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Lipopolysaccharide acts synergistically with the dengue virus to induce monocyte production of platelet activating factor and other inflammatory mediators

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ABSTRACT

Background: Platelet Activating Factor (PAF) has been shown to be an important mediator of vascular leak in acute dengue. Antibody dependent enhancement (ADE) and microbial translocation has also shown to contribute to severe dengue. Since monocytes are one of the primary targets of the dengue virus (DENV) we sought to investigate if monocytes were a source of PAF, and the effect of ADE and microbial endotoxin (LPS) on DENV infected monocytes.

Methods: PAF and cytokine levels were evaluated in serial blood samples, in patients with acute dengue infection. The effect of ADE and LPS in production of PAF and cytokines from DENV infected primary human monocytes derived macrophages (MDM θ) was assessed. Gene expression analysis was undertaken to investigate mechanisms by which LPS potentiates PAF and cytokine production by DENV infected MDM θ .

Results: Serum PAF levels significantly correlated with both TNF- α ($p < 0.0001$) and IL-1 β ($p < 0.0001$) in patients with acute DENV infection. Although primary human MDM θ produced inflammatory cytokines following infection with the DENV, they did not produce PAF following *in vitro* DENV infection alone, or in the presence of dengue immune serum. Levels of PAF produced by DENV infected MDM θ co-cultured with LPS was significantly higher than uninfected MDM θ s co-cultured with LPS. Although TLR-4 was upregulated in uninfected MDM θ s co-cultured with LPS, this upregulation was not significant in DENV infected MDM θ . Only expression of RIG-I was significantly up regulated ($p < 0.05$) when DENV infected MDM θ were co-cultured with LPS.

Conclusion: LPS acts synergistically with the DENV to induce production of PAF and other inflammatory cytokines, which suggests that microbial translocation that has shown to occur in acute dengue, could contribute to dengue disease severity.

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1. Introduction

Dengue viral infections are one of the most important mosquito borne viral infections in the world, and estimated to infect 390 million individuals annually (Bhatt et al., 2013). Since the year 1990,

the global incidence of dengue has doubled every decade and apart from the morbidity due to acute illness, disability due to post dengue related chronic fatigue was estimated to be 186,000 to 1,415,000 years lived with disability in 2013 (Stanaway et al., 2016). Although dengue causes a significant mortality and morbidity, severe forms of dengue such as dengue hemorrhagic fever (DHF) occur only in a proportion of those with clinically apparent infections (WHO, 2011).

Endothelial dysfunction leading to increased vascular permeability is the hallmark of severe dengue infection (Martina et al., 2009). Although the exact timing of fluid leakage is not known, it

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becomes clinically detectable around 3–7 days of onset of illness. The critical phase of dengue infection is thought to last for 24–48 h, following which the leaked fluid is reabsorbed and the patient usually recovers (WHO, 2011). Due to the temporary nature of the fluid leakage in dengue, it is thought that increased vascular permeability is likely to be due to changes in the endothelial electrical resistance and gap junction protein expression, rather than damage to the endothelium (Martina et al., 2009; Srikiatkachorn and Kelley, 2014). Many cytokines, protease mediators and more recently dengue NS1 have been shown to associate with vascular leak in acute dengue (Appanna et al., 2012; Srikiatkachorn et al., 2007; Gomes et al., 2014; Beatty et al., 2015).

We recently reported that platelet activating factor (PAF) is an important mediator of vascular leak and also implicated altered sphingosine 1-phosphate levels as a further contributory mechanism (Gomes et al., 2014; Jeewandara et al., 2015). We found that decrease in expression of the gap junction protein ZO-1 and reduction of the trans-endothelial electrical resistance by sera of patients with acute dengue infection, was significantly inhibited when human umbilical endothelial cells were pre-treated with a PAF receptor blocker (Jeewandara et al., 2015). PAF is a phospholipid mediator with many biological functions including induction of increased vascular permeability (Walterscheid et al., 2002). It has been shown that human monocytes produce PAF in a bi-phasic pattern when stimulated with lipopolysaccharide (LPS), which was shown to be due to the effects of cytokines such as TNF α and IL-1 β (Valone and Epstein, 1988; Han et al., 2002). PAF has been shown to activate transcription of NF- κ B, resulting in expression of many inflammatory cytokines such as TNF α and IL-1 β (Valone and Epstein, 1988; Han et al., 2002; Im et al., 1997). We too have observed that PAF is produced in a bi-phasic pattern in patients with acute dengue (Jeewandara et al., 2015). Since LPS was the main stimulus that resulted in bi-phasic production of PAF and other cytokines, it is possible that LPS plays a similar role in acute dengue infection. It has been shown that patients who develop plasma leakage during dengue infection have significantly higher levels of LPS than those who did not have plasma leakage (van de Weg et al., 2013; van de Weg et al., 2012). It was also shown that higher levels of LPS in dengue patients correlated with the levels of inflammatory cytokines (van de Weg et al., 2013).

Monocytes and macrophages are one of the cells that are predominantly infected by the dengue virus in acute dengue infection (Torrentes-Carvalho et al., 2009; Wong et al., 2012; Miller et al., 2008). Dengue virus (DENV) infected monocytes have also been shown to contribute to endothelial dysfunction in the presence of enhancing antibodies (Anderson et al., 1997). Monocytes and macrophages are also known to be an important source of PAF (Valone and Epstein, 1988; Mariano et al., 2003). Since monocytes and macrophages are a primary target of the DENV and since they are also known to produce PAF, we set out to investigate if monocytes derived macrophages produced PAF when infected with the DENV and under antibody dependent enhancement (ADE). Since LPS has been shown to act on monocyte derived macrophages (MDM θ) and stimulate PAF in a bi-phasic manner, we also investigated if the LPS levels reported in patients with DHF, stimulated production of PAF from DENV infected MDM θ . DENV infected MDM θ did not produce PAF when infected with the DENV alone or under conditions of ADE, but LPS appeared to act synergistically with the DENV to induce MDM θ to produce PAF and other inflammatory cytokines.

2. Methods

2.1. Patients

In one of our previous studies, we had determined changes in PAF levels and disease severity in 36 adult patients with acute

dengue infection (Jeewandara et al., 2015). The changes in the levels of PAF over time in this cohort of patients with DF and DHF are described in this paper. In order to determine the relationship between PAF and other inflammatory cytokines, we used stored sera of these patients for the current study. In this cohort of 36 patients (described in detail in our previous paper), serial blood samples were taken in the morning (approximately around 6 a.m.) and again at 1.00 p.m. throughout the duration of the hospital stay. The onset of illness was defined as the time of onset of fever. All clinical features and laboratory inducers were recorded several times each day from the time of admission to discharge from hospital. Clinical disease severity was classified according to the 2011 WHO dengue diagnostic criteria (WHO, 2011). Accordingly, patients with a rise in haematocrit above $\geq 20\%$ of the baseline haematocrit or clinical or ultrasound scan evidence of plasma leakage in a patient was classified as having DHF. Shock was defined as having cold clammy skin, along with a narrowing of pulse pressure of ≤ 20 mmHg. Based on this definition 25 patients were diagnosed to have DHF and 11 DF.

2.2. Ethics statement

The ethical approval was granted from the Ethical Review Committee of the University of Sri Jayawardanapura. All healthy individuals (n = 5) who also participated in the study gave informed written consent.

2.3. Confirmation of acute dengue

Acute dengue infection was confirmed in the serum samples using the NS1 early dengue ELISA (Panbio, Australia). All assays were done in duplicate. Dengue was also confirmed in these patients with a commercial capture-IgM and IgG enzyme-linked immunosorbent assay (ELISA) (Panbio, Brisbane, Australia). The ELISA was performed and the results were interpreted according to the manufacturers' instructions. This ELISA assay has been validated as both sensitive and specific for primary and secondary dengue virus infections (Vaughn et al., 1999; Sang et al., 1998).

2.4. Isolation and purification of monocytes

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors (n = 5). The monocytes were positively selected from whole PBMCs using CD14 magnetic beads (Milteny Biotech) using MACS separation columns (Milteny Biotech, USA). The monocyte purity determined by flowcytometry and was between 90 and 95%.

After separation, monocytes were placed into a 96 well U bottom plate (20,000 monocytes/well) in RPMI (Life technologies, USA) supplemented with 10% AB negative human serum (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin (Sigma). All monocytes were incubated with IL-4 (25×10^6 mg/ml) at 37 °C with 5% CO $_2$ for 48 h before infecting with the DENV-3 as previously described (Miller et al., 2008). IL-4 was used as it has been shown to increase the infection rate of MDM θ and dendritic cells (Miller et al., 2008; Schaeffer et al., 2015).

2.5. Virus propagation and titration

The DENV-3 CH53489 isolate was used in all experiments (kindly donated by Prof. Aravinda de Silva). 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 plaque assays on BHK-21 cells and expressed as PFU/ml. Briefly, a BHK-21 monolayer was infected with a 10-fold serial dilution of virus,

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and incubated at 37 °C with 5% CO₂ for 4 days. After 4 days, the monolayer was fixed and stained with crystal violet and the number of plaques counted. All assays were done in triplicate.

2.6. Infection of monocytes with dengue virus

Infection with MDM θ was carried out as previously described (Miller et al., 2008). MDM θ isolated from healthy individuals were washed once with RPMI before infecting with the DENV-3 virus inoculum at an MOI of 1. Uninfected MDM θ were treated similarly to the infected MDM θ and incubated with Leibovitz medium (Sigma) as the negative control for the same duration of time as the infected MDM θ s. After 90 min of virus absorption, the cells were washed again with RPMI and incubated in RPMI supplemented with 10% AB negative human serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin in a 5% CO₂ at incubator 37 °C. The cells were stained with the primary antibody, anti DENV E glycoprotein antibody (Abcam, UK) and the secondary antibody Goat anti-Mouse IgG secondary antibody Alexa Fluor 488 (Abcam, UK). Cells were observed with Olympus X100 microscope (Olympus) after mounting on DAPI stain (Sigma-Aldrich) to confirm infection (Supplementary Fig. 1).

The virus infected and uninfected MDM θ were incubated for a period of 24–96 h in the presence and absence of LPS and dengue immune sera (please see below) to determine the effects of these on production of LPS and other inflammatory mediators. The viability of the infected and uninfected cells was \geq 90% up to 96 h following infection.

2.7. Determining the effect of lipopolysaccharide (LPS) on PAF production

As LPS was found to associate with disease severity in patients with acute dengue (van de Weg et al., 2013; van de Weg et al., 2012) we proceeded to determine its effect on PAF production and production of other inflammatory cytokines in DENV infected monocytes. As the levels of LPS reported in patients with dengue in previous studies were in the range of 1–1000 pg/ml (van de Weg et al., 2013), we used a mean value of 500 pg/ml of LPS in our assays. Infected and uninfected MDM θ (20,000/well) from five healthy individuals were incubated for 96 h in the presence and absence of LPS (500 pg/ml per well) and the culture supernatant was collected at 24, 48, 72 and 96 h post infection (HPI) and stored in aliquots at –80 °C. All experiments were done in duplicate.

To determine the effect of LPS on gene expression infected and uninfected MDM θ (100,000/well) from five healthy individuals were incubated for 24 h in the presence and absence of LPS (500 pg/ml per well). RNA from the MDM θ was extracted and converted to cDNA using TaqMan Cells-to-CT kit (Applied Biosystems, USA) according to the manufacturer's instructions. The cDNA was stored in –20 °C until gene expression analysis was done.

2.8. Determining if the presence of dengue antibodies stimulated production of PAF

The ADE experiments were done as previously described (Chaichana et al., 2014). Briefly, in order to determine the effects of DENV specific antibodies, serum from healthy volunteers with known serotype of past dengue infection was collected and pooled together (Chaichana et al., 2014; Boonnak et al., 2013; Luo et al., 2013). Pooled serum was heat inactivated before used in ADE experiments. The sera were incubated in dilutions of 1:5, 1:10 and 1:100 in RPMI media (Life technologies, USA) containing DENV3 at 37 °C for 1 h prior to incubation with the purified monocytes. The primary human MDM θ were incubated with virus alone, or with virus/antibody complexes for 2 h at 37 °C at 5% CO₂. Cells were

centrifuged and the supernatants harvested at 24, 48, 72 and 96 h to determine PAF and other inflammatory cytokine levels the supernatant. These experiments were done using 5 donors and each experiment was done in duplicate.

2.9. Detection of cytokines and PAF

To measure levels of PAF (Cusabio, China), IL-10 (Mabtech, Sweden), TNF- α and IL-1 β (Biolegend, USA) in serum samples of patients with acute dengue, quantitative ELISA was done in duplicate on all serum samples according to manufacturer's recommendations. In the MDM θ culture supernatants the levels of TNF- α , IL-10, IL-12p40 and IL-6 were determined using Luminex (Millipore, France) according to manufacturer's recommendations while PAF levels was determined using the same ELISA kit.

2.10. Dengue virus quantification by RT-PCR

Viral RNA in MDM θ culture supernatants was extracted using QIAamp Viral RNA Mini Kit (Qiagen, USA) and transcribe to cDNA using High Capacity cDNA reverse transcription kit (Applied Biosystems, USA) as per the manufacturer's protocol. Quantitative real-time PCR was performed as previously described using the CDC real time PCR assay for detection of the DEV (Santiago et al., 2013). Oligonucleotide primers and a dual labeled probe (FAMTM and TAMRATM) for DEN 3 serotype was used (Life technologies, USA) based on published sequences (Santiago et al., 2013). This assay was modified to quantify the DENV apart from quantitative analysis.

Real-time PCR was performed using 4 \times TaqMan[®] Fast Virus 1-Step Master Mix (Applied Biosystems, USA). The reaction was performed in an Applied Biosystems[®] 7500, 96-well plate detection system. Following initial denaturation for 20 s at 95 °C, the reaction was carried out for 40 cycles of 3 s at 95 °C and 30 s at 60 °C. The threshold cycle value (Ct) for each reaction was determined by manually setting the threshold limit. Viral quantification (PFU/ml) of unknown samples was performed using the standard curve. All assays were done in triplicate.

2.11. Quantification of gene expression using real time PCR

The real time PCR reaction was carried out using TaqMan[®] Gene Expression Master Mix Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) to detect expression of following genes: *TLR-4*, *NLR-3*, *MAPK-1*, *MAPK-3*, *MAPK-14*, *NF- κ B* and *DDX-58 (RIG-I)*. The reaction was performed in an Applied Biosystems[®] 7500, 96-well plate detection system. The reaction set was done as 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.

2.12. Statistical analyses

Data analysis was performed using Graph Pad Prism 6.0 software. As the data were not normally distributed, differences in means were compared using the Mann-Whitney *t*-test (two tailed). To determine positive and negative associations, the Spearman's correlation test was used (two tailed).

3. Results

3.1. Association of PAF levels with other inflammatory cytokines in patients with acute dengue

We found that TNF α levels were significantly higher in patients

with DHF, when compared to those with DF, especially during the critical phase (from 120 to 168 h) (Fig. 1A) and also that IL-1 β levels were also higher in patients with DHF (Fig. 1B). Serum PAF levels significantly correlated with both serum TNF α levels (Spearman's $R = 0.56$, $p < 0.0001$) and serum IL-1 β levels (Spearman's $R = 0.35$, $P < 0.0001$). However, serum PAF levels did not show any association with serum IL-10 levels.

3.2. Factors associated with production of PAF from monocytes

Although primary human monocytes were very permissive to DENV infection, we did not observe any PAF production by uninfected primary human MDM θ s or by MDM θ s infected with DENV-3 up to 96 h post infection (Fig. 2A).

Lipopolysaccharide (LPS) is a marker used for identification of microbial translocation (Marchetti et al., 2013) and significantly higher LPS levels have been detected in patients with acute dengue infection, especially in those with plasma leakage (van de Weg et al., 2013; van de Weg et al., 2012). Furthermore, it has been shown that human monocytes produce PAF in a bi-phasic pattern when stimulated with LPS, due to the actions of cytokines such as TNF α and IL-1 β (Valone and Epstein, 1988; Han et al., 2002). Since we did observe a bi-phasic pattern of PAF production in our patients (Jeewandara et al., 2015), and since serum PAF levels significantly correlated with TNF α and IL-1 β , we determined if LPS stimulated PAF production from DEN-V infected MDM θ .

We found that as expected, LPS did stimulate PAF production from uninfected primary human MDM θ . However, the PAF production was significantly increased when LPS was co-cultured with DENV infected MDM θ (Fig. 2A). The PAF levels were significantly higher at 24 and 48 h after infection ($P = 0.005$ and $P = 0.04$ respectively), in DENV infected MDM θ in the presence of LPS when compared to the uninfected MDM θ s. As the DENV and LPS appear to act synergistically to increase PAF production, we next proceeded to determine if the increased production was due to increase in virus infection. Using quantitative real time PCR, we determined the viral load in culture supernatants of primary human MDM θ of DENV infected MDM θ s infected in the presence or absence of LPS up to 96 h post infection. We found that there was no difference in the viral loads in the presence or absence of LPS up to 48 h (Fig. 2B). After 48 h the DENV infected MDM θ s, which were co-cultured with LPS showed a sharp decline.

Since LPS appears to enhance PAF production by DENV infected MDM θ s, we also determined if LPS enhanced production of other inflammatory cytokines. We found that the levels of IL-6 and TNF- α were significantly increased in DENV infected MDM θ s co-cultures

with LPS when compared to uninfected MDM θ s with LPS (Fig. 2C and D). However, there was no significant difference observed with the IL-10 and IL-12 levels in DENV infected MDM θ s in the presence of LPS and those which were co-cultured with LPS alone. Similar to the results seen with PAF, the DENV uninfected or infected MDM θ s in the absence of LPS did not produce any TNF α or IL-6. Although we did assay for IL-1 β , the levels were below the level of detection in all samples. Therefore, LPS appears to act synergistically with the DENV to increase production of PAF, IL-6 and TNF α .

3.3. Effect of antibody dependent enhancement on PAF production by monocytes

ADE has been shown to increase production of many types of inflammatory cytokines such as IL-6, IL-8, IL-1 β , IL-12p70 and TNF α , in DENV infected MDM θ s. Since PAF production was not seen in MDM θ s infected with the DENV alone, we sought to investigate if ADE had any role in stimulating production of PAF from MDM θ s. We found that PAF was not produced from any of the primary human MDM θ s, when incubated with varying concentrations of dengue immune sera. Although production of IL-10 and IL-12p40 cytokines was detected following DENV infection of MDM θ s alone, a significant increase in these cytokine levels or in the production of IL-6 and TNF α were not detected in the levels of sera used in our experiments. (Fig. 3).

3.4. Analysis of the effect of LPS on gene expression in DENV infected monocytes

Since we found that PAF, TNF α and IL-6 production was significantly up regulated in the presence of LPS in DENV infected MDM θ s, we sought to investigate the signaling pathways that are responsible for changes in gene expression. Changes in the expression of the *MAPK1*, *MAPK3*, *MAPK14*, *NLRP-3*, *TLR-4*, *NF- κ B*, *RIG-1* were determined to identify different signaling pathways that are activated by LPS and the DENV: Of these seven genes, a significant difference was only observed in the expression of *DDX58* (*RIG-1*). The expression of *DDX58* (*RIG-1*) was significantly increased ($p < 0.05$) in infected MDM θ s compared to LPS stimulated uninfected MDM θ s (Table 1). The expression of *DDX58* (*RIG-1*) in infected MDM θ s was further significantly increased ($p < 0.05$) by LPS stimulation. Although *TLR-4* expression was significantly increased in the MDM θ s co-cultured with LPS as expected, the expression of *TLR-4* was not significantly increased in the DENV-infected MDM θ s co-cultured with LPS.

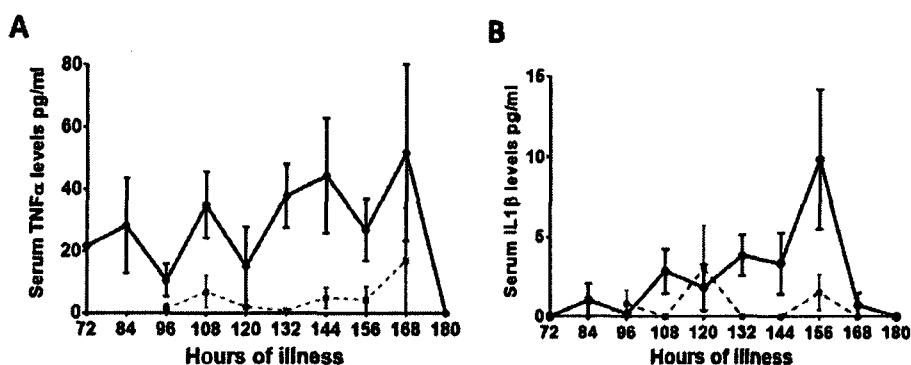


Fig. 1. Levels of TNF α and IL-1 β in patients with acute dengue and their association with serum PAF levels. The solid line represents the levels in patients with DHF and the dotted line patients with DF. A: Serum TNF α levels in patients with DF ($n = 11$) and DHF ($n = 25$), throughout the course of their illness. The dots indicate the mean and the SEM. B: Serum IL-1 β levels in patients with DF ($n = 11$) and DHF ($n = 25$), throughout the course of their illness. The dots indicate the mean and the SEM.

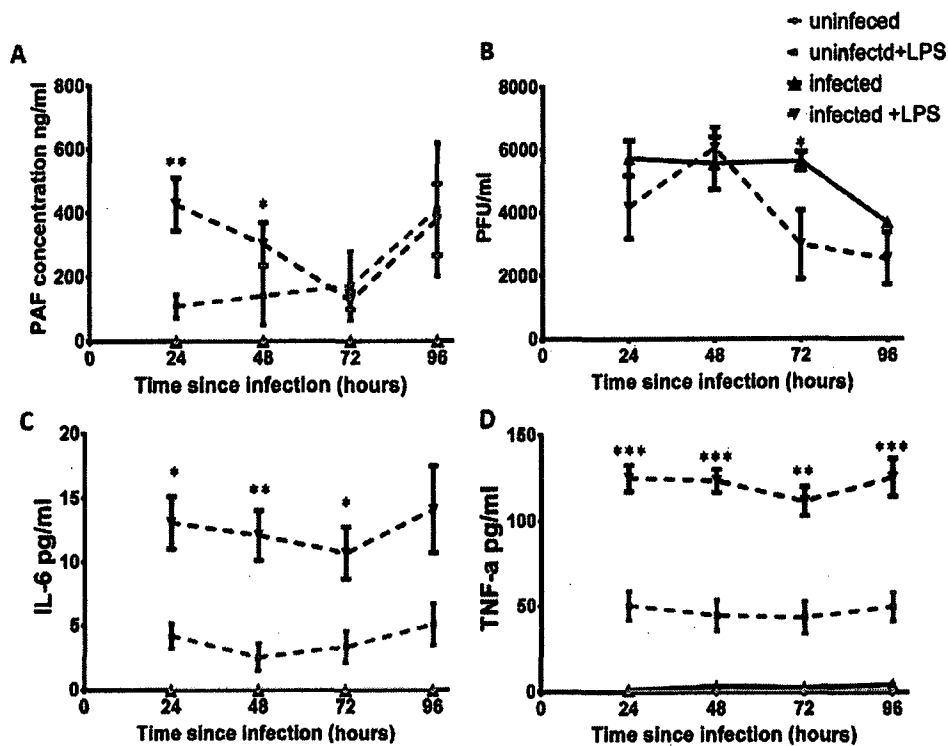


Fig. 2. LPS enhances cytokine production by DEN-V infected monocytes. Monocytes were isolated from healthy donors PBMC by CD14 magnetic beads using MACS separation columns, and infected with DEN-V at MOI of 1. PAF production was assessed in infected and uninfected monocytes in the presence and absence of LPS (500 pg/ml)(A). Viral loads were also assessed in culture supernatants in DENV infected monocytes in the presence and absence of LPS and were expressed as plaque forming units (B). Infected monocytes in the presence of LPS secreted significantly more IL-6 (C) and TNF-α (D) than compared to DENV infection alone. N = 5 Bars show mean and SEM *p < 0.05 **p < 0.01 ***p < 0.005.

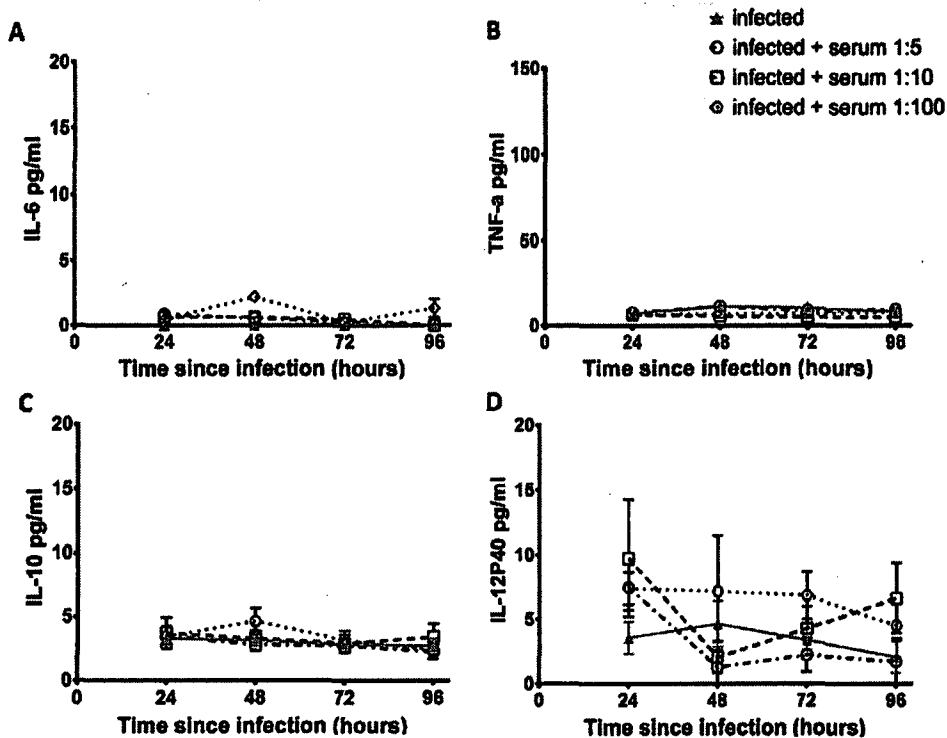


Fig. 3. Pattern of cytokine secretion by DENV-3 Infected monocytes in the presence of varying concentrations of dengue immune sera. Monocytes isolated from healthy donors PBMC by CD14 magnetic beads using MACS separation columns, were infected with the DENV-3 virus at a MOI of 1 in the presence of varying concentrations of DENV immune sera up to 96 h. The levels of (A) IL-6, (B) TNF-α, (C) IL-10 and IL-12p40 (D) were measured in the supernatant by Luminex. N = 5.

4. Discussion

We previously found that PAF was significantly elevated in patients with DHF when compared to those with DF and using human endothelial umbilical cells (HUVECs), it was shown that PAF enhanced vascular leak *in vitro* (Jeewandara et al., 2015). For instance, PAF in serum of patients with acute dengue was associated with reduced expression of tight junction proteins (ZO-1) and reduction in trans-endothelial resistance (TEER) of human endothelial cells (Jeewandara et al., 2015). Therefore, we proceeded to investigate if serum PAF was associated with other inflammatory cytokines such as TNF α , IL-1 β and IL-10 in this previously described cohort of patients with acute dengue. In this study we found that serum PAF correlated with two important inflammatory cytokines, in patients with acute dengue infection. The patterns of variation of TNF α and IL-1 β in patient sera mirrored the same bi-phasic pattern as seen with PAF. Therefore, it is possible that the same stimulus could be driving the production of PAF, TNF α and IL-1 β in patients.

Many cells such as monocytes, mast cells, other leucocytes, endothelial cells and platelets have been shown to produce PAF (Hanahan, 1986). Monocytes and macrophages are one of the main cell types infected with the DENV *in vivo* and are known to produce many types of inflammatory cytokines in acute dengue infection (Martina et al., 2009; Sun and Kochel, 2013). Therefore, we proceeded to investigate if DENV infected MDM θ were a source of PAF and the factors responsible for production of PAF by MDM θ . Although monocytes are highly susceptible to infection with the DENV and in turn produce many inflammatory cytokines, DENV infection alone did not induce production of PAF from MDM θ s.

Many products released from DENV infected monocytes have been shown to induce endothelial dysfunction and to reduce expression of gap junction proteins (Anderson et al., 1997; Lee et al., 2006; Ong et al., 2012). It was shown that endothelial activation, as assessed by expression of adhesion molecules, did not occur by culture supernatants of DENV MDM θ s alone. The increased expression of ICAM-1, VCAM-1 and E-selectin only occurred in the presence of ADE (Anderson et al., 1997). Antibody dependent enhancement (ADE) is thought to be an important mechanism that leads to severe disease in acute dengue, by enhancing infection by the DENV as opposed to neutralization (Acosta and Bartschlagler, 2015; Ng et al., 2014). U937 macrophages infected under ADE conditions have been shown to produce inflammatory mediators that disrupt cell apical junction complexes (Puerta-Guardo et al., 2013). Since we found that PAF was an important mediator of vascular leak in our previous studies, we proceeded to determine if dengue immune sera, simulated production of PAF by DENV infected MDM θ through ADE mechanisms. Although we did not observe any PAF production by DENV infected MDM θ s under ADE conditions, DENV infection alone and ADE did induce the production of some inflammatory cytokines. As TNF α has also been implicated with endothelium activation (Anderson et al., 1997), other inflammatory mediators produced from monocytes apart

from PAF could also lead to endothelium dysfunction.

Secondary bacteremia is known to occur in many patients with acute dengue, especially in those with more severe forms of clinical disease (Premaratna et al., 2015; Lahiri et al., 2008). In addition, considerably high LPS levels have been detected in patients with acute dengue infection, especially in those with plasma leakage (van de Weg et al., 2013; van de Weg et al., 2012). Therefore, it is possible that microbial translocation is associated with severe clinical disease. Since LPS alone stimulates monocytes to produce PAF, we determined the effect of LPS on DENV infected MDM θ s. We found that LPS acted synergistically with the DENV to induce production of PAF, TNF α and IL-6 and it has no effect on IL-12 and IL-10 production. Although a previous report had shown that LPS inhibits DENV infection of monocytes, the LPS levels used in their *in vitro* experiments were 5–10 μ g/ml (Chen et al., 1999), whereas we used 500 pg/ml, which were the mean serum levels reported in patients with DHF (van de Weg et al., 2013; van de Weg et al., 2012). Therefore, LPS could be playing a significant role in inducing production of inflammatory mediators from DENV infected MDM θ s and thereby contributing to vascular leak in the critical phase of infection. As the viral loads did not increase in DENV infected MDM θ s co-cultured with LPS, the increase in production of these inflammatory mediators was unlikely to be purely due to increased viral replication.

As LPS appeared to act synergistically with the DENV in stimulating production of PAF, TNF α and IL-6, we further proceeded to determine the mechanisms by which this is likely to occur. Although we evaluated the gene expression levels of TLR-4, MAP kinase pathways, NF κ B1 pathways, the only relative increase in expression was seen in the RIG-I pathway in DENV infected MDM θ in the presence of LPS. As expected, expression of TLR-4 did increase significantly in MDM θ s co-cultured with LPS, but this increase was not significantly enhanced in DENV infected MDM θ s. RIG-I is a viral sensing pattern recognition receptor, which stimulates production of IFN α and IFN β in infected cells (Kell and Gale, 2015). It has been shown that DENV nonstructural proteins interfere with downstream signaling pathways of RIG-I and thereby inhibit production of IFN α / β (Green et al., 2014). We found that RIG-I was significantly up-regulated in DENV infected monocytes and it was further up-regulated in the presence of LPS. Upon activation of RIG-I, the signaling cascade that follows, leads to activation of IRF3 and NF- κ B (Kell and Gale, 2015). However, we found that NF- κ B expression was less in DENV infected monocytes when compared to monocytes co-cultured with LPS and the levels of NF κ B1 expression were up-regulated in DENV infected cells co-cultured with LPS. Therefore, although RIG-I expression was up-regulated by the DENV, the up-regulation of downstream signaling pathways was less in DENV infected monocytes, which is consistent with the DENV inhibiting downstream signaling of RIG-I.

Although we found that PAF, IL-6 and TNF α levels were significantly increased in DENV infected monocytes co-cultured with LPS, implying that the virus and LPS acted synergistically in

Table 1

Relative expression of different genes in relation to the house keeping gene (*GAPDH*) in DENV infected monocytes, DENV infected monocytes co-cultured with LPS and uninfected monocytes co-cultured with LPS. * indicates when significant changes were observed. The values expressed are all positives and none of the genes were down-regulated relative to *GAPDH*.

Name of gene	Expression in uninfected monocytes + LPS mean (\pm SEM)	Expression in infected monocytes – LPS mean (\pm SEM)	Expression in infected monocytes + LPS mean (\pm SEM)
<i>MAPK1</i>	0.92 (0.16)	0.91 (0.22)	0.92 (0.2)
<i>MAPK3</i>	0.94 (0.25)	0.81 (0.17)	0.54 (0.08)
<i>MAPK14</i>	1.92 (0.9)	1.14 (0.14)	0.99 (0.17)
<i>NLRP-3</i>	2.05 (1.02)	0.82 (0.24)	0.9 (0.27)
<i>NFKβ1</i>	3.88 (1.0)	0.99 (0.26)	3.1 (0.84)
<i>DDX58</i>	5.19 (1.9)	15.37 (4.4) *	18.02 (7.84) *
<i>TLR-4</i>	0.12 (0.11)	1.4 (0.67)	0.77 (0.31)

increase production of these inflammatory mediators, this was not reflected in gene expression analysis. As only RIG-I was upregulated in DENV infected monocytes co-cultured with LPS, upregulation of RIG-I alone is unlikely to be associated with an increase in production of PAF and the other cytokines. Although the mechanisms of interaction of LPS and DENV to induce PAF and proinflammatory cytokine production is not explained by our study, higher levels of serum LPS in patients with acute dengue has shown to correlate with the degree of plasma leakage. Therefore, the mechanisms by which this occurs should be further investigated. Furthermore, since we only determined relative gene expression of RIG-I and other genes, it is possible that the RIG-I protein is not similarly upregulated as our experiments were not designed to determine protein expression levels.

5. Conclusion

In summary, PAF is produced by DENV infected monocytes in the presence of LPS and ADE does not appear to stimulate production of PAF from DENV infected monocytes. In addition, LPS appears to act synergistically with the DENV to induce production of PAF, TNF-α and IL-6. Since microbial translocation has shown to occur in acute dengue infection and as higher LPS levels have been observed in patients with more severe clinical disease, LPS is likely to be important in the pathogenesis of dengue and associated vascular leak.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.antiviral.2016.07.016>.

Glossary

- PAF Platelet activating factor
- LPS Lipopolysaccharide
- DENV Dengue virus
- DHF Dengue Haemorrhagic fever
- DF Dengue fever
- MDM0 monocyte derived macrophages
- ADE Antibody dependant enhancement

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