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Role of Lipopolysaccharide and Cecal Ligation and Puncture on Blood Coagulation and Inflammation in Sensitive and Resistant Mice Models

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The hemostatic system is severely disturbed during endotoxemia, leading to a hypercoagulable state. However, it remains uncertain to what extent hypercoagulability is the critical factor in determining the clinical course rather than just the consequence of a severe systemic inflammatory response. To answer this question, we evaluated the evolution of hemostatic and inflammatory markers, as well as histological features, in mice sensitive and resistant to two models of endotoxemia: lipopolysaccharide-injection and cecal ligation puncture. Genetic (knockout mice) and pharmacological (PJ34) blockade of the nuclear enzyme PARP-1 was used to achieve resistance to the endotoxemia. In both models, endotoxemia resulted in antithrombin deficiency, decreased platelets, and fibrin deposition in organs, which were similar in all groups of mice. By contrast, proinflammatory mediators, inflammatory cell infiltration (especially that mediated by mononuclear cells), and organ degeneration were more intense in sensitive animals. Further studies supported a negative role for the triggering of the coagulation cascade in the mortality associated with the endotoxic shock. Hirudin had a minor effect on cell infiltration and organ damage, despite causing a potent inhibition of fibrin deposition. On the other hand, a sublethal dose of lipopolysaccharide yielded significant fibrin deposition but weak activation of the inflammatory response. Our results suggest that activation of coagulation by endotoxemia is severe and independent of the inflammatory response. However, such activation may act with fibrin deposition to have a minor influence on survival in sepsis. ***(Am J Pathol 2005, 166:1089–1098)***

Sepsis is the most important cause of death among hospitalized patients, with mortality rates ranging from 30 to 70%. Despite advances in supportive care, each year ~750,000 people develop sepsis and 225,000 die in the United States alone, and the incidence of sepsis is rising at rates between 1.5% and 8% per year.[1,2] Sepsis is the result of an acute and systemic immune response to a variety of noxious insults, in particular to bacterial infection. This response leads to the activation of a number of host mediator systems, including the cytokine, leukocyte, and hemostatic networks, each of which may contribute to the pathological sequelae of sepsis.[3] Sepsis has been classically considered as the archetypal clinical condition in which evident molecular links between inflammation and coagulation can be observed.[4,5] Contributing mechanisms to the communication between coagulation and inflammation pathways have been uncovered, including the general appreciation that proinflammatory mediators may regulate coagulation activation, and that products of the clotting cascade may affect inflammation.[4–6]

Multiple hemostatic parameters are affected by sepsis, All of them contribute to a hypercoagulable state that may lead to disseminated intravascular coagulation, which appears to be associated with an unfavorable outcome.[7] Accordingly, the amelioration by means of antithrombotic treatments of experimentally induced disseminated intravascular coagulation in animals decreases the risk of organ failure and, in some cases, death.[7] Despite the apparent association between endotoxemia-associated

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hypercoagulability and the risk of death, however, it remains uncertain to what extent hypercoagulability is the critical factor in determining the clinical course, rather than just a mere consequence of a more severe systemic inflammatory response.

To answer this question we evaluated the evolution of hemostatic and inflammatory parameters in two different models of endotoxemia [injection of lipopolysaccharide (LPS) and cecal ligation puncture (CLP)] both in sensitive and resistant mice. Poly (ADP-ribose) polymerase-1 (PARP-1) is a nuclear DNA-binding protein that has been demonstrated to play a relevant role in cell necrosis and organ failure in various diseases associated with inflammation and reperfusion injury.[8] Accordingly, genetic or pharmacological blockade of PARP-1 in mice results in a significant resistance to LPS-induced endotoxemia[8–10] and protects against acute septic peritonitis.[11] These mice are excellent models to test the molecular link between inflammation and coagulation, and the relevance of hypercoagulability in the clinical course of an endotoxemia.

Materials and Methods

Animals

PARP-1 knockout (PARP-$1^{-/-}$) and their wild-type (PARP-$1^{+/+}$) littermates (strain 129/Sv X C57BL/6) mice were kindly provided by Dr. de Murcia (Strasbourg, France).[12] Polymerase chain reaction genotyping screening was performed using the primer (+/−) 5′-CTTGATGGCCGGGAGCTGCTTCTTC-3′ in combination with oligo (+) 5′-GGCCAGATGCGCCTGTCCAAGAAG-3′for wild-type allele or oligo (−) 5′-GGCGAGGATCTC-GTCGTGACCCAT-3′for mutated allele (Ménissier de Murcia, personal communication). The animals were kept under standardized conditions. Tap water and mouse chow were provided *ad libitum*. Experimental procedures were performed in accordance with University of Murcia approved institutional animal care guidelines.

LPS-Induced Endotoxic Shock

PARP-$1^{-/-}$ and their PARP-$1^{+/+}$ littermates mice (3 months old) were injected intraperitoneally with a lethal dose of LPS from *Escherichia coli* 0111:B4 (40 mg/kg; Sigma, St. Louis, MO) in a volume of 250 μl of sterile saline solution. Pharmacological inhibition of PARP was performed by pretreatment of PARP-$1^{+/+}$ mice with *N*-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-*N*,*N*-dimethyacetamide HCl (PJ34) (10 mg/kg; Alexis, Carlsbad, CA) 1 hour before the LPS treatment. A sublethal dose of LPS (0.4 mg/kg) was injected into a group of wild-type animals. Control mice always received the same volume of sterile saline solution.

Hirudin Treatment

Hirudin specifically blocks thrombin activity through competitive inhibition of its catalytic site. The dose and frequency of hirudin injection was based on a previous study.[13] Briefly, four intraperitoneal injections of recombinant hirudin (1 mg/kg; Lepidurin, Refludin) (Schering, Cambridge, UK) were administered to wild-type animals 30 minutes before LPS, and 30 minutes, 2 hours, and 4 hours after LPS injection (40 mg/kg).

Cecal Ligation Puncture Model

Cecal ligation puncture was performed as described elsewhere.[11] Briefly, PARP-$1^{-/-}$ and their PARP-$1^{+/+}$ littermates mice (3 months old) with and without pretreatment with PJ34 were anesthetized with a mixture of ketamine (30 mg/kg; Parke-Davis, Madrid, Spain), midazolam (0.5 mg/kg; Roche, Foster City, CA), and fentanyl (5 μg/kg; Kern Pharma, Barcelona, Spain). Before surgery, the abdominal skin of the mice was shaved, and under aseptic conditions, a 2-cm midline incision was performed to allow exposure of the cecum with adjoining intestine. The cecum was tightly ligated with a 3.0-silk suture at its base, below the ileo-cecal valve, and was punctured twice with an 18-gauge needle (top and bottom). The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites and returned to the peritoneal cavity. The laparotomy site was then closed with 4.0 silk. Finally, animals were returned to their cages with free access to food and water.

Sample Collection

At the indicated times (0, 1.5, 3, and 12 hours after LPS injection, and at 12 and 22 hours after CLP), mice were anesthetized by using the inhalatory anesthetic isofluorane (Abbott, Madrid, Spain). Blood samples were extracted by cardiac puncture and collected into tubes containing citrate trisodium (0.11 mol/L). Animals were then killed and livers and lungs rapidly dissected. Organs were immediately fixed in 4% formaldehyde (phosphate-buffered saline-diluted), and used for histology and immunohistochemistry analysis, or either rinsed in saline buffer and stored at −70°C until assayed for myeloperoxidase activity.

Whole blood was used for platelet counts. Plasma samples were immediately obtained by centrifugation of blood at 1100 × *g*, aliquoted, and frozen at −70°C until used. In some experiments, small amounts of blood from the same animal were sequentially collected by cardiac puncture before LPS, and at 6 and 24 hours after LPS injection.

Histological and Immunohistochemical Analysis

For histopathological analysis, formaldehyde-fixed tissues were embedded in paraffin, cut in sections of 3 μm, and stained with hematoxylin and eosin (H&E). Scoring of the histopathological changes was performed under light microscopy conditions by two expert pathologists who were blind to the experiment. Mononuclear (MN) and polymorphonuclear (PMN) cells were counted at the level of six pseudorandomized central vein areas (×40 magnification) and the average number of cells was recorded. Fibrin deposition in liver was scored by assigning a subjective value (0, no fibrin deposition; 1, fibrin

deposition limited to endothelial walls of major vessels and/or to minor capillary vessels; 3, widespread fibrin deposition in most capillary vessels within the hepatic lobules and some central/portal veins; 5, widespread fibrin deposition in most capillary vessels within the hepatic lobules and most major central/portal veins). Results were further confirmed by assigning similar scores to slides stained with the rabbit anti-human fibrinogen/fibrin antibody.

Immunohistochemical staining was performed using the DAKO Autostaining Universal staining system (DAKO, Glostrup, Denmark). The tissue sections were deparaffinized and treated with peroxidase-blocking reactive (DAKO) for 5 minutes. The slides were then incubated with 30 μg/ml of rabbit anti-human fibrinogen-fibrin (DAKO) for 20 minutes at room temperature, and sequentially treated using the LSAB+System-HRP (DAKO), following the manufacturer's instructions. After extensive washing, diaminobenzidine chromogen (DAKO) for 5 minutes was used to develop the sections. Finally, after rising in distilled water, the slides were counterstained with hematoxylin, rinsed well with tap water, and mounted. The specificity of the antibody for fibrin was indicated by the absence of staining in all tissues from control mice.

Myeloperoxidase Determination

Myeloperoxidase activity was measured photometrically using 3,3′,5,5′-tetramethylbenzidine (33′55′-TTB) (Sigma) as a substrate.[14] Samples were macerated with 0.5% hexadecyltrimethyl ammonium bromide (Sigma) in 50 mmol/L phosphate buffer (pH 6). Homogenates were then disrupted for 30 seconds by sonication and subsequently frozen in liquid nitrogen and thawed on three consecutive occasions before a final 30-second sonication. Samples were incubated at 60°C for 2 hours and then spun down at 4000 $\times$ *g* for 12 minutes. Supernatants were collected for myeloperoxidase assay. Enzyme activity was assessed photometrically. The assay mixture consisted of 20 μl of supernatant, 25 μl of 33′55′-TTB (final concentration, 1.6 mmol/L), and 25 μl of H_2O_2 (final concentration, 3.0 mmol/L) diluted in 80 mmol/L of phosphate buffer (pH 5.4). The reaction was stopped with HCl 1 N (2 minutes) and absorbance was measured at 450 nm. An enzyme unit is defined as the amount of enzyme that produces an increase of 1 absorbance unit per minute.

Determination of Activity and Antigenic Levels of Antithrombin

Antithrombin activity was determined using a commercial chromogenic (S-2765) anti-factor Xa assay with heparin (Instrumentation Laboratory, Milano, Italy). Antithrombin antigen was measured by two different methods (Laurell and a homemade enzyme-linked immunosorbent assay) using goat and rabbit anti-human antithrombin polyclonal antibodies (Sigma and DAKO). Results obtained using both methods matched each other. The anti-human antithrombin polyclonal antibodies used in our study were able to detect mouse antithrombin, probably because of the high homology of this protein between both species.

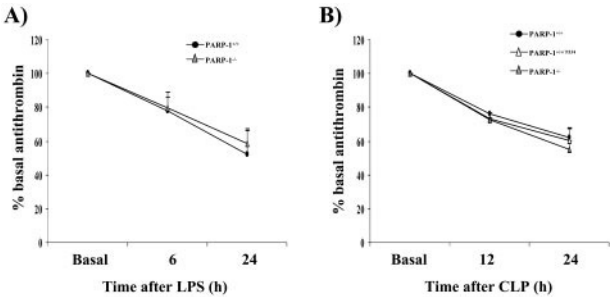

Figure 1. Evolution of antithrombin during endotoxemia. **A:** Plasma antithrombin activity levels in PARP-$1^{-/-}$ and PARP-$1^{+/+}$ littermates. Results represent the percentage of the observed value in the same animal before lethal LPS injection. **B:** Plasma antithrombin activity levels associated with cecal ligation puncture in PARP-$1^{-/-}$, PARP-$1^{+/+}$ littermates, and PARP-$1^{+/+/PJ34}$ littermates. Results represent the percentage of the mean value of five untreated animals.

Platelet Counts

Platelet numbers were determined in fresh citrated blood using an automated counter (KX-21N; Sysmex, Kobe, Japan).

Cytokines Determination

The systemic release of tumor necrosis factor (TNF)-α and interleukin (IL)-6 cytokines was determined by enzyme-linked immunosorbent assay according to the manufacturers' recommendations (Bender MedSystems, San Bruno, CA).

Statistical Analysis

Results are presented as mean values ± SD. Statistical analysis was performed by using the Mann-Whitney nonparametric *t*-test. A P value <0.05 was considered statistically significant.

Results

Antithrombin Antigen and Activity Levels

Sequential follow-up of antithrombin activity and antigen levels were evaluated within each animal after the lethal LPS injection (PARP-$1^{-/-}$, $n = 6$; PARP-$1^{+/+}$, $n = 6$). At the selected time intervals (0, 6, and 24 hours after LPS injection), blood (20 μl) was collected from the heart into tubes containing 2 μl of 0.11 mol/L citrate trisodium. The initial time point, taken before LPS injection (time 0), was considered to represent 100% of antithrombin. The inoculation of LPS resulted in a time-dependent proportional decrease in both antithrombin antigen and activity. Twenty-four hours after the lethal LPS injection antithrombin activities reached values of 52.2 ± 13.9% in PARP-$1^{-/-}$ and 58.7 ± 8.6% in PARP-$1^{+/+}$ when compared to their baseline levels (Figure 1A). Values were similar when analyzing the

Table 1. Evolution of Antithrombin Levels and Activity in PARP-$1^{+/+}$, PARP-$1^{-/-}$, and PARP-$1^{+/+/PJ34}$ Mice after Injection of LPS

		0 hours		3 hours		12 hours	
	LPS	Antigen	Activity	Antigen	Activity	Antigen	Activity
PARP-$1^{-/-}$	40 mg/kg	102.1 ± 6.6	100.5 ± 1.9	93.7 ± 5.2	93.0 ± 1.3	70.6 ± 5.2	63.3 ± 1.0
RP-$1^{+/+/PJ34}$	40 mg/kg	96.4 ± 6.3	102.5 ± 3.2	82.4 ± 2.6	94.0 ± 1.7	64.8 ± 7.6	75.1 ± 6.5
PARP-$1^{+/+}$	40 mg/kg	102.2 ± 5.5	99.0 ± 0.9	90.9 ± 7.6	96.4 ± 1.2	71.1 ± 6.0	70.9 ± 13.9
PARP-$1^{+/+}$	0.4 mg/kg	102.2 ± 5.5	99.0 ± 0.9	98.5 ± 4.6	101.6 ± 4.0	78.6 ± 2.6	86.5 ± 3.5

Results represent the percentage of a pool of five PARP-$1^{+/+}$ untreated mice.

antigen levels at 24 hours after LPS injection (62.0 ± 8.6% in PARP-$1^{-/-}$ and 66.3 ± 12.5% in PARP-$1^{+/+}$). These decreases were time-dependent and were observed in all PARP-$1^{-/-}$, PARP-$1^{+/+}$, and PARP-$1^{+/+/PJ34}$ mice, at 3 and 12 hours after the injection of 40 mg/kg of LPS (n = 6) (Table 1). Sublethal injection of LPS (0.4 mg/kg) (n = 4) caused milder antithrombin reductions than those observed when lethal doses were used ($P < 0.05$) (Table 1). Similarly to the results seen for the LPS lethal model of endotoxemia, CLP resulted in a significant reduction of antithrombin levels after 24 hours in all groups of mice (n = 3) (Figure 1B).

Platelet Counts

As shown in Figure 2, platelet counts dropped from 844 ± 82 × 10^9/L to 376 ± 54 × 10^9/L at 12 hours after the injection of 40 mg/kg of LPS in wild-type mice. Interestingly, in PARP-$1^{-/-}$ and PARP-$1^{+/+/PJ34}$ mice, the drops in platelet counts were almost identical: from 862 ± 83 × 10^9/L to 272 ± 48 × 10^9/L and from 848 ± 97 × 10^9/L to 322 ± 108 × 10^9/L, respectively (Figure 2A). Sublethal injection of LPS in wild-type animals caused a milder decrease in platelet counts (to 451 ± 21 × 10^9/L) at 12 hours after injection ($P < 0.05$) (Figure 2A). In the CLP model, platelet counts also dropped similarly in all groups of mice (Figure 2B).

Fibrin Deposition

Immnuhistochemical and H&E staining achieved similar results. No fibrin deposition was detected in untreated mice (Figure 3). At 3 hours after LPS inoculation, fibrin deposits were evident in livers and lungs of all groups. Figure 3A summarizes the results observed in livers. As indicated by their scores, all mice treated with a lethal dose of LPS displayed similar fibrin deposition. Interestingly, mice treated with a sublethal dose of LPS also presented significant amounts of fibrin at 3 hours. Fibrin deposition occurred mainly at central vein levels in most cases limited to the walls, spreading throughout the hepatic parenchyma, and hardly reaching some of the portal veins. At 12 hours, traces of fibrin could still be found in some blood vessels of all animals (Figure 3).

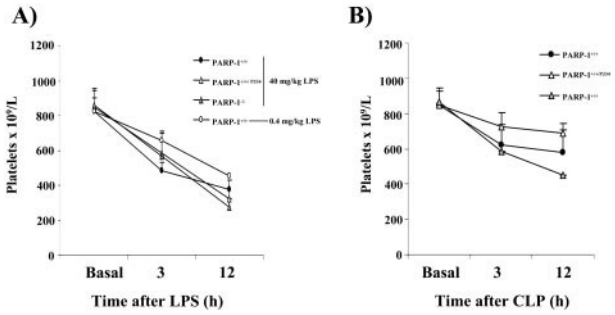

Figure 2. Platelet counts in LPS (**A**)- and cecal ligation puncture (**B**)-induced endotoxemia.

Fibrin deposition in lungs achieved scores and followed patterns similar to all groups. Thin layers of fibrin could be found at 3 hours after lethal LPS inoculation in some pulmonary blood vessels of PARP-$1^{+/+}$, PARP-$1^{-/-}$, and PARP-$1^{+/+/PJ34}$ mice. In all cases, lungs had a patched-like appearance with some areas affected and some normal. By 12 hours after inoculation, fibrin had disappeared from the pulmonary vessels of all groups of animals. Mice treated with a sublethal dose of LPS also displayed lungs with parallel fibrin deposition to that observed in livers (data not shown).

Livers and lungs of all groups were slightly hyperemic at 3 hours. In livers, only portal veins appeared dilated at this stage. Blood flow increased in all cases at 12 hours when the fibrin deposits had disappeared. At this stage, livers and lungs of all groups appeared congestive and all blood vessels were extremely dilated.

As expected, hirudin treatment decreased deposition of fibrin at 3 hours and prevented it at 12 hours after the injection of a lethal dose of LPS (40 mg/kg) both in livers and in lungs (Figure 3).

Distribution patterns of fibrin deposition were different in the CLP model those seen in the LPS-induced endotoxemia. There is a more widespread distribution of fibrin in the CLP model, including fibrin deposition within the central veins that spreads throughout the hepatic lobule and reaching in some cases portal levels. In LPS-treated mice fibrin deposition normally only includes central veins and minor vessels within the liver. However, it is important to point out that all groups of mice (PARP-$1^{+/+}$, PARP-$1^{-/-}$, and PARP-$1^{+/+/PJ34}$) followed the same pattern and displayed similar scores after CLP. Fibrin deposits were abundant throughout the liver parenchyma at 12 (PARP-$1^{+/+}$, 3.00 ± 1.60; PARP-$1^{-/-}$, 4.00 ± 1.42; PARP-$1^{+/+/PJ34}$, 4.00 ± 1.16) and 22 hours (PARP-$1^{+/+}$, 4.33 ± 1.16; PARP-$1^{-/-}$, 4.00 ± 1.41; PARP-$1^{+/+/PJ34}$, 3.67 ± 2.31). All vessels displayed massive fibrin deposits at all times tested after CLP in all groups (Figure 3B). Organs were extremely hyperemic at all stages and in all groups tested. Similar results were observed in lungs of all groups of mice under CLP. At all tested times, fibrin

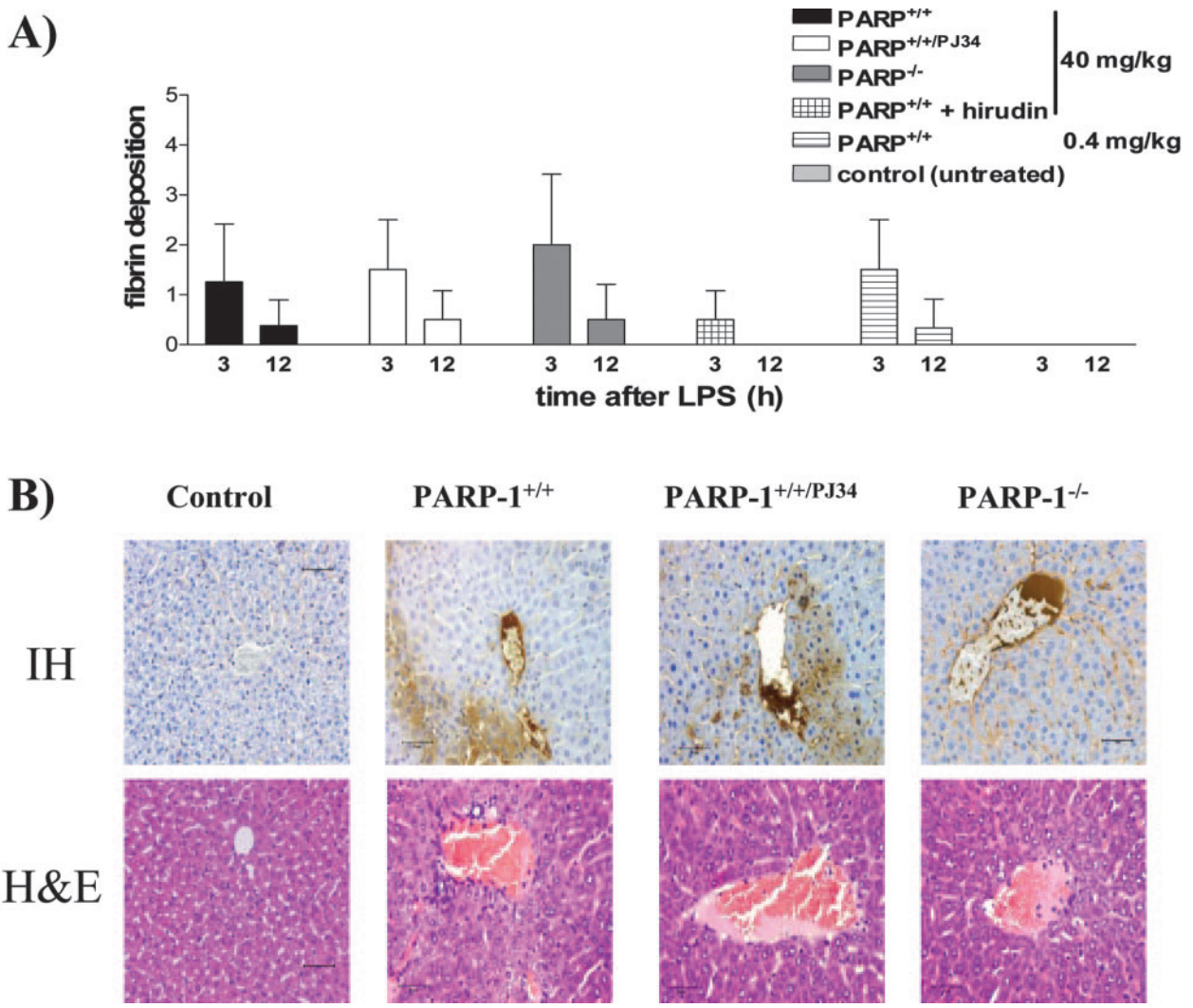

Figure 3. Fibrin deposition in liver induced by LPS- and CLP-induced endotoxemia. **A:** Score of fibrin deposition in LPS-injected mice. **B:** Representative photographs of sections stained with H&E and stained for fibrin by means of immunohistochemistry (IH). Results shown correspond to livers of PARP-$1^{+/+}$, PARP-$1^{+/+/PJ34}$, and PARP-$1^{-/-}$ mice at 22 hours after the cecal ligation puncture, and one control (untreated) wild-type mouse, respectively. Original magnifications, ×40.

deposits appeared in vessels but were also present in some of the alveoli (data not shown).

Inflammatory Mediators

TNF-α concentration reached a peak at 1.5 hours after LPS injection in all groups of mice injected with a lethal dose of LPS. However, the circulating TNF-α levels at this time were statistically higher in PARP-$1^{+/+}$ mice (1762 ± 67 pg/ml) than in PARP-$1^{-/-}$ (1088 ± 84 pg/ml) and PJ34-treated PARP-$1^{+/+}$ mice (862 ± 155 pg/ml) ($P <$ 0.05). Interestingly, pharmacological blockade of PARP-1 was associated with the lowest circulating levels of TNF-α at 1.5 hours after LPS injection, reaching values lower than those observed in PARP-$1^{-/-}$ mice (Figure 4A).

IL-6 displayed a time-dependent increase in response to the LPS challenge in all mice. However, IL-6 expression was significantly inhibited by pharmacological or genetic blockade of PARP-1 (898 ± 18 ng/ml in PARP-$1^{+/+}$ versus 586 ± 47 ng/ml in PARP-$1^{-/-}$, $P < 0.05$, and

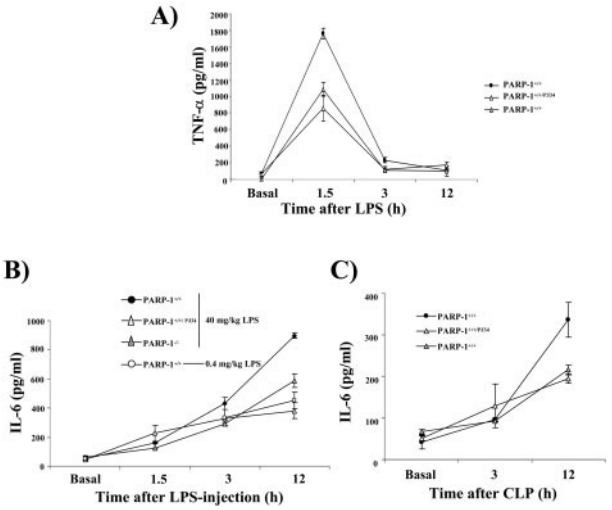

Figure 4. Circulating levels of cytokines in endotoxemia-challenged mice. Plasma levels of TNF-α (**A**) and IL-6 (**B** and **C**) were quantified by enzyme-linked immunosorbent assay at different times after LPS injection or CLP.

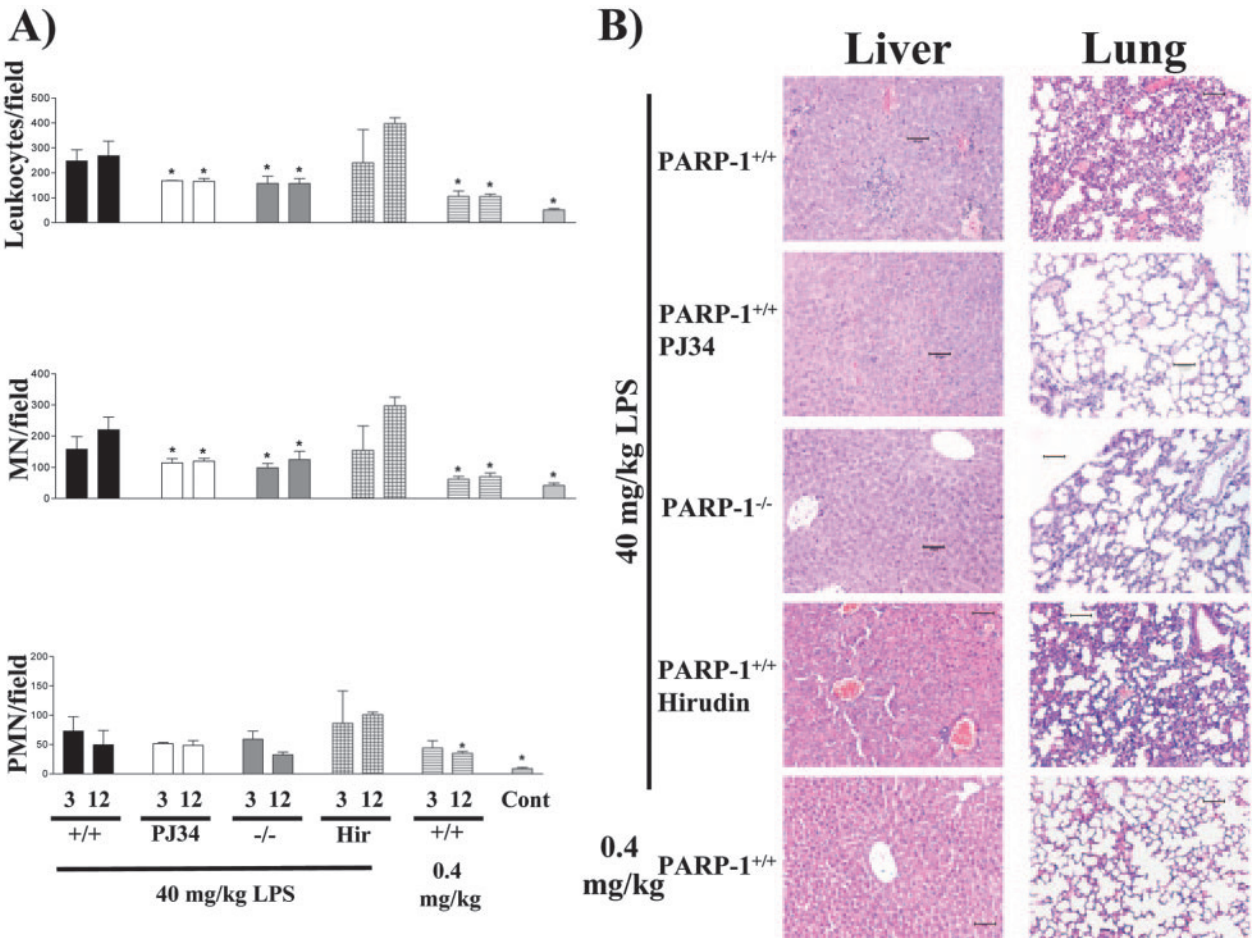

Figure 5. Cell infiltration in LPS-injected mice. **A:** Average number of cells/field in liver. **B:** Example of the histopathological features of livers and lungs of PARP-$1^{+/+}$, PARP-$1^{-/-}$, and PARP-$1^{+/+/PJ34}$ mice at 12 hours after LPS injection as revealed by H&E staining under light microscopy. Nonparametric Student's *t*-tests were performed in appropriate comparisons with PARP-$1^{+/+}$ mice treated with 40 mg/kg of LPS (*, $P < 0.05$). Original magnifications, ×20.

381 ± 53 ng/ml in PARP-$1^{+/+/PJ34}$ mice, $P < 0.05$, at 12 hours after a 40-mg/kg LPS injection). Similar to that seen for TNF-α, this blocking effect was slightly higher in PJ34-treated PARP-$1^{+/+}$ than in PARP-$1^{-/-}$ mice. Sublethal injection of LPS resulted in an increase of IL-6 levels close to those observed in mice previously treated with PJ34 and injected with lethal doses of LPS (Figure 4B). Remarkably, CLP resulted in a significant increase of IL-6 in wild-type animals, but both genetic and pharmacological blockade of PARP-1 reduced such increase ($P < 0.05$) (Figure 4C).

Infiltration and Degenerative Events in the Tissues

A fundamental event in inflammation is the adhesion to endothelial cells and migration of leukocytes to the tissue. Analysis of the histopathological changes in the liver of lethally LPS-treated PARP-$1^{+/+}$ mice at 3 hours after inoculation revealed large infiltrates mainly composed by MN and PMN cells, spreading from portal veins throughout every hepatic lobule. Interestingly, infiltrates in PARP-$1^{-/-}$ or PARP-$1^{+/+/PJ34}$ animals were smaller, with large numbers of monocytes/macrophages and neutrophils (Figure 5). At 12 hours, the numbers of cells/field of infiltrates remained similar to those observed at 3 hours in all groups of mice, slightly increasing in PARP$^{+/+}$ mice (Figure 5). However, PARP-$1^{+/+}$ infiltrates were mainly composed by MN cells (monocytes/macrophages and lymphocytes) that formed sheets surrounding central veins, suggesting the accumulation of this more specialized subset of cells ($P < 0.05$ when compared to both resistant groups) (Figure 5). In contrast, PARP-$1^{-/-}$ or PARP-$1^{+/+/PJ34}$ infiltrates at 12 hours were smaller in numbers and were found in a more diffuse pattern throughout the hepatic parenchyma (Figure 5). Moreover, the numbers of MN and PMN cells did not significantly change from that seen at 3 hours, what suggests that the MN population was not further stimulated by LPS injection at 12 hours (Figure 5).

Findings in lungs were similar to those found in livers. At 3 hours after lethal LPS injection, infiltrates of predominantly MN cells but with rather high numbers of PMN cells could be found in PARP-$1^{+/+}$ animals. By contrast, infiltrates were smaller and had a greater component of PMN cells in lungs of PARP-$1^{-/-}$ or PARP-$1^{+/+/PJ34}$ mice (data not shown). By 12 hours after inoculation, the situation drastically changed. Although infiltrates did not change in size or cellular components in PARP-$1^{-/-}$ or

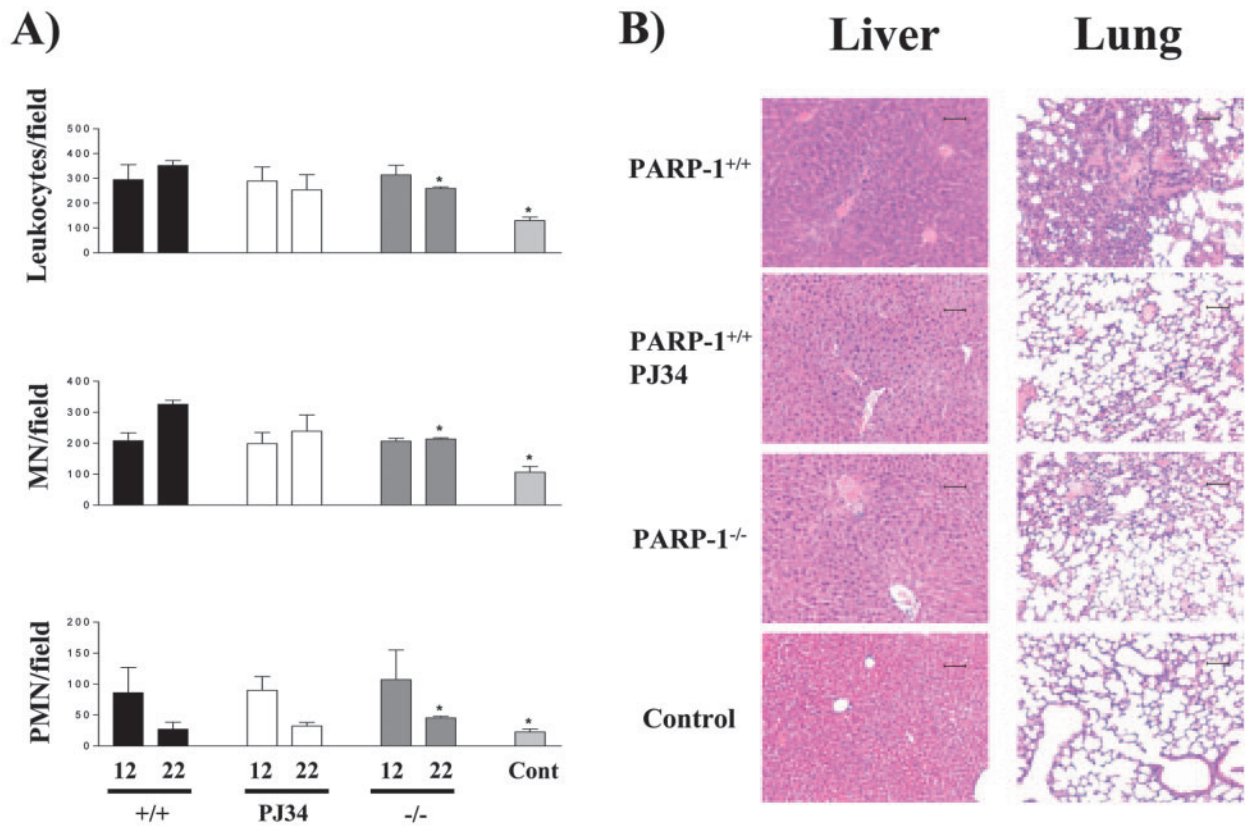

Figure 6. Cell infiltration in the CLP model of sepsis. **A:** Average number of cells/field in liver. **B:** Example of the histopathological features of livers and lungs of PARP-$1^{+/+}$, PARP-$1^{-/-}$, and PARP-$1^{+/+/PJ34}$ mice at 22 hours after CLP as revealed by H&E staining under light microscopy. Nonparametric Student's *t*-tests were performed in appropriate comparisons with PARP-$1^{+/+}$ mice (*, $P < 0.05$). Original magnifications, ×20.

PARP-$1^{+/+/PJ34}$ mice, infiltrates in PARP-$1^{+/+}$ animals massively increased in size and numbers of MNs, but they were very poor in PMN cells (data not shown).

At 3 hours after inoculation, features of apoptosis such as pyknosis, nuclear membrane blebbing, and disruption, were found mainly at central levels within lobules in livers of PARP-$1^{+/+}$ animals. Some features of cytoplasmic degeneration such as vacuolar degeneration could be seen at these levels as well. In hepatic lobules of PARP-$1^{-/-}$ mice, a lower amount of cells displayed features of nuclear and cytoplasmic degeneration, the latter occurring in a much more widespread pattern. Surprisingly, PARP$^{+/+/PJ34}$ animals had features of cytoplasmic degeneration, such as hydroscopic degeneration, even when the numbers of degenerating nuclei were similar to those found in PARP-$1^{-/-}$ mice. At 12 hours, degenerating cells increased in numbers in all groups of mice, PARP-$1^{+/+}$ animals presenting the strongest hepatic degeneration.

In lungs of all groups at 3 hours after LPS injection we observed almost no degenerating cells at the level of the alveolar walls. Transudates and exudates were absent from most alveoli. By 12 hours after inoculation, while cellular degeneration was primarily absent in lungs of PARP-$1^{-/-}$ or PARP-$1^{+/+/PJ34}$ mice, a large amount of degenerating cells and white and red blood cells could be found in the alveolar lumen of the lungs of PARP-$1^{+/+}$ mice, conferring these lungs along with the massive infiltrates a hepatic-like appearance (data not shown).

Numbers of infiltrating cells 3 hours after a sublethal injection of LPS were lower than those found in PARP$^{+/+}$ mice injected with a lethal dose. Interestingly, these values were much similar to those found in both PARP$^{+/+/PJ34}$ or PARP$^{-/-}$ mice injected with a lethal dose (Figure 5). At 12 hours after sublethal LPS, infiltrating cells did not increase in numbers, although a slight increase in MNs similar to that seen in PARP$^{+/+}$ mice treated with lethal doses was detected. However, the numbers of both total leukocytes and MNs were significantly lower than those seen in PARP$^{+/+}$ treated with lethal doses of LPS ($P < 0.05$ for both; Figure 5). By contrast, hirudin treatment of wild-type mice injected with a lethal dose of LPS, almost did not modify the quality and quantity of the cellular infiltration and the organ damage observed in untreated mice injected with the same dose of LPS (Figure 5).

Sepsis produced by the CLP model followed similar stages and patterns to those seen in the LPS model of endotoxemia with some minor differences (Figure 6). In all cases, numbers of infiltrating cells were higher than those found for the corresponding PARP-$1^{+/+}$, PARP-$1^{+/+/PJ34}$, and PARP-$1^{-/-}$ groups of LPS-treated mice. Unlike the LPS model of endotoxemia, infiltrating cells were found in a diffuse pattern spread throughout the hepatic parenchyma, and clusters of cells next to

blood vessels were uncommon (Figure 6). At 12 hours after the CLP, numbers of cells were similar for all groups. This situation changed at 22 hours in a similar pattern to that described for LPS-treated mice at 12 hours. The total number of leukocytes increased in PARP-$1^{+/+}$ mice, but not in PARP-$1^{+/+/PJ34}$ and PARP-$1^{-/-}$ animals, although only reaching statistical significance in the latter. Similarly to the LPS model of endotoxemia, the main differences affected the MN cells, which increased in PARP-$1^{+/+}$ mice, and to a lesser extent in animals with genetic or pharmacological blockade of PARP-1 (Figure 6).

Cellular lesions were the same as found in those LPS-treated animals, although cellular degeneration was more widespread in all cases (Figure 6). Accordingly, the appearance of apoptotic and necrotic features within livers and lungs was much more common than those previously observed in the LPS-induced endotoxemia model (Figure 6). However, degeneration in this model seems to take place later than in the aforementioned model of LPS-induced endotoxemia, although it follows similar patterns throughout the length of the experiment. Myeloperoxidase levels were similar in all groups of mice, supporting the similar numbers of neutrophils found in the histopathological analysis (data not shown).

Discussion

Classically, the association between coagulation and inflammation has been regarded as a two-way process. Inflammation promotes coagulation, but key hemostatic proteins can promote inflammation by direct and indirect mechanisms.[6] This two-way process creates a vicious cycle on a downward spiral leading to vascular injury, organ dysfunction, and death that are often the outcome of severe sepsis.[4,5] Accordingly, an impaired hemostatic response leads to a significant aggravation of endotoxin-induced disseminated intravascular coagulation and cytokine activation, as reported for heterozygous protein C-deficient mice,[15] heterozygous antithrombin-deficient mice,[16] or thrombomodulin-deficient mice.[17] However, the evolution of the hemostatic system has not been analyzed in mice resistant to the lethal consequences of endotoxin. Genetic and pharmacological blockade of PARP-1 protects against LPS-induced endotoxemia,[8–10] resulting in a significant resistance to acute septic peritonitis.[11] However, our results suggest that sensitive and resistant mice display similar procoagulant responses leading to a drop in platelets, deficiency of antithrombin, and relevant fibrin deposition in organs in both models of endotoxemia. In contrast, PARP-1 blockade yielded low counts of infiltrating cells, especially MNs, which might explain the associated resistance to endotoxemia.

Inflammation and coagulation are intricately related processes that may considerably affect each other, but there are still many questions to answer about this cross-talk, especially in sepsis. Thus, blockade of some coagulation molecules (such as thrombin or factor Xa) has minor relevance on the inflammatory response during endotoxemia.[18] Similarly, some inflammatory molecules might have minimal effects on the coagulant response in sepsis. Thus, anti-TNF antibodies did not influence endotoxin-induced activation of the coagulation system despite completely preventing the endotoxin-induced increase in serum TNF activity and profoundly reducing the appearance of IL-6 and IL-8.[19] The effect of other cytokines on coagulation is still controversial.[20] Some results suggest that anti-IL-6 treatments do not affect the induction of the cytokine network by endotoxin or influence the occurrence of a neutrophilic leukocytosis and neutrophil degranulation. However, the same authors showed markedly attenuated endotoxin-induced activation of coagulation.[21] By contrast, a recent study suggested that IL-6 did not appear to mediate early-phase LPS-induced coagulation activation in humans.[22] Similar contradictory results have been observed concerning the role of IL-10. Some authors have suggested that IL-10 apparently inhibited LPS-induced release of TNF, IL-6, IL-8, and IL-12, but did not influence the activation of the coagulation system,[23] but others suggested that IL-10 might inhibit activation of coagulation during human endotoxemia.[24] We found that blockade of PARP-1 attenuates the proinflammatory response to endotoxemia but does not modify the hemostatic consequences of endotoxin, which follow the same pattern in all groups (sensitive and resistant mice), leading to a disseminated intravascular coagulation. Moreover, sublethal doses of LPS were associated with significant fibrin deposition in liver and lung, both at 3 and 12 hours, but only caused a minimal activation of the inflammatory response, similar to that observed in mice treated with PJ34 and injected with lethal doses of LPS (40 mg/kg). Accordingly, our data support that the activation of coagulation could be independent of the inflammatory response in endotoxemia. It is widely accepted that LPS induction of the procoagulant molecule tissue factor within the vasculature and hematopoietic cells causes systemic activation of coagulation and disseminated intravascular coagulation.[7,25]

Interestingly, the severe coagulation response observed in all mice seems to have a minor influence on the survival rate. Thus, the hemostatic parameters in PARP-$1^{-/-}$ and PARP-$1^{+/+/PJ34}$ mice injected with doses of 40 mg/kg of LPS that are lethal for wild-type mice, were severely disturbed, but these mice survived. Additionally, hirudin significantly reduced fibrin deposition in tissues, but did not modify the numbers of infiltrating cells or affect the organic degeneration associated with LPS-induced endotoxemia. Our data are similar to previously published results that suggest that hirudin effectively normalizes coagulation parameters, but does not attenuate LPS-induced arteriolar and venular leukocyte adherence.[26] Moreover, hirudin tends to increase leukocyte adherence after 24 hours and causes a significant deterioration of functional capillary density.[26] The role of anticoagulant treatments in mortality associated with sepsis is controversial. Some reports suggest that hirudin decreases mortality, especially in LPS-induced sepsis,[27,28] but others find no effect,[29,30] or even an increased mortality, especially in bacterial peritonitis (because anticoagulant treatments may enhance the spread of bacteria by preventing localization of the septic focus).[31] Blockade of thrombin formation (by using anti-factor Xa drugs)

also achieved similar results: they attenuate endotoxin-induced coagulopathy, but do not alter endotoxin-induced changes in the fibrinolytic system, cytokine levels, activation of leukocytes, cellular infiltration, and do not increase survival.[32,33] Taken together, all these studies support that inhibition of thrombin could have a minor role in inflammation, cellular infiltration, and organ damage, despite causing a significant improvement of the coagulation parameters. Moreover, our results suggest that both activation of coagulation during sepsis and formation of fibrin per se do not influence the lethal outcome in sepsis. Accordingly, different anticoagulant treatments prevent the coagulation abnormalities in human patients and animal models of sepsis, but failed to protect the host from organ failure or improve the overall survival.[18,34] Recent investigations based on necropsy studies in humans indicate that organ dysfunction is an effect of cell hibernation or stunning rather than thrombosis- and ischemia-induced cell necrosis.[35] Interestingly, recent studies have suggested that strong activation of blood coagulation during the septic episode might even indicate a high level of defense rather than a sign of decompensation.[36] Thus, the procoagulant factor V Leiden polymorphism is associated with a survival advantage in patients with severe sepsis and in mouse endotoxemia,[37] and infusion of low concentrations of thrombin significantly protected animals from endotoxin-induced mortality.[38] These effects could be mediated by the augmented activation of endogenous protein C, explaining the efficacy of recombinant activated protein C for severe sepsis in clinical trials.[39]

PARP-1 blockade might also attenuate the noxious effects of the ischemia caused by the fibrin deposition, but our data support that activation of coagulation is not associated with a fatal outcome in resistant mice. However, we have no conclusive data to discard that coagulation might have contributed to lethality in nonresistant mice. Further studies are required to clarify the influence of hypercoagulability and disseminated intravascular coagulation on the survival of patients with sepsis.

Finally, our results support that MN infiltration of relevant organs during LPS- and CLP-induced endotoxemias, could prove a key factor associated with organ damage and overall survival. Indeed, $PARP^{+/+}$ animals treated with sublethal doses of LPS had similar numbers of PMNs than their lethal dose-treated littermates, but much lower levels of MNs. This could partly explain the different survival rates seen for both dosage regimes. In addition to this, there is emerging evidence that the activation of PARP-1 importantly contributes to the up-regulation of a variety of proinflammatory signal transduction pathways and associated genes.[40] Blocking of PARP-1, by means of inhibiting nuclear factor-κB-dependent gene expression (including iNOS, ICAM-1, TNF-α, MIP-1α, adhesion molecules, and C3), might produce corresponding changes in the nature of the inflammatory leukocyte populations that are recruited.[40,41] Therefore, PARP-1 blockade or therapies aimed at reducing this noxious response of MNs could prove successful as emerging therapeutic strategies in sepsis.

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