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Milk Fat Globule Epidermal Growth Factor-VIII Is Down-Regulated in Sepsis via the Lipopolysaccharide-CD14 Pathway

Hidefumi Komura,* Michael Miksa,* Rongqian Wu,* Sanna M. Goyert,† and Ping Wang2*

Phagocytosis prevents the release of potentially harmful or immunogenic materials from dying cells. Milk fat globule epidermal growth factor (EGF)-factor VIII (MFG-E8) mediates the clearance of apoptotic cells. We have previously shown that the administration of MFG-E8-rich exosomes from immature dendritic cells promotes the phagocytosis of apoptotic cells and improves survival in sepsis. Because endotoxin is elevated in polymicrobial sepsis, we hypothesized that down-regulation of MFG-E8 is mediated via the LPS-CD14 pathway, eventually leading to the accretion of apoptotic cells. Polymicrobial sepsis was induced by cecal ligation and puncture (CLP) in CD14-deficient (CD14−/−), TLR4-mutated and wild-type (WT) mice. In addition, endotoxemia was elicited by i.p. injection of LPS. LPS was also neutralized by pretreating CLP-induced WT mice with polymyxin B. Splenic MFG-E8 expression, phagocytic activity, and apoptosis were assessed 5 and 20 h after CLP or 5 h after LPS administration. In septic WT mice, MFG-E8 mRNA and protein levels were suppressed by 49 and 33%, respectively. Endotoxemia reduced MFG-E8 mRNA expression in a dose dependent manner and the down-regulation of MFG-E8 mRNA expression in CLP-induced sepsis was attenuated by polymyxin B. This CLP-induced suppression was not observed in both CD14−/− and TLR4-mutated mice. CLP significantly decreased phagocytic activity of peritoneal macrophages in WT (by 30%), but not in CD14−/− mice. CLP also induced significant apoptosis in the spleen of WT (by 61%), but less in CD14−/− mice. Thus, MFG-E8 production is down-regulated in sepsis by LPS-CD14 dependent fashion, leading to a reduction of phagocytosis of apoptotic cells. The Journal of Immunology, 2009, 182: 581–587.

Sepsis, defined as systemic inflammatory response syndrome (SIRS), which is caused by infection (1), is the most common case of mortality in the intensive care units (2). Sepsis triggers the secretion of proinflammatory cytokines (3), activation of leukocytes (4), and collapse of coagulation and fibrinolysis (5). Sepsis also leads to an immunocompromised state with decreasing B and CD4 T cells due to apoptosis (6), and increasing anti-inflammatory cytokines (7). Recently, Hotchkiss et al. (8) have shown that pretreatment of animals with apoptotic splenocytes worsens the outcome of sepsis and that overexpression of Bcl-2 or administration of caspase inhibitors protects lymphocyte apoptosis and improves survival in polymicrobial sepsis (9). Hence, immediate removal of apoptotic cells is required for producing beneficial effects in sepsis (10, 11).

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Abbreviations used in this paper: SIRS, systemic inflammatory response syndrome; MFG-E8, milk fat globule EGF-factor VIII; EGF, epidermal growth factor; CLP, cecal ligation and puncture; WT, wild type; BW, body weight; MFI, mean fluorescence intensity; PI, propidium iodine.

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Materials and Methods

Cecal ligation and puncture

Male weight-matched (20–25g) wild-type (WT) (C3H/HeN, Taconic Farms), CD14-deficient mice (C3H/HeN), and TLR4-mutated mice (C3H/HeJ, The Jackson Laboratory) were housed in a temperature-controlled room on a 12-h light/dark cycle and fed Purina chow diet. Before the induction of sepsis, the mice were fasted overnight but allowed water ad libitum. The mice were anesthetized by isoflurane inhalation, and the abdomen was shaved and washed with 10% povidone iodine. The animals were randomly assigned to various groups, and the cecum was...
ligated and double-punctured with a 22-gauge needle. Sham operated animals underwent the same procedure with the exception that the cecum was neither ligated nor punctured. The animals were resuscitated with 1 ml/25 g body weight (BW) isotonic sodium chloride solution (i.c. injection) immediately after surgery. The animals were anesthetized at 5 and 20 h after CLP or sham operation (Sham) for the collection of tissue samples. All experiments were performed in accordance with the National Institutes of Health’s Guidelines for Use of Experimental Animals. This project was approved by the Institutional Animal Care and Use Committee of The Feinstein Institute for Medical Research.

Administration of endotoxin in mice
Endotoxia was induced by i.p. injection of 15 or 45 mg/kg BW LPS (Escherichia coli 055:B5; Difco Laboratories) dissolved in 1 ml of saline. One milliliter of normal saline (0 mg/kg BW) was injected to the control group. Samples were collected at 5 h after LPS injection.

Administration of polymyxin B
At 1 h before CLP, as well as at 10 h after CLP, polymyxin B (Sigma-Aldrich), at a dose of 2000 U/kg BW, or 0.2 ml of normal saline was administered i.m. Polymyxin B is an antibiotic, which binds and detoxifies LPS. In vitro studies have confirmed that polymyxin B neutralizes LPS activity (19). Our previous study has shown that administration of polymyxin B markedly decreased plasma levels of LPS after CLP in vivo (20).

Determination of splenic MFG-E8 gene expression by quantitative PCR
Quantitative PCR was performed to detect splenic MFG-E8 mRNA expression levels in mice. Total RNA was prepared using TRIzol reagent (Invitrogen). Twenty-five milligrams of tissue was homogenized in 1 ml TRIzol and the homogenate was separated into aqueous and organic phases. The aqueous liquid was isolated and followed by chloroform addition and centrifugation. RNA was precipitated from the aqueous phase by addition of isopropanol, and washed with ethanol. The pellet was dissolved in 0.1% diethylpyrocarboxylic-treated, deionized, and distilled water. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm. Reverse transcription was performed on total RNA (4 µg) using Oligo(dT)12-18 primer (Invitrogen). The resulting samples from individual mice were diluted and divided into aliquots for separate PCRs for MFG-E8 and the housekeeping gene β-actin. Mouse MFG-E8 primers were forward 5'-3'-CCG ACA GGG ATC GTC AAT G and reverse 5'-3'-CCG AGA AGG TTC ACC TGG AT. β-actin primer sequences were forward 5'-3'-TGT TAC CAA CTG GGA CGA CA and reverse 5'-3'-GGG GTG TTG AAG GTC TCA AA. Quantitative PCR was performed by 7300 Real Time PCR system (Applied Biosystems) with SYBR Green as detection dye. The reaction was carried in a 25 µl final reaction volume containing 0.2 µM concentration of each forward and reverse primer, 2.5 µl cDNA, 7.5 µl H2O, and 12.5 µl SYBR Green PCR Mast Mix (Applied Biosystems). The thermal profile for the real-time PCR was 50°C for 2 min, 95°C for 10 min, and followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification was calculated according to the ΔΔCt method (User Bulletin Number 2: Relative Quantitation of Gene Expression, Applied Biosystems). In addition, dissociative curve analysis was performed to confirm the specificity of PCR product in this experiment.

Determination of splenic MFG-E8 protein level by Western blotting
Tissue samples (25 mg) were lysed and homogenized in 300 µl of lysis buffer (10 mmol/L Tris-buffered saline, 1 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L sodium orthovanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml Aprotinin, and 1% Triton X-100) for 30 min on ice, and tissue sample lysate was cleared by centrifugation at 14000 rpm for 15 min at 4°C. Samples were diluted in 1% SDS and quantified using the DC protein assay (Bio-Rad). The protein (15 µg) was fractionated on a 4% to 12% Bis-Tris gel and transferred to a nitrocellulose membrane. Nitrocellulose membranes were blocked by incubation in TBST (10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20) containing 5% BSA for 1 h. Membranes were incubated with goat anti-mouse MFG-E8 Ab (AF2805, R&D Systems) overnight at 4°C, washed three times in TBST for 15 min. and incubated subsequently with HRP-conjugated secondary Ab for 1 h at room temperature. Specific proteins were visualized using an ECL system (Amersham Biosciences). Band densities were determined using a Bio-Rad image system.

FIGURE 1. Suppression of MFG-E8 production in the spleen in septic WT mice. Splenic MFG-E8 mRNA expression (A, percentage of Sham), and splenic MFG-E8 protein level (B, percentage of Sham) in WT mice were assessed by quantitative PCR at 5 and 20 h after cecal ligation and puncture (5h CLP and 20h CLP) or sham operation (Sham), or Western blotting at 20 h CLP or Sham. Data are expressed as means ± SEM (n = 4–6/group) and compared by one-way ANOVA and Student-Newman-Keuls test or by Student’s t test: *, p < 0.05 vs sham-operated animals; #, p < 0.05 vs 5h CLP animals.

Bands corresponded to splenic MFG-E8 protein were normalized by splenic β-actin expression.

Ex vivo phagocytosis assay
This novel phagocytosis assay was performed following the method as recently developed in our laboratory. In brief, freshly collected cells by peritoneal lavage with cold HBSS from Sham and 20 h CLP animals were washed twice with PBS and RBC were lysed with ammonia-chloride potassium buffer, and then, cells were plated at a density of 1 × 10⁶ cells in 6 well plate and cultured in DMEM containing 10% heat-inactivated FBS, 10 mmol/L HEPES, 100 U/ml penicillin and 100 mg/ml streptomycin for 2 h at 37°C in a humidified atmosphere containing 5% CO₂.

Autologous thymocytes were cultured in RPMI 1640 medium substituted with 10% heat-inactivated FBS, 10 mmol/L HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin and treated with 10 µM dexamethasone for 24 h (>99% apoptotic CD 90⁺) cells assessed by annexin V) at 37°C in a humidified atmosphere containing 5% CO₂. After washing with PBS twice, apoptotic thymocytes were stained with 20 ng/ml Pfuoduccinimidyl ester (Invitrogen) for 30 min at room temperature, which is a pH-sensitive fluorescent dye that emits light in the red range (approximate fluorescence excitation and emission maxima, 560/585 nm) at an increased intensity with decreasing environmental pH. After washing with PBS, cells were washed with PBS twice thoroughly to remove unengulfed thymocytes, and collected by gentle scraping and stained with both alliphophocyanin-labeled anti-CD11b Ab and Alexa Fluor 700-labeled anti-GR-1 Ab (BD Pharmingen). Analysis was performed by flow cytometry (LSRII, BD Biosciences).

Detection of apoptotic cells
Fresh spleens from sham-operated and septic animals were collected and whole splenocytes were obtained by gentle grinding of spleens between frosted glass slides, lysing RBC with ammonia-chloride potassium buffer, passing cells through a mesh. Collected splenocytes were washed twice in...
FIGURE 2. Effect of LPS on MFG-E8 mRNA expression in the spleen. MFG-E8 mRNA expression in the spleen was assessed by quantitative PCR at 5 h after administration of 0 (normal saline), 15, and 45 mg/kg BW of LPS (A, percentage of control). In the LPS inhibition study, alteration in MFG-E8 mRNA expression in the spleen was assessed by quantitative PCR at 20 h after CLP with normal saline (Vehicle) or polymyxin B (polymyxin B) treatment (B, percentage of control). Data are expressed as means ± SEM (n = 6/group) and compared by one-way ANOVA and Student-Newman-Keuls test: *, p < 0.05 vs 0 mg/kg BW LPS; #, p < 0.05 vs 15 mg/kg BW LPS in A; *, p < 0.05 vs sham-operated animals; #, p < 0.05 vs CLP + Vehicle in B.

PBS, counted, and reconstituted in Ca2+-rich annexin V binding buffer (BD Pharmingen), at a concentration of 105 cells per milliliter. Cell suspension (100 μl) was stained with 2.5 μl annexin V-FITC and/or 1 μl propidium iodine (PI) for 15 min and adjusted to a total volume of 500 μl with binding buffer. Cells were then analyzed by flow cytometry with FACSCalibur (BD Biosciences), and annexin V FITC and/or PI annexin V-FITC and/or 1

Statistical analysis
All data are expressed as means ± SEM and compared by one-way ANOVA and Student-Newman-Keuls test, or Student’s t test. Differences in value were considered significant if p < 0.05.

Results
Suppression of MFG-E8 in the spleen in late phase of sepsis
As shown in Fig. 1A, MFG-E8 mRNA expression in the spleen did not decrease at 5 h after CLP (5h CLP), but significantly decreased by 49% at 20 h after CLP (20h CLP) as compared with sham-operated animals, and by 46% as compared with 5h CLP in WT mice (p < 0.05). Splenic MFG-E8 protein levels also significantly decreased by 33% at 20 h after CLP as compared with sham-operated animals (p < 0.05, Fig. 1B), similar to what we have previously shown in rats (17).

Effect of LPS on MFG-E8 expression in the spleen
We have already reported that LPS down-regulates MFG-E8 production from cultured RAW 264.7 cells (macrophage cell line) in vitro (17). To elucidate whether LPS is responsible for MFG-E8 down-regulation in vivo, we used an animal model of endotoxemia produced by LPS injection. Our result showed that mRNA expression of MFG-E8 significantly decreased, in a dose dependent manner, by 43% at a dose of 15 mg/kg BW and by 80% at that of 45 mg/kg BW at 5 h after LPS injection (p < 0.05, Fig. 2A). To further confirm the down-regulatory effect of LPS on MFG-E8 expression in sepsis, polymyxin B was administered i.m. to septic mice to inhibit LPS activity. With administration of polymyxin B, down-regulation of MFG-E8 gene expression in the spleen was attenuated as compared with vehicle-treated septic animals (p < 0.05, Fig. 2B).

Lack of MFG-E8 suppression in septic CD14−/− mice and in septic TLR4-mutated mice
To further verify that LPS is responsible for the MFG-E8 suppression in polymicrobial sepsis, CD14-deficient (CD14−/−) mice were subjected to CLP. CD14 is an LPS receptor (21) and CD14−/− mice are resistant to LPS (18). As seen in Fig. 3, A and B, both mRNA expression and protein levels of MFG-E8 in the spleen did not significantly change in sepsis. Commercial LPS can stimulate not only TLR4, but also TLR2 (22). TLR4-mutated mice (C3H/HeJ) mice were also subjected to CLP. As seen in Fig. 3, C and D, both mRNA expression and protein levels of MFG-E8 in the spleen did not significantly change in sepsis. These results indicate that the LPS plays an important role in the MFG-E8 suppression in sepsis.

Effect of sepsis on phagocytic activity of peritoneal macrophages against apoptotic cells
MFG-E8 is necessary for the phagocytosis of apoptotic cells (16), and its activity is impaired in sepsis (17). We collected peritoneal cells from both septic WT and CD14−/− mice. Autologous apoptotic thymocytes stained with pHrodo succinimidyl ester, and
Peritoneal cells tagged by CD11b and GR-1 were used to determine the effect of sepsis on phagocytic activity ex vivo. Macrophages are identified by the combination of CD11b and GR-1 expression. CD11b\textsuperscript{high} and GR-1\textsuperscript{int/low} cells were considered as macrophages and thus, analyzed for phagocytic activity. CD11b\textsuperscript{high} and GR-1\textsuperscript{high} cells were considered as granulocytes (neutrophils) (23) and excluded from the analysis. Mean fluorescence intensity (MFI) and phagocytic activity of peritoneal macrophages (phagocytosis index) were significantly decreased in septic WT mice (683.4 $\pm$ 12.6 to 528.3 $\pm$ 19.4 in MFI, Fig. 4A and 7.50 $\pm$ 0.15% to 4.59 $\pm$ 0.15% in phagocytosis index, $p < 0.05$), but was not altered in septic CD14$^{-/-}$ mice compared with Sham (783.1 $\pm$ 20.1 to 745.7 $\pm$ 38.4 in MFI, Fig. 4E and 10.32 $\pm$ 0.49% to 9.91 $\pm$ 0.90% in phagocytosis index, Fig. 4, F–H).

FIGURE 4. Effect of sepsis on phagocytic activity of peritoneal macrophages against apoptotic cells. Peritoneal cells were collected by peritoneal lavage from septic (CLP) or nonseptic (Sham) WT or CD14$^{-/-}$ mice, cultured with pHrodo-stained apoptotic thymocytes for 1 h, and analyzed by flow cytometry. Mean fluorescence intensity (MFI) and phagocytic activity of peritoneal macrophages (phagocytosis index) were assessed in WT (MFI in A, phagocytosis index in B), or CD14$^{-/-}$ (MFI in E, phagocytosis index in F). Representative flow cytometric analysis of CD11b and GR-1 (WT in C left, CD14$^{-/-}$ in H left panels), and phagocytosis assay in the gated area by CD11b and pHrodo succinimidyl ester (WT in C right, CD14$^{-/-}$ in H right panels) are shown. Data are expressed as means $\pm$ SEM (n = 4/group) and compared by Student’s t test: *, $p < 0.05$ vs sham-operated animals.
Effect of sepsis on the clearance of apoptotic cells in the spleen

We have previously shown that MFG-E8 by itself is not anti-apoptotic, but increases the clearance of apoptotic cells by enhancing phagocytosis (17). This results in a reduction of apoptotic cells, and confers beneficial effects in sepsis. Our current results indicate that splenic apoptotic cells were significantly increased by 61% in septic WT mice (15.4 ± 1.5% in Sham, 24.9 ± 2.8% in CLP, p < 0.05, Fig. 5, A and B), but increased only slightly in septic CD14−/− mice (24.2 ± 1.9% in Sham, 30.8 ± 3.8% in CLP, Fig. 5, C and D). This indicates a preserved clearance of apoptotic cells in the spleen of septic CD14−/− mice via normal MFG-E8 levels.

Discussion

Apoptosis is one of the biologically necessary functions for homeostasis (24). However in sepsis, excessive apoptosis exists which induces both immunosuppression (6, 7) and proinflammatory cytokine up-regulations (25). Thus, immediate elimination of apoptotic cells is required to avoid further tissue injury (10, 11). To reduce harmful apoptotic cells, many studies focus on the suppression of apoptosis, for example, by overexpressing Bcl-2, administering caspase inhibitors (9), or blocking complement factor C5a (26). However, promotion of the removal of apoptotic cells by phagocytes before the secondary necrotic cell development, which leads to the release of a variety of proinflammatory cytokines, is critical to reduce apoptotic cells.

Recently, Hanayama et al. (13) have shown that MFG-E8 is one of the bridging molecules between apoptotic cells and phagocytes. This 64 kDa molecule has a unique structure, which is composed of two EGF-like domains (EGF-1 and EGF-2) containing the RGD motif (the amino acid sequence Arg-Gly-Asp) in its N-terminal (13), and is also composed of two coagulation factor VIII-like domains (C1 and C2, discoidin domain) in its C-terminal domain (15). MFG-E8 has been shown to bridge apoptotic cells and phagocytes by binding to phosphatidylserine exposed on apoptotic cells via its coagulation factor VIII homologous domain, and to αvβ3 integrin on phagocytes via its RGD motif domain (13), and plays a critical role in the clearance of apoptotic cells (16). We recently have shown that immature dendritic cell-derived exosomes, which are abundant in MFG-E8, confer beneficial effects in sepsis (17). Furthermore, our recent unpublished study has also shown that the MFG-E8 deficiency increased apoptotic cells because of less phagocytic activity, and worsened the survival in CLP-induced septic mice. Thus, it can be concluded that MFG-E8 played a crucial role in sepsis (our unpublished observation). Previous studies have revealed that the spleen is a major immunological organ to produce MFG-E8 (16), notably, follicular dendritic cells are responsible for the production of MFG-E8 in the spleen rather than tringible-body macrophages (27).

In the present study, we demonstrated that splenic MFG-E8 production decreased in a time-dependent manner in septic WT mice, and endotoxemia suppressed MFG-E8 mRNA expression in the spleen in a dose-dependent manner. The effect of LPS on MFG-E8 suppression in polymicrobial sepsis was attenuated by the administration of polymyxin B, which neutralizes LPS activities. These results strongly suggest that LPS is responsible for the suppression of MFG-E8 production in polymicrobial sepsis. It still remains the possibility that Polymyxin B did not neutralize LPS activities, but weakened the severity of sepsis by working as an antibiotic. To further define this, CD14−/− mice and TLR4-mutated mice were subjected to CLP. CD14-TLR4 receptor complex is one of the most important signaling components in Gram-negative bacterial infections. LPS binds with LPS-binding protein (28) and transduces its signal via the CD14 (29), TLR4 (30), and MD-2 (31) complex. CD14−/− mice are resistant to LPS-mediated SIRS (18), but not to trauma injury-mediated SIRS, for which TLR4 is responsible (32). Our results suggest that the absence of either CD14 or TLR4 prevents the decrease in MFG-E8 mRNA expression and protein levels in the spleen in polymicrobial sepsis. These results strongly support that the LPS-CD14-TLR4 pathway plays an important role in the expression of MFG-E8 in the spleen in polymicrobial sepsis. Our results also exhibited that the phagocytic activity of peritoneal macrophages against autologous apoptotic thymocytes decreased in septic WT, but not in septic CD14−/− mice. Additionally, apoptosis of splenocytes was increased in WT, but not in CD14−/− mice after CLP. This lack of the increase in...
splenic apoptotic cells was possibly due to the maintenance of phagocytic activity in septic CD14<sup>−/−</sup> mice. In a recent study, Chung et al. (33) have shown the role of TLR4 and Fas/FasL in sepsis-induced apoptosis. CD14-TLR4 signaling is not involved in the induction of apoptosis in sepsis. Thus, the apoptosis pathway in sepsis is not dependent on CD14-TLR4, and that the decrease in splenic apoptotic cells observed in septic CD14<sup>−/−</sup> mice is most likely caused by maintaining phagocytic activity.

There are certain limitations of our investigations for phagocytosis assay ex vivo. We used dexamethasone-induced, rather than CLP or LPS-induced, apoptotic thymocytes to measure the phagocytosis of peritoneal macrophages from septic mice. Second, this method measures the phagocytic activity of CLP-treated peritoneal macrophages, which might be different from that of the peritoneal cavity in vivo. In addition, peritoneal cells are dramatically increased after CLP in both WT and CD14<sup>−/−</sup> mice (~7–10 times, data not shown) and the cell population has also been dramatically changed. It has been reported that CD11b<sup>high</sup> and GR-1<sup>low</sup> cells are considered as resident macrophages, CD11b<sup>high</sup> and GR-1<sup>int</sup> cells as recruited (possibly activated) macrophages, and CD11b<sup>high</sup> and GR-1<sup>high</sup> cells as granulocytes (neutrophils) (23). In our experiments, even in sham mice, there were more CD11b<sup>high</sup> and GR-1<sup>int</sup> cells than CD11b<sup>high</sup> and GR-1<sup>low</sup> cells, and CD11b<sup>high</sup> and GR-1<sup>low</sup> cells have been decreased in both septic mice. As expected, these phenomena indicate that most peritoneal cells are activated and recruited in sepsis. In addition, some peritoneal resident macrophages might also be activated after sham operation alone. As such, we could only compare the phagocytic activity of the mixture of resident and activated macrophages between sham and sepsis. Furthermore, sham CD14<sup>−/−</sup> mice have less amount of macrophages in the peritoneal cavity than sham WT mice and most peritoneal cells are neutrophils in septic CD14<sup>−/−</sup> mice in which the phagocytic activity of macrophages is not impaired, so that the total amount of phagocytosis might decrease in septic CD14<sup>−/−</sup> mice. Haziot et al. (34) have also shown a similar result that there is early infiltration of neutrophils in peritoneal cavity after <i>E. coli</i> injection in CD14<sup>−/−</sup> mice. The decrease in macrophages in septic CD14<sup>−/−</sup> mice may possibly cause similar mortality to WT mice (35).

CD14 has also been described as a molecule that tethers apoptotic cells to phagocytes and CD14 deficiency leads to persisting apoptotic cells because of impaired phagocytic activity of CD14-deficient macrophages (36). They conclude that the persistence of apoptotic cells in CD14<sup>−/−</sup> mice is the result of impaired clearance, rather than of increased apoptosis. In our study, the number of basal apoptotic cells was higher in CD14<sup>−/−</sup> mice (Fig. 5), but basal phagocytic activity was not different from WT mice. These different results might be caused by different animal strains, experimental design, and/or the use of the mixture of resident and activated macrophages which were activated by sham operation.

With regard to the relationship between MFG-E8 and mortality, we have shown that MFG-E8 deficiency worsens the survival in septic mice in our unpublished study. Therefore, adequate MFG-E8 levels lead to the maintenance of the phagocytic activity, which could cause the reduction of the mortality in sepsis. With regard to the relationship between LPS-CD14-TLR4 and mortality, further investigations are required. Despite resistance to LPS and lower cytokine levels, it has been reported that the mortality of CD14<sup>−/−</sup> mice in polymicrobial sepsis is similar to that in WT mice (35). There are some reports that TLR4-mutated (C3H/HeJ) mice have a survival benefit over WT (C3H/HePas or C3H/HeN) mice in sepsis (37, 38), and that the phagocytosis of apoptotic cells is not affected in the absence of TLR4 (39). In contrast, other investigators have shown that there is no significant difference of mortality between TLR4-mutated (C3H/HeJ) mice and WT (C3H/HeNj or C3H/OuJ) mice (40, 41). This discrepancy could be due to the difference in strain and/or the severity of CLP. Furthermore, Scott et al. (42) recently have shown that CD14 and TLR4 are required for LPS uptake by hepatocytes. CD14 and TLR4 are important not only for LPS signaling but also for LPS uptake, which is possibly leading to LPS clearance.

Our results show that sepsis down-regulates the production of MFG-E8 in the spleen and the blockade of LPS attenuates that alteration. That down-regulation of MFG-E8 in polymicrobial sepsis has also been attenuated in both CD14<sup>−/−</sup> and TLR4-mutated mice. Especially, in septic CD14<sup>−/−</sup> mice, phagocytic activity is maintained, preventing the increase of apoptotic cells in the spleen. Sepsis-down-regulation of splenic MFG-E8 production is mainly LPS-CD14-TLR4 pathway dependent, and the phagocytosis of apoptotic cells in sepsis is associated with the LPS-mediated down-regulation of MFG-E8. These findings help to further understand the pathophysiological mechanisms in sepsis and further demonstrate the complexity of SIRS. Maintenance of MFG-E8 levels in sepsis may need to be considered for the management of septic patients in the future.

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Disclosures

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