

NANYANG
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**Characterisation of RIG-I-like receptor
agonists for dengue virus therapy or
vaccination**

HO CHIN YONG VICTOR

School of Biological Sciences

2018

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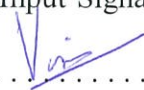
School of Biological Sciences

A thesis submitted to the Nanyang
Technological University in partial fulfilment of
the requirement for the degree of
Doctor of Philosophy

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Luo Nakei

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Acknowledgments

The journey has been a long but fruitful one, and without these people I would not have made it to the end and finished this part of the race:

Many thanks to Dr Katja Fink for her patience and guidance throughout the PhD journey. For giving me the space to grow and also for giving me advice when I need it the most. For providing the best environment for me to be successful and to develop as a scientist and a stronger person.

Thanks to Dr Roland Zuest for teaching me the techniques I needed in the lab when I started and for advice on how to move the project forward.

To everyone in KF lab, for their continual support, the many jokes and snacks and the interesting and deep conversations. To Ying Xiu, many thanks for helping with the skin samples and the neutralisation assays. Thanks also to Dr Gough Au for helping me with the thesis writing process.

Thanks to Dr Luo Dahai for providing the initial stimulus to this project by providing the immRNA used for the characterisation and the ideas that helped guide me through this project.

Thanks to Hui Yee for producing the immRNA and for her patience in doing so as this would have taken some of the time which could have been spent on her own project.

Dr Lisa Ng for volunteering to be on my thesis advisory committee, in spite of her busy schedule, and for all the advice given at TAC meetings.

Dr Tan Ern Yu and her clinical trial team in TTSH for providing the skin samples for the project.

PhD class of 2014 in the NTU/SlgN program, the most lovable, crazy, fun-loving, great looking, intelligent people I know (well at least in the past 4 years). Daniel, Hiroko, Weili, Jelin and Simin, thanks for making the journey an enjoyable one and I hope we will have many more journeys together!

Friends in SlgN who have talked with me, eaten breakfast/lunch/dinner together with me, or even just simply said “hi” when we meet, thanks for making the environment I work in a great one in the past 4+ years.

My family for their unwavering support and their love showered on me throughout the process. For their constant prayers for me and for providing everything I need to pursue my goals and dreams.

And most importantly, I give glory to God for providing me with the grace which I do not deserve, the peace which guards my heart, the joy that is my strength and the love which overcomes every fear. To God be the glory!

*The works of the LORD are great,
studied by all who have pleasure in them*

Psalms 111:2 (NKJV)

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Abbreviations

ADE	Antibody-dependent enhancement
APC	Antigen presenting cells
ATP	Adenosine triphosphate
BCR	B cell receptor
CARD	Caspase activation and recruitment domains
CHIKV	Chikungunya virus
DC	Dendritic cell
DC SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DDC	Dermal dendritic cell
DENV	Dengue virus
DHF	Dengue haemorrhagic fever
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
EBV	Epstein-Barr virus
EC50	Half maximal effective concentration
ED	Envelope domain
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FRNT	Focus reduction neutralization test
GMT	Geometric mean titre
HA	Haemagglutinin
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HM	Hilymax
HMW	High molecular weight
IFN	Interferon
IFNAR	Interferon $\alpha\beta$ receptor

IL	Interleukin
ImmRNA	Immune-modulating RNA
IPS	Interferon β promoter stimulator
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISRE	Interferon-stimulated response element
JEV	Japanese Encephalitis virus
KO	Knock out
LAV	Live attenuated virus
LC	Langerhans cells
LMW	Low molecular weight
LPS	Lipopolysaccharide
MA	Matrix
MAVS	Mitochondrial antiviral-signalling protein
MDR	Multiple Drug Resistance
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MITA	Mediator of IRF3 Activation
MLR	Mixed lymphocyte reaction
MoDCs	Monocyte-derived dendritic cells
MOI	Multiplicity of infection
MP	Macrophage
NA	Neuraminidase
NHP	Non-human primate
NS	Non-structural
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PK	Pharmacokinetics
PRNT	Plaque reduction neutralization test
PRR	Pattern recognition receptor

PVR	Polio virus receptor
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RT	Reverse transcription
SD	Standard deviation
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TCR	T cell receptor
Tfh	T follicular helper cells
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF α	Tumour necrosis factor α
VLP	Virus-like particle
vRNA	Viral RNA
VSV	Vesicular Stomatitis virus
WNV	West Nile virus
YFV	Yellow Fever virus

Abstract

Dengue is a growing problem globally owing to failure in preventing the spread of the virus. Dengue virus (DENV) replication can be blocked through the activation of innate immune responses using RIG-I-like receptor agonists comprising naturally of double-stranded RNA containing a triphosphate group at the 5' end. Using the smallest dsRNA ligand that can activate RIG-I signalling, a short hairpin with a 10 base pair stem (3p10L), we demonstrated that this minimal size immune-modulating RNA molecule (minimal immRNA) is capable of inducing an anti-viral state in human epithelial A549 cells and human monocytic U937 cells. The addition of a guanine nucleotide in position 9 of 3p10L generated a kink near the hairpin loop. This modified molecule called 3p10LG9 was more potent than 3p10L in activating RIG-I dependent type I interferon signalling in human cell lines. Both 3p10LG9 and 3p10L potently inhibited DENV replication in primary human dendritic cells isolated from human skin samples (primary human skin DCs) obtained from healthy donors. When injected with a cationic polymer, 3p10LG9 induced type I interferon production in CD11c-cre IFNAR^{ff} mice and LysM-cre IFNAR^{ff} mice. However, no protective effect was observed when mice were challenged with DENV-2. Overall, these results suggest that 3p10L and 3p10LG9 can activate anti-viral responses and confer short-term protection against DENV. We also found that 3p10LG9 increased the expression of CD80 on human skin APCs and enhanced protection against DENV-2 challenge in CD11c-cre IFNAR^{flox/flox} mice vaccinated with DENV-2 VLPs. Based on these preliminary findings, the ongoing work involves investigating the potential of 3p10LG9 to be used as a vaccine adjuvant and its ability to enhance long-term protection through the adaptive immune response.

1 Introduction

1.1 Dengue Virus

1.1.1 Characteristics and Prevalence

Dengue virus (DENV) is an arbovirus that is transmitted to humans through the bite of an infected *Aedes* mosquito. The two most common vectors of transmission are *A. aegypti* (Neerincx et al., 2012) and *A. albopictus* (Effler et al., 2005), as these vectors have adapted themselves to their urban environment. DENV is part of the *Flaviviridae* family and is a member of the *Flavivirus* genus. This family of viruses comprises other viruses which are known to have posed health problems to the human population, such as Yellow Fever virus (YFV), West Nile virus (WNV) and Japanese Encephalitis virus (JEV) to name a few. DENV has an added complexity in the sense that there are four serotypes that are known to exist (DENV-1, DENV-2, DENV-3 and DENV-4), and these four serotypes are able to cause clinical symptoms of dengue, including the severe Dengue haemorrhagic fever and Dengue shock syndrome (DHF/DSS).

DENV is an enveloped virus that contains a single-stranded, positive sense RNA genome. This viral genome encodes a large polyprotein, which is processed by viral and host proteases into three structural proteins (Capsid, prM and Envelope protein) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (**Figure 1.1**). Immune cells such as monocytes, macrophages and dendritic cells (DCs), are known to be susceptible to DENV infection (Cerny et al., 2014; K. Fink et al., 2009; Kou et al., 2007; Schmid et al., 2014; S. J. Wu et al., 2000). Professional Antigen presenting cells (APCs) in the skin, including Langerhans cells (LCs) that are present in the epidermis, are thought to be particularly important in the establishment of infection as these are the first cells that come into contact with the virus when the host is bitten (Cerny et al., 2014; Schmid et al., 2014; S. J. Wu et al., 2000). However, the question still remains

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as to how the virus infects the LCs found in the epidermis, as the proboscis of the mosquito reaches the dermis and not the epidermis (Kong & Wu, 2009).

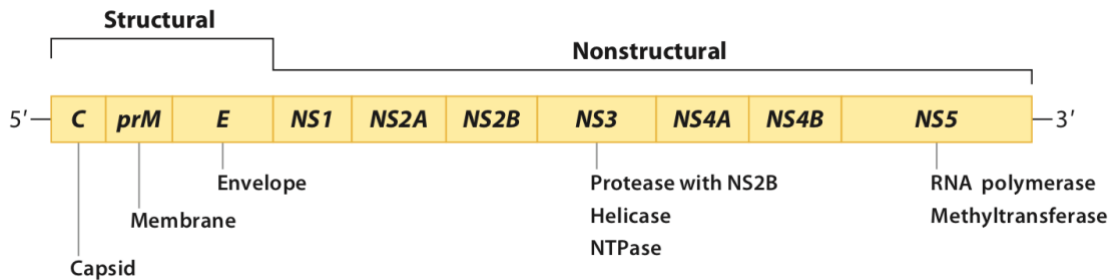


Figure 1.1 Graphical representation of DENV genome.

DENV genome comprises 3 structural proteins (capsid, premembrane and envelope) and 5 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

Figure obtained from Murphy & Whitehead, 2011

1.1.2 Innate immune response against DENV and evasion strategies

The importance of type I interferon production is evident through the observation that DENV replication can only occur in mice deficient in either Type I interferon receptor (IFNAR^{-/-}) or both type I and type II interferon receptor knockout (AG129), leading to high viral loads in these animals (Johnson & Roehrig, 1999; Pinto et al., 2015; Züst et al., 2014). Hence an effective DENV survival strategy should involve ways to inhibit production of type I interferon or exposure to downstream effectors. In *in vitro* experiments, Diamond *et al.* observed that pre-treatment with type I interferon is protective against DENV infection, whereas any treatment post-infection was not as effective in inhibiting viral replication (Diamond et al., 2000). This would suggest that DENV has a way to subvert the anti-viral effects exerted by type I interferon once an established infection has occurred. The Non-structural proteins (NS) of DENV have been found to play important roles in the ability of DENV to replicate once it has entered the host cell. NS2A, NS4A, NS4B and NS5 are involved in the inhibition of various signalling molecules involved in type I interferon signalling, including Signal transducer and activator of transcription (STAT) 1/2 (Ashour et al., 2010; Morrison et al., 2013), Stimulator of interferon genes (STING, also known as

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MITA) (Aguirre et al., 2012; Yu et al., 2012) and TANK binding kinase 1 (TBK1) (Dalrymple et al., 2015). DENV can inhibit the production of type I interferon in human monocyte-derived dendritic cells (MoDCs) through the production of viral protease complex NS2B3, resulting in an impaired Th1 response in the infected host (Rodriguez-Madoz et al., 2010) (**Figure 1.2**). NS5 is a multifunctional protein that comprises of the RNA-dependent RNA polymerase and methyltransferase (MTase) activity (Dong et al., 2012; Egloff et al., 2002; Ray et al., 2006; Tan et al., 1996). Methyltransferase activity results in the formation of a 5' cap on the viral RNA (vRNA), and this function has been demonstrated to have an important role in the survival and replication of the virus (Ray et al., 2006). N-7 methylation is essential for RNA translation and stability, while 2'-O-methylation helps shield the virus from being recognized by host innate immune response mediated through viral RNA sensing Pattern recognition receptors (PRRs) such as RIG-I-like receptors (RLRs) (Daffis et al., 2010). 2'-O-methylation only partially affects viral replication *in vitro*. However, MTase mutant strains are significantly attenuated *in vivo* (Daffis et al., 2010; Züst et al., 2013).

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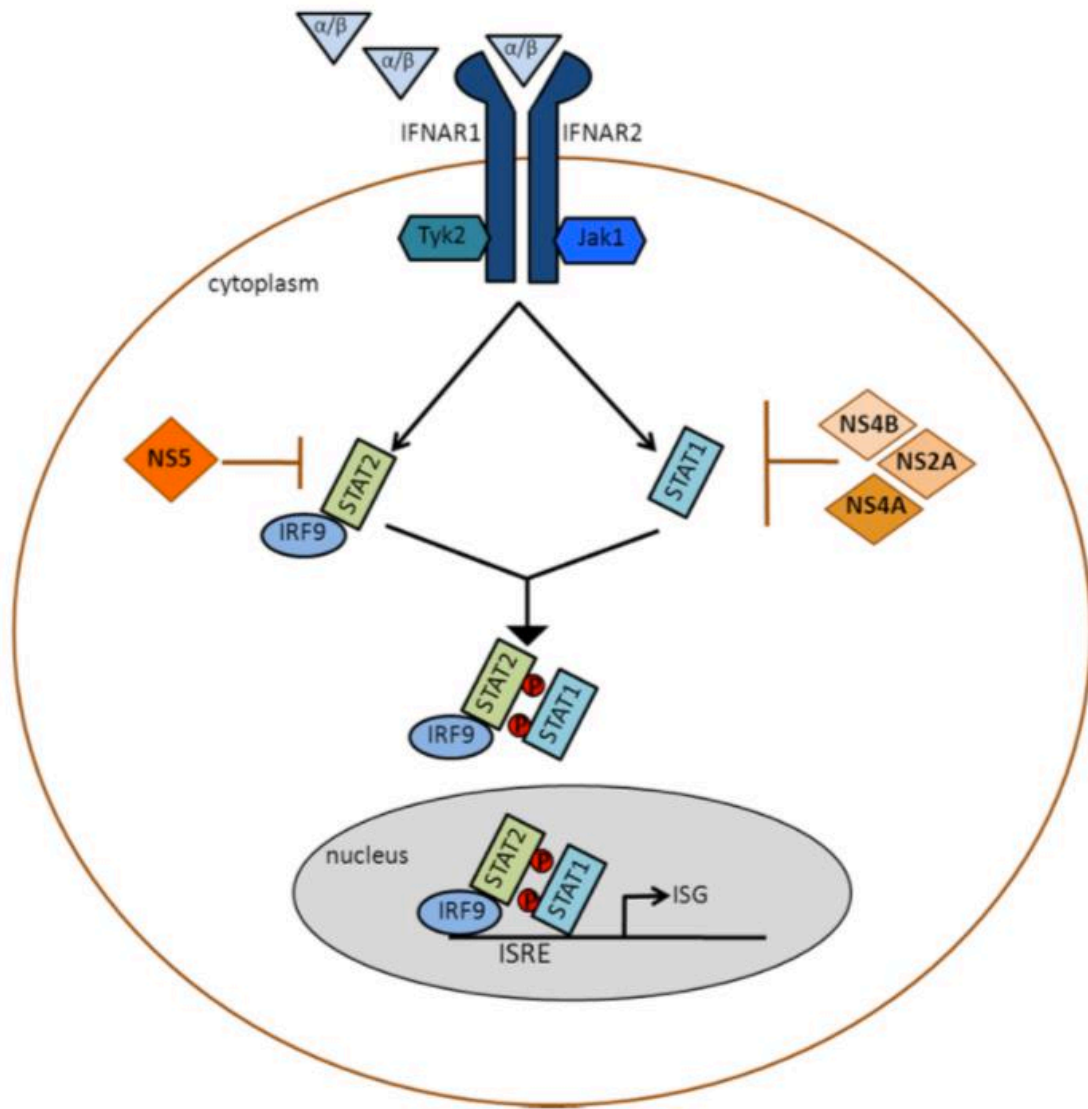


Figure 1.2 Effects on DENV non-structural proteins in evading host innate immunity.

Upon binding of type I interferon (IFN α or IFN β) to Interferon- α/β Receptor (IFNAR), the JAK-STAT signalling pathway is activated, resulting in the phosphorylation of STAT1 and STAT2. Phosphorylated STAT2 recruits Interferon Regulatory Factor 9 (IRF9) and together with phosphorylated STAT1 forms the trimeric IFN-stimulated gene factor 3 (ISGF3) complex. This complex translocates to the nucleus where it binds to Interferon-stimulated response elements (ISRE) in the genome to induce the expression of Interferon-stimulated genes (ISGs). DENV non-structural proteins are able to inhibit specific steps in the activation pathway. NS5 is able to degrade STAT2, and NS2A, NS4A, and NS4B are involved in preventing the activation of STAT1.

Figure obtained from Morrison, Aguirre, & Fernandez-Sesma, 2012.

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1.1.3 Antibody-dependent enhancement (ADE)

Antibody-dependent enhancement (ADE) is an antibody-mediated effect caused by the presence of sub-neutralizing levels of antibodies leading to increased infection and viral burden. These sub-neutralizing antibodies bind to virus, leading to the uptake of the virus-antibody immune complex into cells containing Fcγ receptors and thereby allowing increased virus replication and titres in the host immune cells (**Figure 1.3**). This ADE effect has been shown in DENV pathology both *in vitro* (Boonnak et al., 2008; Halstead, 1977; Littaua et al., 1990) and *in vivo* (Balsitis et al., 2010; Goncalvez et al., 2007; Halstead, 1979; Zellweger et al., 2010), and has been suggested to be the cause of severe dengue in human patients when studying the sero-epidemiology in Nicaraguan children over a period of 12 years (Katzelnick et al., 2017). When studying the effects of how the number of antibodies bound to a particular neutralizing determinant impacts the infectivity of West Nile virus (WNV), Pierson *et al.* (Pierson et al., 2007) found that any antibodies that bind to flaviviruses without neutralization will cause ADE, and this also applies to potent neutralizing antibodies that bind at sub-neutralizing concentrations. The authors mention that differences in the steepness and shape of the dose-response curve for neutralization arise from differences in epitope accessibility. They go on to suggest that E-protein subunit vaccines could be preferentially designed with an appearance similar to exposed regions that have highly accessible epitopes, resulting in the production of antibodies that bind with high occupancy at low concentrations to optimise the protection conferred. Antibodies that bind to PrM and the fusion loop domain of E-protein make up a large fraction of antibodies produced by dengue patients and are found to have little or no neutralizing activity. These antibodies could potentially play a role in ADE as they were observed to enhance infectivity of DENV *in vitro* (Beltramello et al., 2010; de Alwis et al., 2014; Dejnirattisai et al., 2010).

Knowledge on the conditions for ADE to occur and the impact that ADE has on host dengue immunity would have implications for future vaccine design. This

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would help in our understanding of the advantages ADE may have in some vaccine strategies. In a clinical study reported by Chan *et al.*, sequential administration of an inactivated JEV vaccine, followed by a live attenuated YFV vaccine, resulted in improved yellow fever vaccination as a result of prolonged viremia and enhanced immunogenicity. This was attributed to the presence of cross-reactive antibodies against JEV causing ADE, thus increasing the window of time the viral antigen is presented to the adaptive immune system and eventually resulting in the increase in neutralizing antibody titres. Based on these findings, the authors suggest utilising ADE as a potential strategy to improve Live attenuated vaccines (LAV) (Chan *et al.*, 2016). The presence of cross-reactive antibodies have also been shown to enhance vaccination in dengue, based on the observation that pre-exposure to flavivirus improved the efficacy of the first licensed dengue vaccine, Dengvaxia (CYD-TDV) (Capeding *et al.*, 2014; Villar *et al.*, 2015).

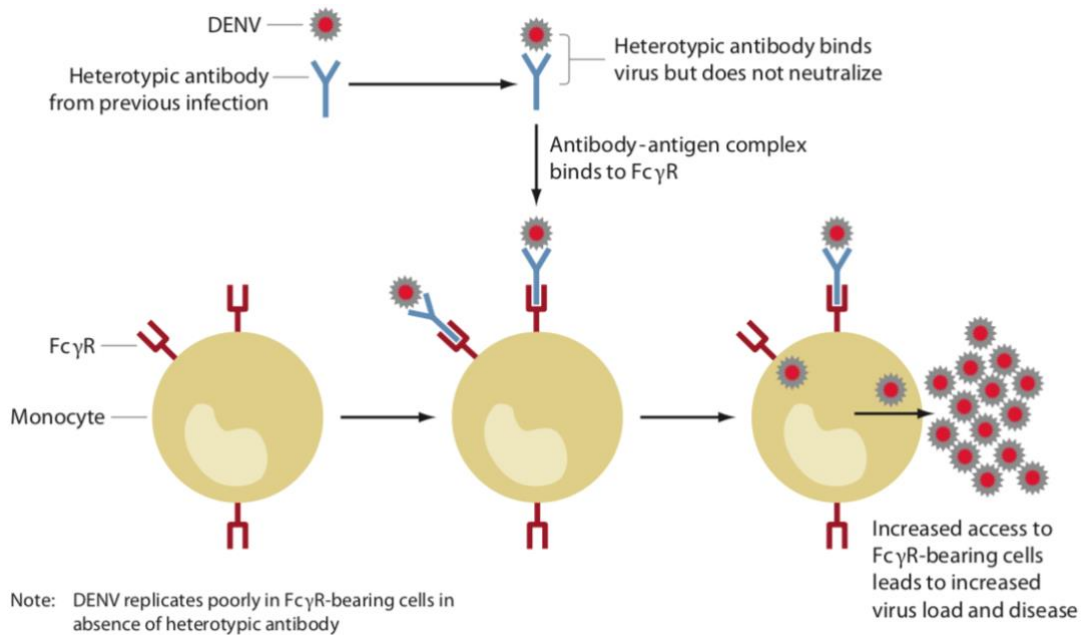


Figure 1.3 Antibody-dependent enhancement of DENV infection.

In this model of ADE, heterotypic antibodies from a previous infection are able to bind to the virus. However, these antibodies are unable to neutralize the virus, and the antibody-antigen complex is subsequently taken up by Fc γ -expressing immune cells, such as monocytes. This gives the virus access to an additional subset of immune cells which were not readily infected in

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the absence of these sub-neutralizing antibodies, thus increasing the virus titre and disease severity.

Figure obtained from Murphy & Whitehead, 2011.

1.1.4 Skin DC infection model to study DENV pathogenesis

The transmission of DENV from mosquito vectors to its human host involves the transfer of virus from the saliva of the mosquito to the dermal layer of skin. Human skin consists of an epidermal and a dermal layer, with the epidermis containing keratinocytes and LCs, a skin resident dendritic cell that is involved in detecting pathogens which penetrate the skin barrier (Kubo et al., 2009). The dermal layer is made up of fibroblasts and immune cells such as macrophages, T-cells and three other subsets of dendritic cells with a network of blood and lymphatic vessels allowing for movement of cells (X.-N. Wang et al., 2014). The three dermal dendritic cell subsets are CD11c dermal dendritic cells (CD11c DDCs) which are also known as CD1c DDCs, CD14 dermal dendritic cells (CD14 DDCs) and CD141 dermal dendritic cells (CD141 DDCs). CD11c DDCs are the most abundant among the three subsets in the dermis. This subset of cells have a high expression of HLA-DR and was able to induce increased T-cell proliferation in Mixed lymphocyte reaction (MLR) assays, indicative of properties of conventional DCs (Zaba et al., 2007). CD14 DDCs have been shown to be monocyte-derived cells that have transcriptional signatures similar to macrophages (McGovern et al., 2014). CD14 DDCs have also been shown to be involved in the induction of T follicular helper cells (Tfh), priming naïve CD4 T-cells into differentiated T-cells capable of activating B-cell class switching to become plasma cells (Klechevsky et al., 2008). CD141 DDCs are specialized DCs that can cross-present antigen to CD8 T-cells to trigger cytotoxic T-cell killing of infected cells (Haniffa et al., 2012).

To study the importance of the initial site of virus entry, Cerny *et al.* used skin from normal healthy skin donors to identify the main targets of DENV from the various subsets of DCs present in the skin (Cerny et al., 2014). They showed that the APCs were the main targets of DENV infection, and that three out of the

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four DC subsets studied were susceptible to infection. These three subsets were the LCs, CD14 DDCs and CD11c DDCs, with the LCs observed to be the most susceptible to infection. This provides a tissue-relevant model that allows us to study the effects of DENV infection on the innate and adaptive immune responses, given that the APCs are the bridge between these two arms of the immune response. Much of the previous work done on dengue pathogenesis has involved the use of *in vitro* MoDCs, which has a mainly inflammatory phenotype. This model might not represent the initial infection state of a DENV target cell, but it rather reflects the response to an infection resulting in a pro-inflammatory immune environment. Sprokholt *et al.* looked at the effects of DENV infection on the immune signalling pathways. They demonstrated that the innate immune response to DENV infection was induced in both CD11c DDCs and CD14 DDCs when infected (Sprokholt *et al.*, 2017b). These subsets showed upregulated type I interferon expression, with CD14 DDCs having a greater response compared to CD11c DDCs. This infection with DENV also led to increased expression of IL-27 in CD14 DDCs, which is important for Tfh formation. Increase in IL-27 expression was abrogated when a DENV RNA replication inhibitor SDM25N was used on the DENV infected CD14 DDCs. These findings using normal healthy skin DCs have helped in our understanding of dengue pathogenesis in the early stages of infection and will prove to be a useful model for future work looking into the downstream effects of DENV infection and possible intervention strategies.

1.2 Innate Immune Response to Infections from RNA viruses

1.2.1 Type I interferon

Viral infections trigger the innate immune response through the initial recognition of Pathogen associated molecular patterns (PAMPs) by their respective Pathogen recognition receptors (PRRs). Some examples of PAMPs include viral/bacterial genomic DNA and single or double stranded RNA molecules. Detection of these PAMPs leads to the expression of key anti-viral cytokines, Interferon- α (IFN α) and Interferon- β (IFN β), which are also known as type I

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interferons. These cytokines are responsible for mediating anti-viral effector molecules and the activation of immune cells, such as T-cells, which aid in the clearance of the virus.

Type I interferon binds to the Interferon- α/β receptor (IFNAR), a heterodimeric receptor comprising two subunits, IFNAR1 and IFNAR2. Upon binding, the JAK-STAT signalling pathway is activated which results in the phosphorylation of STAT1 and STAT2. Phosphorylated STAT1/2 forms a complex with Interferon regulatory factor 9 (IRF9) and this heterotrimeric complex, also known as the Interferon-stimulated gene factor 3 (ISGF3), is translocated into the nucleus. In the nucleus, ISGF3 binds to IFN-stimulated response elements (ISRE), which are regulatory consensus sequences located upstream from Interferon- α/β -inducible genes. This binding would initiate transcription of Interferon-stimulated genes (ISGs), which comprise of a large number of genes (de Veer et al., 2001), and these include the genes encoding proteins with direct anti-viral effects such as *RSAD2* (Viperin) and *OASL*, PRRs which are involved in pathogen sensing such as MDA5 and RIG-I, and chemokines such as CXCL10, which have been shown to be expressed on MoDCs upon treatment with IFN α to result in the recruitment of CXCR3-expressing CD8 T-cells (Padovan et al., 2002). The increased expression of these ISGs help to induce cells into an anti-viral state, resulting in reduced viral replication and spread (**Figure 1.4**). Although type I interferon is commonly recognized as an innate immune signalling molecule, it does play an important role in linking both the innate and adaptive arms of the immune system. Downstream effects of type I interferon-mediated immune activation on APCs include the maturation of DCs through increased expression of costimulatory molecules such as CD80 and CD86, as well as the upregulation of proteins which enhance antigen-presentation such as Major histocompatibility complex (MHC) class I. Type I interferon also leads to the activation of T-cells by acting as a signal 3 cytokine which binds to IFNAR on naïve T-cells, leading to proliferation and maturation of both memory and effector T-cells (reviewed in Crouse, Kalinke, & Oxenius, 2015).

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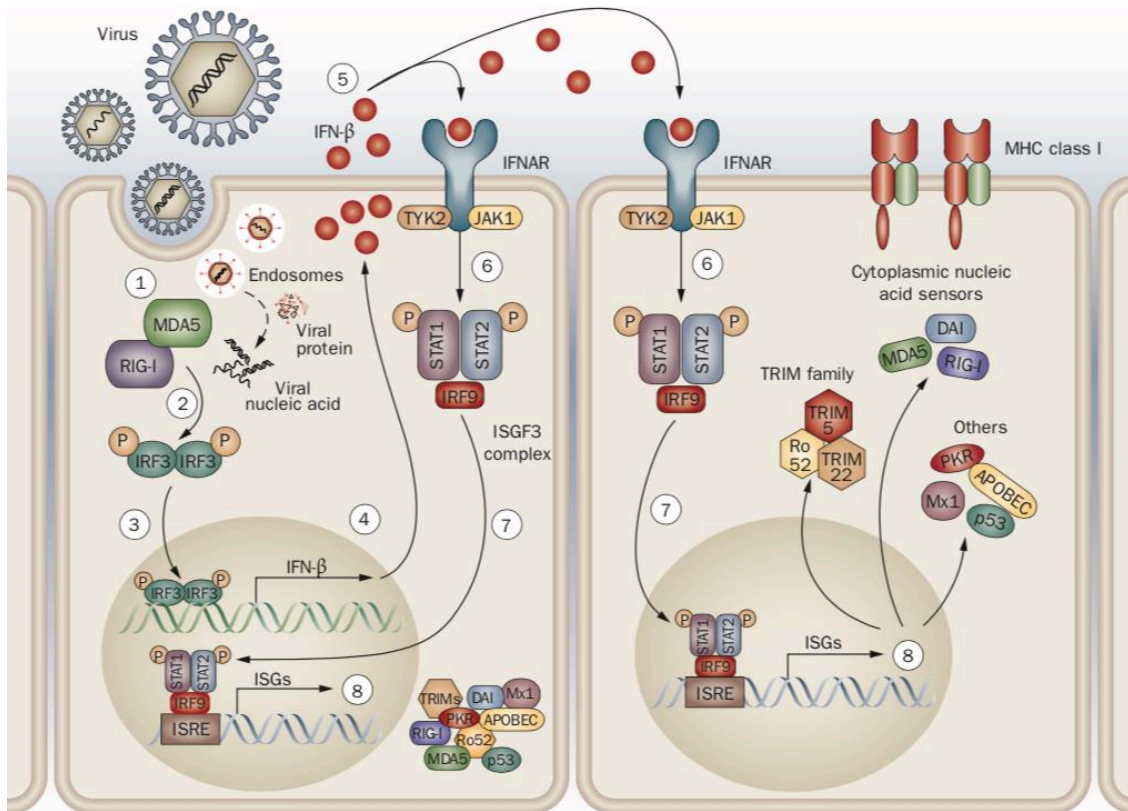


Figure 1.4 Effects of type I interferon in the activation of the innate immune response.

Upon infection, viral nucleic acid is released into the cytosol and these are recognized by Pathogen Recognition Receptors (PRRs) such as RIG-I and MDA5. Binding of viral nucleic acid to its PRRs initiates the innate immune signaling pathway through the phosphorylation and dimerization of IRF3, resulting in its translocation to the nucleus and initiating the transcription of innate immune response genes such as *IFN β* . *IFN β* is produced and secreted by the infected cell, leading to autocrine and paracrine signaling through binding of *IFN β* with IFNAR. This binding leads to a signaling cascade mediated through JAK-STAT signaling, allowing the formation of the Interferon Stimulated Gene Factor 3 (ISGF3) complex made up of phosphorylated STAT1/2 and IRF9. Nuclear translocation of the ISGF3 complex allows binding to Interferon stimulated response elements (ISRE) and results in the expression of Interferon stimulated genes (ISGs) which induce cells to an anti-viral state.

Figure obtained from Hall & Rosen, 2010.

<https://www.nature.com/reprints/permission-requests.html>

1.2.2 Toll-like receptors

Toll-like receptors (TLR) are a family of transmembrane glycoprotein receptors which are able to recognize a wide range of PAMPs (**Table 1**). To date, there are 10 known TLRs in humans and these TLRs have an N-terminal PAMP-

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recognition domain located on the extracellular side and a C-terminal signalling domain located on the intracellular side. The N-terminal is made up of multiple leucine-rich repeats (LRRs) to form a horseshoe structure. The C-terminal signalling domain has structural similarities to the intracellular domain of Interleukin-1 receptor (IL-1R), making this region known as the Toll/IL-1R homology (TIR) domain. Upon binding of the PAMP to the N-terminal domain, the dimerization of TLRs occurs and this results in the cytoplasmic TIR domains being brought together. This leads to the recruitment of adaptor molecules, such as TRIF or MyD88, which then causes the increased expression of type I interferon and pro-inflammatory cytokines mediated through the binding of either IRF3, IRF7 or NFκB transcription factors.

The main TLRs involved in the recognition of RNA viruses are TLR3 (dsRNA), TLR7 and TLR8 (ssRNA). Unlike the other TLRs which can be found on the cell surface, these nucleic acid-recognising TLRs reside in the intracellular compartments of the cell, such as the endoplasmic reticulum (ER), lysosomes and endosomes. These viral RNA-sensing TLRs have similar downstream activation pathways, all resulting in the production of type I interferon and pro-inflammatory cytokines to mediate virus clearance. However, the main difference between them is the adaptor protein which initiates the signalling cascade. TLR7/8 activation pathway is MyD88-dependent whereas TLR3 is MyD88-independent, relying on TRIF binding to initiate signalling rather than MyD88 (Yamamoto et al., 2003). Given the ability to activate type I interferon signalling, TLR agonists have been studied as potential immune activators. The combination of a TLR3 agonist, poly I:C, and a TLR7/8 agonist, R848, seemed to function synergistically in the induction of IL-12 and IL-23, leading to effective priming of Th1 responses (Napolitani et al., 2005). Poly I:C was also observed to be able to activate type I interferon dependent anti-viral pathways in LCs derived from stimulating CD34+ progenitors in human cord blood (Renn et al., 2006; Rozis et al., 2008).

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	PAMP	Species	Ref
TLR 1/2 TLR 2/6	Lipoproteins, peptidoglycan	Bacterial (gram positive)	(Takeuchi et al., 2001; 2002; Underhill et al., 1999)
TLR 3	dsRNA	Virus	(Guillot et al., 2005)
TLR 4	Lipopolysaccharide (LPS)	Bacterial	(Poltorak et al., 1998; Shimazu et al., 1999)
TLR 5	Flagellin	Bacterial	(Hayashi et al., 2001)
TLR 6	Zymosan, beta- glucan	Fungi	(Jouault et al., 2003)
TLR 7	ssRNA	Virus	(Diebold et al., 2004)
TLR 8	ssRNA	Virus	(Diebold et al., 2004)
TLR 9	DNA	Bacterial, Virus, Fungi, Parasite	(Hemmi et al., 2000)
TLR 11 (mouse)	Profilin-like molecule	Parasite (<i>Toxoplasma gondii</i>)	(Yarovinsky et al., 2005)

Table 1: List of TLRs and their respective PAMPs.

Information was obtained from Jensen & Thomsen, 2012; Kawai & Akira, 2011

1.2.3 RIG-I like receptors

RIG-I-like receptors (RLR) are a class of PRRs that recognize pathogenic RNA generated by viruses. There are three members of the RLR family and they are Retinoic acid-inducible gene 1 (RIG-I), Melanoma differentiation-associated gene (MDA5) and Laboratory of genetics and physiology 2 (LGP2) (Kang et al., 2002; Kato et al., 2006; Rothenfusser et al., 2005; Yoneyama et al., 2004). RIG-

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I and MDA5 are similar in structure and function; both have two N-terminal Caspase activation and recruitment domains (CARDs), a central ATPase motor domain and a C-terminal domain that allows viral RNA recognition (Yoneyama et al., 2005). LGP2 does not contain the N-terminal CARDs and is thought to function as a negative feedback regulator (**Figure 1.5**). Upon detection of viral RNA, ATP binds to the vRNA/RIG-I complex and ATPase is activated, followed by the oligomerization of the adaptor protein known as Mitochondrial anti-viral-signalling protein (MAVS) (or IFN β Promoter Stimulator, IPS-1) found on the outer mitochondrial membrane through the exposed CARDs. This results in a signalling cascade through activation of various transcription factors (IRF3, IRF7 and NF κ B) leading to the production of Type I interferon and other cytokines that are involved in clearing the infection (Fujita et al., 2007) (reviewed in Luo, 2014). Studies looking at the importance of the ATPase activity of RIG-I have shown that ATP hydrolysis is not directly involved in immune signalling but instead functions to discriminate self RNA from viral RNA which contains the 5' triphosphate (5'-ppp) moiety (Lässig et al., 2015; Rawling et al., 2015). ATP hydrolysis results in the release of bound RNA, allowing the cell to recycle RIG-I as well as to prevent undesired immune activation leading to autoimmune diseases. Rawling *et al.* also showed that the internal duplex sites alone are not the main determinant of downstream signalling through RIG-I to activate type I interferon expression (Rawling et al., 2015). This was done by using a dumbbell-shaped 14bp RNA construct to show absence of type I interferon signalling as well as a low binding affinity of RIG-I for compared to the blunt-ended dsRNA as demonstrated by Vela *et al.* (Vela et al., 2012).

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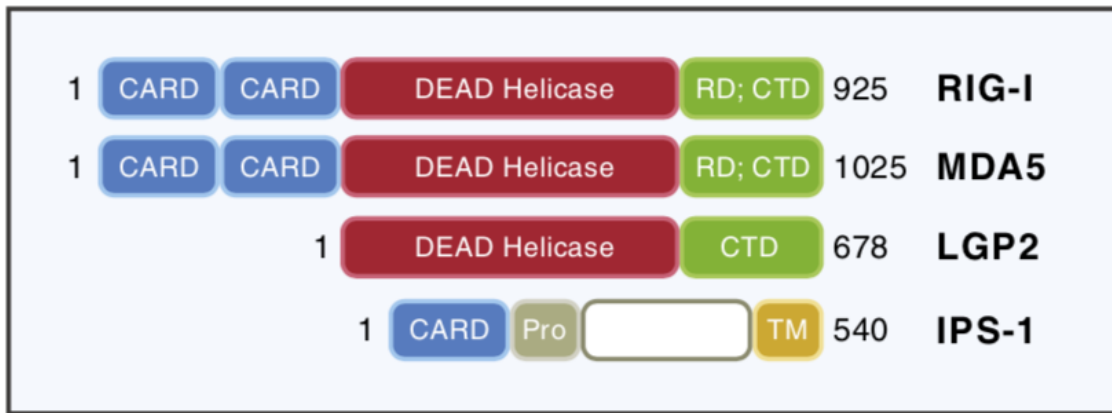


Figure 1.5. Structure of proteins in the RLR family.

Figure obtained from Gale, 2011.

1.3 Functions of RIG-I-like receptors

1.3.1 RIG-I-like receptors in DENV infection

Both RIG-I and MDA5 have differential roles in recognizing viral RNA from various viruses (Kato et al., 2006), with RIG-I preferentially recognizing short duplex RNA with a triphosphate on the 5' end (5'-ppp RNA) and MDA5 recognizing longer duplex RNA with no preferable 5'-end signature (Hornung et al., 2006; Pichlmair et al., 2006). The function of LGP2 and its role in RLR signalling is still unclear. *In vitro* experiments show that LGP2 functions as a negative regulator of RLR signalling when ectopically expressed (Bamming & Horvath, 2009; Yoneyama et al., 2005). However, *Lgp2* deficient mice show attenuated RLR-mediated anti-viral responses, suggesting that LGP2 functions as a positive regulator for RLR signalling instead (Sato et al., 2010; Venkataraman et al., 2007). RIG-I, MDA5 and TLR3 have been observed to be involved synergistically in innate immune activation against DENV-1 in Huh7 cells (Nasirudeen et al., 2011). However, recent data by Chazal *et al.* demonstrated that the 5' region of the DENV genome, containing a triphosphate motif, is recognized by RIG-I and not MDA5 (Chazal et al., 2018). This strongly suggests that RIG-I is the primary cytosolic sensor in DENV infection.

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The recently discovered type III interferon, interferon-lambda, has been reported to be induced by DENV infection and might have a role in DC cell migration to lymphoid organs. This was proposed based on CCL19 and CCL21 production in infected MoDCs (Hsu, Wang, Ho, & Lai, 2016). Using siRNA targeting both RIG-I and MDA5 in MoDCs infected with DENV-2, type III interferon genes *IFNL1* and *IFNL2* activation were reduced, suggesting that RLRs play a role in type III interferon activation (Sprokholt, Kaptein, van Hamme, van Hamme, Overmars, Overmars, Gringhuis, & Geijtenbeek, 2017b).

1.3.2 RIG-I-like receptors in anti-viral therapy

Strategies to prevent DENV replication through the activation of host RIG-I/MDA5 signalling could be explored in the development of an anti-viral therapeutic against DENV, as these PRRs have been observed to be critical in the host anti-viral response (Kato et al., 2006; Loo et al., 2008). Small molecule screening to identify compounds that can trigger the innate immune system have been observed to have anti-viral effects against dengue virus, as well as a wide range of viruses including hepatitis C virus and influenza virus (Bedard et al., 2012; Pattabhi et al., 2015). These anti-viral effects required IRF3 nuclear translocation to activate innate immune signalling resulting in the upregulation of anti-viral genes, pro-inflammatory cytokines (IL-6) and interferon. However, the precise mechanism of these small molecules has yet to be determined.

Given that RNA viruses are recognized by RIG-I to initiate the innate immune response, viral RNA genome sequences of Hepatitis C Virus (HCV) and Vesicular Stomatitis virus (VSV) have been studied in an attempt to use these sequences as initial templates to generate RIG-I ligands (Goulet et al., 2013; Saito et al., 2008). Using synthesized RNA, it was also determined that short (<300bp) sequences which contain the 5'-ppp are required, and these synthetic RNA should be double stranded and blunt at the 5' end (Schlee et al., 2009). Sequences longer than 300bp (300bp and 2000bp) have also been shown to activate RIG-I signalling (Beljanski et al., 2015). In an attempt to determine the

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minimum size of the RIG-I ligand RNA which can induce Type I interferon signalling, Kohlway *et al.* (Kohlway *et al.*, 2013) found that short hairpin structures with a stem of only 10 base pairs adjacent to the 5' end of the RNA are required, along with the presence of the 5'-ppp group (3p10L). dsRNA with 8 base pairs could not activate type I interferon signalling, and a 20 or 30 base pair dsRNA was more efficient than 3p10L. The lack of activation using the 8 base pair dsRNA might suggest that the HEL2i contacts are important as observed by predictive rendering of the structure of RIG-I and 3p10L. The increased type I interferon with the 20 and 30 base pair dsRNA could be attributed to more stable duplexes and thus a longer half-life in the cell, though this was not proven. RIG-I binds 3p10L to generate a monomer comprising of RIG-I and RNA in a 1:1 ratio. Other work done on 3p10L has shown that the type I interferon activity is RIG-I dependent and when injected into mice, 3p10L can induce a large number of genes associated with anti-viral activity, including ISGs (Linehan *et al.*, 2018). The studies mentioned above looking into the effect of length and structural modifications of RIG-I agonists on type I interferon signalling have helped in characterising the properties required in designing RNA that can be used as a ligand for RIG-I activation and signalling. However, the anti-viral effect of 3p10L has not been studied and remains an area for further investigation.

Other RIG-I ligands have been explored as a potential anti-viral strategy, although their application has been limited for use as a prophylaxis and not as a therapeutic in published studies. RIG-I ligands designed based on VSV sequences (5'-ppp RNA) have been used in the DENV and Chikungunya virus (CHIKV) infection model as a potential anti-viral agent. Though the work done has only looked at cell lines (A549 and MRC-5) and human monocytes isolated from PBMCs from healthy donors, the authors found that treatment with the 5'-ppp RNA RIG-I ligand provided protection against infection with DENV and CHIKV and this required an active RIG-I/MAVS/TBK1/IRF3 signalling axis (Olagnier *et al.*, 2014). M8, another RIG-I ligand derived originally from VSV but with modifications made to its sequence (more AU-rich) and length (99nt), was

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also shown to be able to inhibit influenza and dengue virus replication *in vitro* as well as prolong the survival of mice infected with a lethal dose of H5N1 and reduce footpad swelling in CHIKV infected mice (Chiang et al., 2015). Recently it has been suggested that dsRNA could also activate ISG56 gene expression without the need for a triphosphate or diphosphate group on the 5' end (Lee et al., 2018). This 18 base pair dsRNA molecule (CBS-13-BPS) has a phosphorothioate backbone instead of the usual phosphate backbone to increase the stability of the RNA molecule, and 2 wobble sequences that are suggested to enhance ISG56 gene expression. When used to pre-treat A549 human lung carcinoma cells, CBS-13-BPS had potent anti-viral effects against influenza virus PR8 and an oseltamivir-resistant variant as well. These findings support the idea that RIG-I agonists have the potential to be used in anti-viral treatment, although how to effectively apply these agonists *in vivo* remains an area to be explored.

1.3.3 Using RIG-I-like receptors in vaccination as adjuvants

There is a growing interest in utilizing immune-modulating RNAs as an adjuvant for vaccination through the activation of RIG-I signalling and type I interferon expression. An adjuvant is an agent that improves host response to the vaccine. This is done either through the generation of an antigen depot (eg. Alum) or enhanced activation of T-helper cells (Th1 or Th2) upon stimulation by the innate immune response. To characterise the ability of a TLR/RLR agonist to function as an adjuvant, Ziegler *et al.* used a flu vaccine (Influvac) or a VSV-VLP to vaccinate mice along with RNAdjuvant, a 547-nucleotide ssRNA with polyU repeats and a triphosphate at the 5' end stabilized by a cationic carrier peptide. They showed that upon vaccination with either Influvac or VSV-VLP, RNAdjuvant was able to promote the formation of specific IgG2b/c, indicative of a Th1 immune response (Ziegler et al., 2017). This improved Th1 response using RNAdjuvant was observed to enhance neutralising IgG titres in VSV-VLP vaccinated mice and confer protection against lethal challenge with VSV. RNAdjuvant was also observed to only induce local type I interferon induction

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and not systemic induction as shown for poly I:C, suggesting a good safety profile for this TLR/RLR agonist. Using MyD88^{-/-} and Cardif^{-/-} (MAVS^{-/-}) transgenic knockout mouse models, which are adaptor proteins specific to either the RLR or TLR signalling pathway respectively, Ziegler *et al.* also showed that the adjuvant effects of RNAdjuvant was dependent on TLR7 signalling and, to a lesser extent, RLR signalling. There are other groups that have studied the ability of RLR-specific agonists to function as vaccine adjuvants (Beljanski *et al.*, 2015; Kulkarni *et al.*, 2014). A chemically synthesized 19bp RIG-I ligand RNA has been shown to enhance immunological response with Influenza A vaccination in mice when compared with other PRR agonists like alum (targeting NOD-Like Receptor), MPL-A (targeting TLR4) and imiquimod (targeting TLR7/8) (Kulkarni *et al.*, 2014). Mice that were immunized with HA of a 2009 monovalent influenza vaccine along with a RIG-I ligand as an adjuvant showed improved germinal center reaction as well as greater T follicular helper cell induction as compared to those who received nothing or other PRR agonists as an adjuvant. The mice that received the RIG-I ligand adjuvant also showed a dose-sparing effect, requiring lower antigen doses during vaccination to achieve optimal humoral response to Influenza A infection. Beljanski *et al.* have also shown the enhancement of influenza vaccination through the use of M8, a RIG-I ligand derived originally from VSV but with modifications made to its sequence (more AU-rich) and length (99nt) (Beljanski *et al.*, 2015). When treated with M8, monocyte-derived dendritic cells (MDDCs) were observed to have upregulated various DC maturation markers at both transcription and cell surface expression levels, suggesting that M8 acts as a potent inducer of DC activation. M8 was then used as an adjuvant for vaccination with influenza Virus-like Particle (VLP), generated through the co-expression of three viral proteins—Matrix (MA), Hemagglutinin (HA) and Neuraminidase (NA) expressed in HEK-293T cells. By doing so, Beljanski *et al.* found that the M8 RIG-I ligand helped to protect against lethal H5N1 challenge through increased antibody levels, formation of germinal center B cells and induction of a strong Th1 response. M8 RIG-I ligand also generated higher levels of germinal center B cells when compared to other adjuvants like alum and Addavax, which the authors suggested was due to RIG-

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I ligand being able to induce a stronger Th1 response compared to the other adjuvants which are more Th2-response inducing. Using human monocyte-derived dendritic cells, the activation of RLRs in MoDCs transfected with poly I:C or 5'ppp RNA was observed to induce IL-27 secretion, Tfh polarization and IgM/IgG production by B cells (Sprokholt, Kaptein, van Hamme, van Hamme, Overmars, Overmars, Gringhuis, & Geijtenbeek, 2017a). All these results mentioned here suggests that activation of RLRs through RIG-I agonists leads to the induction of Th1 and Tfh responses through crosstalk with type I interferon signaling, linking viral epitope recognition to induction of robust and long-lived antibody responses.

1.4 Dengue vaccination strategies

1.4.1 Live attenuated vaccines

The first licensed dengue vaccine, Dengvaxia (CYD-TDV), was tested by Sanofi Pasteur in two large multi-centre Phase 3 trials after a long period of research and development (Capeding et al., 2014; Villar et al., 2015). This live attenuated vaccine (LAV) was initially created by cloning the prM and E genes from a clinical DENV-2 strain (PUO-218) into the YF-17D vaccine backbone to generate a DENV-2 chimeric strain named ChimeriVax-DENV-2. ChimeriVax-DENV-2 LAV was tested in rhesus monkeys, which demonstrated a neutralising antibody response and complete protection from DENV-2 challenge (Guirakhoo et al., 2000). Eventually, the tetravalent formulation of this LAV was developed for all four serotypes, using the same strategy of cloning the prM and E genes into the YF-17D backbone. These chimeras were found to be able to replicate to high titres in Vero cells, resulted in no neurovirulence in 4-week old mice when injected intracranially and were immunogenic in rhesus monkeys. Seroconversion was observed in all six monkeys tested after one dose of the ChimeriVax-DENV1–4 vaccine, which made it the first tetravalent dengue vaccine successfully tested in non-human primates (Guirakhoo et al., 2001).

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In Asia, the results of the vaccine efficacies against DENV-1, DENV-2, DENV-3, and DENV-4 were 54.5%, 34.7%, 65.2%, and 72.4% respectively (Capeding et al., 2014). This was unexpected, as the geometric mean neutralizing antibody titre (GMT) as measured by a standard plaque reduction neutralization test (PRNT) showed the highest readings for DENV-2, followed by DENV-3, DENV-1 and DENV-4. Similar trends were observed for the Latin America study. Vaccine efficacies were 50.3%, 42.3%, 74.0% and 77.7% for DENV1-4 respectively, and these were noted not to reflect the observations made with the GMT (Villar et al., 2015). The studies also highlighted the limitation of low numbers of seronegative individuals, especially children. For example, in the Latin American study 10% of the trial population were children, and of whom only approximately 20% were seronegative, making the efficacy and safety estimates for children with seronegative status based on approximately 2% of the study population. This turned out to be important as the increase for more severe disease was two times higher in seronegative individuals, but protection against severe dengue was observed in seropositive population (Sanofi Pasteur, 2017).

Dengue vaccination presents a unique challenge of having to attain a balanced immune response to the four serotypes to eliminate the chances of dominant seroconversion of a single serotype, resulting in reduced protection to certain serotypes and potential of ADE. Live attenuated vaccines often have the complication of differences in replication of individual virus serotypes. Using a technique to deplete specific populations of DENV-reactive antibodies in serum samples from vaccinated subjects, the contribution of serotype-cross-reactive and type-specific antibodies to neutralization of DENV can be estimated. This gives some indication of the replication kinetics of each of the LAV from each of the four serotypes. Serum samples from vaccine subjects enrolled into trials of either the CYD-TDV vaccine (Henein et al., 2016) or another LAV, TDENV (Gromowski et al., 2018), were used to determine the contribution of type-specific and cross-reactive antibodies to immunity against DENV. In a study looking at the replication of CYD-TDV in dengue naïve adult volunteers, serotype 4 generated the highest geometric mean titres (GMTs), followed by serotype 3.

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The authors suggest that vaccine serotype 4 may replicate better than other vaccine serotypes when administered together. However, they highlight caution in attempting to link this observation with the efficacy results in the previously mentioned phase 3 studies conducted in Asia and Latin America, as these volunteers were recruited in a dengue-non-endemic setting with the knowledge that the vaccine virus replication would be high in these subjects (Torresi et al., 2017). These observations reflect the differences in replicative fitness of each of the vaccine virus serotypes in humans, and the challenge of attaining a balanced humoral immune response to all four serotypes.

1.4.2 Virus-like particles (VLP) for Dengue vaccination

Virus-like particles (VLP) have certain advantages over live attenuated vaccines. If prepared properly, these vaccines are safer to use compared to live attenuated vaccines as they eliminate the risk of reversion to pathogenic strains. These VLPs are also less reactogenic compared to live attenuated vaccines given that these VLPs do not replicate. With VLPs, a balanced humoral response to each of the four serotypes can be relatively easily attained by adjusting the composition of each of the VLP components, overcoming some of the problems with LAV mentioned earlier.

Given these advantages, there has been increasing interest in exploring VLPs as potential vaccine candidates (Yan Liu et al., 2014; Metz et al., 2018; Urakami et al., 2017). These VLPs have been generated by co-expressing prM and DENV Envelope protein (E-protein) to produce spherical membrane vesicles, quite similar to the morphology of natural virions. VLPs generated in a *Pichia pastoris* expression system were able to generate neutralising antibodies in wild-type mice as measured with PRNT assays, as well as induce the production of cytokines involved in humoral immunity such as IFN γ , TNF α and IL-10 (Yiliu Liu et al., 2017). When injected into suckling mice, VLPs were able to provide protection against intracranial challenge with DENV as judged by paralysis and mortality. Urakami *et al.* recently produced VLPs in a mammalian expression

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system using 293F cells (Urakami et al., 2017). The initial production yielded low amounts of VLP, which led them to discover the substitution of phenylalanine at position 108 to an alanine (F108A) greatly enhanced DENV-1 VLP production, and that C-terminal region of DENV-1 comprising the EDIII region as well as the stem/transmembrane anchor (ST/TM) allowed greater secretion of DENV-2, DENV-3 and DENV-4 VLPs. These VLPs were then used to immunize wild-type mice along with Alum as an adjuvant. It was found that the tetravalent VLPs were able to elicit high titres of neutralising antibodies against all four DENV serotypes as judged by FRNT assays, and these titres were found to be higher than the previously described VLPs produced in yeast. Immunisation with a plasmid DNA vaccine was also compared, but Urakami *et al.* found that the VLP format of the vaccine was more effective compared to the plasmid DNA version. Using a wide range of well-defined human monoclonal antibodies and immune sera from dengue patients, Metz *et al.* showed that the VLPs produced in mammalian cells were able to effectively display epitopes that were mapped previously in virus particles, suggesting that the immunogenicity toward DENV VLPs would be very similar to what is found on the DENV itself (Metz et al., 2018). Transmission electron microscopy also showed that these DENV VLPs spherical particles are generally similar in size and morphology but still with some heterogeneity. The size ranges from 24 to 40nm in diameter, which is smaller than a virus that is approximately 50nm in diameter (Hsieh et al., 2014).

VLP also potentially allows the priming of cytotoxic T lymphocytes (CTLs), which probably occurs in a process called “cross-presentation” whereby the VLP is processed in the cytoplasm of specialised APCs to be presented on MHC class I molecules to prime the CD8+ CTLs (Schirmbeck, Deml, Melber, Wolf, et al., 1995a; Schirmbeck, Melber, & Reimann, 1995b). The repetitive arrangement of epitopes on the VLPs also allow more efficient cross-linking to B cell receptors (BCR), allowing for better B cell activation and differentiation (Bachmann et al., 1993; Zabel et al., 2014). Even though these VLPs can by themselves initiate an immune response through vaccination, adjuvants have been suggested to be useful in enhancing the effects of VLPs in vaccination by improving the

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recruitment of APCs to sites of administration and enhancing the activation of T and B lymphocytes in secondary lymphoid organs. PRRs have been studied as potential adjuvants, with some such as TLR4 agonists AS04 and AS01 already helping in the licensing of some VLP-based vaccines (reviewed in Cimica, 2017). RLR agonists have also been studied and have been shown to allow dose-sparing effects, such as the proof-of-principle study using a 5'-ppp RNA RIG-I agonist to provide effective protection against Influenza challenge with reduced amounts of VLP antigen for vaccination (Beljanski et al., 2015). The author also suggests that it might be more advantageous to have a Th1-biased CD4 T-helper cell response compared to a Th2-biased response, induced by adjuvants such as Alum, when vaccinating against pathogens that require Th1 cell-mediated immunity, like in the case of viruses such as Influenza. More studies into the impact of adjuvants and VLPs would greatly improve the field of vaccine development, especially with a complex disease such as dengue.

1.4.3 Use of mouse models to study DENV and the effects of anti-viral and vaccine candidates *in vivo*.

One of the main limitations in the search for effective anti-viral and vaccine candidates for DENV therapy has been the lack of a proper mouse model to study the effects of potential interventions on clinical strains of dengue. Wild-type mice with an intact immune system are not susceptible to infection by DENV. To evade the effects of the innate immune response, DENV NS5 has the ability to bind and degrade human STAT2 (Morrison et al., 2013), and DENV NS2B3 has the ability to degrade human STING (Aguirre et al., 2012; Yu et al., 2012). However, both these non-structural proteins are unable to degrade mouse STAT2 (Ashour et al., 2010) or mouse STING and this might explain why DENV cannot replicate effectively in wild-type mice. In an effort to develop a mouse model that allows the *in vivo* study of DENV replication, various groups have made use of mice that either lack the receptors for type I interferon and type II interferon (AG129 mice) (Johnson & Roehrig, 1999), mice that only lack type I interferon receptor (IFNAR^{-/-}) (Prestwood et al., 2012), or mice lacking

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components for effective interferon signalling activation, such as STAT1/2 and IRFs (Ashour et al., 2010; H. W. Chen et al., 2013; Shresta et al., 2005).

The AG129 mouse model (Type I/III interferon receptor knockout) has been the preferred mouse model to study the effects of potential anti-viral therapy candidates that work directly on the virus itself to inhibit replication. Iminosugars have been suggested as having the potential to be used as an anti-viral due to its ability to inhibit the catalytic activity of α -glucosidase I and II, which are enzymes located in the Endoplasmic reticulum (ER) that play an important role in the proper folding of N-glycosylated glycoproteins. AG219 mice infected with DENV showed to have reduced viremia and higher survival rates when treated with these glucosidase inhibitors as a result of improper folding of viral proteins (Chang, Schul, Butters, et al., 2011a; Chang, Schul, Yip, et al., 2011b; Miller et al., 2012; S. T. Perry et al., 2013; Rathore et al., 2011). A number of nucleoside analogues have also been studied as potential anti-viral candidates in AG219 mice, inhibiting viral RNA translation and the synthesis of viral proteins (Y. L. Chen, Yin, Duraiswamy, et al., 2010a; Y. L. Chen, Yin, Lakshminarayana, et al., 2010b; Q.-Y. Wang et al., 2011). AG129 mice have also been used to test the effects of small molecule inhibitors which work directly on DENV proteins such as the capsid (Byrd et al., 2012) and the NS3 helicase (Byrd et al., 2013).

Several vaccine candidates have been developed against DENV and these have been tested in both immune competent wild-type mice as well as immunocompromised mice. AG129 mice and IFNAR^{-/-} mice, which are permissive for virus replication, have been used in proof-of-concept studies to determine the efficacy of live-attenuated virus candidate vaccines (Brewoo et al., 2012; Huang et al., 2003; Züst et al., 2013). Though these mouse models allow for virus replication, the lack of a proper innate immune response raises questions about the effectiveness of these live attenuated virus vaccines in immune-competent individuals and if the efficacy in these studies are accurate, thus still requiring the use of relatively expensive non-human primate (NHP) models to confirm findings in these mouse experiments (Züst et al., 2013).

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Subunit vaccines are usually developed based on the epitopes found on the E-protein that have been identified or predicted to elicit potent neutralising antibody responses (Clements et al., 2010; Etemad et al., 2008; Suzarte et al., 2014; Valdés et al., 2009; Zuest et al., 2015). DENV non-replicative vaccine candidates, such as subunit vaccines, have been tested in immune-competent wild-type mice. Non-replicative vaccines usually require an adjuvant to enhance the immune response in a prime/boost vaccination schedule and IFNAR^{-/-} mice might not be able to generate a protective response when immunized with vaccines containing IFNAR-signal-dependent adjuvants. This is different from live attenuated virus vaccines which trigger IFNAR-dependent and -independent innate immune signalling, leading to protective adaptive immune responses even in AG129 mice (Querec et al., 2006). When comparing the effects of vaccination in wild-type Balb/c mice with AG129 mice using a tetravalent dengue DIIC protein administered with ODN and alum as adjuvants, Zuest *et al.* found that the ability to respond to type I interferon is important for protection against DENV challenge (Zuest et al., 2015). IFNAR^{-/-} mice were able to generate neutralising antibodies but were unable confer protection against DENV challenge which might be due to the lack of a CD8 T-cell response. Hence the authors suggest that AG129 and IFNAR^{-/-} mice are not suitable for the testing of adjuvanted recombinant protein vaccines. This results in problems with determining the efficacy of the vaccine candidate, with the solutions in these cases being a intracranial challenge in WT mice (Clements et al., 2010; Valdés et al., 2009), which does not recapitulate disease pathology, or *in vitro* tests like PRNT assays to determine neutralising antibody titres (Etemad et al., 2008), which is a technique that has been recently called to question as geometric mean neutralizing antibody titre (GMT) measured by PRNT did not correlate with serotype-specific efficacy in phase 3 trials done for the first licensed Dengue vaccine, Dengvaxia (Sanofi Pasteur) (Capeding et al., 2014; Sabchareon et al., 2012; Vannice et al., 2018).

Given the challenges in finding an ideal mouse model to study DENV pathology, we have turned to the use of conditional knockout IFNAR mouse models

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(IFNAR^{ff}). These are mice with a loxP-flanked IFNAR crossed with mice expressing a Cre recombinase under different immune cell-type-specific promoters (Clausen et al., 1999; Züst et al., 2014). CD11c-cre and LysM-cre mice that were crossed with IFNAR^{ff} mice were found to be susceptible to DENV infection, and eventually resolved viremia and fully recovered from infection (Züst et al., 2014). CD4-cre mice crossed with IFNAR^{ff} mice showed minimal signs of disease, thus indicating that loss of type I interferon receptor on mainly macrophages (LysM-cre) or dendritic cells (CD11c-cre), and not CD4-expressing T-cells (CD4-cre), was sufficient to allow DENV replication in mice. Upon infection, CD11c-cre and LysM-cre IFNAR^{ff} mice responded with an effective CD8 T-cell response, whereas there was only a weak CD8 T-cell response in IFNAR^{-/-} mice. These mice were also able to induce a protective immune response to a candidate subunit vaccine against DENV-2, showing that these conditional IFNAR mice are immune-competent enough to be used in vaccine studies. Another group has studied ADE of DENV using LysM-cre IFNAR^{ff} mice and found that ADE of DENV-2 or DENV-3 infection in these mice resulted in clinical manifestations of severe dengue comparable to observations in human disease. They also demonstrate the therapeutic effects of a bispecific anti-EDIII antibody, Fc-DART, and a RIG-I receptor immunomodulatory agonist, M8, in the context of ADE (Pinto et al., 2015). Overall these results indicate that a semi-immunocompetent mouse model is useful for studying vaccine efficacy, as well as therapeutic intervention of severe dengue in the context of ADE. More studies using these conditional IFNAR knockout mouse models would hopefully give us a clearer understanding of the usefulness of such models in learning about DENV pathology and treatment.

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1.5 Research Objectives

Although there has been an increasing interest in the use of RIG-I agonists for viral therapy and vaccination, there is no data currently available on the effects of minimal length (~10bp) immune-modulating RNA (immRNA) as RIG-I agonists in viral treatment and as a vaccine adjuvant. Structural modifications of these minimal immRNA agonists can be achieved with changes in the sequence of these RNA constructs, and through an initial screen we hope to identify a lead candidate that can activate type I interferon signalling with greater potency compared to the parental construct as described by Kohlway *et al.* (Kohlway et al., 2013). Using dengue virus as an infection model, we want to characterise the use of minimal immRNA agonists for dengue therapy and as an adjuvant for vaccination. Through the use of various cell lines, primary human skin cells, and dengue infection mouse models, we want to determine the potential for the development of minimal immRNA agonists as an anti-viral therapy and vaccine adjuvant as well as to gain useful insight into the action of these RIG-I agonists.

2 Materials and Methods

2.1 *In vitro* synthesis of immune-modulating RNA (immRNA)

RNAs were transcribed with annealed primer pairs containing the T7 promoter with chemically synthesized DNA from IDT. Reactions were carried out in 40 mM Tris HCl buffer pH 7.9, 30 mM MgCl₂, 2 mM spermidine, 10 mM DTT 0.01 % Triton-X100, 5-6 mM GTP and 4 mM NTP (CTP, ATP and UTP), 1 μM annealed DNA template, 400 nM T7 RNA polymerase, 0.2 U/mL thermostable inorganic pyrophosphatase for 16 h at 37°C. RNAs transcribed were purified by phenol:chloroform:isoamyl alcohol (25:24:1, v/v) extraction followed by ethanol precipitation. RNA pellet was resuspended in 10mM HEPES buffer pH 7.4 and subjected to further purification by HiTrap Q HP column. The eluted RNAs were subjected to ethanol precipitation and further purified from 20% denaturing PAGE followed by ethanol precipitation. Purified RNA was resuspended in ME 50 buffer containing 10mM MOPS pH 7, 1mM EDTA and 50mM NaCl. Sequences for the RNA are as follows:

3p10L-GACGUACGUUUCGACGUACGUCC.

3p10LG9-GGAUUUCCGCCUUCGGGGGAAAUCC

2.2 Ethics Statement

Healthy human skin tissue and matched blood samples were obtained from mastectomy surgery. The study was approved by the institutional review board (National Health Group Domain Specific Review Board (NHG DSRB 2015/00725 and 2017/00812) and patients gave written informed consent. All skin samples were processed on the day of surgery.

Animal experiments using mice were conducted according to the rules and guidelines of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR), Singapore.

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Experiments were reviewed and approved by the Institutional Review Board of the Biological Resource Centre, Singapore (IACUC protocol number 151018).

2.3 Human skin DC isolation

Protocols for isolating single cells from human skin were described in detail previously (Cerny et al., 2014). For the isolation of human skin cells, 300 μ m dermatome (Piling, USA) sections were incubated in RPMI + 10% heat-inactivated FBS (Gibco) containing 0.8 mg/ml collagenase (Type IV, Worthington-Biochemical) and 0.05 mg/ml DNase I (grade II, Roche) for 12 h in sterile non-tissue culture treated 10cm plates (Thermo Scientific). After overnight incubation, cells were filtered through a 70 μ m strainer to obtain a single cell suspension that was resuspended in RPMI + 10% heat-inactivated FBS + 1% Penicillin/Streptomycin (Gibco) and incubated in a 5% CO₂ incubator at 37°C.

2.4 Cell lines

HEK-293T, U937 and A549 cells (ATCC) were grown in RPMI supplemented with 10% fetal bovine serum (FBS) (Gibco). U937 cells expressing DC-SIGN (U937-DC cells) were generated by lentiviral transfection (Züst et al., 2013). HEK-293T cells expressing MX1P-luc were generated by Georg Koch (Kochs et al., 2009) (University of Freiburg, Germany) and were a kind gift from Matthias Habjan (Max Planck Institute of Biochemistry, Germany). HEK-293T cells containing the ISRE-luc reporter plasmid was generated by transfecting 0.5 μ g of plasmid using 293fectin Transfection Reagent (Thermo Fisher Scientific). ISRE-luc plasmid used to transfect HEK-293T cells was a kind gift from Matthias Habjan.

RIG-I knockout cell lines were generated by lentivirus transduction of U937-DC cells with pRRL-gRNA-Cas9-T2A plasmid containing a gRNA sequence targeting exon 1 of RIG-I. The RIG-I gRNA-containing plasmid was a kind gift

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from Dr Alvin Tan (Genome Institute of Singapore, A*STAR, Singapore). Lentiviral particles were produced on 293T cells by using 293fectin Transfection Reagent (Thermo Fisher Scientific) with the following three plasmids: (i) pMDLg/pRRE, which includes gag, coding for the virion main structural proteins; pol, responsible for the retrovirus-specific enzymes; and RRE, a binding site for the Rev protein which facilitates export of the RNA from the nucleus. (ii) pRSV-Rev, encoding the HIV-1 rev under the transcriptional control of an RSV U3 promoter; (iii) pMD2.G, a VSV-G envelope expressing plasmid. pMDLg/pRRE (addgene #12251), pRSV-Rev (addgene #12253) and pMD2.G were generated by Prof. Didier Trono (Lausanne, Switzerland). Successfully transduced cells were selected by supplementing the culture medium with 2 µg/ml puromycin.

All cells were grown in a humidified 5% CO² incubator kept at 37°C.

2.5 Virus

DENV-2 stain TSV01 (NCBI accession number AY037116.1) used for infection experiments in human cell lines is a patient isolate that have been passaged in C6/36 mosquito cells for 5–20 passages. D2Y98P used in the infection of primary human skin DCs was plaque-purified after passage 20 and was derived from an infectious clone. The enhanced viral RNA synthesis of D2Y98P was mapped to a natural mutation in NS4b protein and this mutation had no effect on the IFN-inhibiting capacity of the virus (Grant et al., 2011).

2.6 RNA screening with type I interferon bioassay

HEK-293T MX1P-luc cells were seeded into white 96-well plates at a density of 2.5×10^4 cells per well and incubated overnight. immRNA was diluted to the appropriate concentrations and transfected with 293fectin transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were incubated for 24h and then lysed and analysed using Bright-Glo Luciferase

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Assay System (Promega) on a GloMax-Multi Microplate Reader (Promega) according to the manufacturer's instructions.

2.7 Quantitative PCR (qPCR)

2.7.1 U937-DC human cell line

U937-DC cells were seeded in a 24-well plate at a density of 3.0×10^5 cells per well in 500 μ l of RPMI with 10% FBS and incubated overnight. immRNA was diluted to the appropriate concentrations and transfected (in triplicate) with Hilymax (Dojindo Molecular Technologies) according to the manufacturer's instructions. After 24h incubation, cells were centrifuged at 500 x g for 4 mins and harvested in Trizol reagent (Thermo Fisher Scientific) and total RNA was harvested according to the manufacturer's instructions. RNA was reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen). PCR primers were purchased from Integrated DNA Technology and quantitative RT-PCR was performed on an ABI 7900 HT Real-Time PCR system (Applied Biosystems) using iTaq Universal SYBR Green Supermix (Bio-rad Laboratories). Primer sequences can be found in Table 2. Analysis of qPCR data was done by relative quantitation by the $\Delta\Delta C_t$ method using beta-actin as the reference gene control.

2.7.2 A549 human cell line

A549 cells were seeded in a 24-well plate at a density of 5.0×10^4 cells per well or in a 96-well plate at a density of 1.0×10^4 cells per well and incubated overnight. immRNA was diluted to the appropriate concentrations and transfected (in triplicate) with 293fectin transfection reagent (Thermo Fisher Scientific). Cells were incubated and, 48h later, harvested in Trizol reagent (Thermo Fisher Scientific). Total RNA was harvested for reverse transcription and qPCR as described above in Section 2.7.1.

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2.7.3 Primary human skin DCs

Primary human skin DCs were seeded in a 96-well plate at a density of 2.0×10^5 cells per well. immRNA was diluted to the appropriate concentrations and transfected with Hilymax (Dojindo Molecular Technologies). After 24h incubation, cells were centrifuged at 500 x g for 4 mins and harvested in Trizol reagent (Thermo Fisher Scientific) and total RNA was harvested. Reverse transcription and qPCR were done as described above in Section 2.7.1.

Gene	Species	Sense/Antisense	Sequence (5' to 3')
<i>IFNB</i>	Human	Sense	CTCTCCTGTTGTGCTTCTCC
<i>IFNB</i>	Human	Antisense	GTCAAAGTTCATCCTGTCCCTG
<i>ACTB</i>	Human	Sense	TCGTGCGTGACATTAAGGAG
<i>ACTB</i>	Human	Antisense	GTCAGGCAGCTCGTAGCTCT
<i>DDX58</i>	Human	Sense	GCCATTACACTGTGCTTGGAGA
<i>DDX58</i>	Human	Antisense	CCAGTTGCAATATCCTCCACCA
<i>CXCL10</i>	Human	Sense	TCTTCTCACCCCTTCTTTTTCATTGT
<i>CXCL10</i>	Human	Antisense	TTCCTGCAAGCCAATTTTGTC
<i>RSAD2</i>	Human	Sense	CACAAAGAAGTGTCTGCTTGGT
<i>RSAD2</i>	Human	Antisense	AAGCGCATATATTCATCCAGAATAAG
<i>OASL</i>	Human	Sense	GGATCTTCTCCCACACTCACATCT
<i>OASL</i>	Human	Antisense	CACCATCAGGATTCTTCACGAA
<i>IFIH1</i>	Human	Sense	GGCACCATGGGAAGTGATT
<i>IFIH1</i>	Human	Antisense	ATTTGGTAAGGCCTGAGCTG

Table 2: Sequences for primers used for qPCR

2.8 Bioassay for human type I interferon production

Supernatant from immRNA-transfected cells was incubated on HEK-293T cells transfected the day before with 0.5µg of ISRE-luc plasmid and plated in a 96-

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well white opaque plate the next day. Supernatant was incubated for 6h before cell lysis and was analyzed by using Bright-Glo Luciferase Assay System (Promega) on a GloMax-Multi Microplate Reader (Promega) according to the manufacturer's instructions.

2.9 Type I interferon bioassay for RLR-expressing HEK-293T cells transfected with immRNA

HEK-293T cells were seeded in a 24-well plate at a density of 1.25×10^5 cells per well in RPMI with 10% FBS. 50ng of pUNO-hRIG-I or pUNO-hMDA5 was transfected using Lyovec (Invivogen) and cells were incubated overnight. HEK-293T cells expressing RLRs were transfected with immRNA. Supernatant from cells was harvested 24h after transfection with dsRNA and type I interferon bioassay using HEK-293T cells expressing ISRE-luc was done. hRIG-I and hMDA5 plasmids were a kind gift from Dr Luo Dahai (Lee Kong Chian School of Medicine, NTU).

2.10 DENV-2 infection and flow cytometry analysis

2.10.1 U937-DC and A549 human cell lines

U937-DC-SIGN and A549 cells were transfected with immRNA as described in previously (**Section 2.7.1 and 2.7.2**). After 24h incubation, transfected U937-DC-SIGN and A549 cells were infected with DENV-2 (TSV01 strain) at a MOI-1 and MOI-5 respectively. Cells were incubated with RPMI containing DENV-2 for 2h. After two washes, infected cells were resuspended in RPMI with 10% FBS and placed in the incubator for 24h. For FACS analysis, washed cells were fixed and permeabilized by resuspending cells in Cytofix/Cytoperm buffer (BD Biosciences). Dengue E protein was stained with anti-E protein antibody (4G2) (ATCC) conjugated to Alexa 647, and anti-NS1 antibody conjugated to Alexa 488. Fluorescence on these cells was measured on a BD FACS Canto II analyser

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(BD Biosciences) and analysis was done using Flowjo (Treestar). Cells that stained positive for both NS1 and E protein were considered infected.

2.10.2 Primary human skin DCs

Human skin DCs were isolated and transfected with immRNA as described previously (**Section 2.7.3**). For prophylactic studies, isolated human skin DCs were infected with DENV-2 (D2Y98P strain) at a MOI-5 24h post-transfection. For therapeutic studies, isolated human skin DCs were infected with DENV-2 (D2Y98P strain) at a MOI-5 and transfection of immRNA was done 4h, 6h and 24h post-infection. Infected cells were analysed 72h post-infection to determine the percentage of infected cells using flow cytometry. 1000U of human recombinant IFN- β (Immunotools) was added to cells at 4h post-infection. Flow cytometry was performed on an LSRII (Becton Dickinson [BD]) and data were analysed using FlowJo (TreeStar). The following reagents for staining of human skin DCs were used: fixable live/dead dye (Thermo Fisher Scientific), anti-CD1a (HI149) (Biolegend), anti-CD11c (B-ly6), anti-CD45 (HI30), anti-HLA-DR (L243) (all from BD Biosciences), anti-CD141 (AD5-14H12) (Miltenyi), anti-CD14 (RMO52) (Beckman Coulter), anti-CD80 (2D10) and anti-E protein (4G2) (ATCC) conjugated to Alexa 647 using a protein labelling kit (Molecular Probes, Invitrogen).

Anti-human antibody	Clone	Dye
anti-CD1a	HI149	Alexa-Fluor 700
anti-CD11c	B-ly6	V450
anti-CD45	HI30	V500
anti-HLA-DR	L243	PE-Cy7
anti-CD141	AD5-14H12	PE
anti-CD14	RMO52	PE-Texas Red (ECD)
anti-CD80	2D10	APC
anti-E protein (4G2)	d1-4g2-4-15	APC

Table 3: Antibodies used to stain human skin DCs for flow cytometry

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2.11 Type I interferon blocking assay with U937-DC cells

U937-DC cells were seeded in a 96-well plate at a density of 0.6×10^5 cells per well and transfected with immRNA as described previously (**Section 2.7.1**). After 6 hours, anti-human IFNAR blocking antibody (PBL Interferon Source) or IgG isotype control (R&D systems) was added at a concentration of 10 ug/mL. After overnight incubation, supernatant was harvested and a bioassay for type I interferon was done as described in **Section 2.8**. U937-DC cells were infected with DENV-2 (TSV01) at MOI-1, and infection was quantified as described in **Section 2.10.1**.

2.12 Breeding of conditional IFNAR knockout mice

LoxP-flanked *ifnar1* ($IFNAR^{fl/fl}$), $IFNAR^{fl/fl}$ -CD11c-cre^{+/-} and $IFNAR^{fl/fl}$ -LysM-cre^{+/-} mice (all on C57BL/6 background) were kindly provided by Prof Ulrich Kalinke (Twincore, Germany). $IFNAR^{fl/fl}$ mice were crossed with either $IFNAR^{fl/fl}$ -CD11c-cre^{+/-} or $IFNAR^{fl/fl}$ -LysM-cre^{+/-} mice and heterozygous mice were used for subsequent experiments. All mice were bred and kept under specific-pathogen-free conditions at the Biomedical Resource Centre, Singapore. Mouse ear skin generated from ear-punches were digested in 200ul of tail digestion buffer (Unison Collaborative, Singapore) overnight at 55°C. The required genotype was determined using PCR of genomic DNA with Taq PCR master mix (Qiagen) following the manufacturer's instructions and using the primers (Integrated DNA Technologies, Singapore) listed in Table 4. PCR products were run on 1% agarose gel (1st BASE Pte Ltd, Singapore).

Name	Forward/Reverse	Sequence (5' to 3')	Size
LysM	Forward	CTTGGGCTGCCAGAATTTCTC	350 bp
LysM	Reverse	TTACAGTCGGCCAGGCTGAC	
LysM	Forward	CTTGGGCTGCCAGAATTTCTC	700 bp
Cre	Reverse	CCCAGAAATGCCAGATTACG	

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IFNAR	Forward	CAGGCCACTCTGCATTTTCCTC	358 bp
LoxP	Reverse	CTTTTTGGATCGATCCATAACTTCG	
CD11c-cre	Forward	ACTTGGCAGCTGTCTCCAAG	300 bp
CD11c-cre	Reverse	GCGAACATCTTCAGGTTCTG	

Table 4: Primers used for genotyping conditional knockout mice

Cycle Step	Temperature (°C)	Time	Notes
1	95	3 mins	
2	94	30 sec	Repeat for 35 cycles CD11c and 40 cycles for LysM
3	56 (CD11c) 58 (lysM)	30 sec	
4	72	30 sec	
5	72	5 mins	
6	4	Hold	

Table 5: Cycling conditions for genotyping PCR

2.13 Conditional IFNAR knockout mice for anti-viral and vaccination experiments

2.13.1 Anti-viral experiments

8 to 12-week-old female or male mice were used for infection experiments. 25ug of immRNA was complexed with *in vivo*-jetPEI (Polyplus Transfection) according to the manufacturer's instructions at a N/P (nitrogen/phosphate) ratio of 8 in a final volume of 50ul and was injected intravenously (I.V). Infection was done after the blood was drawn 6-8h post-treatment with immRNA. D2Y98P virus was diluted in PBS to obtain a final concentration of 10^7 pfu/mL of virus. 100ul of virus was injected intraperitoneally (I.P) per mouse to obtain a final amount of 10^6 pfu per mouse. Blood was drawn 72h post-infection and weights were taken to determine the health of the mice. Mice with more than 20% weight loss considered moribund and were euthanized.

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2.13.2 Vaccination experiments with DENV-2 VLP

12-week-old female or male mice were used for vaccination experiments. 10ug of DENV-2 virus-like particles (The Native Antigen Company) containing prM/E proteins were co-injected with 25ug of immRNA was complexed with *in vivo*-jetPEI (Polyplus Transfection) according to the manufacturer's instructions at a N/P ratio of 8, or with 50ug of VacchiGrade poly I:C (Invivogen). 100ul volume of DENV-2 VLP with or without adjuvant was injected intramuscularly (I.M) with a volume of 50ul given for each quadriceps. Booster injection of the same composition was given 14 days after the initial priming, with blood drawn 21 days and 28 days post-immunisation for ELISA. Challenge with DENV-2 was done 28 days post-immunisation. D2Y98P virus was diluted in PBS to obtain a final concentration of 10^7 pfu/mL of virus. 100ul of virus was injected intraperitoneally (I.P) per mouse to attain a final amount of 10^6 pfu per mouse, and blood was drawn on day 3 post-challenge to determine viremia as described in Section 2.14. Weights were taken to determine the health of the mice. Mice with more than 20% weight loss considered moribund and were euthanized.

2.14 Viral RNA qPCR from mouse plasma

Mouse blood was collected in sodium citrate. Plasma was obtained by centrifuging the blood at 6000 x g for 4 mins. High pure viral nucleic acid kit (Roche) was used according to the manufacturer's instructions to extract viral RNA from the plasma. RNA-to-Ct 1-Step Kit (Thermo Scientific) was used along with primers (IDT) designed to detect DENV-2 viral RNA with methods reported in (Züst et al., 2013).

DENV-2 forward primer: ACACCACAGAGTTCCATCACAGA

DENV-2 reverse primer: CATCTCATTGAAGTCNAGGCC

Probe: CGATGGAATGCTCTC – FAM (ABI)

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Viremia was determined using a DENV-2 standard generated by serial dilution from vRNA extracted from a known number of plaque forming units (pfu) and data is presented as pfu/mL equivalents.

Component	Volume (ul)	Final Concentration
Nuclease-free water	8.04	
2X master mix (ABI, TaqMan® RNA-to-C _T [™] 1-Step Kit)	10	1x
RT enzyme mix (ABI, TaqMan® RNA-to-C _T [™] 1-Step Kit)	0.5	1x
DenV F primer (100uM)	0.18	1uM
DenV R primer (100uM)	0.18	1uM
FAM Probe (10uM)	0.1	0.05uM
RNA sample	1	
Total volume:	20	

Table 6: Components used for PCR mastermix for viral RNA quantification (per reaction)

Cycle Step	Temperature (°C)	Time	Notes
1	48	30 mins	
2	95	10 mins	
3	95	15 sec	Repeat for 40 cycles
4	57	1 min	

Table 7: Cycling conditions used for quantification of viral RNA on Applied Biosystems 7500 real-time PCR system (ABI)

2.15 Bioassay for mouse type I interferon production

50ul of plasma was incubated on LL171 cells (Uzé et al., 1994) (ISRE-luciferase L929 reporter cell line) plated in a 96-well white opaque plate (Greiner Bio-one) the previous day at a density of 2.5×10^4 cells per well. Supernatant was incubated for 6h before being lysed and analyzed by using Bright-Glo Luciferase

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Assay System (Promega) on a GloMax-Multi Microplate Reader (Promega) according to the manufacturer's instructions.

2.16 ELISA assay for DENV-2 binding

UV-inactivated concentrated DENV-2 virus was coated onto half-area plates (Greiner bio-one) in coating buffer overnight at 4°C. Plates were washed with ELISA wash buffer (PBS containing 0.05% Tween 20) before blocking with blocking buffer (wash buffer + 3% skim milk) for 2h at room temperature. After washing, plasma was diluted in blocking buffer by doing 2-fold serial dilutions starting at 1:50 and diluting to 1:51200. Diluted plasma was added to plate and incubated for 1h at room temperature, followed by three washes with wash buffer. Peroxidase-conjugated rabbit anti-mouse IgG in blocking buffer was added, followed by 1h of incubation at room temperature and three additional washes. Tetramethylbenzidine (TMB) was added as the enzyme substrate. Reaction was stopped with 1M HCl and the optical densities were read at 450 nm using an automatic enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan). Endpoint titres were defined as the lowest dilution of plasma in which binding was 4-fold greater than the mean binding observed with the naïve plasma controls.

2.17 Flow cytometry-based neutralization assay

Heat-inactivated mouse plasma was serially diluted and incubated at 37degC for 30 mins with TSV01 DENV-2 virus in RPMI medium. 50ul of this antibody-virus mixture was then added to 0.6×10^5 U937-DC cells plated in a 96-well plate and left on the cells for 2 hours at 37degC, after which RPMI with 10% FBS was added to a final volume of 200ul and incubated overnight in the incubator. For FACS analysis, washed cells were fixed and permeabilized by resuspending cells in Cytotfix/Cytoperm buffer (BD Biosciences). Dengue E protein was stained with anti-E protein antibody (4G2) (ATCC) conjugated to Alexa 647, and anti-NS1 antibody conjugated to Alexa 488. Fluorescence on these cells was

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measured on a BD FACVerse analyser with HTS (BD Biosciences) and analysis was done using Flowjo (Treestar). Cells that stained positive for both NS1 and E protein were considered infected and the data was plotted and analyzed with GraphPad Prism 7.0 software to determine the 50% neutralization titre (NT50).

2.18 Graphs and statistical analysis

Graphs and statistical calculations were done using GraphPad Prism 7.0 (Graphpad Software). EC50 values were generated using GraphPad Prism 7.0 by generating a sigmoidal fit response curve with least squares regression fitting method. P-values < 0.05 were considered significant.

RESULTS

3 Results

3.1 Screening and identifying potent immRNA in human cells

3.1.1 Screening the interferon-inducing ability of different minimal immRNAs in human cells yields a potent lead candidate 3p10LG9

The minimal length of the signalling competent hairpin RNA is a 10 base pair stem of a hairpin RNA, as shown previously (Kohlway et al., 2013) . Based on this work, various modifications were carried out on these minimal length immune-modulating RNA constructs (minimal immRNA) to determine if any modification in the stem region could enhance type I interferon production in human cells when compared to the original 10 base pair stem construct (3p10L). Human embryonic kidney (HEK-293T) reporter cells were directly transfected with the various modified immRNA constructs. The luciferase reporter in these cells is driven by the MX1 promoter, an IFN-stimulated gene downstream of IFN- α or β binding to the type I IFN-receptor. One of the modified immRNA constructs, 3p10LG9, had a higher efficacy in inducing type I interferon production when compared to the parental construct 3p10L ($p=0.3699$). 3p10LG9 is similar in size than the parental construct and differs in the presence of a kink near the hairpin loop created by a guanine nucleotide in position 9 of the RNA construct. The EC₅₀ value for 3p10LG9 was 1.1nM, which is a 6-fold improvement compared to OHY23 that has an EC₅₀ of 6.6nM (**Figure 3.1**). To confirm this finding, we tested the effect of 3p10LG9 in different human cell lines. We transfected human lung fibroblast cell line A594 and the monocytic cell line U937 stably expressing DC-SIGN (U937-DC-SIGN) with immRNAs 3p10LG9 and 3p10L. As a negative control, the 3p10LG9 construct without 5' phosphorylation was used (G9neg). The 5' phosphate group is known to be essential for RIG-I activation (Hornung et al., 2006). In both human cell lines, 3p10LG9 was able to activate the IFN response, and was observed to do so more efficiently than 3p10L in U937-DC cells in a dose-dependent manner (**Figure 3.2 A and B**)

RESULTS

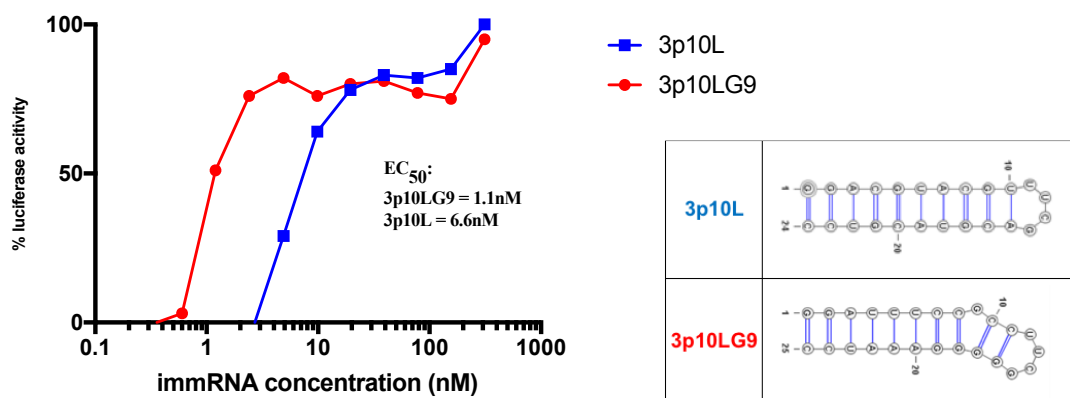


Figure 3.1: 3p10LG9 is a potent type I interferon activator in human cells.

Immune-modulating RNA (immRNA) was transfected into HEK-293T cells that contain a stably integrated luciferase reporter driven by a MX1 promoter (MX1P-luc). RNA was transfected at various concentrations as indicated in the x axis and luminescence was measured 24h after transfection. Results are represented as the percentage of the RLU measured for 312nM treatment of 3p10L (parental construct). Each symbol represents one data point. Structure and sequence of 3p10L and 3p10LG9 are as shown in the table. Statistical significance was determined using one-way ANOVA ($p=0.3699$).

RESULTS

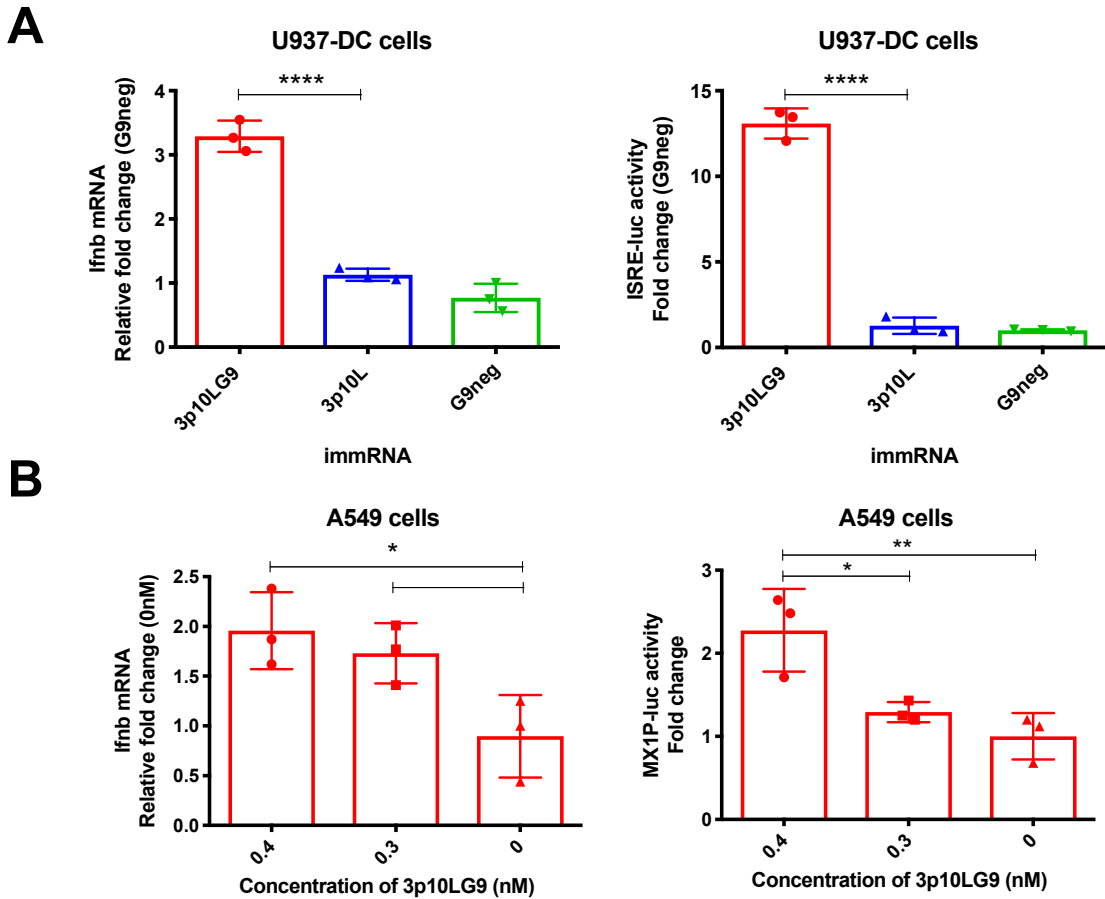


Figure 3.2: 3p10LG9 is a potent type I interferon activator in U937-DC and A549 human cell lines.

(A) U937-DC-SIGN cells were transfected with 10nM of 3p10L, 3p10LG9 or G9neg and harvested 24h after transfection for qPCR (left panel). Supernatant was incubated on HEK-293T cells containing a luciferase reporter driven by Interferon-stimulated Response Element (ISRE-luc) and luminescence was measured after 6h (right panel). Results are presented as fold change compared to G9neg (10nM). Each symbol shows a transfection done in triplicate from one experiment and bars show means \pm SD. Statistical significance was determined using a student T test (**** $P \leq 0.001$)

(B) A549 cells were transfected with 3p10LG9 and harvested 48h after transfection for qPCR (left panel). Supernatant was incubated on HEK-293T MX1P-luc cells and luminescence was measured after overnight incubation (right panel). Results are presented as fold-change compared to samples treated with transfection reagent alone. Each symbol shows transfections done in triplicate from one experiment and is representative of two independent experiments. Bars show means \pm SD. Statistical significance was determined using a student T test (* $P \leq 0.05$, ** $P \leq 0.01$)

RESULTS

3.1.2 Anti-viral effects of lead candidate immRNA 3p10LG9 in U937-DC and A549 human cell lines against DENV-2 infection

To determine if immRNA-induced type I interferon activation could protect human cells against DENV infection by activating anti-viral genes and signalling pathways, we transfected U937-DC-SIGN cells with different concentrations of 3p10L and 3p10LG9 and infected the cells with DENV-2 24h post-transfection. The percentage of infected cells was quantified by flow cytometry using E-protein and NS1 protein-specific fluorescently labelled antibodies to detect intracellular viral proteins. The supernatants of immRNA-transfected cells were transferred to luciferase-reporter HEK293T cells. In U937-DC cells, 3p10LG9 was observed to be approximately 4-fold more potent in inducing interferon-induced gene expression when compared to 3p10L, with the EC50 values being 4.78nM for 3p10LG9 and 15.1nM for 3p10L (** p=0.0013). Both 3p10LG9 and 3p10L were able to significantly reduce DENV infection in a dose-dependent manner. 3p10LG9 had an estimated EC50 of 3.78nM, about 4 times lower than the EC50 of 3p10L, which was 12.9nM (**** p<0.0001) (**Figure 3.3**). However, the EC50 for 3p10LG9 might be underestimated due to the plateau not being achieved at the lowest concentration tested. It is worth pointing out that the transfection of greater than 62nM of either immRNA for the U937-DC SIGN cells had reduced efficiency in inducing type I interferon activity as well as anti-viral effects. A similar trend was observed in A549 cells. 3p10LG9 had an EC50 of 0.27nM and 3p10L had an EC50 of 0.75nM in the DENV infection assay (**** p<0.0001), with luciferase EC50 values being 0.59nM and 1.74nM for 3p10LG9 and 3p10L respectively (** p=0.0058) (**Figure 3.4**) These results showed that immRNA have anti-viral effects preventing DENV-2 infection in human cell lines by inducing an anti-viral state as a result of the interferon signal and interferon-stimulated gene activation. 3p10LG9 was observed to be more potent in inducing this anti-viral state in human cell lines when compared to 3p10L.

RESULTS

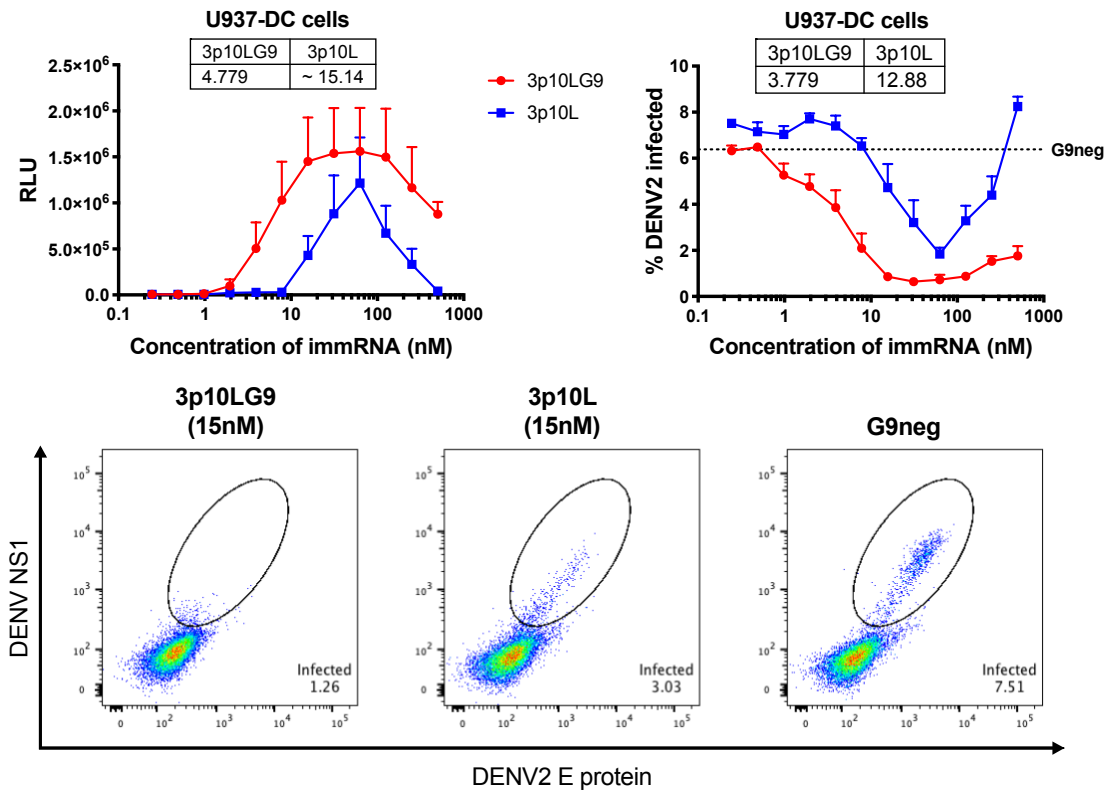


Figure 3.3: 3p10LG9 is effective as prophylaxis against DENV-2 infection in U937-DC-SIGN cells.

3p10L, 3p10LG9 or G9neg was transfected into U937-DC-SIGN. Supernatants were harvested and incubated on ISRE-luc HEK-293T reporter cells. Luminescence was measured 6h after incubation with the supernatant (left). Transfected cells were infected with DENV-2 (TSV01) at MOI 1 and stained for FACS with antibodies specifically binding NS1 and the E-protein (4G2) 24h after infection (right). Tables show EC₅₀ values (nM). Representative FACS plots for infected U937-DC-SIGN cells are shown below the graphs. Each symbol represents n=6 transfections done at various concentrations from two independent experiments. Bars show means \pm SEM. Statistical significance for luciferase assay (** $P=0.0013$) and infection (**** $P < 0.0001$) was determined using two-way ANOVA.

RESULTS

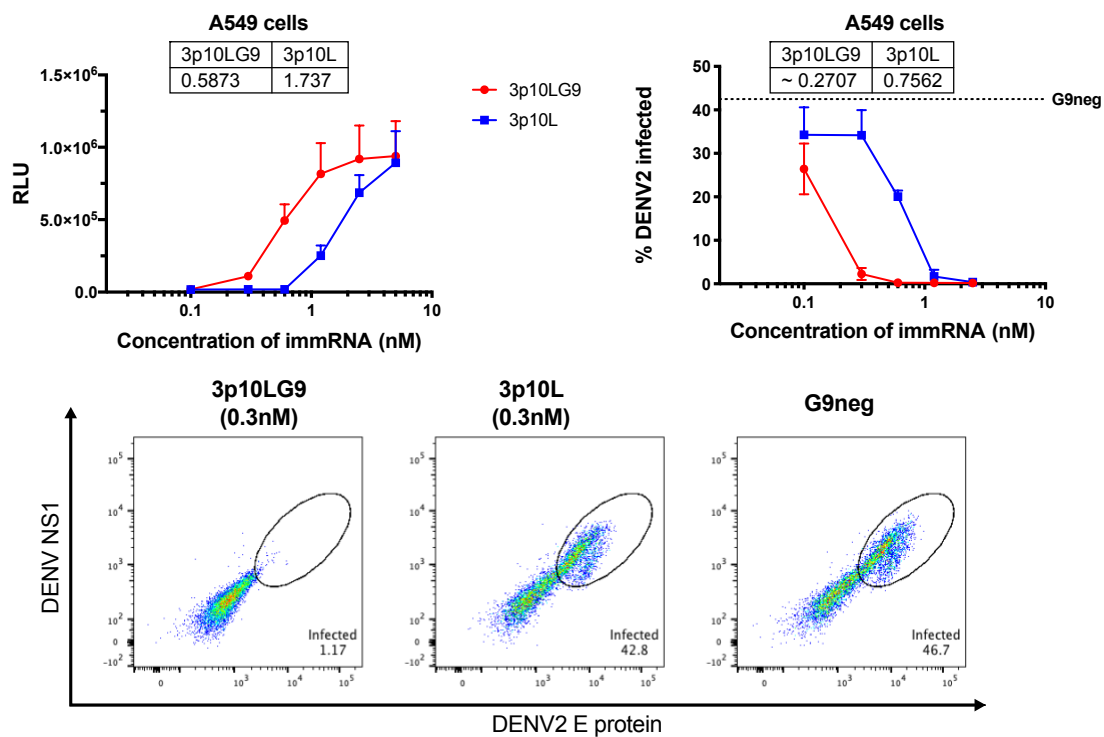


Figure 3.4: 3p10LG9 is effective as prophylaxis against DENV-2 infection in A549 cells.

3p10L, 3p10LG9 or G9neg was transfected into A549 cells. Luciferase and infection experiments were done as in Figure 3.3. Each symbol represents $n=4$ transfections done at various concentrations from two independent experiments. Bars show means \pm SEM. Statistical significance for the luciferase assay (** $P=0.0058$) and infection (**** $P < 0.0001$) was determined using two-way ANOVA.

RESULTS

3.1.3 Anti-viral effects of 3p10LG9 in human cell lines is RIG-I dependent

While it has been proposed that short hairpin RNA molecules bind to RIG-I and are unlikely to bind to MDA5, we wanted to test this experimentally. We generated RIG-I knockout (RIG-I KO) U937-DC-SIGN cells using CRISPR-cas9 mediated gene knockout with a gRNA designed to target exon 1 of human RIG-I (**Figure 7.2**). These RIG-I KO U937-DC cells were unable to upregulate ISG expression when transfected with 3p10LG9 or 3p10L, suggesting that interferon signalling was significantly impaired by the lack of RIG-I expressed in the RIG-I KO cells (** $P \leq 0.001$, **** $P \leq 0.0001$) (**Figure 3.5**). Using HEK293T ISRE-luciferase reporter cells, we measured type I interferon production in the presence or absence of RIG-I and found that 3p10LG9 activation of type I interferon signalling was dependent on RIG-I. When High or Low Molecular Weight (HMW or LMW) poly I:C was transfected using Hilymax (HM) into U937-DC cells, the effects on type I interferon signalling were similar to what was observed with the RIG-I agonists, suggesting that these TLR3 agonists also function in a RIG-I dependent manner in U937-DC cells (**Figure 3.5**). Free HMW poly I:C was unable to stimulate type I interferon production or reduce DENV replication in U937-DC cells, whereas TLR4 agonist Lipopolysaccharide (LPS) was able to do both (**Figure 7.1**). We also co-transfected the immRNA constructs with either RIG-I overexpressing plasmids or MDA5 overexpressing plasmids and found that 3p10LG9 activation of type I interferon signalling was enhanced by the overexpression of RIG-I and that this enhancement was greater when compared to overexpression of MDA5 (**Figure 3.6**). These results demonstrate that 3p10LG9 is a more potent inducer of type I interferon signalling compared to the parental construct 3p10L, and that interferon induction is dependent on the presence of RIG-I.

To determine if the anti-viral effects of these minimal immRNAs as observed previously in cell assays of DENV-infection (**Figure 3.3 and Figure 3.4**) are RIG-I dependent, we infected both parental U937-DC cells and RIG-I KO U937-DC cells that were prophylactically treated with either immRNA or poly I:C, and

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quantified the proportion of infected cells using flow cytometry. RIG-I KO U937-DC cells had a higher proportion of infected cells compared to the parental U937-DC cells. Pre-treatment with both minimal immRNAs (3p10LG9 and 3p10L) or poly I:C (LMW and HMW) could significantly inhibit DENV replication in the parental U937-DC cells (***P* ≤ 0.001) but not in RIG-I KO U937-DC cells (**Figure 3.7**). Although it is difficult to directly compare the efficacy given the large difference in the molecular weight of both agonists, it might be worth noting that 10nM of 3p10LG9 is equivalent to a concentration of about 0.08 ug/mL, and the concentration of poly I:C used in these experiments is 0.5 ug/mL. Overall, these results show that the anti-viral effects of 3p10LG9 is RIG-I dependent and this is mediated through the induction of type I interferon signalling.

RESULTS

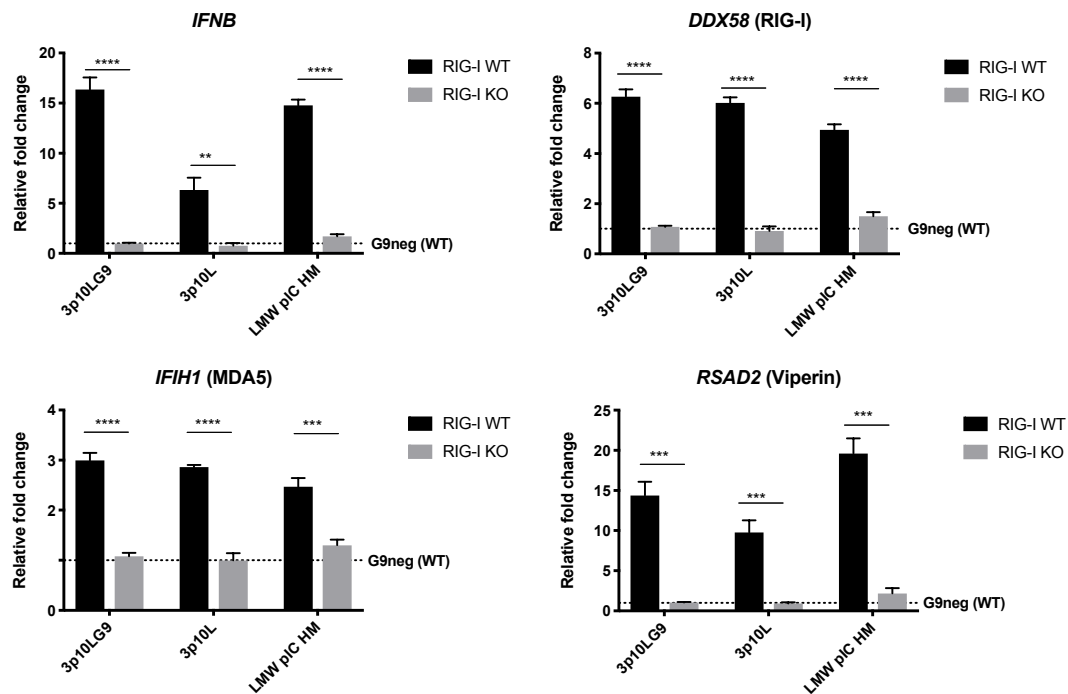


Figure 3.5: Interferon signalling activation induced by 3p10LG9 is RIG-I dependent.

3p10LG9, 3p10L, G9neg or LMW poly I:C was transfected into either RIG-I knock-out U937-DC cells (KO) or the parental U937-DC cells (WT). Gene expression analysis on mRNA extracted from transfected U937-DC cells was done for *IFNB1*, *DDX58* (RIG-I), *IFIH1* (MDA5) and *RSAD2* (Viperin). Data are represented as fold-change compared to the mean of G9neg treated RIG-I WT sample (dotted line). Bars show means \pm SD of triplicate transfections and data is representative of two independent experiments. Statistical significance was determined using a two-tailed student t-test (** $P \leq 0.001$, **** $P \leq 0.0001$).

RESULTS

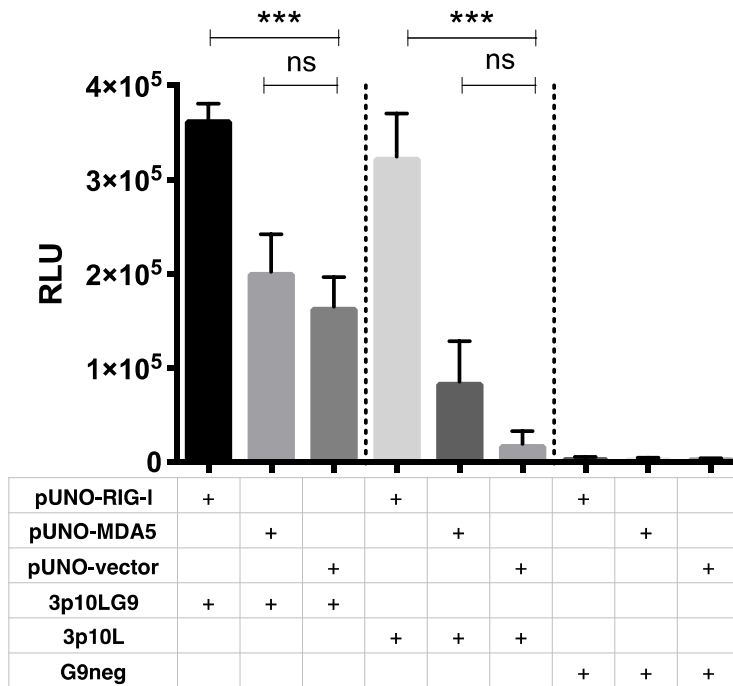


Figure 3.6: Overexpression of RLRs in HEK-293T cells with immRNA transfection

HEK-293T cells were transfected with 50ng of pUNO-hRIG-I or pUNO-hMDA5. These cells were then transfected with 10nM of either 3p10LG9, 3p10L or G9neg. Supernatant from transfected HEK-293T cells was incubated on the HEK-293T cells that contain a luciferase reporter driven by Interferon-Stimulated Response Element (ISRE-luc). Luminescence was measured 6h after incubation with the supernatant. Bars show means \pm SD of triplicate transfections from one experiment. Statistical significance was determined using two-tailed student t-test (** $P \leq 0.001$, ns, not significant).

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3.1.4 Anti-viral effects of 3p10LG9 in human cell lines is type I interferon dependent

To determine if the anti-viral effects observed with 3p10LG9 are type I interferon dependent, we transfected U937-DC cells with 3p10LG9 and used a IFNAR blocking antibody to prevent innate immune activation through type I interferon that is produced in response to RIG-I signalling. In the presence of anti-IFNAR antibody, type I interferon responses to 3p10LG9 was blocked whereas the type I interferon signalling was still present when treated with the isotype control (**Figure 3.8 A**). Anti-IFNAR blocking antibody also abolished the anti-viral effects of 3p10LG9 as DENV-2 replicated as effectively as G9neg-treated U937-DC cells when IFNAR was blocked (**Figure 3.8 B**). Through this we showed that the anti-viral effects observed in U937-DC cells with 3p10LG9 is type I interferon dependent.

RESULTS

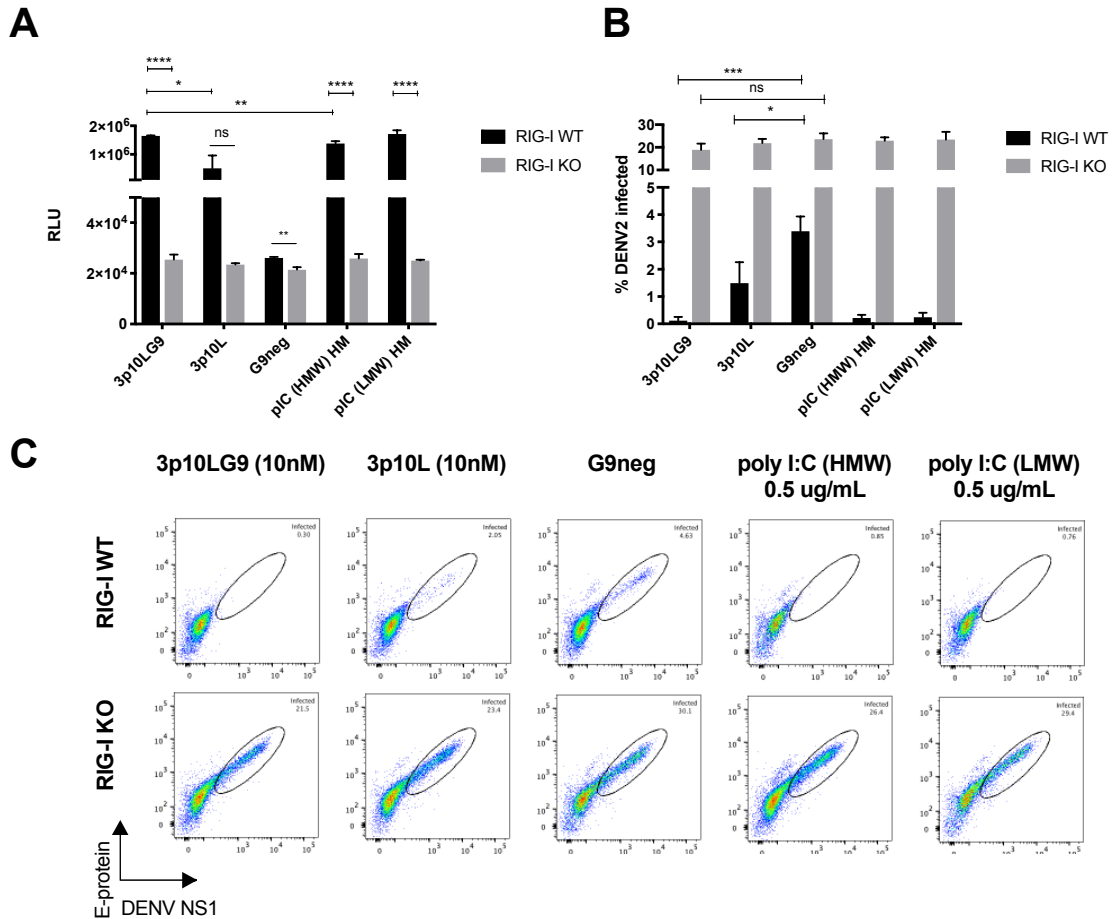


Figure 3.7: Anti-viral effects of 3p10LG9 are RIG-I dependent.

(A) Supernatant from U937-DC cells transfected with either immRNA or poly I:C was incubated on the HEK-293T cells that contain a luciferase reporter driven by Interferon-Stimulated Response Element (ISRE-luc). Luminescence was measured 6h after incubation with the supernatant. Bars shown means \pm SD of triplicate transfections and data is representative of two independent experiments. Statistical significance was determined using a two-tailed student t-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

(B) U937-DC cells pre-treated with either immRNA or poly I:C for 24h were infected with DENV-2 TSV01 (MOI-1). Infected viable cells were quantified using flow cytometry with antibodies targeting NS1 and the E-protein fusion loop (4G2) 24h after infection. Bars shown means \pm SD of triplicate transfections and data is representative of two independent experiments. Statistical significance between the treatment methods within each cell type was determined using a two-tailed student t-test (* $P \leq 0.05$, *** $P \leq 0.001$).

(C) Representative FACS plots of viable U937-DC cells stained with antibodies targeting NS1 and the E-protein (4G2).

RESULTS

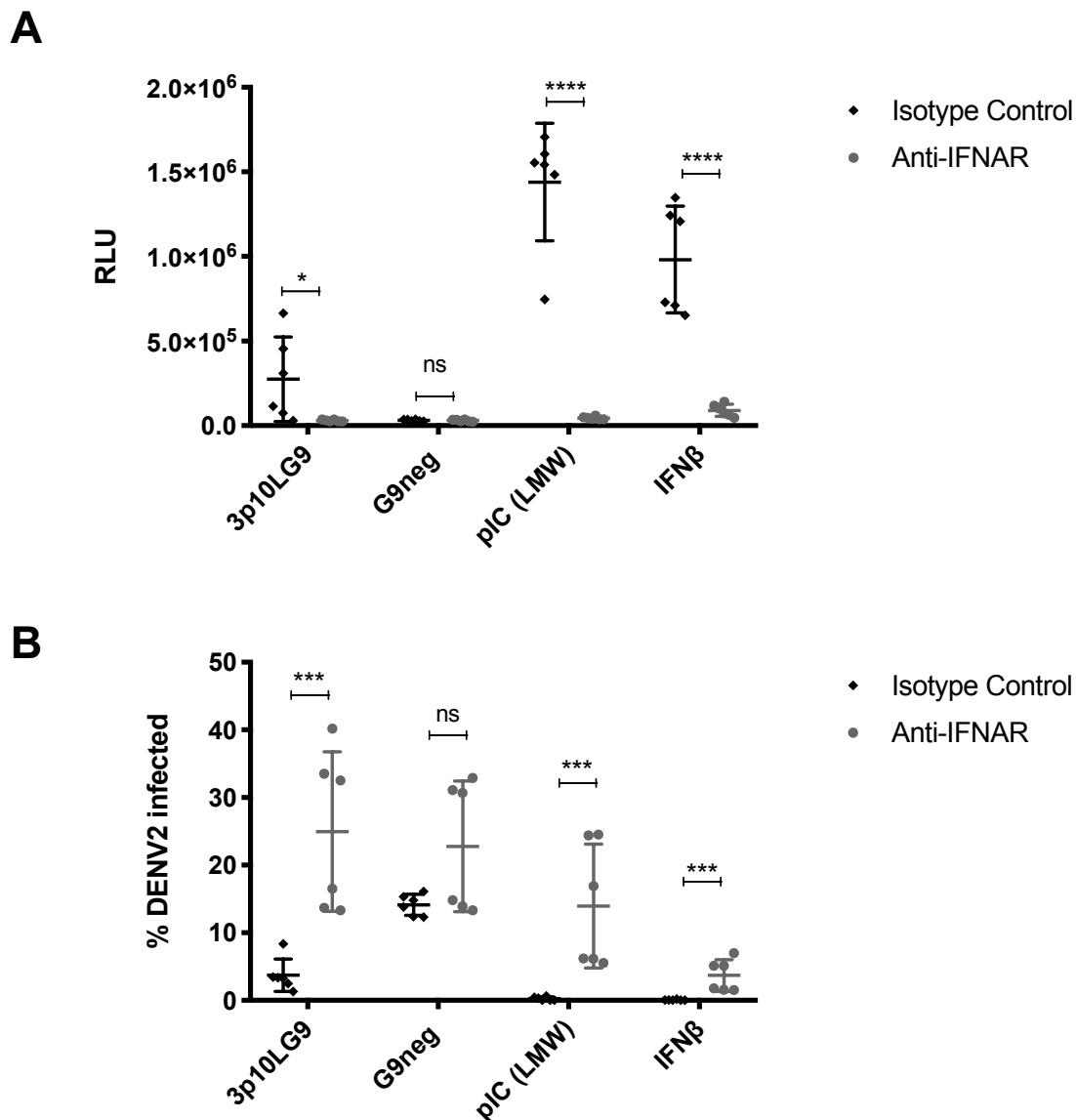


Figure 3.8: Anti-viral effects of 3p10LG9 are type I interferon dependent

(A) U937-DC cells were transfected with immRNA, poly I:C or treated with IFN β for 6 hours before the addition of 10 μ g/mL of either anti-IFNAR blocking antibody or isotype control. Supernatant was collected after overnight incubation and was incubated on the HEK-293T cells that contain a luciferase reporter driven by Interferon-Stimulated Response Element (ISRE-luc). Luminescence was measured 6h after incubation with the supernatant. Bars shown means \pm SD of triplicate transfections from two independent experiments. Statistical significance was determined using two-tailed student t-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.0001$).

(B) U937-DC cells in (A) were infected with DENV-2 TSV01 (MOI-1). Infected viable cells were quantified using flow cytometry with antibodies targeting NS1 and the E-protein fusion loop (4G2) 24h after infection. Bars shown means \pm SD of triplicate transfections from two independent experiments. Statistical significance was determined using two-tailed student t-test (*** $P \leq 0.001$, **** $P \leq 0.0001$).

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3.2 Studying the potential of 3p10LG9 as an anti-viral prophylactic using a human skin infection model

3.2.1 Efficient uptake of immRNA by dermal dendritic cells and Langerhans cells in skin

DENV infection of immune cells found in the skin suggests that these immune cells could play an important role in the systemic spread of DENV as they travel from the site of infection to secondary lymphoid organs such as lymph nodes (Cerny et al., 2014; Schmid et al., 2014). To test whether immRNA could block infection in primary human skin cells, healthy skin samples were processed to prepare single cell suspensions that could be used for transfection with immRNA and infection. We have previously described the method of skin cell preparation and infection with DENV (Cerny et al., 2014). Using flow cytometry, we are able to distinguish macrophages (MP), CD14 DDCs, CD11c DDCs, CD141 DDCs and LCs amongst the various skin cells (**Figure 3.9**). Three of these populations of skin cells, CD11c DDCs, CD14 DDCs and LCs, were susceptible to DENV infection similar to what was described previously (Cerny et al., 2014) (**Figure 3.10 B**). We initially tested which cells are most efficiently transfected with immRNA using a fluorescently labelled version of 3p10LG9 (3p10LG9-RED) that can be traced by flow cytometry. All cell types were transfected but uptake was most efficient in CD14 DDCs, followed by CD11c DDCs and LCs, with CD141 DDCs showing the least efficient uptake. When immRNA-RED was added to the cells without transfection reagent the uptake was minimal, demonstrating that immRNA uptake by phagocytosis was minimal (**Figure 3.10 A**).

3.2.2 Increased expression of interferon-stimulated genes (ISGs) in skin cells after immRNA transfection

To test the ability of immRNA to activate RIG-I in primary human skin cells, we transfected them with immRNA and determined if ISGs were upregulated as a result. In response to 3p10LG9 transfection, type I interferon response genes

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such as *IFNB* and *DDX58* (RIG-I) were found to be increased at least 7-fold when compared to G9neg treated skin cells. Genes involved in inducing an anti-viral state in cells, such as *CXCL10*, *RSAD2* (Viperin) and *OASL* were also observed to be upregulated in human skin cells upon 3p10LG9 transfection. Only a slight upregulation in the expression of anti-viral genes was observed in 3p10L-transfected human skin cells (**Figure 3.11**). Supernatant from 3p10LG9 transfected skin cells were able to induce type I interferon activity in ISRE-Luc reporter HEK293T cells, unlike in the case of 3p10L transfected cells, which showed nearly undetectable changes in interferon activity compared to G9neg treated cells (**Figure 3.12 A**). These data show that 3p10LG9 efficiently primes a type I interferon response in primary human skin cells through the upregulation of ISGs.

RESULTS

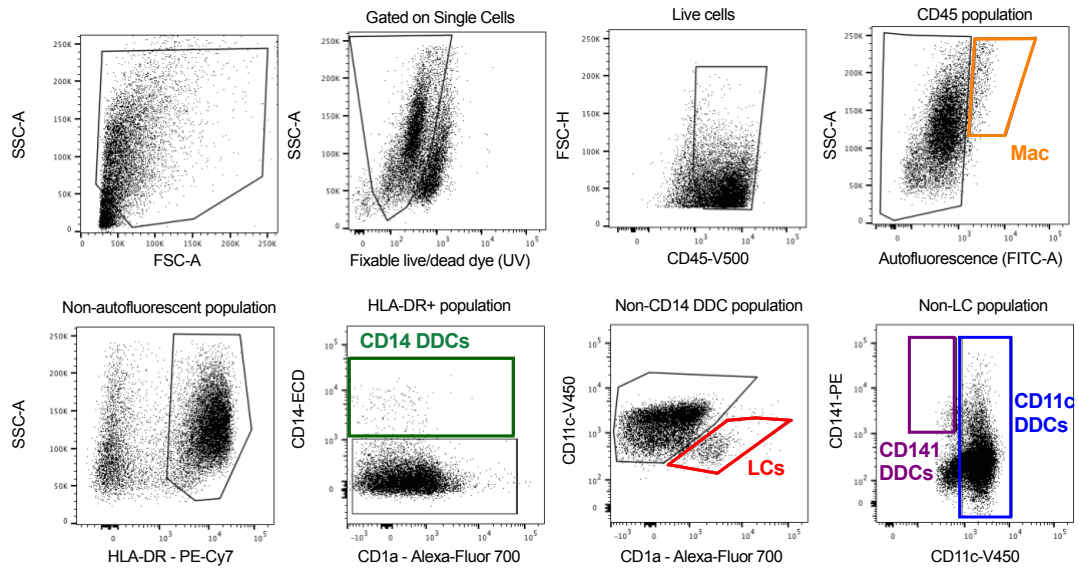


Figure 3.9: Gating strategy to identify primary human skin DC subsets

Gating strategy to identify human skin DC subsets taken from healthy human skin tissue after collagenase digestion. Subsets identified are Macrophages (Mac, orange), CD14+ dermal dendritic cells (CD14 DDCs, green), Langerhans cells (LCs, red), CD11c+ dermal dendritic cells (CD11c DDCs, blue) and CD141+ dermal dendritic cells (CD141 DDCs, purple).

RESULTS

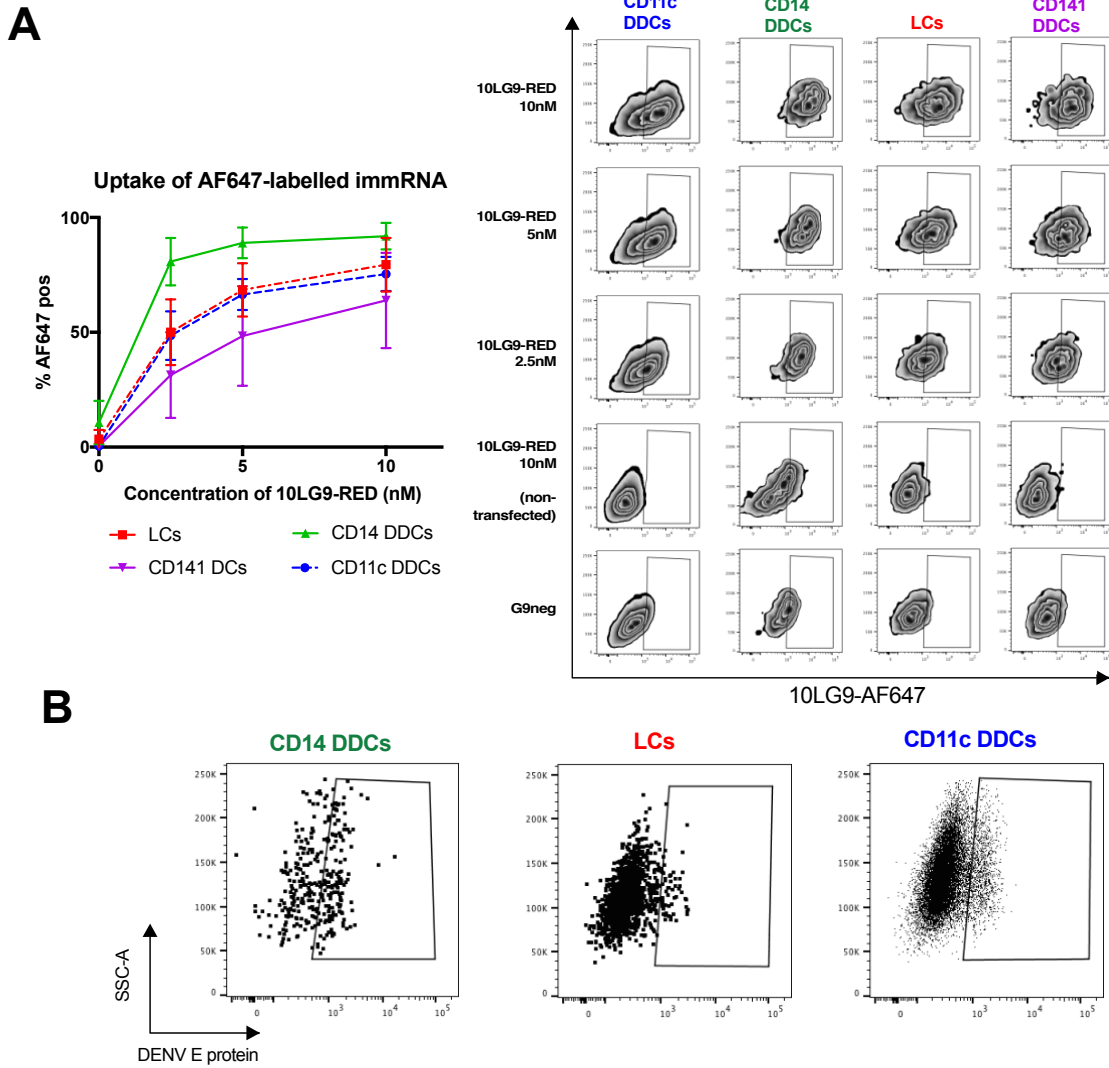


Figure 3.10: immRNA uptake and DENV replication in primary human skin DC subsets

(A) Healthy human skin cells were transfected with various concentrations of 3p10LG9 labelled with Alexa-Fluor 647 (10LG9-RED). Cells were analysed using flow cytometry 24h post-transfection. Data shown was collected from $n=3$ donors. Statistical significance between the different cell types with varying concentrations of 3p10LG9-red was calculated using repeated measures two-way ANOVA (** $p=0.0026$).

(B) Healthy human skin cells were infected with DENV-2 D2Y98P (MOI 5) in a single-cell suspension for 48h. Infected viable cells were quantified using an antibody targeting E-protein (4G2).

RESULTS

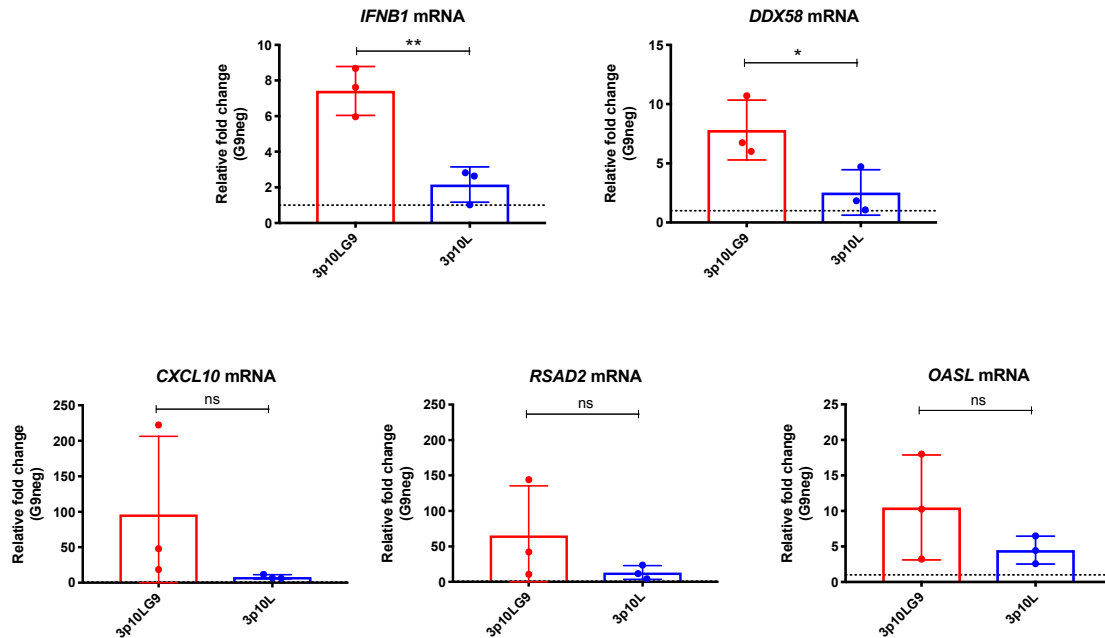


Figure 3.11: immRNA primes the innate immune response in primary human skin DCs through increased expression of interferon-stimulated genes (ISGs)

Gene expression analysis with mRNA extracted from single-cell suspension of primary human skin cells transfected with either 3p10LG9, 3p10L or G9neg was done for *IFNB1*, *DDX58*, *CXCL10*, *RSAD2* and *OASL*. Data is represented as fold-change compared to G9neg treated sample (dotted line). Each point represents a single donor (n=3). Each donor was tested in an independent experiment. Bars show means \pm SD. Statistical significance was determined using a two-tailed student T-test (* $P \leq 0.05$, ** $P \leq 0.01$)

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3.2.3 3p10LG9 is a more potent prophylactic against DENV compared to the parental 3p10L in skin DC

To determine if APCs isolated from human skin were protected from DENV infection, similar to cell lines, we treated skin single cell suspensions with 250nM, 125nM and 62nM of 3p10LG9, 3p10L and G9neg. After 24h we infected the cells with DENV at an MOI of 5. 48h after infection we stained the cells for flow-cytometry-based quantification of infected skin APCs using fluorescently labelled antibodies against E-protein as described previously (Cerny et al., 2014). We observed that prophylactic treatment of human skin APCs with immRNA protected the cells from DENV infection in a dose-dependent manner. 3p10LG9 was more potent compared to 3p10L, having a lower EC50 value in all three subpopulations of APCs studied (**Figure 3.12 B**). At the lowest concentration tested (62nM), 3p10LG9 was observed to be able to potently reduce the number of DENV-2 infected cells in all three dermal DC subsets, with 3p10LG9 significantly more effective than 3p10L in reducing the number of infected CD11c DDCs and Langerhans cells (** $P \leq 0.05$). (**Figure 3.13**). These results showed that pre-treatment of human skin DCs with immRNA 3p10LG9 and 3p10L were able to activate anti-viral signalling responses through type I interferon activation in subsets of DCs susceptible to DENV infection.

RESULTS

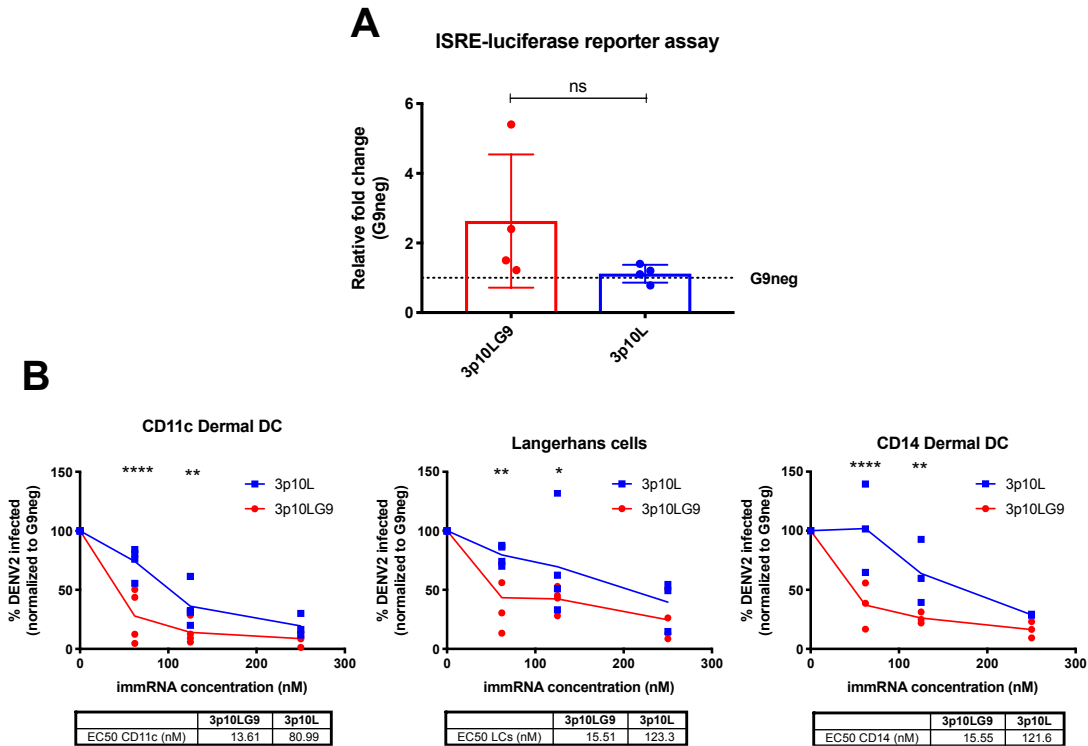


Figure 3.12: 3p10LG9 has a higher efficacy as a prophylaxis against DENV-2 infection compared to 3p10L in primary human skin DC subsets.

(A) Supernatant from primary human skin cells transfected with 62nM of either 3p10LG9, 3p10L or G9neg was incubated on the HEK-293T cells that contain a luciferase reporter driven by Interferon-Stimulated Response Element (ISRE-luc). Luminescence was measured 6h after incubation with the supernatant. Fold change was determined relative to G9neg treated cells (dotted line). Data shown are from n=4 donors. Bars show means \pm SD. Statistical significance was determined using a student's t-test (ns: not significant).

(B) Primary human skin cells were transfected with 250nM, 125nM and 62nM of 3p10LG9 or 3p10L and infected with DENV-2 at MOI 5 for 48h. Flow cytometry was used to quantify the percentage of DENV-2 infected cells in each subpopulation of skin DCs by intracellular staining with 4G2 antibody. Each dot on the graph represents one reading from one donor measured in independent experiments. Statistical significance ($p < 0.05$) was determined using a two-way ANOVA with Dunnett's multiple comparisons test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$)

RESULTS

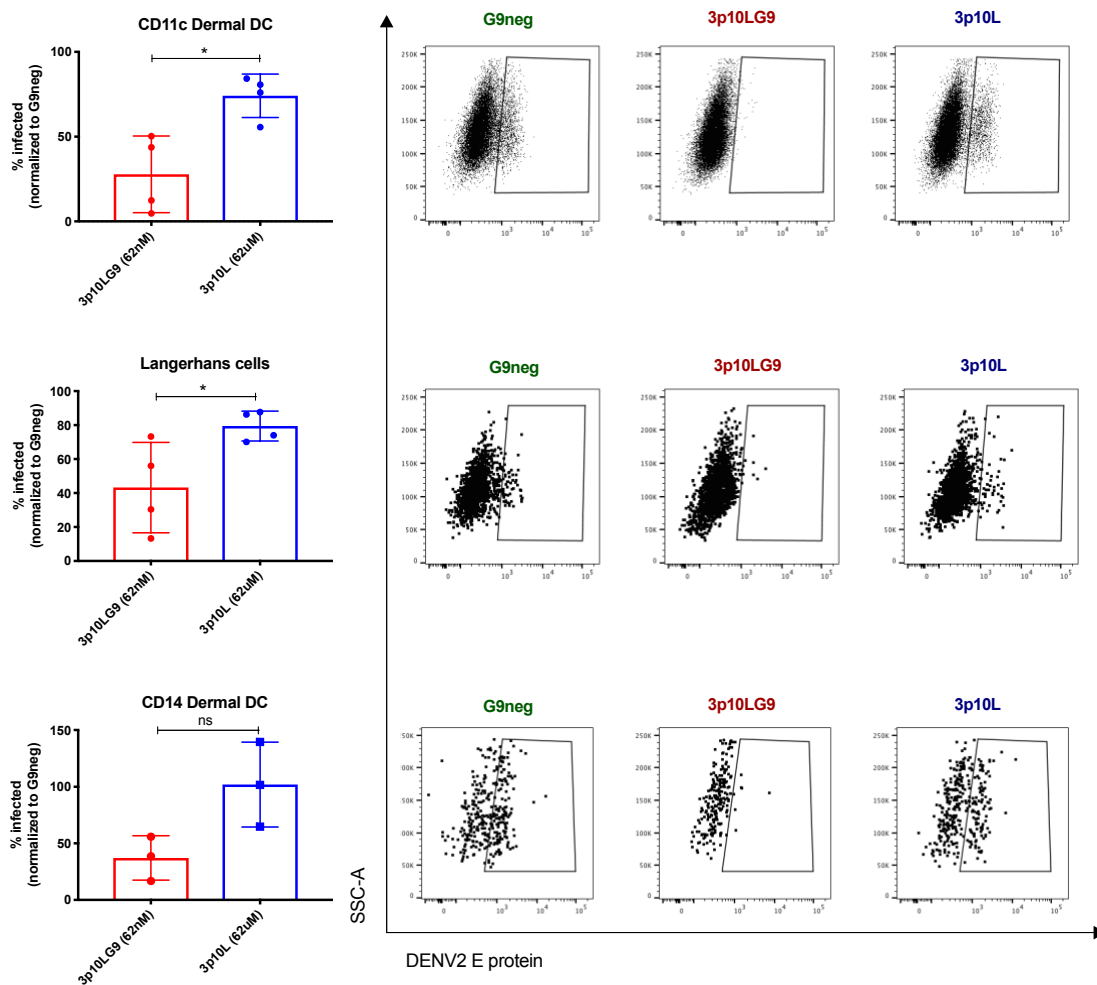


Figure 3.13 3p10LG9 has a higher efficacy as a prophylaxis against DENV-2 infection compared to 3p10L in primary human skin DCs at lowest concentration tested.

Percentage of infected cells for CD11c DDCs, LCs and CD14 DDCs was taken from the data shown in Figure 3.12 after transfection with 62nM of 3p10LG9 or 3p10L (left), with the percentage of cells infected for each condition normalized to G9neg.. Representative FACS plots are shown on the right. Each dot on the graph represents one reading from one donor measured in independent experiments. Bars show means \pm SD. Statistical significance ($p < 0.05$) was determined using a two-tailed student T-test (* $P \leq 0.05$, ns: not significant)

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3.3 Studying the potential of 3p10LG9 as an anti-viral therapeutic using a human skin infection model

3.3.1 3p10LG9 has modest therapeutic effects against DENV in skin DC

To determine if immRNA can act as a therapeutic for DENV infected skin APCs, we infected skin single cell suspensions with DENV at MOI 5 and then treated these cells with 62nM of 3p10LG9 at various time points after infection. Cells were stained 48h later for flow-cytometry-based quantification of infection. Interestingly, 3p10LG9 significantly reduced the percentage of infected cells only in LCs, and only at early time points of 4h and 6h post-infection (* $P \leq 0.05$). Similarly, treating the skin DCs with IFN β for 4h post-infection as a positive control resulted in a reduction of infected LCs, but not the other cell types (**Figure 3.14 B**). Modest therapeutic effects were seen in the CD11c DDCs (**Figure 3.14 A**) and CD14 DDCs (**Figure 3.14 C**) at the early timepoints, though these results were not statistically significant. These results suggest that there might be differential responses to type I interferon signalling between the various DENV-susceptible skin DC subsets, and minimal immRNA 3p10LG9 is only able to induce a modest therapeutic response in LCs.

RESULTS

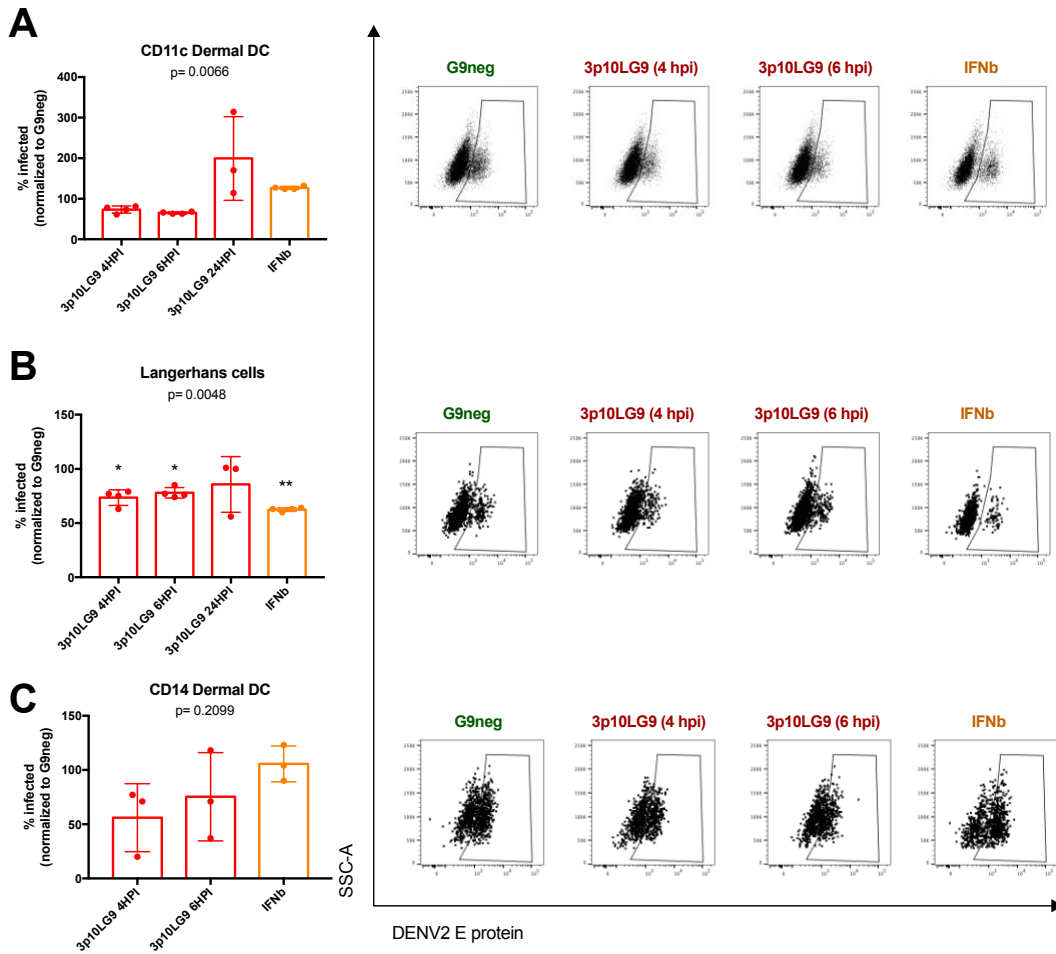


Figure 3.14: 3p10LG9 has modest therapeutic effect against DENV-2 infection in primary human skin APCs.

(A-C) Human skin DCs were infected with DENV-2 at MOI 5 and transfected with 62nM of 3p10LG9, 3p10L or G9neg at the indicated time points post-infection. Summary of the percentage of infected cells within each subpopulation of skin DCs for multiple donors (left) and representative FACS plots (right). Percentages of cells infected were normalized to G9neg control for each donor and the respective condition for (A) CD11c DDCs, (B) LCs, (C) CD14 DDCs. Each dot on the graph represents one reading from one donor measured in independent experiments. Bars show means \pm SD. Statistical significance was determined using a one-way ANOVA with multiple comparisons (* $P \leq 0.05$, ** $P \leq 0.01$). HPI: Hours post-infection

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3.4 Studying the potential of 3p10LG9 as an anti-viral prophylactic using an *in vivo* mouse model

3.4.1 3p10LG9 activates type I interferon signalling, but has no prophylactic effects against DENV in IFNAR conditional knockout mouse models

To test the effects of 3p10LG9 in an *in vivo* model, 3p10LG9 was mixed with a cationic polymer delivery system (in vivo-jetPEI) and injected intravenously (I.V) into conditional knockout IFNAR^{ff} mice crossed with either CD11-cre mice (CD11c-cre IFNAR^{ff}) or LysM-cre mice (LysM-cre IFNAR^{ff}) to delete IFNAR expression primarily on dendritic cells and monocytes/macrophages, respectively. Linehan *et al.* had previously determined that the optimal amount used to inject wild-type C57Bl/6 mice was 25ug (Linehan et al., 2018). Thus we decided to use this amount to start initial experiments to determine the efficacy of 3p10LG9 in our *in vivo* model, given that 3p10L and 3p10LG9 are not too different in size. In both mouse models, we observed that a significant increase in type I interferon activity was detected in both CD11c-cre IFNAR^{ff} (***) $P \leq 0.001$) and LysM-cre IFNAR^{ff} mice (* $P \leq 0.05$) treated with 3p10LG9 both and not with G9neg using a ISRE-luciferase mouse reporter cell line LL171. The plasma concentration of type I interferon was approximately 50 pg/mL in CD11c-cre IFNAR^{ff} mice (**Figure 3.15 B**) and 700 pg/mL in LysM-cre IFNAR^{ff} mice (**Figure 3.16 B**). However, when challenged with DENV-2 (D2Y98P), 3p10LG9 pre-treatment did not confer any protective effects compared to G9neg pre-treated mice in both CD11c-cre IFNAR^{ff} mice (infected with 10^6 pfu per mouse) and LysM-cre IFNAR^{ff} mice (infected with 10^5 pfu per mouse) when measuring viremia in the blood 3 days post-infection (**Figure 3.15 C and Figure 3.16 C**). No significant differences were observed for survival and weight loss between the 3p10LG9 pre-treated and G9neg pre-treated mice for both conditional IFNAR knockout mouse models (**Figure 3.15 D and E**) (**Figure 3.16 D and E**). These data suggest type I interferon activation in mice were treated with 3p10LG9 was

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insufficient to confer protection to DENV challenge in either CD11c-cre IFNAR^{ff} mice or LysM-cre IFNAR^{ff} mice in the conditions tested.

3.5 Studying the potential of 3p10LG9 as a vaccine adjuvant using a human skin DC model and conditional IFNAR knockout mouse models

3.5.1 3p10LG9 increases expression of co-stimulatory molecule CD80 on CD11c dermal dendritic cells and Langerhans cells in human skin

APCs are activated by pathogens such as viruses. The activation of T cells by activated APCs involves the binding of a cognate T-cell receptor (TCR) with a peptide antigen presented on surface MHC class I and class II molecules of the activated APC. However, this is not enough to activate the naïve T-cell and a second activation signal is necessary. This second signal is sent to naïve T-cells through the interaction of CD28 on T cells with co-stimulatory molecules expressed on the surface of the APC. Examples of co-stimulatory molecules are two transmembrane glycoproteins called CD80 and CD86, and high expression of these proteins, along with upregulated MHC molecules, are hallmarks of mature APCs. To determine if 3p10LG9 has any effects in promoting the maturation of APCs, we transfected primary human skin DCs with 250nM of 3p10LG9 and compared the expression levels of CD80 with G9neg-treated or IFN β treated skin DCs using flow cytometry. Treatment with 3p10LG9 increased the MFI of CD80 in CD11c DDCs and LCs (* $P \leq 0.05$) (**Figure 3.17**). The addition of recombinant IFN β had no significant effects in increasing the MFI of CD80 in any of the skin DC subsets. These results show that 3p10LG9 might promote the maturation of CD11c DDCs and LCs.

RESULTS

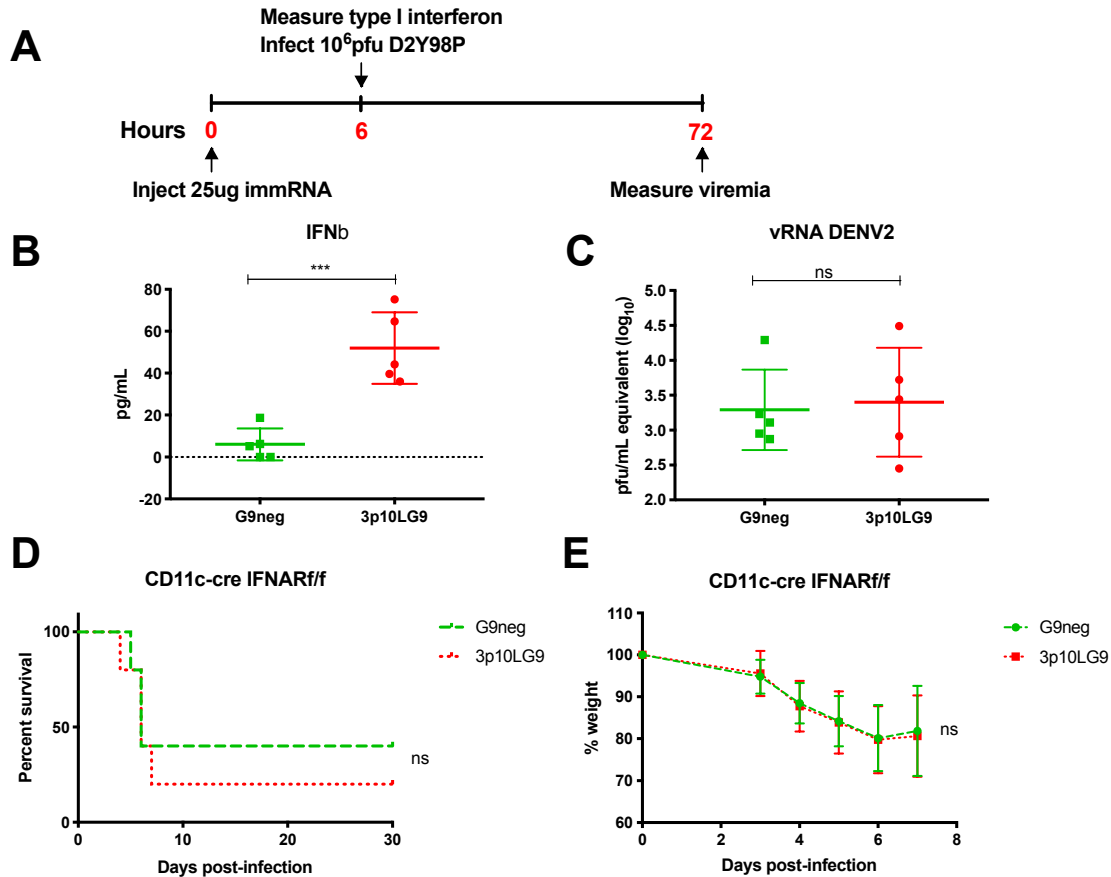


Figure 3.15: 3p10LG9 induces type I interferon signalling in CD11c-cre IFNAR^{fl/fl} conditional knockout mice without having any prophylactic effects against DENV replication

25ug of 3p10LG9 or G9neg was mixed with a cationic polymer delivery system (in vivoJET PEI) and injected intravenously into CD11c-cre IFNAR^{fl/fl} mice. Data was obtained from a single experiment (n=5 per group).

(A) Treatment and challenge schedule for prophylactic experiments.

(B) Plasma from mice obtained 6h post-treatment was incubated on ISRE-luc reporter LL171 mouse cell line and luciferase readout was obtained and plotted against a standard curve of known IFN β concentrations. Bars show means \pm SD. Statistical significance was determined using a two-tailed student's t test (***) $P \leq 0.001$.

(C) Viral RNA (vRNA) was extracted from plasma of immRNA pre-treated mice infected with 10^6 pfu of DENV-2. Taqman assay was used to determine the concentration of vRNA in the plasma. Bars show means \pm SD. Statistical significance was determined using a two-tailed student's t test (ns: not significant).

(D) Survival curves were generated using the Kaplan-Meier method and significance of differences was calculated by using a log rank test. (ns; not significant)

(E) Weights were measured over a period of 7 days post-infection and plotted as % of initial weight. Mice with more than 20% weight loss were considered moribund and were euthanized. Bars show means \pm SD. Statistical significance ($p < 0.05$) was determined using a two-way ANOVA. (ns; not significant).

RESULTS

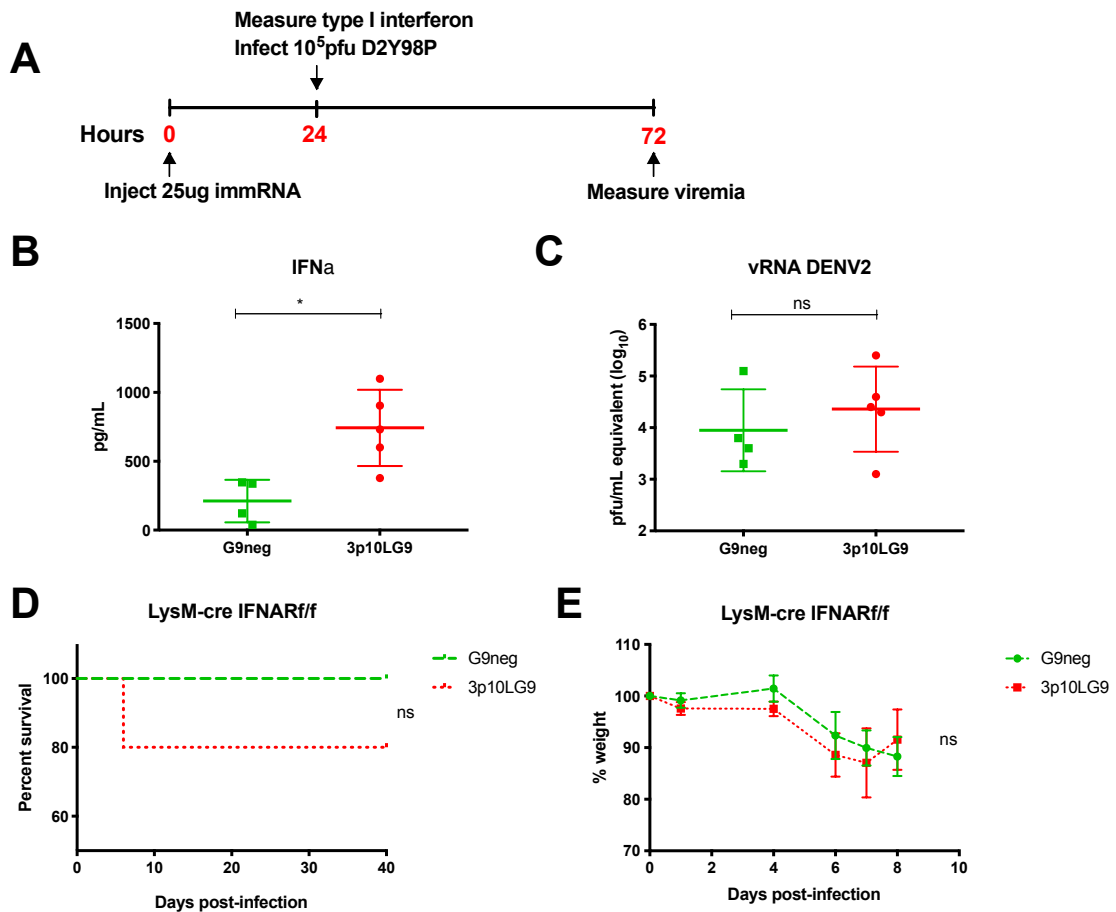


Figure 3.16: 3p10LG9 induces type I interferon signalling in LysM-cre IFNAR^{ff} conditional knockout mice without having any prophylactic effects against DENV replication

25ug of 3p10LG9 or G9neg was mixed with a cationic polymer delivery system (in vivoJET PEI) and injected intravenously into LysM-cre IFNAR^{ff} mice (n=5 per group). Data was obtained from a single experiment (n=4-5 per treatment).

(A) Treatment and challenge schedule for prophylactic experiments.

(B) Plasma from mice obtained 6h post-treatment was incubated on ISRE-luc reporter LL171 mouse cell line and luciferase readout was obtained and plotted against a standard curve of known IFN α concentrations. Bars show means \pm SD. Statistical significance was determined using a two-tailed student's t test (* P \leq 0.05).

(C) Viral RNA (vRNA) was extracted from plasma of immRNA pre-treated mice infected with 10^5 pfu of DENV-2. Taqman assay was used to determine the concentration of vRNA in the plasma. Bars show means \pm SD. Statistical significance was determined using a two-tailed student's t test (ns; not significant).

(D) Survival curves were generated using the Kaplan-Meier method and significance of differences was calculated by using the log rank test. (ns; not significant)

(E) Weights were measured over a period of 8 days post-infection and plotted as % of initial weight. Mice with more than 20% weight loss were considered moribund and were euthanized. Bars show means \pm SD. Statistical significance (p<0.05) was determined using a two-way ANOVA. (ns, not significant).

RESULTS

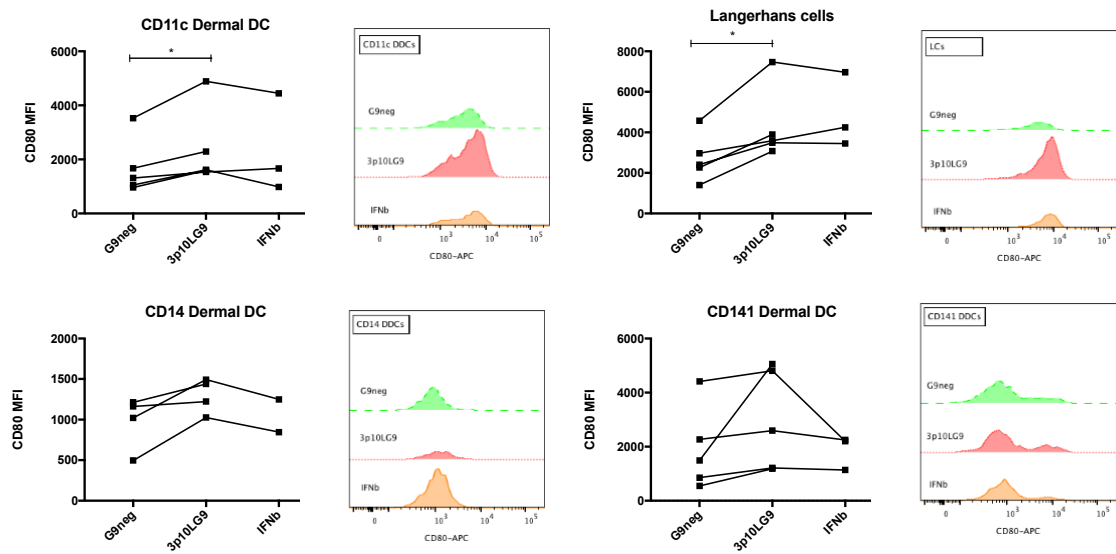


Figure 3.17: 3p10LG9 increases expression of CD80 in primary human skin DC subsets.

Primary human skin cells were transfected with 250nM of 3p10LG9 or G9neg, or treated with 1000U of recombinant human IFN β . After 72h, the mean fluorescent intensity (MFI) of CD80 expression in each subpopulation of skin DCs was measured. Each connecting line represents a single donor (n=4). Statistical significance was determined using a paired two-tailed student's t test (* P \leq 0.05).

RESULTS

3.5.2 3p10LG9 as a vaccine adjuvant enhances antibody production in vaccinated mice

To study the potential for 3p10LG9 as a vaccine adjuvant, we injected 8-10 week old wild-type C57BL/6 mice with monomeric E-protein together with 3p10LG9 or G9neg mixed with a cationic polymer using a prime/boost vaccination schedule. We found that 3p10LG9 was able to induce greater antibody titres against the monomeric E-protein immunogen when compared with G9neg, and antibody production levels were similar to TLR3 agonist poly I:C (**Figure 3.18 B**). This difference in the induction of DENV-2 E-protein antibodies between 3p10LG9 and G9neg was significant at day 21 and day 28 post-immunization (***) $P \leq 0.001$) (**Figure 3.18 C**). This suggests that 3p10LG9 could have potential to be used as an adjuvant for dengue vaccination. However, when flow-cytometry based DENV neutralisation assay was done for plasma that was taken 35 days post-vaccination, no neutralisation of virus was observed (**Figure 3.18 D**).

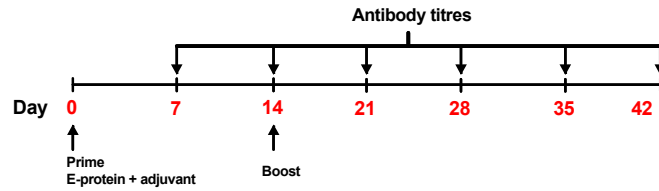
Given the suggestion that VLPs can be used as a potential dengue vaccine strategy, we decided to use VLPs mixed with 3p10LG9 to determine the potential of 3p10LG9 as a vaccine adjuvant. To be able to do a direct challenge in an *in vivo* model, we used the CD11-cre IFNAR^{ff} mouse model to do our vaccination experiments. For this experiment, we used a prime/boost schedule, with a single booster injection given 14 days after initial priming, and challenged the mice 14 days later with D2Y98P (DENV-2) (**Figure 3.19 A**). Viremia was significantly reduced in mice that received the VLP with 3p10LG9 compared to mice that received the VLP alone or with G9neg. Poly I:C adjuvant had highly variable results, showing a non-significant reduction in viremia compared to mice receiving the VLP alone (**Figure 3.19 B**). When measuring levels of antibody in a DENV-2 ELISA assay (with whole virus coating), all three adjuvants showed increases in antibody titres that can bind to DENV when compared to adjuvant alone. Interestingly, even G9neg adjuvant resulted in an increase in anti-DENV antibody titres (**Figure 3.19 C**). A similar trend was observed with the neutralization assay done with plasma obtained at day 28 post-vaccination.

RESULTS

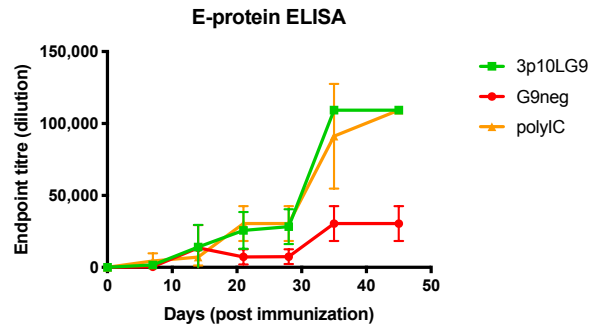
However, the differences in the EC50 values were not significant (**Figure 3.19 D**). When looking at the weight loss and survival data, only 3p10LG9 showed significant protection against DENV infection, with all mice surviving the challenge by DENV-2. G9neg and poly I:C had modest effects in protection, with one mouse in each treatment group not surviving the challenge (**Figure 3.19 E**). 3p10LG9-treated mice had no significant weight loss ($p=0.5173$, ns) at 5 days post-challenge. G9neg (** $P=0.0021$) and poly I:C (* $P=0.0420$) showed significant weight loss but still less compared to VLP alone treated mice (**** $P=0.0001$) (**Figure 3.19 F**). However, all three conditions improved the protection of mice to DENV-2 challenge when compared to the non-adjuvanted VLP vaccination, with all mice succumbing to DENV challenge (**Figure 3.19 E and F**). Although preliminary, these results suggest that 3p10LG9 could potentially be utilized as an adjuvant when coupled with a DENV VLP as an immunogen.

RESULTS

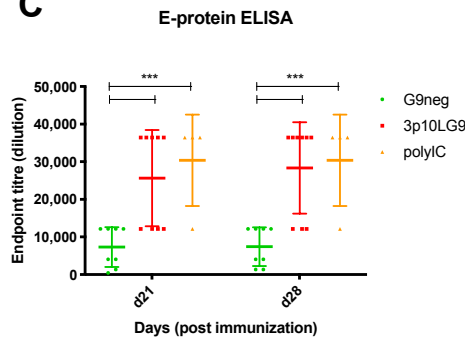
A



B



C



D

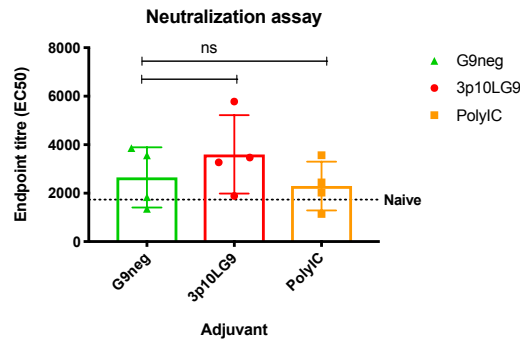


Figure 3.18: 3p10LG9 as an adjuvant is able to enhance antibody production in wild-type C57BL/6 mice

(A) Immunization schedule for vaccination experiments. 8-10 week old C57BL/6 mice were injected intramuscularly (I.M) with 5ug of monomeric DENV-2 E-protein along with either 50ug of poly I:C; or 25ug of 3p10LG9 or G9neg coupled with a cationic polymer delivery system (in vivoJET PEI). Mice were bled on days 7,14,21, 28, 35 and 42 post-immunization and the plasma was used for E-protein ELISA.

(B) Endpoint titre DENV-2 E-protein ELISA with plasma from DENV-2 E-protein-vaccinated mice. Data was obtained from a single experiment (n=4 per treatment). Bars show means \pm SD. Statistical significance was determined using a two-way ANOVA with multiple comparisons (**** $P \leq 0.0001$).

(C) Endpoint titre DENV-2 E-protein ELISA with plasma from mice at 21 and 28 days post-immunization. Data was obtained from two independent experiments (n=9 per treatment). Bars show means \pm SD. Statistical significance was determined using a two-tailed student T-test (***) $P \leq 0.001$

(D) Plasma from day 35 post-immunization was used for flow-cytometry-based U937-DC neutralization assay with DENV-2. Data was obtained from a single experiment (n=4 per treatment). Bars show means \pm SD. Statistical significance was determined using a two-tailed student T-test (ns, not significant)

RESULTS

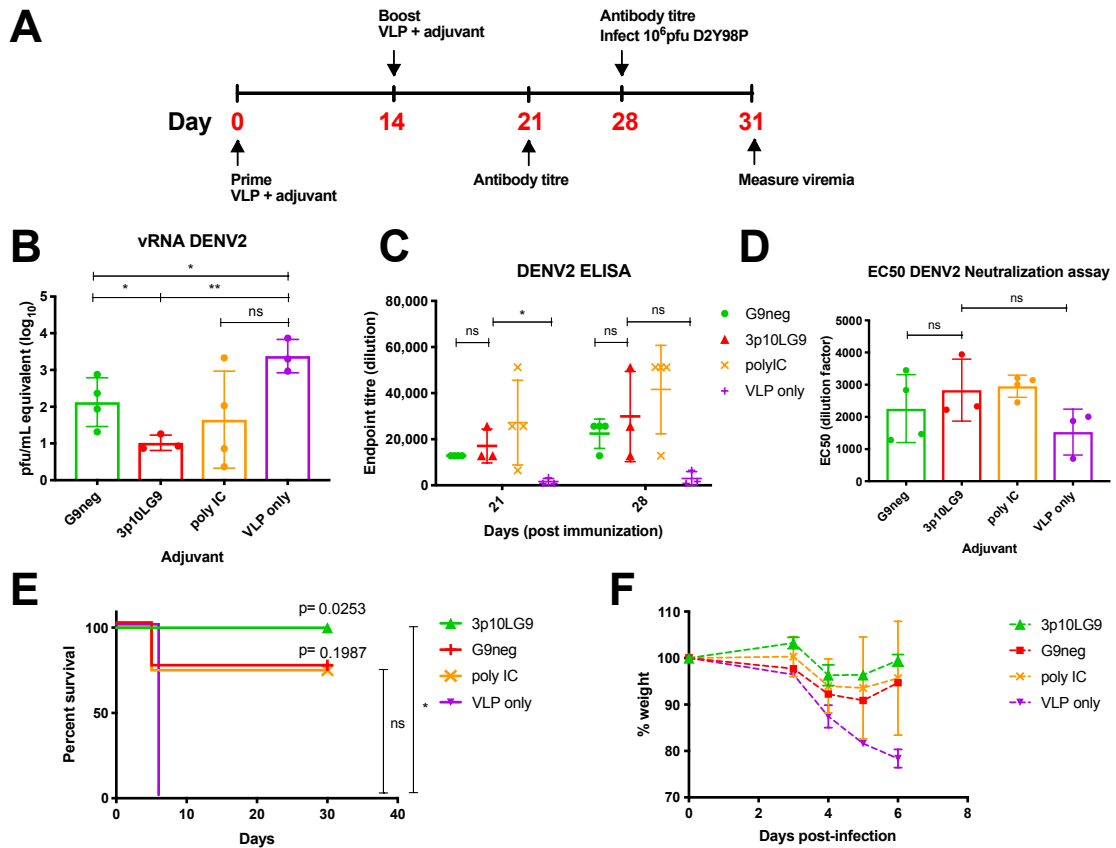


Figure 3.19: 3p10LG9 able to enhance antibody production and protection as an adjuvant in CD11c-cre IFNAR^{ff} DENV challenge mouse model

(A) Immunization and challenge schedule for vaccination experiments. 10ug of DENV-2 VLP was injected with 25ug immRNA mixed with in vivoJET-PEI or poly I:C and injected intramuscularly into CD11c-cre IFNAR^{ff} mice. Data was obtained from a single experiment (n=3-4 per treatment)

(B) Viral RNA (vRNA) was extracted from plasma of DENV-2 VLP-vaccinated mice (n=3-4) infected with 10⁶ pfu of DENV-2. Taqman assay was done to determine the concentration of vRNA in the plasma. Bars show means \pm SD. Statistical significance was determined using a two-tailed student T-test (* P \leq 0.05, ** P \leq 0.01, ns: not significant).

(C) Endpoint titre DENV-2 ELISA with plasma from DENV-2 VLP-vaccinated mice bled on day 21 and 28 post-vaccination. Bars show means \pm SD. Statistical significance was determined using a two-tailed student T-test (* P \leq 0.05, ns: not significant).

(D) EC50 values from neutralization assay done on day 28 plasma from DENV-2 VLP-vaccinated mice. Bars show means \pm SD. Statistical significance was determined using a student T-test (ns: not significant).

(E) Survival curves were generated using the Kaplan-Meier method and significance of differences was calculated by using the log rank test (* P \leq 0.05, ns: not significant).

(F) Weights were measured over a period of 6 days post-infection and plotted as % of initial weight. Mice with more than 20% weight loss considered moribund and were euthanized. Bars show means \pm SD. Statistical significance was determined using a two-way ANOVA with multiple comparisons (* P \leq 0.05, ** P \leq 0.01).

4 Discussion

4.1 Development of RIG-I agonists for anti-viral therapy

4.1.1 Structural modifications to enhance activity for RIG-I agonists

RLRs have been known to play an important role in sensing viral infection, resulting in the initiation of the host anti-viral immune response through the production of type I interferon and pro-inflammatory cytokines (Kato et al., 2006; Loo et al., 2008). In an attempt to define the minimum size for a functional immRNA that can activate RIG-I signalling (minimal immRNA), Kohlway *et al.* found that an RNA molecule of 10 base pairs with a triphosphate group on the 5' end of the duplex RNA (3p10L) is sufficient to result in RIG-I activation (Kohlway et al., 2013).

Based on the structure of the minimal functional immRNA, our collaborator Dr. Dahai Luo made changes to the original sequence of 3p10L, resulting in modifications to the structure of the immRNA. These changes involve the insertion of a single nucleotide in between two base pairs along the double-stranded RNA to generate a kink in the structure. Kinks were introduced in various regions of the dsRNA and through our initial screen we found that a kink in the 9th nucleotide from the 5' end generated by the addition of a guanine nucleotide was able to enhance type I interferon activation when introduced into cells (3p10LG9). This construct was able to do so with at least 6-fold higher potency in human cells when compared to the parental construct 3p10L. It was previously shown by others that various structural modifications made to 5'-triphosphate RNA was able to enhance RIG-I mediated activation of type I interferon and anti-viral activity. In particular, longer sequences as well as the addition of polyU sequences along the stem of the RNA duplex have been shown to enhance the immune activation (Chiang et al., 2015; Kohlway et al., 2013). It also has been shown that the addition of two pairs of wobble sequences along the RNA duplex backbone allows for the recognition of the RNA molecule by

RIG-I resulting in ISG56 expression, and this was independent of a 5'-triphosphate group. Kinked RNA and bulge RNA structures without 5'-triphosphate activated the interferon response, although the potency of these structures to activate RIG-I-dependent ISG expression was not compared directly to the 5'-triphosphorylated versions (Lee et al., 2018). We have also observed that this increased potency of 3p10LG9 compared to the parental 3p10L with regard to type I interferon also translates to increased potency in preventing DENV replication in human cells, and this effect is both RIG-I dependent as well as type I interferon dependent as shown with experiments involving RIG-I KO cells and anti-IFNAR blocking antibody respectively.

Although outside the scope of this project, it is interesting to note that by studying the effect of 3p10L bound to a RIG-I K270 mutant protein, which has its ATP hydrolysis ability abolished, Rawling *et al.* showed that ATP binding and not hydrolysis plays an important role in activating RIG-I signalling (Rawling et al., 2015). Given that 3p10LG9 has some structural similarities to 3p10L, it is possible to speculate that the potency of 3p10LG9 in activating RIG-I signalling could be due to improved ATP binding and thus resulting in the effective release of CARDs from the RIG-I/RNA complex. Using hydrogen/deuterium exchange coupled to mass spectrometry (HDX-MS), Hui Yee *et al.* compared the interaction between RIG-I coupled with either 3p10L or 3p10LG9. They found that the CARDs domain had higher deuterium incorporations and tighter binding of 3p10LG9 to the Hel1 domain on RIG-I compared to 3p10L, suggesting that the stronger RNA-protein interaction leads to more exposed CARDs and thus increased RIG-I mediated signalling with 3p10LG9 (Yong Hui Yee, manuscript in revision). It might be worthwhile to compare the interaction of 3p10LG9 with what is already known for 3p10L, giving additional insight into how the structure of these minimal immRNA agonists play a role in RIG-I activation. Our findings, together with the findings from others, suggest that the structural design and the RNA sequence play an important role in activation of type I interferon responses in host cells. This knowledge can be used to design optimal RIG-I ligands for immune-stimulatory purpose.

4.1.2 Prophylactic anti-viral effects of minimal immRNA agonists in human cells against DENV

Activity of immRNA in human cells is crucial for a potential therapeutic application. Cerny *et al.* previously established a human skin cell assay as a model to study DENV infection of various DC subsets *in vitro* (Cerny et al., 2014). This model is different from the conventionally used MoDCs, which are more representative of an inflammatory type of APCs. MoDCs are not relevant as initial hosts of infection, but are more of a secondary infection target once the infection is established (Cerny et al., 2014; Schmid et al., 2014). The main DC subsets that are susceptible to DENV infection are the CD11c DDCs, CD14 DDCs and LCs. Human skin cells pre-treated with immRNA were effectively primed through type I interferon production and upregulation of ISGs, resulting in the inhibition of DENV replication within the three virus-susceptible DC subsets. Similar to our observation in A549 and U937 cells, 3p10LG9 was more potent than the parental 3p10L in inducing an anti-viral state in human skin DCs. We have shown that minimal immRNA of ~10 bps in length is sufficient to prime an anti-viral response against DENV in human cell lines and primary human skin DCs through the increased expression of ISGs.

However, it is worth noting that the curves generated in experiments using U937-DC human cell line were bi-phasic. After an increase in the ISRE-luc signal with increasing concentrations of immRNA, inhibition was observed above 62nM (**Figure 3.3**). Best-fit curves are therefore only an approximation of EC50. This effect was only observed in the U937-DC cells and not in the skin DCs (**Figure 3.12**). It is unclear what is the cause of this effect. One possible explanation would be the upregulation of factors involved in negative regulation of interferon signalling, such as Ubiquitin specific peptidase 18 (USP18) (Honke et al., 2011), NOD-like receptor family CARD domain containing 5 (NLRC5) (Cui et al., 2010) or LGP2 (Malur et al., 2012). Future studies could look into the role these negative regulators play in regulating RIG-I dependent type I interferon signalling

through the use of RIG-I agonists. Induction of apoptosis has been found to be a strategy for host defence against viral infection, and this is mediated through type I interferon expression (reviewed in Malireddi & Kanneganti, 2013). Hence another possible explanation could be an increase in cell death as a result of increased interferon expression, leading to an overall reduction in interferon activity as measured by our ISRE-luciferase assay.

Human skin cells pre-treated with immRNA were found to upregulate the expression of ISGs. The data presented here reflects the ISG expression found in a heterogeneous mixture of single cells found in the skin, and this might explain the large variation found with the qPCR data. It would be more informative to determine the ISG expression levels in the individual subsets of skin DCs and this can be done either through single-cell RNA sequencing or direct reverse-transcription (direct RT) of a fixed number of sorted cells (V. Ho et al., 2013). This data can be compared with existing gene expression profiles generated through microarray and single-cell RNA sequencing of DENV infected cells generated from cell lines and patients (J. Fink et al., 2007; Zanini et al., 2018). This information can be useful in determining if the gene expression profiles between RIG-I activated anti-viral signalling and anti-viral activation from an acquired DENV infection are similar, and if there are any differences between both type I interferon triggers.

Another limitation of the data from this heterogeneous mixture of cells is that we are uncertain which subset of skin immune cells are directly responding to the RIG-I agonist by producing type I interferon, and whether there is a subset of cells that only respond to but do not produce any type I interferon. One strategy which might be used to address this question is to sort the individual DC subsets right after treatment with immRNA and look at their transcriptome to determine which subset can produce type I interferon. However, we are limited by the number of cells available after sorting each subset, as well as the uncertainty of the viability and health of these subsets of cells after the sorting process. To overcome the difficulty working with a limited number of cells, we could do single-

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cell RNA sequencing of each subset of DCs after treatment with immRNA, or sort 500 cells per subset in triplicate for direct RT RNA sequencing to study the gene expression profile and determine which subsets of DCs respond early to immRNA activation. This might give us some clue as to which skin DC subset is likely to directly respond to a RIG-I agonist by activating type I interferon signalling. Other groups have also reported that aside from the canonical antigen presentation functions for T cell priming in lymph nodes, LCs derived from stimulating CD34+ progenitors in human cord blood were able to mediate host defence against viral infection through the production of IFN-inducible chemokines such as CXCL9 and CXCL11 in response to TLR3 ligand poly I:C (Renn et al., 2006; Rozis et al., 2008). Although this presents an artificial system to study LCs, it does suggest that DC subsets might be able to not just present antigen for T cell priming, but also be involved in generating a microenvironment that is conducive for effective anti-viral responses. These RNA sequencing experiments are currently ongoing and will give us useful information.

As of now, we know that when the skin DCs are transfected with immRNA, the four main subsets of skin DCs are efficiently transfected as indicated by the detection of fluorescent-labelled RNA in each subset (**Figure 3.10**). This indicates that the immRNA has the potential to bind to RIG-I located in the cytoplasm of these cells if RIG-I is present in these cells. Through transcriptomic profiling of human skin DC subsets, McGovern *et al.* showed that the CD14 DDC population is very similar to human monocytes and macrophages (McGovern et al., 2014). This could be a possible explanation why the CD14 DDC population was very effective in the uptake of immRNA compared to the other subsets of DCs in the skin. Studying the transcriptome would also allow us to have an indication as to how effective the uptake of RIG-I agonist is between each skin DC subset, using type I interferon stimulated gene upregulation as a readout.

4.1.3 Use of RLR agonists and TLR agonists in human cells

RIG-I expression is suggested to be ubiquitous, albeit at low levels of expression unless activated by external stimulus such as a viral infection (Yoneyama et al., 2005). In work done on human Poliovirus receptor (PVR) transgenic mice, RIG-I mRNA can be detected at high levels in most non-infected tissues, except for the brain and spinal cord which only had low levels (Ida-Hosonuma et al., 2005). Although no extensive study on the differential expression levels of RIG-I in various human cells has been carried out, PRRs that recognise dsRNA, such as TLR3 and RLRs, could play different roles in various cells and tissues. In our study, we observed that the addition of free HMW polyI:C to U937-DC human cell line without transfection could not activate type I interferon signalling or prevent DENV replication. However, the addition of LPS, a TLR4 agonist, to U937-DC cells could activate type I interferon signalling and this was observed in both U937-DC cells with and without RIG-I. Interestingly, the anti-viral effects of LPS against DENV-2 were only observed in the absence of RIG-I (**Figure 7.1**). There is work showing that TLR agonists, such as poly I:C, could reprogram murine APCs by simultaneously increasing the sensitivity of endosomal TLRs and inhibiting the activation of RLRs in an IFN β -dependent manner (Hotz et al., 2015). The authors suggest that these responses to infection allows anti-viral immunity pathways in the host to function, and at the same time limits pathological inflammation caused by this activation. In their study, the authors have only activated TLR3 signalling and looked at the effect of RLR ligands, but the effect of RLR activation on TLR signalling had not been studied. Results shown here might suggest that only in the absence of RLR signalling is there an activation of anti-viral signalling through TLR activation. However, this conclusion is too preliminary based on our findings alone and requires more experiments to determine the relationship between RLR and TLR activation in the context of infection.

We have also shown that there was no or limited cellular uptake of free fluorescent-labelled immRNA by skin DCs, suggesting that phagocytosis or

endocytosis may not have occurred or might be minimal in these cells. Lack of activation with free polyI:C could be due to polyI:C not being able to have access to the endosomal TLR3. Hence, the addition of free polyI:C to these cells could be ineffective as these DCs might not have functional TLR3 signalling. A RIG-I agonist could be an alternative method to activate innate immune signalling pathway in these skin DCs. Given our observation that polyI:C was able to stimulate RIG-I dependent type I interferon activation when transfected, this could also be an alternative method to activate innate immune signalling in these cells.

We also cannot rule out the possibility that the reason for the lack in activation is simply due to these skin DCs having low expression levels of TLR3. When comparing the transcriptome of APCs (LCs, CD14⁻ and CD14⁺ dendritic cells) found in either human vagina or human skin, Duluc *et al.* observed that the expression levels of RIG-I and MDA5 are similar in all vaginal and skin DC subsets (Duluc *et al.*, 2014). Interestingly, TLR3 expression was below detectable levels in their study and this might indicate that there could be differential expression of RIG-I and TLR3 between DC subsets, with RLRs being more ubiquitously expressed compared to TLRs. However, the authors did cite some limitations to their study, such as the skin samples obtained being lipid-rich due to the source being from individuals with a high body mass index going through cosmetic surgery, thus potentially affecting the transcriptional profiles obtained. Also, the surface markers used to identify the DC subsets were quite few and the gating strategy employed in this study was simple. With the discovery and use of more surface markers to identify more subsets of DCs in skin, along with the power of single-cell RNA sequencing (Haniffa *et al.*, 2012; McGovern *et al.*, 2014), greater understanding of the transcriptome of these DC subsets can be obtained to determine the effective use of innate immune agonists such as RLR and TLR agonists in treatment against infection.

4.1.4 Therapeutic effects of minimal immRNA agonists in human cells

The importance of type I interferon production is evident through the observation that DENV replication can only occur in mice deficient in either type I interferon receptor (IFNAR^{-/-}) or both type I and type II interferon receptor knockout (AG129), leading to high viral loads in these animals (Johnson & Roehrig, 1999; Pinto et al., 2015; Züst et al., 2014). DENV has developed strategies to subvert the anti-viral effects exerted by its host through either inhibiting production of type I interferon or exposure to downstream effectors (Dalrymple et al., 2015). In *in vitro* experiments, it was observed that pre-treatment with type I interferon is protective against DENV infection, whereas any treatment done post-infection was not as effective in inhibiting viral replication (Diamond et al., 2000). Although useful in giving us insight into the cell-intrinsic mechanisms in response to DENV infection, *in vitro* studies are unable to give us an idea of how type I interferon signalling through PRR activation would impact the host microenvironment and if or any bystander activity is present and effective in controlling DENV replication. To our knowledge, there has not been any studies looking at the effects of paracrine signalling through type I interferon activation within the different subsets of cells found in the skin, which is the initial site of infection. We decided to study the effects of inducing type I interferon response after DENV infection using 3p10LG9, and whether we could observe differences in response to type I interferon between the different skin DC subsets. Interestingly, the only significant decrease in DENV replication was observed in the LCs (* P ≤ 0.05), which are located in the epidermis. It is still unclear to what extent LCs come into contact with the virus once the host is bitten by an infected mosquito, since the proboscis of the mosquito probes for blood vessels in the dermis and not in the epidermis (Cerny et al., 2014; Schmid et al., 2014; S. J. Wu et al., 2000). Regardless, our results suggest that LCs are better responders to RIG-I mediated innate immune activation compared to CD11c DDCs or CD14 DDCs, even after DENV infection has been established. However, it remains to be established to which extent the cell-type specific efficacy of immRNA translates into a therapeutic application. One limitation we have is the high MOI required to

achieve detectable levels of DENV E-protein on flow cytometry. This could lead to us underestimating the effects of RIG-I mediated innate immune activation on DENV replication in CD11c DDCs and CD14 DDCs as the reduced DENV replication was observed, but the differences were not statistically significant. More sensitive approaches to virus detection, such as viral RNA sequencing on sorted DC subsets could give us greater insight into the therapeutic effects of immRNA and RIG-I mediated immune activation.

One unexpected finding from looking at the effects of 3p10LG9 on human skin DCs in a therapeutic setting was the enhancement of infection observed when cells were treated with 3p10LG9 24h post-infection. *In vitro* work done by Olagnier *et al.* and Chiang *et al.* looking at the effects of a RIG-I agonist in a therapeutic setting have shown that inhibition of CHIKV or DENV replication can only be observed when the RIG-I agonist is administered less than 8h post-infection (Chiang *et al.*, 2015; Olagnier *et al.*, 2014). However, these studies did not show any data looking at the effects of a RIG-I agonist at timepoints later than 8h post-infection. This still allows the possibility that the introduction of a RIG-I agonist at later timepoints post-infection (>8h) could enhance virus replication. There is no clear explanation why this enhancement occurs, and given the large variation in the result with the small sample size, more experiments would need to be conducted to determine the extent of introducing a RIG-I agonist at late timepoints post-infection and how this would impact a therapeutic application or LAV vaccination. One approach could be the aforementioned RNAseq experiments, but with skin DC subsets treated with RIG-I agonists at later timepoints to study the effects on viral replication in these skin DCs by looking at both the transcriptional profile and vRNA transcript levels. Another unexpected enhancement in DENV infection (1%) was also observed in U937-DC cells with either very low (<10nM) or very high (>1uM) concentration of 3p10L. Similar to the therapeutic experiments, there is no clear explanation why this enhancement occurs, although with 3p10LG9 there is a larger therapeutic window which can be used to reduce the percentage of cell infected

with DENV-2. This would be an area of further investigation to determine the cause of such an enhancement in the absence of antibodies.

This enhancement in infection might have certain translational application in the area of LAV vaccines, whereby the enhancement of replication of these LAV could potentially improve vaccination. We could study the feasibility of this strategy by introducing RIG-I agonists at different timepoints post-infection and seeing if we could enhance *in vivo* efficacy of LAV vaccines such as the methyltransferase mutant live-attenuated DENV vaccine (Züst et al., 2013). This idea of using enhancement of infection to improve LAV immunogenicity is similar to the idea suggested by *Chan et al.* in utilizing the enhancement of replication observed with ADE for the improvement of LAV vaccination (Chan et al., 2016). However, proper characterisation of this effect on enhancing infection as well as extensive pharmacokinetic studies should be done, given the ability of a RIG-I agonist to inhibit replication and thus rendering it ineffective when administered with a LAV vaccine.

4.1.5 Studying the anti-viral effects of minimal immRNA agonists using conditional IFNAR knockout mice

One of the limitations in studying the pathogenesis of DENV and the development of therapeutics or vaccines against DENV has been the lack of a relevant mouse model. Immunocompetent wild-type mice are not susceptible to infection by DENV and one of the reasons could be that DENV NS5 and DENV NS2B3 from clinical isolates are unable to degrade mouse STAT2 (Ashour et al., 2010) or mouse STING (Aguirre et al., 2012; Yu et al., 2012) respectively. To overcome this limitation and allow the *in vivo* study of DENV replication, mice that either lack the receptors for only type I interferon (IFNAR^{-/-}), both type I and type II interferon receptors (AG129 mice), or mice lacking components for effective interferon signalling activation, such as STAT1/2 and IRFs, have been used for various studies involving DENV replication (Ashour et al., 2010; H. W.

Chen et al., 2013; Johnson & Roehrig, 1999; Prestwood et al., 2012; Shresta et al., 2005).

However, these models mentioned also have their limitations in the study of DENV pathogenesis. As mentioned earlier in Section 1.4.3, current studies looking into the anti-viral effects against DENV using AG129 mice have looked at drug candidates that target the virus directly, and these include glucosidase inhibitors resulting in the improper folding of viral proteins, nucleoside analogues that inhibit viral RNA translation and the synthesis of viral proteins and small molecule inhibitors which work directly on DENV proteins such as the capsid and the NS3 helicase. However, AG129 and IFNAR^{-/-} mice models cannot be used to effectively study the effect of agonists and molecules that enhance the host innate immune response such as STING agonists, RLR agonists as well as TLR agonists.

To study the host innate immune response against DENV challenge, we used conditional knockout IFNAR mouse models that are generated by crossing mice which contain a loxP-flanked IFNAR (IFNAR^{ff}) with mice expressing a Cre recombinase under different immune cell-type-specific promoters (Clausen et al., 1999; Züst et al., 2014). Using CD11c-cre and LysM-cre mice that were crossed with IFNAR^{ff} mice, we studied the effect of a RIG-I agonist, 3p10LG9, on DENV replication in mice lacking IFNAR on monocytes/macrophages (LysM-cre) or dendritic cells (CD11c-cre). In experiments looking at the effects of 3p10L in inducing type I interferon response *in vivo*, Linehan *et al.* had determined that the optimal amount used to inject wild-type C57Bl/6 mice was 25ug and using ELISA for IFN α and TNF α they found that this amount was able to induce an interferon response in the first 6 hours upon injection, which subsequently got reduced back to baseline levels 24h post-injection (Linehan et al., 2018). We decided to use this amount as the basis to start initial experiments to determine the efficacy of 3p10LG9 in our *in vivo* model, given that 3p10L and 3p10LG9 were not too different in terms of mode of action as well as size. We found that 3p10LG9 was able to induce significant type I interferon signalling in both

CD11c-cre IFNAR^{ff} mice (***) $P \leq 0.001$) and LysM-cre IFNAR^{ff} mice (* $P \leq 0.05$) compared to G9neg. However, this increase in type I interferon signalling did not correlate with protection against DENV challenge, with no significant changes in viremia, weight loss and lethality between the 3p10LG9 pre-treated mice and the G9neg pre-treated mice. It is worth noting that the data for the LysM-cre IFNAR^{ff} and CD11c-cre IFNAR^{ff} experiments was obtained from a single experiment done for each mouse model. A repeat experiment with a greater number of mice would be necessary for confirmation of these findings. Optimal experimental conditions to determine the dose required for efficacy of 3p10LG9 to function as a prophylactic or therapeutic in our mouse model could also be obtained with a more thorough pharmacokinetic (PK) study and thus we cannot rule out the possibility that 3p10LG9 has anti-viral effects *in vivo* and this could be addressed in future work. This would involve the testing various dosages to determine the amount of 3p10LG9 that could be administered to induce type I interferon activation and anti-viral effects without the introduction of adverse effects in the mice as assessed by physical appearance and weight loss.

One possible explanation for the lack of protective effects of 3p10LG9 is that the levels of type I interferon induced by 3p10LG9 in these conditional knockout IFNAR mouse models is below the levels for effective anti-viral effects. In these experiments, the concentration of type I interferon measured in the plasma was approximately 50pg/mL for CD11c-cre IFNAR^{ff} mice and 600pg/mL for LysM-cre IFNAR^{ff} mice. In wild-type C57Bl/6 mice treated with 3p10L, Linehan *et al.* (Linehan *et al.*, 2018) showed that the plasma concentration of IFN α was approximately 30 ng/mL, which is about 50x greater than what was observed for LysM-cre IFNAR^{ff} mice. No challenge experiments were done in their study so we cannot determine if this concentration of type I interferon would elicit protection against a viral challenge. Goulet *et al.* did an *in vivo* challenge experiment with their 67-mer 5'ppp-RNA oligonucleotide derived from the untranslated regions (UTRs) of VSV (Goulet *et al.*, 2013). When pre-treated with their 5'ppp-RNA, wild-type C57Bl/6 mice had IFN β plasma concentration of 1000

pg/mL, which is 20x greater than what we have observed for CD11c-cre IFNAR^{ff} mice treated with 3p10LG9. This IFN β plasma concentration of 1000 pg/mL was found to be sufficient in protecting mice from lethal influenza infection and the reason why we do not see prophylactic protective effects against DENV challenge in our CD11c-cre IFNAR^{ff} mice could be due to the concentrations of IFN β being low. Another group has studied ADE of DENV using LysM-cre IFNAR^{ff} mice and have demonstrated the therapeutic effects of RIG-I receptor immunomodulatory agonist, M8, in the context of ADE (Pinto et al., 2015). In their study they did not measure the plasma concentration of type I interferon, so we are unable to determine how much was required to elicit protection. Another key difference with their infection model is that they induced ADE of DENV with in their *in vivo* experiments. Although their mouse genotype is similar to ours, there might be inherent differences in the interpretation of the results as their infection model differs from ours.

These findings might point toward these conditional knockout IFNAR mouse models being useful for determining the efficacy of immune-modulating anti-viral therapeutics against DENV only in the context of ADE. However more studies would be necessary in understanding the usefulness and limitations of these mouse models, and this includes a detailed PK study to optimise the dosing schedule and amount of 3p10LG9 to show efficacy as a prophylactic or therapeutic.

4.1.6 Side effects/adverse events associated with type I interferon activation as a therapy

Given the ability of a RIG-I agonist to activate type I interferon signalling, one concern would be the overexpression of inflammatory cytokines that could lead to unwanted side effects and adverse events. An example of how severe complications could arise from immunotherapy leading to increased cytokine production was the use of an anti-CD28 monoclonal antibody, TGN1412, for T-cell immune therapy. In this case, high levels of tumour necrosis factor α (TNF α)

was observed in healthy trial volunteers within one hour after TGN1412 infusion. This was followed by an increase in the level of IL-2, IL-6, IL-10 and interferon- γ within 4 hours post-infusion. Side effects started to develop within 4 hours of infusion, with all six volunteers complaining of headaches and various degrees of nausea, vomiting, diarrhoea and hypotension. This cytokine storm eventually led to all volunteers becoming critically ill, with pulmonary infiltrates leading to lung injury, renal failure and disseminated intravascular coagulation (Suntharalingam, Perry, & Ward, 2006). Rodent models used in the pre-clinical testing TGN1412 only showed moderate increase in serum cytokine levels, unlike in the human clinical trials where the increase was dramatic (Müller et al., 2008). Use of PBMCs from cynomolgus monkeys to test the effects of TGN1412 did not induce large amounts of TNF α and IL 6 or 8, unlike in the tests done using human PBMCs (Stebbing et al., 2007). Hence, in retrospect, PBMC tests would have provided a warning signal of a potential adverse reaction in human trials, that might not have been observed with cells from other species or in mice.

Using a mouse model with transgenic expression of picornaviral RNA-dependent RNA polymerase (RdRP), Painter *et al.* observed that in mouse tissues, ISGs were found to be highly elevated, up to 300-fold, and this elevated ISG profile protected RdRP mice from viral infection. Interestingly, these RdRP mice were found to be healthy with normal longevity despite life-long, constitutive MDA5-mediated innate immune system activation caused by to presence of endogenous long dsRNA (Painter et al., 2015). The authors propose that in inflammatory disease, high chronic upregulation of type I interferon levels is not the main contributor to clinical disease. Instead, properly regulated interferon environment through the expression of negative regulators, such as USP18, NLRC5 and LGP2, helps to control the constitutively activated RdRP-induced anti-viral signalling and avoid adverse effects for the host. Painter *et al.* found that these genes involved in negative regulation were upregulated in the gene expression data, adding weight to the importance of negative regulation of interferon signalling in preventing chronic inflammatory disease and undesirable side effects in the host.

In our mouse model studies using 3p10LG9, we did not observe any severe pathology in the mice such as massive weight loss or ruffled fur. However, we would need to do more studies to determine both efficacy and safety of 3p10LG9 as a therapeutic. Other groups that have done similar studies on RIG-I agonists using mouse models also have not reported any severe adverse effects on the mice, though it cannot be excluded that the findings were not reported (Chiang et al., 2015; Olganier et al., 2014; Pinto et al., 2015). However, this does not address the question as to whether mouse models can accurately represent human physiology and pathology, and if greater emphasis should be placed on using species relevant models to further test safety and efficacy of immunotherapies. In addition to proper pre-clinical testing, the risk of adverse effects through the use of RIG-I agonists such as 3p10LG9 in therapeutics can be further reduced through the method of delivery. These agonists could be used topically to reduce the severity of adverse effects (Huen & Rook, 2014), or packaged into nanoparticles (Tasciotti et al., 2008), or targeted to specific cell types using antibodies or protein antigens (Barbuto et al., 2013; Ilyinskii et al., 2014). Reactivity tests on human PBMCs should also be conducted to confirm the safety of using such RIG-I agonists in human trials as a prophylactic or therapeutic. Though it might improve the efficacy of 3p10LG9, multiple dosing could also lead to adverse side effects due to reduced tolerability and thus can only be considered if it can pass safety studies in mice and human cells or tissues.

Given the ability of RIG-I agonists to enhance the adaptive immune response when delivered with a protein subunit vaccine, 3p10LG9 could be used as a vaccine adjuvant to enhance vaccination. Beljanski *et al.* had shown that M8, a RIG-I agonist, was able to induce a strong Th1 response when used as a vaccine adjuvant with an influenza VLP vaccine. M8 was observed to induce higher levels of germinal center B cells compared to other adjuvants like alum and Addavax (Beljanski et al., 2015). The authors attributed this observation to the stronger Th1 response compared to the other adjuvants which are more Th2-response

inducing, suggesting that it might be advantageous to have a Th1-biased CD4 T-helper cell response. Although having a strong Th1 response could help improve vaccine efficacy, it could also lead to some unwanted side effects as observed in a phase I clinical trial of a tetravalent recombinant subunit vaccine for dengue (Manoff et al., 2018). In this study, the use of an investigational saponin-based adjuvant, ISCOMATRIX, was observed to promote improved immunogenicity compared to an alum adjuvant and unadjuvanted compositions of the vaccine as judged by Focus reduction neutralisation test with 50% neutralisation cut-off (FRNT50) in dengue naïve participants. However, a high proportion of participants in this study who were in the ISCOMATRIX group suffered adverse events such as erythema, pain and swelling at the injection site, and fatigue, fever, and headaches. There were no severe adverse events or deaths in this study, and this finding highlights the importance of determining the tolerability of an immune agonist, such as 3p10LG9, for use as a vaccine adjuvant, and whether the benefits would outweigh the side effects.

4.2 Development of RIG-I agonists as an adjuvant for dengue vaccination

4.2.1 Studying the vaccine adjuvant effects of minimal immRNA agonists using human skin DCs

Three signals are required for the activation of APCs. Activated APCs can support the differentiation and proliferation of naïve T-cells to become effector T-cells. Signal 1 involves the presenting of processed antigen on the surface of an MHC class I/II molecule to the T-cell receptor (TCR) found on naïve T-cells (Cella et al., 1997; Norbury et al., 1995; Pierre et al., 1997; Regnault et al., 1999). Signal 2 occurs when costimulatory molecules present on the APC, such as CD80 and CD86, interact with CD28 on the surface of T-cells. These co-stimulatory molecules are present only in the case of immune activation and signal 1 without signal 2 on T-cells would result in T-cells becoming anergic (Lanier et al., 1995). Signal 3 is through the secretion of cytokines by APCs, resulting in the

differentiation of activated antigen-specific T-cells into an effector T-cell subtype (Cella et al., 1997) (reviewed in Banchereau & Steinman, 1998).

Hence, we wanted to determine if 3p10LG9 is able to induce the upregulation of co-stimulatory molecules on APCs found in human skin. We found that CD80 expression was increased significantly in CD11c DDCs (* $P \leq 0.05$) and LCs (* $P \leq 0.05$) and only moderately in CD14 DDCs and CD141 DDCs with no statistical significance. Haniffa *et al.* showed that these DC subsets found in skin have a much higher co-stimulatory molecule expression compared to the same subsets found in blood, with the exception of LCs which were not studied (Haniffa et al., 2012). Given the already high baseline expression levels of CD80, there may not have been much dynamic range for 3p10LG9 to have an effect in increasing CD80 expression, resulting in only modest changes in CD80 expression levels. Future work with primary human skin DCs will look into the effects of 3p10LG9 in activating autologous T-cells from skin donors, either using EBV peptides specific for HLA-A11 to present to T-cells or a universal MHC class II peptide designed based on a chimera of epitopes from tetanus toxoid and diphtheria toxoid to present to CD4 T-cells (Fraser et al., 2014).

4.2.2 Studying the vaccine adjuvant effects of minimal immRNA agonists in conditional IFNAR knockout mice

Pathogen recognition receptors (PRRs) are attracting increasing interest in the discovery of novel and effective vaccine adjuvants. Upon pathogen-associated molecular pattern (PAMP) binding to PRRs, transcription factors associated with an innate immune response, such as NF- κ B, IRF-3, and IRF-7, translocate to the nucleus and initiate the expression of genes encoding cytokines, chemokines, and costimulatory molecules that eventually play a role in the priming, expansion, and activation of adaptive immune responses. As mentioned in the introduction Section 1.3.3, there have been groups that have utilized RLR agonists in activating these immune pathways and have demonstrated that they produce dose-sparing effects and more effective humoral immune responses in mice

(Beljanski et al., 2015; Kulkarni et al., 2014). However, these groups have tested these adjuvants in an influenza model using VLPs generated from the expression of virus HA and/or NA to vaccinate wild-type mice. Thus far there has not been a study to determine if a RLR agonist could enhance dengue vaccination, possibly due to the challenge in establishing a working mouse model that closely mimics dengue pathogenesis. For this reason, we used our conditional IFNAR knockout mouse model to determine if the RIG-I agonist 3p10LG9 could enhance humoral immunity, given that we and others have found that these minimal immRNA agonists could activate type I interferon signalling in mice (**Figure 3.15**) (Linehan et al., 2018). Preliminary results indicate that 3p10LG9 is able to induce greater protection in CD11c-cre IFNAR^{fl/fl} mice when used as an adjuvant with DENV-2 VLPs as the immunogen. Mice vaccinated with 3p10LG9 as an adjuvant were observed to have lower viremia, reduced weight loss and better overall survival compared to mice treated with the VLP alone. 3p10LG9 showed slightly better efficacy as an adjuvant compared to poly I:C, a TLR3 agonist. Interestingly, VLPs injected with G9neg coupled with a cationic polymer, in vivojet-PEI, was able to enhance anti-DENV-2 antibody titres as well as confer increased protection compared to just VLP alone. The effect of cationic polymer mixed with VLPs is unknown, as previous groups that used this delivery system for their *in vivo* experiments did not include a control group with only in vivojet-PEI (Beljanski et al., 2015; Kulkarni et al., 2014). It has been suggested that VLPs are able to activate specialised APCs which are able to cross present antigen on MHC class I to CTLs (Schirmbeck, Deml, Melber, Wolf, et al., 1995a; Schirmbeck, Melber, & Reimann, 1995b). Hence, one explanation for the enhanced protection of VLP mixed with the cationic polymer compared to VLP alone could be due to increased amount of VLP being carried to the cytoplasm of APCs to allow greater cross-presentation and priming of CTLs. Future experiments would require addition of the cationic polymer with VLP to confirm that G9neg has no effects in this enhanced immune response.

Another interesting observation is that even though the effects of 3p10LG9 in reducing the viremia (* $P \leq 0.05$) and increasing the survival (* $P \leq 0.05$) of

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vaccinated mice was significant, the effects on increasing the amount of DENV-2-binding antibodies was only modest and not statistically significant. The *in vitro* neutralization assay showed no significant differences in the effects on antibodies in mouse plasma on blocking DENV-2 replication in U937-DC cells, although the trend looks similar to the DENV-2 binding ELISA assay. This would suggest that 3p10LG9 might have a role that is independent of anti-DENV-2 antibody titres and that these *in vitro* assays might not be sufficient in measuring correlates of protection. Previous work on using a recombinant EDIII vaccine comparing the protection against DENV challenge in CD11c-cre IFNAR^{ff}, LysM-cre IFNAR^{ff} and ifnar^{-/-} mice showed significant differences in protection of CD11c-cre IFNAR^{ff} mice compared to ifnar^{-/-} mice when looking at survival and viremia levels. However these observations also did not correlate with anti-DENV antibody titres and NT50 neutralisation results, as the differences were observed to be non-significant in these assays (Züst et al., 2014). These results highlight the challenge in determining correlates of protection, as the commonly used *in vitro* neutralization assay is now being questioned as to its value in vaccine evaluation given its failure to predict the vaccine efficacy in human trials (Capeding et al., 2014; Sabchareon et al., 2012; Vannice et al., 2018).

Although preliminary, these results show proof-of-concept that 3p10LG9 RIG-I agonist is able to enhance protection against DENV challenge using VLPs as an immunogen. Future work would involve looking at the effects of serotype-specific protection against other serotypes using a tetravalent formulation of VLPs against all four serotypes. Additionally, the effects of IFNAR expression on immune subsets and the impact on vaccination could also be determined by comparing the effects of vaccination with or without RIG-I agonists in mice with IFNAR-deficient myeloid cells and their wild-type littermates.

4.3 Current development of RIG-I agonists in broader biomedical applications

Aside from their application as anti-viral therapy or as vaccine adjuvant for viral subunit vaccines, RIG-I agonists have been studied as potential anti-cancer agents in various cancer models such as breast cancer, pancreatic cancer, leukaemia and melanoma (Besch et al., 2009; Duewell et al., 2014; Elion et al., 2018; Li et al., 2017). Besch *et al.* (Besch et al., 2009) have observed that RIG-I agonists could induce the proapoptotic signalling pathway independent of Type I Interferon and this RIG-I dependent signalling required MAVS (IPS-1) and led to the induction of proapoptotic BH3-only proteins PUMA and NOXA. This resulted in Caspase-9 and APAF-1 activation, leading to induction of mitochondrial apoptosis in both malignant cells and non-malignant cells, with non-malignant cells being less sensitive to apoptosis than the melanoma cells. Endogenous Bcl-xL was able to rescue the non-malignant cells from apoptosis, but not the melanoma cells as these cells were unable to upregulate Bcl-xL. This suggests that RIG-I agonists might be able to selectively kill melanoma cells, having an attenuated effect on normal non-malignant cells.

Multiple drug resistance (MDR) is known as the resistance of a tumour to several anti-cancer drugs. This is mediated by the activity of an ATP-binding-cassette transporter known as P-glycoprotein (P-gp), which is an efflux pump protein that expels anti-cancer drugs from cells. This effect has led to the ineffective treatment of various cancers such as leukaemia. In an effort to inhibit the effect of MDR and at the same time trigger the innate immune response through RIG-I mediated signalling, Li *et al.* (Li et al., 2017) designed short interfering RNAs (siRNAs) which target MDR1 gene that encodes for P-gp and that contains a 5'-triphosphate end (3p-siRNA-MDR1). They show that 3p-siRNA-MDR1 was able to enhance the secretion of interferon-gamma-inducible protein 10 (IP-10 or CXCL10) and increase the expression of MHC class I on drug-resistant HL-60 and K562 human leukaemia cell lines. 3p-siRNA-MDR1 also promoted caspase-mediated cell apoptosis in these human leukaemia cell lines, and work

synergistically with Doxorubicin, a known chemotherapy drug, to induce enhanced cell killing.

The activity of immune cells in the tumour microenvironment also plays a vital role in controlling tumour growth. Treatment of murine pancreatic cancer cell lines with a RIG-I agonist, a 31bp dsRNA with a 5'-triphosphate, induced type I interferon signalling as well as cell-intrinsic apoptosis, displaying features of immunogenic cell death such as the release of High mobility group box 1 (HMGB1) protein and translocation of calreticulin to the outer cell membrane (Duewell et al., 2014). Interestingly, CD8a⁺ DCs were observed to be able to engulf the apoptotic tumour material and cross-present the tumour-associated antigen to naïve CD8⁺ T-cells. Duewell *et al.* also showed that sub-lethal doses of RIG-I agonists upregulated Fas and MHC class I expression on tumour cells, sensitising these cells to Fas-mediated apoptosis and cytotoxic T lymphocyte (CTL)-mediated killing. Elion *et al.* (Elion et al., 2018) observed the activation of apoptosis and pyroptosis, a highly immunogenic form of cell death, in breast cancer cell lines treated with a RIG-I agonist. Using a 4T1 mammary tumour mouse model, they observed an increase in tumour infiltrating leukocytes in the tumour microenvironment when these mice had an intra-tumoral injection of a RIG-I agonist coupled with a pH-responsive nanoparticle optimized for oligonucleotide delivery *in vivo* (Wilson et al., 2013). Infiltrating leukocytes included tumour-associated macrophages, CD4 and CD8 T-cells, natural killer T-cells (NK-T) as well as inflammatory dendritic cell (DC) populations. When injected in combination with α PD-L1, the tumour growth was decreased to a greater extent compared to either the agonist alone or α PD-L1 alone. These findings by both Duewell *et al.* and Elion *et al.* would suggest that RIG-I agonists, such as 3p10LG9, have the potential to increase the immunogenicity of the tumour microenvironment through the recruitment and activation of immune cells such as macrophages, NK cells, DCs and T-cells. These RIG-I agonists could be used in conjunction with other cancer immunotherapies to enhance the treatment efficacy through the creation of a more immunogenic tumour microenvironment.

Biotechnology companies have been started looking into the use of RIG-I agonists as cancer therapeutics. Rigontec (Bonn, Germany) is a company looking at developing a RIG-I agonist RGT100, which is a 20-24 nucleotide RNA construct with a 5'-triphosphate group, as an anti-cancer treatment for melanoma and other cutaneous tumours, eventually planning to treat solid tumours with the compound (Bouchie & DeFrancesco, 2016). Rigontec was eventually acquired by Merck (Kenilworth, New Jersey, USA) in 2017 in a deal to be worth €464M. Another approach to use a RIG-I agonist involves a small molecule identified from a screen of small, drug-like molecules that drive IRF3 activation and innate immune antiviral activity (Bedard et al., 2012; Pattabhi et al., 2016). This approach has led to the founding of Kineta (Seattle, USA), and have developed KIN131 as a small molecule agonist of the RIG-I pathway. To date, preclinical data of the work in two mouse models, CT26 colon carcinoma and B16F10 melanoma, have been presented.

Future work involving 3p10LG9 will look into exploring its use in anti-cancer therapy by studying both cell-intrinsic effects, such as the ability to induce type I interferon response, as well as indirect effects, such as the ability to recruit and activate immune cells to the tumour microenvironment. Given the observation that 3p10LG9 was more potent in inducing type I interferon response compared to 3p10L, it would be interesting to see how this minimal immRNA would compare with other published molecules. The increased potency could potentially help to reduce the amount of RIG-I agonist required to be efficacious as an anti-cancer drug while limiting any potential harmful effects.

5 Conclusion

RIG-I agonists comprise of RNA molecules with a triphosphate group on its 5' end, and these 5'-ppp RNA have been known to be able to induce type I interferon in host cells. Studies to determine the minimum size of the RIG-I ligand RNA which can induce type I interferon signalling found that only 10 base pairs

adjacent to the 5' end of the RNA is sufficient, along with the presence of the 5'-ppp group. An initial screen of various minimal immune-modulating RNA (immRNA) with modifications in their sequence and structure led to the identification of a lead candidate (3p10LG9) that is able to induce type I interferon responses more potently compared to the parental construct (3p10L).

So far there has been no characterisation of these minimal immRNA agonists as an anti-viral treatment or vaccine adjuvant. Given the potency of 3p10LG9, we investigated the ability of this immRNA to induce human cells to enter an anti-viral state and be primed against infection from DENV. We found that these minimal immRNA agonists were able to prime both human cell lines (U937-DC and A549) as well as primary human skin DCs to enter an anti-viral state through the upregulation of ISGs, and these effects were observed to be both RIG-I dependent as well as type-I interferon dependent. These immRNA also showed modest therapeutic effects after DENV infection in primary human skin DCs, by reducing viral replication specifically in LCs. Preliminary results also show that 3p10LG9 can be used as an adjuvant when administered with VLP as an immunogen to enhance humoral immune responses and protection against DENV-2 challenge in conditional IFNAR KO mice. In primary human skin DCs, 3p10LG9 was able to increase CD80 expression on APCs, priming these cells for more effective antigen-presentation to T-cells. These results demonstrate the potential that minimal immRNA agonists, such as 3p10LG9, have as an anti-viral drug or adjuvant. This warrants further investigation into the application of 3p10LG9 in anti-viral therapy as well as a potential vaccine adjuvant.

6 References

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<http://doi.org/10.1128/JVI.03827-13>

7 Appendix

7.1 Supplemental figures

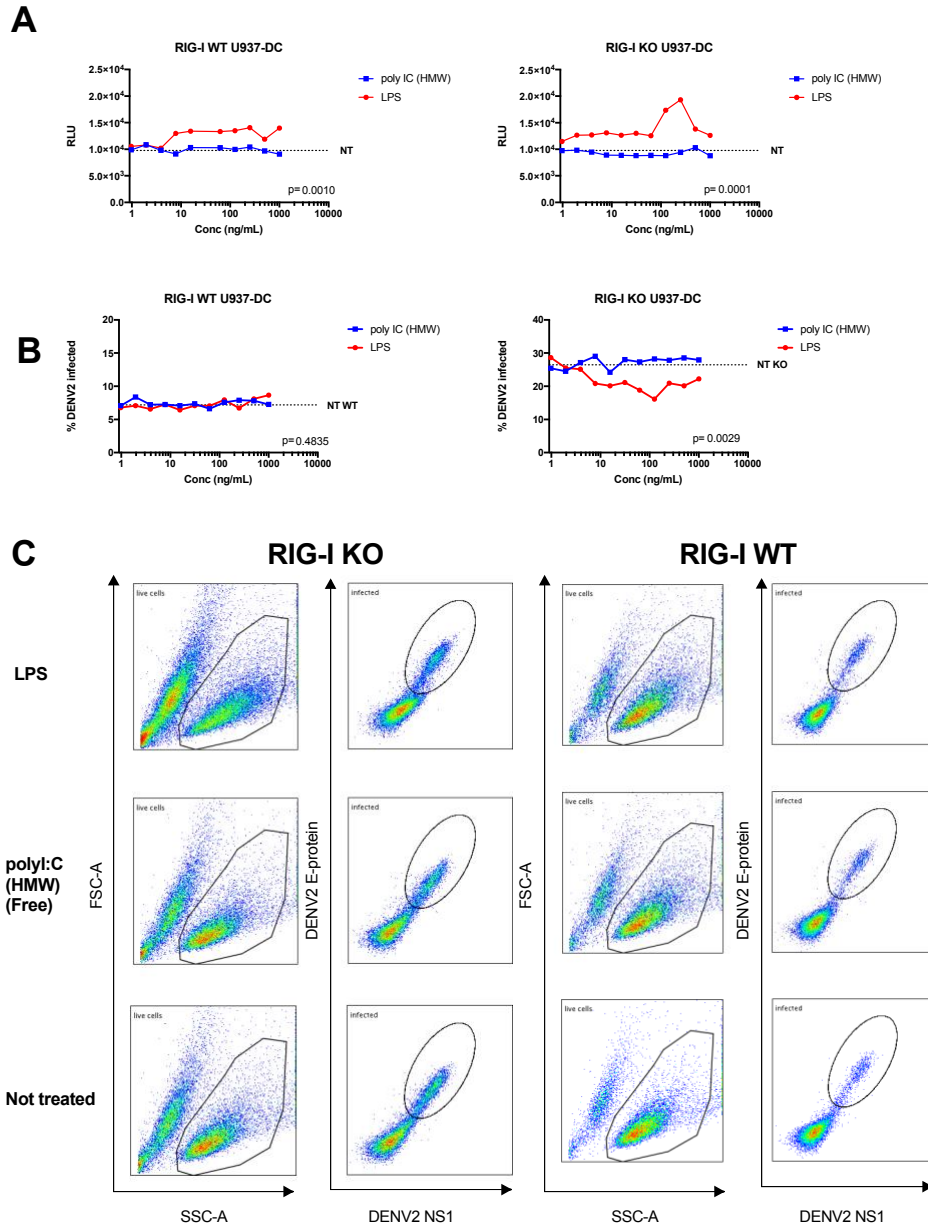


Figure 7.1: LPS, not free-poly I:C, has effect on type I interferon activation and DENV-2 replication in RIG-I KO U937-DC cells.

(A) Supernatant from RIG-I WT or RIG-I KO U937-DC cells treated with either LPS or free poly I:C (HMW) was incubated on the HEK-293T cells containing a luciferase reporter driven by Interferon-Stimulated Response Element (ISRE-luc). Luminescence was measured 6h after incubation with the supernatant. Dotted line indicates the RLU of non-treated (NT) cells. Data is representative of 2 independent experiments. Statistical significance ($p < 0.05$) was determined using a two-way ANOVA with Dunnett's multiple comparisons test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$)

APPENDIX

(B) RIG-I WT or RIG-I KO U937-DC cells pre-treated with either LPS or free poly I:C (HMW) for 24h was infected with DENV-2 TSV01 (MOI 1). Infected viable cells were quantified using flow cytometry with antibodies targeting NS1 and the E-protein fusion loop (4G2) 24h after infection, as represented in **(C)**. Data is representative of 2 independent experiments. Statistical significance ($p < 0.05$) was determined using a two-way ANOVA with Dunnett's multiple comparisons test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$)

APPENDIX



Figure 7.2: Sequencing on U937-DC cells with wild-type *ddx58* (WT) or knockout *ddx58* (KO)

Sequencing results from DNA extracted from U937-DC cells which have either wild-type *ddx58* (WT), or have been transduced with lentivirus containing CRISPR/cas9 with gRNA against exon 1 of *ddx58* (KO). Start codon (ATG) for exon 1 is highlighted with a blue box.

8 Publications and Patents

Title of patent: Immunomodulatory RNA (ImmRNA) For Use As Adjuvant Or Anti-viral Agent

Ref: PAT/289/17/19/PCT

Inventors: 1) LUO Dahai; 2) YONG Hui Yee; 3) Katja FINK (SIgN); 4) HO Chin Yong Victor (SIgN)

Filing date: 17 January 2018

Singapore provisional patent application number: 10201800434S

Ho, V., Yong, H.Y., Chevrier, M., Narang, V., Lum, J., Toh, YX., Lee, B., Chen, J., Tan, E.Y., Luo, D., and Fink, K., (2019), "RIG-I activation by a designer short RNA ligand protects human immune cells against dengue virus infection without causing cytotoxicity". (Manuscript in revision)

9 Posters, awards, invited talks

2016

11th ENII EFIS EJI Summer School on Advanced Immunology, Sardinia, Italy – Oral presentation and poster

5th Singaporean Society for Immunology (SgSI) PhD student retreat, Singapore – Poster presentation

8th International Singapore Symposium of Immunology, Singapore – Poster presentation

2017

17th International Congress of Virology (International Union Of Microbiological Societies), Singapore – Oral presentation and poster presentation

2018

7th NIF Winter School on Advanced Immunology, Osaka, Japan – Oral presentation and poster presentation

6th Singaporean Society for Immunology (SgSI) PhD student retreat, Singapore – Oral and poster presentation