

# The prognostic impact of SIGLEC5-induced impairment of CD8<sup>+</sup> T cell activation in sepsis



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## Summary

**Background** Sepsis is associated with T-cell exhaustion, which significantly reduces patient outcomes. Therefore, targeting of immune checkpoints (ICs) is deemed necessary for effective sepsis management. Here, we evaluated the role of SIGLEC5 as an IC ligand and explored its potential as a biomarker for sepsis.

**Methods** *In vitro* and *in vivo* assays were conducted to both analyse SIGLEC5's role as an IC ligand, as well as assess its impact on survival in sepsis. A multicentre prospective cohort study was conducted to evaluate the plasmatic soluble SIGLEC5 (sSIGLEC5) as a mortality predictor in the first 60 days after admission in sepsis patients. Recruitment included sepsis patients (n = 346), controls with systemic inflammatory response syndrome (n = 80), aneurism (n = 11), stroke (n = 16), and healthy volunteers (HVs, n = 100).

**Findings** SIGLEC5 expression on monocytes was increased by HIF1 $\alpha$  and was higher in septic patients than in healthy volunteers after *ex vivo* LPS challenge. Furthermore, SIGLEC5-PSGL1 interaction inhibited CD8<sup>+</sup> T-cell proliferation. Administration of sSIGLEC5r (0.8 mg/kg) had adverse effects in mouse endotoxemia models. Additionally, plasma sSIGLEC5 levels of septic patients were higher than HVs and ROC analysis revealed it as a mortality marker with an AUC of 0.713 (95% CI, 0.656–0.769; p < 0.0001). Kaplan–Meier survival curve showed a significant decrease in survival above the calculated cut-off (HR of 3.418, 95% CI, 2.380–4.907, p < 0.0001 by log-rank test) estimated by Youden Index (523.6 ng/mL).

**Interpretation** SIGLEC5 displays the hallmarks of an IC ligand, and plasma levels of sSIGLEC5 have been linked with increased mortality in septic patients.

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### Research in context

#### Evidence before this study

The adaptive immune system of septic patients is markedly immunosuppressed, which has been linked to the role of immune checkpoints (ICs). A majority of these ICs and their ligands show Ig-like-V-type domains, which are the structures through which they interact. SIGLEC family members, such as SIGLEC5, exhibit this Ig-like-V-type domain, with some being identified as having a role in sepsis. SIGLEC5 shows a wide recognition range of sialic acid structures and its ability to interact with highly sialylated proteins gives it a high potential to regulate the immune response in sepsis and act as a potential therapeutic target. However, there is a limited understanding of the role of SIGLEC members in sepsis and their potential for therapy. Hence, exploring the relevance of SIGLEC5 in sepsis is an important area for research, as it may lead to biomarkers for early disease prognosis and therapeutic targets.

#### Added value of this study

Our study revealed that SIGLEC5 is expressed at high levels in neutrophils and classical monocytes, with increased presence following stimulation with LPS. We determined that HIF1 $\alpha$  was responsible for the overexpression of SIGLEC5. Additionally, our data demonstrated a significantly heightened expression of SIGLEC5 in both monocytes and plasma of septic patients, particularly those who perished within 60 days of infection. The deleterious effects of SIGLEC5 on survival was further validated using *in vivo* mouse endotoxemia models. Moreover, we discovered that SIGLEC5 obstructs CD8<sup>+</sup> T cell proliferation *in vitro* without initiating apoptosis.

#### Implications of all the available evidence

Our study has shown that sSIGLEC5 levels can be used as a biomarker for prognosis in patients with sepsis. In addition, SIGLEC5 could be potentially involved in pharmacological strategies in sepsis.

## Introduction

Sepsis is perceived as a leading cause of death in intensive care units (ICU) worldwide.<sup>1</sup> Numerous therapeutic strategies, other than antimicrobial and fluid resuscitation treatments, have failed in clinical trials; hence, solid biomarkers of sepsis evolution remain to be identified. Though gold standards to define boundaries amongst sepsis and systemic inflammatory response syndrome (SIRS) are still needed, an increasing number of studies have indicated that patients with systemic response to infection show most of the hallmarks of immunosuppression. In addition, in the complex dynamics of sepsis, two phases have been recognized: an early inflammatory phase and a late immunosuppressive stage<sup>2,3</sup>; however, these two phases can overlap.<sup>3-7</sup>

Studies have demonstrated that monocytes and macrophages are key players in generating an immunosuppressive phenotype during sepsis, which can result in the development of secondary infections and, in many cases, death.<sup>3,8</sup> T cell exhaustion, through the crosstalk between immune checkpoint (IC) ligands expressed on the monocytes and macrophages surfaces and their counterparts on T cells, is one of the main mechanisms through which this immunosuppressive phenotype occurs.<sup>9</sup> In this regard, we and other authors have demonstrated the role of Programmed cell death-protein 1 (PD-1) and its ligand (PD-L1) in the context of sepsis.<sup>10-13</sup>

Several teams have evidenced that sialic acid-binding immunoglobulin-type lectins (SIGLECs) play a crucial role in modulating the immune response, and can be

involved in the pathogenesis and treatment of sepsis.<sup>14-17</sup> These lectins present sialic acid recognition sites in an Ig-like-V-type domain. Interestingly, that domain is described in other proteins known and postulated as ICs or ICs ligands, such as PD-1 and PD-L1, being the structure through which they interact.<sup>18</sup>

Some members of the SIGLEC family have already been identified as playing a role in sepsis, particularly SIGLEC1, a receptor mainly expressed on monocytes and macrophages, which has been linked to increased production of TGF- $\beta$  in macrophages *in vitro*.<sup>19</sup> SIGLEC2, predominantly expressed on B cells, appears to be associated with the development of sepsis, as a soluble fragment of SIGLEC2 was found to be elevated in patients with gram-negative bacterial sepsis.<sup>20</sup> However, SIGLEC2<sup>-/-</sup> mice did not demonstrate any difference in severity, survival rate, or bacterial clearance upon infection with *Staphylococcus aureus*.<sup>21</sup> SIGLEC9 (or SIGLEC-E in mice) is mainly expressed on monocytes, macrophages, neutrophils, and dendritic cells, and its expression is known to increase upon LPS stimulation. This aids in the endocytosis of TLR4 and helps to modulate the inflammatory response during sepsis.<sup>22</sup> SIGLEC10, expressed on macrophage subsets, dendritic cells, and most B cells, is thought to inhibit DAMPs-induced inflammation and assist in regulating the immune response to infection.<sup>23,24</sup> The paired system SIGLEC5/SIGLEC14, mainly expressed on neutrophils and monocytes, has an opposite effect on regulating the immune response despite having almost identical sialic-acid site domains. SIGLEC5 has been

shown to mediate the impairment of phagocytosis and reactive oxidative species production in an infection with group B Streptococcus (GBS),<sup>25</sup> while SIGLEC14, through interaction with adaptor proteins such as DAP12, can activate the p38-mitogen-activated protein kinase (MAPK) and Akt pathways, having a bidirectional action.<sup>26</sup> However, since SIGLEC14 is not widely expressed,<sup>27</sup> it is difficult to study this molecule in the general population. Despite this, our current understanding of the role of SIGLEC members in sepsis and their potential as therapeutic targets is still limited. Therefore, further research is required, including the use of clinically relevant mouse models for sepsis such as polymicrobial cecal ligation and puncture (CLP), to gain a better understanding of the role of these SIGLEC members in sepsis.

SIGLEC5 stands out among the fifteen members of the SIGLEC family reported in humans, due to its potential to regulate the immune response in cases of sepsis and its potential as a therapeutic target. This is because SIGLEC5 has a broad recognition range of sialic acid structures, including  $\alpha$ 2-3,  $\alpha$ 2-6, and  $\alpha$ 2-8 linkage conformations, as well as N-acetylneuraminic and N-glycolylneuraminic variants of sialic acid, and is able to interact with proteins highly decorated with sialic acid.<sup>28,29</sup> Additionally, SIGLEC5 plays a multifaceted physiological role, being involved in cell-cell interactions, pathogen recognition, clearance of apoptotic cells, negative cellular signaling through soluble factors (e.g. Hsp70), and endocytosis of ligands.<sup>30,31</sup> Furthermore, its soluble form, sSIGLEC5, has been shown to interact with the highly sialylated P selectin glycoprotein ligand-1 (PSGL1) molecule, exhibiting robust anti-inflammatory activity.<sup>32</sup> Moreover, PSGL1 has been reported to induce CD8<sup>+</sup> T-cell dysfunction by acting as an IC in a murine model of chronic virus infection.<sup>33</sup>

Considering these assertions, we applied a combination of *in vitro*, *ex vivo* and *in vivo* approaches to study the role of SIGLEC5 in the context of sepsis. We show circulating neutrophils and monocytes as the main cell types expressing surface SIGLEC5, the latter being able to modulate its expression in a greater manner upon LPS stimulation. Additionally, SIGLEC5 expression is governed by the transcription factor HIF1 $\alpha$ . We demonstrate that SIGLEC5 acts as a CD8<sup>+</sup> T cell-specific immune checkpoint ligand that impairs their proliferation. Besides, metalloproteinases drive the generation of a soluble form of SIGLEC5 (sSIGLEC5) that can be detected in plasma and also diminishes CD8<sup>+</sup> T cell proliferation. Along these lines, the administration of sSIGLEC5 exacerbates the disease in two mouse models for sepsis in a CD8 $\alpha$  T cell-dependent manner. Finally, in septic patients both monocyte-membrane-bound and sSIGLEC5 are significantly increased compared with healthy volunteers, indicating that sSIGLEC5 acts as a biomarker of severity in this pathology.

## Methods

### Study design

This study aimed to reveal the role of SIGLEC5 in the context of sepsis. This protein belongs to the SIGLEC family, which plays a relevant role in the modulation of the immune response and could have an important effect on the sepsis disease. Monocytes and macrophages are determinants in orchestrating the immunosuppressive phenotype in sepsis, we hypothesized that SIGLEC5, mainly expressed on monocytes and neutrophils, could be acting as an immune checkpoint in this disease. The combination of *in vitro*, *ex vivo*, and *in vivo* approaches to study the impact of SIGLEC5 in the context of sepsis compared to healthy volunteers suggest that SIGLEC5 plays a role in the immunosuppressive effect, dampening the proliferation capacity of CD8<sup>+</sup> T cells, accelerating the mortality in mouse models of endotoxemia, and showing a prognostic effect of mortality through its soluble form in plasma.

### Healthy volunteers and patients' samples

Patients older than 18 years meeting the diagnostic criteria for sepsis according to the Third International Consensus Definitions for Sepsis and Septic-Shock<sup>34</sup> (Sepsis-3) were enrolled in this study upon arrival to Emergencies (Table 1 and Supplementary Table S1, n = 426). Sepsis picture was primarily identified by SOFA (tachypnea, altered level of consciousness and hypotension) and then thoroughly corroborated following International Sepsis Definitions Conference criteria<sup>35</sup> and classified according to their severity in sepsis and septic shock (operationally defined as requiring vasopressor therapy to maintain a mean arterial blood pressure of >65 mmHg and an increased plasma lactate level of >2 mmol/L). Patients were followed up for 60 days and classified according to their outcome into survivors or *exitus*. Blood samples were promptly obtained from patients upon their admission to the Emergency Department of the University Hospital La Paz (Madrid, Spain) within the first hour of their arrival and peripheral blood mononuclear cells (PBMCs) and plasma were isolated by standardized procedures.<sup>36</sup> Plasma samples from septic patients collected during the first hour of patient admission to the Emergency Department as well as clinical data and evolution information were provided by the University Hospital La Paz (n = 104), the Sepsis Bank of Vall d'Hebron University Hospital Biobank (n = 303) and Virgen de la Arrixaca University Clinical Hospital Biobank (n = 19) (Spanish National Biobanks Network). As controls, healthy volunteers (HV, n = 100) were recruited in person from the Blood Donor Services of La Paz University Hospital and patients with aneurysm (n = 11) and stroke (n = 16) were also enrolled from The Emergency Department and Department of Neurology of La Paz University Hospital, respectively (see Supplementary Table S2). All of them were free of

Characteristic <sup>a</sup>	All patients (n = 426)	Sepsis (n = 188)	Septic shock (n = 158)	SIRS <sup>b</sup> (n = 80)	p-value <sup>c</sup>
Age-yr	67.4 ± 16.2	67.2 ± 17.2	69 ± 15.1	65 ± 15.7	
Male sex—no. (%)	240 (56.47)	106 (56.68)	89 (56.33)	45 (56.2)	
APACHE II Score	17.9 ± 7.6	17.3 ± 6.2	22.2 ± 8.3	13.3 ± 4.4	<0.0001
SOFA Score	5.3 ± 3.2	4 ± 2.4	7.5 ± 3.3	4 ± 2.1	<0.0001
Lactate, nmol/L	3.28 ± 3.21	2.95 ± 2.39	4.49 ± 4.28	1.66 ± 0.6	<0.0001
CRP, mg/L	73.37 ± 105.39	75.65 ± 103.17	70.62 ± 107.95	–	
PCT, ng/mL	25.73 ± 51.73	20.52 ± 51.54	31.94 ± 51.35	–	<0.0001

APACHE II: Acute Physiology and Chronic Health Evaluation II; CRP: C-reactive protein; INR: International Normalized Ratio; PCT: Procalcitonin; SIRS: Systemic Inflammatory Response Syndrome; SOFA: Sequential Organ Failure Assessment. <sup>a</sup>Data are presented as mean ± SD, or number (%). <sup>b</sup>Non-infectious SIRS. <sup>c</sup>p values were calculated by ANOVA test.

**Table 1: Baseline characteristics of SIRS and septic patients included in the study.**

pathogen colonization and recruited randomly but using an age and gender matching. Considering that our study is not a clinical trial, we employed Andrew Fisher's Formula<sup>37</sup> to calculate our sample size. We used a confidence level of 90% (corresponding to a Z-score of 1.65), a standard deviation of 0.5, and a confidence interval (marginal error) of ±5%.

#### Mouse strains

Wild-type female C57BL/6 mice (6–9 weeks old) were purchased from Charles River Laboratories (France). *Rag1*<sup>-/-</sup> female C57BL/6 mice (8–9 weeks old) were a gift from Carlos del Fresno (CNIC, Madrid, Spain). Mice were properly housed in temperature and light-regulated rooms (12:12 h light/dark cycle, 21–24 °C) with food and water ad libitum. All mice were randomly assigned to experimental groups. Mice were followed-up for survival considering mice to have died when they reached the humane endpoint, in our case, when they lost more than 10% of their initial weight. In addition, post-mortem samples were collected.

#### Cell culture conditions

PBMCs and plasma from healthy volunteers and patients were isolated by Ficoll-Plus (GE Healthcare BioSciences) gradient. Monocytes were enriched by: (1) adherence by plating PBMCs in FBS-free RPMI 1640 media with antibiotics for 1 h, and then non-adherent cells were removed by washing with Phosphate Buffered Saline (PBS) three times; or (2) negative isolation using magnetic beads of the Monocyte Isolation Kit (Mintenyi Biotec) according to the manufacturer's instructions. The purity of the monocyte cultures was tested by CD14 labelling and FACS analysis (average 89% and >95% of CD14<sup>+</sup> cells, respectively). Neutrophils isolation was performed using 3% Dextran 500 in NaCl 0.9% of the polymorph nuclear and red cells phase after the isolation of the PBMCs by Ficoll-Plus gradient. All primary human and mice cultures were incubated at 37 °C 5% CO<sub>2</sub> in RPMI 1640 media containing 10% foetal bovine serum (FBS), 25 Mm HEPES, L-glutamine and 1% Penicillin and Streptomycin Mix (Gibco).

#### ELISA protocol

Concentrations of sSIGLEC5 in supernatants of human monocyte cultures and plasma samples were determined using a commercially available ELISA kit (Sigma-Aldrich), following the manufacturer's instructions.

#### Antibodies and flow cytometry analysis

FACS analysis for all *in vitro* assays were developed using specific anti-human antibodies (Abs) to the following surface molecules: CD8-Allophycocyanin (APC, RRID:AB\_11140266), CD14-APC (RRID:AB\_11140663), HLA-DR-Fluorescein-isothiocyanate (FITC, RRID:AB\_11140598) (all three from ImmunoStep), CD3-Brilliant Violet (BV)-786 (RRID:AB\_2739260), CD4-Peridinin-chlorophyll-A protein (PerCP, RRID:AB\_393791), CD8-BV510 (RRID:AB\_2722546), CD14-BUV395 (RRID:AB\_2744288), PSGL1-Phycoerythrin (PE, RRID:AB\_396325) (all five from BD Biosciences) and SIGLEC5-PE (RRID:AB\_2905383, Miltenyi Biotec), as well as specific mouse CD8α-APC (RRID:AB\_2728039, Miltenyi Biotec). Cells were stained with proper Abs for 30 min at 4 °C in the dark, and washed with Phosphate Buffered Saline (PBS) twice. All antibodies used were titrated and matched isotype antibodies were used as negative controls. For all *in vitro* assays, samples were run in FACS Calibur or FACS Celesta (BD Biosciences) flow cytometers, and data were analysed with FlowJo (TreeStar) v. X.07 software or BD FACSDiva v8, respectively.

For the SIGLEC5 expression characterization in the main immune populations, whole blood was lysed by Pharm Lyse 1X (BD) for 20 min, washed with PBS twice and then were stained with fluorochrome-conjugated antibodies to a multi-colour panel of surface markers listed in [Supplementary Table S3](#). Dead cells were excluded using LIVE/DEAD Blue fluorescent reactive dye purchased from Invitrogen and True-Stain Monocyte Blocker (BioLegend) reagent was added prior to the label protocol to block the nonspecific binding of some fluorochrome on monocytes. Labelled cells were acquired on a Cytex Aurora Spectral Cytometer (Cytex Biosciences). Data were analysed using FlowJo (TreeStar) v10.6.2 software.

### **In vitro LPS stimulation**

Monocytes or neutrophils ( $3 \times 10^5$  cells per well) were stimulated or not with 10 ng/mL lipopolysaccharide (LPS, from *Escherichia coli*, Sigma-Aldrich) for 16 h in flat bottom 24-wells plates, in a final volume of 0.5 mL. For the time-course of extracellular SIGLEC5 expression, monocytes were treated or not with 10 ng/mL LPS, for periods ranging from 3 to 48 h. For the matrix metalloproteinase (MMP) and ADAM-family inhibition, the specific inhibitor GM6001 (Merck Millipore) was added 6 h after the LPS challenge, and cultures were maintained for a maximum of 48 h. For HIF1 $\alpha$  inhibition, the potent inhibitor PX-478 (Cayman Chemical) was added for 3 h, and then challenged or not with LPS (10 ng/mL) for another 16 h.

### **In silico analysis of genomic and protein sequences**

Bioinformatics analysis, such as protein and genomic structure of SIGLEC5, were performed using the ENSEMBL, Uniprot and NextProt online tools. The proximal promoter region and flanking promoters of the human SIGLEC5 were localized using the ENSEMBL online tool, and the Hypoxia Response Elements (HREs) using a self-generated Python script with the consensus site to the binding of the HIF1 $\alpha$  factor (5'-ACGTG-3'). The multiple sequence alignments were performed with ClustalW (NCBI). To check the sequence identity and similarity of SIGLEC5 with the other proteins, the EMBOSS-Needle online tool was used.

### **Monocyte nucleofection procedure**

Magnetic negative sorted monocytes by Pan Monocyte Isolation Kit (Miltenyi Biotec) were used for HIF1 $\alpha$  and SIGLEC5 overexpression assays (see Cell Culture above) by nucleofection as previously described,<sup>38</sup> using an Alexa Nucleofector (Lonza Group). HIF1 $\alpha$  overexpression was done with an expression vector, which was a kind gift from Dr. del Peso (Institute for Biomedical Research Alberto Sols). SIGLEC5 overexpression was done with an expression vector (pUNO1-hSIGLEC5, InvivoGen).

### **Chromatin immunoprecipitation (ChIP) assay**

Human/Mouse HIF1 $\alpha$  ExactaChIP Chromatin IP Kit (R&D Systems) was used to perform ChIP on lysates from isolated human blood monocytes, previously nucleofected or not with a HIF1 $\alpha$  overexpression vector.<sup>38</sup> Resultant DNA was isolated using either an unspecific IgG antibody or a specific antibody against HIF1 $\alpha$ , in order to perform the immunoprecipitation according to the manufacturer's protocol.

The products (Hypoxia Response Elements [HRE] sites) were amplified with primers shown in [Supplementary Table S4](#), using the NZY Jaq 2X Colourless Master Mix, in an Eppendorf AG Mastercycler Thermocycler. The following program was used: a first denaturation step at 94 °C for 5 min, 40 cycles of

denaturation at 94 °C for 1 min (primer annealing at 56 °C for 50 s) with primer extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min after completion of the cycling steps.

### **RNA isolation and RT-qPCR**

Adherent monocytes were washed twice with PBS and the RNA was extracted using the High Pure RNA Isolation Kit (Roche Diagnostics). The RNA concentration of each sample was measured using a NanoDrop 2000 (Thermo Fisher Scientific). cDNA was synthesized from 0.25  $\mu$ g total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCRs were performed using the QuantiMix Easy SYG Kit (Biotools) according to the manufacturer's instructions. Gene expression levels were analysed using the LightCycler system (Roche Diagnostics). Reactions were run in triplicate and expression level of  $\beta$ -ACTIN housekeeping was used as internal standard to normalize data. The cDNA copy number of each gene of interest was determined using a 7-point standard curve. All the primers were synthesized by Eurofins Genomics. Specific primers for each gene are shown in [Supplementary Table S5](#).

### **Lymphocyte proliferation assays**

Carboxyfluorescein succinimidyl ester (CFSE) was purchased from ThermoFisher Scientific, and used following the manufacturer's protocol to assess T cells lymphocytes proliferation. Isolated or nucleofected monocytes ( $10^4$  cells per well) were seeded into round bottom 96-wells plates, and then stimulated or not with LPS (10 ng/mL) for 16 h. Afterwards, monocytes were washed once with PBS and autologous CFSE-labelled T cells were added to a ratio 1:5 (monocyte:lymphocyte) in fresh complete RPMI 1640 medium. Cells were then stimulated or not with Pokeweed (PWD, 2.5  $\mu$ g/mL), and treated or not with fully mouse anti-PSGL1 antibody (0.25  $\mu$ g/mL) or anti-SIGLEC5 antibody (1  $\mu$ g/mL).

### **Apoptosis assay**

PBMCs ( $2 \times 10^5$  cells per well) from HVs were cultured with or without sSIGLEC5r (500 or 1000 ng/mL) for 24 h, and then stained with AnnexinV-FITC and Propidium Iodide (PI), for measuring apoptosis by flow cytometry, following manufacturers' recommendations (ImmunoStep).

### **Binding assay protocol**

A polyclonal, FITC-labelled antibody against human IgG-Fc (Abcam), hereinafter  $\alpha$ -Fc-FITC, was purchased. A recombinant human SIGLEC5 protein, with a crystallisable fragment (Fc) region of antibody domain (R&D Systems), hereinafter sSIGLEC5r, was purchased.

Binding of sSIGLEC5r was performed as previously reported.<sup>39</sup> Briefly, PBMCs from HVs or septic patients, treated or not with neuraminidase (NM, 0.01 U/mL for



24 h), were washed twice with 10 mL of ice-cold Ligand Binding Buffer (LBB, PBS with 1% BSA, 0.05% Sodium Azide and 0.1 mM  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ ), fixed using 4% paraformaldehyde (PFA), and  $10^6$  cells resuspended in 1 mL LBB. Afterwards, fixed PBMCs were washed twice with ice-cold LBB and incubated with the recombinant protein sSIGLEC5r (500 ng in 100  $\mu\text{L}$  of LBB) or LBB alone for 1 h and hand-mixed each 15 min on ice. Next, sSIGLEC5r binding was detected using  $\alpha\text{-Fc-FITC}$  (1  $\mu\text{L}$  in 100  $\mu\text{L}$  of LBB). Possible Fc-Receptors were blocked using a Fc-blocking (ImmunoStep) for 30 min before incubation with the recombinant protein. PBMCs incubated only with  $\alpha\text{-Fc-FITC}$  were used as the negative control.

#### Mouse endotoxin *in vivo* model

C57BL/6, 8–9 weeks old (purchased to Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain) female mice were administered a peritoneal injection with either LPS of *E. coli* (055:B5, Sigma Aldrich) (20 mg/kg), sSIGLEC5r (0.8 mg/kg) or both LPS (20 mg/kg) plus sSIGLEC5r (0.8 mg/kg) using sterile saline solution (0.90% w/v of NaCl) as vehicle. To deplete CD8 $\alpha$  T cells, an anti-mouse CD8 $\alpha$  antibody (2.43 clone, Bioxcell) was administered by intraperitoneal injection (20  $\mu\text{g}$  per mice) 24 h before the peritoneal injection of LPS and/or sSIGLEC5r. Mice were followed up for survival and post-mortem lung samples were collected.

#### Mouse cecal ligation and puncture (CLP) *in vivo* model

C57BL/6 wild type (8–9 weeks old) female mice and *Rag1*<sup>-/-</sup> C57BL/6 (8–9 weeks old; purchased to Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain) were modelled. Cecal ligation and puncture protocol was used to induce polymicrobial sepsis as described.<sup>40</sup> Briefly, a 2-cm midline incision was performed to exteriorize the caecum, before being ligated with a 3.0-silk suture at 1 cm of its base, then punctured once with a 25-gauge needle and a small drop of cecal content from the perforation site extruded. Caecum was then returned to the peritoneal cavity and the abdominal incision closed with 4.0-silk sutures. Mice were administered or not a retro-orbital injection of sSIGLEC5r (0.8 mg/kg) using sterile saline solution (0.90% w/v of NaCl) as vehicle.

#### Histological analysis

Following sacrifice, lungs from mice were fixed in 4% paraformaldehyde embedded in paraffin and cut into 5  $\mu\text{m}$  sections in a microtome (RM2255, Lefor histopathological analysis, Leica Biosystems, Wetzlar, Germany). Sections were stained with haematoxylin and eosin (H&E) and images were captured using light microscopy (BX41, Olympus Optical Co., Ltd., Tokyo, Japan). Briefly, the acute lung injury score of mice, on a

scale of 0 (optimal) to 12 (severe), were estimated in accordance with combined assessments of alveolar congestion, haemorrhage, fibrin and infiltrates as previously described.<sup>41</sup> Five randomly selected fields from each slide were analysed by three independent observers.

#### Statistical analysis

Data are presented as numbers and percentages, means and standard deviations. D'Agostino & Pearson Normality test was performed to all the studied variables. Accordingly, differences among the groups were evaluated with the use of Chi-square test, Student's t-test or an analysis of variance (ANOVA) model, followed by the Kruskal–Wallis or Friedman test was used to evaluate multiple comparisons in the ANOVA models, without assuming a Gaussian distribution or normality after a pairing or non-pairing experimental design, respectively. To ascertain the accuracy of the results, Dunn's multiple comparison test was implemented.

For patients with sepsis, Receiver-Operating Characteristic (ROC) curve analysis and its area under the curve (AUC) were used to determine whether the sSIGLEC5 levels could be used as a predictor of mortality in patients with sepsis. The area under the ROC curve measures how well the model discriminates between Survival and *Exitus* groups of patients using only the sSIGLEC5 levels in plasma. We also indicated sensitivity and specificity and the optimal threshold (in terms of maximum sensitivity and specificity) in the primary study cohort were determined on by the Youden Index. Considering sSIGLEC5 levels, we used the Kaplan–Meier method to estimate survival rate over time (60 days) and hazard ratio.

Pearson's analysis was then applied to analyse correlations between plasmatic sSIGLEC5, lactate levels and two severity scores (APACHE-II and SOFA) to determine how variables behave in relation to one another; through the use of the normality test, each variable was ascertained to have a normal distribution. The correlation coefficient (*r*) was interpreted based on prior studies without having to utilise any distinct cut-off.<sup>42</sup>

The Kaplan–Meier method was employed to assess the survival rate of mice after injection of bacterial lipopolysaccharide (endotoxin) and when surgery (CLP) had been initiated, up to a humane endpoint of 10% weight loss. Data for hazard ratio was collected over a period of 168 h.

p values of less than 0.05 were considered to indicate statistical significance. All p-values are two-sided, and the 95% Confidence Intervals (95% CI) are also presented. Statistical analyses were conducted using Prism 8.0 (GraphPad), SPSS version 23 (IBM) software. Youden Index was calculated with MedCalc statistical software. Sample sizes are detailed in the figure legends.

## Ethics

This study was conducted following the guidelines of the 1975 Declaration of Helsinki and was approved by the Local Committee for Ethics (La Paz University Hospital, Madrid, PI-3761). Informed consent was acquired from all participants, and all data collected were treated with the utmost confidentiality. The animal experiments within this study were designed and performed following approval from the Institutional Animal Care and Use Committee at Biomedical Research Institute (IIB, PI-2599).

## Role of the funding source

This research project was conducted with its funders having no oversight of the study design, data collection, data analysis, data interpretation, report writing, or the decision to submit the article for publication. All stages of the project were carried out independently of the research funding, guaranteeing the integrity of the results, conclusions, and interpretations.

## Results

### LPS induces SIGLEC5 expression in human monocytes in a HIF1 $\alpha$ -dependent manner

Based on the values reported in the Monaco database,<sup>43</sup> Fig. 1a illustrates a high *SIGLEC5* RNA expression in human neutrophils, monocytes, dendritic cells, and a milder expression in B cells. We confirmed the expression of membrane-bound SIGLEC5 by full-spectrum flow cytometry on a wide panel of blood circulating immune populations stimulated or not with lipopolysaccharide (LPS), simulating an encounter with Gram-negative bacteria. Fig. 1b shows a strong SIGLEC5 expression on neutrophils and classical and intermediate monocytes. Note that a low expression on B cells (CD19<sup>+</sup>CD20<sup>+</sup>) and both myeloid and plasmacytoid dendritic cells (mDC and pDC) was also detected (Supplementary Figure S1a). In addition, an increase in SIGLEC5 expression was observed when both monocytes and neutrophils were stimulated with LPS (Fig. 1b and c), with a greater increase in monocytes than neutrophils (Fig. 1d).

Due to the important role of classical monocytes and macrophages in orchestrating the immunosuppressive phenotype during sepsis<sup>3,8</sup> and the significant increment of SIGLEC5 on these cells after LPS stimulation, we focused on characterizing the regulation of SIGLEC5 transcription in these cells. Previously, we reported a crucial role for HIF1 $\alpha$  in the control of several key factors during sepsis, including the negative regulator of inflammation IRAK-M, the NLRP3 inflammasome activation and the IC ligand PD-L1.<sup>10,11,44,45</sup> Thus, to explore whether the *SIGLEC5* gene was a direct target of HIF1 $\alpha$  in human monocytes, we searched for potential HIF1 $\alpha$  binding sites, known as Hypoxia Response Elements (HRE), in the proximal promoter region of

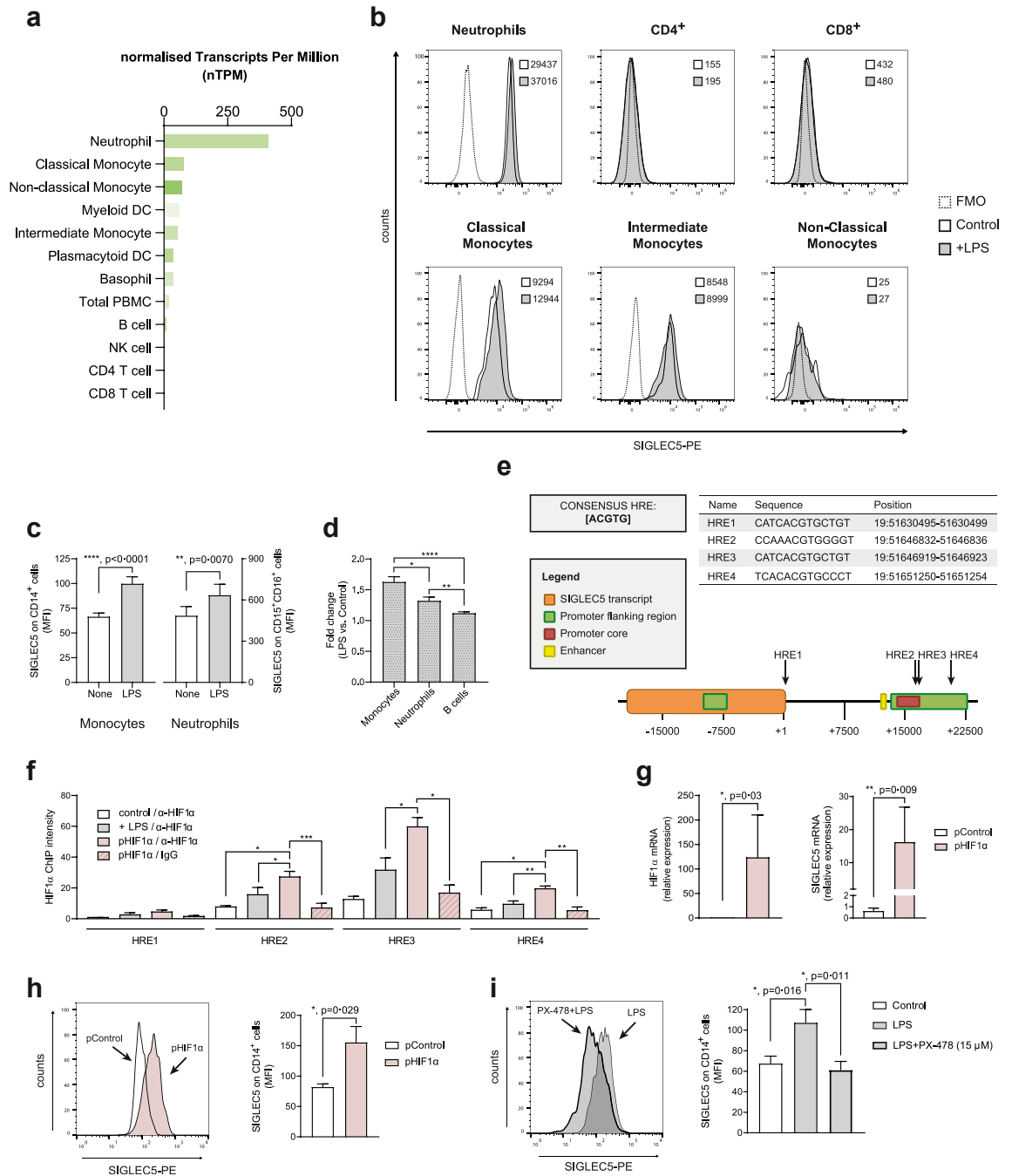
human *SIGLEC5* using bioinformatics approaches. As Fig. 1e shows, four potential HREs containing the consensus sequence (5'-ACGTG-3') were found; three of them were identified within the *SIGLEC5* promoter and the last one close to the transcript region. Then, by a chromatin immunoprecipitation (ChIP) assay, we demonstrated HIF1 $\alpha$  binds to three of the reported HREs in the promoter region of *SIGLEC5* in human monocytes (Fig. 1f). Along these lines, human HIF1 $\alpha$ -transfected monocytes exhibited high expression of SIGLEC5 at both mRNA (Fig. 1g) and protein levels (Fig. 1h). Eventually, SIGLEC5 expression on LPS-stimulated monocytes was reduced in the presence of PX-478 (a potent HIF1 $\alpha$  inhibitor)<sup>46</sup> (Fig. 1i and Supplementary Figure S1b). These results indicate that myeloid circulating cells induce SIGLEC5 expression upon LPS stimulation in a HIF1 $\alpha$ -dependent manner.

### SIGLEC5 displays IC ligand hallmarks

To study the potential immunomodulatory effect of SIGLEC5 on T cells, we stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells with the classical mitogen-lectin pokeweed (PWD) in the presence of unstimulated or LPS-primed autologous monocytes from healthy volunteers (HVs) (Fig. 2a). We observed that LPS-stimulated monocytes from HVs reduced both CD8<sup>+</sup> and CD4<sup>+</sup> T cell proliferation (Fig. 2b and Supplementary Figure S2a). Besides, this effect was reverted in the presence of a SIGLEC5 blocking antibody in CD8<sup>+</sup> (Fig. 2b) but not in CD4<sup>+</sup> T cells (Supplementary Figure S2a). Moreover, an important reduction of CD8<sup>+</sup> (Fig. 2c–e) but not CD4<sup>+</sup> T cell (Supplementary Figure S2b) proliferation was observed when monocytes were transfected with a SIGLEC5 expression plasmid and co-cultured with autologous lymphocytes in presence of PWD. This data supports a specific role for SIGLEC5 in restraining the proliferation of CD8<sup>+</sup> but not CD4<sup>+</sup> T cells.

Since this activity resembles IC hallmarks, we performed a multiple sequence alignment analysis of the Ig-like V-type domain between SIGLEC5 and other known proteins postulated as ICs or IC ligands. As Supplementary Figure S3 illustrates, human SIGLEC5 and these proteins share a canonical Ig-like V-type ICs domain in its primary structure with pairwise identities and similarities that range from 14 to 25% and from 22 to 38%, respectively. This homology was even higher than that exhibited amongst ICs or IC ligands such as the B7 family of proteins, supporting the role of SIGLEC5 as IC or IC ligand.<sup>18,47,48</sup>

We next studied how SIGLEC5 interacts with T-cells (Fig. 2f). Fig. 2g shows the specific binding of the recombinant soluble SIGLEC5-FC protein (sSIGLEC5-FC) to CD8<sup>+</sup> T cells. Curiously, this binding was reduced in presence of neuraminidase (NM), a glycoside-hydrolase enzyme that cleaves the glycosidic linkage of neuraminic acids (Fig. 2g). It should be recalled that PSGL1 has been reported as a receptor for



**Fig. 1: LPS induces SIGLEC5 expression on human monocytes under Hypoxia Inducible Factor 1 alpha (HIF1α) control.** (a) Normalized SIGLEC5 RNA expression on the main immune cells in the blood from the Monaco database.<sup>43</sup> (b) SIGLEC5 expression on the cell surface of a panel of blood circulating immune populations stimulated (grey filled line) or not (black clear line) with lipopolysaccharide (LPS), including neutrophils, T lymphocytes, classical monocytes (CD14<sup>+</sup>CD16<sup>-</sup>), intermediate monocytes (CD14<sup>+</sup>CD16<sup>+</sup>) and non-classical monocytes (CD14<sup>-</sup>CD16<sup>+</sup>); MFI of LPS-stimulated or not conditions were indicated into the plots. A fluorescence minus one (FMO) was used as negative control of SIGLEC5 expression represented in dotted line. (c) SIGLEC5 expression on monocytes (left panel) and neutrophils (right panel, n = 14) from HVs challenged or not with 10 ng/mL of LPS for 16 h. (d) Fold change of SIGLEC5 expression on monocytes, neutrophils and B cells from HVs challenged stimulated with LPS vs. control without stimulation. (e) Hypoxia Response Elements (HRE) in the human SIGLEC5 sequence (Ensembl, ENSG00000268500) based on the consensus sequence [5'-ACGTG-3']. Sequences and positions are shown. (f) A Chromatin immunoprecipitation (ChIP) assay was conducted on: control monocytes (white column), LPS-stimulated monocytes for 16 h (grey filled



SIGLEC5 on T cells.<sup>33</sup> In this context, a blocking antibody against P-Selectin Glycoprotein Ligand 1 (PSGL1) reverted CD8<sup>+</sup> T cell proliferation when these cells were co-cultured with monocytes previously transfected in order to overexpress SIGLEC5 (Fig. 2h); however, this effect was not observed in CD4<sup>+</sup> T cells (Supplementary Figure S2c). These results suggest that SIGLEC5 is acting as IC through PSGL1 engagement on CD8<sup>+</sup> T cells.

### Soluble SIGLEC5 specifically impairs CD8<sup>+</sup>, but not CD4<sup>+</sup> T cell proliferation

We extended the study of the modulation exerted by SIGLEC5 on the T cell response by looking at the potential role played by the soluble form of SIGLEC5 (sSIGLEC5) in this context. Thus, a soluble recombinant protein (sSIGLEC5r) reduced CD8<sup>+</sup> but not CD4<sup>+</sup> T cell proliferation *in vitro* in a dose-dependent manner (Fig. 3a). These differences increased in a sSIGLEC5r-dependent manner (Fig. 3a and c). Remarkably, we found no significant differences in apoptosis of any of the T cell subsets in these experiments (Fig. 3d). Note that sSIGLEC5 levels were higher in monocytes than neutrophils upon the same experimental conditions, both in steady-state (none) and after LPS stimulation, which reinforced our focus on monocytes (Fig. 3e).

Eventually, as Fig. 3f–h illustrate, the presence of the pan-metalloproteinase inhibitor (GM6001) increased and stabilized SIGLEC5 expression on the surface of monocytes and reduced sSIGLEC5 levels in supernatants, pointing to enzymatic cleavage by metalloproteinases as the molecular mechanism for the generation of sSIGLEC5, as described for other membrane-anchored proteins such as TREM-1.<sup>49</sup>

### SIGLEC5 reduces survival in mouse models of sepsis

To better understand the role of SIGLEC5 in response to infection, two *in vivo* mouse models were assessed. First, we performed a cecal ligation and puncture (CLP) protocol where wild-type (WT) and *Rag1*<sup>-/-</sup> mice were simultaneously administered with either sSIGLEC5 or saline intraperitoneally. Compared with CLP mice injected with saline, CLP mice treated with sSIGLEC5r displayed a reduced survival ( $\chi^2 = 7.591$ ,  $p = 0.0059$ , Hazard ratio CLP + sSIGLEC5r vs. CLP + saline of

3.521; 95% CI, 1.005–12.34, Fig. 4a). However, this effect was not observed in *Rag1*<sup>-/-</sup> mice, which lack mature B and T cells, where sSIGLEC5r administration did not influence on the survival rate ( $\chi^2 = 0.064$ ,  $p = 0.801$  (non-significant), Hazard ratio *Rag1*<sup>-/-</sup> CLP + sSIGLEC5r vs. *Rag1*<sup>-/-</sup> CLP + saline of 0.844; 95% CI, 0.210–3.382, Fig. 4b). No statistically significant differences were found in survival rates between wild-type and *Rag1*<sup>-/-</sup> mice after CLP model ( $\chi^2 = 0.910$ ,  $p = 0.340$  (non-significant), Hazard ratio WT CLP + saline vs. *Rag1*<sup>-/-</sup> CLP + saline of 1.830; 95% CI, 0.442–7.569, Fig. 4b). This suggests that sSIGLEC5r suppressive effect is dependent on adaptive response, even though this immunity was not required for the CLP model.

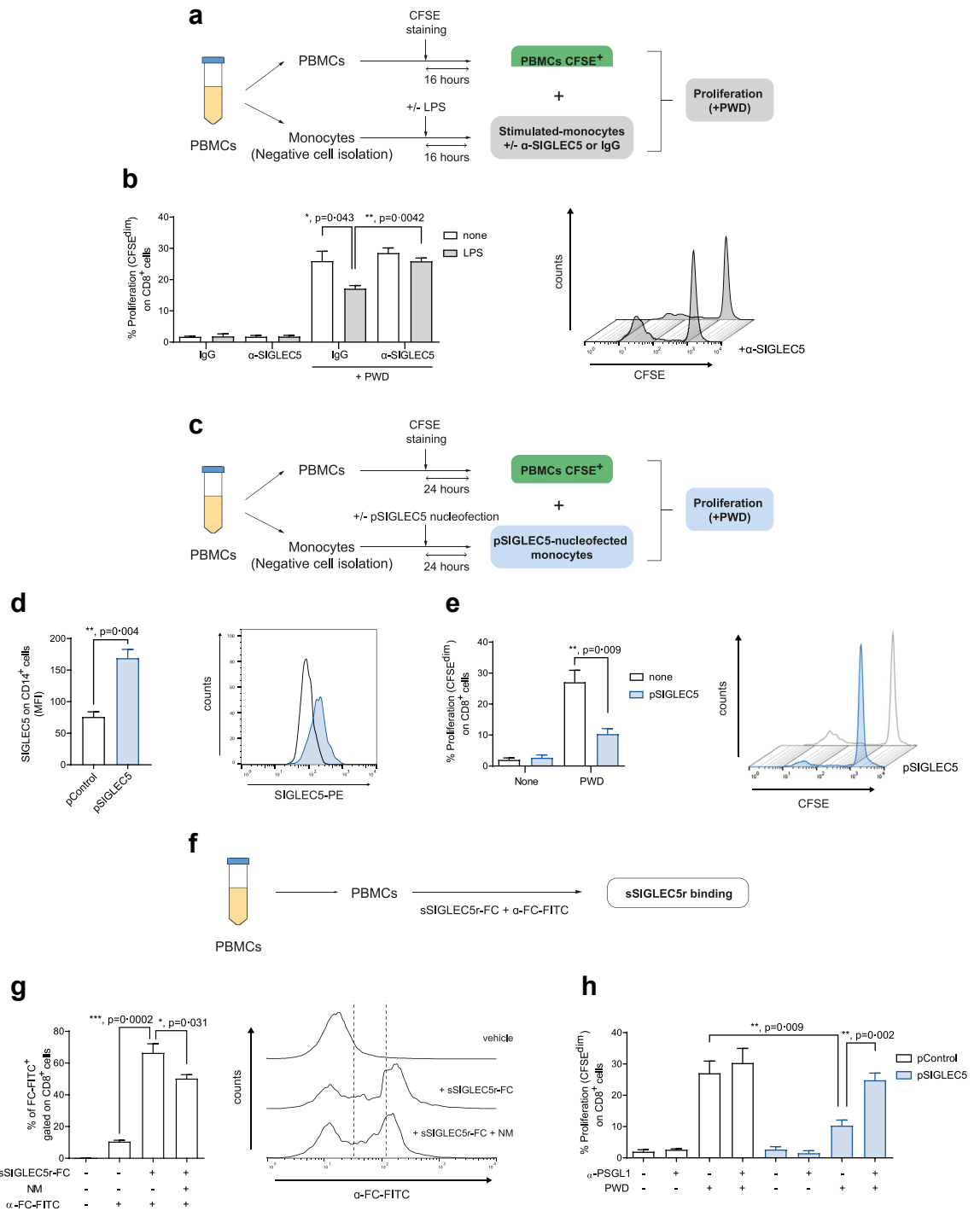
Through haematoxylin/eosin staining, we corroborated a substantial lung injury caused by CLP in both WT and *Rag1*<sup>-/-</sup> mice reaching comparable damage. The lung insult worsened when mice were additionally injected with sSIGLEC5r in WT but not *Rag1*<sup>-/-</sup> mice (Fig. 4c and Supplementary Figure S4a and b). Together, these findings suggested that sSIGLEC5 modulates the severity of sepsis relying on lymphocytes.

In addition, mortality was also evaluated in the context of endotoxin challenge using lipopolysaccharide (LPS) from *E. coli*. Fig. 4d shows that mice treated simultaneously with LPS and sSIGLEC5r died earlier than those challenged only with LPS ( $\chi^2 = 5.432$ ,  $p = 0.019$ , Hazard ratio LPS + sSIGLEC5r vs. LPS + saline of 7.123; 95% CI, 1.366–37.13). However, this effect was reversed in mice administered with a CD8 $\alpha$ -depleting antibody, considering that there was not differences between LPS + saline vs. LPS +  $\alpha$ CD8 $\alpha$  (Fig. 4e and f). Moreover, haematoxylin/eosin staining of lungs sections showed that sSIGLEC5r increased acute lung injury caused by LPS (Fig. 4g and Supplementary Figure S4c), which regressed in CD8 $\alpha$ -depleted mice. These results highlight the impact of sSIGLEC5 on the mortality of sepsis and its dependence on CD8<sup>+</sup> T cells.

### Monocyte from septic patients modulate CD8<sup>+</sup> T cell proliferation through SIGLEC5

To explore the impact of these results in patients with sepsis, we evaluated SIGLEC5 expression on blood

column), HIF1 $\alpha$ -transfected monocytes (burgundy filled column), all of them immunoprecipitated with an antibody against HIF1 $\alpha$ , and HIF1 $\alpha$ -transfected monocytes immunoprecipitated with an unspecific IgG antibody (burgundy filled and lined column). HREs band intensities of PCR products normalized to one of the negative controls ( $n = 4$ ). (g) Relative expression (mRNA) by RT-qPCR of *HIF1 $\alpha$*  (left panel) and *SIGLEC5* (right panel) on CD14<sup>+</sup> cells from HVs nucleofected with pHIF1 $\alpha$  (burgundy filled column) or a control plasmid (white column) ( $n = 6$ ). (h) Representative histogram overlay of SIGLEC5 expression (left panel) on CD14<sup>+</sup> cells nucleofected with pControl (white histogram) or pHIF1 $\alpha$  (burgundy histogram) and MFI of SIGLEC5 (right panel) on CD14<sup>+</sup> cells after 16 h of nucleofection with pControl (white column) or pHIF1 $\alpha$  (burgundy filled column) ( $n = 4$ ). (i) Representative histogram overlay of SIGLEC5 on CD14<sup>+</sup> cells pre-treated (grey thickest line) or not (grey thinnest line) with a specific inhibitor (PX-478) of HIF1 $\alpha$  for 3 h, and then challenged with LPS for 16 h (left panel), and MFI of SIGLEC5 on CD14<sup>+</sup> cells pre-treated (grey filled columns) or not (black clear column) with a specific HIF1 $\alpha$  inhibitor (PX-478) for 3 h, and then challenged (grey filled and thickest line) or not (grey filled and thinnest line) with LPS for 16 h (right panel) ( $n = 7$ ). Data shown as mean  $\pm$  SEM. (c, d, and g–i) Paired t-test (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ ). (f) One-way ANOVA test (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ ).



**Fig. 2: SIGLEC5 on monocytes impairs CD8<sup>+</sup> T cell proliferation, which is restored after blocking the SIGLEC5/PSGL1 axis.** (a) Scheme of the proliferation assay of monocytes stimulated or not with LPS for 16 h in presence of a blocking antibody against SIGLEC5 (α-SIGLEC5) or an unspecific IgG, and then co-cultured with autologous lymphocytes stimulated or not with PWD (2.5 μg/mL) for 5 days. (b) Proliferation levels (CFSE<sup>dim</sup>) of CD8<sup>+</sup> cells from HVs, stimulated or not with PWD and co-cultured for 5 days with autologous monocytes pre-challenged (grey filled columns) or not (white columns) with 10 ng/mL of LPS for 16 h. In some indicated conditions, a blocking antibody against SIGLEC5 (α-SIGLEC5) or an unspecific IgG was added (n = 3, left panel). Representative histogram overlay of proliferation levels (CFSE<sup>dim</sup>) of PWD-stimulated CD8<sup>+</sup> cells from HVs, and co-cultured for 5 days with autologous monocytes pre-challenged with 10 ng/mL of LPS for 16 h, in presence or not of a blocking antibody against SIGLEC5, α-SIGLEC5 (right panel). (c) Scheme of the proliferation assay of monocytes nucleofected or not with

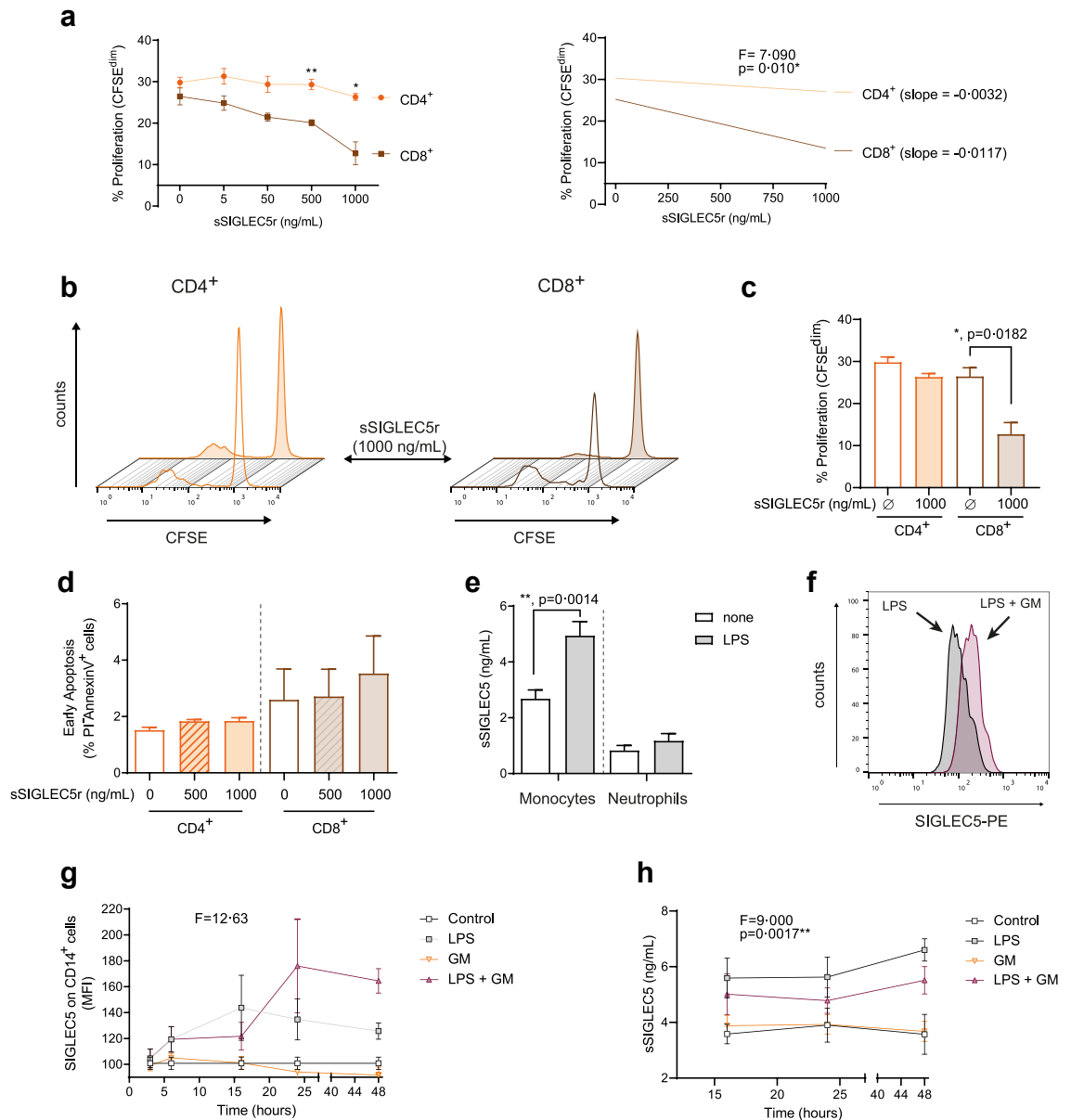
monocytes from septic patients at the time of admission and prior to any treatment to compare it with healthy volunteers (HVs). Septic patients (sepsis or septic shock) exhibited higher SIGLEC5 expression on their circulating monocytes than HVs (Fig. 5a and b), which could be further increased after *ex vivo* LPS stimulation (Fig. 5c). Interestingly, septic patients with positive bacterial haemocultures showed higher expressions of SIGLEC5 on CD14<sup>+</sup> cells than those with negative bacterial haemocultures. Moreover, even in those haemoculture-positive septic patients, the *ex vivo* LPS stimulation further increase SIGLEC5 expression (Fig. 5d). As expected, SIGLEC5 not only binds CD8<sup>+</sup> T cells from HVs (Fig. 2g) but also those from septic patients (Fig. 5e). Accordingly, CD8<sup>+</sup> lymphocytes from septic patients showed an impaired proliferation after PWD stimulation compared to HVs (Fig. 5f). This diminished CD8<sup>+</sup> T cell proliferation was even more accused when monocytes were stimulated *ex vivo* with LPS, which was significantly reverted in presence of either anti-SIGLEC5 or anti-PSGL1 blocking antibodies (Fig. 5g). Despite CD4<sup>+</sup> cells from septic patients bound sSIGLEC5r-FC, their proliferation was not affected (Supplementary Figure S2d and e).

### SIGLEC5 is a biomarker of prognosis in septic patients

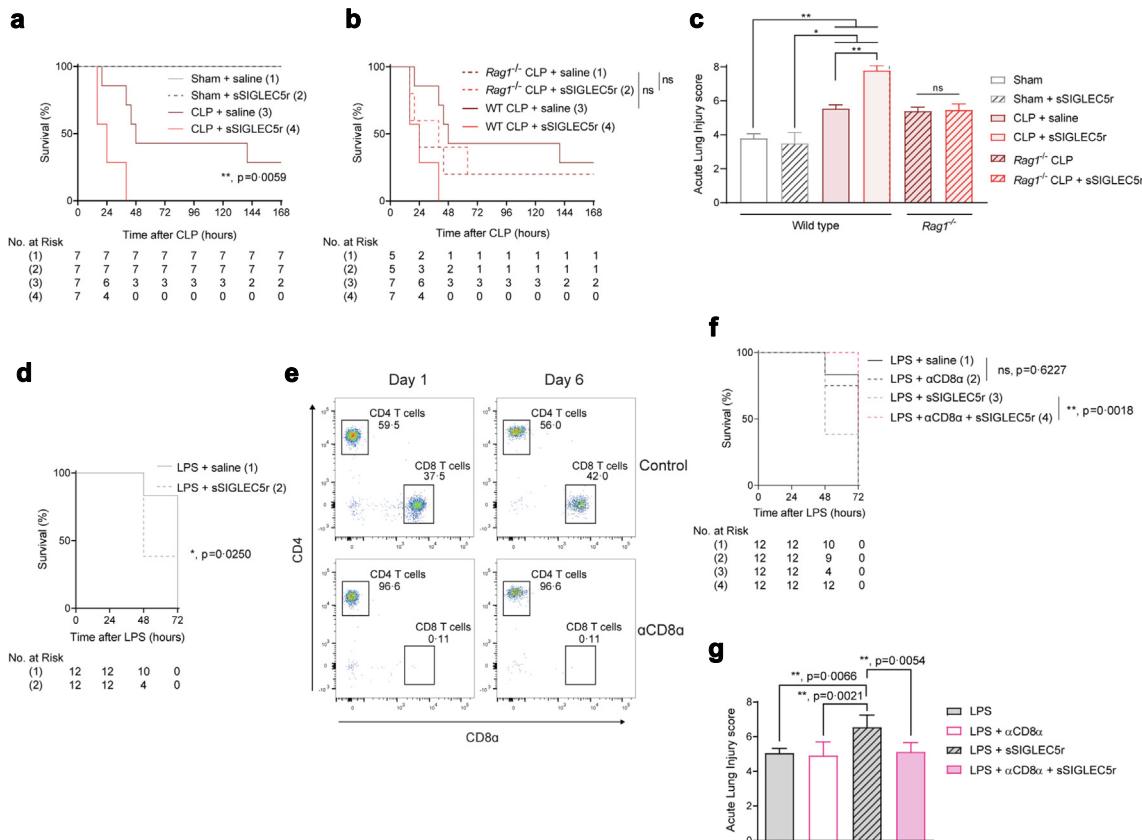
Considering the observed role for SIGLEC5 in monocytes from septic patients, and the presence of its soluble form cleaved from the membrane by metalloproteinases (Fig. 3), we sought to investigate the performance of sSIGLEC5 as a clinical biomarker. Thus, a total of 425 patients with systemic inflammatory response criteria were studied. Patients were classified as septic (44%), septic-shock (37.2%) and non-infectious SIRS (18.8%) according to their clinical parameters and severity.<sup>34,35</sup> They were followed up for 60 days to establish their survivor or *exitus* condition (Table 1). As controls, HVs and also patients suffering aneurysm or stroke were assessed (Supplementary Table S1).

Higher levels of sSIGLEC5 were detected in plasma from septic patients (a multicentre cohort) compared with HVs (Fig. 6a). Moreover, once septic patients were further classified according to their severity into sepsis and septic shock,<sup>34</sup> the latter presented increased levels sSIGLEC5 in their plasma than the former (Fig. 6b). Furthermore, we observed that the levels of sSIGLEC5 were also higher than those of patients with SIRS or non-infectious diseases such as aneurysm or stroke (Fig. 6b). On the one hand, when septic patients classified according to whether they required admission to the ICU, levels of sSIGLEC5 were higher in those patients requiring intensive care (Fig. 6c), with greater values in those patients with the worst outcome (Fig. 6d). On the other hand, septic patients were classified according to their evolution into survivors or *exitus*, observing higher levels of sSIGLEC5 in *exitus* than survivor patients (Fig. 6e). To differentiate between both outcomes, a receiver-operating characteristic (ROC) analysis of plasmatic sSIGLEC5 levels was performed (Supplementary Figure S5). The area under the curve (AUC) for *exitus* was 0.713 (95% confidence interval [CI], 0.656–0.769; *p* < 0.0001), while the AUC for their admission to ICU was 0.642 (95% confidence interval [CI], 0.577–0.706; *p* < 0.0001). The optimal cut-offs, estimated by Youden index, were 523.6 ng/mL and 663.2 ng/mL, respectively, indicating that patients with a circulating sSIGLEC5 level above both Youden indexes exhibit a poor outcome (Supplementary Figure S5a and b). We evaluated the predictive power of sSIGLEC5 in comparison to commonly used clinical indicators such as C-reactive protein and procalcitonin, in order to assess its advantage as an indicator of severity and mortality. Supplementary Figure S5c demonstrates that the predictive power of sSIGLEC5 is significantly higher than that of the other clinical indicators. In addition, sSIGLEC5 showed a weak positive correlation with lactate and moderate positive correlations with clinical prognosis indicators such as APACHE-II and SOFA scores (Supplementary Figure S5d). Kaplan–Meier survival curve showed a significant decrease in the survival

SIGLEC5 expression vector (pSIGLEC5) and then co-cultured with autologous lymphocytes stimulated or not with PWD (2.5 µg/mL) for 5 days. (d) MFI of SIGLEC5 on CD14<sup>+</sup> cells 16 h after nucleofection with either an expression vector of SIGLEC5, (pSIGLEC5), or a control vector, pControl (*n* = 4) (left panel). Representative histogram overlay of SIGLEC5 expression on CD14<sup>+</sup> cells 16 h after nucleofection with either an expression vector of SIGLEC5, pSIGLEC5 (blue histogram), or a control vector, pControl (white histogram) (right panel). (e) Proliferation levels (CFSE<sup>dim</sup>) of CD8<sup>+</sup> cells from HV, stimulated or not with PWD and co-cultured for 5 days with autologous monocytes pre-nucleofected (blue filled column) or not (white column) with an expression vector of SIGLEC5 (pSIGLEC5) (*n* = 4) (left panel). Representative histogram overlay of proliferation levels (CFSE<sup>dim</sup>) of PWD-stimulated CD8<sup>+</sup> cells from HVs, and co-cultured for 5 days with autologous monocytes pre-nucleofected (blue filled line) or not (white line) with pSIGLEC5 (right panel). (f) Scheme of the binding assay of recombinant sSIGLEC5r-FC to CD8<sup>+</sup> T cells. (g) Quantification of sSIGLEC5r (sSIGLEC5r-FC) binding to CD8<sup>+</sup> T cells from HVs pre-treated or not with Neuraminidase (NM, 0.01 U/mL) for 24 h (*n* = 6). Binding was revealed with α-FC-FITC and analyzed by flow cytometry, using the secondary antibody alone as control (left panel). Representative histogram overlay of sSIGLEC5r-FC binding to CD8<sup>+</sup> cells from HVs pre-treated or not with NM (0.01 U/mL) for 24 h and unspecific control using the antibody α-FC-FITC only (vehicle, right panel). (h) Proliferation levels (CFSE<sup>dim</sup>) of CD8<sup>+</sup> T cells from HVs in the presence or not of PWD and co-cultured for 5 days with autologous monocytes pre-nucleofected with an expression vector of SIGLEC5 (pSIGLEC5, blue filled columns) or an empty vector (pControl, white columns) (*n* = 6). In some conditions, a blocking antibody against PSGL1 (α-PSGL1) was added. Data shown as mean ± SEM. (b, d, e, g, and h) Paired t-test (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001).



**Fig. 3: Soluble SIGLEC5 impairs CD8<sup>+</sup> T cell proliferation.** (a) Proliferation levels (percentage of CFSE<sup>dim</sup> cells) (left panel) and linear regression (right panel) of PWD-stimulated CD4<sup>+</sup> (orange) and CD8<sup>+</sup> (mocha) cells from HVs in presence of different concentrations of human recombinant sSIGLEC5r for 5 days (n = 5). (b) Representative histograms overlays of the proliferating CFSE<sup>dim</sup> PWD-stimulated CD4<sup>+</sup> (left panel) and CD8<sup>+</sup> (right panel) cells from HVs in presence (filled line) or not (clear line) of human sSIGLEC5r for 5 days. (c) Proliferation levels (percentage of CFSE<sup>dim</sup> cells) of PWD-stimulated CD4<sup>+</sup> (orange) and CD8<sup>+</sup> (mocha) T cells from HVs in presence or not of the highest concentration of sSIGLEC5r used (1000 ng/mL) for 5 days (n = 5). (d) Percentage of apoptotic (PI/AnnexinV<sup>+</sup>) CD8<sup>+</sup> T cells isolated from HV and incubated (filled column) or not (clear column) with sSIGLEC5r for 24 h at 500 and 1000 ng/mL (n = 5). (e) Soluble SIGLEC5 (sSIGLEC5) levels in supernatants of monocytes (n = 14) and neutrophils (n = 6) treated (grey filled column) or not (white column) with 10 ng/mL of LPS for 16 h. (f) Representative histogram overlay of SIGLEC5 expression on LPS-stimulated CD14<sup>+</sup> cells in absence (grey filled) or presence (wine filled) of a pan-inhibitor of metalloproteinases (GM6001) added 6 h before the LPS stimulation for 16 h. (g) MFI of SIGLEC5 on CD14<sup>+</sup> cells from HVs exposed to LPS (grey), GM6001 (orange), and combinations (wine) for the indicated times (n = 3). (h) sSIGLEC5 levels in the supernatants of monocytes from HVs exposed to LPS (grey), GM6001 (orange) and combinations (wine) for indicated times (n = 3). A control without any stimulation is represented in white. Data shown as mean ± SEM. (a, left panel) Two-way ANOVA test (\*\*p < 0.01). (a, right panel) Spearman linear regression test (\*p < 0.05). (c–e) Paired t-test (\*p < 0.05; \*\*p < 0.01). (g and h) One-way ANOVA test (\*\*p < 0.01; \*\*\*p < 0.001).



**Fig. 4: SIGLEC5 reduces survival in endotoxemia mouse models in a CD8 $\alpha$ -dependent manner.** (a) Kaplan–Meier estimation of survival from CLP-mice injected or not with sSIGLEC5r ( $\chi^2 = 33.20$ ,  $p < 0.0001$  for all four groups comparison: Sham, Sham + sSIGLEC5r, CLP + saline and CLP + sSIGLEC5r;  $\chi^2 = 7.591$ ,  $p = 0.0059$ , hazard ratio [HR] CLP plus sSIGLEC5r vs. CLP groups comparison of 8.302; 95% CI, 1.842–37.42) ( $n = 7$  per group). (b) Kaplan–Meier estimation of survival from CLP-WT or CLP-*Rag1*<sup>-/-</sup> mice injected or not with sSIGLEC5r ( $\chi^2 = 0.064$ ,  $p = n.s.$ , Hazard ratio [HR] *Rag1*<sup>-/-</sup> CLP vs. *Rag1*<sup>-/-</sup> CLP + sSIGLEC5r of 0.828, 95% CI, 0.191–3.582) ( $n = 5$  per group in WT mice and  $n = 7$  per group in *Rag1*<sup>-/-</sup> mice). (c) Acute lung injury evaluated on Haematoxylin/Eosin-stained lung sections of Sham or CLP-mice in the background wild-type or *Rag1*<sup>-/-</sup> injected or not with sSIGLEC5r ( $n = 7$  per group). (d) Kaplan–Meier estimates of survival from mice injected with LPS (20 mg/kg) with or without CD8 $\alpha$  depletion ( $\chi^2 = 0.242$ ,  $p = 0.6227$ , HR of 0.920, 95% CI, 0.413–2.049) or injected with LPS and sSIGLEC5r with or without CD8 $\alpha$  depletion ( $\chi^2 = 9.731$ ,  $p = 0.0018$ , HR of 14.21, 95% CI, 2.681–75.29) ( $n = 12$  per group). (e) Flow cytometry-gating strategy for CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup> T cells in blood from control and CD8 $\alpha$ -depleted mice at day 1 and 6 after injection of a depleting CD8 $\alpha$  antibody (20  $\mu$ g/mice). (f) Kaplan–Meier estimates of survival from mice injected with LPS (20 mg/kg) with or without CD8 $\alpha$  depletion ( $\chi^2 = 0.242$ ,  $p = 0.6227$ , HR of 0.920, 95% CI, 0.413–2.049) or injected with LPS and sSIGLEC5r with or without CD8 $\alpha$  depletion ( $\chi^2 = 9.731$ ,  $p = 0.0018$ , HR of 14.21, 95% CI, 2.681–75.29) ( $n = 12$  per group). (g) Acute lung injury evaluated on Haematoxylin/Eosin stained lung sections of the endotoxemia mouse model with LPS injection (20 mg/mL), CD8 $\alpha$ -depleted or not, and injected or not with sSIGLEC5r ( $n = 12$  per group). (a, b, and d–f) Kaplan–Meier estimation of survival test (\* $p < 0.05$ ; \*\* $p < 0.01$ ). (c and g) Data shown as mean  $\pm$  SEM. Paired t-test (ns, non-significant; \*\* $p < 0.01$ ).

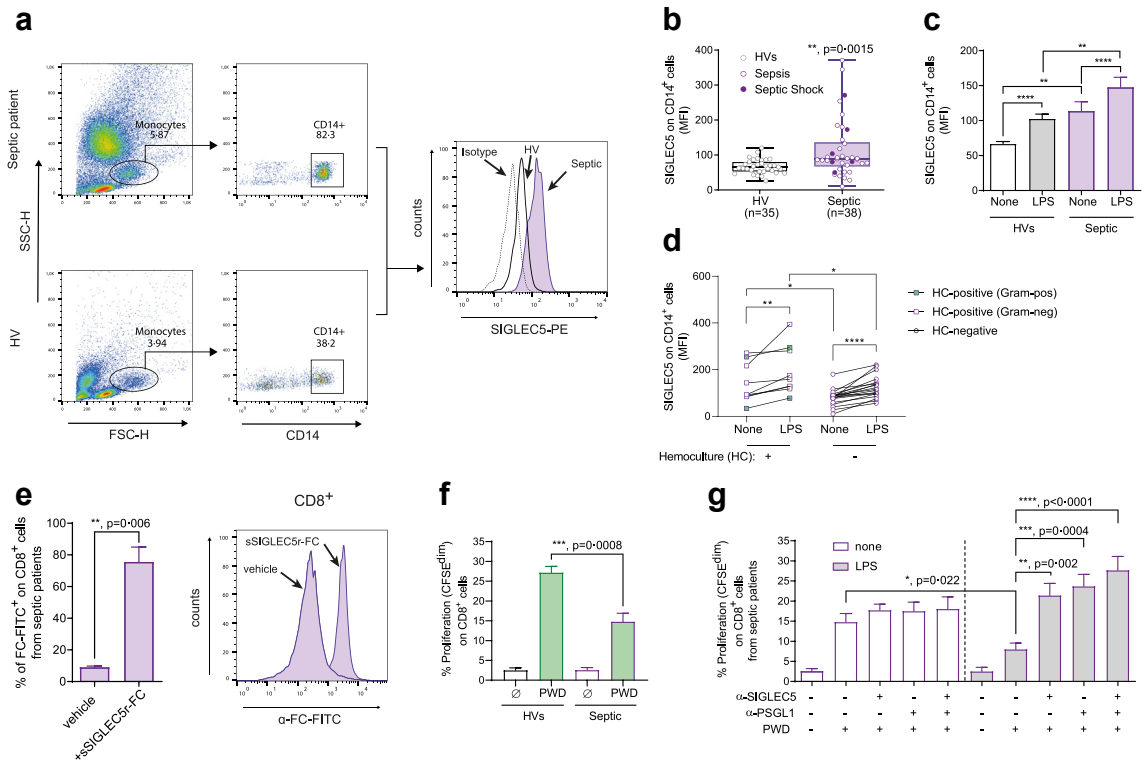
of septic patients with levels of plasmatic sSIGLEC5 above the calculated cut-off (Hazard Ratio [HR] of 3.418, 95% CI, 2.380–4.907,  $p < 0.0001$  by log-rank test, Fig. 6f). Collectively, these data illustrate that plasma concentrations of sSIGLEC5 increase in septic patients compared with non-infectious health conditions, while acting as a severity and *exitus* predictor in septic patients.

## Discussion

We report SIGLEC5 is mainly expressed in myeloid cells, including neutrophils, monocytes, dendritic cells

and at low levels in B cells, as it has been previously described in the Human Atlas Protein database<sup>43</sup> and some other reports.<sup>50,51</sup> However, SIGLEC5 expression is only upregulated on circulating neutrophils and monocytes when they are stimulated with LPS from *E. coli*, simulating an encounter with Gram-negative bacteria. This data matched the high levels of SIGLEC5 expression on circulating monocytes during sepsis, suggesting a critical role in this clinical condition. Nevertheless, neutrophils express the highest levels of SIGLEC5, being the most frequent cell type in circulation and their numbers increased during sepsis.<sup>52</sup> Note that, although the basal expression of SIGLEC5 on neutrophils is





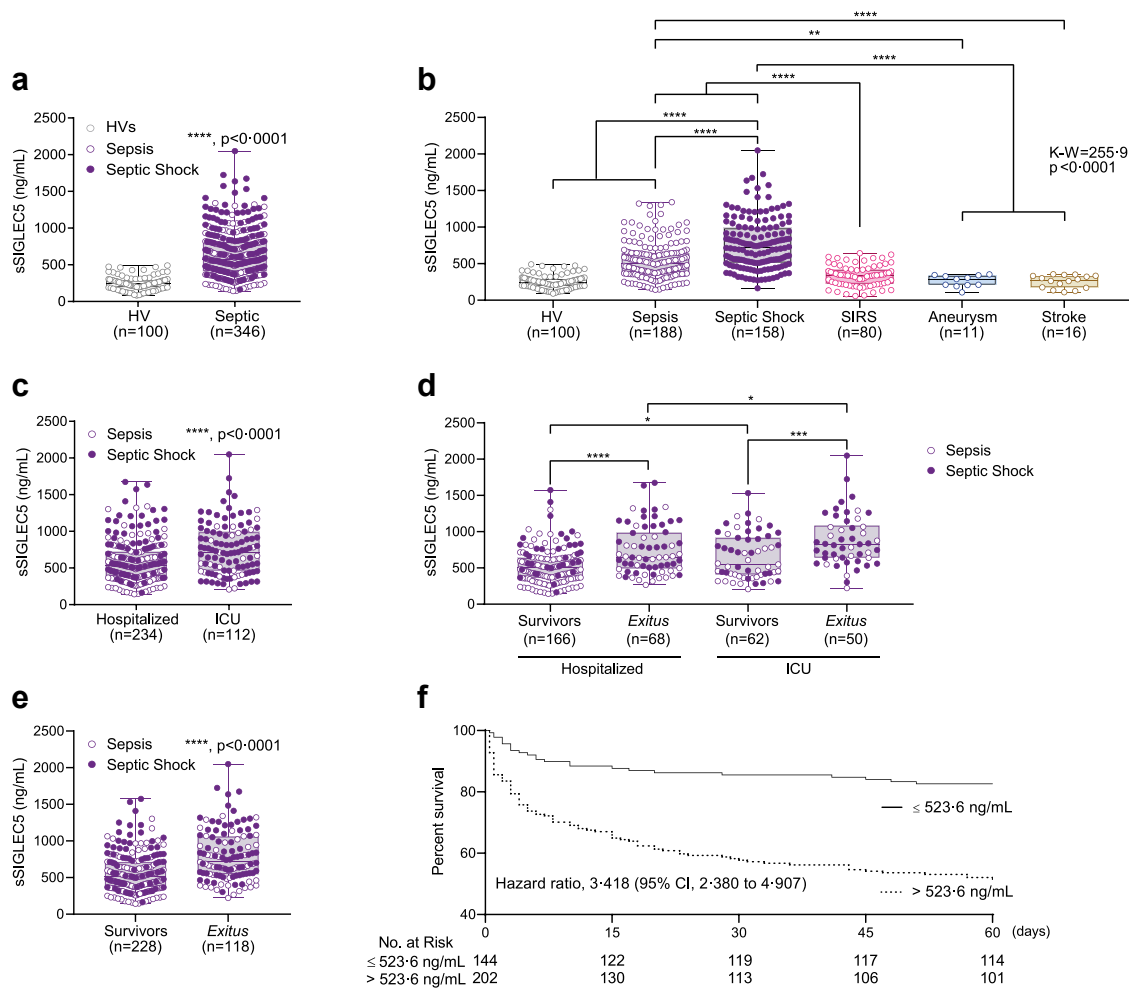
**Fig. 5: Septic patients overexpress SIGLEC5 on monocytes, and blockade of the SIGLEC5/PSGL1 axis restores CD8<sup>+</sup> T cell proliferation.** (a) Flow cytometry gating strategy for CD14<sup>+</sup> cells from both HVs and septic patient (left panels). Representative histogram overlay of SIGLEC5 expression on CD14<sup>+</sup> cells from a HVs (white line) and a septic patient (violet filled line); a dotted line was used as isotype control (right panel). (b) MFI of SIGLEC5 on CD14<sup>+</sup> cells from HVs (grey clear box, n = 35) and septic patients (violet filled box, n = 38). (c) SIGLEC5 expression on monocytes from HVs (grey) and septic patients (violet) challenged or not with 10 ng/mL of LPS for 16 h. (d) SIGLEC5 expression on monocytes from hemoculture-positive (n = 9) and -negative (n = 19) septic patients challenged or not with 10 ng/mL of LPS for 16 h (Violet dots: Hemoculture-negative, violet squares: Hemoculture-positive with gram-negative bacteria, violet squares green filled: Hemoculture-positive with gram-positive bacteria). (e) sSIGLEC5r-FC binding quantification on CD8<sup>+</sup> T cells from patients with sepsis (n = 4) (left panel) and a representative histogram overlay (right panel). (f) Proliferation levels (CFSE<sup>dim</sup>) of CD8<sup>+</sup> T cells from HVs and patients with sepsis in the presence (green filled column) or not (white column) of PWD for 5 days. (g) Proliferation levels (CFSE<sup>dim</sup>) of CD8<sup>+</sup> T cells from patients with sepsis in the presence or not of PWD and co-cultured for 5 days with autologous monocytes pre-challenged (grey filled column) or not (white column) with 10 ng/mL of LPS for 16 h in the presence of anti-SIGLEC5 blocking antibody and/or anti-PSGL1 blocking antibody (n = 6). Data shown as mean ± SEM. (b-d and f) Unpaired t-test (\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001). (c-e and g) Paired t-test (\*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001).

higher than on monocytes, this relationship is reversed in the presence of LPS. Moreover, the interaction of monocytes with T cells during the antigen presentation and the regulation of T cell proliferation by them makes monocytes the best candidate for studying the role of SIGLEC5 in the context of infectious diseases.

We have previously demonstrated the involvement of HIF1α in the regulation of the innate and adaptive immune response during sepsis under hypoxic stress, where blood oxygen saturation and tissue perfusion can be compromised, controlling the transcription of a set of genes including *IRAK-M*, *VEGFA*, and *MMP*.<sup>10,11,44</sup> Herein, we demonstrated the presence of three HREs in the human *SIGLEC5* promoter, and our data confirmed that this gene upregulation in response to LPS is mediated by HIF1α. Furthermore, SIGLEC5 is

cleaved by metalloproteinases, generating a soluble form of this protein, which is significantly upregulated in supernatants of LPS-stimulated monocytes but not in neutrophils.

By exploring the potential role of SIGLEC5 in the regulation of lymphocyte functionality during an infectious process, our findings indicated that the binding of SIGLEC5 to CD8<sup>+</sup> cells is PSGL1-dependent. The latter had previously been described as an immunoglobulin-like cell adhesion molecule that promotes CD8<sup>+</sup> T-cell exhaustion in a murine model of chronic virus infection.<sup>33</sup> However, its ligand was unknown until recently.<sup>32</sup> Our data suggest SIGLEC5 behaves as a ligand for PSGL1, whose binding relies on sialic acid motifs/residues. Moreover, blockade of the SIGLEC5/PSGL1 axis was able to revert the impaired CD8<sup>+</sup> proliferation



**Fig. 6: Soluble SIGLEC5 classified septic patients on admission.** (a) Soluble SIGLEC5 (sSIGLEC5) levels in plasma from HVs (grey clear dots,  $n = 100$ ) and septic patients (sepsis in violet clear dots and shock septic in purple filled dots,  $n = 346$ ). (b) sSIGLEC5 levels in plasma from HVs ( $n = 100$ ) and patients with sepsis ( $n = 188$ ), septic shock ( $n = 158$ ), SIRS ( $n = 80$ ), aneurysm ( $n = 11$ ) and stroke ( $n = 16$ ). (c) sSIGLEC5 levels in plasma from septic patients classified according to their hospitalization ( $n = 234$ ) or their admission to ICU ( $n = 112$ ). (d) sSIGLEC5 levels in plasma from septic patients hospitalized survivors ( $n = 166$ ), hospitalized exitus ( $n = 68$ ), admitted in ICU survivors ( $n = 62$ ) and admitted in ICU exitus ( $n = 50$ ). (e) sSIGLEC5 levels in plasma from patients with sepsis (violet clear dots) and septic shock (purple filled dots) classified according to their outcome after 60 days as Survivors ( $n = 228$ ) and Exitus ( $n = 118$ ). (f) Septic patients were dichotomized according to the optimal cut-off, estimated by Youden index for plasmatic sSIGLEC5 concentration to be 523.6 ng/mL. Kaplan-Meier survival curves from diagnosis to day 60 according to baseline plasmatic sSIGLEC5 ( $\chi^2 = 33.20$ ,  $p < 0.0001$ ) are shown. Data shown as mean  $\pm$  SEM. (a and c-e) Unpaired t-test ( $*p < 0.05$ ;  $***p < 0.001$ ;  $****p < 0.0001$ ). (b) One-way ANOVA test ( $****p < 0.0001$ ). (f) Kaplan-Meier estimation of survival from septic patients according Youden index ( $****p < 0.0001$ ).

without affecting their apoptosis, suggesting this could be a potential pharmacological target in sepsis.

Previous work has focused on the role of SIGLEC5 expressed on lymphocytes, where its expression is triggered upon TCR activation and dampens immune activation.<sup>53</sup> Here, in line with its structural similarities to well-known immunoglobulin-like cell adhesion molecules, we showed that CD8<sup>+</sup> T lymphocyte proliferation could be modulated by sSIGLEC5 or membrane-bound SIGLEC5 on monocytes. Curiously, several authors have

indicated that the number of these effector cytotoxic cells decreases in patients with septic-shock on admission.<sup>54</sup> It is widely accepted that the composition of the naïve pathogen-specific CD8<sup>+</sup> cell repertoire is essential for both the clearance of infection and the generation of memory CD8<sup>+</sup> cells in response to pathogens.<sup>55</sup> This is compatible with SIGLEC5 providing a negative signal for migration of leukocytes to the infected sites, contributing to the immunosuppression stage of sepsis.<sup>33</sup> Thus, an impaired CD8<sup>+</sup> T cell proliferation

together with their low viability can compromise the recovery of septic patients, increasing their risk of suffering from secondary infections and death.

Regarding mouse models used, both endotoxemia and cecal ligation and puncture (CLP) are considered gold standards in sepsis research,<sup>56</sup> with similar mortality rates, but with significant differences in the kinetics and magnitude of cytokine storms. In this context, exogenous sSIGLEC5r has been observed to increase mortality and acute lung injury in both CLP and mice administered with LPS from *E. coli*. Interestingly, when lymphocyte-deficient mice (*Rag1*<sup>-/-</sup>) were subjected to both CLP and injected with exogenous sSIGLEC5r, they did not exhibit any differences in mortality rate. Furthermore, CD8 $\alpha$  depletion was found to protect mice administered with LPS and sSIGLEC5r, thus preventing the accelerated death observed in this model. Thus, it can be concluded that the impact of sSIGLEC5r on the mortality of endotoxin mouse models is dependent on the presence of lymphocytes, specifically on CD8 $\alpha$  T cells.

The data from *in vivo* experiments suggest that the suppression induced by sSIGLEC5r depends on an adaptive immune response, although this response is not necessary for the CLP model. This concept has been observed in other inflammatory settings<sup>57</sup> where the inhibition of a specific protein expressed in a particular population produces a suppressive effect, although the absence of this population does not have any observable effect.

It is important to note the absence of B and T cells (both CD4 and CD8) in *Rag1*<sup>-/-</sup>. We hypothesize that the effects of these populations on CLP-induced polymicrobial sepsis are offsetting one another. Our first CLP *in vivo* model supported the impact of sSIGLEC5 on adaptive immunity (Fig. 4a–c), leading us to develop a more precise and regulated *in vivo* model involving the administration of LPS and specific depletion of CD8 $\alpha$  T cells (Fig. 4d–g). The survival rates between LPS + saline and LPS + anti-CD8 $\alpha$  in this model were not significantly different (Fig. 4f), demonstrating the redundancy of CD8 $\alpha$  T cells in LPS-induced mortality. However, this model also confirmed the specific targeting of sSIGLEC5r on these cells, as the inhibitory effect of sSIGLEC5r was eliminated when CD8 $\alpha$  T cells were depleted (Fig. 4e–g).

Unfortunately, SIGLEC5 does not have a homolog in mice. Both potential candidates, SIGLEC-F and SIGLEC-E, have insufficient levels of identity with the human SIGLEC5. SIGLEC-F shares 45.3% of the sequence with human SIGLEC5, but is mainly expressed in eosinophils in mice, which implies that its functional counterpart is human SIGLEC8. In contrast, SIGLEC-E is thought to be a functional human paralogue of SIGLEC9, primarily expressed on NK cells, or SIGLEC12 due to its high homology with SIGLEC5 (52.4%). This means we are not able to use an

anti-SIGLEC5 antibody in mice models of endotoxemia or CLP.

*In vivo* results support the specific effect observed *in vitro* on human cells, where monocyte-expressed SIGLEC5 or its soluble form is found to attenuate CD8<sup>+</sup> T cell proliferation. Collectively, these outcomes suggest that SIGLEC5 could be functioning as an immunoregulatory ligand.

The relevance of all these experimental data was reflected in the clinic. Monocyte-bound and soluble SIGLEC5 levels in plasma are increased in septic patients but not in patients with non-infectious Systemic Inflammatory Response Syndrome (SIRS), aneurysm or stroke, or healthy donors. It is worth noting that non-infectious SIRS patients suffer from a pro-inflammatory phenotype that is not produced by any endotoxin, such as burn patients, trauma, or ischemia, which suggests that the SIGLEC5 induction is LPS-dependent or involves pathways with similar downstream signals. Interestingly, the expression of SIGLEC5 on CD14<sup>+</sup> cells was markedly higher in septic patients with positive bacterial haemocultures than in those with negative bacterial haemocultures. Moreover, *ex vivo* LPS stimulation further augmented SIGLEC5 expression in the haemoculture-positive septic patients, implying that SIGLEC5 has a broad range of expression which spans from untreated healthy volunteers to LPS-treated septic patients, as shown in Fig. 4c. In addition, plasma levels of soluble SIGLEC5, generated by metalloproteinase shedding, are increased in septic patients compared to healthy volunteers (HVs), with higher levels in septic shock than sepsis patients. We emphasize that the above was demonstrated using a multicentre cohort of patients. It could additionally serve as a biomarker of sepsis evolution, being higher in patients with poor prognosis. Note that the soluble form of SIGLEC5 has been identified by ourselves as a prognosis indicator in some cancer contexts, confirming it can be used as plasmatic biomarker in several diseases with immune component associated.<sup>58,59</sup>

Along these lines, ROC analysis showed a robust AUC for sSIGLEC5 as predictor of requirement of admission to the ICU and as an *exitus* predictor in sepsis. Thereby, the optimal cut-off values, calculated based on the Youden index, revealed that sSIGLEC5 plasma levels on admission and before any treatment, indicated a poor prognosis of septic patients. These data could constitute a feasible score to incorporate into routine analysis of patients achieving sepsis criteria upon arrival in hospital admission.

Therefore, we have reported an IC ligand, transcriptionally regulated by HIF1 $\alpha$ , which could be therapeutically targeted in septic patients. Our results show SIGLEC5 is acting as an IC ligand on T cell lymphocytes through PSGL1, especially on CD8<sup>+</sup> cells, which could be potentially blocked with anti-SIGLEC5, anti-PSGL1 antibodies or other small molecules. Furthermore, two

mouse models of sepsis show that soluble SIGLEC5 decreases survival in a CD8 $\alpha$ -dependent manner. In terms of clinical application, one of the first questions to emerge would be identifying which septic patients could benefit from the blockade of the SIGLEC5/PSGL1 axis. All in all, our data support that the determination of sSIGLEC5 at admission could be used as prognosis biomarker as well as the therapeutic potential of SIGLEC5/PSGL1 blocking strategies in sepsis.

#### Contributors

López-Collazo, E. performed the conceptualisation of the study. Lozano-Rodríguez, R. and Avendaño-Ortiz, J. performed the main experiments and investigation. Pelegrin, P., García-Palenciano, C., Ruiz-Rodríguez, J.C., Ferrer, R., González-López, J.J., Fàbrega, A., Martín-Quirós A., and Maroun-Eid, Ch. recruited the septic patients and followed-up them. Gutiérrez-Fernández, M. and Alonso-López, E. recruited patients with stroke. Cubillos-Zapata, C. contributed samples from patients with aneurysms. Lozano-Rodríguez, R., Montalbán-Hernández, K., Terrón-Arcos V., and Pérez de Diego R. performed specific *in vitro* experimental assays. Lozano-Rodríguez, R., del Fresno, C., Terrón-Arcos, V., and Avendaño-Ortiz, J. performed *in vivo* assays. Lozano-Rodríguez R. and Avendaño-Ortiz, J. performed statistical analyses. López-Collazo, E., del Fresno, C., Lozano-Rodríguez, R., Cueto, F.J., and Avendaño-Ortiz, J. discussed the results. López-Collazo, E. and Lozano-Rodríguez, R. wrote the manuscript. del Fresno, C., Cueto, F.J., Pérez de Diego, R., Pelegrin, P., and Fernández-Velasco, M. performed a critical review of the manuscript. All authors read and approved the final version of the manuscript. In addition, the authors declare no competing financial interests. Note that more than one author has accessed and verified the data, in this case: Lozano-Rodríguez, R., Avendaño-Ortiz, J., del Fresno, C., and López-Collazo, E.

#### Data sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

#### Declaration of interests

We declare that none of the authors have any conflicts of interest with the content of this manuscript.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2023.104841>.

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