

**From Guardian to Gateway: B cell receptor-dependent  
enhancement (BDE) of dengue virus infection in  
dengue-reactive B cells**

Chad Gebo

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# Abstract

From Guardian to Gateway: B cell receptor-dependent enhancement (BDE) of dengue virus infection in dengue-reactive B cells

Author: Chad J. Gebo

Sponsor: Adam T. Waickman

Dengue virus (DENV) is the causative agent of dengue, a mosquito-borne disease that is responsible for approximately 400 million infections every year. One of the defining features of dengue is immune-mediated enhancement. The leading mechanistic explanation for this phenomenon is antibody dependent enhancement (ADE). Multiple independent groups have shown that B cells are the largest reservoir of virally infected cells in circulation during acute dengue and have been shown to be poorly susceptible to infection through conventional means or ADE. In this thesis, we characterize a novel mechanism of DENV entry into specific subsets of B cells via the B cell receptor (BCR), known as BCR-dependent enhancement (BDE). We show that expression of a DENV-reactive BCR can render a cell susceptible to infection and that the pool of susceptible B cells increases following primary exposure to DENV. We also show that DENV entry into cells via this mechanism requires early, proximal BCR signaling and leads to productive DENV infection. These findings, alongside ADE, work to fill in mechanistic gaps within the model of ADE and have implications in the establishment of infection during secondary disease as well as identify insights into potential mechanisms of DENV immunopathogenesis.

# Table of Contents

<b>Abstract.....</b>	<b>ii</b>
<b>Table of Contents.....</b>	<b>iii</b>
<b>Table of Figures .....</b>	<b>vii</b>
<b>Acknowledgements .....</b>	<b>ix</b>
<b>CHAPTER 1: INTRODUCTION PART I: DENGUE HISTORY AND CLINICAL OBSERVATIONS .....</b>	<b>1</b>
<b>Dengue Background and Emergence .....</b>	<b>2</b>
<b>Dengue Virus Proteins and Replication .....</b>	<b>4</b>
Overview of DENV infection and replication cycle .....	4
Capsid protein.....	6
Membrane protein.....	7
Envelope Protein.....	8
NS1 .....	8
NS2A and NS2B.....	9
NS3 .....	9
NS4A and NS4B.....	10
NS5 .....	11
<b>Dengue outcomes and pathogenesis.....</b>	<b>11</b>
Clinical Manifestations.....	11
Clinical Manifestations – Differences in viral genetics.....	13
Pathogenesis – Viral .....	15
Pathogenesis – Host.....	16
<b>Predictors of Disease Severity .....</b>	<b>20</b>
<b>Dengue Countermeasures.....</b>	<b>23</b>
Vector Control .....	23
Vaccines.....	24
Antivirals .....	26
<b>Conclusions .....</b>	<b>27</b>
<b>References:.....</b>	<b>29</b>

<b>CHAPTER 2: INTRODUCTION PART II: DENV CELLULAR TROPISM AND B CELL INFECTION.....</b>	<b>43</b>
<b>Chapter Introduction .....</b>	<b>44</b>
<b>Receptor-mediated Entry .....</b>	<b>44</b>
Attachment Factors.....	45
Mononuclear Phagocyte Infection.....	45
Liver and Epithelial Cell Infection .....	47
<b>Antibody Dependent Enhancement.....</b>	<b>48</b>
ADE History .....	48
ADE Epitopes .....	50
Extrinsic ADE.....	51
Intrinsic ADE.....	52
<b>DENV Tropism.....</b>	<b>53</b>
<b>B cell infection.....</b>	<b>56</b>
<b>BCR Dependent Enhancement .....</b>	<b>59</b>
BCR Structure and function .....	60
FcγR Structure and function .....	61
Evidence of BCR specific infection .....	63
<b>Conclusion.....</b>	<b>64</b>
<b>References:.....</b>	<b>65</b>
<b>CHAPTER 3: B CELL RECEPTOR DEPENDENT ENHANCEMENT OF DENV INFECTION.....</b>	<b>72</b>
<b>ABSTRACT .....</b>	<b>74</b>
<b>INTRODUCTION.....</b>	<b>75</b>
<b>MATERIALS AND METHODS .....</b>	<b>77</b>
Cells and Cell lines .....	77
Generation of Immortalized B cell lines.....	77
Viruses and RVPs.....	78
K562 ADE assay .....	78
DENV-specific ELISA and neutralization assays .....	79
Generation and expression of transmembrane antibody constructs .....	79
hCMV infection.....	79

Single cell RNA sequencing.....	80
5' gene expression analysis and visualization .....	80
DENV/Ig/BCR crosslinking infection.....	81
Statistical analysis.....	82
<b>RESULTS.....</b>	<b>83</b>
Expression of a DENV-specific BCR renders cells susceptible to DENV infection	83
DENV-specific B cells are susceptible to DENV infection. ....	87
Frequency of DENV-infectible B cells increases following primary DENV Infection.....	92
<b>DISCUSSION .....</b>	<b>95</b>
<b>References .....</b>	<b>97</b>
<b>CHAPTER 4: MOLECULAR AND BIOPHYSICAL REQUIREMENTS FOR B CELL RECEPTOR DEPENDENT ENHANCEMENT OF DENGUE VIRUS INFECTION .....</b>	<b>123</b>
<b>ABSTRACT .....</b>	<b>125</b>
<b>INTRODUCTION.....</b>	<b>126</b>
<b>MATERIALS AND METHODS .....</b>	<b>129</b>
Viruses & RVPs.....	129
Cell lines .....	129
Flow cytometry .....	130
DENV-4 tagRFP657 RVP Infection of human B cells .....	131
DENV-2 recombinant E Dimer infection blockade .....	131
Inhibition of viral entry by pharmacological inhibitor treatment. ....	131
Detection of DENV internalization and endosomal localization by microscopy....	133
Quantification of infectious DENV .....	134
Statistical Analysis. ....	135
Acknowledgements. ....	135
Data availability.....	136
Funding.....	136
<b>RESULTS.....</b>	<b>137</b>
BCR antigen-specificity determines DENV susceptibility of B cells. ....	137
BCR signaling is required for BDE-mediated viral entry .....	141
Endosomal localization of DENV in B cells. ....	145

B cells are productively infected with DENV via BCR-mediated infection. ....	148
<b>DISCUSSION</b> .....	<b>152</b>
<b>References</b> .....	<b>155</b>
<b>CHAPTER 5: DISCUSSION</b> .....	<b>165</b>
<b>Summary of Findings</b> .....	<b>166</b>
<b>Significance</b> .....	<b>167</b>
<b>Limitations</b> .....	<b>168</b>
Cell Model .....	168
BCR Isotype .....	169
Alternative Methods of Entry .....	170
<b>Is BDE unique to DENV?</b> .....	<b>171</b>
<b>Functional Consequence of B cell Infection</b> .....	<b>173</b>
Survival.....	174
B cell function .....	176
Memory Generation.....	180
<b>DENV Envelope-specific B cells as a clinical marker</b> .....	<b>181</b>
<b>Concluding Remarks</b> .....	<b>182</b>
<b>References</b> .....	<b>183</b>

# Table of Figures

Figure 1.1. DENV genome and replication cycle.....	5
Figure 1.2. Design of licensed Dengue Virus Vaccines.....	24
Figure 2.1. Expression of DENV-specific BCRs renders cells susceptible to DENV- infection .....	85
Figure 2.2. DENV-specific B cells are susceptible to ex vivo DENV infection .....	90
Figure 2.3. Frequency of DENV-infectible B cells increases following primary DENV Infection .....	93
Supplemental Figure 2.1. Gating scheme and DENV-1 RVP infection of tmlgG transfected 293T cells.....	101
Supplemental Figure 2.2. Gating scheme and DENV-1 RVP infection of tmlgM and tmlgA transfected 293T cells .....	103
Supplemental Figure 2.3. Expression of HCMV specific tmlgG.....	105
Supplemental Figure 2.4. Characterization and DENV-infection of 7B9 and 2F3 B cell lines .....	107
Supplemental Figure 2.5. Quality control metrics and integrated UMAP projections of scRNA seq data.....	109
Supplemental Figure 2.6. DENV infection of polyclonal B cells by BCR/DENV cross- linking.....	111
Supplemental Figure 2.7. Gating scheme for DHIM-3 PBMC analysis.....	113
Supplemental Table 2.1. Quality control metrics for scRNAseq data .....	114
Supplemental Table 2.2. Infected cell characteristics across culture conditions.....	114
Supplemental Table 2.3. Differentially expressed genes, DENV-2 infected cells and uninfected mock cells .....	118
Supplemental Table 2.4. Differentially expressed genes between DENV-2 infected 7B9 cells and uninfected mock cells .....	122
Supplemental Table 2.5. Reagents for flow cytometry analysis .....	122

Figure 3.1. BCR-dependent enhancement in DENV-reactive B cells is highly sensitive to DENV Envelope-specific interactions .....	139
Figure 3.2. Proximal BCR signaling required for viral entry in DENV-reactive B cells. ....	143
Figure 3.3. Internalization of DENV by DENV-specific B cells.....	146
Figure 3.4. Generation of infectious DENV by DENV-specific B cells .....	150
Supplemental Figure 3.1. Flow Cytometry Gating of immortalized B cells and DENV recE dimer. ....	159
Supplemental Figure 3.2. Flow Cytometry Gating of immortalized B cells and Raji DC-SIGN+ Cells. ....	161
Supplemental Figure 3.3. Characterization of pharmacologic inhibitors in immortalized B cells. ....	163

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# **CHAPTER 1**

## **INTRODUCTION PART I: DENGUE HISTORY AND CLINICAL OBSERVATIONS**

## Dengue Background and Emergence

Dengue is a vector borne viral febrile infection transmitted by *Aedes* mosquitoes within tropical and subtropical climates. Today, around half of the global population live in dengue endemic regions, where there has been a rapid rise in the number of WHO reported cases over the last few decades, from half a million cases in 2000 to over 14 million cases reported in 2024. Though it is estimated roughly 100-400 million people are infected every year [1]. The causative agent of dengue disease is infection by dengue virus (DENV), a flavivirus that circulates in four genetically and antigenically distinct serotypes: DENV-1, -2, -3, and -4. Reinfection by the same serotype, known as homotypic infection, can provide long-term durable protection from clinical infection. However, it is reinfection by a different serotype, or heterotypic infection, that is associated with increased risk for severe disease [2, 3]. Sylvatic transmission of DENV in non-human primates is estimated to have originated roughly 1,000 years ago [4]. Through phylogenetic analysis and sequence evolution, zoonotic transmission has been estimated to begin around 300 years ago, with urban transmission beginning roughly 200 years ago [5]. The rapid expansion of dengue has been attributed to a variety of factors, namely: viral evolution, climate change, and societal factors like globalization of trade and rapid urbanization [6].

DENV circulates in both low epidemiological impact sylvatic cycles that rarely cross over to human transmission. While some serotypes or even grouping of strains within serotypes commonly referred to as genotypes, can lead to higher virulence and high epidemiological impact even driving out lower impact strains [7]. It is not

completely understood what the determinants of virulence are, though there is evidence of modifications to domain III of DENV envelope protein that may improve fitness to naïve hosts [4]. Climate change has likely also played a significant role in the increase in dengue incidence. DENV vector *Aedes aegypti* show increased vector competency with increasing temperatures [8]. Additionally, increases in global temperatures expand the natural habitat for mosquito vectors, increasing the effective range of the vector, thus increasing the number of individuals exposed to mosquito vectors [6, 9, 10].

Expansion of human urbanization and travel is arguably one of the most significant factors in the global expansion of vector-borne diseases. Until the 1940s, dengue was only reported in small regions of the world in limited outbreaks across longer intervals of 10-40 years [11]. The mobilization of troops during World War II likely led to a rapid increase in dengue endemicity within Asian countries. With the advent of air travel, rapid global population growth and urbanization, paired with lack of effective vector control, this expansion in dengue endemicity also occurred in the Americas in the 1960s and 70s [6, 11]. Presently, dengue virus is endemic in over 80 countries, representing a significant proportion of the global population at risk of DENV exposure [12].

## Dengue Virus Proteins and Replication

Dengue virus is a single stranded, positive sense RNA virus and is a member of the Flaviviridae family. DENV has a roughly 11kb genome that encodes for a single polyprotein that is cleaved post-translation into 10 individual proteins, 3 structural and 7 nonstructural (**Figure 1A**). The structural proteins compose the physical structure of the virion: the capsid (C) functions as the nucleocapsid and surrounds genomic RNA, while the membrane and envelope (E) embed a host-derived lipid bilayer to surround the genome containing capsid [13]. The seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) serve a variety of functions ranging from replication and virion assembly to immune evasion and controlling host responses [14].

### *Overview of DENV infection and replication cycle*

In humans, following successful deposition of DENV after mosquito blood feeding, DENV attaches to a target cell through E protein interactions with one or more of a variety of cell surface receptors like DC-SIGN, mannose receptor, heparan sulfate, CD14, and more [15, 16]. DENV is then internalized via endocytosis, during which time a drop in the pH of the endosome triggers irreversible conformational changes in E protein allowing the fusion loop region of domain II to fuse with the endosomal membrane and allow escape of the capsid encased viral genome into the cytosol [15, 16]. Once in the cytosol, capsid protein is ubiquitinated and degraded allowing for the release of viral genome into the cell [17]. The encoded positive-sense RNA viral genome is flanked by a

5' 7-methyl guanosine capped untranslated region (UTR) with a non-polyadenylated 3' UTR, allowing the genome to be recognized as mRNA and localized to ribosomes in the ER lumen for initial translation [18, 19]. The DENV genome encodes for a single polypeptide that is then cleaved by

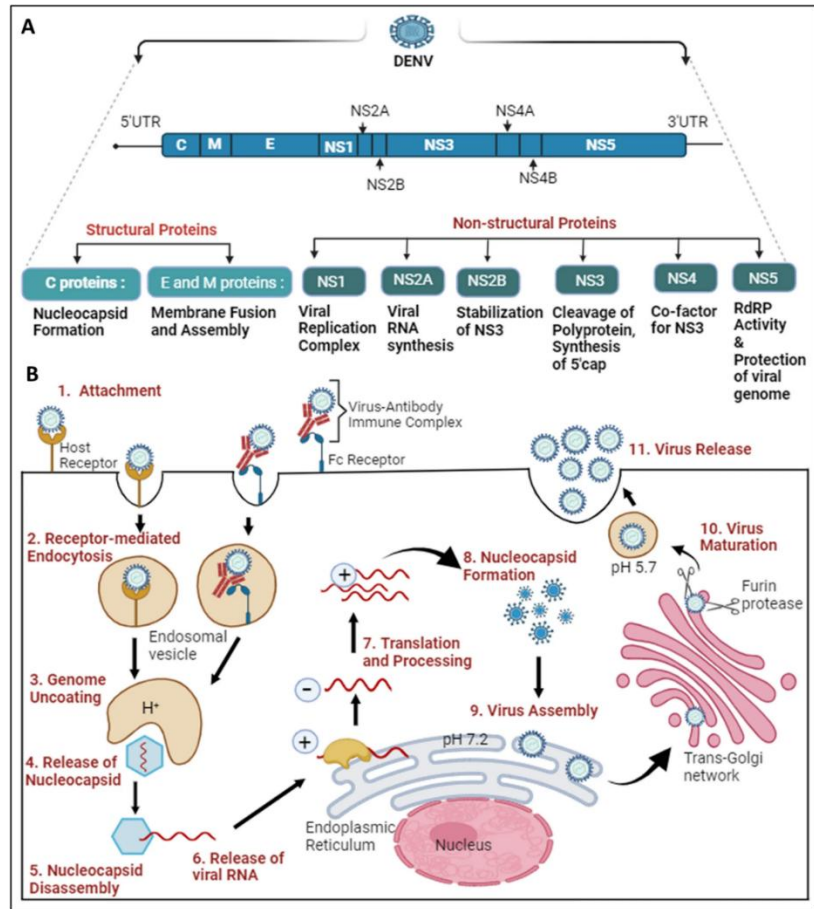


Figure 1.1. DENV genome and replication cycle. From [19].

both host and DENV proteases into specific viral proteins, some of which then assemble into a replication complex allowing for synthesis of negative strand, allowing for the generation of additional copies of positive sense RNA to be translated into additional DENV proteins [20]. Following synthesis the C-prM-E polyprotein is guided and anchored to the ER membrane, positive charges on the capsid allow binding of negatively charged viral RNA to form the nucleocapsid, where the capsid proteins are then cleaved by the NS2B-NS3 protease complex [21]. This genome containing nucleocapsid is then surrounded by the budding vesicle's lipid bilayer, embedded with prM and E proteins [22]. These immature particles are then transported to the trans-Golgi network for final

processing and maturation. Immature particles contain heterodimers of prM and E proteins, with prM acting as a shield to prevent premature irreversible conformational changes to E protein [23]. The particles are then fully processed through the action of host-derived furin, cleaving prM from E protein [24]. Mature particles then leave the cell through exocytosis [25]. A fully matured virion is composed of 90 E protein homodimers creating a smooth surface over a cleaved M protein embedded lipid bilayer surrounding the nucleocapsid containing a single copy of the DENV RNA genome [21]. Depending on cell type, flavivirus RNA synthesis can be detected within 3-6 hours, with mature virus being detectable between 10-24 hours postinfection [26]. In humans, virus detection varies by serotype but typically is detected in serum about 5 or 6 days after infection and is detectable until around 10-15 days post infection [27-29].

### *Capsid protein*

The capsid protein is the first protein encoded in the DENV genome. Following processing by NS3, the capsid aggregates once it binds RNA. After binding, this protein functions as an RNA chaperone, promoting folding of RNA molecules and preventing misfolding of the RNA [30]. Capsid-RNA formation of the nucleocapsid is the first step of DENV assembly, where it is proposed that DENV replication complexes form ER invaginations, capsid protein is recruited to these sites to form nucleocapsid, followed by budding from the ER lumen, where envelope and membrane proteins have been incorporated [22, 31]. Once progeny virions infect another cell, following release of

nucleocapsid into the cell cytoplasm, capsid protein is ubiquitinated and degraded releasing of viral RNA into the cell [17].

### *Membrane protein*

The second protein encoded in the DENV genome is the membrane protein. In a fully assembled virion, there are 180 copies each of the membrane and envelope proteins. How these proteins are layered and interact depends on the maturation state of the virion, with processing of the membrane protein playing an important role [32]. The membrane protein contains two major segments M and prM. Cleavage of prM from the membrane protein is mediated by furin protease during viral assembly through the trans-Golgi network. Due to the acidic nature of transit through the trans-Golgi network, it is believed that processing of prM is important in shielding E protein from premature irreversible conformational changes prior to egress from the cell [32-34]. A fully matured virion contains 90 homodimers of E protein creating a smooth surface on the virion, with the processed M protein remaining as a transmembrane glycoprotein within the virion's lipid bilayer and is considered an infectious particle [32]. Failure to process prM leads to heterodimer formation between membrane and envelope proteins, resulting in a "spiky" virion surface, with fully immature virions typically considered non-infectious outside of the context of antibody-dependent enhancement [32, 35, 36].

### *Envelope Protein*

The third structural protein is the envelope protein. E protein is a class II membrane fusion protein and is the primary glycoprotein responsible for virion attachment to cell surface receptors as well as fusion following internalization into the endosome [37, 38]. E protein contains three  $\beta$ -sheet rich domains: Domain I, II, and III. Domain I primarily functions in stabilizing E protein structure and aids in conformational changes following pH changes [39]. Domain II contains the highly conserved fusion loop region responsible for mediating fusion with endosomal membrane in response to the drop to acidic pH [34, 37-39]. E protein at neutral pH in a mature virion exists as a homodimer. When the pH is lowered, shifting of domain I and domain II of E protein initiates the formation of E protein fusion trimers, allowing the fusion loop to be inserted into the endosomal membrane, followed by successful entry of DENV genome into the cell [37]. Domain III is cited as the primary mediator of receptor binding with no specific cognate receptor interactions required to mediate this binding. Instead, DENV E protein interacts with a wide range of receptors including sulfated glycosaminoglycans, lectin receptors like DC-SIGN, laminin-binding proteins, and glycosphingolipids [16].

### *NS1*

The first of seven nonstructural proteins is NS1, which is typically found either intracellularly as a dimer or is secreted from the cell as a hexamer. Intracellular NS1 primarily functions in viral replication, where it has been shown to require species

specific interactions with NS4A [40, 41]. It is also postulated that dimeric NS1 functions in recruitment of cholesterol and triglycerides to the replication complex, as well as interaction with E and prM proteins within the ER lumen to aid in nucleocapsid envelopment [41-43]. Secreted NS1 functions in immune evasion, through suppression of complement [44] or acting as a decoy for antibody binding and prevent antibody-mediated function on DENV-infected cells [45].

### *NS2A and NS2B*

The next set of encoded nonstructural proteins are NS2A and NS2B. Each are membrane proteins with transmembrane domains and have roles in viral replication and assembly and are part of the replication complex (consisting of NS2A, NS2B, NS4A, NS4B) [46]. NS2A can recruit genomic RNA through binding to 3' UTR sequences. NS2A is also important in virion assembly through interaction with prM and E to aid in RNA packaging to the nucleocapsid [47]. NS2B's primary role is regulating NS3 protease activity, where it acts as a cofactor to stabilize NS3's protease domain and recruit NS3 to the ER membrane [48, 49]. NS2B also plays a role in immune evasion through targeted degradation of cGAS to avoid detection of mitochondrial DNA during viral infection [50].

### *NS3*

NS3 is the primary helicase and protease encoded in the DENV genome. NS3 is responsible for the proteolytic cleavage of the DENV polyprotein at NS2A/NS2B,

NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions [51]. NS3 also contains ATPase and helicase that are necessary for DENV replication [52]. NS3 is also thought to aid in regulation of cell metabolism through inhibition of GAPDH glycolytic activity [53].

#### *NS4A and NS4B*

NS4A and NS4B are the next encoded proteins. Each have their own functions post polyprotein cleavage and also exist as an intermediary separated by a 2K sequence, which is used as a signaling peptide for NS4B translocation to the ER lumen. The intermediary protein, NS4A-2K-NS4B, has been shown with other flaviviruses like West Nile Virus and Kunjin Virus to regulate cytoplasmic membrane organization and Golgi trafficking [54, 55]. Post-processing, NS4A functions in viral replication, immune modulation, and cell survival. NS4A is a necessary component of the DENV replication complex, responsible for anchoring the replication complex to the ER membrane through cholesterol recognition motifs [56]. NS4A has also been shown to modulate interferon (IFN) responses. In ZIKV infection, NS4A represses retinoic acid-inducible-gene I (RIG-I)-like receptors (RLRs), specifically through competitive binding to the CARD domain of MAVS to reduce downstream signaling via MAVS [57]. While in DENV infections, NS4A and NS4B have been linked to reduction in activation of ISRE-54 promoter, with NS4B antagonizing STAT1 phosphorylation [58]. NS4A has also been shown to modulate autophagy in a PI3K-dependent fashion to protect from cell death during viral replication [59]. NS4B function is primarily reliant on interactions with other

DENV proteins. It is a necessary component for viral replication through interactions with NS4A [60]. And its interactions with NS3 enhance helicase activity to unwind dsRNA [61].

## *NS5*

NS5 is the largest of the DENV proteins. The major function of NS5 is as an RNA-dependent RNA polymerase, which is necessary for DENV replication, where it complexes with NS3 in the ER [62]. NS5 also functions as an RNA methyltransferase, capping RNA during DENV polyprotein translation to prevent recognition of viral RNA from RNA sensors such as RIG-I [63, 64]. Following replication, NS5 dissociates from NS4 to translocate to the nucleus where its function still needs to be characterized further and has been associated with increased IL-8 production [65]. In addition to immune evasion from RNA-sensors, NS5 inhibits induction of the infected cell's IFN responses through binding and subsequently degradation of STAT2 [66].

## **Dengue outcomes and pathogenesis**

### *Clinical Manifestations*

Dengue disease manifests in a variety of clinical outcomes ranging from: asymptomatic or clinically inapparent illness experience by up to 75% of individuals, mild or uncomplicated infection, to severe disease manifesting in severe hemorrhage and

shock [28, 29]. Symptoms typically begin to manifest around the same time viremia occurs, roughly 5 days post infection [29]. The most common mild manifestation of dengue is fever, which lasts from 3-7 days. Accompanied by fever are symptoms including: retroorbital headache, arthralgia, malaise, chills, nausea, and a non-painful rash that does not itch [1]. Most acute mild symptoms resolve alongside fever. Severe disease has two classifications under WHO definitions, dengue with warning signs (DWS) and severe dengue. DWS is characterized by abdominal pain/tenderness, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy/restlessness, liver enlargement, and increased hematocrit paired with a rapid decline in platelet count. These symptoms indicate the need for increased risk and monitoring needed with possible progression to severe disease. The major symptomatology of severe dengue manifests as severe plasma leakage, shock, respiratory distress, severe bleeding, and severe organ involvement including AST/ALT over 1000 [67]. Part of what makes clinical management of dengue difficult is due to the delayed timing of severe symptomatology. These symptoms typically occur after fever defervescence and viremia resolution, a period of time typically thought to be when symptoms are resolving. This symptom manifestation post resolution of infection indicates that the host immunologic response may play a significant role in dengue pathology, with the increase in vascular leakage/endothelial cell permeability attributed at least in part to the generation of a cytokine storm of proinflammatory cytokines [68, 69]. There are many different proposed contributors and mechanisms involved in dengue pathophysiology. Factors

such as infecting serotype, influences of specific DENV proteins, the immune response to infection, and host genetics have all been implicated in progression to severe outcomes.

#### *Clinical Manifestations – Differences in viral genetics*

DENV is classified under four different genetically and antigenically distinct serotypes, each sharing roughly 65-70% sequence homology [70]. Broadly, it is important to understand how different serotypes, and even genotypes and specific strains, manifests. Serotype and genotype differences are complicated to study due to the geographic differences in circulating serotypes across the world and even within countries. This becomes further muddled when trying to account for variables such as force of infection, existing immunity, and the order of heterologous reinfections. In a study in Singapore conducted between 2005 and 2011, infection differences between each serotype were observed. DENV-4 numbers were excluded due to low number of infections. This study found that DENV-1 infections were more likely to progress to severe disease, developed red eyes, higher viremia, and were more likely to develop symptoms during primary infection. While DENV-2 was more likely to develop joint pain and low platelet count. While not significant, the study observed the lowest lymphocyte counts in DENV-3 cases [71]. In 2022, a small study was performed in the Western Maharashtra region of India looking at confirmed DENV infection in 118 individuals looking at infection primary with DENV-1 and DENV-2, though there were a small number of DENV-3 infections. Consistent with the Singapore study, they observed higher

rates of joint pain and lower platelet counts, but did not observe any significant increases in severity or outcome based on serotype [72]. In Nicaragua, a 17-year cohort study was conducted between 2004 and 2022, where they observed infections with all four serotypes; however, had low rates of DENV-4 infection only present in patients with inapparent infections. This study found that DENV-3 primary infections typically were more likely to be symptomatic and severe compared to DENV-1 or DENV-2 [73]. From 2005-2010 a study was conducted across Peru, Bolivia, Ecuador, and Paraguay and observed all four serotypes but with minimal DENV-2 infections and focused primarily on non-hemorrhagic clinical manifestations and did not evaluate differences in disease severity. This study found that DENV-3 patients experienced more headaches, malaise, and musculoskeletal and gastrointestinal manifestations. While DENV-4 patients experience more cutaneous and respiratory manifestations [74].

Even within serotypes there are differences in virulence and pathogenesis and has been a focus of the field for years. A 1997 phylogenetic analysis looking at DENV-2 transmission in the Americas posited that introduction of a Southeast Asian genotype of DENV-2 displaced the native, American genotype potentially leading to the appearance of dengue hemorrhagic fever in the Americas [7, 75]. A study in 2003 showed that replacement of the 5' and 3' UTRs paired with a single envelope mutation across genotypes of DENV-2 led to changes in the replicative fitness and viral output within human cells compared to mosquito cells [7, 76]. More recently, a study wanted to understand what factors led to the observance within the South Pacific where the island of Tonga saw few mild cases of dengue while the rest of the region experienced major

dengue outbreaks [77]. Epidemiological and phylogenetic analysis showed that virus derived from the Tonga region segregated to a different clade compared to other DENV-2 virus circulating in the South Pacific. They found that a single substitution within the prM protein was able to attenuate virus translation within mammalian cells, but not insect cells. These substitutions potentially allowed for silent transmission of DENV-2 within the region, providing protection from the more virulent strain circulating in the South Pacific [77].

These studies highlight the global heterogeneity of symptomatology and pathogenesis across serotypes, emphasizing the need to understand study demographics, circulating serotypes, and timing when comparing results between studies.

#### *Pathogenesis – Viral*

Dengue virus nonstructural proteins play an important role in viral replication and immune evasion, while also contributing significantly to dengue pathogenesis. NS1 has been shown to have both direct and indirect pathogenic effects during infection. Secreted NS1 can be detected in serum around day 1 of fever onset and persists past fever defervescence typically until around 9-12 days post fever onset and can be found in high concentrations, typically 500ng/mL-2ug/mL [28, 78, 79]. NS1 induces endothelial hyperpermeability through direct binding to endothelial cells, triggering cathepsin L mediated release of heparan, leading to disrupting the endothelial cell glycocalyx [80].

NS1 has also been shown to interact with TLR4 on PBMC, triggering activation and release of proinflammatory cytokines like TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8, and chemokines that disrupted endothelial cell monolayer integrity and induced vascular leakage in mice when administered alone [81, 82]. NS2A have been shown to impact cytopathic effects in vitro, though the mechanism is still unknown [83]. NS2A and NS2B have been shown to exhibit viroporin activity to alter membrane permeability and has been associated with mitochondrial damage through cleavage of mitochondrial fusion proteins MFN1 and MFN2 as part of the NS2B-NS3 protease complex [84-86]. DENV E protein has also been implicated in pathogenesis, with recombinant domain III capable of inducing highly inflammatory NETosis through NLRP3 inflammasome induction [87]. Another study used recombinant domain III to prime mice later challenged with platelet auto-antibodies, an attempt to mimic the response observed in cases of severe dengue, and found that this sequential challenge triggered hemorrhage and endothelial cell death via apoptosis in mice [88].

### *Pathogenesis – Host*

In addition to the direct and indirect effects of viral protein on dengue pathogenesis, immunity generated against the invading pathogen has been shown to significantly contribute to damage during infection. Host-mediated damage is especially important to discern in dengue infection due to severe disease manifesting after signs of acute viral infection like viremia and fever have resolved [70]. Within dengue disease,

immune cells and the effector molecules they secrete have been implicated in pathogenesis of disease. It has been proposed that during secondary infection, preferential activation of lower avidity DENV cross-reactive CD4 T cells may lead to an aberrant inflammatory profile, causing an increase in the secretion of proinflammatory cytokines such as TNF- $\alpha$ , IL-2, IL-6, and IFN- $\gamma$ , leading to cytokine storm observed during severe disease [69, 89-91]. While it has been shown that there is an increase in the CD8 and CD4 T cell responses during severe secondary illness, more work needs to be done to elucidate how cytokine secretion from T cells contribute to pathogenesis due to the complexity and contributions of other innate and adaptive immune cells [89, 92].

Both membrane-bound and secreted NS1 have been shown to activate complement, likely contributing to vascular leakage during severe disease and is further enhanced by the presence of anti-NS1 antibodies [93]. Antibodies directed towards NS1 have also been shown to be cross-reactive against human epitopes like endothelial cells and platelets. Cross-reactive NS1 antibodies toward endothelial cells has been suggested to induce endothelial cell apoptosis in a caspase-dependent manner [94]. In mice, platelet auto-antibodies generated in response to NS1 treatment have been shown to cause aberrant platelet activation and induce thrombocytopenia [95].

Antibody dependent enhancement (ADE) is one of the most commonly cited explanations for increased pathogenesis during secondary illness. This process will be explained more in depth in later chapters; however, this phenomenon is the observance that IgG antibodies generated during a previous DENV infection fail to neutralize the virus, opsonize it, and allow for increased uptake and infection by Fc $\gamma$  receptor (Fc $\gamma$ R)

bearing cells [3, 96, 97]. The mechanism behind this increased pathogenesis remains to be described, though some posit this process leads to generation of more virus and subsequently this increases damage done by infection [98]. There are conflicting studies on the impact of viremia titers on disease progression, with some observing a correlation between viral burden and disease outcome [99], while others find no significant correlation or even lower levels of viremia in those with severe disease compared to mild illness [100, 101]. Alternatively, it is proposed that this differential route of infection leads to an intrinsic difference in receptor-mediated infection vs FcγR-mediated infection, leading to a change in the cytokine response to infection [102]. Recently, it has been shown that there is a qualitative difference in the transcriptional profile between receptor-mediated infection and ADE within monocyte-derived macrophages, regardless of viral burden, showing changes in metabolism and interferon responses [103]. But more remains to be elucidated on this phenomenon and the mechanisms and impacts surrounding it.

Host genetic factors also play a role in susceptibility to DENV infection as well as risk of developing severe disease. An important genetic contribution to susceptibility are the Human Leukocyte Antigen (HLA) alleles. Studies comparing clinical outcomes with frequency of HLA class-I and class-II alleles have shown patterns such as B\*52 being associated with progression to dengue fever in secondary infections with DENV-2, or A\*24 being associated with severe primary disease [104, 105]. Meanwhile, polymorphisms with TNF- $\alpha$  and IL-10 genes have been associated with an increased susceptibility to severe secondary DENV-2 infection [106]. Polymorphisms within human

platelet antigens (HPAs), specifically HPA-1a and HPA-2b, have been associated with increased pathogenesis through increased generation of auto-antibodies to HPA antigens may mediate this increased risk [107, 108]. There have also been studies looking at the role of host miRNAs and their ability impact DENV replication. For example, upregulated miR-146a and downregulated miR-155 have been shown to promote viral replication through dampening the IFN- $\beta$  response [109, 110].

Outside of immunity and genetics, other host factors also play a role in host susceptibility to DENV infection. In areas with a high force of infection, a significant portion of the at risk population has been observed to be children, which has been attributed to ADE [73, 111, 112]. In areas that have experienced effective vector control measures paired with vaccination like Singapore, where the force of infection has decreased but is still present, the risk of hospitalization is shown to be increased in individuals over the age of 45 where previously this group of adults showed higher rates of inapparent DENV infection [113, 114]. Other studies have shown the while elderly individuals experience fewer classical dengue symptoms, they shown increased incidence of severe dengue [115]. Sex differences have also been observed with DENV infection both for risk and pathogenesis. Depending on study region, more DENV infections tend to be observed in young males over the age of 15 or between the ages of 19-35 [116, 117]. While these studies shown increased incidence in men, they have shown differences in disease manifestation, with men experiencing more acute liver injury while women more severe hematological changes [117]. Patient comorbidities also contribute significantly to disease pathogenesis. Diabetes has been shown to have

four times greater risk of mortality as well as increased risk of developing severe disease. Other comorbidities such as obesity, kidney disease, and pulmonary disorders [115, 118, 119].

Understanding the mediators of dengue pathogenesis is a complex topic. With many different factors like viral and host contributions, it can be difficult to pinpoint what is the cause of specific symptoms and outcomes. And of these observations, it needs to be parsed out what can be globally applied to DENV infection versus what is specific to the infecting serotype or population impacted by an outbreak at that time. Understanding this will help in the development of future countermeasures and effective treatments of dengue disease.

### **Predictors of Disease Severity**

One of the challenges faced by researchers and clinicians during DENV infection is being able to predict what risk factors predispose patients to severe disease and what factors offer protection. As mentioned previously, it is not until after the start of fever defervescence that a patient begins to exhibit the symptoms associated with severe disease, requiring close monitoring of patients for signs of shock and hemorrhage [28, 120]. Many studies have been conducted analyzing different risk factors of severe dengue, two meta-analyses in 2021 pooled together existing meta data to understand which clinical predictors were the most significant. Both analyses found the risk of progressing to severe disease was increased in children, secondary infections, and

individuals with pre-existing diabetes or renal disease [112, 121]. In addition, both studies reported elevated clinical signs that have been described under the 2009 WHO classification of dengue warning signs [112, 121, 122]. Even with these understandings, there is still much to consider on an individual basis who is at risk because in endemic regions children and individuals experiencing secondary infection are still significant portions of the populations. There is still a need to be able to predict within those groups who will progress to severe disease. Even understanding the risk factors, the risk of developing severe disease is estimated to be 1 in 20, leaving a large burden on hospitals just in patient monitoring during the critical phase of infection.

While not all cases of severe disease are secondary infection, depending on the region there is a two-to-three-fold increase in risk of developing severe infection when an individual has experienced a prior infection [123-125]. This increased risk during secondary infection is widely regarded as some form of immune-mediated enhancement of disease. The leading explanation for this enhancement is the theory of antibody-dependent enhancement. Specifically, that if neutralizing antibodies wane to a point they become sub-neutralizing and instead opsonize the virion to enhance infection of FcγR bearing cells. Many studies have looked at neutralizing antibody titers to evaluate protection or risk of disease progression. While these studies show correlation of antibody titer with protection, it is difficult to determine precise titers necessary to provide protection or increased risk, nor how durable that protection is [126, 127]. Though at an overall population level, protective antibody titers on average appear cross-serotype protective for symptomatic disease for roughly 1.5-2 years after infection

and cross-serotype protect against severe disease for roughly 2.5 years. The timing for this window varies depending on DENV endemicity and overall force of infection for the region [128]. Emphasizing that antibody titer alone is insufficient in disease prediction, especially once confounding factors such as: serotype and strain infection history within the region, cell type used for neutralization, virus strain and maturation state, and antibody source (IE serum vs plasma) [128, 129]. These factors require further study into harmonizing methods as well as understanding other correlates of risk.

Outside of antibodies, it has been proposed to look at other potential immune correlates of protection. Memory B cells and long-lived plasma cells are potentially more direct quantifications related to antibody production, specifically for looking at pre-exposure qualifiers of risk for secondary infection; however, the circulating pool of these cells does not account for the breadth of the B cell memory pool as many of these reside within lymph nodes and other respective niches [128]. T cells may be a perspective pool to analyze, as mentioned earlier the hyper reactive low affinity CD4+ T cell responses have been associated with severe disease, CD4+ T cells have also shown to have a protective role [130]. Additionally, CD8+ T cells have shown to have a protective role in symptom outcome in school-aged children; however, analysis of epitope-specific subsets have been inconsistent [131].

Overall, the field has been trending towards finding answers and consensus on what factors could be used to help quantify risk or protection; however, as it stands these are still largely undefined due to the difficulty of generalizing observations across populations and cohorts.

## **Dengue Countermeasures**

The goal with any pathogen of public health relevance is to develop countermeasures to either provide sterile protection against the pathogen or protection against symptomatic illness. For dengue virus, a multi-pronged approach is necessary to combat its growing presence requiring vector control, generation of population immunity, and antivirals for prevention or treatment of disease.

### *Vector Control*

Until relatively recently, the only countermeasures available to the field were vector control based: chemical treatments like pesticide and larvicide to control the mosquito population, biological controls like Wolbachia infection of mosquitos to alter vector fitness to support DENV replication, and aggressive vector surveillance paired with community engagement to reduce mosquito breeding areas [132]. Vector control alone is insufficient in preventing the spread of dengue virus due to many factors such as the rise in pesticide-resistance in mosquitos, the cost and maintenance of control strategies like continual release of Wolbachia-infected mosquitoes and keeping public buy-in for home surveillance of potential mosquito breeding grounds [111, 132, 133].

## Vaccines

The biggest challenge to developing an effective vaccine to DENV is the associated risk of severe disease with secondary heterologous infection. With endemic serotypes of dengue constantly shifting and overtaking one another, taking development of a single serotype vaccine off the table. Instead, the field has focused on the generation of tetravalent, live attenuated vaccines due to their ability to induce multicellular responses to both structural and nonstructural proteins [134]. It wasn't until late 2015 that the first dengue vaccine, Dengvaxia, was first licensed. This vaccine was built on the backbone of a successful flavivirus vaccine targeted towards Yellow Fever Virus (YFV). Each component used the non-structural and capsid protein from the YFV vaccine, and swapped in the prM and E segments of each DENV serotype. Following national rollout in the Philippines and Brazil, safety signals were discovered in children who were

seronegative (no prior dengue history) before vaccination. There was an increased in Dengue-related hospitalizations observed in children three years after vaccination [135].

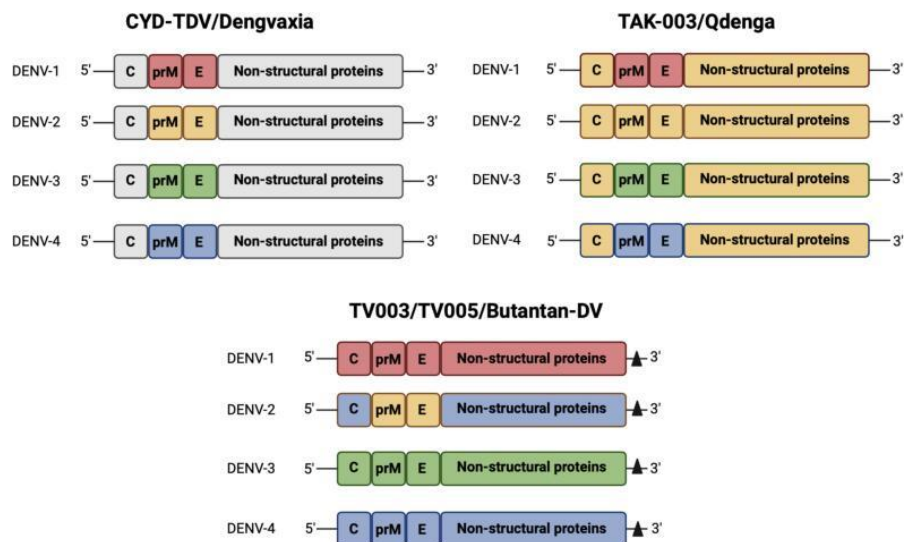


Figure 1.2. Design of licensed Dengue Virus vaccines. Dengvaxia with Yellow Fever backbone and DENV-1-4 prM and E proteins. Qdenga with a DENV-2 backbone and DENV-1, -3, and -4 prM and E. Butantan-DV with a full length, serotype specific composition except DENV-2 composed of a DENV-4 backbone with DENV-2 prM and E. From [134]

Analysis also showed a biasing of the response towards the DENV-4 arm of the vaccine [136]. Some postulate that the poor efficacy may be tied to poor dengue-specific CD8+ responses, likely due to the chimeric yellow fever non-structural protein backbone [137]. Currently, the vaccine is only recommended for seropositive recipients in dengue endemic regions.

There are currently two other dengue vaccines approved for use outside of the US, Qdenga and Butantan-DV. The components of the Qdenga vaccine are similar to Dengvaxia, with the exception that the YFV backbone was replaced with the backbone of an attenuated clinical strain of DENV-2. While this vaccine has not seen increased safety signals in seronegative children, there has been an observed decrease in vaccine efficacy 3 years after vaccination, with DENV-2 efficacy remaining the highest and a particular decrease in efficacy against DENV-3 in seronegative individuals [138]. A third vaccine, Butantan-DV, has recently completed Phase 3 trials and has been approved for use in Brazil with a planned rollout in 2026 and is recommended for people between the ages of 12-59. In an effort to balance the in-host serotype replication and immune generation, this tetravalent vaccine is the closest to being composed of the full-length DENV genome for each serotype in individual components, except the DENV-2 component is composed of a DENV-4 nonstructural and C backbone [139]. After an average of 3-7 years of follow-up, the trial was only able to report efficacy for DENV-1 and DENV-2 due to insufficient data for DENV-3 and DENV-4. The total three-year efficacy reported regardless of serotype and age was 67%, with a bias toward DENV-1 when stratifying by serotype to 75% and 60% for DENV-2. Once age was taken into

consideration, DENV-1 efficacy remained high at 78% and DENV-2 efficacy drops to 40%. While the study showed overall efficacy data by serostatus for the total study population, they did not share efficacy data by serostatus within their age group classifications.

### *Antivirals*

At this time there are no licensed antivirals approved to treat or prevent dengue infections. In large part, this is due to the fact that there are no animal models that completely mimic dengue symptoms or clinical manifestations [140]. Even without a perfect animal model, there are many studies looking at various host and virus targeted antivirals. There are 3 drugs currently being evaluated in clinical trials for potential antiviral effects: NS3/NS4B antagonist JNJ-64281802, Melatonin, and neuraminidase inhibitor Zanamivir [141].

JNJ-64281802, also known as Masnodenvir, is described as an oral pan-DENV small-molecule inhibitor. JNJ-64281802 works to prevent the interaction between NS3 and NS4A, which as mentioned before NS4A acts to enhance NS3 helicase activity, which is essential for DENV replication. In the initial Phase 2a trial results, the study reports that the drug showed dose-dependent antiviral activity against an attenuated DENV-3 challenge in human participants, preventing viremia as well as generation of symptoms and antibody responses to the virus if taken before administration of DENV [142]. The Study suggests potential use as a prophylaxis for non-dengue experienced individuals

traveling to DENV endemic regions. The other drug in beginning its Phase II clinical trial is Melatonin, a naturally secreted hormone known for its ability to regulate circadian rhythm and as an anti-inflammatory treatment [143]. In vitro, Melatonin has been shown to inhibit DENV infection through the activation of SIRT1-mediated interferon pathways [144]. While there is currently no efficacy data in humans for its ability to inhibit DENV, there have been other studies on its effect as an antiviral in the treatment of host-mediated inflammatory responses by acting as an immunoregulator and antioxidant [145]. At this time Zanamivir, also known as Relenza, is a neuraminidase inhibitor that is currently used in the treatment and prevention of Influenza A and B viruses [146]. A clinical trial to evaluate its effects on DENV infection is currently recruiting for Phase II studies in humans. In mice, this drug has been shown to protect mice from NS1 mediated vascular leakage through inhibition of sialidase [147].

## **Conclusions**

In this chapter, I have introduced general background dengue virus emergence, replication, and functions of its encoded proteins. I have discussed the clinical manifestation of dengue disease, our current understanding of viral and host mediators of dengue pathogenesis, and predictors of disease outcome. Finally, I have discussed the current status of dengue countermeasures, including vector control, vaccines, and antivirals currently under clinical study. In the next chapter, I will describe dengue cellular tropism, provide a more in-depth explanation on our current understanding of

antibody-dependent enhancement, along with the current mechanistic idiosyncrasies between the ADE model and what has been observed in clinical observations, specifically the observation on B cell infection. I will then introduce a novel mechanism of viral entry, called BCR-dependent enhancement (BDE), that may synergize with our understanding of ADE and help explain some of the mechanistic gaps under the ADE theory. I will then provide a more mechanistic evaluation of BDE within chapters 3 and 4 of this thesis.

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## **CHAPTER 2**

### **INTRODUCTION PART II: DENV CELLULAR TROPISM AND B CELL INFECTION**

## **Chapter Introduction**

In the first chapter of this dissertation, I provided a broad overview of dengue virus infection: virus replication cycle, viral proteins and their potential contribution to disease, and risk factors associated with disease outcome. In this section, I will discuss the known entry mechanisms described for dengue (including both receptor mediated and antibody-dependent enhancement), the observed cellular tropism of DENV and how this aligns with the ADE model, and finally discuss DENV infection of B cells. The purpose of this section is to provide the necessary background for the data presented in later chapters of this dissertation describing the novel phenomenon of BCR-dependent enhancement of infection by DENV.

## **Receptor-mediated Entry**

A fully assembled, mature dengue virion is spherical, enveloped virus roughly 50nm in diameter. As mentioned previously, the outside surface of the virion is layered in 90 envelope protein homodimers. Because of this, the main interactions of DENV with host cells and their receptors are mediated via interactions with DENV E protein. DENV E protein is composed of three functional domains: domain I which functions in stabilization of the E protein and functions as a hinge region for domains II and III; domain II is the fusion loop domain responsible for mediating membrane fusion with host cell following endocytosis; and domain III which is likely responsible for receptor recognition to allow attachment to host cells [1, 2]. Even with this understanding,

specific cognate receptors for DENV have not been identified. However, many putative receptors have been described in mammalian cells [2, 3].

#### *Attachment Factors*

Glycosaminoglycans (GAGs) are described as attachment factors of DENV binding to host epithelial cells. In vitro studies utilizing epithelial cell lines such as Vero (African green monkey kidney cells) and BHK-21 (baby hamster kidney cells) have shown that DENV is able to bind to receptors like heparan sulfate that contain heavily sulfated regions via two lysine residues on E protein domain III [4]. Pre-treatment of virus with heparin or soluble heparan sulfate, or pre-treatment of cells with heparinase to remove surface expression of heparan sulfate led to decrease in DENV binding [5, 6]. Heparan sulfate containing GAGs are present on a wide variety of cells, but have only been shown as required attachment factors in epithelial cells.

#### *Mononuclear Phagocyte Infection*

The main receptors cited for DENV entry are the lectin receptors like DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), L-SIGN, and the Mannose Receptor [7, 8]. Studies have shown that the carbohydrate recognition domain of DC-SIGN is able to recognize and bind to mannose-rich N-glycan on DENV E protein. DC-SIGN is primarily expressed in myeloid-derived immune cells such as dendritic cells and macrophages, though can also be found on platelets. While its homologue L-SIGN (also

known as DC-SIGNR) is primarily expressed on liver and lymph node epithelial cells [2, 3]. DENV virions produced by infected DC-SIGN cells no longer contain the mannose-rich glycosylate on its E protein, instead they contain distinct N-linked complex glycans, impairing the virion's ability to bind DC-SIGN but still allowing for L-SIGN mediated entry [9]. The observations highlight that the source of virions can alter cell tropism.

Mannose receptor mediated infection has been shown in macrophages, mediated by receptor recognition of glycosylation present on DENV E protein. This effect can be inhibited through antibody blockade of the receptor or removal of the glycosylation on DENV E protein in macrophages [10]. Other receptors implicated in the infection of monocytes and macrophages are the LPS receptor CD14 or heat-shock proteins HSP70 and HSP90. Early studies showed that blocking of CD14 with LPS, but not CD14-blocking antibodies, prevented DENV infection of monocytes. Alternatively, it was proposed that LPS treatment of the cells removed essential HSP70 and HSP90, this study showed that HSP70 and HSP90 bound DENV and that treatment of cells with anti-HSP90 or treatment of virus with recombinant HSP90 was able to block infection and that this process was likely due to receptor complex formation within lipid rafts on the cell [11].

### *Liver and Epithelial Cell Infection*

Other entry receptors that have begun to be characterized in cells types outside of DC-SIGN and Monocytes are: GRP78, Laminin, TIM and TAM receptors, and claudin-1. GRP78 is an ER resident intracellular protein that can also be expressed on the surface of hepatocytes and has been shown to mediate infection by DENV-2, where GRP78 blocking antibodies can prevent infection [12]. Interestingly, a study showed that GFP78 blockade was unable to prevent DENV-1 infection in hepatocytes, where they found that DENV-1 infection of hepatocyte line HepG2 was mediated by the Laminin Receptor and that this route of entry appeared serotype-specific for DENV-1 [13]. In a study utilizing a range of epithelial cell lines, they found that expression of TIM and TAM transmembrane receptors, responsible for phosphatidylserine-dependent phagocytic engulfment and apoptotic cell removal, were capable of mediating DENV entry into these cells. This study found that DENV utilized these receptors and the apoptotic cell clearance pathway to infect target cells [14]. DENV E protein is not the only DENV protein associated with cell entry. Membrane protein, expressed on the surface of immature virions, has been shown to interact directly with claudin-1. This protein is integral to tight junction formation between epithelial cells [15, 16]. Studies suggest that this interaction may allow infection of immature virion into epithelial cells, where immature virions are typically thought to be non-infectious outside of the ADE phenomenon.

Overall, the consensus within the field is that DENV does not have a single cognate receptor. However, there are many variables to consider when trying to

understand DENV entry including the source of the DENV virions, DENV serotype, and cell type.

## **Antibody Dependent Enhancement**

### *ADE History*

Antibody-dependent enhancement (ADE) is the leading theory surrounding the observance of increased risk of severe disease during an individual's reinfection, typically referred to as secondary infection, by a different serotype of dengue virus compared to their first or primary infection. This theory first originated from a series of seminal studies surrounding outbreaks of hemorrhagic fever in Thailand in the 1960s. Halstead and colleagues observed that individuals with a pre-existing antibody response towards dengue were more likely to develop severe disease. The authors speculated that this risk may be due to a hypersensitivity reaction to DENV/antibody immune complexes, and that this was antibody mediated and not sensitized cells because this was also observed in infants who would not have been exposed to virus previously, but would have the presence of maternally-derived antibodies [17].

The group later went on and performed both in vivo and in vitro work to characterize this phenomenon. In non-human primates, they performed either subsequent challenges or passive immunization through the transfer of immune serum followed by challenge with DENV [18-20]. They found that viremia was higher in animals that experienced secondary challenges or received the DENV antibody transfer

compared to normal cord-blood [18, 19]. They also observed increased virus in the tissues of animals experiencing secondary infection compared to primary [20]. And that there was an increase in virus in tissue towards the end of the viremic period, aligning with the timing of the onset of shock observed in humans [20]. Further supporting that this enhancement of DENV production was related to existing antibody immunity against dengue virus.

The *in vitro* studies this group performed setup the baseline mechanism for the current understanding of ADE. Utilizing leukocytes sourced from rhesus macaques and human peripheral blood, they observed a concentration window at which immune sera would lead to increased viral replication [21]. They observed that this enhancing effect was only observed in serum that contained IgG, not IgM. And through generation of F(ab)<sub>2</sub> fragments of anti-DENV IgG, they observed Fc interactions were necessary to mediate this effect [21]. In a follow up paper, they decided to identify what populations of peripheral blood leukocytes were infected via ADE and found that mononuclear phagocytes were the principal cells infected via ADE [22]. Leading to the modern understanding of ADE, where sub-neutralizing concentrations of anti-DENV IgG antibodies can opsonize dengue virions and lead to increased uptake and subsequent infection of Fc receptor (FcR) bearing cells, namely dendritic cells, monocytes, and macrophages. Many studies have utilized these methods and observations to recapitulate ADE *in vitro* to characterize this phenomenon. These observations have led to the theory that there are both extrinsic and intrinsic factors of ADE that influence the observed increase in virus production and pathology during secondary illness.

### *ADE Epitopes*

Much work has gone into understanding what viral epitopes lead to the ADE enhancing effect. The initial observations by Halstead and colleagues highlighted that any DENV immune serum at some window of concentration will see enhancing effects [21], implying that there is a delicate balance between neutralizing effect and antibody concentration to elicit enhancement. The majority of antibodies elicited to DENV are against different epitopes within DENV E and prM proteins [23, 24]. Anti-DENV antibodies are grouped into two major classifications, serotype-specific and cross-reactive. Serotype-specific antibodies tend to be disregarded when studying ADE due to the low risk of developing severe disease upon homotypic reinfection. Whereas cross-reactive antibodies have variable neutralization capacity and can bind to two or more serotypes of dengue. Domain III antibodies are typically serotype specific and highly neutralizing, likely due to domain III's role in attachment to other cellular receptors [24]. Within the field, domain II antibodies are typically regarded as cross-reactive due to high conservation within this region across serotypes, but are poorly neutralizing, likely due to shielding of this region in mature virions until conformational changes made at low pH [24]. Domain II and prM antibodies are the most commonly used targets when studying ADE effect, especially in immature virions, which are normally non-infectious unless under ADE conditions.

### *Extrinsic ADE*

Extrinsic ADE explains that some of this increased viral production during disease is due to the increased population of cells susceptible to DENV infection. In primary dengue, the only cell populations that would be susceptible to infection would be cells that express the receptors mentioned previously like DC-SIGN, L-SIGN, etc... With the introduction of anti-DENV antibodies at sub-neutralizing levels, the pool of cells susceptible to infection would expand to those that express an Fc gamma receptor (Fcγ). Subsequently, it is theorized that the FcγR interactions with DENV/IgG immune complexes experience higher affinity interactions, resulting in increased viral uptake by these cells on a cell-to-cell basis [25, 26]. There is little dispute in the contributions of extrinsic ADE in diversifying the pool of susceptible cells to infection in vitro. However, it is difficult to parse if the increase in virus production observed in culture is due to higher viral burden from increased entry or if engagement of the FcγR leads to internal cell signaling, altering the fitness of the cell to support viral infection, a phenomenon termed intrinsic ADE.

### *Intrinsic ADE*

Intrinsic ADE theorizes that the differential interactions during entry via the FcR lead to altered signaling and change in cellular responses that lead to a more permissive cellular environment to promote DENV replication, increasing the amount of virus released by the cell [26-28]. The first in vitro observation of this was in Ross River virus (RRV) infections. Studies showed that ADE infection macrophages observed similar initial infection, followed by increased viral production in ADE infected conditions compared to virus alone. In concurrence with this observation, there was an observed suppression of LPS-stimulated antiviral response through decrease in TNF- $\alpha$ , IFN- $\beta$ , IP-10, and IRF-1 [29, 30]. To align with this in DENV infection, it has been observed that ADE infections of cells appear to lead to a decrease in IFN- $\gamma$  production compared to virus alone conditions [31]. And other studies have shown an altered inflammatory profile in ADE infected cells [32, 33]. These observations have been made within bulk cultures, with little discrimination between infected cells and uninfected “bystander” cells within the same culture. Recently, a study performed single-cell RNA sequencing on primary human macrophages infected on virus only and ADE conditions, then looked for the presence of RNA within cells to identify infected vs uninfected populations of cells [26]. This study found that cells infected under ADE conditions showed a transcriptional profile highlighting decreased immune signaling, IFN responses, and mitochondrial dysfunction. They observed that the decreased IFN responses did not correlate with intracellular viral RNA levels, suggesting that the presence of virus alone, but differential signaling likely through entry route may have played a role in this suppressed IFN response [26]. This

combination of functional analysis within bulk cultures and single-cell transcriptomic analysis within identified ADE infected cells highlights how typical receptor-mediated entry differs compared to entry via ADE. Intrinsic ADE requires much more characterization in order to understand the mechanism and precise impacts.

### **DENV Tropism**

With this understanding on the two major interactions responsible for DENV entry into cells, it is important to understand what has been shown in vitro, in vivo, and ex vivo as cellular targets of DENV infection. As the first site of exposure, many types of skin-resident cells have shown susceptibility to infection. Ex vivo infection of human skin explants show that Langerhans cells, dermal dendritic cells, and macrophages are likely early targets of infection [34, 35]. Some studies have also implicated Mast cells as targets for infection via ADE [36-38]. Further studies show that while Mast cells are susceptible to some degree, intracellular detection of viral RNA through RIG-I suggests they are likely resistant to direct infection [39]. Additionally, within the skin, keratinocytes have been shown to be susceptible to infection. In an ex vivo infection study utilizing abdominal and breast tissue, keratinocytes composed around 60% of NS3+ cells, indicating active viral replication. This leads to the recruitment of susceptible myeloid cells such as dendritic cells and macrophages [40]. Once infection is acquired in the skin, DENV has been shown to be lymphotropic, where infection of skin immune cells traffic virus to

secondary lymphoid organs, allowing exposure of virus to monocytes, macrophages, dendritic cells, NK cells, B cells, and T cells.

A study in Malaya, Malaysia, utilized an array of tissue samples and peripheral blood clot samples were analyzed for the detection of DENV through immunohistochemistry (IHC) and in situ hybridization (ISH) [41]. The IHC work utilized hyperimmune mouse ascitic fluid to detect the presence of DENV antigens. In these samples the group was able to detect antigen positive cells across all tissue sources: liver, spleen, kidney, lung, bone marrow, and bone clot. Within tissue, infection has been observed in a range of cell types including: Kupffer cells, endothelial cells, monocytes, macrophages, and lymphocytes [41]. However, the detection of viral antigen utilizing hyper immune serum alone is insufficient to assume infection as this could indicate the presence of bound or internalized virus. To detect the presence of DENV RNA, the study utilized both sense and anti-sense DENV RNA probes for their in-situ hybridization tests. This method was able to detect RNA positive monocytes, macrophages, and lymphocytes within the spleen and blood clots [41]. Another study wanted to look at detection of NS3 within samples collected from autopsy to get a better idea on what cells showed evidence of viral replication. They found NS3 present in phagocytes in the lymph node and spleen, macrophages in the lungs, hepatocytes in the liver, and endothelial cells in the spleen [42].

Infection within monocytes, macrophages, and dendritic cells has been widely studied and is the basis for many assays performed within the field today. Infection of these primary immune cells was first characterized by Halstead and colleagues, where

they observed mononuclear phagocytes isolated from whole blood were permissive to infection [22]. Primary Monocytes have been shown to be difficult to infect directly unless under high MOI conditions or provided stimulatory cytokines, skewing towards macrophage differentiation [43, 44]. Primary monocytes have been shown to be susceptible through antibody dependent enhancement [45].

Most infected cell studies during acute infection are made utilizing circulating peripheral blood mononuclear cells (PBMC). One study performed flow analysis on PBMC and utilized a combination of prM and NS3 to identify infected cell populations. They observed that activated monocytes appeared to be the dominant population of infected cells and identified around 20% of CD14 negative cells showed evidence of infection and speculated this may be a combination of B cells, NK cells, and dendritic cells, though did not perform the staining to confirm [46]. Conversely, another study sorted T cells, monocytes, NK cells, and B cells from patients experiencing acute DENV infection and then cultured the isolated cells alongside known susceptible cells and found that B cells harbored higher levels of infectious virus, with little infectious virus in the other subpopulations. This study also confirmed through IHC distribution of virus both on the surface and internalized by the cells [47].

While much work has been done in vitro to characterize what population of cells are susceptible to infection, it is important for context to understand what has been observed in vivo in humans during acute infection. It is difficult to get a full picture of the infected cell types during infection due to feasibility of collection and timing restraints. Much of what has been observed are through analysis of circulating PBMC or samples

collected post recovery or post-mortem [48]. While there are some in vivo studies in mice and non-human primates, neither animal models are capable of reproducing disease symptomatology and pathogenesis as seen in human, so the observations may not be directly translatable to humans.

### **B cell infection**

Within the model of ADE, we would anticipate the observation that FcγR bearing cells like monocytes, macrophages, and dendritic cells would be the primary populations of cells that are infected during acute illness. Over the years, there have been multiple studies analyzing infected PBMC that have found a significant burden of viral infection within the B cell population. This section will discuss the evidence surrounding B cell infection and the field's current understanding of B cell susceptibility and permissivity to DENV infection.

DENV infection of B cells has been a topic of discussion for almost 30 years. In 1999, as mentioned previously, King et. al. observed that sorted B cells derived from infected patient PBMC appeared to harbor a significant portion of infectious replicating virus [47]. Another study utilizing samples collected from patients in the Philippines within 5 days of fever onset, utilized flow cytometry to analyze circulating PBMC. They were able to detect DENV E protein in 20-80% of the B cell population [49]. A study in 2012 performed RT-qPCR analysis on PBMC sourced from children in Thailand. They observed detectable RNA within B cells, T cells, and monocyte populations. Specifically,

they found that B cells contained significantly higher levels of DENV RNA levels/cell compared to T cells or Monocytes. They also found significantly elevated intracellular DENV RNA levels during secondary infection, though detected minimal negative sense RNA within their peripheral blood samples [50].

With the advent of single-cell RNA sequencing, multiple studies have analyzed PBMC populations for the presence of DENV RNA. These studies show that, at least in circulation, B cells harbor a significant burden of viral RNA, both positive and negative sense [51-53]. In the study from Zanini and colleagues, patients were not included if they were already showing signs of severe dengue, though not excluded if they subsequently progressed to severe disease. After screening, the study was able to analyze PBMC samples from 6 patients with confirmed dengue infections. This study included dengue-specific primers to enrich for detection of DENV RNA, and found roughly 45% of the circulating B cells were positive with viral RNA in the two subjects that progressed to severe disease. There are caveats with this finding as a hallmark of dengue symptomatology is leukopenia, which will lead to greatly altered cell population proportionality. In Sanborn et. al. PBMC was sampled from an individual experiencing a natural secondary DENV-1 infection [51]. The purpose of the study was to compare including the use of DENV-specific primer vs oligo(dT) 5' capture primer and results were verified utilizing sourced PBMC. During this testing, this study found that both naïve and memory B cell populations showed evidence of DENV infection during multiple days of acute infection [51]. In Ghita et. al. PBMC samples were analyzed from a Colombian cohort of patients [52]. This study used a combination of single-cell RNAseq

quantification of negative sense RNA, flow cytometry detection of DENV E protein, and co-culture with known susceptible cells to confirm DENV infection within the cell populations. They found that B cells showed a greater abundance of viral RNA. As well as were able to make transcriptional observations with these infected B cells and found downregulation of IFN responses, and upregulated transcription for CD69 and CXCR4, which denote antigen internalization and signal homing to germinal centers [52]. These results highlight that B cells may likely be the primary circulating reservoir of infected cells.

With the observations that B cells are infected, a longstanding question within the field is what is the mechanism behind DENV entry within these cells. The majority of B cells do not express the typical receptor utilized for direct infection like DC-SIGN or the mannose receptor. And while they do express an FcγR, known as FcγRIIb, it is an inhibitory form of the receptor that has been shown to be incapable of mediated ADE [54, 55]. Over engagement of FcγRIIb in B cells has been shown to lead to B cell anergy and even death, typically used in the regulation of auto-reactive B cells [56-58]. Despite these restrictions and limited studies, B cells have been shown to be susceptible and permissive to viral infection. Correa and colleagues provided were able to directly infect B cells in vitro with live DENV2. They confirmed infection through detection of DENV E and NS1 via flow cytometry, as well as RT-qPCR. They found that while susceptible, they appeared poorly permissive to DENV infection compared monocytes infected in vitro. As well as found that infection with DENV2 was sufficient to stimulate B cell activation through CD81 [59]. Upasani and colleagues utilized a Cambodian cohort to assess B cell

infection and identify a potential entry receptor [54]. Utilizing flow cytometry and RT-qPCR, they confirmed DENV infection of B cells through detection of NS3. Through direct infection, they found that B cells were susceptible to infection, and that ADE conditions could not alter their susceptibility [54]. Additionally, they found that infected B cells produced infectious virus, albeit to a lesser extent compared to infected monocytes. Finally, they identified CD300a as a potential entry receptor through using CD300a blocking antibodies, but only saw a reduction in infection and not complete abrogation, indicating the likelihood of alternative routes of infection [54].

These findings at the surface might conflict with the ADE model of infection that would expect FcγR bearing cells to bear the viral burden during acute infection. Understanding B cell infection, while separate from ADE, may help in consolidating our current understanding of DENV tropism and the model proposed by ADE.

### **BCR Dependent Enhancement**

Currently, it is known that during acute dengue illness B cells become infected, but the mechanism of entry and consequence of infection is understudied. The data presented in later chapters will provide evidence for a novel entry mechanism for DENV into specific subsets of the B cell population. We propose that B cell infection may be mediated in a mechanistically similar phenomenon to antibody-dependent enhancement. We hypothesize that expression of a surface-bound DENV-reactive antibody known as the B cell receptor (BCR) may be able to render a subset of the B cell

population, primarily the memory B cell pool, highly susceptible to infection in a manner reminiscent of the process that has been defined for ADE mediated entry. We have termed this process BCR-dependent enhancement (BDE).

### *BCR Structure and function*

While B cells lack expression of most/all canonical DENV entry receptors, they do express another unique receptor specialized in antigen recognition and phagocytic uptake: the B cell receptor (BCR). B cells recognize their cognate antigens via BCR which are generated in a process of germline recombination and somatic hypermutation, resulting in a nearly infinite diversity of receptor sequences and specificities. The interaction of a BCR expressed on the surface of a B cell triggers receptor-mediated endocytosis of the antigen complex bound to the receptor [60-63]. This process is strikingly similar to what occurs when an IgG-opsonized antigen interacts with Fc $\gamma$ R expressed on FcR expressing phagocytes [64-69].

The BCR is part of a receptor complex with expression of the encoded B cell's antibody containing a transmembrane domain to anchor it to the cell surface, alongside two signaling co-receptors: CD79a and CD79b (also referred to as Ig $\alpha$  and Ig $\beta$  respectively) [61]. These co-receptors contain an immunoreceptor tyrosine-based activation motif (ITAM) domain, responsible for initiating downstream signal necessary for receptor internalization as well as cell activation. These ITAM motifs become phosphorylated following antigen binding to the BCR, leading to BCR clustering [60].

Following antigen binding the engaged BCRs will become endocytosed, typically through recruitment of clathrin, though other endocytic processes have been described. Once internalized, the endosome containing the bound antigen is trafficked to highly acidic lysosomes to allow for processing of the antigen into peptides to allow for presentation on MHC class II [60, 61]. Concurrently, the ITAM domain on an engaged BCR leads to the recruitment of kinases such Src-family kinases Lyn and tyrosine kinase Syk. Leading to downstream activation and survival signaling pathways of the B cell through PLC- $\gamma$ 2, MAPK, and PI3K [70].

#### *Fc $\gamma$ R Structure and function*

Fc $\gamma$ R function in binding to the Fc portion of IgG antibodies. This group of receptors are expressed in three major classes: Fc $\gamma$ RI also known as CD64, Fc $\gamma$ RIIIa, b, or c also known as CD32, and Fc $\gamma$ RIIIa or b or CD16. With the exception of Fc $\gamma$ RIIb due to its inhibitory function, most Fc $\gamma$ Rs have been implicated in mediating ADE in vitro [71-73]. While structures vary slightly across each type of Fc $\gamma$ R, the overall structure of these receptors contain an extracellular domain used to recognize IgG antibodies with an intracellular cytoplasmic ITAM domain, except Fc $\gamma$ RIIb which contains the inhibitory ITIM domain [74]. Opsonization of an antigen with IgG antibodies allow for multiple points of interaction with Fc $\gamma$ Rs, leading to clustering of these receptors to allow for downstream signaling to occur through ITAM phosphorylation and clathrin recruitment [67]. Clathrin recruit allows for receptor/antigen internalization into the endosome, where it is then

trafficked to lysosome for MHCII processing [69]. Similar to BCR engagement, concurrently with receptor internalization, ITAM phosphorylation leads to the recruitment of Src family kinases and Syk. These lead to downstream signaling of activation and survival pathways such as PLC $\gamma$  and PI3K [74]. While in Fc $\gamma$ RIIb, phosphorylation of the ITIM domain leads to the recruitment of Lyn and Src homology 2 domain-containing protein tyrosine phosphatase (SHP-1), SHP-2, or SH2-domain-containing inositol polyphosphate 5' phosphatase (SHIP) which are capable of inhibitor PLC $\gamma$  and Bruton's Tyrosine Kinase (BTK) activity, suppressing their activating effects [74].

The similarities in structure and function to BCR and Fc $\gamma$ R engagement highlight an interesting potential route of infection for dengue virus. With what has already been characterized within the model of ADE, DENV is adept at escaping Fc gamma receptor internalization, with both BCR and FcR internalization within an acidified endosome which is a necessary part of the DENV lifecycle. Meanwhile, there are many similarities in the downstream signaling following BCR or FcR engagement through the same pathways. These pathways likely lead to similar metabolic responses that may align with what is hypothesized in the intrinsic ADE model in shaping the cellular environment to one with better fitness for DENV replication.

### *Evidence of BCR specific infection*

The concept of BCR mediated viral entry is fairly new to the field in general with the work presented in this dissertation being the first to describe this phenomenon in relation to DENV. There have been a limited number of studies beginning to look at preferential infection of cells that express an antigen specific BCR. In a 2014 study looking to understand the impacts of Flu virus interactions with Flu-specific B cells [75]. Dougan and colleagues generated a mouse model that produced B cells with BCRs specific for flu hemagglutinin (HA). Both in vitro and vivo they observed that exposure to flu virus led to preferential infection of Flu-specific B cells over other B cells and observed that this infection led to the killing of infected cells [75]. Another study in 2024 wanted to look at the impact of pseudotyping a lentivirus with modified sindbis virus envelope proteins to preferentially transduce desired cells types [76]. In their initial testing, they utilized immunocompromised mice and so, wanted to provide further testing in mice with functional T and B cell populations. They found that this pseudotyped lentivirus preferentially infected B cells specific to the pseudotyped sindbis virus envelope protein. They also replicated this with a more prototypical lentivirus pseudotype of VSV-G and observed similar effects. Additionally, when blocking BCR antigen interaction with anti-mouse Fab, they were able to reduce BCR-specific infection [76].

## Conclusion

In this chapter I have described the known routes of DENV through typical receptor mediated entry or antibody-dependent enhancement. I have discussed the history of antibody-dependent enhancement within the dengue field and described how factors both extrinsic and intrinsic impact cellular susceptibility to DENV infection. With the current understanding of DENV entry, I described what has been observed in regards to DENV cellular tropism and how in vivo observations during acute infection are inconsistent with what we would expect if the ADE model was the sole mediator of increase dengue risk. During the discussion of cellular tropism, I highlighted B cell infection and how infection of this population of cells does not align with our current understanding of dengue viral entry and proposed a novel method of entry: BCR-dependent enhancement. This theory is supported by the structural and functional similarities between antigen engagement with a BCR and antigen immune complex interaction with the FcγR. Finally, I provided examples of preferential infection of BCR-specific B cells by other viruses. In the upcoming chapters, I will provide data supporting the overall hypothesis of this dissertation. A subset of the memory B cell population is highly susceptible to infection mediated by DENV-reactive BCRs and that infection through this route relies on early BCR mediated signaling. In chapters 3 and 4, I provide evidence for the mechanistic and biophysical requirements of BCR-mediated infection in regard to DENV. In the final chapter I will discuss how this model may help explain some of the mechanistic gaps within ADE and potential future implication of BDE.

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## **CHAPTER 3**

# **B CELL RECEPTOR DEPENDENT ENHANCEMENT OF DENV INFECTION**

## **B cell receptor dependent enhancement of dengue virus infection**

Chad Gebo<sup>1</sup>, Céline S.C. Hardy<sup>1</sup>, Benjamin D. McElvany<sup>2</sup>, Nancy R. Graham<sup>2</sup>, Joseph Lu<sup>1,3</sup>, Shima Moradpour<sup>1</sup>, Jeffrey R Currier<sup>4</sup>, Heather Friberg<sup>4</sup>, Gregory D Gromowski<sup>4</sup>, Stephen J. Thomas<sup>1,3</sup>, Gary C. Chan<sup>1</sup>, Sean A. Diehl<sup>2</sup>, Adam T. Waickman<sup>1,3</sup>,

<sup>1</sup>Department of Microbiology and Immunology, State University of New York Upstate Medical University, Syracuse, NY 13210, USA.

<sup>2</sup>Department of Microbiology and Molecular Genetics, The University of Vermont Larner College of Medicine, Vaccine Testing Center, Burlington, Vermont, USA.

<sup>3</sup>Global Health Institute, State University of New York Upstate Medical University, Syracuse, NY 13210, USA.

<sup>4</sup>Viral Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, MD, 20910, USA

**Author contributions.** Conceptualization: C.G., J.R.C., H.F., G.D.G., A.T.W. Formal analysis: C.G., C.S.C.H., J.Q.L, A.T.W. Funding acquisition: S.J.T., A.T.W., S.A.D. Investigation: C.G., C.S.C.H., B.D.M., N.R.G., S.A.D., A.T.W., J.Q.L., S.M. Resources: S.J.T., G.C.C., A.T.W. Visualization: C.G., A.T.W. Writing – original draft: C.G. and A.T.W. Writing – review & editing: all authors

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## **ABSTRACT**

Dengue virus (DENV) is the causative agent of dengue, a mosquito-borne disease that represents a significant and growing public health burden around the world. A unique pathophysiological feature of dengue is immune-mediated enhancement, wherein preexisting immunity elicited by a primary infection can enhance the severity of a subsequent infection by a heterologous DENV serotype. A leading mechanistic explanation for this phenomenon is antibody dependent enhancement (ADE), where sub-neutralizing concentrations of DENV-specific IgG antibodies facilitate entry of DENV into FcγR expressing cells such as monocytes, macrophages, and dendritic cells. Accordingly, this model posits that phagocytic mononuclear cells are the primary reservoir of DENV. However, analysis of samples from individuals experiencing acute DENV infection reveals that B cells are the largest reservoir of infected circulating cells, representing a disconnect in our understanding of immune-mediated DENV tropism. In this study, we demonstrate that the expression of a DENV-specific B cell receptor (BCR) renders cells highly susceptible to DENV infection, with the infection-enhancing activity of the membrane-restricted BCR correlating with the ADE potential of the IgG version of the antibody. In addition, we observed that the frequency of DENV-infectible B cells increases in previously flavivirus-naïve volunteers after a primary DENV infection. These findings suggest that BCR-dependent infection of B cells is a novel mechanism immune-mediated enhancement of DENV-infection.

## INTRODUCTION

Dengue virus (DENV) is a prevalent arboviral pathogen that poses a significant global public health burden. Transmitted by the bite of infected *Aedes* family mosquitoes, DENV co-circulates as four genetically and antigenically distinct types: DENV-1 to -4. While the majority of DENV infections resolve without the need for medical intervention, dengue can quickly progress in some patients to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [1–3]. The risk factors associated with progressing to severe dengue are complex and incompletely understood. However, the risk of developing severe disease increases significantly in individuals experiencing secondary/heterologous infections [4,5].

The leading mechanistic explanation for this phenomenon is antibody-dependent enhancement (ADE). This model posits that DENV-specific IgG antibodies elicited by a prior heterotypic DENV infection can opsonize DENV without neutralizing infectivity, facilitating uptake by FcγR bearing phagocytes [6–9]. This mechanism is supported by strong epidemiological data and in vitro infection studies and has been a foundational model of dengue immunopathogenesis for over 40 years [10–12]. However, an underappreciated facet of the DENV lifecycle is that B cells are the most frequently infected cell type observed in circulation during acute DENV infection [13–17]. This observation has been substantiated by multiple groups using a wide array of assays including flow cytometry, qRT-PCR, mosquito-inoculation, and scRNAseq [13–17]. While B cells are known to be phagocytic [18,19] their antigen-specific receptors provide an additional, specialized means of internalizing extracellular antigens. Accordingly, we

hypothesize that binding of a DENV-specific BCR to DENV could facilitate virion uptake in a manner analogous to ADE.

In this study, we demonstrate that the expression of a DENV-specific B cell receptor renders cells highly susceptible to DENV infection. We posit that this process of BCR-dependent enhancement (BDE) of DENV infection represents an additional mechanism of immune-mediated enhancement which may fill some critical gaps in our current understanding of dengue immunopathogenesis.

## **MATERIALS AND METHODS**

***Cells and Cell lines.*** PBMC for flow cytometric analysis were obtained from a previously described phase 1, open-label dengue human challenge study [28]. This DENV-3 human challenge study and all associated analysis was approved by the State University of New York Upstate Medical University (SUNY-UMU) and the Department of Defense's Human Research Protection Office. 293T cell line were maintained DMEM media (Gibco, # 11965092) supplemented to 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. K562, and U937 DC-SIGN cell line RPMI. Raji cell line was purchased through ATCC and maintained in complete IMDM media (Gibco, # 12440053).

***Generation of Immortalized B cell lines.*** 7B9 cells were generated from a flavivirus-naïve volunteer who was vaccinated with DENV1 $\Delta$ 30 and challenged 270 days later with DENV2 $\Delta$ 30 Tonga (NCT02392325). B cells were isolated 90 days following DENV2 $\Delta$ 30 challenge. This study was approved by the Johns Hopkins University Institutional Review Board (IRB). 2F3 cells were obtained from a DENV-exposed volunteer (DT165) about 1 year following a secondary ZIKV infection (approved by University of Vermont IRB). Written informed consent was obtained from volunteers enrolled in both studies. Both 7B9 and 2F3 cells were generated by transduction of IgM-CD27+ memory B cells with a retrovirus encoding human B cell lymphoma (BCL)-6 and BCL-xL and enhanced green fluorescent protein (GFP) and culture on CD40L-expressing L cell fibroblasts and rhIL-21 (25 ng/mL, Peprotech) as described [23]. Surface IgG expression was performed on live B cells (near-IR fluorescent reactive dye, Invitrogen, cat no. L34975A), that were positive

for GFP and CD19-PerCP.Cy5.5 (Clone HIB-19, Biolegend, #302230) using APC anti-human IgG Fc (Biolegend, cat. no. 410712) on a Beckman CytoFlex flow cytometer.

**Viruses and RVPs.** DENV-1 (strain Wp74) dsRed Reporter virus (RVP) was prepared as previously described [20,21]. RVPs were titrated on U937 DC-SIGN cells, infectious units (IU) calculated based on the dilution of virus needed to infect 10–15% of cells as previously described [29]. DENV-4 (strain D4-1036) stock was prepared by propagating a low-passage inoculum in C6/36 cells. DENV-2 (strain Thailand/NGS-C/1944) stock was prepared by propagating a low-passage inoculum in Vero cells. RVP infection assays were performed using an approximate MOI of 0.1, with cells incubated with the RVP for 6hrs, followed by and an additional 18hrs of incubation prior to analysis by flow cytometry. Surface staining was performed in PBS + 2% FBS using the reagents and dilutions shown in **S5 Table**. Aqua Live/Dead (ThermoFisher, L34957) or ZombieUV (Biolegend, 423107) was used for live/dead cell discrimination. Data collection was performed on a BD LSRII or Fortessa flow cytometer and analyzed using FlowJo v10.2 (Becton Dickinson).

**K562 ADE assay.** ADE assays were performed using K562 cells as previously described [30,31]. In short, serial dilutions of monoclonal antibodies were incubated with the DENV-1 RVP (in sufficient amounts to infect 10%–15% of U937-DC-SIGN cells) at a 1:1 ratio for 1 h at 37°C. This mixture was then added to a 96-well plate containing  $5 \times 10^4$  K562 cells per well in duplicate. Cells were cultured for 18–20 hr overnight in a 37°C, 5% CO<sub>2</sub>, humidified incubator, followed by analysis by flow cytometry.

**DENV-specific ELISA and neutralization assays.** These assays were performed as previously described [32]. Briefly, virus was captured by plate-adsorbed mouse cross-reactive anti-DENV envelope (E) protein monoclonal antibody 4G2. IgG-containing cell culture supernatant was then added and positive DENV binding was detected by alkaline phosphatase-conjugated goat anti-human IgG (Fc) antibody (Millipore Sigma) and p-nitrophenyl phosphate substrate (Millipore Sigma). Reaction color change, indicating DENV-binding, was measured by spectrophotometry as OD<sub>405</sub>. Serum neutralizing antibody titers against DENV1-4 and ZIKV were determined by plaque reduction neutralization test (PRNT), using lowest serum dilution that yielded a 50% reduction in viral plaques (PRNT50) as previously described [33,34].

**Generation and expression of transmembrane antibody constructs.** Sequences for each monoclonal antibody were appended with an Ig transmembrane domain appropriate to the mAb isotype and cloned into pcDNA3.1(+) expression constructs. Transient expression of all tmIg constructs was performed in 293T cells using Lipofectamine (Invitrogen, L3000001). Cells were incubated for 18hrs before use in binding and infection assays. Antigen-specificity of the DENV-specific BCR was assessed by staining with biotin-labeled DENV-2 E protein pre-incubated with AF488-tagged streptavidin.

**hCMV infection.** hCMV strain TB40E-GFP [35] was diluted in complete RPMI media then incubated with transfected cells at an MOI of 1 at 37C for 6 hours. Cells were then washed before incubating at 37C for an additional 18 hours before quantification of infection by flow cytometry.

**Single cell RNA sequencing:** Samples were prepared for single-cell RNA sequencing according to the 10x Genomics RNA-seq protocol. Cells were resuspended at a concentration of 1400 cells/uL in PBS and loaded for a target of 6000–9000 cells per reaction. Cells were loaded for Gel emulsion bead (GEM) generation and barcoding. Construction of 5' gene expression libraries was performed using the Next GEM Single Cell 5' reagent kit, Library Construction Kit, and i7 Multiplex Kit (10x Genomics, CA). was used for reverse transcription, complementary DNA amplification and construction of gene expression libraries. The quality of gene expression libraries was assessed using an Agilent 4200 TapeStation with High Sensitivity D5000 ScreenTape Assay and Qubit Fluorometer (Thermo Fisher Scientific) according to the manufacturer's recommendations. Sequencing of 5' gene expression libraries was performed on an Illumina NextSeq 2000 (Illumina) using P3 reagent kits (100 cycles). Parameters for sequencing were set at 26 cycles for Read1, 10 cycles for Index1, and 90 cycles for Read2.

**5' gene expression analysis and visualization:** Gene expression alignment, sample demultiplexing, alignment, and barcode/UMI filtering was performed using the Cell Ranger software package (10x Genomics, CA) and bcl2fastq (Illumina, CA) using the commands mkfastq and count. The human reference genome (Ensembl GRCh38.93) was combined with the DENV2 genome as an additional chromosome (NC 001474.2). Sequenced transcripts were aligned to a human reference library created using the Cell Ranger mkref command, combined human and DENV2 reference genome, and custom Ensembl GRCh38 DENV2 GTF. Reads were mapped to both the positive and negative

sense DENV2 genome. Multi-sample integration, data normalization, dimensional reduction, visualization and differential gene expression were performed in R studio (v4.3.2) using R package Seurat (v4.4.0). The data set filtered to exclude genes expressed in fewer than 3 cells, and to contain cells with less than 22% mitochondrial RNA content and between 500–7,500 unique features. The resulting dataset was normalized and scaled using the Seurat functions `NormalizeData()`, `ScaleData()`, and `FindVariableFeatures()`. Data was normalized and scaled, and principal component analysis was performed using `RunPCA()`. Clustering was based on the first 10 principal components and a resolution parameter of 0.1 using `FindNeighbors()` and `FindClusters()`, respectively.

Cells labelled as infected were identified by the presence of both DENV (+) and (-) RNA with expression of both transcripts >0. Differentially expressed genes were identified by applying the `FindMarkers()` command and the Wilcoxon rank-sum test to the normalized gene expression dataset. A default minimum logFC value of 0.1 and min.pct of 0.01 were used. Genes with a corrected p-value by Bonferroni correction of <0.05 were considered significant. Ingenuity Pathway Analysis (IPA, Qiagen) was performed using genes with a corrected p-value <0.01 and log fold change <-0.25 or >0.25.

***DENV/Ig/BCR crosslinking infection.*** DENV/antibody immune complexes were generated by mixing DENV-4 ( $6.25 \times 10^7$  IU/mL) using an MOI of 62 with 1 $\mu$ g/mL of VDB33-IgM and anti-IgM in 96-well plate.  $5 \times 10^4$  Raji cells or primary B cells isolated from flavivirus-naïve healthy donors using MojoSort Human Pan B cell Isolation Kit (BioLegend, 480082) were then added and incubated for 24 hours. Supernatants were harvested at 24hrs for

cytokine analysis, while cells were washed and incubated an additional 24 hours before analysis. Cells were intracellularly stained with FITC-labelled DENV E-reactive monoclonal 4G2 antibody at 4µg/mL to quantify DENV infection.

***Statistical analysis.*** Statistical analyses were performed using GraphPad Prism 9 (La Jolla, CA) with a p-value < 0.05 considered significant.

## RESULTS

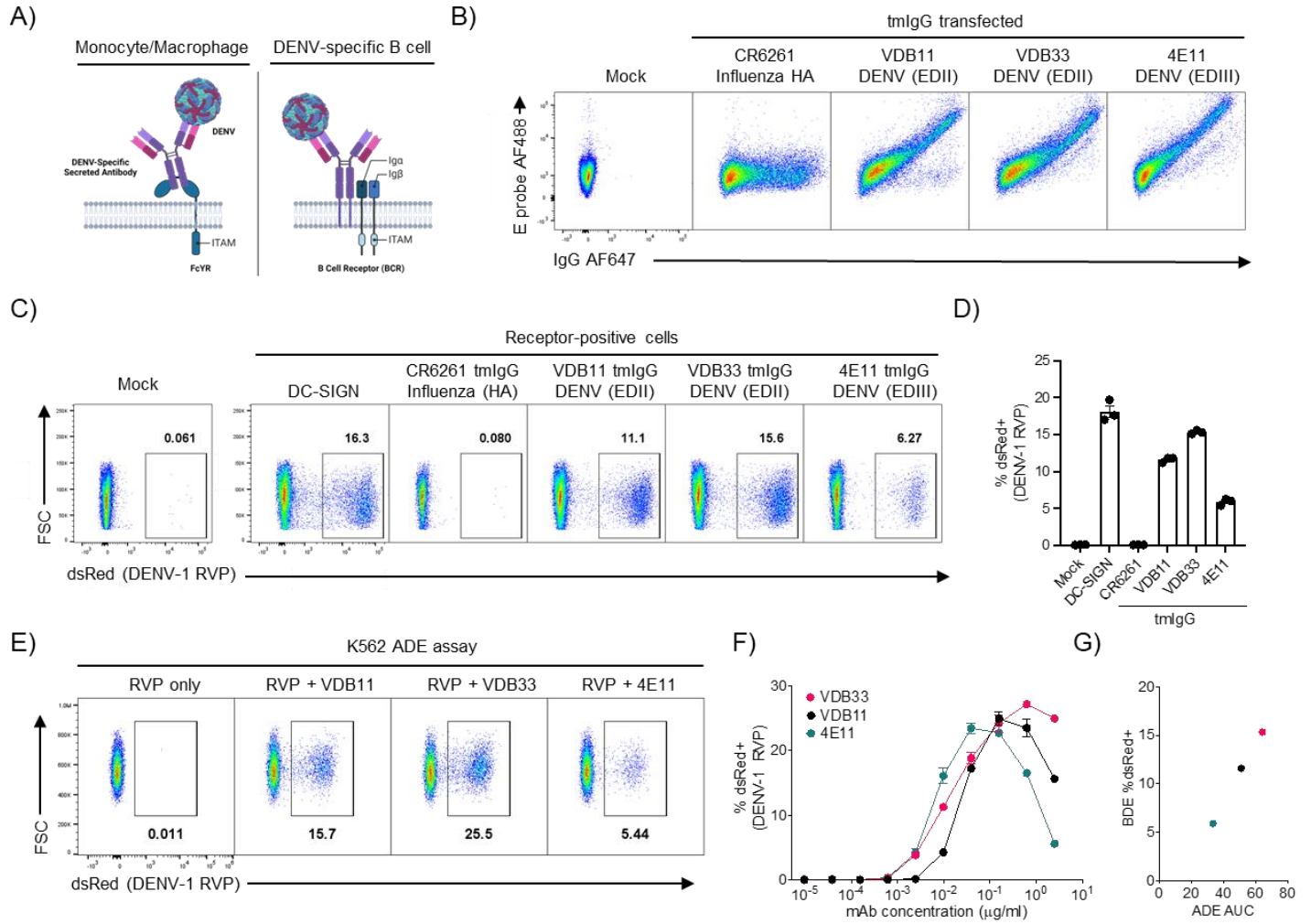
***Expression of a DENV-specific BCR renders cells susceptible to DENV infection.*** The motivation for this study was the appreciation that the structure of an FcγR receptor engaged with a DENV/IgG complex resembles that of a DENV-specific B cell receptor bound to DENV (**Fig 3.1A**). Accordingly, we hypothesized that the expression of a DENV envelope-specific BCR would render a cell susceptible to DENV infection, representing an alternative method of DENV-specific ADE. To test this hypothesis, we generated a panel of transmembrane Ig (tmIg) constructs encoding three well-characterized DENV-specific monoclonal antibodies as well as a control HA-specific tmIg antibody (**Fig 3.1B, S3.1 Fig**). Cells expressing DENV-specific tmIgG constructs retained their DENV-E protein binding activity, while no DENV-specific binding activity was observed for the HA-specific tmIg construct (**Fig 3.1B**).

To evaluate the contribution of DENV-specific tmIgG expression on cellular susceptibility to DENV infection, we exposed tmIgG expressing cells to a DENV-1 pseudotyped flavivirus reporter virus particle (RVP) encoding a dsRed fluorescent reporter [21,22], alongside cells transfected with the canonical DENV-entry receptor DC-SIGN (**Fig 3.1C and 3.1D**). A reporter virus platform was utilized in this analysis to allow for the accurate discrimination of true cellular infection from tmIgG-mediated binding of virions to the exterior of the target cell, as expression of the dsRed fluorescent reporter encoded by the RVP will only occur following intracellular translation of the RVP genome.

Expression of DENV-specific tmlgG receptors significantly increased susceptibility to infection by the DENV-1 RVP, to levels similar to cells expressing DC-SIGN (**Fig 3.1C and 3.1D**). The ability of DENV-specific tmlg constructs to facilitate infection was not limited to IgG isotype BCRs, as a similar pattern was observed in cells expressing tmlgM and tmlgA versions of DENV-specific antibodies (**S3.2 Fig**). Notably, the expression of a virus-specific tmlgG is not a universal mechanism for enhancing the susceptibility cells to viral infection, as the expression of HCMV-specific tmlgG constructs did not increase the susceptibility of cells to infection by HCMV. (**S3.3 Fig**).

While the expression of all DENV envelope-specific tmlgG constructs increased the susceptibility of cells to DENV infection, there was some heterogeneity observed between the constructs. To ascertain if this was due to the specificity of the antibodies for their cognate antigens, we compared the rate of BCR-dependent infection observed in our assay to the rate of ADE achieved with soluble IgG versions of the same mAbs. Strikingly, we observed the same rank-order of ADE and BDE activity of the antibodies when analyzed using a conventional K562 ADE assay (**Fig 3.1D-F**). Given that ADE and BDE were in the same rank-order (**Fig 3.1G**), these results suggest that many of the features of IgG isotype antibodies that are associated with ADE risk may be directly translatable to BDE risk.

**Figure 3.1:**



**Fig 3.1. Expression of DENV-specific BCRs renders cells susceptible to DENV-infection.**

**A)** Schematic representation of DENV/IgG immune complex binding to an FcγR (left), and a DENV virion binding to a DENV-specific BCR (right)<sup>30</sup>. **B)** Expression and DENV E protein binding activity of 293T cells transfected with the indicated tmlgG constructs. **C)** DENV-1 RVP infection of 293T cells transfected with the indicated tmlgG constructs. DC-SIGN and tmlgG conditions gated on receptor-positive cells. **D)** Quantification of DENV-1 RVP infection of 293T cells transfected with DC-SIGN or the indicated tmlgG constructs. Error bars +/- SEM. Experiments were conducted across three biological replicates in triplicate. **E)** ADE activity of the indicated DENV-specific IgG mAbs at a 1ug/ml concentration in K562 cells utilizing a DENV-1 RVP. **F)** Titration of ADE activity of the indicated DENV-specific IgG mAbs in K562 cells utilizing a DENV-1 RVP. **G)** Plot of the ADE and BDE results described above. Error bars +/- SEM.

***DENV-specific B cells are susceptible to DENV infection.*** To extend our results showing that transgene-expressed DENV-specific BCRs increase the susceptibility of a permissive cell line to DENV infection, we next examined this in human B cells with endogenous BCR expression. To this end, we leveraged a panel of human memory B cell lines derived from flavivirus-immune donors that were immortalized via genetic reprogramming with BCL-6/BCL-xL encoding retroviruses and maintained with the T-cell derived stimuli CD40L and IL-21 (**S3.4 Fig**) [22]. BCL6/BCL-xL-immortalized human memory B cells maintain surface Ig expression, ability to bind their cognate antigen, engage proximal BCR signaling, as well the ability to secrete soluble immunoglobulin [22]. Two cell lines were selected for this analysis: clone 7B9—which expresses an IgG isotype BCR that binds (but poorly neutralizes) DENV-1 to -4 and ZIKV—and clone 2F3 –which expresses a ZIKV-specific IgG isotype BCR but exhibits no DENV-binding activity (**Figs 3.2A-C and S3.4**).

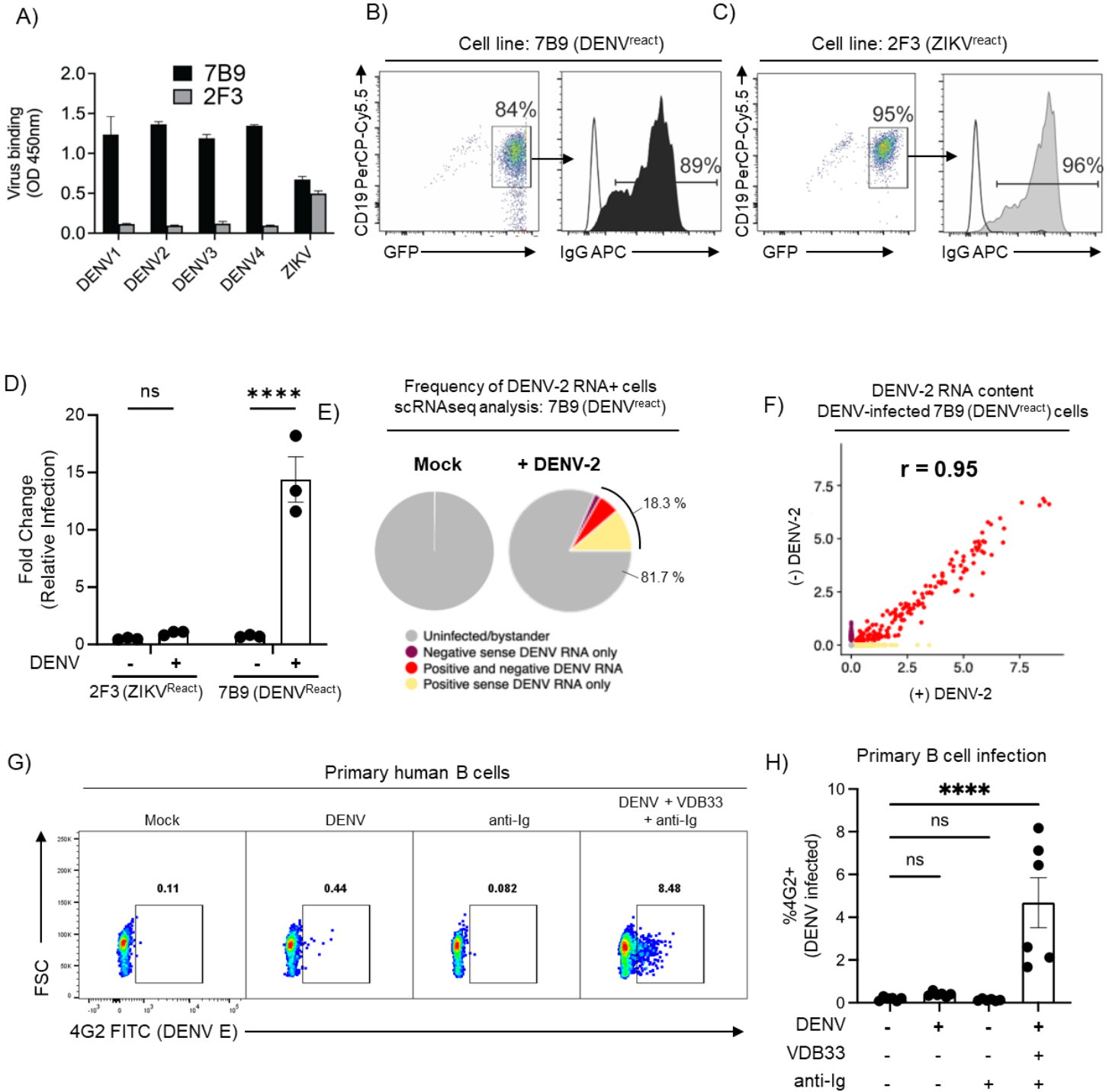
To determine the impact of BCR specificity on the susceptibility of these cells to DENV infection, both the 7B9 (DENV<sup>react</sup>) and 2F3 (ZIKV<sup>react</sup>) cell lines were exposed to live DENV-2 for 24 hours, followed by extensive washing and additional incubation for 24 hours to allow for viral replication. Cells were then fixed/permeabilized and analyzed by flow cytometry to quantify the abundance of DENV-infected cells within the culture (**S3.4 Fig**). Consistent with the results obtained with transgenic expression of DENV-specific BCRs, the DENV-specific 7B9 cell line exhibited a significant increase in the frequency of DENV-infected cells relative to the ZIKV-specific 2F3 line (**Fig 3.2D**). To better characterize the nature of DENV-infection with this B cell system, scRNAseq analysis was performed on both control and DENV-2 exposed 7B9 (DENV<sup>react</sup>) cells (**S3.5**

**Fig and S3.1–S3.4 Tables**). This analysis revealed a similar infection rate (defined as the presence of DENV-2 RNA) within the DENV-2 exposed culture as was observed by flow cytometric analysis of the same culture (**Fig 3.2E**). Furthermore, both positive- and negative-sense DENV-2 RNA was detected within the cells containing the most significant quantity of DENV-2 RNA, indicating active replication in DENV-specific B cells. (**Fig 3.2F**). Differential gene expression analysis between DENV-2 infected cells and uninfected cells demonstrated a transcriptional signature consistent with acute lymphocyte activation and cytokine production (**S3.5 Fig**).

To assess whether primary human B cells are susceptible to DENV infection, we developed a BCR-dependent DENV infection assay that bypasses the need for cells to express a DENV-specific BCR. In this experiment, DENV is bound to an enhancing IgM (VDB33-IgM) and these DENV/Ig immune complexes are crosslinked to the BCR of polyclonal B cells using an anti-IgM secondary antibody (**S3.6 Fig**) and infection is assessed by intracellular DENV antigen with the pan-DENV mouse mAb 4G2. We first tested this in Raji cells, which are poorly susceptible to DENV infection even at high MOI yet are highly permissive if transfected with a scavenging receptor such as DC-SIGN [23]. We observed minimal DENV infection in non-transfected Raji cells in all culture conditions except when all components of the DENV/Ig/BCR crosslinking cocktail were present (**S3.6 Fig**). To survey the infectibility of primary human B cells we exposed purified B cells from a flavivirus-naïve donor to the same infection conditions described above and assessed the frequency of B cell infection by flow cytometry. As was observed for the Raji cells, significant infection of the primary B cells was only observed in

conditions containing the full DENV/Ig/BCR crosslinking cocktail (**Figs 3.2G, 3.2H and S3.6**).

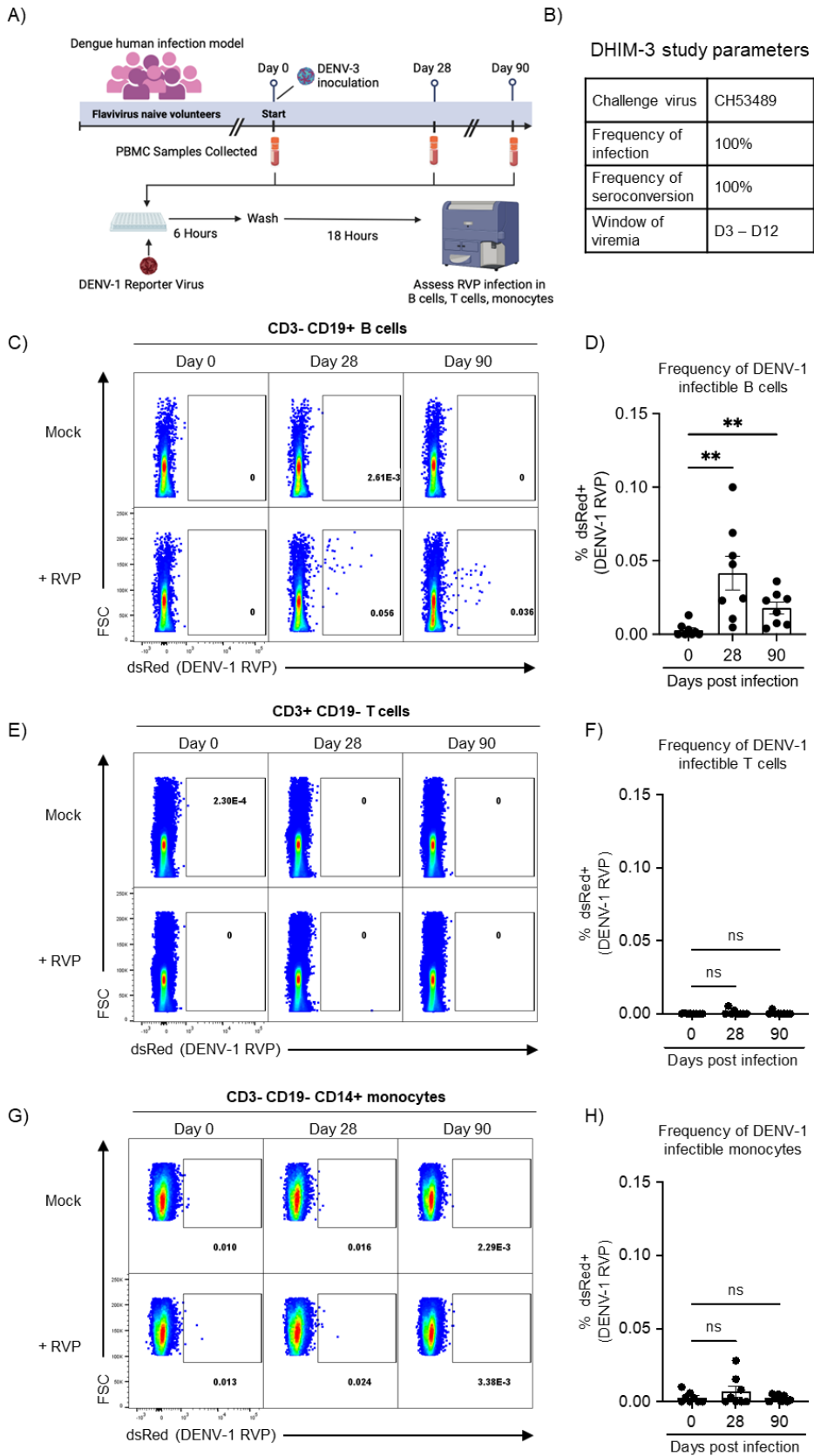
**Figure 3.2:**



**Fig 3.2. DENV-specific B cells are susceptible to *ex vivo* DENV infection.** **A)** Whole virus binding profiles of IgG antibody (1 µg) from cross-reactive 7B9 and ZIKV-specific 2F3 mAb to DENV1-4 and ZIKV by ELISA. **B)** Surface IgG surface expression of immortalized 7B9 and **C)** 2F3 B cells. **D)** Quantification of DENV-2 infection of immortalized 7B9 (DENV<sup>react</sup>) and 2F3 (ZIKV<sup>react</sup>) B cells at MOI of 10. Error bars +/- SEM. Experiments were conducted across two biological replicates in triplicate **E)** Pie charts of DENV-2 infected 7B9 B cell proportions in control and DENV-2 inoculated conditions as assessed by scRNAseq. **F)** Correlation plot of DENV (+) and DENV (-) sense RNA expression in DENV-2 exposed 7B9 B cells as assessed by scRNAseq. **G)** Representative flow cytometry plot showing the frequency of DENV-4 infected CD19+ B cells under the indicated culture conditions. Detection of DENV-infected cells was performed by staining fixed/permeabilized cells with a FITC-conjugated 4G2 antibody. **H)** Quantification of DENV-4 infected CD19+ B cells under the indicated culture conditions. Error bars +/- SEM. \*\*\*\* p < 0.001, One-way ANOVA with Dunnett's multiple comparisons test, with a single pooled variance. Experiments were conducted across three biological replicates in triplicate.

***Frequency of DENV-infectible B cells increases following primary DENV Infection.*** A key prediction of the BDE model described above is that the frequency of DENV-infectible B cells in a flavivirus naïve individual should be quite low, but that this frequency should increase following a primary DENV infection due to the accumulation of DENV-specific memory B cells. To address this hypothesis, we analyzed PBMC collected from a dengue human challenge study, wherein flavivirus naïve volunteers were exposed to the attenuated DENV-3 strain CH53489 (**Fig 3.3A and 3.3B**). We exposed PBMC collected on days 0, 28, and 90 post DENV-3 infection from 8 volunteers to a DENV-1 RVP, followed by overnight incubation and flow cytometric analysis (**Figs 3.3A and S3.7**). We observed a significant increase in the number of DENV-infectible B cells on days 28 and 90 post DENV-3 infection relative to pre-infection (**Fig 3.3C and 3.3D**). No such increase in DENV-infectability was observed in either the T cell (**Fig 3.3E and 3.3F**) or monocyte compartment (**Fig 3.3G and 3.3H**). These results suggest that the accumulation of DENV-specific immunologic memory can alter the susceptibility of an individual's B cells to DENV infection.

**Figure 3.3**



**Fig 3.3. Frequency of DENV-infectible B cells increases following primary DENV**

**Infection. A)** Schematic representation of the dengue human infection model and *in vitro* DENV-1 RVP infection assay<sup>33</sup>. **B)** Key DHIM study characteristic and performance parameters. **C)** Representative flow cytometry plots showing the frequency of DENV-1 RVP infectible viable B cells (CD3-CD19+) in PBMC samples obtained 0-, 28-, and 90-days post DENV-3 infection. **D)** Quantification of DENV-1 RVP infectible B cells from all subjects included in this analysis. **E)** Representative flow cytometry plots showing the frequency of DENV-1 RVP infectible viable T cells (CD3+CD19-) in PBMC samples obtained 0-, 28-, and 90-days post DENV-3 infection. **F)** Quantification of DENV-1 RVP infectible T cells from all subjects included in this analysis. **G)** Representative flow cytometry plots showing the frequency of DENV-1 RVP infectible viable monocytes (CD3-CD19-CD14+) in PBMC samples obtained 0-, 28-, and 90-days post DENV-3 infection. **H)** Quantification of DENV-1 RVP infectible monocytes from all subjects included in this analysis. Error bars +/- SEM. \*\*  $p < 0.01$ , paired one-way ANOVA with correction for multiple comparisons (Friedman test with Dunn's multiple comparisons test).

## DISCUSSION

In this study, we demonstrate that the expression of a DENV envelope-specific B cell receptor renders cells highly susceptible to DENV infection, and that the frequency of DENV-infectible B cells within a given individual can be modified based on DENV immune status. While an extensive body of literature supports the concept that B cells are a significant circulating reservoir of infectious virus during acute dengue [13–17]—and CD300a has been posited to be a B cell entry receptor for DENV [17]—we believe these are the first results to suggest that B cell susceptibility to DENV infection can be dynamically impacted by prior DENV infections.

Although numerous other viruses exhibit tropism for B cells, we posit that several fundamental features of DENV uniquely position it as amenable to BCR-mediated infection. First, DENV does not have—or require—a single cognate entry receptor. Rather, DENV can utilize a broad range of non-specific scavenging receptors or immunoreceptors to gain access to acidified endosomes of phagocytes, resulting in the viral/endosomal membrane fusion and release of genomic material. Indeed, this is exemplified by prior work which demonstrated that simply tethering DENV to the surface of permissive cells using bi-specific antibodies was sufficient to cause efficient infection, similar in magnitude to what can be achieved via ADE in cells expressing FcγRs [24]. These prior results suggest that the expression of a DENV-specific BCR may render a cell susceptible to DENV infection simply by tethering the virion to the surface of the cell, thereby facilitating viral entry during the process of normal membrane recycling without the need to engage full BCR signaling. However, it is also possible that that BCR-mediated

entry of DENV could activate the B cell via multivalent ligation of the BCR complex, thereby increasing protein production and increasing the efficiency of infection in a fashion analogous to what has been described as “intrinsic ADE” for FcγR-mediated entry. Additional work will be required to disentangle these two non-mutually exclusive processes.

An intriguing implication of this model of BCR-mediated entry of DENV is that it may offer some insight into the heterogeneity of risk associated with post-primary DENV exposures. Increased risk for severe disease following a secondary, heterotypic infection has been associated with a narrow range of low level preexisting DENV antibody titers— but only a small fraction of these individuals actually develop severe disease [5,25,26]. Given the extraordinary stability of memory B cells [27], one potential immunopathological mechanism contributing to severe dengue disease may be the relative abundance of DENV-specific antibodies and DENV virion-specific memory B cells circulating after a primary DENV infection. This may result in a situation where the population of highly-susceptible DENV-specific memory B cells are protected from infection with DENV until neutralizing antibody titers wane below a protective threshold. Accordingly, while the frequency of DENV-specific/DENV-infectible B cells identified in our analysis appears to be greatest 28 days after a primary DENV infection, the high neutralizing antibody titers present at this time point make it unlikely that a secondary infection event could occur. Additional analyses are required to determine if—and how—this mechanism may contribute to risk or protection from DENV infection.

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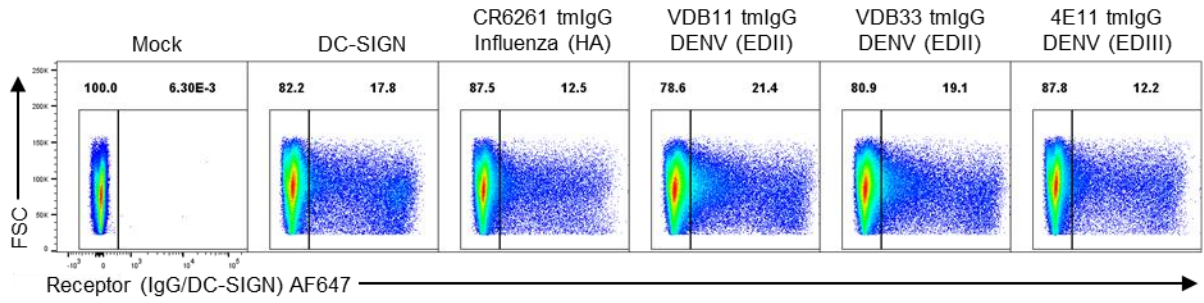
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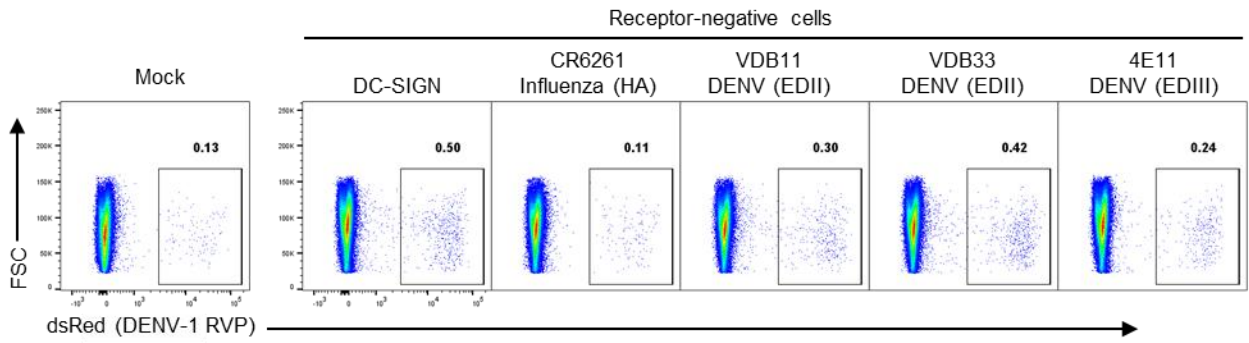
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### S3.1 Fig.

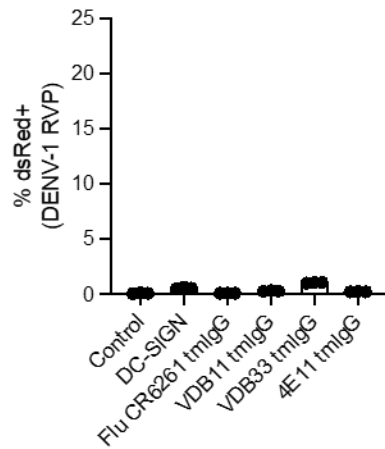
A)



B)

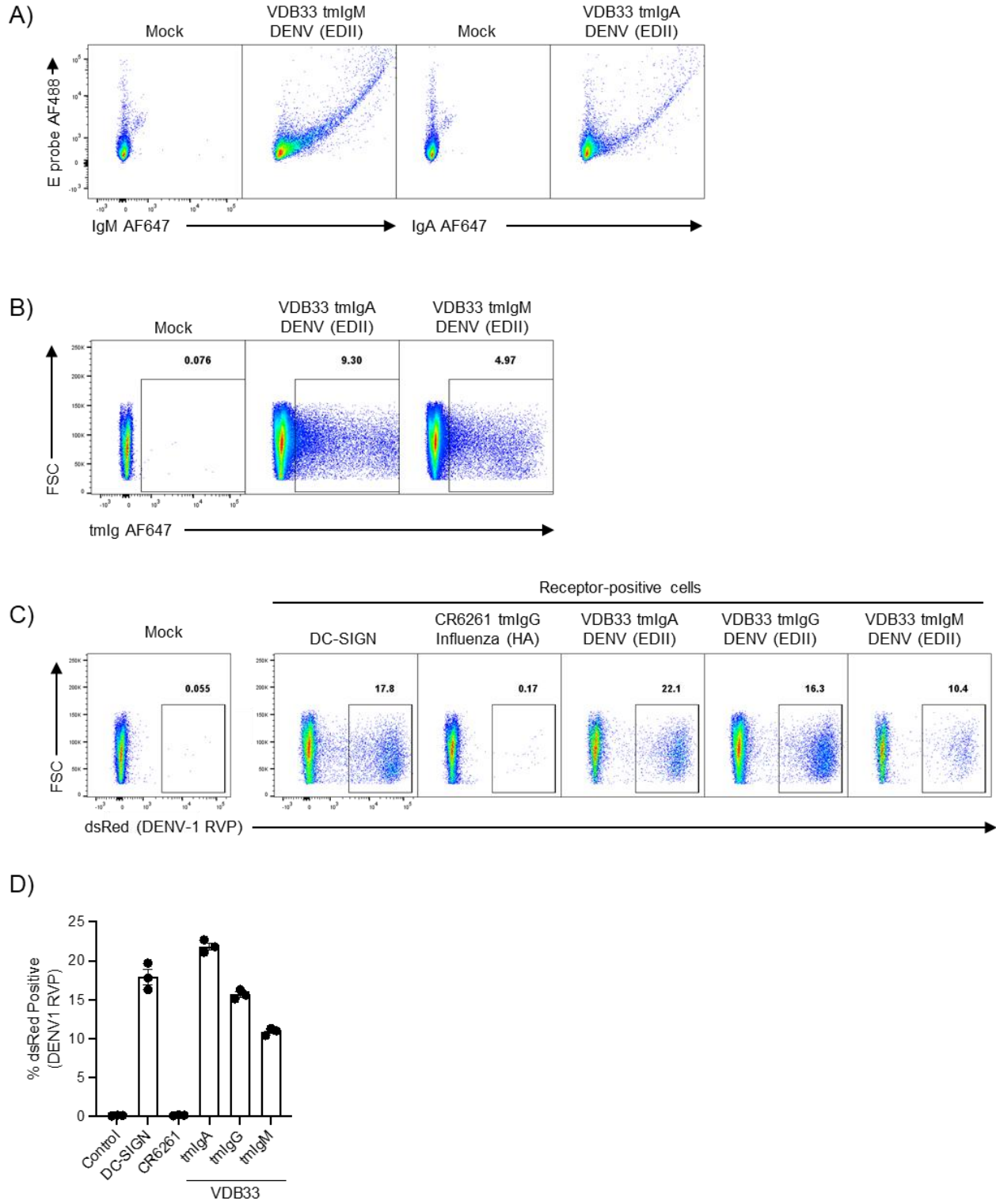


C)



**S3.1 Fig. Gating scheme and DENV-1 RVP infection of tmIgG transfected 293T cells. A)** Expression and gating of tmIgG and DC-SIGN in transfected 293T cells **B)** Representative flow cytometry plots showing the frequency of DENV-1 RVP infected cells within the receptor-negative gate of tmIgG and DC-SIGN in transfected 293T cells 24hrs after RVP exposure **C)** Quantification of DENV-1 RVP infected cells within the receptor-negative gate of tmIgG and DC-SIGN in transfected 293T cells 24hrs after RVP exposure

### S3.2 Fig

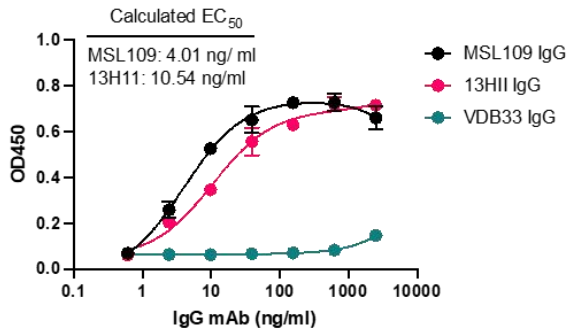


**S3.2 Fig. Gating scheme and DENV-1 RVP infection of tmIgM and tmIgA transfected 293T cells** **A)** Expression and DENV E protein binding activity of VDB33 tmIgM and tmIgA expression constructs. **B)** Expression and gating of tmIgM and tmIgA expression in transiently-transfected 293T cells **C)** Representative flow cytometry plots showing the frequency of DENV-1 RVP infected cells within the DC-SIGN, CR261 tmIgG, VDB33 tmIgA, VDB33 tmIgG, and VDB33 tmIgG positive 293T cells 24 hours after infection **D)** Quantification of DENV-1 RVP infected cells within the DC-SIGN, CR261 tmIgG, VDB33 tmIgA, VDB33 tmIgG, and VDB33 tmIgG positive 293T cells 24 hours after infection

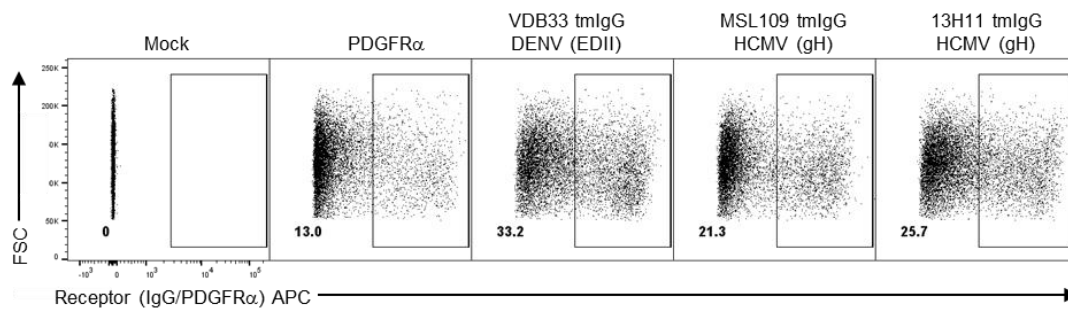
S3.3 Fig.

A)

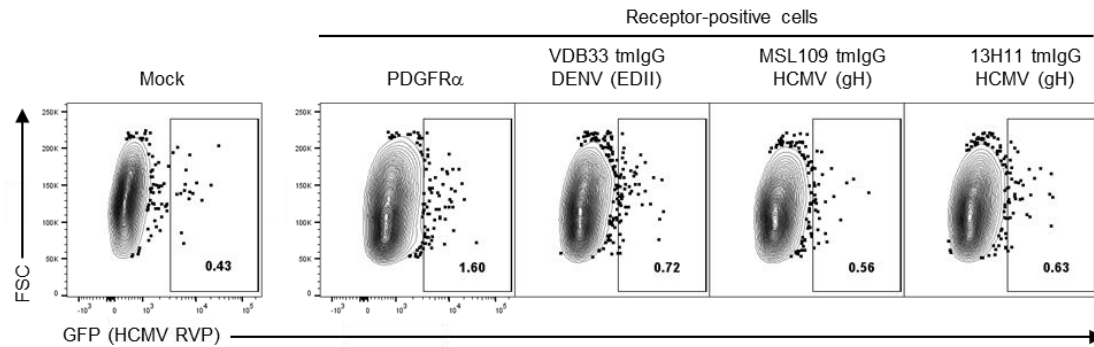
**Recombinant HCMV gH ELISA**



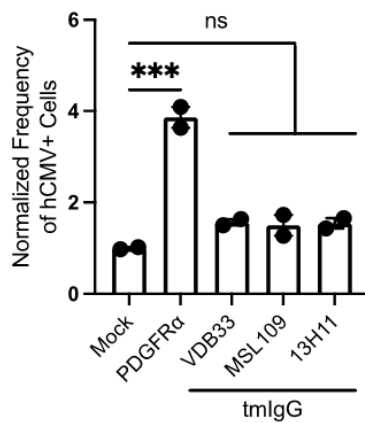
B)



C)

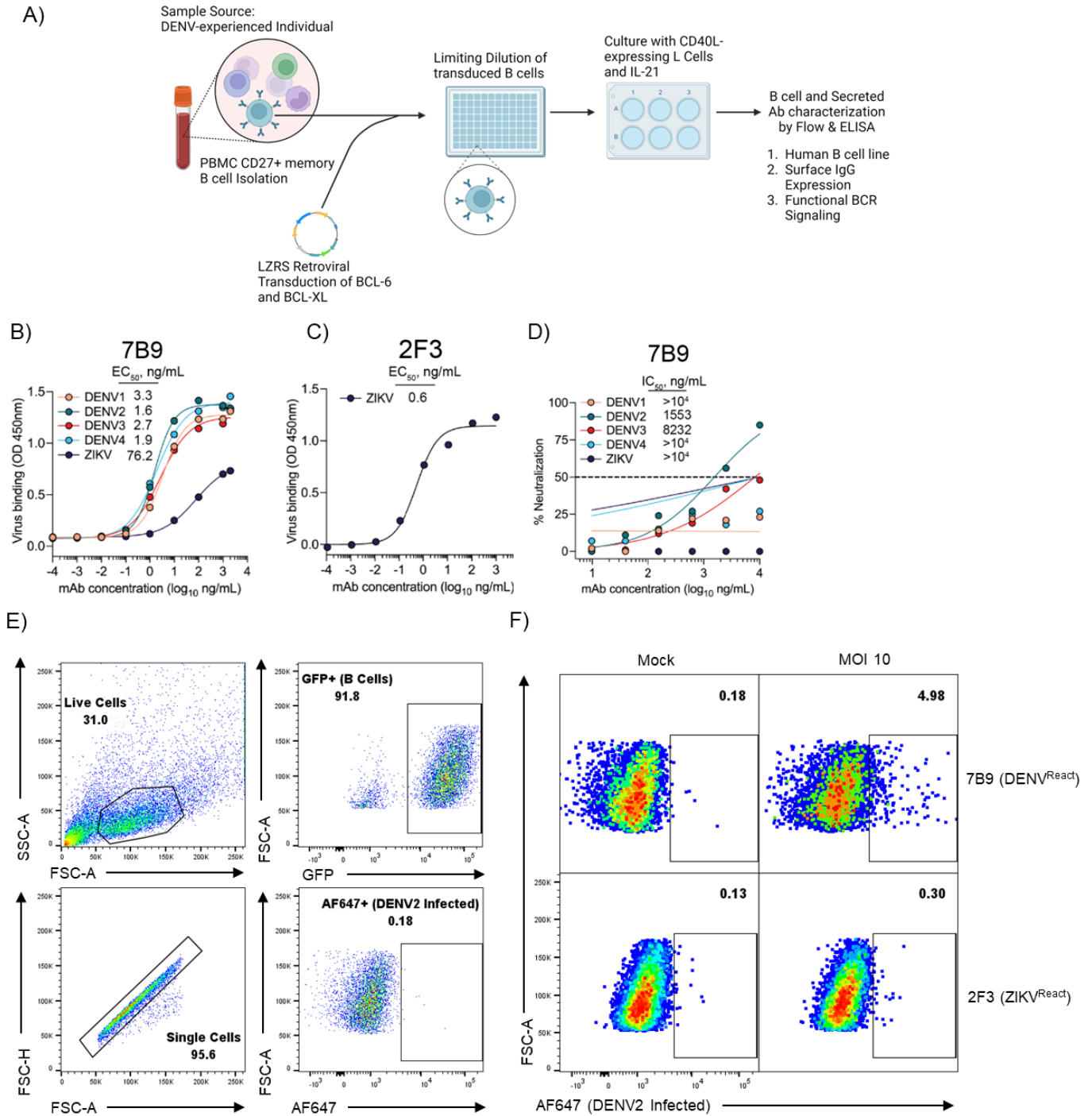


D)



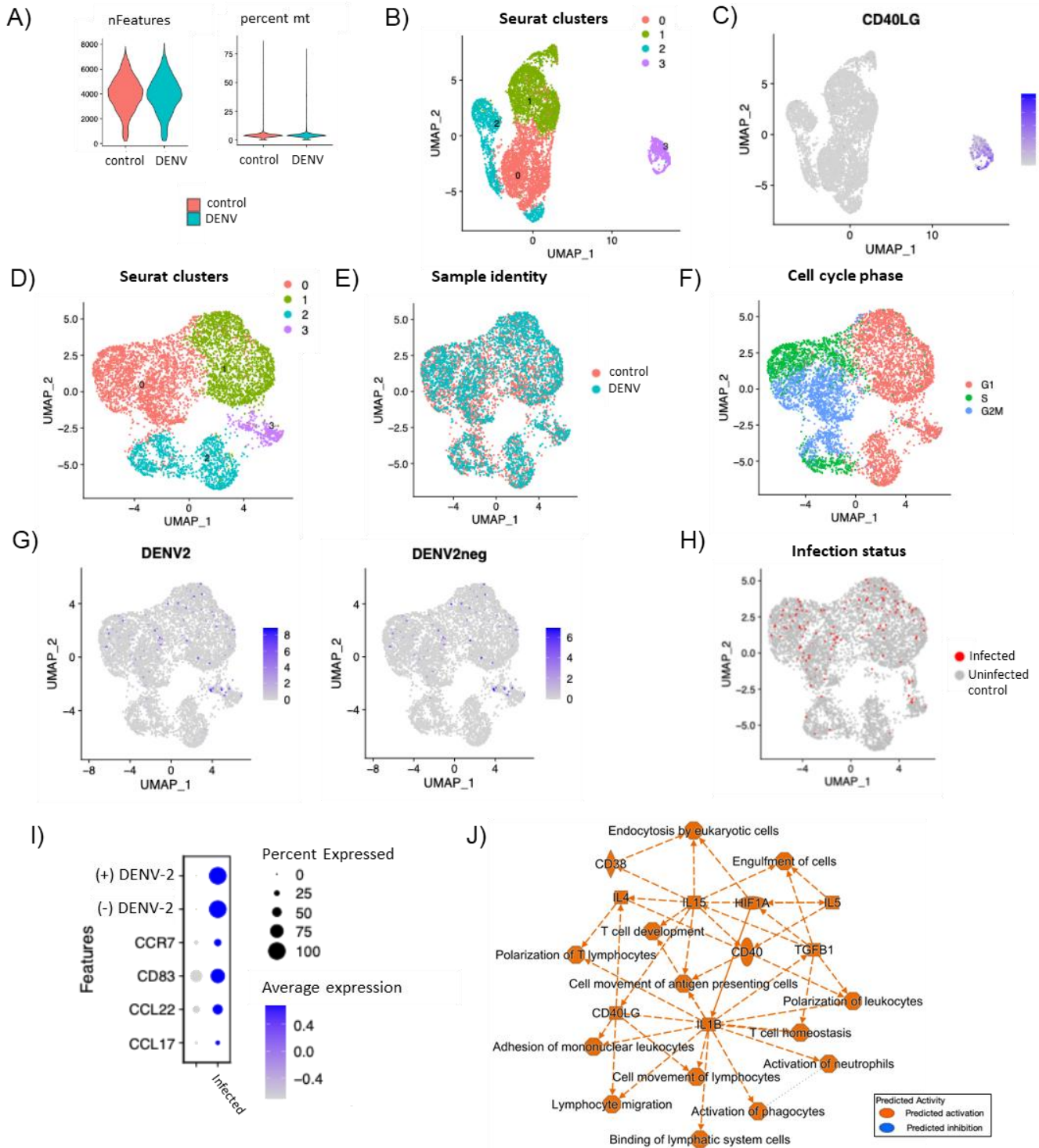
**S3.3 Fig. Expression of HCMV specific tmlgG. A)** Binding of the indicated IgG isotype mAbs to recombinant/purified HCMV gH protein as quantified by ELISA. **B)** Expression and gating of tmlgG and PDGFRa expression in transiently-transfected 293T cells **C)** Representative flow cytometry plots showing the frequency of HCMV SV40-GFP infected cells within the receptor-positive gate of tmlgG and PDGFRa transfected 293T cells 24hrs after virus inoculation. Cells infected at an MOI of 1 **D)** Quantification of HCMV SV40-GFP infected cells within the receptor-negative gate of tmlgG and PDGFRa in transfected 293T cells 24hrs after virus inoculation

### S3.4 Fig



**S3.4 Fig. Characterization and DENV-infection of 7B9 and 2F3 B cell lines.** **A)** Schematic representation of 7B9 and 2F3 cell line generation and maintenance. Created in BioRender. Gebo, C. (2024) BioRender.com/h94e139. **B)** Virus binding ELISA data for mAbs expressed by 7B9 and **C)** 2F3 cell lines. **D)** DENV and ZIKV neutralization profiles of mAb expressed by cell line 7B9. **E)** Gating scheme for flow cytometry analysis of DENV-infected 7B9 and 2F3 cell lines. **F)** Representative flow cytometry plots showing the frequency of DENV-infected 7B9 and 2F3 cell lines after DENV-2 exposure.

S3.5 Fig.

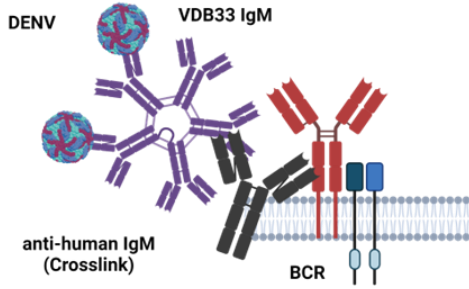


### **S3.5 Fig. Quality control metrics and integrated UMAP projections of scRNA seq data.**

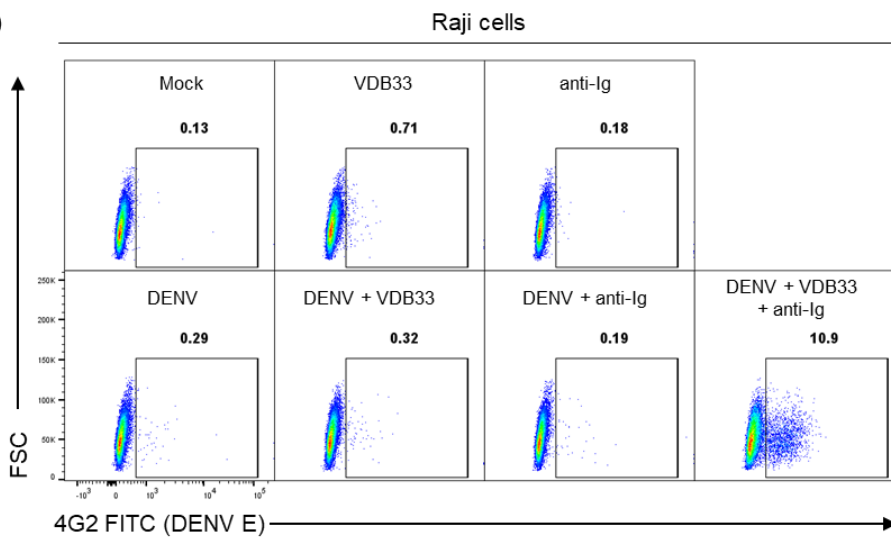
**(A)** Violin plots indicating number of features (nFeatures) and percentage mitochondrial content (percent mt) pre-filtering of scRNAseq data. **(B)** Integrated UMAP projection of cells derived from all conditions prior to subsetting for removal of CD40 ligand positive feeder cell population, and **(C)** Feature plot indicating CD40 ligand (CD40LG) expression, highlighting the feeder cell population (cluster 3) which was removed for subsequent analysis. **(D)** UMAP projections of scRNAseq data indicating Seurat clusters, **(E)** Sample origin, and **(F)** Cell cycle phase. **(G)** Feature plot indicating DENV (+) and (-) sense RNA expression. **(H)** Imputed cell labelling of infected (positive for both DENV positive and negative sense RNA) and uninfected control/bystander cell populations. **(I)** Dot plot highlighting selectively upregulated and downregulated DEGs in infected (expressing both DENV (+) and (-) RNA) compared to uninfected control cells (lacking both DENV (+) and (-) RNA expression). Average expression and percent of cells expressing a given transcript are indicated. **(J)** Ingenuity Pathway Analysis (IPA) Graphical summary of predicted pathway enrichment in infected cells compared to uninfected controls based on differential gene expression between infected (expressing both DENV (+) and (-) RNA) compared to uninfected control cells (lacking both DENV (+) and (-) RNA expression).

S3.6 Fig.

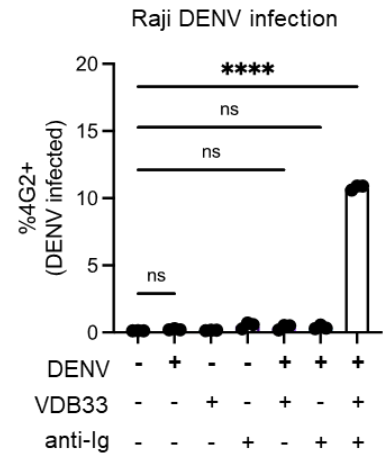
A)



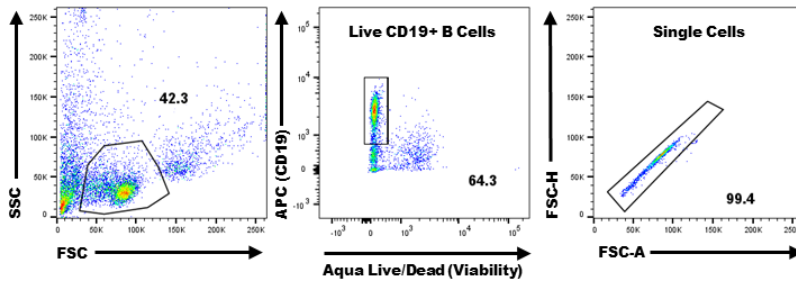
B)



C)



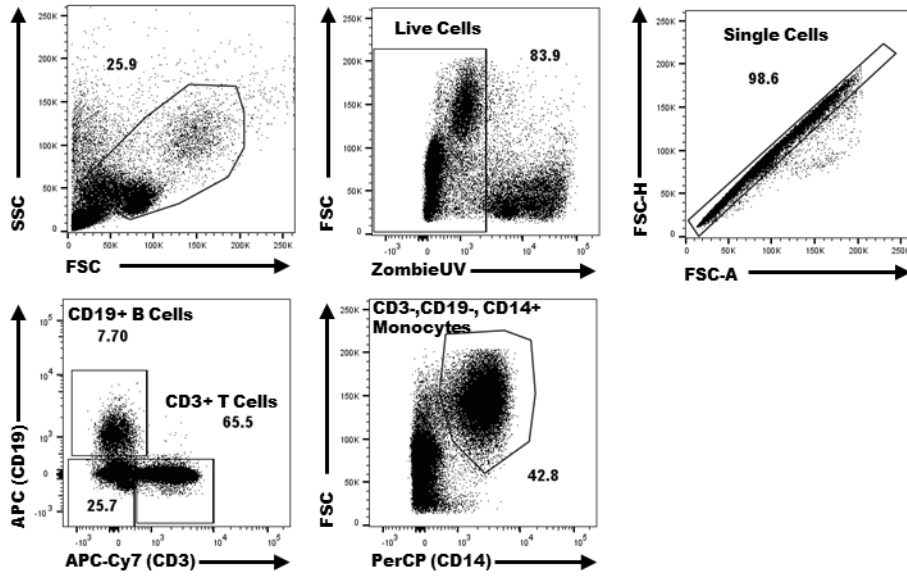
D)



**S3.6 Fig. DENV infection of polyclonal B cells by BCR/DENV cross-linking. A)** Schematic representation of DENV/Ig/BCR crosslinking assay. Created in BioRender. Gebo, C. (2024) [BioRender.com/u98x026](https://BioRender.com/u98x026). **B)** Representative flow cytometry plot showing the frequency of DENV-4 infected Raji cells under the indicated culture conditions. Detection of DENV-infected cells was performed by staining fixed/permeabilized cells with a FITC-conjugated 4G2 antibody. **C)** Quantification of DENV-4 infected Raji cells under the indicated culture conditions. **D)** Gating scheme for B cell infection analysis utilizing the DENV/Ig/BCR crosslinking assay.

### S3.7 Fig. Gating scheme for DHIM-3 PBMC analysis

A)



**Supplemental Table 3.1.** Quality control metrics for scRNAseq data.

<b>Sample</b>	<b>Number of reads</b>	<b>Estimated number of cells</b>	<b>Mean reads per cell</b>	<b>Median genes per cell</b>	<b>Median UMI counts per cell</b>
<b>Control</b>	239 120 923	3 419	69 939	4 004	17 248
<b>DENV-2</b>	293 412 699	3 274	89 619	3 933	17 115

**Supplemental Table 3.2.** Infected cell characteristics across culture conditions.

<b>Sample</b>	<b>Total cells n</b>	<b>(+) RNA only n (%)</b>	<b>(-) RNA only n (%)</b>	<b>(+) and (-) RNA n (%)</b>	<b>Percentage productively infected cells (%)</b>
<b>Control</b>	3089	4 (0)	0 (0)	3 (0)	<b>0</b>
<b>DENV</b>	2874	323 (11.2)	46 (1.6)	158 (5.5)	<b>5.5</b>

**Supplemental Table 3.3.** Differentially expressed genes, DENV-2 infected cells and uninfected mock cells

Gene	p_val	avg_log2FC	pct.1	pct.2	p_val_adj
DENV2	0	7.78946458	1	0	0
DENV2neg	0	5.64984623	1	0	0
RPS29	7.25999011232681e-83	1.35628816	0.981	0.986	1.46564680387654e-78
RPS21	1.4044602476356e-57	0.57383511	0.987	0.993	2.83532434792676e-53
MT-ATP8	3.02340174175545e-54	1.01227813	0.994	0.97	6.10364343625591e-50
RPL37A	1.16825240324941e-53	0.53082581	0.981	0.994	2.35846795167992e-49
ATP5ME	1.74628912311265e-53	0.88281685	0.962	0.914	3.52540848173981e-49
RPS27	1.52768057031352e-48	0.56638913	0.994	0.993	3.08408153534894e-44
MT-ND3	1.39160781456075e-47	0.73113374	0.994	0.987	2.80937785603525e-43
RPL38	1.39618780958835e-46	0.61665388	0.994	0.983	2.81862394999695e-42
RPL37	2.10120092693786e-46	0.46669258	0.987	0.993	4.24190443130216e-42
RMRP	3.98916886228975e-46	0.86657363	0.728	0.251	8.05333409919055e-42
NDUFB1	4.39891030636231e-38	0.71358662	0.949	0.863	8.88052012648424e-34
RPL26	1.08898352981008e-34	-0.5123385	0.968	0.99	2.19843994998059e-30
UBD	5.77634064960661e-33	0.34065402	0.487	0.141	1.16612765034258e-28
ATP5F1E	9.27021771196101e-33	0.45398847	0.968	0.988	1.87147155169069e-28
RPS15A	2.4407716955149e-28	-0.4029856	0.987	0.994	4.92742989890547e-24
NME2	1.06047926068114e-27	0.52578956	0.949	0.863	2.14089553146308e-23
OAZ1	1.56817937998613e-24	-0.4862034	0.981	0.985	3.165840532316e-20
RPS28	1.27749003607066e-22	0.33160919	0.994	0.994	2.57899688481945e-18
COX16	3.40763827400769e-22	0.46417728	0.867	0.654	6.87934014756672e-18
ROMO1	9.06172504820304e-22	0.46921768	0.949	0.945	1.82938105273123e-17
RPL36	1.61912291452079e-21	0.29645018	0.994	0.992	3.26868533983457e-17
MIF	2.06282658931158e-21	0.42464314	0.975	0.984	4.16443431850221e-17
ALDOA	2.80330944601422e-21	0.48325969	0.975	0.937	5.6593211096135e-17
EIF5B	5.69182820695662e-21	0.51527792	0.956	0.89	1.1490662784204e-16
RPL39	1.11630036014419e-20	0.36510245	0.981	0.992	2.25358716705909e-16
NDUFA3	3.76229184241984e-20	0.45464489	0.924	0.81	7.59531477147716e-16
PTPRCAP	4.82252370572321e-20	0.26526699	0.62	0.285	9.73571085711401e-16

Gene	p_val	avg_log2FC	pct.1	pct.2	p_val_adj
MTRNR2L12	1.60868694252421e-19	-0.6386891	0.994	0.992	3.24761719956788e-15
YWHAE	1.29109630290322e-18	0.38938592	0.975	0.97	2.60646521630102e-14
RPSA	2.78908460063567e-18	-0.2891266	0.987	0.989	5.63060399176328e-14
DDT	1.49190884199643e-17	-0.4452174	0.873	0.929	3.01186557022239e-13
ARPC1B	2.75616116535174e-17	0.36627286	0.873	0.658	5.56413816061209e-13
NEDD8	1.15235661387404e-16	-0.4267738	0.88	0.928	2.32637753208891e-12
RPS3A	2.22088635006074e-16	-0.2973643	0.987	0.996	4.48352536350263e-12
TMA7	1.0108239307923e-15	0.33225826	0.994	0.985	2.0406513514835e-11
EIF3J	9.70854194640473e-15	0.38694512	0.861	0.659	1.95996044814019e-10
RPL34	2.71187397446657e-14	0.28326956	0.987	0.994	5.47473117965312e-10
TOMM5	3.61786651933212e-14	0.43725135	0.943	0.874	7.30374892922769e-10
PSMA6	6.41696007303664e-14	0.37102156	0.911	0.757	1.29545589954464e-09
ATP5MD	1.00706280856607e-13	0.33729591	0.968	0.957	2.03305839793318e-09
SLIRP	1.2755346583616e-13	0.42725472	0.956	0.93	2.5750493683004e-09
CMSS1	2.74354398831438e-13	0.25976057	0.677	0.429	5.53866660360908e-09
PET100	7.84949360704804e-13	0.3116727	0.88	0.712	1.58465576939086e-08
RUNX3	7.85331975575218e-13	0.37087152	0.886	0.721	1.58542819229125e-08
DDX1	8.27141834192928e-13	0.34850161	0.823	0.646	1.66983393486868e-08
MIR155HG	1.47645514321639e-12	0.53260008	0.797	0.545	2.98066764312524e-08
TNFAIP2	1.7574352966517e-12	0.60251258	0.937	0.82	3.54791037688044e-08
ABCF1	3.57749450564282e-12	0.37302376	0.899	0.75	7.22224590799173e-08
SAMSN1	5.97179491289947e-12	0.43817783	0.804	0.575	1.20558595701614e-07
NDUFA11	7.30134428300667e-12	-0.3284874	0.785	0.842	1.47399538385339e-07
PA2G4	8.73064851011436e-12	0.44609962	0.949	0.928	1.76254332122189e-07
METAP2	1.25492811923672e-11	0.3386349	0.911	0.811	2.53344888711509e-07
EIF2S2	2.25054359597641e-11	0.33105807	0.943	0.91	4.54339741155719e-07
COX6A1	2.27161015403552e-11	-0.2942879	0.981	0.982	4.58592657896692e-07
NARS	2.62334462180443e-11	0.26499175	0.829	0.606	5.29600812249878e-07
PPA1	2.71720292700571e-11	0.30753662	0.968	0.959	5.48548926903913e-07
PPDPF	4.02509472606887e-11	-0.3969425	0.924	0.945	8.12586123298784e-07
RPS4X	5.87743488249973e-11	-0.2558316	0.994	0.995	1.18653655407905e-06

Gene	p_val	avg_log2FC	pct.1	pct.2	p_val_adj
S100A4	7.41867516922651e-11	0.51334713	0.772	0.577	1.49768214316345e-06
H3F3A	8.32096806568795e-11	-0.2919772	0.975	0.991	1.67983703310108e-06
MRPL23	1.16849639390254e-10	-0.2976701	0.405	0.586	2.35896052001044e-06
TAF11	1.32539947611917e-10	0.25587334	0.759	0.526	2.67571646238939e-06
PPP2CA	1.3578764262815e-10	-0.3642489	0.658	0.768	2.7412809293771e-06
H3F3B	1.84321826352206e-10	-0.3096507	0.987	0.993	3.72108903039833e-06
MT-ATP6	2.7545535464703e-10	-0.32063	1	0.994	5.56089269961423e-06
DENR	6.15790294291304e-10	0.27764285	0.886	0.756	1.24315744611528e-05
SLC25A5	9.78785086703137e-10	-0.3025437	0.943	0.979	1.97597133303629e-05
NDUFA1	1.21867464662305e-09	0.27854151	0.968	0.941	2.46026037660262e-05
MT-ND2	1.36006841469263e-09	0.30662285	1	0.985	2.74570611558149e-05
AL138963.3	2.63463105579513e-09	-0.5445581	0.741	0.804	5.31879317543921e-05
CCR7	3.42496449185524e-09	0.70978491	0.354	0.172	6.91431831615736e-05
SRSF9	3.52210730495601e-09	-0.2780663	0.968	0.966	7.11043022724518e-05
CORO1A	6.55693077567514e-09	-0.3485382	0.949	0.958	0.00013237
DPYSL2	8.88444384567165e-09	0.28215948	0.772	0.568	0.00017936
SNRPB2	1.05693364179265e-08	0.30624269	0.892	0.797	0.00021337
PRDX2	1.21359193980804e-08	-0.3082327	0.949	0.966	0.000245
ERH	2.13954803762635e-08	-0.2748098	0.962	0.978	0.00043193
IGLC3	3.01192707291274e-08	0.40509046	0.93	0.752	0.00060805
FNBP1	3.04446929763137e-08	0.29256032	0.905	0.813	0.00061462
NAPSA	4.76436719908735e-08	-0.3482562	0.538	0.639	0.00096183
ARPC5	7.09231545556128e-08	-0.2946798	0.968	0.979	0.0014318
MKNK2	1.2468912670071e-07	0.3506819	0.861	0.722	0.00251722
CD83	1.31044635033675e-07	0.41216608	0.804	0.647	0.00264553
HLA-C	1.34908219653009e-07	-0.3426838	0.956	0.966	0.00272353
DDX21	1.74955084302371e-07	0.33254476	0.918	0.9	0.00353199
PPP1CA	1.85579640066829e-07	-0.2562549	0.937	0.949	0.00374648
ATP6V1G1	1.88510744852717e-07	-0.3312708	0.943	0.952	0.00380565
GPR157	1.9375995077652e-07	0.27679599	0.437	0.261	0.00391163
LCP1	2.25436168594684e-07	0.2871279	0.987	0.985	0.00455111

Gene	p_val	avg_log2FC	pct.1	pct.2	p_val_adj
MALAT1	2.26555259303704e-07	0.33674625	1	0.995	0.0045737
SKP1	2.3382582128986e-07	-0.2617133	0.943	0.95	0.00472048
CCNI	2.5862007400521e-07	-0.274419	0.937	0.954	0.00522102
IGKV3-15	2.64167659909777e-07	-1.3226319	0.082	0.252	0.00533302
PDCD11	2.80322090233665e-07	0.2694218	0.728	0.547	0.00565914
CCNG1	3.13780313320918e-07	-0.3096497	0.918	0.908	0.0063346
WDR83OS	3.33462018465226e-07	-0.2857171	0.924	0.933	0.00673193
TMEM14B	4.34447239092933e-07	-0.2755085	0.842	0.871	0.00877062
IGKV3D-11	5.36806135548254e-07	-1.1740944	0.082	0.25	0.01083704
DYNLL1	5.37537015403036e-07	-0.3371743	0.93	0.964	0.0108518
LIMD2	5.69800488403979e-07	-0.2836384	0.975	0.963	0.01150313
ANP32B	6.29284332714697e-07	-0.2804014	0.968	0.968	0.01270399
IGKV3-11	7.62445584680517e-07	-1.5906087	0.095	0.256	0.01539225
TNNI1	8.02541581968897e-07	0.3400626	0.519	0.317	0.01620171
PLEC	8.52500839120461e-07	0.35477235	0.753	0.591	0.01721029
PSMD9	9.01146687812747e-07	-0.2714013	0.582	0.69	0.01819235
EIF3H	9.90101493396531e-07	-0.2639272	0.956	0.967	0.01998817
CD44	9.91814559212962e-07	0.38893039	0.241	0.114	0.02002275
IGKC	1.15333776199585e-06	-1.2112693	0.114	0.276	0.02328358
SCD	1.87330420715242e-06	0.26534152	0.854	0.761	0.03781827
CCL17	1.88548054439542e-06	0.59604529	0.19	0.08	0.03806408
FTL	1.90599002852873e-06	-0.3101528	0.981	0.994	0.03847813
IGHV5-51	1.99311771255404e-06	-1.49403	0.095	0.254	0.04023706
FLNA	2.02226636035301e-06	0.31834453	0.911	0.87	0.04082551
TIMP1	2.02410921494935e-06	0.30435698	0.924	0.851	0.04086272
CCL22	2.14564901093193e-06	0.60350096	0.532	0.345	0.04331636
HYOU1	2.15838041353401e-06	0.25599756	0.829	0.695	0.04357338

**Supplemental Table 3.4.** Differentially expressed genes between DENV-2 infected 7B9 cells and uninfected mock cells

Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score
Eukaryotic Translation Initiation	23.3	0.156	1.606
Response of EIF2AK4 (GCN2) to amino acid deficiency	19.6	0.155	1.5
EIF2 Signaling	19.3	0.087	1.667
Nonsense-Mediated Decay (NMD)	18.7	0.137	1
Eukaryotic Translation Termination	18.6	0.16	1.291
Eukaryotic Translation Elongation	18.5	0.158	1.291
Selenoamino acid metabolism	17.7	0.14	1.291
SRP-dependent cotranslational protein targeting to membrane	17.2	0.13	1.291
Major pathway of rRNA processing in the nucleolus and cytosol	16.8	0.0914	1.698
Electron transport, ATP synthesis, and heat production by uncoupling proteins	12.1	0.0938	1.732
Regulation of eIF4 and p70S6K Signaling	10.2	0.0652	#NUM!
Oxidative Phosphorylation	9.92	0.0893	1.265
mTOR Signaling	9.44	0.0561	#NUM!
Sirtuin Signaling Pathway	6.92	0.0378	-1.89
Coronavirus Pathogenesis Pathway	6.31	0.0441	-0.333
Granzyme A Signaling	5.89	0.0811	-1.633
Neutrophil Extracellular Trap Signaling Pathway	5.56	0.0275	0.905
Mitochondrial Dysfunction	5.31	0.0291	-1.265
Cristae formation	4.9	0.129	1
Binding and Uptake of Ligands by Scavenger Receptors	4.85	0.0536	-0.816
rRNA processing	4.84	0.125	#NUM!
Signaling by the B Cell Receptor (BCR)	4.82	0.0412	-1.134
Hematoma Resolution Signaling Pathway	4.54	0.031	1.414
Fc epsilon receptor (FCERI) signaling	4.29	0.034	-1.134
HIPPO signaling	4.26	0.0575	#NUM!
C-type lectin receptors (CLRs)	4.21	0.0414	0
Fc gamma receptor (FCGR) dependent phagocytosis	4.02	0.0382	-0.816
Parkinson's Signaling Pathway	4.01	0.0261	-1.414
Eumelanin Biosynthesis	3.89	0.5	#NUM!

<b>Ingenuity Canonical Pathways</b>	<b>-log(p-value)</b>	<b>Ratio</b>	<b>z-score</b>
Iron uptake and transport	3.81	0.069	-2
NIK-->noncanonical NF-kB signaling	3.75	0.0667	-1
Mitotic G2-G2/M phases	3.46	0.0302	-0.816
Neutrophil degranulation	3.41	0.0189	0.333
Glucocorticoid Receptor Signaling	3.4	0.0172	#NUM!
Cell surface interactions at the vascular wall	3.33	0.0284	0
TP53 Regulates Metabolic Genes	3.11	0.0455	0
Degradation of beta-catenin by the destruction complex	3.04	0.0435	-1
ABC-family proteins mediated transport	2.89	0.0396	1
Cell Cycle Checkpoints	2.76	0.0221	0
Macrophage Alternative Activation Signaling Pathway	2.7	0.0263	1.342
FAT10 Signaling Pathway	2.62	0.0526	#NUM!
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	2.59	0.0248	-1.342
Metabolism of polyamines	2.58	0.0508	#NUM!
Estrogen Receptor Signaling	2.51	0.0171	#NUM!
Mitotic G1 phase and G1/S transition	2.48	0.0305	#NUM!
Complement cascade	2.43	0.0296	#NUM!
Regulation of RUNX2 expression and activity	2.32	0.0411	#NUM!
Glioma Invasiveness Signaling	2.32	0.0411	#NUM!
Mitotic Metaphase and Anaphase	2.31	0.0213	-0.447
p70S6K Signaling	2.24	0.0138	#NUM!
Neddylation	2.23	0.0203	-0.447
Epithelial Adherens Junction Signaling	2.19	0.0253	1
Signaling by NOTCH4	2.16	0.0361	#NUM!
Inhibition of ARE-Mediated mRNA Degradation Pathway	2.15	0.0245	#NUM!
Phagosome Maturation	2.14	0.0244	#NUM!
Cyclins and Cell Cycle Regulation	2.12	0.0349	#NUM!
Regulation of mitotic cell cycle	2.09	0.0341	#NUM!
KEAP1-NFE2L2 pathway	2.05	0.033	#NUM!
Crosstalk between Dendritic Cells and Natural Killer Cells	2.05	0.033	#NUM!
Protein Ubiquitination Pathway	2.04	0.0183	-0.447

<b>Ingenuity Canonical Pathways</b>	<b>-log(p-value)</b>	<b>Ratio</b>	<b>z-score</b>
Actin Nucleation by ARP-WASP Complex	2.03	0.0323	#NUM!
Transcriptional regulation by RUNX3	1.99	0.0312	#NUM!
S Phase	1.94	0.03	#NUM!
RHO GTPases Activate WASPs and WAVEs	1.91	0.0556	#NUM!
Protein Kinase A Signaling	1.9	0.0146	#NUM!
Gene and protein expression by JAK-STAT signaling after IL-12 stimulation	1.89	0.0541	#NUM!
Pyrophosphate hydrolysis	1.86	0.333	#NUM!
TCF dependent signaling in response to WNT	1.85	0.0201	0
Regulation of TP53 Expression and Degradation	1.85	0.0513	#NUM!
Mitotic Prometaphase	1.82	0.0197	0
Hedgehog 'off' state	1.8	0.0265	#NUM!
Regulation of Actin-based Motility by Rho	1.78	0.0261	#NUM!
Synthesis of DNA	1.74	0.0252	#NUM!
ERK/MAPK Signaling	1.74	0.0186	1
Interleukin-10 signaling	1.73	0.0444	#NUM!
RHO GDI Signaling	1.71	0.0182	-1
TCR signaling	1.67	0.0238	#NUM!
Interleukin-1 family signaling	1.65	0.0233	#NUM!
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.63	0.0392	#NUM!
Response to elevated platelet cytosolic Ca <sup>2+</sup>	1.62	0.0227	#NUM!
Regulation of Apoptosis	1.6	0.0377	#NUM!
Signaling by TGF-beta Receptor Complex	1.6	0.0377	#NUM!
RAC Signaling	1.58	0.0219	#NUM!
B Cell Development	1.57	0.0123	#NUM!
Iron homeostasis signaling pathway	1.57	0.0217	#NUM!
Intrinsic Pathway for Apoptosis	1.57	0.0364	#NUM!
RHO GTPases Activate Formins	1.56	0.0216	#NUM!
Communication between Innate and Adaptive Immune Cells	1.52	0.00966	-1
Circadian Clock	1.51	0.0339	#NUM!
Semaphorin Signaling in Neurons	1.48	0.0328	#NUM!
Primary Immunodeficiency Signaling	1.48	0.0328	#NUM!

Ingenuity Canonical Pathways	<b>-log(p-value)</b>	<b>Ratio</b>	<b>z-score</b>
Cytoprotection by HMOX1	1.47	0.0323	#NUM!
UFMylation Signaling Pathway	1.47	0.0323	#NUM!
IL-15 Signaling	1.44	0.0114	#NUM!
Hedgehog ligand biogenesis	1.43	0.0308	#NUM!
FcγRIIB Signaling in B Lymphocytes	1.42	0.0113	#NUM!
TNFR2 non-canonical NF-κB pathway	1.4	0.0294	#NUM!
Remodeling of Epithelial Adherens Junctions	1.4	0.0294	#NUM!
OAS antiviral response	1.39	0.111	#NUM!
Sucrose Degradation V (Mammalian)	1.39	0.111	#NUM!
Cell Cycle: G1/S Checkpoint Regulation	1.39	0.029	#NUM!
Huntington's Disease Signaling	1.36	0.0141	#NUM!
Netrin Signaling	1.33	0.0173	#NUM!
Cellular response to hypoxia	1.32	0.0267	#NUM!
Caveolar-mediated Endocytosis Signaling	1.32	0.0267	#NUM!
WNT/β-catenin Signaling	1.32	0.0172	#NUM!

**Supplemental Table 3.5.** Reagents for flow cytometry analysis

<b>Antibody</b>	<b>Clone</b>	<b>Dilution</b>	<b>Catalog #</b>	<b>Lot #</b>
Anti-human IgA AF647	N/A	1:200	Southern Biotech, 2050-31	G0919-V490B
Anti-human IgG AF647	N/A	1:200	Southern Biotech, 2040-31	B3919-M950B
Anti-human IgM AF647	N/A	1:200	Southern Biotech, 2020-31	D2219-R121
Anti-human CD19 APC	HIB19	1:200	Biolegend, 302212	B386154
Anti-human CD14 PerCP	M5E2	1:50	Biolegend, 301848	B371416
Anti-human CD3 APC-Cy7	OKT3	1:200	Biolegend, 317342	B367892
Anti-human DC-SIGN	9E9A8	1:200	Biolegend, 330112	B386835
Anti-human PDGFRα	16A1	1:200	Biolegend, 323512	B388290
ZombieUV	N/A	1:200	Biolegend, 77474	B385840
Aqua Live/Dead	N/A	1:500	Invitrogen, L34957	2204201
DENV Envelop (4G2) FITC	N/A	4ug/mL	Envigo Bioproducts, Inc, CON004	N/A
Anti-mouse Ig AF646				
IgG				
CD19				

## **CHAPTER 4**

# **MOLECULAR AND BIOPHYSICAL REQUIREMENTS FOR B CELL RECEPTOR DEPENDENT ENHANCEMENT OF DENGUE VIRUS INFECTION**

## **Molecular and biophysical requirements for B cell receptor dependent enhancement of dengue virus infection**

Gaby Madrigal<sup>1,\*</sup>, Chad Gebo<sup>2,\*</sup>, Elizabeth A. Kurtz<sup>2</sup>, Benjamin D. McElvany<sup>1</sup>, Nathan Roy<sup>2</sup>, Sean A. Diehl<sup>1,†</sup>, Adam T. Waickman<sup>2,3,†</sup>

<sup>1</sup>Department of Microbiology and Molecular Genetics, Vaccine Testing Center, University of Vermont Larner College of Medicine, Burlington, Vermont, United States of America.

<sup>2</sup>Department of Microbiology and Immunology, State University of New York Upstate Medical University, Syracuse, New York, United States of America.

<sup>3</sup>Global Health Institute, State University of New York Upstate Medical University, Syracuse, New York, United States of America.

\*These authors contributed equally to this work

†To whom correspondence should be addressed: [Sean.Diehl@med.uvm.edu](mailto:Sean.Diehl@med.uvm.edu),  
[waickmaa@upstate.edu](mailto:waickmaa@upstate.edu)

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## **ABSTRACT**

Dengue virus (DENV) is the causative agent of dengue, a mosquito-borne disease that represents a significant and growing public health burden around the world. A unique pathophysiological feature of dengue is immune-mediated enhancement, wherein preexisting immunity elicited by a primary infection can enhance the severity of a subsequent infection by a heterologous DENV serotype. A leading mechanistic explanation for this phenomenon is antibody dependent enhancement (ADE), where sub-neutralizing concentrations of DENV-specific IgG antibodies facilitate entry of DENV into FcγR expressing cells. Accordingly, this model posits that phagocytic mononuclear cells are the primary reservoir of DENV. However, multiple independent groups have shown that B cells are the largest reservoir of virally infected cells in circulation during acute dengue, representing a disconnect in our understanding of immune-mediated DENV tropism. In response to this persistent knowledge gap, our team has previously identified a novel mechanism of immune-mediated enhancement we have termed BCR-dependent enhancement (BDE) of DENV infection. In this study, we show that DENV infection of DENV-reactive B cells is highly sensitive to BCR/DENV envelope (E) protein interactions. DENV entry into this subset of B cells is dynamin-mediated and requires proximal BCR signaling. Finally, we show that DENV-reactive B cells are productively infected by live DENV, capable of supporting active viral replication and dissemination. We propose that BDE provides an additional layer of pathogen-specific immune-mediated infection risk that complements existing models of ADE and offers additional insight into potential mechanisms of DENV immunopathogenesis.

## INTRODUCTION

Dengue virus (DENV) is a prevalent arboviral pathogen that represents a significant public health burden in many regions of the world. An estimated 3.6 billion people live in regions with sustained DENV transmission, with the virus infecting an estimated 400 million individuals every year [1, 2]. Transmitted via the bite of infected *Aedes* mosquitoes, DENV co-circulates as four genetically and immunologically distinct serotypes: DENV-1, -2, -3, and -4. Approximately 25% of DENV infections are thought to result in some form of symptomatic illness, ranging in severity from dengue fever - a mild flu-like presentation of infection – to life-threatening severe dengue, dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [3-5]. Currently, there are no accurate prognostic indicators of which patients will progress to develop severe dengue. However, the risk of developing severe dengue increases in patients previously infected by a different (heterologous) serotype of dengue compared to DENV-naïve individuals [1, 6]. This increased risk of severe disease following heterologous reinfection strongly suggests a mechanism of immune-mediated enhancement of DENV infection.

While the etiology of severe dengue is complex and incompletely understood, *in vitro* studies and epidemiological modeling suggest that antibody-dependent enhancement (ADE) of DENV infection may play a significant role in both the risk of DENV infection and in dengue pathogenesis [6-9]. Waning titers of cross-reactive DENV-specific antibodies to sub-neutralizing levels have been posited to result in opsonization of the virion and subsequent uptake by Fcγ-receptor (FcγR) bearing cells such as

monocytes, macrophages, and dendritic cells [10, 11]. This has been suggested to result both in an increase in the number of infected cells early after inoculation, as well as a qualitatively altered intrinsic response to infection in these susceptible phagocytes [10, 12-14]. The delicate balance between protective and enhancing levels of immunity in dengue is a hallmark of the disease and has complicated dengue control efforts for decades.

While the ADE model of dengue immunopathogenesis has been a cornerstone of the field for nearly 50 years, numerous observations strongly suggest that other immunologic mechanisms must also contribute to DENV pathology. Most notably, while in vitro infection studies and histological analysis of samples collected from individuals who have succumbed to severe dengue have shown a high DENV burden in FcγR expressing phagocytes [12, 15-18], ex vivo analysis of PBMC collected during acute DENV infection instead shows that B cells are the dominant circulating cellular reservoir of DENV [12, 19-24]. This observation has been recapitulated by multiple groups - including our own - using an array of assays including flow cytometry, scRNAseq, intrathoracic inoculation of mosquitoes with purified cell populations, and qRT-PCR [12, 19-24]. While B cells express an FcγR, the specific isoform of this receptor (FcγRIIb) has been shown to be incapable of facilitating ADE and instead results in anergy and apoptosis in B cells when engaged [20, 25]. Accordingly, there is need for the field to reconcile these disparate observations and assumptions regarding DENV cellular tropism [12, 20, 22].

Recently, our team identified a novel DENV entry mechanism specifically within DENV-reactive B cells, a process we have termed B cell receptor-dependent

enhancement (BDE). Our group, as well as others, have shown that viruses are able to utilize B cell receptor (BCR) antigen specificity in order to preferentially infect subsets of the B cell population in a highly antigen-specific fashion [26, 27]. We have shown in vitro that B cells expressing a DENV envelope protein-specific BCR were susceptible to DENV infection, and that the frequency of DENV-infectible B cells in PBMCs increased significantly after a primary DENV infection (26). However, questions remain regarding the signaling pathways and cellular processes required for BCR-mediated infection of DENV-specific B cells.

In this study, we define the cell-intrinsic mechanistic requirements for BCR-mediated entry of DENV into DENV-specific B cells utilizing a unique set of immortalized flavivirus-specific human B cells. Using this model system, we confirm that the susceptibility of these cells is dependent both on the antigen-specificity of the BCR as well as canonical BCR signaling and endocytic pathways. In addition, we demonstrate that this antigen-specific endocytic process results in localization of DENV into late endosomes in DENV-specific B cells, an essential part of the DENV entry cycle. Finally, we confirm the ability of DENV-reactive B cells to support productive DENV replication. Overall, our results highlight the entry and signaling requirements in BDE and emphasizes how generation of DENV-specific immunologic memory may play a critical cell-intrinsic role in determining DENV susceptibility at an organismal level.

## MATERIALS AND METHODS

**Viruses & RVPs.** DENV-2/NGC & DENV-4/Dominica81 stocks were prepared by propagating a low-passage inoculum (MOI = 0.5) in Vero cells maintained in OPTI-PRO serum free medium (Invitrogen, cat. no.12309019) supplemented with 2% fetal bovine serum (Atlanta Biologicals/R&D Systems, cat. no. S11150) and GlutaMAX (Gibco, cat. no. 35050061) at 37C, 5% CO<sub>2</sub> in a humidified incubator. Viruses were titrated by focus forming unit (FFU) assay as described [38]. DENV-2 (strain Eden-2) stock was prepared by propagating a low-passage inoculum in Vero cells. Infectious particles quantified by plaque assay. DENV-4 (strain Dominica81) tagRFP657 Reporter virus particles (RVP) were prepared as previously described [29]. RVPs were titrated on Raji DC-SIGN+ cells, infectious units (IU) calculated based on the dilution of virus needed to infect 15-20% of cells as previously described [39].

**Cell lines.** Immortalized memory B cells were previously generated by transduction of CD27<sup>+</sup>IgM<sup>-</sup> memory B cells with a retrovirus encoding human B cell lymphoma (BCL)-6 and BCL-xL and enhanced green fluorescent protein (GFP) [26, 37]. 7B9<sup>DENV</sup> are an immortalized human monoclonal B-cell line expressing an IgG isotype BCR that binds, but poorly neutralizes, DENV-1 to -4 and ZIKV generated from a flavivirus-naïve volunteer who was vaccinated with DENV-1Δ30 and challenged 270 days later with DENV-2Δ30 Tonga (NCT02392325). B cells were isolated 90 days following DENV-2Δ30 challenge. 2F3<sup>ZIKV</sup> are an immortalized human monoclonal B-cell line expressing a ZIKV-specific IgG isotype BCR that binds ZIKV, but not DENV and was obtained from an exposed volunteer (DT165) approximately 1 year following ZIKV infection. Cells were

maintained in Iscove's modified Dulbecco's medium (IMDM, Invitrogen, cat. no.12200-028) supplemented with 8% fetal bovine serum (FBS) and 1% pen/strep in 24-well plates with 100,000 irradiated CD40L-expressing mouse L cell fibroblasts and 25 ng/mL rhIL-21 (Peprotech, cat. no. 200-21-1MG) as previously described [37]. Raji cells overexpressing DC-SIGN+ (CD209L) (BEI, ARP-9945) were cultured in complete RPMI-1640 medium (complete RPMI) supplemented with 10% FBS and 100 U/ml penicillin–streptomycin. All cells cultured at 37C, 5% CO<sub>2</sub>, and 85% humidity.

**Flow cytometry.** Immortalized B cells were stained for viability (L/D) with Fixable Viability Dye eFluor 780 (Invitrogen, cat. no. 65-0865-14) according to the manufacturer's protocol and washed with FACS buffer (PBS + 1% BSA). 100 ng of AviTagged and biotinylated DENV-2 or DENV-4 recombinant E protein stabilized dimers (DV2 SC.14 and DV4 SC, respectively, a kind gift of Brian Kuhlman and Aravinda DeSilva, University of North Carolina, Chapel Hill, [28]) were labeled with 0.5  $\mu$ L of streptavidin-PE (Invitrogen, cat. no. 12-4317-87) in a volume of  $\sim$ 8  $\mu$ L for 30 minutes on ice. 100,000 B cells were then stained with PE-labeled recE on ice for 30 min. Following a wash with FACS buffer and final staining with anti-human IgG-APC (clone: M1310G05, BioLegend, cat. no. 410712) for 30min. Events were captured on a Beckman Cytoflex at the Microscopy Imaging and Cytometry at the University of Vermont (RRID# SCR\_018821) and analyzed with FlowJo version 10.

DENV-4 RVP infection assays were collected on a ThermoFisher Attune NXT flow cytometer, detecting 7B9DENV or 2F3ZIKV cells through detection of GFP. RVP infection was detected through the expression of tagRFP657. Data collection was performed on a

ThermoFisher Attune NXT flow cytometer and analyzed using FlowJo v10.2 (Becton Dickinson).

***DENV-4 tagRFP657 RVP Infection of human B cells.*** 7B9<sup>DENV</sup> or 2F3<sup>ZIKV</sup> cells were plated at  $5 \times 10^4$  cells per well. DENV-4 RVP was titrated in two-fold serial dilutions. Cells were incubated for 4 hours at 37C, followed by wash and trypsinization to remove remaining surface bound RVP. Then cells were incubated an additional 20 hours at 37C. Cells were washed twice with PBS + 2% FBS then fixed using 4% PFA and detected by flow cytometry. MOI calculation for titration was back-calculated based on IU calculations on Raji DC-SIGNR cells.

***DENV-2 recombinant E Dimer infection blockade.*** DENV-2 recombinant Envelope protein dimers (recE) were received under MTA from Aravinda M. de Silva at University of North Carolina, Chapel Hill. 7B9<sup>DENV</sup> or Raji DC-SIGN<sup>+</sup> cells were plated at  $5 \times 10^4$  cells per well and chilled to 4C before no treatment or addition of 0.1 $\mu$ g/mL or 1.0 $\mu$ g/mL of the DENV-2 recE. Cells were incubated at 4C for 30 minutes, followed by the addition of DENV-4 RVP at an MOI of 10. Cells were incubated an additional 30 minutes at 4C before three washes using cell culture medium. Cells were resuspended in fresh cell culture media and incubated for 24 hours at 37C. Cells were washed twice with PBS + 2% FBS before fixation using 4% PFA and analyzed by flow cytometry.

***Inhibition of viral entry by pharmacological inhibitor treatment.*** 7B9<sup>DENV</sup> cells were plated at  $5 \times 10^4$  cells per well and pre-treated for 30 minutes at 37C with DMSO or various concentrations of pharmacological inhibitors: 12.5 $\mu$ M, 50 $\mu$ M, and 200 $\mu$ M of

dynamin inhibitor Dynasore (Abcam, cat. no. ab120192); 1.56 $\mu$ M, 12.5 $\mu$ M, and 50 $\mu$ M of BTK inhibitor Ibrutinib (Selleck, cat. no. S2680); or 1.56 $\mu$ M, 12.5 $\mu$ M, and 50 $\mu$ M of PI3K inhibitor LY294002 (Selleck, cat. no. S1105). Following pre-treatment, DENV-4 RVP was added at an MOI of 2.5 and incubated for 4 hours in the presence of drug. Cells were then washed twice using cell culture medium and trypsinized then washed again to remove surface bound RVP before resuspension in fresh cell culture media and incubated at 37C for 20 hours. Cells were then washed twice with PBS + 2% FBS before fixation using 4% PFA and analyzed by flow cytometry.

Inhibitor treatment effect on cell viability in the absence of RVP was performed under the same conditions as described above then assessed by metabolic conversion of MTT tetrazolium into formazan utilizing the CellTiter 96<sup>®</sup> Non-Radioactive Cell Proliferation Assay (MTT) (Promega, cat. no. G4000). 1% Triton-X (Thermofisher, cat. no. A16046.AE) was used as a positive control for cell death. Data was collected on a Biotek  $\mu$ Quant plate reader at 570 nm.

Pharmacological inhibition of PI3K was assessed via flow cytometry through the detection of phosphorylated ribosomal protein S6 at Ser235 and Ser236 as a direct downstream measurement of activated PI3K. Cells were treated with DMSO (Thermofisher, cat. no. 036480.AP) or 10 $\mu$ M LY294002 for 30 minutes. Then washed twice with PBS + 2% FBS. Then fixed using 4% PFA and stained utilizing AF647 labelled phospho-S6 monoclonal cupk43k (Thermofisher, cat. no. 740057MP647) diluted 1:1000 in PBS + 2% BSA. Data collection was performed on a ThermoFisher Attune NXT flow cytometer and analyzed using FlowJo v10.2 (Becton Dickinson).

***Detection of DENV internalization and endosomal localization by microscopy.*** Ibidi 8-well chamber slides (Ibidi, cat. no. 80826-120) were coated with Histogrip (ThermoFisher, cat. no. 008050) to promote cell adhesion to slide. 7B9<sup>DENV</sup> or 2F3<sup>ZIKV</sup> cells were chilled to 4C prior to the addition of DENV-2 EDEN live virus at MOI of 10. Cells were incubated for 1 hour at 4C to allow surface binding before washing. Cells were then plated at  $1 \times 10^5$  per well and allowed to adhere at 4C for an additional 1 hour. Cells were then temperature switched and incubated for 15, 30, and 45 minutes at 37C then fixed using 4% PFA. Cells were then permeabilized using PBS supplemented with 0.1% Triton-X and 1% BSA. Cells were stained overnight at 4C with primary antibodies diluted in perm buffer: mouse cross-reactive anti-flavivirus envelope (E) protein monoclonal antibody 4G2 diluted to 4 $\mu$ g/mL (Envigo, cat. no. 80216718); and rabbit anti-human LAMP1 monoclonal antibody D2D11 diluted 1:100 (Cell Signaling, cat. no. 9091). Followed by two washes in perm buffer before secondary staining in perm buffer, each diluted 1:1000: donkey anti-mouse IgG AF647 (Jackson ImmunoResearch Inc, cat. no. 715-605-151); donkey anti-rabbit IgG AF568 (ThermoFisher, cat. no. A10037). After staining, cells were washed twice in PBS. Intracellular GFP was utilized to detect immortalized B cells and used as cytoplasm dye. Images were collected on a Marianas microscopy system (3i) enclosed in an environmental chamber (Okolab) consisting of a Zeiss Axio Observer 7 equipped with a X-Cite mini + light source (Excelitas) and a Prime BSI Express CMOS camera (Photometrics). Images were taken at 63 $\times$  magnification, with each image created using SlideBook 6 (3i) before exporting to Fiji for analysis. Individual

cells were then analyzed for colocalization of LAMP1 and DENV by Pearson Coefficient utilizing Coloc 2 function in Fiji.

**Quantification of infectious DENV.** Immortalized B cells (250,000 cells) were cultured in complete IMDM in 24-well plates on CD40L cell (100,000 cells) monolayer and supplemented with rhIL-21. Irradiated CD40L-expressing L cells (350,000 cells, to account for total cell number in immortalized B cell conditions) cultured in complete IMDM, and Vero cells (350,000 cells) cultured in OPTI-MEM 2% FBS and GlutaMAX, were included as negative and positive controls respectively. Cells were exposed to DENV at MOIs of 10 or 0.1 at 37C for 24 hours or 1 hour as described in figure legends. Infected cells were then washed three times with DPBS (Invitrogen, cat. no. 14190-144) and cultured in complete IMDM+ rhIL-21 at 37C for an additional 24, 48, 72, or 96 hours. Supernatants were collected at each timepoint and frozen at -80C until further use. Supernatants were then diluted in a range of 10-fold dilutions (neat,  $10^{-1}$  to  $10^{-7}$ ) in virus titration diluent (Opti-MEM- no glutamax (Invitrogen cat. no. 31985-070), 2% heat inactivated FBS, and 10mL L-glutamine 200mM). 100 $\mu$ L of each dilution were applied to confluent Vero cell monolayers in 24 well plates for 1 hour at 37oC, followed by addition of 1 mL of overlay media (Opti-MEM + Glutamax (Gibco, cat. no. 51985.091), 5g Methylcellulose, 2% heat inactivated FBS, and 0.5 mL Gentamicin 50 mg/mL (Gibco, cat. no. 15750.060)) and incubation for 4 days at 37C. Next, overlay medium was removed by flicking and tapping plates, and monolayers were washed twice with 1 X PBS, and fixed with 50%/50% Methanol/Acetone for 40 min at 4C, followed by blocking for 10 min with Antibody Dilution Buffer (5% Oxoid non-fat dry milk in 1 $\times$  PBS), washed again twice with

PBS, and incubated with 4G2 (0.8 ng/mL, HB112 hybridoma from ATCC) in blocking buffer, followed by incubation at 37°C for 1 hour. Plates were then washed twice with PBS and incubated with HRP-labeled secondary goat anti-mouse antibody (1:2000, KPL, cat. no. 074-1806) at 37°C for 1 hour, washed with PBS, air-dried for 5 minutes, and treated with 160 µL TrueBlue substrate (SeraCare, cat. no. 5510-0030), allowing foci to develop for ~15 minutes. The developed plaques were then counted by eye, and virus titers calculated as previously described [38].

***Statistical Analysis.*** All statistical analyses were performed using GraphPad Prism Software (GraphPad Software, La Jolla, CA, USA). A P value <.05 was considered significant. ANOVA was used for the comparison of multiple conditions.

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**Data availability.** The authors confirm that the data supporting the findings of this study are available and included within this article.

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## RESULTS

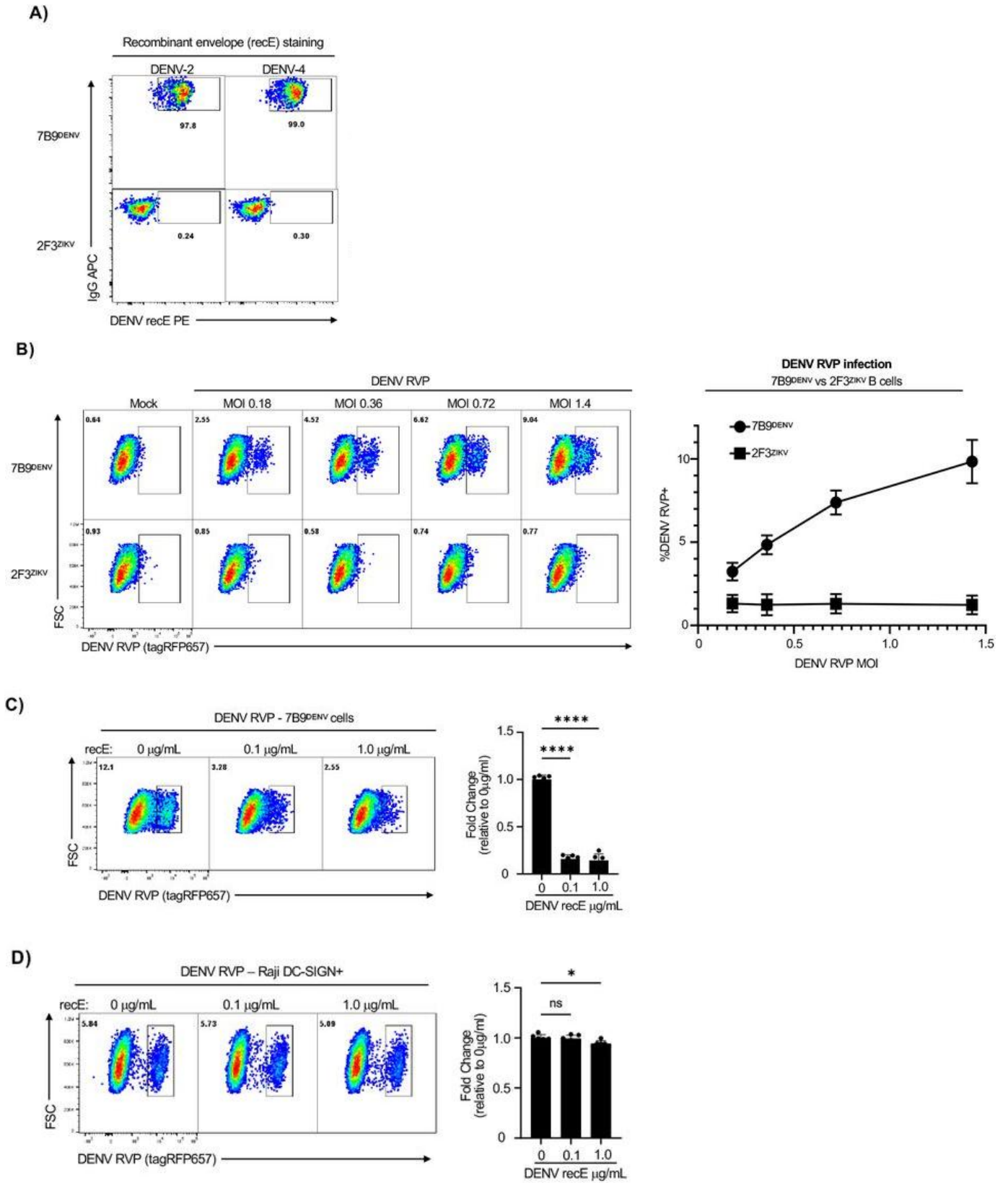
***BCR antigen-specificity determines DENV susceptibility of B cells.*** In our prior work, we demonstrated that ectopic expression of DENV-specific BCRs rendered normally non-infectible cells highly susceptible to DENV infection [26]. Furthermore, we determined that immortalized human B cell lines naturally expressing DENV-specific BCRs were uniquely and potently susceptible to DENV infection [26]. To further define the mechanistic requirements for this BCR-dependent infection process, we further examined these flavivirus-reactive immortalized B cells, generated from individuals previously infected with DENV-1 and DENV-2 (7B9<sup>DENV</sup> cells) or Zika virus (ZIKV, 2F3<sup>ZIKV</sup> cells).

Consistent with the binding specificity of the secreted antibody produced by these cells [26], we observed robust binding of recombinant DENV-2 and DENV-4 stabilized E dimer proteins (recE) [28] to the surface of the 7B9<sup>DENV</sup> cells, but not 2F3<sup>ZIKV</sup> cells (**Fig. 4.1A, and Sup. Fig 4.1**). Mechanistically, if this epitope/paratope interaction contributed to the process of BDE, we hypothesized that blocking DENV-E protein binding sites on the BCR of DENV-specific B cells, would result in a reduction in the susceptibility of DENV-reactive B cells to DENV infection. To facilitate the specific quantification of productive infection – rather than antigen-binding/uptake – we utilized a DENV-pseudotyped reporter virus particle (RVP) platform. This system encodes a fluorescent reporter expressed only upon successful cytoplasmic entry and translation, allowing specific quantification of productive infection rather than surface binding alone [29, 30]. Consistent with our previously published results [26], the 7B9<sup>DENV</sup> cells were

highly susceptible to DENV RVP infection, while the 2F3<sup>ZIKV</sup> cells exhibited no RVP infection across all tested infection conditions (**Fig. 4.1B, and Sup. Fig. 4.2**).

We next incubated 7B9<sup>DENV</sup> cells with recE prior to the addition of DENV RVP. As hypothesized, we observed a dose-dependent reduction in the frequency of RVP-infected cells in the presence of recE blockade (**Fig. 4.1C**). To confirm this reduction in infection was due to the BCR specificity of the 7B9DENV cells and not due to other antagonistic interactions, we repeated this recE blockade in a known DENV-susceptible Raji DC-SIGN<sup>+</sup> cell line. DC-SIGN/DC-SIGNR-mediated DENV uptake, unlike BCR-mediated uptake, depends on low-affinity, high-valency interactions with E protein carbohydrates rather than specific epitope recognition [31, 32]. Accordingly, we predicted— in contrast to what was observed for BCR-mediated infection – that DC-SIGN/DC-SIGNR-mediated infection would be minimally impacted by the exogenous addition of soluble recE. Consistent with this model, we only observed a modest reduction in RVP infection of DC-SIGNR expressing Raji cells (**Fig. 4.1D**), emphasizing the specificity and potency of BCR-mediated DENV uptake and subsequent infection.

**Figure 4.1**



**Figure 4.1. BCR-dependent enhancement in DENV-reactive B cells is highly sensitive to DENV Envelope-specific interactions.** **A)** Binding of conformational recombinant E dimers (recE) from DENV-2 (left) and DENV-4 (right) to 7B9<sup>DENV</sup> and 2F3<sup>ZIKV</sup> immortalized B cells. **B)** Flow cytometric analysis of DENV-4 pseudotyped reporter virus particle (RVP) titration on 7B9<sup>DENV</sup> and 2F3<sup>ZIKV</sup> cells. Data collected 24 hours post infection. **C)** Flow cytometric analysis of DENV-4 RVP infected 7B9<sup>DENV</sup> cells pre-treated with 0.1 mg/mL or 1.0 mg/mL of DENV-2 recE before addition of DENV-4 RVP. **D)** Flow cytometric analysis of Raji DC-SIGN+ cells pre-treated with 0.1 mg/mL or 1.0 mg/mL of DENV-2 recE before addition of DENV-4 RVP. Error bars +/- SEM. ns = not significant, \*  $P < 0.05$ , \*\*\*\*  $P < 0.0001$ , One-way ANOVA with Dunnett's multiple comparisons test, with a single pooled variance. Experiments were conducted across two biological replicates in triplicate.

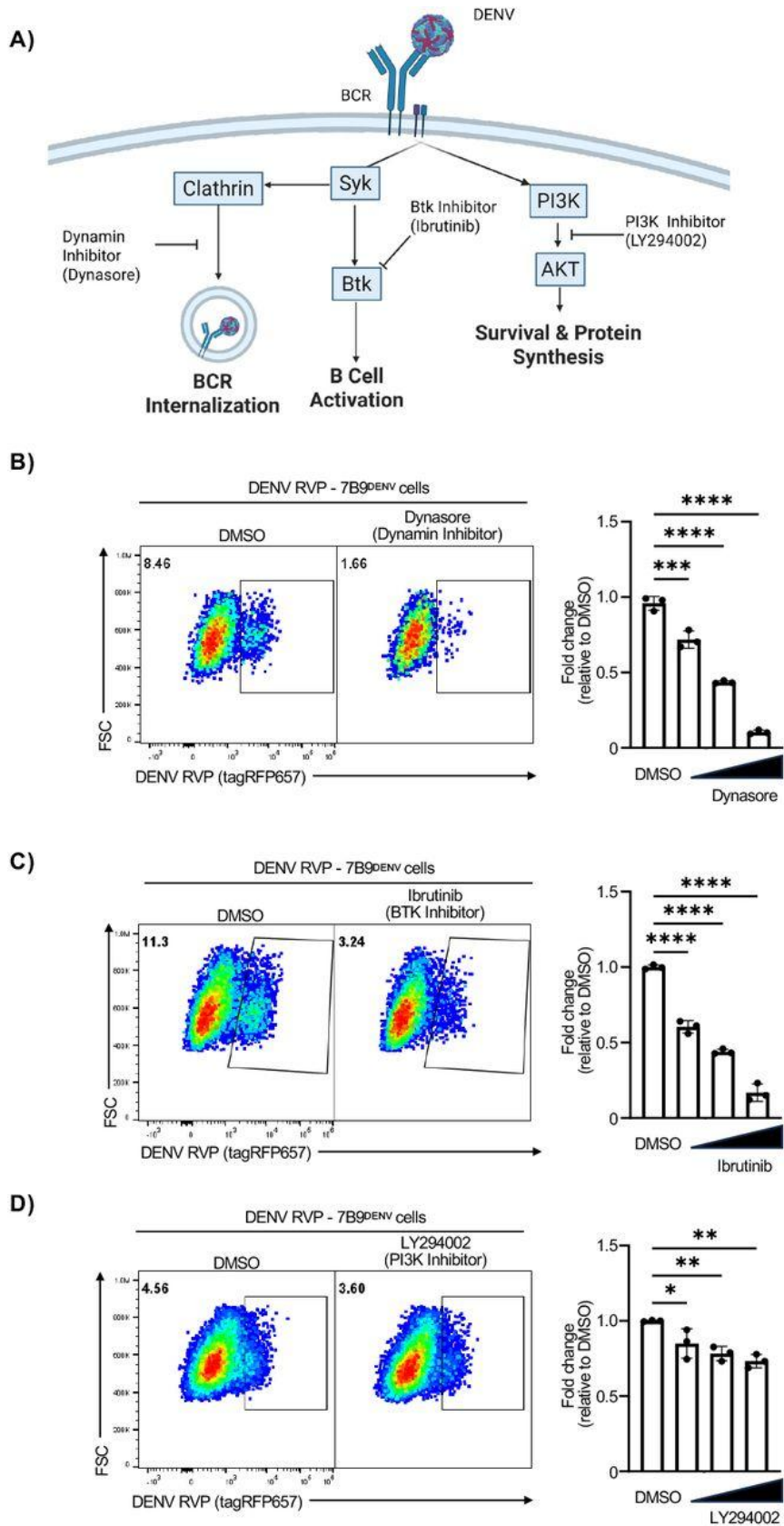
***BCR signaling is required for BDE-mediated viral entry.*** Having demonstrated the role for BCR specificity in DENV infection of immortalized human B cells, we next sought to define the cell-intrinsic signaling and biophysical processes required for BDE. We posited that BCR-mediated infection of DENV-specific B cells would be dependent on canonical B cell signaling and endocytic processes including dynamin-mediated endocytosis and Bruton's tyrosine kinase (BTK) signaling. However, we predicted that other downstream BCR signaling processes not directly involved in endocytosis – such as PI3K signaling – would be dispensable for the initial entry of DENV into the B cell, although these pathways may be involved in regulating the permissivity of the cell to DENV infection due to their role in regulating protein translation and cell cycle progression.

To address these questions, we utilized pharmacological inhibitors of endocytosis and proximal BCR signaling, specifically targeting dynamin-mediated endocytosis, Bruton's tyrosine kinase (BTK) signaling, and phosphoinositide 3-Kinase (PI3K) signaling (**Fig. 4.2A**). 7B9<sup>DENV</sup> cells were pre-treated for 30 minutes with the indicated inhibitors prior to the addition of the DENV RVP followed by an additional 4 hour incubation in presence of inhibitors for infection. Following extensive washing and trypsin-treatment to remove any remaining surface bound RVP, cells were incubated for an additional 20 hours and analyzed by flow cytometry to quantify the frequency of DENV RVP infected cells.

Consistent with our hypothesized mechanism of action for BDE, inhibition of dynamin-mediated endocytosis with the drug Dynasore resulted in a significant and dose-dependent reduction in DENV RVP infected 7B9DENV cells (**Fig. 4.2B**). This

observation is consistent with the endocytic pathways utilized by DENV in other cell types [33, 34]. Next, we sought to assess the contribution of proximal BCR-specific signaling on BDE and observed a similar reduction in the frequency of RVP infected cells upon inhibition of BTK signaling with ibrutinib (**Fig. 4.2C**). However, only a modest – albeit statistically significant - reduction in RVP infection was observed in cells treated with the PI3K inhibitor LY294002 (**Fig. 4.2D**), despite significant reduction of downstream ribosomal S6 phosphorylation (**Sup. Fig. 4.3A-C**). This suggests that while PI3K activity can be induced following BCR ligation by DENV, it is mostly dispensable for the initial infection of B cells via BDE. Notably, cell viability was not significantly impacted in the treated cells, with the exception of the highest concentration of Dynasore (**Sup. Fig. 4.3D**). To confirm the reduction in DENV RVP signal observed upon drug treatment was not due to an overall decrease in protein translation or altered cellular homeostasis, we also quantified expression of GFP within the 7B9<sup>DENV</sup> cells, which is driven by a constitutive promoter contained within the retrovirus utilized to immortalize the cells. GFP expression remained stable across all conditions, indicating that the observed reductions in RVP signal resulted from decreased viral entry rather than global disruption of protein translation or cellular homeostasis (**Sup. Fig. 4.3E**).

Figure 4.2



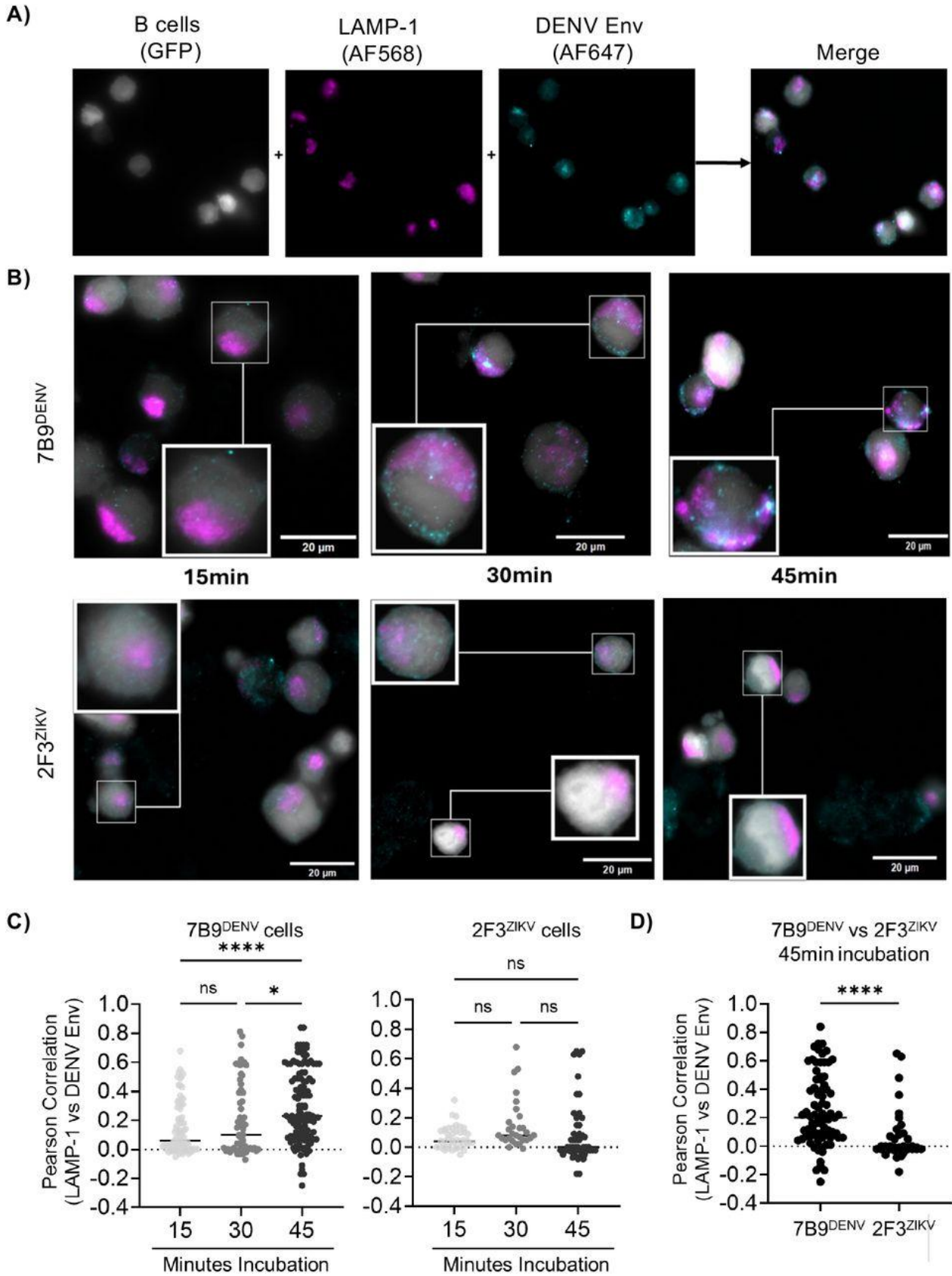
**Figure 4.2. Proximal BCR signaling required for viral entry in DENV-reactive B cells. A)**

Schematic representation of BCR signaling pathways and targets of pharmacological inhibitors. Cells were pre-treated with selected inhibitor for 30 minutes prior to the addition of DENV-4 RVP. **B)** Flow cytometric analysis of DENV-4 RVP infection of 7B9<sup>DENV</sup> B cells pre-treated with dynamin inhibitor Dynasore. **C)** Representative flow plots of DENV-4 RVP infection of 7B9<sup>DENV</sup> B cells pre-treated with BTK inhibitor ibrutinib. **D)** Flow cytometric analysis of DENV-4 RVP infection of 7B9<sup>DENV</sup> B cells pre-treated with PI3K inhibitor LY294002. Error bars +/- SEM. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ , One-way ANOVA with Dunnett's multiple comparisons test, with a single pooled variance. Data are representative of two biological replicates each performed with 3 technical replicates.

**Endosomal localization of DENV in B cells.** Having demonstrated the importance of BCR specificity and proximal BCR signaling to the process of BDE, we investigated the timing and subcellular trafficking of DENV following BCR engagement. We hypothesized that DENV/BCR engagement would lead to receptor internalization and subsequent localization to the late endosome, a necessary part of the DENV entry lifecycle.

7B9<sup>DENV</sup> and 2F3<sup>ZIKV</sup> cells were incubated with DENV-2 at 4C for 1 hour to allow for surface binding of the virion, but not internalization. Cells were then washed, plated, temperature shifted to 37C, and subsequently fixed at 15-, 30-, and 45-minutes post temperature shift and analyzed DENV localization and endosomes by immunofluorescence microscopy using 4G2 and LAMP-1 staining, respectively. (**Fig. 4.3A, 4.3B**). We observed a significant increase in DENV-2 and LAMP-1 colocalization over time in the DENV-specific 7B9<sup>DENV</sup> cells compared to the 2F3<sup>ZIKV</sup> cells (**Fig. 4.3B-D**). Over time, we saw diffuse DENV signal begin to form condensed puncta alongside LAMP-1 signaling inside these cells, with strong colocalization at 45 minutes post temperature shift. Despite higher levels of background signal, likely due to non-specific, low affinity binding to cells, we did not observe any significant changes in DENV-2/LAMP-1 localization in the 2F3<sup>ZIKV</sup> cells (**Fig. 4.3B-D**). These results show that over time, DENV-2 bound to antigen-specific BCRs become internalized and subsequently localize to late endosomes.

**Figure 4.3**



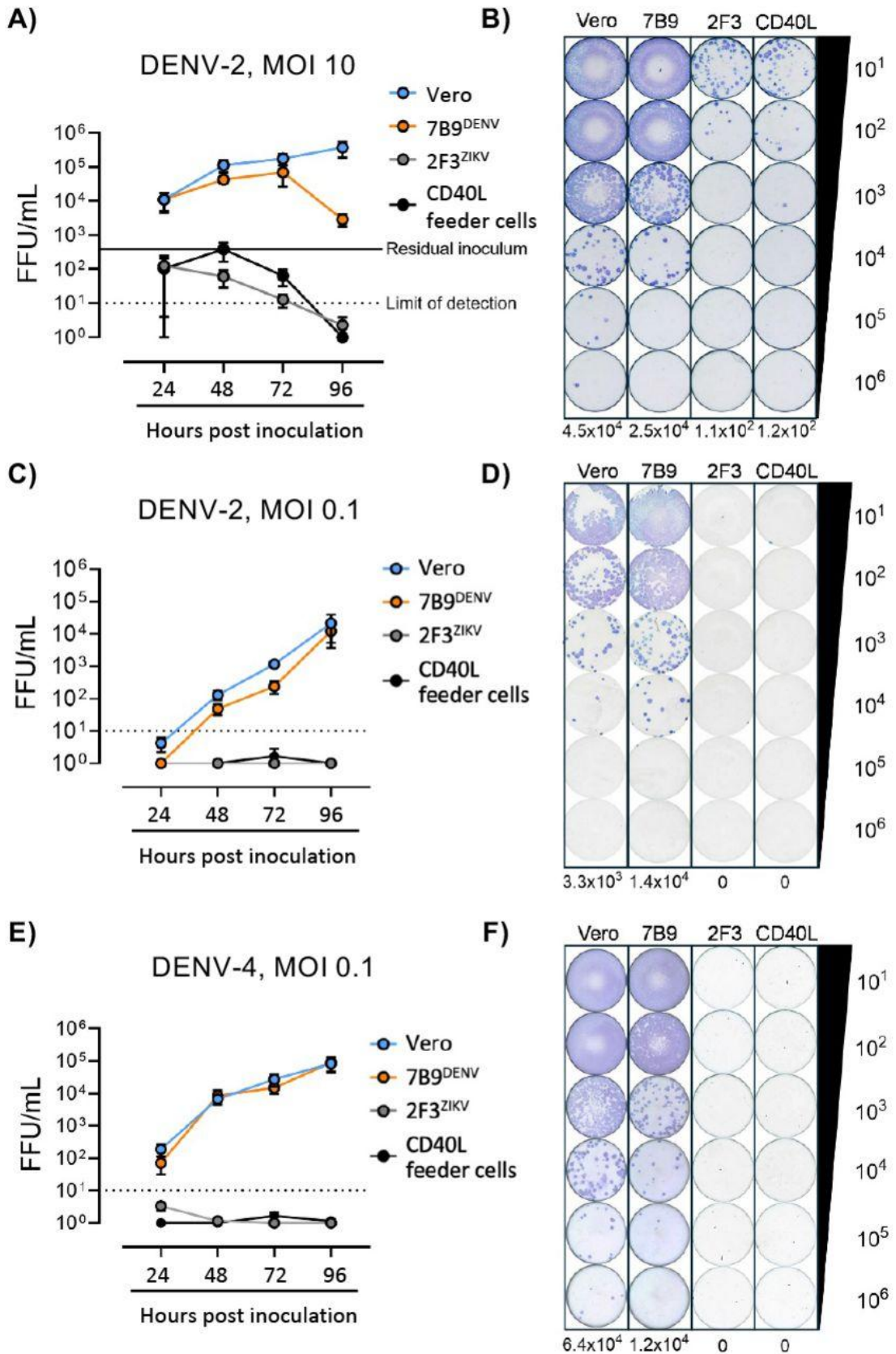
**Figure 4.3. Internalization of DENV by DENV-specific B cells.** **A)** Overlay fluorescent microscopy image of GFP+ B cells (grey) stained for DENV (cyan) and LAMP-1 (magenta). **B)** DENV Internalization. Cells were infected at 4C for 2hr. Cells were fixed 15, 30, or 45min after incubation at 37C to allow for internalization, then permeabilized (1% BSA, 0.1% Triton) overnight. Cells were stained with 4G2 (mouse anti-DENV), and rabbit anti-LAMP-1 for primary staining, followed by anti-mouse (Cyan) and anti-rabbit (Magenta). **C)** Colocalization correlation image of B-cells infected through time. **D)** Colocalization correlation 45min p.i. Correlation was calculated by colocalization analysis using the Fiji image processing package for ImageJ (Coloc2). A minimum of 30 cells from each sample (n = 33 – 125) in different frames were screened, Pearson's R value (no threshold) was scored, then plotted for grouped comparisons. Results represent pooled analysis of two independent technical replicates. ns= not significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . One-Way Anova with correction for multiple comparisons.

***B cells are productively infected with DENV via BCR-mediated infection.*** In our previous work, we demonstrated the presence of positive- and negative-strand DENV RNA in immortalized B cells expressing a DENV-reactive BCR that were exposed to DENV-2 [26]. In contrast, no DENV RNA was detected in B cells expressing a ZIKV-reactive BCR following incubation with DENV-2. These results suggested that B cells may be capable of supporting active DENV replication. Additionally, since BCR signaling was involved in early infection steps and DENV was internalized in 7B9<sup>DENV</sup> cells, we hypothesized that these cells may complete DENV replication, resulting in infectious virions. To test this, we exposed both 7B9<sup>DENV</sup> cells and 2F3<sup>ZIKV</sup> cells to DENV-2 (New Guinea C) at MOI = 10 for 24hrs, consistent with the conditions utilized in our previously published scRNAseq analysis [26]. Irradiated CD40L-expressing L cell fibroblasts used as feeder cells for B-cell maintenance were exposed in a similar fashion as a negative infection control, while Vero cells were utilized as a positive control. Following DENV exposure and washing, cells were incubated to allow for viral replication and supernatants inoculated onto Vero cells to determine presence of infectious DENV by a focus forming unit assay (FFU). Strikingly, we observed robust DENV-2 production from the 7B9<sup>DENV</sup> cells starting 24 hours after initial inoculation, similar in magnitude to what was observed from inoculated Vero cell cultures (**Fig. 4.4A, 4.4B**). There was also substantial infectious DENV-2 observed in 2F3<sup>ZIKV</sup> cell and CD40L feeder cell cultures, likely representing residual inoculum from this high MOI experiment (**Fig. 4.4A, 4.4B**).

Therefore to better characterize the production of infectious DENV from these cells, we next employed more stringent infection conditions to avoid potential

cytotoxicity or cell stress associated with high viral levels and to reduce residual carry-through of viral inoculum. Accordingly, we incubated cells at an MOI of 0.1 with DENV-2 for 1 hour, followed by extensive washing and additional incubation of inoculated cultures for 24, 48, 72, and 96 hours. Under these more stringent conditions, we observed no carry-through viral inoculum (CD40L-feeder cell condition) or production of infectious DENV-2 by 2F3<sup>ZIKV</sup> cells. In contrast, 7B9<sup>DENV</sup> cells exhibited sustained production of infectious DENV-2 at levels similar to that observed for Vero cells inoculated under the same conditions (**Fig. 4.4C, 4.4D**). Given that the BCR of 7B9<sup>DENV</sup> cells binds to DENV-4 in addition to DENV-2, we next assessed the capacity of 7B9<sup>DENV</sup> cells to support replication of DENV-4. Using our stringent infectivity assay of MOI = 0.1 and 1 hr incubation with virus, we observed generation of infectious DENV-4 by 7B9<sup>DENV</sup> cells to levels similar to Vero cells, while no infectious virus was produced by inoculated 2F3ZIKV cells (**Fig. 4.4E, 4.4F**). These results demonstrate that DENV-specific B cells can support the replication of multiple DENV serotypes for at least 96 hours in vitro, producing infectious virus at similar levels to that observed from the commonly used Vero cell line.

Figure 4.4



**Figure 4.4. Generation of infectious DENV by DENV-specific B cells. A)** Vero cells, 7B9<sup>DENV</sup> B cells, 2F3<sup>ZIKV</sup> B cells, or CD40L-expressing fibroblast feeder cells were inoculated with DENV-2/NGC at MOI = 10 for 24 hrs at 37C, washed and cultured for the indicated times. Infectious virus titers in supernatants was determined by passage on Vero cells and assessed using a standard focus assay and data presented as focus forming units (FFU)/mL. **B)** Representative DENV-2 focus assay at MOI = 10 inoculum from 72 hr supernatants. **C)** Cells were infected with DENV-2/NGC at MOI = 0.1 for 1 hr at 37C, washed and cultured for determination of viral titers on Vero cells in supernatants at the indicated timepoints following infection. **D)** Representative DENV-2 focus assay at MOI = 0.1 inoculum from 72 hr supernatants. **E)** Cells were infected with DENV-4/Dominica81 for 1 hr at MOI = 0.1 at 37C followed by washing, and determination of viral titer on Vero cells at the indicated timepoints after culture. **F)** Representative DENV-4 focus assay at MOI = 0.1 inoculum from 72 hr supernatants. Experiments were performed in triplicate with two biological replicates and mean  $\pm$  standard deviation is shown. Dashed lines in A, C, E indicate FFU assay limit of detection. Solid line in A indicates residual carry-through of viral inoculum.

## DISCUSSION

In this study, we provide direct evidence that dengue virus (DENV) can productively infect human B cells in a fashion dependent on B cell receptor (BCR) specificity and proximal BCR signaling. These findings expand the current paradigm of dengue immunopathogenesis, which has historically emphasized antibody-dependent enhancement (ADE) in FcγR-bearing myeloid cells, by providing mechanistic insight into how B cells themselves serve as a cellular reservoir for DENV replication. Previous studies have reported non-neutralizing DENV-specific antibodies facilitating ADE, but the role of B cells as direct targets of infection has remained underappreciated. By demonstrating productive replication and infectious particle release from DENV-specific B cells, our work provides mechanistic support for clinical observations of B cell tropism.

These results support a growing body of literature emphasizing B cells as the principal circulating cellular reservoir of DENV-infected cells [12, 19-24]. Our findings work in conjunction with existing models of ADE, expanding on our understanding of DENV cellular tropism and explaining why we see such a large prevalence of DENV-infected, but non-ADE susceptible cells in circulation during acute illness. We posit that these highly susceptible B cells generated after the initial exposure aid in the establishment of DENV infection during secondary infection, where the frequency of these highly susceptible cells, paired with other risk factors such as waning neutralizing antibody titers could synergize to increase the risk of severe disease.

While this study provides additional mechanistic insight into BDE, several facets of the phenomenon require further characterization. Although we observed that proximal BCR signaling is required to establish infection, it remains unclear whether BCR signaling also influences the intrinsic permissivity of DENV-specific B cells to DENV infection. An analogous process, referred to as "intrinsic" ADE, has been described in which differential signaling through the FcR induces metabolic changes that create a more favorable environment for DENV replication. Understanding how BCR engagement impacts this process will be important, particularly given our observation that BTK inhibition decreases viral entry. Although we observed that PI3K inhibition did not affect entry, DENV can induce or dysregulate PI3K activation in other cell types [35]. Given PI3K's critical role in regulating cell survival, cellular metabolism, and protein translation, this pathway likely influences DENV replication and maturation within BDE-infected cells. Beyond these intrinsic effects, it will also be important to understand how DENV infection via BDE impacts B cell function and immunologic memory, particularly given the shift from poorly neutralizing, type-specific responses to broadly neutralizing, cross-reactive responses observed following secondary infection [36].

There are some limitations to this study that highlight the need for additional analysis. Namely, the DENV-specific human B cells utilized for this analysis were immortalized by retroviral transduction. These cells were indispensable for the analysis described in this manuscript due to the specificity of their BCR, which allowed for minimal manipulation, as well as their functional BCR signaling [26, 37]. However, these immortalized IgG memory cells are maintained in a partial plasmablast-like state through

persistent culturing alongside CD40L-expressing cells and IL-21 [26, 37]. These in vitro cultures do not recapitulate the heterogeneity of primary human B cells, requiring further characterization in more biologically relevant models. Additionally, BCR engagement and internalization activate a complex signaling network; further study of other BCR signaling components—such as Lyn, Syk, SHP1, and Ca<sup>2+</sup> flux—is required to understand the full impact of BCR signaling on DENV entry. Finally, the downstream consequences of B cell infection, including effects on antibody production, apoptosis, and immune regulation, remain to be elucidated.

In conclusion, this study has further characterized the phenomenon of BCR-dependent enhancement as a novel mechanism of viral entry for DENV. This mechanism expands our understanding of how immunologic memory shapes DENV susceptibility, and how the presence of DENV-specific memory B cells may influence an individual's risk of infection or progression to severe disease. This study highlights the need to understand not only the contribution of antibodies as mediators of protection and pathogenesis in dengue, but how B cells themselves may contribute to risk and protection from DENV infection.

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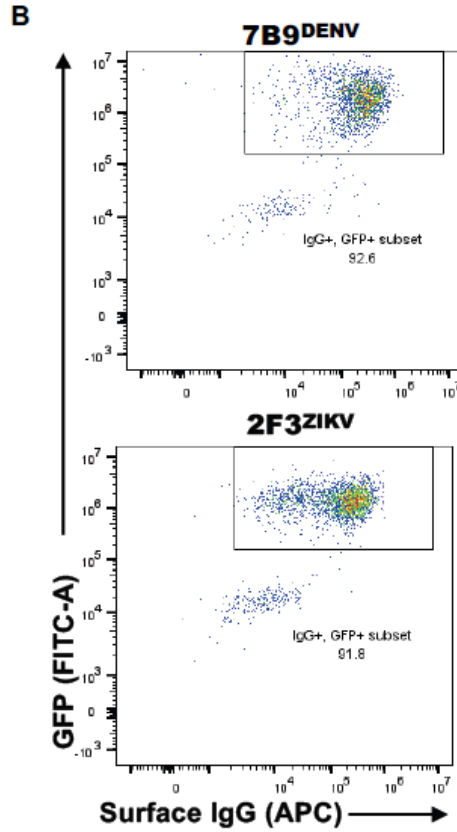
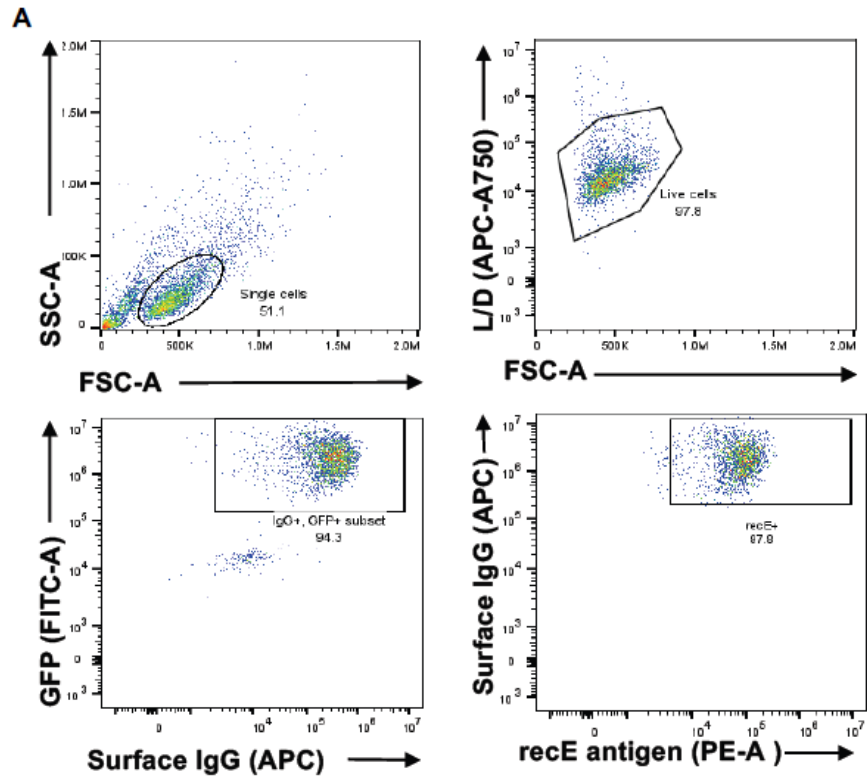
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Supplemental Figure 4.1

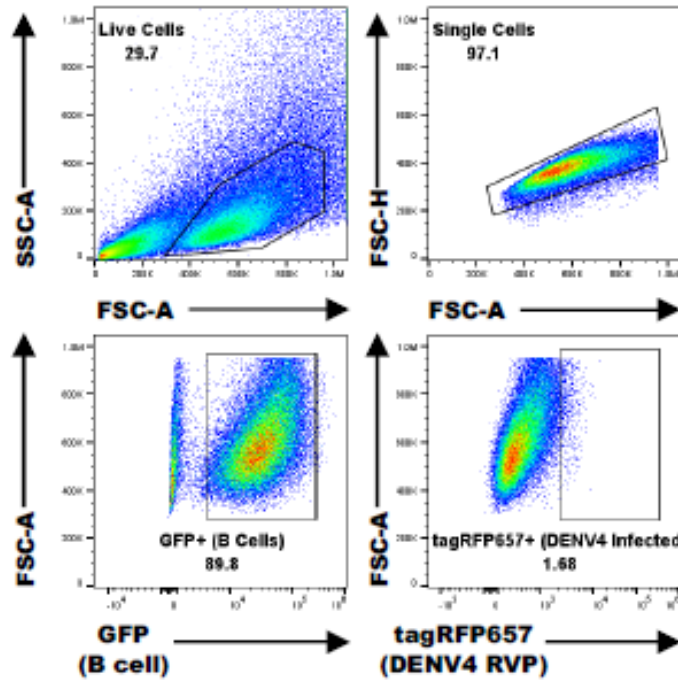


**Supplemental Figure 4.1. Flow Cytometry Gating of immortalized B cells and DENV**

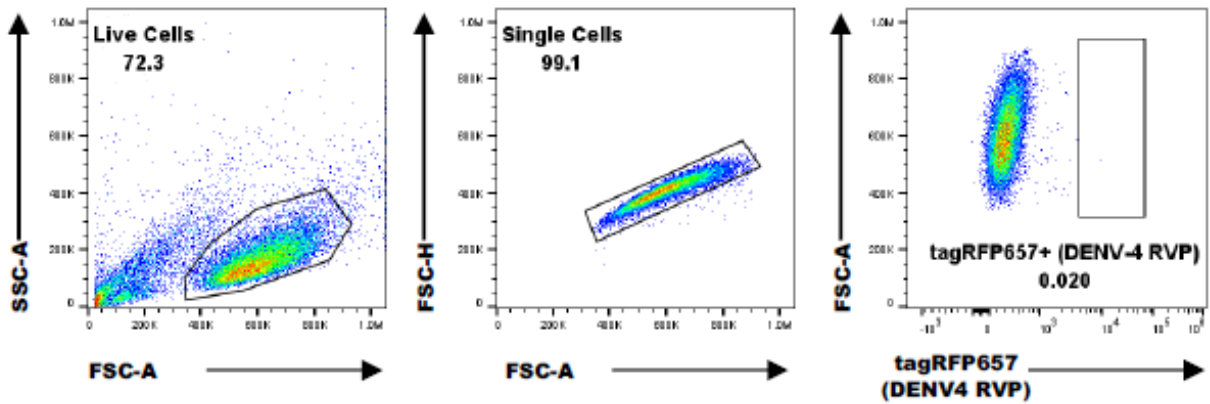
**recE dimer. (A)** Representative flow plots of gating utilized for DENV-2 and DENV-4 recE binding experiments in immortalized B cells. Immortalized cells are incubated with L/D APC-750 for 40min, washed, then incubated with 100ng of either recE-DV2 or recE-DV4 on ice for 30min. Followed by wash and final staining with anti-human IgG-APC for 30min. DENV recombinant envelope protein stabilized dimer (recED) is incubated with Streptavidin-PE for 30min before staining. **(B)** GFP and Surface IgG (BCR) is expressed in both sets 7B9DENV and 2F3ZIKV- cells.

Supplemental Figure 4.2

**A**

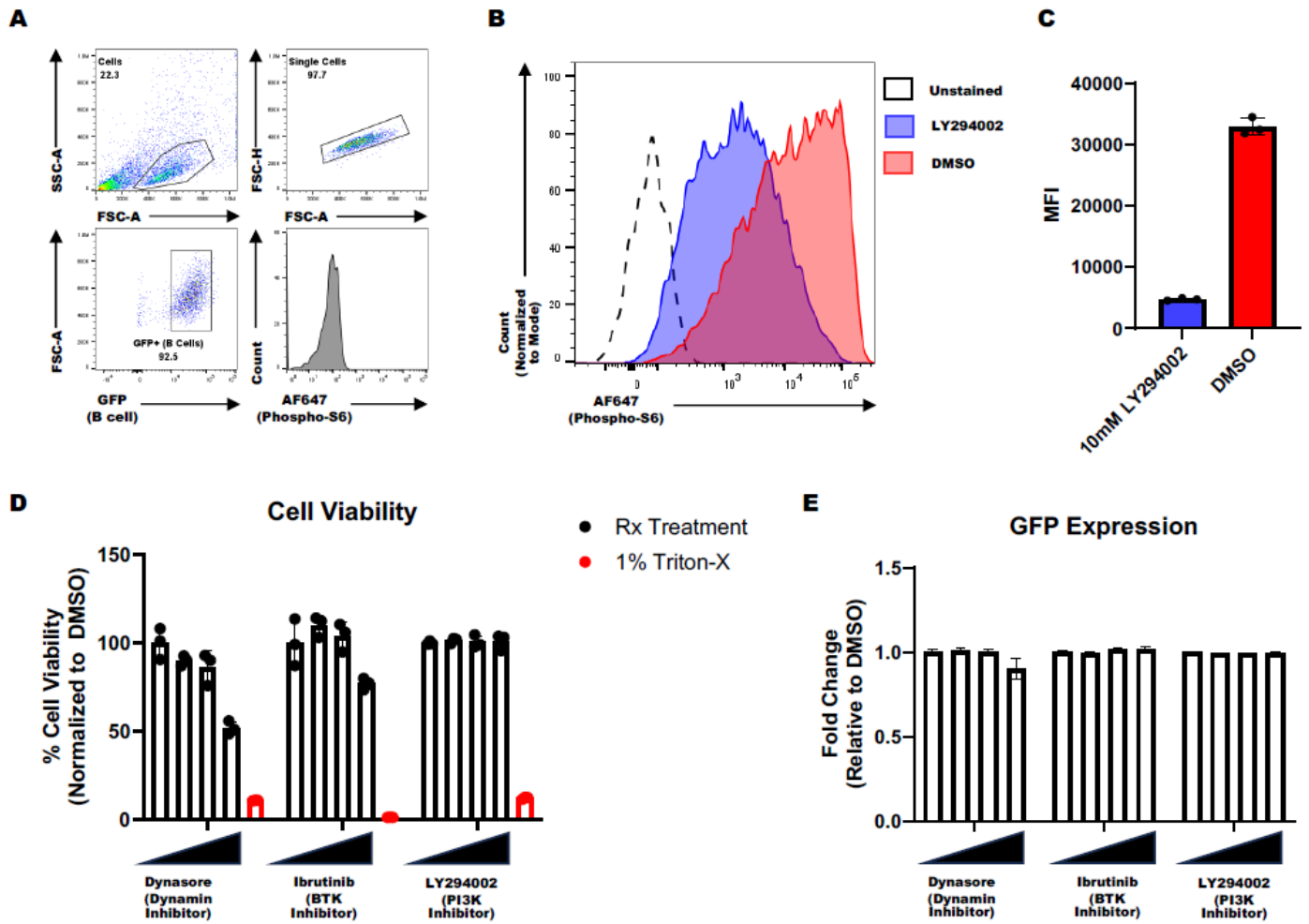


**B**



**Supplemental Figure 4.2. Flow Cytometry Gating of immortalized B cells and Raji DC-SIGN+ Cells. (A)** Representative flow plots of gating utilized for DENV-4 infection experiments in immortalized B cells. Immortalized B cells are co-cultured with CD40L expressing L cells, immortalized B cells are identified by GFP expression. DENV4 RVP Infection detected by expression of tagRFP657. **(B)** Representative flow plots of gating utilized for DENV-4 infection experiments in Raji DC-SIGN+ cells.

### Supplemental Figure 4.3



**Supplemental Figure 4.3.** *Characterization of pharmacologic inhibitors in immortalized B cells.* **(A)** Representative gating on immortalized B cells to detect PI3K activation quantified by expression of phosphorylated ribosomal protein S6. Detected using AF647 labelled anti-human phospho-S6 monoclonal cupk43k. **(B)** Representative histogram detecting phosphorylated S6 in immortalized human B cells treated with 10uM PI3K inhibitor LY294002 for 30 minutes. **(C)** Bar graph quantification of the mean fluorescence intensity of AF647 labelled anti-human phospho-S6 monoclonal cupk43k in DMSO (red) vs LY294002 treated (blue) cells. **(D)** Bar graph quantification of cell viability by MTT assay in inhibitor treated cells. Cells treated for 4 hours with: DMSO, Dynasore (12.5µM, 50µM, 200µM), Ibrutinib (1.56µM, 12.5µM, 50µM), LY294002 (1.56µM, 12.5µM, 50µM), or 1% Triton (red). **(E)** Bar graph quantification of GFP expression in immortalized B cells following treatment with inhibitors described in D. Data collected 24 hours after treatment. Error bars +/- SEM. Experiments were conducted across single biological replicate in triplicate.

**CHAPTER 5**  
**DISCUSSION**

## Summary of Findings

The purpose of this dissertation is to identify a novel mechanism surrounding B cell infection by DENV and begin to characterize the early molecular and biophysical requirements of BCR-dependent enhancement. In this dissertation I have shown: 1) expression of a DENV-specific BCR is capable of rendering a cell susceptible to DENV-infection; 2) this BCR-mediated infection correlates with its secreted form's ability to mediate ADE; 3) the pool of DENV-susceptible B cells increases following an individual's first exposure to DENV; 4) infection of DENV-reactive B cells is highly sensitive to DENV envelope protein-specific interactions; 5) Proximal BCR signaling is necessary for BCR-mediated entry; 6) DENV localizes to endosomes during early stages of DENV infection in DENV-reactive B cells; 7) DENV-reactive B cells are productively infected by DENV.

This understanding of B cell infection works to reconcile differences the field has observed with in vivo DENV cellular tropism and the tropism we would expect under the phenomenon of antibody-dependent enhancement. Our observations also bring to light the need to understand the contributions of alternative potential mechanisms of immune-mediated enhancement outside of secreted antibodies within DENV infection. In this section I will discuss the significance of these findings as well as potential implications and future directions based on the data presented in chapters 3 and 4.

## Significance

The mechanism of BCR-mediated entry for DENV or any other pathogen, viral or otherwise, is a fairly novel observation with only a handful of studies describing this phenomenon [1, 2]. Our initial data highlights the feasibility and early requirements of BCR-mediated infection, but raises many more questions. What other pathogens does this apply to? What happens to B cells infected through this route, and how does this play into B-cell mediated adaptive immunity?

Within the DENV field, this data supports the observation that B cells could be a significant circulating reservoir of infectious virus during acute infection [3-5]. Since its early characterization by Halstead and colleagues in the 1970s, much of the focus in the field surrounding immune-mediated enhancement during DENV infection has been on the contribution of antibody-dependent enhancement [6-8]. These observations have greatly expanded what we understand about infection within phagocytic cell populations and how route of entry can drastically impact viral fitness and replication. However, focus on ADE has left some significant observations to remain understudied, including that of B cell infection. With the understanding on how antibodies can impact infection, it is also important to understand how those antibody-producing B cells are impacted by infection in order to get proper insight into the contributing risk factors to severe disease and develop appropriate countermeasures and antiviral therapies to combat the expanding threat of dengue virus.

## Limitations

### *Cell Model*

One of the major limitations within the data presented in this dissertation is that the bulk of the work characterizing BCR-dependent enhancement is performed within IgG memory B cell that were immortalized through retroviral transduction of BCL-6 and BCL-xL that are cultured alongside CD40L expressing cells in the presence of IL-21. BCL-6 functions as a transcriptional repressor, specialized in B cell survival through suppression of the response to DNA damage which is inherent to the process of somatic hypermutation and class-switching [9]. BCL-xL directly functions as an anti-apoptotic protein where it inhibits apoptotic activity through competitive interactions with BAK and BAX, interactions with  $Ca^{2+}$  channels to prevent  $Ca^{2+}$  mediated apoptotic signaling, and has been implicated in suppression of autophagosome activity though further study is needed [10]. Both of these proteins function in the survival of these immortalized B cells. While the direct interactions we have observed may not be impacted, these may mask any apoptotic effect during infection. The combination of CD40 engagement and IL-21 skew these B cell responses to adopt a profile similar to antibody secreting plasma cells. This treatment pre-emptively activates these immortalized B cells and likely results in a differential metabolic profile compared to that of quiescent memory B cells. While CD40 engagement is not required for BCR-mediated signaling, it has been shown to synergize with BCR engagement to enhance B cell activation [11]. These interactions potentially set up a more intrinsically beneficial environment to allow for DENV infection and replication.

Conversely, we have shown some direct infection data in primary B cells through cross-linking DENV to BCRs via anti-Ig antibodies. However, this method likely leads to the formation of large immune complexes, significantly increasing the levels of BCR crosslinking than would occur through direct binding of virus with antigen-specific BCRs. Additionally, the data presented in chapter 3 evaluating primary B cell susceptibility within patient derived PBMC utilizes DENV-pseudotyped reporter virus. The BCR interactions with these virions would mimic interactions with live DENV, as they are composed of the same structure proteins and differ only in the encoded genome, supporting that virus alone would be sufficient to mediate infection. Taken in hand, further work is needed in order to describe the initial entry within resting primary B cells.

### *BCR Isotype*

The majority of this study focused on IgG expressing B cells. This makes sense from an initial characterization standpoint because of the essential role IgG plays in antibody-dependent enhancement. However, memory B cells can also be found to express other BCR isotypes, including IgA and IgM. While IgA has been shown to have a protective role in inhibiting ADE, likely due to the reduced capacity for IgA to interact with Fc $\gamma$ Rs and the anti-inflammatory nature of the Fc $\alpha$ R [12, 13]. Because of this, we cannot assume that IgA isotype BCRs will be unable to mediate this effect. Within the 293T data presented in Chapter 3, we expressed transmembrane IgA and IgM versions of

VDB33 (a DENV envelope domain II specific antibody). We observed that each isotype of these were capable of mediating BDE, even though their counterparts are insufficient to mediate ADE. This does have a caveat because this model system is not within a B cell and so does not contain the appropriate downstream BCR signaling and activation. These preliminary results highlight the need to understand the breadth of B cells susceptible to BDE.

### *Alternative Methods of Entry*

Our data focuses on BCR-mediated infection. However, this likely is not the sole means of DENV entry into B cells. As mentioned previously, CD300a has also been implicated in B cell infection [5]. In addition, the proportion of B cells found to be infected during acute illness is quite significant, ranging from 20-60% of circulating B cells [14-17]. After initial exposure to a pathogen and the B cell response has equilibrated, the frequency of antigen-specific memory B cells is typically around 0.01-0.1% of the circulating B cell population [18, 19]. Following exposure to an antigen, memory B cells migrate to germinal centers within secondary lymphoid organs, where they undergo robust clonal expansion. Even with this expansion, DENV-specific B cells during acute infection will likely not comprise the majority of the B cell population, though some studies project up to 30% of the circulating lymphocyte population can be composed of DENV induced plasmablasts [20]. Multiple factors may impact population proportions within specific compartments. A characteristic of acute DENV illness is

lymphopenia, causes of which can include bone marrow restriction, migration to sites of infection, and cell death [21, 22]. Depending on if DENV infection directly influences B cell migration and survival, this could dramatically alter the proportions of cells we see in circulation or within the germinal centers.

### **Is BDE unique to DENV?**

One of the natural progressions of identifying antigen-specific BCRs as a potential viral entry receptor is understanding if this phenomenon is unique to dengue virus or if it may be seen in other types of infection. The general process of receptor mediated viral entry involves: 1) attachment to cell via receptor interactions; 2) endocytosis of the receptor/virus complex into the cell; 3) escape from the endosome [23]. As discussed in chapter 2, a cognate receptor has not been identified for dengue virus. While domain III of its envelope protein has been implicated in receptor recognition, ADE has been shown to expand the interactions capable of mediating infection. It has been shown that attachment of DENV to alternate receptors utilizing bispecific antibodies that have been fragmented to remove the Fc region (responsible for binding to Fc receptors) is sufficient to mediate DENV infection of a cell. The study targeted receptors such as  $\beta 2$  microglobulin, CD15, and CD33 and suggested that Fc $\gamma$ R signaling is not necessary to DENV entry [24]. Once internalized, the signal to trigger DENV fusion with endosomal membrane is a drop in pH, where it is then able to release DENV genome in the cell

cytosol [25]. DENV requires few receptor interactions in order to mediate entry into the cells.

For other viruses to support BCR-mediated infection, they will likely need limited host-receptor interactions to initiate entry and fusions, as well as require B cell permissivity in order to support active viral replication within these cells. However, these features may not necessarily be a requirement. The epitope bound by the BCR may be able to function as the receptor binding site necessary for conformation changes required by other viruses. Or downstream BCR signaling may be able to render the B cell permissive to infection when normally it may not be able to support viral entry. As mentioned earlier, there are limited studies involving other viruses that have shown to be capable of infecting B cells via antigen-specific BCRs, both influenza virus and pseudotyped lentiviruses [1, 2]. While B cells appear to be susceptible to infection by influenza virus, the study showed this infection does not appear to be productive and leads to rapid B cell death within 18 hours post exposure to virus [1]. Lentiviruses have been shown to be pseudotyped to allow preferential cell infection and have broad cellular tropism in terms of what cells are capable of supporting lentiviral replication which is why they are an important tool in retroviral transduction, in addition to their efficiency as retroviruses to integrate into host DNA [26, 27]. Other flavivirus like West Nile Virus and Zika Virus would be likely candidates of being able to mediate this BCR-mediated infection as these also have broad receptor recognition and cellular tropism. Other likely candidates would also be viruses that are capable of supporting ADE like cocksaackie virus, SARS-CoV-2, and ebola virus [28-30]. ADE with these viruses has only

been shown in vitro and prior immunity is not implicated in their pathogenesis, with little evidence of tropism within B cells making them unlikely targets in vivo for active infection via BDE. It is possible B cell pool might not support active viral replication of a particular pathogen but entry could still impact B cell function and shape how immunity is generated.

These findings in relation to BCR-mediated infection are novel to the field. Requiring further characterization to understand the requirements for pathogens to subvert the antigen recognition function of the B cell receptor. While I have described this process as requiring promiscuous receptor recognition, there are many potential factors at play. It is possible many pathogens are able to utilize this route of infection. This could go unnoticed for a variety of reasons: existing dogma on cellular tropism or disease pathogenesis narrowing the focus on what cell populations to study; the rarity of antigen-specific B cells masking the impact of this population on infection; or even the potential that infection of these B cells might not produce a phenotype that is able to contribute meaningfully or significantly to disease pathogenesis.

### **Functional Consequence of B cell Infection**

The focus of this dissertation has been surrounding how a subset of the B cell population may become infected. As well as provide evidence that B cells are capable of supporting DENV replication and production of viral progeny. Much more work needs to be done in order to evaluate and characterize the impact of DENV infection on B cell

survival and function. As key mediators of the adaptive immune response, it is important to understand impacts to their ability to produce antibodies, the impact on generation of immune memory, as well as other functions and how they may contribute to DENV inflammatory pathology. There have been limited studies in B cell infection; however, we can utilize a combination of what has already been characterized in addition to what has been observed in other cell types to speculate on likely outcomes.

### *Survival*

The delicate balance of cell survival and death signals is crucial in any viral infection. DENV has been shown to exhibit both pro- and anti-apoptotic effects within host infected cells. Much of the research across cell types have characterized pro-apoptotic effects of various dengue viral protein. Membrane and capsid proteins have been shown to trigger mitochondria-mediated apoptosis through activation of p53 in epithelial Huh-7 cell line [31]. DENV-2 infection of vascular endothelial cells exhibited increased apoptosis markers within 24 hours of infection including XAF1, cleaved caspase 3, and poly-ADP-ribose polymerase [32]. Conversely, DENV-2 has been shown to modulate downstream signaling of the unfolded protein response via PERK and IRE1 to prevent apoptosis during early DENV infection [33]. DENV has also been shown to mediate the mechanistic target of rapamycin (mTOR) pathway to modulate apoptosis. NS5 has been shown to bind mTORC2 and lead to mTORC2 activation, leading to downstream phosphorylation of AKT to promote cell survival and proliferation [34]. In

megakaryocytes, DENV infection has been shown to dysregulate the mTOR signaling pathway. Where DENV infection led to decreased PI3K activation and subsequently AKT phosphorylation leading to increased markers of apoptosis [35]. When analyzing circulating B cells within individuals experiencing acute DENV infection, they observed increased expression of pro-apoptotic marker CD95 across B cell subsets except antibody secreting cells and that NS1 is capable of eliciting increased CD95 expression [36]. However, this study compared expression of these markers to healthy individuals. A more appropriate control of cells sourced from other febrile patients might provide a better idea on if this is a DENV specific effect as CD95 can be increased following B cell activation [37].

Overall, while DENV has been shown to exhibit anti-apoptotic properties, this is likely to prevent cell death during early DENV replication and allow cell survival until virion production is possible. In Chapter 4, we have shown that PI3K is dispensable during the initial entry of DENV via BDE. More study needs to be done to understand impacts of PI3K on the later stages of the DENV lifecycle. Given what is understood about DENV infection in other cells types, DENV will likely modulate survival through multiple pathways during early replication and will eventually lead death of the infected cell.

### *B cell function*

B cells exhibit a variety of functions in response to encountering their target antigen. After initial binding the antigen is endocytosed initiating activation signals as well as processing of the antigen for presentation on MHC class II, allowing the B cell to function as an antigen presenting cell where it is able to interact with other immune cells like T cells to aid in response against pathogens [38-40]. Following activation, B cells migrate to germinal centers within secondary lymphoid organs in order to undergo rapid clonal expansion, where their progeny are fated to become typically short-lived antibody producing plasma cells or long-live memory B cells [40, 41]. Infection can greatly impact cellular function, so it is important to understand how DENV infection might impact B cell activation, function, and differentiation.

Analysis of circulating B cells during acute infection show evidence of B cell activation, which would be expected during secondary illness. A notable observation with secondary dengue infections is the increased prevalence of plasmablasts during acute illness [42-44]. A study looking at PBMC from dengue patients in Thailand revealed that the secondary plasmablast response is dominated by highly cross-reactive E specific B cells [42]. This is consistent with our observation that BDE-mediated infection follows similar infection kinetics to that of ADE, where highly cross-reactive antibodies are more capable of mediating ADE. It is possible that initial infection of highly cross-reactive memory B cells via BDE could induce proliferation and differentiation of this subpopulation of B cells. This is supported by observation within the Cambodian study conducted by Upasani and colleagues looking at B cell infection. They observed evidence

of increased proliferation of DENV-infected B cells sourced from DENV patients through expression of proliferation marker Ki-67. Additionally, during in vitro infection they observed an increased propensity for infected cells to differentiate into plasmablasts upon stimulation [5]. However, there are other factors that can contribute to this phenotype. DENV has been shown to directly induce activation of B cells in vitro through induction of CD81-mediated MAPK signaling [45]. Additionally, it has been shown the DENV-infected monocytes are capable of inducing plasmablast generation through infected cell upregulation of BAFF, APRIL, and IL-10 [46].

With the observance of increased plasmablasts, there have been additional studies characterizing the plasmablast population and antibodies generated during secondary infection. Waickman and colleagues performed transcriptomal analysis on PBMC samples from DENV confirmed patients in Thailand [47]. They found a relatively steady population of naïve and memory B cells in both primary and secondary infections during acute illness. The study noted a significant shift in plasmablast population from a balance IgA, IgG, and IgM during primary infections to heavily skewed IgG population during secondary infection. Which would be consistent with a plasmablast population generated from stimulation of existing memory B cells [47]. Another study showed that there is significant levels of somatic hypermutation within these plasmablast populations, consistent with levels observed during repeated exposure to pathogens like annual flu vaccination or infection [42]. This would suggest that if these populations are the result of DENV infection, they likely may not impact antibody class-switching or hypermutation. This phenotype may likely be due to repeated pathogen exposure and

stimulation of the memory B cell pool. A noticeable change in antibody phenotype observed during secondary illness that trends alongside disease progression is the fucosylation status of IgG antibody and that levels of afucosylated IgG are increased during the early stages of illness during secondary infection who develop severe disease [48]. During antibody synthesis within B cells, there are post-translational modifications of the Fc region of the antibodies through glycosylation that can impact antibody interactions with receptors and complement [49]. Antibodies that are afucosylated lack a fucose core at the glycosylation sites and have been shown to have increased binding to FcγRIIIa and likely contribute to higher affinity receptor interactions that may enhance ADE [48, 50, 51]. With evidence of DENV impacting the cellular metabolic environment [52], it is possible the DENV infection of B cells during the initial stages of infection could impact the fucosylation status of their secreted antibodies. It is also possible this fucosylation status is a pre-existing factor from the initial primary infection in dengue pathogenesis and not directly caused by DENV infection of B cells as afucosylation levels are increased after convalescence in individuals experiencing primary dengue infections [48].

With such a high DENV RNA burden within the B cell compartment, there are a few potential explanations as to why this is observed. As mentioned before, DENV is likely to infect B cells through more routes than just BDE. With DENV likely modulating B cell survival due to its ability to regulate these pathways in other cells, paired with evidence of increased proliferation in direct B cell infection studies. It is possible that part of the phenotype is influenced by robust expansion of DENV-infected B cells

following BCR engagement. Additionally, DENV infection has been shown to alter cell chemotaxis either through either direct or indirect pathways. Early DENV infection shows marked elevation of leukocyte recruitment to infection sites, as well as evidence of increased expression of CD69 within B cells, which is typically a marker of B cell activation and important in retention of lymphocytes in tissues [52, 53]. More analysis is needed on the combination of tissue homing receptors and B cell infection, but if uninfected B cells exhibit this increased CD69 expression, paired with robust proliferation of infected cells, this could be partially responsible for the increased proportion of infected cells observed within circulation during acute infection. Alternatively, CD69 expression could be upregulated in infected cells, but timing of this expression would be important to determine if the B cells would be retained within germinal centers or another target tissues.

Overall, there is significant evidence of plasmablast generation during acute secondary illness. Preliminary B cell infection studies indicate the likely involvement of infection on skewing B cells towards proliferation and this plasmablast profile. These plasmablasts exhibit a highly cross-reactive, E-specific BCR profile indicating potential early targets for BCR-dependent enhancement that may initiate the generation of this response. Additionally, this early infection could potentially impact the B cell metabolic environment, which may influence post-translational modification of secreted antibody like afucosylation which may be able to contribute to early enhancement of DENV infection via ADE. While class switching and somatic hypermutation profiles are consistent with what is observed during secondary exposure to pathogens, this does not

discount the potential contribution of DENV infection to antibody production and maturation during acute illness.

### *Memory Generation*

As mentioned before, after engagement and homing to germinal centers, activated B cells are fated to differentiate in antibody secreting cells or memory B cells. It has been shown that the plasmablast response during acute infection shared little overlapping CDR3 sequences with DENV-reactive memory B cells post recovery, indicating they did not share common clonal ancestors [54]. Additionally, studies have shown that plasmablasts generated during acute infection are largely cross-reactive and envelope-specific. While DENV-specific memory B cells post-secondary infection are more broadly binding with complex epitopes [42, 54]. Finally, following secondary infection individuals typically experience protection from symptomatic dengue across all serotypes of dengue [55, 56]. Paired with our hypothesis that DENV-infection would likely lead to death of the infected cell, it is possible that infection of DENV envelope-reactive memory B cells may preferentially differentiate these cells into plasmablasts and eventual culling of this susceptible population and allowing for more broadly reactive, non-envelope-specific memory to take over and decrease susceptibility of the B cell population. Alternatively, as discussed in early sections, DENV has been shown to stimulate plasmablast generation either directly or via infection of monocytes. Irrespective of infection, the typical fate of a plasmablast is death following resolution of disease, which is important of shaping the memory response during acute infection and post-resolution of infection.

## **DENV Envelope-specific B cells as a clinical marker**

As discussed in Chapter 2, there are no clear immune correlates of protection or risk, due to the complicated nature of immunity involvement in DENV disease pathogenesis. In light of the data presented in this dissertation, where ex vivo analysis of PBMC before and after primary infection show that the B cell population has increased susceptibility to DENV infection following primary infection. Indicating that the accumulation of DENV-specific immunologic memory may alter the susceptibility of the B cell population. We propose that further analysis should be performed on the abundance of envelope-reactive B cells prior to secondary infection to observe infection outcomes. I hypothesize that increased abundance of these potentially highly susceptible population of B cells may aid in the early establishment of infection and thus contributing to disease outcome. Within immunocompetent mouse models of infection, evidence of DENV viral proteins have been found to be localized in B cell follicles within germinal centers<sup>57</sup>. And these germinal centers show high level of B cell activation and proliferation. There is also evidence of CD69 expression, leading to potential retention of these B cells within the germinal centers [52, 53]. This may provide an ideal pathway for infected B cells to home to germinal centers and undergo early replication and virus production necessary to establish infection. Other studies have shown that the prevalence of DENV-reactive plasmablast during acute infection are significantly elevated in patients experiencing severe secondary infection [44]. And that these plasmablast numbers do not correlate with the antibody profile during acute infection [44]. Indicating that it is important to separate the B cell response from antibody secretion.

Under current technological constraints of characterizing the memory B cell pool, it would be difficult to utilize this as a clinical marker primarily due to the technical involvement as well as the time it takes to stimulate these cells to provide sufficient numbers for characterization as this typically takes up to a week [58]. DENV-reactive memory B cells could be analyzed in the context of clinical trials to be assessed as a potential marker of vaccine efficacy and risk assessment.

### **Concluding Remarks**

In this section, I have discussed the potential significance and limitations of the work presented in this dissertation. I have begun to characterize the novel entry mechanism of BCR-dependent enhancement of DENV infection with the DENV-specific B cell population. I have discussed how DENV may be suited to utilize this method of entry, as well as speculated on the characteristics of viral binding and entry that may be required for other viruses to support BDE. I have speculated on what the consequences of DENV infection may have on B cells, including stimulation of the DENV plasmablast response seen during secondary illness and how this may shape immune memory following infection. Finally, I proposed the quantification of DENV Envelope-specific memory following primary infection and before secondary infection to begin to understand the contribution of B cells to disease outcome following reinfection.

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