Fractalkine-Induced MFG-E8 Leads to Enhanced Apoptotic Cell Clearance by Macrophages

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Clearance of apoptotic cells is crucial to maintain cellular function under normal and pathological conditions. We have recently shown that administration of immature dendritic cell-derived exosomes to septic animals promotes phagocytosis of apoptotic cells and improves survival by providing milk fat globule epidermal growth factor-factor VIII (MFG-E8). MFG-E8 acts as an opsonin for apoptotic cells to be engulfed by phagocytosis. In the present study we investigated whether the CX_3C-chemokine fractalkine (CX_3CL1) promotes apoptotic cell clearance through the induction of MFG-E8 in peritoneal macrophages. Cultured rat peritoneal macrophages (pMϕ) and RAW264.7 macrophages were stimulated with LPS and CX_3CL1. MFG-E8 expression was assessed by Western blot, cytokine secretion was assessed by ELISA, and phagocytosis of apoptotic thymocytes was determined by microscopy. For in vivo studies, cecal ligation and puncture (CLP) was used to induce sepsis in rats and mice. LPS significantly decreased MFG-E8 levels and phagocytosis of apoptotic cells, whereas CX_3CL1 induced MFG-E8 expression in both nonstimulated and LPS-stimulated pMϕ, without affecting TNF-α and IL-6 release. Anti-MFG-E8 blocking antibodies completely abrogated the prophagocytic effect of CX_3CL1. Twenty hours after the induction of sepsis in rats via CLP, plasma CX_3CL1 levels as well as MFG-E8 production in peritoneal macrophages decreased by 21% and 56%, respectively. Administration of CX_3CL1 on the other hand induced MFG-E8 and prevented tissue injury. We conclude that CX_3CL1 induces MFG-E8 in vitro and in vivo and enhances clearance of apoptotic cells in an MFG-E8-dependent manner. These findings suggest a possible novel treatment for patients in sepsis.

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INTRODUCTION

Fractalkine (CX_3CL1), a molecule with the dual function of an adhesion molecule and a chemokine, is mainly derived from nonhematopoietic cells (1,2). It also has a unique structure in that the CX_3C motif-bearing chemokine domain is carried by a mucin-like stalk attached to the cell surface membrane. CX_3CR1 has been identified as a cognate receptor for CX_3CL1 that is responsible for both adhesion and chemotaxis (3). The proapoptotic tumor suppressor protein p53 can induce CX_3CL1, suggesting a role in eliminating damaged or transformed cells by attracting immune cells (4). To act as a chemokine, the membrane-bound protein CX_3CL1 must be released from the surface by cleavage via the protease TNF-α converting enzyme (TACE) (5). CX_3CL1 has been found to be highly expressed in endothelial cells, neurons, and astrocytes, and its receptor CX_3CR1 is expressed on lymphocytes, monocytes and macrophages, dendritic cells, and mast cells (6,7). Activation of endothelial cells by TNF-α, for example, induces CX_3CL1 expression on the surface of the cells leading to the adhesion and transmigration of macrophages (8,9). Cleavage of CX_3CL1 by TACE (5,10) or release from injured cells leads not only to a gradient-dependent chemoattraction of macrophages and other immune cells through a CX_3CR1-dependent mechanism, but also to inhibition of apoptosis in these macrophages (11). Interestingly, soluble CX_3CL1 can also protect cells from glutamate-mediated excitotoxicity in neurons that normally do not express CX_3CR1 but experience an upregulation upon activation (9,12). CX_3CL1 is also necessary for normal brain development, during which apoptosis naturally occurs (13).

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Although CX_3CL1 upregulation and release have been found to be associated mainly with infections or inflammatory diseases (14-20), this protein had also been shown to be crucial in normal immune response and in the induction of antitumor cytotoxic T cells (21-23).

Recently, soluble CX_3CL1 has been found to induce milk fat globule protein (MFG)-E8 mRNA expression in microglia (24), indicating an intriguing role in the interaction between injured neurons and cerebral macrophages. It was proposed that injury triggers the release of CX_3CL1 from neurons, leading to the upregulation of MFG-E8 in the surrounding macrophages (24). MFG-E8 is an opsonin for apoptotic cells and acts as a bridging protein between phosphatidylserine on apoptotic cells and $\alpha_v\beta_3/\alpha_v\beta_5$ integrins on phagocytes, thus promoting phagocytosis by macrophages (25,26). Clearance of apoptotic cells by phagocytosis is crucial in inflammation and sepsis. We have recently shown that immature dendritic cell-derived exosomes, which contain abundant MFG-E8, promote phagocyto-

sis of apoptotic cells and improve survival in septic rats (27). We have also found that under septic conditions MFG-E8 expression is significantly downregulated in the spleen and liver. We hence hypothesized that in sepsis, CX_3CL1 plays an important role in the regulation of MFG-E8-dependent clearance of apoptotic cells and that CX_3CL1 can induce MFG-E8 in nonmicroglial macrophages from different species and promote phagocytosis of apoptotic cells.

MATERIALS AND METHODS

Cecal Ligation and Puncture (CLP)

In 6 male Sprague-Dawley rats (275-325 g) or C57BL6/J mice (25-30 g, 5 vehicle treated, 5 treated with 100 μg/kg BW CX_3CL1 in intravenous normal saline), the cecum was ligated and double-punctured with an 18-gauge needle as previously described (28). Six sham-operated rats and 5 mice (i.e., control animals) underwent the same procedure with the exception that the cecum was neither ligated nor punctured. The animals were resuscitated with 3 mL/100g BW normal saline solution administered subcutaneously, and 20 h later animals were killed for the collection of blood samples and peritoneal macrophages. The survival rate of rats undergoing cecal ligation and puncture (CLP) and cecectomy at 20 h after CLP is about 50% over a period of 10 d, with virtually all deaths occurring within the first 72 h. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Experimental Animals and approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research.

RAW 264.7 and Peritoneal Macrophage Culture

The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), and peritoneal macrophages were isolated by peritoneal lavage with 4 °C cold Hanks Balanced Salt Solution (GIBCO, Invitrogen, Carlsbad, CA, USA) and enriched by plastic adherence on a polystyrene culture dish for two hours. Cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum, 10 mmol/L 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), 100 U/mL penicillin, and 100 mg/mL streptomycin (complete culture medium) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were incubated for 24 h with lipopolysaccharides (LPS, *Escherichia coli* 055:B5; Difco AQ2 Laboratories, Detroit, MI, USA) with or without recombinant rat CX_3CL1 (R&D Systems, Minneapolis, MN, USA) at different concentrations.

MFG-E8 Western Blotting

Protein was extracted by lysing and homogenizing cells in 1 mL of lysis buffer (10 mM Tris-buffered saline, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 0.2 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 1% Triton X-100) for 30 min on ice followed by centrifugation at 400 × g for 15 min at 4 °C. Samples were quantified using the DC Protein Assay (Bio-Rad, Hercules, CA, USA); 8 μg of protein was fractionated on a 4%–12% Bis-Tris gel and transferred to 0.2-mm nitrocellulose membrane. Blots were blocked for one hour with TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 10% bovine serum albumin, incubated overnight at 4 °C with polyclonal anti-MFG-E8 IgG (G-17, cross-reactive with mouse and rat MFG-E8, 1:100, Santa Cruz Biotech., CA, USA) and washed 5 times with TBST. The specificity of this antibody was tested using an MFG-E8 blocking peptide provided by the manufacturer. Blots were then incubated with horseradish peroxidase-labeled secondary IgG, and ECL (Amersham, GE Healthcare, Buckinghamshire, UK) was applied according to manufacturer's instructions. Membranes were briefly exposed to radiograph film and band densities were analyzed using the Bio-Rad Imaging system.

Phagocytosis Assay

Phagocytosis of apoptotic cells was assayed using terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) staining of apoptotic cells that were phagocytized by macrophages after a modification of previously described procedures (29). Briefly, freshly collected peritoneal macrophages from healthy adult male Sprague Dawley rats or RAW 264.7 cells were plated at a density of 2.5×10^4/well on a 16-well chamber slide (Lab Tek, Nalge Nunc Intl., Rochester, NY, USA) and cultured in complete culture medium. Freshly collected thymocytes were cultured at a concentration of 10^7 cells/mL in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated fetal bovine serum (Mediatech, Herndon, VA, USA), 10 mM HEPES, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10 μM dexamethasone for 24 h at 37 °C and 5% CO_2. This procedure yielded more than 99% apoptotic (annexin V^+/PI^-) thymocytes ($CD90^+$), verified by TUNEL (fluorescein in-situ cell death detection kit, Roche Diagnostics, Indianapolis, IN, USA) and analysis on a FACSCalibur (BD Biosciences, San Jose, CA, USA). Macrophages were preincubated with LPS (10 ng/mL) and CX_3CL1 (10 nM and 100 nM) for 24 h prior to phagocytosis. After they were washed twice with PBS, the macrophages were incubated with apoptotic thymocytes as targets at a ratio of 4:1 (apoptotic cells:macrophages) for 1.5 h. The firmly adherent macrophages were thoroughly washed three times with PBS to remove loosely adherent thymocytes, fixed with 4% paraformaldehyde, stained with TUNEL (green fluorescence, staining apoptotic cells only), and covered mounting medium containing propidium iodine (PI, red fluorescence, staining all nuclei, including macrophages). In additional experiments, thymocytes were either preincubated with 1 μg/mL recombinant mouse MFG-E8 (R&D Minneapolis, MN, USA) or 1 μg/mL anti-MFG-E8 $F_{ab'}$ (prepared by enzymatic cleavage of the G-17 antibody, using the antibody $F_{ab'}$ preparation kit from Pierce Biotech, Rockford, IL, USA) for one hour prior to incubation with apoptotic cells. Slides were analyzed by phase contrast and fluores-

cent microscopy using a Nikon Eclipse E600 microscope (Nikon Inc., Melville, NY, USA). The ratio of phagocytized apoptotic thymocytes to macrophages was assessed independently and the phagocytosis index expressed as a percentage of the normal ratio under control conditions.

Cytokine Assays

TNF-α, IL-6, and IL-10 levels in culture supernatant or heparinized plasma were quantified using specific enzyme-linked immunosorbent assay (ELISA) kits (BD Pharmingen, San Diego, CA, USA). CX_3CL1 levels in plasma samples from sham and CLP animals were measured using the rat CX_3CL1 duo set ELISA kit (R&D Systems).

Lactate and Liver Enzyme Levels

Plasma levels of lactate, AST, and ALT were quantified using the assay kits according to the manufacturer's instructions (Pointe Scientific, Lincoln Park, MI, USA). Values were obtained by comparison to standards run alongside the samples (lactate) or via kinetic measurements of ΔOD (AST and ALT). Values were given in millimoles per liter (lactate) or units per milliliter (AST, ALT).

MFG-E8 ELISA

This newly developed ELISA was performed by using 0.05 μg/mL of a monoclonal antimurine MFG-E8 capture antibody (clone 2422, MBL Woburn, MA, USA) in 50 mM carbonate buffer (pH 9.6) for the coating of a 96-well plate overnight at 4 °C. After three washes with PBS-Tween 20 (0.05%) the plate was blocked with 3% BSA in PBS for one hour at 37°C. Following another washing step, EDTA-plasma samples were directly applied alongside with standards (recombinant murine MFG-E8, R&D Biosciences, Minneapolis, MN, USA) diluted in the Can Get Signal enhancer Solution 1 (Toyobo, Osaka, Japan) over a range from 0 to 250 ng/mL and incubated at 4°C overnight. After three additional washes with PBS-Tween 20 (0.05%), the plate was incubated with 0.1 μg/mL goat polyclonal antimurine MFG-E8 antibody (R&D Biosciences) diluted in Can Get Signal Solution 1 for one hour at 37°C. The plate was washed again and incubated with donkey antigoat IgG HRP diluted 1:20,000 in Can Get Signal Solution 2 for one hour at 37 °C. The reaction was performed with a commercial TMB substrate solution (R&D Biosciences) according to the manufacturer's recommendations. The ELISA was repeated three times and consistently detected MFG-E8 levels with a detection threshold of 0.1 ng/mL.

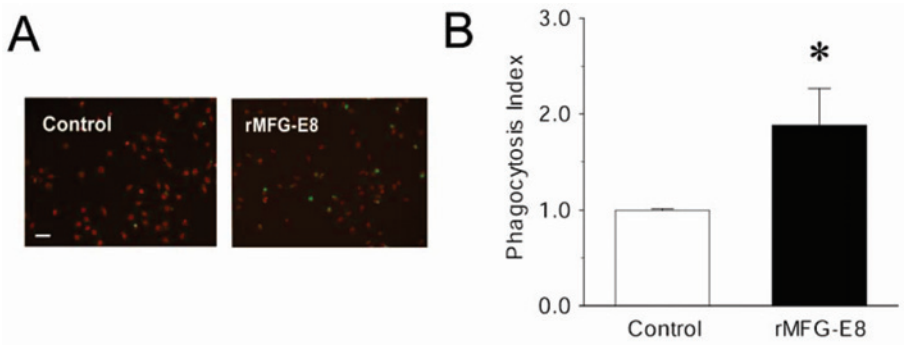

Figure 1. Murine recombinant MFG-E8 induces phagocytosis of apoptotic cells by rat peritoneal macrophages. Phagocytosis assay was performed with or without prior opsonization of apoptotic thymocytes with rMFG-E8 (1 μg/mL). Macrophages were incubated with apoptotic cells as targets for 1.5 h, thoroughly washed three times, and stained with TUNEL. (A) Representative fluorescent micrograph showing macrophages in red (PI) and phagocytized apoptotic cells in green (TUNEL). (B) Quantification of phagocytosis index (average control = 1), Means ± SEM, *$P < 0.05$ vs. control, Student *t*-test, $n = 6$. Size bars = 20 μm.

Statistical Analysis

Data are expressed as means ± _SEM. Values were compared by Student *t*-test. Analysis of variance (ANOVA) and Student-Newman-Keuls' (SNK) test were used to analyze differences between more than two groups. Differences in values were considered significant if $P < 0.05$.

RESULTS

Recombinant MFG-E8 Promotes Phagocytosis of Apoptotic Cells in Rat Macrophages

MFG-E8 in mice and rats are 86% similar with identical binding sites for intergrin $\alpha_v\beta_{3/5}$ (RGD-motif) and phosphatidylserine (factor V/VIII-like domain). To demonstrate that recombinant murine MFG-E8 (rMFG-E8) can promote phagocytosis of apoptotic cells in the rat, we isolated resident rat peritoneal macrophages through plastic adherence after peritoneal lavage. We morphologically verified by Giemsa staining that >95% of cells were macrophages. Thymocyte apoptosis was induced by dexamethasone treatment (10 μM) for 24 h, and apoptotic thymocytes were preincubated with either PBS or 1 μg/mL rMFG-E8 and then added to the resident peritoneal macrophages. The phagocytosis index of rMFG-E8 opsonized apoptotic thymocytes increased on average by 90% compared with nonopsonized thymocytes (Figure 1A and B), showing that murine recombinant MFG-E8 has a prophagocytic effect on rat peritoneal macrophages.

CX_3CL1 Induces MFG-E8 Expression in Peritoneal Macrophages

Because sepsis is commonly associated with endotoxemia, we investigated the role of LPS in the downregulation of MFG-E8. RAW 264.7 macrophages and freshly isolated peritoneal rat macrophages were incubated with 10 ng/mL LPS for 24 h. This resulted in a 24% decrease of MFG-E8 protein levels in RAW264.7 macrophages and a 39% decrease in resident peritoneal macrophages (Figure 2 A and B). Preincubation of cells with CX_3CL1 (100 nM),

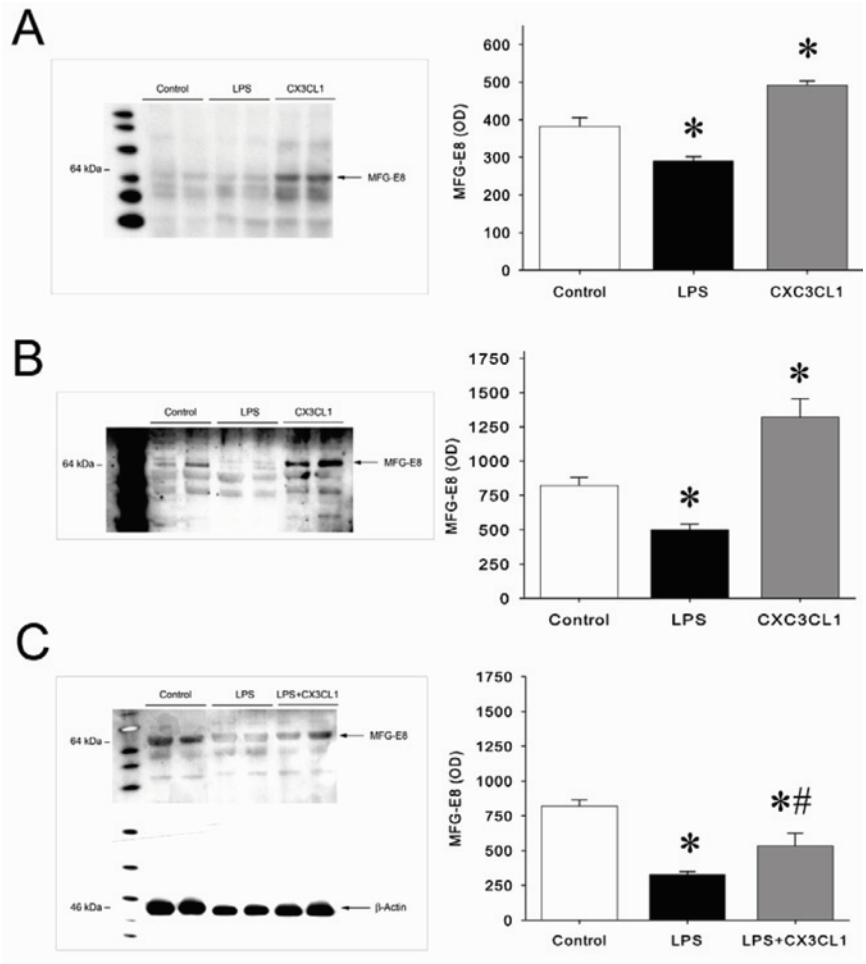

Figure 2. CX_3CL1 induces MFG-E8 expression in peritoneal macrophages. (A) 5×10^6 RAW 267.4 macrophages or (B) freshly isolated rat peritoneal macrophages were incubated for 24 h with either LPS (10 ng/mL) or fractalkine (CX_3CL1 100 nM) and protein from whole cell lysates was used for MFG-E8 detection by Western blotting. *$P < 0.05$ vs. control, ANOVA and Student-Newman-Keuls test (SNK), $n = 3$. (C) CX_3CL1 prevents LPS-induced suppression of MFG-E8. Freshly collected rat peritoneal macrophages were stimulated with 10 ng/mL LPS ± treatment with 100 ng/mL fractalkine for 24 h. MFG-E8 protein levels were assessed by Western blotting (representative blots of two separate experiments shown). . *$P < 0.05$ vs. control, #$P < 0.05$ vs. LPS alone, ANOVA and Student-Newman-Keuls test (SNK), $n = 3$.

however, increased MFG-E8 levels in RAW264.7 cells by 22% and in resident peritoneal macrophages by 56% compared with controls (see Figure 2 A and B). CX_3CL1 also counter regulated LPS-mediated suppression of MFG-E8 in rat peritoneal macrophages. Although it did not prevent LPS-dependent decrease of MFG-E8 completely at the dose used in the present study, CX_3CL1 significantly reduced the LPS-mediated suppression of MFG-E8 expression (Figure 2C).

CX_3CL1 Promotes Phagocytosis of Apoptotic Cells by Inducing MFGE-8 in Macrophages

LPS-mediated decrease of MFG-E8 levels was associated with a decrease in phagocytosis of apoptotic cells (Figure 3 A and C). Preincubation with CX_3CL1, however, increased phagocytosis of apoptotic cells by 2.1-fold compared with untreated macrophages, indicating that CX_3CL1-induced MFG-E8 upregulation plays a role in the phagocytosis of apoptotic cells. CX_3CL1 was also able to partially restore LPS-induced suppression of phagocytosis of apoptotic cells (see Figure 3 A and C). Inhibition of MFG-E8 on CX_3CL1-stimulated macrophages with anti-MFG-E8 IgG $F_{ab'}$ almost completely abrogated MFG-E8-dependent phagocytosis of apoptotic cells to a phagocytosis index of 1.2 ± 0.1 (Figures 3B-C), demonstrating that CX_3CL1-induced phagocytosis of apoptotic cells is likely MFG-E8 dependent.

CX_3CL1 Does Not Alter Proinflammatory Cytokine Response in Macrophages

To investigate whether CX_3CL1 modulates proinflammatory cytokines in naïve and LPS-stimulated macrophages, we investigated the poststimulatory secretion of TNF-α and IL-6. Although both cell types used were responsive to LPS, CX_3CL1 did not change TNF-α or IL-6 cytokine release from RAW264.7 (Figure 4 A and B) or resident peritoneal macrophages (Figure 4 C and D). Additionally, TNF-α and IL-6 secretion from peritoneal macrophages after stimulation with LPS was not altered by CX_3CL1 preincubation at a dose of 100 nM (data not shown). This result indicates that CX_3CL1 does not directly influence proinflammatory cytokine responses. However, to investigate whether antiinflammatory mediators are altered by fractalkine, we measured IL-10 levels in the supernatants of peritoneal macrophages that were stimulated with LPS and fractalkine in vitro for 4 h. Although, as expected, LPS induced IL-10 secretion (0.8 ± 0.1 ng/mL, vs. 0.03 ± 0.02 ng/mL in controls), fractalkine alone or in conjunction with LPS significantly augmented IL-10 levels to 1.3 ± 0.2 and 1.6 ± 0.3 ng/mL, respectively ($P < 0.05$, ANOVA and Student Newman Keuls' test, $n = 6$).

Plasma Levels of CX_3CL1 and MFG-E8 Decrease in Sepsis

To investigate whether sepsis is associated with altered CX_3CL1 levels, we induced sepsis in rats by CLP and mea-

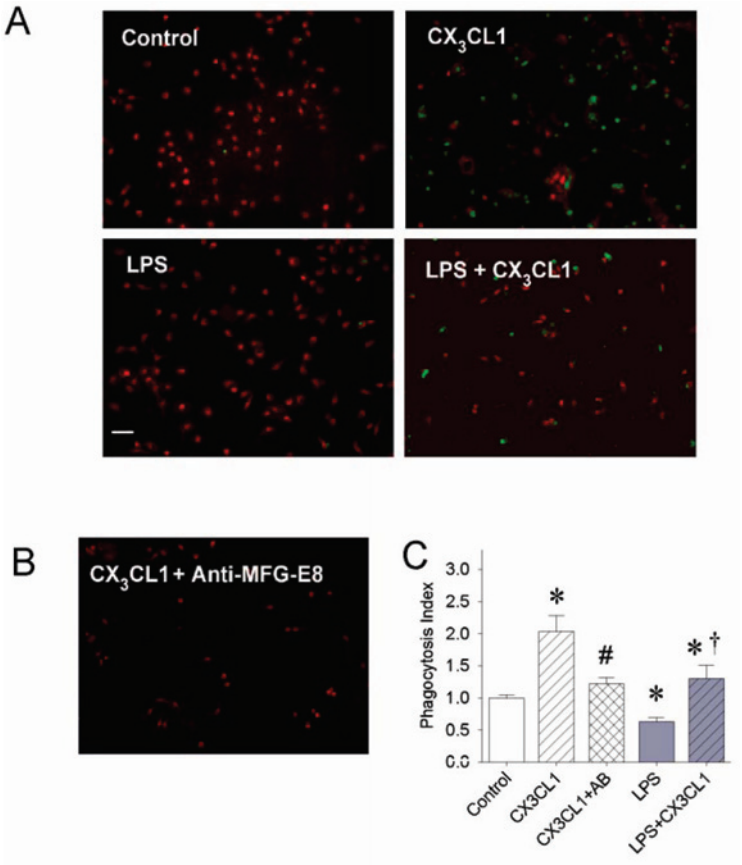

Figure 3. CX_3CL1 promotes phagocytosis of apoptotic cells in peritoneal macrophages. The assay for the phagocytosis of apoptotic cells was performed by incubating freshly collected rat peritoneal macrophages with apoptotic cells for 1.5 h after preincubation with LPS, fractalkine (CX_3CL1), or both for 24 h. (A) Immuno-fluorescent micrographs show macrophages in red (PI) with engulfed apoptotic thymocytes in green (TUNEL, size bars = 20 μm). (B) Resident peritoneal macrophages were stimulated with fractalkine (100 nM) for 24 h, washed and blocked with polyclonal anti-MFG-E8 Ab $F_{ab'}$ (1 μg/mL) for one hour prior to incubation with apoptotic thymocytes for 1.5 h. (C) Phagocytosis index (apoptotic cells / macrophages). Means ± SEM, *$P < 0.05$ vs. control, # $P < 0.05$ vs. CX_3CL1 only, †$P < 0.05$ vs. LPS only, ANOVA and Student Newman Keuls' test, n = 3-5.

sured its plasma levels. Circulating levels of liver enzymes (AST and ALT) and lactate increased significantly 20 h after CLP, indicating organ damage and disturbance of tissue oxygenation associated with sepsis (data not shown). While the proinflammatory cytokines TNF-α and IL-6 increased significantly after CLP (data not shown), plasma CX_3CL1 levels decreased by 20.5% (P = .015, Figure 5A). At an earlier time point (i.e., five hours after CLP) CX_3CL1 levels were slightly elevated (by 12.5% to 2,171.5 ± 135.2 pg/mL) compared with sham operated animals, without reaching a statistically significant difference (data not shown). The late decrease in plasma CX_3CL1 was associated with a dramatic decrease (by 55.5%) in MFG-E8 protein levels in peritoneal macrophages of septic animals (Figure 5B). Using a newly developed MFG-E8 ELISA method, we were able to detect blood levels of MFG-E8 in C57BL6/J mice and found that they significantly decreased from 3.14 ± 0.15 ng/mL in sham operated mice to 1.39 ± 0.20 ng/mL 20 h after CLP ($P < 0.05$, t-Test, n = 5) (Figure 5C). Focusing on the possible beneficial effect of CX_3CL1, we injected 100 μg/mL of CX_3CL1 to mice at the time of CLP. Twenty hours later we found not only improved, but completely restored, plasma levels of MFG-E8 in the septic animals compared with vehicle-treated mice (Figure 5C). This outcome was associated with a complete reversal of sepsis-induced increases of blood lactate levels in the septic mice (Figure 5D) and associated reduction of liver injury and proinflammatory markers (data not shown).

DISCUSSION

In an experimental sepsis model, we found that plasma CX_3CL1 levels significantly decreased, a response that was paralleled by a dramatic decrease in MFG-E8 in peritoneal macrophages and blood. MFG-E8 is crucial for efficient clearance of apoptotic cells. While LPS suppressed MFG-E8 expression and phagocytosis of apoptotic cells, CX_3CL1 improved phagocytosis by upregulating MFG-E8 in macrophages. However, CX_3CL1 by itself did not induce proinflammatory cytokine release from non-stimulated or LPS-stimulated peritoneal macrophages.

CX_3CL1 is upregulated under a variety of pathological conditions, such as vascular injury and tissue damage (30). It is responsible for the recruitment of natural killer cells to the brain during experimental autoimmune encephalitis (31), involved in asthma-associated mast cell chemotaxis (19), and chemotaxis of leukocytes in experimental autoimmune myositis (18). It is also associated with the induction of an antitumor Th_1 response (21,32). Inflammation has been reported to induce NF-κB-dependent CX_3CL1 upregulation in different tissues, through TNF-α, LPS or advanced glycation end-products (30,33,34). This findings suggests that CX_3CL1 plays an important role in inflammation; it also has a function under physiological conditions, however. CX_3CL1 is associated with the guidance of mucosal dendritic cells for the uptake of intestinal antigens leading to tolerance (22,35), and with neuromodulation (13)

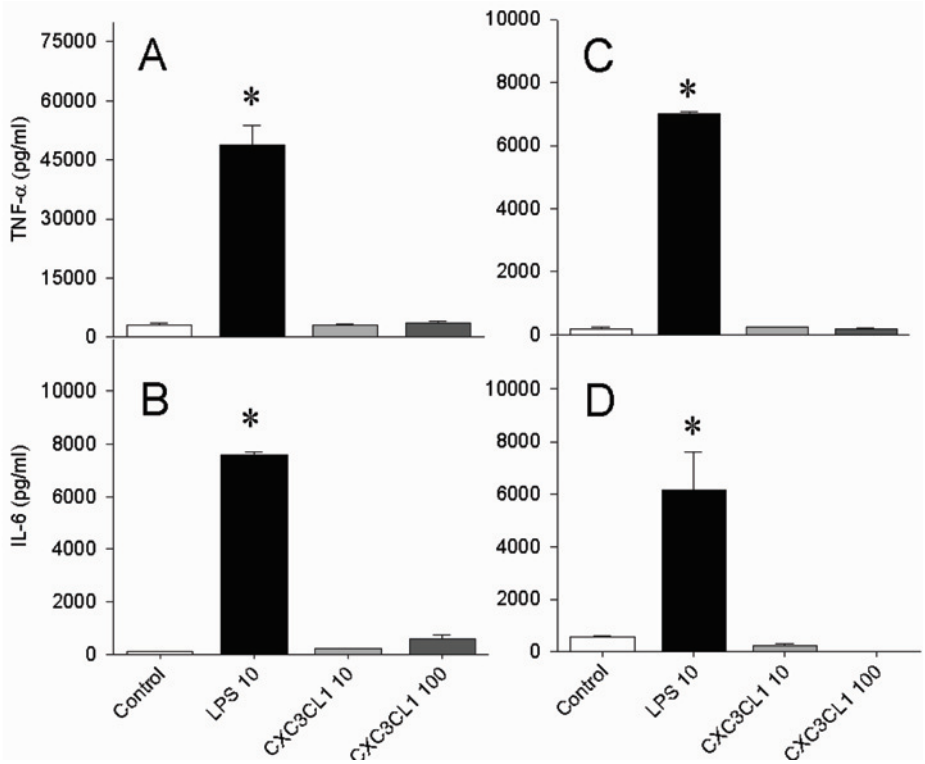

Figure 4. CX_3CL1 does not induce inflammation in cultured macrophages. RAW264.7 cells (A, B) and freshly isolated peritoneal macrophages (C, D) were stimulated with LPS (10 ng/mL) or fractalkine (CX_3CL1, 10 and 100 nM without LPS) for four hours. Panels show concentrations of TNF-α (A, C) and IL-6 (B, D) for both macrophage populations. Means ± SEM, *$P < 0.05$ vs. control, ANOVA and Student-Newman-Keuls' test, $n = 5$.

and postnatal neuronal development (16). CX_3CL1 has even been shown to be beneficial in certain circumstances, because it attenuates glutamate-mediated neurotoxicity in the brain (11,12,36).

Fractalkine surface expression and release from endothelial cells have been shown to be stimulated by IL-1β, TNF-α, and LPS in vitro. Although increased concentrations of these aforementioned proinflammatory mediators play an important role in sepsis, data on fractalkine levels in sepsis were unavailable. Sung et al. described how TNF-α and IL-1β stimulate fractalkine release peaking at 4-6 h after stimulation with a steady decline in fractalkine concentrations thereafter (37). Five hours after CLP, plasma levels were slightly elevated compared with sham operated animals. In late CLP-induced sepsis, which is marked by a systemic inflammatory response and organ damage, we found that circulating CX_3CL1 levels decreased significantly. This finding corresponds well with previously described phenomena in vitro (37). The reason for such a decrease in late sepsis remains unknown. In vitro studies indicate, however, that LPS at least transiently downregulates TACE expression on the cell surface of human monocytes (38). This effect could account for the decrease in soluble CX_3CL1 in the circulation in the late phase of sepsis.

In addition, decreased CX_3CL1 is associated with a decrease in MFG-E8 levels which has been previously shown to be associated with impaired apoptotic cell clearance and poor outcome in sepsis (27). CLP-induced sepsis has been shown to lead to suppressed MFG-E8 levels in the spleen and liver (27), and we show for the first time that sepsis is also associated with systemically decreased MFG-E8 levels. We have also demonstrated the downregulation of MFG-E8 expression by LPS in vitro, which acts through the Toll-like receptor 4. In this respect, Jinushi et al. have recently shown that the surface expression of MFG-E8 on macrophages can also be reduced by the stimulation with peptidoglycan, poly I:C, or CpG, ligands for three other Toll-like receptors 2, 3, and 9, respectively (39). However, a more detailed investigation of the mechanism responsible for the downregulation of MFG-E8 in sepsis is needed. This phenomenon can be found across at least two species (mice and rats), strengthening the possibility that this decrease is also relevant in other mammals, including septic human patients. We previously reported that peritoneal macrophages collected from rats 20 h after CLP displayed a slight but significant decrease of phagocytic capability regarding the clearance of apoptotic cells ex vivo (27), which coincides with the decreased cellular levels of MFG-E8 in these macrophages. Similarly, freshly isolated macrophages from healthy animals show a decreased release of MFG-E8 (by 55%) four hours after stimulation with 100 ng/mL LPS (data not shown).

As a soluble chemokine, CX_3CL1 induces clearance of apoptotic cells, as we have shown in this study. Blocking the effect of MFG-E8, which is uregulated by CX_3CL1, abrogated the prophagocytic effect of CX_3CL1. Although the plasma concentrations of fractalkine were about 1% of the concentrations used in the in vitro experiments for the stimulation of MFG-E8 and phagocytosis of apoptotic cells, we believe that this chemokine plays a crucial role under in vivo conditions. Lower plasma concentrations may represent only a fraction of the actually active fractalkine, indicating a possible localized/paracrine effect. It may also indicate that other factors play a role under in vivo conditions that are not present in vitro, which can be overcome only by the use of supraphysiological concentrations of fractalkine. Although the exact mechanism of CX_3CL1-mediated MFG-E8 in-

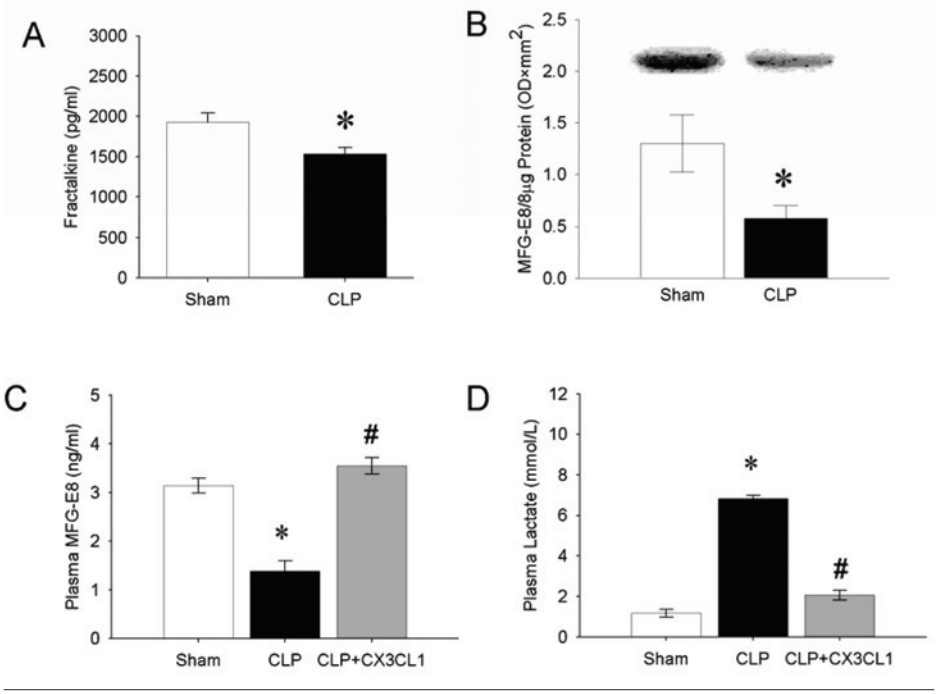

Figure 5. Sepsis-induced decrease of CX_3CL1 and MFG-E8. Rats (A, B) or mice (C, D) were subjected to sham operation or CLP, and blood and peritoneal macrophages were collected at 20 h. (A) Plasma fractalkine (CX_3CL1) levels were assessed in blood plasma by ELISA. Means ± SEM, **P* < 0.05 vs. Sham, Student *t*-test, *n* = 6. (B) MFG-E8 levels in peritoneal macrophages were assessed by Western blotting. A representative blot is shown. Means ± SEM, **P* < 0.05 vs. Sham, Student *t*-test, *n* = 6. (C) Plasma MFG-E8 levels were assessed using ELISA in septic mice 20 h after CLP with or without concurrent treatment with recombinant murine CX_3CL1. (D) Lactate levels were assayed in the same mice using EIA. **P* < 0.05 vs Sham, #*P* < 0.05 vs. Vehicle, ANOVA and Student Newman Keuls' test, *n* = 5.

duction remains to be elucidated, our data indicates that this chemokine is able to induce MFG-E8 protein expression in rat resident peritoneal macrophages and a murine macrophage cell line. Similar results have been reported in the resident macrophages of the brain (i.e., microglia) (24). It has been proposed, but not verified, that the induction of MFG-E8 is associated with an increased clearance of apoptotic cells after an insult (24). We have provided evidence for the first time that this is true in resident peritoneal macrophages and show that this induction leads to an increase in MFG-E8-dependent phagocytosis of apoptotic cells.

The exact mechanism still needs to be elucidated, but activation of the G-protein coupled cognate cell-surface receptor CX_3CR1, which induces an intracellular Ca^{2+} surge, is likely to play a role. CX_3CL1 has also been reported to induce the extracellular signal-regulated kinase (ERK) and the phosphoinositide-3 kinase (PI3K)/Akt pathways without inducing the stress kinases p38 or c-Jun N-terminal kinases (JNK) (11,12,33,36,40). The cytokine profile of macrophages after incubation with CX_3CL1 showed no induction of TNF-α or IL-6 in vitro, which is consistent with the signal-transduction pathways reported earlier. On the other hand, IL-10 levels were significantly increased in fractalkine-stimulated peritoneal macrophages, pointing toward a possible antiinflammatory effect and an involvement of the Stat3 pathway. However, the precise intracellular signal transduction for the up-regulation of MFG-E8 by CX_3CL1 needs further investigation.

As we have shown, CX_3CL1 enhances the phagocytosis of apoptotic cells via the induction of MFG-E8 in macrophages. In both primary peritoneal macrophages and the murine macrophage cell line RAW267.4, cells showed responsiveness to fractalkine by upregulation of MFG-E8 protein expression. Furthermore fractalkine induced phagocytosis of apoptotic cells in an MFG-E8-dependent manner. Although Leonardi-Essmann et al. showed that MFG-E8 is upregulated in microglial cells (24), evidence of a functional enhancement of apoptotic cell clearance was not provided. Our data indicate, however, that this is very likely. Other tissue macrophages such as Kupffer cells or alveolar macrophages that also display a highly phagocytic function may show similar effects. This needs to be investigated in the future. These results, in conjunction with our in vivo data showing CX_3CL1-mediated upregulation of MFG-E8 in septic mice, provide sufficient evidence that the MFG-E8 levels are directly linked to CX_3CL1. Studies in which recombinant CX_3CL1 is administered to septic mice lacking MFG-E8 would provide further evidence of a causative link between the two proteins and should be conducted in the future. While endothelial cell-surface upregulation of CX_3CL1 leads to a higher transmigration of immune cells into inflammatory tissues, the soluble chemokine CX_3CL1 seems to improve clearance of apoptotic cells by macrophages through the induction of the opsonin MFG-E8. This function can be well used in the treatment of sepsis.

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