Manipulation of host signalling for the characterisation and control of dengue fever

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Abstract

Dengue fever is a mosquito-transmitted disease of the tropics and sub-tropics that is caused by dengue virus (DENV). There are an estimated 60-100 million clinical cases of dengue fever per year, resulting in at least 10,000 deaths. Most clinical cases of dengue are characterised by flu-like symptoms. However, for unknown reasons, a small proportion (1-2%) of clinical cases progress to a life-threatening form of disease referred to as "severe dengue". Severe dengue is characterised by cytokine storms, heightened endothelial permeability and associated sequelae such as shock and haemorrhage. During the onset of severe dengue, viraemia and viral antigenaemia are sharply declining or absent. Therefore, it is logical to deduce that dysregulated host signalling is the underlying cause of the cytokine storm phenotype and symptoms of severe dengue. However, although many host factors have been characterised in the context of DENV infection, the root cause of this signalling dysregulation is still poorly understood. Furthermore, there are currently no drug treatments available for the treatment of severe dengue, and although there is a licensed dengue vaccine, it confers only moderate protection, and administration of this vaccine to dengue naïve individuals is contraindicated by the World Health Organisation.

In the first part of this thesis, I characterised how genetic disruption of key host signalling pathways altered the response of macrophages and mice to DENV infection. I found that infection of cells and mice that had a co-deletion of genes encoding cellular inhibitor of apoptosis proteins (cIAPs) resulted in decreased production of virus, and an exaggerated production of inflammatory cytokines.

In the second part of this thesis, I determined whether clinical stage cancer therapeutics could be repurposed as treatments for severe dengue. To investigate this, I established an *in vivo* mouse model of severe dengue and treated these mice with anti-inflammatory compounds. However, these drug treatments did not reduce clinical manifestations of infection or improve the survival of the infected mice.

These studies suggest that cIAPs facilitate the efficient replication of DENV. In addition, I hope that the negative results from my therapeutic experiments can inform future experimental plans, and contribute to reducing the worldwide burden of severe dengue.

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Declaration

This is to certify that this thesis comprises only my original work, except where indicated in the preface. All the work detailed herein was performed following the commencement of my PhD candidature on the 27th of July 2015. Due acknowledgements have been made to individuals who contributed to the work in the preface and in the body of the thesis, including tables and figures. This thesis is less than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

Wasan Otis Forsyth

March 12th, 2020

Preface

The work described in this thesis was undertaken at the Walter and Eliza Hall Institute of Medical Research (WEHI) under the supervision of Professor Marc Pellegrini and Dr Gregor Ebert. During the course of this work the author was a recipient of the Australian Postgraduate Award.

I duly acknowledge the important contributions of individuals to the work presented within this thesis. Contributions are as listed below:

General Contributions

Professor Paul Young and Dr David Muller (University of Queensland) provided me with the original DENV2 stock. This virus stock was subsequently expanded and used for all experiments of this study.

Associate Professor Jill Carr provided me with the plaque assay protocol. In addition, Dr Carr provided me with the MON601 DNA plasmid, and a protocol for *in vitro* RNA transcription for MON601 genomic RNA.

Dr Stephen Wilcox and Ms Isabella Kong helped me to establish the quality and concentration of MON601 RNA, which was then used to establish the standard curve for the DENV2 qPCR assay.

Dr Greg Ebert established the culture protocol I used to produce all stocks of DENV2 for this study. Dr Ebert also helped me establish the DENV2 qPCR protocol.

Dr Joanna Groom provided me with the $IFNAR^{-/-}$ mouse strain.

Chapter 3

Dr Simon Preston helped me to establish the flow cytometry assay used for the detection of viral NS1 antigen.

Dr Turgut-Esad Aktepe (Peter Doherty Institute) provided me with 4G2 (anti-DENV envelope) and 4G4 (anti-DENV NS1) hybridoma supernatants.

Dr Jody Peters and Professor Roy Hall (University of Queensland) provided me with 4G2 (anti-DENV envelope) and 4G4 (anti-DENV NS1) hybridoma supernatants (in bulk).

The WEHI Antibody Facility helped purify antibodies from hybridoma supernatants obtained from the Hall Lab (University of Queensland).

Dr Tan Nguyen provided me with SIDT2$^{-/-}$ mice.

Chapter 4

Dr Cody Allison performed pilot experiments which helped to establish the protocol for *in vivo* MAR1-5A3-mediated suppression of type I interferon signalling.

Professor John Silke and Dr Holly Anderton provided me with TRAF2^{LysM} mice.

Chapter 5

Dr Simon Preston organised the infection and LCL-161 drug treatment of IFNAR$^{-/-}$ mice. This experimental data was used for Figure 5.3.

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It has been my pleasure throughout this PhD to work with Marc Pellegrini, my primary supervisor. He made it easy for me to put hard work into this project because I knew that there was always someone there to help me out with direction, support, and insight. I don't know where this project would have gone without him, and I don't want to find out.

In addition, Greg Ebert, my secondary supervisor, helped me produce virus after I tried and failed to do so for many months. Therefore, all of this work would not have been possible without him, and he also helped me with my qPCR, and in the way that I record and think about my results. I can honestly say that working with Greg has made me a better scientist.

Simon Preston and Marcel Doerflinger have always been supportive of my research and have helped me with numerous useful suggestions throughout the course of this PhD. Simon in particular has helped to shape the direction of this project. Marcel probably thinks he is a world-class humourist and occasionally he does make a good joke. Cody Allison has also patiently assisted me with numerous technical questions throughout this project. I have also received so much support from the rest of the Pellegrini Lab and the ID2 division.

Waihong Tham and Joseph Torresi have been exemplary committee members and have provided me with great mentorship and scientific advice. Their contributions were very much appreciated.

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To all of my animal technicians, past and present (Merle Dayton, Carolina Alvarado, Liana Mackiewicz, Shannon Oliver): you are all superstars. Thank you for taking the time to help me with procedures and training.

I am lucky enough to have many friends inside and outside of WEHI. Thank you all for helping me get through this tough 4 years. In particular, thanks to Hannah Layman and Julia Gorman for taking me in off the street and giving me a stable home to hide away from the madness. To my climbing friends Will Russell, Tom Perkins, Davina Mann, Amelia Caddy, Georgia Wilkinson, Katalina Bobowik, Jeremi Blouin and Maja Flower-y'all are great. Also, it would have been nowhere near as fun to be at WEHI without Jessica Brewster, Connie Li Wai Suen, Michelle Clark, Lisa Verzier, Ushma Ruparel and Ben Seager and also everybody at Classic Curry. Finally, I would like to thank Amal Varghese who is always up for adventures, and my overseas friends Annie Yang, Anthony Schneider, Harry Peirse and Elizabeth Well-Thulin.

Finally, thank you to my parents, Ra and Stewart who are very supportive but also let me know when I'm being dumb. It's always great to see you guys.

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Abbreviations

ADE	Antibody Dependent Enhancement
ALT	Alanine Transaminase
ANOVA	Analysis of Variance
AP1	Activator protein 1
AST	Aspartate Transaminase
ATP	Adenosine Triphosphate
BIR	Baculovirus IAP Repeat
BMDMs	Bone Marrow Derived Macrophages
CD, e.g. CD3	Cluster of Differentiation, e.g. Cluster of Differentiation 3
cFLIP	Cellular FLICE-inhibitory Protein
cGAS	cyclic GMP-AMP Synthase
CHX	Cycloheximide
cIAP	cellular Inhibitor of Apoptosis Protein
CNS	Central Nervous System
CYD	Chimaeric Yellow Fever Virus - Dengue
DCs	Dendritic Cells
DENV	Dengue Virus
DF	Dengue Fever
DHF	Dengue Haemorrhagic Fever
DKO	Double Knockout
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded Deoxyribonucleic Acid
dsRNA	Double-stranded Ribonucleic Acid
DSS	Dengue Shock Syndrome
DUBA	Deubiquitinating Enzyme A
ELISPOT	Enzyme-linked Immunosorbent Spot
EMCV	Encephalomyocarditis Virus
EPO	Erythropoietin
FACS	Fluorescence Activated Cell Sorting
FADD	Fas-Associated Death Domain
FasL	Fas Ligand
FCS	Foetal Calf Serum
FcγR	Fc receptor for IgG
FDA	Federal Drug Administration
G-CSFR	Granulocyte Colony Stimulating Factor Receptor
GE	Genomic Equivalents
GPCR	G-protein Coupled Receptor

GVHD	Graft Versus Host Disease
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HEPES	N-2-hydroxyethylpiperazine-N-ethanesulfonic acid
HIV	Human Immunodeficiency Virus
HOIL-1	Haem-oxidized IRP2 Ubiquitin Ligase-1
HOIP	HOIL-1 interacting protein
HPI	Hours Post Infection
HSV	Herpes Simplex Virus
HTLV-1	Human T-Cell Leukaemia Virus Type 1
IAV	Influenza A Virus
IC_{50}	Half Maximal Inhibitory Concentration
IFN	Interferon
IFNAR	Interferon-α/β Receptor
IFNGR	Interferon-γ Receptor
IgG	Immunoglobulin G
IKK	Inhibitor of Nuclear Factor κB Kinase
IL, e.g. IL-3	Interleukin, e.g. Interleukin-3
IRF	Interferon Response Factor
ISG	Interferon Stimulated Gene
ISGF3	Interferon Stimulated Gene Factor 3
ISRE	Interferon Stimulated Response Element
IκB	Inhibitor of Nuclear Factor κB
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
kDa	Kilodaltons
KIR	Kinase Inhibitory Region
LCMV	Lymphocytic Choriomeningitis Virus
LDH	Lactate Dehydrogenase
LOD	Limit of Detection
LPS	Lipopolysaccharide
LUBAC	Linear Ubiquitin Chain Assembly Complex
LysM	Lysozyme M
MAPK	Mitogen Activated Protein Kinase
MAR1	MAR1-5A3 (an IFNAR-blocking antibody clone)
MAVS	Mitochondrial Antiviral Signalling Protein
MDA-5	Melanoma Differentiation Associated Protein-5
MEFs	Mouse Embryonic Fibroblasts
miRNA	micro Ribonucleic Acid
MOI	Multiplicity of Infection
NEMO	NF-κB Essential Modulator

NF-κB	Nuclear Factor κB
NHP	Non-human Primate
NK Cell	Natural Killer Cell
NLRP3	NOD-, LRR- and Pyrin domain-containing Protein 3
NS1	Non-Structural Protein 1
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
pfu	Plaque Forming Unit
pg	Picograms
PRR	Pattern Recognition Receptor
qPCR	Quantitative Polymerase Chain Reaction
RIG-I	Retinoic-Acid Inducible Gene I
RING	Really Interesting New Gene
RIP1	Receptor-interacting serine/threonine-protein kinase 1
RIP3	Receptor-interacting serine/threonine-protein kinase 3
RLRs	Retinoic-Acid Inducible Gene I-Like Receptors
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
SH2	Src Homology 2
SHARPIN	SHANK-associated RH Domain Interacting Protein
SIDT2	SID1 Transmembrane Family Member 2
siRNA	Short Interfering RNA
SMAC	Second Mitochondrial Activator of Caspases
SOCS	Suppressor of Cytokine Signalling
SP1	Proximal Specificity Protein 1
ssRNA	Single-stranded Ribonucleic Acid
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of Interferon Genes
sVEGFR2	Soluble Vascular Endothelial Growth Factor Receptor 2
T. gondii	*Toxoplasma gondii*
TAK1	Transforming growth factor β-activated kinase 1
TBK1	Tank-Binding Kinase 1
TDV	Tetravalent Dengue Vaccine
T_{FH} Cell	T Follicular Helper Cell
TGF-β	Transforming Growth Factor β
Th1 Cell	T Helper Type 1 Cell
Th2 Cell	T Helper Type 2 Cell
TLR	Toll Like Receptor
TNFR1	Tumour Necrosis Factor Receptor 1
TNFR1 SC	TNFR1 Signalling Complex
TNFR2	Tumour Necrosis Factor Receptor 2

TRADD	TNF Receptor Associated Death Domain
TRAF	Tumour Necrosis Factor (TNF) Receptor-Associated Factor
TRAIL	Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon-β
Tyk2	Tyrosine Kinase 2
VCD	Virologically Confirmed Dengue
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
WT	Wild Type
XIAP	X-linked Inhibitor of Apoptosis Protein

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Chapter 1 - Literature Review

1.1 Background and epidemiology

Dengue is a mosquito-borne disease of the tropics and sub-tropics. Dengue cases normally present with flu-like symptoms, but these can occasionally progress to a more serious form of disease for reasons that are not well-understood. The causal agent of dengue is Dengue Virus (DENV), which is transmitted by female mosquitoes of the genus Aedes (Rothman, 2011). Due to its potentially severe symptoms and wide geographic spread, dengue was listed by the world health organisation as a top ten threat to global health in 2019 (World Health Organization, 2019).

The annual incidence of dengue has been estimated to be 400 million cases worldwide, with the majority of cases occurring in Southeast Asia and other tropical regions (Bhatt et al., 2013). Most cases of dengue are asymptomatic, with estimates putting the number of clinical cases at between 58-100 million per year, resulting in 10,000 deaths (Bhatt et al., 2013; Stanaway et al., 2016). Between 1990-2013, the incidence of dengue increased more than seven-fold, with most of this increase driven by human population growth and spread (Messina et al., 2019; Stanaway et al., 2016). Currently, 53% of the human population resides in areas suitable for dengue transmission, and this figure is projected to increase to 60% by the year 2080 (Messina et al., 2014).

There are four related DENV serotypes (DENV1-DENV4) that share 60-70% similarity at the amino acid level and all cause similar disease. The four serotypes of DENV emerged relatively recently, with the most recent common ancestor of the four serotypes estimated to be approximately 1000 years ago (Twiddy, Holmes, & Rambaut, 2003). The transmission cycle for DENV was mostly sylvatic over last 1000 years, with hosts of the families *Macaca, Presbyti* and *Erythrocebus*, before the virus became endemic in human populations over the last several few hundred years (Holmes & Twiddy, 2003).

1.2 The DENV lifecycle

DENV is a member of the *Flavivirus* genus of the Flaviviridae family, which includes other mosquito-borne viruses such as yellow fever virus, zika virus and west nile virus

(Campos, Mongkolsapaya, & Screaton, 2018). After a human host is bitten by an infected female *Aedes* mosquito, DENV enters host cells by endocytosis. Subsequently, acidification of the endosome allows the viral envelope to fuse with the endosomal membrane, liberating the RNA genome into the cytoplasm. The DENV genome is comprised of positive sense, single-stranded RNA (ssRNA), and is approximately 10.7kb in length. The DENV genome is divided into 3 structural genes (C, prM and E), and 7 non-structural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (see Figure 1). The genome is translated as a single polypeptide, which is then cleaved by DENV and host proteases to form mature proteins. Viral replication takes place in the ER, resulting in the production of immature virions. These immature virions then mature as they pass through the trans-Golgi network and exit the host cell, and this involves cleavage of the host protein prM by the host enzyme furin (see Figure 1) (Campos et al., 2018).

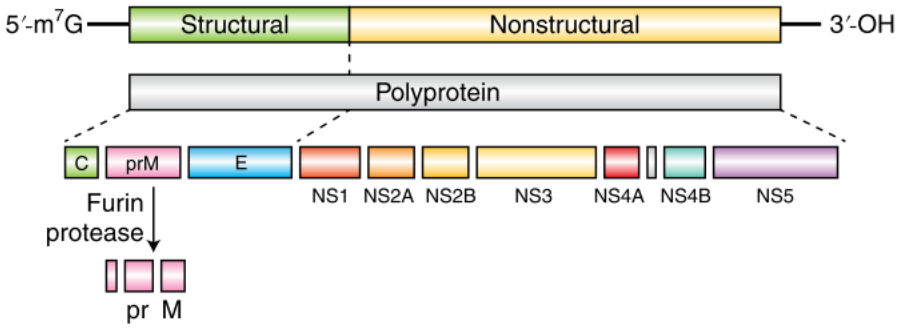


Figure 1.1 Organisation of the DENV RNA genome and polypeptide processing. Reproduced from (Campos et al., 2018).

Studies of intradermally injected mice and human skin explants show that dendritic cells, macrophages and Langerhans cells are the initial cells to be infected by dengue (Cerny et al., 2014; Schmid & Harris, 2014; S. J. Wu et al., 2000). However, after the disease becomes systemic, flow cytometry profiling has shown that monocytes are the main infected cells in peripheral blood (Durbin et al., 2008; Kou et al., 2008).

1.3 Clinical description and classification

The clinical course of dengue is often divided into febrile, defervescent and recovery

phases (World Health Organisation, 2009). During the febrile stage of dengue there is acute onset of a high fever that can be accompanied by flu-like symptoms such as myalgia, rash, nausea, headache and mild mucosal bleeding. This initial stage of the disease generally lasts for 3-5 days and coincides with high levels of viraemia. During defervescence, temperature returns to normal and viraemia levels drop sharply (Duyen et al., 2011; Fox et al., 2011). In a small subset of people the disease can progress at defervescence to severe dengue, characterised by plasma leakage, bleeding, and organ involvement (Simmons, Farrar, Van Vinh Chau, & Wills, 2012). The molecular mechanisms underpinning the progression to severe dengue will be discussed below (section 1.4). During recovery phase, platelet counts and haematocrit return to homeostatic levels (Simmons et al., 2012).

In an effort to improve dengue case management and triage, the World Health Organisation released a classification scheme for dengue severity in 1997 (World Health Organisation, 1997). This divided dengue into dengue fever (DF), and dengue haemorrhagic fever (DHF) of grades I-IV, with grades III and IV also referred to as dengue shock syndrome (DSS) (Table 1.1). In an effort to improve treatment further, the WHO issued an altered classification scheme in 2009, which divided cases into dengue without warning signs, dengue with warning signs, and severe dengue (Table 1.2).

Because of this change, studies have reported their findings using either the 1997 criteria, the 2009 criteria, or both. For simplicity, when referring to the literature during this thesis, I will use the term “dengue fever” to encompass dengue fever (1997 scheme), and dengue with / without warning signs (2009 scheme). I will use the term “severe dengue” to encompass DHF/DSS (1997 scheme) and severe dengue (2009 scheme).

Table 1.1 WHO Dengue Classification Scheme (1997)

Dengue Fever (DF) / Dengue Haemorrhagic Fever (DHF)	Grade	Symptoms	Laboratory Picture
DF		Fever with two or more of the following signs: headache, retro - orbital pain, myalgia, arthralgia	Leukopenia occasionally. Thrombocytopenia may be present. No evidence of plasma loss
DHF	I	Above signs plus positive tourniquet test	Thrombocytopenia < 100,000 Hct# rise > 20%
DHF	II	Above signs plus spontaneous bleeding	Thrombocytopenia < 100,000 Hct# rise > 20%
DHF	III*	Above signs plus circulatory failure (weak pulse, hypotension restlessness)	Thrombocytopenia < 100,000 Hct# rise > 20%
DHF	IV*	Profound shock with undetectable blood pressure and pulse	Thrombocytopenia < 100,000 Hct# rise > 20%

* DHF Grades III-IV are also referred to as Dengue Shock Syndrome (DSS), # haematocrit (hct); adapted from (World Health Organisation, 1997).

Table 1.2 WHO Dengue Classification Scheme (2009)

Dengue Without Warning Signs	Warning Signs	Severe Dengue
Laboratory Diagnosis OR	Abdominal Pain or tenderness	Severe plasma leakage leading to: • Shock (DSS) • Fluid accumulation with respiratory distress
History of residence in / travel to endemic area AND fever AND 2 of the following criteria:	Persistent Vomiting	**Severe bleeding** as evaluated by clinician
Nausea, vomiting	Clinical fluid accumulation	Severe organ involvement • Liver: AST* or ALT# >=1000 • CNS+: Impaired consciousness • Heart and other organs
Rash	Mucosal bleed	
Aches and Pains	Lethargy, restlessness	
Tourniquet test positive	Liver enlargement > 2cm	
Leuokopaenia	Laboratory: increase in haematocrit concurrent with rapid decrease in platelet count	
Any warning Sign		

* aspartate transaminase (AST), # alanine transaminase (ALT), + central nervous system (CNS); adapted from (World Health Organisation, 2009).

1.4 Molecular pathogenesis of severe dengue

1.4.1 Inflammatory mediators, inflammation and vascular leak

In a small proportion of dengue fever patients, symptoms progress at the defervescent stage to severe dengue. Here, I will describe the molecular pathogenesis of severe dengue. Subsequently, I will discuss the causes of severe dengue (section 1.6).

During severe dengue, high concentrations of inflammatory mediators cause transient alterations in endothelial permeability (Simmons et al., 2012). This results in the cardinal signs of severe dengue which are haemorrhage and plasma leakage. Currently, the only clinical tool available to manage these symptoms involves the replacement of fluids to prevent shock and associated sequelae (World Health Organisation, 2009). Failure to properly manage fluids during the defervescent phase of disease can result in lasting organ damage or lethal shock (World Health Organisation, 2009).

The molecular basis of severe dengue is often described as a cytokine storm, similar to the states of inflammation generated by the 1918 influenza or sepsis. The "cytokine storm" moniker implies that all inflammatory mediators associated with severe dengue are host-derived cytokines. While cytokines are very important to the pathogenesis of severe dengue, a number of non-cytokine inflammatory mediators are also associated with severe dengue, including the viral NS1 protein.

A list of inflammatory mediators associated with severe dengue has been collated and is presented as Table 1.3. These mediators meet two criteria. Firstly, the level of these mediators was altered in severe dengue relative to dengue fever in at least one human cohort study. Secondly, studies were selected only if they measured levels of mediators as concentrations of protein, as opposed to measurement of changes at the transcriptional level. Given that the focus of this thesis is on the contribution of signalling pathways to pathogenesis, a description of receptors and signalling adaptors has also been included for each mediator. In addition, it has been noted whether levels of these mediators are increased or decreased in severe dengue, relative to dengue fever. A brief description of the main groups of inflammatory mediators with altered levels in severe dengue is provided below.

Chemotactic cytokines (chemokines) act to mobilise and recruit immune cells and are highly upregulated during severe dengue (Table 1.3). MCP-1 is a powerful regulator of monocyte chemotaxis and adhesion (Deshmane, Kremlev, Amini, & Sawaya, 2009; Gerszten et al., 1999). MIP-1β and RANTES help to regulate the chemotaxis of macrophages and natural killer (NK) cells, and interactions between dendritic cells and T cells (Sokol & Luster, 2015). IL-8 stimulates the recruitment of neutrophils, and IP-10 regulates T cell function and recruits NK cells (Sokol & Luster, 2015). Early production of chemokines is mediated largely by macrophages, mast cells, and dendritic cells, as these cell types express a wide array of pattern recognition receptors (PRRs) and are therefore excellent detectors of pathogen-associated molecular patterns (PAMPs) (Sokol & Luster, 2015). Macrophages, mast cells and dendritic cells can produce chemokines that have been associated with severe dengue; all three types of cells have been reported to produce MCP-1, MIP-1b, RANTES, IL-8 and IP-10 (Griffith, Sokol, & Luster, 2014; Sokol & Luster, 2015). Many of these chemokines can also be produced by cytokine-activated endothelium, although the contribution of these cells is thought to be relatively minor (Griffith et al., 2014).

During the course of severe dengue, immune cells are recruited via high concentrations of chemokines and establish a pro-inflammatory milieu. Immune cells mediate this in part via the production of pro-inflammatory cytokines. Type I interferons (IFN-α/β) are key regulators of the acute anti-viral immune response (Platanias, 2005). Plasmacytoid dendritic cells are the main producers of type I interferon during viral infection (Webster et al., 2018). One of the functions of type I interferon signalling during DENV infection is to drive rapid type II interferon production from NK cells (Webster et al., 2018). Type II interferon (IFN-γ) has an important role in the acute immune response, but is also critical for chronic immune responses, in part through promotion of Th1 differentiation (Paul, 2015). Ex vivo restimulation assays have shown that $CD4^+$ and $CD8^+$ T cells have also been shown to produce IFN-γ (Kurane et al., 1991; D. Weiskopf et al., 2013; Daniela Weiskopf, Bangs, et al., 2015). Collectively, interferons signal via autocrine and paracrine pathways to drive the transcription of hundreds of genes and induce a pro-inflammatory and anti-viral programme (for more information, see section 1.7).

In addition to interferons, IL-1α/β, IL-6, IL-7, GM-CSF, and TNF are all pro-inflammatory

cytokines that are significantly upregulated during severe dengue (Dinarello, 2013; Naugler & Karin, 2008; Pellegrini et al., 2011; Shi et al., 2006). Monocytes, macrophages and other innate immune cells produce all of these cytokines (Auffray, Sieweke, & Geissmann, 2009; Duque & Descoteaux, 2014; Shi et al., 2006) with the exception of IL-7, which is produced by thymic stromal cells and peripheral tissues such as the skin and lymph nodes (Kim, Hong, & Park, 2011). IL-4 controls Th2 differentiation (Paul, 2015), and together with IL-13, is a component of the allergic response (Price et al., 2010). IL-4 and IL-13 are produced by Th2 cells, and other cells involved in allergic responses such as eosinophils and mast cells (Price et al., 2010).

Non-cytokine mediators also promote inflammation during severe dengue. NS1 is a viral glycoprotein that is secreted from DENV-infected cells as a hexamer (Somnuke, Hauhart, Atkinson, Diamond, & Avirutnan, 2011) and exerts a pro-inflammatory effect by stimulating Toll-like receptor (TLR) 4 signalling, which in turn induces the production of TNF and IL-6 (Modhiran et al., 2015). The alternative complement pathway is also activated during severe dengue via the upregulation and downregulation of the regulatory proteins factor D and factor H, and may drive inflammation (Nascimento et al., 2009). In conclusion, the combined effects of cytokines, NS1 and complement activation combine to drive inflammation during severe dengue.

Many inflammatory mediators have also been implicated in the development of vascular leakage during severe dengue. The cytokines TNF and IL-1β have been reported to be upregulated in severe dengue (see Table 1.3) and have been shown to act on endothelial monolayers and increase permeability *in vitro* (London et al., 2010). Recently, it was shown that NS1 treatment could increase the permeability of endothelial cell monolayers and induce vascular leak in mice (Beatty et al., 2015; Modhiran et al., 2015). Furthermore, an elegant follow-up study showed that this hyperpermeability was caused directly by NS1, and not via the actions of pro-inflammatory cytokines expressed upon NS1 treatment (Glasner et al., 2017). Moreover, TNF has been implicated in increasing the permeability of endothelial cells either directly, or indirectly via upregulating the production of reactive oxygen and nitrogen species (Shresta, Sharar, Prigozhin, Beatty, & Harris, 2006; Yen, Chen, Lin, Shieh, & Wu-Hsieh, 2008). Vascular endothelial growth factor (VEGF) is produced by monocytes and macrophages (Auffray

et al., 2009; Duque & Descoteaux, 2014) and is a potent promoter of vascular permeability (Gavard & Gutkind, 2006). The activity of VEGF can be at least partially antagonised soluble decoy receptors such as soluble VEGF receptor 2 (sVEGFR2) (Pavlakovic, Becker, Albuquerque, Wilting, & Ambati, 2010). VEGF is able to alter permeability by altering the stability of intercellular adherens and tight junctions (Gavard & Gutkind, 2006). Srikiatkhachorn and colleagues found that levels of the sVEGFR2 were decreased during severe dengue, and this correlated with increased levels of free (unbound) VEGF, which could contribute to the vascular leak phenotype (Srikiatkhachorn et al., 2007).

Intercellular junctions have also shown to be altered in the context of DENV infection by the mast cell-derived protease tryptase (Rathore et al., 2019). Tryptase is released upon mast cell degranulation and functions together with another mast cell protease, chymase, to disrupt tight junctions. In a mouse model of DENV infection, tryptase inhibition decreased vascular permeability. Moreover, in two human cohorts, tryptase levels directly correlated with the severity of dengue (Rathore et al., 2019). Therefore, similarly to the development of inflammation, many inflammatory mediators drive the development of vascular leak during severe dengue.

Unfortunately, the results of dengue inflammatory mediator studies are heterogeneous, and consistent support for the involvement of specific mediators is often lacking. This is well illustrated by the differences in study findings summarised in Table 1.3 for IFN-α, MIP-1β, NS1 and RANTES. This heterogeneity is driven by a wide range of factors including: methodological factors including study design and classification of disease; viral factors such as serotype and transmission rate; and host factors such as the age and degree of immunity of the study population (Y. H. Lee, Leong, & Wilder-Smith, 2016; Salje et al., 2018; Soo et al., 2017).

Meta-analyses have been conducted in an attempt to identify consistently significant associations of individual inflammatory mediators with severe dengue. Lee and colleagues found that the anti-inflammatory cytokine IL-10 was consistently upregulated in severe dengue relative to dengue fever, with the peak difference occurring around the time of defervescence (Y. H. Lee et al., 2016). They also found that

levels of IFN-γ were also significantly different between severe dengue and dengue fever cases. However, 3 studies found IFN-γ was upregulated, and 3 found it was downregulated so this finding needs to be clarified. Soo and colleagues ran a separate meta-analysis of inflammatory mediators associated with severe dengue (Soo et al., 2017). In a similar finding to Lee et al., Soo and colleagues determined that IL-10 was routinely upregulated during severe dengue infection. In addition to this, they determined that IL-8 and IL-18 were also upregulated during severe dengue. Soo and colleagues also found that levels of RANTES, IL-7, TGF-β and VEGFR2 were decreased during severe dengue (Soo et al., 2017). In summary, although many different inflammatory mediators have been implicated in the pathogenesis of severe dengue (Table 1.3), only a handful of mediators have been associated with disease in a reproducible and statistically significant manner. Studies with consistent sampling and reporting procedures are required to address this shortcoming in the literature (Y. H. Lee et al., 2016; Soo et al., 2017).

Table 1.3. Inflammatory mediator signalling increased / decreased during severe dengue, relative to dengue fever

Mediator	Receptor(s)	JAK(s)	Adaptor(s)	Literature reference: signalling pathway	Literature reference: involvement in severe dengue	Increased / decreased in severe dengue
GM-CSF	GM-CSFR	Jak2, Jak2		(O'Shea et al., 2015; Shi et al., 2006)	(Bozza et al., 2008; Patro et al., 2019)	↑
IFN-α/β	IFNAR	Jak1, Tyk2		(O'Shea et al., 2015)	(De La Cruz Hernández et al., 2014; Libraty, Endy, et al., 2002)	↓; n.s.
IFN-γ	IFNGR	Jak1, Jak2		(O'Shea et al., 2015)	(Bozza et al., 2008; Patro et al., 2019; L. Zhao et al., 2016)	↑
IL-7	IL-7R, γ_c	Jak1, Jak3		(O'Shea et al., 2015)	(Bozza et al., 2008)	↑
IL-10	IL-10R	Jak1, Tyk2		(Moore, de Waal Malefyt, Coffman, & O'Garra, 2001)	(Patro et al., 2019; L. Zhao et al., 2016)	↑
IL-1α	IL-1R1		MyD88	(Dinarello, 2013)	(L. Zhao et al., 2016)	↑
IL-1β	IL-1R1		MyD88	(Dinarello, 2013)	(Bozza et al., 2008)	↑
IL-4	IL-4R, γ_c	Jak1, Jak3		(O'Shea et al., 2015)	(Bozza et al., 2008)	↑
IL-6	IL-6R, gp130	Jak1, Jak2, Tyk2		(Naugler & Karin, 2008)	(Bozza et al., 2008)	↑
IL-8 (CXCR8)	CXCR1, CXCR2			(Griffith et al., 2014)	(L. Zhao et al., 2016)	↑

Mediator	Receptor(s)	JAK(s)	Adaptor(s)	Literature reference: signalling pathway	Literature reference: involvement in severe dengue	Increased / decreased in severe dengue
IL-13	IL-4Ra, IL-13Ra1	Jak1, Jak2, Tyk2		(O'Shea et al., 2015; Paul, 2015)	(Bozza et al., 2008)	↑
IP-10 (CXCL10)	CXCR3			(Griffith et al., 2014)	(L. Zhao et al., 2016)	↑
MCP-1 (CCL2)	CCR2			(Griffith et al., 2014)	(L. Zhao et al., 2016)	↑
MIP-1β (CCL4)	CCR5			(Griffith et al., 2014)	(Bozza et al., 2008; Patro et al., 2019)	↓↑
NS1	TLR4		TRIF, MyD88	(Kawasaki & Kawai, 2014)	(Libraty, Young, et al., 2002; Perdomo-Celis, Salgado, & Narváez, 2017)	↑; n.s.
RANTES (CCL5)	CCR1, CCR3, CCR5			(Griffith et al., 2014)	(Patro et al., 2019; L. Zhao et al., 2016)	↑↓
TNF	TNFR		TRAF2, TRAF5	(Tada et al., 2001)	(L. Zhao et al., 2016)	↑
Tryptase	NA				(Rathore et al., 2019)	↑
Complement Factor D	NA				(Nascimento et al., 2009)	↑
Complement Factor H	NA				(Nascimento et al., 2009)	↓
VEGFR2	NA		Vav2 / Src	(Gavard & Gutkind, 2006)	(Srikiatkhachorn et al., 2007)	↓

*n.s. not statistically significant

1.4.2 The role of cell death in severe dengue

Apoptosis is a form of programmed cell death which is thought to have evolved as a form of defence against intracellular pathogens (Ke et al., 2018; Strasser & Vaux, 2018). Two convergent signalling pathways are capable of inducing apoptosis (Shalini, Dorstyn, Dawar, & Kumar, 2015). The intrinsic pathway is regulated by the BCL-2 family of proteins that sense and respond to cellular stress (Shalini et al., 2015). The extrinsic pathway is triggered when death ligands such as TNF, Fas ligand (FasL) and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) ligate their cognate receptors. Activation of either the intrinsic or extrinsic pathway results in the activation of initiator caspases, such as caspase 8, that can then activate effector caspases resulting in the cleavage of hundreds of substrates and cell death. These pathways are strictly controlled at multiple levels to prevent excessive activation and uncontrolled cell death (Shalini et al., 2015). Apoptosis involves the packaging of intracellular components into membrane-bound vesicles that are then digested by professional phagocytes (Jacobson, Weil, & Raff, 1997). Therefore, apoptosis does not result in the uncontrolled release of cellular components and is generally a non-inflammatory process (Jacobson et al., 1997).

The controlled and regulated nature of apoptosis contrasts with the process of necrosis. Necrosis is an uncontrolled form of cell death which results in the uncontrolled and inflammatory release of intracellular components (Jacobson et al., 1997).

Pyroptosis is a form of controlled but inflammatory cell death that has a role in the clearance of intracellular pathogens (Doerflinger et al., 2020; Shalini et al., 2015). During pyroptosis, chromatin does not condense but remains diffuse; caspase-3 is not activated and membrane integrity is lost (Brennan & Cookson, 2000).

Several studies have shown that apoptosis is active during severe dengue. PBMCs have been shown to undergo increased rates of apoptosis during severe dengue, relative to patients with dengue fever (Jaiyen, Masrinoul, Kalayanarooj, Pulmanausahakul, & Ubol, 2009; Myint et al., 2006), or relative to healthy controls (Torrentes-Carvalho et al., 2014) and this has been associated with increased expression of Fas and TNFR1 death receptors and their cognate ligands (Jaiyen et al., 2009; Torrentes-Carvalho et al., 2014). Several studies have found that levels of the death ligands TRAIL and FasL are increased

in dengue fever relative to severe dengue, suggesting that activation of the extrinsic pathway is involved in restricting the severity of symptoms (Gandini et al., 2013; Limonta et al., 2014). However, other groups have found that levels of soluble Fas were higher in subjects with severe dengue than dengue fever (Myint et al., 2006).

In vitro studies have shown that primary human monocytes undergo apoptosis when infected with DENV2 (Espina, Valero, Hernández, & Mosquera, 2003; Torrentes-Carvalho et al., 2009). However, it is difficult to quantify the role that monocyte apoptosis plays in severe dengue, as this process has not been studied *in vivo* or *ex vivo*.

Several groups have reported that endothelial cells can be infected by DENV *in vitro* or in mouse models, and cause apoptosis (Avirutnan, Malasit, Seliger, Bhakdi, & Husmann, 1998; H.-C. Chen, Hofman, Kung, Lin, & Wu-Hsieh, 2007). However, autopsy studies of human dengue patients show only limited involvement of endothelial cells in disease. While two groups reported limited detection of DENV antigen in endothelial cells, these authors concluded that this finding probably had minimal relevance to severe dengue pathogenesis (Balsitis et al., 2009; Jessie, Fong, Devi, Lam, & Wong, 2004) and other autopsy examinations have found no evidence of endothelial infection (Aye et al., 2014; Couvelard et al., 1999).

Interestingly, several autopsy studies have reported the presence of liver necrosis in subjects that succumbed to severe dengue (Aye et al., 2014; Bhamarapravati, Tuchinda, & Boonyapaknavik, 1967; Couvelard et al., 1999). Two large studies, of 100 cases and 13 cases respectively, found evidence of moderate to severe necrosis in the majority of livers they examined (Aye et al., 2014; Bhamarapravati et al., 1967). Aye and colleagues concluded that the degree of liver damage correlated poorly with levels viral antigen and viral RNA, and therefore hypoxia or inflammatory mediators might account for the damage observed as opposed to the cytopathic effects of DENV infection (Aye et al., 2014).

A relatively small body of *in vitro* has implicated pyroptosis in the pathogenesis of dengue (Cheung, Sze, Chan, & Leung, 2018; Tan & Chu, 2013; M. Wu et al., 2013). Rather than studying the direct impact of DENV infection on the initiation of pyroptosis, these

investigators have mainly relied on indirect measurements of pyroptosis such as the effect of caspase-1 inhibitors on lactate dehydrogenase (LDH) cell death assays and IL-1β production, which generally requires caspase-1. The exception to this is the paper by Tan et al. who examined late-stage (>24hpi) cell death of DENV-infected primary macrophages using electron microscopy, and found morphology that was supportive of pyroptosis-mediated cell death. In future, i*n vivo* infections of mice deficient in caspases or downstream pyroptosis effectors such as gasdermin proteins would help to clarify the role of pyroptosis in dengue pathogenesis.

In conclusion, there is some evidence suggesting that severe dengue may be associated with PBMC apoptosis, and there is some evidence that this is mediated by the extrinsic pathway of apoptosis. There is no evidence to support causation and it is counterintuitive to suggest that apoptosis contributes to disease. Indeed, apoptosis evolved as a mechanism to eliminate intracellular pathogens (Ke et al., 2018; Strasser & Vaux, 2018). On the other hand, necrotic liver damage occurs relatively frequently, at least in fatal cases, and this damage appears to occur as a result of circulatory failure or excessive production of inflammatory mediators – i.e. a consequence of severe dengue infection but not the cause. The role of pyroptosis in the pathogenesis of dengue remains to be properly defined.

1.5 Primary and secondary DENV infections, ADE and risk of severe dengue

Severe dengue can occur during primary or secondary DENV infection, but the risk of severe dengue is much higher during secondary infection (Anantapreecha et al., 2005; Kenji, Keisuke, Taro, & Hiroshi, 2014). This observation can be explained by a phenomenon referred to as antibody-dependent enhancement (ADE). Primary DENV infection induces the production of antibodies that are able to neutralise DENV and prevent reinfection. However, in the absence of reinfection, levels of these neutralising antibodies steadily decline over time (Guzman et al., 2007; Sabin, 1952). Neutralising antibodies can be further sub-classified into serotype-specific and cross-neutralising antibody responses; however, discussion of this area of dengue biology is beyond the scope of this review, but this subject has recently been well-discussed in a l reviewed by

St. John and Rahore (St. John & Rathore, 2019). Human cohort studies have shown that low titres of neutralising antibodies correspond to a high risk of severe dengue, and this risk is much greater than the risk of severe dengue in the complete absence of neutralising antibodies (Katzelnick, 2017; Salje et al., 2018). .

In vitro and *in vivo* studies have shed light on the precise mechanism that underpins ADE. Fc receptor for IgG (FcγR) is expressed on myeloid cells such as monocytes and macrophages (Guilliams, Bruhns, Saeys, Hammad, & Lambrecht, 2014), which are DENV host cells (Cerny et al., 2014; Durbin et al., 2008). FcγR-expressing cells endocytose antibody-DENV complexes with high efficiency (Kou et al., 2008; Littaua et al., 1990). Consequently, DENV infection of FcγR-expressing cells in the presence of sub-neutralising concentrations of anti-DENV antibody results in high levels of cell infection (Kou et al., 2008; Littaua et al., 1990). In addition, infection of DENV mouse models with sub-neutralising concentrations of anti-DENV antibody has been shown to increase rates of infection of myeloid cells and increase disease severity (R M Zellweger, Prestwood, & Shresta, 2010). Therefore, it is believed that a high level of myeloid cell infection underpins the increased risk of severe dengue in individuals with low levels of neutralising antibodies, such as during secondary dengue infection. Furthermore, FcγRs directly modulate innate immune signalling directly during ADE, and this phenomenon has recently been reviewed by Sprokholt and colleagues (Sprokholt, Helgers, & Geijtenbeek, 2018).

In areas where DENV is endemic, titres of cross-neutralising antibodies increase following repeated infection, and the risk of severe dengue is decreased as titres reach levels capable of efficient neutralisation (Katzelnick, 2017; Salje et al., 2018). Therefore, the risk of severe dengue is believed to be lower in tertiary and quaternary infection relative to secondary infection, as high titres of cross-neutralising antibodies eliminate the prospect of ADE, and mitigate levels of myeloid cell infection via neutralisation (Halstead, 2019; Katzelnick, 2017; Salje et al., 2018) . However, it should be noted that although high titres of neutralising antibodies have been definitively correlated with a decreased risk of severe dengue, these results do not rule out the possibility that humoral and cellular immune responses could cooperate to control DENV infection after repeat dengue exposure.

1.6 What causes severe dengue?

A causative agent or mechanism for severe dengue must fulfil several criteria. Firstly, the agent or mechanism must be able to directly or indirectly cause vascular permeability, as this results in the symptoms of severe dengue. Secondly, levels of a causal agent should correlate with disease severity and should be high at the onset of severe dengue, which normal occurs at days 4-6 of dengue illness. Thirdly, a causal agent or mechanism should be able to cause severe dengue during both primary and secondary DENV infection.

1.6.1 Do viral factors cause severe dengue?

Recently, it has been argued that DENV NS1 could be the causative agent for severe dengue (Halstead, 2019). As described above (section 1.4), NS1 is able to induce the production of TNF and IL-6 through ligation of TLR4 and is also able to act independently to cause vascular leak (Beatty et al., 2015; Glasner et al., 2017; Modhiran et al., 2015). Moreover, human studies have shown that higher NS1 levels were correlated with extended coagulation time (Perdomo-Celis et al., 2017), and a lower platelet nadir (Duyen et al., 2011). Therefore, NS1 fulfils the first criteria laid out above: it can cause increased vascular permeability.

It would be expected that levels of a causative agent would generally increase during secondary infection, as the rate of severe dengue is higher during secondary infection than primary infection (Katzelnick, 2017; Kenji et al., 2014). However, several groups have shown that levels of NS1 usually decline more rapidly during secondary dengue infection than primary infection (Duyen et al., 2011; Perdomo-Celis et al., 2017). Furthermore, while one study found that NS1 levels were correlated with disease severity (Libraty, Young, et al., 2002), other studies have not been able to replicate this association (Fox et al., 2011; Perdomo-Celis et al., 2017). In conclusion, NS1 has clearly been shown to stimulate inflammation and vascular leak. However, cohort studies do not support the argument that NS1 is the causative agent of severe dengue. While NS1 does not fulfil the criteria for causation, I would argue that NS1 is a potent soluble mediator that contributes to the cytokine storm that is triggered by a dysregulated host immune response, and results in severe dengue.

It has also been hypothesised that high levels of viraemia could cause or contribute to severe dengue. Several studies have observed higher viraemia in the plasma of patients with severe dengue than in dengue fever (Libraty, Endy, et al., 2002; Vaughn et al., 2000). However, other groups have reported that there is no difference in the levels of viraemia detected in the plasma of patients with dengue fever or severe dengue (Perdomo-Celis et al., 2017; Talarico et al., 2017). Furthermore, symptoms of dengue are most severe at approximately 4-6 days after fever onset, and this correlates with the time of defervescence (Halstead, 2019), when the plasma viral load is dropping or absent (Duyen et al., 2011; Fox et al., 2011). The lack of a clear correlation between viral load and severity, and the low or absent viral load during severe dengue suggest that hyper-viraemia does not cause severe dengue. n

1.6.2 Do host factors cause severe dengue?

As outlined above (section 1.5), ADE can neatly explain how the risk of DENV is elevated in secondary infections above the level of risk in primary, tertiary and quaternary infections. Theoretically, ADE could cause severe dengue by increasing the size of the infected cell mass, resulting in increased pro-inflammatory and permeability-inducing cytokine production (Halstead, 2019). However, it is still possible for naïve adults to develop severe dengue during primary infection, in the absence of ADE (Anantapreecha et al., 2005). Therefore, ADE can be ruled out as a cause of severe dengue.

The phenomenon of original antigenic sin has also been put forward as a cause of severe dengue. Original antigenic sin occurs after the immune system has developed a memory response after encountering a novel antigen, and experiences a secondary exposure to a similar antigen (Rothman, 2011). What can occur in this scenario is that the secondary adaptive immune response is directed mainly against the primary antigenic target, leading to the proliferation of B and T cell clones that are only weakly specific for the secondary antigen. In the case of dengue, this is thought to occur during secondary dengue during infection with a heterologous serotype. Specifically, original antigenic sin is hypothesised to cause severe dengue as pro-inflammatory B and T cell responses of low avidity are directed against the initially encountered serotype, and are only partially capable of neutralising the serotype responsible for secondary infection

(Rothman, 2011).

In support of the original antigenic sin hypothesis, several groups studying secondary DENV infections have reported that antibody titres are higher against non-infecting serotypes (Midgley et al., 2011; Salje et al., 2018). In addition to this, one group used tetramer staining to observe that $CD8^+$ T cell responses during acute dengue infection were directed against non-infecting serotypes (Mongkolsapaya et al., 2003). However, another group observed wide-spread crossreactivity in $CD8^+$ T cell responses during primary and secondary infection, with no evidence of original antigenic sin (Friberg et al., 2011). Finally, Weiskopf and colleagues used an *ex vivo* IFN-γ ELISPOT assay to examine the $CD8^+$ T cell response of a Sri Lankan cohort who reside in a hyper-endemic region. They found evidence for original antigenic sin, but found that this did not affect the multifunctionality or avidity of CD8 T cells during secondary infection (D. Weiskopf et al., 2013). Therefore, there is some evidence that original antigenic sin, involving the skewing of the adaptive response to target the initially encountered DENV serotype, occurs during secondary DENV infection. However, the ability of original sin to contribute to the severity clinical dengue is not proven, and this phenomenon cannot account for cases of severe dengue during primary infection.

As there is no convincing evidence that viral antigenaemia or viraemia causes severe dengue, it can be concluded that the hyper-production of inflammatory mediators during severe dengue is mediated by a host mechanism. However, current host mechanisms that have been shown to occur during severe dengue such as ADE and original antigenic sin are not capable of explaining the occurrence of severe dengue during primary infection, and have not been shown to cause severe dengue even during secondary infection. Therefore, new host mechanisms of immune activation that are capable of causing severe dengue must be discovered and characterised.

Dysregulation of host signalling has been shown to be key to the pathogenesis of other human diseases that are characterised by hyperimmunity and cytokine storms. The cytokine storm phenotype in influenza has been shown to be positively modulated by TLR4 and sphingosine-1-phosphate signalling (Imai et al., 2008; Walsh et al., 2011), and negatively modulated by the suppressor of cytokine signalling (SOCS) 4, which is thought

to act by repressing epidermal growth factor signalling (Kedzierski et al., 2014). Similarly, TLR4 signalling in a human LPS-challenge model of sepsis results in a cytokine storm (Rossignol & Lynn, 2002). Furthermore, activation of the Robo4 signalling pathway during influenza and sepsis can prevent the degradation of adherens junctions, and therefore prevent cytokine-mediated vascular leakage (London et al., 2010).

Importantly, characterisation of these host pathways has allowed the development of therapeutic strategies that can control cytokine storms and reduce symptoms of influenza and sepsis in animal models. For instance, antagonism of TLR4 signalling can mitigate symptoms of influenza disease in mice (Shirey et al., 2013), and of sepsis in humans (Rossignol & Lynn, 2002). Moreover, promotion of Robo4 signalling was able to strengthen endothelial barrier function in animal models of both influenza and sepsis (London et al., 2010).

Given the established nature of host-driven cytokine storms to drive disease, and the lack of satisfactory evidence suggesting that viral factors cause severe dengue, I argue that severe dengue is a disease characterised by a dysregulation of host immune signalling. In support of this, transcriptomic studies have routinely found an upregulation of innate immune signalling and type I interferon signalling in severe dengue cases (Banerjee et al., 2017; P. Sun et al., 2013). Numerous checks and balances normally regulate immune signalling, and prevent overproduction of inflammatory mediators that could result in a poor outcome for the host. I hypothesise that dysregulated signalling in severe dengue could be caused by the loss of function of specific host factors that regulate host immune signalling.

1.7 Key signalling pathways activated during severe dengue

1.7.1 PRRs sense infection and promote antiviral signalling

During DENV infection, ssRNA is activates PRRs, which go on to activate pro-inflammatory signalling via associated adaptor proteins. There are three main families of PRRs that are important for detecting DENV PAMPs. Firstly, the cytoplasmic RNA-helicases RIG-I and MDA5, also known as RIG-I like receptors (RLRs), detect the 5' end of the DENV ssRNA and recruit the adaptor MAVS (Chazal et al., 2018). Secondly,

endosomal Toll-like receptors (TLRs) are also important sensors of viral RNA. TLR3 is thought to detect the double-stranded RNA intermediates produced during viral replication and signals through TRIF (Nasirudeen et al., 2011) and TLR7 recognises ssRNA and signals via MyD88 (P. Sun et al., 2009). Finally, recent studies have shown that DENV infection induces the release of mitochondrial DNA, and that this induces antiviral immunity via the PRR cGAS and the adaptor STING (Aguirre et al., 2017; B. Sun et al., 2017). The result of PRR signalling is the activation of type I interferon and NF-κB signalling; these events will be outlined below.

1.7.2 Type I interferon signalling acts to promote inflammation and control viral infection

Host cell interferon pathways, particularly type I interferon signalling, act in *cis* and *trans* to abrogate viral replication in infected cells and reduce spread of virus to uninfected cells through direct and indirect mechanisms (Platanias, 2005). Interferon regulatory factors (IRFs) are the key mediators causing transcriptional activation of numerous antiviral interferon regulated genes (ISGs) (G. N. Zhao, Jiang, & Li, 2015). IRF-3 is constitutively expressed in most cell types, whereas IRF-7 is expressed highly by plasmacytoid dendritic cells and macrophages, but at a low level by most other cell types (Marie, Durbin, & Levy, 1998; Sato et al., 1998; G. N. Zhao et al., 2015).

Upon PRR activation, IRF-3 is phosphorylated, leading to homodimerisation (Honda, Takaoka, & Taniguchi, 2006). IRF-7 is also phosphorylated and homodimerises or heterodimerises with IRF3. The dimers of phosphorylated IRF-3 and IRF-7 then relocate to the nucleus where they stimulate relatively low-level transcription of a select group of ISGs including cytokines and the interferons IFN-α4 and IFN-β. Binding of these type I IFNs to the Interferon-α/β Receptor (IFNAR) receptor results in the phosphorylation of the receptor associated Janus Kinases (JAKs) JAK1 and Tyk2. These JAKs phosphorylate the Signal Transducer and Activator of Transcription (STAT) proteins STAT1 and STAT2. The phosphorylated STATs then form a complex with IRF-9 which is referred to as the interferon stimulated gene 3 (ISGF3) complex. The active ISGF3 complex translocates to the nucleus, and induces the transcription of a subset of ISGs (Honda et al., 2006). One of the ISGs strongly upregulated by ISGF3 is IRF-7 (Marie et al., 1998; Sato et al., 1998). Higher levels of IRF-7 strongly increase the transcription of IFN-β and multiple subtypes

of IFN-α, thereby amplifying the initial type I IFN signalling in a positive feedback loop.

In summary, in response to type I interferon signalling, the transcription of hundreds of ISGs creates an antiviral state.

1.7.3 NF-κB signalling results in pleiotropic responses

The NF-κB pathway is a major regulator of cellular activation and determines whether a cell dies or survives in response to numerous external and internal cellular stresses. Viruses such as Human Immunodeficiency Virus (HIV) and Human T-Cell Leukaemia Virus Type 1 (HTLV-1) critically require host cell NF-κB activity for a robust transcriptional permissive state to support viral replication (Bachelerie, Alcami, Arenzana-Seisdedos, & Virelizier, 1991; Z. L. Chu, Shin, Yang, DiDonato, & Ballard, 1999) . Additionally, NF-κB promotes inflammatory cytokine responses and in some situations, counterintuitively, this inflammation can promote infectious disease pathogenesis.

There are five different NF-κB subunits, and these can form a variety of homo- and hetero-dimers. In unstimulated cells, NF-κB subunits are sequestered in the cytoplasm by ankyrin repeat-containing proteins such as IκBα and IκBβ (Tran, Merika, & Thanos, 1997). During canonical NF-κB signalling, the removal of IκB inhibition allows the translocation of NF-κB dimers to the nucleus (T. Liu, Zhang, Joo, & Sun, 2017). The most important NF-κB dimers for canonical NF-κB signalling are the p50-p65 and p50-c-Rel heterodimers (T. Liu et al., 2017).

1.8 Known DENV host restriction factors

During infection, viruses co-opt various components of the host cell machinery to promote their replication and spread. These pro-viral host components are referred to as host dependency factors, and infection of cells lacking host dependency factors results in decreased viral production. Conversely, numerous host factors impede viral replication via diverse mechanisms. These antiviral host components are referred to as host restriction factors, and infection of cells lacking host restriction factors results in increased viral production. Numerous DENV host restriction factors have been identified, and these are summarised in Table 1.4.

Clearly, the type I interferon response is of the utmost importance to the control of DENV infection. Accordingly, many of the components of this pathway such as the IFNAR receptor (Shresta et al., 2004), STAT1 and STAT2 (Stuart T. Perry, Buck, Lada, Schindler, & Shresta, 2011), IRF-3, IRF-7, as well as IRF-1 and IRF-5 (Carlin et al., 2017; H.-W. Chen et al., 2013) are known host restriction factors. Underlining the importance of this pathway, many factors that are able to influence type I interferon signalling such as haeme oxygenase-1 (C. K. Tseng et al., 2016), DDX3X (G. Li, Feng, Pan, Shi, & Dai, 2015), and MIR-30E* (Zhu et al., 2014) have also been identified as host restriction factors.

Replication of the DENV ssRNA genome is clearly essential for viral replication, and therefore it is not surprising that a second broad group of host restriction factors are involved in this process. These factors include MIR-133 (Castillo et al., 2016), TRIM56 (B. Liu et al., 2014), RSAD2 (Helbig et al., 2013; Jiang et al., 2010) and IRAV (Balinsky et al., 2017).

The manner in which many host restriction factors control DENV infection is still unknown (Table 1.4), and this highlights the need for more study in this area. Moreover, none of the host restriction factors identified to date can explain the cytokine storm phenotype that underpins the symptoms of severe dengue, and this is an area that will be addressed by this thesis.

Table 1.4 Known DENV host restriction factors

Gene Name	Host Restriction Factor	Mechanism	ISG*	Reference
DDX3X	DDX3X	Positive regulator of Type I interferon Signalling	Yes	(G. Li et al., 2015)
FAM134B	FAM134B	Selective autophagy of endoplasmic reticulum	Yes	(Lennemann & Coyne, 2017)
HMOX1	HO-1	Positive regulator of Type I interferon	Yes	(C. K. Tseng et al., 2016)
MIRLET7C	Let-7c	Positive regulator of HO-1	No	(Escalera-Cueto et al., 2015)
IFITM2	IFITM2 / IFITM3	Disruption of viral entry / uncoating	Yes	(Jiang et al., 2010)
IFNAR1	IFNAR	Type I interferon Signalling	Yes	(Shresta et al., 2004)
IFNGR1	IFNGR	Type II interferon Signalling	No	(Shresta et al., 2004)
FLJ11286	IRAV	Processing of Viral RNA	Yes	(Balinsky et al., 2017)
IRF1 / *IRF3* / *IRF5* /*IRF7*	IRF proteins (-1, -3, -5, -7)	Transcription of ISGs	Yes	(Carlin et al., 2017; H.-W. Chen et al., 2013)
ISG20	ISG20	Viral protein / RNA synthesis	Yes	(Jiang et al., 2010)
MAVS	MAVS	Type I interferon Signalling	No	(S. T. Perry, Prestwood, Lada, Benedict, & Shresta, 2009)
MCM3	MCM3	Unknown	No	(Hafirassou et al., 2017)

Gene Name	Host Restriction Factor	Mechanism	ISG*	Reference
MIR133A1-3	MIR-133	Indirect regulation of RNA replication	No	(Castillo et al., 2016)
MIR223	Mir-223	Unknown	No	(N. Wu et al., 2014)
MIR30E	MIR-30e*	Positive regulator of Type I interferon	No	(Zhu et al., 2014)
MIR3614	miR-3614-5p	Unknown	Y	(Diosa-Toro et al., 2017)
NOMO1	NOMO1	Unknown	No	(Hafirassou et al., 2017)
PHB2	PHB2	Unknown	No	(Hafirassou et al., 2017)
PML	PML	Unknown	Yes	(Giovannoni, Damonte, & García, 2015)
STAT1 / *STAT2*	STAT1 / STAT2	Interferon Signalling	Yes	(Stuart T. Perry et al., 2011)
TNFSF10	TRAIL	Activation of extrinsic apoptosis	Yes	(Warke, R., Xhaja, K., Martin, K., Fournier, M., Shaw, S., Brizuela, N., Bosch, N., Lepointe, D., Ennis, F., Rothman, A & Bosch, 2003)
TRIM56	TRIM56	Impairment of viral RNA replication	Yes	(B. Liu et al., 2014)
RSAD2	RSAD2	Impairment of viral RNA replication	Yes	(Helbig et al., 2013; Jiang et al., 2010)
YBX1	YB-1	Repression of translation	No	(Paranjape & Harris, 2007)

* Note: the determination of whether or not individual host restriction factors are / are not ISGs was performed by consulting the literature cited in this table, or by consulting the Interferome database (accessed March 2020) (Rusinova et al., 2013).

1.9 Animal models of dengue

One of the main challenges facing the dengue research community is that there is no widely used, immunocompetent animal model of dengue disease. Here, I will describe three main types of animal model that have been used for DENV pathogenesis research. Nonhuman primates (NHPs) can develop clinical signs of haemorrhage and thrombocytopaenia when infected with high doses of DENV2 (Kwissa et al., 2014; Onlamoon et al., 2010). NHP models have been used to investigate immune responses to TLR agonists and tetravalent DENV vaccines (Sariol et al., 2007; Strouts et al., 2016).

Secondly, immunocompetent mouse models exert strong control of DENV infection. These models can develop clinical signs of dengue that are relevant to human disease such as thrombocytopaenia and haemorrhage (H.-C. Chen et al., 2007; Huang et al., 2000). However, an unnaturally large infectious dose of DENV is required to produce such signs, and some manifestations of these high-dose models, such as paraplegia, are not associated with clinical dengue (H.-C. Chen et al., 2007; Huang et al., 2000). This limits the applicability of these models to human disease.

Finally, mice with genetic knockouts of the interferon system are the most widely used animal models for dengue research. Mouse knockout models have shown that IFN-α and IFN-β play a key role in the early control of DENV infection, whereas IFN-γ works in concert with IFN-α/β to control infection at later time points (Shresta et al., 2004). However, the severe and global immunodeficiency of these models, particularly in regard to interferon signalling, limits the relevance of these models. In addition to a key role in the early antiviral response, type I interferon signalling contributes to a variety of other functions such as B-cell activation (K. Fink et al., 2006), inflammatory cell trafficking (Salazar-mather, Lewis, & Biron, 2002) and regulation of mucosal T cell responses (Giles et al., 2016).

1.10 Vaccines for the prophylactic prevention of dengue

The use of vaccines holds promise for controlling the spread of dengue infection at a population level. However, given that ADE enhances the risk of severe dengue during secondary infection (Katzelnick, 2017), vaccines must stimulate neutralising immunity

against all four serotypes simultaneously to avoid putting vaccinees at increased risk. Such vaccines are referred to as tetravalent dengue vaccines (TDVs). The only vaccine that is currently licensed for the prevention of dengue is chimeric yellow fever virus–DENV (CYD)-TDV (commercial name Dengvaxia).

Phase III clinical trials measured the ability of CYD-TDV to prevent virologically confirmed dengue (VCD) in Asian (Capeding et al., 2014) and Latin American (Villar et al., 2015) paediatric cohorts. These studies determined the efficacy, or ability of CYD-TDV to reduce cases of symptomatic VCD, to be 56.5-64.7% (Capeding et al., 2014; Villar et al., 2015). Vaccine efficacy was routinely higher in children that were seropositive at baseline (Capeding et al., 2014; Villar et al., 2015), indicating a protective effect of repeated exposure to DENV antigen. However a follow-up analysis found that CYD-TDV vaccination increased the risk of seronegative individuals (individuals with no molecular evidence of previous dengue infection) developing symptomatic VCD (Sridhar et al., 2018), especially in children under the age of 9. Due to these serious concerns, the WHO recommended in September 2018 that CYD-TDV should only be administered to individuals with evidence of previous dengue infection (World Health Organization, 2018). Other vaccine candidates are currently undergoing clinical trials (Biswal et al., 2019; Magnani et al., 2017).

1.11 Drugs that have been trialled for the treatment of dengue

At the time of writing, no drugs exist to prevent or treat dengue (Low, Gatsinger, Vasudevan, & Sampath, 2018). To date, clinical trials have been conducted to assess the therapeutic ability of seven drugs to treat symptoms of dengue. Interestingly, all of these drugs already had other applications and were repurposed to treat symptoms of dengue. The seven drugs trialled had a variety of mechanisms of action. Initially, steroid treatment was used in an attempt to manage the symptoms of dengue. Steroids are potent anti-inflammatory drugs that mainly exert their effects by targeting host cell signalling (Barnes, 2006), although some research has also shown that these drugs can stabilise the endothelial glycocalyx (Chappell et al., 2010, 2007). An early trial assessed the ability of treatment with hydrocortisone to improve the outcomes of patients diagnosed with dengue shock syndrome, but found no evidence of efficacy (Sumarmo,

Talogo, Asrin, Isnuhandojo, & Sahudi, 1982). In a second clinical trial, in which prednisolone treatment was given prior to the development of shock, there was no reduction in complications associated with DENV infection (Tam et al., 2012). Lovastatin has been reported to alter viral assembly, perhaps by reducing cholesterol synthesis or altering glycosylation (Rothwell et al., 2009). Interestingly, statin treatment has also been found to alter host signalling by reducing levels of pro-inflammatory cytokines (Tousoulis et al., 2014). However, while effective in a pre-clinical mouse model (Martinez-Gutierrez, Correa-Londoño, Castellanos, Gallego-Gómez, & Osorio, 2014), no beneficial effect was observed in a clinical trial of lovastatin, although the trial was not powered to assess efficacy (Whitehorn et al., 2016).

In contrast to steroid and statin treatments that can exert their effect via alteration of host signalling, most drugs trialled for the treatment of dengue have mechanisms that manipulate the host cell in a more direct fashion to yield their antiviral effect. Chloroquine, as a weak base, has been reported to change endosomal pH and alter the processing of flavivirus prM protein by the host enzyme furin, and to alter post-translational glycosylation of proteins including putative viral entry receptors (Randolph, Winkler, & Stollar, 1990; Vincent et al., 2005). In addition, chloroquine treatment reduced levels of viraemia and pro-inflammatory cytokines in a non-human primate DENV2 infection model (Farias, Machado, Muniz, Imbeloni, & Da Fonseca, 2015), although chloroquine treatment did not reduce viraemia or NS1 antigenaemia in a clinical trial (Tricou et al., 2010). In a similar fashion, trials of the nucleoside analogue balapiravir failed to show efficacy in a clinical trial (N. M. Nguyen et al., 2013). Celgosivir, an α-glucosidase inhibitor, was shown to interfere with the post-translational modification of DENV proteins, resulting in misfolding of viral proteins and improper trafficking of viral NS1 (Rathore et al., 2011). Furthermore, these authors were able to show celgosivir treatment dramatically reduced mortality in a mouse model of DENV2 infection (Rathore et al., 2011). Although celgosivir treatment of patients in a phase 1b trial did not reduce fever burden or viraemia (Low et al., 2014). Unfortunately, a planned trial with a different celgosivir dosing regimen was recently withdrawn due to a lack of funding (Trial Identifier: NCT02569827). Results of a trial of ribavirin, a nucleoside-depleting agent used to treat hepatitis C virus (HCV), have not yet been

reported (reviewed in (Low et al., 2018)). A recent clinical trial (2019) assessed the pharmacokinetics and pharmacokinetics of the nuclear import inhibitor ivermectin in paediatric dengue ((Trial Identifier: NCT03432442). Previously, clinical trials in 2014-2017 assessed the efficacy of ivermectin (Trial Identifier: NCT02045069) and the mast-cell stabilising agent ketotifen (Trial Identifier: NCT02673840); to the extent of my knowledge the results of these recent clinical trials are yet to be published.

In addition to repurposing existing drugs, the dengue research field has used unbiased, high-throughput screening in an attempt to identify novel antiviral compounds (De Wispelaere et al., 2016; van Cleef et al., 2013; Yang et al., 2019). However, the compounds identified in these screens are yet to progress to clinical trials.

1.12 Aims of this PhD thesis

It is apparent that viraemia or NS1 antigenaemia are not the sole contributors to severe dengue. Given that dysregulated host immune signalling causes the cytokine storms characteristic of severe influenza and sepsis, I propose that an identical mechanism underpins the cytokine storms associated with severe dengue. Furthermore, while many DENV host restriction factors have been identified, the current state of knowledge is unable to explain how changes in host immune signalling result in severe dengue. Therefore, to expand our knowledge of dengue pathogenesis, there is a need to characterise novel host restriction factors in the context of DENV infection.

Furthermore, therapies targeting host immune signalling have shown efficacy in reducing morbidity and mortality by targeting cytokine storms in preclinical models of influenza and sepsis. Therefore, it is important to see whether a similar approach can be used to control symptoms of severe dengue.

The body of work that comprises this thesis was designed to follow three lines of investigation:

1) Characterisation of novel host restriction factors that control responses to DENV infection using *in vitro* models.

2) Characterisation of novel host restriction factors that control responses to DENV infection using *in vivo* models.

3) Use of candidate therapeutics to treat *in vivo* models of dengue.

Chapter 2 - Materials and Methods

2.1 Cell culture

2.1.1 Vero Cells

For viral production, Vero cells were grown in high-glucose (4.5g/L) Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, USA) supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich, USA), without antibiotics. For plaque assays, Vero cells were grown in low-glucose (1g/L) DMEM (Thermofisher Scientific, USA) supplemented with 10% FCS and penicillin/streptomycin (henceforth referred to as complete DMEM). Vero cells were grown at 37°C in 5% CO_2.

2.1.2 Production of L929-conditioned media

L929 cells were grown in complete DMEM at 37°C in 5% CO_2. To produce conditioned media, cells were expanded and split into T150 flasks (Corning, USA) at 5 million cells per flask with 45mL per flask of complete DMEM. At days 6-9 after seeding, when cells sloughed off with gentle agitation, cell supernatants were pooled, clarified, and frozen in aliquots at -20°C for future use.

2.1.3 Bone Marrow Derived Macrophages (BMDMs)

The method of Weischenfeldt and Porse was used for the generation of murine BMDMs (Weischenfeldt & Porse, 2008). Briefly, mice were euthanised by cervical dislocation or CO_2 asphyxiation. Bone marrow from femurs and tibias was flushed into complete DMEM. Cells were pipetted up and down to create a single cell suspension, and this was passed through a cell strainer to remove debris. $2.4X10^7$ total cells were plated in 10cm uncoated bacteriogical petri dishes (Thermofisher, USA) in complete DMEM supplemented with 15% L929-conditioned media (henceforth referred to as complete BMDM media) at $2X10^6$ cells/mL. Three days after seeding, media was removed and plates were gently washed with PBS to remove non-adherent cells before addition of fresh complete BMDM media. At day 6 or 7 after seeding, cells were detached using a cell lifter, counted, and re-plated in complete BMDM media at $4X10^5$ cells/mL for use in downstream assays.

2.2 Virus production

All viral stocks used in these studies were originally derived from a clinical isolate of DENV2 from East Timor. The original stock of this virus was kindly provided by Professor Paul Young (University of Queensland, Australia). For the production of virus, Vero cells were grown to 90% confluence as described above. Cells were washed with PBS and infected with DENV2 at a MOI of 0.035 pfu per cell in high-glucose serum free DMEM at 37°C in 5% CO_2. Flasks were gently rocked from side to side every 15 minutes to ensure the even distribution of virus. After 90 minutes, high-glucose DMEM with 2% FCS was added to each flask, with the final concentration of FCS reaching 1.4%. Infected cells were then cultured for five days at 37°C in 5% CO_2.

At day 5 post infection, supernatants from flasks were pooled and clarified by centrifugation at 2500g for 10 minutes at 4°C. Clarified supernatants were loaded into Amicon Ultra-15 centrifugal filters (Merck Millipore, Germany), and centrifuged at 3750g for 40 minutes at 4°C. After this initial spin, retentates were pooled and kept on wet ice. Amicon columns were then reloaded, and spun for 4000g for 65 minutes at 4°C. Following this second spin, all retentates were pooled and clarified again by spinning at 2000g for 5 minutes at 4°C. Clarified, concentrated virus was then aliquoted and frozen at -80°C. Each viral batch was titrated prior to use by plaque assay.

2.3 Titration of infectious virus by plaque assay

Samples of interest were serially diluted in low-glucose (1g/L) DMEM (Thermofisher Scientific, USA) without serum. Serial dilutions were used to infect Vero cell monolayers seeded the night before into 6 well plates at $4X10^5$ cells per well. During the infection, plates were gently rocked from side to side every 15 minutes to ensure even distribution of virus. After 90 minutes, a 1:1 mixture of 2x DMEM (produced in-house) and 2% Seaplaque low melting point agarose (Lonza, Switzerland) was used to overlay the infected cells (the inoculum was not removed before this overlay was added). Infected cells were cultured for 5 days at 37°C in 5% CO_2. On Day 5, another layer of overlay containing neutral red dye (Sigma-Aldrich, USA) with a final concentration: 0.036%, was added to the wells. Neutral red accumulates in the lysosomes of living cells, therefore allowing the visualisation of plaques without additional fixation or counter-staining. On

Day 10, plaques were counted, allowing the determination of viral titre in pfu/mL.

2.4 BMDM experiments

2.4.1 *In vitro* blockade of type I interferon signalling

WT and IFNAR$^{-/-}$ BMDMs were treated overnight with varying concentrations (range: 10μg/mL to 0.1μg/mL) of MAR1-5A3 (henceforth referred to as MAR1) IFNAR-blocking antibody (Leinco Technologies, USA) or 10μg/mL of an IgG_1 isotype control (Leinco Technologies, USA). The next morning, BMDMs were stimulated with 100 units of recombinant murine interferon-β (PBL Assay Science, USA) for 20 minutes and lysates from these cells were probed by Western blot (see section 2.11).

2.4.2 DENV2 *in vitro* infection of BMDMs

BMDMs were treated overnight with MAR1 or IgG_1 isotype control. For Chapter 3 section 2.4 "MAR1 antibody treatment facilitates infection of WT BMDMs" MAR1 and isotype control antibodies were used at 1μg/mL; for all other BMDM infections these antibodies were used at 2μg/mL. The next day, cells were infected with DENV2 at a MOI of 1 in the presence of 5uL/mL 4G2 hybridoma supernatant (opsonising antibody; Hall Lab, University of Queensland) and MAR1 or isotype control in serum free DMEM for 90 minutes at 37°C. Following infection, media was replaced with complete BMDM media and either MAR1 or isotype control. Cells were then incubated at 37°C in 5% CO_2 for 24-48 hours before cells and or supernatants were harvested and used for downstream applications.

2.4.3 Drug treatment of BMDMs

For dose titration experiments, WT BMDMs were treated with two-fold dilutions of LCL-161 (MedChem Express, USA) or BAY 11-7082 (Sigma-Aldrich). For both drugs, the titration used was from 5μM (highest concentration) to 0.16 μM (lowest concentration). After 48 hours, BMDMs were prepared for flow cytometry as described below (section 2.12).

For other experiments, WT and IFNAR$^{-/-}$ BMDMs were treated with 0.63μM LCL-161 either 2hr prior to infection or immediately following infection. Similar experiments were also carried out using 2.5μM BAY 11-7082. Infections were carried out as

described in section 2.4.2. After 48 hours, BMDMs were prepared for flow cytometry (section 2.12)

2.4.4 Cell death control for BMDM flow cytometry experiments

I used apoptotic BMDMs as a positive control for my zombie aqua viability staining. To induce apoptosis, I treated WT BMDMs with 20μg/mL cycloheximide (CHX) and 100ng/mL TNF treatment of WT BMDMs for 48 hours. For staining, cells were then processed in parallel with live cells according to the protocol described in section 2.12.

2.5 Animal Ethics Statement

All mouse experiments were conducted according to the recommendations in the National Statement on Ethical Conduct in Animal Research of the National Health and Medical Research Council, and were reviewed and approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee (AEC2017.016).

2.6 Mouse strains

All WT mice and gene-targeted animals were on a C57BL/6 background. Generation of $SOCS3^{LysM}$ mice (B. A. Croker et al., 2003), $P50^{-/-}$ mice (Sha, Liou, Tuomanen, & Baltimore, 1995), $cIAP1^{-/-}$ mice (Gardam et al., 2011), $cIAP2^{-/-}$ mice (Gardam et al., 2011), $cIAP1^{LysM}/cIAP2^{-/-}$ mice (Lawlor et al., 2015; W. W. L. Wong et al., 2014), $SIDT2^{-/-}$ mice (T. A. Nguyen et al., 2017) $IFNAR^{-/-}$ mice (Nagarajan et al., 2008) and $TRAF2^{fl/fl}$ mice (Grech et al., 2004), on a C57BL/6 genetic background has previously been described. $TRAF2^{fl/fl}$ mice were crossed to LysM Cre (Clausen, Burkhardt, Reith, Renkawitz, & Förster, 1999) transgenic mice to generate $TRAF2^{LysM}$ animals. All strains of gene-targeted mice were backcrossed to C57BL/6 mice for > 6 generations.

2.7 Genotyping

DNA was prepared from tail biopsies which were digested overnight at 55°C in DirectPCR (Tail) buffer (Viagen Biotech, USA), then heated to 85°C for 45 minutes. 1μL of crude lysate was then used for genotyping polymerase chain reactions (PCRs). Bands were resolved by gel electrophoresis of reaction products using 1% agarose gels (Agarose powder, Bioline, UK).

Table 2.1 Genotyping Primers

Specific For	Primer Name	Sequence (5'-3')
cIAP1^{fl}	Thong Forward	AGACCCTTTCTAAGTAGACTGGTT
cIAP1^{fl}	WT cIAP1 Rev	TTATAGAAATGTTCAGATGTC
cIAP1^{fl}	KO cIAP1 Rev	TCAATCCTCCATGCCACAATAAACAT
cIAP2^{frt}	Sandal Forward	CCCTCAGGGTGTGTGGCTTA
cIAP2^{frt}	WT cIAP2 Rev	TGTGCATGGCTTCTGGTCGG
cIAP2^{frt}	Sandal Rev	CACTGGCTGTTCTTTCAGAGA
Common Cre	Common Cre Fwd	GCTCGACCAGTTTAGTTACCC
Common Cre	Common Cre Rev	TCGCGATTATCTTCTATATCTTCAG
IFNAR KO	IFNAR Fwd	CTC CTC CCG GAC AAG ACG GG
IFNAR KO	IFNAR Rev	TGG TGC TTA TAC ACT GCA CAG TGC
IFNAR KO	IFNAR Neo	GAG GCA GCG CGG CTA TCG TG
P50 KO	WT-50	GCAAACCTGGGAATACTTCATGTGACTAAG
P50 KO	HH neo	AAATGTGTCAGTTTCATAGCCTGAAGAACG
P50 KO	BS-7	ATAGGCAAGGTCAGAATGCACCAGAAGTCC
SIDT2 KO	T2KO_5	GCGAGTGTCTGTGAATGTCC
SIDT2 KO	T2KO_6	CCGCTGATATCTATGGTCCAAG
SIDT2 KO	T2KO_Neo3A	GCAGCGCATCGCCTTCTATC
SOCS3^{fl}	Olg 160	GAGTTTTCTCTGGGCGTCCTCCTAG
SOCS3^{fl}	Olg 161	TGGTACTCGCTTTTGGAGCTGAA
SOCS3^{fl}	Olg 162	GATAACTGCCGTCACTCCAACG
TRAF2^{fl}	TRAF2$^{fl/fl}$ Fwd	CCTACAGTCAAGGCTCAGTGACAA
TRAF2^{fl}	TRAF2$^{fl/fl}$ Rev	GCAAGCAC GTGGGGCAGAAGGTA

2.8 Mouse experiments

Mice were used between the ages of 6-16 weeks. A small exception to this was that two mice each of SOCS3^{LysM} and cIAP2$^{-/-}$ strains, and three mice of the IFNAR$^{-/-}$ strain, were used at ages of 16-28 weeks, and this data forms part of Chapter 4. Mice were housed under specific pathogen free conditions, with a 12-hour day/night cycle, and were provided with access to food and water *ad libitum*.

2.8.1 *In vivo* infections, safety and dosing

For infections, pre-titrated DENV2 stocks were thawed on wet ice and diluted in sterile PBS to obtain the concentration required for intravenous injection. All mice were infected by intravenous injection via the tail vein, using an injection volume of 100μL.

These experiments were approved by relevant WEHI safety officers. One condition of this approval involved the collection and autoclaving of biological waste. Furthermore, to conform to the Institute dengue safety policy, the animal technicians and research staff involved in this study in circumstances where they could potentially be required to handle replication-competent virus or infected animals were required to provide written consent and to check their dengue serostatus before being cleared to participate in experiments.

For all experiments performed in Chapter 4, WT and gene-targeted animals, including IFNAR$^{-/-}$ mice, were infected with 2X10^7 pfu of DENV2 per mouse. For all experiments in Chapter 5, WT mice were infected as in chapter 4, but IFNAR$^{-/-}$ mice were infected with 1X10^5 pfu of DENV2 per mouse. The rationale for this decision is provided in Chapter 5, section 2.

2.8.2 Isolation of plasma from whole blood samples

Mouse whole blood was collected by cardiac / mandible / retro-orbital bleed into EDTA-coated tubes and stored on wet ice. Within an hour of collection, whole blood was centrifuged at 2000g for 10 minutes at 4°C and clarified plasma was aliquoted as required into 1.5mL centrifuge tubes. Centrifuge tubes containing clarified plasma were then stored at -80°C before being used for downstream cytokine and qPCR assays.

2.8.3 Monitoring of weight and temperature; ethical endpoints

For all *in vivo* experiments, weight and temperature of mice was measured daily. For weight measurements, mice were placed into a plastic beaker on a scale. Temperature was measured by rectal probe (Oakton Instruments, USA). The relevant ethical endpoint for these experiments was two consecutive readings of ≥ 15% weight loss (relative to baseline) within a 24-hour period. E.g. for a mouse that weighed 20g at baseline, two consecutive readings of 17g or less within a 24-hour period would require the mouse to

be humanely euthanised. The institute animal ethics committee did not require a complete clinical score monitoring for these experiments.

2.8.4 MAR1 suppression of type I interferon signalling *in vivo*

With the exception of IFNAR$^{-/-}$ positive controls, WT and gene-targeted animals were treated with MAR1 both prior to and following infection according to the dosing protocol presented in Table 2.2. Therefore, mice were treated with 500μg of MAR1, 24 hours prior to infection, and 250μg of MAR1, 2 hours prior to infection, with further doses of 250μg at 24, 48, and 72 hours post infection. MAR1 was administered by intraperitoneal injection.

For animals which were culled at 48 hours post infection, the final two doses of MAR1 were omitted (animals received 1μg / mouse).

Table 2.2 Dosing schedule of MAR1 relative to the time of infection

Timing relative to infection	-24 hours	-2 hours	+24 hours	+48 hours	+72 hours
Dosage of MAR1 (per mouse)	500μg	250μg	250μg	250μg	250μg

2.8.5 Supportive saline treatment for IFNAR$^{-/-}$ mice

Intravenous saline is used as a supportive treatment for severe dengue (World Health Organisation, 2009). In Chapter 5, DENV2 infected IFNAR$^{-/-}$ mice were administered supportive fluid replacement therapy by intraperitoneal injection of 1mL of sterile, 0.9% normal saline. This was performed from day 2-7 post infection.

2.8.6 LCL-161 treatment of mice

For all experiments except for the data presented as figure 5.3, mice were administered 100mg/kg LCL-161 (MedChem Express, USA) or vehicle by oral gavage. For the experimental data presented as figure 5.3 mice were instead administered 400mg/kg LCL-161 or vehicle. The vehicle was prepared by combining 0.1 M HCl with 100mM Sodium Acetate (pH 4.63) at a 3:7 ratio, to create a solution with a final pH of 4.3-4.6.

2.8.7 Ruxolitinib and tofacitinib treatment of IFNAR$^{-/-}$ animals

For the dose titration study, vehicle (2% DMSO, 30% polyethylene glycol 300, deionised H_2O) or drug was administered to mice by oral gavage at 36 and 48 hours post infection. For "low" doses, I used 1.04mg/kg for tofacitinib and 4.16mg/kg for ruxolitinib. These are allometrically scaled from the human dosing regimens for tofacitinib and ruxolitinib of 5mg and 20mg doses, respectively, administered twice daily. The recommendations of Nair and Jacob were used for allometric scaling calculations (Nair & Jacob, 2016). For "high" doses, I used 30mg/kg for tofacitinib and 90mg/kg for ruxolitinib. Mice were humanely euthanised at 50hr post infection, and spleen lysates were prepared for western blotting (see section 2.11).

For the time-to-euthanasia study, mice were treated with vehicle, or "high" doses of ruxolitinib or tofacitinib twice daily (morning and evening) by oral gavage. Treatment was initiated the morning after mice were infected and continued until mice were humanely euthanised.

2.8.8 WEHI-112 treatment of IFNAR$^{-/-}$ animals

Mice were treated with vehicle (2.5% DMSO in PBS) or 30mg/kg WEHI-112. Drug was administered 1x daily by intraperitoneal injection. Treatment was initiated the morning after mice were infected and continued until mice were humanely euthanised.

2.9 Preparation of RNA standard for quantitative PCR (qCPR)

A preparation of a DNA plasmid encoding a laboratory isolate of DENV2, New Guinea C strain, termed MON601 (Pryor et al., 2001), was linearised by overnight digestion with the restriction enzyme XbaI (New England Biolabs, USA). Following this, *in vitro* transcription of MON601 RNA was performed using the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, USA) according to the manufacturer's instructions. The RNA reaction production was purified using an RNEeasy Plus kit (Qiagen, Germany), and residual DNA was removed by a digestion with amplification grade DNAse I (Sigma-Aldrich, USA). The removal of plasmid DNA was confirmed by conducting a qPCR (see below) without reverse transcriptase, and detecting no amplification. Finally, the concentration of RNA was determined using a Qubit RNA High Sensitivity Assay kit

(Thermofisher, USA) and a Qubit Fluorometer (Thermofisher, USA) in accordance with the manufacturer's instructions.

2.10 Quantification of viral load by qPCR

A one-step reverse transcription polymerase chain reaction was used to quantify DENV2 RNA. The chemistry used was the Power Sybr Green 1-step kit (Life Technologies, USA). Samples were run in a 384-well plate format on a LightCycler II machine (Roche, Switzerland). Serial dilutions of purified MON601 RNA (see section 2.9 above) were used to construct a standard curve (10^8 -> 10^1 MON601 genomes per 20uL reaction). The absolute quantification mode of LightCycler II software (Roche, Switzerland) was then used to determine the number of DENV2 genomic equivalents (GE) per sample. Sadon and colleagues described the primers used for the qPCR assay; these primers amplify the DENV capsid gene (Sadon et al., 2008). Forward Primer (5'-3'): CTGCARGGACGAGGACCATT. Reverse primer (5'-3'): GGGATTGTTAGGAAACGAAGGA.

Reaction mix

Component	Volume (μL)
Forward Primer (10μM)	0.4
Reverse Primer (10μM)	0.4
2x Power Sybr Master Mix	10
Reverse Transcriptase	0.16
PCR-grade H_2O	5.04
Template	4
Total	**20**

2.11 Western blotting

BMDMs were trypsinised or scraped and washed with PBS prior to lysis. Mouse spleen sections were homogenised using a Tissue Lyser II (Qiagen, Germany). Cells were lysed in protein lysis buffer: 20mM Tris-HCl pH 7.5, 135mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 1% Triton X-100 (Sigma-Aldrich, USA), 10% glycerol (Ajax Finechem, Australia), Complete

mini EDTA-free Protease Inhibitor Cocktail Tablets and PhosStop phosphatase inhibitor tablets (Roche, Switzerland) for 30 minutes on wet ice. Lysates were spun at 10,000g for 5 minutes at 4◦C to pellet debris. Clarified lysates were quantified using Bio-Rad Protein Assay (Biorad, USA). 30-50μg/lane of lysate was boiled for 5 min in SDS-containing (denaturing) loading buffer (Invitrogen, USA) and separated using 4-12% Bis-Tris pre-cast gels (Thermofisher, USA). Proteins were transferred onto nitrocellulose membranes (Thermofisher, USA) using an iBlot dry transfer system (Thermofisher, USA) and detected using the primary and secondary antibodies detailed in Table 2.3. Primary and secondary antibodies were made up in 5% BSA (Bovogen Biologicals, Australia) and 0.1% Tween 20 (Sigma-Aldrich, USA) in PBS. Primary antibody incubations were performed overnight at 4◦C, secondary antibody incubations were performed for 2 hours at room temperature. All incubations were performed with gentle rolling agitation. Chemiluminescence was detected by ChemiDoc (Biorad, USA).

Table 2.3 Antibodies used for Western Blotting

Antigen	Clone	Dilution	Source	Conjugate	Manufacturer
STAT1	Polyclonal	1:1000	Rabbit		CST*
pSTAT1	58D6	1:1000	Rabbit		CST
STAT3	Polyclonal	1:1000	Rabbit		CST
pSTAT3	D3A7	1:1000	Rabbit		CST
cIAP1	1E1-1-10	1:500	Rat		Enzo#
Rabbit IgG	Polyclonal	1:5000	Goat	HRP	GE Healthcare, USA
Rat IgG	Polyclonal	1:1000	Goat	HRP	Southern Biotech$
β-actin	13E5	1:10,000	Rabbit	HRP	CST

*Cell Signalling Technologies, USA; #Enzo Lifesciences, USA; $Southern Biotech, USA

2.12 Flow Cytometry

2.12.1 NS1 and Zombie staining of BMDMs

Viable BMDMs were detached from untreated cell culture plates (Corning, USA) using cell lifters. Apoptotic controls were generated as described in section 2.4.4 above. BMDMs were washed with PBS prior to staining with Zombie Aqua live/dead stain (Biolegend, USA) at 1:500 in PBS for 15 minutes at room temperature. Cells were then

pelleted by centrifugation at 400g for 5 min at 4◦C and washed with PBS supplemented with 2% FCS. Cells were then fixed and permeabilised using the FOXP3 Staining Kit (Thermofisher, USA) according to manufacturer's instructions. Cells were stained using 30ng/well (1:500 of a 2mg/mL solution) of mouse anti-NS1 in FOXP3 Staining Kit permeabilisation buffer for 1hr at 4◦C. Cells were then washed twice with FOXP3 Staining Kit permeabilisation buffer (400g for 5 min at 4◦C) and stained with a 1:500 dilution of goat anti-mouse IgG Dylight 549 in FOXP3 Staining Kit permeabilisation buffer for 30 minutes at 4◦C. All flow cytometry data was collected using a Fortessa X20 analyser (Becton Dickinson, USA) and analysed using FlowJo software (Version 10, FlowJo LLC, USA). Note: for antibody details see Table 2.4.

2.12.2 Immunophenotyping by flow cytometry

PBMCs isolated from peripheral blood or spleens of animals were stained for 30 minutes at 4◦C with antibodies to detect the following antigens: CD4, CD8, MHC II, CD11b, CD11c, Ly6G. Cells were acquired using a Fortessa X20 analyser (Becton Dickinson, USA) and analysed using FlowJo software (Version 10, FlowJo LLC, USA). Note: for antibody details see Table 2.4.

<u>Defining Immune Cell Populations using Flow Cytometry</u>

All cell populations were initially gated to remove doublets and to isolate the WBC population using forward and side scatter.

$CD4^+$ T cell: $CD4^+$, $CD8^-$

$CD8^+$ T cell: $CD4^-$, $CD8^+$

B Cell: $CD4^-$, $CD8^-$, $CD19^+$

DCs: $CD4^-$, $CD8^-$, $CD19^-$, MHC Class II^+, $CD11c^+$

Monocytes: $CD4^-$, $CD8^-$, $CD19^-$, $CD11b^+$, $Ly6G^{Lo}$

Inflammatory Macrophages: $CD4^-$, $CD8^-$, $CD19^-$, $CD11b^+$, $Ly6G^{Int}$

Granulocytes: $CD4^-$, $CD8^-$, $CD19^-$, $CD11b^+$, $Ly6G^{Hi}$

Table 2.4 Antibodies used for flow cytometry

Antigen	Conjugate	Clone	Isotype	Company / Source
CD4	AF700	RM4-5	Rat IgG2a, κ	BD*
CD8α	BV510	53-6.7	Rat IgG2a, κ	BD
MHC Class II	BV650	M5/114.15.2	Rat IgG2b, κ	Biolegend
CD11b	FITC	M1/70	Rat IgG2b, κ	BD
CD11c	PE-Cy7	HL3	Hamster IgG_1, $\lambda 2$	BD
Ly6G	APC/Cy7	1A8	Rat IgG2a, κ	Biolegend
NS1	NA	4G4	Mouse IgG_1	UQ / WEHI[#]
Mouse IgG	Dy549	Polyclonal	NA	Biorad

*BD - Becton Dickinson, [#]UQ / WEHI – hybridoma supernatant sourced from University of Queensland, purified by the WEHI Monoclonal Antibody Facility

2.13 Multiplex immunoassays

Immunoassays were purchased from Procartaplex, a subsidiary of Thermofisher, USA. A custom panel was used to obtain the cytokine results presented in Chapter 3 and the $IFNAR^{-/-}$ data presented as Figure 5.2. The Cytokine & Chemokine 26-Plex Mouse ProcartaPlex™ Panel 1 (Catalogue # EPX260-26088-901), supplemented with the IFN alpha Mouse ProcartaPlex™ Simplex Kit (EPX01A-26027-901) was used to obtain the cytokine results presented in Chapters 4 and 5 (with the exception of figure 5.2).

Assays were run to detect cytokines in BMDM supernatants or in mouse plasma according to the manufacturer's instructions. 50μL BMDM samples were run neat, while 25μL plasma samples were diluted two-fold using Universal Assay Buffer (Thermofisher, USA). Beads were quantified using a Bioplex 200 analyser (Biorad, USA). Specific analytes of individual samples with a bead count lower than 5 were excluded from downstream analyses.

2.14 Statistical Analysis

All statistical analyses were performed using Prism 8 (Graphpad Software, USA). The use of particular statistical tests is outlined as is relevant in the subsequent results chapters.

Chapter 3 - Characterisation of Novel DENV Host Restriction Factors Using *In Vitro* Models.

3.1 Introduction

An understanding of cell intrinsic factors that impact DENV infection would provide insight into important host molecular pathways that could be targeted prophylactically to either reduce or prevent viraemia in people who travel to endemic areas. Furthermore, an understanding of key DENV host factors could form the basis of interventions that break transmission during seasonal epidemics. This chapter describes the experiments I performed to identify such targetable pathways. My hypothesis was that some host cell signalling molecules may be required for DENV replication whilst others may impede viral turnover. Inhibiting the former and promoting the activity of the latter could result in novel prophylactic or therapeutic avenues.

Type I interferon signalling is quickly and robustly stimulated by DENV infection (Chapter 1, sections 1.4 and 1.7.2) and most known host restriction factors are components of this signalling pathway or modulate its function (Chapter 1, section 1.8). I speculated that additional host cell signalling molecules, independent of or intersecting with interferon signalling, may impact on the ability of DENV to replicate and establish infection. I focused my attention on three major host cell signalling hubs that have been implicated in host cell survival and activation, sensitivity to cytokine signalling and detection of viral PAMPS.

NF-κB signalling has many functions during viral infection, including upregulation of pro-inflammatory gene transcription and promotion of cellular survival (Chapter 1, section 1.7.3). The most important NF-κB dimers for canonical signalling are the p50-p65 and p50-c-Rel heterodimers (T. Liu et al., 2017). Generally, p65 is a transcriptional activator and p50 in many instances is a transcriptional repressor (Bohuslav et al., 1998; Udalova et al., 2000), however this is highly context dependent. The role of p50 in supporting or abrogating DENV replication has not been described.

Several cytokines, death ligands and pathogen-associated molecular patterns can activate the NF-κB pathway through engagement of upstream cognate receptors. Signal

transduction through these receptors is critically dependent on a set of proteins called cellular inhibitor of apoptosis (cIAP) proteins. cIAPs are essential for the activation of canonical NF-κB signalling and the downstream transcriptional activation of genes that drive cytokine production, cell activation and survival (Mahoney et al., 2008; Varfolomeev et al., 2008). In the absence of cIAPs, oligomerisation of death receptors and PRRs causes apoptosis (reviewed in (Lalaoui & Vaux, 2018)). Cytokine production, death ligands and apoptosis have all been implicated in aspects of DENV disease pathogenesis. Despite being a central hub in coordinating these processes, the role of cIAPs in promoting or abrogating DENV infection has not been described in the literature.

One of the major DENV PAMPs is dsRNA which activates cytosolic toll-like receptors (Chapter 1, section 1.7.1). Recent data suggests that extracellular viral dsRNA, released during the course of infection, can be internalised by uninfected cells into an endosomal compartment. A molecule called SIDT2 is capable of transporting the dsRNA out of the endosomes and into the cytoplasm to allow activation of cytoplasmic TLRs (T. A. Nguyen et al., 2017). The consequence of this TLR activation, as alluded to above, is the activation of cellular antiviral and proinflammatory responses. The role of SIDT2 in abrogating DENV infection has not been described in the literature. One of the many consequences of TLR activation and NF-κB activation is the production of proinflammatory cytokines that act in *cis* and *trans* to promote cellular responses in infected and uninfected cells.

The SOCS family of proteins represent an important signalling hub that regulate cellular responses to cytokines. SOCS3 is transcriptionally regulated by cytokine signalling and it forms a negative feedback loop by antagonising IL-6 family cytokine signalling through inhibition of active JAKs (Babon, Varghese, & Nicola, 2014). I hypothesised that the signalling hubs, described above, could have roles in promoting or restriction DENV infection and this chapter explores their contribution in permitting viral replication *in vitro*.

3.2 Establishment of an *in vitro* DENV infection / replication system

3.2.1 Type I interferon is a major restriction factor for murine DENV infection

To initially characterise novel host factors that may impact DENV infection or replication I set out to establish an *in vitro* model using primary murine cells. Such a model would allow me to utilise primary cells derived from existing gene-targeted animals that are deficient in the pathways I described above. Monocytes and macrophages are the main cell type that DENV infects (Blackley et al., 2007; Pham, Langlois, & tenOever, 2012) so I chose to generate primary bone marrow derived macrophages (BMDMs) and infect these with DENV. Using such a system I could quantify the number of infected cells, examine the kinetic of viral production, assess cell viability and quantify cytokine responses. In order to enhance the level of infection that is observed during *in vitro* culture, I used the previously described ADE-facilitating anti-DENV envelope antibody 4G2 (Henchal, Gentry, McCown, & Brandt, 1982; Littaua et al., 1990).

When WT BMDMs were infected with DENV, there was only a modest increase in cell death (Figure 3.1). However, the proportion of WT cells that were infected was less than 10 % (Figure 3.1). To measure the proportion of cells that were infected, I used a flow cytometry assay to detect cells that expressed the DENV NS1. Murine cells are known to be refractory to DENV infection and this is mediated through type I interferon signalling (Shresta et al., 2004; Züst et al., 2014). I examined whether type I interferon signalling was abrogating my ability to infect primary BMDMs by comparing infection kinetics in WT cells to cells derived from gene-targeted mice that lacked IFNAR. Primary macrophages that were deficient in IFNAR were highly susceptible to DENV infection and had a modestly increased propensity to die during infection (Figure 3.1).

Interestingly, the ability of murine type I interferon signalling to restrict DENV infection is different than what is observed during human infections. In clinical cases of mild dengue fever and severe dengue, high serum levels of type I interferon are often recorded (Talarico et al., 2017; Y. Tang et al., 2010). It should however, be duly acknowledged that plasma levels of interferon during human infection are highly dynamic, and consistently high levels of type I interferon have not been identified by meta-analyses (see section 1.4).

In vitro studies have also shed light on the effect of type I interferon treatment on human cells in the context of DENV infection. Upstream regulators of human interferon signaling such as cGAS act to restrict DENV replication by promoting interferon production (Aguirre et al., 2017), and direct pre-treatment of human HEPG2 cells with type I interferon is antiviral (Wati, Li, Burrell, & Carr, 2007). However, a growing body of evidence shows that DENV is able to cleave components of the human type I interferon signaling cascade, but not orthologous proteins from other species (Ashour et al., 2010; Stabell et al., 2018), suggesting that on balance, the type I interferon response is probably less restrictive to viral replication in human cells than in mouse cells.

Since type I interferon appears to act as a particularly potent host restriction factor in mouse, I needed to ameliorate type I interferon signaling to facilitate robust infection of my gene-targeted BMDMs that were deficient in signalling hubs not directly related to interferon. I reasoned that removal of type I interferon signalling in my *in vitro* system would allow me to dissect other important host cell signalling hubs that were made redundant in mice because of interferon responses but could be important in humans where interferon does not play such a dominant role.

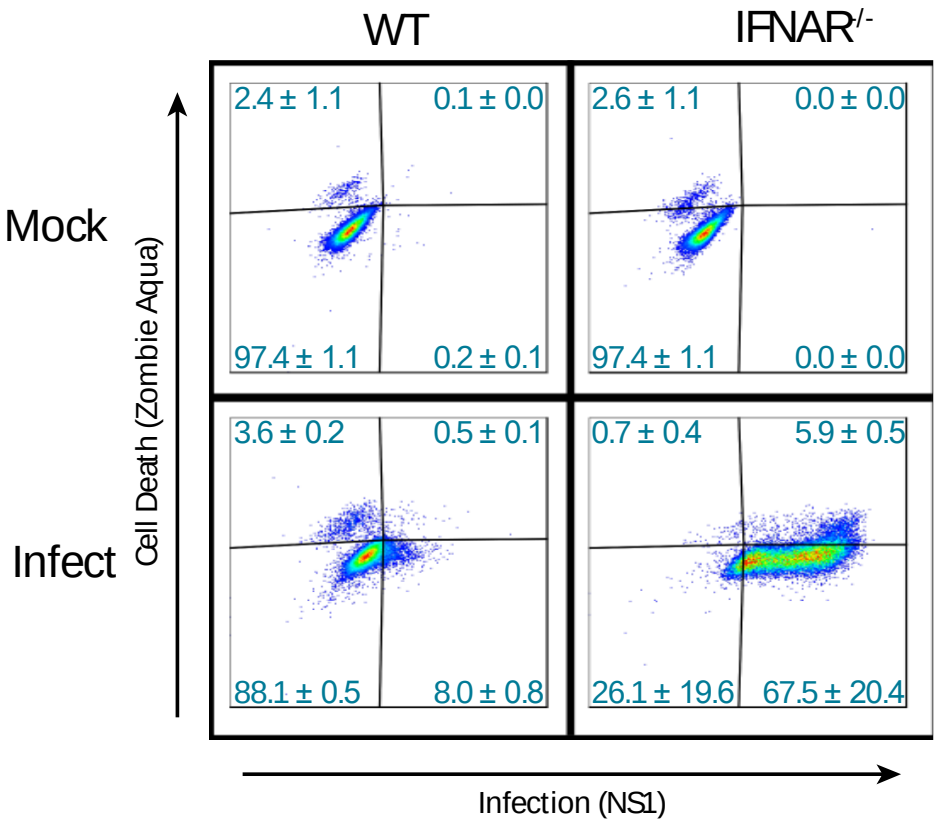


Figure 3.1 | Murine type I interferon is a restriction factor precluding robust DENV infection *in vitro*. WT and IFNAR-/- BMDMs were either left uninfected (mock) or were infected with DENV2 (MOI of 1) in the presence of 4G2 enhancing antibody. Cells were harvested 48 hours after infection and analysed by flow cytometry. The proportion of cells in each quadrant are indicated. These data are representative of 2 independent experiments. The mean ± SEM for the proportion of cells in each quadrant was calculated across the 2 experiments and is indicated in the relevant region.

3.2.2 IFNAR-blocking antibody diminishes type I interferon signaling in uninfected BMDMs and permits robust DENV infection.

To assess whether an IFNAR-blocking antibody could enhance DENV infection, I co-cultured cells prior and during infection with a well characterised anti-IFNAR monoclonal antibody, clone MAR1-5A3 (henceforth referred to as MAR1) (Sheehan, Dunn, Bruce, & Diamond, 2006; Sheehan, Lazear, Diamond, & Schreiber, 2015). To initially determine the effectiveness of this antibody to block IFNAR signaling, WT BMDMs were stimulated with recombinant interferon-β. This strongly induced downstream interferon signaling as evidenced by a robust induction of phosphorylated STAT1 (pSTAT1) (Figure 3.2).

However, when cells had been pre-incubated with MAR1, the expression of pSTAT1 was abolished. This strong inhibition of type I interferon signaling with MAR1 replicated what was observed in IFNAR$^{-/-}$ cells (Figure 3.2). This confirmed the notion that MAR1 may be able to enhance DENV infection in WT BMDMs. Next, I wanted to determine if MAR1 could block IFNAR signaling during *in vitro* infection with DENV.

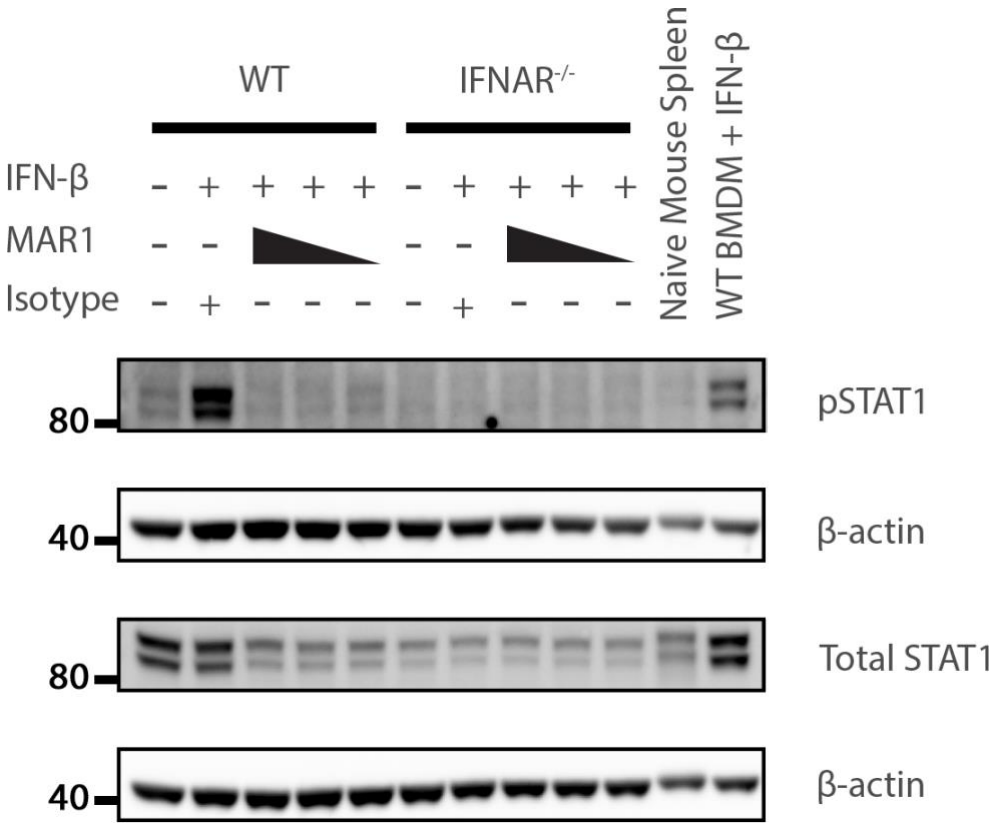


Figure 3.2 MAR1 treatment blocks type I interferon signalling *in vitro*. WT and IFNAR$^{-/-}$ BMDMs were cultured overnight with 10μg/mL of an isotype control antibody or with varying amounts of MAR1 antibody (range: 10μg/mL to 0.1μg/mL). BMDMs were subsequently stimulated with recombinant murine interferon-β (100U/mL) or PBS control for 20 minutes and lysates from these cells were probed by Western blot. Numbers to the left of the panel represent the positions of protein size markers (in kDa). Data are representative of 3 independent experiments.

3.2.3 MAR1 treatment diminishes type I interferon signalling in DENV infected WT BMDMs

In order to determine if MAR1 antibody treatment might facilitate DENV infection in WT

BMDMs, I first needed to confirm that this treatment ameliorated IFNAR signaling during *in vitro* infection. WT and IFNAR$^{-/-}$ BMDMs were treated with 1μg/mL of MAR1 or isotype control overnight. BMDMs were then infected for 90 minutes in the presence of MAR1 or isotype control. Cells were harvested at 24-hour intervals and lysates were probed for pSTAT1 as a marker of type I interferon signalling. Compared to isotype control treated WT BMDMs, MAR1 treatment drastically reduced the expression of pSTAT1 one day after DENV infection (Figure 3.3). However, this effect was greatly diminished by day two post infection, with both isotype and MAR1 treated cells showing similarly pronounced levels of pSTAT1. As a negative control, there was no pSTAT1 observed in IFNAR deficient BMDMs at any timepoint during DENV infection (Figure 3.3). As I had confirmed the effectiveness of using MAR1 to block type I interferon signaling, I next wanted to determine whether MAR1 treatment could enhance the susceptibility of WT BMDMs to DENV infection.

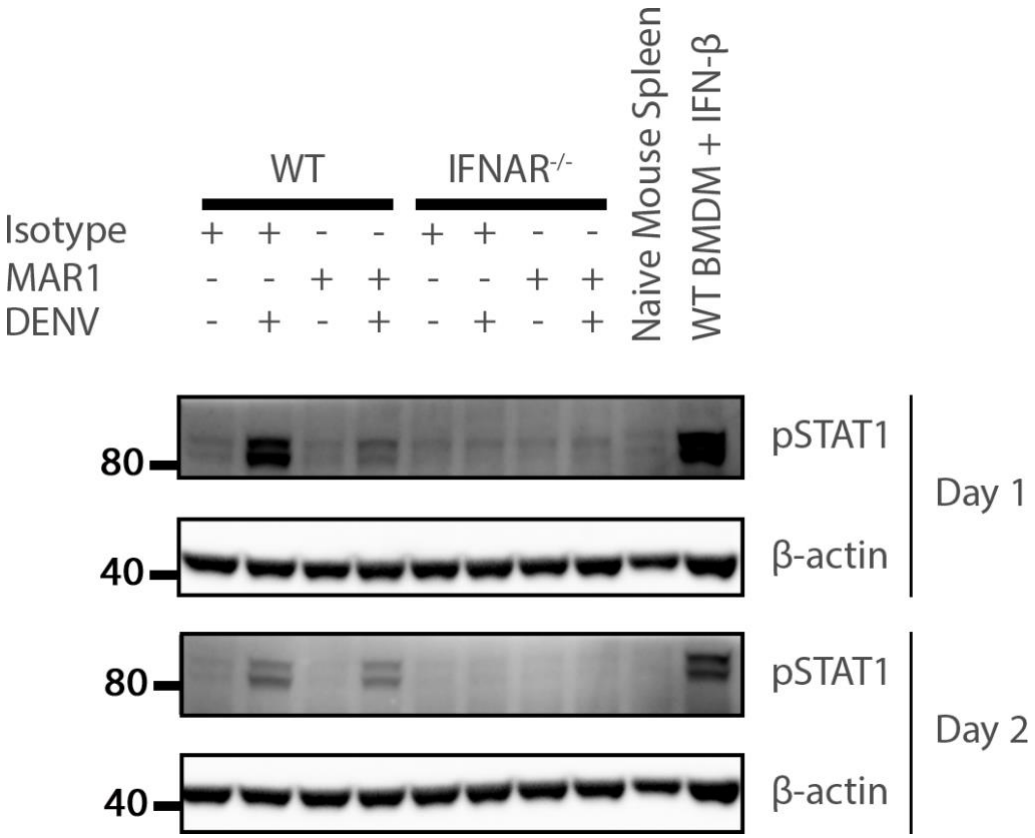


Figure 3.3 MAR1 treatment ameliorates type I interferon response to DENV infection *in vitro*. WT and IFNAR$^{-/-}$ BMDMs were co-cultured with 1μg/mL of MAR1 antibody or isotype control and / or DENV2, MOI of 1, for 1-2 days. Lysates from these cultures were analysed by Western blot. The cell lysate from splenocytes of naïve WT mice (negative control) or WT BMDMs stimulated with recombinant interferon-β (positive control) were used as controls. Numbers to the left of the panel represent the positions of protein size markers (in kDa). Data is from one experiment.

3.2.4 MAR1 antibody treatment facilitates infection of WT BMDMs

I showed that blocking murine type I interferon via MAR1 treatment greatly ameliorates downstream IFNAR signalling, and I next wanted to determine if MAR1 treatment could facilitate the infection of WT BMDMs. MAR1 treatment enhanced the proportion of WT BMDMs infected with DENV2 at 48 hours post infection, as measured by NS1 staining (Figure 3.4). WT BMDMs treated with MAR1 had 4-fold as many infected cells as compared to isotype control treated cells. Compared to mock infected cells, infection with DENV2 induced a modest increase in cell death in WT BMDMs, as measured by Zombie Aqua staining, which has a greater affinity for dead / dying cells (Figure 3.4).

Although MAR1 greatly enhanced the proportion of infected WT BMDMs, it was still only approximately half of what was observed in IFNAR deficient cells (Figure 3.4). Approximately 80% of IFNAR$^{-/-}$ BMDMs stained positive for NS1, regardless of whether they were pre-treated with isotype or MAR1, relative to 35% of WT BMDMs treated with MAR1. The fact that MAR1 treatment enhances the proportion of NS1-positive WT BMDMs but not to the same degree as IFNAR deficient cells is most likely due to the marginal reduction in IFNAR signaling at 48 hours (Figure 3.3). This reduction in signalling is most likely due to the homeostatic turnover of IFNAR on the surface of cells, which leads to newly synthesised IFNAR capable of ligation with type I interferons.

Given that I had developed an *in vitro* DENV infection system that augmented a robust infection in WT BMDMs, I then sought to screen a range of primary BMDMs with genetic deficiencies in different signalling pathways. This would enable me to determine which pathways promote or abrogate viral replication.

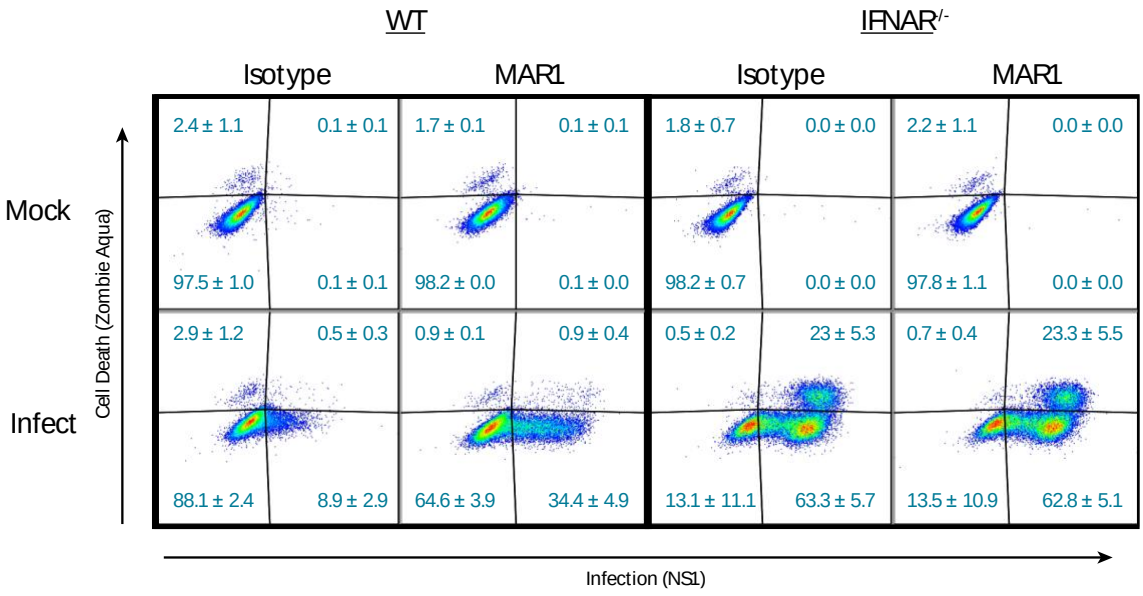


Figure 3.4 MAR1 treatment augments WT BMDM infection *in vitro*. WT and IFNAR$^{-/-}$ BMDMs were pre-treated with 2μg/mL of the indicated antibodies overnight, and subsequently infected with DENV2 (MOI of 1) or mock-infected with PBS in the presence of 2μg/mL of the indicated antibodies. Cells were cultured before staining and analysis by flow cytometry. Viability was determined using Zombie Aqua staining and infection determined by NS1 staining. These data are representative of 2 independent experiments. The mean ± SEM for the proportion of cells in each quadrant was

calculated across the 2 experiments and is indicated in the relevant region.

3.2.5 BMDM endpoint screening

I chose the following endpoints as relevant markers to quantify the magnitude and consequences of infection in gene-targeted BMDMs:

Table 3.1. Endpoints of DENV *in vitro* screen

Endpoint	Method of Assessment
Infected Cells	NS1 FACS
Cell Death	Zombie Aqua FACS
Viral Production	Plaque assay
Cytokine Production	Multiplex ELISA

3.2.6 A note on data analysis

In this chapter, I present flow cytometry data in the form of staining for viral antigen (NS1 staining) and cell viability (zombie aqua staining). This data was collected over multiple experiments, examining multiple genotypes of genetically targeted BMDMs. This flow cytometry was then pooled to form one dataset. For statistical analysis, I have used a one-way ANOVA with the Dunnett post-hoc test for multiple comparisons to compare the mean levels of viral infection (using the surrogate marker NS1) and cell death values of each genotype of BMDMs to the mean levels of viral infection and cell death values for WT controls. For the sake of clarity, I have presented this analysis in the form of graphs comparing one individual genotype to pooled WT and IFNAR positive controls (e.g. Figure 3.5 below), as opposed to presenting the entire dataset all at once.

The same statistical approach was also taken to analyse plaque assay data (Figure 3.13) and cytokine data (Figure 3.14)

3.3 Investigating the effect of p50 genetic knockout on *in vitro* susceptibility to DENV infection

NF-κB signalling has powerful effects on diverse outcomes such as cell survival and innate immunity. The NF-κB pathway is activated by both RLR and TLRs in response to DENV infection, reviewed in (Green, Beatty, Hadjilaou, & Harris, 2014). In addition, DENV infection activates the cGAS-STING signalling axis (Aguirre et al., 2017), which can also induce NF-κB signalling (Q. Chen, Sun, & Chen, 2016). As described in the literature review, NF-κB subunits form hetero- or homo-dimers. Importantly, the different properties of the subunits can influence the transcriptional activity of the resulting dimer. The most important NF-κB dimers for canonical NF-κB signalling are the p50-p65 and p50-c-Rel heterodimers, and these dimers are potent activators of transcription (T. Liu et al., 2017). p50 homodimers can bind to some κB sites and stimulate transcription (Baek et al., 2002; Fujita, Nolan, Ghosh, & Baltimore, 1992). However, in most instances p50 acts as a transcriptional repressor (Bohuslav et al., 1998; Udalova et al., 2000). Therefore, the presence of the p50/p50 homodimer could mitigate pro-inflammatory NF-κB signalling.

Several groups have examined the impact of p50 deficiency on mammalian disease *in vivo*. Interestingly, these results have indicated that p50 deficiency can result in strikingly different outcomes, depending on the nature of the pathogenic challenge. For instance, when Sha and colleagues infected $p50^{-/-}$ mice with *L. monocytogenes*, mice were able to clear extracellular bacteria, but had a reduced ability to clear intracellular bacteria, perhaps due to an inability of infected macrophages to respond to T cell cytokines (Sha et al., 1995). $p50^{-/-}$ mice infected with *E. coli* did not show changes in bacterial loads but succumbed earlier than controls due to acute lung injury caused by increased levels of pro-inflammatory cytokines (Mizgerd et al., 2003).

Conversely, p50 deficiency rescued animals challenged with encephalomyocarditis virus (EMCV) from mortality (Sha et al., 1995). Initially, this resistance to EMCV infection was attributed to an increase in type I interferon production from stromal cells (Sha et al., 1995). However, a subsequent study found that mice with a compound $p50^{-/-}$ $IFNAR^{-/-}$ deficiency were still more resistant to EMCV-induced mortality than WT mice (Schwarz

et al., 1998). Schwarz and colleagues argued that without p50, and by extension, pro-survival p65-p50 transcription, cells were unable to inhibit apoptosis. With increased apoptosis, $p50^{-/-}$ mice had reduced viral titres, relative to WT, and were able to survive the EMCV infection (Schwarz et al., 1998).

Preliminary findings from our laboratory have also shown that $p50^{-/-}$ mice succumb to lymphocytic choriomeningitis virus (LCMV) due to increased endothelial cell permeability, manifested as vascular leak (Cody Allison, unpublished work).

In the first instance I decided to examine the effect of p50 deficiency in DENV infection using my BMDM model. To determine the role of p50 during *in vitro* DENV infection, I infected $p50^{-/-}$ BMDMs and analysed them by flow cytometry. At 48 hours post infection, the proportion of BMDMs infected was similar between WT and $p50^{-/-}$ BMDMs (Figure 3.5). I also infected $IFNAR^{-/-}$ BMDMs as a positive control. As p65-p50 mediated upregulation of transcription has been proposed to be important for the pro-survival function of NF-κB (Schwarz et al., 1998), I sought to determine if p50 deficiency affected the viability of DENV infected BMDMs. There was, however, no difference in the viability of $p50^{-/-}$ BMDMs compared to WT cells (Figure 3.6). I also co-cultured WT BMDMs with the protein synthesis inhibitor cycloheximide (CHX) and TNF, to promote cell death and act as a positive control for the assay. Although I was unable to observe any difference in the infectivity or viability in DENV infected BMDMs in the absence of p50, it's likely that the *in vitro* system is unable to recapitulate many aspects of the *in vivo* environment, especially as I hypothesise that loss of p50 could promote loss of endothelial integrity. I addressed the role of p50 during DENV infection *in vivo* in Chapter 4.

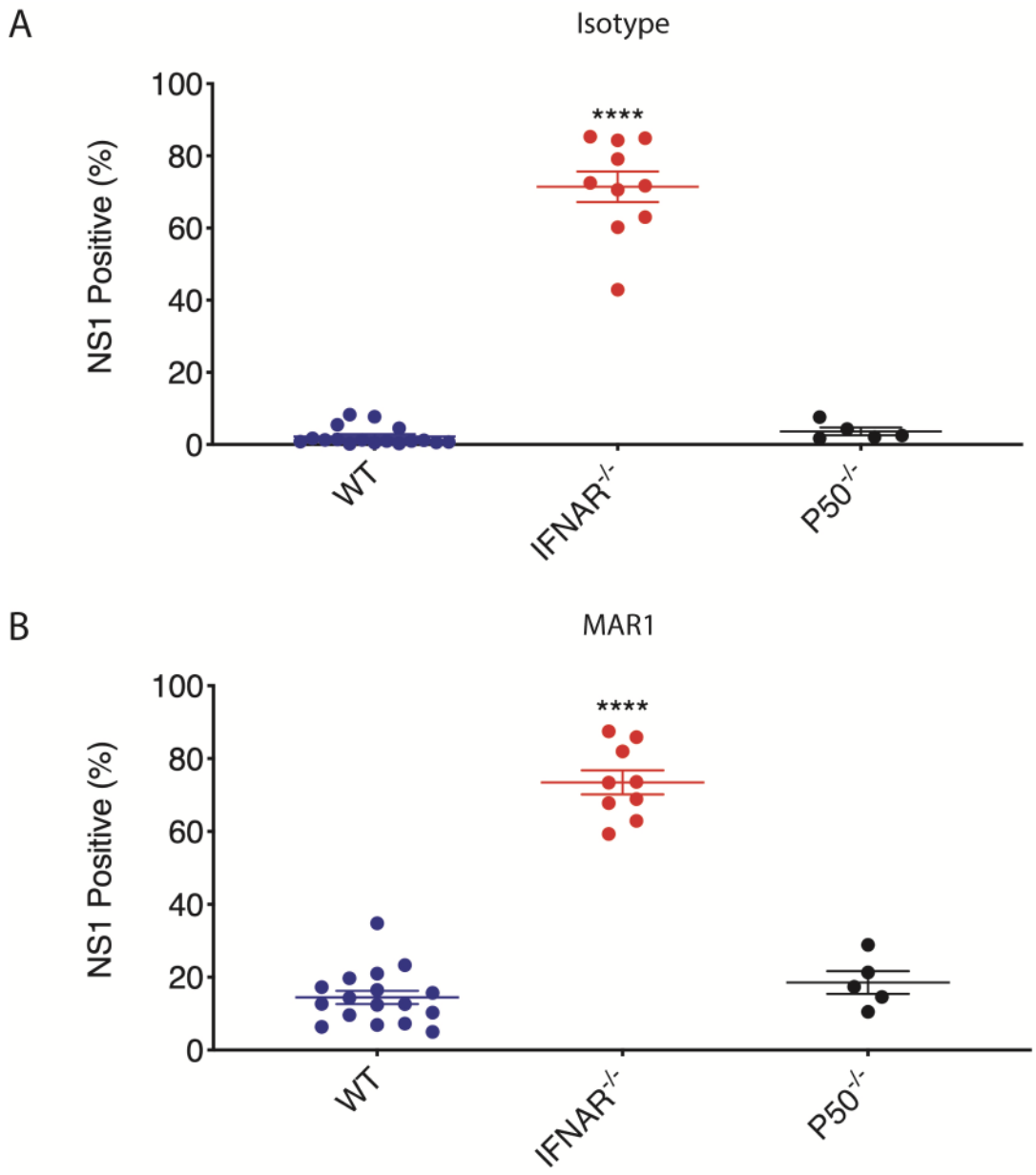
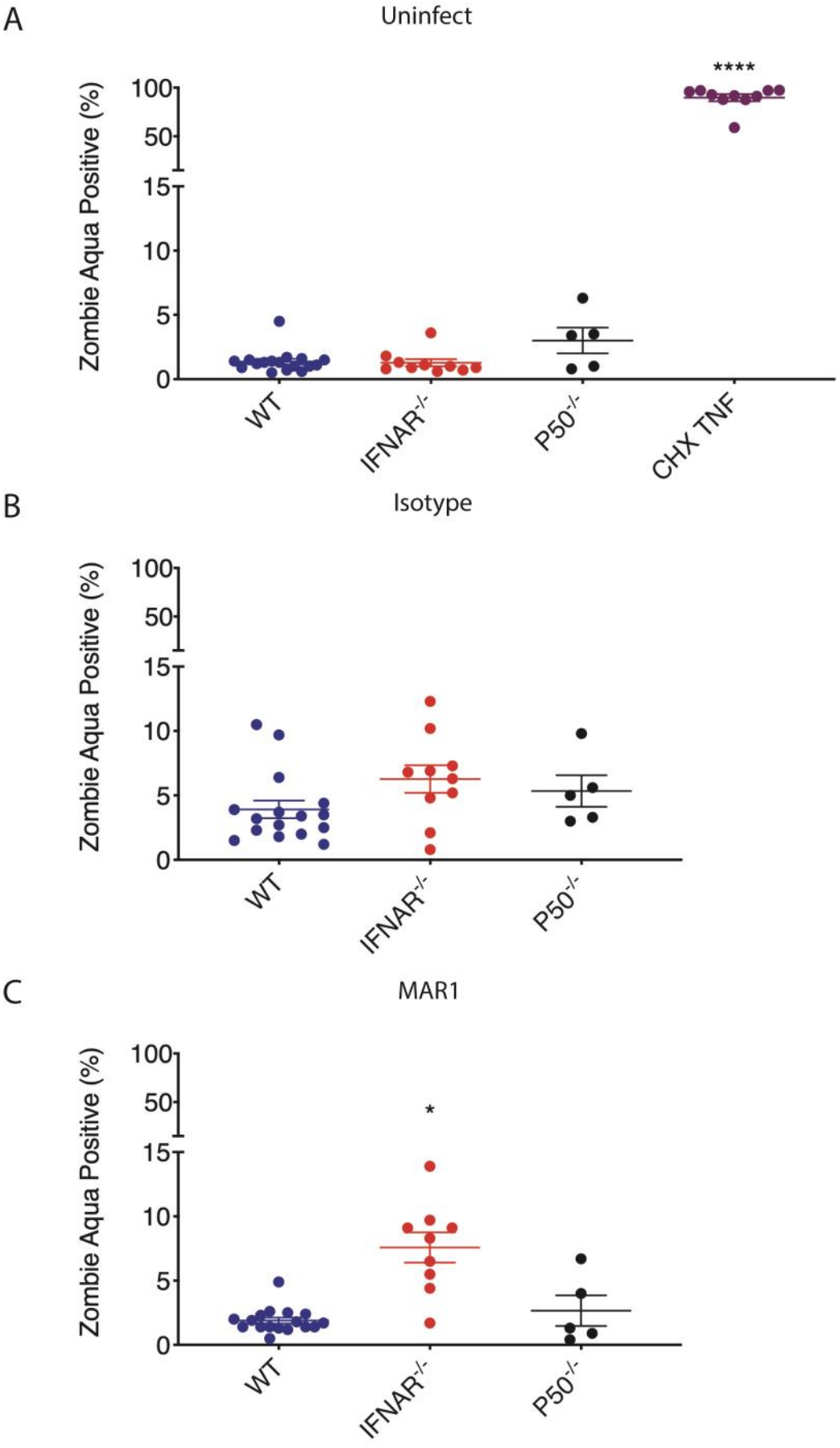


Figure 3.5 p50$^{-/-}$ BMDMs do not have an enhanced susceptibility to DENV infection. Flow cytometric analysis of BMDMs, pre-treated with (A) isotype control or (B) MAR1 antibody 48 hours post infection with DENV2. Cells were stained with NS1 to determine the proportion of infected cells. Every dot represents an individual biological replicate. p50$^{-/-}$ data is combined from 5 independent experiments. Error bars indicate the mean and SEM. Statistical analysis: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons. **** $p < 0.0001$.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100
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0
*
WT
IFNAR-/-
P50-/-

Figure 3.6 Loss of p50 does not affect the viability of DENV infected BMDMs.

Cell death was quantified by flow cytometry in BMDMs that were left (A) Uninfected or were infected with (B, C) DENV2. DENV2 infected cells were either treated with (B) isotype antibody (C) MAR1. All BMDMs were harvested 48 hours after infection for analysis. Every dot represents BMDMs from an independent biological replicate. BMDMs treated with CHX and TNF were used as a positive control for the death assay. $p50^{-/-}$ data is combined from 5 independent experiments. Error bars indicate the mean ± SEM. Statistical analysis: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons. * $p < 0.05$, **** $p < 0.0001$.

3.4 Investigating the effect of SOCS3 genetic knockout on *in vitro* DENV infection

SOCS3 is one of eight SOCS proteins that act to restrain cytokine signalling mediated by the JAK-STAT pathway. SOCS3 is induced by numerous cytokines, including G-CSF, GM-CSF, EPO, interferon-γ, leptin, IL-6, and IL-10 (Babon et al., 2014; Bjørbaek, Elmquist, Daniel Frantz, Shoelson, & Flier, 1998; Ito et al., 1999; Schneider, Chevillotte, & Rice, 2014; Starr et al., 1999). SOCS3 is essential for fetal erythropoiesis and placental formation, with full body knockout resulting in embryonic lethality (Marine et al., 1999; Roberts et al., 2002). Conditional knockout mouse models have also confirmed that SOCS3 has roles in neural leptin signalling, granulopoiesis and regulation of G-CSF and IL-6 signalling (B. A. Croker et al., 2012, 2004; Mori et al., 2004; P. K. K. Wong et al., 2006). SOCS3 functions by negatively regulating the activity of JAK proteins.

There are four mammalian JAKS: JAK1, JAK2, JAK3 and Tyk2. In a resting state, JAKs are found bound to cytokine receptors. Upon ligand binding, receptor activation leads to tyrosine phosphorylation of the cytoplasmic tail of the receptor, and subsequent phosphorylation and activation of the bound JAK molecule (Babon et al., 2014). The SH2 domain of SOCS3 allows it to recognise the phoshotyryosine residues of specific receptors such as gp130, leptin-R and G-CSFR (B. A. Croker et al., 2004; Mori et al., 2004; Nicholson et al., 2000). The KIR domain of SOCS3 allows it to bind JAK1, JAK2 and Tyk2 but not JAK3 (Babon et al., 2014). The dual interaction of SOCS3 with receptor SOCS3-binding sites and bound JAKs allows the inhibition of SOCS3 to be confined to certain

receptors, as opposed to targeting all JAK1, JAK2 and Tyk2 signalling. The exquisite specificity of this system can be obscured by protein over-expression. For example, SOCS1 was originally described as an *in vitro* inhibitor of IL-6 signalling (Naka et al., 1997). However, subsequent characterisation of SOCS1$^{-/-}$ cells and mice showed that SOCS1 had no effect on IL-6 signalling, and instead inhibited interferon signalling (Alexander et al., 1999; B. A. Croker et al., 2003).

In particular, SOCS3 has been shown to be a key regulator of IL-6 signalling. IL-6 signalling results in the phosphorylation of STAT3, and to a lesser extent STAT1. Administration of IL-6 to mice or macrophages deficient in SOCS3 results in an increase in the duration of STAT1 and STAT3 phosphorylation (B. A. Croker et al., 2003; Lang et al., 2003; Whitmarsh et al., 2011). However, no prolonged phosphorylation was observed when mice or cells were administered with interferon-γ or IL-10, demonstrating the specificity of SOCS3 inhibition (B. A. Croker et al., 2003; Lang et al., 2003; Whitmarsh et al., 2011).

In addition, SOCS3 can qualitatively alter IL-6 signalling. Loss of SOCS3 has been shown *in vitro* and *in vivo* to result in increased transcription of genes characteristically associated with interferon-γ signalling (B. A. Croker et al., 2003; Lang et al., 2003). Some groups have also suggested that loss of SOCS3 can result in anti-inflammatory responses to IL-6 (Whitmarsh et al., 2011; Yasukawa et al., 2003), although this view has been contested (B. a Croker et al., 2012).

As a key inhibitor of cytokine signalling, SOCS3 has an important, but complex, role in fine-tuning the host response to pathogenic infection. *In vitro* studies have demonstrated that SOCS3 is upregulated upon infection with the parasite *Toxoplasma gondii* (*T. gondii*); the DNA viruses Herpes Simplex Virus 1 (HSV-1) and Hepatitis B Virus (HBV), and the ssRNA viruses Influenza A (IAV) and DENV (Koeberlein et al., 2010; Pothlichet, Chignard, & Si-Tahar, 2008; Tsai et al., 2014; Whitmarsh et al., 2011; Yokota et al., 2004). Although some groups have argued that SOCS3 acts to inhibit type I interferon signalling during viral infection, these results are based on protein overexpression and should be regarded with caution until they can be replicated *in vivo* (D. Liu et al., 2015; Pothlichet et al., 2008; Yokota et al., 2004).

IL-10 is an anti-inflammatory cytokine that is an inducer of SOCS3 (Ito et al., 1999). Several studies have suggested that an IL-10-SOCS3 axis has a role in the pathogenesis of severe dengue. *in vitro* studies have shown that THP-1 monocyte cells produce IL-10 upon DENV infection, and PBMCs from severe dengue patients upregulate SOCS3 to a higher degree than dengue fever patients (Ubol, Phuklia, Kalayanarooj, & Modhiran, 2010). More convincingly, two independent meta-analyses found that elevated IL-10 is a potential prognostic marker for severe dengue (Y. H. Lee et al., 2016; Soo et al., 2017).

To determine the effect on dengue infection of potentiating cytokine signalling, I infected SOCS3 deficient macrophages in the presence of MAR1. As $SOCS3^{-/-}$ mice are embryonic lethal, bone marrow from $SOCS3^{fl/fl}$ LysM-Cre ($SOCS3^{LysM}$) mice was used to generate BMDMs. The LysM-Cre conditionally deletes targeted genes in myeloid cells such as monocytes and macrophages (Clausen et al., 1999). $SOCS3^{LysM}$ BMDMs were treated and infected in an identical fashion as the $p50^{-/-}$ BMDMs described above. Cells were harvested at 48 hours post infection and prepared for flow cytometry. Flow cytometric analysis of $SOCS3^{LysM}$ BMDMs revealed that these cells did not have statistically significant differences compared to WT cells in terms of their expression of DENV NS1 (Figure 3.7). There was also no difference in the viability of $SOCS3^{LysM}$ and WT cells in the context of infection (Figure 3.8). It is possible that a loss of SOCS3 in myeloid cells could affect the wider host immune response to DENV infection. For example, previous work has shown that the loss of SOCS3 in myeloid cells of mice infected with *T. gondii* can alter T cell activation and cytokine production (Whitmarsh et al., 2011). Therefore, I also examined the disease course of DENV2 infection of $SOCS3^{LysM}$ mice, and these results are presented in Chapter 4.

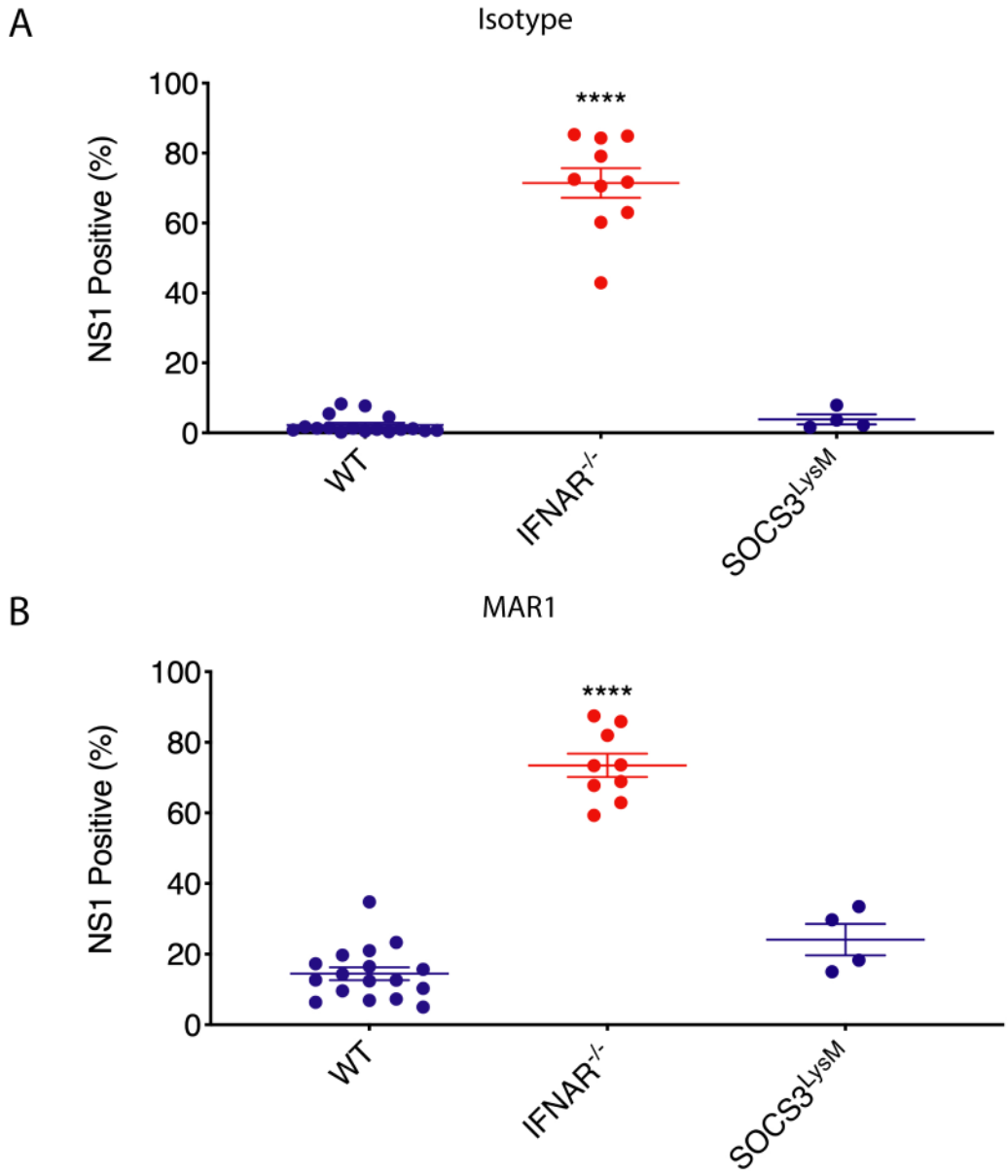
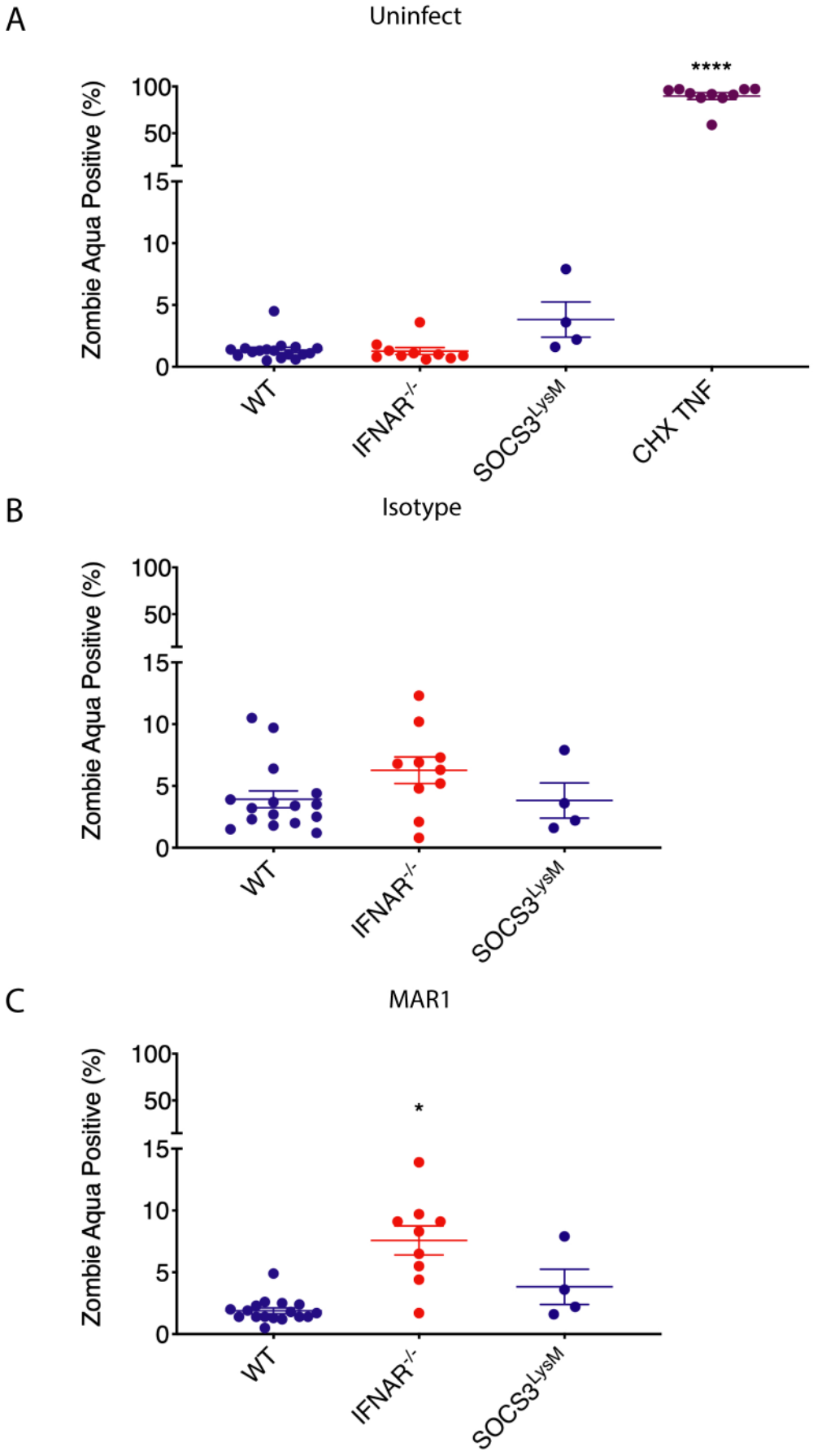


Figure 3.7 SOCS3^{LysM} BMDMs do not have an enhanced susceptibility to DENV infection. Flow cytometric analysis of BMDMs, pre-treated with (A) isotype control or (B) MAR1 antibody 48 hours post infection with DENV2. Cells were stained with NS1 to determine the proportion of infected cells. Every dot represents an individual biological replicate. SOCS3 data is combined from 4 independent experiments. Error bars indicate the mean and SEM. Statistical analysis: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons. **** $p < 0.0001$.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IFNAR-/-
SOCS3LysM

Figure 3. 8 Loss of SOCS3 does not affect the viability of DENV infected BMDMs. Cell death was quantified by flow cytometry in BMDMs that were left (A) Uninfected or were infected with (B, C) DENV2. DENV2 infected cells were either treated with (B) isotype antibody (C) MAR1. All BMDMs were harvested 48 hours after infection for analysis. Every dot represents BMDMs from an independent biological replicate. BMDMs treated with CHX and TNF were used as a positive control for the death assay. SOCS3 data is combined from 4 independent experiments. Error bars indicate the mean and SEM. Statistical analysis: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons. * $p < 0.05$, **** $p < 0.0001$.

3.5 Investigating the effect of SIDT2 genetic knockout on *in vitro* DENV infection

DENV and many other ssRNA viruses produce dsRNA intermediates during viral replication (Dansako et al., 2013; Welsch et al., 2009). These dsRNA species act as PAMPs that are recognised by various PRR families such as the RLRs and TLR3 (Kawasaki & Kawai, 2014; Yoneyama et al., 2004). PRR signalling in response to dsRNA results in activation of the transcription factors IRF3, IRF7 and NF-κB and the upregulation of an antiviral transcription programme (Kawasaki & Kawai, 2014; Yoneyama et al., 2004). Crucially, this antiviral state can be spread to neighbouring cells by the paracrine action of type I Interferons, reviewed in (Ivashkiv & Donlin, 2014).

Recently, several groups have showed that PRR signalling can be induced in bystander cells via the uptake of dsRNA. Early research showed that the viral dsRNA analogue poly(I:C) could be taken up by clathrin-mediated endocytosis (Itoh, Watanabe, Funami, Seya, & Matsumoto, 2008). It was recently reported that extracellular HCV dsRNA could trigger TLR3 signalling and restrict HCV replication (Dansako et al., 2013). Another group found that extracellular dsRNA from ssRNA and dsDNA viruses could be trafficked from endo-lysosomes to the cytoplasm by the transmembrane protein SIDT2 (T. A. Nguyen et al., 2017). SIDT2$^{-/-}$ mice infected with EMCV succumbed rapidly, produced more virus, and had lower serum levels of interferon-β than WT control mice. In addition, SIDT2$^{-/-}$ cells were unable to activate RLR signalling in bystander cells, due to their inability to transport endocytosed dsRNA to the cytoplasm (T. A. Nguyen et al., 2017).

Interestingly, EMCV and DENV are both ssRNA viruses that have observable dsRNA intermediates (Weber, Wagner, Rasmussen, Hartmann, & Paludan, 2006; Welsch et al., 2009). EMCV and DENV are both also lytic viruses, and lysis of infected cells has been hypothesised to increase levels of extracellular viral dsRNA (T. A. Nguyen et al., 2017). This led me to hypothesise that SIDT2 could be a host restriction factor for DENV infection. To examine this hypothesis, I infected SIDT2$^{-/-}$ BMDMs.

SIDT2$^{-/-}$ BMDMs were treated and infected in an identical fashion as the p50$^{-/-}$ BMDMs described above. In a similar fashion to p50$^{-/-}$ and SIDT2$^{-/-}$ BMDMs, flow cytometry analysis of SIDT2$^{-/-}$ BMDMs revealed that these cells did not have statistically significant differences to WT cells in terms of their expression of DENV NS1 (Figure 3.9). Interestingly, Nguyen and colleagues found that SIDT2$^{-/-}$ mouse embryonic fibroblasts (MEFs) were more susceptible to infection with HSV-1 than WT MEFs and SIDT2$^{-/-}$ mice were also more susceptible to infection with HSV-1 and EMCV than WT controls (T. A. Nguyen et al., 2017). My result suggests that the SIDT2 channel, in my BMDM model, is not an important factor restricting DENV infection. Flow cytometry was also used to look for differences in levels of cell death between WT and SIDT2$^{-/-}$ BMDMs. However, no differences were observed (Figure 3.10).

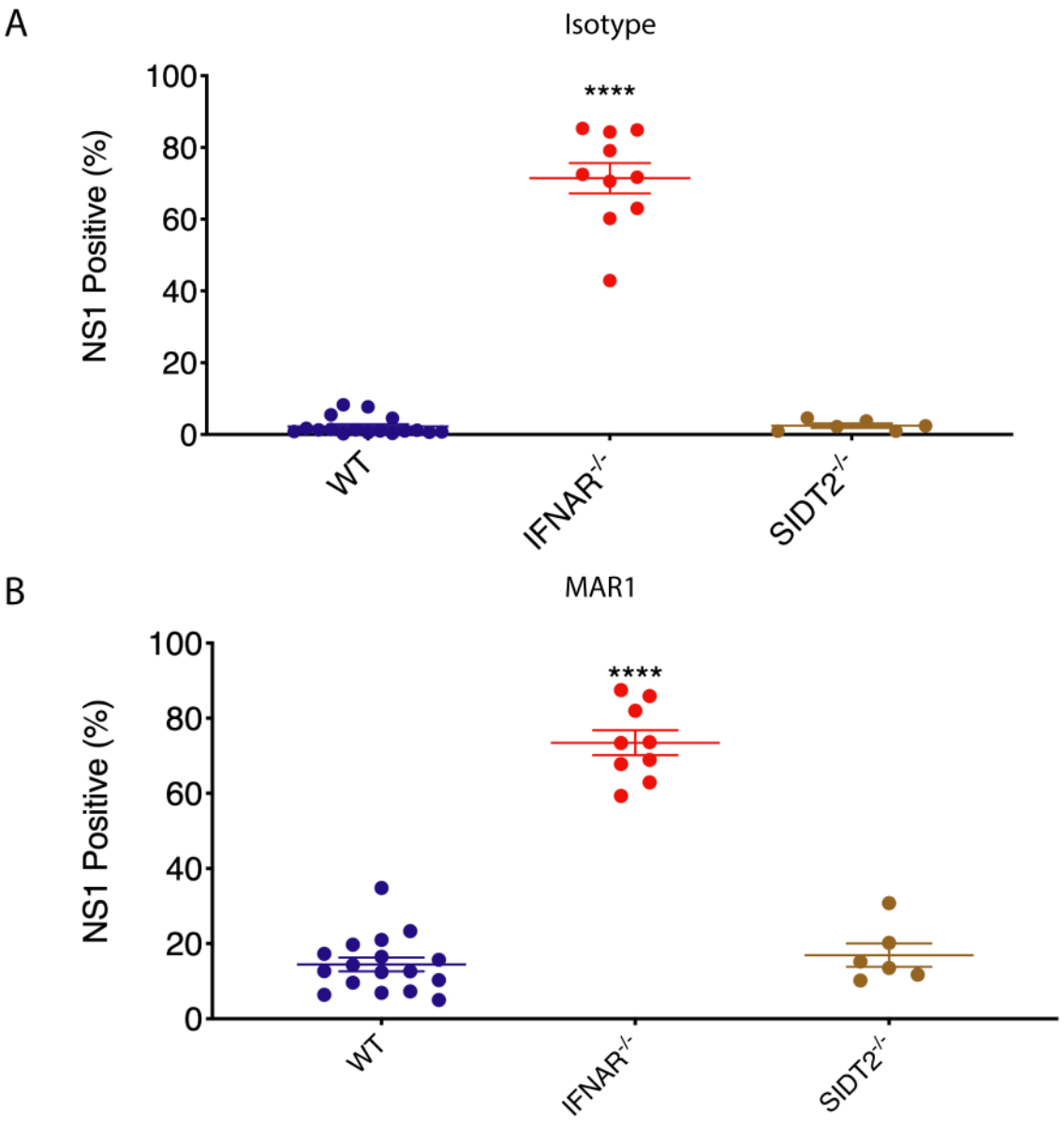


Figure 3.9 SIDT2$^{-/-}$ BMDMs have a normal level of susceptibility to DENV infection relative to WT BMDMs. BMDMs were treated with isotype control (A) or MAR1 (B) and infected with DENV2 at a MOI of 1. Cells were harvested at 48 hours post infection and prepared for flow cytometry. Quantification of NS1 is shown above. Every dot represents BMDMs from an independent biological replicate. SIDT2$^{-/-}$ data is combined from 4 independent experiments. Statistical analysis: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons. **** $p < 0.0001$.

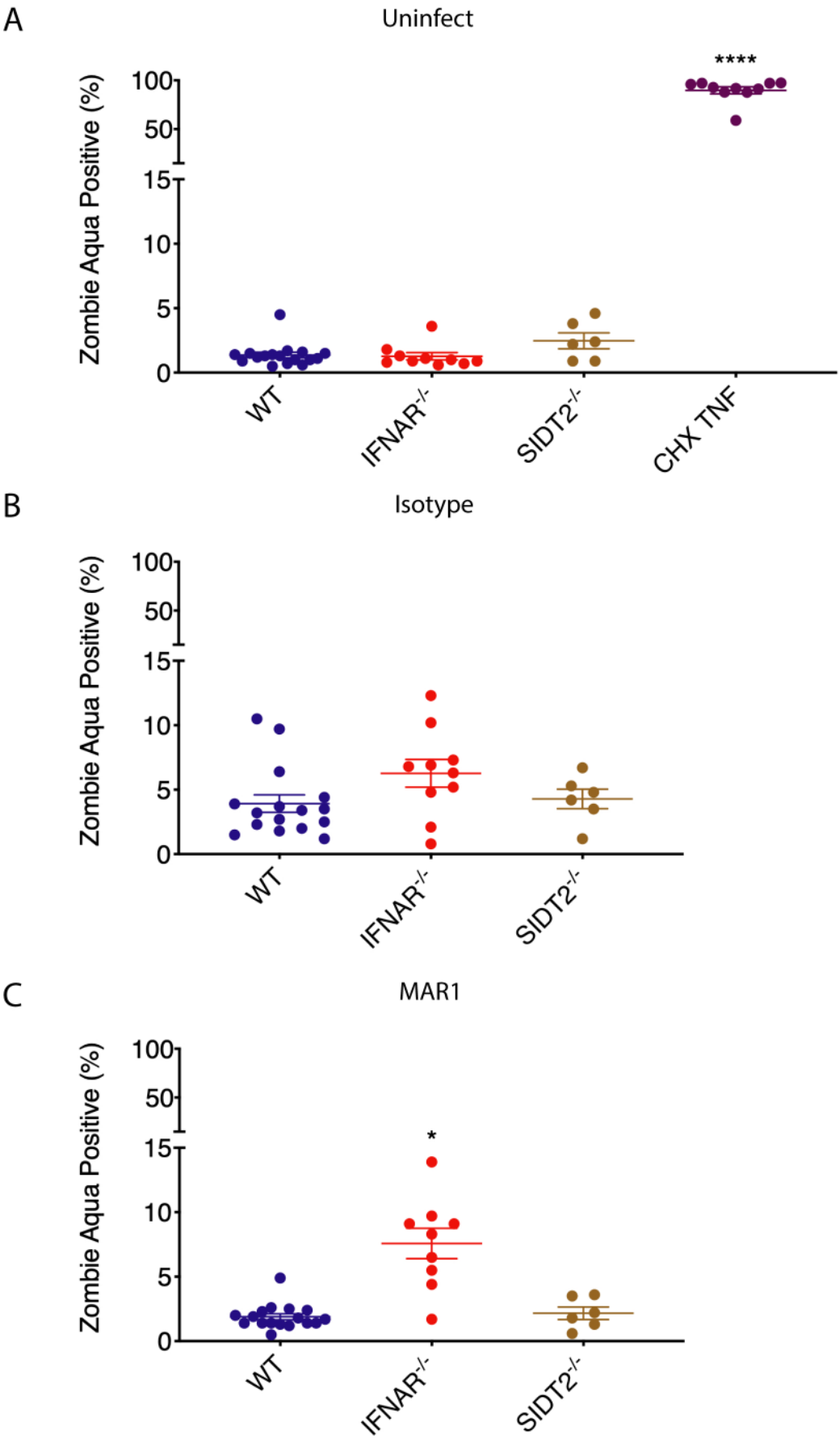


Figure 3.10 SIDT2$^{-/-}$ BMDMs do not have changed levels of cell death in response to

DENV infection. Cell death was quantified by flow cytometry in BMDMs that were left (A) Uninfected or were infected with (B, C) DENV2. DENV2 infected cells were either treated with (B) isotype antibody (C) MAR1. All BMDMs were harvested 48 hours after infection for analysis. Every dot represents BMDMs from an independent biological replicate. WT BMDMs treated with CHX and TNF were used as a positive control for the death assay. SIDT2 data is from 4 independent experiments. Error bars indicate the mean and SEM. Statistical analysis: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons. * $p < 0.05$, **** $p < 0.0001$.

3.6 Investigating the effect of IAP deficiency on *in vitro* DENV infection

Pro-survival and pro-inflammatory NF-κB signalling can be activated by TNF. When TNF binds to TNFR1, cIAP1 and cIAP2 and associate with the TNFR signalling complex by binding to the adaptor TRAF2 (Vince et al., 2009). The cIAPs are able to attach K11, K48 and K63 ubiquitin linkages to TRAF2 (Li X, Yang Y, & Ashwell JD., 2002), RIP1 (Bertrand et al., 2008; Dynek et al., 2010; Mahoney et al., 2008), NEMO (E. D. Tang, Wang, Xiong, & Guan, 2003) and the cIAPs themselves (Conze et al., 2005). This allows recruitment of the LUBAC complex, comprised of HOIL, HOIP and SHARPIN (Gerlach et al., 2011; Haas et al., 2009; Tokunaga et al., 2009). The attachment of linear ubiquitin chains stabilises the TNFR1 signalling complex and is required for efficient NF-κB and JNK signalling by the complex (Haas et al., 2009). Following LUBAC recruitment, the TAK1-TAB1-TAB2 kinase and IKK complexes are able to dock to NEMO and facilitate removal of IκB inhibition via site-specific phosphorylation (reviewed in (Adhikari, Xu, & Chen, 2007)). Therefore, cIAPs play a crucial role in the transduction of canonical NF-κB signalling. NF-κB activation initiates the transcription of pro-inflammatory cytokines such as IL-6 and interferon-β, and the transcription of pro-survival genes such as cFLIP (Micheau, Lens, Gaide, Alevizopoulos, & Tschopp, 2001; Pahl, 1999). During DENV infection, NF-κB activation downstream of the PRR pathways is purported to be an important component of the anti-DENV immune response.

When cIAP proteins are absent, RIP1 cannot be ubiquitinated downstream of receptor ligation, and consequently, NF-κB cannot be activated (Bertrand et al., 2008;

Varfolomeev et al., 2008). As NF-κB signalling upregulates the transcription of potent anti-apoptotic factors such as cFLIP (Micheau et al., 2001), a reduction in NF-κB signalling sensitises cells to apoptosis. Therefore, the loss of cIAPs sensitizes cells to apoptosis downstream of death receptor and TLR ligation (Lalaoui & Vaux, 2018). As described in the introduction, apoptosis appears to be active during dengue, but does not play a causal role in the pathogenesis of severe dengue (Chapter 1, section 1.4.2).

Given the key role of cIAPs in promoting NF-κB signalling, host transcriptional activity and cell survival, I hypothesised that the lack of cIAPs would impair DENV replication and increase levels of cell death. To determine the function of cIAPs in dengue virus infection, I infected IAP deficient BMDMs in the presence of MAR1.

Flow cytometric analysis showed that there was a statistically significant increase in NS1 staining at 48 hours post infection (hpi) when cIAP2$^{-/-}$ BMDMs were infected with DENV2 in the presence of MAR1 (Figure 3.11). The mean ± SEM of NS1 positive cells was 27.6 ± 4.18 percent for cIAP2$^{-/-}$ BMDMs compared with 14.5 ± 1.81 percent for WT BMDMs. There was no statistically significant increase in NS1 staining when cIAP2$^{-/-}$ and WT cells were infected in the presence of isotype control (Figure 3.11). Contrastingly to the cIAP2$^{-/-}$ MAR1 result, when cIAP1$^{-/-}$ or c1^{LysM}/cIAP2$^{-/-}$ BMDMs were infected in the presence of MAR1 no statistically significant differences in NS1 staining were observed relative to WT controls (Figure 3.11).

It was surprising that cIAP1$^{-/-}$ and cIAP2$^{-/-}$ cells did not have the same susceptibility to DENV2 when infected in the presence of MAR1. cIAP1 and cIAP2 are both E3 ubiquitin ligases that have closely overlapping roles in control of apoptosis and TNF signalling. It has been well documented that these proteins are able to compensate for each other in the event of genetic deletion of the other (Mahoney et al., 2008; Moulin et al., 2012; J. Zhang et al., 2019). Therefore, it would be plausible to expect that any phenotype associated with lack of one cIAP would also be apparent when the other cIAP was deleted.

As described above, when the pro-survival ubiquitin ligase function of cIAPs is impaired or absent, TNF can stimulate cell death by the extrinsic apoptotic pathway. However,

no differences in levels of cell death were observed relative to WT controls when cIAP1$^{-/-}$, cIAP2$^{-/-}$, or c1^{LysM}/cIAP2$^{-/-}$ BMDMs were infected in the presence of isotype or MAR1 (see figure 3.12). It was surprising to see a lack of cell death when both cIAPs were deleted. One possible explanation was that cIAP1 was not being efficiently deleted *in vitro* by the LysM-Cre system. However, efficient deletion of cIAP1 was subsequently confirmed by western blot (Figure 3.15). My observation that there was a low level of cell death during DENV infection of IAP deficient BMDMs would make sense if no TNF was present in the system to trigger cell death. However, this would be unexpected as TNF has previously been reported to be produced by macrophages upon DENV infection (Y.-C. Chen & Wang, 2002; M. Wu et al., 2013). I determined levels of cytokine production during DENV infection of BMDMs, and these results are reported below in section 3.8.

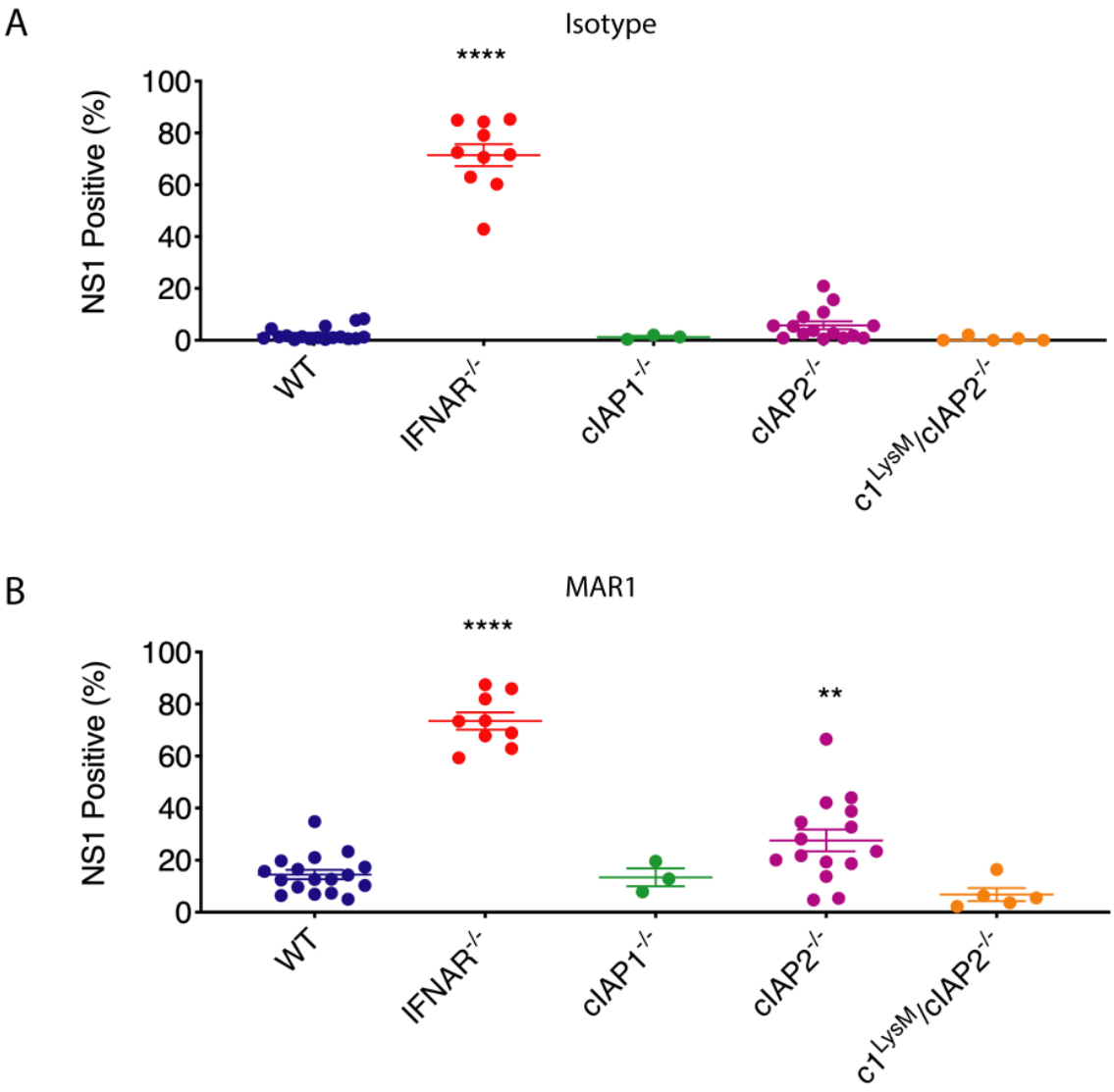


Figure 3.11 cIAP2$^{-/-}$ BMDMs, but not cIAP1$^{-/-}$ or c1^{LysM}/cIAP2$^{-/-}$ BMDMs, are more susceptible to DENV infection than WT BMDMs. BMDMs were treated with isotype control (A) or MAR1 (B) and infected with DENV2 at a MOI of 1. Cells were harvested at 48 hours post infection and prepared for flow cytometry. Quantification of NS1 is shown above. Every dot represents BMDMs from an independent biological replicate. cIAP1$^{-/-}$ data is from 3 independent experiments. cIAP2$^{-/-}$ data is from 8 independent experiments. c1^{LysM}/cIAP2$^{-/-}$ data is from 3 independent experiments. Statistical analysis: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons. ** $p < 0.01$, **** $p < 0.0001$.

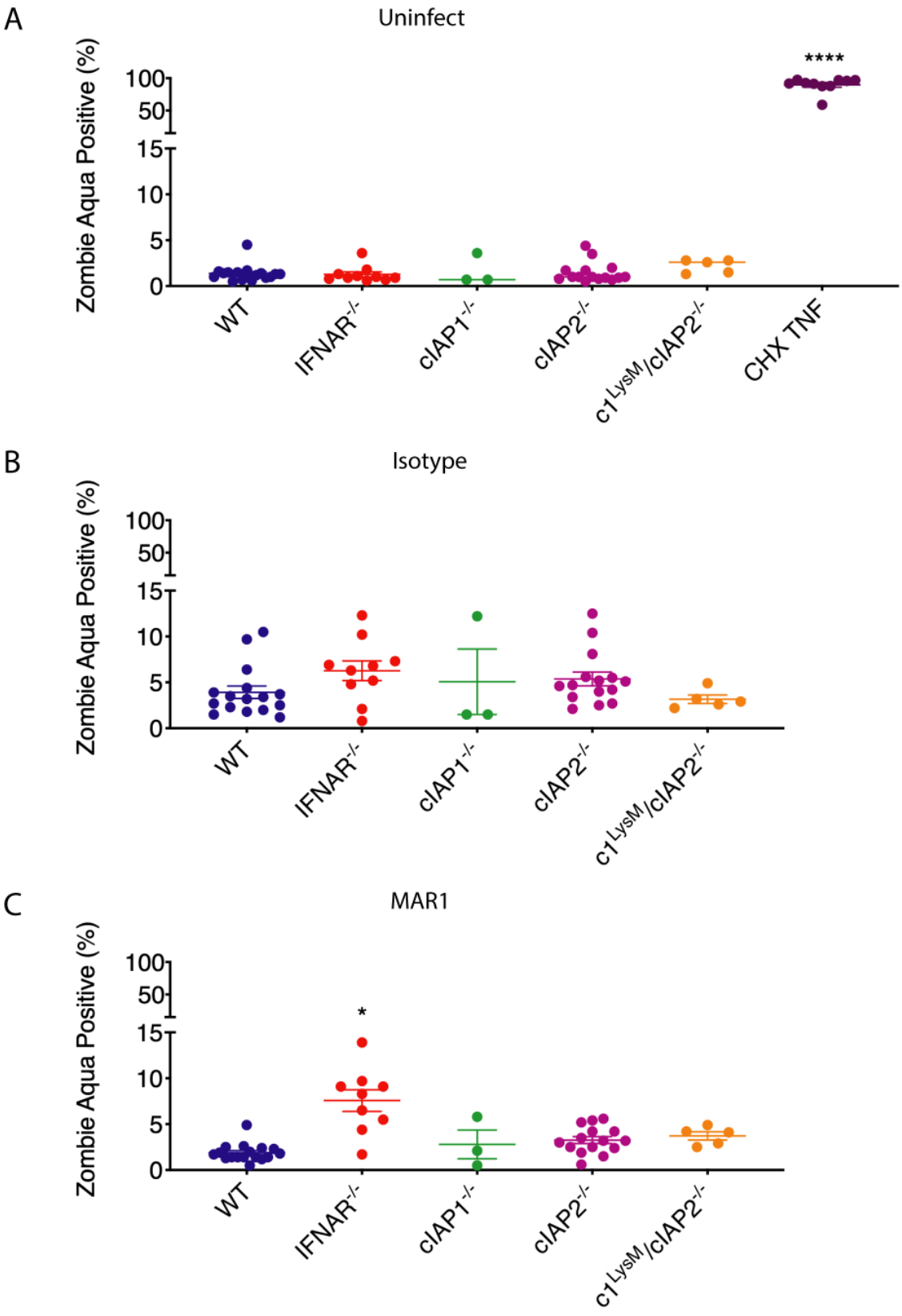


Figure 3.12 cIAP1$^{-/-}$, cIAP2$^{-/-}$ and c1^{LysM}/cIAP2$^{-/-}$ BMDMs do not have changed levels of cell death in response to DENV infection. Cell death was quantified by flow cytometry

in BMDMs that were left (A) Uninfected or were infected with (B, C) DENV2. DENV2 infected cells were either treated with (B) isotype antibody (C) MAR1. All BMDMs were harvested 48 hours after infection for analysis. Every dot represents BMDMs from an independent biological replicate. WT BMDMs co-treated with CHX and TNF were used as a positive control for the death assay. cIAP1$^{-/-}$ data is from 3 independent experiments. cIAP2$^{-/-}$ data is from 8 independent experiments. c1^{LysM}/cIAP2$^{-/-}$ data is from 3 independent experiments. Error bars indicate the mean and SEM. Statistical analysis: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons. * $p < 0.05$, **** $p < .0001$.

3.7 Investigating the effect of IAP deficiency on *in vitro* DENV production using plaque assays

My flow cytometry results showed that cIAP2$^{-/-}$, but not cIAP1$^{-/-}$ or c1^{LysM}/cIAP2$^{-/-}$ BMDMs had increased levels of NS1 when infected in the presence of MAR1 antibody (Figure 3.11). To further investigate the effect of IAP deficiency on DENV infection, I chose to quantify the ability of IAP deficient BMDMs to produce replication-competent DENV. To do this, I took the supernatant from MAR1-treated WT and IAP deficient BMDMs at 48hr post infection and determined levels of viral production by plaque assay.

Surprisingly cIAP2$^{-/-}$ did not produce significantly more virus than WT cells and double knockout cells produced less virus than WT cells (see figure 3.13). The log-transformed mean viral production ± SEM, in pfu/mL, was 3.97 ± 0.14 for cIAP2$^{-/-}$ BMDMs; and 2.82 ± 0.33 for c1^{LysM}/cIAP2$^{-/-}$ BMDMs; compared with 3.7 ± 0.15 for WT BMDMs. Supernatants from IFNAR$^{-/-}$ positive controls produced roughly two orders of magnitude more virus (6.220 ± 0.16) than WT BMDMs.

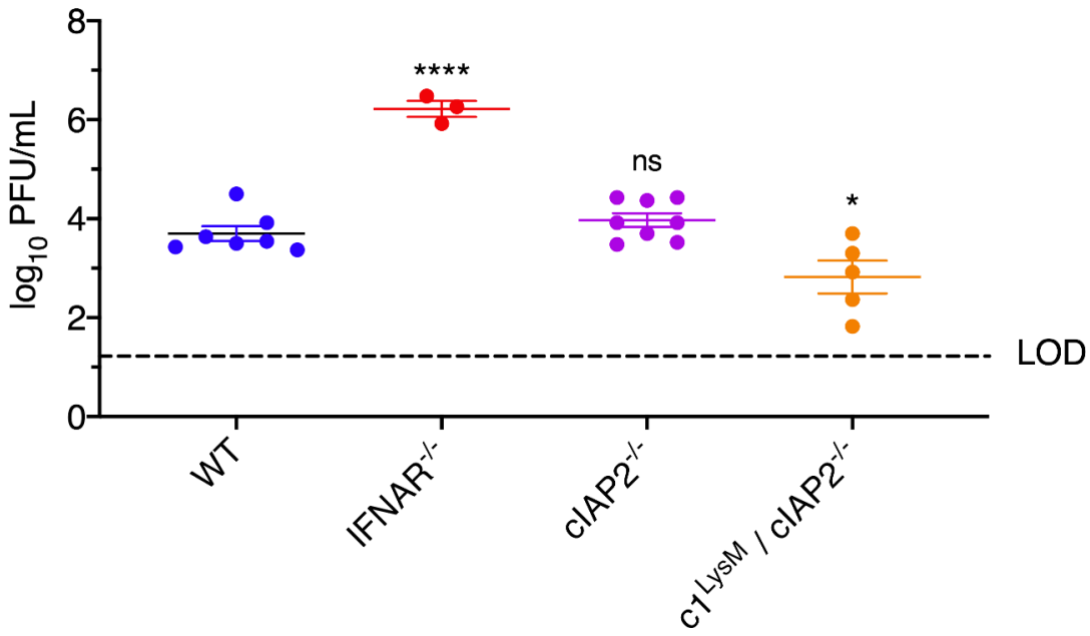
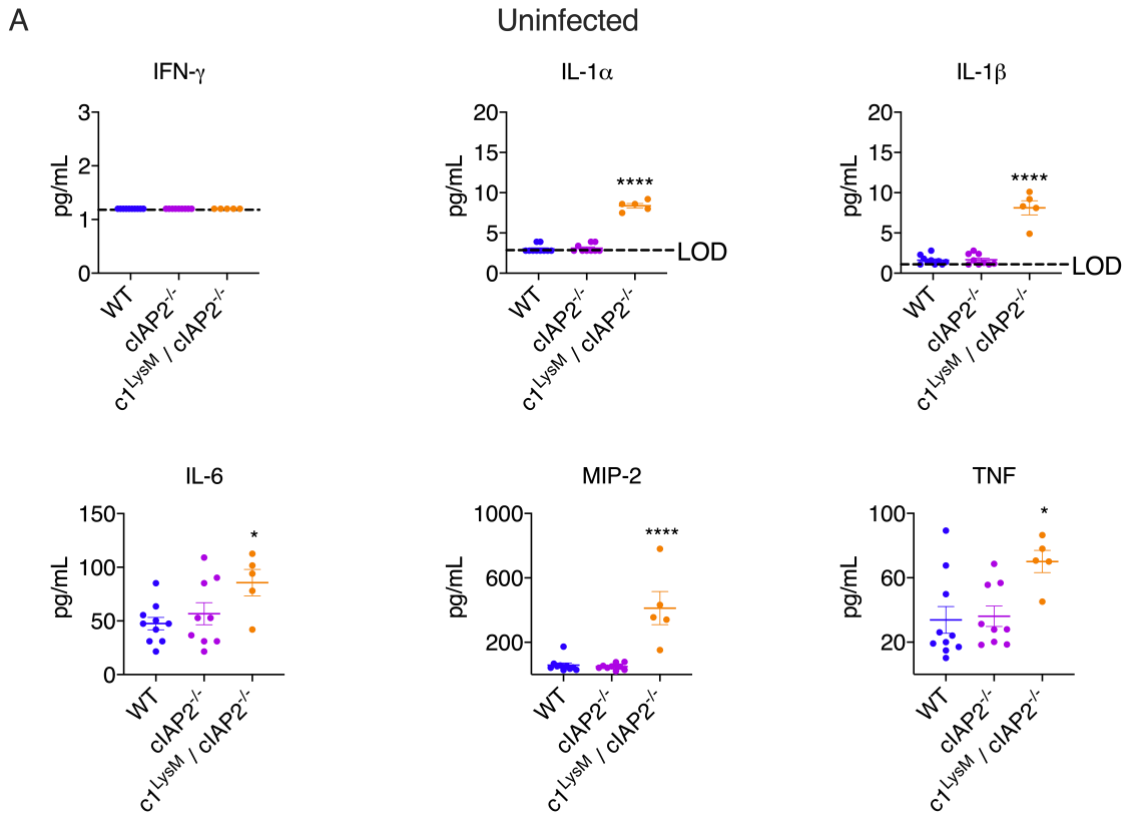


Figure 3.13 cIAP1^{LysM}/cIAP2$^{-/-}$ BMDMs produce less virus than WT controls. Supernatants from MAR1-treated BMDM cultures at 48hr post infection were titrated by plaque assay. Every dot represents BMDMs from an independent biological replicate. BMDM supernatants were collected from three independent experiments. Statistical analysis: ordinary one-way ANOVA of log-transformed data followed by Dunnett's test for multiple comparisons. * $p < 0.05$, **** $p < 0.0001$.

3.8 Investigating the effect of IAP deficiency on cytokine production during DENV infection

During severe dengue, cytokine storms cause transient changes in endothelial permeability and subsequent haemorrhage and hypotension (Rothman, 2011). The cytokine TNF can also trigger the extrinsic pathway of apoptosis if cells have compromised pro-survival NF-κB signalling (Mahoney et al., 2008; Varfolomeev et al., 2008). To further dissect the effect of IAP deficiency on the response to DENV infection, levels of pro-inflammatory cytokines were assessed in supernatants from uninfected BMDMs, and infected BMDMs treated with MAR1 (Figure 3.14). Levels of interferon-γ produced from uninfected and infected cells were universally low. This was expected as interferon-γ is predominantly produced by T cells and NK cells, and not by macrophages. Unexpectedly, levels of other quantified cytokines were increased in the supernatants of uninfected cIAP1^{LysM}/cIAP2$^{-/-}$ BMDMs (Figure 3.14A). This was

surprising as cytokine release from naïve BMDMs has been demonstrated previously, but required the combined loss of cIAP1, cIAP2 and X-linked Inhibitor apoptosis Protein (XIAP) (W. W. L. Wong et al., 2014). Significantly more IL-1β was detected in the supernatant of infected cIAP1^{LysM}/cIAP2$^{-/-}$ BMDMs relative to WT, and significantly more TNF was detected in the supernatant of infected cIAP2$^{-/-}$ BMDMs, relative to WT (Figure 3.14B).



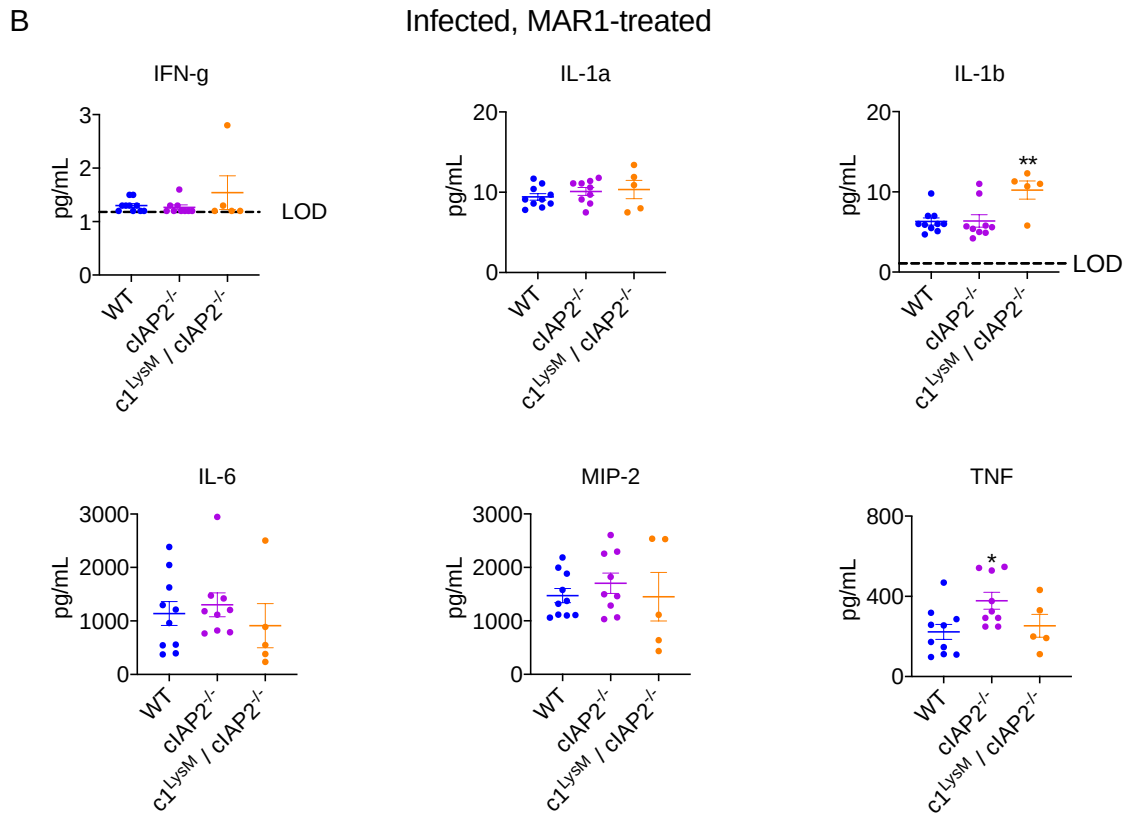


Figure 3.14 Cytokine levels of uninfected BMDMS, and of infected, MAR1-treated BMDMs at 48 hours post infection. Levels of cytokines in the supernatants of A) uninfected and, B) infected MAR1-treated BMDMs, were assessed by multiplex immunoassay. Every dot represents supernatant taken from an independent biological replicate. WT supernatants were collected from four independent experiments; cIAP2$^{-/-}$ and c1^{LysM}/cIAP2$^{-/-}$ supernatants were collected from three independent experiments. Statistical analysis: ordinary one-way ANOVA of log-transformed data followed by Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

3.9 The effect of drug-mediated NF-κB inhibition on *in vitro* DENV infection

To summarise previous experiments briefly, NS1 results and viral production results were non-concordant. NS1 levels of cIAP2$^{-/-}$ BMDMs were increased above baseline, although NS1 levels of double-deficient BMDMs were not statistically increased (Figure 3.11C). However, viral production of cIAP2$^{-/-}$ BMDMs was indistinguishable to WT cells, and viral production of c1^{LysM}/cIAP2$^{-/-}$ was reduced compared to WT cells (Figure 3.13).

Also, when I examined the ability of MAR1-treated, infected WT and IAP deficient BMDMs to produce cytokines, no clear differences were observed in patterns of cytokine production were observed, although production of individual cytokines were sometimes found to be increased (Figure 3.14B).

One possible explanation for the observed decrease in viral production from c1$^{LysM/}$cIAP2$^{-/-}$ could be that DENV requires a certain level of NF-κB activity to drive viral replication. In the absence of cIAPs, NF-κB signalling can be blunted (Mahoney et al., 2008). As one of the most important signalling pathways in mammalian cells, the NF-κB pathway is often hijacked by viruses. For instance, the Tax protein of HTLV-1 is able to bind the IKK complex and constitutively activate NF-κB signalling in infected T cells (Z. L. Chu et al., 1999). It is thought that this is required to maintain the transformed phenotype of HTLV-1 infected cells (Z. L. Chu et al., 1999). In addition, monocytes chronically infected with HIV show pronounced NF-κB induction and increased activation of the HIV enhancer region (Bachelerie et al., 1991).

I previously examined the effect of NF-κB signalling on *in vitro* DENV infection by infecting p50$^{-/-}$ BMDMs. p50 homodimers can activate transcription in some circumstances (Baek et al., 2002; Fujita et al., 1992), but normally repress transcription (Bohuslav et al., 1998; Udalova et al., 2000). Accordingly, the hypothesis behind these experiments was that removal of transcriptional repression could result in increased transcription and apoptosis. However, I found that p50$^{-/-}$ BMDMs had no differences in their susceptibility to DENV infection and virus induced cell death (see Figures 3.5 and 3.6). However, it is possible that some degree of genetic compensation, e.g. overexpression of other NF-κB subunits, exists in p50$^{-/-}$ BMDMs.

My *in vitro* c1$^{LysM/}$cIAP2$^{-/-}$ plaque assay results (see Figure 3.13) suggest that cIAPs could be used by DENV to transduce NF-κB signalling, and establish a transcriptionally active environment in the cell which is favourable for viral replication. To investigate this hypothesis, I decided to attempt to block NF-κB signalling in BMDMs during DENV infection. I used two compounds to block NF-κB signalling. LCL-161 is a monovalent IAP antagonist that can efficiently degrade cIAP1, and to a variable effect cIAP2 and XIAP (K. F. Chen et al., 2012; Ramakrishnan et al., 2014). As a monovalent IAP antagonist, LCL-

161 can be given orally, and has been well tolerated in clinical oncology trials at doses of up to 1200mg weekly (Chesi et al., 2016). BAY 11-7082 (henceforth referred to as "BAY") acts to potently inhibit NF-κB signalling by blocking the degradation of IκB molecules, therefore preventing translocation of the p50-P65 heterodimer to the nucleus (Pierce et al., 1997).

3.9.1 LCL-161 degradation of cIAP1

To confirm that LCL-161 was active in my *in vitro* system, I treated WT BMDMs with DMSO or LCL-161. I found that LCL-161 was able to potently degrade cIAP1 (Figure 3.15), as per previous reports (K. F. Chen et al., 2012; Ramakrishnan et al., 2014). A recent paper has discussed the mechanism by which LCL-161 is likely to trigger the degradation of cIAP proteins (K. F. Chen et al., 2012).

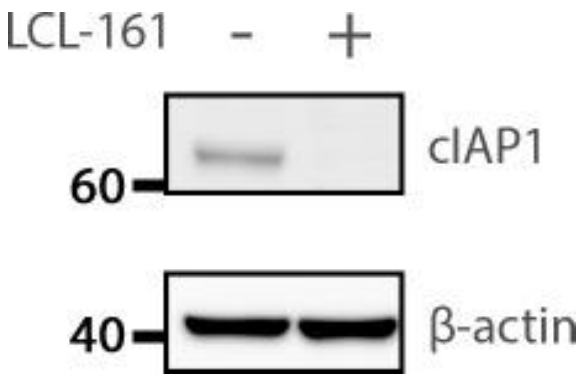


Figure 3.15 Confirmation that LCL-161 treatment potently degrades cIAP1 in BMDMs. WT BMDMs were treated with DMSO or 0.63μM LCL-161. 60 minutes after treatment, cells were harvested and levels of cIAP1 and β-actin were determined by western blot. Data is representative of one independent experiment.

3.9.2 Determination of optimal LCL-161 dosage for *in vitro* experiments

To select a dose of LCL-161 that was not toxic for cells, I infected WT BMDMs with DENV2 for 90 minutes in the presence of MAR1, and treated wells with a two-fold dilution series of LCL-161 (Figure 3.16). After 48 hours, cells were harvested, stained for NS1 and zombie aqua, and analysed by flow cytometry. I selected a dose of 0.63μM as optimal for future experiments, as concentrations higher than this resulted in high levels of cell death as quantified by zombie aqua staining. In addition, this dose was sufficient to trigger degradation of cIAP1 (see figure 3.15 above).

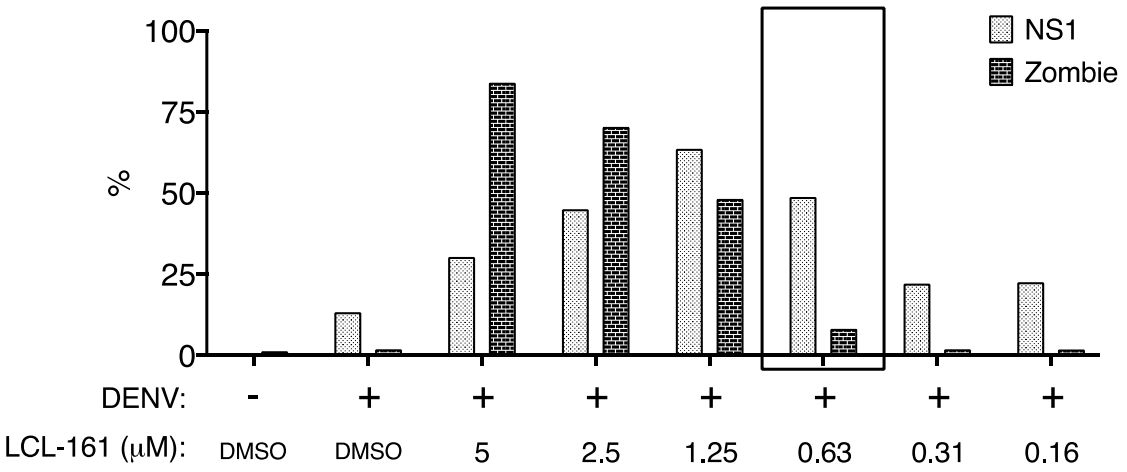


Figure 3.16 Selection of a working dose of LCL-161 for *in vitro* assays. WT BMDMs were treated with MAR1 antibody alone, or infected with DENV2 (MOI 1) in the presence of MAR1. Following infection, media was changed and more MAR1 was added. BMDMs were treated with the concentrations of LCL-161 indicated; cultures not treated with LCL-161 were treated with 1% DMSO. After 48 hours, cultures were stained for viral antigen (NS1) and cell death (zombie aqua) and analysed by flow cytometry. The concentration of drug selected for future experiments is shown by the rectangle. Results are from one independent experiment.

3.9.3 Treatment of BMDMs with LCL-161 does not reduce viral production

To determine whether LCL-161 treatment could reduce viral replication during *in vitro* DENV infection, I treated WT and IFNAR$^{-/-}$ BMDMs with LCL-161 either "Pre" or "Post" infection and analysed them by flow cytometry. To enhance susceptibility to infection, mock and infected BMDMs were also treated with MAR1 before and following infection. There was a trend for LCL-161 to increase the susceptibility of WT BMDMs to infection, regardless of whether or not LCL-161 was applied pre- or post- infection (Figure 3.17A). However, this trend did not reach statistical significance in either case. Contrastingly, treatment of IFNAR$^{-/-}$ BMDMs with LCL-161 either prior to or following infection did not increase levels of NS1 staining (Figure 3.17B).

Treatment of WT and IFNAR$^{-/-}$ BMDMs with LCL-161did not increase levels of cell death, relative to a DMSO-treated WT control (Figure 3.17C and 3.17D). This finding is

consistent with the lack of cell death observed when I infected c1$^{LysM/}$cIAP2$^{-/-}$ with DENV2 (Figure 3.12C).

Finally, I assessed the effect of LCL-161treatment on viral production. To determine levels of viral production, frozen supernatants collected at 48hpi from both WT and IFNAR$^{-/-}$ BMDMs were titrated by plaque assay. The plaque assay results revealed that treatment of BMDMs with LCL-161prior to or post infection did not alter levels of virus production relative to a DMSO only control (3.17E and 3.17F). This finding was in contrast to the reduction in viral production observed when c1$^{LysM/}$cIAP2$^{-/-}$ BMDMs were infected with DENV2 in the presence of MAR1 (Figure 3.13).

Overall, these results show that, although LCL-161may have some effect on production of viral NS1, production of infectious virions was unchanged. There could be several explanations for this. Firstly, although I confirmed that LCL-161potently degraded cIAP1, to date there is not a high-quality antibody that can detect mouse cIAP2 by western blot. Other groups have shown in studies of human cancer cells that the ability of LCL-161 to degrade cIAP2 *in vitro* is variable (K. F. Chen et al., 2012; Ramakrishnan et al., 2014). Therefore, I cannot rule out the possibility that NF-κB signalling is active in these cells despite LCL-161 treatment. Secondly, it is possible that c1$^{LysM/}$cIAP2$^{-/-}$ BMDMs have developed a degree of genetic compensation for the lack of cIAPs, and this compensation is absent in the context of acute IAP degradation triggered by LCL-161 treatment, and this difference contributes to the different ability of these different cells to produce virus.

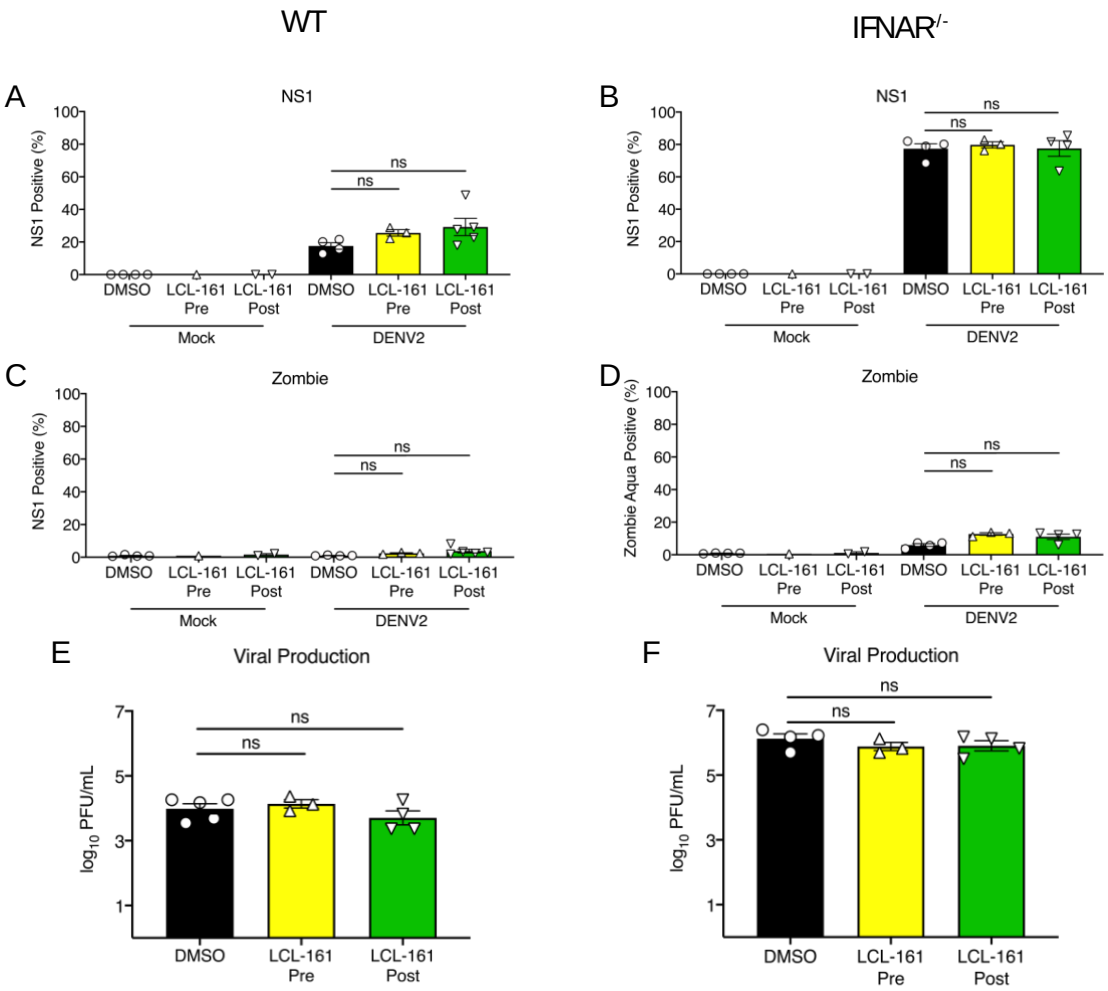


Figure 3.17 LCL-161 treatment does not alter DENV infection of BMDMs. WT and IFNAR$^{-/-}$ BMDMs were treated with DMSO or LCL-161 for either 2hr prior to ("Pre") or immediately following "Mock" or "DENV" infection ("Post") with a MOI of 1. All BMDM cultures were also treated with MAR1. At 48hpi, cells were processed for flow cytometry. The susceptibility of WT (A) and IFNAR$^{-/-}$ cells (B) to infection was assessed by staining for viral NS1. Measurement of zombie aqua staining by flow cytometry was used to determine levels of cell death for WT (C) and IFNAR$^{-/-}$ (D) BMDMs. Finally, supernatants harvested at 48hpi were used to determine levels of viral production from WT (E) and IFNAR$^{-/-}$ (F) BMDMs. Every dot represents cells (A-D) or supernatants (E and F) taken from an independent biological replicate. For A-D, n=2-5 from at least two independent experiments per condition with the exception of WT and IFNAR$^{-/-}$ mock-infected "Pre" treated BMDMs, where n=1. For E and F, n= 3-5 and data are pooled from 1-3 independent experiments. Statistical analysis of NS1 and Zombie flow cytometry data: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons.

Statistical analysis of plaque assay data: ordinary one-way ANOVA of log-transformed data followed by Dunnett's test for multiple comparisons.

3.9.4 Determination of optimal BAY dosage for *in vitro* experiments

To further dissect the importance of NF-kB signalling to DENV infection *in vitro*, I performed experiments using the NF-kB inhibitor BAY. As for the *in vitro* LCL experiments described above, I infected WT BMDMs with DENV2 in the presence of MAR1 and performed a 2-fold titration of BAY (Figure 18). Cells were harvested at 48 hours post-infection, stained for NS1 and zombie, and analysed by flow cytometry. I selected a dose of 2.5μM for usage the following experiments, as treatment of cells with 5μM resulted in >10% of cells staining positive for zombie aqua, compared to <5% with the 2.5μM dosage.

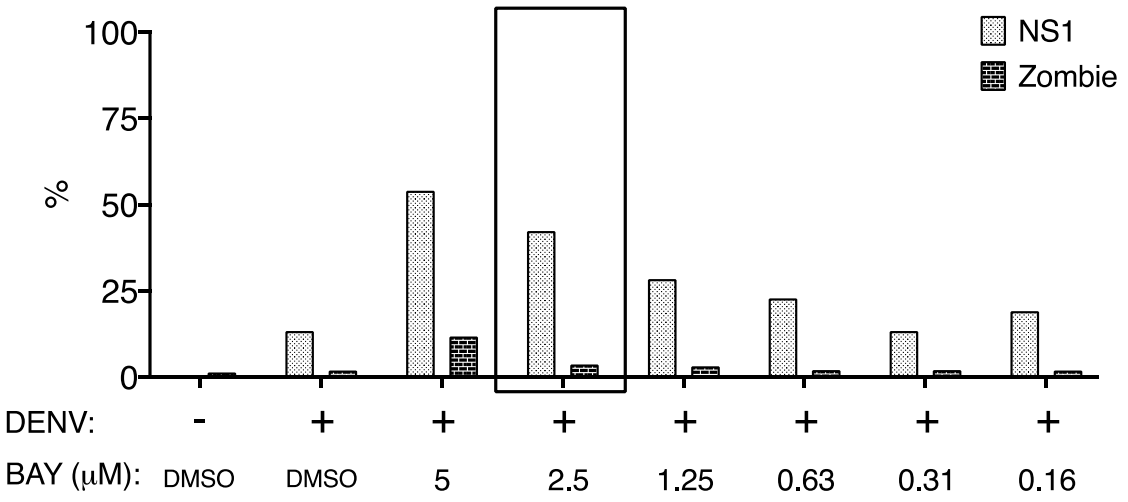


Figure 3.18 Selection of a working dose of BAY 11-7082 ("BAY") for *in vitro* assays. WT BMDMs were treated with MAR1 antibody alone, or infected with DENV2 (MOI 1) in the presence of MAR1. Following infection, media was changed and more MAR1 was added. BMDMs were treated with the concentrations of BAY indicated; cultures not treated with BAY were treated with 1% DMSO. After 48 hours, cultures were stained for viral antigen (NS1) and cell death (Zombie Aqua) and analysed by flow cytometry. The concentration of drug selected for future experiments is shown by the rectangle. Results are from one independent experiment.

3.9.5 Treatment of BMDMs with BAY does not reduce viral production

After optimising the dosage of BAY, I went on to examine the effect of this compound on susceptibility to infection, cell death and virus production. WT and IFNAR$^{-/-}$ BMDMs were treated with 2.5μM BAY either 2hr prior to infection ("Pre") or immediately following ("Post") infection with DENV2 at a MOI of 1. Mock and infected BMDMs were also treated with MAR1 before and following infection. Treatment of WT BMDMs with BAY either before or after infection increased levels of NS1 staining above that of DMSO-treated controls (Figure 3.19A). Contrastingly, NS1 staining of IFNAR$^{-/-}$ BMDMs was not altered by BAY treatment (Figure 3.19B). Interestingly, application of BAY prior to infection, but not post infection, increased levels of cell death for both WT and IFNAR$^{-/-}$ BMDMs (Figure 3.19C-3.19D). Viral production was not altered by BAY treatment of WT or IFNAR$^{-/-}$ BMDMs (Figure 3.19E-F).

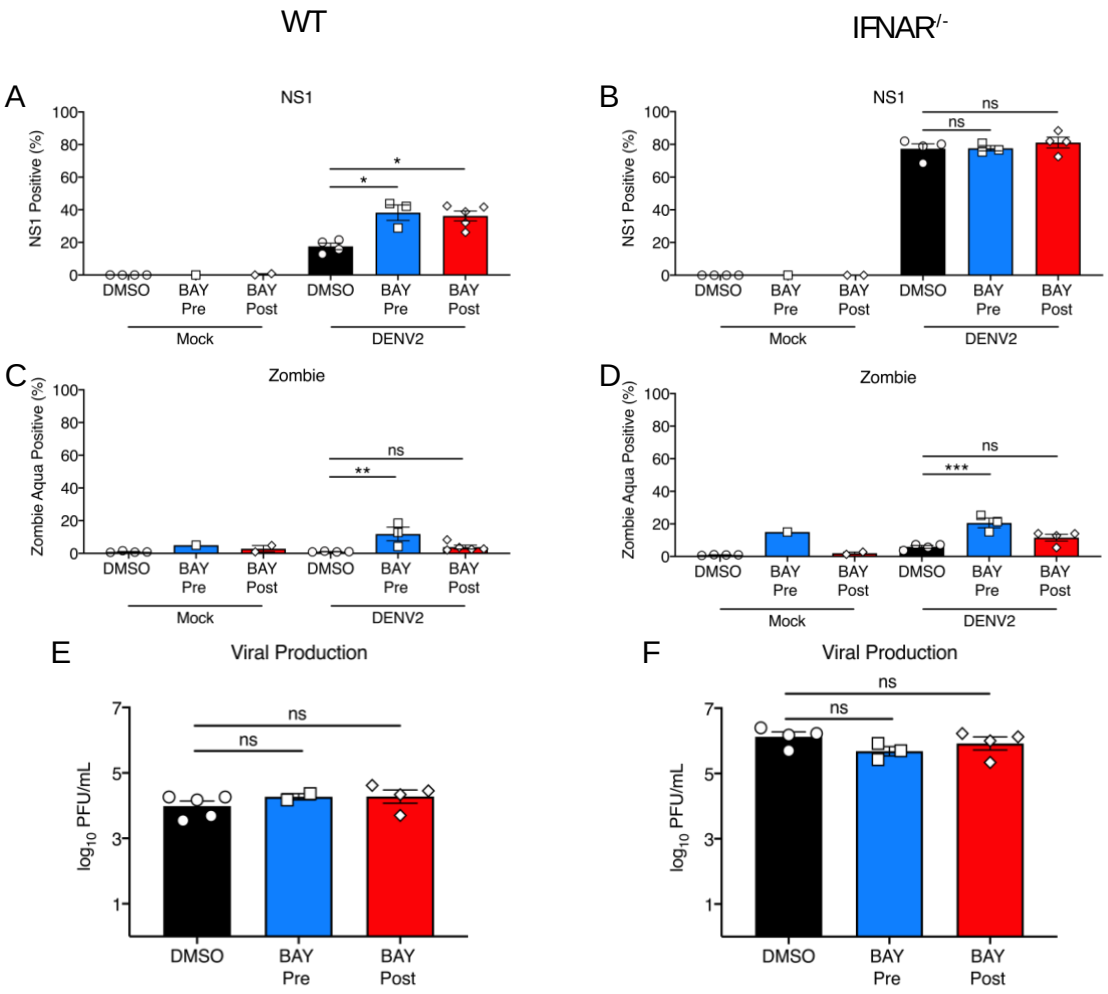


Figure 3.19 BAY treatment increases the susceptibility of BMDMs to infection, and can increase cell death. WT and IFNAR$^{-/-}$ BMDMs were treated with DMSO or BAY for either 2hr prior to infection ("Pre") or immediately following mock or DENV2 infection ("Post") with a MOI of 1. All BMDM cultures were also treated with MAR1. At 48hpi, cells were processed for flow cytometry. The susceptibility of WT (A) and IFNAR$^{-/-}$ cells (B) to infection was assessed by staining for viral NS1. Measurement of zombie aqua staining by flow cytometry was used to determine levels of cell death for WT (C) and IFNAR$^{-/-}$ (D) BMDMs. Finally, supernatants harvested at 48hpi were used to determine levels of viral production from WT (E) and IFNAR$^{-/-}$ (F) BMDMs. Note, for all panels of this figure, DMSO control data has been re-used from Figure 16, as LCL and BAY data was collected simultaneously, but has been presented separately for the purpose of clarity. Every dot represents cells (A-D) or supernatants (E and F) taken from an independent biological replicate. For A-D, n=2-5 from at least two independent experiments per condition with the exception of WT and IFNAR$^{-/-}$ mock-infected "Pre" treated BMDMs, where n=1.

For E and F, n= 2-5 and data are pooled from 1-3 independent experiments. Statistical analysis of NS1 and Zombie flow cytometry data: ordinary one-way ANOVA followed by Dunnett's test for multiple comparisons. Statistical analysis of plaque assay data: ordinary one-way ANOVA of log-transformed data followed by Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.10 Discussion

Although many DENV host restriction factors have been previously identified (Chapter 1, section 1.8) the nature of the host signalling pathways that are dysregulated during severe dengue is still poorly understood. To shed light on this, an *in vitro* screen of genetically targeted BMDMs was conducted. No alterations in cell death or susceptibility to infection were observed when $p50^{-/-}$, $SOCS3^{LysM}$, or $SIDT2^{-/-}$ BMDMs were screened. Therefore, at the level of susceptibility and cell death, these pathways did not appear to be important for cell intrinsic control of DENV infection. Because the cells of these genotypes lacked a positive phenotype during flow cytometry screening, I did not go on to screen these cells for virus production and cytokine production. However, as the *in vitro* screening work was conducted on pure cultures of BMDMs, it is entirely possible that some of these mutations could cause profound signal dysregulation *in vivo*. To this end, $p50^{-/-}$ and $SOCS3^{LysM}$ mice were studied using an *in vivo* model of DENV infection (see Chapter 4).

Mammalian cIAP proteins have dual, interlinked roles as transducers of NF-κB signalling and determinants of the apoptotic threshold. Removal of both cIAP1 and cIAP2 in some cell types can almost completely disrupt canonical NF-κB signalling (Mahoney et al., 2008; Varfolomeev et al., 2008). Although cIAP proteins do not directly inhibit mammalian caspases (Eckelman & Salvesen, 2006), they exert a potent anti-apoptotic effect by ubiquitinating key substrates associated with the TNFR1 complex such as TRAF2, RIP1 and the cIAPs themselves, (Bertrand et al., 2008; Li X et al., 2002; Mahoney et al., 2008; Samuel et al., 2006; Varfolomeev et al., 2008). Specifically, cIAP1 and cIAP2 play a crucial role in linking K63 ubiquitin chains to RIP1, allowing recruitment of pro-survival TAK1 and NEMO, and preventing from RIP1 assuming a pro-death adaptor function (Bertrand et al., 2008; Ea, Deng, Xia, Pineda, & Chen, 2006; Varfolomeev et al.,

2008).

Given the pro-survival ubiquitin ligase function of cIAPs, it is not surprising that, in some cell types, genetic or pharmacological ablation of both cIAP1 and cIAP2 dramatically sensitises cells to TNF-mediated killing. However, previously it was shown that efficient TNF-mediated killing of BMDM cells requires deletion of the protein XIAP in addition to the deletion of cIAP1 and cIAP2 (Lawlor et al., 2015; W. W. L. Wong et al., 2014). This may explain why I did not observe substantial cell death when I infected $c1^{LysM/}cIAP2^{-/-}$ BMDMs.

My examination of NS1 staining and viral production in IAP deficient BMDMs yielded non-concordant results. NS1 levels in $cIAP2^{-/-}$ were increased above baseline, and NS1 levels of double-deficient BMDMs were not statistically increased. However, viral production in $cIAP2^{-/-}$ cells was indistinguishable compared to WT cells, and viral production by $c1^{LysM/}cIAP2^{-/-}$ was reduced compared to WT cells.

Viral production is the more informative result as NS1 expression is simply a surrogate marker of the former. Potently increased levels of NS1 staining could represent an increase in levels of defective virus, or a defect in the budding pathway that allows infectious viral particles to be liberated from the host cell. In contrast to intracellular NS1, the plaque assay is the gold standard for measuring the production of infectious, viral particles. Therefore, the decrease in the production of virus from $c1^{LysM/}cIAP2^{-/-}$ BMDMs has direct implications for the understanding of how changes to immune signaling can contribute to dengue pathogenesis.

As outlined above, the compound genetic or pharmacological ablation of cIAP1 and cIAP2 can dramatically restrict canonical NF-κB signalling (Mahoney et al., 2008; Varfolomeev et al., 2008). As canonical NF-κB signalling is exploited to facilitate the replication of important human viruses such as HTLV and HIV (Bachelerie et al., 1991; Z. L. Chu et al., 1999; Faure, Lecine, Imbert, & Champion, 1996) I hypothesised that a deficiency in canonical NF-κB signalling could be responsible for the observed reduction in DENV production from $c1^{LysM/}cIAP2^{-/-}$ BMDMs. However, treatment of WT and $IFNAR^{-/-}$ BMDMs with the IAP antagonist LCL-161 did not reduce the ability of these cells to

produce DENV (Figures 3.17E and 3.17F).

BAY 11-7082 has been shown to inhibit both NF-κB signalling (Rauert-Wunderlich et al., 2013; Varley, 2017). However, this drug did not affect viral production in WT or IFNAR$^{-/-}$ BMDMs (Figures 3.19E and 3.19F). The LCL-161 and BAY data could be interpreted to indicate that efficient DENV production does not require NF-κB signalling. Alternatively, and more plausibly, the doses and protocols I used did not robustly inhibit NF-κB signalling. To clarify this point, further experiments aiming to confirm that these pharmacological treatments are directly inhibiting NF-κB signalling are proposed in Chapter 6.

3.11 Conclusion

I screened BMDMs to identify host restriction factors that could contribute to DENV infectivity and viral replication. I found that suppression of type I interferon signalling was necessary to establish DENV2 infection of BMDMs. cIAP1^{LysM}/cIAP2$^{-/-}$, but not cIAP2$^{-/-}$ BMDMs showed a reduction in viral production, as determined by plaque assay of cell supernatants. In an effort to clarify the genetic results obtained, WT and IFNAR$^{-/-}$ cells were treated with either the SMAC mimetic LCL-161 or the NF-κB inhibitor BAY prior to, or following, DENV infection. Interestingly, LCL-161 or BAY did not reduce the production of DENV2 from WT or IFNAR$^{-/-}$ BMDMs, and BAY increased NS1 staining in WT, but not IFNAR$^{-/-}$ cells.

Chapter 4 - Characterisation of Novel DENV Host Restriction Factors Using *In Vivo* Models

4.1 Introduction

For this chapter, I sought to extend the *in vitro* work presented in chapter 3 by infecting mice deficient in key immune signalling components and examining the effects of these deficiencies *in vivo*. As for Chapter 3, I hypothesised that the hypersensitivity of DENV to murine interferon could eliminate any chance I had of observing novel phenotypes when infecting conditional or full body knockout mice.

To circumvent this problem, I hypothesised that I could use the MAR1 IFNAR blocking antibody to reduce interferon signalling *in vivo.* Research groups studying DENV and other flaviviruses have had success in increasing the susceptibility of immunocompetent mice to infection using the MAR1 IFNAR-blocking antibody (Carlin et al., 2017; Govero et al., 2016; Roth et al., 2019). The benefit of this approach is that it increases the susceptibility of mice to infection, but, as the blocking effect of the antibody is less absolute than that of a genetic knockout, avoids the global immunosuppression observed in type I interferon pathway knockout models. Furthermore, in earlier results I successfully used MAR1 to increase the infection rate of BMDMs *in vitro* (see Chapter 3). Although the partial abrogation of type I IFN signalling represents a caveat, this proposed approach may allow the identification of ancillary and cooperative host pathways that may impact clinical outcomes during DENV infection. To assess the feasibility of this approach I first examined the virological and clinical outcomes in DENV2 infected mice that were treated with MAR1 IFNAR-blocking antibody.

4.2 MAR1 antibody treatment increases the susceptibility of WT mice to DENV2 infection.

To further explore the role of p50, SOCS3 and cIAP deficiency *in vivo* I wanted to infect genetic knockout mice. However, immunocompetent mice, upon DENV challenge, clear virus quickly and do not normally show signs of disease, reviewed in (Raphaël M. Zellweger & Shresta, 2014). Type I interferon signalling appears to be a powerful restriction factor for DENV infection in mice, whereas in humans DENV infection can

proceed in the presence of high concentrations of these cytokines (see Chapter 3). Therefore, I examined whether administration of MAR1 antibody could increase the susceptibility of immunocompetent mice to DENV infection. To address this question, wild type C57BL/6 mice were treated with MAR1 or isotype control and infected with DENV2. The weight and temperature of infected mice was measured daily. Although I observed trends towards enhanced weight loss and hypothermia in MAR1 treated mice, these differences were not statistically significant (Figure 4.1A-B). MAR1 treated mice had much higher plasma viral loads, as measured by qCPR, at Day 2 post-infection (Figure 4.1C). At day 2, the log-transformed mean viral load ± SEM, in genomic equivalents (GE)/mL, was 6.34 ± 0.10 for MAR1 treated animals compared to 3.42 ± 0.42 for isotype treated animals (viraemia was sampled at other timepoints in subsequent experiments, see Figure 4.2).

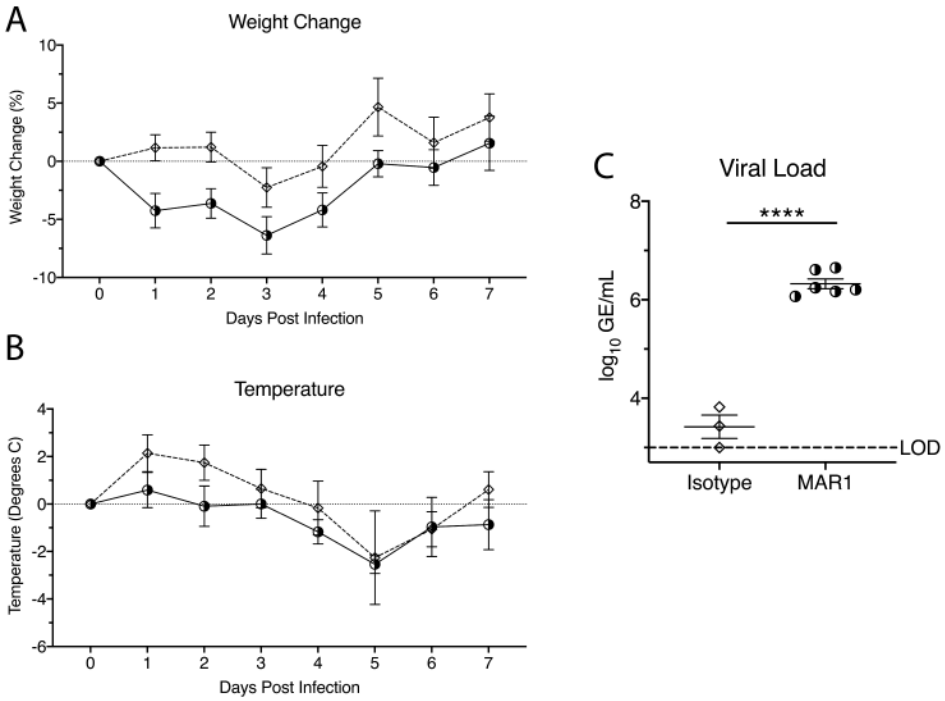


Figure 4.1 | MAR1 antibody treatment increases the susceptibility of WT mice to DENV2 infection. WT C57BL/6 mice of both sexes, 6-16 weeks in age, were treated with IgG Isotype control (diamond symbols) or MAR1 (circle symbols) prior and subsequent to infection with $2X10^7$ pfu of DENV2. Antibody treatment (both Isotype control and MAR1) comprised 1.5μg of antibody per mouse, with doses administered i.p. at 24 hour intervals starting the day prior to infection and concluding three days post-infection; for

details see section 2.8.4. Mice were monitored for signs of illness; the weight (A) and temperature (B) of mice was monitored daily. Plasma viral load (C) was quantified by qPCR at day two post-infection. Each dot represents an individual mouse. Isotype: n=3; MAR1: n=6. Data was obtained from one experiment. Error bars indicate the mean ± SEM. Statistical analysis: weight and temperature datasets were analysed by multiple unpaired t-tests with the Holm-Sidak correction for multiple comparisons. Raw qPCR data was log-transformed and analysed by unpaired, two-tailed t-test. **** $p < 0.0001$.

4.3 An immunocompetent mouse model of DENV2 infection

Using MAR1 I was able to successfully increase the susceptibility of immunocompetent WT mice to DENV2 infection (Figure 4.1). I then proceeded to screen cohorts of mice with the following genotypes: $p50^{-/-}$, $SOCS3^{Lysm}$, $cIAP2^{-/-}$, and $c1^{LysM}/cIAP2^{-/-}$ and $TRAF2^{LysM}$. All mice were on a C57BL/6 genetic background. For each experiment, C57BL/6 (WT) and $IFNAR^{-/-}$ mice were included as negative and positive controls, respectively.

WT and $IFNAR^{-/-}$ mice were infected with the same dose of DENV2. WT mice had remarkably stable weight and temperature measurements over the course of infection (Figure 4.2A, 4.2B). In contrast to WT mice, $IFNAR^{-/-}$ quickly lost weight and developed a transient fever (Figure 4.2A, 4.2B). $IFNAR^{-/-}$ mice reached a pre-determined ethical endpoint at Day 2 post-infection and were humanely euthanised.

$IFNAR^{-/-}$ mice had much higher plasma viral loads at Day 2 post-infection than WT MAR1 mice (Figure 4.2C). At day 2, the log-transformed mean viral load ± SEM, in GE/mL, was 6.10 ± 0.07 for WT animals compared to 7.63 ± 0.10 for $IFNAR^{-/-}$ animals. At days 4 and 7 post-infection, only a small proportion of WT animals (31% and 25% respectively) had viral loads above the limit of detection for the qPCR assay.

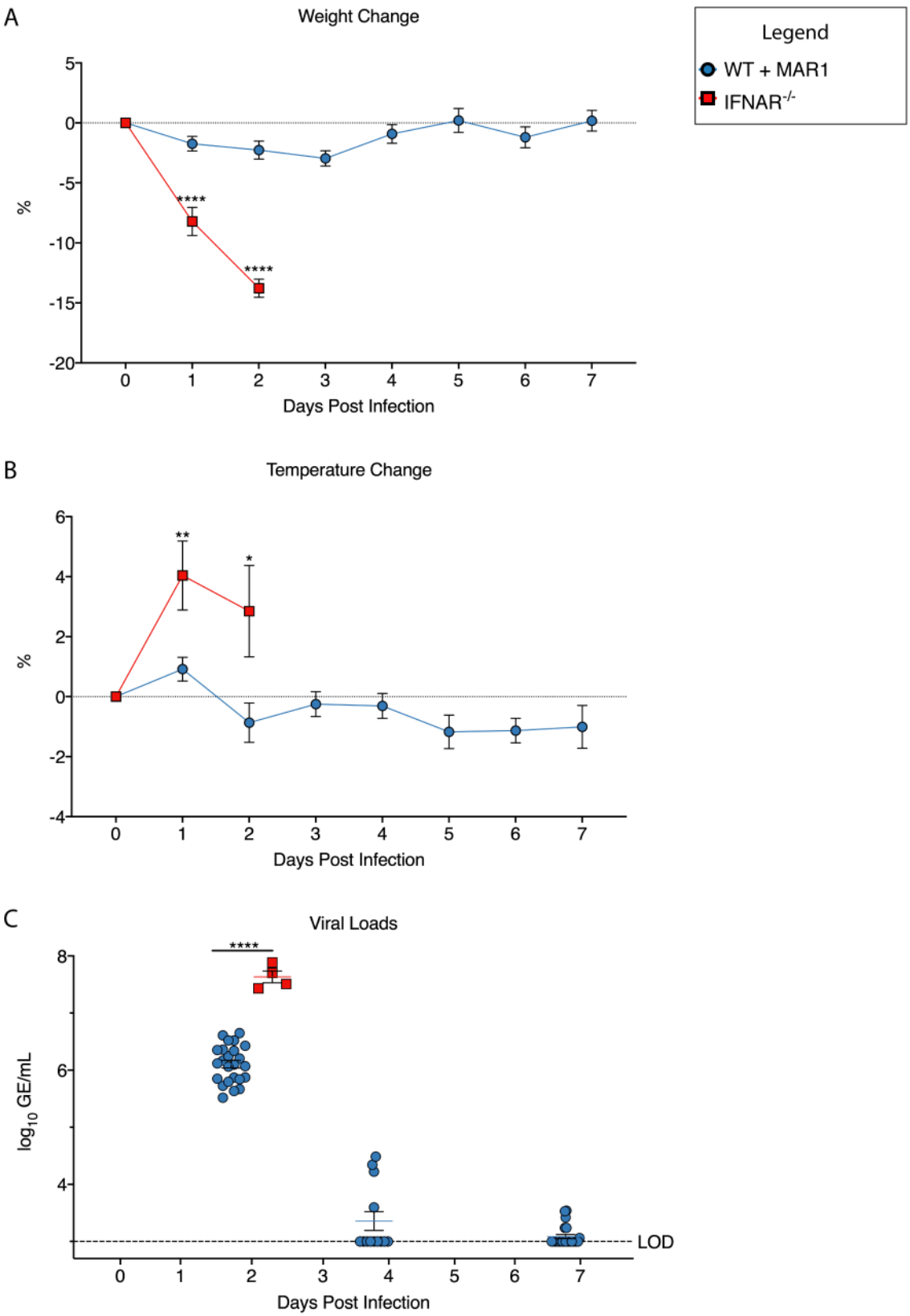


Figure 4.2 | Characterisation of a novel immunocompetent mouse model of DENV2 infection. WT mice were treated with MAR1 prior and subsequent to infection with DENV2. IFNAR$^{-/-}$ mice were included as a positive control. Mice were monitored for

signs of illness: weight (A) and temperature (B) were determined daily. Mice were euthanised when they reached a pre-determined ethical endpoint. Plasma viral load was quantified for surviving mice at days 2, 4 and 7 post-infection (C). For panel C, dots represent individual mice. WT: n=24; IFNAR$^{-/-}$: n=4. Data is pooled from 4-5 independent experiments per genotype. Error bars indicate the mean ± SEM. Statistical analysis: weight and temperature datasets were analysed by multiple unpaired t-tests with the Holm-Sidak correction for multiple comparisons. Raw qPCR data was log-transformed and analysed by one-way ANOVA with Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

This model of DENV disease has several strengths. Firstly, during the course of human disease, viraemia is transient with peak viral loads occurring in the first few days following the onset of fever (Duyen et al., 2011; Libraty, Endy, et al., 2002). Crucially, symptoms of dengue are most severe at approximately 4-6 days after fever onset, and this correlates with the time of defervescence, when the plasma viral load is generally sharply declining (Duyen et al., 2011; Fox et al., 2011). Therefore, unlike other dengue models where virus can be detected for weeks following infection (Mota & Rico-Hesse, 2009; Shresta et al., 2004), infection of MAR1 treated immunocompetent animals results in a viraemia which more closely mimics human disease. Secondly, this model does not utilise mouse strains that have a global defect in interferon signalling. Many studies of dengue use mouse models that have either single (IFNAR$^{-/-}$) or compound (IFNAR$^{-/-}$ IFNGR$^{-/-}$) defects in interferon signalling (reviewed in (Raphaël M. Zellweger & Shresta, 2014)). Therefore, the ability of findings to be generalised to clinical disease is somewhat limited. Although IFNAR signalling is actively being suppressed in this MAR1 model, it is clear, given the vastly different outcomes of WT mice and IFNAR$^{-/-}$ mice, that some degree of type I interferon signalling remains intact in these animals. Finally, the viraemia achieved with this MAR1 model is not dependent on the use of mouse-adapted strains of dengue that have mutated during serial passaging and therefore are biologically distinct from human infectious virus (Atrasheuskaya, Petzelbauer, Fredeking, & Ignatyev, 2003; J.-P. Chen et al., 2006; Orozco et al., 2012).

4.3.1 A note on data analysis

Viral load data from the different genotypes (WT, IFNAR$^{-/-}$, p50$^{-/-}$, SOCS3^{Lysm}, cIAP2$^{-/-}$, and c1^{LysM}/cIAP2$^{-/-}$) was collected over multiple experiments and pooled. All qPCR values of viral loads were compared to the WT control group. As such, I have used a one-way ANOVA with the Dunnett post-hoc test for multiple comparisons (e.g., see Figure 4.2C) for the analysis of this data.

However, for reasons that were not entirely apparent, when TRAF2^{LysM} mice were infected, the Day 2 viral titre for WT mice in the experiment was considerably lower than titres recorded for other experiments. To account for this, TRAF2^{LysM} viral data was not compared to the pooled data as for other genotypes. Instead, TRAF2^{LysM} titres were compared to intra-experimental controls (Section 4.6).

4.4 Investigating the effect of p50 genetic knockout on DENV infection *in vivo*

Previously, I infected BMDMs to investigate the effect of p50 NF-κB subunit deficiency *in vitro* (see Chapter Three). I found that p50 deficiency did not affect the susceptibility of BMDMs to DENV infection or change levels of cell death. I decided to extend my *in vitro* study of the role of p50$^{-/-}$ in DENV pathogenesis by infecting p50$^{-/-}$ knockout mice. The rationale for studying the effect of p50 deficiency *in vivo* was three-fold. Firstly, I hypothesised that a lack of p50-homodimers could remove transcriptional repression and promote pro-inflammatory cytokine signalling and associated pathology. Secondly, as p50-P65 and p50-cRel heterodimers are the main mediators of pro-survival canonical NF-κB signalling (T. Liu et al., 2017), I hypothesised that removal of p50 could sensitise cells to undergo apoptosis upon DENV infection. To summarise the first two parts of my hypothesis: I set out to investigate whether, given the increased complexity of an *in vivo* model, a phenotype related to cytokine signalling or apoptosis might be apparent *in vivo* that was not apparent in my *in vitro* system.

Thirdly, unpublished work from our lab showed that LCMV infection of p50$^{-/-}$ mice triggered endothelial cell permeability and vascular leak (Cody Alison, unpublished work). As one of the most dangerous outcomes of severe dengue, the pathogenesis of vascular leak is not well understood. Various host factors such as chymase, leukotrienes,

platelet activating factor and TNF (Dewi, Takasaki, & Kurane, 2004; Souza et al., 2009; St John, Rathore, Raghavan, Ng, & Abraham, 2013), in addition to viral NS1 (Modhiran et al., 2015; Puerta-Guardo, Glasner, & Harris, 2016) have been implicated in the development of vascular leak during DENV infection. Given that LCMV infection triggered vascular in $p50^{-/-}$ mice I was interested to see whether DENV infection of $p50^{-/-}$ mice could also result in vascular leak.

Despite a lack of phenotype in BMDM *in vitro* experiments it was eminently possible that p50 could have clinically relevant functions or contributions in other cellular compartments that would only be manifest *in vivo* and in the context of complex cellular interactions. To investigate the effect of p50 deficiency on *in vivo* I turned to the MAR1 model (described above). These mice, with a complete complement of immune cells and complete vasculature, allowed the investigation of the effect of p50 deficiency during DENV infection in a way that the study of BMDMs could not. While vascular leak was not directly examined by using Evans blue or similar techniques, animals were closely monitored to detect any serious side effects of DENV infection in this particular genetic background.

MAR1-treated $p50^{-/-}$ mice were infected with DENV. To determine whether these mice developed disease after infection, weight and temperature was assessed daily. Although I noted divergent trends in weight and temperature relative to WT mice these differences did not reach statistical significance (Figure 4.3A and 4.3B). To assess the kinetics of viraemia WT and $p50^{-/-}$ viral titres were determined at days 2, 4 and 7 post-infection (Figure 4.3C). No significant differences in viral load for WT and $p50^{-/-}$ animals were observed.

If p50 deficiency was causing subtle endothelial leakage in experimental animals then it was relatively minor and did not impact disease outcomes. Further analysis of these animals would be prudent including an examination of serum cytokine production given that aberrations in cytokine signalling have been implicated in clinical disease.

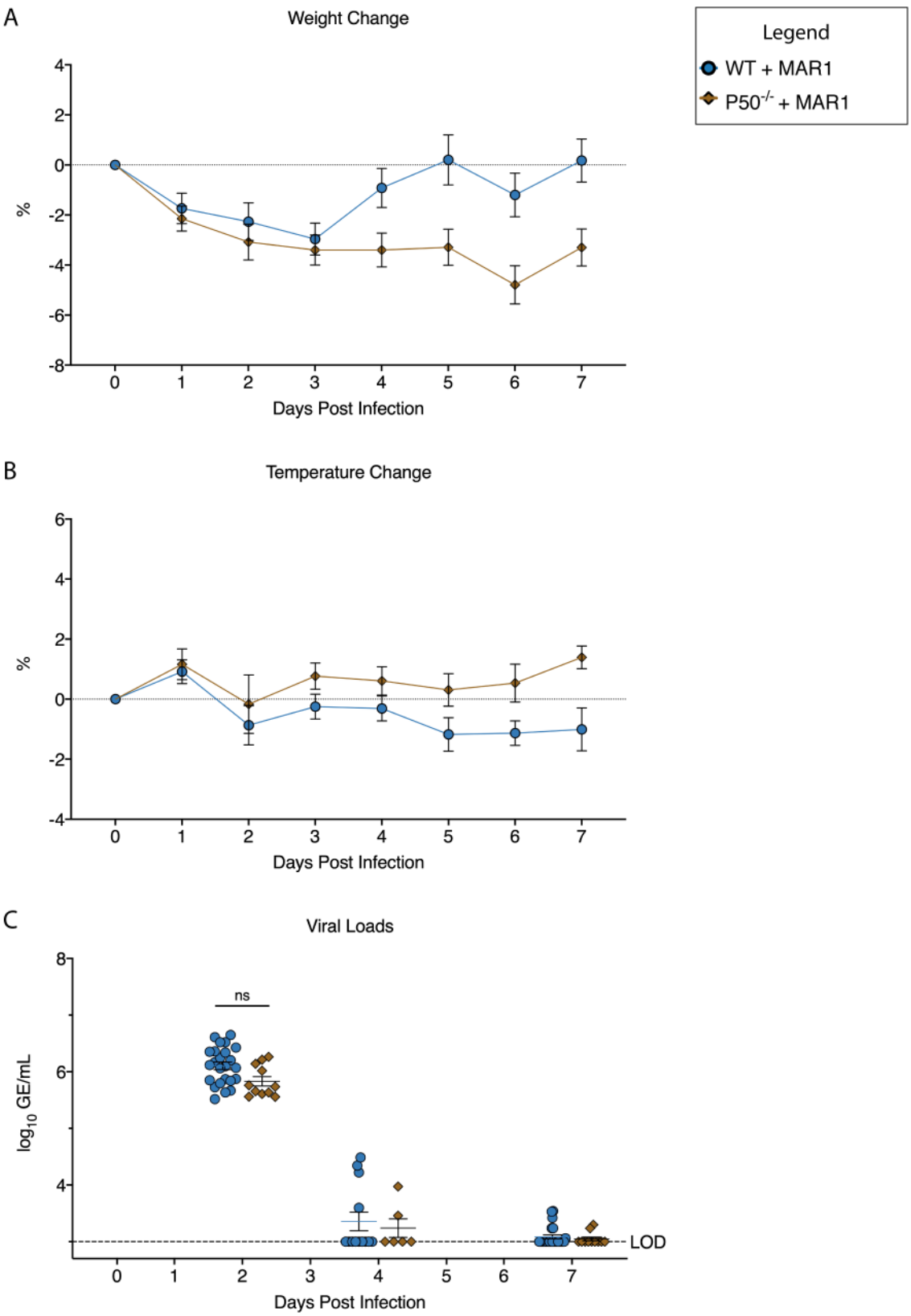


Figure 4.3 | p50$^{-/-}$ mice show no changes in weight, temperature or viral load when infected with DENV2. WT C57BL/6 and p50$^{-/-}$ mice were treated with MAR1 prior and subsequent to infection with DENV2. Mice were monitored for signs of illness: weight

(A) and temperature (B) were determined daily. Plasma viral load was quantified at days 2, 4 and 7 post-infection (C). For panel C, dots represent individual mice. WT n=24; $p50^{-/-}$: n=11. WT data is pooled from 4-5 independent experiments, and is re-used from figure 2. $p50^{-/-}$ data is pooled from 2 independent experiments. Error bars indicate the mean ± SEM. Statistical analysis: weight and temperature datasets were analysed by multiple unpaired t-tests with the Holm-Sidak correction for multiple comparisons. Raw qPCR data was log-transformed and analysed by one-way ANOVA with Dunnett's test for multiple comparisons.

4.5 Investigating the effect of SOCS3 conditional knockout on *in vivo* DENV infection

In Chapter Three, I showed that the loss of SOCS3 in BMDMs did not increase the susceptibility of these cells to infection or change levels of cell death in response to DENV infection. However, several groups have reported that *in vitro* and *in vivo* $SOCS3^{LysM}$ infections can result in drastically different outcomes. For instance, Carow et al. infected $SOCS3^{LysM}$ BMDMs with *Mycobacterium tuberculosis* (*M. tuberculosis*) and found that they did not produce more *M. tuberculosis* than WT controls (Carow et al., 2013). However, these cells produced less IL-12p40. *In vivo* this deficiency of IL-12 production from myeloid cells resulted in a delay in the activation of T or NK cells to produce IFN-γ, and a reduction in survival (Carow et al., 2013). A similar decrease in survival due to impaired IL-12 production, and delayed T cell activation, was observed when $SOCS3^{LysM}$ mice were infected with *T. gondii* (Whitmarsh et al., 2011). Given the ability of SOCS3 to change the recruitment and response of the immune system to infection *in vivo*, I was interested to examine the role of SOCS3 in my MAR1 model of *in vivo* DENV infection. As full-body SOCS3 knockouts do not survive to maturity (Marine et al., 1999), and given that the primary host cells of DENV are monocytes and macrophages of the monocyte lineage, I chose to infect $SOCS3^{LysM}$ mice.

$SOCS3^{LysM}$ mice were treated with MAR1 and infected with DENV2. Similar to $p50^{-/-}$ mice, I noted a divergence in in weight and temperature in $SOCS3^{LysM}$ mice relative to WT mice, with a statistically significant, albeit transient, increase in temperature in $SOCS3^{LysM}$ animals at day 6 post-infection (Figures 4.4A and 4.4B). When log-

transformed viral titres were compared by one-way ANOVA, there were no significant differences in titres at days 2, 4 and 7 post-infection (Figure 4.4C). Although subtle, the phenotypes of both p50 and SOCS3^{LysM} animals could be further explored to examine cytokine levels.

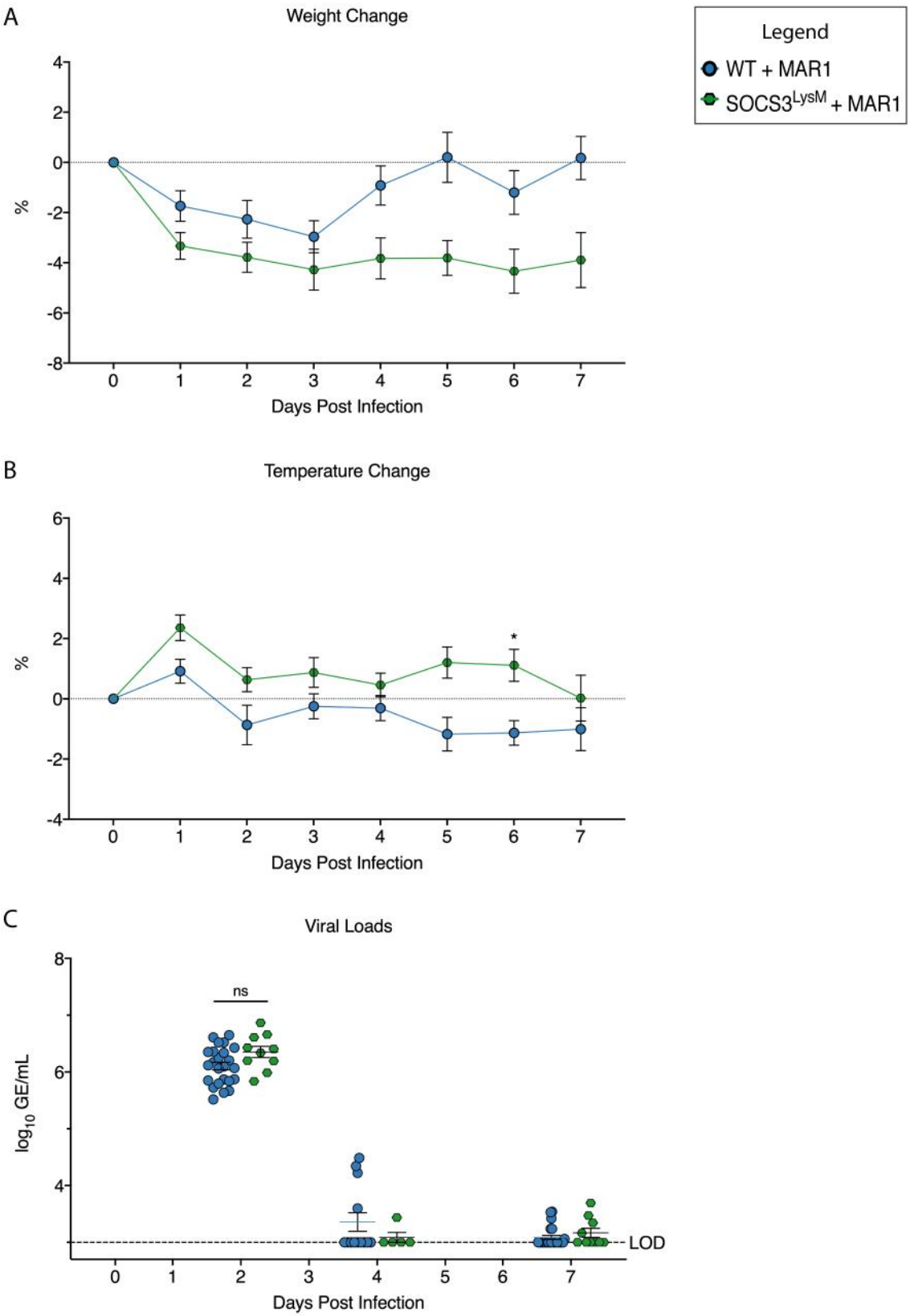


Figure 4.4 | SOCS3^{LysM} mice show no changes in weight, a minor change in temperature and no change in viral load when infected with DENV2. WT C57BL/6 and SOCS3^{LysM} mice were treated with MAR1 both prior and subsequent to infection with DENV2. Mice

were monitored for signs of illness: weight (A) and temperature (B) were determined daily. Plasma viral load was quantified at days 2, 4 and 7 post-infection (C). For panel C, dots represent individual mice. WT n=24; SOCS3$^{LysM\ -/-}$: n=10. WT data is pooled from 4-5 independent experiments, and is re-used from figure 2. SOCS3$^{LysM\ -/-}$ data is pooled from 2 independent experiments. Error bars indicate the mean ± SEM. Statistical analysis: weight and temperature datasets were analysed by multiple unpaired t-tests with the Holm-Sidak correction for multiple comparisons. Raw qPCR data was log-transformed and analysed by one-way ANOVA with Dunnett's test for multiple comparisons. * $p < 0.05$

4.6 The role of TRAF2 during *in vivo* DENV infection

One of the key proteins of the TNFR1 signalling complex (TNFR SC) is TRAF2. TRAF2 is an adaptor protein that has roles in promoting NF-κB and JNK signalling, and in regulating apoptosis. During TNFR1 signalling, TRAF2 binds to TRADD, and is able to recruit cIAP1 and cIAP2 to the nascent TNFR SC via their BIR domains (Zheng, Kabaleeswaran, Wang, Cheng, & Wu, 2010).

Together with the cIAP proteins, TRAF2 possesses a RING domain and exhibits E3 ubiquitin ligase activity. Specifically, TRAF2 has been shown to attach K63- ubiquitin chains to RIP1, cIAP1 and cIAP2 (Vallabhapurapu et al., 2008; Wertz et al., 2004). However, whereas the ubiquitin ligase activity of the cIAPs is crucial to their role in NF-κB signalling (Mahoney et al., 2008; Varfolomeev et al., 2008), it appears that TRAF2 enables NF-κB signalling in an alternative manner. Early work showed that NF-κB signalling was normal in MEFs deficient in either TRAF2$^{-/-}$ or TRAF5$^{-/-}$, but defective in TRAF2/5 double knockout (DKO) MEFs (Tada et al., 2001). Further study showed that transient transfection of DKO MEFs with a RING domain-deleted form of TRAF2 (TRAF2$\triangle$RING) was able to rescue NF-κB signalling (Vince et al., 2009). The dispensable nature of the TRAF2 RING domain suggests that the main role of TRAF2 during NF-κB signalling is to act as a scaffold for cIAP recruitment. The precise activity of TRAF2 in NF-κB signalling appears to be cell-type dependent, as it was recently reported that TRAF2 was required for normal TNF-mediated canonical NF-κB and MAPK signalling in MEFs, fibroblasts and keratinocytes but not in macrophages (Etemadi et al., 2015). Moreover,

i*n vitro* and *ex vivo* studies have also demonstrated an important role for TRAF2 in JNK signalling (S. Y. Lee et al., 1997; Tada et al., 2001; Yeh et al., 1997).

In addition to mediating NF-κB and JNK signalling, TRAF2 has an independent function as an inhibitor of apoptosis. Although Etemadi et al., found that TRAF2 deficient macrophages were still able to signal normally via NF-κB and MAPK pathways, they also showed that TRAF2$^{-/-}$ BMDMs were especially prone to apoptosis (Etemadi et al., 2015). This is in agreement with historical *ex vivo* studies of TRAF2 deficient thymocytes that also demonstrated an increased rate of apoptosis (S. Y. Lee et al., 1997; Yeh et al., 1997). Furthermore, full body TRAF2$^{-/-}$ deletion *in vivo* causes a degree of embryonic lethality as homozygous null offspring from crosses are not born at the expected Mendelian ratios (Piao et al., 2011; Yeh et al., 1997). TRAF2$^{-/-}$ offspring that survive to term are runted, and die within the first few weeks of life, at least in part due to increased apoptosis of colonic epithelial cells (Piao et al., 2011; Yeh et al., 1997). *In vitro*, the anti-apoptotic activity of TRAF2 was shown to be dependent on proper function of the TRAF2 RING domain (Vince et al., 2009; L. Zhang, Blackwell, Shi, & Habelhah, 2010). The exact anti-apoptotic role of the TRAF2 RING domain was clarified when Gonzalvez et al. demonstrated that the TRAF2 RING domain could link K48 ubiquitin chains to caspase-8 (Gonzalvez et al., 2012). TRAF2 deficient cells had an inability to degrade caspase-8, and were therefore sensitised to death (Gonzalvez et al., 2012).

My previous *in vitro* work showed that the cIAPs, which have important roles in NF-κB signal transduction and apoptotic control, seem to have an accessory role facilitating DENV production. Given that, in some cell types, TRAF2 has an important role in NF-κB and JNK signal transduction, and also a distinct role in setting the cellular apoptotic threshold, I decided to investigate the effect of TRAF2 deficiency on *in vivo* DENV infection. As TRAF2$^{-/-}$ mice are not born at mendelian ratios, and surviving pups do not survive for more than a few weeks (Yeh et al., 1997), TRAF2^{LysM} mice were used.

WT and TRAF2^{LysM} mice were treated with MAR1 and infected with DENV2 as for p50$^{-/-}$ mice, and weight and temperature were monitored daily. TRAF2^{LysM} animals did not undergo significant changes in weight (Figure 4.5C), and temperature was normal except for a slight elevation at Day 4 post-infection (Figure 4.5B). For reasons that were not

entirely apparent, WT viral titres at Day 2 post-infection were lower than titres recorded for other experiments. To account for this, TRAF2^{LysM} viral data was not compared to the pooled data as for other genotypes. Instead, TRAF2^{LysM} titres were compared to intra-experimental controls (Figure 4.5C). When log-transformed WT and TRAF2^{LysM} titres were compared, there was no difference in viral load at Day 2 post-infection. However, TRAF2^{LysM} titres were significantly higher at Day 4 post-infection. This suggests that absence of TRAF2 in myeloid cells could potentiate the DENV2 infection *in vivo*. A caveat is that this data was generated from one experiment, with 3-4 animals per group. Nonetheless it is interesting to speculate that the absence of TRAF2 may abrogate TNF signalling and or NF-κB antiviral responses and examination of cytokine responses in these animals may be informative.

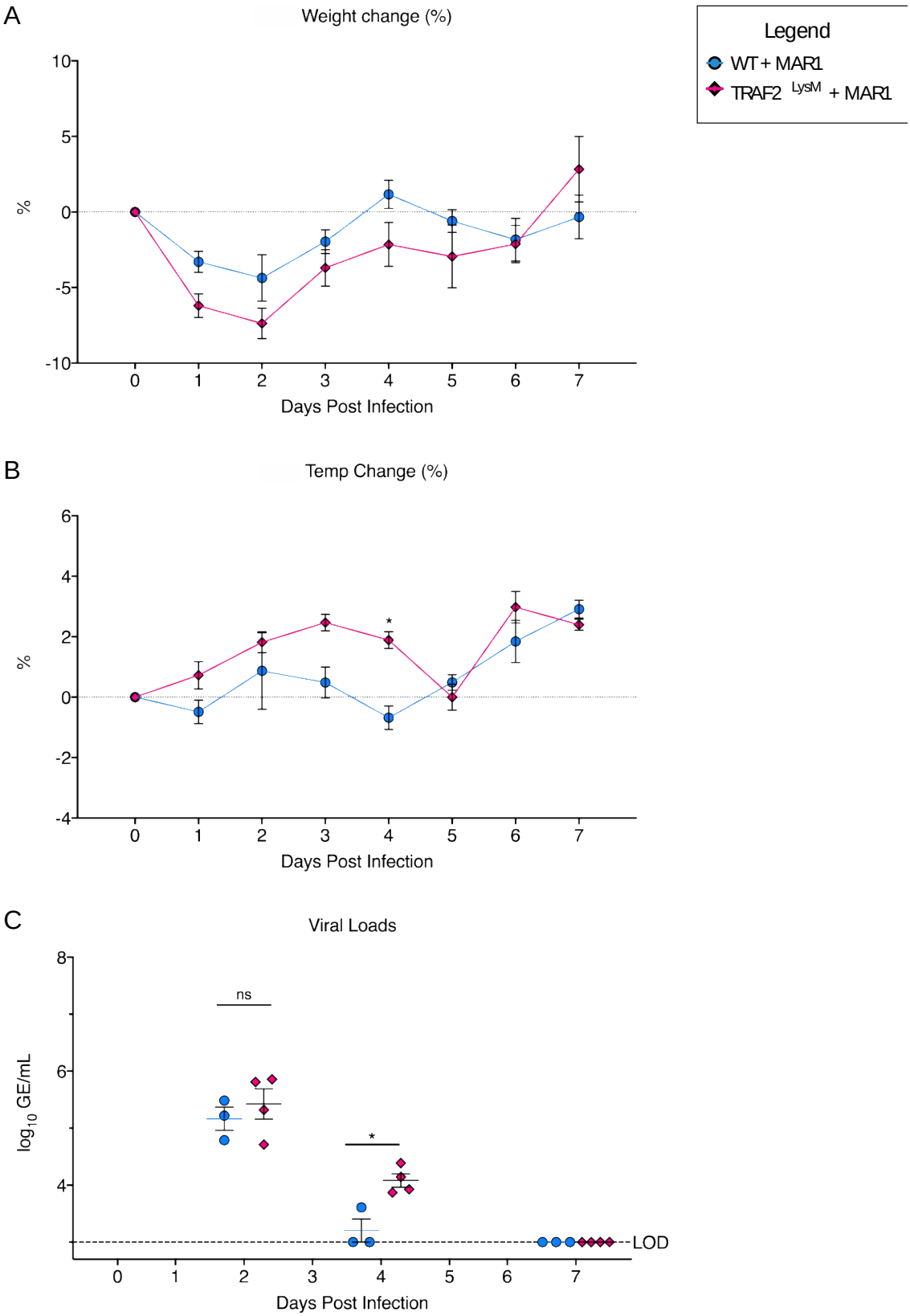


Figure 4.5 | TRAF2^{LysM} mice show no changes in weight, a minor change in temperature and delayed viral clearance when infected with DENV2. WT C57Bl/6 and TRAF2^{LysM} mice were treated with MAR1 prior and subsequent to infection with DENV2. Mice were

monitored for signs of illness: weight (A) and temperature (B) were determined daily. Plasma viral load was quantified at days 2, 4 and 7 post-infection (C). For panel C, dots represent individual mice. WT n=3; TRAF2$^{LysM\ -/-}$: n=4. Data is from one independent experiment. Error bars indicate the mean ± SEM. Statistical analysis: weight and temperature datasets were analysed by multiple unpaired t-tests with the Holm-Sidak correction for multiple comparisons. Raw qPCR data was log-transformed and analysed by two-tailed t test. * $p < 0.05$

4.7 Investigating the effect of IAP deficiency on *in vivo* DENV infection

Previously, I showed that cIAP2$^{-/-}$ BMDMs but not c1^{LysM}/cIAP2$^{-/-}$ BMDMs produced more NS1 when infected with DENV2 than WT controls (Chapter 3, section 3.6). Conversely, I showed that c1^{LysM}/cIAP2$^{-/-}$ BMDMs produced less DENV2 than WT BMDMs, whereas there was no significant difference in virus production when cIAP2$^{-/-}$ BMDMs were compared to WT controls. Degradation of IAPs prior to, or immediately following infection with the IAP antagonist LCL-161 did not affect levels of NS1 staining or virus production of WT or IFNAR$^{-/-}$ BMDMs (Chapter 3, section 3.9.3). However, use of the NF-κB inhibiting compound Bay 11-7082 increased NS1 staining of WT but not IFNAR$^{-/-}$ cells (Chapter 3, section 3.9.5).

To further analyse the role of IAPs and NF-κB signalling in DENV infection, I infected MAR1-treated cIAP2$^{-/-}$ and c1^{LysM}/cIAP2$^{-/-}$ mice. c1^{LysM}/cIAP2$^{-/-}$ animals and cIAP2$^{-/-}$ animals showed transient periods of weight loss, relative to wild type controls, at day 1 and day 4 post infection respectively (Figure 4.6A). In addition, double deficient animals showed transient increases at temperature at day 1 and 3 post infection relative to wild type controls (Figure 4.6B). When I examined the viral load of these MAR1 treated mice at day 2 post infection, double deficient animals had a decreased viral load (Figure 4.6C) just as we observed *in vitro* by plaque assay (Figure 3.13). The log-transformed mean ± SEM Day 2 viral load was 6.10 ± .07 GE/mL for WT animals, and 5.50 ± .09 GE/mL for c1^{LysM}/cIAP2$^{-/-}$ mice. No difference was observed with WT and CIAP2$^{-/-}$ mice, and this was again in agreement with the *in vitro* plaque assay results.

The finding that deficiency of cIAP1 and cIAP2 reduces viral production *in vitro* (see Chapter 3) and *in vivo* provides convincing evidence that NF-κB activity, and presumably, an active transcriptional state in the cell, is required for DENV2 production. Alternatively, cIAPs may simply promote survival of infected cells to allow sufficient time for viral replication and completion of the viral lifecycle. Subsequently, the cause of this reduced viral load was further explored by flow cytometry (see section 4.8 below).

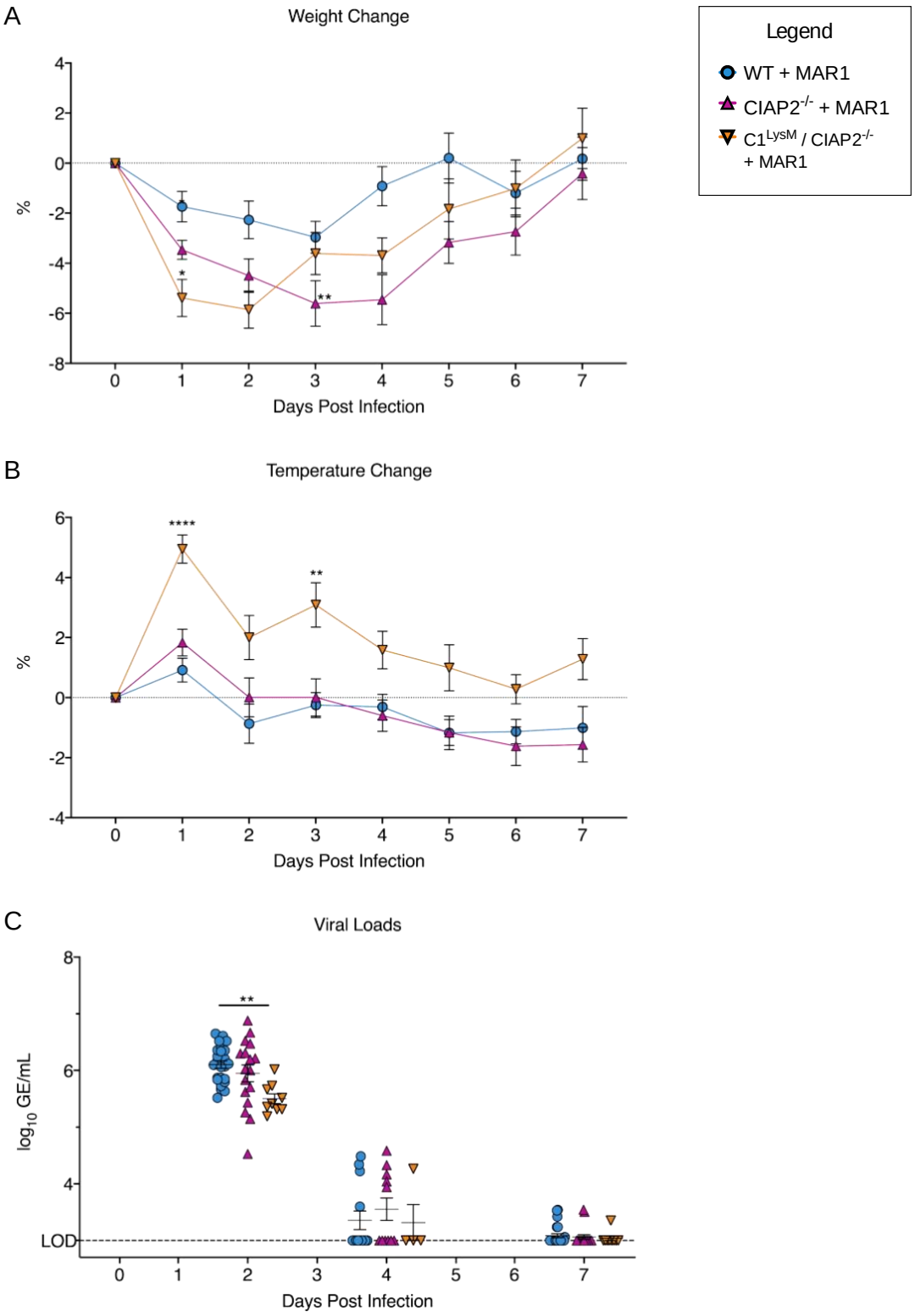


Figure 4.6 | DENV2 infection of IAP-deficient mice results in transient changes in

weight and temperature. c1^{LysM}/cIAP2$^{-/-}$ mice produce less DENV2 than WT mice. WT C57Bl/6, cIAP2$^{-/-}$ and c1^{LysM}/cIAP2$^{-/-}$ mice were treated with MAR1 prior and subsequent to infection with DENV2. Mice were monitored for signs of illness: weight (A) and temperature (B) were determined daily. Plasma viral load was quantified at days 2, 4 and 7 post-infection (C). For panel C, dots represent individual mice. WT n=24, cIAP2$^{-/-}$ n=17, c1^{LysM}/cIAP2$^{-/-}$ n=9. WT data is pooled from 4-5 independent experiments, and is re-used from figure 2. cIAP2$^{-/-}$ and c1^{LysM}/cIAP2$^{-/-}$ data is pooled from 3 and 2 independent experiments, respectively. Error bars indicate the mean ± SEM. Statistical analysis: weight and temperature datasets were analysed by multiple unpaired t-tests with the Holm-Sidak correction for multiple comparisons. Raw qPCR data was log-transformed and analysed by one-way ANOVA with Dunnett's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

4.8 Further investigation of the role of IAPs *in vivo*

I decided to use flow cytometry to further investigate the outcomes of DENV infection in IAP deficient mice. There were several reasons for using flow cytometry for this purpose. Firstly, it would allow me to determine if there were differences in the number of monocyte and macrophage host cells required for DENV replication. This was important to determine, as DENV titre is thought to be correlated to the size of the infected cell compartment (reviewed in (Halstead, 2019)). Secondly, given that severe dengue is driven by dysregulation of inflammatory cytokines, I wanted to quantify the size of different compartments of cytokine-producing myeloid cells. Analysis of the myeloid compartments of cIAP2$^{-/-}$ mice at day 2 post-infection revealed that there were significantly upregulated levels of monocytes and inflammatory macrophages relative to WT controls (see Figure 4.7A and 4.7B). The upregulation of monocytes and inflammatory macrophages is attributable to DENV2 infection, and not the loss of cIAP2, as previous analysis of cIAP2$^{-/-}$ mice did not detect any changes in the myeloid compartment (Conte et al., 2006). For details on the flow cytometry markers used to define particular immune subsets, please see section 2.12.2.

It was interesting to observe that although there was an increase in monocytes and macrophages, which serve as the primary host cells for DENV *in vivo*, there was no

difference in viral load between WT and cIAP2$^{-/-}$ mice.

To extend this analysis further, I infected WT and c1^{LysM}/cIAP2$^{-/-}$ mice with DENV2. At 48 hours post infection, myeloid cells were isolated from the blood and the spleen and prepared for flow cytometry. Previously, Lawlor and colleagues discovered that c1^{LysM}/cIAP2$^{-/-}$ mice spontaneously develop arthritis (Lawlor et al., 2015). This disease was characterised by significantly increased levels of TNF, and trends toward higher levels of IL-1β and IL-6 (Lawlor et al., 2015). In addition, c1^{LysM}/cIAP2$^{-/-}$ mice had elevated numbers of splenic neutrophils, and a tendency towards increased numbers of splenic inflammatory monocytes (Lawlor et al., 2015). My findings cannot be exactly compared to the previous publication, as I measured levels of granulocytes and inflammatory macrophages rather than neutrophils and inflammatory monocytes. However, I found statistically elevated proportions of granulocytes in the spleen and blood (Figure 4.8A and 4.8B), and statistically elevated numbers of inflammatory macrophages in the spleen (Figure 4.8B). Surprisingly, the proportion of monocytes was significantly lower in the blood of c1^{LysM}/cIAP2$^{-/-}$ mice than in WT controls, although no difference was observed in the spleen. In addition, there was a trend towards there being a lower proportion of dendritic cells in the blood of c1^{LysM}/cIAP2$^{-/-}$ mice relative to WT controls, although this did not reach statistical significance (Figure 4.8A).

Overall, my results suggest that upon DENV2 infection, there is a degree of upregulation of the monocyte and inflammatory macrophage compartments of cIAP2$^{-/-}$ animals. However, in c1^{LysM}/cIAP2$^{-/-}$ animals, the proportion of monocytes is sharply lowered relative to controls, and a trend is also present for a decrease in the proportion of dendritic cells in these gene-targeted mice a caveat with this data is that the reduction may just represent redistribution of cells rather than an absolute decrease in numbers. More comprehensive necropsies would be required to exclude this.

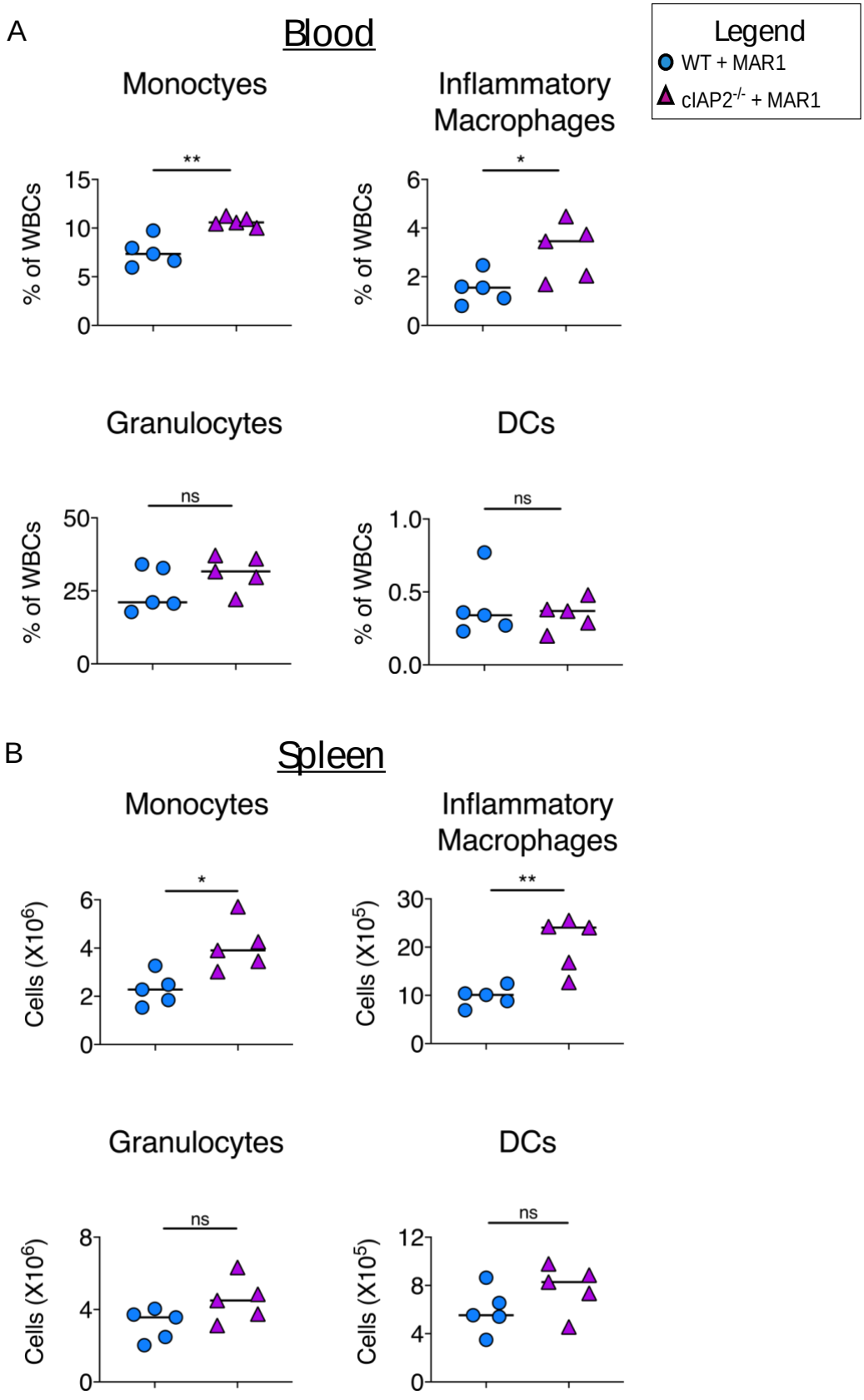


Figure 4.7| Quantification of myeloid cell populations in WT and cIAP2$^{-/-}$ mice. WT and

cIAP2$^{-/-}$ mice were treated with MAR1 prior and subsequent to DENV2 infection. At Day 2 post infection mice were euthanised and flow cytometry was used to quantify the myeloid cell populations in the spleen and peripheral blood. Each dot represents an individual animal. n=4 animals per group, data are from one independent experiment. For the spleen, total numbers of cells were determined by multiplying total leukocyte counts with the percentages of myeloid cell subsets determined by flow cytometry. Statistical analysis: groups were compared by unpaired, two-tailed t-test. Error bars indicate the mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

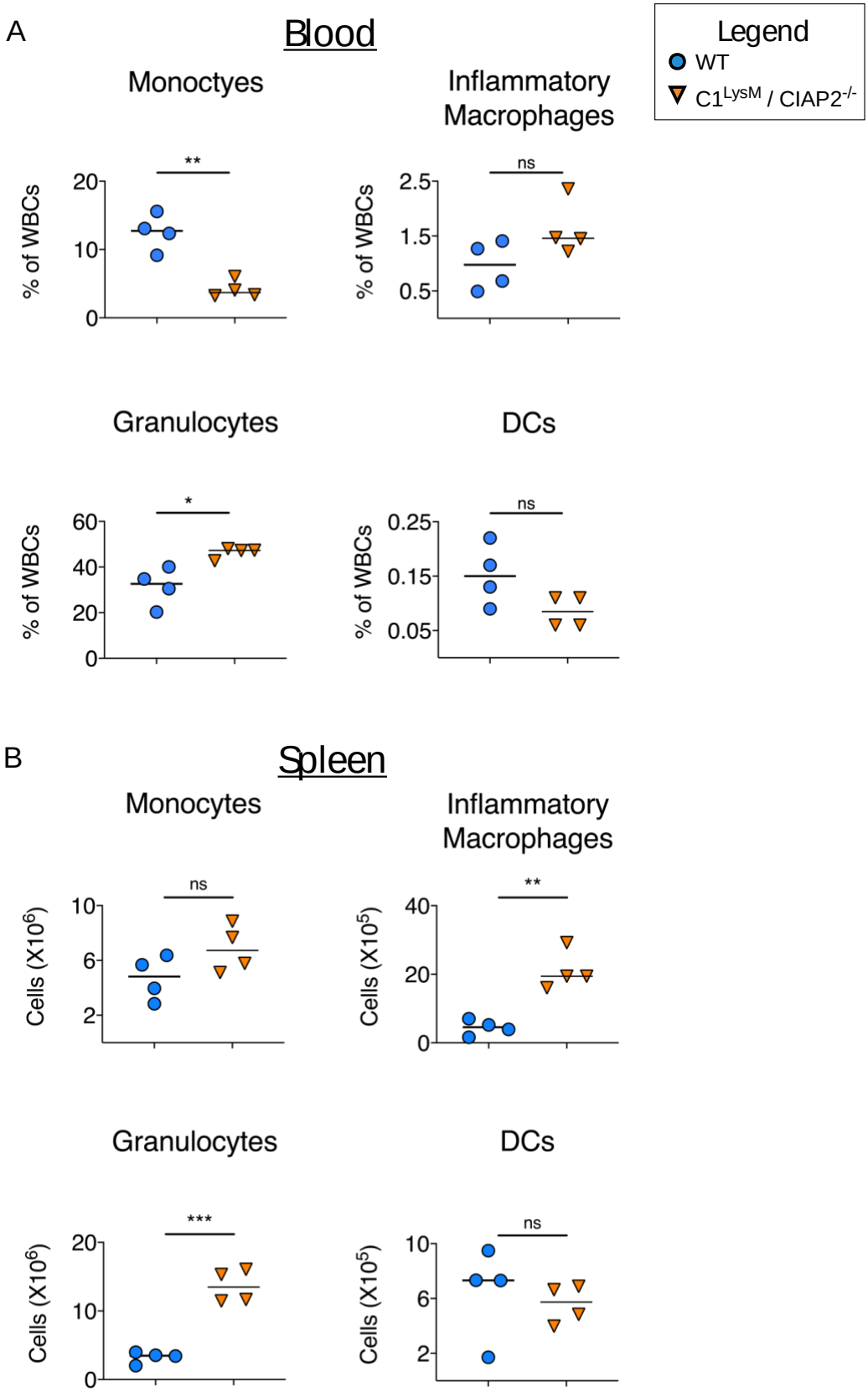


Figure 4.8 | Quantification of myeloid cell populations in WT and c1^{LysM} / cIAP2$^{-/-}$ mice.

WT and c1^{LysM} / cIAP2$^{-/-}$ mice were treated with MAR1 prior and subsequent to DENV2 infection. At Day 2 post infection mice were euthanised and flow cytometry was used to quantify the myeloid cell populations in the spleen and peripheral blood. Each dot represents an individual animal. n=4 animals per group, data are from one independent experiment. For the spleen, total numbers of cells were determined by multiplying total leukocyte counts with the percentages of myeloid cell subsets determined by flow cytometry. Statistical analysis: groups were compared by unpaired, two-tailed t-test. Error bars indicate the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.9 The cytokine response to DENV infection in MAR1 treated WT mice

As the pathologic symptoms of dengue are largely driven by a cytokine storm phenotype, I characterised the cytokine response in WT mice. This was an important piece of model characterisation in the sense that this WT dataset could act as a baseline, that would subsequently allow me to determine whether different genetically-targeted animals developed a more pronounced cytokine storm phenotype in response to DENV infection. To assess the cytokine response to DENV2 infection in my model, I took plasma from WT animals prior to infection (note: naïve animals were not MAR1 treated), and from MAR1-treated WT animals at days 2 and 4 post-infection. Levels of 27 cytokines were quantified by a commercial multiplex immunoassay. Longitudinal profiling over the course of infection for each of these cytokines is presented below (Figure 4.9A-B), and a summary of statistical differences relative to baseline levels (figure 4.9C).

At day 2 post infection, IFN-α and the pro-inflammatory chemokines IP-10 and RANTES were upregulated. Surprisingly, the Th1 cytokine IL-2 was significantly downregulated, although this result was transient and not seen when day 4 values were compared to naïve controls.

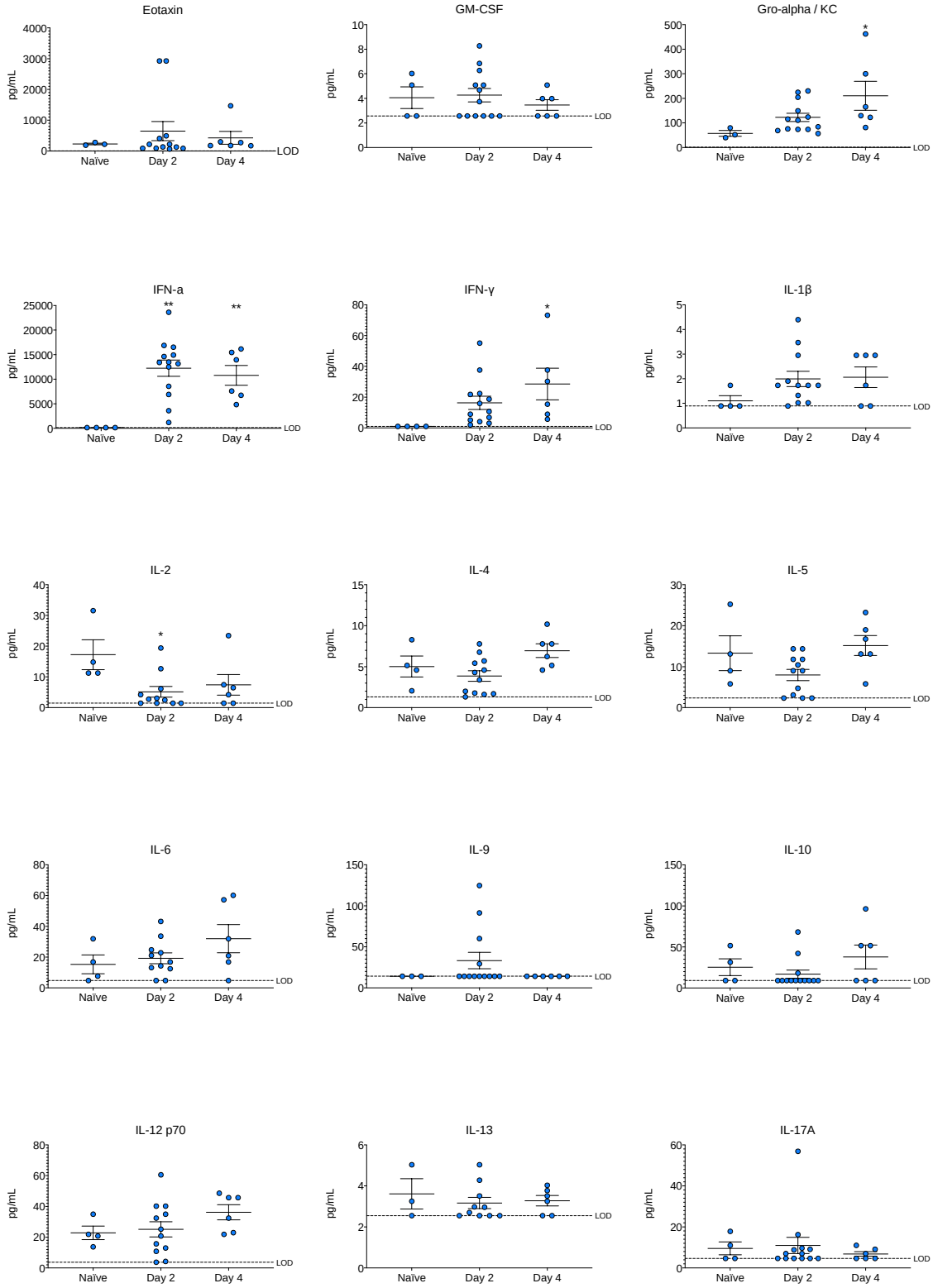
At day 4 post-infection, a broader pro-inflammatory cytokine response was observed. Mean concentrations of IP-10 and RANTES increased, while the concentration of IFN-α remained approximately constant. The other cytokines that were upregulated, relative to baseline, at day 4 post infection were: the hallmark Th1 cytokine IFN-γ; the pro-inflammatory cytokines IL-18 and TNF; and the chemokines Gro-alpha, MCP-1, MCP-3

and MIP-1β. Therefore, the overall state of the cytokine milieu at day 4 post infection is strongly reflective of an acute anti-inflammatory response. IFN-α, TNF, Gro-alpha, MCP-1, MCP-3 and MIP-1β, RANTES and IP-10 are released by innate immune cells such mast cells and macrophages (Ivashkiv & Donlin, 2014; Sokol & Luster, 2015), and therefore it could be these cells that are driving the innate immune response in this model. There was no statistically significant increase in the concentration of the Th2 cytokines IL-4, IL-5 or IL-13, or the Th17 cytokine Th17.

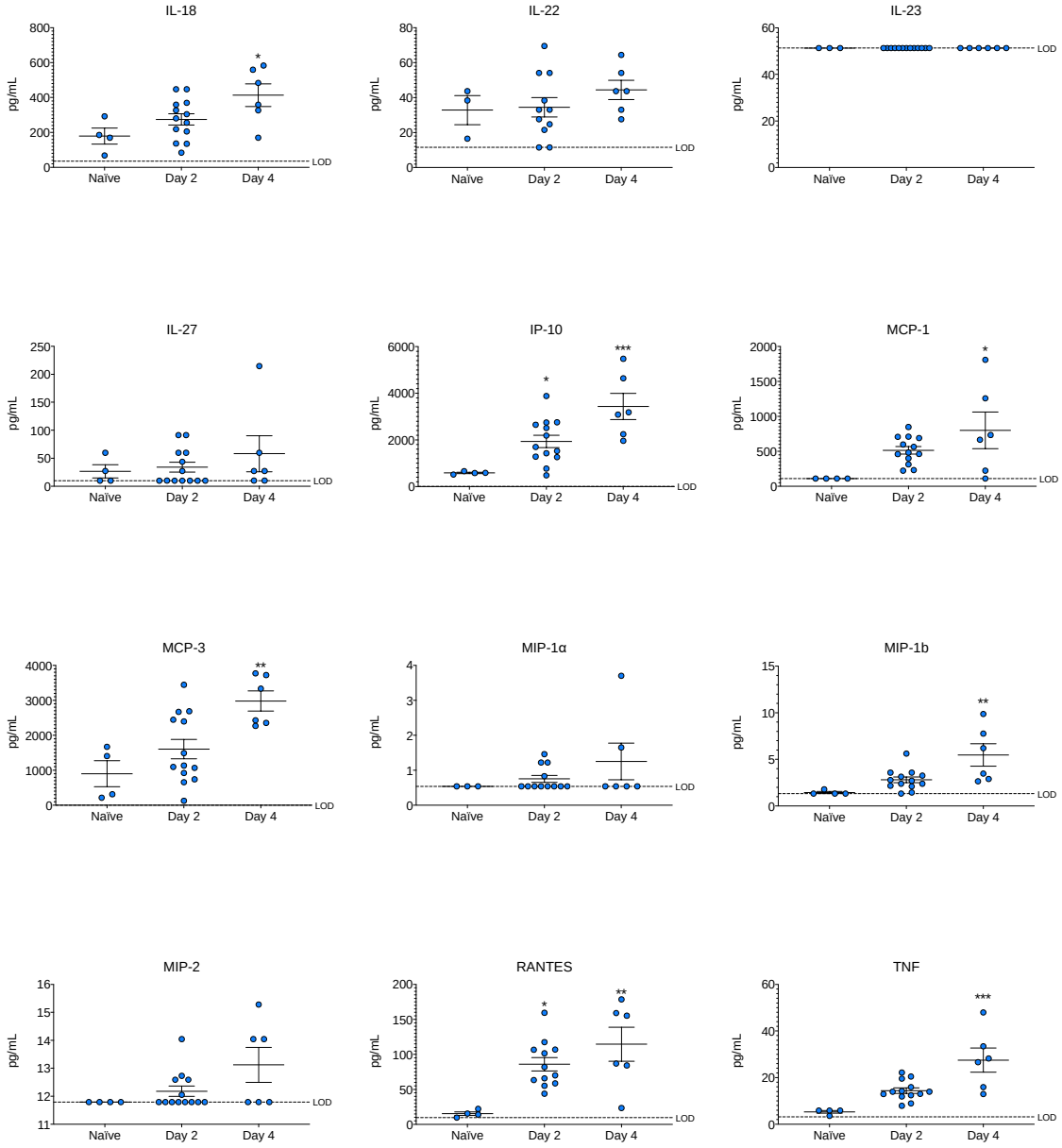
As described in the introduction, characterisation of the cytokine storm that occurs during severe dengue has been difficult. This difficulty has been due to several factors, including heterogeneity in the reporting and sample collection practises of different groups, and differences between different human host populations and viral serotypes (Y. H. Lee et al., 2016; Soo et al., 2017). Therefore, the only cytokines that have been shown to be consistently upregulated in severe dengue, according to meta-analysis, have been IL-8, IL-10, IL-18, while RANTES, IL-7, TGF-β and VEGFR2 are consistently down-regulated (Y. H. Lee et al., 2016; Soo et al., 2017).

In this model I found that IL-18 was upregulated at day 4 post-infection, which is in agreement with meta-analysis results (Figure 4.9A) (Soo et al., 2017). While there is no direct mouse orthologue for human IL-8, mouse KC and MIP-2 belong to the same chemokine cluster, and have similar roles in neutrophil chemotaxis (Hol, Wilhelmsen, & Haraldsen, 2010). I found that Gro-α / KC was significantly upregulated at day 4 post-infection (Figure 4.9A), while there was no significant upregulation of MIP-2. IL-10 has been repeatedly and consistently shown to be upregulated in severe dengue (Y. H. Lee et al., 2016; Soo et al., 2017), however, I did not find evidence that this was upregulated during infection in my model (Figure 4.9A). Interestingly, I found that RANTES was upregulated at both day 2 and day 4 post-infection, whereas meta-analysis has shown that RANTES is downregulated during severe dengue in humans.

A


Eotaxin
GM-CSF
Gro-alpha / KC
IFN-a
IFN-γ
IL-1β
IL-2
IL-4
IL-5
IL-6
IL-9
IL-10
IL-12 p70
IL-13
IL-17A
pg/mL
Naïve
Day 2
Day 4
LOD

B


IL-18
IL-22
IL-23
IL-27
IP-10
MCP-1
MCP-3
MIP-1α
MIP-1b
MIP-2
RANTES
TNF
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Day 4

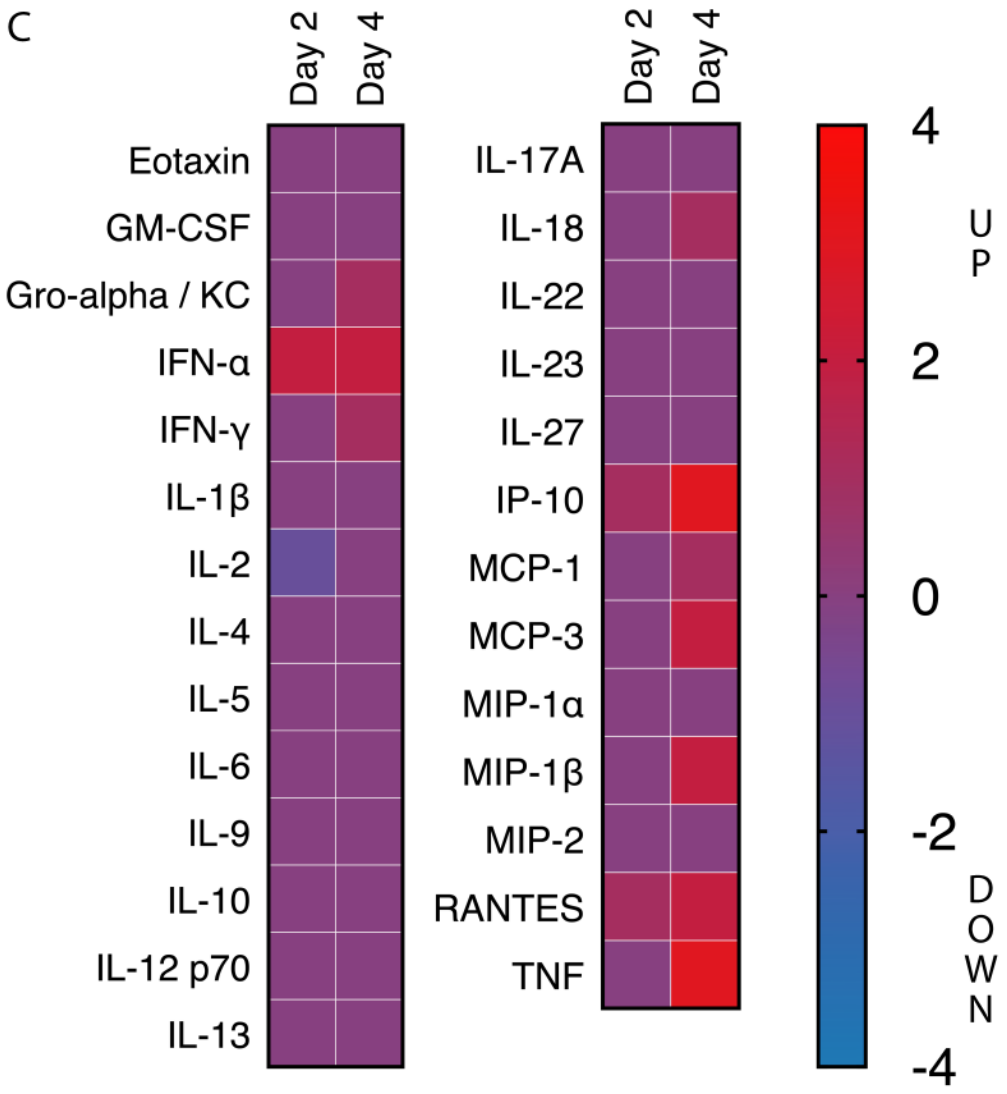

Figure 4.9 | Statistical differences in plasma cytokine levels of DENV2-infected WT mice, relative to naïve WT controls. Plasma was isolated from naïve animals or MAR1-treated, DENV2 infected animals at day 2 and day 4 post-infection. Cytokine levels were determined by multiplex bead array. Cytokines are graphed in alphabetical order, accordingly A) depicts results for Eotaxin through to IL-17A; B) depicts results for IL-18 through to TNF. Error bars indicate the mean ± SEM. Data is representative of 3-13 animals per group. Plasma samples for "Day 2" and "Day 4" were obtained from 3 independent experiments. The mean values for Day 2 and Day 4 data were compared to the mean of the naïve group by one-way ANOVA, using Dunnett's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. C) this heatmap summarises statistical differences when Day 2 and Day 4 samples were compared to naïve samples. "0" = ns, "1" $p < 0.05$, "2" $p < 0.01$, "3" $p < 0.001$, "4" $p < 0.0001$. Negative values indicate that levels of the

cytokine are downregulated relative to naive controls.

4.10 The cytokine response to DENV infection in MAR1 treated gene-targeted mice

After the characterisation of the cytokine response to DENV2 infection in MAR1-treated WT animals, I compared cytokine data from MAR1-treated gene-targeted animals to the equivalent data from WT controls. For naïve controls, bleeds were obtained from animals that had not been treated with MAR1. Ordinary one-way ANOVA with Dunnett's post-hoc test for multiple comparisons was used to determine cytokines that were statistically up- or down-regulated in gene-targeted animals compared to WT controls. In a similar fashion to the summary of the WT cytokine data, I have used heatmaps to graph the degree of statistical difference between gene-targeted and WT animals for each group. For example, $SOCS3^{LysM}$ results at Day 2 post infection are compared to WT results at Day 2 post infection (Figure 4.10A-B). Unfortunately, naïve $SOCS3^{LysM}$ plasma and $TRAF2^{LysM}$ plasma from Day 4 post-infection was not available for this cytokine analysis, and hence these columns are missing from Figure 4.10.

4.10.1 The cytokine response of $p50^{-/-}$ mice to DENV2 infection

There were no significant differences in the levels of surveyed cytokines between naïve WT and $p50^{-/-}$ animals. At Day 2 post-infection, levels of IFN-α, MCP-1 and MCP-3 were downregulated in $p50^{-/-}$ vs WT animals. There is no clear explanation for the decrease of type I interferon in the $p50^{-/-}$ animals. *In vitro*, one group has reported that $p50^{-/-}$ MEFS produced increased IFN-β mRNA relative to WT controls, consistent with a role for p50 homodimers as repressors of IFN-β expression (Gough, Messina, Clarke, Johnstone, & Levy, 2012; Sha et al., 1995). *In vivo*, as previously mentioned , p50 deficiency seems to confer a degree of resistance to infection with EMCV, ostensibly due to a decreased apoptotic threshold reducing the ability of the virus to replicate (Schwarz et al., 1998; Sha et al., 1995). To the extent of my knowledge, no groups have measured levels of type I interferon in a $p50^{-/-}$ murine model to date.

MCP-1 and MCP-3 have been reported to be ISGs (de Veer et al., 2001). In addition, an NF-κB site, along with SP1 and AP1 sites, has been reported to control expression of MCP-1, while overexpression of IκBa was able to significantly reduce production of MCP-

1 and MCP-3 following LPS stimulation of human monocyte-derived macrophages (Ciesielski, Andreakos, Foxwell, & Feldmann, 2002; Lim & Garzino-Demo, 2000). This suggests that the reduced expression of MCP-1 and MCP-3 could be due to a combination of firstly, reduced levels of type I interferon; and secondly, changes in NF-κB activity in these p50 deficient animals in response to DENV2 infection.

4.10.2 The cytokine response of SOCS3^{LysM} mice to DENV2 infection

Deletion of SOCS3 in the myeloid compartment did not lead to significant increases in production of the cytokines examined, with the exception being an upregulation of MCP-1 at Day 2 post-infection. Perhaps this lack of cytokine dysregulation was to be expected, as SOCS3 deficiency, at least in the case of IL-6 signalling, has been shown to qualitatively alter the responses to gp130 cytokine signalling rather than altering cytokine production *per se* (B. A. Croker et al., 2012). Previously, a group was able to reduce production of MCP-1 in response to LPS treatment *in vitro* by treating cells with a cell-permeable version of SOCS3 (Jo, Liu, Yao, Collins, & Hawiger, 2005). Therefore, it is not entirely surprising that plasma levels of MCP-1 should be increased during DENV infection of SOCS3^{LysM} mice.

4.10.3 The cytokine response of TRAF2^{LysM} and IAP deficient mice to DENV2 infection

The cIAPs and TRAF2 play key roles in the transduction of NF-kB signalling and the control of cell survival. However, I found that patterns of cytokine expression in DENV-infected c1^{LysM}/cIAP2$^{-/-}$ mice and TRAF2^{LysM} mice were quite different. When naïve bleeds plasma from c1^{LysM}/cIAP2$^{-/-}$ and WT animals were compared, it was evident that c1^{LysM}/cIAP2$^{-/-}$ mice had a broad pro-inflammatory phenotype, with upregulation of pro-inflammatory cytokines TNF and IL-18, and pro-inflammatory chemokines IP-10, MCP-1, MCP-3, MIP-1β and RANTES (Figure 10A and 10B). In addition, c1^{LysM}/cIAP2$^{-/-}$ animals showed upregulation of IFN-γ, indicative of a chronic Th1-mediated response, and the anti-inflammatory cytokine IL-10. In stark contrast, no cytokines were upregulated in naïve plasma collected from TRAF2^{LysM} and cIAP2$^{-/-}$ mice.

At day 2 post-infection, c1^{LysM}/cIAP2$^{-/-}$ animals had elevated levels of IL-6, RANTES, and TNF relative to WT animals. The reduction, compared to naïve measurements, in the number of cytokines that were upregulated in c1^{LysM}/cIAP2$^{-/-}$ at day 2 post infection,

relative to WT, probably reflects the broad induction of pro-inflammatory cytokines in WT animals in response to infection (see figure 9A-9C). Surprisingly, levels of IFN-α were significantly lower in c1^{LysM}/cIAP2$^{-/-}$ animals (mean ± SEM of 1.72 ± 0.42 ng/mL) relative to WT (mean ± SEM of 12.26 ± 0.17 ng/mL). At day 2 post infection, TRAF2^{LysM} mice also had a lower level of IFN-α (4.93 ± 1.02 ng/mL) than WT, and a decreased level of MCP-1. In contrast to the c1^{LysM}/cIAP2$^{-/-}$ animals, no levels of cytokines were increased, relative to WT, in TRAF2^{LysM} animals at day 2 post infection. cIAP2$^{-/-}$ animals had upregulated expression of IL-6 and MIP-1β, relative to WT at this day 2 time point.

At day 4 post-infection, the c1^{LysM}/cIAP2$^{-/-}$ animals still exhibited increased levels of RANTES and decreased levels of IFN-α, but levels of all other cytokines were not statistically different from WT control values. Levels of cytokines in TRAF2^{LysM} and cIAP2$^{-/-}$ animals were also not statistically different from WT control values.

Therefore, the overall picture is one of profound dysregulation of pro-inflammatory cytokine expression in c1^{LysM}/cIAP2$^{-/-}$ animals, with increased inflammatory cytokine production in the naïve state and at day 2 post-infection, and some pro-inflammatory cytokines were also elevated at this timepoint in cIAP2$^{-/-}$ animals. This dysregulation was not matched in TRAF2^{LysM} animals, despite the fact that TRAF2 and the cIAPs have similar biological functions. In addition, c1^{LysM}/cIAP2$^{-/-}$ and TRAF2^{LysM} animals showed decreased production of IFN-α following infection perhaps consistent with the decreased viral load in the former mice.

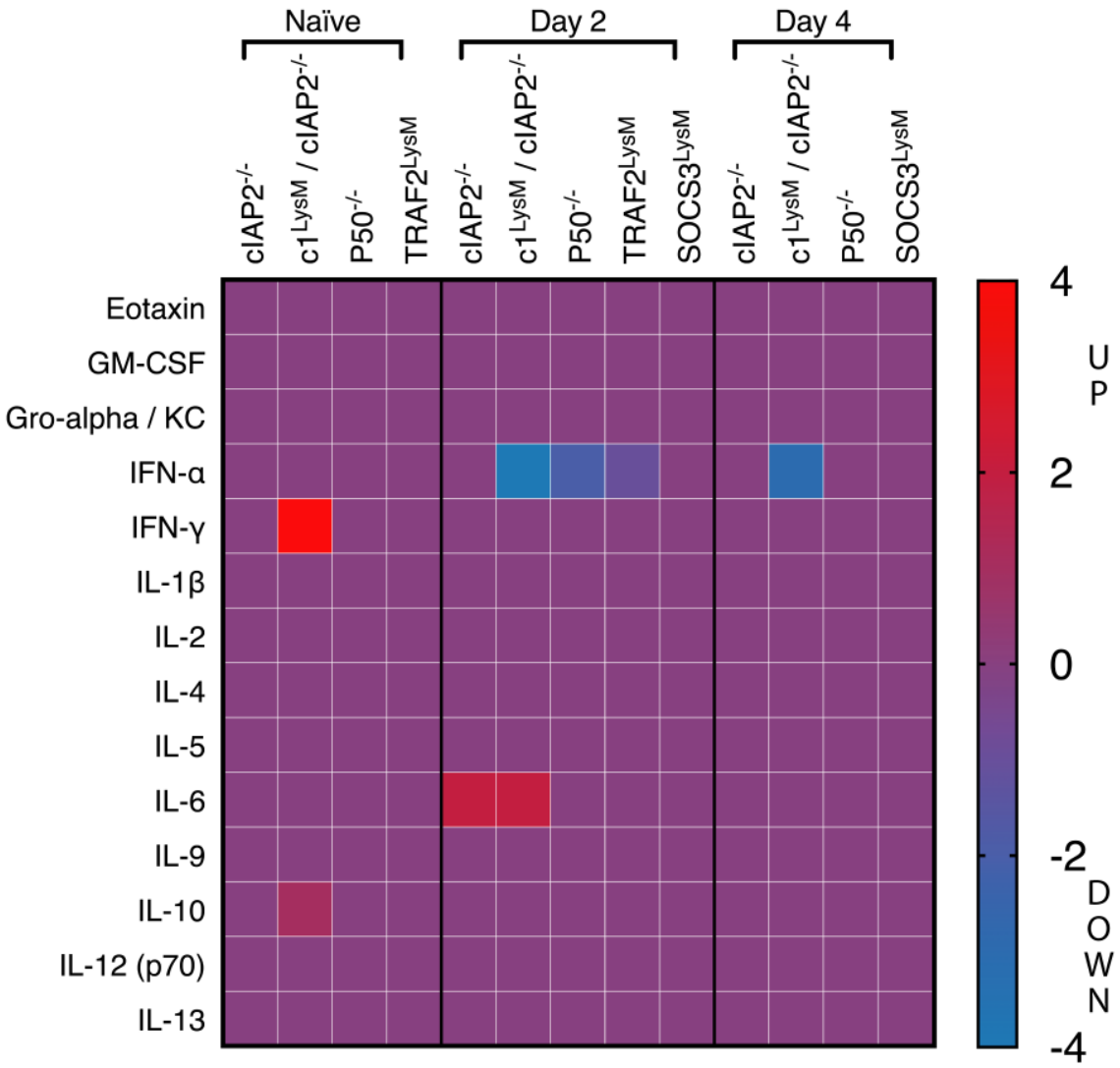

Figure 4.10| Statistical differences of cytokine levels in the plasma of DENV2-infected genetically-targeted mice, relative to WT control (this figure is continued below). Plasma was isolated from naïve animals, or MAR1-treated, DENV2 infected animals at day 2 and day 4 post-infection. Cytokine levels were determined by multiplex bead array. Cytokine levels are coloured according to their statistical difference relative to a WT control group, with red representing upregulation and blue representing downregulation. For each timepoint, data was analysed by ordinary one-way ANOVA, with Dunnett's post-hoc test for multiple comparisons. Data is representative of 3-13 animals per group, with the exception of $p50^{-/-}$ Day 2 samples for IL-5, IL-6 and MIP-1α, where data was only recovered for two animals. Plasma samples for each group were obtained from 1-4 independent experiments. "0" = ns, "1" $p < 0.05$, "2" $p < 0.01$, "3" $p < 0.001$, "4" $p < 0.0001$.

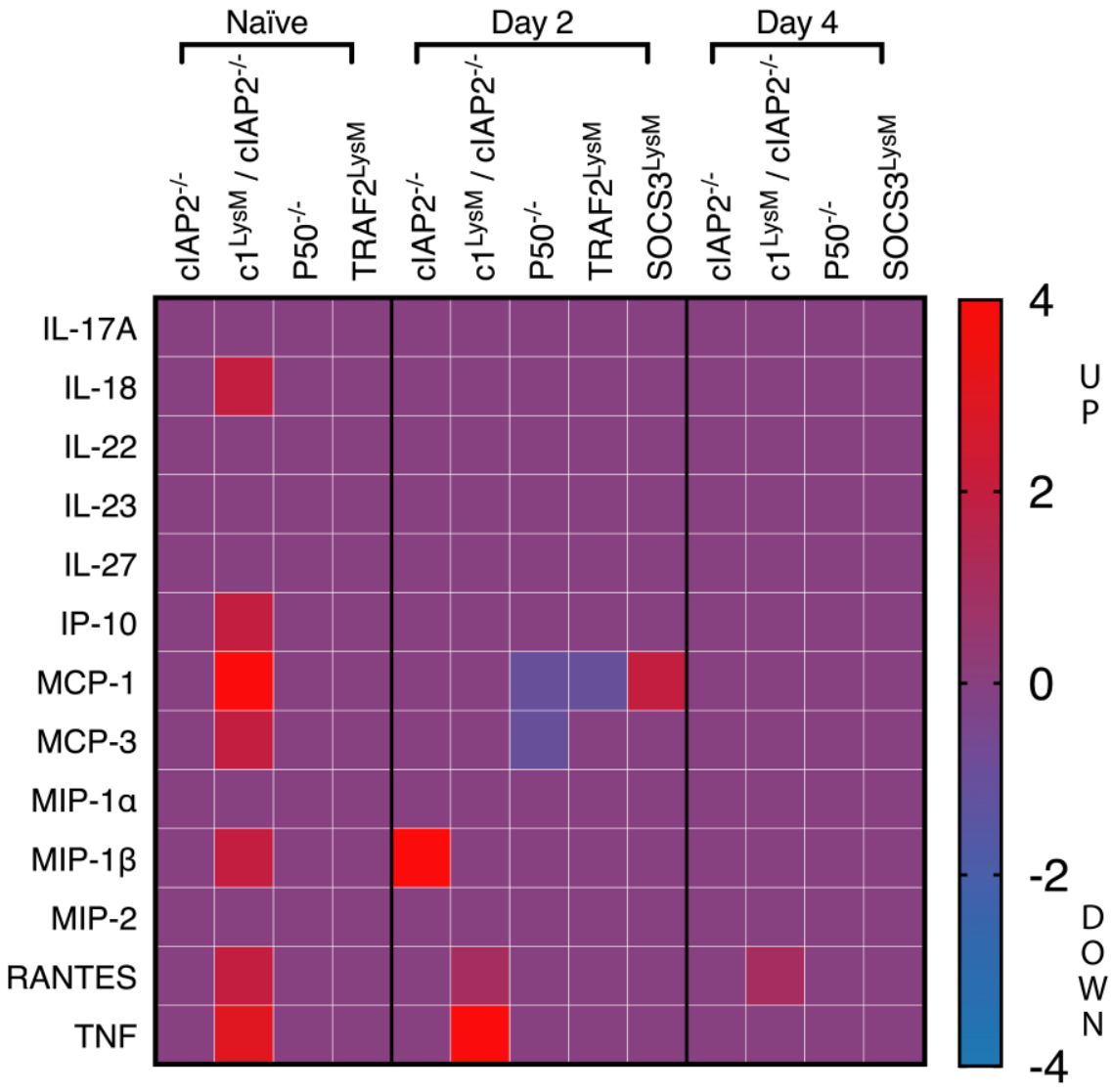

Figure 4.10 (continued) | Statistical differences of cytokine levels in the plasma of genetically-targeted mice, relative to WT control.

4.11 Discussion

Given that our knowledge of the exact aetiology of severe dengue is lacking, and that the cytokine storms of severe dengue are caused by dysregulated host signalling, I set out to attempt to identify and characterise potential host restriction factors *in vivo*. In the first instance, I used MAR1 antibody to suppress the type I interferon responses of a panel of gene-targeted animals. These animals were subsequently infected with DENV2 and followed to determine whether they developed clinical signs of infection. Isolated instances of weight and temperature change were observed in SOCS3^{LysM},

TRAF2^{LysM} and cIAP2$^{-/-}$ animals (see figures 4.4B, 4.5B and 4.6A, respectively). Interestingly, c1^{LysM}/cIAP2$^{-/-}$ animals showed a transient decrease in weight, and repeated spikes in temperature early during the course of infection (days 1-3 post-infection, Figure 4.6A-B) coincident with aberration in cytokines observed in these animals.

Furthermore, c1^{LysM}/cIAP2$^{-/-}$ animals displayed a decrease in viral load at day 2 post infection (Figure 4.6C), consistent with the BMDM *in vitro* results presented earlier (Chapter 3, Figure 3.13). Given the importance of the IAPs to NF-κB signalling (Mahoney et al., 2008; Varfolomeev et al., 2007), it is logical to conclude that a decrease in NF-κB activity in the myeloid DENV host cells in c1^{LysM}/cIAP2$^{-/-}$ animals contributes to the lower viral load observed in these animals. In addition, a decreased proportion of monocytes, and a trend towards a decreased proportion of dendritic cells, was found in the blood of c1^{LysM}/cIAP2$^{-/-}$ mice, relative to WT mice.

Given that monocytes and dendritic cells are preferred host cells for DENV, an argument can be made that the decreased proportions of these populations in c1^{LysM}/cIAP2$^{-/-}$ animals could explain the consistently lower viral titres observed upon DENV2 infection of these animals (Figure 4.6C). However, in this case it would be reasonable to expect that cIAP2$^{-/-}$ animals, which have expanded monocyte and inflammatory macrophage blood compartments upon DENV2 infection (Figure 4.7A), would correspondingly have a higher viral load than WT animals, and this does not seem to be the case (Figure 4.6C). To further examine the effect of IAPs on DENV infection *in vivo*, I treated WT MAR1-treated mice with IAP antagonists; these results are presented in Chapter 5.

Determination of the plasma cytokine levels in WT animals revealed that DENV2 infection induced a pro-inflammatory response. There was a general trend for pro-inflammatory cytokines levels to increase above the level of naïve samples at day 2 post infection, before elevating still higher at day 4 post infection (Figure 4.9A-B). This was reflected in the larger number of cytokines that were significantly elevated above the levels of naïve controls as the infection progressed (summarised in Figure 4.9C). Overall, most of the genetically targeted animals did not have pro-inflammatory cytokine profiles that were markedly different to that of the WT controls, with the

exception of the c1^{LysM}/cIAP2$^{-/-}$ mice which showed a consistently more pro-inflammatory response than WT controls (Figure 4.10A-B). However, despite the widespread elevation of pro-inflammatory cytokines, there was no noticeable effect the weight and temperature of these animals, with the exception of c1^{LysM}/cIAP2$^{-/-}$ animals which manifested moderate signs of fever and weight change (Figure 4.6A-B

Given that the cIAPs and TRAF2 have similar roles in transducing NF-kB signalling and restraining cell death, it was interesting to observe that high levels of pro-inflammatory cytokines were detected in the plasma of c1^{LysM}/cIAP2$^{-/-}$ animals, but not in TRAF2^{LysM} animals (Figure 4.10). Recently, in addition to their well-documented role in preventing cell death, many reports have shown that IAPs have a key role in restraining inflammation. Depletion of IAPs can result in the activation of caspase 8 by TNF signalling (Varfolomeev et al., 2007). In part, this is due to the reduced production of cFLIP, a protein which heterodimerises with caspase 8, and in doing so, restricts the number of substrates that caspase 8 can cleave. Therefore, reduced cFLIP equates to an increase in the substrates that can be processed by caspase 8 (Lalaoui & Vaux, 2018). In addition, there is evidence that in some cell types, an absence of cIAP activity favours the assembly of the ripoptosome, a death signalling complex constituted of RIPK1, FADD, cFLIP and caspase 8 (Lawlor et al., 2015). The ripoptosome is capable of activating caspase 8 in the absence of TNF signalling (Feltham & Silke, 2017). Activated caspase 8 can then drive inflammation by cleaving pro-IL-1β or driving apoptotic cell death (Lawlor et al., 2015; J. Zhang et al., 2019). Alternatively, deficiency of IAPs has been shown to result in activation of the NLRP3 inflammasome, and cleavage of IL-1β by caspase 1 (Vince et al., 2012).

TRAF2 has also been reported to have an anti-inflammatory function. When Jin and colleagues treated WT and TRAF2^{LysM} mice with dextran sodium sulphate to induce colitis they observed that TRAF2^{LysM} mice had higher levels of mortality and inflammation of the distal colon (Jin et al., 2015). Naïve TRAF2$^{-/-}$ animals have been reported to have higher levels of serum TNF than WT controls (L. T. Nguyen et al., 1999), and TRAF2$^{-/-}$ macrophages produce more pro-inflammatory cytokines than WT controls (Jin et al., 2015; L. T. Nguyen et al., 1999)

However, the crossover in functionality between TRAF2 and the cIAPs is not absolute. While a small fraction of TRAF2$^{-/-}$ mice can survive until 2-3 weeks of age before succumbing to colitis or wasting ((Piao et al., 2011; Yeh et al., 1997), cIAP1$^{-/-}$/cIAP2$^{-/-}$ full body knockout mice succumb at approximately E10 to cardiovascular failure and haemorrhage due to excessive activation of RIP kinases (Moulin et al., 2012), which can drive inflammation and cell death in the absence of cIAPs (Vince et al., 2012). Furthermore, mortality of TRAF2$^{-/-}$ mice could be rescued by concurrent deletion of TNFR1 (Piao et al., 2011),, although this phenotype is partially strain-specific (L. T. Nguyen et al., 1999). In contrast, cIAP1$^{-/-}$/cIAP2$^{-/-}$ TNFR1$^{-/-}$ mice are only able to survive until day 2 after birth (Moulin et al., 2012). Finally, NF-κB signalling was largely normal, albeit with delayed kinetics, in TRAF2$^{-/-}$ thymocytes and MEFs (S. Y. Lee et al., 1997; Yeh et al., 1997). In contrast, NF-κB signalling is minimal in cIAP1$^{-/-}$/cIAP2$^{-/-}$ MEFs and hepatocytes (Mahoney et al., 2008; Varfolomeev et al., 2007).

Collectively, these data suggest that a degree of inflammation-dampening IAP-mediated ubiquitination is able to be maintained in the absence of TRAF2, and by extension, is present in the myeloid cells of TRAF2^{LysM} mice. Some weight is given to this argument by the finding that the kinetics and magnitude of IκBα degradation in response to TNF is identical in WT and TRAF2^{LysM} macrophages, suggesting intact IAP-mediated ubiquitination (Etemadi et al., 2015). In contrast, IAPs are deficient in the myeloid cells of cIAP1^{LysM}/cIAP2$^{-/-}$ mice, therefore inflammatory processes are not restrained and these mice produce higher concentrations of pro-inflammatory cytokines in response to DENV infection.

A single knockout of cIAP2$^{-/-}$ would be predicted to have much less of a pro-inflammatory effect, as substantial ubiquitin-mediated control of pro-inflammatory machinery should be maintained, and in keeping with this, cIAP2$^{-/-}$ animals had a lesser degree of inflammation than cIAP1^{LysM}/cIAP2$^{-/-}$ mice at all examined timepoints.

I also observed that IFN-α levels were decreased at Day 2 post infection for c1^{LysM}/cIAP2$^{-/-}$ and TRAF2^{LysM} mice, but not for cIAP2$^{-/-}$ mice. Furthermore, c1^{LysM}/cIAP2$^{-/-}$ also had decreased levels of IFN-α at day 4 post infection. Although TRAF2^{LysM} macrophages have been shown to have intact canonical NF-κB signalling, they were reported to be more

susceptible to apoptotic stimuli (Etemadi et al., 2015). Likewise, $c1^{LysM}$/$cIAP2^{-/-}$ BMDMs have also been shown to be sensitised to undergo apoptosis (Lawlor et al., 2015). Therefore, it is possible that increased apoptosis of IFN-α generating cells could be driving this reduction in IFN-α. In support of this, flow cytometry revealed that there were fewer monocytes in the blood of $c1^{LysM}$ / $cIAP2^{-/-}$ mice at day 2 post-infection, relative to WT controls (see Figure 4.8A). There was also a trend towards reduced numbers of dendritic cells in the blood. Dendritic cells, in particular plasmacytoid DCs that are found in peripheral blood, are very potent producers of type I interferon in response to viral stimulation (Swiecki & Colonna, 2015). Alternatively, the decrease in viral loads in IAP deficient may abrogate production of type I IFN.

Another possibility is that the IAPs and / or TRAF2 are controlling the induction of IFN-α by PRRs. One important pathway for the induction of type I interferon involves the cytoplasmic PRR RIG-I and the adaptor protein MAVS (Seth, Sun, Ea, & Chen, 2005; Yoneyama et al., 2004). In this pathway, RIG-I senses viral RNA, and this signal is transduced by MAVS, resulting in K63-linked ubiquitination of TRAF3, activation of IRF-3 and IRF-7, and the upregulation of type I interferon (Mao et al., 2010; Sharma et al., 2003). cIAP-mediated ubiquitination has been described as an important component of this pathway. Mao and colleagues found that a reduction of levels of cIAP1 and cIAP2 by genetic manipulation or by treatment with an IAP antagonist resulted in reduced levels of TRAF3 ubiquitination, and a reduced type I interferon production in response to Sendai Virus infection (Mao et al., 2010). Therefore, it is possible that decreased ubiquitination in the absence of cIAPs or their adaptor protein TRAF2 ameliorates RIG-I – MAVS signalling, and hence the induction of IFN-α. The role of the cIAPs in controlling type I interferon production was further explored when IAP antagonist treated mice were infected with DENV2, and these results are presented in Chapter 5.

One of the caveats of this *in vivo* model of DENV2 infection is that, over the timepoints assessed, IL-10 levels in WT mice did not significantly increase above baseline (Figure 4.9A), and there was no statistically significant upregulation of IL-10 in other genotypes, relative to WT control, at days 2 and 4 post infection (Figure 4.9C). Meta-analyses have found that IL-10 is consistently upregulated in cases of severe dengue, relative to control patients that have standard dengue fever (Y. H. Lee et al., 2016; Soo et al., 2017). IL-10

is a key anti-inflammatory cytokine that is produced mainly by monocytes, macrophages and T cells (Moore et al., 2001). IL-10 upregulates expression of the JAK signalling regulator SOCS3, and exerts its effects primarily via reducing production of the potent pro-inflammatory cytokines IL-1 and TNF (Ito et al., 1999; Moore et al., 2001; Qasimi et al., 2006). IL-10 was statistically elevated in $c1^{LysM}$/$cIAP2^{-/-}$ animals at baseline; however, the significance of this upregulation was lost in infected mice (Figure 4.9C).

Previously, other groups have investigated cytokine production in the context of murine DENV infection. Cytokine levels generally correlated with the degree of viral control that could be achieved using different strains of virus and different transgenic mice. For example, Zust and colleagues infected mice strains that had the IFNAR gene selectively deleted from different cell populations using Cre recombinase (Züst et al., 2014). They found that deletion of the IFNAR gene in myeloid populations, including the DENV monocyte, macrophage and dendritic cell host cells, resulted in high viral load and cytokine expression relative to infection of mice with a Cre-mediated deletion of IFNAR in lymphocytes, which are not DENV host cells, or WT mice (Züst et al., 2014). DENV infection of AG129 mice, which have a compound deletion of the type I and type II interferon receptors, results in very high levels of inflammatory cytokines and IL-10 (Balsitis et al., 2009; Sarathy et al., 2015), as does infection of mice with mouse-adapted virus (Atrasheuskaya et al., 2003). The MAR1 model of DENV2 presented in this chapter is characterised by high viraemia at day 2, which is rapidly falling at day 4. The higher degree of viral control achieved in this immunocompetent model, compared to the other models referenced above, seems likely to account for the lower levels of cytokine production observed in this model, and perhaps the relatively low concentrations of IL-10.

A caveat of this work is that the examination of the effects of DENV infection in these animal models could have been more thorough. Analysis was restricted to measurement of weight, temperature, plasma viremia, flow cytometry and cytokine levels. These analyses could have been complemented in several informative ways. For instance, a vascular leakage analysis could have been performed to determine whether differences in inflammatory mediators observed with genetic deficiency of cIAP1 and cIAP2 had an effect on endothelial integrity. Furthermore, viremia was only determined

in the blood, and it could be possible, though unlikely, that the kinetics of viral clearance are different in solid organs e.g. the spleen or the brain. Unfortunately, due to constraints involved with the quantities of MAR1-blocking antibody and the numbers, of genetically deficient animals available, these experiments were not performed.

Collectively my data indicate that IAPs may be involved in DENV disease pathogenesis and this should be further explored by formal necropsy assessment and histopathology. In the next section I used clinical stage inhibitors of IAP to determine impact on clinical outcomes and to complement the data in this chapter.

Chapter 5 - Preclinical Testing of Therapeutic Candidates Using *In Vivo* Models

5.1 Introduction

As outlined in the literature review, many clinical trials have been conducted to assess the ability of repurposed therapeutics to improve outcomes of patients suffering from dengue. Given that none of the drugs trialled against dengue to date have shown efficacy in clinical trials, I wanted to explore the ability of novel drugs to treat clinical indications of dengue.

However, before I could explore the function of potential therapeutic drugs, I wanted to develop a suitable *in vivo* model to assess efficacy. IFNAR$^{-/-}$ mice have several features that make them attractive as a model for the study of severe dengue. IFNAR$^{-/-}$ mice are relatively susceptible to DENV infection and develop high levels of viraemia in comparison to WT mice (Züst et al., 2014). Unlike WT animals, IFNAR$^{-/-}$ mice exhibit clinical signs of infection such as weight loss, and therefore the ability of candidate therapeutics to prevent these signs can be assessed (Züst et al., 2014). Finally, and most importantly, IFNAR$^{-/-}$ mice produce high levels of pro-inflammatory cytokines in response to DENV infection such as TNF, IL-6 and IP-10 that are also found in human disease (Züst et al., 2014).

However, several important caveats apply to the use of IFNAR$^{-/-}$ mice as a model for DENV infection. As previously mentioned, type I interferon signalling is important for many aspects of the immune response such as dendritic cell maturation (Montoya et al., 2002), and the enhancement of humoral antiviral responses (K. Fink et al., 2006). Secondly, titres of DENV in the plasma of IFNAR$^{-/-}$ animal are normally very high when mice are severely ill (Züst et al., 2014). In contrast, during human infection, peripheral levels of DENV are normally rapidly declining at the point of defervescence (Duyen et al., 2011; Fox et al., 2011), which coincides with the most severe symptoms of DENV (Halstead, 2019). Finally, the clinical signs exhibited by IFNAR$^{-/-}$ mice, such as weight loss, do not always correlate with symptoms of DENV observed in humans. Therefore, it is important to keep these caveats in mind when interpreting the results of

experiments using IFNAR$^{-/-}$ mice.

The initial section of this chapter will detail the characterisation of an IFNAR$^{-/-}$ mouse model of severe dengue. The remainder of this chapter will briefly summarise the properties and the rationale for the use of various host-directed therapeutic drugs to treat dengue, before outlining the results of these studies. Studies in this chapter were conducted using either IFNAR$^{-/-}$ mice, or the WT mouse MAR1 model of dengue, which was characterised in experiments presented in Chapter 4.

5.2 Establishment of an *in vivo* model of severe dengue

I should note that in Chapter 4, I presented data that showed that IFNAR$^{-/-}$ infected with DENV2 at the same dose given to MAR1-treated WT and gene-targeted mice (20,000,000 pfu/mouse) succumbed very rapidly to the infection. For the experiments presented in this chapter, I infected IFNAR$^{-/-}$ mice with a lower dose of DENV2 (100,000 pfu/mouse). This resulted in a less aggressive course of disease, which afforded me an extended window where I could treat mice with candidate therapeutics.

In order to characterise an *in vivo* model of severe dengue, I infected IFNAR$^{-/-}$ mice and monitored mice daily to determine the clinical course of infection. IFNAR$^{-/-}$ mice rapidly lost weight (Figure 5.1A), and had a small increase in temperature (Figure 5.1B). Given that fluid replacement therapy is the mainstay of clinical management for severe dengue (World Health Organisation, 2009), these mice were provided with ongoing fluid replacement of 1mL of 0.9% normal saline daily as supportive treatment. At Day 4 post-infection mice exhibited a relatively high viral load of 2.54 X10^6 ± 0.69X10^6 pfu/mL (Figure 5.1C). These results were similar to those resulted previously by another group (Züst et al., 2014).

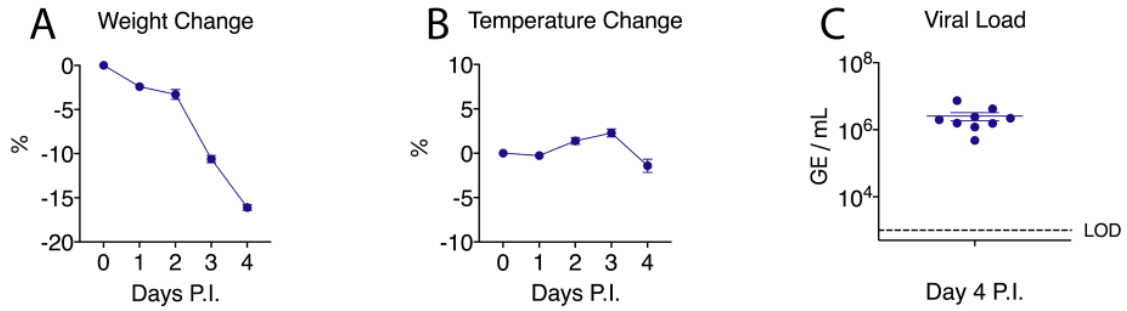

Figure 5.1 | Initial characterisation of an IFNAR$^{-/-}$ mouse model of severe dengue. IFNAR$^{-/-}$ mice were infected intravenously with 1X10^5 pfu of DENV2. Weight (A) and temperature (B) were monitored daily. Mice were humanely euthanised at 4 days post infection. Plasma isolated at Day 4 post infection was used to determine viral loads (C). n=12 mice for weight and temperature; n=9 mice for viral load data. Data is combined from two independent experiments. Error bars indicate the mean ± SEM. For panel C, dots represent data from individual mice.

To confirm that there was a pro-inflammatory cytokine milieu in this IFNAR$^{-/-}$ mouse model, I isolated plasma from peripheral blood at Day 4 post-infection, and quantified cytokine levels using multiplex ELISA (Figure 2). I found significantly increased levels of IFN-γ (A), IL-1β (B), IL-6 (C), IP-10 (D), MIP-2 (E) and TNF (F). After this initial characterisation, I proceeded to use this mouse model of severe dengue to assess the efficacy of novel host-directed therapeutics.

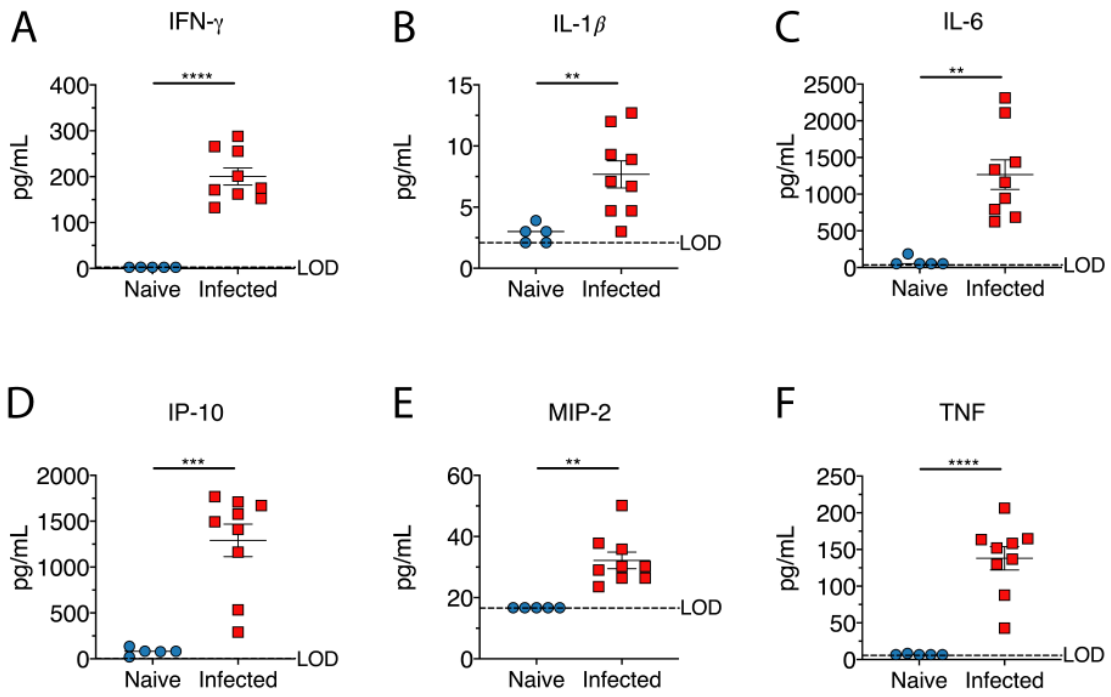

Figure 5.2| Determination of pro-inflammatory cytokine levels in an IFNAR$^{-/-}$ mouse model of severe dengue. IFNAR$^{-/-}$ mice were infected intravenously with 1X10^5 pfu of DENV2. Mice were humanely euthanised at 4 days post infection. A multiplex bead array was run to quantify levels of pro-inflammatory cytokines from plasma isolated at Day 4 post infection. Plasma isolated from the blood of naïve IFNAR$^{-/-}$ mice was included as a negative control. The cytokines analysed were: IFN-γ (A), IL-1β (B), IL-6 (C), IP-10 (D), MIP-2 (E) and TNF (F). n=5 and n=9 for naïve and infected mice, respectively, and data is from one independent experiment. Statistical analysis: samples from naïve and infected plasma for each cytokine was compared by unpaired two-tailed t-test. Error bars indicate the mean ± SEM. Circles and squares represent data from individual mice. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3 Use of LCL-161 to treat *in vivo* DENV infection

Previously, my *in vitro* and *in vivo* studies revealed that a loss of cIAP proteins could reduce viral production (Chapters 3 and 4). Therefore, I decided to use the clinical stage anti-cancer compound LCL-161 to degrade cIAPs in *in* vivo models of DENV infection, and assess the effect of this treatment on disease progression and viral load. For a brief

summary of the LCL-161 literature, please refer to Chapter 3.

For the experiments presented in this chapter, LCL-161 was administered by oral gavage either prophylactically as a single dose of 400mg/kg, or therapeutically as 1-2 doses at 100mg/kg. Previously, our lab has shown that a single dose of LCL-161 administered to mice by oral gavage at 50mg/kg was sufficient to trigger marked degradation of cIAP1 levels in the liver (24 hour time point) (Ebert et al., 2015). Therefore, it can be assumed that the doses of LCL-161 used to treat the animal models of DENV2 infection presented subsequently in this chapter were sufficient to trigger the degradation of IAP proteins.

5.3.1 Can LCL-161 treatment improve the outcomes of DENV2 infected IFNAR$^{-/-}$ mice?

Initially, I decided to test LCL-161 using the IFNAR$^{-/-}$ model of severe dengue (see above). IFNAR$^{-/-}$ animals were pre-treated with a single dose of vehicle or LCL-161 prior to infection with DENV2. After infection, the weight of LCL-161 treated animals declined significantly more rapidly than vehicle-treated controls (Figure 5.3A), although this weight loss normalised from day 3 post infection. LCL-161 treated mice did not show differences in temperature relative to wild type controls (Figure 5.3B). Finally, log-rank analysis revealed that there was no significant difference in survival between the vehicle and LCL-161 groups (Figure 5.3C). It should be noted that a measurement of viral load was not performed for these animals. This was because animals were not euthanised on a set day, but instead were euthanised when they reached a pre-determined ethical endpoint.

In conclusion, LCL-161 did not improve survival outcomes in IFNAR$^{-/-}$ animals; in fact, there was a trend for LCL-161 treated mice to succumb earlier than vehicle treated animals, although this trend did not reach statistical significance.

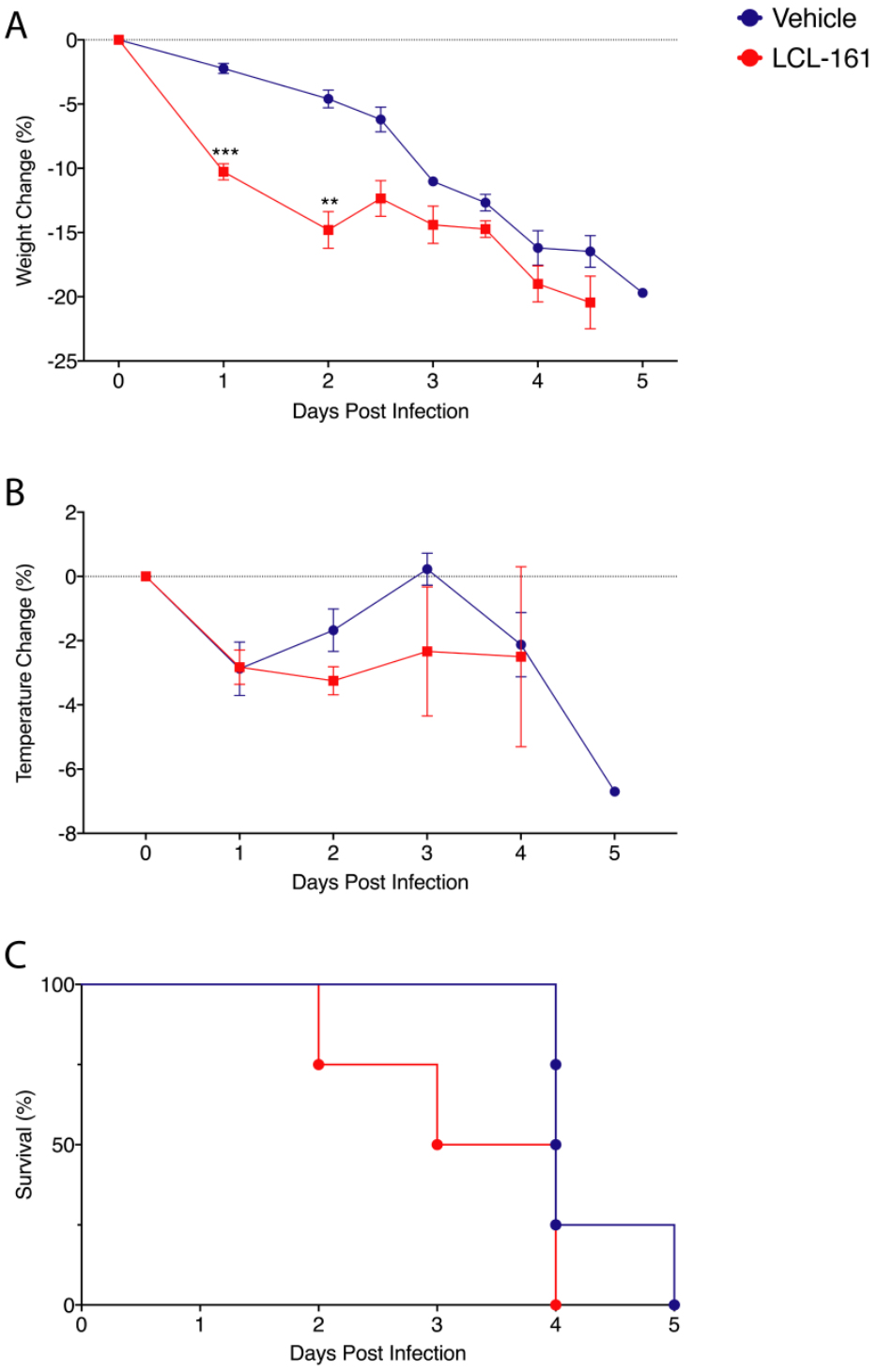

Figure 5.3 | LCL-161 treatment does not improve the survival of IFNAR$^{-/-}$ mice. IFNAR$^{-/-}$ mice were administered 400mg/kg LCL-161 or vehicle by oral gavage 2 hours prior to infection with 1x10^5 pfu per mouse of DENV2. Weight (A) and temperature (B) were monitored daily. Mice were euthanised when they reached a pre-determined ethical endpoint, and the resulting survival curves were plotted (C). n=4 mice per group; data is from one independent experiment. Statistical analysis: log-rank test. Error bars

indicate the mean ± SEM. ** $p < 0.01$, *** $p < 0.001$.

5.3.2 How does LCL-161 treatment alter the myeloid compartments and viral load of MAR1-treated WT mice infected with DENV2?

To further examine the effect of LCL-161 treatment on DENV2 infection *in vivo*, WT mice treated with MAR1 were infected with DENV2. At 24 hours post-infection, mice were treated with a single dose of vehicle or LCL-161. At 48 hours post-infection, LCL-161 mice had developed profound weight loss (Figure 5.4A) while temperature was not significantly altered compared to the control group (Figure 5.4B).

I wanted to determine the size and nature of different myeloid cell subsets in the context of LCL-161 treatment and DENV infection. As DENV host cells, the size of these myeloid cell subsets has been correlated to viral load and disease severity (Halstead, 2019), and these cells also have an important role in producing cytokines that contribute to the pathogenesis of severe dengue. At 48 hours post infection, mice were humanely euthanised. Cells were processed for flow cytometry. There were no differences between vehicle and LCL-161 groups when myeloid cell subsets were examined in the blood (Figure 5.4C), and in the spleen (Figure 5.4D). In addition, an examination of plasma viral load at 48 hours post infection showed that there was no difference between treatment groups (Figure 5.4E).

To extend these results, I repeated this experiment but administered two doses of vehicle / LCL-161 to mice at 4 hours and 30 hours post infection. Again, LCL-161 treated mice lost weight (Figure 5.5A); however, due to the small number of animals in this experiment (n=3 per group), the differences did not reach statistical significance. Mice were euthanised at 48 hours post infection and organs were processed for qPCR and flow cytometry as previously.

Administration of two doses of LCL-161 resulted in the depletion of monocytes and dendritic cells in the blood, while elevating levels of inflammatory macrophages (Figure 5.5C). In the spleen, LCL-161 treatment decreased the numbers of all myeloid subsets examined (Figure 5.5D). Because two doses of LCL-161 was able to reduce the size of the important monocyte, macrophage and DC host compartments (with the exception of inflammatory macrophages in the blood), it was surprising to see that this treatment

had no impact on viral load (Figure 5.5E).

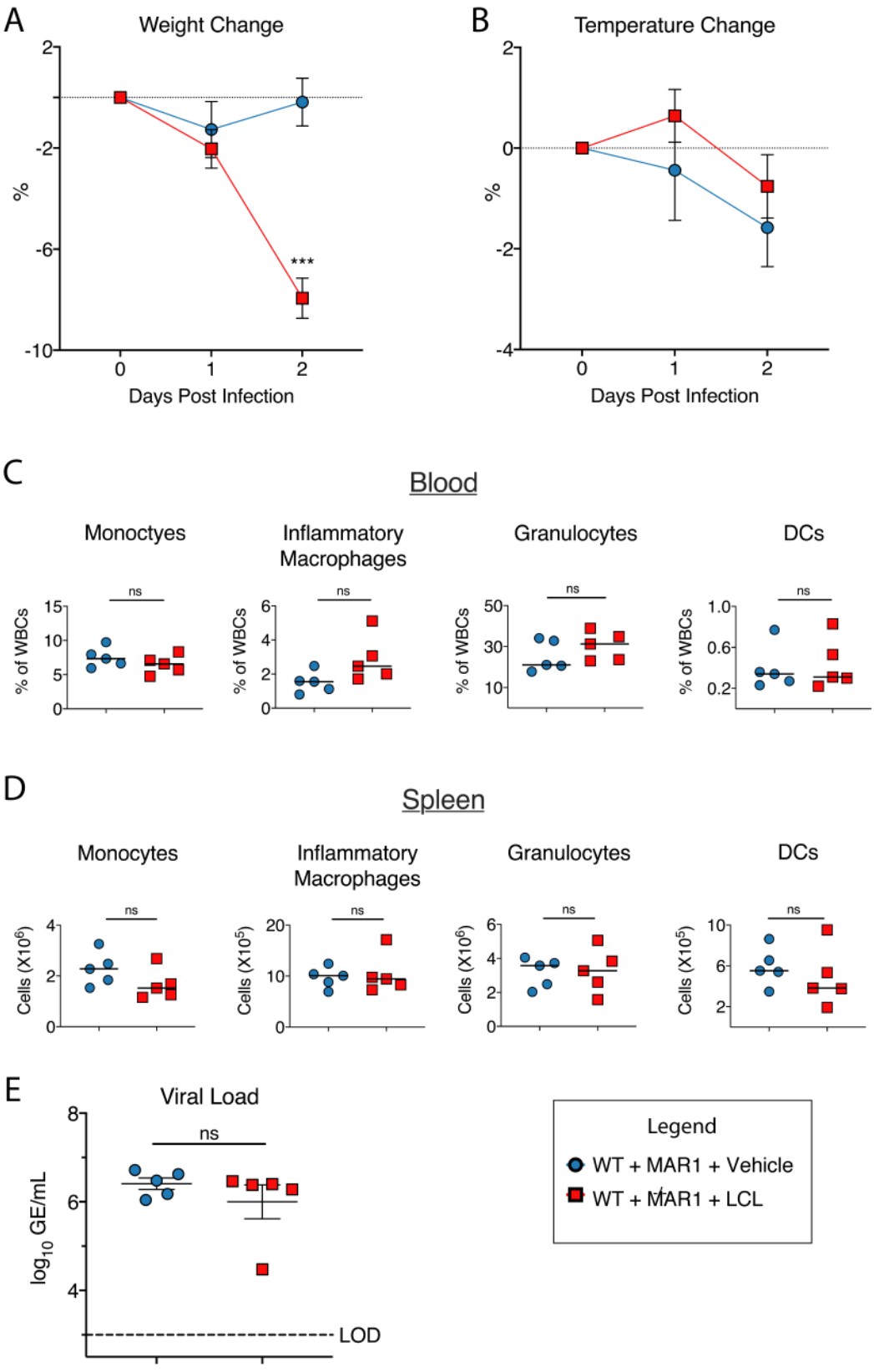

Figure 5.4 | One dose of LCL-161 does not reduce viral load in the WT MAR1 mouse

model of DENV infection. WT mice were treated with MAR1 IFNAR-blocking antibody (500μg at 24 hours prior to infection, 250μg 2 hours prior to infection, 250μg at 24 hours post-infection) and infected with $2x10^7$ pfu DENV2 per mouse. At 24 hours post-infection, mice were administered vehicle or 100mg/kg LCL-161 by oral gavage. Weight (A) and temperature (B) were monitored. Mice were humanely euthanised at 48 hours post infection. Flow cytometry was used to enumerate the proportions and absolute cell counts of the different myeloid cell compartments of the blood (C), and spleen (D), respectively. In parallel, plasma isolated at 48 hours post infection was used to determine the viral loads of vehicle and LCL-161 treated mice (E). n=5 mice per group, data is from one independent experiment. The following statistical tests were used to analyse the presented data. Differences in weight and temperature were assessed by multiple unpaired t-tests with the Holm-Sidak correction for multiple comparisons. Flow cytometry myeloid cell compartment data and log-transformed qPCR viral load data was analysed using two-tailed unpaired t-tests. Error bars indicate the mean ± SEM. For panels C-E, circles and squares represent data from individual mice. *** $p < 0.001$.

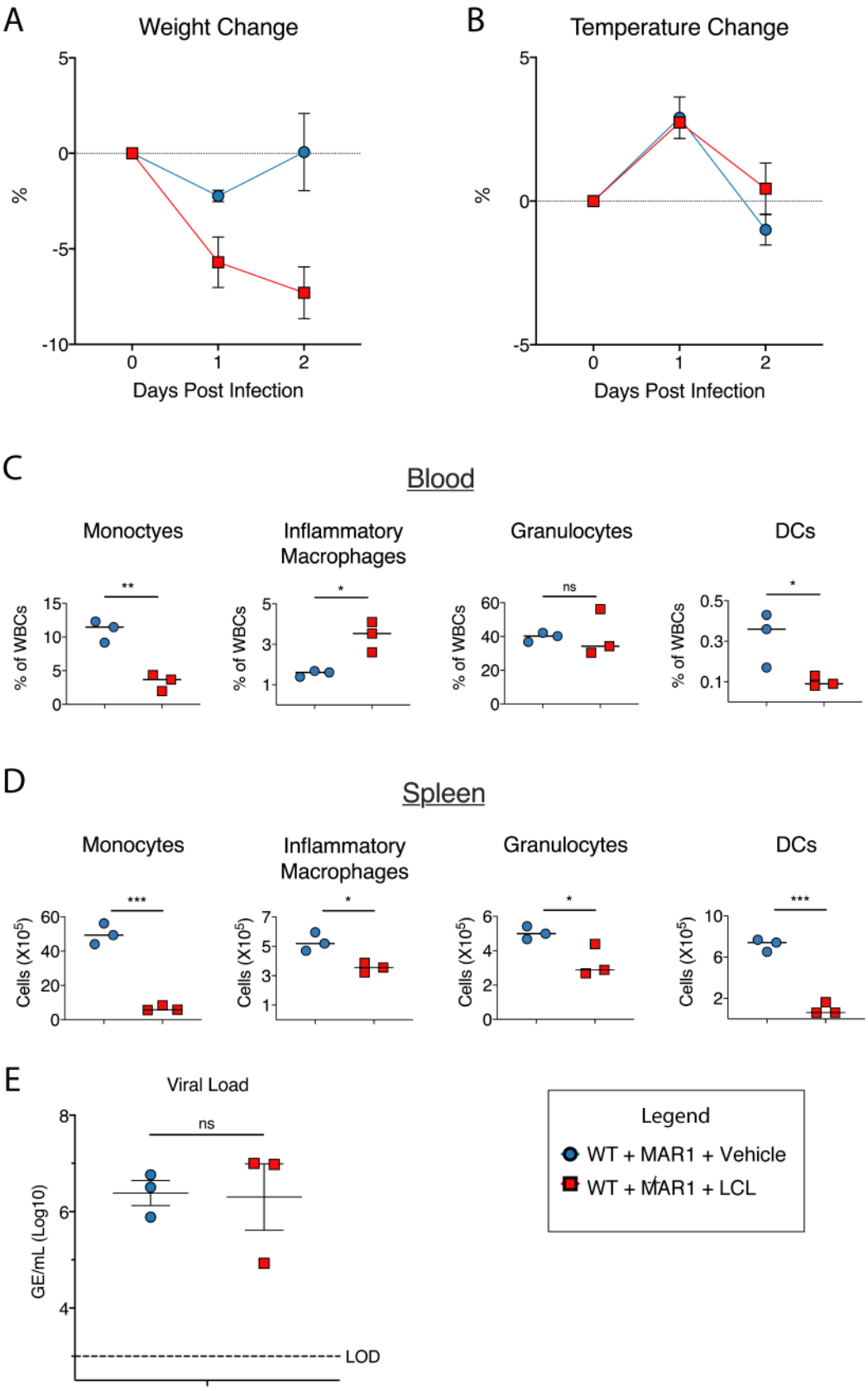

Figure 5. 5 | Two doses of LCL-161 does not reduce viral load in the WT MAR1 mouse model of DENV infection. WT mice were treated with MAR1 IFNAR-blocking antibody

(500μg at 24 hours prior to infection, 250μg 2 hours prior to infection, 250μg at 24 hours post-infection) and infected with $2x10^7$ pfu DENV2 per mouse. At 4 hours and 30 hours post-infection, mice were administered vehicle or 100mg/kg LCL-161 by oral gavage. Weight (A) and temperature (B) were monitored. Mice were humanely euthanised at 48 hours post infection. Flow cytometry was used to enumerate the proportions and absolute cell counts of the different myeloid cell compartments of the blood (C), and spleen (D), respectively. In parallel, plasma isolated at 48 hours post infection was used to determine the viral loads of vehicle and LCL-161 treated mice (E). n=3 mice per group, data is from one independent experiment. The following statistical tests were used to analyse the presented data. Differences in weight and temperature were assessed by multiple unpaired t-tests with the Holm-Sidak correction for multiple comparisons. Flow cytometry myeloid cell compartment data and log-transformed qPCR viral load data was analysed using two-tailed unpaired t-tests. Error bars indicate the mean ± SEM. For panels C-E, circles and squares represent data from individual mice. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$.

5.3.3 The effect of LCL-161 treatment on plasma cytokine levels in MAR1-treated WT mice infected with DENV2

Previously, I showed that MAR1-treated WT mice produced substantial levels of pro-inflammatory cytokines and chemokines in response to DENV2 infection (Chapter 4). Given that two doses of LCL-161 could significantly reduce the size of several myeloid compartments (Figure 5.5C-D), I wanted to see whether this treatment could also reduce levels of cytokine production in response to DENV2 infection. Therefore, I used a commercial multiplex immunoassay to quantify levels of 27 cytokines and chemokines in the plasma of mice that had been treated with either vehicle, one dose of LCL-161 or two doses of LCL-161. All bleeds were taken at 48 hours post infection. I used ordinary one-way ANOVA with Dunnett's test for multiple comparisons to identify levels of cytokines in the plasma of LCL-161 treated mice that were significantly increased or decreased relative to vehicle only control. A summary of these statistical differences is presented below (Figure 5.6).

Not surprisingly, I saw several parallels between the cytokine profile of LCL-161 treated mice and the cytokine profile of $c1^{LysM}/cIAP2^{-/-}$ mice. Previously, I showed that levels

of MCP-3 and IL-18 were elevated in naïve c1^{LysM}/cIAP2$^{-/-}$ mice relative to WT controls (Chapter 4, figure 4.10). However, no statistically significant difference in MCP-3 and IL-18 expression was observed in c1^{LysM}/cIAP2$^{-/-}$ at 48 hours post infection (Chapter 4, figure 4.10). I found that mice treated with a single dose of LCL-161 had increased levels of MCP-3 and IL-18 at 48 hours post-infection (Figure 5.6). I should note that there was also a trend toward elevated concentrations of MCP-3 and IL-18 in the plasma of mice treated with two doses of LCL-161 ($p=0.08$, $p=0.29$ respectively). These values probably did not reach the threshold of statistical significance ($p<0.05$) due to the small number of mice included in this experimental arm (n=3). As the loss of cIAPs has been associated with inflammation (Vince et al., 2012; J. Zhang et al., 2019), this probably explains the increased levels of MCP-3 and IL-18 in mice treated with a single dose of LCL-161.

In contrast, levels of IFN-α, were significantly lower at 48 hours post infection in the plasma of mice treated with one or two doses of LCL-161 treated mice (Figure 5.6), and this finding was also found in c1^{LysM}/cIAP2$^{-/-}$ mice (Chapter 4, Figure 4.10). Surprisingly, levels of type I interferon declined significantly with one dose of LCL-161 (Figure 5.6), even though one dose of LCL-161 did not significantly decrease the size of the myeloid cell compartment (Figure 5.4C-D). The concentration of IFN-α in the plasma of mice treated with one dose of LCL 161 (mean ± SEM 6344 ± 862.4 pg/mL) was higher than that of mice treated with two doses of LCL-161 (mean ± SEM 2793 ± 1162 pg/mL).

There was also an upregulation of IL-22 in mice that had been treated with one dose of LCL-161 and IL-5 in mice that had been treated with two doses of LCL-161 (Figure 5.6). IL-22 is a member of the IL-10 cytokine family that acts to control innate immune responses in epithelial cells (Hakemi, Eskandari, Yazdani, Farahani, & Sherkat, 2014). IL-5 is a Th2 cytokine that promotes B-cell development and regulates eosinophil production (Takatsu & Nakajima, 2008). These results imply that the presence of cIAPs may have a role in regulating production of IL-22 and IL-5 during DENV infection, although this needs more investigation.

In conclusion, LCL-161 treatment was unable to extend the survival of IFNAR$^{-/-}$ mice. In addition, although treatment of MAR1-suppressed WT mice with two doses of LCL-161 was able to alter the composition of myeloid subsets in the peripheral blood and spleen,

this did not translate to a difference in viral load. Finally, treatment of DENV-infected mice with LCL-161 caused an increased level of pro-inflammatory cytokines such as MCP-3 and IL-18, and reduced levels of IFN-α.

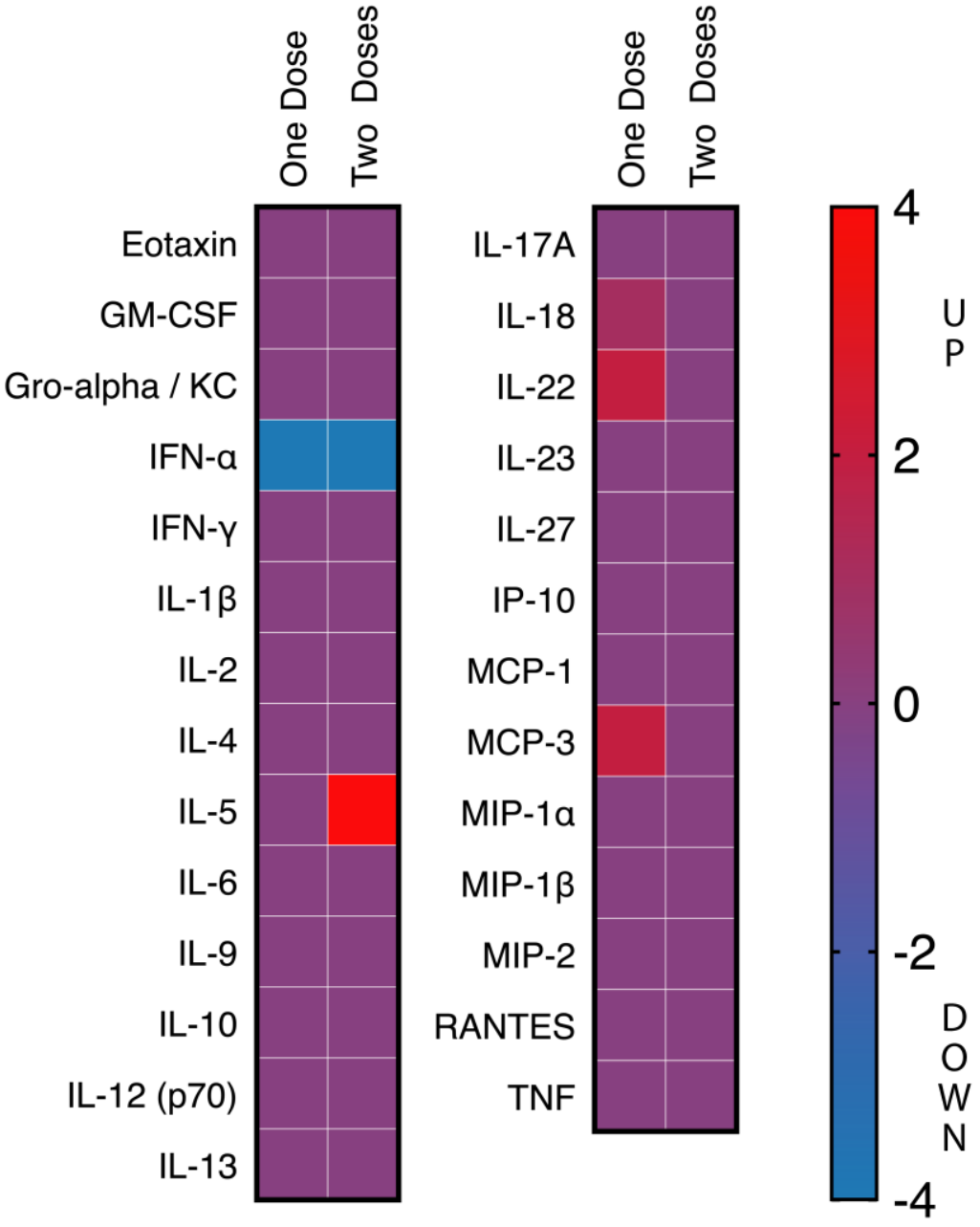

Figure 5.6 | Statistical differences in the cytokine production of WT mice treated with one or two doses of LCL-161, relative to the cytokine production of vehicle-treated controls. WT mice were treated with MAR1 IFNAR-blocking antibody and either vehicle or LCL-161. Plasma cytokines were quantified at 48 hours post infection by multiplex bead array. Plasma samples were obtained from 1-2 independent experiments. Vehicle

control: n=8; one dose LCL: n=5; two dose LCL: n=3. Statistical analysis: the mean values for “One Dose” and “Two Dose” groups were compared to the mean of the naïve group by one-way ANOVA, using Dunnett’s post-hoc test. “0” = ns, “1” $p < 0.05$, “2” $p < 0.01$, “3” $p < 0.001$, “4” $p < 0.0001$.

5.4 Assessment of jakinibs as novel therapeutics for the treatment of *in vivo* DENV infection

The JAK-STAT pathway is important for the signalling of class I and class II cytokine receptors. Cytokines that signal via these receptors include interferons, many interleukins, colony stimulating factors, erythropoietin, thrombopoietin, prolactin, leptin and growth hormone (O’Shea, Holland, & Staudt, 2013). JAKs are found bound to the cytoplasmic domains of these cytokine receptors, and upon cytokine binding, go on to phosphorylate STATs, allowing STAT proteins to dimerise, translocate to the nucleus, and upregulate gene transcription. The ability of specific cytokine receptors to activate particular combinations of JAK and STAT proteins allows different receptors to induce the transcription of different genes. Regulation of JAK-STAT signalling is regulated by several families of proteins such as the SOCS proteins (reviewed in Chapter 3), protein tyrosine phosphatases and protein inhibitor of activated STATs (reviewed in (Villarino, Kanno, & O’Shea, 2017)). In addition, some cytokine receptors can signal by JAK-STAT signalling, and by other pathways. For instance, IL-6 receptor signalling primarily involves JAK1 phosphorylation of STAT3, but also can involve JAK2, Tyk2, STAT1 and the MAP Kinase ERK (Naugler & Karin, 2008; O’Shea et al., 2015).

Knockout mouse models have provided key insights into the role of specific JAKs. $JAK1^{-/-}$ mice die within 24 hours of birth, and $JAK1^{-/-}$ cells are unable to signal via Class II cytokine receptors, common γc receptors and gp130 receptors (Rodig et al., 1998). $JAK2^{-/-}$ cells are unable to transduce signals from erythropoietin, thrombopoietin, IL-3, IFN-γ or GM-CSF, and JAK2 deficiency results in embryonic lethality due to a lack of definitive erythropoiesis (Neubauer et al., 1998; Parganas et al., 1998). In contrast, $JAK3^{-/-}$ and $Tyk2^{-/-}$ mice are viable and fertile (O’Shea et al., 2015). JAK3 deficiency in humans and mice results in severe combined immunodeficiency (O’Shea et al., 2015). JAK3 associates with the common γc, and consequently, the phenotype for deficiency of the

common γc is almost identical to the phenotype of JAK3 deficiency (Cao et al., 1995; Russell et al., 1994) this phenotype is almost identical to knockout of the common γc, and can be explained by the association of JAK3 with the common γc. Tyk2$^{-/-}$ mice have reduced responses to IFN -α, -β, -γ, and IL-12 (Karaghiosoff et al., 2000).

Given that JAKs have wide-reaching roles in controlling growth, inflammation, and other immune responses, they are attractive drug targets. Ruxolitinib and tofacitinib are two FDA-approved JAK inhibitors (jakinibs). These drugs are small molecules with excellent solubility and permeability, that act via their competitive, reversible inhibition of JAK ATP-binding sites (Winthrop, 2017).

Pre-clinical data showed ruxolitinib inhibited JAK1 and JAK2 with an IC_{50} of <5nM, with higher values for Tyk2 and JAK3 (Quintás-Cardama et al., 2010). Ruxolitinib could block JAK1 signalling *ex vivo* to reduce STAT3 phosphorylation, and reduced levels of IL-6 and/or TNF in mouse models of myeloproliferative neoplasm and graft versus host disease (GVHD) (Quintás-Cardama et al., 2010; Spoerl et al., 2014). Ruxolitinib has been approved for the treatment of the BCR-ABL (Philadelphia chromosome) negative myeloproliferative neoplasms. In two independent phase 3 trials, ruxolitinib reduced spleen volume and improved quality of life compared to placebo or best available treatment (Harrison et al., 2012; Verstovsek et al., 2012). Importantly, both of these trials reported sustained decreases in levels of the inflammatory cytokines TNF and IL-6 (Harrison et al., 2012; Verstovsek et al., 2012). Two studies have also shown that ruxolitinib treatment was able to improve outcomes and lower plasma IL-6 levels in patients with GVHD (Spoerl et al., 2014; Zeiser et al., 2015).

Whereas ruxolitinib exhibits a degree of specificity for JAK1 and JAK2, *ex vivo* whole blood assays showed that tofacitinib exhibited a 5-100 fold specificity for JAK1 and JAK3 over JAK2 (Meyer et al., 2010). Similar results were obtained by another group using T-cells (Ghoreschi et al., 2011), and in pre-clinical models of arthritis, tofacitinib treatment was able to reduce levels of pro-inflammatory cytokines including IL-6 and IP-10 (Dowty et al., 2013; Ghoreschi et al., 2011; Meyer et al., 2010). Phase II trials have shown that tofacitinib was efficacious for treating atopic dermatitis (Bissonnette et al., 2016), but

showed no benefit as a treatment for Crohn's disease (Sandborn et al., 2014). Furthermore, phase three trials have shown that tofacitinib is efficacious for treatment of ulcerative colitis (Sandborn et al., 2017) and rheumatoid arthritis (E. B. Lee et al., 2014). Importantly, treatment of rheumatoid arthritis patients with IL-6 decreased levels of IL-6, while having a variable effect on IP-10, TNF and IL-8 (Hodge et al., 2016). Given that ruxolitinib and tofacitinib are both potent anti-inflammatory compounds with proven clinical safety profiles, I investigated the ability of these drugs to treat severe dengue *in vivo*.

Initially, I conducted a titration experiment to determine doses of ruxolitinib and tofacitinib that showed efficacy. Ruxolitinib and tofacitinib have been shown to decrease levels of IL-6 in human cohorts of myelofibrosis (Harrison et al., 2012; Verstovsek et al., 2012) and rheumatoid arthritis patients (Hodge et al., 2016), respectively. Given that I had shown that there were high levels of IL-6 in the serum of DENV-infected IFNAR$^{-/-}$ mice (Figure 5.2C), and IL-6 mainly signals via STAT3 (Naugler & Karin, 2008), I decided to examine the ability of ruxolitinib and tofacitinib to block IL-6 signalling *in vivo*, using STAT3 phosphorylation as a surrogate marker for IL-6 signal transduction. While gp130 signalling can stimulate phosphorylation of both STAT1 and STAT3, it is widely accepted that IL-6 signalling favours activation of STAT3, and therefore the decision was made to use STAT3 phosphorylation as the surrogate marker (Schaper & Rose-John, 2015). I infected IFNAR$^{-/-}$ mice with DENV2 and treated mice at 36 hours and 48 hours post infection with either vehicle, low doses of ruxolitinib or tofacitinib, or high doses of ruxolitinib and tofacitinib. Two hours after the final drug treatment, mice were humanely euthanised and whole spleen lysates were probed for phosphorylated STAT3 by western blot. I found that only high doses of ruxolitinib and tofacitinib were able to block STAT3 phosphorylation in the spleen of IFNAR$^{-/-}$ mice (Figure 5.7). Therefore, I elected to assess the ability of high doses of ruxolitinib and tofacitinib to improve relevant severe dengue outcomes in IFNAR$^{-/-}$ mice.

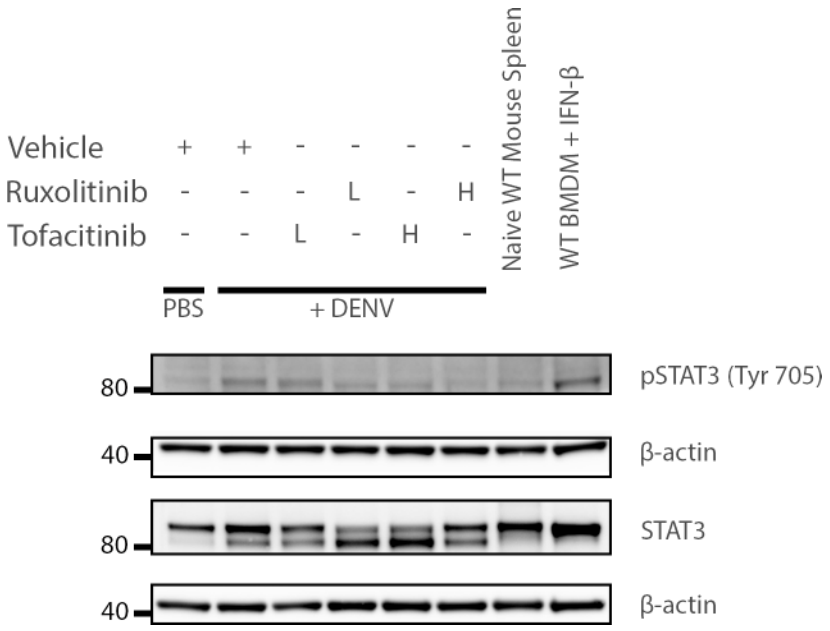

Figure 5.7 | High doses of jakinibs can dramatically reduce STAT3 phosphorylation in DENV-infected IFNAR$^{-/-}$ mice. IFNAR$^{-/-}$ mice were infected with 1X10^5 pfu of DENV2 or sham infected with PBS. At 36 and 48hr post infection, mice were treated with PBS, or low (L) or high (H) doses of ruxolitinib or tofacitinib. At 50hr post-infection, mice were humanely euthanised and spleens were taken. Whole spleen lysates were used for western blotting for pSTAT3 and STAT3. β-actin was used as a loading control. Data is from one independent experiment.

After establishing correct dosing, I proceeded to investigate the ability of ruxolitinib and tofacitinib to improve disease outcomes in DENV-infected IFNAR$^{-/-}$ mice. Given the relatively short half-life of ruxolitinib (X. Chen, Williams, Sandor, & Yeleswaram, 2013; Shilling et al., 2010) and tofacitinib (Dowty et al., 2013; Krishnaswami, Boy, Chow, & Chan, 2015), DENV-infected mice were treated with drug twice daily. During the course of the experiment, mice were humanely euthanised when they reached a pre-defined ethical endpoint. Treatment with ruxolitinib or tofacitinib did not prevent the onset of rapid weight loss that is characteristic of this IFNAR$^{-/-}$ model of DENV infection (Figure 5.8A). Jakinib treated mice also had similar temperature plots to control animals (Figure 5.8B). However, mice that survived past day 8 rapidly regained weight. Log-rank tests showed that ruxolitinib treatment conferred no significant benefit to survival (p=0.18) (Figure 5.8C). Tofacitinib treatment also resulted in no survival benefit (p=0.40) (Figure

5.8D). Plasma was extracted from animals when they were euthanised or at the end of the experiment (Day 11). Subsequently, this plasma was used to determine the terminal viral loads for these animals (Figure 5.8E). As animals from different treatment groups were euthanised at different times after they reached a pre-determined ethical endpoint, it is hard to draw firm conclusions from this data. However, the general trend is the same for each group, with a high viral load evident at days 4-6, when most animals succumbed, and a viral load near or at the limit of detection from Day 8 onwards. As mentioned above, this high viral load co-incident with severe illness is a caveat of this $IFNAR^{-/-}$ model, and is different from the scenario that occurs during most human cases, in which viral load is sharply declining at the time of severe illness (Duyen et al., 2011; Fox et al., 2011). IFN-γ has been shown to increase in serum and spleen at Day 5 post infection in a DENV2 mouse model (Fagundes et al., 2011). The ability of $IFNAR^{-/-}$ mice to clear virus probably reflects the onset of IFN-γ signalling, and subsequent engagement of adaptive immunity, which have both been shown to be relevant for DENV clearance in mice (Fagundes et al., 2011; Shresta et al., 2004).

Therefore, treatment of $IFNAR^{-/-}$ with high-doses of ruxolitinib and tofacitinib was unable to impact on clinical signs of illness in these animals, and was unable to improve survival outcomes. However, it should be noted that this study was somewhat underpowered to detect differences between treatment and vehicle groups. To have an 80% chance of detect a 400% difference in outcome (i.e. if death in the vehicle group was hypothetically 80%, and death in the treated group was 20%), this would require sample sizes of 11 mice per group, whereas due to logistical constraints I was only able to generate data from 4 mice per group (Sullivan, Pezzullo, Dean, & Mir, 2013).

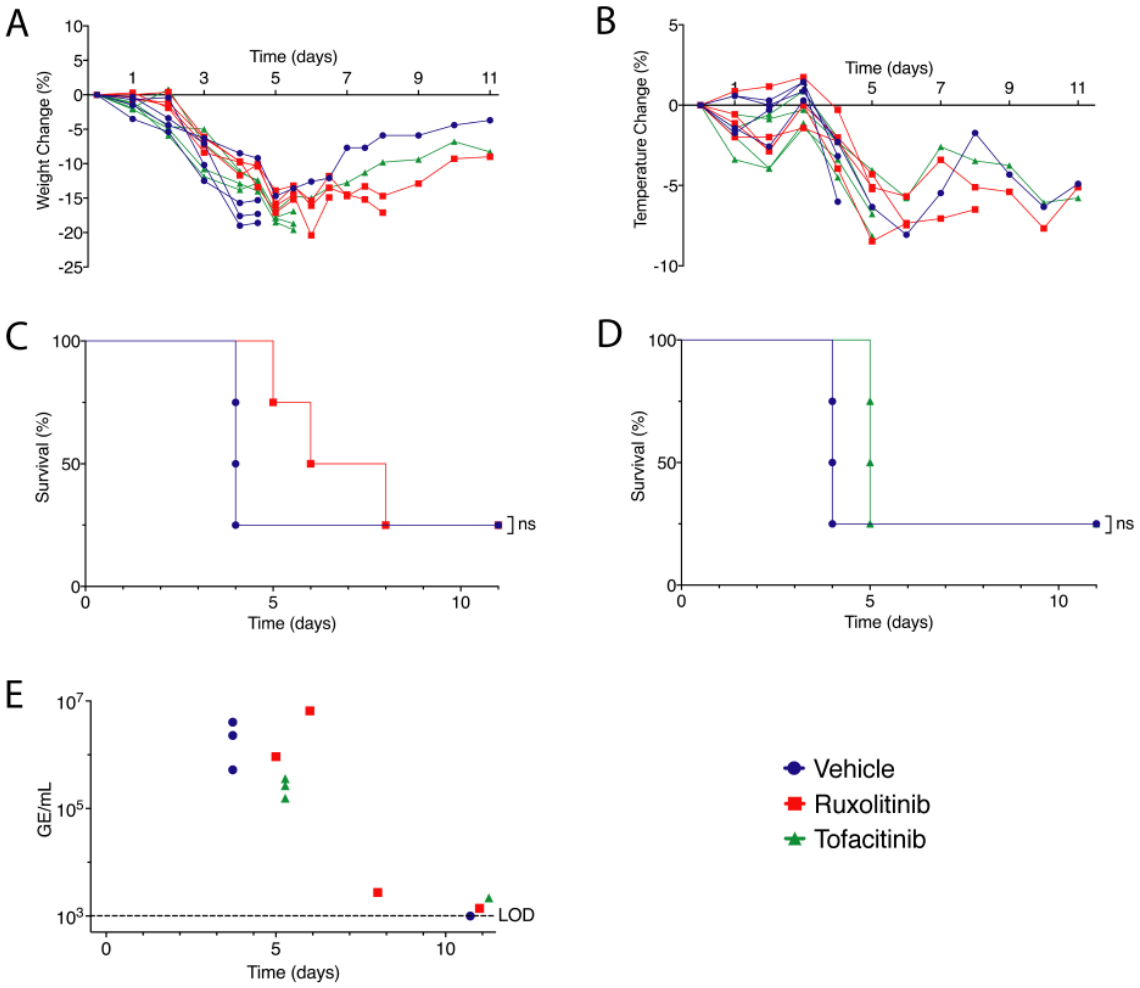

Figure 5.8 | High doses of Jakinib drugs do not increase the survival of DENV-infected IFNAR$^{-/-}$ mice. DENV2-infected IFNAR$^{-/-}$ mice were treated with vehicle or high doses of ruxolitinib or tofacitinib. Twice-daily drug treatment was initiated at approximately 12hr post infection, and continued for 7 days for a total of 14 doses. Weight (A) and temperature (B) were monitored daily. Mice were euthanised when they reached a pre-determined ethical endpoint, and the resulting survival curves are presented for ruxolitinib (C) and tofacitinib (D). Terminal viral loads in plasma were determined by qPCR (E). n=4 mice per group; data is from one independent experiment.

5.5 Assessment of a TBK1 inhibitor as a novel dengue therapeutic

During DENV infection, PAMPs including ssRNA and dsRNA are detected by PRRs, resulting in an antiviral response, as described in detail in my literature review. A key activator of type I interferon and NF-κB signalling which is activated by PRR signalling during viral infection is Tank Binding Kinase 1 (TBK1). TBK1 is a non-canonical IKK that

is activated downstream of the adaptors MAVS, TRIF and STING (S. Liu et al., 2015). Sharma and colleagues showed that TBK1, and the related kinase IKKε, were able to phosphorylate IRF-3 and IRF-7 to stimulate the transcription of a type I interferon gene programme and drive antiviral immunity (Sharma et al., 2003).

In addition to stimulating type I interferon signalling, TBK1 can also stimulate pro-inflammatory and pro-survival NF-κB signalling. TBK1 is able to phosphorylate IκBα at serine (Ser) 36, but not Ser 32, and is therefore not able to trigger IκBα degradation (Tojima et al., 2000). However, TBK1 can directly phosphorylate IKKβ, resulting in upregulation of a pro-inflammatory and pro-survival transcriptional programme (Tojima et al., 2000). Furthermore, TBK1 deficiency severely abrogated the *in vitro* NF-κB signalling response of MEFs to TNF and IL-1 stimulation (Bonnard et al., 2000). In addition, a proteomics study also showed that TBK1 was recruited to the TNFR1 signalling complex, and mediates production of the pro-inflammatory chemokine RANTES (Kuai et al., 2004). TBK1$^{-/-}$ mice do not survive to birth due to advanced liver apoptosis (Bonnard et al., 2000). This phenotype is very similar to the phenotype mice deficient in IKK-β (Q. Li, Van Antwerp, Mercurio, Lee, & Verma, 1999), IKK-γ (Rudolph et al., 2000) and p65 (Beg, Sha, Bronsont, Ghosht, & Baltimore, 1995). Moreover, this phenotype can be rescued by co-deletion of TNFR1 (Bonnard et al., 2000), so collectively these results strongly suggest that, in addition to an antiviral role, TBK1 acts *in vivo* to promote pro-survival TNF-mediated activation of NF-κB.

WEHI-112 is a small molecule inhibitor with nM affinity for TBK1 and the related protein IKKε (Louis et al., 2019). WEHI-112 was recently shown to be efficacious in a collagen-induced arthritis model. WEHI-112 reduced IL-6 in total lymph node cells at the transcript level, reduced inflammation as measured by bioluminescence, and reduced the clinical scores of treated animals (Louis et al., 2019). Given the important role of TBK1 in facilitating the pro-inflammatory NF-κB-mediated antiviral response, I decided to use WEHI-112 in an attempt to improve the outcomes of IFNAR$^{-/-}$ mice infected with DENV. IFNAR$^{-/-}$ animals were used for these experiments as these animals develop robust morbidity in response to DENV infection (see above). Please note that while TBK1 can drive inflammation by inducing type I interferon signalling, this signalling axis is

inactive in the IFNAR model. Therefore, the focus of these experiments was on assessing the ability of WEHI-112 treatment to mitigate pro-inflammatory cytokine signalling driven by TBK1-mediated activation of NF-κB.

When IFNAR$^{-/-}$ mice were treated daily with TBK1 they still exhibited profound weight loss in response to DENV infection (Figure 5.9A). A comparison of vehicle and WEHI-112 weight loss data using Holm-Sidak-corrected multiple unpaired t-tests found no significant differences. As for the jakinib experiments, mice that survived past day 4-6 were able to recover weight. Furthermore, there were no significant difference in temperature between vehicle and WEHI-112 groups (Figure 5.9B). Log-rank analysis revealed that there was no significant difference in survival between vehicle and WEHI-112 groups (p=0.21) (Figure 5.9C). Terminal viral loads from the plasma of euthanised vehicle and WEHI-112 treated mice (Figure 5.9D) showed a similar trend to the equivalent dataset from jakinib-treated IFNAR$^{-/-}$ mice (Figure 5.8E). Viral load was high when the majority of mice succumbed at day 4 post infection, and progressively declined until the experiment was ended (day 8). At day 4, when the majority of animals reached the ethical endpoint and were euthanised, an unpaired, two-tailed t-test of log-transformed qPCR data showed that there was no difference in titres between vehicle and WEHI-112 groups (p=0.22).

In summary, in this model, WEHI-112 did not mitigate surrogate markers of dengue illness, did not improve survival outcomes, and did not alter terminal viral loads.

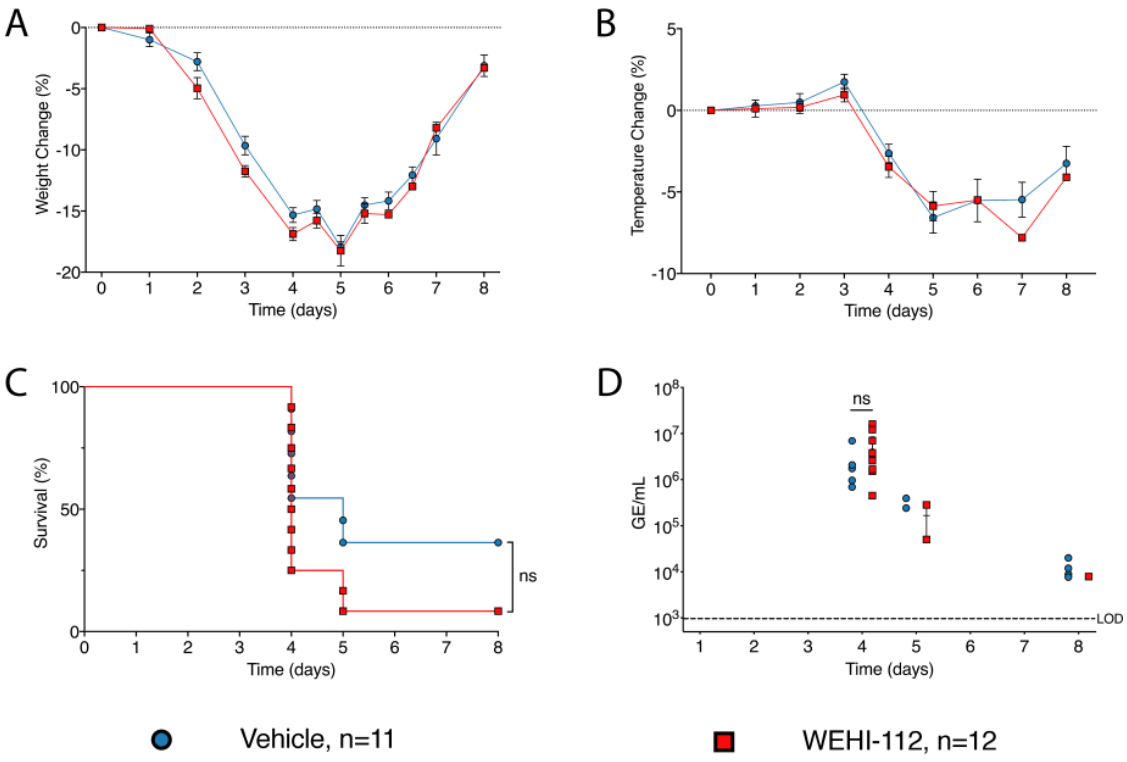

Figure 5.9 | TBK inhibition does not improve the survival of DENV2 infected IFNAR$^{-/-}$ mice. DENV2-infected IFNAR$^{-/-}$ mice were treated with vehicle or WEHI-112 daily for 7 days, with treatment starting approximately 12 hours after infection. Weight (A) and temperature (B) were monitored daily. Mice were euthanised when they reached a pre-determined ethical endpoint, and the resulting survival curves were plotted (C). Terminal viral loads in plasma were determined by qPCR (D). For panel D, circles and squares represent data from individual mice. n=11 and n=12 for vehicle and WEHI-112 groups respectively. Data is pooled from two independent experiments. Error bars indicate the mean ± SEM.

5.6 Discussion

As outlined in the literature review (section 1.11), clinical trials of drugs for DENV treatment have largely focused on targeting pathways that are key for viral replication. For instance, celgosivir and balapiravir directly or indirectly inhibit RNA replication, whereas the effect of chloroquine is mediated by changes to endosomal acidification (Farias et al., 2015; Rathore et al., 2011). In comparison, few clinical trials have been conducted to assess the effects of host-targeted therapies to treat dengue. Of these, the most prominent are the trials of corticosteroid drugs such as prednisolone which act

primarily by influencing NF-κB signalling (Barnes, 2006). However, trials to date have failed to identify drugs that can be used for the prophylactic or therapeutic treatment of dengue.

Given that the effects of severe dengue are mediated by dysregulated host cell signalling, and that the targeting of host cell signalling remains a largely unexplored avenue in the context of dengue research, I decided to investigate the ability of drugs that target host cell signalling to improve outcomes in *in vivo* models of DENV infection.

As genetic deficiency of cIAPs, which are key transducers of NF-κB signalling**,** resulted in decreased viral production *in vitro* and *in vivo* (Chapters 4 and 5)*,* I hypothesised that prophylactic treatment of IFNAR$^{-/-}$ mice with LCL-161 prior to DENV infection could potentially reduce viral burden, reduce disease severity and extend survival. LCL-161 treated animals lost significantly more weight than vehicle treated control animals at 24 and 48 hours post-infection, although this difference was not significant at later time points (Figure 5.2A). I did not observe that any differences in weight and temperature (Figure 5.2B). There was a trend towards a decreased time to euthanasia with LCL-161 treatment (Figure 5.2C). It is possible that the weight loss observed with LCL-161 treatment could be related to cytokine release syndrome, as this has been reported as side effect of high dose LCL-161 treatment in human clinical trials (Infante et al., 2014). LCL-161 is generally well tolerated in mice at doses of 100mg/kg e.g. see reports regarding leukaemia by Houghton et al. and myeloma by Chesi et al (Chesi et al., 2016; Houghton, 2012). However, the dose used for this experiment using IFNAR$^{-/-}$ (400mg/kg) was somewhat higher. As animals were euthanised only when they reached a pre-determined ethical endpoint, a viral load measurement was not performed, as the number of animals culled at any one time point was small, and therefore statistical comparison of vehicle and LCL-161 datasets would not be possible.

To further explore the effect of pharmacological IAP depletion on DENV infection *in vivo*, I treated MAR1 treated WT animals with one or two doses of LCL-161 (100mg/kg) and analysed the composition of myeloid cell compartments and plasma viral load at 48 hours post infection. While LCL-161 treatment of WT mice with one dose of LCL-161 did not affect cell populations in the blood or spleen (Figure 5.4C-D), treatment of mice with

two doses dramatically decreased myeloid cell populations. Specifically, treatment with two doses of LCL-161 decreased levels of monocytes and dendritic cells in the blood (Figure 5.5C) and levels of monocytes, inflammatory macrophages, granulocytes and dendritic cells in the spleen (5.5D). This decrease in myeloid populations is probably due to cell death, given that cIAP proteins are important transducers of pro-survival NF-kB signalling. These results could therefore indicate that LCL-161 treatment drastically reduced NF-kB signalling in this model of DENV infection. It would be informative to perform future experiments to confirm that this decrease in myeloid populations is due to cell death and not due to a downregulation of myeloid cell markers or a distribution of these cells to neighbouring tissues.

Previously, I hypothesised that a lack of NF-kB signalling could decrease DENV production by reducing the levels of cellular transcriptional activity (Chapters 3 and 4). Moreover, monocytes, macrophages, and dendritic cells are important replicative niches for DENV. Therefore, it was surprising that treatment of WT mice with either one or two doses of LCL-161 did not decrease plasma viral load, relative to WT animals, at 48 hours post infection (Figure 5E). In summary, the results of my genetic deletion studies, which suggest that deficiency of IAPs reduces viral production, and my pharmacological studies, which suggest that deficiency of IAPs do not impact viral production, are non-concordant. Further experiments designed to clarify this important discrepancy are outlined in Chapter 6.

Previously, I observed that $c1^{LysM}/cIAP2^{-/-}$ and $TRAF2^{LysM}$ mice produced much less IFN-α in response DENV infection than WT mice (see Chapter 4). Therefore, it was interesting to see that treatment of WT mice with either one or two doses of LCL-161 also drastically reduced levels of IFN-α following DENV infection. In Chapter 4, I proposed that reduced levels of IFN-α could be due to either a decreased proportion of IFN-α producing myeloid cells in the blood, or due to reduced ubiquitination of components of the RIG-I-MAVS-TRAF3 pathway. The results of my IAP antagonist experiments show that even when mice were treated with one dose of LCL-161, and did not have statistically reduced levels of myeloid cells in blood or spleen (Figure 5.4C and 5.4D), they had plasma levels of IFN-α that were substantially lower than vehicle-treated control animals (Figure 5.6). This suggests that a decreased number of IFN-α producing

myeloid cells, in particular potent IFN-α producing plasmacytoid dendritic cells (Swiecki & Colonna, 2015), is not the cause of reduced IFN-α concentrations in the plasma of both genetically and pharmacologically IAP deficient animals. An alternative explanation is that in the absence of cIAPs, a decrease in ubiquitination decreases the activity of the RIG-I-MAVS-TRAF3 signalling axis (see Chapter 4). An outline of future experiments designed to interrogate this experimental hypothesis are presented in Chapter 6.

In addition to my studies on LCL-161, I also investigated the ability of the FDA-approved jakinibs ruxolitinib and tofacitinib to improve disease outcomes in the IFNAR$^{-/-}$ model of severe dengue. Interestingly, treatment of IFNAR$^{-/-}$ mice with high doses of these drugs failed to prevent infection-associated weight loss (Figure 5.8A), or improve the survival of these animals (Figure 5.8C-D).

My decision to treat DENV-infected IFNAR$^{-/-}$ mice with jakinibs was due to the wide array of JAK signalling that is active during severe dengue infection. Mouse models (Shresta et al., 2004; Züst et al., 2014) and human data (Afroz, Giddaluru, Abbas, & Khan, 2016; Kwissa et al., 2014; Zanini et al., 2018) indicate that interferon-signalling plays a key role in dengue infection. In addition to this IL-4, IL-6, IL-10, IL-13, and GM-SCF have been associated with severe dengue and signal via the JAK-STAT pathway (see Chapter 1, Table 3). Therefore, there is definitely hyperactivation of JAK-STAT signalling during cases of severe dengue.

However, many inflammatory mediators that have been reported to play a key role during severe dengue do not signal via the JAK-STAT pathway. The chemokines IL-8, IP-10, MIP-1β, MCP-1 and RANTES signal via different GPCRs (see Chapter 1, Table 3). TNF signal transduction is via TNFR1 and TNFR2 and stimulates several signalling pathways including NF-κB whilst signalling of the IL-1 family cytokines IL-1α and IL-1β is via IL-1R1 and MyD88. Soluble viral NS1 can ligate TLR4, resulting in signalling via MyD88 and TRIF, and enzymes such as tryptase do not signal via a specific receptor. Therefore, given that there are a large number of pro-inflammatory mediators that do not signal via the JAK-STAT pathway, it is possible that some pro-inflammatory signalling cannot be targeted by this strategy, and this could explain the lack of efficacy of these compounds in the

IFNAR$^{-/-}$ model.

Finally, I used the IFNAR$^{-/-}$ mouse model to examine the effect of TBK1 inhibition on severe dengue. The TBK1 inhibitor WEHI-112 did not improve the survival of DENV2-infected IFNAR-/- mice (Figure 5.9C). One explanation for this is that a certain level of pro-inflammatory TBK1 signalling is required to control DENV infection. In support of this argument, Dalrymple and colleagues reported that NS2A and NS4B could inhibit TBK1 phosphorylation and consequent IFN-β production (Dalrymple, Cimica, & Mackow, 2015).

In conclusion, LCL-161 treatment of IFNAR$^{-/-}$ was not able to improve the survival of these mice. Treatment of MAR1-suppressed WT mice with two doses of LCL-161 was able to reduce numbers of key DENV host cells such as monocytes and dendritic cells. LCL-161 treatment resulted in the production of pro-inflammatory cytokines such as MCP-3 and IL-18, but decreased levels of IFN-α. LCL-161 treatment was not able to reduce viral load. MAR1-suppressed WT animals. Treatment of IFNAR$^{-/-}$ mice with ruxolitinib, tofacitinib and WEHI-112 did not increase the survival of these animals.

Chapter 6 - General Discussion

Dengue is the arbovirus that causes the greatest disease burden worldwide, and can present clinically as a mild or severe form (Messina et al., 2019). Frustratingly, the molecular mechanisms that cause the disease to manifest as a severe rather than mild form are not well understood. Because severe dengue manifests at a time when viral load is steeply declining or absent (Duyen et al., 2011; Fox et al., 2011), and is characterised by a cytokine storm (Rothman, 2011), severe dengue can be defined as a disease of dysregulated host immune signalling. I hypothesised that the genetic deletion of key immune signalling hubs could result in a generalised, hyperactive immune response to DENV infection.

My investigation of genetically-targeted *in vitro* and *in vivo* infection models revealed a reduction in the ability of $c1^{LysM}$/$cIAP2^{-/-}$ cells and mice to produce virus following infection (Chapters 3-4). The most plausible explanation for this reduced viral production is that DENV requires highly active NF-κB signalling for efficient replication, and in the absence of IAPs the transcriptional activity of myeloid cells is reduced. This theory is bolstered by the observation that many other viruses such as HTLV-1 and HIV rely on NF-κB activity for efficient replication (Bachelerie et al., 1991; Z. L. Chu et al., 1999).

The role of NF-κB in dengue infection has not been clearly defined. Some *in vitro* studies have assigned a pro-apoptotic role to NF-κB signalling (Lin et al., 2014; Marianneau et al., 1999; Netsawang et al., 2014), and autopsy studies have shown that apoptosis is an active process during dengue infection (Aye et al., 2014; Bhamarapravati et al., 1967; Póvoa et al., 2014). However, the transient and fully reversible nature of severe dengue (World Health Organisation, 2009), implies that any pro-apoptotic role of NF-κB signalling in dengue pathogenesis is of relatively little consequence. It should also be noted that NF-κB signalling normally acts to promote cell survival as opposed to cell death (Beg & Baltimore, 1996).

Transcriptome studies have shown that NF-κB signalling is one of the main pathways activated during dengue fever (J. Fink et al., 2007; Hoang et al., 2010). To my knowledge, only one study has looked at the effect of NF-κB signalling on DENV replication. Fink

and colleagues treated DENV-infected HepG2 cells with dexamethasone (J. Fink et al., 2007). This study found that dexamethasone treatment prevented the expression of the NF-κB induced chemokines IP-10 and I-TAC, but had no effect on viral replication (J. Fink et al., 2007).

Interestingly, and in agreement with the results of Fink and colleagues, my attempts to disrupt NF-κB signalling *in vitro* by pharmacological depletion of IAPs or inhibition of NF-κB in WT cells also did not alter viral production (Chapter 3). Furthermore, *in vivo* LCL-161 treatment of WT mice was also unable to reduce viral load relative to a vehicle-only control (Chapter 5). Therefore, there is a non-concordance in the results I have obtained from c1^{LysM}/cIAP2$^{-/-}$ BMDM and mouse infections (Chapters 3-4), which show that viral production is impaired, and from LCL-161 treated WT BMDMs and mice, which show no defect in viral production (Chapters 3 and 5).

To resolve this apparent contradiction, it would be interesting to determine definitively whether NF-κB signalling in response to DENV infection is reduced in c1^{LysM}/cIAP2$^{-/-}$ cells compared to WT cells using Western blot analysis to examine phosphorylation for p65 and its nuclear translocation. I could complement this with a similar analysis of LCL-161 treated cells. The caveat with this work is that it focuses on one cell type (BMDMs) and *in vivo* there are likely many cellular compartments that contribute to viral production.

I also observed that the proportion of monocytes in the blood was lower in c1^{LysM}/cIAP2$^{-/-}$ mice than for WT controls (Chapter 4). This could indicate that IAP-deficient monocytes are undergoing apoptosis in response to DENV infection, and that this is contributing to the reduction in viral load observed in c1^{LysM}/cIAP2$^{-/-}$ mice *in vivo*. Apoptosis is known to destroy both the host cell and the intracellular pathogen. Evidence to support this notion could be accrued by examining the cellular compartments that produce virus in mice. I could sort a spectrum of cells from WT and gene-targeted mice (monocytes, neutrophils, dendritic cells etc) and determine the number and contribution of viral genomes in each compartment. It would be interesting to determine if c1^{LysM}/cIAP2$^{-/-}$ monocytes *in vivo* are unable to tolerate high levels of viral production compared to WT cells and hence succumb to apoptosis. This would likely be an idiosyncrasy of the *in vivo* system (and physiologically relevant) because

many death ligands are only produced *in vivo* and are absent *in vitro*. Collectively, the aforementioned experiments will provide good mechanistic insight.

To study the role of immune signalling hubs in the cytokine storm phenotype, I characterised cytokine production from gene-targeted BMDMs and mice by using multiplex immunoassays (Chapters 3-4). I found that DENV infection of $c1^{LysM}/cIAP2^{-/-}$ cells and mice resulted in a moderate increase in cytokine production. Specifically, $c1^{LysM}/cIAP2^{-/-}$ cells produced more IL-1β following infection, whereas $c1^{LysM}/cIAP2^{-/-}$ animals had increased plasma levels of IL-6, RANTES and TNF following infection. Interestingly, WT mice that were treated with LCL-161 upregulated a different set of cytokines upon DENV infection, with IL-18, IL-22 and MCP-3 upregulated when mice were treated with one dose of LCL-161, and IL-5 upregulated when mice were treated with two doses of LCL-161 (Chapter 5). Several recent publications have reported that loss of IAPs may result in the production of pro-inflammatory cytokines through indirect mechanisms (Vince et al., 2012; J. Zhang et al., 2019).

I also observed a striking and consistent decrease in IFN-α production upon infection of $c1^{LysM}/cIAP2^{-/-}$ mice and LCL-161 treated WT mice (Chapters 4-5). Flow cytometry of WT mice treated with a single dose of LCL-161 showed that there was no decrease in the populations of myeloid cells in the spleen or blood (Chapter 5). Therefore, the reduction in interferon could not be attributed simply to a reduction in IFN- α producing cells. I also observed that $TRAF2^{LysM}$ mice also have a deficiency in IFN-α production (Chapter 4). TRAF2 often serves a cytoplasmic scaffold that facilitates the ubiquitin ligase activity of cIAP 1 and cIAP2 (Vallabhapurapu et al., 2008). Given that IAP deletion and possible disruption of cIAP function via loss of TRAF2 results in a common phenotype, I searched for an explanation that could explain these findings. I believe the most likely explanation for these observations is that deletion of cIAPs is resulting in a defect in ubiquitination that is required for efficient induction of type I interferon production by the RIG-I – MAVS signalling axis.

Previously, it has been shown that active RIG-I – MAVS signalling results in the K63-linked ubiquitination of TRAF3 (Oganesyan et al., 2006). This ubiquitination is essential for the activation of IKKε and TBK1 and the production of type I interferon (Oganesyan

et al., 2006). K63-linked ubiquitination of TRAF3 has been shown to be directly mediated by cIAP1 and cIAP2 (Mao et al., 2010), and is also mediated by autoubiquitination in a process regulated by DEAD-box helicase 3 (Gu, Fullam, McCormack, Höhn, & Schröder, 2017; P. H. Tseng et al., 2010).

It would be interesting to clarify the role of cIAPs in type I interferon production in response to viral infection. One way to further explore the importance of the cIAPs to this process would be to infect WT and $c1^{LysM}/cIAP2^{-/-}$ BMDMs with DENV, and assess levels of TRAF3 K63-linked ubiquitination. If $c1^{LysM}/cIAP2^{-/-}$ BMDMs had less K63-linked ubiquitination of TRAF3 than WT controls, this would clarify the mechanism by which cIAPs were regulating type I IFN during DENV infection. Another way to examine the role of cIAP-mediated ubiquitination in RIG-I – MAVS signalling and the production of interferon would be to use the MAR1 model that allows a degree of infection of immunocompetent mice. Negative regulation of TRAF3 ubiquitination downstream of MAVS is mediated via deubiquitinating enzyme A (DUBA) (Kayagaki et al., 2007). DUBA removal of K63-linked ubiquitin chains from TRAF3 reduced the interferon response to Sendai virus infection (Kayagaki et al., 2007). Therefore, if TRAF3 ubiquitination was involved in the production of type I interferon in response to DENV infection, infection of DUBA-deficient mice would be expected to result in increased type I interferon production. Therefore, *in vitro* assessment of cIAP-mediated TRAF3 ubiquitination, and DENV infection of DUBA mice would help to determine whether my hypothesis is correct, and cIAPs are in fact regulating type I interferon production following DENV infection by controlling ubiquitination of the RIG-I – MAVS signalling axis.

One obvious contradiction in my data is that $c1^{LysM}/cIAP2^{-/-}$ mice have a reduced viral load, but also reduced levels of type I interferon (Chapter 4). Given the potent anti-viral effect of type I interferon, it would be reasonable to expect that mice with a reduced level of type I interferon would have a higher viral load than WT controls. There are several points to consider with regards to these findings. Firstly, in mice there are 14 IFN-α subtypes (Gibbert, Schlaak, Yang, & Dittmer, 2013). The beads I used for my multiplex immunoassay detected only IFN-α2 and IFN-α4. Therefore, it is possible that while levels of IFN-α2 and IFN-α4 are low, there are high levels of other isotypes and/or IFN-β that are being produced that are acting in conjunction with depressed NF-κB

signalling (discussed above) to depress viral load. However, I consider this scenario relatively unlikely, as IFN-α and IFN-β are transcribed early in the anti-viral response and feedback via upregulation of IRF-7 to induce further transcription of multiple IFN-α subtypes and IFN-β (Marie et al., 1998; Sato et al., 1998). Therefore, it is likely that a reduced level of IFN-α4 would result in a lack of subsequent type I interferon upregulation, and consequently, that a depressed level of IFN-α2 and IFN-α4 represents a global reduction in type I interferon levels.

Although the level of type I interferon detected in the plasma of c1^{LysM}/cIAP2$^{-/-}$ at day 2 post infection was significantly lower than WT controls, it was still relatively high (>1000pg/mL). Also, as noted, before, although these mice were treated with MAR1, comparison of viral load of MAR1-treated mice with isotype control-treated mice and IFNAR$^{-/-}$ mice strongly implies that there is a reduced, but not absent, type I interferon signalling active in MAR1-treated DENV-infected mice. Therefore, it is possible that the plasma level of type I interferon in c1^{LysM}/cIAP2$^{-/-}$ was above a threshold level required for control of DENV infection, and this, combined with reduced NF-κB signalling, was able to reduce viral load below the level observed in WT, MAR1-treated control animals.

In addition to DUBA, another gene that would be interesting to investigate using the MAR1 *in vivo* model is IL-10. IL-10 has been found to be consistently upregulated in severe dengue, as opposed to regular dengue fever (Y. H. Lee et al., 2016; Soo et al., 2017). *in vitro* experiments have shown that IL-10 is produced by monocytes in response to DENV infection, and has been implicated in the stimulation of plasmablast differentiation, IgM secretion and the regulation of RLR signalling via upregulation of SOCS3 (Kwissa et al., 2014; Ubol et al., 2010). However, to the best of my knowledge, no studies have been done to investigate the effect of IL-10 deficiency on DENV infection *in vivo*. IL-10$^{-/-}$ mice are healthy until the age of three months, when they develop colitis, and therefore could be used for this investigation if they were MAR1-treated and infected soon after maturation, i.e. at approximately 6 weeks of age (Berg et al., 2002). However, given that IL-10$^{-/-}$ animals show a marked sensitivity to the TLR4 ligand LPS (Siewe et al., 2006), and given DENV NS1 is a TLR4 ligand (Modhiran et al., 2015), these experiments will need to be conducted with close monitoring of infected animals to ensure proper standards of animal welfare are maintained.

As well as assessing the function of putative host restriction factors *in vitro* and *in vivo*, I assessed the potential of several drug compounds to act as dengue therapeutics (Chapter 5). The results of my experiments using LCL-161 I have already discussed above. Here, I will discuss my experiments using the clinical stage JAK inhibitors ruxolitinib and tofacitinib, and the pre-clinical TBK-1 inhibitor WEHI-112 as novel dengue therapeutics. I hypothesised that the broad anti-inflammatory activity of ruxolitinib and tofacitinib could serve to dampen the excessive inflammation observed during DENV infection of IFNAR animals. I demonstrated that high doses of ruxolitinib and tofacitinib could potently block the high level of STAT3 phosphorylation observed during the course of DENV infection of IFNAR$^{-/-}$ animals. However, twice-daily treatment of IFNAR$^{-/-}$ animals with either of these JAK inhibiting compounds did not improve survival outcomes. The lack of benefit observed during ruxolitinib and tofacitinib treatment could be due to an inability of this strategy to suppress pro-inflammatory signalling mediated by non-JAK-STAT pathways (see chapter 5 discussion). In addition, WEHI-112 treatment was unable to improve the survival outcomes of DENV-infected IFNAR$^{-/-}$ animals. Perhaps this is also due to the inability of this compound to block signalling that is not mediated by NF-κB. Alternatively, perhaps the broader immunosuppressive effect of these drugs is impeding viral clearance which is an important contributor to disease in the models I used. In future experiments it could also be useful to infect these mice with a lower dose of DENV to see whether this might allow potential therapeutic activity of JAK or TBK-1 inhibition to come to the fore.

A caveat to the results produced in this thesis is that all of the results were generated using a single strain of DENV2 (see section 2.2). In the future, it would be desirable to try to repeat key experiments from this thesis using other serotypes of DENV to ensure that the results are broadly applicable, and not specific to DENV2.

Currently, drug trials of several anti-dengue compounds are underway (see Chapter 1). In addition to these trials, several other intervention strategies are being investigated. Release of mosquitoes infected with the bacteria *Wolbachia* has been shown to result in a long-term absence of epidemics in the Australian city of Cairns (Ryan et al., 2019). Furthermore, a Malaysian group reported a decrease in dengue incidence at study sites where *Wolbachia* was released in Kuala Lumpur (Nazni et al., 2019). In addition,

although there are issues with the current CYD-TDV dengue vaccine (see Chapter 1), two novel TDVs are currently being developed by the Butantan Institute (São Paolo, Brazil) and the Takeda Pharmaceutical Company (Japan). These vaccines use a similar principle as CYD-TDV but use an attenuated DENV2 backbone in place of the YFV17D backbone (Biswal et al., 2019; Magnani et al., 2017). The hope is that these TDVs will be able to generate a more robust T cell response than CYD-TDV, and there is some evidence for this (H. Chu, George, Stinchcomb, Osorio, & Partidos, 2015; Daniela Weiskopf, Angelo, et al., 2015). Phase III results of a trial of the Takeda vaccine have recently been published, and show promise (Biswal et al., 2019).

Overall, this body of work has characterised the role of several putative host restriction factors, and identified that $c1^{LysM}$/$cIAP2^{-/-}$ cells and mice produce less virus and higher levels of pro-inflammatory cytokines than WT controls. While my efforts to reduce the clinical signs of dengue in mice were unsuccessful, I hope that the methods that I have established, and the interesting observations that I have made, can lay a foundation for future productive research into anti-inflammatory and anti-viral therapies.

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