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Infection-Induced Myelopoiesis during Intracellular Bacterial Infection Is Critically Dependent upon IFN-γ Signaling

Katherine C. MacNamara,* Kwadwo Oduro,† Olga Martin,* Derek D. Jones,* Maura McLaughlin,* Kyunghee Choi,‡ Dori L. Borjesson,‡ and Gary M. Winslow*

Although microbial infections can alter steady-state hematopoiesis, the mechanisms that drive such changes are not well understood. We addressed a role for IFN-γ signaling in infection-induced bone marrow suppression and anemia in a murine model of human monocytic ehrlichiosis, an emerging tick-borne disease. Within the bone marrow of *Ehrlichia muris*-infected C57BL/6 mice, we observed a reduction in myeloid progenitor cells, as defined both phenotypically and functionally. Infected mice exhibited a concomitant increase in developing myeloid cells within the bone marrow, an increase in the frequency of circulating monocytes, and an increase in splenic myeloid cells. The infection-induced changes in progenitor cell phenotype were critically dependent on IFN-γ, but not IFN-α, signaling. In mice deficient in the IFN-γ signaling pathway, we observed an increase in myeloid progenitor cells and CD11b<sup>+</sup>Gr1<sup>lo</sup> promyelocytic cells within the bone marrow, as well as reduced frequencies of mature granulocytes and monocytes. Furthermore, *E. muris*-infected IFN-γR-deficient mice did not exhibit anemia or an increase in circulating monocytes, and they succumbed to infection. Gene transcription studies revealed that IFN-γR-deficient CD11b<sup>+</sup>Gr1<sup>lo</sup> promyelocytes from *E. muris*-infected mice exhibited significantly reduced expression of *irf-1* and *irf-8*, both key transcription factors that regulate the differentiation of granulocytes and monocytes. Finally, using mixed bone marrow chimeric mice, we show that IFN-γ-dependent infection-induced myelopoiesis occurs via the direct effect of the cytokine on developing myeloid cells. We propose that, in addition to its many other known roles, IFN-γ acts to control infection by directly promoting the differentiation of myeloid cells that contribute to host defense. The Journal of Immunology, 2011, 186: 1032–1043.
(HME), an emerging infectious disease caused by the tick-borne pathogen *Ehrlichia chaffeensis*. The *Ehrlichia*iae are obligate intracellular bacteria that infect mononuclear cells (21). HME is a febrile illness and patients exhibit a severe loss in hemoglobin and platelets (21, 22). Infection of C57BL/6 mice with a closely related pathogen, *Ehrlichia muris*, causes hematological abnormalities, including anemia and thrombocytopenia (23), similar to what is observed in HME patients. We have proposed that the hematological changes that occur during *ehrlichiosis* are a consequence of alterations in hematopoiesis (23). In this study, we extend our previous studies by demonstrating that *ehrlichial* infection induces changes in the phenotype and function of myeloid progenitor cells and augments the production of granulocytes and monocytes, and that these changes require direct IFN-γ signaling. These studies highlight a novel and important role for this factor in host defense during bacterial infection.

Materials and Methods

**Mice**

The mice used in these studies were obtained from The Jackson Laboratory (Bar Harbor, ME) or were bred in the Animal Care Facility at the Wadsworth Center under microisolator conditions. C57BL/6 mice were used as a congenic control strain. The following transgenic and gene-targeted strains were used: IFN-γR−/− mice (a gift of Dr. Susan Swain, Trudeau Institute, Saranac Lake, NY), IFN-γR1−/− mice (B6.129S7-Ifggr1tm1Agt/J, referred to as IFN-γR−/− mice), enhanced GFP (EGFP)-transgenic mice (C57BL/6-Tg(ActB-EGFP)1Osb/J), and a C57BL/6 congenic strain of mice (B6.SJL-PepRb/PepRb/Joy). All animal studies were performed in accordance with Wadsworth Center Animal Care and Use Committee guidelines.

**Bacteria and infections**

Mice were infected, via i.p. injection, between 6 and 12 wk of age, with 50,000 copies of the *E. muris* bacterium. The inoculum was generated from spleen mononuclear cells that were harvested from infected mice and stored at −80 °C, as previously described (24). Bacterial copy number was determined by real-time quantitative probe-based PCR, as previously described (23, 25).

**Flow cytometry and Abs**

Mononuclear cells were harvested from spleens by homogenization of the tissue between frosted glass slides in HBSS. Bone marrow mononuclear cells were harvested by flushing the narrow from femurs using a 26.5-gauge needle and 2 ml HBSS. Tissue homogenates were passed through a 70-μm filter. Erythrocytes were removed by lysis in a hypotonic buffer containing 0.84% ammonium chloride. The cells were washed with a buffer containing 5% FCS and 0.01% sodium azide in 1× PBS (wash buffer). Prior to staining, the cells were incubated in blocking buffer (wash buffer containing 1 μg/ml 2.4G2 [FcγRIIb/I; anti-CD16/[CD32]) for 15 min at 4 °C. The Abs used for flow cytometry included the following: FITC- and biotin-conjugated lineage markers specific for CD3 (clone 17A2), CD11b (M1/70), Ly-6G (RB6-8C5), Ter119 (Ly-76), and CD45R (RA3-6B2). Other Abs used were PE-conjugated Sca-1 (D7), PE-cyochrome-7–conjugated Sca-1 (D7), PerCP-Cy5.5–conjugated CD45.2 (104), PE-cy5.5–conjugated CD127 (A7R34), allophycocyanin-conjugated c-Kit (2B8), PE-Gr-1 (RB6-8C5), allophycocyanin-cyochrome-7-streptavidin, and eFluor 450-streptavidin (eBioscience, San Diego, CA), as well as Pacific Blue-CDillé (M1/70; BioLegend, San Diego, CA). Unstained cells were used as negative controls to establish the flow cytometer voltage settings, and single-color positive controls were used for adjustment of the compensation. The flow cytometric data were acquired using FACSCalibur or LSR II flow cytometers (BD Biosciences), and data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

**In vitro hematopoietic progenitor cell assays**

Bone marrow cells were plated at 2.5 × 10^5 cells per 35-mm tissue culture dish, in duplicate, and cultured in methylcellulose media (MethoCult GF M3434; StemCell Technologies, Vancouver, BC, Canada) along with recombinant cytokines for colony assays of murine cells, following the manufacturer’s instructions. Total colonies derived from granulocyte-macrophage (CFU granulocyte, macrophage), and multipotent (CFU granulocyte, erythrocyte, macrophage, megakaryocyte) progenitor cells were scored after 7 d incubation at 37 °C in 5% CO₂.

**Generation of bone marrow chimeric mice**

C57BL/6 and IFN-γR−/− deficient (CD45.2⁺) mice were lethally irradiated (950 rad administered in two doses, 3 h apart). After irradiation, the hematopoietic compartment of the irradiated mice was reconstituted with 2 × 10⁶ bone marrow cells obtained from CD45.1 congenic mice. To generate the mixed chimeras, CD45.1⁺ mice were irradiated as above and reconstituted with a 1:1 ratio of bone marrow (2 × 10⁶ cells) harvested from EGFP-expressing and IFN-γR−/− deficient mice (both CD45.2). After irradiation and reconstitution, all mice were administered antibiotics in their drinking water for 1 wk, and 6 wk postreconstitution, the mice were bled and screened to determine the degree of hematopoietic chimerism. The chimeric mice were infected with *E. muris* ~8 wk postreconstitution.

**Flow cytometric cell sorting**

Promyelocytes were identified in bone marrow by low surface expression of CD11b and Gr-1, using CD11b-FITC and Gr-1-PE. Bone marrow cells were purified by flow cytometry from day 15 *E. muris*-infected C57BL/6 mice and IFN-γR−/− deficient mice, using an Aria II equipped with FACSDiva software (BD Biosciences).

**Gene expression analyses**

RNA was extracted from flow cytometrically sorted CD11b⁺Gr-1⁺ cells by the RNeasy method (Qiagen, Valencia, CA). RNA integrity was analyzed with an Agilent bioanalyzer (Agilent Technologies, Foster City, CA), and only RNA preparations with an RNA integrity number of 8.0 or higher were used for gene expression studies. Genomic DNA was removed from RNA samples with TurboDNA-free DNase (Ambion, Austin, TX), and cDNAs were generated with a first-strand synthesis kit (SA Biosciences, Frederick, MD). Gene expression analyses were performed using an IFN-γ gene array assay (SA Biosciences; APMM-064).

**Statistical analyses**

Statistical analyses were performed with a Student t test, using GraphPad Prism software (GraphPad Software, La Jolla, CA); a p value <0.05 was considered significant.

**Results**

*Type II IFN-dependent alterations in bone marrow progenitor cells during infection.*

*E. muris* infection of C57BL/6 mice provides a model in which to investigate the pathogenesis of HME (21). In C57BL/6 mice, bacterial burden is maximal between days 8 and 9 postinfection in the spleen, and it declines to very low numbers by day 30 postinfection (21, 26). *E. muris* causes marked anemia and thrombocytopenia, two hallmarks of HME (21, 22). We previously demonstrated that these blood abnormalities, which are maximal during the second week of infection, occur concomitantly with a decrease in bone marrow colony-forming cells (23). Additional studies revealed that infection induced the mobilization and/or depletion of B220⁺ lymphocytes from the bone marrow, which was coincident with an increase in the frequency of immature and mature granulocytes at this site (23). Significant changes in the cellular composition of the bone marrow begins by day 8 postinfection and persists through day 15 postinfection, which prompted us to use these time points for our analyses. In this study, we have extended our previous observations by examining hematopoietic progenitor cell populations in the bone marrow.

In uninfected mice, expression of IL-7Rα (CD127), Sca-1, and c-Kit on lineage-negative (Lin−) bone marrow cells can distinguish common lymphoid progenitors (CLPs; IL-7Rα⁺c-Kit⁺Sca-1−) and myeloid progenitors (IL-7Rα c-Kit⁺Sca-1−) (27, 28). Using this criteria, we determined that *E. muris* infection caused a modest reduction in phenotypically defined CLPs in C57BL/6 mice: a small decrease in frequency was observed on day 8 postinfection (Fig. 1A), although the number of CLPs was unaffected (data not shown).
In contrast, there was almost a complete loss of the IL-7Rα−c-Kithi
Sca-1− myeloid progenitor cells by day 8 postinfection, and a population of Sca-1+ cells emerged.

Sca-1 expression is known to be regulated by IFN-γ signaling (29), and recent studies have implicated a role for IFNs in driving the differentiation of hematopoietic progenitor and stem cells (19, 30, 31). To determine whether IFN signaling was responsible for the loss of IL-7Rα−c-KithiSca-1− cells and the emergence of the Sca-1+ population, we examined the bone marrow of infected mice deficient for either the IFN-α or IFN-γR. In the absence of IFN-α signaling, little effect was noted on the phenotype of Lin−IL-7Rα−progenitor cells during E. muris infection, as compared with infected C57BL/6 mice (Fig. 1B). However, in the absence of IFN-γ signaling, reduced frequencies and numbers of IL-7Rα−c-KithiSca-1+ cells were observed postinfection, relative to infected C57BL/6 mice (Fig. 1C, D). Moreover, we observed an ~8-fold decrease in the frequency and numbers of IL-7Rα−c-KithiSca-1− cells in both C57BL/6 and IFN-αR−deficient mice postinfection, but no significant decreases in this population in IFN-γR−deficient mice. Thus, the alterations in bone marrow progenitor cells observed during infection were dependent on IFN-γ, but not IFN-α, signaling.

Infection-induced alterations in bone marrow progenitor cells are correlated with anemia and changes in leukocyte frequencies in the peripheral blood

To further investigate the role of IFN-γ signaling on infection-induced hematopoiesis, we next examined the bone marrow progenitor cell populations in greater detail. During infection there was a paucity of Sca-1− cells in C57BL/6 mice. As Sca-1 is known to be induced by IFN signaling, we used two additional markers, CD34 and FcγRII/III (CD16/32), to characterize myeloid progenitor subsets within the Lin−IL-7Rα−c-KithiSca-1− and Sca-1+ fractions of cells (as shown in Fig. 1B). In uninfected mice, cells

**FIGURE 1.** E. muris infection causes a loss of CMPs in the bone marrow. A, Lin−IL-7Rα+ and IL-7Rα− bone marrow cells from mock-infected or E. muris-infected mice (left panels) were analyzed for c-Kit and Sca-1 expression. The regions in the right panels demarcate the CLPs (Lin−IL-7Rα+c-Kithi Sca-1+), CMPs (Lin−IL-7Rα+c-Kitlo Sca-1−), and the infection-induced Lin−IL-7Rα−c-KithiSca-1− progenitor cells. B, The Lin−IL-7Rα− cells were analyzed for expression of c-Kit and Sca-1 in C57BL/6, IFN-αR−deficient, and IFN-γR−deficient mice. C, The frequencies of the CLPs, CMPs, and Lin−IL-7Rα−c-KithiSca-1+ progenitor cells in the bone marrow were determined in C57BL/6, IFN-αR−deficient, and IFN-γR−deficient mice. The error bars represent the means and SD. D, The number of Sca-1− and Sca-1+ cells within the Lin−IL-7Rα−c-Kithi population is indicated for each strain of mouse. The lineage markers used were the following: CD3, B220, CD11b, Gr-1, and Ter119. The error bars represent the averages and SEM. The bone marrow was harvested from both femurs. The data are representative of at least three separate experiments, where each experiment included at least three mice per group. A Student t test was used to evaluate statistically significant differences between the indicated groups, and significant differences are indicated as follows: *p < 0.05; **p < 0.001. dpi, day postinfection; n.s., not significant.
that are Lin<sup>−</sup>IL-7R<sup>−</sup> c-Kit<sup>hi</sup>Sca-1<sup>−</sup> and CD34<sup>−</sup>FcyRII/III<sup>−</sup> are megakaryocyte-erythrocyte progenitors (MEPs) and the CD34<sup>+</sup>FcyRII/III<sup>+</sup> fraction are common myeloid progenitors (CMPs); the CD34<sup>+</sup>FcyRII/III<sup>+</sup> population has granulocyte and monocyte potential and are thus referred to as granulocyte monocyte progenitors (GMPs) (28). We observed that, in mock-infected mice, expression of FcyRII/III is very low on the Lin<sup>−</sup>IL-7R<sup>−</sup> c-Kit<sup>hi</sup>Sca-1<sup>−</sup> cells but is increased during infection in both C57BL/6 and IFN-γR-deficient mice. We gated on total Lin<sup>−</sup>IL-7R<sup>−</sup> c-Kit<sup>hi</sup>Sca-1<sup>−</sup> cells and quantified the numbers of classically defined MEPs, CMPs, and GMPs (Fig. 2A, 2B). We observed that the frequency and number of each of these myeloid populations was reduced during E. muris infection, with significant reductions in the frequency of CMPs and GMPs in C57BL/6 mice. A significant reduction in the number of CMPs was also noted in IFN-γR-deficient mice. We next examined the frequency and numbers of infection-induced Sca-1<sup>−</sup> MEPs, CMPs, and GMPs (Fig. 2C, 2D). As Sca-1 is not normally expressed on MEPs, CMPs, and GMPs, each population in this study is referred to as “infection-induced.” The numbers of infection-induced MEPs, CMPs, and GMPs were significantly increased in C57BL/6 mice during infection, whereas IFN-γR-deficient mice exhibited an increase in the CMP population only. The increase in Sca-1<sup>−</sup> myeloid progenitor cells mirrored the loss of Sca-1<sup>−</sup> cells, which suggested that the surface expression of Sca-1 was induced on the classically defined myeloid progenitors, as was previously documented during bacterial infection (2). Thus, IFN-γ signaling contributed to the infection-induced loss of classically defined myeloid progenitors and the emergence of a Sca-1<sup>−</sup> population of progenitor cells.

Because the identification of myeloid progenitor cell subsets relies on Sca-1 expression, which can be altered during inflammation, we next evaluated bone marrow colony formation to determine whether myeloid progenitor cells were altered in the absence of IFN-γ-dependent signaling. E. muris infection suppresses bone marrow colony formation (23), which is consistent with the reduced numbers of classically defined myeloid progenitors that we have observed. In contrast to C57BL/6 mice, E. muris infection did not inhibit colony forming activity in IFN-γR-deficient mice; in fact, bone marrow harvested from E. muris infected IFN-γR-deficient mice exhibited increased colony formation in vitro (Fig. 3A). These data suggest that the IFN-γ-dependent changes in the phenotype of myeloid progenitor cells observed during infection are associated with functional differences in these bone marrow cells.

We next addressed whether the alterations in progenitor populations within the bone marrow correlated with changes in the peripheral blood during infection. Hemoglobin levels began to diminish on day 8 postinfection, and they were significantly reduced by day 15 postinfection in C57BL/6 mice but not in IFN-γR-deficient mice (Fig. 3B). Platelet numbers were also reduced on days 8 and 15 postinfection in C57BL/6 mice, but platelets did not decrease substantially in the IFN-γR-deficient mice until day 15 postinfection (Fig. 3C). In C57BL/6 mice, hemoglobin concentration and platelet numbers were restored to nearly normal levels by day 30 postinfection (23). Thus, in IFN-γR-deficient mice the onset of anemia and thrombocytopenia occurred around the time of death in infected mice. These data reveal that thrombocytopenia was associated with reduction or loss of classically defined MEPs in C57BL/6 mice but not in IFN-γR-deficient mice, and they suggest that the loss of the progenitor cell population is at least in part responsible for the decline in platelet number in the periphery.

We also observed changes in the frequency of WBC populations in the peripheral blood of infected mice. Most striking was the observation that infection induced an apparent expansion of blood monocytes and neutrophils, as well as a contraction of lymphocytes, in the C57BL/6 mice (Fig. 3D). In contrast, IFN-γR-
deficient mice exhibited a much reduced expansion of the blood monocytes, but increased neutrophils. These data are consistent with our hypothesis that the observed change in progenitor cell phenotype during infection is a consequence of IFN-γ-dependent changes in hematopoietic progenitor cell function and altered myeloid differentiation.

Altered infection-induced myelopoiesis in mice lacking IFN-γ signaling

During *E. muris* infection, the bone marrow becomes dedicated to the production of granulocytes (23). To determine whether the IFN-γ-dependent change in the number and phenotype of myeloid progenitors had a direct impact on the differentiation of myeloid cells, we examined the bone marrow of C57BL/6 and IFN-γ-deficient mice for its ability to support granulocyte production and differentiation. To determine the maturation status of the bone marrow granulocytes, we monitored cell-surface expression of CD11b and Gr-1 (Fig. 4A). Promyelocytes express intermediate levels of both cell surface molecules (i.e., are CD11b<sup>lo</sup>Gr-1<sup>lo</sup>), whereas immature neutrophils are CD11b<sup>hi</sup>Gr-1<sup>hi</sup> and mature neutrophils are CD11b<sup>lo</sup>Gr-1<sup>hi</sup> (7). The maturation status of these populations from mock and infected mice was confirmed cytologically (data not shown).

During the first 2 wk of infection, the frequency of granulocytes in the bone marrow increased from ∼40% to nearly 50% in C57BL/6 mice (Table I). However, due to the decrease in bone marrow cellularity during infection (Ref. 23 and data not shown), the change in number of total granulocytes was not significantly different between uninfected and infected mice. We observed a significant decrease in the number of CD11b<sup>hi</sup>Gr-1<sup>hi</sup> cells in C57BL/6 mice on day 8 postinfection, although the numbers returned to normal by day 15 postinfection (Fig. 4B). In contrast, the frequency of granulocytes increased from 37% to nearly 80% in the bone marrow of IFN-γR–deficient mice (Table I). In infected IFN-γR–deficient mice, we observed a significant increase in the numbers of CD11b<sup>lo</sup>Gr-1<sup>hi</sup> promyelocytes in the bone marrow by day 15 postinfection (Fig. 4B). In the infected IFN-γR–deficient mice, the CD11b<sup>lo</sup>Gr-1<sup>hi</sup> promyelocytic cell population constituted more than half of all cells within the bone marrow. The apparent expansion of the CD11b<sup>lo</sup>Gr-1<sup>hi</sup> cells was only noted during infection, as uninfected C57BL/6 mice exhibited very similar frequencies of granulocytes at each developmental stage. It is possible that the infection-induced expansion of the CD11b<sup>lo</sup>Gr-1<sup>hi</sup> cells could have resulted from increased proliferation, decreased cell death, and/or a block in differentiation. Infected IFN-γR–deficient mice had fewer B220<sup>+</sup> B cells in the bone marrow, as was observed in C57BL/6 mice (Ref. 23 and data not shown), indicating that the infection-induced alterations in bone marrow B lymphocytes were unaffected by the loss of IFN-γ signaling. Thus, our data indicate that IFN-γ signaling is required for myeloid, but not lymphoid, differentiation during infection.

The infection-induced alterations in the bone marrow were reflected in the periphery, as infection increased the frequency and number of granulocytes in the spleens of both C57BL/6 and IFN-γR–deficient mice (Fig. 4C, 4D, Table I). Neutrophils normally represent a small fraction (<2%) of the cells in the spleens of both C57BL/6 and IFN-γR–deficient mice. However, *E. muris* infection induced a 1.5- to 2-fold increase in the frequency of spleen granulocytes in C57BL/6 mice, but this corresponded to a 10- to 12-fold increase in the numbers of CD11b<sup>lo</sup>Gr-1<sup>hi</sup> and CD11b<sup>lo</sup>Gr-1<sup>hi</sup> cells due to splenomegaly (Fig. 4D). This difference was more pronounced in infected IFN-γR–deficient mice, which exhibited an ∼40-fold increase in the number of CD11b<sup>lo</sup>Gr-1<sup>hi</sup> promyelocytic cells, relative to uninfected mice; ∼15% of the cells in the spleens of IFN-γR–deficient mice were CD11b<sup>lo</sup>Gr-1<sup>lo</sup> cells. Thus, ehrlichial infection causes an expansion of immature and mature granulocyte populations, and these changes are greatly exacerbated in the absence of IFN-γ signaling. These findings are consistent with related studies of *Mycobacterium*-infected mice (16).

Cytological examination of neutrophils in spleen samples from the infected mice revealed that the splenic neutrophils in the IFN-γR–deficient mice, but not C57BL/6 mice, harbored inclusions containing intracellular ehrlichiae (Fig. 4E). This observation was unexpected, as the ehrlichiae are largely monocyctotropic and have never been reported to infect neutrophils (22). Given that many more phenotypically immature neutrophils were detected in the
spleens of IFN-γR-deficient mice, the altered ehrlichial cell tropism in the IFN-γR-deficient mice may also explain the higher degree of bacterial infection. We interpret the altered bacterial tropism in the IFN-γR-deficient mice to suggest that neutrophils that develop during infection, in the absence of IFN-γ signaling, can phagocytose but not kill the intracellular bacteria. An alternative explanation is that the cells identified as neutrophils in the spleens of infected IFN-γR-deficient mice have some characteristics of monocytes, making them permissive to infection with *E. muris*; however, these possibilities remain to be addressed.

**FIGURE 4.** Infection-induced granulopoiesis is altered in mice incapable of IFN-γ signaling. *A*, Bone marrow harvested from C57BL/6 or IFN-γR-deficient mice was analyzed for expression of CD11b and Gr-1. *B*, The numbers of CD11b<sup>−</sup>Gr-1<sup>−</sup>, CD11b<sup>−</sup>Gr-1<sup>+</sup>, and CD11b<sup>+</sup>Gr-1<sup>−</sup> cells identified in *A* are indicated. *C* and *D*, Splenocytes from the same mice were analyzed as in *A*, and the numbers of each population are indicated. *E*, Spleen cell preparations from mock and day 15 *E. muris*-infected mice were generated by touch impression and were analyzed by Giemsa staining. Neutrophils in the IFN-γR-deficient (bottom panels), but not in C57BL/6 mice (top panels), contained ehrlichia morulae (arrows). None of the neutrophils in the C57BL/6 mice were infected, but 24.7% of the IFN-γR-deficient neutrophils contained morulae (5 of 19, 3 of 17, and 10 of 33 cells enumerated contained morulae in the IFN-γ-deficient mice; three individual fields were counted from three separate mice; original magnification ×100).

A direct role for IFN-γ signaling in infection-induced hematopoiesis

It was possible that the persistence of myeloid progenitors, as well as the enhanced granulopoietic response observed in the absence of IFN-γ signaling, was mediated indirectly, perhaps by signaling on
nonhematopoietic cells within the bone marrow. To address whether our observations were due to direct IFN-γ signaling on hematopoietic cells, we first generated bone marrow chimeric mice by irradiating IFN-γR–deficient and C57BL/6 mice and reconstituting them with congenic wild-type bone marrow. Eight weeks post-reconstitution, the chimeric mice were infected with *E. muris* and bone marrow cells were analyzed on day 8 post-infection for progenitor cell phenotype. We found that the increased frequency of Lin^−^c-Kit^hi^Sca-1^−^ cells that emerged in infected wild-type mice was observed in both C57BL/6 and IFN-γR–deficient recipients (Fig. 5), supporting the idea that IFN-γ acts directly on hematopoietic cells, and not indirectly, via interaction with nonhematopoietic stromal bone marrow cells.

It was possible that the enhanced granulopoietic response observed in the absence of IFN-γ signaling could also be a consequence of higher bacterial infection and inflammation in the IFN-γR–deficient hosts. To address this concern we next generated radiation-induced mixed bone marrow chimeric mice. Irradiated congenic mice were reconstituted with a 1:1 ratio of GFP-expressing C57BL/6 (βACT-EGFP; CD45.2) and IFN-γR–deficient (CD45.2) bone marrow. Donor cells and host cells were identified by CD45 allelism; wild-type cells (CD45.2^GFP^) were distinguished from IFN-γR–deficient cells (CD45.2^GFP^−) on the basis of GFP expression (Fig. 6A). When mixed with C57BL/6 cells, the bone marrow-derived IFN-γR–deficient cells were fully reconstituting recipients mice, and at 6 wk postreconstitution, IFN-γR–deficient cells dominated in the peripheral blood (40.85 ± 6.38% wild-type versus 55.91 ± 6.43% IFN-γR–deficient). Moreover, IFN-γR–deficient T lymphocytes were found at higher frequency than wild-type T cells and, notably, double-positive CDIlb^+^ and Gr-1^+^ myeloid cells were predominantly of wild-type origin (data not shown).

The chimeric mice were infected with *E. muris*, and the frequency and phenotype of wild-type and IFN-γR–deficient cells were analyzed within the bone marrow. Bacterial infection in the chimeric mice was similar to those in C57BL/6 mice, indicating that the chimeric mice controlled infection (Fig. 6B). We next addressed whether the donor cells were of wild-type or IFN-γR–deficient origin in the myeloid progenitor cell populations. In mock-infected mice, we observed an increased frequency of IFN-γR–deficient donor cells, relative to wild-type donor cells, within the Lin^−^IL-7Rα^−^c-Kit^hi^Sca-1^−^ (i.e., CMP) population (Fig. 6C, 6D). However, a greater proportion of the Lin^−^IL-7Rα^−^c-Kit^hi^Sca-1^−^ cells was of wild-type origin. Postinfection, we found that the frequency of wild-type cells was greatly diminished within both the c-Kit^hi^Sca-1^−^ and c-Kit^hi^Sca-1^−^ fractions of Lin^−^IL-7Rα^−^ cells; these two populations of Lin^−^IL-7Rα^−^ myeloid progenitors were instead composed almost entirely of IFN-γR–deficient cells. These data suggest that hematopoietic cells unable to signal via the IFN-γ pathway are retained as immature cells within the bone marrow, perhaps as a consequence of their inability to undergo proper differentiation. In contrast, wild-type cells, capable of receiving IFN-γ–induced signals, were not retained in the bone marrow, possibly because they differentiate and/or egress from this site. Thus, we propose that in the absence of IFN-γ signaling, myeloid cells are prevented from completing their differentiation in response to infection-induced stress.

We next examined the bone marrow and spleens of mock-infected and *E. muris*-infected chimeric mice to determine the proportions of wild-type and IFN-γR–deficient cells within immature and mature granulocytic populations. In mock-infected chimeric mice, IFN-γR–deficient cells predominated within the CDIlb^+^Gr-1^+^ bone marrow population, but postinfection wild-type cells increased in proportion (Fig. 6F). There was little change in the composition of the CDIlb^+^Gr-1^+^ population postinfection, and the IFN-γR–deficient cells predominated. However, within the mature granulocyte population (CDIlb^+^Gr-1^+^) in the bone marrow, we found that *E. muris* infection resulted in a significant increase in the frequency of wild-type cells. Moreover, the higher proportion of wild-type CDIlb^+^Gr-1^+^ cells was also observed in the periphery: CDIlb^+^Gr-1^+^ cells were almost entirely of wild-type origin in the chimeric mice, both prior to and after infection (Fig. 6J). There was little effect of IFN-γ signaling.

### Table I. Frequencies of granulocyte subsets in the bone marrow and spleens of *E. muris*-infected mice

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Tissue</th>
<th>dpi</th>
<th>CDIlb^+^Gr-1^+^</th>
<th>CDIlb^+^Gr-1^+^</th>
<th>CDIlb^+^Gr-1^+^</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Bone marrow</td>
<td>0</td>
<td>12.15 ± 1.78</td>
<td>27.13 ± 3.11</td>
<td>0.66 ± 0.49</td>
<td>39.94</td>
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<td></td>
<td>Bone marrow</td>
<td>8</td>
<td>6.26 ± 3.08</td>
<td>33.90 ± 2.96</td>
<td>0.49 ± 0.11</td>
<td>40.66</td>
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<tr>
<td></td>
<td>Bone marrow</td>
<td>15</td>
<td>18.30 ± 3.70</td>
<td>28.27 ± 1.67</td>
<td>0.96 ± 0.21</td>
<td>47.50</td>
</tr>
<tr>
<td>IFN-γR^−/−</td>
<td>Bone marrow</td>
<td>0</td>
<td>11.86 ± 2.86</td>
<td>25.03 ± 3.04</td>
<td>0.59 ± 0.24</td>
<td>37.48</td>
</tr>
<tr>
<td></td>
<td>Bone marrow</td>
<td>8</td>
<td>17.73 ± 1.19</td>
<td>27.63 ± 5.48</td>
<td>0.49 ± 0.25</td>
<td>45.86</td>
</tr>
<tr>
<td></td>
<td>Bone marrow</td>
<td>15</td>
<td>56.60 ± 2.8</td>
<td>21.73 ± 2.83</td>
<td>0.37 ± 0.14</td>
<td>78.70</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Spleen</td>
<td>0</td>
<td>0.30 ± 0.09</td>
<td>0.46 ± 0.03</td>
<td>0.02 ± 0.003</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>15</td>
<td>0.07 ± 0.07</td>
<td>0.82 ± 0.072</td>
<td>0.06 ± 0.023</td>
<td>1.53</td>
</tr>
<tr>
<td>IFN-γR^−/−</td>
<td>Spleen</td>
<td>0</td>
<td>0.44 ± 0.11</td>
<td>0.63 ± 0.014</td>
<td>0.02 ± 0.001</td>
<td>1.084</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>15</td>
<td>12.40 ± 1.27</td>
<td>8.07 ± 5.48</td>
<td>0.62 ± 0.17</td>
<td>21.09</td>
</tr>
</tbody>
</table>

Data indicate average frequency ± SD. dpi, day postinfection.
on lymphoid lineage chimerism in mock-infected and *E. muris*-infected mice (data not shown), suggesting that IFN-γ acts on myeloid, but not lymphoid, differentiation during infection. Our data indicate that IFN-γ signaling is not required for the generation of CD11b<sub>lo</sub>Gr-1<sub>lo</sub> cells, but is important for mediating the terminal differentiation of neutrophils.

**IFN-γ-mediated transcriptional control of infection-induced myelopoiesis**

Although our data suggested that IFN-γ acts directly on hematopoietic progenitor cells, we wanted to address whether the IFN-γ signaling pathway was activated in the bone marrow promyelocytic CD11b<sup>lo</sup>Gr-1<sup>lo</sup> cells that accumulated during infection. Therefore,
CD11b<sup>B</sup>Gr-1<sup>B</sup> cells were purified by flow cytometry from mock- and day 15-infected wild-type and IFN-γR-deficient mice (>89% purity). Next, these cells were evaluated for the expression of genes known to be regulated by IFN-γ, including the IFN regulatory factors (IRFs), a family of transcription factors that are critical for various aspects of hematopoiesis and immune cell function. The expression of several irf genes was induced during infection, including irf-1, irf-3, irf-4, irf-5, irf-7, and irf-8. Compared to their expression in wild-type CD11b<sup>B</sup>Gr-1<sup>B</sup> cells, transcription of several of the irf genes was not induced in the IFN-γR-deficient cells harvested from infected mice (Fig. 7A), in particular irf-1 and irf-8. We also examined expression of a number of additional IFN-γ-regulated genes, including adenosine deaminase acting on RNA (Adar), Irgm1, Ifng, Ifngr1, and others (Fig. 7B). Most of these genes, known to be regulated by IFN-γ, were induced in wild-type CD11b<sup>B</sup>Gr-1<sup>B</sup> promyelocytes during infection, but not in IFN-γR-deficient cells. Thus, infection-induced IFN-γ directly enhances myelopoiesis, likely via its ability to induce the expression, in developing promyelocytes, of transcription factors known to be involved in hematopoiesis and granulopoiesis. These data also illustrate that IFN-γ is solely responsible for inducing transcription of irf-1, irf-8, Adar, Irgm1, Cxcl10, Mx1, Csf3, and Csf2rb during infection, as these genes were not induced in cells that could not signal via this pathway.

**Discussion**

In this study, we demonstrate that an intracellular bacterial infection causes major changes in the differentiation of hematopoietic progenitor cells via direct IFN-γ signaling. We conclude that while homeostatic production of monocytes and granulocytes is not dependent upon IFN-γ signaling, infection elicits the production of both populations of myeloid cells, and that this infection-induced process is critically dependent on IFN-γ signaling. These findings reveal a proposed, but largely unrecognized, role for IFN-γ in the direct modulation of hematopoiesis during bacterial infection. Because many pathogens induce IFN-γ production, these findings have implications for understanding host defense against a wide range of microbial infections. This conclusion is supported by a report that IFN-γ induces the expansion and proliferation potential of Lin<sup>-</sup> Sca-1<sup>-</sup> c-Kit<sup>+</sup> cells (30). Moreover, studies of hematopoiesis during malaria infection revealed the emergence of a unique population of Lin<sup>-</sup>IL-7Rα<sup>-</sup>c-Kit<sup>hi</sup> progenitor cells that was found to require IFN-γ signaling (20). We propose that these observations together provide a paradigm whereby inflammatory cytokines, such as IFN-γ, act to modulate the early differentiation of hematopoietic progenitor cells, thereby enhancing the production of the mature blood cells required to combat infection.

Although our findings have demonstrated an important role for IFN-γ in vivo, they contrast with earlier studies that described a negative effect of IFN-γ on hematopoiesis. In those studies, IFN-γ inhibited in vitro colony formation of human granulocyte-macrophage progenitor cells (14, 15, 32), and even low levels of IFN-γ inhibited hematopoiesis in long-term bone marrow cultures (13). We observed that the frequency of in vitro bone marrow colony-forming cells decreased during infection in wild-type mice. Although this finding would appear to be consistent with the earlier studies that demonstrated hematopoietic suppression, we propose instead that in vitro colony formation potential decreases because IFN-γ promotes hematopoietic progenitor cell differentiation, thus depleting the bone marrow of progenitor cells. In our studies, the classically defined myeloid progenitor cells were diminished postinfection in wild-type mice but not in IFN-γR-deficient mice, and in the wild-type mice, the frequency of Lin<sup>-</sup>IL-7Rα<sup>-</sup>c-Kit<sup>hi</sup>Sca-1<sup>-</sup> progenitors increased.

Thus, one possible explanation is that the Sca-1<sup>-</sup> cells have a greater ability to form colonies in vitro, whereas the infection-induced Lin<sup>-</sup>IL-7Rα<sup>-</sup>c-Kit<sup>hi</sup>Sca-1<sup>-</sup> population requires additional signals not provided in the standard in vitro culture conditions. Furthermore, in our studies of hematopoiesis in mixed bone marrow chimERIC mice, we observed that infection caused a loss of wild-type cells within the Lin<sup>-</sup>IL-7Rα<sup>-</sup>c-Kit<sup>hi</sup>Sca-1<sup>-</sup> and Sca-1<sup>+</sup> populations; in these mice the progenitor cells were mostly derived from IFN-γR-deficient donor cells. We think that this finding is consistent with our interpretation that the wild-type cells differentiated in response to infection-induced inflammation. An alternative explanation is that these cells were mobilized and emigrated from the bone marrow. We also observed high frequencies of wild-type-derived granulocytes within the bone marrow and spleen of infected mice, suggesting that the wild-type cells that were capable of IFN-γ signaling proliferated and differentiated. Under these same conditions, differentiating promyelocytes that could not signal via IFN-γ accumulated in the bone marrow and in the spleen. Thus, we propose that IFN-γ promotes, not suppresses, hematopoietic differentiation in vivo.

Our studies reveal an important role for IFN-γ in directing the development of both granulocytes and monocytes during infection. Although our previous studies of the bone marrow granulocyte population prompted our investigation of these cells, we noted alterations to the monocyte population as well. The expansion of blood monocytes observed in C57BL/6 mice was reduced in IFN-γR-deficient mice, suggesting that IFN-γ is required for the production of infection-induced monocytes. IFN-γR-deficient mice also exhibited pronounced neutrophilia postinfection, as compared with C57BL/6 mice, suggesting that granulopoiesis does not require IFN-γ signaling. However, more careful analysis of the
infection-induced granulocytes in the IFN-γR–deficient mice revealed that this population of cells was defective, as these cells were infected with *E. muris*. Additionally, wild-type cells that expressed the IFN-γR dominated the population of mature granulocytes in the chimeric mice. Thus, our data support a requirement for IFN-γ–dependent signaling in the terminal differentiation of both granulocytes and monocytes during ehrlichial infection.

These studies also highlight a critical role for IFN-γ in driving the transcriptional regulation of hematopoiesis during infection. IFN-γ mediates a range of signaling events within target cells. As our knowledge of IFN-γ signaling has come primarily from studies of macrophages and other differentiated cell types in vitro, how such signals are regulated in hematopoietic progenitor cells is unknown. CD11b<sup>lo</sup>Gr-1<sup>lo</sup> cells from infected IFN-γR–deficient mice expressed reduced amounts of *irf-1* and *irf-8* mRNA, relative to cells from wild-type mice. IRF-1 and IRF-8 (also known as IFN consensus sequence binding protein) are both known to play critical roles in hematopoiesis (33). IRF-1 deficiency results in increased numbers of immature granulocytic precursors (33), an observation that is similar to what we observed in infected IFN-γR–deficient mice. IRF-8 is required for normal monocyte and dendritic cell development (34, 35), which is consistent with our observation that fewer blood monocytes, but increased neutrophils, were detected postinfection in the IFN-γR–deficient mice. IRF-8 deficiency also results in myeloid hyperplasia (36) and in increased numbers of myeloid-derived suppressor cells (37); additionally, human myeloid malignancies show much lower than normal IRF-8 expression (38). IRF-8 also regulates the proliferation-inducing effects of GM-CSF by inducing the expression of *neurofibrin 1*, a tumor suppressor (39, 40). IRF-8 is also required for host defense: IRF-8–deficient mice are highly sensitive to Mycobacterium tuberculosis and *Plasmodium chabaudi* infections (41, 42). IRF-8 acts to promote phagocyte development and maturation by inducing the expression of the phagocyte oxidase proteins gp91<sup>phox</sup> and gp67<sup>phox</sup> (43). Our observation that a high frequency of granulocytes contained morulae in the spleens of IFN-γR–deficient mice is consistent with the role of IRF-8 in promoting phagocyte development during infection. Thus, the induction of *irf-8* expression during ehrlichia infection likely plays an important role in regulating myelopoiesis by controlling proliferation, differentiation, and oxidative activity of phagocytes, and it is likely to be directly responsible for the defects in myelopoiesis in our infection model. Additionally, our studies show that IFN-γ–mediated signaling is critical for regulating *irf-8* gene expression during infection.

IFN-γ–driven hematopoiesis can be viewed as a favorable response to infection, due to the production of pathogen-killing leukocytes. However, several studies have suggested that IFN signaling in HSCs may also have a negative effect. In this study, we have reported direct action of IFN-γ on myeloid progenitor cells, but it is likely that IFN-γ also acts on HSCs during ehrlichial infection, as several IFN-γ–inducible genes are vital to HSC maintenance (17, 18). The immunity-related GTPase *Irgm1* (also known as Lrg-47) was induced in HSCs during infection and was required for the maintenance of the HSC pool (17). The IFN-γ–inducible gene *Adar1* was also shown to be critical for HSC maintenance: loss of ADAR1 in hematopoietic cells led to rapid apoptosis and the induction of IFN-responsive genes (18). These studies provide strong evidence that IFN-γ acts directly on HSCs, and it was recently shown that IFN-γ signaling leads to HSC exhaustion during chronic mycobacterial infection (31). This conclusion is consistent with the observation that HSCs lacking *Irf-2*, a transcriptional repressor of type I IFN signaling, undergo exhaustion when chronically stimulated with IFN-α (44). Thus, although IFN-γ signaling may be an important driving force in responding to infection, this factor may also deplete stores of stem and progenitor cells within the hematopoietic compartment.

Although it is not clear how Sca-1 functions in progenitor cells, it is thought that Sca-1 can regulate different signaling pathways by modulating lipid raft composition, and it has been shown that overexpression of Sca-1 can inhibit myeloid differentiation (45). This observation is inconsistent with our hypothesis that IFN-γ drives myelopoiesis during infection. We propose that other genes, expressed during infection, modify the effects of Sca-1 signaling in vivo, as it is well known that IFN-γ signaling is context-dependent. Indeed, the observation that Lin “Sca-1<sup>−/−</sup>Kit<sup>+</sup>” cells can be induced to proliferate in response to IFN-γ (30) suggests that the outcome of IFN-γ signaling may differ in less differentiated progenitor cells. In support of this idea, it has been shown that Sca-1 is required for IFN-α–driven HSC proliferation (19).

Several reports have also revealed that TLR signaling is critical for HSC and progenitor cell differentiation (1, 3, 46–48), indicating that some pathogens interact directly with stem and progenitor cells. For example, monopoiesis was impaired in *Listeria monocytogenes*-infected MyD88-deficient mice (48). We observed that MyD88-deficient mice exhibited a phenotype similar to that of *E. muris*-infected IFN-γR–deficient mice with respect to the reduced Sca-1 upregulation on bone marrow progenitor cells (K. C. MacNamara and G. M. Winslow, unpublished observations). However, *E. muris* is not known to encode canonical TLR ligands (i.e., LPS and peptidoglycan), and we failed to detect any differences in hematopoiesis in infected mice deficient in TLR2, TLR4, or TLR9 (K. C. MacNamara and G. M. Winslow, unpublished observations). The TLR-associated signaling molecule MyD88 forms a physical association with the IFN-γR, and MyD88 can stabilize IFN-γ–induced gene transcripts (49). Thus, we propose that an alternative TLR-independent role for MyD88 in infection-induced hematopoiesis is via the IFN-γ pathway or in IL-1β or IL-18 signaling.

The role for IFN-γ specifically during periods of stress and infection is highlighted by the fact that under steady-state conditions the bone marrow, blood, and spleen have nearly identical frequencies of leukocytes in both IFN-γR–deficient mice and C57BL/6 mice. However, infection of IFN-γR–deficient mice resulted in enhanced frequencies and numbers of myeloid progenitor cells, as compared with C57BL/6 mice. In one model of why myelopoiesis is enhanced in the absence of IFN-γ, it was proposed that IFN-γ acts to suppress the innate immune response by acting on myeloid progenitor cells (16). This model is in conflict with our finding that in mice containing both wild-type and IFN-γR–deficient cells, most mature neutrophils were found to be of wild-type origin. Our data support the notion that IFN-γ induced during infection is required for the production of mature neutrophils. However, an alternative possibility is that IFN-γ acts to increase the lifespan of mature neutrophils, thus favoring the accumulation of wild-type cells in the spleen. We propose a model whereby IFN-γ acts directly on progenitor cells and immature cells, thereby accelerating their movement out of the pool of earlier progenitor cells. This would explain why the pool of Lin IL-7Rε<sup>−/−</sup>Kit<sup>hi</sup> cells is composed of IFN-γR–deficient cells in the infected chimeric mice: in the absence of IFN-γ signaling they fail to differentiate and, thus, dominate this pool.

Our data suggest that the hematological alterations observed during ehrlichiosis are due to the direct effects of inflammation on hematopoietic stem and progenitor cells. We propose that anemia and thrombocytopenia, which are hallmarks of ehrlichiosis, occur at the expense of promoting the production of granulocytes. The observation that the onset of these hematological abnormalities is...
delayed in IFN-γ–deficient mice, while we also see a reduced loss of classically defined myeloid progenitor cells, supports our conclusion that anaemia and thrombocytopenia are a direct result of altered functional capacity of myeloid progenitor cells. The cost of producing more granulocytes may therefore be the transient loss of hemoglobin and platelet production; this outcome may be favorable to the host, as wild-type mice survive infection and IFN-γ–deficient mice are unable to control the infection.

Most infectious diseases cause inflammation and induce the expression of IFN-γ, so similar alterations in hematopoiesis likely occur in many infections. A better understanding of how infections alter hematopoiesis and progenitor cell function and capacity is critical not only for our overall understanding of immunity, but for our general knowledge of how hematopoiesis is modulated in the face of inflammation and immunologic stress.

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Disclosures
The authors have no financial conflicts of interest.

References

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