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(54) Titre : PREVENTION ET TRAITEMENT D'INFLAMMATION ET DE LESION D'ORGANE APRES ISCHEMIE/REPERFUSION EN UTILISANT MFG-E8

(54) Title: PREVENTION AND TREATMENT OF INFLAMMATION AND ORGAN INJURY AFTER ISCHEMIA/REPERFUSION USING MFG-E8

(57) Abrégé/Abstract:
Provided are methods for preventing and treating inflammation and organ injury after ischemia/reperfusion, and methods for treating lung injury, comprising administering a milk fat globule epidermal growth factor-factor VIII (MFG-E8) to a subject. Also provided are pharmaceutical compositions comprising MFG-E8 in dosage form for preventing and treating inflammation and organ injury after ischemia/reperfusion, and for treating lung injury, and methods of preparing a pharmaceutical composition for preventing and treating inflammation and organ injury after ischemia/reperfusion, and for treating lung injury, comprising formulating MFG-E8 in a pharmaceutical composition.

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(54) Title: PREVENTION AND TREATMENT OF INFLAMMATION AND ORGAN INJURY AFTER ISCHEMIA/REPERFUSION USING MFG-E8

(57) Abstract: Provided are methods for preventing and treating inflammation and organ injury after ischemia/reperfusion, and methods for treating lung injury, comprising administering a milk fat globule epidermal growth factor-factor VIII (MFG-E8) to a subject. Also provided are pharmaceutical compositions comprising MFG-E8 in dosage form for preventing and treating inflammation and organ injury after ischemia/reperfusion, and for treating lung injury, and methods of preparing a pharmaceutical composition for preventing and treating inflammation and organ injury after ischemia/reperfusion, and for treating lung injury, comprising formulating MFG-E8 in a pharmaceutical composition.

PREVENTION AND TREATMENT OF INFLAMMATION AND ORGAN INJURY AFTER ISCHEMIA/REPERFUSION USING MFG-E8

FIELD OF THE INVENTION

[0003] The present invention relates to using milk fat globule EGF-factor 8 (MFG-E8) for prevention and treatment of inflammation and organ injury after ischemia/reperfusion, especially after gut or intestinal ischemia/reperfusion, and for treatment of lung injury, such as acute lung injury.

BACKGROUND OF THE INVENTION

[0004] Throughout this application various publications are referred to in parenthesis. Full citations for these references may be found at the end of the specification preceding the claims.

[0005] Reperfusion injury refers to damage to tissue caused when blood supply returns to the tissue after a period of ischemia. Intestinal ischemia can occur in a variety of clinical conditions, including small bowel transplant, superior mesenteric artery occlusion, cardiac insufficiency with associated low flow state, and hemorrhagic shock and necrotizing enterocolitis.

[0006] Mesenteric ischemia remains a critical problem, resulting in a mortality as high as 60-80% (1). Multiple organ failure, including acute lung injury (ALI) is a common

complication of intestinal ischemia/reperfusion (I/R) injuries and contributes to its high death toll (2). ALI is caused by a systemic inflammatory response due to the release of pro-inflammatory cytokines and bacteria-derived endotoxins from reperfused ischemic tissue (3-6). So far, only a limited number pharmacologic treatment options have been found that provide some benefit in I/R and acute lung injury, most of them targeting inflammatory mediators and oxidative stress pathways (7). 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 macrophages (2, 8-11). Apoptosis is associated by a marked up-regulation of Fas and Fas-ligand, and the activation of caspase-3 in lung epithelial cells (12, 13). Proinflammatory cytokines like IL-1β or TNF-α seem to play a major role in apoptosis induction involving Bid, Bax upregulation and Bcl-2 downregulation (9, 14, 15).

[0007] While balanced apoptosis and phagocytosis maintain normal function, deficient clearance of apoptotic cells after ischemia potentially leads to increased inflammation and impaired tissue repair (16, 17). Apoptotic cells expose phosphatidylserine (PS) that can be recognized by soluble molecules and receptors, thereby enabling their phagocytosis (18). One of these molecules is milk fat globule EGF-factor 8 (MFG-E8), which is crucial for apoptotic cell clearance (19). Hanayama *et al.* found for example that the effective clearance of apoptotic B-cells in the spleen prevents inadequate pro-inflammatory immune responses and the development of auto-antibodies (20). In a rat sepsis model using cecal ligation and puncture, MFG-E8 is downregulated in spleens and livers. This was associated with impaired apoptotic cell clearance and increased mortality in these animals (21). Similar to sepsis, gut I/R injury is accompanied by a systemic inflammatory response.

[0008] Milk fat globule EGF-factor 8 (MFG-E8) is a potent opsonin for the clearance of apoptotic cells and is produced by mononuclear cells of immune competent organs including the spleen and lungs. Ischemia-reperfusion (I/R) injuries of the gut induce apoptosis, severe inflammation, and remote organ damage including acute lung injury (ALI). Whether enhancing apoptotic cell clearance is beneficial under such conditions has been unknown. There is a clear need for improved treatment and prevention of I/R injuries.

SUMMARY OF THE INVENTION

[0009] The invention is directed to methods of preventing and/or treating inflammation and/or organ injury after ischemia/reperfusion in a subject comprising administering to the

subject milk fat globule epidermal growth factor-factor VIII (MFG-E8) in an amount effective to prevent and/or treat inflammation and/or organ injury.

[0010] The invention is also directed to pharmaceutical compositions comprising milk fat globule epidermal growth factor-factor VIII (MFG-E8) in dosage form for preventing and/or treating inflammation and/or organ injury after ischemia/reperfusion.

[0011] The invention further provides methods of preparing a pharmaceutical composition for preventing and/or treating inflammation and/or organ injury after ischemia/reperfusion, the method comprising formulating milk fat globule epidermal growth factor-factor VIII (MFG-E8) in a pharmaceutical composition in an amount effective to prevent and/or treat inflammation and/or organ injury after ischemia/reperfusion.

[0012] The invention also provides methods of treating lung injury, such as acute lung injury, in a subject comprising administering to the subject a milk fat globule epidermal growth factor-factor VIII (MFG-E8) in an amount effective to treat lung injury in the subject.

[0013] The invention provides pharmaceutical compositions comprising milk fat globule epidermal growth factor-factor VIII (MFG-E8) in dosage form for treating lung injury.

[0014] The invention further provides methods of preparing pharmaceutical compositions for treating lung injury, the methods comprising formulating milk fat globule epidermal growth factor-factor VIII (MFG-E8) in a pharmaceutical composition in an amount effective to treat lung injury.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1A-1B. Suppression of splenic MFG-E8 after intestinal I/R. The superior mesenteric artery (SMA) was occluded for 90 min followed by 4h of reperfusion. (A) MFG-E8 mRNA levels in the spleens were measured by qPCR, (B) MFG-E8 protein levels were assessed by Western blot. Data are expressed as means ± SEM, *$P<.05$ vs. Sham by Student's t-test, $n=6$.

[0016] Figure 2A-2F. Attenuation of organ injury by recombinant murine MFG-E8 (rmMFG-E8) after intestinal I/R. The SMA was occluded for 90 min followed by 4h of reperfusion. (A) H&E staining of the small intestine after intestinal I/R revealed widespread mucosal injury (middle panel), while treatment with rmMFG-E8 showed beneficial local effects (right panel). Left panel: sham control. (B) Lactate, (C) LDH, (D) ALT, (E) AST, and

(F) creatinine were measured 4h after reperfusion. Data are expressed as means ± SEM, **P*<.05 vs. Sham, #*P*<.05 vs. Vehicle by One-Way ANOVA and Student Newman Keul's test, *n*=6.

[0017] Figure 3A-3C. Suppression of plasma cytokines by rmMFG-E8 after intestinal I/R. The SMA was occluded for 90 min followed by 4h of reperfusion. Blood cytokines (TNF-α, IL-1β, and IL-6) were assessed by ELISA. Data are expressed as means ± SEM, **P*<.05 vs. Sham, #*P*<.05 vs. Vehicle by One-Way ANOVA and Student Newman Keul's test, *n*=6.

[0018] Figure 4A-4C. Attenuation of acute lung injury (ALI) by rmMFG-E8. The SMA was occluded for 90 min followed by 4h of reperfusion. Lungs were fixed and stained with H&E. (A) Representative micrographs at original 100x and 400x magnification (inlet). (B) Tissue injury was scored as described in the methods section. (C) Neutrophil activity was assessed by MPO assay. Data are expressed as means ± SEM, **P*<.05 vs. Sham, #*P*<.05 vs. Vehicle by One-Way ANOVA and Student Newman Keul's test, *n*=6.

[0019] Figure 5A-5C. Decreased pulmonary MFG-E8 after intestinal I/R and restoration of apoptotic cell clearance by rmMFG-E8 treatment. (A) MFG-E8 mRNA levels were measured by qPCR, (B) MFG-E8 protein levels were assessed by Western blotting. Data are expressed as measns±SEM, **P*<0.05 vs. Sham by Student's *t* test, *n*=6. (C) Lungs were stained with TUNEL and counterstained with propidium iodine.

[0020] Figure 6A-6B. MFG-E8 deficiency worsens the inflammatory response in the lung of mice after gut I/R. 4h after reperfusion, pulmonary tissues were collected and assessed for (A) IL-1β and (B) IL-6. Data are expressed as measns±SEM, **P*<.05 vs. Sham, #*P*<.05 vs. WT by Two-Way ANOVA and Student Newman Keul's test, *n*=6. WT: wild-type mice; KO: knockout mice.

[0021] Figure 7A-7C. MFG-E8 deficiency deteriorates ALI. 5 The SMA was occluded for 90 min followed by 4h of reperfusion. Lungs were fixed and stained with H&E. (A) Microphotographs at original 100x and 400x magnification (inlet). (B) Tissues were scored as described in the methods section. (C) Neutrophil activity was assessed by MPO assay. Data are expressed as means ± SEM, **P*<.05 vs. Sham, #*P*<.05 vs. WT by Two-Way ANOVA and Student Newman Keul's test, *n*=6.

[0022] Figure 8. Survival benefit in rmMFG-E8-treated mice after gut I/R injury. The SMA was occluded for 90 min followed by 4h of reperfusion. Each group of mice received

one dose of rmMFG-E8 or normal saline (vehicle) i.p. at the beginning of reperfusion and was observed for 24h. *P<.05 vs. Vehicle by Kaplan Meyer Log-rank test, n=15.

DETAILED DESCRIPTION OF THE INVENTION

[0023] The invention is directed to a method of preventing and/or treating inflammation and/or organ injury after ischemia/reperfusion in a subject comprising administering to the subject a milk fat globule epidermal growth factor-factor VIII (MFG-E8) in an amount effective to prevent and/or treat inflammation and/or organ injury. Ischemia/reperfusion includes, but is not limited to, ischemia/reperfusion of the gastrointestinal tract, liver, lung, kidney, heart, brain, spinal cord and/or crushed limb. In particular, the invention is directed to a method of preventing and/or treating inflammation and/or organ injury after intestinal ischemia/reperfusion in a subject comprising administering to the subject a milk fat globule epidermal growth factor-factor VIII (MFG-E8) in an amount effective to prevent and/or treat inflammation and/or organ injury.

[0024] Preferably, the method prevents or reduces serum elevation of one or more of tumor necrosis factor-α, interleukin-6, interleukin-1β, aspartate aminotransferase, alanine aminotransferase, lactate, or lactate dehydrogenase.

[0025] Preferably, the organs in which organ injury is prevented or treated include, but are not limited to, one or more of gastrointestinal tract, liver, lung, kidney, heart, brain, spinal cord, and crushed limb. The lung injury can be acute lung injury (ALI).

[0026] Preferably, the survival of the subject is improved.

[0027] Preferably, the MFG-E8 has an amino acid sequence at least 80%, more preferably at least 90%, even more preferably 95%, still more preferably, at least 99% identical to SEQ ID NO:1 or SEQ ID NO:2, which are the sequences of a human MFG-E8 and mouse MFG-E8, respectively. Most preferably, the MFG-E8 sequence is completely homologous to SEQ ID NO:1. These methods can be used with any mammal, including humans.

[0028] The MFG-E8 in these embodiments can be in purified form, e.g., as a protein purified from a natural source or as a transgenic protein expressed from a recombinant cell. Alternatively, the MFG-E8 can be only partially purified (e.g., further comprising cellular components, such as in the form of MFG-E8-rich exosomes derived from bone marrow dendritic cells or from other mammalian cells, including cells transformed with transgenic

MFG-E8). Where the MFG-E8 is from MFG-E8-rich exosomes, the exosomes are preferably from the same species as the treated mammal; more preferably, the exosomes are from the same individual. The MFG-E8 can have a wild-type sequence from any mammalian species, or can comprise mutations, provided the mutations do not eliminate the protein's activity to prevent and/or treat inflammation and/or organ injury, Such mutants could be made without undue experimentation. The activity of those mutants can also be easily determined by known methods and the methods described herein.

[0029] The MFG-E8 can also comprise peptidomimetics. As used herein, an amino acid mimetic or peptidomimetic is a compound that is capable of mimicking a natural parent amino acid in a protein, in that the substitution of an amino acid with the peptidomimetic does not significantly affect the activities of interest of the protein, in this case, the therapeutic activity of exogenous MFG-E8. Proteins comprising peptidomimetics are generally poor substrates of proteases and are likely to be active *in vivo* for a longer period of time as compared to the natural proteins. In addition, they could be less antigenic and show an overall higher bioavailability. The skilled artisan would understand that design and synthesis of aqueous-soluble proteins comprising peptidomimetics would not require undue experimentation (e.g., 39-41).

[0030] The invention also provides a pharmaceutical composition comprising milk fat globule epidermal growth factor-factor VIII (MFG-E8) in dosage form for preventing and/or treating inflammation and/or organ injury after ischemia/reperfusion. Ischemia/reperfusion includes, but is not limited to, ischemia/reperfusion of the gastrointestinal tract, liver, lung, kidney, heart, brain, spinal cord and/or crushed limb. Intestinal ischemia/reperfusion is a preferred form of ischemia/reperfusion.

[0031] The invention further provides a method of preparing a pharmaceutical composition for preventing and/or treating inflammation and/or organ injury after ischemia/reperfusion, the method comprising formulating milk fat globule epidermal growth factor-factor VIII (MFG-E8) in a pharmaceutical composition in an amount effective to prevent and/or treat inflammation and/or organ injury after ischemia/reperfusion. Ischemia/reperfusion includes, but is not limited to, ischemia/reperfusion of the gastrointestinal tract, liver, lung, kidney, heart, brain, spinal cord and/or crushed limb. Intestinal ischemia/reperfusion is a preferred ischemia/reperfusion.

[0032] The above-described MFG-E8 preparations are preferably formulated in a pharmaceutical composition. These compositions can be formulated without undue experimentation for administration to a mammal, including humans, as appropriate for the particular application. Additionally, proper dosages of the compositions can be determined without undue experimentation using standard dose-response protocols.

[0033] Accordingly, the compositions designed for oral, lingual, sublingual, buccal and intrabuccal administration can be made without undue experimentation by means well known in the art, for example with an inert diluent or with an edible carrier. The compositions may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the pharmaceutical compositions of the present invention may be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums and the like.

[0034] Tablets, pills, capsules, troches and the like may also contain binders, recipients, disintegrating agent, lubricants, sweetening agents, and flavoring agents. Some examples of binders include microcrystalline cellulose, gum tragacanth or gelatin. Examples of excipients include starch or lactose. Some examples of disintegrating agents include alginic acid, corn starch and the like. Examples of lubricants include magnesium stearate or potassium stearate. An example of a glidant is colloidal silicon dioxide. Some examples of sweetening agents include sucrose, saccharin and the like. Examples of flavoring agents include peppermint, methyl salicylate, orange flavoring and the like. Materials used in preparing these various compositions should be pharmaceutically pure and nontoxic in the amounts used.

[0035] The compositions of the present invention can easily be administered parenterally such as for example, by intravenous, intramuscular, intrathecal or subcutaneous injection. Parenteral administration can be accomplished by incorporating the compositions of the present invention into a solution or suspension. Such solutions or suspensions may also include sterile diluents such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents. Parenteral formulations may also include antibacterial agents such as for example, benzyl alcohol or methyl parabens, antioxidants such as for example, ascorbic acid or sodium bisulfite and chelating agents such as EDTA. Buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose may also be added. The

parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

[0036] Rectal administration includes administering the pharmaceutical compositions into the rectum or large intestine. This can be accomplished using suppositories or enemas. Suppository formulations can easily be made by methods known in the art. For example, suppository formulations can be prepared by heating glycerin to about 120° C., dissolving the composition in the glycerin, mixing the heated glycerin after which purified water may be added, and pouring the hot mixture into a suppository mold.

[0037] Transdermal administration includes percutaneous absorption of the composition through the skin. Transdermal formulations include patches (such as the well-known nicotine patch), ointments, creams, gels, salves and the like.

[0038] The present invention includes nasally administering to the mammal a therapeutically effective amount of the composition. As used herein, nasally administering or nasal administration includes administering the composition to the mucous membranes of the nasal passage or nasal cavity of the patient. As used herein, pharmaceutical compositions for nasal administration of a composition include therapeutically effective amounts of the composition prepared by well-known methods to be administered, for example, as a nasal spray, nasal drop, suspension, gel, ointment, cream or powder. Administration of the composition may also take place using a nasal tampon or nasal sponge.

[0039] The invention also provides for the use of milk fat globule epidermal growth factor-factor VIII (MFG-E8) for preventing and/or treating inflammation and/or organ injury after ischemia/reperfusion. The invention further provides for the use of milk fat globule epidermal growth factor-factor VIII (MFG-E8) for the preparation of a pharmaceutical composition for preventing and/or treating inflammation and/or organ injury after ischemia/reperfusion.

[0040] The invention also provides methods of treating lung injury, such as for example, acute lung injury, in a subject comprising administering to the subject a milk fat globule epidermal growth factor-factor VIII (MFG-E8) in an amount effective to treat lung injury in the subject. The invention provides pharmaceutical compositions comprising milk fat globule epidermal growth factor-factor VIII (MFG-E8) in dosage form for treating lung injury. The invention further provides methods of preparing pharmaceutical compositions for treating lung injury, the methods comprising formulating milk fat globule epidermal growth

factor-factor VIII (MFG-E8) in a pharmaceutical composition in an amount effective to treat lung injury.

[0041] The invention also provides for the use of milk fat globule epidermal growth factor-factor VIII (MFG-E8) for treating lung injury, such as for example, acute lung injury. The invention further provides for the use of milk fat globule epidermal growth factor-factor VIII (MFG-E8) for the preparation of a pharmaceutical composition for treating lung injury.

[0042] This invention will be better understood from the Experimental Details, which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

EXPERIMENTAL DETAILS

Materials and Methods

[0043] *Experimental model:* Ischemia was induced in C57BL/6J wild-type (WT) mice and MFG-E8 knockout (KO) mice by clamping the superior mesenteric artery (SMA) for 90 minutes under general anesthesia using isofluorane. Mice were resuscitated with 0.5 ml saline and were treated with recombinant murine MFG-E8 (rmMFG-E8) (0.4 mg/kg in 0.5 ml normal saline i.p.) or normal saline (Vehicle). Control animals underwent the same operative procedure with the exception of the SMA clamping (Sham I/R) (n=6). 4 h after reperfusion, animals were anesthetized and EDTA blood (for plasma) and tissue samples were harvested, frozen immediately in liquid nitrogen and stored at -80°C until measurements. Additional experiments for observation of survival over the course of 24h were performed (n=15). All experiments were performed in accordance with the guidelines for the use of experimental animals by the National Institutes of Health (Bethesda, MD) and were previously approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research (Manhasset, NY).

[0044] *MFG-E8 Western blotting and gene expression:* 25 μg of protein from spleen and lung samples was fractionated on a Bis-Tris gel and transferred to a 0.22-μm nitrocellulose membrane. Blots were blocked with 5% BSA in Tris-buffered saline containing 0.1%v/v Tween™ 20 and incubated with hamster anti-mouse MFG-E8 mAb (clone 2422, MBL, Nagoya, Japan). After the incubation with HRP-labeled goat-anti hamster IgG (Santa Cruz, CA) in 5% BSA-TBST and washing with TBST, bands were detected using a

chemiluminescent peroxidase substrate (ECLplus, Amersham, Little Chalfont, Buckinghamshire, UK) and exposure on a radiograph film. RNA was extracted from spleen and lung tissue samples using TRIzol Reagent (Invitrogen, Carlsbad, CA). 5μg of RNA was reverse transcribed to cDNA using murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) and amplified by qPCR using SYBR™ green PCR Master Mix (Applied Biosystems). The following primer sets were used: mouse MFG-E8 (forward: 5'-GGG CCT GAA GAA TAA CAC GA-3' (SEQ ID NO:3); reverse: 5'-AGG GCA ACT TGG ACA ACA AC-3' (SEQ ID NO:4)); mouse β-actin (endogenous control; forward:5'-TGT TAC CAA CTG GGA CGA CA-3' (SEQ ID NO:5); reverse: 5'-GGG GTG TTG AAG GTC TCA AA-3' (SEQ ID NO:6)).

[0045] *Cytokines and Organ Injury Parameters:* TNF-α, IL-1β, and IL-6, were quantified using specific mouse ELISA kits (BD Pharmingen, Franklin Lakes, NJ) in EDTA plasma, small intestine and lung tissues. AST, ALT, LDH, lactate, and creatinine blood plasma levels were determined using commercial assay kits (Pointe Scientific, Canton, MI).

[0046] *Histopathology:* Samples of the small intestine (non-necrotic areas) and lungs were fixed in 10% formalin and embedded in paraffin. Tissue blocks were sectioned at a thickness of 5 μm, transferred to glass slides, and stained with hematoxylin/eosin. Morphologic examinations were performed using light microscopy and lung injury analyzed by a blinded, experienced investigator for absent, mild, moderate or severe injury (score 0-3) based on the presence of exudates, hyperemia/congestion, neutrophilic infiltrates, intra-alveolar hemorrhage/debris, and cellular hyperplasia (22). The sum of scores of different animals was averaged. Intestinal injury was scored according to Stallion *et al.*, assessing villus-to-crypt ratio (normal: 5 to 1), lymphocytic infiltrates, epithelial degeneration/necrosis, erosions, glandular dilatation and transmural changes (score 0-4) (23).

[0047] *Apoptosis assay:* Tissue samples were de-waxed, incubated with proteinase K, stained using a green fluorescent-tagged TUNEL kit (Roche Diagnostics, Indianapolis, IN) counterstained with propidium iodide and examined under a fluorescent microscope. Apoptotic cells appeared green fluorescent on a red background staining.

[0048] *Tissue myeloperoxidase (MPO) assay:* Tissues were homogenized in KPO_4 buffer containing 0.5% Hexa-decyl-trimethyl-ammonium bromide (60°C for 2h). After centrifuging, supernatant was diluted in reaction solution and ΔOD was measured at 460 nm to calculate MPO activity (24).

[0049] *Statistics:* Data were expressed as means ± SEM and compared by ANOVA using Student-Newman-Keuls' test. Student *t* test was used if only two groups were present. The survival study was analyzed using the Kaplan-Meier Log-rank test. Differences were considered significant if $P<0.05$.

Results

[0050] *Suppression of MFG-E8 in the spleen after gut I/R:* To investigate whether MFG-E8 levels are altered after intestinal I/R injury, MFG-E8 mRNA and protein levels were measured in WT mice 4h post reperfusion after 90-min. ischemia. The results indicate that splenic mRNA levels dropped significantly by on average 63% (Fig. 1A) and protein levels by 68% (Fig. 1B) after gut I/R.

[0051] *Administration of rmMFG-E8 attenuates multiple organ injury after intestinal I/R:* Gut I/R caused widespread macroscopic necrosis with severe enteric mucosal injury (average score 2.25±0.14) in intestinal areas juxtaposed to the macroscopically ischemic bowel (Fig. 2A). Similarly, multiple blood markers of remote organ damage were significantly elevated, including lactate (80% increase), lactate dehydrogenase (LDH) (17.5-fold increase), alanine aminotransferase (ALT) (4.8-fold increase), aspartate aminotransferase (AST) (18.3-fold increase) and creatinine (2-fold increase) compared to sham-operated animals (Figs. 2B-F), indicating the systemic scale of injury induced in this model. Treatment with one dose of rmMFG-E8 (0.4 μg/20g BW i.p.) at the beginning of reperfusion largely attenuated I/R-induced multiple organ injury. Histopathologically even large parts of intestine were protected from secondary mucosal damage after treatment (injury score: 1.25±0.14) (Fig. 2A). Treatment with rmMFG-E8 also entirely blocked the elevation of lactate, AST and ALT, while it suppressed LDH and creatinine levels by 58% and 21%, respectively. Hence rmMFG-E8 has a primary protective effect on intestinal mucosa, general perfusion, liver injury, and, to a lesser but still significant extent, on general tissue damage and renal function (Figs. 2B-F).

[0052] *Administration of rmMFG-E8 suppresses the systemic inflammatory response after intestinal I/R:* Pro-inflammatory cytokines are major contributors in the injury of remote organs after intestinal I/R. Hence it was investigated whether the three cytokines tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) were affected by the treatment with rmMFG-E8. While blood levels of the cytokines increased significantly

after gut I/R (TNF-α by 5.6-fold, IL-1β by 3.9-fold, and IL-6 by 96-fold), rmMFG-E8 dramatically reduced the pro-inflammatory response (by 72%, 42%, and 48%, respectively, Figs. 3A-C). To investigate whether MFG-E8 influences cytokine production in tissues, cytokine protein levels were analyzed in the small intestine and the lungs using ELISA. Similar suppressive effects of rmMFG-E8 could be found on tissue cytokine levels, suggesting that MFG-E8 confers its anti-inflammatory effect by decreased cytokine production and release from the source of injury as well as from target organs (Table 1).

Table 1. Local cytokine suppression by rmMFG-E8 after intestinal I/R

Organ	Cytokine	Intestinal I/R		
		Sham	Vehicle	rmMFG-E8
Gut	TNF-α (pg/mg)	6.5±0.5	17.3±3.2*	6.7±1.4#
	IL-6 (pg/mg)	5.3±0.4	14.3±2.1*	9.6±2.6
	IL-1β (pg/mg)	24.4±2.6	62.5±12.7*	32.5±8.4#
Lung	TNF-α (pg/mg)	6.2±0.5	7.7±1.7	5.0±0.2
	IL-6 (pg/mg)	15.9±3.3	33.0±4.5*	14.7±2.9#
	IL-1β (pg/mg)	33.7±2.3	96.1±15.8*	36.6±2.8#

C57BL6/J mice underwent SMA I/R for 90 min followed by 4h reperfusion. 6 mice also received rmMFG-E8 (0.4 μg/20g BW i.p.) at the beginning of the reperfusion time. Tissue cytokine levels were determined by ELISA and are presented as means±SEM, *P<0.05 vs. Sham, #P<0.05 vs. Vehicle by One-Way ANOVA and Student Newman Keul's test, n=6.

[0053] *Administration of rmMFG-E8 attenuates ALI after intestinal I/R:* The lungs are one of the organs most severely affected by intestinal I/R injury (25). Histopathological analysis of the lungs showed moderate to severe injury with exudates, congestion, cellular infiltrates and intracellular hemorrhage averaging to a histopathological score of 9.7±0.8 (Figs. 4A-B). The cell morphology and timing of cellular lung infiltrates were suggestive of a neutrophilic nature. Therefore, myeloperoxidase activity was assessed in the lungs, which was equally elevated by 3.3-fold after gut I/R (Fig. 4C). Treatment with rmMFG-E8 significantly reduced acute lung injury, histopathologically, as well as biochemically (Figs. 4 A-C). The histopathology score was reduced by 38% and MPO activity was suppressed by 47% (Figs. 4 B-C).

[0054] *Pulmonary MFG-E8 suppression and apoptosis after intestinal I/R:* Although Northern blotting revealed the spleen as the major immune competent organ to produce

MFG-E8 (20), the lung still expresses significant amounts of MFG-E8 mRNA (about 50% of spleen levels; unpublished observation). Jinushi *et al.* showed that under normal conditions alveolar macrophages contain most of the MFG-E8 protein in the lungs (26). Quantitative PCR and Western blotting indicated that gut I/R also suppresses MFG-E8 levels by 48% on an mRNA level and by 49% on a protein level (Figs. 5A-B). At the same time, increased numbers of apoptotic cells were found in the pulmonary tissue by TUNEL staining (Fig. 5C), which was associated with caspase 3 activation (data not shown). Treatment with rmMFG-E8, however, significantly suppressed the number of detectable apoptotic cells in the lungs after gut I/R injury (Fig 5C).

[0055] *MFG-E8 deficiency increases pulmonary inflammation and deteriorates ALI:* To further elucidate the role of MFG-E8 in gut I/R-mediated ALI, the inflammatory response and lung tissue injury were investigated in MFG-E8-deficient mice. Compared to WT mice, MFG-E8 KO mice produced nearly two-fold increased IL-1β and IL-6 protein levels in the lungs after gut I/R (Figs. 6A-B). This dramatic increase in pro-inflammatory cytokine production in the lungs was associated with a further deteriorated ALI in MFG-E8 KO mice compared to their WT controls, including increased congestion, exudates, interstitial cellular infiltrates and consolidation (Figs. 7A-B). Pulmonary neutrophil activity was also significantly increased in MFG-E8 KO mice after gut I/R (Fig. 7C). Overall, this clearly indicates that the absence of MFG-E8 causes more severe inflammation and damage to the lungs in this model.

[0056] *Treatment with rmMFG-E8 improves survival after intestinal I/R injury:* The above results suggest that MFG-E8 is beneficial in I/R-mediated injury. Therefore, a survival study was performed in 15 mice receiving rmMFG-E8 at the beginning of reperfusion and compared to control mice treated with normal saline. All of the control mice died within 24h with a median survival time of 8h (95% CI: 5.5-10.5h, Fig. 8). 7 of the 15 animals treated with rmMFG-E8, however, were still alive 24h after gut I/R (median survival time: 20h, Fig. 8). This significant improvement in survival time showed that MFG-E8 is protective against multiple organ failure and death caused by bowel I/R injury.

Discussion

[0057] The present study demonstrates that intestinal I/R negatively affects lung morphology, increases MPO, and local cytokine production consistent with ALI and that

treatment with rmMFG-E8 attenuates organ injury, inflammation, and improves survival. Treated animals also displayed less apoptosis in their lungs, suggesting enhanced MFG-E8-mediated clearance of apoptotic cells. MFG-E8 KO mice showed a dramatically deteriorated ALI and inflammation, providing further evidence for the crucial role of MFG-E8 in I/R-mediated remote organ injury.

[0058] Acute lung injury is a syndrome of respiratory failure resulting from acute pulmonary edema and inflammation (2). The incidence of ALI and ARDS combined in the United States was estimated by the National Institutes of Health to be 75 per 100,000 population, and more recent data from Scandinavia showed that ALI alone accounts for roughly 18 per 100,000 population (25, 27). Although mortality rates from ALI and ARDS decreased in the past 20-30 years, they still remain unacceptably high (25). Multiple causes can lead to ALI in patients, including sepsis, severe trauma with shock and multiple transfusions (25). Under these pathological conditions, ALI is driven by an overwhelming systemic inflammatory response and often occurs in conjunction with failure of other organs (3). Intestinal ischemia due to transient obliteration of the SMA, for example, causes vast local tissue injury and the reperfusion of the ischemic bowel leads to a tremendous activation of the inflammatory response, leading to a very severe clinical picture with multiple organ failure including ALI (28). Pathophysiologically, ALI is associated with alveolar exudates and bleeding, influx and activation of immune cells with the release of abundant cytokines and enzymes, which can be further complicated by infection and ventilation-induced injury (3, 25). Impaired barrier function of the lung epithelium and endothelium play a major role in the development of ALI and ARDS and one key aspect of this failure is the loss of cells through apoptosis (2). Particularly type II pneumocytes undergo apoptosis mediated by the activation of the Fas-ligand – Fas pathway and the activation of caspase-3 (12, 13), and it is very likely that the nature of apoptotic cells in the present experiments is epithelial, too. Especially cytokines like IL-1β or TNF-α have been reported to play a major role in apoptosis induction in the lungs (9, 14, 15). Mesenteric I/R injury is predominantly mediated by proinflammatory mediators released from the reperfused intestine, possibly contributing to the induction of apoptosis in remote organs (29). However, the role of reactive oxygen species in the induction of post I/R pulmonary cell death should not be underestimated (30). Apoptosis of type II alveolar pneumocytes directly causes pulmonary edema and reduces the production of surfactant. However, apoptosis plays a role beyond the initial phase of tissue

injury, as it is central to the imbalances between inflammatory resolution and the progression of ALI later in the disease (31). Controlled tissue repair through the modulation of apoptosis or apoptotic cell phagocytosis may prevent lung fibrosis, the progression in the disease and its overall severity (2, 12, 25, 31).

[0059] MFG-E8 is a secretory molecule and mainly produced in the spleen. It can, however, also be found in significant amounts in lymph nodes and in the lungs (20, 26). It is mainly produced by macrophages and dendritic cells and has so far been mainly linked to the opsonization of apoptotic cells. Hanayama *et al.* discovered that MFG-E8 plays a major role in the clearance of apoptotic B cells in the spleen, which prevents the development of autoimmune diseases (16, 19, 20). In mice, MFG-E8 is a 64kDa glycoprotein with two EGF-like domains (E1 and E2) containing an RGD-motif that can bind certain integrins (vitronectin receptor, αvβ3 or αvβ5) that are highly expressed on macrophages and other phagocytic cells. This domain is separated by a P/T-rich region from two coagulation factor V/VIII-like domains (C1 and C2) that have a strong affinity to phosphatidylserine (PS). These properties make it an important factor in binding apoptotic cells that express high amounts of PS on their surface to phagocytes (19). While PS-expressing apoptotic cells can also bind to other receptors on macrophages, including the putative PS receptor and CD36, the binding to αvβ3 or αvβ5-integrins via MFG-E8 is required to induce their engulfment (19). At very high concentrations, MFG-E8 has also been shown to modulate the intrinsic coagulation cascade due to its competition for PS binding sites and to increase the prothrombin time by 50% (32). It is also involved in the VEGF-dependent neovascularization (33) and in the migration of enterocytes and intestinal repair (34). It has been proposed to be beneficial in atherosclerosis (35) and Alzheimer's disease (36), although the mechanism is still unclear. MFG-E8 clearly plays an important role at the interface between phagocytosis of apoptotic cells and inflammation in chronic and acute inflammatory diseases. MFG-E8-dependent increase in apoptotic cell clearance can prevent deaths from sepsis (21).

[0060] Sepsis and mesenteric I/R injury share the fact that both lead to a systemic inflammatory response (23). Gut I/R has also been shown to lead to increased microbial translocation and the release of bacterial toxins into the blood stream (4, 5). Activation of toll-like receptors has indeed been shown to suppress MFG-E8 levels *in vitro* and *in vivo* ((26) and unpublished data). So far, only granulocyte/monocyte colony stimulating factor is known to modulate MFG-E8 expression (26). Whether other cytokines have the potential to

change MFG-E8 expression and at what kinetics is currently unknown. The present studies have shown a clear suppression of MFG-E8 in the spleen and in lungs 4h after reperfusion of the 90-min-ischemic gut. The suppression of MFG-E8 by 50%, as found in the lungs of gut I/R mice, appears to be sufficient to impair phagocytosis of apoptotic cells as previously reported in a sepsis model (21). Although the number of apoptotic cells in the lung was not very high, the cells affected are most likely type II pneumocytes, which would contribute to the progression of ALI (9). Treatment with rmMFG-E8 reduced the number of apoptotic cells and suppressed local inflammation. Decreased apoptosis is not mediated by a direct antiapoptotic effect but through the stimulation of apoptotic cell clearance (21). The present studies also found that the pulmonary injury after gut I/R was attenuated after treatment with rmMFG-E8 as evidenced by improved tissue injury and decreased neutrophil activivty. While the proinflammatory cytokines were generally suppressed in the small intestine, lungs, and blood after treatment with rmMFG-E8, there was no difference in the cytokine levels of the intestine and the blood between MFG-E8 KO mice and their WT controls 4h after reperfusion of the ischemic bowel. The lungs, however, demonstrated a 2-fold increase of IL-6 and IL-1β in the MFG-E8 KO mice indicating that in this model deficiency of MFG-E8 mostly affects the lungs. This is quite interesting as this implies that the lungs are not only a victim of the systemic inflammatory response but greatly contribute to the inflammation.

[0061] How this anti-inflammatory effect of MFG-E8 works in this ALI model still remains unknown. The clearance of apoptotic cells clearly suppresses the inflammatory responsiveness of macrophages (17, 31). As alveolar macrophages are potentially the cells that lose MFG-E8 after I/R, these may be primed by the deficit of the suppressive effect of apoptotic cell phagocytosis to produce and release more inflammatory cytokines (17). This could explain the increased inflammation and injury in the lungs and the release of inflammatory mediators into the circulation. The mechanism could involve intracellular anti-inflammatory pathways in the alveolar macrophages, the release of anti-inflammatory cytokines (such as IL-10 and TGF-β) (31, 37) and the generation of immunosuppressive cells, such as regulatory T cells (26, 35). The mere removal of dying cells that have the potential to release toxic and proinflammatory contents may be another potential mechanism by which MFG-E8 confers its beneficial effect (38).

[0062] The present studies have clearly shown that MFG-E8 is anti-inflammatory *in vivo* and protects from multiple organ dysfunctions including lung injury after gut I/R. This is

associated with a significantly improved chance of survival. 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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Appendix - SEQ ID NOs

SEQ ID NO:1 - human MFG-E8 - from GenBank NP005919

1 mprprllaal cgallcapsl lvaldicskn pchngglcee isqevrgdvf psytctclkg
61 yagnhcetkc veplgmengn iansqiaass vrvtflglqh wvpelarlnr agmvnawtps
121 snddnpwiqv nllrrmwvtg vvtqgasrla sheylkafkv ayslnghefd fihdvnkkhk
181 efvgnwnkna vhvnlfetpv eaqyvrlypt schtactlrf ellgcelngc anplglknns
241 ipdkqitass syktwglhlf swnpsyarld kqgnfnawva gsygndqwlq vdlgsskevt
301 giitqgarnf gsvqfvasyk vaysndsanw teyqdprtgs skifpgnwdn hshkknlfet
361 pilaryvril pvawhnrial rlellgc

SEQ ID NO:2 - mouse MFG-E8 - from GenBank NP032620

1 mqvsrvlaal cgmllcasgl faasgdfcds slclnggtcl tgqdndiycl cpegftglvc
61 netergpcsp npcyndakcl vtldtqrgdi fteyicqcpv gysgihcete tnyynldgey
121 mfttavpnta vptpaptpdl snnlasrcst qlgmeggaia dsqisasyvy mgfmglqrwg
181 pelarlyrtg ivnawhasny dskpwiqvnl lrkmrvsgvm tqgasragra eylktfkvay
241 sldgrkfefi qdesggdkef lgnldnnslk vnmfnptlea qyirlypvsc hrgctlrfel
301 lgcelhgcle plglknntip dsqmsasssy ktwnlrafgw yphlgrldnq gkinawtaqs
361 nsakewlqvd lgtqrqvtgi itqgardfgh iqyvesykva hsddgvqwtv yeeqgsskvf
421 qgnldnnshk knifekpfma ryvrvlpvsw hnritlrlel lgc

The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. Use of milk fat globule epidermal growth factor-factor VIII (MFG-E8) for the preparation of a medicament for preventing or treating inflammation in a tissue after ischemia/reperfusion, wherein the MFG-E8 comprises an amino acid defined by sequence SEQ ID NO:1 or SEQ ID NO:2.

2. Use of milk fat globule epidermal growth factor-factor VIII (MFG-E8) for the preparation of a medicament for preventing or treating organ injury after ischemia/reperfusion, wherein the MFG-E8 comprises an amino acid defined by sequence SEQ ID NO:1 or SEQ ID NO:2.

3. Use of claim 2, wherein the organ is one or more of liver, lung, kidney, heart, or brain.

4. Use of claim 1, wherein the tissue is one or more of gastrointestinal tract, spinal cord or crushed limb.

5. Use of claim 2, wherein the organ injury is acute lung injury.

6. Use of milk fat globule epidermal growth factor-factor VIII (MFG-E8) for preventing or treating inflammation in a tissue after ischemia/reperfusion, wherein the MFG-E8 comprises an amino acid defined by sequence SEQ ID NO:1 or SEQ ID NO:2.

7. Use of milk fat globule epidermal growth factor-factor VIII (MFG-E8) for preventing or treating organ injury after ischemia/reperfusion, wherein the MFG-E8 comprises an amino acid defined by sequence SEQ ID NO:1 or SEQ ID NO:2.

8. Use of claim 7, wherein the organ is one or more of liver, lung, kidney, heart, or brain.

9. Use of claim 6, wherein the tissue is one or more of gastrointestinal tract,

spinal cord or crushed limb.

10. Use of claim 7, wherein the organ injury is acute lung injury.

11. Use of any one of claims 1 to 10, wherein the MFG-E8 is recombinant MFG-E8.

12. Use of any one of claims 1 to 11, wherein the ischemia/reperfusion is one or more of gastrointestinal tract, liver, lung, kidney, heart, brain, spinal cord or crushed limb ischemia/reperfusion.

13. Use of claim 12, wherein the ischemia/reperfusion is intestinal ischemia/reperfusion.

14. A milk fat globule epidermal growth factor-factor VIII (MFG-E8) for use in preventing or treating inflammation in a tissue after ischemia/reperfusion, wherein the MFG-E8 comprises an amino acid defined by sequence SEQ ID NO:1 or SEQ ID NO:2.

15. A milk fat globule epidermal growth factor-factor VIII (MFG-E8) for use in preventing or treating organ injury after ischemia/reperfusion, wherein the MFG-E8 comprises an amino acid defined by sequence SEQ ID NO:1 or SEQ ID NO:2.

16. The milk fat globule of claim 15, wherein the organ is one or more of liver, lung, kidney, heart, or brain.

17. The milk fat globule of claim 14, wherein the tissue is one or more of gastrointestinal tract, spinal cord or crushed limb.

18. The milk fat globule of claim 15, wherein the organ injury is acute lung injury.

19. The milk fat globule of any one of claims 14 to 18, wherein the MFG-E8 is recombinant MFG-E8.

20. The milk fat globule of any one of claims 14 to 19, wherein the ischemia/reperfusion is one or more of gastrointestinal tract, liver, lung, kidney, heart, brain, spinal cord or crushed limb ischemia/reperfusion.

21. The milk fat globule of claim 20, wherein the ischemia/reperfusion is intestinal ischemia/reperfusion.

22. A pharmaceutical composition comprising a pharmaceutically acceptable diluent or carrier and milk fat globule epidermal growth factor VIII (MFG-E8) for use in preventing or treating inflammation in a tissue after ischemia/reperfusion, wherein the MFG-E8 comprises an amino acid defined by sequence SEQ ID NO:1 or SEQ ID NO:2.

23. A pharmaceutical composition comprising a pharmaceutically acceptable diluent or carrier and milk fat globule epidermal growth factor VIII (MFG-E8) for use in preventing or treating organ injury after ischemia/reperfusion, wherein the MFG-E8 comprises an amino acid defined by sequence SEQ ID NO:1 or SEQ ID NO:2.

24. The pharmaceutical composition of claim 23, wherein the organ is one or more of liver, lung, kidney, heart, or brain.

25. The pharmaceutical composition of claim 22, wherein the tissue is one or more of gastrointestinal tract, spinal cord or crushed limb.

26. The pharmaceutical composition of claim 23, wherein the organ injury is acute lung injury.

27. The pharmaceutical composition of any one of claims 22 to 26, wherein the MFG-E8 is recombinant MFG-E8.

28. The pharmaceutical composition of any one of claims 22 to 27, wherein the ischemia/reperfusion is one or more of gastrointestinal tract, liver, lung, kidney, heart, brain, spinal cord or crushed limb ischemia/reperfusion.

29. The pharmaceutical composition of claim 28, wherein the ischemia/reperfusion is intestinal tract ischemia/reperfusion.

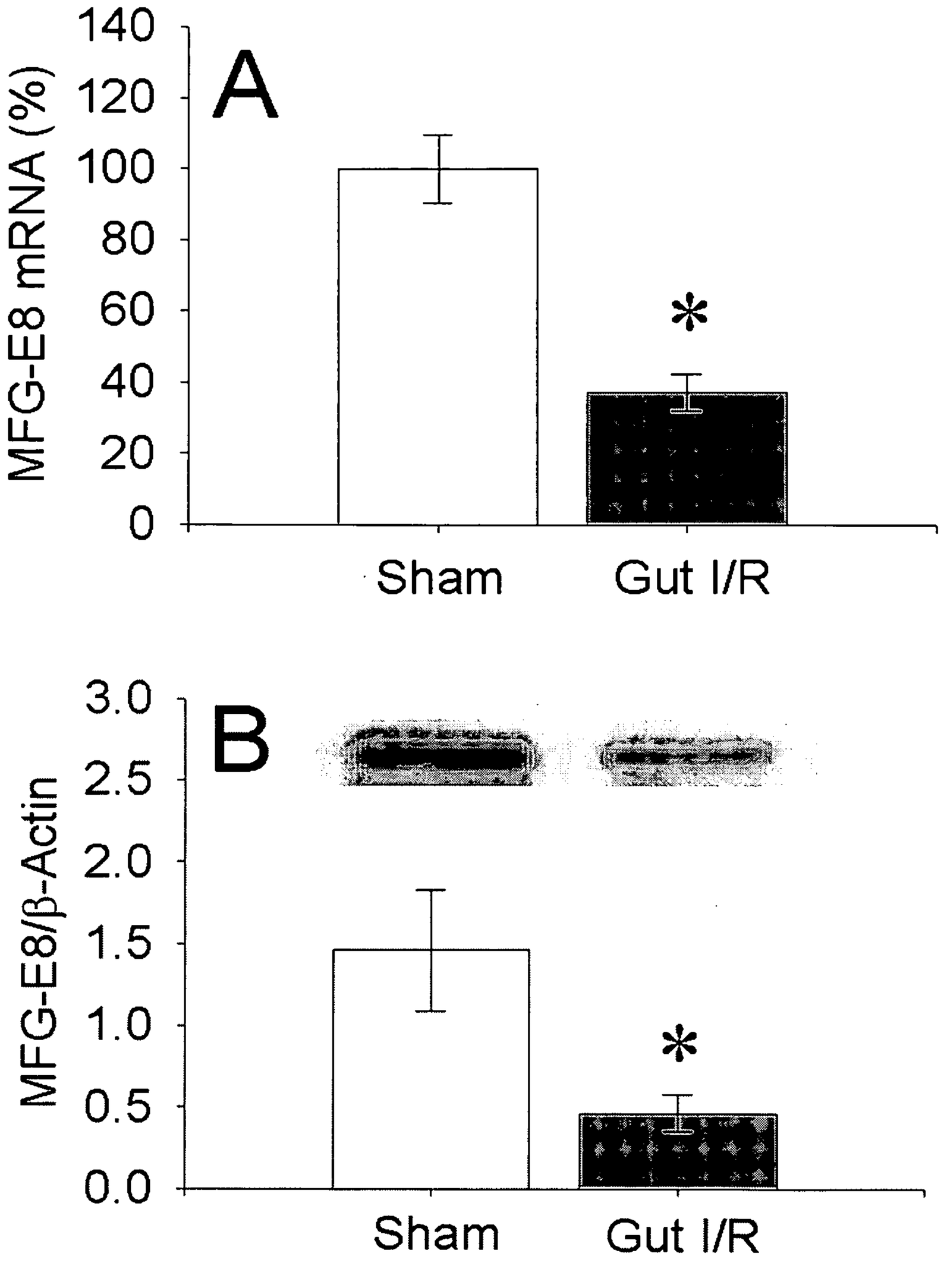
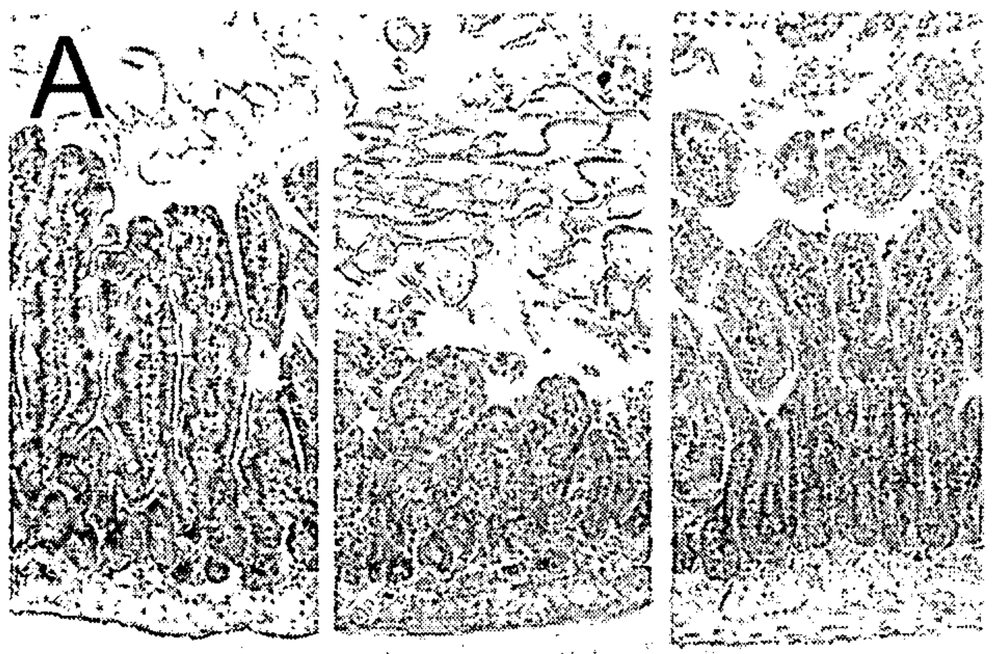
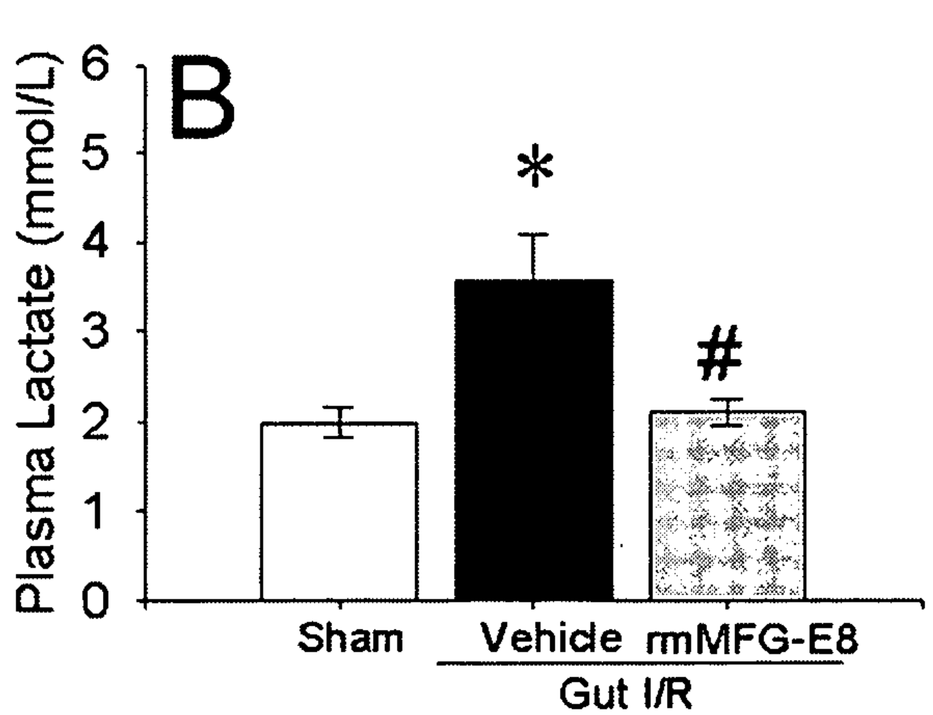
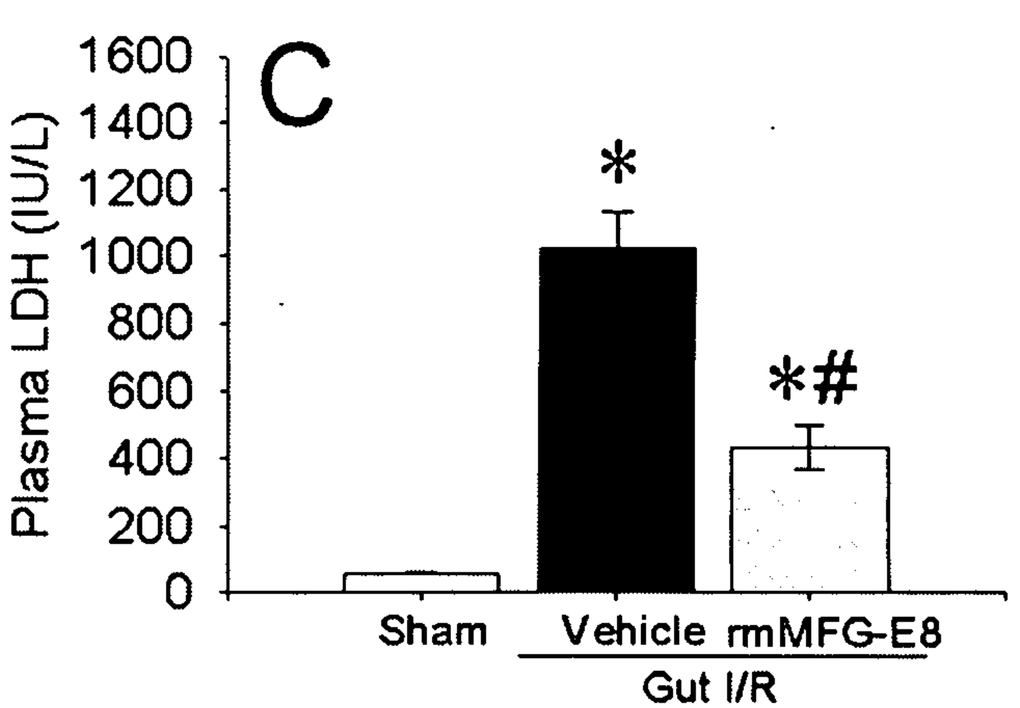
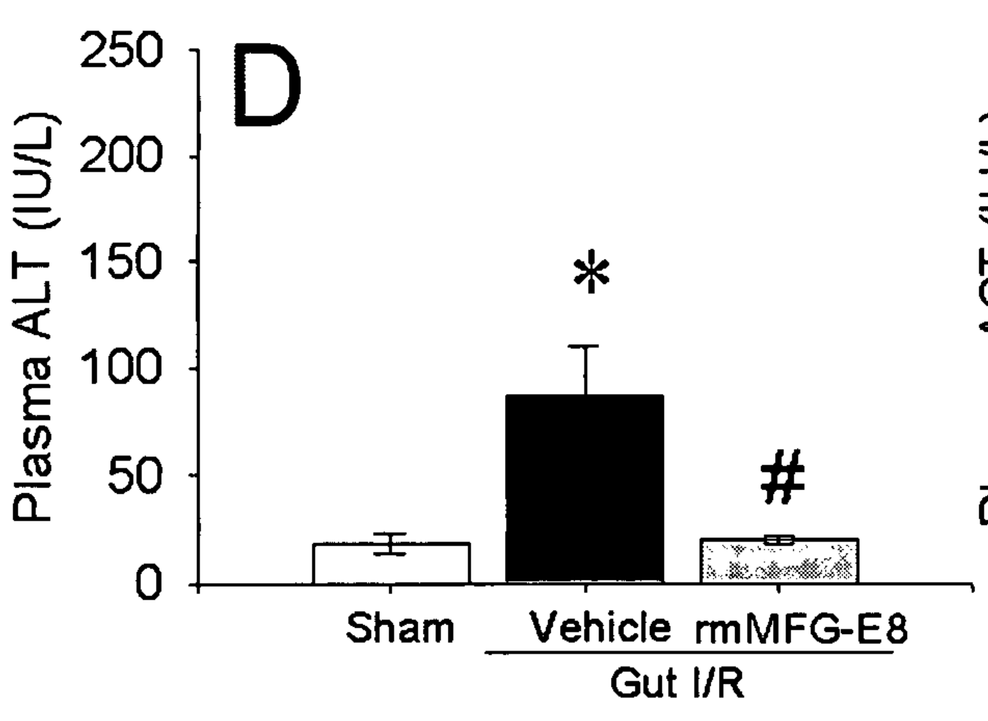

A
MFG-E8 mRNA (%)
140
120
100
80
60
40
20
0
*
Sham
Gut I/R
B
MFG-E8/β-Actin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Sham
Gut I/R

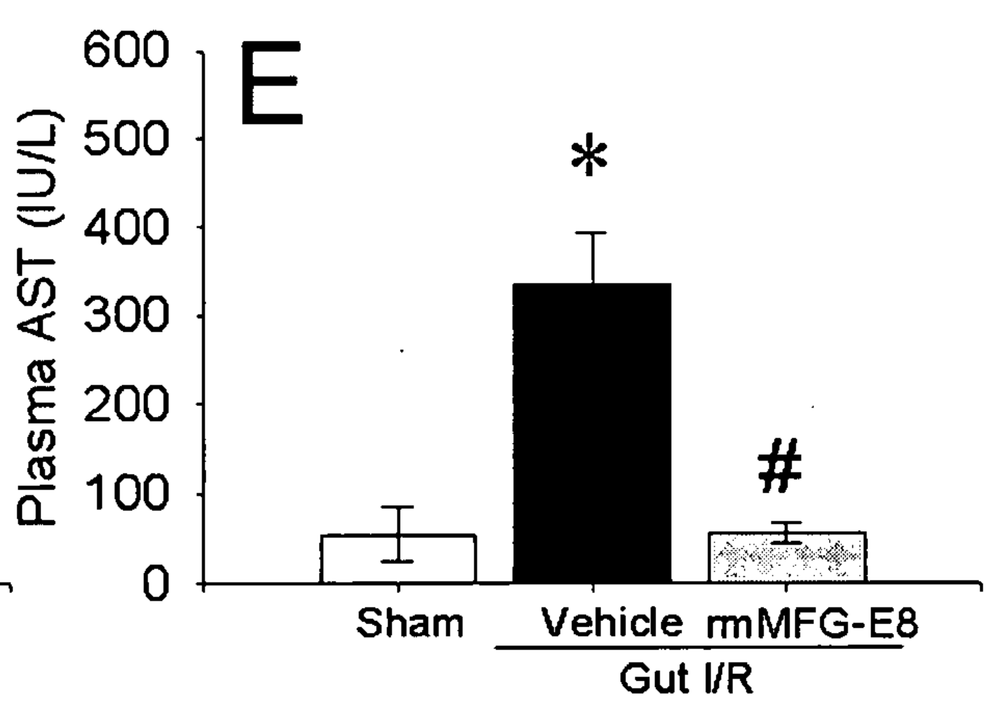
FIGURE 1A-1B


A


B
Plasma Lactate (mmol/L)
0
1
2
3
4
5
6
*
#
Sham
Vehicle
rmMFG-E8
Gut I/R


C
Plasma LDH (IU/L)
0
200
400
600
800
1000
1200
1400
1600
*
*#
Sham
Vehicle
rmMFG-E8
Gut I/R


D
Plasma ALT (IU/L)
0
50
100
150
200
250
*
#
Sham
Vehicle
rmMFG-E8
Gut I/R


E
Plasma AST (IU/L)
0
100
200
300
400
500
600
*
#
Sham
Vehicle
rmMFG-E8
Gut I/R

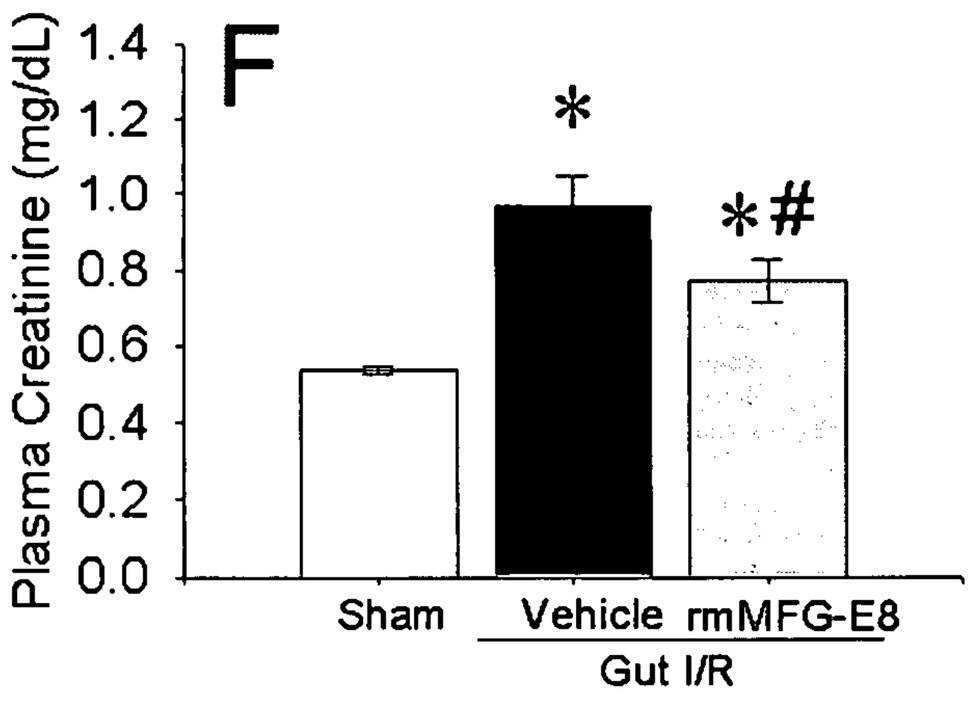

F
Plasma Creatinine (mg/dL)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
*
*#
Sham
Vehicle
rmMFG-E8
Gut I/R

FIGURE 2A-2F

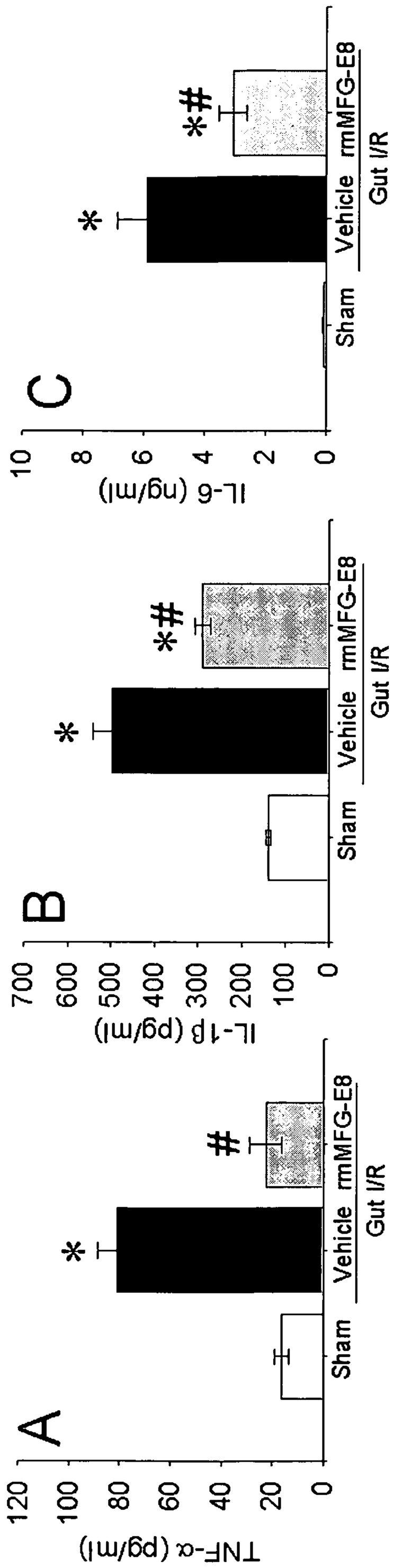

A
TNF-α (pg/ml)
120
100
80
60
40
20
0
Sham
Vehicle
rmMFG-E8
Gut I/R
*
#
B
IL-1β (pg/ml)
700
600
500
400
300
200
100
0
Sham
Vehicle
rmMFG-E8
Gut I/R
*
#
C
IL-6 (ng/ml)
10
8
6
4
2
0
Sham
Vehicle
rmMFG-E8
Gut I/R
*
*#

FIGURE 3A-3C

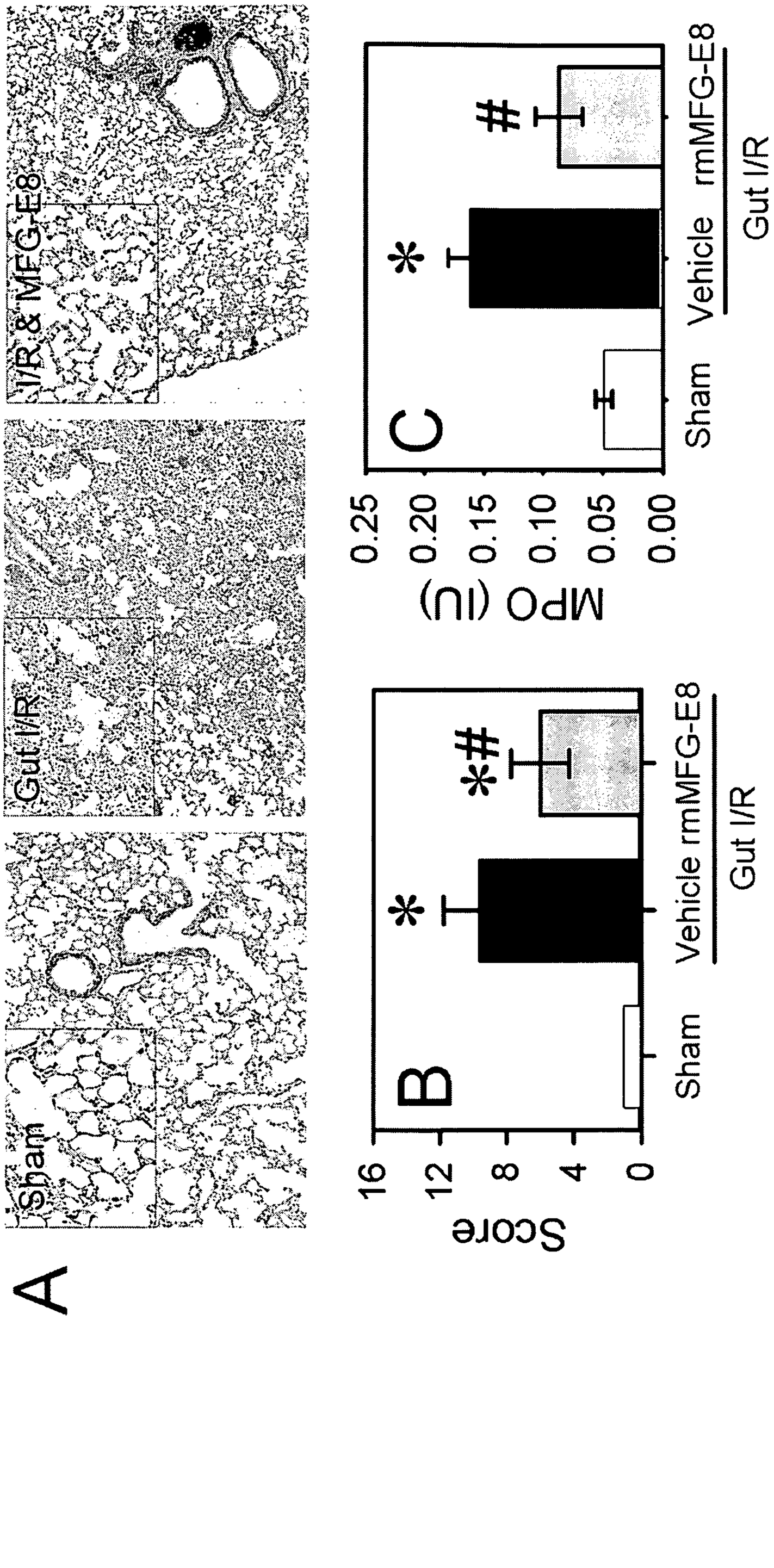

Sham
Gut I/R
Gut I/R & MFG-E8
A
Score
16
12
8
4
0
B
*
*#
Sham
Vehicle
rmMFG-E8
Gut I/R
MPO (IU)
0.25
0.20
0.15
0.10
0.05
0.00
C
*
Sham
Vehicle
rmMFG-E8
Gut I/R

FIGURE 4A-4C

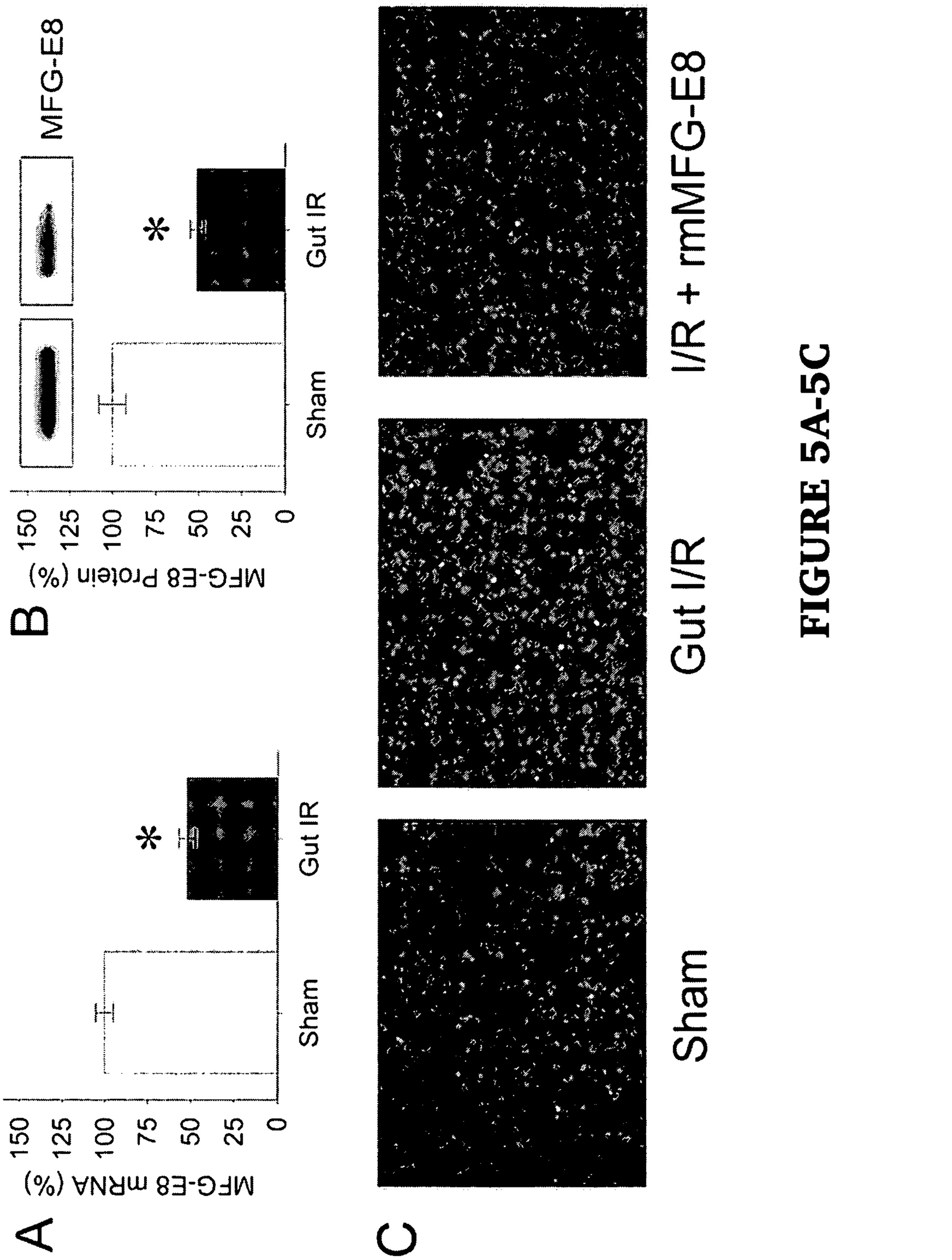
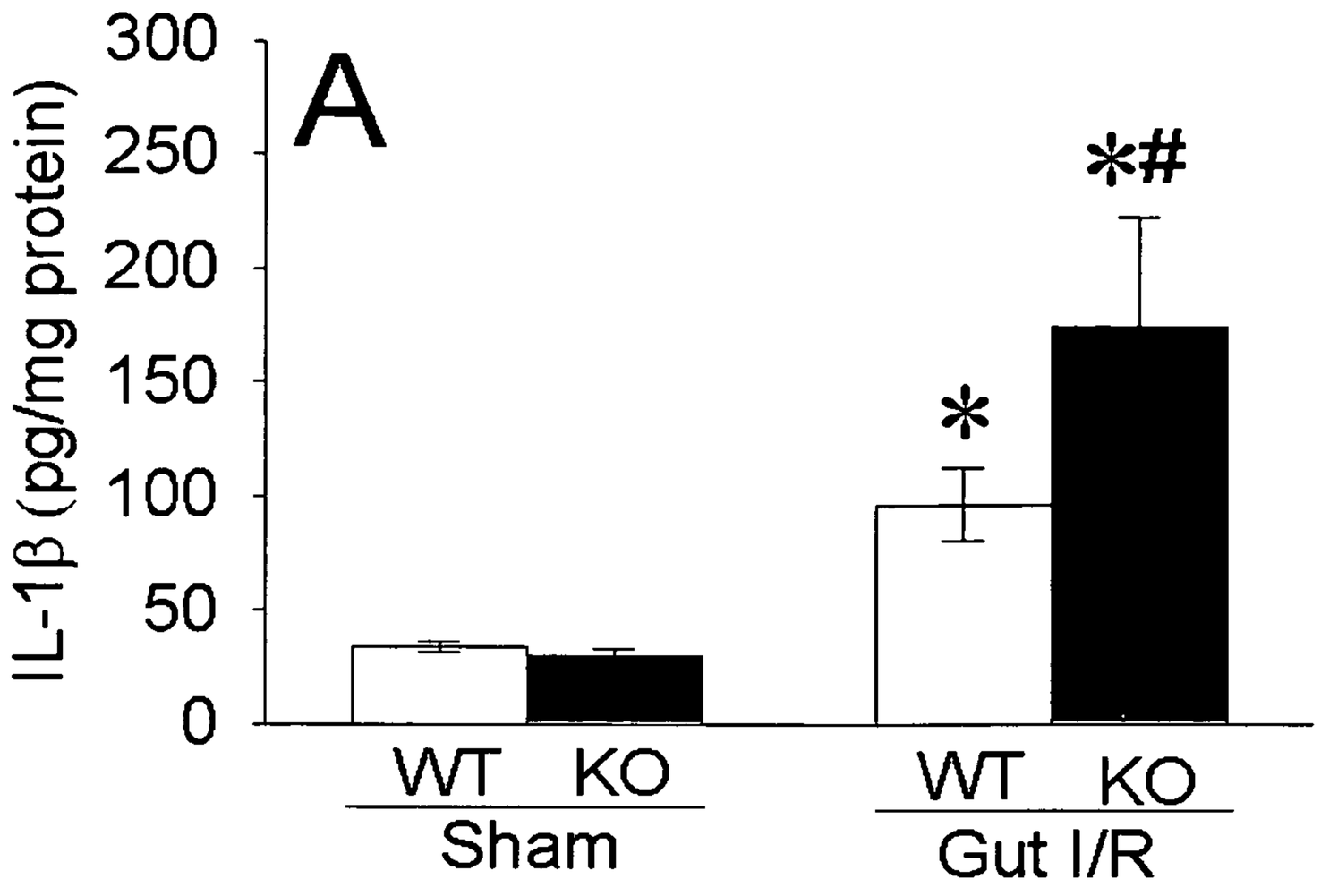

A
MFG-E8 mRNA (%)
150
125
100
75
50
25
0
*
Sham
Gut IR
B
MFG-E8 Protein (%)
150
125
100
75
50
25
0
*
Sham
Gut IR
C
Sham
Gut I/R
I/R + rmMFG-E8
MFG-E8

FIGURE 5A-5C


A
IL-1β (pg/mg protein)
300
250
200
150
100
50
0
*
*#
WT
KO
WT
KO
Sham
Gut I/R

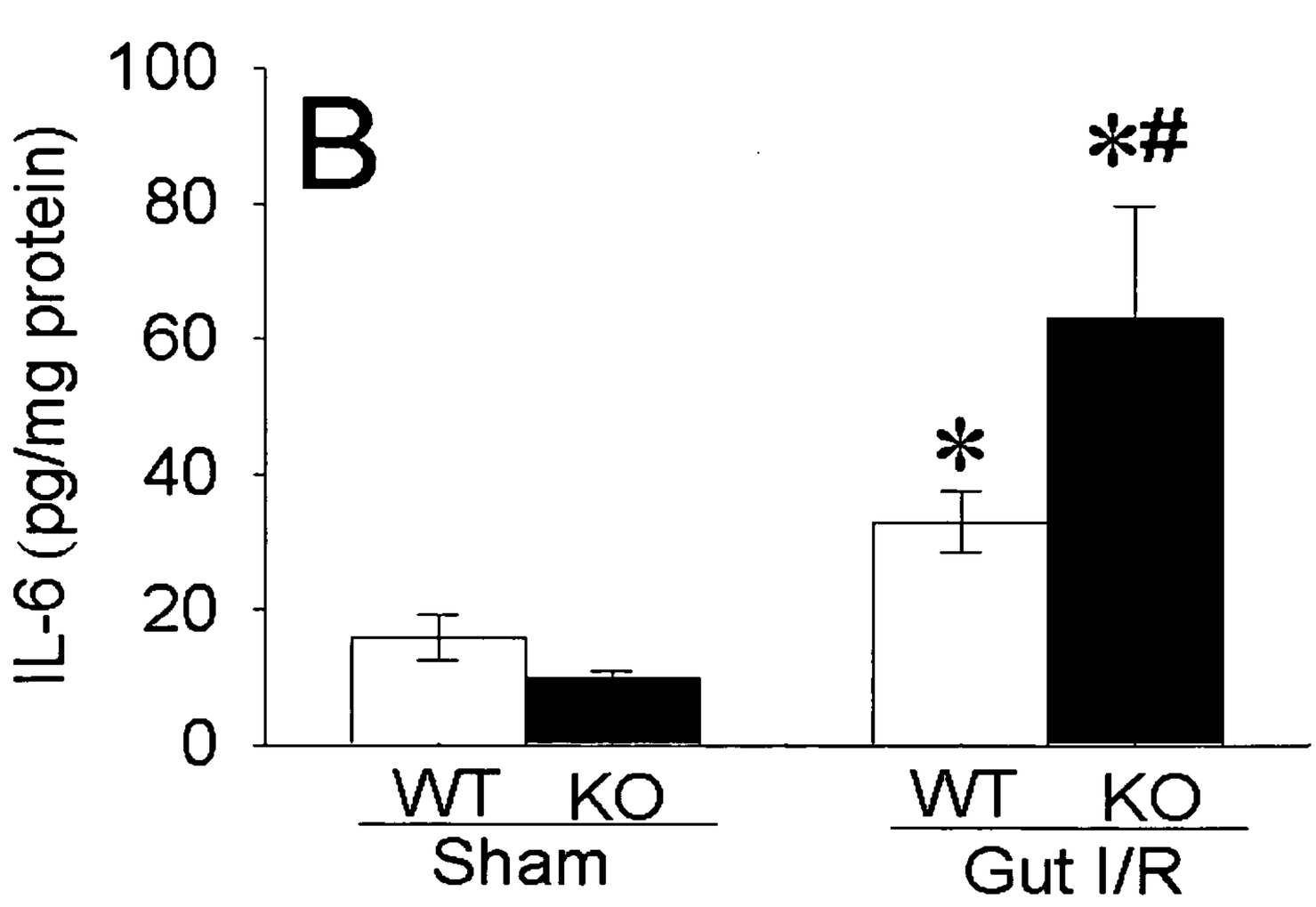

B
IL-6 (pg/mg protein)
100
80
60
40
20
0
*
*#
WT
KO
WT
KO
Sham
Gut I/R

FIGURE 6A-6B

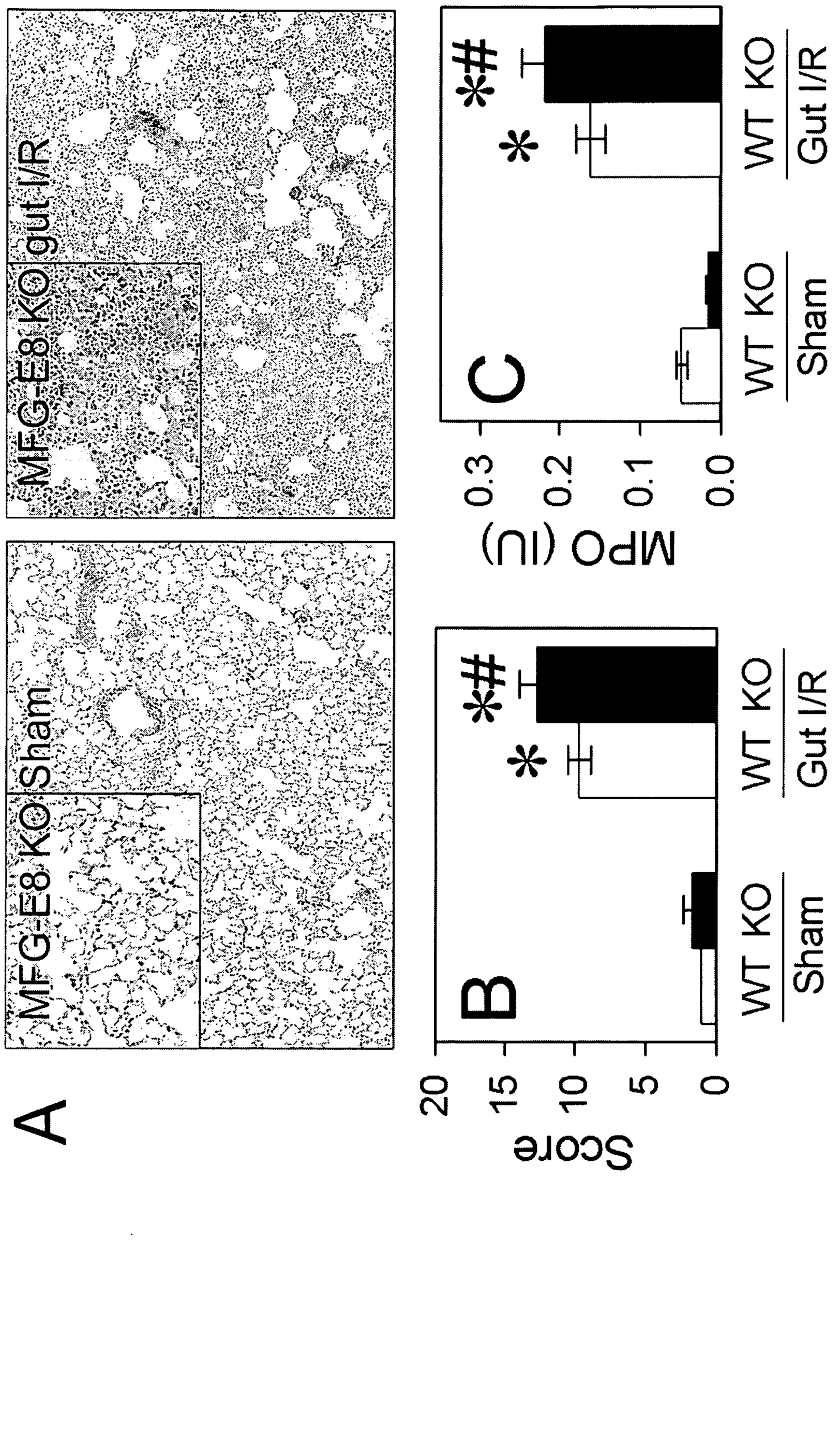

A
MFG-E8 KO gut I/R
MFG-E8 KO Sham
B
Score
20
15
10
5
0
WT KO
Sham
WT KO
Gut I/R
*
*#
C
MPO (IU)
0.3
0.2
0.1
0.0
WT KO
Sham
WT KO
Gut I/R
*
*#

FIGURE 7A-7C

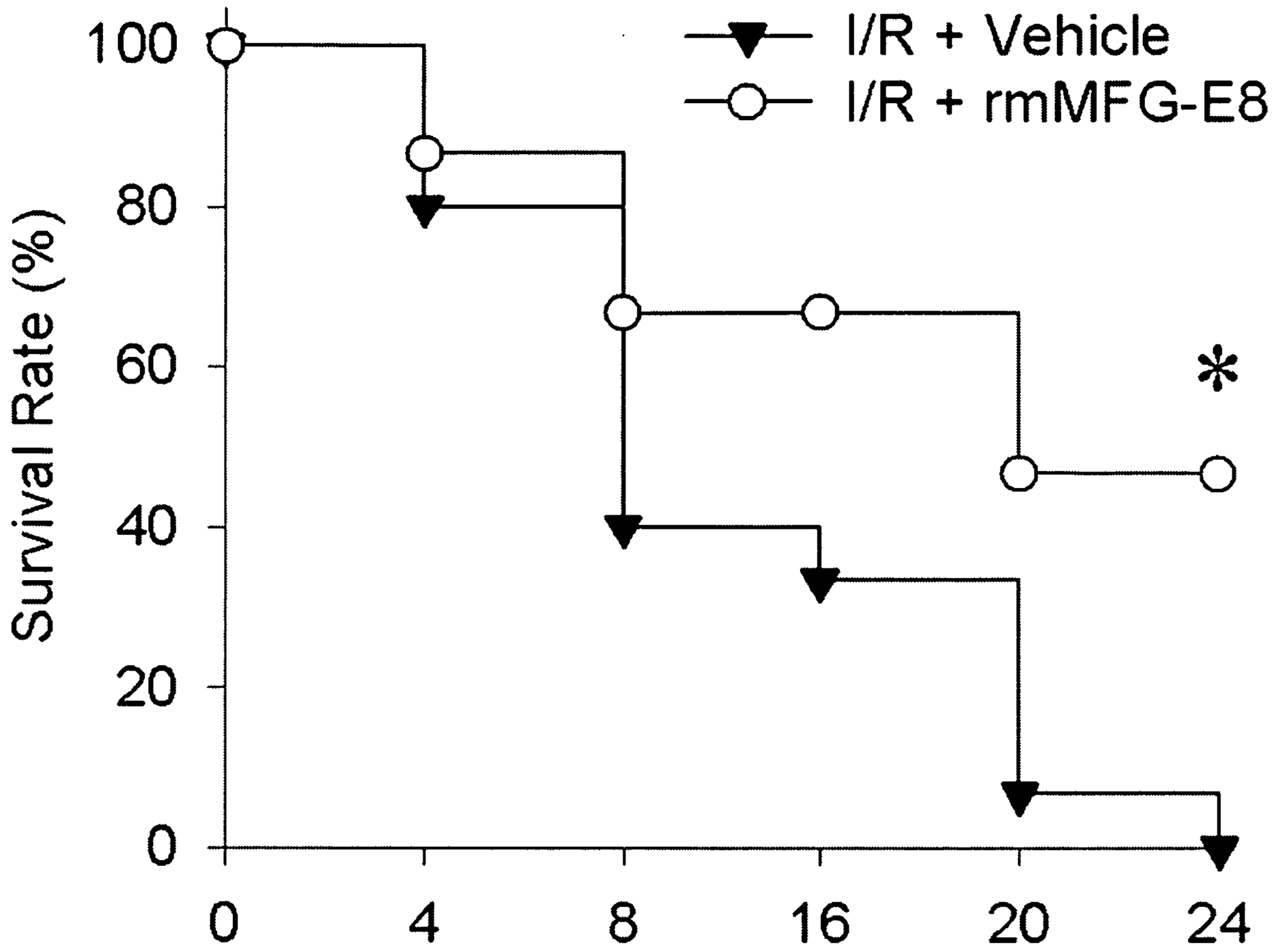

I/R + Vehicle
I/R + rmMFG-E8
Survival Rate (%)
100
80
60
40
20
0
*
0
4
8
16
20
24
Hours after Intestinal I/R

FIGURE 8