EMERGING CONCEPTS IN DENGUE PATHOGENESIS AND HOST INNATE IMMUNE RESPONSE

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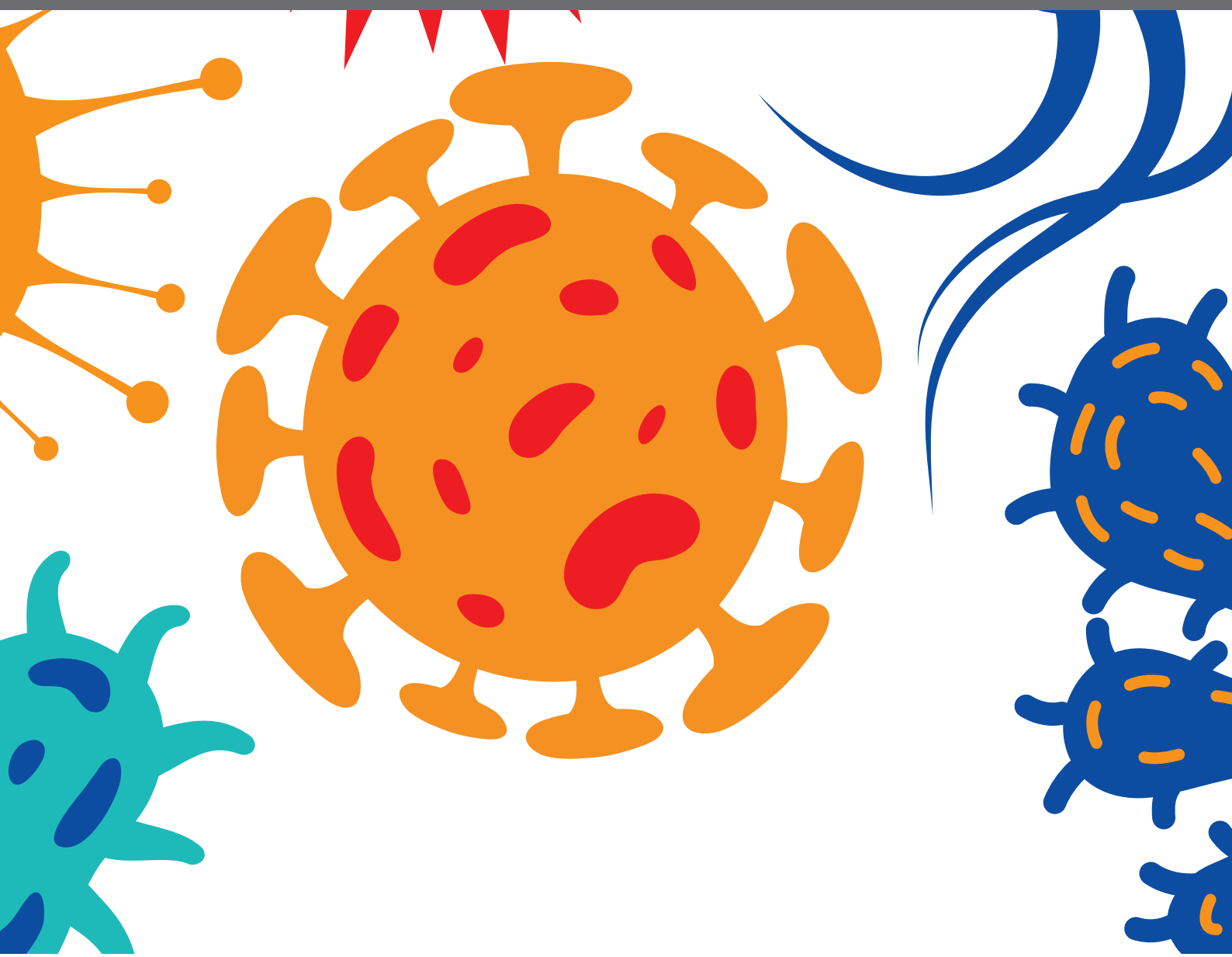

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EMERGING CONCEPTS IN DENGUE PATHOGENESIS AND HOST INNATE IMMUNE RESPONSE

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EDITORIAL
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Editorial: Emerging Concepts in Dengue Pathogenesis and Host Innate Immune Response

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Editorial on the Research Topic

Emerging Concepts in Dengue Pathogenesis and Host Innate Immune Response

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Dengue is one of the most rapidly advancing arthropod-borne viral disease present in over 100 countries. Dengue virus (DENV) infections are a serious health concern around the world. In a recent modelling-based report it was estimated that around half of the world's population is at the risk of this disease. Dengue fever is generally self-limiting, however in certain cases it can progress to more complicated forms like Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) with high mortality rates, as well as significant economic burdens. Moreover, subsequent infections of Dengue are more severe due to a phenomenon known as Antibody Dependent Enhancement (ADE) where pre-existing antibodies help the virus with different serotypes to infect monocytes more efficiently thereby contributing to the enhanced pathogenesis and spread of the disease.

Dengue viruses have evolved multiple strategies to escape the host innate immune system by both modifying viral RNA and viral protein or by post-translational modification of the host cellular proteins. The virus has been reported to trigger the host RLR as well as cGAS-STING axis to modulate the innate immune signaling in infected cells.

There are no specific therapies against this virus and thus novel antivirals to inhibit Dengue infections are highly desired. Although multiple strategies have been used to design vaccine candidates against this virus, these approaches have failed due to the complexity and epidemiology of the disease. To this end, there is an urgent need to identify novel cellular pathways and antiviral therapies that can be used to inhibit this virus.

The topics covered in this Research Topic helped gain insights into some of the very important aspects of Dengue biology. Hsieh et al. studied the effect of ageing on Dengue viral replication. They observed increased viral replication in human monocytic cells that were treated with D-galactose an inducer of cellular senescence. Further investigations revealed that induction of cellular senescence led to increased expression of DC-SIGN. Being one of the receptors for Dengue replication, increased expression of DC-SIGN following D-galactose treatment might contribute towards the enhanced replication of Dengue virus.

Another study by Zheng et al. tried to investigate molecular mechanisms contributing towards severe Dengue. Authors performed high throughput analysis on Dengue infected cells and identified several differentially regulated long non-coding RNAs. They focused on ERG-associated non-

coding RNA (ERGAL lncRNA) as its expression was dependent on time post Dengue infection rather than viral dose. siRNA mediated knockdown of ERGAL suggested that it might regulate vascular endothelial barrier function *via* ERG. Dual luciferase data further suggested that ERGAL could bind to miR-183-5p thereby acting as a sponge and suppressing the effect of miR-183-5p. The study identified interplay among ERGAL and miR-183-5p and their effect in regulating vascular endothelial barrier functions.

The role of platelet cytokines in Dengue biology was reviewed by Singh et al. Authors provided an in-depth analysis of platelet biology and their fate during Dengue infection. They discussed platelet cell surface receptors contributing towards Dengue infection. Authors further discussed the role of various chemokines and cytokines including CCR1-CCL2 axis, CCR1-CCL5 axis, IL6, CXCL8, CXCL10, CXCL11 and IL1b following Dengue infection of platelets.

Apart from vascular leakage, neurological disorders are also one of the hallmark of Dengue virus infection. This aspect was studied by Shen et al. by infecting immunocompetent outbred ICR mouse model with Dengue virus. First of all the authors investigated the presence of Dengue virus following infection of ICR suckling mice. Among various organs, authors could successfully detect NS1 in liver and brain of infected mice though infection was very severe in brain as compared to liver. Then authors conducted single cell immune profiling of brain cells following Dengue infection and observed increased expression of NK-1 NK cells and levels of several cytokines including IL2, IL15, IL4, IL5, IL10, IL13IL1b, IL6 were increased. The data was in conjunction to human patients where transcriptomic studies revealed negative correlation of NK cells with viral load. The study provides insights into the possible mechanism of immune erosion in Dengue patients.

Antibody dependent enhancement contributes towards Dengue severity in pre-exposed individuals. The phenomenon was extensively reviewed in two different reports by Shukla et al. and Narayan and Tripathi. Shukla et al. discussed the biology of DENV, the antibody response against DENV, and the challenges of vaccine development against DENV. In addition, they have proposed have EDIII as a dengue vaccine candidate that is capable of eliciting higher type-specific antibodies with lower ADE potential. Similarly, the article by Narayan et al. outlines the main concept of intrinsic ADE during DENV infection that contributes to the severe dengue pathogenicity. The article provides a good overview on the literature of *in vitro* and cell-signaling studies and propose that drugs against effectors of intrinsic ADE could be used as prophylactic treatment options to minimize disease severity in both adults and infants infected with DENV.

Recent advances on DENV research have shown the role of inflammasomes in contributing towards disease severity. Here, Shrivastava et al. discussed the contribution of inflammasomes in fueling Dengue severity. Authors discussed activation and induction of NLRP3 (one of the widely studied inflammasome) following viral infections. Moreover, inflammasomes specific to various cells including macrophages, dendritic cells, and platelets were also discussed. Apart from this, Dengue ADE and Dengue protein mediated inflammasome induction was also extensively discussed.

Dengue viral proteins have been shown to interact with various cellular proteins. Virus host interactions of one of the most important Dengue protein NS5 was discussed by Bhatnagar et al. They compiled various studies involving Dengue NS5 and host protein interactions to understand the interactome of the viral protein. Their analysis revealed that Dengue-NS5 associated with several host proteins that were involved in various cell signaling pathways including JAK-STAT, RNA processing, cell-cycle, protein synthesis and processing. They concluded that Dengue NS5 interacting partners could be exploited to design antiviral drugs against DENV.

Innate immune responses have been shown to be critical in regulating viral infections. However, viruses have been shown to deploy various strategies to evade host innate immune responses. Innate immune responses against Dengue virus were discussed by King et al., in their review article, have discussed extensively about the inflammatory responses to DENV infection due to innate immune cells, their effector functions and clinical course of the disease. They have talked about the DENV infection in two waves; in the first wave the tissue-resident phagocytes and keratinocytes are productively infected; while in the second wave the monocyte-derived macrophages and DCs are recruited and infected. They have also discussed how the DENV have evolved to both escape from the pre-existing adaptive immunity from antibodies as well as from host innate immune responses.

Several innate immune cells play critical role in regulating Dengue infections. Here Malavige et al. studied the fate of various innate immune cells following Dengue infections. Monocyte transcriptomics from Dengue patients revealed the importance of these cells in shaping Dengue severity. Monocytes are also involved in Dengue ADE which leads to suppression of interferon system in these cells. Secretion of various mediators by mast cells following Dengue infection has been implicated in vascular leakage which was also discussed in this article. The fate of T cells in Dengue severity was also debated in the article.

Lastly, Non-coding RNAs have been recently implicated in regulating host responses. Rajput et al. discussed the role on non-coding RNAs in regulating Dengue pathogenesis. These non-coding RNAs could either directly modulate the virus by binding to its genome or indirectly regulate viral pathogenesis by modulating host innate immune responses. The role of non-coding RNAs in Dengue severity was also discussed by the authors.

Overall, this Research Topic shed light on the current research advancements made in the area of dengue virus research. While it is imperative to have an in-depth understanding of the host innate immune response to dengue virus infection, it is more important to understand how viruses have evolved to evade these responses and establish successful infection. A better understanding of the host pathogen interactions is fundamental to the discovery and

implementation of antiviral strategies, the development of candidate vaccines against dengue viruses.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Senescence in Monocytes Facilitates Dengue Virus Infection by Increasing Infectivity

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Aging and chronic condition increase the incidence of dengue virus (DENV) infection, generally through a mechanism involving immunosenescence; however, the alternative effects of cellular senescence, which alters cell susceptibility to viral infection, remain unknown. Human monocytic THP-1 cells (ATCC TIB-202) treated with D-galactose to induce cellular senescence were susceptible to DENV infection. These senescent cells showed increased viral entry/binding, gene/protein expression, and dsRNA replication. The use of a replicon system showed that pharmacologically induced senescence did not enhance the effects on viral protein translation. By examining viral receptor expression, we found increased expression of CD209 (DC-SIGN) in the senescent cells. Interleukin (IL)-10 was aberrantly produced at high levels by the senescent cells, and the expression of the DENV receptor DC-SIGN was increased in these senescent cells, partially via IL-10-mediated regulation of the JAK2-STAT3 signaling pathway. The results demonstrate that a senescent phenotype facilitates DENV infection, probably by increasing DC-SIGN expression.

Keywords: dengue virus, senescence, IL-10, DC-SIGN, monocytes

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INTRODUCTION

Mosquito-borne dengue virus (DENV), which is a positive-sense single-stranded RNA virus of the genus *Flavivirus*, causes infection in an estimated 50–100 million people annually (Guo et al., 2017). Infection with DENV causes dengue diseases, which range from mild dengue fever (DF) to more severe dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS), central nervous system impairment, and multiorgan involvement (Guzman et al., 2016; Muller et al., 2017). In severe dengue, the exacerbated disease progression and increased case-fatality rate observed in patients have been associated with aging-related issues and common chronic diseases, such as allergies, diabetes, and hypertension (Wang et al., 2019). To obtain a better understanding of the complicated viral pathogenesis of DENV, several hypotheses, including the effects of viral load and virulence, the induction of cytotoxicity and pathological changes, and the immunopathogenesis of host immune alteration and autoimmunity, have been proposed (Diamond and Pierson, 2015; Guzman et al., 2016; Katzelnick et al., 2017). To improve dengue treatment and prevention, the development of safe and effective antiviral agents and vaccines is urgently needed.

During DENV infection, many host cells, including monocytes/macrophages, dendritic cells (DCs), B cells, T cells, basophil/mast cells, endothelial cells, epithelial cells, and hepatocytes, can be targeted by virions through different viral receptors (Cruz-Oliveira et al., 2015). Immune cells, such as monocytes/macrophages and DCs, that express dendritic cell-specific intracellular adhesion molecule 3 grabbing non-integrin (DC-SIGN), which is also known as CD209, are usually the target cells of DENV infection (Tassaneetrithep et al., 2003). The overexpression of DC-SIGN by cells increases the infectivity of DENV (Liu et al., 2017). After DC-SIGN-mediated infection, DENV-infected DCs may survive, become activated, and induce inflammation by increasing the production of multiple cytokines (Ho et al., 2001). Aberrant immune alterations, including immune cell activation, cytokine production, complement activation, inflammatory mediator production, antibody-dependent enhancement (ADE) of infection, and autoimmunity, have been proposed to participate in the immunopathogenesis of dengue (Martina, 2014; Begum et al., 2019).

Because older dengue patients accompanied by chronic condition have an elevated risk of developing severe multi-organ dysfunction/failure compared to younger dengue patients (Lin et al., 2017), there is an urgent need to explore the causes of severe dengue in susceptible individuals. In Singapore and Taiwan, susceptible cases tended to older adults are the main population at high risk of DENV infection, and this population suffers from severe disease burden (Rowe et al., 2014; Hsu et al., 2017; Wang et al., 2019). In gerontology, the reduction of host immunity and the induction of immunosenescence may cause older individuals to be susceptible to infection (Kline and Bowdish, 2016; Yao and Montgomery, 2016). Cellular senescence, particularly in cells of the immune system, is the hallmark of elderly adults (Salvioli et al., 2013; Nikolich-Zugich, 2018); however, the possibility of immunosenescence-enhanced DENV infection has not been explored. In this study, by using a chemical inducer of cellular senescence (Elzi et al., 2016), we examined the possible effects of D-galactose treatment on DENV infection in monocytes. The regulation of immunosenescence-enhanced DENV infection was also investigated.

MATERIALS AND METHODS

Cells, Virus Strains, and Reagents

Cell lines used for this study, including human THP-1 monocytic cells (ATCC TIB-202), baby hamster kidney (BHK)-21 cells (ATCC, CCL10), and *Aedes albopictus* C6/36 cells (ATCC, CRL1660), were cultured by the standard procedures according to the previous works (Tsai et al., 2014). For cell culture, RPMI medium 1640 (RPMI; Invitrogen Life Technologies, Rockville, MD) and Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies) were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technologies). A Taiwanese human isolated DENV2 PL046 was propagated in C6/36 cells and quantitated by using the BHK-21 cells. Reagents and antibodies used in these studies were as follows: D-galactose, 4,6-diamidino-2-phenylindole (DAPI), JAK2 inhibitor AG490, STAT3 inhibitor niclosamide, and a mouse monoclonal antibody (mAb) specific for β-actin (Sigma-Aldrich, St. Louis, MO); antibodies against dsRNA (English and Scientific Consulting, Szirák, Hungary); antibodies against p16, p21, p53, pSTAT3 (Tyr705), STAT3, SOCS3, and DC-SIGN (Cell Signaling Technology, Beverly, MA); antibodies against DENV NS1 (GeneTex, San Antonio, TX); neutralizing antibodies against DC-SIGN, IL-10, mouse IgG, and rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Abcam, Cambridge, MA); and Alexa Fluor 488-conjugated goat anti-mouse antibodies (Invitrogen, Carlsbad, CA).

Cell Viability and Cytotoxicity

For the detection of cell viability and cytotoxicity, a colorimetric Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) and Cytotoxicity Detection kit assays (Roche Diagnostics, Lewes, UK) were respectively assessed according to the manufacturer's instructions. The concentration of formazan WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium,monosodium salt] and lactate dehydrogenase in each sample is then directly determined from the increase in absorbance at 460 and 340 nm, respectively, by using a microplate reader (SpectraMax 340PC; Molecular Devices Corporation, Sunnyvale, CA, USA).

β-Galactosidase Activity

The cellular senescence of D-galactose-treated THP-1 cells was examined using a β-Galactosidase Detection Kit (Abcam) according to the manufacturer's instructions. The galactosidase induced cleavage of fluorogenic fluorescein digalactosidease can be detected with fluorescence instruments equipped with a fluorescein isothiocyanate filter set in spectra of excitation/emission = 490/515 nm. Images were observed and captured using a fluorescence microscope (BX51; Olympus, Tokyo, Japan), and the average relative fluorescence units (RFU) were calculated for all the samples.

Cell Cycle Analysis

The cell cycle was analyzed using propidium iodide (PI; Sigma-Aldrich) staining, and then, the cells were analyzed using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA) with excitation at 488 nm and emission detected in the FL-2 channel (565–610 nm). The percentages of cells were analyzed by using CellQuest Pro 4.0.2 software (BD Biosciences).

Western Blotting

Western blotting was performed according to our previous studies (Tsai et al., 2014; Kao et al., 2018). Briefly, harvested cells were lysed with a buffer and then centrifuged at 12,000 rpm at 4°C for 20 min. Lysates were boiled in sample buffer and then subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk in PBS, membranes were incubated with diluted primary antibodies and then incubated with diluted HRP-conjugated secondary antibodies for ECL development.

Plaque Assay

Measurement of the viral titers was performed with a plaque assay according to previous studies (Tsai et al., 2014). Briefly, in

DENV-infected BHK-21 cells, we fixed and stained the infected cellular monolayer by using 1% crystal violet (Sigma-Aldrich). The formation of plaques was counted in each well to calculate the titer of viral stock samples in terms of plaque forming units (pfu) per milliliter.

Fluorescent DENV

DENV was labeled with Alexa Fluor 594 succinimidyl ester (AF594SE, Molecular Probes, Invitrogen) according to a previous study (Zhang et al., 2010). The labeled viruses were purified using Amicon Ultra filter (Millipore) to remove unbound dye and stored in 50 μl aliquots at −80 °C prior to use.

Reverse-Transcription (RT)-Polymerase Chain Reaction (PCR) and Quantitative (q)PCR

A TRIZol (Invitrogen) RNA extraction reagent was used to extract total RNA, and a PrimeScriptTM RT reagent kit (Takara, Tokyo, Japan) was utilized to prepare complementary (c)DNA. Following a qPCR reaction conducted by using KAPA SYBR FAST qPCR Master Mix (Life Technologies and Kapa Biosystems, Woburn, MA), the PCR was performed using a StepOnePlusTM real-time PCR system (Applied Biosystems, Foster City, CA) with the following pair of specific primers: primer sequences for NS1 (forward): 5′-ATGGATCCGATAGTGGTTGCGTTGTGA-3′ and NS1 (reverse): 5′-ATCTCGAGGGCTGTGACCAAGGAGTT-3′.

Immunostaining

Immunostaining of dsRNA was carried out according to our previous studies (Kao et al., 2018). Briefly, in fixed cells, anti-dsRNA antibodies were incubated overnight at 4°C followed by an incubation with secondary antibodies for 30 min. Cells were counterstained with 1 μg/ml DAPI in PBS for 5 min and washed three times with PBS. The cells were visualized under a fluorescence microscope (BX51; Olympus, Tokyo, Japan) or analyzed using flow cytometry (FACSCalibur). The mean fluorescence intensity (MFI) and the percentage of positive cells were analyzed by using CellQuest Pro 4.0.2 software.

Reporter Assay

The viral protein translation activity was examined by using BHK-21 cells harboring the luciferase-expressing DENV replicon (BHK-D2-Fluc-SGR-Neo-1) according to a previous study (Kao et al., 2018). Briefly, cells were lysed and the luciferase activity was evaluated according to the manufacturer's instructions (Luciferase Assay System, Promega). The luciferase activity was measured by microplate reader (SpectraMax 340PC) at an absorption wavelength of 640 nm.

ELISA

Expression of interleukin (IL)-10 was measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, MN) according to the manufacturer's instructions.

Statistical Analysis

Data, presented as the mean ± standard deviation (SD), were analyzed by an unpaired Student's *t*-test or by one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test. Statistical significance was defined as $p < 0.05$.

RESULTS

Treatment With D-Galactose Induces Senescence and Enhances DENV Replication in Human Monocytic THP-1 Cells

In our previous studies, we established an *in vitro* model of DENV infection by using human monocytic THP-1 cells (Tsai et al., 2014, 2017). In this study, the monosaccharide sugar D-galactose was used to induce senescence (Elzi et al., 2016). MTT and LDH assays showed that D-galactose treatment significantly induced cell growth inhibition and cytotoxicity ($p < 0.05$) (**Figure 1A**). A propidium iodide-based flow cytometric analysis revealed the induction of cell cycle arrest at the G1 phase in the D-galactose-treated THP-1 cells (**Figure 1B**). Detection of the senescence-associated β-galactosidase activity by using imaging (**Figure 1C**) and activity assays (**Figure 1D**) demonstrated its significant senescent responses 48 h of D-galactose (250 mM) poststimulation. Additionally, following D-galactose treatment, the senescence-associated p16, p21, and p53 proteins, which are highly associated with growth arrest during senescence (Rufini et al., 2013), were increased (**Figure 1E**). A plaque assay showed that DENV caused significant infection of senescent THP-1 cells *in vitro* ($p < 0.05$) (**Figure 1F**). These results indicate the enhanced infection of DENV in senescent THP-1 cells.

D-Galactose Treatment Facilitates DENV Infection by Enhancing Viral Entry, Viral Gene/Protein Expression, and dsRNA Replication in THP-1 Cells

To investigate the possible effects of the senescent responses on facilitating DENV infection, the infectious steps of the viral life cycle, including binding/entry, viral protein expression, and dsRNA replication, were assessed (Rodenhuis-Zybert et al., 2010). By studying infection with fluorescently labeled DENV by fluorescence microscopy (Zhang et al., 2010), enhanced viral binding/entry could be identified in the D-galactose-treated THP-1 cells (**Figure 2A**). Furthermore, flow cytometric analysis revealed an increased percentage of cells infected with DENV2 in the senescent group (**Figure 2B**). Following binding/entry, Western blot analysis (**Figure 2C**) and quantitative PCR (**Figure 2D**) showed significantly increased expression of the viral NS1 protein and gene at 24 h postinfection ($p < 0.05$). Furthermore, immunostaining of dsRNA at 6 h postinfection showed that the senescent response significantly ($p < 0.05$) enhanced viral dsRNA replication (**Figure 2E**). The data confirm that senescent conditions enhance DENV infection in THP-1 cells.

To further validate the effects of the senescent response on enhancing viral infection, we next examined the effects of

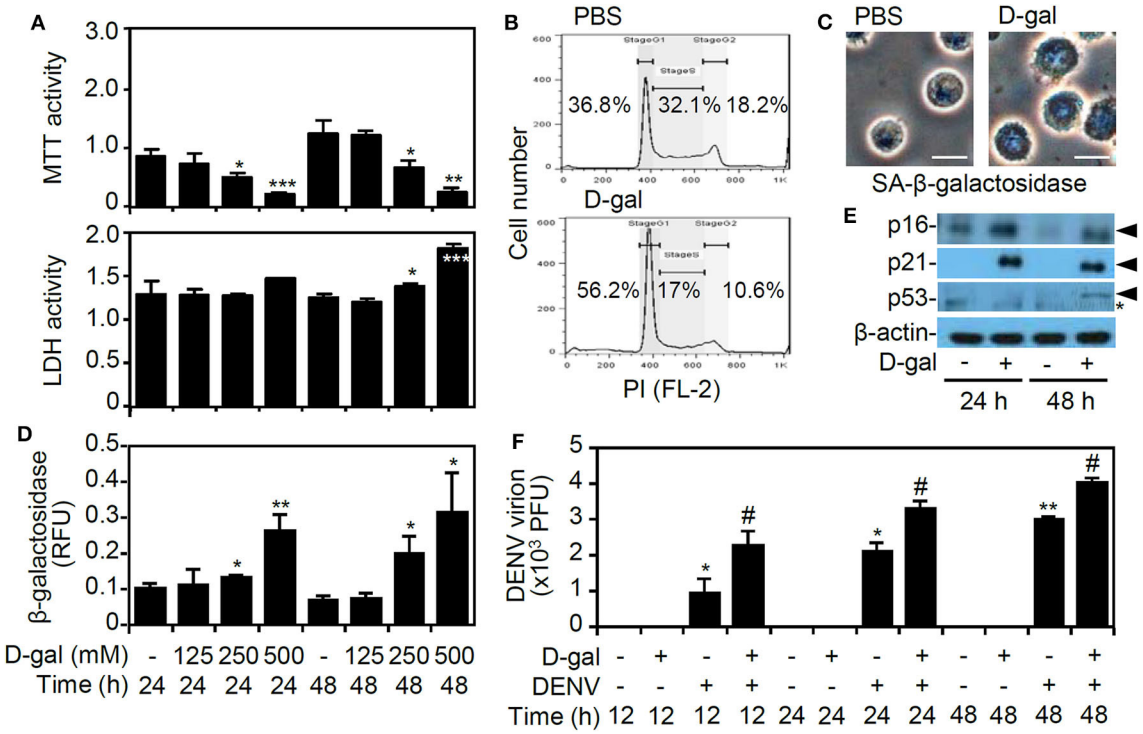

FIGURE 1 | D-galactose treatment induces senescent responses and enhances dengue virus (DENV) serotype 2 PL046 (DENV2) replication in THP-1 cells. **(A)** The MTT and lactate dehydrogenase (LDH) assays showing cell viability and cytotoxicity in D-galactose (D-gal)-treated human monocytic THP-1 cells at the indicated doses and times. **(B)** Flow cytometry analysis showing the cell cycle phases in the D-gal (250 mM)-treated cells 48 hours poststimulation. A representative histogram and the percentages of cells are shown. **(C)** Microscopy analysis, **(D)** β-galactosidase activity assay, and **(E)** Western blot analysis of p16, p21, and p53 expression (arrowheads) showing senescent responses in D-gal (250 mM)-treated THP-1 cells. *, non-specific binding. **(F)** Plaque assays showing the level of viral replication in DENV2 (at a multiplicity of infection of 1)-infected THP-1 cells pretreated with or without D-gal (250 mM) for 48 hours. The quantitative data are shown as the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared to the untreated cells. $^{\#}p < 0.05$, compared to the DENV-infected cells.

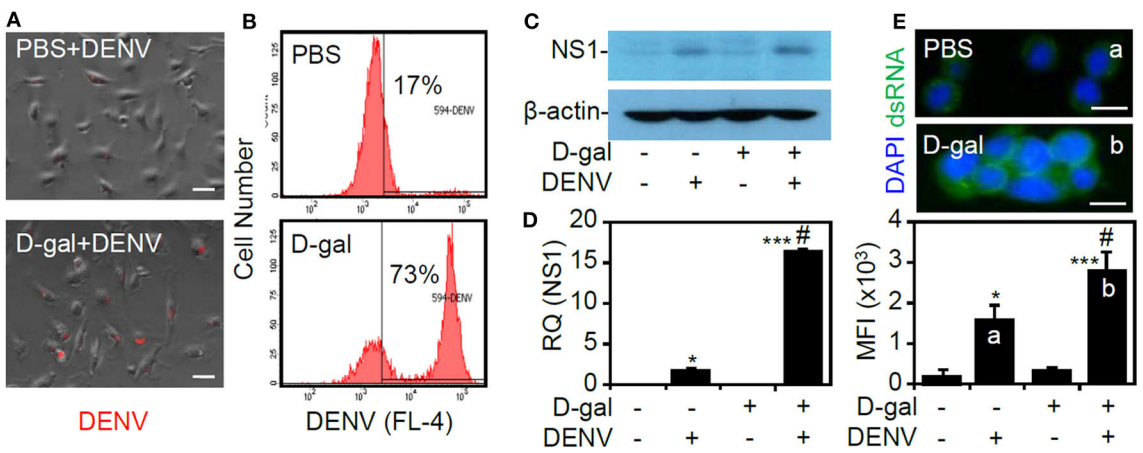

FIGURE 2 | D-galactose treatment enhances dengue virus (DENV) serotype 2 PL046 (DENV2) infection, including viral entry, viral gene/protein expression, and double-stranded (ds)RNA replication in THP-1 cells. **(A)** Fluorescence microscopy and **(B)** flow cytometry were used to measure the expression and percentage of THP-1 cells infected with fluorescent Alexa-594-labeled DENV2 (at a multiplicity of infection of 1) for 2 h pretreated with or without D-galactose (D-gal; 250 mM). In D-gal-stimulated THP-1 cells 48 h posttreatment followed by DENV infection for further 24 h, **(C)** representative Western blot analysis showing viral non-structural protein 1 (*NS1*) expression. β-actin was used as an internal control. **(D)** In addition, qPCR showing the mRNA expression of NS1. **(E)** Immunocytochemistry and the relative mean fluorescence intensity (*MFI*) of viral dsRNA (*green*) in D-gal-stimulated THP-1 cells 48 h posttreatment followed by DENV infection for further 6 h. DAPI staining indicates nuclei (*blue*). For all the images, the representative data were selectively obtained from three individual experiments. The quantitative data are shown as the mean ± SD. $^{*}p < 0.05$ and $^{***}p < 0.001$, compared to the untreated cells. $^{\#}p < 0.05$, compared to the DENV-infected cells.

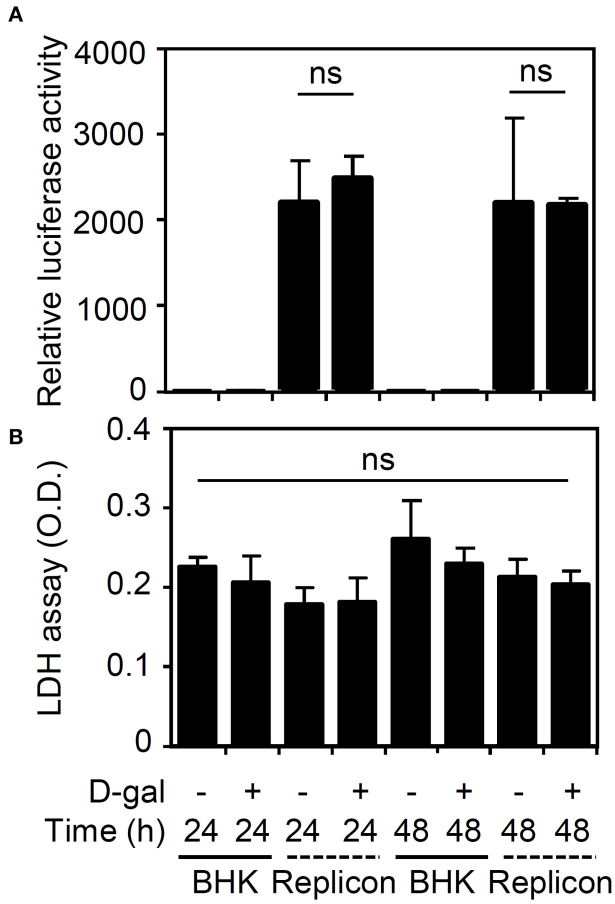

FIGURE 3 | Treatment with D-galactose does not repress firefly luciferase activity in BHK-D2-Fluc-SGR-Neo-1 cells. **(A)** Luciferase activity and **(B)** lactate dehydrogenase (LDH) assays in D-galactose (D-gal; 250 mM)-treated parental BHK-21 and BHK-D2-Fluc-SGR-Neo-1 cells (replicons) 24 and 48 h posttreatment. The quantitative data are shown as the mean ± SD of three independent experiments. ns, not significant.

senescence on viral protein translation by measuring firefly luciferase activity in BHK-D2-Fluc-SGR-Neo-1 cells. We found that treatment with D-galactose caused no inhibitory effects on viral translation (**Figure 3A**) or cytotoxicity in the cells (**Figure 3B**). These results indicate that the senescent response facilitates DENV infection by a mechanism that is independent of the direct promotion of viral protein translation.

Induction of Senescence by D-Galactose Causes Increased Expression of DC-SIGN in THP-1 Cells

According to our results that the senescent response facilitated DENV binding/entry to enhance viral replication, we next examined the possible effects of D-galactose on the expression of the DENV receptor. Notably, THP-1 cells become susceptible to DENV infection in a DC-specific ICAM-3 grabbing non-integrin (DC-SIGN)-mediated manner (Tassaneetrithep et al., 2003). Senescent primary $CD14^+CD16^+$ monocytes exhibit increased expression of DC-SIGN and become further activated (Merino et al., 2011). The results of immunostaining followed by flow cytometric analysis demonstrated a significant increase ($p < 0.01$) in DC-SIGN expression in senescent THP-1 cells (**Figure 4A**). Treatment of neutralizing antibody against DC-SIGN significantly ($p < 0.05$) reversed D-galactose-enhanced DENV infectivity (**Figure 4B**). The findings reveal that the senescent response in monocytes causes the increased expression of DC-SIGN to facilitate DENV infection.

Expression of IL-10 Is Increased in Senescent THP-1 Cell, and Pharmacologically Targeting the IL-10-JAK2-STAT3 Signaling Pathway Eliminate DC-SIGN Expression and DENV Infectivity

In this study, the chemical D-galactose was used to induce senescence in THP-1 cells. Because the cytokine IL-10 has been utilized to trigger senescence in primary $CD14^+CD16^+$ monocytes (Merino et al., 2011), we next examined the involvement of IL-10 in promoting THP-1 cell senescence following D-galactose treatment. First, the ELISA results revealed a significant and time-dependent increase ($p < 0.001$) in IL-10 expression in the THP-1 cells treated with D-galactose (**Figure 5A**). Western blot analysis further demonstrated the activation of signaling molecules downstream of IL-10, including the phosphorylation of STAT3 at Tyr705 and SOCS3, in the senescent THP-1 cells 48 h poststimulation (**Figure 5B**). To verify the role of IL-10-induced JAK2-STAT3 signaling in modulating DC-SIGN expression, the cells were treated with the JAK2 inhibitor AG490 and the STAT3 inhibitor niclosamide. These inhibitors effectively blocked the D-galactose-induced DC-SIGN expression, as demonstrated by immunostaining followed by flow cytometric analysis (**Figure 5C**). Exogenous treatment of anti-IL-10 neutralizing antibody significantly ($p < 0.05$) reversed D-galactose-enhanced DENV infectivity (**Figure 5D**). These results confirm that DC-SIGN expression is induced in senescent THP-1 cells via IL-10-mediated regulation of the JAK2-STAT3 signaling pathway.

DISCUSSION

The present work investigated the possible mechanisms of the association between cellular senescence and the risk of DENV infection and demonstrated, for the first time, that senescence enhances DENV infection *in vitro*. Treatment of D-galactose may induce senescence in monocytes/macrophages *in vitro* (Zhang et al., 2019) and naturally aged mice (20–22-month-old) present accumulated senescent macrophages *in vivo* (Cai et al., 2020). Based on these findings, it is important to validate the increased infectivity of DENV in senescent primary monocytes/macrophages with increased DC-SIGN expression in older adults or aged mice in future studies. As summarized in **Figure 6**, the results showed that the chemical sugar D-galactose efficiently induced cellular senescence in human

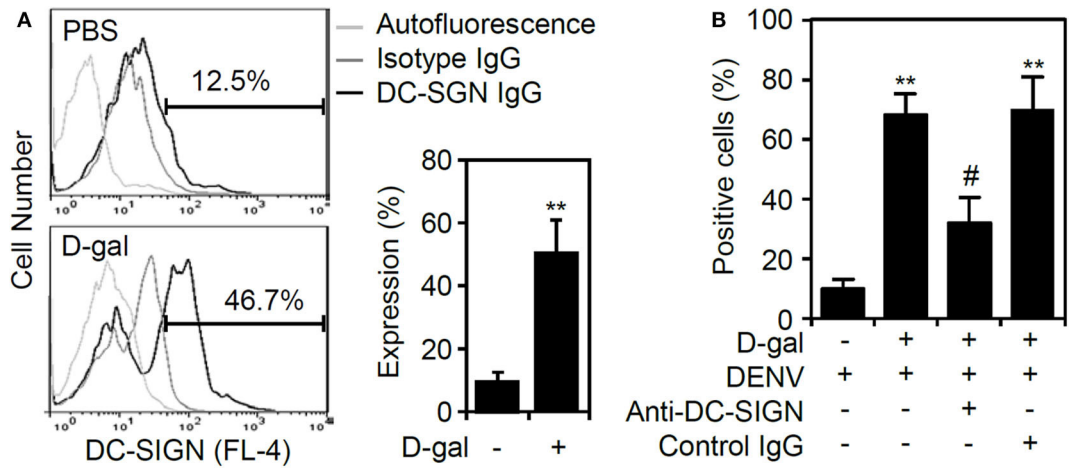

FIGURE 4 | Increased expression of DC-SIGN in D-galactose-treated THP-1 cells. **(A)** Immunostaining followed by flow cytometric analysis showing the expression of DC-SIGN in the D-gal-stimulated THP-1 cells 48 h posttreatment. A representative histogram and the percentage of positive cells are shown, and the data are the mean ± SD of three independent experiments. **(B)** Flow cytometry measured the infectivity of fluorescent Alexa-594-labeled DENV2 (at a multiplicity of infection of 1) 2 h post-infection in D-galactose (D-gal; 250 mM)-treated THP-1 cells in the presence and absence of neutralizing anti-DC-SIGN and control IgG (5 μg/ml). $^{**}p < 0.01$ compared to the DENV-infected cells. $^{\#}p < 0.05$, compared to the DENV-infected senescent cells.

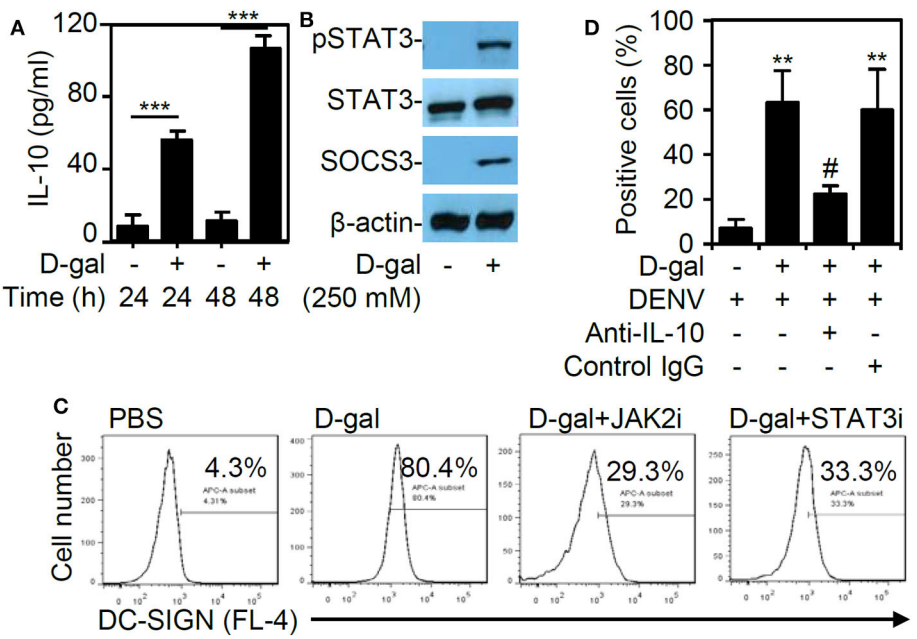

FIGURE 5 | D-galactose treatment induces IL-10 production and activation followed by JAK2-STAT3-regulated DC-SIGN expression. **(A)** ELISA data showing the IL-10 expression in the THP-1 cells treated with D-galactose (D-gal; 250 mM). The quantitative data are shown as the mean ± SD of three independent experiments. $^{***}p < 0.001$. **(B)** Representative Western blot analysis of pSTAT3 (Tyr705), STAT3, and SOCS3 protein expression 48 h poststimulation. β-actin was used as an internal control. **(C)** Cotreatment with or without the JAK2 inhibitor AG490 (JAK2i; 100 μM) and the STAT3 inhibitor niclosamide (SATA3i; 0.1 μM), immunostaining followed by flow cytometric analysis showing the expression of DC-SIGN in the D-gal-stimulated THP-1 cells. A representative histogram and the percentage of positive cells are shown. **(D)** Flow cytometry measured the infectivity of fluorescent Alexa-594-labeled DENV2 (at a multiplicity of infection of 1) 2 h post-infection in D-galactose (D-gal; 250 mM)-treated THP-1 cells in the presence and absence of neutralizing anti-IL-10 and control IgG (5 μg/ml). $^{**}p < 0.01$ compared to the DENV-infected cells. $^{\#}p < 0.05$, compared to the DENV-infected senescent cells.

monocytic THP-1 cells. Notably, the senescent THP-1 cells were susceptible to DENV infection, indicating that senescence enhanced the infection process. Through the induction of the IL-10-activated JAK2-STAT3 signaling pathway, senescence promoted the increased expression of DC-SIGN to facilitate the infectivity of DENV.

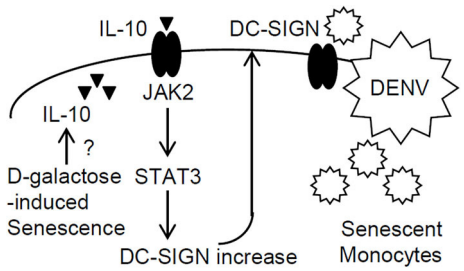

FIGURE 6 | A hypothetical model of enhanced DENV infection in senescent monocytes. In this study, the chemical D-galactose-induced senescent response in monocytic THP-1 cells caused IL-10 production through an unknown mechanism. Then, IL-10 triggered JAK2-STAT3 signaling-regulated DC-SIGN expression to enhance DENV infection.

In the elderly, immune cell senescence has been linked to poor responses to infections, vaccines, and immune therapy and has been associated with chronic low-grade inflammation, which increases morbidity and mortality (Salvioli et al., 2013; Nikolich-Zugich, 2018; Oh et al., 2019). Through the immune profiling of isolated classical monocytes from younger and older adults, the number of circulating classical monocytes is reduced in the older population, while intermediate and non-classical monocytes are increased with age (Pence and Yarbro, 2018). Notably, senescent intermediate monocytes exhibit proinflammatory activity, which is highly associated with chronic inflammatory disorders (Hanai et al., 2008; Merino et al., 2011). Until now, no further reports have shown the infectivity of DENV in senescent monocytes. However, DENV infects primary monocytes *in vitro* and induces cell death to trigger a pro-inflammatory response (Tan and Chu, 2013). We showed that chemical induction of cellular senescence increases DENV infection in THP-1 cells *in vitro*. It is speculated that an increased infectious rate is observed in older adults due to the presence of senescent monocytes.

During immunosenescence, resistance and a decreased antiviral interferon response have been demonstrated (Molony et al., 2018; Oh et al., 2019). Therefore, the lack of immune defense renders senescent cells susceptible to viral infection. The expression of immune sensors, such as Toll-like receptors (TLRs) and cytoplasmic retinoic acid-inducible gene I-like receptors, is poorly upregulated in response to pathogens. However, there are no further investigations on the effect of cellular senescent responses on routes of infection. In this study, we provided evidence to show that the induction of DC-SIGN facilitates DENV infection in senescent THP-1 cells. As a main viral receptor that mediates DENV binding to/entry into DCs and some specialized/differentiated monocytes/macrophages (Tassaneetrithep et al., 2003), the findings of this study indicate that senescence enhances DENV infection in monocytes in a DC-SIGN-mediated manner. Additionally, it is speculated that increased DC-SIGN expression in senescent monocytes may also facilitate other microbial infections, including infections with viruses such as influenza virus, human immunodeficiency virus, Ebola virus, hepatitis C virus, cytomegalovirus, and SARS coronavirus; infections with bacteria such as *Mycobacterium tuberculosis* and *Helicobacter pylori*; and infections with parasites such as *Leishmania pifanoi*, and that these pathogens need DC-SIGN as an infectious receptor (Khoo et al., 2008). Notably, most of the infections with these pathogens are observed in older adults.

The regulation of DC-SIGN expression is multifaceted. In response to TLR activation, the bacterial endotoxin lipopolysaccharide causes bone marrow monocyte differentiation into DCs, which are characterized by increased the expression of CD80, CD86, and DC-SIGN (Cheong et al., 2010). In addition, stimulation by the helper T cell cytokines IL-4, IL-10, and IL-13 upregulates DC-SIGN expression on monocyte-derived macrophages and DCs (Relloso et al., 2002; Soilleux et al., 2002; Merino et al., 2011). Aberrant IL-10 expression has been demonstrated to be pathogenic in DENV infection by facilitating DENV replication following an immunosuppressive mechanism (Malavige et al., 2013; Tsai et al., 2013). In D-galactose-treated THP-1 cells, IL-10 is unexpectedly overproduced and triggers the JAK2-STAT3 signaling pathway to regulate DC-SIGN expression. Although the mechanism of IL-10 induction by D-galactose in THP-1 cells remains unknown, these findings not only confirm the regulation of DC-SIGN by IL-10 but also reveal an effect of senescence on enhancing DC-SIGN-mediated DENV infection. Furthermore, the induction of IL-10 by senescent stress may also confer promoting effects on facilitating infectivity not only in increasing DC-SIGN in monocytes/macrophages but also in causing immunosuppressive effects scape from antiviral immunity both in immune and non-immune cells.

Senescence is present not only in older adults but also in chronic disorders, and it is a key factor that facilitates microbial infection (Salvioli et al., 2013). In addition to older adults, patients with several chronic underlying diseases are also susceptible to DENV infection (Hsu et al., 2017; Wang et al., 2019). Additionally, several hyperendemic countries in South America and Southeast Asia, an increased infectivity in non-aged patients show the involvement of environmental conditions and immunopathogenesis (Campos et al., 2015; Phanitchat et al., 2019; Bavia et al., 2020). Both in aged and non-aged population, senescent response may also distress immunopathogenesis of DENV infection on extrinsic ADE by affecting Fc receptor expression and signaling as well as on intrinsic ADE by enhancing IL-10 production and activation (Halstead et al., 2010). Either in dengue patients or in DENV-susceptible people, who are aging and chronic diseases' population, the presence of senescent monocytes/macrophages must be examined to validate their pathogenic role in facilitating DENV infection. In conclusion, based on the findings of this study, senescent monocytes aberrantly express DC-SIGN to increase the infectivity of DENV. It is thought that IL-10 expression and activation regulate the DC-SIGN expression that facilitates DENV infection. Targeting senescence and IL-10 may diminish

DENV-susceptible monocytes and may be an approach for future anti-dengue therapy.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

T-HH and T-TT performed most of the experiments and interpreted the results. C-LC and C-FL participated in the design and supervision of the projects. T-JS and M-KJ conducted the virus experiments. P-CT contributed to flow cytometry analysis. T-HH, T-TT, and C-FL designed the concept of the project and wrote the manuscript. All authors reviewed and approved the manuscript.

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frontiers in Cellular and Infection Microbiology

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ERG-Associated lncRNA (ERGAL) Promotes the Stability and Integrity of Vascular Endothelial Barrier During Dengue Viral Infection via Interaction With miR-183-5p

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Dengue virus (DENV) continues to be a major public health problem. DENV infection will cause mild dengue and severe dengue. Severe dengue is clinically manifested as serious complications, including dengue hemorrhagic fever and/or dengue shock syndrome (DHF/DSS), which is mainly characterized by vascular leakage. Currently, the pathogenesis of severe dengue is not elucidated thoroughly, and there are no known therapeutic targets for controlling the disease effectively. This study aimed to further reveal the potential molecular mechanism of severe dengue. In this study, the long non-coding RNA, ERG-associated lncRNA (lncRNA-ERGAL), was activated and significantly up-regulated in DENV-infected vascular endothelial cells. After knockdown of lncRNA-ERGAL, the expression of ERG, VE-cadherin, and claudin-5 was repressed; besides, cell apoptosis was enhanced, and cytoskeletal remodeling was disordered, leading to instability and increased permeability of vascular endothelial barrier during DENV infection. Fluorescence *in situ* hybridization (FISH) assay showed lncRNA-ERGAL to be mainly expressed in the cytoplasm. Moreover, the expression of miR-183-5p was found to increase during DENV infection and revealed to regulate ERG, junction-associated proteins, and the cytoskeletal structure after overexpression and knockdown. Then, ERGAL was confirmed to interact with miR-183-5p by luciferase reporter assay. Collectively, ERGAL acted as a miRNA sponge that can promote stability and integrity of vascular endothelial barrier during DENV infection via binding to miR-183-5p, thus revealing the potential molecular mechanism of severe dengue and providing a foundation for a promising clinical target in the future.

Keywords: lncRNA, ERGAL, dengue virus, severe dengue infection, permeability, miRNA-microRNA

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INTRODUCTION

Dengue virus (DENV) is a single positive-strand RNA virus belonging to *Flaviviridae* family, and is spread by *Aedes* mosquitoes. DENV has four virus serotypes (DENV-1–4), all circulating worldwide, including in Asia, Africa, and the Americas. A total of 390 million cases of dengue infection are estimated to occur per year, of which 96 million clearly show severe clinical or subclinical manifestations, ranging from mild fever to potentially fatal syndromes such as dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) (Guzman et al., 2010; Bhatt et al., 2013), which is the most severe complication of DENV infection. Severe dengue, referred to as the occurrence of DHF/DSS, whose main pathological feature and clinical manifestation is the increased vascular permeability, results in the paracellular leakage of plasma fluids and protein and exhibits clinical symptoms like bleeding or diffuse intravascular coagulation and subsequently death. It is estimated that the mortality rate can be as high as ∼50% in infants or secondary heterotypic infections (Martina et al., 2009). However, the pathogenesis of severe dengue has not been fully elucidated to date.

Vascular endothelial cells form a vascular barrier to prevent blood cells from extravasation and maintain a dynamically stable tissue environment. However, the paracellular barrier is the preferred route, when cells and solutes migrate or leak from the blood vessels due to pathophysiological changes (Vestweber et al., 2009). The paracellular barrier, which is also defined as the connection between endothelial cells, is mainly formed by adherent junctions and tight junctions that dynamically regulate permeability of the endothelium (Aghajanian et al., 2008). Adherent junctions, composed of transmembrane vascular endothelial (VE)-cadhesions, can interact with VE-cadherin expressed in adjacent cells via a homotropic mechanism to regulate paracellular permeability (Vestweber, 2008). Claudin-5, a member of the claudin family constituting the tight junctions, is enriched specifically in endothelial cells, where its induced expression alone is sufficient to reconstitute the paracellular barrier and block large molecules from entering the leaky endothelial cells (Morita et al., 1999; Soma et al., 2004; Aghajanian et al., 2008; Vandenbroucke et al., 2008). Besides, expression and clustering of VE-cadherin at junctions are required to up-regulate the transcription of claudin-5 (Taddei et al., 2008), all mentioned above revealing that both VE-cadherin and claudin-5 are key junctional proteins in cell junctions, playing crucial roles in regulating vascular permeability.

Vascular development and angiogenesis require the ETS transcription factor family (Randi et al., 2009). ETS-related gene (ERG) was found to express in the endothelium and regulates vascular homeostasis, angiogenesis, and stability, as well as monolayer integrity and cell growth (Birdsey et al., 2012, 2015). Moreover, it is also involved in modulating permeability and cell survival by regulating the transcription of VE-cadherin or claudin-5 (Birdsey et al., 2008; Yuan et al., 2012). In other words, cytoskeleton and associated proteins can also function as effective regulatory elements of the endothelial barrier (Wang et al., 2010), such as F-actin, which is also known to be particularly important in maintaining integrity and regulating epithelial junction remodeling, affecting the permeability of tissue barriers (Wang et al., 2015).

Only 2% of human genes can encode proteins, while the rest consist of transcriptional regulatory elements and non-coding RNAs. Of them, long non-coding RNAs (lncRNAs) are a kind of non-coding RNAs with at least 200 nucleotides, accounting for ∼80% of all non-coding RNAs (Collins, 2004; Costa, 2005; The FANTOM Consortium, 2005). LncRNAs reportedly play important roles in regulating chromatin remodeling, controlling gene transcription, participating in mRNA transcription, regulating protein function, and participating in intercellular signaling (Mercer et al., 2009). The viruses, like human immunodeficiency viruses, hepatitis B virus, influenza virus, and Epstein–Barr virus, could induce dysregulated expression of lncRNAs, thereby altering the normal function of the host cell. Such differentially expressed lncRNAs are shown to regulate cell proliferation and differentiation as well as to be involved in innate immunity and signal transduction for regulating viral infection (Iwakiri and Takada, 2010; Peng et al., 2010; Lau et al., 2014; Saayman et al., 2014). What is more, lncRNA is reported to localize to the nucleus and/or cytoplasm, and their subcellular localization pattern is considered to indicate their biological basis and potential molecular roles (Derrien et al., 2012; Fort et al., 2014). A new regulatory mechanism has been suggested, involving cytoplasmic lncRNAs that act as natural microRNA sponges to interfere with miRNA pathways, thus reducing the binding of endogenous miRNAs to target genes for fulfilling their functions at post-transcriptional levels (Quinn and Chang, 2015).

In our previous study, monolayers of primary endothelial cells had been used as a model for analyzing the function of the endothelial barrier during DENV infection. We had reported that DENV infection could induce a mass of differentially expressed lncRNAs in vascular endothelial cells; furthermore, the corresponding target genes predicted were mainly involved in important biological processes closely related to severe dengue, hence indicating that differentially expressed lncRNAs were potentially related to the regulation of pathology of severe dengue (Zheng et al., 2019). Of the differentially expressed lncRNAs, the lncRNA with non-code transcript ID: NONHSAT190967 and non-code gene ID: NONHSAG082924 (**Supplementary Table 1**) was found to be increased during viral infection and was located downstream of the transcription factor ERG (GenBank:NM_182918) in cis-regulation. Since *ERG* is associated with vascular permeability, we regarded this as the putative lncRNA that might be associated with vascular permeability. Measurements with respect to lncRNA and ERG were conducted, and a strong correlation was identified; therefore, we proposed its name as "*ERG*-associated lncRNA (ERGAL)," considering its special location and strong correlation. Functional experiments were conducted thereafter, and lncRNA-ERGAL was found to be involved in the regulation of endothelial barrier permeability during DENV infection by binding to miR-183-5p.

The present study highlights an important role of lncRNA-ERGAL in promoting integrity and stability of endothelial

barrier during DENV infection and in the significant interaction between lncRNA-ERGAL and miR-183-5p as a regulator, which might be considered a promising target for treating severe dengue in future.

MATERIALS AND METHODS

Cell Culture and Viral Infection

Aedes albopictus mosquito (C6/36) cells were preserved in our laboratory and were cultured in minimum essential medium (MEM) (Thermo Scientific, CA, USA) with 8% fetal calf serum (Gibco, Carlsbad, CA, USA) and 0.1% penicillin–streptomycin solution (Gibco) at 28°C. Human umbilical vascular endothelial cells (HUVECs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured using the ECM Bullet Kit (ScienCell Research Laboratories) in an incubator (Thermo Scientific, USA) at 37°C and 5% CO_2.

DENV-I strain GD/FS/01/2014 was propagated in C6/36 cells, and virus titers were measured using the $TCID_{50}$ assay. HUVECs were cultured in 6-, 24-, and 96-well plates for infection with or without transfection for 24 h. The infection procedure included addition of DENV at the desired multiplicity of infection (MOI) followed by incubation for 2 h at 37°C. The following assays were conducted at 24 h post-infection. Cells were also incubated with the culture supernatant as mock-infected control.

Quantitative Reverse Transcription PCR (qRT-PCR)

After the intervention, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, followed by quantitative and qualitative RNA analyses using the NanoDrop 2000 (Thermo Scientific, USA). RNA was reverse transcribed to cDNA using the PrimeScript RT Master Mix kit (Takara, Japan) according to the manufacturer's instructions for detecting the expression of ERGAL, VE-cadherin, and claudin-5, while the Evo M-MLV RT Kit (Accurate Biotechnology, China) was used for analyzing microRNA. qPCR was performed using the TB Green Premix Ex TaqII kit (Takara, Japan) on a CFX96 Real-Time PCR system (BIO-RAD, USA). The relative expression of lncRNA, mRNA, or miRNA was calculated using the comparative ΔΔCt method. Primer sequences used for the qRT-PCR assay are provided in **Supplementary Table 2**.

Western Blotting

Cultured cells (5×10^6) were lysed on ice using PMSF lysate buffer. Samples containing 40–60 μg of protein were subjected to thermal denaturation and SDS-PAGE and were then transferred to PVDF membranes. After the membrane was sealed with a blocking solution (TBST containing 5% skim milk) for 60 min, it was incubated with the primary antibody: anti-ERG (Abcam, England, Ca#133264), anti-VE-cadherin (Abcam, Ca# ab33168), and anti-claudin-5 (Abcam, Ca# ab15106) overnight and then with the HRP-labeled secondary antibody (Proteintech, USA, Ca# SA00001-1) at 37°C or 1 h. Finally, the BeyoECLPlus chemiluminescence reagent (Beyotime, China, P0018) was used to visualize the protein bands.

Immunofluorescence Microscopy

Huvecs were washed once using phosphate-buffered saline (PBS; pH 7.4), fixed with 4% paraformaldehyde for 20 min, and blocked with PBS containing 10% goat serum, 0.3 M glycine, 1% BSA, and 0.1% Tween for 1 h at room temperature. Cells were incubated with the following primary antibodies: anti-VE-cadherin antibody (Abcam, England, Ca# ab33168) at a 1/500 dilution and anti-claudin-5 antibody (Abcam, Ca# ab15106) at a 1/200 dilution at 4°C overnight. Cells were then washed with PBS-Tween-20 (PBST) three times and incubated with fluorophore-conjugated secondary antibodies at a 1/100 dilution for 1 h at room temperature. DAPI was used to stain the cell nuclei for 5 min. Samples were imaged on an Axio Imager Z1 (ZEISS, Germany).

Cytoskeletal Staining

Phalloidin-fluorescein isothiocyanate (Sigma-Aldrich, Ca# P5282) was used to label actin filaments. Cells were washed with PBS and fixed with 3.7% formaldehyde solution in PBS for 5 min and again washed extensively in PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS and stained with a 50 mg/ml fluorescent phalloidin conjugate solution in PBS (containing 1% DMSO from the original stock solution) for 40 min at room temperature. The samples were washed several times with PBS to remove unbound phalloidin conjugate. Samples were imaged on an Axio Imager Z1 (ZEISS, Germany).

Transfection

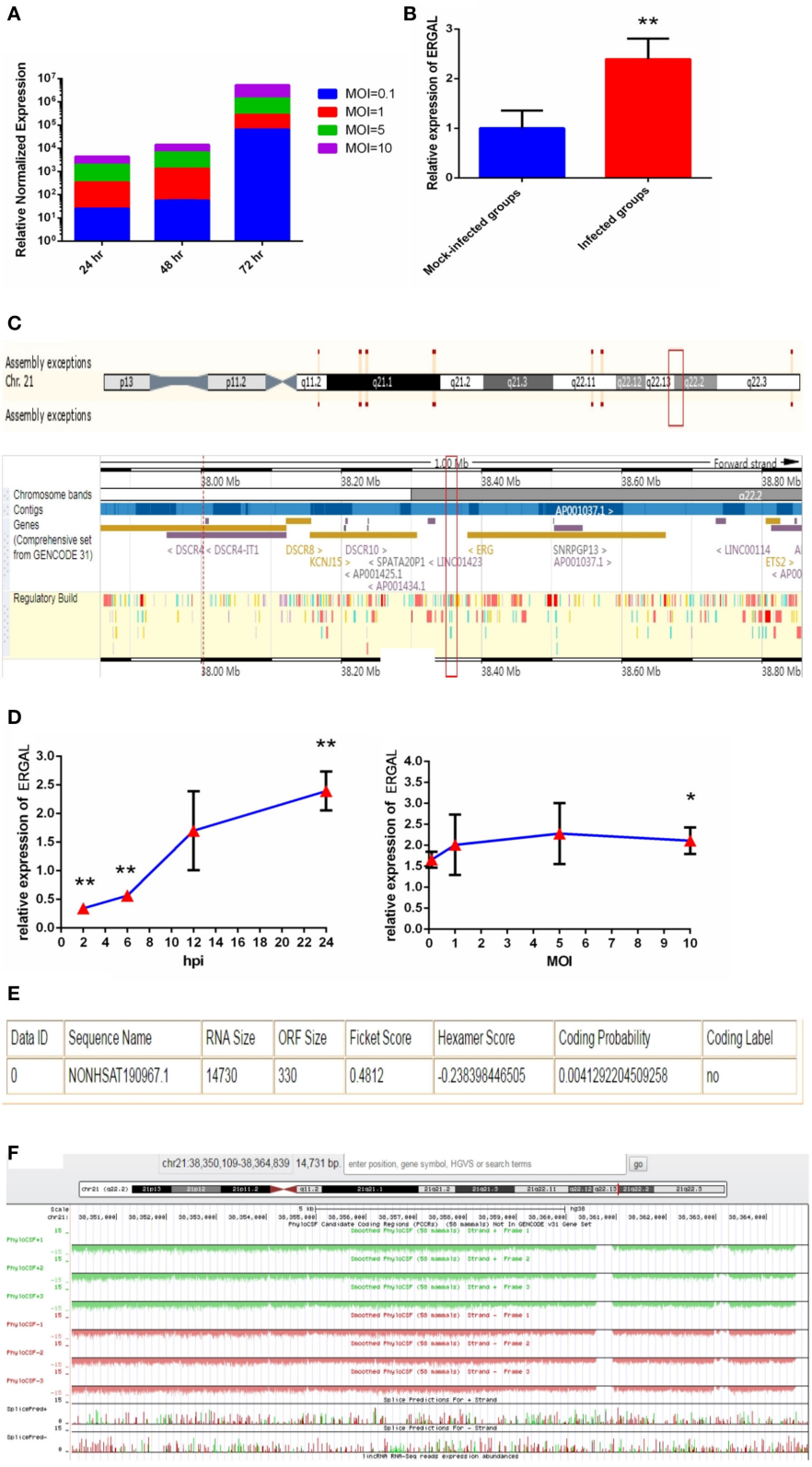
Transfections were performed using Lipofectamine RNAiMAX (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Short interfering (siRNA) targeting lncRNA-ERGAL and miR-183-5p inhibitors were transfected into HUVECs at a final concentration of 100 μM, whereas themiR-183-5p mimic was introduced at a final concentration of 50 μM. si-lncRNA, si-NC, miR-183-5p mimic, miR-183-5p inhibitor, and miR-NC were all designed and synthesized by RiboBio (RiboBio Co., Guangzhou, China) (**Supplementary Table 3**). Cells were then infected with DENV at 24 h after transfection.

Apoptosis Assay

Apoptosis was detected by flow cytometry using the Annexin V-FITC/PI cell apoptosis detection kit (Transgen biotech, Guangzhou, China). After stimulating the cells, they were washed twice in PBS before being resuspended in 100 μl of binding buffer and 5 μl of Annexin V-FITC; then, 5 μl of PI was added in dark conditions to stain the cell for 15 min according to the manufacturer's instructions.

Transwell Assay

An FITC-dextran/transwell assay was used to assess monolayer cell permeability, as described previously (Sedgwick et al., 2002; Irwin et al., 2005). HUVECs were seeded in the upper chamber. After transfection and viral stimulation, the culture medium of the upper chamber was switched to a non-serum culture medium with 5 μg/ml FITC-dextran (Chondrex, Washington, USA, Ca #4009). A culture medium containing 5% FBS was added to the lower chamber and incubated for 1 h at 37°C. The fluorescence



Data ID	Sequence Name	RNA Size	ORF Size	Ficket Score	Hexamer Score	Coding Probability	Coding Label
0	NONHSAT190967.1	14730	330	0.4812	-0.238398446505	0.0041292204509258	no

FIGURE 1 | The expression of ERGAL was up-regulated after DENV infection. **(A)** The viral load in HUVECs showed to be in dose-dependent and time-dependent manner. The expression of E gene of dengue virus in HUVECs was detected by RT-qPCR at different infectious MOIs (a MOI from 0.1 to 10) and time intervals (24 hpi, *(Continued)*

FIGURE 1 | 48 hpi, and 72 hpi) ($n = 3$) compared with those mock-infected groups. **(B)** The expression of ERGAL was increased after DENV infection. The expression levels of ERGAL were detected by RT-qPCR in HUVECs infected with DENV(MOI = 10, 24 hpi). Values were means ± SD ($n = 6$). $^{**}P < 0.01$ vs. mock-infected control group. **(C)** ERGAL genome was located on chromosome 21(start site from 38350109 to end site 38364839) predicted at Ensembl website (http://asia.ensembl.org/index.html). **(D)** The expression pattern of ERGAL was in a time-dependent manner but virus dose-independent. The expression levels of ERGAL were assessed in HUVECs by RT-qPCR at different infectious time intervals (2hpi, 6 hpi, 12 hpi, and 24 hpi with MOI = 10) or different infectious MOIs (MOI = 0.1, 1, 5, and 10 with 24 hpi). Values were means ± SD ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. uninfected control groups. **(E,F)** ERGAL had no protein-coding potential. The bioinformatics prediction of coding protein function of lncRNA-ERGAL was analyzed with Coding-Potential Assessment Tool (http://lilab.research.bcm.edu/cpat/index.php) and PhyloCSF output value assessment at UCSC Genome Browser Gateway (http://genome-asia.ucsc.edu/cgi-bin/hgGateway). The experiments were performed independently at least three times with similar results.

energy value (with an excitation wavelength of 494 nm and an emission wavelength of 520 nm) was detected by SpectraMaxM5 (Molecular Devices, USA). FITC-dextran fluorescence energy values from the upper and lower compartments were obtained; further, the permeability coefficient of dextran (Pd) in HUVECs was calculated according to the following formula: Pd (cm/s) $= ([A]/t) \times (I/A) \times (V/[L])$, where $[A]$ is the FITC-dextran fluorescence energy value of the lower chamber, t (s) is the time interval, A (cm^2) is the transwell surface area, V (m^3) is the volume of the solution in the lower chamber, and $[L]$ is the fluorescence energy value of FITC-dextran in the upper chamber. The ratio of Pd corresponds to either the si-lncRNA group or si-NC group divided by the NC group.

Fluorescence *in situ* Hybridization (FISH)

The subcellular localization of lncRNA in DENV-infected HUVECs was identified by a FISH assay. The lncRNA-ERGAL probe for the assay was designed, and the protocol was conducted using the *in situ* Hybridization kit (Exon Biotechnology, Guangzhou, China) according to the manufacturer's instructions. Images were acquired by laser scanning confocal microscopy LSM710 (ZEISS, Germany).

Double Luciferase Reporter

The mutated lncRNA-ERGAL sequence comprising seven mutated nucleotides within the miR-183-5p binding sites was inserted into a vector. Each construct was co-transfected with the indicated miRNAs (RiboBio Co., Guangzhou, China) into 293T cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 48 h. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, WI, USA) according to the manufacturer's instructions. Luminescence signals were quantified using the BioTek Synergy HTX multi-mode reader, and luciferase activity was presented as the relative hRluc/hluc ratio.

Statistical Analysis

All data analyses were carried out using SPSS20.0 statistical software. The data were presented as the means ± standard deviation (SD) of at least three independent experiments. Comparison between the two groups was made using the Student's t-test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Expression of lncRNA-ERGAL Is Induced by DENV in a Time-Dependent Manner

Since the titer peak of viremia in the early stages of infection is closely correlated with the severity of DENV infection in humans (Vaughn et al., 2000), we performed a qRT-PCR assay to measure the viral load in HUVECs subjected to different virus doses and infectious periods to optimize the infectious dose and duration of infection. We found the viral load in HUVECs to increase notably from 24 to 72 h post-infection, and it tended to increase with MOI values ranging from 0.1 to 10 (**Figure 1A**). An MOI value of 10 could acquire the peak viral load in HUVECs. Owing to the rapid growth of cells and dynamic viral load, we identified infected HUVECs with an MOI value of 10 at 24 h post-infection as a model for conducting high-throughput sequence analyses regarding severe dengue.

Sequence analysis revealed that DENV infection could induce a mass of differentially expressed lncRNAs, of which lncRNA-ERGAL was specifically activated and overexpressed post-infection compared to that in the mock-infected control groups. qRT-PCR was used to verify the expression of ERGAL. As shown in **Figure 1B**, expression of ERGAL was up-regulated after DENV infection. Bioinformatics analysis showed ERGAL to be located on chromosome 21, from site 38,350,109 to 38,364,839, within 20-kb downstream of the transcription factor *ERG* (**Figure 1C**). ERGAL was found to be induced specifically by DENV, suggesting the possible association of ERGAL with the pathology of virus infection. The expression pattern of ERGAL was explored further and found to be increased significantly between 2 and 24 h post-infection; however, it was unrelated to the viral dose administered (**Figure 1D**), indicating that the up-regulation of ERGAL occurred in a time-dependent manner (from 2 to 24 h post-infection) rather than dose-dependent. Analysis using the Coding-Potential Assessment Tool (Wang et al., 2013) and PhyloCSF (Lin et al., 2011) indicated this lncRNA to have no protein-coding potential (**Figures 1E,F**).

LncRNA-ERGAL Was Correlated With the Expression of ERG

As we mentioned above, lncRNA-ERGAL was located within 20-kb downstream of the *ERG* transcription factor; thus, we hypothesized that this lncRNA could be related to the expression of *ERG*. First, as shown in **Figure 2A**, the expression of *ERG*

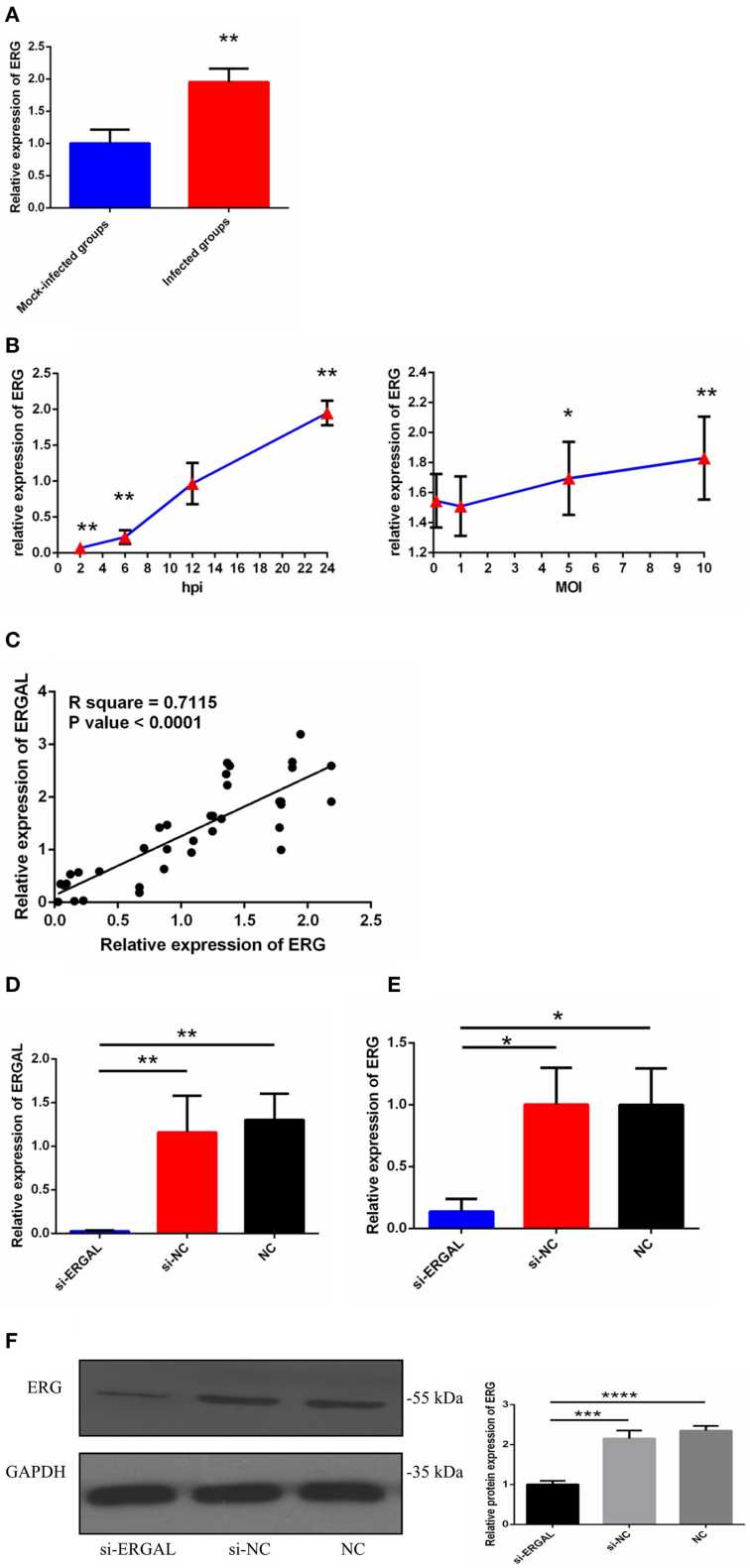


FIGURE 2 | LncRNA-ERGAL was correlated with ERG expression. **(A)** The expression of ERG was up-regulated after DENV infection. The expression levels of ERG were detected in HUVECs by RT-qPCR (MOI = 10, 24 hpi). Values were means ± SD ($n = 6$). $^{**}P < 0.01$ vs. mock-infected groups. **(B)** The expression pattern of

(Continued)

FIGURE 2 | ERG was in a time-dependent manner but virus dose-independent. The expression levels of ERG were assessed in HUVECs by RT-qPCR at different infectious time intervals (2 hpi, 6 hpi, 12 hpi, and 24 hpi with MOI = 10) or at different infectious MOIs (MOI = 0.1, 1, 5, and 10 with 24 hpi). Values were means ± SD ($n = 4$). *$P < 0.05$, **$P < 0.01$ vs. uninfected group. **(C)** ERGAL was strongly correlated with ERG expression. The relationship between ERGAL and ERG was assessed by Pearson correlation analysis. **(D)** The expression of ER GAL was remarkably inhibited after DENV infection by si-RNA transfection. The efficiency of knocking down ERGAL was tested by RT-qPCR after transfecting siRNA 24 h then infecting with MOI = 10 at 24 hpi. Values were means ± SD ($n = 4$). **(E)** The expression of ERG was correspondingly reduced in line with knockdown of ERGAL after infection. The expression of ERG in HUVECs was detected by RT-qPCR after transfecting 24 h then infecting with MOI = 10 at 24 hpi. Values were means ± SD ($n = 4$). *$P < 0.05$; **$P < 0.01$ vs. si-NC groups (negative siRNA control groups) and NC groups (infected control groups). **(F)** The protein level of ERG was decreased with knockdown of ERGAL. The protein level of ERG in DENV-infected HUVECs was evaluated by western blotting. ***$P < 0.001$; ****$P < 0.0001$. The experiments were performed independently at least three times with similar results.

in HUVECs was found to increase during DENV infection. Then, the expression pattern of *ERG* was measured throughout infection and its expression level was found to be similar to that of lncRNA-ERGAL. *ERG* expression also occurred in a time-dependent manner rather than in a virus dose-dependent manner (**Figure 2B**). Since *ERG* was located near the lncRNA and presented a similar expression pattern, we concluded that there could be a certain degree of correlation between them. Pearson's correlation analysis between ERGAL and *ERG* demonstrated ERGAL expression to be strongly correlated with that of *ERG* (**Figure 2C**, $P < 0.0001$, $R^2 = 0.715$).

We used the infectious model (24 h post-infection with MOI = 10) to conduct the subsequent functional experiments due to simulating the early hyperviremia in humans infected with DENV (**Figure 1A**). RNA interference (RNAi) was used to knock down the expression of ERGAL in order to investigate its functional involvement in DENV infection. The efficiency of RNAi for silencing ERGAL was tested first, and short interfering RNA (siRNA) was found to effectively suppress the expression of ERGAL in infected HUVECs compared to that in control cells without siRNA transfection (**Figure 2D**). Additionally, the gene and protein expression of *ERG* were also repressed when ERGAL was silenced (**Figures 2E,F**), indicating once again the association of ERGAL with *ERG* expression.

Knocking Down lncRNA-ERGAL Impaired Cell–Cell Junctions by Inhibiting the Expression of Junctional-Related Proteins

Because both VE-cadherin and claudin-5 were key junction-related proteins that play vital roles in regulating permeability of the endothelial barrier, we further explored the effects of ERGAL on the adherent junctions and tight junctions between cells. qRT-PCR assay was used to measure the gene expression level of VE-cadherin and claudin-5. Results demonstrated that both of them were repressed significantly during DENV infection after lncRNA-ERGAL was knocked down, compared to that in the si-NC and NC groups (**Figure 3A**). As shown in **Figure 3B**, silencing ERGAL also inhibited the protein expression of VE-cadherin and claudin-5 during DENV infection. Additionally, Immunofluorescence analysis demonstrated a comparatively continuous distribution of VE-cadherin along the cell border of HUVECs in both the NC and si-NC groups, whereas its distribution was obviously discontinuous in the si-ERGAL group (**Figure 3C**). Moreover, a mass reduction was observed, along with more severe fractures among the cell–cell junctions in the silenced lncRNA-ERGAL group, as shown by red arrows (**Figure 3C**). Analogously, immunofluorescence results revealed the expression of claudin-5 to be remarkably suppressed in the membrane and in cytoplasm in the group with ERGAL knockdown compared to that in the si-NC and NC groups (**Figure 3D**). Taken together, knockdown of lncRNA-ERGAL aggressively impaired the cell–cell junctions of the vascular endothelial barrier by inhibiting the expression of VE-cadherin and claudin-5 during DENV infection.

lncRNA-ERGAL Knockdown Promoted Apoptosis of Vascular Endothelial Cells and Increased Monolayer Cell Permeability

As shown in **Figure 4A**, flow cytometry analysis with annexin V-FITC/PI fluorescence staining was used to detect early and late apoptosis. The finding demonstrated that the suppression of ERGAL significantly enhanced cell apoptosis, especially promoting early apoptosis ($P < 0.01$), thus indicating ERGAL as possibly an indispensable element for repressing the endothelial cell death caused by DENV infection. Furthermore, DENV-infected HUVECs with knockdown of ERGAL were sparsely and loosely rearranged, the gaps among these cells being notably expanded compared to those in the control groups (**Figure 4B**). Transwell permeability assays were performed to measure the permeability of a HUVEC monolayer. Results demonstrated the permeability coefficient of dextran (P_d) of the si-ERGAL group to be higher than that of the other control groups, representing comparatively elevated permeability after silencing ERGAL in the monolayer endothelial cells. These findings suggested that the knockdown of ERGAL aggravated the breakage of vascular endothelial cell barrier during DENV infection (**Figure 4C**).

In HUVECs that were not infected, cytoskeletal F-actin was clearly structured and neatly arranged (**Figure 4D**: mock-infected group). After DENV infection, F-actin fibers became disassembled and dispersed drastically throughout the cytoplasm, as well as re-polymerizing into stress fibers along the cell edge (**Figure 4D**: infected group and si-NC group), indicating that DENV infection could induce the reorganization of F-actin in the cytoskeleton. When ERGAL was repressed by siRNA, F-actin was almost completely depolymerized and less dispersed in the cytoplasm. The original cytoskeletal structure was also severely disrupted (**Figure 4D**: si-ERGAL group). This finding implied that ERGAL was necessary for F-actin cytoskeleton remodeling during DENV infection.

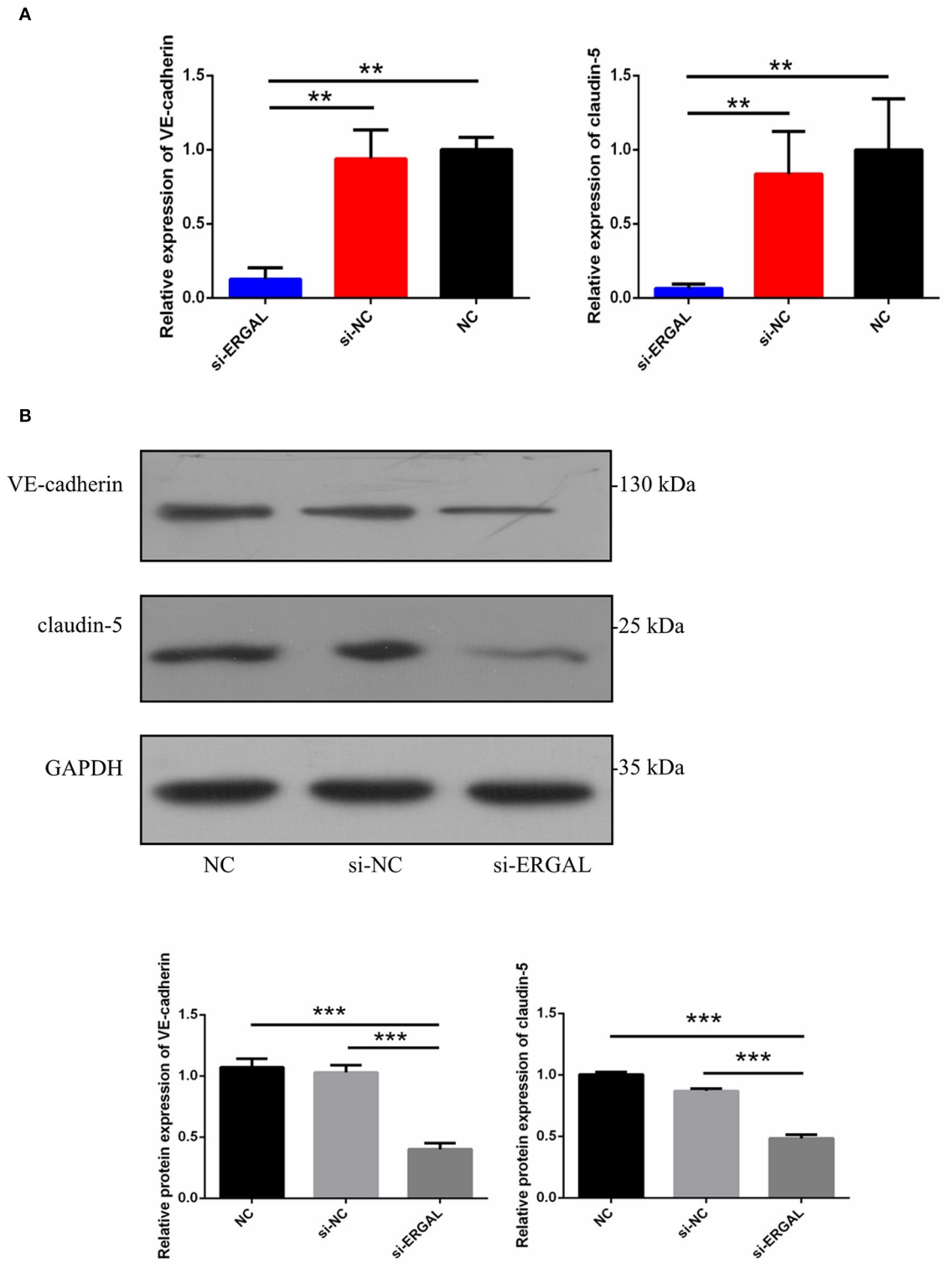


FIGURE 3 | Continued

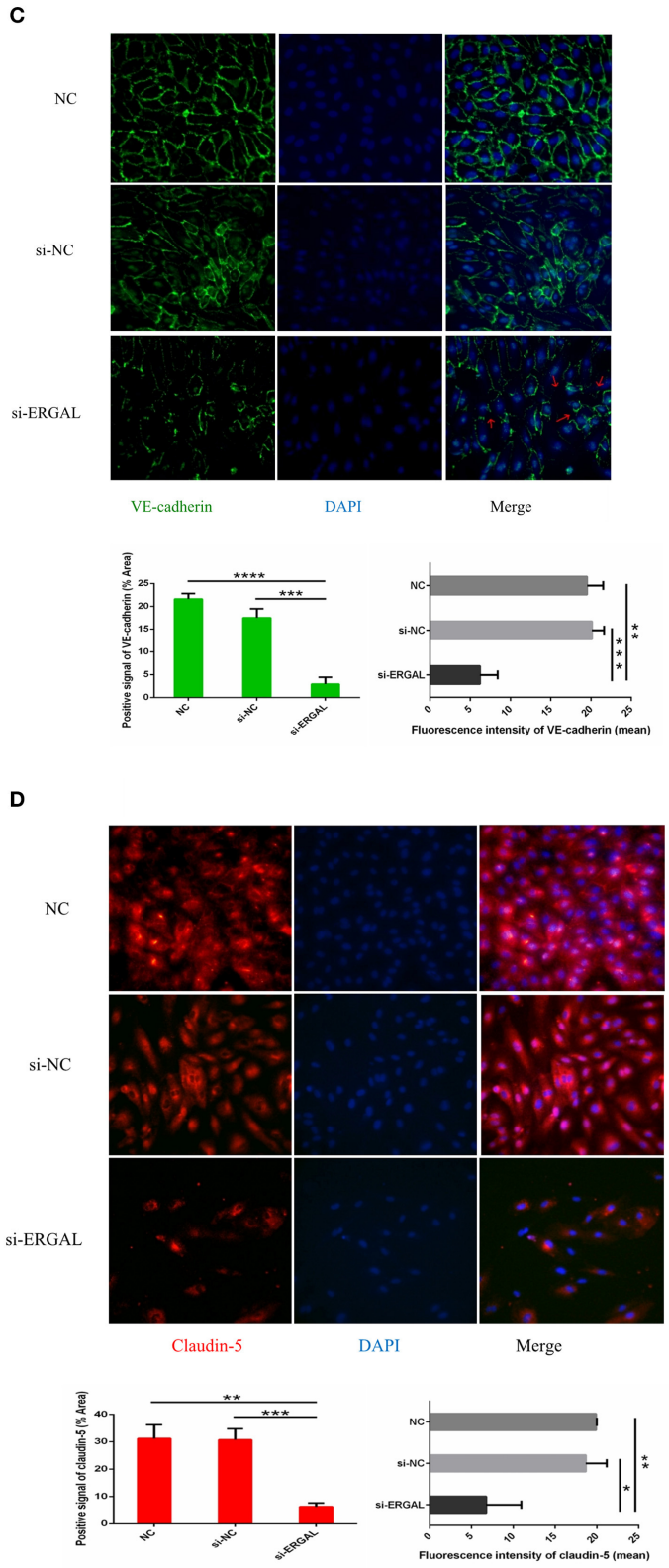


FIGURE 3 | Knockdown of lncRNA-ERGAL impaired the adherens junction and tight junction of HUVECs infected with DENV. **(A)** The gene expression of VE-cadherin and claudin-5 was reduced after DENV infection (MOI = 10, 24 hpi) with knockdown of lncRNA-ERGAL. Relative mRNA expression of VE-cadherin and Claudin-5 was

(Continued)

FIGURE 3 | detected in HUVECs by RT-qPCR with knockdown of lncRNA-ERGAL. Values were means ± SD ($n = 3$). **(B)** The protein level of VE-cadherin and claudin-5 was inhibited after infection (MOI = 10, 24 hpi) with knockdown of lncRNA-ERGAL. Relative protein of VE-cadherin and claudin-5 were measured by western blotting. **(C,D)** The distribution and expression of VE-cadherin and claudin-5 decreased after infection with knockdown of lncRNA-ERGAL. Immunofluorescence analysis of VE-cadherin or claudin-5 in HUVECs. Nuclei were stained blue (DAPI), and VE-cadherin or claudin-5 were stained green or red, respectively. Positive signal represented the area of protein distribution, and the mean fluorescence intensity (Integrated density/Area) represented Semi-quantitative protein expression. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ vs. siNC groups or NC groups. Original magnification: 40x. The experiments were performed independently at least three times with similar results.

MiR-183-5p Regulates the Vascular Endothelial Barrier via Junction-Associated Proteins and the Cytoskeleton

In order to explore the potential regulatory mechanism, the location of ERGAL was confirmed first by RNA FISH, revealing it to be mainly expressed in the cytoplasm (**Figure 5A**). $RegRNA_{2.0}$ (Chang et al., 2013) was further utilized to predict the ERGAL sequences, showing that it contained multiple miRNA binding sites (**Figure 5B**). The above predictions suggested that ERGAL possibly acted as a miRNA sponge, regulating miRNA availability for binding the target mRNA (Tay et al., 2014). While we noted miR-183-5p to be widely reported as an important regulator for many diseases, including cancer and infection, no such report is available to date for DENV infection. We first measured the expression of miR-183-5p in DENV-infected HUVECs and observed that it was highly elevated (over 4-fold) than in mock-infected cells (**Figure 5C**), which implied its involvement in regulating DENV infection. Based on the aberrant expression of miR-183-5p and its predicted relationship with ERGAL, we assumed that miR-183-5p might be possibly involved in the regulation of endothelial barrier during DENV infection. As shown in **Figure 5D**, overexpression of miR-183-5p significantly reduced the protein levels of ERG, VE-cadherin, and claudin-5 in DENV-infected HUVECs. Furthermore, we performed a fluorescence assay to visually assess the effects of miR-183-5p on cell junctions and the cytoskeleton. As shown in **Figure 5E**, VE-cadherin was discontinuously distributed and mostly degraded at the cell–cell junction in the group overexpressing miR-183-5p, whereas it had a comparatively continuous distribution along the cell border when miR-183-5p was inhibited. Similarly, overexpression of miR-183-5p suppressed the expression and distribution of claudin-5 remarkably, whereas knockdown of miR-183-5p increased the abundance of claudin-5 (**Figure 5F**). In addition, F-actin in the cytoskeleton was depolymerized and degraded in the miR-183-5p mimic group, whereas the filaments were well-built and cytoskeleton was remodeled in the miR-183-5p inhibitor group (**Figure 5G**). Consistent with our hypothesis, these results indicated miR-183-5p to be involved in the endothelial barrier by regulating the expression of VE-cadherin and claudin-5, as well as remodeling of F-actin cytoskeleton in DENV infection.

LncRNA-ERGAL Interacts With miR-183-5p to Regulate ERG

The specific binding sites of ERGAL and miR-183-5p were obtained by bioinformatics prediction (**Figure 6A**). A dual-luciferase reporter gene assay was used to verify their binding relationship. As shown in **Figure 6B**, overexpression of miR-183-5p remarkably decreased the luciferase activity of ERGAL-WT vector ($P < 0.0001$), though not of the empty vector. Mutation of the ERGAL pairing sequence in miR-183-5p abolished the interactions between ERGAL and miR-183-5p, and consequently, miR-183-5p overexpression failed to reduce the luciferase activity of ERGAL-MUT vector, thereby indicating ERGAL to function as a sponge for miR-183-5p. The finding suggested that ERGAL/miR-183-5p, as a combination, plays an important involvement in regulating the permeability of vascular endothelial barrier during DENV infection.

DISCUSSION

Dengue has been a major public health problem for years and threatens almost every tropical country, with heavy casualties and economic loss during each epidemic. Severe dengue is about the development of the severe dengue complications, such as DHF/DSS, and has a high mortality rate; it frequently occurs in secondary heterotypic infection or infant infection. Due to the complex pathology and heterotypic cross, severe dengue poses a special challenge to the development of an effective and affordable vaccine. Thus, there is an urgent need to develop novel efficient therapeutic strategies and exploit new kinds of molecular vaccines against the four serotypes simultaneously. Based on a better understanding of the underlying pathogenic mechanisms, it has thus far remained unclear. Wang et al. (2017) reported that the differentially expressed lncRNAs induced by DENV-2 infection in hepatic cells revealed that lncRNA may serve as a new diagnostic marker and therapeutic target for DENV-induced liver damage. Additionally, the expression of lncRNA-NEAT1 was reduced in peripheral blood of patients with severe dengue, which is related to the phenotype of severe dengue fever, suggesting that it would be helpful to understand the progress of DENV-induced diseases by monitoring the expression of NEAT1 and IFI27 in peripheral blood (Pandey et al., 2017). Therefore, the study of lncRNA contributed to dig out its pathogenic mechanism and explore therapeutic targets in severe dengue.

The distinguishing feature of severe dengue is vascular leakage that implies damage to the vascular endothelium, which constitutes the major permeability barrier for the vessel wall (Ramirez et al., 1984). Owing to the many important characteristics that resemble the human microvascular endothelium, a modified HUVEC monolayer that is suitable for studies on dengue hemorrhagic fever was used as a vascular endothelial barrier model (Jacobs and Levin, 2002). On the other hand, HUVECs can be infected with DENV because the results of immunofluorescence assay showed that DENV antigens were distributed in the nucleus and cytoplasm in the infected HUVECs

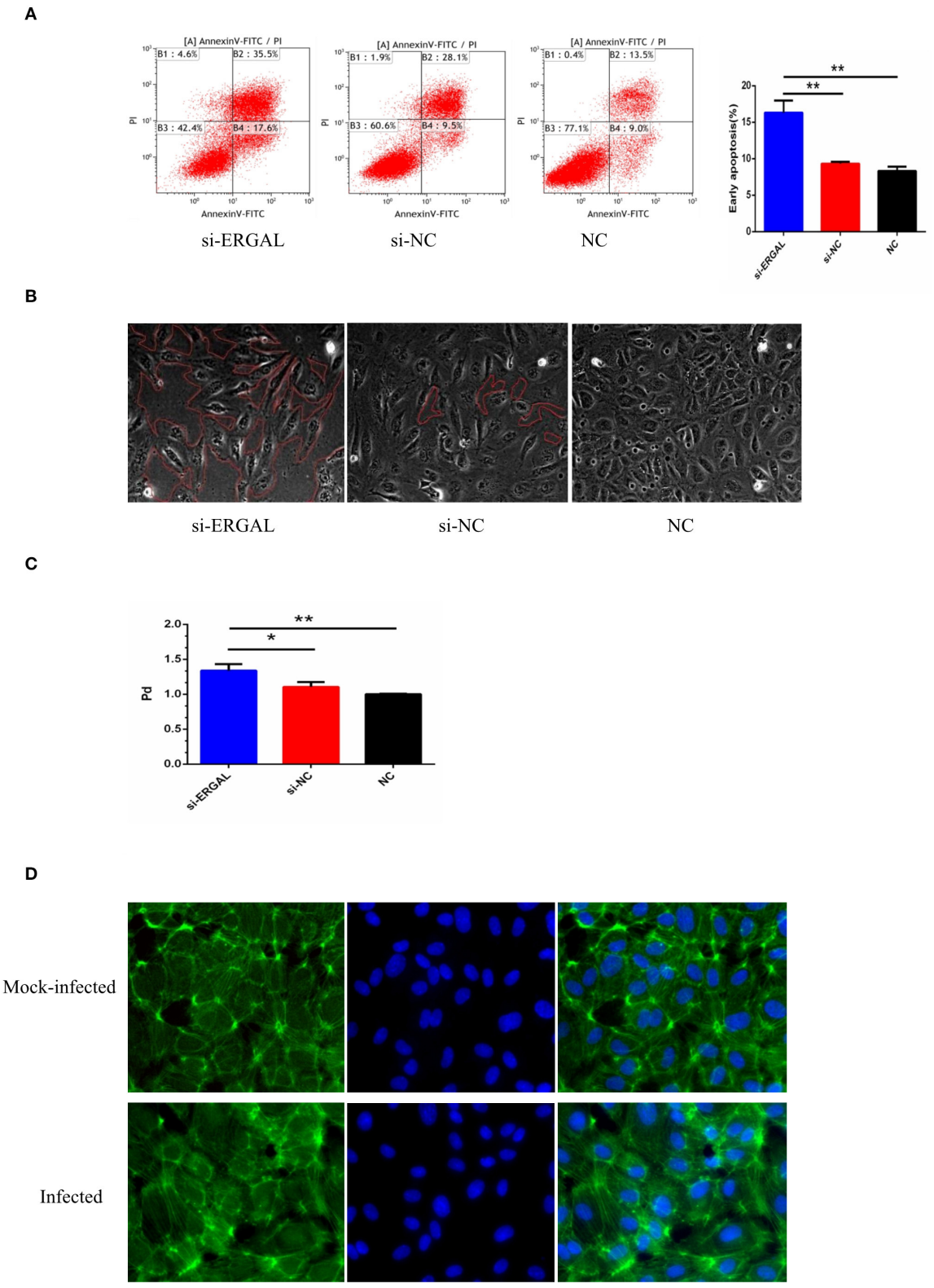


FIGURE 4 | Continued

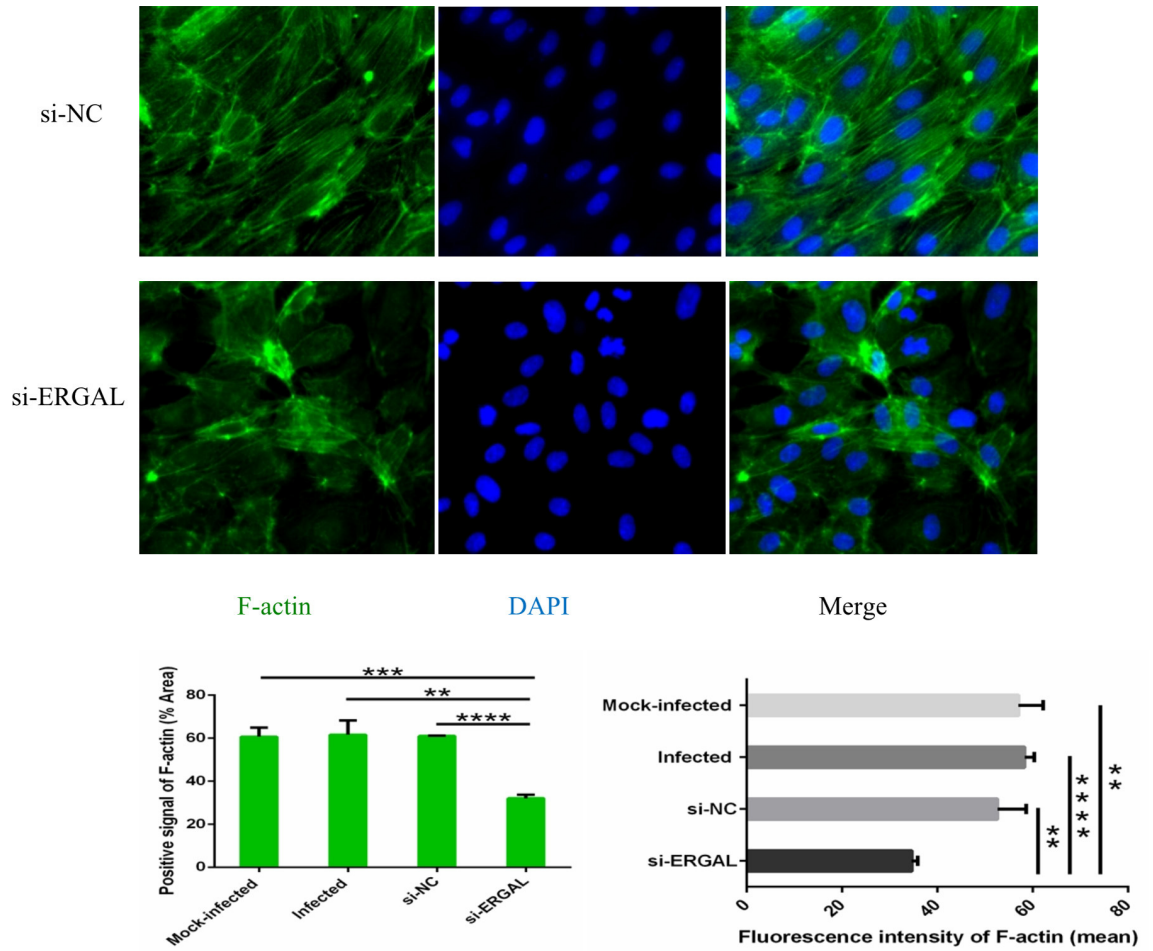


FIGURE 4 | Knockdown of lncRNA-ERGAL promoted vascular endothelial cells apoptosis, increased monolayer cell permeability, and obstructed cytoskeleton remodeling. **(A)** Knockdown of ERGAL enhanced early apoptosis induced by DENV. Apoptotic statues of HUVECs were assessed by flow cytometry. Values were means ± SD ($n = 3$). **(B)** The arrangement of DENV-infected HUVECs with the siRNA-mediated knockdown was sparsely and loosely rearranged, and the gaps among cells were observed to become notably expanded. The gaps among cells were shown under optical microscope (40x), and the gap size was drawn in red. **(C)** The knockdown of ERGAL coincided with increased permeability of HUVECs monolayer to 40 kDa FITC-dextran after DENV infection(MOI = 10, 24 h post-infection). The permeability assays were performed by detected the FITC-dextran permeability coefficient. Values were means ± SD ($n = 3$). **(D)** The distribution and expression of F-actin decreased, and cytoskeleton remodeling disordered after infection with knockdown of ERGAL. Immunofluorescence analysis of F-actin cytoskeleton was performed in HUVECs. Nuclei were stained blue (DAPI), and F-actin were stained green. Positive signal represented the area of protein distribution, and the mean fluorescence intensity (Integrated density/Area) represented Semi-quantitative protein expression. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ vs. siNC groups or NC groups. Original magnification: 40x. The experiments were performed independently at least three times with similar results.

(**Supplementary Figure 1**). More importantly, the viral load in cells was measured with different MOIs (MOI = 0.1, 1, 5, and 10) at different time periods (24 h, 48 h, and 72 h) in order to optimize the infectious model based on the fact that early hyperviremia in patients with dengue fever is related to the development of severe dengue (Vaughn et al., 2000). The results showed that the higher viral load was tested in the cells with the larger MOI and the longer infectious time (**Figure 1A**). HUVECs were likely to develop growth inhibition within 48–72 h, leading to aging and decline and reducing cell viability. Besides, we also found that the virus load in cells was not significantly different between 24 and 48 h with MOI = 10. Therefore, HUVECs with MOI = 10 at 24 h post-infection were selected as the infection model for all the following experiments. Not only could it simulate the early hyperviremia in cells of DENV-infected patients, it also relatively maintained cell viability. We infected this model with DENV (MOI = 10, 24 h post-infection) for high-throughput RNA sequencing and found that the infection could significantly induce a mass of differentially expressed lncRNAs. Furthermore, the differentially expressed lncRNAs and the predicted corresponding target genes were mainly involved in important biological processes closely related to severe dengue, indicating that these lncRNAs might regulate the pathology of severe dengue (Zheng et al., 2019).

In the present study, we discovered a DENV-specific-induced lncRNA (non-code transcript ID: NONHSAT190967, non-code

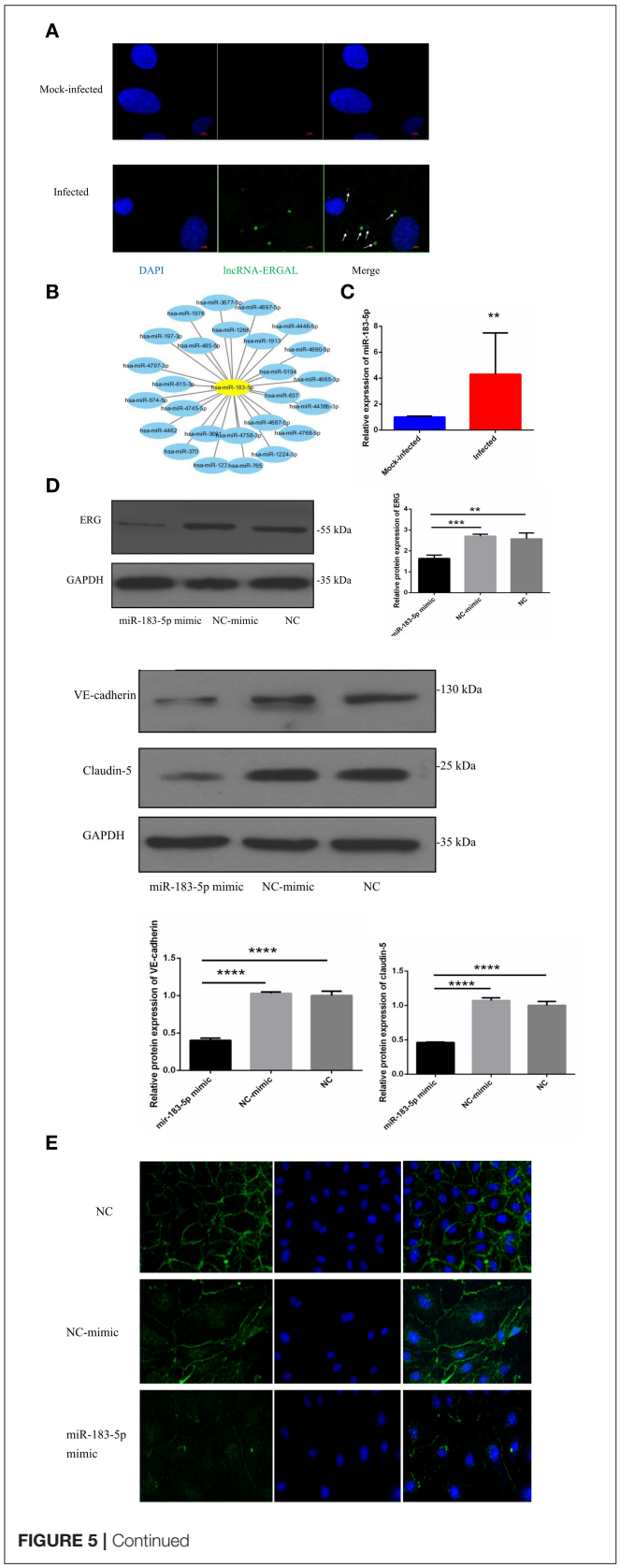
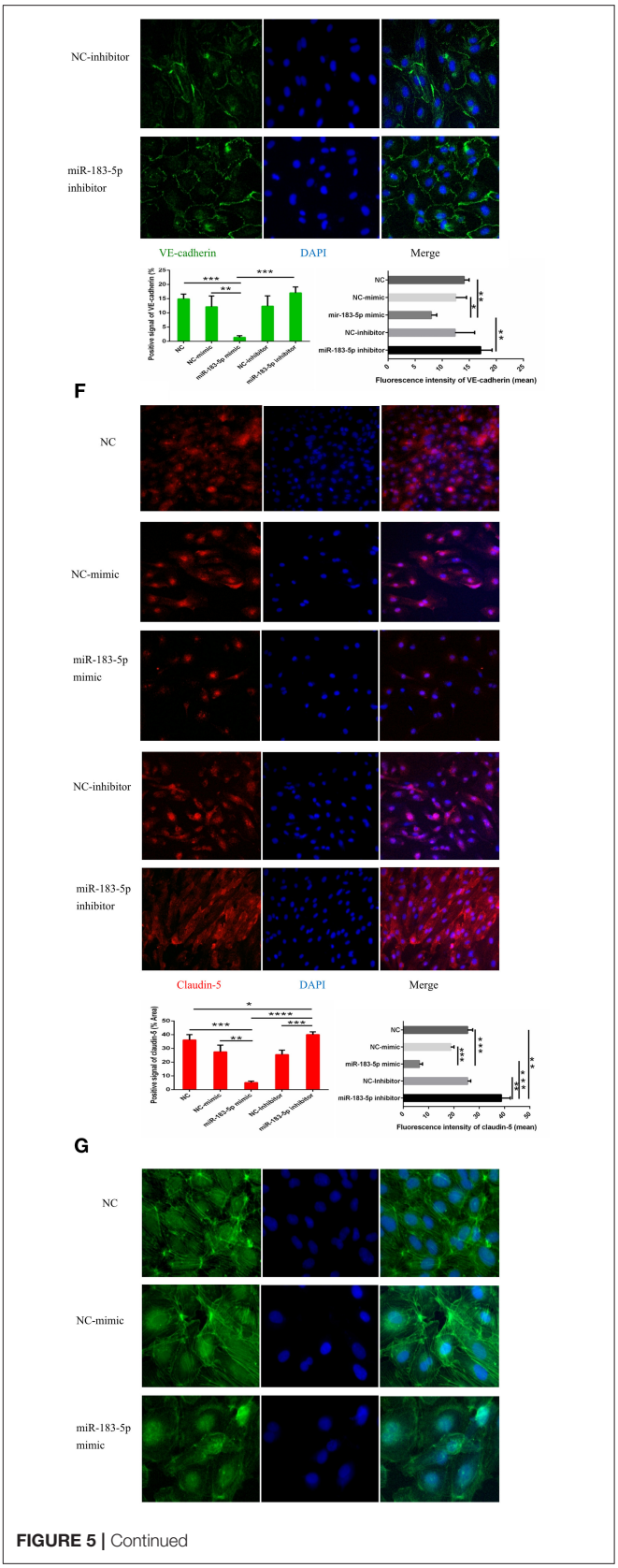


FIGURE 5 | Continued



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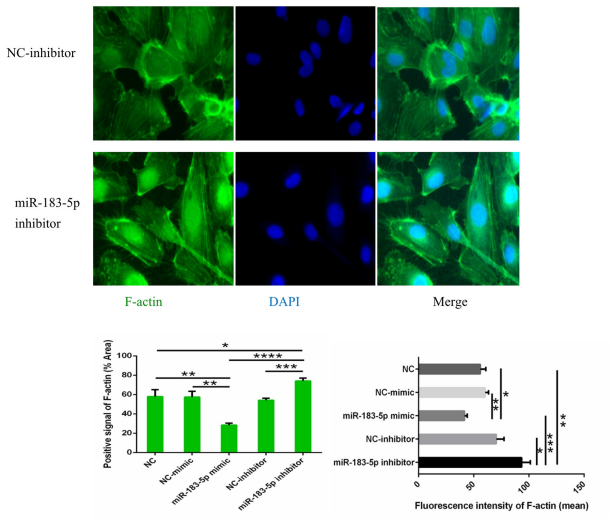

FIGURE 5 | miR-183-5p involved in functioning the endothelial barrier via regulating junction associated proteins and cytoskeleton. **(A)** ERGAL was mainly expressed in the cytoplasm. Localization of ERGAL was detected by FISH in HUVECs. Nuclei were stain blue (DAPI) and ERGAL were stained green (white arrow). Scale bars: 5 μm. **(B)** ERGAL sequence contained multiple miRNAs binding site. The putative microRNAs binding ERGAL were predicted on $RegRNA_{20}$ (http://regrna2.mbc.nctu.edu.tw/). **(C)** The expression of miR-183-5p was significantly increased after DENV infection. The expression levels of miR-183-5p were detected in HUVECs by RT-qPCR (MOI = 10, 24 hpi). Values were means ± SD ($n = 6$). **(D)** The protein level of VE-cadherin and claudin-5 was inhibited in HUVECs with miR-183-5p overexpression and DENV infection. The protein level of VE-cadherin and claudin-5 was measured by western blotting. **(E)** The protein level of ERG was decreased with overexpression of miR-l 83-5p. The protein level of ERG in DENV-infected HUVECs was evaluated by western blotting. **(F, G)** MiR-183-5p regulated the expression of VE-cadherin and claudin-5, F-actin, and cytoskeletal remodeling after DENV infection. Immunofluorescence analysis of VE-cadherin, claudin-5 in DENV-infected HUVECs transfected with microRNA mimic or inhibitor. Nuclei were stained blue (DAPI), VE-cadherin, and F-actin were stained green, claudin-5 were stained red and F-actin were stained green. Positive signal represented the area of protein distribution, and the mean fluorescence intensity (Integrated density/Area) represented Semi-quantitative protein expression. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Original magnification: 40x.

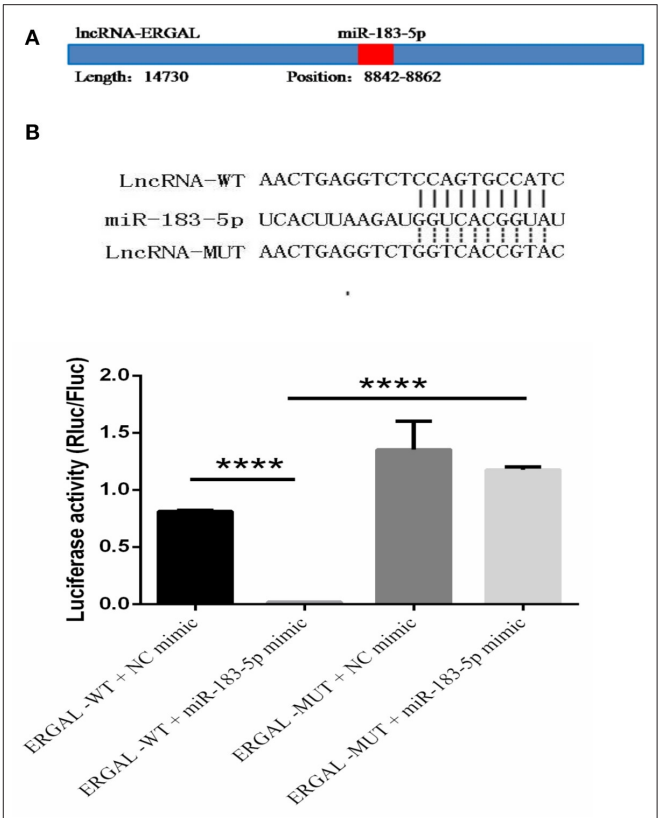

FIGURE 6 | LncRNA-ERGAL interacted with miR-183-5p to regulate the vascular barrier. **(A)** ERGAL was predicted to bind to miR-183-5p from 8842 to 8862 sequence site. The panel showed schematic representation of the predicted binding site for miR-l 83-5p in ERGAL. **(B)** LncRNA could act as miRNA sponge by binding with miR-183-5p. Upper panel presents the alignment or mutation of potential miR-183-5p binding sites in ERGAL transcript. Lower panel showed the interaction between lncRNA-ERGAL and miR-183-5p was proven by luciferase assay. Values were means ± SD ($n = 3$). $^{****}P < 0.0001$.

gene ID: NONHSAG082924) associated with the function of the vascular endothelial barrier. This lncRNA, named *ERG*-associated lncRNA (ERGAL), increased 2-fold in DENV-infected HUVECs in a time-dependent manner instead of a virus dose-dependent manner when compared with mock-infected cells, indicating that it involved during DENV infection. ERGAL was identified as a full-length 14-kb untranslated RNA molecule and was transcribed from chromosome 21, with no predicted protein-coding potential; further, it was found to locate 20 kb downstream of the transcriptional factor *ERG*. We further noticed that ERGAL was silenced without DENV infection, suggesting that it might be activated by DENV as a virus-specific lncRNA. Due to the long length of ERGAL, it was currently difficult to construct a suitable overexpression vector; hence, short interfering RNA (siRNA) was used for knocking down ERGAL to detect its functions. Notably, ERGAL knockdown suppressed both the gene and protein expression level of *ERG*. Previous reports indicated that *ERG* is highly expressed in the endothelium and is essential for endothelial cell homeostasis and angiogenesis (Birdsey et al., 2008, 2015; McLaughlin et al., 2010; Shah et al., 2017), as well as regulating junction stability through transcriptional activation of genes encoding junctional proteins, whereas its loss could increase endothelial permeability (Yuan et al., 2009). Our study assessed and showed the strong correlation between *ERG* and ERGAL, suggesting that ERGAL might play a potentially important role in regulating the vascular endothelial barrier function via *ERG* during DENV infection.

Studies had shown that high DENV load infection altered the junctional integrity of brain and lung microvascular endothelial cell (MEC) lines during early infection (24 h post-infection), with the differential expression of adherent proteins, junctional proteins, gap proteins, and adhesive molecules, of which VE-cadherin was up-regulated within the first few hours of infection with a high MOI of DENV, while the expression slowly decreased later (Soe et al., 2017). This might be a protective mechanism

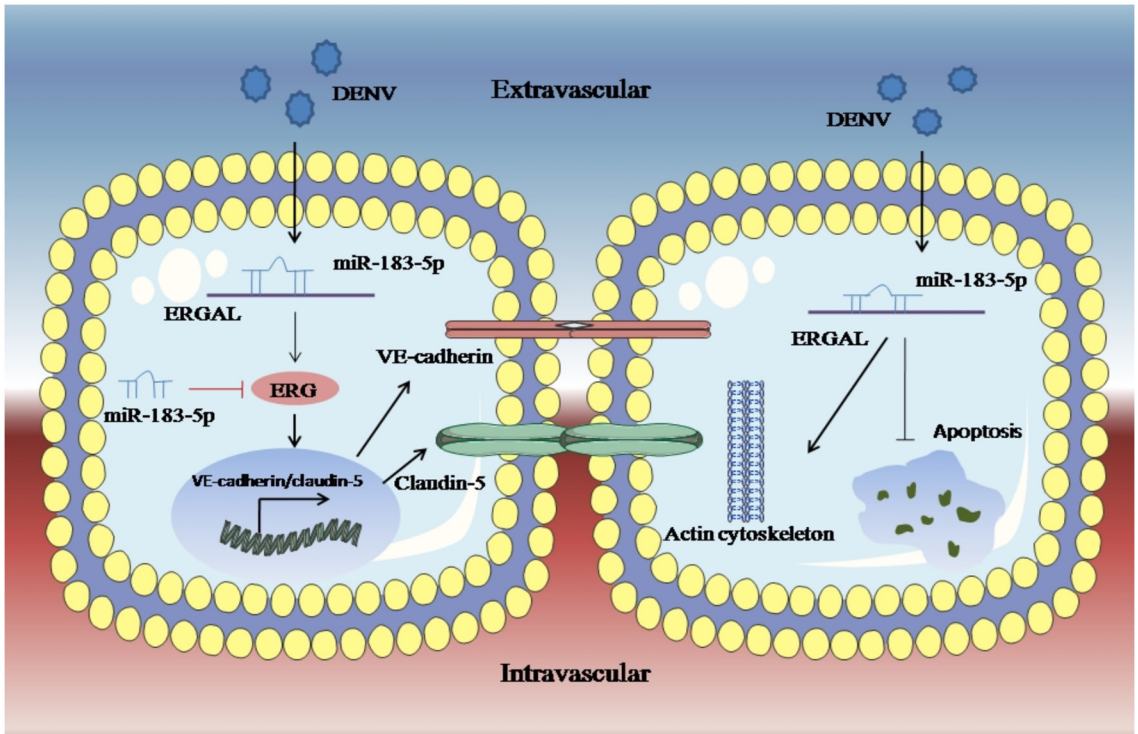

FIGURE 7 | LncRNA-ERGAL was induced by DENV and involved in regulating vascular endothelial barrier during early infection. Dengue virus can activated ERGAL, which could bind with miR-183-5p to attenuate its inhibition on expression of ERG, VE-cadherin, and claudin-5, besides ERGAL could reduce the early apoptosis and promote cytoskeleton remodeling for promoting the stability and integrity of endothelial barrier against DENV challenge.

against the DENV infection during early infection, possibly by increasing anchorage of VE-cadherin to the actin cytoskeleton, as well as by up-regulating the expression of tight junction proteins (Taddei et al., 2008). However, ERGAL silencing dramatically down-regulated the gene and protein expression of VE-cadherin and claudin-5, leading to intercellular gap formation, as well as disruptions in the cytoskeletal structure, all of which resulted in the imperfection and instability of vascular endothelial barrier during early infection. Moreover, ERGAL knockdown greatly promoted early apoptosis of endothelial cells, thus aggravating endothelial injury leading to damage of the blood vessel lining. In our sequencing analysis, we had predicted that multiple lncRNAs could regulate the XAF1 gene (NM_199139, up-regulated 5.6-fold in the sequencing analysis), which contributed to induce apoptosis in vascular endothelial cells in DENV-infected HUVECs (Long et al., 2013); thus, we believed that apoptosis of vascular endothelial cells were more likely to be related to the regulation of multiple lncRNAs during DENV infection. There was no doubt that apoptosis could cause the decreased expression of VE-cadherin and claudin-5 to some extent. However, as shown in immunofluorescence assays (**Figures 3C,D**), VE-cadherin was obviously discontinuous and a mass reduction was observed, along with severe fractures among the cell–cell junctions in the silenced ERGAL groups. Similarly, the expression of claudin-5 was remarkably suppressed in the membrane and cytoplasm in the group with ERGAL knockdown, so we assumed that the reduced expressions of VE-cadherin and claudin-5 were mainly caused by ERGAL knockdown rather than apoptosis. Furthermore, previous reports have revealed that *ERG* and VE-cadherin were associated with apoptosis resistance and antiapoptotic signals related to network stability (Birdsey et al., 2008). Thus, we considered that ERGAL might play an essential role in cell survival via regulating *ERG* and VE-cadherin. Taken together, ERGAL contributed to promote the stability and integrity of the vascular endothelial barrier during DENV infection by regulating *ERG*, junctional proteins, cytoskeletal remodeling, and cell survival.

An RNA FISH assay was performed and found that ERGAL was mainly expressed in cytoplasm, indicating that ERGAL might participate in the post-transcriptional regulation. Furthermore, bioinformatics prediction showed that the ERGAL sequence contained multiple microRNA biding sites, suggesting that it could act as miRNA sponge to interfere with miRNA pathways. Notably, miR-183-5p, which belongs to the miR-183 family, is involved in tumor progression by acting as an oncogene, tumor suppressor, and a biomarker (Rizos et al., 2015; Cheng et al., 2016; He et al., 2018; Meng and Zhang, 2019), and is closely associated with the development of cancer. Additionally, the miR-183 family has been revealed to target a diverse set of host mRNA, some of which have potential effects on viral replication by changing the behavior or resilience to stress in the infected cells. It was also reported that the miR-183 family

plays roles in the pathogenicity of viral infection like Epstein–Barr virus (EBV) or human cytomegalovirus (HCMV) (Stark et al., 2012; Dambal et al., 2015; Oussaief et al., 2015), but its role in DENV infection has been unknown thus far. Therefore, miR-183-5p attracted our interest and attention. In our study, miR-183-5p was dramatically up-regulated after DENV infection. Furthermore, its overexpression damaged the endothelial barrier by suppressing *ERG*, VE-cadherin, and claudin-5, as well as destructing the cytoskeleton during DENV infection, whereas inhibition of miR-183-5p presented the opposite effects. As ERGAL and miR-183-5p showed opposite functions with regard to the vascular endothelial barrier during DENV infection, we conducted a dual luciferase reporter gene assay and confirmed that ERGAL could target and bind miR-183-5p, thereby acting as an miRNA sponge to regulate the permeability of vascular barrier during DENV infection (**Figure 7**). However, there were also some limitations of our study. We used mock-infected cells that were incubated with culture supernatants as the control group without infection when detecting the expression of some genes after DENV infection. Since we could not detect the expression of ERGAL in mock-infected cells, only the infected cells were used as the control groups including NC groups (normal control groups with infection) and si-NC groups (negative siRNA control groups) for functional verification of ERGAL. We also use the NC-mimic groups (negative microRNA overexpression control groups) and NC-inhibitor groups (negative microRNA inhibition control groups) as control groups when detecting the function of miR-183-5p after its overexpression and inhibition. In order to simulate the early hyperviremia in cells of patients and maintain cell viability, only one time point (24 h post-infection) with MOI = 10 was used for all the experiments rather than multiple time points. The possible role of ERGAL in natural infection in human patients was also necessary to be further explored through subsequent animal experiments. Though ERGAL was found to be induced by DENV and regulate the vascular permeability during early DENV infection *in vitro*, it is also very essential to verify ERGAL functions *in vivo*.

Overall, based on what we found in the present study, ERGAL was induced by DENV and promoted the stability and integrity of endothelial barrier during 24 h infection by binding with miR-183-5p, which could further reveal ERGAL/miR-183-5p as regulators involved in the molecular mechanism of pathogenicity, as well as might provide a foundation for a promising target for the clinical treatment of severe dengue.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LJ, JZ, and ZJ conceived the study. HW, JZ, HY, and DF provided instructions during the experiment. BZ, GC, LS, and QG designed and performed the experiment. BZ wrote the manuscript. JZ and HW revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00477/full#supplementary-material

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REVIEW
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Inflammasome Fuels Dengue Severity

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Dengue is an acute febrile disease triggered by dengue virus. Dengue is the widespread and rapidly transmitted mosquito-borne viral disease of humans. Diverse symptoms and diseases due to Dengue virus (DENV) infection ranges from dengue fever, dengue hemorrhagic fever (life-threatening) and dengue shock syndrome characterized by shock, endothelial dysfunction and vascular leakage. Several studies have linked the severity of dengue with the induction of inflammasome. DENV activates the NLRP3-specific inflammasome in DENV infected human patients, mice; specifically, mouse bone marrow derived macrophages (BMDMs), dendritic cells, endothelial cells, human peripheral blood mononuclear cells (PBMCs), keratinocytes, monocyte-differentiated macrophages (THP-1), and platelets. Dengue virus mediated inflammasome initiates the maturation of IL-1β and IL-18, which are critical for dengue pathology and inflammatory response. Several studies have reported the molecular mechanism through which (host and viral factors) dengue induces inflammasome, unravels the possible mechanisms of DENV pathogenesis and sets up the stage for the advancement of DENV therapeutics. In this perspective article, we discuss the potential implications and our understanding of inflammasome mechanisms of dengue virus and highlight research areas that have potential to inhibit the pathogenesis of viral diseases, specifically for dengue.

Keywords: dengue (DENV), NLRP3 inflammasome, innate immune response, cytokine storm, IL-1β, mosquito borne disease, vascular leakage, pyroptosis

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INTRODUCTION

Dengue fever is an acute febrile disease triggered by the dengue virus (DENV). DENV is positive sense, single stranded, RNA virus, belongs to the *Flaviviridae* family, genus *flavivirus*. *Aedes* mosquitoes, *Aedes aegypti* and occasionally *Aedes albopictus* transmit DENV to humans.The disease is caused by mainly four DENV serotypes, DENV 1-4. DENV imposes risk for more than 2.5 billion people with likely 50 million infections per year of infection, majorly in tropical and subtropical countries (Guha-Sapir and Schimmer, 2005; Brady et al., 2012; Bhatt et al., 2013). Constant circulation of different serotypes of the virus further leads to cross-reactivity and imposes added risk on successive dengue infections (secondary infection). DENV infection leads to several clinical symptoms including dengue fever (DF) that is asymptomatic mild flu, to coagulopathy, enhanced vascular fragility, and thrombocytopenia, well-known as Dengue Hemorrhagic fever (DHF), that lead to hypovolemic shock called Dengue Shock Syndrome (DSS), a more severe condition (Murphy and Whitehead, 2011; Afroz et al., 2016). Several factors contribute to DENV pathogenesis such as substantial cytokine secretion ("cytokine storm"), host

genetics, heterotypic secondary infection, and antibody-dependent enhancement (ADE) (Pang et al., 2007; Katzelnick et al., 2017). Although, dengue pathogenesis remains elusive, the cytokine storm has been considered to be one of the primary and crucial causative factors (Hatch et al., 2011; Rothman, 2011). IL-1β, a very strong cytokine that is intensely regulated and stimulated by DENV infected macrophages and monocytes, considered as an important component in cytokine storm (Chang and Shaio, 1994; Dinarello, 2009; Wu et al., 2013b). Several evidences have demonstrated the elevated level of IL-1β in serum cytokine and gene expression profiles in severe dengue patients suggesting the contribution of IL-1β in the severity of dengue pathology (Bozza et al., 2008; Jaiyen et al., 2009). IL-1β increases the vascular permeability, especially in concurrence with TNF-α and IFN-β in many severe dengue profiles (Netea et al., 2000; Dinarello, 2004). As a part of its role as an endogen pyrogen (fever-causing), IL-1β stimulates lymphocytes and foster leukocytes infiltration to the inflammation site, thereby regulating local and systemic inflammation (Dinarello, 2011). Two-step activation pathways are required in the production of IL-1β: "priming signal" fuels the induction of transcription and synthesis of pro-IL-1β, and subsequent "activation signal" assembles and activates inflammasome and caspase-1 (Schroder and Tschopp, 2010). "Inflammasomes" are multimeric protein complexes of innate immune system that form in the cytosol after detecting PAMPs (pathogen associated molecular patterns) or DAMPs (damage associated molecular patterns). Inflammasome protein complex comprises of a sensor proteins; NLR [nucleotide-binding domain (NBD) and leucine-rich-repeat-(LRR)-containing] family, AIM2 like receptor, ALR, an adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and an inactive zymogen, procaspase-1 (Martinon et al., 2002; Rathinam et al., 2012). Activation of inflammasomes in response to PAMP and DAMP signals leads to autocleavage process, which in turn process pro-caspase-1 to active caspase-1. Activated caspase-1 then converts pro-IL-1β and pro-IL-18 into their active forms (IL-1β and IL-18, respectively) (Martinon et al., 2002). Inflammasome plays a vital function in the host innate immune system by regulating the secretion of proinflammatory cytokines (IL-1β and IL-18) and subsequent induction of "pyroptosis," a form of programmed cell death, activated by inflammatory caspases (Fink and Cookson, 2006; Lamkanfi and Dixit, 2014). Although a variety of pathogen senses the PAMP and DAMPs and activate diverse inflammasomes, study shows a range of virus infection that activates inflammasomes such as the NLRP3, AIM2, and RIG-I and mediate the robust host immune response (Shrivastava et al., 2016). Inflammasomes protect the host from the attack of microbial pathogens and endogenous danger signals, thereby playing a crucial role in host defense. Irrespective of the location in cytosol, inflammasome creates an efficient immune response against extracellular and intra-cellular foreign invaders. Although the optimum inflammasome activation is extremely favorable to the security of the host, dysregulation of inflammasome activation can leads to the worsening of symptoms in infectious diseases and results in the autoimmune and inflammatory disorders development (Lamkanfi and Dixit, 2014). It has been shown that increased serum levels of IL-1β and IL-18 correlates with the gravity of dengue infection (Mustafa et al., 2001; Bozza et al., 2008). This finding suggests that inflammasome play a critical role in the pathogenesis of dengue infection. In this perspective, we will discuss the inflammasomes role during DENV infection and the mechanism through which inflammasome imparts a central role in dengue severity.

VIRUS-INDUCED HOST INFLAMMASOME

Numerous studies have emphasized the significance of inflammasome activation in the regulation of virus infection. A range of upstream sensing receptors comprising the AIM2-like receptor, the NOD-like receptor, and the RIG-I-like receptor determine the inflammasome activation during viral infection. These receptors regulate inflammasome activation while present in different cellular compartments i.e., cytoplasm and the nucleus (Schroder and Tschopp, 2010). Among all the known inflammasomes, the NLRP3 inflammasome is the most significantly studied in viral infections and suggested to play a decisive role in both inflammation and antiviral responses (Chen and Ichinohe, 2015). Viral components as well as several stimuli by virus infection are sensed by host innate immune system and activate NLRP3 inflammasome. There are several stimuli which induce cytosolic DAMP signals, such as protein aggregates, mitochondria injury, and aberrant ion mobilization. Two-step process is prerequisite to trigger the NLRP3 inflammasome. The priming step: TLR, NLR, or RIG-I-like receptor families, IFNAR or by a cytokine receptor activate the priming step that leads to transcriptional upregulation of NLRP3, pro-caspase-1, pro-IL-1β, and pro-IL-18. The activation step: signal for inflammasome activation is triggered in response to infection, tissue damage, metabolic imbalances or diverse stress signals coupled with host sterile cell/tissue damage. Stress signal include pore-forming toxins, nucleic acids, Ca^{++} mobilization, K^{+} efflux, ATP lysosomal damage, crystalline substances and invading pathogens (Lamkanfi and Dixit, 2014; Swanson et al., 2019). Several studies have demonstrated that NLRP3 senses the known activators indirectly due to their structural variability (Bauernfeind et al., 2009; Latz, 2010). Structurally, NLRP3 Inflammasome is made of NLRP3, ASC (adaptor protein apoptosis-associated speck-like protein containing CARD) and procaspase-1. Structurally, NLRP3 has three components i.e., PYD–NOD–LRRs in the order of its domains from N-terminus to C-terminus. During NLRP3 Inflammasome activation, NOD domain arranges the NLRP3 as a homo-oligomer and further links with ASC through its PYD domain. ASC binds with procaspase-1 through its CARD domain to form the complete structure. Thus, NLRP3 inflammasome activation leads to the activation of caspase-1 and secretion of mature IL-1β and IL-18 (Latz, 2010). Subsequently, IL-1β potentially recruits neutrophils to the site of inflammation to assist in the removal of infecting viruses (Niu et al., 2019). Furthermore, IL-1β and IL-18 are also involved in the consequent initiation of the adaptive immune response (Dinarello et al., 2013; Joosten et al., 2013).

Although the NLRP3 inflammasome provides the host antiviral status, irregular NLRP3 inflammasome activation leads to severity of pathological injury during virus infection. As an example, in an Influenza A virus (IAV) infection model, NLRP3 inflammasome activation in juvenile mice leads to severe lung injury independent of viral titer, while sustained elevated levels of type I IFNs exist (Coates et al., 2018). IAV infection also shows the high mortality in IL-1RI$^{-/-}$ mice as compared to wild type mice (Schmitz et al., 2005). Likewise, IL-18$^{-/-}$ shows mortality with increased viral load as compared to wild type mice (Liu et al., 2004). In Herpes simplex virus 1 (HSV-1) infection, increased viral load was observed in IL-1β$^{-/-}$ mice as compared to wild-type mice with decreased immune response (Sergerie et al., 2007). In other studies, IL-18 administration prior to HSV-1 infection protect the HSV-1-infected mice and increase survival (Fujioka et al., 1999), revealing the immune control mechanism of IL-1β and IL-18 against the HSV-1 infection. On the other hand, IL-1β and IL-18 protect the host against HSV-1-induced encephalitis. In addition, NLRP3 inflammasome mediates the chronic intrahepatic inflammation and liver injury during HCV infection (Negash et al., 2013). Inflammasome leads to immunopathology progress and recruits excessive inflammatory cells that leads to pyroptosis mediated cell death (McAuley et al., 2013; Haque et al., 2016). Numerous studies have established the activation of NLRP3 inflammasome by viruses with *in vivo* relevance for control of virus infection (Chen and Ichinohe, 2015; Zhao and Zhao, 2020). Viral pathogens such as Encephalomyocarditis virus (ECMV), Hepatitis C virus (HCV), Sendai virus, Human immunodeficiency virus-1 (HIV-1), Vesicular stomatitis virus (VSV), Human chikungunya virus (CHIKV), Japanese encephalitis virus, Respiratory syncytial virus (RSV), Human rhinovirus (HRV), Zika virus (ZIKV), Influenza A virus (IAV), West Nile virus (WNV), Dengue virus (DENV), Rift Valley fever virus (RVFV), and Coronavirus (SARS-CoV) have been reported to activate inflammasomes (Lupfer et al., 2015; Shrivastava et al., 2016; Zhao and Zhao, 2020). Thus, the outcomes of NLRP3 inflammasome activation are very much virus specific (*in vivo* and in clinical settings) and deliver substantial defense against some pathogenic viruses however can also play a detrimental role in immunopathology of others.

RESPONSE OF HOST INFLAMMASOME TO DENV INFECTION

Dengue infected patients shows a plethora of clinical manifestations including sudden-onset fever, hepatosplenomegaly, headache, muscle and joint pain (Setiawan et al., 1998; Wu et al., 2004). Endogen pyrogens (EP) mediate the fever onset which suggests that DENV may trigger high amounts of endogenous pyrogens (e.g., IL-1β and TNF-α) and lead to high fever onset in the patients.

Macrophages Induced Inflammasome

Studies have demonstrated that macrophages (Mϕ) serve as major target cells for DENV infection, replication and the major source of inflammatory cytokines (Chen and Wang, 2002; Jessie et al., 2004; Balsitis et al., 2009). Mϕ are heterogeneous in nature and during DENV infection, M-Mϕ (resting macrophages) and GM-Mϕ (inflammatory macrophages) display distinctive cytokines expression profiling and innate immunity receptors/sensors. High expression of CLEC5A, mannose receptor, and NLRP3 were found in GM-Mϕ as compare to M-Mϕ, supporting the hypothesis that these two subsets display distinct functions (Wu et al., 2013a). In the continuation, a striking study has suggested that the functions of human macrophage subsets and corresponding signaling events are instrumental in the production of IL-1β and IL-18. This study demonstrated that CLEC5A serves as a myeloid PRR for DENV, and DENV replicates more competently in GM- Mϕ (inflammatory macrophages) than in M-Mϕ (resting macrophages) (Wu et al., 2013b). DENV infection activates the NLRP3 inflammasome in GM-Mϕ that results in caspase-1 activation and subsequent IL-1β and IL-18 release. Interestingly, DENV infection in GM-Mϕ induces the increased expression of NLRP3 without affecting other sensors i.e., NLRC4 and NLRP1, while siRNA targeted to NLRP3 obstructs DENV stimulated IL-1β and IL-18 secretion. Additionally, DENV infection in GM-Mϕ leads to increased level of TNF-α with decreased level of IL-10. Moreover, released cytokines increase body temperature and stimulate the release of soluble factors that result in vascular permeability during dengue infection (Wu et al., 2013b). These results suggest the important function of CLEC5A in dengue severity as another study also shows that CLEC5A is critical for DHF and DSS (Chen et al., 2008). The study also indicated the crucial role that CLEC5A plays in NLRP3 activation, as blockade of CLEC5A blocks the NLRP3 inflammasome activation, and subsequent ablated DENV induced IL-1β production and caspase-1 mediated proptosis in GM-Mϕ (Wu et al., 2013b). Apart from facilitating innate immune response, IL-1β and IL-18 display a crucial function in fostering adaptive immune response during DENV infection. IL-1β secretion further enhances the production and release of IL-23 and IL-6 and the association of IL-18, IL-1β, and IL-23 stimulate Th17/γδ T cells to generate pro-inflammatory cytokines (GM-CSF, IL-17A, IL-17F, IL-22,) that create the stage for host adaptive immune responses during DENV infection (Wu et al., 2013a). Thus, the macrophage is also considered as the primary target of DENV since DENV induced thrombocytopenia releases IL-18 via the activation of NLRP3 Inflammasome. Epidemiologic study also supports this notion as the severity of DF/DHF were correlated with elevated level of GM-CSF in the plasma of DENV patients (Bozza et al., 2008). This study indicates the probable switch mechanism of resting macrophages to inflammatory macrophages by GM-CSF during DENV infection, that affects the gravity of clinical outcomes. This warrants further investigation as the inhibition of CLEC5A may diminish the severity of dengue induced clinical symptoms in patients. Notably, a study demonstrated the importance of immunosuppressive status based on different age group that influences the differential induction of IL-1β by monocytes during DENV infection (Valero et al., 2014). Monocytes/macrophages from neonatal, adult, and elderly was infected with all four DENV types and cytokine profile was analyzed. The study observed the highest cytokine production

(TNF-α, IL-6, and IL-1β) by adult monocytes as compare to neonates and elderly subjects depicting the presence of immunosuppressive condition at both sides of life and may be due to different physio pathological mechanisms (Valero et al., 2014).

Dendritic Cells and Inflammasomes

DENV also targets, human dendritic cells (DCs), a proficient antigen-presenting cell with immune sensors that mounts the immune response in the peripheral blood and in the lymphoid (Ho et al., 2001) and trigger IFN signaling (Hsu et al., 2016). It has been reported that TLR9 in endolysosomes in DC senses "self-DNA," binds to it and triggers signaling events, including NF-κB and mitogen- activated protein kinase (MAPK) p38 signaling pathways (Sasai et al., 2010). Mitochondria plays a crucial role in TLR9 mediated pathways as DAMPs released from mitochondria during tissue injury, stress conditions, and cytokine production due to trauma, trigger inflammatory responses through activation of the TLR9 signaling pathway (Zhang et al., 2010; Caielli et al., 2016; Lood et al., 2016). mtDNA that serves as a DAMP, engages several pattern-recognition receptors present on diverse cell types and initiates potent inducement of the proinflammatory immune response and IFN production (West and Shadel, 2017). Another study demonstrated that DENV infects DC and induces NLRP3 inflammasome activation, triggering ROS production in mitochondria that leads to the discharge of mitochondrial DNA (mtDNA) into the cytosol. mtDNA further triggers the production of interferons (IFNs) by activating TLR9 signaling pathways (Lai et al., 2018). These incidents results in the productions of several anti-viral cytokines IFNs, such as IFN-λ3, IFN-λ2, IFN-λ1. Inhibition of inflammasome diminished the release of mtDNA in the cytosol during DENV infection subsequent TLR9 activation. These data support the crucial roles of DENV-induced inflammasome activation and further DENV induced mtDNA-TLR9 axis mediated IFN production and their following incidents.

DENV Induced Inflammasome in Platelets

During DENV infection, increased IL-1β also correlates with thrombocytopenia (Bozza et al., 2008). A study reports that DENV infects the platelets via DC-SIGN receptor that further leads to platelets activation, mitochondrial dysfunction and activation of the apoptosis caspase cascade, that leads to the development of thrombocytopenia in patients with dengue (Bozza et al., 2008). Another study has tested the hypothesis, whether platelets activation by dengue induce the synthesis and processing of IL-1β, that can be capable of disrupting the endothelial cell barrier function. DENV infection activate the NLRP3 inflammasome, and subsequent activation of caspase-1, and caspase-1 dependent secretion of IL-1β in platelets microparticles from patients with dengue as well as when platelets get exposed to DENV *in vitro*. DENV induced inflammasome activation and subsequent platelet released IL-1β-rich microparticles associated with increased vascular permeability and this increase were found to be IL-1β dependent. In addition, RIP1/RIP3-mediated mitochondrial ROS generation are required for NLRP3 inflammasome activation and subsequent release of mature IL-1β cytokine (p17) and the shedding of IL-1β MPs, indicating the role of RIP1/RIP3-mediated mitochondrial ROS generation in dengue vasculopathy (Hottz et al., 2013).

Neutrophils Activation, NETs Formation, and Inflammasome

A new mechanism has been demonstrated regarding the role of platelets in DHF. The study demonstrated that DENV activates mouse and human platelets via the CLEC-2 receptor, thereby triggering the release of extracellular vesicles (EVs), including microvesicles (MVs) and exosomes (EXOs). Further, EXOs (DV-EXOs) and MVs (DV-MVs) upregulate the CLEC5A and TLR2 on neutrophils and macrophages, resulting in the formation of a neutrophil extracellular trap (NETs) and subsequent release of proinflammatory cytokine. *In vivo* mouse model, DENV induced NETs formation and inflammasome activation was impaired in CLEC5A$^{-/-}$ and TLR2$^{-/-}$ mouse neutrophils. Furthermore, DENV triggered systemic vascular permeability and lethality were reduced dramatically. Further, *in vivo* study demonstrated the role of NETs in complex interface among macrophages, platelets, and neutrophils during DENV infection as DNase I mediated exclusion of NETs provided certain defensive effect in *stat1*$^{-/-}$ mice (35% survival). Furthermore, DNase I has no effect in the survival of DENV-infected *stat1*$^{-/-}$*clec5a*$^{-/-}$ mice, suggesting the NETs formation induced pathogenic effect in DENV infection by increasing systemic vascular permeability (Sung et al., 2019). NETs induce the interruption of vascular endothelial cell layer and vascular leakage in dengue infection, due to the presence of metalloproteinases and histones in the NETs (Saffarzadeh et al., 2012; Carmona-Rivera et al., 2015). NETs formation outcome varies greatly in virus to virus infections and specific mechanism for NETs formation and the factors that decide the functions of NETs in viral infection are still elusive. Specifically, while NETs show their beneficial roles in preventing viral dissemination (murine pox virus) (Jenne et al., 2013) and trapping (HIV) (Saitoh et al., 2012), formation of disproportionate NETs turn in exacerbated allergic airway inflammation during rhinovirus (RV) infection (Toussaint et al., 2017) and airway obstruction during respiratory syncytial virus (RSV) infection (Cortjens et al., 2016). Another *in vivo* study also demonstrated the neutrophil activation and neutrophil extracellular trap (NETs) formation during an acute DENV infection. *In vitro* incubation of NETs results in reduced DENV infectivity. Notably, NETs components were more predominantly present in the serum patients with hemorrhagic fever (DHF), but not acute phase of the infection (Opasawatchai et al., 2019). Other evidence that demonstrates the excessive inflammation during DENV infection indicates that neutrophils activation and NETs formation can contribute significantly in disease pathogenesis (Costa et al., 2013). This suggest that somehow NETs are inhibited during acute phase, however, in the more severe phase, NETs formation is induced, implying the dual roles of NETs in DENV infection. Although, the NETs formation directly by DENV particle is still debatable

due to different views from different studies. Where, in a study, NETs formation has shown to be found in healthy neutrophils during DENV infection (Yost et al., 2016), another study suggests the inhibition of NETs formation due to DENV-2 (Moreno-Altamirano et al., 2015). There could be a link of inflammasome and neutrophil activation and NETs formation, as a study found the overexpression of CD66b in neutrophils, a marker of granulocytes activation and increased ROS production during DENV infection. As ROS production is directly linked to trigger inflammasome, the study observed the increased ROS production in granulocytes, in response to *ex vivo* PMA stimulation during acute dengue infection. This phenomenon is suggested due to TNFα and IL-8 cytokines that present during acute DENV infection. ROS is instrumental in ROS-dependent NETs formation, neutrophil antimicrobial activity and inflammasome activation (Opasawatchai et al., 2019). Another independent study demonstrates the role of GSDMD (Gasdermin D), a pore-forming protein and an initiator of pyroptosis with NETs formation. It has been reported that in non-canonical inflammasomes activation, neutrophils trigger the release of NETs and it was found to be GSDMD dependent. It is reported that neutrophils get several unknown stimuli that activate the protease that further cleaves GSDMD to implement NETosis. Furthermore, ROS is required during classical NETosis that activates serine proteases that too cleave GSDMD (Chen et al., 2018). Further, GSDMD acts as a feed forward loop and includes protease activation and nuclear expansion (Sollberger et al., 2018). How these mechanisms exist during DENV infection is not known and more research understanding regarding the neutrophil activation, NETs formation, inflammasome activation and their interconnection functions are warranted. Overall, this data unravels the central role of CLEC5A/TLR2 and CLEC2 in "neutrophil–platelet interactions" that fuels the enhanced inflammatory reactions during DENV infection (Sung et al., 2019).

Inflammasome Stages the Adaptive Immune Response

Another study emphasizes the inflammasome role in mounting the immune response in dengue virus infection. It has demonstrated that primary human γδ T cells (freshly isolated) react promptly to DENV infected DC (dendritic cells) and produces IFN-γ and upregulates CD107a. This anti-DENV IFN-γ is controlled by DENV infected DC that induces type 1 IFN and IL-18 (Tsai et al., 2015). To note, dengue infected patients plasma demonstrated the elevated levels of type I IFN and IL-18 (Mustafa et al., 2001; Gandini et al., 2013) and these cytokines (type I IFN and IL-18) by producing IFN-γ by γδ T display a physiologically phenomenon in fostering an effective anti-DENV Th1 adaptive-immune response and an important determinant of dengue disease severity. Further, the inhibition of inflammasome activation (due to extracellular ATP) weakened the IFN-γ response of γδ T cells. Notably, monocytes serve as an accessory cells as it induces the IL-18 that further triggers the γδ T cells during infected DC response to DENV (Tsai et al., 2015). A wide range of cells shows the activation and expression of both CC and CXC chemokines due to monocytes/macrophages induces IL-18 that augment neutrophil activity, IFN-γ production and natural killer (NK) cell cytotoxicity by NK cells (Dinarello et al., 2013). Therefore, to gain the full anti DENV activity of γδ T cells, two activation checkpoints are required: Secretion of type I IFN through IRF-dependent pathways (e.g., detection of viral RNA by TLR3 and RIG-like receptors) (Loo et al., 2008; Nasirudeen et al., 2011) and the recognition of released extracellular ATP (due to cell damage) lead to activation of inflammasome (Dinarello and Fantuzzi, 2003).

Inflammatory Mediators of Dengue Disease Biomarker

Monocytes activation and aberrant inflammasome activation have demonstrated a central role in the advancement of severe form of dengue disease that also serves as a biomarker of dengue patients. Recently, a study investigated 20 plasma biomarkers from dengue virus infected patient including chemokines, cytokines, along with additional inflammatory mediators that forecast the severity of dengue disease according to the WHO 2009 classification. This study identified IL-18, LBP (LPS binding protein) and sCD14 as a best predictive value, particularly at the febrile phase as their presence is significantly higher than any other marker tested (Yong et al., 2017). The phenomenon of microbial translocation (MT) has been described where LPS translocate from gut into the blood stream under inflammatory conditions. Microbial translocation play a vital role in HIV disease (Brenchley et al., 2006), inflammatory bowel syndrome (Rojo et al., 2007), chronic liver disease (Pinzone et al., 2012) and even end stage kidney disease (Wang et al., 2012). More recently, MT has been reported among dengue patients (van de Weg et al., 2012) and levels of LPS were accompanied with progression of dengue disease as LPS has been found to act along with DENV in triggering IL-6, PAF, and TNF-α (van de Weg et al., 2013; Kamaladasa et al., 2016). In the context of LBP, LBP is a soluble acute phase protein that initiates immune responses by binding directly to bacterial LPS and relocate it to monocyte/macrophages expressing sCD14 and TLR4 on cell surface (Opal et al., 1999), as LPS stimulation to monocyte and macrophages triggers sCD14 secretion (Landmann et al., 1995). The study, therefore, advocates the central role of MT in intensifying the inflammatory response in dengue infection. As systemic immunity and non-canonical inflammasome is thought to be activated by LPS, an increase of LPS in plasma in DWS+ (dengue with symptoms) and SD (severe disease) patients further supports the LPS induced inflammatory responses. This indicates that apart from viral factors, elevation of LPS in plasma also contributed its part to boost cytokine storm along with other danger-associated molecule patterns (DAMPs). Therefore, prediction of biomarkers early in dengue patients fosters proficiently patient triage and permit improved healthcare support for the population specially during dengue outbreaks.

DENV Induced ADE and Inflammasome

ADE (antibody-dependent enhancement) has been proposed to be one of the most important reasons for cytokine storm. ADE develops when preexisting antibodies from a primary (first)

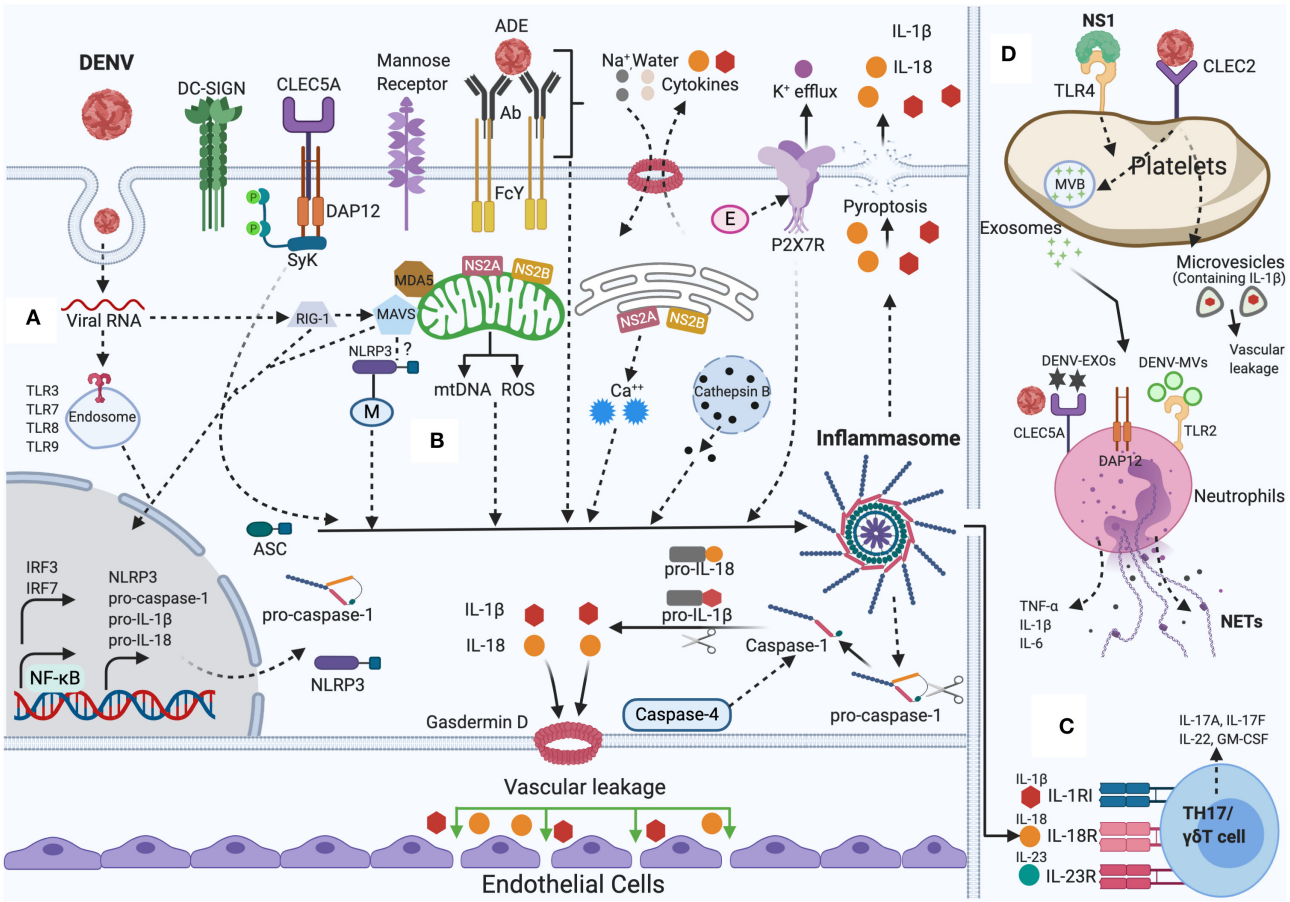

FIGURE 1 | NLRP3 inflammasome activation by DENV: During DENV infection in host cells (Monocytes, Macrophages, Dendritic cells, Endothelial cells, among others). **(A)** Priming (Signal 1): Viral RNA is sensed by cytosolic PRRs (e.g., TLR3, TLR7, TLR8, TLR9, RIG-I, and MDA-5) and triggers the NF-κB signaling, that increases the transcription of pro-caspase-1, ASC, inflammasome receptor (NLRP3), pro-IL-1β, and pro-IL-18. **(B)** Activation (Signal 2): The inflammasome complex is formed and initiated by a secondary stimulus, sensed within the cytosol such as ADE activation, CLEC5A-DAP-12-Syk mediated response, K^+ efflux, ROS generation, mtDNA release, Cathepsin B release, Ca^{++} mobilization, DENV viral proteins (E, M, NS2A, NS2B) mediated DAMPs. Activated NLRP3 inflammasome stimulates the auto-cleavage of pro-caspase-1. Activated caspase-1 further triggers the proteolytic process of pro-IL-1β, pro-IL-18 into its active form as well as gasdermin D (GSDMD) that induces vascular leakage and cell death (pyroptosis). **(C)** DENV induced IL-1β also stimulates the generation of IL-23 and IL-6. IL-1β, IL-18, along with IL-23 stimulates Th17/γδ T cells to release pro-inflammatory cytokines that mounts adaptive immune responses to DENV infection. **(D)** DENV activates platelets and induces the secretion of EVs (DV-EXOs and DV-MVs), that results in the CLEC5A and TLR2 mediated release of proinflammatory cytokines and NETs formation in neutrophils contributing to vascular leakage during dengue infection. NS1 protein, through autocrine loop triggers TLR4 signaling on platelets and thereby amplifies the platelets mediated inflammasome response to DENV infection. Figure generated with Biorender.com.

DENV infection, present in the body, bind to an infecting heterologous DENV particle during re-infection. The antibodies from the primary infection do not have neutralizing capacity against the virus, rather they form Ab–virus complex that binds to Fcγ receptors (FcγR) on circulating monocytes. This results in the receptor mediated endocytosis of the Ab–virus complex, further augmenting the dengue infection and resulting in dengue severity (Whitehead et al., 2007; Raj Kumar Patro et al., 2019). During secondary dengue infection, antibody-dependent enhancement (ADE) has been an anticipated mechanism in explaining dengue hemorrhagic fever (DHF; Guzman and Vazquez, 2010) and studies have observed higher IL-1β level in DHF patients as compared to DF patients (Cui et al., 2016; Kamaladasa et al., 2016). A study has demonstrated the ADE triggered cytokines including IL-1β were studied in primary human monocytes utilizing the anti-DENV mAbs from patient. The data demonstrated that DENV-2 infection in monocytes mediated by ADE induces mature IL-1β secretion in 4 h, independent of DENV replication although it requires the stimulation of spleen tyrosine kinase (Syk). Pharmacological blockade of caspase-1 as well as genetic blockade of NLRP3 drastically ablated DENV derived IL-1β secretion (Callaway et al., 2015). Of note, weak NLRP3 inflammasome activation was observed in monocytes in this study as compared to inflammatory macrophages (Wu et al., 2013b), and it may be due to differential expression of CLEC5A in monocytes vs. inflammatory macrophages as later shows significantly very high expression of CLEC5A (Batliner et al., 2011). DENV

immune complexes induced activation of Syk can have wider effects as activation of Syk also upregulates the TNF and IL-6 expression. It is important to note that activating ERK1/2 by Syk elevates IL-1β secretion, and inhibition of Syk and ERK1/2 diminished the ADE-triggered IL-1β release (Callaway et al., 2015). In addition to the role of Syk activation during ADE in DENV infection, downstream to C-type lectin receptor activation of SyK activation play a crucial role in activating inflammasome during fungal pathogens and helminths infection (Gross et al., 2009; Poeck and Ruland, 2010; Ritter et al., 2010). In addition, malarial hemozoin upon being engulfed by phagocytes, induces Syk mediated inflammasome activation (Tiemi Shio et al., 2009). This suggest that Syk could be a potential therapeutic focus to restrict the "pathogenic cytokine storm" of severe dengue. Furthermore, a recent study has setup a secondary infection experiment to mimic ADE to demonstrate the immune response of human monocytes to the DENV infection. Human individual monocytes were selected based on their past exposure to severe disease or non-severe dengue and further exposed to each four DENV serotypes subsequent incubation with autologous serum. Results exhibited the marked increased viral load, viral sensing gene expression (NLRP3, RIG-1, IFN-β) and production of inflammatory cytokines, specifically IL-1β in the monocytes of individuals with past severe dengue when compared to monocytes of individuals with past non-severe dengue advising the influence of initial innate immune responses and inflammasome components NLRP3, IL-1β in disease outcome (Kamaladasa et al., 2019).

Inflammasome and Vascular Leakage

Although we have discussed above the role of inflammasome and dengue severity, a recent study demonstrated the mechanism regarding the relationship between augmented inflammatory response and damage of vascular barrier integrity. The study supports that inflammasome induced IL-1β plays a central role in tissue injury and vascular leakage. DENV infection mediated IL-1β activation are found in infected patient blood samples, human peripheral blood mononuclear cells (PBMCs) and monocyte-differentiated macrophages (THP-1) as well in C57BL/6 mice and mouse bone marrow derived macrophages (BMDMs). Evidence demonstrates that IL-1β augments tissue injury and leakage in IFNAR-/- C57BL/6 mice, although IL-1 receptor antagonist (IL-1RA) protects from this injury (Pan et al., 2019b). Together, the collected results reveal the mechanism of DENV pathogenesis and establish the contribution of IL-1β in DENV-associated pathology and recommend the consideration of IL-1RA for effective therapeutics in DENV patients' treatment. The summary of the discussed mechanisms is shown in **Figure 1**.

DENV Induced Pyroptosis

Pyroptosis is a programmed cell death (inflammatory) that engages the inflammasomes activation and subsequent caspase-1 activation. Caspase-1 facilitates the processing of pro-IL-1β, pro-IL-18 into the mature IL-1β and IL-18 forms that are released to outside of the cell (Man and Kanneganti, 2016). Caspase-1 further cleaves between C and N domains of Gasdermin D, and the N-terminal domain further attaches to membrane lipids and forms pores that results in cell lysis that facilitates the influx of water molecules leading to cell swelling and subsequent rupture (pyroptosis). Pyroptosis cell death involves the damage of cell membrane integrity and upregulation of the caspase-1 and an upsurge in IL-1β and IL-18 production (Zhao et al., 2018). During dengue infection, apoptosis mediated cell death has been reported, however, evidence has also demonstrated the pyroptosis (inflammatory cell death) in monocytes. Monocytes displayed the activation of caspase-1 and subsequent production of IL-1β accompanied with release of cellular content by 96 h during DENV-2 infection (Tan and Chu, 2013). As we have discussed above, DENV infection induces the inflammasome activation and subsequent cell death. Cell death has been linked to the upregulation of CLEC5A in inflammatory macrophages (Wu et al., 2013a). Of note, upregulation of CLEC5A has been reported in murine monocytes after DENV infection (Cheng et al., 2016), supporting the notion that CLEC5A plays a crucial role in monocyte cell death by pyroptosis. Another study demonstrated a unique mechanism where DENV-2 infection to primary macrophages activates caspase-1 and caspase-4, and subsequent release of IL-1β. Further, loss of cell viability along with the release to the lactate dehydrogenase was observed in extracellular medium. This study demonstrated that caspase-4 activates the caspase-1 and that caspase-4 serves as upstream activator of caspase-1 as inhibitor of caspase-4 ablated both caspases activity and subsequent secretion of IL-1β (**Figure 1**). This study shows the new role of caspase-4 in activation of inflammasome and pyroptosis during DENV-2 infection (Cheung et al., 2018). This study shows that DENV-2 infection in macrophage results in decreased viability and increased LDH, revealing cell damage and lysis; however, macrophages as beneficial or pathogenic role in cell death remains controversial.

Dengue Viral Protein Triggers Inflammasome

Several viral proteins have displayed the strong influence on inflammasome activation and elicited the host immune response; as demonstrated in the influenza A virus M2 protein and encephalomyocarditis virus (EMCV) 2B protein (Ichinohe et al., 2010; Ito et al., 2012). The wide range of viral proteins with their modulating effects on inflammasome have previously been discussed in detail (Lupfer et al., 2015; Shrivastava et al., 2016). In the context of DENV, EDIII is regarded as a highly immunogenic protein. In THP-1, EDIII activates NLRP3 inflammasome via NF-κB pathway resulting in caspase-1 activation and subsequent IL-1β and TNF-α secretion. Increased ROS production and potassium was suggested to be the mechanism behind EDIII protein mediated IL-1β production and release (Khan et al., 2019). Moreover, DENV M protein that plays an important role in DENV packaging and egress (Junjhon et al., 2008; Lin et al., 2011), also displays a role in imparting host innate immune response. This study shows that DENV M induces NLRP3 inflammasome and IL-1β release that further induces the endothelial permeability and vascular leakage in mice. More importantly, M protein stimulates tissue injuries in wild-type (WT) mouse tissues, however the effect of M

protein was inhibited in NLRP3 knockout (NLRP3$^{-/-}$) mouse tissues, indicating a vital role of M protein-stimulated vascular leakage. The effects of M protein in triggering inflammasome activation and IL-1β secretion and subsequent pathological effect is suggested due to the interaction of M protein with NLRP3 (Pan et al., 2019a). However, the molecular mechanism through which M protein binds to NLRP3 is still elusive and warrants more investigation. NS1 is a non-structural protein of DENV and plays a role in DENV replication complex along with other non-structural proteins (Muller and Young, 2013). NS1 is specific in a particular way; this is the only non-structural protein secreted in extracellular milieu (Thiemmeca et al., 2016) and increased NS1 in patient serum is considered as a marker of severe dengue (Libraty et al., 2002). Furthermore, NS1 serves as viral PAMP that triggers TLR4 on the surface of endothelial cells and leukocytes which leads to endothelial dysfunction as well as inflammation (Beatty et al., 2015; Chen et al., 2016; Puerta-Guardo et al., 2016). Recently, NS1 translation and secretion has shown to complement DENV induced inflammasome. The study shows that DENV triggers abortive viral infection in platelets, where DENV translate and replicate its genome without releasing the viral particle. During this infection process, NS1 augments the platelets response via autocrine loop to the DENV infection by caspase-1 mediated secretion of IL-1β and translocating and releasing the stored factors. Notably, NS1 doesn't trigger the direct release of IL-1β, however NS1 triggers the TLR4 on platelets and amplifies the platelets response to DENV, indicating the complex interplay between host and viral factors that leads to the dengue severity (Quirino-Teixeira et al., 2020). In addition, DENV non-structural proteins, NS2A and NS2B have been reported to trigger NLRP3 inflammasome activation. DENV NS2B plays a role in replication, viral protease co-factor and in degradation of cGAS (Aguirre et al., 2017). In addition, NS2B as a protease complex (NS2B/NS3) inhibits mitochondrial fusion by cleaving the mitochondrial fusion proteins i.e., Mfn1 and Mfn2 (Yu et al., 2015). DENV NS2A, is a hydrophobic transmembrane protein with a diverse role in the virus life cycle. NS2A displays a regulatory role in replication, viral assembly, and viral release and most notably by hindering the JAK-STAT and interferon pathways (Leung et al., 2008; Xie et al., 2013, 2015). Moreover, additional findings have demonstrated that DENV-2 NS2B and DENV-2 NS2A as "viroporins" permeabilize host cell membranes (León-Juárez et al., 2016; Shrivastava et al., 2017). Viroporins from several virus that can increase host cell membrane permeability, have shown to activate the NLRP3 inflammasome through various mechanism and thereby regulate antiviral innate immune responses (Guo et al., 2015; Farag et al., 2020). This study initially demonstrated that DENV-2 induces NLRP3 activation, caspase-1 activation and subsequent IL-1β secretion in endothelial cells (HMEC-1). NS2A and NS2B have shown to be co-localized in the endoplasmic reticulum and mitochondria. Furthermore, NS2A and NS2B were sufficient to trigger the activation of NLRP3 inflammasome, and IL-1β secretion through caspase-1 activation in endothelial cells. Pharmacological inhibition of NLRP3, caspase-1 and CRISPR knockout of ASC in endothelial cells showed that NS2A and NS2B mediated inflammasome and IL-1β secretion was NLRP3 and caspase-1 specific. Lastly, the study also suggested that NS2A triggering NLRP3 inflammasome activation is due to Ca^{++} homeostasis and/or mitochondrial disruption /ROS production (Shrivastava et al., 2020). In summary, these evidences show the dengue virus proteins induced mechanism in activating NLRP3 inflammasome. Furthermore, this mechanistic insight with more detailed study would shed more light on DENV pathogenesis understanding (**Figure 1**). These studies may help in designing and developing new therapeutic candidates against dengue virus.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

DENV infection comprises immune dysfunction that leads to leakage of vascular fluids and cytokine storm which are suggested as main contributors to the pathogenesis of DENV, culminating in life-threatening hypovolemic shock (Culshaw et al., 2017). Numerous studies have aimed to understand the role of inflammasome in pathogenesis of virus. Inflammasome activation has been accepted as a crucial player in the outcome of DENV infection. Inflammasome induced by DENV facilitates caspase-1 activation and synthesis and secretion of IL-1β and IL-18. Thus, the discussed investigation founded a promising direct linkage between inflammasome activation and dengue severity, while considering the contribution of caspase-1, IL-18 and IL-1β, to DENV-mediated patient pathology. Particularly, IL-1β plays an indispensable role in the severe dengue complex immunopathological condition. Therefore, a question arises, whether therapeutics targeting IL-1β may be useful in the treatment of DENV associated diseases? Inflammasome activation plays a crucial role in controlling numerous pathogens, and thus loss of IL-1β can lead to impaired immune defense. In clinic settings, drugs directed to inhibit NLRP3 or IL-1β are now being utilized for numerous autoimmune diseases, however not enough clinical research has supported the use of such treatments in viral infection. Though, the precise mechanisms underlying DENV induced damage sensing are still debatable, the existing evidence suggests that NLRP3 ligand directly senses the DENV. The precise mechanism of sensing is still elusive and warrants more research. Whether NLRP3 senses the DENV directly or by some other means and if so, how? NLRP3 often acts in concurrence with other inflammasomes sensors such as AIM2 and IFI16, however, whether these ligands sense DENV along with NLRP3 needs further examination. In the context of IL-18 in dengue infection, whether IL-18 confers protective or detrimental role is still unknown. Although pyroptosis might be relevant for viral clearance of infected cells, research regarding the role of DENV-induced pyroptosis is still in a primitive phase. More studies are warranted to understand DENV-induced proptosis in different cell culture models as well as in clinical settings. Mitochondria have been regarded as an important component in inflammasome activation either by ROS generation, mtDNA release, autophagy, or cell death (West et al., 2011; Zhou et al., 2011). Moreover, it has also been demonstrated that mitochondria adapter protein (MAVS) localizes the NLRP3 inflammasome on mitochondria membranes (Subramanian

et al., 2013). In DENV infection, mitochondria have also been demonstrated to play a crucial role in regulating host immunity either by ROS generation or by mtDNA release (discussed above in this review). Does DENV induced inflammasome occur on mitochondria membrane and if so, how? Another question is whether DENV viral factors interact directly with mitochondria components? Therefore, more studies are required to unravel the link between DENV infection and mitochondria as it can provide several insights to regulate the inflammatory response during infection. Research should also focus on several host molecules that have been suggested to play a crucial role in regulating the dengue severity such as CLEC5A, TLR2, TLR9, inhibitors of the tyrosine kinases Syk and Btk (discussed above in text). In addition, a deeper understanding of immune evasion molecules evolved by viruses that can inhibit the function of inflammasome will expect to uncover novel concepts and may eventually identify targets in the treatment and prevention of DENV severity. Notably, a better understanding of the equilibrium between detrimental vs. favorable inflammasome activation is also indispensable, as host activation of inflammasome is a "double-edged sword" to defense viral infection. As the systematic vision of the inflammasome rises, prospects to create new therapeutic interventions for patients with DENV severity enhance proportionately. It is important to mention that although inflammasome activation does appear to contribute to dengue, other mechanisms such as an early antiviral response (IFN response), also plays a major contributor along with high levels of immunosuppressive cytokines during dengue virus pathogenesis. Moreover, a different and very strong approach against arbovirus has also been considered by targeting the mosquito vector. DENV virus enters the host along with mosquito saliva at the skin epidermis and dermis level during mosquito bite. To date, various findings have reported the capacity of *Ae. Aegypti* saliva that enable blood feeding, pathogen transmission and regulate host innate and adaptive immune responses through its several pharmacologically active proteins that saliva contains (Pingen et al., 2016, 2017; Wichit et al., 2016; Manning et al., 2018). For example, 34 kDa protein from *Ae. aegypti* was found to prevent the expression of IRF-3, resulting in the inhibition of type I IFN production (Surasombatpattana et al., 2014). Aegyptin protein from *Ae. aegypti*, decreases DENV titers in mice along with increased GM-CSF, IFN-γ, IL-5, and IL-6 concentrations in serum as compared to solitary DENV inoculated to mice, displaying the impacts of Aegyptin in DENV infectivity through activation of the immune response (McCracken et al., 2014). More research with enhanced knowledge regarding the interactions between saliva proteins and primary host immune cells will help to identify the key cell populations/molecules/pathways controlling the infection efficiently. Increased characterization and improved functional understanding of the potential role of saliva proteins on DENV infection could offer unique targets that may support in the development of mosquito vector protein based novel therapeutics and vaccines.

AUTHOR CONTRIBUTIONS

GS wrote the manuscript. GS, EC, and PV wrote the final version of the manuscript and approved the submitted version. All authors contributed to the article and approved the submitted version.

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CNS Immune Profiling in a Dengue Virus-Infected Immunocompetent Outbred ICR Mice Strain

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Dengue virus (DENV) infection in the brain causes severe dengue disease with neuropathic complications. In addition to viral effects, immunogenic or pathogenic central nervous system (CNS) inflammation can be induced during DENV infection. By using an immunocompetent outbred ICR (Institute of Cancer Research) mouse model for investigating CNS immunity upon DENV infection, we conducted single-panel immune cell profiling and a multiplex cytokine assay. The ICR mice infected with DENV presented with progressive hunchback posture, limbic seizures, limbic weakness, paralysis, and lethality. When the virions were released, the viral non-structural protein 1 was expressed in the brain in a time-dependent manner. Isolated brain CD45-positive cells revealed a significant population of resident CD14-positive cells, which was considerably decreased 8 days post-infection. We found an unexpected time-kinetic decrease in CD19-positive cells and CD11c/MHC II-positive cells and an increase in NK1.1-positive cells. Further assays showed the time-dependent induction of proinflammatory and NK1.1-associated cytokines in the DENV-infected brains. These results indicate a CNS immune profile of DENV infection and hypothetical CNS immunity in response to DENV infection.

Keywords: dengue virus, mice, CNS, cytokines, immune cells

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INTRODUCTION

Dengue virus (DENV), a mosquito-borne *Flavivirus*, contains a positive-sense RNA genome that encodes three structural proteins (pr-M, envelope, and capsid proteins) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 proteins). The virus causes mild dengue fever (DF) and severe dengue diseases, including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), worldwide. Most DENV-infected people are asymptomatic or have mild symptoms, such as fever, headache, nausea, and joint pain. Unfortunately, some patients present with life-threatening warning signs, such as vascular leakage, internal bleeding, central nervous system (CNS) impairment, and multiorgan failure, that can result in death (Islam et al., 2015; Guzman et al., 2016; Ajlan et al., 2019).

Many studies have shown that DENV can infect various human organs (Shresta et al., 2004; Povoa et al., 2014; Milligan et al., 2015). The DENV negative-sense RNA and viral protein NS3 are detectable in the hearts of the deceased (Shresta et al., 2004). In addition, DENV positive-sense RNA and another viral antigen, NS1, can also be detected in human spleen, lung, liver, and CNS tissues (Povoa et al., 2014). Upon DENV infection, AG129 mice show a widespread distribution of infectious viruses in multiple organs, including their livers, spleens, and large intestines, as well as their brains and spinal cords (Shresta et al., 2004; Milligan et al., 2015). Sarathy et al. also demonstrated the dynamic changes in viral loads in DENV 3-infected AG129 mice. High levels of viral titers were found in livers and spleens starting on the first day of infection. The productive virions had increased in the large intestines and brains at 2 and 3 days post-infection (d.p.i.), respectively. Lethal dengue disease is characterized by the robust induction of cytokines and chemokines in mouse serum, such as interleukin (IL)-6, IL-10, and interferon (IFN)-γ, showing a high correlation with infectious disease progression (Sarathy et al., 2015).

In DENV-infected patients, aberrant production of proinflammatory cytokines and chemokines, such as tumor necrosis factor (TNF)-α, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, IL-8, IL-10, and C-X-C motif chemokine 10 (CXCL-10), which is the critical marker of disease severity. Hypothetically, endothelial cells, monocytes, macrophages, dendritic cells (DCs), natural killer (NK) cells, and T cells are thought to produce these soluble inflammatory factors in DENV-infected patients and mice (Durbin et al., 2008; Costa et al., 2013; Schmid and Harris, 2014; Singla et al., 2016; Patro et al., 2019). Many studies have indicated that DENV can infect the neural system and induce neurological symptoms in humans and mice (Amaral et al., 2011; Verma et al., 2014); however, no reports have shown a CNS inflammation profile for DENV infection. The goals for a systemic study to monitor the kinetic changes of DENV virions and host immune responses have therefore been unmet.

In this study, as shown through the use of an immunocompetent ICR suckling mouse model similar to that created previously (Shen et al., 2019), DENV infection induced severe disease in the mouse CNS, which caused neural symptoms. An immune profiling analysis of the brain revealed dynamic changes in the immune cells and inflammatory responses.

MATERIALS AND METHODS

Ethics Statement

The Ethics Committee approved all experimental procedures used in the animal work on the Institutional Animal Care and User Committee of National Defense Medical Center, Taipei, Taiwan, protocol IACUC 16-261.

Cells and Virus

Dengue virus serotype 2 (DENV2, strain PL046) was obtained from Center Disease Control in Taiwan. We propagated in the monolayer of *Aedes albopictus* clone mosquito C6/36 cells (ATCC, CRL1660) at a multiplicity of infection (MOI) of 0.01 and incubated the cells at 28°C in 5% CO_2 for 5 days. The viral supernatants were collected and filtered with a 0.22 μm filter and then stored at −80°C until use. The C6/36 cells were maintained in minimum essential medium (MEM; Thermo Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (FBS, Biological Industries), 1% penicillin-streptomycin (Thermo Fisher Scientific), 1% sodium pyruvate, 1% 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES; Thermo Fisher Scientific), and 1% non-essential amino acids (NEAA; Thermo Fisher Scientific) at 28°C in 5% CO_2. Baby hamster kidney (BHK)-21 fibroblasts (ATCC, CCL10) were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific), 10% heat-inactivated FBS, and 1% penicillin-streptomycin at 37°C in 5% CO_2.

Infectious Animal Model

To establish the animal model as in previous studies (Tsai et al., 2016; Ho et al., 2017; Jhan et al., 2018; Kao et al., 2018; Shen et al., 2019), we inoculated 7-d-old ICR suckling mice (BioLASCO, Taiwan) with 2.5×10^5 and 7.5×10^5 plaque-forming units (pfu) of DENV 2 (PL046) by simultaneous intracerebral and intraperitoneal injections, respectively. The survival rate and disease score of the mice were monitored daily and recorded. The following disease scoring guidelines were used: 0 for healthy mice; 1 for mice with minor symptoms of illness, including weight loss, reduced mobility, and a hunchback body orientation; 2 for mice that exhibited limbic seizures; 3 for mice that showed limbic weakness, including difficulty moving and anterior or posterior limb weakness; 4 for paralysis; and 5 for death.

Antibodies

Antibody against DENV NS1 (Cat#GTX124280) was purchased from GeneTex (San Antonio, TX); antibody against mouse β-actin (Cat#A5441) was purchased from Sigma-Aldrich (St. Louis, MO); horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cat# 7074S), HRP-conjugated horse anti-mouse IgG (Cat# 7076S) were purchased from Cell Signaling Technology (Beverly, MA); antibodies against mouse CD45 (Cat# 56-0451-82), CD3 (Cat# 48-0031-82), CD11c (Cat# 12-0114-82), CD14 (Cat# 17-0141-82), CD19 (Cat# 69-0193-82), MHC class II (Cat#67-5321-82), and NK1.1 (Cat# 69-5941-82) were purchased from Thermo Fisher Scientific.

Western Blotting

The murine organs were homogenized and extracted with lysis buffer containing a protease inhibitor mixture (Sigma-Aldrich). The extracted proteins were quantified to be of equal concentration and mixed with protein dye. Then, the processed proteins were separated by SDS polyacrylamide gel electrophoresis for 2 h, followed by transfer to a polyvinylidene difluoride (PVDF) membrane (Millipore). The PVDF membrane was blocked with 5% non-fat milk in Tris-buffer-based saline containing 0.05% Tween-20 (TBS-T) at room temperature for 1 h. The membrane was washed three times with TBS-T buffer. Next, the membrane was immunohybridized with the indicated primary antibodies at 4°C for 16–18 h. Then, the membrane was washed with TBS-T buffer three times and

then incubated with the indicated HRP-conjugated secondary antibodies. PVDF membranes containing antibody-protein complexes were detected using an ECL Western blot detection kit (PerkinElmer). Then, the relative densities of the identified proteins were quantified using ImageJ software (Fiji Software).

Plaque Assay

BHK-21 cells were seeded in a 12-well plate (7×10^4 cells/ml) and allowed to form a monolayer overnight. Samples were diluted at 10^{-1}-10^{-5} and then incubated with BHK-21 cells at 37°C in 5% CO_2 for 2 h. Next, the virus inoculum was discarded and replaced with DMEM containing 4% FBS and 0.5% methylcellulose (Sigma-Aldrich) for 5 days. The wells containing methylcellulose were washed with 2 ml PBS twice and then fixed and stained overnight with a crystal violet solution containing 1% crystal violet (Sigma-Aldrich), 0.64% NaCl, and 2% paraformaldehyde (Sigma-Aldrich). The stain was removed with a water wash, and the plates were dried at room temperature. Viral plaques were counted by visual observation.

Multiplex Assay

Mouse brains were homogenized with 400 μl of iced-PBS on ice, followed by centrifugation at 12,000 rpm for 30 min. Supernatants were harvested and quantified to an equal concentration of 1 μg/μl in at least 35 μl. The assay was performed by using an Immunology Multiplex Assay MTH17MAG-47K (Millipore) according to the manufacturer's instructions. Briefly, 200 μl of wash buffer was added to each well of the 96-well plate and shaken at room temperature for 10 min, and then, the wash buffer was removed. Then, 25 μl of standard/control and 25 μl of assay buffer were added to the indicated wells and the background/sample wells, respectively. Next, 25 μl of an appropriate matrix solution was added to the standards, controls, and background wells. Twenty-five microliters of each sample and 25 μl of antibody-immobilized beads were added to the wells. After overnight incubation at 4°C, the supernatant was gently removed, and the plate was washed twice with 200 μl of wash buffer. Then, 25 μl of detection antibody was added to each well, and the plate was incubated at room temperature. After a 1-h incubation, 25 μl of streptavidin-phycoerythrin was added to each well and incubated at room temperature for 30 min. The supernatant was gently removed, and the plate was washed twice with 200 μl of wash buffer. Then, 150 μl of sheath fluid was added per well. After incubation of the plate on a shaker for 10 min, the reactions were detected and analyzed by using Luminex MAGPIX® with xPONENT® software.

Flow Cytometry

Mouse brains were homogenized with 5 ml of iced wash buffer (HBSS + 10% FBS) and then centrifuged at 400 × g for 5 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 ml of digestion buffer (ACCUTASE® solution). The samples in the digestion buffer were gently rotated at room temperature. After a 1-h incubation, the samples were washed with 1 ml of wash buffer and centrifuged at 400 × g for 5 min at 4°C. Then, the samples were gently mixed with 5 ml of 25% density gradient medium (nine parts of Percoll with one part of 10× HBSS as a 100% isotonic density gradient stock medium, which was then further diluted with HBSS containing 3% FBS) and centrifuged at 600 × g for 20 min at 4°C. The myelin coat and the supernatant were carefully removed. One milliliter of wash buffer was added to the cell pellet, and the samples were centrifuged at 400 × g for 5 min at 4°C. After washing the brain samples, 1 ml of staining buffer was added, and the cells were counted. The samples were immunoblocked (Mouse BD Fc Block™) at 4°C. After 30 min, the cells were immunohybridized with specific antibodies against cell surface immune markers, including CD45, CD3, CD11c, MHC class II, CD14, CD19, and NK1.1, at 4°C for 1 h. Then, the cells were washed with 500 μl of iced staining buffer and resuspended in 500 μl of iced staining buffer. The samples were analyzed using a flow cytometry (Attune Nxt) system.

Statistical Analysis

Experimental data were analyzed using GraphPad Prism (Version 8.3.0). Unpaired *t*-test and one-way ANOVA (Tukey's multiple comparisons test) were used to determine experiments involving two and various groups, respectively. The survival rate followed a log-rank test. Values are means ± standard deviation (SD). All *p*-values are for two-tailed significance tests. A *p*-value of <0.05 is considered statistically significant.

RESULTS

DENV Infection Induces CNS Impairment and Death in ICR Suckling Mice

We established a DENV infection model in immunocompetent mice, according to our previous study (Shen et al., 2019). Seven-days-old ICR suckling mice were simultaneously infected with 2.5×10^5 and 7.5×10^5 PFU/ml of DENV by intracerebral and intraperitoneal injections, respectively (**Figure 1A**). Compared with that shown by the mock group, the DENV-infected mice showed significantly increased disease severity with symptoms ($p < 0.01$), including reduced mobility, limbic seizure, and paralysis within 6 d.p.i. (**Figure 1B**). Moreover, the survival rates were significantly different, with the DENV-infected mice dying after 8 d.p.i., and all mice dying within 11 d.p.i. ($p < 0.01$) (**Figure 1C**). The results reveal the establishment of a reporter DENV-infected murine model with disease progression involving CNS disorders.

DENV Significantly Causes Infection in the Mouse CNS

Based on the successful infection of this murine model, we next evaluated the infectious target of DENV. We harvested various organs/tissues for viral protein detection using Western blot analysis. According to the results, there was detectable viral NS1 protein in the livers of the mice post-infection but not in the other organs, including the hearts, lungs, spleens, and kidneys. However, in the mouse brains, NS1 was significantly ($p < 0.001$) expressed at 6 d.p.i., and the expression was significantly ($p < 0.05$) decreased at 8 d.p.i. (**Figure 2A**). To confirm the infectivity and viral replication in the murine CNS,

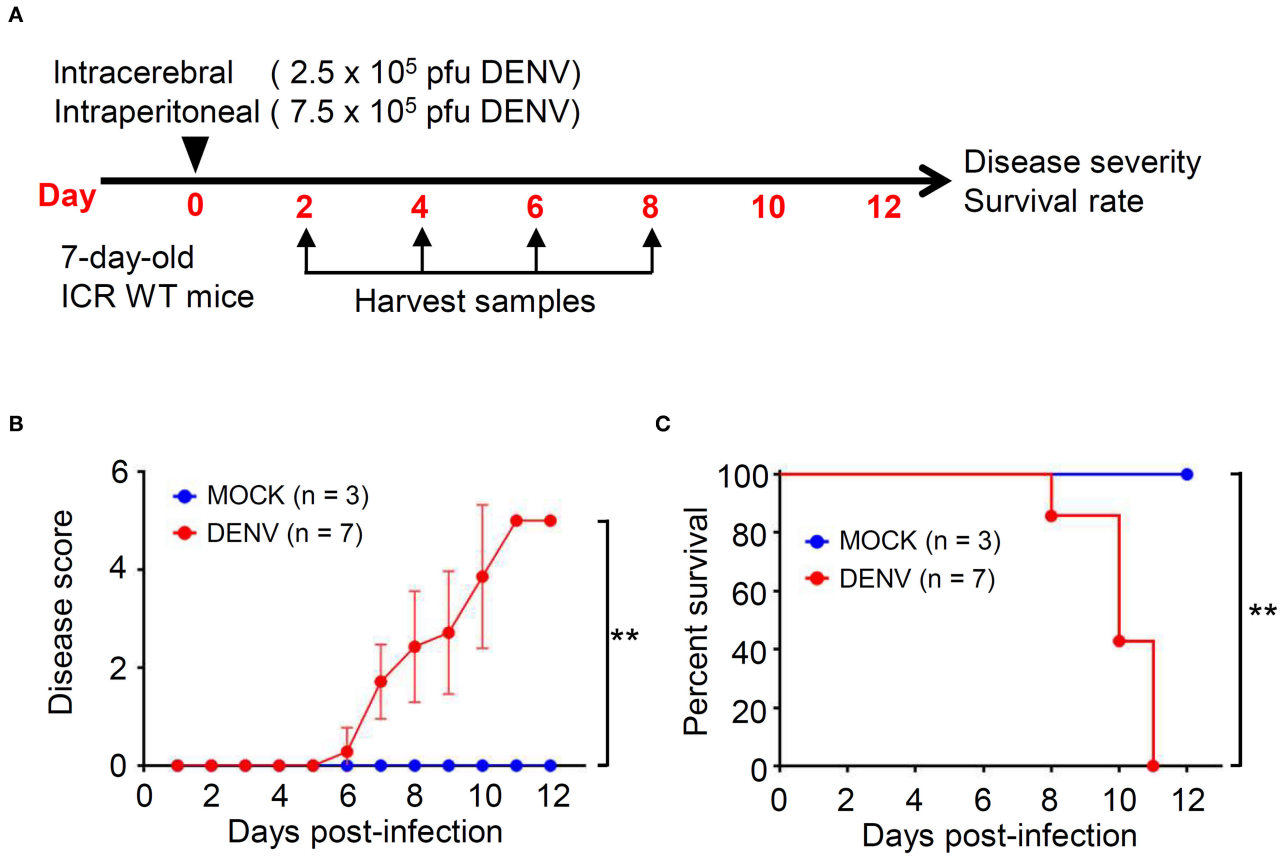

FIGURE 1 | DENV infection causes symptoms and death in ICR immunocompetent mice. **(A)** A concurrent intracerebral and intraperitoneal injection model of DENV infection was generated in 7-days-old ICR mice. Organs/tissues were harvested at the indicated day post-infection. We monitored **(B)** the disease score and **(C)** the survival rate of mice for 12 days after infection. Wilcoxon signed-rank test and log-rank test analyzed the disease score and the survival rate, respectively. The values are presented as means ± SD. $^{**}p < 0.01$.

a plaque assay was conducted, and the results demonstrated a titer of productive virions in the brain representing a remarkable increase at 6 d.p.i. ($p < 0.05$) and a decrease at 8 d.p.i. ($p < 0.05$), findings that closely matched the NS1 expression results (**Figure 2B**). In addition, although the NS1 protein was recognized, the results of the plaque assay showed that there was no detectable virion in mouse liver (data not shown). The data show that DENV could infect the CNS and effectively replicate it.

DENV Infection Causes Changes in the Immune Cell Profile of the Mouse Brain

According to the levels of viral proteins and viral titers (**Figure 2**), we created a successful DENV infection in the CNS of immunocompetent mice. To further investigate using single-shot immune cell profiling of the CNS in the mice, immunostaining of a panel of CD45, CD3, CD11c, MHC class II, CD14, CD19, and NK1.1 was performed in DENV-infected brain cells isolated from suspension. A gating strategy was applied to the CD45-positive cells to distinguish the indicated cell populations (**Figure 3A**). According to the results, the percentage of CD19-positive cells (**Figure 3B**) remained relatively unchanged. The percentage of CD3-positive cells (**Figure 3C**) and CD11c and MHC class II-positive cells (**Figure 3D**) was significantly ($p < 0.05$) decreased, while that of the NK1.1-positive cells (**Figure 3E**) was significantly ($p < 0.001$) increased within days of DENV infection. The significant population consisted of resident CD14-positive cells (**Figure 3F**), likely microglia, in the DENV-infected mouse brains, which increased at 6 d.p.i. but remarkably decreased at 8 days post-infection. Collectively, the results demonstrate that DENV infection orchestrates the composition of immune cells in the CNS of immunocompetent mice.

DENV Infection Induces Cytokine and Chemokine Storms in the Mouse CNS

After we found that high viral protein and viral loads were associated with neurological and physiological changes in the infected mice, we determined the immune profile of the mouse CNS. Because of the increased expression of NK1.1-positive NK cells in the DENV-infected murine brain, the levels of other immune parameters needed to be validated. Elevated

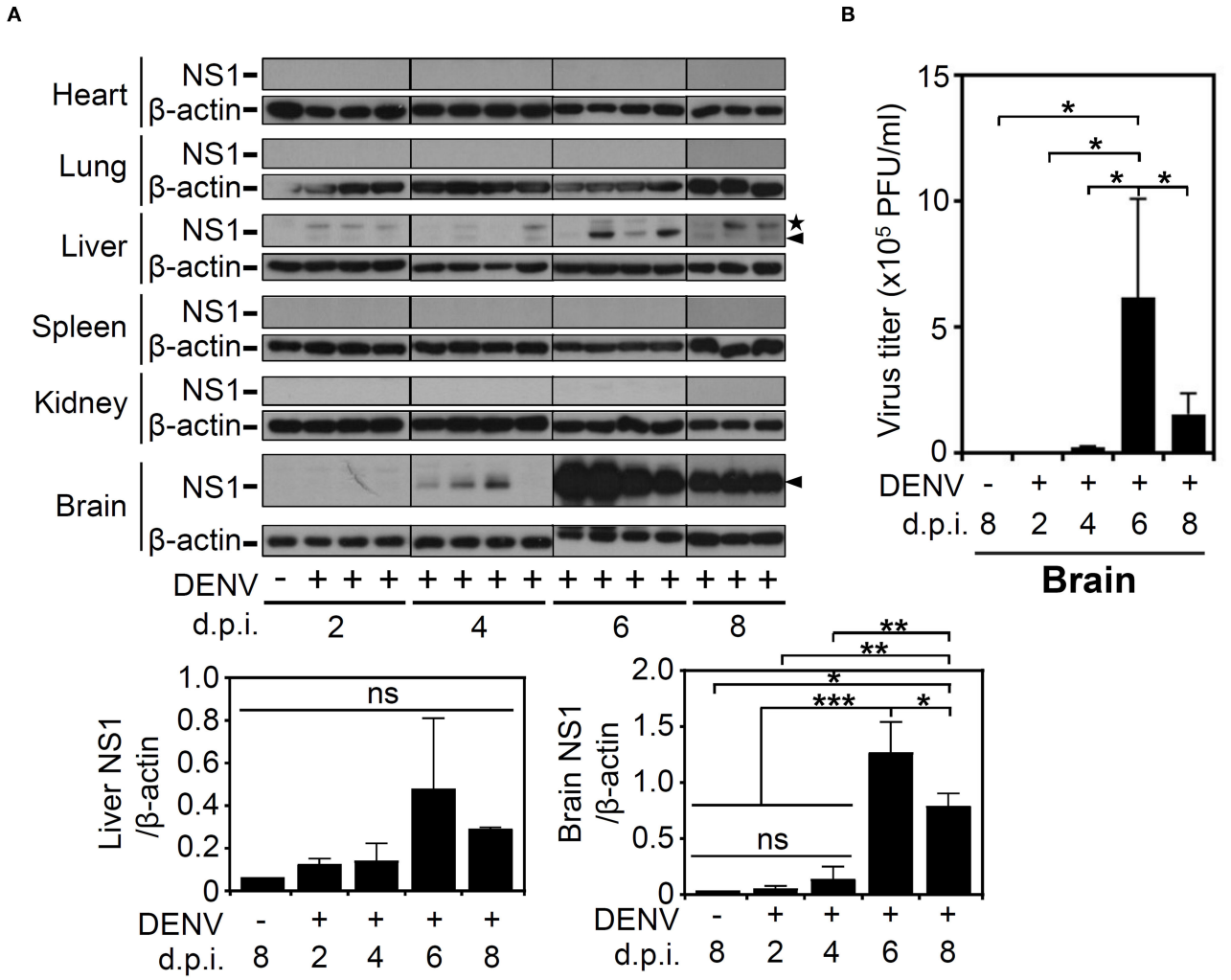

FIGURE 2 | DENV infection causes NS1 protein expression in multiple organs, especially induces virion production in mouse brains. **(A)** Western blot analysis showed viral NS1 protein expression in various organs of the mice. β-actin was used as internal control. Arrowhead (▲): NS1 protein. Star (★): non-specific protein. **(B)** The plaque assay determined the viral titer in the infected brains. Data are presented as the means ± SD based on at least three mice. *$p < 0.05$. Data are presented as the means ± SD based on at least three mice. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ns: not significant.

levels of cytokines and chemokines, including IL-10, IFN-γ, TNF-α, GM-CSF, and MIP-1β, are potential biomarkers of disease severity in DENV patients (Patro et al., 2019). In consideration of an increased NK cell response, the expression of cytokines/chemokines associated with NK cell activation was hypothetically measured according to methods used in previous works (Peritt et al., 1998; Biron et al., 1999; Cooper et al., 2001; Fauriat et al., 2010). The multiplex assay was performed to measure the production of cytokines/chemokines in the DENV-infected mouse brains. Compared to those of the mock group, the levels of cytokines and chemokines, including NK-associated type 1 cytokines (IL-2, IL12p70, IL-15, and IFN-γ) (**Figure 4A**), type 2 cytokines (IL-4, IL-5, IL-10, IL-13, and GM-CSF) (**Figure 4B**), and proinflammatory factors (IL-1β, IL-6, MIP-3a/CCL20, TNF-α, and TNF-β) (**Figure 4C**), were significantly increased ($p < 0.05$) within days of induced DENV infection. These results demonstrate that DENV infection can induce the overproduction of inflammatory cytokines/chemokines in the brain.

DISCUSSION

An animal model of DENV infection is crucial for exploring the pathogenesis of dengue diseases and for evaluating the antiviral strategies, particularly vaccine development (An et al., 1999; Guabiraba and Ryffel, 2014; Sarathy et al., 2015, 2018). Despite the limitations on immunocompromised mice, when monitoring the immune response is necessary, an immunocompetent murine model is essential. We created a murine model of DENV infection in immunocompetent mice (Tsai et al., 2016; Shen et al., 2019). Through a time-kinetic analysis, this work not

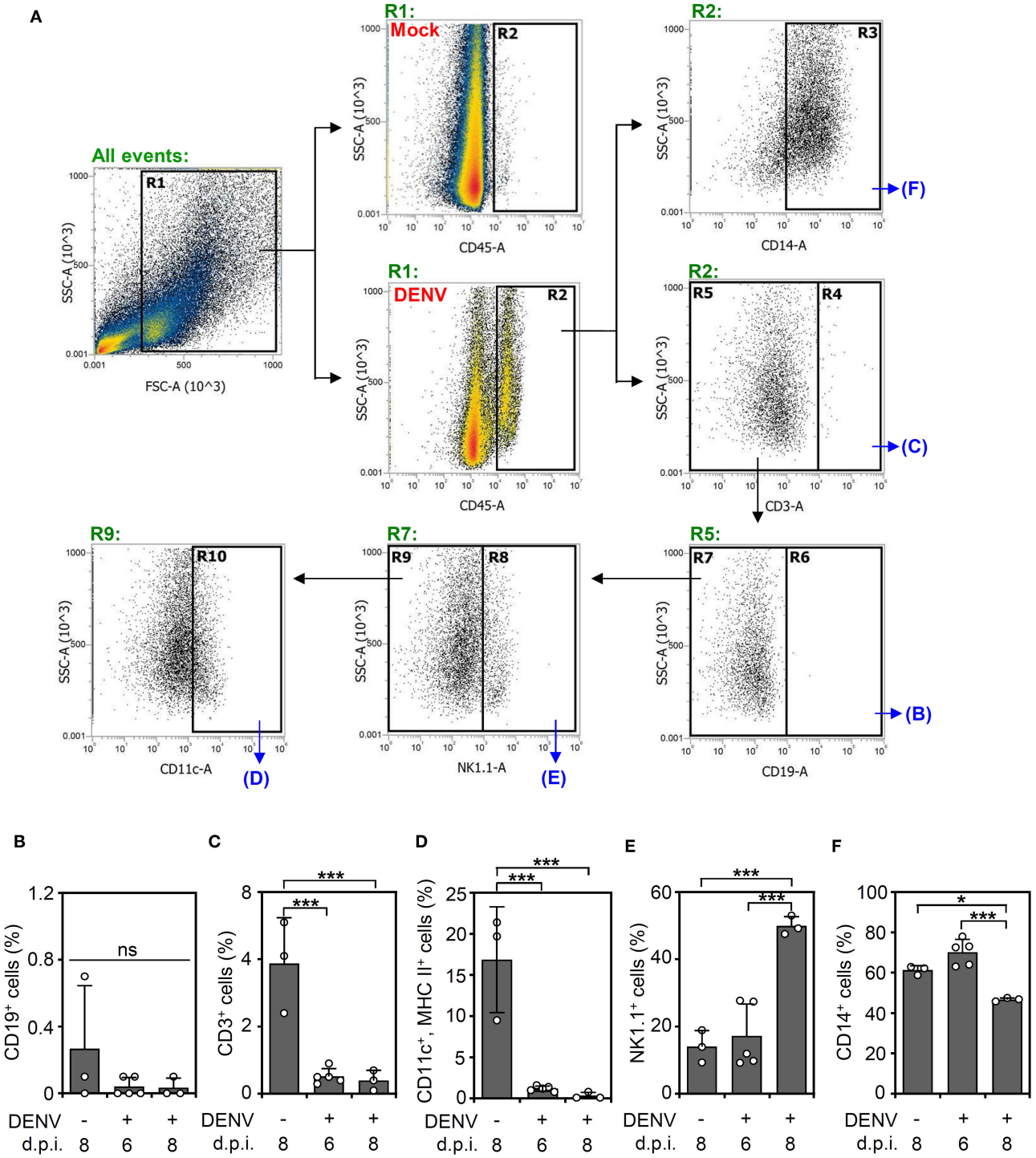

FIGURE 3 | DENV infection causes dynamic changes in immune cell populations in the brain. **(A)** A gating immunostaining strategy was used for the indicated cell populations. The percentage of **(B)** CD19-positive cells, **(C)** CD3-positive cells, **(D)** CD11c and MHC II-positive cells, **(E)** NK1.1-positive cells, and **(F)** CD14-positive cells in the immune cell populations of the mouse brains on the indicated day post-infection were shown. Each point represents a mouse sample. Data show the means ± SD of based on at least three mice. *$p < 0.05$; ***$p < 0.001$. ns: not significant.

only demonstrated a significant replication of DENV in the CNS but also identified the possible immune parameters, including immune cells and cytokines/chemokines, related to viral replication *in vivo*. The increase in NK1.1-positive cells and their regulatory cytokines and chemokines was demonstrated in this infectious model, and it was closely associated with viral

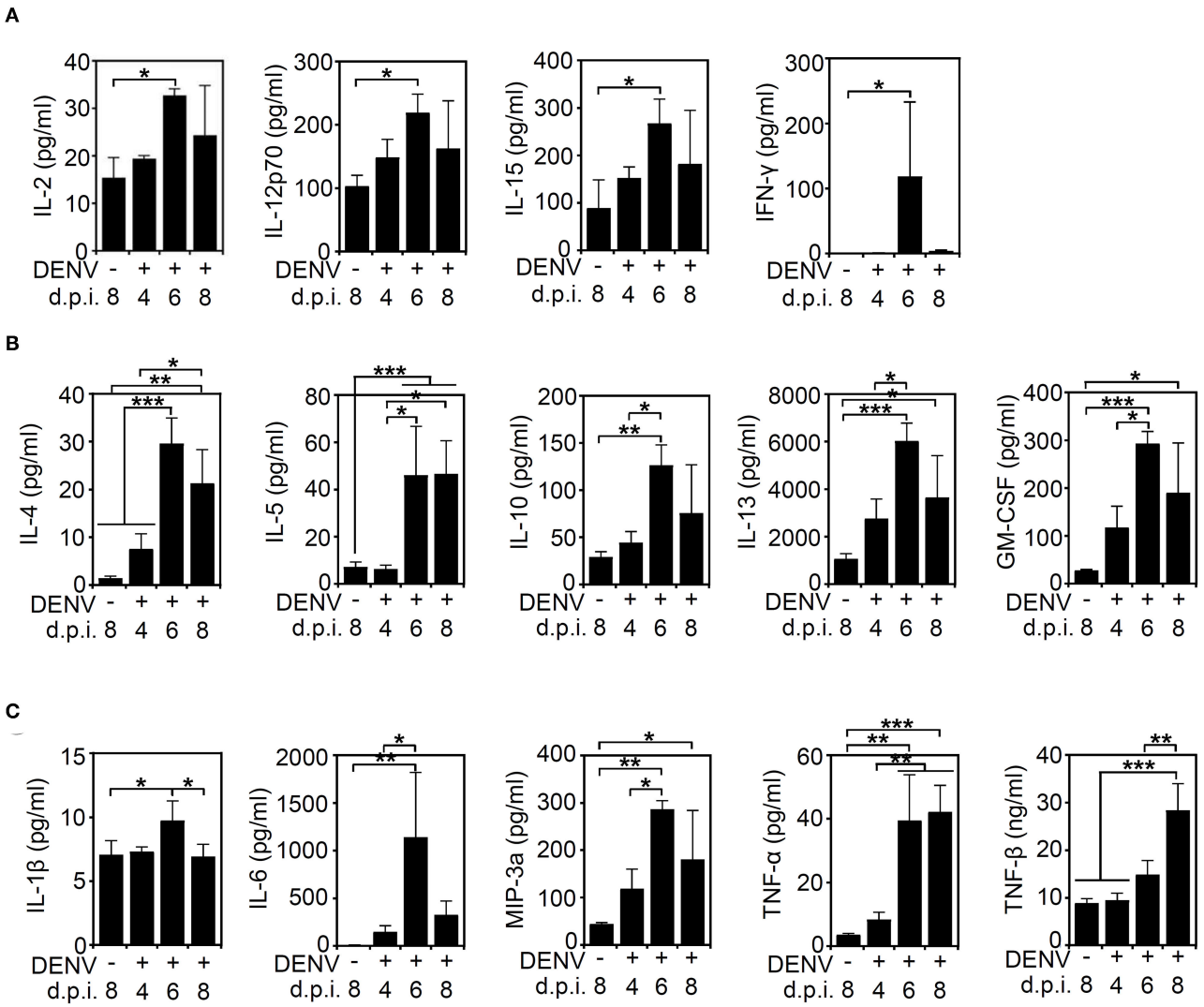

FIGURE 4 | DENV infection induces cytokine/chemokine production in the brain. A multiplex assay showed the levels of **(A)** type 1-like cytokine/chemokines (IL-2, IL12p70, IL-15, and IFNγ), **(B)** type 2-like cytokine/chemokines (IL-4, IL-5, IL-10, IL-13, and GM-CSF), and **(C)** proinflammatory proteins (IL-1β, IL-6, MIP-3a/CCL20, TNF-α, and TNF-β) produced in the brains of mice on the indicated day post-infection. Data show the means ± SD based on at least three mice. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. ns, not significant.

load. Immune profiling of the DENV-infected brain may provide findings showing possible viral immunity in the CNS following DENV infection.

Innate immune responses, such as the induction of type 1 and 2 IFNs, are crucial for the host defense against DENV infection, while IFN-triggered antiviral responses reduce viral replication and viral dissemination (Liang et al., 2011; Suzuki et al., 2016; Balinsky et al., 2017; Sprokholt et al., 2017). Studies widely use immunocompromised AG129 mice, which lack IFN-α/β/γ receptors, to investigate DENV infection. In these mice, mosquito bites lead to natural DENV infection in various organs/tissues, including the lung, liver, spleen, intestine, and brain (Shresta et al., 2004; Milligan et al., 2015; Sarathy et al., 2015, 2018). However, the results of the current study showed a mild infection in the liver and a severe infection in the brain of infected mice. Through an intravenous route of infection using immunocompetent C57BL/6 mice, Chen et al. found that dengue viral RNA was detectable in the spleen, liver, and brain. Additionally, the production of IFN-γ and the levels of helper and cytotoxic T cells have been found in the spleens of infected mice (Chen et al., 2004). Immunocompetent mice present disorders similar to those shown in the clinic, including viral hepatitis and neuropathy, by patients with severe dengue disease (Povoa et al., 2014; Verma et al., 2014). Further pathological studies related to CNS immunity are required to support the application of the present model of DENV infection, particularly concerning the liver and CNS.

Notably, CNS symptoms have been identified as critical signs of severe DENV disease since 2009 (WHO, 2009). Severe

dengue diseases include the involvement of neuropathy and viral encephalitis (Carod-Artal et al., 2013; Verma et al., 2014). The construction animal models of DENV infection with neural complications are urgently needed for further investigation. According to our current work, which is similar to that of previous works using immunocompetent BALB/c mice (Amorim et al., 2019) or C57BL/6 mice (Chen et al., 2004), viral RNA was significantly increased in the brain 1 week after DENV was inoculated through intravenous and intracerebral routes of infection. These infection models demonstrated the successful viral infection and replication in the CNS, indicating a viral enhancement in immunocompetent mice under precise immune-privileged conditions. As noted in the mice that died from viral encephalitis-like symptoms, neurotoxicity caused by viruses may be directly and indirectly affected by excessive CNS inflammation (Verma et al., 2014). Additionally, our previous works demonstrated that activated microglia act as antigen-presenting cells (APCs) for controlling type 1 antiviral immune responses (Tsai et al., 2016). CNS immunity may be pathogenic under neurotoxic conditions and protective in antiviral responses.

According to the immune cell profiling results, we showed that $CD45^+CD14^+$ cells were the major immune cell components in the brains of the naïve mice, while there was a significant decrease in this cell population at 8 d.p.i. DENV infection. Microglia, immune cell resident in the brain, express CD14 as an immune organizer in response to CNS infection and inflammation (Janova et al., 2016). However, under IFN-γ and IL-14 stimulation, the expression of CD14 is significantly decreased by an extremely flexible regulation response. The suppression of CD14 is speculated to be required to weaken the damage response and to favor a strong defense in response to infection. Additionally, activated macrophages, which express downregulated CD14, are involved in macrophage polarization (Genin et al., 2015). Our previous studies and those of others also demonstrated the induction of microglia-derived APCs and the presence of macrophages during antiviral activity (Fink et al., 2009; Tsai et al., 2016). The decreased CD14-positive cells may indicate flexible changes in CNS immunity and require further investigation.

Transcriptomic analysis of dengue patient blood showed a positive correlation of monocytes, macrophages, DCs, and neutrophils, and a negative correlation of NK cells, $CD4^+$ T cells, $CD8^+$ T cells, and B cells with viral loads (Kwissa et al., 2014). Our results also showed late production of NK1.1-positive cells that ultimately constituted a relatively high percentage of the immune cell populations in the brains of the DENV-infected mice, while the viral load was relatively diminished. In general, NK cells play diverse roles in defending against infection (Freud et al., 2017). Many Dengue studies have demonstrated that antiviral NK cells cooperate with IFNs and TNF-α to eliminate virus from DENV-infected DCs (Lim et al., 2014) and DENV-infected mice (Costa et al., 2017). However, infiltrating NK cells have been reported to cause liver cell death (Sung et al., 2012). Therefore, more exploration is needed to examine the critical roles of immune cells, such as NK1.1-positive cells and CD14-positive monocytes/macrophages/microglia, during DENV infection, especially in the CNS.

Generally, TNF-α, as well as IL-4, IL-6, IL-8, and IL-10, are increased in dengue patients (Butthep et al., 2012). We previously demonstrated that DENV infection causes overt TNF-α production during the progression of viral encephalitis (Jhan et al., 2018). The cross-talk between aberrant cytokines/chemokines and immune cells in the CNS needs to be explored. To validate the induction of high NK1.1-positive cell levels in the brains of DENV-infected mice, we analyzed the elevated production of cytokines/chemokines that are highly correlated with intensive NK cell infiltration (Peritt et al., 1998; Biron et al., 1999; Cooper et al., 2001; Fauriat et al., 2010). Our results showed that all NK-associated type 1 cytokines (IL-2, IL12p70, IL-15, and IFNγ), type 2 cytokines (IL-4, IL-5, IL-10, IL-13, and GM-CSF), and proinflammatory factors (IL-1β, IL-6, MIP-3a/CCL20, TNF-α, and TNF-β) were increased within 6 d.p.i. of DENV infection while the level of NK cells was significantly increased at 8 d.p.i. The role of NK1.1-positive cells was undefined in this work, but IL12p70 and IFNγ may have induced the NK cell differentiation toward the type 1 form, and the type 2 form may have been induced by IL-4 (Peritt et al., 1998; Kimura and Nakayama, 2005).

In conclusion, our findings provide a possible link among CNS immunity, viral tropism, and host immune responses during DENV infection in the brain. The induction of dynamic cytokine/chemokine response and changes in immune cell profiling may indicate a link between viral load and CNS impairment and/or protection for CNS immunity. For future studies, particularly for evaluating the efficacy of the vaccine and immune therapy, solving the whole puzzle of viral pathogenesis and host responses in CNS upon DENV infection must be completed by comparing different sources of virus, infectious routes, viral strains, viral inocula, and animal models.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed for this study are available from the corresponding author upon request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and User Committee of National Defense Medical Center, Taipei, Taiwan, protocol IACUC 16-261.

AUTHOR CONTRIBUTIONS

T-JS performed most of the experiments and interpreted the results. C-FL and C-LC participated in the design and supervision of the projects. T-JS and M-KJ conducted the mouse experiments. P-CT contributed to the flow cytometry analysis. T-JS and C-FL

designed the concept of the project and wrote the manuscript. All authors reviewed and approved the manuscript.

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Role of Platelet Cytokines in Dengue Virus Infection

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Platelets are anucleated blood cells derived from bone marrow megakaryocytes and play a crucial role in hemostasis and thrombosis. Platelets contain specialized storage organelles, called alpha-granules, contents of which are rich in cytokines such as C-X-C Motif Chemokine Ligand (CXCL) 1/4/7, (C-C motif) ligand (CCL) 5/3, CXCL8 (also called as interleukin 8, IL-8), and transforming growth factor β (TGF-β). Activation of platelets lead to degranulation and release of contents into the plasma. Platelet activation is a common event in many viral infections including human immunodeficiency virus (HIV), H1N1 influenza, Hepatitis C virus (HCV), Ebola virus (EBV), and Dengue virus (DENV). The cytokines CXCL8, CCL5 (also known as Regulated on Activation, Normal T Expressed and Secreted, RANTES), tumor necrosis factor α (TNF-α), CXCL1/5 and CCL3 released, promote development of a pro-inflammatory state along with the recruitment of other immune cells to the site of infection. Platelets also interact with Monocytes and Neutrophils and facilitate their activation to release different cytokines which further enhances inflammation. Upon activation, platelets also secrete factors such as CXCL4 (also known as platelet factor, PF4), CCL5 and fibrinopeptides which are critical regulators of replication and propagation of several viruses in the host. Studies suggest that CXCL4 can both inhibit as well as enhance HIV1 infection. Data from our lab show that CXCL4 inhibits interferon (IFN) pathway and promotes DENV replication in monocytes *in vitro* and in patients significantly. Inhibition of CXCL4 mediated signaling results in increased IFN production and suppressed DENV and JEV replication in monocytes. In this review, we discuss the role of platelets in viral disease progression with a focus on dengue infection.

Keywords: platelets, cytokines, PF4, immune modulation, dengue infection

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DENGUE INFECTION

Dengue is one of the most prevalent arboviral diseases (Simmons et al., 2012) affecting ∼390 million people around the globe (Bhatt et al., 2013) causing an estimated 20000 deaths each year (Simon et al., 2015). Mainly this virus affects the tropical and subtropical regions of the world (Wilder-Smith et al., 2019). India accounts for the largest number of dengue cases, with ∼33 million apparent and another 100 million asymptomatic infections occurring annually (Bhatt et al., 2013). Since the 1990s, dengue epidemics have become recurrent in several parts of India, at a rate of 34.81 per million of the population in 2010–2014. Thus, indicating that the number of dengue cases has increased markedly in recent times (Mutheneni et al., 2017). The principle vectors for transmission of the disease are mosquitoes *Aedes aegypti* and *Aedes albopictus* (Kraemer et al., 2015). Whereas, non-vector transmission of the virus is also possible through blood transfusion,

organ transplantation, needle stick injuries and mucosal splashes (Wilder-Smith et al., 2009; Busch et al., 2016; Sabino et al., 2016). Dengue virus (DENV) is a Flavivirus that belongs to the family Flaviviridae and has four serotypes, DENV1–4. The virus genome consists of a single-strand of positive-polarity RNA which codes for three structural proteins (capsid C, membrane M, and the envelope E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Noble and Shi, 2012). The clinical features of the disease range from asymptomatic infection, undifferentiated febrile illness (dengue fever) to severe complication including Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), characterized by plasma leakage and coagulopathy (Kalayanarooj, 2011; Simmons et al., 2012). The pathogenesis of severe dengue is the result of a complex interaction between viral and host factors. The four DENV serotypes (DENV 1–4) share homology, but each serotype possesses specific immunoreactivity. Primary infection with one DENV serotype increases the risk of severe dengue upon secondary infection with other DENV serotype. This is because the sub-neutralizing cross-reactive antibodies opsonize mature and immature virus particles, causing infection of mononuclear phagocytes via Fc-receptor (FcR) thus enhancing DENV infection, termed as antibody dependent enhancement (ADE) (Littaua et al., 1990; Dejnirattisai et al., 2010; Schmidt, 2010; Guzman et al., 2013). This is characterized by persistent high fever, thrombocytopenia, event of DHF or DSS and elevated IgG/IgM ratio (Innis et al., 1989; Shu et al., 2003; Prince et al., 2011; Cucunawangsih and Kurniawan, 2015). Complement activation by virus-antibody complexes and T-cell mediated immune response have also been reported in the progression of secondary dengue infection. Independent of primary or secondary infection, platelet activation is the hallmark of dengue infection. Thrombocytopenia (decrease in platelet count) is another common symptom of dengue infection and is associated with hemodynamic instability and progression in severity of dengue fever (Krishnamurti et al., 2001; Schexneider and Reedy, 2005; Mourão et al., 2007; Bozza et al., 2008).

PLATELET FUNCTIONS

The primary and most important role of platelets is to maintain hemostasis (Clemetson, 2012). Platelets are mediators of thrombosis too (Kaplan and Jackson, 2011). Besides, platelets also contribute to non-canonical functions such as immune modulation, atherogenesis, tissue repair and regeneration, angiogenesis, and metastasis (Lindemann et al., 2007; Nurden, 2011; Semple et al., 2011; Eisinger and Langer, 2018; Koupenova et al., 2018; Schlesinger, 2018). Platelets mediate innate immune response and contribute to antimicrobial activity either by releasing antimicrobial proteins or by modulating immune response of other immune cells such as neutrophils and monocytes (Flad and Brandt, 2010). Platelet dysfunction can lead to impaired immune or inflammatory responses and tissue damage. During infection, platelet activation can lead to serious pathophysiological conditions such as Infective Endocarditis and Disseminated Intravascular Coagulation (Beynon et al., 2006; Kitchens, 2009). Platelet activation followed by thrombocytopenia is found to be associated with patients of several infections and sepsis (Akca et al., 2002; Claushuis et al., 2016; Levi, 2016). Platelet activation is the hallmark of many viral infections including dengue, HIV, HCV, H1N1, and Ebola (Geisbert et al., 2003; Chaipan et al., 2006; Afdhal et al., 2008; Assinger et al., 2014; Ojha et al., 2017, 2019).

TABLE 1 | Contents of platelet granules.

Granules	Contents	Function
α-granules		
Chemokines	CXCL1 (GRO-α), CXCL4 (PF4), CXCL4L1 (PF4alt), CXCL5 (ENA-78), CXCL7 (PBP), CXCL8 (IL-8), CXCL12 (SDF-1α), CXCL14 (BRAK) CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL15, CCL17 (TARC), CCL18 (PARK)	Chemotactic for different immune cells, modulate activation and differentiation of Monocytes, regulate thrombopoiesis, hematopoiesis, and T cell development
Growth factors	Epidermal Growth Factor (EGF), Hepatocyte Growth Factor (HGF), Transforming Growth Factor-β (TGF-β), Insulin like Growth Factor (IGF), Platelet derived Growth Factor (PDGF), Vascular endothelium growth Factor (VEGF)	Regulates growth
Microbicidal proteins	Thymosin-β4, Thrombocidins 1 and 2, Kinocidins, Defensins 1 and 2	Antimicrobial peptides
Dense granules	Bioactive amines (serotonin, histamine), Phosphates (polyphosphate, pyrophosphate), cations, ADP, ATP.	Hemostasis, thrombosis
Lysosome	Protein degrading enzyme (collagenase, elastase, cathepsins), carbohydrate degrading enzyme (glucosidase, galactosidase), phosphatases.	Degradation

Blair and Flaumenhaft (2009), Karshovska et al. (2013), Heijnen and van der Sluijs (2015), Gremmel et al. (2016).

PLATELET GRANULES AND CYTOKINES

Platelets contain α and dense granules, also known as storage granules. These granules store huge numbers of proteins, RNAs, antimicrobial peptides and growth factors (Manne et al., 2017). α granules are ∼500 nm in diameter, round in shape and around 50 granules are present in each platelet (Yadav and Storrie, 2017). Different cytokines, growth factors and antimicrobial peptides present are listed in **Table 1**. Upon platelet activation the α-granules release these contents into the plasma (Jonnalagadda et al., 2012). The dense granules on the other hand are smaller in size and contain Adenosine nucleotides (ATP, ADP), serotonin, calcium, magnesium and pyrophosphate (Rendu and Brohard-Bohn, 2001).

IMMUNE FUNCTION OF PLATELETS

Platelets are modulators of innate immunity and possess both surface and intracellular receptors for detection of pathogens in the bloodstream. Receptors include Toll-like receptors (TLRs),

NOD-like receptors (NLR), C-type Lectins and integrins for detection of pathogen associated molecular patterns (PAMPs), and Fc receptors and complement receptors for antibody opsonized pathogens (McDonald and Dunbar, 2019). Platelets express TLR 1–4, 6, 7, and 9 (Hamzeh-Cognasse et al., 2018; McDonald and Dunbar, 2019). On activation the TLRs promote secretion of interferons and proinflammatory cytokines such as IL-6, CXCL8, TNF-α, and CCL5 which further activate other cells resulting in increased inflammation. CD40L, expressed on platelets as a result of activation (Henn et al., 1998), mediates its interaction with CD40 present on B cells, Monocytes, Dendritic cells, Macrophages, endothelial cells and modulate their activity. CD40 and CD40L interaction leads to recruitment of TNF receptor associated factors (TRAFs) which further lead to activation of canonical and non-canonical NF-kB pathways (Bishop et al., 2007). Platelets via CD40L induce B cell isotype switching and augment CD8+ T cell response (Elzey et al., 2003). Activated platelets cause Dendritic cell maturation and activation by direct surface contact through CD40L or through soluble effectors (Czapiga et al., 2004; Hagihara et al., 2004; Martinson et al., 2004). In Dendritic cells CD40 expression tends to increase the expression of MHC class II along with essential co-stimulatory molecules such as CD58, CD80, and CD86. This further leads to improved T cell activation by better presentation of the antigen. Platelets possess an active proteasome and can process and present exogenous antigens which has been shown both *in vitro* and *in vivo* using Experimental Cerebral Malaria (ECM) mouse model (Chapman et al., 2012). An increase in surface expression of HLA class 1 has been observed on platelets obtained from patients with Dengue infection (Trugilho et al., 2017). However, the actual mode of presentation of DENV antigen still needs to be investigated.

Recently it has been shown that platelets can differentiate between bacterial LPS isoforms and as a result between pathogens (Berthet et al., 2012). This helps in providing a pathogen specific response by secreting different cytokines. It has been shown that platelets upon activation with thrombin trigger complement activation. Complement factor C3b bound to bacteria gets detected by GPIb receptor on platelet surface resulting in phagocytosis of the bacteria-platelet complex (Verschoor et al., 2011). Platelets also respond to Damage Associated Molecular Patterns (DAMPs) (Fuchs et al., 2011).

Platelets get activated upon interacting with pathogens directly through their surface receptors or indirectly through plasma proteins such as fibrinogen, vWF, complement and IgG (Fitzgerald et al., 2006; Cox et al., 2011). Upon activation by bacterial pathogens platelets release antimicrobial substances such as ROS (reactive oxygen species), antimicrobial peptides, defensins, thrombocidins and proteases (Suzuki et al., 2001; Tang et al., 2002; Trier et al., 2008; Wiesner and Vilcinskas, 2010; Yeaman, 2014). Platelets support phagocytosis and intracellular killing of bacteria by Neutrophils and release of Neutrophil Extracellular Traps (NETs) (Miyabe et al., 2004; Assinger et al., 2011; Kim and Jenne, 2016). In disease conditions such as malaria, platelets have a direct lethal effect on all major *Plasmodium species* (Kho et al., 2018). Platelets bind with erythrocytes and cause killing of intraerythrocytic *Plasmodium* along with intracellular accumulation of platelet CXCL4 (McMorran et al., 2012). In patients with *mycobacterium tuberculosis*, infected platelets along with other immune cells are responsible for pulmonary inflammation and tissue damage leading to morbidity and spread of infection (Fox et al., 2018). Thus, platelets help to fight infection but at times can also help in disease progression.

IMMUNE RESPONSE OF PLATELETS DURING VIRAL INFECTIONS

CXCL4 is highly abundant in platelets. CXCL4 can associate with CCL5 to modulate monocyte functions. CCL3, CCL5, CCL7, CCL17, CXCL1, CXCL5, and CXCL8 are some of the chemokines among the whole pool of chemokines released by platelets which attract leukocytes causing further activation of platelets (Gear and Camerini, 2003). A comparative study reports that CXCL4 in conjunction with M-CSF supports HIV-1 replication in immune cells including monocytes and macrophages. It was found that CXCL4 derived macrophages can be infected with macrophage-tropic HIV-1 that uses either CCR5 or CXCR4 as a co-receptor for viral entry. CXCL4 increases HIV-1 replication in M-CSF-derived human macrophages after virus adsorption on to the cells (Schwartzkopff et al., 2009). CXCL4 has also been identified as a broad spectrum inhibitor of HIV-1 (Auerbach et al., 2012). In another study, it was found that CXCL4 inhibitory effect lies in a concentration range where it exists in monomeric form. As a monomer it binds with the viral envelope protein resulting in inhibition of HIV-1 attachment to the cell surface. But oligomerization initiated upon increasing concentration; the tetrameric or higher-order forms promoted viral replication *in vitro* (Parker et al., 2016). CXCL4 and beta thromboglobulin (β-TG) have been reported as prognostic markers in Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) infections. A decreased serum CXCL4 and increased β-TG were reported in the study (Poon et al., 2012). CXCL4 is very well-known for its regulatory functions in immune response and inflammation. Role of CXCL4 has been studied in pulmonary influenza infection, and it was found that it helps in protecting the mice from H1N1 virus infection (Guo et al., 2017). CXCL4 up-regulated in HCV induces liver fibrosis both *in vitro* and *in vivo* (Zaldivar et al., 2010). CXCL4 also increases replication and propagation of dengue and Japanese encephalitis viruses (JEV) in monocytes by downregulating the type-I interferon production, thus leading to increased viral load (Ojha et al., 2019).

Platelet derived CCL5 and CCL3 have been reported as major HIV-suppressive factors (Cocchi et al., 1995). CCL5 is also reported as inhibitor of Influenza A (Wareing et al., 2004) and HCV infection (Katsounas et al., 2011). CCL5 is reported to be involved in viral lung diseases (Culley et al., 2006). CCL17 is released by activated platelets and functions in further platelet activation in an autocrine manner by binding to its receptor CCR4 present on platelets (Gear and Camerini, 2003). A recent study reported that co-culture of monocyte derived dendritic cells (MoDCs) and HCV infected cells lead to the expression of CCL17 and CCL22 which attract regulatory T cells at the

site of infection (Riezu-Boj et al., 2011). Co-culture of MoDCs with HBV transfected cells induces the expression of CCL17 and CCL22 which recruits IL-17 secreting T-cells (Zhang et al., 2020).

IMMUNE RESPONSE OF PLATELETS TO DENGUE INFECTION

Dengue Infection in Platelets

The presence of DENV has been reported in circulating platelets of dengue patients (Noisakran et al., 2009). Platelet activation and thrombocytopenia are the hallmarks of dengue infection. Platelet activation results in the release of the granular constituents. Platelets from dengue patients present signs of activation, mitochondrial dysfunction and activation of apoptosis caspase cascade resulting in thrombocytopenia. Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) also termed as CD209 has been shown as a critical receptor involved in DENV-dependent platelet activation (Hottz et al., 2013b). DENV directly binds to the surface receptors DC-SIGN, heparan sulfate proteoglycan (HSP) and CLEC-2 (C-type-lectin-like receptor 2) on platelets and replicates inside the platelets (Simon et al., 2015; Sung and Hsieh, 2019). One of the findings suggests that platelets attach to the histones (H2A) present in systemic circulation of the dengue patients, which lead to their activation (Trugilho et al., 2017). Platelets have specialized FcγRII receptors for binding of immunoglobulin coated virus particles. Antibody dependent enhancement of DENV occurs via these FcγRII receptors on platelets (Wang et al., 1995) upon secondary dengue infection. This leads to platelet activation and thrombocytopenia which results into severe dengue diseases, DHF, and DSS.

Immunoregulation of Dengue Infection by Platelets

Platelets are the key players involved in immunoregulation of dengue disease as there is platelet activation upon dengue infection and release of the granular contents. Cytokines are released from alpha granules, and have been shown to play regulatory roles in dengue virus infection. The CCR1-CCL2 axis plays an important role in the pathogenesis of dengue infection whereas CCR1-CCL5 axis was found to show a protective role in dengue infection (Sierra et al., 2014). A study has suggested that low levels of CCL5 and high levels of CXCL8 during early dengue infection could serve as a marker for severe dengue disease (Patra et al., 2019). Upon DENV infection there is activation of endothelial cells leading to increased expression of E-selectin on the endothelial cells. E-selectin and P-selectin helps in platelet adhesion to endothelial cells (Krishnamurti et al., 2002). P-selectin expressed on the surface of activated platelets promote interaction of platelets with monocytes and neutrophils leading to aggregate formation (Onlamoon et al., 2010). The endothelial cells upon dengue infection secrete CXCL8, IL-6, CXCL10, CXCL11, and CCL5 (Avirutnan et al., 1998; Dalrymple and Mackow, 2012; Kelley et al., 2012). Platelets also secrete IL-1β, CXCL8, and CCL5 and contribute to the total cytokine pool. These cytokines together help in increasing the vascular permeability and possess chemoattractant properties which progress inflammation and plasma leakage *in vivo* thus leading to dengue disease severity (Kelley et al., 2012).

Increased platelet monocyte aggregates have been reported in dengue patients having thrombocytopenia and increased vascular permeability. Platelet binding modulates cytokine release by monocytes in dengue infection. Interaction of platelets isolated from dengue patients with monocytes from healthy individuals lead to the synthesis and secretion of IL-1β, CXCL8, and IL-10 by the monocytes. Also interaction of monocytes with apoptotic platelets leads to secretion of IL-10 through P selectin and phosphatidylserine recognition in platelet-monocyte aggregates. IL-10 secretion requires platelet-monocyte contact but not phagocytosis of platelets by the monocytes. Activated and apoptotic platelets aggregate with monocytes during dengue infection and cause specific cytokine responses contributing to the pathogenesis of dengue. The cytokines IL-1β, CXCL8, and IL-10, released by monocytes in response to interactions with platelets from dengue patients, are frequently observed to be increased in plasma of severe dengue patients (Hottz et al., 2014).

Vascular leakage is one of the hallmarks of DHF, and an increased CCL2 level is reported in DHF. CCL2 leads to disrupted distribution of tight junction protein on the cell membrane of human umbilical vein endothelial cells (HUVEC) leading to increased vascular permeability (Lee et al., 2006). Platelets release IL-1β upon DENV infection by recruiting nucleotide-binding domain leucine rich repeat containing protein (NLRP3) inflammasome and caspase-1 which is highly correlated with increased vascular permeability and activation of innate immunity in dengue infection (Hottz et al., 2013a, 2014; Guabiraba and Ryffel, 2014).

Macrophage migration inhibitory factor (MIF) is a pleiotropic proinflammatory cytokine that mediates several immune responses, serum levels of MIF, IL-6, and IL-10 are reported to be higher in DHF patients as compared to DF patients (Chen et al., 2006). MIF is secreted mainly by T cells, and also by other immune and non-immune cells such as macrophages, endothelial cells, epithelial cells, neutrophils and monocytes (Lai et al., 2020). It is also secreted by platelets (Wirtz et al., 2015). Mif–/– mice show lesser DENV induced inflammation, thrombocytopenia and viral load suggesting it as one of the crucial cytokines involved in dengue pathogenesis (Assunção-Miranda et al., 2010). MIF helps in enhancing dengue viral replication in host cells, it also causes endothelial hyperpermeability upon DENV infection, and also modulates the functions of immune cells. MIF inhibition during dengue infection also leads to decreased TNF-α and IL-6 production which otherwise lead to vascular hyperpermeability. MIF release by neutrophils leads to neutrophil extracellular trap (NET) formation and inflammation which further helps in dengue pathogenesis. It can also be a potential therapeutic target against dengue infection (Lai et al., 2020). In comparison with DENV infection, secretory NS1 protein released upon DENV infection and exogenous NS1 protein stimulation also leads to a partial inflammatory phenotype in platelets. Similar to infected platelets, NS1-stimulated platelets also release the stored

cytokines/chemokines CXCL4, CCL5, and MIF but, in contrast, they do not secrete IL-1β. NS1 induces pro-IL-1β synthesis in platelets but does not induce caspase-1 activation for IL-1β processing and secretion, which occurs upon classical DENV infection providing all necessary signals for IL-1β synthesis, caspase-1 activation, and IL-1β release. There is increased caspase-1 activation in platelets stimulated with either ATP, NS1, and ATP, or DENV, but not in platelets stimulated with NS1 alone (Quirino-Teixeira et al., 2020). DENV activated platelets deliver inflammatory signals to monocytes leading to secretion of CXCL8, IL-10, and IL-6, and also show increased accumulation of lipid droplets (LD) in the monocytes after 18 h of interaction. Stimuli-specific-activated platelets can cause phenotypic changes and metabolic reprogramming in monocytes. Activated platelets exposed to DENV *in vitro* form aggregates with monocytes and signal to LD formation and CXCL8, IL-10, CCL2 and prostaglandin E2 (PGE2) secretion. Pharmacologic inhibition of LD biogenesis prevents PGE2 secretion, but not CXCL8 release, by platelet-monocyte complexes. Mechanistically LD formation in monocytes exposed to DENV-activated platelets is partially dependent on platelet-produced MIF. LD formation is higher in monocytes, which have platelets adhered on their surface, suggesting the importance of adhesion besides paracrine signaling. Activated platelets aggregate with monocytes during DENV infection and lead to LD biogenesis and release of inflammatory mediators (Barbosa-Lima et al., 2020).

CXCL4 in Dengue Infection

CXCL4 is the most abundant chemokine present in platelet α granules, released upon platelet activation (Gleissner et al., 2008). A quantitative proteomic study has investigated the protein content of platelets in dengue patient samples and healthy controls. In dengue patients, activated platelets release a significant amount of CXCL4 in plasma as compared to healthy volunteers (Trugilho et al., 2017). CXCL4 has been identified as a prognostic tool for classifying acute and severe dengue patients (Fragnoud et al., 2015). A comparative study between dengue and leptospirosis (caused by *Leptospira* bacteria) revealed that out of the 19 biomarkers assessed CXCL4 was the one higher in dengue fever compared to leptospirosis (Conroy et al., 2014). A recent work from our lab also showed that CXCL4 is one of the abundant proteins present in blood plasma upon dengue infection. It also helps in propagating the virus inside monocytes *in vitro*. Binding of CXCL4 to CXCR3 leads to increased phosphorylation of p38-MAPK and diminished activation of STAT-2 and IRF-9. This decreases the synthesis and secretion of IFN-α by the DENV2 infected monocytes, resulting in 3–4-fold increase in virus replication. Also, blocking CXCL4 using neutralizing CXCL4 antibodies or its receptor CXCR3 through inhibitor AMG487 reversed the above signaling pathway and significantly restored the IFN-α production, thus inhibiting the DENV propagation in monocytes. The study also showed a decrease in the levels of proinflammatory cytokines TNF-α, IL-1β, and IL-6 in monocytes upon treatment of the cells with rhCXCL4, which was rescued upon treating the cells with anti-CXCL4 antibody or antagonist AMG487, concluding that CXCL4 significantly impacts dengue virus replication (Ojha et al., 2019).

Therapy for Dengue

Till date, there is no specific treatment for Dengue infection. Although few studies describe the development of drugs targeting host factors required for DENV propagation. Chloroquine (CQ) has immunomodulatory effects by suppressing release of TNF-α and IL-6 (Tricou et al., 2010). Although the *in vitro* study showed some promising results but a randomized trial in patients did not show significant reduction in the development of DHF (Savarino et al., 2003). Another drug, Celgosivir is an alkaloid castanospermine derived from the Moreton Bay Chestnut tree showed some inhibitory effects on all four serotypes (Durantel, 2009). Celgosivir was tested in Phase I and II trials as a possible treatment for HIV and hepatitis C infection and was found to be safe (Durantel, 2009; Rathore et al., 2011). In a recent study we have shown that inhibition of CXCL4-CXCR3 interaction by an antagonist AMG487 significantly reduced the replication of DENV and JEV, since both viruses use CXCL4 for their rapid replication in host immune cells *in vitro*. The AMG487 treatment reduced the JEV infection in mice and increased the mice survivability suggesting the CXCL4-CXCR3 axis as the therapeutic target for prophylaxis of dengue (Ojha et al., 2019).

IFNs play major antiviral roles. IFN-α/β have proved to be of considerable value in some chronic virus infections, particularly hepatitis and papillomavirus infections (Finter, 1994). A study suggests the anti-viral activity of IFN-α and ribavirin as a combination therapy against DENV (Piresde Mello et al., 2018). Studies also suggest that manipulating IL-10, an anti-inflammatory cytokine may serve as an effective antiviral treatment in addition to the development of a safe dengue vaccine (Tsai et al., 2013).

A live attenuated vaccine known as Dengvaxia, developed by Sanofi, was approved by the Food and Drug Administration (FDA) in the United States licensed in May 2019, for use in children 9–16 years old living in an area where dengue is common such as US territories Samoa, Puerto Rico and Virgin Islands. Another live attenuated vaccine, named TetraVax-DV, developed by Butantan, is currently in clinical trial phase 3 in Brazil (Durbin et al., 2011). The Dengue tetravalent vaccine, developed by Panacea Biotec Ltd, is in phase 2 trial in India (TDB Panacea Agreement, 2017).

AUTHOR CONTRIBUTIONS

PG has conceptualized the approach. All authors wrote the article.

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Intrinsic ADE: The Dark Side of Antibody Dependent Enhancement During Dengue Infection

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Dengue fever is an *Aedes* mosquito-borne illness caused by any one of the four different dengue virus (DENV) serotypes (1–4) and manifests in the form of symptoms ranging from mild or asymptomatic to severe disease with vascular leakage, leading to shock, and viral hemorrhagic syndrome. Increased risk of severe disease occurs during secondary infection with a virus serotype distinct from that of prior dengue infection. This occurs by antibody dependent enhancement (ADE) of infection, wherein sub-neutralizing antibodies against the virus particles opsonize dengue virus entry via formation of immune complexes that interact with fragment crystallizable gamma receptors (FcγR) on monocytes, dendritic cells, and macrophages. The ADE phenomenon has two components: Extrinsic and Intrinsic ADE. While extrinsic ADE contributes to enhanced virus entry, intrinsic ADE results in heightened virus production by inhibition of type1 interferon and activation of interleukin-10 biosynthesis, thereby favoring a Th2 type immune response. Intrinsic ADE has greater contribution in enhancing Dengue replication as compared to extrinsic ADE. Detailed elucidation of intrinsic ADE during secondary dengue infection can increase our understanding of DENV-pathogenesis and aid in the development of host-targeting antivirals. Here we review literature focusing on intrinsic factors contributing to severe dengue pathology and suggest possible avenues for further research.

Keywords: dengue, pathogenesis, antibody dependent enhancement (ADE), extrinsic ADE, intrinsic ADE

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INTRODUCTION

Dengue fever results from infection with any of the four DENV serotypes via the bite of infected *Aedes* sp. mosquitoes. They are single stranded positive sense RNA viruses belonging to the family *Flaviviridae*. A lipid bilayer membrane surrounds the virus particle and contains the envelope (E) and membrane (M) proteins embedded on the surface. The DENV E protein binds to specific receptors on susceptible cells to gain entry, followed by which the viral RNA genome uncoats and begins replication in the cytoplasm (Guzman et al., 2016). Translation of viral proteins also ensues and virus assembly occurs in the endoplasmic reticulum before being transported through the trans-Golgi network and released extracellularly as mature virus particles (Wilder-Smith et al., 2019).

The disease is widespread in tropical and sub-tropical regions of the world and although a vaccine exist for this disease, its use has not been approved in all countries (Aguiar and Stollenwerk, 2018). As per estimates for the year 2013, about 58.4 million symptomatic dengue cases were

reported globally, which amounted to US$8.9 billion (Shepard et al., 2016). A study by Murhekar et al. estimated the total number of Dengue infections in India during the year 2017 to be 12,991,337 (Murhekar et al., 2019; Wilder-Smith and Rupali, 2019).

Whilst dengue fever can manifest as an asymptomatic or mild febrile illness, a more severe form of the disease ensues during secondary infection whereby increased vascular permeability and thrombocytopenia causes gastrointestinal hemorrhage and plasma leakage that results in Dengue Hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) (Halstead, 2015). During secondary dengue infection by a heterologous DENV serotype, antibody response in humans is primarily directed against the virus serotype from previous infection(s) and the antibodies thus produced are often non-neutralizing against the heterologous serotype compared to the original infecting serotype from primary infection. The phenomenon is termed original antigenic sin and was first described in Dengue by Halstead et al. (1983). These sub-neutralizing antibodies in turn aid in ADE of dengue infection by catalyzing virus entry via interactions with FcγR on the cell surface (Wilder-Smith et al., 2019).

It should be noted that original antigenic sin in the context of dengue fever may refer to enhancement of DENV pathogenesis by either sub-neutralizing antibodies against the infecting serotype during secondary infection or due to aberrant B and T cell responses targeted against the DENV serotype from primary infection (Rothman, 2011). Severe dengue can also occur during primary DENV infection in infants born to dengue immune mothers. In this case, the initially protective antibody titers decline over the first year after birth and leads to increased infection due to ADE (Libraty et al., 2009).

EXTRINSIC VS. INTRINSIC ADE

Two types of ADE have been defined, namely extrinsic and intrinsic ADE. Extrinsic ADE, as its name suggests, refers to phenomena that are extrinsic to mononuclear phagocytes, like enhanced rates of receptor interaction and internalization of virus-immune complexes. In fact, extrinsic factors were thought to be solely responsible for the adverse effects of dengue ADE associated pathogenesis, until studies on Ross River Virus (RRV) revealed a different scenario. Incubation of RRV infected cells with anti-virus IgG resulted in ADE mediated persistent productive infection of macrophages for prolonged time periods, brought about by innate immune suppression (La Linn et al., 1996; Halstead, 2015). This phenomenon is called intrinsic ADE, that involves modulation of innate immune effectors by internalized virus-immune complexes to favor increased replication and release (Halstead, 2015). In other words, intrinsic ADE increases the "burst size" of infected cells i.e., virus release from an infected cell, and extrinsic ADE enhances infected cells mass (Flipse et al., 2016a). Here we focus on intrinsic ADE during dengue infections and the myriad ways by which this phenomenon enhances DENV replication and release.

INNATE IMMUNE RESPONSES DURING PRIMARY DENGUE INFECTION

Severe pathogenicity associated with secondary dengue infection due to intrinsic ADE occurs primarily by evasion of host innate immune responses. A proper understanding of immune response occurring canonical/primary dengue infection is vital to properly appreciate the immune-evasion mechanisms associated with intrinsic ADE. Canonical DENV entry occurs via receptor-mediated endocytosis and recognition of invading pathogen is first detected by pathogen recognition receptors (PRRs). While Toll like receptor (TLR)−3 and TLR-7 detect the virus in endosomes, the low pH dependent escape of viral RNA from these vesicles is recognized by MDA5 (melanoma differentiation-associated gene 5) and RIG-I (retinoic-acid inducible gene 1) (Chen et al., 2008) Activation of both TLR-dependent and independent pathways ensue and promote expression of pro-inflammatory cytokines like IL-12, IL-8, IFN-γ, and IFN-α (Ubol and Halstead, 2010). This coupled with activation of STAT1 by IFNs results in production of nitric oxide (NO) radicals and helps limit DENV replication and spread (Flipse et al., 2016a).

INTRINSIC ADE AND ANTIVIRAL IMMUNITY

Evasion of Innate Immunity

The route of DENV entry into cells and early events of virus replication occurring thereafter are critical factors that influence the host response to infection (Flipse et al., 2013; Chan et al., 2019). As opposed to canonical dengue infections, entry of antibody-opsonized DENV during ADE is thought to occur via a phagocytosis-like pathway (Ayala-Nunez et al., 2016) via the cross-linking of activating FcγR present on the surface of monocytes, macrophages and dendritic cells. Such receptor engagement generally triggers a type-1 interferon stimulated gene (ISG) response, but DENV evades this antiviral mechanism by causing the co-ligation of leukocyte Ig-like receptor-B1 (LILRB1), a tyrosine-based inhibition motif-bearing immunoreceptor. This results in the dephosphorylation of spleen tyrosine kinase, a key regulator of FcγR signaling (Chan et al., 2014), thereby inhibiting the type1 ISG response. Yet another study by Chan et al. reported the use of fluorescently labeled DENV-2 to elucidate changes in the host transcriptome as a result of both canonical receptor-mediated endocytosis and the FcγR coupled antibody mediated entry pathway (Chan et al., 2019). Similar viral loads were used to induce virus entry via either of these two pathways and Gene Ontology (GO) terms analysis was used to identify changes in the host transcriptome. Dengue ADE mediated virus entry predominantly caused the enrichment of differentially expressed genes (DEGs) that regulate RNA processing and many of these factors are known to interact with DENV viral RNA (Viktorovskaya et al., 2016). Cross-referencing of this data with previously published genome wide CRISPS/cas9 screens revealed that dengue ADE modulates the transcriptome of monocytes to upregulate genes that are responsible for vesicular transport and mRNA processing. While canonical dengue infection causes

downregulation of DEGs responsible for host protein translation, antibody-mediated DENV entry reverses this effect to aid in translation of viral proteins (Chan et al., 2019).

In contrast to canonical dengue infection in the absence of immune-complexes, ADE during secondary infection with heterologous serotype inhibits TLR expression and signaling. The expression of negative regulators of toll like receptor (TLR)-expression such as TANK (TAF family associated NF-κB activator) and SARM (Sterile-alpha and Armadillo Motif containing protein) during dengue ADE results in downregulation of TLR-signaling molecules and TLR-3,4, and 7 expression in DENV infected cells (Modhiran et al., 2010; Flipse et al., 2013). A consequence of this is the abrogation of IRF1 and IRF3 production by activation of NF-κB and Inhibitor of κB kinases (IKKs), respectively, during downstream TLR signaling pathway. The upregulation of TANK signaling pathway facilitates complex formation between canonical IKKs and IKK related kinases which culminates in a shift to TLR signaling via a non-NF-κB pathway instead of the canonical NF-κB mediated signaling (Kawagoe et al., 2009; Clark et al., 2011).

While intrinsic and extrinsic mechanisms of ADE immunopathogenesis has been associated with increased DENV entry in susceptible cells, studies on primary macrophages has shown that this is not always the case (Malavige et al., 2012). The antibody-mediated entry of DENV particles in primary macrophages results in enhanced membrane fusion potential within the endosomes, thereby enhancing virus replication and translation. Since virus entry into these cells remains unaltered, the internalized DENV particles go largely unnoticed by the endogenous interferon pathway and enabling enhanced viral replication during early stages of infection (Flipse et al., 2016a).

Effects on Virus Replication and Pathogenesis

Many innate immune effectors have been shown to be modulated in DHF/DSS, out of which the upregulation of IL-10 biosynthesis via intrinsic ADE plays a pivotal role in dampening host mediated innate and adaptive immune responses (Tsai et al., 2013). Dengue induced immunosuppression has been studied *in vitro* by incubating DENV with serum obtained from dengue infected patients, followed by addition of the virus-antibody mixture to THP-1 cells (human monocytic cell line constitutively expressing FcγR). In addition to promoting virus replication, dengue induced ADE was shown to induce a TH2-type immune response as evidenced by increased production of IL10 and IL6. This causes over expression of SOCS3 (Suppressor of cytokine signaling 3 gene) thereby inhibiting the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway and production of IFN-γ. A direct consequence of this is the abrogation of NO synthesis, which facilitates increased dengue viral RNA synthesis. Moreover, studies in K562 cells (human chronic myelogenous leukemia cell line) has shown that inhibition of NO synthesis using specific inhibitors increased virus production during dengue-ADE (Flipse et al., 2013). The enhancement of anti-inflammatory cytokine synthesis and subsequent inhibition of Th1-type cytokines IL-12 and IFN-γ during dengue ADE by this intrinsic mechanism produces a Th2-type biased immune response (Chareonsirisuthigul et al., 2007; Ubol et al., 2010). **Figure 1** summarizes the effects of DENV infection during ADE and non-ADE conditions.

Effects on Adaptive Immune Response

A balanced Th-1 and Th-2 type immune response to any infection is crucial for the effective clearance of pathogens (Berger, 2000). While the elicitation of Th-1 type response leads to the production of pro-inflammatory cytokines and increased phagocytic activity, Th-2 type response results in heightened anti-inflammatory cytokine production characterized by type-2 or antibody-mediated immunity. The Th-2 cytokines IL-1, IL-10, and IL-13 promote B-cell proliferation and thereby stimulates antibody production (Spellberg and Edwards, 2001). In the case of dengue-ADE, a skewed Th-2 type immune response serves only to exacerbate the already worse situation by promoting the production of sub-neutralizing antibodies that aid in immune complex-mediated DENV entry into permissible cells (Ubol and Halstead, 2010).

ADE IN OTHER VIRUSES

Apart from DENV, the effect of ADE on enhancement of virus pathogenesis has been shown to true in the case of few other viruses. The classic example is that of HIV-1 wherein increased viral RNA and protein synthesis ensues when cells are infected in the presence of HIV-1 specific antibodies as compared to untreated cells (Robinson et al., 1989). Similar results also have been reported for other viral diseases like West Nile fever (Gollins and Porterfield, 1985), Ross River fever (Lidbury and Mahalingam, 2000) feline infectious peritonitis, porcine reproductive and respiratory syndrome (PRRS) and Aleutian disease of mink (Halstead et al., 2010). Enhancement of Zika virus infection in the FcγR positive K562 cells was shown to be enhanced in the presence of DENV specific antibodies (Castanha et al., 2017). This effect was seen in $STAT2^{-/-}$ mouse model, where sera from dengue and West Nile positive patients enhanced Zika virus infection and diseases in FcγR dependent manner (Bardina et al., 2017). Studies done using primary macrophages revealed that Zika virus infection resulted in the downregulation of IFNβ and reactive nitrogen intermediates (Hueston et al., 2017). Large scale epidemiological investigations to closely monitor the clinical relevance of such findings in important. For instance, immune correlates of severe dengue was matched with data from a long term study on Nicaraguan children and it was shown that intermediate levels of dengue antibodies lead to exacerbation of disease compared to low (non-protective) or high (protective) levels of antibodies (Katzelnick et al., 2017). Such studies could also help gain better insights into dengue immunity and rationale of vaccine designs (Waggoner et al., 2016). The modulation of Dengue/Zika pathogenicity by DENV antibodies and the prospects of using therapeutic antibodies for protection against both these diseases has been reviewed elsewhere (Khandia et al., 2018).

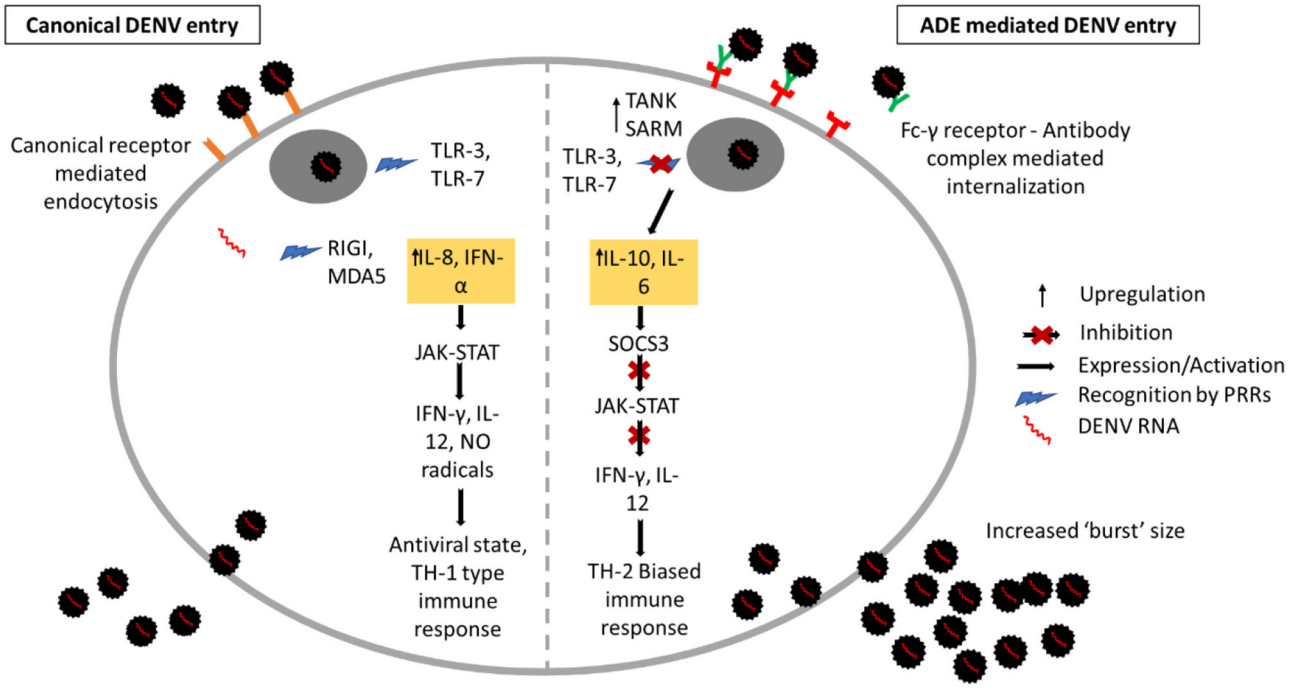

FIGURE 1 | Innate immune response during ADE and non-ADE dengue infection. Canonical non-ADE mediated entry occurs via receptor-mediated endocytosis. Upon entry, the DENV particles are internalized in endosomes and are recognized by the pathogen recognition receptors TLR-3 and 7. Release of viral RNA from endosomes is recognized by RIGI and MDA5 which triggers production of pro-inflammatory cytokines IFN-γ and IL-8. This activates the JAK/STAT pathway resulting in expression of IFN-γ, IL-12, and Nitric Oxide radicals. Virus entry via FcγR-antibody in dengue-ADE caused expression of TANK and SARM which inhibits TLR signaling. Production of anti-inflammatory cytokines IL-10 and IL-6 ensues and expression of SOCS3 as a result inhibits JAK/STAT pathway. This results in inhibition of pro-inflammatory cytokine production and causing a TH-2 biased immune response and increased "burst" size.

More recently, a study employing the use of pseudotyped virus particles has reported that SARS Corona virus entry in permissive cells is enhanced in the presence of neutralizing monoclonal antibodies (MAbs) against Middle East Respiratory Syndrome (MERS) coronavirus spike. Binding of these MAbs with virus particles functionally mimics the virus receptor and mediates entry by causing conformational changes that make the spike protein more prone to undergo proteolytic activation (Wan et al., 2020). With respect to the current COVID-19 pandemic caused by SARS CoV-2 virus (Mitchell, 2020), it is imperative that careful testing and validation of prospective vaccines and MAb-based therapies be done to avoid adverse consequences as a result of coronavirus ADE (Wang and Zand, 2020). A summary of the different animal and/or cell models used, and the specific phenotypes studied in different publications is given in **Table 1**.

CONCLUSIONS AND PERSPECTIVES

Gaining better insights at the genomic level is imperative to truly understand the intrinsic manipulation of host immune responses during dengue ADE. This may be achieved with the use of Genome wide CRISPR/cas9 screens in myeloid cell lines. Generation of such genome wide CRISPR/Cas9 knockout screening libraries can be used to elucidate host intrinsic factors that are indispensable for the modulation of host immune system during dengue infections. Results from such experiments can then be compared with already available data to identify targets for antiviral therapy.

In addition, more focus needs to be addressed toward the use of primary myeloid cells isolated from dengue infected and naïve donors. Host response to dengue ADE can vary depending on the type of cells being studied (Boonnak et al., 2011) and although studies using dendritic cells (Flipse et al., 2016b) and monocytes (Sun et al., 2011) have been reported, it is imperative that DENV research done using lab adapted continuous cell lines are compared with results from primary cells as well in order to get a detailed picture.

Studies on the transcriptional downregulation of antiviral genes associated with intrinsic ADE can be supplemented with proteomics studies to gain more insights into the virus-host interactions during severe dengue fever. Cell-targeting drugs against effectors of intrinsic ADE could be used as a prophylactic treatment option for both adults and infants suffering from severe dengue fever. The application of live imaging studies for the elucidation of non-canonical route of DENV cell entry and early events occurring therein, can aid in the identification of targets for both antiviral and for cell-targeting drugs.

The main theme of this mini review is the intrinsic immune evasion mechanisms of DENV infection in humans, leading

TABLE 1 | Summary of the animal models and phenotypes studied in select publications cited in this manuscript.

	Model used for study	Virus and phenotype studied	References
1	Mice–Bone marrow derived macrophages, STAT1$^{-/-}$ mice	DENV serotypes 1,2,3, and 4 replication kinetics, secretion of pro/anti-inflammatory cytokines	Chen et al., 2008
2	Primary human macrophages	DENV2 infection kinetics, virus-macrophage fusion potential, gene profiles, and IFN signature	Chan et al., 2014; Flipse et al., 2016a
3	Human primary monocytes	Entry of fluorescent labeled DENV2 particles in monocytes, transcriptome analysis	Chan et al., 2019
4	THP-1 cell line	DENV2 infectivity, pro/anti-inflammatory cytokine production, NO radicals synthesis	Chareonsirisuthigul et al., 2007
5	K562 cell line	Zika virus PE/243 and DENV-2 16681 strains, ADE of Zika infection	Castanha et al., 2017
6	K562 cell line, Stat2$^{-/-}$ C57BL/6 mice	Zika virus infection and pathogenicity in mice; ADE, neutralization *in vitro*	Bardina et al., 2017
7	Primary human macrophages	ZIKV MR766 strain ADE mediated cytokine production, reactive nitrogen intermediates	Hueston et al., 2017

to severe dengue pathogenicity. On that account, we have summarized the relevant literature highlighting the inherent differences between extrinsic and intrinsic ADE and suggest key points for further research.

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REVIEW
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Antibody-Dependent Enhancement: A Challenge for Developing a Safe Dengue Vaccine

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In 2019, the United States Food and Drug Administration accorded restricted approval to Sanofi Pasteur's Dengvaxia, a live attenuated vaccine (LAV) for dengue fever, a mosquito-borne viral disease, caused by four antigenically distinct dengue virus serotypes (DENV 1-4). The reason for this limited approval is the concern that this vaccine sensitized some of the dengue-naïve recipients to severe dengue fever. Recent knowledge about the nature of the immune response elicited by DENV viruses suggests that all LAVs have inherent capacity to predominantly elicit antibodies (Abs) against the pre-membrane (prM) and fusion loop epitope (FLE) of DENV. These antibodies are generally cross-reactive among DENV serotypes carrying a higher risk of promoting Antibody-Dependent Enhancement (ADE). ADE is a phenomenon in which suboptimal neutralizing or non-neutralizing cross-reactive antibodies bind to virus and facilitate Fcγ receptor mediated enhanced entry into host cells, followed by its replication, and thus increasing the cellular viral load. On the other hand, antibody responses directed against the host-cell receptor binding domain of DENV envelope domain-III (EDIII), exhibit a higher degree of type-specificity with lower potential of ADE. The challenges associated with whole DENV-based vaccine strategies necessitate re-focusing our attention toward the designed dengue vaccine candidates, capable of inducing predominantly type-specific immune responses. If the designed vaccines elicited predominantly EDIII-directed serotype specific antibodies in the absence of prM and FLE antibodies, this could avoid the ADE phenomenon largely associated with the prM and FLE antibodies. The generation of type-specific antibodies to each of the four DENV serotypes by the designed vaccines could avoid the immune evasion mechanisms of DENVs. For the enhanced vaccine safety, all dengue vaccine candidates should be assessed for the extent of type-specific (minimal ADE) vs. cross-reactive (ADE promoting) neutralizing antibodies. The type-specific EDIII antibodies may be more directly related to protection from disease in the absence of ADE promoted by the cross-reactive antibodies.

Keywords: dengue, dengue virus (DENV), dengue vaccine, live attenuated vaccine, Dengvaxia, antibody-dependent enhancement (ADE), virus-like particle

INTRODUCTION

Daily more than a million people are infected with any of the four distinct serotypes of dengue viruses (DENV-1, −2, −3, and, −4). The world needs a dengue vaccine for all age groups, regardless of whether they may, or may not have been previously exposed to one of the four dengue viruses. In 2019, Sanofi Pasteur's Dengvaxia, a live attenuated tetravalent dengue vaccine, which does not give complete protection, after a three-dose regimen spread over 1 year (Arredondo-García et al., 2018; Thomas and Yoon, 2019), was granted a limited approval by the United States Food and Drug Administration. This approval was for its use in 9–16 year-old children with laboratory-confirmed previous dengue infection, living in dengue-endemic areas (United States Food & Drug, 2019). Efficacy trials of Dengvaxia in several dengue-endemic countries of Asia and Latin America, in ∼35,000 2–16 year-old children (Sabchareon et al., 2012; Capeding et al., 2014; Villar et al., 2015), showed that vaccine efficacy (VE) i.e., the capacity to prevent symptomatic virologically confirmed dengue (VCD), varied by serotype, and was the lowest against DENV-2. Overall VE was 65.6% (95% CI 60.7–69.9) in 9–16 year olds, while in 2–8 year olds it was 44.6% (95% CI 31.6–55), at 2 years following administration of the first dose of the vaccine. At 3 years post-dose 1, vaccinated children in the 2–5 year age group, were found to be nearly 8 times likely to be hospitalized for severe dengue, compared to children in the placebo group (Hadinegoro et al., 2015). VE was subsequently found to be related to the pre-vaccination serostatus of the trial subjects. While VE against VCD at 2 years post-dose 1 was 76% (95% CI 64–84) in >9 year-old children, who had been exposed to dengue infection before vaccination (seropositive), it was only 39% (95% CI−1 to 63) in children who were dengue-naïve (seronegative) at the beginning of the trial (Sridhar et al., 2018). Long-term follow-up studies until 5 years reveal that in seronegative recipients, there is increased risk of severe dengue from the 3rd year onwards, post-dose 1. Clearly, developing a safe and efficacious dengue vaccine constitutes quite a formidable challenge. Several unique factors, associated with the biology, and pathogenesis of dengue, taken together with lessons of the Dengvaxia experience, necessitates exploring alternate dengue vaccine development options. There are additional whole virus-based dengue vaccines in advance stages of clinical trials (Clinicaltrials.gov, 2020; Tricou et al., 2020). Moreover, a few recombinant dengue vaccine candidates are also at various stages of development (Vannice et al., 2015; Swaminathan and Khanna, 2019; Deng et al., 2020). It is great that the pipeline of dengue vaccines continue to increase. A safe and effective dengue vaccine could soon become a reality.

DENGUE: THE VIRUS AND THE DISEASE

DENVs contain a positive sense RNA genome within a glycoprotein shell and are members of the Flaviviridae family, which includes other human pathogenic viruses such as yellow fever virus (YFV), Japanese encephalitis virus (JEV), and Zika virus (ZIKV) (Pierson and Diamond, 2013; Poland et al., 2018). The DENV genome, which is similar in organization to that of the other flaviviruses, encodes ten viral proteins, three of which are structural: the capsid (C), envelope (E), and membrane (M) and the remaining non-structural (NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Two of the structural proteins, the envelope (E) and the pre-membrane (prM) proteins, form the glycoprotein shell of the virus. The E protein is organized into three discrete domains, envelope domain I (EDI), EDII, and EDIII (Modis et al., 2003). The EDI participates in the confirmation changes required for virus entry. The EDII has a fusion loop (FL) which is required for fusion with the host membrane, as a prelude to release of the DENV RNA into the cytosol of the infected cell, and EDIII is believed to be responsible for interaction with the host-receptor molecule (Crill and Roehrig, 2001; Hung et al., 2004; Huerta et al., 2008; Hidari and Suzuki, 2011; Modis, 2014). The prM protein helps mask the FL of EDII to avoid premature fusion and release into cytosol during virus maturation within the infected cell. The immature virus is decorated with spikes of trimers of prM-E dimers. As a final step in virus maturation in the trans-Golgi network, prM is cleaved by host-encoded furin, leaving a peptide (pr) still covering the FL. Upon secretion to the outside of the infected cell, pr peptide dissociates from the virion, which is now fully mature and smooth (Screaton et al., 2015).

In most clinically apparent cases, DENVs cause a self-limiting febrile illness known as dengue fever. However, a small proportion of DENV infections cause severe dengue. This is a potentially fatal form of dengue disease, characterized by increased capillary permeability leading to plasma leakage and shock (Simmons et al., 2012). Severe dengue has often been associated with sequential infection with DENVs of different serotypes. It has been proposed that antibodies to a given DENV serotype induced during a first infection, bind to, but do not neutralize, a different DENV serotype, encountered during a subsequent infection (Tsai et al., 2015). In fact, the cross-reacting or neutralizing antibodies at suboptimal levels facilitate increased uptake of the non-neutralized DENV into monocytes and macrophages, considered to be the *in vivo* sites of DENV replication, via their Fcγ receptors. This phenomenon is termed antibody-dependent enhancement (ADE) (Halstead and O'Rourke, 1977; Dejnirattisai et al., 2010). The DENV-induced prM and fusion-loop Abs facilitate immature DENV entry through Fcγ receptor and mediate enhanced DENV uptake into cells, facilitating subsequent increased viral replication (Extrinsic ADE). The DENVs immune complexed with these antibodies upon entry via Fcγ receptor results in the suppression of intracellular cytokine signaling, causing a favorable environment for enhanced replication of DENVs (Intrinsic ADE). The Fcγ receptor-mediated DENV entry to host cells seems to be ten times more productive than the DENV entry through its host-cell receptor, thus increasing the DENV load (Halstead et al., 2010). On the contrary, the host cell receptor-mediated DENV entry induces PRR (Pattern recognition receptors) signaling, resulting in the suppression of DENV viral replication, thus controlling the DENV load (**Figure 1**).

There are several *in vitro* and *in vivo* studies that support ADE mediated enhanced disease outcome. Dengue virus-induced sera or monoclonal antibodies increase the DENV infection of Fcγ

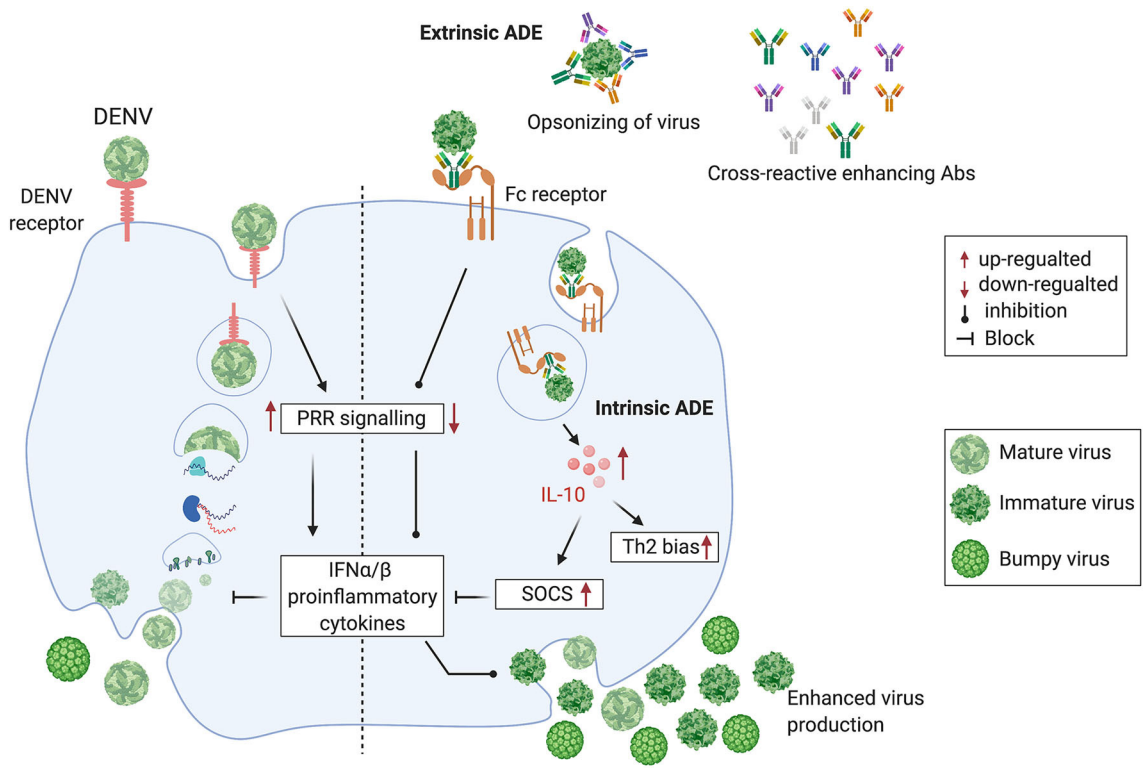

FIGURE 1 | Cross-reactive, prM and fusion-loop Abs facilitate immature DENV entry through Fcγ receptor and mediate enhanced DENV replication by following the intrinsic ADE pathway. **(Left half)** DENV attaches to a host cell surface and is endocytosed followed by virus-endosomal membrane fusion leading to the release of viral genome. Post-release, viral RNA is translated and the viral genome is replicated. Virus assembly occurs on the surface of the endoplasmic reticulum, and immature viral particles mature into their infectious form in the Golgi network. These mature viruses are then released from the cell and ready to infect other cells. **(Right half)** Cross-reactive Abs bind with immature non-infectious particles turning into infectious virus-Ab immune complexes (V-Ab IC) which then bind with the Fc receptor bearing cells. This assembly down-regulates the DENV-specific pattern recognition receptor (PRRs) signaling, inhibits type I interferon (IFNα/β) release and activates production of Interleukin-10 (IL-10) which causes up-regulation of Suppressor of Cytokine Signaling (SOCS) family. Henceforth, controlled mature DENV production is lost, resulting into manifold increase in wide-range of immature viruses which leads to the extrinsic-ADE pathway by infecting other cells via binding with cross-reactive Abs (Figure is adapted from Halstead et al., 2010 under Copyright license, # 4852311472038 and generated in Biorender.com).

receptor-bearing cells (Goncalvez et al., 2007). Non-human primates passively immunized with dengue virus antibodies promoted higher level of viremia as compared to dengue virus infection in the absence of antibodies (Muhammad Azami et al., 2020). A similar outcome was reported earlier using AG129 mouse model, where, passively transferred DENV-induced antibodies enhanced non-lethal DENV infection into a lethal infection, associated with vascular leakage and cytokine storm (Watanabe et al., 2015).

Apart from this, two longitudinal clinical studies from Thailand and Nicaragua (Katzelnick et al., 2017; Salje et al., 2018) evaluated the risk of severe dengue disease following primary and secondary infection. These clinical studies provide strong evidence that highest risk of severe dengue is associated with low levels of pre-existing dengue antibodies. These studies have revealed that low level pre-existing antibody levels are correlated with an increased likelihood of severe dengue disease only during secondary heterotypic infections (Katzelnick et al., 2017). However, ADE is not observed during secondary homotypic infection. This is because highly neutralizing type-specific Abs elicited during primary infection can neutralize a secondary homotypic infection even at low Ab concentrations, preventing the incidence of ADE (Ripoll et al., 2019). In homotypic infection, neutralization is determined by type-specific antibodies and ADE is limited to a very low level of type-specific antibody concentration. During heterotypic infection both type-specific and cross-reactive antibodies contribute to virus neutralization, but at a lower efficiency. Molecular simulations have shown that rough form of the virus become particularly pathogenic in case of heterotypic infection, as the neutralization is determined by cross-reactive Abs (Ripoll et al., 2019).

GENETIC DIVERSITY

There are four antigenically distinct serotypes of DENVs, differing in amino acid (aa) identity of their E proteins by ∼40%. Each of these four DENV serotypes can cause dengue disease ranging from mild to severe manifestations (Simmons et al., 2012). Within each serotype there are several genotypes, with genomic sequences differing by as much as 6% (Rico-Hesse,

2003). Further, when DENVs replicate within a host, the error-prone viral RNA replication machinery, generates an array of genetically related, yet distinct genomic variants, giving rise to intrahost diversity (Parameswaran et al., 2012, 2017).

MORPHOLOGICAL DIVERSITY

It has become increasingly apparent that the DENV maturation process is far from complete as the virions exocytosed from the infected cell display a high degree of heterogeneity. This appears to be the result of incomplete prM cleavage, resulting in an entire spectrum of virion particles ranging from fully mature "smooth" virions (100% prM cleavage) to fully immature, "spiky" virions (0% prM cleavage). Partially mature virions contain varying proportions of "smooth" and "spiky" surfaces (Junjhon et al., 2008, 2010). Further diversity stems from the structural flexibility of the E proteins on DENVs, termed as "breathing," which makes the virion structurally dynamic, with profound effects on epitope exposure. This is inferred from time- and temperature-dependent accessibility of certain virion epitopes to antibodies (Dowd et al., 2014; Kuhn et al., 2015).

ANTIBODIES INDUCED BY DENVs

The immune responses to DENV infections are mainly targeted against structural proteins i.e., E and prM and one non-structural protein NS1 (Rey et al., 2018). The immune response against NS1 is prone to cross-react among all the DENV serotypes. Studies report that NS1 directly triggers vascular hyperpermeability by inducing pro-inflammatory vasoactive response via activation of Toll like receptor−4 (Modhiran et al., 2015) and disruption of endothelial glycocalyx (Puerta-Guardo et al., 2016; Slon-Campos et al., 2019). Apart from above, studies by (Chuang et al., 2014, 2016) reported the proposed participatory role of NS1 in severe DENV pathogenesis by promoting bleeding diathesis through inhibition of thrombin activity and enhancement of fibrinolysis. However, complete contrary results are also reported, where, mouse raised polyclonal NS1 antiserum or anti-NS1 mAbs protects mice from the lethal dose of DENV-2 and also reduces the vascular leakage (Beatty et al., 2015). Thus, further studies are required to delineate the role of NS1 associated outcomes during dengue virus infection. The immune response against the DENV structural proteins are summarized in **Table 1**. Natural DENV infections elicit both protective as well as pathogenic antibodies (Dejnirattisai et al., 2010; Chan et al., 2011). The pathogenic antibodies, which facilitate entry of non-neutralized and immature DENV into monocytes and macrophages, are essentially disease-spreading (DENV ADE promoting) antibodies. Investigations have revealed that prM and the FL epitope (FLE) are particularly immunodominant and elicit cross-reactive, disease-spreading antibodies (Beltramello et al., 2010; Slon Campos et al., 2018). An analysis of the memory B cell responses in DENV-infected individuals showed that ∼60% of the human antibody response is directed toward the prM protein (Dejnirattisai et al., 2010). These are highly cross-reactive antibodies, which are capable of recognizing prM of all four DENV serotypes. Further, anti-prM-antibodies are poor neutralizers of DENV infectivity, but potent promoters of ADE (Beltramello et al., 2010; Dejnirattisai et al., 2010; Smith et al., 2012). These antibodies can actually make non-infectious immature virions infectious, by opsonizing them and facilitating their intracellular entry via the Fcγ receptor pathway. Once within the cell, these immature virions can undergo maturation and become inherently infectious, and spread to other cells. Likewise the anti-FLE antibodies (accounting for 20–30% of the antibody response to DENV), are also cross-reactive and tend to be weak neutralizers but strong promoters of ADE (Beltramello et al., 2010; Smith et al., 2012, 2014). The FLE, which is conserved among flaviviruses, is normally buried in the mature virion, but becomes accessible upon virus breathing (Cockburn et al., 2012; Pierson and Kuhn, 2012; Fibriansah et al., 2013). In the presence of prM and FLE antibodies, the partially immature and immature DENVs become fully infectious and increase the cellular viral load due to ADE (Halstead et al., 2010; Rodenhuis-Zybert et al., 2010) (**Figure 1**).

Protective antibodies appear to be elicited by non-immunodominant epitopes, and are either type-specific or pan-DENV specific. The type-specific antibodies against DENVs are predominantly directed against EDIII (Crill and Roehrig, 2001), which is implicated in host cell receptor recognition and viral entry (Hung et al., 2004; Chin et al., 2007; Gromowski et al., 2008; Modis, 2014). Anti-EDIII antibodies constitute only 5–10% of the total immune responses in DENV infected individuals (**Table 1**) (Wahala et al., 2009). However, EDIII antibodies possess the highest DENV neutralizing capacity with minimal or no disease enhancing potential, when tested using *in vivo* dengue sensitive mouse models (Watanabe et al., 2015; Ramasamy et al., 2018).

Studies from the National University of Singapore demonstrate that the fully or partially neutralized immune complexes (ICs) of a mouse adapted DENV-2 strain (D2 S221) formed with DENV cross-reactive monoclonal antibody (mAb) against FLE (4G2) cause lethal disease in dengue sensitive AG129 mice (Watanabe et al., 2015). The high mortality was accompanied by intestinal pathology, vascular leak, increased cytokine storm, and small intestinal tissue virus load. However, fully or partially neutralized ICs of DENV-2 with type-specific EDIII mAb (3H5) showed protection and no disease enhancement under similar conditions (Watanabe et al., 2015) (**Figure 2**). This observation may explain the failure of Sanofi's vaccine to provide protection against DENV-2 in *in-vitro* assays despite exhibiting DENV-2 neutralizing activity.

Moreover, an anti-EDIII monoclonal antibody has been shown to be capable of protecting humanized mice from all symptoms associated with severe dengue (Robinson et al., 2015). Several potently protective mAbs isolated from DENV-infected individuals have been characterized. These mAbs recognize conformational epitopes, found only when E is displayed on the virion surface, and are known as quaternary epitopes. Many of these are serotype-specific (Teoh et al., 2012; Fibriansah et al., 2014, 2015a,b; Screaton et al., 2015). In addition, broadly neutralizing mAbs, which target conserved quaternary epitopes among the four DENV serotypes, have

TABLE 1 | Immune responses to DENV infections against the DENV structural proteins.

Immunogenic DENV structural Antigens	Elicited human immune response	Antibody response (%)	Reference
Membrane protein (prM)	Cross-reactive Non-neutralizing, disease enhancing antibodies, that enhance the ability of non-infective immature DENV to become infectious (ADE)	~60	Beltramello et al., 2010; Dejnirattisai et al., 2010; Smith et al., 2012
Envelope protein fusion loop epitope (FLE)	Cross-reactive neutralizing antibodies. These antibodies provide transient protection against the heterotypic DENVs, but seem to later (after 2–3 months) enhance replication of heterotypic DENVs (ADE)	20–30	Beltramello et al., 2010; Smith et al., 2012, 2014
DENV Envelope- dimer epitope (EDE)	EDE Ab cross-neutralizes all four DENV serotypes as well as ZIKV with low ADE potential	0–70[#]	Dejnirattisai et al., 2015; Barba-Spaeth et al., 2016; Slon-Campos et al., 2019
DENV Envelope host cell receptor binding domain (EDIII)	Type-specific highly potent DENV neutralizing antibodies, which seem to provide life-long homotypic protection with low ADE	5–10	Wahala et al., 2009; Chen et al., 2016

[#] *E-dimer epitope antibodies are highly variable in convalescent DENV plasma.*

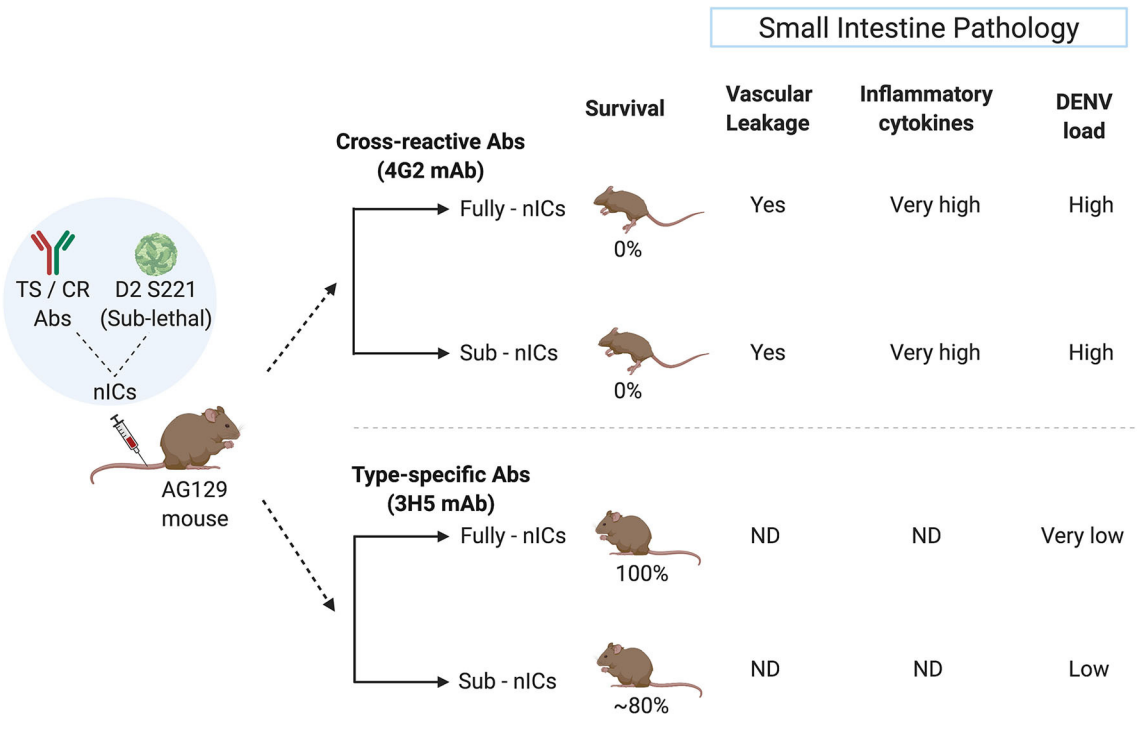

FIGURE 2 | Type-specific (TS) monoclonal antibody (mAb) does not cause ADE in the mouse model whereas cross-reactive (CR) mAb does. The sublethal dose of D2 S221 was inoculated as fully- and sub-neutralized immune complexes ("Fully-nICs" and "Sub-nICs," respectively) made with either cross-reactive (4G2) or type-specific (3H5) mAbs. Investigators (Watanabe et al., 2015) found 100% mortality and elevated levels of different ADE related parameters in the small intestine (Vascular leakage, inflammatory cytokines, and viral load) in both fully and sub-neutralized ICs made with 4G2. On the contrary, fully neutralized TS-ICs exhibited full protection accompanied by very low virus load. TS sub-neutralizing ICs showed a very minimal level of mortality accompanied by low virus load. ND, data not available (The illustrative figure created with Biorender.com).

been identified recently (Dejnirattisai et al., 2015; Rouvinski et al., 2015). However, mimicking quaternary epitopes for a dengue vaccine candidate has been highly challenging. Recently, investigators have successfully displayed DENV neutralizing quaternary structures as covalently stabilized Envelope Dimer Epitopes (EDE) (Rouvinski et al., 2017; Thomas et al., 2020). Further studies show EDE antibodies to be dominant toward the infecting serotype with lower avidity against the other serotypes (Thomas et al., 2020) Interestingly, these EDE antibodies or EDE-Mab were able to cross-neutralize ZIKV infection *in vitro* and showed protection in a lethal mouse model (Swanstrom et al., 2016; Fernandez et al., 2017).

DENV CELLULAR IMMUNE RESPONSES

Several excellent publications on cellular immune responses during DENV natural infection are available (Mathew and Rothman, 2008; Yauch et al., 2009; Zellweger et al., 2015; Elong Ngono et al., 2016; St. John and Rathore, 2019; Tian et al., 2019). Nonetheless, precise nature of a protective T cell response in the context of DENV infection and vaccination is yet to be delineated. Like the antibody response, T cell responses also are implicated both in protection as well as pathogenesis (Beaumier et al., 2008; Friberg et al., 2011; Weiskopf et al., 2013). However, unlike in the case of antibodies for which there is greater clarity on the nature of epitopes that elicit protective vs. pathogenic antibodies, similar information on T cell epitopes is not available. One of the key hypothesis to explain the sub-optimal performance of the Dengvaxia is that it had YFV specific T-cell epitopes and did not contain the DENV specific T-cell epitopes. Nonetheless, a few publications (Simmons et al., 2005; Weiskopf et al., 2013, 2015) indicate that YFV and DENV proteomes do carry several highly conserved cross-reactive T-cell epitopes in the NS3 and NS5 regions. Similar to the B-cell cross-reactive epitopes, the Dengvaxia and the other whole virus-based dengue vaccines do carry some level of cross-reactive T-cell epitopes. Thus, the sub-optimal performance of Dengvaxia cannot be solely attributed to the absence of DENV specific T-cell epitopes. Additional research is required to shed more light on the specific features of both arms of the adaptive immune system in the context of their opposing roles in protection and pathogenesis.

VIRAL INTERFERENCE

The success of the yellow fever LAV, based on the attenuated YFV variant YF17D (Monath, 2005; SAGE Working Group, 2013), provided the paradigm for a dengue LAV. Due to the ADE phenomenon, the dengue LAV needs to be tetravalent, so that it may provide immunity against all four DENV serotypes. However, mixing four monovalent dengue LAVs into a tetravalent formulation is associated with one component replicating better at the expense of the others, leading to a phenomenon known as viral interference (Dittmar et al., 1982; Edelman, 2011). Intuitively, one may posit a role for the dynamics of intra-host microevolution, referred to above, in viral interference. However, this remains to be experimentally ascertained.

Viral interference was historically noticed by Thai (Kanesa-Thasan et al., 2001; Sabchareon et al., 2002) and US Army (Edelman et al., 2003) researchers during initial attempts at developing tetravalent dengue LAVs. The consequence of such interference is that the immune response tends to be skewed toward one DENV serotype. This can lead to the situation wherein, though the LAV is a physically tetravalent mixture, it is essentially immunologically monovalent. The resulting partial protection can prime one to ADE in future, upon natural DENV exposure. In fact, the observation that Dengvaxia predisposed seronegative recipients to increased risk of severe dengue, starting at 3 years after the first dose (Hadinegoro et al., 2015), lends support to the occurrence of ADE (Halstead, 2017). The subsequent finding that Dengvaxia

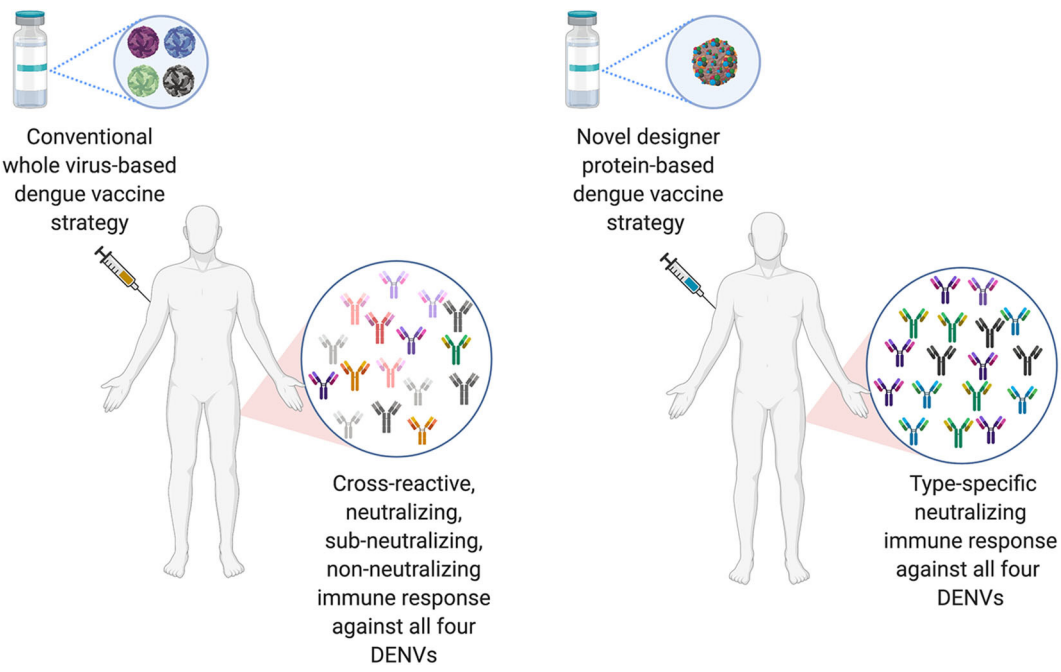

FIGURE 3 | Comparison of conventional DENV vaccine strategy vs. Designer DENV vaccine candidate immune responses. Conventional DENV vaccine generally have the mixture of live-attenuated all four DENV serotypes, whereas, ongoing pre-clinical designer DENV vaccine candidate (s) has single entity expressing all four DENV component. The whole DENV-based vaccine inherently poses to induce imbalance immune responses with high load of cross-reactive Abs against all four DENV serotype, suggests to explore designer DENV vaccine strategy, which may induce non-enhancing type-specific neutralizing immune response in human (Figure designed with Biorender.com).

elicited antibodies predominantly specific to DENV-4 (Henein et al., 2017), is consistent with the vaccine having simulated a primary monotypic infection. It is to be expected that, like the natural DENVs, Dengvaxia, being a viral vaccine encoding prM and E, would also elicit predominantly prM- and FLE-specific antibodies, which are efficient ADE promoters. This would undoubtedly be true for other whole virus-based vaccines as well. This makes ADE evaluation a mandatory step during pre-clinical vaccine development.

Most live attenuated, killed or chimeric whole dengue virus-based vaccine candidates will mimic natural DENV infections and thus will elicit predominantly cross-reactive disease enhancing antibodies with limited type-specific protective antibodies. Therefore, there is a need for designing a dengue vaccine candidate that elicits predominantly protective antibodies (like anti-EDIII) in the absence of pathogenic antibodies (anti-prM and anti-FLE) (Lam et al., 2016; Screaton and Mongkolsapaya, 2018).

The ideal dengue vaccine should be tetravalent and generate long lasting, type-specific neutralizing antibodies against all the four-dengue virus serotypes. Therefore, EDIII seems like an ideal target for dengue vaccine development, as anti-EDIII antibodies have higher type-specific neutralization capacity with lower ADE potential (Ramasamy et al., 2018). Comparative immune responses of novel designer protein-based DENV vaccine strategy over the conventional whole virus-based DENV vaccines are highlighted in **Figure 3**.

A recent study on molecular simulations of dengue virus infection and experimental data suggest that the interplay between epitope accessibility, Ab specificity, Ab affinity, Ab concentration, and mature content of the virus significantly influence the degree of ADE (Ripoll et al., 2019). Since both Ab concentration and type specificity are critical host determinants of ADE, it is important to quantify not only the neutralizing antibody titer but also fine specificity (type-specific vs. cross-reactive) when assessing future dengue vaccine candidates. For the safety and efficacy of a vaccine, it is vital that the vaccine immunogen should be well-characterized However, the maturation states of dengue serotypes in a whole virus-based dengue may be difficult to control. Various maturation states of DENV serotypes may affect its ability to provoke protective Ab responses without creating conditions that increase the risk of severe dengue disease (Ripoll et al., 2019). Vaccine candidates capable of eliciting predominantly type-specific immune responses in the absence of FL and prM directed antibodies could exhibit higher virus neutralization with lower ADE potential (Ramasamy et al., 2018).

ADE: *IN VITRO* VS. *IN VIVO* ASSAYS

Often DENV infection-enhancing activity of antibodies is evaluated *in vitro* using cell lines expressing Fcγ receptors, such as THP-1, U937, or K562. These cell lines differ in their dengue-susceptibility, due to differences in the types and levels of Fcγ receptors expressed by them. To measure ADE, immune complexes (ICs), generated by pre-incubating DENV with the antibody, are added to these cells in culture, followed by measuring the amount of cellular DENV uptake. However, due to the diversity of these cell lines, the *in vitro* ADE assay is unlikely to reflect the true *in vivo* situation (Krol et al., 2019).

In this regard, ADE assays, based on dengue-sensitive mouse models, offer a closer approximation of the *in vivo* situation. The *in vivo* ADE assay is based on mice with genetic defects in the innate immune signaling pathway. Typically, an antibody whose ADE potential is to be evaluated, is either introduced into the mouse by passive transfer, followed by challenge with a sub-lethal dose of a mouse adapted DENV strain or pre-incubated with the mouse-adapted DENV strain to generate ICs, which are then injected into the mouse, followed by survival monitoring, as well as analysis of vascular leakage and cytokine production (Watanabe et al., 2015; Shukla et al., 2020).

The *in vivo* (mouse model) and *in vitro* (Fcγ receptor-bearing cell lines) ADE assays score differently when evaluated

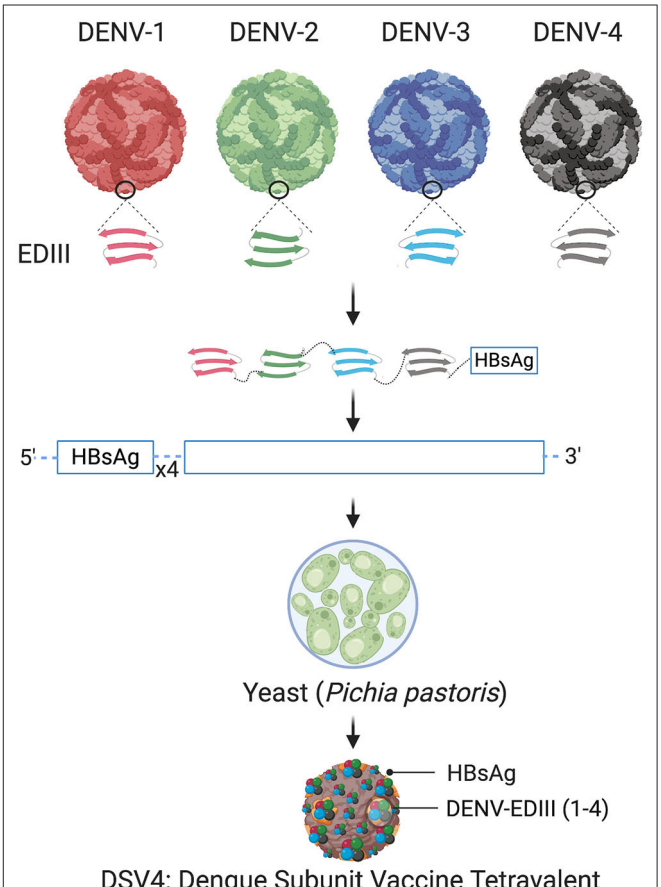

FIGURE 4 | Design of DSV4. Top panel shows schematic diagram of all four DENV, encircled parts represent envelope domain III (EDIII) component of all four viruses. These EDIIIs components were linked with hexa-glycine linkers, fused to n-terminus of Hepatitis-B surface antigen (HBsAg) and further cloned in the background of four copies of HBsAg. The cloned expression cassette including DENV-EDIIIs, was integrated into the yeast expression host, *Pichia pastoris*, and DSV4 antigen was purified from the recombinant host for further immunological studies. The figure approach is adapted from Ramasamy et al. (2018) and created with Biorender.com.

with cross-reactive vs. the type-specific DENV mAbs. This was evidenced in a recent study using the interferon α/β and γ receptor double knock-out AG129 mouse model (Watanabe et al., 2015). Highly neutralized ICs made by pre-incubating the pan-DENV cross-reactive FL-specific mAb 4G2 or EDIII DENV-2 type-specific mAb 3H5 and a mouse-adapted DENV-2 strain, did not manifest ADE in a THP-1 cell line-based *in vitro* ADE assay. However, unlike the ICs made with 3H5 mAb, the ICs generated with 4G2 mAb revealed potent lethality in the dengue-sensitive AG129 mouse, without enhancing serum viremia. This finding is further affirmed with a recent report published in EBioMedicine by Shukla et al. (2020). The investigators showed that both Dengue and Zika virus infections are enhanced by live attenuated dengue vaccine that majorly elicits FL and prM cross-reactive antibodies but not by a recombinant tetravalent Dengue Subunit Vaccine candidate, capable of inducing predominantly EDIII directed type-specific antibodies in the absence of antibodies to FL and prM epitopes in murine models. This study provides the first "head-to-head" experimental comparison of *in vivo* ADE potential between an approved yellow fever virus-based recombinant dengue vaccine "Dengvaxia" and a designed EDIII-based Dengue Subunit Vaccine Tetravalent (DSV4) candidate. In this study, investigators also included an "in-house" version of the whole DENV-based surrogate vaccine candidate by utilizing a physical mixture of tetravalent DENVs (TV DENV). The study revealed that antibodies elicited by Dengvaxia and TV DENV in BALB/c mice were predominantly cross-reactive and failed to offer protection against lethal DENV challenge in AG129 mouse model. Moreover, the completely neutralized immune complexes of DENV-2 made with the virus-based dengue vaccine sera promoted ADE of DENV-2 infection and caused mortality despite virus neutralization. On the other hand, DSV4-induced predominantly type-specific mouse antibodies not only provided significant protection against the lethal DENV-2 challenge but also did not promote ADE of DENV infection when evaluated in the AG129 mouse model. Anti-Dengvaxia and anti-TV DENV antibodies were directly associated in the elevation of intestinal pro-inflammatory cytokines (TNF-α & IL-6) production as well as viral load which leads to intestinal vascular leakage and ultimately death of mice. The study provides crucial insight that type-specific neutralizing Abs are vital for protection without ADE. Thus, testing *in vivo* ADE potential of neutralized DENV ICs in a small animal model offers a superior strategy to de-risk experimental dengue vaccine candidates during pre-clinical development.

DENV/ZIKV INTERACTION AND ADE

A key challenge in dengue vaccine development stems from the interaction between DENV and Zika virus (ZIKV), another human flaviviral pathogen transmitted by the same mosquito vector (Musso et al., 2015). More recently, concerns that DENV antibodies could enhance infection of ZIKV have been raised because: (1) ZIKV is phylogenetically related to DENV, (2) ZIKV outbreaks have occurred in DENV endemic regions around the world, and (3) DENV antibodies can enhance ZIKV infection both *in vitro* as well as *in vivo* in mice. Thus, the worry that a DENV vaccine could also enhance ZIKV disease in humans is a serious concern.

Studies show that the DENV-induced anti-FLE antibodies can interact with Fcγ receptors to mediate ZIKV uptake into susceptible cells. In fact, the recent ZIKV outbreaks associated with Guillain Barre syndrome in adults and microcephaly in infants, have occurred in regions of high DENV endemicity (Lessler et al., 2016; Culshaw et al., 2017), suggesting a role for ADE of ZIKV mediated by cross-reactive anti-DENV antibodies (Dejnirattisai et al., 2016; Bardina et al., 2017). This notion has received strong support from very recent work which shows that DENV-specific antibodies introduced into ZIKV-infected pregnant Stat2$^{-/-}$ mice significantly increased placental damage, fetal growth restriction, and fetal resorption (Brown et al., 2019). Interesting findings were reported using the murine sera obtained by immunization with Dengvaxia, Tetravalent mixture of 1-4 DENVs (TV-DENV) and the recombinant DSV4 immunogens. The Dengvaxia and TV-DENV-based surrogate vaccine candidate anti-sera cross-neutralized ZIKV *in vitro* and also induced ADE of ZIKV infection in adult Stat2$^{-/-}$

TABLE 2 | Comparison of designer dengue vaccine candidate over whole DENV-based vaccine or vaccine candidates.

Features	Whole DENV-based vaccine/candidate (s)	Designer dengue Vaccine Candidate	
		DSV4	E-dimer
Immunogen	Mix of 4 viruses	4-in-1 VLP	Quaternary epitope
Viral interference	Yes	Not applicable	Not applicable
Viral breathing	Yes	Not applicable	Not applicable
FLE,	Present	Absent	Buried
prM, NS1	Present	Absent	Absent
Type-specific Abs responses	Minimal	Maximum	Fair
In vivo DENV ADE	High	Absent	Low
In vivo ZIKV ADE	Present	Absent	Low
Expression host	Mammalian cells	Yeast	Transient mammalian cells

mice. Moreover, enhanced ZIKV infection were observed in several organs of Stat2$^{-/-}$ mice inoculated with anti-Dengvaxia and anti-TV DENV sera. However, DSV4-induced anti-serum neither cross-neutralized ZIKV *in vitro* nor promoted ADE *in vivo* (Shukla et al., 2020). The pre-immunity against ZIKV is also a serious concern for developing the dengue vaccine. Recent Nicaraguan prospective pediatric cohorts study suggested that prior ZIKV infection can enhance severe dengue disease in future (Katzelnick et al., 2020).

DESIGNER VACCINES

It is becoming increasingly evident that alternate dengue vaccine strategies need to be explored (de Silva and Harris, 2018; Rey et al., 2018; Screaton and Mongkolsapaya, 2018), given the formidable challenges that are intrinsically associated with whole virus-based vaccines. It is quite obvious that successful dengue vaccines must be designed to target potently neutralizing epitopes (EDIII and quaternary epitopes such as EDE) while avoiding pathogenic epitopes (prM and FLE). Based on the recent knowledge on dengue virus biology and immunology, several dengue experts are veering to the view that a safe and effective dengue vaccine can be designed using recombinant DNA technology. These findings suggest that a designed EDIII-based VLP platform and stabilized E-Dimer Epitope (EDE)-based dengue vaccine could provide the basis for a safe and effective vaccine candidate capable of eliciting predominantly type-specific antibodies.

Recently, investigators have successfully engineered a stabilized EDE as a novel subunit DENV vaccine candidate. This has been achieved by interfacing the two E-monomers to form a E-dimer and lock this E-dimer via covalent disulphide linkages. The stabilized EDE protein was recognized by Mabs specific to the DENV quaternary epitopes. Moreover, the FLE was unavailable on the surface of these stabilized EDE, thus avoiding a significant level of cross-reactive antibodies to this FLE that are elicited by immunizations with E-monomers. This EDE design resulted in a higher level of serotype-specific immune responses as compared to the E-monomers (Thomas et al., 2020). The DENV cross-reactive antibodies elicited by EDE exhibited potent neutralization of all four DENV serotypes due to the conserved region of EDE (Barba-Spaeth et al., 2016; Fernandez et al., 2017; Rouvinski et al., 2017; Abbink et 'al., 2018; Thomas et al., 2020). Studies show that anti-dengue EDE mAbs exhibit protection against ZIKV infection in pregnant and non-pregnant immunocompromised C57BL/6 mice (Fernandez et al., 2017).

A recently published pre-clinical results of a virus-like particle (VLP) vaccine candidate known as DSV4 (**D**engue **S**ubunit **V**accine **Tetra**valent) are very promising (Ramasamy et al., 2018). This candidate is based on EDIII. Unlike domains EDI and EDII, which elicit largely flavivirus cross-reactive and weakly-neutralizing or non-neutralizing antibodies, EDIII elicits potent serotype-specific virus-neutralizing antibodies. The "four-in-one," tetravalent vaccine candidate incorporates the EDIIIs of all four DENVs spliced together through flexible linkers in a single translational reading frame. Further, it is genetically fused with Hepatitis-B surface antigen (HBsAg) and co-expressed with four expression cassettes of HBsAg in order to display EDIIIs on the surface of HBsAg virus-like-particles (VLPs).

The schematic representation of DSV4 design is shown in **Figure 4**. DSV4 assembles into VLPs and displays critical DENV neutralizing epitopes of all 4 serotypes. It is immunogenic in mice and macaques with aluminum hydroxide as adjuvant. It elicits serotype-specific neutralizing antibodies against all four DENVs in mice. These antibodies exhibit breadth of neutralization against various genotypes of each serotype (Ramasamy et al., 2018). The lack of *in vivo* ADE in the recombinant DSV4 design is a crucial differentiator from the whole virus-based dengue vaccine strategies. **Table 2** compares the key features of DSV4 with other whole DENV-based vaccine strategies.

CONCLUSIONS

Antibodies elicited by DENVs play roles both in protection against, and pathogenesis of, dengue disease. Protective immunity is determined by the balance between these two opposing antibody roles. A safe and efficacious dengue vaccine must confer durable protection against all four DENV serotypes, without the risk of ADE. Further, it should be suitable for all age groups, irrespective of pre-vaccination serostatus. LAVs have been the focus of most efforts, with one partly protective vaccine already licensed, one which has completed Phase 3 trials and a third one due to complete efficacy trials soon. However, the unique features of DENVs and the predominantly ADE-prone nature of antibodies they elicit, coupled to the issues of viral interference rendering physically tetravalent LAVs, functionally monovalent, could continue to pose a challenge for a risk-free dengue vaccine. In this regard, recombinant subunit vaccine strategies that can facilitate selective retention of neutralizing epitopes, while eliminating ADE-associated epitopes, offer alternate promising options that must be explored. Subunit vaccines for preventing infections by other viruses such as hepatitis B, hepatitis E and human papilloma viruses have been licensed (Shukla et al., 2019). Learning from the Dengvaxia experience, it is very critical to assess the *in vivo* ADE potential of all vaccine candidates early on, using the available dengue-sensitive mouse ADE model. The safety and efficacy are inter-linked attributes and both must be evaluated.

AUTHOR CONTRIBUTIONS

All authors worked on the study conception and design, analyzed, and interpreted the data, read, and approved the submitted version.

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REVIEW
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Dysfunctional Innate Immune Responses and Severe Dengue

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Although infection with the dengue virus (DENV) causes severe dengue, it causes a mild self-limiting illness in the majority of individuals. There is emerging evidence that an aberrant immune response in the initial stages of infection lead to severe disease. Many inflammatory cytokines, chemokines, and lipid mediators are significantly higher in patients with severe dengue compared to those who develop mild infection, during febrile phase of illness. Monocytes, mast cells, and many other cells of the immune system, when infected with the DENV, especially in the presence of poorly neutralizing antibodies, leads to production of pro-inflammatory cytokines and inhibition of interferon signaling pathways. In addition, production of immunosuppressive cytokines such as IL-10 further leads to inhibition of cellular antiviral responses. This dysregulated and aberrant immune response leads to reduced clearance of the virus, and severe dengue by inducing a vascular leak and excessive inflammation due to high levels of inflammatory cytokines. Individuals with comorbid illnesses could be prone to more severe dengue due to low grade endotoxemia, gut microbial dysbiosis and an altered phenotype of innate immune cells. The immunosuppressive and inflammatory lipid mediators and altered phenotype of monocytes are likely to further act on T cells and B cells leading to an impaired adaptive immune response to the virus. Therefore, in order to identify therapeutic targets for treatment of dengue, it would be important to further characterize these mechanisms in order for early intervention. In this review, we discuss the differences in the innate immune responses in those who progress to develop severe dengue, compared to those with milder disease in order to understand the mechanisms that lead to severe dengue.

Keywords: dengue, innate immunity, monocytes, mast cells, antibody dependent enhancement, endotoxin, T cells, B cells

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INTRODUCTION

Dengue infections represent one of the most important vector-borne diseases in the world, resulting in significant morbidity and mortality. It is estimated than 105 to 390 million individuals are infected with one of the four dengue viruses (DENV) annually leading to 51 to 96 million apparent dengue infections (Bhatt et al., 2013; Cattarino et al., 2020). Although the infection is asymptomatic or mild in most infected individuals, it can lead to severe forms of disease such as dengue hemorrhagic fever (DHF) and death in some individuals (Cattarino et al., 2020). DHF is shown to occur in ∼23.2% of symptomatic individuals, and if untreated, is reported to have case fatality rates (CFRs) of ∼20% (Guo et al., 2017). Although CFRs are <1% in most countries due to intense monitoring of patients who develop dengue and meticulous fluid management, CFRs are reported to be around 2.6% in some countries (Guo et al., 2017; Murhekar et al., 2019).

Initial infection with a particular DENV serotype is known as a primary infection, while subsequent infection with any other serotype is known as a secondary dengue infection (WHO, 2011). Secondary dengue infection is thought to be one of the most important risk factors for development of severe disease, in part due to antibody dependent enhancement (ADE) in which poorly neutralizing, highly cross-reactive antibodies enhance infection in FcγR-expressing cells (Chareonsirisuthigul et al., 2007; Chau et al., 2008; Guzman et al., 2013; Syenina et al., 2015; Katzelnick et al., 2017). However, not all secondary dengue infections result in symptomatic infection or DHF and only 15% of secondary dengue infections progress to DHF (Wang et al., 2017). Subclinical dengue infection occurs in an equal proportion of those experiencing a primary or secondary dengue infections (Grange et al., 2014). Therefore, many host factors apart from the presence of poorly neutralizing cross-reactive antibodies could lead to protection or pathogenesis.

In those who develop a symptomatic illness, there can be sudden onset of fever along with headaches, myalgia and arthralgia. This initial febrile phase (early illness) typically lasts for 3 to 6 days and the patient recovers in most instances. However, ∼25% of individuals progress to develop complications around day 3 to 6 of illness, which is characterized by fluid leakage leading to pleural effusions, ascites, shock, organ dysfunction and if untreated, can lead to death (WHO, 2011; Malavige and Ogg, 2017). There is emerging evidence that an aberrant immune response in the initial stage of infection, results in endothelial dysfunction and cytokine storms leading to vascular leakage and thus DHF. In this review, we discuss the differences in the innate immune responses in those who progress to develop DHF compared to those with milder disease in order to understand the mechanisms that lead to severe dengue.

CYTOKINE AND CHEMOKINES SIGNATURES IN EARLY ILLNESS LEADING TO SEVERE ILLNESS

Many different chemokines and cytokines have been shown to be elevated in severe dengue such as IFN-γ, GM-CSF, IL-10, MIP-1β, IL-1β, IL-8 TNFα, IP-10, MCP-1, and IL-18 (Bozza et al., 2008; Malavige et al., 2013a; Fernando et al., 2016; Kamaladasa et al., 2016; van Wilgenburg et al., 2016; Patro et al., 2019). Certain cytokines such as TNFα and IL-1β have shown to directly cause vascular leak (Hottz et al., 2014; Kamaladasa et al., 2016), while both these cytokines along with IL-18, IP-10, IL-8, and MIP-1β are potent inflammatory cytokines produced by many immune cells (Tang et al., 2020). IL-10 on the other hand is a potent immunosuppressive cytokine, which has been associated with severe dengue (Malavige et al., 2013a,b). Due to the presence of high levels of these cytokines during the critical phase and due to the presence of cross reactive DENV-specific T cells during acute dengue, T cells were implicated as the main source of these cytokines and the drivers of the cytokine storm (Mongkolsapaya et al., 2006; Appanna et al., 2007; Dong et al., 2007). However, more recent data have shown that innate immune cells such as monocytes are one of the main sources of such cytokines and chemokines, while DENV-specific T cells are likely to be protective (Weiskopf et al., 2013, 2015; Singla et al., 2016; Wijeratne et al., 2018). Those who proceeded to develop severe disease had higher levels of many types of inflammatory markers such a C-reactive protein, increased inflammatory lipid mediators and cytokines in very early illness (initial period of the febrile phase), before they proceeded to develop severe disease or vascular leak (Fernando et al., 2016; Jeewandara et al., 2017; Vuong et al., 2020). For instance, CRP levels of >34 mg/L within the first 3 days of illness (before any patients had developed severe disease) was associated with an increased risk of progression to severe illness (Vuong et al., 2020). Similar findings were observed with an inflammatory lipid enzyme mediator sPLA2, where the enzyme activity was highest during early illness in those progressed to develop DHF (Jeewandara et al., 2017).

Although cytokines and chemokines have been extensively studied in in dengue, only a few have studied their changes during different clinical phases of dengue. Due to the dynamic changes in the clinical and laboratory features along with cytokines and chemokines during the febrile phase (before the onset of vascular leak), is likely to be different than those in the critical phase. A study carried out in a relatively small sample of patients showed that IP-10, MCP-1, and MIP-1β were significantly higher in those who progressed to develop plasma leakage, again during the febrile phase compared to the critical phase (Rathakrishnan et al., 2012). A more recent study, done in a large cohort of patients showed that while many cytokines are elevated in patients with dengue during the febrile phase, it was IL-10 that most significantly associated with subsequent development of DHF (Dayarathna et al., 2020, under review). Therefore, collectively these data suggest that the events that trigger the release of these inflammatory mediators occur very early in illness (initial period of the febrile phase), potentially due to the differences in the innate immune responses to the virus.

Several proteins of the DENV have been shown to inhibit IFN signaling pathways, while NS1, which is a secretory protein has shown to directly cause disease pathogenesis (Castillo Ramirez and Urcuqui-Inchima, 2015; Modhiran et al., 2015; Adikari et al., 2016; Puerta-Guardo et al., 2016; Kao et al., 2018). NS1 Ag, which exists in a hexametric form, has been shown to trigger cytokine release from PBMCs through binding to TLR4 and also to contribute directly to vascular leak by disruption of the endothelial glycocalyx (Modhiran et al., 2015; Glasner et al., 2017). NS1 is a major target of antibodies that develop during natural DENV infections (Dejnirattisai et al., 2010) and mice immunized with NS1 or given polyclonal sera of mice immunized with NS1 were shown to have significantly less vascular leak, when challenged with the DENV (Beatty et al., 2015). However some studies have shown that NS1-NS1 antibody complexes contribute to vascular leak by activation of complement and by causing endothelial apoptosis (Avirutnan et al., 2006). Studies in acute dengue have shown that NS1 antibody levels rise in those with severe dengue compared to those with milder forms of illness, during acute illness and that those with milder dengue appeared to recognize different epitopes than those with severe illness (Jayathilaka et al., 2018). The lower than expected efficacy of some dengue vaccines, have been attributed

to lack of generation of NS1 antibodies (Halstead, 2018, Nascimento et al., 2018).

The non-structural proteins NS2A and NS3 have been shown to inhibit type I interferon production by degrading the stimulator of interferon genes (STING) and IRF3, which in turn impairs RIG-I and TLR-3 signaling pathways (Castillo Ramirez and Urcuqui-Inchima, 2015). Both NS3, NS2A along with NS4A, NS4B, and NS5 also further inhibit interferon signaling by preventing STAT-1 phosphorylation (Castillo Ramirez and Urcuqui-Inchima, 2015; Kao et al., 2018). DENV strains which produce higher levels of subgenomic RNA and therefore are more capable of inhibiting TRIM-25 activation of RIG-I, have shown to have higher transmission rates and able to cause epidemics (Manokaran et al., 2015). Therefore, inhibition of IFN production appears to be an important mechanism of immune evasion by the DENV, which is associated with increased clinical disease severity and epidemiological fitness. However, inhibition of IFN signaling by the DENV does not explain the occurrence of severe disease in some individuals, and mild/asymptomatic infection in others, who are infected with the same strain of the DENV. It is possible that those who develop more severe illness have higher viral replication within the host cells due to increased viral entry into such cells due to ADE and/or other genetic or immunological factors which influence the type I IFN response, and therefore, more inhibition of IFN signaling pathways.

MONOCYTE/MACROPHAGE RESPONSES IN SEVERE DENGUE

Although the DENV is known to infect many different types of cells, it was shown that monocytes are the cells most commonly infected by the virus (Zanini et al., 2018). Single cell transcriptomics in PBMCs of patients during early illness, before they progressed to severe disease, showed that cells of those who subsequently had severe disease had a unique transcriptomic signature (Zanini et al., 2018). Of the gene signatures associated with progression to severe disease, *IFITI* and *CD163* expression in $CD14^{+}CD16^{+}$ monocytes and *MX2* in B lymphocytes had the highest predictive value, highlighting the important role of monocytes in disease pathogenesis (Zanini et al., 2018). Soluble CD163 which is a marker of macrophage activation syndrome has been previously shown to differentiate those who have severe dengue from DF (Ab-Rahman et al., 2016). Therefore, macrophage activation associated with various cytokines such as TNFα, IL-6, and IL-10 or by oxidative stress appears to be a feature of DHF.

Apart from direct infection by the DENV, monocyte infection is further facilitated in acute dengue, by ADE (Chareonsirisuthigul et al., 2007; Syenina et al., 2015; Katzelnick et al., 2017). Internalization of antigen-antibody complexes is known to further suppress antiviral responses (Ubol et al., 2010; Tsai et al., 2014; Wang et al., 2017). *In vitro* infection of monocytes with the DENV, in the presence of sub-neutralizing concentrations of DENV-specific antibodies resulted in increased production of IL-10 and suppression of IFNβ and iNOS (Ubol et al., 2010). The high levels of IL-10 resulted in upregulation of negative regulators such as activation of suppressor of cytokine signaling system (SOCS), specifically SOCS-3 and inhibition of JAK-STAT pathways (Ubol et al., 2010) thereby suppressing antiviral defenses (Tsai et al., 2014) (**Figure 1**).

Certain FcγRs are more efficient at ADE than others. FcγRI, FcγRIIA, and FcγRIIIA are all shown to facilitate ADE while FcγRIIB is less efficient (Boonnak et al., 2008, 2013). FcγRIIA has shown to be more efficient than FcγRI in ADE in the presence of DENV-IgG immune complexes (Rodrigo et al., 2006). Downstream signaling pathways that are activated by engagement of the activating FcγRs (FcγRI and FcγRIIA), has shown to reduce production of TNFα, IL-12, and IFNβ through inhibition of interferon regulatory factor 1 and NFκB gene expression, while increasing the production of IL-10 (Ubol and Halstead, 2010). Engagement of immune complexes with the activating FcγRs in acute DENV infection results in activation of two suppression pathways, which leads to overall impairment of antiviral immune responses (Ubol and Halstead, 2010).

The FcγRIII receptor, which is another activating FcγR, which is important in ADE, has 2 isoforms, FcγRIIIA and FcγRIIIB (CD16a/CD16b). FcγRIIIA is known to bind to immune complexes more efficiently that other FcγRs (Ogonda et al., 2010). Increased expression of FcγRIIIA on non-classical monocytes ($CD14^{+}CD16^{++}$), has been linked to occurrence of severe anemia and other complications during *Plasmodium falciparum* infection (Ogonda et al., 2010). Higher levels of afucosylated Fc IgG1 isoforms, which have an enhanced capacity to engage with the activating FcRIIIA, was associated with DHF and shock in those with acute dengue suggesting antibody binding to FcγRIIIA is likely to be associated with severe disease (Wang et al., 2017). We recently showed that differences in antibodies to dengue NS1 antigen could also be associated with protection or severe disease (Jayathilaka et al., 2018). Indeed, in dengue mouse models, anti-NS1-Abs activated NLRP-3 by binding to FcγRIIIs resulting in severe disease (Lien et al., 2015). Therefore, it would be important to further investigate the role of different antibody subclass binding to different FcγRs and association with clinical disease severity.

Monocytes of healthy individuals who had varying severity of dengue in the past also appear to respond significantly different to infection with the DENV. We showed that monocytes of healthy individuals who had severe dengue in the past when infected with different serotypes of the DENV produced higher viral loads, higher levels of IL-10, IL-6, TNFα, and IL-1β and increased expression of RIG-I and NLRP3 when compared to monocytes of those with past non-severe dengue (Kamaladasa et al., 2019). These monocytes produced higher levels of IL-1β in the presence of autologous serum, in the presence of serum from those with past non-severe dengue and in the presence of seronegative serum suggesting that this aberrant responses by monocytes of those with past severe dengue could be an inherent characteristic (Kamaladasa et al., 2019). Although the reasons for this are not clear, it could be epigenetic reprograming in innate immune cells in such individuals due to exposure to different pathogens or other stimuli (Netea et al., 2016).

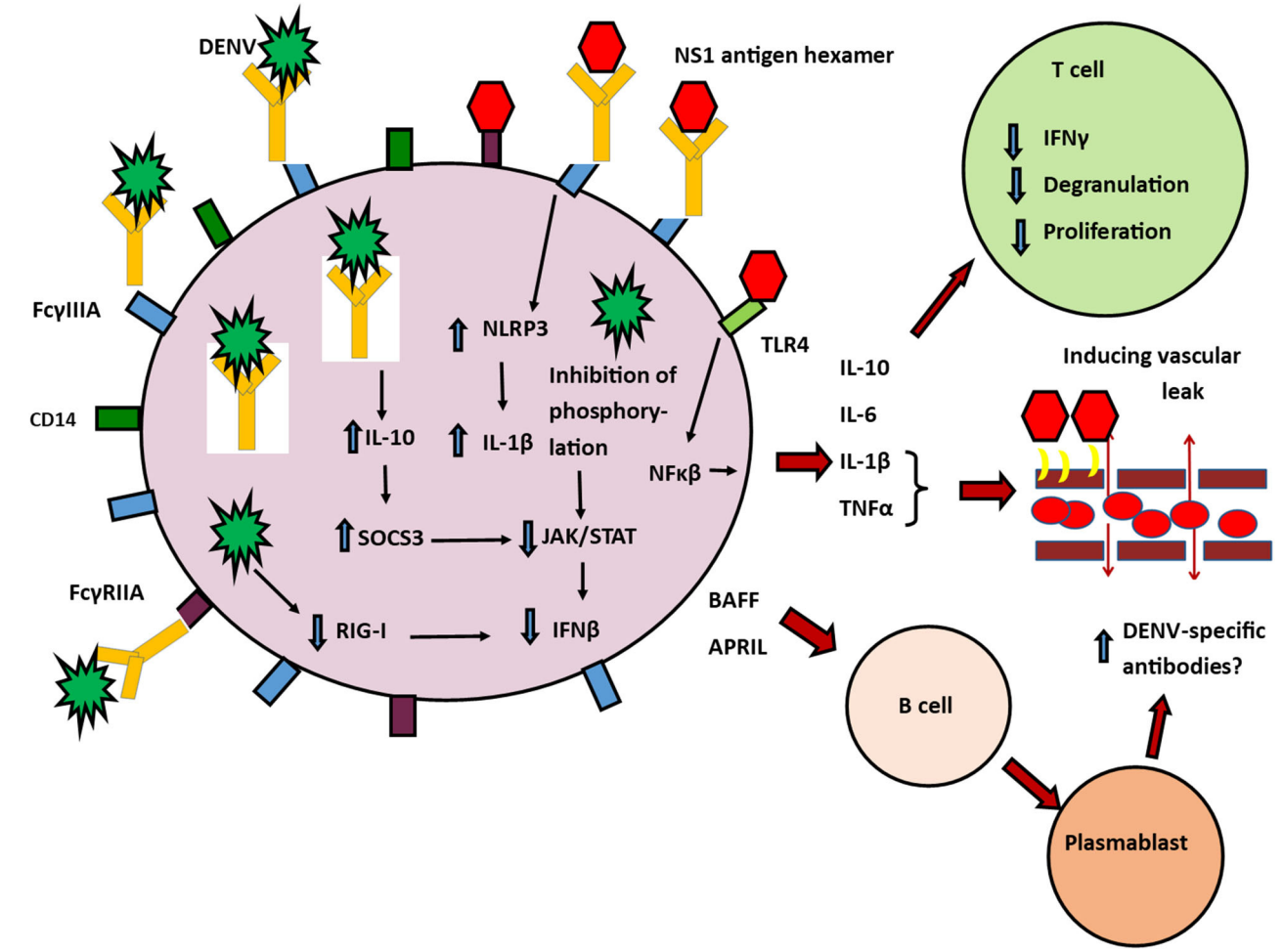

FIGURE 1 | Possible role of monocytes in pathogenesis of severe dengue. Classical and non-classical monocytes have many types of activating FcγRs, that facilitate antibody dependent enhancement (ADE) such as FcγRI, FcγRIIA, and FcγRIIA. FcγRI and FcγRIIA downstream signaling inhibits many antiviral responses by the cell, including inhibition of RIG-I/MDA5 induced type I IFN, TNFα, and IL-12 production. Non-classical ($CD14^{+}CD16^{++}$ monocytes) are more susceptible to infection through the DENV through ADE by binding of immune complexes to their FcγRIIIA (CD16a) receptors. This leads to induction of IL-10 and reduction of IFNβ and activation of iNOS. High levels of IL-10 induce SOCS3 which further inhibits JAK/STAT signaling pathways and inhibition of IFN production. DENV NS1 induced production of inflammatory cytokines after engagement through TLR-4 and contributes to vascular leak by disruption of the endothelial glycocalyx. Other DENV structural proteins leads to degradation of IFN signaling molecules resulting in impaired RIG-I signaling. DENV non-structural proteins also inhibit STAT-1 phosphorylation and thereby also contributing to reduction of IFN production. NS1 antibody-antigen immune complexes can also bind through FcγRIIIA (CD16a) receptors and induce NLRP3. This altered antiviral defense mechanisms within the cell leads to increased production of inflammatory cytokines and IL-10. IL-1β and TNFα contribute to vascular leak. IL-10 suppresses T cell activation, degranulation and cytokine production. These non-classical monocytes also produce BAFF and APRIL which act on resting B cells and stimulate them to transform into plasma cells, which are possibly responsible for further production of DENV-specific antibodies.

MAST CELLS IN DENGUE

Mast cells are shown to be permissive to infection by the DENV, which is enhanced in the presence of DENV-specific antibodies (St. John et al., 2013; Syenina et al., 2015). Many proteases and inflammatory mediators produced by mast cells such as chymases, tryptases, platelet activating factor (PAF) and vascular endothelial growth factor (VEGF) have been implicated in vascular leak and disease pathogenesis (Furuta et al., 2012; Jeewandara et al., 2015; Kamaladasa et al., 2016; Tissera et al., 2017; Inokuchi et al., 2018; Malavige et al., 2018). As mast cells reside in tissues and around blood vessels, it has been difficult to determine their infectivity rates by the DENV and phenotypic changes (St. John, 2013). As they also reside in the skin, they can be readily infected and activated during very early infection, when the mosquito injects the DENV into the dermis. Although

certain mediators such as PAF and VEGF can also be produced by other cells types such as macrophages, monocytes and endothelial cells (Walterscheid et al., 2002), chymases and tryptases are only produced by mast cells (Krystel-Whittemore et al., 2015). Both chymase and tryptase have been shown to be high in patients with acute dengue during early illness and shown to be an important predictor of progression to severe dengue, suggesting that mast cells indeed do have an important role in disease pathogenesis (Tissera et al., 2017; Rathore et al., 2019). Chymase levels were shown to be higher in those who progressed to develop DHF after day 3 of illness (Rathore et al., 2020). However, it is not yet clear whether the differences between those with primary and secondary dengue associate with increased mast cell activation. Serum tryptase has been shown to be higher in patients with DHF and this rise was especially seen during day early illness (Jeewandara et al., 2017; Rathore et al., 2019). Tryptase has been shown to induce vascular leak by acting on endothelial gap junctions and was shown to induce plasma leakage and shock in animal models, suggesting that it indeed is likely to have an important role in inducing vascular leak in dengue (Rathore et al., 2019). In acute dengue, no differences were observed in tryptase levels in those with primary and secondary dengue (Jeewandara et al., 2017), suggesting that the role of DENV-specific antibodies activating mast cells through their FcγRs should be further investigated.

PAF levels were also shown to be high in those with vascular leak and are known to induce vascular leak (Jeewandara et al., 2015). A PAFR blocker (rupatadine), was shown to inhibit the reduction of trans endothelial electrical resistance and reduce the effect of sera of patients with DHF on endothelial tight junction (Malavige et al., 2018). In addition, it also showed dose dependent effects on reducing the rise in hematocrit in mouse models and reduction in the extent of plasma leakage in patients with acute dengue (Malavige et al., 2018). PAF is also known to activate NFκB and thereby induce production of many inflammatory cytokines and further contributing to disease pathogenesis (Choi et al., 2003). Although the source of PAF is not known in patients with acute dengue, it is possible that many different cell types including mast cells could be contributing to its production. PAF has varying effects on many other immune cells such as reduction in dendritic cell maturation, T cell anergy and reduction in T cell and depending on the levels either increase or a decrease in T cell proliferation (Kelesidis et al., 2015). Therefore, PAF appears to have many potential impacts on antiviral immunity other than inducing vascular leak; and therapeutics that block PAF are may be beneficial by acting through many pathways.

METABOLIC DISEASE AND INNATE IMMUNITY

Individuals with comorbidities such as metabolic diseases and asthma are more likely to develop DHF and organ dysfunction (Guo et al., 2017; Pang et al., 2017; Wang et al., 2019). The presence of diabetes mellitus along with other metabolic diseases or poorly controlled diabetes mellitus were independent risk factors for development of severe dengue and shock (Lee et al., 2018). However, the mechanisms of severe dengue of those with comorbidities are not known. Patients with metabolic diseases have been shown to have low grade endotoxemia (Neves et al., 2013) due to gut microbial dysbiosis (Shin et al., 2015). Those with DHF have shown to have higher levels of serum lipopolysaccharide (LPS), which can result in activation of many innate immune cells through TLR-4 (van de Weg et al., 2012, 2013). While LPS alone induces many immune cells to produce inflammatory cytokines, LPS was shown to act synergistically with the DENV to further increase cytokine production and PAF, which can contribute to vascular leak and DHF (Kamaladasa et al., 2016). Therefore, such low grade endotoxemia in patients with metabolic disease, could be instrumental in inducing an unfavorable immune response to the DENV resulting in severe dengue. Gut microbial dysbiosis has shown to significantly influence the antiviral responses to vaccines (Lynn and Pulendran, 2018; Lynn et al., 2018). For instance, those who were given antibiotics just before the influenza vaccine, had reduced neutralizing and influenza specific IgG, and dysregulated immune responses, which was attributed to gut microbial dysbiosis (Hagan et al., 2019). Therefore, gut microbial dysbiosis may have a potential to induce severe dengue due to development of a poor quality, suboptimal antibody response.

Metabolic disease and obesity is associated with chronic, low grade inflammation and is associated with differences in monocyte subsets (Friedrich et al., 2019; Figueroa-Vega et al., 2020). While all $CD14^{+}CD16^{+}$(classical monocytes), $CD14^{dim}CD16^{+}$ (intermediate) and $CD14^{-}CD16^{++}$(non-classical) monocytes are susceptible to infection by the DENV, the non-classical monocytes are the main produced of inflammatory cytokines and IL-10, which are implicated in SD (Wong et al., 2012). *IFITI* and CD163 expression in CD14+CD16+ monocytes, was shown to precede the development of severe dengue (Zanini et al., 2018). As those with metabolic disease and obesity have a higher frequency of the non-classical types of monocytes (Friedrich et al., 2019), increased infection of this subtype by the DENV in such individuals could lead to severe dengue. Increase in mast cell numbers are also seen in adipose tissues of obese individuals, which are thought to contribute to low grade inflammation associated with metabolic syndrome and obesity (Elieh Ali Komi et al., 2020). Therefore, increase infection and activation of mast cells in those with metabolic disease could also lead to more severe disease.

EFFECT OF INNATE IMMUNE RESPONSES ON VIRUS SPECIFIC T CELLS AND B CELLS

Although the role of T cells in dengue have been debated, emerging evidence suggests that they are likely to have a protective role (Weiskopf et al., 2013, 2015; Wijeratne et al., 2018, 2019). Highly cross reactive T cells are seen in acute dengue, which were thought to contribute to disease pathogenesis by production of high levels of inflammatory cytokines (Mongkolsapaya et al., 2003; Appanna et al., 2007;

Dong et al., 2007). Indeed, it has been shown that higher levels of cross-reactive T cells that produce inflammatory cytokines were seen in those with more severe forms of illness rather than those with milder illness (Mongkolsapaya et al., 2006). However, such cross reactive T cells producing high levels of inflammatory cytokines were only seen 14 days since the onset of illness, when the patient was in the recovery phase (Mongkolsapaya et al., 2006). In fact, T cell responses were very low or absent in patients during the critical phase, where vascular leak and the cytokine storm occurs (Mongkolsapaya et al., 2006; Malavige et al., 2013b). This paucity of the DENV-specific T cells in acute dengue, could be due to the massive T cell apoptosis that has been reported in many studies (Mongkolsapaya et al., 2003; Malavige et al., 2012). Indeed, prolonged viremia and persistence of NS1 antigen is seen in those with severe dengue (Wang et al., 2006; Guilarde et al., 2008; Adikari et al., 2016), while early appearance of DENV-specific T cells was associated with milder disease and early clearance of the virus (Wijeratne et al., 2018). Patients with DHF had significantly less DENV-specific T cell responses (Wijeratne et al., 2018), than those with DF and the virus specific T cell responses were restored *in vitro*, with IL-10 blockade (Malavige et al., 2013b). Many studies have shown that T cell responses are absent or suboptimal in those with DHF during acute illness (Mongkolsapaya et al., 2006; Chau et al., 2008). Since early appearance of IL-10 was the main cytokine that was associated with subsequent progression to severe dengue, IL-10 is likely to also contribute to disease pathogenesis by inhibiting virus specific T cell responses and therefore, a delay in virus clearance.

The expansion of non-classical $CD14^{+}CD16^{+}$ monocytes has shown to associate with severe dengue (Kwissa et al., 2014; Zanini et al., 2018). DENV-infected $CD14^{+}CD16^{+}$ monocytes were found to be more efficient at stimulating resting B cells to transformation into plasmablasts by production of BAFF and APRIL (Kwissa et al., 2014). Increase in the frequency of plasmablasts have shown to associate with dengue disease severity (Wrammert et al., 2012; Appanna et al., 2016; Wijesinghe et al., 2020). Although the mechanisms by which the plasmablasts contribute to disease pathogenesis is unclear, it is possible that the increased antibody production by these cells, could contribute to severe disease by ADE.

SUMMARY

There is increasing evidence that a dysfunctional innate immune response leads to severe dengue by impaired production of interferons and increased production of inflammatory cytokines and lipid mediators. This dysregulated and aberrant immune response leads to reduced clearance of the virus, and severe dengue by inducing a vascular leak and by inducing excessive inflammation. Individuals with comorbid illnesses could be prone to more severe dengue due to low grade endotoxemia, gut microbial dysbiosis, and an altered phenotype of innate immune cells. The immunosuppressive and inflammatory lipid mediators and altered phenotype of monocytes are likely to further act on T cells and B cells leading to an impaired adaptive immune response to the virus. Therefore, in order to identify therapeutic targets for treatment of dengue, it would be important to further characterize these mechanisms in order for early intervention.

AUTHOR CONTRIBUTIONS

GM and GO: conceptualization, critical review, and writing the manuscript. CJ: writing the manuscript. All authors: contributed to the article and approved the submitted version.

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REVIEW
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Mobilization and Activation of the Innate Immune Response to Dengue Virus

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Dengue virus is an important human pathogen, infecting an estimated 400 million individuals per year and causing symptomatic disease in a subset of approximately 100 million. Much of the effort to date describing the host response to dengue has focused on the adaptive immune response, in part because of the well-established roles of antibody-dependent enhancement and T cell original sin as drivers of severe dengue upon heterotypic secondary infection. However, the innate immune system is a crucial factor in the host response to dengue, as it both governs the fate and vigor of the adaptive immune response, and mediates the acute inflammatory response in tissues. In this review, we discuss the innate inflammatory response to dengue infection, focusing on the role of evolutionarily conserved innate immune cells, their effector functions, and clinical course.

Keywords: dengue, innate immunity, macrophages, mast cells, interferon, inflammation, pathogenesis, clinical symptoms

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INTRODUCTION

Dengue Virus and Clinical Spectra

Dengue virus (DENV) is an arbovirus transmitted by the mosquito vectors *Aedes aegypti* and, to a lesser extent, *Aedes albopictus* (Scott and Morrison, 2010). Dengue virus belongs to the family Flaviviridae and is a single stranded, positive sense, enveloped, RNA virus. The genome is approximately 11 kb and encodes 10 proteins. Upon infection the viral genome is delivered to the cytoplasm and translated into one long polyprotein that is then cleaved by both host and viral specific proteases to yield 10 individual proteins. Three are structural proteins (envelope, core, and membrane) and seven are non-structural (NS) proteins (NS1, NS2a and NS2b, NS3, NS4a and NS4b, and NS5). Dengue is endemic in tropical and subtropical regions of the world where 2.5 billion people are at risk for infection. With approximately 400 million infections annually (WHO, 2009; Bhatt et al., 2013), dengue disease is a serious public health threat with no specific treatments currently available. There are currently four circulating serotypes (DENV-1 to 4) that exhibit up to 70% sequence homology (Blok, 1985; Green and Rothman, 2006). All four serotypes can cause a spectrum of disease with manifestations ranging from a subclinical infection to a mild febrile illness termed dengue fever (DF). In a subset of infections, severe hemorrhagic manifestations or shock syndrome known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (WHO, 2009) can develop. While the majority of patients develop only mild symptoms and recover after defervescence, approximately 5% develop life threating vascular dysfunction (Gubler, 1998;

Halstead, 2007). The pathogenesis of severe dengue disease has been the focus of countless studies, and some progress in understanding disease associations and mechanisms has been made. What is known is that severe dengue disease most often occurs during a secondary DENV infection with a heterologous serotype (Halstead, 1994; Gubler, 1998; Halstead, 2007). This phenomenon is thought to involve antibody-dependent enhancement (ADE) which is characterized by the enhanced infection of target cells *via* Fcγ receptor bearing cell-mediated internalization of IgG coated virus. The hypothesis suggests that cross-reactive antibodies that bind virus are not neutralizing, or are at sub-neutralizing concentrations, (Halstead and O'Rourke, 1977a) thereby facilitating infection, rather than preventing. Several *in vivo* lines of evidence support this hypothesis (Halstead and O'Rourke, 1977a; Halstead and O'Rourke, 1977b; Zellweger et al., 2010). Both *in vitro* experiments in K562 cells and *in vivo* experiments with juvenile rhesus macaques demonstrated that ADE led to increased titers, with up to a 1000-fold increase *in vitro* and a 100-fold increase *in vivo* (Goncalvez et al., 2007). Higher levels of viremia are correlated with increased dengue disease severity in humans (Vaughn et al., 2000). There is also evidence that immature DENV virions are rendered highly infectious by anti-prM antibodies (Goncalvez et al., 2007; Rodenhuis-Zybert et al., 2010). Moreover, F_c receptor signaling during immune complex binding is not restricted to the internalization event; other signaling includes suppression of IFN-gamma transcription and translation, increased synthesis of IL-6, and downregulation of IRF-1 and STAT1 [reviewed in (Halstead et al., 2010)]. F_c receptor engagement also reportedly downregulates RIG-I/MDA5 signaling and decreases production of type I interferon (Chareonsirisuthigul et al., 2007).

The host-specific immune responses to DENV likely play a large role in the pathophysiology of disease and subsequent clinical manifestation of dengue infection. Dengue disease is a complex viral-host interaction with not only cross reactive antibody and T cell immunity as important determinants of severity (Mongkolsapaya et al., 2003; Friberg et al., 2011; Midgley et al., 2011), but also host genetics including polymorphisms in the TNF and lyphotoxin receptor (Fernandez-Mestre et al., 2004; Vejbaesya et al., 2009)and MHC class I alleles (Stephens et al., 2002; Zompi and Harris, 2012). These studies have found that several polymorphisms in these alleles are associated with more severe dengue disease, while others, particularly in the MHC alleles can be associated with less severe disease. For example, HLA A*0203 is associated with less severe dengue fever, while HLA*0207 is associated with more severe DHF and DSS in secondary infection. By contrast, HLA B44, B62, B76, and B77 are associated with protection against developing clinical disease after secondary dengue infection (Stephens et al., 2002). Virus virulence factors are also associated with severity of disease including the sequence of specific serotypes causing infection (Halstead et al., 1983; Morens and Halstead, 1987; Halstead, 1998). The wide range in clinical presentation is likely the result of the interaction of many variables, both virus and host. Clinical presentation can include severe headache; retro-orbital eye pain; muscle, joint, and bone pain; nausea; vomiting, macular or maculopapular rash; a positive tourniquet test; or other hemorrhagic manifestations such as petechia, ecchymosis, purpura, epistaxis, bleeding gums, and hematuria. Severity is associated with warning signs including abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy, restlessness, and liver enlargement (Deen et al., 2006). The hallmark for DHF and DSS is plasma leakage characterized by endothelial damage and leakage of intravascular plasma to the extravascular space (Srikiatkhachorn, 2009; Rothman, 2010).

In this review, we focus on the innate inflammatory responses to DENV infection by innate immune cells, their effector functions and clinical course.

MODELS FOR DENGUE VIRUS IMMUNE RESPONSE

DENV, and accompanying clinical disease, are almost entirely restricted to humans and non-human primates, and the latter are largely asymptomatic. Few small animal models exist (Yauch and Shresta, 2008; Zellweger and Shresta, 2014), as rodent cells are generally not permissive to DENV infection, and each presents significant challenges for use and extrapolation to humans. To circumvent the issue of infectability, mouse models are genetically modified to be permissive to virus infection, most often by targeting the IFN system, and by adapting the virus. As such, immunocompromised mice are a common model for studying DENV pathogenesis and immunity. The most widely used model is the AG129 IFNα/β/γ receptor knockout mouse that when infected with a mouse-adapted DENV strain, recapitulates aspects of severe dengue disease (Yauch et al., 2009; Watanabe et al., 2012) including vascular leak and ADE (Shresta et al., 2006; Balsitis et al., 2010). The NOD/SCID/IL-2RγKO mice engrafted with human CD34+ stem cells develop symptoms of mild DF (Bente et al., 2005; Mota and Rico-Hesse, 2011), while the RAG-hu mouse model develops fever only (Kuruvilla et al., 2007). These models are not ideal in which to study the immunopathogenesis of a human-constrained virus. Dengue does not naturally infect rodents. Moreover, the use of these models requires adaptation of human dengue strains in order to establish any infection. This, coupled with the required immune knockouts to generate infection and disease, makes it difficult to apply knowledge gained in immune deficient murine systems to the events occurring in human hosts with intact immune systems. Much of the pathogenesis of dengue disease is thought to be due to activation of the immune system, and these models do not recapitulate a fully functioning immune system. However, immunocompetent C57BL/6j and BALB/c Mice infected IP with a mouse adapted passaged clinical isolate of DENV 3 exhibit severe disease and die by day 6–7 post-infection, recapitulating many of the observed clinical signs of severe dengue including thrombocytopenia, decrease in systolic blood pressure, increased liver enzymes, and viremia (Goncalves et al., 2012).

These animal models are not sufficient to test antivirals, understand mechanisms of clinical symptoms, or select vaccine candidates. Limited knowledge of the range and complexity of the immune response generated in humans makes it particularly challenging to design an effective vaccine. Illustrating this, the only available vaccine, licensed in 2019, was shown to sensitize some seronegative recipients to more severe dengue disease upon infection with wild-type virus (Biswal et al., 2019). These limitations arise, in part, from an incomplete understanding of the human immune response to DENV, which is essential to exploit when designing a vaccine. Importantly, the early innate immune response governs the fate and vigor of the subsequent adaptive immune response (Fearon and Locksley, 1996), which confers the long-term protection desired from a vaccine. Compounding this problem, epidemiological studies in dengue-endemic areas are limited in several ways: they cannot control for many important factors in their subjects (e.g. prior flavivirus exposure), and are limited to enrolling virologically-confirmed cases of dengue who present with symptoms. These limitations leave a substantial gap in our knowledge of the early innate immune response to DENV.

Controlled human infection models (CHIMs) have been used successfully and safely for a number of human pathogens including cholera, influenza, malaria, typhoid and other enteric pathogens. CHIMs for DENV challenge studies have been historically and currently used as a safe means to test vaccine product viability (Thomas, 2013; Endy, 2014). The Walter Reed Army Institute of Research (WRAIR) initiated the development of a DENV human infection model (DHIM) in 2001 using previous DENV vaccine viruses that were found to be too reactogenic for a vaccine, but safe in human trials and potential candidates for a challenge virus (Lyons, 2014). The first study was conducted in 2001 in 15 volunteers. Two subjects received DENV-1, three subjects received DENV-2, three subjects received DENV-3, four subjects received DENV-4, and three subjects received placebo. DENV-1 strain 45AZ5 was administered subcutaneously (SC) at a dose of 0.5 ml containing 1.6×10^4 PFU (Mammen et al., 2014). Challenge resulted in a mild dengue fever-like illness with fever, chills, myalgias, arthralgias, headache, eye pain, malaise, anorexia, backache, abdominal pain, pruritus, photosensitivity, lymphadenopathy, and loose stools. Also observed were a morbilliform truncal rash, lymphadenopathy, leukopenia, neutropenia, and small perihepatic effusion. A follow-up study was performed in 2008 using both previously vaccinated and non-vaccinated volunteers. In those who received DENV-1, all 5 subjects previously vaccinated with a tetravalent live-attenuated dengue vaccine were protected against DENV-1 virus challenge (Sun et al., 2013). In volunteers who did not receive the vaccine, dengue fever like symptoms and laboratory findings occurred. In both trials, volunteers had resolution of their symptoms and viremia without serious adverse events. We recently completed a phase I study of 12 healthy adult volunteers using a challenge virus, DENV-1-LVHC, strain 45AZ5 (Endy et al., 2020). All subjects developed neutralizing antibody to DENV-1, and 11 of the 12 developed viremia with peak viral loads similar to wild-type DENV infection. There were no serious consequences to infection and all recovered without problems. The DHIM offers a platform in which to test the viability of candidate vaccines and therapeutics. Equally important, it offers a reproducible model in which to study the viral-host interactions and the innate and adaptive immune response to DENV infection.

INNATE IMMUNE SYSTEM

The ability to respond to and orchestrate effective defenses against invading pathogens is a key element of survival. The human immune system achieves this by effectively controlling dangerous pathogens and ignoring the rest. Composed of two arms, the innate arm and the adaptive arm, The innate arm is activated in response to both injury and infection; the adaptive arm is recruited and activated in response to innate immune activation and direction, is specific, and is tailored for individual pathogens [reviewed in (Dorothy and Lewis)].

Evolutionarily conserved, the innate system harnesses the power of molecular and danger patterns to activate a highly specific inflammatory response aimed at alerting and mobilizing immune cells, with the ultimate goal being to clear the invading pathogen and/or repair the damage that initiated the response [reviewed in (Janeway and Medzhitov, 2002; Barton, 2008)]. By contrast, the adaptive system is highly specific, with recognition mediated by and activation tailored to antigen encountered, and requires appropriate secondary signals. The effectors of the adaptive immune system include T cell and B cells. The innate system is more complex [reviewed in (Douglas and McDonald, 2019)]; it is composed of monocytes, macrophages, neutrophils, mast cells, basophils, eosinophils, NK cells and ILCs. Many of these are granulocytes that store preformed mediators, including specific inflammatory proteases and cytokines, for immediate release. These granulocytes also *de novo* synthesize a plethora of highly inflammatory cytokines, chemokines, and Cox-2- and 5-lipoxygenase-derived lipid mediators (Alvarez et al., 2010) that are necessary to induce and direct an appropriate adaptive immune response. Activation of tissue resident macrophages and mast cells at the area of wound or pathogen entry initiates a cascade of events aimed ultimately at healing and pathogen clearance, often with the help of the adaptive arm. The direct effect of these inflammatory mediators is to modify endothelial tight junctions and adhesion molecules to allow for influx of immune cells from the circulation, to recruit other innate immune effectors to the site, and to activate nearby tissue resident cells to mount a response against the insult. Upon activation of mast cells, dendritic cells are activated and directed to egress from the site, homing to the draining lymph node to activate the adaptive arm of the immune system (Jawdat et al., 2006; Suto et al., 2006; Dudeck et al., 2019). The evolutionary importance of these tissue resident cells is underscored by the finding that both phagocytes (macrophages) and granulocytes (mast cells) are highly conserved, found in a range of Kingdoms and species including invertebrates and primitive chordates (Rhodes et al., 1982; Crivellato and Ribatti, 2010; Wittamer et al., 2011). It is

these tissue resident cells that initiate the acute inflammation required to alert and instruct both the innate and adaptive arms of the immune system to the danger/pathogen, allowing for effective mobilization of the appropriate cellular compartment to the site.

PROFESSIONAL PHAGOCYTES OF THE SKIN: MACROPHAGES, DCS, LANGERHANS CELLS

There are several valid ways of subcategorizing phagocytic immune cell populations. The macrophage/DC/LC field is currently undergoing a shift away from defining these cell types and subtypes by their functional/phenotypic properties, and towards defining the populations by ontogeny (developmental lineage) and transcription factor expression/transcriptome profiles. In consequence, LCs/DCs and subsets and macrophages have not always been defined identically. Where appropriate, we have denoted specific subsets with their definitive CD antigens in order to facilitate comparisons between human and mouse data, as well as to aid in comparing studies where immune cell subtypes may not have been defined identically.

Dendritic cells are phagocytic cells derived from $CD34^+$ hematopoietic cell precursors that give rise to both myeloid and lymphoid precursors. Dendritic cells are considered the most efficient antigen-presenting cell whose canonical function is to activate naïve T cells. In this capacity, dendritic cells capture, process and present antigens in MHC Class II to CD4 + T cells [reviewed in (Banchereau et al., 2000)]. They express high levels of MHC Class II and CD11c in addition to a range of other surface markers that identify distinct subtypes. As tissue-resident cells, they are relatively short-lived, and require replenishment from bone marrow-derived precursors in order to maintain their numbers in peripheral organs [reviewed in (Doebel et al., 2017; Collin and Bigley, 2018; Macri et al., 2018)]. Dendritic cells exist in two functional states, immature and mature [reviewed in (Worbs et al., 2017)]. In tissues they are immature, with a decreased ability to induce naïve lymphocyte effector responses but with a robust ability to capture antigen. Dendritic cells undergo a maturation program both as a result of macrophage and mast cell activation-derived signals and also by sensing of danger- or pathogen-associated molecular patterns. Maturation activates dendritic cells to migrate to secondary lymphoid organs where they function to present antigen to T cells and promote the initiation of adaptive immunity [reviewed in (Patente et al., 2019)]. Langerhans cells are epidermal innate immune cells of myeloid origin. They bear some functional similarities to dendritic cells, including the capacity to migrate to lymph nodes and stimulate T cells. However, classified according to developmental origin (ontogeny), they are considered a specialized subset of macrophages: they arise from embryonic precursors rather than bone marrow. Langerhans cells are long-lived in tissue and self-maintain their population without replenishment (Doebel et al., 2017).

THE INFECTION EVENT: ANATOMY AND MOSQUITO FACTORS

DENV is introduced into the skin *via* saliva deposition as a mosquito takes a blood meal (**Figure 1**, Step 1). The mosquito proboscis must necessarily penetrate through the epidermis and into the dermis in order to access the capillary beds, though the specific distribution of virions introduced during the feeding event is unclear. Some sources suggest that DENV is directly injected into the dermis instead of the epidermis (Begum et al., 2019); others assert that the virus is introduced into both layers (Garcia et al., 2017; Rathore and St John, 2018); and still others claim that virions are introduced directly into the bloodstream, with "spillover" into both the dermis and epidermis (Martina et al., 2009). As discussed below, susceptible and permissive cells from both layers are infected soon after inoculation, supporting the notion that DENV is not restricted to a specific layer of the skin after the inoculation event.

In addition to viral particles, feeding mosquitos also inject several salivary proteins. These, in addition to local anticoagulant activity, have been shown to facilitate the establishment and virulence of several flaviviruses. Inoculation of C57BL/6 mice with West Nile Virus with infected *Culex tarsalis* mosquitoes results in increased viremia 24 and 48 h.p.i. compared to needle injection of a comparable inoculum (Styer et al., 2011). While the precise mechanism was not determined, possibilities include local immunomodulation at the bite site increasing WNV tropism, and/or leukocyte infiltration increasing the number of WNV-susceptible cells at the bite site. C57BL/6 mice passively immunized against the *Aedes aegypti* salivary factor NeSt1 showed reduced early ZIKV replication, reduced macrophage infiltration at the bite site, and increased 30-day survival; this is possibly due to reduced levels of pro-IL-1β and CXCL2 at the bite site (Hastings et al., 2019). Humanized (NSG) mice infected by *A. aegypti* mosquitoes exhibit prolonged DENV viremia; moreover, a mosquito bite containing no virus induced an innate immune cytokine response comprising TNF, IL-4, and IL-10, underscoring that mosquito saliva is itself immunomodulatory in the absence of virus (Cox et al., 2012). From a mechanistic standpoint, the *A. aegypti* salivary protein CLIPA3 has been implicated as a specific factor contributing to increased DENV infectivity, facilitating attachment of viral particles to cell surface receptors and cell migration *via* digestion of extracellular matrix (Conway et al., 2014). These experiments have not elaborated the range of mosquito salivary factors involved, but point to the initial inoculation conditions, and thus the innate immune response of the skin, as an important variable influencing the course of infection. It should be noted that much of the dengue field involving mouse or non-human primates, as well as the current human infection models, do not account for this variable when inoculating with DENV.

Once deposited, DENV is capable of infecting and replicating in keratinocytes and fibroblasts (Garcia et al., 2017) as well as the several populations of professional skin-resident phagocytic cells: Langerhans cells, dendritic cells, and macrophages (Wu et al., 2000; Cerny et al., 2014; Schmid and Harris, 2014; Garcia et al.,

2017; Rathore and St John, 2018). A specific entry receptor or family of receptors for DENV, and thus the range of cellular tropism, has not yet been established. Aside from antibody-dependent enhancement, native cell-surface candidates for DENV internalization include L-SIGN, DC-SIGN, C-type lectins, the mannose receptor, glycosaminoglycans such as heparin sulfate, TIM-1, TAM, CD14, and CD300a (Crill and Roehrig, 2001; Cruz-Oliveira et al., 2015; Ngono and Shresta, 2018). The DENV E protein structural domain III is the most likely candidate for binding the cellular entry receptor (Crill and Roehrig, 2001; Cruz-Oliveira et al., 2015) but, owing in part to the range of putative entry receptors, the exact binding motif(s) remains unknown.

FIRST WAVE: TISSUE-RESIDENT PHAGOCYTES AND KERATINOCYTES PRODUCTIVELY INFECTED

Work primarily done in C57BL/6 mice lacking the interferon alpha receptor (*Ifnar* $^{-/-}$) (Schmid and Harris, 2014) and thoroughly reviewed in (Rathore and St John, 2018), demonstrated that there are two "waves" of cells infected with DENV following inoculation. In the first wave (infection by the initial inoculum), mast cells, macrophages, CD103+ DCs, and Ly6C-CD11b+ DCs—analogous to human CD141+ conventional (myeloid) dendritic cell subtypes 1 and 2, respectively (St John et al., 2011; Collin and Bigley, 2018)—are infected with DENV (**Figure 1**, Step 2). Other studies employing non-human primates and cadaveric human skin explants, in addition to the above studies in *Ifnar* $^{-/-}$ mice, have shown that Langerhans cells are among those initially infected (Taweechaisupapong et al., 1996a; Taweechaisupapong et al., 1996b; Wu et al., 2000).

While the infection of LCs, DCs, and macrophages is well known, there is some disagreement as to which layer of the skin carries the highest burden of infection. Studies done in intradermally inoculated *Ifnar* $^{-/-}$ mice show up to 100-fold more DENV-infected cells in the dermis as compared to the epidermis at 72 h post-inoculation (Schmid and Harris, 2014; Schmid et al., 2014). These flow cytometry data were gated on CD45+ cells taken from the skin of the mice, thereby excluding from consideration keratinocytes, which do not express CD45. By contrast, subsequent work by Duangkhae and colleagues demonstrated that keratinocytes alone accounted for up to 60% of DENV-infected cells in a human skin explant model, and that the epidermis contained approximately six-fold more DENV-infected cells than the dermis at 48 hpi (Duangkhae et al., 2018).

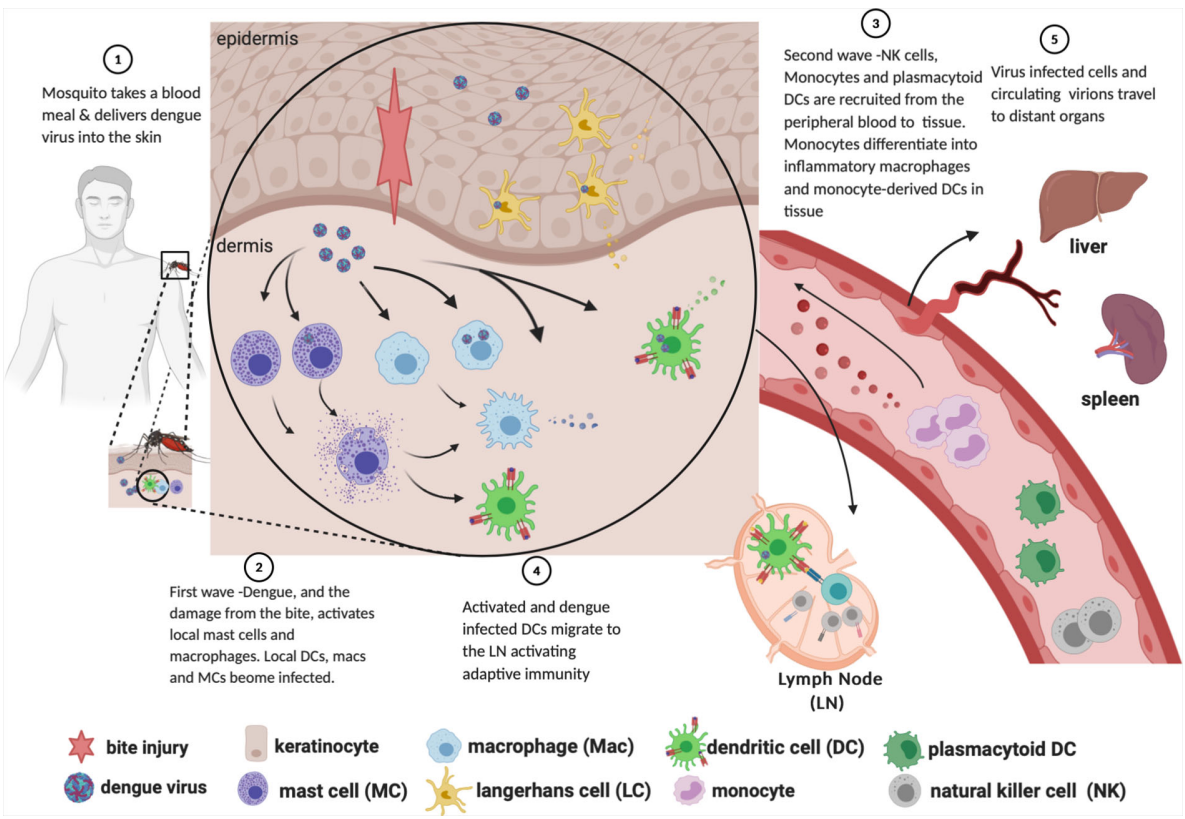

FIGURE 1 | Path of dengue infection from mosquito bite to dissemination to organs. Created with Biorender.com.

While a consensus of infection burden between the skin layers has yet to be reached, the available evidence is generally clear that keratinocytes and skin-resident phagocytes are infected after introduction of virus and prior to development of viremia. These infected cells, while mounting an innate immune response against the virus, simultaneously create a milieu conducive to propagating the number of infected cells. For example, administration of neutralizing Ab to IL-1β into skin at 2 h.p.i. decreased the burden of infected dermal cells by 65% (Duangkhae et al., 2018). These data illustrate the feed-forward effect that leads to the second wave of infection.

General Cellular Host Response to DENV and Clinical Consequences

During viral replication, intracellular DENV ssRNA and dsRNA, intermediates of viral genome replication, are recognized as pathogen-associated molecular patterns (PAMPs) by host cell pattern recognition receptors (PRRs) including MDA5, RIG-I, TLR3, and TLR7 (Pichyangkul et al., 2003; Rothman et al., 2003; Morrison and García-Sastre, 2014; Cumberworth et al., 2017; Ngono and Shresta, 2018; Uno and Ross, 2018; Tremblay et al., 2019). Engagement of these receptors triggers an antiviral response, which is initially characterized by the induction of the type 1 interferon (IFN) response. Briefly, intracellular signaling pathways downstream of the above PAMP receptors culminate in activation of the master innate immune transcription factors IRF3, IRF7, and NF-κB, which direct the transcription and secretion of IFN-α and IFN-β. These proteins in turn act in both an autocrine and paracrine fashion to produce a general antiviral intracellular milieu hostile to virion production (Rothman et al., 2003; Morrison and García-Sastre, 2014; Cumberworth et al., 2017; Uno and Ross, 2018; Tremblay et al., 2019). Thus, the initial stages of DENV infection proceed in a positive-feedback cycle of an increasing burden of cells infected, as well as an increasing capacity to mount a type 1 interferon response.

The cellular immune response at large—not limited to type 1 IFN secretion—as well as an increasing burden of infected cells contributes to the subsequent clinical manifestations of dengue. In one human subject who received a tetravalent live DENV vaccine, infected dendritic cells and Langerhans cells were found in a cutaneous rash distant from the injection site (Wu et al., 2000). The investigators performed immunohistochemical staining for DENV glycoprotein from a skin biopsy and confirmed the presence of infected DCs and LCs, indicating an association between presence of DENV and clinical symptoms manifesting in the skin. Production of the pyrogen IL-1β begins with the first wave of infected cells; additionally, production of TNF, responsible for pyrexia, myalgia and appetite suppression, could begin at the stage of skin infection as well (Schaeffer et al., 2015). More broadly, type 1 interferon contributes to a gamut of clinical symptoms, the range of which is clearly delineated during administration of exogenous interferon in a therapeutic setting: fever, chills, headache, and fatigue (Todd and Goa, 1992; Sleijfer et al., 2005; Owen et al., 2013; Torkildsen et al., 2016).

DENV: Subversion of the Interferon Response

Though the IFN response is critical to dampen DENV replication (Diamond et al., 2000), it is insufficient to completely suppress production of infectious DENV. Indeed, historical studies in humans [reviewed in (Snow et al., 2014)] as well as human data from our lab (Endy et al., 2020) suggest that not every DENV inoculation results in detectable viremia. However, the typical finding of viremia in symptomatic cases, and the presence of DENV antigen in several cell types beyond the site of infection (Begum et al., 2019), indicates that infection is not routinely confined to the skin. This is likely due to subversion of the human interferon response by DENV. At a tissue level, data from human skin explants show transient, rather than sustained, production of IFN-α by skin cells after DENV infection, with peak levels occurring less than 12 h.p.i. (Duangkhae et al., 2018). At the cellular level, several DENV nonstructural (NS) proteins suppress both induction and downstream signaling of interferon. NS2a, NS4a, and NS4b block TBK1 phosphorylation, preventing the transcription of IFN-β (Dalrymple et al., 2015). NS2b targets cGAS for degradation, and NS2b/3 cleaves the intracellular DNA sensor STING, representing multiple points of disruption to the cGAS-STING pathway which would otherwise induce interferon (Lee et al., 2018). NS5 2'-O-methylates the 5' cap of the DENV genome, which prevents detection by RIG-I (Lee et al., 2018). NS4a binds to the CARD-like and transmembrane domains of MAVS, preventing binding of RIG-I and therefore activation of IRF3 and consequent interferon induction (He et al., 2016).

Not all DENV antagonism of the IFN response occurs upstream of interferon production itself. This is important when considering that pDCs (discussed below in the *Second Wave* section) produce IFN without being infected. Thus, DENV also has the capability to disrupt the signaling downstream of the IFNAR: NS2a, NS4a, and NS4b inhibit STAT1 phosphorylation, and NS5 mediates proteasomal degradation of STAT2 (**Figure 2**) (Morrison and García-Sastre, 2014; Tremblay et al., 2019). DENV has genome size constraints and limited coding capacity expressing 10 proteins, seven of which are non-structural; five of those function to target an aspect of the IFN system. This intense focus of interfering with the IFN system underscores how important this system is in controlling DENV infection. Together, these mechanisms suppress IFN activation and downstream signaling, enabling DENV to replicate locally and to produce virions that infect cells beyond the bite site.

Tissue Resident Mast Cells Are Activated During Dengue Infection

Mast cells (MCs) are long-lived, CD34+ (Maaninka et al., 2013) tissue-resident innate immune inflammatory cells that reside in almost every tissue and organ, including in high levels in the skin (Galli et al., 2005a). In skin, mast cells are positioned in the dermis, adjacent to microvessels, where they serve as sentinels and sense blood borne and tissue localized pathogens to direct immune cell recruitment to the area. The perivascular location

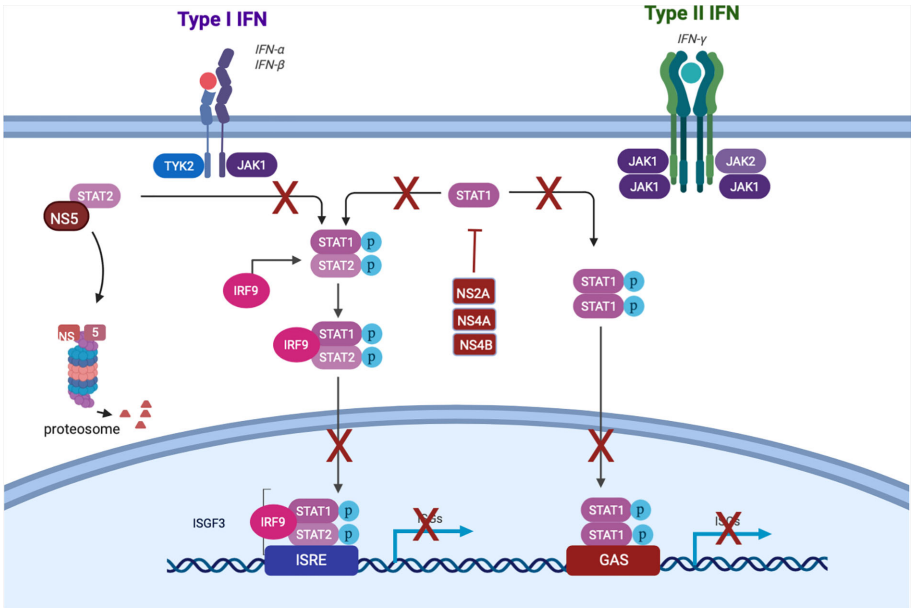

FIGURE 2 | Dengue subversion of interferon signaling. Created with Biorender.com.

also facilitates MC-driven modulation of EC function (Klein et al., 1989; Brown et al., 2011; Kunder et al., 2011), and several mast cell specific -mediators have been shown to promote EC activation (Compton et al., 1998; Frossi et al., 2004), a response necessary for immune cell recruitment. MCs recognize and become activated by a wide range of stimuli, including foreign pathogens [reviewed in (Marshall and Bienenstock, 1994; Marshall and Jawdat, 2004; Abraham and St John, 2010)] to drive a rapid, pro-inflammatory response (Galli et al., 2005b; Metz et al., 2007; Metz and Maurer, 2007) involving protease release, eicosanoid synthesis and release, and *de novo* synthesis of cytokines, chemokines and growth factors, including Type 1 IFNs.

Mast cell activation *in vivo* in tissue and *in vitro* by DENV induces degranulation with release of granule contents (**Figure 1**, Step 2) (St John et al., 2011; Troupin et al., 2016) that stimulates activation of the endothelium (Brown et al., 2011; Kunder et al., 2011) and likely contributes to the clinical rash often seen during the clinical course of infection (Nakamura et al., 2009; Mishra et al., 2018). Activated mast cells synthesize and release CXCL2, a potent chemotactic factor for neutrophils, and have been shown to promote neutrophil activation and recruitment from the periphery (Zhang et al., 1992; Abraham and Malaviya, 1997). *In vivo* mouse studies and *in vitro* human models have demonstrated mast cells are permissive to dengue virus infection *via* ADE (King et al., 2000). One of the key responses is *de novo* synthesis of pro-inflammatory cytokines TNF (Brown et al., 2011) and IL-1β (King et al., 2000), and the chemokines CCL3, CCL4 and CCL5 (King et al., 2002), CXCL12, and CXCL1 (St John et al., 2011). Mast cells respond to DENV antibody-mediated infection with a robust IFN expression by 12 h post-infection that is maintained up to 72 h (Brown et al., 2011). *Via* degranulation, release of Type 1 IFNs, and chemotactic factors, MCs initiate local acute immune activation, providing the required signals for activation and chemotactic recruitment of circulating monocytes, local tissue macrophages, NK cells and neutrophils. Subsequent work with MC-deficient mice demonstrated the importance of mast cell-driven recruitment of natural killer and natural killer T cells into the infected skin. In this model, mast cells were critical for containing DENV *in vivo*, and without which there was increased viral burden within draining lymph nodes after subcutaneous infection compared to MC-sufficient mice (St John et al., 2011).

Human studies have demonstrated that mast cells exhibit an extensive activated phenotype with degranulation in the rash of infected subjects (Asher AN et al., 2002) and dengue disease severity associated with levels of mast cell granule stored mediators including chymase (Avirutnan and Matangkasombut, 2013; Tissera et al., 2017), histamine (Tuchinda et al., 1977), VEGF (Furuta et al., 2012), and tryptase (Rathore et al., 2019). Disease severity is also associated with IgE, a classical mast cell activating antibody (Koraka et al., 2003). Together, these data strongly suggest a role for dengue-driven mast cell mediator release in clinical symptoms of dengue disease. In addition, a large histological study of DHF patients in Thailand determined that mast cells in connective tissue showed evidence of activation including swelling, vacuolation of the cytoplasm, and loss of granule integrity (Bhamarapravati et al., 1967). Mast cell activation [reviewed in (Valent, 2013; Afrin et al., 2017)] is known to mediate several of the common clinical symptoms associated with dengue patients including rash, diarrhea, vomiting, headache, eye pain/inflammation and muscle pain. Given the potency of mast cell activation and the resultant inflammation and clinical symptoms, targeting vasoactive mast

cell mediators such as histamine or tryptase may have a significant impact of clinical course. In a small study in Pakistan, treatment with anti-histamines and steroids dramatically reduced dengue symptoms, shortening duration to 3–5 days, as compared to the other treatment groups of 7–10 days (Siddique et al., 2008). An older study on 24 patients in the Armed Forces found even low dose targeting of histamine reduced the duration of clinical symptoms, suggesting that interfering with mast cell mediators or activation may have a role in treating dengue disease (Hoffman et al., 1954). More recently, a randomized clinical trial in 200 dengue patients demonstrated that a single daily dose of 10 mg montelukast, a cysteinyl leukotriene inhibitor that blocks leukotriene C4, D4, and E4 eicosanoids from binding their cognate cysteinyl leukotriene receptor 1, significantly reduced incidence of severe dengue shock syndrome by 71%, as compared to the control group (Ahmad et al., 2018). Montelukast is a standard cysteinyl leukotriene receptor inhibitor given to asthma patients to help control the mast cell-derived inflammatory leukotriene responses to reduce airway inflammation. Given that dengue virus induces degranulation of tissue resident mast cells, that rash exists at sites distant to inoculation, and that in our human infection model rash is present both before and after resolution of DENV viremia (Endy et al., 2020), we suggest that perhaps DENV is present at high levels in skin sites throughout disease. In this context, rash would then be a direct clinical sign of DENV virus replication, as has been observed in other severe viral infections, including measles and pox virus skin lesions.

SECOND WAVE—MONOCYTE-DERIVED MACROPHAGES AND DCS RECRUITED AND INFECTED; PLASMACYTOID DCS RECRUITED AND ACTIVATED

Following the first wave of cells infected in the skin, local myeloid cells, monocyte-derived macrophages, monocyte-derived dendritic cells (moDCs, Ly6C+CD11b+), and circulating plasmacytoid dendritic cells (pDCs, CD123+) are recruited into the skin *via* chemokine signals (**Figure 1**, Step 4). These signals are secreted from tissue-resident macrophages, mast cells and other initially infected resident cells. Myeloid cells are recruited to the site of inflammation *via* CCL2, IL-1β and CCL20 (Dieu-Nosjean et al., 2000; Rider et al., 2011; Schmid and Harris, 2014; Schmid et al., 2014; Duangkhae et al., 2018); monocyte extravasation from peripheral blood is facilitated by CCL1 and CCL5 (Shi and Pamer, 2011); and pDC recruitment to inflamed skin is facilitated by CCL2, among others (Swiecki and Colonna, 2015).

Upon arrival, moDCs and macrophages are productively infected with DENV (Schmid and Harris, 2014), with some *in vitro* evidence suggesting that moDCs are up to ten times more permissive to infection than either monocytes or macrophages (Navarro-Sanchez et al., 2003). Of interest, dengue virions shed by mosquito cells have a different tropism for human cells than those shed by human DCs, with the former able to bind both DC-SIGN and L-SIGN, and the latter only able to bind L-SIGN (Dejnirattisai et al., 2011). This factor, which is not controlled for in many experimental models, complicates the analysis of the kinetics and infected cell burden of the second wave. pDCs, on the other hand, are not productively infected themselves; they are instead activated by DENV-infected cells in a contact-dependent manner. Once activated, they are the predominant producers of type 1 interferons (Navarro-Sánchez et al., 2005; Webster et al., 2016; Webster et al., 2018; Assil et al., 2019). Though the roles of pDCs in viral immunopathology is complex (Swiecki and Colonna, 2015), contraction of the pDC compartment in peripheral blood was associated with a higher risk of severe dengue in children (Rothman et al., 2003) suggesting that pDCs are critical to the successful control of dengue virus.

NK Cells Are Activated and Recruited to Tissues During Infection

NK cells are bone marrow-derived innate immune lymphocytes that can kill virally infected cells, tumour cells, and antibody-opsonized cells/pathogens through a mechanism termed antibody-dependent cellular cytotoxicity (ADCC). Tissue resident cells including moDCs, macrophages [reviewed in (Michel et al., 2012)] and mast cells [reviewed in (Portales-Cervantes et al., 2019)] activated by initial infection (**Figure 1**, Step 2) and during the first "wave" rapidly recruit NK cells into infected organs and tissues in response to both DAMPS and viral infection, by secreting chemokines and cytokines (**Figure 1**, Step 3). Recruitment involves selective chemokine production in the microenviroment and corresponding receptor expression on NK cells. Several chemokines have been shown to mediate chemotaxis of NK cells to tissues including those typically known to direct chemotaxis and activation of monocytes [reviewed in (Shi and Pamer, 2011)] and mast cells [reviewed in (Halova et al., 2012)]. These include CCL2, CCL3, CCL4 (Morrison et al., 2003), CXCL2 (Burke et al., 2008), CXCL12 (Bernardini et al., 2008), and IFN (Paolini et al., 2015). Several chemokine receptors have been shown to mediate recruitment into infected tissues including CCR5, CXCR3, (Morrison et al., 2003; Huang et al., 2006), and sphingosine 1-phosphate receptor (Walzer et al., 2007), further expanding the chemokine ligands that can recruit NK cells.

Activation of NK-cell function is achieved by two distinct mechanisms: integration of signaling through a variety of inhibitory and activation receptors present on both the NK cells themselves and on host cells (Yokoyama et al., 2004; Jonsson and Yokoyama, 2009) and cytokine stimulation (Biron et al., 1999; Andrews et al., 2003; Cooper and Caligiuri, 2004). NK cell killing occurs by resting NK cells but is enhanced in response to cytokine stimulation. Typically, NK cells recognize "missing self" on infected cells when the host cell exhibits a down regulation in surface MHC class I molecules (Karre, 1995; Gasser and Raulet, 2006). NK cell killing activity is augmented by cytokine stimulation, including IL-12, IL-15, IL-18, and IL-21 (Cooper et al., 2001; Zwirner and Domaica, 2010; Brandstadter and Yang, 2011; Boieri et al., 2017) whereby cytokine-activated NK cells display enhanced cytotoxic

activities (Henney et al., 1981; Boieri et al., 2017) and *de novo* cytokine synthesis that effectively amplifies the local immune response. Limiting infection spread early on is key to a rapid, effective, resolution and clearance of pathogen. NK are key effectors in controlling viral infections [reviewed in (Jost and Altfeld, 2013)] and are activated during dengue virus infection. In skin, St. John and colleagues, demonstrated early NK cell recruitment and activation at the site of DENV 2 inoculation in mouse footpads (St John et al., 2011). NK cell recruitment was dependent on mast cell activation and underscored how the innate immune system works in concert to mount an effective defense. More recently, human skin studies in DENV infected patients demonstrated CD69+CLA+ CXCR3+ CCR5+CD56 +NK cell recruitment to the skin during acute infection. These cells expressed proliferation markers and were increased during the febrile stage of illness, declining post-febrile, and in convalescence (Zimmer et al., 2019).

In several human studies circulating NK cells were found to express an activated phenotype with enhanced expression of CD69, a type II C-lectin receptor and marker of lymphocyte activation. Homchampa et al. found evidence of NK cell cytotoxicity in children with acute dengue that correlated to disease severity (Homchampa et al., 1988) and later increased frequencies of circulating activated CD56+ CD69+ NK cells was observed in pediatric patients from Thailand with severe dengue disease as compared to patients with milder disease (Chen R. et al., 1999). In subsequent work, plasma from convalescent patients obtained after primary infection was found to mediate ADCC an *in vitro* NK cell killing assay (Laoprasopwattana et al., 2007) suggesting in the context of dengue, ADCC is likely also occurring.

An analysis of DENV patients from Brazil found an increase in circulating CD56+ NK cells during the acute phase of disease, defined as days 1–5 after onset of symptoms, with the majority of NK cells displaying early markers for activation including CD69, HLA-DR, and CD38, and increased expression of cytotoxic granule, TIA-1 (Azeredo et al., 2006) as compared to the late acute (days 6–10), or convalescence (days >11) patients. The group also showed that NK cell activating cytokine IL-15 was elevated in a significant number of patients during early acute infection (Azeredo et al., 2006).

NEUTROPHILS

Neutrophils are phagocytic granulocytes that are primarily involved in control of bacteria. Neutrophils are activated by DAMPs, PAMPs and pro-inflammatory cytokines and complement split products, including CXCL2, TNF, C5a, and C3a [reviewed in (Silvestre-Roig et al., 2016)]. The roles of neutrophils during viral infections, and in relation to outcome, are not well understood. One of the most potent outcomes of neutrophil activation is the release of NETs. NET formation (NETosis) is characterized by nuclear decondensation and delobulation, rupture of the plasma membrane, and release of DNA fibers with antimicrobial peptides and histones (Brinkmann et al., 2004) NETosis is a potent anti-microbial mechanism, but excessive formation of NETs, or the inability to clear NETs from the circulation, contributes to pathogenesis of both autoimmune diseases (Kaplan and Radic, 2012; Knight et al., 2012) and exacerbation of several different viral infections. Excessive NET formation results in exacerbated allergic airway inflammation during rhinovirus (RV) infection (Toussaint et al., 2017) and airway obstruction during respiratory syncytial virus (RSV) infection (Cortjens et al., 2016). Influenza virus infection also induces NETs in lungs of infected mice, though inhibition of NET formation did not affect infection outcomes (Hemmers et al., 2011). Together, the emerging data suggest that a neutrophil response to a viral infection may be more detrimental than beneficial, though more research is needed.

Recent work has demonstrated that neutrophils are activated during DENV infection. Studies in Vietnamese children with severe dengue have demonstrated neutrophil activation at the transcriptomic level (Hoang et al., 2010). DENV-infected patients display an increased number of circulating neutrophils during infection, suggesting that they are being activated (Thein et al., 2014 #37)]. More recently, neutrophil elastase activity (a key component of neutrophil granules) was increased DENV-infected patients, as compared to healthy controls, and levels were associated with severity of disease (Kunder et al., 2018). These data suggest that neutrophils may play an unrecognized role in dengue disease. To fully understand any relationship more work is needed.

DENV: ESCAPE FROM SKIN AND REPLICATION IN LYMPH NODES LEADS TO DEVELOPMENT OF VIREMIA

DENV escape is marked by migration of Langerhans cells and conventional dendritic cells stimulated by IL-1β and TNF (Stoitzner et al., 1999) out of the skin towards draining lymph nodes (Wu et al., 2000; Navarro-Sánchez et al., 2005; Cerny et al., 2014; Schmid and Harris, 2014; Duangkhae et al., 2018) (**Figure 1**, Step 4). Though some commentators presume that the mosquito feeding event results in viral particles being introduced directly into the bloodstream (Martina et al., 2009), this rarely, if ever, leads directly to viremia. Analysis of data from humans infected with DENV by mosquito bite has established a median intrinsic incubation period of 5.9 days, with 95% of subjects developing viremia between days 3 and 10 (Chan and Johansson, 2012). Therefore, it is much more likely that detection of DENV in the peripheral blood is a consequence of the egress of infected cells from the skin. This migration of infected phagocytes into regional lymph nodes 1) initiates the adaptive immune response, 2) precipitates further DENV replication in lymph node-resident and recruited mononuclear phagocytes, and 3) allows infectious DENV virions into the peripheral blood and the monocyte compartment (Castillo et al., 2018).

PERIPHERAL BLOOD—MONOCYTES

There is some disagreement as to which circulating cell type comprises the majority of DENV-infected cells. Early work using flow cytometry and immunocytochemistry analysis of blood from acutely ill dengue patients claimed that B cells were the main mononuclear cell fraction containing DENV (King et al., 1999). Later reports directly contradicted that, claiming that monocytes were the predominant infected cell type (Durbin et al., 2008; Schmid et al., 2014). More recently still, virus-inclusive single-cell RNA seq of peripheral blood from a limited number of dengue-infected patients showed that the majority of cells containing DENV RNA were B lymphocytes (Zanini et al., 2018). Importantly, the authors noted that the gene expression profiles of the sequenced B cells suggested that the virus may not be actively replicating. Despite perhaps not being the most numerous DENV-infected circulating cell type, monocytes are critical cellular players in dengue pathogenesis, Monocytes are the main target for DENV replication in peripheral blood (Halstead et al., 1977; Chen Y.-C. et al., 1999; Durbin et al., 2008; Zanini et al., 2018), primarily entering *via* the mannose receptor [reviewed in (Reyes-del Valle et al., 2014)].

Infection of monocytes occurs at a higher frequency in secondary dengue infection as a result of ADE. Functionally, ADE results in an increased proportion of infected monocytes, at least a 14-fold increase in *in vitro* experiments with DENV-1; ADE in this model also resulted in an increase in TNF secretion (Halstead and O'Rourke, 1977b). Infection of peripheral monocytes activated endothelial cells in a TNF dependent manner (Anderson et al., 1997), suggesting increased monocyte infection may lead to enhanced levels of TNF, driving more severe clinical disease.

DISTANT SITES-TISSUE MACROPHAGES

Following dissemination of DENV from the skin to the peripheral blood (**Figure 1**, Step 5), and other organs in the body, other populations of specific tissue-resident macrophages are infected with DENV in the course of disease. These include Kupffer cells of the liver (Aye et al., 2014), and macrophages in the spleen (Balsitis et al., 2009). The infection of Kupffer cells and the resultant acute inflammation of the immediate area likely contributes to the observed elevation in liver enzymes ALT and AST (Fernando et al., 2016).

THE ADAPTIVE IMMUNE RESPONSE

The adaptive immune response is composed of both a humoral and a cell mediated component and is absolutely essential for controlling viral pathogens. Dengue is known to activate B cells and results in the production of virus-specific IgM, IgG, and IgA antibodies, a portion of which bind the viral envelope protein and neutralize virions, thereby preventing entry into target cells. IgE is produced during dengue infection, and as noted in the mast cell section, would serve to activate innate immune cells through the high affinity Fc epsilon receptor one expressed at high levels on mast cells and upregulated on activated dendritic cells. Importantly, prior infection and antibody levels are a major risk factor for development of more severe disease as discussed in the *Introduction*: (dengue virus and clinical spectra). In the case of a heterotypic infection with a different dengue serotype, patients are at increased risk to develop dengue hemorrhagic fever and/or dengue shock syndrome. In these cases, sub-neutralizing levels of cross reactive antibodies facilitate entry into increased numbers of target cells *via* Fc receptor mediated uptake (Halstead, 1979; Halstead, 1988; Halstead, 1994). This is thought to increase the overall infection burden, leading to higher titers of circulating virus and greater inflammation. The cell-mediated arm of the adaptive system consists of CD4+ helper T cells and CD8+ cytotoxic T cells that function to promote B cell activation and killing of virally infected host cells. CD8+ and CD4+ T cells are known to be activated in large numbers during dengue virus infection (Tian et al., 2019). Several studies have demonstrated that T cell epitopes are present across the viral proteome and T cell activation can result in both protective and pathogenic outcomes. Significant evidence suggests that dengue can induce cross reactive T cell activation, termed T cell original sin (Mongkolsapaya et al., 2003; Rothman, 2011). In this setting cross-reactive T cells, specific for the primary infecting serotype, become predominant during a secondary heterologous infection. This expansion of preexisting cross-reactive and low-affinity memory T cells is thought to hamper effective viral control and contribute to severe disease through enhanced production of inflammatory cytokines. However several studies have also demonstrated a protective role for dengue specific T cells in controlling infection. In murine models both CD4+ T cells and CD8+ T cells play a protective role against DENV infection preventing severe disease and facilitating viral clearance (Yauch et al., 2009; Zellweger et al., 2010; Zellweger et al., 2014; Ngono and Shresta, 2018). The protective role of T cells during dengue infections is underscored by studies both in murine model and in humans, that identified protective HLA alleles that are associated with strong and multifunctional T cell responses (Stephens et al., 2002; Stephens, 2010).

DISCUSSION

It is thought that the DENVs evolved into four distinct serotypes approximately 1,000 years ago and each of these four serotypes emerged into a cycle of transmission between humans and its mosquito vector approximately 125 to 320 years ago (Holmes and Twiddy, 2003; Twiddy et al., 2003a). Phylogenetic analysis suggests that the DENVs are rapidly evolving with major clade replacements and genetic shifts occurring in populations endemic for DENV (Holmes, 2003; Twiddy et al., 2003b; Zhang et al., 2005; Holmes, 2006). Asia in particular in this century has been pivotal in the evolution of DENV as the location of the first cases of the more severe form of DENV

infection, dengue hemorrhagic fever (DHF), which made its first appearance in the 1950's first in the Philippines then in Thailand (Halstead et al., 1967). This event was a hallmark denoting a change in the severity and pathogenesis of DENV viral-host interactions. The current Asian genotypes of each serotype are considered more severe and result in more severe dengue illness than the American genotypes (Kochel et al., 2002). Evidence suggests that DENV circulation in Asia due to its population growth and urbanization, high vector burden, and high level of pre-existing flavivirus seroprevalence, has contributed to the increase in genetic diversity of DENV which is estimated as increasing at a factor between 14 and 20 in the last 30 years (Twiddy et al., 2003a). The overall picture of DENV evolution in Asia and now the Americas is the active transmission of viruses in individuals who are highly flavivirus antibody experienced causing evolutionary pressure on the virus to evolve to escape and utilize pre-existing flavivirus immunity. By its nature the current evolving DENVs are adept at escaping heterologous neutralizing antibody and using it as a means to attain high viral load levels and more severe disease through antibody-dependent enhancement. Furthermore, in addition to the need to escape pre-existing adaptive immunity from antibody, the DENVs have evolved unique means to escape the host innate immune response as discussed above. Much needs to be understood about dengue pathogenesis and the viral-host interactions that result in severe disease. Utilization of the human infection model, improved techniques to understand the host genetic and immune response, and additional prospective human studies will further our understanding with application towards developing better drugs and vaccines to treat and prevent DENV infection.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct. and intellectual contribution to the manuscript, and approved it for publication.

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Regulation of Host Innate Immunity by Non-Coding RNAs During Dengue Virus Infection

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An estimated 3.9 billion individuals in 128 nations (about 40% of global population) are at risk of acquiring dengue virus infection. About 390 million cases of dengue are reported each year with higher prevalence in the developing world. A recent modeling-based report suggested that half of the population across the globe is at risk of dengue virus infection. In any given dengue outbreak, a percentage of infected population develops severe clinical manifestations, and this remains one of the "unsolved conundrums in dengue pathogenesis". Although, host immunity and virus serotypes are known to modulate the infection, there are still certain underlying factors that play important roles in modulating dengue pathogenesis. Advanced genomics-based technologies have led to identification of regulatory roles of non-coding RNAs. Accumulating evidence strongly suggests that viruses and their hosts employ non-coding RNAs to modulate the outcome of infection in their own favor. The foremost ones seem to be the cellular microRNAs (miRNAs). Being the post-transcriptional regulators, miRNAs can be regarded as direct switches capable of turning "on" or "off" the viral replication process. Recently, role of long non-coding RNAs (lncRNAs) in modulating viral infections *via* interferon dependent or independent signaling has been recognized. Hence, we attempt to identify the "under-dog", the non-coding RNA regulators of dengue virus infection. Such essential knowledge will enhance the understanding of dengue virus infection in holistic manner, by exposing the specific molecular targets for development of novel prophylactic, therapeutic or diagnostic strategies.

Keywords: innate immunity, lncRNA, miRNA, sfRNA, circRNA, dengue virus, lincRNA

INTRODUCTION

Dengue, a mosquito-borne viral disease, is prominent in tropical and sub-tropical regions across the world. It is caused by the dengue virus (DENV) that circulates in the form of four serotypes, DENV-1 to 4. In the past decades, the incidence of dengue has strikingly increased, such that almost half of the global population remains at risk of this infection. The annual estimates for DENV infections are

between 100 and 400 million (WHO, 2020). As it is with any other communicable disease, community involvement remains the most sustainable control measure along with the effective vector control efforts. Furthermore, in conjunction to the non-pharmacological control measures, understanding of the biology of dengue is of utmost importance; in order to continue development of novel therapeutic and prophylactic strategies. While the RNAi- mediated regulation of genes has been known for quite a while now; the back-end control of gene regulation has been of much interest recently. For instance, the role of long non-coding RNAs (lncRNAs) (that themselves act as precursors to miRNAs) in modulation of important immune responses is being investigated. Also, the extent of viral non-coding RNAs in modulation of host innate immunity is also being understood. The present review is an attempt to comprehend the role of non-coding RNAs in modulation of hosts' innate immune defense during DENV infection.

OVERVIEW OF DENGUE VIRUS STRUCTURE AND INFECTION

DENV has been placed in the *Flaviviridae* family under the genus *Flavivirus* and circulates worldwide (endemic in >100 countries) in the form of four serotypes, all of which are assumed to have been originated and later on independently evolved from the strains circulating in the Asian-Oceanic region (Wang et al., 2000). The virus is spherical and enveloped exhibiting the icosahedral symmetry, a lipid bilayer and a nucleocapsid core coating the positive-sense single-stranded RNA (ssRNA) genome (Kuhn et al., 2002). The viral genome (about 10,700 bp) codes for a single precursor poly-protein (approx. 3,411 amino acids long) from which the other functional viral proteins (three structural and seven non-structural) are processed. Of the three structural proteins, *viz*., capsid, precursor membrane, and envelope, the envelope glycoprotein remains the focus of interaction with the neutralizing antibodies as it is involved in receptor attachment and fusion facilitating viral entry into the host cells (Chambers et al., 1990). The precursor membrane protein along with the envelope protein forms a trimeric protrusion in immature virions' surface (Zhang Y. et al., 2003). The capsid protein attached with the viral RNA genome is located beneath the outer protein coat and the lipid bilayer (Zhang W. et al., 2003). The non-structural proteins, *viz*., NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5, have found to be primarily related to evasion of host's immune responses and viral replication (Uno and Ross, 2018).

DENV can infect a variety of cell types, like, dendritic cells (DCs), endothelial cells, fibroblasts, keratinocytes, macrophage, mast cells, and monocytes (Garcia et al., 2017), and hence is known to utilize a diverse range of host surface receptors [like, heparan sulfate- lectins, DC-SIGN, mannose receptor of macrophages, lipopolysaccharide (LPS) receptor CD14, Heat-shock proteins 70 and 90, endoplasmic reticulum chaperonin GRP78, TIM-1, AXL, Claudin-1 proteins, etc.] to enter into the host cell (Cruz-Oliveira et al., 2015). Among the known target cell types, DENV antigens are expressed on cell surfaces of lymphocytes, monocytes and macrophages, as revealed by human post mortem studies. An important characteristic feature of DENV infection is that the DCs are the direct targets of profound infection by DENV, while in other viral hemorrhagic fever cases, endothelial cells are the direct targets. In studies involving human dengue cases, it has been observed that the endothelial cells of lungs and spleen express viral proteins at a relatively lower frequency, but definitely not the viral RNA (Srikiatkhachorn and Kelley, 2014). The DENV envelope glycoprotein interacts with one of such available host cell receptors to enable its entry *via* clathrin-mediated endocytosis; following which a decline in endosomal pH occurs leading to a conformational change in the virion, fusion of the membranes, and eventual release of the viral genome into the cytoplasm (Heinz and Allison, 2000).

Owing to the positive sense orientation, the viral RNA genome, once inside the host cell cytoplasm, gets readily translated into polyprotein by the host ribosome (Polacek et al., 2009). This follows cleavage of the polyprotein by both DENV and host proteases into functional viral proteins. The progeny virion assembly takes place in the golgi apparatus where an inefficient process, i.e., cleavage of precursor membrane protein by host furin protease happens (Welsch et al., 2009; Junjhon et al., 2010). The process is considered inefficient as it generates immature and partially mature virions along with the infectious mature virions that finally exit the host cell *via* exocytosis.

INNATE IMMUNE RESPONSES TO DENGUE VIRUS INVASION

The DENV initially propagates in skin cells (keratinocytes and Langerhans cells) (Garcia et al., 2017) inducing the innate arm of the immune system. DCs, macrophages, and monocytes are quick to respond by recognition of pathogen-associated molecular patterns *via* pattern recognition receptors (PRRs) (Akira and Takeda, 2004; Loo and Gale, 2011), *viz*., cytoplasmic retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), along with endosomal Toll-like receptor 3 (TLR3) and TLR7 (**Figure 1**) (Wang et al., 2006; Nasirudeen et al., 2011). This, in turn, stimulates type I interferon responses, secretion of cytokines and chemokines, eventually establishing an antiviral state. The RIG-I and MDA5 [both of which belong to RIG-I like receptors (RLRs)], upon identification of the DENV *RNA in the cytoplasm* translocate to mitochondrial membrane, and cause stimulation of mitochondrial antiviral signaling (MAVS) protein, leading to activation of TANK-binding kinase 1 (TBK1), IκB kinase- ϵ (IKKϵ), phosphorylating IFN regulatory factors (IRF3), and IRF7. These molecules translocate into the infected cell's nucleus and trigger production of type I IFNs (Seth et al., 2005). The TLR responses work a little differently; in a sense that the *double stranded* and *single stranded RNA* molecules of

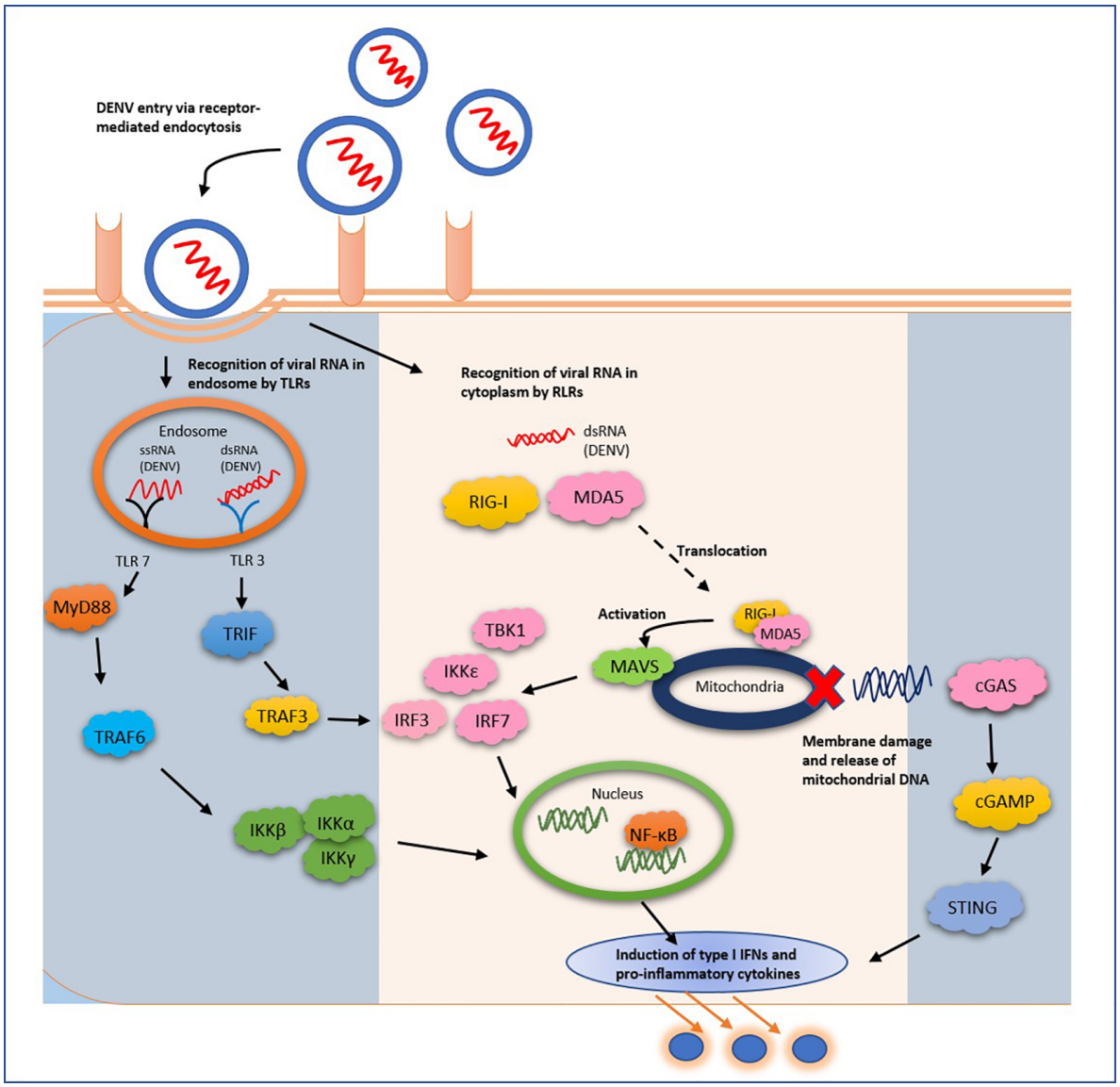

FIGURE 1 | Initial establishment of anti-viral state *via* innate immune responses to DENV infection. As soon as the DENV invades host cells, the TLRs (blue panel, left) and RLRs (pink panel) recognize the viral RNA and induce a series of signals to establish a state of emergency. As described in the text, the series of signaling lead to mitochondrial damage and release of the organelle DNA (blue panel, right), furthering type I interferon responses. This antiviral state in the initial host cells is able to extend up to the adjoining cells *via* IFN signaling. Various non-coding RNAs (as described in the text) regulate different targets in the innate immune responses (demonstrated above) including the complement system pathway (not included in the above figure).

DENV are identified *in endosomes* and *DC endosomes* by TLR3 and TLR7, respectively (Baum and García-Sastre, 2010). The TLR3 functions in sync with the RLRs in establishing an antiviral state against the viral invasion (Nasirudeen et al., 2011). The activated TLR3 induces IFN-α/β-stimulating-genes (ISGs) and chemokines *via* interaction of phosphorylated TRIF (TIR-domain-containing adapter inducing IFNβ), TRAF3 (TNF-receptor-associated factor 3) and TBK1/IKKε (Akira and Takeda, 2004). On the other hand, TLR7 stimulates secretion of pro-inflammatory cytokines *via* the MyD88 (myeloid differentiation primary response gene 88) dependent signaling. The pathway involves TRAF6-mediated activation of nuclear factor-κB (NF-κB) (Wang et al., 2006). In another series of responses, the STING (stimulator of IFN gene) pathway gets activated by the cyclic GMP-AMP synthase (cGAS) PRR. It is important to note that the STING pathway recognizes the cytoplasmic DNA (Sun et al., 2017). During infection by the DENV, the STING pathway gets activated due to the release of mitochondrial DNA (mtDNA) into the cytoplasm, following DENV-led damage of the mitochondria (Aguirre et al., 2017), eventually causing production of type I IFNs. Under *in vitro* conditions, the presence of mtDNA has been shown to stimulate

another endosomal PRR, the TLR9, which enables identification of DNA harboring non-methylated CpG motifs in human DCs (Lai et al., 2018).

Furthermore, innate immune responses have been shown to regulate severity of DENV infection. A study involving human challenge experiments revealed the role of sustained induction of IFN-gamma in acute DENV infections (Gunther et al., 2011). Similarly, the C-type lectin domain family 5, member A (CLEC5A) protein has been shown to regulate cytokine storm in dengue-infected mice model (Chen et al., 2008; Sung et al., 2019). Single cell RNA-seq of dengue-infected patients revealed that MX1 and IFIT1 were highly upregulated in DENV patients before the development of severe disease (Zanini et al., 2018).

The establishment of this initial antiviral state and the subsequent type I IFN responses somehow protect the other monocytes from the DENV threat (Diamond et al., 2000). This is attained by activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway and secretion of ISGs in adjoining cells, in order to maintain and enhance the antiviral state (Morrison and García-Sastre, 2014).

The DENV infection also attracts reaction from the complement system. As the name suggests, the mannose-binding-lectin (MBL), which is considered as an important soluble PRR of the innate immunity, binds to the mannose-harboring glycans on the DENV. This leads to activation of the MBL pathway that ultimately leads to neutralization of the DENV (Avirutnan et al., 2011) and inflammation (Fujita et al., 2004).

ROLE OF NON-CODING RNAs IN INNATE IMMUNE RESPONSES TO DENV INFECTION

Acting as Antivirals

Apart from the traditional innate immunity-led defense against the DENV infection, host or viral non-coding RNAs are also shown to project protection. During viral infection, the host miRNA along with the RNA-induced silencing complex (RISC) identifies and degrades the viral RNA (Jeang, 2012). An *in vitro* study demonstrated that the DENV infection leads to suppression of the host RNAi agents, *viz*., Dicer, Drosha, Ago1, Ago2; and that an inhibition of these RNAi factors led to elevated DENV titer in the human hepatocellular carcinoma "Huh7" cells. The researchers found that the transmembrane domain 3 (TMD3) of the non-structural 4b protein caused this suppression of RNAi during infection by any of the four DENV serotypes (Kakumani et al., 2013). Similarly, the role of hsa-mir-126-5p in negative regulation of DENV infection was also studied (Kakumani et al., 2016). In a study, it was shown that transfection of miR-126-5p (an miRNA mimic of has-126-5p) in Huh-7 cells led to increase in hsa-126-5p levels by 3.41 fold and a simultaneous 70% decline in vRNA levels after 24 h of virus infection (Kakumani et al., 2016). Different miRNAs, *viz*., let-c, miRNA-30e*, and miRNA-126-5p, are reportedly modulated during DENV infection. MicroRNAs also identify or be identified by the TLRs or the RLRs (RIG-I and MDA5), eventually modulating the innate immunity to DENV infection (Urcuqui-Inchima et al., 2017). In a microarray expression analysis, 89 dysregulated miRNAs were found to be associated with 499 potential targets during treatment of critical DENV infection. Among the top-hit targets, DDX3X (DEAD-Box Helicase 3, X-Linked) and PTEN (Phosphatase and Tensin Homolog) were speculated to have important roles in DENV infection. While, the DDX3X is an important regulator of cell proliferation and is able to induce IFN promoter branches during DENV-infected cells, indicating its antiviral effect being modulated by respective miRNA (Shahen et al., 2018), PTEN is known to exhibit antiviral effect against dengue (Wagstaff et al., 2012). Likewise, miR-30e* is also known to exert antiviral impact by furthering the production of IFN-β *via* the NF-κB pathway during dengue (Zhu et al., 2014). A small RNA-seq analysis revealed differential expression of five miRNAs in DENV-infected and -exposed but non-infected human primary macrophages. The DENV-non-infected macrophages expressed elevated levels of miR-3614-5p, which acted as an antiviral agent by targeting a DENV pro-viral protein, adenosine deaminase acting on RNA 1 (ADAR1) (Diosa-Toro et al., 2017).

In another interesting analysis, it was found that over-expression of hsa-miR-133a negatively regulates replication of all four serotypes of DENV (Castillo et al., 2016) in Vero cells, probably *via* the polypyrimidine tract binding protein (PTB). The PTB is known to have role in IRES-independent translation of viral/cellular RNA. In case of dengue, PTB binds to the 3′-UTR of the viral genome and furthers the viral RNA replication, probably by acting as a RNA helicase (De Nova-Ocampo et al., 2002). The miR-133a was speculated to target PTB specifically during the initial hours of DENV infection in the Vero cells. As a counter mechanism, all four serotypes of DENV suppress the endogenous levels of miR-133a so as to allow high expression of PTB leading to viral replication during about 12 h of infection (Castillo et al., 2016), pointing towards negative regulation of DENV by miR-133a. Such an interplay highlights important role of non-coding RNA mediated modulation of dengue.

Overexpression of some of the other non-coding RNAs, *viz*., miR-548g-3p (Wen et al., 2015), miR-484 and miR-744 (Castrillón-Betancur and Urcuqui-Inchima, 2017), inhibit replication of all the four DENV serotypes. MiR-548g-3p interferes with the expression of viral proteins as well. The miR-548g-3p targets the 5′-UTR of DENV genome, specifically at the stem loop A promoter region and leads to suppression of virus propagation in an interferon-independent manner (Wen et al., 2015).

Acting as Pro-Virals

The best known pro-viral miRNA, which is highly expressed in monocytes during dengue, is the miR-146a (Wu et al., 2013). MiR-146a is a known regulator of innate immunity, inflammatory responses and viral replication (Li et al., 2010). Wu and colleagues hypothesized that the miR-146a elevates replication of DENV serotype-2 by decreasing the host IFN-β production *via* targeting tumor necrosis factor receptor (TNFR)–associated factor 6 (TRAF6) (Wu et al., 2013). However, an

overexpression of the miR-146a substantially inhibited DENV-2 *via* autophagy (Pu et al., 2017). It was also found that an antagonist, LNA-antagomir-146a was able to suppress the miR-146a effect and restore the host IFN activity (important host antiviral defence mechanism) against the virus. In a later study, the serum levels of miR-146a seemed to be reduced along with a negative correlation with serum AST/ALT levels in dengue subjects. This indicated a possible role of miR-146a in liver inflammation (Ouyang et al., 2016). Similarly, miR-21 is also a known inhibitor of pro-inflammatory response (Sheedy, 2015) and hence its expression is upregulated during dengue Ouyang et al., 2016). The miR-21 also augments DENV-2 replication in HepG2 cells (Kanokudom et al., 2017). The miR-21 is thought to target NS1 protein of DENV-2 (Wong et al., 2020), which is known to evade the complement innate immune responses by blocking the classical pathway C3 convertase (Avirutnan et al., 2010), and also escape the MBL-mediated neutralization (Thiemmeca et al., 2016).

Apart from the host factors, viral non-structural 3 (NS3) protein is also known to regulate biogenesis and function of host miRNAs in human embryonic kidney (HEK) 293T cells. Amazingly, the negative regulation of host miRNAs exerted by the DENV NS3 was found to be stage-specific to enable up-regulation of the viral host factors, *viz*., up-regulation of TAZ (tafazzin) and SYNGR1 (synaptogyrin 1), facilitating DENV replication (Kakumani et al., 2015).

A high throughput RNA sequencing analysis revealed a significant up- or down-regulation of various lncRNAs in L-02 liver cells post DENV infection. Upon analysis of the lncRNA-mRNA co-expression networks, 68 and 50 interacting nodes were identified by infection of DENV serotype 1 and 2, respectively. The differentially expressed lncRNAs were observed to be potential precursors to mature miRNAs, *viz*., hsa-mir-29b-2, -29c, -22, -1268a, and -3648, and were found to be associated with various biological processes in host cells during DENV infection, such as, biosynthesis, nucleic acid related processes, estrogen signaling, cytoskeleton reorganization, stimulation of apoptosis, to point a few (Wang et al., 2017). In another genome-wide profiling analysis of mRNA and lncRNA expression during dengue and dengue haemorrhagic fever (DHF), 215 and 225 lncRNAs were differentially expressed in dengue and DHF, respectively. Upon, functional analysis, *MAGED1*, *STAT1*, and *IL12A* genes were found to be significantly dysregulated. *MAGED1* has been linked to severe dengue (Silva et al., 2013), *STAT1* is known to supplement protective antiviral interferon responses in presence of schisandrin A against DENV replication (Yu et al., 2017), and *IL2A* stimulates IFN-γ production and differentiation of Th1 and Th2 cells (Lamont and Adorini, 1996). The role of lncRNA in dengue disease progression has also been studied. RNA sequencing was used to investigate and compare the expression profiles of various lncRNAs and protein-coding genes in samples collected from dengue patients exhibiting different extent of severity and in samples from patients presenting with other febrile illnesses. Nuclear Enriched Abundant Transcript 1 (NEAT1), which is a non-coding RNA, and the coding gene Interferon alpha-inducible protein 27 (IFI27) were highly co-expressed and negatively associated with the degree of dengue severity (Pandey et al., 2017). NEAT1 is an important regulator of innate immunity, as it affects the transcriptional regulation of several anti-viral genes (Ahmed and Liu, 2018). This might explain NEAT1 as a differentiating bio-marker of severe dengue from dengue infection (Pandey et al., 2017). In another study, the transcript levels of long intergenic non-coding RNA (lincRNA) in DENV2 infected mosquitos showed 32% decrease in lincRNA post infection in *Aedes aegypti* whereas majority of lincRNAs were over-expressed. The transcription levels of 72 lincRNAs were up-regulated post infection. Supporting the role of certain lincRNA through RNAi mediated silencing of lincRNA_1317 in Aa20 cells and then infected by DENV2 increased the viral replication and infection progression proving that this lincRNA is important for anti-viral response, it was also over expressed in infected mosquitos rather than non-infected ones (Etebari et al., 2016).

Another set of interesting non-coding RNAs are the circular RNAs (circRNAs) that lack free 5′ and 3′ ends and have a closed loop instead. CircRNAs are referred to as transcriptional products that are developmentally regulated at tissue or cell type levels (Barrett and Salzman, 2016). The exact role of circRNAs is not clear at present (Barrett and Salzman, 2016). With respect to human diseases, it has been shown that the levels of certain circRNA vary as per the disease profile. For instance, expression of hsa_circ_0015962 and miR-133b were reportedly elevated in post-treatment group than the pre-treatment group of dengue fever patients. The treatment, here, refers to administration of pain relievers, intra-venous fluids and critical care at hospital to severe dengue patients. Expression of hsa_circ_0006459 and miR-4683 was found to be lower in the post-treatment group than in the pre-treatment group. Furthermore, it was demonstrated that the hsa_circ_0015962 binds and negatively modulates the expression of miR-4683, while the hsa_circ_0006459 targets and negatively regulates miR-133b (He et al., 2019).

In addition to the non-coding RNAs expressed by host, some are expressed by viruses also. During replication of flaviviruses, the uncapped genomes are digested by the host 5′ to 3′ exoribonuclease, however, the process gets stopped when a pseudoknot RNA structure is encountered in the 3′ UTR region. This results in formation of about 0.3 to 0.5 kb sized sub-genomic flavivirus RNA (sfRNA). Elevated levels of sfRNA lead to inhibition of TRIM25 gene and can deactivate RNA binding proteins which are crucial for innate immunity. Reduced guide RNA (gRNA) levels may cause lower stimulation of RIG-I/MDA5, which are the initial drivers of innate IFN responses. Briefly, the DENV-2 clade (PR-2B) sfRNA interacts with TRIM25 (which is an E3 ubiquitin ligase and also an RNA-binding protein), averting ubiquitin-specific peptidase 15 (USP15) to deubiquitinylate TRIM25. This, in turn, stops TRIM25 to polyubiquitinate RIG-I causing a drop in IFN production. This can ultimately render several host cells susceptible to DENV causing viremia (Manokaran et al., 2015). The available literature indicates that the higher sfRNA production and also the structure or sequence of the sfRNA may have important implications on epidemiological fitness of

DENV-2. For instance, the 1994 dengue outbreak in Puerto Rico can be understood in terms of the sfRNA-gRNA ratios. Higher levels of the sfRNA and the decreased levels of gRNA lead to inhibition of TRIM25 and suppressed trigger of RIG-I/MDA5 responses, respectively. And it is quite well-known that the early interferon responses are important to combat establishment of the viral infection and halt the mosquito-borne transmission.

Further, DENV also encodes functional viral small RNAs (vsRNAs). One such vsRNA, DENV-vsRNA-5, was found to act similar to miRNA and exhibited important role in "autoregulation" of virus replication. DENV-vsRNA-5 targets the virus nonstructural protein 1 (NS1) gene and suppresses DENV-1, -2, and -4 replication in mosquito cells (Hussain and Asgari, 2014). This is an interesting finding of virus autoregulation mechanisms and may be of interest for further exploration and use of small RNAs as antiviral agents.

DIFFERENTIATION OF MILD VS. SEVERE DENGUE

Severe dengue is reported to be strongly linked with "cytokine storm", a condition when the pro- and anti-inflammatory mediators get imbalanced. SOCS family of proteins have been known to negatively regulate various signaling pathways. SOCS1, a controller of several cytokines, is negatively regulated by miR-150 during Dengue Haemorrhagic Fever (DHF) (Chen et al., 2014). Chen and colleagues observed that whereas miR-150 was highly induced in DHF patients, levels of SOCS1 were reduced in the same thereby pointing toward the reciprocal interplay among SOCS1 and miR-150. Another study further confirmed these findings and observed enhanced levels of miR-150 in severe dengue patients (Hapugaswatta et al., 2020). SOCS1 was further identified in patients with acute dengue infections (Hoang et al., 2010). The reports suggest that SOCS-1 protein is dysregulated in dengue patients and might be one of the contributing factors toward cytokine storm during dengue pathogenesis. Decreased expression of miR-106b, miR-20a, and miR-30b during DENV-2 is also thought to elevate production of pro-inflammatory cytokines (Qi et al., 2013). MiR-let-7e possibly regulates IL-6 and CCL3, while miR-451 and miR-4279 are deemed as modulators of CCL5 and CXCL1 expression. Low miR-106b expression might lead to increased secretion of CCL5, which is one of the important host factors during viral replication, especially DENV2 infection. Also, the chemokine CCL5 is rapidly produced by mast cells when activated by the DENV-antibody complexes (Brown et al., 2012) and also related to DENV- triggered hepatic dysfunction (Conceicao et al., 2010).

Some interesting studies have been aimed at deciphering the role of non-coding RNAs in distinguishing mild dengue with severe dengue and the related complications. One such interesting study involved comparison of miRNA profiling in blood specimens of dengue and influenza patients. The two categories of diseased cases were taken to enable identification of unique miRNA signatures of dengue. As speculated, of the 106 dysregulated miRNAs associated with acute dengue, 14 miRNAs displayed similar expression profiles in both the diseases, while 12 were unique to acute dengue, i.e., within 0-4 days of the illness. Upon functional analysis, these 12 miRNAs (hsa-miR-450b-5p, -491-5p, -499a-3p, -512-5p, -615-5p, -624-5p, -892b, -1204, -1225-5p, -3121-3p, -4259, and -4327) were found to regulate the P13K/AKT survival pathway. Among these, hsa-miR-1204, 491-5p, and 512-5p seemed to have important roles in apoptosis, P13K/AKT pathway and indirect modulation [via NOD2 (nucleotide-binding oligomerization domain containing 2) gene] of NF-kB, JNK, and MAPK pathways, interleukins, and cytokines, respectively. Moreover, 17 miRNAs were also identified that specified complications arising from dengue, for instance, liver complications, abdominal pain, and capillary leak excluding shock (**Figure 2**) (Tambyah et al., 2016). The miRNAs hsa-miR-24-1-5p, miR-512-5p, and miR-4640-3p were significantly varied in expression profiles between dengue fever (DF) and dengue with liver complications (DFL) patients. Also, the hsa-miR-383 showed 6.3 fold upregulated levels in DF than in the dengue fever with clinical fluid accumulation (DHF) subjects. The expression of miRNAs, *viz.*, hsa-miR-624-5p,

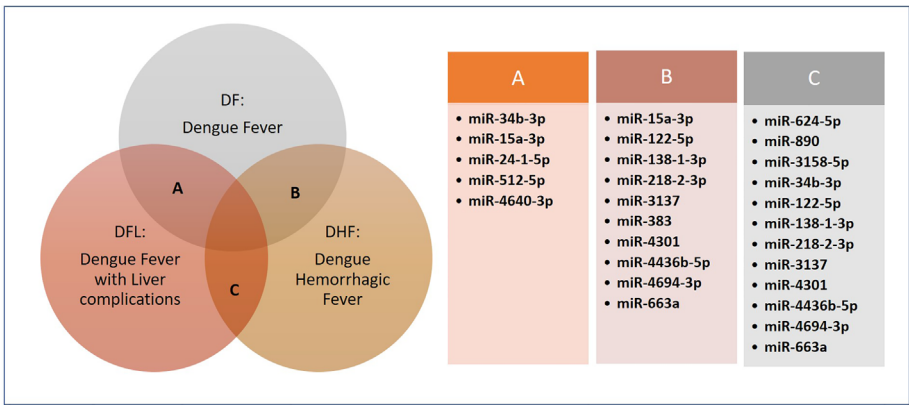

FIGURE 2 | Differentially expressed miRNAs in mild vs. severe dengue conditions.

miR-890 and miR-3158-5p, varied among the severe dengue DFL and DHF categories also. An interesting finding was the expression of hsa-miR-15a-3p, which was highly (48 fold; $p < 0.0005$) downregulated in DF subjects, but only mildly (approx. 5-fold) downregulated severe dengue (DFL and DHF) patients. These observations are crucial indicators that the miRNAs and their extent of dysregulation can be important differentiators of mild and severe dengue (Tambyah et al., 2016).

The previously discussed antiviral miR-744 (Castrillón-Betancur and Urcuqui-Inchima, 2017) may also be a differentiating marker of mild vs. severe dengue, as it targets an important inflammation regulatory protein, TGF-β1 (Martin et al., 2011). TGF-β1 gene polymorphism (−509 CC genotype) is known genetic marker for DHF susceptibility and high viral load (Chen et al., 2009).

A recently published report utilized NGS based approaches to identify circulating miRNAs among DENV infected patients. The study could successfully correlate the dysregulation of several miRNAs among mild and severe dengue patients. (Saini et al., 2020). Specifically, the expression profile of hsa-miR-122-5p in plasma specimens was found to be an important differentiator of dengue infection stage. The miRNA could also demarcate between dengue-negative subjects from other febrile illnesses.

CONCLUSION

The endemic state of dengue in many tropical and sub-tropical nations imposes a serious risk for the other similar climate areas. The symptoms, generally, start surfacing after 4–5 days of incubation period. Since its origin, still there is no effective treatment or vaccine for dengue. Hence, modulation of the molecular regulators, like the various dysregulated non-coding RNAs seem to be lucrative therapeutic option. Further investigations in this area would definitely garner confidence and effective practical use of this approach.

AUTHOR CONTRIBUTIONS

RR and VS conceived and designed the study. RR collected the data, prepared the initial draft and figure, proofread, revised, and approved the final manuscript. JS provided substantial intellectual input, proofread and revised the manuscript, and edited the figure for final approval of the manuscript. MN collected data for initial draft and provided intellectual inputs. MK proofread and provided critical inputs. PA proofread and contributed to the manuscript's structure. VS conceived the work, proofread, provided substantial intellectual inputs, and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Dengue Virus Non-Structural Protein 5 as a Versatile, Multi-Functional Effector in Host–Pathogen Interactions

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Dengue is emerging as one of the most prevalent mosquito-borne viral diseases of humans. The 11kb RNA genome of the dengue virus encodes three structural proteins (envelope, pre-membrane, capsid) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), all of which are translated as a single polyprotein that is subsequently cleaved by viral and host cellular proteases at specific sites. Non-structural protein 5 (NS5) is the largest of the non-structural proteins, functioning as both an RNA-dependent RNA polymerase (RdRp) that replicates the viral RNA and an RNA methyltransferase enzyme (MTase) that protects the viral genome by RNA capping, facilitating polyprotein translation. Within the human host, NS5 interacts with several proteins such as those in the JAK-STAT pathway, thereby interfering with anti-viral interferon signalling. This mini-review presents annotated, consolidated lists of known and potential NS5 interactors in the human host as determined by experimental and computational approaches respectively. The most significant protein interactors and the biological pathways they participate in are also highlighted and their implications discussed, along with the specific serotype of dengue virus as appropriate. This information can potentially stimulate and inform further research efforts towards providing an integrative understanding of the mechanisms by which NS5 manipulates the human-virus interface in general and the innate and adaptive immune responses in particular.

Keywords: Flavivirus, NS5, moonlighting proteins, signaling pathways, protein–protein interactions (PPIs), antiviral immunity, apoptosis, spliceosome

INTRODUCTION

Dengue is a global epidemic resulting in over 100 million clinical cases globally each year with symptoms ranging from fever to hemorrhage and/or shock that can be fatal, especially among children (Guzman et al., 2010; Bhatt et al., 2013). The disease is caused by four distinct dengue virus (DENV) serotypes (DENV-1, 2, 3, 4). DENV is a positive-strand RNA virus that belongs to the genus Flavivirus, family Flaviviridae. The genome encodes three structural (Env, PreM, Capsid) and

seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. Of these NS1 interacts with NS4A/B and promotes viral replication (Chen et al., 2018; Płaszczyca et al., 2019), NS3 performs helicase and protease functions (Swarbrick et al., 2017), NS4A induces autophagy (McLean et al., 2011), and NS4B facilitates dissociation of NS3 helicase from viral RNA (Umareddy et al., 2006).

NS5 is the largest and the most conserved DENV protein. It serves two important functions: one is the RNA-dependent RNA polymerase (RdRp) activity that is required for viral replication (Iglesias et al., 2011). The second is RNA methyltransferase (MTase) activity important for RNA capping during polyprotein translation (Liu et al., 2010; Klema et al., 2016). Additionally, NS5 forms an RNA replicase complex with NS3 in the endoplasmic reticulum during viral replication. After replication, NS5 dissociates from NS3 and translocates to the nucleus (Kapoor et al., 1995). So far, nuclear translocation has been reported for DENV-2 and -3 serotypes (Brooks et al., 2002; Hannemann et al., 2013). Yeast two-hybrid (Y2H) studies suggest that nuclear translocation may occur because the nuclear import receptor importin-β competes with DENV-NS3 for binding with NS5 (Johansson et al., 2001). While the nuclear accumulation of NS5 does not seem to be essential for viral replication (Kumar et al., 2013), it appears to be linked to an increase in the production of the cytokine IL-8 that has been historically correlated with severe dengue (Medin et al., 2005).

Given that NS5 is important for viral replication and serves as a major target for cytotoxic T cell responses (Duangchinda et al., 2010; Alves et al., 2016), there has been much interest to target it for vaccine development and anti-viral interventions. Mutational studies on the NS5-MTase domain identified several residues that are likely to be critical in viral replication (Kroschewski et al., 2008). 2′-O-methylation of the viral RNA is crucial for the dampening of host immune responses at the early stages of the viral life cycle. Abrogation of the 2′-O-MTase by changing a single amino-acid (E216A) results in an earlier activation of anti-viral responses exemplified by RIG-I (a sensor of foreign RNA), IL-8 (a pro-inflammatory cytokine), and IFIT2 (an interferon-induced protein that inhibits translation) leading to viral attenuation (Chang et al., 2016). Several inhibitors of MTase and RdRp activities have been identified by large-scale *in vitro* screening (reviewed by (Lim et al., 2015)). Additionally, NS5 interacts with host proteins such as STAT2 that are critical for type 1 interferon (IFN-I) signaling and innate responses and inhibits host anti-viral responses (reviewed recently by (Ashour et al., 2009; El Sahili and Lescar, 2017). In addition to such well-studied instances, recent high-throughput studies in a variety of experimental systems, as well as bioinformatic analyses, suggest that NS5 interacts with a diverse spectrum of host proteins (Rawlinson et al., 2006; El Sahili and Lescar, 2017; Amemiya et al., 2019). The goal of this review is to provide the interested researcher with a consolidated, annotated list of known and potential NS5-interacting human proteins obtained from multiple studies, highlight significant candidate interactors and situate them in specific biological contexts wherever possible. Additionally, information on the serotype of the viral strain (DENV1–4) used in the cited studies have been retained and highlighted wherever appropriate.

COMPILATION OF NS5-INTERACTING HOST PROTEINS FROM THE LITERATURE

While some of the NS5 interacting host proteins such as STAT2 are well-known, and extensively reviewed (Ashour et al., 2009; El Sahili and Lescar, 2017) the goal of our efforts here is to compile a comprehensive list of NS5 interacting host proteins. We approached this by compilation of NS5 interacting human proteins a) discovered by experimental pull-down studies reported in the literature; b) curated in databases (bioinformatics and Y2H studies). We briefly elaborate on each of these approaches followed by a list of NS5 interacting proteins compiled through these approaches. Finally, we comment on the gaps in our understanding of the role of these interactions and directions that future research in the field could take.

Pull-Down Studies

Typically, pull-down studies have used cell lines that are infected with defined DENV serotypes and/or strains or transfected by DENV-NS5 protein. While this approach has the advantage of direct evaluation of protein-protein interactions (PPIs) the result may be influenced by the cell line used, and the serotype/strain used for infection/transfection. One study infected HEK 293T and Huh7 cells with strep-tagged full-length DENV-2 (strain 16681) and determined 53 binding partners (De Maio et al., 2016). Another study transfected HEK 293T cells with NS5 of DENV-2 (strain 16681) followed by affinity purification-mass spectroscopy (AP-MS) (Shah et al., 2018), and the data so generated were analysed using MiST (mass spectrometry interaction statistics (Verschueren et al., 2015) and CompPASS (Comparison of Multiple Protein Alignments with Assessment of Statistical Significance (Sadreyev and Grishin, 2003). This resulted in the identification of 26 NS5-interacting host proteins. Another study by Carpp et al. (2014) identified 53 interactors of NS5 using HEK293-T cell line. As the addition of affinity tags to the coding sequences of NS5 and NS3 prevented the production of recombinant virions, they used the I-DIRT (isotopic differentiation of interactions as random or targeted) immunoaffinity purification method (Tackett et al., 2005). Cell lines grown in the normal medium were transfected with GFP-tagged NS3/NS5 followed by DENV-2 infection. Cell lines growing in media containing isotopically labeled arginine and lysine ($^{13}C_6$, $^{15}N_4$) were mock-transfected followed by DENV2 infection. After lysis of both samples, equal amounts of the extracts were mixed. This approach distinguishes between pre-lysis and post-lysis interactions by identifying non-specific post-lysis interactions due to the increased proportion of heavy relative to light isotopes (Carpp et al., 2014). In a fourth pull-down study (Poyomtip et al., 2016), a full-length DENV-2 construct (strain 16681) with tandem affinity purification (TAP)-tagged NS5 containing a poly-histidine and FLAG tags (inserted following N173 in MTase domain of NS5) was

propagated in BHK21 cells followed by infection in Huh-7 cells. The NS5 complexes were isolated *via* FLAG-IP and analyzed by mass spectroscopy. This study revealed 97 NS5 interactors, prominent among them being heterogeneous nuclear ribonucleoproteins (hnRNPs) and proteins involved in lipid metabolism (Poyomtip et al., 2016).

Information From Databases and Yeast Two-Hybrid Studies

We used P-HIPSTer (pathogen-host interactome prediction using structure similarity; http://phipster.org) which is a database of computationally predicted PPIs compiled for a set of 1,001 fully sequenced human-infecting viruses. The predictions are based on protein structural similarity and homology modeling, exploiting both sequence and structure-based information to infer interactions between pathogen and human proteins (Lasso et al., 2019). This database employs the extensively validated Pre-PPI (predicting protein-protein interactions) algorithm for its predictions. Additionally, we also used DenvInt (https://denvint.000webhostapp.com/) which is a dengue-specific database of serotype-related experimental evidence of PPIs based entirely on experimental evidence (Dey and Mukhopadhyay, 2017). It curates data from Y2H, bacterial two-hybrid, pull-down, and co-localization experiments (Khadka et al., 2011; Le Breton et al., 2011; Mairiang et al., 2013). This database indicates that of all DENV proteins, NS5 interacts with the largest number of human proteins (152).

Based on databases and published studies, we have compiled a total of 377 proteins that are known/predicted to interact with DENV-NS5 protein. **Figure 1A** depicts the number of interacting proteins identified by each of the above-stated methods of discovery. **Supplementary Table 1** provides an extensive annotated list of these different NS5 interactors along with the serotype and method by which these are deduced. The minimal overlap of the NS5 interactors deduced by these different approaches may be due to the differences in overall methodologies. Pull-down studies use specific cell lines, viruses, or viral strains as explained above. Overexpression of target proteins in cell lines through transfection does not mimic the actual viral infection scenario, pull-down studies can lead to the precipitation of protein complexes, whose components may not all directly interact with the target protein. Extensive washing steps involved in this protocol may lead to dissociation of weak or transitory interactors. Yeast two-hybrid, though a rapid technique for large scale screening of PPIs, does not truly reflect the sub-cellular localization of the expressed protein or the abundance of the interacting proteins inside the cell. However, though bioinformatics analysis has the advantage of taking into consideration many viral variants and the conserved amino acids among them, which is usually not feasible in experimental systems that rely on a limited set of viral strains, it can produce potentially false-positive results.

Using the available data from the combination of approaches described above, we determined the biological pathways that these interactors are potentially involved with using the KEGG database, Gene Ontology (GO) analysis, and WEB-based GEne SeT AnaLysis Toolkit (Liao et al., 2019) available at http://www.webgestalt.org/. **Supplementary Figure 1** provides top biological processes, cellular components, and functions of these NS5 interactors. **Supplementary Table 2** provides an extensive list of the top pathways with a false discovery rate < 0.05. **Figure 1B** outlines some of the major pathways that are enriched for NS5 interacting host proteins pertaining to JAK-STAT signaling pathway, spliceosome, cell cycle, protein processing in ER, necroptosis, and protein synthesis. These are further elaborated in the section below.

CRITICAL COMMENTS ON THE GROWING LIST OF NS5-INTERACTING HUMAN PROTEINS

The most studied NS5 interactor is STAT2. Su et al. reported that SUMOylation of DENV-NS5 is vital for suppressing STAT2-mediated IFN responses (Su et al., 2016). Excellent reviews are available on this subject and thus we are not elaborating on this aspect further (El Sahili and Lescar, 2017). Interestingly, the expanding list of NS5 interactors started revealing several other proteins that are involved in JAK-STAT signaling as outlined in **Figure 1B**, some of which are deduced by pull-down studies (STAT2, MTOR), some by Y2H studies (PIAS1, PIAS3, IFNAR2, TYK2 IFNGR1, IFNGR2), and the others by bioinformatics approaches (STAT1, STAT3, GRB2, EP300) (See **Figure 1B**). It is interesting to note that dengue NS5 not only interacts with IFN-α/β Receptor Subunit 2 (IFNAR2) but also interacts with interferon-gamma receptors 1 and 2 (IFNGR1, IFNGR2). This raises the possibility that NS5, in addition to interfering with the JAK-STAT signaling pathway (Best, 2017), may also interfere with the action of Type I IFN's or IFNγ, which are the key innate and adaptive anti-viral cytokine respectively. Notably, a case-control study that sequenced the DENV-1 NS5 gene in 31 patients of varying disease severity found that polymorphisms corresponding to amino-acids 124 and 166 (I124M and G166S respectively) correlated with increased disease severity in what was designated as viral "clade 2" relative to "clade1" by the researchers. Computational analysis of these amino acid variants indicated that this effect was probably due to the stronger interaction of clade 2 NS5 with the type-I interferon receptor and Janus kinase-1 (JAK-1), eventually suppressing JAK-STAT signaling (Delgado-Enciso et al., 2018) thereby dampening key pathways of the innate immune response. Further studies are needed to understand which domain of NS5 interacts with these different proteins, and what the direct and indirect effects of these interactions are.

It is interesting to note that the list of NS5 interactors constitutes a large number of proteins involved in the spliceosome machinery. Pre-mRNA splicing is a critical mechanism of gene regulation in eukaryotic cells since a majority of protein-encoding transcripts are alternatively spliced (Lee and Rio, 2015). As mRNA splicing is altered in various pathological conditions, it is a potential target for therapeutic intervention using small molecules (Effenberger et al., 2017). De Maio et al. (2016) showed NS5 binds to

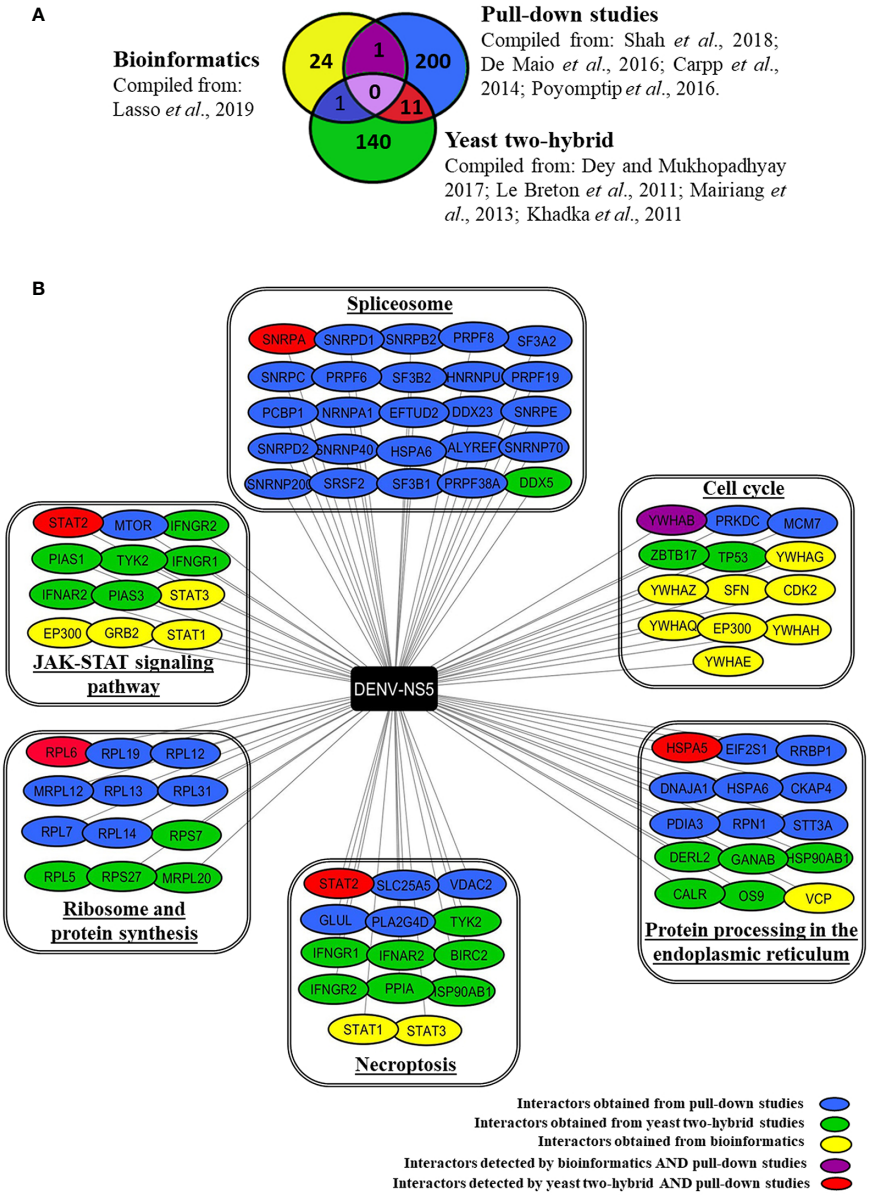

FIGURE 1 | Human interacting partners of DENV-NS5 curated from various experimental studies and databases. **(A)** The Venn diagram indicates the number of DENV-NS5 interacting proteins that are shared with and/or unique to PPI studies in the literature viz., yeast-two-hybrid studies, pull-down studies, and bioinformatics. Yeast-two-hybrid data were curated from the DenvInt database, bioinformatics-based data was obtained from P-HIPSTer, and pull-down data has been derived from published data sources. All cited sources and extended data are compiled and listed in **Supplementary Table 1**. **(B)** Some of the NS5 interactors involvement in key KEGG pathways as obtained using the WEB-based GEne SeT AnaLysis Toolkit. The interactors are grouped in boxes based on the key pathways that they are involved in as obtained from KEGG. The proteins are color-coded according to the method used for their identification. A list of all significant pathways with a false discovery rate (FDR) < 0.05 is given in **Supplementary Table 2**. The complete results of the GO filtering are shown in **Supplementary Figure 1**. SPTAN1 is the only protein detected by both bioinformatics and yeast two-hybrid experiments but has not been shown here because it was associated with a false discovery rate > 0.05 which is the threshold for our compilation.

spliceosome complexes and reduces the efficiency of pre-mRNA processing. Using proteomic analysis and functional experiments, this study demonstrated that NS5 interacts with CD2BP2 and DDX23 from the U5 small nuclear ribonucleoprotein (snRNP) particle to modify the inclusion/exclusion ratio of alternative splicing events, altering the mRNA isoform abundance of known antiviral factors such as CFTR, EDI, and Bclx (De Maio et al., 2016). DENV-NS5 targets nuclear RNA-binding protein 10 (RBM10) for proteasomal degradation. RBM10 regulates alternative splicing, favoring anti-viral mRNA isoforms of proteins such as spermidine/spermine-N1-acetyltransferase (SAT1) (Pozzi et al., 2020). Its

degradation favors pro-viral isoforms, aiding viral replication; however, it is unknown whether this is a direct interaction or not. Interestingly, in this regard, it is interesting to note that NS5 reduces the splicing efficiency of endogenous RIG-I mRNA, and also increases the expression of dominant-negative forms of IKKε during DENV infection, all leading to maintenance of the pro-viral conditions in the cell (De Maio et al., 2016). The NS5 protein of ZIKA and JEV has also been shown to interact with spliceosome-associated proteins (Kovanich et al., 2019). Considering these, it is proposed that NS5 interaction with the spliceosome machinery could be an immune suppression strategy (De Maio et al., 2016). Some recent studies have shed new light on other NS5-interacting human proteins. For example, a ChIP assay study of DENV-2 NS5-transfected HEK293 cells found increased binding of NF-κB on the RANTES promoter than in cells mock-transfected with the empty vector (Khunchai et al., 2015). Elevated RANTES expression in NS5 transfected HEK-293 was validated at both mRNA and protein levels using real-time PCR and ELISA respectively (Khunchai et al., 2015).

NS5 interacts with a host protein, death domain associated protein 6 (Daxx) competitively, which dissociates the Daxx-NF-kB complex. This leads to an increased availability of NF-kB to bind with RANTES promotor and increases RANTES expression (Khunchai et al., 2012). This is very interesting given the observation that NS5 upregulates RANTES which is a key cytokine produced in severe dengue cases (Khunchai et al., 2015; Soo et al., 2017). However, a different study showed that NS5 transfection of HEK293 cells led to upregulation of IL-8 *via* activation of CAAT/enhancer-binding protein (c/EBP) (Medin et al., 2005). Further studies are needed to understand how NS5 transfection influences NF-κB given that NF-κB is a pleiotropic factor that can affect multiple biological processes such as cytokine production, transcription, translation, and apoptosis. In this regard, it is interesting to note that many of the apoptosis-related proteins (e.g., BIRC2; SPTAN1; TUBAL3, etc.) are also shown to interact with dengue NS5 (**Supplementary Table 1**).

Interestingly, NS5 interacts with several proteins that are typically associated with lipid metabolism (fatty acid synthase, hydroxysteroid (17β) dehydrogenase 12, pyruvate carboxylase, ATP citrate lyase). This indicates that NS5 may have a direct role in influencing lipid metabolism (Heaton and Randall, 2010; Carpp et al., 2014; Poyomtip et al., 2016). Further understanding of the role of NS5 in these pathways is important given that lipid metabolism is necessary for viral replication (Melo et al., 2018).

Some of the recent emerging studies are beginning to indicate that NS5 has a causal link in autophagy *via* influencing a host deubiquitinase protein, USP42 expression *via* increased microRNA, miR-590 (Mishra et al., 2019) and TRAF-6 (Pu et al., 2017). However, the interacting partners of NS5 involved in these processes are yet to be identified.

An interesting line of studies in the recent past suggests that NS5 also interacts with promyelocytic leukemia-nuclear bodies (PML-NBs) that are typically involved in several cellular processes including antiviral response (Lallemand-Breitenbach and de Thé, 2010; Khunchai et al., 2012; Giovannoni et al., 2015).

These various lines of evidence indicate that besides the well-known dampening of the initial anti-viral response, NS5 can interact with several other host proteins to influence other aspects of host cell physiology as well. The precise effect of these NS5-host protein interactions on the overall survival and propagation of the virus as well as on the host innate and adaptive immune responses remains to be determined.

FUTURE PROSPECTS

DENV-NS5 interactors participate in a variety of biological processes, most importantly JAK-STAT signalling, RNA processing, cell cycle progression, necroptosis, protein synthesis, and protein processing in the ER among others. DENV-NS5 is an attractive target for drugs and small molecules to inhibit viral replication (Rawlinson et al., 2006; Lim et al., 2015; Shimizu et al., 2019; Troost and Smit, 2020). RNA interference (RNAi)-based approaches have been explored for therapeutic potential against a variety of viral infections, including dengue [reviewed in (Stein and Shi, 2008; Arbuthnot, 2010; Levanova and Poranen, 2018)]. Validating the top hits among the listed NS5-interactors by RNAi in human cell lines and observing the effect of such inhibition of specific host proteins on viral viability or pathogenesis could rapidly identify promising host proteins for disease management. Stepwise investigation of the utility of knocking down interactor-protein levels *via* RNAi and/or deploying interactor decoys to hamper the NS5-interaction with specific host proteins suggest themselves as potential avenues for further clinical research. Some of the NS5-interactors that modulate immune functions or lipid metabolism may serve as potential targets (Canard, 2011). The choice of host protein(s) would be critical, and those involved in more specialized pathways like necroptosis or cytokine production may be preferred over those involved in essential processes like protein synthesis or RNA processing to minimize collateral damage to the host. In case of dengue, RNAi approaches have obtained promising results by targeting TNF-α in cell culture and mice (Subramanya et al., 2010). Furthermore, cell line-based RNAi studies targeting Hsp60 (Padwad et al., 2009), proteins involved in membrane trafficking (Ang et al., 2010) and protein processing in the ER (Savidis et al., 2016), and the IFN-λ receptor 1 (Hsu et al., 2016) indicate that an appropriate choice of host protein, can favorably influence the course of viral infection and disease pathogenesis. Since most of the experimental data on NS5-interacting host proteins available to date are for DENV-2, it would help to learn about serotype-specific differences to fine-tune drug usage. Further investigation of NS5-host protein interactions and their outcomes vis-à-vis viral infection and disease pathogenesis can potentially open novel avenues for effective viral therapy and/or clinical management.

AUTHOR CONTRIBUTIONS

KM-K, AC, and RS contributed to the conception and design of the review. PB organized the database and performed the analysis.

PB and GS wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.574067/full#supplementary-material

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