Title

Normal free IL-18 plasma levels in dengue: the need to measure both total IL-18 and IL-18 binding protein levels

Running title

IL-18 and IL-18BP in dengue

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Abstract

Background: Activated monocytes/macrophages and T-lymphocytes that produce a cytokine storm are assumed to play a pivotal role in the pathogenesis of dengue. Interleukin (IL)-18 is a pro-inflammatory cytokine that is increased during dengue and known to induce interferon (IFN)-γ, which is crucial for dengue-immune response. No data are available regarding the balance between IL-18 and its natural inhibitor IL-18 binding protein (IL-18BP), and how they interact within the inflammatory reaction of patients with dengue infections.

Methods: Circulating levels of IL-18, IL-18BP, free biologically active IL-18, the IL-18-dependent pro-inflammatory cytokine IFN-γ, monocyte-derived cytokines, and ferritin were assessed in adult Indonesian dengue patients (n=95). Healthy individuals (n=22), leptospirosis (n=19) and enteric fever patients (n=6) served as controls.

Results: Total IL-18 levels were increased during dengue, leptospirosis and enteric fever compared to healthy controls. However, due to a concurrent increase in IL-18BP levels, biologically active IL-18 levels remained similar in the different phases of dengue and in patients with leptospirosis. Biologically active IL-18 levels were also similar in patients with severe and non-severe dengue.

Discussion: High total IL-18 and IL-18BP levels concur in dengue virus infections, leptospirosis and enteric fever. This resulted in unchanged levels of free, biologically active IL-18 in dengue and leptospirosis, which underlines the importance to measure both IL-18 and IL-18BP when studying the role of IL-18 in diseases.
Keywords: IL-18, IL-18 binding protein, dengue, leptospirosis, enteric fever, ferritin, IFN-γ, IFN-α, TNF-α, IL-6, IL-1Ra, IL-1α.

Introduction

Dengue has become one of the most important arboviral infections in the world (1). Dengue virus infection usually manifest as a self-limiting febrile illness. A subset of patients, however, develops life-threatening complications, which include plasma leakage, bleeding and/or severe organ failure (2, 3). These complications usually occur during or shortly after defervescence in the so-called critical phase. The precise pathogenesis of these complications is not yet fully understood, but a massive secretion of cytokines by activated monocytes/macrophages and T-lymphocytes is assumed to play a pivotal role (3-6).

Interleukin-18 (IL-18) is a member of the IL-1 family of cytokines. It is synthesized as an inactive precursor requiring processing by caspase-1 into an active cytokine. The precursor is constitutively expressed by nearly all cells in humans. Dengue virus has been shown to activate the inflammasome and induce the production of IL-18 by human macrophages (7).

IL-18, together with IL-12, plays a major role in the production of interferon-γ (IFN-γ). This was also shown in a murine dengue model, in which IL-18 acted in synergism with IL-12 to induce IFN-γ and other T-helper 1 cytokines, which are considered central mediators in dengue host defense (8, 9).

Previous studies in patients with acute dengue have reported high serum IL-18 levels, which correlated with disease severity (10-12). However, the activity of IL-18 is balanced by a
naturally occurring IL-18 binding protein (IL-18BP). IFN-γ stimulates the production of IL-18BP (13) prompting a classical feedback loop whereby IL-18BP neutralizes excessive IL-18 and attenuates the IFN-γ response. Hyperferritinemia has recently been reported to be a common feature of dengue (14). Hyperferritinemia is also commonly observed in conditions with macrophage activation such as adult onset Still’s disease and IL-18 has been implicated in the pathogenesis of these conditions (15). To gain a better insight in the biological activity of IL-18 during dengue, we determined plasma levels of IL-18, together with levels of IL-18BP, IFN-γ and other pro-inflammatory cytokines in a cohort of Indonesian dengue patients. We also determined the association with ferritin levels and included a group of healthy individuals and patients suffering from leptospirosis and enteric fever as controls.

Methods

Study design

This study was part of a prospective study performed between March 2011 – March 2012 in Rumah Sakit Hasan Sadikin, an academic referral hospital in Bandung, Indonesia. Patients clinically suspected for dengue, leptospirosis or *Salmonella typhi*/*Salmonella paratyphi* infection were eligible to be included in the study. Blood was drawn at admission for routine laboratory investigations and diagnosis. Samples of clinically dengue suspected cases were tested for the presence of viral RNA by reverse-transcriptase (RT) PCR and for dengue specific IgM and IgG (Panbio, Windsor, Australia). A positive RT-PCR, minimum 4-fold IgM and/or IgG titer increase and/or an IgM and/or IgG conversion, was considered a proven
dengue infection. Samples that were IgM positive in at least one sample and/or with IgG levels comparable to a HI titer of at least 1:2560 (the IgG cut off point set by the manufacturer to detect secondary infection) were considered highly suggestive dengue cases (2). Patients with a proven or highly suggestive dengue infection were included and retrospectively classified as non-severe or severe dengue according to WHO 2009 criteria (2). Dengue patients were systematically evaluated by daily history, physical examination, laboratory investigation and ultrasonography to detect plasma leakage. Additional blood sampling was scheduled once in each clinical phase of dengue infection: the febrile phase (temperature of 37.5 °C or more on that day), the critical phase (period within 48 hours after defervescence and before platelet counts increased again), early recovery phase (increasing platelet counts and clinical improvement) and convalescence phase (> 2 weeks after discharge).

Patients with a clinical leptospirosis diagnosis in combination with both a positive IgM rapid test (16) and ELISA IgM (Panbio Windsor, Australia) were included, as well as patients with a clinical diagnosis of enteric fever who had a positive blood culture (Bactec Blood Culture System; BD diagnostics, Sparks, MD, USA) for *Salmonella typhi* or *Salmonella paratyphi*. Furthermore, a group of healthy volunteers, recruited among hospital staff, served as healthy controls.

The study was approved by the local Medical Ethical Committee and written informed consent was obtained before enrollment from all patients and healthy controls.
Plasma was obtained from citrate anti-coagulated whole blood, which was immediately centrifuged at 1700g for 15 minutes and stored at -80°C until further analysis. IL-18, IFN-α, IFN-γ, TNF-α, IL-6, IL-1 receptor agonist (IL-Ra) and ferritin levels were determined using commercially available MagPix Milliplex kits (Merck Millipore). A minimum of 50 beads was acquired with a Luminex Magpix instrument (Luminex Corporation, Austin, USA). The minimum detectable concentration was 9.8 pg/mL for IL-18; 2.4 pg/mL for IFN-α, IFN-γ, TNF-α, IL-6, IL-1Ra and 0.03 ng/mL for ferritin, respectively. Plasma IL-1α (Genprobe Dioclone) and IL-18BP (IL-18BP-α, R&D Systems) were determined by commercially available ELISA kits according to the manufacturer’s instructions and had minimum detection limits of 0.03 ng/mL and 0.1 ng/mL respectively. The level of free, bio-active IL-18 was calculated based on the mass-action law, using a dissociation constant of 400 pM and a stoichiometric ratio of 1:1 (17).

Statistical analysis

Data are expressed as medians with interquartile ranges (IQR) or numbers with percentages. Differences in non-continuous data of 2 groups were analyzed by Pearson’s Chi-square test or by Fisher’s exact test in case of expected counts < 5. Continuous variables between 2 groups were analyzed by unpaired t-test in case of normally distributed data; and by Mann Whitney U test in case of non-parametric data. Relationships between continuous data were examined by Spearman’s correlation for non-parametric data. P < 0.05 was considered statistically significant.
Results

Clinical characteristics

A total number of 95 patients with acute dengue, 19 patients with acute leptospirosis, 6 patients with enteric fever and 22 healthy controls were enrolled. Of the dengue patients, 80 (84%) were retrospectively classified as having non-severe dengue and 15 (16%) as severe dengue (2). All patients with severe dengue had plasma leakage in combination with shock; two patients with severe dengue also had respiratory distress accompanied by pleural effusion. None of the severe dengue cases had severe bleeding or organ failure.

Characteristics and baseline data of the enrolled subjects are presented in table 1.

The RT-PCR for dengue was positive in 33 (35%) patients with dengue virus (DENV)-2 being the most common serotype (n=16), followed by DENV-3 (n=8), DENV-1 (n=5) and DENV-4 (n=4). In the remainder of the dengue patients, the diagnosis was based on results of the serological tests. Most dengue patients were admitted in the critical phase (n=76; 80%), the others in the febrile (n=15; 16%) phase and the early recovery phase (n=4; 4%). Plasma leakage in the form of ascites, pleural effusion and/or significant hematocrit changes occurred in 51 (54%) of the patients. Ferritin levels were most increased in dengue and enteric fever patients and to a lesser extent increased in leptospirosis patients, compared to healthy controls.

IL-18BP neutralizes increased IL-18 levels in early dengue and in leptospirosis

Patients with acute dengue, leptospirosis and enteric fever all had significantly higher total median IL-18 plasma levels compared with levels in healthy controls (Figure 1A). However,
plasma IL-18BP levels were also significantly increased in all 3 patient groups. This resulted in a calculated free, biologically active IL-18 level that was unchanged in dengue and leptospirosis patients compared with healthy controls, while patients with enteric fever had increased free IL-18 levels despite a simultaneous increase in IL-18BP (Figure 1 A-C). The critical phase of dengue occurs around defervescence and is the phase of dengue in which most severe complications take place. This phase was defined as the period within 48 hours after defervescence and before platelet counts increased again. Total IL-18, IL-18BP and biologically active IL-18 plasma levels did not differ between non-severe and severe dengue (Figure 2 A-C), or between patients with and without plasma leakage and/or bleeding in the critical phase of dengue (data not shown). In the critical phase of dengue, median total IL-18 levels were 218.5 and 163.0 pg/mL (interquartile ranges 104.5-354.0 and 101.5-258.5 pg/mL), median IL-18BP levels were 14.6 and 16.7 ng/mL, (interquartile ranges 9.4-22.1 and 12.9-22.8 ng/mL), and median biologically active IL-18 levels were 77.4 and 58.1 pg/mL (interquartile ranges 44.9-158.1 and 37.2-77.3 pg/mL) for severe and non-severe dengue patients, respectively. There was a moderate positive correlation between total IL-18 as well as IL-18BP levels with ferritin levels in dengue patients ($R_s=0.36$, $p=0.001$; $R_s=0.29$, $p<0.05$, respectively).

IFN-$\gamma$ and TNF-$\alpha$ increased, while IL-6, IL-1Ra and IL-1$\alpha$ levels remained low during dengue. Plasma levels of IFN-$\gamma$, IFN-$\alpha$ and TNF-$\alpha$ were all increased during early dengue infection (figure 3 A-C).Remarkably, high TNF-$\alpha$ levels were found in dengue patients, while IL-6 remained below the detection limit of 2.4 pg/mL, except for 7/13 (54%) patients in the febrile phase (figure 3 D). The same trend was observed for IL-1Ra (figure 3E) and IL-1$\alpha$ (data on February 8, 2021 by guest http://cvi.asm.org/ Downloaded from
not shown; 156 patients and control samples were below the detection limit, therefore no further analysis of the remaining samples was performed. Compared to patients with non-severe dengue, patient with severe dengue had similar IFN-γ levels (medians 6.3 and 4.4 pg/mL, interquartile ranges 2.4 - 19.1 and 2.4 - 10.5 pg/mL, respectively), but decreased IFN-α levels (medians 4.6 and 11.1 pg/mL, interquartile ranges 2.4 - 10.9 and 6.4 - 27.9 pg/mL, respectively, p<0.05) and increased TNF-α levels (medians 14.6 and 9.6 pg/mL, interquartile ranges 8.9 – 19.9 and 5.0 – 14.3 pg/mL, respectively, p<0.05) in the critical phase. Total IL-18 was moderately associated with TNF-α (Rs= 0.28, p<0.01) and IL-18BP with IFN-α (Rs=0.29, p<0.01) but not with IFN-γ, whereas biologically active IL-18 was not associated with any of the determined cytokines.

Enteric fever patients also had increased IFN-γ, IFN -α and TNF -α plasma levels (figure 1 D-F), while leptospirosis was associated with increased IL-6 and IL-1Ra. In leptospirosis, IL-18BP positively correlated with TNF-α levels (Rs=0.68, p=0.001) (data not shown).

Discussion

Three clinical studies documented high IL-18 levels in dengue infected patients (10-12). However, the interpretation of these data is limited by the fact that no information on its natural antagonist IL-18BP is given as well. The same applies to the in vitro and animal studies that also suggested an important role for IL-18 in the pathogenesis of dengue (7-9). In the present study, we document high plasma IL-18 levels concurring with increased IL-18BP levels in dengue, especially during the febrile and critical phase of dengue and to a
lesser extent in the recovery phase of dengue. As a result, the calculated levels of circulating biologically active IL-18 levels did not increase during any of the phases of dengue. Furthermore, IL-18BP and biologically active IL-18 levels were not related to dengue severity during any of the phases of dengue infection. Our findings therefore suggest that the importance of high IL-18 levels in dengue should be put into perspective.

The concurrent elevation of IL-18 and IL-18 BP was not specific for dengue infection, but also occurred in our cohort of leptospirosis and enteric fever patients. Only enteric fever patients ultimately had increased biologically active IL-18 levels, although this group was too small to draw any firm conclusions.

IFN-γ is a central cytokine in the host defense against dengue. IFN-γ production can be induced, as shown in a murine dengue model, by the combined action of IL-18 and IL-12, while blocking IL-18 activity by administration of recombinant IL-18BP results in lower IFN-γ, higher TNF-α and IL-6 serum levels and more severe disease (8). In contrast, the present study showed that IFN-γ was increased during the febrile phase of dengue, while IL-18 levels were unchanged. Our findings that circulating levels of biologically active IL-18 were not increased in dengue does not preclude a role for IL-18 in IFN-γ production, because IL-18 secreting macrophages are especially located in liver and spleen and levels in the peripheral blood may not reflect local IL-18 production. Furthermore, we cannot exclude that IL-18 independent IFN-γ production by the adaptive part of the immune system, especially specific Th1 memory cells, also plays a role.

Apart from dengue, IL-18BP levels were also measured previously in patients with acute chikungunya infection. IL-18BP levels were higher in acute sera, compared to convalescence,
but levels in that study were significantly lower compared to our data (18). In patients with sepsis total IL-18, IL-18BP and calculated biologically active IL-18 were all increased (19), while the clearly elevated IL-18BP levels again did not reach similar levels as we found. These studies, together with the current study, indicate that biologically active IL-18 should be
determined when analyzing the role of IL-18 in the pathogenesis of diseases.

A limitation of this study is that patients were admitted relatively late in the course of
dengue, enteric fever and leptospirosis, probably due to late help-seeking behavior of the
patient and the study- setting in an academic referral hospital. We cannot rule out that IL-
18BP was induced significantly later than IL-18, and that higher biologically active IL-18 levels
may have been present earlier in the course of dengue.

In conclusion, in this study we report that increased IL-18 levels concur with increased levels
of its antagonist IL-18BP, resulting in unchanged biologically active IL-18 levels in dengue
virus infections. This demonstrates the need for both total IL-18 and IL-18BP measurements
when studying the role of IL-18 in diseases. Increased IL-18 and IL-18BP levels seem not to
be specific for dengue infection as our data in leptospirosis and enteric fever patients
indicate.

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References


Figure Legends

**Figure 1. IL-18, IL-18BP, and free, biologically active IL-18 levels in dengue, leptospirosis, enteric fever and healthy controls.**

Plasma levels of (A) total interleukin(IL)-18, (B) IL-18 binding protein (IL-18BP) and (C) free, biologically active IL-18 in Indonesian adults with dengue fever (n = 95) during the febrile (n = 14), critical (n = 77), early recovery (n = 72) and convalescence phase (n = 54); leptospirosis (n = 19); enteric fever (n = 6) and in a control group of healthy adult Indonesians (n = 22). The minimum detectable concentration was 9.8 pg/mL for total IL-18 and 0.1 ng/mL for IL-18BP. The level of free, bio-active IL-18 was calculated based on the mass-action law, using a dissociation constant of 400 pM and a stoichiometric ratio of 1:1. The dots represent the individual measurements, the horizontal lines represent median values, the whiskers represent interquartile ranges. Outliers were left out of the figure in: (A) 1 febrile dengue, 1 leptospirosis and 1 enteric fever patient (IL-18 = 2077, 2207 and 1367 pg/mL respectively) and in (B) 1 leptospirosis patient (IL-18BP = 441 ng/mL). P values were determined by Mann-Whitney U test for comparison of dengue, leptospirosis and enteric fever patients with the healthy control group. A p value < 0.05 was considered significant. The * indicates a p value < 0.05; ** <0.01 and *** <0.001.

**Figure 2. IL-18, IL-18BP, and free, biologically active IL-18 levels in non severe versus severe dengue cases**

Plasma levels of (A) total interleukin(IL)-18, (B) IL-18 binding protein (IL-18BP), (C) free, biologically active IL-18 in Indonesian adults with non severe dengue fever (n = 65, n = 4
missing) and severe dengue fever (n = 12, n= 3 missing) during the critical phase. The minimum detectable concentration was 9.8 pg/mL for total IL-18 and 0.1 ng/mL for IL-18BP.

The level of free, bio-active IL-18 was calculated based on the mass-action law, using a dissociation constant of 400 pM and a stoichiometric ratio of 1:1. The dots represent the individual measurements, the horizontal lines represent median values, the whiskers represent interquartile ranges. P values were determined by Mann-Whitney U test for comparison of dengue, leptospirosis and enteric fever patients with the healthy control group. A p value < 0.05 was considered significant.

Figure 3. Cytokine levels in dengue, leptospirosis, enteric fever and healthy controls.

Plasma levels of (A) interferon(IFN)-γ, (B) IFN-α, (C) tumor necrosis factor(TNF)-α, (D) IL-6 and (E) IL-1 receptor antagonist(Ra) in Indonesian adults with dengue fever (n = 95) during the febrile (n = 14), critical (n = 77), early recovery (n = 72) and convalescence phase (n = 54); leptospirosis (n = 19); enteric fever (n = 6) and in a control group of healthy adult Indonesians (n = 22). The minimum detectable concentration was 2.4 pg/mL for all cytokines. The dots represent the individual measurements, the horizontal lines represent median values, the whiskers represent interquartile ranges. P values were determined by Mann-Whitney U test for comparison of dengue, leptospirosis and enteric fever patients with the healthy control group. A p value < 0.05 was considered significant. The * indicates a p value < 0.05; ** <0.01 and *** <0.001.
Table 1. Patient and healthy control characteristics and baseline data.

Data are represented in medians (interquartile range) for continuous data or in numbers (n) with percentage (%) for non-continuous data. Mann-Whitney U tests or chi-square tests were performed for comparison of each group with healthy controls. A p-value < 0.05 was considered significant and was indicated as *** (p<0.001); ** (p<0.01); or * (p<0.05). Abbreviations: ALAT: alanine aminotransferase; NA: not applicable; ND: not determined.

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<th>Leptospirosis</th>
<th>Enteric fever</th>
<th>Healthy control</th>
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<tr>
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<td>n=80 (84%)</td>
<td>n=15 (16%)</td>
<td>n=19</td>
<td>n=6</td>
<td>n=22</td>
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<td>23 (16-30)</td>
<td>47 (39-55)***</td>
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<td>Male sex; n (%)</td>
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<td>6 (40)</td>
<td>14 (74)**</td>
<td>3 (50)</td>
<td>7 (32)</td>
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<td>7 (5-9)</td>
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<td>151 (62-293)**</td>
<td>107 (62-159)**</td>
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Table 1. Patient and healthy control characteristics and baseline data.

Data are represented in medians (interquartile range) for continuous data or in numbers (n) with percentage (%) for non-continuous data. Mann-Whitney U tests or chi-square tests were performed for comparison of each group with healthy controls. A p-value < 0.05 was considered significant and was indicated as *** (p<0.001); ** (p<0.01); or * (p<0.05). Abbreviations: ALAT: alanine aminotransferase; NA: not applicable; ND: not determined.