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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 because of 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-1β 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 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.

Neonatal sepsis is caused by systemic inflammatory response because of 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 because of 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

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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 phoshatidylserine 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.

1. Materials and methods

1.1. 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.

1.2. 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 CO_2 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 7000 *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.

1.3. 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 MFGE8KO 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 did not expect any mortality in those mice.

1.4. 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-1β (BD Biosciences) were used for cytokine measurements.

1.5. 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 μm 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.

1.6. 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 (KPO_4) 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.