Ping Wang *Editor*

MFG-E8 and Inflammation

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Preface

Milk fat globule-epidermal growth factor-factor 8 (MFG-E8), also known as breast epithelial antigen (BA46) or lactadherin, is a cysteine rich secretory glycoprotein, cloned by Dr. Gordon Parry from the University of California, in 1990 as a mammary epithelial cell surface protein. This protein contains two epidermal growth factor (EGF)-like domains at the N-terminus and two discoidin domains similar to the sequence of the blood coagulation factor-V/VIII at C-terminal site. Soon after revealing its genomic sequences, Drs. Tsukasa Matsuda and Kenzi Oshima extensively studied MFG-E8's expression and its functional regulations at the mammary glands as well as in other tissues. Hence, in Chap. 1, Matsuda and his team presented immensely valuable information on the introductory overviews of MFG-E8. They have compared the sequence homology of MFG-E8 among nine different species and also provided exciting microarray data containing 69 human cell lines and thousands of various human carcinoma samples, which I believe to be tremendously helpful to understand about MFG-E8 biology. During this time, Dr. Jan Trige Rasmussen extensively worked on to reveal MFG-E8's ability to recognize phosphatidylserine (PS) in cell membranes which has been recognized as an excellent tool to determine apoptotic cells in a number of studies. In Chap. 2, Blans and Rasmussen discussed on the usefulness of MFG-E8/lactadherin in monitoring cell health in vitro and in vivo, for detecting cell-derived PS exposing microparticles, or for exploring mechanisms in apoptosis. Moreover, they also defined MFG-E8/lactadherin as a non-invasive marker in the clinic for imaging of apoptotic events which lined up a new dimension for establishing it as a potential imaging and diagnostic tool to aid physicians and pharmacologists. The intestinal epithelium provides a physical barrier against pathogens. In Chap. 3, Dr. Xiao-Di Tan and his colleagues outline our current understanding about the role of MFG-E8 in maintaining the homeostasis of intestinal epithelial cell lining and highlight potentials for applications of MFG-E8 in gastroenterological diseases. From the hematological point of view, in Chap. 4, Dr. Perumal Thiagarajan and his colleague described the role of MFG-E8 in blood, especially in platelets and red blood cell homeostasis and coagulation, which further implicates a new area on MFG-E8 to emphasize. Dr. Ziad Mallat is the first to elucidate the role of MFG-E8 in the vascular system. In Chap.

5, Mallat and colleagues describe how MFG-E8 promotes neovascularisation through identifying a novel pathway involving VEGF/Akt axis during ischemic, ocular, and inflammatory disorders to maintain vascular integrity required for the repair process of damaged tissues.

The enthusiasm on MFG-E8 research came into light when Drs. Shigekazu Nagata and Rikinari Hanayama discovered the novel function of MFG-E8 for efficient engulfment of apoptotic cells by the professional phagocytes which eventually rewrite the mechanism of the pathogenesis of autoimmune diseases, as well as other acute and chronic inflammatory diseases. In Chap. 6, Hanayama demonstrated the role of MFG-E8 in autoimmune diseases which undoubtedly improves our understanding on the development and prevention of autoimmune diseases. Cellular apoptosis is one of the serious consequences for the worst outcome in sepsis and its related acute systemic inflammatory diseases. If they are not cleared efficiently, they may undergo necrosis and exaggerate tissue injury and severe inflammation, leading to fetal complications. In early 2000s, our laboratory started to evaluate the effect of MFG-E8 in rodent models of sepsis and its related disorders. We later approached towards translational research on MFG-E8 which ultimately led us to propose it as an outstanding therapeutic candidate for the successful treatment of sepsis, organ ischemia-reperfusion, and hemorrhagic shock by removing excessive apoptotic cells from the affected organs, as well as limiting systemic inflammation by down-regulating the potent inflammatory cytokines/chemokines via nuclear factor-κB (NF-κB) modulation. My colleague Dr. Asha Jacob nicely reviewed our work in Chap. 7 to improve our perception for the development of MFG-E8 as a potent therapeutic potential against inflammatory diseases. The anti-inflammatory role of MFG-E8 in a murine model of inflammatory bowel disease (IBD) was first elucidated by Drs. Monowar Aziz and Shunji Ishihara. In Chap. 8, Ishihara and colleagues outline the noteworthy function of MFG-E8 in improving the chronic intestinal inflammation in murine colitis by modulating the hyperactive functions of pro-inflammatory cytokines, via inhibiting NF-κB activities. In Chap. 9, my colleague Dr. Monowar Aziz describes a novel study to delineate the role of MFG-E8 in protecting acute lung injury (ALI) and identified a previously unexplored mechanism involving GRK2/CXCR2 modulation by which MFG-E8 attenuates neutrophil chemotaxis and improves disease status. In order to reveal the roles of MFG-E8 in brain related diseases, my group as well as others have recently elucidated the novel protective function of MFG-E8 in mitigating the complications of cerebral ischemia, Alzheimer's disease and the prion like disease in terms of promoting the engulfment efficacy of the neuronal apoptotic cells and also protection from cellular damage by inducing the expression of anti-apoptotic genes, which are discussed at the end of the book in Chap. 10.

Our goal as the editor of this book is to compile evidences of the latest innovations on MFG-E8 relating to the sector of inflammatory diseases that would help us in proceeding basic as well as translational research towards novel drug discoveries. Moreover, this book will help researchers and clinicians by providing valuable bedside to bench resources to carry out functional studies on MFG-E8. In this edition, we tried to cover all the aspects of inflammatory diseases where MFG-E8 research

has undertaken, and since this is an expanding field we wished to update the ongoing studies on several other inflammatory diseases in a timely manner.

I would like to extend my sincere thanks and deep appreciation to all the distinguished contributors of this book for their time and effort in including latest findings and lucid discussions on MFG-E8 relating to the various inflammatory diseases that they have explored in their laboratories. I am greatly thankful to my colleague Dr. Monowar Aziz who did a splendid job on helping me to design the chapter concept, communicate to the contributing authors, as well as co-edit the book. Without Monowar's dedication, we will not be able to complete this project in a timely fashion. For what Monowar has been done for this book, I am very grateful. I am also thankful to the Springer publishing group for considering MFG-E8 as an interesting molecule to share its essences at the close vicinity to the readers' door. I would also like to thank my present and former laboratory members for their contribution to make MFG-E8 as a potential novel therapeutic compound against various inflammatory diseases (preclinical stages). Finally, I thank my wife Mian and daughters Stephanie and Christie for their strong support.

Manhasset, NY, USA Ping Wang, MD

About the Editor

Ping Wang, MD, is the Vice Chairman for Research at the Department of Surgery, and Professor of Surgery and Molecular Medicine at Hofstra North Shore-LIJ School of Medicine. He is also the Head of the Center for Translational Research at the Feinstein Institute for Medical Research. Previously, Dr. Wang has held positions of Associate Professor of Surgery at Brown University School of Medicine and Professor of Surgery, Pathology, Physiology and Biophysics at University of

Alabama at Birmingham (UAB), as well as Professor of Surgery at Albert Einstein College of Medicine. His laboratory has been continually funded by the NIH since 1995. Dr. Wang has published over 300 peer-reviewed articles in the field of inflammation research, and was the program chair for Shock Society Meeting, 2013.

Dr. Wang's laboratory has done extensive and pioneering work on milk fat globule-epidermal growth factor-factor 8 (MFG-E8). Among other findings, Dr. Wang's laboratory has shown that MFG-E8 is downregulated in sepsis and that treatment of sepsis with recombinant MFG-E8 provided important beneficial effects such as attenuating pro-inflammatory responses, increasing apoptotic cell clearance, and improving survival in sepsis. Dr. Wang's laboratory has also revealed that MFG-E8 treatment reduces acute lung injury caused by either endotoxin instillation or an intestinal ischemia and reperfusion injury model. His long-term goal is to develop better therapies to prevent the progression of systemic inflammatory processes, which include sepsis, acute lung injury, hemorrhagic shock, ischemia and reperfusion injury, radiation injury and stroke. In line with this long-term goal, studies are now being done at Dr. Wang's laboratory to establish the efficacy and optimal dosage of human recombinant MFG-E8 with the objective to develop this agent as a novel therapy for sepsis and other inflammatory diseases. In addition to his work on MFG-E8, Dr. Wang's research interests include other molecules with therapeutic potential in systemic inflammation. As an example, Dr. Wang and his colleagues have recently identified a novel endogenous pro-inflammatory molecule, cold inducible RNA binding protein (CIRP), which triggers systemic inflammation and exacerbates organ injuries. This noteworthy discovery was published in *Nature Medicine* in 2013.

Contents

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Chapter 1
MFG-E8: Origin, Structure, Expression, Functions and Regulation

Kenzi Oshima, Takehiko Yasueda, Shunsuke Nishio, and Tsukasa Matsuda

Abstract Milk Fat Globule-Epidermal Growth Factor-Factor 8 (MFG-E8) with bivalent binding activity to integrin receptors and acidic phospholipids is a secretary glycoprotein exhibiting versatile functions in cell physiology affecting health and diseases. Recent progress in genomics and structural biology studies, in addition to long time accumulation of classical biochemical studies, has been showing its unique molecular structure advantageous to the link between cells and their target cells as well as molecular complexes and its critical roles in regulating inflammation and immunity. MFG-E8 is expressed and secreted by a variety of cells and tissues, especially professional and non-professional phagocytes such as macrophages, immature dendritic cells and the epithelial cells of mammary glands and epididymis. Although MFG-E8 expression has been shown to be up-regulated by a lactogenic hormone, prolactin (PRL), and neuronal chemokine, fractalkine, molecular mechanisms involved in MFG-E8 expression in health and disease states remain largely unknown. The secreted MFG-E8 specifically recognizes the acidic phospholipids and opsonized the targets to be cleared by phagocytosis mainly in paracrine and occasionally in endocrine manners. In addition to the enhancing role in phagocytosis, MFG-E8 binds to its target cells expressing MFG-E8 receptors and acts as a ligand, which modulates inflammatory responses. Moreover, MFG-E8 alone shows ligand activity to intestinal epithelial cells locally exposing the acidic phospholipids and vascular endothelial cells expressing the integrins.

Keywords Acidic phospholipid • Phosphatidylserine • BA46 • Lactadherin • Mammary gland • Mammary epithelial cell • Del-1 • Edil 3 • RGD motif • Three dimensional (3D) structure • Medin • Amyloid • Alzheimer's disease • Zona pellucida • Pulmonary fibrosis • Keratinocyte • Retina pigment epithelium • Expression

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Atlas • Transcription profiling • CX(3)CL1 • LPS-CD14 pathway • p63 • PPARs • Immature dendritic cells • Alpha(v)beta(3) integrin • Alpha(v)beta(5) integrin • Exosome • Systemic lupus erythematosus (SLE) • Autoimmune disease • Autoantibody • Polyunsaturated fatty acid • Unsaturated aldehyde • Lipid peroxidation • MAPKs • CD14 • TLR4 • SOCS3 • GM-CSF • MHC class II • Angiogenesis • ERK • VEGF • Wound-healing • Sepsis • Epididymis • Mastitis

1 Origin, Primary Structure and Molecular Evolution

MFG-E8 with bivalent binding activity to integrin receptors and acidic phospholipids is a secretary glycoprotein exhibiting versatile functions in cell physiology, e.g., recognition of target cells and membrane vesicles by phagocytes [1, 2], development of male reproductive organs and cells [3, 4], cell reorganization in mammary gland development and involution [5, 6], and regulation of inflammatory responses such as macrophage activation [7, 8] and neutrophil infiltration [9]. The name, milk fat globule-epidermal growth factor-factor 8 (MFG-E8), was first given to a protein of mammary epithelial cells, when a cDNA clone cording for this mammary epithelial cell protein was obtained and its increased expression in lactating mammary glands was observed [10]. It was named after its origin and structural properties, i.e., its origin in milk fat globule and its sequence homology to epidermal growth factor-like (EGF-like) domains of *Drosophila* Notch protein and C-terminal domains of human coagulation factors VIII and V (F5/8-type C domain). At nearly the same time as the mouse protein identification, human orthologue protein, named BA46 (afterward lactadherin), was identified by cDNA cloning as a human breast tumor antigen with molecular mass of 46 kDa [11, 12].

Before the naming as MFG-E8 or BA46 for mouse and human proteins, this protein had been studied on several mammalian species, mainly domestic ruminant animals, for a long time as a membrane glycoprotein of milk fat globule membrane. This biological membrane is surrounding lipid droplets, named milk fat globules, which are specifically synthesized in the cytosol of mammary gland epithelial cells and secreted by a budding-off mechanism into lumen of lactating mammary glands. Therefore, MFG-E8 of milk fat globule membrane had been thought to originate from plasma membrane of mammary epithelial cells [13]. However, some recent studies suggest that MFG-E8 is present in whey (milk serum) fraction and at least in part binds subsequently to the membrane of milk fat globules in breast milk stored within mammary gland lumen [14–16]. The cDNA cloning of mouse MFG-E8 and human BA46 was followed by that of the other animal species in not only mammals, including bovine [17, 18], porcine [19], rat [20], but also non-mammalian species such as bird (NCBI: CD218834).

On the other hand, around the same time, a mouse embryonic endothelial protein structurally similar to but distinct from MFG-E8 was identified and named Del-1 (a protein coded in a developmentally regulated endothelial cell locus, also known as Edil3, EGF-like repeats and discoidin I-like domain 3) [21]. Del-1 was originally

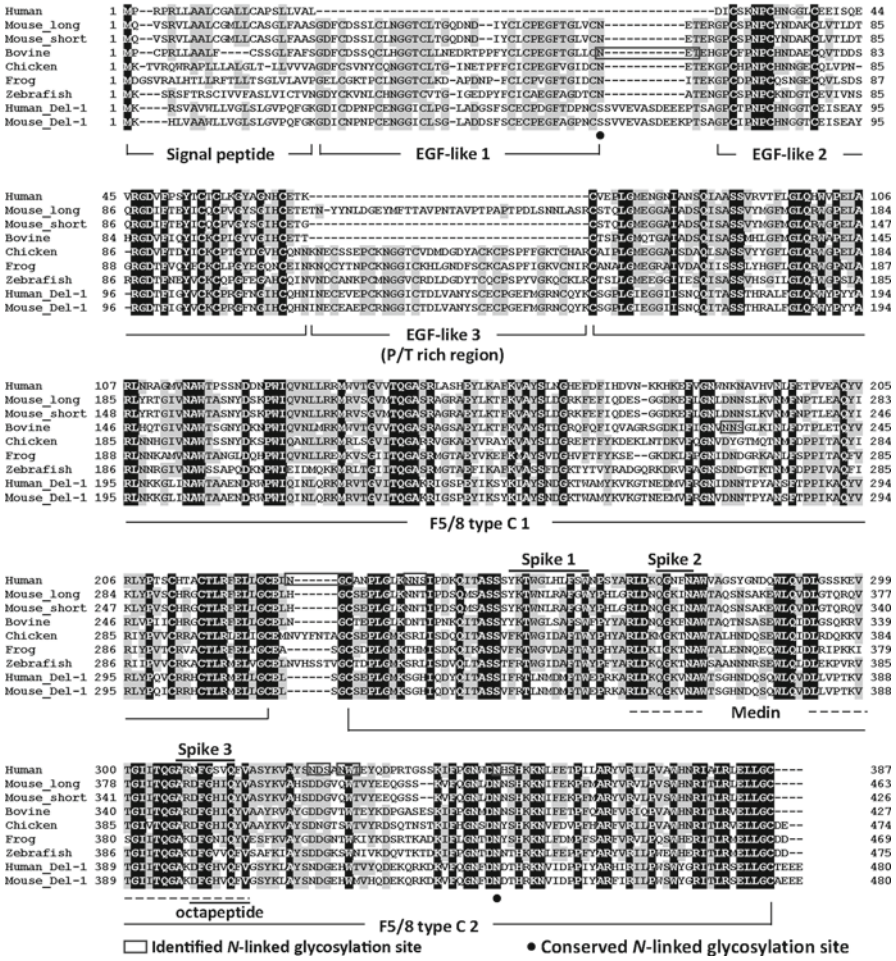

Fig. 1.1 Sequence alignment of vertebrate MFG-E8 and its paralogue Del-1 Amino acid residues conserved among the nine sequences and the five or more are highlighted with *black* and *grey* backgrounds, respectively. MFG-E8 sequences are of human (Human, NP_005919), mouse (Mouse_long, NP_032620; Mouse_short, NP_001038954), bovine (Bovine, NP_788783), chicken (Chicken, NP_001264039), Xenopus laevis (Frog, NP_001089989), and fish (Zebrafish, NP_001014321). Del-1 sequences are of human (NP_005702) and mouse (NP_001033076)

characterized to be a matrix protein and relate to endothelial cell adhesion, and later reported to show molecular functions similar to MFG-E8 [22]. As the great expansion of genomic sequences for a variety of animal species, it has become evident that the two genes coding MFG-E8 and Del-1 are present in most of vertebrates' genome [23].

An alignment for seven MFG-E8 and two Del-1 amino acid sequences, including putative ones, of representatives in mammals, birds, amphibians and fish are shown in Fig. 1.1. Overall sequence homology is high, and cysteine residues are

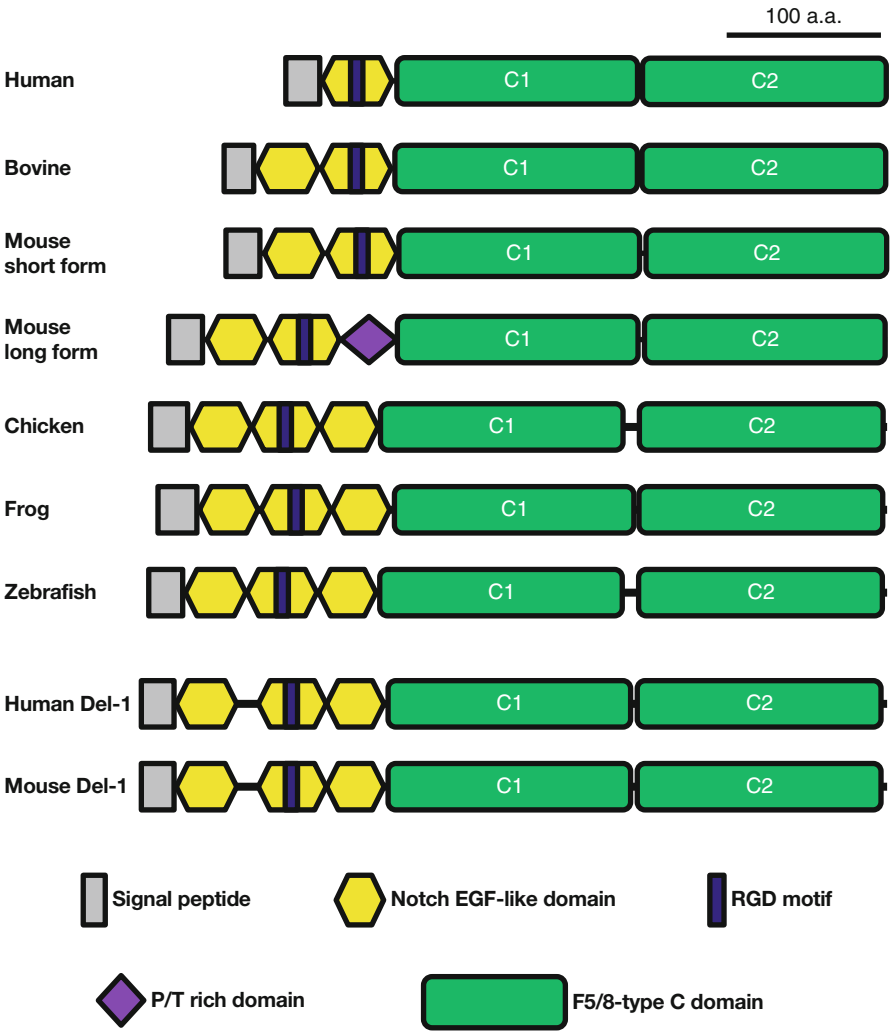

Fig. 1.2 Domain structure of vertebrate MFG-E8 and Del-1

well conserved among the nine sequences. The number in the repeat of N-terminal Notch EGF-like domains of MFG-E8 differs among species, whereas the C-terminal tandem F5/8-type C domains (F5/8-C1 and -C2 domains) are common. As schematically shown in Fig. 1.2, three EGF-like domains are present in the MFG-E8 sequences of bird, amphibian and fish, while two are in those of mammalian species other than humans, in consequence of deletion of the third EGF-like domain. In mouse and rat, an isoform of MFG-E8 has been found with a proline and threonine (P/T)-rich mucin-like domain replaced with the third

EGF-like domain [1, 24]. This P/T-rich domain is almost the same in residue number as the third EGF-like domain but completely differ in the sequence, e.g., no cysteine residues in addition to the rich proline and threonine residues. Human MFG-E8 has further lost the first EGF-like domain and results in remaining of the second EGF-like domain only, in which a functionally important RGD-motif is conserved among the species [11, 12]. An evolutional analysis of MFG-E8 gene from 11 mammalian species indicated that the loss of the first EGF-like domain and amino acid replacements in the second EGF-like domain occurred in ancestral primates, suggesting that MFG-8 underwent accelerated evolution in ancestral primates possibly by positive selection and MFG-E8 function might change during primate evolution [25].

2 Glycosylation

Amino acid sequence of MFG-E8 indicates that MFG-E8 is a typical secretory protein, which has potential to be glycosylated in Golgi, with a signal sequence and without any hydrophobic putative transmembrane regions. As shown in Figs. 1.1 and 1.3, there are several potential N-glycosylation sites (NXT/S motifs) in the MFG-E8 sequences. In fact, two NXT/S sites with N-glycosylation have chemically been identified in bovine MFG-E8 [18, 26]. One is located at the C-terminal end of the first EGF-like domain and the N-glycosylation motif at this site (C-NXT-) is conserved by MFG-E8, but not Del-1, from fish to mammals except humans, which has lost the first EGF-like domain. In the Del-1 sequences, this site is not conserved but, instead, a motif NCS is present in the two-residue away from the site of MFG-E8. Five N-glycosylation sites were identified in human MFG-E8 by MS analysis of its glycopeptides [27]. One is located nearly at the C-terminal end of the F5/8-C2 domain and the N-glycosylation motif at this site (NXS/T) is conserved by MFG-E8 from fish to mammals, and human and mouse Del-1 as well. Interestingly, an unusual N-glycosylation at an NGC site is present between F5/8-C1 and C2 domains of human MFG-E8 [27].

Two O-glycosylation sites have been identified in bovine MFG-E8 [18]. One site in the first EGF-like domain, Thr17, is conserved among vertebrate species except humans. The long isoform with a P/T-rich mucin-like domain found in mouse MFG-E8 has some O-glycans as with other mucin and mucin domains [24].

A deletion mutant of MFG-E8 found in a few patients with systemic lupus erythematosus (SLE) autoimmune disease is aberrantly glycosylated and sialylated [28]. The 82 residues at the C-terminus are deleted in this human MFG-E8 mutant, which may cause defective folding of the F5/8-C2 domain and abnormal trafficking in subcellular secretion pathway, resulting in the aberrant glycosylation. The aberrantly glycosylated MFG-E8 mutant is resistant to blood clearance, probably due to the differential glycaosylation, when it is injected intravenously into mice [28].

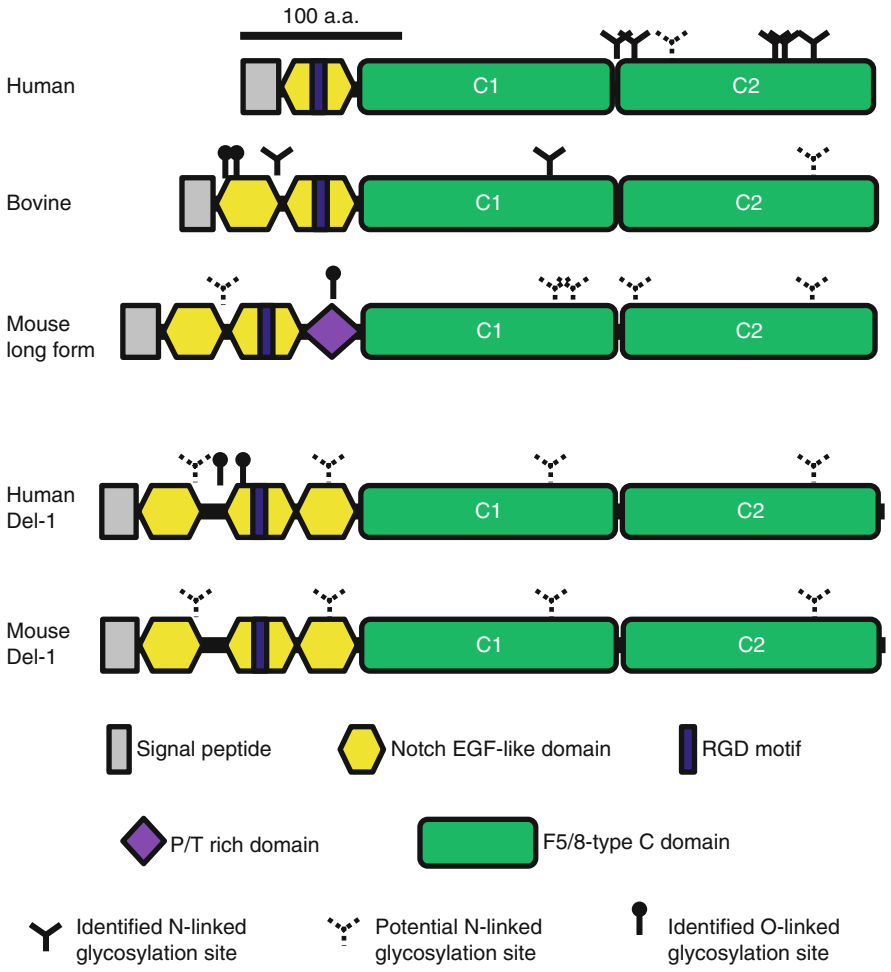

Fig. 1.3 Potential and identified glycosylation sites of MFG-E8 and Del-1

3 Domain Structure and Molecular Functions

Biological roles of MFG-E8 have long remained unknown in mammary gland and some other tissues and cells. One breakthrough study was reported in 2002 on the critical role of MFG-E8 in linking apoptotic cells to phagocytes [1]. MFG-E8 secreted by activated macrophages recognizes phosphatidylserine exposed on apoptotic cells and, on another front, the MFG-E8 in association with the apoptotic cells binds to phagocytes expressing integrin, resulting in MFG-E8-mediated linkage of apoptotic cells, but not healthy cells, to phagocytes and eventual clearance of apoptotic cells by phagocytosis. Before this important finding on MFG-E8 physiological

function and its mechanism of action, many data have been accumulated on molecular functions such as biophysical interaction of the MFG-E8 molecule and its domains with cellular receptors as described below.

3.1 The EGF-Like Domain with RGD Motif

MFG-E8 consists of two functional domains, i.e., the N-terminal Notch EGF-like repeat domain with ligand activity to a cell surface receptor, integrin, and the C-terminal tandem F5/8-type C domain with anionic phospholipid binding [10, 29–31]. Del-1, a probable paralogue protein of MFG-E8, has sequence and domain structure similar to MFG-E8, also shows the same biochemical functions of the divalent binding activity to cell membrane molecules [21, 22]. The RGD motif in the second EGF-like domain is conserved between MFG-E8 and Del-1, both of which show binding to cells expressing alpha(v)beta(3) and alpha(v)beta(5) integrins.

The binding to cell surface integrin was first demonstrated using human MFG-E8 (lactadherin) purified by Triton X-114 phase partitioning [30]. The purified human milk MFG-E8 enhanced cell attachment of several cell lines from human, green monkey and mouse, and a monoclonal antibody to alpha(v)beta(3) integrin inhibited the attachment of the monkey kidney cell (MA104) to MFG-E8 coated on cell culture plates [30]. The alpha(v)beta(5) integrin was affinity-purified from detergent extract of bovine mammary tissue using solid-phased bovine MFG-E8 and a synthetic RGD peptide [29]. The RGD-dependent binding of MFG-E8 to alpha(v)beta(3) and alpha(v)beta(5) integrins was confirmed using recombinant bovine MFG-E8 and its RGE-mutant, the later of which showed no binding to the integrin [31].

Based on the location of the conserved RGD motif between putative two anti-parallel beta-strands in the MFG-E8 sequence, the second EGF-like domain was speculated to be a scaffold for RGD presentation to cell surface integrin receptors [12]. Recently, the crystal structure of the EGF-like repeat domain in human Del-1, a possible MFG-E8 paralogue, has been solved and demonstrates that the RGD motif of the second EGF-like domain forms beta-turn structure at the tip of a long protruding loop (named “the RGD finger”) [32] (Fig. 1.4a). High sequence homology on the second EGF-like domain between MFG-E8 and Del-1 (about 50 % identical including six cysteine residues and 65 % conserved residues in human as well as mouse) (Fig. 1.4b) suggests these EGF-like domains of two paralogue proteins fold into a similar structure. Using a protein-modeling program from sequence with induced fit, MOE (Molecular operating environment, Chemical Computing Group Inc.), three-dimensional (3D) structure of human MFG-E8 EGF-like domain can be predicted as shown in Fig. 1.4c. The predicted 3D structure suggests that the EGF-like domain of human MFG-E8 can form a similar structure including the RGD finger to interact with cellular integrin receptors. At the same time, although the second EGF-like domain is well conserved, evolutional deletion of the first and

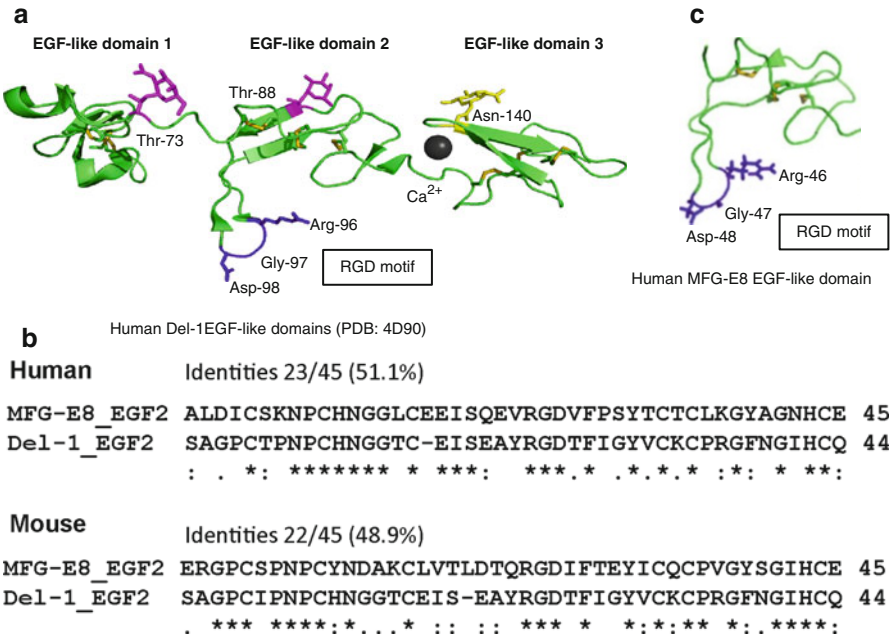

Fig. 1.4 Notch EGF-like domains of human Del-1 and MFG-E8. (**a**) Crystal structure of human Del-1 EGF-like domains (1 ~ 3). Thr73 and The88 are O-glycosylated with GalNAc and Fuc, respectively. Asn140 is N-glycosylated with GlcNAc. Whole structure of glycans are unknown. (**b**) Sequence alignment of Del-1 and MFG-E8 second EGF-like domains from human and mouse. (**c**) A predicted model of human MFG-E8 EGF-like domain

third EGF-like domains from non-mammalian MFG-E8 may indicate some physiological roles in human MFG-E8 distinct from Del-1. MFG-E8 and Del-1 are expressed respectively in different sets of macrophages among primary macrophages and macrophage cell lines [22], suggesting that macrophage subsets use MFG-E8 or Del-1 differently to select target cells and materials to be phagocytosed and recognize them marked with MFG-E8 or Del-1 as integrin ligands.

3.2 The F5/8-type C Domains

Binding of MFG-E8 to phospholipids had been speculated based on the finding that MFG-E8 has domains with homology to phosphatidylserine-binding domains of blood coagulation factor VIII and factor V [10, 11]. The phosphatidylserine-binding activity of MFG-E8 was first shown in a biochemical experiment using several kinds of phospholipids coated on ELISA plates [29]. Purified bovine MFG-E8 (formally PAS-6/7) bound to the wells coated with phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol, and their precursor, phosphatidic acid, but not phosphatidylcholine, indicating specific binding of MFG-E8 to acidic

phospholipids but not neutral ones. The recombinant F5/8-C2 domain of bovine MFG-E8 binds to plate-coated phosphatidylserine with an affinity similar to native MFG-E8 (Kd = 1.8 nM), and MFG-E8 enhanced attachment of alpha(v)beta(5) integrin-expressing MCF-7 cells to the plate coated with phosphatidylserine [31]. These results suggest the binding of MFG-E8 to the artificial phospholipid membrane through the F5/8-C2 domain independent of the binding to the cell surface integrin through the second EGF-like domain having an RGD motif.

Mouse MFG-E8, but not its F5/8-C1 or F5/8-C2 domain deletion mutants, expressed in COS-7 transfectants is detectable on cell surface using immunofluorescence microscopy or a cell surface biotin-labeling method, and also distributed in the membrane vesicle fraction of the cell culture supernatant, suggesting that both of the C1 and C2 domains are required for the binding of MFG-E8 to biological cell membranes of phospholipid bilayer [33]. Bovine MFG-E8 binds in a saturable manner with phosphatidylserine-containing unilamellar membrane supported by glass microspheres with an apparent Kd of about 3 nM in a Ca^{2+} independent manner, and MFG-E8 can bind more in number to small membrane vesicles (microspheres) than to large ones [34]. As for natural membrane vesicles, MFG-E8 promotes the phagocytotic clearance by macrophage of platelet-derived microvesicles [35], which are pro-coagulant phosphatidylserine-rich microvesicles released from activated platelet. From a practical view point, because of its specific binding to the membrane containing a small amount of phosphatidylserine, MFG-E8 is expected to be a useful marker for detection of slight exposure of phosphatidylserine to cell surface reflecting cell stress in addition to apoptosis [36–38].

Crystal structure of the bovine MFG-E8 F5/8-C2 domain has been determined, showing that the F5/8-C2 domain has a beta-barrel core with three loops, designated spikes 1, 2 and 3, at the end of the beta-barrel [39] (Fig. 1.5a). The spikes 1 and 3 protrude from the beta-barrel core and display water-exposed hydrophobic residues, which were shown to be critical for the phospholipid binding based on mutagenesis studies. Moreover, 3D structure of mouse MFG-E8 F5/8-C2 domain in solution has recently been determined by nuclear magnetic resonance (NMR) spectroscopy [40] (Fig. 1.5b), indicating similarity of the previously determined crystal structure of bovine MFG-E8 C2-domain to the structure of mouse MFG-E8 C2-domain in solution. The binding between the F5/8-C2 domain and phosphatidylserine was characterized by ^{31}P-NMR, verifying that positively charged and aromatic amino acid residues clustered in the spikes 1 and 3 plays key roles in the binding, and presumably in the recognition of apoptotic cells exposing phosphatidylserine [40]. Using the MOE program, 3D structure of human MFG-E8 F5/8-C1 and -C2 domains can also be predicted, and is shown together with the predicted EGF-like domain in Fig. 1.6a. The protruding and phosphatidylserine-interacting spikes 1 and 3 of the F5/8-C2 domain are sterically placed on the flip side of the EGF-like domain with the RGD finger. As shown in Fig. 1.6b, the amino acid sequences of the F5/8-C1 and -C2 domains, which had presumably been produced by gene duplication, are similar to each other, and the positively charged, hydrophobic and non-polar amino acid residues are also present at the corresponding position in the F5/8-C1 domain sequence. These conserved basic and hydrophobic residues are also located at the

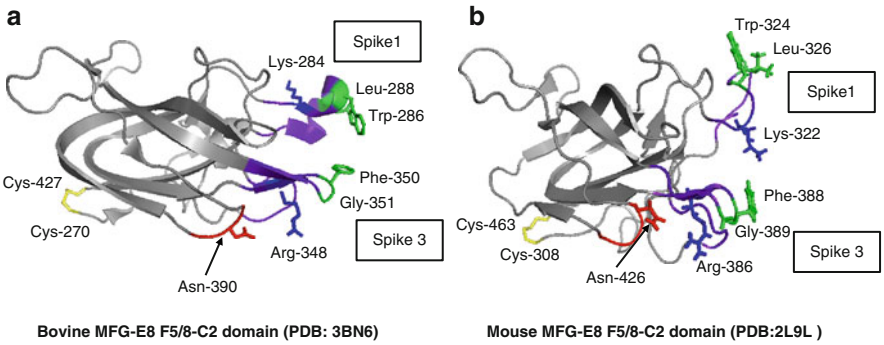

Fig. 1.5 3D structure of the bovine and mouse MFG-E8 F5/8-C2 domains. (**a**) The crystal structure of bovine F5/8-C2 domain obtained by X-ray crystallography. (**b**) The 3D structure of mouse F5/8-C2 domain in solution obtained by an NMR study. Three protruding hydrophobic region (*spikes*) are *purple-colored* with the side chains of conserved positively charged (*blue*) and hydrophobic (*green*) residues in spikes 1 and 3. The Asn residue in the conserved N-glycosylation site is *red-colored*. The *yellow stick* shows the intradomain di-sulfide bridge between the two Cys residues at N- and C-termini of the domain, respectively

loops protruding from the beta-barrel core of the F5/8-C1 domain, suggesting the possible functions of this region as the second site interacting with membrane phospholipids or some other molecules.

3.3 Medin Peptide and Amyloid

A peptide consisting of 50-amino acid residues corresponding to the central part of the human MFG-E8 F5/8-C2 domain (see Fig. 1.1) was identified as a main constituent of age-associated arterial amyloid, and named medin after aortic medial amyloid [41]. Not only the medin peptide but also intact MFG-E8 was detected in the proteins extracted from amyloid-rich aortic media of aged individuals [42]. An octapeptide (NFGSVQFV) corresponding to the C-terminus of human medin forms amyloid-like fibrils *in vitro* [41]. The sequence, NFGSVQFV, is mapped just to the protruding loop (spike 3) including the exposed and phosphatidylserine-interacting hydrophobic and non-polar residues (Phe^{310} and Gly^{311}) in the 3D model of human MFG-E8 F5/8-C domains (see Figs. 1.1 and 1.6a). This coincidental match of MFG-E8-derived amyloid peptide with the phosphatidylserine-interacting loop may indicate that the MFG-E8 C2-domain, and possibly the C1 domain as well, interacts through this water-exposed hydrophobic loop with not only phospholipid membrane but also some proteins on cell surface or extracellular matrix. In fact, medin amyloid co-localizes with elastic fibers of arteries, and both medin and MFG-E8 bind to tropoelastin (pro-elastin) *in vitro* [43]. Moreover, the formation of amyloid-like fibrils by MFG-E8, medin and the medin-derived octapeptide may be due to their self-association through this hydrophobic loop common to these protein and peptides.

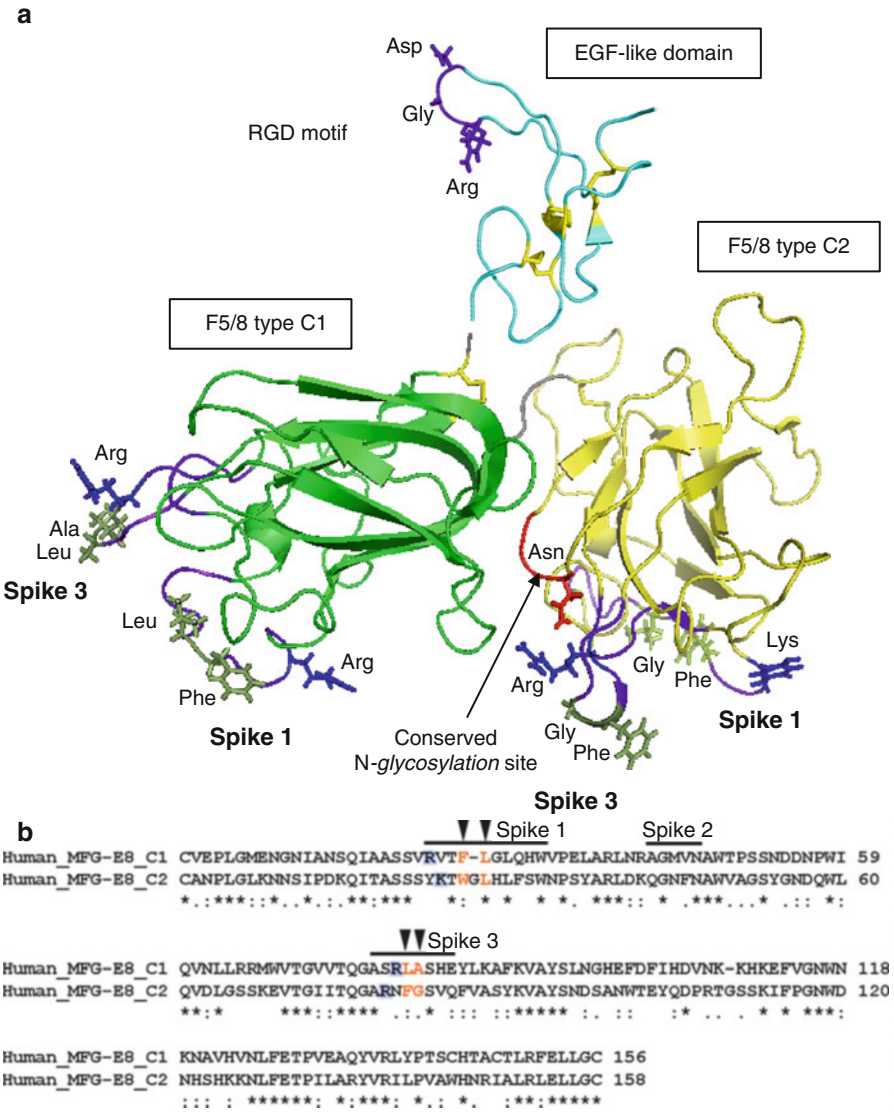

Fig. 1.6 A 3D model of human MFG-E8 predicted using MOE based on known 3D structures including bovine MFG-E8 and human Del-1. (**a**) The 3D models of EGF-like and F5/8-C1-C2 domains are predicted independently and placed arbitrarily. Exposed hydrophobic and positively charged residues in the spike regions are highlighted in addition to the RGD motif. (**b**) Sequence comparison between human MFG-E8 C1 and C2 domains. In the spikes 1 and 3, conserved hydrophobic residues are highlighted with arrowheads and basic residues are highlighted as well

In contrast to the involvement of MFG-E8 in amyloid formation, interaction of MFG-E8 with amyloid beta-peptide has been reported [44]. MFG-E8 is detected in the brains of individuals with and without Alzheimer's disease and, interestingly,

strong MFG-E8 signals are detected in area without amyloid beta-peptide but not in senile plaque-rich area. The weak MFG-E8 signals in the senile plaque-rich area, where the amyloid peptide is accumulated, may indicate that low expression of MFG-E8 causes defective clearance of the amyloid, resulting in the formation of senile plaque. In fact, a direct protein-protein interaction between MFG-E8 and the amyloid peptide was demonstrated using surface plasmon resonance, and a significant decrease to 65 % in MFG-E8 mRNA expression in the brains of patients with Alzheimer's disease was observed in comparison with age-matched control [44].

The consecutive phosphatidylserine-interacting residues, Phe-Gly, in the spike 3 of MFG-E8 are well conserved among all vertebrate species, and also in the sequences of mouse and human Del-1 (see Fig. 1.1). The evolutionally conserved amino acid sequence and 3D structure of the F5/8-C2 domain of MFG-E8 and Del-1, in contrast to accelerated evolutional changes in the EGF-like domains, suggest that the F5/8-C2 domain of both MFG-E8 and Del-1 play critical and common roles in interaction with phospholipid membrane and/or some other molecules.

3.4 Binding with Extracellular Matrix

It is the egg-specific extracellular matrix known as *zona pellucida* that was first found to be a binding target of MFG-E8 other than phospholipid cell membrane [19]. A bore 47-kDa protein (p47) peripherally associated with sperm plasma membrane was identified to be MFG-E8 by the isolation using an affinity column with immobilized *zona pellucida* glycoproteins. Later, MFG-E8 was identified as a sperm protein required for mouse sperm binding to *zona pellucida* surrounding oocyte and egg [4]. It was shown that MFG-E8 (also termed SED1, a secreted EGF repeat discoidin-domain protein) was expressed in spermatogenic cells, secreted also by the caput epididymis and eventually localized on the sperm plasma membrane. Mouse MFG-E8 binds specifically to the unfertilized oocytes, but not to that of fertilized eggs. Recombinant MFG-E8 and its F5/8-C domain fragment inhibit the sperm-egg binding. The sperm from *Mfge8*$^{-/-}$ mice do not bind to the *zona pellucida in vitro*, and the *Mfge8*$^{-/-}$ male mice are subfertile. Domains and regions of MFG-E8 responsible for the binding to *zona pellucida* remain uncertain.

Another extracellular matrix interacting with MFG-E8 is excess fibrous connective tissue mainly consisting of type-I collagen secreted from fibroblast in pulmonary fibrosis. MFG-E8 binds to the collagen through its F5/8-C domain and enhances uptake of collagen by macrophages, leading to the removal of accumulated collagen and the suppression of tissue fibrosis [45]. In a mouse model for bleomycin-induced lung injury, *Mfge8*$^{-/-}$ mice had enhanced symptoms of pulmonary fibrosis and defect in collagen turnover. MFG-E8 directly binds to collagen, and macrophages from the *Mfge8*$^{-/-}$ mice show defect in collagen phagocytosis. The defective collagen phagocytosis by the *Mfge8*$^{-/-}$ macrophages can be rescued by recombinant MFG-E8 and even by deletion mutants with a single F5/8 C-domain, suggesting the MFG-E8 binding to collagen through its F5/8-C domain.

3.5 P/T-Rich Domain of Murine MFG-E8

A long form splice variant of mouse [1, 24, 46] and rat [47] MFG-E8 contains a P/T-rich mucin-like domain, instead of the third EGF-like domain of MFG-E8 from non-mammalian species (see Fig. 1.1). Addition of the P/T-rich domain presumably results from in-flame inclusion of an extra exon by alternative splicing, and this domain is O-glycosylated at some threonine residues [24]. The polypeptide length of this domain is almost the same with the third EGF-like domain of MFG-E8 in birds, flog and fish. Although biological meanings of these MFG-E8 splice variants found in murine remain unknown [24, 47], insertion of the P/T-rich domain between the EGF-like and F5/8-C1 domains of mouse MFG-E8 increased the binding activity of MFG-E8 to plate-coated phosphatidylserine [1, 33] and enhanced uptake of apoptotic cells bound with MFG-E8 by macrophages [1]. The mouse MFG-E8 variant with the P/T-rich domain is expressed abundantly in mammary epithelial cells of lactating mice [24]. The MFG-E8 splice variants, especially the P/T-rich domain-bearing form, are expressed also in undifferentiated keratinocytes during tissue development [46] and in the retina and pigment epithelium [47]. Cultured mouse keratinocyte stem cells consistently express both MFG-E8 variants and the secreted MFG-E8 proteins with and without the P/T-rich domain are detected respectively in the culture supernatant as a soluble form and the cell fraction as a cell-associating form [46]. These differential expression and localization of the murine MFG-E8 two variants may indicate some roles of the O-glycosylated P/T-rich domain in modification of MFG-E8 function and/or extracellular distribution.

4 Gene Expression

4.1 Expression Profiling by Genomic Studies

After the initial identification of MFG-E8 protein as a component of milk fat globule membrane proteins and the subsequent cDNA cloning from mouse [10] and human [11] mammary glands at lactation stages, ubiquitous but regulated expression of *Mfge8* gene has been shown in various tissues, organs and cells from humans, mice and other vertebrate species [48]. In a gene expression database, Expression Atlas, by EMBL-EBI, detailed expression profiles of human *Mfge8* is available (http://www-test.ebi.ac.uk/gxa/gene/ENSG00000140545?ef=organism_part). Expression of *Mfge8* has been examined in transcription profiling by array in 271 experiments, where 78 organism parts, 68 diseases, 69 cell types and 282 cell lines are analyzed, and 71 compound treatments and 36 other conditions are tested. Some excerpts from the *Mfge8* expression profiles in Expression Atlas are shown in Tables 1.1, 1.2, and 1.3. Cell- and tissue-specific expression of *Mfge8* under normal and pathological conditions was reviewed recently in detail [48].

Table 1.1 Transcription profiling by array of human post mortem tissue samples

Organism part	Up/down	T-statistic	P-value
Coronary artery	Up	15	$<1\times10^{-10}$
Saphenous vein	Up	11	$<1\times10^{-10}$
Mammary gland	Up	9.5	$<1\times10^{-10}$
Urethra	Up	8.9	$<1\times10^{-10}$
Myometrium	Up	7.7	$<1\times10^{-10}$
Nipple cross-section	Up	6.8	1.8×10^{-9}
Heart ventricle	Up	6.3	2.19×10^{-8}
Ovary	Up	6.1	6.48×10^{-8}
Cervix	Up	6.1	6.73×10^{-8}
Dorsal root ganglion	Up	4.9	2.36×10^{-5}
Vagina	Up	4.9	$2\ 6\times10^{-5}$
Heart atrium	Up	4.2	3.28×10^{-4}
Adipose tissue omental	Up	3.8	0.001
Endometrium	Up	3.4	0.005
Adrenal gland cortex	Up	3.3	0.006
Adipose tissue subcutaneous	Up	3.2	0.009
Bronchus	Up	2.9	0.02
Hippocampus	Down	−7.1	4.02×10^{-10}
Cerebellum	Down	−6.7	2.76×10^{-9}
Vestibular nuclei superior	Down	−6.6	6.79×10^{-9}
Kidney cortex	Down	−6.5	1×10^{-8}
Bone marrow	Down	−6.2	4.56×10^{-8}
Liver	Down	−6.1	8.32×10^{-8}
Thalamus	Down	−5.9	2.66×10^{-7}
Corpus callosum	Down	−5.7	4.7×10^{-7}
Spleen	Down	−5.5	1.79×10^{-6}
Temporal lobe	Down	−5.4	2.06×10^{-6}
Ventral tegmental area	Down	−5.4	$2\ 7\times10^{-6}$
Hypothalamus	Down	−5	1.33×10^{-5}
Pituitary gland	Down	−4.9	2.53×10^{-5}
Cerebral cortex	Down	−4.8	3.11×10^{-5}
Substantia nigra	Down	−4.8	3.13×10^{-5}
Midbrain	Down	−4.8	3.71×10^{-5}
Kidney medulla	Down	−4.7	6×10^{-5}
Salivary gland	Down	−4.7	6.03×10^{-5}
Occipital lobe	Down	−4.6	7.75×10^{-5}
Spinal cord	Down	−4.4	1.46×10^{-4}

Roth et al. [98]
In a gene expression database, Expression Atlas, by EMBL-EBI, detailed expression profiles of human mfg-e8 is available, (http://www-test.ebi.ac.uk/gxa/gene/ENSG00000140545?ef=organism_part)

Transcription profiling by array of human post mortem tissue samples shows that *Mfge8* gene transcription is up-regulated relatively in blood vessels (both artery and vein), female reproductive organs (mammary gland, ovary, uterus and vagina), heart and adipose tissue, and down-regulated in brain (hippocampus, cerebellum, thalamus, hypothalamus, pituitary gland, etc.), spinal cord, kidney, bone marrow, liver and spleen (Table 1.1).

Table 1.2 Transcription profiling by array of humna 69 cell types

Cell type	U/D	Experiments
Fibroblast	D 1 U 5 U/D 2	E–GEOD–17549, E–GEOD–3920, E–GEOD–14897, E–GEOD–37258, E–GEOD–13548, E–GEOD–23402
Mesenchymal stem cell	U 3	E–GEOD–40613, E–GEOD–9894, E–MEXP–466
Stromal cell	D 2 U 3	E–MEXP–2034, E–MEXP–1327, E–GEOD–3998
Embryonic stem cell	D 1 U 2	E–MEXP–930, E–GEOD–23402
T cell	D 3 U 2	E–GEOD–13987, E–GEOD–7307
CD8+ naive CD8 T cell	U 1	E–TAB M–40
Cardiac myocyte	U 1	E–TABM–145
Granulosa cell	U 1	E–MEXP–3641
Leukocyte	D 1 U 1	E–GEOD–21909
M1 macrophage	U 1	E–GEOD–5G99
B cell	D 1 U 1 U/D 2	E–GEOD–3982, E–GEOD–7307
Monocyte	D 3 U 2 U/D 3	E–GEOD–17549, E–MEXP–583, E–GEOD–7307, E–GEOD–21909, E–GEOD–11755
Neutrophil	D 1 U 1	E–GEOD–12662
iPSC	D 1 U 1	E–GEOD–37258
Monoblast	D 1	E–MEXP–3810
Pulmonary endothelial cells	D 1	E–GEOD–40613
Whole blood	D 1	E–GEOD–3Q26
Cumulus cell	D 2	E–MTAB–1670, E–MEXP–3641
Mononuclear	D 2	E–GEOD–13987, E–GEOD–11057
Epithelial cell	D 4 U 3	E–GEOD–4483, E–MEXP–2034, E–GEOD–13548

Expression Atlas, by EMBL-EBI, http://www-test.ebi.ac.uk/gxa/gene/ENSG00000140545?ef=organism_part

The expression profile of human 69 cell types suggests that *Mfge8* gene transcription is up or down-regulated even in the same cell type depending on the cell and experimental conditions used (Table 1.2). Mesenchyme cells (fibroblast, mesenchymal stem cell and stromal cell) seemingly express *Mfge8* at relatively high levels. Among ovarian follicle cells, *Mfge8* is up-regulated in granulosa cells, surrounding the oocyte, but down-regulated in cumulus cells. As for leukocytes (T and B cells, monocyte, and neutrophil), *Mfge8* expression may be regulated by complex mechanisms involving cell differentiation and environmental conditions.

Multiple genome-wide microarray studies performed to measure the transcriptomics profile of various cancer cell lines [49] indicate that several cancer cell lines derived from cancer tissues of mammary gland, ovary, brain, and uterus show

Table 1.3 Transcription profiling by array of human multiple cancer cell lines (950 samples) E-MTAB-37

Cell lines	Up/down	T-statistic	P-value	Organism part	Disease state	Clinical information	Bio source provider
C32TG	Up	16	$<1\times10^{-10}$	Skin	Amelanotic skin melanoma	ICDO; 44	ATCC
WI38	Up	15	$<1\times10^{-10}$	Lung	Normal	ICDO: 34	ATCC
HCC2157	Up	15	$<1\times10^{-10}$	Breast	Carcinoma	ICDO: 50	ATCC
DMS114	Up	13	$<1\times10^{-10}$	Lung	Small cell lung carcinoma	ICDO: 34	ATCC
SKMEL1	Up	13	$<1\times10^{-10}$	Skin	Melanoma	ICDO: 44	ATCC
Malme3M	Up	13	$<1\times10^{-10}$	Skin	Melanoma	ICDO: 44	ATCC
HCC1599	Up	11	$<1\times10^{-10}$	Breast	Carcinoma	ICDO: 50	ATCC
NCIH295R	Up	11	$<1\times10^{-10}$	Brain	Carcinoma	ICDO: 71	ATCC
CaOv3	Up	9.6	$<1\times10^{-10}$	Ovary	Carcinoma	ICDO: 56	ATCC
SW900	Up	9.6	$<1\times10^{-10}$	Lung	Small cell carcinoma	ICDO: 34	ATCC
D6TRG05MG	Up	9.3	$<1\times10^{-10}$	Central nervous system	Glioblastoma	ICDO: 71	ATCC
A172	Up	9.1	$<1\times10^{-10}$	Brain	Brain glioblastoma	ICDO: 71	ATCC
NCIH838	Up	8.9	$<1\times10^{-10}$	Lung	Lung adenocarcinoma	ICDO: 34	ATCC
SKLMS1	Up	8.8	$<1\times10^{-10}$	Vulva	Sarcoma	ICDO: 49	ATCC
KLE	Up	8.7	$<1\times10^{-10}$	Uterus	Carcinoma	ICDO: 55	ATCC
DKMG	Up	8.6	$<1\times10^{-10}$	Central nervous system	Glioblastoma	ICDO: 71	DSM2
SW1353	Up	8.5	$<1\times10^{-10}$	Bone	Sarcoma	ICDO: 49	ATCC
NCIH2052	Up	8.4	$<1\times10^{-10}$	Lung	Mesothelioma	ICDO: 34	ATCC
SJSA1	Up	8.3	$<1\times10^{-10}$	Bone	Osteosarcoma	ICDO: 49	ATCC
NCIHI65I	Up	8.2	$<1\times10^{-10}$	Lung	Lung adenocarcinoma	ICDO: 34	ATCC
DU4475	Down	−11	$<1\times10^{-10}$	Breast	Carcinoma	ICDO: 50	DSMZ
HuPT4	Down	−11	$<1\times10^{-10}$	Pancreas	Carcinoma	ICDO: 25	ECACC
CORL88	Down	−11	$<1\times10^{-10}$	Lung	Lung carcinoma	ICDO: 34	ECACC
HT1376	Down	−11	$<1\times10^{-10}$	Bladder	Carcinoma	ICDO: 67	ATCC
Daudi	Down	−11	$<1\times10^{-10}$	Hematopoietic and lymphatic system	Burkitt lymphoma	ICDO: 42	ATCC
EB3	Down	−11	$<1\times10^{-10}$	Hematopoietic and lymphatic system	Burkitt lymphoma	ICDO: 42	ATCC
RL	Down	−11	$<1\times10^{-10}$	Hematopoietic and lymphatic system	Non-Hodgkin lymphoma	ICDO: 42	ATCC

NCIHI87	Down	−10	$<1\times10^{-10}$	Lung	Lung carcinoma	ICDO: 34	ATCC
MCCAR	Down	−10	$<1\times10^{-10}$	Hematopoietic and lymphatic system	Plasma cell myeloma	ICDO: 42	ATCC
HT	Down	−10	$<1\times10^{-10}$	Hematopoietic and lymphatic system	B-cell neoplasm	ICDO: 42	ATCC
MV4II	Down	−10	$<1\times10^{-10}$	Hematopoietic and lymphatic system	Myeloid leukemia	ICDO: 42	ATCC
Y79	Down	−10	$<1\times10^{-10}$	Eye	Retinoblastoma	ICDO: 69	ATCC
RPMI6666	Down	−10	$<1\times10^{-10}$	Hematopoietic and lymphatic system	Hodgkin lymphoma	ICDO: 42	ATCC
JAR	Down	−10	$<1\times10^{-10}$	Placenta	Choriocarcinoma	ICDO: 58	ATCC
THP−1	Down	−10	$<1\times10^{-10}$	Hematopoietic and lymphatic system	Acute myeloid leukemia	ICDO: 42	ATCC
COLO668	Down	−9 9	$<1\times10^{-10}$	Lung	Colorectal adenocarcinoma	ICDO: 34	ECACC
SUDHL16	Down	−9.8	$<1\times10^{-10}$	Hematopoietic and lymphatic system	B-cell neoplasm	ICDO: 42	DSMZ
DB	Down	−9.7	$<1\times10^{-10}$	Hematopoietic and lymphatic system	B-cell neoplasm	ICDO: 42	ATCC
BT474	Down	−9.7	$<1\times10^{-10}$	Breast	Carcinoma	ICDO: 50	ATCC
JEG3	Down	−9.6	$<1\times10^{-10}$	Placenta	Choriocarcinoma	ICDO: 58	ATCC

Expression Atlas, by EMBL-EBI, http://www-test.ebi.ac.uk/gxa/gene/ENSG00000140545?ef=organism_part

up-regulated expression of *Mfge8* (Table 1.3), which is consistent with the expression profile of human post mortem tissue samples (see Table 1.1).

4.2 Regulation of Gene Expression

Although regulation of *Mfge8* gene expression has been studied using various cells, animal models and human biopsies, details of molecular mechanisms underlining MFG-E8 expression remain to be investigated (Table 1.4). Tissue- and stage-specific expression of MFG-E8 in lactating mammary glands suggests that *Mfge8* gene expression is dependent on lactogenic hormones as is the case with major milk proteins like caseins [50]. However, regulation of *Mfge8* gene expression is presumably regulated by more complicated mechanisms, even in the mammary gland, because high expression of *Mfge8* is maintained after weaning or at early stages of mammary gland involution, at which milk protein gene expression has been shut down [5, 14]. Up-regulation of *Mfge8* by a defined ligand was found first in microglia, macrophage cells in brain, using gene chip analysis, suggesting removal of apoptotic neurons by microglia [51]. Fractalkine (also known as CX(3)CL1), a member of the CX(3)C-chemokine family, is expressed by and released from neurons, whereas its receptor, CX(3)CR1, is primarily expressed by the microglia. Following this finding, CX(3)CL1 was shown to up-regulate *Mfge8* expression also in rat peritoneal macrophages [52]. In the same study, it was shown that LPS was a ligand responsible for down-regulation of *Mfge8* in macrophages and CX(3)CL1 was able to reduce the LPS-induced *Mfge8* down-regulation in the macrophages *in vitro* and in rats with CLP-induced sepsis *in vivo*. Using *CD14*$^{-/-}$ and *TLR4*-mutated mice, the LPS-induced down-regulation of *Mfge8* in the mouse sepsis model was shown to be mediated via the LPS-CD14 pathway [53].

Prolactin (PRL) induces up-regulation of the PRL receptor gene and also *Mfge8* in macrophages [54]. A luciferase reporter gene assay and a gel mobility-shift analysis showed that the C/EBPbeta binding site was responsible for PRL-induced activation of the *Mfge8* gene promoter. PRL, which is secreted also by stromal cells of human endometrium, has been suggested to modulate *Mfge8* expression in the endometrium during the window of implantation (WOI) [55]. Using whole endometrial biopsies, MFG-E8 was localized mostly to the endometrium and *Mfge8* expression was up-regulated in the luteal phase and highest during the WOI. Moreover, PRL treatment of cultured primary epithelial cells significantly increased MFG-E8 protein intracellular expression.

Human MFG-E8 (termed BA46) was originally identified as a breast cancer antigen and shown to be up-regulated in some cancer cell lines as described above (see Table 1.3). However, molecular mechanisms involved in the MFG-E8 up-regulation in cancer remain largely unknown. In an *in vitro* study using keratinocyte and a squamous cell carcinoma line [56], reporter gene assays using *Mfge8* gene promoter regions and chromatin immunoprecipitation experiments showed that the transactivator (TA) isoforms of p63 activated *Mfge8* transcription through a p53/p63

Table 1.4 Regulation and mechanism of MFG-E8 expression

Up/down regulation	Cells *in vitro*	Cells and tissue *in vivo*	Ligands	Mechanisms	Year, references
Up	Rat microgria		fractalkine CX(3)CL1		2005, [51]
Up	Rat oeritoneal macrophage mouse RAW264.7	Rats and mice prevention of CLP-induced tissue injury	CX(3)CL1		2007, [52]
Down	Peritoneal macrophages	Spleen mice with CLP- induced sepsis	LPS	LPS-TLR4-CD14 pathway	2009, [53]
Up	Macrophage		Prolactin	PRL-receptor CEBP/beta	2008, [54]
Up	Human primary endometrial cell	Human endometrial biopsies	Prolactin		2011,[55]
Up	Human keratinocyte a squamous cell carcinoma line			Trans-activator (TA) isoforms of p63	2008, [56]
Down	Mouse macrophage		Apoptotic cells, PPAR-delta ligands		2009, [57]

motif at −370. Moreover, p63 siRNA treatment of the squamous carcinoma cells suppressed production of MFG-E8 protein, leading to suppression of the cell adhesion.

Among three mouse PPARs (alpha, beta and delta) only PPAR-delta is up-regulated, when primary bone marrow-derived macrophages phagocytosed apoptotic cells [57]. Fatty acids including oxidized ones, rich in apoptotic cells, act as PPAR-delta ligands and regulate some opsonin-relating genes including *Mfge8* in a PPAR-delta dependent manner, leading to effective clearance of apoptotic cells by phagocytosis.

5 Physiological Function

5.1 Opsonization and Enhancement of Phagocytosis

Phagocytes rapidly engulf and remove apoptotic cells, but not healthy cells, in order to prevent the release of potentially immunogenic intracellular materials. To distinguish the dead cells from living ones, professional and non-professional phagocytes, such as activated macrophages, immature dendritic cells, mammary epithelial and retinal pigment epithelial (RPE) cells, secrete MFG-E8 that opsonizes apoptotic cells as the "eat-me" marker [1]. Cells undergoing apoptosis externalize phosphatidylserine, which is kept extensively on the inner leaflet of the lipid bilayer in the living cells, onto the outer leaflet of cell membrane presumably because of losing directional phospholipid transport activity and activation of phospholipid scramblase(s) [58, 59]. Secreted MFG-E8 specifically binds to apoptotic cells by recognizing phosphatidylserine, preferentially to oxidized phosphatidylserine [60]. The phagocytes expressing alpha(v)beta(3) or alpha(v)beta(5) integrin engage MFG-E8 on the apoptotic cells via the RGD motif. Engagement of apoptotic cells through alpha(v)beta(5) integrin recruits the CrkII-DOCK180 module to activate Rac1-small GTPase, which is involved in the formation and remodeling of actin-based cell structures such as lamellipodia and promotes phagocytic process[61]. Immature dendritic cells express both MFG-E8 and DOCK180. MFG-E8 activates Rac1 through the alpha(v)beta(5) integrin, and the MFG-E8-dependent Rac1 activation and the engulfment of apoptotic cells are abrogated by suppression of DOCK180 expression [62]. Transglutaminase 2 has binding affinity to MFG-E8 and stabilizes the formation of the MFG-E8-integrin-mediated recognition complexes on macrophages to apoptotic cells [63]. A homologous gene product, Del-1/Edil3, is expressed in the distinct subset of macrophages from MFG-E8 expressing ones and similarly enhances the phagocytic activity against apoptotic cells [22].

MFG-E8 also promotes engulfment of other biological materials. MFG-E8 is involved in phagocytosis of amyloid beta-peptide in brain as mentioned above [44]. Membrane vesicles containing cellular materials are potentially immunoreactive to induce autoimmune response. The photoreceptor cell sheds its photoreceptor outer

segment fragments (POS) for renewing materials once a day. The apical side of RPE cells extends microvilli containing alpha(v)beta(5) integrin and contacts with the intact outer segments of photoreceptor cells. MFG-E8 is secreted from RPE cells and binds to POS to promote engulfment of POS through alpha(v)beta(5) integrin receptor [64]. Various cells release membranous small particles into fluids, known as microvesicles and exosomes. MFG-E8 binds to the platelet-derived phosphatidylserine-rich microvesicles and promotes their cellular uptake by phagocytosis. *Mfge8*$^{-/-}$ mice increase the platelet-derived microvesicles in their plasma and generate more thrombin [35]. MFG-E8 has been reported as the major component of exosomes derived from various type cells and to regulate targeted engulfment of them by receiving cells [65–67]. Exosomes contain the antigen/MHC complexes and transfer them to dendritic cells. Targeting of exosomes to dendritic cells is partially mediated by MFG-E8 on exosomes and alpha(v)beta(3) integrin on dendritic cells [67]. Tumor antigen combined with the F5/8-C domain of MFG-E8 accumulates on exosomes. Adenovirus-mediated expression of this recombinant protein in mice improves antigen specific immune responses and anti-tumor effects [66].

5.2 Autoimmunity, Macrophage Regulation and Immunosuppression

Release of immunogenic intracellular materials of apoptotic cells such as nucleus is related to onset of autoimmune diseases. Consistent with this, deficiency of the *Mfge8* gene in mice was reported to result in the systemic lupus erythematosus (SLE)-like phenotype in an age dependent manner [2]. In this study, many apoptotic cells were not engulfed in the germinal centers of the spleen and lymph nodes, and thus the deficient mice developed splenomegaly with formation of a lot of germinal centers. The 40 week-old *Mfge8*$^{-/-}$ mice, particularly females, were shown to raise high concentrations of autoantibodies against double stranded DNA and nuclear. Intravenous injection of the D89E mutant of MFG-E8, carrying a point mutation in an RGD motif, also induced the production of autoantibodies [68]. At the age of 40 weeks, the kidney of the *Mfge8*$^{-/-}$ female mice had a massive deposition of the immune complex, which is the typical pathogenic consequence of circulating autoantibody production, and most these mice suffered from glomerulonephritis with the high protein concentration in the urine [2]. This phenotype is quite similar to that of human SLE. Actually, human *Mfge8* gene in a part of female SLE patients was reported to have an intronic mutation that causes a cryptic exon from intron 6 to be included in the transcript [28]. The cryptic exon provides a termination codon into the F5/8-C2 domain, resulting in the production of a C-terminal truncated protein. Although the mutant type protein has the ability to bind to phosphatidylserine and to enhance the phagocytosis of apoptotic cells comparable to the wild type one, repeated intravenous administrations of the mutant MFG-E8 protein causes the production of autoantibodies in mice. Double null mutation of *Mfge8* and *Timd4*, which

is another phosphatidylserine recognizing protein on the resident peritoneal macrophages, has a synergistic effect on the autoimmune development [69].

The mechanism in which MFG-E8 prevents the onset of the autoimmune diseases largely remains unclear. Reduction of MFG-E8 activity leads to the production of autoantibodies against double stranded DNA, nucleus, and phospholipids [1, 68]. One simple mechanism is immediate elimination of the immunoreactive intracellular materials from the apoptotic cells to prevent presentation of these materials as the autoantigen by antigen-presenting cells (APCs). Elevation of antoantibodies reacting with the oxidized lipoproteins is associated with autoimmune diseases including SLE. A recent study showed that the *Mfge8*$^{-/-}$ mice raised the level of IgM against the protein-bound 4-oxo-2-nonenal (ONE), a highly reactive aldehyde generated from the peroxidation of ω6 polyunsaturated fatty acids [70]. The monoclonal IgM antibodies specific to the ONE-modified proteins recognized the late apoptotic and necrotic cells and enhanced phagocytosis of apoptotic cells, probably due to cross-reaction with phosphatidylserine or aldehyde-modified proteins. Thus, prolonged retention of the dead cell exposing phosphatidylserine in the tissues and bloods possibly plays a positive role in the induction of innate antibodies reacting to the proteins modified with unsaturated aldehydes derived from the lipid peroxidation. Cellular materials in the apoptotic cells engulfed by the APCs are processed in the endocytic pathway and then presented on the cell surface as the MHC-antigen complex to responding helper T cells, which induces autoantibody production. MFG-E8 expressed in the immature dendritic cells also has been reported to control intracellular processing of ingested apoptotic cells into MHC-antigen complexes [71]. Engulfed apoptotic cells are rapidly delivered into lysosomes in the wild type dendritic cells. In contrast, small cell fragments were shown to remain for 24 h in some endosomal compartments of the *Mfge8*$^{-/-}$ dendritic cells, leading to enhanced self-antigen presentation.

Apoptotic cells cause immunosuppressive effect by increasing secretion of anti-inflammatory cytokines and decreasing release of the pro-inflammatory cytokines from LPS-stimulated phagocytes [72]. The D89E mutant of MFG-E8 losing the integrin-binding activity inhibits phagocytosis-mediated enhancement of IL-10 production from macrophages [68]. Matured resident peritoneal macrophages (RPM) express less amounts of MFG-E8 than thioglycolate-elicited peritoneal macrophages (TGPM). RPM has the low capacity to phagocytose the apoptotic cells because of low expression of MFG-E8 and thus lacks phagocytosis-induced immunosuppression [73]. In the mice suffering from intestinal and subsequent systemic inflammation after intestinal ischemia, MFG-E8 expression decreases, and intraperitoneal administration of recombinant MFG-E8 protein suppresses production of pro-inflammatory cytokines (TNF-alpha, IL-6, and IL-1beta) and tissue injury [74]. MFG-E8 is released from apoptotic endothelial cells in a caspase-3 dependent manner, and causes reprogramming of macrophages to adopt a high anti-inflammatory and low pro-inflammatory cytokine/chemokine balance. Phosphorylation and activation of STAT3 occurs in peritoneal leukocytes *in vivo* when the peritonitis-induced mice are pre-treated with conditioned media from apoptotic endothelial cells containing MFG-E8 [8]. MFG-E8 mediated phagocytosis of apoptotic cells inhibits phosphorylation of MAPKs, activation of NFkappaB, and TNF-alpha release in

responding to LPS in TGPM, causing immunosuppressive effect. Treatment with recombinant MFG-E8 depressed the LPS-induced pro-inflammatory cytokine TNF-alpha expression in the cultured and peritoneal macrophages [7]. In these cells, pSTAT3 and its downstream target SOCS3 were induced, and NF-kappaB p65, downstream molecule of TLR4 signaling, was reduced probably due to increased degradation by SOCS3. Conversely, deficiency of *Mfge8* potentiated release level of TNF-alpha protein induced by LPS *in vitro* and *in vivo*. These reports suggest that MFG-E8 is a direct anti-inflammatory molecule, which mediates the immunosuppression signal from apoptotic cells into the engulfing macrophages.

The homologous gene product Del-1 expressed by endothelial cells attenuates inflammation as the integrin antagonist limiting leukocyte adhesion and infiltration in various tissues [75–77]. When acute lung injury was induced by intratracheal injection of LPS, *Mfge8*$^{-/-}$ mice exhibited extensive lung inflammatory damage due to exaggerated infiltration of neutrophils and production of TNF-alpha, MIP-2, and myeloperoxidase [9]. Different from the Del-1 cases, MFG-E8 reduces neutrophil migration through up-regulation of G protein-coupled receptor kinase2 (GRK2) and following down-regulation of chemokine receptor CXCR2 on the cell surface. In the murine experimental colitis model, MFG-E8 ameliorated this by reducing inflammation and improving disease symptoms in an integrin-dependent manner [78]. In this situation an extracellular matrix (ECM) protein, osteopontin, increased in the early phase of colitis induction. Osteopontin positively regulated IL-1beta and TNF-alpha induction through alpha(v)beta(3) integrin-mediated focal adhesion kinase phosphorylation in LPS-stimulated peritoneal macrophages. MFG-E8 antagonized osteopontin activity in the integrin signal propagation and abolished pro-inflammatory cytokine induction, probably by interfering with osteopontin binding to alpha(v)beta(3) integrin in the similar way of Del-1.

GM-CSF enhances protection against tumors and is required for the expression of MFG-E8 in APCs [79]. Engulfment of apoptotic cells by macrophages influence regulatory T cell (Treg) function in immune tolerance induction [80]. In this process MFG-E8 is essential for TGF-beta-dependent MHC class II-mediated Treg expansion. MFG-E8 expression in the hematopoietic cells increases TGF-beta level and down-regulates pro-inflammatory cytokines *in vivo* [79].

MFG-E8 expression declines in systemic inflammatory condition caused by sepsis, whole body irradiation, and ischemia-reperfusion injury, in which LPS-CD14-TLR4 pathway works. Exogenous MFG-E8 administration into the disease model mice significantly improves systemic inflammatory responses [81]. Thus, immunosuppressive effect of MFG-E8 offers a novel therapeutic treatment for systemic inflammation.

5.3 Angiogenesis and Tumorigenesis

While MFG-E8 functions as a secretory protein from phagocytes, this protein is deposited around blood vessels and smooth muscles. MFG-E8 has a role in VEGF-induced neovascularization after ischemia in the adult mouse, while this gene is

dispensable for basal vascularization during development and basic FGF-dependent angiogenesis [82]. During VEGF-dependent blood vessel growth in the ischemic model, endogenous MFG-E8 positively regulates phosphorylation of Akt, a downstream molecule of VEGF signal, via alpha(v)beta(3) and alpha(v)beta(5) integrins but not another downstream molecule ERK. MFG-E8 is expressed in pericytes and pericyte precursors wrapping endothelial cells and capillaries rather than endothelial cells in the melanomas and retinas of mice with oxygen-induced retinopathy [83]. The *Mfge8*$^{-/-}$ mice diminished tumor- and retinopathy-associated angiogenesis, and inhibition of MFG-E8 in the surrogate pericytes/pericyte precursors selectively attenuated PDGF-driven cell migration in an alpha(v) integrin dependent manner. In pericytes/pericyte precursors responding to PDGF-BB stimulation, MFG-E8 transiently associates with PDGFRbeta and diminishes ubiquitin-dependent degradation, resulting in cell surface retention of PDGFRbeta and consolidation of focal adhesion kinase association [84].

Human MFG-E8, BA46, was originally identified as a breast tumor antigen [11]. MFG-E8 is up-regulated not only in the breast cancer cells, but also various tumors including melanomas and the bladder tumor [85, 86] (Table 1.3). Nevertheless, functional contribution of MFG-E8 to tumorigenesis had been unclear for a long time. Provocation of angiogenesis is critical for solid tumor growth. In *Rip1- Tag2* transgenic mice, a cancer model in which VEGF-mediated angiogenesis is important, angiogenic islets and tumors increase MGF-E8 expression. *Mfge8*$^{-/-}$ mice reduce frequency of angiogenesis and tumor formation. Interestingly, compensatory expression of Del-1 is up-regulated in the *Mfge8*$^{-/-}$ mice, suggesting the relevant function of these proteins [87]. MFG-E8 also enhances tumorigenicity and metastatic capacity of melanoma by triggering an epithelial-to-mesenchymal transition through Akt-dependent and Twist-dependent pathway, and induces Tregs associating with innate and adaptive antitumor cytotoxicity [88]. Cancer stem cells (CSCs) induce MFG-E8 expression from tumor-associated macrophages. In the tumor microenvironments, MFG-E8 activates STAT3 pathway and induces Smoothened expression which is a downstream regulator of Sonic Hedgehog pathway, and consequently gives CSCs anticancer drug resistance [89].

5.4 Maintenance of Epithelium Integrity (Intestinal and Epididymal Epithelia)

MFG-E8 is constitutively expressed in the macrophages localizing at lamina propria of mouse small intestine and plays a crucial role in maintenance and repair of murine intestinal epithlium [90]. In an *in vitro* wound-healing assay, rat intestinal epithelial cells (IEC-18) spontaneously migrate after wounding by physical scrapping of the cell monolayer, and treatment of the cells with MFG-E8 (5 nM) markedly enhanced the cell migration into the denuded area through PKCepsilon signaling pathway. At the wound edge of IEC-18 cell monolayers, MFG-E8-binding

to the cells through membrane phosphatidylserine and the reorientation of actin cytoskeleton are observed. Intraperitoneal administration of anti-MFG-E8 antibody to mice results in the delay of the migration of BrdU-labeled epithelial cells along the crypt-villus axis in the small intestine. In addition, the epithelial cell migration is markedly reduced in *Mfge8*$^{-/-}$ mice compared with wild-type control. At the intestinal mucosal tissue of the *Mfge8*$^{-/-}$ mice, a mild focal mucosal injury at villous tips is observed with a few necrotic epithelial cells. Thus, MFG-E8 has been suggested to play an important role in the maintenance of intestinal epithelial homeostasis.

In a mouse sepsis model induced by cecal ligation and puncture (CLP), *Mfge8* expression in the intestinal tissue is down-regulated, and concurrently typical pathology is induced including intestinal injury and interrupted epithelial cell migration. In this experimental sepsis model, administration of recombinant MFG-E8 restores the cell migration and depletion of MFG-E8 results in delay in repair of the intestinal epithelium [90]. MFG-E8 constitutively expressed by macrophages at lamina propria has thus been suggested to contribute to the promotion of mucosal healing after wounding. In consistent with these results, some recent studies on the effect of MFG-E8 administration on the wound healing of intestinal epithelium have also suggested that MFG-E8 has roles in attenuation of intestinal injury and promotion of hearing in animal models, such as acute radiation syndrome induced by whole body irradiation of rats [91], acute colitis induced by oral administration of dextran sodium sulfate (DSS) to mice [78, 92]. Roles of MFG-E8 in intestinal inflammation and its regulation have recently been reviewed in detail [93].

Mouse MFG-E8 (also known as SED1) is secreted into the lumen of epididymis, in which sperms mature to acquire the fertilization ability, binds to sperm plasma membrane there, and eventually plays important roles in sperm-egg binding at the initial stage of fertilization [4]. In addition to this role in sperm maturation and adhesion, MFG-E8 is suggested to have intrinsic role in the epididymis [94]. In the epididymis of *Mfge8*$^{-/-}$ male mice, pathological detach of epithelia and extravasate of sperm are observed, indicating that MFG-E8 play roles in the maintenance of epididymal epithelium integrity. MFG-E8 is localized in the basolateral domains of epididymal epithelial cells lining mouse epididymal duct, while MFG-E8 is secreted into both apical and basolateral sides of polarized epithelial cell monolayers *in vitro*. In the same way as described previously on human breast carcinomas MCF-7 cells [31], mouse epididymal epithelial cells adhere effectively to MFG-E8-coated cell substratum through the RGD-integrin interaction [94]. MFG-E8 secreted basolaterally from the epididymal epithelial cells may enhance the cell adhesion to epididymal basal lamina utilizing its EGF-like domain and F5/8-C domains for cellular integrin receptors and extracellular matrix of basal lamina, respectively.

In the epididymis of *Mfge8*$^{-/-}$ male mice, not only the breakdown of epididymal epithelium but also aberrant epididymal fluid with hypo-osmosis and alkaline pH is observed, suggesting disrupted fluid re-absorption and pH regulation in the epididymis in the absence of MFG-E8 [95]. These changes in the epididymal fluid were suggested not to be the secondary consequences of defective testis duct or improper epididymal differentiation.

5.5 Mammary Gland Development and Involution

MFG-E8 is expressed in mammary epithelial cells predominantly at lactation and early involution stages, suggesting yet unknown roles in differentiated mammary glands. In addition, MFG-E8 has important roles in mammary gland morphogenesis during mammary gland development [6]. In the developing mammary glands of *Mfge8*$^{-/-}$ female mice, severely reduced branching morphogenesis is observed from both epithelial ducts and terminal end buds. In the developing epithelial ducts, both luminal and myoepithelial cells express MFG-E8, which auto-activates myoepithelial cells through binding to cellular integrin receptors leading to MAPK activation and cell proliferation. From these observations, some sort of MFG-E8 contribution was suggested to the intercellular interaction and signaling between luminal epithelial and myoepithelial cells in developing mammary glands [6].

MFG-E8 is expressed in mammary epithelial cells and residential or migrating macrophages in mammary glands. At some stages of mammary gland involution, apoptotic epithelial cells are phagocytosed and cleared by neighboring epithelial cells and/or macrophages. As a large number of mammary epithelial cells result in apoptotic cell death in the involuting mammary glands, a deficiency of MFG-E8 causes the accumulation of apoptotic cells due to defective phagocytosis by macrophages and epithelial cells. Milk fat globule-like lipids are also accumulated in the mammary ducts during involution, suggesting MFG-E8-dependent phagocytosis of milk fat globules surrounded with cell plasma membrane. After the involution periductual mastitis is observed in the mammary glands of *Mfge8*$^{-/-}$ mice even under specific pathogen free (SPF) conditions, indicating that residual apoptotic cell debris and lipids may be relevant to the non-infectious mastitis [5].

MFG-E8-dependent phagocytosis of milk fat globules by mammary epithelial cells after natural and forced weaning was suggested by the microscopic observation of lactating and involuting mouse mammary glands [14]. In the immunofluorescence analysis of mouse mammary gland tissue sections, MFG-E8 surrounding milk fat globules is detected around the epithelium of involuting mammary glands, whereas such immunofluorescence signals of MFG-E8 are detected on milk fat globules stored in the alveolar lumen at lactation stages. In the tissue of involuting mammary glands, but not lactating glands, a small number of CD68/MFG-E8 double-positive cells (activated macrophages) were also detected but in small numbers in the post-weaning mammary glands, suggesting milk fat globules are phagocytosed by migrating macrophages. However, the number of MFG-E8-positive- and CD68-negative signals are much more than that of the double-positive signals, indicating that clearance by phagocytosis of residual milk fat globules could be ascribed mainly to the mammary epithelial cells as non-professional phagocytes rather than macrophages.

Milk fat globules are phagocytosed in fact by HC11 mouse mammary epithelial cells *in vitro*, and milk fat globules recovered from involuting mammary glands are taken up by HC11 cells more effectively than those from lactating mammary glands [15]. Interestingly, MFG-E8 is present on some, but not all, milk fat globules in

breast milk at lactation stages, whereas most of MFGs are MFG-E8 positive at early involution stages [15, 16]. Anti-MFG-E8 antibody remarkably decreases milk fat globules taken up by HC-11 cells. Thus, milk fat globules are taken up by mammary epithelial cells in an MFG-E8-dependent manner *in vitro*. Post-weaning up-regulation of milk ceruloplasmin, which catalytically removes hydrogen peroxide and lipid hydroperoxides [96], may also indicate a rapid increase in oxidative stress in the involuting mammary glands [97].

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Chapter 2
MFG-E8 as a Marker for Apoptotic, Stressed and Activated Cells

Kristine Blans and Jan Trige Rasmussen

Abstract Milk fat globule-epidermal growth factor-factor 8 (MFG-E8)/lactadherin's ability to specifically recognize phosphatidylserine (PS) in membranes has been recognized as an excellent tool in a variety of scientific and clinical contexts. An asymmetric pattern of phospholipids across cellular membranes in eukaryotes is a fundamental property in maintaining normal cell function. However, randomization of phospholipids is an equally important event when cells are activated leading to exposure of the otherwise hidden PS crucial in orchestrating downstream events in apoptosis and coagulation. Lactadherin has in recent years been recognized as a sensitive PS binding protein for visualizing apoptosis and as an anticoagulant. Compared to the benchmark PS-probe, annexin V, lactadherin seems to be superior in several PS binding properties. Numerous studies show the usefulness of lactadherin in monitoring cell health *in vitro* and *in vivo*, in detecting cell-derived PS exposing microparticles, or for exploring mechanisms in apoptosis. Moreover, radio-labeled lactadherin has been proposed as a non-invasive marker in the clinic for imaging of apoptotic events. Lactadherins PS recognition owes to the proteins C-domains, and has been used in recombinant exosome engineering in addressing proteins of interest to surfaces of nano-membrane particles. This chapter outlines the use of lactadherin as a PS binding protein, based on several publications where many of these are conducted in collaboration with us, and reflects our experimental experiences with the protein over several years.

Keywords Phospholipids • Phosphatidylserine • Phosphatidylethanolamine • Lipid asymmetry • Lipid transporters • Scramblases • Flippases • P_4-ATPases • Floppases • ABC transporters • PS display • Annexin V • PS recognition • PS detection • Exosomes • Microparticles • Diagnostic marker • Radio isotope-labeled lactadherin • Coagulation • Factor V • Factor VIII • Anticoagulant • Platelets • PS-blocker

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Abbreviations

FITC	Fluorescein isothiocyanate
HYNIC	Hydrazinonicotinamide
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine

1 Phospholipid Asymmetry in Cellular Membranes

Each side of the eukaryotic cells plasma membrane has different biophysical properties, each influencing a great number of crucial cellular functions. These includes vesicle budding, membrane curvature, fertilization, phagocytosis, exocytosis, apoptosis, blood coagulation, regulation of the activities of membrane-associated proteins, intracellular signaling, and the maintenance of membrane integrity and impermeability. The asymmetric pattern of lipids and other molecules across the bilayer is therefore of great importance for the cell to maintain normal function. Phospholipids accounts for approximately 70 % of the total lipid content of mammalian cells, whereas the remaining comprises cholesterol, sphingomyelin, and glycosphingolipids. Phosphatidylcholine (PC) is the most common phospholipid accounting for 40–50 %. Phosphatidylethanolamine (PE) is the second most common comprising 20–45 %, whereas phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatic acid only represent minor constituents in the plasma membrane. The PS level in cellular membranes ranges from 1 to 12 %, where the plasma membrane contains the highest percentage of PS. PS and PE are preferentially found in the cytoplasmic leaflet, while PC and SM are enriched on the extracellular or luminal leaflet. Even though PS is of low abundance in membranes, it is outweighed by its physiological importance. By its serine head group PS is characterized as the most negatively charged phospholipid in the eukaryotic plasma membrane, and its exposure to the outside is tightly regulated, since its display to the environment is associated with crucial signaling events.

Although asymmetric lipid synthesis is an ongoing process as well as chemical modifications contributes to the unequal distribution, lipid transporters is the primary mechanism for generating and maintaining lipid asymmetry. Lipid synthesis takes place in the cytoplasmic leaflet of the endoplasmatic reticulum, resulting in an unequal distribution of lipids across the bilayers. Here, lipid transporters named scramblases helps in counteracting the asymmetric biosynthesis. From here, small vesicles are budding and fusing with membrane organelles. In Golgi, lipid sorting and lipid processing events takes place, including the generation of lipid asymmetry. This section will give a short introduction to the proteins that sustain phospholipid asymmetry in cellular membranes, and the significance of PS when presented to the surrounding environment on the outside of the cell. More detailed

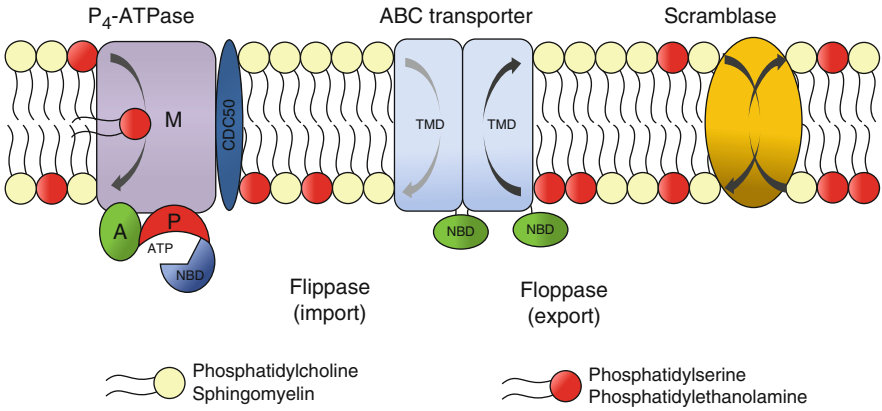

Fig. 2.1 Transporters in regulation of membrane lipid asymmetry. An asymmetric distribution of *e.g*. phospholipids is of great importance for normal cell function. PC and sphingomyelin are primarily found in the extracellular leaflet, while PS and PE are kept on the cytoplasmic leaflet. Three distinct families of membrane transporters have been identified to transport lipids between membrane bilayers. *P4-ATPases* are flippases working as lipid importers. This membrane transporter adopts a five domain structure consisting of two membrane-embedded domains; M, 10 membrane spanning helices, and a CDC50 β-subunit. Moreover, this flippase includes three cytoplasmic domains; *A* Actuator, *P* Phosphorylation, *NBD* Nucleotide binding domain. A, P and NBD subdomains works together in utilizing the energy from ATP in flipping lipids from the outer to the inner membrane bilayer. *ABC transporters* have primarily been characterized as floppases exporting lipids to the extracellular leaflet, but lipid import might also be a function of the transporter. Structurally, ABC transporters consist of two transmembrane domains (*TMD*) and two cytoplasmic nucleotide binding domains (*NBD*). Binding of ATP to the nucleotide binding domains lead to a rearrangement of the transmembrane domains and lipid flopping. *Scramblases* are energy independent acting to dissipate lipid asymmetry by randomizing lipid distribution

descriptions on the subject can be found in the following reviews [1–3]. Disruption of the asymmetric pattern of lipids is associated with cell activation or pathological conditions, why a delicate system of molecular mechanisms is present to maintain the unequal distribution characterizing a quiescent cell. Especially PS is carefully retained in the inner leaflet of all viable cells, since its exposure to the environment is the key to a number of carefully regulated signaling events. Passive flip-flop of phospholipids is a very inert event, hampered by a thermodynamic barrier not making the presence of phospholipids polar head groups favorable in the hydrophobic membrane interior. Therefore much more efficient transport systems have been discovered that maintain, regulate or dissipate the lipid gradient across the plasma membrane. Three classes of transport proteins have been found to be implicated in translocating phospholipids across cellular membranes, and have been named scramblases, flippases, and floppases [3]. Figure 2.1 summarizes structural and functional aspects of protein transporters involved in maintaining membrane phospholipid asymmetry and enabling rapid translocation of PS upon cellular changes, stimuli, or activation.

Activation of blood platelets is a well-known example in rapid loss of lipid asymmetry in cell membranes, resulting in exposed PS that initiates the coagulation cascade, in order to make a primary arrest of bleeding. The rapid loss of lipid asymmetry is suggested to be mediated by scramblases. This type of lipid transporters is characterized by nonspecific bi-directional flipping of all types of lipids across membrane bilayers without using energy. Since cytoplasmic Ca^{2+} is an important regulatory component, and likewise has been shown to activate scramblases resulting in PS exposure, Ca^{2+} has been suggested to participate in the regulation of lipid scrambling. The rate of lipid scrambling also seems to vary with cell type, which has been suggested to be linked to the efficiency of scramblase activation [4]. Accordingly, scramblases should orchestra dissipation of membrane lipid asymmetry leading to the physiological important translocation of PS to the outside of *e.g*. apoptotic cells. Cells undergoing apoptosis exposes PS as an important "eat me" signal to its surroundings for proper removal of cell corpses [2]. Proteins that has been suggested to possess scramblase activity is the human phospholipid scramblase family belonging to the ATP-independent class of phospholipid translocators, activated by divalent cations [5]. However, this protein family has later been found not to be dedicated scramblases, and doubt has later been placed on their actual role as scramblases [4, 6]. Anoctamin 6, a ubiquitous protein, which expression is defect in patients with Scott syndrome has also been suggested to work as a scramblase. Scott syndrome is an inherited bleeding disorder that is characterized by a phospholipid-scrambling defect leading to impaired blood clotting. Studies have shown that anoctamin 6 enhances apoptosis-induced phospholipid scrambling, without being an essential factor in the process. In line with that, the gene encoding anoctamin 6, TMEM16F, have been identified with recessive mutations, in patients with Scott syndrome [7]. Moreover, members of the TMEM16 protein family has besides their role in Cl^- channeling lately been suggested as calcium dependent phospholipid scramblases possessing different affinities towards different phospholipid classes [8].

Flippases, also named P_4-ATPases, are able to translocate specific phospholipids, including PS, unidirectional from the outer to the inner leaflet of membranes. P_4-ATPases belongs to the P-type ATPase superfamily, utilizing the energy from ATP hydrolysis to translocate ions and lipids across membranes and is found in all kingdoms of life. However, the lipid transporting P_4-ATPases are phylogenetically diverse from other P-type ATPases since none from this subfamily has been identified in prokaryotes so far, whereas they are found all over the eukaryotic kingdom. Entire 14 P_4-ATPases have until now been identified in humans and comprises the largest subfamily in most eukaryotes, in contrast to the four known genes encoding the Na^+, K^+-ATPase [9].

Structural studies on the phospholipid transport process of P_4-ATPases has shown parallel dephosphorylation and release of phospholipids similar to the dephosphorylation of the Na^+, K^+-ATPase by K^+. However, P_4-ATPases is not working alone, but is known to be associated with an accessory beta-subunit named CDC50, resulting in a heterodimeric complex thought to be necessary for optimal phospholipid transport. The precise role of CDC50 proteins in lipid transport is not yet clear, but it is suggested to be required for endoplasmic reticulum exit of the

pumps. Studies on substrate specificity of P_4-ATPase transporters have primarily identified PS and PE as substrates, underscoring the role of P_4-ATPases in maintaining PS unexposed to the extracellular environment. Interestingly, several members of this group of lipid transporters have recently been linked to severe human disorders. A second function of the P_4-ATPases is in assisting budding of transport vesicles in the endocytic and secretory pathways [3, 9].

While flippases transports lipids to the inner leaflet of membranes, floppases transports lipids in the other direction. Outward lipid transporters belong to the family of ABC (ATP-binding cassette) transporters, which are known to transport a wide variety of compounds across cell membranes. Using ATP hydrolysis this protein family actively exports compounds, including phospholipids, to acceptor proteins or to the extracellular environment by extrusion. However, new studies indicate that some ABC transporters also functions as importers, questioning their sole role as exporters. ABC transporters are composed of four principal functional domains: two transmembrane domains and two cytoplasmic ATP-binding cassettes, which typically are synthesized in one single polypeptide chain. However, some holds additional domains for activity regulation. Insight into the transport mechanisms of ABC transporters have been eased by structural studies of several exporters in addition to biochemical studies of their function, and a transport cycle model has been proposed for phospholipid export. The human genome encodes entire 49 genes for ABC proteins, in which a large number of mutations have been linked to severe human diseases emphasizing their physiological importance [3]. Still, information about their substrate specificity, mechanism and regulatory properties is inadequate, leaving enough unanswered questions for futures studies, including their role in flopping PS for exposure to the environment.

2 MFG-E8 as a Phosphatidylserine Recognition Tool

Exposed PS on cell surfaces is as mentioned a well-known feature of apoptotic cells and on activated blood platelets, where the phospholipid in both cases is a crucial tag in sustaining homeostasis and hemostasis, respectively. A controlled shutdown of cellular processes by cell shrinkage, nuclear condensation, and membrane blebbing are known characteristics for apoptosis. Cells that undergo this death-program are under healthy circumstances rapidly removed and degraded by phagocytes without causing necrosis or inflammation [10]. PS displayed on apoptotic cells is an early signal to the environment tagging cell corpses for engulfment by phagocytes [11, 12]. Detection of this specific anionic phospholipid on cell surfaces has become a standard tool in cell biology *e.g.* aiding to monitor the health of cells grown in culture, test the viability of examined cells or tissue living, or during the investigation of the complex mechanisms in initiation and regulation of apoptosis. A baring principle of many of the described methods is the use of proteins and molecules with PS binding properties. Annexin V was one of the first proteins discovered with pronounced PS binding ability [13, 14]. Few years later, it was shown that annexin

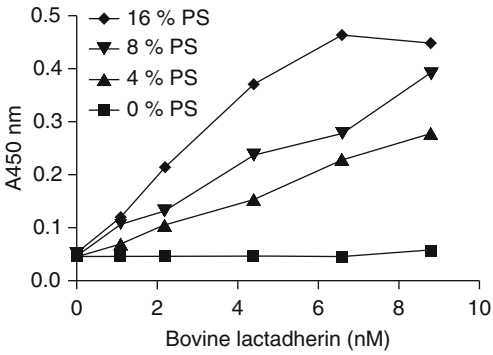

Fig. 2.2 Representative example of lactadherin's PS binding by solid-phase ELISA. The PS binding by lactadherin can be demonstrated in an assay similar to ELISA. Wells coated with ascending percent's of PS balanced with the neutral PC demonstrates how proportional amounts of lactadherin bind according to the PS percent. Bound lactadherin was following detected using an anti-lactadherin antibody and a secondary labeled antibody

V has the ability to identify PS exposed on apoptotic cells before morphological changes during apoptosis can be detected [15]. Numerous of commercial kits do now rely on labeled-annexin V for PS imaging.

Ability to bind PS specifically has successively been demonstrated as a feature for the protein MFG-E8/lactadherin as well (Fig. 2.2). Physiologically, MFG-E8/lactadherin has among others been shown to operate as a linker between PS exposing cells/cell remnants and consuming macrophages. This functions was suggested in 2000 [16] and proven a few years later [11]. The protein has been allotted different names mostly due to its discovery in different species, however, throughout this chapter it will be referred to as lactadherin if nothing else is stated. A simple way to show lactadherin PS binding is by the use of a solid-phase binding assay [17]. Structural investigations have revealed that the capacity to bind PS in a phospholipid membrane primarily owes to the proteins C2 domain [16, 18].

Two studies on bovine lactadherin have found no significant difference in PS affinity of either the C2 domain alone or full-length lactadherin using different methodologies [16, 18]. However, a recent study reports a reduced affinity of the C2 domain alone compared to the full-length protein in displacement and anticoagulant studies [19], suggesting a role of lactadherin C1 domain for high affinity binding. Structural information on the lactadherin C2 domain has provided important information on critical lactadherin residues for PS recognition. Shao et al. obtained in 2008 a crystal structure of the bovine lactadherin C2 domain at 1.7 Å resolution that showed a core-structure very similar to that of the C2 domain of the blood coagulation factor V and VIII [20]. Three loops or spikes were in Shao's study identified to carry water exposed hydrophobic residues (Trp26, Leu28, Phe31 and Phe81) and was by mutagenesis studies confirmed to play a significant role in lactadherin-C2 membrane binding. This detailed report on structural information about the bovine lactadherin-C2 domain was published after a preceding work of Lin and colleagues [21].

A mechanism for lactadherins membrane binding has also been proposed for the protein using synthetic phospholipid vesicles and bovine lactadherin in stopped-flow kinetic experiments. The kinetic data was consistent with a two-step binding mechanism proposed to consist of an initial fast binding event followed by a slower

step that either involves a conformational change or an altered degree of membrane insertion [18]. Membrane-docking models were also proposed by Shao et al. based on their structural information on the C2 domain, suggesting that a slight conformational change in C2 maybe can enhance the preference for PS [20].

Indeed, the inherent structural recognition features of lactadherin have been found particular effective in highlighting PS exposing cells. Accordingly, several studies comparing PS binding abilities has led to the supposition that lactadherin holds PS binding features superior to annexin V. e.*g*., the PS binding of annexin V is Ca^{2+} dependent, making it a rather unfavorable probe in many experimental set-ups [22], whereas lactadherin PS binding is calcium independent [23]. The annexin V requirement for Ca^{2+} should in particular be considered in flow cytometry set-ups when studying PS exposing microparticles, since calcium-phosphate microprecipitates have been reported to mimic microparticles. Microprecipitates can thereby increase the annexin V signal and potentiates false-positive cytometry results [24]. In addition, annexin V is under some conditions internalized by both viable and apoptotic cells by a PS dependent mechanism also potentiating imaging of false-positive cells [23, 25, 26].

Lactadherin is remarkable sensitive to very low percent of PS in membranes. Studies on lactadherins PS binding kinetics by stopped-flow fluometry, have shown that lactadherin is capable of detecting as little as 0.03 % PS in synthetic phospholipid vesicles [18]. But, lactadherin also seems to reflect the membrane amount of PS more competently than Annexin V. Part of the evidence for that comes from experiments investigating the binding of fluorescent labeled annexin V and/or –lactadherin using glass bead supported synthetic membranes with increasing PS content balanced with the neutral phospholipid, PC [23]. The PS binding of the two probes were evaluated by flow cytometry and demonstrated that lactadherin bound PS over the entire interval (2–12 % PS), while annexin V first detected PS when the amount in the membrane reached 8 % (Fig. 2.3a). However, when the PS content was supplemented with an increasing amount of phosphatidylethanolamine (PE), the PS threshold for annexin V was lowered to approx. 2.5 % (Fig. 2.3b) [23]. Earlier reports has in accordance with this study showed that annexin V's membrane binding is enhanced when PE is present in the membrane [27], whereas lactadherin has been reported not to exhibit any altered binding to PS regardless of the presence of PE [28]. However, later studies have contrary reported that PE does increase lactadherins affinity for vesicles but only in the presence of PS [18]. Lactadherin binding to vesicles containing the likewise anionic phospholipid, phosphatidylglycerol, have also been tested, however, no interaction could be detected [18]. Another feature of lactadherins PS binding, also valid for its C2 domain alone, is its preference for PS in highly curved membranes [18, 19, 29]. This binding-property is often observed on apoptotic membrane blebs with high curvature, where the lactadherin stain is particular prominent (Fig. 2.4). Annexin V, prefers in contrast to lactadherin flat membrane patches [30].

Detection of PS exposure on apoptotic cells using labeled lactadherin has in several immortalized cell lines showed PS display at earlier states in apoptosis than the amount detectable by annexin V [23, 26, 31, 32]. Moreover, procoagulant PS

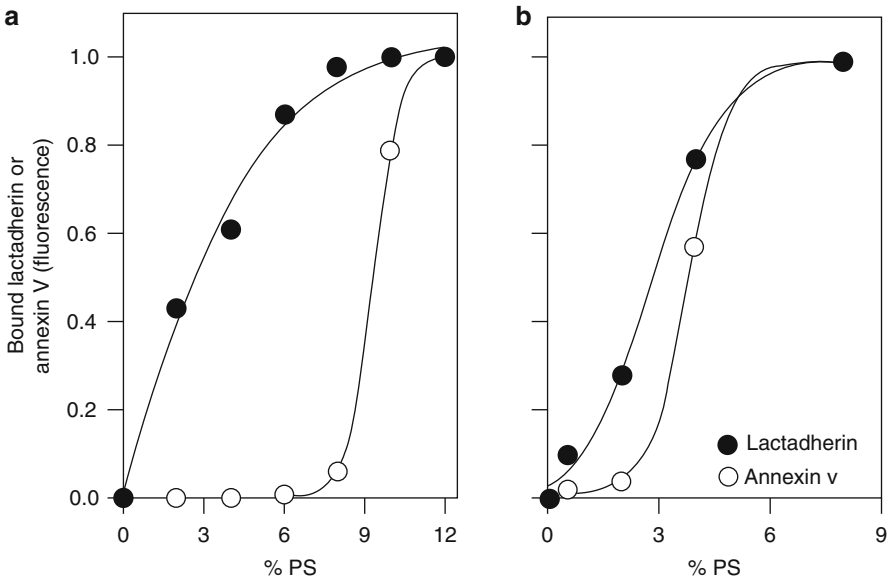

Fig. 2.3 Binding studies on lactadherin and annexin V on synthetic phospholipid vesicles supported by glass beads. (**a**) Lactadherin detects PS over the entire interval while annexin V needs 8 % PS before it reports PS on the vesicles. In this experiment the vesicles were supplemented with a constant level of 2 % PE. (**b**) However, supplementing the vesicles with PE:PS ratio of 4:1, the PS recognition of annexin V is enhanced (With permission from Shi et al. [23])

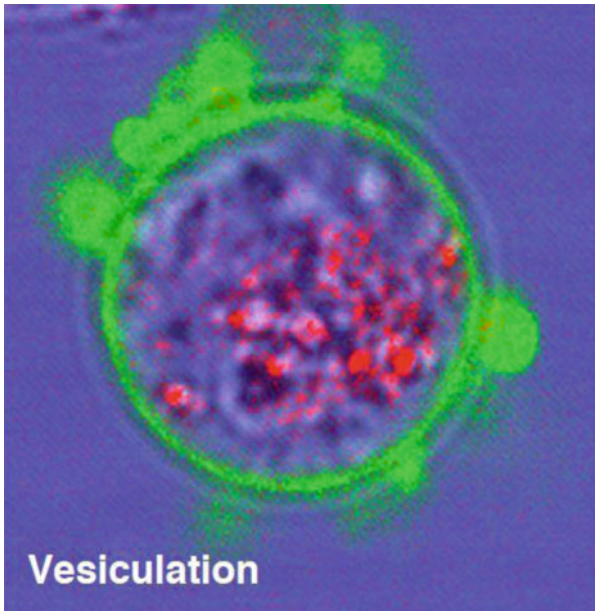

Fig. 2.4 Lactadherin and annexin V stain on apoptotic HL-60 cells. PS exposure and topography detected on apoptotic HL-60 cells by co-staining with fluorescent labeled lactadherin (*green*) and annexin V (*red*). The picture is an overlay of confocal and phase contrast microscopy (With permisson from Shi et al. [23])

exposure on activated platelets and red blood cells is also detected earlier using lactadherin compared to that detected by annexin V [31]. Figure 2.4 shows how fluorescently labeled lactadherin (green) and annexin V (red) stains exposed PS on apoptotic HL60 cells after 24 h induction with etoposide. While lactadherin stains the whole surface of the apoptotic cell, and especially prefers apoptotic membrane blebs of high curvature, annexin V localized to internal bodies as its primary location. Later in apoptosis, annexin V likewise stains the dying cells plasma membrane [23]. That annexin V localizes to internal bodies is in agreement with the fact that the protein have been identified to be obligate intracellular, binding to the intercellular part of the β_5 integrin receptor subunit [33]. Also preapoptotic HeLa cells treated with staurosporine is detected by lactadherin at an earlier time point than annexin V [32]. Besides stain on plasma membrane rims and blebs revealed by both probes, lactadherin did in this study also identify PS on long filopodia-like structures, whereas annexin V was observed in granule-like structures in agreement with the above mentioned study in apoptotic HL60 cells.

A number of small molecules and peptides have been developed for PS detection. The design of these molecules has mostly been based on annexin Vs PS binding motif [34–37]. Few years ago, cyclic peptides mimicking lactadherin (cLac) were designed, and found specific and effective in staining PS containing membranes as well as early apoptotic cells [22]. The group designing these peptides focused on the critical hydrophobic residues for lactadherins PS binding identified by Shao et al.'s structural studies, as well as the hydrophilic residues proposed to be engaged in specific polar interactions with the PS head group, providing the lipid specificity of the lactadherin C2 domain. Due to lactadherins excellent abilities in detecting PS in membranes, numerous studies have applied the protein or derivatives of its C2 domain to explore processes in which PS is involved. Light has been shed on the mechanism of apoptotic PS exposure, showing that the exposure is graded during the cell death program in different adherent cell lines. Initially, PS is localized on ruffled regions on membrane bound projections and small vesicles, in accordance with lactadherins preference for highly curved membranes [29]. Later, PS exposure is increased and generalized over the cell surface, as the membrane asymmetry collapses and enables intense lactadherin staining [23, 26]. The initial partly exposure of PS suggest that a more regulated mechanism exists other than complete and deathly collapse of the phospholipid asymmetry. Controlled PS exposure might be performed by floppase lipid transporters or by less efficient scramblases shown to vary in activity in different cell types [4].

As an anti-inflammatory protein, lactadherin has also been suggested to inhibit the inflammatory component and PS dependent, secretory phospholipases A2, in digesting membrane phospholipids in apoptotic human leukemia cells [38].

The PS distribution in cells organelles has also been explored in recent years by applying a lactadherin-C2 domain fused to green fluorescent protein transfected into different eukaryotic cell lines [39, 40]. By this procedure, Yeung and colleagues reported that the endogenously expressed PS-probe was able to visualize accessible PS molecules on the cytosolic leaflet of the plasma membrane, and that PS also is found on endosomes and lysosomes. Neither mitochondria, nor the Golgi complex

or the endoplasmic reticulum stained significantly with PS in this study [39]. That might indicate that PS is confined to the luminal side of organelles early in the synthesis of the phospholipid, and before they reach the endocytic transport system, in agreement with the externalized PS on endosomes. A few years after, another study investigating the intracellular PS distribution reported similar findings [40]. PS was here found to be associated with caveolae and the trans-Golgi network. Additionally this labeling pattern was compared to the cellular distribution of PS visualized by a novel on-section technique. This technique revealed PS in the interior of the endoplasmic reticulum, Golgi complex, and mitochondria [40], supporting the model in which PS first is exposed by transmembrane flipping to the cytosol in the trans-Golgi network where cargo is shipped off into the endocytic transport system.

In later years, even more versatile use of lactadherins PS recognition has been demonstrated. PS exposing exosomes, nanometer-sized membrane vesicles, have been recognized as significant vehicles for intercellular communication, as potential diagnostic markers, and structures that can be engineered to carry therapeutic molecules and direct them to reach a target destination. Besides exosomes, circulating microparticles are known to correlate with increased risk for thrombosis in cardiovascular diseases [41]. A common feature characterizing shed membrane vesicles is their PS display, why lactadherin have been found helpful in identifying their presence in biological samples [42]. FITC labeled lactadherin have moreover been used to study microparticles in patients with antiphospholipid syndrome reporting an increased level of PS exposing microparticles in patients compared to control individuals [43]. In a case of amyotrophic lateral sclerosis, a progressive neurodegenerative disorder, a patient was with FITC-labeled lactadherin found to display over 100 times more PS exposing microparticles in his cerebrospinal fluid than healthy control subjects [44]. The authors suggested that the microparticles reflected PS exposing fragments from dying motorneurons, and that it might be used as a biomarker for early diagnostic purposes, as the significant number of PS exposing particles contribute to the inflammatory processes. Another recent study using FITC label and flow cytometry as well, have implied that patients diagnosed with schizophrenia have an elevated number of PS exposing microparticles in the blood in comparison with healthy control subjects [45].

3 Lactadherin as a Diagnostic Marker

Apoptosis, and the following disposal of apoptotic bodies, is an everyday-event in multicellular organisms with indisputable significance for tissue homeostasis. Dysregulation of apoptotic processes can be triggered by a variety of pathophysiological stimuli and is associated with many human diseases [46]. Moreover, medical treatment can by itself induce apoptosis, *e.g.* by chemotherapeutics or radiation therapy. Obviously, visualization and information about locations of apoptotic events in a non-invasive manner, is a desirable technique in diagnostics. Naturally, lactadherins PS binding properties have been suggested for application in the development of such a probe.

In 2007, the first synthesis of radio isotope-labeled lactadherin was reported for apoptosis detection. Lactadherin purified from bovine milk was conjugated to the complexing agent for technetium-99m, HYNIC (Hydrazinonicotinamide) and subsequently labeled with ^{99m}Tc. ^{99m}Tc-HYNIC-lactadherin was found to be stable for at least 5 h when supplemented with 1.5 mg/mL BSA. The radiolabeled lactadherin was found to retain its native PS binding capacity, which was verified by its binding to apoptotic HL60 leukaemia cells. This binding could almost completely be prevented by adding a surplus of unlabeled lactadherin, showing that the binding is mediated by properties equivalent to native lactadherin. Moreover, the binding was reported to be unaffected by the addition of high amounts of RGD tripeptides, indicating that the lactadherin probe's binding was not mediated by integrins [47]. In 2010, a study took the lactadherin probe a step further in the direction for *in vivo* quantification of apoptosis by reporting data on its tracer properties [48]. Moreover, the same publication also reported an improved procedure for increasing the radiochemical purity of labeled lactadherin that neglected the need for stabilizing the isotope with BSA. Radio-labeled ^{99m}Tc-HYNIC-lactadherin was injected into 15 mice and the biodistribution of the probe was followed over 3 h. Mice were divided into three groups, and sacrificed after 10, 60 and 180 min after injection. Using a gamma counter the distribution of ^{99m}Tc-HYNIC-lactadherin was evaluated in the different mouse organs. ^{99m}Tc-HYNIC-lactadherin was found primarily to localize to the liver (60 %) and kidneys (4–5 %). Due to the high uptake in the liver, the obvious conclusion was that the applied version is a less suitable probe for *in vivo* studies of apoptosis in the liver and adjacent organs, *e.g.* the heart. The biodistribution of an identical labeled version of annexin V was in the same study found primarily in the kidneys of nine mice injected with the annexin V probe [48].

Recently, a comparative study has looked into the kinetics of ^{99m}Tc-HYNIC-lactadherin and ^{99m}Tc-HYNIC-Annexin V in pigs [49]. This study reported that ^{99m}Tc-HYNIC-lactadherin was cleared four times faster from the pigs' plasma than ^{99m}Tc-HYNIC-AnnexinV. However, similar to the mouse study, ^{99m}Tc-HYNIC-lactadherin preferentially localized to the liver, whereas ^{99m}Tc-HYNIC-Annexin V was primarily taken up by the kidneys. This publication also reported that the estimated effective dose after a single injection of ^{99m}Tc-HYNIC-lactadherin is in the clinically acceptable range, although recommendations regarding its clinical use must await studies in patients [49]. The high uptake of the lactadherin probe in the liver might be due to the organs specialized macrophages named Kupffer cells. Lactadherin functions as an opsonin of PS displaying cells and membrane vesicles by bridging them to integrin receptors on phagocytosing cells, e.g. the $\alpha_v\beta_3$ integrin on Kupffer cells in the liver [50]. Lately, ^{99m}Tc-HYNIC-lactadherin has been tested as tracer for renal cell injury in a pig model. However, the lactadherin probe was in this study not able to detect apoptosis in the post-ischemic kidneys despite an elevated number of caspase-3-positive cells. No difference was found in the uptake between a blood flow marker and lactadherin, suggesting that lactadherin might just have followed the blood supply why lactadherin probably is a less functional PS-probe under conditions like those described in the experimental set-up [51].

In rabbit hearts, ischemia followed by reperfusion results in PS exposure on myocytes without signs of apoptosis or necrosis [25]. Accordingly, radio-labeled lactadherin have also been tested for its function as a marker for ischemic areas at risk. Another set of new results suggest that ^{99m}Tc-HYNIC-lactadherin can be used as a sensitive marker for area at risk in myocardial ischemia [52]. This study showed that the myocardial uptake of radio-labeled lactadherin closely correlated with the area at risk (visualized by Evans blue) and the infarct size when injected 30 min after reperfusion in a pig model of myocardial ischemia. Accordingly, these results emphasizes that imaging of PS exposure by radio-labeled lactadherin might also be a novel technique in quantifying area at risk following ischemia and reperfusion, without apoptosis and necrosis as a matter of course, but also as a marker for stressed cells.

Cell communication via soluble mediators or cell-cell contact is well documented. However, in recent years a tremendous attention has been put on PS exposing extracellular vesicles in nanoscale and their importance in shipping proteins and nucleic acids between cells over short and long distances. Cell-derived vesicles have been shown to induce immune tolerance, T cell apoptosis, metastasis and angiogenesis. In particular membrane particles named exosomes, characterized by a diameter of 50–100 nm and a density between 1.1 and 1.19 g/mL, have received great attention since evidence exists for their role in shipping molecules with importance in tumor progression to target cells [53]. Even though science is getting closer to understand their physiological function, membrane particles is still mysterious nanosized parcels transferring rather blurred messaged between cells. However, the identification of membrane particles in cell communication has given drug delivery scientist a new vehicle to work with. Given that exosomes are nature's own creation in transporting molecules prone to degradation, ways to engineer exosomes have largely been studied for drug delivery purposes [54] where lactadherins PS binding features have been found beneficial. Exosome display is a technology developed to manipulate exosomal display of proteins in order to tailor exosomes with desirable properties, *e.g*. generating antibody responses against difficult targets. Exosomes are known to display PS on their surface, and is also characterized to be associated with lactadherin [53]. Delcayre discovered in 2005 that a murine lactadherin C1C2 domain was capable of addressing soluble antigens or protein domains not naturally found on exosomes to the exosomal membrane (Fig. 2.5). They did this by fusing the lactadherin C1C2 domain, including its N-terminal secretion signal, to a protein of interest. The chimeric construct was then expressed in exosome producing cell lines, resulting in the production of recombinant exosomes that bare the desired construct on their surface. The biological activity of the purified recombinant and protein-loaded exosomes was also confirmed in this study [55].

By using the Delcayre method, tumor antigens have been targeted to PS exposing membrane vesicles, and shown to induce potent antigen-specific antitumor immune responses. Importantly, presenting the antigen on exosome membranes has been found more effective in promoting immune responses compared to the response seen for the same soluble antigen alone [55–57]. Exosomes have been shown to be taken up by antigen presenting cells, and it has therefore been proposed that targeting intracellular antigens to exosomes would increase antigen-trafficking to these cells and stimulate immunogenicity towards the received antigens [55]. Presenting

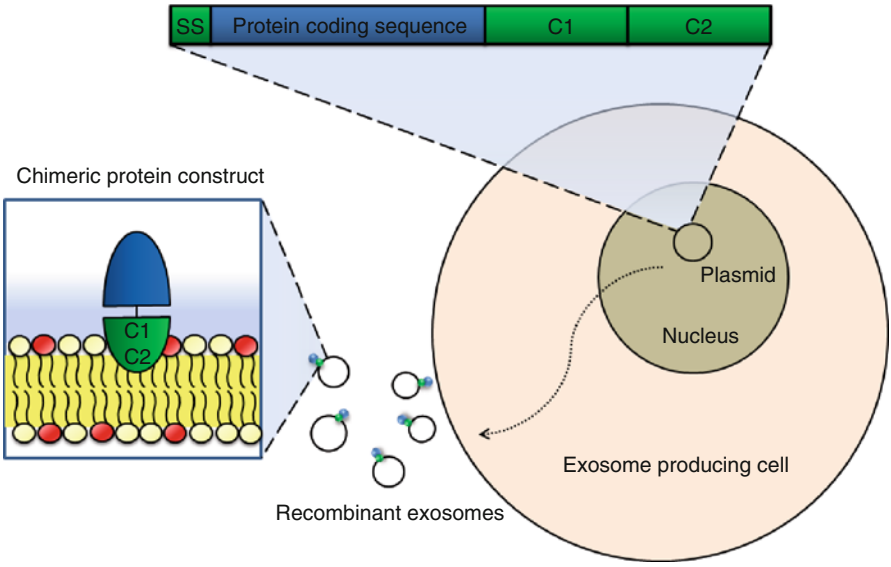

Fig. 2.5 Recombinant exosome targeting strategy using the lactadherin-C1C2 domain. A chimeric construct is generated consisting of the secretion signal from lactadherin (*SS*), a coding sequence of the desired protein, and the C1C2 protein of murine lactadherin. An exosome producing cell line is transfected with the construct whereupon the secretion signal and C1C2 domain directs the chimeric protein to produced PS exposing exosomes, generating recombinant exosomes carrying the protein of interest

antigens on exosomes using lactadherin C1C2, have contributed to the development of a candidate immunotherapy product for the treatment of prostate cancer (MVA-BN® PRO), which is currently in the second half of Phase I clinical trials [58]. MVA-BN® PRO is based on a recombinant virus containing tumor-associated antigens fused to the murine C1C2 domain, which exosome targeting effect has let to at striking increase in immune response, compared to other soluble versions of the prostate-antigens [59]. Another recent publication reports the use of a lactadherin based construct in visualizing and *in vivo* tracking of exosomes in mice for pharmacokinetic analysis. This publication reports the design of a fusion protein consisting of the N-terminal secretion signal of lactadherin, Gaussia luciferase (gLuc), and the C1C2 domain of murine lactadherin [60]. B16-BL6 murine melanoma cells were transfected with the gLuc-lactadherin-expressing plasmid vector and the recombinant exosomes were subsequently isolated and injected into BALB/c mice. The recombinant exosomes were detected by luciferace activity mainly to distribute to the liver and later to the lungs, and to be cleared rapidly from the circulation. When only the gLuc-lactadherin construct was injected, a somewhat uniform distribution of label was observed throughout the mouse body, showing that the chemiluminescence signal did not derive from release of free label from the exosomes. Therefore, a chimeric construct consisting of gLuc and lactadherin-C1C2 might work well as a novel exosome tracer.

4 Lactadherin and Blood Coagulation

Coagulation is an important and quite complex part of hemostasis, which is the physiological process occurring to stop bleeding after damage to a blood vessel. Platelets are tiny cells without a nucleus circulating in blood that helps in forming clots when activated at sites of vessel injury [61]. Upon activation of platelets by collagen and thrombin, or non-physiological levels of Ca^{2+}, rapid dissipation of phospholipid asymmetry leads to PS exposure on their surface by mechanisms equivalent to the above described. PS display on platelets is a major event of hemostasis because it supports clotting procoagulant function by its anionic surface, providing a platform for binding of blood clotting enzyme complexes, leading to the production of thrombin and fibrin clots [62, 63]. Factor V and VIII are membrane binding blood coagulation factors recognizing exposed PS on activated platelets through their C domains, which are homologous to the C1 and C2 domains of lactadherin. These two coagulation factors are the foundation of the Xase and the prothrominase complex, respectively, working together in the coagulation cascade [64]. Naturally, annexin V has been the probe of choice in monitoring procoagulant PS exposure [65, 66]. A number of comparison studies between the annexin V and lactadherin have been performed in order to evaluate their potential as investigative tools and behavior during blood coagulation and anticoagulant characteristics. A whole line of studies have been performed using fluorophore-conjugated bovine lactadherin and recombinant annexin V monitored with flow cytometry or visualized by fluorescence microscopy. The first report from 2003 on the anticoagulant properties of lactadherin revealed that the bovine derived protein efficiently inhibited the prothrombinase and factor Xase complexes by competing for PS binding sites with factor VIII and V on synthetic glass bead supported phospholipid membranes. In comparison, the tests showed that annexin V less efficiently inhibited the binding of the enzyme complexes opposed to lactadherin. Adding lactadherin to whole blood did also result in a prolonged clotting time, confirming lactadherins potential as an anticoagulant [28]. Consistently, bovine lactadherin has lately been shown to compete for both factor X and Xa binding sites, limiting factor X activation and forcing the release of bound factor Xa from membranes [67]. Figure 2.6 outlines the coagulation cascade and lactadherins anticoagulant action points. Correspondingly, the bovine lactadherin C2 domain alone has been reported to compete for Factor VIII binding sites, inhibiting most of factor Xase activity, as well as for factor V binding sites, resulting in an 82 % decrease in prothrombinase activity. However, full length lactadherin still showed a superior anticoagulant ability in the same study [19].

Limited PS amounts, compared to a total exposure of the phospholipid on viable platelets, have also been shown to support coagulation reactions [68, 69]. As much as 95 % of purified platelets were in 2008 reported in a publication by Shi et al. to express low quantities of PS detected by fluorescence-labeled lactadherin in flow cytometry when stimulated with thrombin or low concentrations of a calcium ionophore. However, the low PS display was only detected by lactadherin and not annexin V, underscoring that absence of measurable annexin v not should be

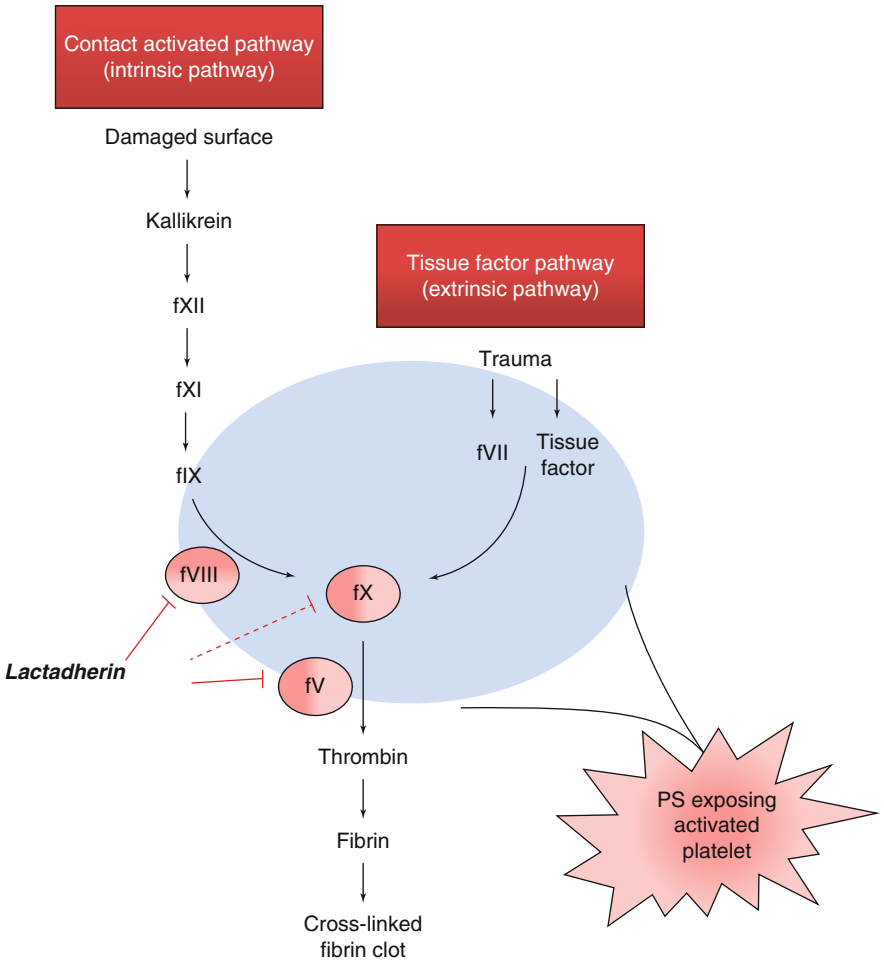

Fig. 2.6 The coagulation cascade of secondary hemostasis. Two coagulation pathways lead to fibrin formation; a contact activated pathway and a tissue factor pathway. Both pathways consist of a series of reactions involving the downstream activation of inactive coagulation factors. Activated platelets expose PS on their surface that provides a platform for coagulation complexes to form allowing their conversion into active serine proteases. Lactadherin is capable of inhibiting the binding and activity of complexes containing the membrane binding factor VIII and factor V, thereby inhibiting the activity of the factor Xase complex that generates thrombin

interpreted as absence of PS exposure[70]. Shi et al. also reported the anticoagulant properties of lactadherin in a mouse thrombosis model. In addition, the *in vivo* pattern of platelet PS exposure was shown to be partial in damaged blood vessels in thrombotic mice. The PS exposure was only detectable by lactadherin, while annexin V still was not capable in staining the low amount of the exposed negatively charged phospholipid. Furthermore, blockade of PS with lactadherin inhibited platelet prothrombinase and factor Xase activity, prolonging tail bleeding time, and the time before carotid artery thrombosis in these mice [70].

Underestimation of PS exposure on platelets by annexin V has also been addressed in two thorough studies looking into PS exposure during storage of platelet concentrates [71, 72]. Using flow cytometry, FITC conjugated lactadherin and annexin V were compared in monitoring platelet activation in up to 9 days old platelet concentrates. The conclusion of the two independent studies was that lactadherin detected a significantly higher number of PS exposing platelets, and procoagulant microparticles than annexin V managed to do. Compared to previous reports, 10 % more platelets were shown to expose PS by using lactadherin as probe vs. annexin V at the end of the storage time [71]. During platelet senescence a time dependent incline in PS exposure starts when platelets has been stored for 3 days. After 5 days, 17 %, followed by 33.6 % on day 7, and 41 % were reached on day 9 [72]. Since high platelet PS exposure equals high procoagulant activity in stored platelet concentrates, the shelf life of stored platelets probably should be reconsidered for avoiding coagulopathy in clinical settings. The publication by Hou et al. also reports the morphological changes associated with PS exposure on platelets and microparticles which were similar to the ones observed when lactadherin stains apoptotic cells [29]. Also Hou et al. reported lactadherins superior abilities in inhibiting procoagulant activities of stored platelets compared to annexin V.

PS display on platelets, detected by lactadherin, has also contributed in demonstrating that platelet cytrochrome c release and the subsequent activation of caspase-3 and ROCK1 mediates the exposure of PS on stored platelets, and that this mechanism is distinct from activation-induced PS exposure. This knowledge could contribute to the development of new strategies in preventing PS exposure improving the recovery of stored platelets after transfusion [73]. The PS probe has also contributed to show a significant change in apoptosis regulation in cold-rewarming of stored platelets, involving changes in glycoprotein Ibx-distribution, and downstream events favoring apoptosis induction, including PS exposure [74]. Also deterioration of long-term storage of red blood cells has been addressed as a procoagulant problem with high relevance in transfusion medicine. In 2011 a significant correlation was reported between PS exposure and procoagulant activity of red blood cells starting after 14 days storage, which demonstrated that red blood cells most likely have a critical role on their own as procoagulant squads [75]. The anticoagulant properties of lactadherin were in this study shown to be transferable to the stored PS exposing red blood cells.

Increasingly awareness about lactadherins excellent abilities in reflecting and highlighting PS exposure, as well as being a useful PS blocker with anticoagulant properties, is stated in the increased number of publications reporting its use in hemostasis research. The following references in hemostasis studies have all applied lactadherin in either PS detection, as a PS blocker, or as both, in investigating the significance of PS in blood coagulation under normal and pathological conditions [50, 76–82]. Awareness of the characteristics of lactadherin has come a long way since the first descriptions of its abilities to bind PS specifically in phospholipid membranes. Studies have been performed addressing the hypothesis that bovine lactadherin effectively can be used as a tool for imaging of PS exposure; especially done in comparison with the hitherto known benchmark PS-probe annexin V.

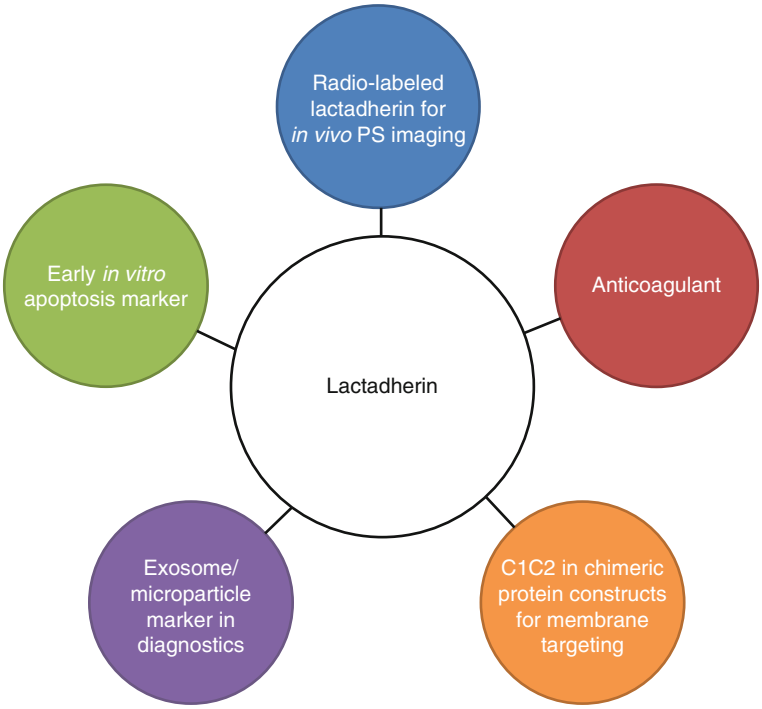

Fig. 2.7 Outline of lactadherins versatile uses as a PS binding protein

Experiments have clearly shown that lactadherin is a reliable probe for PS exposed on *e.g* activated, stressed and/or dying cells. In some cases it might even give a more true reflection of the amount of exposed PS. Forthcoming studies will hopefully challenge this current impression, and it will be interesting to see whether the applicability of lactadherin as PS-probe improves or undiscovered knowledge will call for lead to more limited use. Figure 2.7 Outlines lactadherins versatile use as a PS binding protein.

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Chapter 3
Role of MFG-E8 in Protection of Intestinal Epithelial Barrier Function and Attenuation of Intestinal Inflammation

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Abstract The intestinal epithelium is a continuous single layer of cells that lines the intestinal tract. It provides a physical barrier that separates internal body compartments and the harmful environment of the gut lumen. Defects in epithelial barrier and immune functions can lead to infections with opportunistic and pathogenic microbes and contribute to the pathogenesis of inflammatory bowel disease (IBD). Recent studies have shown that macrophages in the underlying intestinal tissue produce milk fat globule-EGF factor 8 (MFG-E8) which directly targets intestinal epithelial cells and regulates the integrity of intestinal epithelial barrier function.

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This chapter outlines our current knowledge about the role of MFG-E8 in maintaining the homeostasis of intestinal epithelial cell lining and highlights potentials for applications of MFG-E8 in gastroenterological diseases.

Keywords MFG-E8 • Macrophages • Intestine • Homeostasis • Inflammation

1 Introduction

Milk fat globule-epidermal growth factor-factor 8 (MFG-E8, lactadherin homolog in humans, BA46 or SED1), a membrane associated integrin-binding glycoprotein, was first discovered in milk and mammary epithelial cells [1]. Shortly after its discovery, MFG-E8 was shown to inhibit the replication of rotavirus in tissue culture and prevent the gastroenteritis in an animal model system [2]. This initial finding led to an increased interest in MFG-E8 and researchers around the globe started to unravel its structure, properties and functions.

MFG-E8 (53- and 66-kDa) contains two EGF (epidermal growth factor)-like domains on the N-terminal side and two factor-VIII-homologous (C1 and C2) domains on the C-terminal side [3]. It has two alternative splice variants based on the presence or absence of a proline/threonine-rich domain [4]. The RGD (arginine-glycine-aspartate) motif in the second EGF-like domain of MFG-E8 facilitates the binding to cells expressing some members of the integrin family of receptors, including $\alpha_v\beta_3$ and $\alpha_v\beta_5$. It recognizes phosphatidylserine through its C1 and C2 domains [5].

A decade of research has revealed the presence of MFG-E8 in various cells and tissues. MFG-E8 is expressed by splenocytes, monocytes, dendritic cells, macrophages and other cell types [4, 6–14]. On the other hand, a low or no expression of MFG-E8 is detected in thymocytes [15]. Under normal condition, MFG-E8 mRNA is expressed ubiquitously in almost all organs including brain, lung, mammary glands, spleen, lymph nodes, and intestine [16] but its protein level is undetectable by western blot in normal mouse liver (Reviewed in [15]). MFG-E8 is expressed as short and long isoforms in mouse and rat tissues [4, 17]. The short isoform is produced more abundantly than the long one. Although a single isoform was reported in humans earlier, recent NCBI gene bank data has suggested the existence of two different isoforms [15].

2 MFG-E8 and Apoptotic Cell Clearance

Probably the best characterized function of MFG-E8 till date is its role in mediating phagocytosis by macrophages. Molecular characterization and cloning of MFG-E8 was first reported in the early 90s. However, recognition of the role of MFG-E8 as

an opsonin for the phagocytosis of apoptotic cells by macrophages did not come until 2002 [4].

Apoptosis occurs in the physiological state and inflammatory diseases. Apoptotic cells can release potentially noxious or immunogenic intracellular materials which are able to cause persistent inflammation and necrosis of surrounding tissues. Thus, it is critical to rapidly eliminate dying cells from tissues to prevent or limit inflammation. Cells generally maintain an asymmetric distribution of phospholipids across the plasma membrane bilayer, restricting phosphatidylserine to the inner leaflet of the plasma membrane. In dying cells, this asymmetric transbilayer distribution is lost, thus exposing phosphatidylserine on the outer leaflet of the plasma membrane where it acts as 'eat-me' signals for engulfment by phagocytes. It has been demonstrated that MFG-E8 binds apoptotic cells by recognizing phosphatidylserine through its C2 domain. When MFG-E8 is bound to phosphatidylserine, it also binds to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins expressed on phagocytes via an RGD motif in its second EGF domain. Thus, MFG-E8 forms a bridge between apoptotic cells and phagocytes and promotes the phagocytosis of apoptotic cells [4]. By providing indispensable role in the scavenging of apoptotic cells by macrophages, MFG-E8 has proved to be a critical factor in the resolution of inflammation (Reviewed in [15]).

Using MFG-E8 knockout mouse model, Hanayama et al. demonstrated the accumulation of apoptotic cells in spleen, which subsequently resulted in dysregulated immune functions with abnormal homeostasis [16]. The MFG-E8 deficient mice show autoimmune disease-like human systemic lupus erythematosis due to the impaired uptake of apoptotic cells by macrophages and the accumulation of these apoptotic cells [16, 18]. Interestingly, we found that MFG-E8 deficient mice have no defect in clearing apoptotic cells in the intestine under normal physiological state [19], suggesting that the role of MFG-E8 in the gut is not related to promoting the phagocytosis of apoptotic cells.

3 Intestinal Epithelial Barrier

A 20 μm thick intestinal epithelium is a single layer of cells lining the gut lumen constituting one of the most important physical barriers separating the lamina propria from the gut lumen where more than 500 different species of bacteria thrive [20]. The differential permeability and integrity of the epithelial barrier is of principal importance as it allows the absorption of water and nutrients while preventing the intraluminal bacteria, toxins, and potentially antigenic material from gaining access into the underlying tissue. Intestinal epithelial cells proliferate rapidly at the crypt and the new cells generated then move from the crypt towards the tip of the villi differentiating themselves along the way. Following maturation, the cells are sloughed off into the intestinal lumen and then replaced by new cells [21]. A delicate and tightly controlled regulation of epithelial cell proliferation and cell death maintain intestinal epithelial layer homeostasis. Abnormal increase or decrease in epithelial cell proliferation or cell death may compromise the epithelial barrier and may also cause cells to amass into tumors [22].

Epithelial cells in the intestinal epithelial layer are joined together by tight junctions (TJ). TJ is a complex of membrane-bound proteins, e.g., occludin and claudins, and their adaptor and scaffolding proteins, e. g., junctional adhesion molecule, ZO-1, ZO-2 and ZO-3. These proteins form a structure at the boundary of two adjacent cells working as a barrier within the epithelial cellular space. The TJ proteins are dynamic rate-limiting step in the paracellular pathway which selectively facilitates the passage of solutes, fluid and other nutrition elements while preventing the translocation of luminal bacteria and their toxins [23]. Over the past decade, increasing evidence has suggested that the altered intestinal epithelial barrier function is a central predisposing factor in the development of inflammation in the gut mucosa and remote sites. However, the mechanisms involved in maintaining homeostatic intestinal barrier integrity remain undefined.

4 MFG-E8 and Intestinal Epithelial Barrier Function

4.1 MFG-E8 Plays an Important Role in Maintaining Intestinal Epithelial Homeostasis

Among various essential glycoproteins found in the intestine, e.g., mucin, dickkopf-1 and lactase, MFG-E8 is one of the most important and less studied one. We have shown that MFG-E8 is constitutively expressed by macrophages in the lamina propria of murine small intestine under normal physiological conditions [19]. Furthermore, we found that the physiological function of MFG-E8 in the gut is involved in promoting intestinal epithelial cells migration along crypt-villus axis. Using a wound-healing assay, MFG-E8 was shown to promote the migration of intestinal epithelial cells through a PKCε-dependent mechanism. MFG-E8 bound to phosphatidylserine and triggered reorientation of the actin cytoskeleton in intestinal epithelial cells at the wound edge. Moreover, we demonstrated that intestinal MFG-E8 is downregulated in septic mice, which in turn impairs intestinal mucosal repair in sepsis. Together, our data indicate that MFG-E8 plays an important role in the maintenance of intestinal epithelial homeostasis and the promotion of mucosal healing.

4.2 MFG-E8 and Acute Intestinal Injury

Acute gastrointestinal (GI) barrier dysfunction remains a critical clinical problem. Acute intestinal injury contributes to morbidity and mortality of hospitalized patients in the intensive care unit and surgical department. Critical conditions such as burn and major trauma can result in intestinal inflammation and subsequent mucosal injury leading to the development of severe systemic inflammation and multiple organ failure [24].

During sepsis, MFG-E8 expression has been found to be decreased in multiple organs such as intestines, lungs, and the spleen [19, 25, 26]. Previously, we demonstrated that microscopic injury of the small intestine occurred under septic condition in mice [19]. In addition, we showed that sepsis-triggered intestinal injury is associated with a downregulation of intestinal MFG-E8 and a delayed enterocyte migration along the crypt-villus axis [19]. Furthermore, it was found that treatment with MFG-E8 results in restoring intestinal epithelial migration along the crypt-villus axis in cecal ligation and puncture (CLP)-challenged mice [19]. Together, these findings suggest that MFG-E8 is essential for the maintenance of intestinal mucosal homeostasis and integrity in septic condition. Recombinant MFG-E8 may have a great potential in the prevention and treatment of intestinal injury in patients with severe systemic inflammatory response.

Several lines of evidence indicate that intestinal barrier is impaired following acute alcohol exposure [27, 28]. Acute alcohol exposure severely inhibits phagocytosis and killing of bacteria by macrophages. It enhances sepsis-induced MFG-E8 down-regulation *in vivo* [29]. We demonstrated that acute alcohol exposure inhibits MFG-E8 promoter activity and gene expression in macrophages via an oxidant-dependent mechanism [30], which may mitigate the role of macrophage maintaining intestinal barrier function.

Inflammation of the GI tract can also be caused by bacterial and viral infection. It is manifested by the sudden onset of nausea, vomiting, and diarrhea. Bacteria that cause gastroenteritis include *Escherichia coli*, *Salmonella*, *Campylobacter*, and *Shigella*. Viruses that trigger gastroenteritis include adenovirus, calcivirus, astrovirus, norovirus and rotavirus. Both bacterial and viral gastroenteritis can be transmitted to humans through contact with an infected person or ingestion of contaminated food or water. While bacterial gastroenteritis can be treated with antibiotic regimen, there is no effective treatment for viral gastroenteritis. Viral gastroenteritis can be fatal in infants, elderly and people with compromised immune system [31]. A high incidence of morbidity and mortality among infants and young children throughout the world is attributable to acute intestinal infections triggered by rotaviruses. Highly stable triple layer capsid of rotaviruses facilitates its transmission by the fecal-oral route into the small intestinal villi where it infects mature enterocytes resulting in diarrhea [2, 32]. While there is no specific treatment for a rotavirus infection, lactadherin/MFG-E8 was found to inhibit rotavirus binding and infectivity [2]. Feeding animals with milk components, containing MFG-E8, completely prevented the rotavirus induced gastroenteritis [2]. Furthermore, Newburg et al. revealed the role of lactadherin/MFG-E8 in protection against symptomatic rotavirus infection in humans [33]. However, molecular mechanisms by which MFG-E8 inhibits rotavirus replication and prevents gastroenteritis remain to be explored.

Gut ischemia-reperfusion (I/R) injury is associated with high morbidity and mortality in surgical and trauma patients. Ischemia causes interruption of blood supply resulting in tissue injury, whereas restoration of blood flow or reperfusion to the ischemic tissue exacerbates the injury. I/R injury induces systemic inflammatory response, exceeds the original ischemic insult, and causes damage to the intestinal mucosa [34]. Wu et al. found that intestinal levels of MFG-E8 decreased after gut

I/R injury correlated with increased apoptosis and impaired barrier function [35]. Treatment with recombinant MFG-E8 mitigated mucosal destruction, loss of villi and epithelial cells, hemorrhage, infiltration of inflammatory cells and bacterial translocation [35]. Intestinal I/R injury not only decreased intestinal levels of MFG-E8 but it also resulted in suppression of MFG-E8 expression in spleen and lung. Following intestinal I/R, inflammation and injury was not restricted to intestine alone; it caused severe widespread injury and inflammation of lungs, liver and kidneys. Treatment with recombinant MFG-E8 significantly suppressed pro-inflammatory mediators TNF-α, IL-6, IL-1β and neutrophil infiltration in these organs [36]. Accumulation of apoptotic cells potentiates organ injury after intestinal I/R. However, treatment with recombinant MFG-E8 enhances the apoptotic cell clearance resulting in overall survival of the animals after intestinal I/R injury.

Acute radiation syndrome (ARS) is an acute illness caused by exposure of the body to a high dose of ionizing radiation in a very short period of time. One of the three classical ARS syndromes is GI syndrome. In its prodromal stage, the GI syndrome occurs within a few hours after radiation exposure resulting in anorexia, severe nausea, vomiting, cramps and diarrhea. This subsequently leads to loss of intestinal crypts and breakdown of mucosal barrier predisposing patients to further infection [37]. Interestingly, apoptosis does not play a role in the death of intestinal epithelial cells following GI syndrome, nevertheless it is regulated by tumor suppressor protein p53 [38]. Treatment of rats with recombinant MFG-E8 after ARS confers a therapeutic advantage by up regulating p53. While preserving intestinal structure and function, treatment with recombinant MFG-E8 following ARS improves overall survival rate [39]. *In vitro* studies also revealed that recombinant MFG-E8 protected IEC-6 cells from radiation-induced cell death [39].

4.3 MFG-E8 and Chronic Intestinal Injury

Chronic GI diseases and disorders account for substantial morbidity, mortality and financial burden in the United States [40]. Ulcerative colitis is a chronic inflammatory disease affecting the large intestine. It is associated with diarrhea, abdominal pain, bloating, and blood in the stool [41]. It is a chronic lifelong condition with no definitive cure. Anti-inflammatory drugs and immune system suppressors provide temporary relief to patients [41].

Although the role of MFG-E8 in IBD/ulcerative colitis has been demonstrated in Chapter-8 of this book, herein we provide a snapshot highlighting interesting findings from our lab as well as others. Previously, we showed the therapeutic efficacy of MFG-E8 in experimental colitis [42]. We and others found that down regulation of MFG-E8 expression occurs in inflamed colons in an animal model of dextran sulphate sodium (DSS)-induced colitis [42–44]. Administration of recombinant MFG-E8 protected mice against colitis [42–44]. In addition, Zhao et al. found that intestinal MFG-E8 is decreased in patients with ulcerative colitis and thereby causes increased apoptosis and impaired wound healing [45]. Recently, Mishiro et al.

reported that butyric acid attenuates intestinal inflammation in murine DSS-induced colitis model via MFG-E8 [46]. Down regulation of epithelial MFG-E8 is a risk factor in colitis associated with increased apoptosis; recombinant MFG-E8 administration can improve the inflammation associated with colitis.

Expression of MFG-E8 mRNA and protein increase significantly in inflamed colons in the early phase of the DSS treatment period, while it is down-regulated in the late phase of the DSS treatment period [42]. MFG-E8 was shown to have an anti-inflammatory effect on experimental colitis not only by removal of apoptotic cells but also by down-regulating proinflammatory cytokines, e.g., interleukin-1β and TNF-α, through $\alpha_v\beta_3$ integrin-mediated nuclear factor-κB inhibition [43, 44]. Taken together, it appears that MFG-E8 could be a therapeutic compound for treatment and attenuation of colitis.

5 Summary

MFG-E8, a multi-functional protein, is now well recognized as a critical factor for its important role in maintaining homeostatic balance in various organ systems particularly the intestine. In the gut, MFG-E8 protects intestinal barrier and maintains intestinal epithelial homeostasis via promotion of intestinal epithelial restitution and attenuation of inflammation. Human MFG-E8 (i.e. lactadherin) has anti-rotavirus activity. The essential contribution of MFG-E8 in the maintenance of intestinal barrier homeostasis makes it a suitable candidate for continued research interest. The mechanisms of action of the extraordinary molecule MFG-E8 should be further investigated and future efforts can be directed to explore and identify potential strategies for therapeutic intervention to ameliorate tissue inflammation and related disorders.

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Chapter 4
MFG-E8 in the Blood Cell Homeostasis and Coagulation

Swapan Kumar Dasgupta and Perumal Thiagarajan

Abstract Milk Fat Globule-EGF Factor 8 (MFG-E8), a multifunctional glycoprotein originally identified as a product of mammary epithelial cells, has diverse functions in the clearance of apoptotic blood cells, regulation of immune function, microglial phagocytosis, angiogenesis, sperm-egg interaction and possibly in many other physiological functions. With a phosphatidylserine (PS) binding domain at the amino-terminus and a integrin binding motif in the carboxy-terminus, it can anchor PS containing membranes to integrin expressing cells. Current chapter focuses mainly on its anticoagulant effect and on its role on the clearance of PS expressing platelet and red blood cell. It has imperative to define its role in the blood stream as it is being developed as an imaging event to detect apoptosis in vivo and possibly as an anticoagulant.

Keywords MFG-E8 • Blood • Vascular system • Coagulation • Integrin

1 Introduction

Lactadherin or Milk Fat Globule-EGF Factor 8 (MFG-E8) is a 47-kDa glycoprotein, originally described as BA-46, a tumor antigen (BA-46) for breast cancer [1, 2]. It is a component of milk fat globule, a membrane-enclosed collection of proteins and fats that buds from mammary epithelial cells during lactation. Human lactadherin contains an epidermal growth factor (EGF)-like domain at the amino terminus and two C-domains

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at the carboxy terminus that share homology to discoidin-type lectin domains similar to the phosphatidylserine (PS)-binding domains of blood coagulation factors V and VIII [3–5]. The EGF domain contains a RGD (Arg-Gly-Asp) sequence, which is recognized by the integrin receptors such as $\alpha_V\beta_3$ and $\alpha_V\beta_5$ [6–9]. Unlike human lactadherin, bovine and murine lactadherin (MFG-E8) have two amino terminal EGF domains and the second EGF domain has the RGD motif. Though lactadherin is expressed abundantly in lactating mammary gland [6], it is detected in number of other tissues, including activated macrophages [10], brain, lung, heart, kidney and spleen and in and around blood vessels [11–13]. It is present in the plasma at a concentration of $\approx$ 10 ng/ml [8, 14–16]. Hanayama et al. also identified lactadherin as an opsonin that mediates the binding of PS expressing apoptotic lymphocytes to macrophages [10]. Lactadherin-deficient mice have impaired clearance of apoptotic cells and develop autoimmunity [17]. Lactadherin was also identified as a factor facilitating sperm adhesion to the egg coat during fertilization [18]. In addition, Lactadherin is also shown to have role in the clearance of Aβ amyloid by astrocytes [19], diurnal clearance of fragments of photoreceptor outer segments in the retina [20], clearance of apoptotic cells in atherosclerotic plaque [21] and in facilitating phagosome function in dendritic cells [22, 23]. Aortic medial amyloid is a form of localized amyloid that occurs in elderly and its main component, a 5 kDa peptide called medin is derived from lactadherin [11]. Lactadherin is a multifunctional protein with diverse physiological roles. In this chapter, we will concentrate on the role of lactadherin on coagulation and blood cell clearance.

2 Phospholipid in Coagulation

Anionic phospholipids such as PS play an essential role in normal hemostasis by providing sites for the assembly of enzyme-cofactor complexes involved in the enzymatic cascade that results in the generation of fibrin clot [24]. Anionic phospholipid surface accelerates two distinct reactions in the coagulation cascade, activation of factor X by factor IXa (in the presence of factor VIIIa and Ca^{2+}) and activation of prothrombin by factor Xa (in the presence of factor Va and Ca^{2+}), by providing sites for the assembly of enzyme substrate complexes [25–27]. Soluble PS also enhances factor Xa proteolytic activity to a similar extent [28]. This activation occurs below the critical micellar concentration (CMC). Binding of soluble PS to factor Va also enhances its binding to factor Xa. This study indicates that apart from providing a negatively charged surface, PS also allosterically modify factor Xa and Va by binding to their active sites (reviewed in [29]). All these studies establish a principal role of PS in blood coagulation. In vivo, PS is provided by activated platelets or platelet derived microvesicles [29, 30]. In resting platelets, PS and other anionic phospholipids are located on the inner aspect of the membrane bilayer [31]. Following platelet activation PS moves from the inner to the outer leaflet of platelet plasma membrane [30]. This movement is associated with an increase in the activation of prothrombin and factor X [32] Also, the extent of PS exposure on platelets correlates with the thrombin generation. ADP does not lead to PS exposure and its

effect on activation of prothrombin and tenase complexes is minimal. Non physiological agonist calcium ionophore, C5b-9 and thrombin plus collagen causes maximum PS exposure with concomitant increase in tenase and prothrombinase activity [33–37] As mentioned above, factors VIIIa and Va are integral component of the tenase and protrombinase complexes [38, 39]. Both the factors VIII and V are homologous in their amino acid sequence [40–44] and have two discoidin type lectin domains in their light chain that binds stereo selectively and with high affinity (Kd about 2nM) to PS [24, 25, 43, 44]. Furthermore, the vitamin K dependent coagulation factors (prothrombin, factor X and factor IX) also bind anionic phospholipids via the vitamin K dependent GLA domain [27].

3 Lactadherin Binding to PS Resembles Factor VIII and Factor V Binding to Phospholipid

The C domains of lactadherin are highly homologous to the corresponding C domains of cofactors V and VIII. The sequence homology predicts similar structural and functional properties. Lactadherin binds to surface immobilized PS and to vesicles containing physiologic concentration of PS [9, 45, 46] with high affinity similar to factor V and VIII [47–49]. Lactadherin binds phospholipid vesicles containing PS/PE (phosphatidylethanolamine)/PC(phosphatidylcholine) (4 %/20 %/76 %) saturably and with high affinity(KD ~3 nM) [46]. Competition binding studies utilizing small (small unilamellar vesicles, SUV), medium (large unilamellar vesicles, LUV) and large (large multilamellar vesicles, LMV) vesicles with different PS composition suggest that like factor V and VIII [44, 50], lactadherin binds with similar affinity to surfaces with varying degree of curvature. However, smallest vesicles (highest curvature) provided highest binding sites followed by LUV and LMV. Experiments with different concentration of PE and fixed concentration of PS (1 %) indicate that unlike factor VIII [51], PE does not alter the affinity of lactadherin towards PS. Other anionic phospholipids such as phosphatidylinositol does not support binding to lactadherin. PS has two stereoisomers, L-PS and D-PS, both of which have two chiral centers, and they are not enantiomers, but rather called diasteromers. Similar to factor V and factor VII [52, 53], lactadherin binding to PS is stereoselective. Lactadherin binds avidly to L-PS containing SUV coated glass microspheres (lipospheres) whereas binding to comparable D-PS containing lipospheres is similar to control vesicles containing only PE and PC. Diastereomers have same chemical properties but they could differ in physical properties. However, the quantifiable physical properties like calcium mediated aggregation of vesicles containing L-PS or D-PS are similar [54]. In addition, the head group of L-PS, phospho-L-serine reacts preferentially to lactadherin than phospho-D-serine. Hence, the difference in binding between the two stereoisomers is attributed to stereoselectivity. Favorable spatial arrangement of L-PS probably allows it to interact with lactadherin preferentially over D-PS. In addition, lactadherin-PS binding does not

require calcium. It is noteworthy that another PS binding protein, annexin A5, widely used to study apoptosis and PS exposure, required calcium for its binding to PS and in sharp contrast to lactadherin, binds to less curved phospholipid surfaces (bigger vesicles) containing more than 4 % L-PS [46].

High-resolution crystal structure of the C2 domains of the clotting factors V and VIII and lactadherin have revealed a striking similarity in their architecture and PS binding [55–58]. All the three proteins have an oval shaped barrel with a core composed of a of beta sheets facing each other. Of the two beta sheets, one is composed of three and the other is composed of five anti parallel beta strands. A 22 amino acid peptide resembling the extreme C-terminal sequence in C2 domain that was initially shown to be responsible for PS binding is in fact buried inside a beta barrel and hence cannot take part in PS binding. The base of the barrel has three protrusions or spikes that contain solvent exposed hydrophobic residues and primarily involved in membrane interaction. Structurally and functionally, spikes 2 and 3 are very similar in all three proteins. At least four hydrophobic residues of factor VIII and two of factor V in spike 1 and 3 are involved in membrane binding. In case of lactadherin besides hydrophobic residues, glycine also could be a potential binding partner. The solution structure of the C2 domain of lactadherin by nuclear magnetic resonance spectroscopy revealed that the positively charged residues are uniquely positioned to bind electrostatically to the negatively charged phospho-head group of PS and the hydrophobic residues at the tip of the loops inserted into the phospholipid membrane to further stabilizes this interaction [59]. In spite of having striking structural similarities among these proteins, there are some vivid structural differences like spike 1 in lactadherin is more extended and more complex and diverse than the corresponding spikes in the clotting factors. These differences in spike 1 in lactadherin C2 could possibly be responsible for the differences seen in membrane interaction between lactadherin and two clotting factors. For example, the affinity of C2 domain of factor VIII for L-PS containing surface is about ten times lower than that of lactadherin. When spike one of lactadherin C2 domain was replaced by that of factor VIII the binding affinity decreases considerably suggesting a role of spike 1 in C2 domain of lactadherin. Membrane binding affinity of intact lactadherin is 100 fold more compared to C2 domain of lactadherin [46]. This suggests that although C2 domain of lactadherin is undoubtedly involved in membrane interaction, other domains of lactadherin could have a potential role in membrane binding. A relatively new study, however, suggest that lactadherin binding to vesicles is a two step process and it involves only the C2 domain [60].

4 Lactadherin as an Anticoagulant

Apart from the proteins involved in coagulation, there are a few like annexin A5 and β2G1 which though not homologous to coagulation factors but can bind phospholipid and influence blood coagulation. However, physiological relevance of these

proteins in coagulation is not known. On the other hand, the C terminal C1 and C2 domains of lactadherin, is highly homologous to the corresponding domains of factor V and factor VII. The remarkable sequence homology and similarity in the three dimensional architecture allows it to compete successfully with factor V and factor VII for the sites on PS containing surface [61]. In a competition assay performed on a model lipid membrane comprising of 4 % PS, 20 % PE and 76 % PC, lactadherin displaces factor VIII and factor V with a half maximal displacement at lactadherin concentration of 1–4 nM. In line with this observation, lactadherin inhibits the formation of tenase and prothombinase complexes. Half-maximal inhibition of tenase complex formation requires 6 nM lactadherin. More than 90 % inhibition of thrombin was achieved with lactadherin in a prothrombinase assay. The displacement of both coagulation factors by lactadherin suggest that its binding to the membrane, unlike factor V and factor VIII, does not follow a stringent guideline. Lactadherin also inhibits factor VIIa-tissue factor complex. Prothrombinase and tenase assays utilizing activated platelet surface also showed that lactadherin inhibits the generation of both factor Xa and thrombin [62]. The validity of the above observation was confirmed by noting the clotting time of whole blood in presence of lactadherin. In this assay, anticoagulant treated fresh whole blood was allowed to react with calcium and tissue factor in absence or presence of lactadherin. Lactadherin prolong the clotting time in all concentrations tested and three folds increase in clotting time was observed when 100 nM lactadherin was used. To further establish the role of lactadherin as an in vivo anticoagulant, lactadherin was administered through mouse-tail vein. It prolonged the tail bleeding time and in a carotid artery injury model lactadherin prolonged the time of occlusion by about two folds [62]. Interestingly many of the experiments were also carried out in presence of annexin A5 and it was inferior to lactadherin as an anticoagulant.

Lactadherin is involved in the clearance of apoptotic cells from the body. Inhibition of this process led to accumulation of cell debris in the body. An optimal level of lactadherin is required for efficient phagocytic clearance by macrophages because over or under expression of lactadherin has been shown to inhibit the clearance of apoptotic cells. Lactadherin deficient mice develop lupus which could be due to improper clearance of apoptotic cells [17]. Lactadherin is present in blood in miniscule quantity. Activated macrophages, not the resting macrophages, only secrete lactadherin. Its concentration in the plasma increases only in pathological conditions [8, 16]. It seems under normal condition lactadherin expression needs to be tightly regulated. Though lactadherin has potential to be an anticoagulant, the above findings will argue against it.

5 Lactadherin Deficient Mice are Prothombotic

Lactadherin deficient mice have normal phenotype. They are fertile and have normal fecundity [17]. Lactadherin null mice develop antiphospholipid antibodies, splenomegaly and immune complex glomerulonephritis, apparently because of

impaired phagocytosis of apoptotic lymphocytes. These mice subsequently develop lupus [17]. Hemostatic functions in lactadherin deficient mouse are studied in a light/dye-induced endothelial injury/thrombosis model in cremasteric venules. The time for occlusion in lactadherin-deficient mice was significantly shorter compared to control group (5.93 ± 0.43 min versus 9.80 ± 1.14 min) [14]. Since lactadherin inhibits the formation of tenase and prothrombinase complexes, the hypercoagulable state seen in lactadherin deficient mice could be due to the absence of natural anticoagulant effect of lactadherin. Indeed, the plasma from lactadherin-deficient mice generated twice as much thrombin as did their wild-type littermate controls (41.5 nM versus 20.4 nM in 30 min). However, when microvesicles were removed from plasma of the lactadherin-deficient mice and their wild-type littermates by ultracentrifugation, no difference in thrombin generation was observed. This observation suggests that microvesicles are responsible for the increased thrombin generation. Further studies show that lactadherin is involved in the clearance of microvesicles and impaired clearance of microvesicles in lactadherin deficient mice is responsible for the hypercoagulable state (this topic will be further discussed when we will talk about lactadherin mediated clearance of blood cells).

6 Lactadherin and Clearance of Blood Cells

PS, exposed on apoptotic cell surface, is a well-known tag for their recognition by macrophages. Several macrophage receptors bind PS directly or indirectly through a bridging molecule on apoptotic cell surface for efficient phagocytosis [63, 64]. Several years ago, Fadok et al. [65] described PSR1. However, it has since been revealed that the protein recognized by the antibody is a nuclear protein [66]. Park et al. have identified Bai1 (brain-specific angiogenesis inhibitor 1), a transmembrane protein [67], while Miyanishi et al. identified Timd4 (T cell immunoglobulin and mucin domain containing molecule 4) as a cell surface receptor on macrophages that directly recognizes PS on the surface of apoptotic cells [68]. Some studies have suggested that LDL-receptor related protein 1 and calreticulin are involved in the phagocytosis of PS-expressing apoptotic cells [69]. In addition to the direct recognition of PS on cell surface, several soluble proteins have been identified that promotes phagocytosis of PS expressing apoptotic cells. These proteins bind PS and anchor them to macrophage. Such bridging molecules are collectively termed opsonins.

Lactadherin contains integrin binding epidermal growth factor (EGF)-like domain at the amino terminus and PS binding C-domains at the carboxy terminus. Because of the presence of the two different binding sites at the opposing ends, lactadherin can act as an opsonin. Lactadherin mediates clearance of apoptotic lymphocytes by bridging lymphocytes and macrophages. Lactadherin binds to apoptotic lymphocytes by recognizing exposed PS via the C-domains. The integrin $\alpha_V\beta_3$ in macrophage binds the RGD sequence in the EGF domain of lactadherin resulting in the phagocytosis of lactadherin-bound lymphocytes.

7 Red Blood Cell Senescence and PS Exposure

Asymmetrical distribution of phospholipids in cell membrane is a universal property of all mammalian cells. Since the pioneering studies by Bretscher and van Deenan, the red blood cell has been extensively studied for lipid asymmetry [70, 71]. Normal red blood cells exhibit an asymmetric distribution of phospholipids in the membrane such that the anionic phospholipids reside in the inner leaflet while the neutral or zwitterionic phospholipids are enriched in the outer leaflet [30]. For example, 70 % of phosphatidylcholine and more than 80 % of sphingomyelin are in the outer leaflet, while 85 % of phosphatidylethanolamine and almost all the PS are in the inner leaflet. A number of factors have been proposed in the maintenance of this asymmetry.

An increase in intracellular Ca^{2+} causes a rapid bidirectional movement of the plasma membrane phospholipids between leaflets, resulting in exposure of PS and phosphatidylethanolamine[72]. The molecular mechanism(s) underlying this intracellular Ca^{2+}-initiated scrambling of phospholipid remains poorly understood. Recently, two transmembrane proteins were identified that are involved in transbilayer movement of anionic phospholipids during apoptosis [73–75].

Red blood cells survive in circulation for about 120 days after which they are phagocytosed by macrophages. Ageing of red blood cells is associated with a number of physicochemical changes (reviewed in [76]). These include a decrease in cell size due to vesiculation with minimal loss of hemoglobin, change in shape from discocyte to spherocyte, decrease in ATP and enzymes content [77] loss of membrane asymmetry with concomitant PS exposure in the outer leaflet [78], increase in membrane bound lipid peroxidation products [79], desialylation of membrane glycoprotein [80], exposure of cryptic senescent antigens [81], and aggregation band 3 protein [82] and an increase in cell surface-bound immunoglobulins and complement [83, 84]. All of these observations were made based on density that correlates with red blood cell ageing. With ageing red blood cells become smaller and denser due to microvesiculation. For instance, it was shown that denser and supposedly older red cells expose more PS in the outer leaflet of the membrane bilayer [85]. However, studies with biotinylated red blood cells have shown that old cells were neither denser [86, 87] nor ATP depleted, and their deformity was only slightly increased [88, 89]. However, with ageing the exposure of PS was increased that correlates with their removal from circulation. As in many other cells, PS on aged red blood cells could be a tag for macrophage recognition clearance from circulation. This hypothesis is further supported by the fact that PS laden red blood cells were cleared much faster that untreated cells [78].

8 PS Exposure in Sickle Red Blood Cells

In 1981, Chiu et al. first showed the presence of PS in the outer leaflet of sickle red blood cells [90, 91]. De Jong et al. have shown that the sickle RBCs can expose PS at several stages during their life span [92, 93]. The PS-exposing subpopulation

includes both sickle reticulocytes, and more mature RBCs that vary with respect to density, RNA content and shape. These investigators proposed that factors other than multiple sickling events play a role in their generation. In their study, the PS exposure correlates with the loss of aminophospholipid translocase activity. In an another study, Yasin et al. [94] examined the relationships among PS externalization, fetal hemoglobin content, hydration state, and cell age. Sickle red blood cells exhibit a wide range of PS externalization. Those with low-level exposure (type 1 PS+) include many young transferrin-receptor-positive cells. This is not specific for sickle cell disease because many nonsickle transferrin-receptor-positive cells are also PS positive. RBCs with higher PS exposure (type 2 PS+) appear to be more specific for sickle cell disease. Their formation is sickling dependent because type 2 PS positive dense sickle cells have a lower percentage of fetal hemoglobin (HbF) than PS negative cells in the same density fraction. In vivo, experiments using biotin-labeled sickle cells showed a sharp decrease in the percentage of circulating, labeled PS-positive cells in the first 24 h after reinfusion. This decrease was confined to type 1 PS-positive cells and was attributed the reversal of PS exposure in very young cells. These studies indicate that PS externalization in sickle cells may be low level, as observed in many immature cells, or high level, which is associated with dehydration and appears to be more specific for sickle red blood cells.

9 Role of Lactadherin in Red Blood Cell Clearance

The shortened red blood cell survival in sickle cell anemia is due to extravascular destruction mediated by the macrophages. Several mechanisms, including the exposure of PS, have been proposed as tags for macrophage recognition. Lactadherin is present in small amounts in blood [8] and stimulated macrophages secrete lactadherin [10]. Since senescent red blood cells as well as sickle red blood cells expose PS (Fig. 4.1), the role of lactadherin in their clearance was studied. Fifty days old stored red blood cells expose about 30 % PS and lactadherin dose dependently increased the phagocytosis of these cells by THP-1 derived macrophages [95] (Fig. 4.2). THP-1 cells express lactadherin and significant level of phagocytosis was observed in the absence of lactadherin. An antibody to lactadherin inhibited this basal phagocytosis dose dependently (Fig. 4.3) proving further the role of lactadherin in red blood cell phagocytosis. Splenic macrophages isolated from lactadherin deficient mice also showed significant impairment in phagocytic ability toward calcium ionophore treated mouse red blood cells (Fig. 4.4). It is to be mentioned here that calcium ionophore has previously been shown to increase the PS exposure in red blood cells [96]. Also, in presence of lactadherin a significantly higher proportion of sickle red blood cells than normal red blood cells were phagocytosed by human monocyte derived macrophages [97]. An antibody to integrin $\alpha_V\beta_3$ inhibited the phagocytosis of sickle red blood cells while a monoclonal antibody to Fcγ receptor II (IV3) had no significant effect under similar conditions (Fig. 4.5). These results indicate that the lactadherin-induced phagocytosis is mediated by integrin

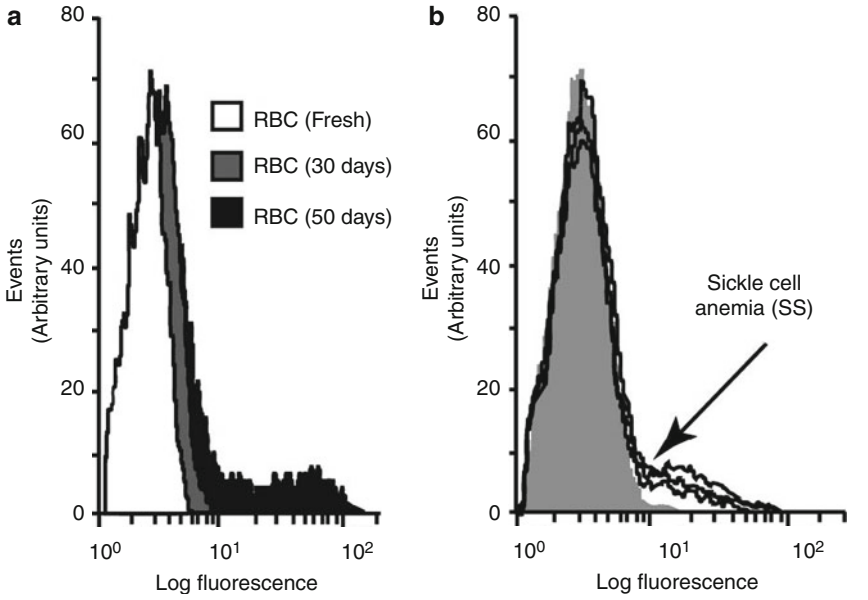

Fig. 4.1 Panel (**a**) Age dependent binding of FITC lactadherin to red blood cells. Washed red blood cells of different *in vitro* ages were incubated with the FITC-lactadherin and fluorescence associated with red blood cells were analyzed by flow cytometry using FITC-lactadherin. Panel (**b**) FITC-Lactadherin binding to sickle red blood cells. Washed red blood cells from normal (*gray*) and three patients with sickle cell anemia were incubated with lactadherin (100 nM) and the fluorescence associated with red cells were measured (Modified from Dasgupta et al. [132] with permission)

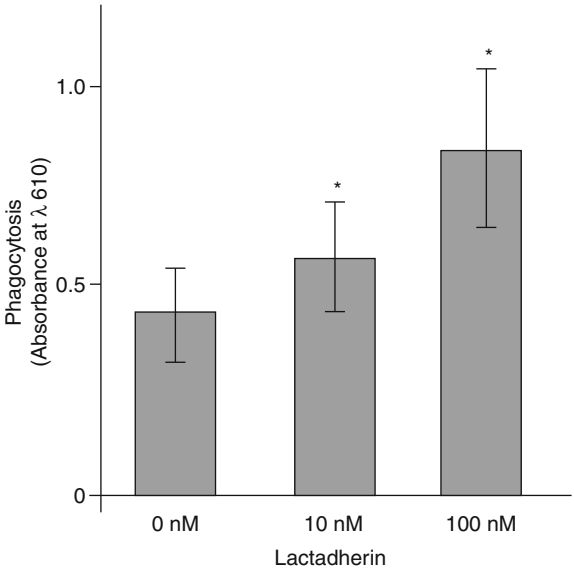

Fig. 4.2 Phagocytosis of Red blood cells in presence or absence of lactadherin. Red blood cells stored for 50 days were incubated with THP-1 derived macrophages at various concentrations of lactadherin for 90 min at 37 °C. The phagocytosis was quantified by the pseudoperoxidase activity of hemoglobin towards diaminofluorene (Modified from Dasgupta et al. [132] with permission)

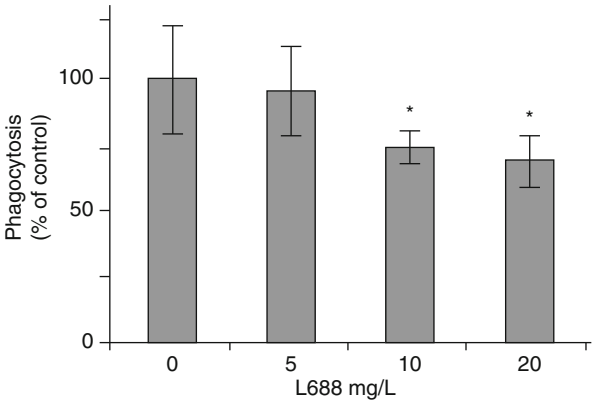

Fig. 4.3 Effect of anti lactadherin antibody on red blood cell phagocytosis. Red blood cells stored for 50 days were incubated with THP-1 derived macrophages at various concentrations of L688 for 90 min at 37 °C. The phagocytosis was quantified by the pseudoperoxidase activity of hemoglobin towards diaminofluorene (Modified from Dasgupta et al. [132] with permission)

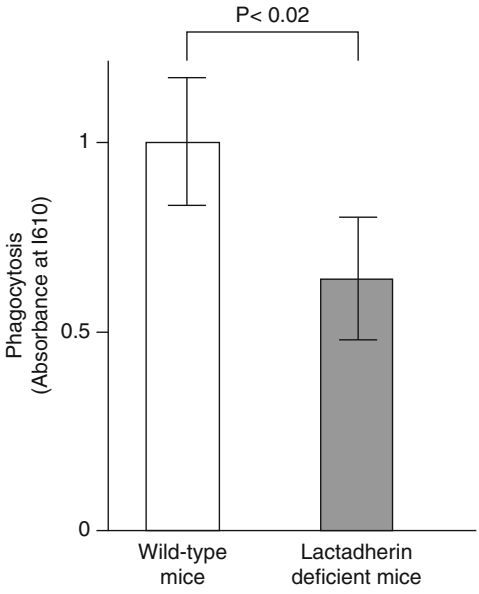

Fig. 4.4 Phagocytosis by splenic macrophages. Washed red blood cells were treated with N-ethyl maleimide and calcium ionophore to induce phosphatidylserine expression and incubated with splenic macrophages from wild type or lactadherin-deficient mice for 90 min at 37 °C and engulfed red blood cells were quantified by the pseudoperoxidase activity of hemoglobin towards diaminofluorene. The mean and ± SD of triplicate measurements are shown (Modified from Dasgupta et al. [132] with permission)

$\alpha_V\beta_3$ on macrophages and that the Fcγ receptor does not play a major role. Though lactadherin plays a role in red blood cell phagocytosis in vitro, lactadherin deficient mice have normal level of PS expressing red blood cells in circulation and there in vivo survival is also normal. Impairment in in vivo clearance was seen only when surface level of PS on red blood cells was increased by incubation with PS micelles [95]. In this experiment, after infusion of PS-coated red blood cells into mice, the percentages of PS-labeled red blood cells was determined in the circulation at 5, 10, and 20 min after injection. In wild-type mice, PS-coated cells were rapidly cleared. In lactadherin-deficient mice the percentages of the PS-labeled red blood cells at 5 and 10 min were significantly higher compared to the control. No significant difference was noted at 20 min (Fig. 4.6). Taken together, these results indicate that under

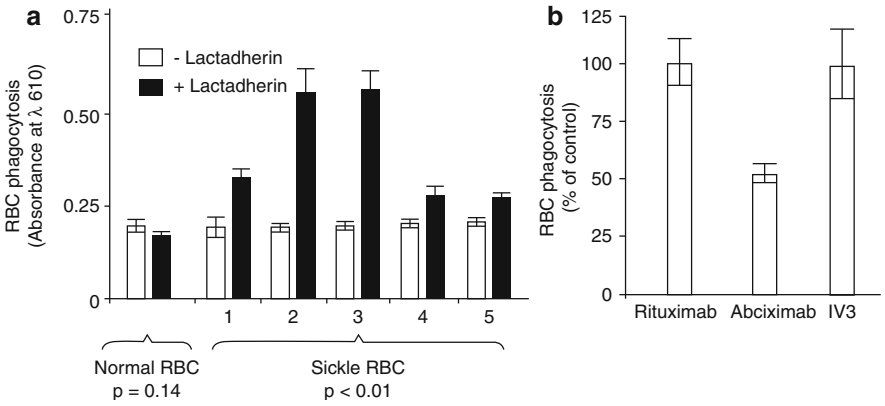

Fig. 4.5 Panel (**a**) Effect of lactadherin on the phagocytosis of red blood cells by macrophages. Washed red blood cells from a normal and five patients with sickle cell anemia were incubated with monocyte-derived macrophages with and without lactadherin (100 nM) and the engulfed red blood cells were quantified by measuring the cell associated pseudoperoxidase activity of hemoglobin. The mean and ± s.e.m of triplicate measurements are shown. $p < 0.01$ in all sickle patients. Panel (**b**) Effect of antibodies on lactadherin-induced phagocytosis of red blood cells. 10 μg/ml of abciximab (anti-Integrin V3), control antibody (rituximab, anti-CD20), IV3 (anti-Fcγ receptor II) were incubated with the macrophages and the effect of lactadherin-induced phagocytosis was determined as in Fig. 4.4. The phagocytosis in the absence of any antibodies was considered 100 % (Modified from Dasgupta et al. [97] with permission)

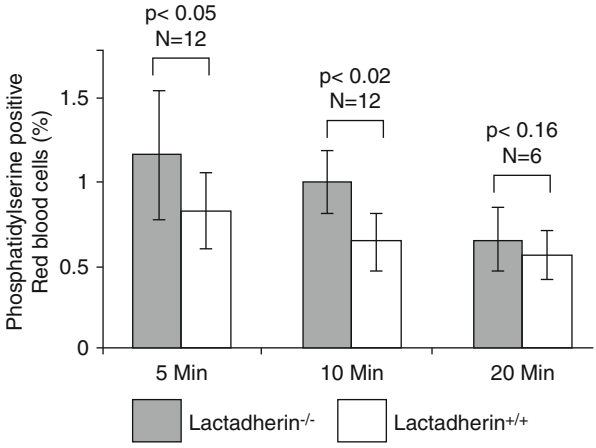

Fig. 4.6 In vivo clearance of phosphatidylserine-labeled red blood cell in mice. Phosphatidylserine expressing murine red blood cells were infused via the tail vein. Percentages of phosphatidylserine positive red blood cells in the peripheral circulation of wild-type and lactadherin deficient mice were determined by flow cytometry. Values are means and ± SDs of at least six mice in each group

normal circumstances, there could be more than one pathway for red blood cell clearance. Only when challenged with large excess of PS exposing red blood cell, lactadherin plays an important role.

The clearance of apoptotic cells is a fundamental process and involves multiple redundant clearance pathways. As lactadherin deficiency leads only to mild impairment in the clearance of senescent red blood cells from circulation similar,

redundancy could be involved in this case as well. A few other PS binding proteins, such as, gas-6 [98], del-1 [99] and complement components [100] are also involved in the clearance of apoptotic cells. These proteins, as well as other pathways not involving PS could also be involved in the clearance of senescent red blood cells.

10 Microvesicles in Circulation

Microvesicles (also called microparticles) are submicron size vesicles released from cells in response to activation or during apoptosis. Platelet-derived microvesicles, released from surface blebs of activated platelet, constitute a major fraction of microvesicles in the circulating blood. Microvesiculation is accompanied by externalization of the anionic phospholipid, PS, which is normally present only in the inner leaflet of membrane bilayer. These microvesicles, initially termed as "platelet dust" promote coagulation. The precise molecular mechanism of microvesicles formation is not well understood. Increase in intraplatelet calcium, reorganization of cytoskeletal proteins and the loss of lipid asymmetry (due to transbilayer movement of PS from the inner to outlet leaflet of membrane bilayer) are an essential requirements for microvesicles formation.

Microvesicles are highly procoagulant due to the presence of PS on their outer surface. It has been suggested that microvesicles also play a role in initiating coagulation by providing a source of tissue factor [101]. Scott syndrome is a rare disease characterized by deficiency of platelet procoagulant activity due impaired exposure of PS and microvesicles formation [35, 73, 102, 103]. These patients have a lifelong bleeding disease. A less well-defined bleeding disorder, in which a defect in microvesiculation with normal PS exposure, has also been described [104, 105].

Increase in circulating platelet microvesicles have been detected in antiphospholipid antibody syndrome [106], cancer associated deep vein thrombosis [107], heparin-induced thrombocytopenia [108], thrombotic thrombocytopenic purpura [109], acute coronary syndromes [110], ischemic stroke [111], diabetes [112], cirrhosis [113] and sepsis [114, 115]. All these conditions are associated with a procoagulant state and/or vascular dysfunction.

In addition to their well-known role in hemostasis, platelet microvesicles play a role in intercellular communications, atherosclerosis, angiogenesis and in the spread of HIV infection [116–119]. Platelet microvesicles can transfer the adhesion molecules from platelets to endothelial cells [120] or to tumor cells [121], conferring pro-adhesive properties to them, and elicit cytokine responses from synovial fibroblasts [122]. Platelet microvesicles have a very short half-life in the circulation. In rabbits, the infused microvesicles are cleared within 10 min [123]. Microvesicles were elevated in dogs containing artificial shunts and the levels starts normalizing within 3 h of removal [124]. These findings mean microvesicles are continuously formed in the circulation. Furthermore, microvesicles also originate from megakaryocytes [125]. Clearly, the generation and the clearance of procoagulant

particles must be balanced. At least three proteins that are involved in microvesicles clearance have been identified. Here, we will discuss only the role of lactadherin in microvesicles clearance.

11 Lactadherin and Microvesicles

Endothelial cells, monocytes, red blood cells and platelets are the primary source of circulating microvesicles but majority of it is derived from platelets. Flowcytometric and immunoblot analysis showed that lactadherin is associated with circulating microvesicles [14]. In vitro assay, utilizing differentiated human monocytic THP-1 cells, showed an enhancement of platelet derived microvesicles phagocytosis in presence of lactadherin. THP-1 cells were incubated in presence and absence of fluorescent platelet derived microvesicles. About 20 % THP-1 cells were found to be positive for microvesicles fluorescence at the basal level. Lactadherin promoted the phagocytosis of microvesicles dose dependently with half maximal effect at about 5 ng/ml. The C1C2 fragment of lactadherin which has only the PS binding site inhibited the phagocytosis. An antibody, abciximab, which specifically binds β3 subunit of integrins also inhibited the phagocytosis. These results suggest that lactadherin mediates the clearance of microvesicles by binding PS on the microvesicles and integrin on the macrophage. Similarly, lactadherin also promoted the phagocytosis of microvesicles derived from endothelial cells and monocytes. In vivo significance of these findings was assessed by quantifying the circulating microvesicles in lactadherin deficient mice. Flowcytometric analysis of plasma of lactadherin deficient and control mice revealed that lactadherin deficient mice had twice as much microvesicles in circulation compared to control mice. Macrophages isolated from the spleens of lactadherin deficient mice showed a significant impairment of phagocytic ability compared to macrophages isolated from wild type littermates. Microvesicles posses clot promoting activity. Plasma from lactadherin deficient mice indeed generated significantly more thrombin compared to compare to wild type plasma. When microvesicles were removed from plasma by ultra centrifugation, there was no difference in thrombin generation between lactadherin deficient or normal wild type plasma. This experiment clearly demonstrates the role of microvesicles in thrombin generation. As explained in the previous section, lactadherin deficient mice are in hypercoagulable state which could be attributed to the increased microvesicles in circulation due to impaired clearance of microvesicles [14].

12 Lactadherin and Atherosclerosis

Atherosclerotic narrowing of blood vessels results in >19 million deaths annually in the world. Sudden rupture of atherosclerotic plaque results in myocardial infarction and stroke and these acute events accounts for the majority of death. Since only

certain plaques (vulnerable plaques) undergo rupture, understanding the development of plaque rupture is of critical importance. A number of physicochemical characteristics have been associated with vulnerable plaque, including the presence of apoptotic macrophages [126]. Atherosclerotic plaques also contain PS-expressing microparticles [127]. A role for lactadherin in plaque development is suggested by the finding that lactadherin deficiency leads to increases in lesion size with accumulation of apoptotic cells in mouse models of atherosclerosis [21]. Lactadherin may play a role as a vasoprotective agent against the progression of atherosclerosis by scavenging PS expressing microparticles and/or macrophages. Alteration in protective immune response is also postulated for the increase size of plaques in lactadherin deficient mice. Since platelet-derived microvesicles are mitogenic [128]. It is possible that elevated microvesicles in lactadherin-deficient mice [14] may also contribute to the enhanced plaque development.

13 Conclusion

Lactadherin even though originally identified as a product of mammary epithelial cells it has a wide range of functions in the clearance of blood cells, regulation of immune function, microglial phagocytosis [129] and sperm-egg interaction [130], angiogenesis [13] and possibly in many other physiological functions. This review focuses mainly on blood cells. It has imperative to define its role in pathophysiological mechanism as it is being developed as an imaging event to detect apoptosis in vivo [131] and possibly as an anticoagulant.

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Chapter 5
MFG-E8 in the Vascular System

Xuan Li, Nicolas Deroide, and Ziad Mallat

Abstract Milk fat globule-epidermal growth factor-factor 8 (MFG-E8) is a secretory glycoprotein which not only enhances the clearance of the apoptotic cells/bodies from the injured vessels, but also promotes neovascularisation through VEGF-Akt signalling pathway during ischemic, ocular, and inflammatory disorders to maintain vascular integrity required for the repair process of damaged tissues. However, the pro-angiogenic properties of MFG-E8 may also enhance tumor cell survival, invasion, and contribute to local immune suppression. Hence, systemic MFG-E8 blockade may intensify the antitumor activities of existing therapeutic regimens through coordinated cell-autonomous and immune-mediated mechanisms. In this regard, there remains an unmet need of improving our understanding on the fine tuned balance between the beneficial wing of MFG-E8 for tissue repair and its detrimental features for tumor progression to establish MFG-E8 as potential therapeutic target against inflammatory and malignant disorders. In this current chapter, we therefore aim to describe the role of MFG-E8 in vascular system with emphasis given on its molecular mechanisms and therapeutic implications in various vascular diseases.

Keywords MFG-E8 • Vascular system • VEGF • AKT • Del-1 • PDGF

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1 Introduction

MFG-E8 plays multiple regulatory roles in the vascular system. The function of MFG-E8 in this setting is not only limited to its well-known capacity of assisting the clearance of apoptotic cells in diseased vessels [1]. Through its diverse roles in angiogenesis, smooth muscle cell proliferation, collagen turnover, inflammasome activation, and regulation of innate and adaptive immune responses, MFG-E8 stands as a multi-faceted player controlling vascular homeostasis and vascular response to injury.

2 MFG-E8 and Angiogenesis

Angiogenesis is the process of the growth (sprouting) of new blood vessels from pre-existing ones [2]. By the function of carrying oxygen to tissues, it is essential for organ growth and repair. Disturbance of this process contributes to pathogenesis of many disorders. After the embryonic developmental stage, most blood vessels remain quiescent. However, angiogenesis is mostly known to be switched on during malignant, ischemic, ocular, and inflammatory disorders to maintain vascular integrity in the repair process of damaged tissue and in the formation of collateral vessels in response to tissue ischemia. Angiogenesis is also important for diabetes, obesity, autoimmune disease, asthma, cirrhosis, multiple sclerosis, endometriosis, AIDS, bacterial infections and many other diseases [3]. During angiogenesis, vascular endothelial growth factor (VEGF) is a key regulator of vascular growth, whereas platelet-derived growth factor (PDGF) recruits mural cells around endothelial channels [3].

The role of MFG-E8 in angiogenesis has been explored in several models. The results varied among studies, which might reflect subtle differences in the functions and roles of this protein in distinct tissues. MFG-E8 is structurally and functionally homologous to angiogenic protein developmentally regulated endothelial locus-1 (Del-1). Both of them consist of a signal sequence, epidermal growth factor domain, RGD motif, and discoidin I-like domain. MFG-E8 and Del-1 are glycoproteins, which can be secreted from different subsets of macrophages, and they both play an important role in assisting phagocytes engulfing apoptotic cells [4]. Del-1 is expressed in endothelial cells during embryological vascular development and in ischemic tissue, and it is known to initiate angiogenesis and postischemic neovascularization through its RGD motif and discoidin-I-like domain [5]. MFG-E8 possesses the same structure and therefore was speculated to have a similar angiogenic role. The role of MFG-E8 in angiogenesis was first demonstrated in a VEGF-dependent manner [6]. VEGF signalling represents a major crucial rate-limiting step in both physiological and pathological angiogenesis [7], and MFG-E8 was proved to be required for the pro-angiogenic effect of the key VEGF by using in vivo matrigel migration and angiogenesis assay, in which administration of

MFG-E8 antibody totally abrogated VEGF-induced cell in growth [6]. MFG-E8 was expressed in and around blood vessels and blockade of MFG-E8 function through genetic deficiency or administration of a neutralising antibody markedly inhibited post-ischaemic neovascularisation [6]. At the molecular level, VEGF-induced Akt phosphorylation was markedly reduced in MFG-E8-deficient mice, suggesting a crucial role of MFG-E8 in the VEGF-Akt signalling pathway [6]. In the absence of exogenously added growth factors, over expression of MFG-E8 showed pro-angiogenic activities and might have potential therapeutic relevance in the treatment of ischemic disease [6].

In other studies, MFG-E8 was also found to be expressed in pericytes [8, 9]. In melanomas and oxygen-induced retinopathy, tumor and retinopathy associated angiogenesis were diminished in MFG-E8 knockout mice. Alongside with reduced level of angiogenesis, pericyte coverage of neovessels was reduced in these mice too [8]. Using pericyte/pericyte-like 10T1/2 cells in vitro, the promoting role of MFG-E8 in angiogenesis has been linked to its association with PDGFRβ and focal adhesion kinase after PDGF treatment. This association results in several interesting features, including cell surface retention of PDGFRβ, delaying PDGFRβ degradation, potentiating downstream signalling, and enhancing migration [9].

The relevance of angiogenesis in tumor biology is well established, and modulation of angiogenesis is one of the major therapeutic targets for tumor regression. Using a cancer model, Rip1-Tag2 tansgenic mice in which angiogenesis is critical, Neutzner et al. demonstrated that MFG-E8 was pro-angiogenic and promoted tumorgenesis in these mice [10]. They inferred that MFG-E8 exerted its influence on tumorigenesis primarily through potentiation of VEGF signalling in endothelial cells by engaging integrins. Apart from promoting angiogenesis, MFG-E8 enhanced tumor cell survival, invasion, and contributed to local immune suppression [11]. MFG-E8 can therefore regulate disease progression through coordinated $\alpha_v\beta_3$ integrin signalling in tumor and host cells, and through modulation of both angiogenesis and anti-tumor immune responses. Systemic MFG-E8 blockade may also intensify the antitumor activities of existing therapeutic regimens through coordinated cell-autonomous and immune-mediated mechanisms [11]: in cooperation with cytotoxic chemotherapy, molecularly targeted therapy, and radiation therapy to induce destruction of various types of established mouse tumor, MFG-E8 blockade induced extensive tumor apoptosis that was coupled to efficient dendritic cell cross-presentation of dying tumor cells, which engendered potent antitumor effector T cells and inhibited FoxP3 (+) Treg cells, thereby achieving long-term protective immunity.

The role of MFG-E8 in promoting angiogenesis is not universal. In a mouse model of carcinogen-induced bladder carcinoma, MFG-E8-deficient animals developed less advanced tumor but angiogenesis was similar in carcinogen-treated MFG-E8-expressing or deficient bladders, thus ruling out a major contribution of the pro-angiogenic function of MFG-E8 to its pro-tumoral effect. This study suggested that MFG-E8 promoted development of bladder tumor at least partially through an immune system-dependent mechanism [12]. Similarly, in a mouse model of post-ischemic cerebral injury [13], MFG-E8 deficient mice exhibited comparable level

of angiogenesis when compared with MFG-E8 expressing mice, while significantly dampening inflammasome-dependent activation of the innate immune response (see below). Therefore, MFG-E8 might exert distinct functions in different pathophysiological settings.

3 MFG-E8 and Atherosclerosis

3.1 Anti-atherogenic Properties of MFG-E8

Throughout the development of atherosclerosis, macrophages, smooth muscle cells, and endothelial cells undergo apoptotic programmed cell death. Under normal physiological conditions and in order to maintain organ integrity, all those apoptotic dead cells are efficiently cleared by efferocytosis, a cellular process used by neighbouring phagocytes to ingest dead cells and prevent secondary cellular necrosis and inflammation. Several atherosclerosis risk factors such as hyperglycemia, oxidized low-density lipoproteins, and immune injuries induce endothelial cell apoptosis [14], which may trigger the release of MFG-E8 thereby re-programming macrophages into an anti-inflammatory phenotype [15]. When macrophages are exposed to conditioned media from serum-starved apoptotic endothelial cells, they adopt a high anti-inflammatory, low pro-inflammatory cytokine/chemokine secreting phenotype that disappears when MFG-E8 is absent from the media [15]. These data highlights an MFG-E8-dependent mechanism of macrophage programming by the micro-environment.

Defective phagocytic clearance of dying cells is linked to the progression of advanced atherosclerotic lesions. A well-recognized characteristic of atherosclerotic plaques is the accumulation of cell debris during plaque progression towards advanced stages: when lesions develop, phagocytic removal of apoptotic cell becomes inadequate which can progressively generate secondary necrosis, promote loss of cellular integrity, expand plaque necrotic core, and finally cause susceptibility to rupture leading to a severe disease condition such as atherothrombosis [16–18]. Phagocytic clearance of apoptotic cells by professional scavengers is the physiological consequence of apoptosis in vivo and is the most important determinant of steady state by suppressing pro- inflammatory signalling and activating anti-inflammatory pathways [19]. A set of molecules are involved in the clearance of apoptotic cells during lesion development, and the key molecular regulators of efferocytosis in atherosclerotic lesion development include MFG-E8 [17], complement C1q [20] and Mer receptor tyrosine kinase (Mertk) [21].

Since a 2007 publication from Mallat's lab showing that lack of MFG-E8 in bone marrow-derived cells impacts necrotic core formation and lesion inflammation [22], the studies of MFG-E8's effect on atherosclerosis have been extended to endothelial [15] and vascular smooth muscle cells (VSMC) [23, 24]. MFG-E8 was identified in human coronary vessels by using direct tissue proteomics (DTP)

from paraffin-embedded samples [25]: it is a method of extracting proteins from slide sections, followed by tandem mass spectrometry and bioinformatics data processing and analysis. MFG-E8 was shown to be expressed in both normal and atherosclerotic arteries from humans and mice, but particularly with lower abundance in advanced atherosclerotic vessels [22]. MFG-E8 was expressed in endothelial cells, smooth muscle cells and macrophages, but the expression was lower in macrophages located in the cholesterol-rich lipid core of advanced plaques, where apoptotic debris accumulates. The lower expression of MFG-E8 around the necrotic core can be related to increased accumulation of free cholesterol in the cells [26]. In advanced human lesions, apoptotic cell death detected by TUNEL staining occurred more frequently in areas with low MFG-E8 expression [22].

Myeloid-derived MFG-E8 is considerably protective in atherosclerotic development: disrupting bone-marrow-derived MFG-E8 leads to substantial accumulation of apoptotic cells systemically and within the developing lipid lesions, impairs the regulatory immune response, and accelerates lesion development [22]. An *Ldlr*$^{-/-}$ mouse model, rather than *Apoe*$^{-/-}$ mouse model, was used to examine the importance of myeloid-derived MFG-E8 on the development of atherosclerosis [22], for a reason that apolipoprotein E (ApoE) is known to modulate clearance of apoptotic cells both *in vitro* and *in vivo*, which can result in a systemic pro-inflammatory state in this strain [27]. Although *Ldlr*$^{-/-}$ mice with *Mfge8*$^{+/+}$ bone marrow developed fatty streak lesion with marginal accumulation of smooth muscle cells and collagen after 8 weeks of high-fat diet, the early lesions from those mice had barely detectable cell death. In contrast, lesions of mice reconstituted with *Mfge8*$^{-/-}$ bone marrow displayed large cores with cell debris, which was associated with a marked 70 % increase in lesion size. The phenotype persisted after a prolonged duration on high-fat diet. Those results are consistent with and partially explain the unexpected substantial increase of atherosclerotic lesions in mice with $\alpha_v\beta_3$ deficiency [28], an integrin receptor required for MFG-E8-mediated clearance of apoptotic cells via RGD domain of MFG-E8 [1].

Dendritic cells (DCs) have a central role in immune regulation [29]. Interestingly, Ait-Oufella's paper reported reduced DC tolerogenicity in the absence of MFG-E8. Using a co-culture regulatory T cell suppression experiment, the investigators unravelled a DC-dependent alteration of the suppressive properties of Treg cells when DCs were recovered from *Mfge8*$^{-/-}$ mice compared with MFG-E8-expressing DCs. Consequently, defective apoptotic cell phagocytosis in *Mfge8*$^{-/-}$ mice has contributed to the pro-inflammatory phenotype of these animals by altering the regulatory T-cell function that controls immune homeostasis. Absence of MFG-E8 was associated with reduced T-cell-derived IL-10 and increased level of IFN-γ *in vivo*, suggesting a switch of immune response towards a Th1 pro-inflammatory phenotype. This is in agreement with data from other investigators who reported an exquisite role for MFG-E8-mediated uptake of apoptotic cells in mediating GM-CSF-dependent immune tolerance through increased generation of Tregs [30].

3.2 A Pathogenic Role for MFG-E8 in Vascular Disease?

Not all studies found MFG-E8 to be protective in atherosclerosis. MFG-E8 may have a pathogenic role in atherosclerotic processes because its involvement in the following aspects of disease development: (i) collagen turnover, (ii) vascular smooth muscle cell (VSMC) invasion, (iii) advanced glycation end products (AGEs)-dependent endothelial dysfunction, and (iv) angiogenesis. However, those results are quite preliminary and proof of the detrimental effect of MFG-E8 in those specific settings needs to be backed up with additional data.

Despite the protective role of MFG-E8 in the removal of apoptotic debris and the preservation of an athero-protective immune response, MFG-E8 deficiency in the studies mentioned above had also led to the accumulation of collagen in the fibrous cap [22], a major plaque-stabilising and protective mechanism. The finding was not explained until the discovery by another group that MFG-E8 was able to bind collagen directly and facilitates the removal of accumulated collagen through at least one discoidin domain [31]. Thus, the role of MFG-E8 in clearance of collagen during atherosclerotic development and its potential implications for lesion stability need to be further investigated.

MFG-E8 has been proposed to be involved in arterial wall inflammatory remodeling during aging [24], hypertension [32], and diabetes mellitus [33, 34]. Advancing age is an independent risk factor for the epidemic of human cardiovascular disease [35]. Age-related arterial remodeling is associated with increased VSMC invasion and proliferation and accompanied by pro-inflammatory processes. Angiotensin II (Ang II) signaling cascade is a central feature of arterial aging [36]. Ang II and MFG-E8 within VSMCs were closely associated and both markedly increased within the aortic wall, particularly in the thickened aged intima [24]. MFG-E8 was demonstrated to be induced in young VSMCs after Ang II treatment and to work downstream of Ang II-initiated signaling. MFG-E8 was required for VSMC invasion, and knock-down of MFG-E8 by RNA interference successfully reduced invasive capability of both young and old VSMCs. Data from this research indicated that arterial MFG-E8 significantly increased with aging and was a pivotal relay element within the Ang II/MCP-1/VSMC signaling cascade [24]. MFG-E8 co-localized with MCP-1, preferentially in the thickened intima, and invasive capacity of VMSCs was partially potentiated through MCP-1 [37]. In another study, MFG-E8 signaling was linked to a modulation of VMSC, leading to increased VSMC proliferation [23]: an age-associated increase in MFG-E8 and its integrin receptor $\alpha_v\beta_5$ via autocrine or paracrine signaling promoted the expression of cell cycle activators and of the powerful mitogen PDGF and its receptors, enabling increased proliferation of old VSMCs. These data imply that the targeting of MFG-E8 within this signaling axis pathway might limit age-associated vascular remodeling. It should be noted that the effects of MFG-E8 on VSMCs of the atherosclerotic plaque have not been addressed. Should MFG-E8 have any proliferating potential in those cells, this would result in better plaque stabilization.

Atherosclerosis is one of the major complications of type 2 diabetic patients. Advanced glycation end products (AGEs) are correlated with diabetic vascular risk, lead to endothelial dysfunction and apoptosis, and thus contribute to atherosclerosis [38]. Li et al. found that in patients with type 2 diabetes mellitus (T2DM), MFG-E8 serum level was significantly correlated with enhanced aortic pulse-wave velocity, and measuring it was a useful clinical surrogate prognosticating aortic stiffness. They also found MFG-E8 was significantly increased in diabetic rat aorta compared to control aorta and was substantially reduced after administration Grape seed procyanidin B2 (GSPB2) in vivo [33, 34]. The same team found that adjunction of AGEs up-regulated MFG-E8 expression in human umbilical vein endothelial cells (HUVECs), and inhibition of MFG-E8 with siRNA increased HUVEC viability. Accordingly, MFG-E8 over expression increased HUVEC death through an up-regulation of Bax/Bcl-2 ratio, cytochrome c release and caspase 3 and 9 activation [39]. They hypothesized that AGEs-dependent endothelial dysfunction was mediated through MFG-E8 and that GSPB2 could prevent this process through down regulation of MFG-E8. They treated db/db mice with GSPB2 or MFG-E8 RNAi for 4 weeks and found that both suppressed endothelial injury and VSMC proliferation. Opposite effects were found after treatment with recombinant MFG-E8. They attributed those effects to the modulation of both MCP-1 and ERK1/2 signaling [34]. It has to be noted that the db/db mice were not fed with high fed diet and did not develop significant atherosclerotic lesions. This mouse model is more representative of an early diabetic endothelial dysfunction model than an atherosclerosis model. Moreover, GSPB2 also significantly decreased the body weight of the diabetic mice and therefore possibly induced bias.

The role of angiogenesis in the development of atherosclerosis still carries controversial debate, and it remains unclear whether angiogenesis plays any role in the development of atherosclerosis or its complications [40]. The best-known role of angiogenesis in atherosclerosis is its occurrence within the vessel wall in advanced lesions. Plaque neovascularization is comprised of a network of capillaries that arise from the adventitial vasa vasorum and extend into the intimal layer of atherosclerotic lesions. The functions of these plaque capillaries are not well understood but are proposed to be important regulators of plaque growth and lesion instability [41]. Therefore, further exploration of the angiogenic role of MFG-E8 in atherogenesis needs to be properly designed and examined.

4 MFG-E8 and Aneurysm

Amyloid deposits are common in arteries media and one of the main amyloid fibril proteins in human arteries is Medin, a 50 amino-acid residue derived from MFG-E8 [42]. Amyloidosis may induce media degeneration and thus worsen thoracic aneurysms [43]. Peng et al. found that Medin amyloid deposit was significantly lower in patients with aneurysms compared to control material. However, immunostaining of Medin not associated with amyloid aggregates increased in the diseased group

[44]. Interestingly, pre-fibrillar oligomeric aggregates may be more cytotoxic than fibrillar aggregate [45]. In vitro, Medin was found to induce smooth muscle cell death and matrix metalloprotease 2 (MMP2) production. Thus, it is possible that Medin pre-fibrillar oligomers, found in the diseased group, have a detrimental effect on aortic aneurysms. How Medin is cleaved from MFG-E8 and the putative therapeutic interest of modulating its generation in this setting are still unknown [46]. Abdominal aortic aneurysms (AAA) are a more frequent disease of the aging population. MFG-E8 RNA level was found down regulated in human AAA tissues [47]. However the relevance of MFG-E8 in the pathogenesis of AAA is virtually unknown.

5 MFG-E8 and Ischemia/Reperfusion (I/R) Injury

MFG-E8 is also involved in I/R injuries (see the related Brain and Intestinal I/R chapters). Hallmarks of I/R injuries include notably cell death and sterile inflammation, processes where MFG-E8 is classically involved. In a murine renal I/R model, administration of recombinant MFG-E8 (rMFG-E8) improved renal function recovery and dampened inflammatory response and tubular cell death [48]. Survival rate at 60 h was also improved, highlighting the long-term benefit of rMFG-E8 in this model. Recently, the same team found rMFG-E8 to be also beneficial in a liver I/R model [49]. Function of MFG-E8 in stroke has been the subject of three studies, two of them finding a neuroprotective effect [13, 50, 51].

MFG-E8 expression in I/R injuries is associated with anti-inflammatory effects [13, 48–50]. However, because of its many pleomorphic functions, the precise protective mechanism of MFG-E8 differs according to the model studied. In I/R injuries, MFG-E8 has been found to down-modulate NF-κB, to lessen macrophage and neutrophil infiltration, to dampen TNFα, IL6, IL1β and MIP-2 production and to increase TGFβ expression. Using a murine cerebral ischemia model, we found that absence of MFG-E8 was indeed associated with an impaired efferocytosis and a pro-inflammatory cytokine environment [13]. More interestingly, we found that the neuroprotective effect of MFG-E8 in our model was linked to a direct down-regulation of inflammasome activation, independent of efferocytosis. MFG-E8 decreased inflammasome-dependent IL1β production both in vitro and in vivo. MFG-E8 inhibited necrotic cell-induced and ATP-dependent IL1β production by macrophages/microglia through mediation of integrin β_3 and P2X7 receptor interactions. The in vivo relevance of our results was supported by the fact that MFG-E8 neuroprotective effect was abrogated in *P2x7r*$^{-/-}$ and *Itgb3*$^{-/-}$ mice or in mice treated with IL1-receptor antagonist [13]. In renal and liver I/R injury models, a MFG-E8-dependent NF-κB regulation mechanism was proposed by the authors [48, 49]. Interestingly, the multitude of MFG-E8 anti-inflammatory effects, both on innate and adaptive immunity, makes MFG-E8 a very interesting target for immunomodulation strategy in cardiovascular diseases. Some anti-apoptotic effects have also

been reported in stroke, liver I/R and renal I/R with an up-regulation of PPAR-γ and of Bcl2/bax ratio [48–50]. However, it is still uncertain if this anti-apoptotic property of MFG-E8 is a direct effect of MFG-E8 or a consequence of its anti-inflammatory role in the context of I/R injury.

As in atherosclerosis, some of MFG-E8 effects could be detrimental in I/R injury. MFG-E8 mediates efferocytosis of apoptotic cells and, thus, leads to beneficial anti-inflammatory effects. However, in a specific endothelin 1-induced rat cerebral ischemia model, MFG-E8 was found to worsen functional impairment through efferocytosis of phosphatidylserine (PS)-positive but viable neurons by activated microglia [51]. Of interest, this stroke model induces a small ischemia with a relatively large penumbra and a small ischemic core where the inflammatory reaction is modest and, probably, less relevant.

6 Conclusion

The studies of MFG-E8 in the cardiovascular field have led to the identification of diverse roles for this glycoprotein in several pathophysiologic processes that control the development and complications of cardiovascular diseases, including vascular cell proliferation and survival, angiogenesis and the regulation of innate and adaptive immune responses. As a result, MFG-E8 is proposed to play either protective or detrimental effects dependent on the pathophysiological setting and the relevance of each of its modulatory roles to the distinct disease context. A better understanding of these context-dependent regulatory effects is needed to guide the development of disease-specific therapeutics.

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Chapter 6
Autoimmune Diseases and the Role of MFG-E8

Rikinari Hanayama

Abstract Clearance of apoptotic cells is an essential process to maintain homeostasis in both physiological and pathological circumstances. Accumulation of apoptotic cells as a result of inappropriate clearance has been implicated in the release of self-antigens that may cause inflammation and autoimmune diseases. MFG-E8 is a factor that links apoptotic cells to phagocytes. MFG-E8 is strongly expressed by macrophages in the germinal centers of the spleen and lymph nodes, and the deficiency of MFG-E8 causes impaired clearance of apoptotic cells by these macrophages. Uncleared apoptotic cells undergo post-apoptotic (secondary) necrosis and release intracellular contents that cause harmful inflammatory responses. As a result, MFG-E8-deficient mice spontaneously develop autoimmune diseases that resemble systemic lupus erythematosus (SLE). This chapter discusses the current understanding of the clearance of apoptotic cells, particularly *via* MFG-E8, in the prevention of autoimmune diseases.

Keywords Apoptosis • Phagocytosis • Macrophage • MFG-E8 • Phosphatidylserine • Inflammation • Self-antigens • Autoimmune diseases • SLE

1 Introduction: Clearance of Apoptotic Cells

In our body, billions of cells undergo apoptosis every day and are rapidly cleared away to maintain the integrity and functions of their surrounding tissues [1]. The clearance of apoptotic cells is mediated by neighboring amateur phagocytes (such as fibroblasts, epithelial cells and endothelial cells) and/or professional phagocytes (such as macrophages and immature dendritic cells) that ingest and digest the

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dying cells. This process is called phagocytosis. The phagocytes approach the apoptotic cells by sensing the "find me" signals secreted from the dying cells, and engulf the cells (but not healthy ones) by recognizing the "eat me" signals exposed on the surface of the dying cells.

1.1 "Find-Me" Signals

At the early stage of apoptosis, the apoptotic cells secret chemotactic factors ("find me" signals or "come-and-get-me" signals) that stimulate the attraction of motile phagocytes (monocytes, macrophages and dendritic cells) to ensure the quick and efficient removal of apoptotic cells as intact cell corpses. Lysophosphatidylcholine (LPC), ATP/UTP, sphingosine-1-phosphate (S1P), and CX3CL1 (fractalkine) have been proposed as the "find me" signals; they are released from apoptotic cells in a caspase-dependent manner, bind specific receptors on phagocytes and trigger chemotaxis of the phagocytes [1]. LPC is generated by the caspase-3 mediated activation of the phospholipase A2, which converts phosphatidylcholine into LPC [2], and is released by the ATP-binding cassette transporter A1 [3]. LPC binds to G2A, a G protein-coupled receptor expressed on phagocytes, and induces attraction of the phagocytes [4]. Likewise, ATP/UTP and S1P recruit phagocytes by binding to P2Y2 and S1P receptors, respectively [5, 6].

A chemokine, CX3CL1/fractalkine is also released from apoptotic cells, playing an active role in the chemotaxis of phagocytes to apoptotic cells [7]. It is rapidly processed and released in a caspase-dependent mechanism from apoptotic cells in various tissues, such as apoptotic neurons in the central nervous system or apoptotic B cells in germinal centers of spleen and lymph nodes. It binds to the specific receptor, CX3CR1, on phagocytes, and activates them for chemotaxis. Notably, CX3CL1/fractalkine up-regulates the expression of MFG-E8 in phagocytes, which concomitantly enhances the phagocytosis of apoptotic cells [8, 9]. Thus, "find me" signals can ensure the prompt and efficient clearance of apoptotic cells, not only by attracting phagocytes but also by up-regulating the pro-phagocytosis machinery in the phagocytes.

1.2 "Eat-Me" and "Don't Eat-Me" Signals

Phagocytes engulf apoptotic cells but not healthy cells, indicating that the dying cells display 'eat-me' signals to phagocytes and the phagocytes recognize the signals using specific receptors. The best-studied, and most likely 'eat-me' signal is an aminophospholipid of cell plasma membranes, phosphatidylserine [10]. Masking phosphatidylserine on the apoptotic cells inhibits the phagocytosis of apoptotic cells by a wide variety of phagocytes, suggesting a pivotal role for phosphatidylserine [11]. Phosphatidylserine is a component of cell plasma membrane and is kept exclusively on the inner leaflet of the lipid bilayers in healthy cell by the action of

ATP-dependent aminophospholipid translocases [12]. When cells receive apoptotic signals, the translocases are inactivated, causing the randomization of the membrane leaflets and the exposure of phosphatidylserine on the outer leaflet of the plasma membranes.

Another player involved in the exposure of phosphatidylserine during apoptosis is phospholipid scramblases [13]. Putative phospholipid scramblases are activated by the increased intracellular calcium levels in the dying cells. Activated scramblases randomize the phospholipid in the plasma membranes, causing the exposure of the phosphatidylserine. However, the proteins responsible for scrambling activity have been disputed. In human, a family of four homologous proteins was named as PLSCR1-4 for phospholipid scramblase. However, all PLSCRs have a nuclear localization signal, and PLSCR1 binds to the promoter of the inositol 1, 4, 5-triphosphate receptor type 1 gene to enhance the expression of the receptor, suggesting a possible nuclear function [14]. In addition, the *PLSCR1* and *PLSCR3-deficient* mice do not show any abnormality in the phosphatidylserine exposure on apoptotic cells or platelets [15, 16]. These data suggest that PLSCRs are not the scramblase, nor is it required for scramblase activation or activity.

Recently, Nagata and colleagues have found that Xk-related protein 8 (Xkr8) mediates phospholipid scrambling that promotes the exposure of phosphatidylserine in response to various apoptotic stimuli [17]. Xkr8 contains six transmembrane regions that are flanked by cytosolic N and C termini with a conserved caspase-3 recognition site near the C terminus. Xkr8 is directly activated by caspase-cleavage upon various apoptotic stimuli, and promotes the externalization of phosphatidylserine to the surface of apoptotic cells. CED-8, the only Xk-family ortholog in *C. elegans*, also promotes both apoptotic phosphatidylserine exposure and apoptotic cell clearance, indicating that Xk-family proteins have evolutionarily conserved roles in promoting the externalization of phosphatidylserine as an "eat me" signal for phagocytosis.

On healthy cells, the "eat me" signal is counteracted by a separate "don't eat me" signal, such as CD47, a membrane protein with five membrane-spanning regions. When CD47-deficient red blood cells are injected into mice, they are more rapidly cleared by macrophages in the spleen than CD47-positive cells [18]. As a "don't eat me" signal, CD47 on healthy cells prevents the phagocytosis by macrophages by binding to the inhibitory receptor, signal regulatory protein alpha (SIRPalpha). However, on apoptotic cells, the CD47 expression is not lost, yet the apoptotic cells are efficiently engulfed by macrophages, indicating that an "eat me" signal overcomes the "don't eat me" signal [19].

1.3 Phosphatidylserine Receptors

The phosphatidylserine on apoptotic cells are recognized by phagocytes directly through surface receptors or indirectly through soluble bridging proteins that bind to both phosphatidylserine on apoptotic cells and specific receptors on phagocytes [1]. Recently, three surface phosphatidylserine receptors have been identified, namely, Tim-4, BAI1, and Stabilin-2 [20].

T-cell immunoglobulin- and mucin-domain-containing molecule 4 (Tim-4) is a type I transmembrane protein that consists of a signal sequence, an immunoglobulin (Ig) V domain, a mucin-like domain, a transmembrane and a cytoplasmic region [21]. Tim-4 is expressed by macrophages and dendritic cells in various places including spleen, lymph nodes and peritoneum. Tim-4 specifically binds to phosphatidylserine on apoptotic cells *via* its extracellular IgV domain, and promotes the uptake of the apoptotic cells. However, Tim-4 has a short cytoplasmic region that carries no obvious signaling motif, and this region is not necessary to enhance the phagocytosis of apoptotic cells [22]. Tim-4 may therefore require a signaling partner to promote the uptake of apoptotic cells.

Brain-specific angiogenesis inhibitor 1 (BAI1) was identified as another candidate of phosphatidylserine receptors [23]. BAI1 belongs to the G-protein coupled receptor family, with five thrombospondin type 1 repeats (TSRs) in its extracellular region, a seven-transmembrane region and a 392 amino acids-cytoplasmic tail. BAI1 binds to phosphatidylserine on apoptotic cells *via* its TSRs and promotes the engulfment of apoptotic cells by interacting with Dock180/ELMO1 complex, a guanine nucleotide exchange factor for the small GTPase Rac1.

The third phosphatidylserine receptor is Stabilin2 [24]. It is a multifunctional receptor containing a large extracellular domain that consists of seven FAS1 domains and four EGF-like domain repeats. Stabilin2 recognizes phosphatidylserine through extracellular EGF-like domain repeats and activates Rac1 *via* interactions with GULP adaptor protein through the NPXY motif in the cytoplasmic tail. However, it is still unclear whether these surface phosphatidylserine receptors (Tim-4, BAI1 and stabilin2) play essential roles in the engulfment of apoptotic cells *in vivo*, and whether these receptors are functionally redundant, additive, or synergistic.

Two secreted bridging proteins (MFG-E8 and Gas6/ProS) have been identified as molecules that bind phosphatidylserine on apoptotic cells and promote their engulfment by phagocytes. Growth arrest-specific 6 (Gas6) and its related protein, Protein S (ProS), are vitamin K-dependent proteins containing a gamma-carboxyglutamic acid (Gla) domain at its N-terminus, which binds phosphatidylserine [10]. In the C-terminus, Gas6 and ProS have a receptor binding domain that is responsible for binding to TAM receptor family members (Tyro3, Axl and Mer). TAM receptor family members carry a tyrosine kinase domain in their cytoplasmic region which induce tyrosine phosphorylation of various signaling molecules including focal adhesion kinase (FAK). FAK then associates with the cytoplasmic tail of β_5 integrin, which promotes the internalization of apoptotic cells [10]. Thus, Gas6/ProS-TAM receptor pathway may share partially overlapping functions with MFG-E8-integrin pathway and undergo crosstalk in a similar fashion for phagocytosis.

2 MFG-E8

Milk fat globule EGF factor 8 (MFG-E8, also called as lactadherin or SED1), originally found associated with milk fat globules in mammary glands, is a glycoprotein secreted from a subset of phagocytes that actively engulf apoptotic cells [25].

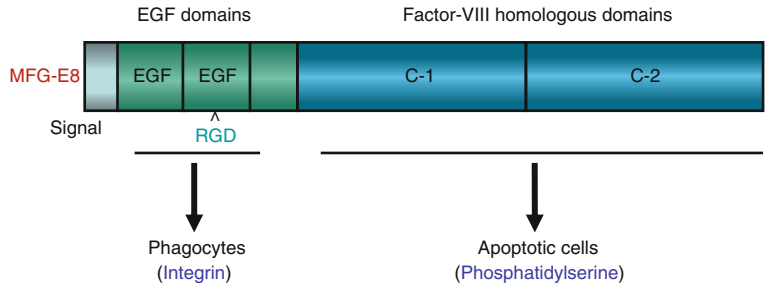

Fig. 6.1 The structure of MFG-E8. MFG-E8 binds to phosphatidylserine on apoptotic cells *via* C1 and C2 domains, and to integrins on phagocytes *via* an RGD motif in the second EGF domain

MFG-E8 consists of a signal sequence, two epidermal growth factor (EGF) domains, a proline/threonine (PT) rich domain and two factor-VIII-homologous domains (C1 and C2, also called as discoidin domains) from its amino terminus, but lacks a membrane-spanning region (Fig. 6.1). In particular, this protein contains RGD (arginine-glycine-aspartate) motif in the second EGF domain, which can be recognized by some members of the integrin family [26].

2.1 MFG-E8 Links Apoptotic Cells to Phagocytes

MFG-E8 was originally identified as a surface protein of mammary epithelial cells but its physiological roles had been unknown for a long time [27]. In 2002, we found that MFG-E8 secreted from activated macrophages and immature dendritic cells works as a bridge between apoptotic cells and phagocytes [25, 28]. MFG-E8 does not bind to healthy cells, but when cells undergo apoptosis exposing phosphatidylserine on their surface, MFG-E8 strongly bind the phosphatidylserine *via* its C1 and C2 domains. MFG-E8 has a high affinity for phosphatidylserine with a dissociation constant (Kd) of 2nM, but has no affinity for other phospholipids on the cell plasma membrane, including phosphatidylinositol, phosphatidylcholine or phosphatidylethanolamine, which accounts for the specificity of MFG-E8 for apoptotic cells, but not healthy cells. MFG-E8 has an alternatively spliced variant that lacks the PT rich domain. The full-length MFG-E8 is predominantly expressed in inflammatory macrophages, immature dendritic cells and mammary epithelial cells. On the other hand, the short variant is ubiquitously expressed in various tissues [28], but it has a weaker affinity for phosphatidylserine [29].

While engaged with apoptotic cells, MFG-E8 can also interact with phagocytes to stimulate the uptake of the apoptotic cells. MFG-E8 contains an RGD motif in the second EGF domain, which is a ligand for $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin expressed on various phagocytes (including both amateur and professional phagocytes). An unequivocal role for $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin in apoptotic cell clearance has been well established, but neither of these molecules directly binds phosphatidylserine [30]. MFG-E8 solves this dilemma by linking $\alpha_v\beta_3$ or $\alpha_v\beta_5$ to the phosphatidylserine with its

bivalent arms. Local secretion of MFG-E8 from macrophages acts as an opsonin between phosphatidylserine on apoptotic cells and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin on phagocytes. Thus, MFG-E8 promotes the phagocytosis of apoptotic cells not only by the MFG-E8-secreting macrophages but also by neighboring amateur phagocytes expressing integrin.

The binding of MFG-E8 to integrin stimulates a signaling cascade involving FAK, p130, CrkII and Dock180/ELMO1complex, which leads to the activation of the Rac1 for the internalization of the bound apoptotic cells [10]. This signaling cascade has evolutionarily conserved roles in promoting apoptotic cell clearance, since their orthologs in *C.elegans* (ced-2/CrkII, ced-5/Dock180, ced-10/Rac1, and ced-12/ELMO1) were originally identified as key regulators of apoptotic cell clearance in *C. elegans* by genetic screenings.

2.2 Expression of MFG-E8 in Germinal Centers

In contrast to Gas6 and ProS, both of which are abundant in serum, MFG-E8 is a macrophage-derived opsonin produced at the site of apoptosis, where serum proteins may be excluded. MFG-E8 is mainly expressed by inflammatory macrophages (M1 macrophages) but not by anti-inflammatory macrophages (M2 macrophages), suggesting that the protein acts at sites of inflammation where massive apoptosis may occur [31]. In the presence of MFG-E8, the recruited inflammatory macrophages engulf the apoptotic cells, and release anti-inflammatory molecules that will limit the tissue destruction. The expression of MFG-E8 can be up-regulated in phagocytes during chemotaxis by the "find-me" signal (such as CX3CL1) released from the apoptotic cells.

To explore the physiological roles of MFG-E8, the expression of MFG-E8 *in vivo* was examined [32]. MFG-E8 is predominantly expressed by macrophages in spleen and lymph nodes, Langerhans cells in skin, epithelial cells in mammary gland, and microglia and astrocytes in brain [28, 32–34]. Interestingly, although there are many subsets of macrophages and dendritic cells in spleen and lymph nodes [35], the expression of MFG-E8 is restricted to follicular dendritic cells and tingible body macrophages in germinal centers [32, 36].

Germinal centers are sites of B cell activation, proliferation and maturation during antibody responses [37]. Activated B cells that strongly bind the antigens presented on the follicular dendritic cells can be selected as future plasma cells and memory B cells, but those with low affinity for the antigen undergo apoptosis and are swiftly removed by the tingible body macrophages in the germinal centers (Fig. 6.2) [38]. These macrophages contain many condensed chromatin fragments from apoptotic cells (tingible bodies) inside them. Follicular dendritic cells produce MFG-E8 to opsonize the dying B cells and target them for removal by tingible body macrophages [36]. Thus, follicular dendritic cells appear to regulate both survival of germinal center B cells and their removal once these cells have undergone apoptosis.

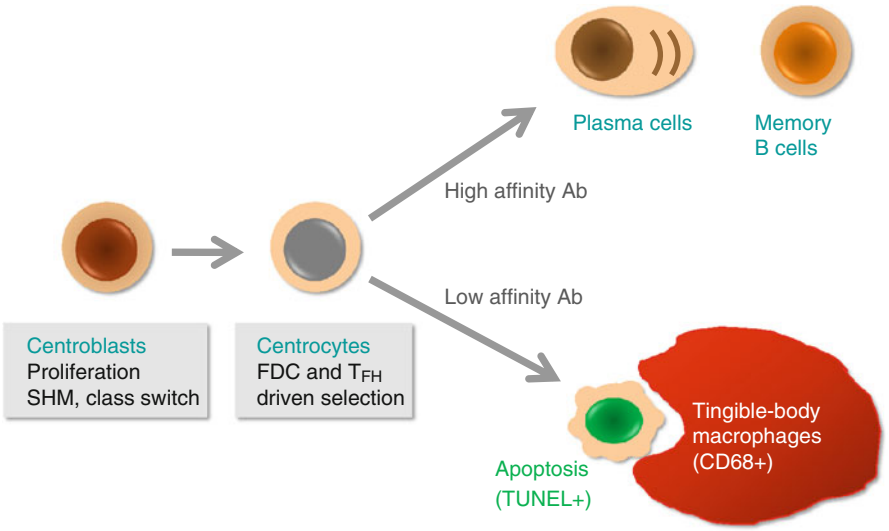

Fig. 6.2 Life of B cells in germinal centers. Activated B cells (centroblasts) migrate into germinal centers, and undergo proliferation, somatic hypermutation (*SHM*) and class switching. The maturing B cells (centrocytes) whose antibodies (*Ab*) strongly bind the antigens presented on the follicular dendritic cells (*FDC*) can survive and differentiate into plasma cells or memory B cells with a support from follicular helper T cells (T_{FH}). However, those with low affinity antibodies for the antigen die by apoptosis and are cleared by the tingible body macrophages

2.3 MFG-E8-Mediated Clearance of Apoptotic Cells in Germinal Centers

To examine the functions of MFG-E8 in germinal centers, MFG-E8-deficient mice were generated [32]. These mice grow normally, but as they grow older, MFG-E8-deficient mice develop splenomegaly with enlarged white pulps and germinal centers. Then the ability of tingible body macrophages to engulf apoptotic cells was examined. In the spleen of aged wild-type mice, tingible body macrophages in the germinal centers are associated with none or only few apoptotic cells. On the other hand, in aged MFG-E8-deficient mice, tingible body macrophages are enlarged and associated with many apoptotic cells. When B cells are activated by immunization with keyhole limpet hemocyanin (KLH) and complete Freund's adjuvant, the enlargement of the tingible body macrophages and their association with apoptotic cells become much more apparent (Fig. 6.3). That is, tingible body macrophages in wild-type mice engulf several apoptotic B cells that underwent apoptosis after activation on the stimuli. On the other hand, tingible body macrophages in MFG-E8-deficient mice carry a large number of apoptotic B cells and become greatly enlarged. To confirm the localization of the apoptotic cells, the spleen sections were analyzed by electron microscopy. It revealed that tingible body macrophages in wild-type mice carry many condensed nuclei from apoptotic cells inside them and

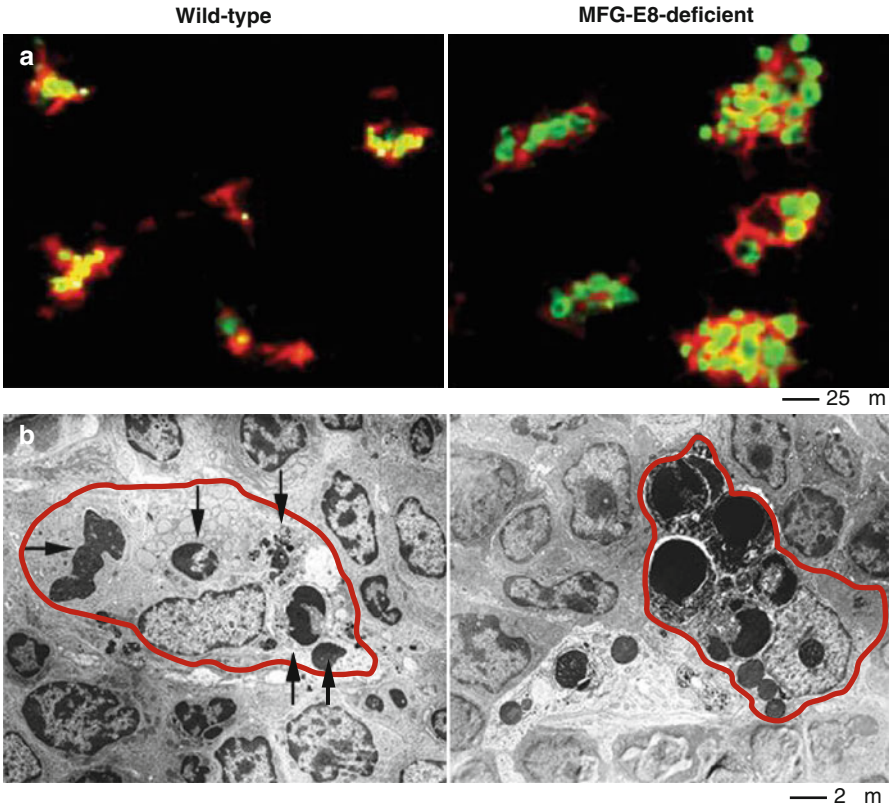

Fig. 6.3 Impaired uptake of apoptotic cells in MFG-E8-deficient mice. (**a**) Apoptotic cells (*green*) are efficiently engulfed by tingible body macrophages (*red*) in wild-type mice. On the other hand, a number of apoptotic cells seem to be present extracellularly on the surface of the tingible body macrophages in MFG-E8-deficient mice. (**b**) EM analysis of spleen sections revealed that the tingible body macrophages (*encircled by red line*) in wild-type mice have condensed nuclei from apoptotic cells inside them (*arrows*), and some of the apoptotic cells are degraded. In the MFG-E8-deficient mice, apoptotic cells were also localized to the tingible body macrophages, but they have intact plasma membranes, indicating that these apoptotic cells are not engulfed by the macrophages

apoptotic bodies are degraded. In contrast, in the MFG-E8-deficient mice, apoptotic cells are also associated with the tingible body macrophages but they have intact plasma membranes, indicating that these apoptotic cells are not engulfed and located outside the MFG-E8-deficient macrophages.

2.4 Two-Step Phagocytosis of Apoptotic Cells

A possible explanation of this finding is "tethering and tickling" two-step model for phagocytosis [39]. In this model, apoptotic cells are first tethered to macrophages *via* specific ligands and receptors, but another tickling (stimulating) signal is

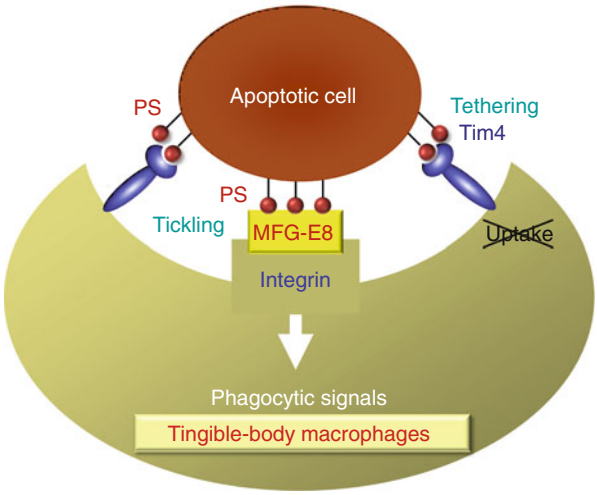

Fig. 6.4 Two-step engulfment of apoptotic cells. By binding phosphatidylserine (*PS*), Tim4 may tether apoptotic cells to tingible body macrophages without their uptake. The MFG-E8-integrin pathway stimulates (tickles) the phagocytic signals for the uptake of the bound apoptotic cells

required to initiate the engulfment. MFG-E8 binds to phosphatidylserine on apoptotic cells and stimulates the phagocytic signals through integrin, thus working as the tickling signal. In MFG-E8-deficient mice, macrophages are still able to associate with apoptotic cells *via* tethering receptors but unable to engulf them due to the lack of the tickling signaling. It resulted in the persistence of a number of apoptotic cells on the surface of the MFG-E8-deficient macrophages.

On the other hand, the tethering receptors on the tingible body macrophages have been unknown, but recently, Tim-4 has been found on the tingible body macrophages [40]. As explained, Tim-4 is a phosphatidylserine receptor that strongly binds to apoptotic cells but does not transduce the uptake signaling by itself [22], raising the possibility that Tim4 might serve as the tethering receptor on the tingible body macrophages. To prove this idea, the engulfment of apoptotic cells was reconstituted *in vitro* by using a mouse pro-B cell line, Ba/F3, which ordinarily do not engulf apoptotic cells [41]. Ba/F3 transformants expressing Tim4 efficiently bind apoptotic cells in a phosphatidylserine-dependent manner but do not engulf them at all. However, Ba/F3 transformants expressing both Tim4 and the $\alpha_v\beta_3$ integrin bind to and efficiently engulf apoptotic cells in the presence of MFG-E8. These results support the idea that the engulfment of apoptotic cells proceeds in two steps: Tim4 tethers apoptotic cells, and the MFG-E8-$\alpha_v\beta_3$ integrin mediates engulfment of the bound apoptotic cells (Fig. 6.4).

3 Autoimmune Disease Caused by the Deficiency of MFG-E8

Uncleared apoptotic cells undergo secondary necrosis and lose their membrane integrity. Then the necrotic cells release intracellular self-antigens such as nucleosomes [42], as well as internal danger signals that activate the immune system. As a consequence, the MFG-E8-deficient mice, particularly of the B6/129-mixed background,

spontaneously produce autoantibodies in an age-dependent manner. No autoantibody can be detected in young MFG-E8-deficient mice in their serum. However, as they grow older, MFG-E8-deficient mice produce high titers of anti-double stranded DNA antibody and anti-nuclear antibody (ANA) in their sera. Female mice produce much more severely than male mice, which is a common feature of many autoimmune diseases [43]. When young female mice receive immunization twice with KLH to activate B cells, MFG-E8-deficient mice start to produce ANA. This result indicates that the inefficient removal of apoptotic B cells can cause the autoantibody production. Due to the high concentrations of autoantibodies, aged MFG-E8-deficient mice develop glomerulonephritis with a massive deposition of IgG, IgM, C1q, C4 complements in the glomeruli and hypercellularity [44]. These autoimmune properties in MFG-E8-deficient mice resemble those of human systemic lupus erythematosus (SLE).

3.1 Impaired Apoptotic Cell Clearance Causes SLE

SLE or lupus is a chronic autoimmune disease which predominantly affects women by harming multiple organs including the heart, joints, skin, lungs, blood vessels, liver, kidney, and nervous system [45]. Patients with SLE have autoantibodies in their sera against nuclear components (anti-ribonucleoprotein and anti-DNA antibodies) and sometimes exhibit circulating DNA or nucleosomes [46]. The relevance of defective clearance of apoptotic cells to the development of SLE has been implicated for long time [47]. For example, monocyte-derived macrophages from patients with SLE have impaired ability of phagocytosis of apoptotic cells [48]. As observed in MFG-E8-deficient mice, patients with SLE often have a defect in the engulfment of apoptotic B cells by tingible body macrophages in the germinal centers [49]. Thus, the SLE-type autoimmune phenotype observed in MFG-E8-deficient mice will provide a good model system to study the pathophysiology of SLE.

Like MFG-E8-deficient mice, some mutant mice in which apoptotic cells persist as a result of impaired clearance also develop an SLE-type autoimmune disease [50]. These include mice deficient in the first component of complement (C1q) and Mer tyrosine kinase [51, 52]. Most apoptotic cells are rapidly cleared while in a stage of early apoptosis. However, if apoptotic cells persist longer in the tissues because of either impaired engulfment or overwhelming generation of apoptotic cells, the complement system functions in clearance of dead cells. C1q is the most studied complement component that binds dead cells in the later stages of apoptosis and also necrotic cells. Notably, almost all individuals deficient in C1q develop severe SLE [53]. In C1q-deficient mice, unengulfed dead cells persist in the glomeruli of kidneys, and develop an SLE-like phenotype mainly in lupus-prone mouse strains such as MRL/MP [53]. The mice have a defect in clearance of apoptotic cells, suggesting that C1q-mediated engulfment is a back-up system for the clearance of dead cells. In this regard, it may be interesting to cross C1q-deficient mice with the MFG-E8-deficient mice to enhance the defect in engulfment of apoptotic cells.

Loss of Mer, a member of TAM receptor family, in macrophages also leads to impaired removal of apoptotic cells and causes the development of an SLE-type

autoimmune disease [52]. Recently, like MFG-E8, Mer has been found on tingible body macrophages, and a deficiency of Mer caused the accumulation of apoptotic bodies in germinal centers [54]. These phenotypes are especially interesting, since crosstalk between Gas6/ProS-TAM receptor pathway and MFG-E8-integrin pathway were already demonstrated [10, 55]. However, how these two pathways cooperate with each other *in vivo* remains to be studied.

3.2 Released Self-Antigens Activate the Immune System

What are the mechanisms of how accumulated apoptotic cells cause the SLE-type autoimmune disease in MFG-E8-deficient mice? Apoptotic cells are usually cleared by macrophages without inflammation [56]. In contrast, persistent apoptotic cells undergo secondary necrosis, which allows the release of pro-inflammatory or immunogenic self-antigens [57]. For example, it has been reported that a DNA-binding protein, high-mobility group protein B1 (HMGB1) is passively released from necrotic cells and triggers inflammation [58]. Once released into the extracellular milieu, it functions as a cytokine that activates monocytes and macrophages to produce inflammatory cytokines (TNFα, IL-1β, IL-6) [59]. The pro-inflammatory cytokine activity of HMGB1 is mostly attributed to its ligation with the receptor for advanced glycation end products (RAGE), which is expressed on monocytes, macrophages and other cell types like epithelial cells and endothelial cells [60]. Aberrant and prolonged inflammation may drive autoimmune responses. In addition, HMGB1 blocks MFG-E8-mediated phagocytosis of apoptotic cells by binding to its receptor, $\alpha_v\beta_3$ integrin [61]. Thus, increased appearance of apoptotic cells further worsen the clearance of apoptotic cells.

Another possible mechanism is that self-DNA released from uncleared apoptotic cells form complexes with IgG and activate autoreactive B cells, which accidentally emerge during somatic hypermutation but normally remain quiescent [62]. The persistent apoptotic B cells in the germinal centers may release self-DNA to activate and proliferate the autoreactive B cells in a B cell receptor and Toll-like receptor dependent manner (Fig. 6.5). This activation of autoreactive B cells may be further enhanced by cytokines produced by macrophages that are also stimulated by the self-antigens and danger signals released form the necrotic cells. In any case, the MFG-E8-deficient mice may provide a good model system for studying the molecular mechanisms by which endogenous cellular components activate the immune system extracellularly.

3.3 Immunosuppressive Roles of MFG-E8

As stated above, the SLE-type autoimmunity in MFG-E8-deficient mice is triggered by the self-antigens released from uncleared apoptotic cells, but also might be enhanced by the defects of immunosuppressive functions of MFG-E8-mediated

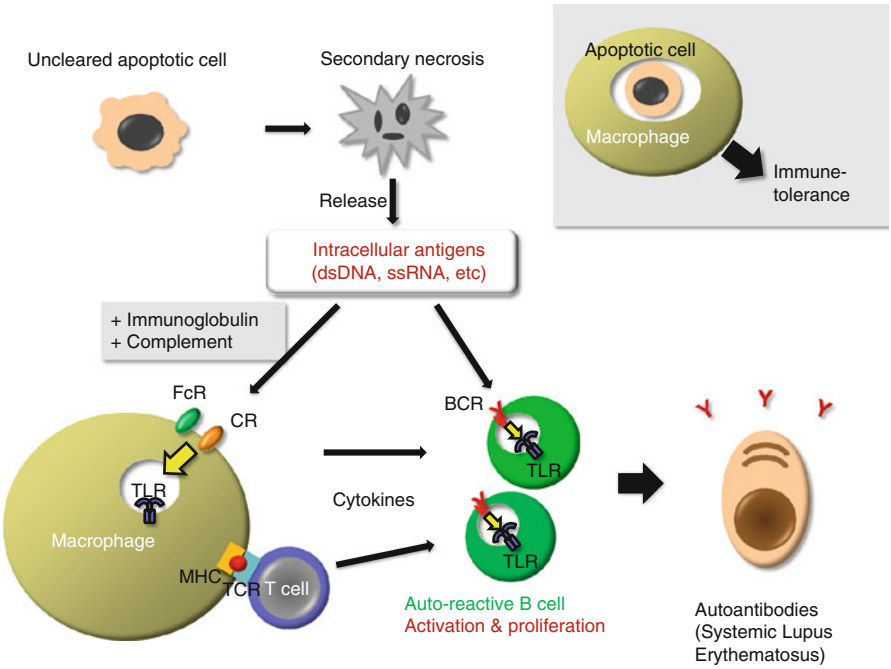

Fig. 6.5 Development of autoimmune diseases caused by impaired clearance of apoptotic cell. The engulfment of apoptotic cells ordinarily leads to immune-tolerance in macrophages (*inset*). However, if phagocytosis is impaired, uncleared apoptotic cells undergo secondary necrosis. The necrotic cells release intracellular antigens that activate and proliferate the autoreactive B cells in a B cell receptor (*BCR*) and Toll-like receptor (*TLR*) dependent manner. These antigens, together with immunoglobulin or complement, are also taken up by macrophages through Fc receptors or complement receptors (*CR*) and stimulate TLRs in endosomes. The activated macrophages or T cells release cytokines that enhance the activation and proliferation of autoreactive B cells. Activated B cells differentiate into plasma cells and secrete autoantibodies that cause SLE

phagocytosis. Binding or uptake of apoptotic cells to phagocytes induces production of anti-inflammatory cytokines such as transforming growth factor β (TGFβ) and IL-10 *in vitro* [63]. These anti-inflammatory cytokines inhibit the lipopolysaccharide-induced expression of tumor necrosis factor α (TNFα). In addition, MFG-E8 inhibits production of inflammasome-induced inflammatory cytokines through mediation of integrin [64]. Accordingly, in the spleen of MFG-E8-deficient mice, production of IL-10 decreases, while IFN-γ level increases, suggesting a switch of the immune response to Th1 proinflammatory phenotype in the absence of MFG-E8 [65].

The production of anti-inflammatory cytokines can be blocked by a dominant-negative mutant of MFG-E8, which cannot bind $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin, indicating the ligation of MFG-E8 to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin transduce the signal that up-regulates the expression of anti-inflammatory cytokines [11]. However, the mechanisms how the engulfment of apoptotic cells leads to the production of anti-inflammatory

cytokines have been unclear. Recently, MFG-E8 has been found to play critical roles in reprograming macrophages into anti-inflammatory macrophages which produce TGFβ and IL-10, by increasing the levels of STAT3 phosphorylation in the macrophages [66]. Thus, phagocytosis *via* MFG-E8, secreted from inflammatory macrophages, might induce anti-inflammatory responses that lead to the resolution of the inflammation.

By producing anti-inflammatory cytokines, MFG-E8-mediated phagocytosis also causes the expansion of regulatory T cells (Tregs), and the reduction of cytotoxic T cells [67]. Therefore, blockage of the engulfment of apoptotic cells by a dominant-negative mutant of MFG-E8 inhibits Tregs, thus expanding cytotoxic T cells. Tregs play an important role in the maintenance and regulation of immune tolerance and in the prevention of autoimmunity. Recent studies have demonstrated a deficiency in number and function of Tregs in SLE patients [68]. The development of SLE in MFG-E8-deficient mice could also be attributed to the impaired functions of Tregs. As tumor immunity is often regulated by Tregs and cytotoxic T cells, MFG-E8 or its dominant-negative mutant could be used clinically to treat tumor patients, by regulating the number of Tregs and cytotoxic T cells,

3.4 Altered Processing of Apoptotic Antigens by MFG-E8-Deficiency

Recently, another mechanism to develop autoimmunity by MFG-E8-deficiency has been proposed [69]. In the absence of MFG-E8, dendritic cells cannot engulf intact apoptotic cells efficiently, and late apoptotic cell debris, which are much smaller cell fragments start to accumulate. MFG-E8-deficient dendritic cells ingest these debris *via* endocytic pathway. As a result, the relative proportion of cell debris engulfed by MFG-E8-deficient dendritic cells increases, and the composition of the apoptotic material differs significantly between wild-type and MFG-E8-deficient dendritic cells. As MFG-E8-mediated phagocytosis stimulates phagosome maturation, wild-type dendritic cells contain predominantly intact apoptotic cells in phagosomes that fuse with acidic lysosomes for digestion. On the other hand, cell debris in MFG-E8-deficient dendritic cells persist in endosomal compartments and remain outside of lysosomes. The lack of phagosome maturation in MFG-E8-deficient dendritic cells causes the increase of apoptotic cell-associated antigens outside lysosome, enhancing the preservation of these self antigens. This, in turn, facilitates self-peptide access to the cross-presentation machinery in cytosol, and enhances the activation of self-reactive cytotoxic T cell responses in MFG-E8-deficient mice. The cytotoxic T cells activated by self antigens infiltrate into skin, causing the spontaneous dermatitis in MFG-E8-deficient mice. This is an intriguing model explaining how altered trafficking of apoptotic cell-associated antigens leads to the development of autoimmunity.

4 MFG-E8 in Human SLE

In mouse and rat, there are two splice variants for MFG-E8 mRNA: the long and short forms [70]. The long form of MFG-E8 has an extra exon that encodes a proline/threonine (PT) rich domain. However, in human, only a short MFG-E8 variant is expressed [71]. The human MFG-E8 consists of one EGF domain containing an RGD motif, and shares 65 % homology to the factor-VIII-homologous domains (C1 and C2 domains) of murine MFG-E8 that are responsible for phosphatidylserine binding, but the ability of human MFG-E8 to promote phagocytosis of apoptotic cells is comparable to that of murine MFG-E8.

4.1 Genetic Polymorphism on MFG-E8

As MFG-E8-deficiency has been linked to the development of the SLE-type autoimmune disease in a mouse model, a case–control study of MFG-E8 genetic polymorphism was performed on 147 SLE patients and 146 non-lupus control subjects in Taiwan to determine whether genetic variations of MFG-E8 are associated with SLE [72]. Single nucleotide polymorphisms (SNPs) in the coding sequence of human MFG-E8 gene were investigated, and genetic polymorphism on MFG-E8 residue 76 in C1 domain correlated significantly to SLE. Individuals with MFG-E8-76^{Met} allele, which is found in 25 % of SLE patients, displayed a predisposition to SLE in a recessive mode (odds ratio: 2.1, $P=0.020$). On the other hand, carriage of MFG-E8-76^{Leu} were negatively associated with SLE. The MFG-E8 genotypic combinations with 3^{Ser} and 76^{Leu} showed the most pronounced protective effect on SLE when compared to the most predisposing genotype 3^{Arg}–76^{Met}, which is found in 15 % of SLE patients (OR: 0.29, $P=0.007$). According to these results, MFG-E8 polymorphism is associated with SLE predisposition. As mutations in C1 domain often impairs the binding ability to phosphatidylserine [31], this result implicates that the impairment of MFG-E8-mediated clearance of apoptotic cells compromises a significant risk factor for the development of human SLE.

4.2 MFG-E8 as a Therapeutic Target for SLE

For the treatment of human SLE, corticosteroids are routinely used to control symptoms that occur in SLE, including inflammation, pain and tissue damage throughout the body. The anti-inflammatory effects of corticosteroids have been attributed to the suppression of the immune system that produce the pro-inflammatory cytokines. Recently, Herrmann and colleagues have proposed an additional mechanism of corticosteroids to reduce inflammation [73]. SLE patients with chronic inflammation receiving high-dose prednisone therapy displayed substantially increased MFG-E8 mRNA levels in

circulating monocytes. Several corticosteroid responsive elements were found within the promoter region of MFG-E8 that mediate the transactivation of MFG-E8 expression *via* corticosteroid receptors. The corticosteroid-mediated transactivation of MFG-E8 expression caused the concomitant enhancement of apoptotic cell clearance that results in the reduction of inflammation. Thus, MFG-E8 could be a prospective target for the future treatment of SLE. However, it should be noticed that administration of excess amount of MFG-E8 can also cause an SLE-type autoimmune disease (see below) [11].

4.3 Autoimmune Disease Caused by an Excess of MFG-E8

As inferred from its characteristic as a bridging molecule, the effect of MFG-E8 on engulfment is dose-dependent but bell-shaped: MFG-E8 enhances the engulfment of apoptotic cells at lower concentration, but it inhibits the engulfment at higher concentrations [71]. It seems that if MFG-E8 is present in excess, phosphatidylserine on apoptotic cells, and integrin on phagocytes are occupied by different MFG-E8 molecule, preventing its role as a bridge. An excess of MFG-E8 may inhibit not only the MFG-E8-dependent phagocytosis but also the phagocytosis with the other phosphatidylserine receptor systems by masking phosphatidylserine on apoptotic cells. Accordingly, injection of the recombinant MFG-E8 into mice causes an SLE-type autoimmune disease [11]. Thus, either shortage or excess of MFG-E8 leads to the development of autoimmune diseases by preventing the engulfment of apoptotic cells. Using a sensitive ELISA system for human MFG-E8, 16 of the 72 childhood-onset SLE patients and 17 of the 172 adult SLE patients were found to carry a high level of MFG-E8 in their serum (3–40 ng/ml), which is ordinarily undetectable in healthy individuals [71]. Although it is not clear whether the high level of MFG-E8 is the cause of the disease or the secondary effect by the disease, the high level of MFG-E8 would certainly worsen the disease. A more detailed analysis of MFG-E8 gene expression at the transcriptional or translational level will be required to understand what causes its overexpression in these SLE patients.

An excess of MFG-E8 is also caused by an alteration of protein stability. Recently, the MFG-E8 gene in Japanese female SLE patients has been analyzed, and two out of 322 female patients were found to carry a heterozygous intronic mutation. It causes production of aberrant MFG-E8 with a cryptic exon from intron 6 to be included in the transcript, resulting in an aberrantly spliced MFG-E8 mRNA in mononuclear cells of the patients [74]. The cryptic exon contains a premature termination codon, generating a C-terminally truncated MFG-E8 protein. The truncated MFG-E8 can bind to phosphatidylserine and enhance the phagocytosis of apoptotic cells as well as wild-type MFG-E8 does. Interestingly, the truncated MFG-E8 is aberrantly glycosylated and sialylated, which prolongs protein's half-life *in vivo*. As a result, when intravenously administrated into mice, the truncated MFG-E8 is sustained longer than wild-type protein in the blood. This prolong half-life of truncated MFG-E8 may cause an excess of MFG-E8 in blood, which inhibits the apoptotic cell clearance and leads to the development of autoimmune diseases. Taken together,

these findings suggest that not only a loss of MFG-E8 but also an excess of MFG-E8 may cause the development of an SLE-type autoimmune disease. Optimal expression of MFG-E8 is essential to prevent the development of autoimmune diseases.

5 MFG-E8 in Other Tissues

MFG-E8 is widely expressed in phagocytes in various tissues. Among them, the roles of MFG-E8 in microglia in brain have been extensively studied. MFG-E8 is up-regulated in microglia by CX3CL1/fractalkine released from damaged neurons to remove the apoptotic cells and debris in ischemic or degenerative brain tissue [75]. In Alzheimer's disease, microglia produce various neuroprotective factors including MFG-E8 that increases microglial neuroprotective activity against amyloid-β-peptide-induced neuronal cell death, by accelerating phagocytosis of neuronal debris and amyloid-β-peptide [76]. On the other hand, it has recently been reported that MFG-E8 from microglia mediates phagocytosis of viable neurons that express phosphatidylserine in neuroinflammation and Alzheimer's disease, leading to the death of the neurons by phagocytosis [77, 78]. These findings suggest MFG-E8 may be a potential therapeutic target to prevent neuronal loss in neuroinflammatory and neurodegenerative diseases.

The roles of MFG-E8 in the prevention of metabolic diseases have been reported. MFG-E8 is expressed by macrophages that infiltrated into atherosclerotic lesion, and the deficiency of MFG-E8 causes accumulation of apoptotic cells that accelerate the development of atherosclerosis [65]. Similarly, the deficiency of MFG-E8 accelerates the onset of disease in a mouse model of diabetes with increased numbers of cytotoxic T cells that attack the pancreatic beta cells [69].

In mammary glands, the expression of MFG-E8 is up-regulated in late pregnancy and increases further after the glands undergo involution, which is caused by loss of the suckling stimulus [29]. During the involution process, mammary epithelial cells undergo apoptosis and are cleared by neighboring epithelial cells and macrophages that have migrated into the glands. MFG-E8 promotes the clearance of apoptotic mammary epithelial cells by both cell types. MFG-E8 is also indispensable for clearing milk fat globules, minute globules carrying fat, which expose phosphatidylserine on their surface. In MFG-E8-deficient mice, a large number of milk fat globules were not cleared in the mammary ducts, and the mice acquire mammary duct ectasia and periductal mastitis, causing the impaired redevelopment of the mammary glands. These findings suggest that MFG-E8 plays an important role in efficient involution of mammary glands, and that proper involution is essential for the redevelopment of the mammary glands.

6 Future Prospects

The innate immunity is an immune reaction in which cells respond to bacterial and viral pathogens to produce various cytokines such as IFNγ, IL-1β, and TNFα

to combat with these pathogens. As discussed here, endogenous components released from uncleared apoptotic cells can also activate the innate immunity. How the cellular components released from dead cells in MFG-E8-deficient mice lead to the development of an SLE-type autoimmune reaction has not been completely elucidated. The DNA and RNA released from the apoptotic cells might stimulate TLR9 and TLR7 in endosomes, respectively, but it is still unclear how these TLRs, that normally recognize bacterial DNA and viral RNA, can be activated by the mammalian self-DNA and self-RNA in the form of protein complexes and how this process breaks tolerance to the self-antigens. The genetic polymorphisms of TLR7 and TLR9 have been associated with the development of SLE [79]. It would be intriguing to study how the polymorphisms of TLRs enhance binding affinity to the self-components and concomitantly increase the susceptibility to SLE.

SLE is a complex autoimmune disorder, affected by many genetic and environmental factors. Not only TLRs, recent genome-wide association studies and other more targeted investigations have revealed numerous novel genetic polymorphisms associated with SLE disease susceptibility [80]. The autoimmune phenotype caused by MFG-E8-deficiency is also influenced by the genetic background. Autoantibody production in MFG-E8-deficient mice is very prominent on autoimmune-prone B6/129-mixed background, but is attenuated on non-autoimmune-prone B6 background, suggesting the existence of additional strain-specific genetic modifiers [81]. Recently, genetic polymorphisms have been found between the B6 and 129 alleles of the β_3 integrin gene that may affect splicing, mRNA stability or post-transcriptional control [34]. This finding suggest that MFG-E8/$\alpha v\beta 3$ integrin-dependent phagocytosis may be affected by β_3 integrin allotypes. Although MFG-E8 polymorphism alone is well-associated with human SLE predisposition [72], more detailed analysis that examine the combination of genetic polymorphisms between MFG-E8 and β_3 integrin will be further required.

In any case, our knowledge about how apoptotic cells are recognized, engulfed, transferred to lysosomes and degraded is still very primitive. The clarification of these processes will help us understand the pathophysiology of various human diseases, especially autoimmune diseases. The increase of potential for modulating apoptotic cell clearance will lead to the development of new therapeutic strategies for treating autoimmune diseases.

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Chapter 7
Novel Therapeutic for Systemic Inflammation: Role of MFG-E8

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Abstract Systemic inflammation associated with diverse clinical conditions is a vital problem in critical care medicine with significant morbidity and mortality. In this chapter, we describe exclusively on systemic inflammation caused by sepsis, ischemia and reperfusion injury, and trauma hemorrhagic shock. Despite all efforts in the clinical arena treatment for these indications remain limited. The only FDA approved drug as a treatment for sepsis, Xigris (drotrecogin alfa [activated]) has recently been voluntarily withdrawn by Eli Lily. There is an unmet and urgent clinical need exists for novel therapies for these conditions. There are pathological similarities as well as differences exist among these conditions. Even though all three pathologies are initiated by different means, all leads to exaggerated inflammatory response and multi-organ failure. Therefore, therapies developed to dampen the exaggerated systemic inflammation could be beneficial for all three pathologies. Milk fat globule-epidermal growth factor-factor 8 (MFG-E8) is first identified as a bridging molecule that accelerated the interaction between apoptotic cells and phagocytes and facilitates the engulfment of apoptotic cells. We then, demonstrated that MFG-E8 plays a significant role in sepsis, ischemia and reperfusion injury, and trauma hemorrhagic shock. In this chapter, we will briefly review the different systemic inflammatory conditions and describe the key evidence for the role of MFG-E8 and highlight the notion that MFG-E8 could be developed as a potential therapeutic for these indications.

Keywords Sepsis • Ischemia-reperfusion • Hemorrhagic shock • MFG-E8 • Systemic inflammation

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1 Introduction

Systemic inflammation caused by sepsis, ischemia/reperfusion injury and trauma/hemorrhage is a critical problem causing significant morbidity and mortality [1]. Sepsis and septic shock is the second leading cause of death in the non-coronary intensive care units and is in the top 10 leading causes of deaths overall in the United States [2]. The mortality rate for severe sepsis and septic shock is about 30 % annually, with over 200,000 deaths per year [3] which is similar to the number of people dying with acute myocardial infarction. Similar to sepsis, ischemia and reperfusion (I/R) injury also results in high morbidity and mortality. Gut or mesenteric ischemia remains a critical clinical condition, resulting in mortality as high as 60 % [4]. Hepatic ischemia-reperfusion (I/R) damage occurs in diverse clinical settings including liver transplantation and liver resection [5, 6]. Acute renal failure (ARF) caused by renal I/R injury is quite common in hospitalized patients, affecting 3–7 % of general admissions, and as much as 25–30 % of patients in intensive care units [7, 8]. Trauma is the fifth leading cause of death overall and the number one cause of death for patients between the ages of 1 and 40 [9, 10]. As such, development of therapies for such systemic inflammatory conditions is an unmet need exists for efficient patient care.

There are pathological similarities as well as differences exist among these conditions. Sepsis is defined as a systemic host response to an infectious origin which eventually leads to systemic inflammatory response and multi-organ failure (MOF) [11, 12]. Ischemia and reperfusion (I/R) injury is a pathological condition characterized by an initial restriction of blood supply to a specific organ followed by subsequent restoration of perfusion and reoxygenation. I/R injury is manifested as an initial tissue hypoxia due to occlusion of the arterial blood supply and a subsequent tissue injury and exacerbation of inflammatory response as a consequence of the reperfusion. The major organs that are affected by I/R injuries due to varied clinical conditions are the gut, the liver and the kidneys. I/R injury typically occurs in a sterile environment and eventually leads to exaggerated inflammation and MOF [13]. While exsanguinations and head injury continue to account for a large number of early trauma deaths, the majority of late trauma deaths occur as a result of infection and/or MOF [9, 10]. Even though all three pathologies are initiated by different means, all leads to exaggerated inflammatory response and MOF. Therefore, therapies developed to dampen the exaggerated systemic inflammation could be beneficial for all three pathologies.

Milk fat globule-epidermal growth factor-factor 8 (MFG-E8) was first identified by Hanayama et al. [14] as a bridging molecule that accelerated the interaction between apoptotic cells and phagocytes and facilitates the engulfment of apoptotic cells. We then, demonstrated that MFG-E8 is decreased in sepsis and the reduction in its expression leads to impairment of apoptotic cell clearance resulting in increased mortality. Administration of exogenous MFG-E8 in septic animals increased apoptotic cell clearance, reduction in inflammatory response, and improved survival [15–17]. We also showed beneficial effect of MFG-E8 in a number of other organ

injury conditions [18–21]. In this chapter, we will briefly review the different systemic inflammatory conditions and describe the key evidence for the role of MFG-E8 in these indications. With the available data, we highlight the notion that MFG-E8 could be developed as a potential therapeutic agent for sepsis, ischemia and reperfusion injury, and trauma hemorrhagic shock.

2 MFG-E8 and Sepsis

Sepsis is a critical problem causing significant morbidity and mortality [1]. It continues to be the second leading cause of death in non-coronary intensive care units, and is in the top 10 leading causes of deaths overall in the United States [2]. It is estimated that there are more than 1,000,000 cases of sepsis among hospitalized patients each year in the US. The incidence of sepsis among hospitalized patients is increasing by 8.7 % per year. Numerous reports have shown that the incidence of sepsis and severe sepsis is increasing in excess of the growth of the population [12]. The mortality rate for severe sepsis and septic shock is about 30 % annually, with over 200,000 deaths per year [3] which is similar to the number of people dying with acute myocardial infarction. Sepsis is defined as a systemic host response to an infection caused by bacteria, virus or fungi [11]. Sepsis tends to occur from specific and consistent sources such as respiratory infections, genitourinary and abdominal sources of infection with primary bacteremia, and other unknown sources. The occurrence of severe sepsis is related to the source of infection, as in the cases of patients with respiratory infection who are at high risk for developing respiratory related organ dysfunction. Regardless of the time and the organisms, the treatment of infection is the primary antisepsis therapy. From a clinical perspective, antimicrobial therapy is the chosen method of treatment. However, the choice of antibiotics, and the timing of their administration are extremely critical for successful outcome. Thus, there has been a substantial amount of work ranging from analyzing triage decisions made for intensive care unit admissions [22] to evaluating cortisol as a potential treatment against sepsis [23]. Despite these efforts, the treatment of sepsis however, has remained elusive. The only FDA approved drug as a treatment for sepsis, Xigris (drotrecogin alfa [activated]) [24, 25] has recently been voluntarily withdrawn by Eli Lily. There is an unmet and urgent clinical need exists for a sepsis therapy.

During inflammation and sepsis, systemic increases in pro-inflammatory cytokines have been shown to increase mortality [26]. During sepsis and other states with systemic inflammatory response, several cell types (e.g., B cells, CD4 T cells, dendritic cells (DCs), vascular endothelial cells and enteric epithelial cells) undergo apoptosis [27–31]. Apoptotic cells that are not cleared are likely to undergo secondary necrosis [32], thereby continuing to release harmful and toxic mediators and worsening sepsis. Studies have shown that phagocytic function of macrophages is impaired in late sepsis [33, 34]. Hanayama et al. [35] have discovered that lack of

clearance of apoptotic B cells in the spleen potentially leads to autoimmune diseases which underscores the importance of clearing apoptotic cells from organism [36]. MFG-E8, a 64-kDa secretory protein that is mainly produced by the spleen, was responsible for removal of apoptotic cells. Without MFG-E8, engulfment and removal of apoptotic cells were impaired which led to the release of autoantibodies [35]. MFG-E8 was originally identified as a component of milk-fat globules [37] but secreted by activated macrophages and immature dendritic cells [38]. 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 [14, 39]. One unique characteristic of apoptotic cells is to expose their phosphatidylserine (PS) from its inner leaflet membrane to the outer surface. This is termed "eat me" signal which can allure distinct opsonins (i.e., MFG-E8), to recognize and bring apoptotic cells to the close vicinity of phagocytes [40]. MFG-E8 has a strong binding affinity to the exposed PS of apoptotic cells and facilitates phagocytic engulfment via $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrins. This 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 [41, 42]. Thus, MFG-E8 promotes the engulfment of apoptotic cells by working as a bridging molecule between those cells and phagocytes [14].

In an animal model of cecal ligation and puncture (CLP)-induced sepsis, we showed that MFG-E8 levels were decreased by 45 % in the blood during late sepsis (i.e., 20 h after CLP) indicating the systemic scale of its depletion under septic conditions [16]. A 48–70 % reduction was observed in the spleen and liver tissues [15]. This decrease in the MFG-E8 expression in late sepsis was associated with impaired phagocytosis of apoptotic cells or apoptotic cell clearance [15]. Splenic macrophages from MFG-E8 deficient (*Mfge8*$^{-/-}$) mice showed a dramatically decreased ability to phagocytose apoptotic cells under normal conditions as compared to wild type mice, suggesting a critical role for MFG-E8 in this process. Interestingly, *Mfge8*$^{-/-}$ mice accumulated higher amounts of apoptotic cells as compared to the WT mice during late sepsis. These data clearly demonstrated that the clearance of apoptotic cells is directly regulated by MFG-E8 [16]. Endotoxemia also reduced splenic MFG-E8 expression in a dose dependent manner and the downregulation of MFG-E8 expression in CLP-induced sepsis was attenuated by the LPS inhibitor, polymyxin B. The CLP-induced suppression was not observed in either CD14$^{-/-}$ or TLR4-mutated mice. These studies indicated that MFG-E8 production is downregulated in sepsis by LPS-CD14 dependent fashion, leading to a reduction of phagocytosis of apoptotic cells [43].

MFG-E8 is secreted from DCs in exosomes that resemble milk fat globules in size and membrane lipid composition [38, 44]. These tiny vesicles (50–100 nm in diameter) are derived from multivesicular bodies, intermediates in the endosome maturation between endosome and endolysosome [44, 45]. Fusion of these multivesicular bodies with the plasma membrane leads to the release of MFG-E8 containing exosomes. In this regard, we isolated MFG-E8 containing exosomes from rat bone marrow immature DCs. Treatment of rats with MFG-E8 containing exosomes at the time of CLP, reduced the presence of apoptotic cells by 33 %. Peritoneal

macrophages from exosome-treated rats displayed a 2.8-fold increased ability to phagocytose apoptotic thymocytes [16]. Thus, the reduced presence of apoptotic cells in exosome-treated septic rats could have been due to the increase in apoptotic clearance. Treatment also reduced plasma tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels and improved survival from 44 % in the saline treated animals to 81 % in the treatment ones [15]. Similarly, treatment of septic rats with recombinant murine MFG-E8 (rmMFG-E8) attenuated the inflammatory response during sepsis, increased apoptotic cell clearance, and improved survival [16]. To develop MFG-E8 as a therapeutic agent against sepsis, recombinant human MFG-E8 (rhMFG-E8) was expressed in bacterial system, purified and confirmed of having biological activity similar to the mouse counterpart in abilities of mediating the phagocytosis of apoptotic cells by macrophages [46]. Treatment with the purified rhMFG-E8 in septic rats significantly reduced, organ injury indicators (AST, ALT, creatinine, lactate), serum IL-6 and TNF-α, and plasma HMGB-1 levels [17]. In a 10-day survival study in septic rats, vehicle-treated rats produced 36 % survival rate, while rhMFG-E8 treatment significantly improved survival rate to 68–72 %. Treatment with rhMFG-E8 significantly reduced the number of apoptotic cells detected suggesting increased apoptotic cell clearance. In addition to its role in apoptotic cell clearance, a recent study showed that MFG-E8-mediated potential therapeutic benefits in sepsis and intestinal injury were not solely dependent on the enhanced clearance of apoptotic cells, but also due to diverse cellular events to maintain epithelial integrity and healing of the injured mucosa [47]. In this regard, we have shown that the pre-treatment with rmMFG-E8 followed by endotoxemia showed significant attenuation of TNF-α levels in circulation and in splenic tissues suggesting an anti-inflammatory role of MFG-E8. In contrast, endotoxemia in the *Mfge8*$^{-/-}$ mice caused greater increase in TNF-α than those in WT mice [48]. Aziz et al. [48] further demonstrated that MFG-E8-mediated decrease in TNF-α is regulated by pSTAT3/SOCS3 leading to downregulation of NF-kB and subsequent decrease in TNF-α. Nevertheless, these findings taken together clearly provided evidence to develop rhMFG-E8 as a therapy for patients suffering from sepsis (Fig. 7.1).

3 MFG-E8 and Ischemia/Reperfusion Injury

3.1 MFG-E8 and Gut I/R

Gut or mesenteric ischemia remains a critical clinical condition, resulting in mortality as high as 60 % [4]. Intestinal ischemia and subsequent reperfusion are encountered in a variety of clinical conditions, including acute mesenteric ischemia, intestinal obstruction, incarcerated hernia, small intestine volvulus and necrotizing colitis. The consequences of mestenteric ischemia are devastating to the patient and usually results in severe diarrhea, malabsorption, short bowel syndrome, and death.

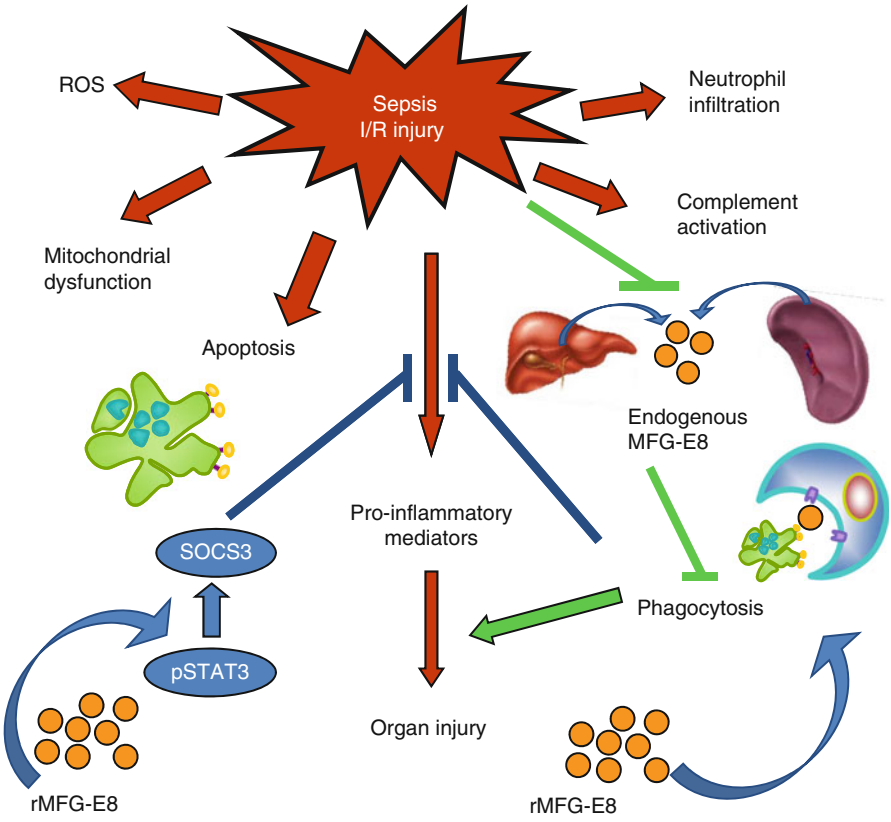

Fig. 7.1 Potential mechanism of MFG-E8 in sepsis and I/R injury. Sepsis and I/R injury is caused by a number of factors including neutrophil infiltration, complement activation, ROS, mitochondrial dysfunction and apoptosis leading to increase in proinflammatory mediators and organ injury. During sepsis, downregulation of the endogenous MFG-E8 in the tissues, i.e., spleen, liver and kidneys, attenuates apoptotic cell clearance (phagocytosis) and exacerbates organ injury. Administration of recombinant MFG-E8 (rMFG-E8) enhances the phagocytic activity and attenuates inflammation and decreases organ injury. In macrophages, rMFG-E8 upregulates pSTAT3/SOC3 signaling pathway and attenuates proinflammatory cytokines and decrease organ injury (Schematic illustration of data compiled from references [15, 16, 18–20, 48])

The pathophysiology of gut ischemia/reperfusion (I/R) involves tissue ischemia followed by cellular damage due to resumption of blood (reperfusion). Tissue ischemia initiates a series of events that can ultimately lead to cellular dysfunction and necrosis, and subsequent reperfusion causes more tissue damage including remote organ injury and subsequent death [13, 49–58]. A common complication of gut I/R is acute lung injury (ALI) and it contributes to the high mortality rate observed in gut I/R injuries. ALI is caused by a systemic inflammatory response due to the release of proinflammatory cytokines and bacteria-derived endotoxins from reperfused ischemic tissue [59, 60]. The mechanism of ALI involves a complex cross-talk among various cellular components of the alveolar microenvironment, their

secretory products, and leukocyte recruitment from the vascular bed in regions of inflammation. Activated neutrophils release proteolytic enzymes, such as elastase and myeloperoxidase (MPO) and reactive oxygen species, including hydrogen peroxide and superoxide. Excessive production of these factors not only destroys invaded pathogens, but also engages in the disruption of the endothelial barrier and promotes tissue damage. These events lead to ALI that is clinically manifested as acute respiratory distress syndrome followed by multiple organ dysfunction syndrome [61–64]. Even though numerous treatment modalities have been implicated in reducing ALI-induced mortality, none have been successful [65]. Thus, the development of novel and effective therapies for ALI is crucial for the improvement of patient outcome.

The gut is one of the most sensitive organs to I/R injury [55, 66]. Ischemia initiates a series of events that can ultimately lead to cell dysfunction and necrosis, and resumption of blood (reperfusion) causes more tissue damage [49–58]. The lungs are among the organs that are most severely affected by gut I/R-induced injury [67]. Ischemia or I/R induces apoptosis in various organs [68–70]. Apoptosis has been considered as the principal mode of cell death during I/R [66, 71–73]. Apoptotic cells stimulate inflammatory responses if they are not removed by phagocytes [74]. Deficient clearance of apoptotic cells leads to inflammation and tissue injury [39, 75]. MFG-E8 plays a crucial role for the engulfment of apoptotic cells by phagocytes [14, 35]. In this regard, we have shown that in a mouse model of gut I/R induced by superior mesenteric artery occlusion followed by reperfusion, as compared to the WT mice, *Mfge8*$^{-/-}$ mice produced much severe ALI after gut I/R [18]. MFG-E8 levels were markedly reduced in the spleen, gut and lungs by 50–70 %, suggesting impaired apoptotic cell clearance [76]. Treatment with rmMFG-E8 in gut I/R-induced WT mice significantly decreased lung apoptosis, improved lung morphology, and reduced neutrophil infiltration into the lungs. Treatment also suppressed tissue injury and inflammation as evidenced by reduction in liver enzymes (AST, ALT), lactate and creatinine, decreased proinflammatory cytokines (TNF-α, IL-6, IL-1β), and improved survival. Thus, MFG-E8 may serve as a novel treatment option for gut I/R-induced ALI.

3.2 MFG-E8 and Hepatic I/R

Hepatic ischemia-reperfusion (I/R) damage, which occurs in diverse clinical settings including liver transplantation, trauma, hemorrhagic shock, or liver surgery, is a serious clinical complication that may compromise liver function because of extensive hepatocellular loss. I/R injury represents a complex series of events that result in cellular and tissue damage. It involves the transient deprivation of blood flow and oxygen, and the return of blood flow during reperfusion with concomitant release of reactive oxygen species (ROS), inflammatory mediators, adhesion molecules, adenosine triphosphate (ATP) depletion, and derangements in calcium homeostasis. Finally, these functional changes induce cell death due to apoptosis as

well as necrosis [5, 6]. Despite the fact that hepatic injury is a major clinical problem, no reliable therapies have been established. The development of a therapy for hepatic I/R would indeed benefit patients undergoing liver surgery and liver transplantation.

It has been shown that programmed cell death or apoptosis of liver sinusoidal cells and hepatocytes is a prominent feature of liver I/R injury, in both experimental models and clinical transplantation [5, 77, 78]. Historically, apoptosis has been seen as an ordinary process of cell suicide that, unlike necrosis, does not elicit inflammation [32]. Studies have shown that if the removal process of apoptotic cells fails, apoptotic cells undergo secondary necrosis, which enables to release potentially cytotoxic intracellular contents, followed by inflammation and impaired tissue repair [79, 80]. In a rat model of hepatic I/R, liver and plasma levels of MFG-E8 were significantly decreased. Administration of rhMFG-E8 significantly improved liver injury, suppressed apoptosis, attenuated inflammation and oxidative stress, and downregulated the NF-κB signaling pathway. In a survival study conducted using *Mfge8*$^{-/-}$ mice and WT mice, the survival rate of the *Mfge8*$^{-/-}$ mice was markedly reduced as compared to that of the WT mice indicating that the *Mfge8*$^{-/-}$ mice were more susceptible to hepatic I/R-mediated mortality than the WT mice. In contrast, exogenous administration of rhMFG-E8 in WT mice improved the survival rate after hepatic I/R from 31 % in the saline treated animals to 70 % in the treatment ones [19]. Furthermore, it has been demonstrated that MFG-E8-mediated therapeutic potential is not only dependent on enhancement of phagocytosis, but also on multiple cellular events associated with tissue remodeling [47, 81, 82]. MFG-E8-mediated multiple physiological events may represent an effective therapeutic option in tissue injury following an episode of hepatic I/R.

3.3 MFG-E8 and Renal I/R

Acute renal failure (ARF) is a critical clinical problem posing significant economic and financial burden on the society. ARF is quite common in hospitalized patients, affecting 3–7 % of general admissions, and as much as 25–30 % of patients in intensive care units. Renal ischemia-reperfusion (I/R) injury causes ARF in various clinical settings, including kidney transplantation and cardiopulmonary and aortic bypass surgery. Renal I/R injury is associated with high mortality and morbidity [7, 8]. Current strategies used to prevent ARF consist mainly of fluid resuscitation and diuretics, and/or prevention of the insinuating factor. Despite these efforts, the mortality remains unacceptably high and has not improved in several decades. There is an urgent need to develop therapeutics to fight this pathological condition.

During renal I/R injury, renal damage begins immediately from the onset of ischemia. Upon restoration of perfusion, however, the tissues undergo further injury. Reperfusion injury involves the accumulation of neutrophils, generation of free oxygen radicals, and cytokine activation. These changes may also be seen

histopathologically, as demonstrated by the loss of the brush border, tubular disruption, and cast formation [83]. Renal damage due to I/R injury occurs as early as 5 h following injury as evidenced by a rising serum lactate, TNF-α, IL-6 and TGF-β levels, as well as decreasing systemic venous oxygen levels [84]. In the clinical setting, serum markers such as blood urea nitrogen and creatinine, are regarded as gold standards for renal compromise, but these markers may not become elevated until 24 h after the initial injury. Studies looking at early biomarkers, such as keratinocyte-derived chemokine (KC) and neutrophil gelatinase-associated lipocalin (NGAL), demonstrate that increases in these markers are associated with the development of ARF [85, 86]. With a better understanding of the pathophysiology of ARF as well as the identification of new biomarkers, one is able to determine the actual time point in the evolution of renal compromise pharmacological or hormonal therapy would be beneficial.

Ischemia typically damages renal tubular epithelial cells and also glomerular cells and is characterized by several hallmark features at the cellular level: Profound intracellular ATP depletion and a fall in tissue oxygen and glucose content with a concomitant rise in intracellular calcium [87, 88]. Although ischemic events alone may lead to necrosis and apoptosis in the kidneys, reperfusion occurs upon restoration of blood flow and is associated with increased apoptosis and necrosis in addition to the production of reactive oxygen species (ROS) and inflammatory mediators [89, 90]. Renal I/R injury can be ameliorated by inhibiting molecules involved in apoptosis, necrosis, or inflammation, suggesting that multiple injury and death mechanism may be involved in renal I/R injury [91]. Among these, the coexistence of apoptosis and necrosis in renal tissues is a characteristic feature of renal I/R injury. Both types of cell death have been implicated significantly in the pathogenesis of ARF, which is marked by a loss of tubular epithelial cells and subsequent renal dysfunction [92, 93]. Moreover, additional mechanisms which contribute to the ongoing pathogenesis of I/R injury-induced ARF has been reported. For instance, renal vascular endothelial injury and dysfunction, due to increases in renal vascular resistance and persistent reductions in renal blood flow, exacerbates hypoxia and play an important part in extending renal tubular epithelial injury and subsequent cell death [94]. In a rat model of bilateral renal ischemia followed by reperfusion (renal I/R) [20], MFG-E8 mRNA and protein expressions were significantly decreased in the kidneys and spleen. Treatment with rmMFG-E8 recovered renal dysfunction, significantly suppressed inflammatory responses, reduced apoptosis and necrosis, and improved capillary functions in the kidneys. In a 60 h survival study, survival rate after renal I/R injury decreased significantly from 44 % in the WT mice to 11 % in the *Mfge8*$^{-/-}$ mice. Interestingly, the exogenous treatment with rmMFG-E8 in the WT mice showed significant improvement in survival rate to 73 %. These data collectively demonstrated that the protective effect of MFG-E8 is mediated through the enhancement of apoptotic cell clearance and improvement of capillary functions in the kidneys. Thus, MFG-E8 could be developed as a novel treatment for renal I/R injury.

3.4 MFG-E8 and Hemorrhagic Shock

Trauma is the fifth leading cause of death overall and the number one cause of death for patients between the ages of 1 and 40 [9, 10]. In the US, about 90,000 people die annually due to traumatic injuries and complications. It is estimated that 10–20 % of the deaths are potentially preventable and nearly 80 % of these occur due to hemorrhage and it occurs within the first 24 h after injury [9, 95–97]. Immediate hemorrhage control and adequate fluid resuscitation are the key components of early trauma care. While fluid resuscitation decreases the risk of death in severe hemorrhage, it increases the risk of death in less severe hemorrhage. Despite the fact that fluid infusion at a pre-determined rate has shown to reduce organ injury and reduce mortality, the best approach recommended is to avoid unnecessary field interventions and focus on fast and efficient transport of the patient to hospital [98]. Advances in trauma care systems and emergency medical services have resulted in a significantly large percentage of patients who survive to hospital admission [96]. Another strategy implemented is the hypotensive resuscitation that showed some reduction in the risk of death. Hypotensive resuscitation at a fixed rate of 60–80 cc/kg/h generally maintains the systolic blood pressure of 80–90 mmHg and mean arterial pressure of 40–60 mmHg. Although the data suggest this strategy of infusion rates is beneficial in hemorrhagic shock, it requires monitoring of hemodynamic changes which would be difficult to accomplish in the field. Another strategy for resuscitation is the use of hypertonic saline. An number of pre-clinical studies have demonstrated that hypertonic saline modulate the immune response and leads to attenuation of immune mediated cellular injury [99–108]. However, in two recent multicenter clinical trials, hypertonic saline treated patients experienced early high mortality in comparison to normal saline treatment and thus, hypertonic saline is not recommended for resuscitation in trauma patients [109].

Observational data from trauma centers and the battlefield suggest that early administration of component therapy containing fresh frozen plasma and platelets may be beneficial [110, 111]. Based on battlefield experience, US Army instituted a policy of using a 1:1:1 ratio of packed red blood cells:fresh frozen plasma:platelets in the battlefield for those who meet the criteria for massive resuscitation. However, no study has identified the optimal ratios of blood components to be used for resuscitation [109]. In addition, although advance in viral screening have markedly decreased the risk of infectious transmissions, blood transfusion remains to be associated with numerous side effects. Blood transfusion has shown to cause early immune activation resulting in systemic inflammatory response syndrome and immune suppression which predisposes the patients to infection [112–116]. In addition to fluid resuscitation, a wide range of pharmacological agents including neuroendocrine agents, calcium channel blockers, prostaglandins, sex steroids, immune modulators, and histone deacetylase inhibitors have been tested in pre-clinical trials. Although majority of these agents show beneficial effects in animal models, none have been in clinical use as resuscitative agents. Thus, there is an urgent unmet need in the development of novel therapies for hemorrhagic shock.

Exsanguination and head injury continue to account for a large number of early trauma deaths, the majority of late trauma deaths occur as a result of infection and/or multi-organ failure. The clinical association of late trauma deaths and the development of multi-organ failure have been established as early as in the 1970s. However, only within the past few decades that the focus has been directed towards inflammatory response and how it may predisposes the body to infection and multi-organ failure. Hemorrhagic shock induces a surge of inflammatory cytokines including IL-6 and TNF-α which is associated with increased mortality [26, 117]. Prolonged and severe hemorrhagic state leads to tissue hypoxia and the presence of apoptotic cells [32]. If these apoptotic cells are not cleared, they will likely undergo secondary necrosis and release harmful agents and worsens hemorrhagic shock.

Apoptotic cell death is prevalent in gastrointestinal associated intestinal epithelial cells [118–120]. These cells are already prone to apoptosis after noxious stimuli exposure because these cell types normally undergo a rapid physiological turnover that is believed to be a result of apoptosis. Since bowel is the primary organ responsible for inflammatory response in trauma and shock, if accelerated cell death occurs in the intestine of patients with trauma and shock, important pathologic consequences could result. During trauma and shock, the intestinal wall loses its barrier function which results in the leakage of endotoxin and bacteria into the circulation causing a systemic inflammatory response. Apoptosis of intestinal epithelial tissues occur as rapidly as 2–3 h after initial injury and it compromises bowel wall integrity and becomes the primary mode for bacterial or endotoxin translocation into the systemic circulation [121]. In contrast, increased apoptosis of peripheral blood neutrophils is associated with reduced incidence of infection in trauma patients with hemorrhagic shock [122]. Clearance of apoptotic peripheral blood neutrophils by the liver and spleen inhibit inflammatory response thereby sparing the other organs such as the lung, which is among the most common sites of infection following serious trauma that leads to multi-organ failure and death.

Ingestion of apoptotic cells by macrophages results in the release of anti-inflammatory mediators, including TGF-β1 and PGE_2 and suppresses the production of pro-inflammatory cytokines such as IL-8, TNF-α and thrombaxane A_2 [123, 124]. In this regard, MFG-E8 has been identified as a bridging molecule between professional phagocytes via the $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrins and apoptotic cells via PS, which accelerates the engulfment of apoptotic cells [41, 42]. In a mice model of pressure-controlled (25 ± 5 mmHg) hemorrhagic shock [21], MFG-E8 levels in the plasma, lungs and spleen were significantly decreased at 4 h after hemorrhage. Resuscitation with rhMFG-E8 significantly improved apoptosis at 4 h as evidenced by a reduction in TUNEL positive cells and cleaved caspase-3 expression. Neutrophil infiltration into the lungs and spleen were also blunted. Pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) were reduced significantly in plasma (64–73 %), lungs (24–58 %) and spleen (49–76 %). In a 7 day survival study, a significant improvement (83 % vs. 43 %) with one-time dose of rhMFG-E8 as compared to normal saline treated mice after hemorrhage was observed. These data taken together suggest that rhMFG-E8 could be developed as a treatment for hemorrhagic shock.

4 Future Perspectives

In this chapter, we clearly demonstrated that MFG-E8 could be developed as a novel therapy for sepsis, ischemia and reperfusion injury, and trauma hemorrhagic shock. The data described further indicates that MFG-E8 could be functioning as a tether between apoptotic cells and phagocytes for efficient engulfment of apoptotic cells and thereby reduce inflammation and improve survival. It is also implicated that MFG-E8 could function directly by binding to $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrins and upregulate or downregulate signaling components causing the reduction in inflammation. Regardless of its mechanism of action, it is clear that administration of MFG-E8 is beneficial in attenuating the exaggerated inflammatory response associated with systemic inflammation caused by sepsis, ischemia and reperfusion injury, and trauma hemorrhagic shock. Thus, MFG-E8 treatment could be a potential therapy for patients suffering from complications associated with such conditions.

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Chapter 8
Anti-Inflammatory Role of MFG-E8 in the Intestinal Tract

Shunji Ishihara, Ryusaku Kusunoki, and Yoshikazu Kinoshita

Abstract Engulfment of apoptosis cells is an essential process for maintaining immune homeostasis, which is regulated by a variety of molecules. Milk fat globule-epidermal growth factor-factor 8 (MFG-E8), a secreted glycoprotein, enhances engulfment of apoptotic cells by forming a link between phosphatidylserine on apoptotic cells and $\alpha_v\beta_3$-integrin on phagocytes. MFG-E8-mediated engulfment of apoptotic cells contributes to preserving the intestinal physiological condition. In addition to this scavenging function, MFG-E8 also directly regulates the functions of macrophages and intestinal epithelial cells via $\alpha_v\beta_3$-integrin on their cell surfaces, which attenuates inflammation and enhances regeneration of injured intestinal tissues. This chapter highlights recent findings regarding the role of MEG-E8 in the intestinal tract and demonstrates its therapeutic potential for gut inflammatory diseases.

Keywords MFG-E8 • Apoptosis • $\alpha_v\beta_3$-integrin • Intestinal inflammation • Inflammatory bowel disease • Ulcerative colitis • Crohn's disease

1 Introduction

Efficient clearance of apoptotic cells by phagocytes contributes to avoiding release of numerous inflammatory mediators from dying cells, which is an essential function for maintaining immune homeostasis [1]. Several unique molecules expressed on the surfaces of apoptotic cells and phagocytes regulate engulfment of apoptotic cells as ligands and receptors [2–4]. The glycoprotein milk fat globule-epidermal growth factor-factor 8 (MFG-E8), originally identified as a mammalian milk fat

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globule membrane component, is secreted by mammary epithelial cells, as well as macrophages and dendritic cells (DCs) [5, 6]. This glycoprotein accelerates the engulfment of dying cells by forming a bridge between phosphatidylserine (PS) on apoptotic cells and $\alpha_v\beta_3$-integrin on phagocytes [7, 8]. Accumulation of apoptotic cells in MFG-E8-knockout (KO) mice leads to development of various inflammatory diseases with abnormal immune homeostasis [9, 10].

The role of MFG-E8 has been reported mainly in regard to its immunoregulatory functions associated with clearance of apoptotic cells in various organs under physiological conditions. Apart from this scavenger effect, recent studies have also indicated that MFG-E8 is involved in the pathogenesis of various diseases including sepsis, ischemia, and atherosclerosis, as well as neurodegenerative disorders, intestinal inflammation, and malignancies [11–14]. Of those disease conditions, we have focused on intestinal inflammation and investigated the role of MFG-E8 in the pathogenesis of inflammatory bowel diseases (IBD). The aim of the present chapter is to highlight recent findings regarding the role of MEG-E8 in intestinal pathophysiology as well as its therapeutic potential for treatment of intestinal inflammation.

2 Expression and Role of MFG-E8 in Intestinal Tract Under Physiological Conditions

Although MFG-E8 maintains host immune homeostasis by enhancing the clearance of apoptotic cells, little is known regarding its expression and function in the intestinal tract under physiological conditions. We previously investigated the basal levels of MFG-E8 expression in different compartments of mice gut tissues, and found that its level was relatively higher in the colon as compared to the stomach and small intestine [15]. Furthermore, MFG-E8 expression was detected in lamina propria mononuclear cells in mice colonic sections in immunohistochemical examinations.

A previous report revealed that MFG-E8 KO mice spontaneously develop lupus-like autoimmune diseases due to impaired phagocytosis of apoptotic cells even in the absence of pathological stress [10]. However, the structure and function of the intestinal tract in this model mouse under physiological conditions remain largely unknown. Thus, we employed MFG-E8 KO mice and examined age-related (up to 30 weeks) changes in body weight, and gross and histological findings of the colon in both KO and wild-type (WT) mice without pathological stress induction. No differences regarding these parameters were found between those groups of mice [16].

On the other hand, Bu et al. examined the role of MFG-E8 in the small intestine of mice [17]. They injected a monoclonal anti-MFG antibody (Ab) into WT mice and evaluated BrdU-labeled epithelial migration in histological sections, then compared the results to those of mice treated with control IgG. Mice treated with the anti-MFG Ab showed a decrease in migration of BrdU-labeled enterocytes from the crypts to villus tips. MFG-E8 KO mice also showed a decrease in migration of enterocytes in the crypt-villus axis, with mild focal mucosal injury noted at the

villous tips. Those findings suggested that MFG-E8 physiologically regulates epithelial cell proliferation in the mouse small intestine. Furthermore, we recently investigated the proliferation of colonic epithelial cells by PCNA staining in WT and KO mice [16]. However, that experiment did not reveal any differences between those two groups of mice, in contrast to the results obtained by Bu et al. using small intestine samples. Further investigations are necessary to confirm the physiological functions of MFG-E8 in the intestinal tract.

3 Expression and Roles of MFG-E8 in Animal Models with Intestinal Injury

Several animal models have been used for evaluating the role of MFG-E8 in intestinal inflammation. This section provides recent evidence obtained from mice models with intestinal inflammation (Table 8.1).

3.1 IBD Models

Ulcerative colitis (UC) and Crohn's disease (CD) are two major forms of IBD characterized by chronic and relapsing intestinal immune-mediated disorders. Although a variety of pathological factors including genetics, immune regulation, and interacting microbial flora have been reported [23–26], their association with the etiology of IBD remains largely unknown. Various studies have been conducted to evaluate new innovative approaches, and we and others have recently demonstrated the role of MFG-E8 as well as its therapeutic potential in mice experimental models.

3.1.1 Dextran Sodium Sulfate (DSS)-Induced Colitis

DSS-induced colitis has been widely used for investigating the pathogenesis of IBD. We examined time course changes of MFG-E8 expression during DSS colitis (3.5 %) and found that it was reduced in inflamed colons during the induction period, while it became upregulated during the healing phase [15]. In mice administered a low concentration of DSS (1.5 %), MFG-E8 expression was not reduced to the same extent as in severe colitis, suggesting that altered expression of MFG-E8 may be dependent on colitis severity. Previous studies have also shown an abrupt decrease in the majority of stress-induced disease conditions. Based on those findings, we investigated the anti-inflammatory effects of recombinant mouse MFG-E8 protein (rmMFG-E8) in DSS colitis. Intravenous injection of rmMFG-E8 significantly reduced body weight loss, and led to shortening of inflamed colon tissues and reduced histological inflammation (lamina propria infiltration by mononuclear cells as well as crypt epithelial damage), as well as increased the colonic tissue contents of

Table 8.1 Roles of MFG-E8 in animal models with intestinal injury

	MFG-E8 expression	MFG-E8 KO-mice (disease activity)	Effect of rMFG-E8 (disease activity)	Functions of MFG-E8	Reference
IBD models					
DSS colitis	Down-regulated	–	Attenuated	Anti-Inflammation (Macrophages)	[15]
DSS colitis	Up-regulated (early phase)	Aggravated (epithelial injury)	Attenuated (inflammation)	Anti-inflammation regeneration	[18]
TNBS colitis	Down-regulated (late phase)	–	Attenuated (healing)	Anti-Inflammation (IECs)	[19]
I/R model	Down-regulated (Small intestine)	–	Attenuated	Apoptotic cell clearance Anti-Inflammation Anti-apoptosis (IECs) angiogenesis	[20, 21]
ARS model	Down-regulated (Small intestine)	–	Attenuated	Anti-apoptosis (IECs)	[22]
Sepsis model	Down-regulated (Colon)	Delayed healing	Attenuated	Regeneration (IECs migration)	[17]

IBD inflammatory bowel disease, *DSS* dextran sodium sulfate, *TNBS* trinitrobenzene sulfonic acid, *I/R* ischemic reperfusion, *ARS* acute radiation syndrome, *IECs* intestinal epithelial cells

inflammatory cytokines as compared to PBS-treated mice given DSS. Those findings indicate a therapeutic benefit of MFG-E8 administration for intestinal inflammation.

Chogle et al. reported that DSS administration induced more severe crypt-epithelial injury with delayed healing of colonic epithelium in MFG-E8 KO mice as compared to WT mice [18]. In particular, they focused on the effect of MFG-E8 on regeneration of colonic epithelium, and found that intraperitoneal administration of rmMFG-E8 during the recovery phase of DSS-induced colitis attenuated mucosal inflammation and enhanced epithelial repair. Thus, experimental results obtained from DSS colitis models suggest that MFG-E8 has important roles in anti-inflammation as well as regeneration of inflamed intestinal tissues.

3.1.2 Trinitrobenzene Sulfonic Acid (TNBS)-Induced Colitis

Intrarectal administration of TNBS stably induces colitis in mice, thus it is also commonly used to produce IBD models. Recently, we examined the anti-inflammatory roles of rmMFG-E8 in such a colitis model [19]. rmMFG-E8 was intra-rectally administered into TNBS-induced colitic mice, after which disease activity was evaluated. Body weight loss, shortening of colon length, histological score, and inflammatory cytokine levels were significantly reduced in mice with rmMFG-E8 treatment, which suggested a direct effect of MFG-E8 on colonic epithelial cells that contributes to attenuating colonic inflammation in our TNBS colitis model (detailed mechanisms are described in a later section).

3.2 Ischemia-Reperfusion Model

Intestinal ischemia is induced by a variety of serious clinical conditions, resulting in necrotizing enterocolitis, superior mesenteric artery occlusion, and small bowel transplantation, which often affects patient mortality. Several studies have revealed that the appearance of apoptotic cells is increased in intestinal mucosa under an ischemic condition. Since excessive apoptosis of mucosal cells induces inflammation and immune activation in the gut, prompt clearance of apoptotic cells is likely important for inhibiting ischemia-induced intestinal injury. In this regard, studies regarding the role of MFG-E8 in intestinal ischemia have been recently conducted.

An ischemia-reperfusion (I/R) model is widely used for evaluating the pathogenesis of ischemia-induced intestinal injury. Wu et al. evaluated this model and found a decrease of MFG-E8 expression in the small intestine after gut I/R [20]. They also indicated that administration of rmMFG-E8 attenuated I/R-induced small intestine injury by inhibiting neutrophil infiltration and myeloperoxidase (MPO) activity. The protective effects of rmMFG-E8 shown in this model were dependent on various changes, including suppression of bacterial translocation to mesenteric lymph nodes (MLN), a decreased number of mucosal apoptotic cells, up-regulation of

Bcl-2, and increased expression of vascular endothelial growth factor (VEGF) in the small intestine. Those findings suggest that enhancing apoptotic cell clearance by rmMFG-E8 mitigates bacterial translocation, inhibits neutrophil infiltration, and promotes tissue repair after gut I/R. Another study published by the same group demonstrated that MFG-E8 deficiency (KO mice) induced severe multiple organ injuries after gut I/R, whereas treatment with rmMFG-E8 attenuated those damages and improved survival [21]. Thus, MFG-E8 contributes to inhibiting I/R-induced injury as well as accelerates tissue regeneration in various organs.

3.3 Radiation-Induced Intestinal Injury Model

Radiation therapy is effectively used to treat patients with gastrointestinal, gynecological, and urinary malignancies. However, radiation-induced intestinal injuries often occur, which decrease patient quality of life. Apart from irradiation for medical use, acute radiation syndrome (ARS) is caused by rapid exposure of the body to a high dose of penetrating radiation. ARS induces serious gastrointestinal damage with depletion of immature parenchymal stem cells, a main cause of early mortality. Although the situation of radiation-induced intestinal damage caused by ARS is different from that by radiation therapy, appropriate studies should be conducted for development of novel therapeutic strategies to be used in patients with these disease conditions.

Ajakaiye et al. investigated the roles of MFG-E8 in intestinal injury and mortality in rats that received high levels of whole body irradiation (WBI) [22]. They examined changes in MFG-E8 expression in the intestines after WBI and found a significant decrease. When rmMFG-E8 was intraperitoneally administered in this model, survival was significantly improved as compared to non-treated control rats. Furthermore, rmMFG-E8 treatment resulted in a reduction in radiation-induced mucosal damage in the small intestine. The authors also indicated that the improved survival with decreased intestinal damage seen in their study may have been dependent on increased expression levels of the anti-apoptotic cell regulators B-cell lymphoma-2 (Bcl-2), p53, and p21 due to rmMFG-E8 treatment. These findings also indicate that MFG-E8 has a protective role in WBI-induced intestinal injury, which may contribute to development of novel therapy for ARS. On the other hand, local and low-dose radiation exposure is used to treat cancer patients. Further studies should be conducted to confirm whether MFG-E8 also protects against radiation-induced intestinal damage in such patients.

3.4 Sepsis-Triggered Intestinal Injury Model

A sepsis-triggered intestinal injury model can be made by puncturing a distal part of the cecum after ligation [17]. In this model, microscopic injury of the small intestine occurs rapidly after cecal ligation and puncture (CLP), while villous necrosis and edema develop 48 h later. Mucosal damage then gradually recovers within the next

48 h. Bu et al. investigated the expression and role of MFG-E8 in CLP-induced small intestine injury. Intestinal MFG-E8 expression was markedly down-regulated within 48 h after CLP, along with decreased migration of epithelial cells. Furthermore, MFG-E8 deficiency (KO mice) resulted in delayed epithelial cell renewal and turnover, whereas treatment with rmMFG-E8 restored enterocyte migration in CLP-induced septic mice. These findings suggest that endogenous MFG-E8 is required for intestinal repair in a septic condition.

4 Anti-inflammatory and Regenerating Roles of MFG-E8: Evidence Obtained from *In Vitro* Studies

Clearance of apoptotic cells is an essential function of MFG-E8 for maintaining immune homeostasis, which contributes to inhibiting excess inflammation as well as repairing inflamed tissues in the intestinal tract. Apart from such scavenger effects, several *in vitro* studies have demonstrated that MFG-E8 also directly acts on macrophages and intestinal epithelial cells (IECs), which are associated with regulation of intestinal inflammation and regeneration.

4.1 Anti-inflammatory Roles

4.1.1 Macrophages

The anti-inflammatory roles of MFG-E8 have been shown in various animal models with intestinal injury. For example, intravenous injection of rmMFG-E8 significantly decreased the expression of pro-inflammatory cytokines in the colonic tissues of DSS-treated colitis mice [15]. Pro-inflammatory cytokines are mainly produced by infiltrating immune cells via NF-κB activation. In addition, integrin signaling pathways are activated during intestinal inflammation, which recognize various ligands to activate the intracellular inflammatory cascade. Osteopontin (OPN), an extracellular matrix phosphoprotein that contains the RGD domain, binds to $\alpha_v\beta_3$-integrin in macrophages and subsequently induces the production of NF-κB-mediated inflammatory cytokines. With that background in mind, we examined the anti-inflammatory effects of rmMFG-E8 on LPS-treated cultured macrophages in the presence or absence of OPN. The results indicated a potential role of OPN to induce inflammatory responses via $\alpha_v\beta_3$-integrin-mediated NF-κB activation. In addition, OPN-mediated activation of $\alpha_v\beta_3$-integrin resulted in recruitment of phosphorylated focal adhesion kinase (pFAK), which exacerbated LPS-stimulated inflammatory signaling. Moreover, LPS stimulated phosphorylation of FAK to enhance binding of OPN to $\alpha_v\beta_3$-integrin. By targeting this pathway, MFG-E8 can inhibit LPS-induced NF-κB activation by blocking OPN binding, as well as modulate $\alpha_v\beta_3$-integrin-dependent and FAK-mediated downstream signaling (Fig. 8.1a).

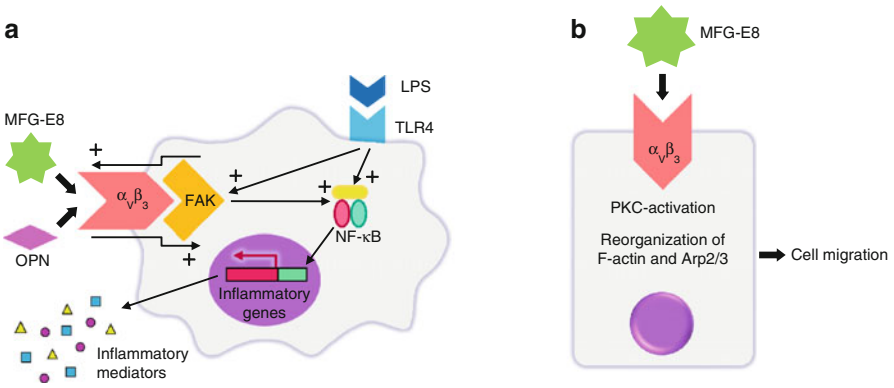

Fig. 8.1 (**a**) Anti-inflammatory roles of MFG-E8 in macrophages. LPS activates a pro-inflammatory response in macrophages via TLR4-NF-κB-mediated signaling. LPS also stimulates $\alpha_v\beta_3$-integrin and promotes the binding of several inflammatory mediators, e.g., osteopontin (*OPN*). After OPN binding, activation of $\alpha_v\beta_3$-integrin results in recruitment of phosphorylated focal adhesion kinase (pFAK), which leads to further augmentation of TLR4-mediated NF-κB activation. By targeting this pathway, MFG-E8 can reduce LPS-induced NF-κB activation by blocking OPN binding, as well as modulation of $\alpha_v\beta_3$-integrin-dependent and FAK-mediated downstream signaling. (**b**) MFG-E8-mediated migration was regulated by activating intracellular protein kinase C (*PKC*) as well as reorganizing the F-actin and Arp2/3 via $\alpha_v\beta_3$-integrin on IECs

4.1.2 IECs

The pathogenesis of intestinal inflammation is closely associated with abnormal interactions between IECs and microbial pathogens. Of the various microbial ligands, flagellin is a specific microbial ligand of Toll-like receptor (TLR)-5 and has been shown to be a potent stimulator of inflammatory cytokines in IECs. We examined the effects of rmMFG-E8 on flagellin-stimulated IECs and found that treatment with rmMFG-E8 reduced flagellin-induced expression of inflammatory cytokines in Colon-26 cells [19]. On the other hand, treatment with RGD domain-mutant rmMFG-E8, a dominant negative form of MFG-E8, did not show a significant anti-inflammatory effect in flagellin-stimulated IECs. Since the RGD domain is a key part of MFG-E8 protein and can bind to $\alpha_v\beta_3$-integrin on the cells, our results suggest that MFG-E8 inhibits flagellin-induced inflammatory responses via $\alpha_v\beta_3$-integrin on IECs.

4.2 Regeneration and Anti-apoptosis Roles of MFG-E8 in IECs

Various studies using animal models with I/R-, sepsis-, or radiation-induced intestinal injury have demonstrated regeneration and anti-apoptosis roles of MFG-E8 in IECs. Bu et al. investigated whether rmMFG-E8 enhances IEC migration in an *in*

vitro wound-healing model (IEC-18 cells) and found that treatment with rmMFG-E8 promoted the migration of IECs by activating intracellular protein kinase C (PKC) (Fig. 8.1b) [17]. They also clarified that rmMFG-E8-mediated migration was regulated by activating intracellular protein kinase C (PKC) as well as reorganizing the F-actin and Arp2/3 via $\alpha_v\beta_3$-integrin on IECs. Furthermore, treatment with rmMFG-E8 reduced cell death in cultured IEC-6 cells after exposure to radiation. Although anti-apoptotic proteins including Bcl-2, p53, and p21 have been suggested to be associated with the beneficial roles of MFG-E8 in treating radiation-induced intestinal injury, other molecular mechanisms remain to be clarified.

5 Expression and Role of MFG-E8 in Human UC

Zhao et al. used colonic biopsy specimens obtained from UC patients and control subjects to examine MFG-E8 expression [27]. They found that MFG-E8 was mainly expressed in colonic epithelial cells. Furthermore, the expression level of MFG-E8 was lower in biopsy samples of inflamed mucosa obtained from UC patients as compared to the controls, which was inversely correlated with histological inflammatory and clinical disease activities. Also, the number of TUNEL-positive apoptotic epithelial cells was reduced in the colonic samples from UC patients. In addition, lentiviral vectors encoding human MFG-E8 targeting short hairpin RNA were established to obtain the MFG-E8 knockdown intestinal epithelia cell lines HT-29 and Caco-2. MFG-E8 knockdown promoted apoptosis in HT-29 and Caco-2 cells, along with a decrease in level of the anti-apoptotic protein Bcl-2 and induction of the pro-apoptotic protein Bcl2-associated protein X (BAX). Moreover, the results of an *in vitro* wound healing assay revealed that MFG-E8 knockdown attenuated restitution of IECs. Those findings supported the results presented in earlier studies. However, this is the first and only study regarding MFG-E8 expression in the human intestinal tract. Additional investigations are necessary for a better understanding of the role of MFG-E8 in human inflammatory intestinal diseases.

6 Regulators of MFG-E8 Expression; Butyric Acid-Induced MFG-E8 Expression in IECs

Prolactin (PRL), fractalkine (a CX3C chemokine), peroxisome proliferator-activated receptor (PPAR)-δ ligand, granulocyte macrophage colony-stimulating factor (GM-CSF), connexin-43, and glucocorticoid have also been reported to induce or suppress MFG-E8 expression [28–33]. In addition, butyric acid (BA), a short-chain fatty acid and one of the main metabolites of intestinal microbial fermentation of dietary fiber, plays important roles for regulating gut immune homeostasis and contributes to inhibition of excess intestinal inflammation [34]. To precisely confirm the beneficial effects of BA, we performed microarray analyses to

reveal potent factors induced by IECs stimulated with BA. Among the upregulated genes, MFG-E8 was elevated by approximately 5-fold and its expression was regulated by acetylation on histone 3lysine 9 (acetyl-H3K9) around the MFG-E8 promoter. Moreover, we found that BA-mediated anti-inflammatory effects were decreased in MFG-E8 KO mice with DSS-induced colitis as compared to WT mice. Those findings suggest that BA-mediated anti-inflammatory effects are dependent on, at least in part, induction of MFG-E8 expression.

7 Conclusions and Future Perspective

In this chapter, we summarized findings obtained from recent studies regarding the role of MFG in the intestinal tract, which indicate that MFG-E8 contributes to inhibit intestinal inflammation and regenerate injured intestinal mucosa. However, those findings were mainly obtained in experiments using animal models with acute and severe intestinal injury, and details of MFG-E8 functions in chronic gut inflammatory disorders and human intestinal diseases remain largely unknown. Furthermore, recent studies have shown that MFG-E8 functions are closely associated with the pathogenesis of malignancies in various organs, including cancer development, growth, and metastasis [35–37]. However, no studies of MFG-E8 functions in the pathogenesis of intestinal cancers have been presented. Further investigations are needed to elucidate the roles of MFG-E8 in intestinal inflammatory and malignant diseases, which will lead to development of novel therapies that utilize this important growth factor.

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Chapter 9
MFG-E8 and Acute Lung Injury

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Abstract Although milk fat globule-epidermal growth factor-factor 8 (MFG-E8) was initially identified as a pivotal factor for the clearance of apoptotic cells, subsequent studies revealed its diverse cellular functions which is not only confined to the development of systemic lupus erythematosus (SLE), but also other systemic and localized acute inflammatory diseases. Herein, we have described the promising role of MFG-E8 in terms of attenuating the consequences of acute lung injury (ALI) in several animal models adopted by either direct lipopolysaccharide (LPS) instillation or indirectly via clinically relevant approaches, *e.g.*, sepsis, ischemia-reperfusion, and hemorrhages. Current chapter emphasizes the pathophysiology of ALI and the implications of MFG-E8 mode of actions towards implementing it as a potential therapeutic target to improve disease prognosis in critically ill patients.

Keywords MFG-E8 • ARDS/ALI • Neutrophil • CXCR2 • GRK2

Abbreviations

ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
CLP	Cecal ligation and puncture
GRK2	G-protein coupled receptor kinase 2
I/R	Ischemia/reperfusion
ICAM-1	Intercellular adhesion molecule-1

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IFN-γ	Interferon-γ
LAD	Leukocyte adhesion deficiency
MFG-E8	Milk fat globule-epidermal growth factor-factor 8
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
NF-κB	Neuclear factor-κB
NOS	Nitric oxide synthase
PS	Phosphatidylserine
RGD	Arginine-glycine-aspartate
ROS	Reactive oxygen species
TLR	Toll like receptor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VCAM-1	Vascular cell adhesion molecule-1

1 Introduction

Milk fat globule-epidermal growth factor-factor 8 (MFG-E8) is a 66 kDa secreted glycoprotein, originally identified as a component of the milk fat globule, a membrane-enclosed collection of proteins and triglycerides that expel out of the apical surface of lactating mammary glands [1]. Although MFG-E8 was initially discovered as a bridging molecule between apoptotic cells and macrophages for the enhancement of phagocytic engulfment of apoptotic cells [2], later a series of studies reveal its diverse cellular functions. Its deficiency not only causes autoimmune diseases [3], but also exaggerates inflammation and tissue injury during sepsis, organ ischemia/reperfusion (I/R) and ulcerative colitis [4, 5]. Recently, a novel finding represents its beneficial role in acute lung injury (ALI) by attenuating excessive neutrophil infiltration in lungs through the modulation of its chemokine receptor, CXCR2 [6]. The primary goal of this chapter is to compile cumulative evidences of MFG-E8-mediated protracted beneficial roles in ALI caused by either direct lipopolysaccharide (LPS) instillation or indirectly due to sepsis, gut I/R, bleomycin-induced insults in relevant animal models. Furthermore, we also aim to focus on the MFG-E8-mediated signal transduction by which it ameliorates ALI not only through the inhibition of neutrophil migration, but also by exhibiting several anti-apoptotic and anti-inflammatory properties. Our approach imposes better understanding of the MFG-E8 role in ALI, and further implicates it as an outstanding therapeutic potential in this deadly inflammatory disease.

2 MFG-E8: Functional Domains

Structural dissection of MFG-E8 reveals its N-terminal region containing a signal peptide sequence which directs it to secret into the extracellular compartment, followed by two epidermal growth factor (EGF) domains with the second EGF-repeat

having a highly conserved arginine-glycine-aspartate (RGD) motif, by which it recognizes $\alpha_v\beta_3/\alpha_v\beta_5$-integrin of macrophages [7]. On the other hand, the C-terminal site of MFG-E8 has factor V/VIII like domains resembling the sequences of the blood coagulation factors which allows it to bind to the apoptotic cells via phosphatidylserine (PS) [7]. Thus, these bi-motif functions of MFG-E8 enable it to serve as a scavenging factor for the removal of apoptotic cells by the professional phagocytes such as, macrophages from various organs [2]. In mouse, a long isotypic form of MFG-E8 containing a proline-threonine (PT) rich domain with unknown function is located between the N- and C-terminal sites [7]. However, it is needed for executing its optimum function as the short form of MFG-E8 devoid of this PT region decreases its phagocytic potential for apoptotic cells [2]. Beside this classical function, several recent studies showing its anti-inflammatory and tissue regenerative roles mediated either through its N-terminal $\alpha_v\beta_3/\alpha_v\beta_5$-integrin binding site or the C-terminal PS recognition domains are reported [3, 7, 8].

2.1 MFG-E8 Expression in Lungs

Although the expression of MFG-E8 was first demonstrated in the lactating mammary glands [1], subsequent studies which is compiled in a review article reveals its ubiquitous pattern of expression in different cells and tissues, especially in lungs [7]. MFG-E8 is found to be expressed in the epithelial cells and immune reactive mononuclear cells, such as, macrophages and dendritic cells [7]. Since lung is composed of both interstitial epithelial cells, as well as alveolar macrophages, it is reasonable to observe its presence in the lungs. However, to define its localization, Atabai et al performed lung tissue immunohistochemistry in adult mice where the strong staining of MFG-E8 can be seen at the alveolar interstitium, pulmonary endothelium, as well as in the alveolar macrophages collected from the bronchoalveolar lavage (BAL) [9]. To correlate immunohistological findings, the total proteins from lung tissues were harvested and carried-out for Western blot analysis which shows concordance with the findings of immunohistochemistry [9]. Interestingly, during ALI induced by a direct LPS instillation shows rapid down-regulation of MFG-E8 expression at the earlier time points, even though it became restored at the later stages [6]. Due to the abundant expression of MFG-E8 in normal lungs, considerable interests are being showed to elucidate its novel functions in acute or protracted lung diseases.

3 Acute Lung Injury

The term acute respiratory distress syndrome (ARDS) was first coined in 1967 by Ashbaugh and colleagues [10]. The acute lung injury and its severe form ARDS are a spectrum of lung diseases characterized by a severe inflammatory processes

causing diffuse alveolar damage and resulting in a variable degree of ventilation perfusion mismatch, severe hypoxaemia, and poor lung compliance imposing a substantial health burden all over the world [11, 12]. Recent estimates indicate approximately 190,000 cases per year of ALI with an associated 74,500 deaths in the United States, annually [13]. In-hospital mortality is 38.5 % for ALI, and 41.1 % for ARDS [13]. Most studies of ALI/ARDS have reported a mortality of 40–60 %, however, low-tidal volume ventilation has been shown to reduce mortality to 31 % in a defined cohort [14, 15]. The common causes of ALI/ARDS are sepsis, pneumonia, burn, organ I/R, haemorrahage, traumatic injury, aspiration, pancreatitis, drug overdose, multiple blood transfusions, smoke or toxic gas inhalation [12]. Even in patients who survive ALI, there is evidence that their long-term quality of life is adversely affected. Recent advances have been made in the understanding of the epidemiology, pathophysiology, and treatment strategy of this disease. However, more progress is needed to further improve clinical outcomes of ALI/ARDS by introducing novel therapies.

3.1 ALI/ARDS Pathophysiology

ALI/ARDS is characterized by an overwhelming inflammatory response leading to alveolar epithelial and vascular endothelial injury in the lungs. During the initial phase of ALI there is alveolar flooding with protein-rich fluids due to increased vascular permeability. Alveolar epithelial injury of type I squamous pneumocytes contribute to the pulmonary edema and the breakdown of this epithelial barrier expose the underlying basement membrane, predisposing to bacterial translocation and sepsis. Injury to type II alveolar septal cells leads to impaired surfactant synthesis and metabolism resulting in increased alveolar surface tension and alveolar collapse. Histopathologically there is diffuse alveolar damage with neutrophil infiltration, alveolar hemorrhage, and hyaline membrane formation. The acute phase is followed by a fibro proliferative phase in some with various degrees of fibrosis, neovascularisation and later resolution. Vascular injury and remodeling may lead to pulmonary arterial hypertension which may compromise right ventricular function, exacerbating hypoxaemia and leading to poor clinical outcome. In the air space, alveolar macrophages secrete cytokines, interleukin-1, -6, -8, and -10, (IL-1, -6, -8, and -10) and tumor necrosis factor-α (TNF-α), which act locally to stimulate chemotaxis and activate neutrophils. IL-1 can stimulate the production of extracellular matrix by fibroblasts. A number of antiinflammatory mediators are also present in the alveolar milieu, including interleukin-1-receptor antagonist, soluble TNF receptor, antibodies against IL-8, and cytokines such as IL-10 and -11. The influx of protein-rich edema fluid into the alveolus leads to the inactivation of surfactant. Neutrophils play a critical role in the pathogenesis of ALI/ARDS and when activated release harmful mediators including cytokines, proteases, reactive oxygen species (ROS), matrix metalloproteinases (MMP), oxidants, proteases, leukotrienes, and other proinflammatory molecules, such as, platelet-activating factor (PAF) leading to further damage of the lung tissues and exaggerate inflammation [11, 12].

3.2 Mechanism of Neutrophil Migration in Lungs

Neutrophils are the earliest innate immune cells to be recruited to the site of injury or inflammation. Neutrophil recruitment into the lungs takes place in the small capillaries by following the cascade of activation, sequestration from the blood to interstitium, and transepithelial migration from interstitium to alveolar airspace [16, 17]. Inflammatory stimuli, primarily those which bind to seven-transmembrane-spanning G-protein linked receptors, such as, the chemokine receptor CXCR2 induce changes in the cytoskeleton of neutrophils that leads to an increase of neutrophil sequestration in the interstitium. Before neutrophils migrate along the endothelium into the interstitium and alveolar airspaces, they tether and roll on the endothelium as mediated by selectins which is followed by slow rolling and arresting on the endothelium mediated by integrins and chemokines [17]. In particular, neutrophils that adhere to the endothelium affect the endothelial cytoskeleton, inducing remodeling of tight junctions (for example, platelet endothelial cell adhesion molecule [PECAM]-1, CD99, VE-cadherin, JAMS). Consequently, this transient remodeling of tight junctions of the endothelium facilitates transmigration of neutrophils [18, 19]. There are several receptors and ligands which play major roles in neutrophil migration into the lungs are: selectins which are a family of transmembrane molecules expressed on the surface of leukocytes and activated endothelial cells, and are essential for initiating the rolling process of neutrophils on the endothelium. This initial step in leukocyte adhesion is called capture, which is governed by interactions between L-, E- and P-selectins and P-selectin glycoprotein ligand (PSGL1). L-selectin is expressed by leukocytes, P-selectin is from inflamed endothelium and platelets, E-selectin is expressed by inflamed endothelium PSGL1 is expressed by leukocytes and endothelium [20, 21]. Integrin-β_2 which mediates neutrophil adhesion by interaction with adhesion molecules (for example, intercellular adhesion molecule [ICAM]-1, ICAM-2, vascular cell adhesion molecule [VCAM]-1) [17]. Deficiency of β_2-integrin in humans is known as leukocyte adhesion deficiency (LAD) and causes recurrent bacterial infections, such as, pneumonia, gingivitis, abscesses or peritonitis. The inhibition of β_2-integrins is known to attenuate neutrophil recruitment and protects tissue from injuries [22].

3.3 Harmful Mediators Released from Neutrophils

Excessive production of several harmful mediators causes hyper inflammation and injury to the lung tissues. Matrix metalloproteinase-2 (MMP-2) also known as gelatinase A and MMP-9 (gelatinase B) stored in tertiary granules of neutrophils and MMP-8 (collagenase 2) from secondary granules of neutrophils are the most extensively studied MMPs in the context of ALI. Although MMPs can be released by resident cells, recent studies demonstrated the pathogenetic role of MMP-released from neutrophils in ALI, and their inhibition reduces lung injury and improves

survival after cecal ligation and puncture (CLP) in rats [23, 24]. In particular, BAL fluid [25] and plasma [26] of patients with ALI/ARDS displayed elevated levels of MMPs which correlated with clinical severity.

The antimicrobial polypeptide LL-37 is released from neutrophil secondary granules in its inactive pro-form hCAP18, which is then activated upon secretion by proteolytic modification by proteinase-3. In addition to its broad antimicrobial activity, LL-37 can promote inflammatory responses by activation of monocytes, neutrophils and T-lymphocytes [27, 28]. Defensins are small, arginine-rich cationic peptides that are divided in two subgroups, α-defensins and β-defensins. Human α-defensins 1-4, also known as human neutrophil peptides (HNPs 1-4) are produced principally by neutrophils and stored in primary (azurophilic) granules [29]. High concentrations of α-defensins have been found in BAL fluids from patients with ARDS correlating with the severity of disease [30]. Besides their microbicidal function, α-defensins act as an effector of cytokine production. In this context it has been shown that HNPs activate macrophages to induce the release of TNF-α and interferon (IFN)-γ and to promote a phenotypic switch towards a more proinflammatory phenotype [31]. In acute lung injury, α-defensins also induce IL-8, a chemokine that potently attracts neutrophils [32]. Moreover, α-defensins increase the permeability of the epithelial monolayer *in vitro* [33]. HNPs also exert chemotactic effects on other innate and adaptive immune cells, such as, immature dendritic cells, T cells and mast cells [17, 34]. Azurocidin (also known as CAP37 or HBP) is stored in secretory vesicles and primary granules of neutrophils and is released upon neutrophil adhesion and during neutrophil extravasation. Its positive charge allows for immobilization on the endothelial cell surface where it induces adhesion of inflammatory monocytes, and also promotes permeability changes [17, 35].

Myeloperoxidase (MPO) is an iron containing heme protein predominantly stored in the granula of neutrophils and a key contributor to the respiratory burst of neutrophils. During extensive inflammation, activated neutrophils release MPO which damage resident lung cells. Recent observations suggest that MPO has proinflammatory properties, independent of its enzymatic activity [36]. Enzymatic activity of MPO results mainly in the conversion of hydrogen peroxide (H_2O_2) and chloride ions into hypochlorous acid (HOCl), leading to increased levels of oxidative stress enhancing tissue damage. MPO was demonstrated to associate with the outer membrane of neutrophils by binding to CD11b/CD18 integrins, which are known to be centrally linked to neutrophil activation [36]. In this manner MPO may contribute to neutrophil recruitment to the site of inflammation. In addition, MPO has been shown to stimulate the production of TNF-α and IFN-γ by peritoneal macrophages and hereby enhance target cell killing by macrophages [37].

Neutrophils produce vast quantities of ROS and reactive nitrogen species (RNS) through their oxidant-generating systems such as the phagocyte NADPH oxidase and nitric oxide synthase (NOS) respectively. ROS is released into the cytosol where they alter the redox state of the cell and modify other cell contents, such as proteins and lipids by oxidation [38]. The membrane-bound multicomponent enzyme complex NADPH oxidase, which is dormant in resting cells and can be activated rapidly by chemoattractant peptides or chemokines, generates much ROS after activation.

Furthermore, the myeloperoxidase, which is found in the neutrophil granules, catalyzes the production of additional ROS species, that is, the hydroxyl radical and hypochlorous acid. Activated neutrophils produce prostaglandine E and F using the arachinodic acid metabolism and this metabolism produces ROS, which is able to regulate other signaling pathways in neutrophils directly or indirectly. Deficiency of NADPH oxidase in humans, known as a chronic granulomatous disease, causes recurrent infections because phagocytic cells fail to produce ROS and to kill engulfed foreign organisms. In animal models of ALI, neutrophil-derived ROS and RNS caused lung injury as shown by histological examination and permeability measurements [17, 39]. A recent study revealed that ROS can disrupt intercellular tight junctions of the endothelium by phosphorylation of focal adhesion kinase [40]. *In vitro*, ROS induced cell apoptosis and necrosis of alveolar type II cells during oxygen exposure [40] ROS may prolong inflammation by modulating neutrophil function. After oxidation of acid spingomyelinase, ROS delays neutrophil apoptosis in a caspase-8-dependent way [41]. Therefore, the neutrophil-derived oxidants are regarded to present a major role in neutrophil-mediated tissue injury, including ALI/ARDS.

3.4 Animal Models of Acute Lung Injury

Animal models serve as a bridge between bedside and laboratory bench. Ideally, the animal models of ALI should reproduce the mechanisms and consequences of ALI in humans, including the physiological and pharmacological changes that occur, and also innovation of the novel therapeutic potentials. The mice are used as a tool to study the biology of ALI, because of the availability of specific reagents and the development of genetically modified mice that can be used to evaluate the physiological function of specific genes. The most widely used and highly reproducible animal model of ALI can be attained by the direct LPS instillation into the lungs through intra-tracheal route. LPS binds to a specific LPS binding protein (LBP), forming an LPS:LBP complex that activates the CD14/TLR4 receptor structure on monocytes, macrophages, and other cells, triggering the production of proinflammatory cytokines and chemokines [TNF-α, IL-1β, IL-6, G-CSF, IL-8, and macrophage chemoattractant protein (MCP-1)] and at the later phase increases in the BAL fluid PMN, monocyte, macrophage, and lymphocyte counts [42]. Beside LPS, intratracheal instillation or aerosolization of bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) results in bilateral increases in permeability and PMN sequestration as well as an increase in proinflammatory mediators [42]. Although the bleomycin model is usually considered a model of pulmonary fibrosis, its administration is also associated with the features of ALI. Bleomycin is an antineoplastic antibiotic drug which forms a complex with oxygen and metals, leading to the production of oxygen radicals, DNA breakes, and ultimately cell death. It can be administered intravenously, intratracheally, intraperitonally, or subcutaneously causing acute inflammatory injury followed by reversible fibrosis [43]. Beside these chemically

induced models, there are several clinically relevant models that directly or indirectly promote ALI in rodents. CLP is the most widely used model of peritonitis. In contrast to models using LPS and live bacteria, in which the effects are almost immediate, the effects of CLP develop over days, and the onset is less abrupt. In CLP the pulmonary hypertension develops within 24–72 h which is featured by hypoxemia, neutrophilic inflammation, and interstitial and alveolar edema, but less hyaline membrane formation [44]. Another most reproducible and clinically relevant model of developing ALI is ischemia followed by reperfusion, either in lungs or in the distant vascular beds, that targets the epithelium and endothelium can lead to ALI. A classic form of injury secondary to lung I/R is developed following lung transplantation. The reimplantation response is characterized by non-cardiogenic pulmonary edema, inflammatory infiltrates, and hypoxia, and is unrelated to rejection [42, 45]. In the pulmonary models of I/R, the lung is subjected to ischemia for varying periods of time by clamping the pulmonary artery, or the hilum before circulation is reestablished. This leads to the structural damage of the alveolar endothelium/epithelium, disruption of the septal capillary walls, vascular permeability, hemorrhage, increased inflammatory mediators and infiltration of PMN [46]. The nonpulmonary I/R models are generated based on the anatomic area subjected to ischemia, e.g., gut and lower torso in rats, mice, rabbits and sheep. These models attribute to severe lung injury characterized by the infiltration of PMN, increased pro-inflammatory mediators, edema, and increased vascular permeability and sometimes hemorrhage [42]. Apart from these, there are several other models which leads to the development of ALI include: mechanical ventilation, hyperoxia, oleic acid and acid aspiration, are summarized in Table 9.1.

3.5 Treatment For ALI

In recent years, the pace of ongoing research in the area of ALI has been quite brisk. Treatment of ALI is based on both ventilatory and nonventilatory strategies. To date, the most significant advances in the supportive care of lung injury patients have been associated with improved ventilator management. However, the use of this technique has adverse effects, including the increased risk of pneumonia, impaired cardiac performance, and difficulties associated with sedation and paralysis [52]. Moreover, application of pressure to the lung, whether positive or negative, can cause damage known as ventilator-associated lung injury (VALI). Therefore, modification of mechanical ventilation so that VALI is kept to a minimum improves survival of patients with ARDS [52]. Several clinical trials involving a large number of pharmacologic agents have been shown to be ineffective in reducing mortality. Recent advances in the understanding of the pathophysiology of ALI have led to investigations of numerous potential pharmacologic treatments. Despite earlier encouraging preclinical evidence, phase III trials have not supported the use of exogenous surfactant, inhaled nitric oxide, intravenous prostaglandin E1, glucocorticoids, Ketoconazole, Lisofylline, N-acetylcysteine, and activated protein C as

Table 9.1 Animal models of acute lung injury

Model	Feature	Reference
LPS	Intrapulmonary/systemic inflammatory cytokine production, with excessive amount of neutrophil infiltration and mild alveolar-capillary permeability	[6]
Bacterial inoculation	Intrapulmonary/intravenous inoculation of *E. Coli*, *P. aeruginosa* edema, congestion, PMN sequestration, increased permeability, less hyaline membrane formation	[47]
Cecal ligation and puncture	Degree of lung injury depends on surgical variability, increased local/systemic inflammation, increased permeability, PMN infiltration, minimal hyaline membrane formation, increased apoptosis	[48]
Pulmonary I/R	Increased pulmonary vascular permeability, infiltration of PMN, hemorrhagic	[42]
Gut I/R	Neutrophil infiltration in lungs, increased gut permeability and intestinal/systemic inflammation, increased rate of cell death	[49]
Trauma/ hemorrahagic shock	Increased PMN influx, lung protein leak, increased chemokine cytokine expression	[50]
Saline lavage	Decreased lung compliance by impaired gas exchange	[42]
Bleomycin	Lung fibrosis, acute inflammation, collagen deposition, no hyaline membrane formation	[9]
Acid aspiration	Disruption of alveolar/capillary architectures, PMN infiltration	[42]
Oleic acid	Acute endothelium damage due to necrosis of type I cells	[51]
Hyperexia	Acute epithelial barrier injury, neutrophil infiltration, type II cell proliferation and scarring	[42]

treatments for ALI [11]. In phase III clinical trials from two different studies utilizing glucocorticoid at the acute phase of the disease did not show any therapeutic benefit [53, 54]. Similarly, the clinical studies using surfactant and N-acetylcysteine in ALI patients were also not effective in terms of improving the survival [55, 56]. However, studies on treatment of glucocortioid at the late phase of ALI showed some benefits in context of reducing mortality [57].

There has been considerable preclinical data supporting the potential value of β-2 agonist therapy for the treatment of ALI [58, 59]. It has been reported that β-2 agonists accelerate the resolution of pulmonary edema by decreasing inflammation and upregulating alveolar salt and water transport, hastening the resolution of alveolar edema. Recently, a large, multicenter, randomized clinical trial of an aerosolized β-2 agonist, albuterol, was stopped early for futility [60].

A novel approach to ALI includes HMG-CoA reductase inhibitors (statins). Normally used for the prevention or treatment of cardiovascular disease, statins also possess significant anti-inflammatory, immunomodulatory, and antioxidant effects. However, it is uncertain how these properties will translate to the human ALI/ARDS population. Several observational studies in the human sepsis model, a known risk factor for ALI, have reported statin users have a decreased severity of sepsis and mortality despite having higher baseline comorbidities [61, 62]. Conversely, Kor

and colleagues found no difference in morbidity and mortality in a retrospective observational study with ALI/ARDS treated with statin [63].

One promising new treatment for ALI is bone marrow-derived mesenchymal stem cells (MSCs). These cells possess the ability to differentiate into many types of cells, including vascular endothelium and alveolar epithelium. MSCs also secrete paracrine factors that reduce the severity of ALI, including growth factors, factors that regulate barrier permeability, and anti-inflammatory cytokines [11, 64]. Recent study reported the MSCs' anti-inflammatory properties in both *in vivo* and *in vitro*. In a mouse model of LPS instillation into the distal airspaces of the lung, the direct intrapulmonary post treatment of MSCs decreases extra vascular lung water, alveolar–capillary permeability, and mortality. The pro-inflammatory response is downregulated, whereas the anti-inflammatory response become upregulated [65]. Several investigators are working on translating these experimental studies to phase I and II clinical trials of patients with severe ALI.

Depending on this observation it is still an unmet requirement to innovate novel therapeutic regimens without adverse effects for the treatment of patients with ALI to reduce morbidity and mortality.

4 MFG-E8 Amiliorates LPS-Induced ALI

ALI/ARDS is characterized by an excessive inflammatory response, representing considerable clinical challenges to the critical care medicine. Despite extensive research, only a few therapeutic strategies for ALI/ARDS have been emerged, and current specific options for treatment remain enigmatic. Excessive neutrophil infiltration to the lungs is a hallmark of ALI. MFG-E8 was originally identified as an indispensable factor for phagocytosis of apoptotic cells [2]. However, subsequent studies reveal its diverse cellular functions in terms of its direct role in anti-inflammation and tissue regeneration [3, 7, 8]. Neutrophil recruitment to the lungs is a critical event to promote pulmonary inflammatory responses associated with ALI [66]. Proteolytic enzymes, such as elastase, MPO, and ROS including hydrogen peroxide and superoxide as released from the activated neutrophils not only kill the invaded pathogens, but also exaggerate epithelial/endothelial barrier dysfunctions leading to tissue damage and uncontrolled inflammation [67, 68]. Neutrophil recruitment into the lungs is mediated by a local production of chemokines released by the alveolar macrophages as well as other cell types in response to inflammatory stimuli [17, 69]. CXC chemokines, such as IL-8 are elevated significantly in the BAL of patients with ARDS, and increased IL-8 levels are associated with increased neutrophil infiltration [70, 71]. In rodents, the IL-8 homologue, CINC-1/2 and MIP-2 regulate neutrophil recruitment into the lungs of experimental ALI via chemokine receptor, CXCR2 [72, 73]. CXCR2 is a seven transmembrane type G protein-coupled receptor (GPCR) whose expression, localization and function in PMN are tightly regulated by the intracellular G protein-coupled receptor kinase 2 (GRK2) [72]. Upon activation, GRK2 phosphorylates CXCR2 and causes receptor

desensitization and internalization, leading to down-regulation of neutrophil chemotaxis [72]. The cellular expression pattern of MFG-E8 reveals it's localization into the macrophages, dendritic, epithelial, and fibroblast cells from various organs [6]. In normal lungs, MFG-E8 is present in the alveolar interstitium and pulmonary vasculature which comprise largely of epithelial and endothelial cells and a few resident macrophages [9]. Even though the expression of MFG-E8 is ubiquitously found in lungs, a marked decrease in its content has been reported in ALI induced by direct LPS instillation, causing exaggerated inflammation and abnormal tissue homeostasis [6]. The decrease production of MFG-E8 may be due to the uncontrolled activation of immune-reactive cells, since MFG-E8 expression is negatively regulated by the activation of toll-like receptor (TLR)-mediated pathways upon LPS challenge [74]. Among several features, cellular apoptosis is markedly observed in ALI [17], therefore predicting a potential scavenging role of MFG-E8 to get rid of the deleterious effects of apoptotic cells before they undergo secondary necrosis. Phagocytosis of apoptotic cells can indirectly regulate pro-inflammatory milieu by modulating the activation of potent transcription factor, NF-κB [75]. Beside this, in immune reactive cells a direct anti-inflammatory role of MFG-E8 by inhibiting the NF-κB-mediated pro-inflammatory cytokine production is demonstrated in recent studies [5, 6, 76]. Although the findings of direct or indirect immunomodulatory roles of MFG-E8 in terms of inhibiting NF-κB may greatly improve our understanding of delineating the protective roles of MFG-E8 against ALI, the underlying mechanisms of resolving excessive neutrophil infiltration should be a critical concern. Recently, the synthetic peptide RGD has been shown to attenuate lung neutrophil chemotaxis in ALI by recognizing $\alpha_v\beta_3$-integrin and modulating down-stream signaling events [77]. Since MFG-E8 has a binding affinity for $\alpha_v\beta_3$-integrin through its RGD motif, the recent hypothesis, therefore consider an additional mechanism by which MFG-E8 attenuates neutrophil migration during ALI to ameliorate LPS-induced ALI.

4.1 MFG-E8 Deficiency Augments ALI

Recent studies by Aziz et al. demonstrated that, in MFG-E8 knock-out (*Mfge8*$^{-/-}$) mice, the TNF-α expression is comparatively higher than the wild-type animals within early time points (4 h) after LPS instillation [6]. The histological images of the lung tissues at 24 h after LPS instillation represent increased alveolar congestion, exudates, interstitial and alveolar neutrophilic infiltrates, intraalveolar capillary hemorrhages, and extensive damage of their epithelial architecture in *Mfge8*$^{-/-}$ mice as compared to the wild-type (WT) counterpart (Fig. 9.1). After LPS treatment which causes dramatic decrease in MFG-E8 expression may lead to the increased neutrophil infiltration into the lungs as reflected by the increased production of MPO, a marker of granulocyte infiltration into the lungs. The direct assessment of cellular contents shows significantly increased numbers of neutrophils in the BAL as compared to the WT mice at 24 h after LPS injection. Furthermore, a

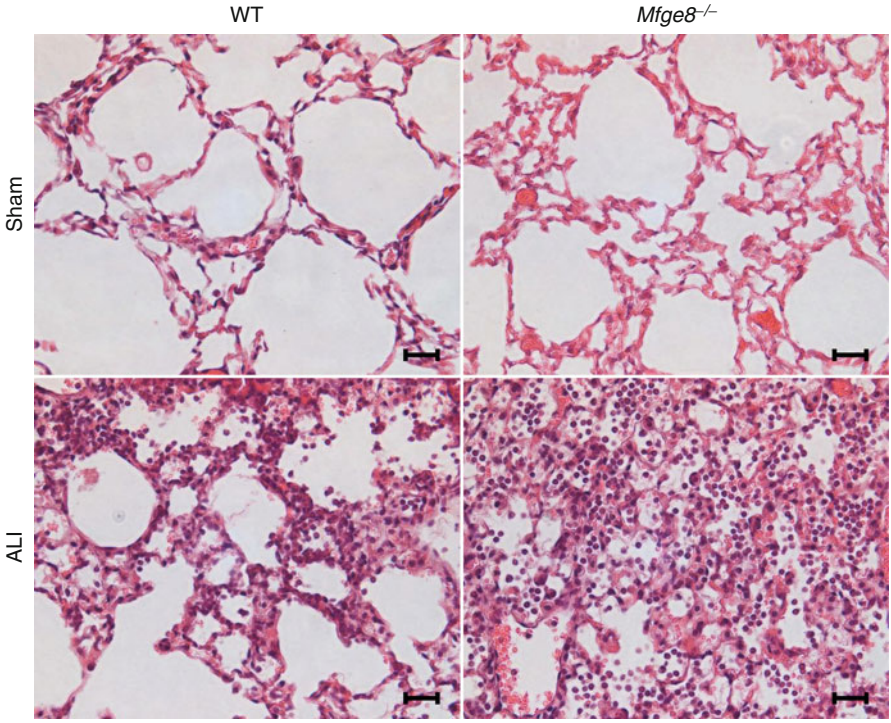

Fig. 9.1 Lung tissue immunohistochemistry after ALI: WT and *Mfge8*$^{-/-}$ mice were subjected to *i.t.* injection of LPS (5 mg/kg). At 24 h after LPS instillation, lung tissues were fixed and stained with H&E. Representative histological images at 400× magnification are shown. Scale bar, 50 μm. Tissue injury are assessed based on the presence of exudates, hyperemia/congestion, neutrophilic infiltrates, intra-alveolar hemorrhage/debris, and cellular hyperplasia. (Reprinted from Aziz et al. [6]. 2012 with permission from The Journal of Immunology)

significant increase in total protein levels is observed in BAL from *Mfge8*$^{-/-}$ mice than WT animals at 24 h of ALI. Beside that, an increased accumulation of apoptotic cells in the lungs of *Mfge8*$^{-/-}$ mice can also be found after ALI with the significantly elevated amount of cleaved caspase-3 in the lungs of *Mfge8*$^{-/-}$ mice, indicating an increased occurrence of cellular apoptosis in *Mfge8*$^{-/-}$ mice than that of WT counterpart. Collectively, these findings demonstrate that the higher amounts of apoptotic cells in the lungs of *Mfge8*$^{-/-}$ mice are due to the reduced phagocytosis and/or increased rate of apoptosis mediated by the activation of caspase-3. Apart from these features, an exaggerated production of MIP-2, a vital chemokine responsible for the neutrophil chemotaxis is significantly elevated in *Mfge8*$^{-/-}$ mice than WT animals at 4 h after LPS treatment which provokes neutrophil migration into the lungs [6]. Since macrophages are one of the major cell types for producing chemokines and cytokines, further studies evaluated that the alveolar macrophages from BALF of *Mfge8*$^{-/-}$ mice release comparatively higher amount of MIP-2 than WT

animals. Conversely, these deleterious effects can be overcome by supplementing recombinant MFG-E8 protein which not only negatively regulates inflammatory mediators but also attenuates neutrophil infiltration to the lungs following LPS-induced ALI. Since LPS instillation causes significant downregulation of MFG-E8 mRNA and protein levels in the lungs, it become worthy of pre-treating them with recombinant MFG-E8 *i.v*. to ameliorate LPS triggered ALI initiation. Therefore, these collective evidences clearly support MFG-E8 as a potential therapeutic molecule to salvage the deficits of endogenous MFG-E8 that occur during ALI. Interestingly, the number of total cells as well as the neutrophil counts in BALF of recombinant MFG-E8 pretreated mice is shown to be significantly lower than those in mice without recombinant MFG-E8 treatment after LPS instillation. Depending on this finding, it can be suggested that the excess in neutrophil infiltration into the lungs may be due to the decreased production of endogenous MFG-E8 during ALI, which can be ameliorated by exogenous treatment of recombinant MFG-E8 protein.

4.2 MFG-E8 Inhibits Neutrophil Migration

In order to carry-out the neutrophil migration assay, the neutrophils are isolated from mice bone marrow using the density gradient centrifugation, followed by the flow cytometric assessment to find the neutrophil population based on the presence of their putative surface receptor, $CD11b^{+}Ly6G^{+}$. Next, to evaluate whether MFG-E8 has any direct effect on the neutrophil migration, the neutrophils isolated from the WT and *Mfge8*$^{-/-}$ mice bone marrow are subjected to *in vitro* migration assay using recombinant IL-8 as a chemoattractant [6]. The study showed significantly higher rate of *Mfge8*$^{-/-}$ neutrophil migration as compared to that of WT mice. CXCR2 serves as the cognate receptor expressed on neutrophils for MIP-2-dependent chemotaxis. By using flow cytometric analysis, the surface expression of CXCR2 in MFG-E8-deficient neutrophils ($CD11b^{+}Ly6G^{+}$) are found to be significantly higher than those of neutrophils from WT mice, indicating that MFG-E8 can be a critical molecule in regulating neutrophil migration by altering the CXCR2 expression. Conversely, the number of migrated cells in recombinant MFG-E8-treated BMDN is markedly reduced, as compared to the vehicle-treated BMDN. Since, $\alpha_v\beta_3$-integrin serves as the receptor for MFG-E8 binding, co-treatment of anti-MFG-E8 neutralizing antibodies with recombinant MFG-E8 abrogates the functions of recombinant MFG-E8 for reducing the migration of BMDN, thus become comparable to the vehicle control. Correspondingly, the recombinant MFG-E8-treated BMDN has a lower CXCR2 expression compared to the vehicle animals and the reduction of CXCR2 expression can be rescued by co-treatment of anti-MFG-E8 neutralizing antibodies. Intracellular GRK2 is a major determinant for surface CXCR2 translocation in neutrophils, whose activation leads to desensitization of CXCR2, resulting in its intracellular translocation, and negative regulation of the neutrophil migration [72, 73]. GRK2 expression in BMDN is increased

significantly upon recombinant MFG-E8 treatment, compared to the vehicle control, while co-treatment of anti-MFG-E8 antibodies diminishes such induction of GRK2 expression [6]. Correspondingly, the BMDN pre-treated with anti-α_v-integrin antibodies abrogates the functions of MFG-E8-mediated inhibition of their migration. It has been further demonstrated that the pre-treatment of BMDN with anti-α_v-integin antibodies blocked the effects of recombinant MFG-E8-mediated down-regulation of CXCR2 and up-regulation of GRK2 [6]. Collectively, these features clearly demonstrate the critical roles of MFG-E8 for GRK2-dependent down-regulation of surface CXCR2 expression in neutrophils through $\alpha_v\beta_3$-integrin-mediated pathway. Therefore, intra-tracheal LPS exposure triggers MIP-2 expression in lungs, which serves as a potent chemoattractant for neutrophil migration into the lungs by recognizing CXCR2, causing widespread inflammation and tissue damage. In neutrophils, recombinant MFG-E8 recognizes $\alpha_v\beta_3$-integrin and then generates down-stream signaling for intracellular GRK2 activation, which leads to the down-regulation of the surface CXCR2 expression, hence attenuating the MIP-2 dependent neutrophil migration during LPS-induced ALI [6] (Fig. 9.2).

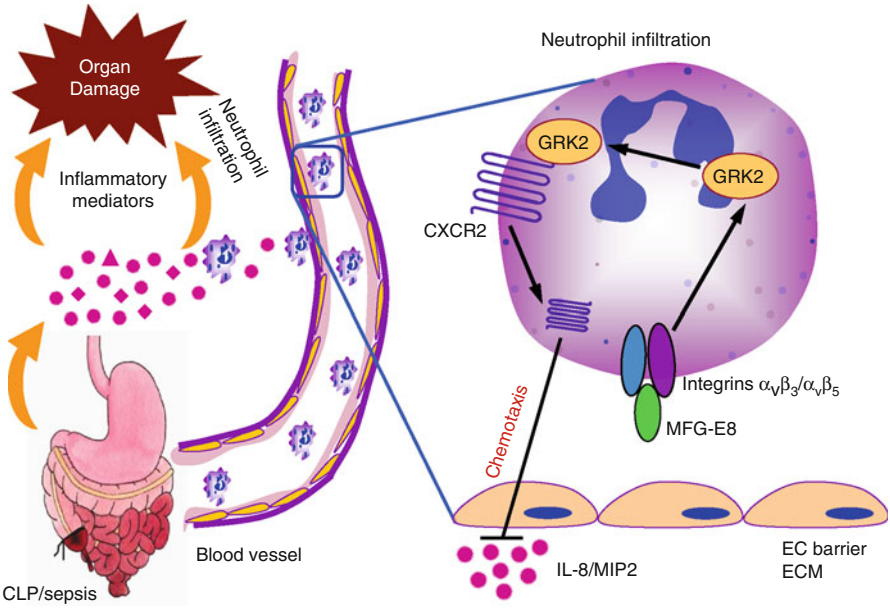

Fig. 9.2 MFG-E8 attenuates lung neutrophil infiltration during ALI: Systemic inflammation caused by CLP triggers inflammatory cytokine and chemokine production in lungs. The chemokine, such as, MIP-2/IL-8 serves as a potent chemoattractant for neutrophil migration into the lungs by recognizing CXCR2 and causes widespread inflammation and tissue damage. In neutrophils, recombinant MFG-E8 recognizes $\alpha_v\beta_3$-integrin and then generates down-stream signaling for intracellular GRK2 activation, which down-regulates the surface CXCR2 expression, hence attenuating the MIP-2/IL-8 dependent neutrophil migration during ALI

5 Role of MFG-E8 in Gut I/R-Induced ALI

Mesenteric ischemia remains a critical problem, resulting in mortality as high as 60–80 % [78]. Multiple organ failure, including ALI, is a common complication of intestinal I/R injuries and contributes to its high mortality rate. A key aspect of I/R injury is the increased occurrence of apoptotic cell death of intestinal and bronchial epithelial cells and of type II alveolar epithelial cells [49, 79, 80]. Apoptosis is associated with a marked up-regulation of Fas and Fas-ligand and the activation of caspase-3 in lung epithelial cells. Proinflammatory cytokines, such as IL-1β or TNF-α, play a major role in apoptosis induction, involving Bid, Bax up-regulation, and Bcl-2 down-regulation [80, 81]. Similar to sepsis, gut I/R injury is accompanied by a systemic inflammatory response, lung neutrophil infiltration and organ damage. Considering the novel role of MFG-E8 for the phagocytic removal of apoptotic cells, Cui et al. revealed the fact that MFG-E8 can attenuate intestinal and pulmonary inflammation after gut I/R through restoring the phagocytic removal of apoptotic cells, which become retarded during stress [49]. The effects of intestinal I/R on endogenous MFG-E8 expression in spleen, and blood and the beneficial role of MFG-E8 for the protection of organ failure, including ALI are shown in their study. In their model, ischemia is induced in C57BL/6 J WT and *Mfge8*$^{-/-}$ mice by bilateral clamping the superior mesenteric artery (SMA) for 90 min under general anesthesia using isofluorane. The vascular clamps are released after 90 min to allow reperfusion. The suppression of MFG-E8 in the spleen after 4 h of intestinal I/R injury is clearly evident from their study. Gut I/R causes widespread macroscopic necrosis with severe enteric mucosal injury in intestinal areas juxtaposed to the macroscopically ischemic bowel. Similarly, multiple blood markers of remote organ damage are significantly elevated, including lactate, LDH, ALT, AST, and creatinine compared with the sham-operated animals, indicating the systemic scale of injury induced in this model. Interestingly, the intra-peritoneal treatment with a single dose of recombinant MFG-E8 (0.4 mg/20 g BW) at the beginning of reperfusion largely attenuates I/R-induced multiple organ injury [49]. Histopathologically, even large parts of intestine are shown to be protected from secondary mucosal damage after recombinant MFG-E8 treatment. Similarly, the treatment with recombinant MFG-E8 markedly reduces the elevation of lactate, AST, ALT, LDH and creatinine levels in blood. Apart from that, the administration of recombinant MFG-E8 significantly decreases the expression of systemic pro-inflammatory mediators after gut I/R. The lungs are among the organs that are most severely affected by intestinal I/R injury. Histopathological analysis of the lungs shows moderate to severe injury with exudates, congestion, cellular infiltrates, and intracellular hemorrhage. The infiltrating cells are suggestive of neutrophilic in nature as determined from their morphology. The assessment of MPO activity in the lungs shows elevated levels after gut I/R. However, the treatment with recombinant MFG-E8 significantly reduces ALI histopathological and biochemical scores [49]. Beside this, the pulmonary MFG-E8 expression is decreased and on the other hand apoptosis is increased after intestinal

I/R. As a measure of apoptosis, the expression of cleaved caspase-3 protein in the lungs is markedly increased after gut I/R-mediated injuries [49]. Treatment with recombinant MFG-E8 decreases pulmonary cleaved caspase-3 levels dramatically to the levels similar to those in sham animals. At the same time, the number of apoptotic cells in the pulmonary tissues is significantly increased as they revealed by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Treatment with recombinant MFG-E8, however, suppresses the number of detectable apoptotic cells in the lungs after gut I/R stress. To further elucidate the role of MFG-E8 in gut I/R-mediated ALI, Cui et al. reveal a marked increase in the inflammatory response and lung tissue injury in MFG-E8–deficient mice [49]. Compared to the WT mice, *Mfge8*$^{-/-}$ mice exhibits significantly elevated levels of IL-1β and IL-6 protein levels in the lungs after gut I/R. This dramatic increase in proinflammatory cytokine levels in the lungs is associated with deteriorated features of ALI in *Mfge8*$^{-/-}$ mice compared with their WT controls, which includes increased congestion, exudates, interstitial cellular infiltrates, and consolidation. Pulmonary neutrophil activity is also significantly increased in *Mfge8*$^{-/-}$ mice after gut I/R injuries. On the other hand to assess the effect of recombinant MFG-E8 on intestinal I/R-induced organ injury in *Mfge8*$^{-/-}$ mice, Cui et al. injected rmMFG-E8 (0.4 mg/20 g BW in 0.5 ml normal saline) or normal saline (Vehicle) intraperitoneally at the end of 90 min SMA clamping, which shows significant decrease in pulmonary proinflammatory cytokine (i.e., TNF-α, IL-1β, and IL-6) levels, inhibits neutrophil infiltration (MPO activities) and apoptosis (cleaved caspase-3 levels) in the lung, and reduces circulating levels of LDH, ALT, and AST after intestinal I/R in *Mfge8*$^{-/-}$ mice. Finally, treatment with recombinant MFG-E8 shows improved survival after intestinal I/R Injury. These novel findings suggest that MFG-E8 can be beneficial in I/R mediated injury. In conclusion, MFG-E8 is found to be a critical factor in controlling gut I/R-mediated ALI in terms of attenuating the neutrophil migration, decreasing pro-inflammatory mediators, as well as protecting tissues from injuries and apoptosis by reducing IL-6 and the caspase-3 activities [49].

6 MFG-E8 Protects Bleomycin-Induced Lung Fibrosis

Tissue fibrosis is characterized by the replacement of normal tissue architecture with collagen-rich matrix, which leads to disrupt organ function [82, 83]. In the lungs, fibrosis can occur due to abnormal remodeling after acute lung injury, in the setting of systemic autoimmune and inflammatory disease, or as an idiopathic process [84]. Collagen turnover, in the form of its production, deposition, and removal is a dynamic process, with the balance between collagen production and removal determining tissue architecture [85]. When persistent collagen production outpaces or overwhelms mechanisms that remove collagen, excess collagen is deposited in the extracellular matrix, leading to tissue fibrosis. Collagen turnover occurs by two pathways, extracellular proteolytic cleavage and endocytosis followed by lysosomal degradation [85, 86]. Extracellular collagen cleavage facilitates subsequent

intracellular uptake and its degradation. However, little is known about the molecules that mediate collagen endocytosis. To reveal MFG-E8 function in collagen turnover in lung fibrosis, Atabai et al. injected bleomycin, as an inducer of lung fibrosis through the intratracheal route in *Mfge8*$^{-/-}$ mice, and the severity of pulmonary fibrosis was evaluated at 28 days [9]. According to their findings, MFG-E8 deficiency results in an exaggerated fibrotic response to bleomycin. Histologically, *Mfge8*$^{-/-}$ mice exhibit more extensive deposition of collagen fibrils than control mice in tissue sections as compared to that of *Mfge8*$^{+/-}$ mice with heterogenous genetic background. To quantify lung fibrosis biochemically, they measured total lung hydroxyproline content 28 days after bleomycin treatment, which reveals significantly greater total lung hydroxyproline contents in *Mfge8*$^{-/-}$ mice than the *Mfge8*$^{+/-}$ littermate controls. To find the mechanism, they suggested that the enhanced fibrosis in *Mfge8*$^{-/-}$ mice is not associated with the impaired clearance of apoptotic cells as measured by the contents of BAL apoptotic cells, rather linked to impaired collagen uptake by surrounding macrophages, even though there is no significant changes in the endogenous expression of collagen levels in *Mfge8*$^{-/-}$ mice as compared to the control [9]. Interestingly, they also revealed that the MFG-E8-mediated collagen endocytosis, in contrast to uptake of apoptotic cells, can be independent of its N-terminal RGD binding domain, but depend on the first discoidin domain of MFG-E8 which exerts an efficient role in binding to collagen and their subsequent uptake in alveolar macrophages. The high prevalence and lack of treatment for fibrotic diseases underscore the importance of a better understanding of the endogenous pathways that mediate removal of collagen from the extracellular matrix. The identification of the novel role of MFG-E8 should effectively be implemented as potential therapeutic potential in lung fibrosis and its associated complications.

7 Role of MFG-E8 in Asthma

Obstruction of airway function is a hallmark of asthma and is primarily caused by an exaggerated response of airway smooth muscle (ASM) to contractile agonists [87]. Severe bronchospasm in asthma is associated with significant morbidity and economic burden. New insights into the pathogenesis of bronchoconstriction, especially as they relate to the ASM, are of significant clinical interest. The cytokines IL-13, IL-17A, and TNF-α, all of which are elevated in the airways of patients with asthma, regulate airway hyperresponsiveness (AHR) by increasing ASM calcium sensitivity [88–90]. The release of contractile agonists during airway inflammation causes excessive bronchoconstriction in sensitized ASM. The protective mechanisms that prevent or attenuate the increase in calcium sensitivity of ASM exposed to cytokines have not been well studied. The force of ASM contraction is regulated by the phosphorylation status of myosin light chain (MLC) [91]. Cytokines enhance ASM calcium sensitivity and contraction through a signaling pathway involving NF-κB, ras homolog gene family, member A (RhoA),

and ROCK2 [88–90]. Activation of this pathway leads to phosphorylation and inactivation of MLC phosphatase (MLCP) by ROCK2 [9, 92]. The protective mechanisms that prevent or attenuate the increase in RhoA activity have not been well studied. Recently, Kudo et al. elucidated that the mice lacking MFG-E8 gene develop exaggerated airway hyperresponsiveness in experimental models of asthma [93]. In their studies, Ova challenged model of asthma was introduced in WT and *Mfge8*$^{-/-}$ mice. They have noticed that the *Mfge8*$^{-/-}$ mice ASM has enhanced contraction after treatment with IL-13, IL-17A, or TNF-α. Correspondingly, recombinant MFG-E8 treatment reduces contraction in murine and human ASM treated with IL-13. Based on their studies, MFG-E8 inhibits IL-13-induced NF-κB activation and induction of RhoA. MFG-E8 also inhibits the rapid activation of RhoA, an effect that can be eliminated by an inactivating point mutation in the RGD integrin-binding site in recombinant MFG-E8, indicating that the N-terminal site of MFG-E8 plays potential role in this event. The C-terminal discoidin domains of MFG-E8 have been shown to bind PS of apoptotic cells. Using annexin V, a competitive binder of PS they also proved that the effects of MFG-E8 for the modulation of ASM contraction is mediated through its discoidin domain after recognizing the PS. In addition, the *Mfge8*$^{-/-}$ mice tracheal rings treated with IL-13 shows increased contraction in response to KCl dependent increased calcium sensitivity which can be opposed by addition of recombinant MFG-E8 through the regulation of Rho induction and activity [93]. To show its clinical relevance, the human subjects with asthma have decreased MFG-E8 expression in airway biopsies compared with healthy controls. These data indicate that MFG-E8 binding to integrin receptors on ASM opposes the effect of allergic inflammation on RhoA activity and identify a novel pathway for specific inhibition of ASM hypercontractility in asthma.

8 MFG-E8's Role in Sepsis and Hemorrhagic Shock-Induced ALI

In addition to the direct ALI models, several clinically relevant models of systemic inflammation indirectly cause deterioration of lung functions due to exaggerated inflammation, neutrophil infiltration and tissue damage. These include CLP-induced sepsis and hemorrhagic shock [4, 50]. It has recently been reported that the *Mfge8*$^{-/-}$ mice with experimental sepsis exhibit severe lung dysfunction as compared to the WT animals [4]. After treatment with the recombinant MFG-E8 in the septic mice significantly attenuate lung malfunction by downregulating pro-inflammatory cytokines, chemokines, and MPO levels as well as reversing cellular apoptosis by inhibiting caspase-3 activity. In order to implement MFG-E8 as therapeutic potential, recent study revealed that the treatment with increasing doses of recombinant MFG-E8 significantly reduces the number of apoptotic cells and markedly attenuates the lung tissue damage [48]. Similarly, in hemorrhagic shock model the systemic MFG-E8 levels become decreased, which reflects reduced endogenous expression of it in

spleen and lung tissues [50]. The administration of recombinant MFG-E8 in hemorrhagic mice attenuates neutrophil infiltration in the lungs, which in turn improves inflammation and tissue damages. Following hemorrhage, the number of apoptotic cells in the lungs and spleen are increased, which can be protected by administrating recombinant MFG-E8 protein that leads to an overall improvement in the survival of the hemorrhagic animals [50].

9 Summary

In the current chapter several noteworthy discoveries related to the role of MFG-E8 for protecting ALI have been demonstrated. The effect of MFG-E8 on ALI and its associated complications is not restricted to a single mechanism, rather portrayed to a broad range of cellular events involving the regulation of neutrophil migration, apoptosis, phagocytosis, innate-immune function and maintenance of the efficient collagen turnover. In addition to demonstrating the roles of MFG-E8 in regulating neutrophil migration, recent findings reveal an exaggerated induction of MIP-2 by LPS stimulation in the lungs and alveolar macrophages due to the deficiency of MFG-E8 [6]. Since it has been previously established that the role of MFG-E8 in down-regulating the pro-inflammatory cytokines is mediated by modulating the intra-cellular signaling cascade [76], it is conceivable that MFG-E8 has an additional role in protecting the inflammatory consequences in ALI through the similar mechanism. This chapter describes a novel and previously unexplored function of MFG-E8 in attenuating lung injury induced by LPS through inhibiting neutrophil infiltration to the lungs. The MFG-E8 effect for decreasing neutrophil migration is mediated by the $\alpha_v\beta_3$-integrin to up-regulate GRK2 expression that results in to a rapid downregulation of surface CXCR2 levels in neutrophils. Under physiological conditions, a secondary (postapoptotic) necrosis of apoptotic cells can be prevented through their quick removal by phagocytes in tissues and circulation [2]. Hence, the potential harm from apoptotic cells by leakage of their damage associated molecular patterns (DAMP) due to secondary necrosis can be abrogated. It is conceivable that excessive apoptosis has pathological consequences on the immune system [94]. Apoptotic cells undergoing secondary necrosis may contribute to the proinflammatory response. In a study by Hotchkiss and colleagues [94], pretreatment of animals with apoptotic splenocytes worsens the outcome in a mouse model of sepsis, pointing out the detrimental effect of accumulated apoptotic cells in the body. Gut I/R induces apoptosis in various cells and decreases apoptotic cell clearance through down-regulation of MFG-E8 at the same time. Accumulated apoptotic cells may undergo secondary necrosis, potentiating lung injury under such a condition. The administration of recombinant MFG-E8 protein enhances apoptotic cell clearance and therefore attenuates lung injury after gut I/R. Thus, MFG-E8 may serve as a novel treatment option for ALI after I/R, or of other etiology, by promoting tissue repair and positively affecting morbidity and mortality in affected patients.

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Conflict of Interest 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. M. Aziz reports no financial conflicts of interest.

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Chapter 10
Role of MFG-E8 in the Brain

Monowar Aziz and Ping Wang

Abstract Neurodegenerative diseases such as Alzheimer's disease (AD), stroke, traumatic brain injury (TBI) attribute to significant morbidity and mortality in young as well as elderly people. Recent studies reveal intriguing features utilizing milk fat globule-epidermal growth factor-factor 8 (MFG-E8) as a potential therapeutic candidate against several brain diseases. Although MFG-E8 discovery, origin, expression, and its physiological functions at different organs and tissues have been well defined in the other chapters of this book, there is still an unmet medical need to clarify its role in the brain. Based on several remarkable findings published from our lab as well as others, the current chapter focuses on the novel roles of MFG-E8 against the development and pathogenesis of brain disorders that leads to generate novel therapeutic potential.

Keywords MFG-E8 • Alzheimer's disease • Cerebral ischemia • MCAO • Prion diseases • Fractalkine

Abbreviations

ABP	Amyloid beta-peptide
AD	Alzheimer's disease
BSE	Bovine spongiform encephalopathy

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CCA	Common carotid artery
CJD	Creutzfeldt-Jakob disease
DCI	Delayed cerebral ischemia
GAS-6	Growth arrest specific gene-6
ICAM-1	Intercellular cell adhesion molecule-1
MCAO	Middle cerebral artery occlusion
MFG-E8	Milk fat globule-epidermal growth factor-factor 8
PPAR-γ	Peroxisome proliferator activated receptor-γ
SAH	Subarachnoid hemorrhage
SLE	Systemic erythematosus lupus
TSE	Transmissible spongiform encephalopathy
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
vCJD	variant Creutzfeldt-Jakob disease

1 Introduction

Milk fat globule-EGF factor 8 (MFG-E8), also known as lactadherin, is a cysteine rich secretory glycoprotein originally identified in mouse milk and mammary epithelium [1]. MFG-E8 is subsequently found to be widely distributed in various tissues in mice and other mammalian species including humans [2]. In the brain, MFG-E8 is expressed in astrocytes, microglia, and microvascular smooth muscle cells [3, 4]. MFG-E8 contains each of these two N-terminal epidermal growth factor (EGF)-like repeats, and C-terminal discoidin domains similar to the blood coagulation factor-V/VIII. MFG-E8 binds $\alpha_v\beta_{3/5}$-integrin heterodimers through an arginine-glycine-aspartate (RGD) motif contained in the second EGF domain. The second factor-V/VIII C-terminal domain of MFG-E8 has high affinity for anionic membrane phospholipids such as phosphatidylserine (PS) which become externalized during apoptosis [5]. Therefore, MFG-E8 has been shown to facilitate phagocytic removal of apoptotic cells by forming a bridge between PS exposed on the apoptotic cell and $\alpha_v\beta_{3/5}$-integrin receptors on phagocytes. This enhanced clearance of apoptotic cells prevents secondary necrosis which could release pro-inflammatory mediators and autoantibodies leading to develop autoimmune disease, such as systemic erythematosus lupus (SLE) [5]. Beside this, MFG-E8 exerts other beneficial functions in tissue injury such as suppression of inflammation and apoptosis in intestinal ischemia, colitis, acute lung injury and sepsis [6–9]. Brain diseases such as Alzheimer's disease (AD), stroke, traumatic brain injury (TBI) attribute to significant morbidity and mortality in young as well as elderly people. It is therefore an imperative need to identify potential therapeutic interventions to overcome the deleterious effects caused by these diseases. Recently, a number of studies have been performed to reveal the protective roles of MFG-E8 in brain diseases, most notably in Alzheimer's disease, cerebral ischemic injury and prion diseases not only by enhancing the phagocytosis of apoptotic neuronal cells, amyloid β-peptide (AβP) and prion fragments, but also by promoting anti-inflammatory and anti-apoptotic

functions through the modulation of intracellular signaling cascades [3, 4, 10]. Based on the recent advancements in the role of MFG-E8 for controlling brain diseases this chapter highlights several novel findings demonstrating potential beneficial roles of MFG-E8 in brain diseases.

2 Clearance of Apoptotic Neurons by Microglia

Apoptotic cells and potentially noxious substances induce neurodegenerative diseases and normal aging processes [11–13]. The efficient removal of apoptotic materials is of utmost importance for protecting the surrounding tissue from damage due to released proteins from dying cells, thus maintaining immune homeostasis. Therefore, it is critical that immune cells are capable of recognizing apoptotic cells so that they may be efficiently removed from the tissues. The mechanics of PS-triggered phagocytosis have been studied in detail in the peripheral immune system, where phagocytes recognize the exposed PS on apoptotic cells either directly through a PS receptor [14] or through opsonized bridging proteins such as MFG-E8 [5] and growth arrest specific gene-6 (GAS-6) [15]. MFG-E8 released by the macrophages, forms a tether between macrophages and apoptotic cells [5] and triggers a conformational change in the integrin receptor that signals the recruitment of the CrkII-DOCK180-Rac1 complex, causing the activation of Rac1 [16]. The result is cytoskeletal reorganization in the macrophage and its transformation into a phagocyte capable of removing the apoptotic cells. However, when interference with this tethering occurs, such as insufficient production of MFG-E8, a proinflammatory state is created. While a significant amount of work supports this mechanism in the peripheral immune system, there is relatively limited number of studies have been done to examine the role for MFG-E8 in the process of apoptotic cell phagocytosis in the brain. Microglia, which are similar to macrophages in the periphery of the body, act as the phagocytic cells in the brain to remove harmful materials and dying cells [17, 18], in which MFG-E8 is shown to play a pivotal role [3]. In an *in vitro* study carried out by Fuller et al. revealed an induction of the release of MFG-E8 in microglial cell line, BV-2 when they were stimulated by apoptotic neuronal cell line, SY5Y [4]. In their study, the authors utilized a co-culture system comprised of the murine BV-2 cells and SY5Y neuroblastoma cells to create the glial-neuronal environment, and then measured MFG-E8 released from the BV-2 cells when co-cultured with either healthy or even dying SY5Y cells. To induce apoptosis in SY5Y cells, SY5Y cultures were exposed to ultraviolet (UV) light prior to co-culture. Apoptotic SY5Y cells resulted in an increased level of MFG-E8 production under this co-cultured environment, indicating that apoptotic cells elicit a robust upregulation in MFG-E8 production and release from a microglial cell line. After they confirmed the increased production of MFG-E8 by microglial cells under co-cultured condition with apoptotic neuronal cells, they further investigated the ability of MFG-E8 to increase the phagocytic capacity of microglia. In this regard, BV-2 cells were untreated or treated with recombinant mouse MFG-E8 or dominant

negative MFG-E8 proteins and observed significantly increased potential in the phagocytosis of the apoptotic neuronal cells as compared to the untreated or mutant MFG-E8-treated condition. The dominant negative MFG-E8 has a point mutation at their RGD sequence, while the C-terminal PS binding domain remain intact. Due to the mutation in RGD, it failed to recognize $\alpha_v\beta_3$-integrin, while there were no difficulties in binding to the PS of apoptotic cells. The addition of excessive dominant negative form of MFG-E8 over floods or masks the PS targets and blocks the binding of normal endogenous MFG-E8 hence impairs phagocytosis. Altogether, these findings support the hypothesis that MFG-E8 binds apoptotic neurons and enables their clearance by microglia through the involvement of potential scavenging receptors, $\alpha_v\beta_3$-integrin and PS. To provide further support for this mechanism, future studies by recruiting primary microglia and neurons will be important.

2.1 Fractalkine Upregulates MFG-E8 Expression in Microglia

Chemokines are a family of small cytokines, or signaling proteins secreted by immune reactive cells. As part of the immune system, they mediate recruitment of leukocytes to the sites of inflammation. There are four families of chemokines, such as, CXC, CC, C and CX3C which possesses four cysteine residues in their conserved locations that are key to forming their 3-dimensional shape [19]. Fractalkine, also known as, neurotactin is the only known member of the CX3C-chemokine family and so is its receptor. Fractalkine is a 95-kDa chemokine expressed by neurons throughout the brain in both membrane-bound and soluble forms [20]. The membrane-bound protein is cleaved to its soluble form following cell stress or injury, which then acts as a chemoattractant for T cells, monocytes, and potentially microglia, hence providing essential innate and adaptive immune functions in brain [21]. Typically, CX3CR1, the fractalkine receptor and other chemokine receptors are expressed by specialized glial cells, such as astrocytes or microglia, the macrophages of the brain, and are upregulated upon injuries or infections [22]. The relatively specific distribution of fractalkine ligand and its receptor raised the interesting hypothesis that upon injury or disease fractalkine may be released from its neuronal site and activate microglia [23, 24]. In a recent study using rat cultured microglial cells upon fractalkine stimulation leads to the upregulation of MFG-E8 expression as revealed by the gene chip analysis [25]. In the periphery, fractalkine stimulates MFG-E8 production by peritoneal macrophages and increases the phagocytosis of apoptotic thymocytes [26]. Likewise, studies report abnormal levels of fractalkine in neurodegenerative diseases such as Alzheimer's disease and acquired immunodeficiency syndrome (AIDS) associated dementia [27, 28], therefore suggesting a potential role for MFG-E8 regulation in the progress of these diseases. Since MFG-E8 exerts beneficial roles in several inflammatory diseases, as an inducer of MFG-E8 expression fractalkine could be a potential therapeutic molecule against neurodegenerative diseases.

3 Role of MFG-E8 in Alzheimer's Disease

Alzheimer's disease is the most common form of dementia. There is no cure for this disease, which worsens as it progresses, and eventually leads to death. It was first described by German psychiatrist and neuropathologist, Alois Alzheimer in 1906 and was named after him [29]. Most often, AD is diagnosed in people over 65 years of age, although the less-prevalent early-onset Alzheimer's can occur much earlier. Worldwide prevalence of AD is estimated to be around 26.6 million people in 2006 [30]. Among them 4.5 million people with AD are from the US populations. By 2050, this number will increase by almost threefold to 13.2 million. Owing to the rapid growth of the oldest age groups of the US population, the number who are 85 years and older will more than quadruple to 8.0 million. The numbers who are 75–84 years old will double to 4.8 million, while the number who are 65–74 years old will remain fairly constant at 0.3–0.5 million [31]. The hallmarks of AD are neuronal loss, extracellular accumulation of senile plaques, the major component of which is the amyloid-β-peptide, and the presence of neurofibrillary tangles. Excess deposition of AβP is involved in neuronal cell death and is thought to result from an imbalance between AβP production, aggregation, and/or clearance. The AβP peptides result from the processing of the AβP-amyloid precursor protein (APP) by the β- and γ-secretases. Pathogenic mutations in the APP and presenilin 1 and 2 genes (involved in the γ-secretase activity) have clearly demonstrated the major role of AβP overproduction in the AD process. Recently reinforcing this role, genetic variability in the ubiquilin gene, which regulates endoproteolysis of presenilin and modulates γ-secretase components, has been associated with familial forms of AD [3, 32].

Recent study has provided a series of data that implicates MFG-E8 in a major process related to the pathophysiology of AD, namely enhancing AβP phagocytosis [3]. Boddaert et al. examined MFG-E8 expression in brain sections of patients with or without AD and studied its role in the phagocytosis of AβP [3]. Quantitative analysis of the level of MFG-E8 showed a dramatic reduction in MFG-E8 mRNA expression in the brains of patients with AD ($n=52$) compared with age-matched controls ($n=58$; $P=0.003$). Interestingly, lactadherin protein was detected in the brains of patients with AD and controls, with low expression in areas rich in senile plaques and marked expression in areas without AβP deposition. Immunohistochemical analysis of the protein also showed reduced expression in AD sections. MFG-E8 expression was mostly detected in astrocytes. Expression was also detected occasionally in CD68-positive cells, and in smooth muscle cells of large arteries [3]. Using surface plasmon resonance, they observed a direct protein-protein interaction between recombinant MFG-E8 and AβP peptide *in vitro*. MFG-E8 deficiency or its neutralization using specific antibodies significantly prevented AβP phagocytosis by murine and human macrophages. Therefore, it is suggested that MFG-E8 plays an important role in the phagocytosis of AβP, and its expression is reduced in Alzheimer's disease, which contributes to the initiation and/or progression of Alzheimer's disease.

4 Cerebral Ischemia

Cerebral ischemia, also known as, brain ischemia, occurs as a result of insufficient blood flow into the brain to meet metabolic demand, leading to the energy crisis, cerebral hypoxia, and death of brain tissues which is referred to as cerebral infarction [33]. It is a sub-type of stroke along with subarachnoid hemorrhage and intracerebral hemorrhage. There are two types of ischemia: focal ischemia and global ischemia. The focal brain ischemia occurs when a blood clot has occluded a cerebral vessel, which causes reduced blood flow to a specific region in the brain, increasing the risk of cell death to that particular area. It can be either caused by thrombosis or embolism. On the other hand, the global brain ischemia occurs when blood flow to the brain is halted or drastically reduced due to cardiac arrest. If sufficient circulation is restored within a short period of time, symptoms may be transient. However, if a significant amount of time passes before restoration, brain damage may be permanent. Although reperfusion may be essential to protect as much brain tissue as possible, it may also lead to reperfusion-mediated injury. Thus, depending on its cause brain ischemia can be further subdivided into thrombotic, embolic, and hypoperfusion, where thrombotic and embolic are generally focal or multifocal in nature while hypoperfusion affects the brain globally. The main symptoms involve impairments in vision, body movement, and speaking. Other effects that may result from brain ischemia are stroke, cardiorespiratory arrest, and irreversible brain damage.

During cerebral ischemia, the brain cannot perform aerobic metabolism due to the loss of oxygen and substrates, resulting in the drop of adenosine triphosphate (ATP) rapidly, which even approaches zero within a few minutes. Therefore, in the absence of biochemical energy, the cells begin to lose the ability to maintain electrochemical gradients. Consequently, there is a massive influx of calcium into the cytosol, a massive release of glutamate from synaptic vesicles, lipolysis, calpain activation, and the arrest of protein synthesis. Additionally, an abnormality in the removal of metabolic wastes can also be noticed in cerebral ischemia [33]. Anticoagulation with warfarin or heparin may be used if the patient has atrial fibrillation. Operative procedures such as carotid endarterectomy and carotid stenting may be performed if the patient has a significant amount of plaque in the carotid arteries associated with the local ischemic events. However, recent research for the treatment of delayed cerebral ischemia (DCI) is a common and serious complication following subarachnoid hemorrhage (SAH) after ruptured cerebral aneurismal. Although a good number of pharmacological agents are being utilized which include, calcium channel blockers, Tirilazad, Statins, magnesium sulfate, endothelin-1 antagonist, Fasudil, antiplatelet therapy, Enoxaparin, albumin, erythropoietin, intracisternal thrombolytics, the morbidity and mortality are not improved noticeably [34]. Therefore, the novel therapeutic potentials are always challenging to overcome the adverse effects of cerebral ischemia.

4.1 Animal Model of Cerebral Ischemic Injury

In rodents, the permanent focal cerebral ischemia is induced by middle cerebral artery occlusion (MCAO) [35, 36]. In order to create cerebral ischemia, rats are fasted overnight with water ad libitum. Anesthesia is imposed in experimental rats with 3.5 % isoflurane and subsequent maintenance by intravenous boluses of pentobarbital, not exceeding 30 mg/kg BW. Body temperature should be maintained between 36.5 and 37.5 °C using a rectal temperature probe and a heating pad. The right common carotid artery (CCA) that is exposed upon ventral midline neck incision is carefully dissected free from the vagus nerve. The external carotid artery is then dissected and ligated. The internal carotid artery (ICA) is isolated and carefully separated from the adjacent vagus nerve, and the pterygopalatine artery is then dissected and temporally occluded with a microvascular clip. Next, the CCA is ligated and an arteriotomy made just proximal to the bifurcation. A 2.5 cm length of 3–0 poly-L-lysine coated monofilament nylon suture with a rounded tip is inserted through the arteriotomy into the ICA and advanced to the middle cerebral artery (MCA) origin to cause occlusion. Occlusion of the MCA is ascertained by inserting the suture to a pre-determined length of 19–20 mm from the carotid bifurcation and feeling for resistance as the rounded suture tip approaches the proximal anterior cerebral artery which is of a relatively narrower caliber. The cervical wound is then closed in layers. One hour post-MCAO each rat should be given an infusion of 1 ml saline as vehicle or confined doses of the therapeutic regimens for treatment. Rats are then allowed to recover from anesthesia in a warm and quiet environment. The intraluminal suture is left in-situ and the rats allowed unrestricted access to food and water. Rats are sacrificed at different time points after MCAO for the assessment of pathophysiology and therapeutic potential of particular drug candidates. The Bederson neurological deficit scoring scale (no deficit ¼ 0, forelimb flexion ¼ 1, decreased resistance to push ¼ 2, circling ¼ 3) can be used to screen rats at 24 h post-operatively for successful MCAO. Rats with a Bederson score of at least 3 are considered to have successful MCAO-induced stroke [37]. On the other hand the rats with lesser Bederson scores should be replaced. Afterwards, the brain tissues are rapidly collected for various analyses. For histopathology, immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), rat brains are transcardially perfused with ice-cold normal saline followed by 4 % paraformaldehyde before removal. Brain samples are then paraffin-embedded and sectioned. Brain tissues for mRNA and protein analysis should be kept in liquid nitrogen until their use.

4.2 MFG-E8 Attenuates Cerebral Ischemic Injury

Promotion of apoptotic cell clearance has been shown to be beneficial in various brain diseases. Therefore, the identification of factors which are essential for the clearance of dead cells are promising and extensively highlighted in recent studies.

The macrophage scavenger receptor A (CD204), which acts similar to MFG-E8 by promoting macrophage clearance of apoptotic cells, has been shown to be neuroprotective in focal cerebral ischemia [38]. Considering this fact, recently the effects of MFG-E8 on cerebral ischemia have been investigated by Cletus Cheuyo and Ping Wang [35]. During permanent focal cerebral ischemia induced by MCAO in mice brain resulted in decreased expression of MFG-E8 at the cerebral tissues at its protein levels. However, they found that the gene expression at its mRNA level of MFG-E8 was not affected by permanent cerebral ischemia. Therefore, it can be suggested that the MFG-E8 protein levels may be post-translationally reduced by proteasomal degradation since MFG-E8 has been shown to undergo proteasomal degradation [39], and proteasome is activated during cerebral ischemia [40]. After MCAO induced cerebral ischemia the animals developed sensorimotor and vestibulomotor deficits; however, the administration of recombinant human MFG-E8 (rhMFG-E8) through a right femoral vein catheter post-MCAO considerably improved left forelimb sensorimotor deficit, measured by the limb placing test. The treatment of recombinant human MFG-E8 also decreased left hindlimb sensorimotor deficit as compared to the non-treated animals. Similarly, vestibulomotor deficit, measured by beam balance test, was significantly reduced by rhMFG-E8 treatment over that of vehicle mice. With rhMFG-E8 treatment in experimental cerebral ischemic mice the infract size as measured by triphenyl tetrazolium chloride (TTC) stain became decreased than the vehicles (Fig. 10.1). Twenty-four hours of cerebral ischemia by MCAO resulted in profound neuronal necrosis in the ischemic core and the penumbra, appearing as eosinophilic neurons on hematoxylineeosin staining. Treatment with rhMFG-E8 protected neurons from necrosis, preserving significantly higher amount of intact basophilic neurons in the penumbra compared to the vehicle. Apart from that, cerebral ischemia caused an upregulation of the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) which are known to cause exaggerated inflammation and tissue damage. On the other hand, the mice treated with rhMFG-E8 significantly downregulated the expression of those pro-inflammatory mediators. Inflammation and apoptosis play crucial roles in tissue damage during cerebral ischemia. In the permanent animal model of cerebral ischemia, IL-6 secretion has been shown to peak at 24 h after cerebral ischemia onset [41]. Cheuyo et al. demonstrated that the anti-inflammatory effects of rhMFG-E8 treatment in cerebral ischemia included suppression of cytokine (IL-6 and TNF-α) release, intercellular cell adhesion molecule-1 (ICAM-1) expression, and cerebral neutrophil infiltration at 24 h after permanent cerebral ischemia. The anti-inflammatory suppression of cytokine release was likely due to the effect of rhMFG-E8 on microglia cells, the resident macrophages in the brain. Cheyuo et al. confirmed this hypothesis by demonstrating that rhMFG-E8 suppressed IL-6 production during BV2 microglia oxygeneglucose deprivation [35]. The exact signaling mechanism of rhMFG-E8-mediated cytokine downregulation is unknown. Upregulation of the ligand-inducible transcription factor, PPAR-γ, could be responsible for the inhibition of cytokine release following treatment with rhMFG-E8. The authors found that focal cerebral ischemia by MCAO suppressed PPAR-γ levels. Treatment with rhMFG-E8 attenuated the ischemia-induced downregulation of PPAR-γ compared

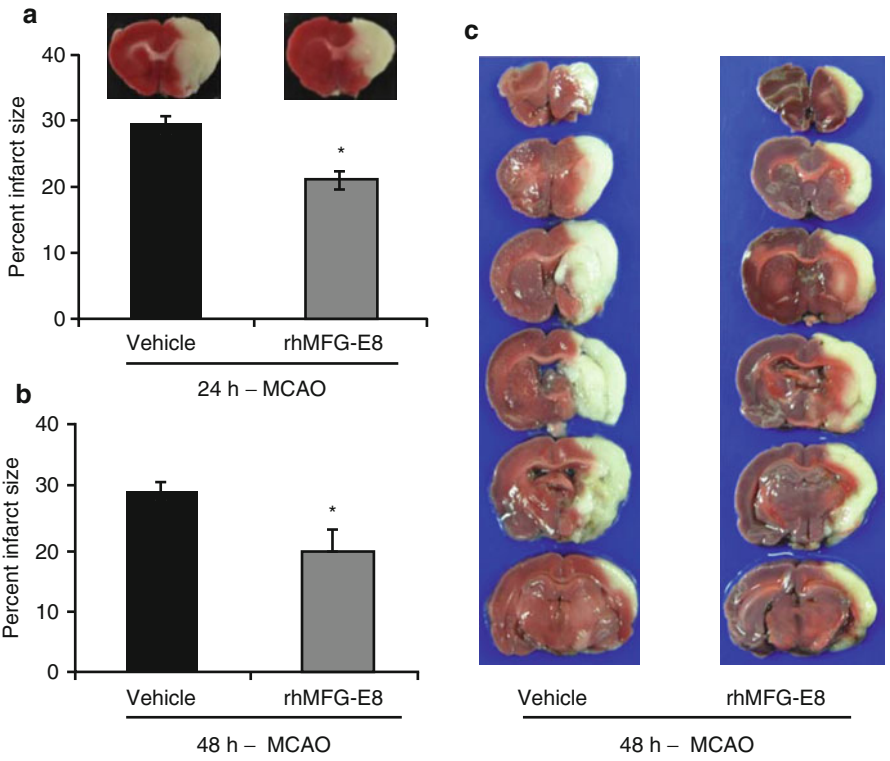

Fig. 10.1 The effect of rhMFG-E8 on infarct size after 24–48 h permanent focal cerebral ischemia. Two millimeter thick coronal slices of fresh brain tissue were stained with TTC and digitally analyzed with NIH ImageJ software. Data are presented as mean ± SE, and analyzed by Student's t-test. (**a**) Treatment with rhMFG-E8 decreased infarct size at 24 h post-MCAO compared with Vehicle (n ¼ 6, *p < 0.05 *vs.* Vehicle). (**b**) rhMFG-E8 treatment decreased infarct size at 48 h post-MCAO compared with Vehicle (n ¼ 6, *p < 0.05 *vs.* Vehicle). (**c**) Representative TTC staining of rostro-caudally sectioned rat brains at 48 h post-MCAO shows that the vehicle grouped developed larger cerebral infarcts (Reprinted from Cheyuo et al. [35]. 2012 with permission from Elsevier)

with vehicle. PPAR-γ is known to suppress NF-κB mediated cytokine production through a variety of mechanisms collectively termed transrepression [42]. PPAR-γ agonists such as the thiazolidinediones have shown neuroprotection in cerebral ischemia [43]. The novel finding of rhMFG-E8-mediated upregulation of PPAR-γ together with suppression of cytokine release is consistent with the work of Zhang et al. who showed that the PPAR-γ agonist, pioglitazone, suppresses NF-kB signaling in permanent focal cerebral ischemia, resulting in neuroprotection [36]. Cheyuo et al. also demonstrated that rhMFG-E8 treatment inhibited apoptosis in cerebral ischemia by downregulating cleaved caspase-3 and bax, and upregulating bcl-2 and bcl-2/bax ratio. Based on their study, they found that bcl-2/bax regulation in permanent focal cerebral ischemia was time-dependent, with a significant decrease in bcl-2/bax ratio in the vehicle group at 48 h post-MCAO compared with sham,

in contrast with an earlier time point of 24 h post-MCAO when there was no difference between bcl-2/bax ratio in sham and vehicle group. rhMFG-E8 treatment had an early effect on bcl-2/bax regulation resulting in a persistent significantly increased bcl-2/bax ratio at both 24 h and 48 h after the onset of cerebral ischemia. The MFG-E8 receptor, $\alpha_v\beta_3$-integrin, has been shown to increase Bcl-2 transcription through a focal adhesion kinase (FAK)-dependent activation of the PI3K-Akt pathway [44]. The anti-apoptotic effect of rhMFG-E8 may further be explained by the upregulation of PPAR-γ. Wu et al. showed that PPAR-γ overexpression inhibited apoptosis in cerebral ischemia. Knockdown of PPAR-γ using small interfering RNA abrogated the anti-apoptotic effects of PPAR-γ [45]. The CX3X chemokine, fractalkine, stimulates the release of MFG-E8 from microglial cells [25]. The neuroprotective effects ofMFG-E8 under conditions of hypoxia/ischemia are further supported by *in vitro* studies by other investigators using fractalkine. Noda et al. showed that fractalkine decreased excitotoxicity by stimulating microglial clearance of apoptotic neurons. Treatment of the microglial cells with anti-MFG-E8 neutralizing antibodies abolished the microglial clearance of necrotic neurons and diminished the neuroprotection [46]. Similarly, Kranich et al. also showed that MFG-E8 protects against neurotoxicity in a model of prion disease [10]. In summary, they have shown in this first assessment of the role of rhMFG-E8 in cerebral ischemia that endogenous brain MFG-E8 levels are post-translationally decreased by cerebral ischemia. Exogenous rhMFG-E8, administered intravenously 1 h post cerebral ischemia onset, improved neurological function by suppressing inflammation and apoptosis at the 24–48 h time points after permanent focal cerebral ischemia onset, without altering the hemodynamic status of animals. In accordance with the recommendations of the Stroke Academic and Industrial Roundtable (STAIR) recommendations [47], the effects of rhMFG-E8 need to be further assessed independently in the transient focal cerebral ischemia model which includes the additional pathophysiology of reperfusion injury.

5 Prion Diseases

Prions are the agents of transmissible spongiform encephalopathy (TSE), with unconventional properties, such as resistance to high temperatures, high pressures, formaldehyde treatment or UV-irradiation [48]. The term ‘prion’ does not have any structural implications other than that a protein is an essential component of the transmissible agent. “PrPC” is the naturally occurring cellular prion protein encoded by the Prnp gene. PrPC is expressed on cells of the central nervous system and on cells of the immune system. In a given cell type PrPC is necessary, but not sufficient for the replication of prions. Alternatively, the “PrPSc” is an abnormal isoform of the PrPC protein and is found in the tissues of TSE patients. The isoform is partially resistant to digestion by proteinase K, is believed to be conformationally distinct from PrPC and is considered to be the main component of the transmissible agent [48, 49]. Prions are primarily distinguished from the cellular prion protein by their three-dimensional structure. The cellular prion protein is predominantly composed

of the α-helix structure and is almost devoid of β-sheet, whereas about 43 % of scrapie prions are composed of β-sheet [49]. Many functions have been attributed to PrPC, including immunoregulation, signal transduction, copper binding, synaptic transmission, induction of apoptosis or protection against apoptosis and many others [49]. However, regardless of its physiological function, the conversion of PrPC to PrPSc culminates in neurodegeneration. Prion infections cause characteristic lesions in the CNS with spongiform vacuolation, accumulation of abnormal PrPSc, neuronal cell loss, microglial activation and proliferation of astrocytes. Neuronal cell loss, microglial activation and proliferation of astrocytes occur in many neurodegenerative diseases, but are particularly evident in prion diseases [48]. In addition, prions colonize the lymphoid compartment of infected organisms. Following intracerebral (ic), oral or intraperitoneal (ip) inoculation, prion replication occurs in many sites of the LRS, including the spleen, lymph nodes, Peyer's patches (PPs) and tonsils [48]. Prion diseases, also known as TSEs, are a group of animal and human brain diseases that are uniformly fatal and often characterized by a multifocal neuropathologic picture of neuronal loss, spongiform changes, and astrogliosis [48]. In human the prion diseases include: Creutzfeldt-Jakob disease (CJD), Variant Creutzfeldt-Jakob disease (vCJD), Gerstmann-Straussler-Scheinker Syndrome, and Fatal Familial Insomnia [50]. Prion diseases attracted much attention and public concern after an outbreak of bovine spongiform encephalopathy (BSE) occurred among cattle in many European countries and scientific evidence indicated the food borne transmission of BSE to humans [51].

6 MFG-E8 Amiliorates Prion Diseases

The molecular mechanisms underlying brain damage in prion diseases are not well understood. Upon prion infection, neurons may experience progressive damage and ultimately undergo apoptosis; during this process, they may secrete apoptotic PS-coated bodies containing prions [52, 53]. However, the inhibition of apoptosis by overexpressing anti-apoptotic gene, Bcl-2 or ablating Bax does not affect the life expectancy of prion-inoculated mice [54]. The prion-infected brain cells may release membrane fragments even when undergoing nonapoptotic death. Furthermore, exosomes may be released by perfectly healthy cells [55] and may conceivably carry prion infectivity. Hence, it is worthy of removing the PS coated apoptotic bodies to safeguard brains from inflammation and injuries. By virtue of its affinity to PS, MFG-E8 helps mediating the removal of apoptotic bodies [5]. Phagocytic cells then bind MFG-E8-opsonized apoptotic cells through $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins [5]. A recent study described a potential involvement of MFG-E8 expressed by human astrocytes, microglia, and smooth muscle cells in the removal of amyloid β plaques [3]. A microarray screen also identified MFG-E8 expression in mouse astrocytes [56]. Another study claimed MFG-E8 expression *in vitro* by the microglial cell line BV-2 [4]. Based on this concept, Kranich et al. suggest that astrocyte-derived MFG-E8 opsonizes these prion-laden apoptotic bodies by binding to PS exposed on their surface, which are then cleared by the phagocytic cells in the

CNS that express the MFG-E8 receptors, $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrin heterodimers [10]. Because microglia antagonize prion replication [57], it appears plausible that MFG-E8-opsonized, prion-laden apoptotic bodies are taken up and degraded by microglia. Because clearance of apoptotic bodies is inefficient in the absence of MFG-E8, prions may persist in the brain and exacerbate PrPSc accumulation, thereby accelerating the disease. Therefore, this novel innovation leads to develop an efficient therapeutic potential against prion diseases utilizing MFG-E8.

7 Concluding Remarks

Herein, based on the recent studies, we describe multiple mechanistic approaches of MFG-E8 in terms of maintaining normal brain functioning. During Alzheimer's disease MFG-E8 not only performs the removal of the apoptotic neuronal cells by the microglia, but also inhibits excessive accumulation of AβP in the brain. Further work on the mechanism of MFG-E8 suppression in AD may uncover potential therapeutic strategies for the treatment of AD and other neurodegenerative diseases. However, it remains to be established that MFG-E8 deficiency directly leads or contributes to Alzheimer's neuropathology in experimental models of the disease. Future studies should examine the effect of MFG-E8 deficiency on the accumulation of senile plaques in animal models of AD. Nevertheless, we believe that the data presented here point to a potentially important and new role for lactadherin in AD and should pave the way for future studies aiming at the comprehension of the precise mechanisms involved in these processes. Furthermore, identification of fractalkine as the novel positive regulator of MFG-E8 in brain leads to develop a new therapeutic target against neuropathogenesis by accelerating MFG-E8 expression. In permanent cerebral ischemia as mediated by the occlusion of common carotid arteries, MFG-E8 exerts beneficial roles by removing apoptotic cells, protecting secondary necrosis, upregulating anti-apoptotic genes and attenuating excessive injury and inflammation via utilizing a novel PPARγ-dependent pathway. The regulation of phagocytosis is complex, and several independent, redundant pathways of phagocytosis are known. MFG-E8 may not be the only mediator of engulfment, and some hitherto unidentified factors may, in certain situations, compensate for its absence. However, identification of several novel properties of MFG-E8 other than the phagocytosis of apoptotic cells comes from the observation in the acceleration of prion pathogenesis seen in MFG-E8 deficient mice where an abnormal accumulation of disease causing prion proteins are noticed. These outstanding achievements utilizing the previously unexplored roles of MFG-E8 contribute to the efficient remedial against fetal brain diseases and improve quality of life expectancies.

Conflict of Interest Ping Wang is an inventor of the pending PCT application #WO/2006/122327: "Milk fat globule epidermal growth factor-factor VIII and sepsis" and PCT application #WO/2009/064448: "Prevention and treatment of inflammation and organ injury after ischemia/reperfusion using MFG-E8". These patent applications cover the fundamental concept of using MFG-E8 for the treatment of sepsis and ischemia/reperfusion injury.

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