Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects

Xu-Ming Dai, Gregory R. Ryan, Andrew J. Hapel, Melissa G. Dominguez, Robert G. Russell, Sara Kapp, Vonetta Sylvestre, and E. Richard Stanley

The effects of colony-stimulating factor 1 (CSF-1), the primary regulator of mononuclear phagocyte production, are thought to be mediated by the CSF-1 receptor (CSF-1R), encoded by the c-fms proto-oncogene. To investigate the in vivo specificity of CSF-1 for the CSF-1R, the mouse Csf1r gene was inactivated. The phenotype of Csf1r−/− mice closely resembled the phenotype of CSF-1-nullizygous (Csf1op/Csf1op) mice, including the osteopetrotic, hematopoietic, tissue macrophage, and reproductive phenotypes. Compared with their wild-type littermates, splenic erythroid burst-forming unit and high-proliferative potential colony-forming cell levels in both Csf1op/Csf1op and Csf1r−/− mice were significantly elevated, consistent with a negative regulatory role of CSF-1 in erythropoiesis and the maintenance of primitive hematopoietic progenitor cells. The circulating CSF-1 concentration in Csf1r−/− mice was elevated 20-fold, in agreement with the previously reported clearance of circulating CSF-1 by CSF-1R-mediated endocytosis and intracellular destruction. Despite their overall similarity, several phenotypic characteristics of the Csf1r−/− mice were more severe than those of the Csf1op/Csf1op mice. The results indicate that all of the effects of CSF-1 are mediated via the CSF-1R, but that subtle effects of the CSF-1R could result from its CSF-1–independent activation.

Introduction

Colony-stimulating factor 1 (CSF-1) regulates the survival, proliferation, and differentiation of mononuclear phagocytic cells and is the primary regulator of mononuclear phagocyte production in vivo.1,2 However, CSF-1 also regulates cells of the female reproductive tract and plays an important role in fertility.3,4 The effects of CSF-1 are mediated by a high-affinity receptor tyrosine kinase (CSF-1R) encoded by the c-fms proto-oncogene.2 The CSF-1R is expressed on primitive multipotent hematopoietic cells,9,10,11 mononuclear phagocyte progenitor cells,12 monoblasts, promonocytes, monocytes,5,6 tissue macrophages,6,13-15 osteoclasts,16 B cells,17,18 smooth muscle cells,19 and neurons.20,21 CSF-1R messenger RNA (mRNA) is expressed in Langerhans cells,22 in the female reproductive tract, in oocytes and embryonic cells of the inner cell mass and trophoderm,23 in decidual cells,24-26 and in cells of the trophoblast.24,25 The expression of the CSF-1R on primitive hematopoietic cells that are unable to proliferate in vitro in response to CSF-1 alone10,11 but are able to proliferate and differentiate if stimulated with combinations of CSF-1 and other hematopoietic growth factors10,11,27 suggests that CSF-1R is involved in the regulation of more primitive hematopoietic cells than those that form macrophage colonies in vitro in response to CSF-1 alone.

Mice homozygous for the mutation osteopetrotic28 possess an inactivating mutation in the coding region of the CSF-1 gene and are devoid of detectable CSF-1.29,30 These Csf1fop/Csf1fop mice are osteopetrotic because of an early and marked deficiency of osteoclasts28 that spontaneously recovers with age,31,32 probably because of the action of vascular endothelial growth factor.33 However, the phenotype of these mice is pleiotropic.7 They are toothless; have low body weight, low growth rate, and skeletal abnormalities; and are deficient in tissue macrophages.2,28,30,34,35 They have defects in both male and female fertility, neural development, the dermis, and syновial membranes.3 The pleiotropic phenotype of the Csf1fop/Csf1fop mouse may be due to a reduction in trophic and/or scavenger functions of the tissue macrophages regulated by CSF-1, secondary to the reduction of their concentration in tissues,7 because outside the female reproductive tract the CSF-1R is primarily expressed in mononuclear phagocytes.13 However, it is possible that some of these effects may also be due to loss of function of other cells such as neuronal cells and muscle precursors, which have also been reported to express the CSF-1R.20,30,36

To address the questions of whether CSF-1 activates other receptors besides the CSF-1R and, conversely, whether the CSF-1R mediates the response to ligands other than CSF-1, we have carried out the targeted inactivation of the CSF-1R gene. The present study describes the phenotype of mice homozygous for a targeted CSF-1R-null mutation and compares it with the phenotype of Csf1fop/Csf1fop mice. The slightly more severe phenotype of Csf1r−/− over Csf1fop/Csf1fop mice indicates that the effects of CSF-1...
are uniquely mediated through the CSF-1R and that subtle effects of the CSF-1R could result from its CSF-1–independent activation.

Materials and methods

Construction of the Csf1r gene-targeting vector

Mouse Csf1r 5′ promoter region and exon 2 primers included cfms-75F, 5′-GGC ACG GGG CTC CCA GCT GCT AGT TCT GTG-3′, and cfms-497R, 5′-AAG GGC AGA TGA GAA AGG TAG TAT GAA GAA TGF-3′; and mouse Csf1r 3′ terminal region and exon 22 sequence primers included cfms-3024F, 5′-CCT CAG CTT GGC CCG ACT CTG ACA ATT CAG-3′, and cfms-3360R, 5′-AGT GAA GGT CAA GAG TAGT TGG CCAATA ATG-3′. These primers were synthesized and submitted to Genome Systems (St Louis, MO) to screen a 129Sv mouse-derived embryonic stem (ES) cell genomic P1 library. Three genomic clones containing the Csf1r gene were obtained. One of these clones was mapped by a combination of restriction enzyme digestion, field inversion gel electrophoresis, Southern analyses, polymerase chain reaction (PCR), and sequencing. A 12-kb AseI fragment spanning exons 2 to 6 was subcloned into the Smal site in the multiple cloning site of pGEM7Z in which the Apal site had been mutated, and the subclone was designated pGEM-Ase-Csf1r. The AseI fragment was subjected to restriction enzyme mapping, PCR, and DNA sequencing to generate the restriction map shown in Figure 1A. A humanized green fluorescent protein (hGFP) sequence and the neomycin resistance (PGKneo) cassette was cloned in-frame into the Apal site in the exon 3 of pGEM-Ase-Csf1r, and a PGK-tk (thymidine kinase) cassette was cloned into the ClaI site in the multiple cloning site of the pGEM-Ase-Csf1r, yielding the final targeting vector (Figure 1A). A unique MluI site in the vector was used for linearization.

ES cell culture and chimeric mouse production

Mouse ES cells (Go Germline; Genome System) (ESVJ-1182) derived from 129SvJ strain were cultured on feeder layer of mitomycin C–treated mouse embryonic fibroblasts (MEFs) in ES cell medium (Dulbecco modified Eagle medium containing leukemia inhibitory factor [LIF]; Gibco BRL, Rockville, MD) at 37°C and 7.5% CO2. MEF preparation, ES cell propagation, electroporation, and selection of recombinants with G418 and gancyclovir (Ganc) were carried out exactly as described in the Genome Systems Manual. Briefly, ES cells were expanded on MEF feeder layers; after trypsinization and preplating to remove MEF, 1 × 10⁶ ES cells were electroporated with 20 μg of the MluI-linearized targeting vector DNA. Twenty-four hours later, G418 and Ganc were added into the LIF-containing ES cell medium for selection over 6 days. G418/Ganc ES clones were then picked and replated onto gelatinized 24-well plates in ES cell medium. After a further 2 days, the ES cells were trypsinized and split in 2, one half being used to expand cells for DNA extraction and the other half cultured for 2 further days before freezing. Genomic DNA was extracted from the expanded cells, digested with HindIII, and subjected to Southern blotting with an intron 7 probe to identify the targeted clones. The correctly targeted allele yielded an 8-kb HindIII band that was clearly resolved from a 7-kb wild-type allele and 8-kb targeted allele HindIII fragments depicted below it. (B) Southern blot analyses of the DNA from individual G418 and gancyclovir-resistant ES cell clones using the probe shown in A. Asterisks mark clones possessing the targeted clones. (C) Anti–CSF-1R Western blot analysis of BMM (upper panel) and 3′-actin (lower panel) are shown. (D) Growth curves of progeny of double heterozygote (Csf1r+/Csf1r−) crosses (n = 5 for each genotype). (E) Serum CSF-1 concentration determined by a radioimmunoassay that selectively detects biologically active CSF-1 (≥ SD; n = 5 for each genotype).

Mice

To obtain germline transmission, chimeras were mated with C57Bl/6J × C3HeBFeJ-a/a strain mice on which the Csf1r allele was maintained. All mice were maintained on this segregating background, behind a barrier at the Albert Einstein College of Medicine animal facility. Csf1r+/Csf1r− and Csf1r+/Csf1r− mice were distinguished from normal siblings at 10 days of age by the absence of incisor eruption and were fed ad libitum. Powdered diet mixture of mouse food and infant milk formula (Esfamil) daily to improve their nutritional status. Control mice received mouse chow ad libitum. Genotyping of the Csf1r allele was carried out as described.

Radiographic analysis of mouse skeletal structure and CSF-1 radioimmunoassay

Radiographs were produced by exposing euthanized or anesthetized mice in a Faxitron pathology specimen x-ray cabinet (Faxitron X-Ray, Buffalo, NY) at a length of 200 μm and a voltage of 34 kV. The right tibiae were cut at metaphysis level and placed in 70% ethanol for 30 min. Samples were scanned using Faxitron X-Ray. The right femur and ilium were used to measure serum CSF-1 concentration using a radioimmunoassay (RIA) kit (Peninsula Laboratories, San Carlos, CA) as described.

Figure 1. Targeted disruption of the mouse Csf1r gene: decreased growth rate and increased circulating CSF-1 in Csf1r+/Csf1r− mice. (A) The targeted region of the Csf1r gene, the Csf1r gene-targeting vector, and the correctly targeted allele, showing exons 1 to 7, restriction enzyme sites (As, Ase; H, HindIII; A, Apal; C, ClaI), PCR primers used (P1–P6, see text), the in-frame humanized green fluorescent protein (hGFP) sequence and the neomycin resistance (PGKneo) and thymidine kinase (PGKtk) cassette above the flanking intron 7 probe were used to identify the 7-kb wild-type allele and 8-kb targeted allele HindIII fragments depicted below it. (B) Southern blot analyses of the DNA from individual G418 and gancyclovir-resistant ES cell clones using the probe shown in A. Asterisks mark clones possessing the targeted alleles (middle panel) and control primers for β-actin (lower panel) are shown. (C) Anti–CSF-1R Western blot analysis of BMM (upper panel) and 3′-actin (lower panel) are shown. (D) Growth curves of progeny of double heterozygote (Csf1r+/Csf1r−) crosses (n = 5 for each genotype). (E) Serum CSF-1 concentration determined by a radioimmunoassay that selectively detects biologically active CSF-1 (≥ SD; n = 5 for each genotype).
Grove, IL). The animals were posed immediately above a fine-grained Polavid 665 instant negative film package. Exposure was set at 50 kV for 1.5 minutes. The negatives were developed and printed according to the manufacturer’s instructions (Polaroid, Cambridge, MA). CSF-1 in mouse sera was determined by a radioimmunoassay that selectively detects biologically active CSF-1.14,15

**Immunohistochemistry and histochemistry**

Rat monoclonal antibody to F4/80 was a gift from Dr David Hume (Department of Microbiology, University of Queensland). For immunostaining with F4/80 antibodies and histochemical localization of tartrate-resistant acid phosphatase (TRAP), siblings of the different genotypes were perfused and tissues were fixed, decalcified (knee joint only), embedded, sectioned, and immunostained as described.1-3 TRAP staining was carried out as described.4 Quantitative histomorphometric analyses of the hema
toxin and eosin–stained sections were performed using a digital camera to capture images. Image analysis was performed by using Image-Pro Plus (Media Cybernetics, Silver Spring, MD). F4/80+ cells in tissue sections of at least 2 mice of a particular genotype at each age were quantitated as described.2 Whole-mount preparations of the 4th inguinal mammary gland were stained with alum carmine as described.4 For the estrus cycle parameters were analyzed by using an automatic blood cell counter. 

**Statistical analyses**

The mean, SD, or SEM of all numeric data was calculated. Data were analyzed by either Student’s *t* test or chi-square test, where appropriate. Comparisons of data sets yielding *P* > .05 were considered not statistically significant.

**Results**

**Targeted disruption of the mouse CSF-1R gene**

A P1 clone containing the entire coding region and flanking DNA of CSF-1R gene was used to prepare the targeting construct (Figure 1A) as described in “Materials and methods.” This vector was linearized and electroporated into 129SV1 strain ES cells, and clones of transfected cells resistant to both G418 and Ganc were screened for homologous recombinants by Southern blotting and PCR (Figure 1B). Of the 164 G418/Ganc-resistant clones obtained, 20 were identified as homologous recombinants and 2 of these (2C5 and 6D3) were injected into blastocysts. Of the 5 chimeras obtained, 3 (2 derived from 6D3 and one from 2C5) were transmitted to the germline. These lines were maintained on the same C57BL/6J x C3HeB/FeJ-a background as the Csf1r+/- mice. Western blot analysis of whole cell lysates of BMM prepared from Csf1r+/- and littermate control mice indicated that the Csf1r+/Csf1r+ cells were devoid of CSF-1R (Figure 1C, top panel). Consistent with these results, RT-PCR analysis using primers P5 and P6 (Figure 1A) indicated that both Csf1r+/Csf1r+ and Csf1r+/ Csf1r+ cells expressed mRNA encoding the CSF-1R–hGFP fusion protein (Figure 1C, lower panels). However, no significant expression of GFP could be detected in Csf1r+/Csf1r- or Csf1r+/Csf1r- cells by fluorescence microscopy or flow cytometry (data not shown).

**Gross phenotype of Csf1r+/Csf1r- mice**

Csf1r+/Csf1r- mice were identical in appearance to Csf1r+/- mice. They were small (see below) and toothless and possessed truncated limbs, a domed skull, and, occasionally, a kinked tail. Studies with Csf1r+/- mice indicate that they have a low body weight and a low growth rate.28,37 Mice homozygous for the Csf1r+/- were deaf (data not shown).

A comparison of the genotypic frequencies for single heterozygous crosses (Csf1r+/- x Csf1r+/- and Csf1r+/Csf1r- x Csf1r+/Csf1r-) is presented in Table 1. At birth, the frequency of the progeny of Csf1r+/- were expected for a nondeleterious gene inherited in a Mendelian fashion. By weaning, however, the survival of Csf1r+/- Csf1r- mice approximated the survival of Csf1r+/Csf1r+ mice, and the survival of both mutant mice was significantly lower than the survival of wild-type mice, as previously reported for Csf1r+/- mice.28 Analysis of the data from double heterozygote crosses at weaning (Table 2) revealed that survival of Csf1r+/- Csf1r+/- mice was not significantly different from the survival of Csf1r+/Csf1r- mice or of double mutant Csf1r+/- Csf1r+/Csf1r+ , except for the survival of Csf1r+/Csf1r+, Csf1r+/- Csf1r+/- mice, which unexpectedly exhibited normal survival, suggesting that the Csf1r+/- Csf1r+/- phenotype might be slightly less severe than the Csf1r+/- Csf1r+/- phenotype. Previous studies from our laboratory indicated that 95% of the circulating CSF-1 is cleared by CSF-1R–mediated endocytosis and intracellular destruction by sinusoidally located macrophages and...
that circulating CSF-1 has a half-life of only 10 minutes.\(^{48}\) We therefore determined the CSF-1 concentration in the sera of \(\text{Csfr}^r/\text{Csfr}^r\), \(\text{Csfr}^r/\text{Csfr}^r\), and \(\text{Csfr}^r/\text{Csfr}^r\) (Figure 1E). Consistent with these previous observations, levels of serum CSF-1 in \(\text{Csfr}^r/\text{Csfr}^r\) mice were elevated approximately 20-fold compared with the levels in \(\text{Csfr}^r/\text{Csfr}^r\) and \(\text{Csfr}^r/\text{Csfr}^r\) littersate control mice. Interestingly, the levels of circulating CSF-1 in the \(\text{Csfr}^r/\text{Csfr}^r\) mice (19.0 ± 5.4 ng/mL) were within normal range (19.9 ± 6.1 ng/mL).

### Comparison of the osteopetrotic phenotypes of \(\text{Csfr}^r/\text{Csfr}^r\) and \(\text{Csfr}^r/\text{Csfr}^r\)/\(\text{Csfr}^r/\text{Csfr}^r\) mice

\(\text{Csfr}^r/\text{Csfr}^r\) mice exhibit impaired bone resorption associated with a reduction in the number of osteoclasts.\(^{28}\) Their inability to remodel bone resulted in general skeletal deformities that, as shown in Figure 2, were shared with the \(\text{Csfr}^r/\text{Csfr}^r\) mice. Compared with wild-type littermates, the long bones of their limbs were short (Figure 2) with increased bone density at the metaphyses (Figure 2), deformities in the flat bony plates result in a domed skull (Figure 2), and the increased bone density in the mandible presumably leads to the failure of tooth eruption (Figure 2).

Interestingly, a radiograph analysis of the temporal changes in the femurs of wild-type and mutant mice with age (Figure 2) indicates that there is significantly more radiopacity in the distal metaphysis of the femurs of \(\text{Csfr}^r/\text{Csfr}^r\) than of \(\text{Csfr}^r/\text{Csfr}^r\) mice. Similar results were obtained with \(\text{Csfr}^r/\text{Csfr}^r\) mice derived from both \(2C5\) and \(6D3\) ES cell lines. All further experiments were carried out with \(\text{Csfr}^r/\text{Csfr}^r\) mice derived from \(2C5\) ES cells.

The metaphyseal radiopacity of the long bones of \(\text{Csfr}^r/\text{Csfr}^r\) and \(\text{Csfr}^r/\text{Csfr}^r\) mice is the result of the failure of osteoclast development at this site. Hematoxylin and eosin staining of longitudinal sections of the distal metaphyseal regions of the femurs of these mice revealed that both \(\text{Csfr}^r/\text{Csfr}^r\) and \(\text{Csfr}^r/\text{Csfr}^r\) mice possessed higher amounts of bony trabeculae than littermate control mice, consistent with impaired bone remodeling (Figure 3A). In addition, \(\text{TRAP}^+\) cells (osteoclasts) were present in low numbers in the bony trabecula regions of \(\text{Csfr}^r/\text{Csfr}^r\) femurs compared with littermate control femurs and even fewer in number in \(\text{Csfr}^r/\text{Csfr}^r\) femurs (Figure 3B). Histomorphometric analyses of the entire bone marrow cavity of the femurs were consistent with these observations, the percentage of trabecular bone in \(\text{Csfr}^r/\text{Csfr}^r\) and \(\text{Csfr}^r/\text{Csfr}^r\) femurs being greater than in wild-type femurs (Figure 3C). In the metaphyseal region, the retention of trabecular bone was greater for \(\text{Csfr}^r/\text{Csfr}^r\) (92%) than for \(\text{Csfr}^r/\text{Csfr}^r\) (83%) mice (compared with wild-type

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**Table 1. Frequency of genotypes of progeny of single heterozygote crosses of \(\text{Csfr}^r/\text{Csfr}^r\) and \(\text{Csfr}^r/\text{Csfr}^r\) mutations**

<table>
<thead>
<tr>
<th>Age of progeny</th>
<th>Cross</th>
<th>Total progeny</th>
<th>Homozygous</th>
<th>Heterozygotes</th>
<th>Homozygous mutant</th>
<th>(\chi^2) test probability*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 d</td>
<td>(\text{Csfr}^r/\text{Csfr}^r\times\text{Csfr}^r/\text{Csfr}^r)</td>
<td>40</td>
<td>12</td>
<td>17</td>
<td>11</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>(\text{Csfr}^r/\text{Csfr}^r\times\text{Csfr}^r/\text{Csfr}^r)</td>
<td>72</td>
<td>21</td>
<td>38</td>
<td>13</td>
<td>0.37</td>
</tr>
<tr>
<td>3 wk</td>
<td>(\text{Csfr}^r/\text{Csfr}^r\times\text{Csfr}^r/\text{Csfr}^r)</td>
<td>214</td>
<td>71</td>
<td>109</td>
<td>34</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>(\text{Csfr}^r/\text{Csfr}^r\times\text{Csfr}^r/\text{Csfr}^r)</td>
<td>393</td>
<td>107</td>
<td>221</td>
<td>65</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*Probability that results do not differ significantly from the 1:2:1 ratio.
†Ratio compared with expected 1:2:1 ratio of an independently segregating gene.

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**Figure 2. Comparison of the skeletal development of wild-type, \(\text{Csfr}^r/\text{Csfr}^r\), and \(\text{Csfr}^r/\text{Csfr}^r\) mice. Radiograms of the heads, bodies, and femurs of mice of the indicated genotypes at different ages (m, months). Each femur is from a different mouse. Arrows indicate regions of increased bone density most easily visualized at this magnification. Also shown is the extent (horizontal line) and fraction (in parenthesis) of the total femur length that is of high radiopacity.**

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**Table 2. Genotypic frequencies as a percentage of total 3-week-old progeny from double heterozygote (\(\text{Csfr}^r/\text{Csfr}^r\times\text{Csfr}^r/\text{Csfr}^r\)), \(\text{Csfr}^r/\text{Csfr}^r\times\text{Csfr}^r/\text{Csfr}^r\), and \(\text{Csfr}^r/\text{Csfr}^r\) crosses**

<table>
<thead>
<tr>
<th>Csfr genotype</th>
<th>(\text{Csfr}^r/\text{Csfr}^r\times\text{Csfr}^r/\text{Csfr}^r)</th>
<th>(\text{Csfr}^r/\text{Csfr}^r\times\text{Csfr}^r/\text{Csfr}^r)</th>
<th>(\text{Csfr}^r/\text{Csfr}^r\times\text{Csfr}^r/\text{Csfr}^r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Csfr}^r/\text{Csfr}^r)</td>
<td>6.25/7.7(a)</td>
<td>12.5/17.6</td>
<td>6.25/3.8</td>
</tr>
<tr>
<td>(\text{Csfr}^r/\text{Csfr}^r)</td>
<td>12.5/15.3</td>
<td>25.0/31.0</td>
<td>12.5/4.2</td>
</tr>
<tr>
<td>(\text{Csfr}^r/\text{Csfr}^r)</td>
<td>6.25/4.2</td>
<td>12.5/16.2</td>
<td>6.25/3.4</td>
</tr>
</tbody>
</table>

*Percentage expected for independently segregating alleles/percentage observed, \(n = 261\).*
mice [43%]), reflecting the greater radiopacity of the Csf1r−/−Csf1r− over Csf1+/-Csf1+ mice in this region (Figure 2).

Consequent to their decreased volume of femoral bone marrow, the total femoral marrow cellularity of Csf1+/-Csf1+ and Csf1r−/−Csf1r− mice was significantly lower than that of the bone marrow cellularity of wild-type mice (Table 3). As previously reported for Csf1+/-Csf1+ mice,31,32 the bone marrow cellularity Csf1r−/−Csf1r− mice recovered to the levels observed in control wild type mice by 8 months of age (Table 3), despite evidence of some residual osteoporosis in both Csf1+/-Csf1+ and Csf1r−/−Csf1r− mice (Figure 2).

Reduced mononuclear phagocyte production in Csf1r−/−Csf1r− mice

Previous reports34,49 have shown that blood monocyte and lymphocyte percentages are reduced in Csf1+/-Csf1+ mice. Fluorescence-activated cell sorter (FACS) analysis of these populations by forward and side-light scatter revealed that there was a decrease in monocytes and lymphocytes and an increase in granulocytes in the circulation of Csf1r−/−Csf1r− mice and that they were not significantly different from Csf1+/-Csf1+ mice in this respect (Figure 4A). Similarly, the total cellularities of the pleural and peritoneal cavities of Csf1r−/−Csf1r− mice were reduced to the same extent as in Csf1+/-Csf1+ mice (Figure 4B) as were the frequencies of Mac1(CD11b)+ cells in these cavities (Figure 4C). Tissue macrophages expressing macrophage-specific cell-surface protein, F4/80, are significantly decreased in many tissues of Csf1+/-Csf1+ mice.2 The F4/80+ cell densities of several such tissues were determined in wild-type, Csf1+/-Csf1+ and Csf1r−/−Csf1r− mice at ages in which the F4/80+ macrophage density had previously been shown to be maximum for the particular wild-type tissue (Figure 5, Table 4).2,50,51 As previously shown,2 the F4/80+ cell densities of the tissues of Csf1+/-Csf1+ mice were significantly lower than those of wild-type control mice (including the Langerhans cells of the epidermis, previously reported to be normal in Csf1+/-Csf1+)

Table 3. Bone marrow cellularity of 7-week-old and 8-month-old mice

<table>
<thead>
<tr>
<th>Age</th>
<th>Wild type</th>
<th>Csf1+/-Csf1+</th>
<th>Csf1r−/−Csf1r−</th>
</tr>
</thead>
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<tr>
<td>7 wk</td>
<td>0.50 ± 0.10</td>
<td>0.25 ± 0.04*</td>
<td>0.23 ± 0.10*</td>
</tr>
<tr>
<td>8 mo</td>
<td>1.57 ± 0.28</td>
<td>1.90 ± 0.14</td>
<td>1.53 ± 0.82</td>
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</table>

*Significantly different from wild type (Student t test P < .01).
mice). There was no difference between the lowered F4/80+ cell densities of these tissues and those of Csf1r−/Csf1r− mice, except that the F4/80+ cell density in the kidney was significantly lower in Csf1r−/Csf1r− mice than in Csf1r+/Csf1r+ mice (Table 4). Thus, apart from this difference, the mononuclear phagocyte numbers were equivalently reduced in Csf1r−/Csf1r− and Csf1+/Csf1+ mice.

Hematopoietic progenitor cells in Csf1r−/Csf1r− mice

Previous experiments have shown that, consistent with their reduced space for marrow hematopoiesis (Table 3, Figures 2 and 3), 6-week-old Csf1r+/Csf1r+ mice have a compensatory splenic hematopoiesis with elevated frequencies of CFU-Cs and HPP-CFCs.31 As in the case of the hematopoiesis with elevated frequencies of CFU-Cs and HPP-CFCs in Csf1r−/Csf1r− mice, splenic weights (milligram per gram of body weight) of 6-week-old Csf1r−/Csf1r− mice (3.6 ± 0.2), the splenic weights (milligram per gram of body weight) of week-old Csf1r−/Csf1r− mice (5.1 ± 0.5) were significantly elevated compared with those of littermate control wild-type mice (3.0 ± 0.3 [± SD], n = 3). The hematopoietic status of the wild-type, Csf1+/Csf1+ mice, Csf1r−/Csf1r−, and Csf1r−/Csf1r− mice was examined by determining bone marrow and splenic density of hematopoietic progenitor cells at 6 weeks of age (Figure 6). Splenic BFU-Es and HPP-CFCs were elevated to a similar extent in both types of mutant mouse. There was no significant effect of either mutation on CFU-GM or CFU-GEMM. As expected, in contrast to the elevated frequency of CFU-C responsive splenic culture in Csf1r−/Csf1r− mice, there were no CFU-1-responsive progenitors in spleens of the Csf1r−/Csf1r− mice. In the bone marrow of both Csf1r−/Csf1r− and Csf1r−/Csf1r− mice, there was no difference in the frequency of these progenitors compared with their frequency in wild-type mice, save for the absence of CFU-Cs in the Csf1r−/Csf1r− mice. These results reflect a negative regulatory role of CSF-1 on the in vivo frequencies of splenic BFU-Es and HPP-CFCs.

Reproductive phenotype of Csf1r−/Csf1r− mice

CSF-1 plays an important role in ovulation, preimplantation, placental function, regulation of the estrus cycle, and lactation,34,52-55 Estrous cycling times are altered in mature Csf1r−/Csf1r− mice, estrus occurring irregularly and more infrequently compared with the estrous times of normal mice (~5 days).53 The duration of estrous in female Csf1r−/Csf1r− mice, determined by the appearance in the vagina of exfoliated anuclear cornified cells and the absence of macrophages, although lower (3.0 ± 0.5, n = 10; Figure 7A) than the previously reported cycling time for Csf1+/Csf1+ mice (~14.5 days), was significantly higher than the duration of estrus in wild-type mice (5.8 ± 0.5, n = 20; Figure 7A). As previously reported for the Csf1+/Csf1+ mice, this increase in cycling time was primarily due to an increase in the duration of the diestrus period (Figure 7B). In pregnant Csf1+/Csf1+ mice, the lactating mammary gland fails to develop normally because of a

Table 4. Tissue F4/80+ cell densities

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age of mice</th>
<th>Wild type</th>
<th>Csf1+/Csf1+</th>
<th>Csf1−/Csf1−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow*†</td>
<td>2 wk</td>
<td>67.9 ± 1.17</td>
<td>210.5 ± 14.8</td>
<td>173.7 ± 41.0</td>
</tr>
<tr>
<td>Liver*†</td>
<td>2 d</td>
<td>67.8 ± 108.4</td>
<td>413.4 ± 13.0</td>
<td>463.7 ± 45.3</td>
</tr>
<tr>
<td>Liver*†</td>
<td>4 mo</td>
<td>372.9 ± 44.2</td>
<td>1243 ± 7.2</td>
<td>150.0 ± 68.2</td>
</tr>
<tr>
<td>Kidney*†‡</td>
<td>2 wk</td>
<td>184.3 ± 83.0</td>
<td>345 ± 1.6</td>
<td>123.3 ± 0.4</td>
</tr>
<tr>
<td>Thymus*†</td>
<td>2 wk</td>
<td>376.4 ± 40.1</td>
<td>110.6 ± 18.1</td>
<td>168.3 ± 15.2</td>
</tr>
<tr>
<td>Langerhans cells*†</td>
<td>2 d</td>
<td>15.8 ± 1.3</td>
<td>3.0 ± 1.4</td>
<td>2.0 ± 1.4</td>
</tr>
<tr>
<td>Dermis*†</td>
<td>2 d</td>
<td>253.1 ± 56.0</td>
<td>15.4 ± 5.1</td>
<td>325 ± 10.0</td>
</tr>
<tr>
<td>Testes*†</td>
<td>2 wk</td>
<td>178.3 ± 83.2</td>
<td>46.7 ± 22.2</td>
<td>36.6 ± 19.4</td>
</tr>
</tbody>
</table>

Densities in cells/mm² average of multiple counts (± SD, n = 4) of tissue sections from at least 2 mice per genotype.

*Significant differences between wild type and Csf1+/Csf1+ (P < .05).
†Significant differences between wild type and Csf1−/Csf1− (P < .05).
‡Significant differences between Csf1+/Csf1+ and Csf1−/Csf1− (P < .05).
§Significant differences between Csf1+/Csf1+ and Csf1−/Csf1− (P < .05).

Significantly elevated compared with wild-type control mice (3.0 ± 0.3 [± SD], n = 3). The hematopoietic status of the wild-type, Csf1+/Csf1+ mice, Csf1r−/Csf1r−, and Csf1r−/Csf1r− mice was examined by determining bone marrow and splenic frequency of hematopoietic progenitor cells at 6 weeks of age (Figure 6). Splenic BFU-Es and HPP-CFCs were elevated to a similar extent in both types of mutant mouse. There was no significant effect of either mutation on CFU-GM or CFU-GEMM. As expected, in contrast to the elevated frequency of CFU-C responsive splenic culture in Csf1r−/Csf1r− mice, there were no CFU-1-responsive progenitors in spleens of the Csf1r−/Csf1r− mice. In the bone marrow of both Csf1r−/Csf1r− and Csf1r−/Csf1r− mice, there was no difference in the frequency of these progenitors compared with their frequency in wild-type mice, save for the absence of CFU-Cs in the Csf1r−/Csf1r− mice. These results reflect a negative regulatory role of CSF-1 on the in vivo frequencies of splenic BFU-Es and HPP-CFCs.
failure of branching morphogenesis of the ductal epithelium. As shown in the whole-mount stained mammary glands in Figure 7C, a similar phenotype was observed for mammary glands from pregnant Csf1r−/Csf1r− mice.

Compared with normal males, Csf1r+/Csf1r+ male mice had low testosterone levels, low libido, and reduced viable sperm numbers, as well as mating infrequently and displaying a long latency between mating when presented serially with female mice in estrus. A similar phenotype was observed for Csf1r−/Csf1r− mice, which mated less frequently with cycling females and produced fewer pregnant females on successive days following daily exposure to different superovulated females than wild-type males (Figure 7D).

Discussion

In this study, we have shown that BMM from mice in which both Csf1r alleles have been targeted by the insertion of a PGKneo cassette containing stop codons into exon 3 fail to express detectable CSF-1R mRNA or protein. In contrast, BMM from wild-type mice express approximately 50 000 cell surface CSF-1R molecules per cell. Consistent with this observation, neither bone marrow nor splenic cells from the Csf1r−/Csf1r− mice are able to form CSF-1–dependent macrophage colonies in agar cultures. Furthermore, the circulating concentrations of CSF-1 in the Csf1r−/Csf1r− mice were 20-fold higher than the wild-type CSF-1 concentration, consistent with a failure of the previously established, CSF-1R–mediated clearance mechanism for circulating CSF-1. These results indicate that the Csf1r−/Csf1r− mice are truly CSF-1R nullizygous.

The phenotype of the Csf1r−/Csf1r− mice was very similar to the phenotype of the Csf1r+/Csf1r+ mice lacking CSF-1. The growth rates and postnatal lethalities of Csf1r−/Csf1r−, Csf1r+/−, and the double-mutant Csf1r−/Csf1r−;Csf1r+/− mice are indistinguishable. Of interest was the demonstration that postnatal lethality of both mutants was not detectable at day 1 but became apparent by 3 weeks of age (Table 1). Both Csf1r−/Csf1r− and Csf1r+/Csf1r+ mice were toothless and severely osteopetrotic with the same osteopetrosis-associated skeletal abnormalities and with a reduced bone marrow cellularity that returns to normal levels by 8 months of age. There was a similar depletion of circulating monocytes, pleural and peritoneal cavity cells, peritoneal cavity Mac1+ cells, and tissue macrophages in both mutant mice. With the exception of CSF-1–dependent CFU-Cs, which cannot be measured in the Csf1r−/Csf1r− mice, there was no difference in their levels of bone marrow hematopoietic progenitor cells, and both mutants had splenic BFU-E and HPP-CFC levels that were significantly higher than in wild-type control mice. Furthermore, both mutants shared previously reported defects in reproductive function, including longer estrus cycles, failure of normal lactating mammary gland development, and a reduced male mating performance. As no phenotype in the Csf1r+/Csf1r+ mice.

Figure 6. Hematopoietic progenitor cell concentrations in the spleen and bone marrow of 6-week-old mice. (A) Splenic progenitor cell colony numbers per 10⁵ cells (BFU-E, CFU-GM, CFU-GEMM, CFU-C) or per 10⁴ cells (HPP-CFC). Means ± SD (3 mice per genotype). *Significantly different from wild type; **Significantly different from wild type and Csf1r+/Csf1r+ (P ≤ .01).

Figure 7. Reproductive phenotype of Csf1r−/Csf1r− mice. (A) Duration of estrus cycle in virgin female mice (5 mice per genotype, 8 cycles/mouse, ± SD). (B) Percentage time in proestrus (P), in estrus (E), in metestrus (M), and in diestrus (D) (± SD). (C) Whole-mount alum-carmine staining of the 4th inguinal mammary gland from 18-day pregnant mice. All panels are at the same magnification, approximately only one third of the wild-type gland is shown. LN indicates lymph node. (D) Percentage of successful pregnancies resulting from the consecutive daily mating of wild-type (open) and Csf1r−/Csf1r− (filled) male mice with superovulated virgin female mice. *Indicates significantly different from wild type; wt, wild type.
was more severe than in the Csf1r−/Csf1r− mice, these results indicate that all of the effects of CSF-1 are mediated via the Csf1R that is encoded by the c-myc gene.

Despite the overall similarity of the Csf1r−/Csf1r− and Csf1op/Csf1op mouse phenotypes, aspects of the Csf1r−/Csf1r− phenotype were more severe than those of the Csf1op/Csf1op mouse. These aspects include a slightly more severe osteopetrosis in the femurs (Figures 2 and 3) and a more severe depletion of F4/80+ cells in the kidney (Figure 5, Table 4). In addition, there was a difference in the postnatal survival of mice homozygous for one allele and heterozygous for the other. Csf1op/Csf1op, Csf1r−/Csf1r− mouse survival was as expected for 2 independently segregating alleles with no associated lethality, whereas the survival of Csf1r+/Csf1r−:Csf1+/Csf1+ mice was one third that rate (Table 2). Because both Csf1r+/Csf1r−;Csf1op/Csf1op and Csf1r−/Csf1r−:Csf1+/Csf1+ mice also survived at one third of the expected rate, this finding suggests that a single copy of the CSF-1R gene may confer an advantage in the absence of CSF-1. This observation, with significant numbers of progeny, supports the concept of a CSF-1–independent protective effect of the CSF-1R, although it is not clear how 2 copies of the CSF-1R gene fail to protect. It is possible that some of these effects were due to partial rescue of the Csf1op/Csf1op mice by transplacental passage of CSF-1 from the heterozygotic mothers, which is not possible in the case of the Csf1r−/Csf1r− mice. However, because no difference was observed between the liver F4/80+ cell densities of 2-day-old Csf1op/Csf1op and Csf1r−/Csf1r− mice and nearly 80% of the fetal hepatic blood flow is derived directly from the umbilical vein, this seems unlikely. Furthermore, the increased severity of Csf1r−/Csf1r− phenotype has recently been confirmed by using the progeny of mice of a single strain on which both alleles have been backcrossed for more than 5 generations. On this background, the Csf1r−/Csf1r− phenotype is even more severe and the Csf1r−/Csf1r− mice all die by 3 weeks of age, whereas the Csf1op/Csf1op survival approximates their survival on the C57BL/6 × C3HeB/FeJ-a/a background. These observations suggest that the CSF-1R can respond to an additional ligand or can function in a ligand-independent fashion in some situations.

It has previously been suggested, in part because of the residual tissue macrophage production seen in certain tissues in Csf1op/Csf1op mice and in part because of the nature of the mutation, that this mouse is not completely devoid of a mutated yet active form of CSF-1. However, as indicated above, the Csf1op/Csf1op and Csf1r−/Csf1r− phenotypes were very similar. In particular, with one exception, every Csf1r−/Csf1r− tissue possessed macrophage concentrations that did not significantly differ from those of the corresponding Csf1op/Csf1op tissue. These observations strongly suggest that the residual tissue macrophage populations in Csf1op/Csf1op mice are not due to the action of a mutated less-efficient CSF-1. Thus, the present study indicates that other growth factors are involved in the regulation of macrophage production observed in Csf1op/Csf1op and Csf1r−/Csf1r− mice. Among the known growth factors most likely to be responsible for macrophage production in these mutant mice are GM-CSF and IL-3 and vascular endothelial growth factor. GM-CSF and IL-3 together are capable of correcting not only the osteopetrotic condition of Csf1op/Csf1op mice but also macrophage deficiencies in several tissues. Vascular endothelial growth factor is also able to correct the osteopetrotic condition of Csf1op/Csf1op mice and appears to be responsible for the age-related increase in osteoclasts in these mice.

It has previously been reported that the percentage of blood monocytes and lymphocytes is reduced and the percentage of blood granulocytes increased in Csf1op/Csf1op mice, without significant changes in the circulating leukocyte concentration. These changes in monocytes and granulocytes are not unexpected, given the specificity of CSF-1 for the mononuclear phagocytic lineage and the existence of progenitors capable of giving rise to both granulocytes and macrophages. However, the decrease in the percentage of blood lymphocytes, which we also observed in both Csf1op/Csf1op and Csf1r−/Csf1r− mice in the present study, is of interest. Recently, it has also been shown that the frequencies of stromal cell– and IL-7–dependent B-cell precursors and CFU–IL-7 in bone marrow were significantly diminished in Csf1op/Csf1op mice, as well as other osteopetrotic mice (Fos-nullizygous and microphthalmia (Mitf−/Mitf−)). However, these precursors could be generated from Csf1op/Csf1op bone marrow precursor cells in vitro incubation with stromal cells in the presence of IL-7. Also, CSF-1 has been shown to reduce apoptosis in precursor B cells in whole mouse bone marrow cultures but not in cultures of B220+ B-lineage cells, implying that CSF-1 can indirectly regulate B lymphopoiesis. These results suggest that the decreased B lymphopoiesis in Csf1op/Csf1op mice results from alterations of the microenvironment rather than from a B-cell autonomous defect. The CSF-1R-nullizygous mice will be useful in analyzing direct and indirect effects of CSF-1 in B lymphopoiesis.

The development of the CSF-1R–nullizygous mouse permits new approaches to be taken to the analysis of CSF-1 action. In studies of CSF-1R signal transduction, a powerful approach to our understanding of CSF-1R structure-function has been to introduce mutated forms of the receptor into fibroblast or myeloid cell lines that do not express the CSF-1R. The functions affected by particular CSF-1R mutations have been found to differ substantially, indicating the importance of cellular context for experiments of this kind (reviewed in Hamilton). By using BMM and progenitor cells from Csf1r−/Csf1r− mice and cell lines derived from them, as well as “knock-in” mutation approaches based on the “knock-out” construct used here, it is now possible to carry out these experiments in the correct cellular context.

Circulating CSF-1 is composed of both proteoglycan and glycoprotein forms. The analysis of these forms and their regulation has been limited by the amounts of mouse serum needed and the low specific activity of serum CSF-1 per unit serum protein. The 20-fold elevation of the concentration (and specific activity) of serum CSF-1 in Csf1r−/Csf1r− mice will greatly facilitate these investigations.

Perhaps the area of most immediate import is the assessment of the role of CSF-1 in the regulation of primitive hematopoietic cells. Previous studies have indicated that, although CFU-Cs are the most primitive cells that can be stimulated to proliferate and differentiate by CSF-1 alone in vitro, CSF-1 can cause the proliferation and differentiation of precursors of the CFU-Cs, by synergizing with other growth factors that alone have limited or no effect on the proliferation of the primitive cells. Indeed, the observation in the present study that splenic BFU-E and HPP-CFC levels in Csf1op/Csf1op and Csf1r−/Csf1r− mice are significantly elevated already suggests that CSF-1, via the CSF-1R, negatively regulates the maintenance of these precursor cells. Consistent with the observed elevation of BFU-Es and HPP-CFCs, CSF-1 has been shown to inhibit erythroid and erythroid/myeloid colony formation in vitro. CSF-1 has also been shown to inhibit the differentiation of ES cells to blood cells other than macrophages.
has recently been shown that ectopic expression of the CSF-1R in the multipotent hematopoietic EML cell line decreases their erythroid potential, raising the possibility that CSF-1R regulates erythroid differentiation and/or commitment. Because of the cell autonomous nature of the Csf1r-/- mutation, it is now possible to study the role of the receptor in the generation of primitive cells of different lineages by using bone marrow transplantation. Such an approach permits testing of CSF-1/CSF-1R regulation of cell proliferation and differentiation in the absence of an endogenous osteopetrotic condition.

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References

Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects

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