ELECTRONIC SUPPLEMENTARY MATERIAL

INCREASED CIRCULATING REGULATORY T CELLS ($CD4^+CD25^+CD127^-$) CONTRIBUTE TO LYMPHOCYTE ANERGY IN SEPTIC SHOCK PATIENTS

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Patients and Methods

Patients

Septic shock patients admitted to the Intensive Care Units (ICUs) at Lyon-Sud University Hospital were prospectively included in the study. The diagnosis of septic shock was based on criteria of the ACCP/SCCM [E1], as defined by an identifiable site of infection, evidence of a systemic inflammatory response manifested by at least two of the following criteria: a) a body temperature >38°C or <36°C, b) a heart rate >90 beats per min, c) a respiratory rate >20 breaths per min, d) a white blood cell count >12,000/mm^3 or <4,000/mm^3, and a blood pressure no higher than 90mmHg despite fluid resuscitation and requiring vasopressor therapy (more than 5µg/kg of body weight of epinephrine and/or norepinephrine and/or dobutamine). Severity of septic shock was assessed by the Simplified Acute Physiology Score II [E2]. Mortality was defined as death occurring within 28 days after diagnosis. To provide a panel of reference values, we included 17 healthy volunteers from our laboratory (age: 22-54, 7 female – 10 males). Whole blood (2ml) was obtained once in EDTA anticoagulant tubes between day 3 and day 7 after the diagnosis of septic shock. All studies were approved by our hospital's institutional review board and carried out in accordance with the human experimentation guidelines for clinical research of our institute.

Flow cytometry on peripheral blood from patients and healthy volunteers

Flow cytometric (FC500, Beckman-Coulter, Hialeah, FL, USA) expression of cell surface and intracellular markers was assessed on EDTA-anticoagulated peripheral blood from patients and healthy volunteers. Monoclonal antibodies and their respective isotype controls were used according to manufacturer's recommendation: ECD-labeled anti-CD4, PE-labeled anti-CD127, PC5-labeled anti-CD25 (Immunotech, Marseille, France), FITC-labeled anti-Foxp3 (eBioscience, San Diego, CA). Red blood cells were lysed using the automated TQ-Prep lysing system or Versalyse reagent (Beckman-Coulter, Miami, FL) in the case of Foxp3 intracellular staining. Monocyte Human Leukocyte Antigen-DR (HLA-DR) expression was measured as already described [E3]. For absolute count measurements, the instrument was calibrated using Flow-count fluorospheres™

according to the recommendations of the manufacturer (Beckman-Coulter, Miami, FL). Results are expressed as percentages of $CD4^+CD25^+CD127^-$ cells out of the total $CD4^+$ lymphocytes and given as the number of lymphocytes per µl of whole blood.

Isolation of human peripheral blood mononuclear cells (PBMCs) from whole blood and cell culture conditions

Depending on the assay to be run subsequently, PBMCs were isolated from either heparinized or EDTA-anticoagulated blood over a Ficoll-Paque density gradient according to the manufacturer's description (PAA laboratories, Austria). Cells were subsequently cultured in RPMI 1640 medium supplemented with 10% bovine fetal serum, 2 nM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Saint-Louis, USA) in 96-well plates. Cells were incubated 4 days at 37°C in a humidified 5% CO_2 atmosphere.

Mixed cell proliferative response of human PBMCs to mitogens

PBMCs were seeded in flat-bottom 96-well microtitre plates (2×10^5 cells / 200 µl) and stimulated with 10 µg/ml phytohaemagglutinin (PHA, Oxoid, Dardilly, France) or 0.1 mg/ml concanavalin A (ConA, Sigma, L'Isle D'Abeau, France) or 20 µg/ml pokeweed (PWD, Sigma). [methyl-^{3}H]-Thymidine (10 µCi/ml, Amersham life Science, Sarclay, France) was added 24 hours before harvesting cells on fibreglass filters using an automated cell harvester (Filtermate, PerkinElmer life and Analytical Sciences, Waltham, MA, USA). Incorporated radioactivity was measured in a direct beta counter (Matrix96 PerkinElmer life and Analytical Sciences). The assays were carried out in triplicate.

Mixed cell proliferative response of purified human $CD4^+CD25^-CD127^+$ T lymphocytes to PHA

Non-$CD4^+$ T cells were stained using a cocktail of biotin-conjugated monoclonal anti-human antibodies against CD8, CD14, CD16, CD19, CD56, CD123, TCRγδ and CD235a (10 µl/10^7 total cells), incubated for 30min at 4°C, magnetically labeled with antibiotin microbeads (20 µl/10^7 total cells), incubated for 30 min at 4°C and depleted over a MACS® MS column (purity of purified $CD4^+$ lymphocytes > 90%). $CD4^+CD25^+$ T cells

were directly labeled with anti-CD25 microbeads (10 µl/10^7 total cells), incubated for 30 min at 4°C and positively selected using MACS® MS column according to manufacturer's recommendation (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was assessed by CD4/CD25/CD127/intracellular Foxp3 staining. $CD4^+CD25^-$ lymphocytes (1×10^5 cells / 200 µl) were immediately cultured in the presence of an increasing number of $CD4^+CD25^+$ lymphocytes and of PHA (10 µg/ml).

Murine model of polymicrobial sepsis

Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) 8 to 10 weeks of age, which had been acclimated in our animal facility no less than 7 to 10 days, were used for this study. All the experiments performed on mice were carried out in accordance with the National Institutes of Health Guidelines on laboratory animals and were approved by the Rhode Island Hospital Committee on Animal Use and Care.

Polymicrobial sepsis was induced in mice using the model of cecal ligation and puncture (CLP) as previously described by our laboratory [E4]. The animals were lightly anesthetized with isoflurane, shaved on the abdomen and scrubbed with betadine. A 1-cm midline incision was made below the diaphragm to expose the cecum. The cecum was ligated with a 4-0 silk so as to prevent intestinal obstruction, and punctured twice with a 21-gauge needle and gently compressed to extrude a small amount of cecal contents. The cecum was then returned to the peritoneal cavity and the abdominal incision was closed in two layers (all incisions were bathed in xylocaine). Saline (4 ml/100 × g body weight) was given subcutaneously to resuscitate the animal. For sham controls, the cecum was extracted but neither ligated nor punctured. The mice were then returned to their cages and provided food and water *ad libitum*.

Sample Collection – Preparation of murine splenocytes

Mice were sacrificed by inhaled CO_2 overdose 24h after CLP/sham. Spleens were harvested as previously described by our laboratory in a sterile fashion and gently ground to produce a single-cell suspension between frosted microscope slides [E5]. After hypotonic lysis of erythrocytes, splenocytes were washed once with PBS and

resuspended in RPMI medium containing L-glutamine, antibiotics and serum. Splenocytes were counted and cell viability was assessed by Trypan Blue exclusion.

Flow cytometry on murine splenocytes

Percentages of $Foxp3^+$ or $CD127^-$ cells among $CD4^+CD25^+$ splenocytes were measured by flow cytometry (BD FACSArray, San Diego, CA). Monoclonal antibodies and their respective isotype controls were used according to manufacturer's recommendation: PECy7-labeled anti-CD4, PE-labeled anti-CD25 and APC-labeled anti-Foxp3 or anti-CD127 (eBioscience, SanDiego CA). For intracellular Foxp3 staining, splenocytes were permeabilized using eBioscience Fixation/Permeabilization kit. Results are expressed as percentages of $CD4^+CD25^+CD127^-$ or $Foxp3^+$ cells out of $CD4^+$ lymphocytes.

Proliferative response of murine splenocytes after transfection with siRNA

Immediately after harvesting, 2×10^6 splenocytes were transfected with 2µM Foxp3 siRNA (sense sequence: 5'-P-GAAUUUGAGUUUCGCAAGAUUdTdT-3'; anti-sense sequence: 5'-P-PUCUUGCGAAACUCAAAUUCUUdTdT-3') or control siRNA (Dharmacon, Lafayette, CO) following manufacturer's instructions for mouse T-cell electroporation kit (Amaxa, Gaithersburg, MD). After overnight incubation (16h), cells were centrifuged, counted again and assessed for viability using Trypan Blue exclusion and for decreased intracellular Foxp3 expression by flow cytometry. Cells were then stained with PKH26 per the manufacturer's instructions (Sigma, St. Louis, MO) and cultured in 96-well plates with or without ConA (0.1 mg/ml - 1×10^6 cell per ml) for 5 days. Proliferation was assessed by flow cytometric measurement of the decrease in PKH26 (yellow) fluorescence on viable cells selected based on SSC/FCS characteristics. Results are presented as proliferation ratio (i.e. [percentage of cells with decreased fluorescence (e.g. divided cells) / percentage of highly fluorescent cells (e.g. non-divided cells)] $\times$ 100).

Statistical analysis

Results are presented as Mean $\pm$ SEM. Comparisons between groups were made using Student t-test, non-parametric Mann-Whitney U test or Wilcoxon paired test. Spearman

rank coefficient was used for correlation test. A p-value < 0.05 was regarded as significant without correction of the number of tests performed.

Supplementary Results

$CD4^+CD25^+CD127^-$ lymphocytes express Foxp3 in healthy individuals and septic shock patients and possess regulatory properties.

CD127 has recently been proposed as a negative marker for Treg [E6-E7]. The goal of our first set of experiments was to verify this result on purified cells from healthy individuals. $CD4^+CD25^+$ lymphocytes were therefore purified from PBMCs using magnetic beads and monitored for CD127 and intracellular Foxp3 expression as well as for their capacity to regulate responder lymphocyte's proliferation. We observed that the positive fraction of $CD4^+CD25^+$ lymphocytes was enriched in $CD127^-$ cells (70%, Figure E1) whereas $CD4^+CD25^+CD127^-$ cells were almost totally depleted from the negative fraction ($CD4^+CD25^-$) of the purification (1%, Figure E1-B). Moreover, the majority of these cells expressed intracellular Foxp3 (>90%, Figure E1-C), whereas we were unable to detect any Foxp3 expression among cells of the CD25 negative fraction (Figure E1-D). Finally, we confirmed that these $CD4^+CD25^+CD127^-$ cells possess regulatory properties that suppress $CD4^+CD25^-$ lymphocytes proliferation in response to PHA in a dose-dependent fashion (Figure E2). This confirmed that $CD4^+CD25^+$ lymphocytes from healthy individuals that do not express CD127 possess the phenotype as well as the regulatory capacity depicted for Treg.

Supplementary References

E1. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ (1992) Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest 101:1644-1655

E2. Le Gall JR, Lemeshow S, Saulnier F (1993) A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. JAMA 270:2957-2963

E3. Monneret G, Lepape A, Voirin N, Bohé J, Venet F, Debard AL, Thizy H, Bienvenu J, Gueyffier F, Vanhems P (2006) Persisting low monocyte human leukocyte antigen-DR expression predicts mortality in septic shock. Intensive Care Med 32:1175-1183

E4. Ayala A, Perrin MM, Kisala JM, Ertel W, Chaudry IH (1992) Polymicrobial sepsis selectively activates peritoneal but not alveolar macrophage to release inflammatory mediators (IL-1, IL-6 and TNF). Circ Shock 36:191-199

E5. Ayala A, Deol ZK, Lehman DL, Herdon CD, Chaudry IH (1994) Polymicrobial sepsis but not low dose endotoxin infusion causes decreased splenocyte IL-2/IFN-gamma release while increasing IL-4/IL-10 production. J Surg Res 56:579-585

E6. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, Kelleher A, Fazekas de St Groth B (2006) Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 203:1693-1700

E7. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF, Bluestone JA (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 203:1701-1711

Supplementary Figures

Figure E1: Flow cytometry phenotyping of lymphocytes purified from healthy individuals.

Peripheral blood mononuclear cells (PBMCs) were harvested from healthy individuals heparinized peripheral blood (n = 2). $CD4^+CD25^+$ and $CD4^+CD25^-$ lymphocytes were subsequently purified using magnetic beads and stained by flow cytometry using a 4-color staining (CD4-ECD – CD25-PC5- CD127-PE – intracellular Foxp3-FITC). Flow cytometry dot-plots of the $CD4^+CD25^-$ (negative fraction – right panel) or $CD4^+CD25^+$ (positive fraction – left panel) fractions for one representative cell purification experiment are shown. **A/B**/ CD25 / CD127dot plots. **C/D/** CD25 / Foxp3 dot plots.

Figure E2: Proliferative response of $CD4^+CD25^-$ lymphocytes purified from healthy individuals.

Peripheral blood mononuclear cells (PBMCs) were harvested from healthy individuals heparinized peripheral blood (n = 2). $CD4^+CD25^+$ and $CD4^+CD25^-$ lymphocytes were subsequently purified using magnetic beads. $CD4^+CD25^-$ lymphocytes (CD25-) were cultured in 96-well plates in complete RPMI medium with PHA (1×10^5 cells / 200 µl - PHA - 10 µg/ml) in the presence of an increasing number of $CD4^+CD25^+$ (Treg - black bars - ratio ranging from 1/1 to 1/10 vs $CD25^-$ cells). Proliferation was measured by the increased incorporation of [methyl-^{3}H]-Thymidine. The assays were carried out in triplicate. Results are presented as mean ± SEM of counts per minute (cpm) of 2 independent experiments.

Figure E1

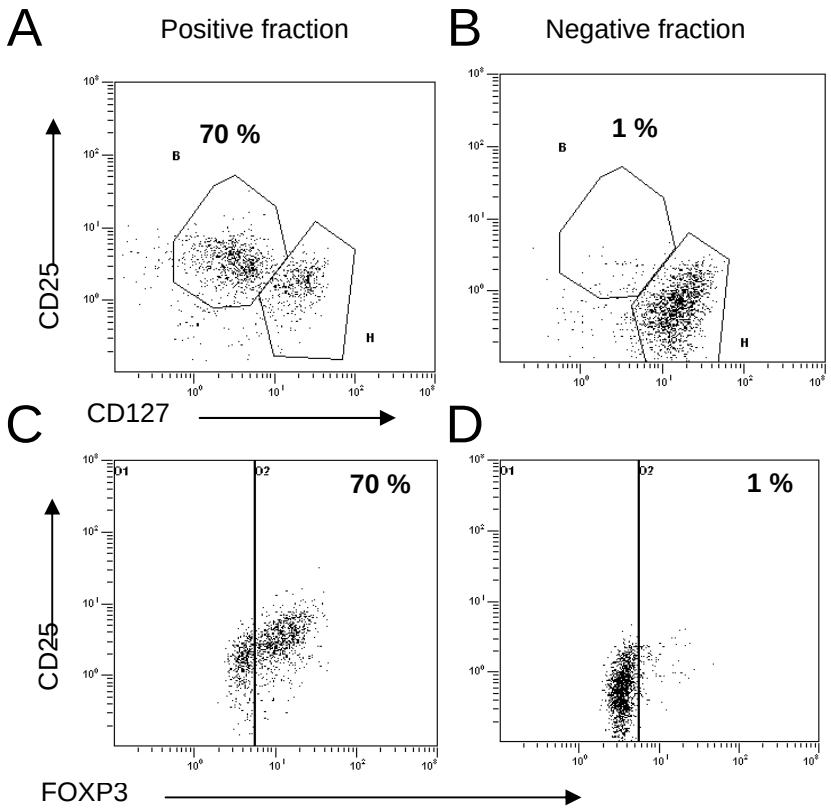
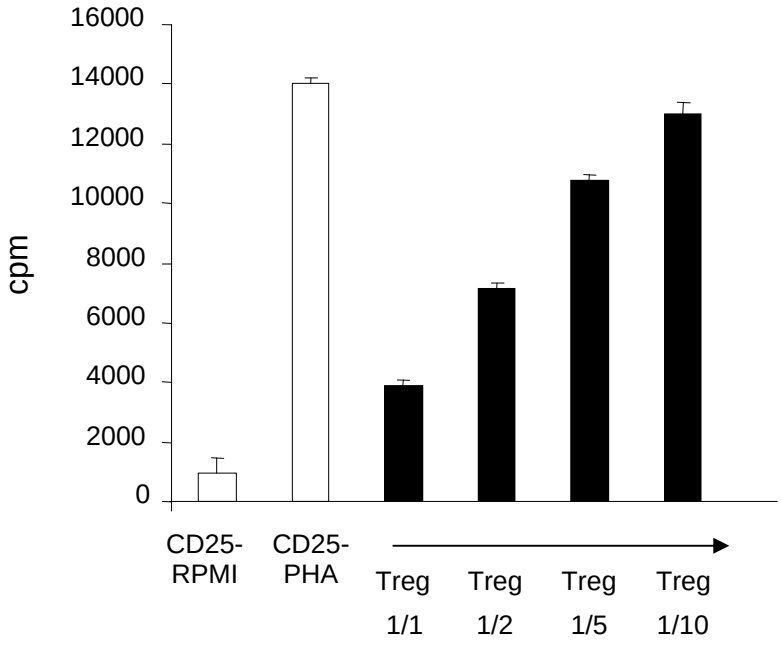

A
Positive fraction
B
Negative fraction
70 %
1 %
CD25
CD127
C
D
70 %
1 %
CD25
FOXP3

Figure E2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