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Pivotal Role of the CC Chemokine, Macrophage-Derived Chemokine, in the Innate Immune Response[1]

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Macrophage-derived chemokine (MDC), a recently identified CC chemokine, has been regarded to be involved in chronic inflammation and dendritic cell and lymphocyte homing. In this study, we demonstrate a pivotal role for MDC during experimental sepsis induced by cecal ligation and puncture (CLP). Intraperitoneal administration of MDC (1 μg/mouse) protected mice from CLP-induced lethality. The survival was accompanied by increased number of peritoneal macrophages and decreased recovery of viable bacteria from the peritoneum and peripheral blood. In addition, mice treated with an i.p. injection of MDC cleared bacteria more effectively than those in the control when 3×10^8 CFU live *Escherichia coli* was i.p. inoculated. Endogenous MDC was detected in the peritoneum after CLP, and neutralization of the MDC with anti-MDC Abs decreased CLP-induced recruitment of peritoneal macrophages and increased the recovery of viable bacteria from the peritoneum and peripheral blood. MDC blockade was deleterious in the survival of mice after CLP. In vitro, MDC enhanced the phagocytic and killing activities of peritoneal macrophages to *E. coli* and induced both a respiratory burst and the release of lysozomal enzyme from macrophages. Furthermore, MDC dramatically ameliorated CLP-induced systemic tissue inflammation as well as tissue dysfunction, which were associated in part with decreased levels of TNF-α, macrophage inflammatory proteins-1α and -2, and KC in specific tissues. Collectively, these results indicate novel regulatory activities of MDC in innate immunity during sepsis and suggest that MDC may aid in an adjunct therapy in sepsis. ***The Journal of Immunology,*** **2000, 164: 5362–5368.**

The innate immune response is the first line of defense to protect a host from invading pathogens, including bacteria, that could threaten survival. The innate immune response, through the actions of phagocytic leukocytes such as neutrophils and macrophages, functions to inactivate and clear bacteria and microbial-related noxious products (1, 2). This first line of defense against an infectious agent involves both the recruitment and activation of leukocytes at an infectious focus, allowing these cells to successfully localize, kill, and clear the pathogen (3, 4). Growing lines of evidence suggest that the recruitment of specific leukocyte populations is governed by cell-specific chemoattractants called chemokines (5, 6). Chemokines basically have been divided into two main families, CXC and CC chemokines, based on the sequence homology and the position of the first two cysteine residues. CXC chemokines are typically chemotactic for neutrophils, whereas CC chemokines attract and activate monocytes and lymphocytes (7).

Macrophage-derived chemokine (MDC),[3] a recently identified member of the CC chemokine family, has been shown to be a potent chemoattractant for dendritic cells, NK cells, and the Th2 subset of T cells (8). MDC binds to CCR4, which is expressed on Th2 cells (9, 10). T cells attracted by MDC generated cell lines predominantly producing Th2-type cytokines such as IL-4 and IL-5 (10). Recently, MDC has been shown to be involved in T cell-mediated allergic airway inflammation and atopic dermatitis (11, 12). In addition, it has been reported that MDC is highly expressed by epithelial cells of normal thymus and attracts CD3- and CD4-positive T cells in the thymus (13). Thus, most of the studies to date have focused on the involvement of MDC in the setting of chronic inflammation and dendritic cell and lymphocyte homing. However, MDC is also known to attract monocytes both in vitro and in vivo (11, 14). The fact that the recruitment and subsequent activation of monocytes into infectious foci have a pivotal role to clear bacteria (4, 15) suggests that MDC may play a crucial role in innate immunity during sepsis induced by bacterial infection. Furthermore, mRNA and protein expressions for MDC from cultured monocytes were induced by IL-4 and IL-13 (16). IL-4 and IL-13 are known to exert beneficial effects in experimental models of sepsis via modulating the production of inflammatory cytokine/chemokines (17, 18). Thus, it is interesting to speculate that IL-4- and IL-13-inducible CC-chemokine MDC may play a beneficial role in innate immunity during sepsis.

In the present study, we explored the role of MDC during sepsis induced by a well-established murine septic peritonitis model, cecal ligation and puncture (CLP) (19). The studies have demonstrated for the first time that MDC induces both a respiratory burst in macrophages and the release of lysozomal enzyme, enhances phagocytosis, increases the bactericidal activity of macrophages, and protects mice from the lethality induced by CLP. In addition, MDC treatment ameliorated systemic tissue inflammation and tissue dysfunction induced by CLP via modulation of the levels of inflammatory cytokines and chemokines in specific tissues. These data indicate that MDC exerts novel immune-regulatory activities during the innate immune response associated with acute septic peritonitis.

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[3] Abbreviations used in this paper: MDC, macrophage-derived chemokine; CLP, cecal ligation and puncture; TSA, thymic shared Ag; AST, aspartate transaminase; BUN, blood urea nitrogen; MPO, myeloperoxidase; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein.

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Materials and Methods

Mice

Specific pathogen-free female CD-1 mice (6–8 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the animal care facility unit (University Laboratory of Animal Medicine, University of Michigan Medical School). The Animal Use Committee at the University of Michigan approved all studies.

Cecal ligation and puncture

The mice underwent CLP surgery, as described previously (19). In brief, the mice were anesthetized with i.p. injection of ketamine HCl (Vetamine; Mallinckrodt, Mundelein, IL) and then inhaled methoxyflurane (Metafane; Mallinckrodt). Under sterile conditions, the cecum was exposed through a 1- to 2-cm incision of the lower left abdomen, ligated with a 3-0 silk suture below the ileocecal valve, and punctured through and through once with a 21-gauge needle. The cecum was replaced in the peritoneum, and the abdomen was closed with surgical staples. The mice were injected with 1 ml of saline s.c. for fluid resuscitation and placed on a heating pad until they recovered from anesthesia.

Experimental protocol

Immediately after CLP, the mice were treated with either i.p or i.v. injection of recombinant murine MDC (1 μg/mouse; R&D Systems, Minneapolis, MN; endotoxin content $<$ 0.1 ng/μg). Equivalent volume of saline was used as a control. In the first set of experiments, the mice were monitored for 7 days after CLP to determine the survival rate induced by CLP. In the next set of experiments, the CLP mice were anesthetized, bled, and euthanized at appropriate intervals after CLP. The peritoneal cavities were washed with 2 ml of sterile saline, and the lavage fluids were harvested. After taking a 10-μl aliquot of lavage fluids for the assessment of bacteria CFU, the fluids were centrifuged at 6000 $\times$ *g* for 1 min at 4°C. Cell-free peritoneal fluids were stored at −20°C, the cell pellets were resuspended in saline, and the cell numbers were counted in a hemocytometer. Differential cell analyses were performed by Diff-Quik-stained cytospin preparations (Dade, Düdingen, Switzerland). The liver, lung, and kidney were excised, weighed, frozen in liquid nitrogen, and stored at −20°C for subsequent analyses.

Passive neutralization of MDC was conducted in some CLP mice by i.p. injection of 0.5 ml of anti-murine MDC antiserum 2 h before CLP. Anti-MDC antiserum was raised by immunizing New Zealand White rabbits with murine MDC (R&D Systems). The polyclonal Abs were titered by direct ELISA, and the Abs recognized MDC even at a dilution of 1×10^{-6}. The Abs completely blocked MDC-induced T cell chemotactic activity in vitro (data not shown). The Abs did not cross-react with a number of other murine cytokines, including CXC and CC chemokines, as seen in the fact that MDC ELISA established with the Abs did not detect any murine cytokines at a concentration as high as 100 ng/ml. As a control, 0.5 ml of pre-immune serum was used. The endotoxin content in both anti-MDC antiserum and control serum was below detection level ($<$0.05 EU/ml; PYROGENT; BioWhittaker, Walkersville, MD).

In other studies, mice received an i.p. inoculation of live *Escherichia coli* (3×10^8 CFU) that were previously recovered from the peritoneum of CLP mice. Immediately after the injection, each mouse received an i.p. injection of either MDC (1 μg/mouse) or saline. Twenty-four hours later, the mice were anesthetized, bled, and euthanized, and the peritoneal cavities were washed with 2 ml of sterile saline. Ten-microliter aliquots of lavage fluids were used for the assessment of bacteria CFU in the peritoneum.

Determination of CFU

A total of 10 μl of peritoneal lavage fluids and peripheral blood from each mouse was placed on ice and serially diluted with sterile saline. Ten microliters of each dilution was plated on thymic shared Ag (TSA) blood agar plates (Difco, Detroit, MI) and incubated overnight at 37°C, after which the number of colonies was counted. Data were expressed as CFU/10 μl.

Phagocytic and killing activities of macrophages in vitro

Peritoneal cells (1×10^6 cells) harvested from normal mice were suspended in antibiotic-free RPMI 1640 containing 5% FCS and were incubated at 37°C in two 24-well culture dishes. Two hours later, nonadherent cells were removed, the medium was replaced, and the adherent cells were preincubated with various concentrations of MDC (0, 20, or 100 ng/ml) for 1 h. The cells were then infected with 1×10^6 CFU of *E. coli* recovered from the peritoneum after CLP. After 1-h incubation, the wells were extensively washed out to remove unphagocytosed bacteria, and the wells in one plate were lysed with sterile 0.5% Triton X-100 for bacterial phagocytosis assay. Wells in the other plate were replaced with prewarmed fresh medium and incubated for an additional 2 h, after which the cells were lysed with 0.5% Triton X-100 for bacterial killing assay. The serially diluted cell lysates were plated on TSA blood agar plates (Difco) and incubated overnight at 37°C, and the numbers of colonies were counted.

Superoxide production

The production of superoxide from cultured macrophages was measured using the reduction of ferricytochrome *c* (20, 21). In brief, peritoneal macrophages (1×10^6 cells) harvested from normal mice were cultured in phenol red-free RPMI 1640 in 24-well culture dishes for 2 h at 37°C. Nonadherent cells were removed and the medium was replaced with fresh medium containing cytochrome *c* (1.3 mg/ml; Sigma, St. Louis, MO). The adherent cells were stimulated with various concentrations of MDC (0, 1, 10, or 100 ng/ml) at 37°C. After 30-min incubation, the plate was shaken gently, and the supernatants were harvested. Superoxide production in the supernatants was measured spectrophotometrically at 550 nm as a function of ferricytochrome *c* reduction. To exclude the presence of other reductants, 15 μl of superoxide dismutase (SOD; 4.5 mg/ml; Sigma) was added as a control for each sample. The amount of superoxide was calculated and expressed as pmol SOD-inhibitable cytochrome *c* reduction/min/10^6 cells according to the following formula (21): ΔA/min $\times$ reaction volume $\times$ 10^6 nmol/mmol $\times$ 1000/(Cy/mmol/L) $\times$ 1000 ml/L = pmol cytochrome *c*, where ΔA/min = change in absorbance per minute and Cy = extinction coefficient of cytochrome *c* (21.1 mM/cm).

Measurement of lysozomal enzyme release

The lysozomal enzyme release from macrophages was determined by lysozyme activity in the culture supernatants. Peritoneal macrophages (1×10^6 cells) harvested from normal mice were cultured in phenol red-free RPMI 1640 containing 5% FCS and antibiotics in 24-well culture dishes for 2 h at 37°C. Nonadherent cells were removed, the medium was replaced, and the adherent cells were stimulated with various concentrations of MDC (0, 1, 10, or 100 ng/ml) for 48 h. Lysozyme activity was assayed by a turbidometric method (22). In brief, 50 μl of culture supernatant was added to 200 μl of the assay mixture, which consisted of 30 mg/ml *Micrococcus lysodeikticus* (Sigma) in 50 mM sodium acetate buffer (pH 6.0) and 0.05% Triton X-100. Chicken egg white lysozyme (Sigma) was used as a standard. The rate of substrate lysis was measured at 540 nm after 30 min. The activity was expressed as micrograms of lysozyme activity/10^6 cells.

Clinical chemistry

Serum levels of aspartate transaminase (AST), blood urea nitrogen (BUN), and creatinine were measured by Clinical Pathology at the University of Michigan Medical School using standardized techniques.

Measurement of cytokines and myeloperoxidase (MPO)

Murine MDC was quantitated using a standard method of sandwich ELISA. In brief, microtiter plates were coated with 50 μl of affinity-purified anti-murine MDC IgG (R&D Systems; 1 μg/ml) in coating buffer (0.6 M NaCl + 0.26 M H_3BO_4 + 0.08 M NaOH (pH 9.6)). Detection and processing were made by using biotinylated rabbit anti-MDC IgG (3.5 μg/ml), streptavidin-peroxidase conjugate (Bio-Rad, Richmond, CA), and chromogen substrate (Bio-Rad). TNF-α, macrophage inflammatory protein (MIP)-1α, MIP-2, KC, and monocyte chemoattractant protein (MCP)-1 were quantitated by specific sandwich ELISA, as described elsewhere (23, 24). The ELISAs employed in this study did not cross-react with other murine cytokines available and consistently detected murine cytokine concentrations above 25 pg/ml. MPO in tissue extracts was measured using an ELISA kit (Calbiochem-Novabiochem, San Diego, CA) according to the manufacturer's instruction. The lower detection limit was 1.6 ng/ml.

Preparation of tissue extracts

The excised tissues (0.1 g) were placed in 1 ml of homogenization buffer (500 mM NaCl + 50 mM HEPES (pH 7.4) containing 0.1% Triton X-100, 0.5 μg/ml leupeptin, 1 mM PMSF, and 0.02% NaN_3) and were homogenized with a Tissue Tearor (model 985-370; Biospec Products, Racine, WI). The homogenates were then subjected to one freeze/thaw extraction before ELISA or three freeze/thaw extractions before MPO assay. The homogenates were centrifuged at 6000 $\times$ *g* for 20 min at 4°C, and the cell-free supernatants were used for measurement of cytokines or MPO.

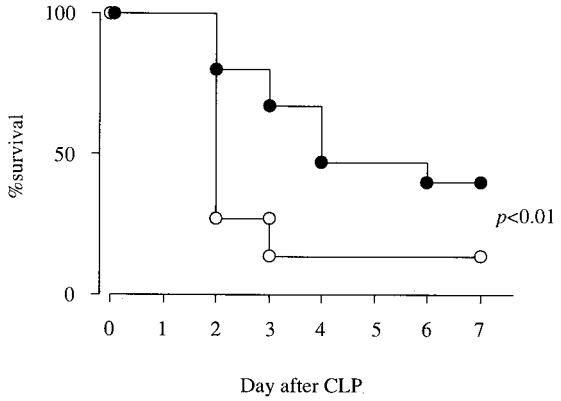

FIGURE 1. MDC protects mice from the lethality induced by CLP. The CLP mice were treated with i.p. injection of either MDC (1 μg/mouse; ●; 15 mice) or vehicle (○; 15 mice) immediately after CLP. The survival rates were monitored for 7 days after CLP. Two different experiments were conducted, and the data were pooled. The mortality rates were very similar in individual experiments.

Statistics

Statistical significance was evaluated by ANOVA. In case of survival curve and CFU count, the data were analyzed by the log-rank test and Mann-Whitney test, respectively. A p value <0.05 was regarded as statistically significant. All data were expressed as mean ± SEM.

Results

MDC protects mice from CLP-induced lethality

To determine whether the CC chemokine MDC is capable of exerting beneficial effects on the lethality induced by CLP, either MDC or vehicle was injected i.p. or i.v. immediately after CLP surgery, and survival rates were monitored. The data in Fig. 1 show the protective effects of MDC on the survival of mice after CLP. When MDC was administered into the peritoneum, the protective effects were seen after 48 h post-CLP. At 48 h after CLP, the mortality rate in control mice was 73% (11/15 mice), whereas only a 20% mortality rate (3/15 mice) was found in mice treated with MDC (Fig. 1). On day 7, 13% of control mice (2/15 mice) survived compared with 40% in MDC-treated mice (6/15 mice). Although the survival rate on day 7 after i.v. injection of MDC was similar to that after i.p. injection (42%; 8/19 mice), the i.v. administration did not affect the survival of mice at 48 h after CLP, causing no statistical significant difference compared with control (mortality rates in control vs MDC-treated mice at 48 h after CLP, 58% (11/19 mice) vs 53% (10/19 mice), respectively). The results clearly indicate a beneficial role of MDC in CLP-induced lethality and suggest that MDC needs to act locally in the peritoneal cavity for mouse survival.

MDC increases the recruitment of macrophages into the peritoneum and decreases viable bacteria recovered from CLP mice

CLP permits the leakage of polymicrobial flora in the peritoneal cavity, which leads to a local inflammatory response directed at eliminating bacteria from the peritoneum (19). Experiments were conducted to assess the local effects of MDC in this model of sepsis. Intraperitoneal injection of MDC increased the recruitment of macrophages, but not of neutrophils, in the peritoneum at 24 h after CLP, compared with control (Fig. 2*A*). The amount of viable bacteria recovered from the peritoneum at 24 h after CLP was markedly decreased in mice treated with MDC compared with control (Fig. 2*B*). The mean CFU in MDC-treated mice (6.3×10^5/10 μl) was 40-fold lower than that in control (2.7×10^7/10 μl; $p < 0.01$). At the same time point (24 h), bacteremia was found in 10 of 11 control mice (mean CFU = 3.3×10^4/10 μl blood), whereas 5 of 14 mice treated with MDC were bacteremic and the mean CFU was 12-fold lower than that in control (2.7×10^3/10 μl blood; $p < 0.05$; Fig. 2*C*). MDC treatment resulted in significant decreases in the peritoneal levels of TNF-α, MIP-2, KC, MIP-1α, and MCP-1 at 24 h after CLP (Table I). Thus, MDC appears to elicit the recruitment of macrophages into the peritoneum independently of other cytokines/chemokines that may attract the cells and also seems to increase bacterial clearance.

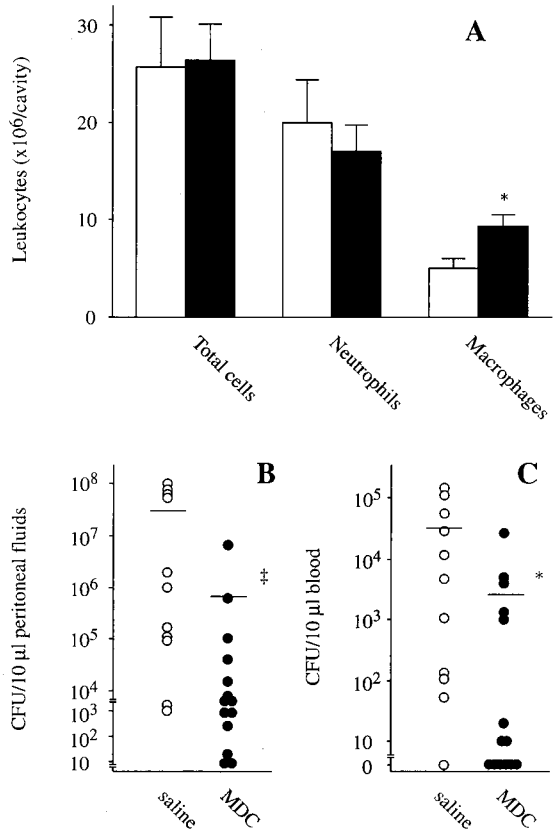

FIGURE 2. Effects of MDC on the leukocyte infiltration and bacterial load in the peritoneum and peripheral blood. MDC (1 μg/mouse) or vehicle was i.p. injected immediately after CLP. Twenty-four hours later, the mice were anesthetized, bled, and euthanized. *A*, The numbers of infiltrating leukocytes in the peritoneum were counted. Differential cell analyses were determined by Diff-Quik-stained smears. □, Control mice (11 mice). ■, MDC-treated mice (14 mice). *B* and *C*, Ten microliters of peritoneal fluids (*B*) and sera (*C*) were serially diluted and plated on TSA blood agar plates, and the CFU was determined. Line represents the median CFU count. ○, Control mice (10 mice); ●, MDC-treated mice (10 mice). Two different experiments were conducted, and the data were pooled. The results were very similar in individual experiments. *, $p < 0.05$; ‡, $p < 0.01$; when compared with control.

MDC enhances phagocytic and killing activities of macrophages to E. coli

To confirm the in vivo effects of MDC in bacterial clearance, either MDC (1 μg) or vehicle was given i.p. immediately after i.p. inoculation of *E. coli* (3×10^8 CFU/mouse). Twenty-four hours later, the bacterial load in the mice was assessed. Fig. 3 shows that MDC treatment lessened the viable bacteria recovered from the peritoneum and blood compared with that from control. The mean

Table I. *Amounts of cytokines in the peritoneal fluids at 24 h after CLP*[a]

Cytokines (ng/cavity)	Control	MDC (1 μg/mouse)
TNFα	16.4 ± 2.2	10.5 ± 1.3*
MIP-2	24.7 ± 2.4	17.7 ± 1.6*
KC	26.2 ± 6.1	10.1 ± 4.3*
MIP-1α	13.5 ± 2.9	5.4 ± 1.4*
MCP-1	88.4 ± 10.4	53.4 ± 8.0*

[a] Mice were treated with either MDC (1 μg/mouse; 14 mice) or vehicle (11 mice) immediately after CLP. At 24 h after CLP, the mice were euthanized, and the peritoneal fluids were harvested. The amounts of cytokines in the peritoneal fluids were measured by ELISA. Two different experiments were carried out, and the data were pooled. The results were very similar in individual experiments.

*, $p < 0.05$, when compared with control.

CFU in the peritoneum in MDC-treated mice (1.6×10^4/10 μl) was 16-fold lower than that in control (2.6×10^5/10 μl; $p < 0.01$; Fig. 3*A*). Bacteremia was found in 70% of control mice (7/10 mice), whereas 20% of mice treated with MDC were bacteremic (2/10 mice) (Fig. 3*B*).

To identify the mechanism whereby MDC cleared bacteria in vivo, peritoneal macrophages were cultured in the presence or absence of MDC, and the phagocytic and killing activities of macrophages to *E. coli* were examined in vitro. As shown in Table II, MDC dose dependently increased the CFU counts inside the cells after 1-h incubation. When the infected cells were cultured for an additional 2 h, the killing rate of bacteria was markedly increased by the treatment with MDC in a dose-dependent fashion. Bacterial killing rate by macrophages treated with 100 ng/ml MDC was five times higher than that with control (Table II). It appeared that MDC cleared bacteria through enhancing the phagocytic and bactericidal activities of macrophages.

Neutralization of endogenous MDC decreases the recruitment of macrophages into the peritoneum and increases viable bacteria recovered from CLP mice

Above-described findings clearly show that MDC treatment was effective in bacterial clearance. We next examined whether endogenous MDC was capable of exerting the bactericidal activity in this model of sepsis. As shown in Fig. 4*A*, the level of MDC in the peritoneal fluids significantly increased with time and remained elevated at 48 h after CLP. MDC blockade with anti-MDC Abs decreased the number of infiltrating macrophages, but not neutrophils, in the peritoneum at 8 h after CLP, compared with the number in control (Fig. 4*B*). At this time point, no differences were found in the peritoneal levels of TNF-α, MIP-2, KC, MIP-1α, and MCP-1 (data not shown). Although the numbers of leukocyte populations at 24 h were similar between the groups, the recovery of viable bacteria from the peritoneum at this time point markedly increased in mice treated with anti-MDC Abs, compared with that of control (Fig. 4*C*). The peritoneal levels of TNF-α, MIP-2, KC, MIP-1α, and MCP-1 at 24 h after CLP were significantly increased by 2- to 3-fold by anti-MDC treatment (data not shown). At the same time point (24 h), bacteremia was found in four of seven mice treated with control Abs, whereas all mice that received anti-MDC Abs were bacteremic (nine of nine mice). The mean CFU in anti-MDC-treated mice was 10^3-fold higher than that in the control (Fig. 4*D*). Furthermore, neutralization of endogenous MDC significantly deteriorated the survival of mice after CLP compared with that of the control (Fig. 5). The data support a host defense role of MDC during experimental sepsis because neutralization of MDC resulted in a reduced ability to clear bacteria from the host.

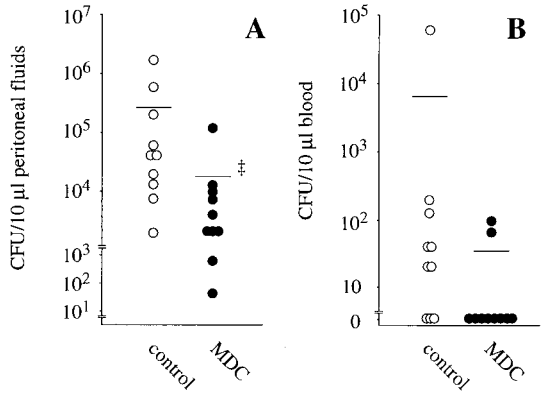

FIGURE 3. MDC enhances bacterial clearance. Either MDC (1 μg/mouse; ●; 10 mice) or vehicle (○; 10 mice) was i.p. injected, immediately after an i.p. inoculation of live *E. coli* (3×10^8 CFU) recovered from CLP mice. Twenty-four hours later, the mice were euthanized and the peritoneal fluids were harvested. Ten microliters of peritoneal fluids (A) and sera (*B*) were serially diluted and plated on TSA blood agar plates, and the CFU was determined. Line represents the median CFU count. Two different experiments were conducted, and the data were pooled. The results were very similar in individual experiments. ‡, $p < 0.01$, when compared with control.

Table II. *MDC enhances phagocytic and killing activities of macrophages in vitro*[a]

MDC (ng/ml)	Incubation (h)	*E. coli* CFU[b] ($\times 10^3$)	Phagocytosis[c] Index	Killing Rate[d] (%)
0	1	41.8 ± 3.9	1.0	
	3	37.2 ± 3.5		11.0
20	1	62.6 ± 7.9	1.5	
	3	39.0 ± 7.5		37.7
100	1	97.1 ± 26.3*	2.3	
	3	48.5 ± 8.4		51.1

[a] Peritoneal macrophages, recovered from normal mice, were preincubated with MDC for 1 h and challenged with *E. coli* (1×10^6) recovered from CLP mice. The data are representative of the two individual experiments ($n = 6$ each). *, $p < 0.05$, when compared with control (MDC = 0 ng/ml).

[b] After 1-h incubation, the wells were extensively washed and the cells were lysed with 0.5% Triton X-100 for phagocytosis assay. Wells were replaced with fresh medium and cultured for an additional 2 h, after which the cells were lysed with 0.5% Triton X-100 for killing assay. The cell lysates were serially diluted and placed on TSA blood agar plates, and CFU within the cells were determined.

[c] Phagocytosis index was standardized by the data obtained from control (MDC = 0 ng/ml).

[d] Killing rate = [1 − (CFU at 3 h/CFU at 1 h)] × 100.

MDC induces respiratory burst and lysozomal enzyme release from macrophages

The ability of MDC to activate macrophages was evaluated next. During activation of macrophages, reactive oxygen products are generated and lysozomal enzymes are released from the granules (25, 26). In the present study, we measured superoxide anion (O_2^-) generation (a NADPH-dependent oxidase enzyme) and lysozyme release (a lysozomal enzyme) from macrophages after the stimulation with MDC. The data in Fig. 6 show that MDC elicited the generation of O_2^- and lysozyme release from macrophages in a dose-dependent manner. Heat-inactivated MDC (100°C for 3 min) did not induce these observations (data not shown). The data clearly indicate that MDC can activate macrophages and likely can enhance intracellular bacterial killing.

MDC ameliorates tissue inflammation and injury induced by CLP

Sepsis frequently causes severe systemic inflammation (27). Next, experiments were conducted to assess the systemic effects of MDC

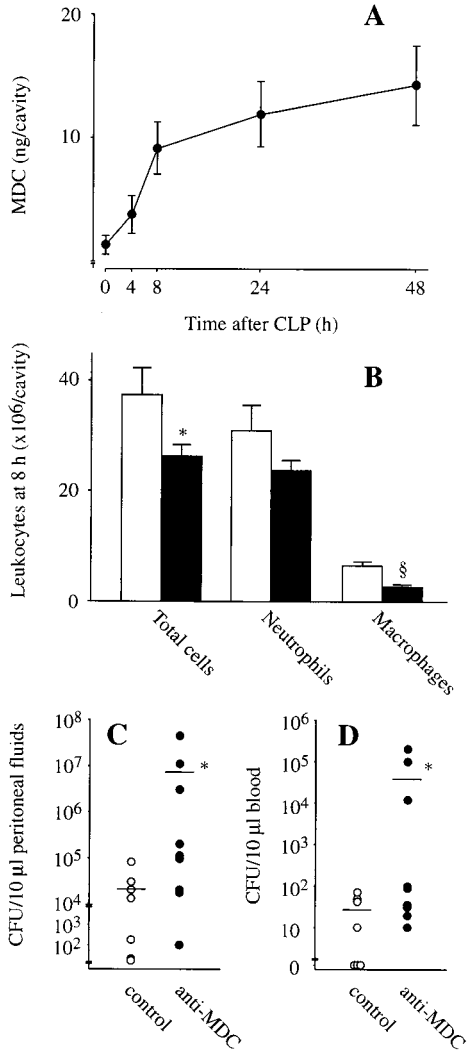

FIGURE 4. MDC blockade decreases the recruitment of peritoneal macrophages and increases viable bacteria recovered from CLP mice. *A*, At indicated intervals after CLP, mice were euthanized and bled, and the peritoneal fluids were harvested. The amounts of MDC in the peritoneal fluids were measured by specific sandwich ELISA. Each point represents the mean ± SEM of eight mice. *B*, Either 0.5 ml of anti-MDC antiserum (■; eight mice) or control serum (□; eight mice) was i.p. injected 2 h before CLP. At 8 h after CLP, mice were euthanized, and the numbers of infiltrating leukocytes in the peritoneal cavity were counted. Differential cell analyses were determined by Diff-Quik-stained smears. *C* and *D*, Either 0.5 ml of anti-MDC antiserum (●; nine mice) or control serum (○; seven mice) was i.p. injected 2 h before CLP. Twenty-four hours later, mice were euthanized, and the peritoneal fluids and sera were harvested. Ten microliters of peritoneal fluids and sera was serially diluted and plated on TSA blood agar plates, and bacterial load in the peritoneum (*C*) and blood (*D*) was determined. Line represents the median CFU count. Two different experiments were conducted, and the data were pooled. The results were very similar in individual experiments. *, $p < 0.05$; §, $p < 0.001$; when compared with control.

in CLP mice by measuring the tissue level of MPO, an indirect marker of the number of neutrophils. Intraperitoneal injection of MDC significantly decreased CLP-induced MPO level in liver, lung, and kidney at 24 h post-CLP compared with that of the control (Fig. 7*A*). The treatment dramatically decreased serum levels of AST, BUN, and creatinine, compared with those in the control (Fig. 7*B*). To identify the mechanism whereby MDC treatment ameliorated the tissue inflammation induced by CLP, the levels of

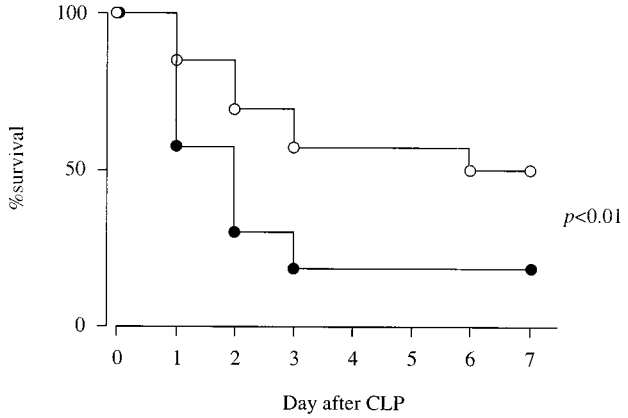

FIGURE 5. MDC blockade reduces survival of mice after CLP. The CLP mice were treated with i.p. injection of either anti-MDC antiserum (●; 30 mice) or control serum (○; 30 mice) 2 h before CLP. The survival rates were monitored for 7 days after CLP. Three different experiments were conducted, and the data were pooled. The mortality rates were very similar in individual experiments.

cytokine/chemokines known to attract neutrophils were examined. Administration of MDC resulted in significant decreases in the levels of TNF-α, MIP-2, KC, and MIP-1α in the liver and lung compared with those in the control (Fig. 8). KC level in kidney was also decreased by MDC treatment (Fig. 8). Conversely, neutralization of MDC dramatically increased CLP-induced MPO level in tissues and serum levels of AST, BUN, and creatinine compared with those in the control, which were accompanied by increased

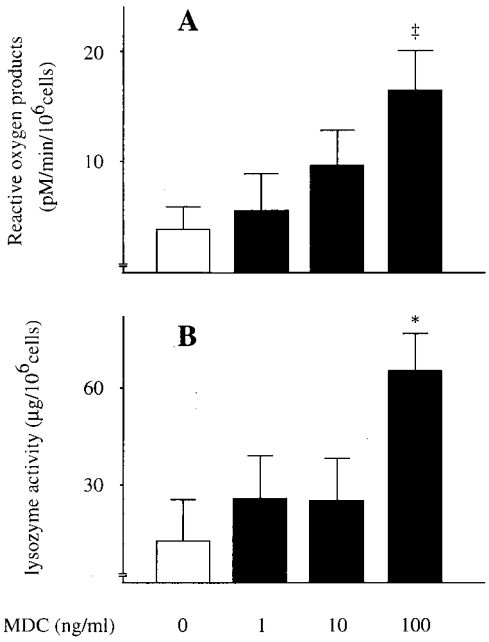

FIGURE 6. MDC induces respiratory burst in macrophages and lysozomal enzyme release from macrophages. Peritoneal cells were harvested from normal mice, the cells (1×10^6 cells) were incubated for 2 h at 37°C, and nonadherent cells were removed. *A*, The adherent macrophages were stimulated with murine MDC. After 30 min, the cultures were assayed for extracellular O_2^- release. *B*, The adherent macrophages were stimulated with murine MDC for 48 h at 37°C, and then the lysozyme enzyme activities in the culture supernatant were measured. The data are representative of the three individual experiments ($n = 8$ each). *, $p < 0.05$; ‡, $p < 0.01$; when compared with control (MDC = 0 ng/ml).

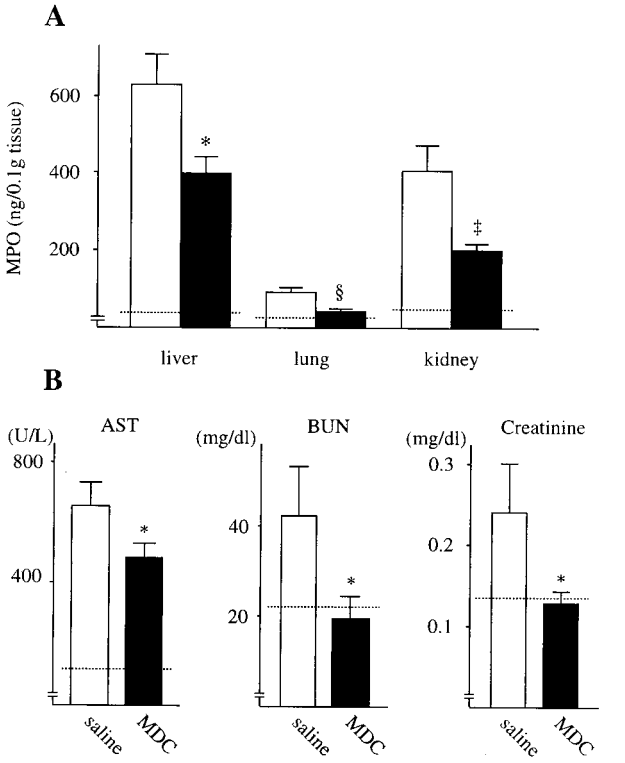

FIGURE 7. Effects of MDC on tissue inflammation and tissue dysfunction induced by CLP. Either MDC (1 μg/mouse; ■; 14 mice) or vehicle (□; 11 mice) was i.p. injected immediately after CLP. At 24 h after CLP, mice were euthanized and bled, and the liver, lung, and kidney were resected. *A*, The tissues were extracted and the amounts of MPO in liver, lung, and kidney were measured by the ELISA. *B*, The amounts of AST, BUN, and creatinine in the sera were measured. Two different experiments were conducted, and the data were pooled. The results were very similar in individual experiments. *, $p < 0.05$; ‡, $p < 0.01$; §, $p < 0.001$; when compared with control. Dotted line represents the mean data obtained from normal mice (eight mice).

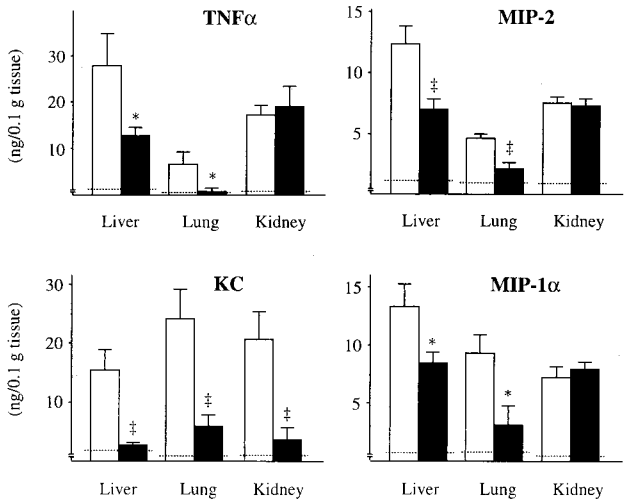

FIGURE 8. Effects of MDC on the production of cytokines in tissues after CLP. The CLP mice were i.p. injected with either MDC (1 μg/mouse; ■; 14 mice) or vehicle (□; 11 mice) immediately after CLP. At 24 h after CLP, the mice were euthanized, and the tissues were excised and then extracted. TNF-α, MIP-2, KC, and MIP-1α in tissue extracts were measured by specific sandwich ELISA. Two different experiments were conducted, and the data were pooled. The results were very similar in individual experiments. *, $p < 0.05$; ‡, $p < 0.01$; when compared with control. Dotted line represents the mean data obtained from normal tissues (eight mice).

levels of TNF-α, MIP-2, KC, and MIP-1α in specific tissues (data not shown). Thus, it appears that MDC ameliorated systemic tissue inflammation as well as tissue injury in part via modulation of the production of inflammatory cytokines/chemokines.

Discussion

Effective host defense against bacterial infection is dependent on the recruitment and activation of phagocytic cells (2, 28). Neutrophils have long been regarded as key phagocytic cells needed to eliminate invading pathogen (3); however, additional evidence suggests that macrophages also participate in this process (4). The recruitment and activation of macrophages appear to be initiated by a group of specific chemoattractants, CC chemokines (5–7). Recently, we have shown that the CC chemokine MCP-1 protected mice from CLP-induced lethality via its effect on the accumulation and activation of macrophages (29). Earlier studies demonstrated that administration of MCP-1 augmented the killing activity of peritoneal macrophages in a different infectious model (30). MCP-1 activates monocytes and causes lysozomal enzyme release (31) and H_2O_2 production (32), both of which are effector molecules for bacterial killing. Although MDC is thought to be involved in T cell-related inflammation and dendritic cell and lymphocyte homing, the CC chemokine MDC also attracts monocytes in both in vitro and in vivo settings (11, 14). In the present study, we explored whether MDC protected mice from CLP-induced lethality. Our findings suggest that MDC provides a major protective role that is linked to its effects on the innate immune response.

The mechanism whereby MDC exerted a protective role in CLP mice appears to be due to its novel activities on phagocytic macrophages. Administration of MDC not only induced the recruitment of macrophages but also served to clear bacteria from CLP mice. Conversely, neutralization of endogenous MDC decreased the recruitment of macrophages and increased bacterial load in CLP mice. Cytokines/chemokines known to attract monocytes directly or indirectly, which include TNF-α, MIP-2, KC, MIP-1α, and MCP-1, were inhibited by MDC treatment. Furthermore, the levels of these mediators were unchanged after anti-MDC treatment by the time when the recruitment of macrophages was inhibited. Thus, MDC appears to elicit macrophages directly in this model of septic peritonitis. The changes of the peritoneal levels of cytokine/chemokines by MDC or anti-MDC appear to be due to the change in the pathogen load that alters cytokine production. In addition to its bactericidal activity in CLP mice, bacterial load in the peritoneum and peripheral blood after inoculation of live *E. coli* was decreased by MDC treatment. In vitro, MDC enhanced bacterial phagocytic and killing activities of macrophages in a dose-dependent fashion. Like MCP-1 (31, 32), MDC was found to induce a respiratory burst in macrophages (O_2^- generation) and to induce lysozomal enzyme release from macrophages (lysozyme enzyme release), both of which are crucial molecules for bacterial clearance (3, 4). Altogether, the data suggest that the accumulation and activation of macrophages by MDC are the main mechanisms underlying the beneficial effects of MDC in our model of sepsis.

Sepsis frequently causes severe systemic inflammation, which leads to multiple organ failure, a condition that is often fatal to the host (27, 33). Interestingly, MDC treatment decreased the intensity of CLP-induced tissue inflammation and also improved the physiologic function of specific tissues. Neutralization of endogenous MDC increased the severity of the tissue inflammation and worsened the dysfunction. The beneficial effects of MDC appear to be dependent on the reduced tissue production of TNF-α, MIP-2, KC,

and MIP-1α in that MDC treatment decreased and, in contrast, anti-MDC treatment increased the levels of these cytokine/chemokines in specific tissues. The above inflammatory cytokines/chemokines are regarded as powerful mediators of inflammation and tissue damage (5, 34). Unlike IL-4, IL-10, and IL-13, all of which reduced LPS-induced production of inflammatory cytokines by monocytes (35–37), MDC failed to inhibit the production of TNF-α, MIP-2, KC, and MIP-1α from LPS-stimulated peritoneal macrophages in vitro (data not shown). As discussed above, MDC played a pivotal role to clear bacteria from the circulation. Thus, it is likely that the modulation of systemic inflammation by MDC treatment is due to a decrease in the pathogen load that alters tissue/organ damage.

Interestingly, the novel activities of MDC on macrophages are similar to those of MCP-1, which have both been shown to be associated with chronic inflammation. As shown in this study and elsewhere (31, 32), both of these CC chemokines are capable of activating macrophages. Administration of MCP-1 6 h before bacteria challenge protected mice in lethal *Pseudomonas aeruginosa* infection model by increasing the killing and clearance of bacteria (30); however, such beneficial effects were not evident when MCP-1 was given at the time of bacteria inoculation. Administration of MCP-1 (1 μg/mouse) failed to protect mice from CLP-induced lethality when treatment was initiated immediately after CLP (A. Matsukawa, unpublished data), although immune-neutralizing studies have demonstrated that MCP-1 played a protective role in the same model of sepsis (29). In this study, we have demonstrated that MDC can enhance bacterial clearance in two types of bacterial peritonitis models and that this chemokine can protect mice from CLP-induced lethality when administered after CLP surgery.

Investigations such as these are important. Sepsis remains a serious disorder, and the mortality due to sepsis and sepsis-mediated multiple organ failure has not significantly improved over the past three decades (38, 39), despite the development of powerful antibiotics and significant advances in the management of intensive care patients and intensive care unit technology. Experimental sepsis employed in the present study possesses a number of the hallmarks of clinical sepsis with peritonitis associated with postsurgical or accidental trauma (19). The novel immune-regulatory activities of MDC that enhance bacterial phagocytic and killing activity of macrophages, leading to the modulation of systemic inflammatory responses in this model, may pave the way for the development of novel therapeutic interventions in sepsis.

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