

## MCP-1 Protects Mice in Lethal Endotoxemia

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### Abstract

The overzealous production of proinflammatory cytokines in sepsis can result in shock, multiorgan dysfunction, and even death. In this study, we assessed the role of monocyte chemoattractant protein-1 (MCP-1) as a mediator of sepsis in endotoxin-challenged mice. Intraperitoneal administration of LPS to CD-1 mice induced a substantial time-dependent increase in MCP-1 in plasma, lung, and liver. The passive immunization of mice with rabbit antimurine MCP-1 antiserum 2 h before endotoxin administration resulted in a striking increase in LPS-induced mortality from 10% in control animals to 65% in anti-MCP-1-treated animals. Importantly, the administration of anti-MCP-1 antibodies to endotoxin-challenged mice resulted in increases in peak TNF- $\alpha$  and IL-12 levels, and also in a trend toward decreased serum levels of IL-10. Conversely, the administration of recombinant murine MCP-1 intraperitoneally significantly protected mice from endotoxin-induced lethality, and resulted in an increase in IL-10 levels, a decrease in IL-12 levels, and a trend toward decreased levels of TNF. In conclusion, our findings indicate that MCP-1 is a protective cytokine expressed in murine endotoxemia, and does so by shifting the balance in favor of antiinflammatory cytokine expression in endotoxin-challenged animals. (*J. Clin. Invest.* 1997. 99:2832–2836.) Key words: monocyte chemoattractant protein-1 • endotoxin • sepsis • cytokines • murine

### Introduction

The sepsis syndrome is an acute systemic illness characterized by shock, coagulopathy, and multiorgan dysfunction, with death occurring in as many as 25–35% of patients with this syndrome (1–3). LPS, a major cell wall component of gram-negative bacterial organisms, mediates many of the pathophysiologic events in sepsis by stimulating the release of host-derived proinflammatory cytokines. TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$

are produced in response to endotoxin exposure, and these cytokines have been shown either to directly or indirectly mediate many of the hemodynamic and inflammatory changes of sepsis (4–8). In addition, the cytokines IL-12 and IL-10 appear to have opposing effects in endotoxin-challenged animals, as IL-12 augments the septic response via the induction of TNF and IFN- $\gamma$  (9, 10), whereas IL-10 downregulates proinflammatory cytokine expression and tissue injury in endotoxemia (11–13). Finally, several members of the C-X-C and C-C chemokine family, including macrophage inflammatory protein-2 (MIP-2),<sup>1</sup> macrophage inflammatory protein-1 alpha (MIP-1 $\alpha$ ), and RANTES (regulated upon activation, normal T expressed and presumably secreted) are expressed in sepsis, and have been shown to exert proinflammatory effects by mediating organ-specific leukocyte influx and/or activation that occurs in endotoxemia (13–16).

Monocyte chemoattractant protein-1 (MCP-1) is a 76-amino acid member of the C-C subfamily of chemokines with chemoattractant activity for monocytes, T cells, mast cells, and basophils (17, 18). MCP-1 is produced by both immune and nonimmune cells in response to various stimuli, including TNF, IL-1 $\beta$ , IL-4, viruses, and endotoxin (17, 19). In addition to its chemotactic properties, MCP-1 is believed to promote inflammation by directly activating specific macrophage effector cell activities, including  $\beta_2$  integrin expression, and tumoricidal activity (20). Recent investigations, however, suggest that MCP-1 may play a broader role in the regulation of antigen-specific immune responses. Specifically, MCP-1 has been shown to promote Th2-phenotype inflammation in a murine model of *Schistosoma mansoni*-induced pulmonary granuloma by enhancing the expression of IL-4, while inhibiting the production of IL-12 by granuloma macrophages in vitro (17). These observations suggest that MCP-1, as opposed to other chemokines, may possess important antiinflammatory properties both in vivo and in vitro.

Treatment of baboons with LPS has been shown recently to result in substantial time-dependent increases in plasma MCP-1 levels (21). Furthermore, MCP-1 levels dramatically increase after the administration of endotoxin to human volunteers (22), and have been found to be elevated in the plasma of patients with sepsis (19). Given the recently recognized immunoregulatory properties of MCP-1, we investigated its contribution to the pathogenesis of sepsis in an established murine model of endotoxemia. The effects of neutralization of endogenously produced MCP-1, as well as the effect of murine re-

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1. *Abbreviations used in this paper:* MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; mr, murine recombinant.

combinant (mr) MCP-1 administration on cytokine expression and outcome in endotoxin-challenged animals, were assessed.

## Methods

**Reagents.** Polyclonal rabbit antimurine MCP-1, IL-4, IL-10, IL-12, TNF, and MIP-2 antisera used in our ELISAs and/or in neutralization studies were produced by immunization of rabbits with recombinant murine cytokines in multiple intradermal sites with CFA (13, 23). Stock LPS (*Escherichia coli* 0111:B4, lot 4130) was purchased from Sigma Chemical Co. (St. Louis, MO). mrMCP-1 was purchased from R & D Systems (Minneapolis, MN). The mrMCP-1, as well as antiserum used for passive immunization experiments, was endotoxin-free as determined by the limulus lysate assay (ICN Biomedicals, Costa Mesa, CA, sensitivity 0.1 ng/ml).

**Murine model of endotoxemia.** A murine model of endotoxemia has been developed in our laboratory (13). Briefly, 20–30-g female CD-1 mice (Charles River Laboratories, Wilmington, MA), maintained under specific pathogen-free conditions were administered either 750 µg or 1 mg LPS i.p. in 1 ml of saline. Control animals received equal volumes of saline. All endotoxin-treated animals appeared acutely ill, displaying lethargy, ruffled fur, and diarrhea. In passive immunization experiments, 0.5 ml rabbit antimurine MCP-1, or preimmune serum, was administered intraperitoneally 2 h before endotoxin treatment. At designated time points, the mice were anesthetized with inhaled ether, and killed. Blood was collected in heparinized tubes, plasma was separated, and various organs were harvested for assessment of cytokine protein expression. Before organ removal, the pulmonary vasculature was perfused with 1 ml of PBS containing 5 mM EDTA via the right ventricle. Lungs were then removed and assessed for cytokine protein. To measure cytokine expression, organs were homogenized in a lysis buffer containing 1% Nonidet P-40, 500 mM NaCl, 50 mM Hepes, 1 mg/ml leupeptin, and 100 mg/ml PMSF using a tissue homogenizer (Biospec Products, Inc., Bartlesville, OK). For assessment of liver and spleen cytokine levels, the caudate lobe of the liver and the entire spleen were removed and homogenized in 2 ml homogenization buffer. Homogenates were incubated on ice for 30 min, and were then centrifuged at 2,500 rpm for 10 min. Supernatants were collected and stored at -20°C for assessment of cytokine levels.

**Cytokine ELISAs.** Immunoreactive murine TNF, MIP-2, IL-10, IL-4, IL-12, and MCP-1 were quantitated using a modification of a double ligand method as previously described (13). In brief, flat-bottom 96-well microtiter plates (Immuno-Plate I 96-F; NUNC A/S, Roskilde, Denmark) were coated with 50 µl/well of purified rabbit antimurine TNF, MIP-2, IL-10, IL-4, IL-12, and MCP-1 antibodies (1 µg/ml in 0.6 M NaCl, 0.26 M H<sub>3</sub>BO<sub>4</sub>, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C, and were then washed with PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer, and diluted (neat and 1:10) cell-free supernatants (50 µl) in duplicate were added, followed by incubation for 1 h at 37°C. Plates were washed four times, followed by the addition of 50 µl/well of biotinylated rabbit antimurine TNF, MIP-2, IL-10, IL-4, IL-12, and MCP-1 antibodies (3.5 µg/ml in PBS, pH 7.5, 0.05% Tween-20 and 2% FCS). Plates were incubated for 30 min at 37°C, and then were washed four times. Streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) was added, and then the plates were incubated for 30 min at 37°C. Plates were again washed four times, and chromogen substrate (Bio-Rad) was added. The plates were then incubated at room temperature to the desired extinction, and the reaction was terminated with 50 µl/well of 3 M H<sub>2</sub>SO<sub>4</sub> solution. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of murine recombinant TNF, MIP-2, IL-10, IL-4, IL-12, and MCP-1 from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected TNF and MIP-2 concentrations > 25 pg/ml, and MCP-1, IL-4, IL-10, and IL-12 concentrations > 100 pg/ml. The ELISAs did not cross-react with IL-1, IL-2, or IL-6. In addition, the ELISAs did not cross-react with other

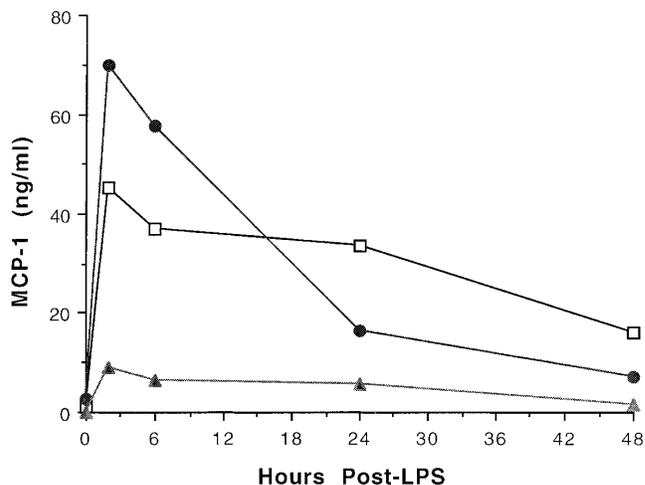
members of the chemokine C-C family, including RANTES, MIP-1α, and MIP-1β, or members of the C-X-C chemokine family, including IL-8, GRO-α, ENA-78, NAP-2, and IP-10.

**Statistical analysis.** All experiments involving cytokine antigen determinations were performed in duplicate. Data were analyzed by a Macintosh II computer (Statview 4.0 statistical package; Abacus Concepts, Inc., Berkeley, CA). Survival data were compared for statistical significance using  $\chi^2$  analysis. All other data are expressed as mean  $\pm$  SEM and compared using a two-tailed Student's *t* test. Data were considered statistically significant if *P* values were less than 0.05.

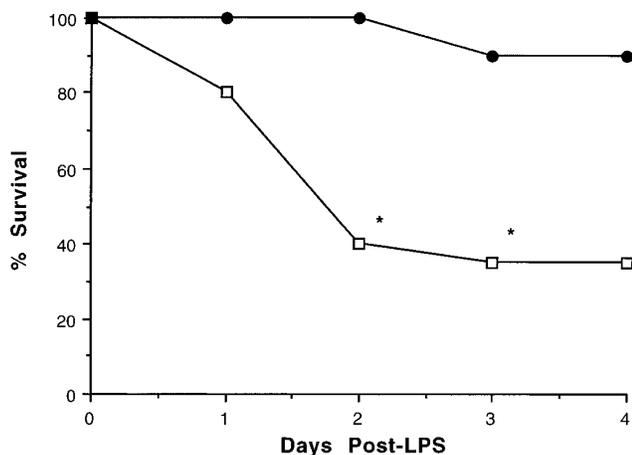
## Results

**Organ-specific production of MCP-1 in endotoxemia.** Experiments were performed to assess the time-dependent production of MCP-1 in murine endotoxemia. CD-1 mice were challenged with LPS 750 µg i.p., and MCP-1 levels were determined in plasma, liver, and lungs at multiple time points after LPS administration. As shown in Fig. 1, LPS administration resulted in a rapid and substantial increase in MCP-1 levels, peaking at 2 h in plasma, lung, and liver, returning toward baseline levels by 48 h after LPS. Maximally, MCP-1 levels in liver and lung represented a 10- and 42-fold increase, respectively, over those levels in animals receiving saline, whereas plasma MCP-1 levels increased from undetectable levels to  $9.03 \pm 1.9$  ng/ml at 2 h after LPS.

**Neutralization of MCP-1 significantly increases endotoxin-induced mortality.** Experiments were performed to determine the contribution of endogenously produced MCP-1 to mortality in mice administered LPS. For these studies, a dose of 750 µg was used, which represented an approximately LD<sub>10-20</sub> dose of endotoxin. As shown in Fig. 2, treatment of animals with rabbit control preimmune serum (0.5 ml/animal), followed 2 h later by LPS 750 µg i.p. resulted in only 10% mortality at 72 h after LPS administration. A striking 65% mortality, however, was observed in animals administered 0.5 ml polyclonal rabbit antimurine MCP-1 serum 2 h before LPS (*P* < 0.01). All animals surviving to 72 h after LPS were long-term survivors.



**Figure 1.** Time-dependent production of MCP-1 in lung homogenates, liver homogenates, and plasma from CD-1 mice after intraperitoneal administration of LPS (750 µg/animal). *n* = 6 per time point. ●, liver; □, lung; ▲, plasma.



**Figure 2.** Effect of anti-MCP-1 serum on survival in endotoxemia. CD-1 mice were immunized passively with rabbit preimmune or rabbit antimurine MCP-1 serum, and then were challenged with 750  $\mu$ g LPS. \* $P < 0.01$  when compared with preimmune-treated animals.  $n = 20$  per group. ●, preimmune + LPS; □, anti-MCP-1 + LPS.

*Passive immunization with anti-MCP-1 serum alters the expression of cytokines during endotoxemia.* We and others have demonstrated that specific proinflammatory cytokines, including TNF and IL-12, promote many of the pathophysiologic events that occur in animal models of endotoxemia, whereas the antiinflammatory cytokine IL-10 is protective in endotoxin-challenged mice (4–6, 9–13). We next performed experiments to determine whether endogenously produced MCP-1 regulated the balance between pro- and antiinflammatory cytokines in endotoxemia. CD-1 mice were pretreated with control serum or anti-MCP-1 serum 2 h before the administration of LPS 750  $\mu$ g, and then plasma was collected 2 h after LPS (time of maximal plasma TNF, IL-12, and IL-10 levels). Interestingly, treatment with anti-MCP-1 serum followed by LPS resulted in a 2- and 2.3-fold increase in plasma TNF and IL-12 levels, respectively, when compared with endotoxin-challenged animals receiving preimmune serum (Table I). Additionally, 1.5- and 1.4-fold increase in lung TNF and IL-12 levels, as well as a 1.7-fold increase in spleen IL-12 levels, was noted in anti-MCP-1-treated animals, when compared with controls ( $P < 0.05$ , data not shown). In contrast, treatment with anti-MCP-1 resulted in a 53% decrease in the LPS-induced peak in plasma IL-10 levels, although this difference did not reach the level of statistical significance ( $P = 0.12$ ). Treatment with anti-MCP-1 antibodies resulted in no significant change in the endotoxin-induced increases in plasma, lung, liver, and splenic levels of the C-X-C chemokine MIP-2. IL-4 was not detected in the plasma of control or LPS-treated animals, and no change in constitutive IL-4 levels was observed in lungs, livers, and spleens from endotoxin-treated animals when compared with mice administered saline. Furthermore, IL-4 levels in these organs were not altered by passive immunization with anti-MCP-1 serum (data not shown). Finally, no differences in TNF, IL-12, IL-10, MIP-2, and IL-4 levels among groups were observed 6 h after LPS, a time at which all cytokines were returning to or had reached preendotoxin levels (data not shown).

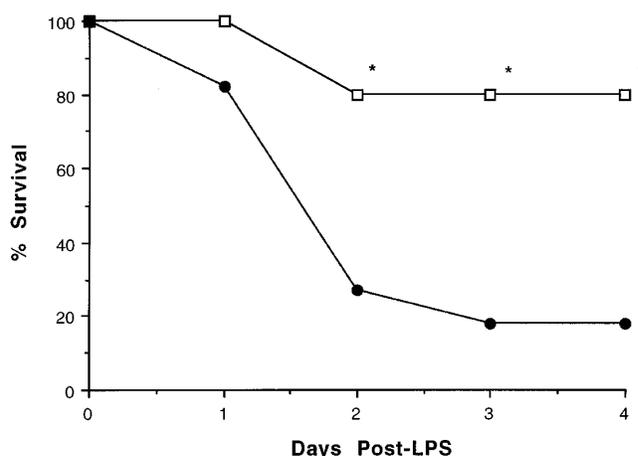
**Table I.** Effect of Rabbit Antimurine MCP-1 Serum on Plasma Cytokine Levels 2 h After LPS Administration

Cytokine	Preimmune + saline	Preimmune + LPS	Anti-MCP-1 + LPS
TNF (ng/ml)	0	1.59±0.22	3.09±0.54*
IL-12 (ng/ml)	0.25±0.03	4.28±0.75	9.38±0.97*
IL-10 (ng/ml)	1.50±0.95	6.59±1.16	4.23±1.27
MIP-2 (ng/ml)	0.14±0.04	9.54±1.00	11.98±2.93
IL-4 (ng/ml)	0	0	0

Animals were pretreated with either 0.5 ml of control or anti-MCP-1 serum 2 h before LPS administration (750  $\mu$ g/animal). \* $P \leq 0.01$  when compared with LPS-treated animals receiving preimmune serum.  $n = 18$  per group.

*Administration of mrMCP-1 decreases endotoxin-induced mortality.* Having demonstrated that neutralization of MCP-1 resulted in substantial increases in endotoxin-induced lethality, experiments were performed to determine whether the administration of mrMCP-1 would protect mice from the lethal effects of endotoxin. Mice were injected with mrMCP-1 500 ng/animal i.p. at the time of endotoxin administration, and then 250 ng/animal i.p. at 24 and 48 h after LPS. To assess for a protective effect of MCP-1, the dose of endotoxin administered was increased to 1 mg/animal (representing an LD<sub>80</sub>–LD<sub>100</sub> dose). An 80% mortality rate was observed in animals receiving vehicle control (PBS + 0.1% human serum albumin) followed by LPS (Fig. 3). In contrast, treatment of animals with mrMCP-1 significantly shifted the survival curve to the right, with only a 20% mortality observed.

*Murine recombinant MCP-1 administration alters the expression of cytokines during endotoxemia.* We next performed experiments to determine the mechanism whereby treatment with MCP-1 protected endotoxemic animals. In these experiments, CD-1 mice were pretreated with mrMCP-1 500 ng/animal i.p. 2 h before the administration of LPS 750  $\mu$ g, and then



**Figure 3.** Effect of mrMCP-1 administration on survival in endotoxemia. CD-1 mice were administered mrMCP-1 500 ng/animal i.p. at the time of endotoxin administration (1 mg/animal), and then 250 ng/animal i.p. at 24 and 48 h after LPS. \* $P < 0.01$  when compared with preimmune-treated animals.  $n = 10$  per group. □, mrMCP-1 + LPS; ●, vehicle + LPS.

Table II. Effect of mrMCP-1 on Plasma Cytokine Levels 2 h After LPS Administration

Cytokine	Saline	LPS + vehicle	LPS + mrMCP-1
TNF (ng/ml)	0	1.17±0.19	0.77±0.08
IL-12 (ng/ml)	0.42±0.14	6.84±0.19	5.58±0.39*
IL-10 (ng/ml)	0.91±0.52	2.99±0.31	4.49±0.54 <sup>‡</sup>

Animals were pretreated with 500 ng mrMCP-1 i.p. 2 h before LPS administration (750 µg/animal). \* $P \leq 0.01$  as compared to LPS-treated animals receiving vehicle control; <sup>‡</sup> $P \leq 0.05$  when compared with LPS-treated animals receiving vehicle control.  $n = 18$  per group.

plasma and lungs were collected 2 h after LPS. As compared to endotoxin-challenged animals pretreated with vehicle control, treatment with mrMCP-1 followed by LPS decreased the induction of TNF and IL-12 by 34 and 20%, respectively, whereas plasma IL-10 levels increased by 1.7-fold (Table II). Similarly, treatment with mrMCP-1 attenuated TNF and IL-12 levels in lung homogenates 2 h after LPS by 23 and 35%, and increased lung IL-10 by twofold when compared with control endotoxemic animals (data not shown). Treatment with MCP-1 resulted in no significant change in the endotoxin-induced increases in the plasma and lung MIP-2 levels (data not shown).

## Discussion

Members of the C-X-C and C-C chemokine family are known to exert potent proinflammatory properties in several animal models of inflammation, including endotoxemia (12–16). Ours is the first study to demonstrate that one member of the chemokine family, MCP-1, has marked antiinflammatory properties in vivo. We, like others (21, 22), found that MCP-1 is produced early in response to endotoxin challenge, as increases in MCP-1 levels were noted 2 h after LPS, and returned to baseline levels after 48 h. In contrast to previous investigations where it was postulated that MCP-1 was harmful in clinical septic shock since its release was proportional to the severity of shock (19), we have shown that MCP-1 is protective in a murine model of endotoxemia. Specifically, we observed a significant increase in endotoxin-induced mortality in animals pretreated with anti-MCP-1 serum, when compared with controls. Furthermore, the administration of mrMCP-1 provided substantial protection against the lethal effects of LPS.

The mechanism(s) whereby MCP-1 protects mice against endotoxin challenge is not entirely clear. Neutralization of MCP-1 resulted in significant increases in plasma and lung IL-12 and TNF levels, concomitant with a trend towards reduction in plasma IL-10 levels. Conversely, administration of mrMCP-1 significantly attenuated plasma and lung IL-12 levels, while increasing the production of IL-10 in both plasma and lung. The balance between pro- and antiinflammatory cytokines appears to be pivotal in determining the magnitude of the septic response and resultant tissue injury. IL-10 is protective when administered to animals challenged with LPS (11, 12). In addition, inhibition of endogenous IL-10 production in endotoxemia results in substantial increases in endotoxin-induced lethality (13). In contrast, endogenous IL-12 expression has been shown to be detrimental to the host in endotoxemia, in part by augmenting the expression of TNF and IFN- $\gamma$  levels (9,

10, 24–26). Our findings are the first to support the notion of MCP-1 as an immunoregulatory cytokine in endotoxemia, as expression or administration of this cytokine appears to shift the balance in favor of an antiinflammatory response by inhibiting the expression of IL-12 and TNF, while at the same time enhancing the expression of IL-10. As IL-12 is a potent inducer of IFN- $\gamma$ , it is likely that anti-MCP-1-induced increases in IL-12 result in enhanced expression of IFN- $\gamma$  in vivo. The effect of anti-MCP-1 antibodies and MCP-1 administration on endotoxin-induced IFN- $\gamma$  production is currently being investigated.

The biological role of MCP-1 has been evaluated in several animal models of infection. The overexpression of this cytokine in MCP-1 transgenic mice results in enhanced susceptibility to intracellular pathogens such as *Listeria monocytogenes* and *Mycobacterium tuberculosis* (27). This effect is speculated to occur as a result of direct macrophage desensitization by high circulating levels of MCP-1. In contrast, MCP-1 appears to be crucial to cell-mediated host defense against *Cryptococcus neoformans* (28). Furthermore, MCP-1 plays an important role in innate immunity required for clearance of bacterial organisms, as mice were protected from *Pseudomonas aeruginosa* and *Salmonella typhimurium* peritoneal infection by treatment with recombinant MCP-1 administered intraperitoneally (29). Similarly, we have observed that depletion of MCP-1 in mice with *Klebsiella pneumoniae* results in significant attenuation of bacterial clearance and the development of early bacteremia (our unpublished observations). Collectively, these findings suggest that MCP-1 may downregulate the overzealous production of proinflammatory cytokines that occurs in endotoxemia, with the potential additional benefit of enhancing the host's ability to clear certain fungal and bacterial pathogens. No other cytokine has been shown to be of benefit in experimental models of both LPS administration and bacterial sepsis. Because endotoxin challenge is a quite different response from that observed in septic patients clinically, the role of MCP-1 in experimental sepsis as a result of infection requires further study.

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