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Milk Fat Globule-Epidermal Growth Factor-Factor VIII Attenuates Sepsis-induced Acute Kidney Injury

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Abstract

Introduction—Acute kidney injury (AKI) is most commonly caused by sepsis in critically ill patients, and it is associated with high morbidity and mortality. The pathophysiology of sepsis-induced AKI is generally accepted to include direct inflammatory injury, endothelial cell dysfunction, and apoptosis. Milk fat globule-epidermal growth factor-factor VIII (MFG-E8) is a secretory glycoprotein with a known role in the enhancement of apoptotic cell clearance and regulation of inflammation. We hypothesize that administration of recombinant mouse MFG-E8 (rmMFG-E8) can protect mice from kidney injuries caused by sepsis.

Methods—Sepsis was induced in 8-week-old male C57BL/6 mice by cecal ligation and puncture (CLP). rmMFG-E8 or PBS (vehicle) was injected intravenously at a dosage of 20 µg/kg body weight at time of CLP (n=5–8 mice/group). After 20 h, serum and renal tissue were harvested for various analyses. The renal injury markers blood urea nitrogen (BUN) and creatinine were determined by enzymatic and chemical reactions, respectively. The gene expression analysis was carried-out by real-time qPCR.

Results—At 20 h after CLP, serum levels of BUN and creatinine were both significantly increased in the vehicle group compared to the sham group, while the mice treated with rmMFG-E8 had a significant reduction in BUN and creatinine levels by 28% and 24.1%, respectively

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CONFLICT OF INTEREST:

Ping Wang is an inventor of the pending PCT application #WO/2006/122327: "Milk fat globule epidermal growth factor-factor VIII and sepsis" and PCT application #WO/2009/064448: "Prevention and treatment of inflammation and organ injury after ischemia/reperfusion using MFG-E8." These patent applications cover the fundamental concept of using MFG-E8 for the treatment of sepsis and ischemia/reperfusion injury. PW is a co-founder of TheraSource LLC. Other authors report no financial conflicts of interest.

AUTHOR CONTRIBUTIONS

PW conceived the idea and supervised the whole project. CC, MA, W-LY, PW designed the experiments. CC performed the experiments. MZ carried-out IHC. CC analyzed the data and prepared the figures. MA wrote the manuscript. JMN, GFC, PW reviewed and edited the manuscript. All authors read and approved the final manuscript.

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(BUN: 197.7 ± 23.6 vs. 142.3 ± 20.7 mg/dL; creatinine: 0.83 ± 0.12 vs. 0.63 ± 0.06 mg/dL; $p < 0.05$). Expression of novel biomarkers of renal tissue injury neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) were also significantly downregulated by 58.2% and 95%, respectively after treatment with rmMFG-E8. Pro-inflammatory cytokine IL-6 and TNF- α mRNA were significantly reduced by 50.8% and 50.3%, respectively, in rmMFG-E8-treated mice compared to vehicle-treated mice. The mRNA levels of the chemokines keratinocyte chemoattractant (KC) and macrophage inhibitory protein-2 (MIP-2) were reduced by 85.1% and 78%, respectively in mice treated with rmMFG-E8 compared to the vehicle mice. In addition, the expression of inter-cellular cell adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) mRNA was downregulated by 35.6% and 77.8%, respectively in rmMFG-E8-treated mice compared to the vehicle animals ($p < 0.05$).

Conclusions—Treatment with rmMFG-E8 reduces renal tissue injury induced by sepsis through inhibiting the production of pro-inflammatory cytokines and chemokine, as well as through the activation of endothelial cells. Thus, MFG-E8 may have a therapeutic potential for treating AKI induced by sepsis.

Keywords

MFG-E8; Sepsis; Acute Kidney Injury; inflammation; neutrophils; ICAM-1; PECAM-1

INTRODUCTION

Sepsis arises due to an overwhelming immune response to infection which may lead to tissue damage, multiple organ failure, and death (1, 2). The incidence of severe sepsis in the United States as recorded in last decade has shown an average annual increase of 13% (3). Despite a number of randomized clinical trials of specific therapies involving multitudes of patients, lack of reproducibility and refractory outcomes of these treatment options have been reported (1, 4). Therefore, early diagnosis, rapid resuscitation of fluid, and timely administration of antibiotics represent the only therapeutic options leading to improved outcomes for patients with sepsis (1, 4–6). Sepsis associated multi-organ dysfunctions strongly attribute to intensive care unit-related morbidity, which includes prolonged length of hospital stay, ventilation, nosocomial infections, and mortality (7–9). Kidney is one of the major organs which become frequently afflicted during sepsis. Acute kidney injury (AKI) occurs in about 19% patients with moderate sepsis, 23% with severe sepsis and 51% with septic shock (9–11). Sepsis-associated AKI reflects a high burden of morbidity and mortality in both children and adults with critical illness.

AKI is often more acute and severe in patients with sepsis compared with non-septic AKI (9). Clinical and basic science studies indicate that sepsis-associated AKI is distinct from AKI without sepsis, driven by a number of characteristic pathophysiological mechanisms. Although sepsis-induced hypoperfusion leading to tubular necrosis has been reported as the primary pathophysiology for AKI, growing body of evidence showing numerous drivers for injury are now recognized as playing a role in sepsis-associated AKI (9, 11, 12). These include ischemia-reperfusion injury to the glomerulus, inflammation in the nephron, hypoxic and/or oxidant stress, cytokine- and chemokine-driven direct tubular injury, and tubular and mesenchymal cell apoptosis (9, 12–14). Despite revealing pathophysiology, no singular

effective therapy for AKI has been identified, understanding of AKI risk and early detection of injury coupled with initiation of appropriate supportive care remain the basis of therapy. Our previous studies demonstrate recombinant milk fat globule-EGF factor VIII (MFG-E8) protein, a magic bullet to protect against a wide range of inflammatory diseases including sepsis, ischemia-reperfusion (I/R)-mediated injuries and inflammatory bowel disease (IBD) (15–18). Although MFG-E8 was shown to improve the overall disease phenomenon of sepsis, its role in protecting local organs especially the kidney from acute inflammation and injury has not been studied previously.

MFG-E8 is a 66 kDa glycoprotein that is expressed in nearly all organs and various cell types including macrophages and dendritic cells (15). It is strongly expressed in mammary glands during lactation, as well as in the spleen, lungs, and liver (15, 19). It was initially identified as a major component of bovine milk fat and is known to play a role in facilitating the phagocytic clearance of apoptotic cells by professional phagocytes (20). MFG-E8 is secreted from cells and binds to $\alpha_v\beta_3$ -integrin on phagocytes and exposed phosphatidylserine (PS) on apoptotic cells via its N- and C-terminal conserved domains, respectively, enhancing phagocytic clearance of apoptotic cells (20). Apart from this generalized function, a direct, MFG-E8-mediated anti-inflammatory role via inhibition of pro-inflammatory cytokines and chemokines has also been reported (15, 21). Hence, MFG-E8 is a pivotal element for maintaining tissue homeostasis by regulating several intracellular signaling events, and its deficiency may lead to severe inflammatory and autoimmune diseases (18, 22, 23). Recent studies have shown that during acute inflammation the expression of MFG-E8 in various organs became decreased, while the replenishment of MFG-E8 deficiency ameliorated systemic inflammation (15, 16). Although the overall beneficial outcomes of MFG-E8 in sepsis have been demonstrated, the impact of sepsis towards developing AKI and the role of recombinant MFG-E8 to attenuate the extent of kidney injuries are largely unknown. In the current study, we therefore investigated the role of MFG-E8 in AKI caused by sepsis. Based on our findings, MFG-E8 could serve as a potent therapeutic agent in sepsis-associated AKI by improving renal functions as reflected by decreased levels of blood urea nitrogen (BUN) and creatinine. Our study also revealed the treatment with recombinant mouse MFG-E8 (rmMFG-E8) attenuated the novel renal function biomarkers, namely neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) which were accompanied by the reduction of renal tissue histological damage, inhibition of the expression of pro-inflammatory cytokines, chemokines and cell adhesion molecules in the renal tissues. Thus, MFG-E8 may serve as an outstanding therapeutic potential for treating AKI induced by sepsis.

MATERIALS AND METHODS

Mouse model for polymicrobial sepsis

Male 8–10 weeks old C57BL/6 mice (21–28 g body weight) were purchased from Taconic (Albany, NY, USA). All experiments were performed in accordance with the guidelines for the use of experimental animals by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of The Feinstein Institute for Medical Research. Mice were anesthetized with isoflurane inhalation and underwent cecal ligation

and puncture (CLP) (24). Briefly, a 1.5 cm midline incision was made to the abdominal wall, and the cecum exposed and ligated 0.5 cm from the tip with 4-0 silk suture. A 22-gauge needle was used to make one puncture through and through to the distal cecum, extruding a small amount of fecal material. The cecum was replaced into the abdomen, and the abdominal wound was closed in two layers with running 6-0 silk suture. The sham mice underwent the same procedure without ligation and puncture of the cecum. Both sham- and CLP-operated animals were resuscitated with 1 ml of normal saline subcutaneously.

Administration of rmMFG-E8 into septic animals

Immediately after performing CLP, a small incision on the neck was made to expose the internal jugular vein (IJ). rmMFG-E8 (Cat. No.: 2805-MF-050; R&D systems, Minneapolis, MN) was delivered by bolus injection through the IJ vein using 29G $\times$ 1/2" U-100 insulin syringe (Terumo Medical Corporation, Elkton, MD) at a concentration of 20 μ g/kg BW in 100 μ l or same volume of PBS as vehicle. The proximal and distal ends of the jugular vein were occluded and the wound was closed with one interrupted 4-0 silk suture. After wound closure, the mouse was given 500 μ l of normal saline (NS) subcutaneous fluid resuscitation. The animals were allowed food and water *ad libitum* until 20 h after the time of intervention and the animals were euthanized. Blood and renal tissue samples were collected for various *ex vivo* analyses.

Analysis of serum renal function markers

Blood samples were centrifuged at 3,000g for 10 min to collect serum and then analyzed for renal injury parameters immediately. BUN and creatinine were measured by using commercial assay kits according to the manufacturer's protocol (Pointe Scientific; Lincoln Park, MI).

Quantitative real-time PCR

Total RNA was extracted from kidney tissue using TRIzol (Invitrogen; Carlsbad, CA) and reverse-transcribed into cDNA using murine leukemia virus reverse transcriptase (Applied Biosystems; Foster City, CA). The PCR reaction was performed in 20 μ l of final volume containing 0.08 μ mol of forward and reverse primer each, 3 μ g of cDNA, and 10 μ l of SYBR Green PCR Master Mix (Applied Biosystems). The thermal profile used by the Applied Biosystems StepOnePlus Real-Time PCR System was: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 seconds, and 60°C for 1 min. Mouse β -actin was used for normalization. Relative expression of mRNA was represented as fold induction in comparison to the sham group. The primer sequences are: NGAL (NM_005564.4) Forward: CTCAGAACTTGATCCCTGCC, Reverse: TCCTTGAGGCCAGAGACTT; KIM-1 (NM_134248.2) Forward: TGCTGCTACTGCTCCTTGTG, Reverse: GGGCCACTGGTACTCATTCT; IL-6 (NM_031168) Forward: CCGGAGAGGAGACTTCACAG, Reverse: GGAAATTGGGGTAGGAAGGA; TNF- α (NM_013693.2) Forward: AGACCCTCACACTCAGATCATCTTC, Reverse: TTGCTACGACGTGGGCTACA; KC (NM_008176) Forward: GCTGGGATTACCTCAAGAA, Reverse: ACAGGTGCCATCAGAGCAGT; MIP-2 (NM_009140) Forward: CCCTGGTTCAGAAAATCATCCA, Reverse: GCTCCTCCTTTCCAGGTCAGT; ICAM-1 (NM_010493) Forward:

GGGCTGGCATTGTTCTCTAA, Reverse: CTTGAGAGGCAGGAAACAGG; PECAM-1 (NM_008816) Forward: ATGACCCAGCAACATTACACA, Reverse: CACAGAGCACCGAAGTACCA; β -actin (NM_007393) Forward: CGTGAAAAGATGACCCAGATCA, Reverse: TGGTACGACCAGAGGCATACAG.

Histological assessment of renal injury

Renal tissue was fixed in 10% formalin and embedded in paraffin. Tissue was sectioned into 5 μ m slices and stained with hematoxylin and eosin (H&E). Using light microscopy, the level of injury was assessed in the outer medulla of the kidney section in the following categories: tubular cell injury, tubular cell detachment, loss of brush border, tubular simplification, and cast formation (25). Histologic changes were assessed in a blinded fashion according to a modified scoring system based on the extent of tissue injury (25). The area of damage was determined by percentage of damaged tubules within total tubules. An injury score was calculated based on 0 representing 0% area of damage, 1 (<10%), 2 (10–25%), 3 (25–50%), 4 (50–75%), and 5 (>75%), for a maximal score of 5. Scores were averaged for each sample over 10 randomly selected fields.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM) and compared via one way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) test for multiple group comparisons. Significance was considered if $p < 0.05$ between the experimental groups.

RESULTS

Treatment with rmMFG-E8 improves renal functions after sepsis-induced AKI

Estimation of kidney function is routinely diagnosed by BUN and creatinine levels in serum. After 20 h of sepsis, blood was collected from sham, vehicle, and rmMFG-E8-treated mice and assessed for the renal function markers, BUN and creatinine. After sepsis, serum levels of BUN and creatinine were both significantly increased in the vehicle group as compared to the sham group, on the other hand the animals treated with rmMFG-E8 had a significant reduction in BUN and creatinine levels by a mean values of 28% and 24.1%, respectively (BUN: 197.7 ± 23.6 vs. 142.3 ± 20.7 mg/dL; creatinine: 0.83 ± 0.12 vs. 0.63 ± 0.06 mg/dL; $p < 0.05$) (Figure 1A, B).

rmMFG-E8 reduces NGAL and KIM-1 expression in renal tissues after sepsis

We further assayed for additional markers of renal tissue injury which have been implicated in recent articles (26, 27). NGAL is part of the lipocalin superfamily of secreted proteins markedly upregulated in activated epithelial cells and may serve as an early biomarker for AKI (26). The mRNA level of NGAL in the renal tissue was dramatically upregulated in the vehicle-treated septic mice compared to the sham animals, while mice treated with rmMFG-E8 showed 58.2% reduction in NGAL expression compared to the vehicle-treated mice (212.2 ± 32.1 vs. 88.6 ± 41.3 ; $p < 0.05$) (Figure 2A). We then looked at the expression of KIM-1 which is a transmembrane protein expressed at a low level in normal kidney but increased dramatically in the post-ischemic kidney (27). The mRNA level of KIM-1 was significantly upregulated in vehicle-treated animals compared to the sham mice, by contrast

mice treated with rmMFG-E8 significantly reduced KIM-1 mRNA by 95% in the kidneys compared to the vehicle-treated mice (232.7 ± 78.1 vs. 11.6 ± 1.2 ; $p < 0.05$) (Figure 2B).

rmMFG-E8 protects renal tissue damage after sepsis

To assess whether treatment with rmMFG-E8 led to lessened renal injury, we evaluated tissue histology with a H&E stain. At 20 h after sepsis, there was a noticeable difference in the amount of tubular cell injury and cast formation in the rmMFG-E8-treated mice compared to the vehicle-treated control mice (Figure 3A). Using semiquantitative histological evaluation, an injury score was calculated based on criteria described in Materials and Methods. A 50% improvement of renal tubular damage was noticed in the rmMFG-E8-treated mice compared to the vehicle-treated group (Figure 3B).

Treatment with rmMFG-E8 attenuates the expression of pro-inflammatory cytokines in renal tissues following sepsis

Sepsis resulted in the significant upregulation of the expression of pro-inflammatory cytokines, IL-6 and TNF- α in the renal tissues compared to sham mice (Figure 4A, B). However, the mice treated with rmMFG-E8 showed significant downregulation of IL-6 and TNF- α expression by 50.8% and 50.3%, respectively (IL-6: 122.5 ± 60.3 vs. 15.3 ± 2.4 folds; TNF- α : 29.6 ± 6.1 vs. 14.7 ± 4.1 folds; $p < 0.05$) (Figure 4A, B). Thus, renal inflammation was significantly decreased when rmMFG-E8 was administered into the septic animals.

rmMFG-E8 downregulates the expression of chemokines in renal tissues after sepsis

To look for the expression of chemokines, we performed qPCR for chemokine (C-X-C motif) ligand-1 (CXCL-1) also known as keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2) in renal tissues after sepsis treated with either PBS as vehicle or rmMFG-E8. mRNA levels of the chemokine KC and MIP-2 were significantly increased in the vehicle-treated animals compared to sham-operated mice; however, the septic mice treated with rmMFG-E8 decreased KC and MIP-2 mRNA expression by 85.1% and 78% compared to vehicle-treated animals, respectively (KC: 712.9 ± 106.5 vs. 115.3 ± 27.1 folds; MIP-2: 2795.7 ± 614.5 vs. 1006.8 ± 356.4 folds; $p < 0.05$) (Figure 5A, B).

rmMFG-E8 inhibits the expression of endothelial cell adhesion molecules in the kidney tissues after sepsis

Cell adhesion molecules expressed on the endothelial cell lining of the blood vasculature play critical role for leukocytes' adherence on the blood vessel walls and trans-migration into the tissue beds during inflammation (28, 29). We therefore aimed to assess the expression of the commonly known cell adhesion molecule, inter cellular cell adhesion molecule-1 (ICAM-1) in the renal tissues after sepsis. The expression of ICAM-1 mRNA at the renal tissues were significantly increased in the vehicle-treated CLP mice as compared to the sham mice, however, the mice treated with rmMFG-E8 showed significant decrease in the expression of ICAM-1 mRNA in renal tissues by 35.6%, compared to the vehicle-treated animals (5.9 ± 0.6 vs. 3.8 ± 0.8) (Figure 6A). While ICAM-1 expression occurs at the epical

surface of the endothelial cells, the expression of platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) on endothelial cells is largely concentrated at junctions between adjacent cells (30). Here, we found that the expression of PECAM-1 in renal tissues was significantly upregulated in vehicle-treated septic animals compared to the sham animals, on the other hand the mice treated with rmMFG-E8 significantly inhibited the expression of PECAM-1 by 77.8% compared to the vehicle-treated mice (1.85 ± 0.32 vs. 1.04 ± 0.22) (Figure 6B). Collectively, these data suggest that rmMFG-E8 could serve as a beneficial molecule to protect against renal injury after sepsis.

DISCUSSION

Sepsis-associated AKI occurs at a high incidence rate in critically ill patients, which remains a significant challenge for clinicians (11, 12). Our incomplete understanding of the pathophysiology of sepsis-associated AKI might leave difficulties to innovate novel therapeutic potential against this disorder. Even though various treatment modalities have been implicated in reducing sepsis-related mortality, none have been successful due to concerns about its safety and efficacy (1, 4). Since sepsis may cause local tissue damage, therapeutic strategies can be adopted by introducing novel drugs which can restore tissue function. In our previous studies using animal model of sepsis we have noticed downregulation of MFG-E8 expression, while the replenishment of MFG-E8 deficit could provide beneficial outcome (17, 31). The beneficial role of MFG-E8 in sepsis was mainly demonstrated in terms of improving lung function, while the status of kidney function has not been focused before. Current study not only validates our murine model to study kidney injury during sepsis, but also depicts MFG-E8's efficacy as a novel therapeutic potential to protect kidney tissues from injury and inflammation.

Our present finding demonstrates treatment with rmMFG-E8 leads to preservation of renal function and a reduction in renal tissue injury after sepsis. This was attained by decreases in BUN, creatinine, NGAL, and KIM-1 in the rmMFG-E8 treated septic mice, which correlated to histological findings. BUN and creatinine are the most widely assessed diagnostic and prognostic markers of renal diseases, but new injury markers such as NGAL and KIM-1 have become promising next-generation biomarkers in clinical nephrology. NGAL was first purified from human neutrophils because of its association with gelatinase, and it is highly upregulated in the early post-ischemic mouse kidney (26). KIM-1 is a transmembrane glycoprotein that promotes the obstruction of the tubule lumen that characterizes AKI (27). In clinical studies, both NGAL and KIM-1 are biomarkers that have shown an ability to predict AKI days before an elevation in serum creatinine (27, 32–34). In the current study, treatment with rmMFG-E8 led to significant reductions in both NGAL and KIM-1, emphasizing the importance of MFG-E8 in reducing kidney injury during sepsis.

Acute inflammation caused by sepsis is considered to be an important pathway for tissue injury. We previously found reduced expression of MFG-E8 in spleen as well as its decreased content in the circulation during sepsis (15, 35). In renal ischemia and reperfusion we have also noticed a decreased expression of MFG-E8 in spleen, kidney and liver (17). Since MFG-E8 is known to exhibit anti-inflammatory function, the decreased production of MFG-E8 as noticed in these acute inflammatory diseases might lead to increased pro-

inflammatory milieu. As expected, in the current study, we have noticed significant upregulation of pro-inflammatory cytokines IL-6 and TNF- α in the renal tissue compared to sham-operated animals, while the administration of rmMFG-E8 in septic animals significantly downregulated the expression of these pro-inflammatory cytokines in the kidney compared to vehicle-treated mice. In addition, the septic mice showed extensive renal tissue damage as demonstrated by their increased histological injury score, which was found to be dramatically reduced after treatment with rhMFG-E8. Therefore, by treating animals with rmMFG-E8 we were able to reduce excessive cytokine production, and decrease tubular epithelial cell injury located in the outer medulla of the kidney tissue, which in turn led to overall improvement after sepsis.

MFG-E8 participates in various physiological functions associated with tissue remodeling and maintenance of homeostasis (15, 22). Hanayama *et al.* have shown that MFG-E8 is one of the bridging molecules between apoptotic cells and phagocytes by binding to phosphatidylserine (PS) exposed on apoptotic cells and to $\alpha_v\beta_3/\alpha_v\beta_5$ integrins on phagocytes (20). Consequently, MFG-E8 deficient mice showed various characteristics of inflammation and autoimmunity and suffered from glomerulonephritis that are specifically due to defects in apoptotic cell engulfment by phagocytes (23). This may suggest MFG-E8-mediated beneficial effects for protecting sepsis-associated AKI could be either directly mediated by recognizing its receptors $\alpha_v\beta_3/\alpha_v\beta_5$ integrins or indirectly through the phagocytic removal of apoptotic cells. As an attempt to reveal a mechanism of renoprotection by MFG-E8 treatment, our previous study utilizing renal I/R model in mice investigated the apoptotic cell death in kidney tissues (17), which is one of the major causes of tissue damage after renal I/R injury. Our previous results showed that treatment with rmMFG-E8 reduced the number of apoptotic cells and suppressed local inflammation after renal I/R injury (17). Aside from the phagocytosis of apoptotic cells, inhibition of certain elements of the apoptotic pathway seemed to ameliorate sepsis and renal I/R injury (36–38). Our previous studies using animal model of sepsis showed inhibition of cleaved caspase-3 activity in lung tissues after treatment with rmMFG-E8 (36, 37, 39), suggesting MFG-E8 to preserve tissue homeostasis by not only facilitating phagocytosis of apoptotic cells, but also inhibiting cellular apoptosis, thus this previously identified mechanism could also be linked with our present finding of protection of kidney tissue damage by MFG-E8 during sepsis.

Several recent studies have shown the efficacy of MFG-E8 for promoting tissue regeneration by inducing AKT- and vascular endothelial growth factor (VEGF)-mediated neovascularization (40, 41). In addition to these, a recent study also revealed the regeneration and maintenance of intestinal tissues by MFG-E8 after sepsis, which was shown to promote through the upregulation of protein kinase C (PKC) pathway (22). Based on these findings it can be suggested that MFG-E8-mediated renal tissue protection from injuries after sepsis could be pursued by inducing AKT-, VEGF- and PKC-dependent pathways.

Towards elucidating the anti-inflammatory mechanism by MFG-E8, our previous studies in acute inflammatory disease conditions have demonstrated that MFG-E8 can attenuate inflammation and improve survival rate through the direct inhibition of inflammation by targeting macrophages and neutrophils (15, 37, 39). In macrophages, MFG-E8 inhibited

LPS-induced inflammation by downregulating the activation of down-stream transcription factor, nuclear factor (NF)- κ B (18). The MFG-E8-mediated inhibition of NF- κ B activation was further identified to be mediated through an upstream pathway involving signal transducer and activator of transcription 3 (STAT3)-dependent suppressor of cytokine signaling 3 (SOCS3) activation (21, 24).

Studies in animal models of AKI have revealed that innate immune cells, such as neutrophils, macrophages, dendritic cells, natural killer (NK) cells and natural killer T (NKT) cells, and adaptive CD4⁺ T cells promote renal injury (25, 28, 30, 42). Previously, we have shown that MFG-E8 protected lung tissues by attenuating infiltration of neutrophils in lungs through the downregulation of chemokine receptor CXCR2 (37). Here we studied the expression of cell adhesion molecule ICAM-1 and PECAM-1 in renal tissues and found that the treatment with rmMFG-E8 during sepsis attenuated their expression in renal tissues, which might therefore imply with the less activated leukocyte infiltration in renal tissue. In addition to this, the reduced expression of chemokines KC and MIP-2 in renal tissues in rmMFG-E8-treated mice could also be correlated with the reduced infiltration of activated leukocytes in kidney tissues to cause kidney inflammation and injury during sepsis. PECAM-1, also known as CD31 is a 130 kDa glycoprotein of the Ig gene superfamily (30). PECAM-1 is expressed to different degrees on most leukocyte sub-types, platelets, and on endothelial cells where its expression is largely concentrated at junctions between adjacent cells (30, 43). PECAM-1 was originally reported to mediate leukocyte migration through endothelial cell junctions (30). In the renal tissue we found an upregulation of PECAM-1 expression after sepsis, while the treatment with rmMFG-E8 significantly downregulated its expression, implicating a novel mechanism mediated by MFG-E8 for inhibition of leukocyte migration in renal tissues during sepsis.

In summary, in the current study we have demonstrated that treatment with rmMFG-E8 reduced renal dysfunction caused by excessive inflammation and tubular cell injury. We have also revealed the beneficial effect of MFG-E8 are likely due to decreased recruitment of leukocytes to the kidney as a result of chemokine and cell adhesion molecule upregulation after sepsis. While the role of MFG-E8 has been globally investigated in the field of sepsis, our current study demonstrating the role of MFG-E8 in local tissue such as kidney will further help improve our understanding the complex pathophysiology of AKI and will also implicate a novel tool for improving renal function during sepsis. Therefore, administration of MFG-E8 after sepsis may be a promising therapeutic option for patients at high risk of renal injury during sepsis.

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ABBREVIATION

AKI	Acute kidney injury
MFG-E8	Milk fat globule-epidermal growth factor-factor VIII

CLP	cecal ligation and puncture
BUN	blood urea nitrogen
NGAL	neutrophil gelatinase-associated lipocalin
KIM-1	kidney injury molecule-1
KC	keratinocyte chemoattractant
MIP-2	macrophage inhibitory protein-2
ICAM-1	inter-cellular cell adhesion molecule-1
PECAM-1	platelet endothelial cell adhesion molecule-1
I/R	ischemia-reperfusion
IBD	inflammatory bowel disease
PS	phosphatidylserine
VEGF	vascular endothelial growth factor
PKC	protein kinase C
STAT3	signal transducer and activator of transcription 3
SOCS3	suppressor of cytokine signaling 3
NK	natural killer

References

1. Aziz M, Jacob A, Yang WL, Matsuda A, Wang P. Current trends in inflammatory and immunomodulatory mediators in sepsis. *J Leukoc Biol.* 2013; 93(3):329–42. [PubMed: 23136259]
2. Angus DC, van der Poll T. Severe sepsis and septic shock. *N Engl J Med.* 2013; 369(21):2063.
3. Gaieski DF, Edwards JM, Kallan MJ, Carr BG. Benchmarking the incidence and mortality of severe sepsis in the United States. *Crit Care Med.* 2013; 41(5):1167–74. [PubMed: 23442987]
4. Dellinger RP, Levy MM, Carlet JM, Bion J, Parker MM, Jaeschke R, et al. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Crit Care Med.* 2008; 36(1):296–327. [PubMed: 18158437]
5. Hall MJ, Williams SN, DeFrances CJ, Golosinskiy A. Inpatient care for septicemia or sepsis: a challenge for patients and hospitals. *NCHS Data Brief.* 2011; (62):1–8.
6. Bernard GR, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med.* 2001; 344(10):699–709. [PubMed: 11236773]
7. Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med.* 2001; 29(7):1303–10. [PubMed: 11445675]
8. Martin CM, Priestap F, Fisher H, Fowler RA, Heyland DK, Keenan SP, et al. A prospective, observational registry of patients with severe sepsis: the Canadian Sepsis Treatment and Response Registry. *Crit Care Med.* 2009; 37(1):81–8. [PubMed: 19050636]
9. Alobaidi R, Basu RK, Goldstein SL, Bagshaw SM. Sepsis-associated acute kidney injury. *Semin Nephrol.* 2015; 35(1):2–11. [PubMed: 25795495]

10. Rangel-Frausto MS, Pittet D, Costigan M, Hwang T, Davis CS, Wenzel RP. The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study. *JAMA*. 1995; 273(2): 117–23. [PubMed: 7799491]
11. Uchino S, Kellum JA, Bellomo R, Doig GS, Morimatsu H, Morgera S, et al. Acute renal failure in critically ill patients: a multinational, multicenter study. *JAMA*. 2005; 294(7):813–8. [PubMed: 16106006]
12. Schrier RW, Wang W. Acute renal failure and sepsis. *N Engl J Med*. 2004; 351(2):159–69. [PubMed: 15247356]
13. Langenberg C, Bellomo R, May C, Wan L, Egi M, Morgera S. Renal blood flow in sepsis. *Crit Care*. 2005; 9(4):R363–74. [PubMed: 16137349]
14. Gomez H, Ince C, De Backer D, Pickkers P, Payen D, Hotchkiss J, et al. A unified theory of sepsis-induced acute kidney injury: inflammation, microcirculatory dysfunction, bioenergetics, and the tubular cell adaptation to injury. *Shock*. 2014; 41(1):3–11.
15. Aziz M, Jacob A, Matsuda A, Wang P. Review: milk fat globule-EGF factor 8 expression, function and plausible signal transduction in resolving inflammation. *Apoptosis*. 2011; 16(11):1077–86. [PubMed: 21901532]
16. Matsuda A, Jacob A, Wu R, Zhou M, Nicastro JM, Coppa GF, et al. Milk fat globule-EGF factor VIII in sepsis and ischemia-reperfusion injury. *Mol Med*. 2011; 17(1–2):126–33. [PubMed: 20882259]
17. Matsuda A, Wu R, Jacob A, Komura H, Zhou M, Wang Z, et al. Protective effect of milk fat globule-epidermal growth factor-factor VIII after renal ischemia-reperfusion injury in mice. *Crit Care Med*. 2011; 39(9):2039–47. [PubMed: 21666453]
18. Aziz MM, Ishihara S, Mishima Y, Oshima N, Moriyama I, Yuki T, et al. MFG-E8 attenuates intestinal inflammation in murine experimental colitis by modulating osteopontin-dependent alphavbeta3 integrin signaling. *J Immunol*. 2009; 182(11):7222–32. [PubMed: 19454719]
19. Hanayama R, Nagata S. Impaired involution of mammary glands in the absence of milk fat globule EGF factor 8. *Proc Natl Acad Sci U S A*. 2005; 102(46):16886–91. [PubMed: 16275924]
20. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. *Nature*. 2002; 417(6885):182–7. [PubMed: 12000961]
21. Aziz M, Jacob A, Matsuda A, Wu R, Zhou M, Dong W, et al. Pre-treatment of recombinant mouse MFG-E8 downregulates LPS-induced TNF- α production in macrophages via STAT3-mediated SOCS3 activation. *PLoS One*. 2011; 6(11):e27685. [PubMed: 22114683]
22. Bu HF, Zuo XL, Wang X, Ensslin MA, Koti V, Hsueh W, et al. Milk fat globule-EGF factor 8/lactadherin plays a crucial role in maintenance and repair of murine intestinal epithelium. *J Clin Invest*. 2007; 117(12):3673–83. [PubMed: 18008006]
23. Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, et al. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science*. 2004; 304(5674):1147–50. [PubMed: 15155946]
24. Cen C, Aziz M, Yang WL, Nicastro J, Coppa GF, Wang P. Milk fat globule-epidermal growth factor-factor VIII downregulates interleukin-17 expression in sepsis by modulating STAT3 activation. *Surgery*. 2015
25. Cen C, Aziz M, Yang WL, Nicastro JM, Coppa GF, Wang P. Osteopontin Blockade Attenuates Renal Injury After Ischemia Reperfusion by Inhibiting NK Cell Infiltration. *Shock*. 2016
26. Mishra J, Ma Q, Prada A, Mitsnefes M, Zahedi K, Yang J, et al. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol*. 2003; 14(10):2534–43. [PubMed: 14514731]
27. Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, et al. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *J Biol Chem*. 1998; 273(7):4135–42. [PubMed: 9461608]
28. Brady HR. Leukocyte adhesion molecules and kidney diseases. *Kidney Int*. 1994; 45(5):1285–300. [PubMed: 8072240]
29. Muller WA. Getting leukocytes to the site of inflammation. *Vet Pathol*. 2013; 50(1):7–22. [PubMed: 23345459]

30. Woodfin A, Voisin MB, Nourshargh S. PECAM-1: a multi-functional molecule in inflammation and vascular biology. *Arterioscler Thromb Vasc Biol.* 2007; 27(12):2514–23. [PubMed: 17872453]
31. Matsuda A, Jacob A, Wu R, Aziz M, Yang WL, Matsutani T, et al. Novel therapeutic targets for sepsis: regulation of exaggerated inflammatory responses. *J Nippon Med Sch.* 2012; 79(1):4–18. [PubMed: 22398786]
32. Devarajan P. Emerging urinary biomarkers in the diagnosis of acute kidney injury. *Expert Opin Med Diagn.* 2008; 2(4):387–98. [PubMed: 19079800]
33. Devarajan P. Neutrophil gelatinase-associated lipocalin--an emerging troponin for kidney injury. *Nephrol Dial Transplant.* 2008; 23(12):3737–43. [PubMed: 18809975]
34. Bonventre JV. Kidney injury molecule-1 (KIM-1): a urinary biomarker and much more. *Nephrol Dial Transplant.* 2009; 24(11):3265–8. [PubMed: 19318357]
35. Komura H, Miksa M, Wu R, Goyert SM, Wang P. Milk fat globule epidermal growth factor-factor VIII is down-regulated in sepsis via the lipopolysaccharide-CD14 pathway. *J Immunol.* 2009; 182(1):581–7. [PubMed: 19109191]
36. Aziz M, Jacob A, Wang P. Revisiting caspases in sepsis. *Cell Death Dis.* 2014; 5:e1526. [PubMed: 25412304]
37. Aziz M, Matsuda A, Yang WL, Jacob A, Wang P. Milk fat globule-epidermal growth factor-factor 8 attenuates neutrophil infiltration in acute lung injury via modulation of CXCR2. *J Immunol.* 2012; 189(1):393–402. [PubMed: 22634615]
38. Kumar S, Allen DA, Kieswich JE, Patel NS, Harwood S, Mazzon E, et al. Dexamethasone ameliorates renal ischemia-reperfusion injury. *J Am Soc Nephrol.* 2009; 20(11):2412–25. [PubMed: 19797168]
39. Cui T, Miksa M, Wu R, Komura H, Zhou M, Dong W, et al. Milk fat globule epidermal growth factor 8 attenuates acute lung injury in mice after intestinal ischemia and reperfusion. *Am J Respir Crit Care Med.* 2010; 181(3):238–46. [PubMed: 19892861]
40. Silvestre JS, Théry C, Hamard G, Boddaert J, Aguilar B, Delcayre A, et al. Lactadherin promotes VEGF-dependent neovascularization. *Nat Med.* 2005; 11(5):499–506. [PubMed: 15834428]
41. Wang, P. MFG-E8 and inflammation. Dordrecht: Springer; 2014. p. xvp. 198
42. Basile DP, Anderson MD, Sutton TA. Pathophysiology of acute kidney injury. *Compr Physiol.* 2012; 2(2):1303–53. [PubMed: 23798302]
43. Anastassiou G, Duensing S, Steinhoff G, Zorn U, Grosse J, Dallmann I, et al. Platelet endothelial cell adhesion molecule-1 (PECAM-1): a potential prognostic marker involved in leukocyte infiltration of renal cell carcinoma. *Oncology.* 1996; 53(2):127–32. [PubMed: 8604238]

Figure 1. rmMFG-E8 improves renal function after sepsis-induced AKI

Serum from sham, vehicle, and rmMFG-E8 treated mice were collected at 20 h after sepsis to measure (A) BUN and (B) creatinine levels. Data are expressed as mean $\pm$ SEM (n=4–8 mice/group) and compared by one-way ANOVA by SNK method. * p <0.05 vs. sham. rmMFG-E8, recombinant mouse milk fat globule-epidermal growth factor factor VIII.

Figure 2. rmMFG-E8 reduces novel renal injury markers

Renal tissue from sham, vehicle, and rmMFG-E8 treated mice were collected to measure (A) NGAL and (B) KIM-1 mRNA by qRT-PCR. Their expression levels were normalized to β -actin, and sham was designated as 1 for comparison. Data are expressed as mean $\pm$ SEM (n=4–7 mice/group) and compared by one-way ANOVA by SNK method. * p <0.05 vs. sham; # p <0.05 vs. vehicle. rmMFG-E8, recombinant mouse milk fat globule-epidermal growth factor factor VIII; qRT-PCR, quantitative real-time polymerase chain reaction; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1.

Figure 3. rmMFG-E8 reduces renal histologic injury

Sections of kidney tissue from sham, vehicle, and rmMFG-E8 treated mice were collected after CLP and stained with H&E. **(A)** Representative sections are shown at 200× magnification. **(B)** Histologic injury grading score as described in Materials and Methods. Data are expressed as mean ± SEM (n=10 microscopic fields/group) and compared by one-way ANOVA by SNK method. *p<0.05 vs. sham; #p<0.05 vs. vehicle. H&E, hematoxylin and eosin; rmMFG-E8, recombinant mouse milk fat globule-epidermal growth factor factor VIII.

Figure 4. rmMFG-E8 reduces kidney inflammation

Renal tissue from sham, vehicle, and rmMFG-E8 treated mice were collected to measure levels of cytokines (A) IL-6 and (B) TNF- α by qRT-PCR. Their expression levels were normalized to β -actin, and sham was designated as 1 for comparison. Data are expressed as mean $\pm$ SEM (n=4–7 mice/group) and compared by one-way ANOVA by SNK method.

* p <0.05 vs. sham; # p <0.05 vs. vehicle. rmMFG-E8, recombinant mouse milk fat globule-epidermal growth factor factor VIII; IL, interleukin; TNF, tumor necrosis factor.

Figure 5. rmMFG-E8 reduces chemokine expression

Renal tissue from sham, vehicle, and rmMFG-E8 treated mice were collected to measure levels of chemokines (A) KC and (B) MIP-2 by qRT-PCR. Their expression levels were normalized to β -actin, and sham was designated as 1 for comparison. Data are expressed as mean $\pm$ SEM (n=6–8 mice/group) and compared by one-way ANOVA by SNK method.

* $p<0.05$ vs. sham; # $p<0.05$ vs. vehicle. rmMFG-E8, recombinant mouse milk fat globule-epidermal growth factor factor VIII; KC, keratinocyte chemoattractant; MIP-2, macrophage inflammatory protein-2.

Figure 6. rmMFG-E8 reduces endothelial activation

Renal tissue from sham, vehicle, and rmMFG-E8 treated mice were collected to measure levels of cell adhesion molecules (A) ICAM-1 and (B) PECAM-1 by qRT-PCR. Their expression levels were normalized to β -actin, and sham was designated as 1 for comparison. Data are expressed as mean $\pm$ SEM (n=6–8 mice/group) and compared by one-way ANOVA by SNK method. * p <0.05 vs. sham; # p <0.05 vs. vehicle. rmMFG-E8, recombinant mouse milk fat globule-epidermal growth factor factor VIII; ICAM-1, intercellular adhesion molecule-1; PECAM-1, platelet endothelial cell adhesion molecule-1.

Table 1

Renal tissue histological injury scores

Experimental groups	Loss of brush border (No. of tubules)	Vacuolization (No. of tubules)	No. of injured tubules	No. of total tubules	Damaged area (%)	Injury Score
Sham	2.15 ± 0.27	0 ± 0.0	2.15 ± 0.27	45 ± 11	5.23 ± 0.62	1 ± 0.0
CLP + Vehicle	9.21 ± 1.33	11.64 ± 2.42	31.28 ± 4.1	36.5 ± 3.6	60.62 ± 5.38	3.9 ± 0.2
CLP + rmMFG-E8	3.95 ± 0.74	3.1 ± 0.94	7.05 ± 1.2	45 ± 4.8	17.13 ± 3.13	1.85 ± 0.23

The data presented in the table represent mean ± SE.