Milk Fat Globule-Epidermal Growth Factor-Factor 8 Attenuates Neutrophil Infiltration in Acute Lung Injury via Modulation of CXCR2

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Excessive neutrophil infiltration to the lungs is a hallmark of acute lung injury (ALI). Milk fat globule epidermal growth factor-factor 8 (MFG-E8) was originally identified for phagocytosis of apoptotic cells. Subsequent studies revealed its diverse cellular functions. However, whether MFG-E8 can regulate neutrophil function to alleviate inflammation is unknown. We therefore aimed to reveal MFG-E8 roles in regulating lung neutrophil infiltration during ALI. To induce ALI, C57BL/6J wild-type (WT) and Mfge8<sup>−/−</sup> mice were intratracheally injected with LPS (5 mg/kg). Lung tissue damage was assessed by histology, and the neutrophils were counted by a hemacytometer. Apoptotic cells in lungs were determined by TUNEL, whereas caspase-3 and myeloperoxidase activities were assessed spectrophotometrically. CXCR2 and G protein-coupled receptor kinase 2 expressions in neutrophils were measured by flow cytometry. Following LPS challenge, Mfge8<sup>−/−</sup> mice exhibited extensive lung damage due to exaggerated infiltration of neutrophils and production of TNF-α, MIP-2, and myeloperoxidase. An increased number of apoptotic cells was trapped into the lungs of Mfge8<sup>−/−</sup> mice compared with WT mice, which may be due to insufficient phagocytosis of apoptotic cells or increased occurrence of apoptosis through the activation of caspase-3. In vitro studies using MIP-2-mediated chemotaxis revealed higher migration of neutrophils of Mfge8<sup>−/−</sup> mice than those of WT mice via increased surface exposures to CXCR2. Administration of recombinant murine MFG-E8 reduces neutrophil migration through upregulation of GRK2 and downregulation of surface CXCR2 expression. Conversely, these effects could be blocked by anti-α<i>c</i>, integrin Abs. These studies clearly indicate the importance of MFG-E8 in ameliorating neutrophil infiltration and suggest MFG-E8 as a novel therapeutic potential for ALI. The Journal of Immunology, 2012, 189: 393–402.

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Abbreviations used in this article: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; ALI, acute lung injury; AMC, 7-amino-4-methylcoumarin; ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; BMDN, bone marrow-derived neutrophil; EGF, epidermal growth factor; GRK2, G protein-coupled receptor kinase 2; IL-8, interleukin-8 homolog; MPO, myeloperoxidase; Mfge8, milk fat globule-epidermal growth factor-factor 8; PI, propidium iodide; RGD, arginine-glycine-aspartate; rm, recombinant murine; WT, wild-type.

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M.A. and A.M. contributed equally to this work.

M.A., A.M., W.-L.Y., and P.W. conceived and designed the experiments; M.A., A.M., W.-L.Y., and A.J. analyzed the data; M.A., A.M., W.-L.Y., and A.J. wrote the paper; and P.W. supervised the project. All authors read and approved the final manuscript.
MFG-E8 attenuates neutrophil infiltration

Experimental model
Male (25–30 g) age-matched wild-type C57Bl/6J (Taconic, Albany, NY) and Mfge8−/− mice (a gift of Dr. Shigekazu Nagata, Kyoto University, Kyoto, Japan) were anesthetized with isoflurane and then instilled with 40 μl sterile saline (PBS) without or with 5 mg/kg body weight LPS (Escherichia coli serotype O55:B5; Sigma-Aldrich, St. Louis, MO) via intratracheal (i.t.) injection using a 28-gauge U-100 insulin syringe. Although the present model of LPS-induced ALI is nonlethal, it can significantly induce lung injury. The rmMFG-E8 (R&D Systems, Minneapolis, MN) was administered i.p. at 20 μg/kg body weight dose 2 h before i.t. LPS instillation. The protocol was approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research.

Lung tissue histology
Lung tissues were fixed in 10% formalin and embedded in paraffin. Tissue blocks were sectioned at a thickness of 5 μm and stained with H&E. Morphological changes were scored by an independent pathologist as absent (0), mild (+1), moderate (+2), or severe injury (+3) based on the presence of exudates, hyperemia/congestion, neutrophil infiltrates, intra-alveolar hemorrhage/debris, and cellular hyperplasia (20). The sum of scores of different animals was averaged.

In situ TUNEL assay. DNA breaks occur late in the apoptotic pathway and can be determined by performing the TUNEL assay. The presence of apoptotic cells in lung tissues was determined using a TUNEL staining kit (Roche Diagnostics, Indianapolis, IN). Briefly, lung tissues were fixed in 10% phosphate-buffered formalin and were then embedded in paraffin and sectioned at 5 μm following standard histology procedures. Lung sections were dewaxed, rehydrated, and equilibrated in TBS. The sections were then digested with 20 μg/ml proteinase K for 20 min at room temperature. Following this, the sections were washed and incubated with a mixture containing TdT and fluorescence-labeled nucleotides and examined under a fluorescence microscope (Nikon Eclipse Ti-S, Melville, NY).

Caspase-3 enzyme activity assay. The caspase-3 activity in lung tissues was assessed using a fluorometric assay kit (Sigma-Aldrich), which is based on the principles of hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety. In brief, lung tissues were homogenized in liquid nitrogen, and equal weights of powdered tissues (~30 mg) were dissolved in 500 μl lysis buffer (10 mM HEPES [pH 7.4], 5 mM MgCl2, 1 mM DTT, 1% Triton X-100, and 2 mM each EDTA and EGTA) and subjected to sonication on ice. Protein concentration was determined by a protein assay reagent (Bio-Rad, Hercules, CA). Equal amounts of proteins in a 5 μl vol were added to the 100 μl assay buffer (20 mM HEPES [pH 7.4], 5 mM DTT, 2 mM EDTA, and 0.1% CHAPS) containing 10 μM Ac-DEVD-AMC substrate and the changes of fluorescence intensity with time at 37°C were measured at excitation (370 nm) and emission (450 nm) in a fluorometer (Synergy H1; BioTek, Winooski, VT). A standard curve was generated using various concentrations of AMC as standard. The results are expressed as nmol AMC/min/mg protein.

Isolation of BALF and cell counts
Mice were euthanized and the trachea was cannulated using a PE50 catheter. PBS (1 ml) was infused into the lungs to collect the lavage fluid five times. The number of total cells in BALF was counted with a hemacytometer. To identify cell population, BALF aliquots were subjected to cytopsin and stained with a Differential Quik Stain Kit (Polysciences, Warrington, PA). Alternatively, the percentage of neutrophils in BALF was determined by gating with cell size and positive staining of Abs allophycocyanin-Ly6G and FITC-CD11b (BD Biosciences, San Jose, CA) in flow cytometric analysis.

Isolation of bone marrow-derived neutrophils
Bone marrow-derived neutrophils (BMDNs) were isolated as described by Boxio et al. (28). Mice were euthanized and femurs from both hind legs were removed. The distal tip of each edge was cut off and bone marrow cells were isolated by flushing the femur with HBSS. Cell suspensions were filtered through a nylon membrane and centrifuged at 1000 rpm for 10 min. Cell pellets were resuspended in HBSS and laid on a three-layer Percoll gradient of 78, 69, and 52% (Amersham Pharmacia, Uppsala, Sweden), followed by centrifugation at 3000 rpm for 30 min without braking. Cells at the 69/78% interface were carefully removed and washed with cold PBS. The identification and purity of isolated BMDNs, which were stained with Abs allophycocyanin-Ly6G and FITC-CD11b, were examined by flow cytometric analysis.

MPO staining and activity assay
Paraffin-embedded lung tissue sections were incubated with rabbit anti-MPO Abs (Abcam, Cambridge, MA), followed by incubation with biotinylated anti-rabbit IgG. Staining was developed by Vectastain ABC reagent and a diaminobenzidine kit (Vector Laboratories, Burlingame, CA). For the negative control, the primary Ab was substituted with normal rabbit IgG. To determine MPO activity, tissues were homogenized in KPO4 buffer containing 0.5% hexa-decyl-trimethyl-ammonium bromide and incubated at 60°C for 2 h, followed by centrifugation. The supernatant was diluted in a

Materials and Methods

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reaction solution, and ΔOD was measured at 460 nm to calculate MPO activity.

Quantitative real-time PCR analysis
Total RNA was extracted from lung tissues by TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA using murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). A PCR reaction was carried out in a 25 μl final volume containing 0.08 μmol each forward and reverse primer, cDNA, and 12.5 μl SYBR Green PCR Master mix (Applied Biosystems). Amplification was conducted in an Applied Biosystems 7300 real-time PCR machine under the thermal profile of 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The level of mouse β-actin mRNA was used for normalization. Relative expression of mRNA was calculated by the $2^{-ΔΔCt}$ method, and results were expressed as fold change in comparison with control group. The sequence of primers used for this study are: TNF-α (NM_013695), forward, 5'-AGACCTCTACACCTAGATCCTCTC-3', reverse, 5'-TGTCACTACGCTGGTCACTACA-3'; MIP-2 (NM_001190), forward, 5'-CATCCAGAGCTTGATGTTGA-3', reverse, 5'-CCTTTGGTTCTTCCGTAGG-3'; MFG-E8 (NM_008594), forward, 5'-CGGGCAAGACAGACTGACATC-3', reverse, 5'-TCTCTCATGTCATTGTCACACAG-3'; and β-actin (NM_007393), forward, 5'-CTGTTAGGTTGA-3', reverse, 5'-TGGTACGACCCAGAGGCATACAG-3'.

Measurements of TNF-α and MIP-2 proteins
Levels of TNF-α and MIP-2 in the lung tissues, BALF, and cell culture supernatants were measured using an ELISA kit specific for mouse TNF-α (BD Biosciences) and MIP-2 (R&D Systems). The assay was carried out according to the instructions provided by the manufacturers.

Western blot analysis
Lung tissues were homogenized and lysed in RIPA buffer (10 mM Tris-HCl [pH 7.5], 120 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN). Protein concentration was determined by a Bio-Rad protein assay reagent. Lysates were electrophoresed on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in TBST buffer (0.1% Tween 20, 20 mM Tris-HCl [pH 7.5], and 140 mM NaCl) and incubated with primary Ab against MFG-E8 (MBL International, Nagoya, Japan), followed by secondary Ab-HRP conjugate and detected using chemiluminescence (GE Healthcare, Buckinghamshire, U.K.) and autoradiography. The immunoblot was reprobed with anti-β-actin Abs as loading control.

Flow cytometric analysis
To examine surface CXCR2 expression, BMDNs with various treatments were stained with propidium iodide (PI) and Abs FITC-CD11b, allophycocyanin-Ly6G, and PerCP/Cy5.5-CXCR2 (BioLegend, San Diego, CA) and subjected to FACS Calibur (BD Biosciences). CXCR2 levels were analyzed in the cell population with PI staining with PE-GRK2 Abs (Abcam). After washing, the stained cells were fixed and permeabilized with IntraPrep (Beckman Coulter, Fullerton, CA), followed by staining with PE-GRK2 Abs (Abcam). Flow cytometry was performed using a FlowJo software (Tree Star, Ashland, OR) with 15,000 events per sample. Isotype controls were used for all the samples.

In vitro neutrophil migration assay
The migration assays were conducted in a modified 24-well (3.0 μm) Boyden chamber (BD Biosciences). Cells (3 × 10^5) were plated in the upper well, and medium with 1 ng/ml rmMIP-2 (R&D Systems) was placed in the lower well as a chemotactic stimulus. After 2 h incubation, the upper surface of the filter was swabbed with cotton to remove non-migratory cells. Migrated cells were fixed with 10% formalin and stained with PI. Five random microscopic fields per well were counted. For blocking experiments, cells were incubated with rmMFG-E8, anti-MFG-E8 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-α, neutralizing Abs (EMD Biosciences, La Jolla, CA) for 2 h prior to plating.

Statistical analysis
All data are expressed as means ± SE and compared by one-way ANOVA and a Student–Newman–Keuls test. A Student t test was used when only two groups were compared. Differences in values were considered significant with p < 0.05.

Results
MFG-E8 deficiency augments pulmonary inflammation and injury induced by LPS
To identify whether MFG-E8 played a role in ALL, we compared the inflammatory parameters between WT and Mfge8−/− mice subjected to i.t. injection of LPS. Within 4 h after LPS instillation, robust induction of TNF-α was noted in the lungs of WT and Mfge8−/− mice (Fig. 1A, 1B). However, the TNF-α mRNA and protein levels in Mfge8−/− mice after 4 h LPS treatment were found to be 1.50- and 1.45-fold higher, respectively, than those in WT mice. Similar trends were also noted at 24 h after LPS exposure, where the Mfge8−/− mice showed 1.62- and 1.86-fold higher amounts of TNF-α mRNA and proteins, respectively, than did WT mice (p < 0.05). Moreover, the TNF-α levels in the BALF collected from Mfge8−/− mice were significantly higher than those from WT mice at 4 and 24 h (Fig. 1C). The histological images of the lung tissues at 24 h after LPS instillation represented increased alveolar congestion, exudates, interstitial and alveolar neutrophilic infiltrates, intra-alveolar capillary hemorrhages, and extensive damage of epithelial architecture in Mfge8−/− mice as compared with the WT counterparts (Fig. 2A). These changes were reflected in a higher lung tissue injury score in Mfge8−/− mice than in WT mice (10.9 versus 7.6; p < 0.05; Fig. 2B).

MFG-E8 deficiency leads to increased neutrophil infiltration to the lungs
To further validate the neutrophil infiltration as observed in the histological analysis (Fig. 2), we first carried out qualitative assessment of MPO, a marker of infiltrating granulocytes in lung...
tissues, by immunostaining, which revealed a stronger intensity of MPO staining in Mfge8<sup>−/−</sup> mice than that in WT mice (Fig. 3A). After quantification by MPO activity assay, we noticed that its activity in Mfge8<sup>−/−</sup> mice was 1.85-fold higher than that in WT mice after 24 h LPS instillation (Fig. 3B). We next analyzed the cells isolated from BALF, which revealed no significant increase in their numbers in either WT or Mfge8<sup>−/−</sup> mice at 4 h after LPS instillation (Fig. 3C). However, at 24 h, the numbers of total cell counts from WT mice reached 3.1 ± 0.27 × 10<sup>6</sup> cells, whereas it was 5.8 ± 0.45 × 10<sup>6</sup> cells in Mfge8<sup>−/−</sup> mice (<i>p</i> < 0.05; Fig. 3C). The major cell type in BALF of sham mice was alveolar macrophages, whereas neutrophils were predominantly found in both WT and Mfge8<sup>−/−</sup> mice after ALI (Fig. 3D). Consistent with the cytospin results, BALF isolated from WT and Mfge8<sup>−/−</sup> mice after 24 h LPS instillation contained ~80% neutrophils as determined by flow cytometry (data not shown). The number of neutrophil counts in Mfge8<sup>−/−</sup> mice was 2.0-fold higher than that in WT mice at 24 h after LPS instillation (Fig. 3E). Furthermore, we detected a significant increase in total protein levels in BALF, which emerged to be comparatively higher in Mfge8<sup>−/−</sup> mice than in WT mice with 24 h ALI (16.4 ± 0.86 versus 11.6 ± 0.85 mg/ml; <i>p</i> < 0.05; Fig. 3F).

**Accumulation of apoptotic cells in lungs after ALI**

MFG-E8 was initially identified as a factor for engulfment of apoptotic cells by professional phagocytes, and its deficiency led to the development of autoimmune disease (16, 27). In this study, we carried out TUNEL assay in lung tissues, which revealed a significant increase in the number of apoptotic cells in Mfge8<sup>−/−</sup> mice as compared with WT mice after ALI (Fig. 4A, 4B), reflecting inadequate clearance of apoptotic cells by the phagocytes. Additionally, following ALI, we noticed increased activation of caspase-3 in the lungs of Mfge8<sup>−/−</sup> mice, which led to a greater increase in cellular apoptosis in Mfge8<sup>−/−</sup> mice than in WT counterparts (Fig. 4C). Collectively, these findings demonstrated that the higher amounts of apoptotic cells in the lungs of Mfge8<sup>−/−</sup> mice were due to reduced phagocytosis and/or an increased rate of apoptosis mediated by the activation of caspase-3.

**Supplement of rmMFG-E8 attenuates neutrophil infiltration to the lungs during ALI**

At 4 h after LPS instillation, MFG-E8 mRNA and protein levels in the lungs decreased by 42 and 57%, respectively, compared with the WT sham controls (Fig. 5). Although at 24 h MFG-E8 expression was rebounded, it was still significantly lower than the WT sham controls (Fig. 5). Considering this fact, we sought to determine the effect of rmMFG-E8 pretreatment to WT mice prior to induction of ALI as a therapeutic regimen to salvage the deficits of endogenous MFG-E8 that occur during ALI. Interestingly, the numbers of total cells and neutrophil counts in BALF of rmMFG-E8–pretreated WT mice were significantly lower than those in WT mice without rmMFG-E8 treatment after LPS instillation (Fig. 6). Based on this finding, we proposed that the excess in neutrophil infiltration into the lungs was due to decreased production of endogenous MFG-E8 during ALI, which could be ameliorated by exogenous treatment of rmMFG-E8.

**Exaggerated production of MIP-2 and TNF-α in lungs, BALF, and alveolar macrophages of Mfge8<sup>−/−</sup> mice during inflammation**

After observing the excessive neutrophil infiltration in Mfge8<sup>−/−</sup> mice, we then examined the levels of MIP-2, a critical chemokine responsible for neutrophil chemotaxis. MIP-2 mRNA and protein levels in Mfge8<sup>−/−</sup> mice were 2.2- and 2.1-fold higher, respec-
tively, than those in WT mice at 4 h, and 2.4- and 2.3-fold higher at 24 h \((p < 0.05\); Fig. 7A, 7B). Similarly, the MIP-2 levels in BALF from \(Mfge8^{-/-}\) mice were significantly higher than those from WT mice at 4 and 24 h (Fig. 7C). Because macrophages are one of the major cell types for producing chemokines and cytokines, we therefore harvested alveolar macrophages from BALF of sham mice and assessed MIP-2 and TNF-\(\alpha\) levels in LPS-treated conditions. After 4 h exposure to LPS, the MIP-2 mRNA and protein levels were increased significantly in alveolar macrophages from \(Mfge8^{-/-}\) mice, being 1.8- and 1.9-fold higher than those from WT mice \((p < 0.05\); Fig. 7D, 7E). Similarly, TNF-\(\alpha\) mRNA and protein levels in alveolar macrophages from \(Mfge8^{-/-}\) mice were also significantly higher than those from WT mice after LPS stimulation (Fig. 7F, 7G).

**MFG-E8 inhibits neutrophil migration through regulating CXCR2 expression**

We further examined whether MFG-E8 had a direct effect on the neutrophil migration by isolating neutrophils from bone marrow of WT and \(Mfge8^{-/-}\) mice. The number of migrated neutrophils from \(Mfge8^{-/-}\) mice was significantly higher than those from WT mice at 4 and 24 h (Fig. 7C). Because macrophages are one of the major cell types for producing chemokines and cytokines, we therefore harvested alveolar macrophages from BALF of sham mice and assessed MIP-2 and TNF-\(\alpha\) levels in LPS-treated conditions. After 4 h exposure to LPS, the MIP-2 mRNA and protein levels were increased significantly in alveolar macrophages from \(Mfge8^{-/-}\) mice, being 1.8- and 1.9-fold higher than those from WT mice \((p < 0.05\); Fig. 7D, 7E). Similarly, TNF-\(\alpha\) mRNA and protein levels in alveolar macrophages from \(Mfge8^{-/-}\) mice were also significantly higher than those from WT mice after LPS stimulation (Fig. 7F, 7G).

**MFG-E8 interacts with \(\alpha_v\beta_3\) integrin for inhibition of neutrophil migration**

It has been well characterized that MFG-E8 can bind \(\alpha_v\beta_3\) integrin in immune cells through its N-terminal domain (16). To examine the utilization of \(\alpha_v\beta_3\) integrin in MFG-E8–mediated suppression of neutrophils from WT mice (Fig. 8B). To validate the role of MFG-E8 in regulating neutrophil migration, BMDNs were treated with rmMFG-E8 before applying the migration assay. As shown in Fig. 8C, the number of migrated cells in rmMFG-E8–treated BMDN was reduced by 40% compared with vehicle-treated BMDNs. Furthermore, cotreatment of anti–MFG-E8 neutralizing Abs with rmMFG-E8 abrogated the functions of rmMFG-E8 for reducing the migration of BMDNs, hence becoming comparable to the vehicle control (Fig. 8C). Correspondingly, the rmMFG-E8–treated BMDNs had a lower CXCR2 expression compared with the vehicle control, and the reduction of CXCR2 expression was rescued by cotreatment of anti–MFG-E8 Abs (Fig. 8D). Intracellular GRK2 is a major determinant for surface CXCR2 flip-flop in neutrophils, whose activation leads to desensitization of CXCR2, resulting in its intracellular translocation, and negative regulation of the neutrophil migration (29–31). GRK2 expression in BMDNs was increased by 44% after rmMFG-E8 treatment, compared with the vehicle control, whereas cotreatment of anti–MFG-E8 Abs diminished such induction of GRK2 expression (Fig. 8E).
of neutrophil migration, anti-αv integrin neutralizing Abs were applied (32). As shown in Fig. 9A, BMDNs pretreated with anti-αv integrin Abs abrogated the functions of MFG-E8–mediated inhibition of their migration. We further demonstrated that the pretreatment of BMDNs with anti-αv integrin Abs blocked the effects of rmMFG-E8–mediated downregulation of CXCR2 and upregulation of GRK2 (Fig. 9B, 9C). Collectively, these features clearly demonstrated the critical roles of MFG-E8 for GRK2-dependent downregulation of surface CXCR2 expression in neutrophils through αvβ3 integrin-mediated pathway.

Discussion

In this study, we demonstrated the novel mechanism of MFG-E8 for regulating chemokine-mediated neutrophil migration in an LPS-induced murine model of ALI, which revealed more severe lung injury in MFG-E8–deficient mice than in WT counterparts. This observation is consistent with our previous study showing the beneficial effects of MFG-E8 for attenuating lung injury induced by intestinal I/R (20). In this study, we noticed exaggerated infiltration of neutrophils into the lungs of Mfge8<sup>−/−</sup> mice, as confirmed by increased levels of MPO in interstitial tissues, as well as neutrophil numbers and protein contents in BALF in comparison with WT mice. We further identified the potential roles of MFG-E8 in inhibiting migration of neutrophils by com-
paring the BMDNs isolated from WT and Mfge8\(^{-/-}\) mice and the effect of rmMFG-E8 administration by modulating the surface exposures of CXCR2 via GRK2-dependent pathways. Because \(\alpha\beta_3\) integrin is a putative receptor of MFG-E8 (16), we finally revealed the novel mechanism of MFG-E8 for regulating neutrophil migration through recognizing \(\alpha\beta_3\) integrin.

FIGURE 8. Inhibition of BMDN migration by MFG-E8 is mediated through regulation of CXCR2 and GRK2. (A and B) BMDNs were isolated from WT and Mfge8\(^{-/-}\) mice. (A) BMDNs were placed in the upper well of a modified Boyden chamber for migration assays. The media containing rmMIP-2 (1 ng/ml) was placed in the lower wells as chemotactic stimulus. After 2 h, migrated cells were fixed and stained with PI. Representative images of migrated BMDNs are shown. Five random microscopic fields per well were counted. Original magnification \(\times 100\). (B) CXCR2 expression in BMDNs was analyzed by flow cytometry. Granulocytes were gated according to forward and side scatter dot plots, followed by identification of neutrophils with staining of FITC-CD11b and allophycocyanin-Ly6G Abs. Representative histograms of expression intensity of CXCR2 in CD11b\(^{+}\)Ly6G\(^{+}\) cells stained with PerCP/Cy5.5-CXCR2 Abs are shown. The average of mean fluorescence intensities (MFI) for CXCR2 in BMDNs is plotted. Data are expressed as means ± SE \((n = 5\) mice/group). \(*p < 0.05\) versus WT. (C–E) BMDNs isolated from WT mice were treated with isotype Abs (1 \(\mu\)g/ml) plus rmMFG-E8 (500 ng/ml) or anti-MFG-E8 Abs (1 \(\mu\)g/ml) plus rmMFG-E8 (500 ng/ml) for 2 h before analysis. (C) BMDNs with various treatments were subjected to a migration assay. Representative images of migrated BMDN with PI staining are shown. Five random microscopic fields per well were counted. Original magnification \(\times 100\). (D) CXCR2 expression in BMDNs with various treatments was analyzed by flow cytometry. (E) Intracellular GRK2 expression in BMDNs with various treatments was analyzed by flow cytometry. Representative histograms of expression intensity of GRK2 in CD11b\(^{+}\)Ly6G\(^{+}\) cells stained with PE-GRK2 Ab are shown. The average of MFI for GRK2 in BMDNs is plotted. Data are expressed as means ± SE \((n = 5\) independent experiments). \(*p < 0.05\) versus PBS control, \(*p < 0.05\) versus isotype Ab plus rmMFG-E8.
We previously demonstrated the roles of MFG-E8 in promoting phagocytosis of apoptotic cells as one of the mechanisms of ameliorating inflammation in animal models of sepsis, renal, and gut I/R (18, 20, 24). We therefore monitored the well-characterized function of MFG-E8 for engulfment of apoptotic cells to maintain the homeostatic balance and observed increased numbers of apoptotic cells in lung tissues of Mfge8<sup>−/−</sup> mice after ALI (Fig. 4). We further noticed significant induction of caspase-3 activity in Mfge8<sup>−/−</sup> mice as compared with the WT animals (Fig. 4), suggesting the increased occurrence of apoptosis in lung tissues following ALI. However, exaggerated infiltration of neutrophils into the lungs is a hallmark of ALI. The initial cascade of the development of ALI is the promotion of neutrophils into the lung tissues, which release inflammatory mediators, proteases, and reactive oxygen species to cause inflammation and epithelial cell apoptosis-mediated lung damage (33). Moreover, substantial research demonstrated that increased rates of epithelial cell death and decreased rates of activated neutrophil apoptosis in lungs are the two potentially important pathological mechanisms of ALI (33). Uncontrolled migration of neutrophils due to ALI deteriorates the disease status, whereas reducing their contents improves lung integrity. Therefore, blocking the neutrophil migration to the lungs by MFG-E8 to attenuate ALI precedes phagocytosis of apoptotic cells. Correspondingly, we recently demonstrated reduced contents of MPO in the lung tissues of rmMFG-E8–treated animals with gut I/R injuries (20). Hence, delineating the direct effects of MFG-E8 on regulation of neutrophil migration could be the dominant mechanism for attenuating ALI.

The therapeutic strategy for ALI is attained by attenuating neutrophil chemotaxis toward the inflammatory sites. CXCR2 expressed on the surface of neutrophils functions as a sensor to lead neutrophils at the inflamed sites (7, 10). The significant role of CXCR2 in contributing to neutrophil infiltration to the inflamed lungs has been well demonstrated by using CXCR2 knockout mice (11). Inhibition or knockout of this chemokine receptor diminished neutrophil influx into the lungs and improved mortality associated with ALI (11, 34, 35). Considering the putative roles of

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**FIGURE 9.** MFG-E8–mediated inhibition of BMDN migration is through α<sub>v</sub>β<sub>3</sub> integrin. BMDNs isolated from WT mice were treated with isotype Abs (1 μg/ml) plus rmMFG-E8 (500 ng/ml) or anti-α<sub>v</sub> integrin Abs (1 μg/ml) plus rmMFG-E8 (500 ng/ml) for 2 h before analysis. (A) BMDNs with various treatments were subjected to a migration assay. Representative images of migrated BMDNs with PI staining are shown. Five random microscopic fields per well were counted. Original magnification ×100. (B) CXCR2 and (C) GRK2 expression in BMDNs with various treatments was analyzed by flow cytometry. Representative histograms of expression intensity of CXCR2 and GRK2 in CD11b<sup>+</sup>Ly6G<sup>+</sup> cells are shown. The averages of mean fluorescence intensities (MFI) for CXCR2 and GRK2 in BMDN are plotted. Data are expressed as means ± SE (n = 5 independent experiments). *p < 0.05 versus PBS control, #p < 0.05 versus isotype Abs plus rmMFG-E8.

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CXCR2 for neutrophil migration, several selective CXCR2 inhibitors have been developed to treat various lung diseases caused by acute or chronic inflammation (36). In this study, we demonstrated that MFG-E8 can regulate surface expression of CXCR2 in neutrophils, which led to inhibition of neutrophil migration and subsequent infiltration to the lungs. Thus, MFG-E8 may serve as a potentially therapeutic agent for attenuating ALI.

We further elucidated the signaling pathway mediated by MFG-E8 for suppressing surface CXCR2 expression. Several studies have demonstrated that phosphorylation and internalization of CXCR2 is tightly controlled by GRK2 in leukocytes (37, 38). Activity of GRK2 is further regulated though its subcellular localization, kinase activity, and expression levels (15). Other than the chemokine receptors, under inflammatory conditions, GRK2 expression can be regulated through activation of TLR2 or TLR4 signaling (30, 31, 39). Likewise, our findings showed MFG-E8-mediated upregulation of GRK2, which can be correlated with reduction of surface CXCR2. Next, we focused on identifying the surface receptors that can transmit extracellular MFG-E8 signaling to regulate GRK2/CXCR2 expression in neutrophils. MFG-E8 has two functional parts: N-terminal EGF domains that bind to αβ2 integrin of mostly hematopoietic cells, whereas the C-terminal discoidin domains can recognize the phosphatidylserine exposed in apoptotic cells (16). In this study, we considered αβ2 integrin as focal receptor for MFG-E8-mediated signal transduction. Structurally, αβ2 integrin is a heterodimeric transmembrane receptor formed by noncovalent association of α and β subunits (40, 41). In this study, we demonstrated that the blocking α5 integrin in neutrophils could effectively diminish the mMFG-E8 effects on GRK2/CXCR2 expression, indicating the involvement of α5 integrin in mediating MFG-E8 activity. Furthermore, these findings add the integrin signaling pathway as another critical factor in controlling GRK2.

Our previous study demonstrated that MFG-E8 can attenuate cytokine release from the peritoneal macrophages after LPS stimulation (25), which is consistent with the present study showing the higher levels of TNF-α expression and release in MFG-E8–deficient mice. In addition to demonstrating the roles of MFG-E8 in regulating neutrophil migration, we also observed that the induction of MIP-2 by LPS stimulation in the lungs and alveolar macrophages was augmented by the deficiency of MFG-E8. Because it has been previously established that the role of MFG-E8 in downregulating the proinflammatory cytokines has been mediated by modulating the intracellular signaling cascade (22, 25), it is conceivable that MFG-E8 has an additional role in protecting the inflammatory consequences in ALI. Future studies are required for further confirmation.

In summary, we have identified another novel function of MFG-E8 in attenuating lung injury induced by LPS through inhibiting neutrophil infiltration to the lungs. The MFG-E8 effect is mediated by αβ2 integrin to upregulate GRK2 expression and results in downregulation of surface CXCR2 levels in neutrophils, leading to decrease of neutrophil migration (Fig. 10). With this regulatory function on neutrophils, MFG-E8 represents a potentially therapeutic regimen for treating ALI.

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Disclosures
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