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Comparative monocyte and T cell responses in DENV-exposed subjects from South-East Asia and DENV-naïve residents in Taiwan

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ABSTRACT

Background/purpose(s): Dengue virus (DENV) is one of the most troublesome mosquito-borne infectious viruses in tropical and subtropical zones. People with secondary/multiple DENV infections are at an increased risk of developing severe dengue. Both monocytes and T cells are known to play important roles in the immune response against DENV. However, the function of monocytes and T cells in individuals with potentially multiple exposures to DENV is rarely reported.

Method: In the present study, we performed a functional analysis of monocytes and T cells from people with previous DENV infection and DENV-naïve people that stimulated with DENV2 *ex vivo*.

Results: Our preliminary analysis indicated that the response of monocytes and T cells to DENV2 restimulation was comparable between DENV-exposed and DENV-naïve individuals. Furthermore, the cytokine expression profiles in monocytes from both naïve individuals and previously DENV-exposed subjects were similar after DENV2 stimulation. In addition, it was observed that the function of T cells was also equivalent when monocytes were present as antigen-presenting cells for dengue antigen, NS3, in terms of cell proliferation, interferon-gamma (IFNγ) secretion, and memory response.

Conclusions: Based on the results, it was observed that previously DENV-exposed monocytes and T cells seemed to be anergic during DENV reinfection. However, whether the impaired response of monocytes and T cells against DENV in people with a history of previous DENV infection leads to severe dengue upon secondary infection in endemic areas requires further investigation.

1. Introduction

Dengue virus (DENV) belongs to the family *Flaviviridae*, genus *Flavivirus*. There are four serotypes of DENV, from serotypes 1–4.[1] According to the World Health Organization (WHO), there are 390 million DENV infectious cases and 96 million individuals develop DENV symptoms annually.[2] Also, 500,000 develop severe disease, leading to 22,000 deaths.[3] In South-East Asia (SEA), approximately 1.3 billion people are at risk of DENV infection, resulting in 2.9 million dengue cases and 6000 deaths annually.[4–6] In Taiwan, there were two dengue outbreaks causing 15,732 and 43,784 dengue cases in 2014 and 2015, respectively.[7]

In the Southeast Asia (SEA) region, there is a notably higher incidence of secondary DENV infections compared to non-SEA regions, particularly involving DENV2.[8] A study conducted in the Philippines observed that 92 % of 90 dengue patients (aged 2–37 years) experienced secondary DENV infections.[9] Similarly, research from Thailand found that over 90 % of 154 symptomatic dengue-infected children had secondary DENV infections.[10] Another Thai study revealed that more than 75 % of confirmed dengue cases across all serotypes involved secondary DENV infections among 2200 cases examined.[11] These studies collectively underscore the significant prevalence of secondary DENV infections in the SEA region. There is no doubt that DENV poses a threat to public health. The diverse clinical symptoms of DENV infection arise

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from the complex interplay between the human immune and the virus.[1,12,13]

The human immune system, comprising both innate and adaptive immunity, plays a critical role in combating DENV infections. Innate immunity provides rapid protection against DENV through mechanisms such as inflammation, cytokine production, chemokine release, and interferon (IFN) production.[14–16] Antigen-presenting cells (APCs), including dendritic cells and macrophages, are vital in initiating the adaptive immune response by presenting antigens to naive T cells. Upon activation, these T cells further differentiate into various types of helper T cells. Consequently, the adaptive immune system is activated to eliminate DENV and DENV-infected cells.[1,17]

Human monocytes play a critical role in the immune response to DENV infection. They perform several functions against the virus, including cytokine production and the enhancement of T-cell function and proliferation.[18,19] During DENV infection, pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-α), and macrophage migration inhibitory factor (MIF), are highly expressed to induce fever and enhance immune cell function. Besides, interleukin-10 (IL-10), a major anti-inflammatory cytokine, plays an essential immunoregulatory role in DENV infection.[20–23] Previous studies revealed that these cytokines are highly expressed in DENV patients and correlated with disease severity.[23]

T cells are involved in both humoral and cell-mediated immunity. Response of T cells is also crucial in DENV infection. Mature T cells can be distinguished as cytotoxic T-cells ($CD8^+$ T cells) and helper T-cells ($CD4^+$ T cells).[24] The memory response is a characteristic of adaptive immunity. Some effector T cells can transit into memory T cells upon viral clearance. Memory T cells exhibit robust immune responses upon encountering identical pathogens.[25] These memory T cells can be categorized as central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). It was stated while secondary infection occurs, T_{CM} in blood would migrate to lymphoid organs to proliferate into new effector T cells against pathogens. Moreover, studies indicated that circulating T_{EM} could rapidly respond to virus reinfection through cytokine production.[26] T cells act as a double-edged sword in acute DENV infection owing to their controversial role. $CD8^+$ and $CD4^+$ T cells were found to control DENV infection through cytokine secretion and enhancing B-cell response.[27] However, few reports suggest that the 'original antigenic sin' theory may contribute to severe secondary DENV infection due to impaired T-cell function after previous DENV infection.[28,29]

Thus, to understand how monocytes and T cells respond to repeated DENV infection, we explored the functions of monocytes and T cells in DENV-naïve individuals and previously DENV-exposed subjects upon DENV infection *ex vivo*.

2. Methods

2.1. Ethics statement

Written informed consent was obtained from volunteers who had previously lived in DENV-endemic countries, including the Philippines, Vietnam, Indonesia, Malaysia, and Thailand in SEA, and from local residents of Taiwan (IRB number: A-ER-106-417). All volunteers reported being in good health by themself before blood withdrawal and the sample were all collected from 2018 to 2019. The age of donors ranges from 22 to 27 in DENV-naïve individuals and 20 to 38 in previously DENV- exposed subjects, respectively (Table 1). These studies were approved by the Institutional Review Boards at the National Cheng Kung University Hospital in Taiwan.

2.2. Isolation of PBMCs (peripheral blood mononuclear cells) and plasma

Peripheral blood was extracted from donors. Briefly, 20–30 mL of peripheral blood was collected and then centrifuged at 300 r.c.f. for 8 min to separate plasma and blood cells. The following step of RBC lysis was performed to obtain PBMCs. PBMCs were resuspended in 10 % FBS-containing RPMI and counted for further experiments.

Table 1
Information of recruited naïve individuals and previously DENV-exposed subjects.

Group	Donor ID	Age	Rapid Test Results[a]			Anti-Dengue IgG[b]
			NS1	IgM	IgG	
Naïve individuals	#1	22	–	–	–	–
	#2	23	–	–	–	–
	#3	27	–	–	–	–
	#4	26	–	–	–	–
	#5	23	–	–	–	–
	#6	22	–	–	–	–
Previously DENV-exposed subjects	#1	20	–	–	–	+
	#2	32	–	–	–	+
	#3	36	–	+	+	+
	#4	24	–	+	+	+
	#5	42	–	–	–	+
	#6	28	–	–	–	+
	#7	25	–	–	–	+
	#8	26	–	–	–	+
	#9	25	–	–	–	+
	#10	28	–	–	–	+
	#11	38	–	–	–	+

[a] Results were obtained by using rapid test kits (CTK Biotech, USA).
[b] DENV IgG antibody detected using ELISA detection kit (Focus Diagnostics, USA).

2.3. DENV rapid antigen/antibody test

Dengue OnSite Duo Ag-IgG/IgM Rapid Tests (CTK Biotech, USA) were used to detect DENV NS1 antigens and anti-DENV IgM/IgG in serum samples. The manufacturer's instructions followed all the processes.

2.4. Evaluation of DENV-specific IgG

The anti-DENV IgG ELISA (Focus Diagnostics, USA) was used to assess previous DENV exposure. The manufacturer's index suggested a cutoff value of >1.00 to classify the specimens as positive, indicating the presence of DENV-IgG. Seventeen specimens were tested and divided into naïve individuals and previously DENV-exposed subjects.

2.5. Extraction of viral RNA and PCR

Human plasma was subjected to viral RNA extraction by commercial kits (GeneDirex, USA). Viral RNA was used to synthesize cDNA by using the SuperScrip III kit (Thermo Scientific, USA). Next, cDNA was used as a template in the PCR amplification. Mastermix Taq DNA polymerase (Ampliqon, Denmark) was used in each reaction. The primer targeted nonstructural-protein 5 (NS5) of the DENV genome was designed by Prof. Shainn-Wei Wang at NCKU. Amplification was performed by using a Mastercycler Nexus Thermal Cycler (Eppendorf, Germany). Thermal cycling parameters were as follows: heating at 94 °C for 2 min, reaction in 35 cycles of 15 s at 94 °C, 40 s at 55 °C, and 2 min at 72 °C, and extension at 72 °C for 5 min. The PCR products were electrophoresed on 1.2 % agarose gel and the results were visualized on the MS UVCI-2300/2400 imaging system.

2.6. Plaque assay and plaque reduction neutralizing assay (PRNT)

BHK21 cells (3×10^5 cells/well) were seeded in 6-well plates and incubated at 37 °C overnight. The collected sample was subjected to serial dilution from 10^{-1} to 10^{-6} to prepare the diluted sample. 400 μL of diluted sample was added and incubated at 37 °C for 2 h. Then, 1 % methylcellulose medium was added and incubated at 37 °C for 5–7 days. Plaques were counted after crystal violet staining of the cells. The

highest viral titer during the infection period was calculated and defined as the peak of viral titer. Viral titer (PFU/ml) = (Count of plaque) × $\frac{1000\ \mu l}{400\ \mu l} \times \frac{1}{dilution\ factor}$. For PRNT assay, serum from donors was heat inactivated for 30 min at 56 °C before use, followed by two-fold serial dilution of plasma was performed from 80-fold to 1280-fold. DENV1 to DENV4 was diluted in 2 % FBS-contained DMEM to yield 50–100 plaques/well. Virus-plasma mixture was incubated in a 37 °C incubator for 30 min. Then, plaque assay was performed to evaluate the neutralizing effect of serum.

2.7. Isolation of human monocytes and T cells

Human monocytes and T cells were isolated by magnetic-bead sorting. Briefly, 1×10^8 PBMCs were resuspended in the MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14 (monocytes) and CD3 (T cells) antibody-conjugated magnetic beads (Miltenyi Biotec, Germany) were added following the vendor manual. LS column (Miltenyi Biotec, Germany), with strong magnetic field, was used to separate $CD14^+$ monocytes and $CD3^+$ T cells, respectively.

2.8. Ex vivo DENV2 stimulation in human monocytes

Monocytes (1×10^7) were collected and separated into mock (uninfection) and DENV (DENV2 infection) groups. DENV2 was added at multiplicity of infection (MOI) of 1. After 2 h for infection, DENV-stimulated monocytes were distributed into 24-well plates and incubated at 37 °C. Monocytes were harvested at 2, 24, 48, and 72 h. Then, the supernatant and the pellet were collected, respectively.

2.9. Cytokine detection

Purified monocytes were stimulated with LPS at 100 ng/mL (positive control, **S1 Fig**) and stimulated with DENV2 at MOI of 1 (experimental group). Cells were cultured with growth media and supernatant were collected at specific time points (2, 24, 48, and 72 h) to measure IL-6, IL-8, IL-10, TNFα, and MIF levels. ELISA kits for IL-6, IL-8, IL-10, and TNFα were obtained from Invitrogen (Waltham, USA), and MIF was obtained from R&D Systems (Minneapolis, USA). All measurements were executed according to the manufacturer's protocols. The concentrations were measured by spectrophotometry at 450 nm.

2.10. Analysis of RNA sequencing

Isolated $CD14^+$ monocytes were separated into an uninfected group (mock) and a DENV-infected group. Then, monocytes were infected with DENV at MOI of 1 for 2 h. After infection, monocytes were dissolved in 1 mL TRIzol to release cell RNA. The solution was submitted to Welgene Biotech (Taipei, Taiwan) to conduct RNA microarray for differential gene expression analysis. Briefly, RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). High-quality RNA samples were then subjected to microarray analysis by using the SurePrint chip (Agilent Technologies, USA).

2.11. Differential gene expression and pathway enrichment analysis

Differential gene expression analysis between uninfected (mock) and infected (DENV2) monocytes was performed using the limma package in R. Genes with an adjusted p-value <0.05 and an absolute log2 fold change >1 were considered significantly differentially expressed. Pathway enrichment analysis was conducted using the Gene Set Enrichment Analysis (GSEA) tool with gene sets from the Molecular Signatures Database (MSigDB). The enrichment score (ES) for each gene set was calculated based on the overrepresentation of genes at the extremes of the ranked list. The normalized enrichment score (NES) was computed to account for gene set size differences.

2.12. Ex vivo coculture of monocytes and T cells

Monocytes and T cells were sorted from PBMCs by using CD14 and CD3 magnetic beads, respectively. Purified monocytes were stimulated with DENV2 at MOI of 1 for 2 h. Coculture was conducted in 24-well plates with 1×10^6 DENV-stimulated monocytes and 1×10^6 T cells at 37 °C for 2, 24, 48, and 72 h. The cell pellet and supernatant were collected at indicated time points and stained with specific cell markers. For T-cell activation, CD3/CD28 T-cell activator Dynabeads (Life Technologies, USA) was added according to the standard protocol. Brefeldin A (BioLegend, USA), an inhibitor of intracellular transport of secretory proteins, was added 2–4 h prior to each time point for subsequent interferon-gamma (IFNγ) staining.

2.13. FACS analysis

To detect markers of monocyte-derived antigen-presenting cells (APCs), PBMCs were extracted from donors and separated into uninfected group (mock) and DENV2-stimulated group. After DENV2 stimulation, cells were stained with anti-CD11c FITC, anti-HLA-DR APC-H7 and anti-DC-SIGN BV421. To perform a comprehensive analysis of T cells following NS3 peptide stimulation *ex vivo*, PBMCs (1×10^6) were stimulated with DENV NS3 peptides (1 μg/mL) for 5 h at 37 °C in the presence of brefeldin A (10 μg/mL). After stimulation, the cells were stained with anti-CD3 Alexa488, anti-CD4 BV605, anti-CD8 APC, anti-CD45RO APC-H7, anti-CD45RA PE-Cy7 and anti-CCR7 V450 to determine memory T-cell population. Then, the cells were fixed and permeabilized by using Fixation/Permeabilization diluent (eBioscience, USA), anti-IFNγ PE for intracellular IFNγ evaluation and anti-Ki67 PerCP-Cy5.5 for cell proliferation. All of antibodies were purchased from BD Biosciences (BD Biosciences, USA). FACS analyses were performed on Canto II flow cytometer (BD Biosciences, USA) and analyzed by using Kaluza software (Beckman Coulter, USA).

2.14. Statistical analysis

Graphs of the results were presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed by using *t*-test, Mann-Whitney *U* test, and significant differences were denoted as p-value <0.05(*), <0.01(**), <0.001(***), and <0.0001(****). Correlation tests were done by using Spearman correlation test. All results and statistical calculations were performed by using GraphPad Prism Version 9.

3. Results

3.1. Anergic function of monocyte in previously DENV-exposed subjects

Firstly, to classify the DENV infection history, serum sample from volunteers were subjected into NS1 antigen and DENV-antibodies detection. Therefore, the participants were categorized into: (1) dengue-naïve individuals: negative DENV antigen and antibodies (Table 1); (2) previously DENV-exposed subjects: negative antigen but positive antibodies for DENV (Table 1). All volunteers tested negative for dengue viremia through PCR screening (Table 2). In order to realize the infection history in our enrolled volunteers, we attempted to clarify the infected serotype via plaque reduction neutralizing test (PRNT). The

Table 2
DENV NS5 PCR detection for enrolled donors.

Serology[a]	PCR	
	Positive	Negative
IgG negative (n = 6)	0	6
IgG positive (n = 11)	0	11

[a] DENV IgG antibody detected using Focus ELISA detection kits.

result showed that some of donors was indeed infected by DENV, and the titer of $PRNT_{50}$ was calculated (Table S1).

We then sought to elucidate the function of monocytes by analyzing the correlation between the $CD14^+$ monocyte population and the peak viral titer, which represents the highest viral burden in PBMCs following DENV infection. The results revealed the similar trend ($r = -0.360$, $p = 0.0999$) in both naïve individuals (Fig. 1A) and previously DENV-exposed subjects ($r = -0.2553$, $p = 0.1814$) (Fig. 1B). We also compare the $CD14^+$ monocyte levels in both naïve individuals and previously DENV-exposed subjects. The results show a trend of higher $CD14^+$ monocyte in previously DENV-exposed subjects compared to naïve individuals ($p = 0.1039$, data not shown). However, the higher levels of $CD14^+$ monocytes were not associated with viral control in previously DENV-exposed subjects.

Previous reports indicated that DENV2 is highly associated with secondary infections in Southeast Asia.[8,30] Therefore, the DENV2 16681 strain, which belongs to the Asian lineage of DENV2, will be used for monocyte stimulation in the following experiment. The cytokine profiles of DENV2-stimulated monocytes will be analyzed using ELISA. This analysis will assess the levels of inflammatory cytokines TNFα, IL-6, IL-8,

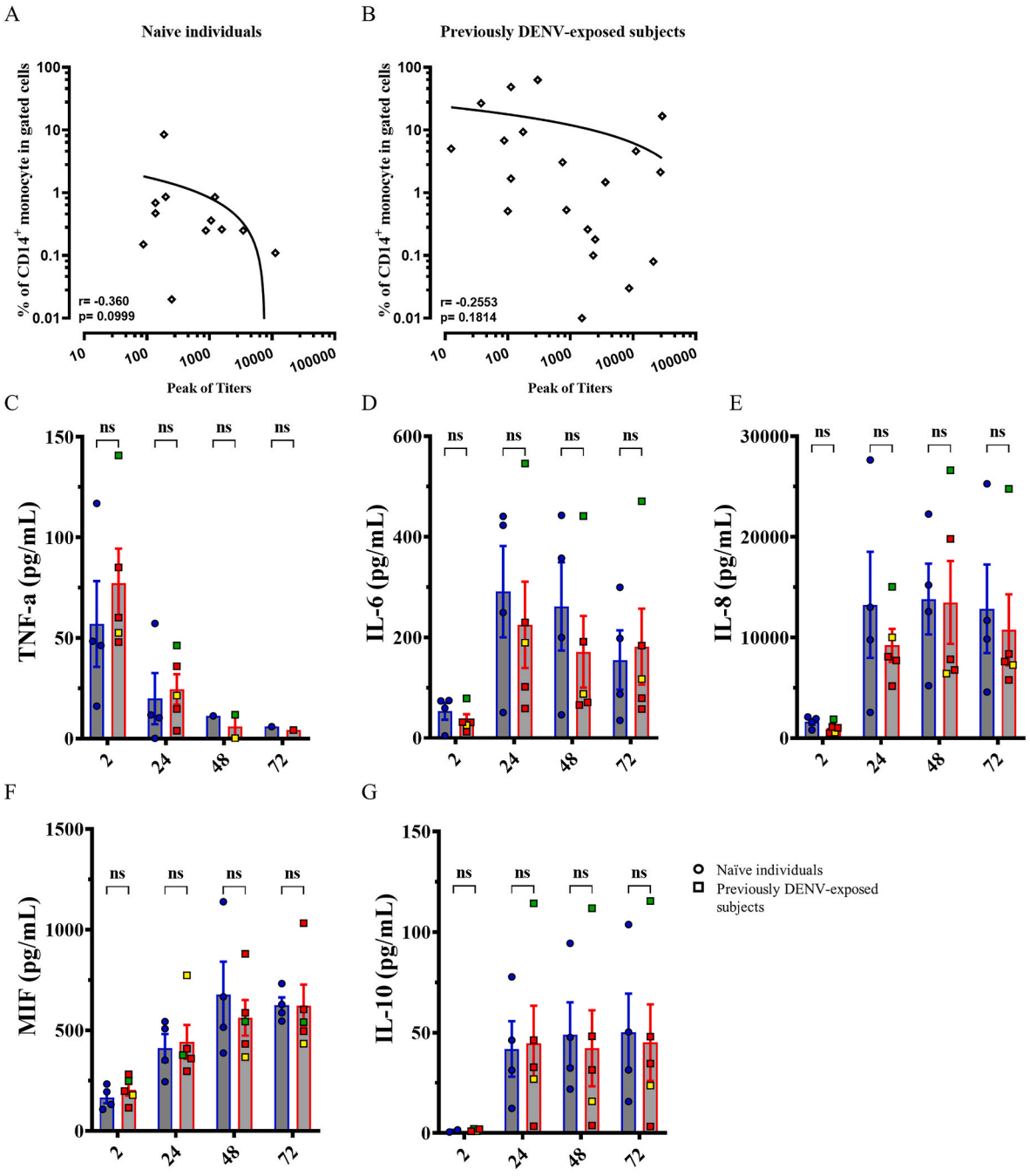

Fig. 1. Cytokine expression profile in monocytes from naïve individuals and previously DENV-exposed subjects upon DENV2 restimulation
The correlation between the percentage of $CD14^+$ monocytes and viral peak titer in healthy individuals (A) and previously DENV-exposed subjects (B) was studied. Human monocytes were stimulated with DENV2 at MOI of 1. Supernatants from different time points (2, 24, 48, and 72 h) were harvested for the detection of cytokine expression by using the commercial ELISA kits, including proinflammatory cytokines, (C) tumor necrosis factor alpha (TNF-α), (D) interleukin-6 (IL-6), (E) interleukin-8 (IL-8), (F) macrophage migration inhibitory factor (MIF) and anti-inflammatory cytokine (G) interleukin-10 (IL-10), both in naïve individuals (n = 4) and previously DENV-exposed subjects (n = 5). The yellow and green dots represent Donor #3 and Donor #4, respectively. The data are shown as the mean ± SEM.

and MIF, as well as the anti-inflammatory cytokine IL-10. Similar expression levels of the evaluated cytokines were observed in TNF-α (Fig. 1C), IL-6 (Fig. 1D), IL-8 (Fig. 1E), MIF (Fig. 1F), and IL-10 expression (Fig. 1G). The results indicated that monocytes from DENV-naïve and previously DENV-exposed subjects exhibited equivalent cytokines secretion upon DENV2 restimulation.

Expression of specific markers on monocyte-derived antigen-presenting cells after DENV2 restimulation.

Apart from releasing cytokines, the monocytes also function as antigen-presenting cells (APCs). As APCs, the monocytes present antigens to promote the adaptive immunity. Hence, we attempted to verify the expression level of Human Leukocyte Antigen – DR isotype (HLA-DR) that the molecule presented by monocytes to activate T cells, in antigen-presenting monocytes from both groups of volunteers. The gating strategy is shown in Fig. 2A. The results revealed that regardless of DENV2 stimulation, HLA-DR was increased in naïve individuals, but decreased in previously DENV-exposed subjects after 72 h of DENV2 stimulation (Fig. 2B). However, there was no statistically significant change. Next, we aim to uncover the expression of the dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) which involved in pathogen recognition and uptake in monocyte-derived APCs. The gating strategy is shown in Fig. 2C. The results indicated the increased trend of DC-SIGN expression, which was not statistically significant, after DENV2 stimulation in previously DENV-exposed subjects compared to naïve individuals (Fig. 2D). However, DC-SIGN expression was significantly increased in previously DENV-exposed subjects after 72 h DENV2 infection within groups (**S3 Fig**). Thus, it was suggested that the antigen-presenting marker expression in monocytes from previously DENV-exposed subjects was enhanced when compared with the naïve individuals after DENV2 restimulation.

Proliferation and IFNγ expression of T cells from previously DENV-exposed subjects upon DENV2 restimulation.

We first observed that monocytes from previously DENV-exposed subjects did not exhibit apparent activation upon DENV2 stimulation. Next, we analyzed the genetic alteration of monocytes during DENV2 stimulation. The results of RNA array analysis suggested that the monocytes from previously DENV-exposed subjects tended to express the signaling associated with adaptive immunity (Fig. 3A). In addition, the upregulated genes were linked to immune cell activation signaling pathways. Notably, the activation pathways of T cells were enhanced in these monocytes following DENV2 stimulation. (Fig. 3B). Consequently, we sought to investigate the impact on T cells following coculture with corresponding DENV2-stimulated monocytes. The cocultured T cells were collected and FACS analysis was conducted to characterize the T-cell populations, assess Ki67 for T-cell proliferation, and evaluate IFNγ expression for T-cell function. The results indicated that the Ki67 of $CD4^+$ T cells from previously DENV-exposed subjects was significantly decreased after coculture with DENV2-stimulated monocytes for 72 h (Fig. 3C). The expression of Ki67 in $CD8^+$ T cells from previously DENV-exposed subjects was also decreased after coculture with DENV2-

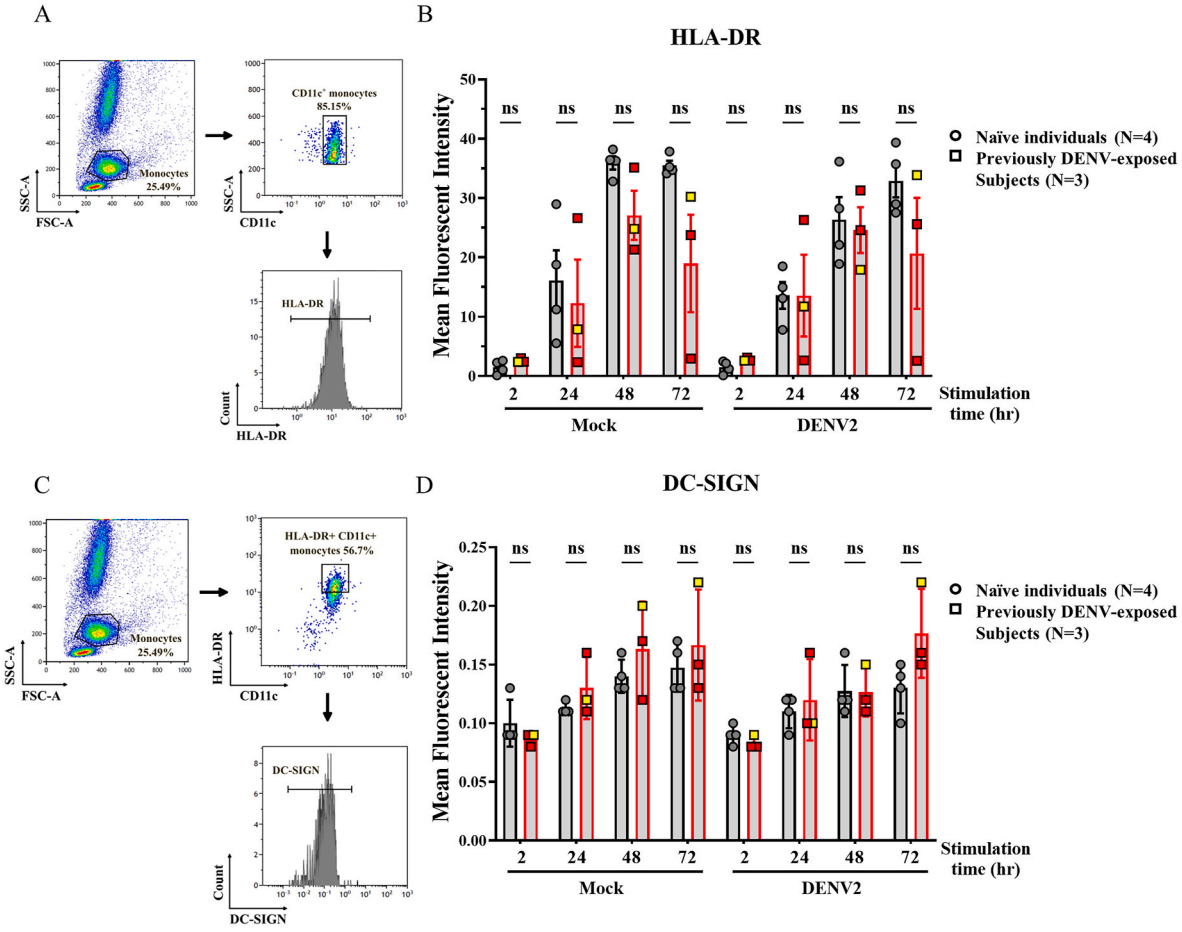

Fig. 2. Expression of specific markers on monocyte-derived antigen-presenting cells after DENV2 restimulation
The monocytes were isolated from peripheral blood and separated into unstimulated group (mock) and DENV2-stimulated group (DENV2 infection at MOI = 1). Cell pellets were collected from the indicated time points and stained with the surface marker for APCs (HLA-DR). (A) Gating strategy for the assessment of HLA-DR expression. (B) MFI of HLA-DR in monocytes. Naïve individuals (n = 4) and previously DENV-exposed subjects (n = 3) (C) Gating strategy for FACS analysis of the DC-SIGN MFI in $CD11c^+HLA\text{-}DR^+$ monocytes. (D) MFI of DC-SIGN expression in $CD11c^+HLA\text{-}DR^+$ monocytes from naïve individuals (n = 3) and previously DENV-exposed subjects (n = 3). The yellow dot represents Donor #3. Data are shown as the mean ± SEM.

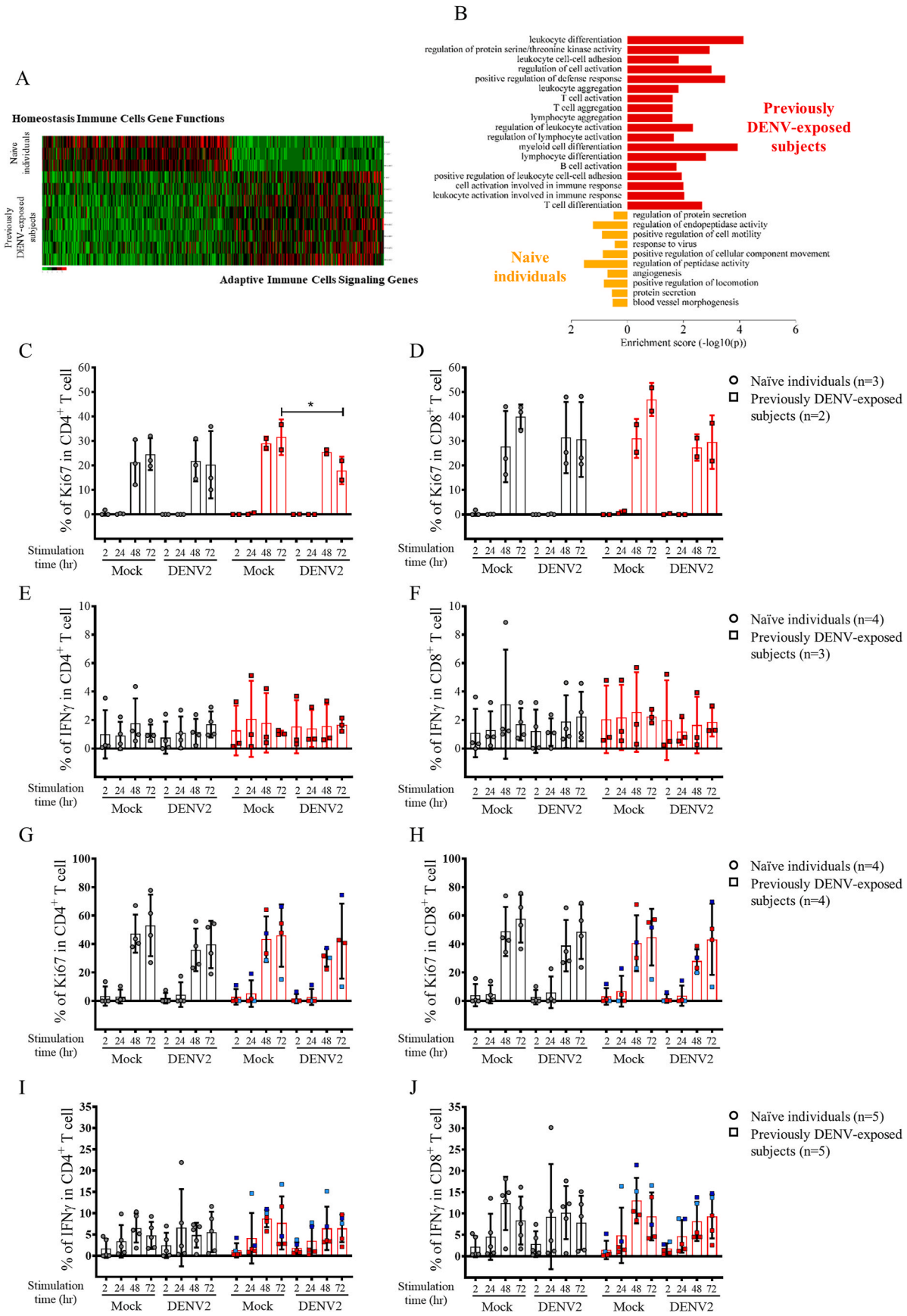

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Fig. 3. Proliferation and IFNγ expression of T cells from previously DENV-exposed subjects upon DENV2 restimulation
(A) RNA array analysis of DENV2-restimulated monocytes from naïve individuals and previously DENV-exposed subjects. (B) Biological pathway analysis of DENV2-stimulated monocytes from naive individuals and previously DENV-exposed subjects. (C) $CD4^+$ and (D) $CD8^+$ T-cell proliferation marker, Ki67 expression levels after coculture with unstimulated monocyte (mock) and DENV2-stimulated monocytes (DENV2 infection at MOI = 1) in the absence of T-cell activators. Naïve individuals (n = 3) and previously DENV-exposed subjects (n = 2). (E) $CD4^+$ and (F) $CD8^+$ T cells IFNγ expression levels after coculture with unstimulated monocyte (mock) and DENV2-stimulated monocytes (DENV2 infection at MOI = 1) in the absence of T-cell activators. Naïve individuals (n = 4) and previously DENV-exposed subjects (n = 3). (G) $CD4^+$ and (H) $CD8^+$ T-cell proliferation marker, Ki67 expression levels after coculture with unstimulated monocyte (mock) and DENV2-stimulated monocytes (DENV2 infection at MOI = 1) in the presence of CD3/CD28 T-cell activators. Naïve individuals (n = 5) and previously DENV-exposed subjects (n = 5). (I) $CD4^+$ and (J) $CD8^+$ T cells IFNγ expression levels after coculture with unstimulated monocyte (mock) and DENV2-stimulated monocytes (DENV2 infection at MOI = 1) in the presence of CD3/CD28 T-cell activators. Naïve individuals (n = 5) and previously DENV-exposed subjects (n = 5). Cells were harvested at the indicated time points, and Ki67 and IFNγ were stained and analyzed by FACS. The levels of Ki67 and IFNγ were calculated in T-cell populations. The light blue and bright blue dots represent as Donor #2 and Donor #5, respectively. Data are shown as the mean ± SEM. Tainan.

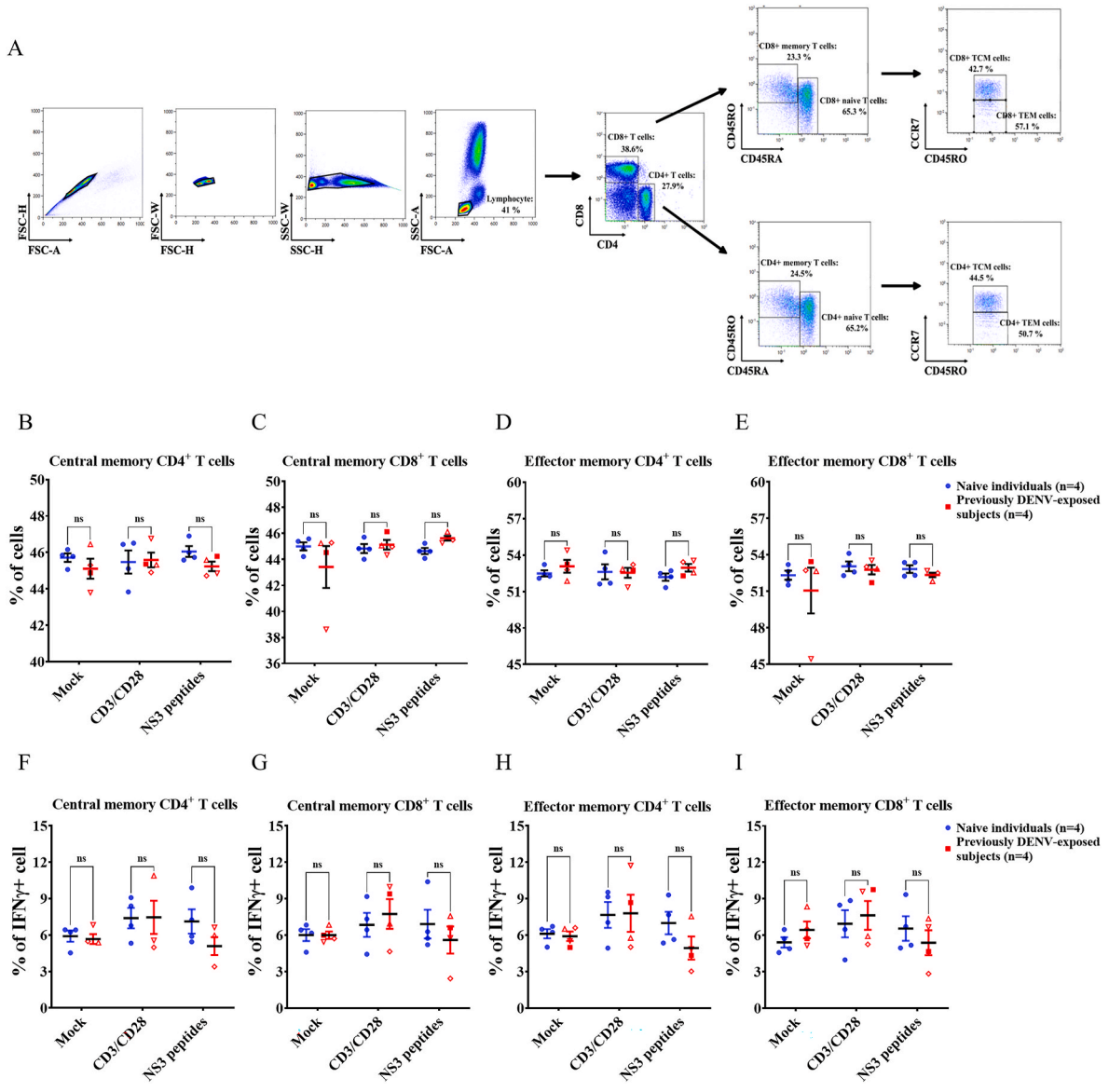

Fig. 4. Memory response of T cells after DENV2 NS3 peptide stimulation
(A) Flow cytometric gating strategy for memory T cells. (B) The population of memory T cells, including (B) central memory $CD4^+$ T cells, (C) central memory $CD8^+$ T cells, (D) effector memory $CD4^+$ T cells and (E) effector memory $CD8^+$ T cells in naïve individuals (n = 4) and previously DENV-exposed subjects (n = 4). Mock: unstimulated group; CD3/CD28: T cells activator. (F–I) The expression of intracellular IFNγ in each population of memory T cells measured after DENV2 NS3 peptide stimulation, including (F) central memory $CD4^+$ T cells, (G) central memory $CD8^+$ T cells, (H) effector memory $CD4^+$ T cells and (I) effector memory $CD8^+$ T cells. Naïve individuals (n = 4) and previously DENV-exposed subjects (n = 4). Mock: unstimulated group; CD3/CD28: T cells activator. ⋄: Donor #5, with DENV2 and DENV3 infection history; ▽: Donor #7, with DENV1 infection history; △: Donor #8, with DENV2 and DENV3 infection history. Data are shown as the mean ± SEM. Tainan.

stimulated monocytes for 72 h when compared with those coculture with unstimulated monocytes, but there was no statistically significant (Fig. 3D). IFNγ-secreting T cell populations remained unchanged in both $CD4^+$ and $CD8^+$ T cells, indicating that DENV2-stimulated monocytes did not induce IFNγ secretion from T cells in either group of volunteers (Fig. 3E-F).

Next, instead of DENV2-stimulated monocytes, CD3/CD28 Dynabeads were utilized to ensure T-cell activation. The Ki67 and IFNγ were assessed after T-cell activation. The results demonstrated that Ki67 expression was increased in both $CD4^+$ and $CD8^+$ T cells over time (Fig. 3G-H). Furthermore, the presence of CD3/CD28 T-cell activators induced the IFNγ production from T cells. In naïve individuals, IFNγ expression in $CD4^+$ T and $CD8^+$ T cells was maximum at 24 h and 48 h, respectively. Whereas, in the previously DENV-exposed subjects, the peak of IFNγ was maximal at 48h in $CD4^+$ T cells and at 72h in $CD8^+$ T cells. (Fig. 3I-J). The results showed the delayed IFNγ secretion of T cells in previously DENV-exposed subjects. Cumulative results suggested that monocytes from previously DENV-exposed subjects insufficient to induce IFNγ secretion. Additionally, T cells with previous exposure to DENV might show delayed IFNγ secretion compared with DENV-naïve individuals.

3.2. Similar level of memory T cells response after DENV2 NS3 peptide stimulation

We next aimed to investigate T-cell memory response by DENV2 nonstructural-protein 3 (NS3) peptides stimulation. Although NS3 was reported as the common antigen to T cells in dengue patients, its true role remains controversial.[31–33] The NS3 peptide was designed by the research team of Laura Rivino.[34,35] The analyzed strategy is represented in Fig. 4A. The result revealed similar populations of T_{CM} and T_{EM} after NS3 peptide stimulation (Fig. 4B–E). However, both T_{CM} and T_{EM} in $CD4^+$ and $CD8^+$ T cells from previously DENV-exposed subjects expressed lower IFNγ after NS3 peptide stimulation, but not statistically significant, when compared with naïve individuals (Fig. 4F–I). The results suggested that T cells from previously DENV-exposed subjects exhibited slightly-reduced responsiveness to DENV infection in comparison to naïve individuals. Thus, it was observed that monocytes and T cells from previously DENV-exposed subjects exhibited comparable response with naïve individuals to DENV infection.

4. Discussion

Our pilot study demonstrated that monocytes and T cells from previously DENV-exposed subjects exhibited comparable immune response with DENV-naïve resident in Taiwan upon DENV infection *ex vivo*. Cytokine expression from monocytes and monocyte-derived APCs markers showed similar level in our study. Furthermore, comparable memory T cell responses were assessed through IFNγ evaluation and DENV NS3 peptide stimulation.

Previous studies reported that the monocytes play an important role in viral clearance and adaptive immune activation during DENV infection. However, little was known about monocytes in previously DENV-exposed subjects. In our pilot study, we explored monocyte function in individuals previously exposed to DENV from DENV-endemic area. The results showed similar responses between monocytes from previously DENV-exposed subjects and DENV-naïve individuals. (Fig. 1A–B). The expression of MIF was significantly increased after 24, 48 h DENV2 infection in both naïve individuals and previously DENV-exposed subjects. The IL-8 was only enhanced in previously DENV-exposed subjects after 48 h DENV2 infection within groups (**S2 Fig**). Generally, similar cytokine expression profile was revealed in monocytes from previously DENV-exposed subjects and DENV-naïve individuals upon DENV2 restimulation *ex vivo* (Fig. 1C–G). In addition, comparable of HLA-DR and DC-SIGN expression from monocytes were also observed, representing a similar antigen-presenting function for monocytes (Fig. 2B and D). However, HLA-DR expression seems decreased but DC-SIGN was significantly enhanced by DENV2 infection for 72 h in the group, respectively (**S3 Fig**). This phenomenon may suggest that monocytes from previously DENV-exposed subjects exhibit an enhanced capacity for antigen capture, whereas their ability to stimulate T cells remains unchanged after 72 h DENV2 stimulation. We also found the similar expression level of HLA-DR and DC-SIGN between mock and DENV2 stimulated group (Fig. 2B and D). Our experiment aimed to establish continuous time-point culture conditions. Consequently, prolonged incubation may stimulate immune cells, potentially increasing the expression of relevant functional proteins. However, RNA microarray analysis revealed that the $CD14^+$ monocytes from DENV-experienced subjects still maintained the ability to activate adaptive immunity upon DENV2 restimulation. The underlying mechanisms of antigen presentation in previously DENV-exposed subjects after secondary DENV infection still require further investigation.

Circulating monocytes have a relatively short lifespan, approximately 1–3 days as half-life. Hence, isolation of monocytes from donors who recently experienced DENV infection becomes challenging. Therefore, the underlying mechanism of how DENV infection affects newly differentiated monocytes needs further study. Previous studies reported lower IL-6 secretion from monocytes after coculture with corresponding regulatory T-cells (Tregs) from acute DENV patients,[36] indicated that Tregs might be the potential monocyte regulators. Additionally, our previous research revealed that hematopoietic stem cells (HSCs) could be the reservoirs for DENV.[37,38] Furthermore, progenitor cells of monocytes was found significantly decreased after DENV infection in HSCs.[38] Hence, the detailed mechanism of how DENV interacts with HSCs and affects the newly differentiated monocytes requires further investigation.

T cell response plays a critical role in combating both primary and secondary DENV infection. Our results implied that T cells from previously DENV-exposed subjects showed comparable response in terms of IFNγ expression and memory response with DENV-naïve individuals. This phenomenon was compatible with the "original antigenic sin effect" regarding that serotype-specific T cells could not elicit an adequate immune response against secondary infection with different DENV serotypes. Previous study has shown that memory T cells expansion peaks earlier in primary DENV infection than in secondary infection subjects, as observed using *ex vivo* DENV A11-NS3133 tetramer stimulation.[39] Pieces of evidence from studies in endemic areas suggested impaired immune responses upon secondary DENV infection. Research from Thailand detected the frequency of intracellular $IFN\gamma^+$ T cells after DENV1-4 stimulation in subjects who subsequently developed subclinical or symptomatic DENV infections, finding that over 50 % were IFNγ non-responders.[40] Another study stated that the T cells from subjects with prior mild/sub-clinical DENV infection tended to produce granzyme B, but not IFNγ and TNF after NS3 peptide stimulation *ex vivo*.[41] This result supported our findings of lower $IFN\gamma^+$ T cell frequency in previously DENV-exposed subjects. An intriguing question arises whether the NS3 peptide act as a negative regulator of memory T-cell response in previously DENV-exposed subjects. Further investigation is needed to elucidate the detailed mechanisms involved.

The primary limitation of our research was the small number of previously DENV-exposed subjects, which restricted the number of samples we could collect and analyze. To better understand the infection history of these subjects, we attempted to classify the infecting serotype using PRNT. However, we were unable to identify the infected serotype in all donors by retrospectively checking the serotype-specific neutralizing antibody. In our study, four participants had neutralizing antibodies against at least one DENV serotype, suggesting past exposure to DENV (Table S1). The limited sample size restricted our study, as we could not collect samples across all age groups. A previous report from Vietnam indicated that higher age is associated with an increased risk of symptomatic dengue development in both primary and secondary DENV infections.[42] Conversely, a report from Singapore suggested that

increasing age in secondary DENV infections results in a higher incidence of dengue fever, rather than dengue hemorrhagic fever, among most adult dengue patients.[43] These findings imply that the immune response to DENV infection may vary across different age groups and remain somewhat controversial. Therefore, to enhance the comprehensiveness of future studies, it is essential to recruit DENV-naive donors residing in SEA and local residents from Taiwan with a history of DENV infection, covering all age groups as well as their naïve counterparts. This would allow for a more detailed analysis of the functions of monocytes and T cells, as well as the exploration of the underlying mechanisms in secondary DENV infections.

In conclusion, our pilot study provides the evidences about comparable immune response between previously DENV-exposed people from SEA and DENV-naïve residents in Taiwan. Further investigation is essential to determine whether the impaired response of monocytes and T cells in individuals with previous DENV infection leads to severe dengue upon secondary/multiple infection in endemic areas.

CRediT authorship contribution statement

Sheng-Hsuan Wang: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Yun-Erh Chuang:** Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Sia-Seng Tan:** Validation, Software, Methodology, Investigation, Formal analysis. **Tzu-Chuan Ho:** Software, Methodology, Investigation, Conceptualization. **Oscar Guey Chuen Perng:** Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Po-Lin Chen:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors have declared no conflicts of interest in the article.

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Appendix A. Supplementary data

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