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C. Cen
Northwell Health

M. Aziz
Northwell Health

W.L. Yang

J. Nicastro
Hofstra Northwell School of Medicine

G. F. Coppa
Hofstra Northwell School of Medicine

See next page for additional authors

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MFG-E8 Downregulates IL-17 Expression in Sepsis by Modulating STAT3 Activation

Cindy Cen, MD[1], **Monowar Aziz, PhD**[1,2], **Weng-Lang Yang, PhD**[1,2], **Jeffrey Nicastro, MD**[1], **Gene F. Coppa, MD**[1], and **Ping Wang, MD**[1,2]

[1]Department of Surgery, Hofstra North Shore-LIJ School of Medicine, Manhasset, NY

[2]Center for Translational Research, The Feinstein Institute for Medical Research, Manhasset, NY

Abstract

Background—Milk fat globule-epidermal growth factor-factor VIII (MFG-E8) is a secretory glycoprotein with a known role in inflammation. In sepsis, interleukin-17 (IL-17) acts as a proinflammatory cytokine to exaggerate systemic inflammation. We hypothesize that MFG-E8 downregulates IL-17 expression in sepsis.

Methods—Sepsis was induced in 8-week-old male C57BL/6 mice by cecal ligation and puncture (CLP). Recombinant mouse MFG-E8 (rmMFG-E8) at a dosage of 20 μg/kg body weight or PBS was concurrently injected. After 10 h, blood and spleen samples were harvested for analysis. For *in vitro* studies, splenocytes isolated from healthy mice pre-treated with rmMFG-E8 and splenocytes from MFG-E8 knockout (*mfge8*$^{-/-}$) mice were stimulated with phorbol myristate acetate (PMA) and ionomycin, followed by measurement of IL-17 expression with either qPCR or ELISA.

Results—At 10 h after CLP, rmMFG-E8 inhibited the elevated levels of IL-17 protein in serum by 31%, compared to the vehicle. In the spleen, rmMFG-E8 reduced the upregulated IL-17 mRNA and protein levels by 81% and 51%, respectively. This correlated with a significant reduction in

Address for correspondence, proofs, and reprint requests to: Ping Wang, MD, Head, Center for Translational Research, The Feinstein Institute for Medical Research, Professor and Vice Chairman for Research, Department of Surgery, Hofstra North Shore-LIJ School of Medicine, 350 Community Dr., Manhasset, NY 11030, Tel: (516) 562-3411, Fax: (516) 562-2396, pwang@nshs.edu.

Author Contributions

CC designed and performed the experiments. CC, MA, WL-Y helped in data analysis and interpretation. CC, MA wrote and edited the manuscript. JN, GFC, PW reviewed the manuscript and provided valuable comments. PW conceived the idea, and supervised the whole project. All authors read and approved the final manuscript.

Conflict of Interest

One of the authors (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.

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organ injury markers AST and ALT in sepsis after administration of rmMFG-E8. *In vitro* treatment of splenocytes isolated from healthy mice with rmMFG-E8 showed significant downregulation in PMA/ionomycin-induced IL-17 expression. In contrast, CD4 T-cells from *mfge8*$^{-/-}$ mice showed significant upregulation of IL-17 compared to wild-type mice. The phosphorylated level of STAT3 was downregulated in spleen tissue of septic mice treated with rmMFG-E8. Conversely, *mfge8*$^{-/-}$ mice showed increased pSTAT3 compared to wild-type mice after sepsis.

Conclusion—Our findings demonstrate MFG-E8-mediated downregulation of IL-17 expression, implicating its potential as a novel therapeutic agent against sepsis.

INTRODUCTION

Sepsis is a pervasive and constant human health concern that affects approximately 3 million people in the United States every year [1, 2]. Mortality is estimated between 15 % and 30%, at a cost to our healthcare system for hospital care totaling more than $24 billion each year [2–4]. Injury from sepsis occurs as a result of an exaggerated or uncontrolled inflammatory response and mortality stems from injuries sustained by the various organ systems.

Interleukin-17 (IL-17) is a family of cytokines first found to be expressed in activated CD4 T- cells, and includes IL-17A, -B, -C, -D, -E, and -F [5–7]. They were initially thought to only be produced from Th17 cells, but later found to be produced in other immune cells including neutrophils, lymphocytes, inducible Natural Killer T (iNKT) cells, γδ T cells, and Paneth cells [5, 6, 8]. IL-17A has been linked to severity of inflammation in tissues, and seen to predominantly interact with endothelial cells, epithelial cells, fibroblasts, and macrophages, producing proinflammatory mediators such as IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) [5]. IL-17 is involved in varied processes that include bacterial defense, rheumatoid arthritis, allograft rejection, tumor modulation, and asthma and allergic reactions [9]. Although it is in these latter autoimmune processes that IL-17 has been more widely studied, IL-17 has recently attracted attention as a regulator of innate immunity in host defense.

Regulation of IL-17 expression in activated T-cells is mediated via a wide range of cytokines, including IL-23, transforming growth factor-β1 (TGF-β1), IL-6, IL-1β, and IL-21 [6, 10]. Differentiation of IL-17-producing Th17 cells requires a novel set of transcription factors, including signal transducer and activator of transcription 3 (STAT3), retinoic acid receptor related orphan receptor γ (RORγ), and nuclear factor kappa B (NF-κB) [6, 10]. Conversely, IL-10 is well known for suppressing IL-17 expression in Th17 cells and macrophages [11]. Mice deficient in IL-10 will exhibit higher levels of IL-17 due to STAT3 and RORγ upregulation [11]. Similarly, IL-18, an IL-1 family epithelial-derived cytokine, has recently been shown to regulate IL-17 expression during homeostatic and inflammatory conditions likely by antagonizing IL-1R1 signaling in Th17 cells [12].

In an experimental model of sepsis using cecal ligation and puncture (CLP), IL-17 was identified to promote high levels of proinflammatory mediators and bacteremia [9]. Levels of IL-17 rose in a time-dependent manner after CLP, and *in vitro* incubation of macrophages with lipopolysaccharide (LPS) and IL-17 increased production of TNF-α, IL-1β, and IL-6 [9]. Furthermore, targeting of IL-17 with neutralizing antibodies showed a protective effect, with

reduced bacteremia and increased survival [9]. Additionally, neutralization of peritoneal IL-17 after CLP resulted in markedly improved neutrophil infiltration and decreased levels of proinflammatory cytokines [13].

We have previously shown that administering recombinant milk fat globule-EGF factor VIII (MFG-E8) during sepsis provides beneficial effects [14, 15]. MFG-E8 is a 66 kDa glycoprotein that is expressed in nearly all organs and various cell types that include macrophages and dendritic cells [16]. It is strongly expressed in mammary glands, as well as in the spleen, lungs, liver, lymph nodes, and brain [17, 18]. It was initially identified as a major component of mouse milk fat and is known to play a role in facilitating the phagocytic clearance of apoptotic cells by professional phagocytes [16, 17]. 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 engulfment of dead cells [16, 17]. Apart from this universal function, a direct, MFG-E8-mediated anti-inflammatory role via inhibition of pro-inflammatory cytokines and chemokines has recently been reported [19, 20]. Hence, MFG-E8 is pivotal for maintaining tissue homeostasis by regulating several cell signaling events, and its deficiency can lead to severe inflammatory disorders [17, 18, 21, 22]. Although beneficial outcomes of MFG-E8 treatment in sepsis and in other inflammatory disorders have been demonstrated, its molecular mechanism has yet to be fully elucidated. In this study, we investigated the role of MFG-E8 in decreasing the pro-inflammatory cytokine, IL-17 in sepsis. Based on our findings, MFG-E8 could serve as a therapeutic agent in sepsis by reducing IL-17 expression both locally and systemically.

MATERIALS AND METHODS

Animal model of sepsis

Male 8–10 weeks old C57BL/6 mice (21–28 g) purchased from Taconic (Albany, NY, USA) were anesthetized with isofluorane and underwent cecal ligation and puncture (CLP) [23]. Briefly, a 1.5 cm 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 two punctures through and through to the distal cecum, extruding a small amount of fecal material. The cecum was replaced into the abdominal cavity, and the abdomen was closed in two layers with running 6-0 nylon suture. The sham mice underwent the same procedure with the exception that the cecum was neither ligated nor punctured. Animals were resuscitated with 1 ml of normal saline subcutaneously.

In vivo administration of recombinant mouse MFG-E8 (rmMFG-E8)

Immediately after performing CLP, a small incision on the neck was made to expose the internal jugular vein (IJ). Commercially available recombinant mouse MFG-E8 (rmMFG-E8) (Cat. No.: 2805-MF-050; R&D systems, Minneapolis, MN) was delivered by bolus injection through the IJ vein using 29G × 1/2″ U-100 insulin syringe (Terumo Medical Corporation, Elkton, MD) at a concentration of 20 μg/kg BW in 100 μl or same volume of PBS as vehicle. A survival study performed with this model in mice determined the lethal dose (LD50) to occur at day 7 in the 10 day monitoring period [24]. The proximal and distal ends of the jugular vein were ligated and the wound was closed with one interrupted 4-0

nylon suture. After wound closure, the mouse was given 500 μl of normal saline (NS) subcutaneous fluid resuscitation. The animals were allowed food and water *ad libitum* until 10 hours after the time of intervention and the animals were euthanized. Blood and tissue samples were collected for various *ex vivo* analyses. All experiments were performed in accordance with the guidelines for the use of experimental animals by the National Institute of Health and were approved by the Institutional Animal Care and Use Committee of The Feinstein Institute for Medical Research.

Quantitative real-time PCR analysis

Total RNA was extracted from spleen 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 25 μl of final volume containing 0.08 μmol of forward and reverse primer, cDNA, and 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems). The thermal profile used by the Applied Biosystems 7300 real-time PCR machine 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 change in comparison to the sham group. The primer sequences are: IL-17A (NM_010552): Forward: CAGGGAGAGCTTCATCTGTGT, Reverse: AGGAAGTCCTTGGCCTCAGT; β-actin (NM_007393): Forward: CGTGAAAAGATGACCCAGATCA, Reverse: TGGTACGACCAGAGGCATACAG.

Western blot analysis

Spleen tissue was homogenized in lysis buffer (10 mM Tris-HCl, pH 7.5; 120 mM NaCl; 1% NP-40; 1% sodium deoxycholate; and 0.1% sodium dodecyl sulfate) containing protease inhibitor (Roche Diagnostics; Indianapolis, IN), then sonicated (Fisher Scientific; Waltham, MA). Protein concentrations were determined by Bio-Rad protein assay reagent (Hercules, CA). Total lysate was electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then blocked with 0.1% casein in 0.2× PBS and then incubated with anti-IL-17A (Cat. No. ab79056; Abcam, Cambridge, MA), phosphorylated STAT3 (Tyr705) (Cat. No.: 9131; Cell Signaling Technology, Beverly, MA), or β-actin (Cat. No.: A2228; Sigma, St. Louis, MO) primary antibodies. After washing, the membranes were incubated with a fluorescently-labeled secondary antibody (Cat. No.: 925-68020, 925-32211, LI-COR; Lincoln, NE). The Odyssey image system (LI-COR) was used to scan the membranes, and the Odyssey densitometric software used to measure band intensities.

Analysis of organ injury markers

Blood samples were centrifuged at 2,000*g* for 15 min to collect serum and then either analyzed for injury parameters immediately, or stored at −80°C. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) levels were measured using assay kits from Pointe Scientific (Canton, MI).

In vitro splenocyte and CD4 T-cell isolation and stimulation

For *in vitro* studies, the spleen was removed from normal C57BL/6 mice and cell suspensions were prepared by disruption using frosted glass slides in RPMI medium with 10% FBS. The isolated cell suspensions were then passed through a 70 μm nylon mesh (BD Falcon, Durham, NC), and red blood cell (RBC) lysis was conducted with RBC lysis solution (10 mM $KHCO_3$, 150 mM NH_4Cl, 0.1 mM EDTA, pH 8.0). After centrifugation at 200*g* for 10 min, the cell pellets were resuspended in RPMI medium with 10% FBS. A total of 2.5×10^6 splenocytes were plated and pre-treated with either rmMFG-E8 (500 ng/ml) or PBS for 90 min. The cells were then stimulated with Phorbol 12-myristate 13-acetate (PMA) and ionomycin at 10 ng/ml and 1 μg/ml, respectively. After 5 h of PMA/ionomycin stimulation, cells were collected by centrifugation at 300*g* followed by mRNA isolation and cDNA preparation for the measurement of IL-17A expression by qPCR.

In another *in vitro* study, a total of 1×10^6 CD4 T-cells/ml isolated from wild-type (*wt)* and MFG-E8 knockout (m*fge8*$^{-/-}$) mice spleens using mouse EasySep CD4 T-cell isolation kit (Stem Cell Technologies, Vancouver, Canada) were stimulated by PMA (10 ng/ml) and ionomycin (1 μg/ml) overnight, followed by the measurement of IL-17 levels in culture supernatants by enzyme linked immunosorbent assay (ELISA) (eBioscience; San Diego, CA). *Mfge8*$^{-/-}$ mice were obtained as a kind gift from Dr. Shigekazu Nagata, Kyoto University, Japan. They were developed by replacing exons 4–6 of the MFG-E8 gene with a neomycin cassette. The chimera mice were then backcrossed to C57BL/6 mice at least 9 times to generate *mfge8*$^{-/-}$ mice [25].

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) and compared via one way analysis of variance (ANOVA) and Dunn's and Student-Newman-Keuls (SNK) test for multiple group comparisons. Student's *t*-test and Mann Whitney Rank Sum test were applied only for a pair comparison. Significance was considered if $p \leq 0.05$ between the experimental groups.

RESULTS

Recombinant MFG-E8 downregulates IL-17A levels in blood after CLP

IL-17 serves as a pro-inflammatory cytokine and its expression is induced in sepsis [5, 9]. To assess the *in vivo* effect of rmMFG-E8 on IL-17A production after CLP, serum was analyzed by western blot. As shown in Figure 1, septic mice treated with vehicle showed a 35% increase in IL-17. Mice treated with intrajugular rmMFG-E8 had significantly decreased levels of IL-17A protein in the plasma, with approximately 31% inhibition compared to the vehicle-injected animals.

Downregulation of IL-17A expression in spleen tissue after recombinant MFG-E8 administration into septic animals

The spleen is a major organ for IL-17 expression. In addition to systemic levels of IL-17A, we wanted to examine local expression. In septic mice, vehicle spleen exhibited a 17-fold and 68% increase in mRNA and protein expression, respectively, compared to sham.

Compared to the vehicle group, intrajugular treatment of rmMFG-E8 significantly inhibited IL-17A mRNA and protein expression by 81% and 51%, respectively (Figure 2A, B).

Recombinant MFG-E8 attenuates organ injury in sepsis

Sepsis causes severe organ injury as reflected by the elevated levels of AST and ALT in serum. To establish whether rmMFG-E8 has any protective effect against the severity of sepsis, we performed CLP on C57BL/6 mice and treated them with rmMFG-E8 or vehicle via intrajugular injection. We then assessed the levels of AST, ALT, and LDH in serum at 10 h after CLP. We found that after sepsis the serum levels of AST, ALT, and LDH were significantly upregulated 2.2-fold, 2.9-fold, and 2.5-fold respectively (Figure 3A–C). In contrast, mice treated by rmMFG-E8 significantly downregulated serum AST and ALT levels by 39% and 43%, respectively, compared to the vehicle-treated CLP animals (Figure 3A, B). For LDH, although we could not attain statistically significance, a considerable inhibition of about 24% in its levels was evident in rmMFG-E8-treated mice as compared to that of vehicle-treated septic mice (Figure 3C).

In vitro treatment of recombinant MFG-E8 directly attenuates IL-17A expression in splenocytes

To evaluate the direct effects of rmMFG-E8, we established an *in vitro* system. Primary splenocyes were pre-treated with rmMFG-E8 and then stimulated using PMA/ionomycin, followed by the assessment of IL-17A expression. PMA and ionomycin are both naturally isolated compounds that are commonly used together to stimulate intracellular cytokine production. As shown in Figure 4, *in vitro* treatment of rmMFG-E8 in splenocytes significantly downregulated IL-17A expression at its mRNA level by 49% as compared with the PBS-treated counterpart.

Deficiency of endogenous MFG-E8 leads to exaggerated IL-17 expression *in vitro*

To examine whether MFG-E8 deficiency causes increased IL-17 expression, we cultured CD4 T-cells isolated from *wt* or *mfge8*$^{-/-}$ mice in the presence of PMA/ionomycin and then assessed IL-17 production in culture supernatants. Of note, we observed significant upregulation of IL-17 production 4.7-fold from the CD4 T-cells of m*fge8*$^{-/-}$ mice compared to *wt* mice, indicating that loss of MFG-E8 might potentiate IL-17 production during inflammation (Figure 5).

Activation of STAT3 in the spleen is attenuated in recombinant MFG-E8-treated septic animals

To investigate the underlying mechanism of rmMFG-E8-mediated downregulation of IL-17 expression in sepsis, we focused on phosphorylated STAT3 (pSTAT3), a vital transcription factor for IL-17 expression. Septic animals showed a 4.7-fold increase in level of IL-17 expression in spleen tissue compared to the sham. Animals treated with rmMFG-E8 showed a 54% inhibition of STAT3 phosphorylation in spleen tissue (Figure 6A).

STAT3 phosphorylation in the spleen is increased in *mfge8*$^{-/-}$ mice after CLP

To further confirm the role of MFG-E8 in regulating STAT3 in sepsis, we induced CLP in *mfge8*$^{-/-}$ mice and analyzed spleen tissue for pSTAT3. As shown in Figure 6B, there is significant upregulation of pSTAT3 levels in both *wt* and *mfge8*$^{-/-}$ mice after CLP as compared to their respective sham animals, 2.7-fold and 4.8-fold, respectively. There is also a 25% increase in the level of pSTAT3 protein between the *wt* and m*fge8*$^{-/-}$ mice. Together with Figure 6A, these data support the notion that inhibition of IL-17 expression by rmMFG-E8 in sepsis is mediated through the downregulation of STAT3 activation.

DISCUSSION

Sepsis is a major clinical problem and a significant burden on resources in our intensive care units. Even though various treatment modalities have been implicated in reducing sepsis-related mortality, none have been successful. There has only been one FDA-approved treatment available on the market, recombinant activated protein C, drotrecogin alfa (Eli Lilly, Indianapolis, IN), but it was withdrawn due to concerns about safety and questions about efficacy [26]. Clinical trials have also been conducted with the aim to counteract individual sepsis-inducing inflammatory mediators—for example, tumor necrosis factor (TNF)-α and interleukin (IL)-1 [27]. Unfortunately, none of the trials demonstrated any benefit from treatment [28]. Attention has therefore shifted toward targeting novel cytokines that regulate other pro-inflammatory molecules in response to invading pathogens [29, 30]. IL-17 is an example of a pleiotropic cytokine that interacts with macrophages, epithelial cells, fibroblasts, and endothelial cells and can initiate production of a wide range of proinflammatory mediators such as IL-1, TNF-α, IL-6, and IL-8. The strategy of neutralizing IL-17 is therefore a distinct approach from previous experiments with IL-1 and TNF-α blockade/inhibitors.

Originally identified as an important factor for clearance of apoptotic cells, MFG-E8 has been shown to be beneficial in sepsis [15, 25, 31, 32]. It has a role in diverse physiological functions, including anti-inflammation, tissue regeneration, and clearance of apoptotic cells to maintain tissue homeostasis, which makes it an important component in various acute and chronic inflammatory diseases [18]. Another protein that also serves as a bridging molecule for the phagocytosis of apoptotic cells, growth arrest-specific protein 6 (GAS6), has been shown to downregulate IL-17 in serum and spleen, and be protective in sepsis [33, 34]. IL-17 is known to cause exaggerated inflammation and organ injury during the course of inflammatory disease, and strategies for targeting IL-17 during experimental sepsis in rodents were found to be beneficial [5, 6, 9, 35]. Direct blockade of IL-17 with intravenously administered antibody, even at 12 h after a severe model of CLP, improved survival and decreased systemic proinflammatory mediators and bacteremia [9]. Similarly, injection with anti-IL-17A antibody into the peritoneal cavity of septic mice also showed a survival advantage [13]. In our previous studies, we have shown a significant improvement in survival with administration of recombinant MFG-E8 in septic rats [32]. We have also shown that m*fge8*$^{-/-}$ mice had significantly increased mortality compared to wild-type mice after CLP [24]. It is therefore reasonable to explore the potential function of MFG-E8 as another

scavenging molecule for apoptotic clearance in the downregulation of IL-17 production in sepsis.

For our model, since other studies have shown that serum levels of IL-17 reached its peak at around 12 h after CLP, we chose to evaluate the efficacy of rmMFG-E8 for regulating IL-17 expression at 10 h, which is close to the above time point [9, 13]. IL-17 levels could also peak as early as 3 h after CLP, occuring in a case of a more severe model [36]. Our study revealed that there is inhibition of IL-17A expression in blood and spleen tissue of septic mice treated with rmMFG-E8, and also direct attenuation of IL-17 expression under *in vitro* conditions. The outcome of rmMFG-E8-mediated downregulation of IL-17 expression strongly correlated with decreased levels of organ injury markers. We established a novel link between MFG-E8 and IL-17 expression during sepsis, and also focused on a new paradigm for exploring a potential role towards controlling the innate immune system.

Our experimental model did not include the use of antibiotics since we were studying the animals for only a short period of time after non-severe CLP. All the mice survived at time of harvest at 10 h, though some appeared sick. These mice had severe systemic inflammation, as shown by elevated levels of pro-inflammatory cytokine IL-17 and injury markers, ALT, AST, and LDH. Since our current study does not evaluate for survival after CLP, but instead involves a non-lethal model of CLP to study the pathogenesis of sepsis, we did not pursue an antibiotic treatment approach.

Male mice were chosen for our experiments instead of mixed gender mice because of recent findings from experimental and clinical studies indicating sex-specific differences in sepsis and infectious disease [37]. Male and female sex steroids exhibit immune-modulating functions in both humoral and cell-mediated immune responses under normal conditions and in varied disease processes [37]. Experimental studies in mice revealed a significantly increased survival rate of proestrus female mice following polymicrobial sepsis induced by CLP compared with male animals [38]. Therefore, male and female sex differences may not be ignored when using animals to study sepsis pathogenesis. To generate reliable and consistent findings, we only used male mice. Furthermore, specific to the purposes of our study, MFG-E8 is a milk-derived glycoprotein that is abundantly expressed and secreted from the mammary gland of female mice [18]. Studies show that the hormone prolactin can induce MFG-E8 expression during the lactation period of female mice [39], which would be difficult to adequately control for in an experiment with mixed gender mice.

Aside from sepsis, MFG-E8 has also been studied in other processes. An indirect role of MFG-E8 for regulating IL-17 expression has previously been shown in a model for tumor growth [40]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) upregulates MFG-E8 via macrophage expression. GM-CSF-deficient macrophages were shown to have markedly decreased MFG-E8 levels in the spleen, lung, and other tissues, which resulted in an altered immune cell and cytokine profile [40]. Co-culturing GM-CSF deficient macrophages with naïve T-cells in the presence of apoptotic cells resulted in Th17 skewing due to impaired phagocytosis of apoptotic cells caused by reduced expression of MFG-E8 [40].

Even in chronic inflammatory disease, MFG-E8 deficiency is correlated with increased IL-17 levels. In a model of periodontitis, loss of MFG-E8 led to increased bone loss which was associated with elevated levels of IL-17 from excised gingival tissues [41]. Similarly, in a study of an MFG-E8 homolog, Del-1, periodontitis was accompanied by a decrease in Del-1 expression, and associated with an increase in neutrophil infiltration and increased IL-17 expression [42]. Knockout mice for Del-1 also showed an increased level of IL-17A and neutrophils specifically in the lung [42].

The mechanism of IL-17 expression and its functions have been extensively studied in recent years [6, 7]. STAT3 serves as a potent transcription factor for the expression of a wide range of pro-inflammatory genes [43]. Activated STAT3 has been well reported as a vital transcription factor for the expression of IL-17 [44]. STAT3 is phosphorylated at Tyr705 which allows dimerization and translocation to the nucleus as a transcription factor to induce target gene expression [45]. To ascertain a mechanistic pathway for IL-17A upregulation, we focused on assessing STAT3 activation in terms of Tyr705 phosphorylation and noticed significantly decreased levels of STAT3 phosphorylation in the spleen of rmMFG-E8-treated mice after sepsis. Although the role of MFG-E8 for regulating STAT3 activation was previously demonstrated in an *in vitro* system utilizing peritoneal macrophages, its clinical relevancy was unknown [19]. In our model of acute systemic inflammation, we discovered the negative regulatory role of MFG-E8 for downregulating IL-17 expression in serum and tissue, and relationship to reducing organ injury markers. Further studies could reveal the missing novel negative regulators that might control STAT3 activation upon MFG-E8 treatment.

Apart from the deleterious role of IL-17, there are also some studies that suggest that IL-17A could be beneficial to the host in sepsis. Studies by Ogiku *et al* showed that mortality in sepsis was increased among IL-17 knock-out mice compared to their *wt* counterparts [46]. Similarly, in other studies involving CLP or *K. pneumonia/T. gondii*-mediated acute inflammatory models, IL-17 receptor (IL-17R) deficient mice had reduced survival due to reduced neutrophil chemotaxis at the infectious foci to clear the bacterial load [47, 48]. These findings reflect the importance of IL-17/IL-17R axis in host defense against infection. Obviously, as a component of the innate immune system, IL-17 and its receptor might be upregulated at a desired level to carry out their designated roles in responding to infection. Loss of expression of IL-17 or IL-17R with knockout mice might not be beneficial due to this impaired response against infection. Therefore, a fine-tuned balance should be emphasized for both host protection and also regulation of the abnormal induction seen in the septic “cytokine storm.”

In summary, our study revealed the inhibition of IL-17A expression in blood and spleen tissue in mice treated with rmMFG-E8 after sepsis, and direct attenuation of IL-17 expression under *in vitro* conditions. This downregulation of IL-17 expression strongly correlated with decreased levels of organ injury markers. Therefore, there is a clearly beneficial role of MFG-E8 in downregulating IL-17 expression via modulating STAT3 activation, implicating its potential as a novel therapeutic agent against sepsis.

LIST OF ABBREVIATIONS

CLP	cecal ligation and puncture
BW	body weight
PBS	phosphate-buffered saline
AST	aspartate aminotransferase
ALT	alanine aminotransferase
LDH	lactate dehydrogenase
SEM	standard error of the mean
SNK	Student-Newman-Keuls
STAT3	signal transducer and activator of transcription 3
pSTAT3	phosphorylated STAT3
MFG-E8	milk fat globule-epidermal growth factor-factor VIII
rmMFG-E8	recombinant murine MFG-E8
IL	interleukin
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear neutrophil
GM-CSF	granulocyte-macrophage colony-stimulating factor

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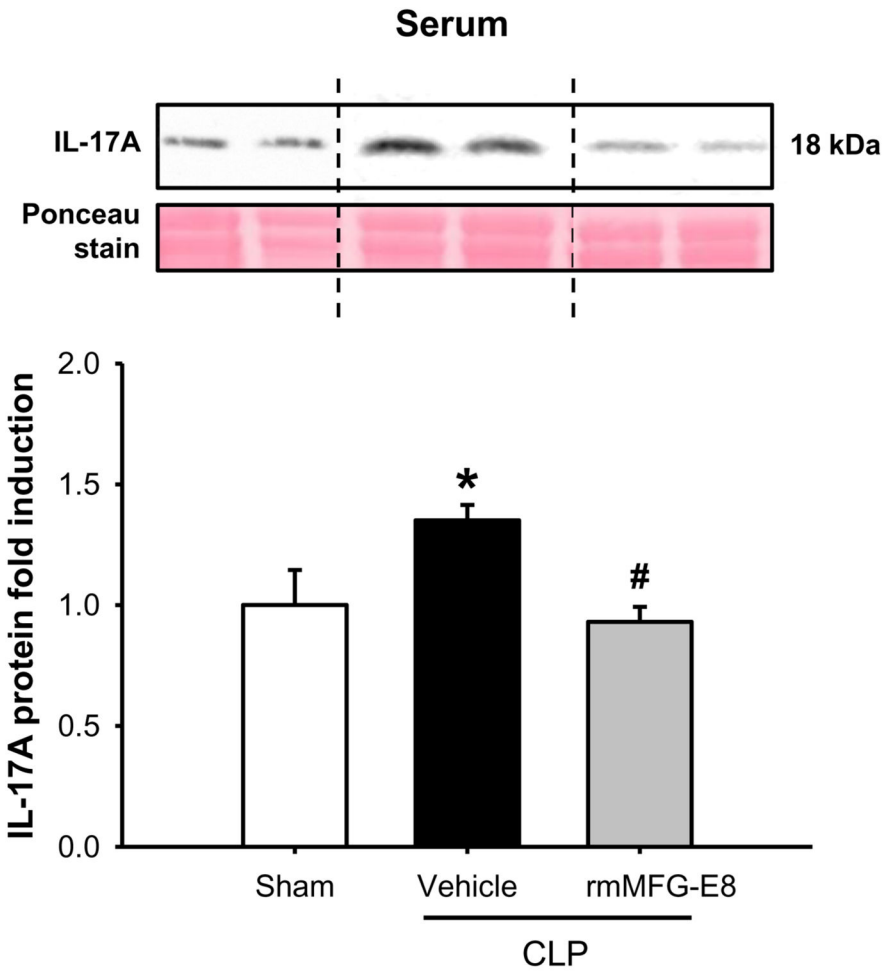

Figure 1. IL-17 expression in blood after sepsis and effect of rmMFG-E8

After 10 h of sepsis, serum was collected from sham, vehicle-, and rmMFG-E8-treated animals. A total of 3 μl of serum from each group was analyzed via western blot. Ponceau staining confirmed equal loading. Compared to the vehicle group, intrajugular treatment of rmMFG-E8 at a dose of 20 μg/kg BW inhibited IL-17 protein levels in the plasma by 31%. Sham, n=3 mice; vehicle-, and rmMFG-E8-treated, n=6 mice/group. Data are expressed as mean ± SEM and compared by one-way ANOVA by SNK method (*$p<0.05$ *vs*. sham; $^{\#}$ $p<0.05$ *vs*. vehicle).

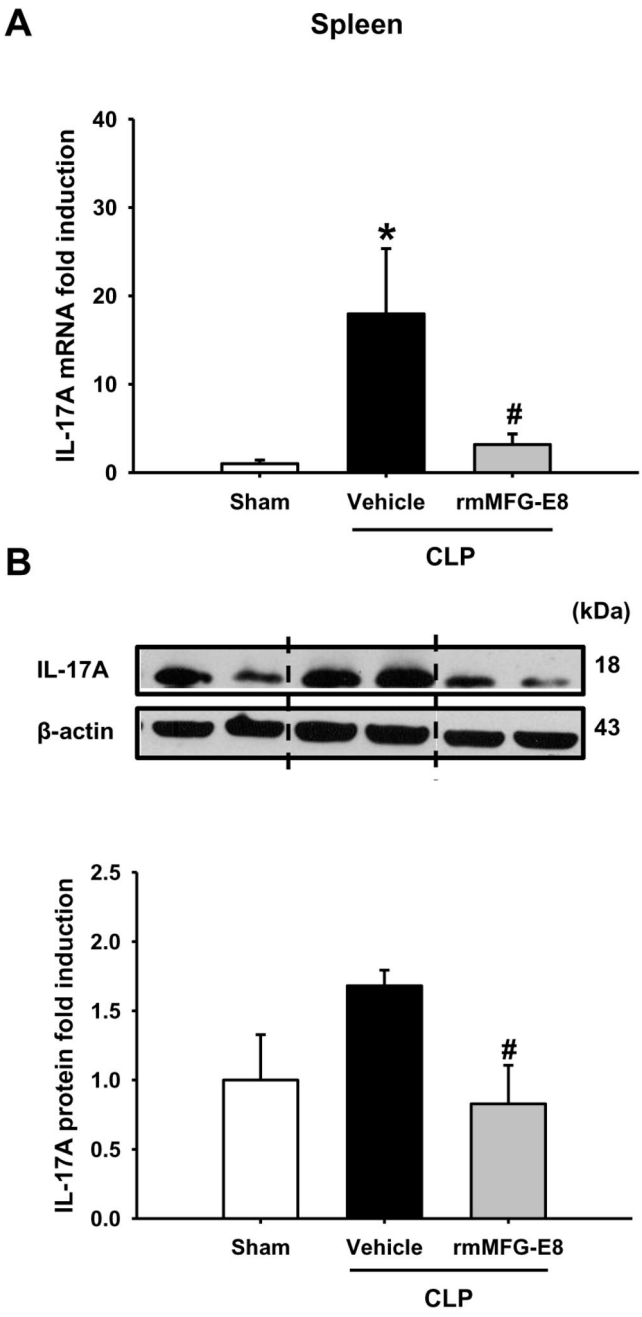

Figure 2. Effect of rmMFG-E8 on IL-17 expression in the spleen

Spleen tissue was isolated from sham, vehicle- and rmMFG-E8-treated animals after 10 h of sepsis. Extracted mRNA and protein were subjected to real-time PCR and western blot to determine IL-17 expression at its transcriptional and translational levels, respectively. Compared to the vehicle, intrajugular treatment of rmMFG-E8 at 20 μg/kg BW inhibited IL-17 **(A)** mRNA, and **(B)** protein expression by 81% and 51%, respectively. Sham, vehicle-, and rmMFG-E8-treated, n=4–8 mice/group. For both real-time PCR and western blot, the results are normalized with β-actin as an internal control and equal loading control for respective experiments. The results are expressed as fold induction in comparison with

the sham. Data are expressed as mean ± SEM and compared by one-way ANOVA by SNK method and Student's *t* test (**p*<0.05 *vs*. sham; # *p*<0.05 *vs*. vehicle).

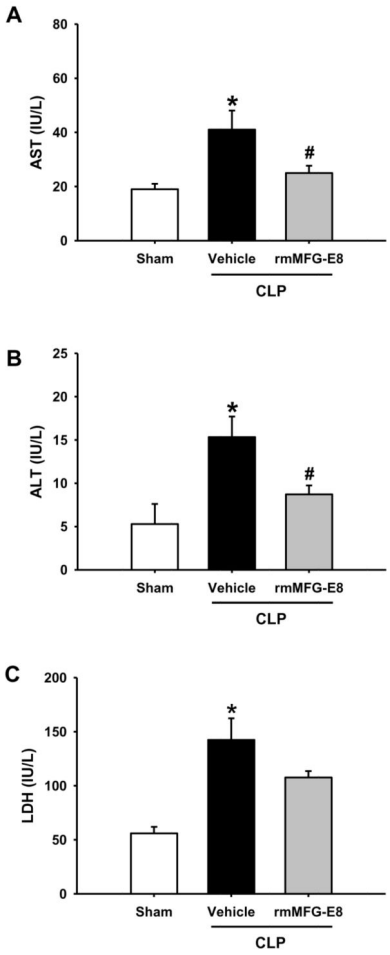

Figure 3. Treatment with recombinant mouse MFG-E8 attenuates organ injury

Serum was collected at 10 h after CLP from sham, vehicle-, and rmMFG-E8-treated (20 μg/kg BW) mice. Levels of **(A)** AST **(B)** ALT, and **(C)** LDH were measured and found to be reduced in the treatment group. Sham, n=3 mice; vehicle-, and rmMFG-E8-treated, n=7 mice/group. Data are expressed as means ± SEM and compared by one-way ANOVA by Dunn's and SNK method, and Mann Whitney Rank Sum test (*$p<0.05$ *vs*. sham; $^{\#}$ $p<0.05$ *vs*. vehicle).

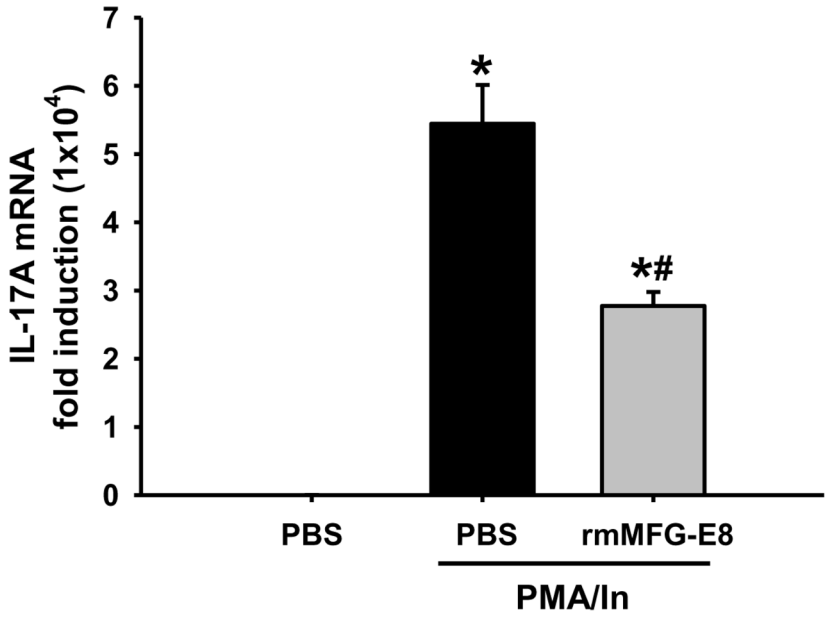

Figure 4. In vitro treatment of rmMFG-E8 in splenocytes decreases PMA/ionomycin-induced IL-17 production

Splenocytes were isolated from C57BL/6 mice and a total of 1.5×10^6 cells/ml in a 24-well culture plate were pre-treated with rmMFG-E8 (500 ng/ml) for 90 min, followed by stimulation with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 5 h. mRNA was then extracted for real-time PCR to quantify IL-17 expression. IL-17 mRNA expression is significantly decreased by 49% with rmMFG-E8 treatment. The ratio obtained in the PBS control was arbitrarily set to 1. The results are obtained from n=3 mice/group with 3 independent experiments. β-actin serves as an internal control for normalization of the results. Data are expressed as mean ± SEM and compared by one-way ANOVA by SNK method (*p<0.05 *vs*. sham; $^{\#}$ p<0.05 *vs*. vehicle).

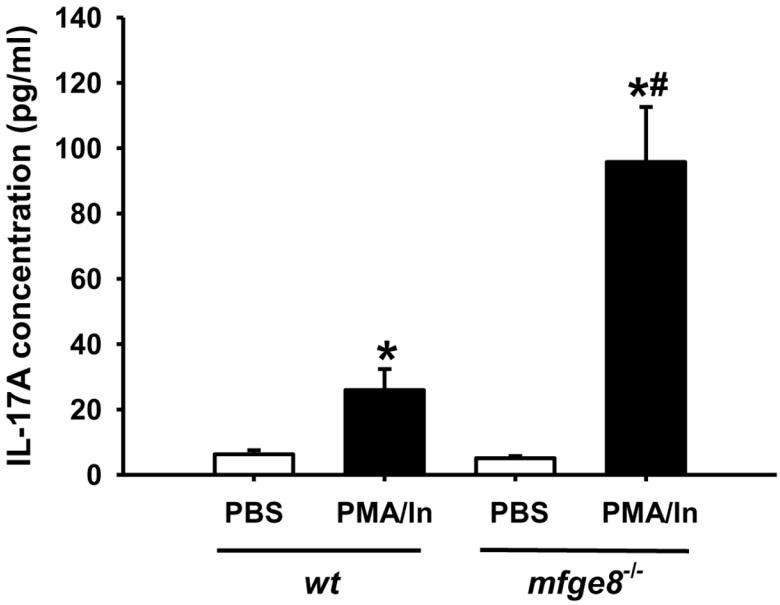

Figure 5. In vitro stimulation with PMA/ionomycin shows increased IL-17 production in CD4 T-cells isolated from MFG-E8 deficient mice

CD4 T-cells from 8–10 week-old male *wt* or *mfge8*$^{-/-}$ mice spleens were isolated by negative selection. A total of 1.5 × 10^6 cells/ml isolated from *wt* or *mfge8*$^{-/-}$ mice in a 24-well culture plate were stimulated by PMA (10 ng/ml) and ionomycin (1 μg/ml) for 5 h, followed by the assessment of IL-17 in culture supernatants by ELISA. Cells from *mfge8*$^{-/-}$ mice exhibited 4.7-fold upregulation of PMA/ionomycin-induced IL-17 production compared to that of *wt* mice. The results are obtained from n=4 mice/group. Data are expressed as mean ± SEM and compared by one-way ANOVA by SNK method and Student's *t* test (*p<0.05 *vs*. sham; $^{\#}$ p<0.05 *vs*. vehicle).

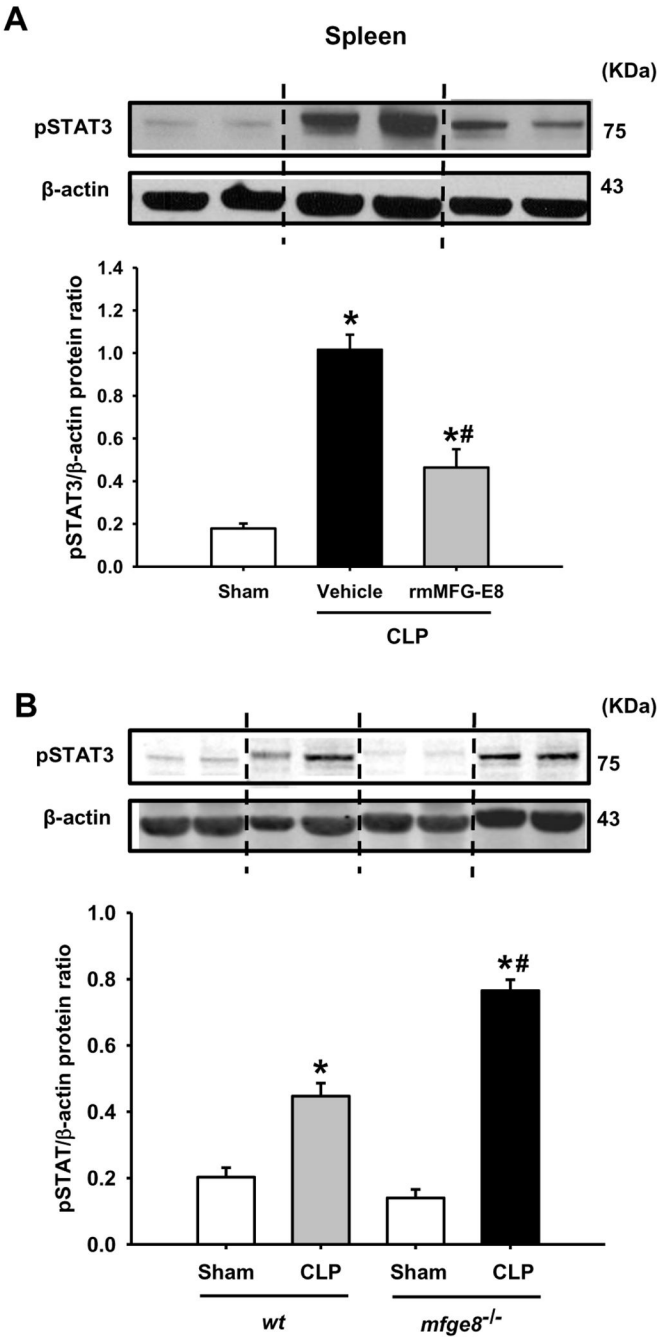

Figure 6. Treatment of rmMFG-E8 into the septic animals downregulates STAT3 phosphorylation in the spleen

(A) Vehicle or rmMFG-E8 (20 μg/kg BW) was injected intravenously into the CLP animals. Following 10 h of CLP, spleen samples were harvested to analyze for phosphorylation of STAT3 by western blot. Mice treated with rmMFG-E8 downregulated STAT3 phosphorylation in spleen tissue by 54%. **(B)** Sepsis was induced in C57BL/6 *wt* and *mfge8*$^{-/-}$ mice by CLP and after 10 h spleen tissue was collected for western blot. *Mfge8*$^{-/-}$ mice showed significantly increased levels of pSTAT3 by 25% compared to *wt* mice. The

representative results for 6A are obtained from sham, n=5 mice; vehicle and rmMFG-E8 treatment, n=6–7 mice/group. The representative results for 6B are obtained from sham, n=3 mice; *wt* and *mfge8*$^{-/-}$, n=5 mice/group. Results are normalized with β-actin as an equal loading control and are expressed as fold induction in comparison with the sham. Data are expressed as mean ± SEM and compared by one-way ANOVA by SNK method(*p<0.05 *vs*. sham; $^{\#}$ p<0.05 *vs*. vehicle).