B cell receptor dependent enhancement of dengue virus infection

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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-infectable B cells increases in previously flavivirus-naïve volunteers after a primary DENV infection, and that the direct infection of B cells by DENV results in a pro-inflammatory cytokine response. These findings suggest that BCR-dependent infection of B cells is a novel mechanism of viral entry during DENV infection, and could contribute to immune-mediated enhancement of dengue pathogenesis.

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 mosquitos, 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 [6-10]. 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 [11-13]. 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 [14-18]. This observation has been substantiated by multiple groups using a wide array of assays. While B cells are known to be phagocytic [19, 20] 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.

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 closely mirrored that of a DENV-specific B cell receptor bound to DENV (**Figure 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 (**Figure 1B, Figure S1A, Figure S1B**). We observed that the 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 (**Figure 1B**).

To evaluate the contribution of DENV-specific tmIgG expression to susceptibility to DENV infection, we exposed tmIgG expressing cells to a DENV-1 RVP, alongside cells transfected with the canonical DENV-entry receptor DC-SIGN (**Figure 1C and 1D**). Expression of DENV-specific tmIgG receptors significantly increased susceptibility to infection by the DENV-1 RVP, to similar levels achieved in cells expressing DC-SIGN (**Figure 1C and 1D**). The ability of DENV-specific tmIg constructs to

facilitate infection was not limited to IgG isotype BCRs, as a similar pattern was observed in cells expressing tmIgM and tmIgA versions of DENV-specific antibodies (**Supplemental Figure 2**). Notably, the expression of a virus-specific tmIgG is not a universal mechanism for enhancing the susceptibility cells to viral infection, as the expression of HCMV-specific tmIgG constructs did not increase the susceptibility of cells to infection by this virus (**Supplemental Figure 3**).

While the expression of all DENV envelope-specific tmIgG 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 (**Figure 1D, 1E, 1F**). 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.

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 (**Figure 2A, Figure 2B**). 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 (**Figure 2A, Supplemental Figure 4**). We observed a significant increase in the number of DENV-infectable B cells on days 28 and 90 post DENV-3 infection relative to pre-infection (**Figure 2A, 2B**). No such increase in DENV-infectability was observed in either the T cell (**Figure 2E, 2F**) or monocyte compartment (**Figure 2G, 2H**). These results suggest that the accumulation of DENV-specific immunologic memory fundamentally and specifically alters the susceptibility of an individual's B cells to DENV infection.

DENV infection of B cells increases pro-inflammatory cytokine production. The low frequency of DENV-infectable B cells observed in even DENV-immune individuals significantly complicates the evaluation of BDE-associated immunopathogenesis. Therefore, we sought to develop a BCR-dependent DENV infection assay that bypasses the need for cells to express a DENV-specific BCR. To this end, we utilized an DENV/Ig/BCR crosslinking strategy, wherein DENV/Ig immune complexes were crosslinked to the BCR of polyclonal B cells using an anti-Ig secondary antibody (**Supplemental Figure 5**). The effectiveness and specificity of this infection strategy was first assessed using Raji cells, which are poorly susceptible to DENV even at high MOI. We observed minimal DENV infection in the Raji cells in all culture conditions except when all components of the DENV/Ig/BCR crosslinking cocktail were present in the inoculum (**Figure 3A, 3B, Supplemental Figure 5**).

Having successfully established an infection assay that facilitates the direct infection of a B cell line in a BCR-dependent fashion, we sought to determine if this assay could be utilized to infect primary B cells from a flavivirus-naïve donor. To this end, we exposed purified primary human B cells 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 (**Figure 3C, 3D, Supplemental**

Figure 5). Furthermore, B cell cultures containing the full DENV/Ig/BCR crosslinking cocktail demonstrated significant IL-8 production, a cytokine associated with acute DENV infection [21], but minimal TNFα production.

DISCUSSION

In this study, we demonstrate that the expression of a DENV envelope-specific B cell receptor renders cells highly susceptible to DENV infection. Furthermore, we observed that the frequency of DENV-infectible B cells within a given individual can be modified based on DENV immune status, and that BCR-dependent infection of B cells results in the production of pro-inflammatory cytokines known to be associated with DENV infection in human patients.

An intriguing implication of this model is that it may offer some insight into the heterogeneity of risk associated with post-primary DENV exposure. The risk of developing severe dengue after a secondary heterotypic infection is associated with having DENV-specific antibody levels from the first infection within a narrow range of [5, 22, 23]. However, only a small fraction of individuals experiencing secondary DENV infection with antibody titers within this window of risk actually experience severe disease [5, 22, 23]. Given the extraordinary stability of memory B cells [24], one potential immunopathological mechanism contributing to severe dengue may be the ratio of serum neutralizing antibody titers to DENV envelope-specific memory B cells circulating after a primary DENV infection. Additional analysis is required to determine if – and how – this mechanism may contribute to risk or protection from DENV infection.

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 [21]. 293T cell line was purchased through ATCC (CRL-3216) and maintained in DMEM media (Gibco, # 11965092) supplemented to 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. U937 DC-SIGN cell line was supplied by WRAIR. Raji cell line was purchased through ATCC (CCL-86) and maintained in complete RPMI media. K562 cell line was purchased through ATCC and maintained in complete IMDM media (Gibco, # 12440053).

Viruses and RVPs. DENV-1 (strain Wp74) dsRed Reporter virus (RVP) was prepared as previously described [25, 26]. 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. DENV-4 (strain D4-1036) stock was prepared by propagating a low-passage inoculum in C6/36 cells. RVP infection assays were performed using an 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. Aqua Live/Dead (ThermoFisher, L34957) or ZombieUV (Biolegend, 423107) PBMC infection experiments. Data collection was performed on a BD LSRII or Fortessa flow cytometer and analyzed using FlowJo v10.2 (Becton Dickinson). ADE assays were performed using K562 cells as previously described [27, 28].

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 FITC-tagged streptavidin.

hCMV infection. hCMV strain TB40E-GFP [29] was diluted in complete RPMI media then incubated with transfected cells at an MOI of 5 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.

DENV/Ig/BCR crosslinking infection. DENV/antibody immune complexes were generated by mixing DENV-4 (6.25×10^7 IU/mL) using an MOI of 62 with 1ug/mL of VDB33-IgM and anti-IgM in 96-well plate. 5×10^4 Raji cells or primary B cells were then added and incubated for 24 hours. Supernatants were harvest at 24hrs for cytokine analysis, while cells were washed and incubated an additional 24 hours before analysis. Cytokine production was assessed using the MSD human U-PLEX Viral Combo 1 kit according to the manufacturer's recommendation. 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.

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Data and material availability. The authors declare that all data supporting the findings of this study are available within this article and its Supplementary Information files, or from the corresponding author upon reasonable request.

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Figure Legends

Figure 1. Expression of DENV-specific BCRs renders cells susceptible to DENV-infection. **A)** Schematic representation of DENV/IgG immune complex binding to an FcgR (left), and a DENV virion binding to a DENV-specific BCR (right). **B)** Expression and DENV E protein binding activity of 293T cells transfected with the indicated tmIgG constructs. **C)** DENV-1 RVP infection of 293T cells transfected with the indicated tmIgG constructs. DC-SIGN and tmIgG conditions gated on receptor-positive cells. **D)** Quantification of DENV-1 RVP infection of 293T cells transfected with DC-SIGN or the indicated tmIgG constructs. Error bars +/- SEM. **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)** Pearson correlation analysis of the ADE and BDE results described above. Error bars +/- SEM

Figure 2. 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. **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 infectable 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 infectable 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 infectable 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).

Figure 3. DENV infection of B cells increases pro-inflammatory cytokine production. DENV/Ig/BCR crosslinking can facilitate DENV infection of polyclonal B cells. **A)** 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. **B)** Quantification of DENV-4 infected Raji cells under the indicated culture conditions. **C)** 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. **D)** Quantification of DENV-4 infected CD19+ B cells under the indicated culture conditions. **E)** Quantification of IL-8 production from DENV-4 infected B cells under the indicated culture conditions. **F)** Quantification of TNFα production from DENV-4 infected B cells under the indicated culture conditions. **G)** Schematic representation of the contribution of BCR-dependent enhancement to DENV infection and immunopathogenesis. Error bars +/- SEM. **** $p < 0.001$, One-way ANOVA with Dunnett's multiple comparisons test, with a single pooled variance

Figure 1

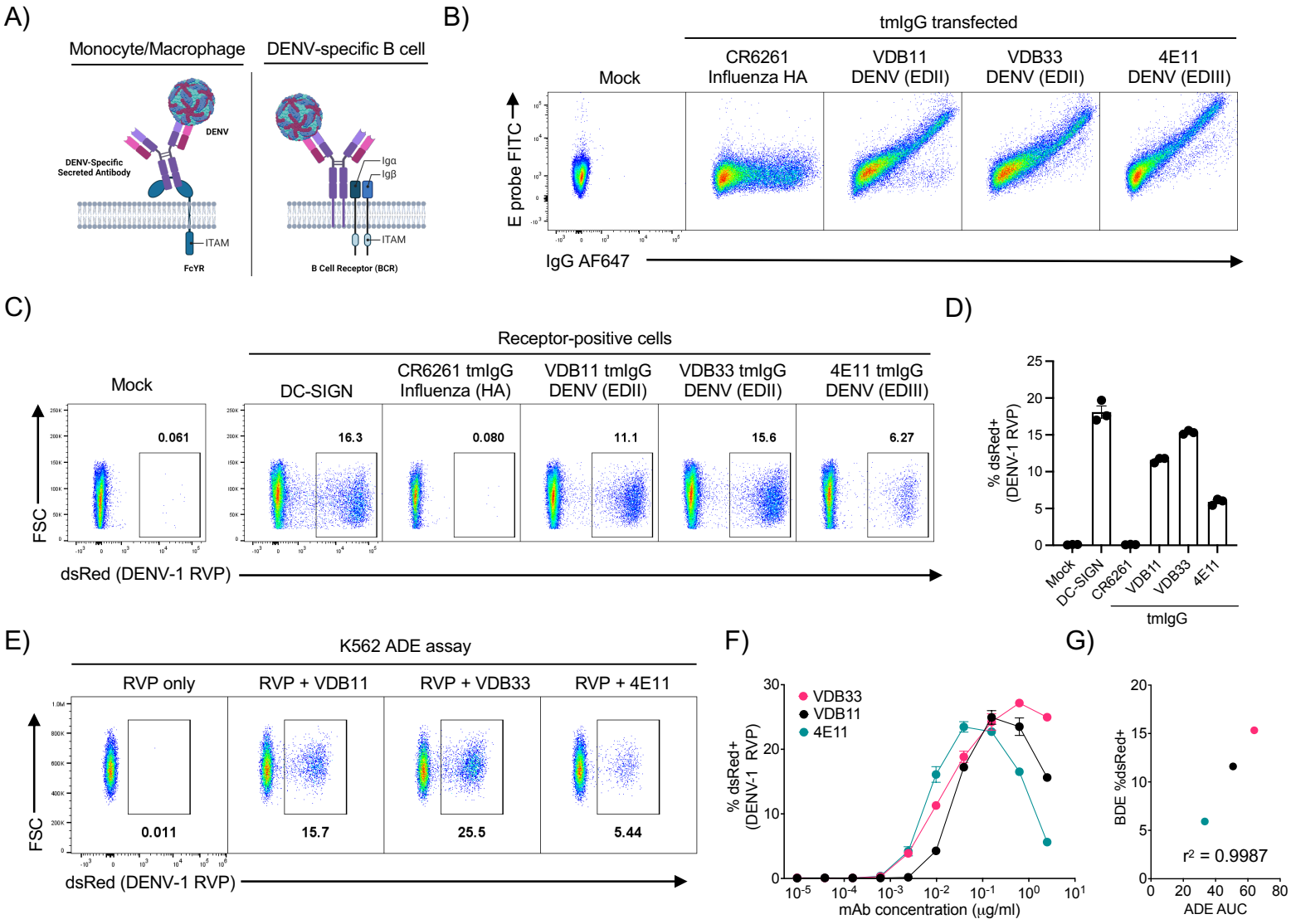
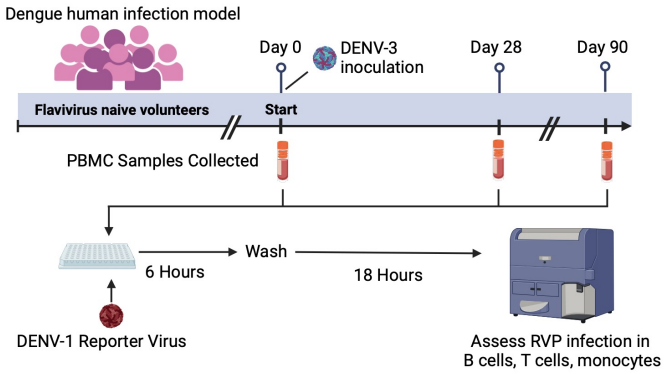

A)
Monocyte/Macrophage
DENV-specific B cell
DENV
DENV-Specific Secreted Antibody
ITAM
FcγR
Igα
Igβ
ITAM
B Cell Receptor (BCR)
B)
tmIgG transfected
Mock
CR6261 Influenza HA
VDB11 DENV (EDII)
VDB33 DENV (EDII)
4E11 DENV (EDIII)
E probe FITC
IgG AF647
C)
Receptor-positive cells
Mock
DC-SIGN
CR6261 tmIgG Influenza (HA)
VDB11 tmIgG DENV (EDII)
VDB33 tmIgG DENV (EDII)
4E11 tmIgG DENV (EDIII)
0.061
16.3
0.080
11.1
15.6
6.27
FSC
dsRed (DENV-1 RVP)
D)
% dsRed+ (DENV-1 RVP)
Mock
DC-SIGN
CR6261
VDB11
VDB33
4E11
tmIgG
E)
K562 ADE assay
RVP only
RVP + VDB11
RVP + VDB33
RVP + 4E11
0.011
15.7
25.5
5.44
FSC
dsRed (DENV-1 RVP)
F)
VDB33
VDB11
4E11
% dsRed+ (DENV-1 RVP)
mAb concentration (μg/ml)
G)
BDE %dsRed+
r² = 0.9987
ADE AUC

Figure 2

A)

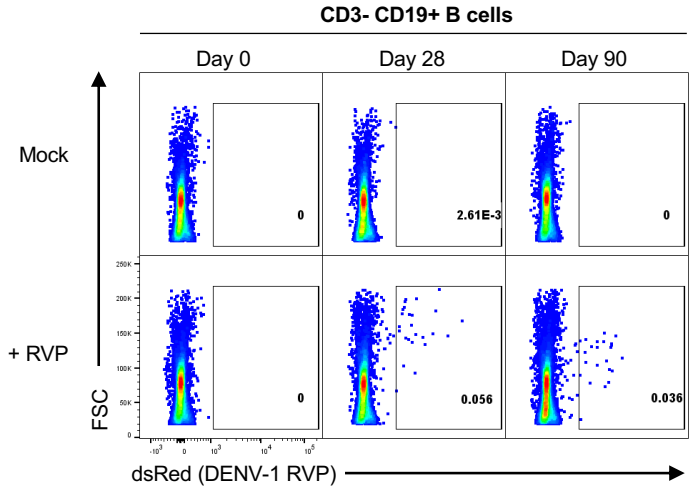


B)

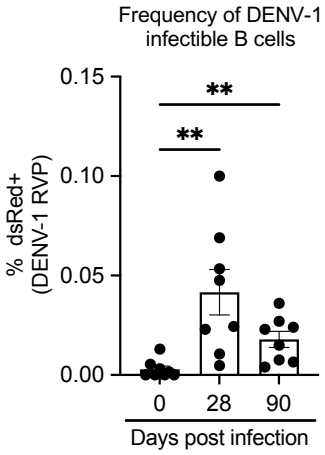
DHIM-3 study parameters

Challenge virus	CH53489
Frequency of infection	100%
Frequency of seroconversion	100%
Window of viremia	D3 – D12

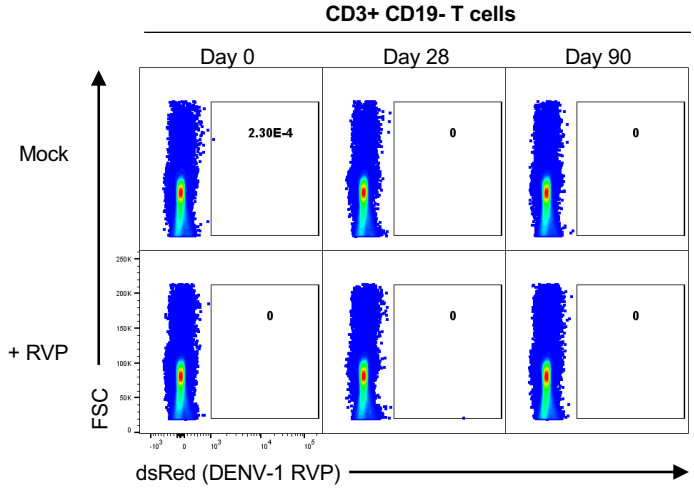
C)



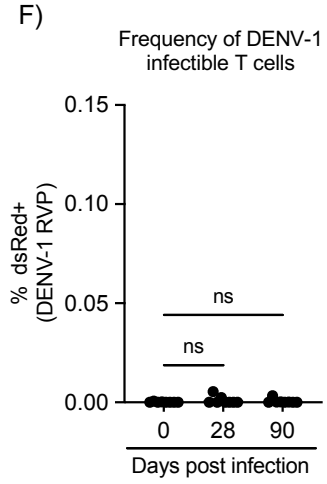
D)



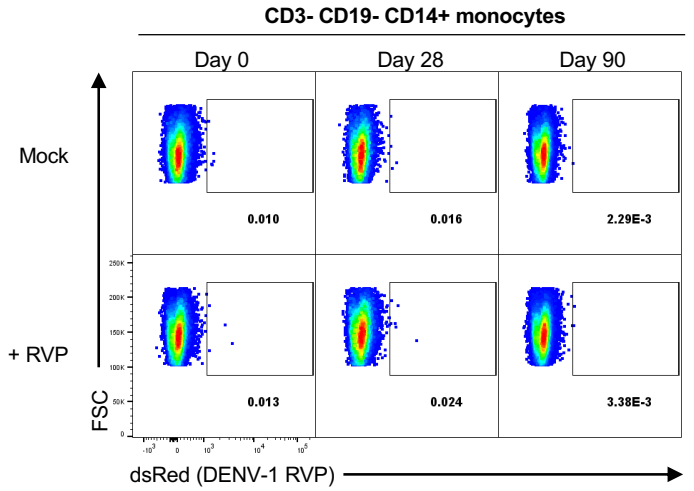
E)



F)



G)



H)

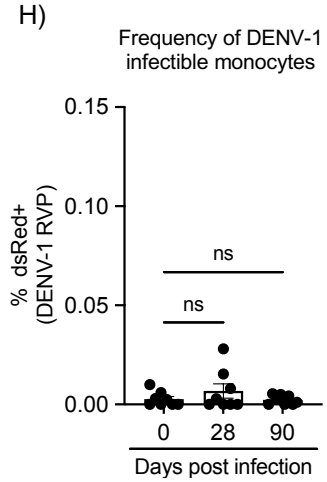
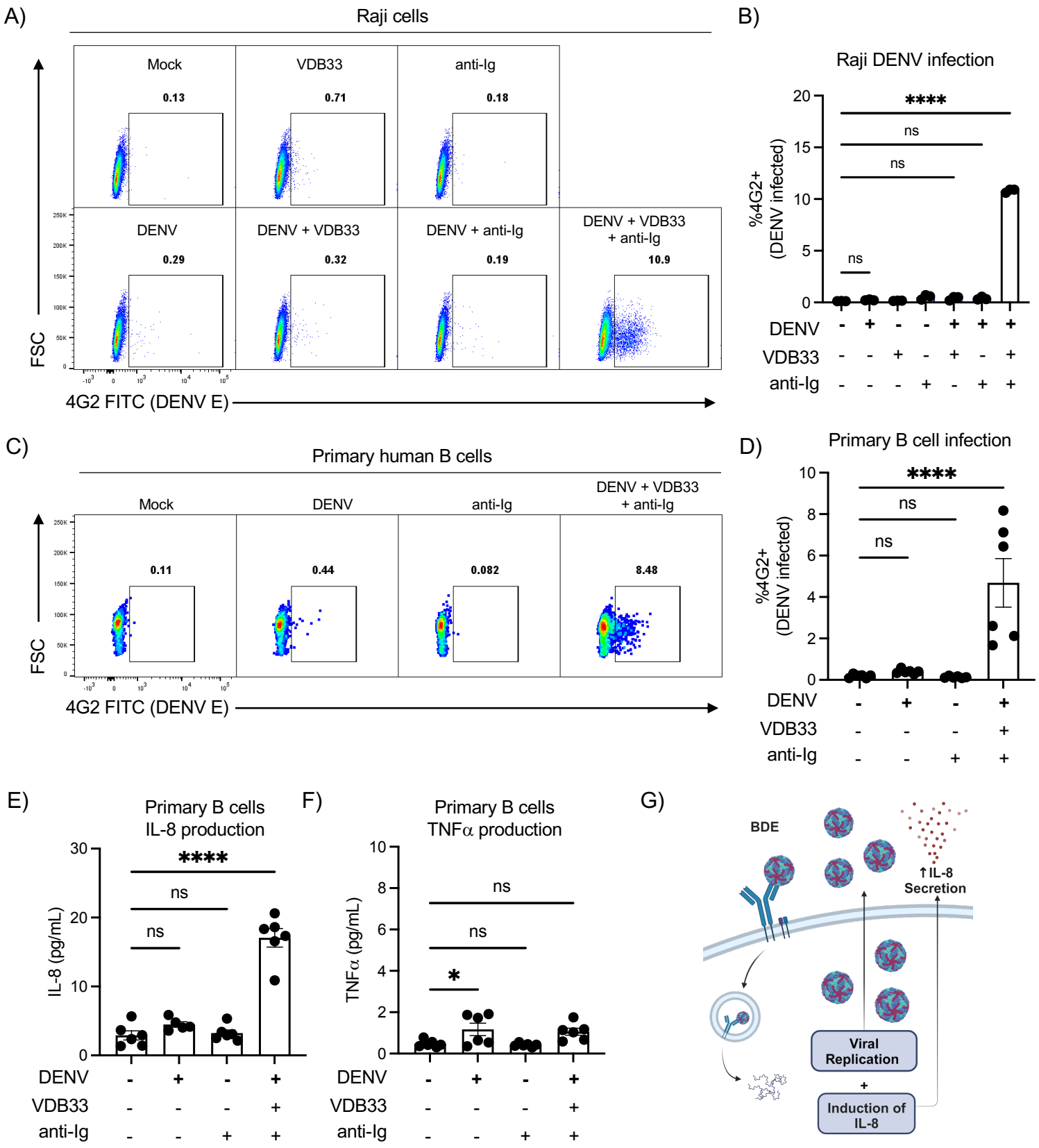


Figure 3


A)
Raji cells
Mock
VDB33
anti-Ig
0.13
0.71
0.18
DENV
DENV + VDB33
DENV + anti-Ig
DENV + VDB33 + anti-Ig
0.29
0.32
0.19
10.9
FSC
4G2 FITC (DENV E)
B)
Raji DENV infection
%4G2+ (DENV infected)
****
ns
ns
ns
DENV
VDB33
anti-Ig
C)
Primary human B cells
Mock
DENV
anti-Ig
DENV + VDB33 + anti-Ig
0.11
0.44
0.082
8.48
FSC
4G2 FITC (DENV E)
D)
Primary B cell infection
%4G2+ (DENV infected)
****
ns
ns
DENV
VDB33
anti-Ig
E)
Primary B cells IL-8 production
IL-8 (pg/mL)
****
ns
ns
DENV
VDB33
anti-Ig
F)
Primary B cells TNFα production
TNFα (pg/mL)
ns
ns
*
DENV
VDB33
anti-Ig
G)
BDE
↑IL-8 Secretion
Viral Replication
+
Induction of IL-8