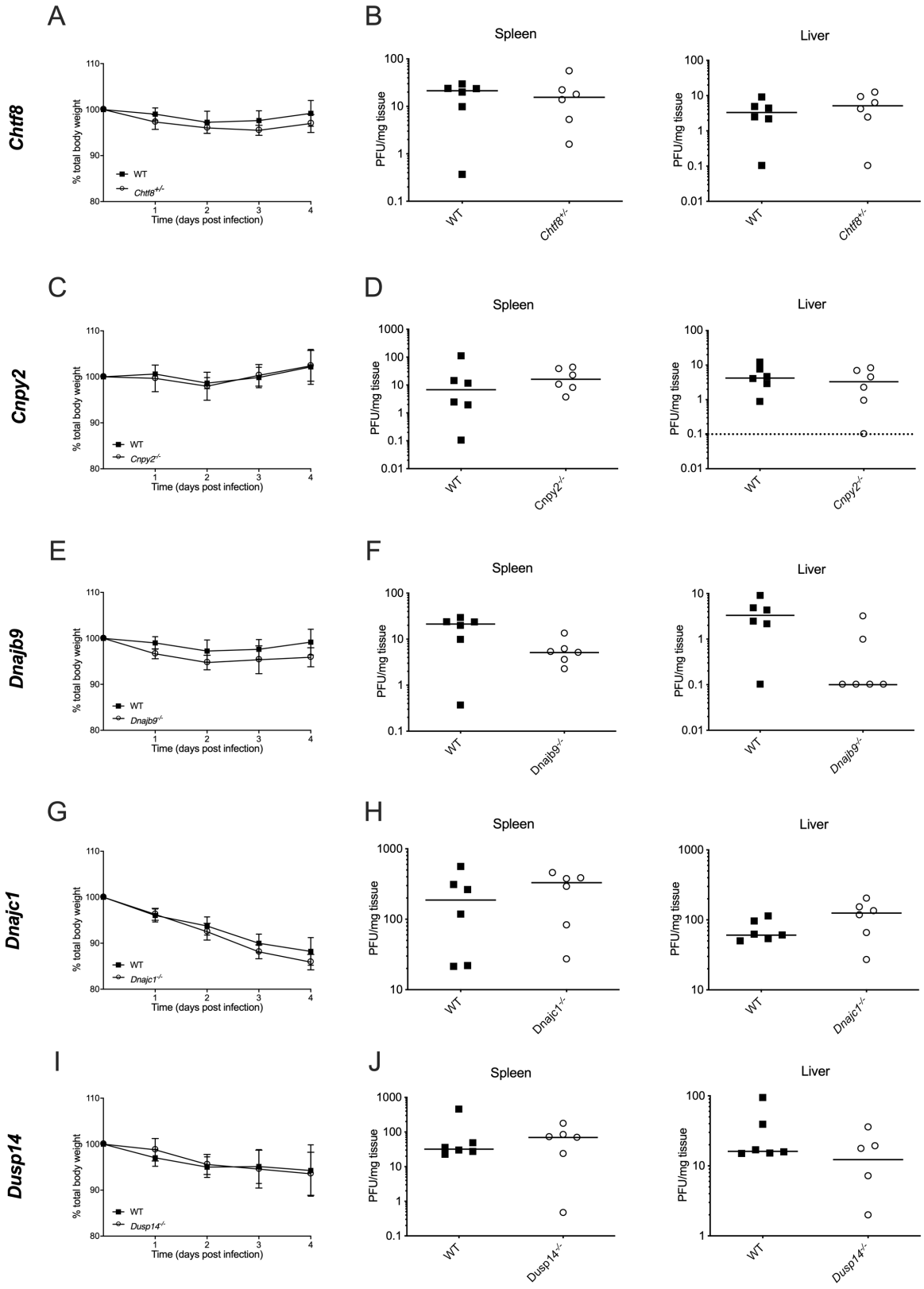

Figure 7. 3 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

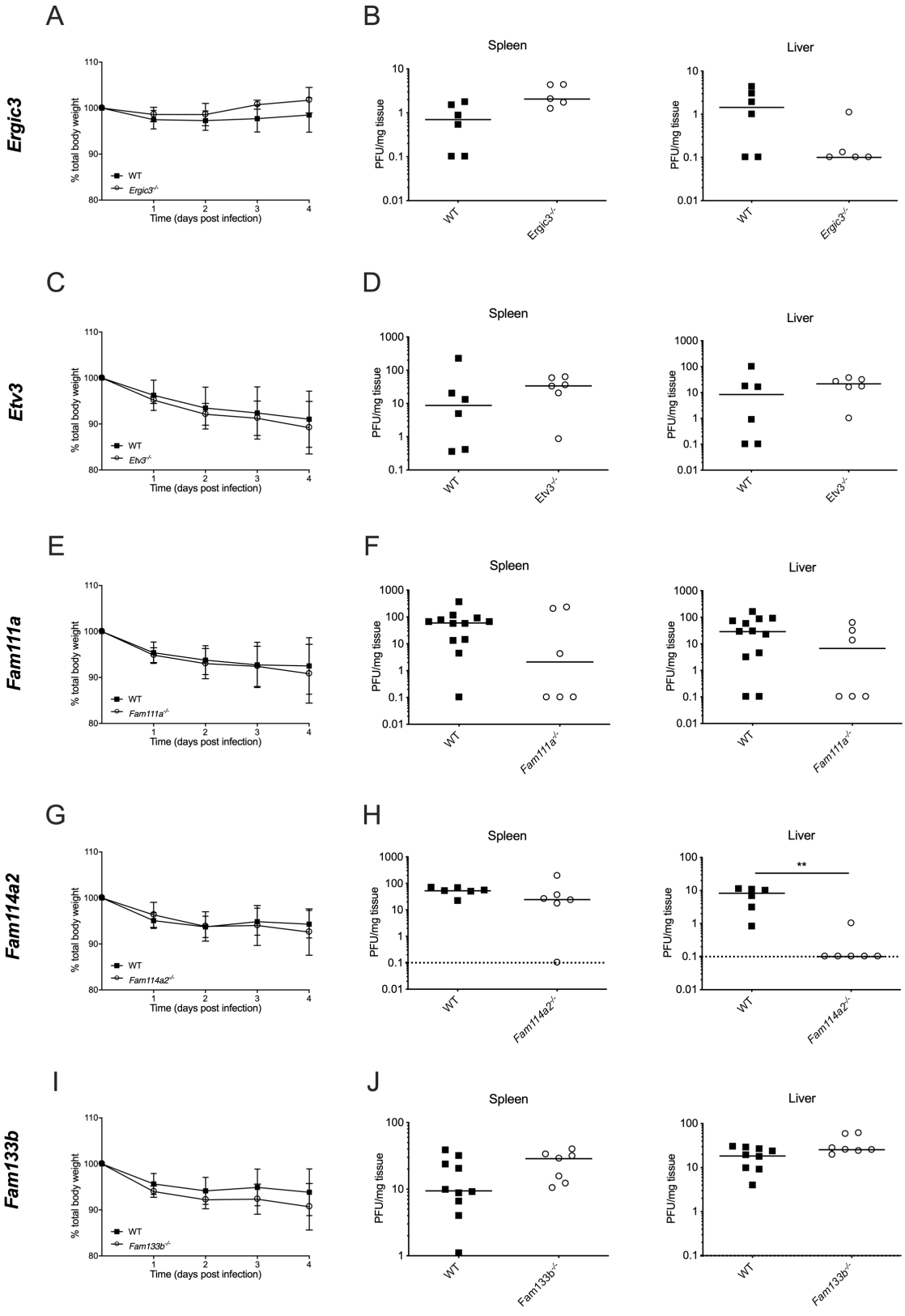

Figure 7. 4 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

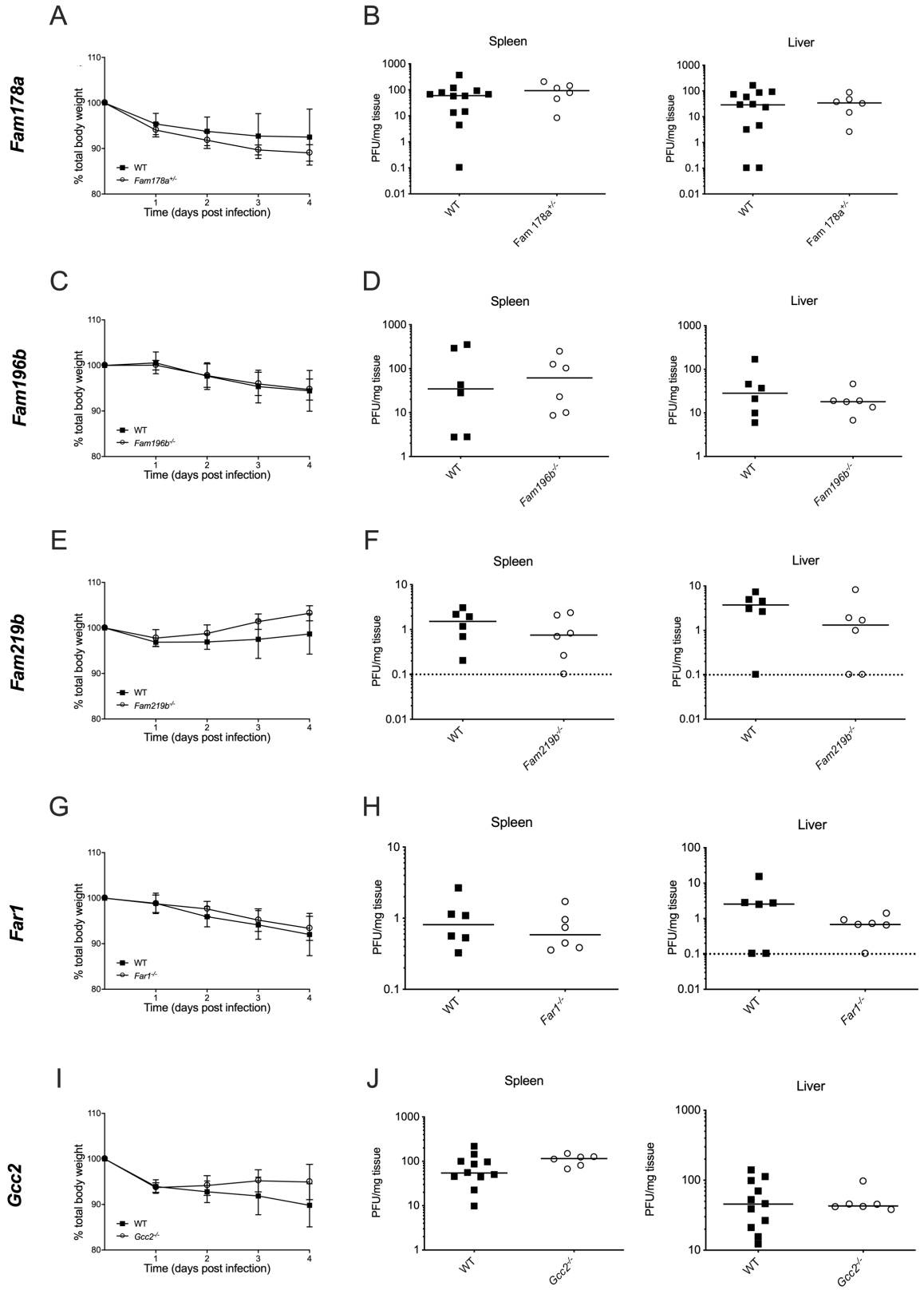

Figure 7. 5 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

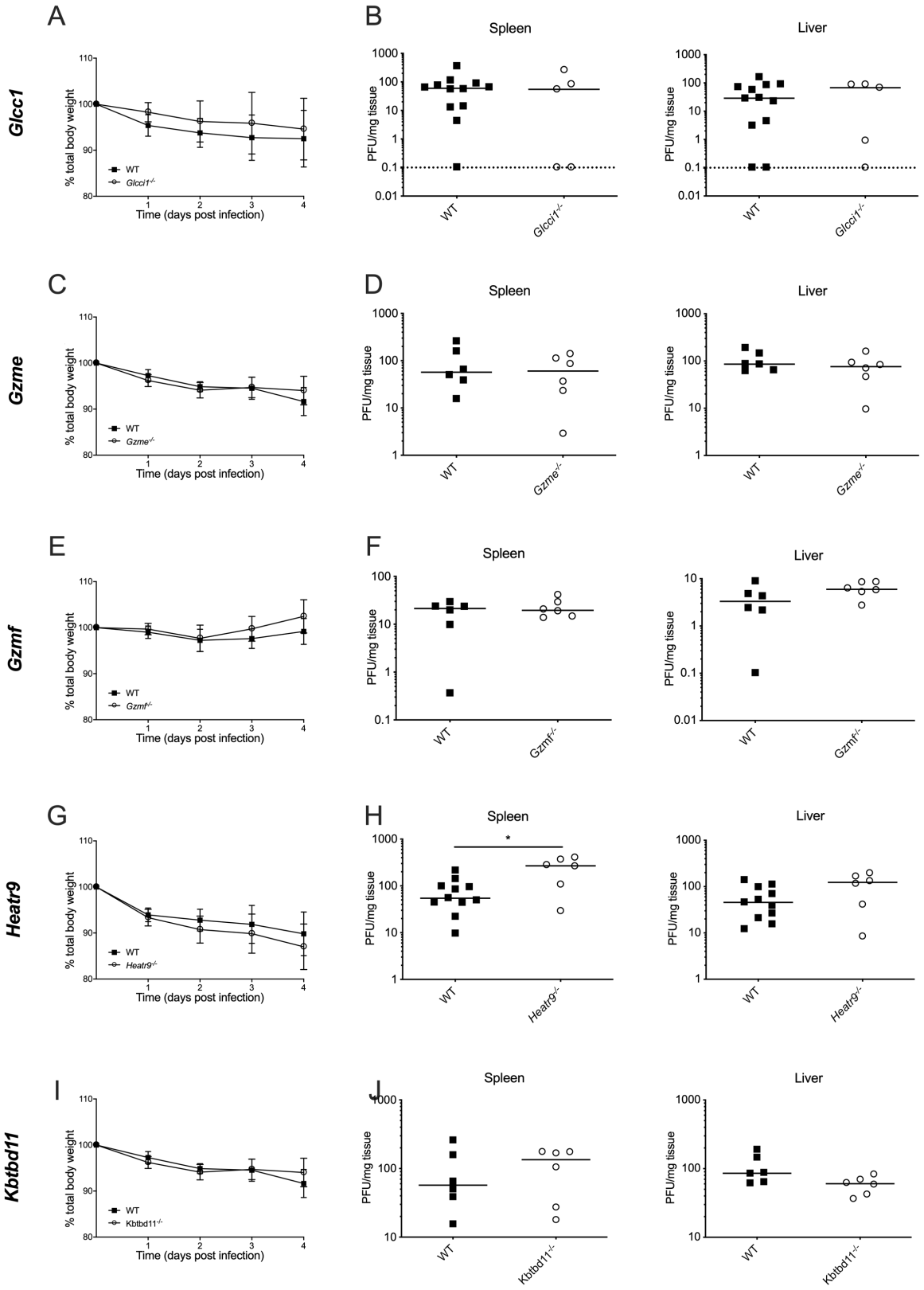

Figure 7. 6 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

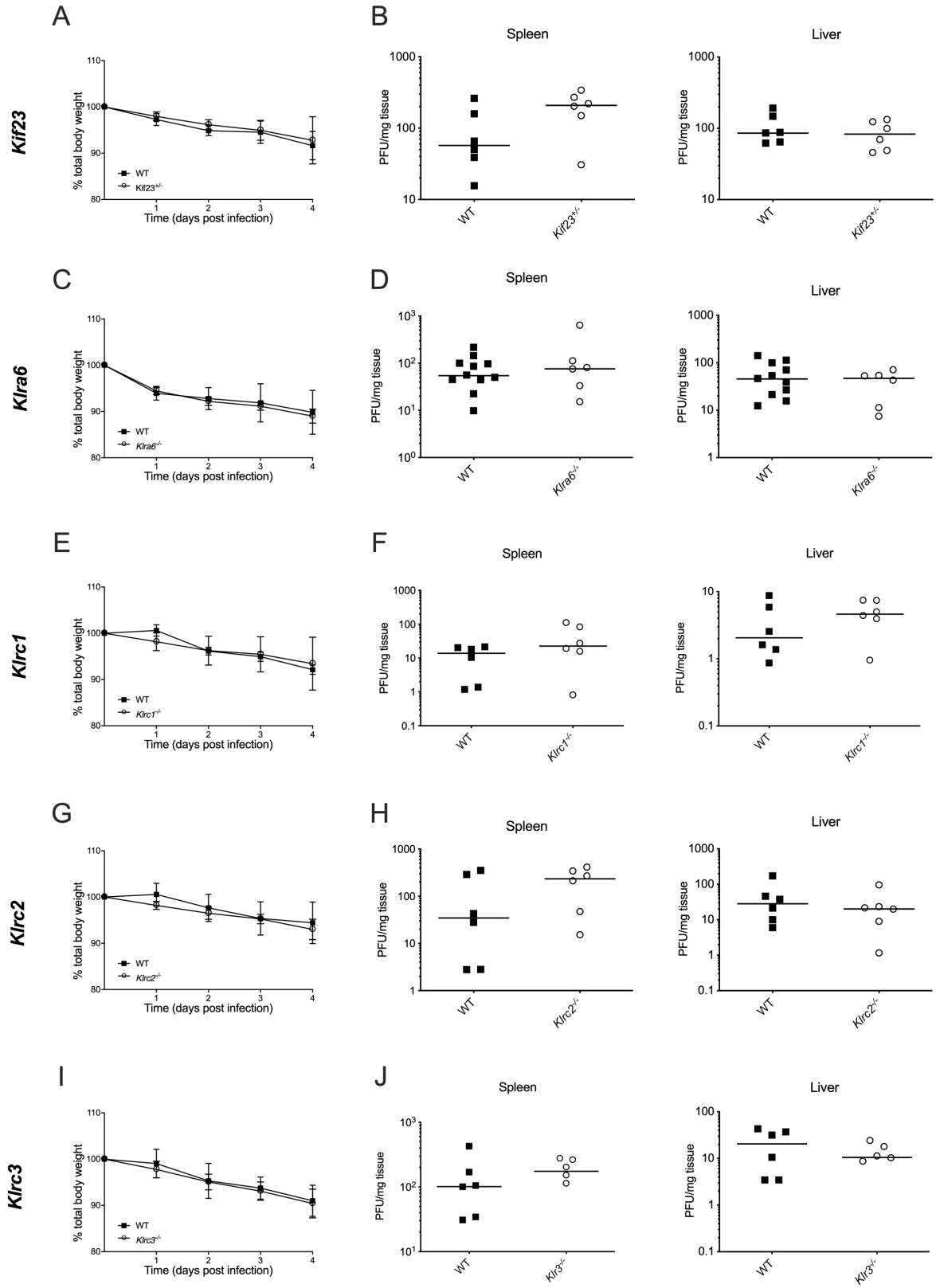

Figure 7. 7 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

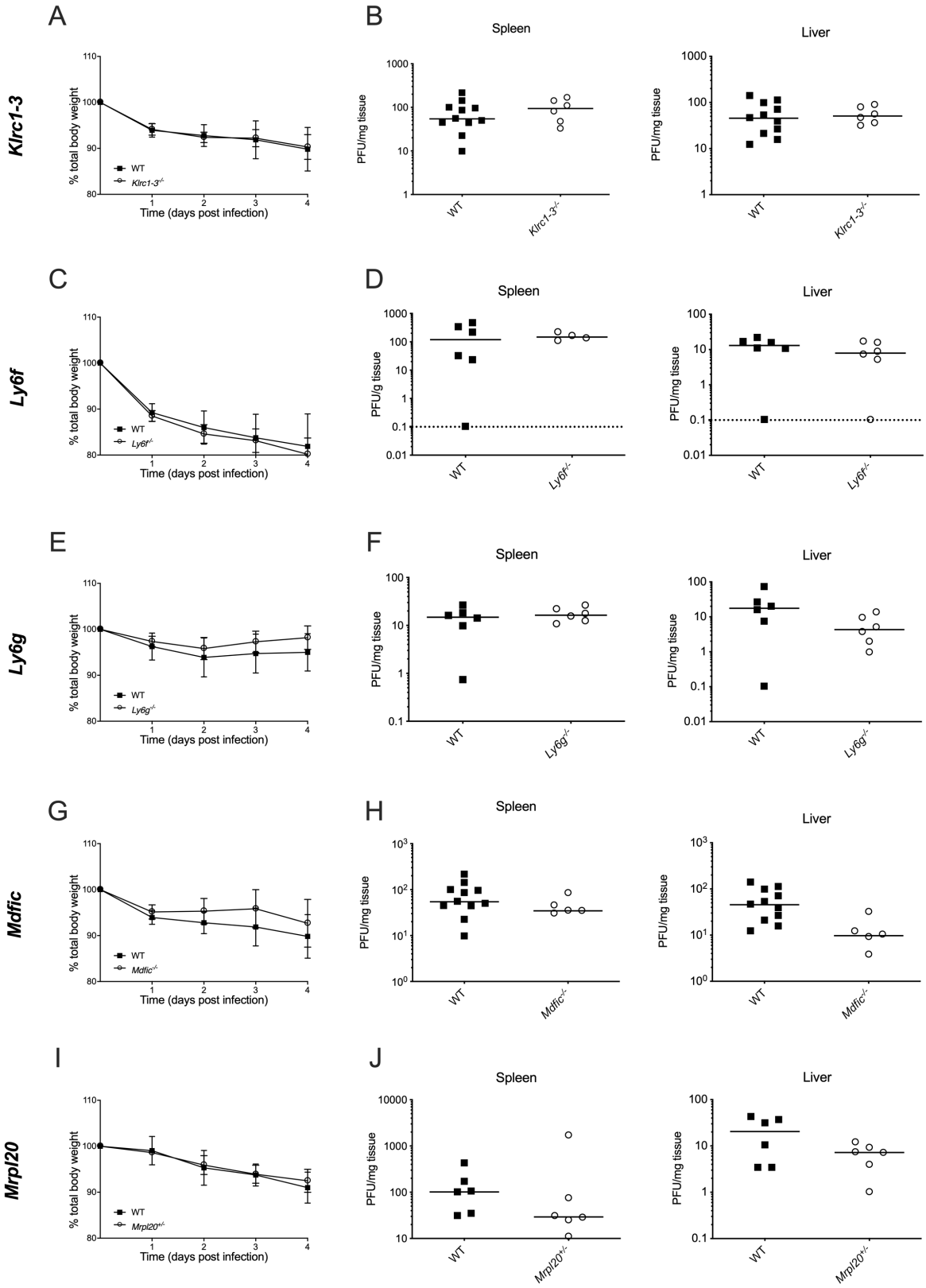

Figure 7. 8 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

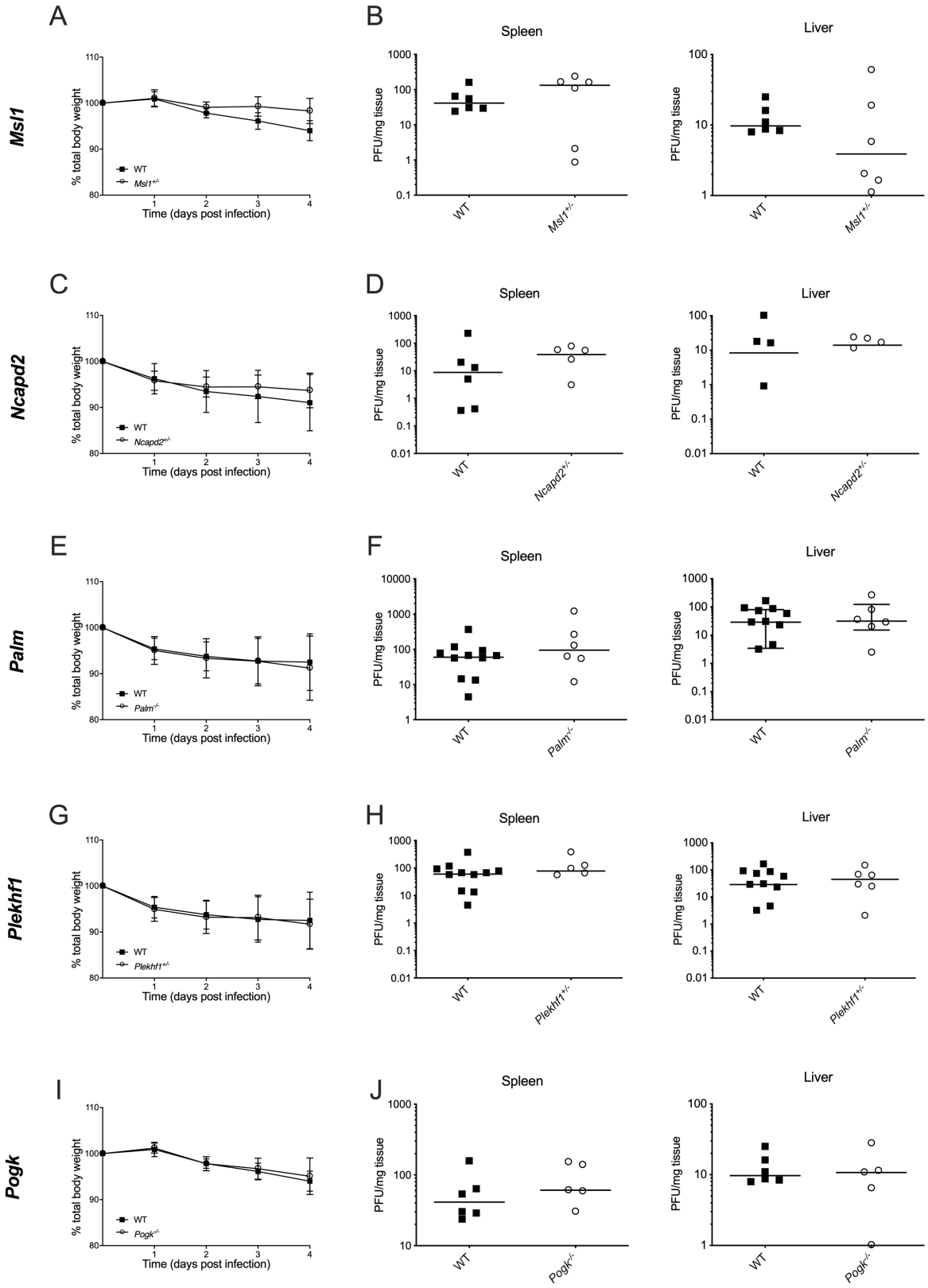

Figure 7. 9 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

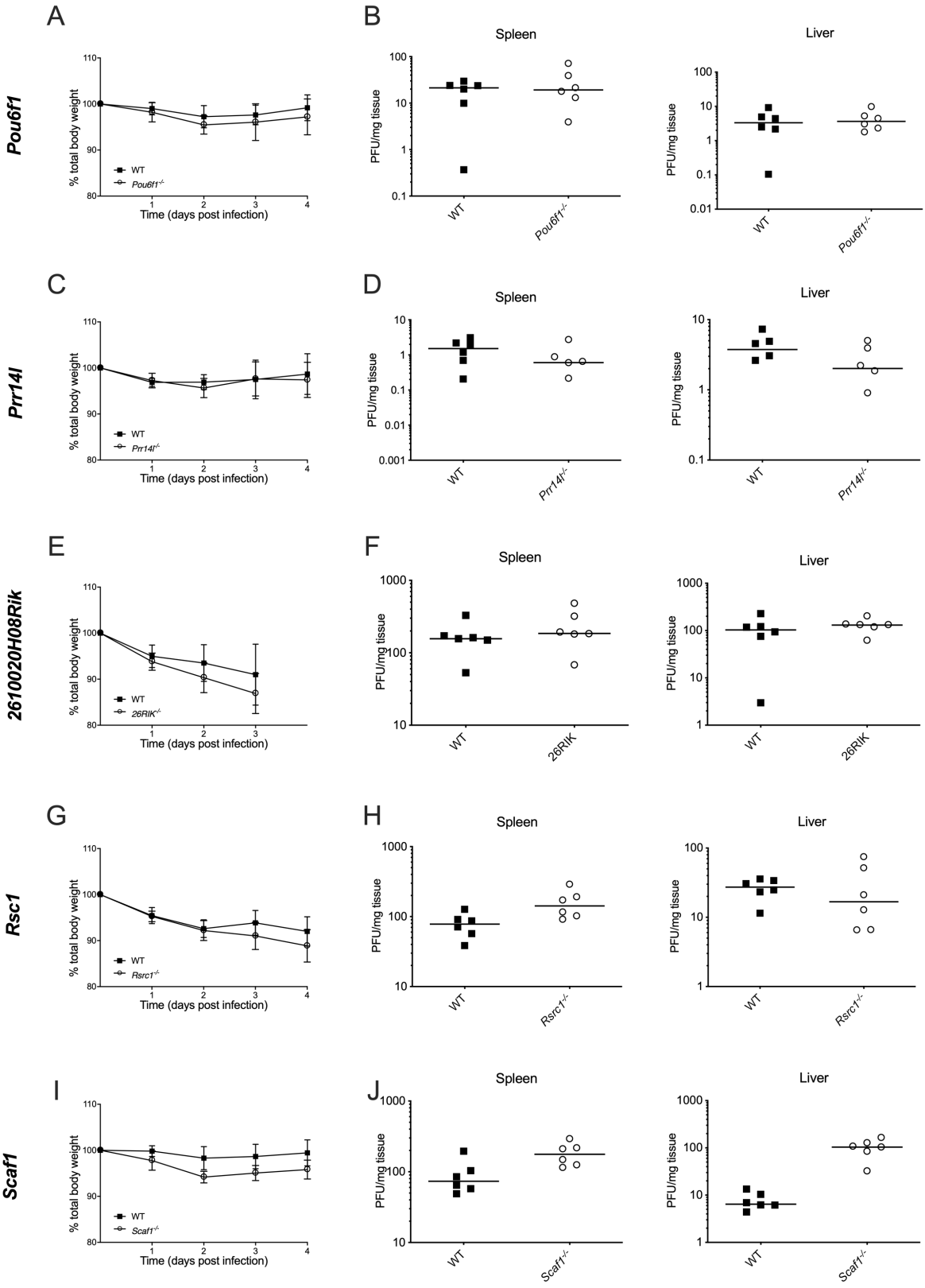

Figure 7. 10 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

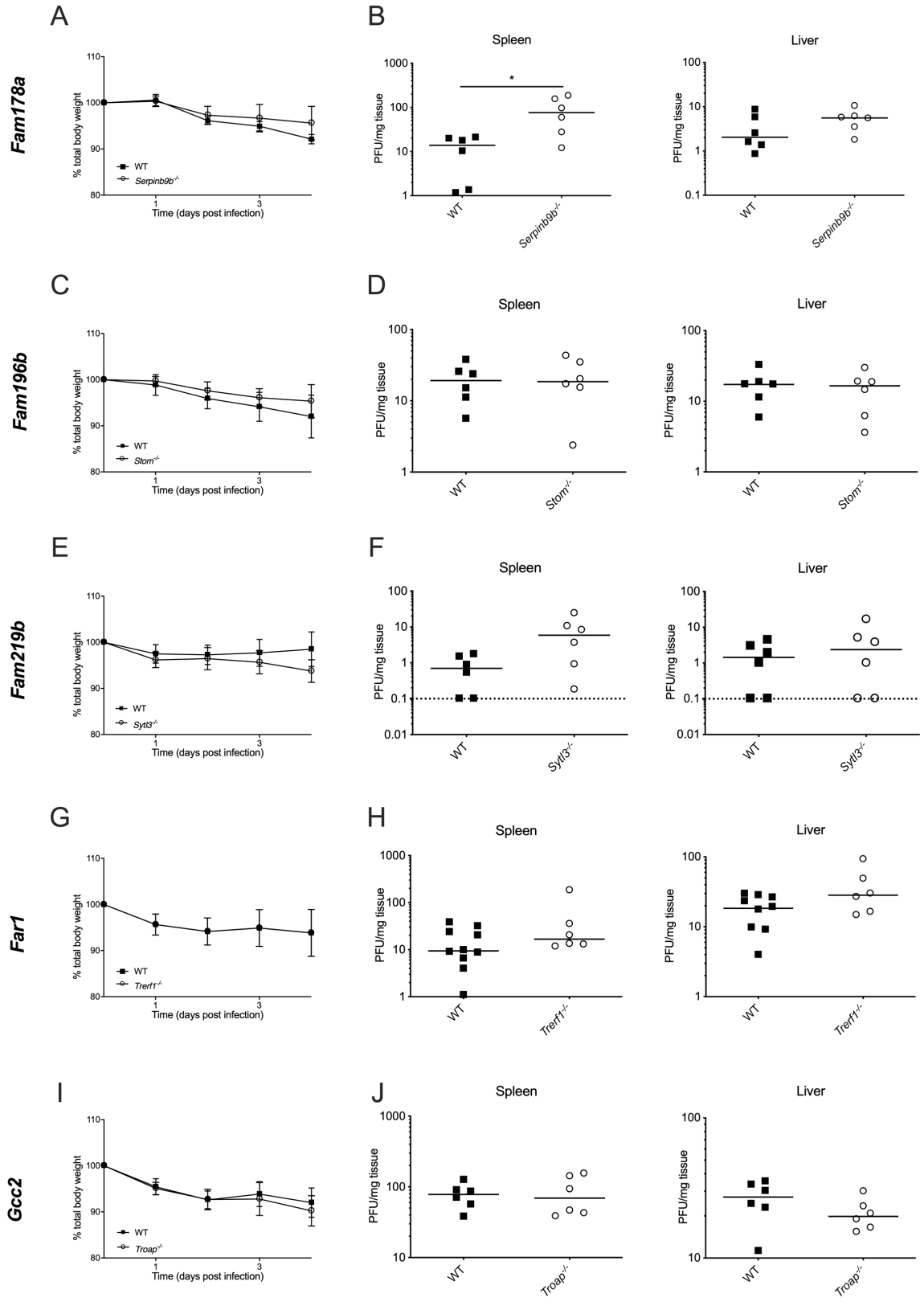

Figure 7. 11 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

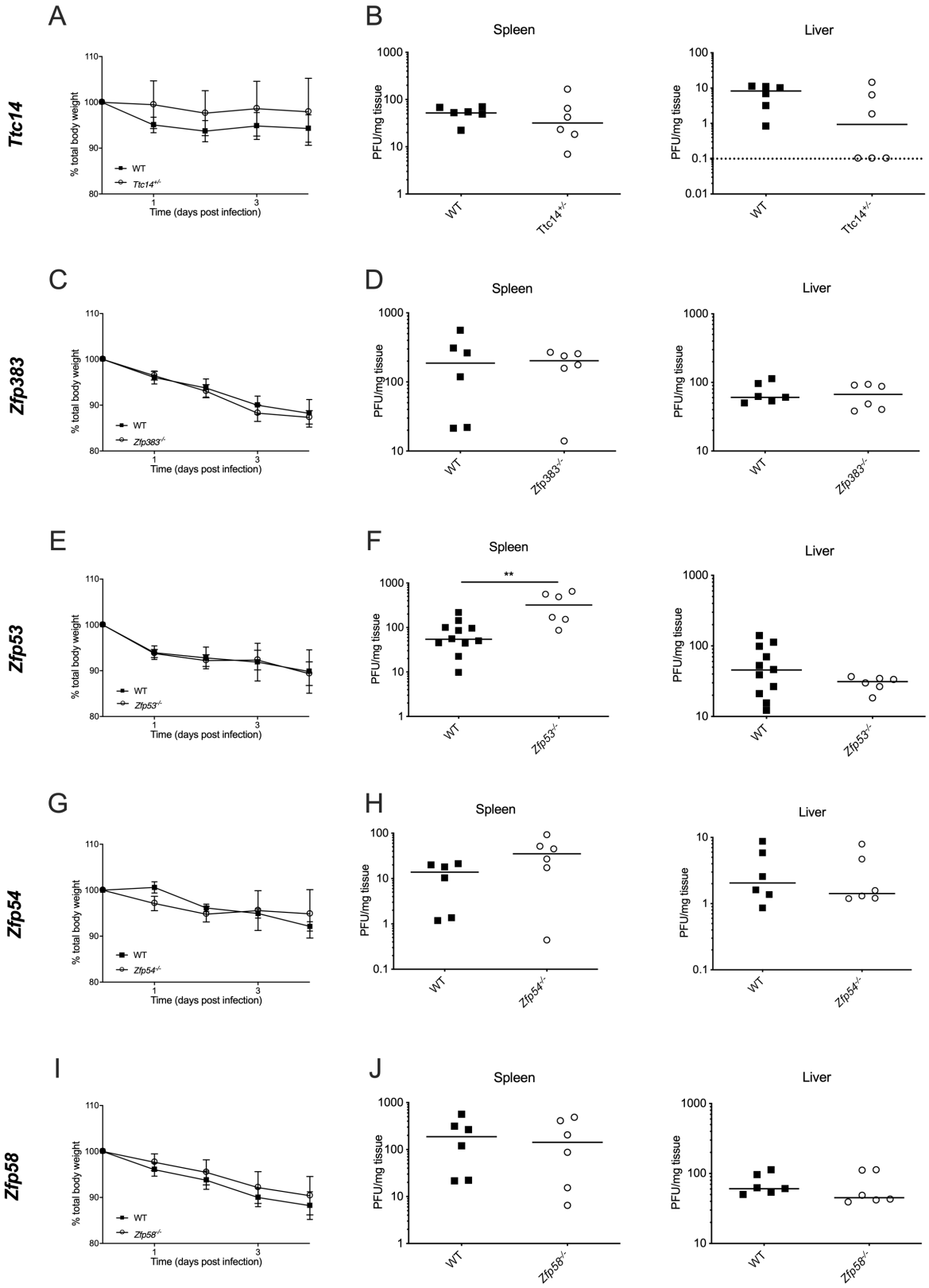

Figure 7. 12 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

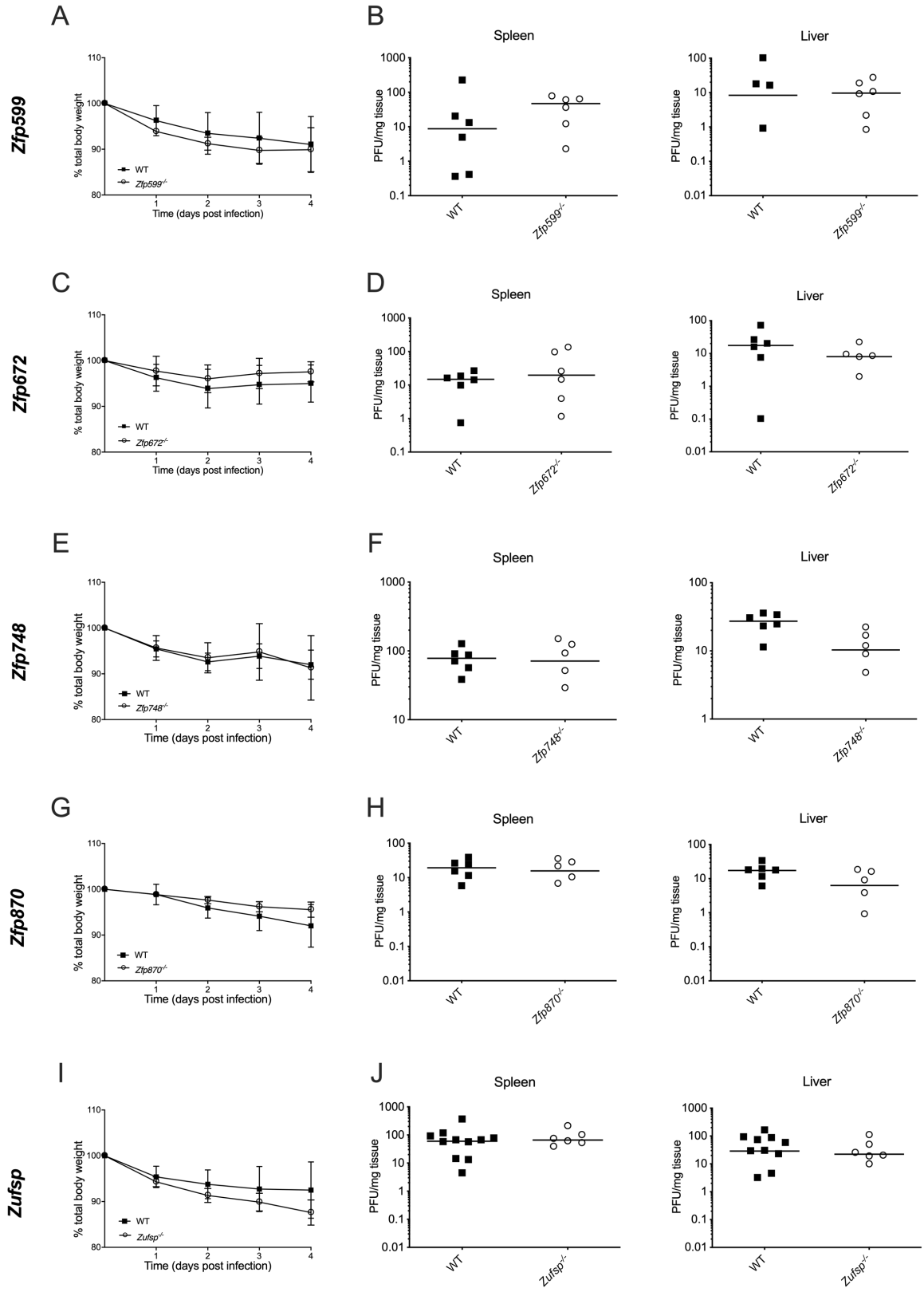

Figure 7. 13 *In vivo mouse* screen weight and viral replication.
(A, C, E, G, I) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H, J) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

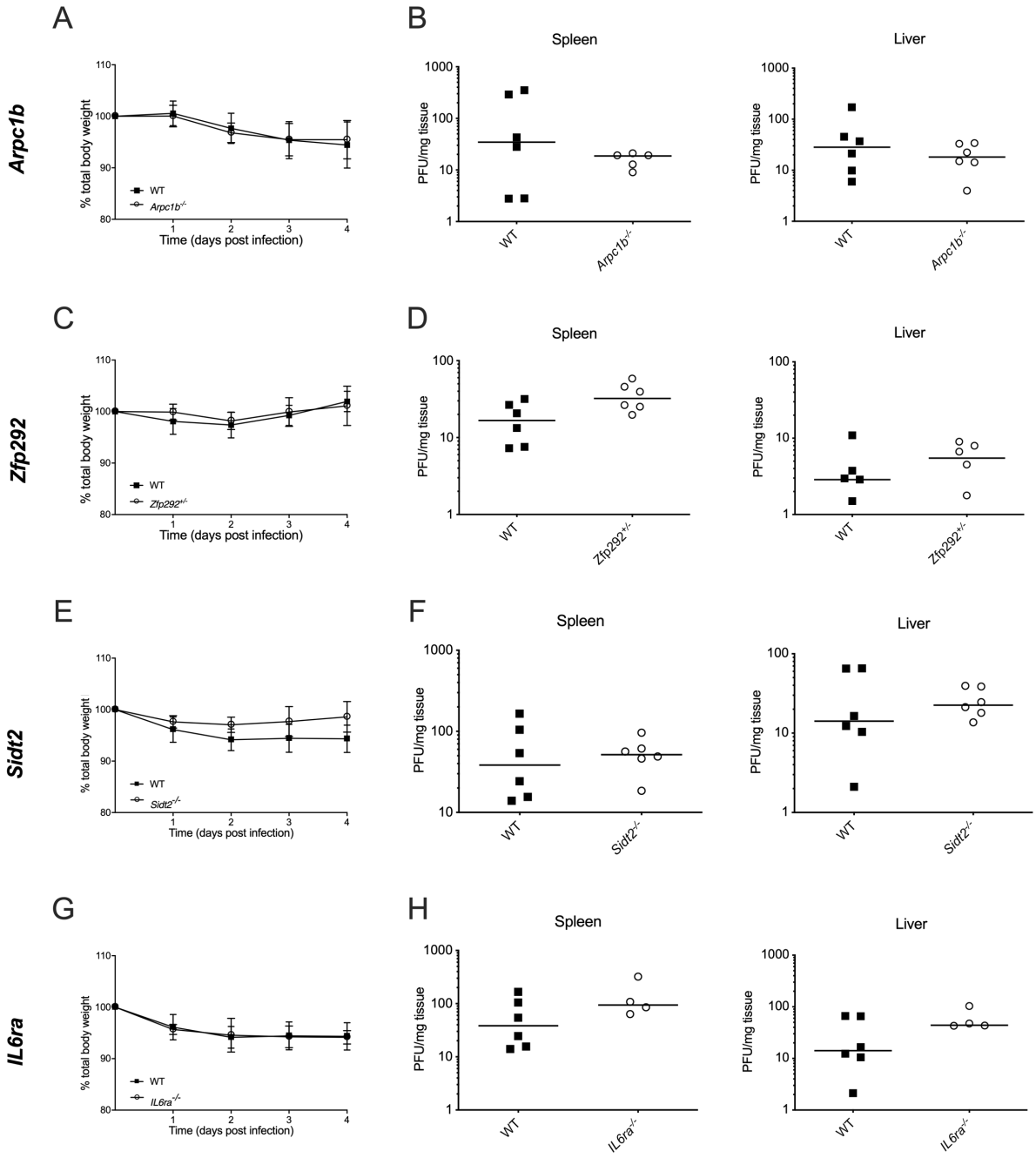

Figure 7. 14 *In vivo mouse* screen weight and viral replication.
(A, C, I G) Percentage total body weight in gene-deficient mice compared to WT 4 days post-MCMV infection. Lines represent mean ± standard deviation. (B, D, F, H) Viral replication titres from WT and gene-deficient mouse spleen and liver. Undetectable titres are expressed as an arbitrary value of 0.1 for display on a logarithmic scale. Individual mice and median are shown (6-12 mice per group).

7.2 Appendix II

Literature reviews published in Oxford Open Immunology as a co-author within the Oxford-Cardiff COVID19 Literature Consortium which was a key activity during lockdowns and when access to the laboratory was limited.