# A mechanistic investigation of FcγR signaling during antibody-enhanced dengue infection

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by

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Duke-NUS Graduate Medical School

Date: 12 February 2015

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Dissertation submitted in partial fulfillment of
the requirements for the degree of
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ABSTRACT

Viruses must evade the host innate defenses for replication and dengue is no exception. During secondary infection with a heterologous dengue virus (DENV) serotype, DENV is opsonized with sub- or non-neutralizing antibodies that enhance infection of monocytes and dendritic cells through Fc-gamma receptors (FcγRs), a process termed antibody-dependent enhancement (ADE). However, cross-linking of activating FcγRs signals an early antiviral response by inducing the type-I interferon-stimulated genes (ISGs). To escape this antiviral response, we demonstrate that antibody-opsonized DENV co-ligates leukocyte immunoglobulin-like receptor-B1 (LILRB1), which recruits Src homology phosphatase-1 (SHP-1) to dephosphorylate spleen tyrosine kinase (Syk) and reduce ISG induction. As Syk also regulates phagosomal maturation, it is possible that compartmentalization of DENV-containing phagosomes could be altered by differential Syk phosphorylation, thereby influencing the outcome of ADE. Indeed, we observed that LILRB1 co-ligation reduced levels of phagosomal acidification, which could be reversed by inhibiting SHP-1. Collectively, we have shown that DENV co-ligates LILRB1 to both attenuate the expression of ISGs and the rapid acidification in the phagolysosomal pathway, ensuring intracellular survival during antibody-enhanced dengue infection.

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**Abstract**

Dengue virus (DENV) continues to put billions at risk of life-threatening disease annually. Infection is enhanced when DENV is opsonized with sub- or non-neutralizing antibodies that augment entry into monocytes and dendritic cells through Fc-gamma receptors (FcγRs), a process termed antibody-dependent enhancement (ADE) of DENV infection. It has been suggested that besides augmenting entry, ADE occurs through other intrinsic factors activated by FcγR-mediated signaling. However, the nature of this pathway and its mechanism of action remain poorly defined.

This thesis explored the molecular pathways governing ADE using two subclones of THP-1 with differential susceptibility to ADE despite similar infection rates. The findings show that co-ligation of activating FcγR leads to Syk phosphorylation, which in turn upregulates the expression of interferon stimulated genes (ISGs) by directly phosphorylating STAT-1. Upregulation of the ISGs led to reduced DENV replication. To overcome this early antiviral response, this thesis demonstrates that DENV co-ligates the inhibitory leukocyte immunoglobulin-like receptor B1 (LILRB1) to inhibit FcγR signaling for ISG induction. Co-ligation of LILRB1 results in the recruitment of the phosphatase SHP-1 that dephosphorylates Syk to attenuate the expression of ISGs, leading to enhanced DENV replication.

As Syk is also a key intermediate of the signaling pathways that control phagosomal trafficking and maturation, we also tested the hypothesis that reduced Syk signaling would lead to differences in the compartmentalization of DENV-containing phagosomes, which may influence the outcome of ADE. Indeed, increased Syk activity led to faster phagocytic trafficking of DENV immune complexes through Rab-5, Rab-7

and LAMP-1 compartments during ADE. This also resulted in higher levels of phagosomal acidification and activation of lysosomal hydrolases like Cathepsin D. Conversely, co-ligation of LILRB1 reduced levels of phagosomal acidification, which could represent a potential viral strategy to escape the phagolysosomal pathway, thus allowing more time for viral fusion.

Collectively, this thesis shows that LILRB1 serves as an important co-factor during antibody-enhanced dengue infection. DENV co-ligates LILRB1 to both attenuate the expression of ISGs and the rapid acidification in the phagolysosomal pathway, ensuring its intracellular survival.

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# Chapter 1. INTRODUCTION

## 1.1 Dengue

### 1.1.1 Dengue epidemiology

Dengue is currently the most prevalent arthropod-borne viral disease worldwide. A recent estimation of the global distribution of dengue using cartographic approaches approximated 390 million infections annually, more than three times the disease burden reported by World Health Organization, and of which 96 million infections resulted in apparent clinical manifestations (Bhatt et al, 2013). Dengue is most prevalent in the tropical and sub-tropical regions of the world, where approximately half of the world's population are at risk for dengue transmission (Guzman et al, 2010). More countries are now hyperendemic for dengue with all four serotypes co-circulating at any time, in contrast to observations made 30-40 years ago where co-circulation of virus serotypes was limited to only two at most (Mackenzie et al, 2004). Hyperendemicity as well as the geographical expansion of the mosquito vectors have culminated in the global re-emergence of dengue which has led to more frequent epidemics that are larger in scale and linked to more severe clinical outcomes (Gubler, 1998; Gubler, 2002).

Dengue virus (DENV) originated from forest sylvatic cycles in Africa and Asia involving transmission of DENV between *Aedes* mosquito vectors and non-human primates (Gubler, 1998). The emergence of an urban transmission cycle in the last three centuries, involving *Aedes* mosquitos and humans, and obviating the need for an enzootic vector, has firmly established dengue as a major public health challenge in urban centres (Gubler, 2002). Its primary vector is the domesticated

*Aedes aegypti*, which feeds on and can infect multiple individuals in a single gonotrophic cycle, rendering it an efficient epidemic vector (Gubler, 1998; Platt et al, 1997). The geographic expansion of both *Aedes aegypti* and its secondary vector, *Aedes albopictus* has largely been driven by increased international trade and travel, thus amplifying regions where DENV could cause epidemics (Simmons et al, 2012).

### 1.1.2 Clinical manifestations of dengue

Dengue infection can lead to a full spectrum of clinical manifestations, ranging from asymptomatic infections to dengue fever (DF), or the clinically severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). A large proportion of infections are asymptomatic in nature. Symptomatic individuals develop acute illness following an incubation period of 3 to 7 days. DF presents with the following symptoms: high fever of sudden onset, accompanied with severe headache, muscle and joint pains, nausea, pain behind the eyes, vomiting or rash (Simmons et al, 2012). These symptoms typically persist for 2 to 7 days, with most individuals recovering without complications.

In a small proportion of patients, their condition deteriorates quickly around the time of defervescence. This phase is characterized by vascular leakage, thrombocytopenia (platelet count below 100,000 cells/$mm^3$), and hemorrhagic manifestations (petechiae, capillary fragility, or bleeding from the mucosa or gastrointestinal tract). Increased vascular permeability leading to plasma leakage into interstitial spaces could result in hypovolemic shock that can be life threatening in patients. The altered vascular permeability is short-lived and is resolved within 48 to

72 hours. During the recovery phase, patients are expected to make rapid improvements in their symptoms although fatigue may persist in adults after recovery (Whitehead et al, 2007).

There are currently no available vaccines or antiviral therapies that can treat this disease. Treatment for the disease remains supportive, with fluid management being the mainstay for reducing mortality to 1% of severe cases (WHO, 2009). There is wide consensus that early and anticipatory treatment can reduce complications and deaths arising from severe dengue. The symptoms that accompany dengue are similar to those observed with other febrile illnesses; hence clinical diagnosis relies on using permutations of a list of symptoms or signs. Dengue case definition in the 2009 WHO classification scheme expands the criteria for severe dengue, which includes severe hemorrhage and organ impairment, in addition to DSS. This revised scheme (Figure 1-1) is expected to aid in triage and better clinical management of dengue infection.

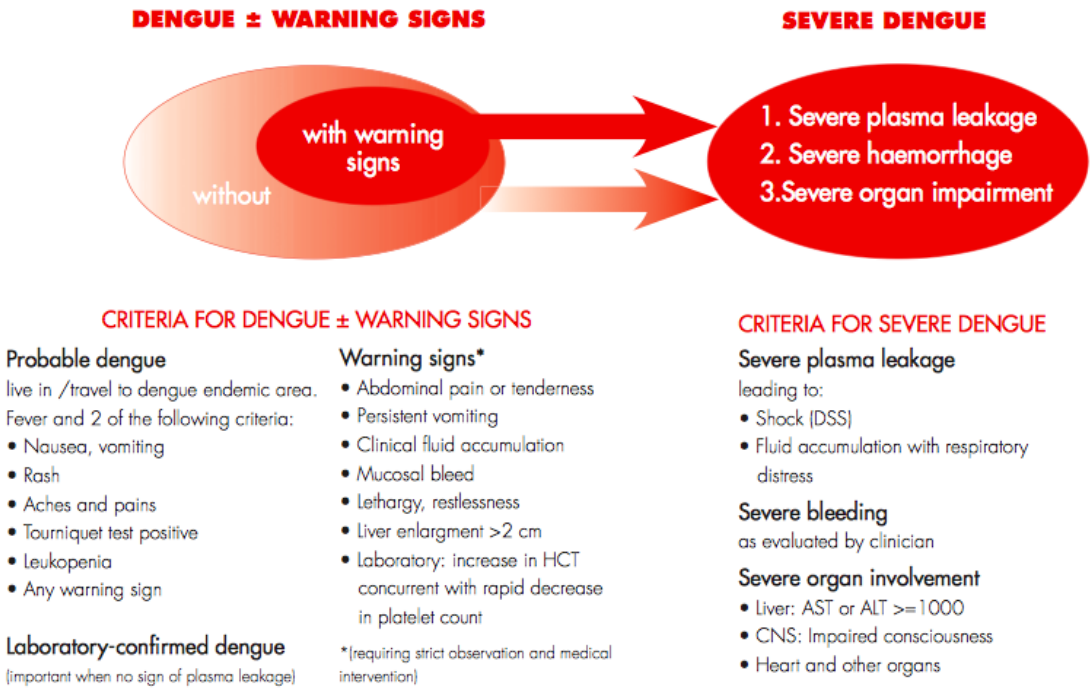



**Figure 1-1. Guidelines for dengue case classification.** The criteria for dengue (with and without warning signs) and severe dengue will aid in the clinical decision as to how intensively the patient is observed and triaged. However, it should be noted that patients without warning signs could still progress to severe dengue. Figure adapted from WHO, 2009.

### 1.1.3 Dengue genome and virion structure

Dengue is a member of the *Flaviviridae* family, which includes other clinically important viruses such as West Nile, yellow fever and Japanese encephalitis viruses. DENV comprises 4 antigenically related but immunologically distinct serotypes which share 65-70% homology. The DENV genome is 11kb in size and consists of a single open reading frame which encodes a polypeptide which must be cleaved by viral and host proteases to yield 3 structural (capsid (C), membrane (M) and envelop (E) proteins) and 7 non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Figure 1-2) (Mukhopadhyay et al, 2005). While the structural proteins provide an architectural form for the virion, the non-structural proteins are essential for viral RNA replication, virus assembly and modulating host cell responses. It is flanked by the 3' and 5' untranslated regions which are imperative in virus replication and regulation of translation (Clyde & Harris, 2006).

The infectious virion is approximately 50nm in diameter. The positive strand RNA genome is complexed with capsid proteins, and is surrounded by a lipid bilayer membrane. Anchored on the outer surface of the membrane are the M and E proteins. The viral surface is composed of 180 copies of E protein (Figure 1-3). The pH of the environment modulates E protein conformation: at alkaline pH, the E proteins are arranged in dimers exhibiting a herringbone pattern and lie flat on the surface. An acidic environment prompts the extrusion of the E proteins into a trimeric conformation, which facilitates fusion of the viral and host cell membranes and release of the viral genome into the cytoplasm (Figure 1-4) (Perera & Kuhn, 2008).

Newly synthesized immature virus budding into the endoplasmic reticulum (ER) lumen has trimers of E-prM heterodimers on the surface, giving the immature virus its characteristically spiky appearance (Zhang et al, 2003). As the immature virus undergoes maturation in the trans-Golgi network, the reduction in pH leads to the rearrangement of E proteins into a flat, dimeric conformation, so that it now has a smooth appearance (Modis et al, 2004). Furin cleavage leads to dissociation of prM and extracellular secretion of the mature virus.

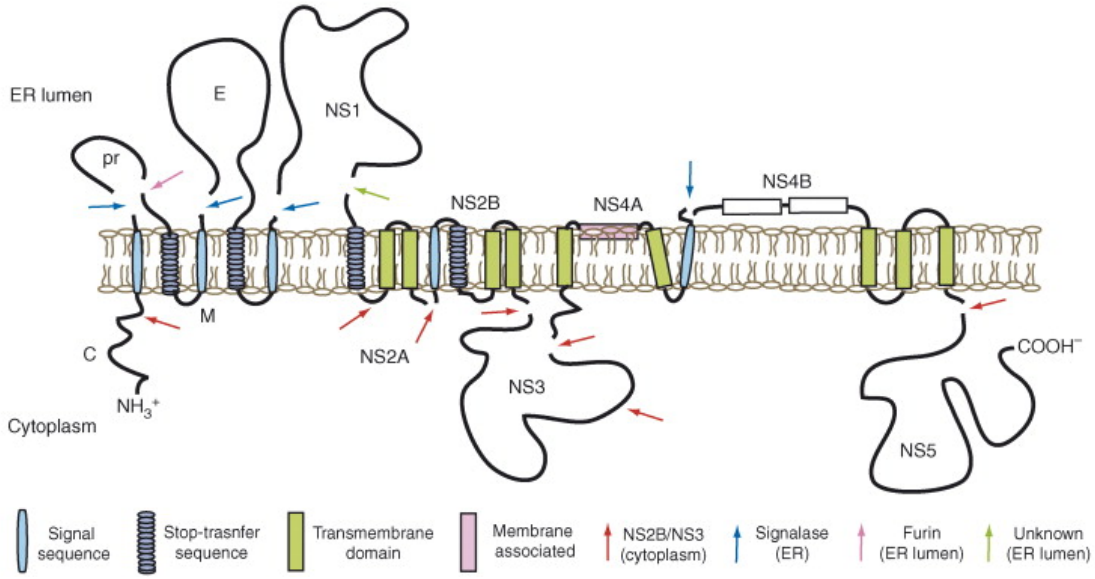



**Figure 1-2. Organization of DENV genome.** DENV is translated as a polyprotein and cleaved by viral and host proteases (denoted by arrows). The 3 structural proteins are released by signalase cleavage in the ER. The non-structural proteins are mostly cleaved by the NS2B-NS3 viral protease in the cytoplasm, except NS1 which is released into the ER by an unidentified protease. The NS proteins are essential for viral replication, assembly and modulation of host cell responses. Figure adapted from Perera and Kuhn, 2008.

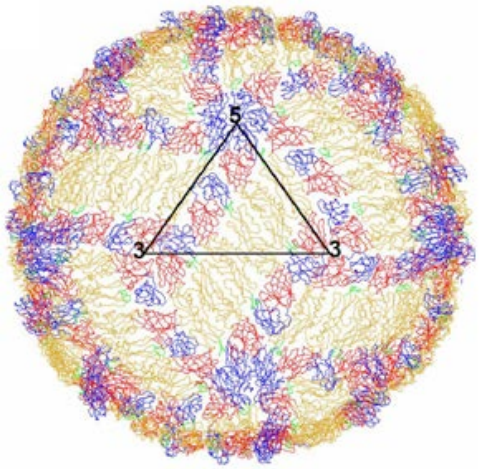



**Figure 1-3. E protein organization on surface of DENV.** DI, DII, DIII and fusion loop on DII are coloured in red, yellow, blue and green respectively. E protein dimers lie flat on DENV surface and are arranged in a herringbone pattern. E proteins are organized in icosahedral symmetry, and the black triangle indicates one asymmetric unit. Figure adapted from Lok, 2014.

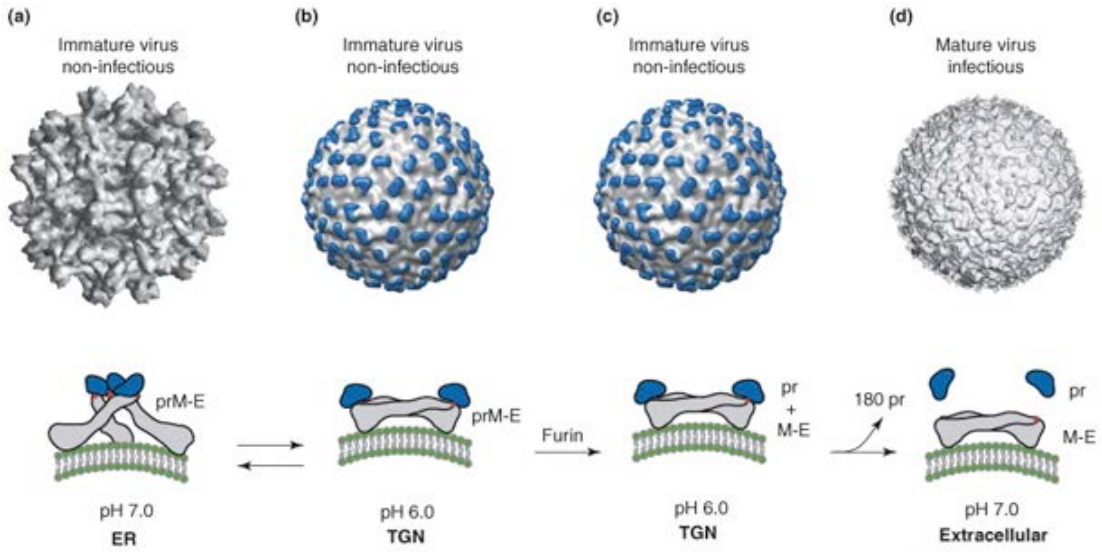



**Figure 1-4. E protein conformations in environments of varying pH.** (a) Surface of immature virus has trimers of prM-E heterodimers, giving the virus a characteristically spiky appearance. (b-c) As the immature virus transits through the trans-Golgi network (TGN), the reduction in pH causes prM-E proteins to dimerize and adopt a flat conformation, giving the virion a smooth appearance. Furin cleavage causes the dissociation of prM protein into M and pr peptide, the latter which remains capped on E protein and the former embedded beneath the E protein (not shown in figure) in the viral membrane. (d) As the pH increases to 7, pr protein is released from the mature virus. Figure adapted from Perera and Kuhn, 2008.

## 1.2 Immune responses to DENV infection

### 1.2.1 Challenges facing DENV vaccine development

Despite more than 70 years of effort, the development of a safe and efficacious dengue vaccine has been elusive. There are considerable challenges facing this daunting task, and with both humoral and cellular responses following DENV infection still under investigation, the paucity of accurate immune correlates of protection has complicated vaccine development (Thomas, 2014). Although vaccine developers have relied on neutralizing antibody titers as an immune correlate of protective efficacy, *in vitro* detection of neutralizing antibodies may not accurately correlate with protection *in vivo*, as exemplified by results of the phase 2b and phase 3 clinical trial for Sanofi Pasteur's CYD-TDV, the most advanced dengue vaccine candidate (Capeding et al, 2014; Sabchareon et al, 2012). Of note, the efficacy observed in the trials appears to be serotype-specific, with lower efficacy observed against DENV-2. The higher overall efficacy of 56% observed with the phase 3 trial, compared to 33% with the phase 2b trial may be due to the lower prevalence of DENV-2 in the phase 3 trial (Capeding et al, 2014; Sabchareon et al, 2012). While the results of the phase 3 trial in Latin America are yet to be published, it is likely that this vaccine offers good protection against DENV-3 and -4, moderate protection against DENV-1 but marginal protection against DENV-2.

This suggests that in order for a vaccine to offer optimal *in vivo* protection, it should induce both humoral and cellular immunity. Thus far, there is incomplete understanding of whether components of cellular immune responses, such as T cell

responses and interferon (IFN)-γ secretion, are involved in DENV vaccine-induced protection.

An efficacious dengue vaccine should confer protection against all four dengue serotypes. However, a significant concern with dengue is antibody-dependent enhancement (ADE), the observed enhancement of disease severity, especially following secondary infection with a heterologous dengue serotype. An ill-conceived dengue vaccine that induces sub-protective levels of anti-DENV antibodies could thus place the vaccine recipient at greater risk of severe disease, although this risk has not yet been demonstrated in field studies (Sabchareon et al, 2012). In addition, the protective antibody response following vaccination should be durable, or else waning levels of the protective antibodies induced could potentially cause ADE in vaccine recipients.

### 1.2.2 T cell responses

T cell responses constitute an important arm of cellular immunity, mediating viral clearance. T cells recognize viral epitopes presented on infected cells by major histocompatibility complex (MHC) molecules, which directs cytotoxicity and release of pro-inflammatory cytokines that restrict viral replication. $CD4^+$ T cells are important in amplification of B cell and $CD8^+$ T cell responses. They enhance the production of pro-inflammatory cytokines, mediate direct cytotoxicity and promote memory responses (Sant & McMichael, 2012). $CD8^+$ T cells restrict viral infection by direct cytotoxicity or via the production of pro-inflammatory cytokines such as IFN-γ and TNF-α (Remakus & Sigal, 2013). A recent study has also highlighted the

different protein targets of the $CD4^+$ and $CD8^+$ T cell responses against DENV (Rivino et al, 2013). While $CD8^+$ T cell responses preferentially target non-structural proteins like NS3 and NS5, $CD4^+$ epitopes are skewed towards E, C and NS1, which are also targets of the human antibody response against DENV (Rivino et al, 2013). In addition, the serotype of infection also prompts differential antigen immunodominance in the T cell response. While the primary DENV-3 response is directed predominantly towards structural proteins like E and prM, the structural proteins constitute a minor component of the primary DENV-2 response (Weiskopf et al, 2014).

*Pathogenic T cell responses*

The original antigenic sin hypothesis has also been used to explain the increased disease severity associated with secondary DENV infections (Duangchinda et al, 2010; Mongkolsapaya et al, 2003). It is hypothesized that cross-reactive T cells raised during a secondary heterologous infection is skewed to the initial infecting serotype. The resultant low avidity cross-reactive memory T cells then dominate the T cell response during secondary infection, more so than naïve T cells which would be of higher avidity for the new DENV serotype. Because of the lower avidity for the secondary infecting virus, they are unable to control the DENV infection (Mongkolsapaya et al, 2003) but instead contribute to the cytokine storm and resultant DENV pathogenesis (Weiskopf & Sette, 2014). However, recent data has demonstrated that during heterologous infections (DENV-2/DENV-3 and DENV-3/DENV-2 infections), the recognition of conserved or cross-reactive epitopes was

either constant or expanded compared to that in homologous infections (Weiskopf et al, 2014). These results seem to suggest that antigenic sin does not impair the quality of T cell responses significantly during secondary infections.

While the role of T cells in the pathogenesis of dengue remains to be fully elucidated, T cells alone cannot fully explain the epidemiological trends observed with DHF/DSS in infants. Infants at 6-12 months of age appear to be at increased risk of DHF/DSS compared to either younger infants or toddlers (Chau et al, 2008; Halstead et al, 2002; Kliks et al, 1988). This phenomenon has been attributed to the presence of DENV-specific maternal antibodies, which is transferred across the placenta and enhance disease after decaying to sub-neutralizing levels (Chau et al, 2008; Halstead et al, 2002; Kliks et al, 1988). In contrast, maternal lymphocytes typically do not cross the placental barrier, thus T cells are not required to produce severe dengue in infants who lack DENV-specific memory T cells. A recent study also showed that there is a temporal mismatch with the production of $CD8^+$ T cells to the time of onset of vascular leakage in children with dengue, suggesting that the mechanism that triggers vascular leakage in children with DHF is independent of $CD8^+$ T cell responses (Dung et al, 2010). However, the possibility that T cell responses could contribute to pathogenesis during secondary infections in older children or adults cannot be excluded at this stage.

*Protective T cell responses*

Several studies have assessed the protective role of T cells during DENV infection in humans, by demonstrating the contribution of DENV-specific $CD4^+$ and $CD8^+$ T cell

responses during secondary DENV infections. In a prospective study that compared the T cell responses of individuals who were subsequently hospitalized or not during secondary DENV infection, levels of IFN-γ to the infecting serotype were significantly higher in non-hospitalized individuals (Mangada et al, 2002). Higher frequencies of DENV-specific T cells were also found in children with subclinical infection, compared to those who developed symptomatic secondary DENV infection (Hatch et al, 2011). The significance of cellular immunity for vaccine-induced protection was also demonstrated in a study which used viral replicon particles (VRP), consisting of a Venezuelan equine encephalitis virus (VEEE) vaccine expression vector encoding the DENV-2 E protein ectodomain, for immunization of wildtype mice (Zellweger et al, 2013). As AG129 mice (type I and II IFN receptor-deficient mice) are extremely susceptible to DENV infection, they were then used as a challenge model to assess the contribution of transferred cells or serum from VRP-immunized wildtype mice. Both passive transfer of VRP-immune serum and adoptive transfer of VRP-immune B cells could increase viral load in AG129 mice upon infection, whereas transfer of T cells from wildtype mice reduced viral load in mice (Zellweger et al, 2013). This study thus demonstrated that vaccine-induced protection should induce both cellular and humoral components of the immune system, and suggests that the role of T cell responses during vaccination should not be ignored.

T cells also play a role in controlling DENV infection, as suggested by studies that associate variations in human leukocyte antigen (HLA) alleles with DENV disease susceptibility or severity (Loke et al, 2001; Malavige et al, 2011; Nguyen et al, 2008). In a study that examined $CD8^+$ responses in a general Sri Lankan

population, involving measurement of *ex vivo* IFN-γ responses associated with more than 400 T cell epitopes, memory T cell responses was found to be protective against DENV. This was demonstrated by the higher magnitude and more polyfunctional responses for HLA alleles associated with decreased susceptibility to severe disease (Weiskopf et al, 2013a).

The positive role for T cell responses in controlling DENV infection as well as in mediating vaccine-induced protection provides an additional dimension to our understanding of dengue pathogenesis. Perhaps the combination of protective HLA alleles and robust antibody response could contribute to optimal protection against DENV. The protective and pathological roles of anti-DENV antibodies will be discussed in the next section.

## 1.3 Paradoxical role of FcγR signaling during DENV infection

### 1.3.1 Antibody-mediated protection

*Protective antibody responses following DENV infection*

The human antibody response to DENV infection has been studied extensively in the hope of identifying neutralizing epitopes that may aid development of a dengue vaccine or therapeutic antibodies. Immunoglobulin M (IgM) antibodies are produced around 5-6 days after a primary DENV infection, peaking around 2 weeks after fever onset and declining to undetectable levels over 2-3 months. Dengue-specific IgG antibodies are first detected 1 week after fever onset and titers increase over time (Guzman et al, 2010). These antibodies are mostly developed against the E, prM and NS1 proteins (de Alwis et al, 2011; Lai et al, 2008). In contrast, during secondary

infections, high levels of cross-reactive IgG antibodies are detected during the acute phase and titers increase rapidly over time. The rapid rise in dengue-specific IgG is attributed to memory B cells that are reactivated upon secondary infection.

Initial studies to characterize the targets of neutralizing antibodies were performed using mouse monoclonal antibodies, and E protein domain III (EDIII) was identified as a major antigenic target (Crill & Roehrig, 2001; Shrestha et al, 2010; Sukupolvi-Petty et al, 2007). Although EDIII-specific antibodies were found to be potently neutralizing *in vitro* and *in vivo*, subsequent studies with human immune sera showed that depletion of EDIII-specific antibodies did not lead to significant reduction in neutralization potency (Wahala et al, 2012; Williams et al, 2012). These studies were the first indication that humans produce neutralizing antibodies that mostly do not bind EDIII. Instead, immune sera collected from DENV infected individuals revealed the antibody response is dominated by weakly neutralizing and cross-reactive antibodies (Dejnirattisai et al, 2010). Only a small fraction of human antibodies are serotype-specific and potently neutralize DENV (Beltramello et al, 2010; de Alwis et al, 2011). These antibodies mostly bind quaternary epitopes on DENV envelope. Examples include 1F4, a potent neutralizing antibody that binds the EDI and EDII hinge region, and HM14c10, a potently neutralizing antibody that recognizes a discontinuous epitope spanning adjacent surfaces of E-protein dimers on DENV-1 (de Alwis et al, 2012; Teoh et al, 2012). Identification of neutralizing conformational epitopes has not been exclusive to DENV – potent neutralizing antibodies against West Nile Virus (WNV) recognize the flexible DI-DII hinge region, which prevents pH-induced rearrangement of the E protein for virus fusion

(Vogt et al, 2009). Lifelong immunity against the homologous DENV serotype is mediated by neutralizing antibodies that develop following acute infection. Thus, continued investigation into identifying neutralizing dengue epitopes could translate into the development of therapeutic antibodies as potential antivirals for DENV.

*"Multiple hit" phenomenon for DENV neutralization*

DENV neutralization is a "multiple hit" phenomenon, in which virions are required to bind multiple antibodies at a stoichiometry that exceeds a required threshold (Pierson & Diamond, 2008; Pierson et al, 2008; Pierson et al, 2007). Molecular modeling studies on E16, a WNV-specific monoclonal antibody (mAb), have estimated the stoichiometric threshold for flavivirus neutralization at 30 mAbs (Pierson et al, 2007). However, this number may vary depending on epitope accessibility and antibody affinity, both of which are principal determinants for neutralization (Figure 1-5).

Epitope accessibility refers to the number of epitopes available for binding and can be influenced by steric constraints from the virus structure, size of the antibody, structural dynamics of the virus and different oligomeric states during virus maturation (Dowd & Pierson, 2011). For example, the neutralizing mAb 1A1D2, binds hidden epitopes in EDIII that are transiently exposed following dynamic movement of DENV E protein at 37°C (Lok et al, 2008). Antibody affinity refers to the fraction of epitopes bound by antibodies at non-saturating concentrations and has been found to correlate with neutralizing potential *in vitro*. Neutralizing activity of antibodies has been shown to vary against different virus strains of the same DENV serotype, and this is attributed to differences in antibody affinity (Sukupolvi-Petty et

al, 2010; Wahala et al, 2010). Collectively, antibodies that bind highly accessible epitopes may exceed the stoichiometric threshold for neutralization by binding the virus at low occupancy, while binding at higher occupancy may be required for antibodies targeting poorly accessible epitopes (Pierson et al, 2007).

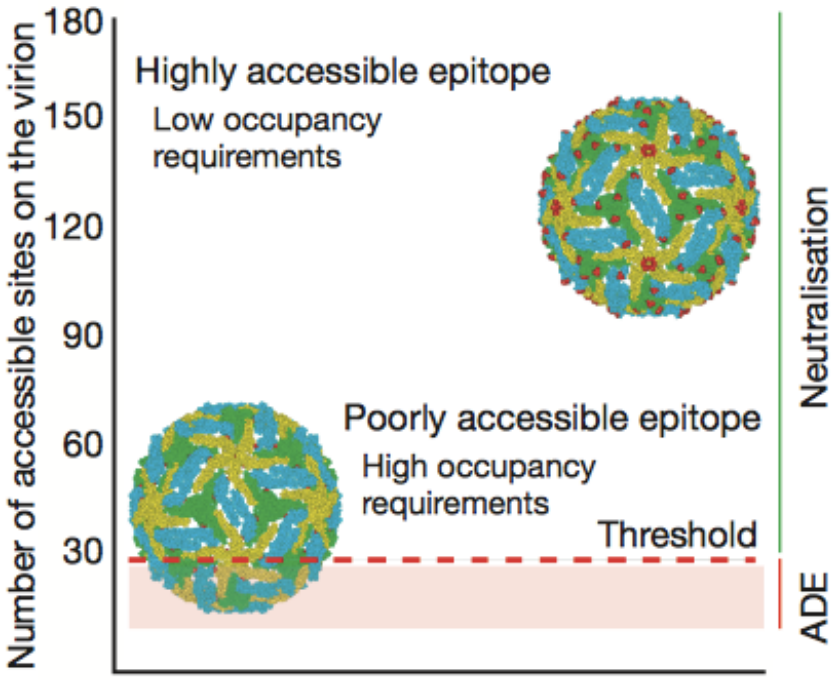



**Figure 1-5. Occupancy requirements and epitope accessibility are determinants for DENV neutralization.** A significant percentage of poorly accessible epitopes must be bound by antibodies to exceed the threshold for neutralization. In contrast, only a fraction of highly accessible epitopes must be bound by the same amount of antibodies for neutralization. Figure adapted from Pierson and Diamond, 2008.

*Mechanisms of antibody-mediated neutralization*

Antibodies can neutralize DENV at different stages of the virus life cycle - either by blocking viral attachment to cellular receptors (Crill & Roehrig, 2001) or by inhibiting intracellular viral fusion (Gollins & Porterfield, 1986). Many of the most potent neutralizing antibodies inhibit infection by disrupting virus attachment to cellular receptors. mAb A12 has been found to be cross-neutralizing against polioviruses of serotypes 1 and 2, and does so by binding the recognition site for the cellular poliovirus receptor CD155 (Chen et al, 2013)

There is a lack of consensus in the field for a bona fide cellular receptor for DENV – candidate receptors include mannose receptor (Miller et al, 2008), C-type lectin domain family 5, member A (CLEC5A) (Chen et al, 2008), heparan sulphate (Chen et al, 1997), CD14 (Chen et al, 1999) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Tassaneetrithep et al, 2003). Identifying antibodies that could block cellular attachment or uptake into host cells as a therapeutic option has been challenging, particularly since most of these studies did not utilize cells that are the main targets of dengue infection in humans. Moreover, there is a possibility that these antibodies, when opsonized to DENV, could enhance infection in myeloid cells via FcγR-mediated phagocytosis. This suggests that antibodies that serve to inhibit intracellular viral fusion could be a more efficacious mechanism for antibody-mediated DENV neutralization.

Antibodies that inhibit intracellular viral fusion prevent nucleocapsid uncoating and release of viral RNA into the cytosol. In the absence of viral fusion, which occurs in Rab-7 positive late phagosomal compartments (van der Schaar et al,

2008), DENV is entrapped in the phagocytic pathway. Subsequent phagosome-lysosome fusion can lead to the degradation of DENV upon exposure to lysosomal hydrolases and the production of superoxide and nitric oxide radicals (Haas, 2007). DENV serotype-specific antibodies, which are associated with long-term immunity in humans, are able to neutralize DENV in the presence of FcγR-mediated phagocytosis (Chan et al, 2011). This reinforces the notion that neutralizing antibodies for DENV should inhibit intracellular viral fusion, in order to serve as a viable therapeutic option (Chan et al, 2013). Antibodies that permit intracellular neutralization have also been demonstrated for viruses such as WNV and human respiratory syncytial virus (RSV). Humanized antibody E16, which binds to EDIII of WNV, is strongly neutralizing as it can block pH-dependent viral fusion (Thompson et al, 2009). Palivizumab, a clinically approved mAb for RSV, neutralizes RSV intracellularly by inhibiting cell-cell or virus-cell fusion (Huang et al, 2010b).

### 1.3.2 Antibody-dependent enhancement (ADE) of DENV infection

*Epidemiological evidence for ADE*

Infection with any one serotype confers lifelong serotype-specific protection against re-infection by the same serotype, but offers limited and short-lived cross-protection to the remaining three serotypes (Sabin, 1952) During World War II (1944-1945), Albert Sabin conducted experimental human challenge studies in which individuals were infected with dengue, in an attempt to develop a dengue vaccine and diagnostic tools. Sabin's work indicated that the duration of cross-protection from a secondary infection was approximately 8 weeks, with significant individual variation (Sabin,

1952). The duration of cross-protection was likely dependent on viral factors and host immune response, although the mechanism to explain this short-lived heterotypic immunity remains to be elucidated. Sabin had described these findings in his publication in broad terms without showing specific data (Sabin, 1952). Recently, Sabin's original laboratory notebooks were reviewed. One of the interesting findings were that patients with secondary infections had a 3-5 day shorter incubation period, compared to primary infections (Snow et al, in press). Although there was no evidence of disease enhancement in the patients with secondary infection, this observation does suggest that cross-reactive antibodies enhance the efficiency of DENV infection. To date, these studies by Sabin represent the most groundbreaking work for investigating the degree of cross-protection and immune enhancement, during secondary infection with a heterologous serotype, at various intervals following a primary infection.

ADE is hypothesized to occur when sub-neutralizing levels of antibody, either acquired from a previous DENV infection or from maternal-fetal transfer, form immune complexes with DENV of a heterologous serotype. The resultant DENV immune complexes are then preferentially taken up by phagocytes and antigen presenting cells through FcγRs, leading to enhanced viral uptake and replication and more severe clinical outcomes (Figure 1-6) (Murphy & Whitehead, 2011).

Epidemiological observations and *in vitro* studies have widely associated secondary infections with a higher risk (15-80 times) of severe clinical outcome. Early studies done in Thailand showed that 14% of children with a primary infection and 41% of children with a secondary infection subsequently developed DSS

(Halstead, 1970; Halstead et al, 1970). These observations were later confirmed in a different setting, the 1981 Cuba DENV-2 epidemic, which was preceded by a mild epidemic of DENV-1 in 1977. The DENV-1/DENV-2 sequence of secondary infections accounted for 98% of DHF/DSS cases. Furthermore, DHF/DSS was absent in children born after the 1977 epidemic as they were only at risk of primary infections (Guzman et al, 1987; Kouri et al, 1989). In the last 20 years, secondary infections as a risk factor for ADE have been reported in studies from Southeast Asia, the Americas and the Western Pacific (Table 1-1).

Conversely, DHF/DSS cases occurring during primary infections as well as the absence of severe clinical disease following secondary infections have also been reported. In Iquitos, Peru, where a DENV-1 epidemic in 1990 was followed by a DENV-2 epidemic in 1995, secondary infections with the American DENV-2 genotype resulted in mild disease (Kochel et al, 2002). Kochel et al. hypothesized that antibodies to DENV-1 acquired from the earlier primary infection were protective against the DENV-2 virus, instead of causing ADE. Indeed, they observed that sera positive for DENV-1 antibodies neutralized the American DENV-1 and DENV-2 viruses more effectively than Asian DENV-2 viruses. This could probably account for the absence of severe dengue in Iquitos during the DENV-2 epidemic in 1995 (Kochel et al, 2002). In a study of 614 patients with confirmed dengue during the 1998 dengue epidemic in Nicaragua, in which the majority of cases stemmed from DENV-3 infections, secondary infections were not significantly associated with DHF/DSS (Harris et al, 2000).

The sequence of DENV infections has been purported to modulate disease severity during secondary infections. We know from epidemiological observations that not all sequences of infections exhibit the same likelihood for severe disease. Early studies in Thailand demonstrated that secondary DENV-2 infection was associated more frequently with DHF than secondary infections with other serotypes (Anantapreecha et al, 2005; Sangkawibha et al, 1984; Vaughn et al, 2000), and this observation has also been corroborated in studies outside Thailand (Guzman et al, 2000; Thomas et al, 2008; Yeh et al, 2006). In contrast, secondary DENV-3 infections have showed low prevalence of severe disease (Harris et al, 2000; Libraty et al, 2009). It is possible that DENV serotypes may benefit differentially from ADE, with a caveat that disease severity is also determined by factors such as genotype of infection and host immunity.

ADE and its resultant clinical outcome of severe dengue is perhaps most uniquely demonstrated in infants with primary infections accompanied by DHF born to dengue-immune mothers (Chau et al, 2008; Halstead et al, 2002; Hammond et al, 2005; Simmons et al, 2007). At birth, these infants possess dengue-specific antibodies which are cross-neutralizing *in vitro*. These maternal antibodies are catabolized with a half-life of 40 days, waning to levels that are no longer neutralizing, but instead enhance dengue infection *in vitro*. (Kliks et al, 1988). In healthy Vietnamese infants, a strong temporal association was demonstrated in the enhancing activity of neat serum and the age-related epidemiology of severe dengue (Chau et al, 2008). These studies in infants are illuminating, as they enable the protective efficacy of dengue–specific antibodies to be studied in a setting whereby interference from pre-existing cellular

immunity can be discounted. Recently, a mouse model of ADE has provided direct experimental evidence for the role of maternal antibodies in enhancing dengue disease severity (Ng et al, 2014). DENV-2 infected mice born to DENV-1 immune mothers resulted in earlier death, accompanied with higher viremia levels and increased vascular leakage, as compared to DENV-2 infected mice born to naïve mothers. This model of ADE also managed to recapitulate earlier epidemiological observations, with an age-dependent propensity for disease enhancement in mice born to DENV-1 immune mothers.

However, not all human studies support the ADE hypothesis. In a prospective nested case-control study involving infants infected with DENV-3 in Philippines, the role of maternal antibodies in ADE was challenged as the authors found no correlation between viremia levels and disease severity (Libraty et al, 2009). Although variation in study design and methods may hinder direct comparison of different studies, there is sufficient evidence to warrant further investigation into the contribution of ADE towards DENV pathogenesis.

**Table 1-1. Secondary infection (SI) as a risk factor for severe dengue.** Table adapted from Guzman, 2014.

| Year | Country | Results | Reference |
|---|---|---|---|
| **1962-1964** | Thailand | Out of 528 children admitted to Bangkok Children's Hospital, 457 had a SI and 71 had a primary infection. 186 (41%) of the former and only 10 (14%) of the latter developed DSS. | (Halstead et al, 1967) |
| **1977-1981** | Cuba | The 1977 DENV-1 epidemic (more than 500, 000 cases reported) in Cuba was followed by the 1981 DENV-2 epidemic. The DENV-1/DENV-2 sequence of secondary infections accounted for 98% of DHF/DSS cases in both children and adults. Furthermore, DHF/DSS was absent in children born after the 1977 epidemic as they were only at risk of primary infections. | (Guzman et al, 1987; Kouri et al, 1989) |
| **1999-2001** | Nicaragua | DENV-2 was the predominant serotype of infection in infants, children, and adults with confirmed DENV in three hospitals in Managua. In children, SI was a risk factor for DHF/DSS. The peak of DHF/DSS in infants 4-9 months is consistent with the theory of maternal antibody enhancement of disease. | (Hammond et al, 2005) |
| **2008** | Martinique | 146 adult patients with confirmed DENV-2 and 4 were studied. The most severe cases of dengue resulted from the combined effects of DENV-2 and SI. | (Thomas et al, 2008) |

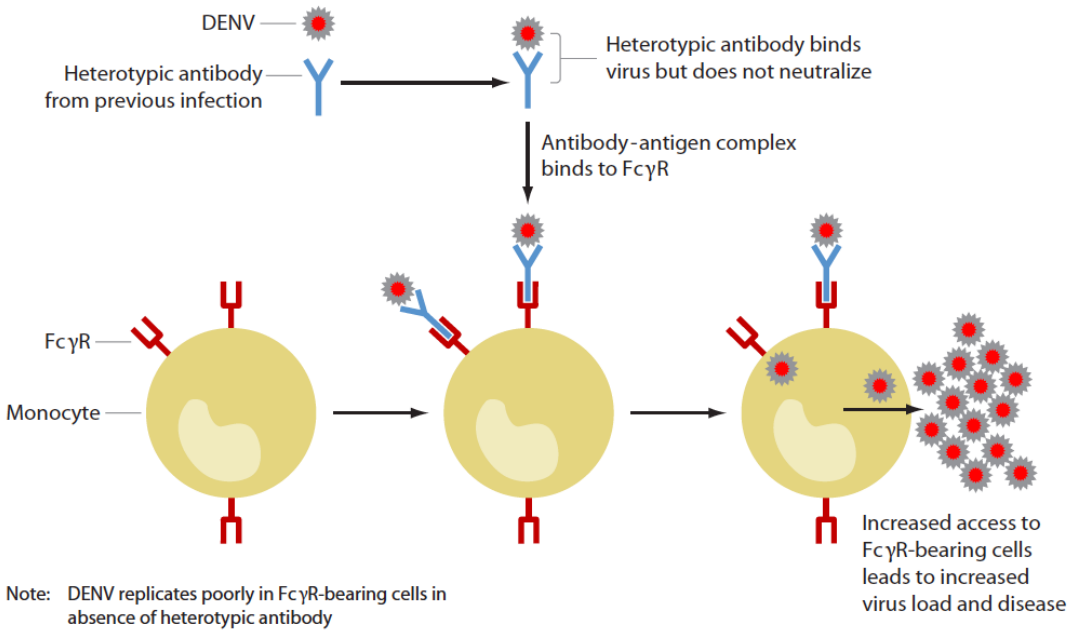



**Figure 1-6 Model of antibody-dependent enhancement (ADE) of DENV infection.** ADE is hypothesized to occur when sub-neutralizing levels of antibody, either acquired from a previous DENV infection or from maternal-fetal transfer, binds to the virus but is unable to neutralize the virus. The resultant antibody-opsonized DENV can infect circulating monocytes via FcγR-mediated entry, facilitating the infection of FcγR-bearing cells that are not readily infected in the absence of antibody. This leads to enhanced viral uptake and virus replication, with the resultant higher levels of viremia being associated with an increase in disease severity. Adapted from Murphy and Whitehead, 2011.

*Pathological role of antibodies in DENV pathogenesis*

The contradictory role of antibodies in DENV pathogenesis is a distinctive feature of the disease. While opsonization of DENV with antibodies at levels above the neutralization threshold effectively clears the virus, DENV opsonized with non-neutralizing or sub-neutralizing levels of antibodies result in ADE in FcγR-bearing cells (Halstead & O'Rourke, 1977). This mechanism increases uptake of antibody-opsonized DENV into FcγR-bearing cells and increases viral replication, leading to higher viremia levels and more severe clinical outcomes like DHF or DSS. Indeed, several studies have demonstrated that peak viremia is higher during secondary DHF cases (Endy et al, 2004; Thomas et al, 2008; Vaughn et al, 2000) and that levels of complement and pro-inflammatory cytokines are also higher in patients with DHF compared to DF (Wang et al, 2006).

Studies characterizing the repertoire of antibodies produced in DENV infected individuals have found the preponderance of cross-reactive and weakly neutralizing antibodies (Beltramello et al, 2010; de Alwis et al, 2011; Dejnirattisai et al, 2010). The majority of antibodies are produced towards the E and prM structural proteins, and the NS1 non-structural protein. Sera derived from DENV infected patients indicate that the bulk of anti-E antibody response are directed to residues in EDII fusion protein (Lai et al, 2008), and that only a small proportion of these antibodies produced are protective (de Alwis et al, 2011). Weakly or non-neutralizing anti-E antibodies could promote ADE by competing, through steric hindrance, with neutralizing antibodies for binding on overlapping epitopes, as previously suggested for influenza virus (Ndifon et al, 2009). Both human and murine mAbs directed

against EDII fusion protein have been shown to enhance viremia, leading to lethality in the AG129 mouse model (Balsitis et al, 2010; Beltramello et al, 2010; Zellweger et al, 2010). Passive transfer of an EDII fusion protein-specific mAb 1A5 at sub-neutralizing concentrations also enhanced DENV infection significantly in juvenile rhesus monkeys (Goncalvez et al, 2007). Lastly, antibodies directed against E protein have also been reported to enhance infectivity of immature DENV particles. Structural analysis have shown that the E protein is exposed in immature DENV and indeed, antibodies like E53, an EDII fusion protein-specific mAb that preferentially binds spikes in immature forms of both DENV and WNV, are able to significantly enhance infectivity of fully immature DENV and WNV *in vitro* (Rodenhuis-Zybert et al, 2011). The same has been observed with other anti-E antibodies that confer infectivity on immature DENV and WNV, causing lethal disease in mice (da Silva Voorham et al, 2012).

A surprising majority of antibodies produced are directed towards prM (Dejnirattisai et al, 2010), which have been shown to target uncleaved prM on immature or partially mature virus particles. Antibodies directed against prM permit binding and cell entry of immature DENV particles into FcγR-bearing cells, enhancing the infectivity of immature or partially mature virus particles, as endosomal furin activity efficiently cleaves prM to M in target cells (Rodenhuis-Zybert et al, 2010). This finding is significant because of the mixture of immature, partially mature and mature virus particles produced in cell culture, which can be enhanced *in vitro* and in mice by anti-prM antibodies (Colpitts et al, 2011; Rodenhuis-Zybert et al, 2010). It is imperative that antibodies with enhancing activity

in immune sera be identified, so that vaccines can be designed with minimal potential for disease enhancement in recipients.

Other than structural proteins, antibodies can also be directed against NS1, a secreted glycoprotein that accumulates to high levels in plasma from DENV-infected individuals. Higher levels of NS1 antigenemia in plasma are frequently observed in children with DHF as compared to those with DF (Libraty et al, 2002; Vaughn et al, 2000). NS1 antibodies have been shown to elicit autoantibodies that react with platelet and extracellular matrix proteins (Falconar, 1997; Oishi et al, 2003; Sun et al, 2007), causing platelet destruction that serves as an additional explanation for thrombocytopenia during severe dengue. However, autoimmunity mediated by anti-NS1 antibodies is incompatible with the clinical picture of immune thrombocytopenia (ITP), an autoimmune condition that results in acute or chronic thrombocytopenia (Lo & Deane, 2014). While chronic ITP can persist longer than 12 months (Lo & Deane, 2014), chronic manifestation of thrombocytopenia associated with DENV infection has never been reported despite lifelong persistence of anti-NS1 antibodies (Murphy & Whitehead, 2011).

Anti-NS1 antibodies also cause damage to endothelial cells via induction of nitric-oxide mediated apoptosis (Lin et al, 2002). The expression of cytokines and chemokines like interleukin (IL)-6, IL-8 and monocyte chemoattractant protein (MCP)-1 are upregulated in endothelial cells following treatment with anti-NS1 antibodies *in vitro* (Lin et al, 2005), which could contribute to pro-inflammatory responses that underlie the vasculopathy in severe DHF/DSS. Soluble NS1 has been shown to bind glycosaminoglycans like heparan sulphate and chondroitin sulphate E

on lung and liver endothelium tissue, and can be targeted by cross-reactive NS1 antibodies. This is hypothesized to contribute to selective vascular leakage during severe disease (Avirutnan et al, 2007). Anti-NS1 antibodies have also been demonstrated to enhance the activation of complement, and the increased plasma levels of MAC and anaphylatoxins observed in patients with DSS could contribute to the pathogenesis of vascular leakage (Avirutnan et al, 2006). The production of antibodies cross-reactive to self-antigens during DENV infection could enhance disease severity through mechanisms such as enhancement of vascular permeability and thrombocytopenia. However, it is difficult to reconcile the kinetics of anti-NS1 antibodies with the short duration of vascular leakage leading to shock, hence the autoimmune hypothesis has remained contentious. Further studies to identify putative self-antigens that could be recognized by anti-DENV antibodies could guide vaccine development, ensuring that memory IgG responses are not triggered against self.

Taken together, it is evident that antibodies targeted to DENV have a dual role in protection and pathogenicity. Continued inquiry into the role of antibodies in DENV pathogenesis could identify fundamental requisites of protective immunity.

### 1.3.3 FcγR usage in neutralization and disease enhancement

*The family of FcγRs*

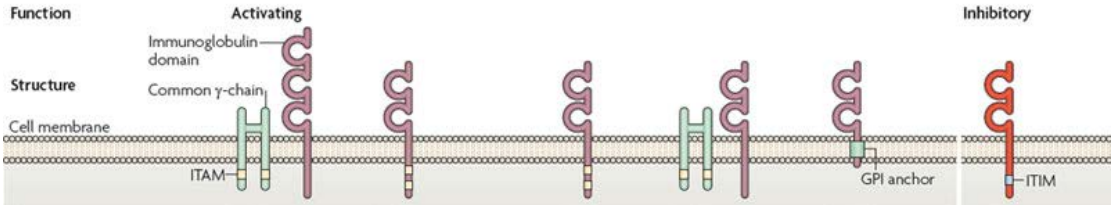
FcγRs are a family of cell surface receptors that specifically bind the Fc region of antibodies. They mediate a myriad of immune responses through simultaneous triggering of activating and inhibitory signaling pathways, allowing fine-tuning of the immune response. As receptors for antigen-antibody immune complexes, they bridge

the innate and adaptive immune systems, serving as a conduit to activate and regulate immunity (Nimmerjahn & Ravetch, 2008).

Human FcγRs are differentiated according to their affinity for the Fc region of antibodies and the signaling pathways they induce (Nimmerjahn & Ravetch, 2011). They can be broadly classified into activating receptors (FcγRI, FcγRIIA and FcγRIIIA) and inhibitory receptors (FcγRIIB) (Figure 1-7). Both activating and inhibitory FcγRs are widely expressed in innate immune effector cells such as monocytes, macrophages, dendritic cells and mast cells. Immune cells that only express activating or inhibitory receptors include natural killer (NK) cells, which solely express activating FcγRIII and B cells, which only express the inhibitory FcγRIIB.

Engagement of activating receptors leads to phosphorylation of the immunoreceptor tyrosine activating motif (ITAM), which triggers activation of downstream signaling cascades. FcγRI, a high affinity receptor for both monomeric IgG and immune complexes, signals through a dimer of γ-subunits containing the ITAM motif. FcγRIIA and FcγRIIIA, which are low affinity receptors that bind only immune complexes, signal through ITAM in the cytoplasmic tail of these receptors. Following aggregation of activating FcγRs by IgG or immune complexes, proto-oncogene tyrosine-protein kinase Src mediates tyrosine phosphorylation of the ITAM. This leads to recruitment of spleen tyrosine kinase (Syk) and phosphorylation of kinases in the downstream signaling cascade (Nimmerjahn & Ravetch, 2008). In contrast, co-aggregation of inhibitory FcγRIIB with activating FcγRs leads to Src kinase Lyn-mediated tyrosine phosphorylation of the immunoreceptor tyrosine

inhibitory motif (ITIM) in its cytoplasmic tail (Smith & Clatworthy, 2010). SH2-domain-containing inositol phosphatases (SHIPs) and SH2-domain-containing protein tyrosine phosphatase 1 (SHP1) are recruited and mediate dephosphorylation of kinases in the activating FcγR signaling cascade.




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Name** | | FcγRI | FcγRIIA | FcγRIIC | FcγRIIIA | FcγRIIIB | FcγRIIB |
| **Expression** | **Lymphoid** | Not expressed | Not expressed | NK cell | NK cell | Not expressed | B cell and plasma cell |
| | **Myeloid** | Monocyte, DC and macrophage | Monocyte, DC, platelet and macrophage | Not expressed | Monocyte, DC and macrophage | Not expressed | Monocyte, DC and macrophage |
| | **Granulocyte** | Neutrophil and eosinophil | Neutrophil | Not expressed | Not expressed | Neutrophil, mast cell and eosinophil | Neutrophil, basophil and mast cell |
| **IgG binding affinity (to cell surface FcγRs)** | | IgG3>IgG1> IgG4>>IgG2 | Variant H131* IgG3>IgG1> IgG2>>IgG4<br>Variant R131* IgG3>IgG1>> IgG2 and IgG4 | IgG3>IgG1 and IgG4; does not bind IgG2 | Variant V158‡ IgG3>IgG1>>> IgG2 and IgG4<br>Variant F158‡ IgG3>>>IgG1; does not bind IgG2 and IgG4 | IgG3>>IgG1; does not bind IgG2 and IgG4 | IgG3>IgG1 and IgG4; does not bind IgG2 |

**Figure 1-7. The family of FcγRs.** FcγRs vary according to their affinity for the Fc protion of the antibody, the signaling pathways induced and their cellular expression. They can be broadly classified into activating receptors (FcγRI, FcγRIIA and FcγRIIIA) and inhibitory receptors (FcγRIIB). While FcγRI and FcγRIIIA signal through the ITAM in the γ-chain, FcγRIIA signals through the ITAM in its cytoplasmic tail. In contrast, the inhibitory FcγRIIB signals through the ITIM in its cytoplasmic tail to counteract activating FcγR signaling. Adapted from Smith and Clatworthy, 2010.

*FcγR usage in DENV neutralization*

While previous studies focused on whether antibodies block viral attachment to cellular receptors or inhibit intracellular viral fusion, more recent studies have examined the contribution of activating and inhibitory FcγR to DENV neutralization. Indeed, the type of FcγR can change the stoichiometric requirement for neutralization. Initial work showed that CV-1 cells transfected with either FcγRI or FcγRIIA, required less and more antibodies for neutralization, respectively, as compared to FcγR-negative untransfected CV-1 cells (Rodrigo et al, 2009). This was validated by Chawla and colleagues, who used THP-1 cells that naturally express FcγR and found that uptake via FcγRI required less antibodies for neutralization, compared to FcγRIIA (Chawla et al, 2013).

The molecular mechanisms for the difference in stoichiometric requirement with different FcγRs are not well understood. A recent work demonstrated that FcγRI-mediated phagocytosis led to activation of phospholipase-D1 and sphingosine kinase-1 to induce trafficking to late endosomes or lysosomes for antigen presentation and the induction of pro-inflammatory cytokines (Dai et al, 2009). In contrast, FcγRIIA-mediated phagocytosis led to increased intracellular calcium levels via activation of phospholipase C-gamma-1 (Dai et al, 2009). It also led to trafficking of immune complexes to intracellular compartments that impaired antigen presentation and pro-inflammatory cytokine response (Dai et al, 2009).

Looking at FcγR usage as an auxiliary parameter for DENV neutralization is a timely move as measurements of neutralizing antibody on epithelial cells result in different titers compared to assays using FcγR-bearing cells (Moi et al, 2010; Rodrigo

et al, 2006). The field lacks an effective tool to determine protective immunity and this has hampered vaccine developments. A recent study indicated that cross-reactive but not serotype-specific antibodies require high antibody concentration to co-ligate FcγRIIB and inhibit phagocytosis of DENV immune complexes (Chan et al, 2011). The ability to distinguish serotype-specific antibodies, which confer lifelong protection, from cross-reactive antibodies, that mediate short-lived humoral protection, could thus be inferred in the presence of FcγR-mediated phagocytosis. This strategy was validated with clinical samples, and found to be able to clarify serologically the serotype of infection more accurately than traditional plaque reduction neutralization tests (PRNTs) (Wu et al, 2012). This could be transformative to how candidate dengue vaccines are assessed for protective immunity and could also inform on therapeutic antibody selection for further development.

*FcγR usage in DENV infection enhancement*

The interaction between anti-DENV antibodies and FcγRs is one that has been imperative in modulating disease enhancement. Whether or not this interaction is with activating or inhibitory FcγRs also has divergent implications for ADE.

FcγR-mediated uptake of DENV immune complexes can be inhibited by altering the Fc portion of anti-DENV antibodies, blocking the interaction of anti-DENV antibodies with FcγRs, or by co-ligation with the inhibitory FcγRIIB. When antibodies with Fc modifications were administered in mice and in rhesus monkeys, therapeutic efficacy against ADE was achieved (Balsitis et al, 2010; Goncalvez et al, 2007). Similarly, blocking FcγRs with monoclonal antibodies in peripheral blood

mononuclear cells (PBMCs) also reduced ADE (Boonnak et al, 2011; Kou et al, 2008). Since the inhibitory FcγRIIB serves to downregulate activating FcγR-mediated phagocytosis and also immune complex induced inflammation, it is tempting to envision a role for FcγRIIB in inhibiting ADE (Tridandapani et al, 2002). Indeed, Chan and colleagues recently demonstrated that DENV opsonized with high concentrations of weakly neutralizing, cross-reactive antibodies forms large viral aggregates that permit co-ligation of FcγRIIB, inhibiting FcγR-mediated phagocytosis and thus ADE (Chan et al, 2011). Importantly, the inhibitory activity of FcγRIIB has been attributed to the ITIM in its cytoplasmic tail, a key feature that discriminates inhibitory from activating FcγRs, which instead contain a cytoplasmic ITAM. Cells engineered to express "swapped" versions of the FcγRII (FcγRIIA-ITIM and FcγRIIB-ITAM) showed equal binding of DENV immune complexes. However, FcγRIIA-ITIM inhibited ADE while FcγRIIB-ITAM restored ADE, showing that the cytoplasmic ITAM/ITIM is a major determinant for ADE (Boonnak et al, 2013).

FcγRIIA seems to be most permissive for ADE (Chawla et al, 2013; Rodrigo et al, 2006) and this could be attributed to divergent internalization pathways following uptake via these receptors. Differences in receptor trafficking and antigen processing have been observed following FcγRI and FcγRIIA-mediated phagocytosis, with antigens taken up by FcγRI trafficked to late endosomal/lysosomal compartments (Dai et al, 2009). Accordingly, DENV immune complexes taken up by FcγRI may be trafficked into compartments that promote virus degradation, while those taken up by FcγRIIA trafficked into compartments that permit viral replication (Chawla et al, 2013).

FcγRIIA has also been hypothesized to play a supporting role in concentrating the DENV immune complex on the cell surface, requiring interaction with other cellular receptors for virus entry (Chotiwan et al, 2014). Chotiwan et al. did not observe enhancement of viral titers when CV-1 cells transfected with FcγRIIA were infected under ADE conditions (Chotiwan et al, 2014). The possibility that downstream mediators of FcγR signaling may not be intact and thus impair FcγR–mediated phagocytosis in CV-1 cells, a FcγR-negative cell line, precludes the authors' assessment that FcγRIIA is insufficient for ADE-mediated DENV entry. Since no experiments were performed to visualize the role of FcγRIIA in concentrating the immune complex on FcγRIIA, this hypothesis is at most, speculative. Experiments to investigate DENV internalization and infection under ADE conditions must attempt to use cell lines or primary cells that naturally express the full spectrum of FcγRs, in order to capture a relevant picture of ADE-mediated DENV infection.

That FcγRs are mechanistically involved in both neutralization and enhancement of DENV infection, underscores the diverse signaling pathways that can be triggered upon engagement of different FcγRs. Both activating and inhibitory FcγRs contribute to the resultant signaling pathway, and this serves as an additional layer of regulation to fine-tune FcγR-mediated immune responses. Recent literature seems to suggest that FcγRI and FcγRIIA are preferentially used for neutralization and enhancement of DENV infection respectively. Although both receptors contain an intracellular ITAM, its location on the associated gamma chain of FcγRI and the cytoplasmic tail of FcγRIIA suggests that this may lead to differences in FcγR-mediated uptake of DENV immune complexes. Differential compartmentalization,

and thus intracellular fate of DENV immune complex which arises from these distinct signaling pathways is an interesting notion that hitherto has not been investigated.

### 1.3.4 Role of antibody effector functions during DENV pathogenesis

Besides removal of immune complexes, antibodies also mediate host defence by recruitment of other FcγR-dependent mechanisms like activation of the classical complement pathway and antibody-dependent cell-mediated cytotoxicity (ADCC) (Jiang et al, 2011). Antibody effector functions undergird their roles in pathogen clearance and protective immunity, as well as deleterious immune reactivity such as allergic reactions and antibody-mediated enhancement of infections.

Protection conferred by neutralizing antibodies has been examined almost singularly from the variable region of the antibody. However, there is renewed insight into how antibody effector functions could provide ancillary mechanisms for antibody-mediated neutralization (Burton, 2002), thus allowing even antibodies that neither block virus-receptor attachment nor fusion with endosomal membranes to confer protection.

*Complement*

An important antibody effector activity is complement dependent cytotoxicity (CDC). The Fc region of the antibody can activate the classical complement pathway by binding the C1q component, a vital initial step of the complement cascade that facilitates CDC. Fixation of complement on virion surface by virus-specific antibodies mediates direct virolysis (Figure 1-8) by triggering the complement

cascade and formation of the membrane attack complex (MAC) C5b-9 (Nakamura et al, 1993). Subsequent binding of antibody- or complement-coated viruses to FcγR or complement receptors leads to phagocytic uptake and clearance of the virus in intracellular compartments (McCullough et al, 1988).

C1q was shown to bind to the Fc region of antibody-opsonized DENV (Mehlhop et al, 2007). Because C1q is a large multimeric protein (Kishore & Reid, 2000), and the binding sites for C1q and FcγR are in close proximity on the Fc region (Idusogie et al, 2001), Mehlhop et al. hypothesized that C1q binding to Fc would consequently interfere with Fc-FcγR interaction, accounting for how C1q could restrict ADE *in vitro* and *in vivo* (Mehlhop et al, 2007). Addition of commercial rabbit complement or fresh sera from healthy humans was also shown to abolish enhancing activity of mouse mAbs against DENV-2 and DENV-4 *in vitro* (Yamanaka et al, 2008). This finding was abrogated when C1q or C3 was depleted from serum or when heat inactivated serum was used (Yamanaka et al, 2008). Collectively, the classical complement pathway can both augment antibody-mediated neutralization as well as reduce ADE of DENV infection. Protective immunity accorded by the classical complement pathway is not limited to DENV and has also been observed in antibodies against measles (Iankov et al, 2006) and influenza (Mozdzanowska et al, 2006).

Soluble and membrane-associated DENV NS1 protein activates complement in the presence of antibodies against NS1 (Avirutnan et al, 2006). The presence of high concentrations of NS1, the complement anaphylatoxin C5a and MAC C5b-9 was detected in pleural fluids from DSS patients, and plasma levels of NS1 and C5b-9 also

correlated with disease severity (Avirutnan et al, 2006). Elevated levels of complement proteins C3, C3a and C5a are important in the recruitment of mast cells and the release of histamine, which locally increases vascular permeability (Dalrymple & Mackow, 2012). C3a and C5a activate platelets, leading to the release of soluble factors with inflammatory properties like matrix metalloproteinase-9 (MMP-9), which enhances the permeability of endothelial cells (Luplertlop et al, 2006). C5a also triggers the release of cytokines like IL-1, IL-8 and TNF-α from monocytes, all of which activate endothelial cells and enhance vascular permeability (Martina et al, 2009). Taken together, complement activation could drive proliferation of the "cytokine storm" and other soluble mediators that are involved in enhancing permeability of endothelial cells, a property that underlies vascular leakage in dengue pathogenesis.

*Antibody-dependent cell-mediated cytotoxicity*

During ADCC, DENV surface antigen expression on infected cells modulates the binding of DENV antibodies, whose Fc region is recognized by FcγRs on effector cells such as natural killer (NK) cells, macrophages, DCs and T cells, which can trigger effector cell-mediated ADCC of infected cells (Figure 1-8) (Kurane et al, 1984). In addition to causing cytolysis of the infected cell, ligation of FcγRs can also lead to the release of antiviral cytokines that aid in pathogen clearance (Russell & Ley, 2002), and this effect can be quantified by either measuring the cytotoxic effect on infected cells or as antibody-dependent cell-mediated virus inhibition (ADCVI)

which considers virus reduction as a result of cytotoxicity, production of antiviral cytokines and other secondary factors (Overbaugh & Morris, 2012).

In dengue infections, studies of ADCC activity for DENV pathogenesis have been relatively few. In a prospective cohort study of Thai school children, ADCC activity was found to correlate with plasma neutralizing antibody levels in both secondary DENV-2 and DENV-3 infections (Laoprasopwattana et al, 2007). However, ADCC was only protective during secondary DENV-3 infections, with lower viral loads attributed to higher ADCC activity (Laoprasopwattana et al, 2007). While ADCC may control viremia levels *in vivo* early after secondary DENV-3 infection, no significant correlation between ADCC activity and plasma viremia levels were observed in secondary DENV-2 infection (Laoprasopwattana et al, 2007).

Recently, the role of non-neutralizing antibodies that rely on Fc-FcγR interaction to mediate neutralization of HIV-1 has also been investigated. The RV144 HIV-1 vaccine trial in Thailand, which reported a vaccine efficacy of 31.2% despite the absence of neutralizing antibodies or cytotoxic T cell responses, raised the hypothesis that Fc effector functions of non-neutralizing antibodies could have contributed to the vaccine efficacy (Haynes et al, 2012). High levels of ADCC activity were found to correlate with decreased risk of infection in RV144 secondary immune correlate analysis (Haynes et al, 2012), corroborating previous reports of an inverse correlation between HIV-1 gp120-specific ADCC antibodies and disease progression (Baum et al, 1996). Moreover, ADCC responses in HIV-1 infected elite controllers with undetectable viremia were higher as compared to infected individuals with viremia (Lambotte et al, 2009). Non-neutralizing antibodies induced following

RV144 vaccination also showed highly coordinated Fc-mediated effector responses by selective induction of highly functional IgG3 (Chung et al, 2014). V1V2-specific IgG3 antibodies were also associated with broad antiviral responses and correlated with decreased risk of infection in the RV144 trial (Yates et al, 2014). These studies agree that antibody subclass and antibody effector functions of non-neutralizing antibodies, coupled with FcγR-mediated adaptive and innate immune functions, could contribute significantly to HIV-1 neutralization.

Recent work by DiLillo and colleagues has also shed light on how broadly neutralizing antibodies (bNAbs) mediate protection against influenza. Antibodies against influenza virus target the two major domains of hemagglutinin (HA): the head domain, where most antigenic variation occurs, and the stalk domain, which is conserved between influenza virus subtypes (Wang & Palese, 2011). Broadly neutralizing antibodies are typically targeted to the stalk domain, allowing it to neutralize different subtypes of influenza virus. The mechanism of action for anti-HA stalk bNAbs involved disruption of the fusion process and efficient interaction with FcγR, conferring protection through NK cell activation and cytotoxicity of infected cells (DiLillo et al, 2014). In contrast, the anti-HA head immune complexes did not interact with FcγRs, and could not mediate cytotoxicity of infected cells (DiLillo et al, 2014). Interestingly, at high bNAb doses, *in vivo* protection is FcγR independent and thus ADCC independent (DiLillo et al, 2014). It is possible that at low doses of the bNAb, its ability to block fusion may be inefficient *in vivo*. This was raised as a possibility following a prospective cohort study that correlated detection of ADCC antibodies to children with clinical influenza (Co et al, 2014). Anti-stalk antibodies

may represent only a small portion of the ADCC antibodies detected and multiple exposures to influenza may be required before protective levels of anti-stalk antibodies are developed (Co et al, 2014). The interaction of bNAbs with FcγRs to mediate ADCC of infected cells thus contributes towards optimal *in vivo* protection. Collectively, this work manages to delineate the multiple paths of antiviral protection taken by anti-HA head and stalk antibodies, which elevates our understanding of how antibody effector functions can be harnessed to enhance mAb-mediated antiviral therapeutics.

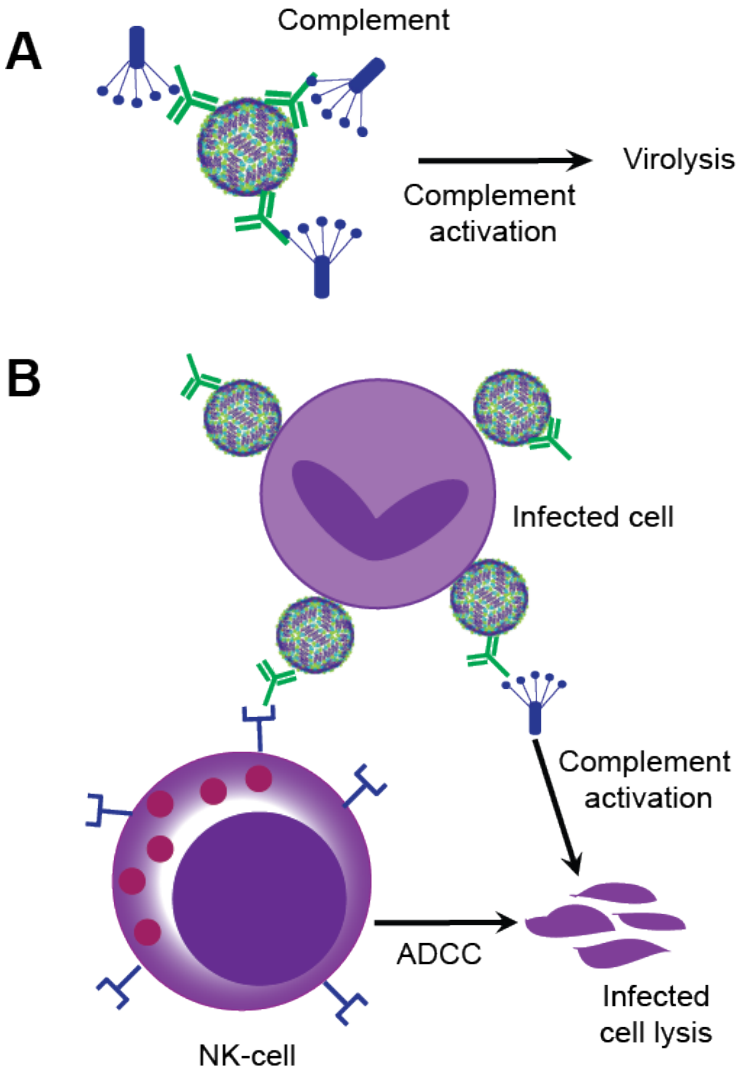



**Figure 1-8. Antibody effector functions against DENV.** (A) The Fc region of DENV-specific antibodies mediates the deposition of complement on the virion surface, which can rupture the virion envelope and lead to direct virolysis of DENV. (B) DENV-specific antibodies can activate complement and NK cells, leading to lysis of infected cells via antibody-dependent cell-mediated cytotoxicity (ADCC). Adapted from Chan, 2013.

## 1.4 Modulation of host innate immunity during ADE

### 1.4.1 Intrinsic ADE

During ADE, host innate immunity is suppressed to favour higher levels of viral replication, a phenomenon termed “intrinsic ADE” by Halstead and colleagues (Halstead et al, 2010). DENV can be detected by pattern recognition receptors like Toll-like receptors (TLRs), transmembrane proteins that recognize viral nucleic acid components either outside of cells or in cytoplasmic vesicles, and are involved in priming host innate immunity upon viral infection (Takeuchi & Akira, 2009). The expression of TLR3, 4 and 7 and TLR signaling molecules like TRAF-6 and TRIF were reduced in the presence of DENV infection under ADE conditions in THP-1 cells. In contrast, negative regulators of TLR signaling, sterile α-armadillo motif containing protein (SARM) and TRAF family member-associated NF-κB activator (TANK), were upregulated, leading to increased viral replication and suppression of innate immunity (Modhiran et al, 2010). This observation was recapitulated in PBMCs of DHF but not DF patients, correlating the suppression of innate immunity with increased disease severity in DHF patients. The expression of TLRs and production of interferon-β (IFN-β) was restored when ADE-infected cells were pretreated with anti-FcγR antibodies, reinforcing the point that FcγR-mediated uptake of antibody-opsonized DENV downregulates TLR signaling and IFN-β production (Modhiran et al, 2010).

Once viral RNA is released into the cytoplasm, it can be detected by cytoplasmic RNA helicases like retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5), which serve to activate type I IFN

production. Infection under ADE conditions in THP-1 cells enhanced expression of dihydroxyacetone kinase (DAK) and autophagy-related 5-autophagy-related 12 (Atg5-Atg12), negative regulators of RIG-I/MDA5 signaling. This led to suppression of type I IFN production and inhibition of IFN-mediated antiviral responses (Ubol et al, 2010). Similarly, PBMCs obtained from DHF patients displayed suppressed levels of RIG-I, MDA5, mitochondrial antiviral signaling protein (MAVS), a downstream adaptor of RIG-I/MDA5 signaling, and plasma levels of IFN-β, as compared to PBMCs obtained from DF patients (Ubol et al, 2010). However, when Rolph and colleagues examined infection under ADE conditions in primary monocyte-derived macrophages, they did not observe a significant reduction in RIG-I or MDA5 expression compared to infection with DENV only (Rolph et al, 2011). It is possible that differential mechanisms and mediators for ADE exist in different cell types. Careful consideration must be conducted before generalizing results from one cell type, or even one DENV strain, to another.

Intrinsic ADE is frequently accompanied by suppression of type I IFN production (Rolph et al, 2011; Ubol et al, 2010). However, in line with the observation that there exist cell type-specific mediators of ADE, different primary myeloid target cells were found to support variable levels of type I IFN production. In primary human macrophages, peak enhancement of viral titers corresponded to reduced type I IFN levels (Boonnak et al, 2011). However, levels of IFN-β correlated with levels of DENV infection in primary human DCs (Boonnak et al, 2011). Type I IFNs were not detected in primary human monocytes during infection with DENV alone or under ADE conditions (Boonnak et al, 2011), while Kou et al. observed with

a vesicular stomatitis virus (VSV) infection inhibition bioassay that type I IFN levels were induced early after infection with DENV alone and to a greater magnitude under ADE conditions (Kou et al, 2011). However, VSV is extremely sensitive to type I IFNs and the higher levels of VSV inhibition observed could merely reflect increased type I IFN production due to higher proportion of DENV-infected to uninfected cells under ADE compared to virus only infection. Thus, type I IFN production may still be lower in an individual cell infected under ADE compared to virus only conditions.

The production of type I IFNs triggers the activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, leading to nitric oxide (NO) synthesis and the activation of transcription factors like STAT-1 for the induction of antiviral interferon stimulated genes (ISGs). NO is a free radical typically found elevated in the sera of DF but not DHF patients (Neves-Souza et al, 2005). While NO has been shown to possess antiviral activity on DENV-infected cells (Takhampunya et al, 2006), lower levels of NO are detected in THP-1 models of ADE infection, due to suppression of STAT-1 and interferon regulatory factor-1 (IRF-1), which are both transcription factors for ISGs (Chareonsirisuthigul et al, 2007; Ubol et al, 2010).

Inhibition of NO synthesis during ADE underscores the immune suppressive state during ADE, which is also amplified by inhibitors of JAK/STAT signaling and the production of immunosuppressive cytokines. Higher levels of suppressor of cytokine signaling-3 (SOCS-3) have been observed during ADE in both THP-1 cells and primary monocyte-derived macrophages, which was mediated by increased levels of IL-10, an immunosuppressive cytokine (Rolph et al, 2011; Ubol et al, 2010). IL-10

blocks both NF-κB and JAK/STAT signaling, effectively impeding both TLR and type I IFN-mediated antiviral responses. Ligation of FcγRs by antibody opsonized-DENV leads to IL-10 production early after ADE infection, which results in low levels of type I IFN production that suppresses the transcription and translation of IL-12, IFN-γ and tumour necrosis factor-α (TNF-α) (Chareonsirisuthigul et al, 2007). Both IL-10 and SOCS-3 have been detected at higher levels in PBMCs of DHF rather than DF patients (Ubol et al, 2010).

IL-10 expression, however, during ADE can be modulated by both cell-type specificity and host genetic polymorphisms. ADE did not augment IL-10 production in primary monocytes, macrophages or DCs (Boonnak et al, 2008; Kou et al, 2011; Rolph et al, 2011). A modest increase in IL-10 production was only observed 72 hours after infection in primary monocytes, which cannot account for the increased virus production observed early after ADE infection (Kou et al, 2011). In a study that did observe IL-10 production during ADE infection of primary monocytes, no significant reduction in ADE infection of cells was seen. This led the authors to hypothesize if IL-10 may modulate ADE via bystander effects, such as inhibiting DC maturation, antigen presentation and general suppression of immune responses. IL-10 promoter polymorphisms also varied IL-10 production, but not viral infectivity of primary monocytes during ADE infection. Donors with a GCC IL-10 promoter haplotype showed highest levels of IL-10 production, while ACC and ATA donors showed intermediate and low levels of IL-10 production respectively. Blocking the activating FcγRs abrogated IL-10 production and markedly reduced ADE infection. (Boonnak et al, 2011).

Host innate immunity is modulated rapidly in response to viral infections. During ADE, the ligation of FcγRs by antibody-opsonized DENV and subsequent FcγR-mediated uptake of the DENV immune complex triggers the activation of signaling cascades whereby kinases are phosphorylated within minutes (Crowley et al, 1997). Single particle tracking of DENV has also revealed that DENV fusion from within late endosomes takes place within 30min of binding to cell surface (van der Schaar et al, 2008). However, the design of experiments to address the hypothesis of intrinsic ADE frequently measures mediators of antiviral activity at late time points (12 hours post-infection or later), which may not be representative of the early innate immune responses during viral entry. Intrinsic ADE could be better addressed by investigating expression of innate immune mediators within the first 6 hours of infection. This would fill important gaps in our understanding of how innate immunity is modulated during ADE.

### 1.4.2 Role of FcγRs in modulating innate immunity

FcγRs are broadly expressed in innate immune effector cells such as monocytes, macrophages, neutrophils, NK cells and mast cells. They determine the activation threshold of innate immune cells and serve to bridge humoral and cell-mediated immunity. Impaired regulation of antibody-mediated effector functions by FcγRs leads to either hyperreactivity or unresponsiveness to either foreign or self-antigens.

The immunomodulatory function of FcγRs has perhaps been most clearly characterized in models of antibody-mediated autoimmune diseases like systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). FcγR polymorphic variants

of low and high responders have been correlated to the pathogenesis of chronic inflammatory diseases, serving as genetic risk factors for disease prognosis (Bournazos et al, 2009). Low responder variants are usually associated with autoimmune pathologies characterized by the presence of circulating immune complexes like SLE. The reduced efficiency of Fc-FcγR interaction compromises the clearance of these immune complexes, which leads to their deposition on peripheral tissues and exacerbation of inflammatory processes (Bournazos et al, 2009; Karassa et al, 2002). Various meta analyses have demonstrated a significant association between the FcγRIIA R131 allele and increased risk of SLE (Karassa et al, 2002; Magnusson et al, 2004; Yuan et al, 2009). The FcγRIIA R131 allele is an example of a low responder variant of FcγRs, which confers reduced binding of IgG2 to FcγRIIA (Parren et al, 1992) and therefore compromises the clearance of immune complexes from circulation. The deposition of immune complexes on various tissues results in inflammation and damage via complement activation (Li et al, 2009).

Conversely, high responder variants result in prolonged Fc-FcγR interactions, which reduce the threshold for effector functions, promoting leukocyte infiltration into tissues accompanied by release of cytotoxic compounds that amplify inflammation and tissue damage (Bournazos et al, 2009). The high responder variant, FcγRIIA H131 has been shown to bind and enable phagocytosis of IgG2-coated particles. (Warmerdam et al, 1990) It confers enhanced capacity for clearance of immune complexes in circulation and increased activation of leukocytes. The FcγRIIA H131 variant has been associated with increased susceptibility to Guillain-Barré syndrome (GBS), a syndrome characterized by nerve infiltration of leukocytes

and autoantibodies directed against nerve components (van der Pol et al, 2000; van Sorge et al, 2005). Patients with GBS frequently have high levels of autoantibodies against ganglioside GM1 in the serum, which interact with FcγRs to trigger effector functions like cytotoxicity, phagocytosis and cytokine release (van Sorge et al, 2003), leading to demyelination and nerve damage. The allelic polymorphism at residue 131 (H/R) in FcγRIIA is clearly demonstrated to affect IgG binding in autoimmune diseases.

There is a possibility that this FcγRIIA polymorphism could also affect the binding and subsequent FcγR-mediated phagocytosis of the DENV immune complex. This could be an important contributing factor for susceptibility to ADE during secondary infection. Indeed, the H/H131 genotype was significantly associated with DF and DHF in Cuban individuals, relative to individuals with subclinical infection (Garcia et al, 2010). This was complemented by a separate study in which an association was reported between the R/R131 genotype and reduced risk of DHF/DSS in Vietnamese children (Loke et al, 2002).

The inhibitory FcγRIIB serves as a negative regulator of immune complex triggered activation, and suppresses autoimmunity by downregulating B cell responses and effector functions. Gene deletion studies have demonstrated that in H-$2^b$ mice, which are non-permissive to type II collagen induced arthritis (CIA), deletion of FcγRIIB was sufficient to render mice susceptible to CIA (Kleinau et al, 2000; Yuasa et al, 1999). The maximal arthritis index was comparable to DBA/1 mice, a strain of mice susceptible for CIA induction (Yuasa et al, 1999). CIA is a model for RA in humans. The development of arthritis is associated with high levels of

autoantibodies to synovial antigens, leading to leukocyte infiltration and induction of inflammatory cytokines such as TNF-α and IL-1 at the joints (Ji et al, 2002; Takai, 2002).

In addition to IgG-FcγR interactions that prompt the release of pro-inflammatory cytokines or induction of cytotoxicity against target cells, recent research has also suggested that the balance of activating and inhibitory FcγRs is important for modulating the type I IFN response programme in human monocytes and DCs (Dhodapkar et al, 2007). Manipulating this balance via antibody-mediated blockade of the inhibitory FcγRIIB in the presence of activating ligands has distinctive effects on gene expression and activation of human monocytes and DCs, including the induction of ISGs and inflammation-related cytokines and chemokines which, interestingly, was not associated with an increase in the expression of the type I IFNs, or addition of exogenous IFNs (Dhodapkar et al, 2007).

IFN-independent induction of ISGs is an example of how the innate immune system utilizes redundant induction pathways for the induction of type I IFNs and ISGs, thus serving as a countermeasure for the viral evasion strategy of IFN antagonism. Like other viruses, DENV has evolved to evade innate immunity by inhibiting various steps of the innate immune response through the expression of viral proteins that also serve to antagonize type I IFN production and signaling (Morrison et al, 2012). The proteolytic activity of the NS2B/3 complex has been shown to inhibit type I IFN production in human monocyte derived DCs by cleaving stimulator of the interferon gene (STING), an adaptor molecule that induces the IFN-β promoter (Aguirre et al, 2012). NS2A, NS4A and NS4B can also inhibit STAT-1

phosphorylation, which directly regulates type I IFN production (Munoz-Jordan et al, 2003). Finally, NS5, which encodes an RNA-dependent RNA polymerase, can also serve as an ISG antagonist in flaviviruses. DENV NS5 binds to and targets STAT-2 for proteasome-mediated degradation (Ashour et al, 2009). Similar mechanisms of NS5-mediated IFN antagonism have been observed in West Nile Virus (Laurent-Rolle et al, 2010) and Japanese encephalitis virus (Lin et al, 2006).

The induction of ISGs upon activating FcγR signaling is mediated by STAT-1 phosphorylation (Dhodapkar et al, 2007), and this is in turn likely activated by upstream adaptors of FcγR signaling such as spleen tyrosine kinase (Syk). Syk is a tyrosine kinase protein that is recruited by the cytoplasmic ITAMs upon ligation of the activating FcγRs. Binding of Syk to phosphorylated ITAM results in Syk phosphorylation and allows Syk to phosphorylate downstream substrates of the FcγR signaling cascade. Phosphorylated Syk has also been shown to directly bind and phosphorylate STAT-1, which stimulates production of IFN-α and ISGs (Dhodapkar et al, 2007; Tassiulas et al, 2004).

Multiple reports have indicated that co-ligation of the inhibitory FcγRIIB by DENV immune complexes requires high antibody concentration, and such co-ligation inhibited the entry of DENV immune complexes into monocytes (Boonnak et al, 2013; Chan et al, 2011). At low antibody concentrations where ADE occurs, the inhibitory FcγRIIB is not co-ligated (Chan et al, 2011). In accordance with observations by Dhodapkar et al., ligation of activating FcγRs by DENV opsonized with sub-neutralizing levels of antibody would thus induce the expression of ISGs. As ISGs are known to inhibit viral replication (Jiang et al, 2010), entry through activating

FcγR would place DENV in an intracellular environment unfavorable for enhanced replication. It is curious how enhancement of DENV infection is permitted, given that cross-linking of activating FcγRs signals an early antiviral response by induction of ISGs (Dhodapkar et al, 2007).

Like all other inhibitory immunoreceptors, the inhibitory FcγRIIB contains an ITIM in its cytoplasmic tail. Ligand engagement by ITIM-bearing receptors results in ITIM phosphorylation by Src and recruitment of phosphatases like SHP-1 and SHIP, which contribute to downregulation of activating pathway effectors. It is conceivable that in addition to FcγRIIB, the presence of other ITIM-bearing receptors on the cell surface may serve as a negative regulator of activating FcγR-mediated signaling if they are at close enough proximity to be co-ligated. However, this notion has not been examined before, and it will be interesting to investigate if engagement of other ITIM-bearing receptors could account for how DENV successfully evades the early antiviral response for enhanced infection.

## 1.5 Gaps in knowledge in FcγR signaling and ADE

This thesis seeks to delineate the distinct FcγR-mediated signaling cascades that are activated following ligation of activating FcγRs by DENV immune complex during ADE. The cross-linking of activating FcγRs has been shown to upregulate the expression of ISGs, unless the inhibitory FcγRIIB is co-ligated (Dhodapkar et al, 2007). Co-ligation of FcγRIIB by DENV immune complexes requires high antibody concentration, and leads to inhibition of FcγR-mediated uptake of the DENV immune complex (Chan et al, 2011). During ADE, the low antibody concentration is insufficient to form a large enough immune complex to co-ligate the inhibitory FcγRIIB (Chan et al, 2011). Activating FcγR-mediated uptake would thus place DENV in an intracellular environment unfavourable for enhanced replication. A mechanistic understanding of how DENV escapes the early induction of ISGs in the absence of FcγRIIB signaling is thus the main objective of this thesis.

The involvement of a variety of activating and inhibitory FcγR also raises a second possible effect that could regulate the outcome of ADE of DENV infection. Intracellular compartmentalization was recently suggested to be a form of cell-autonomous immunity. By trafficking pathogens to specific cellular compartments, the composition of compartments as well as the borders between them helps govern cellular self-defence, conferring effective protection against various pathogens (Randow et al, 2013). It is not known if internalization of DENV immune complexes by distinct activating FcγRs results in differential compartmentalization and thus intracellular fate of the DENV immune complex. The signaling pathways governing these differences in compartmentalization may provide insights on how DENV

subverts innate immunity for enhanced replication during ADE. This could constitute an “intrinsic ADE” event, shedding light on additional cell-intrinsic events which could enhance DENV replication.

The specific aims of this thesis are therefore:

1. Elucidate the FcγR signaling events during ADE of DENV infection in monocytes.
2. Investigate if ITIM-bearing cell surface receptors (other than FcγRIIB) could be involved in enabling DENV to evade the early antiviral response in monocytes.
3. Explore how DENV immune complexes could benefit from differential compartmentalization and how this would affect their intracellular fate.

# Chapter 2. LEUKOCYTE IMMUNOGLOBULIN-LIKE RECEPTOR B1 IS CRITICAL FOR ANTIBODY-DEPENDENT DENGUE

## 2.1 Introduction

Cross-linking of activating FcγRs would lead to induction of downstream signaling cascades and the upregulation of ISGs, unless the inhibitory FcγRIIB is co-ligated (Dhodapkar et al, 2007). Activating FcγR-triggered upregulation of ISG responses was found to be independent of type I IFN secretion, and this is hypothesized to lower the threshold for basal IFN signaling in monocytes (Dhodapkar et al, 2007; Taniguchi & Takaoka, 2001). Engagement of activating FcγRs thus serves as a constitutive signal which 'primes' the monocyte for a rapid and robust immune response, allowing the immune cell to overcome challenges to host defence, such as infection by pathogens (Taniguchi & Takaoka, 2001). FcγR usage or FcγR polymorphisms that affect the binding of immune complexes, factors that alter the balance of activating and inhibitory FcγR signaling, could have a direct impact on the level of basal IFN signaling in the absence of pathogens.

Ligation of activating FcγRs by antibody-opsonized DENV mediates viral entry into monocytes, and also triggers activating FcγR signaling during ADE. In accordance to findings by Dhodapkar et al, this should trigger upregulation of ISG expression, since the inhibitory FcγRIIB is not co-ligated during ADE (Chan et al, 2011; Dhodapkar et al, 2007). Entry through activating FcγR would thus place DENV in an intracellular environment unfavourable for enhanced replication, as ISGs are known to inhibit DENV replication (Jiang et al, 2010).

In this chapter, we seek to elucidate the early FcγR signaling events during ADE of DENV infection in monocytes. Furthermore, to address the conundrum of how DENV evades an early antiviral response in the absence of co-ligation of inhibitory FcγRIIB, we investigated if antibody-opsonized DENV could co-ligate other ITIM-bearing cell surface receptors to downregulate ISG expression for enhanced viral replication.

## 2.2 Materials and Methods

### 2.2.1 Cells

THP-1 and K562 was purchased from ATCC. THP-1 was subcloned by limiting dilution. From ~50 subclones, we selected two (THP-1.2R and THP-1.2S) which showed enhanced uptake of DiD-labelled DENV as compared to THP-1. Primary monocytes were isolated from different individuals under approval by National University of Singapore-Institutional Review Board (Approval Number: NUS 1584). Informed consent was obtained from all subjects. Primary monocytes were cultured as described previously (Zhang et al, 2010). Cell lines used were negative for mycoplasma contamination (Mycoalert, Lonza).

### 2.2.2 Viruses

DENV-1 (06K2402DK1), DENV-3 (05K863DK1) and DENV-4 (06K2270DK1) are clinical isolates from the EDEN study (Low et al, 2006). DENV-2 (ST) is a clinical isolate from the Singapore General Hospital. Viruses were propagated in the Vero cell line, harvested 96h post infection and purified through 30% sucrose. Virus pellets resuspended in HNE buffer were stored at −80 °C until use.

### 2.2.3 Virus infection

3H5 and 4G2 chimeric human/mouse IgG1 antibodies were constructed as previously described (Hanson et al, 2006). DENV was incubated with media, h3H5 (0.39µg/ml), h4G2 (1.56µg/ml) or serum for 1hr at 37˚C before adding to cells at indicated moi.

### 2.2.4 Virus uptake and replication

Uptake was assessed using DiD or Alexa 488 labelled DENV as previously described (Chan et al, 2011; Zhang et al, 2010). Virus replication was assessed at indicated time-points using qPCR. Cells were washed thrice in PBS, followed by RNA extraction using RNAeasy kit (Qiagen), cDNA synthesis (Biorad) and real-time qPCR (Roche) according to manufacturer's protocol. DENV primers used were:

DEN-F 5'-TTGAGTAAACYRTGCTGCCTGTAGCTC,

DEN-R 5'-GAGACAGCAGGATCTCTGGTCTYTC.

Primers used for ISGs and GAPDH were from Origene, and all RNA levels were measured relative to GAPDH. At 72hpi, virus in the culture supernatant was quantified with plaque assay (Chan et al, 2011).

### 2.2.5 Microarray analysis

Following RNA extraction, microarray was performed at the Duke-NUS Genome Biology Core Facility. cRNAs were hybridized to Illumina Human HT-12 v4 Beadchips, according to manufacturer's instructions. Data analysis was performed using Partek software and normalized against GAPDH.

### 2.2.6 Flow cytometry

Cells were resuspended in staining buffer (10% fetal calf serum, 15mM HEPES and 2mM EDTA in PBS). To $1x10^6$ cells per well, human FcγR block (1:10, eBioscience 14-9161) was added and incubated for 25min on ice, followed by a single wash with staining buffer. Subsequently, human monoclonal antibodies, Anti-HLA-

A,B,C(W6/32)FITC (1:25, Biolegend); Anti-CD206(19.2)PE (1:25, BD Pharmingen); Anti-CD274/PD-L1(MIH1)PE (1:50, BD Pharmingen); Anti-CD86(FUN-1)PE-Cy7 (1:50, BD Pharmingen); Anti-CD11b(ICRF44)PE-Cy7 (1:100, BD Pharmingen); Anti-HLA-DR(L243)APC (1:200, BD Pharmingen); Anti-CD11c(S-HCL-3)APC (1:200, BD Pharmingen); Anti-CD80(L307.4)AF700 (1:25, BD Pharmingen); Anti-CD40(5C3)AF700 (1:50, BD Pharmingen); Anti-CD14(M5E2)Pacific Blue (1:100, BD Pharmingen); Anti-LILRB4(ZM 4.1)PE (1:50, eBioscience); 7-AAD Viability Staining Solution (1:25, eBioscience) and Anti-CD16 (CB16)eFluor®605NC (1:20, eBioscience) were incubated with cells for 30 minutes on ice. Anti-LILRB1 (1:200, Abcam ab67532) was incubated with cells for 30 minutes on ice before Alexa 488-conjugated anti-mouse secondary antibody (1:200, A-21202, Life Technologies) was added. Flow cytometry was performed with LSRFortessa™ cell analyzer (Becton Dickinson), acquired with BD FACSDiva and analyzed using FlowJo. Calibration was completed with SPHERO™ Rainbow Calibration Particles (Sphereotech Inc) to maintain consistency between experiments and to remove background fluorescence.

### 2.2.7 Immunoprecipitation and Western blotting

After incubation at indicated time points with DENV-2 or h3H5-opsonized DENV-2, cells were washed once in PBS and resuspended in lysis buffer (1% Nonidet P-40, 150mM NaCl, 50mM Tris, pH 8.0) in the presence of protease inhibitors (Sigma). Total cell extract (100µg) was incubated with mouse anti-Syk monoclonal antibody (1µg, 4D10, Abcam ab3113) at 4°C overnight and then with 50µl of Protein G–sepharose (Pierce) for 2h. For Western blot, proteins were separated by SDS-PAGE,

transferred to PVDF (Millipore) and probed with primary antibody, followed by HRP-conjugated anti-mouse (1:1000, Dako P0447) or anti-rabbit (1:3000, Abcam ab6721) antiserum. Primary antibodies for LAMP-1 (1:500, eBioscience 611043), LILRB4 (1:500, Biolegend 333002), FcγRIIB (1:500, Abcam ab123240), LILRB1 (1:500, Abcam ab67532), SHP-1 (1:500, Abcam ab2020), pSHP-1 (1:500, Abcam ab51171), Syk (1:1000, Abcam ab3113), 4G10 (1:500, Millipore #05-321), MAVS (1:1000, Abcam ab25084), GAPDH (1:3000, Abcam ab8245), IRF3 (1:3000, Cell Signaling 4302) and TRIF (1:3000, Cell Signaling 4596) were used. Thereafter, blots were developed by enhanced chemiluminescence detection reagents (Amersham).

### 2.2.8 Interferon treatment

THP-1.2S was treated with 500 U/ml IFN-α (Millipore) 30min after incubation with h3H5-opsonized DENV-2. ISG expression was assayed 6hpi using real-time qPCR.

### 2.2.9 Receptor blocking

$2x10^5$ cells/ml were pre-treated with 15µg/ml of anti-interferon alpha receptor (IFNAR), 10µg/ml of anti-LILRB1 or their respective isotype controls for 1hr at 4ºC. Subsequently, cells were washed once with maintenance media before adding h3H5-opsonized DENV-2, DENV-2 or 500 U/ml IFNα.

### 2.2.10 Drug assays

$2x10^5$ cells/ml of THP-1.2R were pre-treated for 6hrs with piceatannol (15.6 µg/ml) or DMSO control before adding DENV-2 or h3H5-opsonized DENV-2. For sodium

stibogluconate (0.138 mM) treatment, $2x10^5$ cells/ml of primary monocytes were treated for 6hrs before adding DENV or h4G2-opsonized DENV. Cell viability was assessed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega) according to manufacturer's protocol.

### 2.2.11 Cloning and competition with soluble LILRB1

The extracellular domain of LILRB1 was amplified from cDNA derived from Origene (SC127469) using the primer pairs: 5'-CTAGGCGGCCGCATGCATCATCACCATCACCACATTGAAGGGCGCACCCC CATCCTCACGGTC-3' and 5'-CTAGGCGGCCGCCTAGTGCCTTCCCAGACCACTC-3'. The purified PCR fragments were then digested with *Not*I and ligated into pCMV-XL5 (Origene). To express the protein, these cloned products were transiently transfected in HEK293T with 2µg of the DNA in 6-well plates using JetPRIME® transfection reagent, as described by the manufacturer (Polyplus). 48 hours after transfection, cell pellets were resuspended in ice-cold lysis buffer for 30min, followed by centrifugation at 14,680 rpm for 10min at 4ºC. Proteins were subsequently purified with HisPur™ Cobalt Purification kit (Thermo Scientific) according to manufacturer's protocol. Following which, the eluted fractions were desalted using Zeba™ spin desalting columns (Thermo Scientific) and analysed for yield and purity using SDS-PAGE and Western blot. For competition studies, DENV-2 or h3H5-opsonized DENV-2 were incubated with indicated concentrations of the purified extracellular domain of LILRB1 or

200μM BSA for 1h at 37ºC before adding to THP-1.2S. Infectious titers were assessed using plaque assay 72 hpi.

### 2.2.12 siRNA transfection and overexpression

Knockdown and overexpression studies were performed as previously described (Chan et al, 2011). siRNA targeting FcγRIIB (Qiagen), LILRB1, MAVS, IRF3 and TRIF (SABio) were used while overexpression studies were performed with either empty plasmid, plasmid encoding LILRB1 (Origene) or tyrosine mutant LILRB1, or LILRB4. To generate mutant LILRB1, DNA fragments with nucleotides mutated from tyrosine to phenylalanine in the ITIM tail were synthesized by Bio Basic Canada. Thereafter, restriction enzymes *SbfI* and *BsmI* (New England Biolabs) were used to clone the DNA fragment into the plasmid originally encoding for LILRB1.

### 2.2.13 ELISA

Viruses were coated in MaxiSorp™ plate overnight at 4°C. BSA (0.5 ug per well) or PBS were used as negative controls. Blocking was done with 5% skimmed milk at room temperature (RT) for 2h. Plate was washed with PBS before incubation with indicated concentrations of purified LILRB1 (Origene) diluted in PBS at 37°C for 1h, followed by RT for 1h. After washing with PBS-T (PBS, 0.1% Tween 20), anti-LILRB1 pAb (1:1000, Abcam ab3113) was added to wells for 2h at RT followed by incubation with HRP-conjugated anti-mouse IgG (1:1000, Dako P0447) at RT for 1h. Antibody binding was visualized by addition of 50μl of TMB followed by addition of 50 μl of 1M HCl. The absorbance was read at 450 nm using a plate reader.

### 2.2.14 Statistical analysis

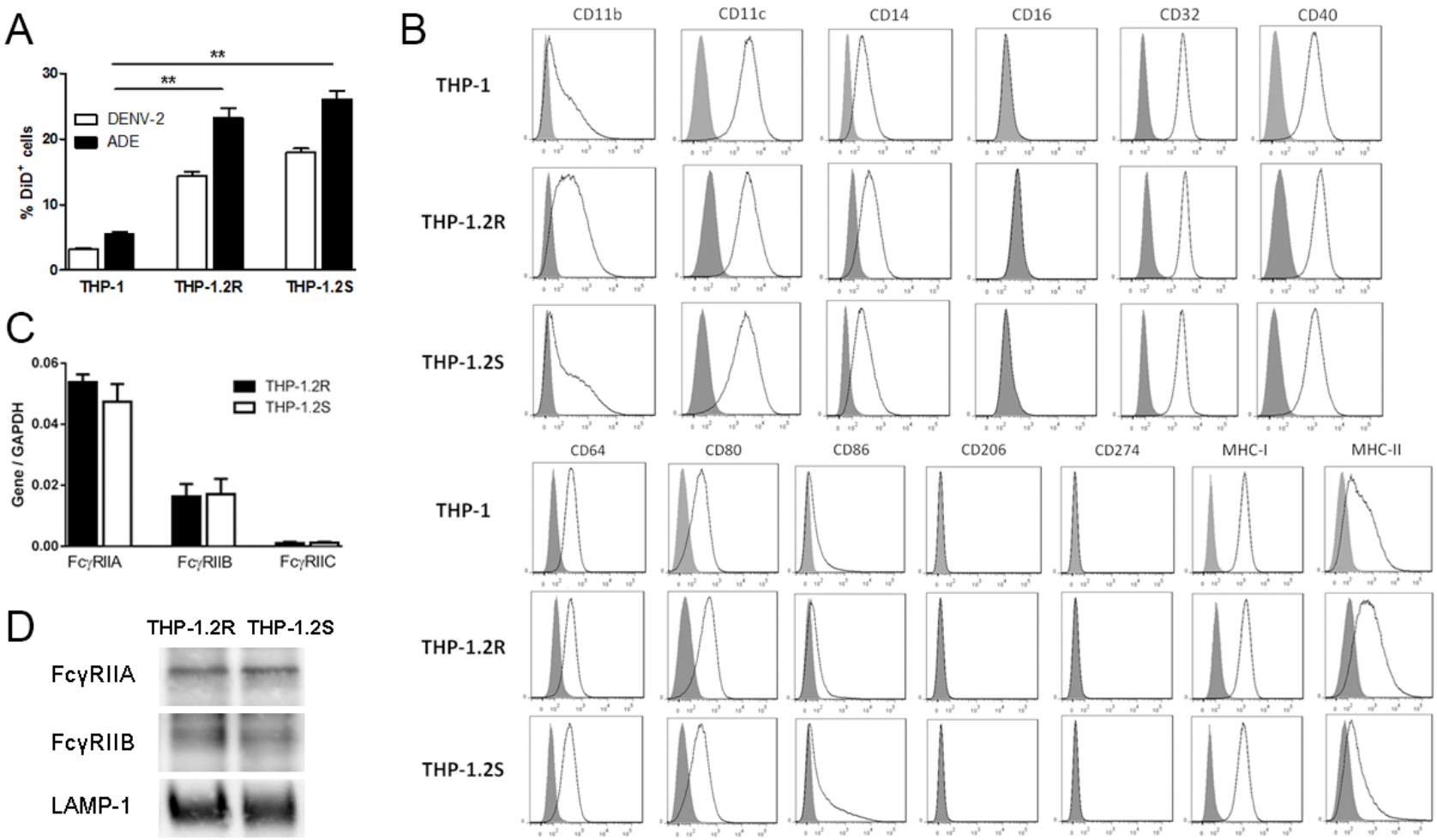
All experiments were conducted with at least 3 biological replicates and repeated at least twice. To compare between any two means, two-tailed unpaired Student t test was performed using GraphPad Prism v5.0 (GraphPad Software Inc) ($P<0.05$).

## 2.3 Results

### 2.3.1 Isolation of THP-1 subclones with increased uptake of DENV immune complex

Our work was enabled by the isolation of subclones of THP-1 cells with different phenotypes to ADE. The low rate of FcγR-mediated phagocytosis in THP-1 cells (~5%) (Chan et al, 2011) had led us to reason that this cell line is genetically heterogeneous, either through the method in which it was derived (Tsuchiya et al, 1980) or through genetic instability resulting from aneuploidy (Sheltzer et al, 2011). Using limiting dilution and *in vitro* expansion, we obtained around 50 subclones that were screened for enhanced phagocytic uptake of DENV immune complexes.

Screening of our newly isolated subclones with DiD (1, 1'-dioctadecyl-3, 3, 3', 3' – tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) labelled DENV-2 alone or opsonized with sub-neutralizing concentrations of humanized 3H5 monoclonal antibody (h3H5) identified two clones (labelled as THP-1.2R and THP-1.2S) that showed increased uptake of DENV immune complexes compared to parental THP-1 (Fig. 2-1A). Monocyte surface marker analysis indicated no significant difference in the expression of FcγRs (FcγRI/CD64, FcγRII/CD32, FcγRIII/CD16) in these sub-clones (Fig. 2-1B). Expression of FcγRIIA, FcγRIIB and FcγRIIC were similar in these subclones, as determined by qPCR (Fig. 2-1C) and Western blot (Fig. 2-1D). Both subclones were also heterozygous for 131H/R FcγRIIA polymorphism (Fig. 2-1E and F). Identical HLA haplotyping confirmed that both subclones were derived from THP-1 and not the result of a contamination with another cell line (Table 2-1).


A
**
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30
20
10
0
% DiD+ cells
DENV-2
ADE
THP-1
THP-1.2R
THP-1.2S
B
CD11b
CD11c
CD14
CD16
CD32
CD40
THP-1
THP-1.2R
THP-1.2S
CD64
CD80
CD86
CD206
CD274
MHC-I
MHC-II
THP-1
THP-1.2R
THP-1.2S
C
0.06
0.04
0.02
0.00
Gene / GAPDH
THP-1.2R
THP-1.2S
FcγRIIA
FcγRIIB
FcγRIIC
D
THP-1.2R
THP-1.2S
FcγRIIA
FcγRIIB
LAMP-1

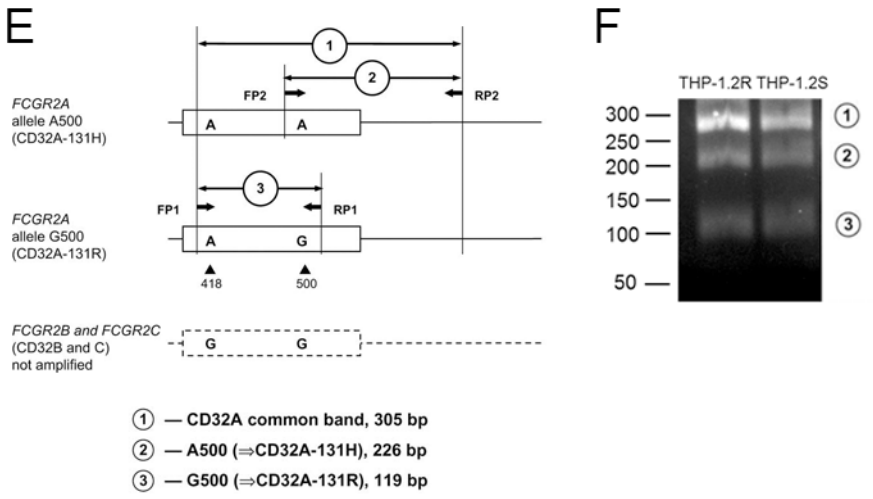


**Figure 2-1. Isolation of 2 THP-1 subclones with increased uptake of dengue immune complex.** (A) Percentage of internalized DiD-labelled DENV-2 30min post-infection under DENV-2 or ADE conditions in THP-1, THP-1.2R and THP-1.2S. (B) Flow cytometry analysis of THP-1, THP-1.2R and THP-1.2S stained with antibodies specific for monocyte surface markers. Live unstained control cells are shaded grey while stained cells have an open histogram. (C) Transcript levels of FcγRIIA, FcγRIIB, FcγRIIC in THP-1.2R and THP-1.2S, determined by qPCR. (D) Protein levels of FcγRIIA and FcγRIIB in THP-1.2R and THP-1.2S, determined by Western blot. LAMP-1 served as a loading control. (E) Schematic showing polymerase chain reaction with confronting two-pair primers method for genotyping of FcγRIIA (131H/R). Using the different specific primers, FcγRIIA, FcγRIIA-131H, FcγRIIA-131R can be detected as 305bp, 226bp and 119bp bands respectively. This figure is adapted from (Vilches et al, 2008). (F) Results obtained with THP-1.2R and THP-1.2S based on schematic presented in (E). Data expressed as mean ± s.d. from three independent experiments. ** $P < 0.01$. Panel B was kindly provided by Nivashini Kaliaperumal and John E. Connolly.

**Table 2-1. HLA haplotyping for THP-1.2R and THP-1.2S** (Data from Prof Chan Soh Ha's Laboratory)

| Name of cell line | HLA-A | HLA-A' | HLA-B | HLA-B' | HLA-DR | HLA-DR |
|---|---|---|---|---|---|---|
| **THP-1.2R** | 02G1 | - | 1511 | - | 0101/18/20/24 | 1501/02/05/07 |
| **THP-1.2S** | 02G1 | - | 1511 | - | 0101/18/20/24 | 1501/02/05/07 |
| **THP-1** | 02G1 | - | 1511 | - | 0101/18/20/24 | 1501/02/05/07 |

### 2.3.2 ADE differs in THP-1 subclones

Despite no significant differences in uptake and production of plaque titers when infected with DENV-2 only, infection under ADE conditions resulted in significantly different DENV-2 titers in THP-1.2R and THP-1.2S (Fig. 2-2A). Similar observations were also made with enhancing titers of convalescent serum (Fig. 2-2B) or other DENV serotypes (Fig. 2-2C). Furthermore, early DENV RNA replication diverged in these two subclones where a significant difference was observed as early as 6 hours post-infection (Fig. 2-2D). Analysis of early gene expression indicated significant upreulation of ISGs in THP-1.2R but not THP-1.2S (Fig. 2-3A to E). These included MX1, MX2, and viperin, which are potent inhibitors of DENV replication (Jiang et al, 2010). The upregulation of ISGs in THP-1.2R, however, was not due to h3H5 (Fig. 2-3F and G) and is independent of interferon (IFN)-α, β and γ signaling as both subclones expressed similar IFN transcript levels (Fig. 2-4A). As expected, addition of antibodies that blocked IFNα receptor (IFNαR) signaling (Fig. 2-4B) did not reduce this early ISG induction in THP-1.2R following infection (Fig. 2-4C). The possibility that THP-1.2S had impaired IFNαR-mediated signaling was also excluded, as ISGs were significantly upregulated in response to exogenous IFN (Fig. 2-4D). These subclones thus serve as exquisite tools to decipher the signaling requirement to overcome the early antiviral responses for successful ADE.

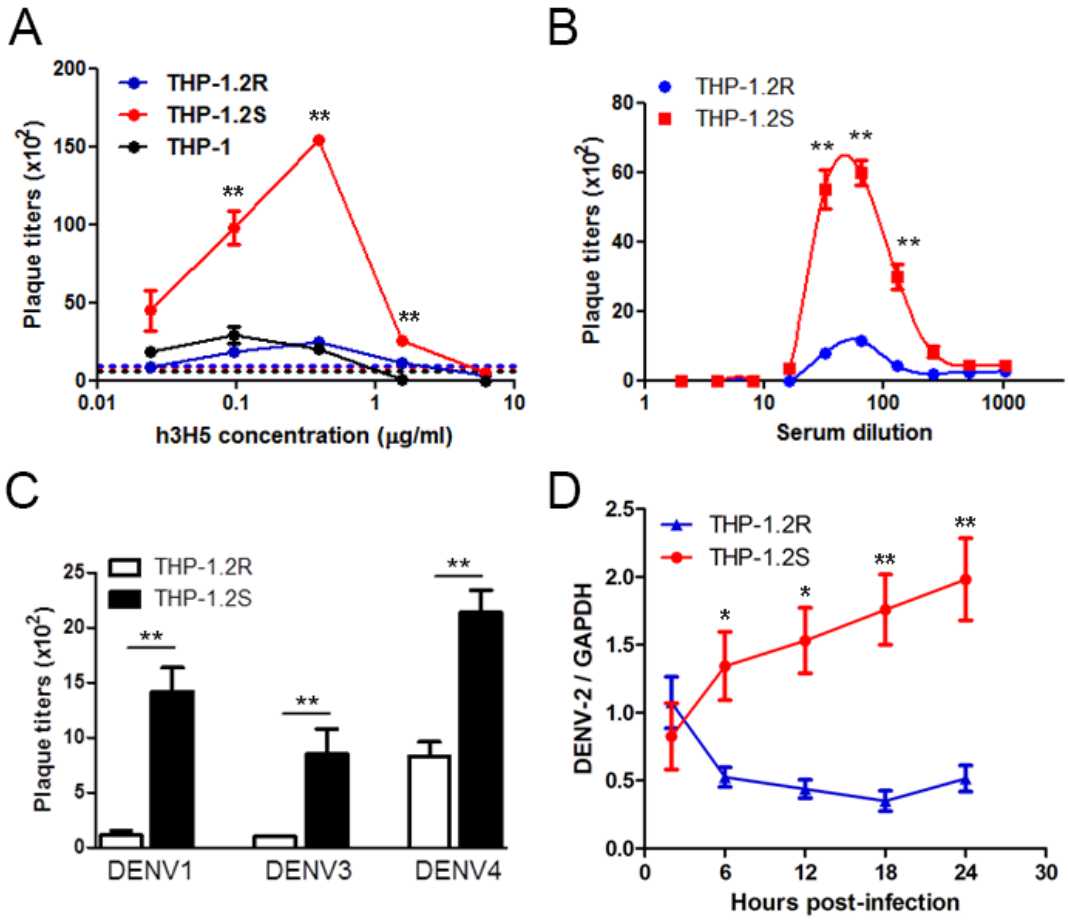


**Figure 2-2. ADE differs in THP-1 subclones.** (A) Plaque titers of THP-1, THP-1.2R or THP-1.2S when infected with DENV-2 opsonized with different h3H5 concentrations 72 hours post-infection (hpi). Dotted lines indicate plaque titers following DENV-2 only infection, with no significant differences observed between the cell lines. (B) Infectious titer of DENV-2 in the culture supernatant of THP-1.2R (blue) and THP-1.2S (red) incubated with DENV-2 (moi 10) reacted with serial two-fold dilutions of convalescent serum. (C) Peak enhancement titers for DENV-1, DENV-3 or DENV-4 (moi 10) opsonized with h4G2 in THP-1.2R or THP-1.2S. Plaque titers were determined 72hpi. (D) Time course of viral RNA copy numbers in THP-1.2R or THP-1.2S under ADE conditions. Data expressed as mean ± s.d. from three independent experiments. ** $P < 0.01$, * $P < 0.05$.

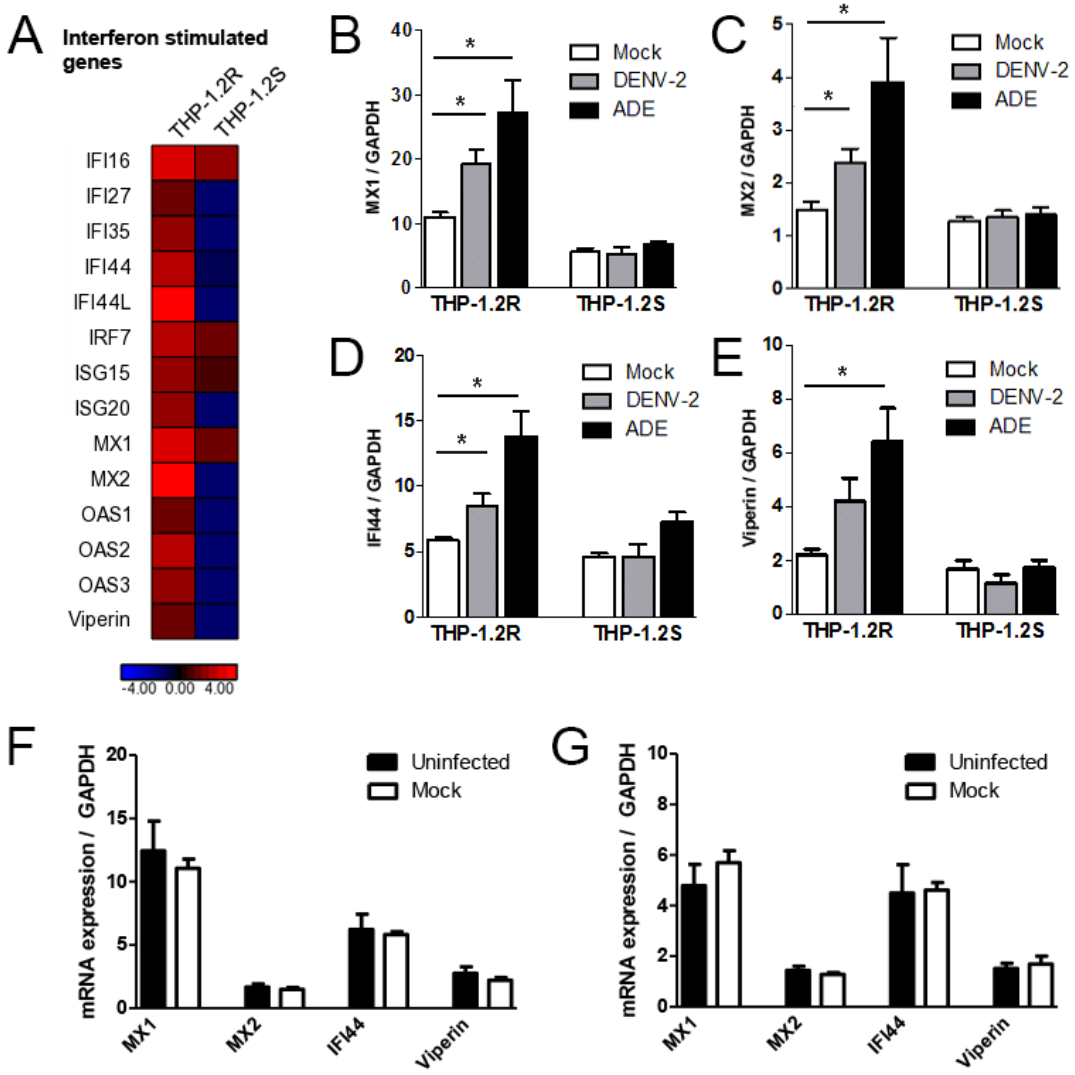


**Figure 2-3. ISGs are upregulated in THP-1.2R.** (A) Heat map showing fold change of ISG expression in THP-1.2R and THP-1.2S at 6hpi under ADE conditions. (B-E) Validation of microarray data in (A) by qPCR. (F) Expression of ISGs 6hpi in THP-1.2R that were either uninfected or mock treated (h3H5 only, without DENV-2). (G) Expression of ISGs 6hpi in THP-1.2S that were either uninfected or mock treated (h3H5 only, without DENV-2). Data expressed as mean ± s.d. from three independent experiments. * $P < 0.05$.

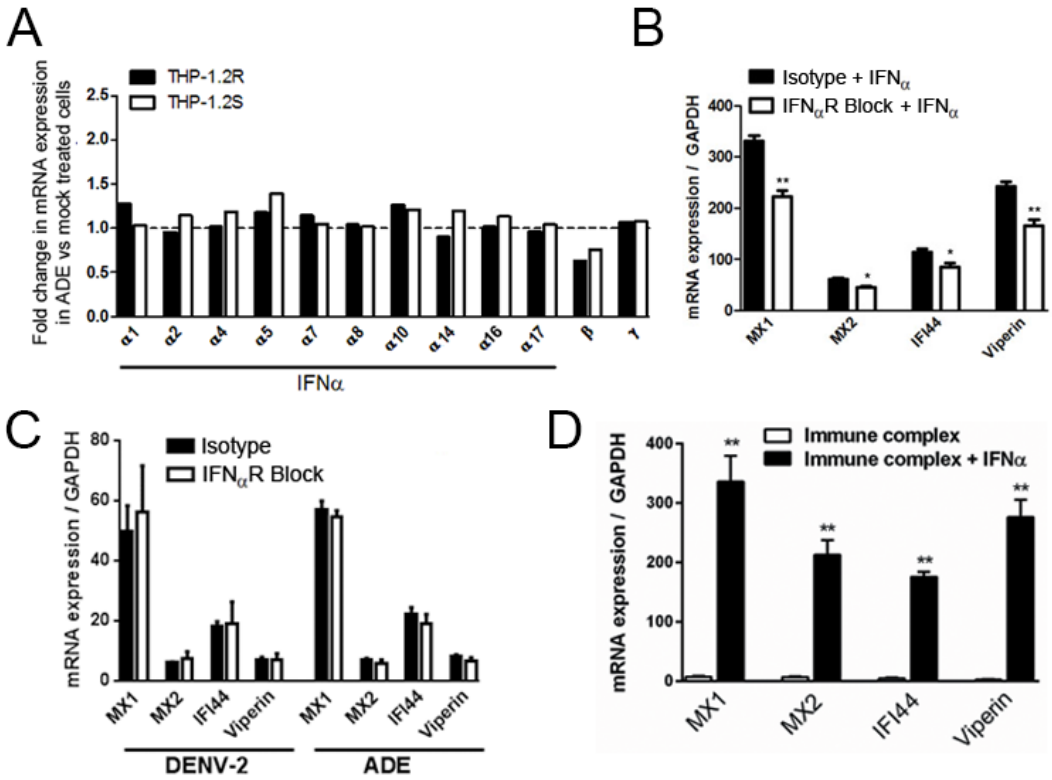


**Figure 2-4. IFN signaling contributes minimally to ISG induction.** (A) Fold change in transcript levels of interferons in THP-1.2R and THP-1.2S 6hpi under ADE conditions. (B) ISG expression in THP-1.2R in the presence of isotype or anti-IFNαR antibodies under 500 U/ml IFN-α treatment. (C) ISG (MX1, MX2, IFI44, viperin) expression in THP-1.2R in the presence of isotype or anti-IFNαR antibodies under DENV-2 or ADE conditions. (D) THP-1.2S treated with or without 500 U/ml IFN-α 30min after incubation with h3H5-opsonized DENV-2. All ISG expression was assayed 6hpi using real-time qPCR. Data expressed as mean ± s.d. from three independent experiments. ** $P < 0.01$, * $P < 0.05$.

2.3.3 Early ISG expression during ADE is independent of RIG-I/MDA5 signaling

Differences in viral entry through ADE and DENV-2 only conditions could have resulted in different intracellular antigenic load and hence resulted in differential ISG expression in the subclones. To identify the specific signaling pathway responsible for early ISG induction in THP-1.2R during ADE infection, we titrated the multiplicity of infection (moi) for DENV-2 only that resulted in equivalent level of infection as ADE (moi 10) to serve as an antigenically equivalent control (Fig. 2-5A and B). Interestingly, lower and higher plaque titers were observed in THP-1.2R and THP-1.2S, respectively, during ADE relative to DENV-2 only (moi 60) conditions (Fig. 2-5C), which corroborates the notion that THP-1.2R has reduced susceptibility to ADE. Immunofluorescence imaging showed nuclear translocation of pSTAT-1 at 3 hours post ADE in THP-1.2R but not in THP-1.2S or during antigenically equivalent DENV only infection (Fig. 2-5D). This early nuclear translocation of pSTAT-1 is transient as little co-localization could be observed at 6 hours post infection.

With similar intracellular antigenic load in ADE and DENV-2 only conditions, we determined if trafficking of DENV-containing phagosomes to cellular compartments enriched with pattern recognition receptors was an explanation for ISG induction in THP-1.2R. This was not the case as reduced expression of adaptor molecules (mitochondrial antiviral signaling protein/MAVS and interferon regulatory factor 3/IRF3) of retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated protein 5 (MDA5) resulted in significantly increased early DENV replication under DENV-2 only but not ADE conditions (Fig. 2-5E). Reduced TIR-domain containing adapter-inducing interferon β (TRIF) did not result in significant change in DENV replication under either condition (Fig. 2-5E). Collectively, these

results indicate that the early induction of ISG in THP-1.2R is unique to infection under ADE condition and is not mediated by RIG-I/MDA5 dependent type-I interferon expression.

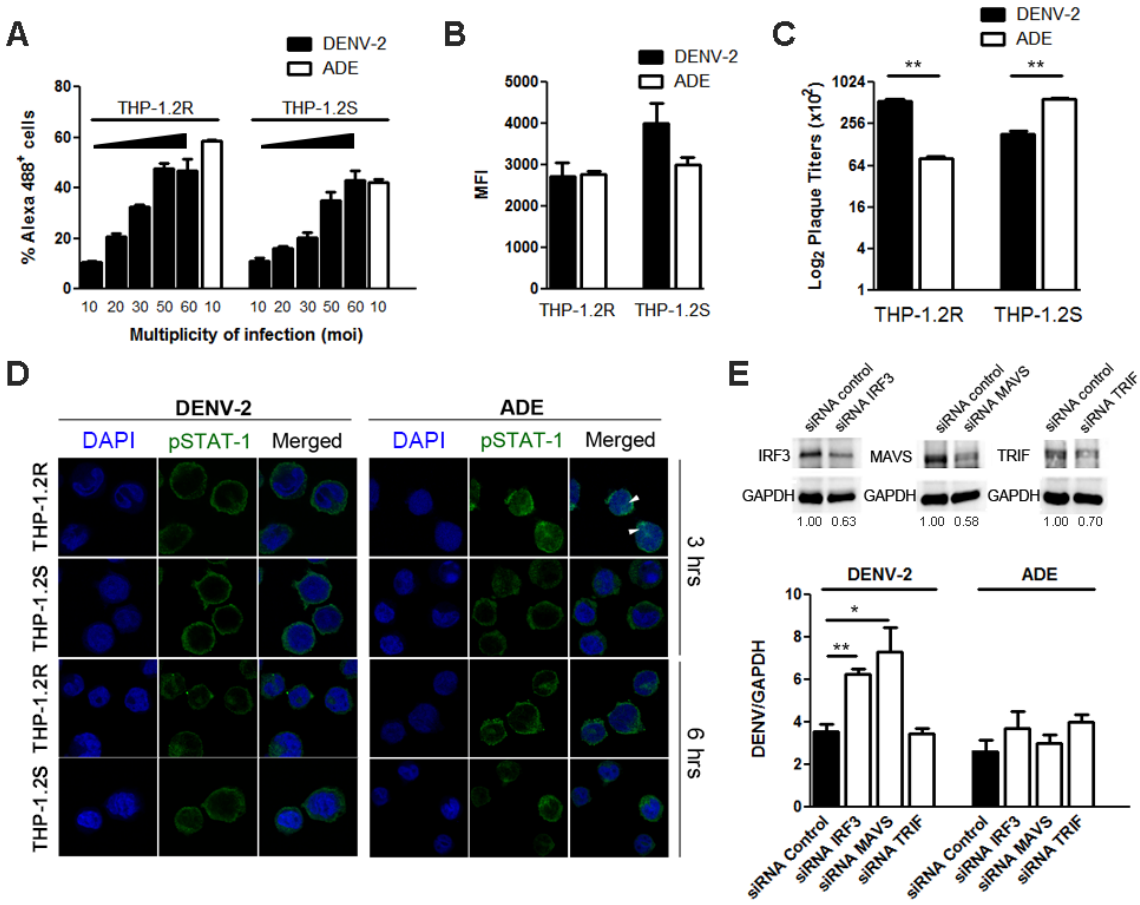


**Fig. 2-5. Early ISG induction during ADE is independent of RIG-I/MDA5-contingent IFN signaling.** (A) Uptake of Alexa 488-labelled DENV-2 under virus only (moi 10 to 60) and ADE (moi 10) conditions 6hpi. (B) Mean fluorescence intensity under virus only (moi 60) and ADE (moi 10) conditions 6hpi. All subsequent experiments were performed under DENV-2 only (moi 60) or ADE (moi 10) conditions. (C) Plaque titers of THP-1.2R and THP-1.2S when infected with DENV-2 only or ADE conditions. (D) Co-localization of pSTAT-1 with DAPI 3hpi and 6hpi under DENV-2 only or ADE conditions. (E) Viral RNA expression determined 6hpi in siRNA treated cells infected under DENV-2 only or ADE conditions. Data expressed as mean ± s.d. from three independent experiments. ** $P < 0.01$, * $P < 0.05$.

### 2.3.4 Activating FcγR-signaling mediates early ISG induction during ADE

The independence of ISG expression from RIG-I/MDA5-mediated signaling thus suggests that activating FcγR signaling (Dhodapkar et al, 2007) through spleen tyrosine kinase (Syk) activation (Tassiulas et al, 2004) is critical in THP-1.2R. We thus quantified Syk activation by Western blot with densitometric measurements. Significant difference in Syk phosphorylation was observed as early as 10min post-infection under ADE but not DENV-2 only conditions in THP-1.2R (Fig. 2-6A). In contrast, no significant difference in Syk phosphorylation was observed under DENV-2 only and ADE conditions in THP-1.2S. Pre-treatment of THP-1.2R with piceatannol, a Syk-selective tyrosine kinase inhibitor resulted in greater reduction of ISG expression under ADE conditions (Fig. 2-6B) and a correspondingly greater increase in DENV replication (Fig. 2-6C) compared to DENV-2 only. Increase in DENV replication was also greater in THP-1.2R than THP-1.2S. These findings suggest that early ISG expression in THP-1.2R is conditioned upon activating FcγR signaling through phosphorylated Syk (Dhodapkar et al, 2007).

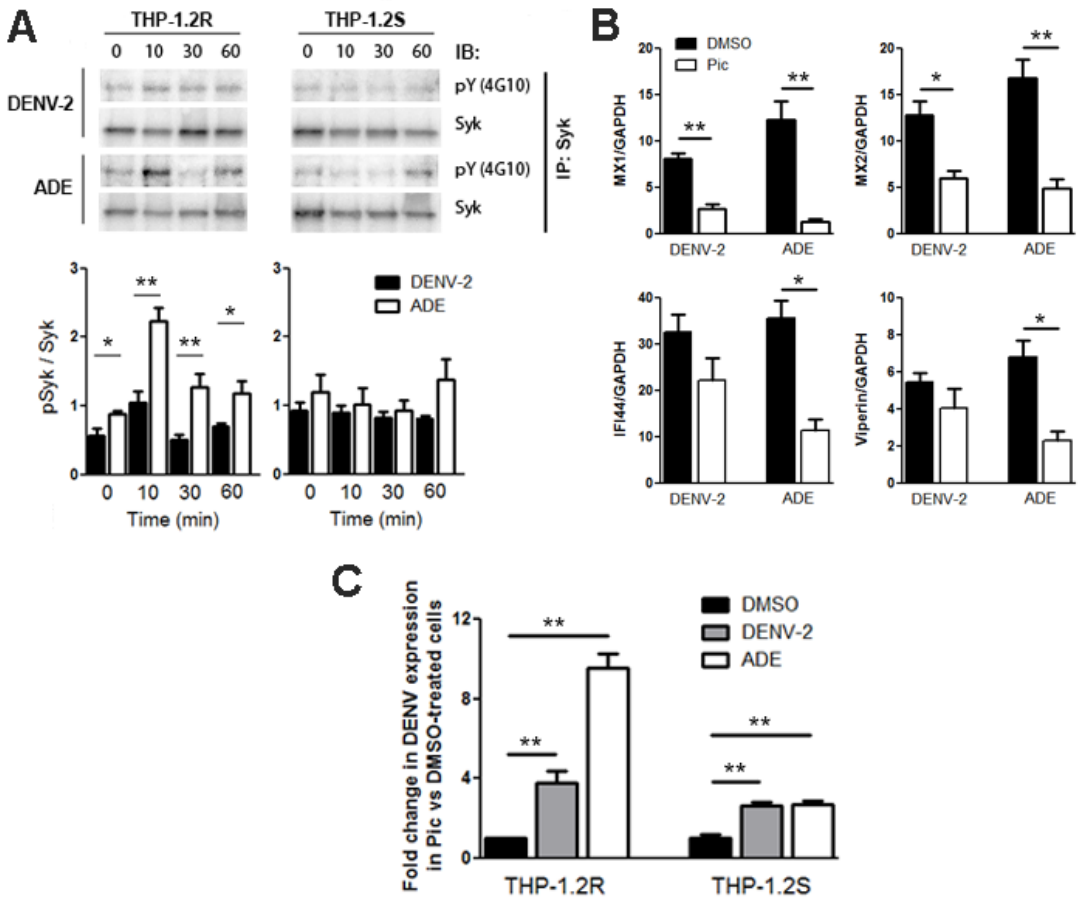


**Figure 2-6. Early ISG induction following ADE requires Syk phosphorylation.** (A) Western blot and quantitative densitometry of pSyk levels using immunoprecipitation with Syk antibody. (B) ISG expression in DMSO- or piceatannol-treated (15.6µg/ml) THP-1.2R under DENV-2 only or ADE conditions 6hpi. (C) Fold change in DENV RNA copy numbers in THP-1.2R and THP-1.2S pre-treated with piceatannol relative to DMSO control. Data expressed as mean ± s.d. from three independent experiments. ** $P < 0.01$, * $P < 0.05$.

### 2.3.5 Identification of LILRB1 as a co-receptor for inhibition of ISG induction

As activating FcγR signals through immunoreceptor tyrosine-based activation motif (ITAM), we postulated that DENV co-ligates an immunoreceptor tyrosine-based inhibition motif (ITIM)-bearing receptor to inhibit Syk activation (Steevels & Meyaard, 2011) in THP-1.2S. Examination of the gene expression data identified two such possible receptors. LILRB1 (also known as CD85j or immunoglobulin-like transcript-2) and LILRB4 were upregulated pre-infection in THP-1.2S relative to THP-1.2R (Fig. 2-7A). Flow cytometry analysis, however, showed that only LILRB1 (Fig. 2-7B and C) displayed higher surface expression on THP-1.2S. Since one of the effects of ITIM phosphorylation is the recruitment and phosphorylation of SHP-1 (Fanger et al, 1998; Scharenberg & Kinet, 1996), we measured phosphorylated SHP-1 in the two subclones. Higher pSHP-1 levels were found in THP-1.2S than THP-1.2R under ADE conditions (Fig. 2-7D and E), suggesting that pSHP-1 dephosphorylated Syk in THP-1.2S.

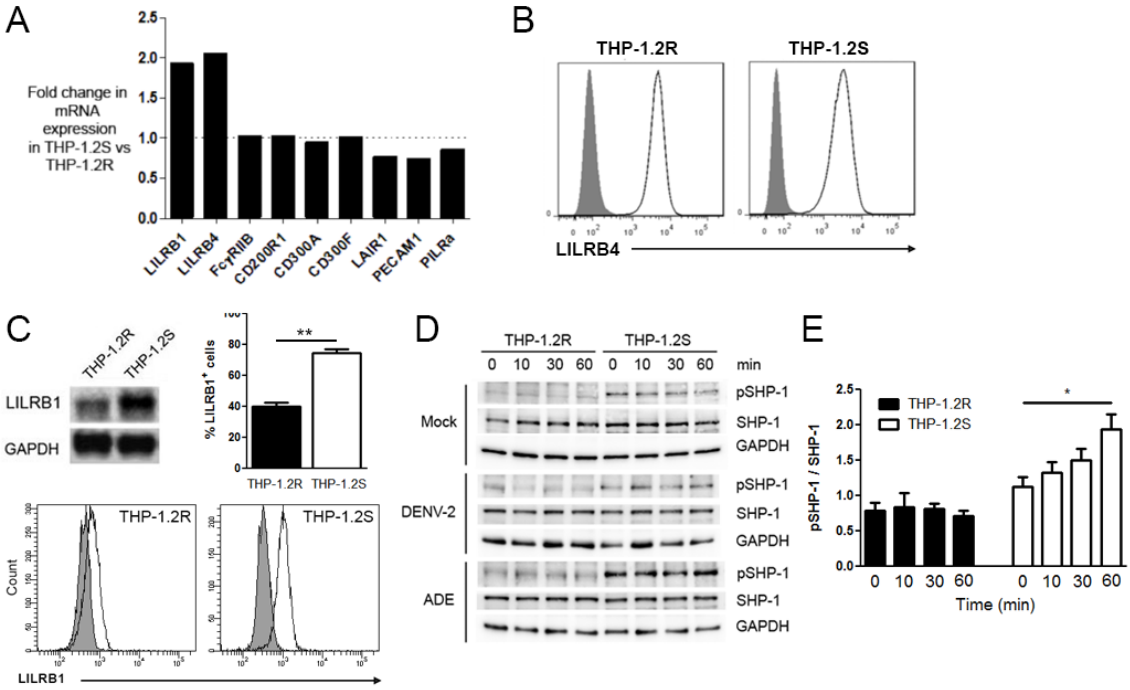


**Figure 2-7. LILRB1 signals through phosphorylated SHP-1 to downregulate ISG induction.** (A) Relative expression of ITIM-bearing receptors in THP-1.2S and THP-1.2R determined by microarray. (B) Surface expression of LILRB4 in THP-1.2R and THP-1.2S determined using flow cytometry. (C) Western blot, % LILRB1$^{+}$ cells, and representative flow cytometry plots of LILRB1 in THP-1.2R and THP-1.2S. Cells were either stained with isotype (grey) or polyclonal anti-LILRB1 antibody (open histogram). (D) Western blot of pSHP-1, SHP-1 and GAPDH at different time points after infection under mock, DENV-2 only and ADE conditions. (E) Quantitative densitometry of pSHP-1 levels under ADE conditions. Data expressed as mean ± s.d. from three independent experiments. ** $P < 0.01$, * $P < 0.05$.

### 2.3.6 Co-ligation of LILRB1 is required for ADE

If LILRB1 is necessary for ADE, then antibody-opsonized dengue should co-ligate LILRB1. Indeed, all 4 DENV serotypes bind to LILRB1, more strongly with whole virus than with E protein ectodomain (Fig. 2-8A and B), suggesting that LILRB1 binds to a quaternary structure-dependent epitope. Furthermore, the addition of soluble extracellular domain of LILRB1 (Fig. 2-8C) successfully competed with native LILRB1 on THP-1.2S to reduce ADE but not DENV-2 only infection in a dose-dependent manner (Fig. 2-8D). As expected, soluble LILRB1 ectodomain did not alter the rate of viral entry as this receptor functions by modulating the antiviral state of the cell rather than increasing DENV entry (Fig. 2-8E and F).

Likewise, reduced LILRB1 expression in THP-1.2S resulted in reduced DENV replication under ADE conditions (Fig. 2-9A), without altering the rate of viral entry (Fig. 2-9B). The lack of any change in DENV replication with FcγRIIB expression also reinforces the notion that sub-neutralizing levels of antibody are insufficient to aggregate DENV to co-ligate FcγRIIB (Chan et al, 2011). Similar observations were made with knockdown of LILRB1 expression in another unrelated human myelogenous leukemia cell line, K562 (Fig. 2-9C and D).

Conversely, over-expression of LILRB1 in THP-1.2R resulted in increased DENV replication under ADE conditions (Fig. 2-9E). As a control, we also over-expressed LILRB4 but this did not result in increased DENV replication. Critically, mutation of the 4 tyrosine residues in the ITIM tail to phenylalanine (Fig. 2-10) abrogated the increased DENV replication (Fig. 2-9E). Taken collectively, these findings indicate that DENV co-ligates LILRB1 to inhibit FcγR-activated early ISG expression for ADE.

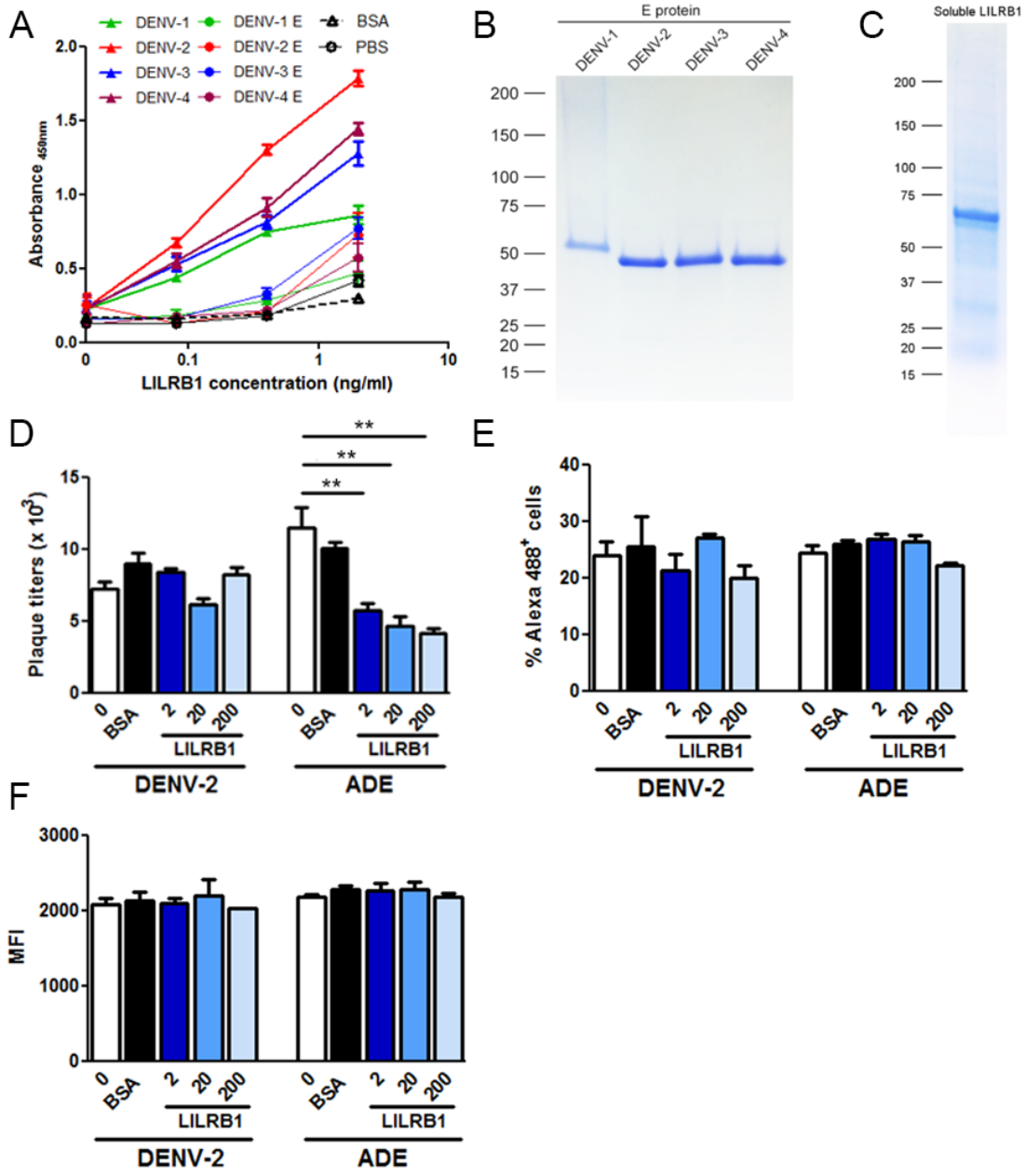


**Figure 2-8. Antibody-opsonized DENV co-ligates LILRB1.** (A) Binding of LILRB1 to whole DENV or DENV E protein ectodomain. (B) Coomassie blue staining of DENV E protein ectodomain used for ELISA assay in (A). (C) Coomassie blue staining of soluble LILRB1 ectodomain used for competition assay in (D). (D) Plaque titers following DENV-2 or ADE infection in the presence of soluble LILRB1 ectodomain (2µM, 20µM, 200µM), 200µM BSA, or no protein control. (E) Percentage positive uptake and (F) MFI of Alexa 488-labelled DENV-2 6hpi in the presence of various concentrations of soluble LILRB1 ectodomain (2µM, 20µM, 200µM), 200µM BSA or no protein control. Data expressed as mean ± s.d. from three independent experiments. ** $P < 0.01$. Panel A was kindly provided by Qian Zhang and Shee Mei Lok.

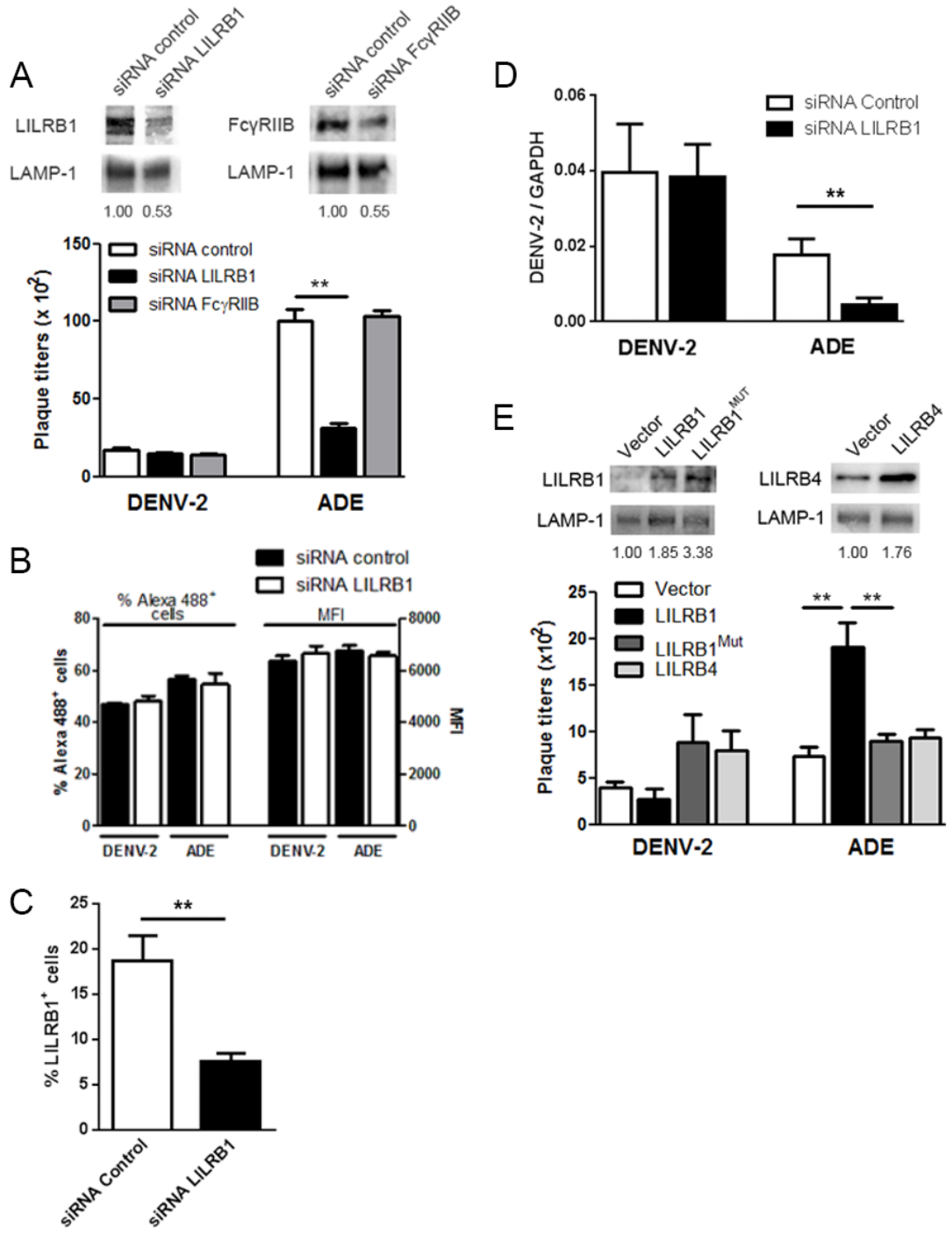


**Figure 2-9. Co-ligation of LILRB1 is essential for ADE.** (A) Plaque titers following DENV-2 or ADE infection after LILRB1 or FcγRIIB knockdown. Numbers below Western blot indicate levels of proteins relative to LAMP-1. (B) Uptake (% positive and MFI) of Alexa 488-labelled DENV-2 6hpi in LILRB1 knockdown cells. (C) Expression of LILRB1 in K562 cells transfected with siRNA control or siRNA against LILRB1, assessed by flow cytometry. (D) DENV RNA copy numbers 6hpi under DENV-2 or ADE conditions in K562 cells transfected with siRNA control or siRNA against LILRB1. (E) Plaque titers following DENV-2 or ADE infection in THP-1.2R transfected with empty vector, vector expressing LILRB1, mutant LILRB1

(LILRB1$^{MUT}$) or LILRB4. Numbers below Western blot indicate levels of proteins relative to LAMP-1. Data expressed as mean ± s.d. from three independent experiments. ** $P < 0.01$.

```
LILRB1           1 mtpiltvlic lglslgprth vqaghlpkpt lwaepgsvit qgspvtlrcq
LILRB1 Mutant    1 ---------- ---------- ---------- ---------- ----------
LILRB1          51 ggqetqeyrl yrekktalwi tripqelvkk gqfpipsitw ehagryrcyy
LILRB1 Mutant   51 ---------- ---------- ---------- ---------- ----------
LILRB1         101 gsdtagrses sdplelvvtg ayikptlsaq pspvvnsggn vilqcdsqva
LILRB1 Mutant 101 ---------- ---------- ---------- ---------- ----------
LILRB1         151 fdgfslckeg edehpqclns qphargssra ifsvgpvsps rrwwyrcyay
LILRB1 Mutant 151 ---------- ---------- ---------- ---------- ----------
LILRB1         201 dsnspyewsl psdllellvl gvskkpslsv qpgpivapee tltlqcgsda
LILRB1 Mutant 201 ---------- ---------- ---------- ---------- ----------
LILRB1         251 gynrfvlykd gerdflqlag aqpqaglsqa nftlgpvsrs yggqyrcyga
LILRB1 Mutant 251 ---------- ---------- ---------- ---------- ----------
LILRB1         301 hnlssewsap sdpldiliag qfydrvslsv qpgptvasge nvtllcqsqg
LILRB1 Mutant 301 ---------- ---------- ---------- ---------- ----------
LILRB1         351 wmqtflltke gaaddpwrlr styqsqkyqa efpmgpvtsa hagtyrcygs
LILRB1 Mutant 351 ---------- ---------- ---------- ---------- ----------
LILRB1         401 qsskpyllth psdplelvvs gpsggpsspt tgptstsagp edqpltptgs
LILRB1 Mutant 401 ---------- ---------- ---------- ---------- ----------
LILRB1         451 dpqsglgrhl gvvigilvav illllllllll flilrhrrqg khwtstqrka
LILRB1 Mutant 451 ---------- ---------- ---------- ---------- ----------
LILRB1         501 dfqhpagavg peptdrglqw rsspaadaqe enlyaavkht qpedgvemdt
LILRB1 Mutant 501 ---------- ---------- ---------- ---f------ ----------
LILRB1         551 rqsphdedpq avtyaevkhs rprremaspp splsgefldt kdrqaeedrq
LILRB1 Mutant 551 ---------- ---f------ ---------- ---------- ----------
LILRB1         601 mdteaaasea pqdvtyaqlh sltlrreate pppsqegpsp avpsiyatla
LILRB1 Mutant 601 ---------- -----f---- ---------- ---------- -----f----
LILRB1         651 ih
LILRB1 Mutant 651 --
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**Figure 2-10. Amino acid sequence of LILRB1 and LILRB1 mutant.** Identical amino acid residues are marked by dashes, and tyrosine residues in the ITIM cytoplasmic tail which were mutated to phenylalanine are highlighted in red.

### 2.3.7 Inhibition of LILRB1 signaling abrogates ADE in primary monocytes

The mechanistic requirement for LILRB1 in ADE suggests that interfering with this pathway would abrogate ADE in primary monocytes. We studied $CD14^{hi}CD16^{-}$ inflammatory monocytes that express both FcγRs and LILRB1 (Fig. 2-11A and B), which form the majority of the circulating monocytes (Passlick et al, 1989). Indeed, pretreatment with sodium stibogluconate, a SHP-1 inhibitor resulted in a dose-dependent reduction in DENV-2 replication under ADE conditions (Fig. 2-11C) Likewise, plaque titers following ADE infection of the other 3 DENV serotypes on primary monocytes obtained from different healthy donors were significantly lower in sodium stibogluconate treated cells as compared to untreated cells (Fig. 2-11D to F). No significant reduction in primary monocyte cytotoxicity was detected with the drug concentrations used (Fig. 2-11G). Pretreatment of primary monocytes derived from peripheral blood mononuclear cells (PBMCs) from 12 different healthy human volunteers with anti-LILRB1 antibodies also resulted in significantly reduced DENV replication compared to isotype antibodies (Fig. 2-11H). Collectively, these findings indicate that co-ligation of LILRB1 is a critical first step for successful antibody-dependent DENV infection (Fig. 2-12).

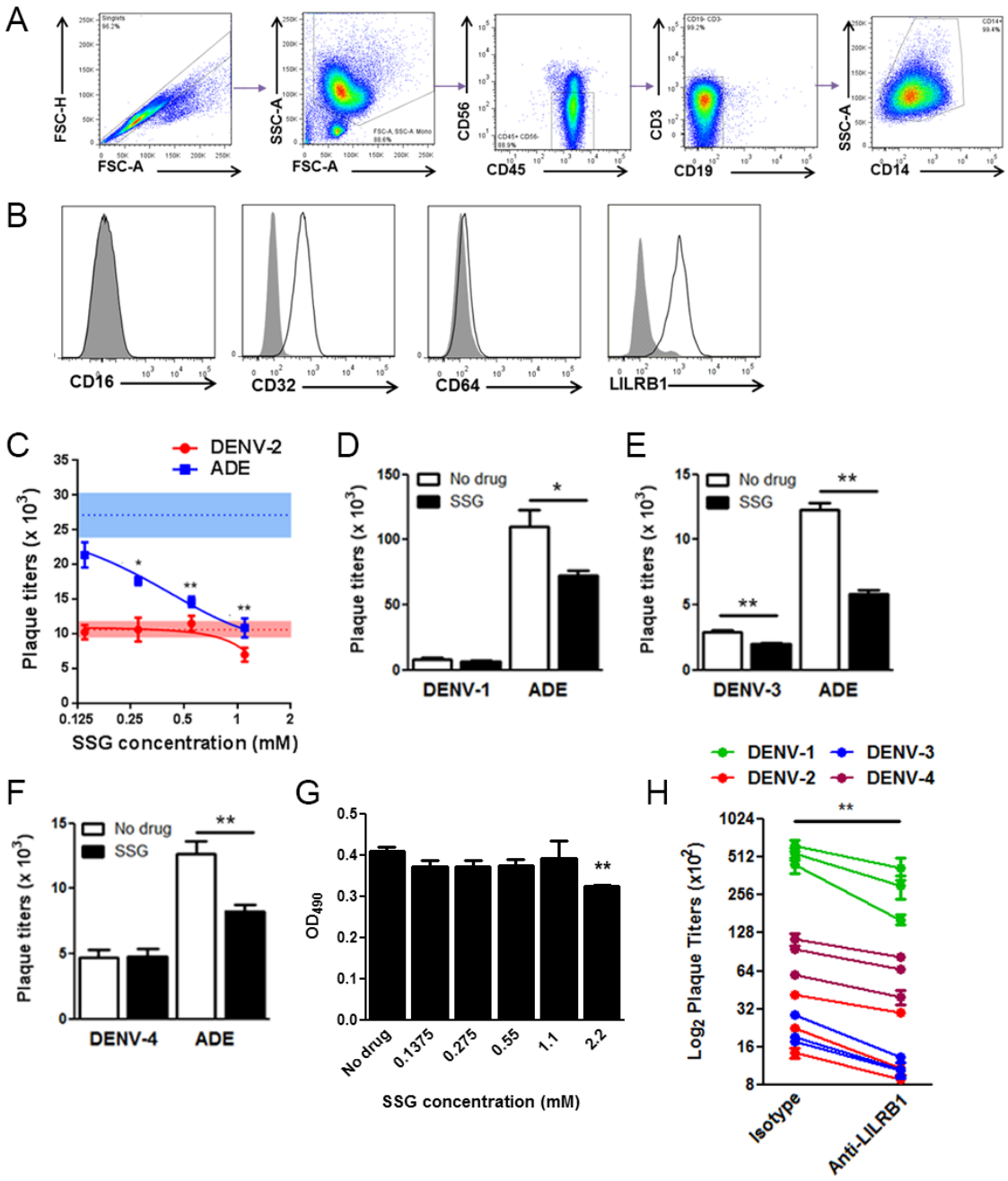



**Figure 2-11. Inhibition of LILRB1 signaling abrogates ADE in primary monocytes.** (A) Gating strategy employed for primary monocyte characterization. (B) Expression of FcγRs (CD16, CD32, CD64) and LILRB1 in primary monocytes using gating strategy displayed in (A). Cells were either stained with isotype (grey) or indicated antibodies (open histogram). (C) Plaque titers following DENV-2 only and ADE infection of primary monocytes treated with sodium stibogluconate (SSG) or PBS control (dashed lines, shaded areas reflect s.d.). (D to F) Plaque titers following DENV-1 (D), -3 (E) or -4 (F) only and ADE infection of primary monocytes treated with SSG (0.138mM) or PBS control. (G) Viability of primary monocytes after treatment with SSG (H) Plaque titers in primary monocytes derived from PBMCs harvested from 12 healthy individuals and infected *in vitro* with either DENV-1

(n=3), DENV-2 (n=3), DENV-3 (n=3) or DENV-4 (n=3) opsonized with h4G2 antibodies at 72hpi. PBMCs were either pretreated with polyclonal anti-LILRB1 antibody or isotype antibody control. Data expressed as mean ± s.d. from three independent experiments. ** $P < 0.01$, * $P < 0.05$. Panels A and B were kindly provided by Nivashini Kaliaperumal and John E. Connolly.

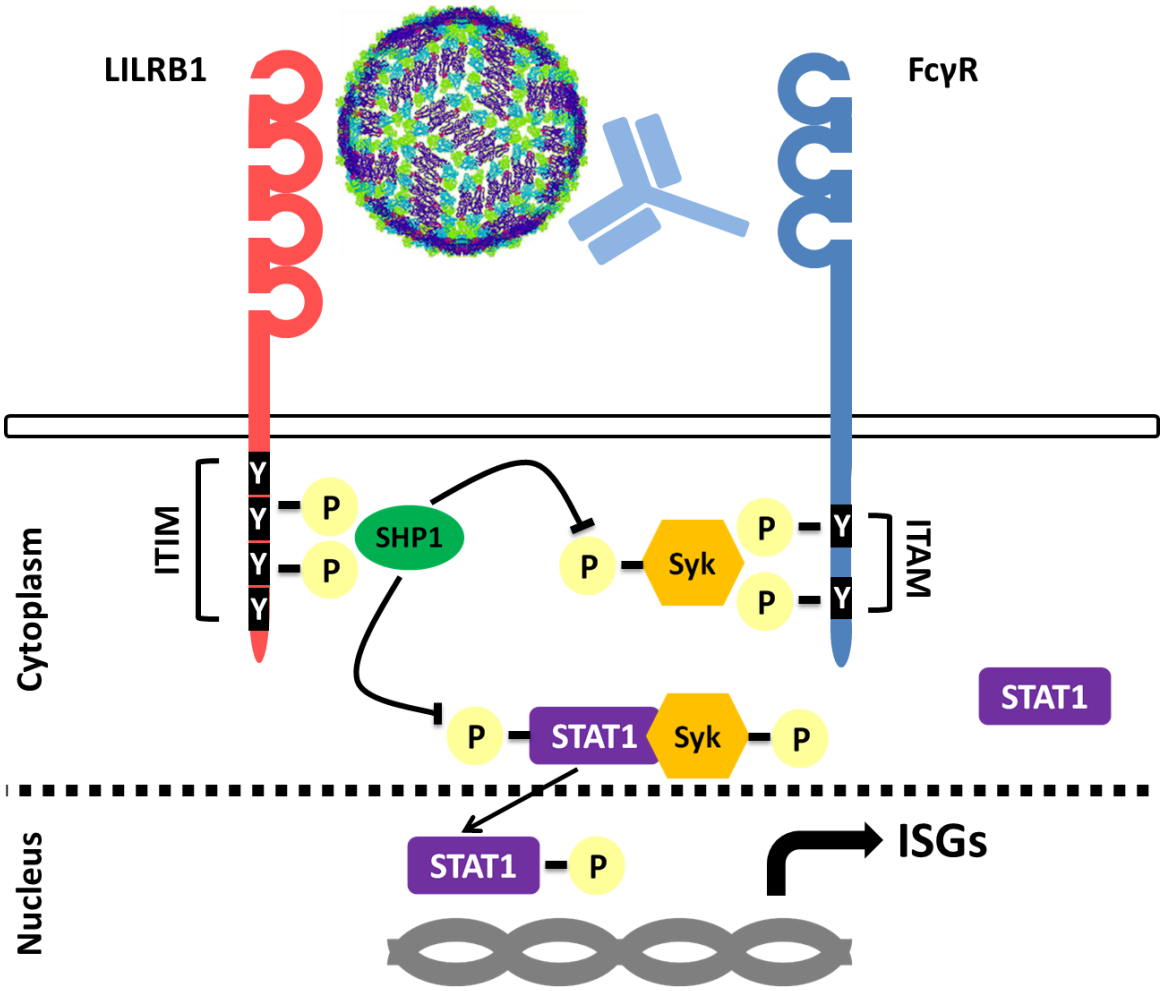



**Figure 2-12. Schematic representation of proposed role of LILRB1 in antibody-dependent infection.** Activating FcγR is represented here as FcγRIIA but should apply to FcγRI as well. However, it remains to be conclusively established whether there is a difference in the outcome of infection when viral entry is mediated through FcγRI or FcγRIIA.

## 2.4 Discussion

### 2.4.1 Role of LILRB1 in ADE of DENV infection

Dhodapkar and colleagues had observed that ligation of activating FcγRs induces expression of ISGs, which could be suppressed only upon co-ligation of the inhibitory FcγRIIB (Dhodapkar et al, 2007). Since the inhibitory FcγRIIB is not co-ligated during ADE (Chan et al, 2011), we addressed how antibody-opsonized DENV could overcome ISG responses triggered upon ligation of activating FcγRs. DENV co-ligates LILRB1, which leads to SHP-1 recruitment and dephosphorylation of Syk, and downregulates the ISG response. This provides a mechanistic explanation for how DENV evades an early antiviral response for enhanced viral replication during ADE (Fig. 2-12).

LILRB1 belongs to the family of leukocyte immunoglobulin-like receptors, which comprise activating and inhibitory receptors that differ in their transmembrane and cytoplasmic domains. LILRB1 is expressed on monocytes, dendritic cells and subsets of NK, B and T cells (Dietrich et al, 2000). It consists of 4 extracellular Ig-like C2-type domains and expresses ITIMs on its cytoplasmic tail, similar to the inhibitory FcγRIIB. LILRB1 is an inhibitory receptor, which serves to prevent excessive activation of the immune response. Binding of LILRB1 to major histocompatibility class I (MHC-I) molecules leads to ITIM phosphorylation, which serves as a docking site for the SH2-domain containing phosphatase SHP-1 (Colonna et al, 1997). SHP-1 recruitment activates negative feedback mechanisms such as inhibition of B cell receptor signaling in B cells, and inhibition of cell killing by NK and T cells (Colonna et al, 1997). When LILRB1 is cross-linked to activating receptors, SHP-1 recruitment dephosphorylates signaling effectors and downregulates

signaling from activating receptors. In experiments using specific monoclonal antibodies to cross-link LILRB1 and FcγRI on monocytes, Fanger and colleagues observed inhibition of tyrosine phosphorylation of the Fcγ chain and Syk, and reduced intracellular calcium mobilization (Fanger et al, 1998).

The inhibitory FcγRIIB inhibits phagocytosis when cross-linked to activating FcγRs, via recruitment of SHIP (Ono et al, 1996; Tridandapani et al, 2002). Overexpression of SHIP leads to inhibition of FcγR-mediated phagocytosis in macrophages (Cox et al, 2001). Furthermore, macrophages that expressed catalytically inactive SHIP or reduced levels of SHIP demonstrated enhanced phagocytosis (Cox et al, 2001). Although LILRB1 and FcγRIIB are both ITIM-bearing receptors, LILRB1 did not alter the rate of viral entry (Fig, 2-8E, Fig. 2-9B) in our experiments. This is because LILRB1 mediated signaling leads to recruitment of SHP-1 instead of SHIP (Ono et al, 1996). The recruitment of differential phosphatases by LILRB1 and FcγRIIB thus determines qualitatively the downstream signaling pathways that are modified. We determined that LILRB1 signaling during ADE modulated the antiviral state of the cell rather than increasing DENV entry, and this was mediated by dephosphorylation of Syk by pSHP-1, resulting in reduced ISG induction (Fig. 2-3A, Fig. 2-7D).

Studies have shown that overexpression of SHP-1, but not catalytically inactive SHP-1, results in downregulation of NFκB-dependent gene transcription in THP-1 cells activated by clustering FcγRIIA (Ganesan et al, 2003). In dendritic cells, LILRB1 ligation inhibited secretion of cytokines like IL-10, IL-12p70 and TGF-β (Young et al, 2008). LILRB1-ligated DCs were also deficient in stimulating T-cell proliferative responses (Young et al, 2008). Our findings that co-ligation of LILRB1

by DENV immune complex during ADE leads to SHP-1 recruitment and downregulation of ISGs thus extends on the observed attenuation of immune responses by this receptor.

Co-ligation of LILRB1 by DENV during antibody-dependent infection suggests that LILRB1 polymorphism may influence outcome of infection. Previous studies have shown that this gene is highly polymorphic (Kuroki et al, 2005). This could influence the diversity of immune responses, as individuals carrying different polymorphic variants of LILRB1 could have altered binding affinity to DENV. Since co-ligation of LILRB1 is crucial for downregulation of ISG responses, functional LILRB1 polymorphisms could thus modulate an individual's susceptibility to ADE. The LILRB1 gene can also be alternatively spliced to produce soluble isoforms of LILRB1 (Jones et al, 2009). By expressing the extracellular domain of LILRB1 as a soluble protein, we showed that soluble LILRB1 competed successfully with native LILRB1 on THP-1.2S to inhibit ADE in a dose-dependent manner (Fig. 2-8D). Soluble LILRB1 could thus serve as a negative regulator of LILRB1 signaling, and individuals with higher levels of soluble LILRB1 could be protected from ADE. However, a recent genome-wide association study did not reveal a significant association between LILRB1 and dengue shock syndrome (Khor et al, 2011). This is not surprising because, although LILRB1 activation is critical for initial replication with FcγR-mediated entry, multiple other host and viral factors contribute to eventual disease outcome (Modhiran et al, 2010; OhAinle et al, 2011; Ubol et al, 2010).

Our findings also suggest that generation of antibodies to quaternary structure-dependent epitopes on DENV that block LILRB1 interaction can reduce ADE. One of the key safety concerns in the development of a dengue vaccine is the enhancement of

dengue infection in FcγR-bearing cells by heterotypic antibodies. Hence, a vaccine that can generate high-titer antibody that binds the quaternary structure-dependent epitopes on DENV to prevent LILRB1 ligation could reduce the risk of vaccine-induced ADE. However, care must be taken in selecting a suitable in vivo model as the LILRB1 gene is deleted in laboratory strains of mice (Kubagawa et al, 1997). Further studies to clarify this could include cryo-electron microscopy (EM) approaches and epitope mapping studies that would allow us to understand how LILRB1 binds DENV. This would be instrumental in creating new opportunities for therapeutic intervention and vaccine development.

### 2.4.2 Role of ITIM-bearing receptors in viral immune evasion

DENV is not the only virus to exploit LILRB1 to modulate the host response to enable an environment more favorable for replication. Although our study focused on inhibitory signaling in monocytes, viruses and other pathogens are known to exploit inhibitory receptor signaling pathways in NK cells, T cells and DCs to evade host immunity. This creates a favourable intracellular environment for replication and transmission, especially if the virus is known to cause persistent infections.

Table 2-2 Immune inhibitory receptors

| Receptor | Cellular distribution | Functional relevance | Reference |
|---|---|---|---|
| LILRB1 | Myeloid, B, subset T, NK | Normal role<br>• Prevents excessive activation of immune system<br>• LILRB1 recognizes MHC-I molecules on target cells to inhibit killing by NK cells<br>• Downregulates B cell proliferation and B cell receptor signaling<br>• LILRB1 inhibits T cell proliferation and T cell activation<br>Role in viral evasion<br>• Downregulates ISG responses for enhanced viral replication during ADE for DENV infection<br>• Binds UL18 to limit antiviral effector functions and NK cell cytotoxicity during HCMV infection<br>• Reduced cytokine secretion and cytotoxicity during persistent EBV infections | (Navarro et al, 1999)<br>(Brown et al, 2004)<br>(Dietrich et al, 2001)<br>(Chan et al, 2014)<br>(Cosman et al, 1997)<br>(Poon et al, 2005) |
| LILRB2 | Myeloid, B, subset T, NK | Normal role<br>• Binds MHC-I to downregulate immune responses and limit autoreactivity<br>Role in viral evasion<br>• Binds B*3503 with high affinity, leading to DC dysfunction *in vitro*; DCs from HIV-1 carriers of B*3503 have impaired functional properties | (Colonna et al, 1999)<br>(Huang et al, 2009) |

| | | | |
|---|---|---|---|
| CD94/NKG2A | NK, T | Normal role<br>• Binds HLA-E to reduce NK cell activation and killing<br>• Binds MHC-I ligands to dampen T cell cytotoxicity and cytokine production<br>Role in viral evasion<br>• Increases HLA-E expression and reduces NK cell cytotoxicity during HCMV infection<br>• Upregulation of CD94/NKG2A in chronic HCV-infected patients. NK cells from these donors were deficient in activating DCs. | (Borrego et al, 2002)<br>(Vivier & Anfossi, 2004)<br>(Tomasec et al, 2000)<br>(Jinushi et al, 2004) |
| KIR3DL2 | NK, T | Normal role<br>• Binds HLA-A alleles to inhibit NK cell lysis<br>Role in viral evasion<br>• KIR3DL2 recognizes HLA-viral peptide complexes which prevents NK cell lysis of EBV-infected cells | (Ravetch & Lanier, 2000)<br>(Hansasuta et al, 2004) |
| PD-1 | NK, T, B | Normal role<br>• Downregulates B cell proliferation and B cell receptor signaling<br>• Reduces T cell proliferation and IFN-γ secretion<br>Role in viral evasion<br>• The HCV core protein upregulates expression of programmed death ligand 1 (PD-L1) on Kupffer cells, which ligate PD-1 to promote T cell dysfunction and development of viral persistence<br>• Upregulation of PD-1 and PD-L1 in monocytes and macrophages during HIV-1 infection impairs CD4+ T cell activation | (Ravetch & Lanier, 2000)<br>(Barber et al, 2006)<br>(Tu et al, 2010)<br>(Said et al, 2010) |

| | | | |
|---|---|---|---|
| TIM-3 | Subset of T cells | Normal role<br>• Binds galactin-9 to reduce IFN-γ secretion through induction of cell death<br>• Induction of peripheral tolerance<br>Role in viral evasion<br>• Increased levels of TIM-3 are correlated with T cell dysfunction and reduced production of IFN-γ and TNF-α during HCV infection<br>• Upregulated on HIV-1 specific $CD4^+$ and $CD8^+$ T cells, reduced T cell proliferation and impaired cytokine secretion | (Zhu et al, 2005)<br>(Sabatos et al, 2003)<br>(McMahan et al, 2010)<br>(Jones et al, 2008) |
| DCIR | DCs, B, monocytes | Normal role<br>• Reduced cytokine secretion in DCs<br>• Inhibits B cell receptor signaling and intracellular calcium release<br>Role in viral evasion<br>• Attachment factor for HIV-1 to promote infection of DCs and $CD4^+$ T cells | (Meyer-Wentrup et al, 2009)<br>(Lambert et al, 2008) |

*Human cytomegalovirus (HCMV)*

Human cytomegalovirus (HCMV) is a highly prevalent β-herpesvirus that causes life-long latent infections. The long co-evolution between the virus and its human host has resulted in the virus possessing an arsenal of genes for subversion of the human immune system. As a strategy to evade the host immune response, HCMV expresses UL-18, a MHC class I (MHC-I) homologue which has approximately 25% homology to classical MHC-I. Despite the low sequence identity, its secondary structure remarkably resembles MHC-I molecules (Beck & Barrell, 1988; Chapman & Bjorkman, 1998).

Like other MHC-I molecules, UL-18 binds endogenously derived peptides and β-microglobulin (Browne et al, 1990; Fahnestock et al, 1995). However, it binds with >1000-fold greater affinity to LILRB1 relative to other MHC-I molecules, and this is postulated to arise from local differences in LILRB1 contact residues between the α3 domains of MHC-I and UL-18 (Chapman et al, 1999; Wagner et al, 2007b). The frequency of $LILRB1^{+}$ NK and T cells are elevated in sero-positive HCMV patients (Guma et al, 2004), which presumably allow HCMV to evade the host immune system. Binding of UL-18 to LILRB1 triggers an inhibitory signaling pathway that limits antiviral effector functions and protects HCMV-infected cells from NK cell-mediated cytolysis (Cosman et al, 1997; Prod'homme et al, 2007; Yang & Bjorkman, 2008). In T cells, increased LILRB1 expression is also associated with reduced cytokine secretion and cytotoxicity (Saverino et al, 2000), and purified UL18Fc proteins inhibited production of IFN-γ (Wagner et al, 2007a).

UL-18 has 13 potential N-liked glycosylation sites, and this distinguishes it from MHC-I molecules that only have 1 N-glycan attached. UL-18 is predicted to be fully glycosylated, with the exception of the binding site for LILRB1 and docking site on β-

microglobulin (Yang & Bjorkman, 2008). The glycan shield for UL-18 prevents itself from antibody-mediated neutralization and preserves the binding site for LILRB1, allowing UL-18 to compete successfully with host ligands for LILRB1 to evade the host immune response. Such a strategy is also exemplified by viruses like HIV and influenza (Julien et al, 2012), which have highly glycosylated envelope proteins for reducing immunogenicity in the host.

The expression of UL-18 in clinical isolates of HCMV underscores its importance for viral survival in the host. UL-18 from different clinical isolates bind LILRB1 with different affinities, compared to AD169, a laboratory strain of CMV (Cerboni et al, 2006; Vales-Gomez et al, 2005), indicating that the immune response to HCMV may be differentially modulated in patients.

Apart from LILRB1 engagement, HCMV also suppresses NK cells through the inhibitory CD94/NKG2A receptor complex. A nonameric peptide derived from the UL-40 glycoprotein serves as a ligand for HLA-E, increasing HLA-E expression on the surface of HCMV infected cells (Tomasec et al, 2000). This facilitates the interaction between HLA-E and CD94/NKG2A receptor, which confers resistance to NK-cell lysis (Tomasec et al, 2000; Wang et al, 2002).

Taken together, HCMV utilizes an array of strategies for the engagement of host inhibitory receptors to undermine the host NK cell response.

*Epstein-Barr virus (EBV)*

EBV belongs to the gammaherpesvirus group of viruses, and infects over 90% of the world's population, establishing life-long persistence in immunocompetent hosts. Analysis of LILRB1 expression in EBV-specific CD8+ effector T-cells during the

primary and persistent phases of EBV infection revealed low LILRB1 expression during the primary phase of infection. However, LILRB1 expression increases during persistent EBV infections, and results in reduced cytokine secretion and cytotoxicity (Poon et al, 2005).

Furthermore, EBV also uses an inhibitory NK receptor to elude the host immune system. The EBV viral protein, EBNA-3A, supplies peptides that bind certain HLA-A allotypes. These HLA-peptide complexes are recognized by inhibitory NK receptor KIR3DL2, which then prevents NK cell lysis of EBV-infected cells (Hansasuta et al, 2004).

*Hepatitis C virus (HCV)*

Acute cases of HCV infection are typically asymptomatic, and persistent infections are usually undetected until chronic liver disease or cancer develops. Underlying the viral persistence in HCV are dysregulated immune responses in NK cells, DCs and T cells.

Inhibition of NK cells may be particularly relevant for chronic infections caused by hepatotrophic viruses like HCV as up to half of all innate lymphocytes in the intrahepatic immune system are NK cells (Doherty & O'Farrelly, 2000). Interaction between HLA-E on DCs and the inhibitory NK cell receptor CD94/NKG2A leads to inhibition of NK cell activation. Both the HCV core protein and cytokines like IFN-γ are known to stabilize HLA-E expression (Cerboni et al, 2001; Nattermann et al, 2005). The expression of inhibitory NK cell receptor CD94/NKG2A was upregulated in chronic HCV-infected patients. NK cells from these donors were deficient in activating DCs, and produced IL-10 and TGF-β when cultured with hepatic cells expressing HLA-E (Jinushi

et al, 2004). High serum levels of TGF-β in chronic HCV patients also led to upregulation of CD94/NKG2A in NK cells (Bertone et al, 1999).

Broad multitypic $CD4^+$ and $CD8^+$ T cell reponses are critical for viral clearance, and this is undermined by immune exhaustion during chronic infection. In persistent HCV infections, anergy and T cell exhaustion is frequently demarcated by the upregulation of inhibitory receptors like programmed cell death protein 1 (PD-1) and T cell Ig and mucin domain-containing molecule 3 (TIM-3), markers for functionally impaired cytotoxic $CD8^+$ T cells (Golden-Mason et al, 2007). The dual expression of PD-1 and TIM-3 on cytotoxic $CD8^+$ T cells is a predictive marker for viral persistence in patients with acute HCV infections (McMahan et al, 2010). Increased levels of PD-1 and TIM-3 are correlated with T cell dysfunction, reduced production of IFN-γ, TNF-α, and CD107a, a marker for cytolytic T cells, as compared to $PD\text{-}1^{lo}/TIM\text{-}3^{lo}$ $CD8^+$ T cells (McMahan et al, 2010).

The HCV core protein upregulates expression of programmed death ligand 1 (PD-L1) on Kupffer cells, which can ligate PD-1 to promote T cell dysfunction and development of viral persistence (Tu et al, 2010). Reduced PD-1 expression on HCV-specific $CD4^+$ and $CD8^+$ T cells is correlated to sustained response to combination IFN-based antiviral therapy (Golden-Mason et al, 2008). In addition to PD-1, coexpression of other inhibitory receptors like 2B4, CD160 and KLRG1 has been observed in T cells that have impaired proliferation and cytokine production (Bengsch et al, 2010).

*Human immunodeficiency virus type 1 (HIV-1)*

Infection with HIV-1 leads to a rapid and progressive immune deficiency in most individuals, leading to a decline of CD4+ T cells and inhibition of functional properties of

plasmacytoid and myeloid DCs (Doitsh et al, 2014; Donaghy et al, 2003). Recent evidence has shown that the disease outcome hinges upon a complex interplay of immunomodulatory mechanisms, which can determine the rate of disease progression. Inhibitory receptor signaling is an integral component of the regulation of immune responses against HIV-1 and can be manipulated by HIV-1 to subvert host immunity for viral replication.

DC dysfunction begins early during disease onset, and differentiates HIV-1 infected individuals from elite controllers, which are a subset of patients with spontaneous control of HIV-1 replication in the absence of antiretroviral therapy. Differential interaction between HLA allele subtypes and MHC-I receptors on DCs have been shown to contribute to HIV-1 disease progression and viral manipulation of host immunity. The B*35-Px molecule, B*3503, was found to bind to LILRB2 on DCs with high affinity, leading to DC dysfunction *in vitro,* which was corroborated by *ex vivo* assessment that DCs from HIV-1 carriers of B*3503 had impaired functional properties (Huang et al, 2009). Overall, the data indicates that HLA-B*35-Px subtype could accelerate disease progression in HIV-1 infected individuals through LILRB2-dependent inhibition of DCs.

Conversely, inhibitory receptors like LILBR1 and LILRB3 have been shown to be elevated on circulating myeloid DCs of elite controllers, who are able to spontaneously control HIV-1 replication. Blocking LILRB1 and LILRB3 using siRNA or monoclonal antibody approaches led to reduced capacity for antigen presentation, while blocking LILRB1 led to reduced secretion of proinflammatory cytokines like IL-12p70, IL-6 and TNF-α (Huang et al, 2010a). Although LILRB1 and LILRB3 are ITIM-bearing receptors conventionally associated with the transduction of inhibitory signals, they seem to

enhance antigen presentation on DCs in elite controllers. This suggests that cellular context is crucial in determining the function of ITIM-bearing receptors.

Inhibitory receptors like DCIR have also been implicated as an attachment factor for HIV-1, facilitating viral replication in DCs and transmission to $CD4^+$ T cells. Binding of HIV-1 to dendritic cell immunoreceptor (DCIR), an ITIM-containing C-type lectin, promotes HIV-1 infection of DCs and $CD4^+$ T cells (Lambert et al, 2008; Lambert et al, 2010). HIV-1 drives expression of DCIR on $CD4^+$ T cells, which is accompanied by enhancement of virus attachment and entry, viral replication and infection of bystander cells (Lambert et al, 2010). In addition to serving as a receptor for viral entry, it is tempting to speculate that DCIR-mediated signaling could contribute to immune evasion by HIV-1 since endocytosed DCIR has been shown to inhibit the production of IL-12 and IFN-α (Meyer-Wentrup et al, 2008; Meyer-Wentrup et al, 2009), both of which are important in inhibition of HIV-1 replication (Hosmalin & Lebon, 2006; Mirani et al, 2002).

Inhibitory signaling events at the interface of DCs and T cells can also lead to T cell inhibition during HIV-1 infection. The inhibitory receptors PD-1 and TIM-3 are coexpressed on $CD4^+$ and $CD8^+$ T cells in chronic HIV-1 and HCV patients (Golden-Mason et al, 2007; Migueles et al, 2002). The HIV-1 accessory protein, Nef, transcriptionally induces PD-1 expression during infection of $CD4^+$ T cells (Muthumani et al, 2008). Upregulation of PD-1 on T cells allows HIV-1 to evade the T cell response, and even cause T cell exhaustion (Day et al, 2006). Increased levels of its cognate ligand, PD-L1 was found on $CD4^+$ T cells and macrophages, and ligation to PD-1 can trigger inhibitory signaling that downregulates T cell responses *in vivo* (Latchman et al, 2004). Control of HIV-1 replication is correlated to reduced levels of PD-1 on $CD8^+$ T cells, and

blocking PD-1 enhances T cell proliferation (Brown et al, 2003). In monocytes and macrophages, both PD-1 and PD-L1 are upregulated upon HIV-1 infection, and can activate PD-1 inhibitory signaling on monocytes to induce high levels of IL-10, which can impair CD4+ T cell activation (Rodriguez-Garcia et al, 2011; Said et al, 2010).

TIM-3 is another inhibitory receptor upregulated on HIV-1 specific $CD4^+$ and $CD8^+$ T cells, as well as T cells pulsed by HIV-1 infected DCs. (Jones et al, 2008; Shankar et al, 2011). T cells that express TIM-3 proliferate poorly and are impaired in cytokine secretion (Jones et al, 2008). Blocking PD-1 and TIM-3 pathways simultaneously *in vivo* leads to a reversal of T cell exhaustion and control of viral replication (Jin et al, 2010).

Multiple examples abound in the literature investigating how viruses exploit inhibitory receptor-mediated signaling pathways for enhanced viral replication. That co-ligation of LILRB1 by DENV immune complex during ADE leads to downregulation of ISG induction and enhanced viral replication underscores the importance of this strategy adopted by viruses to evade host immune responses. We are just starting to gain a mechanistic understanding of how antibody-opsonized DENV subverts host immunity early after infection during ADE. It would be interesting to test if other viruses that benefit from ADE like HIV-1 could utilize LILRB1-mediated suppression of immune signaling for viral replication in the host (Beck et al, 2008). In addition to identifying novel therapeutic targets, knowledge of how the immune system is modulated during ADE could also have important implications for strategies to minimize ADE during vaccine development.

### 2.4.3 Role of ITIM-bearing receptors in modulating antibody effector functions

Antibody effector functions are triggered upon binding of Fc region of antibody to effector molecules or FcγRs expressed on immune effector cells like monocytes, macrophages, DCs, NK cells and T cells. This can include antibody-dependent cell-mediated cytotoxicity, complement-dependent lysis (CDC), phagocytosis, release of reactive oxygen intermediates and inflammatory mediators and antigen presentation. The magnitude of antibody effector functions is dependent on the balance of activating and inhibitory receptor signaling. While the inhibitory FcγRIIB plays a pivotal role in the regulation of antibody effector functions, the lack of FcγRIIB expression on cell subsets like T cells and NK cells (Ravetch & Lanier, 2000) suggest that other ITIM-bearing receptors may play a role in dampening antibody effector functions arising from activating FcγR signaling.

*Antibody-dependent cell-mediated cytotoxicity (ADCC)*

ADCC is a mechanism through which FcγR-bearing cells can recognize and kill antibody-coated target cells expressing pathogen- or tumour-derived antigens on their surface. This typically leads to the release of cytotoxic granules that kill target cells via the perforin or granzyme cell death pathway.

NK cells are the primary effector cells capable of eliciting ADCC, which occurs through cross-linking of the activating FcγRIIIA. This triggers Syk phosphorylation and the activation of downstream pathways involved in calcium mobilization and NK cell granule polarization and release for ADCC activity. Although ADCC is also mediated by FcγRI and FcγRIIA on monocytes, macrophages and neutrophils, less is known about the regulation of signal transduction pathways in these cells.

NK cells facilitate the cytolysis of target cells through the release of lytic granules and mediate apoptosis of target cells by secretion of tumor necrosis family (TNF) ligands and cytokines like IFN-γ. The balance of activating and inhibitory receptors is thus crucial for regulation of NK cell cytolytic activity. To this end, NK cells express an array of inhibitory receptors like leukocyte immunoglobulin-like receptors (LILRs), killer immunoglobulin-like receptors (KIRs) and C-type lectin receptors (CD94/NKG2A) to downregulate FcγRIIIA-mediated ADCC activity.

KIR recognition of MHC-I ligands on target cells inhibits FcγRIII-mediated ADCC by NK cells (Fanger et al, 1999), but this can be abrogated by overexpression of a dominant-negative mutant of SHP-1 in NK cells (Binstadt et al, 1996). Ligation of KIR leads to SHP-1 recruitment, and subsequent dephosphorylation of ZAP-70 and PLC-γ (Binstadt et al, 1996). However, the precise substrates of SHP-1 associated with KIR signaling have not been determined. Tumour-derived antigens on multiple myeloma cells bind KIRs on NK cells as a means of immunoevasion. Blocking inhibitory KIR signaling could thus augment NK cell cytotoxicity against multiple myeloma cells. The anti-KIR antibody IPH2101 has demonstrated safety in a phase 1 trial in patients with multiple myeloma and should provide greater impetus for development of anti-cancer therapeutics that block inhibitory signaling (Benson et al, 2012). While KIRs recognize allele-specific MHC-I molecules, LILRs interact with a broad range of classical and nonclassical MHC-I molecules (Chapman et al, 1999). LILRB1 can recognize MHC-I molecules on the target cell, and this inhibits FcγRIII-mediated NK cell cytotoxicity via the recruitment of SHP-1 (Colonna et al, 1997).

*Complement*

IgG immune complexes can lead to activation of all 3 complement pathways resulting in the generation of C3 and C5 cleavage products that ligate complement receptors (CRs) on immune cells. CRs are often co-expressed with FcγRs on immune cells, and there is increasing evidence that cross-talk between these two signaling systems ensures both synergistic co-operation and feedback control to keep the immune response in check.

Cross-talk between the C5a receptor, C5aR and FcγRs has demonstrated how the complement and FcγR signaling systems are mutually responsive. C5aR acts upstream of FcγRs and is able to modify the ratio of activating to inhibitory FcγRs to alter the threshold for FcγR effector function. In alveolar macrophages, activation of C5aR after induction of C5a led to upregulation of activating FcγRIIIA and downregulation of the inhibitory FcγRIIB, lowering the activation threshold for cytokine production and neutrophil recruitment (Shushakova et al, 2002). This was subsequently attributed to $G_{ai2}$- and PI3K-dependent signaling downstream of the G-protein coupled C5aR which was critical for regulating the expression of FcγRs (Skokowa et al, 2005). This signaling is bidirectional, as galactosylated IgG1 immune complexes that promote the association of FcγRIIB and dectin-1, led to Syk- and SHIP-dependent inhibition of C5aR signaling in macrophages and neutrophils (Karsten et al, 2012). Administration of galactosylated IgG1 immune complexes was able to prevent C5a-mediated inflammation in vivo including development of skin blisters in experimental epidermolysis bullosa acquisita, an autoimmune skin disorder (Karsten et al, 2012).

An increased understanding of how the complement system collaborates with FcγR signaling will allow us to harness these two powerful effector systems for development of therapeutic solutions for human diseases. While augmenting cytotoxicity

and inflammation would benefit therapeutic interventions against cancer or infectious diseases, the converse would be true when devising treatments against autoimmune diseases.

*Phagocytosis*

Activating FcγR-mediated phagocytosis is important for removing immune complexes or pathogens from circulation. Phagocytosis is frequently coupled with the production of reactive oxygen intermediates and inflammatory cytokines, which help to resolve the infection. However, excessive production can also lead to tissue damage. This process is thus subject to regulation by inhibitory FcγRIIB and other intracellular phosphatases, including SHIP-1 and SHP-1, that dampen activating FcγR-mediated signaling back to basal levels.

The ITIM-bearing FcγRIIB inhibits signaling pathways triggered by the ITAM-bearing activating FcγRs. Macrophages deficient in FcγRIIB had enhanced phagocytic capacity of IgG-opsonized particles relative to wildtype macrophages (Clynes et al, 1999). FcγRIIB inhibits phagocytosis by recruitment of SHIP-1, which hydrolyzes PI(3,4,5)P3 (Gupta et al, 1997). This inhibits the activation of key enzymes involved in phagocytosis like Vav, a guanine nucleotide exchange factor for Rho GTPases required for particle internalization, and Bruton's tyrosine kinase, which is involved in intracellular calcium mobilization (Patel et al, 2002; Scharenberg et al, 1998).

Signal regulatory protein α (SIRPα), an ITIM-bearing surface glycoprotein found on NK cells, macrophages and DCs has also been implicated in downregulation of FcγR-mediated phagocytosis (Jaiswal et al, 2010). Upon binding of SIRPα to CD47, a ubiquitously expressed transmembrane protein, the ITIM of SIRPα is phosphorylated and

recruits SHP-1 to the plasma membrane. This precludes the accumulation of myosin at the cell surface, inhibiting phagocytosis (Tsai & Discher, 2008). In cancer cells, the upregulation of CD47 serves as an antiphagocytic signal to prevent tumor cell destruction by macrophages, with increased levels of protein expression correlated to the ability to evade phagocytosis *in vivo* (Jaiswal et al, 2010). Blockade of the SIRPα-CD47 interaction using anti-CD47 antibodies has been shown to enhance rituximab treatment, promoting phagocytosis and elimination of lymphomas in a human non-Hodgkin lymphoma xenotransplant mouse model (Chao et al, 2010). Engineered SIRPα variants with high affinity binding to CD47 have shown remarkable synergy with tumor-specific monoclonal antibodies by enhancing phagocytosis *in vitro* and increasing tumor regression *in vivo* (Weiskopf et al, 2013b).

Inhibitory receptors play a key role as a rheostat of immune cell modulation and are strongly involved in the regulation of antibody effector functions, which could be deleterious to the human host if left unchecked. Unfortunately, viruses have been able to exploit these receptors to evade immune responses that would otherwise limit its spread. This thesis has demonstrated the role that LILRB1 plays in downregulating the ISG response early after DENV infection through the recruitment of SHP-1 to dephosphorylate Syk, thus permitting enhanced viral replication during ADE. A deeper understanding of how inhibitory receptors regulate both immunity and tolerance could offer novel strategies for immunotherapy against infectious diseases, autoimmune diseases and cancer. In conclusion, DENV co-ligates LILRB1 to downregulate the activating FcγR-mediated early ISG expression for successful antibody-dependent infection.

# Chapter 3. SYK-ING PATHWAYS FOR ANTIBODY-ENHANCED DENGUE INFECTION

## 3.1 Introduction

### 3.1.1 Compartmentalization as a means of host defence

Host immunity is governed by the signaling networks and effector functions triggered within specialized immune cells when they are faced with a potential threat. However, this view underplays the capacity of non-immune cells to protect themselves against the threat of potential pathogens. This intrinsic form of self-defence, ubiquitous in most cell lineages across phyla, is also known as cell-autonomous immunity (Randow et al, 2013). The production of cytotoxic gases like nitric oxide is an example of a cell-autonomous effector function conserved in organisms as diverse as Gram-positive bacteria (Gusarov et al, 2009), flies (McGettigan et al, 2005) and humans (MacMicking, 2012). This portion of the thesis will focus on cellular compartmentalization following FcγR-mediated phagocytosis as a platform for cellular self-defence. This is encompassed by the presence of phagosome borders that serve as a physical barrier for invading pathogens. In addition, the expression of pattern recognition receptors on these borders as well as potent anti-pathogen effectors restricted within the phagosome represent “rigged” warning systems for the cell to respond to pathogen invasion (Randow et al, 2013).

### 3.1.2 FcγR signaling and phagocytic trafficking

Ligation of activating FcγRs leads to initiation of the downstream signaling cascade. Firstly, FcγR cross-linking upon receptor ligation leads to activation of Src family kinases, which phosphorylate the tyrosine residues on ITAM. These serve as docking sites for the dual SH2 domains of the non-receptor tyrosine kinase Syk, resulting in

enzymatic activation of Syk and downstream signal transduction (Mocsai et al, 2010). Other than the FcRγ chain, ITAM-bearing proteins include the TCRζ and CD3γ/δ/ϵ chains of T cell receptors (TCRs), the Igα/β chains of B cell receptors and the DNAX activation protein 12 (DAP12) of activating NK receptors. Signaling through any of these receptors or accessory proteins contributes to Syk activation and can induce a productive adaptive immune response (Mocsai et al, 2010).

Syk is highly expressed in haematopoietic cells and is comprised of a kinase domain and dual SH2 domains. Importantly, initial ITAM binding triggers activation of Syk and this also catalyzes autophosphorylation of tyrosine residues in its linker region, which leads to sustained Syk activation and downstream signaling, even after transient ITAM phosphorylation (Furlong et al, 1997; Mocsai et al, 2010). Syk activation can be counteracted by phosphatases like SHP-1, and the balance of Syk and SHP-1 activity contributes to the eventual signaling output (Veillette et al, 2002). Syk can also be ubiquitylated and degraded by the E3 ubiquitin ligase Casitas B-lineage lymphoma (CBL) (Lupher et al, 1998).

Src kinases and Syk are critical for FcγR-mediated phagocytosis, and this has been shown in both primary mouse bone marrow-derived macrophages and primary human monocytes. Macrophages from mice deficient in Src kinases Lyn, Hck and Fgr endocytose soluble IgG-containing immune complexes normally, but are defective in phagocytosis of IgG-coated erythrocytes and activation of Syk (Fitzer-Attas et al, 2000). Some functional redundancy may be present in Src kinases as bone marrow-derived macrophages from mice deficient in Hck and Fgr express higher levels of other Src kinases and Syk, which are subsequently activated during phagocytosis of IgG-coated targets (Majeed et al, 2001). Finally, macrophages derived from mice deficient in Syk

were defective in phagocytosis of IgG-coated targets, and phosphorylation of downstream substrates (Crowley et al, 1997). Furthermore, treatment of human monocytes with piceatannol, a Syk inhibitor, led to reduced FcγR-mediated phagocytosis but not endocytosis (Huang et al, 2006).

During FcγR-mediated phagocytosis, Syk recruitment is followed by mobilization of signaling adaptors such as phosphatidylinositol 3-kinase (PI3-K) and phospholipase C-gamma (PLCγ) which promote lipid modifications during reorganization of the membrane and cytoskeleton for particle internalization during phagocytosis (Goodridge et al, 2012). Local synthesis of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3), a byproduct of PI3-K catalyzed phosphorylation of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2), is required to direct actin remodeling for pseudopod extension during engulfment, by recruiting the actin motor protein myosin X (Cox et al, 2002; Cox et al, 1999). In addition, Syk interacts with Rho GTPases like cell division control protein 42 (Cdc42), Rac1 and Rac2 which regulate intracellular actin dynamics for particle engulfment and phagocytic trafficking (Beemiller et al, 2010; Hoppe & Swanson, 2004). Rab and ADP-ribosylation factor (Arf) GTPases regulate transport between membrane-bound organelles, binding to effector proteins that recruit additional machinery for phagosome maturation (Flannagan et al, 2009). Finally, SNARE (soluble NSF-attachment protein receptor) proteins including VAMP3 and VAMP7 mediate vesicle fusion during the successive steps of phagosome maturation (Flannagan et al, 2009).

Phagosome maturation involves the sequential fusion of the nascent phagosome with early endosomes, late endosomes and lysosomes. This is accompanied by membrane remodeling and the acquisition of an acidic and degradative environment in the eventual phagolysosome. Phagosomal acidification is marked by the recruitment of V-ATPase

proton pumps and the enrichment of mature lysosomal hydrolases. Together with the production of reactive oxygen species (ROS) by the NOX2 NADPH oxidase on the phagosome membrane, these strategies contribute to the elimination of pathogens within phagolysosomes. The involvement of Syk in regulating phagosome maturation through phagosome acidification has been documented more substantively in recent literature regarding Dectin-1, a C-type lectin receptor important for antifungal immunity. Like FcγRs, Dectin-1 ligation leads to activation of the Src kinases and Syk, and signal transduction is mediated through an ITAM-like motif in its cytoplasmic tail (Rogers et al, 2005; Underhill et al, 2005). Dectin-1 is critical for recognition of fungal β-glucans and elimination of fungal pathogens, and also triggers phagocytosis through pseudopod extension for internalization of its target particle (Goodridge et al, 2012). Together with FcγRs, transfection of these receptors is sufficient to instruct phagocytosis of specific targets on non-phagocytic cells (Brown & Gordon, 2001; Indik et al, 1991).

Dectin-1 has been shown to activate Syk in macrophages and is important for reactive oxygen production (Underhill et al, 2005). Activation of Dectin-1 by β-glucan also regulates phagolysosomal maturation via Syk activation as Syk inhibition led to prolonged retention of Dectin-1 on the phagosome and arrest of β-glucan-containing phagosomes at an early endosomal stage (Mansour et al, 2013). As the phagosomal retention of Dectin-1 upon Syk inhibition was correlated to reduced phagosomal acidification (Mansour et al, 2013), Syk phosphorylation may have a role in phagosomal acidification, a hallmark of phagosome maturation. This is in agreement with a separate study showing that Syk inhibition led to defects in CD63 recruitment, an acidification-dependent process (Artavanis-Tsakonas et al, 2006), onto *Aspergillus fumigatus*

phagosomes upon infection of primary monocytes and THP-1 differentiated macrophages (Kyrmizi et al, 2013).

### 3.1.3 ITIM-mediated inhibition of phagocytic trafficking

The inhibitory FcγRIIB is a negative regulator of activating FcγR-mediated phagocytosis. Macrophages deficient in FcγRIIB show enhanced phagocytosis of IgG-opsonized targets compared to wildtype macrophages (Clynes et al, 1999). In addition, while transfection of COS-1 fibroblast cells with ITAM-containing FcγR led to efficient phagocytosis of IgG-opsonized particles, the co-transfection of FcγRIIB led to a decrease in phagocytic efficiency (Hunter et al, 1998). This also suggested that the outcome of phagocytosis depended on the balance of activating and inhibitory FcγR signaling. The mechanism by which FcγRIIB mediates inhibition of phagocytosis involves ITIM phosphorylation by Src kinases and recruitment of Src homology 2 domain-containing inositol phosphatase-1 (SHIP-1) (Gupta et al, 1997). SHIP-1 hydrolyzes PI(3,4,5)P3, which is required for the activation of key enzymes involved in phagocytosis like Bruton's tyrosine kinase (BTK), involved in intracellular calcium mobilization (Scharenberg et al, 1998), and Vav, a guanine nucleotide exchange factor (GEF) for Rho GTPases which is required for Rac activation and particle internalization (Patel et al, 2002).

Ligation of LILRB1 leads to ITIM-mediated recruitment of SHP-1, a protein tyrosine phosphatase (PTP) that could also have important functional consequences for FcγR-mediated phagocytosis. SHP-1 has been shown to inhibit FcγR-mediated phagocytosis through suppression of Rac activity and dephosphorylation of CBL (Kant et al, 2002). Recent work has also begun to understand the role of SHP-1 in phagosome maturation. SHP-1 is recruited to nascent phagosomes and phagosomal acidification is

impaired in macrophages derived from mice deficient in SHP-1 (Gomez et al, 2012). Although this is consistent with the role of other PTPs in regulating signaling and membrane fusion events during phagosome trafficking (Huynh et al, 2004), the mechanism by which SHP-1 modulates phagosomal acidification remains to be clearly elucidated. While Gomez and colleagues suggest that SHP-1 may positively regulate phagosomal acidification by activating membrane fusion events that recruit V-ATPase to the phagosome membrane, others have shown that PTPs can also negatively regulate phagosomal acidifcation. An example for this is the mycobacterial PTP, PtpA, which was shown to dephosphorylate vacuolar protein sorting protein 33B (VPS33B), a key regulator of membrane fusion (Wong et al, 2011). This led to exclusion of V-ATPase from the phagosome during *Mycobacterium tuberculosis* infection, resulting in inhibition of phagosomal acidification (Wong et al, 2011).

Hence, the role of SHP-1 for the maintenance of phagosomal acidification remains controversial. Given that Gomez and colleagues addressed the role of SHP-1 in phagolysosome biogenesis through the use of bone marrow-derived macrophages from SHP-1 deficient mice, evaluating the role of SHP-1 in a human monocytic cell line or human primary monocytes could provide us with new insights on the role of SHP-1 in phagosome maturation following FcγR-mediated phagocytosis.

Based on our observed dephosphorylation of Syk when LILRB1 was co-ligated during ADE (Chan et al, 2014), we hypothesized that recruitment of phosphatases like SHP-1 during LILRB1-mediated signaling could modulate phagosome maturation. It is possible that reduced Syk signaling would result in DENV being trafficked into phagocytic compartments more congenial for replication, thus influencing the outcome of ADE.

## 3.2 Materials and Methods

### 3.2.1 Purification of DENV-containing phagosomes on a step sucrose gradient

Isolation of DENV phagosomes was performed using a protocol previously used for the isolation of latex bead phagosomes (Desjardins et al, 1994). Briefly, DENV was incubated with media or h3H5 (0.39µg/ml) for 1hr at 37˚C before adding to cells at indicated moi. THP-1.2R and THP-1.2S were incubated with DENV immune complex at 4°C for 20min before transferring to 37°C for 2h. After 2h, cells were washed 3 times with PBS before resuspending in homogenization buffer (8.55% (w/v) sucrose in HNE buffer, 3mM imidazole, 1% protease inhibitor solution, pH 7.4). To isolate phagosomes, cells were homogenized on ice in 25 strokes using a 30-G syringe. The homogenate was spun down at 2000 rpm for 5min to remove cell debris and nuclei. The supernatant was collected and mixed with an equivalent volume of 62% (w/v) sucrose to bring it to a final concentration of 40% sucrose. DENV phagosomes were then isolated on a discontinuous sucrose gradient, which was prepared as follows: 3ml 62% sucrose, 2ml of 40% sucrose phagosome suspension, 2ml of 35% sucrose, 2ml of 25% sucrose and 2ml of 10% sucrose. Centrifugation was performed at 4°C in a swinging bucket rotor (SW41; Beckman Instruments) for 1h at 100, 000 x g. Fractions at the interfaces of step sucrose gradients were collected, with 140µl subjected to viral RNA extraction using QiaAmp Viral RNA Mini kit (Qiagen). The phagosomes were resuspended in 10ml cold PBS containing protease inhibitors and pelleted by ultracentrifugation (40,000 x g, 30min) at 4°C in an SW41 rotor. Supernatant was removed and the phagosome pellet was resuspended in 80 to 100µl lysis buffer (1% Nonidet P-40, 150mM NaCl, 50mM Tris, pH 8.0) in the presence of protease and phosphatase inhibitors (Sigma). The protein concentration of the phagosome preparation was determined using Pierce BCA Protein

Assay Kit (Pierce). Phagosome lysates (3μg) were separated on a SDS-PAGE gel for Western blot analysis. For nanoparticle tracking analysis (NTA), washed phagosomes were resuspended at an appropriate dilution in HNE buffer before injection into the LM unit with a 1ml syringe.

### 3.2.2 Purification of DENV-containing phagosomes on a continuous sucrose gradient

For isolation of DENV phagosomes on a continuous sucrose gradient, sucrose gradient was first formed by careful layering of 10% to 60% sucrose in 10% increments, starting with the densest at the bottom. The gradient was allowed to linearize overnight at 4°C. Cell homogenate was layered above the continuous sucrose gradient for ultracentrifugation. After ultracentrifugation, 250μl fractions were collected and subjected to viral RNA extraction using QiaAmp Viral RNA Mini kit (Qiagen).

### 3.2.3 Sucrose gradient purification of latex bead-containing phagosomes

Deep blue dyed latex beads of 0.24μm (Sigma, L1273) were first washed 3 times in PBS before use. 100μl of 10% latex bead suspension was resuspended in 7.5ml of coupling buffer (0.1M 2-(N-Morpholino) ethanesulfonic acid (MES) in PBS, pH 6.1). Opsonization of 6μg human IgG to latex beads was performed overnight at 4°C with constant mixing. Beads were centrifuged (14, 680 rpm, 10min) and washed with PBS 3 times before they are diluted to obtain a 1% latex bead suspension. The 1% bead suspension is then added to $1x10^7$ cells, and incubated at 37°C for 30min for internalization to take place. Thereafter, cells were washed 3 times in PBS (1000 rpm, 3min) to remove non-internalized beads. Cells were further incubated at 37°C for a chase period ranging from 30min to 5.5h to obtain mature phagolysosomes. At the end of the

chase period, cells were washed in PBS 3 times, with a final wash in 8.55% (w/v) sucrose homogenization buffer. The isolation of latex bead containing phagosomes was performed in a manner similar to previously described steps for isolation of DENV containing phagosomes. After ultracentrifugation, latex bead containing phagosomes were collected at the 10%-25% sucrose interface. The phagosomes were resuspended in 10ml cold PBS containing protease inhibitors and pelleted by ultracentrifugation (40,000 x g, 30min) at 4°C in an SW41 rotor. Supernatant was removed and the phagosome pellet was resuspended in 80 to 100µl lysis buffer (1% Nonidet P-40, 150mM NaCl, 50mM Tris, pH 8.0) in the presence of protease and phosphatase inhibitors (Sigma). The protein concentration of the phagosome preparation was determined using Pierce BCA Protein Assay Kit (Pierce). Phagosome lysates (3µg) were separated on a SDS-PAGE gel for Western blot analysis.

### 3.2.4 Nanoparticle tracking analysis (NTA)

The Nanosight LM10 utilizes a single-mode red laser diode with illumination of 638nm wavelength and laser power of 25mW to visualize nanoparticles ranging from 10 to 1000nm in size. It was also customized with a 692nm bandpass filter for use under fluorescence mode. The red laser illuminates DiD-labelled nanoparticles in suspension diluted to between $10^7$ and $10^9$ particles per ml in light scatter mode or fluorescence mode. A high sensitivity scientific CMOS camera tracks individual particles moving under Brownian motion and particle distribution and size, as calculated using the Stokes-Einstein equation, was analyzed with the NTA 2.3 analytical software (Nanosight). The camera settings (shutter, gain, detection threshold and sensitivity) were optimized for

individual samples. For each sample, 5 videos of 90s duration were recorded, with a 5s delay between recordings and chamber temperature recorded at the end of each video.

### 3.2.5 Dual labelling of DENV with pHrodo Red and Alexa Fluor 488

Fresh labelling buffer (0.2M sodium bicarbonate buffer, pH 8.3) was prepared and filter sterilized with 0.2μm syringe filters prior to DENV labelling. Purified DENV was diluted to approximately $3x10^8$ PFU in 1ml of labelling buffer. Both lyophilized Alexa Fluor 488 (AF488) succinimidyl esters and pHrodo Red succinimidyl esters were reconstituted to 2mM in labelling buffer or DMSO respectively, immediately prior to the labelling reaction. pHrodo Red dye was added to the diluted virus at final concentrations of 90μM, 100μM, 110μM, 120μM, 140μM and 150μM, while stirring gently with the pipette tip. The labelling reaction mix was incubated at room temperature for 30min in the dark and mixed by gentle inversions every 15min. Next, AF488 dye was added to the diluted virus at final concentrations of 50μM, 60μM, 80μM, 90μM, 100μM and 110μM, while stirring gently with the pipette tip. The labelling reaction mix was incubated at room temperature for an additional 1h in the dark and mixed by gentle inversions every 15min. Excess dye was removed by gel filtration on a PD-10 column (GE Healthcare). Briefly, the column was equilibrated with 25ml of HNE buffer (5mM Hepes, 150mM NaCl, 0.1mM EDTA, pH 7.4) before use. The labelled virus was applied to the top of the column and collection of flow-through began once the labelled virus entered the matrix. The first 3.25ml of flow-through was discarded, while the following 2ml of labelled virus fraction was collected. The pHrodo/AF488-labelled DENV was stored in 100μL aliquots at −80 °C, away from light source, retitrated by plaque assay, and tested for fluorescence using immunofluorescence assay on Vero cells before use in experiments.

### 3.2.6 Immunofluorescence of virus infection on Vero cells

Equal volumes of pHrodo/AF488-labelled dengue virus were incubated with Vero cells plated on coverslips for 10 minutes at 37°C, washed, fixed with 3% paraformaldehyde (PFA) and permeabilized with 0.1% saponin. The cells were then incubated for 1 hr with anti-E protein h3H5 monoclonal antibody, at room temperature. The cells were washed three times in PBS, followed by incubation with AF647 anti-human IgG antibody for 45min at room temperature. Cells were then washed three times in PBS, rinsed once in deionised water and mounted on to glass slides with Mowiol 4-88. Cells were visualized at 63x magnification on a Zeiss LSM710 confocal microscope and co-localization coefficients were calculated using Zeiss ZEN2011 program. Images were exported in individual colours for processing in Adobe Photoshop CS6 version 13, which involved adjustment of the contrast on the images for clarity. Images in individual colours were then merged using ImageJ.

### 3.2.7 Assessing phagosomal acidification with pHrodo- or pHrodo/AF488-labelled DENV

pHrodo-labelled or pHrodo/AF488-labelled DENV were incubated with sub-neutralizing concentrations of h3H5 (0.391μg/ml) for 1h at 37°C before adding to THP-1.2R, THP-1.2S or primary monocytes (moi 10). Primary monocytes were treated with indicated concentrations of sodium stibogluconate or PBS control for 6h before infection with pHrodo/AF488-labelled DENV. Cells were synchronized on ice for 20min, followed by 2h infection at 37°C and fixed with 3% PFA for 30min at room temperature. Cells were subjected to cytospin at 800rpm for 3min. After washing with PBS, cells infected with

pHrodo-labelled DENV were permeabilized with 0.1% saponin for 30min and incubated with anti-E protein antibody at 4°C overnight. Cells were washed with PBS, and incubated with AF647 anti-human IgG antibody for 45min at room temperature. Cells were then washed three times in PBS, rinsed once in deionised water and mounted on to glass slides with Mowiol 4-88. Cells were visualized at 63x magnification on a Zeiss LSM710 confocal microscope and quantification of the fluorescence intensities of pHrodo, AF488 and AF647 in THP-1.2R, THP-1.2S and primary monocytes was carried out using Zeiss ZEN2011 program. Briefly, fluorescence intensities of DENV phagosomes in 25 to 30 randomly selected cells were calculated. Only DENV phagosomes with AF488 or AF647 fluorescence intensity more than 20 were included in the analysis.

### 3.2.8 Immunoblotting

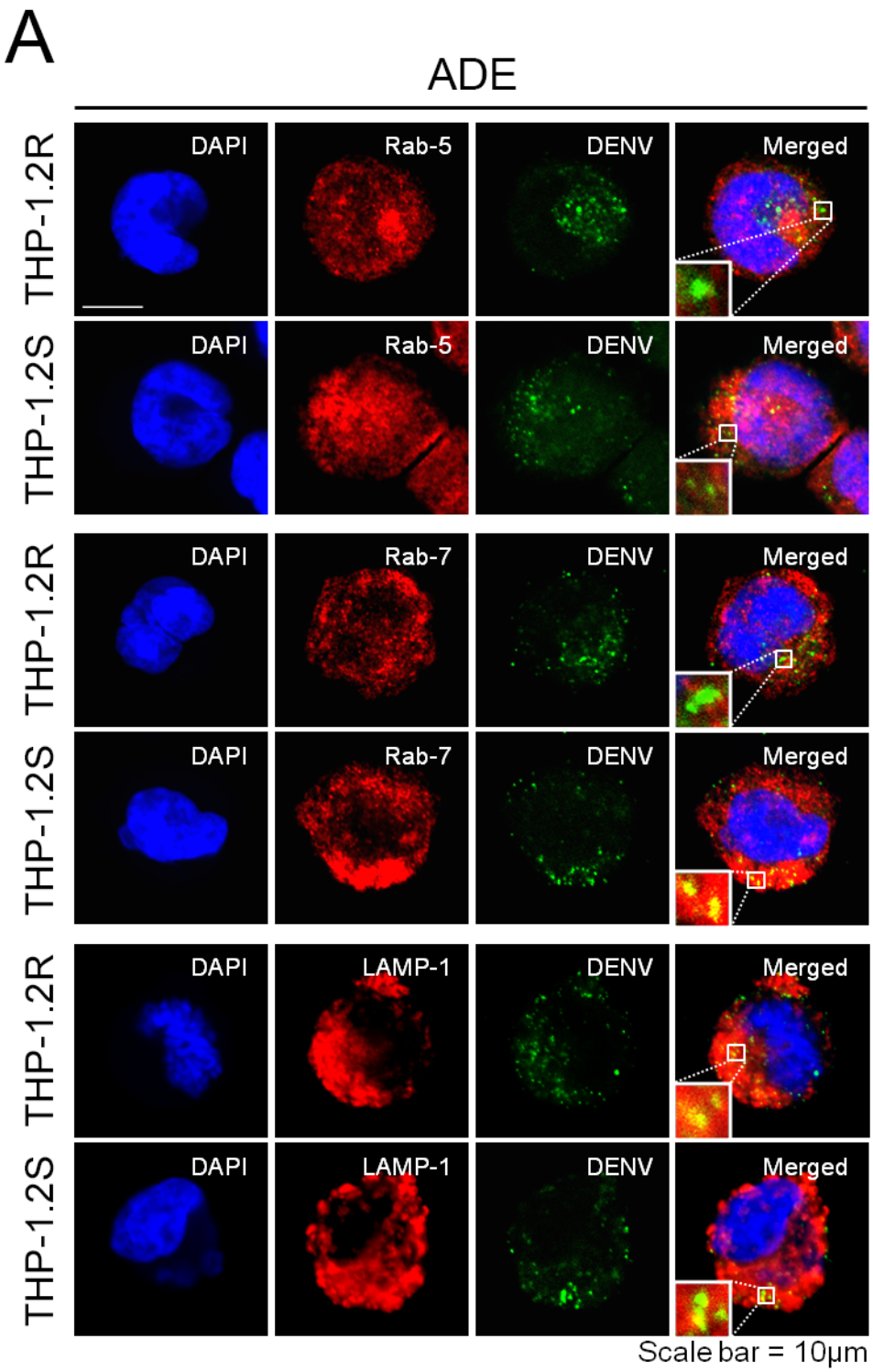
Phagosome lysates (3µg) were separated on a SDS-PAGE gel for Western blot analysis and probed with primary antibody, followed by HRP-conjugated anti-mouse (1:1000, Dako P0447) or anti-rabbit (1:3000, Abcam ab6721) antiserum. Primary antibodies for Rab-5 (1:1000, Abcam ab13253), Rab-7 (1:1000, Abcam ab50533), LAMP-1 (1:1000, eBioscience 611043), Cathepsin D (1:500, Abcam ab6313), EEA-1 (1:1000, Santa Cruz sc33585), BiP (1:1000, Abcam ab21685), calnexin (1:1000, Abcam ab22595), PMP70 (1:500, Abcam ab3421), GM130 (1:10,000, Abcam ab52649) and HSP60 (1:20,000, Abcam ab59457) were used. Thereafter, blots were developed by enhanced chemiluminescence detection reagents (Amersham).

## 3.3 Results

### 3.3.1 Higher degree of phagocytic maturation in THP-1.2R

Since LILRB1 signals through phosphorylated SHP-1 to inactivate Syk in the activating FcγR signaling pathway, co-ligation of LILRB1 during ADE by DENV immune complex could modulate other host responses. Syk is a key regulator of downstream FcγR signaling; in addition to its role in ISG induction, it also triggers signaling pathways that regulate calcium signaling (Kurosaki & Tsukada, 2000), cytoskeletal rearrangement and phagocytosis (Crowley et al, 1997; Greenberg et al, 1996; Majeed et al, 2001), and ROS production (Kyrmizi et al, 2013; Underhill et al, 2005).

We first tracked the transition of DENV using immunofluorescence imaging. Results showed that DENV-containing phagosomes transited through Rab-5, Rab-7 and LAMP-1 compartments more rapidly in THP-1.2R relative to THP-1.2S during ADE. We observed reduced co-localization of Alexa fluor 488 (AF488)-labelled DENV with Rab-7 in THP-1.2R relative to THP-1.2S 3 hours post-infection under ADE conditions (Fig. 3-1A). At the same time, increased co-localization of AF488-labelled DENV was observed with LAMP-1 in THP-1.2R relative to THP-1.2S during ADE (Fig. 3-1A). In contrast, the transition of DENV-containing phagosomes was comparable in THP-1.2R and THP-1.2S under DENV-2 only conditions (Fig. 3-1B). The higher degree of phagosome maturation in THP-1.2R corroborates the higher levels of phosphorylated Syk that were observed in THP-1.2R relative to THP-1.2S during ADE (Fig. 2-6A). These findings reinforce the notion that phagosome maturation is a Syk-dependent process.


A
ADE
THP-1.2R
THP-1.2S
THP-1.2R
THP-1.2S
THP-1.2R
THP-1.2S
DAPI
Rab-5
Rab-7
LAMP-1
DENV
Merged
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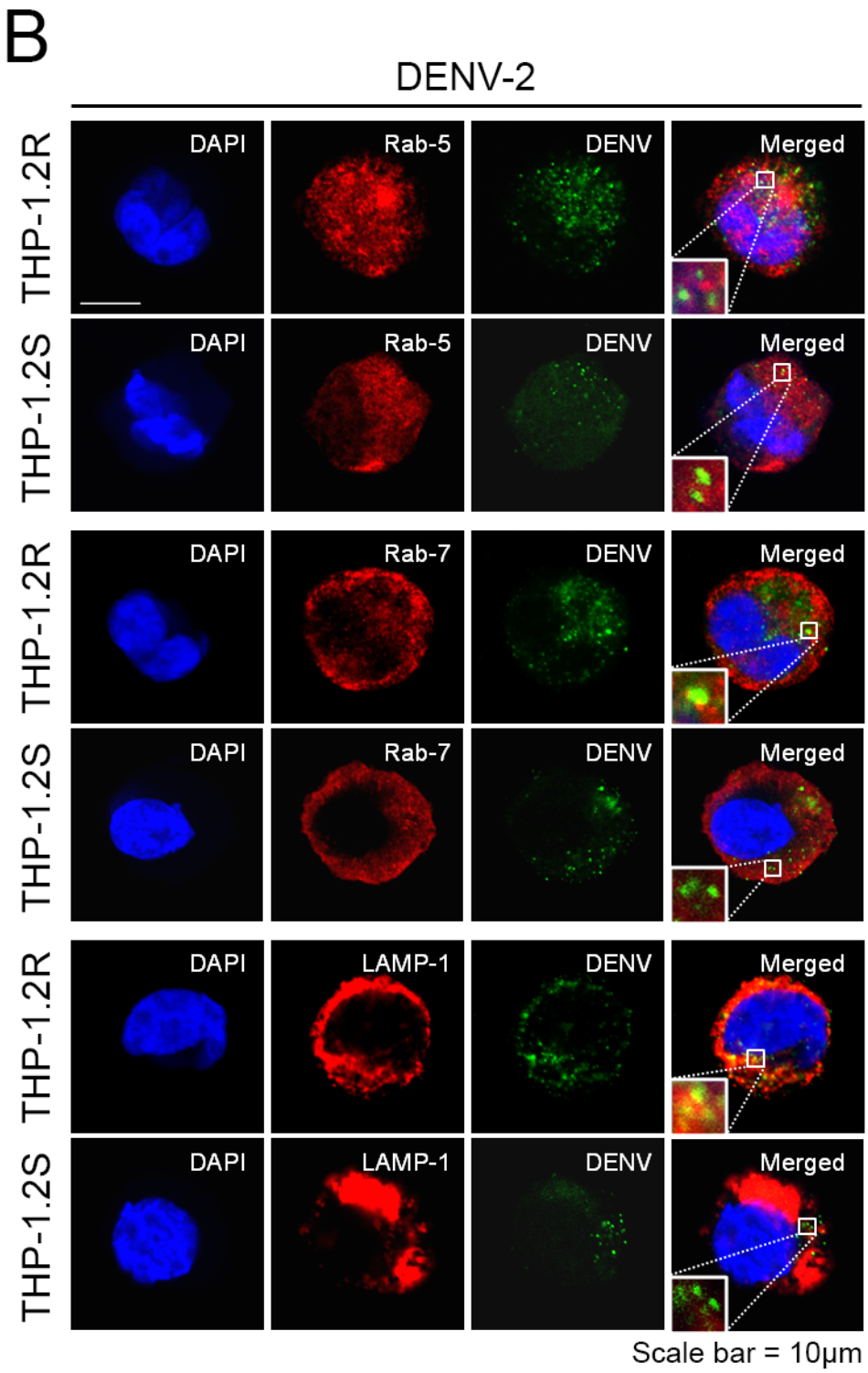



**Figure 3-1. Higher degree of phagocytic maturation in THP-1.2R.** (A) Co-localization of DENV-2 with Rab-5, Rab-7 and LAMP-1 3h post-infection under ADE conditions. (B) Co-localization of DENV-2 with Rab-5, Rab-7 and LAMP-1 3h post-infection under DENV-2 only conditions. Panel was kindly provided by Summer Zhang.

### 3.3.2 Use of latex bead-containing phagosomes as a surrogate to investigate role of differential Syk phosphorylation on compartmentalization

We reasoned that the higher degree of DENV phagosome maturation in THP-1.2R could be due to differences in DENV compartmentalization as a result of differential levels of Syk phosphorylation. The isolation of latex bead-containing (LBC) phagosomes has been used successfully to study FcγR-mediated phagocytosis (Majeed et al, 2001) and the proteomic characterization of phagosomes (Stuart et al, 2007). We thus adapted a protocol developed by Desjardins and colleagues (Desjardins et al, 1994) for phagosome isolation to examine compartmentalization during ADE. Briefly, unopsonized blue-dyed latex beads (LB) or blue-dyed latex beads opsonized with IgG (LB-IgG) were added onto THP-1.2R cells pre-treated with DMSO or piceatannol, a Syk-selective inhibitor. LBC phagosomes were then isolated via flotation on a step sucrose gradient, and could be visualized as a blue layer at the 10%-25% sucrose interface. Phagosomes isolated in this manner have been shown to be devoid of contamination by other cellular organelles (Desjardins et al, 1994) due to the low density of latex.

To verify the purity of isolated LBC phagosomes, we probed the expression of endosomal and organelle markers. As expected, phagosomal fractions (F1) isolated at all indicated time points expressed Rab-7, LAMP-1 and Cathepsin D (Fig. 3-2A). However, phagosomes isolated from piceatennol-treated cells faced deficits in acquiring Rab-7 (Fig. 3-2A). The prolonged retention of Rab-5 in these phagosomes at 1 hour post-uptake (Fig. 3-2A), although transient, is further indication that the Rab-5 to Rab-7 transition is inhibited by piceatannol treatment.

While we did not detect the expression of HSP60 (mitochondria) or GM130 (Golgi apparatus), the expression of calnexin and BiP, endoplasmic reticulum (ER)

membrane and lumen proteins respectively, were detected in the isolated phagosomes (Fig. 3-2B). Rather than contamination with the ER, the presence of ER proteins in LBC phagosome preparations could be attributed to contribution of ER membrane to the maturing phagosome (Gagnon et al, 2002; Garin et al, 2001), although this has been controversial (Touret et al, 2005).

However, piceatannol treatment of THP-1.2R cells may have unintended off-target effects, as demonstrated by reduced expression of organelle markers (HSP60, GM130 and BiP) in the non-phagosomal fraction (F3) (Fig. 3-2B). Since this could potentially confound downstream analysis of how differential levels of Syk phosphorylation affect compartmentalization, we decided to substitute the approach of using piceatannol treatment to disrupt Syk activity, with isolation of DENV-containing phagosomes in THP-1.2R and THP-1.2S, in which Syk phosphorylation levels had previously been characterized post-infection (Fig. 2-6A).

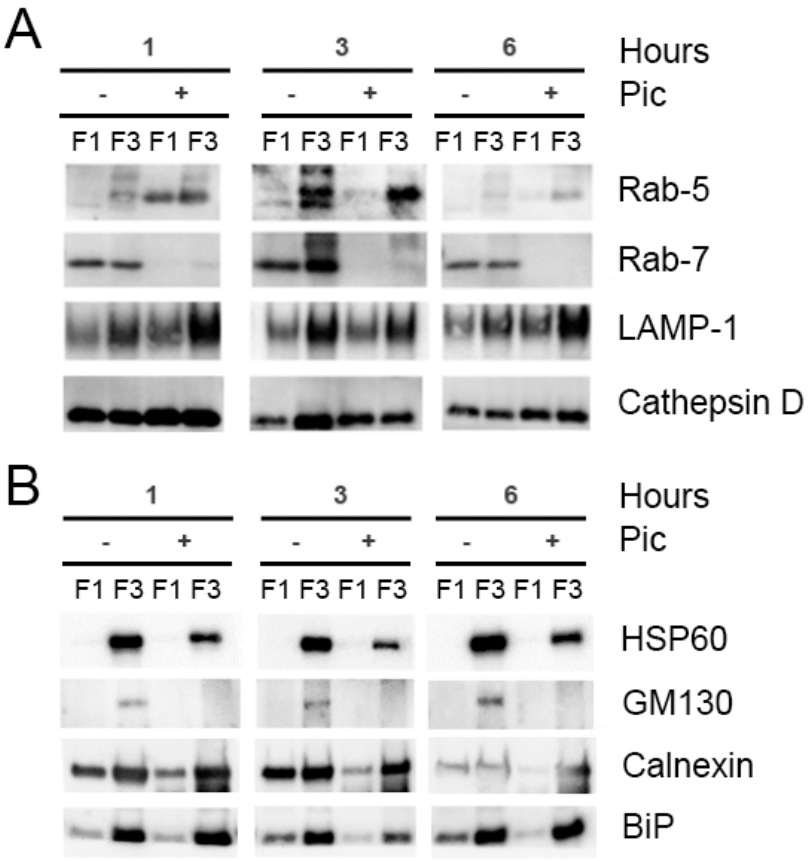



**Figure 3-2. Western blot analysis of subcellular fractions.** (A) Western blot of membrane-bound endosomal markers, Rab-5, Rab-7 and LAMP-1, and a lysosomal hydrolase, Cathepsin D. (B) Western blot of organelle markers for mitochondria (HSP60), Golgi apparatus (GM130), ER membrane (Calnexin) and ER lumen (BiP).

### 3.3.3 Isolation and characterization of DENV phagosomes

We adapted the protocol developed by Desjardins and colleagues (Desjardins et al, 1994) for isolation of DENV phagosomes. As this protocol had not been validated for isolation of DENV phagosomes, this protocol was first tested to ensure reproducible and accurate isolation of DENV phagosomes. Briefly, DENV or DENV opsonized with sub-neutralizing levels of h3H5 were added onto THP-1.2R and THP-1.2S cells. DENV phagosomes were then isolated via flotation on a step sucrose gradient. As a control, similar fractions from uninfected THP-1.2R and THP-1.2S cells were also collected after flotation on a step sucrose gradient. Fractions isolated at step sucrose interfaces were subjected to viral RNA extraction and highest recovery of viral RNA was detected in Fraction 3, followed by Fraction 2 and Fraction 1 (Fig. 3-3A).

To validate the viral RT-PCR findings, we used Nanosight, a technology that enables sizing and quantification of nanoparticles, to characterize isolated fractions from cells infected with DiD-labelled DENV under DENV-2 or ADE conditions. As Nanosight utilizes a high intensity laser beam against a low background optical configuration, it allows particles of sub-micron dimensions to be visualized. Biological samples like endosomes and vesicles typically have a lower refractive index and depending on their size, they may be below the detection threshold of 300nm for most commercially available flow cytometers (Dragovic et al, 2011). Since Nanosight determines particle size from Brownian motion, this measurement is independent of the particle's refractive index (Dragovic et al, 2011). Ultrastructural analysis and super resolution imaging of endosomal and lysosomal compartments have determined the sizes of these subcellular organelles to range from 200nm to 600nm (Firdessa et al, 2014; Shim et al, 2012), and the use of Nanosight to characterize purified subcellular fractions should allow tracking of

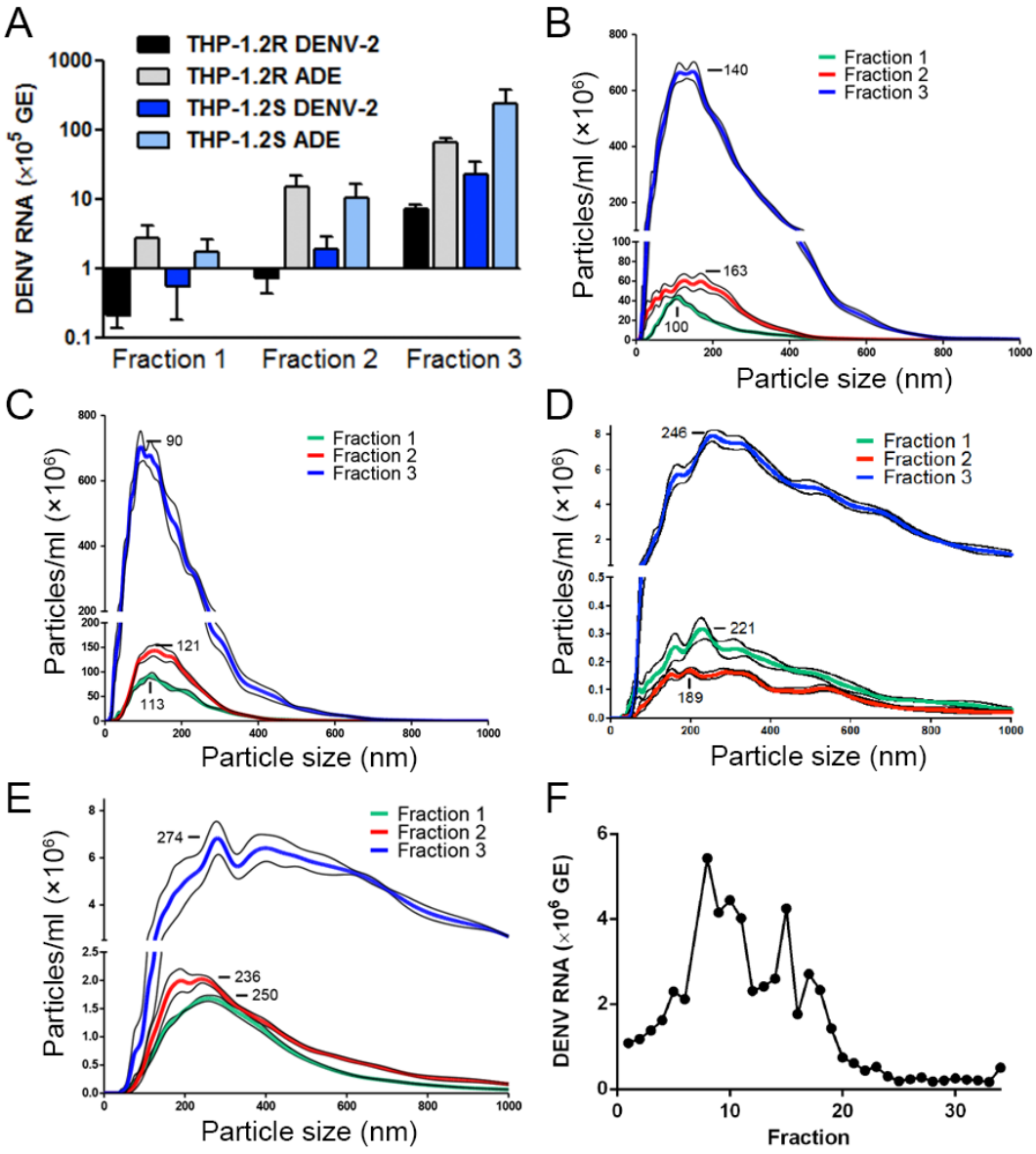
DENV with cellular endocytic machinery.

DENV was first labelled with a lipophilic fluorescent dye, DiD, at concentrations high enough to quench fluorescence intensity. Upon fusion of the viral and host cell membranes, the fluorescence unquenches and this can be detected using Nanosight under fluorescent mode. As fusion events during phagosome maturation allow the transfer of DiD dye onto lipid membranes, this enables the tracking of particles that enagaged in prior fusion events with DiD-labelled DENV. Operation of the Nanosight under fluorescence mode allows discrimination of DiD-particles from non-labelled particles, allowing us to probe the size and number of DiD-particles. The modal size of DiD-particles was 2 to 3 fold larger than modal size of nanoparticles detected under light scatter mode (Fig. 3-3B to E). The modal size of 246nm and 274nm for DiD-particles isolated following DENV only and ADE infection respectively also suggests the possibility that DENV is contained within endosomal or lysosomal compartments (Fig. 3-3D to E). Importantly, the majority of DiD-particles were detected in Fraction 3 during DENV-2 and ADE infection (Fig. 3-3D to E), which corresponds to the bulk of viral RNA recovery from Fraction 3 (Fig. 3-3A).

To ensure the reproducibility of isolating DENV phagosomes using a sucrose gradient, we also validated this protocol using a continuous sucrose gradient that would allow flotation of DENV phagosomes at their buoyant density. Recovery of viral RNA peaked at Fractions 7 and 14, indicating that DENV phagosomes are isolated in fractions with greater density (Fig. 3-3F). This also corroborates the recovery of viral RNA being highest in Fraction 3 (Fig. 3-3A), the densest fraction collected using a step sucrose gradient. As the yield of viral RNA recovery during purification of DENV phagosomes with a step sucrose gradient was considerably higher, this method was subsequently used

for further experiments.

Western blot probing for the expression of endosomal markers indicated that fractions collected expressed low levels of EEA-1 and Rab-5, markers for early endosomes, but higher levels of Rab-7 and LAMP-1, markers for late endosomes (Fig. 3-3G). When considered with the size of DiD-particles from isolated fractions, it suggests the recovery of DENV associated with late endosomal or lysosomal compartments. The expression of endosomal markers was also enriched in the purified fractions relative to whole cell lysate (Fig. 3-3G), providing further validation that isolation of DENV phagosomes can be performed using purification on a step sucrose gradient. Organelle markers were also used to verify the purity of the DENV phagosome isolation. Although we did not detect any expression of GM130 (Golgi apparatus) and PMP70 (peroxisomes), HSP60 (mitochondria) expression could be detected in Fractions 2 and 3 in THP-1.2R and THP-1.2S at enriched levels relative to whole cell lysate (Fig. 3-3H). Although it is not known if HSP60 associates with DENV phagosomes, there is evidence that HSP60 could be important for DENV replication, as siRNA knockdown of HSP60 in U937 monocytic cells led to reduced viral replication and increased IFN-α production (Padwad et al, 2009). Calnexin and BiP, ER membrane and lumen markers could also be detected in Fractions 1 to 3, but at similar levels relative to whole cell lysate (Fig. 3-3H).


A
THP-1.2R DENV-2
THP-1.2R ADE
THP-1.2S DENV-2
THP-1.2S ADE
DENV RNA (×10^5 GE)
1000
100
10
1
0.1
Fraction 1
Fraction 2
Fraction 3
B
Particles/ml (×10^6)
Fraction 1
Fraction 2
Fraction 3
140
163
100
Particle size (nm)
C
Particles/ml (×10^6)
90
121
113
Particle size (nm)
D
Particles/ml (×10^6)
246
221
189
Particle size (nm)
E
Particles/ml (×10^6)
274
236
250
Particle size (nm)
F
DENV RNA (×10^6 GE)
Fraction

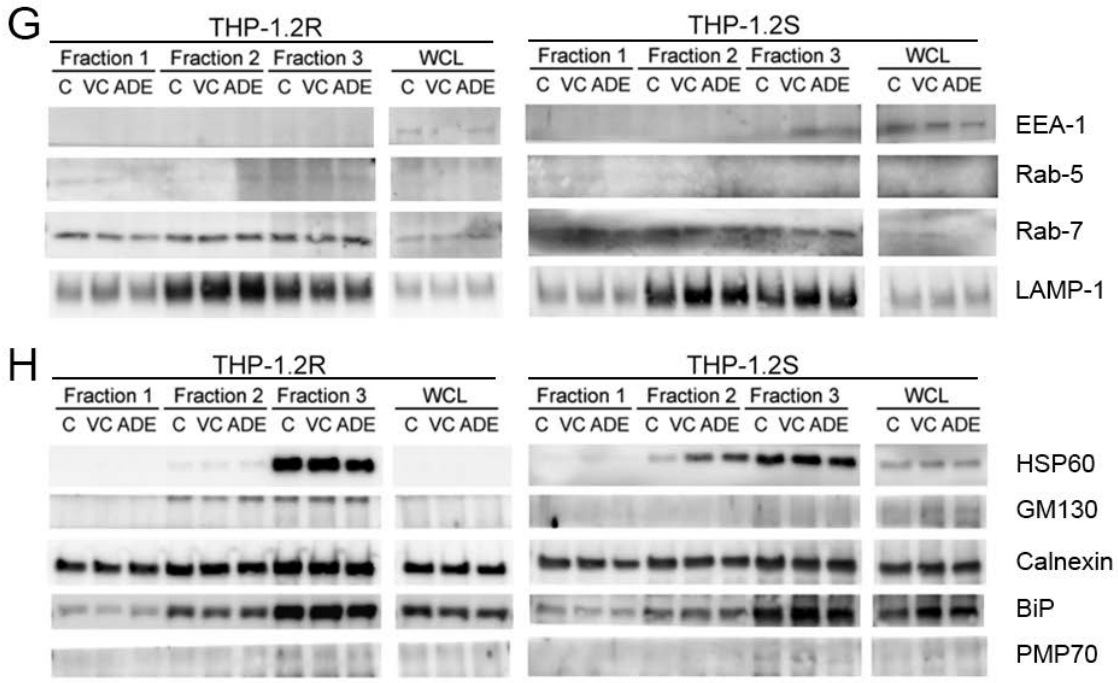


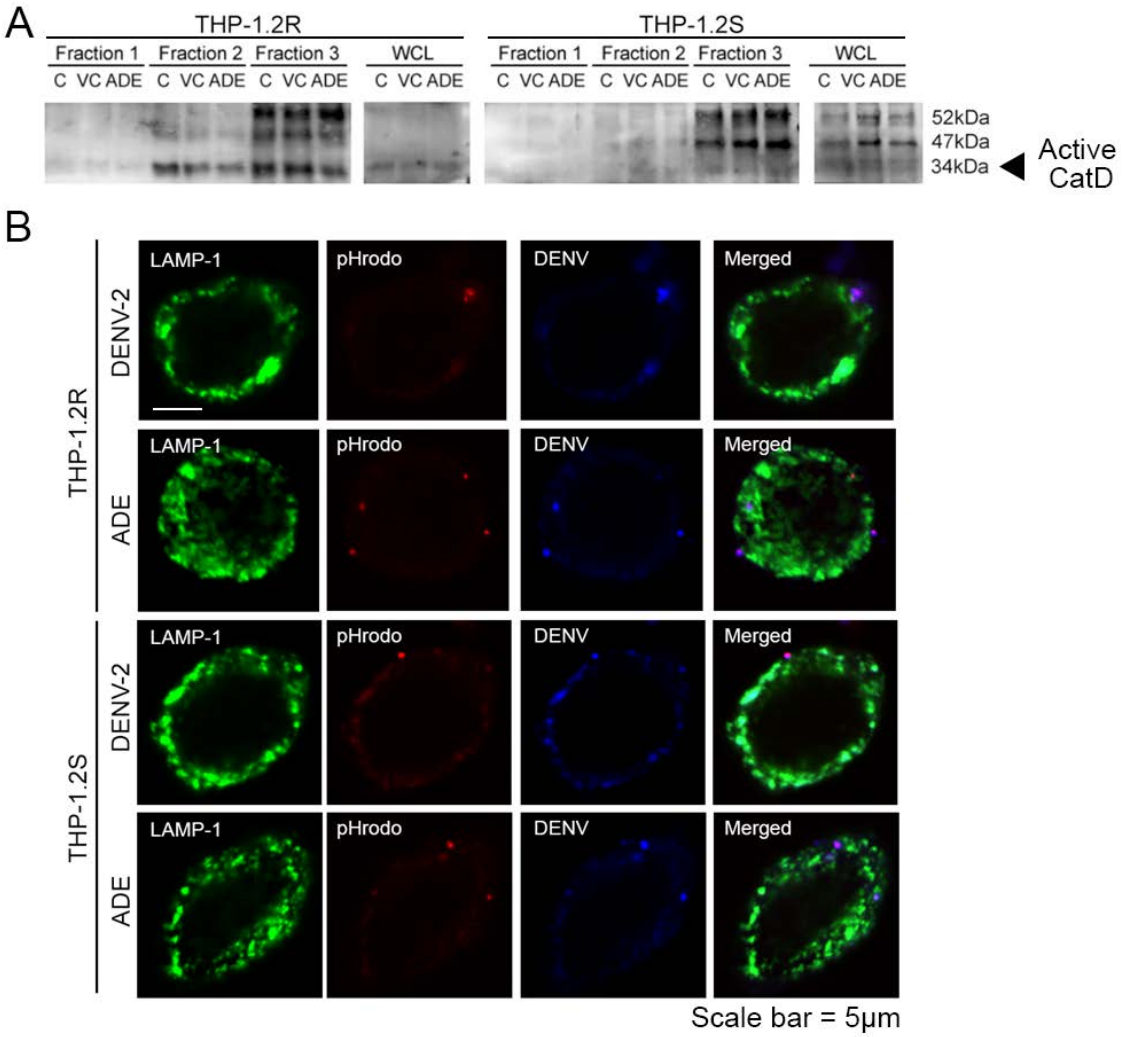

**Figure 3-3. Isolation of DENV-containing phagosomes from fraction 3 of step sucrose gradient.** (A) Recovery of viral RNA from step sucrose gradient purified fractions collected from THP-1.2R or THP-1.2S infected under DENV-2 only or ADE conditions. (B-C) Size and concentration of all nanoparticles from fractions collected from THP-1.2S under DENV-2 only (B) or ADE (C) conditions. (D-E) Size and concentration of DiD-labelled nanoparticles from fractions collected from THP-1.2S under DENV-2 only (D) or ADE (E) conditions. (F) Recovery of viral RNA from continuous sucrose gradient purified fractions collected from THP-1.2R infected under ADE conditions. (G-H) Western blot analysis of subcellular fractions, probed for endosomal markers (G) and organelle markers (H).

### 3.3.4 Higher levels of phagosomal acidification in THP-1.2R

Since the degree of phagocytic maturation was higher in THP-1.2R (Fig. 3-1), we hypothesized that this could also be accompanied by phagosomal acidification, which is considered a hallmark of phagosome maturation. To gain a functional insight into phagosomal acidification, we probed the expression of cathepsin D (CatD), a lysosomal aspartic endopeptidase that is processed to its catalytically active form only upon acidification. After synthesis in the ER, the 52kDa proCatD is targeted to endosomes, phagosomes and lysosomes. Acidic endosomal and lysosomal compartments cause the protein to be cleaved to its 47kDa intermediate active form and further proteolytic processing yields the 34kDa active CatD. Western blot probing the expression of CatD showed higher levels of active CatD in THP-1.2R compared to THP-1.2S (Fig. 3-4A), consistent with the other data showing higher degree of phagocytic maturation in the former subclone.

In order to probe levels of phagosomal acidification in DENV-containing phagosomes, we designed a new approach that enabled us to obtain a relative measure of pH changes in the phagosome. Our approach entailed labelling DENV with the pH-sensitive pHrodo dye, and counterstaining DENV E protein with a pH-insensitive AF647 anti-human IgG antibody to indicate intracellular presence of DENV. Since acidification of the environment increases the fluorescence intensity of pHrodo but not AF647, the ratio of fluorescence intensities between pHrodo and AF647 can serve as a quantitative readout of phagosomal acidification. This ratiometric measurement of phagosomal acidification has previously been used to measure the pH of bacteria-containing compartments (Ip et al, 2010), but our approach has broadened the application of such a method to investigate changes in phagosomal pH in virus-containing compartments.

When pHrodo-labelled DENV was used to infect cells, it co-localized strongly with E protein, indicating that infectivity of pHrodo-labelled DENV was retained post-labelling (Fig. 3-4B). pHrodo-labelled DENV also co-localized with LAMP-1 positive compartments in both THP-1.2R and THP-1.2S under DENV and ADE conditions (Fig. 3-4B). To measure the fluorescence intensities of pHrodo and AF647, a line is drawn across the profile where fluorescence intensity is to be measured. An intensity plot for the profile is generated and the intensity of pHrodo corresponding to peak intensity of AF647 was measured (Fig. 3-4C). By calculating fluorescence intensities of DENV phagosomes in 25 to 30 randomly selected cells, phagosomal acidification was found to be higher in THP-1.2R infected under ADE conditions (Fig. 3-4D). This also corroborates the higher degree of phagosomal maturation observed in THP-1.2R under ADE conditions (Fig. 3-1A).


A
THP-1.2R
THP-1.2S
Fraction 1
Fraction 2
Fraction 3
WCL
C VC ADE
52kDa
47kDa
34kDa
Active CatD
B
THP-1.2R
THP-1.2S
DENV-2
ADE
LAMP-1
pHrodo
DENV
Merged
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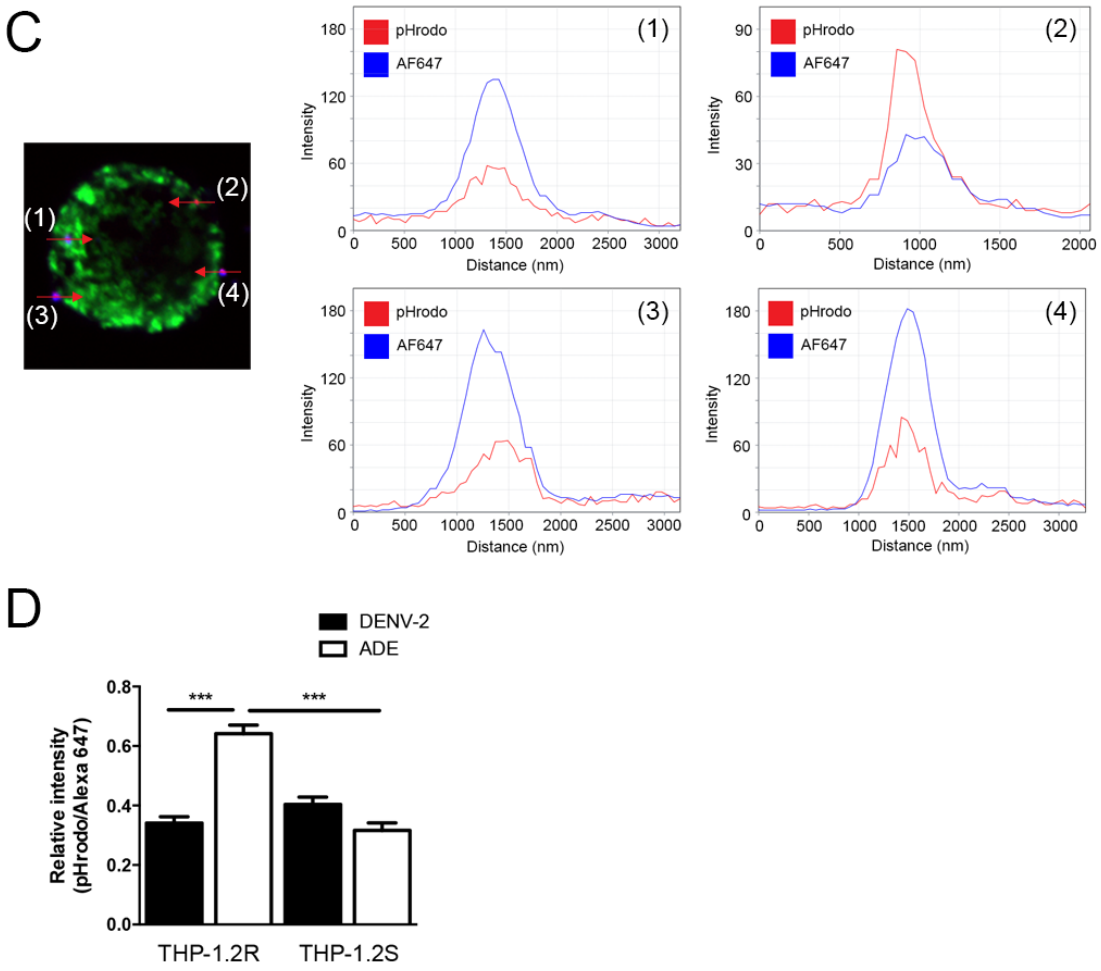



**Figure 3-4. Higher levels of phagosomal acidification in THP-1.2R.** (A) Western blot showing higher levels of active CatD (34kDa) in THP-1.2R. (B) Co-localization of pHrodo-labelled DENV-2 with E protein (AF647) and LAMP-1 in THP-1.2R or THP-1.2S 2h post-infection under DENV only or ADE conditions. (C) Schematic showing how fluorescence intensity was measured. Numbered red arrows indicate the profile where fluorescence intensity was measured and these are represented on the right as intensity plots (1 to 4). For each profile, pHrodo intensity corresponding to peak AF647 intensity was measured. (D) Relative fluorescence intensity of pHrodo and AF647 (E protein) in DENV-containing phagosomes in THP-1.2R or THP-1.2S infected under virus only or ADE conditions. Data expressed as mean ± s.e.m. *** $P < 0.001$.

### 3.3.5 LILRB1 signaling attenuates phagosomal acidification during ADE

The ratiometric assessment of phagosomal acidification using pHrodo-labelled DENV with AF647 secondary antibody against anti-E protein antibody was indeed promising and revealed higher levels of phagosomal acidification in THP-1.2R as compared to THP-1.2S during ADE. However, to enhance the specificity of probing phagosomal acidification, we labelled DENV with both pHrodo and AF488. Direct labelling of DENV with both fluorophores has clear advantages. It eliminates the additional step of counterstaining DENV with a pH-insensitive fluorescent conjugated secondary antibody, which could increase the size of DENV by up to 30nm, or 160% of the size of a virus particle. Moreover, using the same batch of labelled virus would ensure precision and reproducibility over different experiments, as indirect labeling of DENV would not be required.

Furthermore, a potential application of the smaller sized pHrodo/AF488-labelled DENV would be in super resolution microscopy like stimulated emission depletion (STED) microscopy or stochastic optical reconstruction microscopy (STORM). These systems allow image capture with a higher resolution than the light diffraction limit and are gaining traction in studying virus entry and replication. The role of CD81 in influenza A virus uncoating during virus budding was studied recently and it was revealed through the use of STORM that CD81 was recruited to sub-viral locations during virus assembly and may thus play a role in scission of budding virions from the plasma membrane (He et al, 2013). STORM has also been used to demonstrate recruitment of HIV-1 envelope proteins to viral assembly sites near the surface of infected cells, suggesting a role for envelope proteins that extends beyond its well-characterized functions in viral entry (Muranyi et al, 2013). The smaller size of dual labelled DENV would permit imaging at

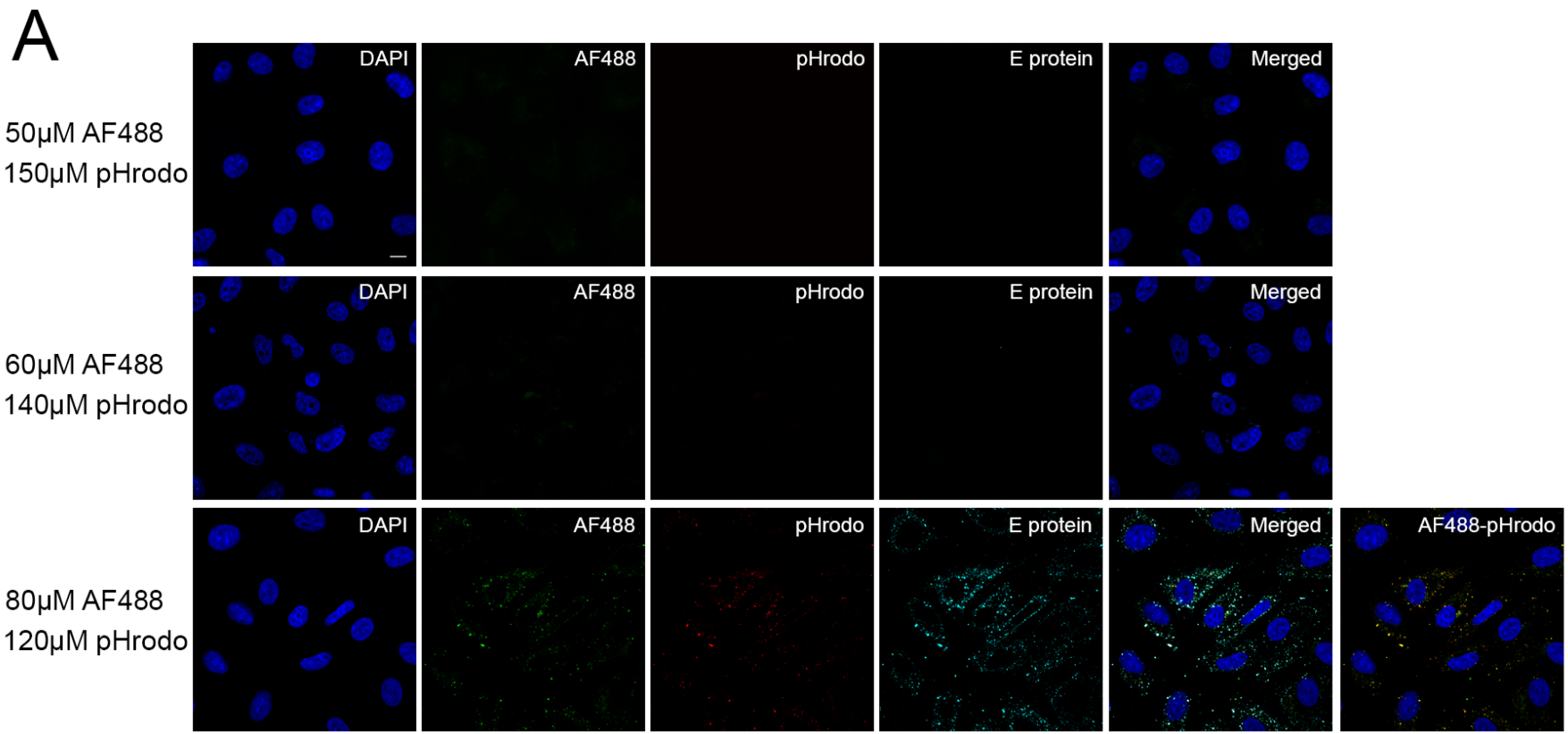
higher resolution and more accurate tracking of the virus as it is internalized and trafficked in host cells. Specifically, it could be applied to answer the critical question of whether pH changes in the phagosome result in degradation or viral fusion, which would determine the intracellular fate of DENV that is taken up via activating FcγRs.

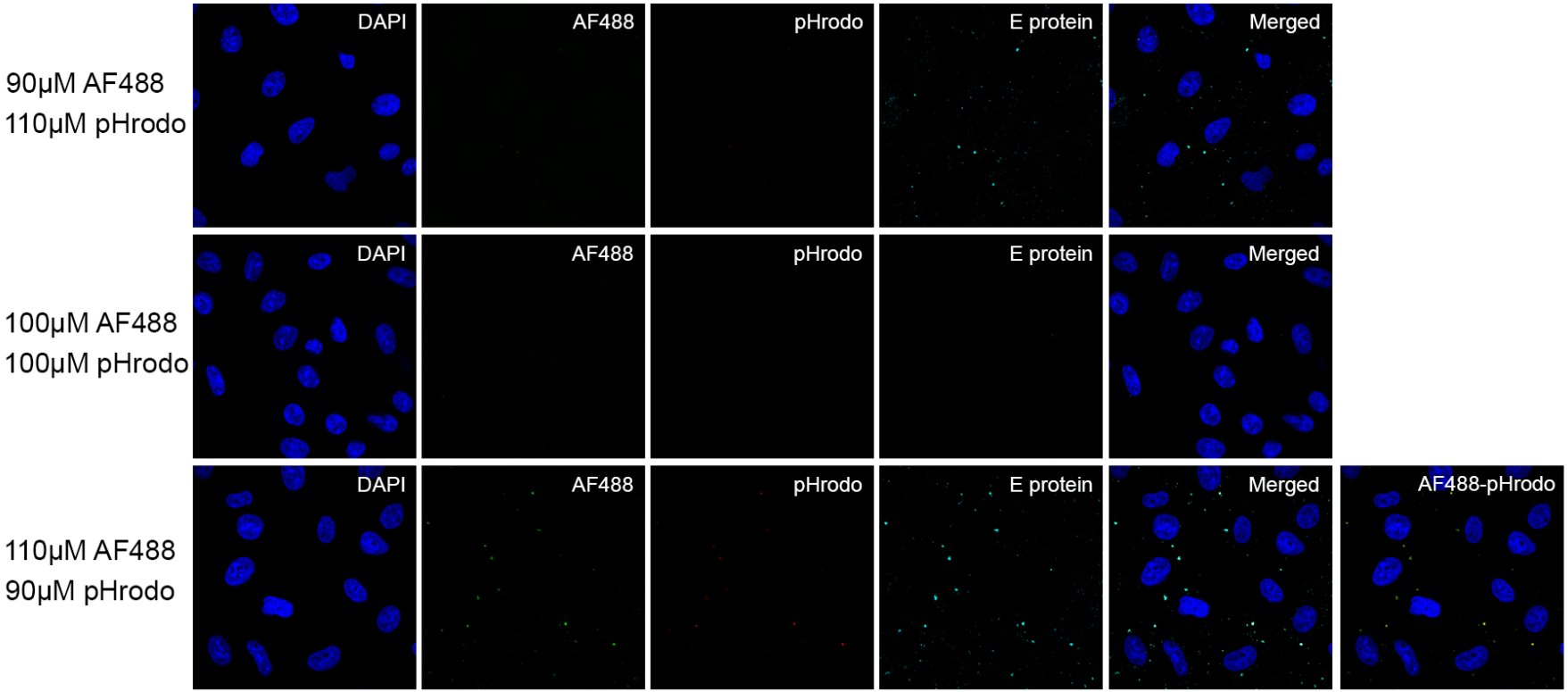
To determine the optimum concentrations of AF488 and pHrodo for labelling DENV to obtain maximum fluorescence and minimal loss in viable virus titers, different combinations of AF488 and pHrodo concentrations were tested. The infectivity of pHrodo/AF488-labelled DENV was assessed by immunofluorescence staining. Vero cells seeded on coverslips were incubated with equal amounts of DENV labelled with different concentrations of pHrodo and AF488. Cells were fixed, stained for E protein and visualized using confocal microscopy. Fluorescence intensities of pHrodo/AF488-labelled DENV were best achieved at final concentrations of either 80μM AF488 and 120μM pHrodo or 110μM AF488 and 90μM pHrodo (Fig. 3-5A). To quantify the co-localization with E protein, we sampled 30 randomly selected cells and calculated the co-localization coefficient. In both of these labelling conditions, AF488 and pHrodo fluorescence co-localized well with E protein staining (Fig. 3-5B). DENV labelled with different concentrations of pHrodo and AF488 was also re-titrated by plaque assay. Approximate 100 to 429-fold reductions in titers were obtained when DENV was labelled with either 80μM AF488 and 120μM pHrodo or 110μM AF488 and 90μM pHrodo (Fig. 3-5C).

In other AF488 and pHrodo concentrations tested, there was a reduction in DENV infectivity, as observed from low fluorescence intensity of AF488 and pHrodo, as well as E protein staining (Fig. 3-5A and B). Upon re-titration of titers by plaque assay, labelled

DENV either failed to form plaques or underwent substantial fold reductions in titers (Fig. 3-5C).

With our dual-fluorophore labeling strategy on DENV, we tested the hypothesis that lower levels of phagosomal acidification in THP-1.2S during ADE (Fig. 3-4D), is due to the dephosphorylation of Syk by pSHP-1 as a consequence of LILRB1 signaling. We pre-treated primary monocytes with different concentrations of sodium stibogluconate (SSG), a SHP-1 selective inhibitor, or PBS before infection under DENV only or ADE conditions. Representative confocal images indicated that similar to what we had observed with Vero cells, pHrodo/AF488-labelled DENV also infected primary monocytes with strong fluorescence, and co-localized with LAMP-1 positive compartments (Fig. 3-6A). Indeed, measuring the ratio of pHrodo and AF488 fluorescence intensities revealed a dose-dependent increase in phagosomal acidification when SSG-treated primary monocytes were infected under ADE conditions (Fig. 3-6B). Statistically significant changes in endosomal acidification were also observed under DENV-2 only infection although these were not dose-dependent (Fig. 3-6C).


A
50μM AF488
150μM pHrodo
60μM AF488
140μM pHrodo
80μM AF488
120μM pHrodo
DAPI
AF488
pHrodo
E protein
Merged
AF488-pHrodo


90μM AF488
110μM pHrodo
100μM AF488
100μM pHrodo
110μM AF488
90μM pHrodo
DAPI
AF488
pHrodo
E protein
Merged
AF488-pHrodo

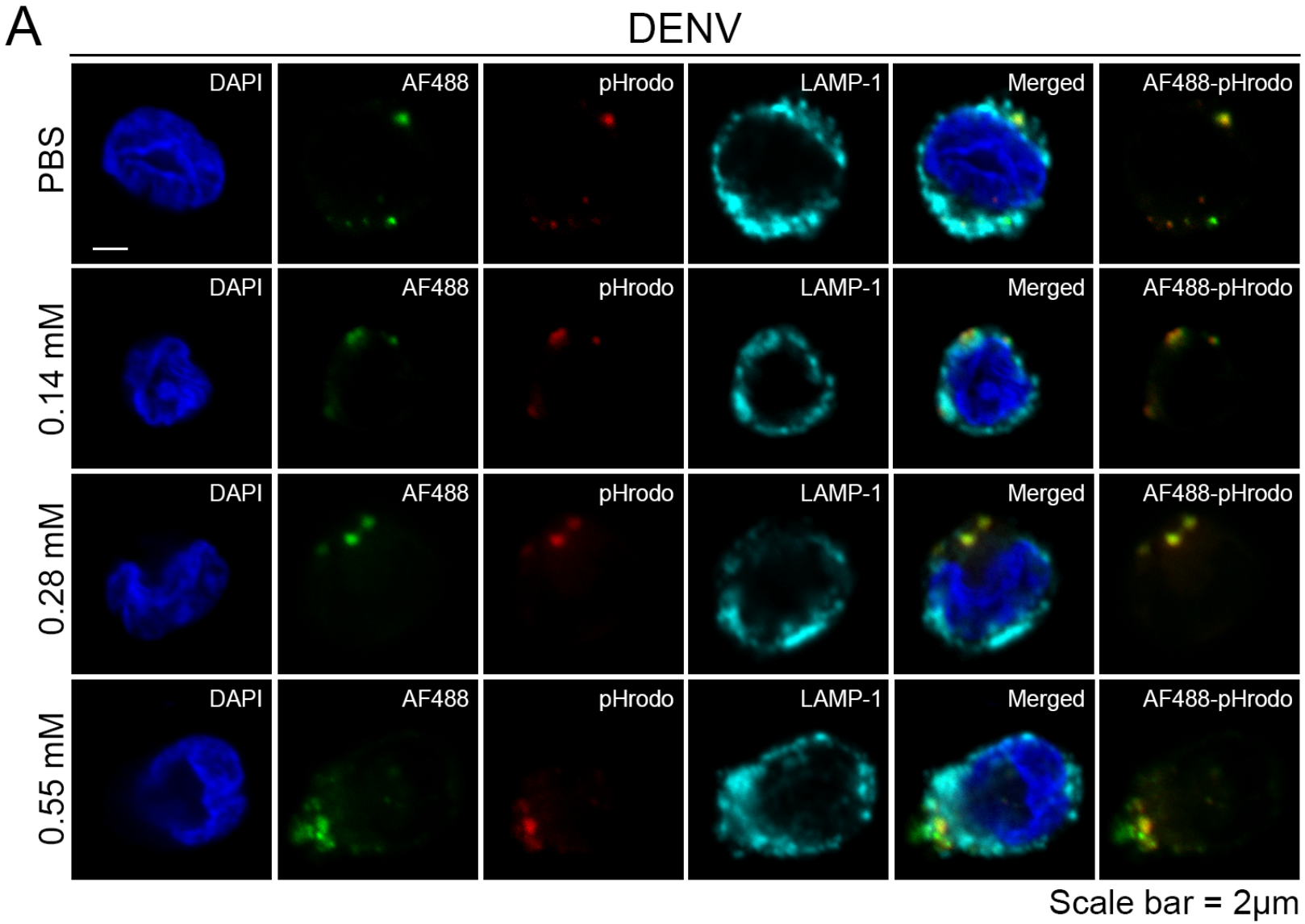
B

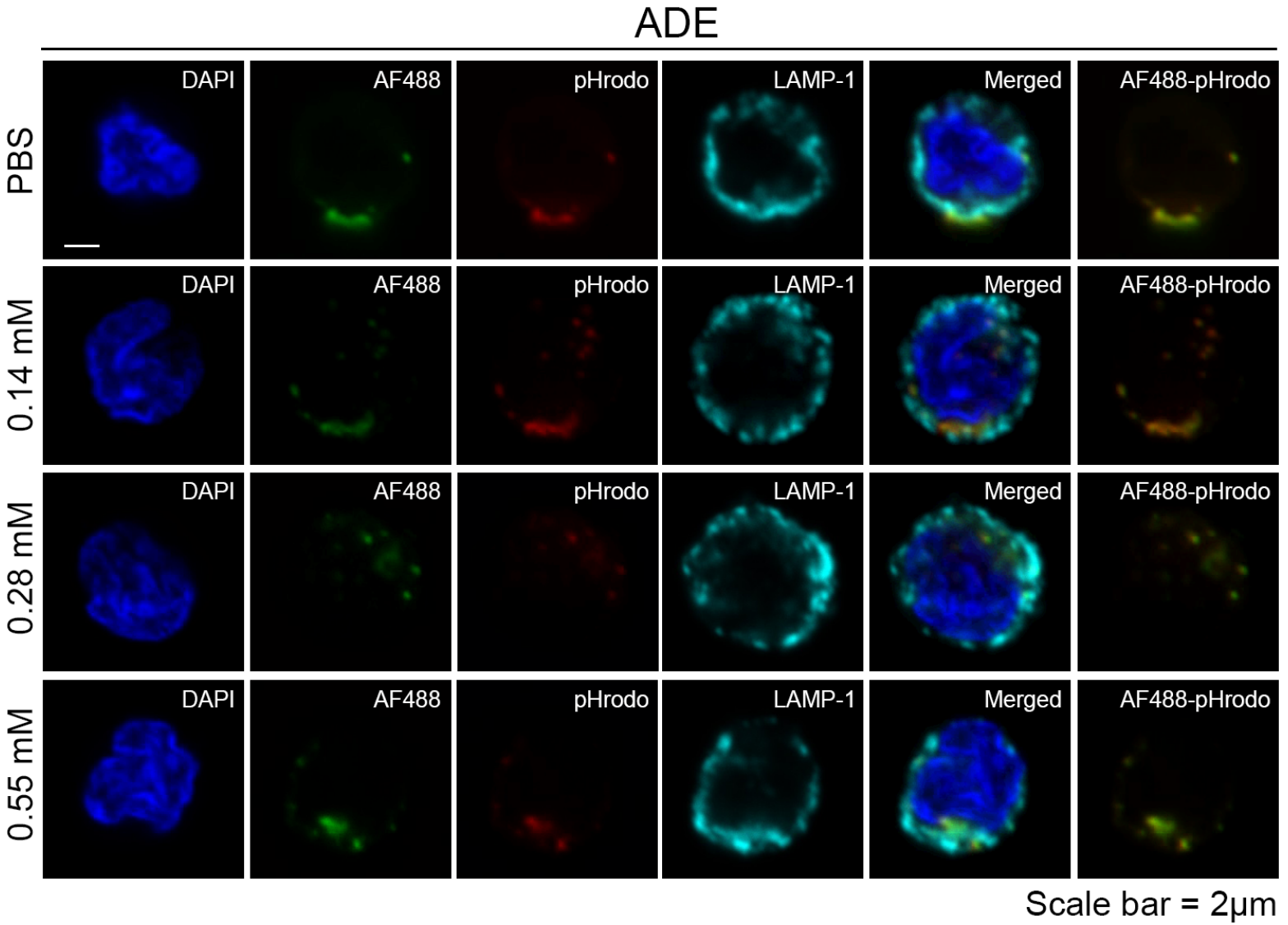
| AF488 | pHrodo | Colocalization Coefficient | | |
|---|---|---|---|---|
| | | AF488-pHrodo | AF488-E protein | pHrodo-E protein |
| 50μM | 150μM | N.D. | N.D. | N.D. |
| 60μM | 140μM | N.D. | N.D. | N.D. |
| 80μM | 120μM | 0.748±0.106 | 0.619±0.130 | 0.699±0.137 |
| 90μM | 110μM | N.D. | N.D. | N.D. |
| 100μM | 100μM | N.D. | N.D. | N.D. |
| 110μM | 90μM | 0.697±0.155 | 0.741±0.091 | 0.788±0.080 |

C

| AF488 | pHrodo | Infectious titers |
|---|---|---|
| 50μM | 150μM | N.D. |
| 60μM | 140μM | N.D. |
| 80μM | 120μM | $3 \times 10^6$ PFU/ml |
| 90μM | 110μM | $2 \times 10^5$ PFU/ml |
| 100μM | 100μM | N.D. |
| 110μM | 90μM | $7 \times 10^5$ PFU/ml |

**Figure 3-5. Viability of DENV post pHrodo and AF488 labelling.** (A) Representative confocal images to test fluorescence intensity of DENV labelled with different concentrations of AF488 and pHrodo. Vero cells were seeded on coverslips one day before infection with labelled DENV for 10 minutes at 37°C. Cells were fixed and labelled with anti-E antibody, and examined for co-localization of E protein (cyan) with AF488 (green) and pHrodo (red) labelling. Scale bar is 10μm. (B) Quantification of co-localization between AF488 and pHrodo on labelled DENV, AF488 and E protein, and pHrodo and E protein. Fluorescence intensity not detected (N.D.) in some labelling conditions. Data expressed as mean ± s.d. (C) Infectious titers of DENV determined post-labelling by plaque assay. Plaque titers not detected (N.D.) in some labelling conditions.


A
DENV
DAPI
AF488
pHrodo
LAMP-1
Merged
AF488-pHrodo
PBS
0.14 mM
0.28 mM
0.55 mM
Scale bar = 2μm


ADE
DAPI
AF488
pHrodo
LAMP-1
Merged
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PBS
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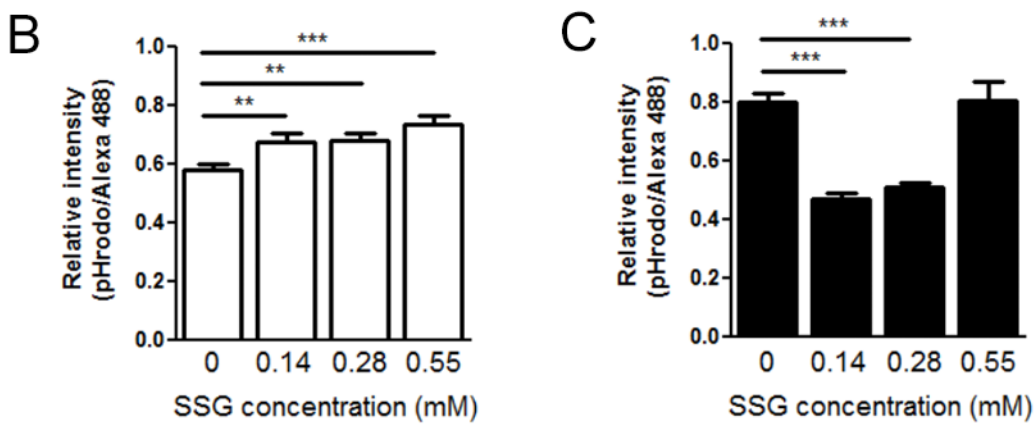


**Figure 3-6. SHP-1 inhibition results in increased phagosomal acidification in primary monocytes during ADE.** (A) Representative confocal images of pHrodo/AF488-labelled DENV co-localized with LAMP-1 in PBS or sodium stibogluconate (SSG)-treated primary monocytes 2h post-infection under DENV only or ADE conditions. Scale bar is 2μm. (B-C) Relative intensity of pHrodo/AF488-labelled DENV in SSG-treated primary monocytes infected under ADE (B) or DENV-2 only (C) conditions. Data expressed as mean ± s.e.m. *** $P < 0.001$, ** $P < 0.01$.

## 3.4 Discussion

### 3.4.1 Role of LILRB1 in modifying DENV compartmentalization during ADE

Chapter 2 has shown that antibody-opsonized DENV ligates activating FcγR and that the ITAM signaling from FcγRs leads to Syk phosphorylation. Apart from inducing antiviral responses, Syk is also known to regulate phagocytosis, cytokine secretion and reactive oxygen species production depending on its binding to downstream substrates (Mocsai et al, 2010). As Syk mediates diverse biological functions, we tested the hypothesis that reduced Syk signaling could lead to differences in DENV compartmentalization, which could also influence the outcome of ADE. Data in this chapter showed that higher levels of phosphorylated Syk permitted faster phagocytic trafficking of DENV immune complexes through Rab-5, Rab-7 and LAMP-1 compartments during ADE (Fig. 3-1). Furthermore, using pHrodo-labelled DENV, we demonstrated that LILRB1 co-ligation reduced levels of phagosomal acidification (Fig. 3-4D), and this could be reversed by SHP-1 inhibition in primary monocytes (Fig. 3-6B). This work has now expanded knowledge of how co-ligation of LILRB1 allows DENV to evade host immune responses – allowing DENV to both overcome the early ISG response (Chan et al, 2014) and possibly avoid degradation via the phagolysosomal pathway for enhanced viral replication.

The finding that LILRB1-mediated signaling could inhibit phagosomal acidification has important implications for pathogen replication and degradation, which are both pH-dependent processes. Pathogens typically exploit endocytic machinery for cellular uptake, and the acidic environment of endocytic vesicles serve as a trigger for fusion for many viruses, allowing efficient entry into the cytosol (White et al, 2008). Low pH is the sole trigger for viral fusion for orthomyxo-, flavi-,

alpha-, rhabdo-, arena-, and bunyaviruses. These viruses enter host cells via endocytosis and fuse from within early or late endosomes, depending on the pH that elicits key conformational changes for fusion to occur (White et al, 2008). Single particle tracking of individual virus particles has revealed that DENV fusion occurs in acidic compartments of the late endosome (van der Schaar et al, 2008). An additional cue for DENV fusion is the lipid composition of endosomal membranes, as fusion requires the target membrane to contain anionic lipids like bis(monoacylglycero)phosphate, which is predominantly found within late endosomes (Zaitseva et al, 2010). These studies were however performed in the absence of antibodies. It is not known if FcγR-mediated entry delivers antibody-opsonized DENV with the same entry route used during DENV only infection or if phagocytic trafficking diverts DENV into a different pathway. We compared the levels of phagosomal acidification using pHrodo-labelled DENV under both DENV only and ADE infection and found higher levels of phagosomal acidification in THP-1.2R compared to THP-1.2S during ADE (Fig. 3-4D). Next, we examined if co-ligation of LILRB1 could be responsible for the reduced levels of phagosomal acidification by inhibition of SHP-1. Indeed, we found that SHP-1 inhibition with sodium stibogluconate led to dose-dependent increase in phagosomal acidification in primary monocytes during ADE but not DENV only infection (Fig. 3-6B and C).

Pathogens have evolved strategies to modify their intracellular compartmentalization to subvert phagosome maturation pathways and ensure survival in host cells. Some of these strategies include preventing phagosomal acidification and restricting phagolysosomal fusion to create an intracellular niche for survival in the host. *Mycobacterium tuberculosis* (Mtb) secretes a protein tyrosine phosphatase

(PtpA) that binds the macrophage vacuolar-$H^+$-ATPase (V-ATPase) complex to inhibit phagosomal acidification (Wong et al, 2011). PtpA also inhibits phagolysosomal fusion by inactivating the host vacuolar protein sorting 33B (VPS33B), which regulates membrane fusion in the endocytic pathway (Bach et al, 2008). It is thus conceivable that recruitment of SHP-1 following ligation of LILRB1 could also lead to dephosphorylation of similar classes of substrates that regulate DENV phagosome maturation. While we have established that SHP-1 inhibition increases phagosomal acidification during ADE in primary monocytes (Fig. 3-6B), future work should aim towards elucidating the pathway for how LILRB1-mediated signaling inhibits phagosome acidification and maturation.

### 3.4.2 Possible roles of Syk in regulating phagosome maturation

Phagosome acidification is an essential component of phagosome maturation. The acquisition of V-ATPase proton pumps, hydrolytic enzymes, and the production of reactive oxygen species (ROS) by the NADPH oxidase complex contribute to rapid acidification of the phagosome. The role of Syk in regulating phagosomal acidification has gained traction in recent studies detailing how Syk activity following phagocytosis of fungal pathogens regulates phagosome maturation. Inhibition of Syk following dectin-1 ligation by *Candida albicans* led to prolonged retention of dectin-1 on the phagosome and reduced phagosomal acidification (Mansour et al, 2013). Similarly, in *Candida glabrata*, Syk activation decayed faster in macrophages with viable *Candida glabrata* that had resided in non-matured phagosomes (Kasper et al, 2014). It is possible that Syk activation is required for full phagosome maturation of viable *Candida glabrata* containing phagosomes.

The V-ATPase proton pumps harness energy from ATP hydrolysis for active transport of protons across the phagosome membrane and serve as the main determinant for phagosome acidification. Recently, synaptosomal associated protein of 23kDa (SNAP-23), a plasma membrane-localized soluble NSF attachment protein receptor (SNARE) was found to regulate phagosome formation and maturation on macrophages (Sakurai et al, 2012). Overexpression of SNAP-23 led to increased ROS production and enhanced FcγR-mediated phagocytosis, as well as functional recruitment of V-ATPase proton pumps and NADPH oxidase complex to phagosomes (Sakurai et al, 2012).

The influx of protons leads to a gradual build-up of electrical potential difference, increasing the resistance for translocation of protons into the phagosome. Counter-ion movement can dissipate the increased electrical potential difference by facilitating the efflux of cations or influx of anions. Macrophages derived from cystic fibrosis transmembrane conductance regulator (CFTR)-null mice possessed defects in phagosome acidification, which was attributed to reduced chloride ($Cl^-$) conductance (Di et al, 2006). The role of chloride ion in opposing the build-up of electrical potential that would inhibit proton accumulation was further demonstrated when knockdown of $Cl^-/H^+$ antiporter ClC-7 inhibited acidification of lysosomes *in vivo*, and was found to be the primary chloride permeation pathway in lysosomes (Graves et al, 2008). This also suggests that limiting counter-ion movement could lead to a stunted pH gradient and insufficient phagosome acidification.

Syk phosphorylation leads to the activation of phospholipase C-gamma (PLCγ) and an increase in cytosolic calcium levels. This triggers calmodulin, which recruits Vps34 to the phagosome membrane and leads to production of PI(3)P that is

necessary for the recruitment of SNAREs that mediate vesicle fusion (Vergne et al, 2003). It is possible that by regulating calcium signaling and the activity of SNARE proteins, Syk can also indirectly mediate the recruitment of V-ATPase proton pumps and anion/$H^+$ antiporters like ClC-7 for enhanced phagosome maturation.

Syk phosphorylation following FcγR-mediated phagocytosis also leads to activation of the NADPH oxidase complex, which is responsible for ROS production. Downstream effects of Syk phosphorylation include mobilization of phosphatidylinositol 3-kinase (PI3-K), which catalyzes the synthesis of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) on the phagocytic cup (Cox et al, 1999). The production of PI(3)P can occur via breakdown of phagosomal PI(3,4,5)P3 by phosphatases like SHIP-1 or by calcium signaling which triggers calmodulin-mediated recruitment of Vps34 on the phagosome membrane (Thi & Reiner, 2012; Vergne et al, 2003). PI(3)P binds the $p40^{phox}$ subunit of NADPH oxidase complex and is crucial for timing the oxidative burst on phagosomes. This was aptly shown in a study in which point mutations of $p40^{phox}$ that disrupted PI(3)P binding led to abrogation of FcγRIIA-stimulated NADPH oxidase activity (Suh et al, 2006).

Besides regulating phagosome acidification, Syk also plays a role in endocytic trafficking of immune receptors like FcεRI and was shown to be required for internalization of FcεRI complexes and their trafficking to lysosomes for eventual degradation. Syk was shown to phosphorylate hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a component of the endosomal sorting complex required for transport (ESCRT-0) (Gasparrini et al, 2012). Phosphorylated Hrs was targeted to membrane compartments, allowing Hrs to regulate the sorting of

ubiquitylated cargo to multivesicular bodies (Gasparrini et al, 2012). In this context, Syk may also regulate phagosome maturation by activating adaptors of endocytic trafficking for fusion with late endosomes or lysosomes.

Examples from the literature have demonstrated possible mechanisms in which Syk could modulate phagosome maturation, one of which is through the activation of acid hydrolases. There is also significant overlap in how Syk-mediated calcium signaling and PI3-K pathways could result in phagosome maturation through their roles in vesicle fusion, recruitment of adaptors of endocytic or phagocytic trafficking, and phagosome acidification. Future work should seek to clarify Syk-driven molecular events for regulating phagosome maturation.

### 3.4.3 *Festina lente* – a model to explain DENV compartmentalization during ADE

The Roman emperor Augustus used the phrase "*festina lente*" to discourage his military commanders from making rash decisions that led to failures on the battlefield. Here, the findings in this thesis suggest that *festina lente* could be a model employed by DENV to escape the phagolysosomal pathway, ensuring its intracellular survival during ADE. We showed that activating FcγR-mediated phagocytosis of antibody-opsonized DENV triggers Syk phosphorylation, leading to higher degree of phagosomal maturation and acidification in DENV-containing phagosomes. This activates lysosomal hydrolases like CatD and other potential candidates (Table 3-1) that are activated only in an acidic microenvironment, which could degrade DENV in the phagolysosomal pathway. Conversely, co-ligation of LILRB1 leads to recruitment of SHP-1 and dephosphorylation of Syk, which results in reduced phagosomal acidification and maturation. While the reduced or slower acidification would also

delay trimerization of DENV E protein for successful uncoating of the nucleocapsid, the overall effect of LILRB1 co-ligation may favor DENV as the virus would be less likely to encounter activated enzymes that would otherwise digest it. This may accord DENV the opportunity to overcome an initial barrier for intracellular survival. *Festina lente* may thus capture the essence of the early events of ADE.

**Table 3-1. pH-dependent lysosomal enzymes.** Out of an estimated 60 proteins found in the lysosomal matrix (Lubke et al, 2009), this list has been curated from the following references (Guha & Padh, 2008; Lubke et al, 2009; Schroder et al, 2010; Sleat et al, 2007) to include lysosomal enzymes that work at an acidic optimum pH and could inactivate DENV trapped in the phagolysosomal pathway.

| Protein | Gene | Protein function |
|---|---|---|
| Arylsulfatase A | ARSA | Hydrolysis of cerebroside sulphates |
| Arylsulfatase B | ARSB | Hydrolysis of the four-sulphate groups of chondroitin and dermatan sulphate |
| N-acetylglucosamine-6-sulfatase | GNS | Hydrolysis of 6-sulphate groups |
| N-acetylgalactosamine-6-sulfatase | GALNS | Hydrolysis of 6-sulphate groups, relevant for degradation of chondroitin sulphate and keratan sulphate |
| α-N-acetylgalactosaminidase | NAGA | Glycosidase; Removes terminal α-N-acetylgalactosamine residues from glycolipids and glycopeptides |
| Lysosomal α-glucosidase | GAA | Glycosidase; degradation of glycogen |
| β-hexosaminidase subunit α | HEXA | Glycosidase; degradation of GM2 gangliosides |
| β-hexosaminidase subunit β | HEXB | Glycosidase; degradation of GM2 gangliosides |
| Lysosomal α-mannosidase | MAN2B1 | Glycosidase; cleaves α-linked mannosyl residues with broad specificity |
| α-galactosidase A | GLA | Glycosidase; hydrolysis of terminal α-D-galactosyl residues in α-D-galactosides |
| α-N-acetylglucosaminidase | NAGLU | Glycosidase; degradation of heparan sulphate |
| β-galactosidase | GLB1 | Glycosidase; cleaves terminal galactosyl residues from gangliosides, glycoproteins and glycosaminoglycans |
| β-glucuronidase | GUSB | Degradation of dermatan and keratan sulphates |
| Cathepsin B | CTSB | Proteolysis |
| Cathepsin D | CTSD | Proteolysis |
| Cathepsin H | CTSH | Proteolysis |
| Cathepsin L | CTSL | Proteolysis |
| Cathepsin S | CTSS | Proteolysis |
| Cathepsin Z | CTSZ | Proteolysis |

| | | |
|---|---|---|
| Acid ceramidase | ASAH1 | Lipase; Hydrolysis of ceramide into sphingosine and free fatty acid |
| Deoxyribonuclease II | DNASE2 | Hydrolysis of DNA |
| Hyaluronidase | HYAL1 | Degradation of hyaluronate |
| Lysosomal acid phosphatase | ACP2 | Phosphatase activity |
| Tissue α-L-fucosidase | FUCA1 | Hydrolysis of α-1,6-linked fucosyl residues in glycopeptides |
| Tripeptidyl-peptidase I | TPP1 | Release of N-terminal tripeptides from polypeptides |

## Chapter 4. CONCLUSION AND FUTURE DIRECTIONS

This thesis provides new insights into the early molecular events during DENV infection under ADE conditions. During ADE, antibody-opsonized DENV cross-links activating FcγRs, which rapidly signals to trigger an antiviral response by inducing ISGs, unless suppressed by co-ligation of the inhibitory FcγRIIB (Dhodapkar et al, 2007). However, earlier work from our laboratory demonstrated that co-ligation of FcγRIIB requires high concentrations of cross-reactive antibodies that form viral aggregates, which also inhibited FcγR-mediated phagocytosis of DENV immune complexes. At sub-neutralizing levels of antibody, FcγRIIB is not co-ligated. In the absence of FcγRIIB-mediated inhibition, entry through activating FcγR would lead to induction of ISGs, which are known to inhibit viral replication. We have shown that in order to overcome this early antiviral response, DENV co-ligates an inhibitory receptor LILRB1, which recruits the phosphatase SHP-1 to dephosphorylate Syk and hence downregulate ISG expression, thus favouring DENV replication. As Syk is a key intermediate of signaling pathways that control phagosomal trafficking and maturation, co-ligation of LILRB1 also appears to modulate DENV compartmentalization during antibody-enhanced DENV infection. Indeed, increased Syk activity led to faster phagocytic trafficking of DENV immune complexes through Rab-5, Rab-7 and LAMP-1 compartments during ADE. This also resulted in higher levels of phagosomal acidification and activation of lysosomal hydrolases like CatD. However, engagement of LILRB1 reduced levels of phagosomal acidification, which could be a potential viral strategy for escaping the phagolysosomal

pathway to allow more time for viral fusion. A schematic to illustrate these findings is shown in Figure 4-1. Collectively, this thesis has demonstrated that LILRB1 serves as a co-factor for antibody-enhanced DENV infection.

Our findings suggest that identification of epitopes on DENV that interact with LILRB1 could facilitate the generation of antibodies that block LILRB1 interaction, reducing ADE. This could guide vaccine development to generate vaccines that induce robust immunity and minimize the risk of ADE following vaccination, even when neutralizing antibodies wane to levels insufficient to prevent infection. Further studies to determine how DENV binds LILRB1 could include generating truncated fragments of the LILRB1 ectodomain and performing ELISA to test for binding against the four serotypes of DENV. Site-directed mutagenesis could also be performed to pin-point the amino acid residues involved in binding DENV. Alternatively, cryo-electron microscopy approaches could be used to map the interaction of quaternary structure dependent epitopes between LILRB1 and DENV.

LILRB1 signaling also dysregulates Syk-mediated phagosomal maturation and acidification of DENV-containing phagosomes. Our data currently shows that SHP-1 inhibition increases phagosomal acidification during ADE, and we aim to further characterize how LILRB1 signaling could govern phagosome maturation. We recently completed a mass spectrometric analysis of DENV phagosomes isolated under DENV only and ADE conditions which could provide us with possible candidates that mediate this process. We will focus on candidates that modify phagocytic trafficking, phagosome acidification and the activation or abundance of antimicrobial effectors. These are factors

that could modulate the intracellular fate of DENV following activating FcγR-mediated uptake. The use of these approaches could be instrumental in improving our understanding of the pathogenesis of ADE and how DENV exploits LILRB1 to evade host immunity. This will create new opportunities for therapeutic intervention and contribute to the design of vaccines that boost immunity while tackling the risk of antibody-enhanced dengue.

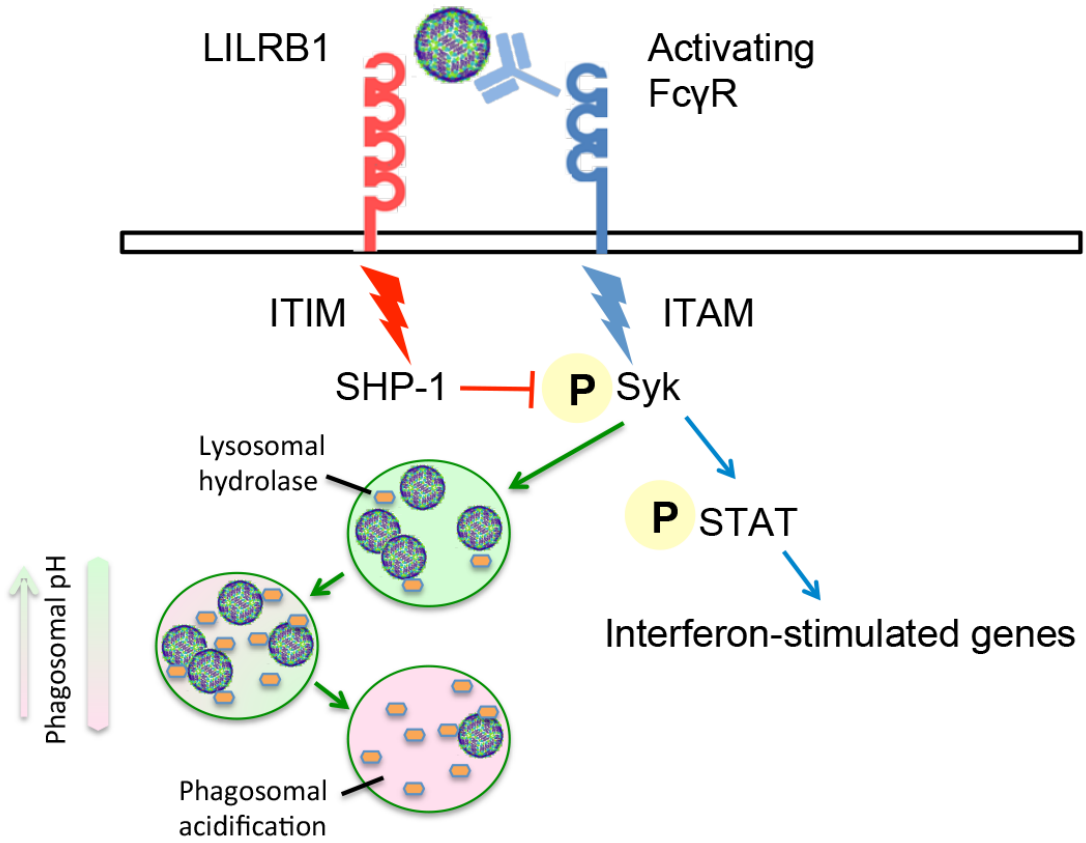


**Figure 4-1. Schematic illustrating key findings.** At sub-neutralizing antibody concentrations, DENV immune complexes ligate activating FcγRs, triggering Syk phosphorylation and ISG induction. DENV overcomes this early antiviral response by co-ligating the inhibitory LILRB1, which recruits the phosphatase SHP-1. SHP-1 dephosphorylates Syk and reduces ISG expression, resulting in enhanced viral replication during ADE. Syk also mediates phagosomal trafficking and maturation, activating lysosomal hydrolases that degrade phagosomal cargo. During ADE, co-ligation of LILRB1 reduces phagosomal acidification, which could delay the activation of lyososomal hydrolases. This allows DENV to escape the phagolysosomal pathway, but benefit from the mildly acidic environment for viral fusion and replication.

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# APPENDIX A

# Leukocyte immunoglobulin-like receptor B1 is critical for antibody-dependent dengue

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**Viruses must evade the host innate defenses for replication and dengue is no exception. During secondary infection with a heterologous dengue virus (DENV) serotype, DENV is opsonized with sub- or nonneutralizing antibodies that enhance infection of monocytes, macrophages, and dendritic cells via the Fc-gamma receptor (FcγR), a process termed antibody-dependent enhancement of DENV infection. However, this enhancement of DENV infection is curious as cross-linking of activating FcγRs signals an early antiviral response by inducing the type-I IFN-stimulated genes (ISGs). Entry through activating FcγR would thus place DENV in an intracellular environment unfavorable for enhanced replication. Here we demonstrate that, to escape this antiviral response, antibody-opsonized DENV coligates leukocyte Ig-like receptor-B1 (LILRB1) to inhibit FcγR signaling for ISG expression. This immunoreceptor tyrosine-based inhibition motif-bearing receptor recruits Src homology phosphatase-1 to dephosphorylate spleen tyrosine kinase (Syk). As Syk is a key intermediate of FcγR signaling, LILRB1 coligation resulted in reduced ISG expression for enhanced DENV replication. Our findings suggest a unique mechanism for DENV to evade an early antiviral response for enhanced infection.**

early innate immune response | innate immune signaling | immune evasion

Despite long-lived serotype-specific immunity upon initial infection, predicted global prevalence of dengue now surpasses World Health Organization estimates by more than threefold with 390 million cases annually (1). Furthermore, the risk of severe disease is augmented by cross-reactive or subneutralizing levels of antibody (2, 3), which opsonize dengue virus (DENV) to ligate Fc-gamma receptor (FcγR) for entry into monocytes, macrophages, and dendritic cells, a phenomenon known as antibody-dependent enhancement (ADE) of DENV infection (4, 5). The resultant greater viral burden leads to increased systemic inflammation that precipitates plasma leakage, a hallmark of dengue hemorrhagic fever (6). However, ligation of the activating FcγRs by immune complexes has been shown to induce type-I IFN stimulated genes (ISGs), independent of autocrine or paracrine IFN activity, unless the inhibitory FcγRIIB is coligated (7). We and others reported recently that coligation of FcγRIIB by DENV immune complexes requires high antibody concentration, and such coligation inhibited the entry of DENV immune complexes into monocytes (8, 9). At low antibody concentrations where ADE occurs, the inhibitory FcγRIIB is not coligated (9). Ligation of the activating FcγRs by DENV opsonized with subneutralizing levels of antibody would thus induce the expression of ISGs and hinder DENV replication (10). Here, we demonstrate that DENV employs a unique evasive mechanism by coligating LILRB1 to down-regulate the early antiviral responses triggered by activating FcγRs for ADE.

## Results

**ADE Differs in THP-1 Subclones.** Our work was enabled by the isolation of subclones of THP-1 cells with different phenotypes to ADE. The low rate of FcγR-mediated phagocytosis in THP-1 cells (~5%) (9) had led us to reason that this cell line is genetically heterogeneous, either through the method in which it was derived (11) or through genetic instability resulting from aneuploidy (12). Screening of our newly isolated subclones with DiD (1, 1'-dioctadecyl-3, 3, 3′, 3′ – tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt) labeled DENV-2 alone or opsonized with subneutralizing concentrations of humanized 3H5 monoclonal antibody (h3H5) identified two clones (labeled as THP-1.2R and THP-1.2S) that showed increased uptake of DENV immune complexes compared with parental THP-1 (Fig. 1*A*). Monocyte surface marker analysis indicated no significant difference in the expression of FcγRs (FcγRI, FcγRII, FcγRIII) in these subclones (*SI Appendix*, Fig. S1*A*). Expression of FcγRIIA,

### Significance

**Dengue virus (DENV) infects almost 400 million people annually and some of these infections result in life threatening disease. An incomplete understanding of pathogenesis, particularly on how non- or subneutralizing levels of antibody augments DENV infection of cells expressing Fc-gamma receptors (FcγRs), has hampered vaccine development. Here, we show that, to overcome the activating FcγR-dependent expression of type-I interferon stimulated genes (ISGs), DENV binds and activates the inhibitory receptor, leukocyte immunoglobulin-like receptor-B1 (LILRB1). LILRB1 signals through its immunoreceptor tyrosine-based inhibition motif cytoplasmic tail to inhibit the expression of ISGs required for successful antibody-dependent DENV infection. Inhibition of DENV activation of LILRB1 could hence be a strategy for vaccine or therapeutic design.**

Author contributions: K.R.C., E.Z.O., and E.E.O. designed research; K.R.C., E.Z.O., H.C.T., S.L.-X.Z., Q.Z., K.F.T., and N.K. performed research; A.P.C.L., M.L.H., S.H.C., J.E.C., M.N.K., S.M.L., B.J.H., and C.-N.L. contributed new reagents/analytic tools; K.R.C., E.Z.O., H.C.T., S.L.-X.Z., Q.Z., K.F.T., N.K., M.L.H., S.H.C., J.E.C., M.N.K., S.M.L., C.-N.L., and E.E.O. analyzed data; and K.R.C., E.Z.O., and E.E.O. wrote the paper.

The authors declare no conflict of interest.

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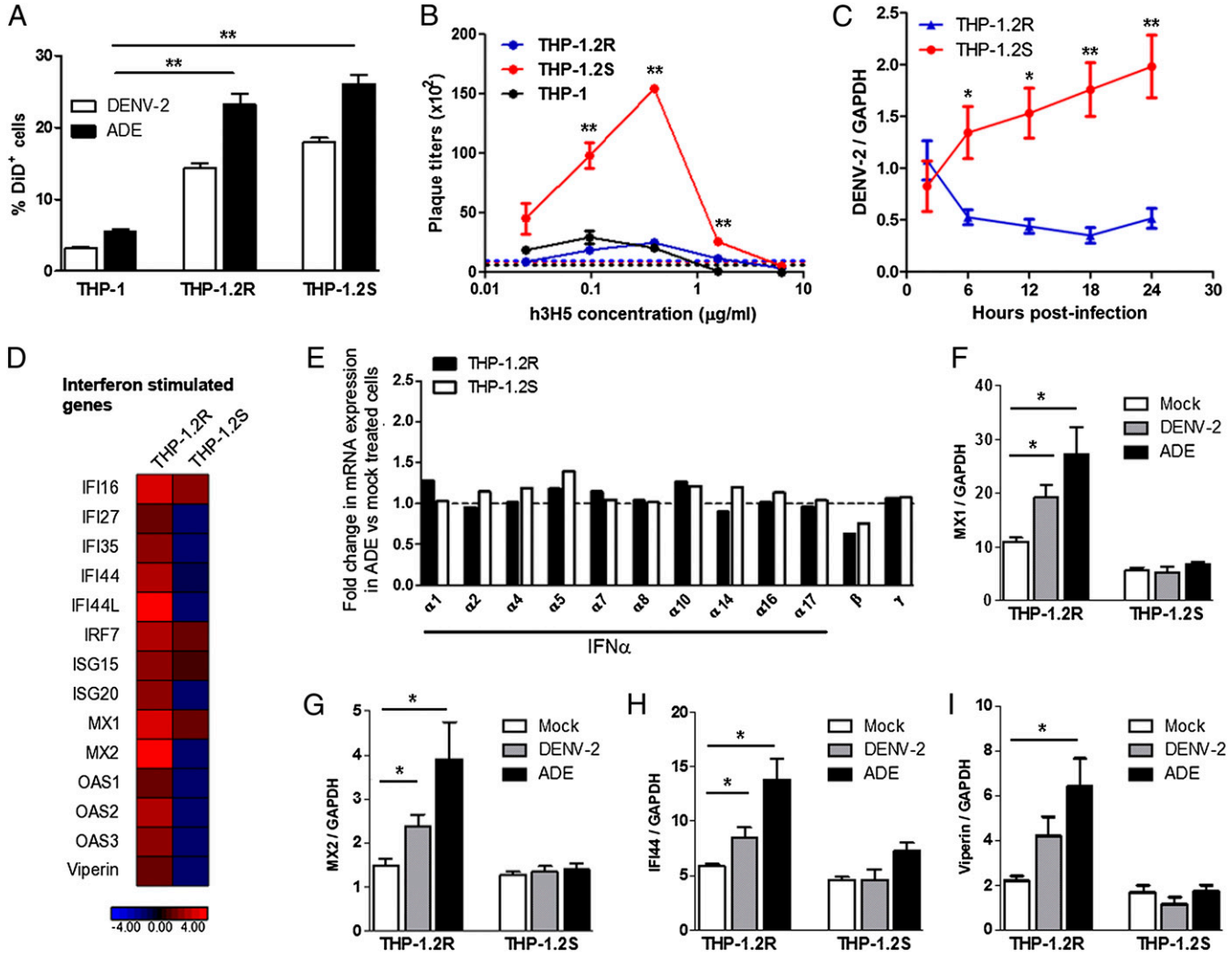


**Fig. 1.** ADE differs in THP-1 subclones. (*A*) Percentage of internalized DiD-labeled DENV-2 30 min postinfection under DENV-2 or ADE conditions in THP-1, THP-1.2R, and THP-1.2S. (*B*) Plaque titers of THP-1, THP-1.2R, or THP-1.2S when infected with DENV-2 opsonized with different h3H5 concentrations 72 h postinfection (hpi). Dotted lines indicate plaque titers following DENV-2–only infection, with no significant differences observed between the cell lines. (*C*) Time course of viral RNA copy numbers in THP-1.2R or THP-1.2S under ADE conditions. (*D*) Heat map showing fold change of ISG expression in THP-1.2R and THP-1.2S at 6 hpi under ADE conditions. (*E*) Fold change in transcript levels of interferons in THP-1.2R and THP-1.2S 6 hpi under ADE conditions. (*F–I*) Validation of microarray data in *D* by quantitative PCR. Data are expressed as mean ± SD from three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$.

FcγRIIB, and FcγRIIC were similar in these subclones (*SI Appendix*, Fig. S1 *B* and *C*). Both subclones were also heterozygous for 131 H/R FcγRIIA polymorphism (*SI Appendix*, Fig. S1*E*). Identical HLA haplotyping confirmed that both subclones were derived from THP-1 and not the result of a contamination with another cell line (*SI Appendix*, Table S1).

Despite no significant differences in uptake and production of plaque titers when infected with DENV-2 only, infection under ADE conditions resulted in significantly different DENV-2 titers in THP-1.2R and THP-1.2S (Fig. 1*B*). Similar observations were also made with enhancing titers of convalescent serum (*SI Appendix*, Fig. S2*A*) or other DENV serotypes (*SI Appendix*, Fig. S2*B*). Furthermore, early DENV RNA replication diverged in these two subclones, where a significant difference was observed as early as 6 h postinfection (Fig. 1*C*). Analysis of early gene expression indicated significant up-regulation of ISGs in THP-1.2R but not THP-1.2S (Fig. 1 *D* and *F–I*). These included MX1, MX2, and viperin, which are potent inhibitors of DENV replication (10). The up-regulation of ISGs in THP-1.2R, however, was not due to h3H5 (*SI Appendix*, Fig. S3) and is independent of IFN-α, -β, and -γ signaling as both subclones expressed similar IFN transcript levels (Fig. 1*E*). As expected, addition of antibodies that blocked IFNα receptor (IFNαR) signaling (*SI Appendix*, Fig. S4*A*) did not reduce this early ISG induction in THP-1.2R following infection (*SI Appendix*, Fig. S4*B*). The possibility that THP-1.2S had impaired IFNαR-mediated signaling was also excluded, as ISGs were significantly up-regulated in response to exogenous IFN (*SI Appendix*, Fig. S4*C*). These subclones thus serve as exquisite tools to decipher the signaling requirement to overcome the early antiviral responses for successful ADE.

**Early ISG Expression During ADE Is Independent of RIG-I/MDA5 Signaling.** Differences in viral entry through ADE and DENV-2–only conditions could have resulted in different intracellular antigenic load and hence resulted in differential ISG expression in the subclones. To identify the specific signaling pathway responsible for early ISG induction in THP-1.2R during ADE infection, we titrated the multiplicity of infection (MOI) for DENV-2 only that resulted in equivalent level of infection as ADE (MOI 10) to serve as an antigenically equivalent control

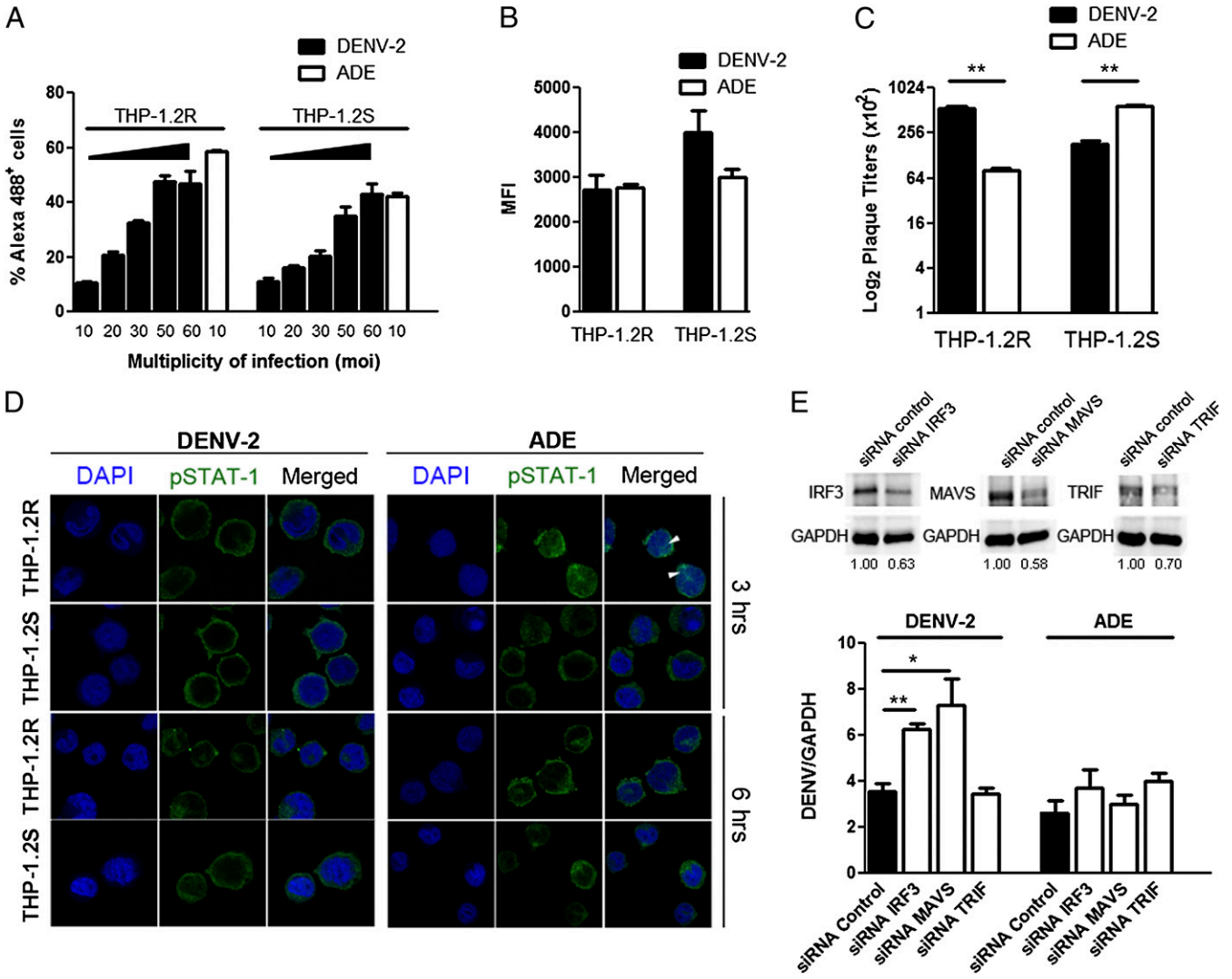


**Fig. 2.** Early ISG induction during ADE is independent of RIG-I/MDA5-contingent IFN signaling. (*A*) Uptake of Alexa 488-labeled DENV-2 under virus-only (MOI 10–60) and ADE (MOI 10) conditions 6 hpi. (*B*) Mean fluorescence intensity under virus-only (MOI 60) and ADE (MOI 10) conditions 6 hpi. All subsequent experiments were performed under DENV-2–only (MOI 60) or ADE (MOI 10) conditions. (*C*) Plaque titers of THP-1.2R and THP-1.2S when infected with DENV-2–only or ADE conditions. (*D*) Colocalization of pSTAT-1 with DAPI 3 hpi and 6 hpi under DENV-2–only or ADE conditions. (*E*) Viral RNA expression determined 6 hpi in siRNA-treated cells infected under DENV-2–only or ADE conditions. Data are expressed as mean ± SD from three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$.

(Fig. 2 *A* and *B*). Interestingly, lower and higher plaque titers were observed in THP-1.2R and THP-1.2S, respectively, during ADE relative to DENV-2–only (MOI 60) conditions (Fig. 2*C*), which corroborates the notion that THP-1.2R has reduced susceptibility to ADE. Immunofluorescence imaging showed nuclear translocation of pSTAT-1 at 3 h post ADE in THP-1.2R but not in THP-1.2S or during antigenically equivalent DENV-only infection (Fig. 2*D*). This early nuclear translocation of pSTAT-1 is transient as little colocalization could be observed at 6 h postinfection.

With similar intracellular antigenic load in ADE and DENV-2–only conditions, we determined whether trafficking of DENV containing-phagosomes to cellular compartments enriched with pattern recognition receptors was an explanation for ISG induction in THP-1.2R. This was not the case as reduced expression of adaptor molecules [mitochondrial antiviral signaling protein (MAVS) and IFN regulatory factor 3 (IRF3)] of retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated protein 5 (MDA5) resulted in significantly increased early DENV replication under DENV-2–only but not ADE conditions (Fig. 2*E*). Reduced TIR-domain containing adapter-inducing IFN β (TRIF) did not result in significant change in DENV replication under either condition (Fig. 2*E*). Collectively, these results indicate that the early induction of ISG in THP-1.2R is unique to infection under ADE condition and is not mediated by RIG-I/MDA5–dependent type-I IFN expression.

**Early ISG Induction Is Mediated by Activating FcγR.** The independence of ISG expression from RIG-I/MDA5–mediated signaling thus suggests that activating FcγR signaling (7) through spleen tyrosine kinase (Syk) activation (13) is critical in THP-1.2R. We thus quantified Syk activation by Western blot with densitometric measurements. Significant difference in Syk phosphorylation was observed as early as 10 min postinfection under ADE but not DENV-2–only conditions in THP-1.2R (Fig. 3*A*). In contrast, no significant difference in Syk phosphorylation was observed under DENV-2–only and ADE conditions in THP-1.2S. Pretreatment of THP-1.2R with piceatannol, a Syk-selective tyrosine

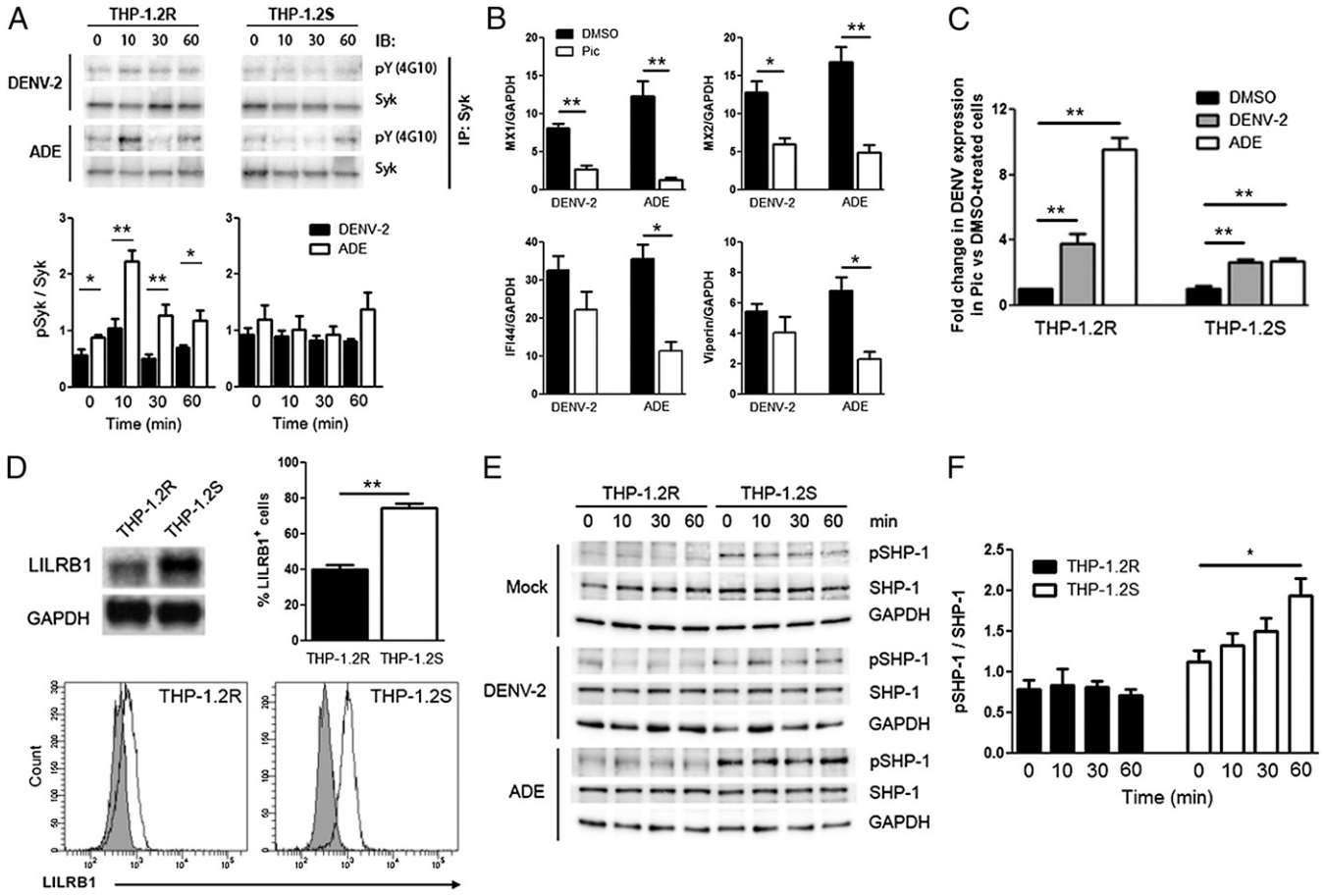


**Fig. 3.** Early ISG induction following ADE requires Syk phosphorylation. (*A*) Western blot and quantitative densitometry of pSyk levels using immunoprecipitation with Syk antibody. (*B*) ISG expression in DMSO- or piceatannol-treated (15.6 μg/mL) THP-1.2R under DENV-2–only or ADE conditions 6 hpi. (*C*) Fold change in DENV RNA copy numbers in THP-1.2R and THP-1.2S pretreated with piceatannol relative to DMSO control. (*D*) Western blot, % LILRB1$^+$ cells, and representative flow cytometry plots of LILRB1 in THP-1.2R and THP-1.2S. Cells were either stained with isotype (gray) or polyclonal anti-LILRB1 antibody (open histogram). (*E*) Western blot of pSHP-1, SHP-1 and GAPDH at different time points after infection under mock, DENV-2–only, and ADE conditions. (*F*) Quantitative densitometry of pSHP-1 levels under ADE conditions. Data are expressed as mean ± SD from three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$.

kinase inhibitor resulted in greater reduction of ISG expression under ADE conditions (Fig. 3*B*) and a correspondingly greater increase in DENV replication (Fig. 3*C*) compared with DENV-2 only. Increase in DENV replication was also greater in THP-1.2R than THP-1.2S. These findings suggest that early ISG expression in THP-1.2R is conditioned upon activating FcγR signaling through phosphorylated Syk (7).

**Coligation of LILRB1 Inhibits ISG Induction.** As activating FcγR signals through immunoreceptor tyrosine-based activation motif (ITAM), we postulated that DENV coligates an immunoreceptor tyrosine-based inhibition motif (ITIM)-bearing receptor to inhibit Syk activation (14) in THP-1.2S. Examination of the gene expression data identified two such possible receptors. LILRB1 (also known as CD85j or Ig-like transcript-2) and LILRB4 were up-regulated preinfection in THP-1.2S relative to THP-1.2R (*SI Appendix*, Fig. S5*A*). Flow cytometry analysis, however, showed that only LILRB1 (Fig. 3*D* and *SI Appendix*, Fig. S5*B*) displayed higher surface expression on THP-1.2S. Because one of the effects of ITIM phosphorylation is the recruitment and phosphorylation of SHP-1 (15, 16), we measured phosphorylated SHP-1 in the two subclones. Higher pSHP-1 levels were found in THP-1.2S than THP-1.2R under ADE conditions (Fig. 3 *E* and *F*), suggesting that pSHP-1 dephosphorylated Syk in THP-1.2S.

If LILRB1 is necessary for ADE, then antibody-opsonized dengue should coligate LILRB1. Indeed, all four DENV serotypes bind to LILRB1, more strongly with whole virus than with E protein ectodomain (Fig. 4*A* and *SI Appendix*, Fig. S6*A*), suggesting that LILRB1 binds to a quaternary structure-dependent epitope. Furthermore, the addition of soluble extracellular domain of LILRB1 (*SI Appendix*, Fig. S6*B*) successfully competed with native LILRB1 on THP-1.2S to reduce ADE but not DENV-2–only infection in a dose-dependent manner (Fig. 4*B*). As expected, soluble LILRB1 ectodomain did not alter the rate of viral entry as this receptor functions by modulating the antiviral state of the cell rather than increasing DENV entry (*SI Appendix*, Fig. S6 *C* and *D*). Likewise, reduced LILRB1 expression in THP-1.2S resulted in reduced DENV replication under ADE conditions (Fig. 4*C*), without altering the rate of viral entry (*SI Appendix*, Fig. S6*E*). The lack of any change in DENV replication with FcγRIIB expression also reinforces the notion that subneutralizing levels of antibody are insufficient to aggregate DENV to coligate FcγRIIB (9). Similar observations were made with knockdown of LILRB1 expression in another unrelated human myelogenous leukemia cell line, K562 (*SI Appendix*, Fig. S7).

Conversely, overexpression of LILRB1 in THP-1.2R resulted in increased DENV replication under ADE conditions (Fig. 4*D*).

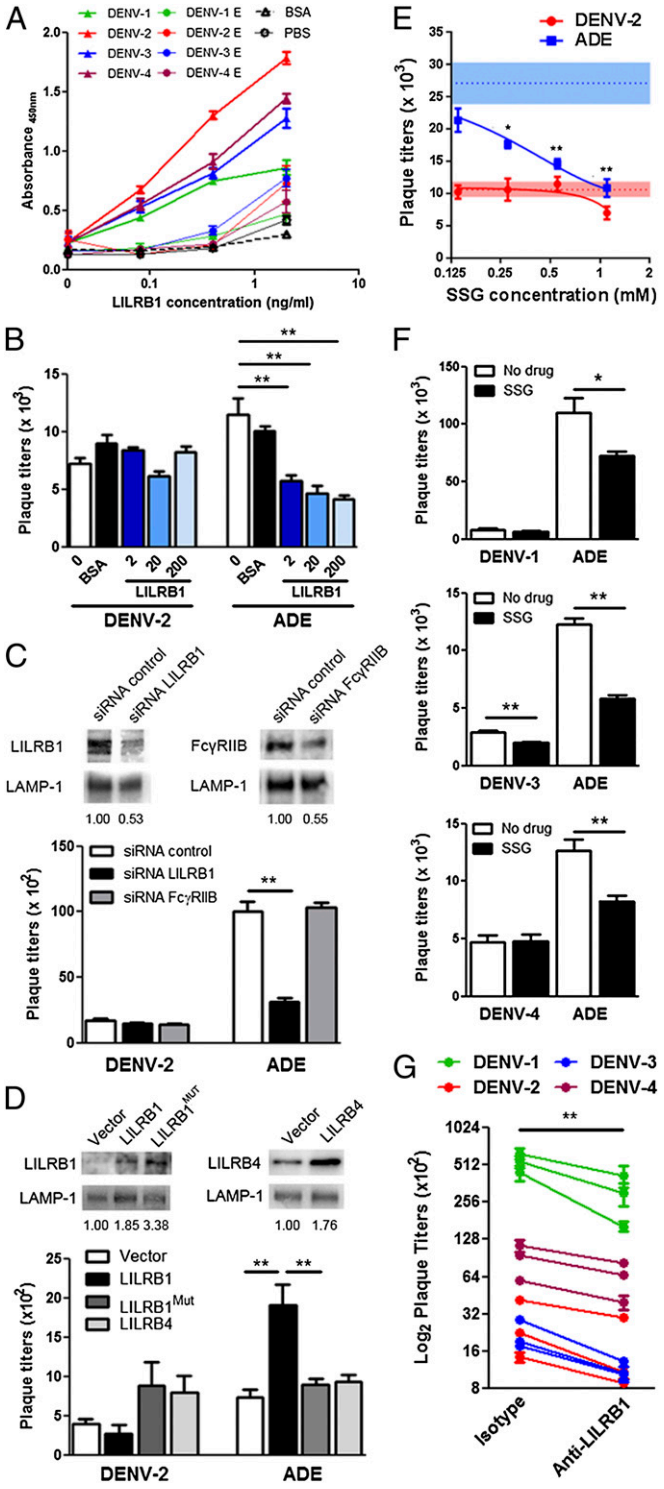


**Fig. 4.** Coligation of LILRB1 is essential for ADE. (*A*) Binding of LILRB1 to whole DENV or DENV E protein ectodomain. (*B*) Plaque titers following DENV-2 or ADE infection in the presence of soluble LILRB1 ectodomain (2 μM, 20 μM, 200 μM), 200 μM BSA, or no protein control. (*C*) Plaque titers following DENV-2 or ADE infection after LILRB1 or FcγRIIB knockdown. Numbers below Western blot indicate levels of proteins relative to LAMP-1. (*D*) Plaque titers following DENV-2 or ADE infection in THP-1.2R transfected with empty vector or vector expressing LILRB1, mutant LILRB1 (LILRB1$^{MUT}$), or LILRB4. Numbers below Western blot indicate levels of proteins relative to LAMP-1. (*E*) Plaque titers following DENV-2–only and ADE infection of primary monocytes treated with sodium stibogluconate (SSG) or PBS control (dashed lines, shaded areas reflect SD). (*F*) Plaque titers following DENV-1–, -3, or -4–only and ADE infection of primary monocytes treated with SSG (0.138 mM) or PBS control. (*G*) Plaque titers in primary monocytes derived from PBMCs harvested from 12 healthy individuals and infected in vitro with either DENV-1 ($n$ = 3), DENV-2 ($n$ = 3), DENV-3 ($n$ = 3), or DENV-4 ($n$ = 3) opsonized with h4G2 antibodies at 72 hpi. PBMCs were either pretreated with polyclonal anti-LILRB1 antibody or isotype antibody control. Data are expressed as mean ± SD from three independent experiments. $^{**}P < 0.01$, $^{*}P < 0.05$.

As a control, we also overexpressed LILRB4, but this did not result in increased DENV replication. Critically, mutation of the four tyrosine residues in the ITIM tail to phenylalanine (*SI Appendix*, Fig. S8) abrogated the increased DENV replication (Fig. 4*D*). Taken collectively, these findings indicate that DENV coligates LILRB1 to inhibit FcγR-activated early ISG expression for ADE.

The mechanistic requirement for LILRB1 in ADE suggests that interfering with this pathway would abrogate ADE in primary monocytes. We studied CD14$^{hi}$CD16$^{-}$ inflammatory monocytes that express both FcγRs and LILRB1 (*SI Appendix*, Fig. S9 *A* and *B*), which form the majority of the circulating monocytes (17). Indeed, pretreatment with sodium stibogluconate, a SHP-1 inhibitor resulted in a dose-dependent reduction in DENV-2 replication under ADE conditions (Fig. 4*E*), with no significant reduction in primary monocyte cytotoxicity (*SI Appendix*, Fig. S9*C*). Likewise, plaque titers following ADE infection of the other 3 DENV serotypes on primary monocytes obtained from different healthy donors were significantly lower in sodium stibogluconate treated cells compared with untreated cells (Fig. 4*F*). Pretreatment of primary monocytes derived from peripheral blood mononuclear cells (PBMCs) from 12 different healthy human volunteers with anti-LILRB1 antibodies also resulted in significantly reduced DENV replication compared with isotype antibodies (Fig. 4*G*).

## Discussion

The ADE hypothesis has been widely used to explain the epidemiological association between secondary DENV infection and severe dengue (18, 19). However, entry through the activating FcγR pathway would pose no replicative benefit to DENV unless it is able to overcome the ITAM–Syk–STAT-1 signaling axis that leads to ISG induction (7, 13). The findings here thus indicate that coligation of LILRB1 is a critical first step for successful antibody-dependent DENV infection (*SI Appendix*, Fig. S10).

LILRB1 is expressed on monocytes, dendritic cells, and subsets of T and NK cells. Its natural function is to activate negative feedback mechanisms upon binding to major histocompatibility complex class I (MHC-I) molecules (20). Consequently, it is conceivable that viruses exploit this pathway to create an intracellular environment more favorable for replication. Besides dengue, human cytomegalovirus (HCMV) also binds LILRB1 through the glycoprotein UL-18 to trigger an inhibitory signaling pathway that limits antiviral effector functions (21, 22). Furthermore, increased LILRB1 expression in CD8$^{+}$ effector T-cells is associated with reduced cytokine secretion and cytotoxicity in persistent HCMV and Epstein–Barr virus infections (22, 23). It would be interesting to test if LILRB1-mediated suppression of immune signaling is also exploited by other viruses.

Coligation of LILRB1 by DENV during antibody-dependent infection suggests that LILRB1 polymorphism may influence outcome of infection. Previous studies have shown that this gene is highly polymorphic (24) and can be alternatively spliced (25). However, a recent genome-wide association study did not reveal a significant association between LILRB1 and dengue shock syndrome (26); this is not surprising because, although LILRB1 activation is critical for initial replication with FcγR-mediated

entry, multiple other host and viral factors contribute to eventual disease outcome.

Our findings also suggest that generation of antibodies to quaternary structure-dependent epitopes on DENV that block LILRB1 interaction can reduce ADE. That heterotypic antibodies can enhance dengue infection in FcγR-bearing cells represents a safety concern in the development of a dengue vaccine. Hence, a vaccine that can generate high-titer antibody that binds the quaternary structure-dependent epitopes on DENV to prevent LILRB1 ligation could reduce the risk of vaccine-induced ADE. Further studies would be needed to clarify this, although care must be taken in selecting a suitable in vivo model as the LILRB1 gene is deleted in laboratory strains of mice (27).

In conclusion, DENV coligates LILRB1 to down-regulate the activating FcγR-mediated early ISG expression for successful antibody-dependent infection.

## Materials and Methods

**Cells.** THP-1.2R and THP-1.2S were subcloned from THP-1 by limiting dilution. Primary monocytes were isolated from healthy donors and cultured as described (9).

**Viruses.** DENV-1 (06K2402DK1), DENV-3 (05K863DK1), and DENV-4 (06K2270DK1) are clinical isolates from the EDEN study (28). DENV-2 (ST) is a clinical isolate from the Singapore General Hospital.

**Virus Infection.** Endotoxin-free (LAL Chromogenic Endotoxin Quantitation kit, Pierce) 3H5 and 4G2 chimeric human/mouse IgG1 antibodies were constructed as described (29). DENV was incubated with media, antibodies, or serum for 1h at 37 °C before adding to cells at indicated MOI. Uptake was assessed using DiD and Alexa 488-labeled DENV as described (9, 30). For drug assays, cells were pretreated with piceatannol (Sigma-Aldrich) or sodium stibogluconate (Santa Cruz Biotechnology) 6 h before infection. Cell viability was assessed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega) according to the manufacturer's protocol. Subsequently, virus replication was assessed using quantitative PCR at indicated time points and plaque assay at 72 h postinfection. Protein and protein phosphorylation levels were assessed using Western blots and analyzed with ImageJ.

**Microarray Analysis.** Following RNA extraction, microarray was performed at the Duke-NUS Genome Biology Core Facility. cRNAs were hybridized to Illumina Human HT-12 v4 Beadchips, according to manufacturer's instructions. Data analysis was performed using Partek software and normalized against GAPDH.

**Competition with Soluble LILRB1 Ectodomain.** The extracellular portion of LILRB1 was cloned into pCMV-XL5 (Origene) and transfected into HEK293T cells for protein expression. The expressed proteins were then purified and incubated with DENV-2 or h3H5-opsonized DENV-2 for 1 h at 37 °C before adding to THP-1.2S.

**siRNA Transfection and Overexpression.** siRNA transfections and overexpression were performed as described (9). siRNA targeting FcγRIIB (Qiagen), LILRB1, MAVS, IRF3, and TRIF (SABio) were used, and overexpression studies were performed with either empty plasmid, plasmid encoding LILRB1 or tyrosine mutant LILRB1, or LILRB4.

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# APPENDIX B

Review

Text

EXPERT REVIEWS

# Therapeutic antibodies as a treatment option for dengue

*Expert Rev. Anti Infect. Ther.* 11(11), 000–000 (2013)

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Dengue is the most prevalent mosquito-borne viral disease globally with about 100 million cases of acute dengue annually. Severe dengue infection can result in a life-threatening illness. In the absence of either a licensed vaccine or antiviral drug against dengue, therapeutic antibodies that neutralize dengue virus (DENV) may serve as an effective medical countermeasure against severe dengue. However, therapeutic antibodies would need to effectively neutralize all four DENV serotypes. It must not induce antibody-dependent enhancement of DENV infection in monocytes/macrophages through Fc gamma receptor (FcγR)-mediated phagocytosis, which is hypothesized to increase the risk of severe dengue. Here, we review the strategies and technologies that can be adopted to develop antibodies for therapeutic applications. We also discuss the mechanism of antibody neutralization in the cells targeted by DENV that express Fc gamma receptor. These studies have provided significant insight toward the use of therapeutic antibodies as a potentially promising bulwark against dengue.

**Keywords:** antibody • antibody-dependent enhancement • dengue • neutralization • therapeutics

Dengue is the most prevalent mosquito-borne viral disease globally [1]. Infection with any of the four dengue virus serotypes (DENV-1-4) can result in a range of syndrome, from self-limiting febrile illness to severe dengue [2]. Out of an estimated 400 million infections that occur globally each year, a quarter of these develop into acute illness [1]. The escalating number of dengue cases worldwide is fuelled by the increased geographical distribution of the mosquito vector from international movement of human and cargo, unplanned and uncontrolled urbanization, migration of dengue susceptible individuals into dengue endemic cities, inadequate domestic water supplies and poor vector control measures in most areas of the tropics [3–6]. Infection with one of the four DENV serotypes results in long-term immunity to the homologous serotype but provides only temporary protection against the remaining three heterologous serotypes [7,8]. Consequently, secondary infections with a heterologous DENV serotype, which can increase the likelihood of severe dengue, are increasingly prevalent [9–12] These trends culminate in dengue becoming a major and growing public health problem throughout the tropical world.

Although dengue transmission can be reduced by vector control, many dengue endemic areas do not employ effective vector surveillance and control programs. This is partly due to the lack of long-term political and financial support for national mosquito surveillance and control programs [13]. Over-reliance on chemical control and poor participation from the community also resulted in short-lived effectiveness in disease prevention [14]. Furthermore, low vector density may not necessarily result in sustainable reduction in dengue incidence. For instance, despite active entomologic surveillance and source reduction efforts in Singapore, the incidence of dengue surged in the 1990s and remains high even at present. Multiple factors contribute to this re-emergence of dengue in Singapore despite vector control. These include lowered herd immunity [15], a shift in virus transmission from a domestic to non-domestic setting [16], more clinically overt infections in adults and reduced emphasis on surveillance in the present vector control program [17]. All of these are a direct consequence of the vector control program [17], which collectively underscores the need for a safe, effective and affordable vaccine for sustainable prevention against dengue.

Although safe and effective vaccines have been developed for other flaviviruses such as yellow fever virus (YFV), Japanese encephalitis virus (JE) and tick-borne encephalitis virus, no

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licensed dengue vaccine is currently available. The development of an effective dengue vaccine has been challenging because of the need to protect against all four DENV serotypes simultaneously. Furthermore, as non-neutralizing or sub-neutralizing levels of antibodies may opsonize DENV and engage fragment crystallisable receptors (FcγR) in myeloid cells for enhanced cellular entry and infection [18,19], the induction of antibodies has to be at levels sufficient to prevent antibody-dependent enhancement (ADE) of DENV infection. This hypothesis is the leading explanation for the association between secondary infection and increased risk of severe dengue. The current leading vaccine candidate is Sanofi Pasteur's ChimeriVax-DENV vaccine, which uses the yellow fever virus 17D vaccine strain as a live vector for the pre-membrane (prM) and envelope (E) genes of the four different DENV serotypes [20,21]. However, although excellent immunogenicity and safety profile of ChimeriVax-based vaccine candidates have been observed [21–23], the recent phase 2b trial on Thai school children indicated that vaccine efficacy was only 30.2% [24], suggesting significant room for improvement. Without sustainable vector control measures or licensed preventive vaccines, management of dengue cases is critical to minimize the disease burden.

Currently, clinical management of dengue is primarily supportive. No licensed antiviral drug against dengue is available. Therapies that can effectively reduce the risk of severe dengue could be transformative to the field. An option to be considered is therapeutic antibodies. Indeed, lifelong immunity against the homologous DENV serotype is largely mediated by the neutralizing antibodies that develop following acute infection [7,8]. This suggests that timely administration of neutralizing antibodies could lower DENV viremia, high levels of which has been shown to be associated with severe dengue [25]. Furthermore, the expanding knowledge on dengue neutralizing epitopes and the increasing popularity of therapeutic antibodies as a treatment option for infectious diseases also work in favor of such an approach to the treatment of dengue.

The main advantages of therapeutic antibodies are that they are well-established and are generally well tolerated by humans [26]. As they are increasingly used as treatment for other infections or diseases, the production cost of therapeutic antibodies has also reduced over the years. Moreover, these antibodies can be modified to improve their efficacy [27,28]. In recognition of these possible benefits, there is an increasing attention to identify and develop therapeutic antibodies against dengue. In this article, we describe the potential of using therapeutic antibodies against dengue and the epitopes that can be targeted to generate potent neutralizing antibodies. With an improved mechanistic understanding of DENV neutralization and ADE, we also describe how recent findings in this area can be applied to augment therapeutic efficacy of these antibodies.

## Therapeutic monoclonal antibodies for infectious diseases

Several human serum immunoglobulin (IgG) preparations have been licensed as passive immunotherapy for a wide range of viruses, indicating that antibody therapy can be effective therapeutically [29,30]. The main advantage of using polyclonal antibody preparations is that they contain a large and diverse population of antibodies that recognize different viral epitopes. These different antibodies can have strong antiviral activity as the presence of different neutralizing antibodies can exert additive or even synergistic effects on neutralization. Targeting multiple epitopes to neutralize DENV also reduces the risk of emergence of neutralization escape mutants. However, polyclonal preparations have batch to batch variations and may carry the risk of blood-borne pathogen transmissions. Moreover, as the vast majority of DENV-specific antibodies are non-neutralizing [31–33], polyclonal preparations will have to be individually screened to ensure that they contain sufficiently high titers of neutralizing antibodies, so as to eliminate any potential risks arising from ADE. Monoclonal antibodies (mAbs), in contrast, can be produced in large quantities and with high consistency. As mAbs can bind to their antigens with high affinity and specificity, the adverse events associated with the use of these antibodies can be greatly reduced.

Rapid production of mAbs suitable for clinical use has been enabled by mouse hybridoma technology [34] as well as transgenic mice engrafted with human immune system or carrying human immunoglobulin genes [35]. The development of methods such as microbial surface display [36] and human memory B-cell immortalization [37] have also contributed to the production of humanized and chimeric antibodies. Several mAbs have been developed for different viruses, including human respiratory syncytial virus (RSV), rabies virus, West Nile virus (WNV) as well as severe acute respiratory syndrome coronavirus. These are currently at different stages of clinical evaluation [29]. The most successful mAb approved for prophylactic use is palivizumab, a humanized mAb that specifically targets the fusion protein of RSV, hence preventing viral entry and infection [38]. Based on two Phase III clinical trials in children, palivizumab prophylaxis in infants was found to significantly reduce the risk of hospitalization due to RSV infection by 55% [39] and 45% [40], respectively. In addition, the palivizumab-treated group had shorter hospitalization with few adverse events, supporting the use of mAbs for prophylaxis in infants. The use of therapeutic antibodies against viruses has also gained popularity in the past decade. The recent use of m102.4 against Hendra virus [41] in humans based only on *in vitro* and *in vivo* efficacy in animal models strengthens the potential of therapeutic mAbs against viruses, particularly during epidemics.

## Targeting neutralizing dengue epitopes

The development of therapeutic antibodies as antivirals has been accelerated by display and screening platforms enabling rapid mapping of neutralizing and non-neutralizing viral epitopes using viral structural proteins as 'bait'. More recently, various groups have succeeded in generating panels of DENV-specific humanized monoclonal antibodies (Table 1). Besides its therapeutic applications, these studies also provide insights on the human antibody response to DENV.

**Table 1. DENV-specific humanized monoclonal antibody (huMAb) preparations.**

| Donor | Number of huMabs | huMAb target | Serotype specific or cross-reactive | DENV neutralizing activity | DENV enhancing activity | Ref. |
|---|---|---|---|---|---|---|
| **Epstein Barr virus transformation of memory B cells** | | | | | | |
| 1 DENV infected patient | 3 (IgG1) | E | Cross-reactive | 1 huMab neutralized DENV1 and DENV3 | Enhances DENV1 infection *in vitro* | [102] |
| 7 DENV infected patients. | 6 (IgG1) | prM | Cross-reactive | Incomplete DENV neutralization | Potently enhancing *in vitro*. | [33] |
| 2 patients with primary DENV infection | 26 (25 IgG1, 1 IgG3) | prM, E | Broadly cross-reactive | Weakly neutralizing | Not tested | [49] |
| | | EDIII | Serotype specific or cross-reactive | Neutralizes homologous serotype | Not tested | |
| 5 patients with primary or secondary DENV infection | 89 (85 IgG1, 2 IgG3 and 2 IgG4) | EDI, EDII, prM | Cross-reactive | Weakly neutralizing | Enhancing activity to all 4 serotypes | [32] |
| | | EDIII | Serotype specific or cross-reactive | Strongly neutralizing | Enhancing activity to all 4 serotypes | |
| | | NS1, NS3, capsid | Serotype specific or cross-reactive | N.A. | N.A. | |
| DENV infected patients (refer [103]) | 3 | Complex epitopes (EDI and EDII hinge region) | Serotype specific | Strongly neutralizing to homologous serotype | Not tested | [52] |
| 12 patients with primary or secondary DENV infection | 37 (35 IgG1, 2 IgG2) | E | Cross-reactive | Weakly neutralizing | Enhances infection *in vitro* | [103] |
| | | prM | Cross-reactive | Weakly neutralizing | Strongly enhancing *in vitro*. | |
| DENV-1 infected patient | 1 (IgG1) | Complex epitope (Spans adjacent surface of E protein dimers) | Serotype specific | Neutralizes DENV1 | Enhancing activity to DENV' | [53,57] |
| 3 convalescent DENV infected patients | 3 (IgG1) | Fusion loop of EDII | Cross-reactive | Neutralizes all 4 serotypes | Enhancing activity to all 4 serotypes | [104] |
| **Fusion of PBMCs with murine-human chimera fusion partner cells (SPYMEG cells)** | | | | | | |
| 8 patients in acute or convalescent phase of secondary DENV infection | 121 acute-phase and 15 convalescent phase huMabs | Mostly directed to E | Cross-reactive | Neutralizes all 4 serotypes | Not tested | [105] |
| 3 patients at acute phase of secondary DENV2 infection | 17 (16 IgG1, 1 IgG4) | E | Cross-reactive | Neutralizes all 4 serotypes | Enhancing activity to DENV2 | [106] |

E: Envelope; ED: Envelope domain; NS: Non-structural; NA: Not applicable; prM: Pre-membrane.

DENV is a positive-sense single-stranded RNA virus. Its 10.7-kb RNA genome encodes for three structural proteins, namely capsid (C), pre-membrane/membrane (prM/M) and envelope (E) as well as seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5). Its nucleocapsid core is surrounded by 180 monomers of E protein organized into 90 tightly packed dimers that lie flat on the surface of the viral membrane [42]. Individual subunits of the E protein form three beta-barrel domains, domains I (EDI), II (EDII) and III (EDIII), with the hydrophobic viral fusion peptide located at the tip of EDII and the receptor binding sites at EDIII [43]. The DENV E protein is the major neutralization target in the human antibody responses following dengue infection. Antibodies against dengue can either be serotype-specific or cross-reactive as the E proteins of the four serotypes are approximately 72–80% identical at the amino acid level [44].

Since long-term immunity to DENV is serotype-specific, much work has focused on characterizing neutralizing antibody responses against the homologous DENV serotype. Using mouse mAbs, several studies have reported that antibodies targeting the lateral ridge or A-strand of EDIII are potent neutralizing antibodies, strongly inhibiting infection *in vitro* and *in vivo* [45–48]. Similarly, by mapping the E protein-specific responses in humans, potent neutralizing mAbs that target the lateral ridge and the A-strand have been identified [49]. However, these antibodies represent a surprisingly small fraction of the antibodies that bind recombinant E-protein [49]. This was substantiated by studies showing that the neutralization activities of sera before and after depletion of EDIII-specific antibodies had no reduction in neutralization potency *in vitro* [50] and *in vivo* [51]. Hence, unlike mice, humans produce neutralizing antibodies that mostly do not bind EDIII epitopes [48].

Instead of EDIII, highly potent mAbs in DENV human immune sera bind quaternary epitopes on DENV envelope [52]. de Alwis and colleagues identified a potent neutralizing antibody that binds the hinge region between EDI and EDII [52]. Teoh and colleagues isolated a potent neutralizing mAb HM14c10 that recognizes a discontinuous epitope spanning adjacent surfaces of E-protein dimers on DENV-1 [53]. These conformational neutralizing epitopes are not only limited to DENV. Potent neutralizing antibodies against WNV have also been reported to recognize the flexible DI-DII hinge region, preventing pH-induced re-arrangement of the E-protein required for virus fusion [54,55]. Therefore, besides using humanized mAbs derived from mouse EDIII-specific antibodies, human antibodies that recognize neutralizing conformational epitopes like the hinge region between EDI and EDII could also be used therapeutically against homologous serotype of DENV.

## Neutralizing epitope variation

An important consideration for the use of mAb as a therapeutic agent is the diversity of DENV strains. The replication of DENVs RNA genome is error prone, which does give rise to diversity in the E protein sequence, including EDIII, within each of the four DENV serotypes [56]. These differences in the E protein can directly influence antibody binding and hence, the efficacy of therapeutic antibodies [44,56]. Compared with EDIII mAbs, however, those that target the complex structural epitopes on EDIII [48] or the hinge region between EDI and EDII [53] can retain strong binding and neutralizing activity against multiple strains within each of the four DENV serotypes. This suggests that antibodies that target the hinge region between EDI and EDII may act against strain differences more effectively.

Another pitfall that has to be addressed is the possible emergence of neutralization escape mutant viruses. Therapeutic mAb could exert a selection pressure on those strains that are able to escape neutralization. This is supported by *in vitro* studies demonstrating that resistant viruses can emerge within three rounds of passaging in cell culture [57]. In the context of acute dengue, the possibility of resistant virus emerging is reduced as viremia, which is typically short-lived. However, to negate this possibility, it may be necessary to consider an antibody cocktail consisting of two or more mAbs for each DENV serotype. Alternatively, bi-specific antibodies or the antibody variable region-based bi-specific dual affinity re-targeting molecules that targets two spatially distinct epitopes on each serotype could be considered [58].

## Models & mechanisms of dengue virus neutralization

Besides binding suitable epitopes, antibody neutralizes DENV only when a sufficient proportion of the epitopes are bound by antibodies [59,60]. This stoichiometric threshold for DENV neutralization is determined by both antibody affinity and epitope accessibility [61–63]. Antibody affinity is defined by the fraction of epitopes bound by antibodies at non-saturating concentrations. Epitope accessibility, in contrast, is the number of epitopes that is accessible for binding. It is affected by steric constraints from virion structure, structural dynamics of virus, differences in oligomeric states during virion maturation and antibody size [60]. Reduced epitope accessibility results in an increased fraction of epitope occupancy required for virus neutralization. Some of the E protein-specific antibodies also rely on the dynamic movement of protein molecules, binding to hidden epitopes that are transiently exposed. For example, optimal binding of mAb 1A1D2 to EDIII requires incubation at 37°C, as these epitopes are transiently exposed at such temperatures [64]. These studies suggest that antibodies that have high affinity to highly accessible epitopes should be prioritized for therapeutic development.

Another consideration that has to be made for selecting antibody for therapeutic development is how the antibody neutralizes DENV. Antibody blocks DENV infection at different stages of the virus life cycle. MAbs can neutralize DENV by either blocking attachment to cellular receptors [47] or blocking viral fusion intracellularly [65]. However, multiple receptors have been identified as candidates for DENV entry. This reflects a lack of consensus in the field for a bona fide cellular receptor for DENV. Candidate receptors include heparan

sulfate [66], heat-shock protein 90 [67], CD14 [68] and C-type lectins such as CLEC5A [69], dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) [70] and mannose receptor [71]. Hence, identifying mAbs that block attachment of DENV to target cells can be potentially challenging, especially since most of these studies did not utilize cells, which are the primary targets of DENV in human infection. Moreover, when opsonized with antibodies, DENV can enter myeloid cells through FcγR-mediated phagocytosis. Thus, therapeutic antibodies must be able to inhibit viral fusion in phagosomes. Indeed, serotype-specific antibodies, which are associated with long-term immunity in humans, appear to be able to neutralize DENV in the presence of FcγR-mediated phagocytosis [72]. In the absence of virus fusion, which typically occurs in Rab7-positive late phagosomal compartments, DENV remains trapped in the phagocytic pathway [73]. Subsequent late phagosome-lysosome fusion leads to degradation of DENV via lysosomal hydrolases and the production of superoxide and nitric oxide radicals [74]. Potent mAbs that inhibit intracellular neutralization have also been shown for other viruses such as WNV and RSV. Thompson and colleagues showed that humanized antibody E16, which binds to EDIII of WNV, is strongly inhibitory because it was able to block pH-dependent viral fusion [75]. The clinically approved mAb, palivizumab has also been shown to neutralize RSV intracellularly by preventing cell-to-cell or virus-to-cell fusion [38].

Besides mediating uptake by professional phagocytes, the Fc region of antibodies also exerts antiviral effects by interacting with other immune cells (Figure 1). Virus-specific antibodies can bind to DENV antigens displayed on infected cells to result in natural killer (NK) cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) [76]. In addition, virus-specific antibodies can mediate complement deposition on the virion surface to result in direct virolysis [77]. The complement component C1q can also bind to the Fc region of antibody-opsonized DENV and activate the classical pathway, triggering a cascade of events that leads to the formation of the membrane attack complex C5b-9. This leads to pore formation in the plasma membranes of virus infected cells, resulting in complement-dependent cytotoxicity (CDC). C1q can also bind Fc to reduce Fc–FcγR interaction, thus minimizing the risk of ADE [78,79]. Collectively, therapeutic antibodies could reduce DENV replication by involving multiple arms of the immune response, thus leading to improved viral clearance.

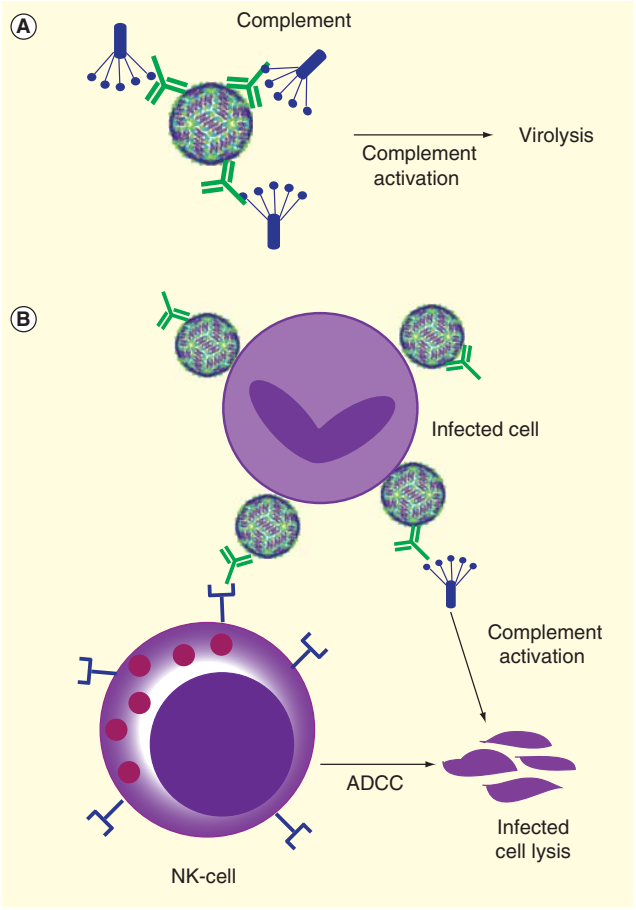


**Figure 1. Effector functions of antibodies against DENV.**
**(A)** The Fc region of DENV-specific antibodies mediates the deposition of complement on the virion surface, which can rupture the virion envelope and lead to direct virolysis of DENV immune complexes. **(B)** DENV-specific antibodies can activate complement and NK cells, leading to lysis of infected cells via complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC).

## The use of serotype-specific or cross-reactive antibodies for therapeutics

Although mAbs can provide great therapeutic potential, careful selection of these antibodies are required to reduce the risk of ADE. The plaque reduction neutralization test (PRNT), first developed by Russel and Nisalak in 1967 [80,81], has been widely used to measure DENV neutralization. However, PRNT is mostly performed on kidney cell lines such as LLC-MK2, Vero and BHK-21. The ability of antibody to prevent DENV infection of these kidney cells may not necessarily inform on the ability of these same antibodies to prevent infection of myeloid cells through FcγR-mediated phagocytosis. Indeed, Endy and colleagues observed in a prospective study that children remained susceptible to dengue despite having neutralizing antibodies, as measured by PRNT, prior to the infection [82]. Since human monocyte is one of the primary targets of DENV, perhaps monocytes may be a more suitable cell to measure DENV neutralization [83]. We have shown that neutralization of homologous DENV serotypes was observed to occur at titers that permit FcγR-mediated phagocytosis while neutralization of heterologous DENV serotypes occur only at titers that aggregate DENV to co-ligate FcγRIIB in human monocytes [72]. These observations were further validated clinically with 30 other convalescent sera [84], suggesting that this approach could better distinguish serotype-specific antibodies from cross-reactive antibodies. That serotype-specific antibody

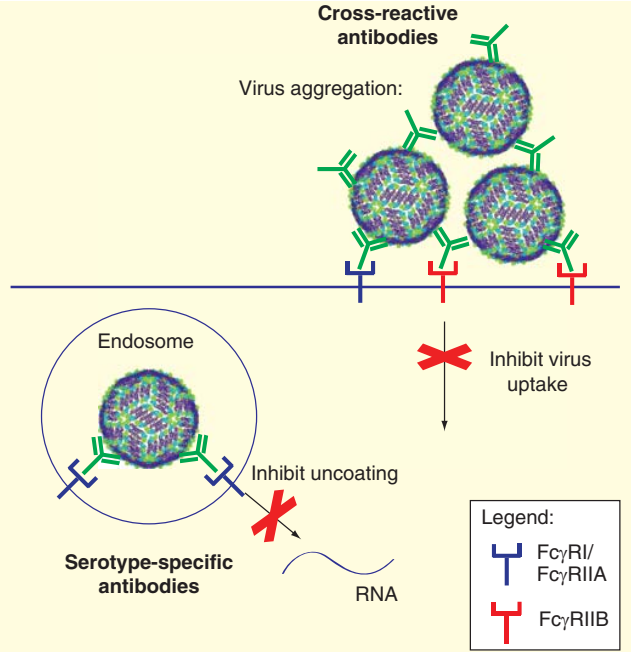


**Figure 2. Mechanisms of DENV neutralization in cells expressing FcγR.** DENV neutralization in FcγR-bearing cells can be mediated by inhibiting virus uptake or intracellular viral fusion. Serotype-specific antibodies neutralize at levels that mediate DENV uptake by inhibiting intracellular viral fusion with host endosomal membrane and viral uncoating, leading to eventual phagosomal degradation of DENV. Cross-reactive antibodies, in contrast, neutralize DENV by forming viral aggregates, which co-ligate FcγRIIB to inhibit phagocytosis of DENV immune complexes.

can inhibit viral uncoating even in the presence of FcγR-mediated phagocytosis, suggests that serotype-specific mAbs should be considered for therapeutic antibodies (Figure 2).

## Cross-reactive therapeutic mAbs

While serotype-specific antibodies have been shown to contribute to long-lasting immunity, cross-reactive antibodies do provide transient protection of approximately 2–3 months [7]. This suggests that antibodies that neutralize more than one DENV serotype may be present at low levels or that neutralization of multiple serotypes require high concentrations of such antibodies. Either possible explanation, however, could be harnessed for therapeutic application.

The possibility that the transient immunity observed by Sabin is due to low prevalence of broadly cross-neutralizing antibody could make isolation of such mAb difficult. Such a problem could potentially be overcome by antibody engineering. Recently, using computational design, Tharakaraman and colleagues were able to identify and change specific nucleotide residues on the gene encoding an existing antibody to obtain a approximately 450-fold increase in affinity to DENV-4 while preserving binding to the other three dengue serotypes [85]. This demonstrates the possibility of engineering antibodies for broad-spectrum application. Improvements in computer-aided antibody design that can further increase binding and specificity of these mAbs could hence play a major role in the future of therapeutic antibody development.

Besides neutralizing multiple serotypes of DENV, cross-reactive neutralizing mAb could also be used to displace non-neutralizing antibodies produced during dengue infections and reduce the risk of ADE. Such a property was embodied by a modified moderately neutralizing antibody that recognizes the fusion loop. This mAb could compete with and displace non-neutralizing antibody that bind to epitopes in the vicinity through stearic hindrance, resulting in reduction of ADE, both *in vitro* and *in vivo* [86].

Another approach to using cross-reactive mAbs therapeutically is to administer at a dose sufficient to aggregate DENV. We have shown recently that in addition to blocking binding to receptor or viral fusion with endosomal membranes, antibodies can also aggregate DENV to co-ligate the inhibitory receptor, FcγRIIB. This receptor signals through an immunoreceptor tyrosine-based inhibition motif (ITIM), which recruits and activates the Src homology 2 (SH2) domain-containing inositol 5'-phosphatase (SHIP) and SH2 domain-containing phosphatase (SHP) that inhibit FcγR-mediated phagocytosis and hence DENV entry into monocytes [72] (Figure 2). This mechanism of inhibiting DENV infection, which is dependent on high antibody concentration, may explain the transient cross-reactive immunity observed by Sabin [7]. Exploiting FcγRIIB-mediated signaling with high dose of mAb could thus be a useful strategy.

An added advantage of exploiting the FcγRIIB pathway therapeutically is that this receptor also signals to down-regulate the pro-inflammatory response. Indeed, intravenous immunoglobulin (IVIG) preparations, which are composed of polyvalent IgG derived from more than a thousand blood donors, have been shown to be effective in reducing TNFα production by inhibiting NF-kB activation [87]. How IVIG mediates this anti-inflammatory effect is less clear. It appears to be dependent on Fc sialylation [88], which suggests that the anti-inflammatory effect is mediated through interaction of Fc with specific receptors. One possible candidate is FcγRIIB. IVIG treatment has also been shown to up-regulate FcγRIIB, which can alter the threshold of activation of inflammatory cells and reduce pro-inflammatory response of monocytes [89]. Therefore, high dose neutralizing antibody could not only serve to impede ADE but also reduce the pro-inflammatory response that underlies pathogenesis of severe dengue [90,91]. Studies testing this strategy for the treatment of dengue could thus be particularly fruitful.

## Fc modifications to reduce risk of ADE & improve half-life

As the administration of dengue antibodies could potentially enhance infection through Fc-FcγR interaction, Fc modifications that reduce interaction with activating FcγRs could alleviate this risk [92,93]. Although Fab fragments could be used therapeutically, their smaller size and hence shorter half-life limits their usefulness [94]. To extend the terminal half-life of these

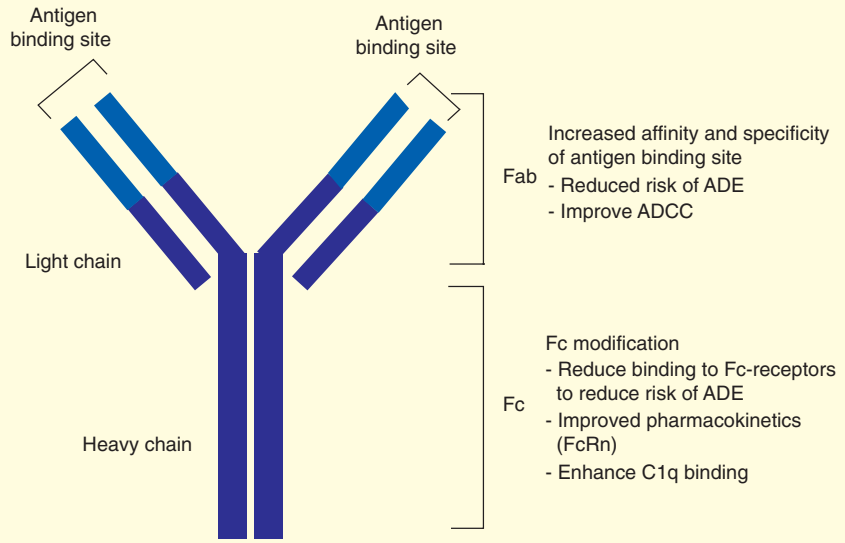


**Figure 3. Modifications that can increase therapeutic efficacy of mAbs.** Fab or Fc regions of antibodies that can be modified to improve antibody effector functions, pharmacokinetics and reduce risk of antibody-dependent enhancement (ADE).

Fab fragments, these molecules can be coupled with molecules such as IgG, serum albumin or with polyethylene glycol [26]. Alternatively, mAbs could be expressed as IgG4 isotype, which has significantly reduced binding to FcγRs compared with IgG1 [95] and has been used in humans [96]. Other mutations or deletions in the Fc-region have also been shown to reduce the risk of ADE of dengue infection *in vivo*. These variations include deletions of nine amino acids [97], mutation of asparagine to glutamate at position 297 (N297Q) [53,86,93] and mutations at positions 234 and 235 from leucine to alanine to form LALA mutants [32]. The modified antibodies were shown to retain binding characteristics to DENV, exhibiting prophylactic and therapeutic efficacy *in vivo*. Such modifications, however, would reduce the other effector immune functions mediated by antibodies, such as ADCC and complement pathways. Future studies will be needed to test the potential of using these modified antibodies for therapeutics in humans.

Besides using mAbs as an antiviral agent, mAbs can be used prophylactically to protect individuals with dengue infection. However, these mAbs will have to be maintained at sufficiently high levels to minimize the risk of ADE. In this case, the Fc-region of mAbs can be exploited to extend the half-life of antibodies, thereby reducing the need for repeated dosing. Antibody half-life can be extended by engineering Fc regions that change binding affinity to its salvage receptor, FcRn. After internalization of antibodies into acidic endosomal compartments in the cells, binding to FcRn diverts antibodies for recycling back to circulation, preventing lysosomal degradation and hence prolonging the serum half-life. Fc mutations at His310 and His435, which bind acidic residues on the surface of FcRn should be avoided to preserve the half-life of mAbs, as an acidic pH environment (pH 6.0–6.5) is critical for the interaction between Fc and FcRn. Based on molecular models from the rat Fc–FcRn complex [98], it was predicted that residues 250, 314 and 428 can have significant effects on Fc–FcRn interactions. Indeed, mutations at positions 250 (Thr250Gln) and 428 (Met428Leu) were found to significantly increase the binding to FcRn and extend the half-life of the antibodies in rhesus monkeys by approximately twofold [99,100], without affecting antigen binding, ADCC and CDC. These mutations indicate that the half-life of these antibodies can be increased without compromising the effector functions of these therapeutic antibodies.

Lastly, as the presence of complement component C1q can inhibit ADE of dengue infection, amino acid substitutions that enhance C1q binding can potentially improve the therapeutic efficacy of dengue mAbs. Importantly, mutations at residues 326 (Lys326Trp) and 333 (Glu333Ser) located in the C1q binding epicenter were observed to enhance C1q binding and CDC activity by fivefold without influencing the ADCC activity [101]. These mutations can thus potentially improve the therapeutic efficacy of dengue mAbs while retaining antigen binding activity. Taken together, Fc modification of antibodies can potentially enhance effector functions while reducing the risk of ADE (Figure 3). However, as most of these functional studies were either performed in monkeys or mice, additional human studies will be required to assess the utility and effectiveness of these Fc modified antibodies.

## Conclusion

Treatment of dengue using therapeutic mAbs can be challenging. Therapeutic mAb preparations must neutralize DENV without increasing the risk of ADE. Nonetheless, its ability to both neutralize DENV and elicit an anti-inflammatory response could be the double-edged sword needed for the treatment of dengue.

## Expert commentary

Current methods to control dengue epidemics primarily rely on vector control, which has been shown to be ineffective over the past decade. The development of an effective dengue vaccine will hence remain a priority for sustainable dengue prevention. In the continued absence of an effective dengue vaccine, antivirals that reduce viremia to alleviate risk of severe dengue would contribute significantly to reducing the overall burden of dengue. Monoclonal antibodies have become an attractive therapeutic option against infectious diseases and have been shown to be well tolerated by humans. Therapeutic antibodies developed against DENV should be able to inhibit infection in cells expressing FcγR, which are the primary targets of infection in humans. With the expanding knowledge on neutralizing and non-neutralizing epitopes, as well as technologies in antibody modification, we believe that therapeutic mAbs against DENV could be developed in the near future. This will be useful for disease management, particularly during dengue epidemics.

## Five-year view

Serotype-specific therapeutic antibody to DENV-1 has been recently identified. Further identification of therapeutic antibodies against the other 3 DENV serotypes will permit cocktail formulations that will be useful for disease management. Improvements in computational design of antibodies that improve binding affinity and specificity across all four DENV serotypes could greatly enhance the development of a cross-reactive therapeutic mAb for dengue. The development of potent therapeutic mAbs could also inform on the design of a dengue vaccine for effective dengue prevention.

### Financial & competing interests disclosure

*The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.*

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## Key issues

- Global prevalence of dengue remains high due to ineffective vector control. There is currently no licensed vaccine or antiviral drug against dengue.
- Therapeutic antibodies are increasingly used for the treatment of infectious diseases as they are well-established and well tolerated by humans.
- Human antibodies that potently neutralize dengue virus (DENV) bind quaternary epitopes on DENV E protein and could be used therapeutically against homologous serotype of dengue.
- Administering an antibody cocktail may lower the risk of neutralization escape viruses. Inclusion of antibodies targeting complex epitopes may act against viral strain differences more effectively.
- DENV neutralizing antibodies prioritized for therapeutic development should possess high affinity for accessible epitopes, and prevent intracellular viral fusion.
- Measurement of DENV neutralization in monocytes better distinguishes serotype-specific from cross-reactive antibodies.
- Serotype-specific antibodies are a good candidate for therapeutic antibodies as they inhibit intracellular viral fusion, and reduce risk of antibody-dependent enhancement (ADE).
- High dose administration of cross-reactive antibodies can also impede ADE and reduce pro-inflammatory responses that underlie severe dengue.
- Fc modifications to improve therapeutic antibody half-life and C1q binding can enhance effector function of antibodies and reduce the risk of ADE.

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**Biography**

I was born 14 January 1987 in Singapore. I attended Nanyang Technological University (NTU) for my undergraduate studies, graduating in August 2010 with a Bachelor of Science with Honours in Biological Sciences. AT NTU, I was privileged to be a recipient of the school's Nanyang Scholarship and also a member of the C. N. Yang Scholars' Programme, a programme for outstanding science and engineering students. I was accepted to do my PhD in Integrated Biology and Medicine at Duke-NUS Graduate Medical School in August 2010. I was supported by the A*STAR Graduate Scholarship (Local) for the course of my PhD studies. During my PhD studies, I published a full-length research paper "Leukocyte immunoglobulin-like receptor B1 is critical for antibody-dependent dengue" (Proceedings of the National Academy of Sciences of the United States of America) and a review paper "Therapeutic antibodies as a treatment option for dengue fever" (Expert review of anti-infective therapy). I was selected to give an oral presentation of my research findings at the 3rd International Conference on Dengue and Dengue Haemorrhagic Fever (Bangkok, Thailand), Agilent Young Scientist Forum 2014 and Singhealth Duke-NUS Scientific Congress 2014. I received the Best Oral Presentation award at the Agilent Young Scientist Forum and Duke-NUS DUNES Symposium in 2014.