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# Expression of Macrophage Colony-Stimulating Factor and Its Receptor in Hepatic Granulomas of Kupffer-Cell-Depleted Mice

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***In mice administered with liposome-entrapped dichloromethylene diphosphonate, which depletes Kupffer cells, the size and the number of zymosan-induced granulomas in the liver were smaller than in untreated mice. The number of macrophage precursors, as detected by the monoclonal antibodies for macrophage precursors, increased after zymosan injection in both groups of mice, proliferated, and differentiated into macrophages. Expression of macrophage colony-stimulating factor (M-CSF), interleukin-1, monocyte chemoattractant protein-1, tumor necrosis factor-α, and interferon-γ mRNA was enhanced in the stage of granuloma formation in the control mouse liver, whereas it was suppressed in Kupffer-cell-depleted mice. However, M-CSF mRNA expression was increased in the Kupffer-cell-depleted mice to form granulomas in the late stages.*** **In situ** ***hybridization demonstrated the expression of M-CSF mRNA and c-*** **fms** ***mRNA in Kupffer cells and monocyte-derived macrophages in the sinusoid and granulomas. The concentration of M-CSF in serum of zymosan-injected control mice was within normal range, but that of zymosan-treated or untreated Kupffer-cell-depleted mice was markedly elevated at day 1. These findings imply that Kupffer cells are indispensable for granuloma formation and produce various cytokines including M-CSF. The local production and consumption of M-CSF in the liver may play a crucial role in granulomatous inflammation. (Am J Pathol 1997, 150:2047–2060)***

Granuloma formation is a kind of inflammatory process accompanied by accumulation of inflammatory cells and production of several cytokines.[1,2] Among the variety of inflammatory cells, macrophages play a central role in granulomatous inflammation.[1–3] Not only monocytes but also tissue macrophages participate in the series of events leading to granuloma formation. To elucidate the role of macrophages, various approaches have been devised. As liposome-encapsulated dichloromethylene diphosphonate (MDP) is highly cytotoxic to macrophages, macrophages in several tissues can be depleted by selecting a suitable administration route. Using this method, Rooijen and other investigators have depleted tissue macrophages and studied their repopulation in macrophage-eliminated tissues.[4–9] However, the kinetics of macrophage repopulation and their role in cytokine production under an inflammatory condition have not been well investigated.

Among several macrophage growth factors, macrophage colony-stimulating factor (M-CSF) or CSF-1 plays an important role in the development, differentiation, proliferation, and function of macrophages.[10–14] In *op/op* mice, macrophage differentiation is defective in physiological and inflammatory conditions[11–13] because of the complete absence of functional M-CSF.[15,16] The number of Kupffer cells in *op/op* mice was reduced to 30% of that of normal littermates, and granuloma formation was impaired in the mutant mice.[12] Supplementation of M-CSF corrects the reduction of Kupffer cells and defective granuloma formation in the mutant mice.[12,13] Together, these observations

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indicate that M-CSF is a key molecule for the development, differentiation, and proliferation of Kupffer cells. It has been shown that fetal and adult livers produce M-CSF[17,18] to provide a microenvironment for Kupffer cell differentiation *in vitro*. However, the local production of M-CSF in the liver has not been clearly demonstrated *in vivo*.

Zymosan contains an insoluble form of β-1,3-glucan, which has a wide range of biological and immunomodulating activities. Zymosan is recognized by glucan receptors[19] and/or the complement receptor type 3 (CR3)[20] on macrophages, and glucan increases the production of M-CSF and granulocyte/macrophage (GM)-CSF in the liver.[21] Glucan and zymosan particles induce granulomas of foreign-body type, and have been used to investigate the role of macrophages in granuloma formation.[22–26] In this study, we performed immunohistochemical and autoradiographic studies on zymosan-induced hepatic granulomas in control mice and mice depleted of Kupffer cells by a single intravenous injection of liposome-entrapped MDP (lipo-MDP) to clarify the role of Kupffer cells in granuloma formation. The role of M-CSF and its receptor c-*fms* in the granuloma formation was also investigated by examining the expression of their mRNAs by reverse transcriptase polymerase chain reaction (RT-PCR) and *in situ* hybridization and measuring the serum concentration of M-CSF.

## Materials and Methods

### Preparation of Liposome-Entrapped MDP

Multilamellar liposomes were prepared as described.[27] In brief, 58.72 mg of phosphatidylcholine (Nihon Seika, Hyogo, Japan), 8.75 mg of dehexadecyl phosphate, and 24.75 mg of cholesterol (Wako, Tokyo, Japan) were dissolved in chloroform in a round-bottom flask and dried under reduced pressure. The lipids were then hydrated in 5 ml of phosphate-buffered saline (PBS) containing 650 mmol/L (0.9455 g) MDP and vortex mixed at room temperature. The liposomes were extruded through polycarbonate membranes (Costar Corp., Pleasanton, CA) with a pore size of 0.8 μm. Nonencapsulated MDP was removed by ultracentrifugation. The liposomes were composed of phosphatidylcholine/dehexadecyl phosphate/cholesterol at a molar ratio of 5:1:4.

### Animals

BALB/c mice were purchased from Charles River Japan (Tokyo, Japan) and maintained under routine conditions at the Laboratory Animal Center of Niigata University School of Medicine. Eight-week-old male mice were intravenously injected with 0.1 ml of lipo-MDP. Ten minutes later, 0.1 ml of zymosan (Sigma Chemical Co., St. Louis, MO) dissolved in physiological saline at a concentration of 1 mg/ml was injected intravenously. The liver was removed at various intervals after lipo-MDP injection. At various time points, three mice were killed by ether anesthesia.

### Blood Cell Count

A small amount of blood was sampled from the tail vein of each animal, and the number of white blood cells was determined. On a blood film, 1000 white blood cells were differentially counted.

### Monoclonal Antibodies

The monoclonal antibodies F4/80, BM8, ER-MP58, and ER-MP20 (BMA Biomedicals, Augst, Switzerland) were used at a dilution of 1:100. These monoclonal antibodies recognize antigens on macrophages and their precursors at different stages of differentiation.[28,29] Monoclonal antibodies Ab-1 (Oncogene Sciences, Cambridge, MA), Thy1.2 (Becton Dickinson, Bedford, MA), and B220 (Pharmingen, San Diego, CA), recognizing murine and human M-CSF, T lymphocytes, and B lymphocytes, respectively, were also used at a dilution of 1:100.

### Immunohistochemistry

The liver was fixed for 4 hours at 4°C in periodate-lysine-paraformaldehyde, washed for 4 hours with PBS containing 10, 15, and 20% sucrose, embedded in OCT compound (Miles, Elkhart, IN), frozen in dry ice/acetone, and cut by a cryostat (Bright, Huntington, UK) into 6 μm-thick sections. After inhibition of endogenous peroxidase activity by the method of Isobe et al,[30] immunohistochemistry was performed using the anti-mouse monoclonal antibodies described above. As a secondary antibody, anti-rat immunoglobulin/horseradish-peroxidase-linked $F(ab)_2$ fragment (Amersham, Poole, UK) was used. After visualization with 3,3′-diaminobenzidine (Dojin Chemical Co., Kumamoto, Japan), nuclear staining with methylene green, and mounting with resin, the positive cells with nuclei per 1 $mm^2$ were counted using a light microscope. Cellular aggregates composed of more than 10 cells were judged as granulomas.

Table 1. *Oligonucleotides Used*

| mRNA | Primers | Sequences (5′-3′) | Products (bp) |
|---|---|---|---|
| M-CSF | Sense | GAGGATCCTGTTTGCTACCTA | 187 |
| | Antisense | GTTGAGCTCCTCATAAGTCCTTGG | |
| MCP-1 | Sense | CTCACCTGCTGCTACTCATTC | 350 |
| | Antisense | GCATGAGGTTGTGAAAAA | |
| TNF-$\alpha$ | Sense | GGCAGGTCTACTTTGGAGTCATTGC | 309 |
| | Antisense | ACATTCGAGGCTCCAGTGAATTCGG | |
| IFN-$\gamma$ | Sense | AGCGGCTGACTGAACTCAGATTGTAG | 213 |
| | Antisense | GTCACAGTTTTCAGCTGTATAGGG | |
| IL-1 $\alpha$ | Sense | CTCTAGAGCACCATGCTACAGAC | 288 |
| | Antisense | GACCATGGGCCATGAGGAACATTC | |
| $\beta$-actin | Sense | TGGAATCCTGTGGCATCCATGAAAC | 348 |
| | Antisense | TAAAACGCAGCTCAGTAACAGTCCG | |

MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin.

## *Immunohistochemical Double Staining*

Immunohistochemical double staining with ER-MP58, ER-MP20, and BM8 was performed according to that described[31] with a minor modification. In brief, after inhibiting endogenous peroxidase activity as described above, cryostat sections were incubated with the primary monoclonal antibody. After incubation with anti-rat Ig/horseradish-peroxidase-linked $F(ab)_2$ fragment, the reaction was stained brown with 3,3′-diaminobenzidine. The sections were washed twice with glycine/HCl buffer for 1 hour and then incubated with the second primary monoclonal antibody. After incubation with anti-rat Ig/horseradish-peroxidase-linked $F(ab)_2$ fragment, they were incubated with nickel chloride solution in a 3,3′-diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA) and processed as above to stain positive cells blue-black.

## *Autoradiography with [$^3$H]Thymidine*

[$^3$H]Thymidine (specific activity, 3.0 TBq/mmol) was obtained from DuPont (Wilmington, DE). Mice were intravenously injected with 1 MBq of [$^3$H]thymidine for 60 minutes at 1, 2, 3, 5, 7, 10, and 14 days after lipo-MDP injection and then killed, and the liver was removed. After immunohistochemical staining with ER-MP20, ER-MP58, and BM8, slides were dipped into a Sakura NR-M2 liquid emulsion (Konica, Tokyo, Japan) diluted 1:2 with water, exposed for 2 weeks at 4°C, and developed. Cells with 10 or more grains over the nucleus above background were considered as labeled.

## *RNA Isolation and mRNA Analysis by RT-PCR*

Total cellular RNA was isolated from the liver of control and lipo-MDP-treated mice at 1, 2, 3, 5, 7, 10, and 14 days after zymosan injection by phenol/chloroform extraction as described previously.[31] PCR amplification was performed by using a TP cycler-100 (Toyobo, Osaka, Japan). All of the PCR primers were made to order by Kurabo Biomedicals (Osaka, Japan). The oligonucleotides used are listed in Table 1. The samples were separated on a 1.5% low-melting agarose gel containing 0.3 $\mu$g/ml ethidium bromide, and bands were visualized and photographed using ultraviolet transillumination.

## In Situ *Hybridization*

Mice were perfused through the heart with 4% paraformaldehyde, and after removal, the liver was refixed in the same fixative for 24 hours. Tissues were dehydrated in ethanol, cleared in chloroform, and embedded in low-melting-point paraffin (Tissue Prep, T565, melting point 56 to 57°C, Fisher Scientific, Fair Lawn, NJ). Sections were cut and mounted onto slides coated with 3-aminopropyl-triethoxysilane (Aldrich Chemical Co., Milwaukee, WI). Paraffin sections of the liver were subjected to *in situ* hybridization by using digoxigenin-labeled (Boehringer Mannheim, Mannheim, Germany) hybridizing (antisense) and nonhybridizing (sense) RNA probes transcribed from pGEM2-MCSF53 and p755 directed against mouse M-CSF and mouse c-*fms*, respectively.[32–35]

## *Measurement of Serum M-CSF Concentrations*

Three groups of mice were prepared: lipo-MDP-injected, zymosan-injected, and lipo-MDP- and zymosan-injected mice. At various time points, blood samples were harvested by cardiac puncture from three mice of each group. Serum M-CSF levels were measured by radioimmunoassay that detects

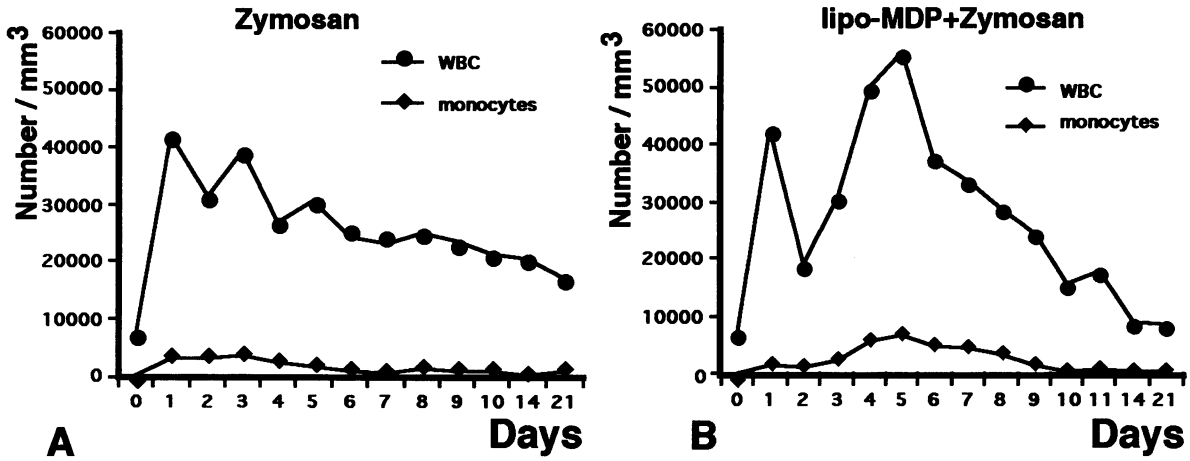


Figure 1. *The numbers of white blood cells and monocytes in peripheral blood after administration of zymosan in control mice* (A) *and Kupffer-cell-depleted mice* (B). *WBC, white blood cells.*

biologically active growth factor at concentrations >120 pg/ml.[36,37]

### Statistics

The significance of the data was evaluated by analysis of variance by repeated measures.

## Results

### Changes in Numbers of White Blood Cells and Monocytes in Peripheral Blood of Mice after Zymosan Injection with or without Lipo-MDP Injection

In untreated normal mice, white blood cell counts ranged from 5000 to 9000 per $mm^3$, and the numbers of monocytes, granulocytes, and lymphocytes were approximately 4, 34, and 62% of the total white blood cells, respectively. In normal mice, the number of white blood cells abruptly increased to approximately 40,000 at 1 day after zymosan injection. Monocytes occupied approximately 9% of the white blood cells from day 1 to day 4 when their number ranged from 2400 to 3500 per $mm^3$, followed by a gradual decrease (Figure 1A). In the lipo-MDP-treated mice, the number of white blood cells increased to nearly 50,000 at day 1, decreased at day 2, and peaked again at day 5, followed by gradual decreases. The monocyte count peaked at approximately 7000/$mm^3$ also at day 5 (Figure 1B).

### Granuloma Formation in the Liver of Lipo-MDP-Treated and Untreated Mice after Zymosan Injection

In normal mice, Kupffer cells were specifically immunostained with F4/80 and BM8. They were found predominantly in the peripheral zone of the hepatic lobules. Three days after zymosan injection, a few clusters of macrophages, neutrophils, and lymphocytes were found in the liver, and several small granulomas were formed in the sinusoids. The size of granulomas was largest between days 7 and 10, and they were most numerous at day 10 (Figure 2). The size and the number of granulomas decreased from 14 days and were almost diminished at 28 days. The number of BM8-positive cells in and out of the granulomas increased until 10 days after zymosan injection (Figure 3). In the granulomas, BM8-positive cells were approximately 30% of granuloma-constituting cells at 3 days and almost doubled at 10 days (Figure 4A). The size of the macrophages became larger, and they transformed into epithelioid cells and multinuclear giant cells after 7 days. T lymphocytes were detectable in and around the granulomas at 5 days and occupied approximately 18% of the granuloma-constituting cells. At 10 days, they were mainly situated at the periphery of the granulomas. Only a few B lymphocytes were present within the granulomas. Both ER-MP20- and ER-MP58-positive cells were mostly round. Although the numbers of ER-MP20- and ER-MP58-positive cells were small in untreated mice, after zymosan injection these macrophage precursor cells were detected both within and outside the granuloma (Figure 4).

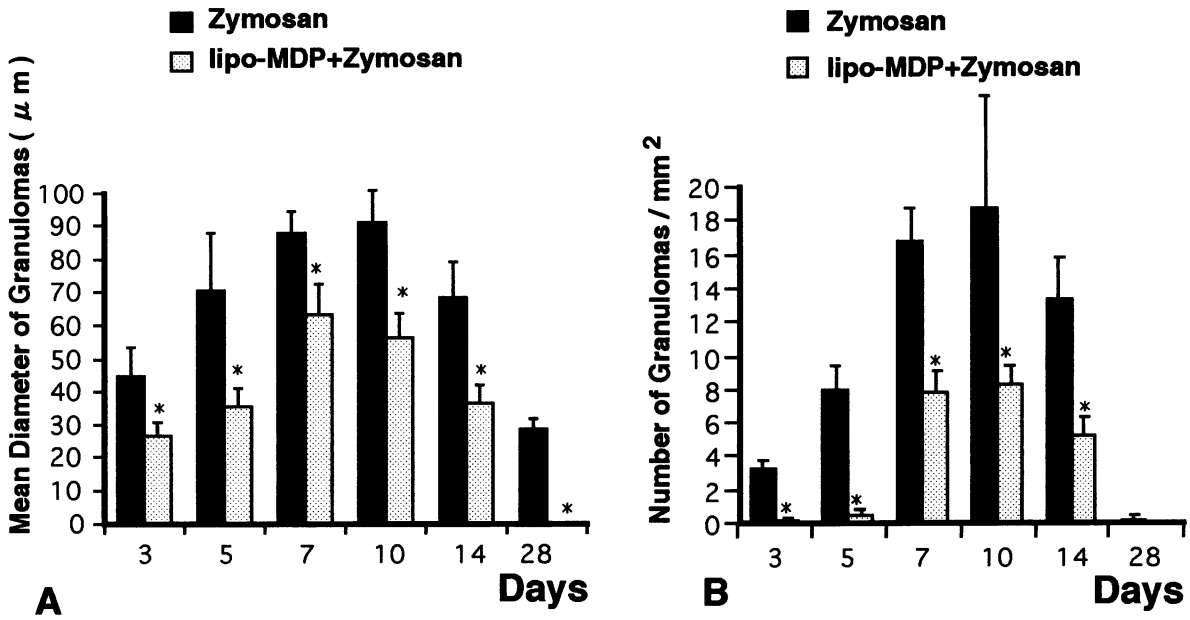


Figure 2. *The size* (A) *and the number* (B) *of granulomas after zymosan injection in both groups of mice. Data are expressed as mean ± SD.* * $P < 0.05$.

In the lipo-MDP-treated mice, the number of Kupffer cells decreased to approximately 12% of the normal mice at day 3, and their distribution was irregular in the hepatic lobules. Two days after injection, no granulomas were formed. Three days after injection, a few small cellular clusters were detected in the liver. At 5 days, a small number of granulomas were formed (Figure 2B). They were significantly smaller than those in control mice (Figure 2A) and consisted of macrophages, macrophage precursors, neutrophils, and lymphocytes. At this period, the number of Kupffer cells outside the granulomas

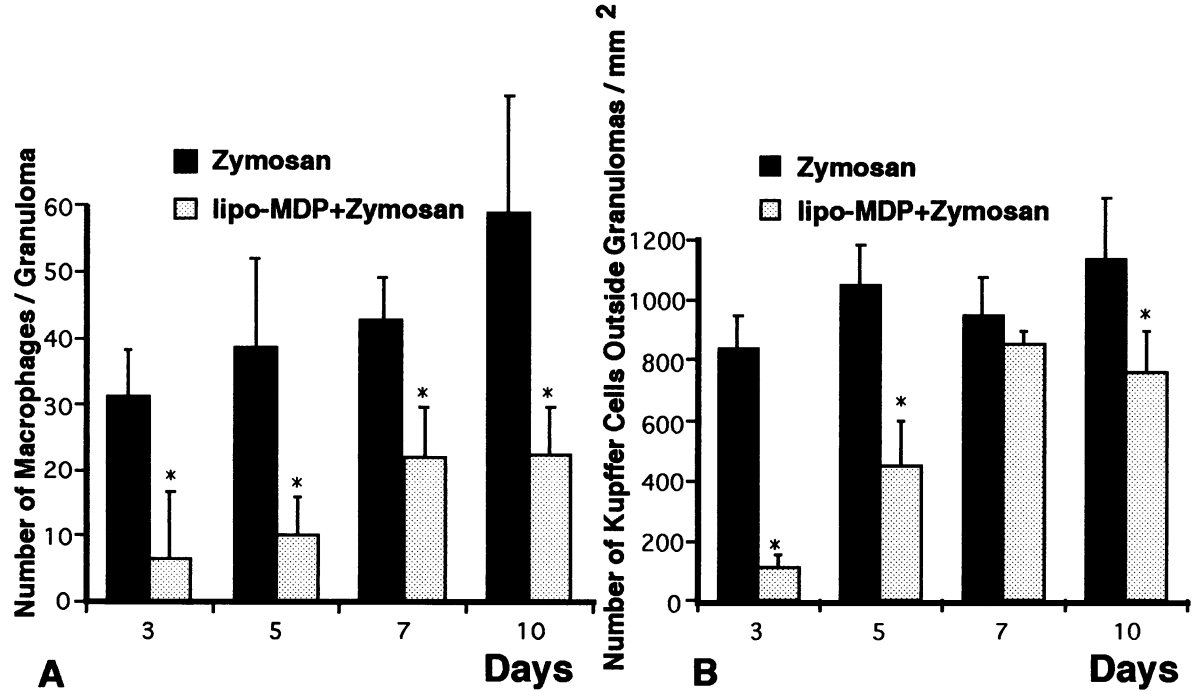


Figure 3. *The number of BM8-positive cells in* (A) *and out* (B) *of the granulomas after zymosan injection in both groups of mice. Data are expressed as mean ± SD.* * $P < 0.05$.

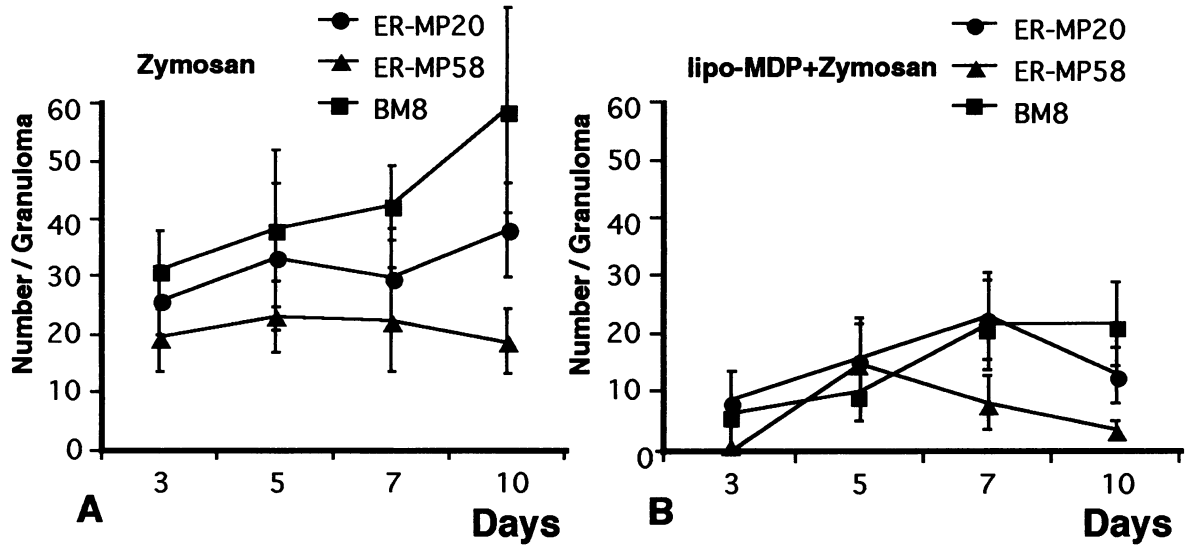


Figure 4. *The number of each cell type in the granulomas of control mice* (A) *and Kupffer-cell-depleted mice* (B) *after zymosan injection. Data are expressed as mean ± SD.*

increased (Figure 3B) and BM8-positive cells occupied nearly 10% of the granuloma-constituting cells. The proportion of T lymphocytes in the granulomas of the MDP-treated mice did not differ from that of untreated mice. The number and the size of the granuloma markedly decreased at 14 days (Figure 2). The numbers of ER-MP58- and ER-MP20-positive cells in (Figure 4B) and out of the granulomas were smaller than those in control mice.

Immunohistochemical double staining demonstrated that the number of ER-MP20-positive cells and BM8-positive cells increased up to 10 days after zymosan treatment, whereas the number of double-positive cells peaked at 5 days, followed by a gradual decrease (Figures 5A and 6, A and B). In lipo-MDP-treated mice, the numbers of BM8-positive macrophages gradually increased until 10 days, but those of ER-MP20-positive cells peaked at 7 days.

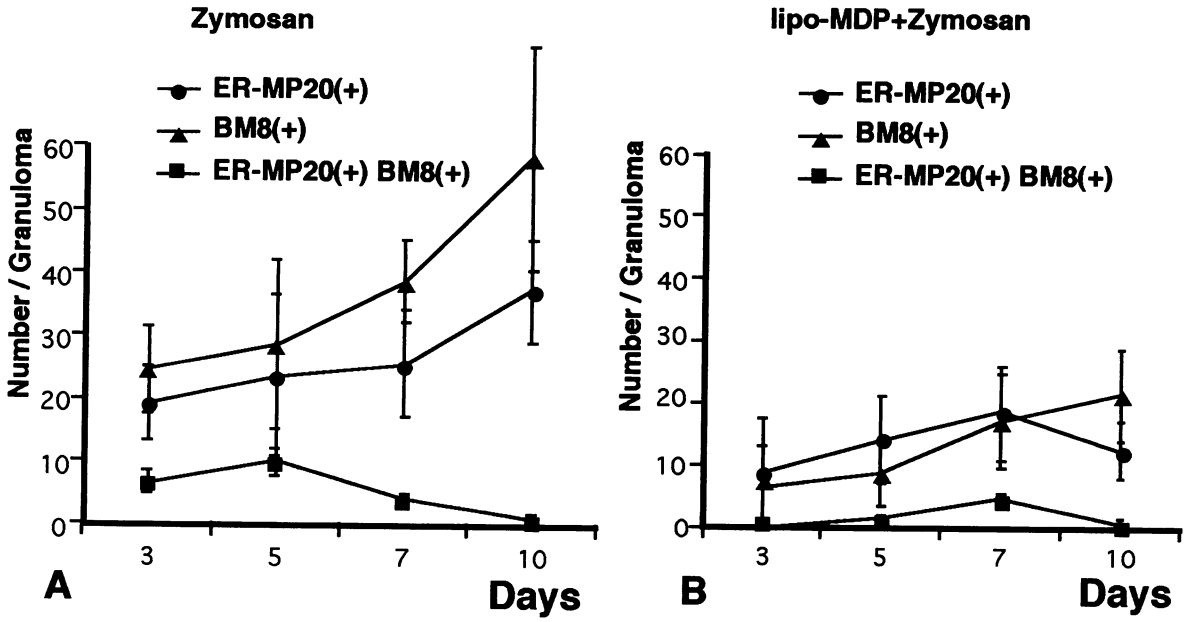


Figure 5. *The number of immunoreactive cells in the granulomas detected by immunohistochemical double staining using ER-MP20 and BM8.* A, *Control mice.* B, *Kupffer-cell-depleted mice. Data are expressed as mean ± SD.*

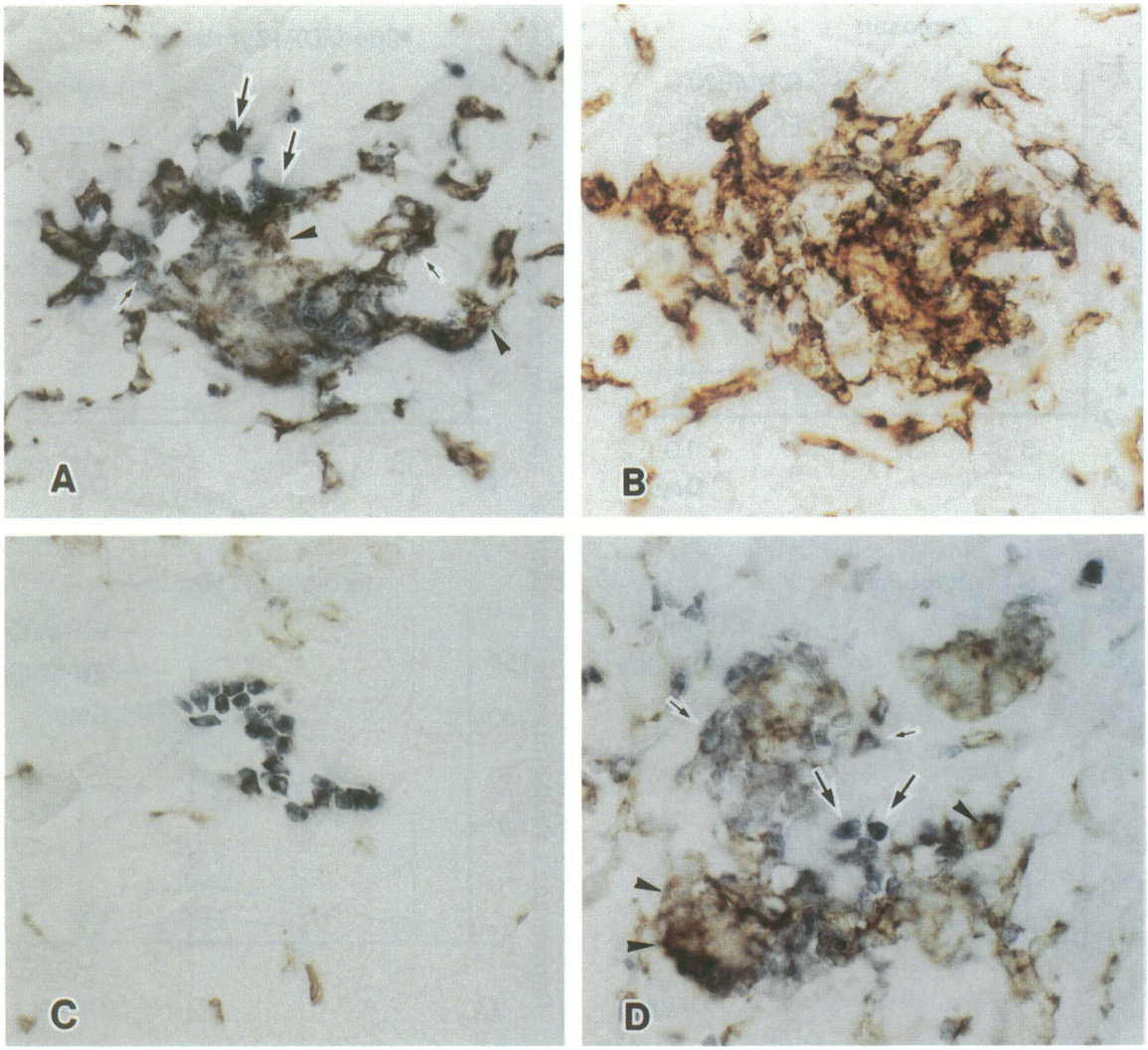


Figure 6. *Immunohistochemical double staining of granulomas formed in a control mouse and a Kupffer-cell-depleted mouse.* A: *ER-MP20-positive (dark blue;* large arrows*) cells, BM8-positive macrophages (brown;* arrowheads*), and double-positive cells (blue-brown;* small arrows*) are admixed in the granuloma at 5 days.* B: *At 10 days, granulomas are composed mainly of macrophages (brown).* C: *Granulomas are mostly composed of ER-MP20-positive cells (dark blue) at 5 days.* D: *At 7 days, most of the granulomas consist of macrophages (brown;* arrowheads*), double-positive cells (blue-brown;* small arrows*), and a few ER-MP20-positive cells (dark blue;* large arrows*). Magnification, ×200.*

The number of double-positive cells peaked at day 7 (Figures 5B and 6, C and D).

## *Proliferative Potential of Macrophages and Macrophage Precursors in Lipo-MDP-Treated and Untreated Mice*

To examine the proliferative potential of Kupffer cells and their precursors, [$^3$H]thymidine was given to mice at various times after lipo-MDP and zymosan injection. In untreated zymosan-injected mice, the percentage of [$^3$H]-thymidine-labeled cells as assessed by autoradiography in BM8-positive Kupffer cells was low and there were few labeled ER-MP20-positive or ER-MP58-positive cells in the granulomas. In mice given lipo-MDP, the [$^3$H]thymidine-labeled ER-MP58- and BM-8-positive cells were markedly increased at 5 days (Figure 7, A and B), followed by a gradual decrease.

Outside the granuloma, there were only a few [$^3$H]thymidine-labeled cells among each cell type in non-lipo-MDP-treated zymosan-injected mice. However, ER-MP58-positive cells showed a marked proliferative capacity in lipo-MDP-treated mice (Figure 7, C and D).

## *Expression of Cytokine mRNAs in the Liver of MDP-Injected and Uninjected Mice*

M-CSF mRNA was the only mRNA detected in the liver of normal mice. Expressions of M-CSF, mono-

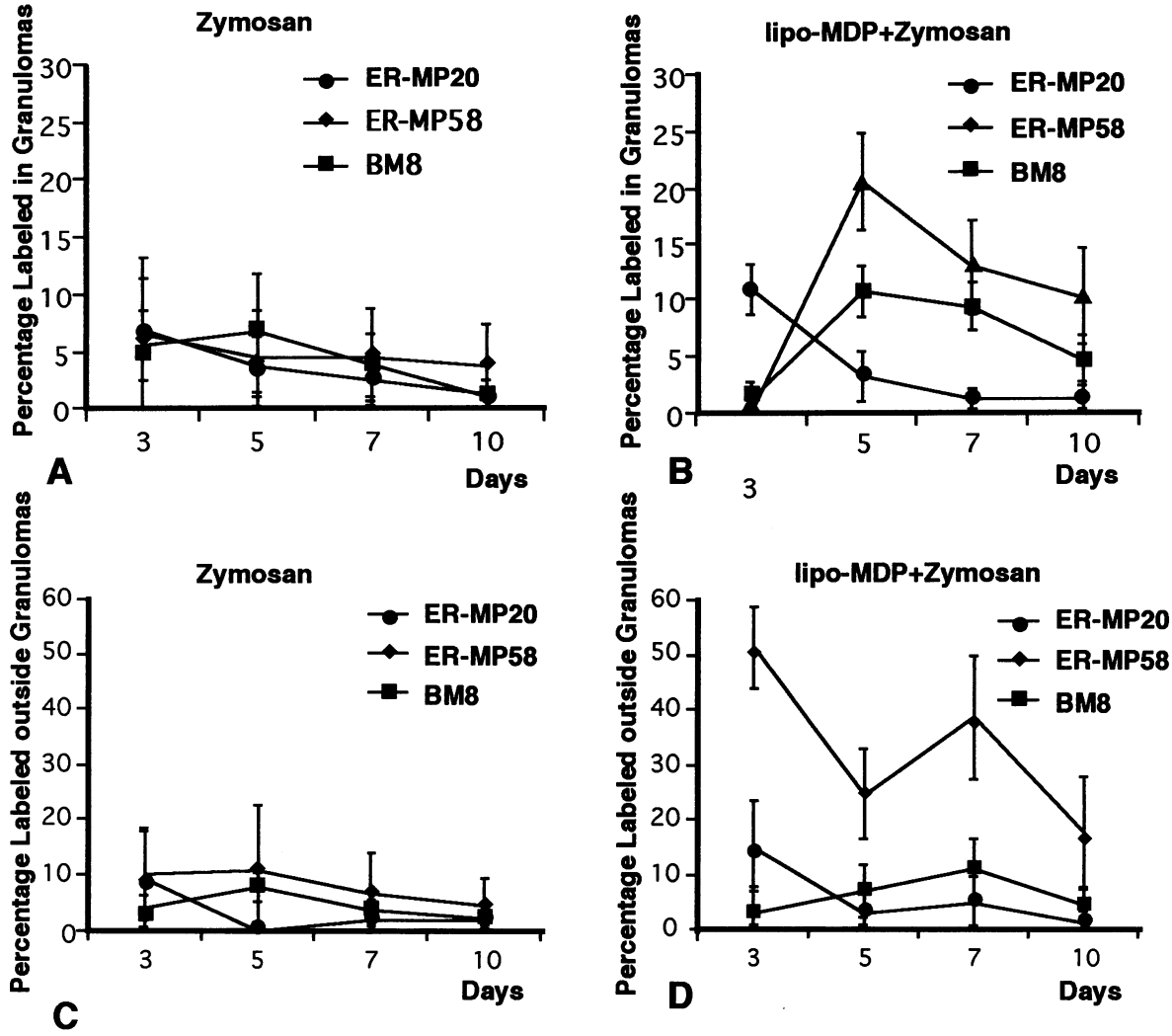


Figure 7. *[$^3$H]Thymidine-labeling rate of each cell type in granulomas after zymosan injection in control mice* (A *and* C) *and Kupffer-cell-depleted mice* (B *and* D). A *and* B: *In granuloma.* C *and* D: *Outside of granulomas. Data are expressed as mean ± SD.*

cyte chemoattractant protein-1, interleukin-1, tumor necrosis factor-$\alpha$, and interferon-$\gamma$ mRNA were enhanced in control mice from 1 to 7 days after zymosan injection. In contrast, cytokine mRNA expression was almost completely suppressed in the liver of lipo-MDP-treated mice. However, M-CSF mRNA expression was increased from 1 to 14 days in lipo-MDP-treated mice as well as control mice (Figure 8).

## *Expression of M-CSF and* In Situ *Expression of M-CSF and c-*fms *mRNAs in the Liver of Lipo-MDP-Injected and Uninjected Mice*

Expression of M-CSF protein was not detected immunohistochemically in any cell type of the liver in normal mice. After zymosan injection in both groups of mice, a few M-CSF-expressing cells were sporadically found. These cells were mononuclear, uniformly small in size and round in shape, and scattered in the sinusoid and granulomas. M-CSF-

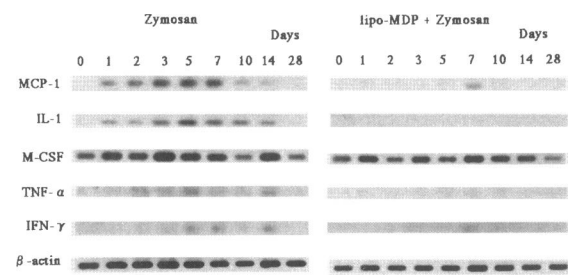


Figure 8. *Expression of cytokine and β-actin mRNA in the liver after zymosan injection in both groups of mice. Expression of cytokine mRNA other than that of M-CSF mRNA was suppressed in Kupffer-cell-depleted mice. RT-PCR.*

positive cells were less than 10% of M-CSF mRNA-expressing cells described below.

M-CSF mRNA expression was not detected in cells in and along the hepatic sinusoids in normal mice (Figure 9A). Three days after zymosan injection to control mice, several cells in the sinusoid showed unequivocally positive signals of M-CSF mRNA. M-CSF-positive cells were also found in the granulomas, but their number was smaller than that in the sinusoid (Figure 9C). In lipo-MDP-treated zymosan-injected mice, M-CSF expression was observed in a few macrophages mainly located outside the granuloma at day 5. A few M-CSF-expressing cells were also found in the granuloma after 7 days (Figure 9E). There were no multinucleated giant cells expressing M-CSF mRNA. Expression of M-CSF mRNA was not detected in endothelial cells or hepatocytes.

In untreated normal mice, c-*fms* mRNA was expressed in Kupffer cells (Figure 9B). After zymosan injection, cells expressing intense signals of c-*fms* mRNA were abundantly found in the sinusoid and in the granuloma in control mice (Figure 9D). Expression of c-*fms* mRNA was prominent in Kupffer cells in the sinusoid and macrophages in the peripheral area of the granuloma. Cells expressing c-*fms* were also found in lipo-MDP-treated mice, but their number was smaller than that of control mice (Figure 9F). Epithelioid cells and multinucleated giant cells expressed lower levels of c-*fms* signals. The numbers of M-CSF- and c-*fms*-positive macrophages in lipo-MDP-treated mice were smaller than those in normal mice. The number of c-*fms*-expressing cells was larger than that of M-CSF-expressing cells. The number and the distribution of M-CSF- and c-*fms*-expressing cells almost paralleled those of BM8-positive macrophages in both groups of mice (data not shown).

### *Humoral Regulation of M-CSF in Zymosan-Induced Granulomatous Inflammation*

In normal untreated mice, the concentration of serum M-CSF ranged from 1559 to 1625 U/ml. After injecting zymosan in control mice, the M-CSF concentration was within the normal range. In lipo-MDP-treated mice, the M-CSF concentration was remarkably high at 1 day after zymosan injection and decreased gradually thereafter. In mice treated with only lipo-MDP, the M-CSF concentration was also higher but not so prominent as in lipo-MDP-treated zymosan-injected mice (Figure 10).

## *Discussion*

The present study demonstrated the expression of M-CSF in macrophages after zymosan injection. M-CSF-producing cells were Kupffer cells and monocyte-derived macrophages, both of which participated in granuloma formation. In our recent study, we demonstrated that lipo-MDP given intravenously was ingested by macrophages and killed them by inducing apoptosis.[38] Using this method, we depleted pre-existing Kupffer cells before zymosan injection to examine the role of Kupffer cells in zymosan-induced granuloma formation in the liver. As a result, we observed that the number and the size of the granulomas were reduced in Kupffer-cell-depleted mice, compared with those of untreated mice. This finding is similar to that of glucan-induced granuloma formation in the Kupffer-cell-deficient M-CSF null mutant *op/op* mice.[12] Granulomas in the Kupffer-cell-depleted mice were formed together with the repopulation of Kupffer cells. These data suggest that pre-existing Kupffer cells form a microenvironment important for macrophage differentiation and granuloma formation.

In the early stage of Kupffer cell repopulation, small immature cells appeared and proliferated in the liver of Kupffer-cell-depleted mice, and their ultrastructural and immunophenotypic features were different from monocytes or resident macrophages.[31] Immunohistochemical and autoradiographic studies showed that ER-MP58-positive cells were highly proliferating macrophage precursors corresponding to M-CSF-responsive granulocyte/macrophage colony-forming cells[28,29] and that most of the ER-MP20-positive cells were regarded as monocytes.[10] The present investigation with immunohistochemical double staining suggested that ER-MP58$^+$ and ER-MP20$^-$ cells became ER-MP58$^+$ and ER-MP20$^+$ cells, ER-MP20$^+$ and BM8$^+$ cells, and then ER-MP20$^-$ and BM8$^+$ cells in the granulomas as reported previously.[31] Although the possibility that these macrophage precursors are native residents in the liver is not ruled out, they are considered to be M-CSF-responsive granulocyte/macrophage colony-forming cells derived from bone marrow and essential precursors for hepatic granuloma formation in Kupffer-cell-depleted mice.

Development, proliferation, and differentiation of macrophages and their precursors appear to be closely associated with the expression of M-CSF. Consistent with the results of previous studies on glucan- or zymosan-induced granuloma formation,[12,21–23] the present study revealed that the proliferative capacity of macrophages was high during

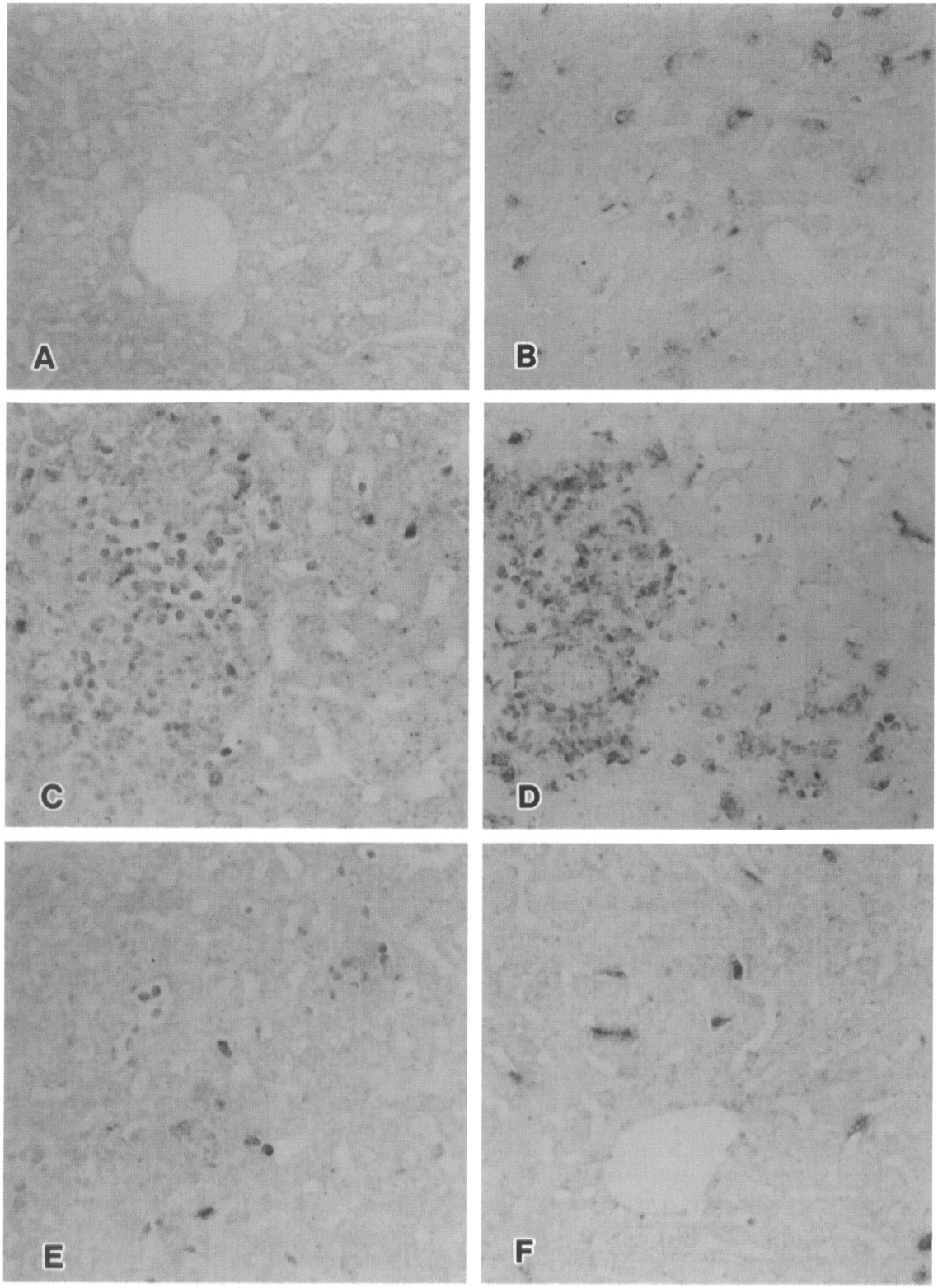


Figure 9. *M-CSF mRNA* (**A**, **C**, *and* **E**) *and c-*fms *mRNA* (**B**, **D**, *and* **F**) *expression by* in situ *hybridization.* **A**: *Signal positive cells are not detected in the hepatic sinusoids in normal mice.* **B**: *c-*fms *mRNA is expressed in Kupffer cells of a normal mouse.* **C**: *At 7 days after zymosan injection, M-CSF mRNA-expressing cells are distributed in the sinusoid and granulomas of a control mouse.* **D**: *Signal positive cells for c-*fms *mRNA located in the sinusoid and granulomas of a control mouse at 5 days after zymosan injection.* **E**: *Signal positive cells for M-CSF mRNA are few in a Kupffer-cell-depleted mouse at 7 days after zymosan injection.* **F**: *c-*fms *mRNA is expressed in a few macrophages in Kupffer-cell-depleted mice at 3 days after zymosan injection. Magnification, ×200.*

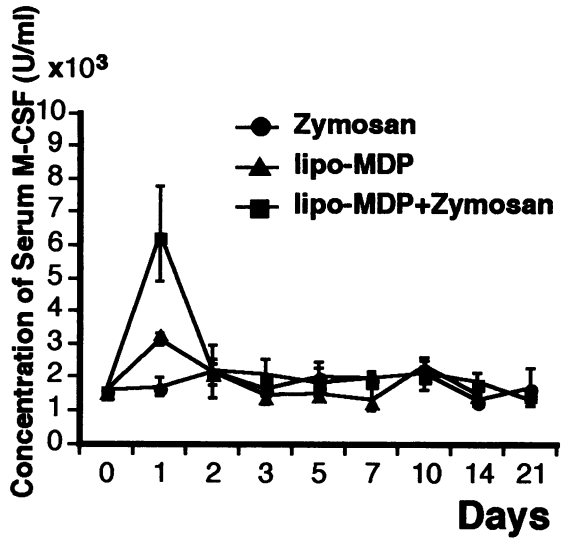


Figure 10. *Serum concentration of M-CSF in mice injected with zymosan, lipo-MDP, and both. Data are expressed as mean ± SD.*

hepatic granuloma formation. Our recent study demonstrated that macrophage precursors proliferated and that M-CSF mRNA expression was augmented during the period of Kupffer cell depletion.[31] In the current study, M-CSF mRNA expression was also enhanced after zymosan injection, and many M-CSF-producing macrophages were found in and outside the granulomas. As M-CSF is required for macrophages to enter the S phase of DNA synthesis,[39,40] it is postulated that the M-CSF served as a proliferation signal for macrophages and for macrophage precursors through autocrine and paracrine mechanisms.

Administration of lipo-MDP markedly suppressed expression of cytokine mRNAs, reflecting the fact that Kupffer cells and monocyte-derived macrophages are the main source of the cytokines produced in the inflamed liver. In contrast to the other cytokines, M-CSF mRNA expression was high after zymosan injection in lipo-MDP-treated and control mice. The present *in situ* hybridization confirmed M-CSF expression at cellular levels. Combining these observations that the expression of cytokines other than M-CSF was greatly suppressed in the liver of Kupffer-cell-depleted mice even in the stage of granuloma formation, M-CSF seems to be more important for granuloma formation than other proinflammatory cytokines examined in this study. This notion may be supported by the fact that M-CSF is both a chemotactic and chemokinetic agent for macrophages, but tumor necrosis factor-$\alpha$ is not.[41]

In this study, we examined the *in loco* production of M-CSF in the liver of mice injected with zymosan with or without lipo-MDP treatment. In general, organ production of M-CSF *in vivo* occurs at a low level under a normal condition,[42] and *in situ* hybridization hardly detects M-CSF mRNA expression in most organs in an unstimulated condition. However, we could clearly detect M-CSF mRNA expression in activated macrophages after zymosan administration. Previous studies reported the production of M-CSF and GM-CSF by hepatocytes and sinusoidal cells.[17,43] Our previous and present findings that M-CSF mRNA expression was detected in normal and Kupffer-cell-depleted livers by RT-PCR[31] and that serum M-CSF concentration was elevated after Kupffer cell depletion support the nonmacrophage source of M-CSF. Such a discrepancy on the source of M-CSF may suggest that macrophages are a major source of M-CSF and the other cytokines produced in inflammatory conditions and that hepatocytes and/or endothelial cells are largely responsible for M-CSF production in normal and Kupffer-cell-depleted conditions.

The M-CSF receptor, c-*fms*, is expressed predominantly in monocyte/macrophages, osteoclasts, and their precursors as well as trophoblasts.[33–35,44,45] M-CSF binds to the receptor, and c-*fms*-M-CSF complexes are rapidly internalized through clathrin-coated pits and targeted to lysosomal digestion.[46,47] In the monocyte/macrophages, receptor numbers increase with cellular maturation from a few hundred per cell on monocytes and their precursors to several thousand on activated macrophages.[48] The present study demonstrated that zymosan injection enhanced the production of M-CSF in monocyte/macrophages and increased the total number of c-*fms*-expressing cells. M-CSF and c-*fms* expressions were prominent in small mononuclear cells, especially outside the granuloma. Epithelioid cells and multinucleated giant cells expressed low levels of M-CSF and c-*fms*. These results suggest that levels of M-CSF and c-*fms* gene expression in macrophages are highest in the early stages of differentiation and decrease thereafter. Thus, Kupffer cells and monocyte-derived macrophages stimulate and recruit neighboring cells as well as themselves to form granulomas by producing and secreting M-CSF after c-*fms* activation.

M-CSF is present at biologically active concentration in the circulation and is maintained at a steady level in serum by c-*fms*-mediated endocytosis and degradation of M-CSF by macrophages.[42] During mouse pregnancy, the concentration of M-CSF increases in serum by only 1.4-fold[36] and by 1000-fold in the uterus,[32] perhaps because a large amount of M-CSF is produced by uterine epithelial cells, uterine

decidual cells, macrophages, embryonic cells, and trophoblasts.[32,34,49] More than 90% of circulating M-CSF is incorporated by macrophages in the liver and spleen.[44] In the present study, M-CSF concentration was stable after zymosan injection in control mice, although local production of M-CSF in the liver was apparently augmented. In contrast, 1 day after depleting Kupffer cells, serum M-CSF concentration was markedly elevated. These results clearly demonstrated that removal of Kupffer cells and splenic macrophages by lipo-MDP administration has resulted in much less use of M-CSF. Furthermore, the M-CSF concentration was much higher in lipo-MDP-treated mice after zymosan injection. The mechanism that caused such a marked elevation of serum M-CSF concentration in this group of mice is unknown. However, it is probable that zymosan stimulates lipo-MDP-resistant macrophages or other cell types to produce M-CSF. Because several forms of M-CSF exist, the soluble, cell-bound, and proteoglycan forms,[42] the significance of each M-CSF isoform in granuloma formation is an interesting subject to be clarified.

In summary, M-CSF and its receptor c-*fms* in macrophages are involved in zymosan-induced granuloma formation. Impaired granuloma formation and suppressed expression of cytokines by Kupffer cell depletion indicate that Kupffer cells play an important role in host defense mechanisms against inflammation in the initial stage.

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