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Osteopetrotic *(op/op)* Mice: An Animal Model for Investigating the Biology of Colony-stimulating Factor-1 (CSF-1/M-CSF)

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Summary. **Macrophage colony-stimulating factor (M-CSF) or colony stimulating factor-1 (CSF-1) functions as an important regulator of macrophage production, differentiation, and survival. This cytokine also plays an important local regulatory role in the uteroplacental unit during pregnancy. Ever since the osteopetrotic *(op)* mutation was demonstrated to be within the coding region of the CSF-1 structural gene itself, the *op/op* mouse has proved an important animal model for investigating the biological regulation of CSF-1. CSF-1-deficient *op/op* mice are severely monocytopenic and show marked reduction and defective differentiation of tissue macrophages. Impaired osteoclast differentiation results in osteopetrosis in the mutant mice. Most of the other tissue macrophage populations are reduced in number and are ultrastructurally immature. However, the degree of reduction in numbers of macrophages in the mutant mice varies among tissues, suggesting that the heterogeneity of macrophages is generated by their different dependency on CSF-1. After daily CSF-1 injections, the numbers of monocytes, tissue macrophages, and osteoclasts increased remarkably; these CSF-1-dependent macrophages showed morphological maturation. In contrast, the numbers of macrophages in the ovary, uterus, omentum, and synovial membrane were not increased. After glucan administration, Kupffer cells in *op/op* mice proliferated, transformed into epithelioid cells and multinucleated giant cells, and participated in low levels of granulomatous inflammation, but failed to show normal levels of granuloma formation. In CSF-1-treated *op/op* mice the process of granuloma formation was similar to that of normal littermates. These results indicate that CSF-1 is a potent inducer of the development and differentiation of CSF-1-dependent monocyte/macrophages and that CSF-1-independent macrophages also play an important role in granuloma formation. Thus, *op/op* mice serve as an excellent model system for clarifying the biological role of CSF-1 in physiological and pathological conditions.**

Key words—osteopetrotic *(op/op)* mouse, colony-stimulating factor-1 (CSF-1/M-CSF), macrophage, osteoclast, differentiation.

Introduction

Interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), and colony stimulating factor-1 (CSF-1) are known to be major hematopoietic growth factors.[1)] Among these growth factors, CSF-1 selectively regulates the proliferation, differentiation, and function of macrophages. CSF-1 has been shown to act on multipotent precursors of the mononuclear phagocyte system. Macrophages are derived from hematopoietic stem cells in bone marrow which differentiate sequentially into monoblasts, promonocytes, monocytes, and macrophages.[2)] Monocytes in the blood reside in each tissue and constitute heterogeneous tissue macrophage populations with respect to their morphologic, functional, and metabolic properties. Mean survival time is also different among each subpopulation. Such a heterogeneity is considered to be produced in response to cell-derived or environmental signals. One mechanism involved in generating macrophage heterogeneity is the unique microenvironment for macrophages due

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to differences in the local production of growth factors. Although GM-CSF and IL-3 have been shown to be less effective than CSF-1, it has not been clarified whether these three growth factors regulate macrophage differentiation synergistically or independently. Besides macrophage proliferation and differentiation, CSF-1 has recently been shown to have an important role in regulating pregnancy.[3-6] From this point of view, osteopetrotic *(op/op)* mice defective in the production of CSF-1 are useful in delineating the role of CSF-1 in normal and pathological conditions. This review describes the regulatory function of CSF-1 in macrophage differentiation based on our investigations using *op/op* mice, and briefly reviews other functions of CSF-1.

Biology of CSF-1 production and receptors

CSF-1, the first CSF to be purified from human urine, was isolated by Stanley et al. in 1970.[7] Murine CSF-1 was purified from L-cell-conditioned medium also by Stanley et al.[8] CSF-1 differs from the other CSFs in several respects. It is a dimeric glycoprotein with extremely variable levels and types of glycosylation. It exists in both soluble and transmembrane cell-associated forms.[9,10] Although both types are biologically active, the role of the cell surface CSF-1 is not clearly understood. Soluble CSF-1 transforms into a proteoglycan form which binds to collagen type I.[11-13] CSF-1 binds to a classical growth factor receptor of the tyrosine kinase type, whereas other CSFs bind to a superfamily of hemopoietin receptors. Several cell types including fibroblasts, macrophages, endothelial cells, stromal cells, T-lymphocytes, uterine decidual cells, osteoblasts, and hepatocytes have been reported to be CSF-1 producing cells. In contrast, CSF-1 receptors encoded by the *c-fms* proto-oncogene are only expressed on macrophages, uterine decidual cells, and trophoblasts.[3-6]

Genetic and phenotypic abnormalities of *op/op* mice

The *op* mutation in the mouse was first described by Marks and Lane as a spontaneous recessive mutation affecting the skeletal system.[14,15] The *op* mutation occurred at the Jackson Laboratory in a stock of mice carrying the dwarf mutation. Some of the skeletal abnormalities in *op/op* mice resembled those found in the known mouse osteopetrotic mutants, i.e. gray-lethal *(gl)*, microphthalmia *(mi)*, and osteosclerotic *(oc)*. Accordingly, the new mutation was named osteopetrosis, and the symbol selected was *op*. The *op* mutation was found to be mapped to the region of chromosome 3 where the CSF-1 gene was subsequently shown to reside.[16] Mice homozygous for the *op* allele are characterized by a severe deficiency in osteoclasts, with their resultant generalized skeletal sclerosis due to a failure of bone resorption and remodeling.[14,15] These mice also have deficiencies in their numbers of monocytes and macrophages.[17-20] Phenotypic abnormalities include the absence of incisors, small body size (about 50 percent of the body weight of their normal littermates), and a domed skull. Because of a narrow bone marrow cavity, numbers of hematopoietic progenitors are decreased in the bone marrow and increased in the spleen.[17] This mutant mouse lacks CSF-1 activity due to a null mutation in the coding region of CSF-1 gene.[16] The insertion of additional thymidine nucleotide 262 base pair from the ATG in the CSF-1 sequence leads to frameshift in the remaining sequence with an inability to transcribe CSF-1 mRNA and an inability of *op/op* cells to produce functional CSF-1. The mutant mice, therefore, have defective differentiations of monocytes or other precursors into osteoclasts and macrophages.

Macrophage subpopulations in *op/op* mice

Reduced numbers of blood monocytes as well as peritoneal and pleural macrophages in *op/op* mice have been reported in earlier studies.[17,20] Our immunohistochemical staining using anti-macrophage antibodies demonstrated reduced numbers of tissue macrophages in *op/op* mice compared with that of normal littermates.[19] Numbers of Kupffer cells in *op/op* mice were about 30% of those in normal littermates (Fig. 1A, B). In addition to quantitative changes in Kupffer cells, Kupffer cells in the mutant mice are morphologically different from those in littermates. Kupffer cells in *op/op* mice have poorly developed lysosomes and show decreased phagocytosis (Fig. 2). The most striking reductions in the numbers of macrophages in *op/op* mice were found in the uterus, ovary, and synovial membrane (Fig. 3). These tissue macrophages were regarded as CSF-1-dependent populations. Most of the other tissue macrophages showed a moderate or partial dependency on CSF-1.

In the spleen, there are discrete macrophage populations detected by a panel of monoclonal antibodies. These populations include red pulp macrophages, marginal metallophilic macrophages, marginal zone macrophages, tingible body macrophages in the germinal center, and dendritic cells in the periarterial lymphoid sheath. In *op/op* mice, MOMA-1-positive marginal metallophilic macrophages and ER-TR9-positive marginal zone macrophages are absent.[21] These facts suggest that the development and differ-

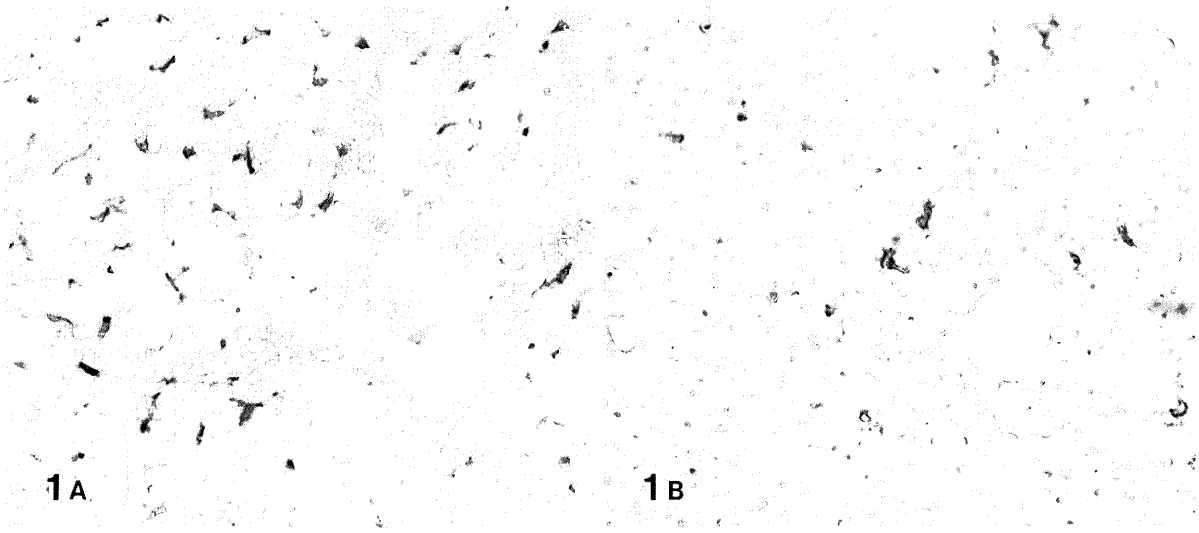

Fig. 1. Kupffer cells in the liver of a normal littermate **A**. and *op/op* mouse **B**. Immunohistochemical staining using an anti-mouse macrophage antibody F4/80. ×100

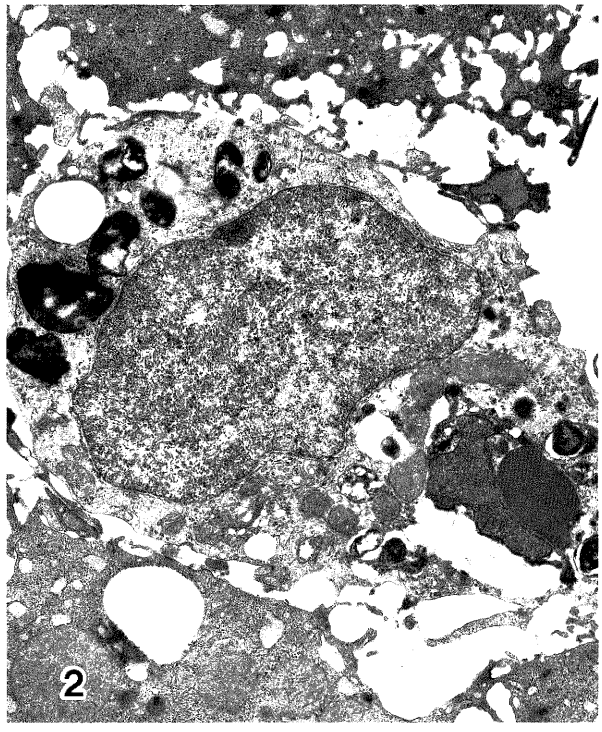

Fig. 2 Kupffer cell in an *op/op* mouse. The Kupffer cell is characterized by a round nucleus, poorly developed intracytoplasmic organelles, and a few short surface projections. ×5,000

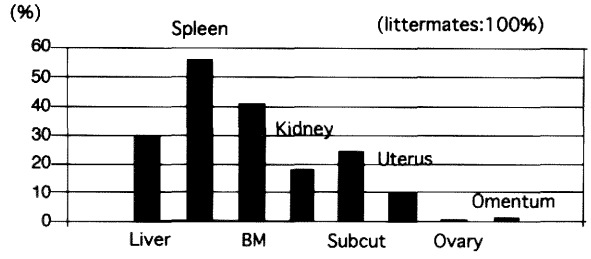

Fig. 3. Percentage of macrophages in various tissues of *op/op* mice normalized to those of normal littermates. BM, bone marrow; Subcut, subcutaneous tissue.

entiation of these macrophage populations is absolutely dependent on CSF-1.

Dendritic cells are macrophage-related cells characterized by a high expression of MHC class II molecules(Ia), poor phagocytic capacity, and potent antigen-presenting cell function in initiating T cell-mediated immune response.[22] Dendritic cell populations include Langerhans cells in the epidermis, veiled cells in lymph, interdigitating cells within the paracortical area of lymph nodes, and dendritic cells in peripheral lymphoid tissues. They are specifically recognized by the monoclonal antibodies, NLDC 145 and MIDC8. Epidermal sheets taken from the skins of *op/op* and normal littermate mice demonstrate a similar distribution of Langerhans cells (Fig. 4). Electron microscopically, Langerhans cells were present among keratinocytes in *op/op* mice and had characteristic granules called Birbeck granules (Fig. 5). Comparing the density of dendritic cells in the skin, spleen, and lymph nodes, there were no significant differences between *op/op* mice and littermates.[23]

These findings indicate that CSF-1 is necessary for most of the macrophage populations. Among CSF-1-dependent macrophages, it seems essential for the dif-

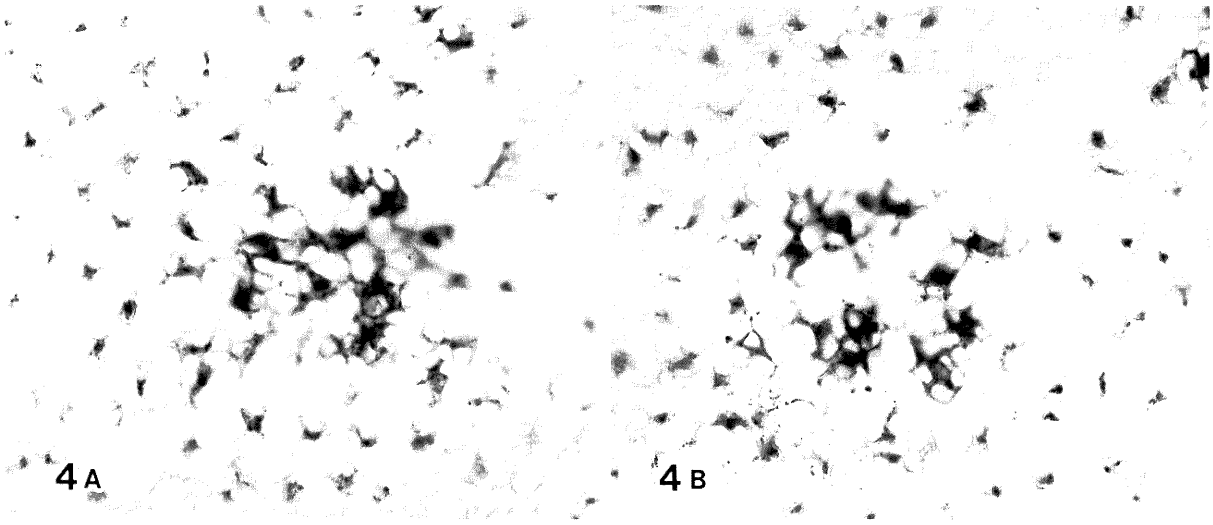

Fig. 4. Langerhans cells in epidermal sheets. **A.** +/? littermate. **B.** *op/op* mouse. Both mice show similar distribution of Langerhans cells. ADPase staining. ×400

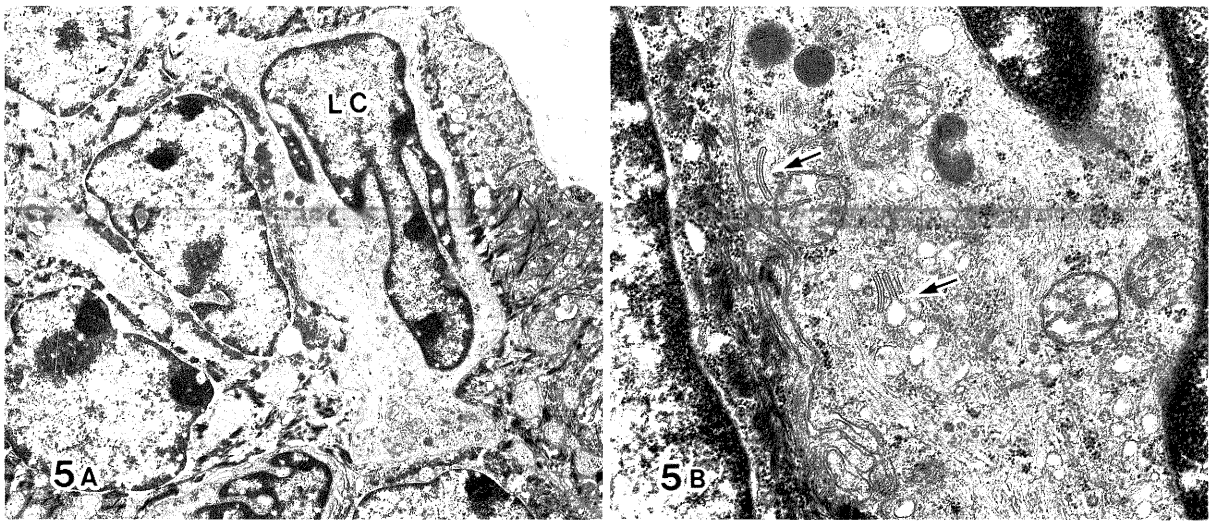

Fig. 5. **A.** Langerhans cell (LC) in an *op/op* mouse and **B.** Birbeck granules *(arrows)* in the cell. **A** ×3,500, **B** ×10,000

ferentiation of monocytes, osteoclasts, and marginal metallophilic macrophages as well as macrophages in the ovary, uterus, and synovium. In contrast, CSF-1 is not required for dendritic cell differentiation.

Macrophages in *op/op* mice after birth

Homozygous *op/op* mice can be distinguished from normal littermates by characteristic phenotypic features appearing about 10 days after birth.[24] Maternal CSF-1, mainly produced from the uterus, is thought to support the early normal development of *op/op* fetuses, which then develop in the absence of CSF-1 after birth. Milk-derived CSF-1 does not seem to play a role in *op/op* development of macrophages. Therefore, postnatal changes in macrophage subpopulations reflect a loss of CSF-1 function.

Just after birth, monocytes are abundant in the peripheral blood both in *op/op* mice and normal littermates. The number of monocytes rapidly decreases in both types of mice. However, *op/op* mice become severely monocytopenic by one week after birth.[25] In most of the tissues of *op/op* mice just after birth, macrophages were present at a normal level. Kupffer cells in one-day-old *op/op* mouse had well developed cytoplasmic projections and phagolysosomes. In gen-

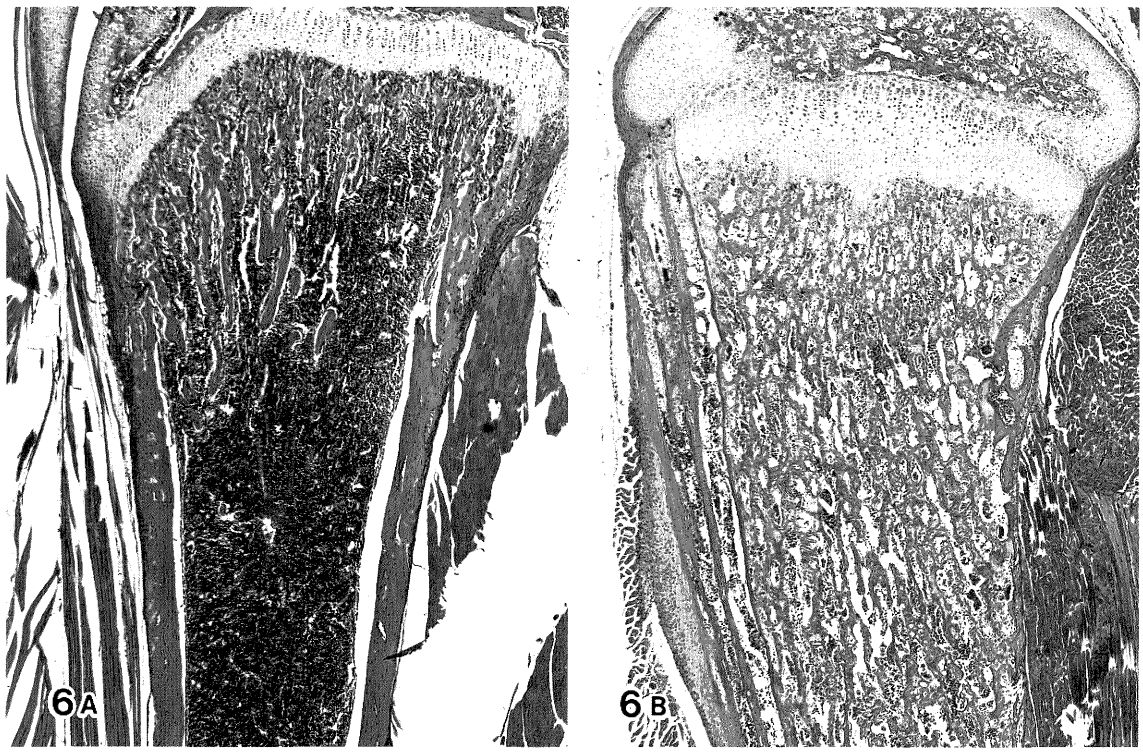

Fig. 6. **A**. Bone marrow of a normal littermate. The bone marrow cavity is filled with numerous hematopoietic cells. Hematoxyline and eosin. ×20 **B**. Bone marrow of an untreated *op/op* mouse. The bone marrow is composed of spongious bone trabeculae. Hematoxyline and eosin. ×20

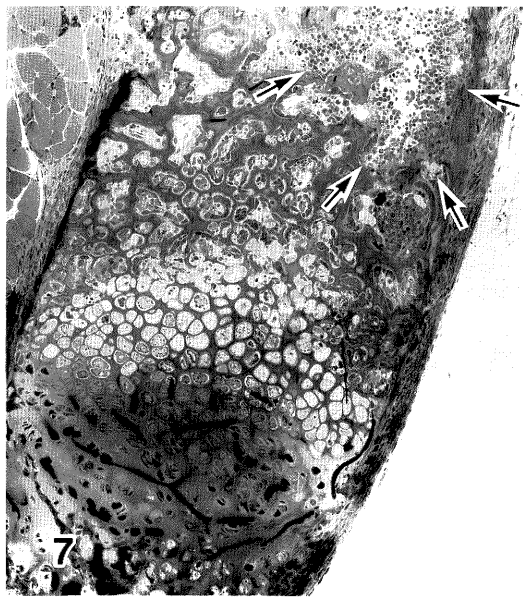

Fig. 7. Femoral bone of an *op/op* mouse one week after daily injections of CSF-1. A bone marrow cavity is newly formed *(arrows)* and filled with abundant hematopoietic cells. H.E. ×40

eral, macrophages in new born *op/op* mice were normal in number and ultrastructure.

The numbers of tissue macrophages in *op/op* mice gradually decreased after birth, with the exceptions of macrophages in the kidney and synovial membrane. Their numbers were few from the beginning as reported by Cecchini et al..[26] Furthermore, osteoclasts were severely depleted even at birth in *op/op* mice and their numbers rapidly decreased thereafter. In the spleen of both newborn *op/op* and normal littermate mice, there were no marginal metallophilic macrophages or marginal zone macrophages. These macrophages gradually developed in littermate mice, but did not appear in *op/op* mice. In contrast, the number of dendritic cells in the spleen was constant and similar between *op/op* mice and littermates.

Macrophages and osteoclasts in *op/op* mice after the administration of CSF-1

Because *op/op* mice are CSF-1-deficient, several studies have focused on effects of CSF-1 injection in *op/op* mice in regard to the responses of macrophages and osteoclasts.[25,28-30] In *op/op* mice, the bone marrow cavity is filled with spongious bone trabeculae

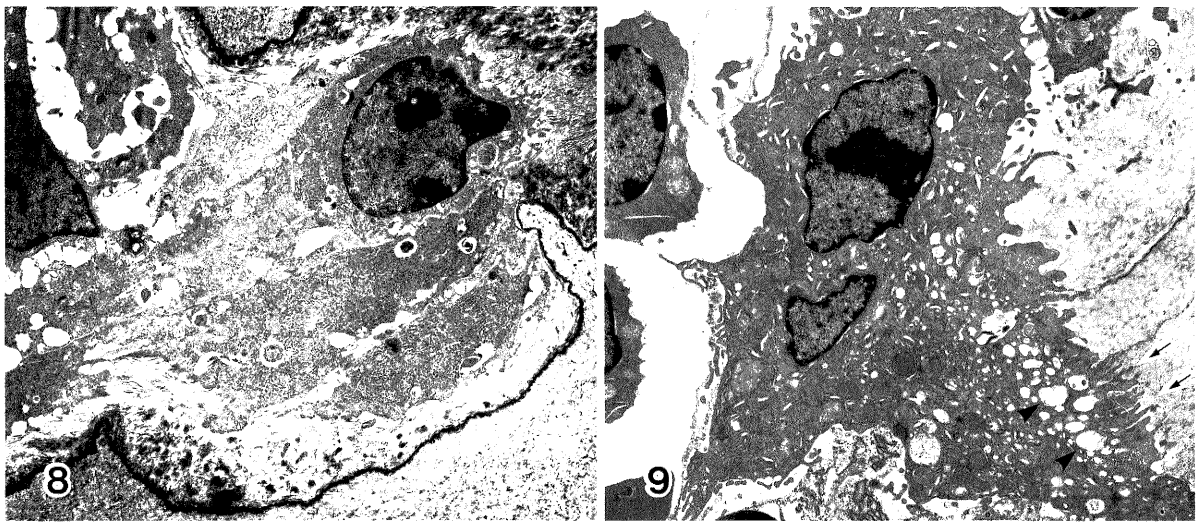

Fig. 8. Mononuclear cell developed on the bone trabecula of an *op/op* mouse two days after daily administrations of CSF-1. ×5,000

Fig. 9. Multinucleated osteoclast developed on the bone trabecula of an *op/op* mouse three days after daily administrations of CSF-1. The cell has well developed ruffled borders *(arrows)* and intracytoplasmic vacuoles. *(arrow heads)* ×5,000

and contains only a few hematopoietic cells (Fig. 6). Osteoclasts cannot be detected by tartrate-resistant acid phosphatase (TRAP) staining. After the initiation of daily CSF-1 injections, the number of monocytes in peripheral blood increases rapidly, reaching maximum levels at day 3 before decreasing to normal levels. CSF-1 injection gradually results in the enlargement of the bone marrow cavity and hematopoietic cell proliferation (Fig. 7). Soon after the initiation of daily administration of CSF-1, TRAP-positive mononuclear cells appear, followed by the development of multinucleated TRAP-positive osteoclasts. Based on this observation, we call the former TRAP-positive cells preosteoclasts.[25]

Ultrastructural studies on *op/op* mice at two days after CSF-1 injection showed mononuclear cells adhering to the surface of the bone matrix (Fig. 8). These mononuclear cells appeared to be preosteoclasts, since they possessed vesicles and extended their cytoplasmic projections as if to embrace or resorb bone matrix. Mononuclear cells on the bony trabecula were sometimes attached to each other, suggesting the fusion of these cells. At three days, multinuclear osteoclasts developed abundant ruffled borders and clear vesicles (Fig. 9). These observations suggest that preosteoclasts differentiate into osteoclasts within one or two days.

Before the development of osteoclasts, myeloid or monocytoid precursors proliferate in the bone marrow. Among the monoclonal antibodies produced against macrophage precursors, ER-MP58 recognized antigens

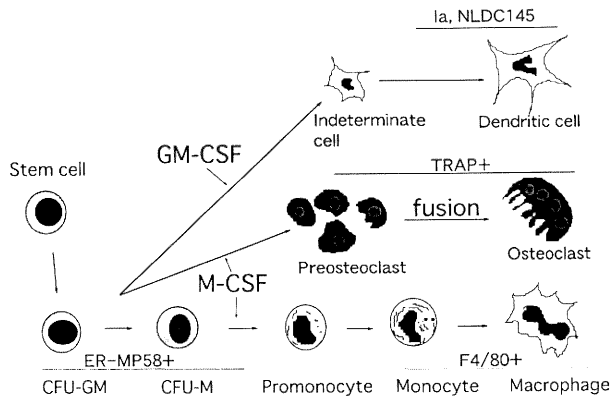

Fig. 10. Schematic presentation of the differentiation pathways of macrophages and macrophage related cells.

on CFU-GM, CFU-M and promonocytes.[31] In our study, ER-MP58-positive cells proliferated in the bone marrow and were characterized by large nuclei as well as a few peroxidase-positive granules.[25] The combined method of immunohistochemistry and ^{3}H-thymidine autoradiography showed that ER-MP58-positive cells remarkably proliferated by the second day after initiating CSF-1 administration. However, TRAP-positive preosteoclasts and osteoclasts were nonproliferating populations. These results indicate that CSF-1 induces the proliferation of ER-MP58-positive precursor cells in bone marrow, the development of preosteoclasts, and their fusion into osteoclasts. Recently, osteoblasts were shown to initiate the production of CSF-1 dur-

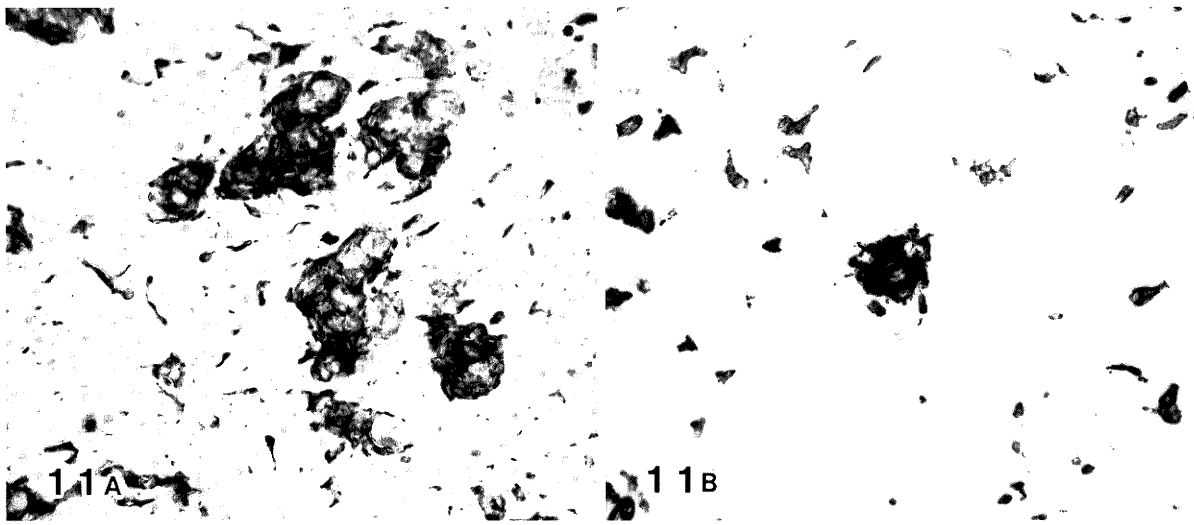

Fig. 11. Glucan-induced granuloma formation at ten days in a normal littermate **A**. and *op/op* mouse **B**. Immunohistochemical staining using F4/80. ×100

ing bone marrow formation in the mouse fetus and the incorporation of CSF-1 into osteoclasts was confirmed.[32] Although the injection of soluble CSF-1 supports osteoclast differentiation, the proteoglycan form of CSF-1 is known to be present in the bone matrix. Osteoclasts often reside adjacent to osteoblasts. Further investigation is required to clarify which form of CSF-1 functions in the support of osteoclast differentiation and how cell-to-cell communication is performed between osteoclasts and osteoblasts.

Macrophages in several tissues respond quickly to the administration of recombinant human CSF-1.[26,27,30] Kupffer cells in *op/op* mice and in littermates proliferated markedly at one and two days after the initiation of CSF-1 injection, but long-term observation revealed that their numbers returned to a normal level soon after CSF-1-treatment (Umeda, unpublished data). However, the numbers of macrophages in the kidneys, uterus, ovary, and synovium did not increase. In the spleen, MOMA-1-positive marginal metallophilic macrophages and ER-TR9-positive marginal zone macrophages appeared after two weeks and increased slowly.[21]

From the degree of response to CSF-1, macrophages can be classified into three categories: highly responsive, partially responsive, and non-responsive populations. The question remains concerning the non-responsive population such as macrophages in the uterus, ovary, omentum, and synovium. Because there are low numbers of such macrophages from birth, they are thought to be CSF-1-dependent macrophages. However, they proved to be CSF-1-non-responding macrophages in our experiment using recombinant CSF-1. Considering this discrepancy, it should be mentioned that there are several forms of CSF-1 which are biologically active. They include soluble, membrane-bound, and proteoglycan forms.[9-13] In this regard, locally- or cell-bound CSF-1 might be of key importance for the development and differentiation of these CSF-1-non-responding macrophages.

Fig. 10 illustrates the macrophage differentiation pathways. CSF-1 induces the proliferation of macrophage precursors and differentiation of CSF-1-responsive macrophage populations, such as osteoclasts, marginal zone macrophages in the spleen, and macrophages in many other tissues. However, dendritic cells are not influenced by CSF-1. Their differentiation is likely to be regulated by GM-CSF.

Glucan-induced granuloma formation in *op/op* mice

Glucan is a polysaccharide extracted from fungus and contains β-1,3 glucosidic linkage. We injected glucan particles intravenously to observe granuloma formation in the liver.[27,33] Glucan particles are phagocytized by macrophages and induce the formation of foreign body granulomas. In glucan-injected normal mice, both monocytes and Kupffer cells transform into epithelioid cells and giant cells. In our glucan-injected *op/op* mice, peripheral blood remained deficient in monocytes and lacked significant monocytosis. Five days following glucan injection, several small granulomas were formed in the liver of normal littermates. In contrast, no granuloma formation was seen in *op/op* mice. Ten days after glucan injection, a small number of irregularly outlined gran-

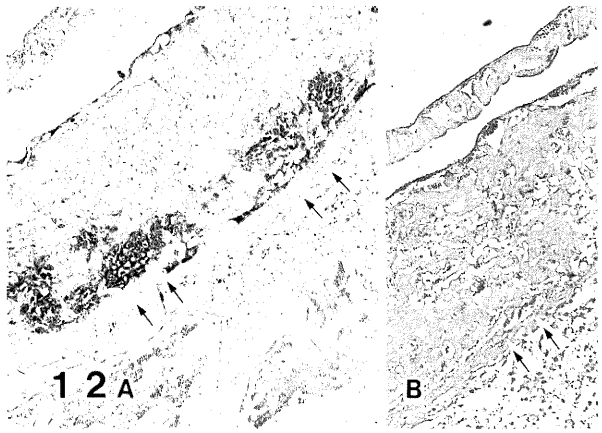

Fig. 12. Localization of *c-fms* mRNA by *in situ* hybridization in trophoblasts of mouse placenta at 12 days of gestation. Hybridized to digoxigenin-labelled antisense *c-fms* RNA **A**. and sense *c-fms* RNA **B**. *Arrows* indicate trophoblasts. ×100

ulomas were formed in the liver of *op/op* mice. In normal littermates, the number and the size of granulomas further increased (Fig. 11). The granulomas in *op/op* mice were composed of macrophages, epithelioid cells and lymphocytes, but not monocytes. Although the numbers of granulomas were small in glucan injected *op/op* mice, CSF-1 treatment resulted in increased numbers of granulomas and increased numbers of Kupffer cells. The proliferation of macrophages, especially outside granulomas, was found in the *op/op* mice and normal littermates in the late stage of granuloma formation.

In glucan-injected *op/op* mice, CSF-1-independent Kupffer cells are thought to transform into epithelioid cells and multinuclear giant cells. Kupffer cell proliferation is particularly important for glucan-induced granuloma formation in a monocytopenic condition. Because glucan injection induces the production of GM-CSF and CSF-1, GM-CSF is thought to be an important growth factor for Kupffer cell proliferation in *op/op* mice. Thus CSF-1-independent tissue macrophages participated in granulomatous inflammation. On the other hand, the process of granuloma formation in CSF-1 treated *op/op* mice was similar to that in normal littermates. The increased number of granulomas observed in the CSF-1-treated *op/op* mice may be due to increased monocytopoiesis and monocyte influx into the granulomas. Thus, CSF-1 is a potent inducer of the development and differentiation of the monocyte/macrophage cell lineage in granuloma formation.

Role of CSF-1 in pregnancy

In contrast to levels of CSF-1 in other tissues and serum, the concentration of CSF-1 in the uterus of pregnant mice increases 1000 fold. It was demonstrated by *in situ* hybridization that CSF-1 mRNA is restricted to the luminal and glandular epithelium through pregnancy.[3-5] The cell types expressing CSF-1 receptor *(c-fms)* mRNA are initially maternal decidual cells, followed by trophoectodermal cells (Fig. 12). Visceral yolk sac cells and macrophages also express *c-fms*. Uterine macrophages produce a variety of cytokines that are thought to immunosuppress the host response to the fetus. These cytokines may act directly on cells of the uteroplacental unit or be involved in regulatory networks during pregnancy. The good correlation between the expression of uterine CSF-1 mRNA and trophoblastic *c-fms* mRNA indicates a regulatory role of CSF-1 for placental development. In this regard, *op/op* mice also serve as an ideal animal model in the study of the biological roles of CSF-1 in pregnancy. Pollard et al. demonstrated that homozygous crosses between homozygotes *(op/op × op/op)*were completely infertile. In contrast, *op/op* males were usually fertile when crossed with heterozygous females, while crosses between an *op/op* female × *op/+*male were less successful.[6] These data suggest the requirement of CSF-1 for pregnancy. The data from this experiment also imply that *+/op* fetuses compensate for the absence of maternally produced CSF-1. Histochemical staining using macrophage differentiation antigens detected the almost complete absence of uterine macrophages in virgin *op/op* mice although these macrophages were abundant in +/? littermate uteri.[6,19] Interestingly, uterine macrophages were initially detected in pregnant *op/op* mice, though they disappeared beyond day 14 of pregnancy.[6] These findings suggest an important local role for uterine macrophages in fertility and/or the maintenance of pregnancy.

Role of CSF-1 in brain development

In the adult brain there is an extensive network of microglia throughout the parenchyma. In general, they are regarded as a member of the mononuclear phagocyte system and play a major role in the cytokine network of the central nervous system. However, their origin, differentiation, and function have remained unknown. In addition to microglia, there has been gathering interest in the role of perivascular cells in the brain. They are characterized by having large granular and autofluorescent materials in the cytoplasm, and express scavenger receptors.[34,35]

However, the biological role of the cells and the relationship between perivascular cells and microglial cells have not been clearly elucidated. We have reported that the number of perivascular cells in *op/op* mice is reduced compared with that in normal littermates.[19] Recently, Wegiel et al. reported that both the number and the length of processes of perivascular cells were normal in the brains of *op/op* mice, but the number of microglial cells in *op/op* mice was reduced to nearly half the values in control mice.[36] Moreover, microglial cells of *op/op* mice were smaller and had shorter processes. Their findings suggest that CSF-1 plays a role in the *in vivo* formation and maturation of microglia. Sawada et al. demonstrated that there are two subpopulations of microglial cells in normal littermate mice as determined by phase brightness, with one subpopulation shown to be absent in *op/op* mice.[37] We have noticed that *op/op* mice do not move around as actively as their normal littermates and do not respond to sound. Pollard et al. recently reported that *op/op* mice bear auditory and visual disturbance.[38] Taking this all together, a future study on *op/op* mice may shed light on the origin, differentiation, and neurobiological significance of microglial cells and perivascular macrophages in the central nervous system.

Conclusion

CSF-1 acts as a critical molecule for inducing certain macrophage populations such as marginal metallophilic macrophages and osteoclasts, while dendritic cell differentiation is not influenced by CSF-1. Several tissue macrophage populations require CSF-1 for their differentiation, but dependency on CSF-1 is variable. CSF-1 and/or other factor(s) synergistically regulate macrophage differentiation to generate heterogeneous macrophage populations *in vivo*. It has been also demonstrated that *op/op* mice are a useful model for analyzing the regulatory role of CSF-1 in pregnancy and possibly in brain development.

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