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## Modulation of LILRB2 protein and mRNA expressions in septic shock patients and after *ex vivo* lipopolysaccharide stimulation



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### ABSTRACT

Septic patients develop immune dysfunctions, the intensities and durations of which are associated with deleterious outcomes. LILRB2 (leukocyte immunoglobulin-like receptors subfamily B, member 2), an inhibitory member of the LILR family of receptors, is known for its immunoregulatory properties.

In a microarray study, we identified LILRB2 as an upregulated gene in septic shock patients. On monocytes primed with LPS *ex vivo*, LILRB2 mRNA and protein expressions were dose-dependently downregulated and subsequently highly upregulated versus non-stimulated cells. This is concordant with clinical data, since both LILRB2 mRNA and protein expressions were significantly increased in septic shock patients at day 3. In a cohort of more than 700 patients, only after septic shock were LILRB2 mRNA levels increased compared with non-infected or less severely infected patients. This was preceded by a phase of downregulated mRNA expression during the first hours after septic shock. Interestingly, the intensity of this decrease was associated with increased risk of death after septic shock.

LILRB2 protein and mRNA expressions are deregulated on monocytes after septic shock and this can be reproduced *ex vivo* after LPS challenge. Considering LILRB2 inhibitory properties, we can hypothesize that LILRB2 may participate in the altered immune response after septic shock.

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### 1. Introduction

Sepsis is a major public health concern, accounting for more than \$20 billion of total US hospital costs in 2011 [1]. The reported incidence of sepsis is increasing [2,3], most likely attributable to aging populations presenting multiple comorbidities and to a greater recognition of the syndrome. Although its true incidence is unknown, conservative estimates indicate that sepsis is a leading cause of mortality and critical illness worldwide [4,5].

The immune response to sepsis is believed to consist of an uncontrolled pro-inflammatory phase in the initial stages of infection and by an anti-inflammatory immunosuppressive response acting as a negative feedback [6]. During the pro-inflammatory

phase, immune cells, including monocytes and neutrophils, release high levels of cytokines, resulting in a severe hyper-inflammatory response and multiple organ failure. If the septic patient survives this initial stage, an immunosuppressive phase enters into play, during which the patient undergoes an apoptosis-induced depletion of immune cells as well as severe functional cell alterations. These immunological alterations compromise the patient's ability to combat invading pathogens and result in primary and secondary opportunistic infections that lead to late death. However, the pathophysiological mechanisms at play in this deregulated immune response remain unknown.

Leukocyte immunoglobulin-like receptors (LILRs), also known as Ig-Like Transcripts (ILTs), are a family of immunoreceptors known to regulate the immune system so as to temper or augment responses [7,8]. LILRs are divided into two sub-families, one designated as LILRA (activators) with 6 members, and LILRB (inhibitors)

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with 5 members, each differing in its expression profile and function [8].

Among these, LILRB2 (i.e. subfamily B, member 2), an inhibitory member of the LILR family of receptors, is known for its immunoregulatory properties in regard to monocyte and dendritic cell functions (pro-inflammatory cytokine production and antigen presentation) and for its capacity to induce tolerogenic dendritic cells (DCs) [9].

In view of the numerous parallels between LILRB2 inhibitory properties and sepsis-induced immune alterations, we launched a research project aimed at evaluating the regulation of LILRB2 protein and mRNA expressions after septic shock and in an *ex vivo* model of lipopolysaccharide (LPS) challenge.

## 2. Patients and methods

### 2.1. Patients and healthy volunteers

#### 2.1.1. IMMUNOSEPSIS cohort

This clinical study was conducted on septic shock patients admitted to the Intensive Care Unit (ICU) of the Centre Hospitalier Universitaire de Lyon Sud and the Edouard Herriot Hospital (Hospices Civils de Lyon, Lyon, France).

Septic shock patients were identified according to the diagnostic criteria of the American College of Chest Physicians/Society of Critical Care Medicine [10]. Exclusion criteria disqualified patients under 18 years of age and subjects with aplasia or immunosuppressive disease (e.g. HIV infection). The onset of septic shock was defined as the beginning of vasopressor therapy in combination with an identifiable site of infection, persisting hypotension—despite fluid resuscitation—and evidence of a systemic inflammatory response manifested by at least two of the following criteria: a) temperature >38 °C or <36 °C; b) heart rate >90 beats/min; c) respiratory rate >20 breaths/min; d) white blood cell count >12,000/mm<sup>3</sup> or <4000/mm<sup>3</sup>.

Biological analyses were performed on residual blood after completing routine follow-up in the ICU except for the PAXgene tube that was sampled during the same blood drawing procedure as routine follow-up. EDTA-anti-coagulated tubes or PAXgene tubes were collected at 3 time points after septic shock onset: day 1–2 (D1), day 3–4 (D3) and day 7–10 (D6). This project is part of a global study on ICU-induced immune dysfunctions. It has been approved by our Institutional Review Board for ethics (“Comité de Protection des Personnes Sud-Est II”), which waived the need for informed consent, because this study did not require specific/additional blood drawing procedure beside routine blood sampling and was classified as “observational with minimal risk for the patients” (#IRB 11236). This study is also registered at the French Ministry of Research and Teaching (#DC-2008-509) and recorded at the Commission Nationale de l’Informatique et des Libertés. Non-opposition to inclusion in the study was recorded for each patient.

#### 2.1.2. MIP-REA cohort

This prospective, multicentric, non-interventional study was conducted in 6 ICUs in Lyon. It has been approved by our ethical Institutional Review Board (Comité d’Ethique des Centres d’investigation Clinique de l’Inter-Région Rhône-Alpes Auvergne – IRB# 5044).

Inclusion and exclusion criteria as well as clinical description of the cohort have been published previously [11].

#### 2.1.3. Healthy volunteers

For *ex vivo* experiments, pouches containing 450 ml of blood (for cell purification experiments requiring large numbers of iso-

lated cells) or 5 ml-EDTA anticoagulated tubes (for every other experiment) collected from healthy donors were obtained from the blood bank of Lyon (EFS de Lyon). According to EFS standardized procedures for blood donation, informed consent was obtained from healthy volunteers and personal data for blood donors were anonymized at the time of blood donation and before the transfer of blood to our research lab.

For transcriptomic studies, peripheral whole blood from 22 healthy volunteers was collected directly in PAXgene Blood RNA tubes (PreAnalytix, Hilden, Germany). Samples were stabilized after collection for at least 4 h at room temperature and stored at –80 °C following manufacturer’s instructions.

### 2.2. Sample processing, RNA extraction and purity

For PAXgene tubes, total RNA was systematically extracted using the PAXgene blood RNA kit (PreAnalytix, Hilden, Germany). For *ex vivo* experiments on purified PBMCs, total RNA was extracted using the Rneasy Mini Kit (Qiagen, Hilden, Germany). The residual genomic DNA was digested using the RNase-Free DNase set (Qiagen Valencia, CA, USA). The integrity of the total RNA was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and samples with RNA integrity number <6 were excluded due to poor quality RNA.

### 2.3. Microarray hybridization

The 73 microarray experiments (51 septic shock patients and 22 healthy donors) were performed as previously described [12]. Gene expressions were generated using GeneChip® Human Genome U133 Plus 2.0 arrays (Affymetrix, Sta. Clara, CA, USA) according to manufacturer’s protocol. Affymetrix GeneChip Operating Software version 1.4 (GCOS) was used to manage GeneChip array data and to automate the control of GeneChip fluidics stations (FS450) and scanner (GeneChip Scanner 3000). These experimental data have been deposited in the National Centre for Biotechnology Information (NCBI) and are available in the GEO DataSets site under access number GSE95233.

### 2.4. Reverse transcription and quantitative PCR

Total RNA was reverse transcribed in complementary cDNA (200 ng in a final volume of 20 µl) using SuperScript® VILO™ cDNA Synthesis Kit as recommended by the manufacturer (Life Technologies, Chicago, IL) for RNA extraction from PAXgene tubes, or using the SuperScript III Strand Synthesis Supermix kit (Life Technologies) for RNA extraction from peripheral blood mononuclear cells (PBMCs).

LILRB2 mRNA expression level was quantified using q-real time polymerase chain reaction (qPCR). qPCR was performed on a Light-Cycler instrument using the standard Taqman Fast Advanced Master Mix PCR kit according to the manufacturer’s instructions (Roche Molecular Biochemicals, Basel, Switzerland). Thermocycling was performed in a final volume of 20 µl containing 0.5 µM of primers and 0.1 µM of probe. qPCR was performed with an initial denaturation step of 10 min at 95 °C, followed by 45 cycles of a touch-down PCR protocol (10 s at 95 °C, 29 s annealing at 68 °C, and 1 s extension at 72 °C). Sequences are for forward primer: CCAGAGCC-CACAGACAGAGG, Reverse primer: TGTCTTCACGGCAGCATAGA and Probe: TGCACTGGAGTCCAGCCAG. The Second Derivative Maximum Method was used with the LightCycler software (Release 1.5.0 SP4) to automatically determine the crossing point for individual samples. Standard curves were generated by using five replicates of cDNA standards and were used to perform efficiency corrected quantification. Relative standard curves

describing the PCR efficiency of selected genes were created and used to perform efficiency-corrected quantification with the Light-Cycler Relative Quantification Software (Roche Molecular Biochemicals).

In case of mRNA extracted from PAXgene tubes, gene expression normalization was performed based on a selected housekeeping gene (HPRT1: hypoxanthine phosphoribosyltransferase 1) and results were expressed as Calibrated Normalized Relative Quantity (CNRQ) [13] to normalize for inter-run variations. Among the different reference genes that were tested in the MIP-REA cohort, HPRT1 was the only one that was not differentially expressed between non-survivors and survivors patients [11]. This reference gene has been previously used by our team to normalize gene expression extracted from PAXGENE tubes sampled from ICU patients [14].

For the *ex vivo* experiments on mRNA extracted from purified PBMCs, qRT-PCR results were expressed as absolute mRNA concentrations. This is because mRNA extractions and qRT-PCR from the same experiment/donor were systematically performed within the same run and identical quantities of extracted mRNA from the same number of cells were systematically engaged in the qRT-PCR. In addition, we verified for each run that cDNA standards were amplified at similar crossing times and that PCR efficiencies were identical.

### 2.5. Cell purification and cell culture experiments

Peripheral Blood Mononuclear cells (PBMCs) were isolated from peripheral blood of healthy donors through Ficoll gradient centrifugation. Residual red blood cells were lysed with an EDTA/NH<sub>4</sub>-Cl/KHCO<sub>3</sub> buffer. After purification, the cells were dispensed at a concentration of  $2 \times 10^6$  cells per ml and stimulated with lipopolysaccharide (LPS, concentrations: 2, 10, and 100 ng/ml). LPS pooled from three different strains of *E. coli* (O111:B4, O55/B5, O127:B8) was purchased (Sigma-Aldrich). Following incubation times ranging from 3 to 24 h, cells were placed in a RLT (guanidine thiocyanate) buffer with beta mercapto-ethanol in order to stabilize mRNA expression, and stored at  $-20^\circ\text{C}$  until RNA extraction. Alternatively, cells were stained with specific fluorochrome-labeled antibodies and analyzed by flow cytometry.

### 2.6. Flow cytometry experiments

The following antibodies were used: Pacific Blue (PB)-labeled anti-CD16 antibody (clone 3G8); allophycocyanin (APC)-labeled anti-CD14 antibody (clone RMO52); phycoerythrin (PE)-labeled anti-LILRB2 antibody (clone 42D1) from Beckman Coulter (Miami, FL). An isotype control for LILRB2 staining (PE labeled rat IgG2a from BD Biosciences) was used. In the case of whole blood staining, red blood cell lysis was performed using Versalys lysing solution (Beckman Coulter, Miami, FL). LILRB2 expression on the surface of PBMCs was measured using the Navios flow cytometer (Beckman Coulter, Miami, FL). Results were analyzed with Kaluza software (Beckman-coulter) expressed as Medians of Fluorescence Intensities (MFI).

In addition, to accurately measure the intracellular level of LILRB2 mRNA in monocytes by flow cytometry, the QuantiGene<sup>®</sup>Flowna Assay was used in accordance with manufacturer's recommendations (eBioscience, San Diego, CA). For this assay, AF647-labeled probe mix targeting LILRB2 mRNA combined with FITC-labeled anti-CD14 and PB-labeled anti-CD45 antibodies were used. As a control for monocyte activation by LPS, similar stainings were performed using AF488-labeled probe mix targeting IL1b mRNA and PB-labeled anti-CD45 antibodies.

### 2.7. Statistical analyses

Results are presented as individual values and box plots in Figures. Clinical data are presented as number of cases and related percentages for categorical variables and medians, and Q1–Q3 interquartile ranges for continuous variables in the Tables. Non-parametric Mann Whitney U tests and Fisher tests were used to compare data between survivors and non-survivors or between septic shock patients and controls. Correlation between parameters was studied with the Spearman correlation test. Statistical analyses and graphics were developed with the use of R-Studio and GraphPad Prism 5 software.

## 3. Results

### 3.1. Microarray study

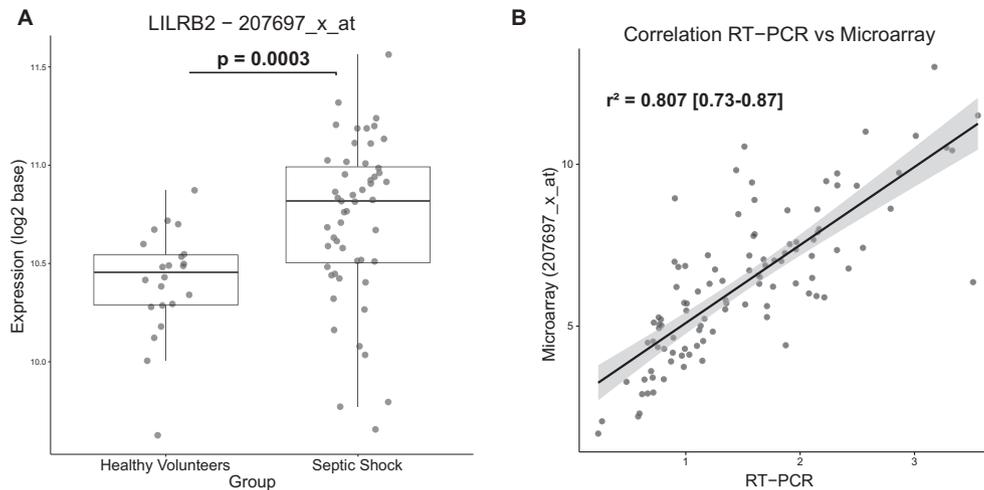
Whole genome expression evaluation was performed on whole blood mRNA extracted from 51 septic shock patients sampled at D3 after the onset of shock (Immunosepsis cohort) and 22 healthy donors. Patients' clinical and demographic characteristics are presented in Table 1. Among the list of differentially expressed genes, LILRB2 gene expression (both probesets available on the microarray, results from one probeset in Fig. 1A) was significantly higher in patients than in healthy donors ( $p = 0.0003$ , Mann Whitney test).

**Table 1**

Clinical and demographic data for the septic shock patients included in microarray study and in the flow cytometry evaluation of LILRB2 expression.

	Microarray study n = 51	Flow cytometric study n = 13
Male	33 (65)	10 (77)
Age (years)	65 [53–74]	74 [62–80]
SAPS II score	51 [43–62]	59 [46–66]
SOFA score	10 [8–12]	10 [9–12]
Comorbidities (per Charlson score)		
0	32 (63)	3 (23)
1	15 (29)	4 (31)
≥2	4 (8)	6 (46)
Type of admission		
Medical treatment	29 (57)	10 (77)
Elective Surgery	3 (6)	0 (0)
Emergency Surgery	19 (37)	3 (23)
Type of infection		
Community-acquired	25 (49)	7 (54)
Nosocomial	26 (51)	6 (46)
Site of infection		
Pulmonary	23 (45)	6 (46)
Abdominal	19 (37)	3 (23)
Urinary	2 (4)	2 (15)
Others	7 (14)	2 (15)
Documentation of infection		
Clinics only	1 (2)	1 (8)
Clinics + Imaging	5 (10)	2 (15)
Clinics + Surgery	4 (8)	0 (0)
Microbiologically documented	41 (80)	10 (77)
Gram-negative	27 (53)	5 (50)
Gram-positive	19 (37)	6 (60)
Fungi	12 (24)	0 (0)
Others	4 (8)	1 (10)

Fifty-one septic shock patients and 22 healthy donors (50% males, age: 57[52–60]) were included in a microarray experiment. Thirteen septic shock patients and 10 healthy donors (40% males, age: 32[25–46]) were included in a flow cytometric evaluation of LILRB2 expression on monocytes and neutrophils. Values are presented as numbers and percentages for categorical variables and as medians and [Q1–Q3] interquartile ranges for continuous variables. SAPSII: simplified acute physiology score II. SOFA: Sepsis-related Organ Failure Assessment.



**Fig. 1.** Microarray study. A. Expression level for LILRB2 probe set 207697\_x-at measured from a pangenomic analysis of whole blood samples collected from healthy donors ( $n = 22$ ) and septic patients ( $n = 51$  sampled at D3 after the onset of shock). Results are presented as individual values and box plots. Statistical analysis was performed using non parametric Mann Whitney  $U$  test. B. Correlation between LILRB2 mRNA expressions measured by microarray experiment (y-axis) and qRT-PCR (x-axis) on samples from healthy donors and septic shock patients ( $n = 73$  total). Statistical analyses were performed using the Spearman correlation test. C. LILRB2 expression.

These results obtained in septic patients were then validated by qRT-PCR. The platform transfer experiment performed on samples from septic shock patients showed a very good correlation between LILRB2 mRNA expression measured on microarrays or by qRT-PCR on the same samples ( $r^2 = 0.807$  [0.73–0.87],  $p < 0.0001$ , Spearman correlation test, Fig. 1B). This technical validation confirms the robustness of our qRT-PCR design to reproduce results observed on the microarray and the specificity of the microarray results.

Considering the immunoregulatory role of LILRB2 and the pathophysiology of septic shock, we decided to further study the regulation of this molecule's expression in patients and healthy donors.

### 3.2. LILRB2 protein expression on circulating leukocytes from healthy donors

First, we sought to identify leukocyte subpopulations that express LILRB2 by monitoring this protein expression by flow cytometry in 10 healthy donors (40% males, 32 [25–46] years old).

LILRB2 expression on lymphocytes was minimal when compared with non-specific staining evaluated by isotype control (data not shown). Conversely, circulating monocytes strongly expressed LILRB2 protein on their surface (MFI medians [Q1–Q3] = 0.15 [0.14–0.16] for isotype control versus 1.9 [1.8–2.0] for LILRB2 staining). We observed a lesser degree of expression of LILRB2 on neutrophils (MFI = 1.3 [1.2–1.4], Fig. 2A, B).

LILRB2 protein expression on monocytes was bimodal with a markedly higher LILRB2 expression on CD16<sup>high</sup> monocytes (MFI = 10.5 [9–13]) compared with CD16<sup>low</sup> monocytes (MFI = 1.9 [1.8–2.0], Fig. 2C) suggesting that the level of LILRB2 protein expression is greater on pro-inflammatory non-classical monocytes than on classical monocytes. To note, this difference of LILRB2 expression between CD16<sup>high</sup> and CD16<sup>low</sup> monocytes was conserved on cells after their isolation through Ficoll gradient centrifugation (Data not shown).

### 3.3. LILRB2 protein expression on circulating neutrophils and monocytes from septic shock patients

Taking the above results, we compared the intensity of LILRB2 protein expression on circulating monocytes and neutrophils

between septic shock patients and healthy donors. This analysis was carried out on blood collected from 13 septic patients at D1, D3 and D6 after onset of sepsis (immunosepsis cohort – Table 1).

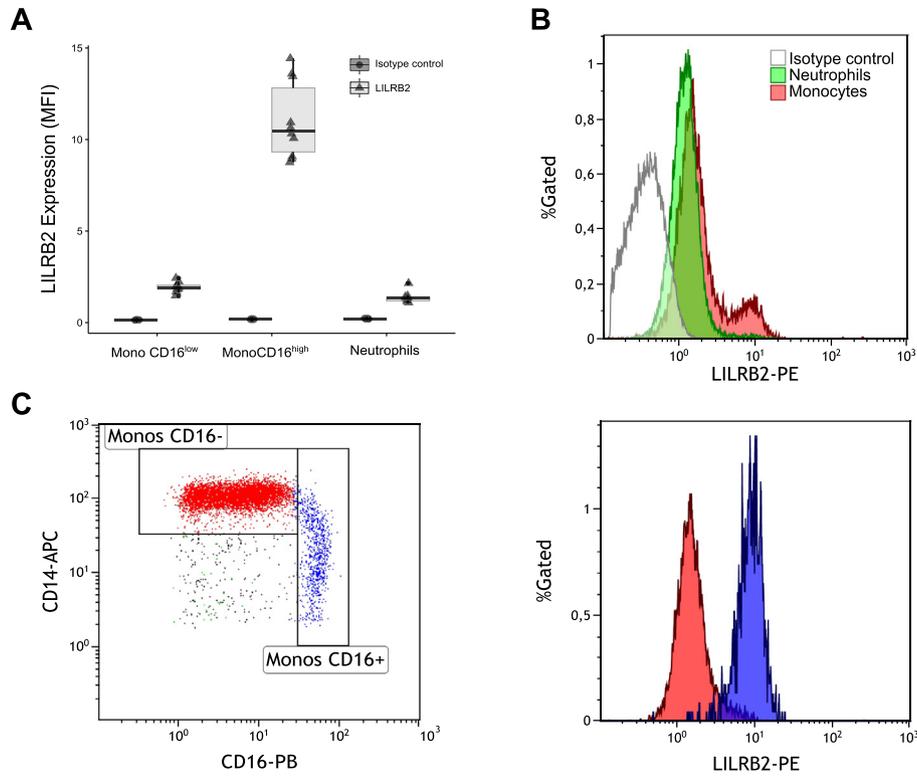
LILRB2 protein expression was increased on neutrophils and monocytes from septic shock patients (LILRB2 MFI on neutrophils = 7.41 [4.8–15.11] in patients at D1) compared with those from healthy donors (Fig. 3). This difference was readily observed at D1 after onset of septic shock and continued at D3 and D6 ( $p < 0.001$  between septic shock patients and controls for all cell populations at every time-point, Mann Whitney test). Interestingly, LILRB2 overexpression on non-classical monocytes (CD16<sup>high</sup>) versus classical monocytes (CD16<sup>low</sup>) was conserved after septic shock (LILRB2 MFI on CD16<sup>high</sup> monocytes = 23.7 [22.4–25.3] versus 4.1 [3.2–5.6] on CD16<sup>low</sup> monocytes in patients at D1, Fig. 3).

### 3.4. LILRB2 expression regulation after LPS stimulation ex vivo

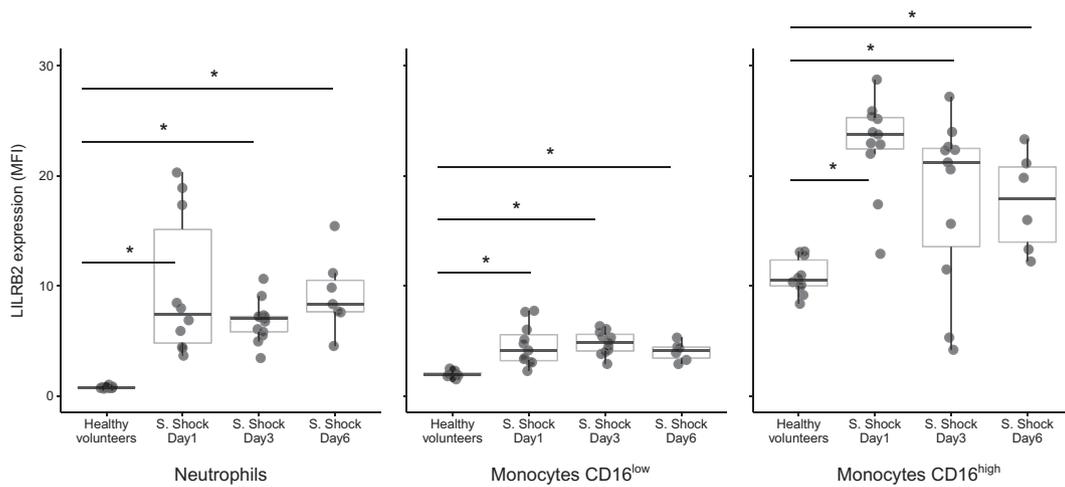
In order to complete previous data obtained in septic shock patients, we studied the regulation of LILRB2 protein and mRNA expressions on mononuclear cells purified from healthy donors after *ex vivo* LPS stimulation. Mononuclear cells purified from healthy volunteers ( $n = 18$ , age range: 37 [35–48] years; 61% male) were stimulated with LPS at concentrations ranging from 2 to 100 ng/ml and for a duration of 3–48 h.

We first observed a significantly increased LILRB2 protein expression on monocytes 48 h after LPS stimulation when compared with non-stimulated cells ( $n = 8$  donors,  $p < 0.05$  between non-stimulated and stimulated cells at 48 h, Wilcoxon paired test, Fig. 4A). This increase was observed after stimulation with LPS at doses of 10 or 100 ng/ml ( $p < 0.05$  between non-stimulated and stimulated cells at 10 or 100 ng/ml LPS, Wilcoxon paired test, Fig. 4B). Interestingly, a slightly decreased LILRB2 protein expression was noted at earlier LPS incubation times (3 h, 6 h, and 24 h, Fig. 4A).

LILRB2 mRNA expression significantly increased in mononuclear cells 24 h after LPS stimulation when compared with non-stimulated cells ( $n = 7$  donors,  $p < 0.05$  between non-stimulated and stimulated cells at 24 h, Wilcoxon paired test, Fig. 4C). Again, a LPS dose effect was observed ( $p < 0.05$  between non-stimulated and stimulated cells at 2, 10 or 100 ng/ml LPS, Wilcoxon paired test, Fig. 4D). Finally, as observed for the protein, a slight and



**Fig. 2.** LILRB2 protein expression on cells from healthy donors. A. Cell surface LILRB2 protein expression was monitored on cells from 10 healthy donors by flow cytometry. Monocytes were first selected based on CD14 expression and then divided according to CD16 expression. Neutrophils were selected based on high CD16 expression. Results are graphically depicted as individual values and box plots of median fluorescence intensities (MFI) of LILRB2 expression on 3 cell subtypes: CD16<sup>low</sup> monocytes, CD16<sup>high</sup> monocytes, and neutrophils. For each cell type, non-specific staining measured after incubation with an isotype control antibody (iso – dark grey boxes) is shown in parallel with results obtained after staining with anti-LILRB2 antibody (LILRB2 – light grey boxes). B. One representative example of LILRB2 expression (x-axis) on total monocytes (x-axis), neutrophil (green area), and non-specific binding (iso – white area) from one healthy donor. C. One representative example of LILRB2 expression (x-axis) on CD16<sup>high</sup> gated monocytes (red area) versus CD16<sup>low</sup> gated monocytes (blue area) from one healthy donor. Both monocyte subpopulations were gated based on the bi-parametric CD14 versus CD16 dot plot shown on the left.

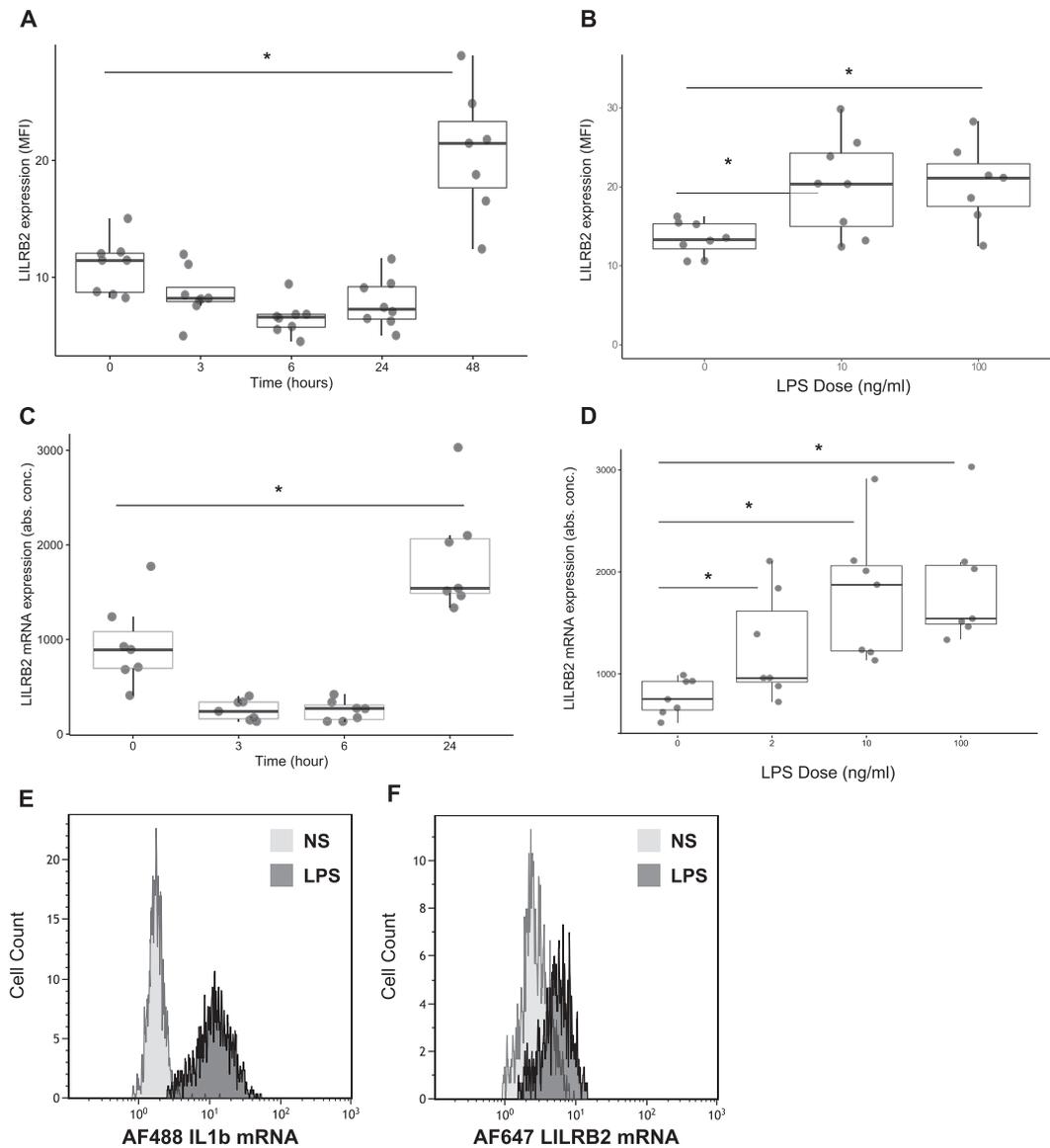


**Fig. 3.** LILRB2 protein expression on monocytes and neutrophils from septic shock patients. Cell surface LILRB2 protein expression was measured by flow cytometry on cells from 13 septic shock patients sampled at D1 (n = 11), D3 (n = 11) and D7 (n = 6) after the onset of shock versus 10 healthy donors. Monocytes were first gated based on CD14 expression and then divided according to CD16 expression. Neutrophils were selected based on high CD16 expression. Results are graphically depicted as individual values and box plots of median fluorescence intensities (MFI) of LILRB2 expression on 3 cell subtypes: neutrophils, CD16<sup>low</sup> monocytes (Monocytes CD16<sup>low</sup>), CD16<sup>high</sup> monocytes (Monocytes CD16<sup>high</sup>). \* p < 0.001 between septic shock patients and healthy controls (Non parametric Mann Whitney U test).

dose-dependent decrease in LILRB2 mRNA expression was observed at 3 and 6 h after LPS stimulation (Fig. 4C).

In order to validate this increased level of mRNA expression in monocytes, we used a documented novel technique that measures

mRNA levels in single cells by flow cytometry [15]. We confirmed that intracellular LILRB2 mRNA content increased in monocytes after LPS stimulation when compared with non-stimulated cells (24 h at 100 ng/ml, Fig. 4E). This was associated with an increased



**Fig. 4.** *Ex vivo* regulation of LILRB2 protein and mRNA expression by lipopolysaccharide stimulation. Peripheral blood mononuclear cells purified from 18 healthy donors were stimulated *ex vivo* with LPS at 100 ng/ml for up to 48 h. A. Cell surface LILRB2 expression was measured on monocytes after 0, 3, 6, 24 and 48 h incubation with LPS (100 ng/ml,  $n = 8$  healthy donors). B. Cell surface LILRB2 expression was measured on monocytes after 48 h incubation with LPS at 10 ng/ml or 100 ng/ml. Results are presented as medians of fluorescence intensities (MFI) of LILRB2 expression measured on CD14 + monocytes and as individual values and box plots ( $n = 8$  healthy donors). C. Transcriptional LILRB2 mRNA expression was measured after 0, 3, 6, and 24 h of incubation with LPS (100 ng/ml,  $n = 7$  healthy donors). D. Transcriptional LILRB2 mRNA expression was measured after 24 h of incubation with LPS at 2 ng/ml, 10 ng/ml or 100 ng/ml. Results are graphically depicted as absolute concentration of LILRB2 mRNA and individual values and box plots ( $n = 7$  healthy donors). \*  $p < 0.05$  between non-stimulated and stimulated conditions (Wilcoxon paired test). E. Intracellular IL-1 $\beta$  mRNA level and F. LILRB2 mRNA level were measured in CD14 + CD45+ monocytes after 24 h stimulation with LPS at 100 ng/ml (dark area – LPS) and in non-stimulated cells (shaded area – NS). These results are from one representative experiment.

mRNA expression of the pro-inflammatory cytokine IL-1 $\beta$  (Fig. 4F). This experiment was reproduced on cells from 3 healthy donors (data not shown).

Overall, these results show that a gram-negative-derived compound can increase LILRB2 protein and mRNA expressions on monocytes, suggesting that the increased LILRB2 expression observed after septic shock may be associated with the initial infectious challenge.

### 3.5. Clinical validation of increased LILRB2 mRNA expression in septic shock patients

In order to compare LILRB2 mRNA expression between infected and non-infected ICU patients, we measured LILRB2 gene expression in a cohort of 725 ICU patients comprising 219 non-septic

severely injured patients, 251 patients with severe sepsis, and 255 septic shock patients (MIP-REA cohort). Patients were sampled at D1, D3, and D6 after ICU admission (Table 2).

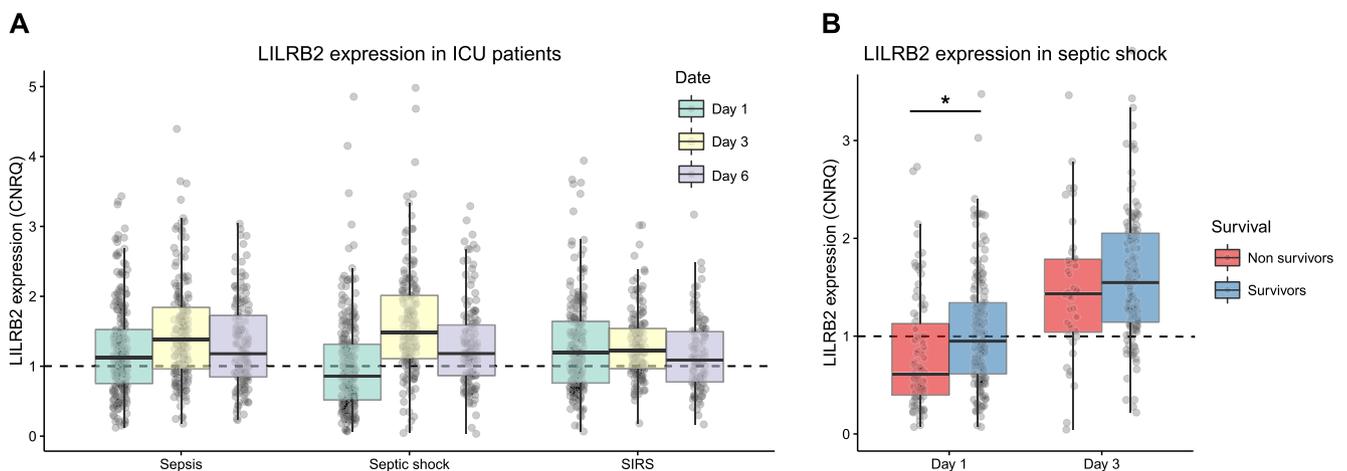
Results showed that LILRB2 mRNA expression was not modified when compared with normal values in septic and non-infected ICU patients (Fig. 5A). Only in septic shock patients did we observe a lower LILRB2 mRNA level at D1 (CNRQ = 0.86 [0.52–1.32]) and greater LILRB2 mRNA level at D3 after ICU admission (CNRQ at D3 = 1.49 [1.11–2.01]) compared with normal values (CNRQ = 1, Fig. 5A). These results confirmed our initial observation in the microarray study and show the same pattern of expression as observed in our *ex vivo* experiments after LPS stimulation.

Finally, when comparing survivors and non-survivors septic shock patients (Supplementary Table 1), we noted that the initial decrease observed at D1 was significantly more pronounced in

**Table 2**

Clinical and demographic characteristics for the severely injured patients included in the MIP-REA cohort. Seven hundred twenty-five severely injured patients were included in the MIP-REA cohort. Values are presented as numbers and percentages for categorical variables and as medians and [Q1–Q3] interquartile ranges for continuous variables. SAPSII: simplified acute physiology score II. SOFA: Sepsis-related Organ Failure Assessment. Comparisons between groups were made by using the Mann Whitney test or the Fisher exact test.

	SIRS n = 219 (30%)	Severe Sepsis n = 251 (35%)	Septic Shock n = 255 (35%)	Total n = 725	p-value
Male	136 (62)	154 (61)	160 (63)	450 (62)	0.949
Age (years)	62 [47–75]	64 [53–75]	67 [59–77]	65 [54–76]	<0.001
SAPS II score	52 [40–68]	50 [39–64]	63 [51–78]	56 [42–69]	<0.001
SOFA score	9 [6–12]	7 [5–9]	11 [9–14]	9 [6–12]	<0.001
Comorbidities (per Charlson score)					<0.001
0	88 (40)	75 (30)	54 (21)	217 (30)	
1	43 (20)	49 (20)	49 (19)	141 (19)	
≥2	88 (40)	127 (51)	152 (60)	367 (51)	
Type of admission					0.003
Medical treatment	145 (66)	195 (78)	162 (64)	502 (69)	
Elective Surgery	13 (6)	10 (4)	9 (4)	32 (4)	
Emergency Surgery	61 (28)	46 (18)	84 (33)	191 (26)	
Type of infection					0.674
Community-acquired		163 (65)	160 (63)	323 (64)	
Nosocomial		88 (35)	95 (37)	183 (36)	
Site of infection					<0.001
Pulmonary		137 (55)	106 (42)	243 (48)	
Abdominal		33 (13)	68 (27)	101 (20)	
Urinary		19 (8)	25 (10)	44 (9)	
Others		62 (25)	56 (22)	118 (23)	
Documentation of infection					<0.001
Clinics only		9 (4)	11 (4)	20 (4)	
Clinics + Imaging		81 (32)	43 (17)	124 (25)	
Clinics + Surgery		8 (3)	15 (6)	23 (5)	
Microbiologically documented		153 (61)	186 (73)	339 (67)	
Gram-negative bacteria		84 (55)	114 (61)	198 (58)	0.423
Gram-positive bacteria		80 (52)	108 (58)	188 (55)	
Fungi		6 (4)	13 (7)	19 (6)	
Others		9 (6)	6 (3)	15 (4)	



**Fig. 5.** LILRB2 mRNA expression in the MIP-REA cohort. A. LILRB2 mRNA expression was measured by qRT-PCR in samples from 219 non-infected patients with systemic inflammatory response syndrome (SIRS), 251 patients with sepsis (Sepsis) and 255 patients with septic shock (Septic shock). Samples were obtained at day 1 (green boxes), day 3 (yellow boxes) and day 7 (purple boxes). B. Among the 255 septic shock patients, LILRB2 mRNA level was measured in non-survivors (n = 86, red boxes) versus survivors (n = 169, blue boxes). Results are expressed as LILRB2 mRNA CNRQ in regard with the reference gene HRPT1, and as individual values. Dotted lines represent normal LILRB2 expression in healthy donors. Statistical analyses were performed using non parametric Mann Whitney U test. \*p < 0.05 between groups.

non-survivors than survivors after septic shock (p < 0.05, Mann Whitney test, Fig. 5B).

#### 4. Discussion

Our results show that both LILRB2 protein and mRNA levels increased after septic shock when compared with non-infected or less severely infected ICU patients and healthy donors. Interest-

ingly, this increase was preceded by a phase of downregulated mRNA expression during the very first hours after onset of septic shock. Our *ex vivo* experiments showed that such an expression pattern can be mimicked when mononuclear cells from healthy donors were stimulated *ex vivo* with LPS. Finally, we observed a link between this dysregulated expression and deleterious outcome after septic shock.

LILRB2, an inhibitory member of the LILR family of receptors, known for its immunoregulatory properties in regard to monocyte

and dendritic cell functions [9], was initially identified as one of the most differentially expressed genes in a microarray study comparing septic shock patients and healthy controls. Considering the numerous parallels between LILRB2 inhibitory properties and sepsis-induced immune alterations, we designed this research project so as to evaluate the regulation of LILRB2 protein and mRNA expressions after septic shock and in an *ex vivo* model of LPS challenge.

In physiological conditions, the inhibitory members of the leukocyte Ig-like receptor family are broadly distributed among cell populations in the immune system [16]. More specifically, LILRB2 expression has been proposed to be restricted to cells of the myelomonocytic lineage, such as monocytes/macrophages and dendritic cells, while LILRB2 is also expressed on the basophils of some individuals [16]. In addition, the expression of LILRB2 on human primary neutrophils has been shown [17,18]. In particular, Baudhuin et al. demonstrated that LILRB2 is the only known receptor for HLA-G expressed on human neutrophils and that this expression is induced during neutrophil differentiation [17]. The authors also showed that an intracellular pool of LILRB2 is rapidly mobilized to the neutrophil surface following stimulation. Our results are in accordance with these findings, since we observed a significant expression of LILRB2 protein on primary human monocytes and, to lesser extent, on neutrophils in healthy donor's blood, and did not detect any expression on lymphocytes. Interestingly, in our results, non-classical CD16<sup>high</sup> monocytes from healthy volunteers expressed higher levels of LILRB2. This is in accordance with results from Allan et al. who showed an intense HLA-G tetramer staining on CD14<sup>low</sup>CD16<sup>high</sup> monocytes correlating with increased LILRB2 expression on these cells [19] as well as with the recently published results of Waschbisch et al. [20].

When comparing LILRB2 protein expression on septic shock patients' cells to healthy donors, we observed that this protein expression was significantly greater on circulating monocytes and neutrophils from patients. This is in accordance with results from Baffari et al. who assessed the cell surface expression of LILRB2 on circulating monocytes from 16 patients with severe sepsis and 16 healthy controls [21]. Similarly to our results, they observed that LILRB2 expression on monocytes from patients was significantly increased in comparison with healthy donors. We extended these results by showing that the differential LILRB2 expression between classical and non-classical monocytes was preserved after septic shock in such way that LILRB2 expression increased after septic shock on both cell types. In addition, we showed for the first time that neutrophils from septic patients also upregulated LILRB2 when compared with normal values.

We next questioned whether the initial infectious challenge could trigger this increased expression in septic shock patients. We sought our response by studying the *ex vivo* regulation of LILRB2 expression following LPS challenge of mononuclear cells derived from healthy donors. We observed increased LILRB2 mRNA and protein expressions at 24 h and 48 h, respectively, after stimulation. The intracellular increased LILRB2 mRNA expression in monocytes was confirmed by flow cytometry. This suggests that initial infection most likely participates in the increased LILRB2 expression measured after septic shock. This also reinforces our observation that, in non-infected critically ill patients, the LILRB2 mRNA level does not increase when compared with controls.

However, we did not observe any significantly increased LILRB2 expression in infected patients not subject to shock, thus suggesting that an infection, in itself, does not suffice to upregulate LILRB2 mRNA levels in patients. In consequence, other factors may be specifically involved in LILRB2 upregulation after septic shock. This is supported by work from Baffari et al. which suggested that LILRB2 increased expression after septic shock may be due, at least in part, to soluble factors present in patients' serum [21]. For exam-

ple, IL-10, an anti-inflammatory cytokine, largely produced after onset of shock, could induce upregulation of LILRB2 on monocytes and LPS-matured DCs [22,23]. In addition, it has been shown that LILRB2 expression is upregulated by HLA-G [24], while increased circulating HLA-G5 concentrations have been observed in septic shock patients [25].

In parallel with this upregulation, we observed a downregulation of LILRB2 expression in the very first hours after LPS stimulation or after onset of shock. This has been detected by very few other studies. Only results from Ju et al. showed that incubation of DCs with CpG-DNA led to a reduction of LILRB2 expression on these cells both at mRNA and protein levels [26]. In the same study, they showed that TNF had no effect on LILRB2 mRNA expression on monocytes/macrophages. However, co-incubation of TNF + PGE2 or sCD40L, PolyI:C and TNF + PGE2 + IL-1 $\beta$  + IL-6 all efficiently downregulated LILRB2 mRNA expression in these cells.

In summary, our results suggest that initial infectious challenge may participate in the up- and downregulation of LILRB2 expression after septic shock. However, the relative roles of other factors, such as soluble mediators, in this specific expression pattern need to be further evaluated.

We can speculate that the increased LILRB2 receptor expression observed after septic shock may play a role in the development of sepsis-induced immune alterations in these patients [6]. Indeed, high-level expression of LILRB2 on monocytes has been shown to inhibit the expressions of co-stimulatory proteins CD80 and CD86 on these cells with a consequential effect on CD4 + T cell proliferation [9]. Similarly, monocytes and DCs, which express high levels of LILRB2, inhibit the activation of primed and unprimed CD4 + T-cells, suggesting that they have acquired a tolerogenic phenotype. Interestingly, LILRB2 has been shown to inhibit cytokine production from monocytic cells when stimulated with CD40L [27]. To date, only 2 studies have investigated the link between increased LILRB2 expression and sepsis-induced immune alterations. Baffari et al. showed that LILRB2<sup>high</sup> monocytes from septic shock patients displayed an alteration in the cytokine response to endotoxin stimulation characterized by reduced IL-12 and increased IL-10 production, as well as a reduced expression of the co-stimulatory molecule, CD86 [21]. In addition, a recent article by Baudhuin et al. reported that LILRB2 mobilization on neutrophil surface is dysregulated in the context of human sepsis, while LILRB2 engagement inhibits neutrophil phagocytic function and ROS production, notably upon engagement with HLA-G in physiologic conditions [17]. Interestingly, their study showed that LILRB2 upregulation upon stimulation on healthy donor neutrophils was impaired in the presence of plasma from septic patients.

We did not investigate the link between altered LILRB2 expression and sepsis-induced immune dysfunctions in our study. However, we observed a larger downregulation of LILRB2 expression in non-survivors after septic shock versus survivors only on the samples collected within the very first hours after the onset of shock. Interestingly, in their evaluation of sHLA-G5 plasma concentration after septic shock, Monneret et al. showed that non-survivors presented a significantly lower sHLA-G5 concentration than survivors [25]. This suggests that the downregulation of this immune regulator within the first hours after the onset of shock is deleterious. This may be possibly confirmed by one genotypic study that showed that critically ill patients who are carriers of +2960IN\_ +3142G\_ +3187A haplotype, associated with a reduced HLA-G expression, are more likely to develop septic shock [28]. We thus propose that the HLA-G/LILRB2 system may be crucially involved in the initial regulation of a massive inflammatory response induced after septic shock. A large release of HLA-G5 and an appropriate maintenance of LILRB2 expression on monocytes would indicate an appropriate and efficient response to the inflammatory

process. Conversely, an extended downregulation of LILRB2 expression associated with insufficient production of its ligand may lead to uncontrolled inflammation and a resulting deregulation of immune system homeostasis, ultimately leading to death. To confirm this hypothesis, experimental investigations are necessary to specifically demonstrate the early involvement of the LILRB2/HLA-G system in the numerous mechanisms that are believed to adequately regulate inflammatory responses during sepsis.

It is to note that patients and healthy donors were not perfectly matched on age in our cohort. With that said, in their article, Baffari et al. observed an upregulation of LILRB2 expression in septic patients compared with age and sex-matched healthy controls [21]. In addition, in the MIP-REA cohort, an increased LILRB2 expression was observed in septic shock patients compared with normal values and non-infectious ICU patients that were in a similar age range than septic shock patients. Thus, although we cannot exclude any effect of age on increased LILRB2 expression; we believe that our results support the up-regulation of this receptor after septic shock.

In summary, our results show that both LILRB2 protein and mRNA expressions increased after onset of septic shock when compared with non-infected or less severely infected ICU patients and healthy donors. This increase was preceded by a phase of downregulated mRNA expression during the very first hours after onset of septic shock. Such an expression pattern can be mimicked when mononuclear cells from healthy donors are stimulated *ex vivo* with LPS. Finally, we observed a link between this dysregulated expression pattern and a deleterious outcome after septic shock.

### Conflicts of interest statement

All authors work in a joint research unit, co-funded by the Hospices Civils de Lyon and bioMérieux. MAC, FV, GM, AL are co-inventors in one patent family covering LILRB2 marker. This does not alter the authors' adherence to all the JOURNAL policies on sharing data and materials.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.humimm.2017.03.010>.

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