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Effects of Macrophage Colony-Stimulating Factor on Macrophages and Their Related Cell Populations in the Osteopetrosis Mouse Defective in Production of Functional Macrophage Colony-Stimulating Factor Protein

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The development of macrophage populations in osteopetrosis (op) mutant mice defective in production of functional macrophage colony-stimulating factor (M-CSF) and the response of these cell populations to exogenous M-CSF were used to classify macrophages into four groups: 1) monocytes, monocyte-derived macrophages, and osteoclasts, 2) MOMA-1-positive macrophages, 3) ER-TR9-positive macrophages, and 4) immature tissue macrophages. Monocytes, monocyte-derived macrophages, osteoclasts in bone, microglia in brain, synovial A cells, and MOMA-1- or ER-TR9-positive macrophages were deficient in op/op mice. The former three populations expanded to normal levels in op/op mice after daily M-CSF administration, indicating that they are developed and differentiated due to the effect of M-CSF supplied humorally. In contrast, the other cells did not respond or very slightly responded to M-CSF, and their development seems due to either M-CSF produced in situ or expression of receptor for M-CSF. Macrophages present in tissues of the mutant mice were immature and appear to be regulated by either granulocyte/macrophage colony-stimulating factor and/or interleukin-3 produced in situ or receptor expression. Northern blot analysis revealed different expressions of GM-CSF and IL-3 mRNA in various tissues of the op/op mice. However, granulocyte/macrophage colony-stimulating factor and interleukin-3 in serum were not detected by enzyme-linked immunosorbent assay. The immature macrophages differentiated and matured into resident macrophages after M-CSF administration, and some of these cells proliferated in response to M-CSF. (Am J Pathol 1996, 149:559–574)

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.[1–3] Homozygotes (*op/op*) lack functional activity of macrophage colony-stimulating factor (M-CSF) or CSF-1, which results from a thymidine insertion in the coding region of the *Cfms* gene.[4] In the *op/op* mice, there is severe depletion of monocytes in the peripheral blood.[5] Production of macrophage colony-forming cells and monocytic cell series (monoblasts, promonocytes, and monocytes) is defective in bone marrow,[6–9] and monocyte-derived macrophage subpopulations and osteoclasts are deficient in tissues.[1–3,5,10–12] Our previous *in vitro* studies have demonstrated a severe disturbance in differentiation of monocytes into macrophages.[11] In various organs and tissues of *op/op* mice, however, immature macrophages are present, suggesting that these M-CSF-independent macro-

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phages are derived from granulocyte/macrophage colony-forming cells or earlier hematopoietic cells.[7,11] These immature macrophages are thought to be regulated by granulocyte/macrophage colony-stimulating factor (GM-CSF).[13] Administration of recombinant murine GM-CSF can induce increases in number of macrophages deficient in various tissues of *op/op* mice.[14] These macrophages are termed a GM-CSF-dependent macrophage population.

Administration of M-CSF into *op/op* mice can induce the development, differentiation, and maturation of osteoclasts in bones and of monocyte-derived macrophages in tissues[6–8,9,12,15–17] as well as the production of macrophage colony-forming cells and monocytic cell series in bone marrow. Recently, Cecchini et al[18] demonstrated that M-CSF administration is effective for the postnatal development and differentiation of some macrophage subpopulations in *op/op* mice when the treatment starts from 3 days after birth. However, the effects of M-CSF administration on the macrophage subpopulations and their related cells in various organs and tissues of adult *op/op* mice remain unclear. Little is known about the relationship between the development and differentiation of macrophage subpopulations and the effects of M-CSF administration on local production of other cytokines.

In this study, we utilized a panel of monoclonal antibodies for macrophages, their subpopulations, or macrophage precursors for immunohistochemistry to investigate the effects of M-CSF administration on these cells in *op/op* mice. In our previous studies of the effects of recombinant human M-CSF on the differentiation and maturation of Kupffer cells in the liver of *op/op* mice[19] or protein-deprived mice,[20] we found marked increases of Kupffer cells in the liver and monocytes in peripheral blood as well as the increased influx of monocytes into the liver in the early stage of daily M-CSF administration, particularly within a week after administration. However, their numbers were maintained at plateau levels similar to those of normal littermates from 1 week on after administration. Based on these data, we examined the development, differentiation, and maturation of macrophages and their subpopulations by immunohistochemistry and electron microscopy at the same time points as those of our previous studies[19,20] after daily administration of M-CSF into 4-week-old mice. The proliferative capacity of macrophages was examined with [^{3}H]thymidine autoradiography at the same time points. Because the previous studies showed a dramatic increase of blood monocytes in *op/op* mice or protein-deprived mice within a week after daily M-CSF administration,[19,20] we examined the changes of myeloid precursor cells in the bone marrow of *op/op* mice for the same period in this study. Using the enzyme-linked immunosorbent assay (ELISA) and Northern blot analysis, we examined serum levels of GM-CSF and interleukin-3 (IL-3) in peripheral blood and the expression on GM-CSF and IL-3 mRNA in various organs and tissues before or at 7 days after daily M-CSF administration. Furthermore, we examined the changes in number of monocytes in peripheral blood or of macrophages in various organs and tissues of *op/op* mice after cessation at 28 days of daily M-CSF administration compared with those of littermate mice.

Materials and Methods

Animals

(C57BL/6J × C3HeB/FeJ) F_2 *op/op* mice were obtained from the Jackson Laboratory and maintained under routine conditions at the Laboratory Animal Center, Kumamoto University School of Medicine. The *op/op* mice were produced from matings of +/*op* heterozygotes. Normal littermates (+/?) consisted of two-thirds +/*op* and one-third +/+. The +/*op* mice and +/+ mice imply heterozygous and homozygous normal offsprings, respectively; the former has a mutant *op* gene and the latter no mutant gene. However, the two genotypes are phenotypically indistinguishable. At 4 weeks after birth, *op/op* mice and normal littermates were injected subcutaneously with 5 μg of recombinant human M-CSF twice a day as described elsewhere[19,20] and killed at 1, 3, 5, 7, 14, 21, and 28 days after initial injection according to the time points in our previous studies.[19,20] This M-CSF was generously provided by Morinaga Milk Industry Co. (Kanagawa, Japan). Bone, bone marrow, liver, spleen, thymus, lymph nodes, intestines, heart, lungs, kidneys, brain, omentum, synovia, and other tissues were excised from the mice. After discontinuation at 28 days of daily M-CSF administration, the animals were killed at 3, 5, 7, 14, 21, and 28 days, and similar tissues were removed. At each time point, at least three mice were examined.

Cell Preparation

Peripheral blood was sampled from the retro-orbital sinus of the animals by puncture. Alveolar cells were obtained by bronchoalveolar lavage, and peritoneal cells were harvested from the peritoneal cavity of the mice after intraperitoneal injection of phosphate-buffered saline (PBS). Bone marrow tissues were

Table 1. *Immunoreactive and Antigen Specificities of Monoclonal Antibodies*

Monoclonal antibodies	Molecular mass (kd)	Isotype	Immunoreactive cells	Reference
F4/80	160	IgG2b	Promonocytes, monocytes, free or tissue-fixed macrophages, Kupffer cells, histiocytes, synovial A cells, microglia, phagocytes on the peri- and endosteal surfaces, and epidermal Langerhans cells	21–23
BM-8	125	IgG2a	Tissue-fixed macrophages and epidermal Langerhans cells	23, 24
MOMA-1	Unknown	IgG2a	Marginal metallophilic macrophages in spleen, certain macrophages in the medulla of thymus, lymph nodes, Peyer patches, and the intestinal villi	23, 25
ER-TR9	Unknown	IgM	Marginal zone macrophages in spleen and certain macrophages in lymph nodes	23, 26, 27
ER-MP20	14	IgG2a	Macrophage colony-forming cells, monoblasts, promonocytes, monocytes, and part of immature macrophages	23, 28, 29
ER-MP12	125–140k	IgG2a	Immature myeloid cells (granulocyte/macrophage colony-forming cells) and T cells	23, 28–32
ER-MP58	Unknown	IgM	M-CSF-responsive immature myeloid cells (granulocyte/macrophage colony-forming cells)	23, 28

obtained from the femoral bones of the animals by flushing with PBS. Bone marrow cell suspensions were prepared by repeated pipetting. Cytospin preparations were prepared from the peritoneal cells, bronchoalveolar lavaged cells, and bone marrow cell suspensions.

Blood Cell Count

A small amount of blood was sampled from the tail vein of each animal, and 1000 white blood cells were examined on a blood film to obtain a differential count.

Monoclonal Antibodies

For immunohistochemistry, the following anti-mouse monoclonal antibodies were purchased from BMA Biomedicals (August, Switzerland): F4/80,[21–23] BM8,[23,24] MOMA-1,[23,25] ER-TR9,[23,26,27] ER-MP20,[23,28,29] ER-MP58,[23,28] and ER-MP12.[23,28–32] Table 1 shows antigen specificities and reactive cells for these monoclonal antibodies.

For direct immunofluorescence staining by flow cytometry, fluorescein-isothiocyanate-conjugated ER-MP20 and biotinylated ER-MP12 were purchased from BMA Biomedicals. Phycoerythrin-conjugated streptavidin was purchased from Serotec (Oxford, UK).

Immunohistochemistry

Cytospin preparations were fixed with acetone at room temperature for 10 minutes. Tissue specimens were fixed with a periodate-lysine-paraformaldehyde fixative at 4°C for 6 hours, washed with PBS containing 10, 15, and 20% sucrose for 4 hours, and embedded in OCT compound (Miles, Elkhart, IN). The specimens were frozen in liquid nitrogen and cut into 5-μm-thick sections by a cryostat. After inhibition of endogenous peroxidase activity according to the method of Isobe et al,[33] cryostat sections were stained by the indirect immunoperoxidase method using anti-mouse monoclonal antibodies mentioned above. As secondary antibody, anti-rat immunoglobulin-horseradish-peroxidase-linked F(ab')$_2$ fragment (Amersham, Poole, UK) was used. After the visualization of peroxidase activity with 3,3'-diaminobenzidine, the sections were stained with hematoxylin and mounted with malinol. As negative control, the same procedures were performed without the primary monoclonal antibodies.

Electron Microscopy

The tissues were cut into small pieces of specimens, fixed in 2.5% glutaraldehyde for 1 hour, and post-fixed in 1.0% osmium tetroxide for 2 hours. After dehydration in a graded series of ethanols, the specimens were processed through propylene oxide and embedded in Epon 812 (E. Fullan, Lathan, NY). Ultrathin sections were cut by an ultrotome Nova (LKB, Uppsala, Sweden) and observed with a JEM-2000EX electron microscope (JEOL, Tokyo, Japan).

Autoradiography with [^{3}H]Thymidine

[^{3}H]Thymidine (specific activity, 185 to 740 GBq/mmol) was purchased from Amersham and stored at 4°C. At daily intervals from 1 to 7 days and 2, 3, and 4 weeks after daily M-CSF administration, the mice were injected intravenously with [^{3}H]thymidine (0.05

MBq/mg body weight) and killed by cervical dislocation after ether anesthesia 60 minutes after the pulse labeling. The liver, spleen, and some other tissues were also removed from the animals, and frozen sections were prepared as above for immunohistochemistry. These sections were stained by the indirect immunoperoxidase method as described above. Stained sections 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 grains on their nuclei above background were judged to be labeled. Percentages of the labeled immunoreactive cells in the total immunoreactive cells were calculated.

Immunofluorescence Staining and Flow Cytometry

The expression of macrophage precursor cell surface antigens was analyzed by flow cytometry as described previously.[29,34] Cells (1×10^6) were prepared from peripheral blood, stained directly with 50 μl of biotinylated ER-MP12 for 20 minutes on ice, and washed three times with PBS containing 5% fetal calf serum. Afterward, they were stained with phycoerythrin-conjugated streptavidin and fluorescein-isothiocyanate-conjugated ER-MP20 (20 μg/ml) for 20 minutes on ice and washed three times with 5% fetal calf serum/PBS. For analysis, FACScan (Becton Dickinson, Mountain View, CA) was used, and only the cells within a leukocyte gate were counted, as described previously.[28,34]

ELISA

Serum GM-CSF and IL-3 levels were measured with murine GM-CSF and IL-3 ELISA kits (Endogen, Boston, MA).

Northern Blot Analysis

Total cellular RNA was isolated from the liver, lungs, thymus, and brain of *op/op* mice and littermates before or at 7 days of daily M-CSF administration and examined by the acid guanidium phenol-chloroform method.[35] As controls, the NFSA murine fibrosarcoma cell line and the WEHI-3 cell line were used; the former is known to produce GM-CSF and M-CSF[36] and the latter produces IL-3.[37] Northern blotting hybridization was performed according to the method of Sambrook et al.[38] A 0.8-kb *Eco*RI fragment that covers the full length of GM-CSF cDNA was used as the GM-CSF probe (Genetics Institute, Cambridge, MA). The IL-3 cDNA probe was kindly provided by Dr. Toshio Suda, Department of Cell Differentiation, Institute for Medical Embryology and Genetics, Kumamoto University School of Medicine. A murine β-actin probe was used as the control (Genetics Institute). Briefly, the frozen sections were homogenized in 4 mol/L guanidine isothiocyanate, 20 mmol/L sodium citrate, and 0.5% *N*-lauroysarcosine solution. RNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated twice with isopropanol and ethanol with sodium acetate. Each RNA sample was applied at 11.2 μg/lane and electrophoresed in a 1% agarose gel containing 17% formaldehyde. The banded RNA was blotted onto nylon filters (Schleicher and Schuell, Dassel, Germany) by capillary blotting overnight in 20X standard saline citrate (1X SSC contains 0.15 mol/L NaCl, 0.015 mol/L sodium citrate). Mouse GM-CSF or IL-3 cDNAs were labeled to a specific activity of 10^9 cpm/mg with [^{32}P]dCTP (110,000 MBq/nmole; Amersham) using a random primer DNA labeling kit (Takara Shuzo Co., Kyoto, Japan). Hybridization was performed in 50% formamide, 4X SSC, 0.1% sodium dodecyl sulfate (SDS), 0.1% Denhardt's solution (2% Denhardt's solution contains 2% Ficoll, 2% bovine serum albumin (Sigma Chemical Co., St. Louis,MO), 2% polyvinylpyrrolidone), 10 μg/ml salmon sperm DNA (Sigma), and labeled DNA probes at 42°C. After they were washed in 2X SSC with 0.1% SDS at room temperature and in 0.2X SSC with 0.1% SDS at 56°C, the filters were exposed at −80°C using intensifying screens.

Cell Enumeration

Numbers of positive cells per 1 mm^2 were determined in various organs and tissues of *op/op* mice and littermates after daily M-CSF administration or after cessation at 28 days of daily M-CSF administration. Percentages of the positive cells in the *op/op* mice relative to those of the littermates were calculated. In the cytospin preparations, percentages of positive cells among total nucleated cells were calculated.

Statistics

The significance of differences between means was evaluated by the Mann-Whitney test.

Results

Effects of M-CSF Administration on Monocytes and Macrophage Precursor Cells in Peripheral Blood of op/op *Mice and Normal Littermates*

In the peripheral blood of 4-week-old *op/op* mice, total white blood cell counts ranged from 2000 to 3000 per mm^3, whereas the number of monocytes was less than 50 per mm^3 (Figure 1). Flow cytometric analysis revealed a minor population of ER-MP12-positive cells but no ER-MP20-positive cells (Figure 2). In the littermates, blood monocytes were approximately 200 per mm^3 (Figure 1) and both ER-MP12-positive cells and ER-MP20-positive cells were detected (Figure 2).

Daily M-CSF injection into 4-week-old *op/op* mice and normal littermates induced numerical increments of monocytes in peripheral blood, and their numbers peaked at 3 days after administration (Figure 1). From 5 days after administration on, the blood monocytes were maintained at the level of normal littermates. After 7 days, numbers of ER-MP12- and ER-MP20-positive cells increased markedly in peripheral blood of both *op/op* mice and littermate mice (Figure 2).

After discontinuation at 28 days of daily M-CSF administration, the number of monocytes in peripheral blood of the *op/op* mice decreased gradually with each day to the level of untreated mutant mice at approximately 1 week. In contrast, the monocyte number was maintained at normal levels in the littermate mice (Figure 1).

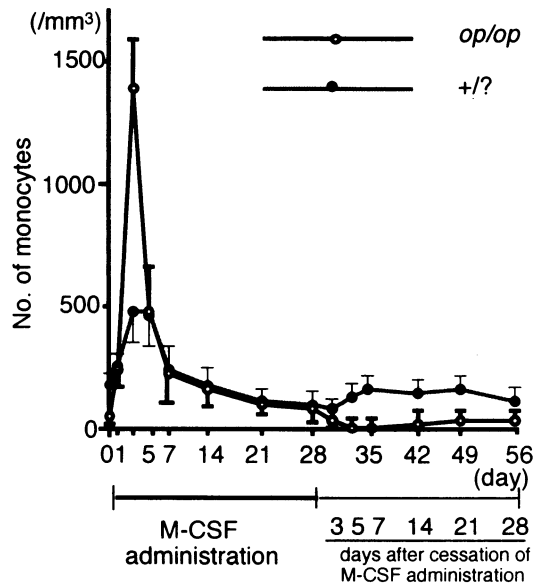

Figure 1. *Changes in number of monocytes in peripheral blood of* op/op *mice and normal littermates (+/?) after daily M-CSF administration and after discontinuation at 28 days of daily M-CSF administration.*

Effects of M-CSF Administration on Macrophage Subpopulations and Their Related Cells in op/op *Mice*

Figure 3 shows the changes in percentage of F4/80-positive macrophages in various organs and tissues of *op/op* mice compared with those of littermates after daily M-CSF administration. Compared with the littermates, the percentage of F4/80- or BM8-positive macrophages in the splenic red pulp of the *op/op* mice increased after daily M-CSF administration, peaked at 2 days, and reached a plateau thereafter (Figures 3 and 4, a and b). In the *op/op* mice, the percentage of F4/80- or BM8-positive macrophages in the liver increased after daily M-CSF administration, doubled at 5 days, and decreased to the level of normal littermates after 2 weeks (Figures 3 and 4, c and d). Compared with the littermates, the number of alveolar macrophages in the lungs of *op/op* mice showed no difference (Figures 3 and 4, e and f), a finding that was also confirmed by flow cytometric analysis of bronchoalveolar lavage cells (Table 2). Alveolar macrophage numbers were also unchanged statistically after daily M-CSF administration (Table 2 and Figure 3). Numbers of F4/80- or BM8-positive perivascular macrophages in the brain and medullary macrophages in the thymus of *op/op* mice were not reduced significantly, compared with the normal littermates. In contrast, F4/80-positive microglia in the brain of the *op/op* mice were not detected and did not appear after daily M-CSF administration. Compared with normal littermates, the numbers of F4/80-positive macrophages were markedly decreased in the uterus, ovaries, and synovial membrane of *op/op* mice and they did not respond or very slightly responded to daily M-CSF administration (data not shown). Macrophages in the bone marrow of the *op/op* mice were 40% of the normal littermates and their number increased slowly after daily M-CSF administration. The number of interstitial macrophages in the kidneys of the *op/op* mice was reduced to 20% of the normal littermates and did not increase after daily M-CSF administration. Compared with the normal littermates, flow cytometric analysis of peritoneal wash cells revealed a statistically significant reduction of peritoneal macrophages in *op/op* mice at 4 weeks of age ($P < 0.05$), and numbers of macrophages were increased at 7

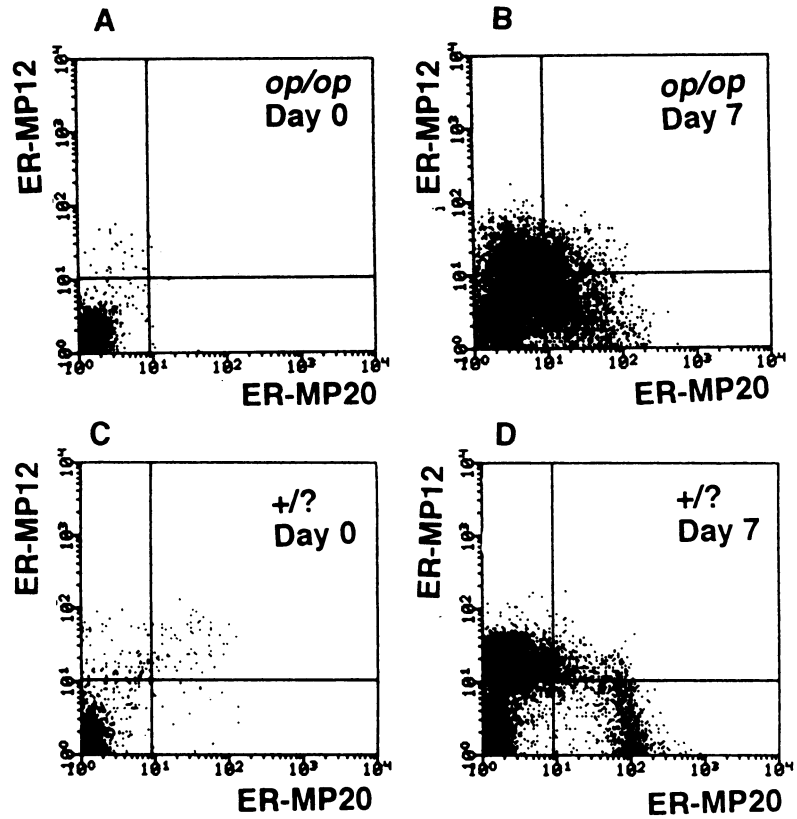

Figure 2. *Flow cytometric analysis of macrophage precursor cells in peripheral blood of* op/op *mice and littermates (+/?) before daily M-CSF administration* (A *and* C) *or at 7 days of M-CSF administration* (B *and* D). *Mouse monoclonal antibodies for macrophage precursors ER-MP12 and ER-MP20 were used.*

days after administration to the nearly same levels of littermates (Table 2).

Figure 5 shows the changes in percentage of F4/80-positive macrophages in various organs and tissues of *op/op* mice relative to those of the normal littermates after discontinuation at 28 days of daily M-CSF administration. Percentages of F4/80-positive macrophages in the liver sinusoids, in the red pulp of the spleen, and in the bone marrow of the *op/op* mice, which had increased to the level of normal littermates after daily M-CSF administration, were reduced abruptly by a week after cessation and slightly thereafter. Similar changes of macrophages were found in the lymph nodes and other lymphoid tissues after M-CSF administration or its discontinuation (data not shown). However, the percentages of pulmonary alveolar macrophages, thymic or brain macrophages, and renal interstitial macrophages were not changed after discontinuation.

In the spleen of *op/op* mice, MOMA-1-positive marginal metallophilic macrophages and ER-TR9-positive marginal zone macrophages were not detected by 2 weeks after administration (Figure 6, a and b). However, both macrophage subpopulations started appearing at low numbers from 3 weeks after administration on, although numbers of these cells were less than 10% of those of littermate mice by 4 weeks after administration. In the normal littermate mice, MOMA-1-positive macrophages were found in the innermost part of the marginal zone in the white splenic pulp, thymic medulla, lymph nodes, and the

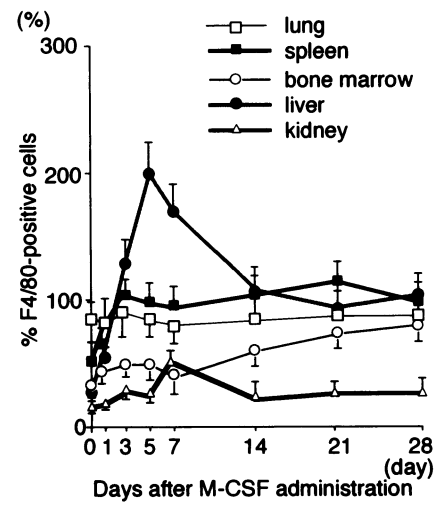

Figure 3. *Changes in percentage of F4/80-positive macrophages in various organs and tissues of* op/op *mice relative to those of normal littermates after daily M-CSF administration. Numbers of F4/80-positive cells were counted per 1 mm² of each organ and tissue of op/op mice and normal littermates, and percentages of the positive macrophages in op/op mice against those of normal littermates were calculated.*

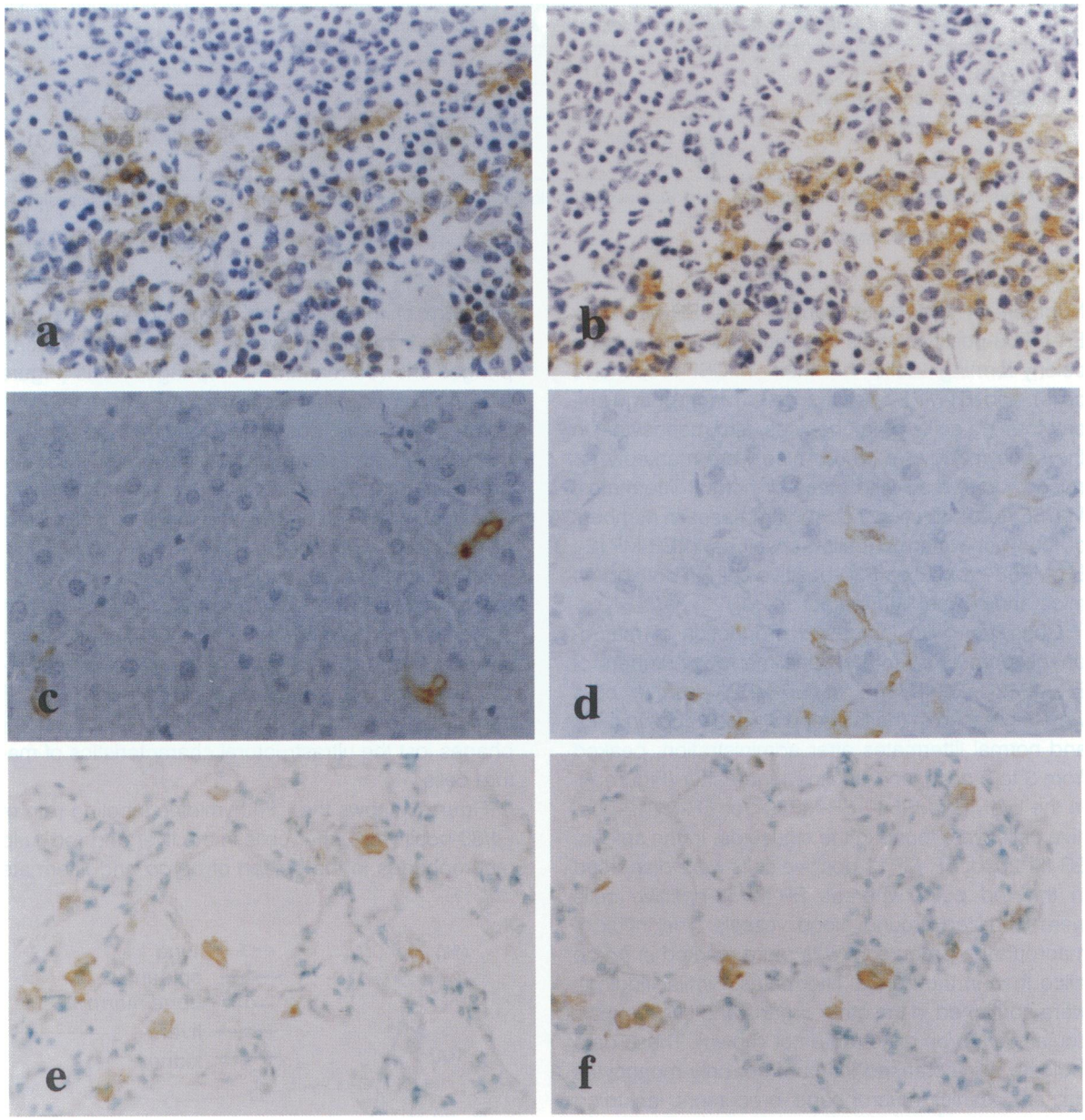

Figure 4. *Compared with untreated* op/op *mice* (**a** *and* **c**), *F4/80-positive cells increase in the red pulp of the spleen* (**b**) *and hepatic sinusoids* (**d**) *of* op/op *mice at 28 days of daily M-CSF administration. Numbers of alveolar macrophages in an untreated* op/op *mouse* (**e**) *are not reduced compared with a littermate* (**f**). *Indirect immunoperoxidase method with F4/80; magnification,* ×*300.*

other peripheral lymphoid tissues and in the uterus, ovaries, omentum, and synovial membrane (Figure 6, c–h); however, those macrophages were absent in any tissues of *op/op* mice and were not detected after M-CSF administration. In the thymic medulla and lymph nodes, ER-TR9-positive macrophages were found in the normal littermates, but none were detected in *op/op* mice and recovered after daily M-CSF administration.

Production of Macrophage Precursors in Bone Marrow and Their Influx into Tissues of op/op *Mice and Normal Littermates after M-CSF Administration*

Table 3 revealed the changes in number of total cells and macrophage precursors in the bone marrow of femurs from *op/op* mice and normal littermates before or within a week after M-CSF administration.

Table 2. *Changes in Percentages of F4/80-positive Macrophages in Peritoneal Wash Lavage and Bronchoalveolar Lavage of op/op Mice and Normal Littermates (+/?) after M-CSF Administration*

M-CSF administration (day)	PWL		BAL	
	op/op	+/?	*op/op*	+/?
0	4.25 ± 0.29	30.27 ± 1.92*	8.01 ± 2.46	8.33 ± 1.61
7	27.41 ± 2.25	32.31 ± 3.06	9.26 ± 1.96	7.90 ± 1.38

Numbers of F4/80-positive macrophages were determined by flow cytometry. PWL, peritoneal wash lavage; BAL, bronchoalveolar lavage.
* $P < 0.05$ *versus* PWL *op/op*.

Numbers of total bone marrow cells, ER-MP12- or ER-MP58-positive cells (granulocyte/macrophage colony-forming cells or their M-CSF-responsive cells), and ER-MP20-positive cells (macrophage colony-forming cells, promonocytes, and monocytes) in the bone marrow of *op/op* mice were markedly reduced, compared with those of normal littermates. M-CSF administration induced increases in numbers of total bone marrow cells as well as in ER-MP12-, ER-MP58-, and ER-MP20-positive cells in both *op/op* mice and normal littermates.

Consistent with increased production of macrophage precursors in the bone marrow, numbers of ER-MP12-, ER-MP58-, or ER-MP20-positive cells were increased in the liver and spleen of *op/op* mice and normal littermates after administration, peaked from 3 to 5 days, and declined thereafter (Figure 7). In the liver, ER-MP58-, ER-MP20-, or ER-MP12-positive cells were found in the sinusoids. In the spleen, ER-MP20- or ER-MP12-positive cells were dispersed in the red pulp, whereas ER-MP58-positive cells were localized around blood vessels. The influx of macrophage precursors was more marked in *op/op* mice than in the normal littermates. Similar findings were confirmed in the other organs and tissues, particularly of *op/op* mice (data not shown). These data indicate the increased influx of not only monocytes but also earlier macrophage precursors including granulocyte/macrophage colony-forming cells into various organs and tissues, particularly in *op/op* mice.

Ultrastructural Differentiation and Maturation of Macrophage Subpopulations and Their Proliferation in op/op *Mice*

In various tissues of 4-week-old *op/op* mice, ultrastructural analysis of macrophages showed an immature phenotype characterized by round euchromatic nuclei, smooth and round cell shape, poor development of lysosomal compartments, rough endoplasmic recticulum, and Golgi apparatus, as well as a few short microvillous projections. The ultrastructure of macrophages in the splenic red pulp was also immature; however, these splenic macrophages showed marked hemophagocytosis (Figure 8a). In contrast, most macrophages were mature in normal littermates, possessed indented heterochromatic nuclei, and showed well developed lysosomal granules, phagocytic vacuoles, rough endoplasmic reticulum, Golgi apparatus, and marked microvillous or cytoplasmic projection.

Immediately after daily M-CSF administration, macrophages in the liver sinusoids, in the red pulp of spleen, and in the other tissues of *op/op* mice started showing maturation of intracellular organelles (Figure 8b). One week after discontinuation of M-CSF administration into *op/op* mice, almost all macrophages lost the ultrastructural characteristics of mature cells.

Figure 9 shows the [^{3}H]thymidine labeling rate of F4/80-positive Kupffer cells in the liver and red pulp macrophages in the spleen of *op/op* and littermate

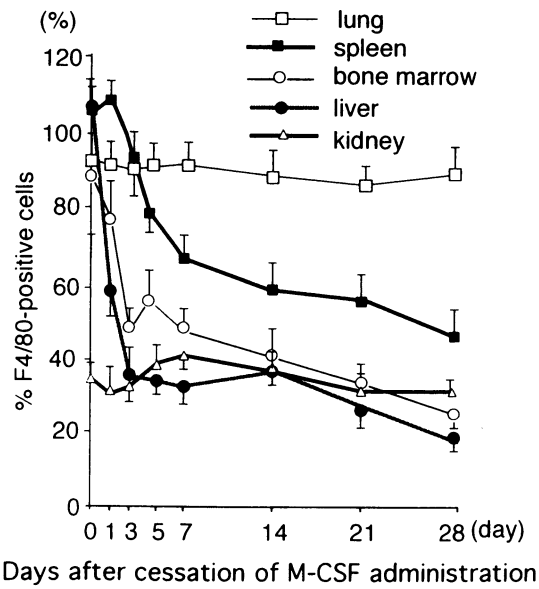

Figure 5. *Changes in percentage of F4/80-positive macrophages in various organs and tissues of* op/op *mice relative to those of normal littermates after discontinuation at 28 days of daily M-CSF administration. Percentages were calculated as in Figure 3.*

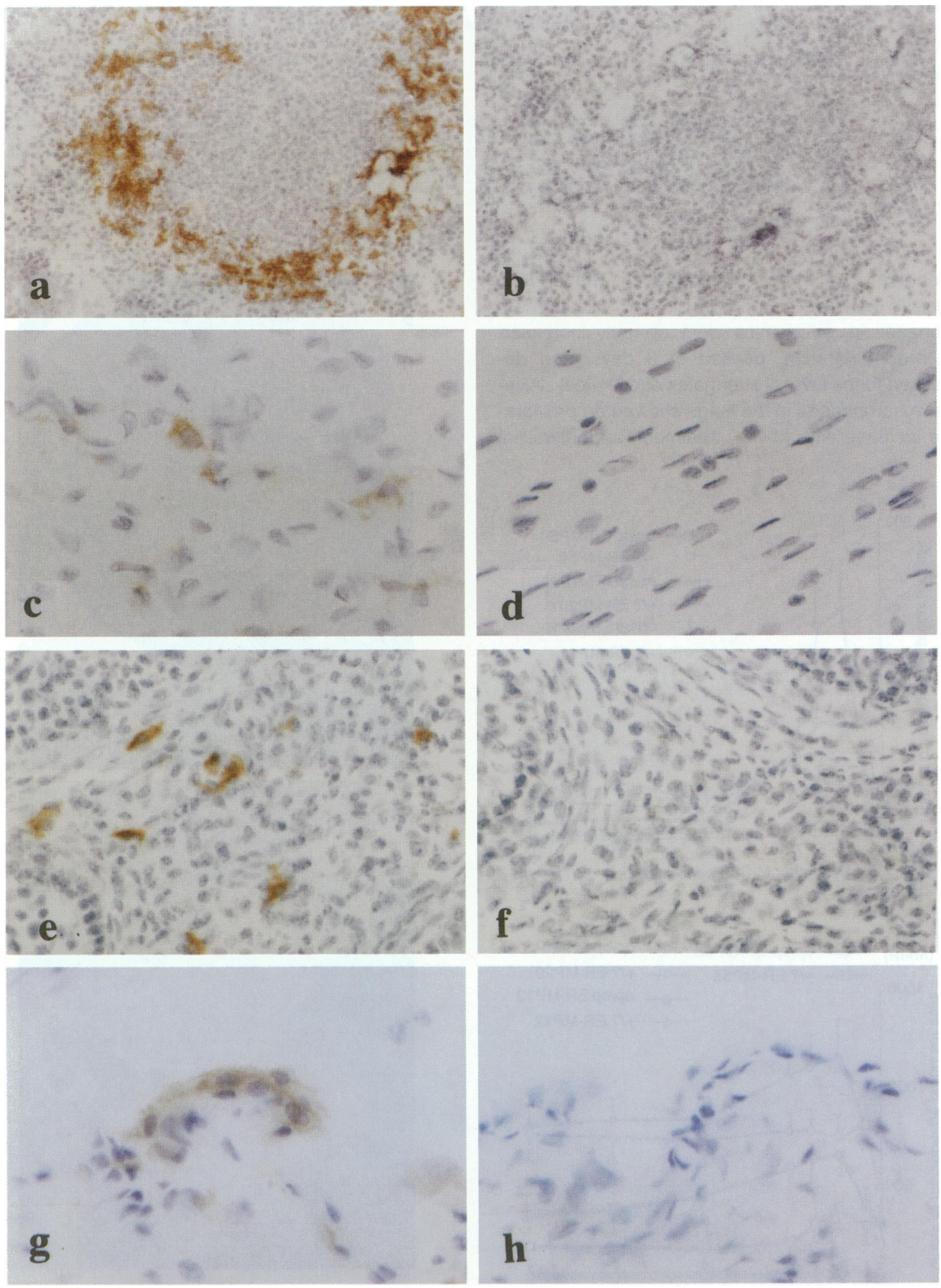

Figure 6. *MOMA-1-positive marginal metallophilic macrophages are well developed in the marginal zone of the white pulp in the spleen of a normal littermate mouse* (a) *and are absent in an* op/op *mouse* (b). *F4/80-positive macrophages are found in the omentum* (c), *ovary* (e), *and synovial membrane* (g) *of a normal littermate and are absent in an* op/op *mouse* (d, f, *and* h). *Indirect immunoperoxidase method with MOMA-1* (a *and* b) *or F4/80* (c *to* h); *magnification,* ×*300.*

Table 3. *Changes in Numbers of Total Bone Marrow Cells, ER-MP12-, ER-MP58-, or ER-MP20-Positive Cells in the Femurs of op/op Mice and Normal Littermates after M-CSF Administration*

M-CSF administration (day)	TBMCs ($\times 10^6$/femur)		ER-MP12 ($\times 10^4$/femur)		ER-MP58 ($\times 10^4$/femur)		ER-MP20 ($\times 10^4$/femur)	
	op/op	+/?	*op/op*	+/?	*op/op*	+/?	*op/op*	+/?
0	1.63 ± 0.12	10.04 ± 2.21	1.96 ± 0.45	14.93 ± 1.95	32.53 ± 4.21	127.68 ± 11.36	50.41 ± 3.96	231.72 ± 23.54
1	1.76 ± 0.22	11.37 ± 2.55	3.24 ± 0.63	18.99 ± 2.01	55.52 ± 6.35	233.08 ± 19.52	55.26 ± 4.75	325.18 ± 27.48
3	3.85 ± 0.28	13.14 ± 3.02	7.74 ± 0.71	22.86 ± 2.33	140.14 ± 12.55	337.69 ± 21.46	132.82 ± 10.35	413.91 ± 34.85
5	4.62 ± 0.33	14.55 ± 3.47	9.47 ± 0.82	23.57 ± 3.65	152.46 ± 14.02	276.45 ± 19.78	158.46 ± 10.86	404.49 ± 37.98
7	4.91 ± 0.23	15.30 ± 3.96	9.50 ± 0.73	25.70 ± 2.97	105.35 ± 10.85	250.92 ± 23.45	173.95 ± 11.65	446.72 ± 40.06

TBMC, total bone marrow cells.

mice after daily M-CSF administration. The labeling rates of the hepatic Kupffer cells and splenic macrophages in *op/op* mice were abruptly increased after administration, peaked at 2 days, and decreased to the level of littermates after 1 week. Alveolar macrophages in the lungs showed no proliferative increase after M-CSF treatment, and interstitial

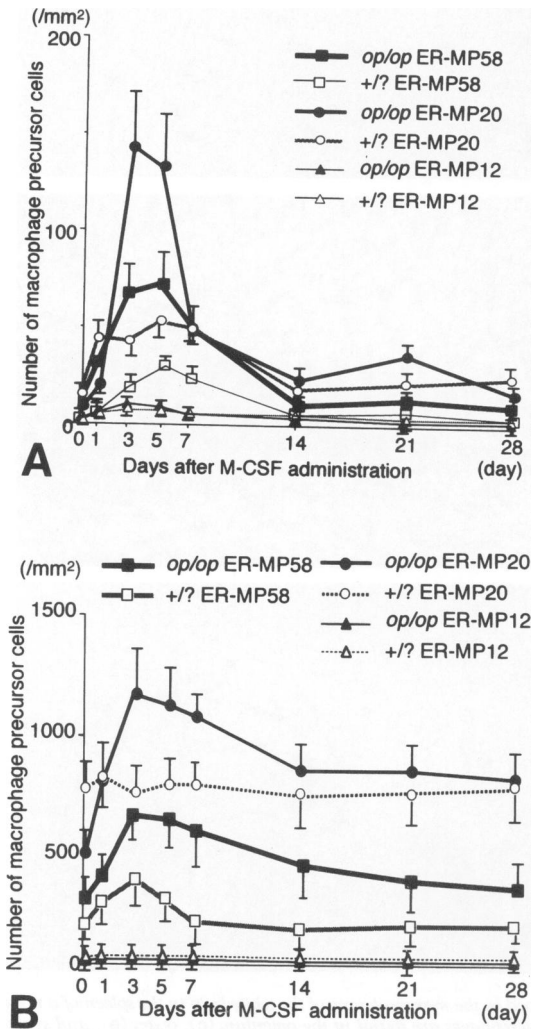

Figure 7. *Changes in number of ER-MP12-, ER-MP58-, or ER-MP20-positive cells in the liver* (A) *and spleen* (B) *of* op/op *mice and normal littermates* (+/?) *after daily M-CSF administration.*

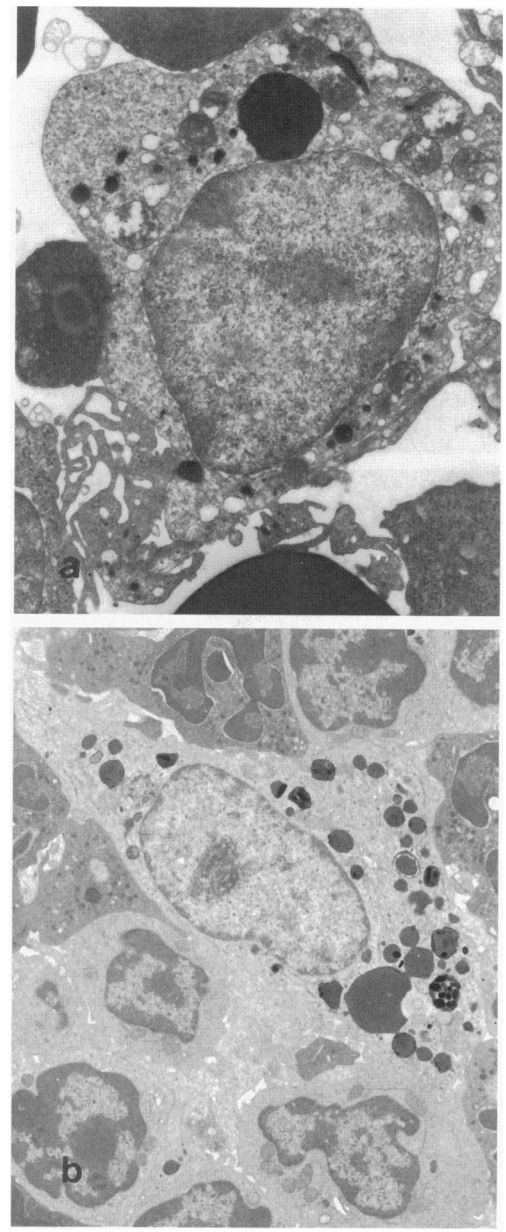

Figure 8. a: *Ultrastructure of a macrophage in the spleen red pulp in an untreated* op/op *mouse shows immature features with poor development of intracellular organelles and cytoplasmic projections. Hemophagocytosis is prominent.* b: *Macrophage in the splenic red pulp of an M-CSF-treated* op/op *mouse is mature, having well developed intracellular organelles and abundant cytoplasmic projections. Magnification,* ×7000 (a) *and* ×6000 (b).

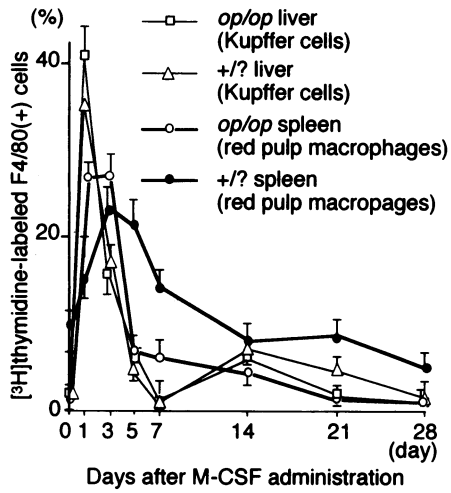

Figure 9. *Changes of the [3H]thymidine labeling rate in macrophages in the liver and spleen of op/op mice and normal littermates (+/?) after daily M-CSF administration. Percentages of labeled F4/80-positive macrophages in the total number of F4/80-positive macrophages were calculated.*

macrophages in the kidneys, synovial A cells, macrophages in the thymus, uterus, and ovaries of *op/op* mice also showed low labeling rates (data not shown).

Levels of GM-CSF and IL-3 in Serum and Expression of GM-CSF or IL-3 mRNA in Various Organs and Tissues of op/op *Mice*

By ELISA, GM-CSF and IL-3 were not detected in serum of either *op/op* or normal littermate mice, except for low levels of IL-3 in two of three M-CSF-treated *op/op* mice (data not shown). Figure 10 shows the expression of GM-CSF and IL-3 mRNA in various tissues of *op/op* mice and littermates before

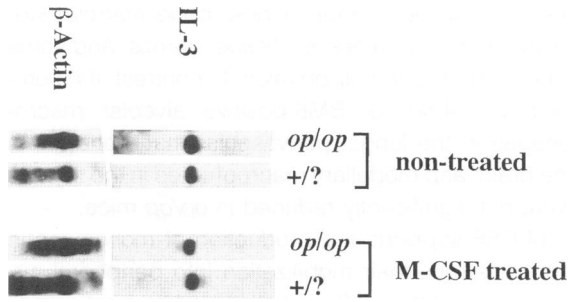

Figure 11. *Expression of IL-3 mRNA in the liver of untreated or M-CSF-treated* op/op *and littermate mice.*

or at 10 days of daily M-CSF administration. Similar to normal littermates, the thymus, brain, and lungs of *op/op* mice showed moderate expression of GM-CSF mRNA, and the liver showed low expression. All these tissues examined expressed IL-3 mRNA before or after daily M-CSF administration. As controls, similar expressions were confirmed in untreated or M-CSF-treated littermate mice. Figure 11 shows no difference in expression of IL-3 mRNA in the liver among untreated or M-CSF-treated *op/op* and littermate mice.

Discussion

The present investigation revealed deficiencies in numbers of monocytes in peripheral blood and monocyte-derived macrophage populations in various tissues, osteoclasts in the bone, and microglia in the brain of *op/op* mice. In addition, MOMA-1-positive and ER-TR9-positive macrophage subpopulations were deficient in the spleen and the other tissues of adult *op/op* mice. Although their numbers were reduced, there were immature macrophages in

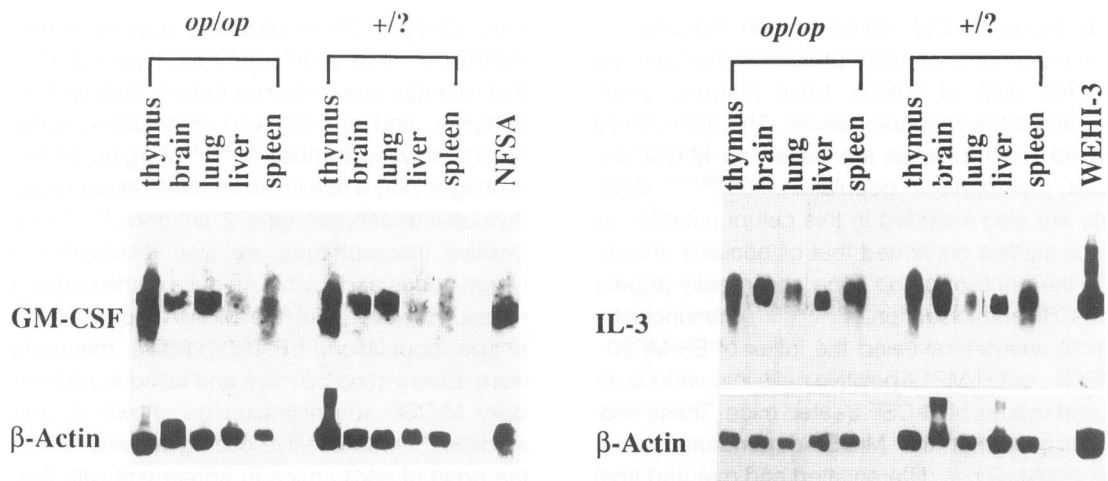

Figure 10. *Different expression of GM-CSF and IL-3 mRNA in various organs and tissues of* op/op *and normal littermate (+/?) mice before or at 7 days after daily M-CSF administration by Northern blot analysis.*

the liver, spleen, lymph nodes, bone marrow, kidneys, brain, subcutaneous tissue, dermis, and some other tissues of the *op/op* mice. In contrast, the numbers of F4/80- or BM8-positive alveolar macrophages in the lungs, perivascular macrophages in the brain, and medullary macrophages in the thymus were not significantly reduced in *op/op* mice.

M-CSF supports the production of monocytes in bone marrow, their mobilization into peripheral circulation, and their differentiation and maturation into macrophages in tissues as well as the survival of macrophages.[39,40] In addition, M-CSF regulates not only the final and intermediate stages of monocyte/macrophage differentiation but also the early stages of hematopoiesis.[41] The present study demonstrated that numbers of total bone marrow cells, ER-MP12- or ER-MP58-positive cells (granulocyte/macrophage colony-forming cells or their M-CSF-responsive cells), and ER-MP20-positive cells (monocytic cell series) in the femur of *op/op* mice were markedly reduced, compared with those of normal littermates. After daily administration of recombinant human M-CSF into *op/op* mice, marked production and expansion of ER-MP58- and ER-MP20-positive cells occurred in the bone marrow. Consistent with these changes in the bone marrow, the number of monocytes increased in peripheral blood, peaked at 3 days, and were maintained at the levels of normal littermates from 10 days of treatment on. In the M-CSF-treated mice, macrophage precursors such as ER-MP20-, ER-MP58-, or ER-MP12-positive cells were also increased in peripheral blood. After discontinuation of M-CSF administration, the monocyte number decreased in a few days and most of the ER-MP20- and ER-MP58-positive cells disappeared thereafter. Consistent with the increased numbers of monocytes and macrophage precursor cells in peripheral blood, M-CSF administration induced numerical increments of macrophages in the liver and in the red pulp of spleen, bone marrow, lymph nodes, and other lymphoid tissues. Therefore, these increased macrophages are called an M-CSF-dependent macrophage population.[11,19,39–44] Osteoclasts are also included in this cell population, as previous studies confirmed that osteoclasts are absent in the bone of *op/op* mice and rapidly appear after M-CSF administration.[6,8,9,12,15,17] Immunohistochemical analysis revealed the influx of ER-MP20-, ER-MP58-, or ER-MP12-positive cells into various organs and tissues of M-CSF-treated mice. These findings suggest that the M-CSF-dependent macrophage population is differentiated and matured from not only monocytes but also from their precursors including granulocyte/macrophage colony-forming cells in tissues by circulating M-CSF after daily administration of this cytokine.

In addition to severe deficiency of M-CSF-dependent and rapidly responsive macrophage populations, the numbers of marginal metallophilic macrophages and marginal zone macrophages in the white pulp of spleen, omental macrophages, macrophages in the uterus, ovaries, synovial membrane (synovial A cells), and microglia in brain were also severely depleted in *op/op* mice. In normal littermates, the marginal metallophilic macrophages are situated in the innermost border of the marginal zone of white splenic pulp,[45] express the sialoadhesin receptor specifically recognized by MOMA-1,[23,25] and are characterized by an intense histochemical staining for nonspecific esterase, staining affinity for silver,[46] and poor phagocytic activity.[47,48] Besides the marginal metallophilic macrophages in spleen, MOMA-1-positive macrophages were detected in the thymic medulla, lymph nodes, Peyer's patches, omentum, synovial membrane, endometrium, and ovaries of normal littermates. In the present study, MOMA-1-positive macrophages were completely absent in *op/op* mice, and their recovery after M-CSF administration was absent or extremely slow, in agreement with our previous data.[44] These data are inconsistent with those in a study of Cecchini et al[18] who reported that marginal metallophilic macrophages were completely recovered in *op/op* mice when daily M-CSF administration started from 3 days after birth. This discrepancy seems due to a difference at the starting point of M-CSF administration.

Marginal zone macrophages are dispersed throughout the marginal zone, particularly in its outer side.[26,27] These macrophages express ER-TR9 and possess a higher phagocytic activity than the splenic red pulp macrophages and marginal metallophilic macrophages. They possess a scavenger receptor (MARCO)[47] that is different from type 1 and type 2 macrophage scavenger receptors, take up bacterial antigens, and are involved in selective uptake of neutral polysaccharides.[28,49,50] Marginal zone macrophages play a role in the immune response against thymus-independent type 2 antigens.[49,51] ER-TR9-positive macrophages are also distributed in the lymph nodes and thymic medulla of the normal littermates. However, like the MOMA-1-positive macrophage population, ER-TR9-positive macrophages were absent in *op/op* mice and failed to appear after daily M-CSF administration as shown in previous studies.[44] F4/80-positive microglia were deficient in the brain of *op/op* mice in agreement with the findings of Cecchini et al in the retina of *op/op* mice.[18] These cells did not respond to M-CSF administration.

In adult *op/op* mice, MOMA-1- or ER-TR9-positive macrophage subpopulations and F4/80-positive microglia did not respond to exogenous M-CSF treatment, suggesting that these cells are regulated by either M-CSF locally produced in tissues, or other causes, such as lack of receptors for M-CSF, may be responsible. In additional studies, the expression of receptors for M-CSF on these macrophage subpopulations in the mutant mice should be clarified.

In *op/op* mice, certain macrophage populations are severely depleted, whereas other macrophage populations are distributed in normal numbers.[11] Compared with normal littermates, numbers of alveolar macrophages and perivascular macrophages were not reduced significantly in *op/op* mice, whereas numbers of Kupffer cells and macrophages in the renal interstitium, subcutaneous tissue, uterus, and ovaries were less than 30% of the normal littermates. These macrophages in *op/op* mice were small and round and showed immature ultrastructural features.[11] As previous studies demonstrated that monocytes in the peripheral blood of *op/op* mice were severely depleted[5] and that *in vitro* differentiation of monocytes into macrophages was completely impaired,[11] the immature macrophages are considered to be derived from macrophage precursor cells earlier than monocytes, promonocytes, and macrophage colony-forming cells. This notion is supported by the results of the present flow cytometric analysis showing the presence of ER-MP12-positive cells (granulocyte/macrophage colony-forming cells) and the absence of ER-MP20-positive cells (monocytes and their precursors) in peripheral blood of *op/op* mice.

To elucidate the differentiation mechanisms of immature macrophages in various tissues of *op/op* mice, we performed ELISA for quantitation of GM-CSF and IL-3 in serum and Northern blot analysis of GM-CSF and IL-3 mRNA in the tissues. Although GM-CSF and IL-3 were not detected in serum from *op/op* mice and littermates before or after daily M-CSF administration, expression of GM-CSF mRNA was normal in the lungs, thymus, and brain, whereas it was reduced in the other tissues such as the liver. In contrast, expression of IL-3 mRNA was found in all of these tissues of *op/op* mice and normal littermates. Therefore, the differentiation of macrophages from granulocyte/macrophage colony-forming cells in various tissues of *op/op* mice was correlated with the expression of GM-CSF mRNA in the tissues. This fact coincides well with the data in previous studies that alveolar macrophages in the lungs of normal mice are sustained with GM-CSF *in vitro*[52,53] and that they are reduced with age in GM-CSF-deficient mice, leading to the development of alveolar proteinosis after 6 months after birth.[54,55] A recent study also reported the development of alveolar proteinosis in IL-3 receptor β-chain knockout mice.[56] This disorder occurs presumably due to the cumulative effect of loss of GM-CSF and IL-5 stimulation,[56] because the receptors for IL-3, GM-CSF, and IL-5 commonly share a β-chain and because GM-CSF and IL-3 have overlapping activities[57,58] and interact with this chain.[59] Taking all these data together, it is suggested that alveolar macrophages are mainly sustained by GM-CSF. In brain, however, macrophages and microglia are considered to be regulated differently *in situ*. The present study demonstrated no significant difference in expression of GM-CSF and IL-3 mRNA in brain between *op/op* mice and normal littermates. In *op/op* mice, brain macrophages were normally developed, whereas microglia in brain were deficient. These data suggest that the microglia do not respond to these colony-stimulating factors in the brain of *op/op* mice *in vivo*. These combined findings provide evidence that the differentiation and maintenance of immature macrophages in various tissues of *op/op* mice are regulated by either GM-CSF and/or IL-3 produced locally in tissue or by other factors such as receptors for these growth or differentiation factors.

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