Granulocyte-Macrophage Colony-Stimulating Factor Is Not Responsible for the Correction of Hematopoietic Deficiencies in the Maturing op/op Mouse


Osteopetrotic (op/op) mice are characterized by an autosomal recessive inactivating mutation resulting in the absence of biologically active colony-stimulating factor-1 (CSF-1). Consequently, young op/op mice have a severe deficiency of macrophages and osteoclasts resulting in excessive bone formation, occlusion of the marrow cavity, and reduced marrow hematopoietic activity. Recently, we showed that the osteopetrosis and hematopoietic deficiencies evident in young op/op mice are not permanent but are progressively corrected with age. There are increases in osteoclast activity; bone resorption; femoral marrow space; and marrow hematopoietic activity, cellularity, and macrophage content. In the present study we show that CSF-1-/- granulocyte-macrophage colony-stimulating factor (GM-CSF)-/- mice also undergo the same pattern of hematopoietic correction as the op/op mouse. Also, like the op/op mouse, the peritoneal cellularity and macrophage content of CSF-1/GM-CSF-deficient mice remains severely reduced. Our data show that the "knockout" of GM-CSF does not change the op/op phenotype, and that GM-CSF is not essential for the correction of the hematopoietic deficiencies in the op/op mouse. Importantly, the data also show that neither GM-CSF nor CSF-1 is an absolute requirement for the commitment of primitive hematopoietic stem cells to the macrophage lineage or for the differentiation of at least some classes of macrophages. This finding suggests that an alternate regulatory factor can be involved in macrophage and osteoclast commitment, differentiation, and function in vivo.

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MICE. Mice deficient in CSF-1 (op/op) and/or GM-CSF, as well as wild-type genotypes, were generated as previously described.22 Homozygous wild-type, heterozygous, and homozygous null genotypes at each locus are reported as +/+, +/−, and −/−, respectively. For these experiments, 5-week-old CSF-1-/- GM-
CSF-/- mice and their wild-type littermates were raised at the Ludwig Institute for Cancer Research, and the 5-week-old op/op mice and their wild-type littermates were raised at the Peter MacCallum Cancer Institute. All experimental work was performed at the Peter MacCallum Cancer Institute.

**Cell suspensions.** Marrow cells from both CSF-1-/-GM-CSF-/- and CSF-1-/-GM-CSF+/+ mice were collected by flushing femoral shafts with cold HEPES-buffered balanced salt solution (BSS) supplemented with 2% bovine calf serum iron-supplemented (BCS). Femurs from osteopetrotic CSF-1-/-GM-CSF-/- and CSF-1-/-GM-CSF+/+ mice were routinely ground using a mortar and pestle, because less than 20% of marrow cells from osteopetrotic mice could be recovered by flushing alone.14,15 The suspensions were gently vortexed to free any cells adhered to bone fragments, and the cell suspension was decanted and pooled, together with three washings of the bone fragments.

Spleen cell suspensions were prepared by macerating the tissue in BSS supplemented with 2% BCS and by dispersion through a mesh sieve.

Peripheral blood was collected from a throat bleed into an EDTA-coated tube and mixed well. Excessive red blood cells containing the samples were lysed with 0.83% ammonium chloride (10 ml per 1 mL of red blood cell pellet) at 37°C for 5 minutes, and cells were then washed twice with BSS-2% BCS.

Peritoneal cells were collected by injecting 10 mL of BSS-2% BCS into the peritoneal cavity using a 23-gauge needle. The peritoneum was gently massaged, without removing the needle tip, to free loosely adherent cells before aspirating the solution. The cells were centrifuged at 1,000 rpm at 4°C for 5 minutes and resuspended in BSS-2% BCS.

Cell counts were performed using a hemocytometer.

**Growth factors.** Partially purified pregnant mouse uterus extract was used as a source of CSF-1.17 Recombinant human interleukin-1 (IL-1; 2.5 × 10^5 U/mg protein) was a gift from Hoffman-La Roche Inc (Nutley, NJ). Recombinant murine IL-3 was prepared as conditioned medium from the genetically altered mouse mammary cell line (C127) expressing the mouse cDNA.20 All factors were used at optimal concentrations.20

**Hematopoietic progenitor cell assays.** Marrow cell suspensions were assayed for low and high proliferative potential colony-forming cells (LPP-CFC and HPP-CFC), using a double-layer nutrient agar culture system exactly as previously described.26 After 14 days of incubation, HPP-CFC typically generated colonies greater than 0.5 mm in diameter consisting of tightly packed cells. Functionally, these cells are defined as primitive cells by their relative resistance to 5-FU, their synergistic growth factor requirements, and their co-purification with long-term reconstituting cells in vivo.22,23 LPP-CFC are macrophage lineage-restricted cells responsive to CSF-1 alone and typically generate colonies measuring less than 0.5 mm in diameter.29

**Monoclonal antibodies (MoAbs).** A panel of rat MoAbs was used to investigate age-related changes in the expression of hematopoietic lineage cell surface antigens in each cell suspension. These antibodies included anti-7/4 (neutrophils, activated macrophages, and macrophage lineage-restricted progenitors);26 anti-B220 (B cells);23 anti-MAC-1 (macrophage subsets); anti-L3T4 (CD4) and anti-Lyt-2 (CD8) (T cells);24,25 and anti-GR-1 (neutrophils).25 Antibodies were diluted in phosphate-buffered saline (PBS) (pH 7.4, 290 mOsm) supplemented with 5% BCS (binding buffer) and used at predetermined optimal dilutions.

**Flow cytometric analysis.** Cells were labeled using equal volumes of primary antibody and binding buffer for 30 minutes on ice and then washed three times and incubated with mouse serum-adsorbed fluorescein isothiocyanate (FITC)-conjugated goat antirat IgG (H+L; Kirkegaard and Perry Laboratories, Gaithersburg, MD) for a further 30 minutes on ice. The cells were washed a further three times, resuspended in PBS supplemented with 0.25% BCS, and held at 4°C before flow cytometric analysis.

Labeled cells were analyzed using a FACStarplus cell sorter (Becton Dickinson, Mountain View, CA) equipped with a 5-W argon ion laser (Coherent Innova 90, Palo Alto, CA) running at 200 mW of power. Light-scatter signals were collected through a 488-nm band pass filter (HEPES-forward light scatter path). Background fluorescence (cell surface binding of FITC-conjugated goat antirat secondary antibody) was less than 5%.

**Preparation of microscopic sections.** Tibiae removed from anesthetized (Penthrane; Abbott Laboratories, North Chicago, IL) mice were immersion fixed in 2% formaldehyde and 2% glutaraldehyde for 48 hours, washed in sucrose buffer, and decalcified in 10% EDTA for 2 weeks. Dehydration in graded ethanol was followed by infiltration and embedding in Polaron embedding medium (Biorad, Cambridge, MA). Three-micrometer sections were cut and stained with Giemsa (BDH Chemicals, Kilsyth, Victoria, Australia).

**Statistics.** Analysis of accumulated data involved testing the difference between paired groups of wild-type mice (CSF-1-/-GM-CSF-/-) from different experiments using the Wilcoxon matched pairs test. Because no significant difference was detected, accumulated data were pooled and the differences between means of each age-matched genotype were evaluated by one-way analysis of variance (ANOVA). Significance was then tested using the Newman-Keul's range test.

## Results

Young (5 weeks old) CSF-1-/-GM-CSF-/- mice weighed significantly less than their wild-type littermates, although, like the op/op (CSF-1-/-GM-CSF+/+) mouse, by 40 weeks of age, no difference could be detected between the weights of CSF-1-/-GM-CSF-/- and either op/op mice or wild-type littermates (Table 1).

**Age-related changes in bone marrow cellularity and hematopoiesis.** Young CSF-1-/-GM-CSF-/- mice had a severe deficiency in marrow cellularity similar to that observed in aged-matched op/op mice. By 5 weeks of age, the femoral marrow cellularity of wild-type mice had attained adult levels. In contrast, by 5 weeks of age, the femoral marrow cellularity of CSF-1-/-GM-CSF-/— mice was only 3.9 × 10^6 cells per femur, or 18% of the cellularity of wild-type femurs (Table 1). However, CSF-1-/-GM-CSF-/- mice subsequently demonstrated an age-related correction in femoral cellularity identical to that observed in the op/op mouse.14 By 40 weeks of age, the femoral marrow cellularity of CSF-1-/-GM-CSF-/- mice had become comparable to that of both op/op and wild-type littermate mice (Table 1).

Flow cytometric analysis of the expression of the cell surface antigen F4/80 also showed a similar age-related correction of the marrow macrophage deficiency observed in young CSF-1-/-GM-CSF-/- mice (Table 1) as that observed in maturing op/op mice.13 At 5 weeks of age, the proportion of F4/80+ cells was approximately 75% that of wild-type marrow. By 40 weeks of age, the proportion of F4/80+ cells was comparable to that of wild-type. Because the femoral bone marrow cellularity in mature CSF-1-/-GM-CSF-/- mice and wild-type mice was also comparable, the total numbers of F4/80+ cells per femur were also equivalent.

The presence of equal proportions of macrophages in mature CSF-1-/-GM-CSF-/-, op/op, and wild-type mice was
confirmed by light microscopy (data not shown). These cells were clearly distinguishable from other cell types by the presence of paracrystalline inclusions, which are characteristic of bone marrow macrophages in normal mice older than 60 days. 20,21

At 5 weeks of age, both young CSF-1−/−GM-CSF−/− and op/op mice had a significant increase in the proportion of MAC-1+ cells and Gr-1+ cells compared with that of their wild-type littermates (Table 1). This increase in the proportion of MAC-1+ cells was most likely caused by the increase in granulocytes, because the MAC-1 antigen is expressed by both macrophages and granulocytes. Also, as previously described, there was a lower incidence of F4/80+ macrophages in both young CSF-1−/−GM-CSF−/− and op/op mice compared with wild-type littermates. There was also no evidence of the upregulation of MAC-1 antigen expression, reflected by a shift in mean channel fluorescence (data not shown). However, the absolute number of Gr-1+ cells per femur in both young CSF-1−/−GM-CSF−/− and op/op mice was markedly reduced compared with wild-type control values (1.9 ± 0.6, 2.9 ± 0.4, and 6.9 ± 1.3 × 106, respectively).

At 5 weeks of age, there was also a significant decrease in both the proportion (Table 1) and total numbers of B220+ lymphocytes in both CSF-1−/−GM-CSF−/− and op/op femurs compared with that of their wild-type littermates (0.5 ± 0.2, 1.1 ± 0.2, and 10.0 ± 1.0 × 106, respectively). Although, by 40 weeks of age, the proportion (Table 1) and total number of B220+ cells was comparable in CSF-1−/−GM-CSF−/−, op/op, and wild-type mice.

The hematopoietic potential of CSF-1−/−GM-CSF−/− femoral bone marrow was evaluated by in vitro clonal assay of committed (LPP-CFC) and primitive (HPP-CFC) progenitors. There were no differences in the incidence of LPP-CFC or HPP-CFC in the marrow of CSF-1−/−GM-CSF−/− and wild-type mice at any age examined (data not shown). Therefore, the total femoral content of these progenitors (Table 2) reflected the age-related changes observed in marrow cellularity (Table 1). By 40 weeks of age, the femoral content in CSF-1−/−GM-CSF−/−, op/op, and wild-type mice was comparable (Table 2).

**Age-related changes in splenic hematopoiesis.** Like young op/op mice, the diminished space for marrow hematopoiesis and the reduced marrow hematopoietic activity in young CSF-1−/−GM-CSF−/− mice correlated with an increase in splenic hematopoiesis. Although young CSF-1−/−GM-CSF−/− mice weighed significantly less than their wild-type littermates (Table 1), there was evidence of splenomegaly, with an average spleen cellularity 1.5 times (Table 3) and a spleen weight 1.3 times that of their wild-type littermates (data not shown). The primitive and committed progenitor cell incidence and content, as evaluated by the in vitro clonal assay of LPP-CFC and HPP-CFC, were also significantly elevated. Young CSF-1−/−GM-CSF−/− mice had an average increase in the incidence of LPP-CFC and HPP-CFC 2- and 10-fold that of wild-type littermates, respectively (Table 4).

Evidence of extramedullary hematopoiesis in the spleen of young CSF-1−/−GM-CSF−/− mice was confirmed by flow cytometric analysis (Table 3). At 5 weeks of age, spleens from CSF-1−/−GM-CSF−/− mice were characterized by an increase in granulocytopenia similar to that seen in the op/op mouse. There was a 39%, 32%, and 76% increase in the

### Table 1. Age-Related Changes in Bone Marrow

<table>
<thead>
<tr>
<th>CSF-1 GM-CSF</th>
<th>5-Week-Old Mice</th>
<th>40-Week-Old Mice</th>
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<tr>
<td>+/-</td>
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<td>+/-</td>
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Mouse weight (g) | 17.5 ± 0.4 | 13.8 ± 0.4* | 10.5 ± 1.0* |
Nucleated cells/femur × 10⁶ | 23.3 ± 1.7 | 5.4 ± 0.6* | 3.9 ± 1.2* |
Ag expression (% positive) | | | |
F4/80 | 44.1 ± 2.5* | 22.7 ± 3.2* | 34.1 ± 3.9* |
MAC-1 | 33.4 ± 2.4 | 55.4 ± 1.7* | 46.5 ± 6.5* |
GR-1 | 34.3 ± 2.7 | 53.2 ± 0.8* | 49.2 ± 3.9* |
B220 | 43.1 ± 3.3 | 20.5 ± 3.4* | 15.2 ± 5.8* |
CD4 | 8.6 ± 1.4 | ND | 13.2 ± 2.3 |
CD8 | 2.0 ± 0.9 | ND | 1.7 ± 0.3 |
CD4/CD8 | 10.8 ± 1.8 | 9.8 ± 0.4 | ND |

Values are the means ± SEM of at least three individual mice.

* P < .05.
† For F4/80, a forward versus perpendicular light scatter window was set to exclude lymphocytic and granulocytic cells that may be nonspecifically labeled.
‡ Values presented for the expression of F4/80 antigen were obtained from both 3- and 5-week-old mice.
§ Anti-CD4 and anti-CD8 were used as an antibody cocktail.

### Table 2. Bone Marrow Hematopoietic Activity in Mature Mice

<table>
<thead>
<tr>
<th>CSF-1 GM-CSF</th>
<th>Femoral Content (×10⁶/femur)</th>
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<tbody>
<tr>
<td>+/-</td>
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<td>+/-</td>
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LPP-CFC | 570 ± 116 | 551 ± 112 | 786 ± 139 | 544 ± 76 |
HPP-CFC | 9.4 ± 2.8 | 8.8 ± 5.6 | 14.8 ± 5.4 | 11.2 ± 3.6 |

Values are the means ± SEM of three replicate dishes for three individual mice at 40 weeks of age. No significant difference was detected at the .05 level.
Table 3. Age-Related Changes in Spleen

<table>
<thead>
<tr>
<th></th>
<th>5-Week-Old Mice</th>
<th>40-Week-Old Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Nucleated cells/spleen x10^9</td>
<td>13.8 ± 1.2</td>
<td>22.1 ± 1.8</td>
</tr>
<tr>
<td>Age expression (% positive)</td>
<td>55.1 ± 2.4*</td>
<td>20.9 ± 3.4</td>
</tr>
<tr>
<td>F4/80</td>
<td>24.1 ± 4.5</td>
<td>20.8 ± 2.8</td>
</tr>
<tr>
<td>MAC-1</td>
<td>9.8 ± 1.1</td>
<td>10.3 ± 1.1</td>
</tr>
<tr>
<td>GR-1</td>
<td>14.3 ± 2.9</td>
<td>14.8 ± 1.8</td>
</tr>
<tr>
<td>7/4</td>
<td>5.5 ± 1.0</td>
<td>6.2 ± 1.8</td>
</tr>
<tr>
<td>B220</td>
<td>47.1 ± 2.9</td>
<td>27.8 ± 2.8</td>
</tr>
<tr>
<td>CD4</td>
<td>20.3 ± 1.2</td>
<td>32.5 ± 1.6</td>
</tr>
<tr>
<td>CD8</td>
<td>10.2 ± 1.6</td>
<td>15.6 ± 1.7</td>
</tr>
<tr>
<td>CD4/CD8t</td>
<td>23.0 ± 0.9</td>
<td>19.8 ± 3.2</td>
</tr>
<tr>
<td>ND</td>
<td>14.5 ± 0.9</td>
<td>15.1 ± 0.6</td>
</tr>
<tr>
<td>ND</td>
<td>14.6 ± 4.0</td>
<td>31.6 ± 1.9</td>
</tr>
<tr>
<td>ND</td>
<td>7.2 ± 1.1</td>
<td>16.5 ± 1.7</td>
</tr>
<tr>
<td>Values are the means ± SEM of analysis in at least three individual mice. Abbreviation: ND, not done.</td>
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</table>

* P < .05.
† Anti-CD4 and anti-CD8 were used as an antibody cocktail.

Table 4. Age-Related Changes in Splenic Progenitor Cell Incidence

<table>
<thead>
<tr>
<th>Progenitors/2,500 Cells</th>
<th>5-Week-Old Mice</th>
<th>40-Week-Old Mice</th>
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<tbody>
<tr>
<td>CSF-1</td>
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<td></td>
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<tr>
<td>GM-CSF</td>
<td>+/+</td>
<td>+/-</td>
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<tr>
<td></td>
<td>+/+</td>
<td>+/-</td>
</tr>
<tr>
<td>LPP-CFC</td>
<td>3.7 ± 0.5</td>
<td>2.7 ± 1.1</td>
</tr>
<tr>
<td>HPP-CFC</td>
<td>0.03 ± 0.03</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>Values are the means ± SEM of three replicate dishes for three individual mice.</td>
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</table>

* P < .05.
related increase in this population was detected in either CSF-1-/-GM-CSF-/- or op/op mice (Table 6).

The proportion of B220+ peritoneal cells was significantly increased in both 5-week-old CSF-1-/-GM-CSF-/- and op/op mice compared with wild-type littermates (Table 6). Although, because of the significant reduction in peritoneal cellularity, the total number of B220+ cells remained significantly lower in both 5-week-old CSF-1-/-GM-CSF-/- and op/op mice compared with wild-type littermates (2.7 ± 0.4, 1.1 ± 0.1, and 5.3 ± 1.6 × 10^5, respectively). Similarly, although no differences in the proportion of B220+ peritoneal cells could be detected by 40 weeks of age, the total number of peritoneal B220+ cells in CSF-1-/-GM-CSF-/- and op/op mice compared with wild-type littermates remained significantly reduced (1.2 ± 0.9, 2.1 ± 0.5, and 50.0 ± 16.3 × 10^5, respectively), because no age-related correction in peritoneal cellularity was observed.

The proportion of CD4+ and CD8+ cells was significantly increased in 5-week-old CSF-1-/-GM-CSF-/- mice compared with wild-type littermates (Table 6). However, as a consequence of the significant reduction in peritoneal cellularity in 5-week-old CSF-1-/-GM-CSF-/- mice compared with wild-type littermates (Table 6), there was no significant difference in the total number of CD4+ and/or CD8+ cells (1.4 ± 0.1 and 1.4 ± 0.3 × 10^5, respectively). By 40 weeks of age, the proportion of CD4+ and/or CD8+ cells in both op/op and CSF-1-/-GM-CSF-/- mice remained significantly higher than wild-type littermates, but, because there was no increase in peritoneal cellularity, no significant difference was detected between the total numbers of CD4+ and/or CD8+ cells in CSF-1-/-GM-CSF-/- and op/op mice compared with wild-type littermates (1.5 ± 0.7, 3.2 ± 1.1, and 17.2 ± 7.5 × 10^5, respectively).

**DISCUSSION**

This study demonstrates that young CSF-1-/-GM-CSF-/- mice have the same hematopoietic characteristics and deficiencies as we previously described for the op/op mouse.14-16 Young CSF-1-/-GM-CSF-/- mice also undergo a similar age-related correction of the hematopoietic deficiencies evident in the young mouse as that described in the op/op mouse.14-16 All measured hematopoietic parameters, except for peritoneal cellularity and macrophage content, became comparable to that of wild-type littermates by 40 weeks of age. Therefore, as with the op/op mouse, the osteopetrosis and hematopoietic deficiencies in young CSF-1-/-GM-CSF-/- mice are not permanent, but undergo a progressive age-related correction.
It is interesting to note that there was a comparable incidence and content of peripheral blood monocytes in CSF-1⁻/⁻ GM-CSF⁻/⁻, op/op, and wild-type mice at all ages examined. In addition, there was no age-related correction of the deficiencies in peritoneal cellularity or macrophage content in either CSF-1⁻/⁻ GM-CSF⁻/⁻ or op/op mice by 40 weeks of age, suggesting an essential requirement for CSF-1 for this macrophage subpopulation. These findings are in agreement with the data recently published by Cecchini et al.,38 whose observations also indicate differences in the dependency of various subpopulations of mononuclear phagocytes on CSF-1.

CSF-1⁻/⁻ GM-CSF⁻/⁻ mice have a pulmonary pathology often complicated by infection23 that may have an impact on the hematopoietic profile we have described. For example, the increase in splenic 7/4⁺ cells can be explained by an increase in activated macrophages, perhaps resulting in part from the pulmonary pathology. This finding is consistent with the large increase in F4/80⁺ splenic cells, both proportionally and numerically.

Other studies have indicated that the daily administration of CSF-1 to newborn op/op mice is required to prevent osteopetrosis and the accompanying hematopoietic deficiencies.12,13 Our previous studies have shown that the hematopoietic system has the capacity to use alternative mechanisms to compensate for the absence of biologically active CSF-1 in op/op mice.14-16 The present study demonstrates conclusively that one of the growth factors that might have been suspected to ameliorate the hematopoietic deficiencies involved, GM-CSF, is not essential for the correction of the hematopoietic deficiencies in the op/op mouse, particularly in macrophage development.

The data also show that neither GM-CSF nor CSF-1 is an absolute requirement for the commitment of primitive hematopoietic stem cells to the macrophage lineage or for the differentiation of at least some classes of macrophages. This finding suggests that an alternate, regulatory growth factor can be involved in macrophage production and function in vivo. This may be an as yet unidentified factor or may be a manifestation of plasticity among the known hematopoietic factors.17

ACKNOWLEDGMENT

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