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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 (MDMθ) was assessed. Gene expression analysis was undertaken to investigate mechanisms by which LPS potentiates PAF and cytokine production by DENV infected MDMθ.

Results: Serum PAF levels significantly correlated with both TNF-α ($p < 0.0001$) and IL-1β ($p < 0.0001$) in patients with acute DENV infection. Although primary human MDMθ 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 MDMθ co-cultured with LPS was significantly higher than uninfected MDMθs co-cultured with LPS. Although TLR-4 was upregulated in uninfected MDMθs co-cultured with LPS, this upregulation was not significant in DENV infected MDMθ. Only expression of *RIG-I* was significantly up regulated ($p < 0.05$) when DENV infected MDMθ 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.

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; Srikiatkhachorn 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; Srikiatkhachorn 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 cytokines such as TNFα and IL-1β (Valone and Epstein, 1988; Han et al., 2002). PAF has been shown to activate transcription of NF-κB, resulting in expression of many inflammatory cytokines such as TNFα and IL-1β (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 biphasic 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 (MDMθ) 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 MDMθ. DENV infected MDMθ 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 MDMθ 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 induces 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 $\leq$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 MDMθ 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,