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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.

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 of 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^3 or <4000/mm^3.

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 isolated 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 LightCycler 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 touchdown 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: CCAGAGCCCACAGACAGAGG, Reverse primer: TGTCCTTCACGGCAGCATAGA and Probe: TGCAGTGGAGGTCCAGCCCAG. 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