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Mice Lacking Three Myeloid Colony-Stimulating Factors (G-CSF, GM-CSF, and M-CSF) Still Produce Macrophages and Granulocytes and Mount an Inflammatory Response in a Sterile Model of Peritonitis

Margaret L. Hibbs, Cathy Quilici, Nicole Kountouri, John F. Seymour, Jane E. Armes, Antony W. Burgess, and Ashley R. Dunn

To assess the combined role of G-CSF, GM-CSF, and M-CSF in myeloid cell production, mice deficient in all three myeloid CSFs were generated (G<sup>−/−</sup>–GM<sup>−/−</sup>–M<sup>−/−</sup> mice). G<sup>−/−</sup>–GM<sup>−/−</sup>–M<sup>−/−</sup> mice share characteristics found in mice lacking individual cytokines: they are toothless and osteopetrotic and furthermore acquire alveolar proteinosis that is more severe than that found in either GM<sup>−/−</sup> or G<sup>−/−</sup>–GM<sup>−/−</sup> mice. G<sup>−/−</sup>–GM<sup>−/−</sup>–M<sup>−/−</sup> mice have a significantly reduced lifespan, which is prolonged by antibiotic administration, suggesting compromised ability to control bacterial infection. G<sup>−/−</sup>–GM<sup>−/−</sup>–M<sup>−/−</sup> mice have circulating neutrophils and monocytes, albeit at significantly reduced numbers compared with wild-type mice, but surprisingly, have more circulating monocytes than M<sup>−/−</sup> mice and more circulating neutrophils than G<sup>−/−</sup>–GM<sup>−/−</sup> mice. Due to severe osteopetrosis, G<sup>−/−</sup>–GM<sup>−/−</sup>–M<sup>−/−</sup> mice show diminished numbers of myeloid cells, myeloid progenitors, and B lymphocytes in the bone marrow, but have significantly enhanced compensatory splenic hemopoiesis. Although G<sup>−/−</sup>–GM<sup>−/−</sup>–M<sup>−/−</sup> mice have a profound deficiency of myeloid cells in the resting peritoneal cavity, the animals mount a moderate cellular response in a model of sterile peritonitis. These data establish that in the absence of G-CSF, GM-CSF, and M-CSF, additional growth factor(s) can stimulate myelopoiesis and acute inflammatory responses. The Journal of Immunology, 2007, 178: 6435–6443.

Myelopoiesis is regulated by a complex network of cytokines and growth factors (1). Although the role of G-CSF in stimulating the production of neutrophils is well-established (2, 3) and G-CSF is used clinically to promote neutrophil production (4, 5), it is clear that other cytokines contribute to the production and/or function of neutrophils. G-CSF-deficient mice (G<sup>−/−</sup> mice), or mice lacking the G-CSF receptor (G-CSF<sup>−/−</sup> mice), are viable but have markedly reduced numbers of circulating neutrophils (6–8), establishing G-CSF as a major regulator of steady-state neutrophil production. Nevertheless, G<sup>−/−</sup> and G-CSF<sup>−/−</sup> mice still produce mature neutrophils. In mice, GM-CSF is dispensable for steady-state hemopoiesis and the maintenance of blood neutrophil levels, but the lungs of mice deficient in either GM-CSF (GM<sup>−/−</sup> mice) or the β-common chain of the GM-CSFR (βc-deficient) manifest pulmonary alveolar proteinosis due to an impaired capacity to clear surfactant (9, 10).

In the context of G-CSF deficiency, other cytokines associated with myelopoiesis, GM-CSF, M-CSF (also known as CSF-1), IL-3, or IL-6, could in principle functionally compensate for the loss of G-CSF. However, mice lacking both G-CSF and GM-CSF (G<sup>−/−</sup>–GM<sup>−/−</sup> mice) still produce neutrophils (11). At birth, G<sup>−/−</sup>–GM<sup>−/−</sup> mice have fewer neutrophils than G<sup>−/−</sup> mice, but this deficiency resolves with age: as adults, G<sup>−/−</sup>–GM<sup>−/−</sup> mice are still neutropenic, but have essentially the same number of bone marrow (BM) and circulating neutrophils, and hematopoietic progenitors as G<sup>−/−</sup> mice (11). This suggests that under certain circumstances, GM-CSF can contribute to steady-state neutrophil production in vivo. The lung abnormalities associated with GM-CSF deficiency are not exacerbated by lack of G-CSF, although G<sup>−/−</sup>–GM<sup>−/−</sup> mice show an increased mortality (11).

IL-6 is a pleiotropic cytokine that has multiple biological activities on a variety of cells (12). Through studies of IL-6-deficient mice, IL-6 has also been found to play a role in emergency granulopoiesis. Although IL-6<sup>−/−</sup> mice have normal steady-state numbers of neutrophils, they are unable to mount a neutrophilia in response to Listeria monocytogenes infection and display a high mortality when challenged with this pathogen using doses that are sublethal in control mice (13, 14). Moreover, IL-6 deficiency superimposed on G-CSFR<sup>−/−</sup> mice leads to a further reduction in neutrophils in blood and BM compared with mice lacking G-CSFR alone, implicating IL-6 as a cytokine that can stimulate granulopoiesis in the absence of normal G-CSF signaling (15).

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Osteopetrotic mice (referred to as op/op or M−/− mice) are naturally occurring mouse mutants that carry a loss-of-function mutation in the M-CSF gene (16). These mice have a limited capacity for bone remodeling and are severely deficient in osteoclasts, monocytes, and mature tissue macrophages (17), as are mice lacking the M-CSFR (18), highlighting the role of M-CSF/M-CSFR signaling in macrophage subpopulation development. GM−/− M−/− mice display all of the features of mice with either growth factor deficiency, however, the alveolar proteinosis associated with GM-CSF deficiency is more severe in the double deficient mice, which are also highly susceptible to bacterial pneumonia (19). Interestingly, GM−/− M−/− mice were consistently found to have a granulocytosis, which may reflect an infection-related granulopoietic growth factor response to pulmonary infection (19). Despite the fact that the mice lack two of the major growth factors implicated in the production and function of monocyte/macrophages, GM−/− M−/− double-deficient mice have peripheral blood monocytes in numbers similar to M−/− mice, and alveolar macrophages from GM−/− M−/− mice retain phagocytic activity (19).

IL-3 displays a spectrum of actions on hemopoietic cells (20), however, deletion of the IL-3 gene via targeting of the alternative subunit does not lead to obvious hemopoietic abnormalities (21). Mice functionally deficient in IL-3, GM-CSF, and IL-5 (generated by intercrossing βc-deficient mice and IL-3−/− mice) lack eosinophils and suffer from alveolar proteinosis; otherwise, hemopoiesis appears normal (22, 23). These animals show no additional pathologies that cannot be attributed to the individual growth factor deficiencies, even when challenged with L. monocytogenes or chemotherapeutic drugs, indicating that an alternative mechanism must exist to produce and activate blood cells in steady-state and emergency hemopoiesis (22, 23).

The roles of cytokines in directing stem and progenitor cells into particular lineages has been studied extensively (24). It is clear that GM-CSF directs stem/progenitor cells toward myeloid pathways (25), but the combined effect of removing all three CSFs (M-, G- and GM-CSF), has not previously been reported. Although the numbers of triple growth-factor-deficient mice born from G−/− GM−/− M−/− parents are in line with Mendelian patterns of inheritance, even under specific pathogen-free (SPF) conditions these mice are prone to infection. Although these animals have detectable circulating neutrophils and monocytes, they have reduced numbers of myeloid cells compared with control, single, or double mutant animals. Nonetheless, G−/− GM−/− M−/− mice are still capable of producing myeloid cells and responding to proinflammatory challenge, indicating that other compensatory mechanisms must exist in these animals.

Materials and Methods

Mice

The generation of G-CSF-deficient (7), GM-CSF-deficient (9), GM−/− M−/− mice (11) has been described. Mice with a loss-of-function mutation in the M-CSF gene (M−/− or op/op mice) have been described (16). Triple CSF-deficient mice (G−/− GM−/− M−/−) were generated by interbreeding G−/− GM−/− M−/− mice and screening progeny for homozygosity at the op allele by PCR as outlined below. Mice were screened for tooth eruption on day 10 and toothless mice (op/op and GM−/− M−/− mice) were fed pureed food (19). All mice were housed in a microisolation facility and maintained as a C57BL/6 × 129/Ola intercross. Animals were regularly tested for specific pathogens, which showed that they carried both rotavirus and Pasteurella pneumotropica, and thus,

FIGURE 1. G−/− GM−/− M−/− mice have poorer survival, are smaller, and have diminished numbers of WBC in blood, spleen, and BM than control mice. A, Kaplan-Meier survival curves of WT, G−/− GM−/−, M−/− (op/op), G−/− GM−/− M−/− (triple) mice, and G−/− GM−/− M−/− mice that were born to parents that had not been treated for 2 wk with the antibiotic baytril (triple + baytril). B, Weight of male WT, G−/− GM−/−, M−/−, and G−/− GM−/− M−/− (triple) mice. Numbers of nucleated cells in (C) blood and (D) BM of WT, G−/− GM−/−, M−/−, and G−/− GM−/− M−/− mice. E, Spleen weight and, F, numbers of nucleated cells in spleen of the animals in C and D. The data are presented as the mean ± SE; n = 8 for WT, G−/− GM−/−, and M−/− mice, and n = 15 G−/− GM−/− M−/− mice.
breeders when first paired were routinely treated for 2 wk by oral application of 25 mg/kg enrofloxacin (bayerl antibiotic; Lyppard Pharmaceuticals). For most experiments, all groups of mice were treated orally at weaning for 2 wk with 25 mg/kg enrofloxacin then allowed to rest for a month before use. At autopsy, animals were assessed for stress on the basis of thymus size and any mice showing a significantly diminished thymus were excluded from data sets. All experiments were performed in accordance with the National Health and Medical Research Council of Australia guidelines for animal experimentation and were approved by the Ludwig Institute for Cancer Research/Department of Surgery Animal Ethics Committee.

Detection of the op allele by PCR

Because our previously published method for detecting the op allele was unreliable (19), we developed a new PCR that involved pairing a 5’sense primer 5’-GCCAAAGGCTATCCACCTCCT-3’ with a 3’antisense primer 5’-CTCATCTATTTGTCGTACGAAAAT-3’ that was immediately upstream of the op mutation. The 3’antisense primer carries a single base-pair mismatch at its most 5’end that was immediately upstream of the op mutation. The 3’antisense primer carries a single base-pair mismatch at its most 5’end that was immediately upstream of the op mutation.

Flow cytometric analyses

Peripheral blood (0.25 ml) was obtained from the retro-orbital venous plexus and depleted of RBC using 0.83% Tris-buffered ammonium chloride before staining. Single-cell suspensions were prepared from spleen using a sieve and passage of cells through a 40-μm filter. Because the op/op and G−/−GM−/−M−/− mice were osteopetrotic, single-cell suspensions of BM were obtained from mice of all genotypes by grinding femurs with a mortar and pestle and filtering cells through a 40-μm filter. FcγRs were blocked with anti-FcγRII Ab (2-4G2) before staining with the following mAbs: RA3-6B2 (B220), 53.7 (CD5), 30-H12 (Thy-1.2), Mac-1 (CD11b), Gr-1 (RB6-8C5) (all from BD Biosciences/BD Pharmingen), F4/80 and AFS98 (c-fms; gift of J. Hamilton, Department of Medicine, University of Melbourne, Melbourne, Australia). Cells were analyzed on a FACScan (BD Biosciences) using propidium iodide to exclude nonviable cells and data were analyzed with Cell Quest software (BD Biosciences).

 Colony assays

Numbers of myeloid progenitors were determined in 1 ml of semisolid 0.3% agar cultures (26). BM cells were plated at 25,000 cells/ml and spleen cells at 100,000 cells/ml in DMEM supplemented with 20% (v/v) FCS and 0.3% (v/v) Bacto agar. Colony formation was stimulated by 10 ng/ml IL-3, 10 ng/ml G-CSF, 10 ng/ml GM-CSF (all from PeproTech), 50 ng/ml stem cell factor (SCF), or 10 ng/ml M-CSF (both a gift of N. Nicola, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). Plates were incubated at 37°C in a fully humidified atmosphere of 10% CO2 in air and colonies were enumerated on day 5 (for G-CSF and SCF) or on day 7 (for all other CSFs).

 Challenge of G−/−GM−/−M−/− mice with thioglycolate

WT, G−/−GM−/−, M−/−, and G−/−GM−/−M−/− mice were given a single i.p. injection of 1 ml of a 3% (w/v) solution of thioglycolate (Difco Laboratories), and euthanized 4, 24, or 72 h after treatment. Peritoneal cavity cells were isolated by peritoneal lavage using 5 ml of DMEM containing 1% (v/v) FCS and untreated mice were used as controls for steady-state numbers of peritoneal cells.

 Histology

Tissues were fixed for light microscopy in Bouin’s solution for 24 h and embedded in paraffin. Sections were stained with H&E according to standard procedures. To objectively grade the severity of the pulmonary phenotype, a total of 59 apparently healthy 8- to 10-wk-old animals had lung tissue harvested, fixed, stained, and examined histologically without knowledge of the genotype. In this analysis, all but two animals were clinically well; one G−/−GM−/− and one G−/−GM−/−M−/− showed small
foci of pulmonary infection. The severity and extent of both alveolar surfactant deposition and pulmonary lymphoid accumulation were scored separately using the previously described reproducible qualitative scale from 0 (normal) to 4 (11). All statistical comparisons were performed using a two-sample t test.

Results

G−/−GM−/−M−/− mice have poor survival

To understand the importance of the three myeloid CSFs—G-CSF, GM-CSF, and M-CSF—for the production of steady-state numbers of myeloid cells, we generated mice simultaneously deficient in all three factors. G−/−GM−/−M−/− mice were generated by interbreeding G−/−GM−/− op/+ mice and then genotyping progeny for the op mutation. In the course of this study, and following genotyping at the op locus, G−/−GM−/−M−/− mice, like M−/− mice, could be confidently identified at 10 days on the basis of a failure of tooth eruption. Of 25 litters that were intact at day 10, 208 mice could be confidently identified at 10 days on the basis of a failure of tooth eruption. Of 25 litters that were intact at day 10, 208 mice were generated that gave rise to 63 G−/−GM−/−M−/−, 91 G−/−GM−/−M−/−, and 54 G−/−GM−/−M−/− newborn mice, indicating that G−/−GM−/−M−/− mice were born at the predicted Mendelian frequency. Although most G−/−GM−/−M−/− mice had short lifespans (Fig. 1A), we occasionally identified G−/−GM−/−M−/− mice that survived longer and when used in breeding programs, we found that male G−/−GM−/−M−/− mice were fertile. Given the established requirement for M-CSF in female fertility (27), we did not attempt to breed female G−/−GM−/−M−/− mice.

In our previous studies of CSF-deficient mice, we housed animals in a conventional facility where they were potentially exposed to numerous environmental pathogens and found that under these conditions, the animals were prone to bacterial infections and succumbed at a young age (9, 11, 19). When housed under conventional conditions, G−/−GM−/−M−/− mice had very poor survival with most animals dying before weaning (data not shown). Accordingly, animals were rederived into a more protected microisolator environment, although regular pathogen testing revealed that they still carried both rotavirus and P. pneumotropica. Under these conditions, survival was improved, although only to a median of 18 days (Fig. 1A). We next investigated whether we could prolong survival by antibiotic treatment of the G−/−GM−/−op/+ breeders. The progeny of these breeders had a marginally improved median survival (22.5 days) and the 20% survival was 39 days compared with 25 days for progeny of untreated breeders (Fig. 1A). G−/−GM−/−M−/− mice (and controls) were then treated with antibiotic (baytril) at weaning for a 2-wk period, which further improved their survival with ~50% of animals surviving at 8 wk of age (data not shown). These data strongly suggest that the poor survival of G−/−GM−/−M−/− mice is in part due to an inability to control bacterial infection. For subsequent experiments in G−/−GM−/−M−/− mice, which were aimed at determining the composition of lymphoid tissue, the numbers of myeloid progenitor cells, and the response of cells to proinflammatory stimuli, newly weaned animals were treated with antibiotic for 2 wk before analysis at 8–9 wk of age. At the data collection stage, mice showing visual signs of illness or stress (assessed by the size of the thymus) were excluded from analysis.

G−/−GM−/−M−/− mice have circulating myeloid cells

Eight-week-old G−/−GM−/−M−/− mice, like M−/− mice, were significantly smaller than WT mice or G−/−GM−/− mice (Fig. 1B). Analysis of the blood of G−/−GM−/−M−/− mice showed that they had lower numbers of circulating white blood cells (WBC) than WT or G−/−GM−/− mice, but numbers were not significantly different to those of M−/− mice (counts given as 106/ml; WT: 5.8 ± 0.7; G−/−GM−/−: 4.6 ± 0.3; M−/−: 3.6 ± 0.3; G−/−GM−/−M−/−: 3.0 ± 0.4; Fig. 1C). G−/−GM−/−M−/− mice, like M−/− mice, were osteopetrotic (see below) and, consequently, had dramatically reduced marrow cellularity (counts given as 109/lemur; WT: 28 ± 4; G−/−GM−/−: 23 ± 4; M−/−: 6 ± 1; G−/−GM−/−M−/−: 4 ± 0.5; Fig. 1D). The spleen to body weight ratio was similar in WT, G−/−GM−/−, and M−/− mice but increased ~1.5-fold in G−/−GM−/−M−/− mice (Fig. 1, B, E, and F).

FIGURE 4. Altered hemopoietic compartment in G−/−GM−/−M−/− mice. Numbers of conventional B cells, T cells, monocytes, and granulocytes in the (A) blood, (B) spleen, and (C) BM of WT, G−/−GM−/−, M−/−, and G−/−GM−/−M−/− (triple) mice. Data represent the mean ± SE. For blood and spleen analysis, data are compiled from n = 8 WT, G−/−GM−/−, and M−/− mice, and n = 17 G−/−GM−/−M−/− mice in three independent experiments. Designations were made on the basis of flow cytometry: B cells were defined as B220+; T cells as Thy-1.2−; monocytes as Mac-1−; and granulocytes as Mac-1− F4/80− and granulocytes as Mac-1− Gr-1−. For BM analysis, data are compiled from n = 5 WT, G−/−GM−/−, and M−/− mice and n = 14 G−/−GM−/−M−/− mice in two independent experiments, and monocyes were defined as Mac-1−c-fms−, granulocytes as Mac-1−c-fms− and B cells as B220+.
peripheral blood of G⁻/⁻GM⁻/⁻M⁻/⁻ mice showed a 3-fold reduction in numbers of monocytes and a 2.2-fold reduction in neutrophils (polymorphonuclear leukocytes (PMNs)) (counts given as 10⁶/ml; WT monocytes: 8 ± 1; WT PMNs: 10 ± 3; G⁻/⁻GM⁻/⁻M⁻/⁻ monocytes: 3 ± 0.5; G⁻/⁻GM⁻/⁻M⁻/⁻ PMNs: 4 ± 1; Fig. 4). Interestingly, mice deficient in M-CSF showed a 6.7-fold reduction in peripheral blood monocytes and G⁻/⁻GM⁻/⁻M⁻/⁻ mice showed a 4.4-fold shortfall in peripheral blood neutrophils (Fig. 4).

G⁻/⁻GM⁻/⁻M⁻/⁻ mice showed reduced numbers of splenic F4/80⁺ macrophages (counts given as 10⁶/spleen; WT: 2.9 ± 0.6; G⁻/⁻GM⁻/⁻M⁻/⁻: 1.8 ± 0.3), but this deficit was not as severe as that seen in M⁻/⁻ mice (M⁻/⁻: 0.7 ± 0.2; Fig. 4). G⁻/⁻GM⁻/⁻M⁻/⁻ mice also showed a deficiency of splenic neutrophils (counts given as 10⁶/ml; WT: 1.9 ± 0.6; G⁻/⁻GM⁻/⁻M⁻/⁻: 1.0 ± 0.3; Fig. 4), but the deficit was no more severe than that observed in G⁻/⁻GM⁻/⁻ mice (G⁻/⁻GM⁻/⁻: 1.0 ± 0.2; Fig. 4).

In the BM, G⁻/⁻GM⁻/⁻M⁻/⁻ mice showed a 6-fold deficit in numbers of monocytes (counts given as 10⁶/femur; WT: 1.9 ± 0.4; G⁻/⁻GM⁻/⁻M⁻/⁻: 0.33 ± 0.06; Fig. 4), whereas M⁻/⁻ mice, which are also osteopetrotic, showed a corresponding 10-fold reduction (M⁻/⁻: 0.19 ± 0.05; Fig. 4). Neutrophil numbers were also severely compromised in G⁻/⁻GM⁻/⁻M⁻/⁻ BM, with the mice showing 5-fold lower numbers than WT mice (counts given as 10⁶/femur; WT: 10.2 ± 2.1; G⁻/⁻GM⁻/⁻M⁻/⁻: 1.9 ± 0.3; Fig. 4). Although both G⁻/⁻GM⁻/⁻ and M⁻/⁻ mice had reduced numbers of BM neutrophils (G⁻/⁻GM⁻/⁻: 5.3 ± 0.7; M⁻/⁻: 2.8 ± 0.5; Fig. 4), their neutrophil deficit was not as severe as that in G⁻/⁻GM⁻/⁻M⁻/⁻ mice.

G⁻/⁻GM⁻/⁻M⁻/⁻ mice, like M⁻/⁻ mice, have reduced numbers of B cells

When we investigated B cell and T cell lineages, we found that numbers of T cells were not affected, however, M⁻/⁻ mice and G⁻/⁻GM⁻/⁻M⁻/⁻ mice had reduced numbers of B cells in all tissues examined (Figs. 3 and 4). The B cell compartment in the blood of M⁻/⁻ mice and G⁻/⁻GM⁻/⁻M⁻/⁻ mice was severely impaired, showing a 5.5- and 4-fold reduction, respectively, compared with WT mice (Fig. 4). A reduction in B cell numbers was also found in spleen, where, compared with WT mice, M⁻/⁻ mice, and G⁻/⁻GM⁻/⁻M⁻/⁻ mice showed a 5.8- and 1.8-fold reduction, respectively. WT mice and G⁻/⁻GM⁻/⁻ mice had similar numbers of B220⁺ B lymphocytes per femur (counts given as 10⁶/femur; WT: 5.3 ± 0.7; G⁻/⁻GM⁻/⁻: 5.5 ± 0.9), whereas B cell numbers per femur were significantly diminished in M⁻/⁻ and G⁻/⁻GM⁻/⁻M⁻/⁻ mice (M⁻/⁻: 0.9 ± 0.3; G⁻/⁻GM⁻/⁻M⁻/⁻: 0.6 ± 0.1). The reduction in B cell numbers in the BM and peripheral tissues is likely to be associated with the fact that M⁻/⁻ and G⁻/⁻GM⁻/⁻M⁻/⁻ mice are severely osteopetrotic and have significantly reduced BM cavities.

**Progenitor numbers in G⁻/⁻GM⁻/⁻M⁻/⁻ mice**

In previous studies of single and double CSF-deficient mice, animals were housed in a conventional facility and were reported to show alterations in hemopoietic progenitors (7, 9, 11). GM-CSF was found to be dispensable for maintenance of normal levels of hemopoietic progenitors (colony-forming cells (CFCs)) in BM, but GM-CSF-deficient mice showed an increased frequency of splenic CFCs (9). Mice lacking G-CSF or both G-CSF and GM-CSF showed reduced hemopoietic progenitors in BM and spleen (7, 11). In the present study, mice were housed in microisolators. Under these conditions, we found that G⁻/⁻GM⁻/⁻ mice showed normal numbers of hemopoietic progenitors, except for a 1.5-fold reduction in BM GM-CSF-CFCs and a 2-fold reduction in splenic SCF-CFCs (Fig. 5). As previously reported, M⁻/⁻ mice, due to

![FIGURE 5. Altered hemopoiesis in G⁻/⁻GM⁻/⁻M⁻/⁻ mice. Myeloid progenitors responsive to the indicated cytokines in (A and B) BM, and (C and D) spleen of WT, G⁻/⁻GM⁻/⁻M⁻/⁻, and triple G⁻/⁻GM⁻/⁻M⁻/⁻ mice. In A, the data are presented as the number of progenitors per 25,000 BM cells; in B, the data shown in A are presented as the total numbers of progenitors per femur following adjustment for total number of cells/femur. In C, the data are presented as the number of progenitors per 10⁶ spleen cells; in D, the data shown in C are presented as the total numbers of progenitors per spleen following adjustment for total number of cells per spleen. Data represent the mean ± SE of six mice in three independent experiments.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
their osteopetrotic phenotype, had significantly impaired BM hematopoiesis (17). Although BM progenitor frequency was not significantly different except for M-CSF-CFCs (Fig. 5A), total numbers of myeloid progenitors per M⁻/⁻ femur were markedly reduced (Fig. 5B). Compared with WT mice, total numbers of GM-CSF-, IL-3-, M-CSF-, and SCF-CFCs in M⁻/⁻ mice were reduced by 7-, 4-, 10-, and 8-fold, respectively. Similarly, while BM progenitor frequency was not significantly different in G⁻/⁻ GM⁻/⁻ M⁻/⁻ mice (Fig. 5A), progenitor numbers were strikingly diminished: GM-CSF-, IL-3-, M-CSF-, and SCF-CFCs in G⁻/⁻ GM⁻/⁻ M⁻/⁻ mice were reduced by 18-, 8-, 16-, and 15-fold, respectively, when compared with WT CFCs (Fig. 5B). In M⁻/⁻ mice and G⁻/⁻ GM⁻/⁻ M⁻/⁻ mice, splenic hematopoiesis was markedly enhanced. M⁻/⁻ mice had both an increase in progenitor frequency and an increase in total progenitor number in spleen (Fig. 5, C and D). When compared with WT mice, total numbers of GM-CSF-, IL-3-, M-CSF-, and SCF-CFCs in M⁻/⁻ spleen were increased by 6-, 9-, 5-, and 3-fold, respectively. G⁻/⁻ GM⁻/⁻ M⁻/⁻ mice also had an increased frequency of IL-3-CFCs in spleen, but the other progenitors appeared to be present at similar frequencies to WT mice (Fig. 5C). When we calculated total numbers of spleen progenitors, we found that G⁻/⁻ GM⁻/⁻ M⁻/⁻ mice have 4-, 6-, 2-, and 1.3-fold increases in GM-CSF-, IL-3-, M-CSF-, and SCF-CFCs, respectively, which is not as elevated as that found in M⁻/⁻ mice. Thus, while G⁻/⁻ GM⁻/⁻ M⁻/⁻ mice have a significant elevation in splenic hematopoiesis, it is not as marked as that seen in M⁻/⁻ mice, and overall numbers of progenitors (BM plus spleen) are still well below the numbers in M⁻/⁻ mice, and much less than the numbers of progenitors in WT mice.

**FIGURE 6.** G⁻/⁻ GM⁻/⁻ M⁻/⁻ mice have significantly reduced numbers of peritoneal cells but mount a moderate response in an animal model of sterile peritonitis. A. Numbers of peritoneal cells in WT, G⁻/⁻ GM⁻/⁻ (GGM), M⁻/⁻, and G⁻/⁻ GM⁻/⁻ M⁻/⁻ (TR) mice at rest (panel 1), and at 4-h (panel 2), 24-h (panel 3), and 72-h (panel 4) following i.p. injection with thioglycolate. Data represent the mean ± SD of six mice in two independent experiments. Representative two-color flow cytometry analysis of peritoneal exudate cells from WT, G⁻/⁻ GM⁻/⁻, M⁻/⁻, and G⁻/⁻ GM⁻/⁻ M⁻/⁻ (triple) mice at (B) 4-h and (C) 72-h after i.p. injection with thioglycolate. Staining was performed with mAbs against Mac-1 and c-fms to determine proportions of granulocytes, and Mac-1 and c-fms to determine proportions of macrophages.

**A Peritoneal Cell Counts**

**B 4-h post-thioglycolate**

**C 72-h post-thioglycolate**
impairment in numbers of circulating neutrophils (Fig. 4A; Ref. 11). Although M−/− mice gave a response 4 h after thioglycolate that was similar to the response of WT mice, the response of G−/−GM−/−M−/− mice was impaired, although the animals showed a dramatic increase in cellularity (36-fold) over that seen in resting peritoneum and it was the typical neutrophilic response as that seen in control animals (Fig. 6, A and B). At 24 h after thioglycolate, the numbers of inflammatory cells in the peritoneum of G−/−GM−/−M−/− mice had stabilized, but were still increasing in the other genotypes. In the G−/−GM−/−M−/− mice 24 h after thioglycolate treatment, the numbers of peritoneal cells were similar to the number observed 4 h after thioglycolate treatment and were 5- to 6-fold lower than in the other groups of mice (Fig. 6A). By 72 h after thioglycolate treatment, the peritoneal exudate of all genotypes was mainly composed of macrophages (Fig. 6C). Although cell numbers were still increasing in WT mice 72 h after treatment, the number of cells in the peritoneal cavities of G−/−GM−/−, M−/−, and G−/−GM−/−M−/− mice was waning (Fig. 6A). Compared with WT mice, G−/−GM−/− and M−/− mice had 3-fold lower numbers, while G−/−GM−/−M−/− mice had a 13-fold lower number of macrophages (Fig. 6A).

Bone and lung histology in G−/−GM−/−M−/− mice

As demonstrated in Fig. 1D, G−/−GM−/−M−/− mice have diminished BM cellularity, and their bones are fragile, consistent with underlying osteopetrosis. When analyzed by histopathology, all G−/−GM−/−M−/− mice show striking bone pathology, with excess woven bone occupying a large extent of the marrow cavity and thus severely reducing the marrow volume (Fig. 7A). This phenotype is similar to that seen in M−/− and GM−/−M−/− mice (17, 19).

G−/−GM−/−M−/− mice were also examined for pulmonary pathology, because previous studies have shown that GM-CSF−/−, GM−/−M−/−, and G−/−GM−/− mice develop pulmonary alveolar proteinosis (9, 11, 19, 28). When directly comparing G−/−GM−/− mice and G−/−GM−/−M−/− mice, the severity of the lung pathology was significantly greater in G−/−GM−/−M−/− mice compared with G−/−GM−/− mice, for proteinosis grade (p = 0.006) and lymphoid infiltration extent (p = 0.002), but the trends for more severe proteinosis extent (p = 0.07) and lymphoid infiltration grade (p = 0.32) were not statistically significant (Table I). The abnormalities comprised consistent widespread intra-alveolar surfactant accumulation (Fig. 7D), as seen in GM−/− and G−/−GM−/− animals (9, 11). However, in addition to more severe surfactant accumulation, other novel changes were also noted. Whereas in both GM−/− and G−/−GM−/− animals, alveolar macrophages were frequent, and typically appeared large with “foamy” distended cytoplasm, alveolar macrophages were infrequent in the lung sections of G−/−GM−/−M−/− mice (Fig. 7C); where present, alveolar macrophages were small but lacked the copious “foamy

Table I. Comparison of lung histology between genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Proteinosis Grade</th>
<th>Proteinosis Extent (%)</th>
<th>Lymphoid Infiltration Grade</th>
<th>Lymphoid Infiltration Extent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0 (0–1)</td>
<td>0 (0–20)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>M−/− (n = 16)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>G−/−GM−/− (n = 8)</td>
<td>1.4 (1–2)</td>
<td>38 (20–70)</td>
<td>1.8 (0–3)</td>
<td>33 (0–50)</td>
</tr>
<tr>
<td>G−/−GM−/−M−/− (n = 24)</td>
<td>2.2 (1–4)</td>
<td>54 (20–90)</td>
<td>2.1 (1–4×)</td>
<td>59 (20–90)</td>
</tr>
</tbody>
</table>

The severity and extent of alveolar surfactant deposition and pulmonary lymphoid accumulation were scored blind using a reproducible qualitative scale from 0 (normal) to 4 as outlined in Materials and Methods and described in Ref. 11. Histologic grading is presented as mean (range) by genotype. All statistical comparisons were performed using a two-sample t test.

a Value of p = 0.0055 comparing G−/−GM−/− mice with G−/−GM−/−M−/− mice.
b Value of p = 0.07 comparing G−/−GM−/− mice with G−/−GM−/−M−/− mice.
c Value of p = 0.0024 comparing G−/−GM−/− mice with G−/−GM−/−M−/− mice.
d Value of p = 0.0024 comparing G−/−GM−/− mice with G−/−GM−/−M−/− mice.
appearing cytoplasm, suggesting that the more severe phenotype in G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice may be attributable to the compound effect of both numerical and functional alveolar macrophage defects. The lungs of G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice also frequently contained interstitial foci of extramedullary hemopoiesis, with rare neutrophils (Fig. 7E), and in the one mouse with small foci of pulmonary infection, morphologically normal neutrophils were seen, but were very rare among inflammatory cells, which contained a predominance of lymphocytes and plasma cells (Fig. 7F). Manifestations of pulmonary emphysema have been described in both aged GM<sup>−/−</sup> and G<sup>−/−</sup> GM<sup>−/−</sup> animals (11), with these changes attributed to IL-3-related increased tissue matrix metalloproteinase activity (29). Areas of emphysema were also evident in G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice, even at the age of 8–10 wk (Fig. 7D).

**Discussion**

Mice with genetic mutations in growth factor genes or their receptors have been invaluable for identifying the roles that hemoipoietic growth factors play in myelopoiesis and blood cell development (20). Because previous studies of compound CSF-deficient mice have suggested that additional myeloid growth factors can regulate granulocyte and monocyte production, we have investigated whether mice simultaneously lacking G-CSF, GM-CSF, and M-CSF can still produce myeloid cells. Our analyses have shown that G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice have circulating monocytes, tissue macrophages and neutrophils, although the total number of myeloid cells is significantly reduced when compared with control, single, or double mutant animals. Interestingly, while G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice show a more severe impairment in numbers of neutrophils than WT, G<sup>−/−</sup> GM<sup>−/−</sup> and M<sup>−/−</sup> mice, they have more monocytes/macrophages in blood, spleen, and BM than M<sup>−/−</sup> mice, and the only region where their macrophage deficit is more severe than M<sup>−/−</sup> mice is in the peritoneal cavity. These data indicate that another monocyte growth factor is most likely responsible for maintaining monocyte numbers in G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice. The data also implies that M-CSF contributes to neutrophil production in G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice as G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice have a more severe deficit of neutrophils than G<sup>−/−</sup> GM<sup>−/−</sup> mice.

G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice also manifest many of the phenotypes associated with deficiency of individual CSFs; like M<sup>−/−</sup> mice, they are osteopetrotic, and they develop alveolar proteinosis albeit more severely than GM-CSF-deficient or G<sup>−/−</sup> GM<sup>−/−</sup> mice. Our analyses of the lungs of triple G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice show both a diminished number of macrophages and an altered appearance, suggesting that the more profound proteinosis is due to both numerical and functional alveolar macrophage defects in G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice. Thus, while deficiency of GM-CSF is the major cause of alveolar proteinosis, clearly loss of both G-CSF and M-CSF on a GM-CSF-deficient background can exacerbate this phenotype suggesting that G-CSF, M-CSF, or both, limit the extent of alveolar proteinosis in GM-CSF-deficient animals.

Similar to M<sup>−/−</sup> mice, G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice show a failure of BM hemopoiesis due to underlying osteopoiesis, and thus have significantly increased splenic hemopoiesis. Intriguingly, M<sup>−/−</sup> mice are better able to compensate for the deficit of BM hemopoiesis having more splenic GM-, IL-3-, M-, and SCF-CFCs than G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice, suggesting that deficiency of G-, GM-, and M-CSF, affects myeloid progenitor cell number and impairs some of the compensatory mechanisms otherwise acting to restore myelopoiesis. Our data also suggest that environment is an important factor in determining progenitor number in CSF-deficient mice. In this study, we have shown that SPF-housed G<sup>−/−</sup> GM<sup>−/−</sup> mice have relatively unchanged numbers of myeloid progenitors (Fig. 5), however, our previous work on conventionally housed G<sup>−/−</sup> GM<sup>−/−</sup> mice showed that they had a significant reduction in both BM and spleen progenitors (11).

In previous studies of G-CSF-deficient, GM-CSF-deficient, G<sup>−/−</sup> GM<sup>−/−</sup>, and GM<sup>−/−</sup> M<sup>−/−</sup> mice, animals were housed in a conventional facility where they were prone to bacterial infections; these mice had shorter life expectancies than WT mice, with median survivals of 70, 71, 56, and 10 wk, respectively (7, 9, 11, 19). Not surprisingly, G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice had extremely poor survival when housed under similar conditions, with few mice surviving to weaning. Rederivation of the animals into an SPF environment improved their survival, although only to a median of 18 days, and antibiotic treatment of parents had only a marginal effect. Direct antibiotic treatment of G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> pups at weaning significantly improved survival, with many animals appearing healthy at 8 wk of age, indicating that the animals are highly susceptible to bacterial infection. Through regular pathogen testing of the SPF facility, we have detected the opportunistic bacterium *P. pneumotropica*, a common pathogen that generally constitutes the natural flora of wild and domesticated animals. It is commonly found in the respiratory tract and conjunctiva of rodents, and while it does not significantly affect the health of immunocompetent animals, immunodeficient animals often develop severe or lethal pneumonia (30, 31). Unsurprisingly, G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice, which harbor a pre-existing lung pathology (alveolar proteinosis) and are severely immunodeficient (diminished monocytes, granulocytes and B lymphocytes), are often found to have eye infections and pneumonia. Collectively, these studies imply that G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice have numbers of myeloid cells below a critical threshold that are required for mounting an effective response to infection with pathogenic organisms.

A general feature of "osteopetrotic" mice is that they show deficiencies in B cell numbers due to their inability to form adequate BM cavities for active hemopoiesis. For example, Fos-deficient mice develop severe osteopoiesis, show altered hemopoiesis, and display lymphopenia due to an impaired BM microenvironment (32–34). Mice lacking RANK are characterized by profound osteopetrosis, a marked B cell deficiency, and enhanced extramedullary splenic hemopoiesis to compensate for an altered BM environment although it is not yet clear whether this is intrinsic to the hemopoietic cell lineage or whether the defect reflects alterations in the stromal environment (35). It is well-established that op/op mice are moderately lymphopenic (17); direct assessment of the B cell lineage has shown that B cell development is impaired and that there is increased apoptosis among precursor B cells (36). Close examination of B lymphopoiesis in marrow showed that the frequency of B cell progenitors (CFU-IL-7) is dramatically reduced, and interestingly, appears to be up-regulated in the liver but not the spleen (37). It was therefore not unexpected that G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice also show a significant diminution of B cells in all tissues examined (BM, spleen, and blood), although B cell numbers in spleen were greater than observed in M<sup>−/−</sup> mice. It will be of interest to determine whether B lymphopoiesis is occurring in the secondary lymphoid tissue of G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice.

A particularly intriguing feature of this study is that, even in the absence of G-, GM-, and M-CSF, mice still produce myeloid cells, indicating that one or other additional growth factors underlies myeloid cell production in G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice. It is of interest that loss of IL-6 in G-CSFR-deficient mice leads to a more profound neutropenia than in G-CSFR-deficient mice alone (8, 15), or in double mutant G<sup>−/−</sup> GM<sup>−/−</sup> mice (11), suggesting that IL-6 may contribute to neutrophil production in G<sup>−/−</sup> GM<sup>−/−</sup> M<sup>−/−</sup> mice. It has also been reported that serum levels of GM-CSF and IL-3 are increased in aged op/op mice.
and that this may underlie the correction of some of the defects in older animals (29, 38). It would be interesting to superimpose IL-3 deficiency in triple G−/− GM−/− M−/− mice, as IL-3 may well contribute to macrophage production in these animals. Our current work is geared toward establishing the identity and mechanism of action of the factor(s) responsible for steady-state and emergency myelopoiesis in CSF-deficient and WT mice.

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Disclosures

The authors have no financial conflict of interest.

References