Levels of soluble ST2 in serum associated with severity of dengue due to tumour necrosis factor alpha stimulation

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The interleukin-1 receptor-like-1 protein (IL1RL1), also known as ST2, has been shown previously to regulate T-cell function and is produced by T cells and endothelial cells. It was reported recently to be elevated in mild dengue patients during acute disease. The ST2 gene encodes several splice products: L (long), V (short) and s (soluble). A cohort of 38 patients with dengue haemorrhagic fever (DHF) and mild dengue fever (DF) were evaluated using a secreted soluble ST2 (sST2) ELISA. The RNA expression of ST2 was evaluated by real-time quantitative RT-PCR using patients’ peripheral blood mononuclear cells (PBMCs) and in vitro using human umbilical vein endothelial cells (HUVECs) exposed to sera from dengue patients. DHF patients had higher levels of serum sST2, tumour necrosis factor alpha (TNF-α), interleukin (IL)-8 and IL-10 compared with DF patients and normal healthy control individuals. However, viraemia was indistinguishable between mild and severe cases. No changes in ST2 mRNA expression were found in PBMCs from these two groups of dengue patients. In vitro, sST2 was elevated in HUVECs treated with patient sera. Neutralization of TNF-α in patient sera by pre-treatment with a TNF-α antibody inhibited the upregulation of sST2 expression in HUVECs. These results implicate serum TNF-α in the modulation of expression of sST2 in an in vitro system, and indicate that sST2 could be associated with the severity of disease. Further studies to determine whether sST2 levels are predictive of the severe form of the disease and the role of sST2 in immune regulation are warranted.

INTRODUCTION

Dengue virus (DENV) is a positive-sense, single-stranded RNA virus in the family Flaviviridae that causes disease in humans. DENV infection results in different clinical manifestations ranking from benign disease [dengue fever (DF)] to severe disease [dengue haemorrhagic fever (DHF)]. In situations where the onset of hypovolaemic shock syndrome or dengue shock syndrome (DSS) persists, DHF can result in a life-threatening infection. DHF is characterized by increased vascular permeability resulting in plasma leakage and coagulation derangements. DHF can be classified further into four degrees of severity, and degrees III and IV are considered to be DSS (WHO, 2005). Due to the lack of efficient biomarkers that define endothelial damage for determining the degree of severity, there is an urgent need to find relevant biological markers of disease, as well as to determine more coherent definitions of the degree of severity in patients. To this effect, many laboratories are combining in vitro studies with clinical studies to find correlates of pathogenesis and disease severity.

All four DENV serotypes (1–4) cause DF or DHF/DSS (Monath, 1994). Secondary infection with a serotype different from the first infection is a risk factor for developing DHF/DSS (Guzmán et al., 1991; Halstead, 1981). Cells carrying the FcγRII receptor, such as monocytes, macrophages and platelets, can also opsonize DENV with non-neutralizing heterotypic antibodies from the first infection. This may lead to an increase in the number of circulating viruses and activation of monocytes and T cells that secrete disproportionate amounts of cytokines.
inducing plasma leakage (Kurane, 2007). A series of cytokines – tumour necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), interleukin (IL)-6, IL-8 and IL-10 – have been shown to correlate with the severity of dengue infection (Bozza et al., 2008; Chakravarti & Kumaria, 2006; Chen et al., 2006; Raghpahputy et al., 1998). Low-avidity T cells cross-reactive to different serotypes may predominate over antigen-specific T cells, thus resulting in an inadequate response to the virus and uncontrolled cytokine production (Mongkolsapaya et al., 2003, 2006). The DHF/DSS pathogenesis model indicates that the disease results from an altered immune reaction in patients.

The interleukin-1 receptor-like-1 protein (IL1RL1), also known as ST2, is a member of the IL1R/Toll-like receptor (TLR) superfamily (Dunne & O’Neill, 2003). The ST2 gene (also designated T1 or DER4) was originally identified as a gene induced by oncogene expression in mouse fibroblasts (Tominaga, 1989). Three isoforms are generated from alternative mRNA splicing: a membrane-anchored long form (ST2L), a secreted soluble form (sST2) and a membrane-anchored variant form (ST2V) (Berger et al., 1994; Iwahana et al., 1999). It has been postulated that both ST2L and sST2 are involved in the control of cytokine expression during inflammatory events to regulate exacerbated inflammatory responses (Trajkovic et al., 2004). Transient ST2L expression in human and mouse cells does not activate NF-κB (Brint et al., 2002), but it has been reported that ST2L blocks this signalling route, probably by sequestration of the MyD88 and Mal adaptor proteins, which are downstream of IL1RL1 and TLR signalling, respectively (Brint et al., 2004). ST2L is considered to be a marker for the activated T helper 2 (Th2) lymphocyte subpopulation (Löning et al., 1998) and could be involved in binding of the Th2 effector cell to the ligand IL-33 (Schmitt et al., 2005). In contrast, sST2 expression occurs in response to increased pro-inflammatory cytokines in mouse fibroblasts and human umbilical vein endothelial cells (HUVECs) (Kumar et al., 1997). sST2 inhibits TNF-α, IL-6, IL-12 and IL-1α production in different in vivo and in vitro models of sterile inflammation (ischaemia model) or infectious inflammation (Escherichia coli lipopolysaccharide [LPS] stimulus or respiratory syncytial virus infection) (Fagundes et al., 2007; Sweet et al., 2001; Takezako et al., 2006; Walzl et al., 2001; Yin et al., 2006). sST2 reduces TLR-4 expression and IL-6 production in different in vivo and in vitro models of sterile inflammation (ischaemia model) or infectious inflammation (LPS stimulus or respiratory syncytial virus infection) (Fagundes et al., 2007; Sweet et al., 2001; Takezako et al., 2006). Higher levels of sST2 have been found in patients having inflammatory disorders such as asthma (Oshikawa et al., 2001), autoimmune diseases (Kuroiwa et al., 2001), idiopathic pulmonary fibrosis (Tajima et al., 2003), sepsis (Brunner et al., 2004) and myocardial infarction (Shimpo et al., 2004). Recently, increased serum sST2 was found in patients having secondary infection and DF, indicating its participation in the disease immunopathology (Becerra et al., 2008).

An association between sST2 and the severity of DENV infection has not been determined. The cell type that expresses sST2 during DENV infection as well as the regulation of this process and its role during infection remain unknown. In the present study, we found that DHF patients had higher levels of sST2, TNF-α, IL-8 and IL-10 compared with DF patients and healthy controls. There were no detectable changes in the mRNA expression of sST2 and ST2L in peripheral blood mononuclear cells (PBMCs). Stimulation of HUVECs with sera from DHF patients induced sST2 but not ST2L mRNA expression; in addition, the amount of secreted sST2 increased. The stimulation of sST2 was dependent on TNF-α. We hypothesize that the increase in sST2 from HUVECs is due to increased inflammation in vivo. sST2 could have predictive value as a marker of severity of disease. Prospective clinical samples can be used to test this hypothesis in the future.

RESULTS

Clinical and demographic data

The clinical characteristics of patients admitted to the protocol are shown in Table 1. According to World Health Organization (WHO) criteria, 21 patients were classified as DF, four patients as moderate DHF (grades I and II) and 13 patients as severe DHF (grades III and IV). According to the serology results for IgG and PCR for viral RNA, 76.2% of patients with DF and 88.2% of patients with DHF had a secondary infection. In addition, 11.8% of DHF patients who had primary infections were children less than 1 year old. X-rays of the thorax showed pleural effusion (76.5% of patients) and hepatomegaly by ultrasound analysis (94.1% of patients), which were both common clinical findings associated with DHF. Thirteen (76.5%) of the 17 DHF patients presented with DSS (DHF grades III/IV). Thrombocytopenia, prolonged prothrombin time and prolonged partial thromboplastin time increased in DHF patients but not in DF patients. Aspartate aminotransferase and alanine transaminase levels increased in 100 and 9% of DHF patients, respectively. The haematocrit of DHF patients was not significantly different from DF patients, possibly because DHF patients were hydrated intravenously prior to obtaining the sample.

Severity and quantification of DENV

Circulating virions infect and activate target cells, which produce a flood of cytokines during DENV infection. Viral RNA was thus quantified by real-time PCR in an attempt to detect DENV infection in PBMCs (Fig. 1a). No significant differences were found between DF and DHF PBMCs [7 068 136 ± 21 144 814 and 2358 ± 7975 copies (μg total RNA)−1, Mann–Whitney U test = 98.5, P = 0.53]. Possible differences in viral load depending on the number of disease days were investigated (Fig. 1b). DHF patients had a greater number of viral RNA copies than those with DF during the early disease days (days 2–4) [13 880 ± 7376

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Clinical profile of the patients enrolled in this study

Table 1.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DF (n=21)</th>
<th>DHF (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age in years [mean (range)]</td>
<td>26 (3–55)</td>
<td>4 (0.3–12)</td>
</tr>
<tr>
<td>Gender (M : F)</td>
<td>11 : 10</td>
<td>7 : 10</td>
</tr>
<tr>
<td>Primary infection (%)</td>
<td>23.8</td>
<td>11.8</td>
</tr>
<tr>
<td>Secondary infection (%)</td>
<td>76.2</td>
<td>88.2</td>
</tr>
<tr>
<td><strong>Clinical sign and symptoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constitutional symptoms†</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>Vascular leakage§</td>
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<td>15</td>
</tr>
<tr>
<td>Petechiae</td>
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<td>3</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Shock</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td><strong>Laboratory parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets (mean ± SD x 10⁷ μl⁻¹)</td>
<td>88 ± 34</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>Leukocytes (mean ± SD x 10⁹ μl⁻¹)</td>
<td>6 ± 2</td>
<td>6.6 ± 2</td>
</tr>
<tr>
<td>PT (mean ± SD, s)</td>
<td>12.5 ± 2</td>
<td>27 ± 47</td>
</tr>
<tr>
<td>PTT (mean ± SD, s)</td>
<td>29 ± 7</td>
<td>49 ± 8</td>
</tr>
<tr>
<td>Haemoglobin (mean ± SD, %)</td>
<td>40 ± 4</td>
<td>36 ± 6</td>
</tr>
<tr>
<td>AST (mean ± SD, U ml⁻¹)</td>
<td>ND</td>
<td>167 ± 148</td>
</tr>
<tr>
<td>ALT (mean ± SD, U ml⁻¹)</td>
<td>ND</td>
<td>45 ± 40</td>
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<tr>
<td>DENV serotype (%)§</td>
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<tr>
<td>DENV1</td>
<td>54</td>
<td>30</td>
</tr>
<tr>
<td>DENV2</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>DENV3</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>DENV4</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>

*Number of patients with each sign/symptom during the period of study.
†Acute fever with two or more of the following manifestations: headache, retro-orbital pain, myalgia, arthralgia, rash.
§Determined by haemoconcentration, pleural effusion or ascites.
§§DENV serotype was not determined in 8% of DF patients and 40% of DHF patients.

vs 319.5 ± 261.3 copies (µg total RNA)⁻¹, Mann–Whitney U test = 6, P = 0.057, but lower during the later disease days (days 5–7) [53.9 ± 27.3 vs 44 713 527 ± 22 357 547 copies (µg total RNA)⁻¹, Mann–Whitney U test = 4, P = 0.053].

Pro-inflammatory cytokine levels associated with severity in dengue patients

Dengue haemorrhagic manifestations result partly from pro-inflammatory cytokines acting on the endothelium. An attempt was made to define patients’ immune response by quantifying cytokines associated with the acute inflammatory response (TNF-α, IL-1β and IL-8), type 1 cytokines (IFN-γ, IL-12 and IL-2) and type 2 cytokines (IL-4, IL-5, IL-2 and IL-10) (Table 2). The concentration of a number of cytokines was significantly higher in DHF compared with DF patients, including TNF-α (Mann–Whitney U test = 69, P = 0.001), IL-8 (Mann–Whitney U test = 69, P = 0.001), IL-10 (Mann–Whitney U test = 75.5, P = 0.002) and IL-4 (Mann–Whitney U test = 112, P = 0.032). No significant differences between patients with DF and DHF were found for IL-5, IL-2, IL-12 and IFN-γ. Control samples had undetectable levels of all of these cytokines (data not shown).

sST2 levels associated with severity in dengue patients

ELISA was used to quantify soluble protein in sera. Ten febrile children from the Universidad El Bosque Clinic, Colombia, between the ages of 2 and 6 years that had acute respiratory illness (ARI), but were negative for DENV-specific IgM and RNA were included in the analysis. sST2 levels were significantly higher in DHF patients (3904 ± 2351 pg ml⁻¹) than in those with DF (1496 ± 1530 pg ml⁻¹) (Mann–Whitney U test = 88.5, P = 0.008), ARI (705 ± 227 pg ml⁻¹) (Mann–Whitney U test = 23, P = 0.002) and the control group (531 ± 515 pg ml⁻¹) (Mann–Whitney U test = 16.5, P = 0.003) (Fig. 2a). A temporal analysis during the course of the disease showed that sST2 levels became progressively lower in patients suffering from DF and DHF (Fig. 2b). Circulating immune cells, such as monocytes, macrophages, B cells and T cells, may express ST2. Accordingly, we evaluated mRNA expression for sST2 and ST2L in patientPBMCs. The results showed no change in the levels of mRNA for either ST2 isoform in PBMCs from DF and DHF patients (sST2 mRNA fold increase: 0.9 ± 1.5 and 0.7 ± 0.9, respectively, Mann–Whitney U test = 386, P = 0.75; ST2L mRNA fold increase: 0.9 ± 1.8 and 0.7 ± 0.6, respectively, Mann–Whitney U test = 361, P = 0.083).

Sera from dengue patients induces sST2 but not ST2L expression in endothelial cells: role of TNF-α

The endothelium plays a central role in dengue disease pathogenesis and may also express ST2 in response to inflammatory stimuli. HUVECs were incubated with patient serum to simulate in vivo DENV infection to evaluate whether this treatment could modulate sST2 and ST2L expression in endothelial cells. This model was validated by measuring intercellular adhesion molecule 1 (ICAM-1) expression on the surface of HUVECs, which is an indicator of endothelial activation. DHF patient sera induced a significant increase in ICAM-1 expression compared with sera from DF patients and controls, and pre-treatment of the sera with a TNF-α neutralizing antibody inhibited almost 70% of ICAM-1 expression (Fig. 3).

Sera from DHF patients produced an almost twofold rise in sST2 mRNA at 24 h compared with sera from DF patients,
controls or DMEM with 20% FCS (P<0.05) (Fig. 4). At 48 h post-treatment, sST2 expression increased when HUVECs were treated with DHF sera compared with that of 20% FCS treatment (P<0.05). Neutralization of serum TNF-α almost completely eliminated sST2 transcription at 24 and 48 h (Fig. 4). Treatment with human recombinant (hr) TNF-α increased ST2 expression; this effect was blocked by pre-treatment with neutralizing antibody but not with the unrelated mouse IgG control. At 48 h after stimulus with DHF sera, there were higher levels of sST2 (2133 ± 171 pg ml⁻¹) in HUVEC medium, which was significantly higher than the sST2 levels found in DF sera-treated cells (1744 ± 77 pg ml⁻¹) or those induced by control sera (P<0.05) (Fig. 5). TNF-α neutralizing antibody treatment also significantly reduced sST2 protein production at 24 h (Fig. 5). A positive correlation was found between circulating TNF-α levels and sST2 in HUVECs (Spearman’s r²=0.35, P=0.004).

H U V E C  treatment with sera from controls and patients resulted in a downregulation of ST2L mRNA. Moreover, a TNF-α blockade caused greater downregulation, which was more prominent when sera from patients were used (Fig. 4). Treatments with DMEM with 20% FCS and hrTNF-α increased ST2L mRNA expression, but only at 24 h of treatment. Only three of the samples from DHF patients and two from DF patients produced infection in HUVECs upon incubation, as viral RNA was detected by quantitative PCR (qPCR). There was no difference in virus replication with ST2 expression (data not shown).

**DISCUSSION**

Epidemiological data have suggested that the presence of heterotypic DENV antibodies before secondary infections is a risk factor for developing DHF (Guzmán et al., 1991; Fig. 1. Viral RNA copies in PBMCs from dengue patients. PBMCs isolated from blood samples collected upon admission (2–7 days after onset of symptoms) were subjected to RNA extraction followed by RT and real-time PCR analysis of viral load. (a) Viral titres were 7.068 ± 136 ± 21144814 and 2358 ± 7975 copies (μg total RNA)⁻¹, respectively, in patients with DF (n=19) and DHF (n=12) (virus was not detectable in all patients). There was no significant difference between groups (Mann–Whitney U test=98.5, P=0.53). Horizontal bars represent the mean number of viral RNA copies for each group. (b) Kinetic changes in the viral load detected in patients with DF (hatched bars) and DHF (open bars). Data are represented as median and percentiles by a box and whisker plot. ○, Outlier. *, Mann–Whitney U test=6, P=0.057; #, Mann–Whitney U test=4, P=0.053.

Table 2. Cytokine profiles in DF and DHF patients

Data presented are medians (interquartile ranges) and P values between DF (n=21) and DHF patients (n=17) as tested by a Mann–Whitney U test.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>DF (pg ml⁻¹)</th>
<th>DHF (pg ml⁻¹)</th>
<th>Mann–Whitney U test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>0 (0)</td>
<td>283.81 (0–835.3)</td>
<td>69</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-8</td>
<td>0 (0)</td>
<td>70.8 (0–208.7)</td>
<td>69</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.14 (0–51.9)</td>
<td>33.5 (0–62.3)</td>
<td>138.5</td>
<td>0.228</td>
</tr>
<tr>
<td>IL-10</td>
<td>30.5 (0–33.7)</td>
<td>193.5 (58.2–442.4)</td>
<td>75.5</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-4</td>
<td>0 (0)</td>
<td>21.5 (0–111.8)</td>
<td>112</td>
<td>0.032</td>
</tr>
<tr>
<td>IL-5</td>
<td>0 (0)</td>
<td>0 (0–36.9)</td>
<td>157</td>
<td>0.397</td>
</tr>
<tr>
<td>IL-2</td>
<td>30.5 (0–33.7)</td>
<td>33.4 (0–62.7)</td>
<td>137.5</td>
<td>0.217</td>
</tr>
<tr>
<td>IL-12</td>
<td>0 (0)</td>
<td>19.5 (0–96.2)</td>
<td>125</td>
<td>0.08</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0 (0)</td>
<td>0 (0–34.5)</td>
<td>158</td>
<td>0.419</td>
</tr>
</tbody>
</table>
Halstead, 1981). In this study, 88.2% of DHF cases had secondary infections and 11.8% had primary infections (Table 1). In the latter group, the patients were less than 1 year old. About 10% of DHF cases occur in primary infection, usually in infants in the second half of the first year of life (Green & Rothman, 2006). It has been postulated that maternal transmission of non-neutralizing DENV antibodies is involved in antibody-dependent enhancement (Kliks et al., 1988). As our study area was highly endemic for DENV (Salgado et al., 2008), the frequency of secondary infections were not different between DF and DHF cases (Table 1) and there was no significant association between primary or secondary infection with other variables, such as sST2 level, cytokines and viral load.

Some studies have shown a relationship between viral load and severity of DHF (Libraty et al., 2002; Wang et al., 2003), whilst others have found no relationship (Chen et al., 2005; Yeh et al., 2006). It is believed that an increase in viral load is an initial event in DHF pathogenesis and has an indirect effect on the endothelium by immune mediators released by infected cells (Green & Rothman, 2006). In this study, there was no significant correlation with disease severity; however, DHF/DSS patients presented higher TNF-α and IL-8 pro-inflammatory cytokine levels. TNF-α is known to act directly on vascular endothelium and cause changes in vascular permeability (Chakravarti & Kumaria, 2006; Chen et al., 2007). IL-8 is known to be associated with changes in vascular permeability in vitro (Talavera et al., 2004), and elevated levels of IL-8 were found in patients with DHF/DSS (Raghuapaty et al., 1998). IL-10 levels were higher in DHF patients than in DF patients. Earlier studies have shown significantly higher levels of IL-10 in patients with DHF/DSS (Chen et al., 2005; Pérez et al., 2004). Taken together, these results suggest that viral load or cytokines probably influence dengue severity, but this depends on other factors such as age of the host, virus serotypes, sequential infections of different virus serotypes and cytokine profile. Mortality was not found among the group of patients studied, even in patients suffering from the most severe DHF, raising the question of whether host factors can control the immune response and whether sST2 could contribute to this control.

Fig. 2. Levels of serum-soluble ST2 in dengue patients. (a) Mean soluble ST2 protein levels in serum were 3904±2351 and 1496±1530 pg ml⁻¹, respectively, in patients with DHF (n=17) and DF (n=21). Mean sST2 levels for ARI patients were 705±227 pg ml⁻¹ (n=10) and for control (C) volunteers (n=8) were 531±515 pg ml⁻¹. *, Significant difference between DHF patients and control (Mann–Whitney U test=16.5, P=0.003), ARI patients (Mann–Whitney U test=23, P=0.002) and DF patients (Mann–Whitney U test=88.5, P=0.008); #, significant difference between DF patients and control (Mann–Whitney U test=41.5, P=0.04). (b) Kinetic changes in sST2 levels in patients with DF (filled bars) and DHF (open bars). *, Mann–Whitney U test=3, P=0.036. Data are sST2 mean values ± sem for each patient group and the different disease days.

Fig. 3. Effect of serum from patients with dengue on the expression of ICAM-1 on HUVECs. HUVECs were cultured for 20 h in the presence of serum samples from DF (n=18) and DHF patients (n=10) or sera from controls (C; n=3), or in the presence of medium supplemented with 0.5% FCS (n=3) or human recombinant TNF-α (hrTNF-α; n=3) (filled bars). After culture, ICAM-1 expression on HUVECs was evaluated using a Cell-ELISA fluorometric assay. Another experiment included serum from patients incubated with mAb against human TNF-α for 1 h (open bars). An isotype control IgG mAb was used (hatched bar). Data are mean values ± sem for each group and each treatment time. *, Mann–Whitney U test=39, P=0.014 between DHF and DF or control; **, Mann–Whitney U test=0.001, P=0.011 between hrTNF-α and DHF. ##, Mann–Whitney U test=5, P=0.002; ##, Mann–Whitney U test=3, P=0.017 for serum versus serum plus mAb against TNF-α.
Fig. 4. Effect of serum from patients with dengue on the expression of ST2 mRNA on HUVECs. HUVECs were cultured for 6, 24 and 48 h in the presence of serum samples from DF (n=21) and DHF patients (n=17) or sera from controls (C; n=8), or in the presence of medium supplemented with 20 % FCS (n=3) or hrTNF-α (n=3) (filled bars). After culture, HUVECs were subjected to RNA extraction and real-time PCR analysis of ST2s and ST2L mRNA expression. Another experiment included serum from patients incubated with mAb against human TNF-α for 1 h (open bars). An isotype control IgG mAb was used as control (hatched bars). Kinetics of the fold increase of sST2 and ST2L mRNA expression was calculated as normalization by β-actin mRNA expression and comparison to HUVEC cultured with medium and 0.5 % FCS. Data are means ± SEM for each group and each treatment time. *P<0.05 between groups; #P<0.05 for serum versus serum plus mAb against TNF-α.

Fig. 5. Effect of serum from patients with dengue on the expression of soluble ST2 protein in HUVECs. sST2 supernatant levels were quantified by ELISA assay from HUVECs cultured for 6, 24 and 48 h in the presence of serum samples from DF (n=21) and DHF patients (n=17) or sera from controls (C; n=8). Medium supplemented with 20 % FCS (n=3) or human recombinant TNF-α (n=3) were used as positive controls and medium supplemented with 0.5 % FCS as background control (BG) (filled bars). Another experiment included serum from patients incubated with mAb against human TNF-α for 1 h before treatment (open bars). An isotype control IgG mAb was used as a control (hatched bars). Data are means ± SEM for each group and each treatment time. *P<0.05 between groups; #P<0.05 for serum versus serum plus mAb against TNF-α.
In this study, higher levels of sST2 were found in sera from severe dengue patients (DHF) compared with sera from mild dengue (DF) and ARI patients (Fig. 2a). These results suggest that sST2 levels correlate with severity in dengue patients. As the DF patients were adults, whilst the DHF patients were children, age could affect the level of sST2. To the best of our knowledge, there are no reports suggesting that age affects ST2 expression, but it is known that a chronic increase in serum levels of inflammatory mediators such as IL-6 and TNF-α and acute-phase proteins is found in the elderly population (Krabbe et al., 2004; Vasto et al., 2007). Furthermore, T cells experience a decline in function with increasing age (Wu & Meydani, 2008). Nevertheless, we were unable to obtain samples from elderly dengue patients, but the levels of sST2 were not different between DF patients >15 years old (n=14) and DF patients ≤15 years old (n=7) (1606±2211 vs 1393±1299 pg ml⁻¹, respectively, Mann–Whitney U test=42.5, P=0.63). ST2 levels in children with DHF (0–15 years, n=17) were significantly higher than child DF patients (2–15 years, n=7), (3904±2351 vs 1606±2211 pg ml⁻¹, respectively, Mann–Whitney U test=24, P=0.032).

Surprisingly, there were no changes in the mRNA expression of ST2 in patient PBMCs, although using one sample per acute phase may not be sufficient to get an accurate correlation value, and it is possible that this change appears later in the disease when LTh2 (via ST2) predominates, as has already been proposed (Trajkovic et al., 2004).

Sera from DHF patients induced marked activation in HUVECs, partly due to the effect of TNF-α, a pro-inflammatory cytokine previously reported to be present during DENV infection (Cardier et al., 2005; Green & Rothman, 2006). It also resulted in a significant increase in sST2 expression. Such data strongly indicate that TNF-α is important for sST2 expression in endothelial cells, although the contribution of other factors in serum cannot be excluded. It is unlikely that sST2 upregulation is due to active virus replication in HUVECs, as in most cases no virus was detected in cells incubated with patient sera. qPCR analysis revealed a strong deregulation of ST2L expression in HUVECs treated with sera from volunteers and patients; however, ST2L was upregulated at 24 h in response to inflammatory (hrTNF-α) and proliferative (DMEM with 20% FCS) stimuli, as reported previously (Kumar et al., 1997). These results indicate that different mechanisms regulate sST2 and ST2L isoform expression. Transcription factors activated by sera from dengue patients could preferentially bind to a promoter that stimulates sST2 expression and consequently become favoured.

sST2 function during DENV infection remains unknown. High sST2 serum levels have been found in patients with severe asthma (Oshikawa et al., 2001). Nevertheless, it is thought that sST2 binds to IL-33 and inhibits its binding to ST2L to block NF-κB activation and Th2 cytokine expression, which suggest that sST2 modulates IL-33 biological activity during allergic inflammation (Hayakawa et al., 2007). Recombinant sST2 also suppresses pro-inflammatory cytokine production following stimulation with LPS in macrophages and monocytes. It also promotes reduced TLR-4 expression and IκB degradation (Sweet et al., 2001; Takezako et al., 2006). Administering sST2 to mice after intestinal ischaemia results in reduced TNF-α production and inhibition of vascular permeability, neutrophilia and haemorrhagia in the intestine and lungs (Fagundes et al., 2007). However, plasmid sST2 expression in vivo markedly inhibits hepatic damage and TNF-α and IL-6 inflammatory cytokine production in a mouse model of ischaemia of the liver (Yin et al., 2006). These examples show that the association between ST2 and different inflammatory diseases is not always causal, and that the functions could be pathogenic or protective. ST2 could be involved as a negative regulatory mechanism in response to TNF-α and/or other pro-inflammatory cytokines to prevent an uncontrolled immune reaction. Nevertheless, such an immunosuppressive effect could be an immune evasion strategy implemented by DENV. Blocking IL-33–ST2L binding could inhibit polarization towards a Th2 response and prolong the Th1 inflammatory response. Preliminary results using qPCR and ELISA did not show increased expression of IL-33 mRNA in PBMCs from dengue patients nor differences in the levels of soluble IL-33 in DHF and DF patient sera (data not shown).

In conclusion, we found that patients with acute-phase DHF/DSS had greater sST2 levels than DF patients or controls, and that sST2 could be a marker of disease severity. For this study group, circulating sST2 may have originated from PBMCs, or may have originated from endothelial cells during the acute phase. This may be a response to immune activation by vasoactive and pro-inflammatory mediators, because HUVECs expressed ICAM-1 and sST2 in response to dengue patient sera, even though ST2L was not expressed and DHF patients had higher TNF-α levels, and experimental blocking reduced sST2 expression in HUVECs, indicating that TNF-α is important for sST2 expression in these cells. Further studies will be directed towards defining the role of sST2 in DENV infection and could provide new insights in interpreting the disease immune pathology.

**METHODS**

**Clinical samples.** Approved human protocols were obtained from the Universidad El Bosque’s Ethics Committee in Colombia. Written, informed consent was obtained from the family for all subjects under age and signed consents were obtained for all other patients and normal donors. Samples were taken from patients at the Paediatric Neiva Hospital, Colombia, between 2003 and 2006. Clinical dengue cases and acute respiratory patients were obtained under signed consent. These were diagnosed by detection of viral RNA and RT-PCR of DENV and by the presence of DENV-specific IgM. Controls consisted of samples from eight healthy volunteer adults who had no fever, no detectable viral RNA and lacked DENV-specific IgM in the serum, and a second paediatric control group consisted of nine serum
samples from children with fever from respiratory infection but negative for DENV. DHF cases were defined according to WHO criteria (WHO, 2005). DENV serotype-specific RT-PCR was performed using the primers described by Chien et al. (2006). Whole blood samples were collected and the serum was separated by centrifugation. Thirty-eight patients with confirmed DENV infections were included in the study. PBMCs from fresh heparin anti-coagulated whole blood were isolated using Ficoll Hypaque (Gibco) and treated with Trizol (Invitrogen) to obtain total cellular RNA. Samples were stored at −70 °C until use.

**DENV antibodies, cytokine quantification and ELISA against ST2.** The Ultra-Micro ELISA (Tecnosuma International) technique was used for detecting IgM and IgG antibodies against DENV in sera. All samples were taken between days 2 and 7 of the disease and were considered at the same immunological moment. Primary infection was defined as positive for the viral genome, as determined by RT-PCR, but without DENV-specific IgG; secondary infection consisted of samples having viral RNA and DENV-specific IgG (Chen et al., 2005). IL-1β, TNF-α, IL-8, IL-4, IL-5, IL-10, IL-12, IFN-γ and IL-2 levels in the serum were quantified using a FlowCytomix multiplex quantification system (BMS710FF; Bender MedSystems) in a Dako flow cytometer. Circulating sST2 was quantified using a DuoSet-ELISA sandwich assay (DY523; R&D Systems).

**Viral RNA quantification by real-time PCR.** cDNA was obtained with random primers and SuperScript III reverse transcriptase (Invitrogen) from 0.5 μg RNA from each sample. A qPCR was carried out as reported previously. The reaction contained 2 μl cDNA plus SYBR Green I Master Mix (DyNaMo; Finnzymes) and primers specific for a 151 bp fragment of the viral capsid gene using an Opticon Monitor (Bio-Rad) detection system. Data were expressed as the means of triplicates and were quantified by extrapolation from a pDV2core plasmid standard curve containing a DENV2 capsid/prM gene fragment. The forward primer was DV2C-L (5'-CAATATGCTGAAACCGAGA-3') and the reverse primer was DV2C-R (5'-TGCTGTGGGATTGTTA-3').

**Endothelial cell culture and activation assay.** HUVECs were isolated from a pool of six umbilical cords by dissociation with 200 U collagenase ml−1 and seeded in 1.5 % gelatin-treated wells. The cultures were maintained in Dulbecco’s minimal essential medium (DMEM) with 20 % fetal calf serum (FCS), 100 U penicillin ml−1 and treated with Trizol (Invitrogen) to obtain total cellular RNA. Samples were stored at −70 °C until use.

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**ST2 mRNA quantification.** qPCR was carried out as described above. sST2 and ST2 mRNA-specific primers were designed using Primer3 software. Product specificity was confirmed on a 2 % agarose gel stained with ethidium bromide and by analysing the dissociation curve (data not shown). sST2 (GenBank accession no. EU181431) and ST2L (GenBank accession no. EU181432) sequence alignment revealed 100 % identity with the previously reported sequence. Relative mRNA expression for ST2 and sST2 in PBMCs from patients or activated HUVECs was calculated using β-actin mRNA for normalization and compared with reference samples (healthy serum controls or HUVECs with 0.5 % FCS in DMEM) using the following equation:

\[
R = \left( \frac{E_{\beta\text{-actin(sample)}}}{E_{ST2\text{-actin(sample)}}} \right) \times \left( \frac{E_{ST2\text{-actin(sample)}}}{E_{ST2\text{-actin(control)}}} \right)
\]

where \(E\) is the efficiency of each gene per well (Schefe et al., 2006). The primers used were forward primer ST2L (5’-AATGATGG-AAAGCTCTATG-3’) and reverse primer ST2L (5’-TCTTTCTATG-TTGGTTTCTC-3’) or forward primer sST2 (5’-GAACCAAGATTCT-AACAAG-3’) and reverse primer sST2 (5’-TCAAGAACACTCCTA-CCTG-3’). The primer sequences for β-actin have been published previously (Xie et al., 2001).

**Statistical analysis.** A non-parametric Mann–Whitney U test was used to determine the significance between groups using the statistic software SPSS 13.0 (SPSS).

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