Soluble ST2 Levels Are Associated with Bleeding in Patients with Severe Leptospirosis

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Abstract

Background: Severe leptospirosis features bleeding and multi-organ failure, leading to shock and death. Currently it is assumed that both exaggerated inflammation and immune suppression contribute to mortality in sepsis. Indeed, several proinflammatory cytokines are reported to be induced during leptospirosis. Toll-like receptors, which play an important role in the initiation of an innate immune response, are inhibited by negative regulators including the membrane-bound ST2 (mST2) receptor. Soluble ST2 (sST2) has been implicated to inhibit signaling through mST2. The aim of this study was to determine the extent of sST2 and (pro-) inflammatory cytokine release in patients with severe leptospirosis.

Methodology and Principal Findings: In an observational study, 68 consecutive cases of severe leptospirosis were included. Soluble ST2 and cytokines (TNF-α, IL-1β, IL-6, IL-8, and IL-10) were repeatedly measured. To determine whether blood cells are a source of sST2 during infection, we undertook an in vitro experiment: human whole blood and peripheral blood mononuclear cells (PBMC) were stimulated with viable pathogenic Leptospira. All patients showed elevated sST2, IL-6, IL-8, and IL-10 levels on admission. Admission sST2 levels correlated with IL-6, IL-8, and IL-10. Thirty-four patients (50%) showed clinical bleeding. Soluble ST2 levels were significantly associated with bleeding overall (OR 2.0; 95%CI: 1.2–3.6) and severe bleeding (OR 5.1; 95%CI: 1.1–23.8). This association was unique, since none of the cytokines showed this correlation. Moreover, sST2 was associated with mortality (OR 2.4; 95%CI: 1.0–5.8). When either whole blood or isolated PBMCs were stimulated with Leptospira in vitro, no sST2 production could be detected.

Conclusions: Patients with severe leptospirosis demonstrated elevated plasma sST2 levels. Soluble ST2 levels were associated with bleeding and mortality. In vitro experiments showed that (white) blood cells are probably not the source. In this regard, sST2 could be an indicative marker for tissue damage in patients suffering from severe leptospirosis.

Introduction

Leptospirosis is a worldwide occurring zoonosis [1], reported to be fatal in up to 50% of cases [2]. The disease is caused by spirochetes that are spread by the urine of infected animals, for example rats, mice and cattle amongst others. Survival of Leptospira is enhanced in a warm and humid environment, where environmental circumstances are most favourable. Hence prevalence is higher in (sub) tropical countries. Severe leptospirosis is featured by bleeding complications and multi-organ failure, which can eventually lead to shock and even death. Necropsy reports confirm widespread haemorrhaging throughout the body, involving most vital organs and tissues [3]. This haemorrhaging could possibly be the result of capillary wall damage.

Several proinflammatory cytokines, such as TNF-α and IL-12p40 are reported to be induced during infection with Leptospira [4,5]. As well, elevated plasma concentrations of TNF-α have been associated with lethal outcome amongst leptospirosis patients [6].

In a hamster model, late expression of the anti-inflammatory cytokines IL-4, TGF-β and IL-10 have been shown [7].

Currently it is assumed that both exaggerated inflammation and immune suppression contribute to an adverse outcome in sepsis [8]. ST2, also designated T1, Fit-1 and DER4, is thought to play a significant role in tuning the host inflammatory response. ST2 is a receptor that is present in two main forms, in the soluble secreted form (sST2) [9] and in a membrane-anchored form (ST2L) [10]. Both are encoded from the ST2 gene regulated by different promoters [11] and are members of the IL-1 receptor family. ST2 gene expression was identified originally in fibroblasts [9,12]. Expression has also been detected in several other cells, including Th2 cells, mast cells and macrophages [13–16].

ST2L has been reported to attenuate downstream IL-1RI and TLR4 signalling by sequestering MyD88 and MAL (MyD88 adaptor-like) [17]. In contrast, previous work has demonstrated that interleukin (IL)-33 is able to activate NF-kB and MAP kinases by signalling through ST2L [10]. IL-33/ST2L signalling in mast
Author Summary
Leptospirosis is a bacterial disease that is mainly spread by rodents and other small mammals. Transmission frequently occurs in (sub-) tropical countries, where environmental circumstances are most favourable. Severe leptospirosis can cause bleeding and vital organ dysfunction. An exaggerated immune response is thought to play an important role in the pathophysiology of leptospirosis. Soluble ST2 (sST2) is thought to inhibit negative regulatory pathways of this response. Soluble ST2 is produced by cells that surround, for example, blood vessels, and several of these blood cells play an important part in the host immune response. In an observational study, we measured the extent of sST2 release in patients suffering from severe leptospirosis. We found that patients that died from leptospirosis displayed higher levels of sST2. Moreover, from this study we have seen that sST2 levels were associated with bleeding, whereas other markers of infection were not. In an experiment, we showed that (white) blood cells did not seem to be the source of sST2 production. Damage to blood vessels is likely to cause bleeding in leptospirosis patients, exposing sST2 producing cells like fibroblasts to the blood stream. Hence, we believe that sST2 may be used as a marker for tissue damage in patients suffering from severe leptospirosis.

Methods

Ethics statement
The study protocol was approved by the medical ethic committees of both the Dr. Kariadi hospital- University of Diponegoro, Semarang, Indonesia and the Slotervaart Hospital in The Netherlands. Written informed consent was obtained from all included subjects.

Patients and design
Consecutive cases of severe leptospirosis were included from February 2005 to September 2006 at the Dr. Kariadi hospital-University of Diponegoro, Semarang, Indonesia. Severe leptospirosis was defined as a hospitalized patient with high clinical suspicion of severe leptospirosis a positive LeptoTek Dri-Dot assay (Biomerieux), presenting with at least one of the following symptoms or signs jaundice, renal failure, thrombocytopenia and/or haemorrhaging. Cases were confirmed by further laboratory testing. Blood samples were taken on hospital admission and during follow up. Plasma was worked up immediately and aliquots were stored at −70°C for further analyses. Twenty control (non-leptospirosis patients) samples were collected among healthy volunteers at the department of internal medicine of the Dr. Kariadi hospital- University of Diponegoro, Semarang, Indonesia.

Measurements and assays
Soluble ST2 was measured by the commercially available ELISA (R&D systems, Minneapolis, MN). Tumor necrosis factor (TNF-α), IL-1β, IL-6, IL-8, IL-10, and IL-12p70 were determined using a cytometric beads array multiplex assay (BD Biosciences, San Jose, CA). The detection limits were as follows, TNF-α, IL-10 (2.5 pg/ml); IL-1β, IL-6, IL-8 (5 pg/ml); IL-12p70 (10 pg/ml); sST2 (15 pg/ml). Leptospirosis was confirmed by either a positive culture or microscopic agglutination test (MAT). Tests were considered positive for the MAT with a titre of ≥1:320 on a single sample, seroconversion or a fourfold or higher increase of the titre in paired samples or a titre ≥1:80 in a single sample from early deceased patients.

In vitro experiments
To determine whether blood cells are an important source of sST2 during infection, we undertook an in vitro experiment. We used either human whole blood or peripheral blood mononuclear cells (PBMCs), which were then stimulated with Leptospira interrogans serovar Bataviae strain M, as this serovar is commonly found in the region. This was a fresh, low passage isolate obtained from the Leptospira Reference Center in Amsterdam, The Netherlands.

For the in vitro experiments, bacteria were washed 3 times with RPMI 1640 (Gibco) and counted using a Helber Counting Chamber (Hawksley, Lancing, Sussex, UK) under darkfield microscopy and then resuspended at concentrations of 2.5×10^7 till 2.5×10^9 bacteria per ml. Shortly before starting the experiment, heparinized blood was sterilely drawn from multiple healthy donors and diluted 1:1 in RPMI. PBMC were obtained using Lymphoprep™ (Axis-Shield) according to the manufacturer’s guidelines. Whole blood (50 μl per well) or PBMC were divided over each well of a 96-well plate before Leptospira concentrates were added. The concentration PBMC was equivalent to 50 μl whole blood per well (approximately 0.5×10^6 monocytes). Plates were incubated for six hours at 37°C, 5% CO2. Following incubation the plates were centrifuged and supernatant was collected and stored at −70°C for further testing. All
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Bleeding is associated with increased sST2 plasma levels

Since bleeding is an important feature of severe leptospirosis we were interested whether this event was also associated with sST2 levels. In total 34 patients (50%) showed signs of bleeding. We found mild haemorrhaging (petechiae, ecchymoses and epistaxis) in 23 cases and severe haemorrhaging (gastrointestinal, melaena, gum bleeding, hemoptysis and hematuria) in 10 cases. To determine whether elevated sST2 levels were associated with bleeding, we performed a binary logistic regression analysis and calculated the area under the ROC curve (AUC), see Table 3. Elevated sST2 levels were significantly associated with overall haemorrhaging (mild and severe) (OR 2.0; 95% CI: 1.2–3.6, p = .01; AUC: 0.70, p = .006) and severe haemorrhaging (OR 5.1; 95% CI: 1.1–23.8, p = .04; AUC: 0.76, p = .009), but not with mild bleeding alone (OR 1.3; 95% CI: 0.76–2.2, p = .3; AUC: 0.6, p = .5). As we log-transformed the original variables, this means that for a ten-fold increase of plasma sST2 levels, the odds of developing mild or severe bleeding will be 2.0 times higher, and the odds of developing severe bleeding 5.1 times higher. None of the cytokines were significantly associated with (severe) haemorrhaging (see Table 3).

Soluble ST2 and cytokine levels are associated with mortality

The association between plasma levels sST2, cytokines and mortality was calculated using a binary logistic regression (OR, 95% CI) and a ROC approach (AUC). The odds of patients with leptospirosis dying increased by 2.4 (95% CI: 1.0–5.8, p = .05) with a ten-fold increase of plasma sST2 levels with an AUC of 0.73 (p = .006). As well the odds of patients with leptospirosis dying increased by 3.2 (95% CI: 1.4–7.7, p = .008) with an AUC of 0.74 (p = .003), with a ten-fold increase of plasma IL-6 levels. With a ten-fold increase of plasma IL-8 there was an odds of 6.9 (95% CI: 1.8–27, p = .005) and an AUC of 0.75 (p = .003). The anti-inflammatory cytokine IL-10 failed to reach significance (OR 1.3; 95% CI: 0.5–3.9, p = .58; AUC: 0.58, p = .40), see Table 3.

Soluble ST2 is not released in whole blood after stimulation with viable Leptospira

To evaluate whether blood cells are an important source of sST2 during infection, we undertook an in vitro experiment. We used a fresh viable pathogenic isolate of Leptospira interrogans serovar Bataviae strain M. When either human whole blood or isolated PBMCs were stimulated with different concentrations bacteria, we could not detect sST2 production after 6 hours incubation (Figure 2). The same held true for the negative controls. In contrast, high levels TNF-α were measured as dose dependent upon stimulation with viable Leptospira.

Discussion

This study reports elevated sST2 levels in patients with severe leptospirosis. Soluble ST2 levels correlated with other indicators of inflammation. A unique, significant association between sST2 and bleeding was observed. As well soluble ST2, IL-6 and IL-8 levels were all associated with poor outcome in leptospirosis patients.

Previous work has reported elevated sST2 plasma levels in fifteen septic patients, but in this study no association with mortality was found [22]. Becerra et al. reported elevated sST2 levels in patients suffering from dengue fever, but in the convalescent samples sST2 levels were normalized [33]. Since all patients in this study survived and had only mild disease, no associations with regard to disease severity and outcome could be found. The data presented here extends these earlier studies, with
Figure 1. Soluble ST2 and cytokine dynamics in patients with severe leptospirosis. The bar graphs show mean soluble ST2 and cytokine plasma levels for survivors (white) and non-survivors (black). The error bars indicate the standard error of the mean (SEM). The horizontal dotted line represents the detection limit of the assays. Asterisks in the figure indicate the strength of the statistical difference from healthy controls (* p < 0.05, ** p < 0.01, *** p < 0.001; Mann-Whitney U test).

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Table 1. Soluble ST2 and cytokines on admission in patient with severe leptospirosis.

| Marker (pg/ml) | All (n = 68) | Survivors (n = 52) | Non-survivors (n = 16) | Controls | p-value |
|---------------|-------------|--------------------|------------------------|----------|---------|
| sST2          | 1480 (502–4378) | 1203 (285–2773) | 3596 (1452–8590) | <15 | 0.006   |
| IL-6          | 45 (17–135) | 27 (16–74) | 133 (52–430) | <5 | 0.003   |
| IL-8          | 40 (16–98) | 32 (51–182) | 81 (51–182) | <5 | 0.003   |
| IL-10         | 7 (4–18) | 6 (4–17) | 8 (4–37) | <2.5 | 0.64 |

Abbreviations: IQR, interquartile range. Values represent medians with the corresponding IQR range. Statistical difference between survivors and non-survivors was calculated using the Mann-Whitney U test. A p-value < 0.05 was considered significant.

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findings of elevated sST2 levels during infection in a larger, homogeneous group of patients.

Leptospirosis patients yielded elevated levels of IL-6 and IL-8 associated with mortality in the present study which were stronger than sST2. From the literature, several studies have found similar associations between sST2 levels and mortality. Weinberg et al. identified IL-1β, IL-6, and IL-12p70 concentrations in the serum of leptospirosis patients that were associated with mortality. These findings were in line with previous findings of our group in which we found that membrane bound ST2 is upregulated on monocytes when whole blood is incubated with LPS, while sST2 remains undetectable in blood plasma after 24 hour whole blood LPS stimulation [38].

In conclusion, in patients with severe leptospirosis we demonstrated elevated plasma sST2 levels that normalized during follow-up and were associated with mortality. Interestingly sST2

Table 2. Correlation between soluble ST2 (sST2), clinical markers and cytokines on day of admission.

| Variable          | Serum sST2 |
|-------------------|------------|
|                   | rho        | p-value   |
| Pulse             | 0.20       | 0.1       |
| RR                | -0.25      | 0.04      |
| Leucocytes        | -0.05      | 0.7       |
| Platelets         | -0.25      | 0.04      |
| Creatinin         | 0.33       | 0.007     |
| AST               | 0.46       | 0.001     |
| ALT               | -0.016     | 0.9       |
| CRP               | 0.50       | <0.0001   |
| Severe            | 0.34       | 0.005     |
| Mild              | 0.10       | 0.4       |
| TNF-α             | 0.17       | 0.2       |
| IL-1β             | 0.14       | 0.3       |
| IL-12p40          | 0.04       | 0.8       |
| IL-6              | 0.45       | 0.001     |
| IL-8              | 0.72       | <0.0001   |
| IL-10             | 0.56       | <0.0001   |

Abbreviations: RR, respiratory rate; CRP, C-reactive protein. The correlation coefficient (rho) is calculated by the non-parametric Spearman’s rank correlation test. A p-value < 0.05 was considered significant. doi:10.1371/journal.pntd.0000453.t002

Table 3. Association between soluble ST2 (sST2) bleeding and mortality.

| Variable         | OR (95% CI) | p-value | AUC     | p-value |
|------------------|-------------|---------|---------|---------|
| 10-log sST2      | 2.0 (1.2–3.6) | 0.01    | 0.70    | 0.006   |
| 10-log IL-6      | 1.8 (0.9–3.7) | 0.1     | 0.61    | 0.1     |
| 10-log IL-8      | 2.6 (1.0–7.4) | 0.06    | 0.62    | 0.08    |
| 10-log IL-10     | 1.3 (0.5–3.3) | 0.6     | 0.55    | 0.5     |

| Variable         | OR (95% CI) | p-value | AUC     | p-value |
|------------------|-------------|---------|---------|---------|
| 10-log sST2      | 5.1 (1.1–24) | 0.04    | 0.76    | 0.009   |
| 10-log IL-6      | 2.0 (0.83–4.9) | 0.1     | 0.66    | 0.01    |
| 10-log IL-8      | 2.4 (0.77–7.7) | 0.1     | 0.65    | 0.01    |
| 10-log IL-10     | 2.0 (0.6–6.6) | 0.3     | 0.61    | 0.3     |

| Variable         | OR (95% CI) | p-value | AUC     | p-value |
|------------------|-------------|---------|---------|---------|
| 10-log sST2      | 2.4 (1.0–5.8) | 0.05    | 0.73    | 0.006   |
| 10-log IL-6      | 3.2 (1.4–7.7) | 0.008   | 0.74    | 0.003   |
| 10-log IL-8      | 6.9 (1.8–27) | 0.005   | 0.75    | 0.003   |
| 10-log IL-10     | 1.3 (0.5–3.9) | 0.58    | 0.58    | 0.4     |

Abbreviations: OR, odds ratio; CI, confidence interval, AUC, area under the ROC curve (receiver operating characteristic). Associations are presented as OR with 95% confidence interval and AUC values. A p-value < 0.05 was considered significant. doi:10.1371/journal.pntd.0000453.t003

Our in vitro experiments with pathogenic Leptospira showed that, at least in the early phase, blood is not the source of sST2 production. These findings were in line with previous findings of our group in which we found that membrane bound ST2 is upregulated on monocytes when whole blood is incubated with LPS, while sST2 remains undetectable in blood plasma after 24 hour whole blood LPS stimulation [38].

In conclusion, in patients with severe leptospirosis we demonstrated elevated plasma sST2 levels that normalized during follow-up and were associated with mortality. Interestingly sST2
was the only marker that was associated with (severe) bleeding. More research is warranted to elucidate the function of sST2 in the innate immune response to Leptospira and to evaluate its value as a marker for tissue damage in severely ill patients.

Supporting Information

Checklist S1 STROBE Checklist

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Author Contributions

Conceived and designed the experiments: JFPW MHG MGAG RAH TvdP CvV ECMvG. Performed the experiments: JFPW MGAG. Analyzed the data: JFPW MHG ML RAH TvdP CvV ECMvG. Contributed reagents/materials/analysis tools: MGAG TvdP CvV. Wrote the paper: JFPW.