RAPID COMMUNICATION

Mice Lacking Both Macrophage- and Granulocyte-Macrophage Colony-Stimulating Factor Have Macrophages and Coexistent Osteopetrosis and Severe Lung Disease

By Graham J. Lieschke, Edouard Stanley, Dianne Grail, George Hodgson, Vincent Sinickas, John A.M. Gall, Roger A. Sinclair, and Ashley R. Dunn

Mice deficient in granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF, CSF-1) were generated by interbreeding GM-CSF−/− mice with M-CSF−/− osteopetrosic mice (genotype M−/−, op/op). Mice deficient in both GM-CSF and M-CSF (genotype GM−/−M−/−) are viable and have coexistent features corresponding to mice deficient in either factor alone. Like M-CSF−/− mice, they have osteopetrosis and are toothless because of failure of incisor eruption. Like GM-CSF−/− mice, they have a characteristic alveolar proteinosis-like lung pathology, but it is more severe than that of GM-CSF−/− mice and is often fatal. In particular, in GM−/−M−/− mice the accumulation of lipo-proteinaceous alveolar material is more marked, and bacterial pneumonia infections are more prevalent and more extensive, particularly involving Gram-negative bacteria. Neutrophilia consistently accompanies pulmonary infections, and some older GM−/−M−/− mice have polycthemia. Survival of GM−/−M−/− mice is significantly reduced compared with mice deficient in either factor alone, and all GM−/−M−/− mice have broncho- or lobar-pneumonia at death. These observations indicate that in vivo, M-CSF is involved in modulating the consequences of GM-CSF deficiency in the lung. Interestingly, GM−/−M−/− mice have circulating monocytes at levels comparable with those in M-CSF−/− mice and the diseased lungs of all GM−/−M−/− mice contain numerous phagocytically active macrophages, indicating that in addition to GM-CSF and M-CSF, other factors can be used for macrophage production and function in vivo.

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THE OVERLAPPING activities of the various hematopoietic growth factors in vitro, both in terms of the populations of cells on which they act and also their effects on those cells, have raised the possibility that there may be redundancy amongst these factors in vivo. One such group of hematopoietic regulators is that influencing the proliferation and function of cells of the monocyte/macrophage lineage, which include granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF, also known as CSF-1). A definitive way to determine if redundancy exists amongst hematopoietic regulators in vivo is to study mice deficient in individual factors and combinations of factors.

In a previous study, we generated mice deficient in GM-CSF by disruption of the GM-CSF gene using gene targeting in embryonal stem cells. GM-CSF−/− mice have relatively unperturbed baseline hematopoiesis in terms of the number of circulating hematopoietic cells and progenitor cell levels in hematopoietic organs. However, GM-CSF−/− deficient mice have an invariable but nonfatal lung disorder characterized by accumulation of alveolar material, and vulnerability to pulmonary infections with a range of opportunistic bacterial and fungal organisms, showing an irreproducible role for GM-CSF in pulmonary physiology.

Mice deficient in M-CSF arose as a result of the spontaneously occurring osteopetrotic (op) mutation, which is a point mutation in the M-CSF gene that results in truncated and inactive M-CSF. M-CSF−/− mice are characterized by osteopetrosis and an age-dependent macrophage deficiency, and have been instructive in defining unique roles for M-CSF in hematopoiesis and bone development in vivo.

It is possible that the consequences of deficiency of one factor may be ameliorated in part by compensatory effects of another factor. For example, the pulmonary consequences of GM-CSF deficiency may be ameliorated by compensatory effects of other hematopoietic growth factors. Although the demonstration of elevated levels of hematopoietic growth factors remaining in GM-CSF−/− mice would provide indirect evidence for this hypothesis, a more definitive approach is to combine deficiency of a second hematopoietic growth factor with GM-CSF deficiency. This genetic approach may result in new or exacerbated phenotypic characteristics in addition to those evident in animals deficient in either factor alone, providing insights to distinguish between the unique and overlapping in vivo roles of the factors in question.

Among the factors that remain in GM-CSF−/− mice, M-CSF is of particular interest, because of its lineage-specific role in augmenting macrophage production and function in vivo, and because large, phagocytically active macrophages are a prominent feature of the pulmonary pathology in GM-CSF−/− mice. Similarly, it is of interest to define the extent to which GM-CSF may be involved in compensating for M-CSF deficiency. Therefore, we interbred GM-CSF−/− mice with M-CSF−/− osteopetrotic mice (genotype M−/−, op/op) to generate the interbreeding GM-CSF−/−M−/−, GM−/−M−/−, GM−/−M−/−, and GM−/−M−/− mice described above.

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teopetric mice to generate mice with combined GM-CSF and M-CSF deficiency.

Although mice deficient in both GM-CSF and M-CSF have coexistent features of both individual deficiency states, they have a number of unexpected features. GM-CSF– and M-CSF–deficient mice still have circulating monocytes and phagocytically active tissue macrophages, indicating that factors other than GM-CSF and M-CSF can be used to support macrophage lineage differentiation in vivo. Furthermore, GM-CSF– and M-CSF–deficient mice have a more severe and often fatal form of the GM-CSF–deficiency lung disease, implicating M-CSF in modulating the pulmonary consequences of GM-CSF deficiency in vivo.

MATERIALS AND METHODS

Mice. In this report, for clarity and consistency, the genotypes of mice are designated as follows: the GM-CSF locus is given as “GM,” the M-CSF (CSF-I, op) locus as “M”: at each locus, homozygous wild-type, heterozygous, and homozygous null genotypes are given as +/+, +/-, and −/− respectively. Homozygous GM-CSF–deficient mice (GM−/− M+/+) and appropriately outbred GM+/+ + M+/+ wild-type controls were generated as described previously.6 Initial GM−/− M+/− mice were generated by interbreeding GM+/+ M−/− and GM+/- M+/+ mice and selecting the GM+/+ M+/− progeny for interbreeding to generate the various genotypes including GM−/− M−/− and GM+/+ M+/+ (ie, op/op). To generate additional GM−/− M−/− animals, GM−/− M+/+ mice were mated. Animals were housed in a conventional animal facility. M−/− animals were identified after 10 days of age by the failure of incisor eruption, but because initially all genotypes were of interest, all pups were retained. Toothless M−/− mice were separated from littersmates at weaning and fed a pureed mixture of powdered mouse food (Imbrot; Hobart, Tasmania, Australia) and powdered nutritional supplement (Ensreau; Abbot Australasia, Kurnell, New South Wales, Australia); littermates were fed mouse food pellets ad libitum. Mice were killed if they developed signs of illness. Some confirmatory observations on M−/− (op/op) mice were made on animals from the animal facility of the Peter MacCallum Cancer Institute (generously provided by S. Nilsson and I. Bertonecillo).

Polymerase chain reaction (PCR) diagnosis of GM-CSF and M-CSF genotype. Mice were genotyped at the GM-CSF and M-CSF loci by PCR analysis of tail DNA after 3 weeks of age, or on DNA prepared from tissues obtained at postmortem examination. The PCR determination of allelic status at the GM-CSF locus was performed as previously described.8 Although op/op (M−/−) mice are readily recognized in litters from M+/− x M+/− matings by their “toothless” phenotype, heterozygous (M+/-) and wild-type (M+/+) littermates are not distinguishable by phenotypic features, and in experiments with op/op (M−/−) mice it has been typical to use a random selection of M+/− and M+/- toothed littersmates as controls without precise definition of their genotypic status.12 To eliminate this imprecision and the heterogeneity that a possible gene-dosage effect might introduce, particularly in the face of GM-CSF deficiency, a PCR-based strategy for defining allelic status at the M-CSF locus was devised. By comparison of the murine cDNA sequence13,14 with the published incomplete genomic structure of the human M-CSF gene,15,16 it was predicted that the op point mutation lay in an exon homologous to human exon 3. Primers complimentary to murine cDNA sequences were used to amplify a fragment from murine genomic DNA corresponding to the intron 5′ of this exon, and 300 bp of the 3′ end of this 1.6-kb PCR-generated fragment was sequenced (G.J.L., unpublished data, March 1993). The diagnostic PCR paired a 5′ sense primer corresponding to sequences from this intronic sequence (5′-TGTTGCTCCTCTCTCAGATTACA-3′) with a 3′ antisense primer corresponding to exonic M-CSF sequences immediately 3′ of the op point mutation (5′-GGCTCTACCTTATTAGCTGTGTACCGCAGC-3′). PCRs (20 μL) contained ~250 ng DNA, 67 mmol/L Tris-HCl (pH 8.8), 16.6 mmol/L (NH₄)₂SO₄, 0.45% Triton X-100, 200 μg/mL gelatin, 2.0 mmol/L MgCl₂, 200 μmol/L of each dNTP, 12.5 ng of each primer, and 1.5 U of Taq polymerase. These primers generated a PCR product of 195 bp or 196 bp, dependent on whether or not the thyminide base insertion of the op mutation was present in template DNA. A 2-bp mismatch in the 3′-antisense primer (underlined), introduced a second Bgl I site into PCR product spanning the extra base of the op mutation that was absent from PCR product from wild-type template. PCR product was digested with Bgl I (10 U/20 μL PCR), which cut at the intrinsic Bgl I site generating a 96-bp product in all cases (confirming restriction enzyme activity in each PCR reaction), and generating either a 99-bp fragment diagnostic for wild-type template, or 70-bp and 30-bp fragments indicative of the extra base in PCR product generated from op template. These small fragments were separated by electrophoresis in 4% Metaphor agarose gels (FMC BioProducts, Rockland, ME) and visualized by ethidium staining. Thus, heterozygous (op/+), homozygous (op/op), and wild-type (M+/+) mice could be distinguished on the basis of this diagnostic PCR alone (Fig 1).

Hematologic analysis. Hemoglobin, total leukocyte, and platelet levels in peripheral blood were determined on 1:4 dilutions of eye-bled samples using a Sysmex K-1000 automated counter (Toa Medical Electronics Co Ltd, Kobe, Japan), and manual 100-cell leukocyte differential counts performed on May-Grünwald/Giemsa–stained smears. To confirm the presence of monocytes, some fresh blood smears were stained with fluorescein isothiocyanate-conjugated F4/80 antibody (Serotec MCA497F; Oxford, England). Histologic analysis. For histologic analysis, formalin-fixed paraffin-embedded sections of mouse organs were stained using standard techniques with hematoxylin and eosin (H&E), and selected sections with the Grocott methamine silver, period-acid-Schiff (PAS), toluidine blue, and Gram stains. Bones were decalcified before mounting and sectioning.

For electron microscopy, random strips of fresh lung tissue were
immersion-fixed in 2.5% glutaraldehyde in 1% cacodylate buffer (pH 7.4), postfixed in 2% aqueous osmium tetroxide, and processed into Araldite-Epon resin using standard techniques. Thin sections were stained with alkaline lead citrate and uranyl acetate and viewed in a Jeol 1200EX electron microscope (Jeol Ltd, Tokyo, Japan).

To assess the range of pulmonary pathology in GM−/−M−/− mice, lung sections from 19 GM−/−M−/− mice were examined in detail (ages 24 to 324 days, median 40 days; 13 were postmortem specimens from mice found dead, 4 were from mice killed because of apparent illness, 2 were from mice killed for unrelated experiments), and compared with sections of lungs in our histology library from over 50 GM−/−M+/+ mice (ages 1 to 297 days), 30 GM−/+M+/+ mice (1 to 297 days) (which formed the basis of our previous report of GM-CSF−deficient mice), 14 GM−/+M−/− mice (35 to 324 days; 1 killed because of distress, 13 killed for unrelated experiments), and 3 GM−/+M−/− mice (35 to 117 days; all killed for nonrespiratory illnesses).

Microbiologic analysis. Specimens of fresh mouse tissues including lungs for microbiologic analysis were obtained at postmortem examination or immediately after killing. They were prepared immediately for examination by microscopy after Gram staining, and cultured for bacteria and fungi according to standard techniques.22

Statistics. Survival data were analyzed by constructing Kaplan-Meier survival curves and compared using the Mantel-Cox statistic. There was no difference between the survival of GM−/−M−/− and 12 GM−/+M−/− mice (P = .23); therefore, these data were pooled for survival comparisons. Other data were compared using the Chi-squared test and unpaired t-test as appropriate. Comparisons of peripheral blood hematologic data were confined to groups of mice of different genotypes sampled on the same day. A P value of <.05 was regarded as statistically significant.

RESULTS

Viability and fertility of GM−/−M−/− mice. From initial interbreeding of GM+/+M+/+ mice, litters of 7 ± 2 pups (n = 15) were born. GM-CSF allelic status was determined by PCR analysis, and M-CSF allelic status by the diagnostic PCR developed for this purpose (Fig 1). The nine possible genotypes were represented in approximately the expected Mendelian ratios at weaning of 144 genotyped pups (Table 1). In particular, GM−/−M−/− were not significantly under-represented relative to wild-type mice, indicating that GM−/−M−/− mice were viable and there was no major fetal or neonatal loss, although amongst this genotype, male mice were over-represented (Table 1).

Subsequent interbreeding of GM−/−M+/+ mice generated litters of which 25/164 (15%) of pups were GM−/−M−/−; this was significantly fewer than expected (P < .05), and as observed in crosses of heterozygous parents, there was again a trend toward male preponderance (19/25) (P < .1). GM−/−M−/− males were fertile; one such male mated with a GM−/−M+/+ female has sired five litters of 5 ± 3 pups. In view of the difficulties experienced by others,3 we have not attempted to breed from M−/− females, regardless of GM-CSF status.

Survival of GM−/−M−/− was compared with that of all other toothless mice (GM−/−M+/− and GM+/+M−/−), mice deficient only in GM-CSF (GM−/−M+/+), and wild-type (GM+/+M+/+) mice. All toothless (M−/−) mice had significantly reduced survival compared with wild-type and GM-CSF-deficient littermates, regardless of whether they were only M-CSF deficient, or were also GM- and M-CSF deficient (Fig 2). Median survivals were: GM−/−M+/+ and GM+/+M−/−, not attained with follow-ups in excess of 300 days; GM+/+M+/+ (231 days (n = 26); GM−/−M−/−, 71 days (n = 38). The median survival of GM−/−M+/+ and GM−/−M−/− mice was significantly shorter than that of GM+/+M+/+ mice (P = .02), and that of GM−/−M−/− mice was significantly shorter than that of other M−/− mice (P = .02) (Fig 2). The difficulties with nutrition of toothless GM+/+ or GM+/− mice contributed to the death of most animals of these genotypes, although one GM−/−M−/− mouse died of acute lobar pneumonia at 231 days, and three were killed for incidental reasons (snout abscess [88 days], an abdominal mass which proved to be a hernia [117 days], and glossitis [118 days]). In contrast, acute broncho- or lobar-pneumonia contributed to the death of most GM−/−M−/− mice (see below); at histologic examination of 19 GM−/−M−/− mice that died or were killed at ages from 24 days to 324 days because of apparent illness, all had severe lung disease and acute lobar pneumonia at the time of death; some of the mice were killed because of apparent distress were tachypneic, suggesting that the lung pathology in these mice was significantly compromising pulmonary function.

| Table 1. Ratio of Genotypes in Litters From Matings of Mice Heterozygous for Both M-CSF and GM-CSF Null Mutations |
|---|---|---|---|---|
| Genotype | Expected Ratio | Observed |
| | of 16 | of 144 | *Total | *Male (%) |
| +/+ | +/+ | 1 | 9 | 10 | 5 (50) |
| +/+ | +/− | 2 | 18 | 20 | 6 (30) |
| +/+ | +/− | 1 | 9 | 10 | 6 (60) |
| +/+ | +/− | 2 | 18 | 20 | 6 (60) |
| +/+ | +/− | 4 | 36 | 40 | 18 (45) |
| +/+ | +/− | 2 | 18 | 20 | 6 (60) |
| +/− | +/− | 1 | 9 | 10 | 9 (90) |
| +/− | +/− | 2 | 18 | 20 | 6 (60) |
| +/− | +/− | 1 | 9 | 10 | 9 (90) |
| +/− | +/− | 2 | 18 | 20 | 6 (60) |

Results from 144 genotyped progeny of 4 double-heterozygous breeding pairs. During this period, genotypes of the following 5 other mice remained in question: 2 GM−/−M−/− males, 2 GM−/−M−/− females, one GM−/−M−/− female.

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levels were not significantly higher than GM+/+M−/− mice. Platelet levels of GM−/−M−/− mice tended to be lower than for mice of other genotypes, although the differences were not marked. There were consistent perturbations of peripheral blood leukocyte populations in GM−/−M−/− mice. A relative lymphopenia was evident in GM−/−M−/− mice of all ages (Table 2), although the degree of lymphopenia was not more marked than that which is typical for GM+/+M+/+ mice (Table 2).11 GM−/−M−/− mice (138 to 170 days) had significantly elevated neutrophil levels compared with age-matched mice deficient in either factor alone (Table 2). The propensity to neutrophilia was apparent at all ages, although statistical significance was not always attained (Table 2). Monocytes were present in the peripheral blood of GM−/−M−/− mice of all ages (Table 2 and Fig 3D), but levels were not consistently different to GM+/+M+/+ or GM−/−M+/+ mice. The presence of circulating monocytes in GM−/−M−/− mice was confirmed by F4/80 immunostaining of blood films (Fig 3E). There were no consistent differences in levels of eosinophils in GM−/−M−/− mice (the 157-day GM−/−M+/+ mice in Table 2 had atypically elevated and varied eosinophil levels).

In the tissues, leukocytes were evident in significant numbers. Despite the lymphopenia in GM−/−M−/− mice relative to GM+/+M+/+ mice, a perivascular lymphoid infiltrate characterized GM−/−M−/− lung pathology, and was similar in distribution to that seen in GM−/−M+/+ mice (Fig 3F). Neutrophils localized to sites of infection, because all GM−/−M−/− examined histologically had acute multifocal bronchopneumonia containing many neutrophils in the inflammatory exudate (Fig 3G). The diseased lungs of older GM−/−M−/− mice contained large numbers of alveolar macrophages (Fig 3H), indicating that tissue macrophages were available for host defenses. These alveolar macrophages had abundant foamy cytoplasm with numerous cytoplasmic particles of diastase-resistant PAS-positive material (Fig 3I), indicating that these particles were likely to be phagocytosed material rather than glycogen granules. On toluidine blue-stained spleen sections, the frequency of mast cells was 2 to 7/mm². In the lungs, mast cells were most common in connective tissue near larger vessels and bronchi and near the serosal surface. Although numbers of pulmonary mast cells were not clearly increased, some aggregates of 10 to 20 mast cells were observed in mediastinal connective tissue of GM−/−M−/− mice. For comparative evaluations with the lung pathology of GM−/−M−/− mice, control sections were selected from the histology library of over 50 age-matched GM+/+M+/+ mice. The lungs of 14 (op/op) GM+/+M−/− mice (35 to 324 days) and 3 GM−/−M+/+ mice (35 to 117 days) showed no consistent abnormal features, although one 321-day GM+/+M−/− mouse was killed because it became unwell had acute lobar pneumonia.

Within the lungs of individual GM−/−M−/− mice, an extensive range of pathologic processes was evident (eg, Fig 4A shows adjacent areas of bronchopneumonia, perivascular lymphoid hyperplasia, and alveoli containing eosinophilic material without inflammatory cells). Although the lungs of GM−/−M−/− mice had many histologic features seen with the lung disease previously observed in GM-CSF-deficient mice, they were also some prominent differences, particularly in the extent of alveolar macrophage accumulation, the prevalence and severity of acute pulmonary consolidation, and the incidence of documented microbial infection.

An invariable feature of the lungs of GM−/−M−/− mice was the presence of a granular, eosinophilic material within alveoli. This material accumulated with age and was already...
MICE LACKING M-CSF AND GM-CSF

Table 2. Peripheral Blood of GM-CSF- and M-CSF-Deficient Mice of Various Ages

<table>
<thead>
<tr>
<th>Age (d)</th>
<th>Genotype</th>
<th>Hemoglobin (g/dL)</th>
<th>Erythrocytes (×10¹²/L)</th>
<th>Platelets (×10¹⁰/L)</th>
<th>Leukocytes (×10⁹/L)</th>
<th>Neutrophils (×10⁹/L)</th>
<th>Lymphocytes (×10⁹/L)</th>
<th>Monocytes (×10⁹/L)</th>
<th>Eosinophils (×10⁹/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29-35</td>
<td>+/+</td>
<td>139 ± 7</td>
<td>7.7 ± 0.7</td>
<td>648 ± 114</td>
<td>6.7 ± 1.7</td>
<td>1.3 ± 0.3</td>
<td>6.3 ± 2.0</td>
<td>0.07 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td>30-32</td>
<td>--/--</td>
<td>149 ± 25</td>
<td>7.3 ± 0.2</td>
<td>412 ± 523</td>
<td>5.1 ± 2.7</td>
<td>3.4 ± 2.7</td>
<td>1.5 ± 0.68</td>
<td>0.17 ± 0.11</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>+/+</td>
<td>154 ± 7</td>
<td>8.2 ± 0.5</td>
<td>737 ± 245</td>
<td>13.6 ± 2.9</td>
<td>2.3 ± 1.0</td>
<td>12.5 ± 2.4</td>
<td>0.17 ± 0.21</td>
<td>0.19 ± 0.15</td>
</tr>
<tr>
<td>49-55</td>
<td>--/--</td>
<td>159 ± 18</td>
<td>8.0 ± 1.1</td>
<td>799 ± 27</td>
<td>7.9 ± 1.45</td>
<td>0.7 ± 0.35</td>
<td>7.0 ± 1.45</td>
<td>0.02 ± 0.04</td>
<td>0.11 ± 0.07</td>
</tr>
<tr>
<td>46-77</td>
<td>+/-</td>
<td>148 ± 05</td>
<td>7.6 ± 0.25</td>
<td>663 ± 237</td>
<td>5.7 ± 1.05</td>
<td>1.5 ± 0.69</td>
<td>4.0 ± 0.51</td>
<td>0.05 ± 0.096</td>
<td>0.08 ± 0.07</td>
</tr>
<tr>
<td>46-77</td>
<td>--/--</td>
<td>162 ± 215</td>
<td>8.5 ± 1.14</td>
<td>624 ± 921</td>
<td>8.4 ± 3.23</td>
<td>4.0 ± 4.1</td>
<td>4.3 ± 1.79</td>
<td>0.13 ± 0.16</td>
<td>0.09 ± 0.13</td>
</tr>
<tr>
<td>157</td>
<td>+/-</td>
<td>175 ± 4</td>
<td>9.8 ± 0.1</td>
<td>741 ± 138</td>
<td>9.3 ± 2.3</td>
<td>1.6 ± 0.6</td>
<td>7.1 ± 1.6</td>
<td>0.21 ± 0.09</td>
<td>0.35 ± 0.26</td>
</tr>
<tr>
<td>124-138</td>
<td>--/--</td>
<td>174 ± 5</td>
<td>9.3 ± 0.5</td>
<td>929 ± 183</td>
<td>8.1 ± 2.1</td>
<td>1.3 ± 0.5</td>
<td>6.5 ± 1.8</td>
<td>0.22 ± 0.11</td>
<td>0.12 ± 0.12</td>
</tr>
<tr>
<td>135</td>
<td>+/-</td>
<td>172 ± 4</td>
<td>10.6 ± 0.51</td>
<td>923 ± 65</td>
<td>6.7 ± 1.4</td>
<td>1.2 ± 0.2</td>
<td>5.2 ± 1.6</td>
<td>0.15 ± 0.06</td>
<td>0.08 ± 0.10</td>
</tr>
<tr>
<td>139-170</td>
<td>--/--</td>
<td>185 ± 104^†</td>
<td>10.9 ± 0.81</td>
<td>604 ± 70^†</td>
<td>6.6 ± 1.15</td>
<td>2.1 ± 0.9^†</td>
<td>4.3 ± 0.9^†</td>
<td>0.05 ± 0.06^†</td>
<td>0.07 ± 0.08^†</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* † Peripheral blood analyses performed on same day.
§ P < .05 compared with GM+/+M+/+.
∥ Total granulocyte count.
# P < .05 compared with GM--/M--/.

extensive by 40 days of age. In some areas it was particulate (Fig 4B), but in other areas it was confluent and contained clefts typical of lipid accumulation (Fig 4C). The alveolar material was PAS-positive and diastase-resistant (Figs 4G and 3I). Particularly in mice younger than 40 days, the amount and extent of granular alveolar material was greater than in age-matched GM--/M+/+ mice.

A lymphoid infiltrate, such as typifies the lung pathology of GM--/M+/+ mice, also occurred in these mice, with a marked perivascular rather than peribronchial distribution (Figs 4A and 3F). In GM--/M--/ lungs the lymphoid infiltrate was a less prominent aspect of the pathologic process than the acute inflammatory alveolar exudate containing neutrophils, in contrast to GM--/M+/+ lungs, where the converse was true.

A major feature of the lungs of all GM--/M--/ mice was acute pneumatic consolidation, which was both more prevalent and markedly more extensive than in GM--/M+/+ mice. Most typically, numerous discrete foci of acute bronchopneumonia occurred throughout all of the GM--/M--/ lungs examined (eg, Figs 4D and 3G), including in lungs of mice that were not distressed but were killed for other experiments. In the lungs examined from GM--/M--/ mice that died, the pneumonia was more extensive, often showed a lobar pattern, and was often bilateral. Acute lung abscesses were found in 2/19 mice (ages 48 and 205 days) (eg, Fig 4E). The abscesses contained neutrophil-rich pus and acute inflammatory cells predominated within the poorly organized walls, although there was a zone of macrophages up to 3 cells deep (Fig 4F).

In the lungs of young (≤40 days) GM--/M--/ mice, alveolar macrophages were sparse compared with age-matched GM--/M+/+ mice, but in older GM--/M--/ mice (>70 days), large alveolar macrophages with foamy cytoplasm were abundant (Fig 3, H and I). Some alveolar macrophages were embedded in homogenous eosinophilic inflammatory exudate. Most macrophages were mononucleated, but binucleated forms occurred (Fig 3H).

Bacteria, predominantly Gram-negative bacilli, were shown by Gram stain of lung sections of 11/19 GM--/M--/ mice (eg, Fig 4G). A variety of bacteria was isolated by microbiologic cultures of 7/7 samples of lung tissue, 5 of which were obtained immediately on killing an animal, and 2 of which were processed immediately after postmortem sampling of dead mice. The bacteria isolated were: Klebsiella oxytoca (3), Pasteurella pneumotropica (2), Pasteurella hemolytica (1), and a mixed infection with Escherichia coli and α-hemolytic streptococci (1); these bacteria were recovered in significant numbers as a pure growth and were seen in Gram stains of tissues submitted for microbiologic analysis. One GM--/M--/ mouse was killed because it developed a facial abscess tracking into the orbit, from which Streptococcus spp. were isolated. The histologic and microbiologic analyses demonstrated bacterial pneumonia to be more prevalent in GM--/M--/ lungs than was observed in GM--/M+/+ lungs from mice held contemporaneously in the same room of a conventional animal house. No fungal organisms were cultured from specimens from GM--/M--/ lungs, and there was no histologic evidence of fungal pathogens among 19 GM--/M--/ lung sections surveyed by Grocott and PAS stains. In particular, there were no foci of infection with the fungal agent identified histologically, such as we have observed in some GM--/M+/+ lungs examined in a similar manner.

Electron microscopy showed aggregates of fibrillary material and numerous type-C lamellar bodies within alveoli (Fig 5A), similar to those seen in GM--/M+/+ lungs. The fibrillar alveolar material was comprised of aggregates of fibrils spaced 49 nm apart, each fibril having four axial ribs on transverse section (Fig 5B); there was a tendency for fibrils to stack in an orderly array. Large alveolar macrophages could be readily identified by their size, nuclear mor-
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...phology, alveolar location, and surface microvilli. Many large alveolar macrophages contained numerous phagocy-
osed type-C lamellar bodies within their cytoplasm (eg, Fig 5A shows a macrophage 18 x 14 μm containing at least 34
type-C lamellar bodies). Some alveolar macrophages also contained amorphous lipid vacuoles* and well-demarcated
long linear crystalloid clefts within their cytoplasm (Fig 5D).

Such clefts were also evident within some alveoli. The appearances of these clefts were typical of those seen in associa-
tion with cholesterol or cholesterol esters (J.M. Papadimitriou, personal communication, April 1994).

DISCUSSION

Analysis of mice deficient in hematopoietic growth factors provides a basis for determining the role of these factors in
regulating physiologic process in vivo. GM-CSF and M-CSF are two hematopoietic growth factors that are both implicated
in regulating the production and function of cells of the monocyte/macrophage lineage. Mice deficient in each of
these factors exist and have characteristic but dissimilar phenotypic features. To investigate the impact of com-
binant deficiency of these two major regulators of cells of the monocyte/macrophage lineage, we generated mice defi-
cient in both factors by interbreeding. Analysis of these doubly deficient mice showed a number of interesting observa-
tions.

Despite lacking both GM-CSF and M-CSF, which have been regarded as two of the major factors influencing the production and differentiation of monocytes and macro-


drophages, GM-/-/M-/-/ mice still have circulating mono-
cytes and tissue macrophages, indicating that at least one other factor can be used for monocyte/macrophage produc-
tion in vivo. Among the factors that may be supporting monocyte and macrophage production in the absence of both
GM-CSF and M-CSF, interleukin-3 (IL-3) is a potential can-
didate. The demonstration of increased IL-3 production by tissues from GM-/-/M-/-/ in vivo and in vitro would
support this notion. The net effect of combined GM-CSF and M-CSF deficiency on IL-3 production is difficult to
predict from studies on mice deficient in the individual factors: increased induced release of GM-CSF and IL-3 were
shown in op/op mice, but we observed reduced IL-3 levels in spleen-conditioned media made from splenocytes from
GM-CSF-deficient mice. An issue complicating the inter-
pretation of this type of analysis is that GM-/-/M-/-/ mice consistently have a granulocytosis, which may reflect an
infection-related granulopoietic growth factor response to pulmonary infections, and levels of granulopoietic growth
factors may be altered for this reason alone. Similar consider-
ations apply to the other factors potentially compensating for combined M-CSF and GM-CSF deficiency, such as IL-
1. Of course, the compensatory role of IL-3 or any other factor could potentially be assessed more directly by super-
imposing deficiency of a third factor on combined GM-CSF and M-CSF deficiency.

It will be of interest to assess progenitor and stem cell levels of all hematopoietic lineages in the marrow and spleen
of GM-/-/M-/-/ mice. Such an analysis will complement studies of factor production in GM-/-/M-/-/ mice by giv-
ing insight at a cellular level into the mechanisms underlying the peripheral blood changes, including the lymphopenia and tendencies to polycythemia and slightly reduced platelet levels. The polycythemia is of particular interest, as it may be

driven in part by increased erythropoietin production second-
ary to hypoxia, reflecting the severity of the lung disease, although this hypothesis requires direct evaluation.

Our analysis shows that the typical GM-CSF-deficiency lung disease, a form of alveolar proteinosis, is exacerbated by concomitant M-CSF deficiency. In particular, in our con-
ventional animal house, M-CSF deficiency transforms the nonfatal GM-CSF-deficiency pulmonary disease into an of-
ten fatal illness, primarily caused by an increased incidence and severity of bacterial acute pneumonia. We have pre-
viously suggested that the accumulation of surfactant phospholipid and proteins may be the primary intrinsic defect in

Fig 2. Hematopoietic cells in GM-CSF- and M-CSF-deficient (GM-/-/M-/-) mice. (A) Osteopetrotic femur of 24-day GM-/-/M-/- mouse with scattered small foci of marrow cells (eg, arrows) (original magnification x 40, H&E). (B) Osteopetrotic tibia of 324-day GM-/-/M-/- mouse showing active granulopoiesis (original magnification x 400, H&E). (C) Circulating monocyte in peripheral blood of GM-/-/M-/- mouse (original magnification x 400, May-Grunwald-Giemsa). (D) Monocytes in thick peripheral blood film shown by immunofluorescence staining with the F4/80 antibody (arrowed, original magnification x 100). (E) Pulmonary lymphocytic infiltrate (eg, arrows) in lung of 68-day GM-/-/M-/- mouse (original magnification x 400, H&E). (F) Neutrophils (eg, arrowed) in acute inflammatory focus in lung of 71-day GM-/-/M-/- mouse (original magnification x 400, H&E). (G) Large foamy intra-alveolar macrophages in lung of 71-day GM-/-/M-/- mouse, including binucleated form (arrowed) (original magnification x 400, H&E). (H) PAS-positive diastase-resistant cytoplasmic granules within intra-alveolar macrophages (arrowed) in lung of 68-day GM-/-/M-/- mouse (original magnification x 400, PAS and diastase). (J) Tissue mast cells (arrowed) in lung of 205-day GM-/-/M-/- mouse (original magnification x 400, toluidine blue).

Fig 4. Lung pathology in GM-CSF- and M-CSF-deficient (GM-/-/M-/-) mice. (A) Low power overview of 68-day GM-/-/M-/- lung showing heterogeneous focal pathologies: perivascular lymphocytic infiltrate (white arrows), eosinophilic inflammatory exudate (black arrows), relatively uneffected area (curved arrow) (original magnification x 40, H&E). (B) Granular PAS-positive alveolar material filling contiguous alveoli of 205-day GM-/-/M-/- lung (original magnification x 400, PAS). (C) Confluent PAS-positive alveolar material containing lipid clefts (eg, arrows) in 324-day GM-/-/M-/- lung (original magnification x 400, PAS). (D) Multifocal acute bronchopneumonia (arrows) in 71-day GM-/-/M-/- lung (original magnification x 200, H&E). (E) Acute pulmonary abscess in 205-day GM-/-/M-/- lung (original magnification x 40, H&E). (F) Gram-negative bacilli were identified in areas of this lung. (F) Detail of macrophages (arrowed) in wall of abscess shown in (F) (original magnification x 400, H&E). (G) Numerous Gram-negative bacilli (arrow) in area of bronchopneumonia (original magnification x 400, Gram stain, same lung as [D]).
the alveolar proteinosis syndrome characterizing GM-CSF–deficient mice, and that infection is a secondary event.\textsuperscript{a} We have further evidence in support of this hypothesis: in GM-CSF–deficient mice reared in cleaner isolated environments, the initial accumulation of alveolar material still occurs, but without the pathologic features such as perivascular lymphocytic infiltrates and focal bronchopneumonia that invariably occur in GM-CSF–deficient mice raised concurrently in a conventional animal house environment (G.J.L., unpublished data, January 1994). To evaluate the impact of infection on the pulmonary pathology and survival of mice with combined GM-CSF and M-CSF deficiency, it will be necessary to reassess these parameters by studying experimentally induced infections in animals reared in a gnotobiotic facility.

Our observations also show that M-CSF–dependent cells contribute to pulmonary host defenses in vivo, at least in the face of GM-CSF deficiency. We hypothesize that the exacerbation of the GM-CSF lung disease by superimposed M-CSF deficiency may be caused in part by a further impairment in phagocyte function or reduction in their numbers, altering the balance of alveolar phospholipid and protein production or clearance. The observation that M-CSF–deficient mice do not have consistent pulmonary pathology although they have reduced numbers of alveolar macrophages\textsuperscript{23} lends support to this hypothesis. It will be interesting to enumerate alveolar macrophages in GM–/–M–/– mice. Although our observations unequivocally implicate M-CSF and M-CSF–dependent cells as pivotally important in ameliorating the pulmonary consequences of GM-CSF deficiency, they do not prove that M-CSF is compensating for GM-CSF deficiency. Compensation by M-CSF for GM-CSF deficiency implies that M-CSF production in GM-CSF–deficient mice occurs at supraphysiologic levels, compared with levels of M-CSF production in wild-type mice. It is also of interest that the infections in GM–/–M–/– mice are not controlled despite the presence of considerable numbers of granulocytes, suggesting either an indisputable role for macrophages and the factors activating them in the control of infection in vivo, or a critical role for GM-CSF in the activation of these granulocytes.

Mice deficient in single or multiple hematopoietic growth factors are an informative model for defining the overlapping and unique roles of these regulators in vivo. Studies of mice deficient in these and other hematopoietic growth factors should significantly further the understanding of their physiologically relevant roles.
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Mice lacking both macrophage- and granulocyte-macrophage colony-stimulating factor have macrophages and coexistent osteopetrosis and severe lung disease

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