

Review: milk fat globule-EGF factor 8 expression, function and plausible signal transduction in resolving inflammation

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Abstract Although the cloning and molecular characterization of MFG-E8 was first reported in the early 90s, breakthrough on MFG-E8 research came into light when it was explored as an outstanding factor for phagocytosis of apoptotic cells by professional macrophages in 2002. Since then numerous studies have been performed on MFG-E8 not only to demonstrate the role of phagocytic clearance of apoptotic cells, but also to focus on a wide range of aspects, even emphasizing on a direct link to innate-immune systems. In terms of its role as therapeutic potentials, our group, as well as others, has shown MFG-E8 to be an essential factor in attenuating inflammation and improving prognosis in several animal models of life threatening diseases. Considering these versatile functions of MFG-E8, several *in vitro* and *in vivo* studies were embarked on to explore the mechanistic pathways exerted by MFG-E8 during inflammation. With the relevant cumulative findings, herein we reviewed the potential roles of MFG-E8 in pathophysiological conditions by highlighting its plausible signal-transduction mechanisms.

Keywords MFG-E8 · Signal transduction · Apoptosis · $\alpha_v\beta_3$ -integrin · Phosphatidylserine · NF- κ B · STAT3 · SOCS3 · HMGB1

Abbreviations

MFG-E8	Milk fat globule-epidermal growth factor-factor 8
RGD	Arginine–glycine–aspartate
CLP	Cecal ligation and puncture
SLE	Systemic lupus erythematosus
PPAR	Peroxisome proliferator-activated receptor
HMGB1	High-mobility group protein B1
AD	Alzheimer’s disease
ABP	Amyloid β peptide
VEGF	Vascular endothelial growth factor
Jak/STAT	Janus kinase/signal transducer and activator of transcription
SOCS3	Suppressor of cytokine signaling 3

Introduction

Justified to its name, milk fat globule-epidermal growth factor-factor 8 (MFG-E8) was initially identified as an indispensable component of the milk fat globule, a membrane-encased collection of proteins and triglycerides that sprout from the apical surface of lactating mammary glands [1]. It is also considered to be so named because of its regions of sequence similarity to two of each epidermal growth factor (EGF) repeats and the blood coagulation factor V/VIII segments, respectively [2]. The N-terminal site of nascent MFG-E8 peptide has a signal peptide sequence which directs it to emit into the extracellular pool. The second EGF-repeat of MFG-E8 contains a highly conserved arginine–glycine–aspartate (RGD) motif, by

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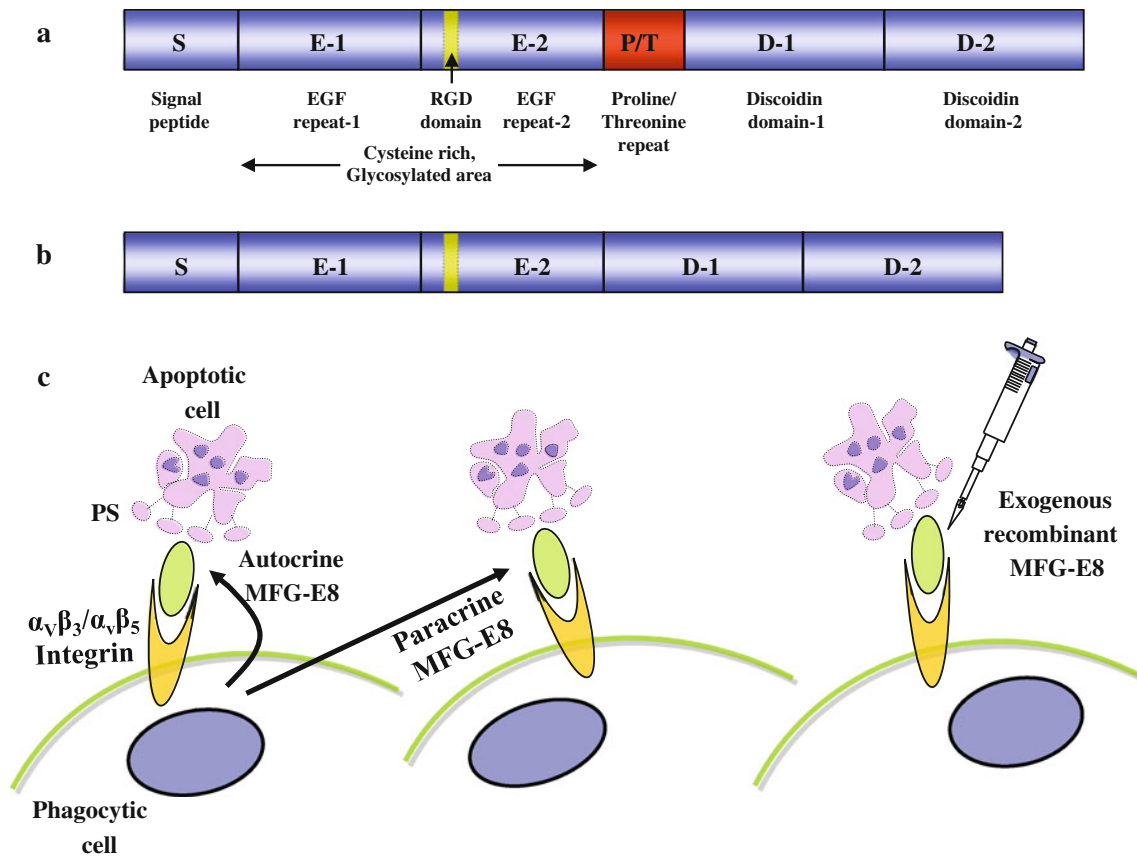


Fig. 1 Structure and universal functions of MFG-E8: The N-terminal site of MFG-E8 contains two EGF like domains, while the C-terminal site has two discoidin like domains with the regions of sequence similarity to blood coagulation factor V/VIII. The nascent murine MFG-E8 has two isoforms **a** long and **b** short forms, based on the presence of the proline/threonine rich repeats (P/T). **c** The

which it recognizes $\alpha_v\beta_3/\alpha_v\beta_5$ -integrin of phagocytic cells, while the C-terminal factor V/VIII like domains enable it to bind to the apoptotic cells via phosphatidylserine (PS). Thus, these bi-motif functions of MFG-E8 facilitate it to scavenge the dying cells from the tissue micro-environment [2] (Fig. 1a, b). With this concept of removing the dying cells from various organs, MFG-E8 has proved to be an essential factor in controlling the progression of various inflammatory diseases. MFG-E8 knock-out mice can spontaneously develop the detrimental autoimmune diseases due to the infiltration of apoptotic cells at the germinal centers of spleen [3]. Sliding away from its classical functions, recent studies showed that MFG-E8-mediated potential therapeutic benefits in sepsis and intestinal tissue injury were not only solely dependent on the enhanced clearance of apoptotic cells, but also relied on diverse cellular events for maintaining the epithelial integrity and healing of the injured mucosa [4].

To date, MFG-E8 research has been promulgated into the various sectors of molecular medicine and innate immune fields to exhibit its potential beneficial roles in

conventional function of MFG-E8 is to make a link between apoptotic cells and phagocytes, thus promoting the engulfment of apoptotic cells in an autocrine and paracrine manner. The functions of MFG-E8 for the phagocytosis of apoptotic cells by macrophages can also be noticeable even with exogenous administration of recombinant MFG-E8

several animal models of inflammatory diseases, e.g., colitis, renal, hepatic and intestinal ischemia/reperfusion (I/R) [5–7]. Some of those reports have delineated the signal transduction events towards implementing its functional outcome. The major aspect of this review is to compile cumulative findings on MFG-E8-mediated signaling systems, as well as center on how it modulates other pathways that are activated by various ligands/factors during inflammatory consequences. In addition, we also aim to focus on MFG-E8 expression profile, functions and how its appearance is regulated by various signaling events. Our approach imposes better understanding of MFG-E8 mechanism of action, and further implicates towards delineating unresolved queries on MFG-E8 roles as therapeutic potentials in various inflammatory diseases.

MFG-E8 expression and localization

Although MFG-E8 was initially identified as a component of milk fat globule membrane protein with abundance of its

expression at the lactating mammary glands [8], a decade of research revealed its ubiquitous pattern of expression in different cells and tissue types. To define its localization, here we discussed some of the striking immunohistochemical studies that clearly demonstrated its appearance at different compartments of experimental tissue sections. At early lactation periods, MFG-E8 was clearly detected around or on the apical surface of the alveolar epithelium of involuting mammary glands [9]. Moreover, in those tissue samples, several CD68 (marker of activated macrophages) positive cells were also detected in the involuting mammary glands with positive staining for MFG-E8, also suggesting its co-localization in mononuclear cells. Interestingly, MFG-E8 was shown to be secreted from the surrounding cells and localized/accumulated into the lumen and in the apical surfaces of epithelium [9]. In another study, immunostaining of MFG-E8 has been performed in both mouse and rat eyes, where it showed intense staining of the inner segment layer of photoreceptor cells and lighter staining of the retinal pigment epithelial cells [10]. In addition, intestinal tissues in mice with acute colitis had an abundant localization of MFG-E8 at the cytoplasmic and extracellular regions of lamina propria mononuclear cells of injured colonic mucosa [5]. In normal mice, the anti-MFG-E8 immunostain persisted in the spinous layer of mice skin, while it was localized in cells between the basal and surface layers of neoplastic skin tissues [11]. In human endometrial biopsies, MFG-E8 was predominantly localized at the glandular as well as surface-luminal epithelial cells [12]. Although the above evidences imply with the notion of its omnipresent distribution in mammals, we classified its expression profile into two major categories based on normal and stressed conditions.

Cell and tissue specific expression of MFG-E8 under normal condition

In mouse and rat tissues, MFG-E8 is expressed as two different isoforms, termed as short and long forms [2, 10]. Between these two types, short form is more abundantly produced than the long one. In humans, although a single isoform was reported earlier, recent NCBI gene bank data also revealed the existence of two different isoforms. Hence, this may impose further study on human MFG-E8 isoforms to unveil their characteristic function. The longer isoform of mouse MFG-E8 transcript possesses 2,143 nucleotides and gives rise to a 463 amino acids (aa) containing mature peptide with the molecular weight ranging between 64 and 75 kDa. On the other hand, the alternatively spliced shorter fragment has 2,032 nucleotides, which lacks the exon 4 of original transcript encoding the proline/threonine rich repeat. The short form of MFG-E8 has a total of 426 aa residues giving rise to a 50–56 kDa sized protein product.

Due to high glycosylation at the post translational modification, the product sizes of each of these two MFG-E8 isoforms may vary within the above ranges. Since both these two isoforms retain their conserved binding domains intact, they exhibit similar function but with varied efficacy [2]. Interestingly, in human a very rare heterozygous intronic mutation was found in female systemic lupus erythematosus (SLE) patients that caused a cryptic exon from intron 6 to be incorporated into the transcript, thus generating a C-terminally truncated MFG-E8 protein due to the presence of a premature termination codon. The mutant MFG-E8 was aberrantly glycosylated and sialylated to generate a shorter sized product than the wild-type [13]. To date, no other truncated or splicing variant of MFG-E8 in mammals has been reported. However, two different SNPs and their genetic linkages to SLE have been recently reported in case-control studies among Taiwanese subjects [14]. MFG-E8 was found to be expressed in primary cells, as well as transformed and non-transformed cell lines. The assessment of MFG-E8 expression at its protein level in cell cultures has been well described in several reports [2, 15]. Majority of them estimated the MFG-E8 expression in cellular lysates by using the western blot technique. To measure the MFG-E8 levels in cell culture supernatants, the collected samples were concentrated by using the ultracentrifugation, followed by the detection of MFG-E8 expression by western blot analysis. Since MFG-E8 may also be secreted as microvesicles, hence the samples were subjected to sucrose density-gradient ultracentrifugation followed by SDS-PAGE and western blotting detection of the fractions with different densities [1]. MFG-E8 expressing primary cell-types that were isolated from various organs and body fluids include mammary epithelial cells, keratinocytes, splenocytes, monocytes, peritoneal macrophages, dendritic cells, glial cells, antigen presenting cells (APCs), stromal cells, fibroblast, osteoblast, vascular smooth muscle cells, and astrocytes [2, 8, 11, 12, 15–20]. Studies also showed considerable amounts of MFG-E8 in intracellular exosomal compartments of immature dendritic cells and in undifferentiated macrophages, while its contents become decreased upon maturation [21]. Conversely, thymocytes detected low or no expression of MFG-E8 [2]. MFG-E8 is also expressed in cell-lines from epithelial and myeloid origins, e.g., mammary epithelial cell-line COMMAID, 3T3-L1 adipocytes, P388D1 macrophages, and RAW264.7 cells [1, 15, 22, 23]. On the other hand, several cell-lines that do not express MFG-E8 include BAM3, J774A.1, and NIH3T3 [2]. Apart from these, MFG-E8 expression was also noted in murine cancer cell lines, such as, Lewis lung carcinoma cells, RMA T-cell lymphoma, MB49 bladder carcinoma and B16 melanoma [24]. In addition, tissue specific expression of MFG-E8 in mice and rats under normal physiologic condition has also been reported, which showed its presence in the brain,

heart, lungs, mammary glands, spleen, intestines, liver, kidneys and reproductive organs [7, 8, 25, 26]. By contrast, in humans MFG-E8 expression in several tissues was considerably less reported, showing its presence in reproductive organs, endothelium, epidermal tissues and blood [11–13].

Altered expression of MFG-E8 in pathological conditions

In majority of cases, MFG-E8 is differentially expressed from its basal level under pathophysiological stress (Table 1). During severe systemic inflammation induced by polymicrobial sepsis, MFG-E8 production was dramatically declined in the spleen and serum at 20 h after cecal ligation and puncture (CLP), an animal model of sepsis [27]. Mesenteric I/R caused severe widespread injury and inflammation of the small intestines and remote organs, and thereby decreased MFG-E8 levels in the spleen and lung tissues [4, 28]. Similarly, in mouse and rat models of renal and hepatic I/R, MFG-E8 levels were down-regulated in the spleen, kidney and liver as compared to sham [6, 7]. On the other hand, although acute alcohol intoxication did not affect the production of MFG-E8 in the spleen, however the levels of MFG-E8 gene expression in pre-alcohol exposed septic animals were even significantly lower than those in sepsis alone animals, suggesting that

alcohol possesses the priming effect that sensitizes the animal more susceptible to the second hit caused by sepsis [29]. Consistent with the above findings, others have also demonstrated a decreased production of MFG-E8 in mice colonic tissues with acute colitis and in advanced atherosclerosis [5, 30, 31]. Moreover, in a mouse model as well as in human Alzheimer's disease (AD) subjects, the decreased expression of MFG-E8 has been reported in senile plaques, while marked expression was seen in uninvolved region [32, 33]. At the early times of its discovery, MFG-E8 was first termed as a breast cancer antigen (BA46), due to its abundant expression in carcinogenic breast tissues [34]. Later, a handful of evidences also confirmed its up-regulation in cells and tissues of the tumor micro-environment [35, 36]. MFG-E8 expression is also altered in autoimmune diseases. Although the deficiency of MFG-E8 production has been reported to develop SLE in mice due to impaired clearance of apoptotic cells [2, 3, 37], serum MFG-E8 levels in human SLE subjects were found to be higher than in the healthy volunteers [13, 38]. These higher levels of MFG-E8 may perturb the phagocytosis of apoptotic cells by masking either PS or $\alpha_v\beta_3$ -integrin and inhibiting the formation of bridges between apoptotic cells and phagocytes for engulfment [38]. To measure the soluble form of human MFG-E8 levels in crude serum or plasma samples, Yamaguchi et al. [38] were the first to

Table 1 At a glance: factors and disease conditions that alter MFG-E8 expression

Factors/diseases	Cells/tissues/organs	MFG-E8 status	References
Prolactin	Mammary epithelial cells	Upregulation	9
	Macrophage	Upregulation	15
	Endometrium	Upregulation	12
Fractalkine	Peritoneal macrophage	Upregulation	23
	Glial cells	Upregulation	19
Connexin 43	Mammary epithelial cells	Upregulation	43
GM-CSF	Macrophage	Upregulation	17
PPAR- δ agonist	Human PBMC	Upregulation	41
LPS	Macrophage, spleen, blood	Downregulation	21, 42
Apoptotic cells	Macrophage	Upregulation	41
Sepsis	Spleen, macrophage, blood	Downregulation	21, 42
Sepsis + alcohol	Spleen	Downregulation	29
Gut I/R	Gut, spleen	Downregulation	28
Intestinal injury	Intestine	Downregulation	4
Colitis	Distal colon	Downregulation	5
Acute lung injury	Lung	Downregulation	28
SLE	Blood	Upregulation	38
Renal I/R	Kidney, liver	Downregulation	6
Hepatic I/R	Liver	Downregulation	7
Atherosclerosis	Endothelial plaque	Downregulation	30
Neurodegenerative disease	Senile plaques	Downregulation	32, 33
Melanoma	Basal layer of skin	Upregulation	11

develop an indirect sandwich ELISA-based technique utilizing mouse anti-human MFG-E8 monoclonal Ab and biotinylated hamster monoclonal Ab as capture and detection purposes, respectively. Currently, the ELISA kits for measuring both the mouse and human MFG-E8 in serum and plasma samples are commercially available from different manufacturers. Studies of MFG-E8 expression in various diseases where inefficient clearance of apoptotic cells is a major phenotype may be able to generate solution to overcome from those disorders.

Factors utilizing the signal transduction mechanisms to regulate MFG-E8 expression

To maintain homeostatic balance, MFG-E8 expression is tightly regulated. To date, considerable number of *in vitro* and *in vivo* studies were done to unveil potential signaling pathways by which several factors can modulate (increase or decrease) MFG-E8 expression (Table 1). During lactation, the hormone prolactin (PRL) is abundantly produced in the mammary glands or in circulation [8, 39]. Implementing the concept of MFG-E8 up-regulation during lactation, *in vitro* studies using mammary epithelial cells and macrophages clearly showed an up-regulation of MFG-E8 expression by PRL [8, 15]. To evaluate the mechanism, murine MFG-E8 promoter was cloned, revealing C/EBP β responsive element to govern PRL-dependent MFG-E8 expression via its receptor PRL-R [15]. Studies using growth hormone, insulin and steroid hormone, and hydrocortisone were also shown to activate MFG-E8 in target epithelial cells [40].

Apart from the hormone dependent activation of MFG-E8, factors of innate-immune system were also known to regulate its expression in mononuclear cells. Fractalkine is an active member of CX(3)C-chemokine family typically expressed by neurons. Leonardi-Essmann et al. [19] was the first to identify fractalkine as a potent inducer of MFG-E8 in microglial cells via its receptor CX(3)CR1. The effect of fractalkine was also extensively studied by Miksa et al. [23] to show MFG-E8 up-regulation in mouse peritoneal macrophages and RAW264.7 cells, thereby enhancing the phagocytosis of apoptotic cells by macrophages. Apoptotic cells are found to be a positive regulator of MFG-E8 expression in macrophages, where the *in vitro* co-culturing experiments with macrophages and apoptotic cells greatly increased the MFG-E8 contents as compared to only macrophage cultures [41]. Since MFG-E8 expression was significantly declined during inflammation, focus was also given to elucidate whether or not the factors that exacerbated tissue injury and inflammation might regulate MFG-E8 expression. Lipopolysaccharide (LPS) is considered as one of the potent stimulators for aberrant innate-

immune functions during inflammation, therefore mice treated with LPS significantly decreased the endogenous MFG-E8 levels in the serum, spleen and other major organs [21, 42]. Consistent with the above *in vivo* findings, Komura et al. [42] delineated the mechanism to show that the LPS effects for down-regulating MFG-E8 expression in mouse peritoneal macrophages and RAW264.7 cells was mediated via TLR4/CD14 pathways.

MFG-E8 research has also been performed in the field of cancer biology, where it plays major roles in cell growth and proliferation. Gap junction protein expression has been reported to control the growth of a variety of transformed cells. Studies using the gap junction protein connexin 43 showed suppression of glioma cell growth by affecting MFG-E8 production, thus it served as a negative regulator of MFG-E8 expression [43]. Granulocyte monocyte-colony stimulating factor (GM-CSF) is a cytokine that functions as a white blood cell growth factor to differentiate stem cells into granulocytes (neutrophils, eosinophils, and basophils) and monocytes. The concept of GM-CSF dependent MFG-E8 expression was raised when Jinushi et al. [17] noticed a failure of apoptotic cell uptake by macrophages of GM-CSF deficient mice. The flow cytometry data confirmed the marked decrease in MFG-E8 expression in macrophages isolated from the peritoneal cavity, spleen, liver, and the lungs of GM-CSF deficient mice. Recently, role of the peroxisome proliferator-activated receptor (PPAR)- δ has been identified as a crucial transcriptional sensor of apoptotic cells, where the PPAR- δ ^{-/-} mice developed autoimmune disease due to accumulation of apoptotic cells. PPAR- δ had been shown to regulate the expression of opsonin gene, e.g., MFG-E8, thereby accelerated the phagocytic potential of apoptotic cells by macrophages [41]. From the above discussions we noticed that the factors which induced the clearance of apoptotic cells by macrophages may in turn act as positive regulators for MFG-E8 expression in maintaining tissue homeostasis.

MFG-E8 function, mechanism of action and related complications due to its deficiency

The most remarkable function of MFG-E8 that made it as one of the pillars of phagocytosis research is its ability to promote the clearance of apoptotic cells by forming a tether between phagocytes and apoptotic cells. Among its diverse cellular functions, our current review is confined to define MFG-E8 roles concerning inflammation, injury and other associated stress conditions. The phagocytosis property of cells fits into the vast field of innate immune system, while malfunctioning of this process may generate immune-mediated disorders. One major characteristic

phenomenon of apoptotic cells is to expose the PS from its inner leaflet membrane to the outer surface. This is termed as “eat me” signal which can allure distinct opsonins to recognize and bring them to the close vicinity of professional phagocytes [44]. MFG-E8 has a strong binding affinity to the exposed PS of apoptotic cells and facilitates phagocytic engulfment via $\alpha_v\beta_3/\alpha_v\beta_5$ -integrin receptor (Fig. 1c). This triggers a conformational change in the integrin receptor that signals the recruitment of the CrkII-DOCK180-Rac1 complex and thus the activation of Rac1 [45, 46]. The result is cytoskeletal reorganization in the macrophage and its transformation into a phagocyte capable of removing the apoptotic cell. In general, MFG-E8 deficiency attributes to SLE due to aberrant production of autoantibodies, and/or glomerulonephritis with abundant deposition of immune complexes at the glomeruli [3, 37]. Based on this fundamental concept, MFG-E8 is also known to accelerate the clearance of various kinds of apoptotic cells, unwanted debris, microvesicles, nuclei and other molecules from different organs.

In the brain tissues, apoptotic cells and potentially toxic materials induce neurodegenerative diseases and normal aging processes [47]. The efficient removal of apoptotic material is of utmost importance for protecting the surrounding tissue from damage due to released proteins from dying cells [48]. Recently, one hypothesis indicated a conserved mechanism for MFG-E8-mediated clearance of apoptotic neuronal cells by the microglial cells in the brain, thereby implicating possible role for this protein in protecting neurodegenerative diseases like AD [32]. Evidence for MFG-E8 roles in AD was also obtained from other studies where MFG-E8 function was established for clearance of excessively produced amyloid β peptide (ABP), which is considered as one of the hallmarks of developing AD [32, 33]. From their studies, a direct protein–protein interaction between recombinant MFG-E8 and A β 1-42 peptide was observed in vitro and MFG-E8 deficiency or its neutralization using specific antibodies significantly prevented ABP phagocytosis by murine and human macrophages leading to cause AD. Prion diseases or transmissible spongiform encephalopathies (TSEs) are a family of rare progressive neurodegenerative disorders that affect both humans and animals [49]. Similarly, MFG-E8-mediated clearance of cerebellar apoptotic bodies in vivo to protect mice from developing another neurodegenerative disorder, e.g., prion disease, has also been reported so far [18].

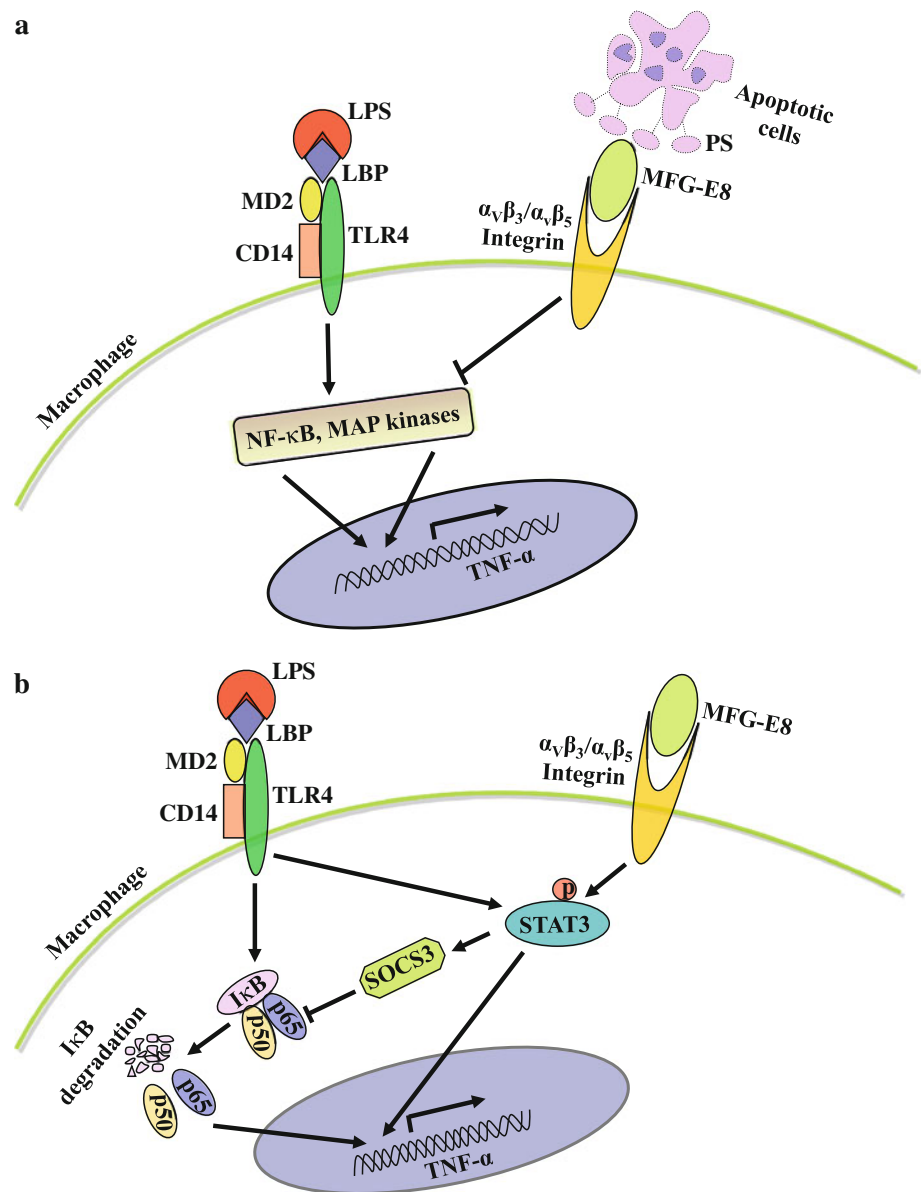
Other than the engulfment of the apoptotic bodies, MFG-E8 was also known to promote excessive collagen uptake by alveolar macrophages from lung tissues, thus diminishing the severity of pulmonary fibrosis [50]. In the lungs, fibrosis can occur due to abnormal remodeling after acute lung injury by the replacement of normal tissue

architecture with collagen-rich matrix, leading to the disruption of organ function. In normal tissues, collagen turnover occurs by two pathways, extracellular proteolytic cleavage and, endocytosis followed by lysosomal degradation. MFG-E8 contains two discoidin domains, homologous to those present in the collagen receptors DDR1 and DDR2, thus facilitated its binding to collagen and promoted endocytosis by alveolar macrophages [50]. Besides these organ/tissue type specific functions, MFG-E8 also plays a vital role in reticulocyte system where the nuclei that are expelled from the erythroid precursor cells are engulfed by the macrophages in the blood island. Fetal liver macrophages are generally efficient in engulfing these nuclei. Masking the PS on these nuclei with the dominant-negative form of MFG-E8 prevented this engulfment by macrophages [51]. Furthermore, MFG-E8 is also facilitated the removal of defective red blood cells (RBC) from the blood where the phagocytosis of sickle red blood cells by macrophages suggesting a potential role of MFG-E8 in sickle RBC clearance. In platelets, the PS is present only in the inner leaflet of the membrane bilayer. During platelet activation, PS moves from the inner to the outer leaflet of the membrane bilayer as PS rich microvesicles. These microvesicles are procoagulant and account for the clot-promoting activity of serum. MFG-E8 promotes the clearance of PS-expressing procoagulant platelet-derived microvesicles, and safeguards from hypercoagulable state occurred in cancer-associated deep vein thrombosis, anti-phospholipid antibody syndrome, disseminated intravascular coagulation, heparin-induced thrombocytopenia, and thrombotic thrombocytopenic purpura [52]. Collective evidences summarized the diverse functional abilities of MFG-E8 and further implicate possibilities to reveal its other unexplored roles.

Novel anti-inflammatory role of MFG-E8: modulation of TLR4 signaling during apoptotic cell engulfment

Phagocytosis of apoptotic cells promotes an immune tolerance state during LPS-triggered condition by decreasing the production of pro-inflammatory cytokines, tumor necrosis factor (TNF)- α and interleukin (IL)-12p40, and increasing the synthesis of anti-inflammatory cytokine IL-10. This phenomenon is mediated by PPAR- δ , which in turn can activate the expression of MFG-E8 and other potential opsonins to promote engulfment of apoptotic cells by phagocytes efficiently [41]. In a co-culturing system with apoptotic cells and macrophages, recombinant (r) MFG-E8 attenuated the LPS induced pro-inflammatory cytokine production by the macrophages [21]. The question then generally arises as to how does MFG-E8 facilitate this effect? To resolve this, Miksa et al. [21] proposed two

Fig. 2 Anti-inflammatory roles of MFG-E8: **a** During inflammation, LPS in combination with the lipopolysaccharide binding protein (LBP) can recognize the TLR4-CD14-MD2 receptor complex to activate the innate-immune response. The process of engulfment of apoptotic cells by the macrophages exerts an immune-tolerant state to the micro-environment. MFG-E8 can promote an anti-inflammatory status indirectly by its extraordinary ability to enhance the phagocytic potentials of apoptotic cells, which in turn ameliorates the inflammation by down-regulating NF- κ B and MAP kinases. **b** MFG-E8 can also generate the direct anti-inflammatory roles in macrophages even without the presence of apoptotic cells or regardless of its canonical functions of phagocytic engulfment of apoptotic cells. Utilizing the STAT3 pathway, MFG-E8 can directly generate its anti-inflammatory effects towards downregulating the LPS-induced TNF- α production in macrophages via activating SOCS3. MFG-E8-induced SOCS3 may in turn target the NF- κ B p65 component and act as a negative regulator of LPS-mediated TLR4 signaling via NF- κ B for TNF- α production



schemes: one way is by the enhancement of the phagocytic potential, while the other one is by the modulation of the LPS triggered intra-cellular signaling events. In this regard, they revealed that the rMFG-E8 treated macrophages co-cultured with apoptotic thymocytes greatly reduced the LPS stimulated phosphorylation and activation of intracellular MAP kinases, p38, ERK1/2, JNK as well as nuclear NF- κ B p65, thereby reducing pro-inflammatory cytokines (Fig. 2a). Riding on these mechanistic tools, exogenously treated rMFG-E8 attenuated inflammation by reducing pro-inflammatory cytokines, TNF- α , IL-6 and IL-1 β in sepsis as well as renal, hepatic and intestinal I/R conditions where LPS-TLR4 signaling is predominant [6, 53]. Moreover, the recovery from tissue/organ injury induced by those distresses was attained by rMFG-E8 via

reducing myeloperoxidase levels and preventing cellular apoptosis through caspase three inhibitions [29].

Direct anti-inflammatory role of MFG-E8 without apoptotic cell clearance: relevant mechanistic approaches targeting TLR4 and $\alpha_v\beta_3$ -integrin

Although in a number of inflammatory diseases where the phagocytosis of apoptotic cells was not impaired or the accumulation of apoptotic cells did not exacerbate inflammation, the treatment with rMFG-E8 to those diseases showed considerable improvement, hence pointing to the possibility of direct anti-inflammatory roles of MFG-E8. In colitic mice, treatment of rMFG-E8 attenuated intestinal

inflammation, which was not due to phagocytic engulfment of apoptotic cells but via directly modulating TLR4 signaling through its binding to $\alpha_v\beta_3$ -integrin [5]. In that report, the authors focused on LPS-TLR4 mediated inside-out and outside-in $\alpha_v\beta_3$ -integrin mechanisms, where rMFG-E8 played a crucial role to attenuate the outside-in signaling by competitive binding with the potent inducer osteopontin to the $\alpha_v\beta_3$ -integrin. Similar concept has also been adopted to implicate MFG-E8 roles in minimizing the deleterious effects of high-mobility group protein B1 (HMGB1) protein during inflammation. HMGB1 is considered to be a cytokine mediator of inflammation and tissue injury during arthritis, colitis, ischemia, sepsis, endotoxemia, and systemic lupus erythematosus. Studies revealed that elevated levels of HMGB1 that were secreted during inflammation impaired macrophage function of apoptotic cell clearance by binding to $\alpha_v\beta_3$ -integrin receptor and exacerbating inflammation and tissue damage. Since MFG-E8 shares the same integrin receptor with HMGB1, MFG-E8 competitively inhibits HMGB1 binding to the $\alpha_v\beta_3$ -integrin and restores phagocytic potential of macrophages and ameliorates inflammation and tissue injury induced by HMGB1 [54].

In an attempt to clarify the direct anti-inflammatory role of MFG-E8, we revealed a distinct signaling pathway where MFG-E8 activates suppressor of cytokine signaling (SOCS) 3 gene expression via STAT3 mediated pathway, which in turn served as a negative regulator for LPS induced TLR4 signaling by targeting NF- κ B p65 component, thereby attenuating the down-stream signaling for TNF- α production [55] (Fig. 2b). Elucidation of the above novel mechanistic findings strengthens the hypothesis of direct potential roles of MFG-E8 for attenuating inflammation in experimental disease models.

MFG-E8 accelerates tissue regeneration and neovascularization via distinct mechanisms: a direct functional strategy against injury and ischemia

As an evidence of direct functional approach other than its conventional role in phagocytosis, MFG-E8 is also involved in maintaining normal tissue and blood vessel integrity by binding to its receptors, $\alpha_v\beta_3$ -integrin and PS. Sepsis-triggered intestinal injury is associated with a downregulation of intestinal MFG-E8 and a delayed enterocyte migration along the crypt-villus axis. Treatment of rMFG-E8 in experimental septic mice accelerated mucosal healing via its binding to the transiently exposed PS of the injured intestinal epithelia. In this regard, MFG-E8 transduced its down-stream signaling for tissue regeneration and mucosal healing by means of activating intracellular protein kinase C (PKC) ϵ [4]. This novel property of

MFG-E8-mediated tissue regeneration can be further implemented in healing of lesions in several ulcerative diseases.

The term therapeutic angiogenesis is viewed as a highly promising strategy to ensure revascularization of ischemic tissues by promoting the growth of new vessels or the maturation of pre-existing ones. Identification of factors that may affect vessel growth is of major therapeutic importance. The crucial role of MFG-E8 in the proangiogenic effect exerted by vascular endothelial growth factor (VEGF) was evaluated in the clinically relevant pathological setting of ischemia, using the surgically induced ischemic hindlimb model in mice [56]. VEGF-induced blood vessel growth in physiological angiogenesis requires integrin-mediated signaling. The angiogenic protein MFG-E8 expressed in and around blood vessels exhibit a crucial role in VEGF-dependent neovascularization in the adult mouse. The molecular mechanism underlying this effect is mediated by the activation of serine-threonine kinase Akt. VEGF-induced Akt phosphorylation was abolished in MFG-E8-deficient animals, suggesting that MFG-E8 has a major role in the VEGF-Akt signaling pathway to promote neovascularization in stressed tissues/vessels [56]. This extraordinary function of MFG-E8 can be an efficient therapeutic tool for vascular repair in ischemic diseases.

Conclusion and perspective

In the context of inflammation, injury and homeostasis, our current discussion extracted the outstanding findings of MFG-E8 research that were done in the past decade, and focus on perspectives that may implicate its role towards resolving several unanswered ailments. Although those studies showed a direct or indirect beneficial anti-inflammatory role of rMFG-E8 in acute disease conditions, there are several points that require further clarification before its clinical use. Since many of the inflammatory disorders are chronic and relapsing, hence the role of MFG-E8 in chronic models should be addressed further. Additionally, the long-term effects of rMFG-E8 in regard to physiological, immunological, and clinical aspects should be evaluated in the future. Recently, Shah et al. [57] successfully evaluated the dose dependent efficacy of human rMFG-E8 protein as therapeutic potential in a rat model of sepsis, thus suggesting a future hope for its use in a broad spectrum of human studies. To date, the gateway of MFG-E8 mediated signal transduction have been reported to transmit via cell surface receptor $\alpha_v\beta_3$ -integrin and finally modulated intracellular MAP kinases and NF- κ B components which implicated the need of future studies to determine the influence of TLR negative regulators and association or dissociation of adaptor molecules into this process.

Moreover, focus should also be given on other lines of signaling which are also become activated during inflammation. In conclusion, MFG-E8 mediated therapeutic potential as assessed by the improvement of clinical, physiological and immunological parameters together with the elucidation of its mechanism of actions build it as an extraordinary molecule towards ameliorating inflammation and its related disorders.

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Conflict of interest One of the authors (P 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. Other authors report no financial conflicts of interest.

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