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

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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),1 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).

1Abbreviations 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; MRI, 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 cut...
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 (s.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

Endotoxemia 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 total RNA. 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% diethylpyrocarbonate-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′-CGG ACA GGG ATC GTC AAT G and reverse 5′-3′-CGC 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 analyzed according to 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 dissolved 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 PVDF membrane using a wet transfer system. Bands corresponding to splenic MFG-E8 protein were normalized by 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 pHrodo succinimidyl 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 used as targets for cultured macrophages at a ratio of 4:1 (apoptotic cells/peritoneal cells) in DMEM containing 1% FBS for 1 h. After coincubating, cells were washed with PBS twice thoroughly to remove unengulfed thymocytes, and collected by gentle scraping and stained with both allophycocyanin-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 1](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)
Statistical analysis

All data are expressed as means ± SEM 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.

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-deficient 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 were assessed by quantitative PCR or Western blotting at 20 h after CLP or Sham. Splenic MFG-E8 mRNA expression (C, percentage of Sham), and splenic MFG-E8 protein level (D, percentage of Sham) in TLR4 mutated mice were assessed by quantitative PCR or Western blotting at 20 h after CLP or Sham. Data are expressed as means ± SEM (n = 6/group in A and B, n = 6/group in C and D) and compared by Student’s t test. No statistical differences were found.

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^{high} and GR-1^{int}/H11002^low cells were considered as macrophages and thus, analyzed for phagocytic activity. CD11b^{high} and GR-1^{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 ± 12.6 to 528.3 ± 19.4 in MFI, Fig. 4A and 7.50 ± 0.15% to 4.59 ± 0.15% in phagocytosis index, p < 0.05, Fig. 4, B–D), but was not altered in septic CD14^{−/−} mice compared with Sham (783.1 ± 20.1 to 745.7 ± 38.4 in MFI, Fig. 4E and 10.32 ± 0.49% to 9.91 ± 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 ± 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 αβ3 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 tringle-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
spleen. Macrophages in septic CD14
treated 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
to CD14
treatment, rather than of increased apoptosis. In our study, the number of resident macrophages between sham 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
to CD14
to CD14


demonstrate the complexity of SIRS. Maintenance of MFG-E8 is important not only for LPS signaling but also 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
to CD14

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Disclosures

The authors have no financial conflict of interest.

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