Online supplemental text

Supplemental methods.

The study protocol received prior approval by both the National Institutes of Health (Bethesda, US) and the Charles Darwin (Paris, France) ethical committees for animal care

Cecal ligation and puncture model

Cecal ligation and puncture model (CLP) was performed in aged (42-44 weeks old) male TG or WT C57BL/6 mice with free access to water and chow before and after surgery. Under isoflurane anesthesia, a 4-0 silk ligature was placed 8mm from the cecal tip. The cecum was punctured twice with a 21- gauge needle and gently squeezed to express a 1mm column of fecal material. Prewarmed normal saline (NS; 1 ml) was injected intraperitoneally. Treatment with fluid and antibiotic was started at 6 h after surgery with subcutaneous injection of imipenem/ cilastatin (14 mg/kg) in 1ml of NS. Animals were euthanized 6h and 24h after surgery for collecting specimens. In the survival study, treatment was continued every 12 h with imipenem/cilastatin (7 mg/kg) in 1ml of NS, during 7 days. A clinical score was assigned to the mice every 12h: depressed respiratory rate (2), apneustic respiration (5), spontaneous activity without stimulus (0), activity in response to tactile stimuli (1), delayed activity in response to tactile stimuli (2), unresponsive to tactile stimuli (5), piloerection (1), and lack of eye grooming (1). Animals with a score > 5 were considered as “pre-mortem” mice, and euthanized.

Calpain activity assay

Fresh tissues were weighted and homogenized in imidazole buffer (100µL/20mg) (63,2mM imidazole, 1mM EDTA, 10mM EGTA, 10mM 2-mercaptoethanol, pH 7.3) then centrifugated at 15 000G for 30min at 4°C. Supernatant (cytosolic fraction) were then diluted (1/5) in

imidazole buffer supplemented with calcium (63, 2mM imidazole, 10mM 2-mercaptoethanol, CaCl2 2mM). These samples, in wells of a 96-well plate, were exposed at 37°C to the substrate N-succinyl-Leu-Tyr-AMC 50µM together with or without the calpain inhibitor N-acetyl-LeuLeu-Norleu-Al à 30µM (Sigma-Aldrich). After a 30-min incubation period, fluorescence was detected at 360 nm excitation and 460 nm emission, using the FLX800 spectrofluorometer (Bio-Tek Instruments, Winoski, UT). Calpain activity was determined as the difference between fluorescences measured without and with calpain inhibitor and expressed as µM AMC using a standard curve (0 to 25 µM) constructed for each assay.

Morphologic evaluation of kidney, liver and lung

A cannula was introduced in the right atrium and mice were perfused with paraformaldehyde. Subsequently, the trachea was cannulated, and paraformaldehyde was perfused through the airways. Kidneys, lungs, spleen and livers from mice from each group were immersed in formalin (4% formaldehyde) solution or snap-frozen. Kidneys, livers and lungs were embedded in paraffin after conventional processing, and 4-µm-thick sections were stained with periodic acid-Schiff reagent (PAS) or with Martius Scarlet Blue staining for fibrin. Tubular damage and histological liver injury were scored as previously described (1). Scoring was performed in a blinded manner on coded slides. Lung injury (septal thickening and cell infiltration) were assessed by quantitative morphometric analysis, using an automated image analyzing system (Image analysis software, Olympus).

Measurement of blood chemistry, TNFα, IL-6, IL-1α, MCP-1, KC and IL-10 and coagulation tests

Serum Cr was measured by HPLC method (2). BUN, serum aspartate aminotransferase, and alanine aminotransferase were measured using an autoanalyzer (Hitachi 917, Boehringer

Mannheim, Indianapolis, USA). Serum TNFα, IL-6, IL-1α, MCP-1, KC and IL-10 were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, USA). Plasma pO2, pCO2, and pH were measured by a blood gas analyser (RapidLab 1265, Siemens) immediately after arterial puncture. Prothrombin times (PTs) and activated partial thromboplastin times (aPTTs) were performed by clotting assays with the STA-R Coagulation Analyzer using STA-neoplastine and STA-PTT (Diagnostica Stago).The activities of Factor (F)VIII, FIX, FXI,FXII, FII, FV, FVII, and FX were determined by clotting assays with the deficient FVIII, FIX, FXI,FXII, FII, FV, FVII, and FX kits, respectively (Siemens). Expression of coagulation factors is in % related to the mean value provided by a panel of 12 normal WT mice.

NF-κB Activation Assay

Nuclear proteins were extracted from fresh kidney samples, and the amounts of activated NF-κB p65 subunit that was contained in these proteins were measured with commercial kits (Nuclear Extract Kit and TransAM; Active Motif, Rixensart, Belgium) - according to the manufacturer's instructions.

Western blotting

Tissues were homogenized in 400 µl of radioimmunoprecipitation assay buffer and used for western blot analysis as described previously (3) . The rabbit anti m-calpain antibody was purchased from Santa Cruz Biotechnology (1/1000).

Isolation, phenotyping and counting of microparticles

Blood was collected from the heart and anticoagulated with 4% sodium citrate. Platelet-poor plasma (PPP) was obtained by centrifuging blood at 1,500g at 23°C for 25 min then

centrifuging for 2 min at 15,000g to remove contaminating cells from the plasma. PPP was stored at -80°C for further analysis. For flow cytometry, MPs were identified by gating of size (less than or equal to 1µm) and specific binding of phosphatidylserine (PS) (Annexin V-PE (BD Pharmingen, San Diego, CA)) (4). The absolute MP count per microliter was established by spiking the sample with a known number of 4µm beads (BD Pharmingen, San Diego, CA). Samples were run on a BDFACSAria I flow cytometer (Becton Dickinson, San Jose,CA). The size of MPs correlates with forward scatter (FS) and their granularity is reflected by the side scatter (SS) parameter. Standard beads of different diameters (0,2-3µm) (Sigma Latex Beads, LB3, LB8, LB30) were used for size calibration. Antibodies were added to samples and incubated on ice for 20 min to stain leukocytes (rat anti-mouse APC CD14, 4 µg/ml, BD Pharmingen, San Diego, CA, USA), erythrocytes (rat anti-mouse FITC TER119, 4 µg/ml, BD Pharmingen), endothelial cells (rat anti-mouse APC CD144, 5 µg/ml, eBiosciences, San Diego, CA, USA) and platelets (rat anti-mouse FITC CD41, 4 µg/ml, BD Pharmingen), with corresponding isotype-matched controls (APC rat IgG1, FITC rat IgG2b, APC rat IgG1, FITC Rat IgG1κ, respectively). CD14, TTER119, CD144, and CD41 positive MPs were identified by fluorescence levels greater than two standard deviations above the isotypic controls.

Thrombin generation assay

The following parameters of thrombogram were analyzed: the lag-time of thrombin generation and the time to reach the peak of thrombin (ttPeak) that reflect the initiation and propagation phase of thrombin generation, the peak of thrombin (Peak), the endogenous thrombin potential (ETP) that reflects the total amount of thrombin activity.

Intravital microscopy

Six hours after CLP, mice were anesthetized (intraperitoneal injection of pentobarbital 50 mg/kg) and TG mice and WT counterparts were maintained at 37°C on a heated microscope stage, using a rectal electrothermometer (CMA). A PE10 catheter (Phymep) was placed in the right femoral vein for venous access and continuous 50 µl/min infusion of 2.5% BSA in 0.9% saline. Following midline abdominal incision, the mesentery adjoining the terminal ileum was carefully placed on a glass pedestal (Klaus Effenberger) and superfused with 0.9% saline. Intravenous injection of 250 µg Rhodamine 6G (R4127, Sigma Aldrich) was performed to label circulating leukocytes. Imaging was performed using a fixed stage upright microscope with a water immersion 40x objective (Axio, Examiner Z1, Zeiss). Single unbranched mesenteric veins ranging from 80 to 120 µm diameter were studied and the microcirculation was recorded over consecutive 20-s periods in at least 3 different mesenteric veins per animal (Axiovision 4 software, Zeiss). The mean duration of the experimental procedure did not exceed 90 min. Quantification of leukocyte adhesion and rolling was performed off-line, during a single-session blinded playback analysis of videotaped images (Image J software). Leukocytes were defined as adherent when immobile for at least 10 s, and were expressed as the number of cells adhering on a 100-µm vein length per 20-s period. Rolling leukocytes were defined as cells that were clearly moving along the endothelium slower than free-flowing blood cells, and expressed as the number of rolling cells per mm and per second.

Bacterial count in blood and peritoneal cavity

The peritoneal cavity was lavaged with 1.5 ml sterile saline, and blood was collected by cardiac puncture 24h after CLP surgery. Serial dilutions of blood or peritoneal fluid were plated onto tryptic soy agar (Remel, Lenexa, KS, USA) and colony counting after 24 h incubation at 37°C. Bacterial counts were log normalized.

Immunohistochemical analysis of activated caspase-3 in spleen

Immunohistochemical staining of 4 μm paraffin sections was performed with anti-activated caspase-3 antibody (Cell Signaling Technology, Beverly, USA) as described previously (5). The number of positive stained cells was determined from the mean of five randomly selected non overlapping 400X fields in each section.

Measurement of apoptosis and necrosis by flow cytometry

Spleen cells from C57BL/6J wild type (WT) and TG mice were isolated by passing the tissue through a 40μm nylon membrane. They were depleted of erythrocytes by 90 sec exposure to ACK (Ammonium-Chloride-Potassium) lysing buffer (Bio Whittaker), washed, and resuspended in RPMI medium supplemented with 10% fetal bovine serum. Lymphocytes were isolated from this preparation using a lymphocyte cell enrichment kit (Lympholyte, Cedarlane). Apoptotic and necrotic lymphocytes were detected by flow cytometry after staining with the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) by the manufacturer's procedure. The method depends on the selective binding of Annexin V to phosphatidylserine on the cell surface of apoptotic cells, and the staining by propidium iodide of cells that have lost membrane integrity. Cells were analyzed with a FACS Quanto flow cytometer (Becton-Dickinson Biosciences).

Adoptive transfer of lymphocytes into RAG $2^{-/-}$ mice.

Lymphocytes from normal TG and WT mice were isolated as described above. Mice that underwent CLP were injected with 1×10^7 lymphocytes or saline intravenously 2h after CLP or 24h before CLP. The three groups of mice received antibiotics as described previously. RAG2-/- mice are on a C57BL/6 background.

Immunofluorescence staining

Immunofluorescence was performed on 3-µm frozen sections from liver and lung sections fixed 3 min in acetone. After washes in PBS, sections were incubated with FITC-conjugated polyclonal rabbit anti fibrin (1/50, Dakocytomation). Microscopy was performed with an Olympus BX51 microscope.

Table E1.

	Sham WT	Sham TG	CLP WT	CLP TG
Platelets (10^3/µl)	1078 (± 56.1)	1100 (± 75.24)	654.3# (± 45.8)	879.5* (± 63)
Hemoglobin (g/dl)	11.76 (± 0.22)	12.18 (± 0.22)	12.56 (± 0.32)	11.92 (± 0.28)
White blood cell count (10^3/µl)	5.82 (± 0.54)	6.42 (± 0.52)	0.98# (± 0.15)	0.99# (± 0.14)

Table E1. Measurement of hemoglobin and white blood cell count 24h after CLP surgery. *P<0.05, versus CLP WT (n=4 in each sham group and n=8 in each septic group); # : P<0.05 versus sham; Results are expressed in mean (±s.e.m)

Table E2.

	CLP WT	CLP TG	CLP TG + MPs
Time after CLP (h)	61 (±13.4)	96 (±15.2)	55 (±8.9)

Table E2. Time points after CLP for "pre-mortem" mice.

Time points (h) after CLP for "pre-mortem" mice in WT, TG mice and TG mice supplemented with WT septic MPs. Animals with a score > 5 were considered as "pre-mortem" mice.

References

1. Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med. 2009 Jan;15(1):42-9.

2. Yuen PS, Dunn SR, Miyaji T, Yasuda H, Sharma K, Star RA. A simplified method for HPLC determination of creatinine in mouse serum. Am J Physiol Renal Physiol. 2004 Jun;286(6):F1116-9.

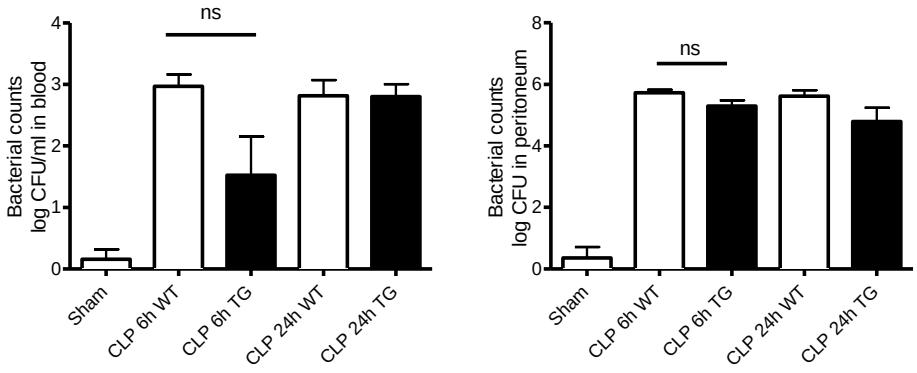
3. Peltier J, Bellocq A, Perez J, Doublier S, Dubois YC, Haymann JP, et al. Calpain activation and secretion promote glomerular injury in experimental glomerulonephritis: evidence from calpastatin-transgenic mice. J Am Soc Nephrol. 2006 Dec;17(12):3415-23.

4. Shet AS, Aras O, Gupta K, Hass MJ, Rausch DJ, Saba N, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood. 2003 Oct 1;102(7):2678-83.

5. Dear JW, Yasuda H, Hu X, Hieny S, Yuen PS, Hewitt SM, et al. Sepsis-induced organ failure is mediated by different pathways in the kidney and liver: acute renal failure is dependent on MyD88 but not renal cell apoptosis. Kidney Int. 2006 Mar;69(5):832-6.

Figure E1.

A.



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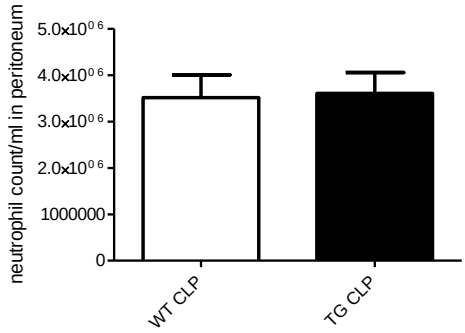

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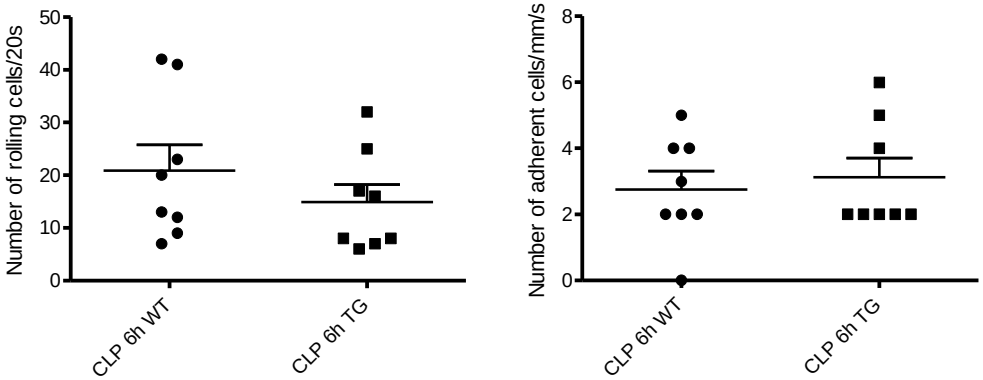


Figure E1. Effect of calpastatin overexpression on bacterial load and neutrophil recruitment during sepsis

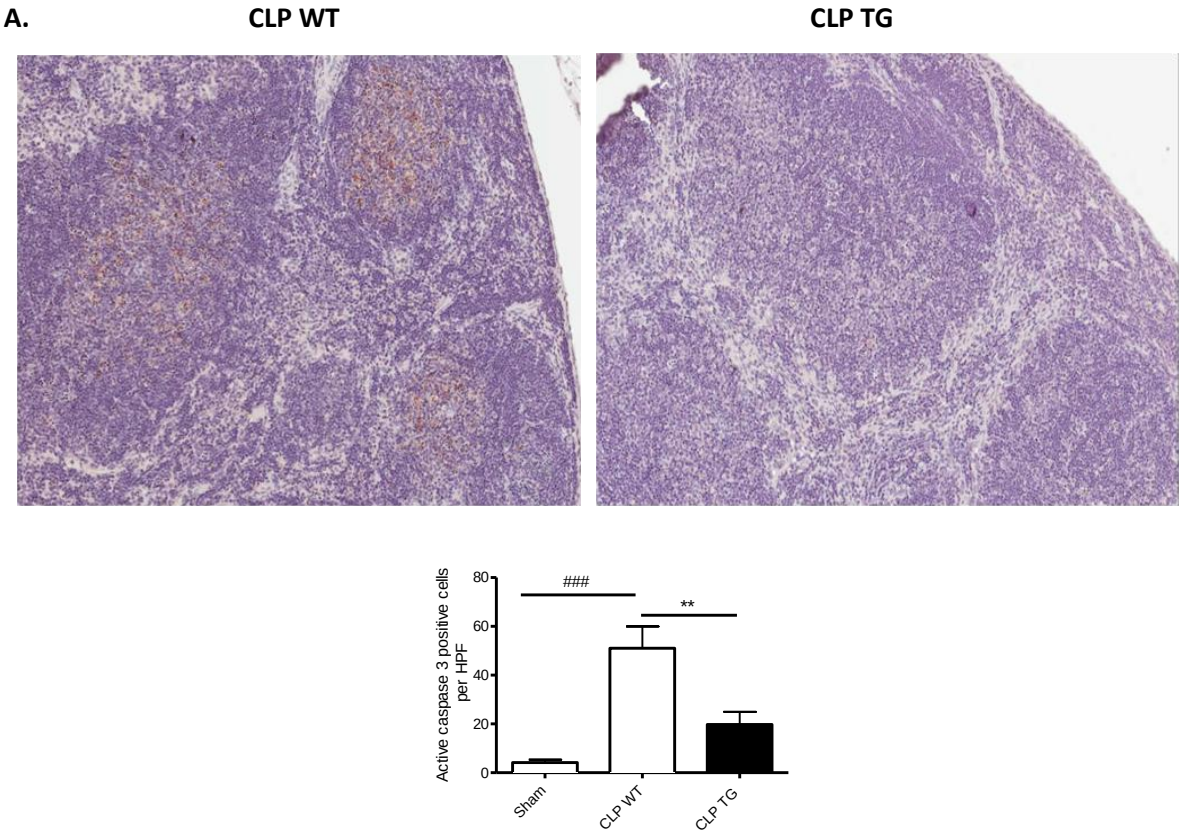
A) Bacterial counts in peritoneal fluid and blood of sham and septic WT or TG mice (n=5 mice per group).

B) Neutrophil counts in peritoneal fluid in septic WT and TG mice 6h after CLP surgery (n=5 per group)

C) Intravital microscopy evaluation of leukocyte rolling and adhesion to endothelium of mesenteric veins 6h after CLP surgery. (n=8 mice per group)

Error bars represent means ±s.e.m ; ns : non significant

Figure E2.



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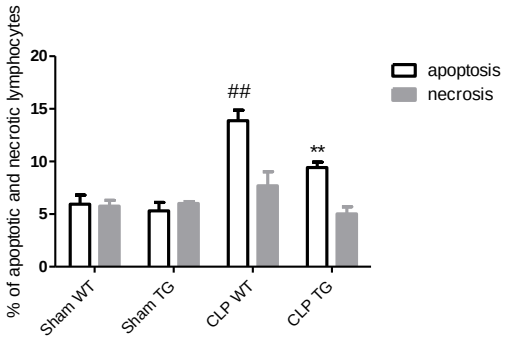

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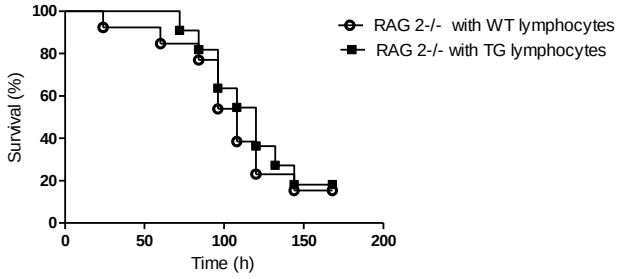


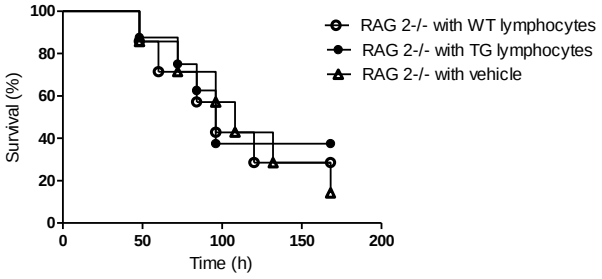


Figure E2. Effect of calpastatin overexpression on lymphocyte apoptosis

(A) Staining of caspase 3 cells in spleen sections of septic WT and TG mice 24h after CLP surgery. (n=10 per group) (original magnification 10X)

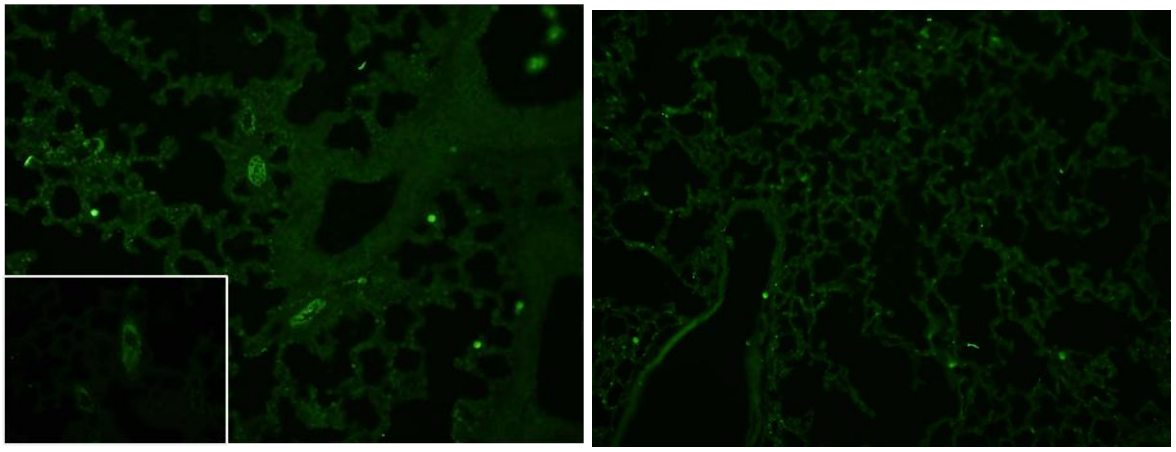
(B) Percentage of necrotic and apoptotic lymphocytes in sham WT, sham TG, CLP WT and CLP TG 24h after CLP surgery determined by flow cytometry (n=5 mice per group).

(C) Left : RAG2-/- mice received 1.1^{07} lymphocytes (2h after surgery) from WT or TG mice. Survival curves showed no difference between the 2 groups (n=12 per group).

Right : RAG 2-/- mice received 1.10^7 lymphocytes from WT and TG mice or vehicle (24h before surgery). Survival curves showed no difference between the 3 groups (n=7 per group).

Error bars represent means ±s.e.m ; *P<0.05 WT vs TG, # : P <0.05 CLP versus sham, ## : P < 0.01 CLP versus sham, ns: non significant

Figure E3.



CLP WT **CLP TG**

Figure E3. Fibrin deposit in lung vessels.

Immunofluorescence staining for fibrin was performed in lung sections from "premortem" WT and TG septic mice (original magnification 20X). Fibrin deposits were observed in lung vessels in WT septic mice.

Figure E4. Impact of MP transfer on organ dysfunction.

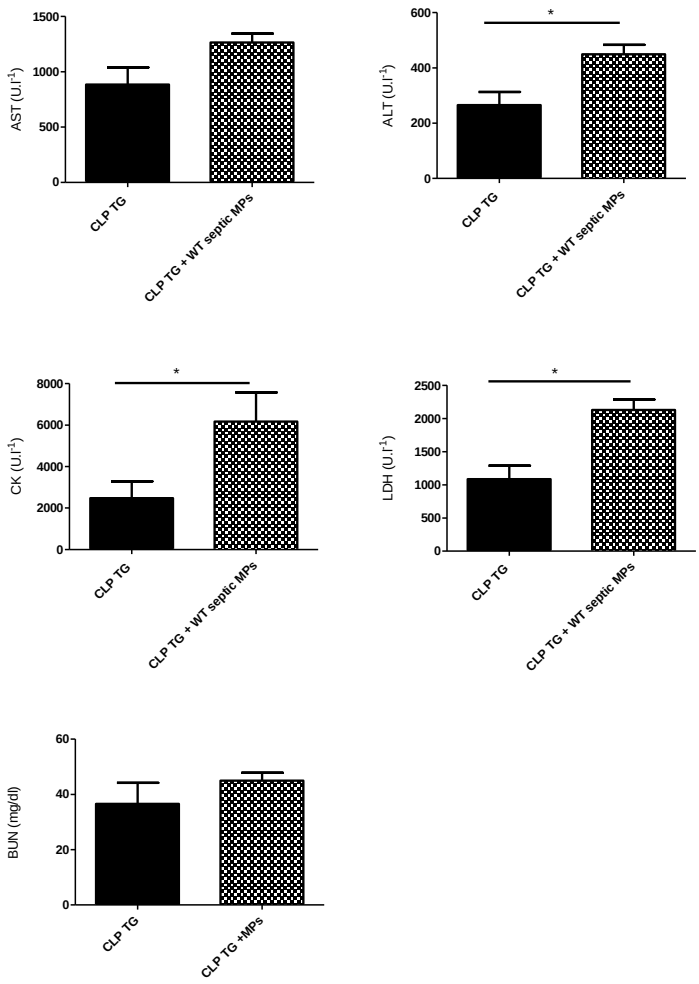


Figure E4. Impact of MP transfer on organ dysfunction.

MPs effect on organ dysfunction as reflected by Alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) and serum urea (BUN) in TG mice and TG mice supplemented with WT septic MPs, 24h after CLP surgery (n=6 per group). Results are means± s.e.m *p<0.05 vs CLP TG.

Figure E5.

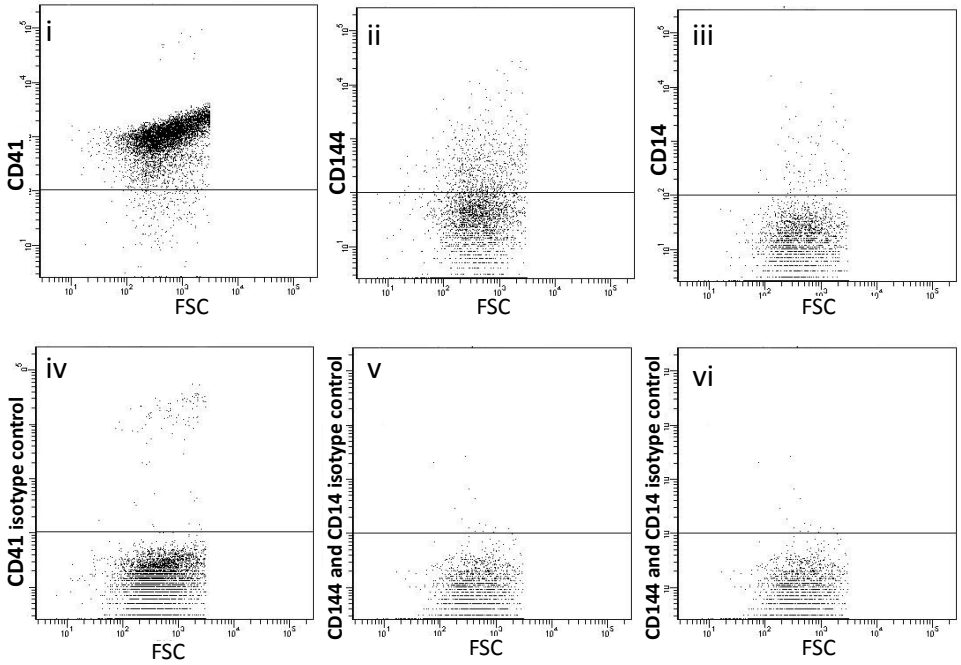


Figure E5. Flow cytometric quantitation of MPs.

Representative fluorescence-activated cell sorter dot plots of MPs from WT mice 24h after CLP surgery (i) MPs labeled with CD41-FITC ; (ii)MPs labeled with APC-CD144 ; (iii) MPs labeled with APC-CD14 ; (iv) MPs labeled with FITC IgG1 , κ isotype control ; (v)(vi) MPs labeld with APC IgG1 isotype control