MFG-E8 and HMGB1 Are Involved in the Mechanism Underlying Alcohol-Induced Impairment of Macrophage Efferocytosis

By: Xiao Wang, Heng-Fu Bu, Wei Zhong, Akihiro Asai, Zhanxiang Zhou, Xiao-Di Tan

Wang X, Bu HF, Zhong W, Asai A, Zhou Z, Tan XD. MFG-E8 and HMGB1 are involved in the mechanism underlying alcohol-induced impairment of macrophage efferocytosis. ***Mol Med***. 2013; 19(1): 170-182.

Made available courtesy of Feinstein Institute for Medical Research:
http://dx.doi.org/10.2119/molmed.2012.00260

Abstract:

Efferocytosis is a unique phagocytic process for macrophages to remove apoptotic cells in inflammatory loci. This event is maintained by milk fat globule-EGF factor 8 (MFG-E8), but attenuated by high mobility group box 1 (HMGB1). Alcohol abuse causes injury and inflammation in multiple tissues. It alters efferocytosis, but precise molecular mechanisms for this effect remain largely unknown. Here, we showed that acute exposure of macrophages to alcohol (25 mmol/L) inhibited MFG-E8 gene expression and impaired efferocytosis. The effect was mimicked by hydrogen peroxide. Moreover, N-acetylcysteine (NAC), a potent antioxidant, blocked acute alcohol effect on inhibition of macrophage MFG-E8 gene expression and efferocytosis. In addition, recombinant MFG-E8 rescued the activity of alcohol-treated macrophages in efferocytosis. Together, the data suggest that acute alcohol exposure impairs macrophage efferocytosis via inhibition of MFG-E8 gene expression through a reactive oxygen species dependent mechanism. Alcohol has been found to suppress or exacerbate immune cell activities depending on the length of alcohol exposure. Thus, we further examined the role of chronic alcohol exposure on macrophage efferocytosis. Interestingly, treatment of macrophages with alcohol for seven days in vitro enhanced MFG-E8 gene expression and efferocytosis. However, chronic feeding of mice with alcohol caused increase in HMGB1 levels in serum. Furthermore, HMGB1 diminished efferocytosis by macrophages that were treated chronically with alcohol, suggesting that HMGB1 might attenuate the direct effect of chronic alcohol on macrophage efferocytosis in vivo. Therefore, we speculated that the balance between MFG-E8 and HMGB1 levels determines pathophysiological effects of chronic alcohol exposure on macrophage efferocytosis in vivo.

Keywords: macrophage efferocytosis | Alcohol | MFG-E8 | HMGB1

Article:

INTRODUCTION

Many cells undergo apoptosis at sites of inflammation. Apoptotic cells are timely removed by phagocytes such as macrophages through a phagocytic process known as efferocytosis (1,2). Clearance of apoptotic cells prevents progression of dying cells to secondary necrosis and, in turn, avoid the release of potentially noxious or immunogenic intracellular contents that amplify inflammation and cause damage to surrounding tissue (3). In addition, phagocytes increase their capacity to synthesize antiinflammatory molecules such as TGF-β, IL-10 and PGE2 after ingestion of apoptotic cells (4,5). Therefore, removal of apoptotic cells by phagocytes limits inflammatory responses and accelerates resolution of inflammation.

Efferocytosis is a dynamic process which consists of recognition and engulfment of apoptotic cells by phagocytes (6). Recognition of dying cells is an early step of efferocytosis that is mediated by a group of bridging molecules and efferocytic receptors (7). It triggers distinctive signal events in phagocytes and then induces cytoskeletal rearrangement as well as uptake of apoptotic cells. Rac1 and RhoA, two small Rho GTPases, have been demonstrated to play opposing roles in regulating engulfment of apoptotic cells: Rac1 enhances, while RhoA and Rho kinase (ROCK, a downstream effector of RhoA) inhibit the process (6).

Evidence has shown that alcohol abuse causes cellular injury and excessive cell death in liver, lung epithelium and myoblastic cells (8–10). It exhibits a variety of effects on immune cell function (11). Acute alcohol exposure profoundly inhibits phagocytosis and killing of bacteria by macrophages (12). Recently, Boé *et al*. demonstrated that alcohol exposure results in impairment of efferocytosis, a process with characteristics that are distinct from engulfment of microorganisms (13). The effect of alcohol on inhibiting efferocytosis is partially mediated by RhoA-independent activation of ROCK (13). However, it remains unclear whether alcohol targets bridging molecules and subsequently disrupts efferocytosis.

Milk fat globule-EGF factor 8 protein (MFG-E8) is a glycoprotein secreted by macrophages (14). It is a well-studied bridging molecule for clearance of apoptotic cells by macrophages (15,16). MFG-E8 plays an important role in maintaining tissue homeostasis and promoting tissue repair (15–21). Previously, we and others have shown that inflammation is associated with decrease in MFG-E8 levels (17,22). Impairment of *MFG-E8* gene expression attributes to delaying tissue repair (16,17,23). Recently, efferocytosis has been revealed to be abolished by high mobility group box 1 (HMGB1), a potent inflammatory mediator (24). The role of MFG-E8 and HMGB1 in resolution of alcohol-induced tissue injury is yet to be determined.

In the present study, we investigated whether alcohol exposure influences *MFG-E8* gene expression in macrophages, if so, whether alcohol-induced change of MFG-E8 levels contributes to alteration of macrophage efferocytosis. We found that acute alcohol exposure inhibited *MFG-E8* gene expression in macrophages via an oxidant-dependent mechanism and, in turn, attenuated

macrophage efferocytosis. By contrast, chronic alcohol exposure *in vitro* enhanced macrophage *MFG-E8* gene expression with increase in efferocytosis. On the other hand, chronic alcohol feeding of mice was found to cause an increase in serum HMGB1 levels, which might block efferocytosis maintained by MFG-E8 *in vivo*. These results advanced our knowledge on understanding how alcohol exposure precisely regulates efferocytosis.

MATERIALS AND METHODS

Animals

C57BL/6 wild-type mice (male, 8–10 wks old) were purchased from the Jackson Laboratory (Bar Harbor, MN, USA) or Harlan (Indianapolis, IN, USA). They were housed in a specific pathogen-free animal facility. All animal experiments were conducted according to the experimental procedures approved by the Institutional Animal Care and Use Committees of Ann and Robert H. Lurie Children's Hospital of Chicago Research Center as well as University of North Carolina at Greensboro.

Materials

All cell culture media were obtained from Life Technologies (Carlsbad, CA, USA) or American Type Culture Collection (ATCC, Manassas, VA, USA). *N*-acetylcysteine (NAC), H_2O_2, regular chemicals and molecular biology reagents were purchased from Sigma Chemical Company and/or Sigma-Aldrich (St. Louis, MO, USA). Alcohol was obtained from AAPER Alcohol and Chemical Company (Shelbyville, KY, USA). Recombinant murine MFG-E8 was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant HMGB1 was provided by eBioscience (San Diego, CA, USA). NAC was dissolved with culture medium to prepare 0.6 mol/L stock solution and the extreme acidity of this stock solution was adjusted to pH 7.0 with NaOH in advance.

Rodent Macrophage Cell Lines and Cell Culture

RAW 264.7 cells were purchased from ATCC. Rat Kupffer cell–derived macrophage cell line, namely RKC1, was obtained from Michel M Murr at the University of South Florida (25). All cells were cultured and maintained in a water-saturated atmosphere with 5% CO_2 at 37°C in Dulbecco's modified Eagle minimum essential medium containing 50 U/mL penicillin, 50 μg/mL streptomycin and 10% fetal bovine serum.

Isolation of Peritoneal Macrophages

We routinely used five mice for preparation of peritoneal macrophages in each experiment. The protocol described by Gordon *et al*. was used (26,27). First, C57BL/6 mice (8 wks old, Jackson Laboratory) were injected intraperitoneally (IP) with 1.5 mL of 3% (w/v) thioglycollate (Sigma) for macrophages elicitation. After 4 d, mice were euthanized with inhalation of CO_2 and used for isolation of peritoneal macrophages as described before (28). Briefly, the peritoneum was

lavaged with cold serum-free Dulbecco's modified Eagle medium (DMEM) several times. The exudate cells were washed, plated at 1×10^6cells/mL on 6-well plates in DMEM with 10% FBS and antibiotics, and incubated for 2 h at 37°C in a humidified air atmosphere containing 5% CO_2. At the end, nonadherent cells were removed by washing plates with culture medium. Adherent cells were then cultured in DMEM containing 10% FBS overnight and used for various experiments.

Exposure of Macrophages to Alcohol

Primary peritoneal macrophages, RKC1 and RAW 264.7 cells were used. For acute alcohol exposure, cells in the experimental groups were treated with culture medium containing alcohol (25 mmol/L) for 18 h. The 25 mmol/L *in vitro* ethanol concentration approximates a 0.1 g/dl blood alcohol level, which is achieved *in vivo* after a dose of moderate drink and is a little above the legal limit of blood alcohol concentration (29). For chronic alcohol exposure, macrophages were treated with medium containing 50 mmol/L alcohol for 7 d and the medium was changed in 2 d intervals. The technique described by Szabo and his associates was applied to avoid the fluctuation of alcohol concentration due to evaporation (29–31). Briefly, cell culture plates were placed in an Incubator Culture Chamber manufactured by C.B.S. Scientific (Del Mar, CA, USA) with twice the alcohol concentration in the bottom of the chamber to saturate the chamber and maintain the alcohol concentration in the wells for the entire duration of stimulation. In each experiment, a group of macrophages that was pair cultured with alcohol-free medium was used as the control.

Analysis of Efferocytosis with Terminal Deoxynucleotidyl Transferase Nick End Labeling (TUNEL) Staining

The procedure consisted of phagocytosis of apoptotic thymocytes by macrophages and detection of macrophages with engulfed apoptotic cells by TUNEL staining. In brief, peritoneal macrophages were plated at a density of 5×10^4/well on 8-well chamber slides, cultured in complete culture medium for 1 d and treated as described in experimental designs. Apoptosis was induced on freshly isolated mouse thymocytes by treatment of cells with 10 μmol/L dexamethasone for 24 h using a protocol described by Torres-Roca *et al*. (32). Before the phagocytosis assay, macrophages and apoptotic thymocytes were washed several times with fresh serum-free medium. Then, apoptotic thymocytes at the ratio of 4:1 (thymocytes:macrophages) were added to culture plates containing macrophages. The plates were incubated at 37°C for 1.5 h. At the end of incubation, culture plates were washed thoroughly three times with PBS to remove loosely adherent thymocytes. Then, macrophages were fixed briefly with 4% paraformaldehyde, permeabilized and processed for labeling of engulfed apoptotic cells with fluorescein isothiocyanate (FITC)-labeled deoxyuridine triphosphate (dUTP) using a TUNEL staining kit (Roche, San Francisco, CA, USA). The protocol provided by the manufacturer was followed. After staining, slides were counterstained

with 4′,6-diamidino-2-phenylindole (DAPI) for visualizing macrophage nuclei, washed thoroughly, and examined under a fluorescent microscope.

Quantitative Analysis of Efferocytosis with Fluorescence-Activated Cell Sorting (FACS)

First, murine peritoneal macrophages were processed for efferocytosis of apoptotic thymocytes as described above. Then, macrophages were thoroughly washed, collected and processed for labeling of macrophages and engulfed apoptotic cells at 4°C. Briefly, cells were blocked with mouse BD Fc Blocker (purified anti-mouse CD16/CD32 mAb; BD Biosciences, San Jose, CA, USA) in FACS staining buffer (2% fetal calf serum [FCS] in phosphate-buffered saline [PBS]) for 10 min, incubated with biotin-conjugated anti-F4/80 mAb (murine macrophage marker; AbD Serotec, Kidilington, UK) for 40 min and allophycocyanin (APC)-labeled streptavidin for 40 min. At the end, we washed cells with PBS, fixed them with 4% paraformaldehyde and followed by permeabilization of fixed macrophages and labeling of engulfed apoptotic cells with FITC-labeled dUTP using a TUNEL staining kit as described above. Finally, stained cells were washed, suspended in FACS buffer and analyzed by FACSCalibur flow cytometer (BD Biosciences). To determine the baseline of spontaneous apoptotic macrophages, a portion of naive macrophages also were stained and processed for FACS analysis. Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Protein Extraction and Western Blot

We used our standard protocol for isolation of total protein from macrophages and immunoblotting (28). Protein extracts (15 μg) were fractionated in NuPAGE 4% to 12% Bis-Tris Gels (precast polyacrylamide gels purchased from Invitrogen/Life Technologies) followed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes. Goat polyclonal antibody against murine MFG-E8 (1:800, R&D Systems) was used to detect MFG-E8 protein in blots. Horseradish peroxidase-conjugated anti-goat IgG polyclonal antibody (1:3,000, Life Technologies) was used as the secondary antibody. After washing with PBS-T, membranes were treated with a development solution supplied in ESL plus kit (Thermo Scientific, Rockford, IL, USA), scanned with Tynphoon 7000 (GE Healthcare, Piscataway, NJ, USA) and analyzed with Image Quant TL7.0 software (IQ, GE Healthcare).

RNA Extraction (23)

Total RNA from macrophages was extracted using the RNeasy kit (QIAGEN, Valencia, CA, USA) according to a protocol provided by the manufacturer. RNA quantity was determined using a Smart Spec plus spectrophotometer (Bio-Rad, Hercules, CA, USA) by measuring optical density at A260 nm wavelength.

cDNA Synthesis and Quantitative Real-Time PCR

Our standard protocol was used (23,33). Briefly, cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) according to a protocol provided by the manufacturer. Total cellular RNA (0.7 μg) from each sample was added to 20 μL of reaction mixture that contains deoxyribonucleotide triphosphates (dNTP) mix, RNase inhibitor, 1× mixture of oligo (dT) and random hexamer primers, 1× reaction buffer, 1 μL of Moloney murine leukemia virus (MMLV)-derived iScript reverse transcriptase. The reaction was run at 25°C for 5 min, 42°C for 30 min and stopped by incubation at 85°C for 5 min. The resulting cDNA was used for the following quantitative real-time PCR procedure.

Specifically, QuantiTect SYBR Green PCR kit (QIAGEN) was used for the study. The reaction system in 25 μL contained mastermix, primers and 2 μL cDNA for each sample. Duplicate reactions were run using a Fast 7500 real-time PCR system (Life Technologies) under the following conditions: 50°C for 2 min; 95°C for 10 min; and then 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). All PCR reactions were performed in 96-well plates. The cycle at which each sample crossed a fluorescence threshold, C_T (at 0.1–0.2 fluorescence units), was determined. The duplicate values for each cDNA were averaged. Sequences for forward (F) and reverse (R) primers for real-time PCR were murine 18S rRNAF 5′-TGCCC TATCAACTTTCGATG-3′, murine 18S rRNAR 5′-GATGTGGTAGCCGTT TCTCA-3′, murine *MFG-E8*F 5′-ATCTA CTGCCTCTGCCCTGA-3′ and murine *MFG-E8*R 5′-CCAGACATTTGGCAT CATTG-3′. Fold changes in expression levels of *MFG-E8*mRNA in each treatment group were calculated with the $2^{-\Delta\Delta}C_T$ method using 18S rRNA as the internal reference (34). The $\Delta\Delta C_T$ value is defined as the C_T difference between the normalized amount of sample and the normalized amount of calibrator.

Generation of Luciferase Reporter Construct and Analysis of *MFG-E8* Promoter Activity

First, we isolated mouse genomic DNA using genomic DNA purification system (Promega, Madison, WI, USA). Then, we obtained a 590-bp DNA fragment containing mouse *MFG-E8* promoter (−444 to +146 in mouse *MFG-E8* gene) using the genomic DNA as a template via a PCR approach as described previously (35). The purified 590-bp DNA fragment was cloned unidirectionally into the pGL3 basic plasmid by using a rapid DNA Dephos and ligation kit (Roche). The characteristic of the inserted DNA fragment was confirmed by DNA sequencing. The new luciferase reporter plasmid was named as pGL3-mfge8$^{-444/+146}$. The activity of pGL3-mfge8$^{-444/+146}$ was verified with a luciferase assay (X Wang, unpublished data).

RAW 264.7 cells and antibiotic-free culture medium were used for experiments. For analysis of *MFG-E8*promoter activity, cells were plated at 0.4×10^5 cells per well in 24-well plates 1 d prior transfection. Then, they were cotransfected with 0.1 μg of pRL-null reporter plasmid and 0.4 μg of pGL3-mfge8$^{-444/+146}$ reporter plasmid using Lipofectamine 2000 transfection reagent (Life Technologies) with the manufacturer's protocol. Twenty-four hours after transfections, cells were treated for an additional 18 h as experimental designs. Then, cells were processed for measuring luciferase activity using Dual-Luciferase Reporter Assay System (Promega) with a

protocol provided by the manufacturer. Wallac Victor2 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA) was used for measuring levels of bioluminescence generated in the assay. Levels of the firefly luciferase activity represented murine *MFG-E8* promoter activity, whereas the Renilla luciferase activity was used for normalization of the transfection efficiency. The luciferase activity of each construct was compared with that of the promoterless pGL3 basic vector.

Protocol for Examining the Effect of *In Vivo* Chronic Alcohol Exposure on HMGB1 Production

We treated mice with alcohol using our standard protocol described previously (36). Briefly, C57BL/6 mice from Harlan at 10 wks old were pair fed with a modified Lieber-DeCarli alcohol liquid diet containing either ethanol or isocaloric maltose dextrin as control for 8 wks. Ethanol concentration (%, w/v) in the liquid diet was 4.8% at the beginning to 5.4% at the end, increasing by 0.2% every 2 wks. At the end of feeding experiment, mice were anesthetized by isoflurane in conjunction with pure oxygen and blood was collected. After clotting, blood samples were centrifuged with an Eppendorff microcentrifuge at 8,000*g* for 15 min at 4°C. Then, serum was removed and stored at −80°C until used.

To determine HMGB1 levels in serum, an enzyme-linked immunosorbent assay (ELISA) kit for murine HMGB1 was obtained from IBL International (Hamburg, Germany). The protocol provided by the manufacturer was followed. Serum samples were used for the assay. Standard curve was generated using recombinant HMGB1 supplied with the assay kit.

Statistical Analysis

All experiments were performed at least twice. Data were expressed as means ± SEM. Analysis of variance and one-way analysis of variance (ANOVA) followed by Fisher least significant difference*post hoc* test were used to assess the significance of differences. $P < 0.05$ was considered significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

Acute Exposure to Alcohol Causes Downregulation of Expression and Promoter Activity of *MFG-E8* Gene in Macrophages

In this experiment, we examined whether alcohol altered *MFG-E8* gene expression. To this end, murine peritoneal macrophages were cultured with either medium alone (control) or a medium containing alcohol (25 mmol/L) for 18 h followed by measuring *MFG-E8* gene expression with a quantitative real-time RT-PCR and Western blot. It was revealed that acute alcohol exposure attenuated *MFG-E8* gene expression at transcriptional (Figure 1A) and translational (Figure 1B) levels.

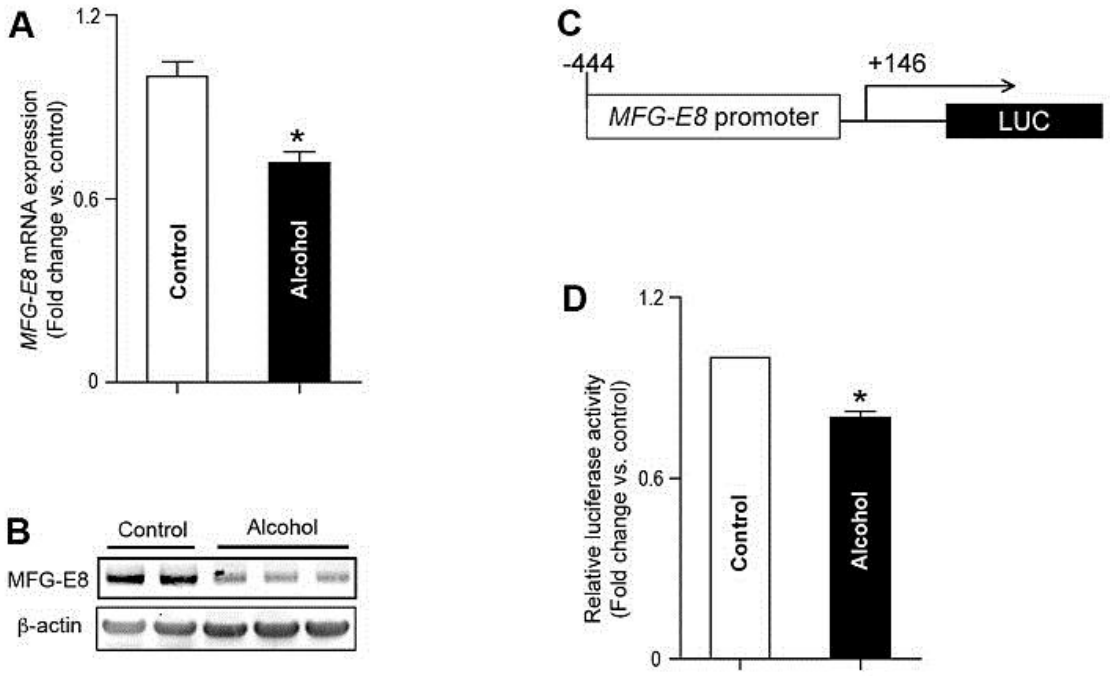


Figure 1. Acute alcohol represses expression and promoter activity of *MFG-E8* gene in macrophages. (A and B) Effect of acute alcohol exposure on *MFG-E8*gene expression. Murine macrophages were treated with culture medium alone (control) or medium containing alcohol (25 mmol/L) for 18 h as indicated in the figure. At the end of treatments, total cellular RNA and protein were extracted from cells. *MFG-E8* mRNA levels (panel A) in total RNA samples were quantified with real-time RT-PCR, whereas MFG-E8 protein (panel B) in total cellular protein samples were determined with Western blot as described in Materials and Methods. (C) Scheme of murine *MFG-E8* gene promoter/luciferase reporter. A fragment of murine MFG-E8 gene promoter (−444 to +146) was cloned into the upstream of the luciferase gene in pGL3-basic reporter plasmid. The construct is named as pGL3-mfge8$^{-444/+146}$. (D) Acute alcohol represses *MFG-E8* promoter activity. RAW 264.7 cells were dual transfected with plasmids of pRL-null and pGL3-mfge8$^{-444/+146}$ reporters. They were subjected to treatment with medium alone (control) or alcohol (25 mmol/L) for 18 h followed by processing for luciferase assay as described in Materials and Methods. The experiments were performed twice with triplicates. Results are expressed as mean ± SEM; $n = 3$. *$P < 0.05$ versus control group.

It is expected that *MFG-E8* gene expression is controlled by a broad spectrum of transcriptional regulators located in the promoter region. Therefore, we further examined whether alcohol suppressed the promoter activity of *MFG-E8* gene. For this purpose, we generated a luciferase reporter construct, namely, pGL3-mfge8$^{-444/+146}$that contained the promoter of murine *MFG-E8* gene (Figure 1C). With the luciferase reporter assay described in the method, we found that acute alcohol treatment significantly inhibited *MFG-E8* promoter activity (Figure 1D). Together, the data indicated that alcohol repressed *MFG-E8* gene promoter activity, which was associated with a decrease in *MFG-E8* mRNA and protein levels in macrophages.

Downregulation of *MFG-E8* Gene Expression is an Underlying Mechanism by Which Acute Alcohol Exposure Impairs Macrophage Efferocytosis

MFG-E8 plays an important role in facilitating macrophage efferocytosis, a phagocytic process for clearance of apoptotic cells (15,16). Previous studies have shown that acute alcohol exposure attenuates macrophage efferocytosis (13). However, the exact molecular mechanisms underlying the effect remain largely unknown. In this study, we first aimed to confirm the effect of acute alcohol exposure on macrophage efferocytosis. With this objective, murine peritoneal macrophages were treated with alcohol (25 mmol/L) for 18 h. As the control, a group of murine peritoneal macrophages were treated with culture medium alone for 18 h. Thereafter, all macrophage cells were coincubated with apoptotic thymocytes for an additional 90 min followed by TUNEL staining for determining ability of macrophages to phagocytize apoptotic cells. Under a fluorescent microscope, we found that alcohol-treated macrophages engulfed fewer apoptotic cells as compared to the control (Figure 2A). We further confirmed this finding using flow-cytometric analysis that was described in Materials and Methods (Figure 2B). Through quantitative analysis of flow-cytometric data, we revealed that alcohol exposure (25 mmol/L, for 18 h) markedly inhibited macrophage activity in efferocytosis (Figure 2C). In addition, we examined the effect of alcohol exposure on efferocytosis using RKC1 cells, a rat Kupffer cell–derived macrophage cell line. Similar to peritoneal macrophages, RKC1 cells decreased their activity in efferocytosis after exposure to alcohol (Supplementary Figure S1).

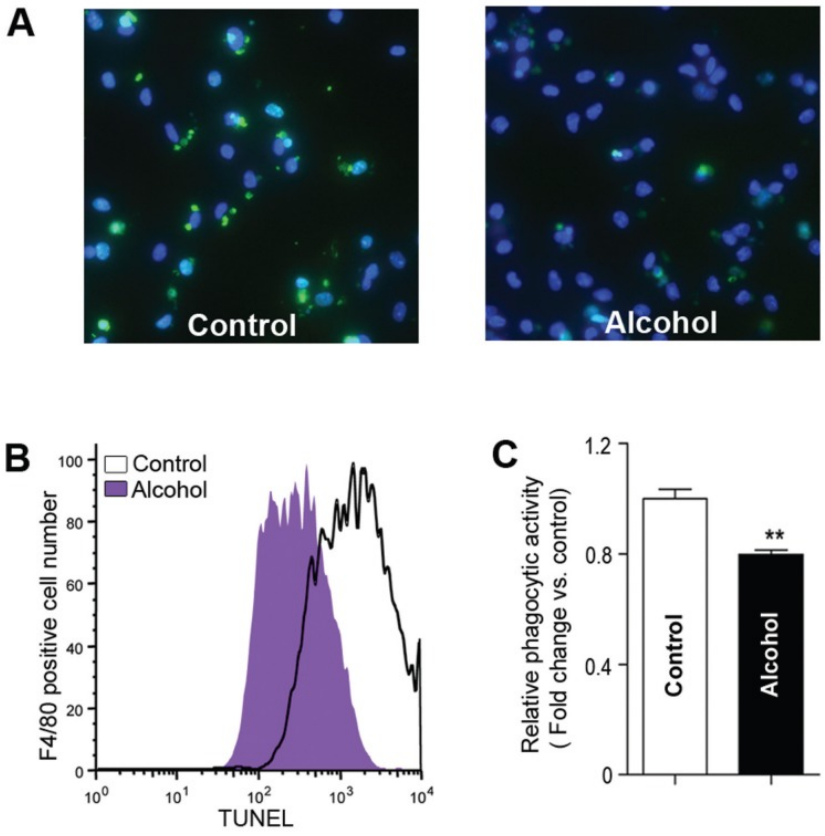


Figure 2. Acute exposure to alcohol decreases macrophage efferocytosis. Peritoneal macrophages were subjected to treatment with culture medium alone (control) or medium containing alcohol (25 mmol/L) for 18 h as indicated in the figure. After treatments, macrophages were cocultured with apoptotic thymocytes in the 1:4 ratio for 90 min, washed with PBS and processed for staining and analysis of macrophages with engulfed apoptotic thymocytes using fluorescent microscopy and flow cytometry as described in Materials and Methods. (A) Representative photomicrographs of TUNEL-stained samples visualized under a fluorescent microscope. Nuclei of apoptotic cells engulfed by macrophages were demonstrated with bright green fluorescence. Blue fluorescent indicated DAPI-stained macrophage nuclei. Original magnification: 10×. (B) Representative histogram of FACS analysis for quantitatively determining macrophages that engulfed apoptotic cells. (C) Quantitative analysis of efferocytotic ability of macrophages in each treatment group with a flow cytometry–based method. Values are mean ± SEM and represent average of findings from three independent experiments with triplicate samples in each group. $^{**}P < 0.01$ versus the control group.

Because inhibition of macrophage efferocytosis by acute alcohol exposure is associated with downregulation of*MFG-E8* gene expression, we hypothesized that an alcohol-induced decrease in *MFG-E8* gene expression attributes to the impairment of macrophage efferocytosis. To test the hypothesis, we examined whether exogenous MFG-E8 rescued macrophages from alcohol-induced impairment of uptake of apoptotic cells. Through analysis of efferocytosis with TUNEL staining, alcohol (25 mmol/L) was found to markedly inhibit uptake of apoptotic cells by macrophages (Figure 3A, middle panel versus left panel). By contrast, alcohol-challenged macrophages regained their ability to engulf apoptotic cells when recombinant MFG-E8 (0.5 μg/mL) was added to the medium for efferocytosis assay (Figure 3A, right panel versus middle panel). This observation was further strengthened by quantitative flow-cytometric assessment (Figures 3B, C). Taken together, the data suggested that alcohol inhibits macrophage *MFG-E8* gene expression, which in turn disrupts the cell efferocytosis.

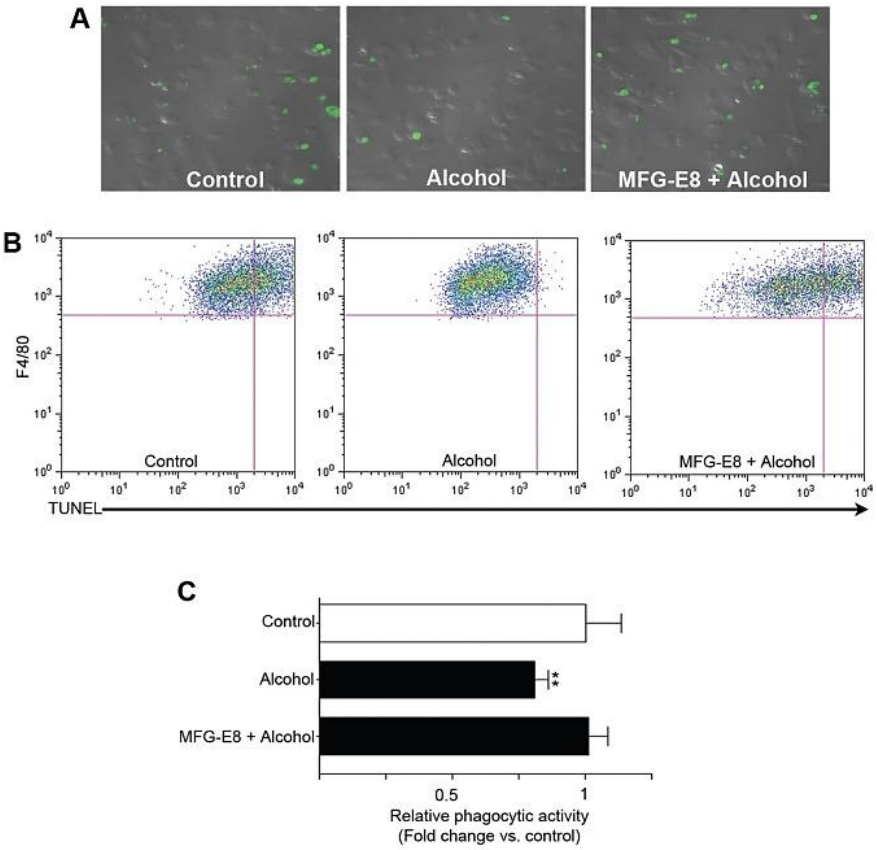


Figure 3. MFG-E8 rescues macrophages from acute alcohol exposure–induced impairment of efferocytosis. Peritoneal macrophages were divided randomly into groups as indicated in the figure. They were subjected to treatment with culture medium alone (control) or medium containing alcohol (25 mmol/L) for 18 h respectively. After treatments, macrophages were further cocultured with apoptotic thymocytes in the 1:4 ratio for 90 min. In the group MFG-E8 + alcohol, the coculture medium contained 0.5 μg/mL of MFG-E8. At the end of treatments, all macrophages were processed for appropriate staining followed by determining macrophages that engulfed apoptotic cells using either a fluorescent microscopy or flow cytometry–based method as described in the Figure 2 legend. (A) Representative photomicrographs of TUNEL-stained samples visualized under a fluorescent microscope. Nuclei of apoptotic cells engulfed by macrophages were demonstrated with bright green fluorescence. Original magnification: 10×. (B) Representative scatter plots of FACS analysis for determining macrophages that engulfed apoptotic cells in each treatment group. (C) Quantitative analysis of efferocytotic ability of macrophages in each treatment group with a flow cytometry–based method. Values are mean ± SEM and represent average of findings from two independent experiments with triplicate samples in each group. $^{**}P < 0.01$ versus the control group.

Reactive Oxygen Species Mediate Impairment of Macrophage *MFG-E8* Expression and Efferocytosis by Acute Alcohol Exposure

In this experiment, we aimed to elucidate mediators which facilitate acute alcohol effect on inhibition of macrophage *MFG-E8* gene expression and efferocytosis. Previous studies have

shown that reactive oxygen species (ROS) contribute to a wide range of adverse health effects of alcohol (37). Therefore, we examined whether ROS played a role in inhibition of *MFG-E8* gene expression and efferocytosis by acute alcohol exposure. First, we investigated whether H_2O_2 and alcohol have similar effects on macrophage *MFG-E8* gene expression and efferocytosis. To this end, murine peritoneal macrophages were subjected to treatment with H_2O_2 (100 μmol/L) and alcohol (25 mmol/L) for 18 h. The dose of H_2O_2 was chosen because our preliminary study confirmed previous finding that H_2O_2 (100 μmol/L) did not cause severe injury in macrophages (38). As the control, a group of murine peritoneal macrophages were pair cultured in medium that contained neither alcohol nor H_2O_2. With quantitative real-time RT-PCR analysis, we revealed that *MFG-E8* mRNA levels in alcohol-as well as H_2O_2-treated cells were decreased markedly compared to the control (Figure 4A). Moreover, alcohol and H_2O_2 repressed *MFG-E8* promoter activity respectively (Figure 4B). With flow cytometry–based efferocytosis analysis, H_2O_2-and alcohol-treated macrophages were found to have lesser amounts of engulfed apoptotic cells than macrophages in the control group (Figure 4C). Together, the data suggested that H_2O_2 mimicked the effect of alcohol on macrophage *MFG-E8* gene expression and efferocytosis.

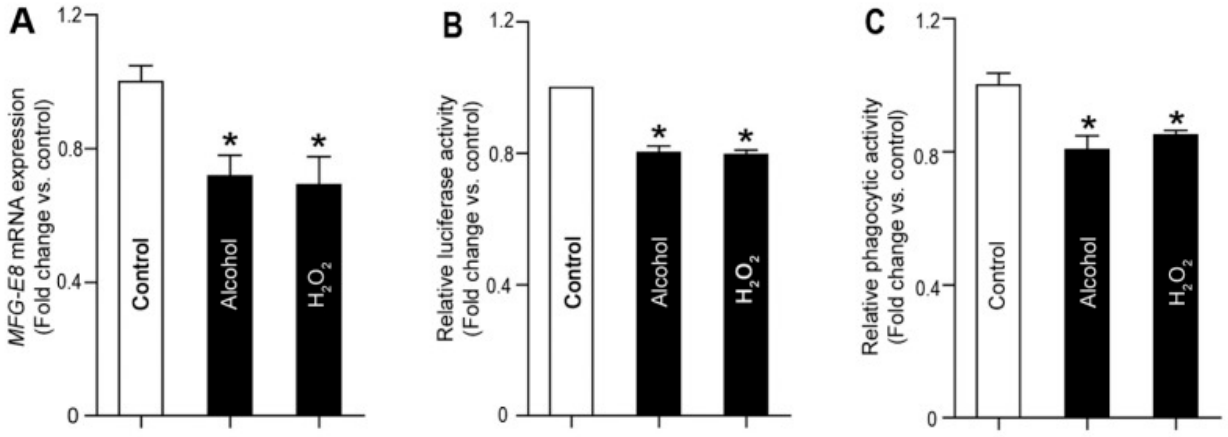


Figure 4. Effect of ROS on macrophage *MFG-E8* gene expression and efferocytosis. (A) ROS mimics the effect of alcohol on suppression of mRNA levels of*MFG-E8* in macrophages. Peritoneal macrophages were subjected to treatment with culture medium alone (control), alcohol (25 mmol/L) or H_2O_2(100 μmol/L) respectively for 18 h as indicated in the figure. After treatments, macrophages were processed for isolation of total cellular RNA followed by determining mRNA transcripts of *MFG-E8* gene with a quantitative real-time RT-PCR method as described in the Figure 1 legend. (B) ROS mimics the effect of alcohol on suppression of *MFG-E8* gene promoter activity. RAW 264.7 cells were dual transfected with plasmids of pRL-null and pGL3-mfge8$^{-444/+146}$ reporters. Twenty-four hours after transfections, cells were then subjected to treatment with culture medium alone (control), alcohol (25 mmol/L) or H_2O_2 (100 μmol/L) for additional 18 h as indicated in the figure. Then, cells were processed for luciferase assay as described in the Figure 1 legend. (C) ROS mimics the effect of alcohol on impairment of macrophage efferocytosis. Peritoneal macrophages were subjected to treatment with culture medium alone (control), alcohol (25 mmol/L) or H_2O_2 (100 μmol/L) for 18 h as indicated in the figure. After treatments,

macrophages were processed for appropriate staining followed by quantitatively determining macrophages that engulfed apoptotic cells using a flow cytometry–based method as described in the Figure 2 legend. Values are mean ± SEM and represent average of findings from two independent experiments with triplicate samples in each group. *$P < 0.05$ versus the control group.

Furthermore, we investigated whether ROS mediated the effect of acute alcohol on downregulation of *MFG-E8*gene expression and inhibition of efferocytosis using an approach involved in neutralizing alcohol-induced ROS production with NAC, an antioxidant. Murine peritoneal macrophages were used in this experiment. First, we confirmed that alcohol impaired macrophage efferocytosis (Figures 5A, B) and *MFG-E8* gene expression (Figure 5C). Then, we revealed that the addition of NAC (10 mmol/L) diminished alcohol's effect on the inhibition of macrophage *MFG-E8* mRNA and efferocytosis. However, NAC treatment alone did not alter macrophage efferocytosis and *MFG-E8* expression. Taken together, the data indicated that acute exposure of macrophages to alcohol blocks *MFG-E8* gene expression and impairs efferocytosis via an ROS dependent manner.

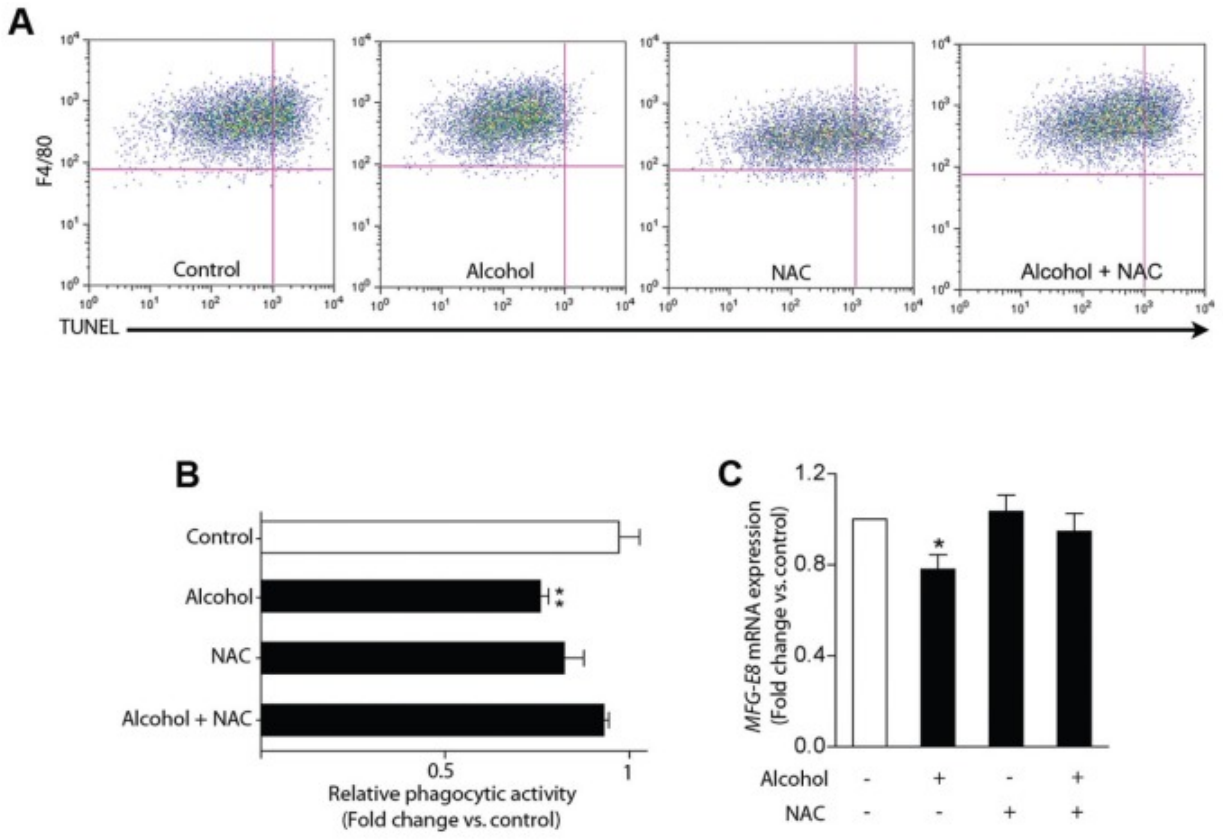
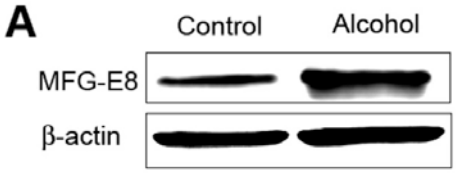


Figure 5. ROS mediates alcohol effect on impairment of macrophage efferocytosis and *MFG-E8* gene expression. Peritoneal macrophages were subjected to treatment with culture medium alone (control), alcohol (25 mmol/L), NAC (10 mmol/L) or alcohol (25 mmol/L) + NAC (10 mmol/L) for 18 h as indicated in the figure. After treatments, part of samples in each treatment group was cocultured with apoptotic cells in the 1:4 ratio for 90 min, stained and followed by analysis of macrophages with engulfed

apoptotic cells using the flow cytometry as described in the Figure 2 legend. The remaining samples were processed for RNA isolation followed by determining mRNA transcripts of *MFG-E8* gene with a quantitative real-time RT-PCR method as described in the Figure 1 legend. (A) Representative scatter plots of FACS analysis for determining macrophages that engulfed apoptotic cells in each treatment group. (B) Results of quantitatively analyzing efferocytotic ability of macrophages in each treatment group with FACS. (C) Results of quantitatively analyzing *MFG-E8* mRNA in macrophages with real-time RT-PCR. In panels B and C, values are mean ± SEM and represent average of findings from two independent experiments with triplicate samples in each group. **P* < 0.05 versus the control group, ***P* < 0.01 versus the control group.

Chronic Alcohol Exposure *In Vitro* Enhances Macrophage MFG-E8 Protein Expression and Efferocytosis

It has been shown that alcohol is able to suppress or exacerbate immune cell activities depending on the length of alcohol exposure (12). Therefore, we further examined the effect of chronic alcohol treatment on macrophage MFG-E8 protein expression and ingestion of apoptotic cells. To this end, murine peritoneal macrophages were challenged with alcohol (50 mmol/L) *in vitro* for 7 d as described in the Materials and Methods. Interestingly, prolonged alcohol exposure for 7 d resulted in an increase in MFG-E8 protein levels in macrophages (Figure 6A) and an enhancement of uptake of apoptotic cells by macrophages (Figure 6B). Collectively, the data indicated that the direct effect of chronic alcohol exposure on macrophage MFG-E8 protein expression and efferocytosis is distinct from acute alcohol treatment *in vitro*.



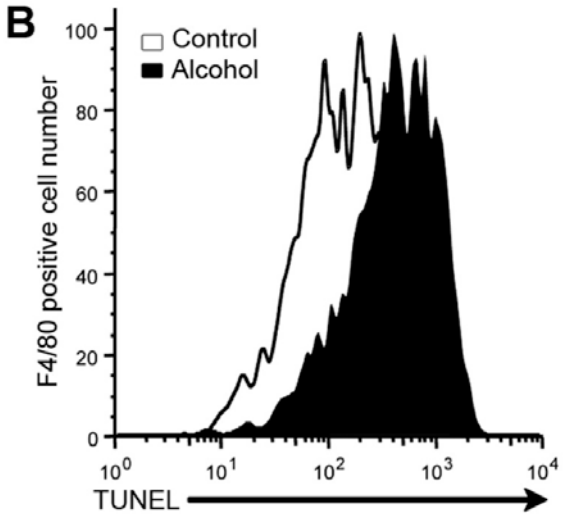


Figure 6. Effect of chronic alcohol treatment on macrophage MFG-E8 gene expression and efferocytosis. Peritoneal macrophages were exposed to alcohol (50 mmol/L) for 7 d as described in Materials and Methods. After treatments, part of the samples in each treatment group was processed for protein isolation followed by determining MFG-E8 protein with Western blot as described in the Figure 1 legend. The remaining samples were cocultured with apoptotic cells in the 1:4 ratio for 90 min, stained and followed by analysis of macrophages with engulfed apoptotic cells using flow cytometry as described in the Figure 2 legend. (A) Chronic alcohol treatment results in increase in MFG-E8 protein levels in macrophages. A representative autoradiograph from two independent experiments is shown. (B) Chronic alcohol treatment enhances macrophage efferocytosis. A representative histogram of FACS analysis from two independent experiments with triplicate samples in each group.

High Mobility Group Box 1 Protein Is a Potent Mediator That Contributes to Chronic Alcohol Effect on Macrophage Efferocytosis *In Vivo*

Although our data showed that chronic alcohol treatment *in vitro* directly increased macrophage engulfment of apoptotic cells, Boé *et al*. recently reported that chronic feeding of mice with alcohol alone did not cause induction of macrophage efferocytosis *in vivo*(13). Therefore, we speculated that *in vivo* chronic alcohol exposure induced a factor which neutralized the direct effect of chronic alcohol exposure on macrophage efferocytosis. High mobility group box 1 protein (HMGB1) is a critical inflammatory mediator (39). It has high affinity to phosphatidylserine (24). HMGB1 is shown to inhibit macrophage efferocytosis through binding to phosphatidylserine on the apoptotic cell surface (24). Thus, we examined whether chronic alcohol intake changed serum levels of HMGB1 *in vivo*. For this purpose, mice were subjected to pair feeding with alcohol or control diet for 8 wks followed by measuring HMGB1 contents in serum. As shown in Figure 7, serum levels of HMGB1 were increased markedly in alcohol-treated mice compared to the pair-fed control group. Furthermore, we examined the role of HMGB1 in regulation of chronic alcohol-treated macrophage efferocytosis using an *in vitro* efferocytosis assay as described in Materials and Methods. To this end, we directly treated apoptotic cells with HMGB1 (1 μg/mL) for 30 min for opsonization of the cells. As the control, a group of apoptotic cells was cultured with vehicle for 30 min instead. Then, apoptotic cells were processed for analysis of macrophage efferocytosis. Macrophages isolated from murine peritoneal cavity were used for the assay. As expected, large amounts of vehicle-treated apoptotic cells (control) were engulfed by macrophages (Figure 8). By contrast, opsonization of apoptotic cells with HMGB1 significantly attenuated the process of macrophage efferocytosis (apoptotic cells^{HMGB1} versus control in Figure 8). Similarly, treatment of peritoneal macrophages with HMGB1 also diminished efferocytosis (macrophages^{HMGB1} versus control in Figure 8).

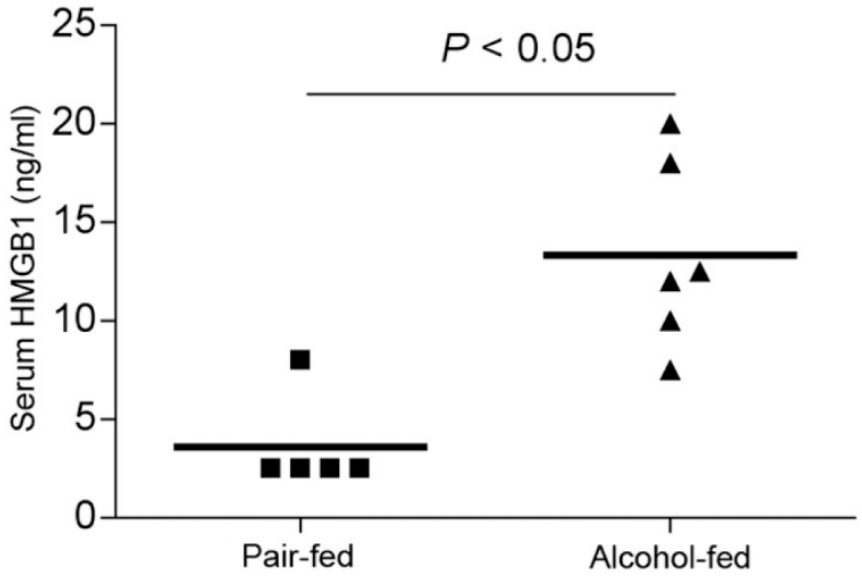


Figure 7. Chronic feeding with alcohol causes an increase in serum levels of HMGB1 in mice. C57BL/6 mice (male, 10 wks old) were subjected to pair feeding with control (n = 5) or alcohol liquid diet (n = 6) for 8 wks. At the end of treatments, blood was collected followed by processing for isolation of serum and measuring HMGB1 levels with ELISA assay as described in Materials and Methods. Values are mean ± SEM.

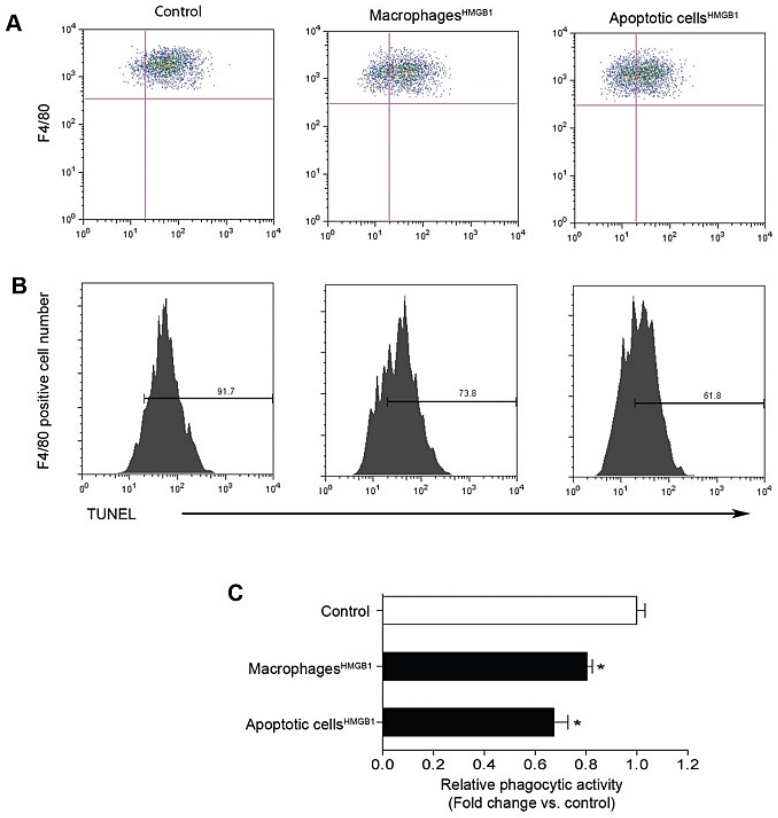


Figure 8. HMGB1 impairs macrophage efferocytosis. Peritoneal macrophages were divided into groups of control, macrophages^{HMGB1} and apoptotic cells^{HMGB1} as indicated in the figure. Macrophages in macrophages^{HMGB1} were treated with HMGB1 (1 μg/mL) for 30 min followed by cocultured with apoptotic thymocytes for 90 min. Macrophages in apoptotic cells^{HMGB1} were treated with culture medium for 30 min followed by coculturing with apoptotic thymocytes that were pretreated with HMGB1 (1 μg/mL) for 30 min. Macrophages in control were treated with culture medium for 30 min followed by coculturing with apoptotic thymocytes that were not pretreated with HMGB1. After treatments, all macrophages were stained and processed for analysis with the flow cytometry as described in the Figure 2 legend. (A) Representative scatter plots in each group. (B) Representative histograms in each group. (C) Statistic analysis of FACS assay data. Values are mean ± SEM and represent average of findings from two independent experiments with triplicate samples in each group. **P* < 0.05 versus the control group.

To further understand the pathophysiological meaning of induction of HMGB1 to macrophage efferocytosis during chronic alcohol exposure, we examined whether HMGB1 interfered with the direct effect of chronic alcohol treatment on macrophage efferocytosis *in vitro*. To this end, peritoneal macrophages were exposed to alcohol (50 mmol/L) for 7 d followed by treatment with HMGB1 (1 μg/mL) for 30 min. As controls, groups of peritoneal macrophages were treated with either medium alone or a medium containing alcohol (50 mmol/L) for 7 d. At the end of treatments, all cells were processed for measuring efferocytosis with a flow cytometry–based method. As shown in Figure 9, HMGB1 diminished the direct effect of chronic alcohol treatment on macrophage efferocytosis. The data suggested that HMGB1 was able to antagonize the effect of chronic alcohol treatment on efferocytosis.

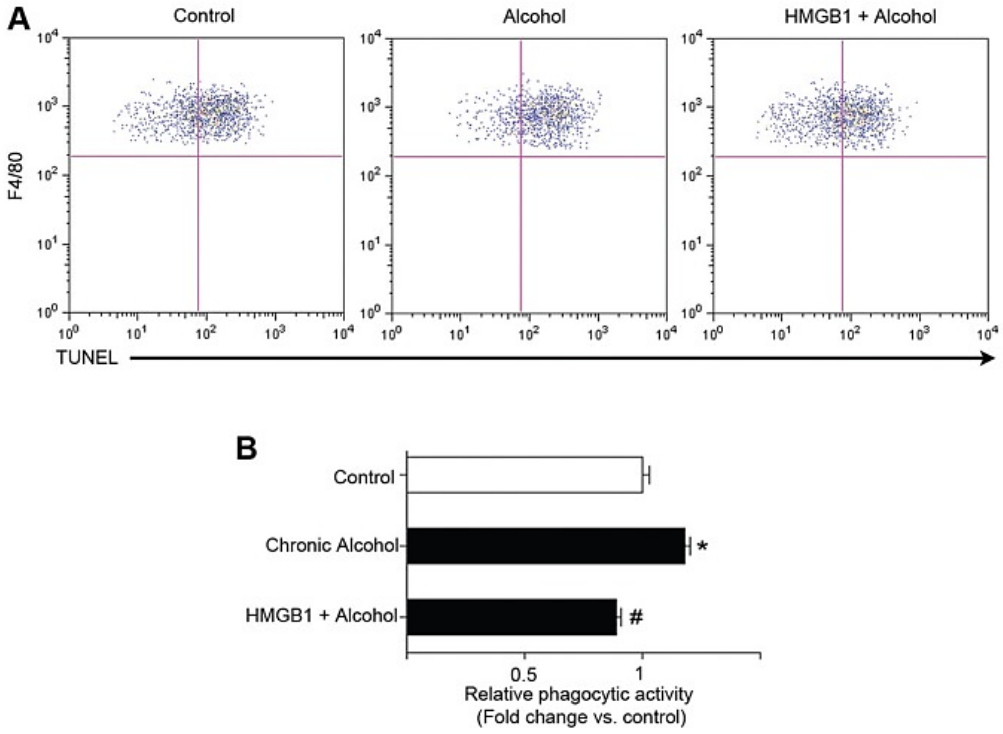


Figure 9. HMGB1 diminishes the direct effect of chronic alcohol on induction of macrophage efferocytosis. Peritoneal macrophages were divided into groups as described in the figure. They were treated with culture medium alone (control) or medium containing alcohol (50 mmol/L) for 7 d. Then, macrophages were cocultured with apoptotic thymocytes from 90 min. The coculture medium contained HMGB1 (1 μg/mL) in the group alcohol + HMGB1, whereas coculture medium in other groups was standard DMEM without HMGB1.

After treatments, all macrophages were stained and processed for analysis with the flow cytometry as described in the Figure 2 legend. (A) Representative scatter plots in each group. (B) Statistic analysis of FACS assay data. Values are mean ± SEM and represent average of findings from two independent experiments with triplicate samples in each group. $^{*}P < 0.05$, chronic alcohol versus the control group. $^{\#}P < 0.05$, chronic alcohol versus alcohol + HMGB1.

DISCUSSION

Macrophage efferocytosis plays a critical role in the resolution of tissue injury and inflammation (6). It is a complex process that requires recognition of apoptotic cells by macrophages (40). Dying cells lose phospholipid bilayer asymmetry and expose phosphatidylserine (PtdSer) on the outer leaflet of the plasma membrane. This unique change results in displaying the key “eat-me” signal on the surface of apoptotic cells. Evidence has shown that numerous cellular factors can bind to eat-me molecules on the surface of apoptotic cells and interact with surface markers of macrophages (7,40). Among them, MFG-E8 and HMGB1 are revealed to bind to PtdSer on the surface of apoptotic cells and have a high affinity to $\alpha_v\beta_3$ integrin on macrophages (15,16,24,41). MFG-E8 has been shown to bridge macrophages to apoptotic cells through binding these structures and thereby facilitates macrophage efferocytosis (15,16). By contrast, HMGB1 diminishes efferocytosis by blocking the binding of MFG-E8 to these structures (24,41). Thus, they are important mediators for clearance of apoptotic cells by macrophages.

In the present study, we investigated the effect of alcohol treatments on efferocytosis and dissected underlying mechanisms by focusing on MFG-E8 and HMGB1. Using peritoneal and Kupffer cell–derived macrophages, we found that acute exposure of macrophages to alcohol *in vitro* impairs efferocytosis. Previously, Boé *et al*. showed that *in vivo* and *in vitro* acute alcohol treatments disrupt alveolar macrophage efferocytosis (13). In conjunction with our data reported here, this suggests that (a) the effect of *in vitro* alcohol exposure on efferocytosis mimics the event *in vivo*; and (b) macrophages derived from different tissue locations can all acquire decreased efferocytic potential when suddenly exposed to alcohol.

In addition, we demonstrated for the first time that impairment of macrophage efferocytosis by acute alcohol exposure is associated with downregulation of *MFG-E8* gene expression in macrophages. It has been shown that macrophage efferocytosis is abnormal in MFG-E8 deficient mice (15,16). Previous studies demonstrated that a decrease in *MFG-E8* expression contributes to impairment of macrophage and microglial cell efferocytosis (42–44). Furthermore, we revealed that recombinant MFG-E8 rescues the activity of alcohol-treated macrophages in

efferocytosis. Meanwhile, our pilot data indicated that acute exposure of macrophages to alcohol has no effect on HMGB1 secretion *in vitro* (Supplementary Figure S2). Taken together, acute alcohol exposure is expected to disrupt efferocytosis mainly through a decrease in MFG-E8 levels but not an increase in HMGB1 production.

By contrast to acute alcohol exposure, chronic alcohol treatment was found to promote macrophage MFG-E8 protein expression directly and to enhance efferocytosis *in vitro* in the present study. On the other hand, we noted that chronic feeding of mice with alcohol resulted in an increase in the levels of serum HMGB1, a highly conserved nonhistone nuclear protein. It has been shown that HMGB1 has multiple intracellular and extracellular physiological functions such as transcriptional regulation and modulation of cell migration (45). HMGB1 is secreted when cells are activated by inflammatory stimuli or undergo apoptosis and necrosis. It has a cytokinelike property, regulates immunity and inflammation, as well as functions as a damage-associated molecular pattern (39). Recently, HMGB1 is found to inhibit macrophage efferocytosis (24,41). In the present study, HMGB1 is revealed to attenuate the direct effect of chronic alcohol exposure on the induction of macrophage efferocytosis *in vitro*. Collectively, we speculate that the balance between MGF-E8 and HMGB1 in tissues determines the pathophysiological effects of chronic alcohol exposure on macrophage efferocytosis *in vivo*.

As stated above, MFG-E8 is shown to bridge apoptotic cells to macrophages via binding PtdSer and $\alpha_v\beta_3$integrin on surfaces of apoptotic cells and macrophages respectively, which in turn facilitates efferocytosis. Interestingly, HMGB1 also has a high affinity to the same molecules that MFG-E8 binds to but disrupts efferocytosis (24,41). Our data further confirmed the inhibitory function of HMGB1 on macrophage efferocytosis during alcohol exposure. However, it remains largely unknown how HMGB1 inhibits efferocytosis. It is possible that HMGB1 inhibits efferocytosis through competing with MFG-E8 on binding to PtdSer and $\alpha_v\beta_3$ integrin (24,41). In addition, efferocytosis requires Rac-1 activation and cytoskeletal rearrangement (6). Banerjee *et al*. recently revealed that intracellular events involved in efferocytosis and those upstream of Rac-1 activation, such as phosphorylation of ERK and focal adhesion kinase (FAK), are increased after knockdown of HMGB1 (46). Furthermore, they demonstrated that HMGB1, through associating with Src kinase and inhibiting interactions between Src and FAK, diminishes the efferocytotic ability of macrophages. Together, we hypothesize that HMGB1 abolishes MFG-E8-mediated efferocytosis via disruption of multiple cellular events associated with macrophage efferocytosis.

Mechanisms underlying alcohol-induced alteration of *MFG-E8* gene expression and efferocytosis in macrophages are not clear. In the present study, we showed that ROS, a product derived from alcohol metabolism, mimicked acute alcohol effect on macrophage *MFG-E8* gene expression and efferocytosis. Moreover, we demonstrated that antioxidants attenuated acute alcohol's activity on *MFG-E8* gene expression and MFG-E8-mediated macrophage efferocytosis. Therefore, we hypothesize that ROS is an important mediator for acute alcohol to disrupt *MFG-E8*gene expression and disturb uptake of apoptotic cells by macrophages.

By contrast with the effect of acute alcohol exposure on macrophage *MFG-E8* gene expression and MFG-E8-mediated efferocytosis, chronic alcohol treatment *in vitro* was shown to induce MFG-E8 levels and to increase efferocytosis activity in the present study. This interesting finding suggests that the pathophysiological state of macrophages may alter during chronic alcohol exposure. Indeed, macrophages exhibit phenotypic and functional heterogeneity (47). Their activation can result in classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages have a proinflammatory profile, while M2 macrophages display antiinflammatory properties (47). It has been reported that chronic feeding of mice with alcohol enhances induction of both M1 and M2 macrophage activations *in vivo*(48). However, whether chronic alcohol exposure directly causes macrophage polarization is not clear. Previously, macrophages have been found to display an increased capacity of efferocytosis when they are polarized to an M2 phenotype (24). In the present study, we demonstrated that direct exposure of macrophages to alcohol for 7 d promoted macrophage uptake of apoptotic cells. This characteristic is similar to macrophages exhibited with M2 markers. However, further studies are required to determine whether chronic alcohol exposure–induced alteration of MFG-E8 protein levels and efferocytosis activity is associated with macrophage M2 polarization.

Finally, evidence has shown that chronic alcohol consumption has diverse and different effects on macrophage cytokine production and activation as compared to acute alcohol exposure. For example, acute ethanol ingestion suppresses the capacity of monocytes and macrophages to generate TNF-α both through decreased signal transduction and through posttranscriptional and posttranslational suppression of TNF-α production (49,50). Opposite to acute alcohol exposure, both clinical and animal studies suggested that chronic alcohol ingestion increased macrophage production of TNF-α (51). Mandrekar *et al*. demonstrated that acute and chronic alcohol modulate macrophage response to stimulation by lipopolysaccharide differently (31). Thus, it is not surprising that the effect of acute alcohol exposure on macrophage *MFG-E8* gene expression and efferocytosis differs from that of chronic alcohol exposure. However, our study revealed that chronic alcohol treatment enhanced production of both MFG-E8 and HMGB1, two molecules that have opposite effects on macrophage efferocytosis. Moreover, we showed that HMGB1 sharply inhibited efferocytosis by macrophages which were chronically treated with alcohol. Thus, the overall effect of chronic alcohol consumption on macrophage efferocytosis might be attributed to changes in cytokine levels *in vivo*. It is expected that cytokine profiles might provide new insights about how alcohol affects tissue injury and repair.

CONCLUSION

In summary, we examined the effect of alcohol exposure on macrophage efferocytosis and explored the role of MFG-E8 and HMGB1 in the regulation of macrophage efferocytosis by alcohol treatment in the present study. We showed that acute and chronic alcohol exposures exhibit opposite effects *in vitro* on macrophage *MFG-E8*gene expression as well as HMGB1 production (Table 1). We revealed that acute alcohol exposure impairs macrophage efferocytosis mainly via inhibition of *MFG-E8* gene expression through an ROS-dependent mechanism. By

contrast, chronic alcohol exposure is found to affect production of both MFG-E8 and HMGB1 in cells. It seems that the total effect of chronic alcohol exposure on macrophage efferocytosis is dependent on the levels of MFG-E8 and HMGB1 in a local area. Together, our findings suggest that MFG-E8 and HMGB1 are important factors contributing to alcohol effect on efferocytosis.

Table 1. Summary of alcohol effect on expression of MFG-E8 and HMGB1.

Alcohol treatment	**MFG-E8 protein**[a]	***MFG-E8* mRNA**[b]	**HMGB1**[c]
Acute	↓	↓	No change
Chronic	↑	↑	↑

↓, Decrease; ↑, increase.
[a]Detected with Western blotting.
[b]Detected with real-time RT-PCR.
[c]Detected with ELISA

ACKNOWLEDGMENTS

This work was supported in part by the Excellence in Academic Medicine Award from Illinois Department of Public Aid (to X-D Tan) and the grants from National Institutes of Health including R21AA020494 (to X-D Tan), R01DK064240 (to X-D Tan) and R01AA020212 (to Z Zhou). Authors thank Michel M Murr for kindly providing the RKC1 cell line.

Footnotes

Online address: http://www.molmed.org

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine,* or other interests that might be perceived to influence the results and discussion reported in this paper.

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