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Altered monocyte response to the dengue virus in those with varying severity of past dengue infection

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ABSTRACT

Objective: We sought to investigate the differences in monocyte immune responses to the dengue virus (DENV) in those who previously had either severe disease (past SD) or non-severe dengue (past NSD) following a secondary dengue infection.

Method: Monocytes from healthy individuals who had either past SD (n = 6) or past NSD (n = 6) were infected at MOI one with all four DENV serotypes following incubation with autologous serum. 36-hours post infection, levels of inflammatory cytokines and viral loads were measured in the supernatant and expression of genes involved in viral sensing and interferon signaling was determined.

Results: Monocytes of individuals with past SD produced significantly higher viral loads (p = 0.0426 and cytokines (IL-10 p = 0.008, IL-1β p = 0.008 and IL-6 p = 0.0411) when infected with DENV serotypes they were not immune to, compared to those who has past NSD. Monocytes of individuals with past SD also produced significantly higher viral loads (p = 0.022) and cytokines (IL-10 p < 0.0001, IL-1β < 0.0001 and IL-6 p < 0.0001) when infected with DENV serotypes they were previously exposed to, despite the monocytes being infected in the presence of autologous serum. A significant upregulation of NLRP3 (p = 0.005), RIG-I (0.0004) and IFNB-1 (0.01) genes were observed in those who had past SD compared to past NSD when infected with non-immune DENV serotypes.

Conclusion: Monocytes from those with past SD appear to show marked differences in viral loads, viral sensing and production of inflammatory mediators in response to the DENV, when compared to those who experienced past NSD, suggesting that initial innate immune responses may influence the disease outcome.

1. Introduction

Dengue viral infections represent one of the most rapidly emerging mosquito borne viral infections in the world, with an estimated annual global cost of $8.9 billion (Shepard et al., 2016). It is estimated that 390 million individuals are infected with the dengue virus (DENV) annually of which 96 million manifest as apparent dengue infections (Bhatt et al., 2013). Intense monitoring with meticulous fluid control is currently the only option in the management of dengue infection, as specific treatments for dengue are not yet available. Therefore, it is important to further understand dengue pathogenesis in order to develop drugs for the treatment of acute dengue infection.

Infection with the DENV is associated with a self-limiting illness in the majority of individuals. However, it can cause severe clinical disease manifestations such as dengue haemorrhagic fever (DHF) and organ involvement in up to 10–25% of individuals, depending on serotype and population (Fernando et al., 2016; Lee et al., 2016). Although disease enhancement due to the presence of non-neutralizing antibodies and possibly cross-reactive T cells is thought to lead to severe disease (Guzman et al., 2013), DHF and fatalities have also been reported in primary dengue infection in the absence of DENV specific antibodies or T cells (Ong et al., 2007; Singla et al., 2016). In addition, it has been shown that inapparent dengue infection occurs in an equal proportion of those experiencing a primary or secondary dengue infection (Grange et al., 2014). Since the likelihood of developing an inapparent infection was shown to be the same for those who have an acute primary or

secondary dengue infection, the contribution of non-neutralizing cross reactive antibodies, which are present in those with a secondary dengue infection, in the pathogenesis of severe clinical disease should be further investigated (Grange et al., 2014). On the other hand, it could be an altered initial immune response to the DENV by immune cells readily infected by the virus, such as monocytes and dendritic cells, that could lead to either severe disease or asymptomatic infection.

Following the bite of a DENV-infected mosquito, the DENV infects immune cells such as dendritic cells, monocytes and mast cells, which in turn produce massive quantities of inflammatory cytokines and lipid mediators (Malavige and Ogg, 2017). The initial phase of viral replication is known as the febrile phase, which is then followed by a critical phase in some individuals that is characterized by fluid leakage (WHO, 2011). Patients who proceed to this vascular leakage phase (critical phase) are known to have developed DHF, whereas those who proceed to the recovery phase without any clinically apparent vascular leakage are diagnosed as having dengue fever (DF) (WHO, 2011). Inflammatory mediators such as platelet activating factor (PAF), IL-1β, TNFα, VEGF, and chymase produced by innate immune cells are thought to act on the vascular endothelium leading to endothelial dysfunction, which subsequently leads to vascular leakage (Malavige and Ogg, 2017; Tissera et al., 2017). Inflammatory mediators that lead to vascular leakage are highest at the critical phase (defervescence) of illness in patients with DHF (Jeewandara et al., 2015b; Kamaladasa et al., 2016; van de Weg et al., 2014). On the other hand, there is contradicting data showing that mediators and enzymes responsible for the production of these mediators are significantly elevated very early (day 2–4 since onset of symptoms) illness in those who proceed to develop DHF (Fernando et al., 2016; Jeewandara et al., 2017; Zanini et al., 2018). Therefore, in order to fully understand the reasons why some individuals develop DHF, while some develop milder clinical disease, it would be important to understand the events that occur during early infection and the differences in responses of immune cells infected with the DENV.

Studies both in vitro and in patients with acute dengue have shown that monocytes are target cells for DENV (Srikiatkhachorn et al., 2012; Zanini et al., 2018). Separately, an expansion of CD14^{+}CD16^{+} monocytes in acute dengue has been shown to be associated with severe clinical disease and to produce high levels of proinflammatory cytokines (Kwissa et al., 2014). In addition, monocytes have also been shown to induce differentiation of B cells into plasmablasts, further modulating disease pathogenesis (Kwissa et al., 2014). In addition, expression of CD163 and IFIT1 by monocytes in early infection (before the critical phase) was associated with severe disease. Therefore, monocytes appear to play a significant role in the development of severe dengue. In this study, to understand the role of monocytes in contributing to severe dengue, we investigated the responses of primary human monocytes of individuals who had developed either past severe dengue or past non-apparent dengue during a secondary dengue infection. Following infection of primary human monocytes in the presence of autologous serum, we examined the quantity of virus, differences in cytokine production and differences in gene expression in these two groups of individuals.

2. Methods

2.1. Human subjects

Twelve healthy adult individuals who previously had either asymptomatic dengue/mild dengue (n = 6) or DHF (n = 6) were recruited for this study. Healthy dengue seropositive individuals, who had never been hospitalized due to a febrile infection and therefore likely had an asymptomatic or mild infection) and were considered as having past non-severe dengue (past NSD). Individuals with past DHF were also healthy individuals who had previous DHF diagnosed according to WHO 2011 guidelines (past SD). Both group of individuals (past NSD

and past SD) were found to respond to, and therefore considered immune to, two serotypes of the DENV, by a T cell-based ELISpot assay (Jeewandara et al., 2018; Malavige et al., 2012b). We have previously validated this ELISpot assay, which can be used to determine the past infecting DENV serotypes (please see below) (Jeewandara et al., 2015a, 2018; Malavige et al., 2012b). Based on this assay, each individual with either past SD or past NSD, responded to two DENV serotypes at the time of recruiting them to this study and were considered to be immune to those DENV serotypes. We felt it was important that individuals with past NSD be immune to two DENV serotypes, as it was evident that they had a nonapparent dengue infection, even when they probably had a secondary dengue infection.

2.2. Ethics approval

The ethical approval was granted from the Ethical Review Committee of the University of Sri Jayewardenepura. All healthy individuals who participated in the study gave informed written consent.

2.3. Isolation and purification of monocytes

Peripheral blood mononucleocytes (PBMCs) were obtained from donors using lymphoprep (Axis-Shield, UK) density gradient centrifugation. The monocytes were positively selected from the PBMCs using CD14 magnetic beads (Milteny Biotech, Germany) using MACS separation columns (Milteny Biotech, Germany). The monocyte purity was determined by flow cytometry and was between 90 and 95%.

2.4. Virus propagation and titration

The following four DENV serotypes (DENV1-4) (kindly donated by Prof. Aravinda de Silva) were used in all experiments: DENV1 - West Pac 74; DENV2 - S16803; DENV3 - CH53489; DENV4- 341750 (TVP-360). The virus was propagated using the C6/36 cell lines and stored in aliquots at −80 °C until used. The concentration of the virus was determined by focus forming assays on Vero-81 cells and expressed as FFU/ml. Briefly, Vero-81 cells (kindly donated by Prof. Aravinda de Silva, UNC, USA) monolayers was infected with a 5-fold serial dilution of virus and incubated at 37 °C with 5% CO_2 for 2–3 days. After 2–3 days, the monolayer was fixed with 4% paraformaldehyde (Alfa Aesar, USA) and blocked with a blocking buffer, which contained 3% normal goat serum (Sigma-Aldrich, USA). To detect foci, a mix of 4G2 and 2H2 monoclonal antibodies (kindly donated by Prof. Aravinda de Silva) were used as the primary antibodies and HRP conjugated goat anti-mouse IgG (KPL, USA) as the secondary antibody. The plates were developed using the True-Blue Peroxidase Substrate (KPL, USA). All assays were done in duplicate.

2.5. Infection of monocytes with dengue virus

Monocytes isolated from healthy individuals were washed once with RPMI before infection with DENV. The four DENVs were first mixed separately 1:1 (v/v) with heat inactivated autologous serum of each individual for an hour. After 1 h, virus-serum mix was used as to infect the monocytes at MOI 1. Monocytes from each individual were infected with all four DENV serotypes separately in duplicate wells. Uninfected monocytes were incubated with Leibovitz medium (Sigma-Aldrich, USA) with autologous serum, as the negative control. After 90 min of virus absorption, the cells were washed again with RPMI and incubated in RPMI supplemented with 10% AB negative human serum (Sigma-Aldrich, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin in a 5% CO_2 at incubator 37 °C for a period of 36 h. The 36 h time point was chosen as several in vitro studies, which have investigated infection of monocytes/PBMC with DENV have shown that viral loads and the cytokines in the culture supernatant can reach a maximum at 48 HPI (Chen et al., 1999; Sun et al., 2011). At the end of

incubation and after collecting the culture supernatants, the monocytes were lysed and RNA was converted to cDNA immediately using a Cell to Ct kit (Life Technologies, USA) for gene expression analysis of these cells. The collected supernatant was stored at −80 °C till further use.

To confirm that monocytes were being infected using this approach, monocytes from two individuals infected with the DENV in the presence and absence of autologous serum were stained with anti DENV E glycoprotein antibody (Abcam, UK) and secondary antibody Goat anti-Mouse IgG-PE (Abcam, UK). The cells were acquired with Guava easyCyte 12-HT flowcytometer (Merck Millipore, UAS) and analyzed using FCS express 4 (De Novo, USA), which showed that < 1% of infection in line with previous studies (Miller et al., 2008).

2.6. Quantification of the DENV in monocyte culture supernatants by real time PCR

Viral RNA in monocyte culture supernatants was extracted using QIAamp Viral RNA Mini Kit (Qiagen, USA) and transcribed to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to manufacturer's protocol. Quantitative real-time PCR was performed as previously described using the CDC real time PCR assay for detection of the DENV (Santiago et al., 2013). Oligonucleotide primers and a dual labeled probe (*FAM™* and *QSY™*) for DEN 3 serotype was used (Life technologies, USA) based on published sequences (Fernando et al., 2016; Santiago et al., 2013).

Real-time PCR was performed using TaqMan® Multiplex Master Mix (Applied Biosystems, USA). The reaction was performed in an Applied Biosystems® *7500,* 96-well plate detection system. The threshold cycle value (Ct) for each reaction was determined by manually setting the threshold limit. Viral quantification (viral copy numbers/ml) of unknown samples was performed using the standard curve, as previously described (Fernando et al., 2016). All assays were done in triplicate.

2.7. Quantification of gene expression using real time PCR

The expression of 15 genes was determined with real time PCR using TaqMan® Gene Expression Master Mix (Applied Biosystems, USA) and TaqMan Gene Expression Assays (Applied Biosystems, USA). The relative expression of the following genes was analyzed: *IFNA2, IFNB1, DDX-58 (RIG-I), DHX-58, TRIM-25, ISG-15, NLRP3, TLR-3, TLR-7, TLR-9, MAPK-1, MAPK-3, MAPK-14,* and *NF-Kb*. The reaction was performed in an Applied Biosystems® 7500, 96-well plate detection system. The reaction set was done according to comparative C_T method, with GAPDH as the endogenous control and the uninfected monocytes as the reference sample. Relative quantification values were taken from the analysis software (Applied Biosystems, USA). All assays were done in triplicate.

2.8. Quantification of cytokines and PAF

The levels of IL-10, IL-1β, IL-6, IL-8, TNF-α, and VEGF were determined using multiplex cytokine bead arrays (Millipore, France) in monocyte culture supernatants while the levels of PAF, IFN-β (Cusabio, China), IFN-α (Mabtech, Sweden) and IL-18 (Abcam, UK) were measured using quantitative ELISA.

Further we measured the levels of IL-4, IL-5 and IL-13 (Biolegend, USA) and the presence of DENV specific IgG antibodies using the indirect dengue IgG capture ELISA (Panbio, Australia) in serum samples (12) of all healthy individuals. The quantity of DENV specific IgG antibodies were semi-quantitatively assessed by this indirect dengue IgG capture ELISA and the antibody titres were expressed as PanBio units.

2.9. T cell-based assay to determine immunity to DENV serotypes

In our previous studies, we carried out cultured ELISpot assays using a T cell-based assay in a large cohort of individuals to determine DENV

serotype immune responses (Jeewandara et al., 2015a). From this cohort of individuals, we selected 12 healthy individuals who either had past SD or past NSD and were immune to only two DENV serotypes (Jeewandara et al., 2015a). Cultured ELISpot assays were performed on these 12 individuals with past SD and past NSD as previously described using a panel of DENV serotype specific peptides from highly conserved regions of the DENV, which did not share any homology with other flaviviruses (Jeewandara et al., 2018; Malavige et al., 2012b). Briefly, PBMCs from each donor were incubated with the pool of 17 peptides serotype specific peptides representing all 4 serotypes of the DENV. There were four peptides specific to DENV-1, five specific to DENV-2, four specific for DENV-3 and four specific for DENV-4. T cell lines were tested individually after 10 days by ELISpot assays for responses to the 17 serotype specific peptides. All peptides that induced an IFN-γ response of more than mean + 3 standard deviations of the control wells were considered positive. All individuals with past SD or non-SD responded to serotype specific peptides of two DENV serotypes and were thus considered to be immune to those two serotypes. The two serotypes that these individuals did not respond to were considered as the DENV serotypes for which these individuals were non-immune.

2.10. Epitope binding studies

ELISA plates were coated with anti-V5 tag antibodies, followed by incubation of V5-tagged wildtype or mutated E proteins produced in S2 cells. This indirect ELISA method facilitates immobilization of E protein as dimers and the method and its validation have been described in detail before (Xu et al., 2016). Each serum sample was incubated on 12 E protein mutants and the WT E protein. Binding was detected with a goat anti-human IgG-HRP antibody (ThermoFisher, Singapore), and TMB substrate was used to quantify binding. For standardization, a mixture of four antibodies binding to different sites of the E protein was used (control). Loss of binding was calculated as follows: (binding of serum X to E protein mutant Y/binding of control to E mutant Y)/ (binding of serum X to WT E protein/binding of control to WT E protein). Using this formula, the value for the binding to WT E protein is 1 for each serum.

2.11. Statistical analyses

Data analysis was performed using Graph Pad Prism 6.0 software. As the data were not normally distributed, differences in in the viral loads and cytokine levels in monocyte culture supernatants were compared using the Mann-Whitney *t*-test (two tailed). The associations between cytokines and viral loads in culture supernatants was determined by using the Spearman rank correlation.

3. Results

3.1. Immunity to DENV serotypes in this cohort of healthy donors

Of the 12 healthy individuals recruited, 6 had had an episode of DHF in the past and were considered to have past SD and were found to be immune to two DENV serotypes (Table 1), based on the results of the cultured T cell ELISpot assays. The other 6 healthy individuals were seropositive for the DENV and were also found to be immune to two DENV serotypes (Table 2), despite them never having been hospitalized for a febrile illness and not being aware of an infection (past NSD). Therefore, each individual (those with past SD and past NSD) were considered to be immune to two DENV serotypes and non-immune to the two remaining of the four DENV serotypes. The details of the type of DENV serotypes those with past SD were immune to and when they were hospitalized due to DHF are shown in Table 1. The serotype specific peptides to which each individual responded are given in Fig. 1.

The DENV serotype responses in those with past NSD are shown in Table 2. The dates of infection due these serotypes are unknown as they

Table 1

DENV serotypes that individuals with past SD were found to be immune to and the dates of hospitalization.

Individuals with past SD	Immune serotypes	Date of hospitalization due to DHF (years before serum analysis for this study)	Duration of the hospital stay (days)
SD1	DENV 2 and DENV 4	January 2010 (7 years)	7
SD2	DENV 1 and DENV 4	May 2016 (1 year)	4
SD3	DENV 1 and DENV 4	June 2016 (1 year)	5
SD4	DENV 1 and DENV 2	June 2016 (1 year)	5
SD5	DENV 2 and DENV 4	June 2012 (5 years)	4
SD6	DENV1 and DENV 4	May 2012 (5 years)	5

Table 2

DENV serotype-specific immunity of those with past NSD.

Individuals with past NSD	Immune DENV serotypes
NSD1	DENV 1 and DENV 3
NSD2	DENV 1 and DENV 3
NSD3	DENV 1 and DENV 3
NSD4	DENV 1 and DENV 3
NSD5	DENV 1 and DENV 2
NSD6	DENV 1 and DENV 2

had never been hospitalized due to a febrile infection in the past.

3.2. Viral loads and inflammatory cytokine production of monocytes of those with past SD or past NSD

A number of studies have shown that a high viral burden before defervescence is associated with development of severe disease in patients with acute dengue (Nunes et al., 2018; Vaughn et al., 2000). It has recently been shown that DENV RNA was detectable predominantly in naïve B cells and monocytes during acute dengue infection in vivo (Zanini et al., 2018). Since monocyte infection by the DENV has been previously investigated (Diamond et al., 2000a; Wong et al., 2012), we focused on infection of primary human monocytes in this study. To address whether different individuals possessed a cell-intrinsic capacity to produce more or less virus when infected with the DENV, we sought to investigate if monocytes of individuals with past SD produced higher viral titers than those with past NSD when infected with different DENV serotypes. Indeed, we found that viral titers were significantly higher in culture supernatants of monocytes from individuals who had past SD when compared to individuals who had past NSD, when infected with either immune (p = 0.036) or non-immune DENV serotypes (p = 0.043). (Fig. 2A).

IL-10, IL-1β, IL-6, IL-8, TNF-a, IL-18, and VEGF and lipid mediators such as PAF are elevated in patients with acute dengue and have been shown to be associated with disease severity (Jeewandara et al., 2015b; Kamaladasa et al., 2016; Malavige et al., 2012a, 2013; Priyadarshini et al., 2010). In contrast, in vitro experiments have shown that type I interferons such as IFNα and IFNβ reduce DENV replication in infected cells and high levels of IFNα levels were seen in patients with DF compared to those with DHF (Diamond et al., 2000b; Ubol et al., 2008). Therefore, we proceeded to investigate if monocytes from individuals with past SD produced more inflammatory cytokines compared to those with past NSD, when infected with the DENV. Uninfected monocytes
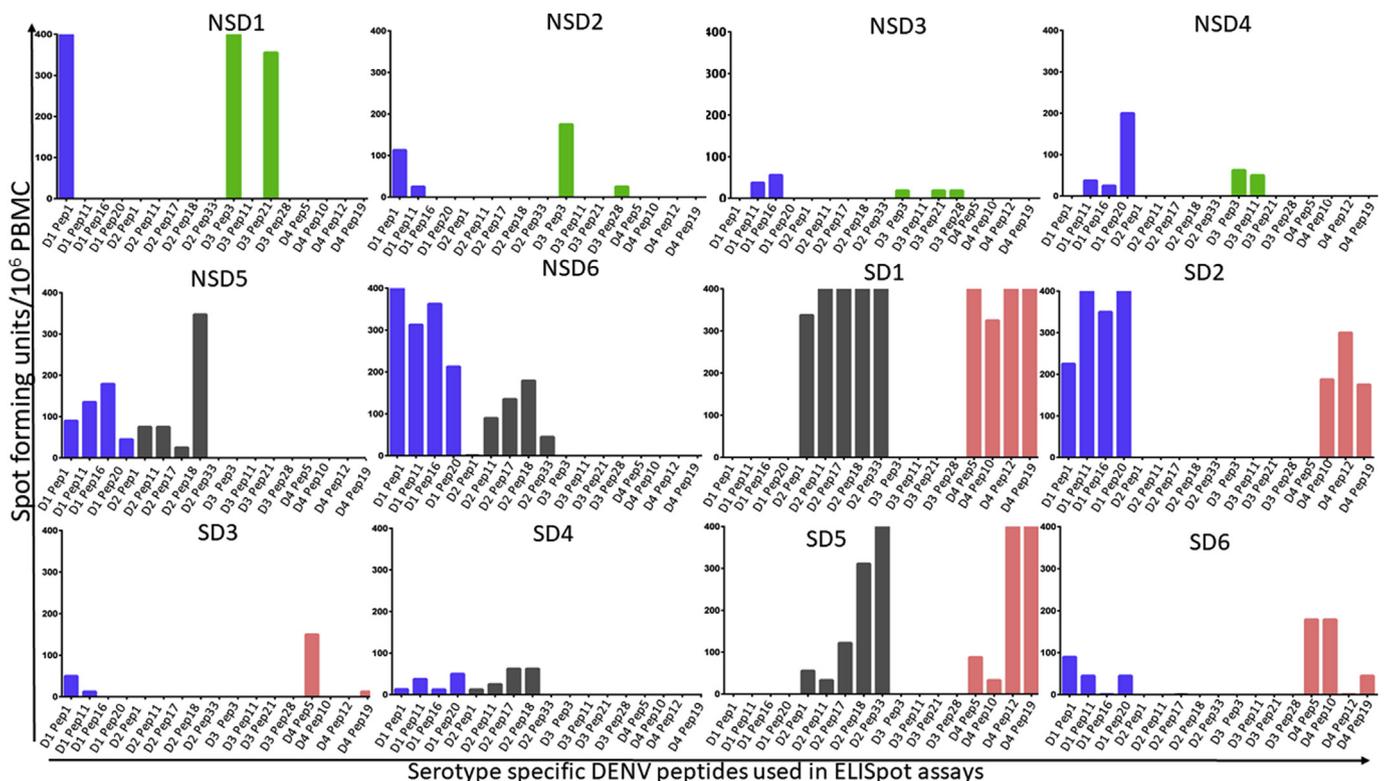

Fig. 1. ELISpot responses as spot forming unit per 10^6 PBMC for each individual for the 17 DENV serotype specific peptides PBMCs from individuals who had had past NSD (n = 6) and past SD (n = 6) were cultured with IL-2 to expand T cells and were stimulated with a panel DENV serotype specific peptides from highly conserved regions of all four DENV serotypes and IFN-γ ELISpots was carried out. Response to a particular peptide was taken as an indicator for a previous DENV infection by that serotype. DENV serotype 1: D1, DENV serotype 2: D2, DENV serotype 3: D3, DENV serotype 4: D4, Pep: Peptide.
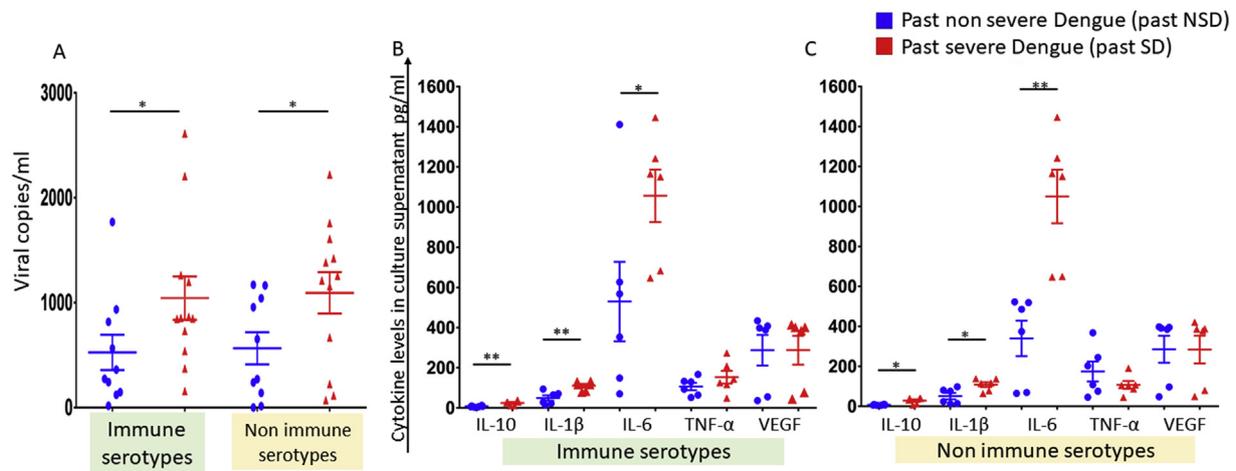
Fig. 2. Viral loads and cytokine levels in the culture supernatant of monocytes infected with DENVMonocytes isolated from individuals who had past NSD (n = 6) and past SD (n = 6) were separately infected with all four DENV serotypes in the presence of autologous serum. After 36 h infection the following were quantified in the culture supernatant A) viral loads, B) and C) levels of IL-10, IL-1β, IL-6, TNF-α and VEGF compared between previously immune and non-immune serotypes. Monocytes of each per person were infected with 4 DENV serotypes, of which two immune DENV serotypes and two were nonimmune serotypes. Symbols represent individual Luminex measurements. Statistical significance based on Mann Whitney test. The line displays the median and Interquartile Range. *p < 0.05 **p < 0.01 ***p < 0.005.

from individuals with both past NSD and SD, were shown to produce very low, insignificant but varying amounts of IL-6, IL-10 and TNF-a. We found that monocytes from individuals with past SD produced significantly more IL-10, IL-1β and IL-6 when infected with both immune (IL-10: p = 0.008, IL-1β: p = 0.008, and IL-6: p = 0.0411) and non-immune DENV serotypes compared to individuals with past NSD (IL-10: p = 0.015, IL-1β: p = 0.041 and IL-6: p = 0.002) (Fig. 2B and C). A higher production (p = 0.043) of TNFα was only seen in monocyte culture supernatants in those with past SD when infected with immune DENV serotypes (Fig. 2B). No significant differences were observed in VEGF and PAF levels in culture supernatants in these two groups irrespective of whether their monocytes were infected with previously immune or non-immune DENV serotypes (Fig. 2B and C). In all culture supernatants IL-8 and IFN-β levels were above the upper limit of detection of the assay, which was 14957 pg/ml and 2000 pg/ml, respectively (data not shown). We did not detect IL-18 and IFN-α in any of the monocyte culture supernatants.

IL-10, IL-1β, IL-6 and VEGF showed a significant and positive correlation with the viral titers in the culture supernatants of monocytes from individuals who has past SD, infected with both immune (IL-10: Spearmans' r = 0.56 P = 0.045, IL-1β: Spearmans' r = 0.74 P = 0.008, IL-6: Spearmans' r = 0.64, P = 0.03, VEGF: Spearmans' r = 0.69, P = 0.02) and non-immune DENV serotypes (IL-10: Spearmans' r = 0.68 P = 0.02, IL-1β: Spearmans' r = 0.71 P = 0.01, IL-6: Spearmans' r = 0.69 P = 0.01, VEGF: Spearmans' r = 0.68 P = 0.01) (Fig. 3B and D and Table 3). However, there was no correlation between IL-10 and IL-6 cytokine levels and viral titers in monocyte culture supernatants of individuals who had past NSD, irrespective of whether their monocytes were infected with immune or non-immune DENV serotypes (Fig. 3A and C). Interestingly, viral titers in monocytes culture supernatants from those who had past SD positively correlated with IL-1β, whereas a negative correlation was seen in those who had past NSD when infected with DENV (irrespective of immune or nonimmune). Therefore, while certain cytokines such as IL-1β, IL-6 and IL-10 showed a positive correlation with the viral titers in those with past SD, IL-1β and IL-6 showed a negative correlation with viral loads in monocyte culture supernatants of individuals with past NSD.

3.3. Expression of genes associated with initial viral recognition in monocytes with immune and non-immune DENV serotype

Since monocytes from individuals with past SD and past NSD showed differences in the degree of infection and the type and quantities of cytokines they produced, we proceeded to investigate if these differences could be attributed to differences in viral recognition and the downstream signaling pathways in monocytes in these two groups of individuals. We investigated the expression of fourteen genes associated with initial viral recognition and downstream signaling in DENV infected monocytes and of these only *RIG-1, NLRP-3* and *TRIM-25* were differentially expressed (Fig. 4). Individuals who experienced past SD showed a significant upregulation of *RIG-I* (P = 0.0004) and *NLRP-3* (P = 0.005) genes compared to individuals who had past NSD when infected with non-immune DENV serotypes. TRIM-25 showed a trend towards upregulation in monocytes of those with past SD irrespective of infecting serotype, but this was not significant.

Interestingly, in monocytes from individuals who experienced past SD, NLRP-3 gene expression showed a significant and positive correlation with IL-1β levels in culture supernatants (Spearman r = 0.4645, P = 0.029) and a significant negative correlation with IL-10 levels (Spearman r = −0.4432, P = 0.038) when infected with non-immune DENV serotypes. However, we did not observe any association with viral titers and NLRP-3 expression when monocytes were infected by immune or non-immune DENVs in these individuals. Although RIG-I was significantly upregulated in monocytes in those with past SD, RIG-I expression levels did not correlate with cytokine levels or viral titers in individuals who experienced past SD.

3.4. Difference in dengue IgG antibody levels, dengue E proteins binding properties between individuals who experience asymptomatic and severe dengue

As we observed that those with past SD had higher viral titers when compared to those who had past SD, when their monocytes were infected with immune or non-immune DENV serotypes, we sought to investigate if this was due to any differences in the type and quantity of DENV antibodies in serum. We assessed the DENV specific IgG levels semi quantitively using a commercial assay quantifying IgG antibody titres as Panbio units. DENV IgG levels were significantly higher (P = 0.015) in the serum of individuals who had past NSD (median,
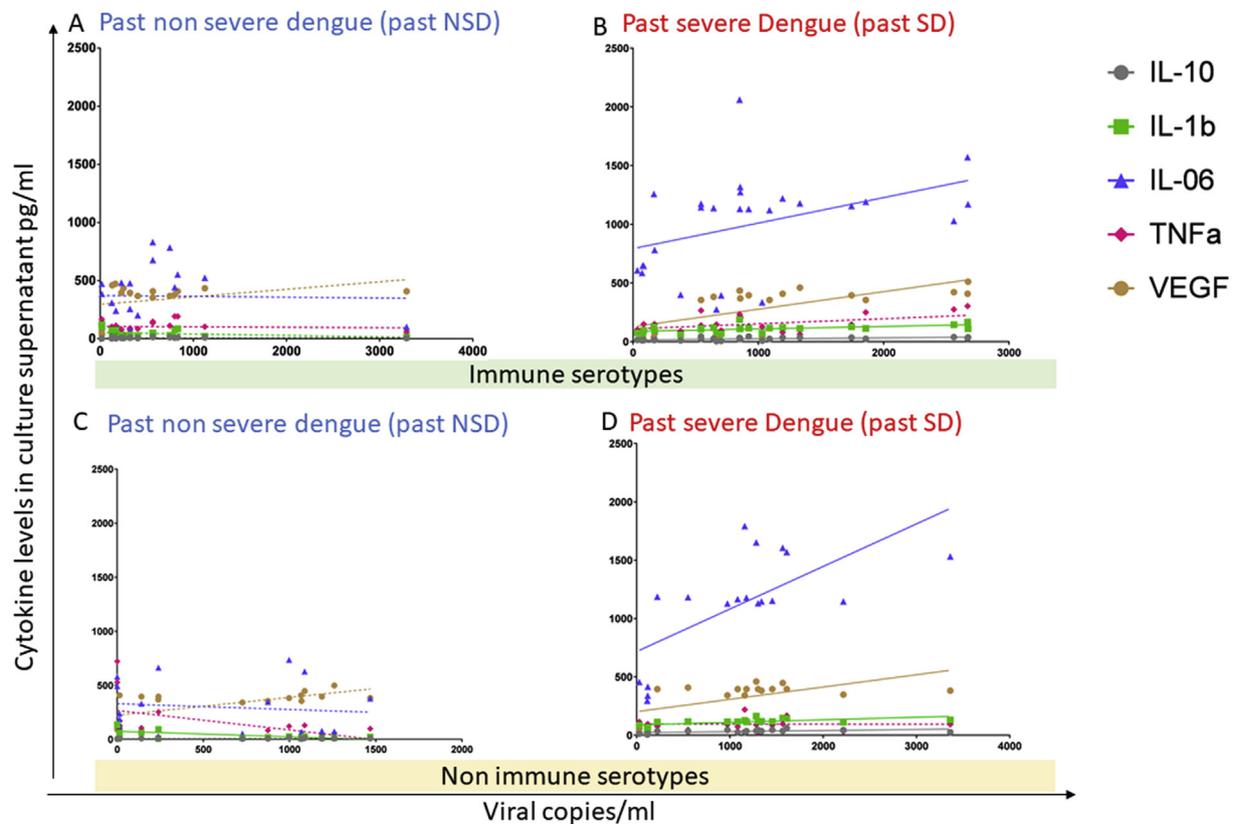
Fig. 3. Correlation of viral loads with cytokines in the monocyte culture supernatant when infected with DENV Viral loads were correlated with cytokine levels in the monocytes culture supernatants in A) past NSD and B) past SD individuals after infections with immune DENV serotypes and C) past NSD and D) past SD individuals after infections with non-immune DENV serotypes. Symbols represent individual Luminex measurements for a given sample for which the viral amount was determined as well. Monocytes of each individual were infected with 4 DENV serotypes, of which two were immune DENV serotypes and two were nonimmune serotypes. Statistically significant correlations are indicated in solid line. Statistically non-significant correlations are indicated in doted lines. Refer to Table 3 for Spearman r and p values.

IQR) compared to individuals who had past SD (median, IQR) (Fig. 5A).

While antibodies to complex epitopes including the hinge region and antibodies to EDIII can be efficiently neutralizing (de Alwis et al., 2012; Messer et al., 2014), antibodies to the fusion loop of the DENV envelope are in general more prone to support antibody dependent enhancement (ADE) (Balsitis et al., 2010; Beltramello et al., 2010; de Alwis et al., 2014). Therefore, we tested whether the serum from individuals with past SD and NSD had different repertoires, particularly with regards to fusion-loop specificity. We used 12 alanine-replacement E protein mutants from DENV-2 to test loss of binding to the mutated E protein dimers in the sera of all individuals (Xu et al., 2016). Three mutations were in the fusion loop whereas the other nine were reported previously to be dominant epitopes of patient-derived monoclonal

antibodies (Dejnirattisai et al., 2015). The epitope binding studies showed that DENV antibodies from all individuals similarly bound to two epitopes in the fusion loop of DENV-2 E protein and to Threonine 155, a glycosylation site in E domain II, regardless of the severe or asymptomatic infection history (Fig. 5B).

Binding to the WT E protein (standardized to 1 for the epitope mapping in Fig. 5B) was different for the individual donors (Fig. 5C) but there was no trend of higher binding in the SD or NSD, although the limitation is that only binding to DENV-2 E dimers was tested.

Table 3
Correlation of viral loads with cytokines in the monocyte culture supernatant when infected with DENV.

| Immune serotypes				Non-immune serotypes			
Cytokine	Spearman r value	P value	Significant level	Cytokine	Spearman r value	P value	Significant level
Past non severe dengue (past NSD)				Past non severe dengue (past NSD)			
IL-10	0.56	0.10	NS	IL-10	0.39	0.26	NS
IL-1β	−0.14	0.71	NS	IL-1β	−0.72	0.02	p = 0.02
IL-6	0.39	0.26	NS	IL-6	−0.16	0.66	NS
TNF-α	−0.30	0.95	NS	TNF-α	−0.61	0.07	NS
VEGF	0.22	0.54	NS	VEGF	0.54	0.11	NS
Past severe Dengue (past SD)				Past severe Dengue (past SD)			
IL-10	0.56	0.046	p = 0.04	IL-10	0.65	0.003	p = 0.01
IL-1β	0.74	0.008	p = 0.007	IL-1β	0.71	0.0008	p = 0.01
IL-6	0.64	0.03	p = 0.02	IL-6	0.58	0.01	p = 0.01
TNF-α	0.39	0.20	NS	TNF-α	−0.013	0.96	NS
VEGF	0.66	0.022	p = 0.02	VEGF	0.49	0.038	p = 0.01
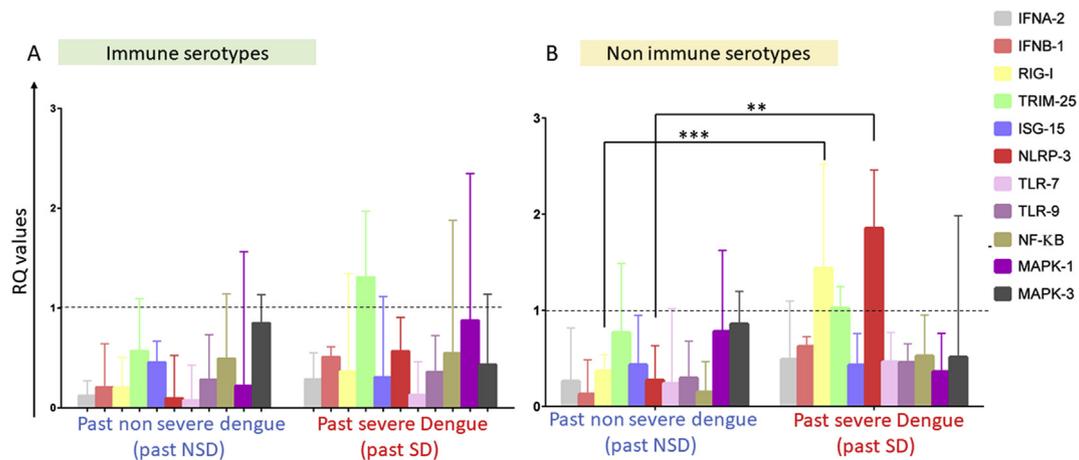
Fig. 4. Expression of genes associated with initial viral recognitions in monocytes incubated with DENV Monocytes isolated from individuals who had past NSD (n = 6) and past SD (n = 6) were separately infected with all four DENV serotypes in the presence of autologous serum. After 36 h infection, monocyte gene expression was quantified for the following conditions: A) infected with immune DENV serotypes B) infected with non-immune serotypes. RQ: Relative Quantification. RQ more than 1 indicate upregulation of the gene. Statistical significance based on Mann Whitney test. Bars display the Median. **p < 0.01 ***p < 0.005.
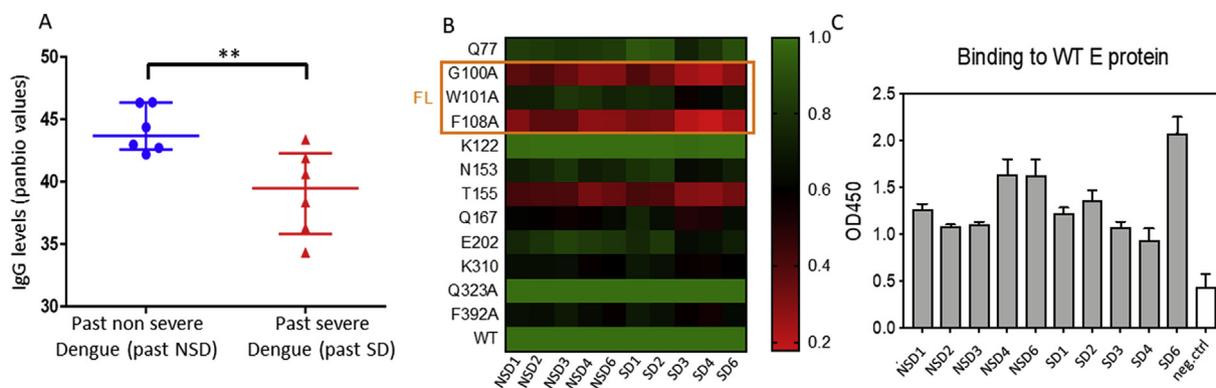

Fig. 5. Dengue- and E-protein specific serum IgG levels in individuals with past asymptomatic or severe dengue. Binding of antibodies in serum of individuals who had past NSD (n = 5) and past SD (n = 5). A) Dengue IgG levels quantified by commercial indirect PanBio Kit B) Serum IgG binding to DENV-2 E protein mutants. C) Serum IgG binding to DENV wildtype (WT) E protein.

3.5. Monocyte responses in those with past SD and NSD when co-cultured with NSD or DENV seronegative sera

Based on the above experiments, although monocytes of those with past SD produced higher viral loads, inflammatory cytokines and increased expression of NLRP3 and RIG-I, and although there was no difference in antibody specificity for the fusion loop, it was still not clear if the differences were due to the monocyte response or due to differences in serum. In order to answer this question, we infected monocytes with DENV1 and DENV2 in the presence of serum from a healthy donor who had past NSD and was found to be immune to DENV1 and DENV3 by the T cell-based assay and by neutralizing antibody assays (Table 2 and Fig. 1, NSD1). The neutralizing antibody titres of this individual for the 4 DENV serotypes were as follows: DENV1 = 957, DENV2 = 385, DENV3 = 860, DENV4 = 243. Serum from a seronegative donor was used as control. The monocytes used in this experiment were from 3 individuals with past SD and from 3 individuals with past NSD.

We found that the IL-1β production by monocytes of those with past SD was higher compared to the monocytes of those with past NSD (n = 3) irrespective of whether the monocytes were co-cultured with serum of a NSD donor or a seronegative donor (Fig. 6A and Table 4). Interestingly, the viral loads of monocytes from individuals with either previous SD or NSD were lower when co-cultured with DENV seronegative serum and when infected with a serotype (DENV-2), which

was a serotype the healthy past NSD donor was not immune to (Fig. 6B). These results suggested that the serum enhanced infection effect was independent of IL-1β production.

We then proceeded to further study if the increased expression of IL-1β by monocytes of those with past SD was due to a serum or monocyte effect by co-culturing monocytes of an individual with past SD and an individual with past NSD with sera of 3 individuals with past SD and 3 individuals with past NSD. We observed that monocytes of the individuals with either past SD or past NSD produced more virus (DENV2) and more IL-1β when co-cultured with sera from donors with past SD compared to serum from donors with past NSD (Fig. 7 A and B). For instance, the IL-1β levels in supernatants of those with past NSD, when infected with DENV2 in the presence of serum from donors from SD was a median of 446.0 (IQR = 183.9–446) pg/ml, compared to when they were infected in the presence of serum from NSD donors (median 339.5, IQR = 319.9–445.6 pg/ml). The median IL-1β production of monocytes of those with past SD, when infected with DENV2 in the presence of serum from donors from SD was 805.5 (IQR = 750.4–873.9) pg/ml compared to when they were in the presence of serum from NSD donors (median 294.1, IQR = 265.4–375.6 pg/ml). Again, the effect of enhanced infection was associated with the serum and likely a result of ADE. The IL-1β production appeared to be more independent of the serum.
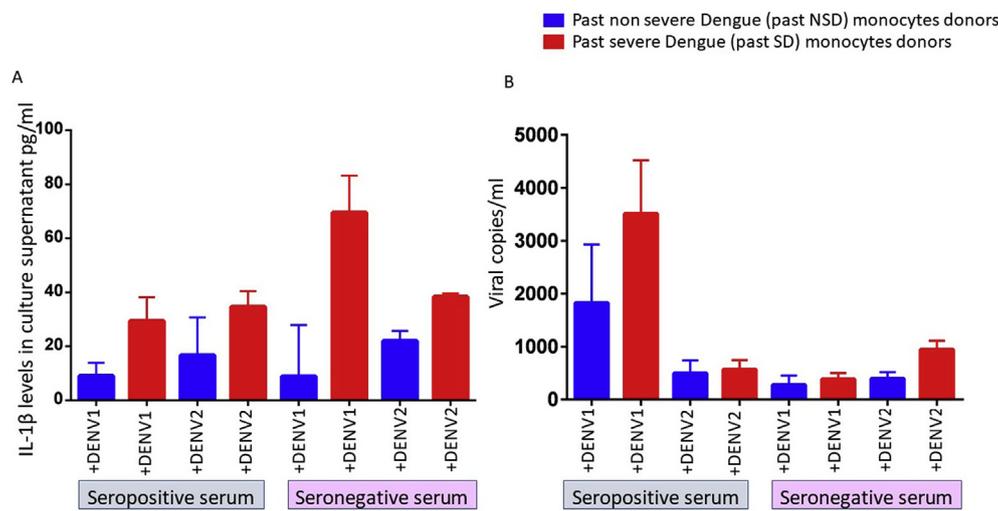
Fig. 6. Viral loads and IL-1β levels in the culture supernatant of monocytes infected with DENV incubated with common serum donor Monocytes isolated from individuals who had past NSD (n = 3) and past SD (n = 3) were separately infected with DENV1 and DENV2 after incubation with dengue seropositive serum of one donor who had past NSD and immune to seropositive for DENV1 AND DENV3 and dengue seronegative serum. After 36 h infection the following were quantified in the monocyte culture supernatant of those with past SD and past NSD A) viral loads and B) IL-1β. The line displays the median and interquartile range, p values are listed in Table 4.

4. Discussion

In this study we have investigated the differences in the responses to the DENV from monocytes from individuals who had past SD and past NSD. Although all individuals were healthy at the time of recruitment and had the episode of DHF more than 1 year ago, at the time of recruitment to the study, marked differences in the responses to the DENV were seen. Monocytes of individuals with past SD produced significantly higher viral loads and inflammatory cytokines (IL-1β, IL-6 and IL-10) when infected with either the DENV they were immune to and were non-immune to, despite them been infected with autologous serum. This observation could be due to the presence of poorly neutralizing, infection enhancing antibodies to the fusion-loop region of the envelope protein (de Alwis et al., 2014; Rodenhuis-Zybert et al., 2011), in those who developed past SD, leading to increased monocyte infection rates and cytokine production. However, at least the type and quantity of fusion loop-specific antibodies present in the sera of those with past SD and past NSD were found to be similar when examining binding to 12 different types of mutations, suggesting the changes in specificity or quantity of antibodies to the DENV envelope fusion loop protein are unlikely to be the cause.

In order to further understand if the differences in monocyte responses to the DENV in those with past SD and NSD were due to certain inherent differences of the monocytes or due to differences in the serum, we tested the same sera from a healthy individual with past NSD immune to DENV1 and DENV3 using different monocyte donors. Again, we observed a trend towards higher production of IL-1β from monocytes of those with past SD when compared to those with past NSD (Fig. 6). These observations were also consistent when DENV seronegative sera was used (Fig. 7). These experiments further suggest that monocytes of those who had past SD respond to the DENV by producing more inflammatory cytokines, possibly due to differences in viral

sensing or due to changes in downstream signaling of these pathways leading to enhanced inflammatory cytokine production. Interestingly, the viral loads of culture supernatants were higher in monocyte culture supernatants when infected with DENV1 in the presence of DENV1 immune sera, likely as a result of enhancement by low titers of cross-reactive antibodies. Although monocytes of those with either past SD or NSD had higher viral loads in the above conditions, viral loads were higher in those with past SD. Monocytes of those with past SD and NSD produced higher viral loads and IL-1β, when co-cultured with sera from individuals with past SD. Collectively, these experiments suggest that the differences in monocyte responses to the DENV in those with past SD are likely to depend on their sera as well as inherent differences within monocytes themselves, whereby the inflammatory IL-1β response seemed to depend more on monocytes than on serum. Since the type of cytokine production and immune responses are known to be different in classical monocytes compared to inflammatory monocytes (Wu et al., 2013a; Yang et al., 2014), it would be important to further investigate the relative proportions of these monocyte populations within these groups and possible epigenetic changes in viral gene transcription pathways.

Although those with past SD and NSD did not differ in the quantity and specificity of antibodies to the fusion loop proteins, those with past NSD had significantly higher DENV IgG antibody titers to DENV envelope protein, probably mostly in its monomeric form, when semiquantitatively measured by using an indirect DENV IgG ELISA (Panbio, Australia). We cannot exclude that the time between infection in the NSD group and the time these assays were done was, on average, shorter compared to the SD group, explaining the higher titers. It is also possible that those with past NSD had higher titres of neutralizing antibodies, which were shown to be associated with occurrence of milder disease (Katzelnick et al., 2016). Unfortunately, due the limited availability of the quantity of sera, we were unable to carry out assays to

Table 4
IL-β levels in the culture supernatant of past SD and past NSD monocyte donors when infected with DENV incubated with seropositive serum or seronegative sera.

|	Past NSD monocyte donors, n = 3 Median (IQR)	Past SD monocyte donors, n = 3 Median (IQR)	P value
Virus was incubated with:			
DENV Seropositive serum			
● Infected with DENV 1	9.0 (7.1–13.8) pg/ml	29.4 (28.6–38.2) pg/ml	P = 0.1000
● Infected with DENV 2	16.7 (13.9–30.7) pg/ml	37.7 (33.7–40.4) pg/ml	P = 0.1000
DENV Seronegative serum			
● Infected with DENV 1	8.8 (6.6–27.89) pg/ml	69.49 (46.8–83.2) pg/ml	P = 0.1000
● Infected with DENV 2	22 (18–25.7) pg/ml	38.4 (37.1–39.5) pg/ml	P = 0.2000

IQR- Inter Quartile Range.
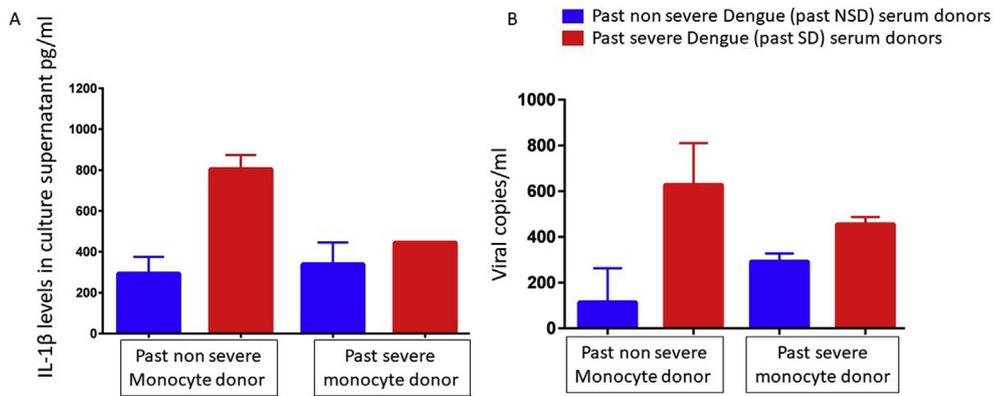
Fig. 7. Viral loads and IL-1β levels in the culture supernatant of monocytes infected with DENV isolated from two individuals Monocytes isolated from one individual who had past NSD and one who had past SD were separately infected with DENV2 after incubating with serum from individuals with past NSD (n = 3) and individuals with past SD individuals (n = 3). After 36 h infection the following were quantified in the monocyte culture supernatants A) viral loads and B) IL-1β. The line displays the median and Interquartile Range.

quantify the neutralizing antibody titers to all 4 DENVs in the individuals for Figs. 1–5. In order to understand how differences in monocyte responses could contribute to severe disease in secondary dengue infections, it would be important to study monocyte responses in a larger cohort of individuals and follow them longitudinally to see which patients develop severe vs asymptomatic secondary dengue.

It has been shown that susceptibility of monocytes to dengue infection is increased by treatment with IL-4 or IL-13 (Miller et al., 2008). The presence of metabolic diseases and allergic diseases such as asthma have shown to associate with development of severe dengue (Pang et al., 2017). Since patients with allergies and asthma are known to have higher type 2 cytokines in their sera (Steinke and Borish, 2001), we assessed if those with past SD had higher IL-4, IL-5 and IL-13 levels in their sera, which could contribute to increased monocyte infection. However, none of the healthy individuals who either had past SD or NSD had detectable levels of any of these cytokines in their sera, suggesting that increased infection rates of monocytes of individuals with past SD were unlikely to be due to baseline presence of such cytokines. As it is difficult to capture samples during asymptomatic infection, it will be difficult to comparatively address levels of type 2 cytokines during acute SD and NSD infection.

The main receptors that sense the DENV are the cytoplasmic retinoid acid inducible gene I (RIG-I), TLR-3 and TLR-7 (Uno and Ross, 2018). Recognition of DENV by RIG-1 leads to activation of many downstream signaling pathways, which ultimately lead to production of type I interferons and inducing an antiviral state (Baum and Garcia-Sastre, 2010; Uno and Ross, 2018). In this study we determined the expression of several of these genes along with interferon inducible genes in the monocytes of those with past SD and non-SD. We did not observe a significant upregulation of any of these genes when the monocytes were infected with the DENV serotypes that these individuals were immune to, possibly due to lower viral titres observed when the monocytes were infected with immune serotypes in the presence of autologous serum. Interestingly, the expression of RIG-1 and NLRP-3 was significantly higher in the monocytes of those who had past SD when infected with non-immune DENV serotypes. As the monocytes of those with past SD had higher viral replication as evidenced by significantly higher viral titers in culture supernatants, this could have led to an increase in RIG-I expression. However, there was no increase in the expression of TLR-3, TLR-7 or any of the interferon inducible genes or in the expression of IFNA and IFNB. Instead, there was a significant upregulation of NLRP-3 expression.

It was previously shown that the DENV activates the NLRP-3 inflammasome through CLEC5A, which is a C-type lectin (Wu et al., 2013b). It was shown in dengue mouse models that blockade of DENV-CLEC5A interaction reduced production of inflammatory cytokines and that anti-CLEC5A monoclonal antibodies impaired DENV associated vascular leakage, haemorrhage and reduced death (Chen et al., 2008). Therefore, NLRP3 inflammasome activation appears to be a key event

that leads to production of proinflammatory cytokines, contributing to immunopathology and vascular leakage. Indeed, we found that IL-1β production, which is dependent on NLRP3 activation, was significantly higher in the monocyte culture supernatants of those with past SD when compared to those with NSD. IL-1β levels in monocyte culture supernatants of those with past SD significantly correlated with expression of NLRP3 and also correlated with the virus titers. In contrast, IL-1β levels showed a significant and inverse correlation with the virus titers in monocyte culture supernatants of those with past NSD. In addition, there was no upregulation of the NLRP3 gene in those with past NSD. Therefore, upregulation of NLRP3 gene expression and subsequent production of inflammatory cytokines only occurred in those with past SD and not in those with past NSD. The reasons why only some individuals appear to have this response to the DENV, while others do not have inflammasome activation should be further investigated.

In summary, in this study we have explored the differences in monocyte responses to the DENV in those with past SD and NSD and found that monocytes of those with past SD produce higher viral titers, higher proinflammatory cytokines and also upregulate activation of the NRLP3 inflammasome. The activation of the NRLP3 inflammasome appears to be an important factor in development of subsequent severe disease and the reasons for its activation in those who developed past SD and not in those with past NSD should be further investigated.

Acknowledgements

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