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Research article

Monocytes as suitable carriers for dissemination of dengue viral infection

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ABSTRACT

Dengue viruses (DENVs) exploit monocytes and macrophages for tropism and replication, therefore, establishing a long-term reservoir. However, their roles in dengue pathogenesis remains unclear. Here, using the human monocytic cell line THP-1, human primary monocytes, and non-human primate models, we show that DENV-infected monocytes represent suitable carriers for circulatory viral dissemination. Monocyte-derived macrophages expressing M2 surface markers at the gene level efficiently replicated, while the productivity of monocyte replication was low. However, attachment of DENVs to the cellular surface of monocytes was similar to that of macrophages. Furthermore, after differentiation with type-2 cytokines, DENV-attached monocytes could replicate DENVs. Productive DENV infection was confirmed by intravenous injection of DENVs into nonhuman primate model, in which, DENV attachment to monocytes was positively correlated with viremia. These results provide insight into the role of circulating monocytes in DENV infection, suggesting that monocytes directly assist in DENV dissemination and replication during viremia and could be applied to design antiviral intervention.

1. Introduction

Dengue, a mosquito-borne viral infection, is a global public health problem and is estimated to infect approximately 100 million people worldwide annually (Guzman and Harris, 2015). The incidence of dengue has spread rapidly around the world in recent decades. In 2019, WHO has listed dengue as a potential threat to global health (Thangaraju and Venkatesan, 2019). The infection of dengue viruses (DENVs) is usually asymptomatic or mild in most patients; however, approximately 23.2% of the infected individuals were reported to develop severe complications such as haemorrhagic fever, shock syndrome, and death (Guo et al., 2017). Although the host's immune system significantly contributes to the progression of dengue pathogenesis, the mechanism of viral dissemination by immune response remains unclear.

Monocytes can strongly contribute to the widespread dissemination of infectious agents, as they circulate in the bloodstream and migrate to the infected sites in response to signals exerted by cytokines and other molecules, following infection by pathogens (Nikitina et al., 2018; Ayala-Nunez et al., 2019). Monocytes can differentiate into macrophages (Mϕs) anytime under suitable conditions; therefore, monocyte infection could significantly contribute to the pathological progression associated with Mϕs. For example, monocytes differentiate/polarize into different Mϕ phenotypes (M1 and M2) that allow the cytomegalovirus to persist in the body (Min et al., 2020) and increase the mobility of Mϕs to spread the zika virus to neurons (Ayala-Nunez et al., 2019). In the case of dengue, the presence of DENV-positive monocytes/Mϕs in the lymph nodes, spleen, and lung in mouse models and human tissue samples suggests that monocytes/Mϕs may contribute to viral replication and spread (Halstead et al., 1977; Kou et al., 2008; Balsitis et al., 2009; Aye et al., 2014). However, how circulating monocytes and the process of their differentiation/polarization in tissues contribute to the replication and dissemination of DENVs are poorly understood.

In the present study, using a human monocytic cell line, primary cells, and nonhuman primate animal model, we examined the relationship

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between DENVs and monocyte/monocyte-derived Mϕs to better understand the pathogenesis of dengue.

2. Results

2.1. THP-1 monocytes had lower efficiency than M2-like Mϕs to replicate DENVs

To explore whether DENVs can efficiently utilize monocytes and monocyte-derived cells for its replication, we used the human monocyte THP-1 cell line. We successfully established a method of cell polarization using phorbol-12-myristate 13-acetate (PMA) and other stimuli to obtain M1-and M2-like Mϕs from THP-1 cells. Cells, differentiated with PMA for 48 h, were polarized to M1-or M2-like Mϕs by incubation with lipopolysaccharide (LPS) + interferon gamma (IFN-γ) and interleukin 4 (IL-4) + IL-13, respectively, for 24 h (Figure 1A). As expected, M1-specific markers including CXCL10 (22,445.8-folds) and IL-6 (2.7-folds) in M1-like cells and M2-specific markers including CD206 (80.1-folds) and CD163 (226.5-folds) in M2-like cells were significantly more than those in other cell types (Figure 1B). The expression of other specific markers, including CCL22, and CD209, was also evaluated (Supplementary data, Figure S1), and therefore, each type of Mϕs was phenotypically identified based on the expression of polarization markers in our *in vitro* system. Next, we investigated how DENVs used these cells for its own replication. We found that DENV-1 infected M2-like Mϕs more robustly than monocytes and M1-like Mϕs, with a 100-fold increase in viral RNA level (Figure 1C). The focus-forming assay also showed that live virus titres were significantly higher in M2-like Mϕs, approximately 100-folds after three days of infection, than those in THP-1 cells (Figure 1D). A similar pattern was observed for the other three DENV serotypes (Supplementary data, Figure S2). Taken together, our data suggest that monocytes, which are believed to be the target cells of DENVs, lack the ability to contribute to DENV replication compared to monocytes-derived cells such as M2-like Mϕs.

2.2. Rate of DENV entry into THP-1 monocytes was lower than that into M2-like Mϕs

We hypothesized that the release of lower amount of DENV RNA and infectious DENV particles by monocytes than by M2-like Mϕs is attributed to the differences in binding or entry of DENVs into monocytes and M2-like Mϕs. To test this hypothesis, we performed a binding/entry assay based on quantitative real-time polymerase chain reaction (qRT-PCR) (Figure 2A). In contrast to the data on DENV susceptibility shown in Figure 1C, similar levels of DENV were bound to monocytes and M2-like Mϕs (Figure 2B). However, the frequency of DENV internalization by M2-like Mϕs was significantly higher than that by monocytes (Figure 2C). Using binding/entry assay, we demonstrated that DENV serotypes other than DENV-1 were capable of binding to monocytes; however, the binding did not affect the replication of these serotypes (Supplementary data, Figure S3). Fluorescent staining clearly indicated the binding of DENV-1 to monocytes and M2-like Mϕs, whereas no binding/entry of DENV-1 was observed in case of M1-like Mϕs (Figure 2D). Therefore, despite their significant binding with DENVs, monocytes have relatively poor efficiency of DENV replication compared to M2-like.

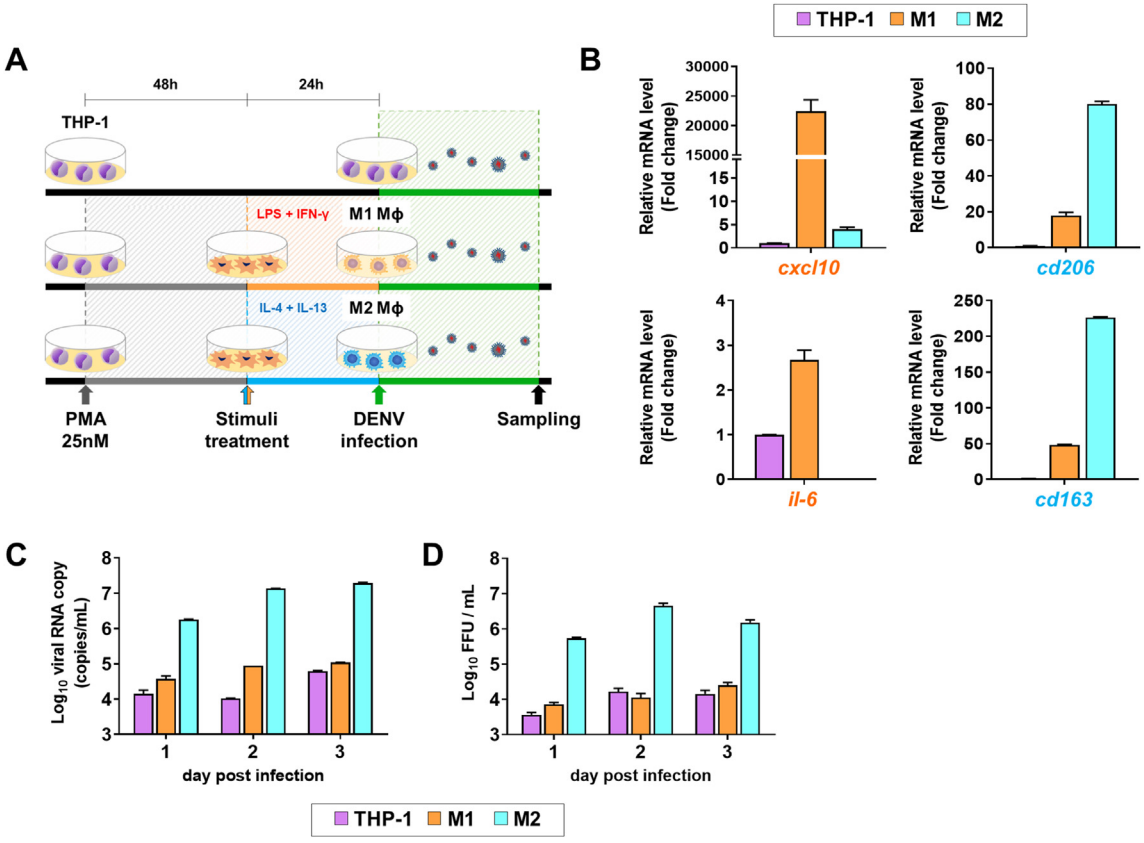

Figure 1. THP-1 monocytes have low efficiency of DENV replication compared to M2-like Mϕs (A) Experimental design of DENV replication in THP-1 monocytes and Mϕs. THP-1 cells were differentiated into Mϕs by PMA treatment for 48 h and then polarized to M1-like (LPS + IFN-γ) and M2-like Mϕs (IL-4 + IL-13) for 24 h (B) Mϕ polarization was determined by assessing the expression of typical Mϕ lineage marker genes by qRT-PCR. Replication of DENV in THP-1, M1-like, and M2-like Mϕs cells was determined. At the indicated time points after infection, the DENV-1 titres in culture supernatants were measured by (C) qRT-PCR and (D) focus-forming assay.

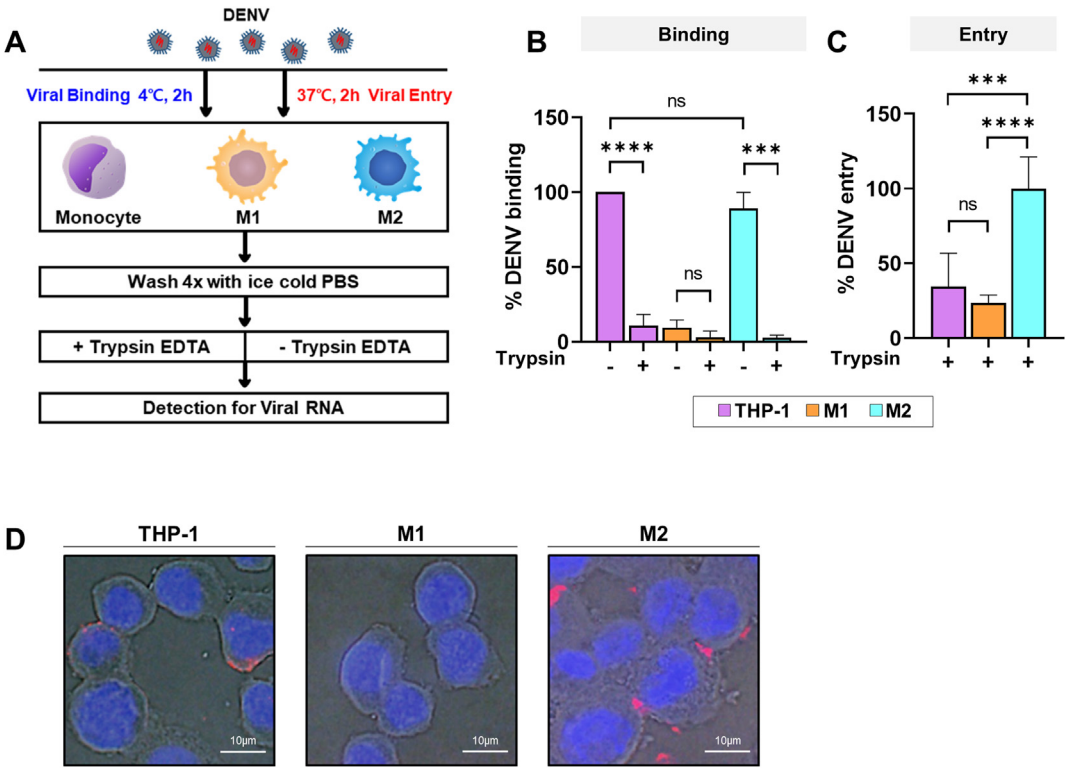

Figure 2. Rate of DENV penetrating THP-1 cells is lower than that into M2-like Mϕs (A) Schematic diagram of DENV-1 binding/entry assay (B) DENV binding assay (C) DENV entry assay. For (B) and (C), the percentages of bound or internalized DENV-1 were determined by qRT-PCR (D) Representative fluorescent images of monocytes, and M1-like and M2-like Mϕs inoculated with AF594-labelled DENV (red) and DAPI (blue). Data are represented as the mean ± standard deviation (SD) of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

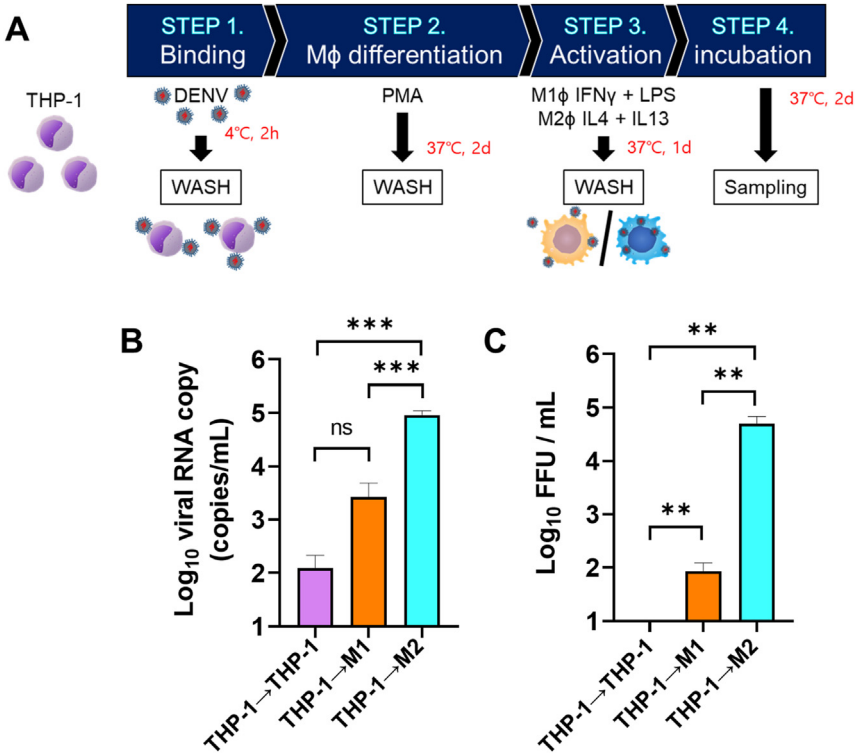

Figure 3. DENV-attached THP-1 can be permissive for DENV replication after differentiation into M2-like Mϕs (A) Experimental design. After incubation of THP-1-derived cells with DENV, the viral titre in the supernatants were determined by (B) qRT-PCR and (C) focus-forming assay. Results are represented as mean ± SD (***$p < 0.001$ and ****$p < 0.001$, by one-way ANOVA).

2.3. DENV-attached THP-1 was used as a reservoir after differentiation into M2-like Mϕs

Monocytes in the blood circulation encounter DENVs anytime during the related viremia. Monocytes are recruited into peripheral tissues during infection and can be exposed to endogenous or exogenous inflammatory factors that influence their differentiation and subsequent polarization to Mϕs. We assessed the suitability of DENV-attached monocytes as viral reservoirs during differentiation (Figure 3A). Following DENV-1 attachment to monocytes, a significantly higher extent of replication in monocyte-derived M2-like Mϕs was observed than that in undifferentiated DENV-attached monocytes. Monocyte-derived M1-like Mϕs did not show a similar extent of replication as did M2-like Mϕs (Figures 3B and 3C). Taken together, the data suggest that DENV-attached monocytes could efficiently replicate DENVs in response to cytokine signals that help in their differentiation into Mϕs.

2.4. Human primary monocytes showed a low efficiency of viral replication but the potential to be a contributor to DENV replication after differentiation into Mϕs

To experimentally validate our phenomenon in human primary cells, we used myelopoietic growth factors and cytokines to differentiate $CD14^+$ blood monocytes into Mϕs (Figure 4A). Human monocytes and Mϕs were infected with DENV-1 at MOI of 10 and incubated for 48 h to analyse viral replication within the cells (Figure 4A). The viral titre in M2-like Mϕs was higher by 10^2 FFU/mL than that in $CD14^+$ monocytes (Figure 4C). The level of viral RNA in M2-like Mϕs was higher by $10^{1.6}$ copies/mL than that in $CD14^+$ monocytes and M1-like Mϕs (Figure 4B); however, more than 10^5 viral genomes were detected in M1-like Mϕs, demonstrating residual viral RNA after the removal of infectious virus. We next tested whether monocytes can be a target of DENV attachment in the circulation. Peripheral blood mononuclear cells (PBMCs) obtained

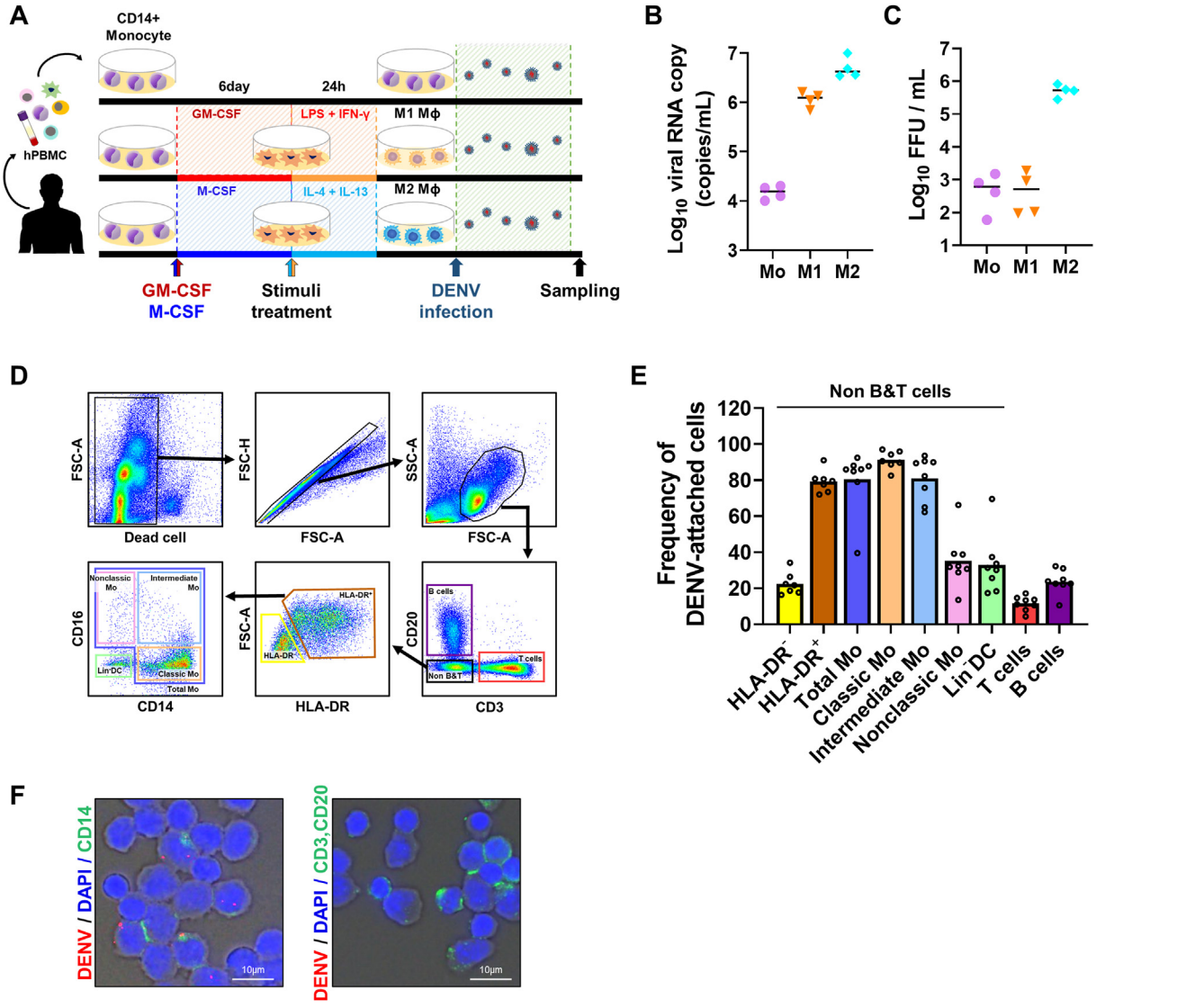

Figure 4. Human primary $CD14^+$ monocytes exhibit a relatively lower rate of viral replication than do M2-like Mϕs, even with high attachment of DENVs (A) Experimental design of DENV replication in human primary $CD14^+$ monocytes and in $CD14^+$-derived Mϕs. $CD14^+$ monocytes were differentiated in the presence of GM-CSF or M-CSF for seven days and then polarized with (IFN-γ + LPS) or (IL-4 + IL-13) for 24 h. For human primary $CD14^+$ monocytes and the derived Mϕs, DENV titres in the culture supernatants were measured using (B) qRT-PCR and (C) focus-forming assay (D) The gating strategy of flow cytometry for immune cell subsets in human PBMCs (E) The DENV-attached proportion of each subset of immune cells in total PBMCs was determined (F) Representative image of DENV-attached cells in PBMCs represented as virus (red), CD14 (green), or CD3/CD20 (green).

from a healthy donor were infected with AF594-labelled DENV-1. A total of nine clusters-including HLA-DR$^-$, HLA-DR$^+$, total monocytes, classic monocytes, intermediate monocytes, nonclassical monocytes, lin$^-$DC, T cells, and B cells were identified using gating strategy with five different surface markers (Figure 4D). Analysis of flow cytometry revealed that DENV-1 was preferentially attached to classical and intermediate monocytes at a higher level (83.4–90.4%), while other cells, including non-classic monocytes, lin$^-$DC, T cells, and B cells, showed a lower proportion extent of DENV-1 attachment (11.6–27.8%) (Figure 4E). Immunofluorescence analysis showed higher expression of DENV-1 by CD14$^+$ cells than by CD3/CD20$^+$ cells, after 2 h of infection (Figure 4F). This result suggests that human monocytes might act as carriers for the dissemination of DENV, although they cannot be effectively used for viral replication. We next tested the possibility of DENV-attached monocytes being used as a potential reservoir for viral replication during differentiation to Mϕ (Figure 5A). The amounts of viral RNA (Figure 5B) and infectious DENV (Figure 5C) were higher in M2-like Mϕs than in M1-like Mϕs differentiated from DENV-attached monocytes. These results were consistent with observations in THP-1 cells, mentioned earlier.

2.5. Monocytes were the targets for DENV attachment, and DENV-attached monocytes exhibited a high transmigration potential in non-human primates

Our final objective was to demonstrate the relevance of our previous results in cynomolgus macaques as a natural host for DENVs. Similar to the results of human PBMCs, compared to other immune cells, the CD14$^+$ cell population including classic and intermediate monocytes in PBMCs isolated from eight non-human primates was found to be a major target for DENV attachment (Figure 6A). This result suggests that the targets for DENV attachment in the immune cell populations of humans and primates were identical. We inoculated two female macaques intravenously with a relatively high dose (1×10^7 FFU) of DENV-1, as described in a previous study (Onlamoon et al., 2010). Peripheral blood was collected at indicated time points to analyse the viral burden and immune cell dynamics (Figure 6B). Plasma viremia was positive one day post infection (dpi) in the two monkeys and peaked at 3 dpi (Figure 6C). However, viral loads quickly resolved at 5 dpi, and viral RNA and released DENVs were undetectable at 7 dpi. The absolute number of monocytes markedly decreased at 1 dpi and recovered thereafter (Figure 6D). Plasma chemokine levels peaked at 1 dpi and decreased thereafter (Figure 6D). The number of other immune cells reduced on day 1 and showed a slow recovery (Supplementary data, Figure S4). The number of PBMC-associated DENVs peaked at 3 dpi (Figure 6F), and plasma viremia was positively correlated with viral RNA load in PBMCs ($p < 0.01$, r = 0.7785, Figure 6G). Together, these results suggest that DENVs could attach to potentially mobile monocytes, which indicates that the efficiency of using monocytes as means of viral transport may be correlated with the amount of DENVs in the circulation.

3. Discussion

Monocytes may be the ideal candidates as mediators for the spread of DENVs during infection because they can readily transport DENVs from the bloodstream to tissues. During DENV infection, monocytes migrate to the tissue in response to the signals originated by host–virus interaction and are differentiated and polarized by environmental factors. Therefore, understanding how these cells contribute to dengue pathogenesis is important to design therapeutic strategies. Here, we determined that circulating blood monocytes are targeted for attachment, rather than replication, by DENVs. This is the first report to show that monocytes exposed to DENVs can transform into the main target for DENV replication in a Th2 environment.

We noticed that circulating monocytes are not attractive targets for DENV replication. Monocytes had significantly lower replication efficiency than M2-like Mϕs, despite a similar level of virus attachment to the

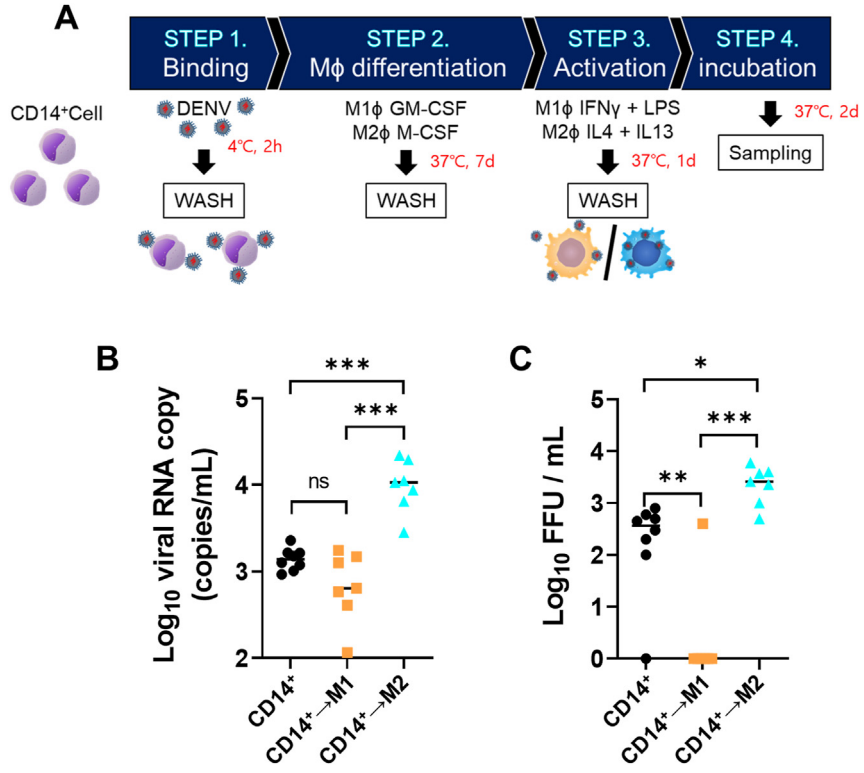

Figure 5. DENV-attached human primary CD14$^+$ monocytes can be permissive for DENV replication after differentiation into M2-like Mϕs (A) Schematic process of the protocol for assessing the role of human CD14$^+$ cells in DENV replication during differentiation. Supernatants were harvested for (B) viral copy number by qRT-PCR and (C) focus-forming assay.

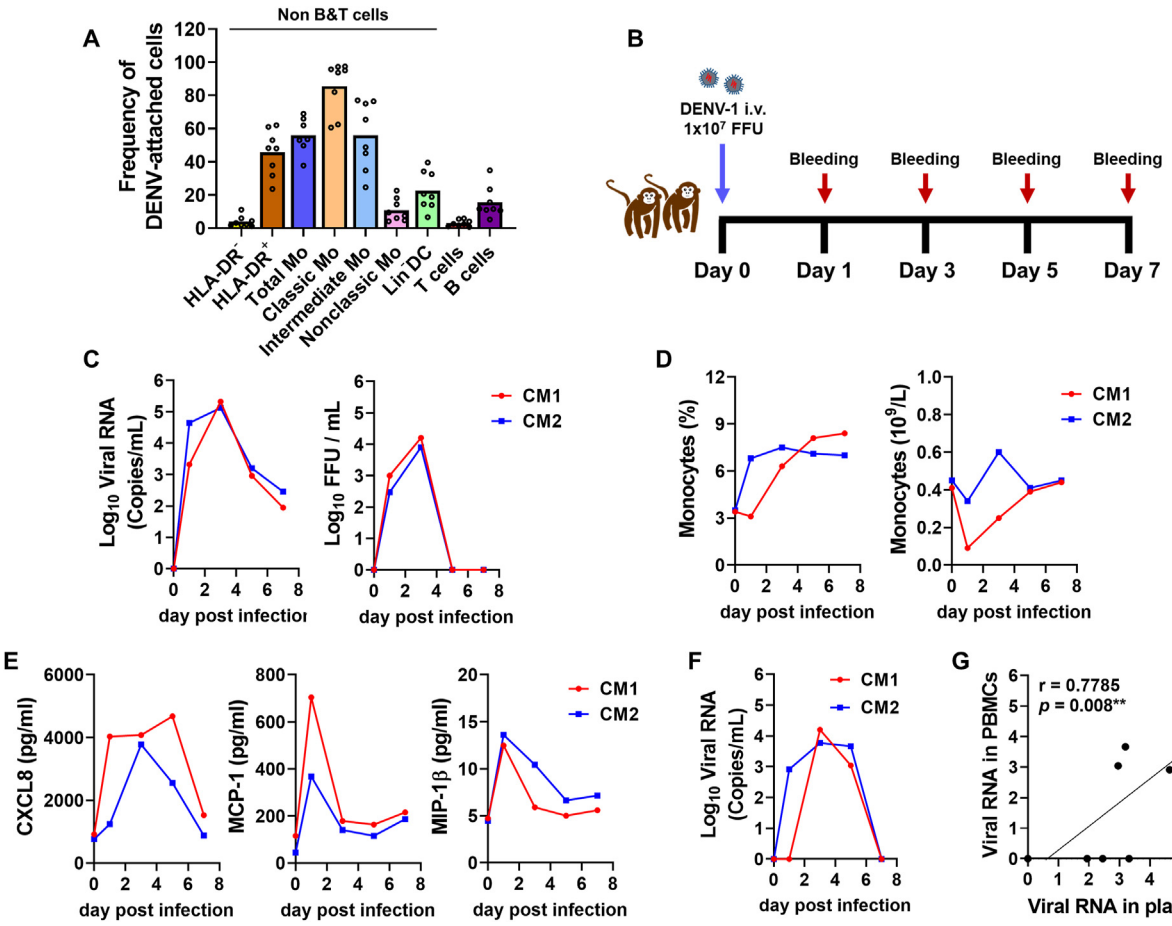

Figure 6. DENV-attached monocytes in the circulation correlate with viremia in a non-human primate model (A) DENV binding to each subset of immune cells was detected in PBMCs isolated from eight cynomolgus macaques (B) Experimental design using the nonhuman primate model infected with DENV (C) In the plasma of cynomolgus macaques, DENV titres were measured by qRT-PCR and focus-forming assay (D) The kinetics of the absolute number of monocytes in the whole blood was determined (E) Plasma levels of CXCL8, MCP-1, and MIP-1β were measured (F) DENV RNA expression in PBMCs of DENV-infected cynomolgus macaques was measured by qRT-PCR (G) Correlation of log (viral RNA) values between PBMCs and plasma. Linear regression is shown. $^{**}p < 0.01$.

cell surface. Studies with human blood $CD14^+$ cells showed that the levels of non-structural proteins representing a positive rate of viral replication were 5-fold lower than that of the structural proteins representing viral adhesion and replication (Durbin et al., 2008). DENV replication was found to be hampered in monocytes by the induction of interferon-stimulated genes, suppression of genes related to protein translation/mitochondrial function, and the induction of inducible nitric oxide synthase (Waickman et al., 2021; Neves-Souza et al., 2005). On the contrary, monocytes have been suggested as the main targets for DENV replication (Kou et al., 2008; Durbin et al., 2008). These studies, using flow cytometry after intracellular staining to assess susceptibility to DENV, suggested that DENV-attached monocytes may be the primary target cells for DENV replication. However, this study could not reflect the chance of abortive replication, in which viral particles are not released from infected cells. We found that about 80% of DENV-attached cells were $CD14^+$ monocytes in nonpermeabilized PBMCs of humans and monkeys; however, these cells had poor efficiency to replicate DENVs. Therefore, the attachment of DENVs to monocytes does not necessarily lead to DENV replication by those monocytes.

However, DENV-attached monocytes could replicate DENV when they differentiated into Mϕs in the Th2 environment. Previous studies have reported that Th2 cytokine-treated monocytes induce an increase in the expression of viral entry receptors and viral burden *in vitro* (Schaeffer et al., 2015; Miller et al., 2008). Of the markers specifically increased in M2-like Mϕs polarized in the Th2 environment, CD206 and CD209 are representative entry receptors for DENVs. Serum levels of Th2 cytokines were reported to be significantly higher in severe dengue patients compared to those in non-severe cases (Chaturvedi et al., 2000; Abhishek et al., 2017). Type II diabetes patients detected with high levels of serum Th2 cytokines were at high risk for developing severe dengue (Lee et al., 2013). Indeed, severe types of dengue are associated with high viremia (Morsy et al., 2020). Therefore, we postulated that in a Th2-biased environment, monocytes are susceptible with an increased viral affinity and, therefore, need to be explored in dengue pathogenesis. To the best of our knowledge, we provided the first evidence for this hypothesis.

The conditions of Mϕ polarization were reported to be different *in vitro*. Wu et al. reported that monocyte-derived M1 Mϕs differentiated with granulocyte-macrophage colony-stimulating factor (GM-CSF) alone are more permissive than M2 Mϕs differentiated with macrophage colony-stimulating factor (M-CSF) alone (Wu et al., 2013). However, CD206, a typical receptor on the surface of M2 Mϕs (Schaeffer et al., 2015; Miller et al., 2008), was also highly expressed on M1 Mϕs, suggesting that M1 Mϕs also have partial characteristics of M2 Mϕs. In fact, monocytes incompletely differentiate into naive M0 Mϕs in response to primary growth factors such as GM-CSF and M-CSF (Hamilton, 2008; Zarif et al., 2016; Hohensinner et al., 2021), and additional stimulation of cytokines is required for complete polarization. For example, typical M1 Mϕs are induced by IFN-γ and LPS owing to signal transducer and activator of transcription (STAT)-1 and nuclear factor kappa B (NF-κB) activation (Su et al., 2015; Zhang et al., 2017), while typical M2 Mϕs are induced by the treatment of IL-4 and IL-13 through the STAT-6 pathway (Zhang et al., 2017; Ishii et al., 2009). In Mϕ subsets that actively

supported DENV replication under our experimental conditions, they were fully differentiated by growth factors and cytokines as confirmed through traditional M2 markers such as CD163, CD206, and CD209.

In conclusion, using *in vitro*, *ex vivo*, and *in vivo* models, we determined that monocytes can play an important role in the dissemination of DENV by viral attachment, rather than viral replication. Additionally, the monocyte-derived Mϕs in the Th2 environment are capable of DENV replication. Overall, our findings provide a better understanding of dengue pathogenesis, and, therefore, may help in developing therapeutic intervention against DENVs.

3.1. Limitations of the study

Our study has three limitations. First, Mϕs derived from the yolk sac were not tested. Second, we focused on the pathogenesis of primary DENV infection; therefore, the role of monocytes in secondary infection has not been elucidated. Further studies on antibody-virus complexes are necessary. Third, the works suffers from a technical limitation of not acquiring enough number of monocytes owing to their rapid reduction following DENV infection.

4. Methods

4.1. Cell culture

Human monocytic leukemic cell line THP-1 (Korean Collection for Type Cultures, KCTC; Jeongeup-si, Jeollabuk-do, Korea) was cultured in RPMI1640 medium (Welgene, Republic of Korea) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% penicillin/streptomycin (P/S), and 0.5 nM 2-mecaptoethanol. THP-1 monocytes were differentiated into Mϕs by treating with 25 nM PMA for 48 h prior to polarization. Human $CD14^{+}$ monocytes (PromoCell, Heidelberg, Germany) were cultured with 50 ng/mL of GM-CSF or M-CSF for seven days to achieve differentiation. All Mϕs were polarized *in vitro* with either LPS (20 pg/mL; Invitrogen, USA) and IFN-γ (20 ng/mL) or IL-4 (20 ng/mL) and IL-13 (20 ng/mL) (Peprotech, USA) for 24 h. Cells were washed thrice with phosphate-buffered saline (PBS) to remove any remaining cytokines. Human PBMCs were purchased from Cellular Technology Limited (CTL; OH, USA). Vero E6 cells were purchased from KCTC and maintained in minimal essential medium (MEM; Welgene, Daejeon, Korea) supplemented with 10% FBS and 1% P/S.

4.2. Viruses

A dengue virus type 1 stock (Indonesia, 2011; NCCP number 43251) was obtained from the National Pathogen Culture Collection (NCCP, Korea). The virus was propagated by infecting Vero E6 cells at a low MOI of 0.05 and collecting supernatants seven dpi. The culture supernatant was harvested when 80% of the cells showed a cytopathic effect and clarified by centrifugation at 1000 ×g for 30 min at 4 °C. The resulting supernatant was passed through a 0.2-μm filter and purified by ultracentrifugation (28 000 ×g, 4 °C, 4 h) through a 20% sucrose cushion. Purified viral pellets were resuspended in PBS and stored as 100-μl aliquots at -80 °C until use. The viral titre in the supernatant was measured by a focus formation assay in Vero E6 cells.

4.3. RNA isolation and qRT-PCR

RNA was isolated using TRIzol® reagent according to the manufacturer's instructions. RNA concentration was determined using NanoDrop 2000 (Thermo Fisher Scientific, USA). Subsequently, 1 μg of RNA was converted to cDNA using a PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara, Japan). Quantitative PCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using QuantiTect SYBR Green PCR Kit (Qiagen, Germany) according to the manufacturer's specifications. The reaction conditions were 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The results were normalized to the THP-1 mRNA and expressed as relative values. Viral RNA in the culture supernatant was extracted with a viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The reaction conditions for qRT-PCR of viral RNA were 50 °C for 30 min and 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. All primer sequences are listed in Supplementary data, Table S1.

4.4. Focus-forming assay

Virus-containing supernatants were used to infect Vero E6 cells for 2 h at 37 °C in 6-well plates. After removal of the inoculum, cells were incubated in 2% MEM (1% P/S, 2% FBS) at 37 °C. After three days, cells were washed, fixed with 4% paraformaldehyde for 30 min at 4 °C, and permeabilized with PBS containing 0.5% Triton X-100 for 15 min at 20 °C. An anti-monoclonal antibody was used for the assay. Cells were incubated with monoclonal antibody (Millipore, USA) (dilution 1:3000) against DENV envelope protein for 1 h at 20 °C followed by incubation with goat anti-mouse IgG HRP conjugate (Abcam, USA) (dilution 1:3000). Foci were developed using TrueBlue peroxidase substrate (KPL, MD, USA), counted in each well to calculate the viral titre, and expressed as $\log_{10}$ FFU/mL.

4.5. Virus-fluorophore conjugation and immunostaining

To visualize virus–cell interaction, DENVs were conjugated with Alexa Fluor 594 succinimidyl ester (AF594) (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions (Zhang et al., 2010) and stored at -80 °C prior to use. The binding of labelled DENV to cells was analysed using a fluorescent microscope (Thermo Fisher Scientific). Immunostaining for the binding assay was carried out at 4 °C, and cells were incubated with AF594-labelled DENV1 (MOI 10) for 2 h to allow binding but not internalization. Cells were washed thrice with cold PBS and subsequently stained with FITC-conjugated CD3 and CD20, or CD14. The stained cells were fixed in 4% paraformaldehyde in PBS at 4 °C.

4.6. Virus binding and entry

The binding assay was performed at 4 °C whereas, the entry assays were performed at 37 °C. Cells were incubated with DENV for 2 h, then washed thrice with cold PBS, and treated with 0.25% trypsin-EDTA for 5 min to remove the bound DENVs. RNA was isolated using TRIzol® Reagent (Invitrogen), and viral RNA was detected by qRT-PCR using a One Step PrimeScriptTM RT-PCR Kit (Takara).

4.7. Flow cytometry

Following infection with AF594-labelled DENVs, PBMCs of humans or non-human primate were stained first with Fixable Viability Stain 575V (BD Biosciences, USA) at 20 °C for live/dead cell gating, followed by surface staining with CD14, HLA-DR, CD16, CD20, and CD3. PBMCs were then analysed using a LSR Fortessa system (BD Bioscience) and FlowJo software v10.7.1.

4.8. Animal model

All monkeys used in this study were maintained at the National Primate Research Center according to the minimal requirements of "The Guide for the Care and Use of Laboratory Animals" by the Institute for Laboratory Animal Research (2010). The *in vivo* studies were approved by the KRIBB Institutional Animal Care and Use Committee (permit no. KRIBB-AEC-21149). Two cynomolgus macaque were challenged with DENVs of 1×10^{7} FFU. Blood samples were collected 0, 1, 3, 5, and 7 dpi. Blood plasma and cells were separated by centrifugation.

4.9. Determination of chemokine levels

The levels of CXCL8, MCP-1, and MIP-1β were determined in the plasma using multiplex kits (Millipore) according to the manufacturer's instructions. Each sample was run in duplicate using 25 μL plasma and measured using a Luminex-200 system (Luminex Corporation, TX, USA).

4.10. Statistical analysis

Data were analysed using statistical methods in GraphPad Prism 8 software (GraphPad Software). Data are presented as mean ± SD.

Declarations

Author contribution statement

Jung Joo Hong: Conceived and designed the experiments, performed the experiments, analyzed and interpreted the data and wrote the paper.

Eun-Ha Hwang: Performed the experiments, analyzed and interpreted the data and wrote the paper.

Bon-Sang Koo, Hanseul Oh, Hoyin Jung and Green Kim: Performed the experiments.

Gyeung Haeng Hur, You Jung An and Jong-Hwan Park: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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