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Deficiency in Milk Fat Globule-Epidermal Growth Factor-Factor 8 Exacerbates Organ Injury and Mortality in Neonatal Sepsis

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Abstract

Introduction—Neonatal sepsis is a systemic inflammation occurring in neonates due to a proven infection within the first 28 days of birth. It is the third leading cause of morbidity and mortality in the newborns. The mechanism(s) underlying the systemic inflammation in neonatal sepsis has not been completely understood. We hypothesize that the deficiency of milk fat globule-epidermal growth factor-factor 8 (MFG-E8), a protein commonly found in human milk, could be responsible for the increased inflammatory response leading to morbidity and mortality in neonatal sepsis.

Methods—Male and female newborn mice aged 5–7 days were injected intraperitoneally with 0.9 mg/g body weight cecal slurry (CS). At 10 h after CS injection, they were euthanized, and blood, lungs and gut tissues were obtained for further analyses. Control newborn mice underwent similar procedures with the exception of the CS injection. In duplicate newborn mice after CS injection, they were returned to their respective cages with their mothers and were closely monitored for 7 days and survival rate recorded.

Results—At 10 h after CS injection, serum LDH in the MFG-E8 knockout (KO) newborn mice was significantly increased by 58% and serum IL-6, IL-1 β and TNF- α in the MFG-E8KO newborn mice were also significantly increased by 56%, 65%, and 105%, respectively, from wild type (WT) newborn mice. There were no significant difference between WT control and MFG-E8 control newborn mice. The lung architecture was severely damaged and a significant 162% increase in injury score was observed in the CS MFG-E8KO newborn mice. The MPO, TUNEL staining, and cytokine levels in the lungs and the intestine in CS MFG-E8KO newborn mice were significantly increased from CS WT newborn mice. Similarly, intestinal integrity was also

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compromised in the CS MFG-E8KO newborn mice. In a survival study, while the mortality rate within 7 days was only 29% in the CS WT newborn mice, 80% of the CS MFG-E8KO newborn mice died during the same time period with the majority of mortality occurring within 48 h.

Conclusion—The deficiency in MFG-E8 caused increases in inflammation, tissue injury, neutrophil infiltration and apoptosis, which led to morbidity and mortality in murine neonatal sepsis. These studies suggest that MFG-E8 has a protective role in fighting against neonatal sepsis.

Keywords

MFG-E8; neonatal sepsis; lung injury; cytokines; MIP-2

Introduction

Neonatal sepsis is caused by systemic inflammatory response due to a proven infection in neonates below the age of 28 days. The incidence of neonatal sepsis in the US is 0.77 to 1 per 1000 live births [1,2]. In 2010, among the 3–7 million deaths reported in neonates by the World Health Organization, 37% of them were due to infectious origin [3]. Anti-microbial agents are the first line of defense against neonatal sepsis. Supportive care such as aggressive fluid resuscitation and vasoactive and inotropic supports are among few of its current treatment strategies. Despite advances in management and supportive care, *E coli* infections have been increasing over the years especially in low birth weight infants [1]. Sepsis in neonates is characterized by persistence and prevalence of proinflammatory mediators up to the third day of diagnosis. High levels of proinflammatory cytokines, TNF- α , IL-1 and IL-6 were observed in neonates with sepsis [4–6] and uncontrolled proinflammatory responses lead to morbidity and mortality in neonatal sepsis. Therefore understanding the mechanism underlying the exaggerated inflammatory response will aid in the development of therapeutic strategies for this devastating condition in neonates.

Milk fat globule-epidermal growth factor-factor VIII (MFG-E8) or lactadherin is a protein commonly found in human milk and is mainly produced by the spleen [7]. The most remarkable function of MFG-E8 is its ability to promote the clearance of apoptotic cells by forming a tether between phagocytes and apoptotic cells [7,8]. The expression of phosphatidylserine on the surface of apoptotic cells is considered as an “eat me” signal which can allure distinct opsonins (e.g., MFG-E8), to recognize and bring apoptotic cells to the close vicinity of phagocytes [9]. MFG-E8 has a strong binding affinity to the exposed phosphatidylserine of apoptotic cells and facilitates phagocytic engulfment via $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins. The binding of MFG-E8 to the integrin triggers a conformational change in the integrin receptor that signals the recruitment of various signaling cascade proteins and transforms the macrophage into a phagocyte capable of engulfment [10,11]. MFG-E8 is differentially expressed under various pathological conditions [12–15]. Decreased expression of MFG-E8 has been observed in disease conditions including, experimental models of adult sepsis, acute colitis, and advanced atherosclerosis [16–19].

Although multiple cell types comprise the innate immune response, neutrophils and antigen presenting cells, i.e., monocytes, macrophages and dendritic cells, are the primary cells involved in neonatal immune response. The neonatal immune response has been considered

“immature” as functional impairment in phagocytosis and bactericidal activity have been seen in the effector cells such as neutrophils and macrophages. Neonatal cytokine responses are Thelper2 (Th2) and Th17-polarized with impairment in Th1 cytokines. As such the immunological profile of the newborn is distinct from adults [20]. Previously we have shown that the deficiency in MFG-E8 exacerbated inflammation and mortality in experimental adult models of injury [18,21]. In this study, we examined whether the deficiency in MFG-E8 could be responsible for the increased inflammatory response leading to increased morbidity and mortality in a murine model of neonatal sepsis.

Materials and Methods

Experimental animals

House-bred, male and female C57BL/6 wild-type (WT) and MFG-E8 knockout (KO) mice, were kept in a temperature controlled room under 12 h light/dark cycle and were fed a standard Purina rodent diet. Newborn mice aged 5–7 days (3–4 g body weight) from each strain were used for all experiments and pups remained with their mothers throughout. Newborn mice used in this study were not identified as either male or female. Experimental procedures were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals. This project was approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research.

Mouse model of neonatal sepsis

Neonatal sepsis was induced by a cecal slurry (CS) method adapted from Wynn et al [22] with some modifications. Cecal slurry was prepared from six adult (3 males and 3 females), house-bred mice (aged 11–13 wks). Briefly, mice were euthanized via CO₂ and cecal contents were collected by laparotomy and cecotomy. After weighing, the cecal contents were suspended in 5% dextrose for a dilution of 84.7 mg/ml. The cecal slurry was filtered through a 70 µm filter, separated as 1 ml aliquots, and stored at –80°C. To administer the CS, newborn mice aged 5–7 days were separated into two groups from their mothers, placed on a 37°C heating pad, and anesthetized using 2.5% inhalational isoflurane. One group was then injected intraperitoneally (IP) with 0.9 mg/g body weight CS with a 29-gauge needle and the other was untreated and served as controls of the experiment. Subsequently, they were returned altogether to their respective cages with their mothers for recovery from anesthesia. A new aliquot of CS was used within 2 h of thawing for each experiment. At 10 h after CS injection, they were anesthetized and euthanized. Blood, lungs, and gut samples were collected. Blood samples were centrifuged at 7,000 *g* for 10 min. Serums were then collected and stored at –80°C until assays. Lungs and gut samples were frozen in liquid nitrogen and stored at –80°C for further analyses.

Experimental groups

For the 10 h time point study, male and female WT control, WT CS, MFG-E8KO control, MFG-E8KO CS newborn mice aged 5–7 days (n=6/group) were used. In general, we obtain 6–8 pups from each mother for both WT and MFG-E8KO mice. Both WT and MFG-E8KO newborn mice used as controls and experimental groups were randomly assigned from the same litter for the respective strain. To achieve statistical power, six pups each from both

strains were used in the study for the 10 h time point and therefore, maximum of two litters each were used. All assays performed were conducted at the same time with n=6/group. For survival study, male and female WT CS (n=14) and MFG-E8KO CS (n=15) were used and therefore, three litters were used for the survival experiments. Control newborn mice from the different strains were not included in the survival study because we didn't expect any mortality in those mice.

Measurement of serum organ injury marker and cytokine levels

To assess organ injury and inflammation, serum levels of lactate dehydrogenase (LDH) and cytokine levels were measured. A Commercial assay kit (Pointe Scientific, Lincoln Park, MI) was used to measure serum levels of lactate dehydrogenase (LDH) and enzyme-linked immunosorbent assay (ELISA) kits specific for TNF- α (BD Pharmingen), interleukin (IL)-6 (IL-6) and IL-1 β (BD Biosciences) were used for cytokine measurements.

Histological examination

Lung and gut tissue samples were fixed in 10% formalin and then stored/archived in paraffin. The lung and gut were segmented into 5 μ m sections, placed onto glass slides and stained with hematoxylin-eosin (H&E). Tissue injury of these sections was evaluated under a light microscope in a blinded manner. Morphological examination of lung injury was analyzed using a scoring system adapted from Matute-Bello and his colleagues [23]. The extent of lung injury was evaluated from 0 to 2 based on the presence of neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces, and alveolar septal thickening. The sum of the scores was weighted using a modified formula described by Matute-Bello et al [23] for a maximum grade of 100 per visual field. The severity of gut damage was graded in 5 fields per section using modified scoring criteria as described by Feinman et al. [24]. Scores per visual field were assessed with 0 as normal, 1 as development of vacuoles and epithelial space at the villus tip, 2 as extension of the epithelial space and lifting of epithelial layer, 3 as vacuolization from tip to middle of the villus, sloughing, and greater epithelial lifting, 4 as epithelial lifting and vacuolization from tip to bottom of the villus, and 5 as mucosa ulceration and disintegration of epithelial layer.

Myeloperoxidase (MPO) activity assay

To evaluate neutrophil infiltration in response to sepsis in various tissues, MPO activity was measured. Briefly, lung and gut tissues were homogenized with a sonic dismembrator in potassium phosphate (KPO₄) buffer containing 0.5% hexa-decyl-trimethyl-ammonium bromide. Samples were centrifuged and the supernatant was diluted in reaction solution (o-dianisidine hydrochloride and hydrogen peroxide). To calculate MPO activity, the rate of change in optical density was measured at 460 nm for 2 min.

TUNEL assay

To evaluate apoptosis in response to sepsis in various tissues, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed. Lung and gut tissue sections were deparaffinized and processed with 20 μ g/ml proteinase K for 20 min at room

temperature. Sections were then stained with green fluorescent, TUNEL kit (Roche Diagnostics, Indianapolis, IN), and counterstained with 4',6-diamidino-2-phenylindole (DAPI). TUNEL-positive cells per villus in the gut and # fields per section in the lungs were counted at 200x magnification under a fluorescence microscope.

Quantitative real-time PCR

To examine sepsis-associated lung inflammation, the lung mRNA expression of IL-6, IL-1 β , and MIP-2 was assessed. Lung and gut tissues were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) with a sonic dismembrator to extract RNA. The total RNA was then reverse-transcribed into cDNA by using murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). A PCR reaction was carried out in 25 μ l final volume containing 0.08 μ mol each forward and reverse primer, complementary DNA, and 12.5 μ l SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Amplification was performed in an Applied Biosystems 7300 real-time PCR machine under the thermal profile of 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min and 72°C for 1 min. Mouse β -actin mRNA levels were used to normalize samples and relative expression of mRNA was quantified by the 2^{-C_t} method. The results were expressed as fold change in relationship to control group. The sequences of primers used for this study consist of: mouse IL-6 5' - CCG GAG AGG AGA CTT CAC AG-3' (forward) and 5' - GGA AAT TGG GGT AGG AAG GA-3' (reverse); mouse IL-1 β 5' - CAG GAT GAG GAC ATG AGC ACC -3' (forward) and 5' - CTC TGC AGA CTC AAA CTC CAC-3' (reverse); mouse MIP-2 5' - CCC TGG TTC AGA AAA TCA TCC A-3' (forward) and 5' - GCT CCT CCT TTC CAG GTC AGT-3' (reverse); mouse β -actin, 5' - CGT GAA AAG ATG ACC CAG ATC A-3' (forward) and 5' - TGG TAC GAC CAG AGG CAT ACA G-3' (reverse).

Measurement of tissue cytokine levels

To assess sepsis-induced gut inflammation, gut IL-6 and IL-1 β protein levels were measured. Frozen gut tissues were crushed into powder in liquid nitrogen and were stored at -80°C. Protein was extracted by homogenizing the tissues with a sonic dismembrator in lysis buffer (10 mM TBS pH 7.5, 1% Triton-X 100, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycoltetraacetic acid), and protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Protein concentrations were then measured with DC Protein Assay kit (Bio-Rad Laboratories) and a standardized amount of protein was loaded on the ELISA plate to measure tissue levels of IL-6 and IL-1 β . The assays were conducted according to the manufacturers' instructions.

Survival study

For survival studies, male and female WT (n=14) and MFG-E8KO newborn mice (n=15), aged 5–7 days, received IP CS injection of 0.3 mg/g. Pups were closely monitored (i.e., every 2 h) for the first 12 h, then twice a day for a total of 7 days. The survival rate was recorded.

Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM). For multiple groups, data were compared using one-way analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) test for between comparisons. Survival study was analyzed by Kaplan-Meier log-rank test. Differences in values were determined significant if $P < 0.05$. Results were analyzed using SigmaPlot11 software (Systat Software Inc., San Jose, CA, USA).

Results

MFG-E8 deficiency increases organ injury and serum cytokines after neonatal sepsis

At 10 h after CS injection, serum LDH levels increased by 72.0% in WT newborn mice compared to WT control mice receiving no CS injection whereas in MFG-E8KO newborn mice, the levels were increased by 172%. The LDH levels in MFG-E8KO newborn mice were equivalent to a significant 57.7% increase from CS WT newborn mice (Fig 1A; $P = 0.03$). Serum IL-6, IL-1 β , and TNF- α levels were significantly increased by 38.4-, 10.0- and 28.9-fold in CS WT newborn mice whereas in the MFG-E8KO these levels were increased by 60.7-, 15.6-, and 60.2-fold, respectively from WT control newborn mice (Fig 1B–D). These levels were equivalent to significant increases of 56.5%, 64.6% and 104.7% in the MFG-E8KO mice in comparison with CS WT mice (Fig 1B–D; $P = 0.016$; $P = 0.006$; $P = 0.003$, respectively). There were no significant difference between WT control newborn mice and MFG-E8KO control newborn mice.

MFG-E8 deficiency increases lung injury, neutrophil infiltration and apoptosis after neonatal sepsis

Tissue histology was evaluated by H&E staining of the lung tissues collected from WT and MFG-E8KO newborn mice. In MFG-E8KO newborn mice, increased presence of neutrophils in the alveolar space and the interstitial tissues, appearance of the hyaline membranes and increased alveolar thickening were observed as compared to the WT newborn mice after CS injection (Fig 2A). The lung injury score in the WT and MFG-E8KO newborn mice after CS injection were increased by 4.0- and 12.0-fold respectively, which was equivalent to a significant 162% increase in the MFG-E8KO newborn mice as compared to the CS WT newborn mice (Fig 2B; $P < 0.001$). Although there was no significant increase in MPO activity in the WT newborn mice after CS injection, MPO was significantly increased by 88% in the MFG-E8KO newborn mice (Fig 2C; $P < 0.001$). While TUNEL positive cells were significantly increased by 5.4-fold in the CS WT newborn mice, there was an 8.4-fold increase in TUNEL positive cells in MFG-E8KO newborn mice which was equivalent to a significant 72.6% increase from the WT CS newborn mice (Fig 2D–E; $P < 0.001$).

MFG-E8 deficiency increases intestinal injury, neutrophil infiltration and apoptosis after neonatal sepsis

The intestinal integrity was evaluated by H&E staining of the gut tissue collected from WT and MFG-E8KO newborn mice. Although the intestinal barrier in the CS injected WT newborn mice appeared normal with no sign of villi disintegration compared to WT control,

the intestinal barrier appeared damaged in the CS MFG-E8KO newborn mice with clear indication of sloughing, epithelial lifting and vacuolization from tip to midsection of villi (Fig 3A). While the intestinal injury score increased by only 8.1% in WT mice after CS injection, the gut injury score in the CS MFG-E8KO newborn mice was increased by 210.7% as compared to CS WT newborn mice (Fig 3B; $P=0.003$). There was no significant increase in MPO activity in the gut between groups in the WT newborn mice but surprisingly a 2.0-fold increase in the gut MPO was seen in the MFG-E8KO control newborn mice as compared to WT control newborn mice. However, a significant 240.8% increase was observed in MFG-E8KO newborn mice from WT ones after CS injection (Fig 3C; $P=0.001$). While there was no significant increase in WT newborn mice in TUNEL positive cells after CS injection, there was a significant 17.0-fold increase in CS MFG-E8KO newborn mice which was equivalent to 12.3-fold increase from CS WT newborn mice (Fig 3D–E; $P<0.001$).

MFG-E8 deficiency accelerates organ inflammation after neonatal sepsis

In the WT mice, IL-6 and IL-1 β mRNA expression was significantly increased by 9.5 and 10.0-fold after CS injection, respectively (Fig 4A, B). While similar trend occurred in the CS MFG-E8KO newborn mice, IL-6 and IL-1 β mRNA expression significantly increased by 34.3- and 37.0-fold in CS MFG-E8KO newborn mice compared to CS WT newborn mice, respectively, which was equivalent to 236% and 244% increase relative to WT newborn mice after CS injection (Fig 4A, B; $P=0.01$; $P<0.001$, respectively). There was a significant increase of MIP-2 mRNA expression in CS WT newborn mice by 66.5-fold in comparison with WT control newborn mice (Fig 4C) whereas in CS MFG-E8KO newborn mice, MIP-2 expression was 185.6-fold and equivalent to 176% increase relative to WT newborn mice after CS injection (Fig 4C; $P<0.001$). Gut IL-6 and IL-1 β significantly increased by 421% and 58.9% in CS WT newborn mice as compared to WT control mice, respectively (Fig 4D–E). In the MFG-E8KO newborn mice, gut IL-6 and IL-1 β levels were significantly increased by 913% and 217% from WT control newborn mice which was equivalent to 94.3% and 99.4% increase as compared to the WT newborn mice after CS injection (Fig 4D–E; $P<0.001$; $P<0.001$, respectively).

MFG-E8 deficiency increases mortality after neonatal sepsis

Within 7 days of CS injection, while the mortality rate was only 29% in the WT newborn mice, 80% of the MFG-E8KO newborn mice died in the same time period with the majority of mortality occurring within 48 h (Fig 5).

Discussion

Neonatal sepsis is a systemic infection occurring in neonates under the age of 28 days and is the third leading cause of morbidity and mortality in newborns. While both Gram positive and Gram negative microbes are associated with neonatal sepsis, Gram negative infections, particularly *E coli* associated ones have been increasing over the years especially in low birth weight infants [1]. The underlying basis of such increase in infections has not been completely elucidated. Understanding the mechanism by which neonatal sepsis causes

mortality in newborns will aid in the therapeutic interventions of such conditions in neonates.

In the current study using an established CS model which simulates neonatal sepsis in mice, MFG-E8 deficiency significantly increased mortality in newborn mice. This suggests that those newborn mice are more susceptible to infection than their wild type counterpart. Serum levels of IL-6, IL-1 β and TNF- α were significantly increased in wild type newborn mice at 10 h after CS injection compared to untreated newborn mice indicating that our model mimics the neonatal sepsis in infants. Sepsis in neonatal infants has been characterized as persistence and prevalence of proinflammatory mediators within the third of diagnosis. High circulating levels of cytokines are observed in neonatal sepsis in infants [4–6]. Increased IL-6 and TNF- α in combination is considered as a highly sensitive marker of sepsis in the immediate postnatal period [6]. The increases in circulating levels of proinflammatory cytokines is a hallmark of systemic inflammatory response syndrome (SIRS). Interestingly, the serum levels of those cytokines were significantly elevated in the MFG-E8KO newborn mice indicating exaggerated inflammatory response in mice deficient in MFG-E8. Increased cytokines and chemokines in the lungs and gut were observed in wild type newborn mice and further increased in the MFG-E8KO newborn mice. The observed increase in cytokines in the lungs and gut in the MFG-E8 deficient newborn mice could lead to increased organ injury. In fact, significant damages to the lung and gut architecture were observed in the MFG-E8KO newborn mice 10 h after CS injection. Lactate dehydrogenase (LDH), an indicator of tissue injury evaluated from circulation was also significantly elevated in CS MFG-E8KO neonates in comparison to the wild type counterpart. These findings suggest that MFG-E8 could be responsible for controlling inflammatory response and organ injury in neonatal sepsis.

Increased neutrophil infiltration to the lungs and gut were observed in MFG-E8 deficient newborn mice. Neutrophils are generally recruited to the infected tissues to clear bacterial debris via phagocytosis. During which reactive oxygen species, cytokines, chemokines and other inflammatory mediators are released from neutrophils facilitating more immune cell recruitment to the tissue and ultimately leading to tissue damage. Exogenous administration of MFG-E8 has shown to ameliorate neutrophil infiltration to the lungs in an experimental model of acute lung injury in adult mice [25] suggesting MFG-E8 could play a role in inhibiting neutrophil infiltration to lungs and gut in neonatal sepsis.

MFG-E8 deficiency led to increased presence of TUNEL positive cells in the lungs and the gut. This could be due to either an increase in apoptosis or the lack of clearance of apoptotic cells. Deficiency in efficient clearance of apoptotic cells can be potentially harmful to the host and those apoptotic cells also release inflammatory and toxic mediators and exaggerate the inflammatory response [7,8]. Since MFG-E8 acts as a tether between phagocytes and apoptotic cells to clear dying cells [7], the observed increase in TUNEL positive cells could be due to the lack of clearance of dying cells as opposed to an increase in apoptotic cell death.

Prior studies have established that there is a direct link between MFG-E8 and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [7]. The integrin α_v (CD51) subunit can form heterodimers with five distinct beta subunits;

β_1 , β_3 , β_5 and β_6 . The integrin $\alpha_v\beta_3$ which is also known as the vitronectin receptor interacts with various extracellular matrix proteins such as osteopontin, fibronectin, fibrinogen, thrombospondin, proteolysed collagen and von Willebrand factor [26]. Therefore in addition to molecular interaction with MFG-E8, $\alpha_v\beta_3$ interacts with extracellular matrix proteins. In the absence of MFG-E8, the $\alpha_v\beta_3$ interaction with these proteins could in fact accelerate the inflammatory response. It is highly unlikely that the mRNA expression of these integrins will be significantly altered due to the deficiency of MFG-E8. However, we have previously shown that integrin β_1 , β_2 , β_3 mRNA expressions were significantly increased in the gut and lungs in an adult ischemia reperfusion injury model [27]. In addition, there is evidence that MFG-E8 directly interacts with the integrin receptor and inhibits LPS-induced TNF- α and IL-1 β production in macrophages [28]. Therefore, it is possible that integrin expression will be increased during sepsis in the WT newborn mice and further elevated in the MFG-E8KO newborn mice. Further studies are needed for such conclusion.

The actual mechanism of action of MFG-E8 in the neonates has not been elucidated. Previously we have shown in adult sepsis that MFG-E8 levels were significantly decreased in the blood, spleen and liver tissues during late sepsis [18]. Splenic macrophages from adult MFG-E8KO mice showed dramatically decreased ability to phagocytose apoptotic cells under normal conditions as compared to WT mice, suggesting a critical role for MFG-E8 in this process [13]. Interestingly, adult MFG-E8KO mice accumulated higher amount of apoptotic cells as compared to the WT mice during late sepsis [18]. These data clearly indicated that MFG-E8 functions to clear apoptotic cells during late sepsis. In an endotoxemia model, adult MFG-E8KO mice caused greater increases in TNF- α than the WT mice and that MFG-E8 mediated decrease in TNF- α is regulated by pSTAT/SOCS3 activation and subsequent downregulation of the NF- κ B pathway [28]. In this regard, the current study demonstrated that in MFG-E8KO newborn mice, there was significant increase in proinflammatory cytokines, i.e., TNF- α , IL-1 β and IL-6, and TUNEL positive cells were also increased at 10 h after CS injection suggesting a strong relation to pathways relatable to adult sepsis. Since the immunological profile of the newborn mice is distinct from adults, it is important to examine the effect of MFG-E8 or lack thereof on the cell types, i.e., neutrophils and antigen presenting cells such as monocytes, macrophages and dendritic cells in newborn mice to pinpoint the actual mechanism of action of MFG-E8 in this unique patient population. Further studies are needed for such evidence.

While we recognize that a true model of NEC or cecal perforation would have been suitable to test the hypotheses using MFG-E8KO newborn mice in the current study, both models are very challenging to execute in a newborn mouse. Therefore, as a proof of concept study to determine whether MFG-E8 has any role in neonatal sepsis, we employed the current CS model. We recognize CS model as a limitation for our study. Additional clinically relevant models such as NEC or cecal perforation model are needed to confirm our findings. Another limitation of our study is the choice of the control group. While both control and experimental groups in WT and MFG-E8KO newborn mice underwent similar procedures such as the use of the heating pad, isoflurane inhalation, euthanasia, collection of blood and tissues for analysis, the controls in the study were not given IP injection of the vehicle, i.e., 5% dextrose. Therefore, the baseline inflammatory mediator values were derived from newborn mice from both strains that were untreated with the vehicle. In addition, we used

frozen aliquots of CS to induce sepsis in the newborn mice. To minimize inter and intra-variations in experiments, all CS aliquots used in various experiments were from the same batch of CS preparation stored as frozen aliquots, thawed once and used within 2 h of thawing.

To assess inflammation and tissue injury at 10 h time point, we chose 0.9 mg/g dose of CS injection. This dose was selected based on our pilot study while we observed 100% mortality within 20 h using 0.9 mg/g dose in MFG-E8KO newborn mice, no mortality was observed in the 0.9 mg/g dose until after 10 h post injection. While we show that MPO was significantly increased in the MFG-E8KO newborn mice in the lungs and which was also correlated with the lung injury score, there was no increase in the MPO in the WT newborn mice at 10 h after CS injection. This discrepancy could be because the 0.9 mg/g dose was sub-optimal for the WT newborn mice and increased CS dose could significantly increase the MPO activity and all other parameters tested. In addition, since we observed 100% mortality with 0.9 mg/g dose within 20 h in the MFG-E8KO newborn mice, the dose was reduced to 0.3 mg/g for the survival study.

Neonates are vulnerable to microbial infections. It has been reported that the innate and adaptive immune responses in the neonates are immature and therefore functionally impaired and that this impairment causes neonates to be more susceptible to microbial infection [29–31]. These studies suggest that neonates have an inherent bias against Thelper1 (Th-1) cytokines such as TNF- α and IL-1 β that are essential to fight off microbial infection and that the responses in neonates are generally Th-2 or Th-17 leaving neonates vulnerable to microbial infection. Our data indicated that both TNF- α and IL-1 β are significantly increased in both WT as well as MFG-E8KO newborn mice after CS injection suggesting Th-1 cytokines are increased after neonatal sepsis. In fact infection-induced proinflammatory cytokines/Th-1 cytokines TNF- α and IL-1 β have been associated with premature labor and pre-term delivery. Other studies have shown that due to inadequate number of T cells newborn mice are vulnerable to TLR4 agonists LPS, and that transfer of T cells to newborn mice are sufficient to control the early response to LPS [32]. However, the actual mechanism of the vulnerability of neonates to microbial infection has not been completely understood.

In summary, our data showed that the deficiency of MFG-E8 in newborn mice caused increases in inflammation and tissue injury which could be responsible for the increased morbidity and mortality in neonatal sepsis. These data further suggest that MFG-E8 could be a novel therapeutic for neonatal sepsis.

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Figure 1. LDH and serum cytokines were increased in MFG-E8KO neonates

Serum LDH (A), IL-6 (B), IL-1 β (C) and TNF- α (D) from wild type (WT) and MFG-E8 knockout (MFG-E8KO) newborn mice of control (Ctrl) and at 10 h after CS injection (CS).

Data are expressed as mean $\pm$ SEM and compared by one way ANOVA and SNK test.

*P < 0.05 vs. WT Ctrl; #P < 0.05 vs. WT CS.

Figure 2. Lung architecture was compromised and neutrophil infiltration to the lungs was increased in MFG-E8KO neonates

H&E stain of lungs from WT and MFG-E8KO newborn mice. A. Representative photographs at 200X magnification. B. Histological lung injury score calculated as described in Materials and Methods. C. Myeloperoxidase (MPO) activity of lung lysates. D. TUNEL staining of lung tissue sections. E. TUNEL positive cells per field calculated from D. Data are expressed as mean $\pm$ SEM and compared by one way ANOVA and SNK test.

* $P < 0.05$ vs. WT Ctrl; # $P < 0.05$ vs. WT CS.

Figure 3. Intestinal morphology was altered and neutrophil infiltration into the intestine was increased in MFG-E8KO neonates

H&E stain of Intestines from WT and MFG-E8KO newborn mice. A. Representative photographs at 200X magnification. B. Histological intestinal injury score calculated as described in Materials and Methods. C. MPO activity of Intestinal lysates. D. TUNEL staining of Intestinal tissue sections. E. TUNEL positive cells per field calculated from D. Data are expressed as mean $\pm$ SEM and compared by one way ANOVA and SNK test.

* $P < 0.05$ vs. WT Ctrl; # $P < 0.05$ vs. WT CS.

Figure 4. Lung and gut tissue cytokines and lung MIP-2 were increased in MFG-E8KO neonates
 Real time reverse transcription-polymerase chain reaction (qPCR) for IL-6 (A), IL-1 β (B) and MIP-2 of total RNA from lungs and fold change calculated against β -actin. Data are expressed as mean $\pm$ SEM and compared by one way ANOVA and SNK test. * $P < 0.05$ vs. WT Ctrl; # $P < 0.05$ vs. WT CS.

Figure 5. Mortality was increased in MFG-E8KO neonates after neonatal sepsis

Survival curve from WT and MFG-E8KO newborn mice aged 5–7 days after CS injection.

Data are expressed as percent survival rate. * $P < 0.05$ vs. WT.