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Abnormal Differentiation of Tissue Macrophage Populations in 'Osteopetrosis' (op) Mice Defective in the Production of Macrophage Colony-stimulating Factor

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Examination of the* op/op *mouse disclosed marked reduction and abnormal differentiation of osteoclasts in the bones and of tissue-specific macrophages in various visceral organs and tissues. Most of these macrophages were immature as judged by ultrastructural criteria. In co-cultures of normal mouse bone marrow cells with fibroblast cell lines prepared from the lungs of the* op/op *mice, a defective differentiation of monocytes into macrophages was confirmed, supporting previous evidence that the fibroblast cell lines of the mutant mouse failed to produce functional macrophage colony-stimulating factor (M-CSF/CSF-1). In such co-cultures, however, a small number of macrophages apparently mature under the influence of granulocyte/macrophage colony-stimulating factor (GM-CSF) produced by the* op/op *fibroblast cell lines. In the mutant mice, the numbers of macrophages in the uterine wall and ovaries were severely reduced. Compared with the tissues of normal littermates, those of the mutants contained about 60% fewer macrophages in many tissues. This suggests that an M-CSF–independent population of macrophages is derived from granulocyte/macrophage–colony-forming cells (GM-CFC) or earlier hematopoietic progenitors. (Am J Pathol 1991, 139:657–667)

Macrophages are heterogeneous populations with respect to their morphologic, functional, and metabolic properties. Such heterogeneity reflects differences in tissue localization, differentiation, maturation, or activation, as macrophages differentiate and mature in response to cell-derived or environmental signals.[1,2] As one of the cell-derived soluble factors (cytokines), colony-stimulating factors (CSFs) preferentially act on specific cell lineages or function at different stages of the 'differentiative hierarchy.' Granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF)/CSF-1 directly regulate proliferation and differentiation of hematopoietic progenitors into macrophage populations at different stages.[3,4] According to the concept of the mononuclear phagocyte system (MPS) advocated by van Furth and his colleagues,[5–7] almost all macrophages, not only those distributed ubiquitously *in vivo* under a normal steady state condition, but also those exudating in inflammatory foci, are derived from blood monocytes that are differentiated through promonocytes from monoblasts originating in bone marrow. In contrast, recent *in vitro* studies with CSFs demonstrated that mature macrophages derived from bone marrow progenitors under the influence of either CSF-1 or GM-CSF differ morphologically and functionally,[2,8] suggesting that heterogeneity of mature macrophage populations might reflect the effects of different CSFs on bone marrow progenitors. The alternative differentiative pathways of such macrophage populations *in vivo*, however, have not yet been demonstrated.[9,10]

Osteopetrosis is an inherited metabolic bone disease of several animal species including mice, rats, rabbits, dogs, cows, and humans. This bone disease is charac-

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terized by a generalized skeletal sclerosis due to failure of bone resorption and remodeling by a functional defect of osteoclasts.[11] In mice, four distinct mutations—gray-lethal (*gl*), microphthalmia (*mi*), osteosclerotic (*oc*), and osteopetrotic (*op*)—are known to be transmitted by autosomal recessive traits.[12,13] Among these mutants, *op/op* mice can be distinguished from their normal littermates at 10 days after birth by the absence of incisors, a distinctly domed skull, short tail, and small body size.[14,15] In addition to a defect in bone remodeling due to marked reduction of functional osteoclasts, deficiencies of monocytes and peritoneal macrophages and reduced peritoneal macrophage function in the *op/op* mouse also have been observed by previous hematocytologic analysis[16] and by functional assessment.[17] In this mutant mouse, the developmental defect of the skeletal bones is not cured by transplantation of normal bone marrow cells or spleen cells,[15] suggesting a primary defect in the abnormal hematopoietic microenvironment but not in the hematopoietic stem cells. Recently the absence of functional CSF-1 in the *op/op* mouse was reported.[18–22] Although macrophage colony-stimulating factor gene (*Csfm*) messenger RNA was present at normal levels, the *op* mutation was demonstrated to be a mutation within the *Csfm* gene itself.[18] Thus the *op/op* mouse serves as a model for investigating the differentiation and maturation of macrophage populations under conditions of defective CSF-1 production.

This article describes the deficiencies of osteoclasts in the skeletal bones and reduction in numbers of macrophages in various tissues, as well as the immunohistochemical and ultrastructural properties of macrophage populations, in *op/op* mice. It also discusses the limited differentiation, maturation, and heterogeneity of macrophage populations in these mutant mice.

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 Medical School. The animals were grouped into pairs of *op/op* mice and normal littermates (+/?). Four-week-old male and female mice as well as 2- and 4-month-old mice were killed under ether anesthesia. Liver, spleen, bone marrow, lungs, ovaries, uterus, and other tissues were excised for further light and electron microscopic studies. For the immunohistochemical comparison of the uterus, seven C3H/He Slc female mice were used as controls.

Light Microscopy

The liver, spleen, bone marrow, kidneys, thymus, uterus, ovary, and certain other tissues were fixed in 10% formaldehyde and embedded in paraffin. Four-micron-thick sections were prepared and stained with hematoxylin and eosin for routine light-microscopic examination. The sections were stained also with toluidine blue and for tartrate-resistant acid phosphatase activity.[23]

Electron Microscopy

Samples of spleen, liver, thymus, and bone marrow were fixed in 2.5% glutaraldehyde for 1 hour and postfixed in 1.0% osmium tetroxide for 2 hours. After dehydration in graded series of ethanol, the specimens 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 electron microscope JEM-2000EX (JEOL, Tokyo, Japan)

Immunohistochemistry

Tissue samples were fixed for 4 hours at 4°C in periodate-lysin-paraformaldehyde solution, washed sequentially for 4 hours with phosphate buffer solutions 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.

After inhibition of endogenous peroxidase (PO) activity by the method of Isobe et al,[24] we performed immunohistochemistry using the anti-mouse panmacrophage monoclonal antibodies F4/80[25,26] and BM8.[27] F4/80 is expressed by promonocytes, monocytes, tissue-fixed macrophages, free macrophages of the body cavities, histiocytes, synovial A cells, Langerhans cells, microglia, and phagocytes on the periosteal and endosteal bone surfaces, but not by osteoclasts.[25,26] BM8 is expressed by tissue-fixed macrophages, epidermal Langerhans cells, inflammatory macrophages, and multinuclear giant cells, but it is absent from monocytes and earlier macrophage precursors.[27] As a secondary antibody, anti-rat Ig-horseradish PO-linked F(ab')2 fragment (Amersham, Amersham, UK) was used. After visualization with 3,3'-diaminobenzidine, nuclear counterstaining with hematoxylin, and mounting with resin, numbers of positive cells per square millimeter were enumerated in the liver, spleen, bone marrow, kidney, subcutaneous tissue, brain, ovary, and uterine wall of the *op/op* mice and normal littermates. In the femoral bones, we counted and

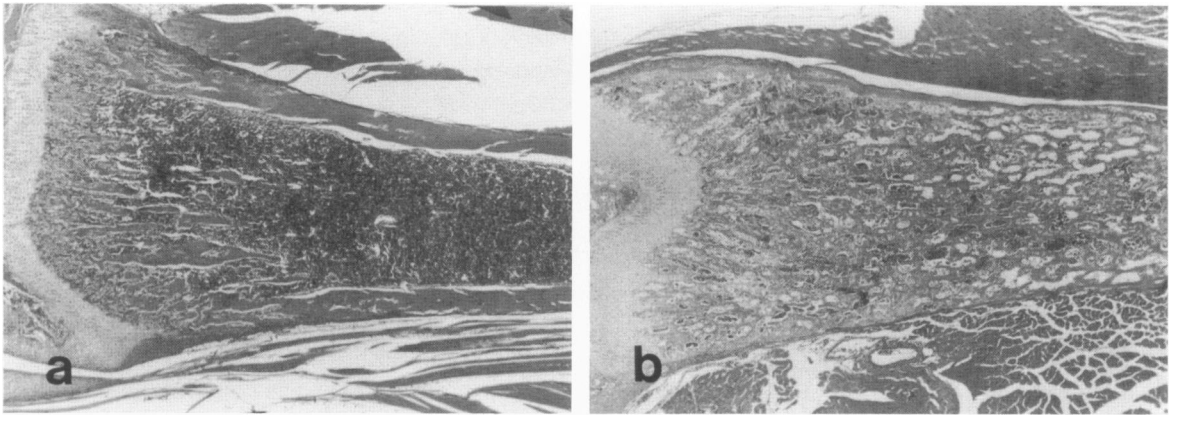

Figure 1. *In Figures 1 and 3 to 9, panel* (a) *represents normal littermates; panel* (b) *represents* op/op *mice.* a: *Longitudinal section of the distal femur from a normal littermate. The distal epiphyseal plate is at the left of the figure. The cortical shaft is well developed and the large marrow cavity is filled with hematopoietic cells.* b: *Longitudinal section of the distal femur from an* op/op *mouse. The bone shaft does not have a well-defined cortex. A definitive marrow cavity is absent and the interior of the bone is filled with primary spongiosa. Hematoxylin and eosin,* ×40.

compared the number of osteoclasts and F4/80-positive mononuclear cells on the endosteal bone surfaces per 10 mm in both groups.

Fibroblast Cultures

Fibroblast cell lines were established from lung tissues.[18] These fibroblast cell lines were tested for their ability to produce factors that would support the proliferation of normal macrophage precursors. BALB/c Cr Slc bone marrow cells were passed twice through Sephadex G-10 columns, and then 5×10^5 cells were co-cultured in plastic flasks (25 cm^2, 50 ml; Nunclon, Roskilde, Denmark) with the confluent cell layer of the fibroblast lines for 3, 7, 10, and 14 days. Cultured cells were fixed with 0.1% glutaraldehyde for 10 minutes, processed by the combined method of ultrastructural PO cytochemistry and immunoelectron microscopy with F4/80 and BM8 as described by Hoefsmit and Beelen,[28] and examined at the ultrastructural level without counterstaining. Numbers of PO-positive cells, as well as F4/80- and BM8-positive macrophages, were counted by electron microscopy, and their percentages of total cells were calculated.

Statistics

The significance of differences between means was evaluated by the Student's *t*-test.

Results

Skeletal Abnormalities and Osteoclasts

In 4-week-old *op/op* mice, osteopetrotic changes were found in all skeletal bones. The proximal end of the tibia and the distal end of the femur were wide, the diaphysis did not have a well-defined cortex, and the zone of hypertrophied chondrocytes occupied half the thickness of the epiphyseal plate. In contrast to the wide bone marrow cavity filled with numerous hematopoietic cells in normal littermates, the bone marrow of the mutant mice was composed of primary spongiosa and showed no definitive marrow cavity. Numbers of hematopoietic cells in the bone marrow of the *op/op* mice were remarkably reduced on account of an excessive amount of bone trabeculae (Figure 1). Multinuclear osteoclasts were found mainly in the metaphysis of long bones and vertebrae of the normal littermates, especially abundantly in the coccygeal vertebrae. In the mutant mice, the multinuclear osteoclasts showed reduced size and their numbers were decreased to one fifth compared with normal littermates (Table 1). Tartrate-resistant acid phosphatase activity was detected in multinuclear osteoclasts. Osteoclasts were predominantly negative for F4/80, but some mononuclear spindle cells along the bone trabeculae and bone cortex adjacent to the periosteum were positive for the antigen. Numbers of these F4/80-positive cells in the mutant mice were less than one-tenth those of the normal littermates (Table 1). No obvious abnormality was found in osteoblasts, which were abundant in the metaphyseal region. There were no signs of remission of

Table 1. *Comparison of the Number of Osteoclasts and Mononuclear Cells on the Endosteal Surface of the Femoral Bones of* OP/OP *and Normal Littermate Mice*

Cells	op/op	+/?
Osteoclasts (multinucleated giant cells)	2*	10
F4/80-positive mononuclear cells	22	52

* Number of cells per 10 mm.

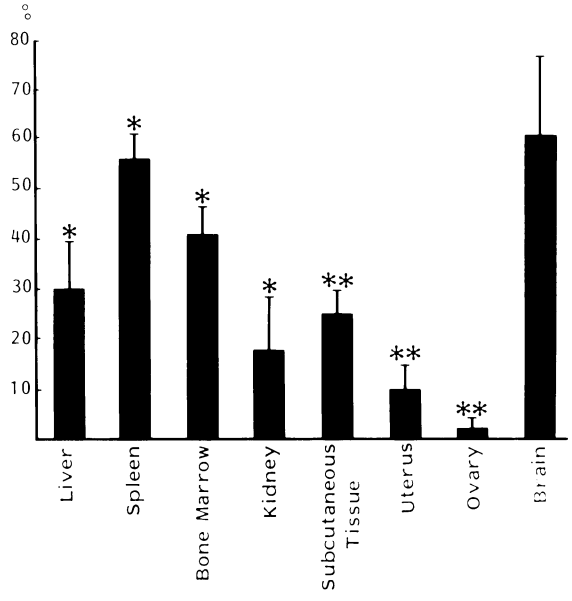

Figure 2. *Percentage of F4/80-positive macrophages in various tissues of* op/op *mice compared to those of normal littermates. Data are means ± SEM of three mice. The reduction of macrophages in various tissues in* op/op *mice is statistically significant.* *P < 0.05 *and* **P < 0.01 *by Student's t-test.*

such abnormal bone architectures, and reduced osteoclasts also were observed in mutant mice with ages up to 4 months after birth.

Pathologic Changes and Distribution of Macrophages in the Visceral Organs and Tissues

Extramedullary hematopoiesis was more extensive in the red pulp of the spleen of *op/op* mice than in normal littermates. In the thymus of these mutant mice, the cortex became markedly attenuated and consisted almost exclusively of thymic epithelial cells. In the atrophic thymic medulla, there were numerous macrophages phagocytizing nuclear fragments. In the uterus, the endometrium and myometrium were hypoplastic and there was poor development of glandular epithelia. In the subcutaneous tissue, several mast cells showing metachromasia by toluidine blue staining were observed both in mutant mice and normal littermates.

Immunohistochemical examination showed that the number of F4/80-positive macrophages was reduced in *op/op* mice compared with the normal littermates (Figure 2). In the mutant mice, numbers of Kupffer cells were about 30% of those in the normal littermates (Figures 2 and 3). Distribution of Kupffer cells in the hepatic lobules was quite irregular. Numbers of F4/80-positive cells in the synovial membrane (Figure 4), bone marrow (Figure 5), red pulp of the spleen (Figure 6), interstitium of the kidney, subcutaneous tissue, lymphatic sinuses of the lymph nodes, thymus, testis, endometrium and myometrium of the uterus, stroma of the ovaries (Figure 7), pericapillary space of the brain, and lamina propria of the stomach were also reduced in the mutant mice. The reduction of macrophages in most of these tissues was statistically significant ($P < 0.05$: liver, spleen, bone marrow, kidney; $P < 0.01$: uterus, ovary, subcutaneous tissue). Such reduction of macrophages in various tissues was confirmed also in 4 month-old mutant mice, and the number of BM8-positive cells was similarly reduced in *op/op* mice. Comparing their immunoreactivities in adjacent sections, F4/80-positive cells simultaneously expressed BM8 antigen. Thus, F4/80-positive cells are considered to be macrophages, because BM8 is expressed on macrophages but not on monocytes.

The most striking reduction in numbers of macrophages in *op/op* mice was evident in the uterine wall and ovary of the mutant mice ($P < 0.01$). There were only 10% normal levels of uterine wall macrophages and 2.5% of ovarian macrophages in *op/op* mice compared with the

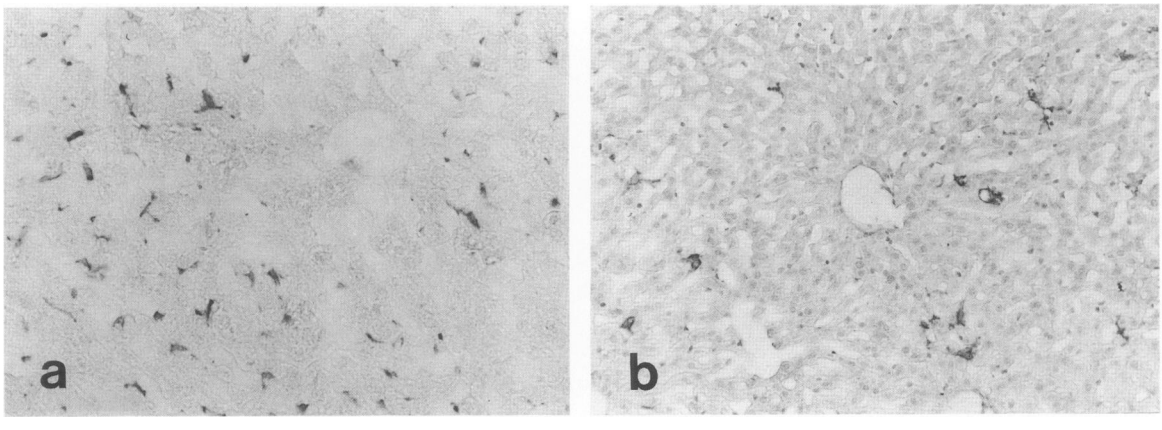

Figure 3. *Figures 3 to 8 show immunohistochemical staining using F4/80. Kupffer cells in the liver are less numerous in* op/op *mice than in normal littermates, ×200.*

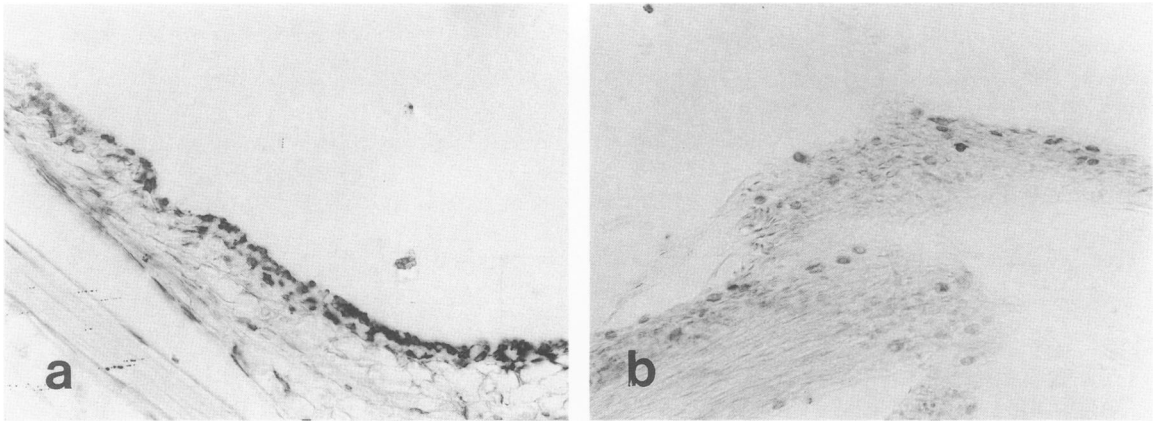

Figure 4. *There were few macrophages in the synovial membrane of* op/op *mice than in normal littermates,* ×*200.*

normal littermates (Figures 2, 7, and 8). Among seven C3H/He Slc mice (three in the secretory phase, four in the interval phase), the number of macrophages in the uterine wall was similar to the littermate +/? mice, indicating no statistical difference between two stages of sexual cycle.

Ultrastructural Difference of Macrophages Between op/op Mice and Normal Littermates

In *op/op* mice, macrophages in the splenic red pulp were uniform in size and showed various degree of phagocytosis. Rough endoplasmic reticulum (rER), lysosomal granules, and microvillous projection, however, were not well developed (Figure 9b). In the normal littermates, macrophages varied in size and shape, and most of them possessed much more developed organelles and cytoplasmic projection than those in the *op/op* mice (Figure 9a). In the liver, Kupffer cells often possessed phagocytized blood cells and electron-dense substance of variable size, but their intracellular organelles and microvillous projection were poorly developed in 4-week-old mutant mice. In Kupffer cells of 4-month-old mutant mice, vacuoles were often found, but phagocytosis was less prominent. Poor development of organelles in macrophages were also observed in the thymus and bone marrow.

Development of Macrophages After Co-culture of BALB/c Bone Marrow Cells with Fibroblast Cell Lines Established from op/op *Mice and Normal Littermates*

After 3 days of co-culture of bone marrow cells from BALB/c mice on fibroblast cell lines established from normal littermates, a few colonies of monocyte/granulocyte series developed. These colonies were composed of F4/80- or BM8-negative cells containing several peroxidase-positive cytoplasmic granules. Monocyte/granulocytes proliferated at 7 days, together with the marked increase of F4/80- or BM8-positive macrophages. After 2 weeks of

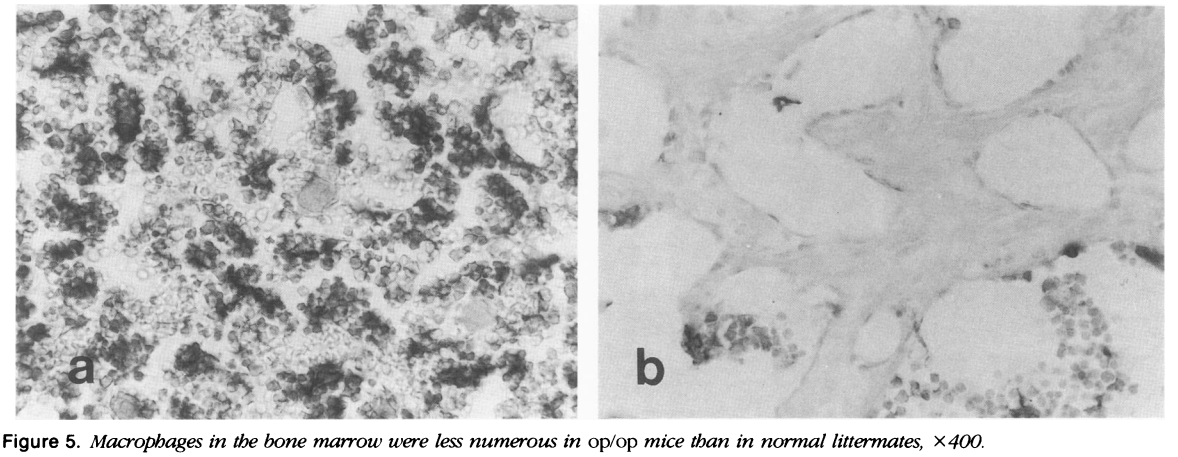

Figure 5. *Macrophages in the bone marrow were less numerous in* op/op *mice than in normal littermates,* ×*400.*

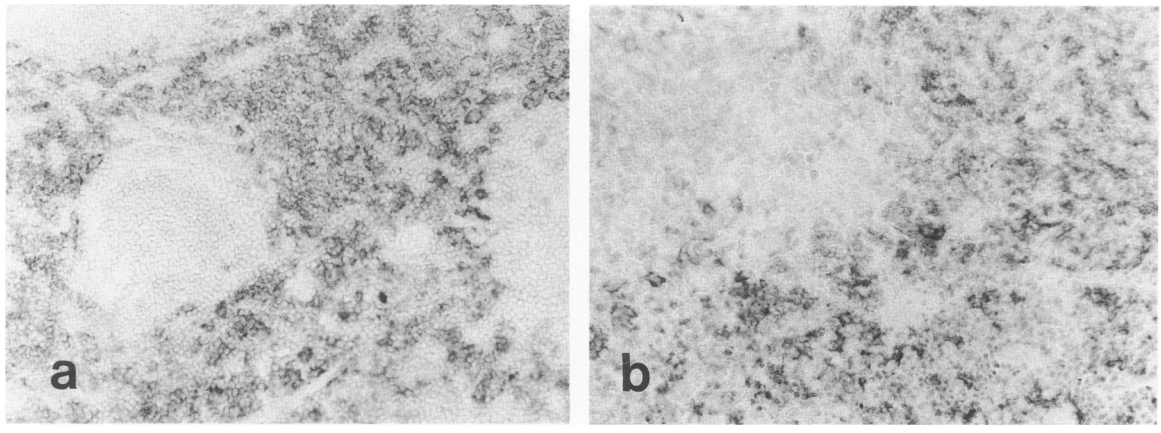

Figure 6. *Macrophages in the red pulp of the spleen were less numerous in* op/op *mice than in normal littermates,* ×200.

co-culture, most of the hematopoietic cells were differentiated into macrophages. Ultrastructurally, 99% of these macrophages expressed F4/80 or BM8 on the cell membrane, projected well-developed microvilli or filopodia from the cell surface, and possessed abundant rER, secondary lysosomes, and phagocytic vacuoles in the cytoplasm (Figure 10a).

In the culture of bone marrow cells on fibroblast cell lines established from the lungs of *op/op* mice, a few monocyte/granulocyte colonies were observed at 3 days. After 7 days, monocytes/granulocytes proliferated rather diffusely with a few macrophages. Even after 10 and 14 days of culture, about 95% of the hematopoietic cells were monocytes and occasional granulocytes (Figure 11). Fine PO-positive granules contained in monocytes at 3 and 7 days after culture were dispersed throughout their cytoplasm (Figure 12a). These granules, however, were localized in the cell periphery at day 14 in culture (Figure 12b). These cells ranged from 6 to 10 μ in diameter (average, 7.9 μ) and contained one to 23 PO-positive granules per cell (average, 13). The size of the granules ranged from 0.07 to 0.12 μ. Macrophages constituted about 5% of the hematopoietic cells. Although large macrophages expressed F4/80 or BM8 clearly, more than half of PO-negative cells showed no or weak immunoreactivity against the antibodies. These cells were small in size, and lysosomes, rER, and microvillous projections were poorly developed at ultrastructural level (Figure 10b).

Discussion

In agreement with the results of previous studies of the *op/op* mouse,[12–17,29,30] this study showed marked deficiency of osteoclasts and severe disturbance of bone resorption and remodeling, resulting in skeletal bone abnormalities characteristic of osteopetrosis after 10 days of life. To compensate for marked reduction of bone marrow hematopoiesis, extramedullary hematopoiesis was prominent in the spleen of the *op/op* mouse. In the mutant mice, osteoclasts and spindle-shaped mononuclear cells were severely reduced on the endosteal bone surfaces. These mononuclear cells were immunoreactive with F4/

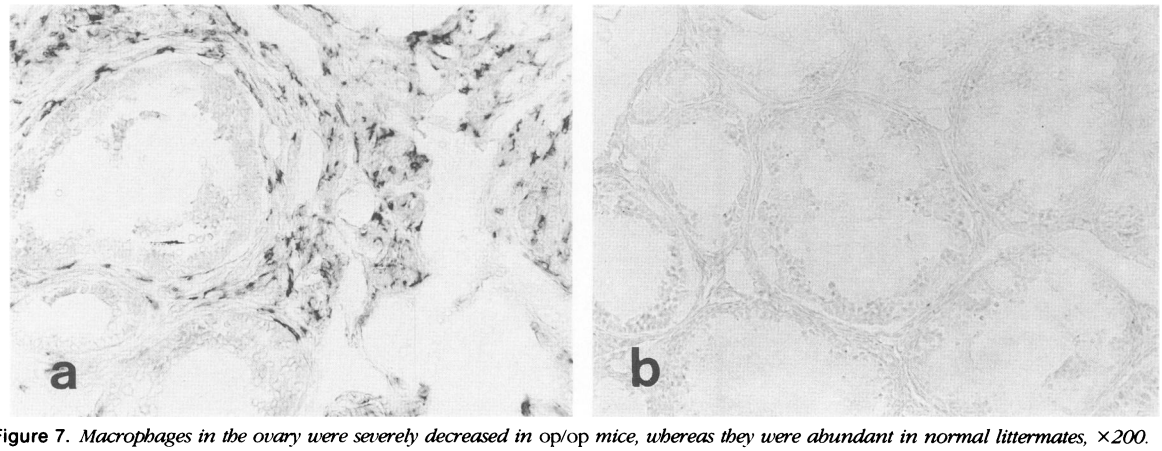

Figure 7. *Macrophages in the ovary were severely decreased in* op/op *mice, whereas they were abundant in normal littermates,* ×200.

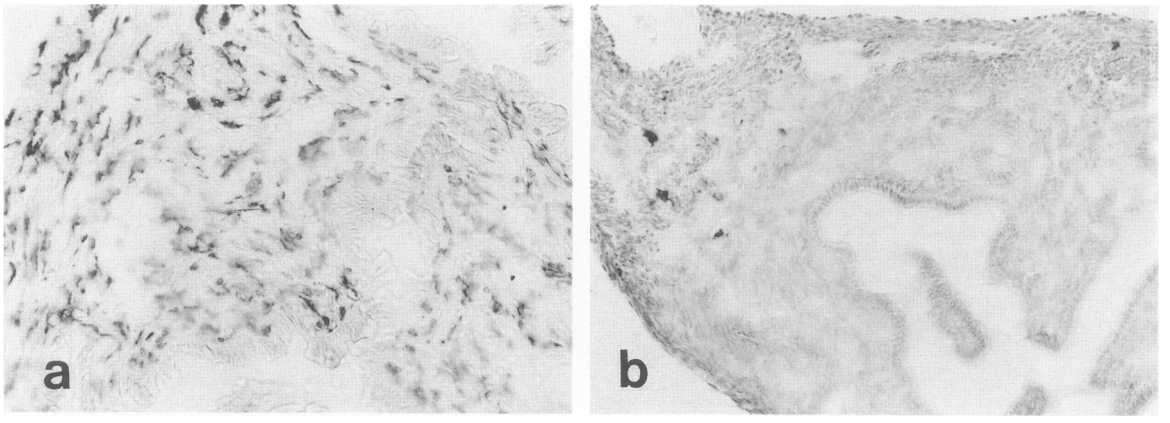

Figure 8. *Macrophages in the uterus were numerous in normal littermates, but were very few in* op/op *mice.* ×*200.*

80, suggesting that they are osteoclast precursors. These findings suggest that osteoclast reduction in the *op/op* mouse is due to a defective differentiation or proliferation of osteoclast precursor cells. In the mutant mice, reduction in numbers of macrophages was detected in the visceral organs and tissues. In the uterine wall, macrophage numbers were reduced to about 10% of those of the normal littermates. The results of previous studies support the conclusion that production of functional CSF-1 is absent in the *op/op* mouse.[18–22] Our approach by the combined method of immunoelectron microscopy and ultrastructural PO cytochemistry in the culture system using the fibroblast cell lines established from the lungs of the *op/op* mice demonstrated that normal mouse bone marrow precursor cells could differentiate into monocytes but not into macrophages in co-culture. These results show that differentiation of monocytes into macrophages is defective in the *op/op* mouse, resulting in a reduction of monocyte-derived macrophage population in various organs and tissues.

It has been postulated that the myeloid-lymphoid stem cells, pluripotential myeloid stem cells, granulocyte-

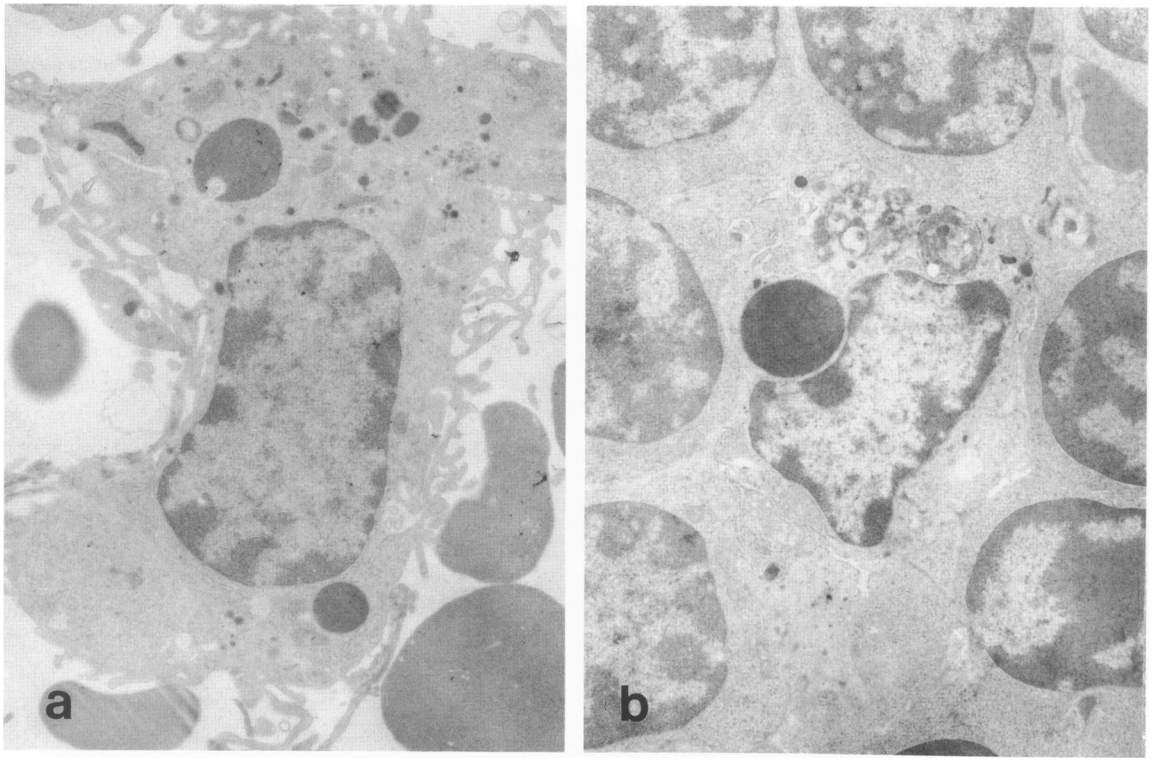

Figure 9. *Macrophages in splenic red pulp.* **a**: *normal littermate.* **b**: op/op *mouse. Most of the splenic macrophages in* op/op *mice are smaller than those in normal littermates. Intracytoplasmic organelles of the macrophage are poorly developed in* op/op *mice,* ×*6000.*

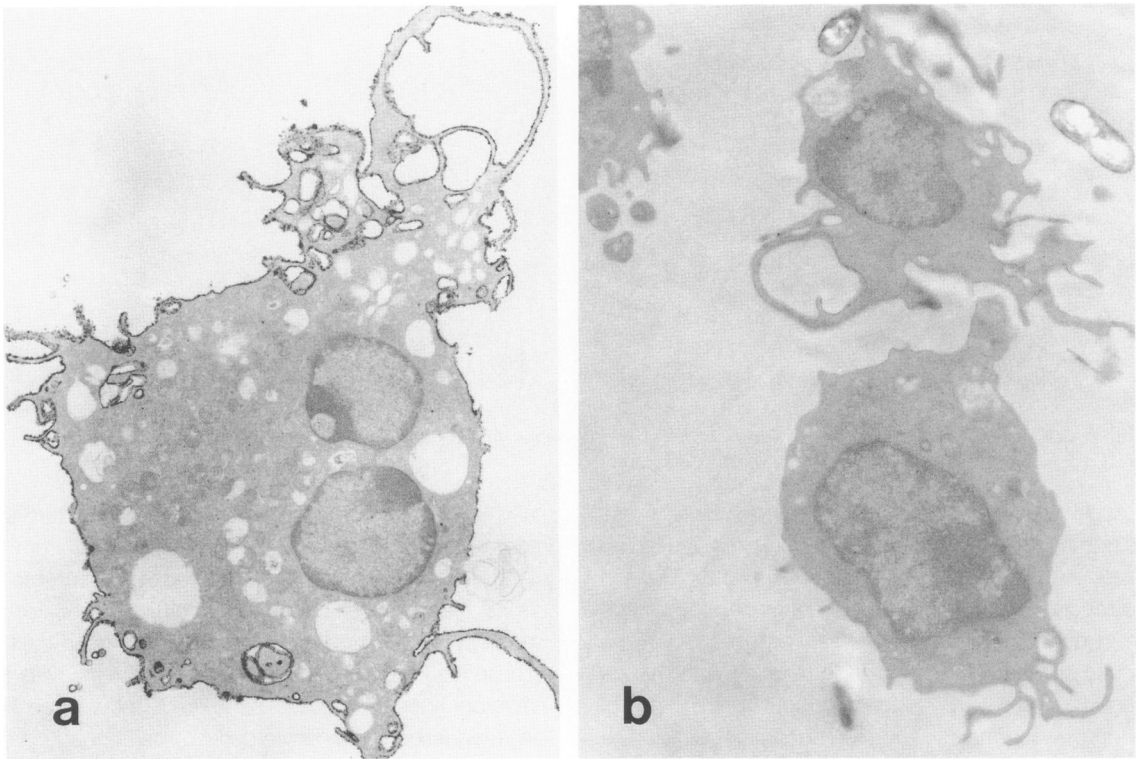

Figure 10. **a:** *Macrophage developed after 2 weeks of coculture on fibroblasts established from normal littermates. The macrophage shows well-developed organelles, microvillous projections, and phagocytic vacuoles and expressed reaction products for F4/80 are present on the cell surface, ×6000. No counterstaining.* **b:** *Macrophage developed after two weeks of coculture on fibroblasts established from an* op/op *mouse. Macrophages only rarely develop in this culture system and show poorly developed cytoplasmic organelles and weak or absent reactivity for F4/80. Combined electron microscopical PO cytochemistry and immunoelectron microscopy using F4/80, ×6000.*

macrophage colony-forming cells (GM-CFC), and macrophage colony-forming cells (M-CFC) appear in the sequential order during the macrophage differentiation process[31] and that macrophages develop as the terminal differentiation of MPS from M-CFC through promonocytes and monocytes.[5–7] Proliferation and differentiation of GM-CFCs and M-CFCs are regulated by GM-CSF and CSF-1, respectively.[2–4] CSF-1 is an essential molecule for inducing the terminal differentiation of MPS. This cytokine is synthesized by a variety of cells in murine organs and tissues including the liver, lungs, brain, heart, and uterus.[32] The severe reduction of macrophages and osteoclasts in the *op/op* mouse is due to the failure in differentiation of monocytes into macrophages because of the absence of functional CSF-1 and because monocytes are completely[21] or nearly completely deficient in peripheral blood.[20] In the mutant mice, macrophage reduction was most severe in the uterine wall and ovaries. Because the differentiation of macrophages in the uterine wall is regulated by CSF-1 produced by the epithelial cells of the endometrium in nonpregnant normal mice,[33] the severe depletion of macrophages in the uterine wall of the *op/op* mice is probably explained by the absence of functional CSF-1 *in situ*. Because the defect in production of CSF-1 in the *op/op* mouse is genetically determined, the peculiar skeletal signs characteristic of the *op/op* mouse would be expected to appear in the fetal stage, but such phenotypical abnormalities are usually first observed after about 10 days of life. The likely explanation for this is that a 10,000-fold increase of mouse uterine CSF-1 and temporal expression of CSF-1 by decidual cells and trophoblasts during pregnancy has been reported,[33–37] and this might prevent the development of the mutant mouse's abnormal pathologic changes in the fetal stage.

Monocyte-derived macrophages are a nondividing and short-lived population that die within a few weeks under a normal steady-state condition.[7,38] In the *op/op* mouse, although macrophage reduction results from the absence of monocyte-derived macrophages, tissue macrophage populations were found in various organs and tissues even after 4 weeks after birth, albeit at reduced levels. Felix et al[20] also recently reported that the number of macrophages in *op/op* mice was dramatically reduced in the bone marrow, whereas dermal macrophages were normal in number.[20] The reason for this is unclear, especially because *op/op* osteoblastlike cells and skin fibroblasts failed to produce CSF-1.[20] These findings suggest that macrophages may exist in various tissues in *op/op* mice, but do not depend on differentia-

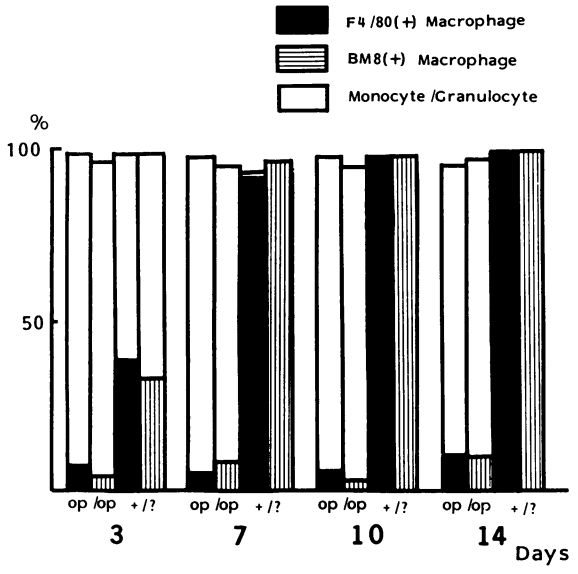

Figure 11. *Percentage of cell types in the co-culture of mouse bone marrow cells on fibroblasts established from* op/op *mice and normal littermates 3, 7, 10, and 14 days after culture.*

tion via CSF-1 stimulation. A similar alternate differentiation process is known to occur in the yolk sac or liver of normal mouse fetuses in early ontogeny.[39–41] As for the origin of such macrophages in the mutant mice, GM-CFC must be considered a valid candidate. As mentioned above, a minor population of macrophages developed in co-culture with fibroblast cell lines of *op/op* mice. Half of such macrophages then appeared poorly differentiated in ultrastructural appearance and with respect to immunoreactivity to F4/80, agreeing with previous *in vitro* studies with GM-CSF.[2,8] In the spleen and other tissues in *op/op* mouse, we confirmed predominance of similar macrophages. Because the fibroblast cell lines from *op/op* mice produce GM-CSF, macrophages from these mice may develop under the influence of GM-CSF *in vitro*.

Previous *in vitro* studies have implicated both CSF-1 and GM-CSF in the formation of osteoclasts.[42–44] In a transplantation study of granulocyte-macrophage progenitors in osteopetrotic rats, Schneider and Relfson[45] demonstrated that granulocyte colony-forming cells and GM-CFC contained osteoclast precursors and were effective in correcting the skeletal defect, whereas M-CFC were not effective.[45] In the *op/op* mouse, we found a severe reduction of osteoclasts and mononuclear osteoclast precursor cells on the endosteal bone surfaces, in agreement with the results of previous studies.[20–22] Two recent studies have demonstrated that osteopetrosis in

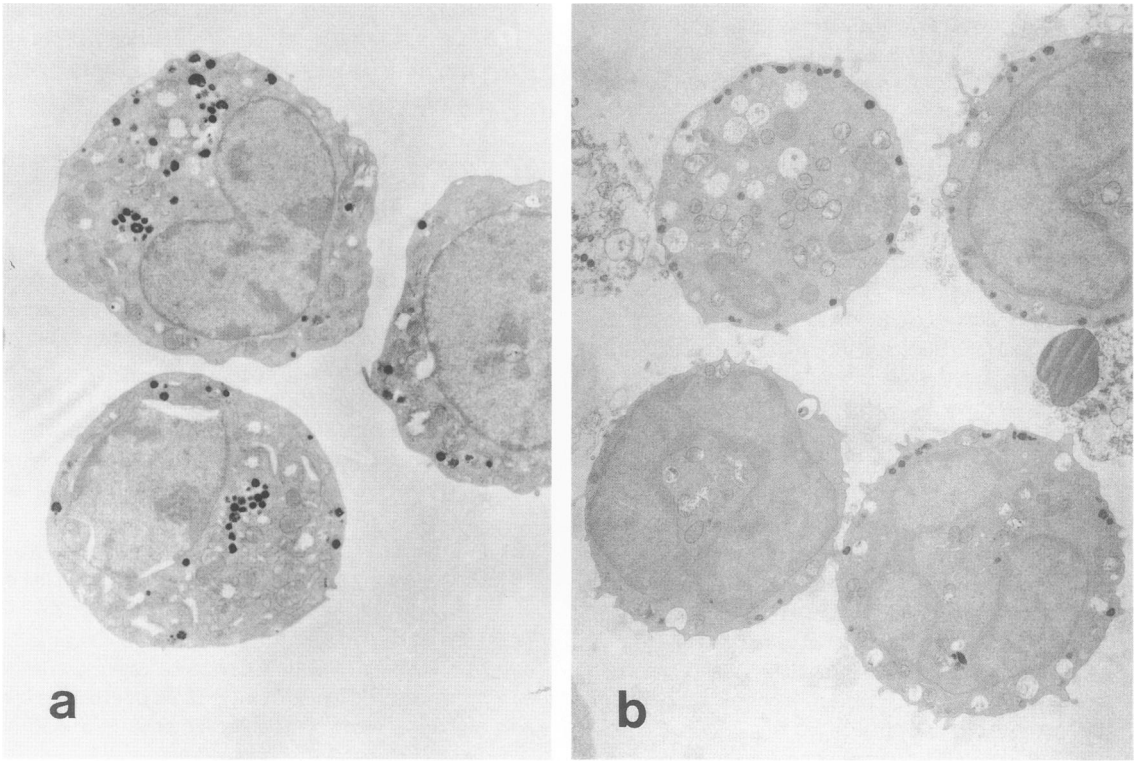

Figure 12. **a**: *Monocytes developed in the coculture on fibroblasts established from an* op/op *mouse. After 3 days of culture, PO-positive granules were present throughout the cytoplasm,* ×*4000.* **b**: *Fourteen days after culture, most of the PO-positive granules were located near the cell surface. They express no F4/80. Combined electron microscopical PO cytochemistry and immunoelectron microscopy using F4/80,* ×*4000.*

op/op mice is cured completely by administration of recombinant human CSF-1.[21,22] Getting together these facts and information, it appears obvious that CSF-1 is one of important factors responsible for the terminal differentiation of osteoclasts under physiologic conditions.

In conclusion, although further approaches are necessary to clarify the differentiation of macrophages and osteoclasts in the *op/op* mouse, there appear to be certain pathways of macrophage differentiation other than monocyte-macrophage differentiation of MPS *in vivo*. In this CSF-1–independent pathway, certain tissue macrophage populations can be differentiated directly from GM-CFC or earlier macrophage progenitors without passing through the stages of promonocytes and monocytes in adults, as shown in early ontogeny.[39–41]

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References

1. Cohn ZA: The activation of mononuclear phagocytes: Fact, fancy and future. J Immunol 1978, 121:813–816
2. Falk LA, Vogel SN: Differential production of IFN-α/β by CSF-1- and GM-CSF-derived macrophages. J Leukocyte Biol 1990, 48:43–49
3. Geissler K, Harrington M, Srivastava C, Leemhuis T, Tricot G, Broxmeyer HE: Effects of recombinant human colony stimulating factors (CSF) (granulocyte-macrophage CSF, granulocyte CSF, and CSF-1) on human monocyte/macrophage differentiation. J Immunol 1989, 143:140–146
4. Morrissey PJ, Grabstein KH, Reed, SG, Conlon PJ: Granulocyte/macrophage colony stimulating factor. A potent activation signal for mature macrophages and monocytes. Int Arch Allergy Appl Immunol 1989, 88:40–45
5. van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL: The mononuclear phagocyte system: A new classification of macrophages, monocytes, and their precursor cells. Bull WHO 1972, 46:845–852
6. van Furth R: Cells of the mononuclear phagocyte system. Nomenclature in terms of sites and conditions, Mononuclear Phagocytes, Functional Aspects, Part I. Edited by R van Furth. The Hague, Martinus Nijhoff Publishers 1980, pp 1–30
7. van Furth R: Origin and turnover of monocytes and macrophages, Cell Kinetics of the Inflammatory Reaction. Edited by OH Invesen. Current Topics in Pathology, Vol 79. Berlin, Springer-Verlag, 1989, pp 125–150
8. Falk LA, Vogel SN: Comparison of bone marrow progenitors responsive to granulocyte-macrophage colony stimulating factor and macrophage colony stimulating factor-1. J Leukocyte Biol 1988, 43:148–157
9. Morrissey PJ, Bressler L, Charrier K, Alpert A: Response of resident murine peritoneal macrophages to *in vivo* administration of granulocyte-macrophage colony-stimulating factor. J Immunol 1988, 140:1910–1915
10. Pennline KJ, Pellerito F, DaFonseca M, Monahan P, Siegel MI, Smith SR: Flow cytometric analysis of recombinant murine GM-CSF (rmuGM-CSF) induced changes in the distribution of specific cell populations in vivo. Cytometry 1990, 11:283–291
11. Brown DM, Dent PB: Pathogenesis of osteopetrosis: A comparison of human and animal spectra. Pediatr Res 1971, 5:181–191
12. Seifert MF, Marks SC: Congenitally osteosclerotic (*oc/oc*) mice are resistant to cure by transplantation of bone marrow or spleen cells from normal littermates. Tissue Cell 1987, 19:29–37
13. Marks SC, Lane PW: Osteopetrosis, a new recessive skeletal mutation on chromosome 12 of the mouse. J Hered 1976, 67:11–18
14. Marks SC: Morphological evidence of reduced bone resorption in osteopetrotic (*op*) mice. Am J Anat 1982, 163:157–167
15. Marks SC, Seifert MF, McGuire JL: Congenitally osteopetrotic (*op/op*) mice are not cured by transplants of spleen or bone marrow cells from normal littermates. Metab Bone Dis Relat Res 1984, 5:183–186
16. Wiktor-Jedrzejczak W, Ahmed A, Szczylik C, Skelly RR: Hematological characterization of congenital osteopetrosis in *op/op* mouse. Possible mechanism for abnormal macrophage differentiation. J Exp Med 1982, 156:1516–1527
17. Chambers TJ, Loutit JF: A functional assessment of macrophages from osteopetrotic mice. J Pathol 1979, 129:57–63
18. Yoshida H, Hayashi S-I, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD, Nishikawa S-I: The murine mutation "osteopetrosis" (*op*) is a mutation in the coding region of the macrophage colony stimulating factor (Csfm) gene. Nature 1990, 345:442–443
19. Wiktor-Jedrzejczak W, Bartocci A, Ferrante AWJr, Ahmed-Ansari A, Sell K, Pollard TW, Stanley ER: Total absence of colony-stimulating factor in the macrophage-deficient osteopetrotic (*op/op*) mouse. Proc Natl Acad Sci USA 1990, 87:4828–4832
20. Felix R, Cecchini MG, Hofstetter W, Elford PR, Stutzer A, Fleisch H: Impairment of macrophage colony-stimulating factor production and lack of resident bone marrow macrophages in the osteopetrotic *op/op* mouse. J Bone Miner Res 1990, 5:781–789
21. Felix R, Cecchini MG, Fleisch H: Macrophage colony stimulating factor restores in vivo bone resorption in the op/op osteopetrotic mouse. Endocrinology 1990, 127:2592–2594
22. Kodama H, Yamasaki A, Nose M, Niida S, Ohgame Y, Abe M, Kumegawa M, Suda T: Congenital osteoclast deficiency in osteopetrotic (*op/op*) mice is cured by injections of macrophage colony-stimulating factor. J Exp Med 1991, 173:269–272
23. Hammarström LE, Hanker JS, Toverud SU: Cellular differences in acid phosphatase isoenzymes in bone and teeth. Cln Orthop 1972, 78:151–156

24. Isobe S, Nakane PK, Brown WR: Studies on translocation of immunoglobulins across intestinal epithelium: 1. Improvements to study the peroxidase-labeled antibody for application to study of human intestinal mucosa. Acta Histochem Cytochem 1977, 10:161–171
25. Hume DA, Robinson AP, Macpherson GG, Gordon S: The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. J Exp Med 1983, 158:1522–1536
26. Hume DA, Loutit JF, Gordon S: The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80: Macrophages of bone and associated connective tissue. J Cell Sci 1984, 66:189–194
27. Malorny U, Michels E, Sorg C: A monoclonal antibody against an antigen present on mouse macrophages and absent from monocytes. Cell Tissue Res 1986, 243:421–428
28. Hoefsmit ECM, Beelen RHJ: The expression of antigen F4/80 and Ia on peritoneal macrophages in normal and BCG immunized mice, Mononuclear Phagocytes, Characteristics, Physiology, and Function. Edited by R van Furth. Dordrecht, Martinus Nijhoff Publishers 1985, pp 19–29
29. Loutit JF, Nisbet NW: Resorption of bone. Lancet 1979, 2:26–28
30. Chambers TJ: Phagocytosis and trypsin-resistant glass adhesion by osteoclasts in culture. J Pathol 1979, 127:55–60
31. Novak JP, Skamene E, Gervais F: Quantitative model of mononuclear phagocyte lineage proliferation in murine bone marrow. J Leukocyte Biol 1989, 46:25–33
32. Kawasaki ES, Ladner MB: Molecular biology of macrophage colony-stimulating factor, Colony-Stimulating Factors, Molecular and Cellular Biology. Edited by TM Dexter, JM Garland, NG Testa. New York, Marchel Dekker, 1990, pp 155–176
33. Rajavashisth TB, Eng R, Shadduck RK, Wahed A, Ben-Avram CM, Shively IE, Lusis AJ: Cloning and tissue-specific expression of mouse macrophage colony-stimulating factor mRNA. Proc Natl Acad Sci USA 1987, 84:1157–1161
34. Pollard JW, Bartocci A, Arceci RJ, Orlofsky A, Ladner MB, Stanley ER: Apparent role of the macrophage growth factor, CSF-1, in placental development. Nature 1987, 330:484–486
35. Bartocci A, Pollard JW, Stanley ER: Regulation of colony-stimulating factor 1 during pregnancy. J Exp Med 1986, 164:956–961
36. Arceci RJ, Shanahan F, Stanley ER, Pollard JW: Temporal expression and location of colony-stimulating factor 1 (CSF-1) and its receptor in the female reproductive tract are consistent with CSF-1-regulated placental development. Proc Natl Acad Sci USA 1989, 86:8818–8822
37. Regenstreif LJ, Rossant J: Expression of the c-fms protooncogene and of the cytokine, CSF-1, during mouse embryogenesis. Dev Biol 1989, 133:284–294
38. van Furth R, Diesselhoff-den Dulk MMC, Sluitere W, van Diessel, JT: New perspectives on the kinetics of mononuclear phagocytes, Mononuclear Phagocytes, Characteristics, Physiology, and Function. Edited by R van Furth. Dordrecht, Martinus Nijhoff Publishers, 1985, pp 201–219
39. Takahashi K, Yamamura F, Naito M: Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: A light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. J Leukocyte Biol 1989, 45:67–96
40. Naito M, Yamamura F, Nishikawa S, Takahashi K: Development, differentiation, and maturation of fetal mouse yolk sac macrophages in cultures. J Leukocyte Biol 1989, 46:1–10
41. Naito M, Takahashi K, Nishikawa S: Development, differentiation, and maturation of macrophages in the fetal mouse liver. J Leukocyte Biol 1990, 48:27–37
42. MacDonald BR, Mundy GR, Clark S, Wang EA, Kuehl TJ, Stanley ER, Roodman GD: Effects of human recombinant CSF-GM and highly purified CSF-1 on the formation of multinucleated cells with osteoclast characteristics in long-term bone marrow cultures. J Bone Miner Res 1986, 1:227–233
43. van de Wijngaert FP, Tas MC, van der Meer JWM, Burger EH: Growth of osteoclast precursor-like cells from whole mouse bone marrow: Inhibitory effect of CSF-1. J Bone Miner Res 1987, 3:97–110
44. Hattersley G, Chambers TJ: Effects of interleukin 3 and of granulocyte-macrophage and macrophage colony stimulating factors on osteoclast differentiation from mouse hemopoietic tissue. J Cell Physiol 1990, 142:201–209
45. Schneider GB, Relfson M: The effects of transplantation of granulocyte-macrophage progenitors on bone resorption in osteopetrotic rats. J Bone Miner Res 1988, 3:225–231