Impact of leukocytes and platelets in mediating hepatocyte apoptosis in a rat model of systemic endotoxia

C. Eipel, R. Bordel, R. M. Nickels, M. D. Menger, and B. Vollmar

Department of Experimental Surgery, University of Rostock, D-18055 Rostock; and Institute for Clinical and Experimental Surgery, University of Saarland, D-66421 Homburg/Saar, Germany

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Eipel, C., R. Bordel, R. M. Nickels, M. D. Menger, and B. Vollmar. Impact of leukocytes and platelets in mediating hepatocyte apoptosis in a rat model of systemic endotoxia. Am J Physiol Gastrointest Liver Physiol 286: G769–G776, 2004. First published January 8, 2004; 10.1152/ajpgi.00275.2003.—Apoptotic hepatocytes have been demonstrated to represent an important signal for transmigration of leukocytes sequestered in sinusoids during endotoxemia in vivo. Beside leukocytes, platelets and their adhesion to endothelial cells and leukocytes have been implicated in inflammatory liver injury. Using in vivo multifluorescence microscopy, we examined the possibility that hepatocellular apoptosis causes both leukocytes and platelets to colocalize within the sinusoidal microvasculature of endotoxemic livers. We further addressed the issue whether cellular colocalization with apoptotic hepatocytes is cause or consequence of apoptosis. Intraportal exposure of rats with LPS (5 mg/kg) induced liver injury after 6 and 16 h, as given by nutritive perfusion failure (20 ± 2 and 21 ± 2%), intrahepatic leukocyte (60 ± 10 and 121 ± 48 cells/mm²), and platelet (12 ± 4 and 34 ± 4 cells/mm²) accumulation as well as parenchymal cell apoptosis (4 ± 1 and 11 ± 2 cells/mm²) and caspase cleavage (4.7 ± 2.4- and 7.0 ± 3.0-fold increase; P < 0.05 vs. saline-exposed controls). Higher doses of LPS (10 mg/kg ip) further increased intrahepatic leukocyte and platelet accumulation but not the extent of parenchymal apoptosis. Detailed spatial analysis revealed colocalization of leukocytes (range 12–24%) but barely of platelets (<6%) with apoptotic hepatocytes in all endotoxemic groups studied. It is of interest, however, that platelets were found at increasing rates in colocalization with leukocytes at 6 and 16 h after LPS exposure (5 mg/kg LPS: 7 ± 3 and 25 ± 6%; 10 mg/kg LPS: 11 ± 4 and 14 ± 1%). Platelet-leukocyte events significantly correlated with the extent of caspase cleavage as an indicator of tissue apoptosis (P < 0.05; r = 0.82). Blockade of apoptosis by a pan-caspase inhibitor caused a significant reduction of leukocyte adherence and platelet-leukocyte colocalization on LPS exposure. On the other hand, leukocyte depletion revealed reduced hepatocyte apoptosis, although values still exceeded those of controls, and in leuko- and thrombocytopenic animals, hepatocyte apoptosis was found reduced to control values. Taken together, LPS-associated hepatocyte apoptosis seems to be initiated by circulating blood cells that become adherent within the liver but might also contribute to further sustain the inflammatory cell-cell response.

intrahepatic fluorescence microscopy; bisbenzimide; lipopolysaccharide; microcirculation; cell-cell interaction

MATERIALS AND METHODS

Animal model and surgical preparation. Sprague-Dawley rats of either sex (250–300 g body wt; Charles River Laboratories, Sulzfeld, Germany) were used for the experiments. Animals were kept on water and standard laboratory chow ad libitum. The experiments were conducted in accordance with the German legislation on protection of animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council). Animals were injected intraperitoneally either with 5 (n = 10) or 10 mg/kg body wt (n = 10) Escherichia coli LPS (serotype 0128:B12; Sigma, Taufkirchen, Germany), respectively. Control animals (n = 5) received equivalent volumes of isotonic saline only. Intrahepatic fluorescence microscopic analysis of the hepatic microcirculation was performed at 6 (n = 10) and 16 h (n = 10) after LPS exposure, respectively. For this purpose, pentobarbital sodium-anesthetized animals (50 mg/kg body wt ip) were tracheotomized to facilitate spontaneous respiration (room air) and placed in supine position on a heating pad for maintenance of defense mechanisms, leukocytes can considerably aggravate tissue injury. Previously, intrasinusoidal sequestration of leukocytes with transmigration into tissue has been identified as a critical step in murine models of endotoxin-induced liver injury (4, 7). After transmigration, neutrophils attach parenchymal cells and cause severe liver cell necrosis (4, 7). Within this process, apoptotic hepatocytes have been shown to function as chemotactic signals, triggering leukocyte transmigration and thus sustaining the cell-dependent inflammatory response (13).

Beside leukocytes, platelets and their adhesion to sinusoidal endothelial cells have also been implicated in inflammatory liver injury on ischemia and reperfusion (26, 37). Platelets carry several adhesion molecules required for cell-cell-interactions, such as P-selectin, PECAM, and integrins (36), and they generate an array of proinflammatory mediators and oxygen radicals, thus being comparably regarded as mediator and effector cells in inflammation (11). The capability of platelets to adhere and directly interact with leukocytes (22) as well as to modulate activation of leukocyte function (19) further underscores the need to broadly integrate these cells in the complex, still incompletely understood series of events leading to inflammatory tissue injury. Using a rat model of systemic endotoxia, we therefore studied by high-resolution multi-fluorescence microscopy whether apoptotic hepatocytes might cause platelets, similar to leukocytes, to seed at the sinusoidal endothelial lining and whether endotoxemia causes a colocalization between platelets and leukocytes. We further addressed the issue whether cellular colocalization with apoptotic hepatocytes is cause or consequence of apoptosis.

THE DEVELOPMENT OF LIVER DYSFUNCTION and liver failure is a significant cause of morbidity and mortality in patients with gram-negative sepsis (2, 16). Endotoxemia/sepsis induces an inflammatory response with leukocytes primarily contributing to hepatocellular injury by the release of a variety of harmful mediators (10, 17). Although, in turn, necessary for vital host...
body temperature at 36–37°C. Polyethylene catheters (PE 50, ID 0.58 mm, Portex, Hythe, UK) in the right carotid artery and Jugular vein allowed for assessment of systemic hemodynamics, injection of fluorescent dyes and platelets, as well as permanent infusion of isotonic saline solution at a rate of 2 ml·kg⁻¹·h⁻¹. After transverse laparotomy, the animals were positioned on their left side, and the left liver lobe was exteriorized and covered with a glass slide for intravital fluorescence microscopy (28, 29). Liver tissue was sampled for subsequent Western blot analysis, histology, and immunohistochemistry (n = 5/group).

To assess whether the colocalization of platelets with leukocytes is of pathophysiological relevance to the liver injury induced by endotoxin, additional animals were treated with nitrogen mustard or busulphan. Nitrogen mustard-treated animals (1.75 mg/kg body wt iv; Sigma) developed jaundice (10% of initial values) within 8–10 days (38) and were then exposed to endotoxin (10 mg/kg body wt ip; LPS/busulphan; n = 3). At 6 h after LPS exposure, intravital fluorescence microscopic analysis of the hepatic microcirculation was performed as described in Intravital fluorescence microscopy, including the injection of fluorescently labeled platelets.

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Platelet preparation. Resting platelets were isolated using the Sepharose column as described previously (33). Briefly, blood was drawn from healthy volunteers, and after centrifugation, platelet-rich plasma was layered on the prepared Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden). Isolated platelets were stained with BCECF (Molecular Probes, Eugene, OR), passed again through the Sepharose column, and were resuspended in PBS to a final concentration of 1 × 10⁷ cells/ml.

Intravital fluorescence microscopy. After the surgical procedure and a 20-min stabilization period, in vivo microscopy was performed using a modified fluorescence microscope with a 100-W mercury lamp (Eclipse E600-FN, Nikon, Tokyo, Japan). In epi-illumination technique, the microscopic images were recorded by a CCD video camera (FK 6990-Q, Pieper, Berlin, Germany) and transferred to a video system (S-VHS Panasonic AG 7350-E, Matsushita, Tokyo, Japan). Using water immersion objectives (×200/0.75W; 40×/0.8W, Nikon, Tokyo, Japan) magnifications of ×332 and ×583 were achieved on the video screen (PVM-20M2E, Sony, Tokyo, Japan).

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Sampling and assays. At the end of in vivo microscopy of the liver, arterial blood samples were taken for the spectrophotometric determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) serum activities that served as an indicator for hepatocellular disintegration. Liver tissue was sampled for Western blot analysis, histology, and immunohistochemistry.

Flow cytometry of whole blood for analysis of platelet-leukocyte aggregates. To evaluate as to whether platelet-leukocyte colocalization represents the accumulation of circulating platelet-leukocyte aggregates induced by endotoxin or the sequential binding of platelets to adherent leukocytes, flow cytometry was performed to assess circulating platelet-leukocyte aggregates. For this purpose, whole blood aliquots were sampled from animals before and 6 h after LPS exposure (10 mg/kg body wt ip; n = 6) for subsequent analysis of circulating platelet-leukocyte aggregates. Fifty microliters of whole blood were incubated for 30 min with 2 μl Lyp20, a mouse monoclonal anti-rat CD62p antibody (Biocytex, Marseille, France). After being washed once with PBS, 2 μl FITC-coupled anti-mouse IgG (Santa Cruz Biotechnology, Heidelberg, Germany) were added for 30 min followed by washing with PBS. Finally, the monoclonal anti-rat phyceroerythrin (PE)-coupled CD45 antibody (Acris, Hidenhausen, Germany) was added for 30 min. The reaction was stopped by diluting the solution with 2 ml PBS, followed by centrifugation at 400 g for 5 min. The aliquots were washed again with PBS, and the pellet was resuspended with 2 ml Cell Fix. Flow cytometry was performed within the next 2 h.

FACScan flow cytometer (Becton-Dickinson) was calibrated with fluorescent standard microbeads (CalIBRITE Beads, Becton-Dickinson) for accurate instrument setting. Leukocytes were identified and gated by their characteristic forward and sideward scatter light (see Fig. 4A) and selectively analyzed for their red fluorescence properties in the upper left quadrant, respectively, using the CellQuest data.
handling program (Becton-Dickinson) with assessment of 3,000 events per sample. Platelet-leukocyte aggregates were identified as those events in the upper right quadrant with both green and red fluorescence. The relative fluorescence intensity of a given sample was calculated by subtracting the signal obtained when cells were incubated with the isotype specific control antibody from the signal generated by cells incubated with the test antibody.

**Histology and immunohistochemistry.** At the end of each experiment, liver tissue was fixed in 4% phosphate-buffered formalin for 2–3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 5-μm sections were cut and stained with hematoxylin and eosin (H&E) for histological analysis of apoptosis. Apoptotic cells (i.e., cells characterized by cell shrinkage and budding, nuclear and cytoplasmic condensation, and nuclear fragmentation) were counted within 50 consecutive high-power fields (HPF; ×400/0.65 objective) and given as cells per square millimeter of observation field, using a Leitz microscope (Biomed; Leitz, Wetzlar, Germany) (24). Leukocytes were stained by the AS-D chloroacetate esterase (CAE) technique and were identified by positive staining and morphology (50 HPF). Phagocytic cells were detected using the monoclonal antibodies ED1 and ED2, discriminating between blood-borne monocytes/macrophages and rat resident macrophages, i.e., Kupffer cells (6, 12). Cells were identified by positive staining and their dense cytoplasmatic labeling (50 HPF).

**Western blot analysis.** For Western blot analysis of cleaved caspase 3, liver was homogenized in lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X 100, 0.02% NaN₃, and 0.2 mM PMSF), incubated for 30 min on ice, and centrifuged for 15 min at 10,000 g. The supernatant was saved as whole protein fraction. Before use, the buffer received a protease inhibitor cocktail (1:100 vol/vol; 10,000 PMSF), incubated for 30 min on ice, and centrifuged for 15 min at 10,000 g. The supernatant was saved as whole protein fraction. Twenty micrograms of protein per lane were separated discontinuously on sodium dodecyl sulfate polyacrylamide gels (12%) and transferred to a polyvinylidifluoride membrane (Immobilon-P, Millipore, Eschborn, Germany). After blockade of nonspecific binding sites, membranes were incubated for 2 h at room temperature with rabbit anti-cleaved caspase-3 (Asp 175; 1:1,000; Cell Signalling Technology, Wiesloch, Germany), followed by peroxidase-conjugated goat anti-rabbit IgG antibody (1:2,000, Cell Signalling Technology) as secondary antibody.

Protein expression was visualized by means of luminol enhanced chemiluminescence (ECL plus; Amersham Pharmacia Biotech, Freiburg, Germany) and exposure of the membrane to a blue light-sensitive autoradiography film (Kodak BioMax Light Film, Kodak-Industrie, Chalon-sur-Saone, France). Signals were densitometrically assessed (Gel-Dokumentationssystem E.A.S.Y. Win32, Herolab, Wiesloch, Germany).

**Statistical analysis.** All data are expressed as means ± SE. After assumption of normality and equal variance across groups was proven, differences between groups were assessed using ANOVA followed by the appropriate post hoc comparison test. Statistical significance was set at *P* < 0.05. Statistics were performed using the software package SigmaStat (Jandel, San Rafael, CA).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5 mg/kg LPS</th>
<th>10 mg/kg LPS</th>
<th>6 h</th>
<th>16 h</th>
<th>6 h</th>
<th>16 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonperfused sinusoids, %</td>
<td>2.3±0.9</td>
<td>20.8±2.0*</td>
<td>21.1±2.1*</td>
<td>23.6±2.9*</td>
<td>33.2±2.2*#</td>
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<tr>
<td>Venular leukocyte rolling, %</td>
<td>12.8±1.7</td>
<td>41.0±3.0*</td>
<td>34.9±2.8*</td>
<td>51.3±7.9*</td>
<td>54.5±7.3*</td>
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</tr>
<tr>
<td>Venular leukocyte adherence, n/mm²</td>
<td>35±11</td>
<td>132±42</td>
<td>178±79*</td>
<td>217±51*</td>
<td>264±47*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Venular platelet adherence, n/mm²</td>
<td>0.2±0.0</td>
<td>3.6±2.4</td>
<td>14.2±10.4*</td>
<td>9.0±9.0</td>
<td>28.2±5.3*</td>
<td></td>
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Values are means ± SE; *P* < 0.05 vs. control, #P < 0.05 vs. LPS 6 h, ANOVA followed by appropriate post hoc comparison test.
Apoptosis and blood cell colocalization in endotoxemic livers of zVAD(OMe)-fmk-treated animals. On treatment of animals with the pancaspase inhibitor z-VAD(OMe)-fmk, hepatocyte apoptosis was found almost absent in livers at 6 h after LPS exposure (LPS/zVAD, cells/mm²: 1.2 ± 0.6, \( P < 0.05 \) vs. LPS: 8.4 ± 2.0). Of interest, intrahepatic adherence of both leukocytes (LPS/zVAD, cells/mm²: 66 ± 9, \( P < 0.05 \) vs. LPS: 155 ± 25) and platelets (LPS/zVAD, cells/mm²: 23 ± 3, \( P < 0.05 \) vs. LPS: 43 ± 18) was found markedly reduced. Moreover, blockade of hepatocyte apoptosis further caused a reduction in colocalization of leukocytes with platelets (LPS/zVAD, %: 7 ± 2, \( P < 0.05 \) vs. LPS: 11 ± 4).

Apoptosis in endotoxemic livers of nitrogen mustard- and busulphan-treated animals. Experimental leukocytopenia was induced by nitrogen mustard with an average value of 8% of its initial values within 48 h. These leukocytopenic animals revealed reduced hepatocyte apoptosis with 4.9 ± 1.2 cells/mm², which, however, was still more pronounced than in control animals without LPS exposure (3.4 ± 0.9 cells/mm²).

Within 8–10 days after busulphan application, systemic leukocyte and platelet counts reached a nadir of <10% of its initial values followings.
initial values \((0.4 \pm 0.2 \times 10^9\) and \(77 \times 10^9\) per liter vs. baseline: \(11.9 \pm 1.0 \times 10^9\) and \(1,150 \times 10^9\) per liter). Noteworthy, in these animals, hepatocyte apoptosis on LPS exposure for 6 h was found reduced to \(3.8 \pm 0.7\) cells/mm\(^2\), reflecting control values, and thus significantly differed from values found in endotoxemic animals with normal circulating blood cell counts.

Histological analysis of endotoxemic livers. In line with in vivo fluorescence microscopic analysis of hepatocellular apoptosis, the number of hepatocytes exhibiting characteristic morphological features of apoptosis in H&E-stained tissue sections was increased in LPS-exposed animals (Table 2). Moreover, analysis of CAE- and ED1/ED2-stained liver tissue sections revealed a markedly increased infiltration of both leukocytes and macrophages (Table 2). Whereas the ratio of ED1\(^+\) to ED2\(^+\) cells remained constant at 6 h, it was found markedly higher at 16 h after LPS exposure due to a disproportionate increase in the numbers of newly invading blood monocyte-derived ED1\(^+\) macrophages (Table 2).

Western blot analysis of endotoxemic livers. As illustrated by Western blot analysis of liver tissue, LPS exposure caused pronounced cleavage of caspase 3 at 6 and 16 h, indicating tissue cell death by apoptosis (Fig. 5). Regression analysis revealed a significant correlation between the colocalization of...
Values are means ± SE. Quantitative assessment of hepatocellular apoptosis was performed in hematoxylin and eosin-stained sections, and leukocyte infiltration was assessed in chloroacetate (CAE)-stained tissue sections. ED1 and ED2 immunohistochemical staining served for analysis of monocyte/macrophage recruitment. *P < 0.05 vs. control, #P < 0.05 vs. LPS 6 h, ANOVA followed by appropriate post hoc comparison test. HPF, high-power field.

## DISCUSSION

Previously, it has been shown by histology that parenchymal apoptotic cell death coincides with leukocyte transmigration in mice with galactosamine/endotoxin-induced liver injury (13). By in vivo high-resolution multiﬂuorescence microscopy, we conﬁrm and extend this observation in that leukocytes, but not platelets, frequently colocalize with apoptotic hepatocytes. By blockade of hepatocyte apoptosis in endotoxemic livers using the pancaspase inhibitor zVAD(Ome)-fmk, we could further demonstrate that colocalized leukocytes are, at least to some extent, the consequence rather than the cause of hepatocyte apoptosis. On the other hand, however, leuko- and thrombocytopenic animals, which were found resistant against induction of hepatocyte apoptosis by endotoxin, provided major evidence for a causal role of intrahepatic cell sequestration for apoptotic tissue injury. It is of interest that in endotoxemic animals, platelets were found at increasing rates in colocalization with leukocytes, most probably representing the sequestration and accumulation of circulating platelet-leukocyte aggregates, as indicated by the present flow cytometric analysis. In line with this, platelet-leukocyte colocalization was also found markedly reduced in endotoxemic livers lacking hepatocyte apoptosis. Thus parenchymal apoptosis does not speciﬁcally attract platelets; however, the adhesion and interaction of platelets with leukocytes might represent a crucial step for leukocyte activation with the risk of aggravation of leukocyte-dependent tissue injury.

Whereas both apoptotic and necrotic hepatocytes can be identiﬁed in endotoxemia (4, 34), it has been recognized that hepatocellular apoptosis may represent an early, general, and possibly causal event, often preceding liver cell necrosis (9, 15). Interventions directed against TNF-α, e.g., the use of neutralizing antibodies or TNF-receptor knockout animals as well as inhibition of TNF-α gene transcription, protected against hepatocellular apoptosis and liver injury and thus...
established a major role for TNF-α in mediating this mode of cell death. However, there is also evidence for TNF-α-independent mechanisms, as given by a recent study (24) that demonstrated the inhibition of endotoxin-induced parenchymal apoptosis by blockade of p53. The fact that in p53-blocked endotoxemic animals, intrahepatic leukocyte accumulation was found reduced (24) supports the view that apoptotic hepatocytes are capable of recruiting leukocytes (13). In line with this, we herein provide direct in vivo evidence for the colocalization of leukocytes with apoptotic hepatocytes. Moreover, the observation that in the zVAD-treated endotoxemic animals lacking hepatocyte apoptosis, intrahepatic leukocyte adherence and platelet-leukocyte colocalization were found markedly reduced, allows us to state that intrahepatic cell sequestration is, at least to some extent, a consequence of hepatocyte apoptosis. In parallel, the fact that thrombo- and leukocytopenic animals failed to show hepatocyte apoptosis on LPS exposure provides major evidence for the impact of those blood cells in mediating apoptotic cell death. Thus it is reasonable to hypothesize that hepatocyte apoptosis may be initiated by cells that become adherent within the liver; however, they may also contribute to further sustain inflammatory cell adherence.

Unexpectedly, platelets did not colocalize with apoptotic hepatocytes, which theoretically could be due to the fact that the platelets we used were resting and missed the appropriate priming by endotoxin. However, although not analyzed quantitatively, we observed by intravital microscopy that adherence of endogenous rhodamine 6G-stained platelets, exhibiting colocalization with leukocytes, also failed to spatially coincide with apoptotic hepatocytes. Thus BCECF-stained platelets seem to reliably mirror flow behavior of this blood cellular component.

By flow cytometry, LPS exposure was shown to cause a significant fall in circulating platelet-leukocyte aggregates, disproving the sequential binding of platelets to adherent leukocytes and favoring the sequestration of circulating platelet-leukocyte aggregates. This might be the explanation for our present observation that platelets were frequently found in colocalization with leukocytes adherent within the microvasculature of endotoxemic livers. A close interaction between platelets and leukocytes has been described in coagulation (3) and vascular inflammation (21). A range of molecules, mainly associated with the platelet surface and/or the platelet granules, regulates the capacity of platelets to cross-talk with other inflammatory cells during the process of inflammation (21). Thereby, synergistic effects, as shown for leukocytes and platelets in mediating sinusoidal endothelial cell apoptosis (27), might enhance cell-dependent tissue injury. Moreover, platelets have been shown to prevent apoptosis and prolong the functional lifespan of neutrophils (1). Because leukocytic apoptosis is associated with loss of distinctive functions, such as degradation and respiratory burst (35), it has been proposed that apoptosis significantly contributes to resolution of inflammation (8, 14). In concert with sequestrated platelet-leukocyte aggregates, endotoxic LPS may directly prolong leukocyte functional longevity by inhibiting the rate of apoptosis in a concentration-dependent fashion (14). Moreover, platelet-selectin-dependent superoxide anion release by leukocytes may increase their tissue-damaging potential (19). Thus platelets coinciding with leukocytes might contribute to apoptotic tissue injury by increasing not only the lifespan but also the harmfulness of leucocytes. Being aware that correlation does not necessarily mean a causative relationship, the significant correlation between the platelet colocalization with leukocytes and the extent of cleaved caspase underlines a potential impact of this cell-cell interaction in the endotoxemic liver. Thereby, both leukocytes and platelets seem to participate in endotoxin-induced tissue injury. In support of this view, animals suffering leukocytopenia were not completely protected against apoptotic tissue injury, whereas animals that underwent induction of both thrombocytopenia and leukocytopenia by application of busulphan exhibited complete blockade of hepatocyte apoptosis at 6 h on LPS exposure. Thus platelets per se seem to be of some pathophysiological relevance in manifestation of endotoxemic liver injury, however, not via direct locally confined cross-talk with hepatocytes.

Previous studies (31, 32) of hepatic inflammatory injury have shown that Kupffer cells participate in LPS hepatotoxicity in that pharmacological modulation of Kupffer cell activity confers protection from tissue injury. Recently, in an ex vivo ischemia-reperfusion model of the rat liver, the synergistic effects of leukocytes and platelets on sinusoidal endothelial cell apoptosis were found almost completely abrogated after Kupffer cell depletion, suggesting a triangular interaction among Kupffer cells, platelets, and leukocytes (27). Although not specifically studied as to whether this cross-talk is also of significant relevance for the endotoxemic liver, the high numbers of invading monocytes and macrophages on LPS exposure imply a certain local demand for phagocytic clearance capacity.

In summary, we could demonstrate in an in vivo model of endotoxemic liver injury that platelets, unlike leukocytes, do not allocate in direct vicinity to apoptotic hepatocytes but, however, might play a peculiar role in the communication with leukocytes. Despite the fact that the allocation of platelets among the inflammatory cells is still at issue and not completely defined yet, this study contributes to our understanding of platelet-associated signaling events and might offer new potential insight into platelet function in inflammatory diseases.

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