Supporting Information

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SI Materials and Methods

Cecal Ligation and Puncture. Mice were lightly anesthetized using isoflurane, a midline incision (~1 cm) was made below the diaphragm, exposing the cecum, then the cecum was ligated distally and punctured through with a 22-gauge needle. In the control animals (sham), the cecum was located and mobilized but was neither ligated nor punctured. The abdominal incision was then closed in layers with an Ethilon 6.0 suture, and the animals were resuscitated with 1.0 ml lactated Ringer’s solution by s.c. injection. Animals were subsequently allowed food and water ad libitum.

Histological Examination. The 10% formalin-fixed, paraffin-embedded kidney sections were stained with periodic acid-Schiff (PAS) reagent. Histological changes in the cortex were assessed by quantitative measurements of tissue damage. Tubular damage was defined as tubular epithelial swelling, flattened lining cells, reduced or absent brush border staining, dilated proximal tubules, vacuolar degeneration, necrotic tubules, desquamation, and cast formation. The degree of kidney damage was estimated at 400× magnification using five randomly selected fields for each animal by the following criteria: 0, normal; 1, areas of damage <25% of tubules; 2, damage involving 25–50% of tubules; 3, damage involving 50–75% of tubules; 4, 75–100% of the area being affected. The slides were evaluated by an investigator blinded to the identities of samples.

Patient Information. Five patients, who were diagnosed with septic shock according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine, were enrolled in this study along with 5 healthy volunteers. The institutional ethics committee of Hospices Civils de Lyon (Lyon, France) reviewed and approved this observational study. Septic shock was defined as tubular epithelial swelling, flattened lining cells, reduced or absent brush border staining, dilated proximal tubules, vacuolar degeneration, necrotic tubules, desquamation, and cast formation. The degree of kidney damage was estimated at 400× magnification using five randomly selected fields for each animal by the following criteria: 0, normal; 1, areas of damage <25% of tubules; 2, damage involving 25–50% of tubules; 3, damage involving 50–75% of tubules; 4, 75–100% of the area being affected. The slides were evaluated by an investigator blinded to the identities of samples.

Flow Cytometry. Peripheral blood leukocytes were isolated from whole blood samples taken from volunteers or patients. Briefly, flow-cytometric (FC500, Beckman-Coulter) expression of monocyte-surface PD-1 was assessed on peripheral whole blood collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes. Monoclonal antibodies were used according to the manufacturer’s instructions; these antibodies were PC5-conjugated anti-CD4 (clone 13B8.2, Beckman-Coulter) and phycoerythrin (PE)-conjugated anti-PD-1 (clone M1H4, BD bioscience). The red blood cells (RBC) were lysed with fluorescent activated cell sorting (FACS) lysing solution (BD Bioscience). The red blood cells (RBC) were lysed with fluorescent activated cell sorting (FACS) lysing solution (BD Bioscience). Monocytocytes were gated on CD4low staining cells. To determine cell surface expression of PD-1 on mouse blood leukocytes, 50 μl of whole blood was mixed with antigen-presenting cell (APC)–conjugated anti-CD11b (clone M1/70), PE-conjugated anti-PD-1 (clone J43), and Fc blocker, and was incubated on ice for 45 minutes. Red blood cells were lysed with ammonium chloride potassium (ACK) buffer and samples were washed and analyzed on a FACSArray flow cytometry (BD Bioscience). Peritoneal leukocytes and splenocytes were isolated and assayed as described. Anti-F4/80 (clone BM8), anti-CD4 (clone RM4-5), anti-CD8 (clone 53–6.7), anti-CD11c (clone N418), and anti-B220 (clone RA3–6B2) antibodies along with the appropriate isotype controls were purchased from eBioscience.

Evaluation of Systemic and Local Bacterial Burden. Blood samples were collected and spread on tryptic soy agar (TSA) blood agar plate (Remel) with dilutions of 1, 10, or 100 μl in 100-μl saline aliquots. Peritoneal lavage fluid was harvested after injecting 2 ml of PBS into the peritoneum and serial dilution in sterile saline. A 100-μl aliquot of each dilution was spread on a TSA blood agar plate. All plates were incubated at 37 °C for 24–48 hours. Colonies were counted and expressed as CFU/100 μl for blood samples or CFU/ml for peritoneal lavage samples.

Phagocytosis Assay. Peritoneal leukocytes were plated in 24-well tissue culture plates at 1 × 10⁶/ml well and incubated in complete RPMI medium 1640 at 37 °C. At 90 minutes later, non-adherent cells were washed off with PBS. Adherent macrophages were then co-cultured with ~1 × 10⁶ fluorescein-conjugated E. coli (Molecular Probes) in PBS at 37 °C for 1 hour and then were washed completely with PBS. The addition of 0.25 mg/ml trypan blue solution quenched the fluorescence of extracellular particles. The phagocytosis of fluorescein-conjugated E. coli by macrophages was analyzed by a Nikon ECLIPSE TE2000-U microscope (Nikon). The mean fluorescence integrated intensity (MFI) per cell was calculated with MetaVue software (Meta Imaging Series 6.1, Universal Imaging) and used as a measure of phagocytosis. Approximately 150–300 cells per field were assessed by transforming individual cells from each image into MFI of fluorescence per cell (two to three mice per group).

Peritoneal Macrophage Isolation and Characterization. Peritoneal lavages were performed using 10 ml of PBS. Lavages containing peritoneal cells from individual mice were processed separately. Cells were pelleted and resuspended at a concentration of 10⁶ macrophages/ml in RPMI 1640 containing 10% fetal calf serum, 2 mmol/l L-glutamine, and 5 μg/ml gentamicin. Macrophages were plated in 24-well tissue culture plates at 1 ml/well and incubated at 37 °C in 5% CO₂ for 90 minutes. Adherent macrophages were cultured with media only, or media containing lipoteichoic acid (LTA) (2 μg/ml) or lipopolysaccharide (LPS; 100 ng/ml) for 24 hours. Supernatants from this in vitro culture were collected and the concentrations of cytokines were determined by enzyme-linked immunosorbent assay (ELISA). Cells left in wells were quantified by a CyQuant proliferation assay kit (Molecular Probes) and cytokine concentrations were adjusted by the ratio of cell numbers from each mouse. mRNA expression was detected by polymerase chain reaction. Primers: PD-1 5′–TTCAAGGCGATGGTCTTG (sense) and 5′–AGAGGCCAAGAACATGTC (antisense); β-actin 5′–GAATCCGTTGCTGACATC (sense) and 5′–GCTGATCCACATCCTGCTG (antisense).

Cytokine, Chemokine, and Serum Chemistry Determination. Concentrations of murine TNF-α, IL-6, IL-1β, IFN-γ, IL-12(p70), IL-10, CXCL2, and CCL2 were measured in cell-free peritoneal lavage
fluid, plasma, and cell culture supernatants using sandwich ELISA (BD Biosciences and R&D Systems) as previously described. Serum levels of creatinine were measured by using the Creatinine Parameter Assay Kit from R&D Systems.

**In Vivo Macrophage Depletion.** Cl2MDP (clodronate) was a gift of Roche Diagnostics (Mannheim, Germany). Clodronate liposomes were kindly provided by Nico van Rooijen (Vrije Universiteit-VUMC, Amsterdam). Mice were injected with 0.2 ml of a suspension of clodronate liposomes or PBS intraperitoneally 48 hours before being subjected to cecal ligation and puncture (CLP) surgery. This treatment was confirmed to deplete >95% of F4/80+ macrophages in the peritoneal cavity from 24 to 72 hours after treatment, as detected by flow cytometry.

**Statistical Analysis.** For survival studies, the log-rank test was used to determine significance. All other data are shown as the mean ± SEM and analyzed with the Mann-Whitney rank-sum test if they were frequency/intensity data. Parametric data were analyzed with the unpaired Student’s t test as indicated. A P value <0.05 was considered statistically significant; p values <0.01 and <0.001 are also indicated.
WT PD-1-/- sham CLP

$\text{p} = 0.038$ $\text{p} = 0.004$

mg/dL

A

Cortex

B

$\text{p} = 0.029$

sham CLP sham CLP

Fig. S1. PD-1–deficient mice displayed less severe renal injury during sepsis in comparison with WT mice. (A) Serum creatinine levels in septic PD-1–deficient mice are significantly lower than levels in septic WT mice. At 48 hours after surgery, mice were killed and bled. Concentration of creatinine in serum was determined by picric acid methods. Graphs depict data (mean ± SEM) pooled from two independent experiments. (sham-treated mice, $n = 4–5$; CLP-treated mice, $n = 6$) (B) The tubular damage score of septic WT mice is significantly higher than that of septic PD-1–/- mice. The tubular damage score of cortex was measured on the periodic acid-Schiff–stained kidney sections. Values are mean ± SEM (sham-treated mice, $n = 2$; CLP-treated mice, $n = 4$) $P$ value is determined by Mann-Whitney test.
Fig. S2. PD-1–deficient mice displayed a less severe cytokine storm and improved bacterial clearance during sepsis in comparison with WT mice. (A) PD-1−/− mice manifested significantly lower systemic levels of inflammatory cytokines at 6 hours and 24 hours post-CLP than did WT mice. At 6 or 24 hours post surgery, mice were killed and bled. Concentration of cytokines in plasma was determined by ELISA. Graphs depict data (mean ± SEM of 4–8 sham-treated and 11–13 CLP-treated mice) pooled from three independent experiments. (B) PD-1−/− mice had lower levels of inflammatory cytokines in the peritoneum than WT mice at 24 hours post-CLP. Graphs depict data (mean ± SEM of 4 sham-treated and 20–21 CLP-treated mice) pooled from three independent experiments. N.D., not detectable. *P < 0.05, **P < 0.01, and ***P < 0.001, CLP-treated PD-1−/− mice vs. CLP-treated WT mice, by Mann-Whitney test. (C) PD-1−/− mice had significantly enhanced local bacterial clearance. At 4 hours (n = 7–10) and 24 hours (n = 10–11) after CLP, mice were subjected to 2-ml peritoneal washes with sterile saline. Serial 10-fold dilutions were spread on TSA plates and incubated at 37 °C for 24–48 hours. Levels of aerobic bacteria were expressed as CFU per milliliter of peritoneal lavage fluid. Graphs depict data pooled from three independent studies showing similar results. Horizontal bar indicates median for each group. P value as indicated by Mann-Whitney test.
Fig. S3. mRNA expression of purified macrophage from sham-treated and CLP-treated mice for 24 hours. (A) Peritoneal macrophages were purified by adhering peritoneal lavage fluid on plastic plate (Left) or by anti-F4/80 antibody positive selection (Right). β-Actin served as internal control. (B) Densitometric quantification of PCR results.
Fig. S4. Change in bacterial phagocytosis observed in peritoneal macrophages from WT as compared with PD-1⁻/⁻ septic mice is antibody independent. Macrophages were fed with opsonized or nonopsonized pHrodo-stained bioparticles. Phagocytosis was assayed by flow cytometry. Data are representative of experiments performed no less than two times each.
Fig. S5. Impaired cytokine production by macrophages was partially prevented by PD-1 deficiency during sepsis. Adhered macrophages were cultured with media only, or media containing LTA or LPS for 24 hours and supernatants were collected for ELISA. Cytokine concentrations are normalized by adhered cell numbers, which were counted by CyQUANT kit. *P < 0.05, **P < 0.01, and ***P < 0.001, septic PD-1−/− macrophage vs. septic WT macrophage by Mann-Whitney rank-sum test. Graphs depict data (mean ± SEM of 5–7 mice in sham-treated group [S] and 10–12 mice in CLP-treated group [P]) pooled from two independent experiments.
Peritoneal macrophages are critical for regulating the cytokine response seen in PD-1−/− mice during sepsis. Concentrations of cytokines in peritoneal lavage fluid (sham n = 4, CLP n = 14–21) were measured by ELISA. *P < 0.05, and **P < 0.01, CLP-treated PD-1−/− mice vs. CLP-treated WT mice; ●, P < 0.05, and ◆◆◆◆ P < 0.001, macrophage depleted PD-1−/− mice vs. macrophage intact PD-1−/− mice by Mann-Whitney test. Graphs depict data (mean ± SEM) pooled from two to three independent experiments.
Fig. S7. PD-1 may be a tolerance marker for circulating monocytes in sepsis. (A) PD-1 expression on circulating mouse blood monocytes was up-regulated after CLP. The percentage of PD-1$^+$ peripheral blood monocytes was significantly increased during sepsis. At 12, 24, and 48 hours after CLP, PD-1 expression on peripheral blood monocytes (gated by CD11b$^+$) was analyzed by flow cytometry. ** $P < 0.01$, CLP-treated vs. sham-treated mice, by Mann-Whitney test. Graphs depict data (mean ± SEM) pooled from three independent experiments (sham-treated mice, $n = 5–8$; CLP-treated mice, $n = 9–11$). (B) Representative data of up-regulated PD-1 expression on blood monocytes from patients with septic shock. CD4$^{low}$ peripheral monocytes (circled; Left) from a healthy volunteer (filled) and septic shock patient (black line; Right).