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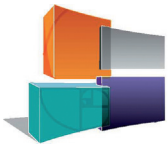

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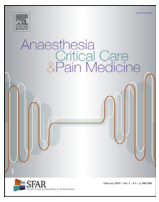

Original Article

# Low interleukin-10 release after ex vivo stimulation of whole blood is associated with persistent organ dysfunction in sepsis: A prospective observational study

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ABSTRACT

*Background:* Sepsis profoundly alters immune homeostasis. Cytokine release after whole blood lipopolysaccharide (LPS)-stimulation reflects cell function across multiple immune cell classes and represents the immune response to LPS. The main goal of this study was to evaluate the prognostic value of ex vivo stimulation of whole blood with LPS in sepsis.
*Methods:* Blood was drawn on day 1 and day 7 after admission, and stimulated ex vivo with LPS. Tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 were measured with and without stimulation. Our primary outcome measure was the persistence of at least one organ dysfunction at day 7. Organ dysfunction was defined according to the SOFA components by a score $\geq 2$.
*Results:* Forty-nine patients with sepsis from a 21-bed intensive care unit, and 23 healthy volunteers were enrolled. The blood of septic patients was less responsive to ex vivo stimulation with LPS than that of healthy controls at day 1 and 7, as demonstrated by lower TNF-α, IL-1β, IL-6 and IL-10 release. Persistent organ dysfunction was more frequent in patients with lower IL-10 release at day 1 but such an association was not found for pro-inflammatory cytokines. A persistent low IL-10 release at day 7 was also associated with persistent organ dysfunction.
*Conclusion:* These data suggest that the capacity to produce IL-10 in response to whole blood ex vivo stimulation early in sepsis, as well as persistent low IL-10 response over time, may help in prognostication and patient stratification. These results will need to be confirmed in future studies.


## 1. Introduction

Immune dysregulation is now a well-recognised phenomenon in Sepsis [1,2]. Improvements in critical care have increased the number of patients who survive the initial hyper-inflammatory phase of sepsis, but also increased the frequency of a chronic critical illness named persistent inflammation, immunosuppression and catabolism syndrome (PICS). PICS has been associated with prolonged intensive care unit (ICU) stays, moderate organ dysfunction, secondary infection, requirement for life support and protein catabolism [3]. Several biomarkers of immunosuppression in sepsis have been proposed to identify the subset of these patients who might benefit from of immune therapies [4,5]. The most studied of these have been human leukocyte antigen (HLA)-DR expression on monocytes, and Tumour Necrosis Factor (TNF)-α release in whole blood lipopolysaccharide

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(LPS)-stimulation assays. Indeed, both decreased HLA-DR expression and decreased release of TNF-α after ex-vivo whole blood LPS-stimulation are associated with higher morbidity and mortality in critically ill patients [5,7,8,9–11]. While cell surface HLA-DR expression in monocytes may be considered a phenotypic marker of sepsis severity, cytokine release after whole blood LPS-stimulation reflects cell function across multiple immune cell classes, and represents the immune response to LPS [12,13]. Ex vivo whole blood LPS-stimulated TNF-α release has been evaluated as a prognostic marker in critically ill patients in a variety of clinical situations, including sepsis patients, and with a range of clinical endpoints [5,6,10,11,14–19]. Although several authors have studied the release of other cytokines, such as interleukin (IL)-1β, IL-6, or IL-10, after ex vivo whole blood LPS stimulation in sepsis [20–23], few have examined the prognostic value of the release of these cytokines in septic patients after ex vivo whole blood LPS stimulation [14,16,17]. We aim, therefore, to examine the prognostic value of release of TNF-α, IL-1β, IL-6, and IL-10 after LPS whole blood stimulation measured within the 24 hours and one week after admission in ICU in patients with severe sepsis and septic shock.

Preliminary data for this study were presented as a poster presentation at the International Symposium of Intensive Care and Emergency Medicine (ISICEM), 20-23 march 2018, Brussels, Belgium.

## 2. Materials and methods

This prospective observational pilot study was performed between November 2012 and September 2014 in a 21-bed ICU at the University Hospital of Rennes (France). The study was approved by the local institutional ethics committee on June 12, 2012 (Comité d'éthique, Centre Hospitalier Universitaire de Rennes, avis n°12-23, Chairperson: Dr Vincent MOREL) and informed consent was obtained from all participants or their next of kin.

### 2.1. Study population

Adults patients admitted in the ICU with a first episode of severe sepsis or septic shock were eligible. Severe sepsis and septic shock were defined based on the criteria proposed by the American College of Chest Physician/Society of Critical Care Consensus Conference Committee [24]. Patients who were pregnant or lactating, receiving immunosuppressive drugs, or who had a history of haematologic malignancy were excluded.

### 2.2. Healthy control

Twenty-three healthy volunteers without medical history were recruited (mean age 33 years, sex ratio M/F 0.9) and informed consent was obtained from all participants.

### 2.3. Blood samples and ex vivo LPS-stimulation

For each patient, 15 mL of whole blood were collected in heparin-tubes within the first 24 hours of ICU admission (day 1), and then 7 days after ICU admission (day 7) if the patient was still in the study hospital. Within one hour of collection, blood was aliquoted in 96-well microplates under sterile conditions and incubated for 30 minutes at 37 °C in an atmosphere of 5% $CO_2$ and 95% air [25]. At the end of the incubation period, LPS from *Escherichia Coli* O55:B5 (product number L2880, Sigma-Aldrich) was added in stimulated wells at 10 μg/mL, whereas unstimulated wells received a sterile saline vehicle only (NaCl 0.9%). After 24 h incubation, the plasma supernatants were gently transferred in polypropylene micro tubes and stored at −80 °C until cytokine assay.

### 2.4. Cytokine assay

The concentrations of TNF-α, IL-1β, IL-6 and IL-10 were determined via enzyme linked-immunosorbent assay (ELISA) according to the manufacturer instructions (Bio-Techne, R&D systems, France). A standard concentration-response curve was run on each microplate to allow determination of cytokine levels in samples. Cytokine concentrations in each sample were determined using an automatic plate reader associated with Genesis software (LabSystems Spectrophotometer, Cambridge, UK) and data were expressed in pg/mL.

### 2.5. Data collection

The following data were collected: age, sex, ICU admission characteristics, comorbidities and overall severity of pre-existing underlying disease based on McCabe's classification [26], information regarding the site, source and type of initial infection, presence of vasopressors infusion, mechanical ventilation and renal replacement therapy, delay between hypotension and first antimicrobial therapy, use of low-dose corticosteroids, acquisition of health-care associated infection in ICU, length of ICU and study hospital stay, 28- and 90-day mortality. The severity of the illness was assessed within 24 hours of ICU admission using the Simplified Acute Physiology Score (SAPS) II. The Sequential Organ Failure Assessment (SOFA) Score and white blood cell count were assessed within the first 24 h and 7 days after the admission in ICU if the patient was still in the study hospital.

### 2.6. Outcome definitions

Our primary outcome measure was the persistence of at least one organ dysfunction at day 7. Organ dysfunction or failure was defined according to the SOFA components by a score $\geq 2$. The secondary outcomes were health-care associated infection in the ICU according to the Centres for Disease Control and Prevention definitions (central line-associated bloodstream infections, catheter-associated urinary tract infections, ventilator-associated pneumonia and surgical site infections), and 28- and 90-day mortality.

### 2.7. Study design

For each patient, the cytokine concentration after LPS stimulation was compared to the concentration in samples incubated without LPS (baseline), and expressed as a ratio (stimulated/baseline). A ratio above 1.5 at day 1 and day 7 was chosen as an arbitrary cut-off point, to reflect a potentially preserved response after LPS-stimulation.

First, the cytokine variations after stimulation were compared between septic patients and healthy controls. Then, the proportion of patients with persistent organ dysfunction at day 7 was compared between patients below and above the 1.5 ratio for each cytokine at day 1. Finally, the relationship between organ dysfunction and the temporal evolution of cytokine release after whole blood LPS-stimulation was studied. For this final analysis, only the patients with a ratio below 1.5 at day 1, for a given cytokine were considered. For each of these groups, the proportion of patients with persistent organ dysfunction at day 7 was compared between patients who recovered a ratio above 1.5 at day 7 and those who did not.

### 2.8. Statistical analysis

All statistical analysis was performed using PRISM 7 (GraphPad Software, La Jolla, Ca, USA). Quantitative data were expressed as

median and interquartile ranges (IQR), while categorical data were expressed as absolute values and percentages. Quantitative data were compared using Mann–Whitney U test. Categorical data were compared using Chi$^2$ tests or Fisher's exact test in case of non-parametric data. A two-sided $P$-value $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Patient characteristics

A total of 49 patients were enrolled in the study, including 37 patients with septic shock (76%). Thirty-one patients (63%) were mechanically ventilated. The origin of sepsis was abdominal in 61% of the cases. The rate of appropriate first antimicrobial therapy was 89% and the median duration between hypotension and initiation of an effective antimicrobial therapy was 0 (0–30) minutes. Other patient characteristics are displayed in Table 1.

### 3.2. Cytokine release

Median concentration of cytokine release with and without LPS-stimulation at day 1 and day 7, in septic patients and healthy volunteers, are displayed in Table 2.

**Table 1**
Patient Characteristics ($n$ = 49).

| | |
|---|---|
| Age, years, median (IQR) | 64 (54–70) |
| Gender, (male) $n$ (%) | 29 (59) |
| MacCabe, $n$ (%) | |
| A | 23 (47) |
| B | 23 (47) |
| C | 3 (6) |
| Type of admission, $n$ (%) | |
| Medical | 10 (20) |
| Surgical emergency | 35 (71) |
| Surgical scheduled | 4 (9) |
| Septic shock, $n$ (%) | 37 (76) |
| Mechanical ventilation, $n$ (%) | 31 (63) |
| Renal replacement therapy, $n$ (%) | 7 (14) |
| Low-dose corticosteroids use, $n$ (%) | 8 (16) |
| Site of initial infection, $n$ (%) | |
| Intra-abdominal | 30 (61) |
| Urinary tract | 7 (15) |
| Lung | 6 (12) |
| Others | 6 (12) |
| Type of Infection, $n$ (%) | |
| Gram-positive | 30 (61) |
| Gram-negative | 25 (51) |
| Fungi | 6 (12) |
| Source of initial infection, $n$ (%) | |
| Community-acquired | 23 (47) |
| Health-care associated | 26 (53) |
| Healthcare associated secondary infection | 5 (10) |
| ICU-acquired, $n$ (%) | |
| SSI | 3 |
| VAP | 2 |
| SAPS II, median (IQR) | 44 (35–56) |
| SOFA Score, median (IQR) | |
| Day 1 | 7 (5–8) |
| Day 7 $n$=46 | 1 (0–3) |
| White Blood Cells day 1, $10^9$/L, median (IQR) $n$ = 46 | 15.9 (10.8–20.8) |
| White Blood Cells day 7, $10^9$/L, median (IQR) $n$ = 35 | 14.7 (10.7–18.4) |
| ICU Free-Days at Day 28, median (IQR) | 22 (16–25) |
| Hospital Mortality, $n$ (%) | 2 (4) |
| Day 90 Mortality, $n$ (%) | 5 (10) |

ICU: intensive care unit; SAPS: simplified acute physiology score; SOFA: sequential organ failure assessment; SSI: surgical site infection; VAP: ventilator-associated pneumonia.

**Table 2**
Median concentrations (pg/mL) of cytokine release in whole blood without (baseline) and with LPS stimulation in septic patients at day 1 and day 7, and in healthy volunteers.

| | Day 1 | | Day 7 | | Healthy volunteers | |
|---|---|---|---|---|---|---|
| | Baseline, $n$ = 49 | LPS | Baseline, $n$ = 35 | LPS | Baseline, $n$ = 23 | LPS |
| TNFα | 44.57 (5.50-204.91), $n$ = 48 | 129.26 (20.09–391.77) | 127.77 (5.50–223.76) | 305.77 (71.10–851.41) | 5.50 (5.50–122.25) | 2461.60 (1690.80–6525.80) |
| IL1β | 2.03 (1.00–37.84) | 50.80 (1.00–251.93) | 136.70 (11.59–369.55) | 904.02 (341.09–1672.65) | 1.00 (1.00–103.45) | 3998.20 (2,637.00–5883.55) |
| IL6 | 1805.35 (404.9–8,326.23), $n$ = 48 | 4,440.10 (1284.15–9839.04) | 4,868.25 (1230.52–8734.45) | 12,194.90 (7039.39–23,868.50) | 9.05 (0.70–311.55), $n$ = 22 | 23,460.80 (16,330.23–35,519.53) |
| IL10 | 119.01 (43.43–373.88), $n$ = 47 | 222.51 (98.47–452.47) | 136.42 (52.99–271.14) | 451.27 (204.64–626.72) | 3.90 (3.90–140.80) | 1000.20 (685.10–1606.30) |

Data are presented as median (IQR). IL: Interleukin; LPS: Lipopolysaccharide; TNF: tumor necrosis factor.

### 3.3. Comparison of the cytokine release after whole blood LPS-stimulation in septic patient with those in healthy controls

At day 1 and day 7, the ratio of cytokine release after whole blood LPS-stimulation to cytokine release at baseline in septic patients were significantly lower than the same ratio in healthy controls (Fig. 1).

When considering only patients with samples available at day 1 and at day 7 ($n$ = 35 except for IL-6 $n$ = 34), a significant increase was seen in this ratio between day 1 and day 7 for IL-1β, IL-6 and IL-10 but not for TNF-α (Fig. 2).

### 3.4. Persistent organ dysfunction at day 7

Considering IL-10 release, a significantly higher proportion of patients with a ratio below 1.5 at day 1 was found to have persistent organ dysfunction (SOFA Score ≥ 2) at day 7 than patients with a ratio above 1.5 (13/24 vs. 4/20 respectively, $P$ = 0.03). However, the proportion of patients with persistent organ dysfunction at day 7 was not significantly different between patients with a ratio below and above 1.5 for IL-1β (8/19 vs. 9/27 respectively, $P > 0.99$), IL-6 (9/21 vs. 7/24, $P$ = 0.12) or TNF-α (11/27 vs. 6/18, $P$ = 0.38) release at day 1. Similarly, when considering cytokine release at day 7, there was no significant relationship between patients with a ratio below and above 1.5 and organ dysfunction for any of the measured cytokines, including IL-10.

### 3.5. Secondary outcomes

Only three patients (6%) died within 28 days, five patients (10%) died within 90 days, and five patients (10%) developed healthcare-associated infection in the ICU. These outcomes were less common than expected, precluding further analysis.

### 3.6. Relationship between persistent organ failure at day 7 and the temporal evolution of cytokine release after whole blood LPS-stimulation

When considering only IL-10 release, patients whose ratio was below 1.5 at day 1 and increased above 1.5 at day 7 were significantly less likely to have persistent organ dysfunction than patients who did not recover IL-10 release at day 7 (6/14 vs. 5/5, respectively $P$ = 0.045). However, no significant difference in organ dysfunction was found between patients whose ratio was below 1.5 at day 1 and increased above 1.5 at day 7 for IL-6 (3/10 vs. 3/4, respectively $P$ = 0.25), IL-1β (5/10 vs. 2/3 respectively, $P > 0.99$) or TNF-α (3/9 vs. 8/13 respectively, $P$ = 0.39).

## 4. Discussion

We observed a significant and prolonged reduction of TNF-α, IL-1β, IL-6 and IL-10 release after whole blood LPS-stimulation in septic patients compared to healthy controls. This reduction in TNF-α, IL-1β, IL-6 and IL-10 release likely reflects the well-described phenomenon of LPS tolerance or immunoparalysis, in which circulating leukocytes in septic patients have a diminished ability to release additional cytokines [22,27]. We also observed that patients with low IL-10 release after LPS stimulation at day 1 had a higher rate of persistent organ dysfunction. Moreover, persistently low IL10 release was associated with higher proportion of organ dysfunction at day 7. The same kind of association was not observed with the other cytokines.

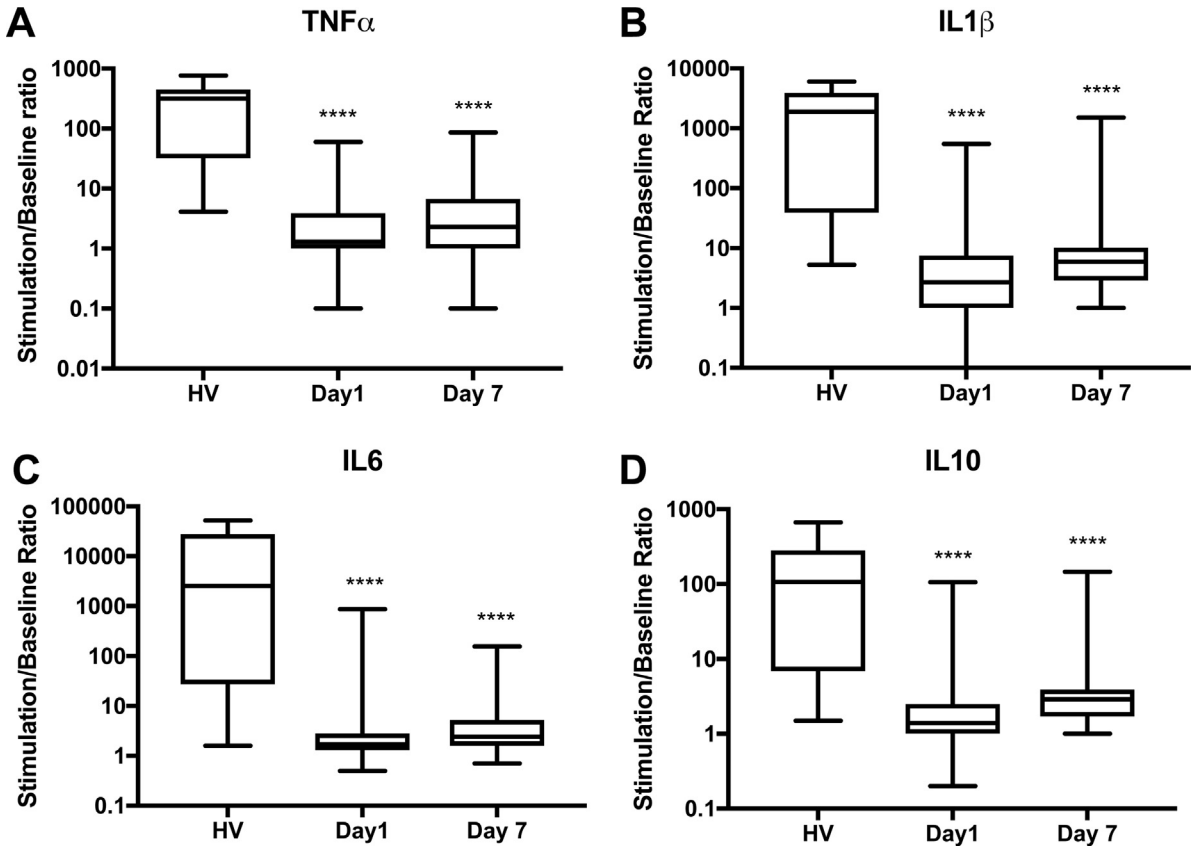


**Fig. 1.** Comparison of the ratio of cytokine release after whole blood lipopolysaccharide-stimulation to cytokine release at baseline at day 1 and day 7 between septic patients and healthy controls. A. Tumor Necrosis Factor(TNF)-α ($n$ = 48 septic patients and 23 healthy controls). B. Interleukin (IL)-1β ($n$ = 49 and 23 respectively). C. IL-6($n$ = 48 and 22 respectively). D. IL-10 ($n$ = 47 and 23 respectively), Data presented as box-plots with minimum to maximum value on a log-scale, Mann-Whitney U test $^{****}P < 0.0001$.

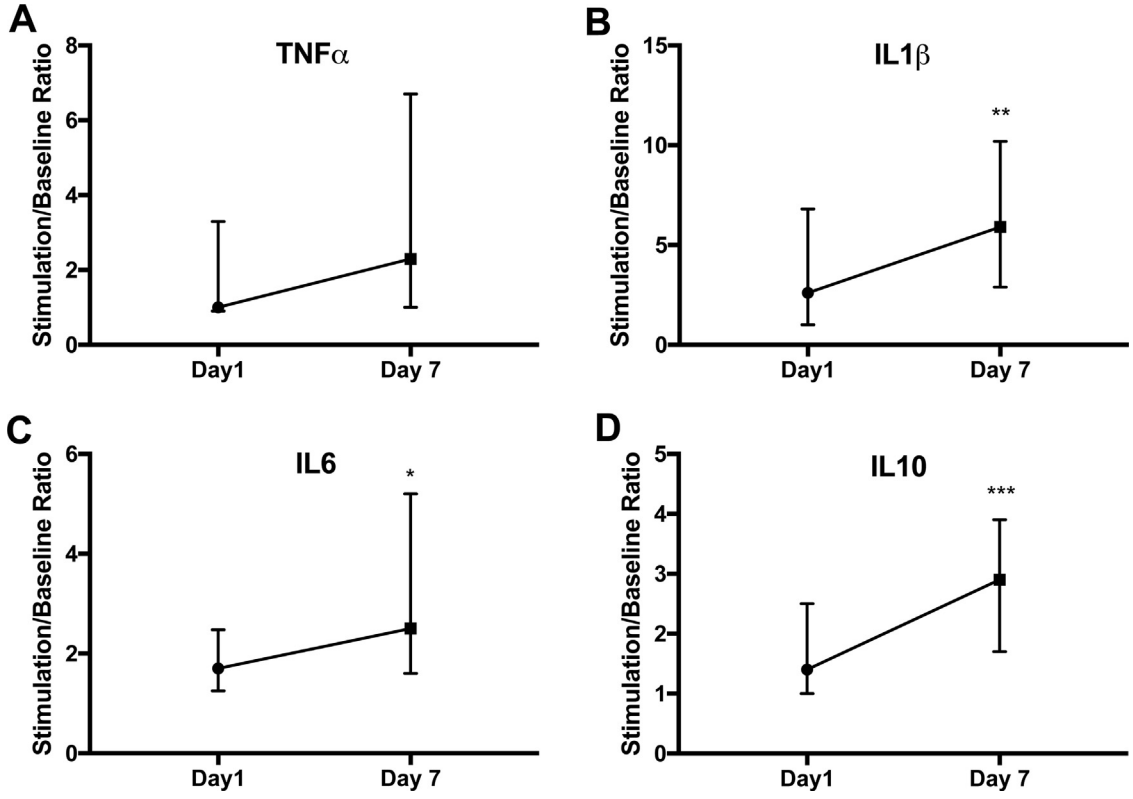


**Fig. 2.** Evolution of the ratio of cytokine release after whole blood lipopolysaccharide-stimulation to cytokine release at baseline between day 1 and day 7 ($n$ = 35, except IL6 $n$ = 34). A. Tumor Necrosis Factor(TNF)-α. B. Interleukin (IL)-1β. C. IL-6. D. IL-10, Median with interquartile range, Mann–Whitney U test * $P$ = 0.03,** $P$ = 0.01 and *** $P$ = 0.0005.

The data available concerning the release of IL-10 after whole blood LPS-stimulation in septic patients are scarce and contradictory. While Marchant et al. found a decrease in IL-10 release in 10 patients with septic shock compared to healthy controls, Rigato and Salomao did not observe any differences in IL-10 release in 20 septic patients (including 15 with septic shock) compared to healthy controls [20,21]. Four hours after experimental endotoxemia in healthy human volunteers, Kox et al. reported a 45% drop in peak IL-10 release after *ex vivo* LPS stimulation [28]. Other authors have reported that, while peripheral blood mononuclear cells (PBMCs) in septic patients released less IL-10 than in healthy controls after LPS stimulation, IL-10 release from the monocytes of septic patients after LPS stimulation was higher than in healthy controls [29,30]. Finally, *ex vivo* stimulation of whole blood from septic patients, with intact *E. coli*, leads to a depressed IL-10 release compared to healthy controls, but stimulation with intact *Pseudomonas aeruginosa* does not [21,23].

It may be that a decreasing ability to release IL-10 in whole blood in response to ex vivo stimulation is the result of significant ongoing IL-10 production in vivo, and may reflect immunosuppression. This explanation is consistent with previous studies, which found that elevated plasma IL-10 concentrations were associated with mortality in severe sepsis and septic shock [31,32,33,34]. Elevated plasma IL-10 was also found to be associated with ICU acquired infections in severe sepsis and septic shock, although other authors did not find any associations [34,35]. In one study, IL-10 release after LPS-stimulation in PBMCs was found to be higher in non-surviving septic patients, the opposite of the pattern that we observed [30]. This difference may be explained by our use of a whole blood stimulation assay, which allows the evaluation not only of PMBCs functionality, but also of other immune cells including granulocytes and platelets [36,37]. Furthermore, the use of whole blood maintains interactions between cellular elements that are required for cell viability, and avoids the potential influence of artificial medium on isolated cells [37].

We did not find any association between inflammatory cytokine release after whole blood LPS-stimulation at day 1 and persistent organ dysfunction. Several studies have previously evaluated TNF-α release after whole blood LPS-stimulation and outcomes in septic patients with contradictory results. Ploder et al. reported that TNF-α release after *ex vivo* stimulation in 19 patients with polytrauma and sepsis was lower in non-survivors than in survivors [15]. Similarly, in 52 critically ill children with Influenza, lower TNF-α release upon ex vivo stimulation within 72 hours of ICU admission was associated with higher mortality and fewer ICU-free days [10]. Finally lower TNF-α release was found to be associated with persistent organ dysfunction in 102 septic children as well as in 24 septic adults [18,19]. In contrast, in 83 adults with severe sepsis, TNF-α release at several time points was not found associated with survival or development of secondary infection [6]. Similarly, no association was found between TNF-α, IL-6 or IL-1β release after whole blood LPS-stimulation in another population of 73 critically ill patient (including 47 septic patients) and subsequent development of ICU-acquired infections [17]. These discrepancies could be explained in part by the lack of standardisation of LPS-induced cytokine production assays. Indeed, TNF-α release was found to be highly dependent on the source and concentration of LPS, duration and temperature of incubation, anticoagulation, and sample dilution [38]. As a result, increased standardisation of LPS stimulation assays is urgently required in order to improve the external validity of studies in this area. In our study, although the stimulation protocol was based on a previous study in COPD patients [39], LPS concentration was higher and incubation time longer than that often reported in

studies performing whole blood LPS-stimulation in septic patients. This study is, to the best of our knowledge, the first to report an association between low release of IL-10 after ex vivo whole blood LPS-stimulation and persistent organ failure in a prospective cohort of severe sepsis patients. Moreover, the cytokine release was evaluated at two different time points to better reflect the dynamic process of inflammation in sepsis. However, several limitations must be underlined. First, as discussed above, our LPS-stimulation protocol differed in several points from the protocols generally used in septic patients, limiting the comparability of our data. Also, our septic population was older than the healthy volunteers, so the comparison could not be age-matched. This is relevant because older age is associated with a state of chronic inflammation, and impaired production of pro-inflammatory cytokines in response to lipopolysaccharide stimulation has been reported in the elderly [40,41]. However, human studies comparing levels of cytokine release between young and older septic patients remains scarce and the data contradictory [41]. Additionally, our sample size is small, as our work was designed as a pilot study. Also, while our study population was severely ill, with 76% having septic shock, and high median SOFA and SAPS II Scores, both the rates of mortality and healthcare associated infections were lower than typically reported [42,43,44,45,46]. These low rates precluded the use of these outcomes as secondary endpoints as previously planned. Moreover, a monocentric design and the enrolment of mainly surgical (80%) patients limit the generalisation of our results. Further studies are needed to confirm our results in a larger and multicentre cohort with a higher ratio of medical patients and with additional endpoints.

## 5. Conclusion

Early low IL-10 release after *ex vivo* LPS stimulation of whole blood in sepsis, as well as persistent low IL-10 response over time, may be associated with persistent organ dysfunction. While these results will need to be confirmed in future studies, they do highlight the potential role of early IL-10 release and evolution of IL-10 release after *ex vivo* LPS stimulation of whole blood in sepsis as both a potential prognostic marker, and a potential means to stratify patients in clinical trials of immunomodulatory therapeutics.

## Ethical statement

The study was approved by the local institutional ethics committee on June 12, 2012 (Comité d'éthique, Centre Hospitalier Universitaire de Rennes, avis n°12-23, Chairperson: Dr Vincent MOREL) and informed consent was obtained from all participants or their next of kin.

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## Authors' contributions

NN and CMC contributed to the study concept and design. NN, CMC, HP, JTR, PSi, YL, SI, EM, PS and YM contributed to the acquisition, analysis, or interpretation of data for the work. CR and NN performed the statistical analysis. NN, CMC, HP, JTR, PS and YM drafted the manuscript, which was revised for important intellectual content by PSi, YL, SI and EM. All authors read and approved the final manuscript.

### Disclosure of interest

The authors declare that they have no competing interest.

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