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The Role of Macrophage Colony-Stimulating Factor in Hepatic Glucan-Induced Granuloma Formation in the Osteopetrosis Mutant Mouse Defective in the Production of Macrophage Colony-Stimulating Factor

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To elucidate the effects of macrophage colony-stimulating factor (M-CSF) on Kupffer cells and monocyte/macrophages in hepatic granuloma formation, we examined granulomas produced by glucan injection in the liver of osteopetrotic mice and littermates with or without M-CSF administration. In the osteopetrotic mice, monocytes were deficient in peripheral blood, and their number did not increase after glucan injection. Hepatic granulomas were formed in the osteopetrotic mice by glucan injection without a supply of blood monocytes. During this process, M-CSF-independent Kuppfer cells proliferated, particularly before the granuloma formation, clustered in the hepatic sinusoid, and transformed into epithelioid cells and multinuclear giant cells. In the M-CSF-treated osteopetrotic mice, glucan injection induced an increase in the number of blood monocytes and formed hepatic granulomas at a nearly similar degree to that of littermate mice. Thus, it is concluded that neither monocytes nor M-CSF are necessary for granuloma formation. In contrast, Kupffer cells play a crucial role as granulomas develop in M-CSF-uninjected osteopetrotic mice. (Am J Pathol 1994, 144:1381–1392)

Based on the concept of a mononuclear phagocyte system, tissue (resident) macrophages are explained to be derived from blood monocytes in a normal steady-state that differentiate from monoblasts via promonocytes in bone marrow. Such monocytes migrate to sites of inflammation, accumulate within tissues, and transform into exudate macrophages, epithelioid cells, and multinuclear giant cells.[1–3] Macrophages are thought to be a short-lived, nondividing population under a normal steady-state condition and to die *in loco*.[2,3] This concept is based mainly upon the results obtained from various experimental studies, including administration of glucocorticoids in a massive dose,[4] radiation chimera,[5] or whole body irradiation with partial shielding of bones.[6] In contrast with this view, recent studies of ^{89}Sr administration[7] or with fractionated radiation[8] have suggested that resident macrophages survive by self-renewal, proliferate in response to stimuli, and participate in inflammation.[7,9–10] In our previous studies on severe monocytopenia induced by the administration of ^{89}Sr to mice, we found that Kupffer cells were capable of surviving without an influx of monocytes. Such cells proliferated and differentiated into epithelioid cells and multinuclear giant cells in the liver after glucan injection.[9,10]

Mice homozygous for the autosomal recessive osteopetrosis (*op*) mutation are characterized by a generalized skeletal sclerosis due to a failure of bone resorption and remolding. Homozygotes (*op/op*) have a defect in osteoclast development and a deficiency in the number of monocytes and macrophages.[11–17] This mutant mouse lacks functional macrophage colony-stimulating factor (M-CSF) or CSF-1 activity resulting from a mutation in

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the coding region of the *Csfm* gene;[13] consequently, it has defective differentiation of monocytes into osteoclasts and macrophages.[12–18] Although monocyte-derived macrophages are known to be deficient in various tissues of *op/op* mice, we demonstrated the existence of immature tissue macrophages at many sites, suggesting that these M-CSF-independent macrophages are derived from granulocyte/macrophage-colony forming cells (GM-CFC) or earlier hematopoietic progenitors.[12] Such immature tissue macrophages are negative for peroxidase (PO) activity on ultrastructural PO cytochemistry.[12] Because the development of the monocytic cell series and its differentiation into macrophages are completely impaired in *op/op* mice,[12,13] this mutant is considered to serve as an animal model for investigating the differentiation and maturation of M-CSF-independent tissue macrophages in a deficient condition of monocytes and monocyte-derived macrophages. Inversely, granuloma formation in *op/op* mice supplemented with M-CSF also provides evidence of the role of M-CSF in chronic granulomatous inflammation.

Using this mouse model, the present study elucidates the behavior and kinetics of M-CSF-independent Kupffer cells, their proliferation and maturation by M-CSF administration, and the role of M-CSF in glucan-induced granuloma formation.

Materials and Methods

Animals

(C57BL/6J × C3HeB/FeJ)F_2-*op/op* mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under routine conditions at the Laboratory Animal Center of Kumamoto University School of Medicine. *Op/op* mice and their normal littermates were produced from the mating of +/*op* heterozygotes. The normal littermates (+/?) consisted of two out of three +/*op* and one out of three +/+; the two genotypes are phenotypically indistinguishable. The animals were grouped into pairs of *op/op* mice and normal littermates. At 6 weeks after birth, 0.1 mg/g body weight of particulate glucan[19] was injected intravenously into a group of *op/op* mice and normal littermates. A second group of *op/op* mice and normal littermates were injected subcutaneously with 5 μg of recombinant human M-CSF twice a day for 2 weeks. M-CSF was generously provided by Morinaga Milk Industry Co. Ltd. (Kanagawa, Japan). At 2 weeks of daily M-CSF administration, glucan at the same dose noted above was injected into *op/op* mice and normal littermates. These mice were also subsequently injected with M-CSF. The liver was removed at 2, 5, 8, and 10 days after glucan injection. Other tissues such as bone marrow, spleen, lymph nodes, lungs, or kidneys were excised for light microscopy. At each time point, three animals were killed with ether anesthesia.

In addition, *op/op* mice and littermate mice were injected subcutaneously with the same dose of recombinant human M-CSF and killed with anesthesia at 1 to 5, 7 and 14 days after M-CSF administration. The liver was removed from each animal and submitted to the following investigations.

Blood Cell Count

For cell counting, a small amount of blood was sampled from the tail vein of each animal, and 1,000 white blood cells were counted on a blood film to obtain a differential count.

Light Microscopy

The liver, bone marrow, spleen, lymph nodes, lungs, kidneys, and other tissues were fixed in 10% formaldehyde and embedded in paraffin. Four-μ-thick sections were prepared and stained with hematoxylin and eosin for routine light microscopic examination.

Immunohistochemistry

The liver was fixed for 12 hours at 4 C in periodate-lysine-paraformaldehyde solution, washed for 4 hours with phosphate buffer solution containing 10%, 15%, and 20% sucrose, embedded in OCT compound (Miles, Elkhart, IN), frozen in dry ice-acetone, and cut by a cryostat into 6-μ-thick sections. The liver was also frozen in liquid nitrogen, cut by a cryostat, and fixed in acetone for 5 minutes. After inhibition of endogenous peroxidase activity by the method of Isobe et al,[20] we performed immunohistochemistry using the anti-mouse macrophage monoclonal antibodies F4/80[21] and BM8[22] (BMA Biomedicals, August, Switzerland), anti-mouse monoclonal antibodies against macrophage precursors ER-MP20 (BMA Biomedicals) or Ia antigens M5/114,[23] anti-mouse T lymphocyte monoclonal antibody Thy1,2 (Becton Dickinson, Mountain View, CA), anti-mouse monoclonal antibody for B cell antigen B220 (6B2; a kind gift from Dr. I. Weissman, Stanford University, Palo Alto, CA), and anti-mouse monoclonal antibody against immunoglobulin M (LO-MM-9; Serotic, Oxford, UK). As a secondary antibody, we used anti-rat immunoglobulin-horseradish peroxidase-linked F(ab′)$_2$ fragment (Amersham, Amersham, UK). After

visualization with 3,3′-diaminobenzidine, nuclear staining with hematoxylin, and mounting with resin, the positive cells with nuclei per 1 mm^2 were counted with a light microscope. We selected the number of positive cells in 100 granulomas composed of more than 10 cells.

Electron Microscopy

The liver specimens were cut into small blocks and the blocks were fixed in 2.5% glutaraldehyde for 1 hour and postfixed in 1.0% osmium tetroxide for 2 hours. After dehydration in a graded series of ethanol, the blocks were processed through propylene oxide, and embedded in Epon 812 (E. Fullan, Inc., Latham, NY). Ultrathin sections were cut by an ultrotome Nova (LKB, Uppsala, Sweden), stained with uranyl acetate and lead citrate, and examined with a JEM 2000EX electron microscope (JEOL, Tokyo, Japan).

Ultrastructural PO Cytochemistry

For cytochemical demonstration of PO activity, the liver was perfused with 1.5% glutaraldehyde through the portal vein for 1 minute according to the method of Wisse.[24] After washing in 0.05 mol/L cacodylate buffer, the tissues were sliced into 50-μ-thick sections by a vibrotome (Lancer, St. Louis, MO) and then incubated according to the method of Graham and Karnovsky.[25] Ultrathin sections were cut, stained with lead citrate, and observed as above.

Autoradiography with [^{3}H]Thymidine

[^{3}H]Thymidine (specific activity, 90 to 135 Ci/mmole) was purchased from Amersham (Amersham, UK) and stored at 4 C. At 1 to 5, 6, 10, and 14 days of daily M-CSF administration and at 2, 5, 8, and 10 days after glucan injection, the mice were injected intravenously with [^{3}H]thymidine, 1.5 μCi/g body weight, and killed 60 minutes after pulse labeling. After immunohistochemical staining with F4/80 or BM8, slides were dipped in 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 positive grains on their nuclei above background were determined to be labeled.

Statistics

The significance of the data was evaluated by the Student's *t*-test.

Results

Changes in Number of White Blood Cells and Monocytes in Peripheral Blood of Op/op *Mice by Daily M-CSF Administration or Glucan Injection*

In *op/op* mice, the white blood cell count ranged from 2,000 to 3,000 per mm^3, and peripheral blood was deficient in monocytes. The number of granulocytes and lymphocytes in the mutant mice was about 60 to 70% of normal littermates. In *op/op* mice, monocyte counts were consistently about 25/mm^3 (Figure 1). Due to daily M-CSF administration, the number of monocytes in peripheral blood increased abruptly and peaked up to 1,300/mm^3 at 3 days. Afterward, it reduced to the level of the normal littermates (Figure 1A). After daily M-CSF administration, the number of monocytes in the normal littermates increased abruptly and peaked at 2 days. Thereafter, it decreased and was about 200/mm^3 at 7 days. From 7 days on, the numbers of monocytes in M-CSF-treated *op/op* mice remained nearly similar to those of the M-CSF-treated littermate mice.

The *op/op* mice treated with M-CSF for 2 weeks showed an increased number of white blood cells and monocytes up to the level of the normal littermates and of those injected daily with M-CSF as shown at day 0 in Figure 1B. After glucan injection, the number of white blood cells in normal littermate mice nearly doubled by day 5. At 2 days after injection, the number of neutrophils had more than doubled. Monocytes comprised 6 to 12% of white blood cells from day 2 to day 10 after injection, and the number of such cells increased remarkably up to more than 500/mm^3 at day 5. After glucan injection, similar leukocytosis and nearly similar increases in number of monocytes to the littermate mice with or without daily M-CSF injection were observed in these M-CSF-treated *op/op* mice (Figure 1B). In contrast, increases in number of monocytes in *op/op* mice after glucan injection were not marked, reached a plateau at 5 days, and remained at about 160 to 170/mm^3 thereafter. The statistically significant difference in number of monocytes between *op/op* mice and the other three groups of mice was confirmed (Figure 1B).

Changes in Number of Kupffer Cells in the Liver of Op/op *Mice because of Daily M-CSF Administration*

In normal littermates, F4/80-positive and BM8-positive Kupffer cells were found predominantly in the

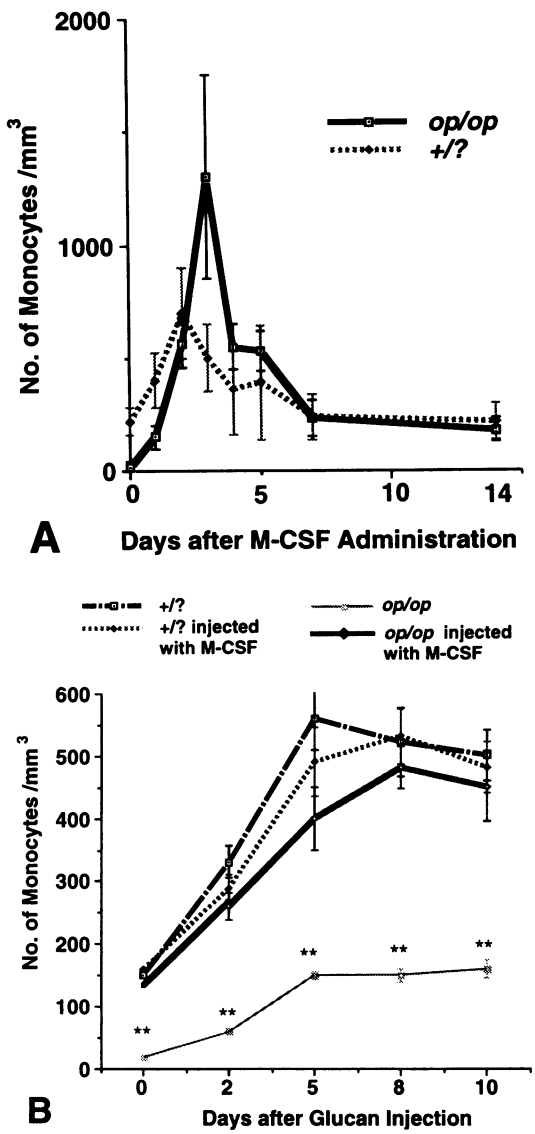


Figure 1. A: *Changes in number of monocytes in peripheral blood of* op/op *mice and +/? littermates after daily M-CSF administration. Data are representative of three mice.* B: *Number of monocytes in peripheral blood of M-CSF-treated and nontreated* op/op *mice and +/? littermates after glucan injection. ** The numbers of monocytes in unmanipulated* op/op *mice were fewer than those of the other groups of mice, which represents statistical significance* ($P < 0.01$ *by Student's* t*-test). Data are representative of three mice.*

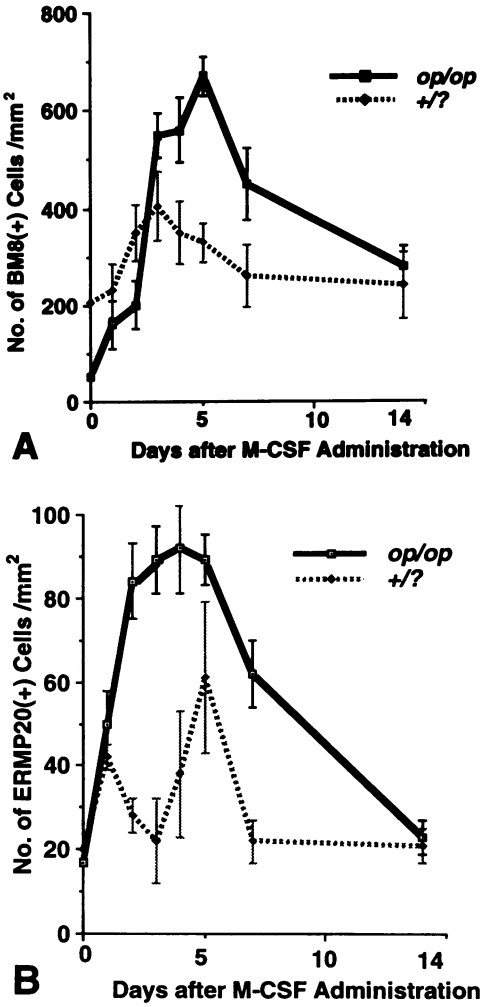


Figure 2. A: *Changes in number of Kupffer cells per 1-mm² section in the liver of* op/op *mice and +/? littermates after daily M-CSF administration. Data are representative of three mice.* B: *Changes in number of ER-MP20-positive monocytes per 1-mm² section in the liver of* op/op *and +/? littermates after daily M-CSF administration. Data are representative of three mice.*

peripheral zone of the hepatic lobules. In *op/op* mice, the number of Kupffer cells was only 30% of normal littermates; their distribution was irregular in the hepatic lobules. An account of daily M-CSF administration, the number of Kupffer cells increased in the liver of the mutant mice, peaking at 3 to 5 days and decreasing thereafter, but maintaining the level of normal littermates (Figure 2A); their intrahepatic distribution was also similar to that of the normal littermates. After M-CSF administration, increases in number of Kupffer cells in the normal littermates were slight compared with those of M-CSF-treated *op/op* mice and returned to the baseline level of normal littermates about 200 per 1 mm² from 2 weeks on. In *op/op* mice, monocytes were rarely found in the hepatic sinusoids, but after M-CSF administration, ER-MP20-positive cells increased in the sinusoids, peaked at 5 days, and decreased thereafter (Figure 2B). Judging from their size and shape, most of the ER-MP20-positive cells were thought to be monocytes.

Granuloma Formation in the Liver of Op/op *Mice and Littermates after Glucan Injection*

In normal littermates, the number of F4/80-positive cells increased 2 days after glucan injection, forming a few clusters together with macrophages, neutrophils, and lymphocytes and producing several small granulomas. Hepatic F4/80-positive cells within and outside of the liver granulomas increased until 10 days after glucan injection; the F4/80-positive cells occasionally showed mitosis, particularly outside the granulomas. The number of granulomas did not change after 8 days (Figure 3A), but they grew with time. Approximately 65% of the cells in the granulomas expressed the F4/80 or BM8 antigens by 2 days after glucan injection, and about 80% were positive by 5 to 10 days. The size of the granulomas increased with time, reaching 700 ± 400 μ in diameter 10 days after glucan injection. Macrophages transformed into epithelioid cells and multinuclear giant cells 5 days after injection (Figure 4A). In the littermate mice, few lymphocytes were found in the small granulomas 2 days after glucan injection. At 5 days, we detected lymphocytes in and around the granulomas, comprising about 15% of the granuloma-constituting cells. Most of these lymphocytes were T cells, as determined by the expression of Thy1,2 antigen, and they were situated in the periphery of the granulomas at 10 days after injection. B220- or immunoglobulin M-positive B lymphocytes were present in the granulomas only occasionally. No M5/114-positive cells were observed before glucan injection. After injection, they were scattered in and outside of the granulomas, and most were Kupffer cells or monocyte-derived macrophages.

In *op/op* mice, monocytes were rarely detected in the sinusoidal lumen before glucan injection. Even after injection, monocytes were rare in the liver, and no granulomas were detectable in the mutant mice 2 days later. Five days after injection, a few small Kupffer cell clusters were detected in the liver (Figure 4B), some in mitosis. Eight days after injection, a small number of irregularly outlined granulomas had formed in the liver; they were smaller than those in the normal littermates. Ten days after glucan injection, the granulomas were 600 ± 210 μ in diameter, consisting of epithelioid cells, multinuclear giant cells, and lymphocytes. The number of Kupffer cells outside the granulomas increased steadily immediately after glucan injection (Figure 3B) with F4/80- and BM8-positive cells comprising nearly 80% of the granuloma-constituting cells. T lymphocytes were present sporadically in the liver until 5 days; they comprised nearly 20% of the granuloma-constituting cells. The proportion of T cells in the granulomas of the *op/op* mice was similar to that of the +/? littermates. Only a few immunoglobulin M- or B220-positive B lymphocytes were dispersed in a few granulomas. M5/114-positive cells appeared 5 days after glucan injection; most were Kupffer cells.

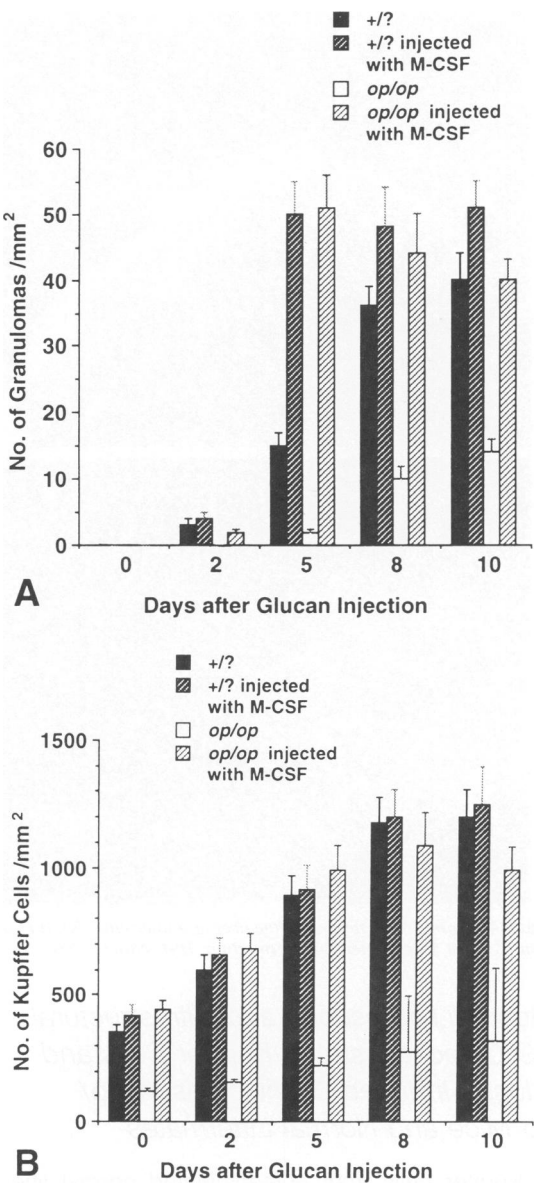


Figure 3. **A:** *Number of granulomas per 1-mm² section in the liver of M-CSF-treated and nontreated* op/op *and littermate +/? mice after glucan injection. Data are representative of three mice.* **B:** *Number of Kupffer cells per 1-mm² section in the liver of M-CSF-treated and nontreated* op/op *and littermate +/? mice after glucan injection. Data are representative of three mice.*

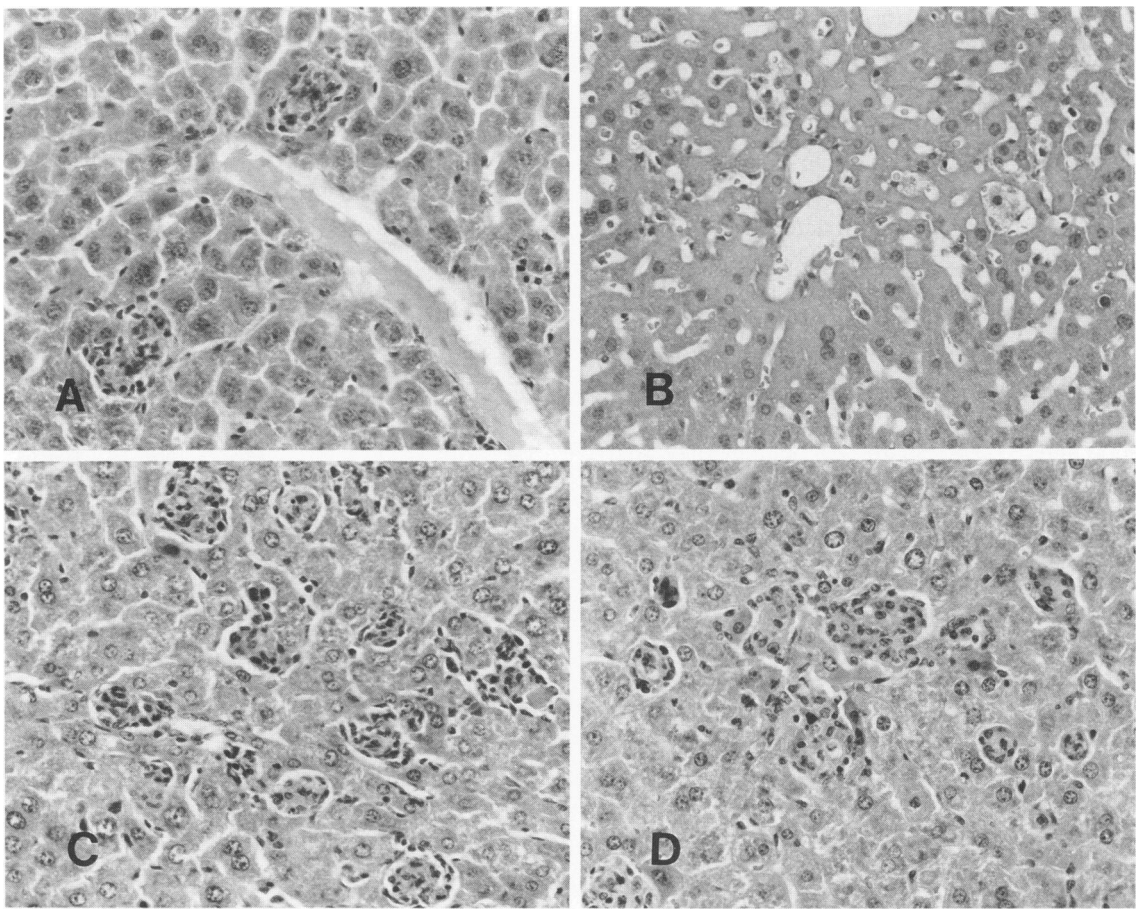
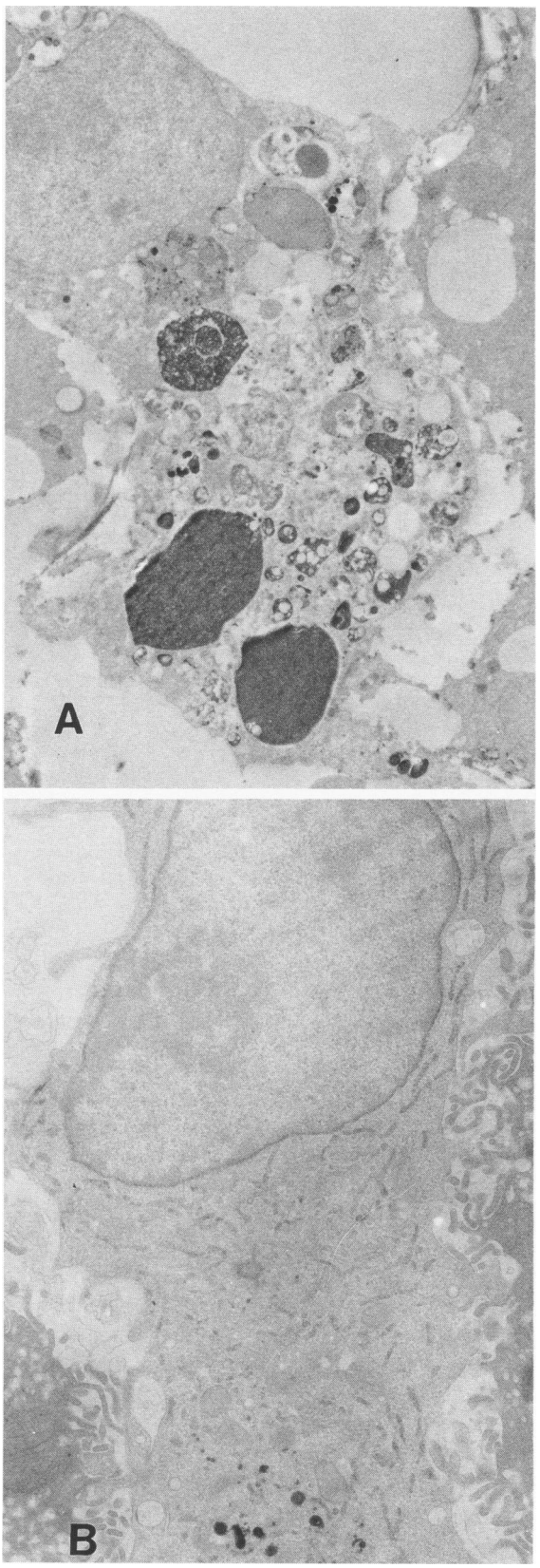


Figure 4. *Granulomas formed in the liver of a littermate* (A), *an* op/op *mouse* (B), *an M-CSF-treated littermate* (C), *and an M-CSF-treated* op/op *mouse* (D) *at 5 days after glucan injection. H&E stain* (×200).

Electron Microscopy and Ultrastructural PO Cytochemistry of Kupffer Cells and Glucan-Induced Granulomas in Op/op *Mice and Normal Littermates*

In Kupffer cells from unmanipulated normal littermates, PO activity was localized in the nuclear envelope and rough endoplasmic reticulum. The cells had well-developed intracellular organelles, including lysosomal compartments, and projected numerous cytoplasmic processes or microvilli. After glucan injection, many monocytes were observed adhering to the sinusoidal wall; neutrophils, lymphocytes, monocytes, and macrophages were also observed in the granulomas. From 2 to 5 days after glucan injection, monocytes and macrophages were clustered, forming granulomas. By ultrastructural PO cytochemistry in the granulomas, we detected various types of macrophages, including macrophages showing PO activity in the nuclear envelope and rough endoplasmic reticulum (resident macrophages or Kupffer cells), monocytes and monocyte-derived macrophages with PO-positive granules (exudate macrophages), PO-negative macrophages, and a few macrophages with PO-positive activity in the nuclear envelope, rough endoplasmic reticulum, and granules (exudate-resident macrophages). However, promonocytes were extremely rare. Five days after glucan injection, the number of PO-negative macrophages increased in the granulomas, followed by the appearance of epithelioid cells and multinuclear giant cells in the center and usually Kupffer cells in the periphery. Cytochemically, the epithelioid cells and multinuclear giant cells showed weak or little PO activity in the nuclear envelope and rough endoplasmic reticulum and had no PO-positive cytoplasmic granules.

In *op/op* mice, Kupffer cells were small and immature with poorly developed intracellular organelles, particularly the lysosomal compartments, though they often showed marked phagocytosis of blood cells or of electron-dense substances of various



sizes. These immature Kupffer cells possessed fewer cytoplasmic processes. By ultrastructural PO cytochemistry, they showed no PO activity in any intracellular organelles, unlike ordinary Kupffer cells in the normal littermates (Figure 5A). In the hepatic sinusoids, monocytes were not detected even after glucan injection, but the number of Kupffer cells increased, and they clustered to form a few small granulomas at 5 days after injection. These granulomas consisted of PO-negative macrophages, epithelioid cells, and multinuclear giant cells (Figure 6). The epithelioid cells and giant cells were situated in the center of the granulomas, with several PO-negative Kupffer cells around them. However, monocytes and exudate macrophages were absent.

Proliferative Potential of Kupffer Cells in Glucan-Induced Granuloma Formation of the Op/op *Mice and Normal Littermates*

In *op/op* mice, the labeling index of [^{3}H]thymidine in F4/80- or BM8-positive Kupffer cells was almost identical to that in the littermates (2 to 5%). Upon daily M-CSF administration, the labeling rate of the Kupffer cells in *op/op* mice was nearly 30% at day 1, peaked to 60% at day 2, and reduced to the baseline level of the normal littermates from day 7 on (Figure 7A). In the normal littermates, no significant increases in the labeling index of [^{3}H]thymidine in Kupffer cells were observed after daily M-CSF administration.

After glucan injection, F4/80- or BM8-positive cells in the normal littermates showed a marked uptake of [^{3}H]thymidine at 2 and 5 days. F4/80-positive cells outside the granulomas were particularly markedly labeled. In *op/op* mice, the [^{3}H]thymidine uptake of Kupffer cells was still low at day 2 after glucan injection, but a marked increase of the uptake was found in macrophages, particularly outside the granulomas (Figure 7B). In both the mutant and control animals, the [^{3}H]thymidine labeling index of epithelioid cells and multinuclear giant cells within the granulomas was low throughout the experimental period. At day 10 after glucan injection, the [^{3}H]thymidine labeling index of macrophages in hepatic granulomas returned nearly to the baseline levels of *op/op* mice before injection, but it was half of the maximum in the littermates.

Figure 5. *Ultrastructural and PO cytochemical findings of Kupffer cells in* op/op *mice with or without M-CSF administration.* **A:** *In* op/op *mouse, Kupffer cell reveals immature ultrastructure with prominent phagocytosis and is negative for PO activity* (×9,000). **B:** *After M-CSF administration, Kupffer cell becomes mature, showing localization of PO activity in the nuclear envelope and rough endoplasmic reticulum* (×9,000).

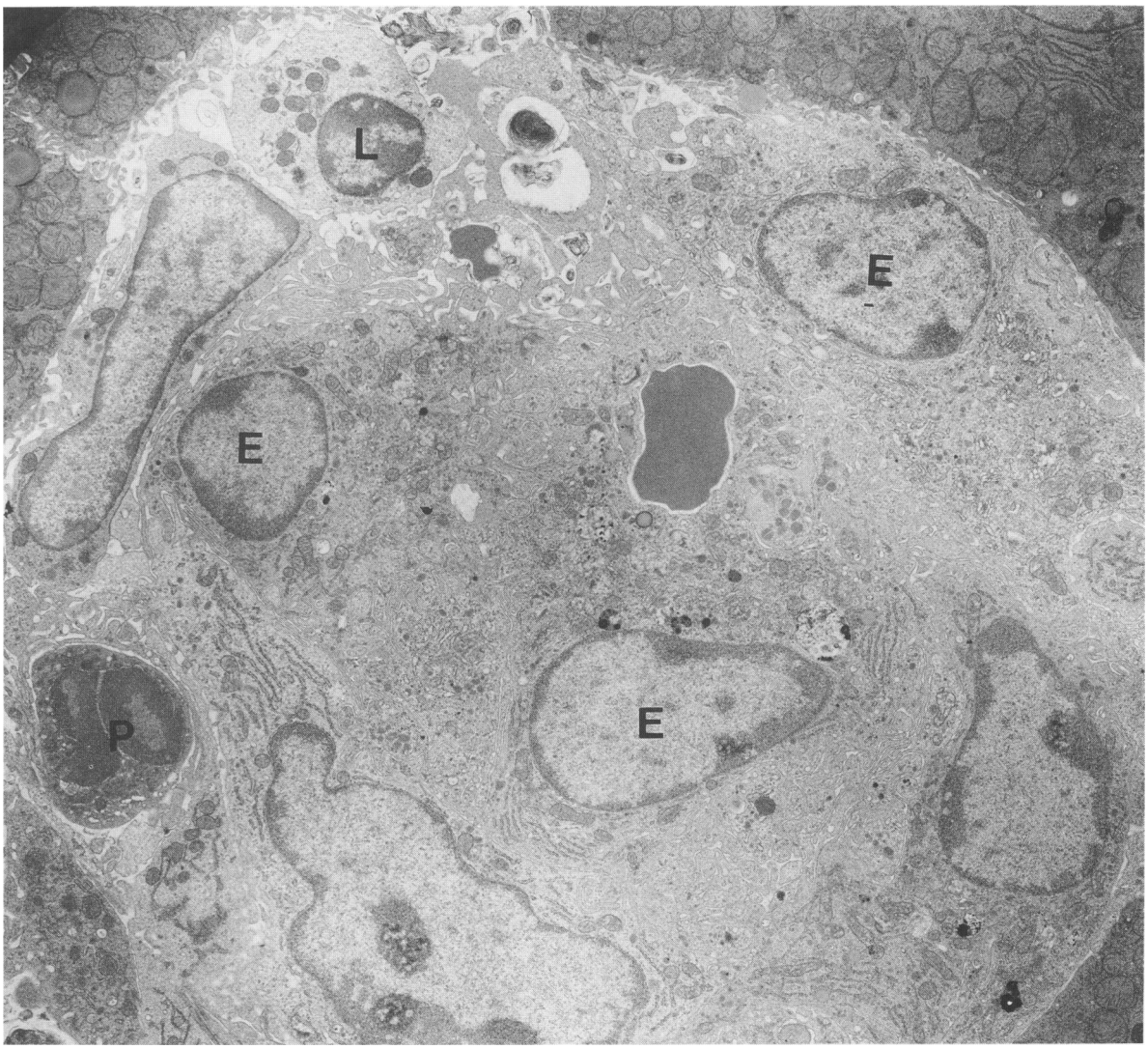


Figure 6. *Electron micrograph of a hepatic granuloma in an* op/op *mouse at 10 days after glucan injection.* **E:** *epithelioid cells,* **L:** *lymphocytes,* **P:** *polymorphonuclear leukocytes* (×5,000).

The Role of M-CSF in Kupffer Cells and Glucan-Induced Granuloma Formation of Op/op *Mice*

After daily M-CSF administration in *op/op* mice, the immature Kupffer cells started showing the cytoplasmic maturation accompanied by the emergence of PO activity in the nuclear envelope and rough endoplasmic reticulum (Figure 5B). This localization of PO activity was observed in nearly 50% of the Kupffer cells at day 1 of M-CSF administration and in more than 90% of the cells at day 2. These PO-positive Kupffer cells showed well-developed intracellular organelles and cytoplasmic projections similar to those of normal littermates. At 2 weeks of daily M-CSF administration, the number of monocytes in peripheral blood and the number of F4/80- or BM8-positive hepatic macrophages had increased markedly in the mutant mice. The number of F4/80-positive cells in the liver of *op/op* mice exceeded that of nontreated normal littermates. In *op/op* mice treated with M-CSF for 2 weeks, Kupffer cells possessed well-developed organelles and showed localization of PO activity in the nuclear envelope and rough endoplasmic reticulum as seen in the normal littermates. In contrast to nontreated normal littermates and *op/op* mice, such Kupffer cells in M-CSF-treated *op/op* mice were positive for M5/114.

In *op/op* mice and littermates injected with M-CSF for 2 weeks, the number of granulomas induced by glucan injection was greater than in nontreated normal littermates. Granuloma formation in the liver of

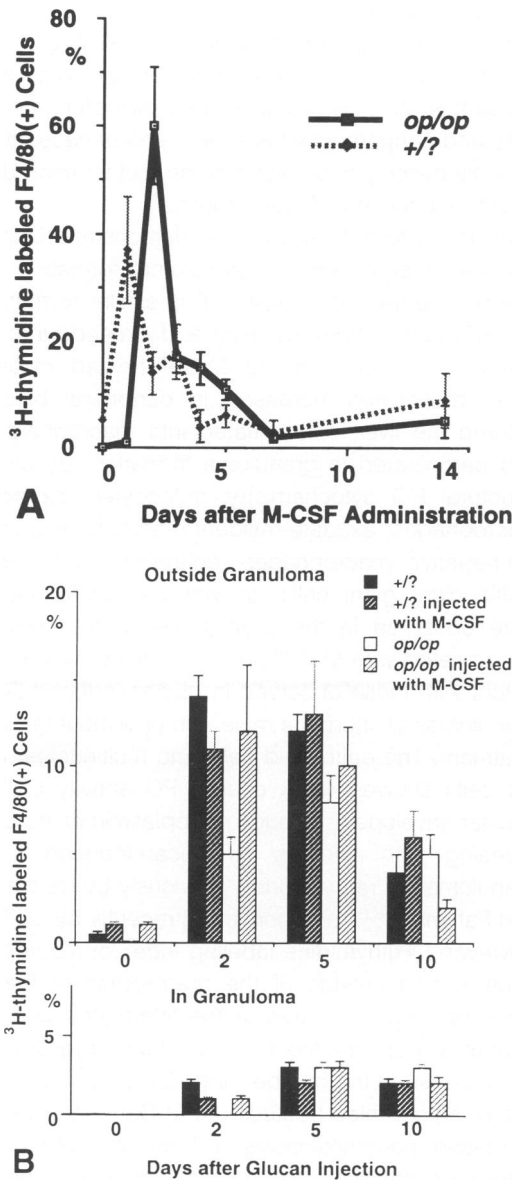


Figure 7. A: *[^{3}H]thymidine labeling rate of F4/80-positive Kupffer cells in* op/op *mice and normal littermates after M-CSF administration.* **B:** *[^{3}H]thymidine labeling rate of F4/80-positive macrophages within and outside of the granulomas in the liver of M-CSF-treated and nontreated* op/op *and +/? littermates after glucan injection. Data are representative of three mice.*

M-CSF-treated *op/op* mice and M-CSF-treated littermate mice was augmented, especially at 5 days after glucan injection (Figures 3A, 4, C, and D); monocytes were found frequently in granulomas of the early stage and differentiated into macrophages, epithelioid cells, and multinuclear giant cells from 5 to 10 days after injection. In this process, the PO-cytochemical findings of M-CSF-treated *op/op* mice were similar to those of nontreated normal littermates as described in the preceding section. The proportion of T and B lymphocytes in the granulomas of M-CSF-treated *op/op* mice and their littermates was almost identical to that of nontreated normal littermates. In the *op/op* mice injected with M-CSF for 2 weeks, most of the macrophages in and outside of the granulomas were positive for M5/114, and the [^{3}H]thymidine labeling index of macrophages in and outside of the granulomas was similar to that of normal littermates (Figure 7B).

Discussion

In agreement with previous studies,[12,26] the present study has revealed that immature Kupffer cells exist in the liver of the *op/op* mouse, though their number is markedly less than that of normal littermates. These Kupffer cells are considered to be a M-CSF-independent population of macrophages,[27] presumably a GM-CSF-dependent macrophage population, because GM-CSF is a major cytokine normally secreted in the *op/op* mouse[13] and because they closely resemble macrophages developed from hematopoietic cells of bone marrow in adult mice *in vitro* in the presence of GM-CSF (GM-CSF-derived macrophages).[28] In the mutant mouse, it is postulated that the GM-CSF-dependent Kupffer cells are derived from GM-CFC or earlier hematopoietic progenitors, but not from monocytes,[12] because monocytes are completely or almost completely absent from peripheral blood[11,12,16,17] and because differentiation of monocytes into macrophages is impaired.[12] In the present study, daily M-CSF administration increased the number of monocytes in peripheral blood and Kupffer cells in the liver of the *op/op* mice, both exceeding the level of normal littermates. Most of the Kupffer cells in the M-CSF-treated mutant mice and littermates showed Ia expression before glucan injection, unlike in the nontreated normal littermate mice. As for the increase of Kupffer cells in M-CSF-treated *op/op* mice, there are two possibilities; one is an influx of monocytes into the liver and their differentiation into macrophages *in loco*; the other is a local proliferation and maturation of immature Kupffer cells. Our present study revealed that the number of monocytes in the hepatic sinusoids of *op/op* mice peaked at 5 days after daily M-CSF administration, which was 2 days after the number of monocytes peaked in peripheral blood. Before the number of monocytes peaked, the preexisting immature Kupffer cells prominently proliferated. Most of them matured and developed intracellular organelles, particularly lysosomal compartments, projected cytoplasmic processes, and showed localization of PO activity identical to that of resident macrophages. These data indicate that

besides the development and differentiation of monocyte/macrophage lineage, M-CSF induces proliferation of M-CSF-independent immature Kupffer cells and their differentiation into mature resident Kupffer cells.

In the present study, granuloma formation was induced by glucan injection in the *op/op* mice. However, it was slower and the granulomas are smaller, fewer, and more irregularly shaped than in the littermates. The differences between the *op/op* mice and normal littermates in the number, size, and cell populations of the glucan-induced hepatic granulomas are quite similar to those between severely monocytopenic mice by ^{89}Sr administration and nontreated control mice.[9,10] These differences are thought to be due to the presence or absence of monocytes or their precursors, including M-CFC. In both monocytopenic animal models, the glucan-induced granulomas consisted of activated or Ia-positive macrophages, epithelioid cells, multinuclear giant cells, neutrophils, and lymphocytes, but not monocytes. In the *op/op* mice, M-CSF-independent immature Kupffer cells are considered to play a major role in the granuloma formation. This is because of the poor development of the monocytic cell series and the complete or nearly complete absence of blood monocytes, and because of the defective differentiation of monocytes into macrophages due principally to the total lack of functional M-CSF activity.[12] At the ultrastructural level, no participation of monocytes in the granuloma formation was detected in the *op/op* mice. There was, however, a marked increase of [^{3}H]thymidine uptake in the Kupffer cells of the mutant mice just before the initiation of granuloma formation, as confirmed in mice whose blood monocytes were severely depleted by ^{89}Sr administration.[9,10] These findings indicate that Kupffer cell proliferation is required for glucan-induced granuloma formation in both monocytopenic mouse models. Although the mechanism for the induction of Kupffer cell proliferation by glucan injection is still unknown, glucan injected *in vivo* induced increases in the number of GM-CFC and M-CFC in bone marrow[9,29] as well as increased extramedullary hepatic hematopoiesis,[30] suggesting that glucan stimulates the production of macrophage growth factors, especially M-CSF and GM-CSF. Besides, the other growth factors such as interleukin-1, interleukin-3, tumor necrosis factor, interferon-β, or T-cell-derived cytokines should be considered, because the hepatic sinusoidal cells produce them[31,32] and interleukin-3 and interferon-β are known to be important in granuloma formation.[33,34] Among these growth factors, GM-CSF is one of the most potent involved in macrophage proliferation during glucan-induced granuloma formation in *op/op* mice, because this factor is produced by liver parenchymal cells and nonparenchymal cells[35] and because it is the only major growth factor in the mutant mice deficient in functional M-CSF protein.[13]

In the present study, we demonstrated that glucan-induced hepatic granuloma formation in *op/op* mice treated with M-CSF is similar to that in M-CSF-treated littermate mice and exceeds that in normal littermates. In the M-CSF-treated mutant mice, monocytes increased in peripheral blood, entered the liver, differentiated into macrophages, and participated in granuloma formation. By ultrastructural PO cytochemistry, monocytes, exudate macrophages, exudate-resident macrophages, and PO-negative macrophages, epithelioid cells, and multinuclear giant cells, as well as Kupffer cells, were observed in the granulomas of the mutant mice treated with M-CSF. These findings were consistent with those observed in glucan-induced liver granulomas of littermate mice with or without M-CSF treatment. The epithelioid cells and multinuclear giant cells showed weak or little PO activity in the nuclear envelope and rough endoplasmic reticulum, agreeing with findings in glucan-induced liver granulomas of rats reported previously by Deimann and Fahimi[30,36,37] and confirmed recently by us.[9,10] However, [^{3}H]thymidine labeling index of macrophages in and outside of the granulomas of these mice was low, as it was in the littermates with or without M-CSF treatment. These data suggest that the increase in the number and size of granulomas in the mutant mice treated with M-CSF was due to increased monocytopoiesis and the influx of monocytes into the liver granulomas, not to enhanced proliferation of Kupffer cells.

In summary, M-CSF-independent Kupffer cells can produce granulomas in the liver of *op/op* mice after glucan injection without a supply of blood monocytes; they are activated, proliferate, and transform into epithelioid cells and multinuclear giant cells. In addition, the development and differentiation of the monocyte/macrophage population can be induced in the mutant mice by M-CSF treatment. This population also participates in the hepatic granuloma formation together with preexisting Kupffer cells. During these processes, M-CSF administration is considered to induce the production of the monocytic cell series, the appearance of monocytes in peripheral blood, and the differentiation of monocytes into macrophages, as

well as the proliferation of M-CSF-independent immature Kupffer cells in *op/op* mice.

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