

## Longitudinal study of cytokine and immune transcription factor mRNA expression in septic shock

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### Abstract

Success in treating severe sepsis will require relevant tools to monitor the patient immunoinflammatory status. This study aimed to investigate the feasibility of measuring a panel of immunological mediator mRNAs in whole blood and to study their prognostic values in septic shock patients. At the onset of shock, compared to healthy volunteers, mRNA levels in septic shock patients were increased for IL-10, IL-1beta, and high mobility group B1 (HMGB1) and decreased for transforming growth factor beta 1, the Th1, and Th2 transcription factors, T-bet and GATA-3, respectively. Single parameter analysis highlighted an increased expression of IL-10 and HMGB1 mRNA in nonsurvivors and a significant rise over time of GATA3 in survivors. Combining the expression levels of four genes, hierarchical cluster analysis showed that up to 95% of the patients with a similar outcome displayed transcriptional similarities. These results illustrate both the potential of whole blood mRNA quantification assays and the interest of a multiparametric strategy to better stratify septic patients.

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**Keywords:** Septic shock; mRNA; Blood; Outcome; qRT-PCR

### Introduction

Severe sepsis and septic shock remain the leading causes of mortality in intensive care units [1]. Septic syndromes have been mainly characterized by systemic symptoms related to inflammatory response [2]. The failure over the past two decades of clinical trials using anti-inflammatory drugs in septic shock has been partly explained through the concept of sepsis-induced immuno-

suppression [3]. The host response consists in a quasi-simultaneous release in blood of pro- and anti-inflammatory mediators, secondly turning off the innate immune system by an unknown mechanism [3,4]. Importantly, patients with immunoparalysis show an increased risk of contracting a secondary nosocomial infection, explaining that most deaths occur during this prolonged hypoimmune state [3,4]. Anti-inflammatory therapies used early in patients with a hyperinflammatory state may improve the likelihood of survival, while it may worsen the outcome if applied during the hypoimmune phase [3]. Thus, a crucial issue for the future will consist in our capacity to stage immune dysfunctions in a very heterogeneous population of patients. Indeed, sepsis is expressed through the interaction of extremely complex networks and amplifica-

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tion cascades since more than 200 mediators are involved and are rapidly changing over time [5].

For such an approach, innovative biological tools able to provide an overview of immune disorders both at the beginning of sepsis and over time are required. In contrast to a large number of studies focused on the measurement of immune mediators in serum samples [6,7], there is little information on the mRNA expression levels in peripheral blood of septic patients. We have therefore carried out an observational study of an immune mRNA panel in peripheral blood of septic shock patients using quantitative real-time RT-PCR (qRT-PCR). The first objective of the present study was to investigate the feasibility of measuring the selected mRNA in blood samples and to study their individual relevance as predictors of septic shock outcome. Moreover, we hypothesized that a cluster of mediators more than a single one could help to stage severe septic patients. The second objective was thus to perform a global analysis of mRNA results using a hierarchical clustering software.

## Materials and methods

### *Study design*

After the approval of our Ethical Committee for clinical research, 42 Caucasian consecutive patients with septic shock admitted in the surgical or medical intensive care units of a university hospital (Lyon, France) were enrolled in the study. All patients or relatives gave informed consents. Septic shock was defined according to the consensus conference of the American College of Chest Physicians/Society of Critical Care Medicine [8,9]. All patients received similar therapies (catecholamines, low dose corticosteroids) as standardized in our ICUs in case of septic shock. Severity was assessed by the Simplified Acute Physiologic Score II (SAPS II) [10]. Each patient was studied along 28 days from the onset of septic shock. Serial blood samples were collected every 2 or 3 days as far as possible until day 13. Eighteen healthy individuals (sex ratio: 1.25 M/F, mean age:  $56 \pm 4$  years), enrolled regularly during the study, served as controls.

### *mRNA extraction and cDNA synthesis*

Blood samples were drawn directly into PAXGene™ Blood RNA tubes (PreAnalytix, Hilden, Germany). Total RNA was extracted using PAXGene™ Blood RNA kit (PreAnalytix). Before RNA elution, residual genomic DNA was digested using RNase-Free DNase set (Qiagen). The integrity and quality of the total RNA were assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) by means of the RNA 6000 Nano Assay (Agilent). Total RNA was reverse transcribed into cDNA

using ThermoScript™ RT-PCR system (Invitrogen) according to manufacturer's instructions.

### *Preparation of standard curves and analysis of mRNA expression by real-time RT-PCR*

The mRNA expression of the selected genes was quantified using qRT-PCR. PCR reactions were performed in a LightCycler™ instrument using the Fast-Start™ DNA Master SYBR Green I real-time PCR kit according to the manufacturer's instructions (Roche Molecular Biochemicals). Thermocycling was performed in a final volume of 20 µl containing 3 mM MgCl<sub>2</sub> and 0.5 µM each of the required primers. PCR was performed with an initial denaturation step of 10 min at 95°C, followed by 40 cycles of a touch-down PCR protocol (10 s at 95°C, 10 s annealing at 68–58°C, and 16 s extension at 72°C).

In order to quantify gene expression, a standard curve was prepared for each selected mRNA. The cDNA calibrator for HMGB1 (accession number: NM\_002128) was prepared from purified PCR amplicons obtained with the following primer combination: forward primer: 5'-GCGGACAAGGCCCGTTA-3', reverse primer: 5'-AGAG-GAAGAAGGCCGAAGGA-3' (amplicon size: 119 pb), as previously described [11]. The cDNA calibrators for the housekeeping gene PPIB (encoding for cyclophilin B) and the other selected mRNAs were investigated by using specific cDNA standards and ready-to-use primer mixes obtained from Search-LC (Heidelberg, Germany). An early [Interleukin (IL) 1β] and a late [high mobility group protein 1 (HMGB1)] proinflammatory cytokine, two anti-inflammatory cytokines [IL10, transforming growth factor beta 1 (TGFβ1)] that may participate in the sepsis-induced host-response, were selected. Moreover, either based on cytokine (IL-4, IL-12B) or transcription factor (T-bet, GATA3) mRNA, we attempted also to investigate the Th1/Th2 balance. The efficiency of using PPIB mRNA levels as reference for target mRNA quantification in human peripheral blood has been previously studied in our institute [12].

The Second Derivative Maximum Method was used with the LightCycler software to determine the crossing point (Cp) for individual samples. Serial dilutions of each cDNA standard were prepared in quadruplicate to generate standard curves as previously described [13]. Relative standard curves describing the PCR efficiency of selected genes and PPIB were created and used to perform efficiency-corrected quantification with the LightCycler Relative Quantification Software (Roche Molecular Biochemicals). All calibration curves showed correlation coefficients >0.99, indicating a precise log-linear relationship. The mean efficiency of the dilution series for all target genes was  $1.89 \pm 0.07$ . Intraassay variability was determined from triplicate measurements of nine patient and four control samples. The mean SDs for Cp did not exceed 0.12 PCR cycles. Interassay variability, calculated from triplicate samples assayed on

three independent series, showed mean SDs for Cp <0.1 PCR cycles.

## Statistical analysis

### Monoparametric analysis

The comparison between patient and healthy donor groups was made with the nonparametric Mann–Whitney *U* test. Due to the lack of serial data for the healthy donors, groups were compared only once using the earliest measurement for each septic patient.

Each marker level and slope over time between survivors and nonsurvivors were compared using linear mixed effects models [14,15]. In these models, individual intercepts and slopes were allowed to vary randomly and to deviate from the group average according to within- and between-individual variances. Moreover, this method adjusted for the within-subject correlation of the repeated observations over time and also allowed for the inclusion of patients with a varying number of measurements. For each marker, the *P* intercept value described the difference between survivors and nonsurvivors at the onset of the syndrome, while the *P* slope value allowed to compare the evolution over time. The statistical analyses were performed with the SPSS software (Illinois, USA).

### Multiparametric analysis

In order to investigate the relationship among mRNA expression patterns, data were imported into the Spotfire Decision Site 7.1 for Functional Genomics software (Spotfire, Gothenburg, Sweden). Both patients and genes were

reordered in a dendrogram based on the similarity between their expression patterns. Pearson's correlation coefficient *r* served as the similarity metric in this clustering approach, and an unweighted average clustering method (UPGMA) clustered the patients according to the calculated similarities in gene expression patterns. To take into account the constitutive difference between gene expression, expression levels were normalized using a standardized normal distribution. Because our goal was to focus on a monitoring approach, the patients that died within the first 48 h after the onset of septic shock (*n* = 6) were excluded from this analysis.

## Results

### Patients

The global mortality rate was 38% knowing that half of the nonsurvivors deceased after day 10. Mean SAPS II admission score and the percentage of community acquired infection were significantly higher in nonsurvivors compared to survivors. None of the other clinical and demographic characteristics described in Table 1 displayed significant differences between the two groups. Fifty percent of the patients had no comorbidity. The most represented comorbidities were chronic obstructive pulmonary diseases (14%) and cancer (11.9%). The number of comorbidities did not show significant differences between survivors and nonsurvivors (*P* = 0.878, chi-squared test).

### Monoparametric analysis of the mRNA expression profile

For patients and healthy donors, the mRNA levels of IL-4 and IL-12B were not interpreted due to the concen-

Table 1  
Demographic, clinical characteristics, microbiology, and type of infection

	Survivors, <i>n</i> = 26 (%)	Nonsurvivors, <i>n</i> = 16 (%)	Total, <i>n</i> = 42 (%)	<i>P</i>
Male	16 (62)	11 (69)	27 (64)	0.887
Female	10 (38)	5 (31)	15 (36)	
Age (years)*	66 (53–76)	63 (57–78)	65 (55–76)	0.525
Length of stay in the ICU	11 (8–16)	8 (3–20)	11 (6–19)	0.337
SAPS II [10] at the admission*	45 (34–51)	63 (47–70)	50 (37–61)	0.011
Microbiologically documented diagnosis	17 (65)	13 (81)	30 (71)	
Bacilli gram (–)	8 (31)	8 (50)	16 (38)	0.687
Cocci gram (+)	6 (23)	5 (31)	11 (26)	
Fungi	5 (19)	6 (38)	11 (26)	
Type of infection				
Community acquired	8 (31)	11 (69)	19 (45)	0.026
Nosocomial [16]	18 (69)	5 (31)	23 (55)	
Site of infection				
Pulmonary	12 (46)	11 (69)	23 (55)	0.773
Abdominal	10 (39)	4 (25)	14 (33)	
Others	4 (15)	1 (6)	5 (12)	

Results are presented both for the whole cohort of septic shock patients (*n* = 42) and for survivors and nonsurvivors at 28 days after the onset of shock. The severity was assessed by the Simplified Acute Physiologic Score II (SAPS II) [10]. Comparison between survivors and nonsurvivors was made either with the nonparametric Mann–Whitney *U* test for the continuous variables or with the chi-squared or Fisher exact test for categorical variables.

\* Median (Q1–Q3).

trations being below or just at the qRT-PCR detection threshold.

At the onset of the follow-up, the gene expression in the global population of septic patients was significantly increased for IL-10, IL-1 $\beta$ , and HMGB1, compared to healthy donors, and significantly decreased for TGF $\beta$ 1, T-bet, and GATA3 mRNA ( $P < 0.001$ ) (Table 2).

At the onset of shock, nonsurvivors showed higher IL-10 and HMGB1 mRNA levels than survivors ( $P$  intercept  $< 0.005$ ) (Fig. 1). In contrast, survivors and nonsurvivors showed comparable expression of TGF $\beta$ 1, IL-1 $\beta$ , T-bet, and GATA3.

Regarding variations over time, the mRNA level of IL-10 and IL-1 $\beta$  decreased progressively in the course of the septic shock. As compared to the healthy donors, IL-1 $\beta$  mRNA levels remained higher in patients during the entire study period, but still with no differences between survivors and nonsurvivors. In contrast, the IL-10 to IL-1 $\beta$  ratio remained increased in nonsurvivors as compared to survivors (data not shown). Both TGF $\beta$ 1 and HMGB1 mRNA levels remained stable throughout the 13 days observation period and thus elevated and diminished, respectively, as compared to the healthy donors. The mRNA levels of both T-bet and GATA3 tended to increase over time in survivors (elevation was significant for GATA 3 only,  $P$  slope = 0.01) while expression seemed to decrease in the nonsurvivors.

#### Multiparametric analysis of the mRNA expression profile

The comparison of the average gene expression of 11 nonsurvivors and 25 survivors served as basis for our hierarchical clustering analysis. Based on the expression data of the six selected genes, all combinations from two to six genes have been tested with the hierarchical clustering analysis, and combinations resulting in the most relevant clusterization of the patients outcome were selected. Only the genes that could improve the reliability of the clusterization were selected.

Our first analysis was based on the earliest profile of each patient, which was studied at days 1–3. We observed that the reliability of the clusterization had the tendency to increase with the number of genes used for the analysis (data not shown). The most relevant clusterization of the survivors

and nonsurvivors was obtained with a combination of four genes: IL-10, IL-1 $\beta$ , TGF $\beta$ 1, and HMGB1. Two distinct subclusters were achieved with these genes (Fig. 2A). Nine nonsurvivors out of 11 (82%) were found in the first subcluster, and 20 survivors out of 25 (80%) were found in the second subcluster.

To compare the two groups in further details over time, a second analysis was performed using the earliest profile (as previously studied at days 1–3) and a second one obtained at days 4–7. Because the death of the patients NS4 and NS11 occurred at day 3 and because the patient S3 left the ICU, these patients could not be included in this analysis. The most relevant clusterization was obtained with a combination of four genes: IL-1 $\beta$  and HMGB1 both measured at days 1–3, GATA3 measured at days 4–7, and TGF $\beta$ 1 measured both at days 1–3 and 4–7. Two subclusters were obtained (Fig. 2B). Eight nonsurvivors out of nine (89%) were found in the first subcluster, and 23 survivors out of 24 (96%) were found in the second subcluster. By performing this additional cluster analysis with measurements obtained later in the patient's illness, we were thus able to properly group the survivor patients 5, 9, 21, and 25, which were misclustered in the prior analysis using measurements only from days 1 to 3.

#### Discussion

The complexity in the host response to an infection is likely one of the main reasons behind the failures with immune therapies in severe sepsis. Success in treating septic patients will rely on mediator-directed therapy and thus will require innovative biological tools to stage and monitor the patient immunoinflammatory status. Here, we report time-related mRNA expression of an immunological mediator panel in whole blood from 42 patients with septic shock using qRT-PCR.

Despite several sources of data variation, the fluorescence-based qRT-PCR remains the most sensitive and specific method for quantitating mRNA levels [17,18]. PAXgene blood RNA system provides an efficient and standardized method for blood collection. This product reduces RNA degradation and prevents against any post-sampling stimulation, which is essential for the investigation

Table 2

Cytokine, Th1, and Th2 marker mRNA expression in whole blood of septic shock patients (survivors  $n = 26$ , nonsurvivors  $n = 16$ ) and healthy volunteers ( $n = 18$ )

		IL-10	TGF $\beta$ 1	IL-1 $\beta$	HMGB1	GATA3	T-bet
Septic shock	Mean	2.6E–02	2.8E–01	1.01	1.48	8.0E–03	5.1E–03
	SEM	5.0E–03	2.0E–02	2.1E–01	8.0E–02	1.0E–03	1.0E–03
Healthy donors	Mean	7.0E–4	1.53	1.9E–01	7.9E–01	6.8E–02	6.5E–02
	SEM	1.2E–04	9.0E–02	1.2E–02	3.5E–02	4.0E–03	6.6E–03
Mann–Whitney $U$ test	$P$ value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Gene expressions were quantified in peripheral blood using real-time PCR. Results are expressed as relative quantification ratio between the selected gene and the housekeeping gene PPIB. The comparison between patient and healthy donor groups was made with the nonparametric Mann–Whitney  $U$  test.

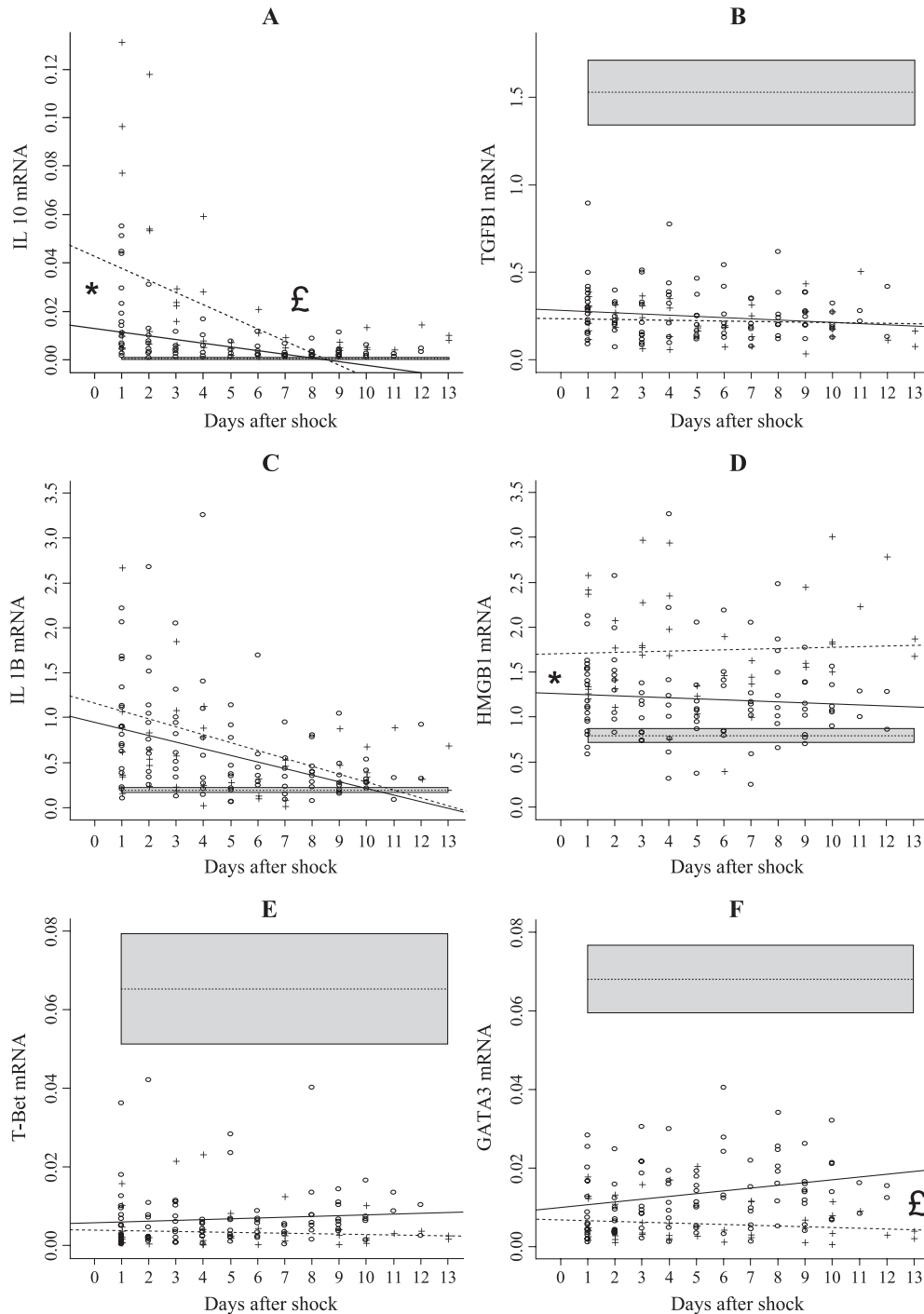


Fig. 1. mRNA expression in the course of septic shock. Gene expressions were quantified in peripheral blood of 26 survivor (circle points) and 16 nonsurvivor (cross points) patients using real-time PCR. Results are expressed as relative quantification ratio between the selected gene and the housekeeping gene PPIB. The evolution of IL-10 (A), TGFβ1 (B), IL-1β (C), HMGB1 (D), T-bet (E), and GATA3 (F) mRNA was modeled for survivor (full line) and nonsurvivor (dotted line) patients using the linear mixed effects models. \**P* intercept value < 0.05 (describing the difference between survivors and nonsurvivors at the onset of the syndrome); £*P* slope value < 0.05 (describing the overtime difference between survivors and nonsurvivors). Normal expression levels were measured once in 18 healthy volunteers. Results are presented by the grey rectangle as mean ± 95% confident interval.

of highly reactive genes such as cytokines [19]. Indeed, both previously published studies and personal data obtained in our institute showed that blood collection in EDTA tubes and cell purification is accompanied by profound changes in mRNA expression [20,21]. However, there are two limi-

tations of this method. First, it is important to point out that accurate quantification of mRNA level does not inform about mRNA stability or protein production and activity. Moreover, the use of RNA isolated from whole blood might make some rare mRNA undetectable. These underlying



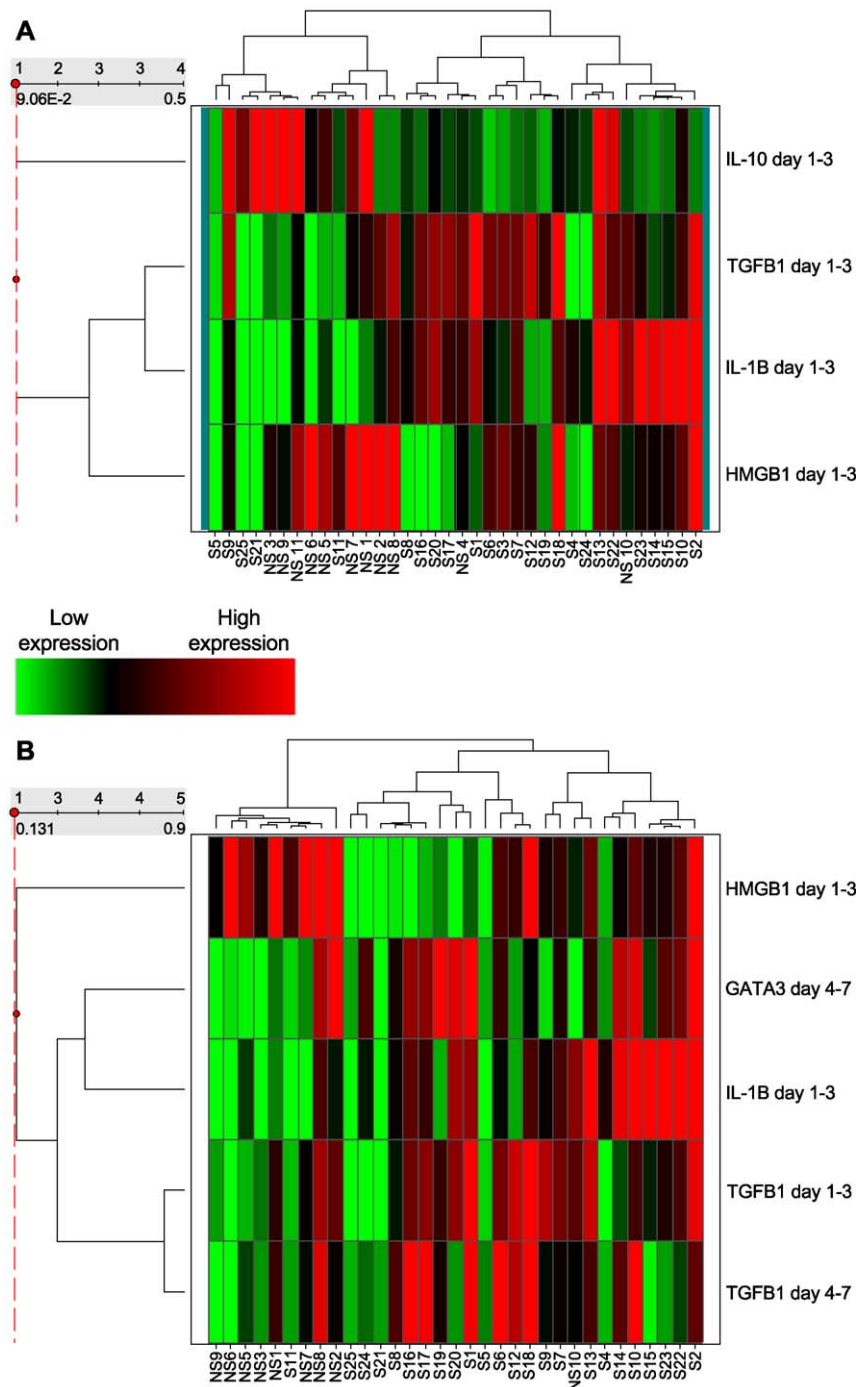


Fig. 2. Hierarchical clustering of 36 patients with septic shock. The expression of four genes measured during the first 3 (A) or 7 (B) days after the onset of septic shock was used. Survivor (S) and nonsurvivor (NS) patients are arranged along columns and genes along rows in a way that patients or genes with similar expression patterns are placed adjacent to each other. The results were obtained by quantitative real-time PCR and expressed as relative quantification ratio between the selected gene and the housekeeping gene PPIB. As shown in the color bar, green indicates low gene expression, black intermediate, and red high expression. The length of the branches of the dendrogram represents similarity distances of expression profiles, and the dotted line divides the column dendrogram into two groups of patients.

technical limitations might explain, at least in part, our inability to quantify the IL-4 and IL-12B mRNAs. Nevertheless, the major part of our results agreed with previous studies investigating serum protein levels in septic patients. In this respect, we found increased mRNA expression of IL-

1 $\beta$ , IL-10, and HMGB1 at the time of diagnosis, whereas the mRNA level of TGF $\beta$ 1 was decreased [21–24]. In accordance, the feasibility of measuring whole blood mRNA levels has been recently illustrated for the monitoring of Human Leukocyte Antigen DR (HLA-DR) in septic patients [25].

Regarding pathophysiological insights, our results illustrate the importance of immunosuppression. Among the various anti-inflammatory cytokines identified in critical ill patients, both IL-10 and TGF $\beta$ 1 may participate in the counterregulation of the inflammatory cascade [6]. According to previous studies investigating proteins [26], we found that IL-10 is likely to be a part of the anti-inflammatory response occurring rapidly in septic shock. Compared to healthy volunteers, IL-10 mRNA expression was continuously increased in patients. In contrast, TGF $\beta$ 1 mRNA levels were down-regulated in septic shock without overlapping normal values and did not give any input in terms of prognosis. It should be noted that the increased production of TGF $\beta$ 1 protein in the course of septic syndromes is less clear than for IL-10 [27–29]. More notably, Sfeir et al. [24] found decreased TGF $\beta$ 1 protein levels in septic shock patients, although with no significant differences from normal controls. Recently, Fumeaux and Pugin [30] have emphasized the importance of IL-10 in the process of monocytic HLA-DR down-regulation, which is described as a reliable marker of immunoparalysis that seems to correlate with an increased risk of fatal outcome [31]. These studies, together with our results, support the idea that IL-10, as compared to TGF $\beta$ 1, is a major actor in sepsis-induced immunosuppression. Moreover, the increased IL-10 mRNA expression and IL-10 to IL-1 $\beta$  ratio observed in nonsurvivors suggested that an early and sustained anti-inflammatory profile is associated with fatal outcome.

Recently, a proinflammatory role of HMGB1 has been described [23,32]. Moreover, studies have demonstrated that extracellular HMGB1 is also a major late-acting mediator of endotoxin lethality, positioning HMGB1 as a therapeutic target for sepsis treatment more accessible than the early pro-inflammatory cytokines TNF $\alpha$  and IL-1 $\beta$  [23,33]. Both at the onset of shock and during the entire follow-up period, we found increased HMGB1 mRNA levels in nonsurvivors as compared to survivors, illustrating HMGB1 as a potential marker of poor outcome. To date, the lack of a reliable assay for measuring serum HMGB1 impedes from investigating its prognostic potential in a large population of septic patients [34]. Nevertheless, and in accordance with our results, Wang et al. [23] detected increased HMGB1 serum levels in septic patients using immunoblot analysis, with a significantly greater increase in patients who succumbed. Since IL-1 $\beta$  has been described as an inducer of HMGB1 release [23], it was surprising to observe such a different pattern of gene expression for these two cytokines. The persistent increased expression of HMGB1 might suggest a posttranscriptional mRNA stabilization. However, taking into account the redundancy of immune mediators, some of them may participate to the sustained HMGB1 expression even after the early peak of the proinflammatory cytokines IL-1 $\beta$  and TNF $\alpha$ .

The assessment of the Th1/Th2 balance using IL-12B and IL-4 mRNA was not conclusive. mRNA levels of both

cytokines were below the qRT-PCR detection threshold. Moreover, the quantification of interferon gamma (IFN $\gamma$ ) mRNA in a subset of 28 patients showed only a weak expression with no difference between survivors and nonsurvivors (data not shown). Thus, we attempted to study the Th1/Th2 balance through the expression levels of T-bet and GATA3, which have been described as subset-specific transcription factors of Th1 and Th2 cells, respectively [35,36]. In this context, qRT-PCR constitutes a real improvement since these mediators are hardly measurable at the protein levels. Compared to healthy volunteers, we found a marked down-regulation of T-bet and GATA3 in septic shock without overlap. Most importantly, we showed that an increasing expression of the Th2 transcription factor GATA3 over time could be a potential marker of recovery. Although further cellular or protein studies are required to confirm this Th1/Th2 pattern, our results are in line with previous studies in trauma or septic patients [37–39]. Measuring in-vitro cytokine production either at the protein or mRNA level, they observed a global anergy of both Th1 and Th2 lymphocytes. In accordance, we reported that Th2 response is decreased after septic shock by monitoring the cell surface expression of prostaglandin D2 receptor CRTH2 and the eotaxin receptor CCR3 [40].

From monoparametric analysis, it was interesting to note that at the onset of shock both elevated mRNA levels of IL-10 and HMGB1 were associated with poor outcome, whereas over time, those of GATA3 and HMGB1 allowed for distinguishing survivors from nonsurvivors. To get further insight in the interpretation of our results, we used a hierarchical clustering method to observe similarities in gene expression patterns among patients. Combining the expression levels of four genes in the first 7 days after the onset of shock, up to 95% of the patients with a same outcome showed similarities in mRNA profiling. As expected, early measurement of IL-10 and HMGB1 and late measurement of GATA3 were selected in the profile in accordance with our monoparametric analysis. Surprisingly, the most relevant combination also included IL-1 $\beta$  and TGF $\beta$ 1, which were not differentially expressed in both groups when interpreted independently. The clusterization results were clearly worse when performed without the expression levels of these two genes. None of the six selected genes, like any other documented marker of septic shock, is able by its own to flawlessly distinguish survivors and nonsurvivors. Indeed, the individual measurement showed systematically an overlap. Our results suggest that important information is hidden by this overlap and illustrate the potential of a global data interpretation compared with the usual monoparametric analysis. Moreover, combining early and late mRNA measurements, the distinction between survivors and nonsurvivors could be further improved. This highlights the importance of carrying out longitudinal monitoring instead of a single measurement.

## Conclusions

In conclusion, regarding the technical aspect, we showed in this first preliminary study that real-time RT-PCR may be a reliable tool for the monitoring of mRNA expression levels in peripheral blood. This method may contribute to describe immune function and stage patients with septic syndromes. It requires now to be further investigated in larger clinical studies. Our results confirmed, at the transcriptional level, the poor prognostic value of a persisting anti-inflammatory profile and the contribution of IL-10 in the process of sepsis-induced immunoparalysis. Together with the decreased mRNA expression of the Th1 and Th2 transcription factors T-bet and GATA3, these results confirm that the reversal or prevention of sepsis-induced immune deficiency must become a major focus of research. Moreover, the constant overexpression of HMGB1 mRNA in nonsurvivors and the increasing level of GATA3 mRNA in patients who recovered illustrated new potential candidate markers for the monitoring of the sepsis-induced host response. Lastly, our findings illustrate the great potential of a multiparametric strategy to classify septic patients rather than monitoring a single marker.

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