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Serum of patients with septic shock stimulates the expression of Trem-1 on U937 monocytes

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Abstract. *Objectives:* To describe the concentrations of sTREM-1 in patients with sepsis and to explore the effects of their serum on the expression of TREM-1 on U937 monocytes.
Methods: Blood was sampled at regular time intervals in 56 patients with sepsis. Concentrations of tumour necrosis factor-alpha (TNFα), interleukin-1beta (IL-1α), IL-6, IL-8, IL-10 and IL-12p70 and sTREM-1 were measured. U937 monocytes were incubated in the presence of serum at sepsis onset.
Results: Median sTREM-1 concentration on day 1 for patients with septic shock was 915 pg/ml and 228.5 pg/ml for those without shock ($p = 0.002$). TNFα, IL-1α, IL-6, IL-8 and IL-10 did not differ between them. A positive correlation was found between changes in sTREM-1 and SOFA scores from day 1 to 7. Sera from patients with septic shock evoked a significant increase in the expression of TREM-1. The concentrations of TNFα and IL-8 in supernatants increased only after stimulation with sera from patients without shock, but not after stimulating with sera of patients with shock.
Conclusions: Levels of sTREM-1 correlated with sepsis severity. sTREM-1 is considerably higher in patients with shock compared to patients without shock. The serum of shocked patients was able stimulate the expression of TREM-1 on U937 monocytes.

Key words: Monocytes – Infection – Shock – Cytokines – Inflammation

Introduction

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a novel receptor highly expressed on neutrophils and monocytes in the event of sepsis and septic shock [1, 2]. Its soluble counterpart, designated as sTREM-1, has been recently described and has been shown to be elevated in patients with septic shock [3, 4]. It has been proposed that sTREM-1 may act as an anti-inflammatory mediator and that it might be implicated in the transition from sepsis or severe sepsis to septic shock [5]. That hypothesis was based on the results of former studies of our group in a cohort of patients with ventilator-associated pneumonia [4, 5]. However, the exact ligand of TREM-1 remains undefined.

Based on the hypothesis that sTREM-1 is a mediator implicated in the generation of septic shock, the present study was performed in a different cohort of septic patients than previously reported. Septic patients were divided in two groups according to the presence or absence of shock. In addition to the estimation of sTREM-1 and other cytokines, sera collected on day 1 were used to stimulate monocytes of the U937 cell line both for the expression of TREM-1 and for the release of pro-inflammatory cytokines. The latter was done in order to investigate whether differences in sTREM-1 between septic patients with and without shock may reflect respective differences of the potency of serum to stimulate TREM-1 expression. The U937 monocytic cell line was selected because it responds to stimulation with the production of pro-inflammatory cytokines by the activation of intracellular pathways similar to those of human cells [6].

Patients and methods

Study design

This prospective study included 56 mechanically ventilated, septic patients hospitalized in the ICU of "Evangelismos" General Hospital during the period January-December 2003. Enrolled patients represent a different population compared to that previously reported [4, 5]. The protocol was approved by the Ethics Committee of the hospital. Exclusion criteria were:

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Table 1. Demographic characteristics of patients enrolled in the study.

	Sepsis	**Severe Sepsis**	**Septic Shock**
Number of patients	16	22	18
Age (years, mean ± SD)	38.31 ± 13.97[a]	52.27 ± 20.07	67.56 ± 15.54[a]
Male/Female	15/1	15/7	8/10
APACHE II score (mean ± SD)	13.12 ± 4.14	20.13 ± 5.22	24.67 ± 4.82[b]
SOFA score (mean ± SD)	6.44 ± 1.45	10.86 ± 3.11	13.77 ± 3.75[b]
Underlying conditions (No (%))			
Multiple trauma	9 (56.3 %)	8 (36.4 %)	4 (22.2 %)
Cerebrovascular accident	4 (25.0 %)	2 (9.1 %)	2 (11.1 %)
Postoperative (Colectomy/lobectomy)	3 (18.8 %)	9 (40.9 %)	11 (61.1 %)
Others	0 (0 %)	1 (4.5 %)	1 (5.5 %)
Type of infection (No (%))			
Ventilator-associated pneumonia	10 (62.50 %)	15 (68.2 %)	5 (27.7 %)
Intrabdominal infection	3 (18.8 %)	5 (22.7 %)	12 (66.7 %)
Others	3 (18.8 %)	2 (9.1 %)	3 (16.7 %)
Pathogens (No (%))			
Acinetobacter baumannii	5 (31.25 %)	8 (36.4 %)	7 (38.9 %)
Pseudomonas aeruginosa	0 (0 %)	5 (22.7 %)	0 (0 %)
Enterococcus spp	1 (6.25 %)	1 (9.1 %)	4 (22.2 %)
Others	4 (25.0 %)	5 (22.7 %)	1 (5.5 %)
Case-fatality (No (%))	1 (6.25 %)	9 (40.9 %)	12 (66.7 %)

[a] $p < 0.05$ compared to both patients with sepsis and severe sepsis
[b] $p < 0.0001$ compared to both patients with sepsis and severe sepsis

a) neutropenia (≤500 neutrophils/mm^3),
b) HIV infection, and c) administration of corticosteroids before enrolment or during ICU stay. Informed consent was taken from first-degree relatives.
Inclusion criteria were the concomitant presence of
a) age ≥18 years, and
b) sepsis or severe sepsis or septic shock for less than 24 hours. For the purposes of the analysis, patients with sepsis and severe sepsis were considered as one group, while patients with shock constituted a separate group. Sepsis was defined as the presence of an infection accompanied by at least two of the following criteria [7]: a) core temperature >38 °C or <36 °C, b) P_{co2} <32mmHg, c) pulse rate >90/min, and d) white blood cells >12,000/μl or <4,000/μl or >10 % of band forms.
Severe sepsis was determined as the acute dysfunction of at least one organ i.e. the acute presentation of at least one of the following [7]:
- Acute Respiratory Distress Syndrome (ARDS): pO_2/FiO_2 below 200 with diffuse shadows in lung X-ray
- Acute renal failure: urine production of less than 0.5 ml/Kg body weight/h for at least two hours provided that the negative fluid balance of the patient was corrected
- Metabolic acidosis: pH <7.30 or any base deficit greater than 5mEq/l and serum lactate at least more than 2 × the normal value
- Acute coagulopathy: platelet count <100.000/μl or INR >1.5

Septic shock was considered if systolic pressure was below 90mmHg requiring the administration of vasopressors for more than one hour provided that the negative fluid balance of the patient was corrected [7].

Enrolled patients were followed-up on a daily basis for a total of 28 days; evaluation comprised chest X-rays, estimation of the APACHE II and SOFA scores, blood cultures and, if necessary, cultures of tracheobronchial secretions (TBS) as well as computed tomography of thorax and/or abdomen. Five milliliters of blood were sampled after puncture of a peripheral vein under sterile conditions on days 1, 3, 5 and 7. Blood was collected into sterile tubes. After centrifugation, serum was kept at –70 °C until assayed.

Ventilator-associated pneumonia (VAP) was diagnosed in any patient presenting with the following [8, 9]:
a) new or persistent consolidation in lung X-ray;
b) purulent TBS; and c) clinical pulmonary infection score (CPIS) more than 6 defined as proposed elsewhere [10]. For patients with signs of VAP, TBS were collected after insertion of a sterile catheter in the endotracheal tube connected to a negative pressure device.
Intrabdominal infection was diagnosed in any patient with the following [11]:
a) core temperature >38 °C or >36 °C;
b) radiological findings compatible with intra-abdominal infection; and
c) white blood cells >12,000/μl or <4,000/μl or >10 % of band forms.

Laboratory techniques

Quantitative TBS cultures were performed immediately after collection; 0.5 ml of TBS was added into a sterile tube with 2 ml of dithiothreitol (Oxoid Ltd, London, UK) and diluted five consecutive times 1:10 in dithiothreitol. Volumes of 0.1 ml of each dilution were plated onto McConkey and blood agar (Becton Dickinson, Cockeysville Md). Dishes were incubated for five days at 37 °C and their count was estimated after multiplying with the appropriate dilution factor. Cultures yielding a pathogen at a count $\geq 1 \times 10^6$ cfu/ml were considered positive [12]. Flasks with blood were incubated for seven days. Identification

Table 2. Concentrations of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), tumour necrosis factor-alpha (TNFα), interleukin-1β (IL-1β), IL-6, IL-8, IL-10 and IL-12p70 on day 1 of sepsis of 56 patients in relation to the presence or not of shock.

	Patients without shock (n = 38)	Patients with shock (n = 18)	p
	Median (IQR, pg/ml)		
sTREM-1	228.5 (698)	915 (1816)	0.002
TNFα	2.5 (2)	2.5 (2)	0.388
IL-1β	34 (86)	51.5 (99)	0.539
IL-6	269 (809)	402 (900)	0.145
IL-8	135 (166)	164 (161)	0.108
IL-10	13 (27)	20 (63)	0.172
IL-12p70	9 (26)	9.5 (11)	0.648

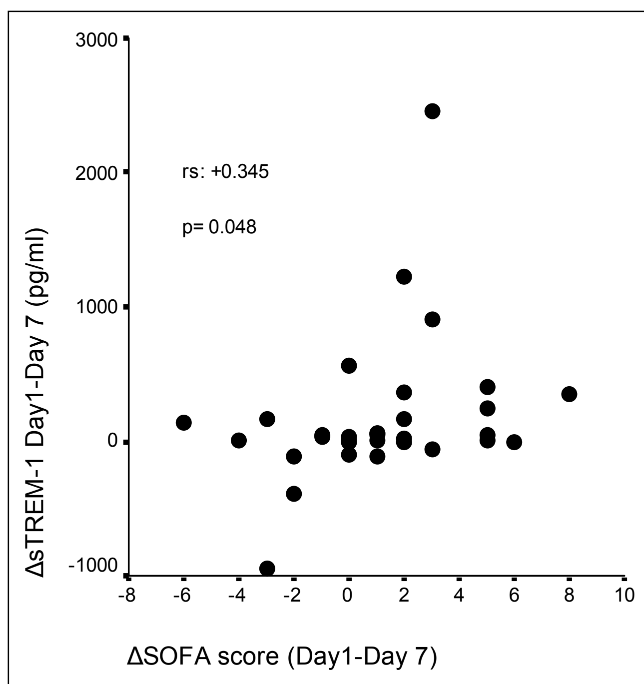

Fig. 1. Correlation of changes in serum levels of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) between days 1 and 7 of follow-up with respective changes in SOFA score of a cohort of 56 septic patients.

of pathogens was performed by the API20E and the AP 20 systems (bioMérieux, Paris, France).

Concentrations of tumour necrosis factor-alpha (TNFα), interleukin-1beta (IL-1α), IL-6, IL-8, IL-10 and IL-12p70 in sera were estimated in duplicate after staining with monoclonal antibodies and passage through FACS Calibur (Becton Dickinson). The lowest limits of detection were 2 pg/ml for TNFα; 3 pg/ml for IL-1β; 3 pg/ml for IL-6; 3 pg/ml for IL-8; 3 pg/ml for IL-10; and 3 pg/ml for IL-12p70.

Estimation of sTREM-1 was performed by a home-made enzyme immunoabsorbent assay. Capture antibody of sTREM-1 (R&D InC, Minneapolis, USA) was diluted to 4000ng/ml and distributed in a 96-well plate at a volume of 0.1 ml per well. After overnight incubation at 25 °C, wells were thoroughly washed with a 0.05 % solution of Tween in PBS (Merck) (pH: 7.2–7.4). Then 0.1 ml of standard concentrations of sTREM-1 (15.1–4000 pg/ml, R&D Inc) or serum was added in wells. After incubation for two hours, wells were washed thrice, and 0.1 ml of one 400ng/ml dilution of sTREM-1 detection antibody (R&D Inc) was added per well. The plate was then incubated for two hours, and attached antibodies were signalled by streptavidin. Concentrations of sTREM-1 in each well were estimated by the optical density detected at 450 nm after addition of one 1:1 solution of H_2O_2: tetramethylbenzidine as a substrate (R&D Inc). All determinations were performed in duplicate; the inter-day variation of the assay was 5.23 %.

The potency of serum drawn on day 1 from patients to stimulate expression of TREM-1 on human monocytes was assessed with the application of the U937 human monocytic cell line. Cells were incubated in 75cm^2 flasks with RPMI 1640 (Biochrom AG, Berlin, Germany) supplemented with 10 % Fetal Bovine Serum (FBS, Biochrom AG) and 2mM of glutamine (Biochrom AG) until they became confluent. The content of flasks was centrifuged and the cell pellet was dissolved in RMPI 1640 supplemented with 2 mM of glutamine. Then cells were distributed in a 96-well plate at a density of 1×10^5 cells per well at a volume of 100 µl. In each well, 100 µl of serum samples were added. The plate was incubated at 37 °C in a 5 % CO_2 atmosphere. After 12 hours the plate was centrifuged for eight minutes at 1,700 g in room temperature; the supernatant of each well was stored at –80 °C and the cell pellet was re-suspended in 200 µl of PBS pH: 7.2. Cells were then incubated with the PE-conjugated monoclonal antibodies anti-IgG1 or anti-TREM-1 (emission 520 nm, R&D, Minneapolis, USA) for 40 minutes at 4 °C. Expression of TREM-1 receptor on the cell membrane of U937 was assessed after analysis through the EPICS XL/MSL flow cytometer (Beckman Coulter Co, Miami, Florida) using unstained cells as negative controls. Experiments were run in duplicate; wells with added serum from six healthy volunteers were also done. TREM-1 was expressed both by its mean fluorescence intensity (MFI) and by the percentage of U937 staining positive after incubation with serum samples.

We hypothesized that if the serum of patients could stimulate the TREM-1 cascade in U937 monocytes that would lead to the production of TNFα and IL-8 [13, 14]. Both cytokines were estimated in supernatants by an enzyme immunoabsorbent assay in duplicate (Diaclone, Paris, France). The lower detection limit of that assay was 2 pg/ml for TNFα and 62.5 pg/ml for IL-8. The concentration of cytokines resulting from cell stimulation was derived after substracting serum concentrations.

Statistical analysis

Results of parameters estimated in serum were expressed as medians and interquartile range (IQR) because they followed a non-normal distribution of values as assessed by Kolmogorov-Smirnov's statistics. Comparisons of cytokines of the same day between survivors and non-survivors were performed by the Mann-Whitney U test. Receiver Operator Curve (ROC) was designed in an attempt to define a threshold of sTREM-1 of

Table 3. Concentrations of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), tumour necrosis factor-alpha (TNFα), interleukin-1 (IL-1β), IL-6, IL-8, IL-10 and IL-12p70 in 56 septic patients in relation to their final outcome.

Days	Survivors (n = 34)	Non-survivors (n = 22)	p
	sTREM-1 [pg/ml, median (IQR)]		
1	267.1 (914)	384.7 (1285)	NS
3	348.0 (935)	328.4 (655)	NS
5	293.0 (750)	426.4 (954)	NS
7	392.2 (944)	229.1 (1231)	NS
	TNFα [pg/ml, median (IQR)]		
1	2.0 (1)	2.4 (2)	NS
3	2.0 (3)	2.0 (4)	NS
5	2.0 (3)	2.0 (2)	NS
7	2.0 (52)	2.0 (2)	NS
	IL-1β [pg/ml, median (IQR)]		
1	43.5 (115)	29.0 (77)	NS
3	22.0 (105)	22.0 (54)	NS
5	10.0 (57)	10.0 (57)	NS
7	7.0 (120)	30.0 (64)	NS
	IL-6 [pg/ml, median (IQR)]		
1	254.5 (359)	807.0 (3877)	0.005
3	183.0 (237)	267.0 (1097)	0.014
5	97.0 (175)	244.0 (347)	0.001
7	105.0 (171)	245.0 (387)	0.041
	IL-8 [pg/ml, median (IQR)]		
1	116.0 (120)	191.5 (334)	0.008
3	83.0 (79)	141.0 (302)	0.020
5	66.0 (60)	100.0 (121)	0.005
7	65.0 (52)	84.0 (173)	NS
	IL-10 [pg/ml, median (IQR)]		
1	13.5 (19)	28.0 (79)	0.044
3	12.0 (11)	15.0 (20)	NS
5	10.0 (8)	11.0 (10)	NS
7	4.0 (7)	9.0 (12)	NS
	IL-12p70 [pg/ml, median (IQR)]		
1	8.0 (20)	11.0 (23)	NS
3	6.0 (19)	6.0 (12)	NS
5	4.0 (16)	7.0 (12)	NS
7	4.0 (15)	9.0 (11)	NS

day 1 for the occurrence of septic shock. The odds ratio (OR) ± 95 %CI at that threshold was estimated by Mantel-Haenzel statistics. Changes of serum levels of parameters between days 1 and 7 were correlated to respective changes of APACHE II and SOFA scores by the non-parametric co-efficient of Spearman (r_s). Results of TREM-1 and cytokines in supernatants were given as means ± SE and compared by ANOVA with post-hoc Bonferroni analysis. Any value of p below 0.05 was considered statistically significant.

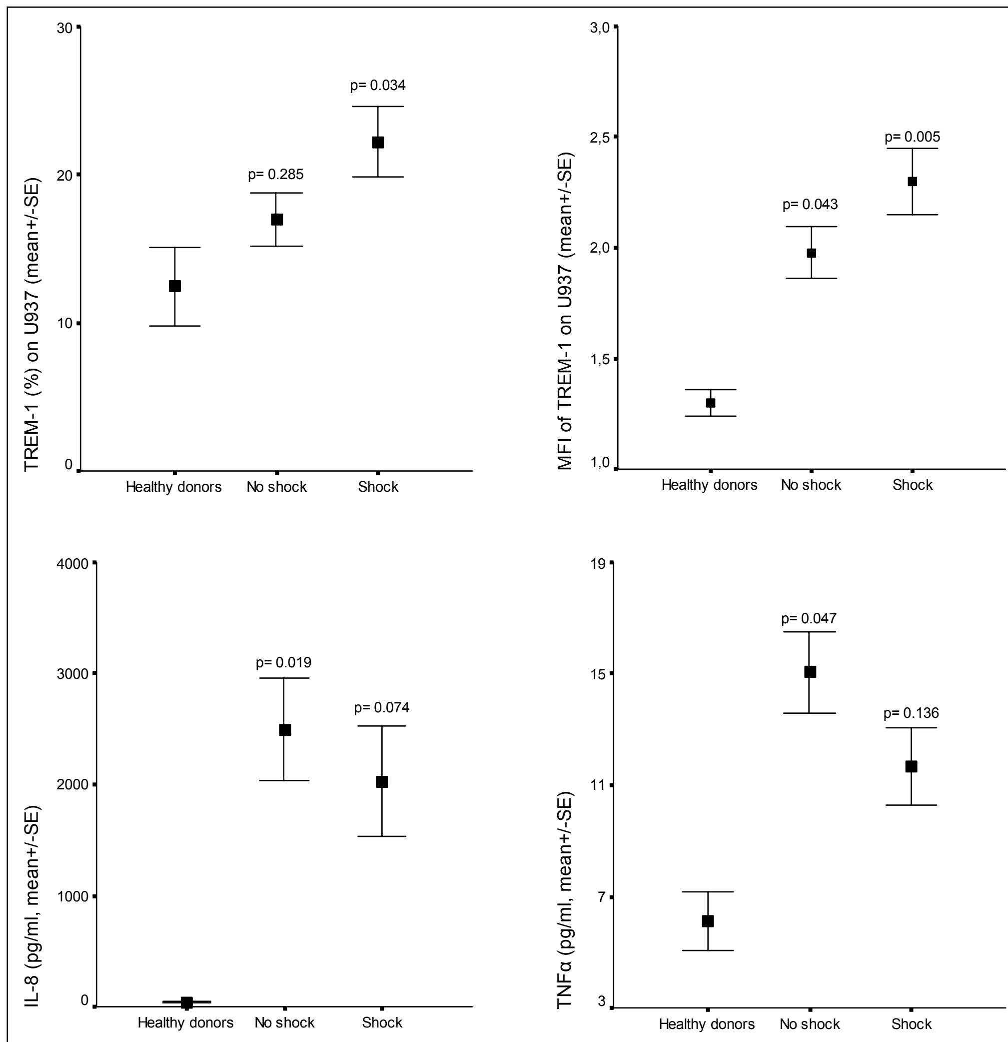

Fig. 2. Expression of triggering receptor expressed on myeloid cells-1 (TREM-1) induced on the cell membrane of U937 monocytes after stimulation with sera sampled on day 1 from septic patients without shock (n = 38) and of patients with septic shock (n = 18) on day 1 of diagnosis. Concentrations of tumour necrosis factor-alpha (TNFα) and of interleukin-8 (IL-8) of U937 supernatants are also shown. P values refer to comparisons with six healthy donors

Results

Patients' characteristics according to the clinical stages of the septic syndrome are shown in Table 1. Patients with septic shock were older and in a more critical state, as reflected by the higher APACHE II and SOFA scores, compared to patients with sepsis or severe sepsis. The commonest sites of infection were the lungs and the abdomen. Mortality rates in severe sepsis and septic shock were higher than sepsis.

Concentrations of the estimated cytokines on the first day of sepsis for septic patients without shock and those with shock are shown separately in Table 2. Concentrations of TNFα, IL-1β, IL-6, IL-8 and IL-10 did not differ between these groups. sTREM-1 was higher in patients with shock ($p = 0.002$ compared to patients without shock). The OR for developing septic shock at concentrations of sTREM-1 higher than 750 pg/ml was 4.53 (95 % CI: 1.34-15.27, $p = 0.015$).

A positive correlation was found between changes in sTREM-1 from day 1 to day 7 and respective changes in SOFA scores (r_s: +0.345, $p = 0.048$, Figure 1). No significant correlations were found between changes in SOFA scores between these days and respective changes in TNFα (r_s: –0.019, pNS); in IL-1β (r_s: +0.041, pNS); in IL-6 (r_s: +0.108, pNS); in IL-8 (r_s: +0.053, pNS) in IL-10 (r_s: +0.109); and in IL-12p70 (r_s: –0.320). No significant correlation was found between changes in any of the estimated parameters and those in APACHE II score.

Concentrations of sTREM-1, TNFα, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 in relation to the final outcome are presented in Table 3. On day 1, non-survivors had higher levels in IL-6, IL-8, and IL-10 compared to survivors.

The expression of TREM-1 induced on the cell membranes of U937 monocytes after their incubation with serum drawn on day 1 is shown in Figure 2. When compared to serum sampled from healthy volunteers, sera of patients with septic shock evoked a higher increase in the expression of TREM-1 on U937 compared to patients without shock. However, concentrations of TNFα and IL-8 of supernatants after stimulation with sera of patients without shock were increased compared to stimulation with serum of healthy donors. This phenomenon did not occur after stimulation with sera of patients with shock.

Discussion

Among the various inflammatory mediators measured, only concentrations of sTREM-1 differed in patients with septic shock compared to patients with lesser degrees of severity of sepsis (Table 2). The remaining pro- and anti-inflammatory mediators were similar on day 1 between these two groups of patients. Serum sTREM-1 greater than 750 pg/ml was associated with a 4.53-fold greater risk for the occurrence of shock. Although its levels did not differ between survivors and nonsurvivors on the same day as was the case for IL-6, IL-8 and IL-10 (Table 3), changes in sTREM-1 followed the changes in sepsis severity. SOFA score is an index of acute multiple organ failure as opposed to APACHE II score that comprises also an assessment of chronic health status. The positive correlation of changes of SOFA score to those of sTREM-1 within the same patients (Figure 1) is an indirect association of sTREM-1 to severity of sepsis. To the best of our knowledge, such a relationship has not been previously reported.

The pathophysiological role of sTREM-1 in the cascade of events in sepsis is largely unknown. sTREM-1 is the soluble counterpart of a receptor which is highly expressed on cell membranes of neutrophils and monocytes of septic patients. Former studies described the hypothesis that sTREM-1 might behave as an anti-inflammatory mediator [3, 5]. This hypothesis is based on the assumption that sTREM-1 binds to the yet undefined ligand of TREM-1 and prevents the induction of pro-inflammatory phenomena.

What causes the stimulation of TREM-1 is not clearly defined. This might be attributed to other cytokines, to endotoxins or to a ligand that is yet undefined [16]. In the present study incubation of sera sampled from patients on day 1 induced the expression of TREM-1 on U937 monocytes. That was higher in patients with shock than in those without shock (Figure 2). However, when TNFα and IL-8 were estimated in supernatants as an indirect assessment of the functional capacity of the stimulated TREM-1 to induce cytokine release [13–15], it was found that cytokine levels were lower after stimulation with serum of patients with shock.

Explanations accounting for these differences remain theoretical. Differences in stimulation of TREM-1 did not seem to derive from serum cytokines because there concentrations were similar in shocked and non-shocked patients. The possibility that the serum of patients with shock induced a limited functional blockade of TREM-1 should be considered. Wong-Baeza et al [16] described that the addition of a TREM-1/IgG fusion molecule decreased the cytokine response of mononuclear cells when stimulated with serum collected from 18 septic patients. Their results were in favour of the presence of a ligand of TREM-1 in serum. The findings of the present study may also be explained if such a ligand exists.

In conclusion, the presented results revealed that concentrations of sTREM-1 were considerably higher in serum of patients with septic shock than in patients without shock and correlated with clinical severity as measured by SOFA score. Moreover, serum of patients in shock could stimulate the expression of TREM-1 receptor on U937 monocytes. Taken together these suggest that both TREM-1 and sTREM-1 contribute to the development of shock in patients suffering from sepsis.

Authors declaration. The authors declare that they have no competing interests

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