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Role of Resident Peritoneal Macrophages and Mast Cells in Chemokine Production and Neutrophil Migration in Acute Inflammation: Evidence for an Inhibitory Loop Involving Endogenous IL-10¹

Maureen N. Ajuebor,* Anuk M. Das,* László Virág,[†] Roderick J. Flower,* Csaba Szabó,[†] and Mauro Perretti^{2*}

The roles played by resident macrophages (M ϕ) and mast cells (MCs) in polymorphonuclear leukocyte (PMN) accumulation and chemokine production within the mouse peritoneal cavity in response to administration of zymosan (0.2 and 1 mg), LPS (1 mg/kg), and thioglycolate (0.5 ml of a 3% suspension) were investigated. A marked reduction (>95%) in intact MC numbers was obtained by pretreatment with the MC activator compound 48/80, whereas resident M ϕ were greatly diminished (>85%) by a 3-day treatment with liposomes encapsulating the cytotoxic drug dichloromethylene-bisphosphonate. No modulation of thioglycolate-induced inflammation was seen with either pretreatment. Removal of either MCs or M ϕ attenuated LPS-induced PMN extravasation without affecting the levels of the chemokines murine monocyte chemoattractant protein-1 and KC measured in the lavage fluids. In contrast, MC depletion inhibited PMN accumulation and murine monocyte chemoattractant protein-1 and KC production in the zymosan peritonitis model. Removal of M ϕ augmented the accumulation of PMN elicited by the latter stimulus. This was due to an inhibitory action of M ϕ -derived IL-10 because there was 1) a time-dependent release of IL-10 in the zymosan exudates; 2) a reduction in IL-10 levels following M ϕ , but not MC, depletion; and 3) an increased PMN influx and chemokine production in IL-10 knockout mice. In conclusion, we propose a stimulus-dependent role of resident MCs in chemokine production and the existence of a regulatory loop between endogenous IL-10 and the chemokine-mediated cellular component of acute inflammation. *The Journal of Immunology*, 1999, 162: 1685–1691.

Chemokines are a class of small polypeptides that are chemotactic for blood-derived leukocytes (1). The great interest generated by the discovery of chemokines lies in the specificity of these proteins such that, for instance, CXC chemokines with the ELR motif are chemoattractants for neutrophils in vivo, whereas CC chemokines are chemotactic for eosinophils and mononuclear cells. The prototype for the former group is IL-8, whereas for the latter group there is MCP-1,³ which causes only monocyte accumulation in vivo. Eotaxin acts predominantly on eosinophils, and MIP-1 α promotes PMN and mononuclear cell recruitment (for a recent review, see Ref. 2).

Many studies have focused on the role played by chemokines in attracting different subsets of leukocytes in human inflammatory pathologies and in their experimental counterparts. Experimentally, several observations have highlighted the strict

relationship between chemokine expression and the intensity of the host inflammatory response as well as their direct link with the appearance of blood-derived leukocytes at the site of inflammation. For example, IL-8 is generated in a rabbit model of joint articular inflammation and promotes PMN extravasation (3). In the mouse, chemokines such as MCP-1, MIP-2, and MIP-1 α contribute to the initiation of articular arthritis and recruit monocytes and lymphocytes (4). A similar link between chemokines and leukocyte recruitment also exists in acute models of inflammation. We have recently shown that MCP-1, MIP-1 α , and KC are expressed at early time points (<4 h) in a murine model of acute peritonitis (5). This is not totally surprising, as some of the genes that produce chemokines were initially identified as early/intermediate response genes (e.g., JE in the mouse, which produces murine (m) MCP-1) (6).

In the present study we use several distinct models of acute inflammation to address the question of which cell type(s) produces these chemokines. Because of the early expression of these mediators, we addressed our attention to M ϕ and MCs, since both are resident cells in close contact with postcapillary venules (7). As demonstrated by selective depletion experiments, M ϕ and MCs produce the initial signals responsible for the accumulation of PMNs, eosinophils, and mononuclear cells in experimental models of inflammation (8–10).

Here, we have 1) monitored the effect of selective removal of either resident cell type on KC (a murine CXC chemokine) and mMCP-1 (CC chemokine) gene and protein expression in the mouse and 2) assessed how their expression is functionally linked to leukocyte migration. Finally, we describe a novel chemokine/cytokine inhibitory feedback loop based upon IL-10 released from the M ϕ .

*Department of Biochemical Pharmacology, The William Harvey Research Institute, London, United Kingdom; and [†]Division of Critical Care Medicine, Children's Hospital Medical Center, Cincinnati, OH 45229

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² Address correspondence and reprint requests to Dr. Mauro Perretti, Department of Biochemical Pharmacology, The William Harvey Research Institute, Charterhouse Square, London, U.K. EC1 M6BQ. E-mail address: m.perretti@qmw.ac.uk

³ Abbreviations used in this paper: MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; m, murine; PMN, polymorphonuclear leukocyte; M ϕ , macrophage; MC, mast cell; wt, wild type; KO, knockout; TG, thioglycolate; Cl₂MDP, dichloromethylene-bisphosphonate.

Materials and Methods

Animals

Male Swiss Albino mice were purchased from Banton and Kingsman (Hull, U.K.). Male C57BL/6 IL-10 wt and C57BL/6 IL-10 KO were obtained from The Jackson Laboratory (Bay Harbor, ME). The animals were fed a standard chow pellet diet and had free access to water. All animals were maintained on a 12-h light, 12-h dark cycle and housed for 1 wk before experimentation. Mice weighed between 26–30 g on the day of the experiment.

Models of inflammation

Zymosan peritonitis was induced as previously reported (5). Briefly, mice were injected i.p. with zymosan A (0.2 or 1 mg in 0.5 ml of saline). At selected time points, animals were euthanized by carbon dioxide exposure, and peritoneal cavities were lavaged with 3 ml of PBS containing 3 mM EDTA. Aliquots of the lavage fluid were then stained with Turk's solution (0.01% crystal violet in 3% acetic acid), and differential cell counts were performed using a Neubauer hemocytometer and a light microscope (B061, Olympus, Melville, NY). The lavage fluids were centrifuged at $400 \times g$ for 10 min, and cell-free supernatants were stored at -20°C before chemokine and cytokine evaluation by ELISA (see below). Cell pellets were then prepared for RNA extraction and RT-PCR analysis as described below.

LPS peritonitis was induced as previously reported (5). Mice received i.p. injections of LPS (1 mg/kg/10 ml of sterile saline). At selected time points, peritoneal cavities were washed, and the lavage fluid was handled as described above.

Thioglycolate (TG) peritonitis was induced as previously reported (11). Mice received i.p. injections of TG (3% (w/v) in 0.5 ml of saline). At designated time points, peritoneal cavities were washed, and lavage fluid was handled as described above.

Chemokines and cytokine ELISA

Immunoreactive mMCP-1, IL-10, TNF- α , and KC were quantified using a commercially available ELISA according to the manufacturer's protocol. In brief, lavage fluids (100 μl) were assayed for mMCP-1 and compared with a standard curve constructed with 0–2.5 ng/ml murine mMCP-1. Similarly, lavage fluids were tested for the murine chemokines KC (standard curve ranging from 0–1 ng/ml) and the cytokines TNF- α (standard curve ranging from 0–1.5 ng/ml) and IL-10 (standard curve ranging from 0–1 ng/ml). The ELISA method consistently detected KC, TNF- α , and murine IL-10 at >1.5 pg/ml and mMCP-1 at >9 pg/ml. The ELISAs showed negligible ($<1\%$) cross-reactivity with several murine cytokines and chemokines (data furnished by the manufacturer).

Detection of chemokine mRNA by RT-PCR analysis

Peritoneal cell pellets (5×10^6) were lysed in 1 ml of Trizol reagent, and RNA was isolated according to the manufacturer's protocol. Briefly, RNA was extracted with chloroform and isopropanol. The RNA was precipitated with ethanol, and the pellet was resuspended in diethylpyrocarbonate-treated water. The yield and the purity of the RNA were then estimated spectrophotometrically at 260 and 280 nm. Total RNA (3 μg) was used for the generation of cDNA. PCR amplification reactions were performed on aliquots of the cDNA. For the mMCP-1 target, the primers were 5'-ACT-GAA-GCC-AGC-TCT-CTC-TTC-CTC-3' and 5'-TTC-CIT-CTT-GGG-GTC-AGC-ACA-GAC-3' (forward and reverse), which amplified a fragment 274 bp in length. For the KC target, primers were 5'-GGA-TTC-ACC-TCA-AGA-ACA-TCC-AGA-G-3' and 5'-CAC-CCT-TCT-ACT-AGC-ACA-GTG-GTT-G-3' (forward and reverse), which amplified a fragment 454 bp in length.

All PCR reactions were performed in a final volume of 25 μl using a Hybad OmniGene thermal cycler (Middlesex, U.K.). For mMCP-1, the PCR profile consisted of one cycle of denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C (45 s), annealing at 57°C (45 s), and extension at 72°C (30 s). For KC, an initial denaturation was performed at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C (45 s), annealing at 54.7°C (45 s), and extension at 72°C (30 s). Amplification products were visualized by ethidium bromide fluorescence in agarose gels. Bands of the expected sizes were obtained. Images were inverted using the Graphic Converter software (version 2.1; Lemke Software, Peine, Germany) running on a Macintosh Performa 6200 (Apple Computer, Cupertino, CA).

Drug treatments

Liposomes. Selective depletion of resident peritoneal M ϕ was achieved by pretreatment of mice with multilamellar liposomes containing dichlo-

romethylene-bisphosphonate (Cl₂MDP or clodronate) and was performed according to a published procedure (12). Briefly, 86 mg of phosphatidylcholine and 8 mg of cholesterol were dissolved in 10 ml of chloroform in a sterile round-bottom flask by vacuum rotary evaporation at 37°C . Cl₂MDP (1.89 g dissolved in 10 ml of sterile PBS) was encapsulated into the preparation of phosphatidylcholine and cholesterol by gently shaking for 10 min. The solution was kept for 2 h at room temperature, then sonicated for 3 min at 20°C and kept for an additional 2 h at room temperature. Free Cl₂MDP was removed by three centrifugation steps ($100,000 \times g$ for 30 min at 4°C) in a Beckman Ultracentrifuge L-80 Optima (Beckman, Palo Alto, CA). Liposomes, layered on top of the supernatants, were collected and washed twice by centrifugation. The liposome pellets were resuspended in 4 ml of sterile PBS. Mice were treated i.p. with 100 μl of liposome preparation for 3 consecutive days; 24 h after the last injection peritoneal cavities were lavaged, and the number of macrophages was determined following staining in Turk's solution. In another set of experiments, zymosan (0.2 or 1 mg), TG (0.5 ml of 3% suspension), or LPS (1 mg/kg) was administered i.p. 24 h after the last liposome administration. Peritoneal cavities were lavaged 4 h later (for zymosan and TG) or 3 and 24 h later (for LPS) for chemokine and PMN determinations as described above.

M ϕ depletion was also confirmed by FACS analysis. In brief, peritoneal cells (1×10^6) were stained for 60 min on ice with 20 $\mu\text{g}/\text{ml}$ of rat anti-mouse F4/80 mAb or rat IgG2b isotype control. After extensive washing, cells were stained with FITC-conjugated anti-rat IgG for 45 min on ice, extensively washed, and analyzed by flow cytometry using a FACScan analyzer (Becton Dickinson, Cowley, U.K.). Distinct populations were based on the forward and side scatter characteristics, and fluorescence associated with them was measured in the on FL1 channel.

Compound 48/80. Resident peritoneal MCs were depleted using a well-characterized protocol (8). Mice received a single dose of compound 48/80 (1.2 mg/kg, i.p.) or sterile saline (100 μl i.p.) 72 h before lavaging the peritoneal cavities: the number of intact MCs in the lavage fluids was determined following staining in 200 μl of toluidine dye. In another set of experiments, 72 h post-treatment, mice were injected i.p. with zymosan (1 mg), LPS (1 mg/kg), or TG (0.5 ml of a 3% suspension). Peritoneal cavities were lavaged 4 h later (for zymosan and TG) or 3 and 24 h later (for LPS) for chemokine and PMN determinations as described above.

Measurement of nitrite/nitrate concentration in the lavage fluids

Nitrite/nitrate production, an indicator of nitric oxide synthesis, was measured in lavage fluids as previously described (13). First, nitrate in the peritoneal lavage fluids was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 mM) at 37°C for 3 h. The nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthalene-1,4-diamine dihydrochloride in H₂O and 1% sulfanilamide in 5% concentrated H₃PO₄ (1:1, v/v)) to 100- μl samples. The OD at 550 nm (OD₅₅₀) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Nitrate concentrations were calculated by comparison with OD₅₅₀ of standard solutions of sodium nitrite prepared in saline solution.

Reagents

Quantikine ELISA kits for murine IL-10, TNF- α , and KC were purchased from R&D Systems (Abingdon, U.K.), whereas the specific murine mMCP-1 ELISA Cytoscreen was obtained from BioSource International (Camarillo, CA). F4/80 mAb (clone CI:A3-1) and FITC-conjugated anti-rat IgG were purchased from Serotec (Oxford, U.K.). LPS (*Escherichia coli* serotype 0111:B4), zymosan A, TG, and all other chemicals were purchased from Sigma (Poole, U.K.). Clodronate was a generous gift from Boehringer Mannheim (East Sussex, U.K.).

Reagents for the PCR reaction were purchased from the following companies: mMCP-1 and KC primers from OligoExpress (Middlesex, U.K.), Trizol reagent (lysis buffer for RNA preparation) from Life Technologies (Paisley, U.K.), and Ready-to-Go T-Primed First-Strand Kit and PCR Beads from Pharmacia Biosystem Europe (St. Albans, U.K.).

Statistical analysis

Data are reported as the mean \pm SEM of n mice per group, and statistical differences were evaluated by one-way analysis of variance once Bartlett's test confirmed the homogeneity of the variances. Post-hoc comparisons were made with Bonferroni's test using InStat software (version 2.04) running on a Macintosh Performa 6200. A threshold value of $p < 0.05$ was taken as significant.

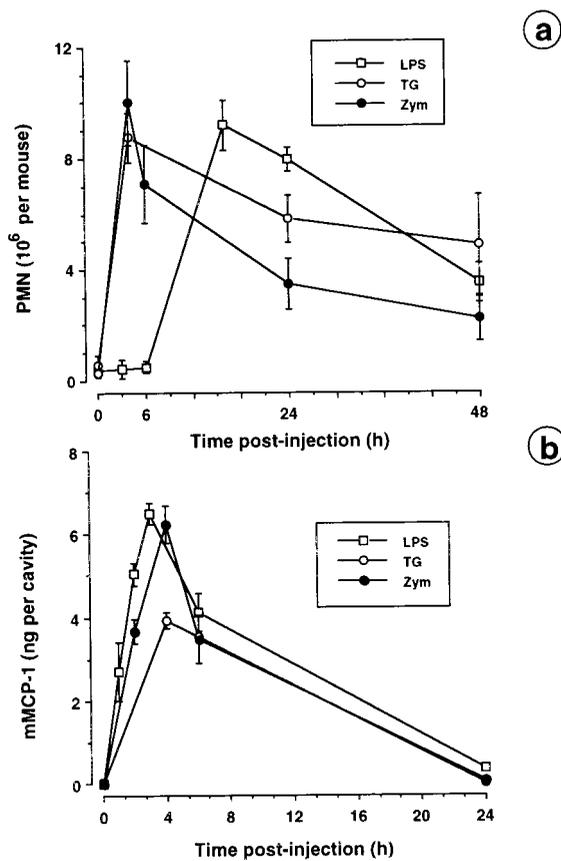


FIGURE 1. Time dependency of zymosan- (zym), LPS-, and TG-induced PMN extravasation and mMCP-1 release in the mouse peritoneal cavity. Mice were treated i.p. with zymosan (1 mg), LPS (1 mg/kg), or TG (0.5 ml of a 3% suspension) at time zero or were left untreated (time zero, control group). Peritoneal cavities were washed at the reported time points, and lavage fluids were handled as described in *Materials and Methods* for quantification of PMN cell numbers (a) and mMCP-1 protein levels (b). Data are the mean \pm SE of 10 mice/group.

Results

Characterization of the inflammatory models

In initial experiments the different inflammatory responses to the three stimuli were characterized. Zymosan injection induced a time-dependent accumulation of PMN into the cavity, with a maximal cell influx at the 4 h point (Fig. 1a). PMN accumulation was almost resolved by 48 h postzymosan administration. Local injection of TG caused a significant extravasation of PMN into the cavity, peaking at 4 h and still elevated above basal levels at 48 h postinjection. In contrast, i.p. injection of LPS induced a delayed influx of PMN, with a high rate of influx between 6–16 h ($\sim 0.9 \times 10^6$ PMN/h). Significant numbers of PMN were still present in the cavity at 24 h, whereas a sharp decline to near basal levels was detected by 48 h (Fig. 1a). In all cases, the large predominance of neutrophils (99%) in the PMN population was confirmed in cyto-spin preparations stained with May-Grunwald and Giesma (data not shown).

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Expression of mMCP-1 in the peritoneal lavage fluids

The level of mMCP-1 in the peritoneal lavage fluid was determined by a specific ELISA. No mMCP-1 protein was found in basal conditions (Fig. 1b). As expected (5), i.p. administration of zymosan resulted in a rapid release of mMCP-1 into the lavage fluids, with significant amounts at 2 and 4 h and a return to basal levels by 24 h. A time-dependent release of mMCP-1 was also observed following i.p. injection of LPS: endogenous mMCP-1 could be detected as early as 1 h postinjection, peaked at 3 h, and

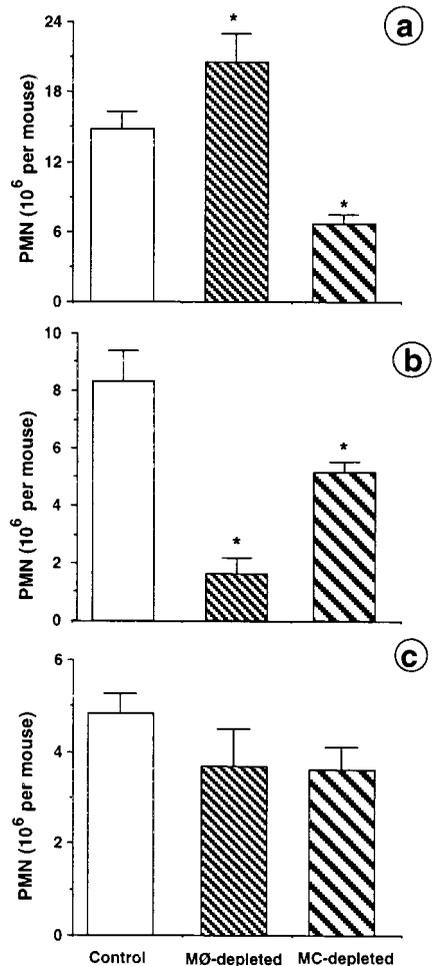


FIGURE 2. Assessment of the role played by resident Mφ or MC in the PMN extravasation induced by zymosan, LPS, or TG. Mice were pretreated with sterile saline (control group), liposomes encapsulating clodronate (to reduce Mφ numbers), or compound 48/80 (to reduce the number of intact MCs) as described in *Materials and Methods*. Animals were then injected i.p. with 1 mg of zymosan (a), 1 mg/kg LPS (b), or 0.5 ml of a 3% suspension of TG (c). PMN accumulation into the peritoneal cavities was quantified 4 h (zymosan and TG) or 24 h (LPS) later. Data are the mean \pm SE of 12 mice/group. *, $p < 0.05$ vs the respective control group.

Table I. Selective depletion of peritoneal MC and Mφ with liposomes and compound 48/80^a

i.p. Pretreatment	Peritoneal Mφ		Peritoneal MC	
	10 ⁶ per cavity	%	10 ³ per cavity	%
Vehicle	3.97 \pm 0.35	100	6.30 \pm 0.58	100
Liposome	0.43 \pm 0.20*	11	6.56 \pm 0.77	104
Vehicle	3.61 \pm 0.28	100	6.70 \pm 0.71	100
Compound 48/80	3.60 \pm 0.45	100	0.36 \pm 0.10*	5

^a Mice were pretreated with sterile saline (vehicle group), liposomes encapsulating clodronate (to reduce Mφ numbers), or compound 48/80 (to reduce the number of intact MCs) as described in *Materials and Methods*. Peritoneal cavities were washed 24 h after last i.p. injection of liposomes (and 72 h postcompound 48/80) and the number of resident Mφ or MCs determined following specific staining and cell counts in a Neubauer chamber. Data are mean \pm SE of 12 mice per group.

* $p < 0.05$ vs respective vehicle group.

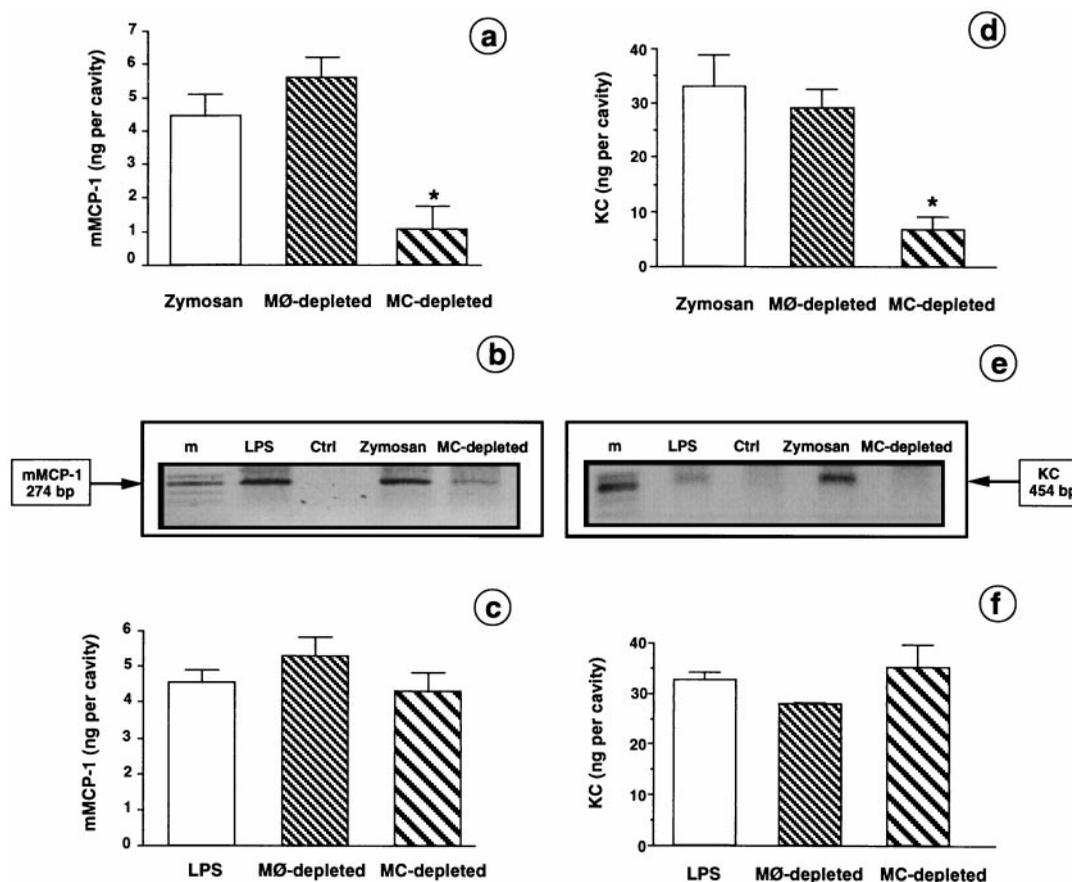


FIGURE 3. Role of resident M ϕ or MC on mMCP-1 and KC generation induced by zymosan and LPS. Mice were pretreated with sterile saline (control group), liposomes encapsulating clodronate (to reduce M ϕ numbers), or compound 48/80 (to reduce the number of intact MCs) as described in *Materials and Methods*. Animals were then injected i.p. with 1 mg of zymosan (a, b, d, and e) or 1 mg/kg LPS (c and f), and peritoneal cavities were washed 4 or 3 h later, respectively. KC and mMCP-1 contents in cell-free fluids were determined by ELISA. Cell pellets were processed to detect mMCP-1 mRNA (274 bp) or KC mRNA (454 bp) by RT-PCR analysis. Data are the mean \pm SE of 12 mice/group. The data in b and e are representative of three distinct RT-PCR analyses. *, $p < 0.05$ vs the respective control group.

then declined to near basal levels by 24 h (Fig. 1b). Administration of TG also induced the release of mMCP-1 protein in the lavage fluids, with peak production measured at 4 h.

Effect of depletion of resident peritoneal MCs or M ϕ

Table I shows that pretreatment of mice with compound 48/80 reduced the number of intact MCs by 95%. Similarly, a marked depletion (86% reduction) of resident peritoneal M ϕ was obtained by a 3-day treatment of mice with liposomes. M ϕ depletion was confirmed by the disappearance of F4/80 staining as assessed by FACS analysis (from 463 ± 36 down to 22 ± 4 mean fluorescence intensity units; $n = 6$; $p < 0.05$).

In the case of zymosan, alteration of the MC population significantly reduced (>60%) the 4 h PMN influx into peritoneal cavities (Fig. 2a). However, significant reductions in the number of resident M ϕ produced an unexpected (and significant) increase in cell extravasation. This was investigated further (see below). The LPS response was also attenuated in MC-depleted mice compared with that in control animals. A significant reduction (80%) in LPS-induced PMN influx at 24 h was obtained following M ϕ depletion (Fig. 2b). PMN accumulation into the peritoneal cavities measured following local injection of TG was not modulated by either MC or M ϕ depletion (Fig. 2c). For this reason, the TG-induced inflammatory response was not used any further.

The role of the resident cells in the release of mMCP-1 (see Fig. 1) and KC (5) induced by zymosan or LPS was then assessed.

Analysis of cell extracts by RT-PCR showed the presence of mMCP-1 and KC mRNAs only in peritoneal cells from zymosan-treated mice and not in control PBS-injected animals (Fig. 3, b and e). A reduction in intact MC numbers reduced the release of both mMCP-1 and KC by >60% as measured in zymosan lavage fluids (Fig. 3, a and d). An apparent reduction in KC and mMCP-1 mRNA was seen in MC-depleted mice. In contrast, no significant modulation was observed of KC and mMCP-1 protein levels in the lavage fluids (Fig. 3, a and d) or of mRNA in the cell pellets (data not shown) of M ϕ -depleted animals. Depletion of either resident peritoneal MCs or M ϕ did not alter the amounts of KC and mMCP-1 released by LPS (Fig. 3, c and f). In this set of experiments TNF- α levels were also measured, and again no modulation by M ϕ depletion was found (420 ± 15 and 430 ± 10 pg/cavity in intact and liposome-pretreated mice, respectively; $n = 8$; not significant).

Endogenous IL-10 plays a tonic inhibitory role in controlling PMN influx and chemokine production in zymosan peritonitis

Based on the results shown above, we decided to characterize further the mechanism(s) underlying the unexpected increased influx of PMN after zymosan injection into M ϕ -depleted mice. Fig. 4 compares the PMN accumulation measured after the injection of 0.2 or 1 mg of zymosan; a twofold increase was seen in the M ϕ -depleted animals treated with the lower dose of zymosan. Lower

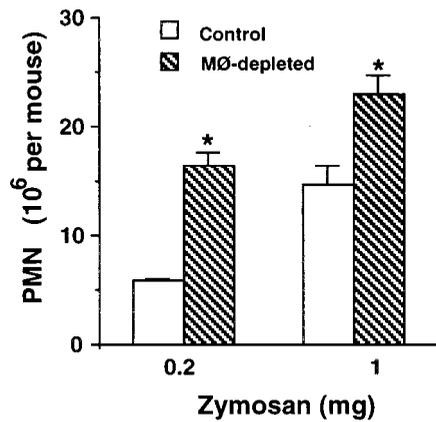


FIGURE 4. Potentiation of zymosan-induced PMN extravasation in M ϕ -depleted mice. Mice were pretreated with sterile saline (control group) or liposomes encapsulating clodronate (to reduce M ϕ numbers) as described in *Materials and Methods*. Animals were then injected i.p. with the reported doses of zymosan, and PMN accumulation into the peritoneal cavities was quantified 4 h later. Data are the mean \pm SE of eight mice per group. *, $p < 0.05$ vs the respective control group.

concentrations of chemokines were released with 0.2 mg of zymosan (compare Table II with Fig. 3). No significant change in mMCP-1, but a pronounced reduction in KC concentrations, between control and M ϕ -depleted mice was detected (Table II).

A time-dependent release of IL-10 was observed after injection of zymosan, with a peak at 4 h and significant amounts at 2, 6, and 8 h post-zymosan injection. The levels of the cytokine returned to basal values by 24 h (Fig. 5a). A marked reduction in resident M ϕ , but not MC, numbers significantly affected the amounts of IL-10 recovered in the lavage fluids at the 4 h point (Fig. 5b).

The pivotal role of IL-10 in modulating PMN accumulation and chemokine production in zymosan-induced peritonitis was confirmed using IL-10 KO mice (Fig. 6). Similar increases in PMN accumulation were induced by 0.2 mg of zymosan in IL-10 KO as well as IL-10 wt mice depleted of peritoneal M ϕ . Depletion of M ϕ in IL-10 KO mice resulted in a further increase in PMN influx (Fig. 6a). Zymosan injection released comparable amounts of chemokines in C57BL/6 wt and Swiss Albino mice. Higher mMCP-1 and KC levels were measured in the lavage fluids of IL-10 KO mice than in IL-10 wt mice. Further, the release of mMCP-1 lasted longer in IL-10 KO animals, with values of 2.0 ± 0.1 and 4.4 ± 0.4 ng/cavity measured in the 8 h lavage fluids in wt and KO mice, respectively ($n = 7$; $p < 0.05$). No modulation by peritoneal M ϕ of mMCP-1 release in either wt or KO animals was seen (Fig. 6, b and c).

To validate the M ϕ depletion protocol in IL-10 KO mice, and since recent evidence shows that nitric oxide is released in zymo-

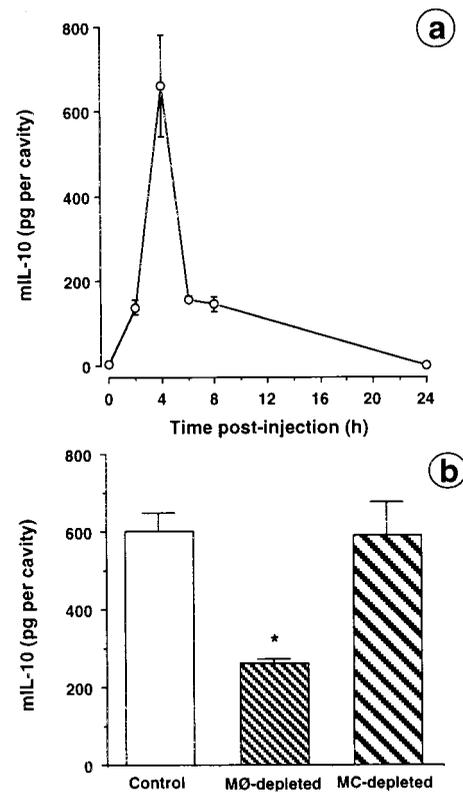


FIGURE 5. Detection of IL-10 in the lavage fluids of zymosan peritonitis. *a*, Time dependency of IL-10 release following i.p. administration of 1 mg of zymosan. Data are the mean \pm SE of six mice per group. *b*, Mice were pretreated with sterile saline (control group), liposomes encapsulating clodronate (to reduce M ϕ numbers), or compound 48/80 (to reduce the number of intact MCs) as described in *Materials and Methods*. Animals were then injected i.p. with 1 mg of zymosan, and immunoreactive IL-10 levels were measured in the lavage fluids 4 h later. Data are the mean \pm SE of six mice per group. *, $p < 0.05$ vs the control group.

san peritonitis (13), we monitored nitrate/nitrite levels as a positive control. A significant reduction in the concentration of nitrate/nitrite was measured after M ϕ removal, from 10.0 ± 1.8 in intact mice to 6.4 ± 0.7 μ M in M ϕ -depleted mice ($n = 5$; $p < 0.05$).

Discussion

In this study we provide evidence for a central role of resident MCs in producing CXC and CC chemokines during experimental inflammation in vivo and identify an unexpected inhibitory role of resident M ϕ on the influx of PMN that is effected via the release of endogenous IL-10.

One of the aims of this study was to identify the potential contribution by resident MCs and M ϕ of the production of selected CXC and CC chemokines during experimental inflammation. We chose to use three inflammogens: 1) particles of zymosan, a phagocytic stimulus for the M ϕ and the MC, which also causes complement activation and produces an intense, but short-lasting, influx of PMN (14); 2) TG broth, which causes a milder and longer lasting accumulation of PMN (11); and 3) LPS, a soluble M ϕ activator that releases endogenous cytokines to produce PMN influx (15, 16).

The i.p. injection of TG and zymosan induced a rapid and time-dependent accumulation of PMNs in the peritoneal cavity. PMN influx in response to LPS was delayed but was also time dependent. Injection of these inflammatory agents also led to the coordinated, rapid, and time-dependent production of mMCP-1 in the

Table II. Effect of peritoneal M ϕ depletion on chemokine production following administration of a low dose of zymosan

Treatment	KC (ng per cavity)	mMCP-1 (ng per cavity)
Vehicle	2.40 ± 0.25	1.64 ± 0.17
M ϕ -depleted	$0.65 \pm 0.19^*$	2.14 ± 0.16

^a Mice were pretreated with sterile saline (vehicle group) or with a clodronate-liposome preparation as described in *Materials and Methods*. A low dose of zymosan (0.2 mg in 0.5 ml of sterile saline) was then injected i.p., and peritoneal cavities were washed 4 h later. Lavage fluids were tested for the reported chemokines using specific ELISA. Data are mean \pm SE of six to eight mice per group.

* $p < 0.05$.

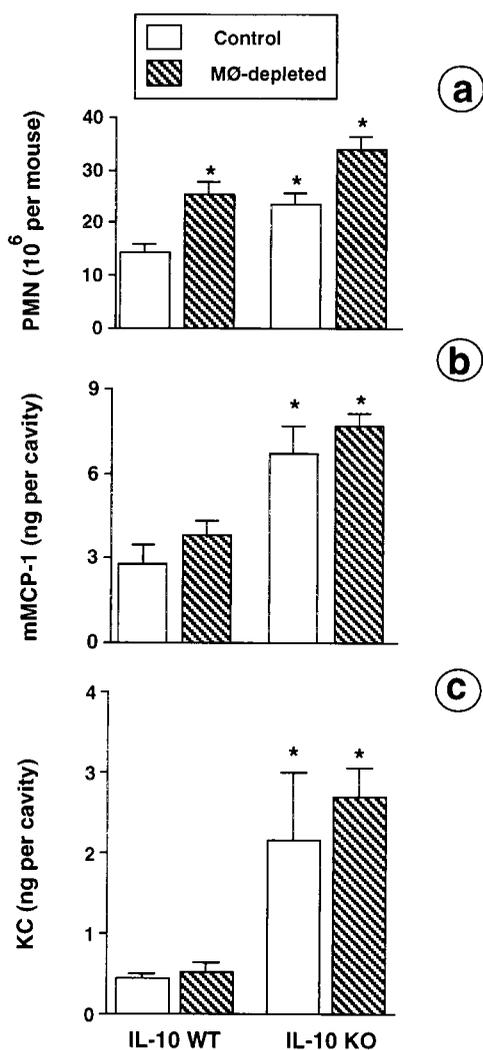


FIGURE 6. Determination of zymosan-induced inflammation in IL-10 KO mice. C57BL/6 wt or C57BL/6 IL-10 KO animals were pretreated with sterile saline (control group) or liposomes encapsulating clodronate (to reduce M ϕ numbers) as described in *Materials and Methods*. Animals were then injected i.p. with 0.2 mg of zymosan, and peritoneal cavities were washed 4 h later and handled for quantification of PMN number, (a), mMCP-1 (b), and KC (c) protein levels (see *Materials and Methods*). Data are the mean \pm SE of five or six mice per group. *, $p < 0.05$ vs control group of wt mice.

peritoneal lavage fluid as assessed by ELISA. Secretion of mMCP-1 by these inflammatory stimuli reached a peak 4 h post-zymosan and TG administration and 3 h post-LPS injection; in all cases mMCP-1 decreased rapidly thereafter, indicating a fast clearance of the chemokine from the peritoneal cavity. The fact that mMCP-1 induction by these inflammatory stimuli was rapid and transient is not unusual in *in vivo* experimental systems (5, 17). The rapidity of mMCP-1 induction can be attributed to the fact that this chemokine is the product of an early activation gene (6), whereas the transient nature of its appearance can be attributed 1) to the ability of mMCP-1 to bind to duffy Ag receptor for chemokines, which has been proposed to function as a sink for chemokines (2); or 2) to the release of endogenous inhibitors that switch off the synthesis of the chemokine. We have previously reported an apparent discrepancy between mMCP-1 protein release in the exudates and gene expression in the peritoneal cell pellet, which suggests the existence of such a control mechanism at the level of mMCP-1 mRNA translation (5).

A marked reduction in resident MCs, achieved using a protocol well validated in our laboratory (8, 10), attenuated the cellular response produced by both zymosan and LPS. Resident M ϕ numbers were reduced according to a validated procedure that implies the selective delivery of a cytotoxic drug by neutral liposomes (12). Liposomes have been reported to produce an anti-inflammatory action *per se* (18); however, this is unlikely to be the case in our experiments because 1) a general anti-inflammatory effect was not seen (compare the diverse effects of M ϕ depletion on the three inflammogens); and 2) the precaution of provoking the inflammatory response 24 h after the last injection of the liposome preparation was taken. As variance from MC depletion, a distinction between zymosan and LPS peritonitis was observed following M ϕ depletion, such that LPS-induced PMN influx was almost abolished ($\sim 90\%$), whereas the response to zymosan was unexpectedly augmented. The former data are not surprising (19), but the data obtained with zymosan prompted a series of experiments that is discussed below. No modulation by either MCs or M ϕ of the TG response was seen; for this reason this inflammogen was not used in subsequent experiments. Overall, these observations confirm the important role that resident MCs play in the initiation of the cellular component characteristic of acute inflammation (7, 8, 10).

To extend these studies, we then investigated whether chemokine generation could also be modulated by MCs *in vivo* as well as by PMN influx. We chose to monitor the archetypal chemokines KC and mMCP-1 because both have been associated with PMN influx in the mouse (5, 17, 20). We found that in the zymosan peritonitis model resident MCs accounted for a large part of the release of these two chemokines. The reduction seen at the protein level after MC depletion is possibly secondary to a repression at the gene level, although due to the qualitative rather than quantitative value of the RT-PCR analysis, this conclusion needs to be corroborated by other studies. The possibility that MCs produce some soluble mediators that, in turn, cause KC and mMCP-1 release from another cell type cannot be excluded. To our knowledge this is the first study that shows evidence for a crucial role of MCs in chemokine release during experimental inflammation *in vivo*. To date, few *in vitro* studies have demonstrated the ability of MCs to produce chemokines. In particular, human skin MCs and a human MC line synthesize *de novo* IL-8, which is then stored in granules and released upon activation (21). Similarly, an anti-IgE-dependent secretion of MIP-1 α from human MCs has been recently observed (22). Finally, expression of multiple chemokine genes, including mMCP-1 and IL-8, has been demonstrated again in a human MC leukemia cell line (23).

In contrast to zymosan, *i.p.* injection of LPS led to the production of KC and mMCP-1, which were not derived from M ϕ or MCs. It is very likely that another cell type(s), such as endothelial cells (24) or mesothelial cells (25, 26), could be responsible for the effect of LPS. Mesothelial cells may be the best candidate, since they have been recently implicated in the production of mMCP-1 in related models of inflammation (25) and can also be present in small numbers in the peritoneal cell pellet (where we could detect LPS-induced KC and mMCP-1 mRNA by RT-PCR analysis). In this particular condition, we have also measured TNF- α levels, but again LPS-induced release of this cytokine was not altered by M ϕ depletion. Interestingly, the large majority of TNF- α produced in murine models of bacteria peritonitis derives from peritoneal MCs rather than M ϕ (27).

The potentiating effect seen in M ϕ -depleted animals was even more evident when a lower dose of zymosan was used, with an approximately twofold increase in PMN extravasation at the 4 h point. It is very likely that the cellular accumulation obtained with 1 mg of zymosan is almost maximal for a single mouse, and it could only be potentiated by 40–50% in M ϕ -depleted animals. In

contrast to the results with 1 mg of zymosan and discussed above, the release of KC measured with the lower dose of zymosan was almost entirely due to the resident M ϕ . Together, these data indicate the existence of at least one endogenous mediator released from the M ϕ that acts to down-regulate PMN accumulation. IL-10 has been shown to reduce PMN elicitation in experimental inflammation (28) and to inhibit the production of proinflammatory cytokines and chemokines (29, 30). In the present study immunoreactive IL-10 was detected in the zymosan exudates, and more importantly, its levels were modulated by M ϕ , but not MC, depletion. IL-10 KO mice have become available to help unravel the numerous biological activities of this cytokine (31, 32). Disruption of the IL-10 gene mimicked the effect of M ϕ removal in IL-10 wt mice in terms of potentiation of PMN influx. A drastic reduction (>85%) in peritoneal M ϕ numbers in IL-10 KO mice resulted in a further increase in 4 h PMN influx, suggesting the involvement of M ϕ product(s), other than IL-10, in these experimental conditions. A marked increase in mMCP-1 and KC levels was also measured in IL-10 KO mice, but this was not further modulated by M ϕ depletion, indicating different mechanisms for control of leukocyte infiltration and chemokine production. This hypothesis seems plausible, since twice as much mMCP-1 protein is found at the 8 h point in IL-10 KO animals.

In conclusion, this study compared three different inflammatory stimuli in terms of 1) the profile of PMN accumulation in the mouse peritoneal cavity, 2) chemokine generation, and 3) role of resident M ϕ and MCs. We conclude that resident peritoneal MCs play a central role in the production of the CXC chemokine KC and the CC chemokine mMCP-1 in the acute inflammatory response induced by i.p. injection of zymosan. In addition, endogenous IL-10 released from resident M ϕ plays a negative modulatory role, with an effect on PMN accumulation and chemokine generation. The existence of a stimulus and dose specificity for the cell type(s) responsible for the rapid production of chemokines during experimental inflammation is also proposed.

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