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MyD88 Signaling in CD4 T Cells Promotes IFN-γ Production and Hematopoietic Progenitor Cell Expansion in Response to Intracellular Bacterial Infection

Yubin Zhang,* Maura Jones,* Amanda McCabe,* Gary M. Winslow,† Dorina Avram,‡ and Katherine C. MacNamara*

Hematopoietic stem and progenitor cell (HSPC) phenotype and function can change in response to infectious challenge. These changes can be mediated by cytokines, IFNs, and pathogen-associated molecules, via TLR, and are thought to promote tailored immune responses for particular pathogens. In this study, we investigated the signals that activate HSPCs during ehrlichiosis, a disease characterized by profound hematopoietic dysfunction in both humans and mice. In a mouse model of ehrlichiosis, we observed that infection-induced proliferation of bone marrow HSPCs was dependent on IFN-γ signaling and was partially dependent on MyD88. However, MyD88 was not required in HSPCs for their expansion during infection, because similar frequencies of MyD88-deficient and wild-type HSPCs proliferated in mixed bone marrow chimeric mice. MyD88-deficient mice exhibited low serum and bone marrow concentration of IFN-γ compared with wild-type mice. We next identified CD4 T cells as the primary cells producing IFN-γ in the bone marrow and demonstrated a nonredundant role for CD4-derived IFN-γ in increased HSPC activation. Using mixed bone marrow chimeric mice, we identified a requirement for MyD88 in CD4 T cells for increased T-bet expression, optimal IFN-γ production, and CD4 T cell proliferation. Our data demonstrate an essential role for CD4 T cells in mediating HSPC activation in response to bacterial infection and illustrate a novel role for MyD88 signaling in CD4 T cells in this process. These findings further support the idea that IFN-γ production is essential for HSPC activation and hematopoietic responses to infection.

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The direct stimulation of hematopoietic progenitors by pathogen-associated molecules was first demonstrated by Nagai et al. (4), who showed that myeloid cells could be generated from hematopoietic progenitors via TLR- and MyD88-dependent signaling. Related studies of vaccinia virus infection demonstrated that the TLR9 ligand CpG can act directly on common lymphoid progenitors to drive dendritic cell production, at the expense of lymphopoiesis (5). Candida albicans was shown to direct the production of myeloid cells in mice, via TLR2, which required intact MyD88 signaling (6, 7). The TLR adaptor protein MyD88 also has been implicated in the maintenance of monocytes, as was shown during Listeria monocytogenes infection (8). Thus, host responses to a wide variety of pathogens involve the infection-induced modification of hematopoiesis via direct TLR- and MyD88-mediated signaling.

In addition to their capacity to directly sense pathogens via TLRs, hematopoietic stem and progenitor cells (HSPCs) also can respond to inflammatory cytokines and IFNs produced during infection. We and others (9) have demonstrated a critical role for IFN-γ in activating HSCs during infection. Intrinsic IFN-γ-mediated signals were essential for functional myelopoiesis during infection with ehrlichia (10) and lymphocytic choriomeningitis virus (LCMV) (11). IFN-γ also has been shown to play a role in the emergence of a unique hematopoietic progenitor cell population during Plasmodium chaubudi infection (12). These findings demonstrate a novel role for IFN-γ in promoting immune responses during infection through its direct action on hematopoietic progenitors.

In this study, we have addressed which cells are responsible for driving IFN-γ-mediated changes in hematopoiesis during ehrlichial infection. Ehrlichia muris is a tick-transmitted, obligate intracellular pathogen, closely related to the causative agent of human monocytic ehrlichiosis (HME), Ehrlichia chaffeensis. HME is...
characterized by profound hematopoietic dysfunction and important clinical manifestations of the disease are severe anemia and thrombocytopenia (13). The ehrlichia do not encode genes for LPS or peptidoglycan synthesis (14), and the cellular host mechanisms by which these organisms elicit innate inflammation are currently unknown. Bone marrow–derived dendritic cells (BMDCs) from TLR-deficient and caspase-1–deficient mice responded to *E. muris* and generated normal amounts of IL-12p40 in vitro. However, MyD88-deficient BMDCs produced less IL-12p40 in response to *E. muris* infection (15), suggesting an important role for MyD88 signaling in production of IL-12, and/or IL-23, in response to ehrlichial infection, although the pathway in which MyD88 is required during ehrlichial infection is not yet known. We also noted that in the absence of the adaptor molecule MyD88, *E. muris*–infected mice exhibited increased susceptibility to infection, which was correlated with significantly reduced IFN-γ production. These findings prompted our investigation of how MyD88 deficiency impacted hematopoietic activity in response to ehrlichial infection. MyD88 signaling was not required in HSPCs for their expansion; rather, MyD88 signaling within CD4 T cells was essential for the production of IFN-γ. These studies are relevant to our understanding of how hematopoeisis is modulated during infection and inflammation and point to an important role for MyD88–dependent mechanisms within T lymphocytes in regulating the functional capacity of hematopoietic progenitors.

**Materials and Methods**

**Mice**

C57BL/6 mice and the following transgenic and gene-targeted strains were obtained from The Jackson Laboratory (Bar Harbor, ME): IFN-γR1–deficient (B6.129S7-Ifngr1tm1Agt/J; described in this study as IFN-γR deficient), IFN-γ-deficient (B6.129S7-Ifngtm1Bst/J), MyD88-deficient (B6.129P2(SJL)-Myd88tm1Defr/J), Ifngr1tm1Agt/J, C3H/HeJ, C3H/HeOuJ, CD4–deficient (B6.129S2-Cd4tm1Mak/J), β-act–EGFP (B6-Tg(CAG-EGFP)31Osbi/LeySop), and a CD45 congenic strain (B6.SJL-Ptprca Pepcb/BoyJ). TLR9-deficient mice were provided by Dr. S. Swain (Trudeau Institute, Saranac Lake, NY), and TLR2-deficient mice were provided by Dr. T. Sellati (Albany Medical College, Albany, NY). All mice were bred in the Animal Resources Facility at Albany Medical College under microisolator conditions.

**Bacteria**

Mice were infected, via i.p. injection, between 6 and 12 wk of age, with 50,000 *E. muris* bacteria obtained from infected mouse splenocytes, as described previously (10).

**PCR quantification for bacterial burden**

DNA from 2 × 10^6 splenic cells was extracted using DNAzol (Molecular Research Center, Cincinnati, OH). The number of bacterial copies was assayed using a real-time quantitative probe-based PCR that measured the copy number of the bacterial *dsb* gene (which encodes a thiodisulfide oxidoreductase gene) (16, 17).

**Flow cytometry and Abs**

Bone marrow mononuclear cells were harvested and prepared as described previously (10). The Abs used for flow cytometry included the following: biotin-conjugated lineage markers specific for CD3 (clone 17A2), CD11b (M1/70), Gr-1 (RB6-8C5), Ter119 (Ly-76) and CD45R (RA3-6B2), Alexa700-CD45.2 (104), EFluor450-streptavidin, and allophycocyanin-CD3 (17A2) (all from eBioscience, San Diego, CA); PE-cychrome-7 (Cy7)-Sca-1 (D7), PE-Scal-1 (D7), PerCP-Cy5.5-CD45.2 (104), PE-CD45.1 (RMV-7), allophycocyanin–c-Kit (2B8), allophycocyanin–Cy7-streptavidin, Pacific Blue (PB)-CD4 (RM4-5), FITC-CD4 (RM4-5), FITC-CD3 (17A2), PE-CD8 (53-67), PerCP-Cy5.5-CD8 (53-67), FITC-B220 (RA3-6B2), PB-CD19 (6D5), PE-CE7-NK1.1 (PK136), allophycocyanin-IFN-γ (XM1G2.12), FITC-CD11b (M1/70), allophycocyanin–CD11c (N418), PE-CD11b (AF589), allophycocyanin–Ly6C (HK1.4), PB-Ly6C (HK1.4), PerCP-Cy5.5-Ly6G (1A3F11), PE-CD4–MHCI class II (AF6-120.1), PE-GR1 (RB6-8C5), allophycocyanin–Cy7-F4/80 (Cl: A3-1), FITC-F4/80 (CF: A3-1), and PE-CD68 (FA-11) (all from BioLegend, San Diego, CA); and V500-CD45.2 (104) (from BD Biosciences). PE-conjugated CD1d tetramer was a gift from Dr. E. Leadbetter (Trudeau Institute, Saranac Lake, NY). Unstained cells were used as negative controls to establish the flow cytometer voltage settings, and single-color positive controls were used to adjust the instrument compensation. The flow cytometric data were acquired using an LSR II flow cytometer (BD Biosciences), and data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

**In vitro hematopoietic progenitor cell assays**

Mice were administered recombinant IFN-γ (PeproTech, Rocky Hill, NJ) via i.v. injection (10 μg/200 μl) on days 8, 9, and 10 postinfection. Bone marrow cells were harvested at day 11 postinfection, plated at 2.0 × 10^6 per 35-mm tissue culture dish, in duplicate, and cultured in methocult media (MethoCult GF M3434; Stem Cell Technologies, Vancouver, BC, Canada). After incubation for 7 d at 37°C in 5% CO2, colonies derived from multipotent granulocyte, erythroid, macrophage, and megakaryocyte progenitors (GEMM), granulocyte-macrophage progenitors (GM), macrophage progenitors (M), and granulocyte progenitors (G) were scored.

**Intracellular cytokine staining**

Bone marrow and splenic cell suspensions were prepared, and erythrocytes were removed by a brief hypotonic lysis. A total of 2 × 10^6 cells were plated in a 96-well plate, and FcRs were blocked by incubation with anti-CD16/32 mAb for 20 min on ice. Cells were incubated on ice for 30 min with specific Abs to stain for surface proteins. Cells were washed and then fixed and permeabilized in Fix/Perm buffer (BD Biosciences). Intracellular IFN-γ was detected by incubating cells in Wash/Perm buffer (BD Biosciences) with anti–IFN-γ–ab (clone XM1G2.12) for 30 min on ice. Cells were washed twice in Wash/Perm buffer, resuspended in simple wash buffer, and analyzed on an LSR II (BD Biosciences).

**Nuclear staining for T-bet**

Bone marrow and splenic cells were prepared, and surface staining was performed as described previously. Thereafter, cells were processed fixed and permeabilized and then stained with PE-Cy5–conjugated anti-T-bet (4B10) Ab (buffers and Abs from eBioscience). Protocols were following the manufacturer’s instructions. Cells were analyzed on an LSR II, and data were analyzed using FlowJo software.

**In vivo proliferation assay**

Mice were administered with BrdU (1 mg/mouse; i.p.) 6 h prior to harvest at day 11 postinfection. Cells were surface stained as described above and then fixed and permeabilized in Fix/Perm buffer (BD Biosciences) and Fix/Perm buffer containing DMSO, respectively. Cells were incubated with DNase I (Sigma-Aldrich) at 37°C for 1 h and then incubated with FITC-conjugated anti-BrdU Ab (PE (53-67); eBioscience) or PE (Bud30; BioLegend)–conjugated anti-BrdU Ab. Flow cytometry was performed using an LSR II flow cytometer, and data were analyzed using FlowJo software.

**Serum and bone marrow cytokine measurements**

Bone marrow homogenates were made in the presence of Nonidet P-40 and protease inhibitors. Serum and bone marrow homogenate cytokine assessment was performed using the Lumisphere platform (Bio-Rad), according to the manufacturer’s instructions. Total protein concentration in bone marrow homogenate was measured by the bicinchoninic acid kit (Pierce, Rockford, IL) according to the manufacturer’s instruction. Cytokines in bone marrow were calculated as picograms per milligram of total protein.

**Generation of mixed bone marrow chimeras**

To generate mixed wild-type and MyD88-deficient chimeric mice, CD45 congenic mice were lethally irradiated (950 rads, administered in two doses, 3 h apart). Irradiated mice received a total of 5 × 10^6 bone marrow cells derived from β-ACT-EGFP and MyD88-deficient mice. Mice were screened for chimerism at 4–6 wk and infected with *E. muris* at 7 wk postreconstitution. To generate chimeras where CD4 cells were unable to produce IFN-γ (referred to as CD4^<IFN-γ<sup>−</sup>−>) mice, IFN-γ–deficient mice were lethally irradiated and reconstituted with 2.5 × 10^6 bone marrow cells from each CD4-deficient and IFN-γ–deficient mice. Control chimeric mice (referred to as CD4^<IFN-γ<sup>−</sup>−>) were generated by irradiating wild-type C57BL/6 mice that were reconstituted with equal numbers of CD4-deficient and wild-type bone marrow cells (total 5 × 10^6 cells).

**Statistical analyses**

Statistical analyses were performed with a two-way ANOVA or a Student t test, as indicated, using Prism GraphPad Software (La Jolla, CA); p < 0.05 was considered to be significant.
We previously identified IFN-γ as a major mediator of hematopoietic function during *E. muris* infection and demonstrated that IFN-γ promoted the production of mature myeloid cells (10). These studies led us to address whether bacterial ligands contributed to direct activation of HSPCs, as has been shown in other models of infection (4). Similar to a previous report, we found no changes in bacterial infection or immunity (data not shown) and similar frequencies of Lin⁻, Sca-1⁺, c-Kit⁺ (LSK) cells in mice deficient in TLR2 or TLR4 (Supplemental Fig. 1A, 1B and Ref. 15), consistent with the fact that *E. muris* lacks the genes required for the synthesis of LPS or peptidoglycan. The primary TLR for bacterial CpG DNA is TLR9 (18), which, we predicted would play an important role in the induction of IFN-γ production during ehrlichiosis. However, we found that bacterial infection was similar in TLR9-deficient mice and C57BL/6 mice, and expansion of the LSK population, greatly enriched for HSPCs, was similar in frequency and number in both strains after *E. muris* infection (Supplemental Fig. 1C). In contrast, we found that mice deficient in the TLR adaptor molecule MyD88 exhibited reduced frequencies and numbers of LSK cells, as compared with C57BL/6 mice (Fig. 1A–C). To test whether the increase in LSK cells was accompanied by cell proliferation, we measured BrdU incorporation in progenitor cells in response to *E. muris* infection. Mice were injected with BrdU 6 h prior to sacrifice, and we found that the BrdU incorporation was significantly higher in the LSK population of wild-type mice, relative to IFN-γR-deficient and MyD88-deficient (Fig. 1D, 1E). Although LSK cells in MyD88-deficient mice exhibited reduced BrdU incorporation, relative to wild-type mice, the proliferative defect in the former strain was of lower magnitude relative to that observed in the absence of IFN-γR-mediated signaling. Increased proliferation may represent an increase in Sca-1 expression on Lin⁻, cKit⁺, Sca-1⁻ myeloid progenitors as well as increased proliferation of Sca-1⁺ cells. These data suggest that MyD88 signaling partially mediates the infection-induced increase of this progenitor cell population during infection.

**Intrinsic MyD88 signaling is not required for progenitor cell expansion**

To test whether MyD88 signaling was required in progenitor cells for the infection-induced increase in LSK cells, we generated radiation-induced mixed bone marrow chimeric mice that contained similar numbers of wild-type cells, derived from β-ACT-GFP mice (CD45.2; GFP⁺), and MyD88-deficient donor cells (CD45.2; GFP⁻). Seven weeks postconstitution, chimeric mice were infected with *E. muris*. On day 11 postinfection, the frequency of BM LSK cells induced by *E. muris* infection in the chimeric mice was equivalent to what we observed in wild-type infected mice but was higher relative to MyD88-deficient mice (Fig. 2A, 2B). We hypothesized that if MyD88 signaling was intrinsically required for the infection-induced increase in LSK progenitor cells, we would observe a reduced frequency of MyD88-deficient LSK cells, relative to wild-type LSK cells, in *E. muris*-infected chimeric mice. However, the LSK population contained equivalent frequencies of wild-type and MyD88-deficient cells (Fig. 2C, 2D). Moreover, in chimeric mice, we observed similar proliferation of both wild-type and MyD88-deficient bone marrow LSK cells during infection (Fig. 2E, 2F). These data reveal that MyD88 signaling in progenitor cells is not required for the infection-induced expansion the progenitor population. We also observed significantly higher bacterial infection in splenocytes from *E. muris*-infected mice that lacked MyD88, as compared with wild-type controls and mixed chimeric mice on day 11 postinfection (Fig. 2G). The increased bacterial burden observed in MyD88-deficient mice was similar to what was observed in mice that lack IFN-γ, suggesting a defect in IFN-γ production or signaling in the absence of MyD88. Although bacteria can be detected in the bone marrow, burden is much lower relative to the spleen. Bacterial burden diminished by day 15 postinfection in all strains; however, it was slightly elevated in MyD88-deficient mice relative to wild-type mice.

**MyD88 signaling promotes IFN-γ production during ehrlichiosis**

MyD88 signaling can augment IFN-γ production, as has been shown in murine models of tularemia, legionella infection, and *Chlamydia* (19–21). Because intrinsic MyD88 signaling was not required for the expansion of the LSK population, we next addressed whether MyD88 signaling was required for IFN-γ production during *E. muris* infection. IL-12p70 was also measured because it is known to induce IFN-γ production (22). Both IFN-γ and IL-12p70 concentrations were significantly increased in the serum of infected mice, relative to mock-infected mice, by day 11.
postinfection (Fig. 3A). In comparison with *E. muris*-infected wild-type mice, MyD88-deficient mice had significantly reduced amounts of IFN-γ and IL-12p70 in sera on day 11 postinfection. Serum concentrations of IFN-γ and IL-12p70 in mock-infected wild-type mice were similar to concentrations observed in MyD88-deficient mice, both expressing comparable amounts of serum MCP (MCP-1), IL-10, IL-6, and TNF-α in mock-infected *E. muris*-infected mice. However, MyD88-deficient mice expressed increased cytokine levels in response to infection, indicating that IFN-γ and/or IL-12p70 production might require MyD88 signaling.

To address whether changes in serum cytokine concentrations were reflected by similar changes within the bone marrow, where HSPCs reside, we next measured cytokine concentrations in bone marrow homogenates. As was observed in the sera, significantly increased IFN-γ was observed in wild-type bone marrow following infection, relative to MyD88-deficient mice; the mixed bone marrow chimeric mice exhibited an intermediate concentration of IFN-γ on day 11 postinfection (Fig. 3B). IL-12p70 concentration was increased in wild-type mice on day 11 postinfection; however, no significant differences were observed between wild-type and MyD88-deficient mice postinfection. Thus, MyD88 plays a role in regulating the production of IFN-γ in response to *ehrlichia* infection.

IFN-γ contributes to LSK expansion, macrophage colony formation, and monopoiesis

To directly test whether reduced LSK expansion in MyD88-deficient mice was due to decreased IFN-γ production during *ehrlichia* infection, we administered rIFN-γ to MyD88-deficient mice on days 8, 9, and 10 postinfection. Relative to *E. muris*-infected MyD88-deficient mice, administration of rIFN-γ resulted in an increase in the frequency and number of LSK cells, such that rIFN-γ-treated MyD88-deficient mice resembled *E. muris*-infected wild-type mice (Fig. 4A, 4B). As we previously demonstrated that infection-induced LSK cells contained increased myeloid potential (23), we next determined the functional significance of IFN-γ-induced changes to progenitor cell phenotype by quantitating hematopoietic progenitors in the bone marrow. Infection elicited an increase in primitive GEMM (CFU-GEMM) and GM (CFU-GM) colonies in both wild-type and MyD88-deficient mice.
of the increase in the presence of MyD88 signaling. To address the functional significance, we analyzed populations. This increase was markedly reduced in the absence of MyD88 signaling. (Fig. 4C) indicating MyD88 signaling is not required for an increase in primitive progenitors. The number of granulocyte colonies was unchanged in infected mice, however, an increase in M colonies (CFU-M) was observed. Whereas MyD88-deficient mice exhibited significantly reduced CFU-M, relative to wild-type mice, rIFN-γ treatment of MyD88-deficient mice resulted in an increase in CFU-M postinfection. In addition to the IFN-γ-dependent increase in macrophage progenitors we observed increased monocytes (CD11b+Ly6C+) in the bone marrow postinfection, a process dependent on IFN-γ (Fig. 4D, 4E). Increased monopoiesis did not require intrinsic MyD88-signaling as equal numbers of wild type and MyD88-deficient monocytes were observed in mixed chimeric mice (Fig. 4F, 4G). These data demonstrate that MyD88 contributes to increased LSK cells, and specifically increased monopoiesis, in an IFN-γ-dependent manner.

Identification of the source of IFN-γ in the bone marrow during infection

IFN-γ can activate dormant HSCs (24), can induce Sca-1 expression (25), and contributes to infection-induced monopoiesis in models of bacterial and viral infection (11, 23); however, the source(s) of IFN-γ within the bone marrow during infection have not been defined. Expression of Ifng in the bone marrow is increased significantly on day 11 postinfection (23), and we demonstrated in this study that total IFN-γ protein also was significantly increased at this time point. As the increase in IFN-γ occurs concomitantly with phenotypic and functional activation of HSCs (23), we sought to identify the cell population(s) responsible for the production of IFN-γ at this time. Populations of cells were purified from the bone marrow of mock- or E. muris–infected wild-type and MyD88-deficient mice on day 11 postinfection, and expression of Ifng was quantitated (Supplemental Fig. 2). We observed relatively high Ifng expression in CD4 T cells from E. muris–infected mice, but very little expression in other cell populations. This increase was markedly reduced in the absence of MyD88 signaling. To address the functional significance of the increase in Ifng in CD4 T cells during infection, we performed intracellular cytokine staining (ICCS) for IFN-γ on CD4 T cells directly ex vivo. Within the bone marrow of wild-type mice, we found that ∼30% of CD4 T cells produced IFN-γ during infection (Fig. 5A). The frequency of IFNγ+ CD4 T cells was significantly higher in the bone marrow, relative to the spleens of infected mice. In the absence of MyD88 signaling, however, frequencies of IFNγ+ CD4 T cells were significantly reduced in response to ehrlichia infection. CD8 T cells in E. muris–infected mice also produced higher amounts of IFN-γ as compared with CD8 T cells obtained from mock-infected mice (Fig. 5B). In contrast to CD4 T cells, however, the increased CD8 T cell-derived IFN-γ in E. muris–infected mice was not dependent on MyD88-mediated signals. Because the frequencies of IFNγ+ CD4 T cells were much higher than anticipated, we sought to validate our ICCS assay by examining IFN-γ production in mice that lacked the Ifng and Ifngr genes. IFN-γ was not detected in IFN-γ-deficient mice, as expected (Supplemental Fig. 3), but we observed significantly increased IFN-γ in mice lacking the Ifngr. Increased IFN-γ expression in CD4 T cells in IFN-γR-deficient mice was not unexpected, based on previous reports that IFN-γ production is elevated in the absence of IFN-γR-mediated signaling (26). Furthermore, CD4 T cells exhibited little to no IFN-γ staining in the absence of permeabilization demonstrating that the IFN-γ detected in our assay was produced and not simply bound to the surface of the IFN-γ cell.

Although MyD88 signaling can modulate T cell migration (20), similar numbers of CD4 T cells were detected within the bone marrow of mock- and E. muris–infected mice, and no differences were observed between wild-type and MyD88-deficient mice (Fig. 5C). In contrast, E. muris infection elicited an increase in CD8 T cells within the bone marrow that was independent of MyD88 signaling. Despite the increase in CD8 T cells in the bone marrow, the number of IFNγ+ CD8 T cells was much lower than IFNγ+ CD4 T cells (Fig. 5D). The number of splenic CD4 and CD8 T cells was also similar between mock- and E. muris–infected mice (Fig. 5E), although, we observed significantly higher numbers of IFNγ+ CD4 T cells in the spleens of infected wild-type mice (Fig. 5F). In the bone marrow in both wild-type and MyD88-deficient mice, IFNγ+ CD4 T cells exhibited a highly activated phenotype (CD44hiCD62Llo) (Fig. 5G). In the spleen, however, wild-type IFNγ+ -producing CD4 T cells exhibited a more activated phenotype, as compared with MyD88-deficient IFNγ+ CD4 T cells. Thus, the bone marrow contains a more activated pool of CD4 T cells. These data demonstrate that MyD88 signaling was partially required for CD4 T cell activation and production of IFN-γ.

Although IFN-γ can be produced by cells of different lineages, it was not detectable in NK cells in the bone marrow (Supplemental Fig. 4A, 4B). In contrast, IFN-γ was detected in NKT cells in both wild-type mice and MyD88-deficient mice (Supplemental Fig 4C); however, E. muris infection did not induce a significant change in NKT cell IFN-γ production in either wild type or MyD88-deficient mice. The number of NKT cells was similar in all groups of mice, whereas a decrease in NK cell number was noted in response to infection, and in the bone marrow of MyD88-deficient mice (data not shown). It has been demonstrated that myeloid cells can produce IFN-γ during invasive group A Streptococcus infection (27), thus we also performed ICCS on neutrophils, monocytes, and macrophages in the bone marrow. We observed a low amount of IFN-γ expression in neutrophils and macrophages; however, this was likely due to surface-bound IFN-γ, as we observed similar IFN-γ staining in the absence of permeabilization (Supplemental Fig 4D, 4F). IFN-γ was not detected in monocytes and immature myeloid cells (Supplemental...
Fig 4E). Similar negative data were obtained for splenic NK cells, NKT cells, and myeloid cells (data not shown). Thus, we have identified CD4 T cells as critical producers of IFN-γ during ehrlichia infection, and a requirement for MyD88-signaling for this response.

CD4 T cell–derived IFN-γ is essential for bone marrow LSK expansion during infection

To test whether CD4 T cell–derived IFN-γ was essential for LSK cell expansion during infection, we generated mixed bone marrow chimeric mice wherein CD4 T cells were unable to produce IFN-γ (CD4 Ifng−/−). A small percentage of T cells can persist even after lethal irradiation (28); thus, IFN-γ–deficient mice were used as recipient mice, to ensure that all CD4 T cells were unable to produce IFN-γ. IFN-γ–deficient mice were irradiated and reconstituted with equal numbers of bone marrow cells from CD4-deficient, and IFN-γ–deficient mice; thus, in the chimeric mice, all cells, other than CD4 T cells, were able to produce IFN-γ. Control chimeric mice in which CD4 T cells were sufficient for IFN-γ (CD4+/+) were also generated. After E. muris infection, reduced frequencies and lower numbers of LSK cells were detected in the bone marrow of CD4 Ifng−/− mice (Fig 6. A–C).

Although it is currently unclear if local production of IFN-γ in the bone marrow is essential for LSK expansion, these data demonstrate that CD4 T cells play a nonredundant role in promoting the optimal expansion of HSPCs via IFN-γ in response to an intracellular bacterial infection.

MyD88 signaling is required in CD4 T cells for robust expression of IFN-γ and cell proliferation

To determine whether MyD88 signaling regulates CD4 T cell function via an intrinsic mechanism, we generated mixed chimeric
mice using wild-type and MyD88-deficient bone marrow. Donor wild-type cells were obtained from \( \beta \)-ACT-GFP mice, which facilitated the detection of both donor wild-type (CD45.2; GFP\(^+\)) and MyD88-deficient T cells (CD45.2\(^+\); GFP\(^2\)). This strategy also allowed us to distinguish wild type (i.e., radioresistant) host-derived T cells (CD45.1\(^+\); GFP\(^2\)). Routine screening at 4 wk posttransplantation and analysis of bone marrow at the time of infection revealed normal numbers of T cells, relative to nonirradiated mice, and equal percentages of donor wild type and MyD88-deficient cells in chimeras (data not shown).

Following infection, reduced frequencies of IFN-\( \gamma \)–producing cells were detected among MyD88-deficient CD4 T cells, relative to wild-type cells in both the bone marrow and the spleen (Fig. 7A, 7C). We also observed that wild-type CD4 T cells exhibited increased BrdU incorporation, relative to MyD88-deficient CD4 T cells, in both the bone marrow and spleen (Fig. 7B, 7D). Because MyD88 signaling is well documented to contribute to activation of APCs, which may also contribute to CD4 T cell function, we also measured expression of MHC class II on populations of APCs in the mixed bone marrow chimeric mice. Very similar class II surface

**FIGURE 5.** Increased IFN-\( \gamma \) expression in CD4 T lymphocytes requires MyD88. ICCS was performed directly ex vivo. Bone marrow and spleen cells were harvested from mock-infected and \( E. \) muris–infected C57BL/6 and MyD88-deficient mice on day 11 postinfection. CD3\(^+\)CD4\(^+\) (A) and CD3\(^+\)CD8\(^+\) (B) cells were analyzed for IFN-\( \gamma \) expression in mock-infected bone marrow (top row), \( E. \) muris–infected bone marrow (middle row), and infected spleens (bottom row). Representative flow plots are shown for C57BL/6 mice (left column) and MyD88-deficient mice (right column), and the numbers above the gated region represent the average frequency and SD of IFN-\( \gamma \)^+ cells among CD4 T cells. Total numbers (C) and numbers of IFN-\( \gamma \)^+ CD4 and CD8 T (D) cells in the bone marrow are shown. Total numbers (E) and numbers of IFN-\( \gamma \)^+ CD4 and CD8 T (F) cells in the spleen are shown. Average numbers and the SD are shown; gray bars represent mock-infected mice, and open bars represent \( E. \) muris–infected mice. (G) Expression of CD44 and CD62L on CD3\(^+\)CD4\(^+\)IFN-\( \gamma \)^+ T cells in the bone marrow (upper row) and spleen (bottom row) in the wild-type mock and \( E. \) muris–infected wild-type and MyD88-deficient mice are shown. Numbers represent the frequencies within each gated region. Statistically significant differences are shown; # indicates differences between strains of mice, whereas * represents differences between mock and infected groups. At least five mice were assayed for each group in two independent experiments.

**FIGURE 6.** CD4 T cell–derived IFN\( \gamma \) is required for LSK expansion during bacterial infection. Radiation-induced mixed bone marrow chimeric mice were generated in which CD4 T cells were unable to secrete IFN-\( \gamma \) (CD4\(^{Ifng^{-/-}}\); control chimeric mice contained wild-type CD4 T cells (CD4\(^{Ifng^{+/+}}\)). (A) Representative flow cytometric plots of lineage^- cells that were analyzed for expression of c-Kit, Sca-1^+ in mock- and \( E. \) muris–infected chimeric mice on day 11 postinfection. The frequency of c-Kit^+ Sca-1^+ cells among total Lin^- cells is shown above each gate, and the average frequencies are shown in (B). (C) Numbers of LSK cells in one leg is shown for mock (\( \bullet \)) and \( E. \) muris–infected (\( \square \)) chimeric mice. Statistically significant differences are shown; # indicates differences between strains of mice, whereas * represents differences between mock and infected groups. These data represent one experiment with between three and seven mice in each experimental group.
expression was detected on both wild-type and MyD88-deficient dendritic cells, macrophages, and B cells in both the bone marrow and spleen (data not shown).

**MyD88 signaling contributes to T-bet expression in CD4 T cells during infection**

T-bet is a critical transcription factor that regulates IFN-γ expression by T cells (29); thus, we determined whether MyD88 signaling was required for T-bet expression in response to infection. Following *E. muris* infection, T-bet expression was significantly increased in CD4 T cells in the bone marrow and spleen; MyD88 signaling was required for high T-bet expression in CD4 T cells, and relatively higher T-bet expression was observed in bone marrow CD4 T cells, as compared with splenic CD4 T cells (Fig. 8A, 8B). Moreover, we found that T-bet expression was significantly higher in wild-type CD4 T cells, as compared with MyD88-deficient CD4 T cells (Fig. 8C, 8D), in *E. muris*-infected mixed chimeric mice, demonstrating an intrinsic requirement for MyD88 in increasing T-bet expression. These data establish that intrinsic MyD88 signaling in CD4 T cells regulates T-bet expression. Thus, our data together identify an intrinsic defect in MyD88-deficient CD4 T cell function that results in reduced HSPC activation and proliferation in response to a bacterial infection.

**Discussion**

In the current study, we found that intrinsic MyD88 signaling contributed to the infection-induced increase in LSK cells by driving IFN-γ production by CD4 T cells. Furthermore, MyD88 signaling was not required in HSPCs for their increase or proliferation during *E. muris* infection. This finding reinforces the idea that cytokine-mediated signaling is an important component of HSPC activation during infection. Because LSK expansion is not completely ablated in the absence of IFN-γ, we propose that additional cytokines may impact HSPC activation and proliferation including TNF-α and IL-6, as has been reported in vitro (30), either alone or in concert with IFN-γ. Our data suggest that direct sensing of pathogens by stem and progenitor cells is not absolutely essential to drive progenitor cell expansion and differentiation. Thus, progenitor cells can respond to infectious agents at sites distant from infection due to the action of soluble factors and activated cells, which may be particularly important for host defense against intracellular pathogens. Extracellular bacteria and fungi may be more able to directly activate progenitor cells that express TLRs. In contrast, hematopoietic responses to intracellular pathogens, such as *E. muris*, may rely on cytokine-mediated activation. The cytokine cues present during different infections, and in response to particular hematopoietic stresses, likely act to shape the hematopoietic response at the stem and progenitor cell level to gear cell production toward generating particular cell types.

In response to adjuvants such as CFA and Alum production of neutrophils is increased, at the expense of lymphopoiesis, a process termed “emergency granulopoiesis” (1, 31). Alum-elicited emergency granulopoiesis depends on IL-1β and TNF-α (1, 32). In contrast to Alum, the primary mediator of LPS-induced emergency granulopoiesis is G-CSF, and it occurs independent of IL-1β-mediated signaling (33). Thus, different adjuvants operate via unique mechanisms to elicit granulocyte production, and the production of granulocytes is likely particularly important for control of extracellular bacterial pathogens. However, myelopoiesis is increased in response to intracellular bacteria and virus infections as well. Ehrlichial infection is characterized by robust...
The mechanisms by which *E. muris* elicits an IFN-γ response are not yet clear, however we found a critical role for MyD88-signaling in CD4 T cells for robust production of IFN-γ. It is also important to note that MyD88-signaling in nonhematopoietic cells, such as mesenchymal stem cells and other stromal cells in the bone marrow, may contribute to optimal HSPC responses during ehrlichiosis. In this study, we demonstrate an intrinsic requirement for MyD88 in the infection-induced increase in T-bet expression in CD4 T cells.

Although IFNγ+ CD8 T cells were also significantly increased during *E. muris* infection, the total numbers of IFNγ+ CD8 T cells were minor compared with the CD4 T cell population. The striking frequency of IFNγ+ CD4 T cells within the bone marrow was nearly triple what was observed in the spleen. It will be interesting to determine whether robust IFN-γ production by CD4 T cells in the bone marrow is common to many different infections or is unique to ehrlichial infection and infections that elicit robust production of monocytes. Another outstanding question is whether CD4 T cells are activated within the bone marrow or traffic to the bone marrow from peripheral sites, which will be an important area of future research. IFN-γ is also responsible for inducing severe blood pathologies, including anemia and thrombocytopenia, during ehrlichiosis and other intracellular infections such as *Toxoplasma gondii* infection (34). The observation of profound hematopoietic dysfunction in HME patients, combined with our data demonstrating that IFN-γ drives many similar changes in the murine model of ehrlichiosis, suggests that a unique feature of HME pathogenesis is the overwhelming activation of a Th1 CD4 T cell response within the bone marrow.

Previously we, and others, have reported that infection-induced activation of HSPCs required IFN-γ signaling (23, 24). Although multiple types of cells including T cells, NK cells, and NKT cells are able to produce IFN-γ in vivo, the source(s) of IFN-γ that drives LSK expansion in the bone marrow during *E. muris* infection had not been defined. Our data show that CD4 T cells are the major producers for IFN-γ in bone marrow and that a very high frequency of IFNγ+ CD4 T cells is present in this tissue.
expression in CD4 T cells. MyD88 is an essential member of the IL-1R/TLR superfamily and mediates TLR, IL-18R, and IL-1ß signaling (as reviewed in Ref. 35). It is unclear whether the CD4 T cell response observed in response to ehrlichial infection is Ag-specific, or whether it occurs via non-specific activation, such as what can occur during Salmonella typhimurium infection when IL-18 is abundant (36). As CD4 T cells do express TLRs, at least at the transcriptional level, it is possible that the reduced IFN-γ production by CD4 T cells is due to impaired TLR signaling. However, the requirement for TLR signaling in mediating infection-induced LSK expansion is not supported by our other data; thus, we favor the possibility that diminished responses to IL-18 in MyD88-deficient CD4 T cells accounts for reduced IFN-γ production by these cells.

T cell–intrinsic defects in the absence of MyD88 have been reported in other infection models, most notably with LCMV. In a model of LCMV-induced neuropathology, MyD88 was shown to play a CD4 T cell intrinsic role in driving pathogenesis in the brain (37). In the absence of MyD88 signaling mice were protected from neuroinflammation and significantly fewer IFNγ+ CD4 T cells were observed in the brain. It was also shown that MyD88-dependent CD4 T cell function was not due to IL-18 or IL-1ß suggesting a novel mechanism by which MyD88 signaling can regulate CD4 T cell function. In addition, the authors demonstrated that CD4 defects were independent of APCs, similar to what we observe in our studies. T cell intrinsic defects in CD8 T cells were also observed during LCMV infection, and the primary defect was seen in survival during clonal expansion (38). MyD88-deficient, Ag-specific CD8 T cells were able to proliferate similarly to wild-type cells but exhibited impaired survival. The authors suggested that MyD88-dependent pathways were linked to the inflammatory signals specific to LCMV infection although they were unable to identify the specific signals involved. In a model of Toxoplasma gondii infection, T cell intrinsic MyD88 was also found to be critical for controlling disease (39). Interestingly, the impact of MyD88 signaling was not through IL-1R or IL-18R, supporting the possibility that direct TLR stimulation of T cells during toxoplasmosis is important for T cell effector function. Thus, MyD88 may operate in T cells in a variety of pathways that may depend on the infectious agent and inflammatory cytokines present in particular tissues during infection.

NKT cells have been reported to be activated by E. muris (40) and were shown to regulate host immunity in response to infection with a related strain of ehrlichia, Ixodes ovatus ehrlichia (IOE) (17). We did not observe a significant difference in NKT cell production of IFN-γ in wild-type and MyD88-deficient mice indicating the decreased IFN-γ observed in MyD88-deficient mice is not due to a defect in NKT cell function. In fact, we observed slightly, although not statistically significant, increased frequencies of IFNγ+ NKT cells in the absence of MyD88 signaling. This is in agreement with the observation that serum IFN-γ concentrations were not significantly reduced in the absence of NKT cells as shown during IOE infection (17). Taken together, we conclude that CD4 T cells are the major IFN-γ-producing cells in the bone marrow during E. muris infection.

The total number of CD4 T cells in spleen and bone marrow are comparable between wild-type mice and MyD88-deficient mice during infection, suggesting MyD88 signaling is not involved in T cell trafficking. In a model of Aspergillus fumigatus infection of the lung, CD4 T cells were found to traffic normally to the lung in the absence of MyD88 (41), supporting the idea that MyD88 is not required for proper trafficking of lymphocytes. This is in contrast to Chlamydia muridarum infection, where MyD88 signaling was critical for recruitment of CD4 T cells into the genital tract during infection (20). This difference in MyD88-dependent CD4 T cell migration may be due to specific requirements for migration into lymphoid versus non-lymphoid tissues, such as the genital tract. Another possibility is that these differences are due to pathogen-specific TLR ligands.

The importance of TLR-mediated signaling in controlling T cell function is primarily thought to occur via MyD88-signaling in APCs. MyD88 signaling in BMDCs was important for IL-12p40 production in E. muris infection in vitro (15). However, our in vivo observation that very similar amounts of IL-12p70 were generated in the bone marrow of wild-type and MyD88-deficient mice in response to E. muris infection, suggest that differences in IL-12p40 do not affect IL-12, but may impact IL-23 production. We observed similar activation of APCs in mixed chimeric mice, supporting our conclusion that the primary defect in control of bacteria in MyD88-deficient mice, is due to the intrinsic defect in CD4 T cells. However, we cannot rule out the possibility that APC function is compromised in the absence of MyD88. Interestingly, it is unclear what pathways require MyD88 signaling, and the MyD88-dependent responses likely differ between cells of the innate and adaptive immune cells. It was recently reported that E. chaffeensis, the agent of HME, induces inflammatory responses in monocytes through MyD88, ERK, and NF-kB, but did not require TLRs, the adaptor TRIF, IL-18R, or IL-1ß (42). These data suggest the existence of previously unrecognized pathogen-associated molecular patterns and cognate receptors.

Our data support a model whereby cytokines, in particular IFN-γ, produced by mature hematopoietic cells can act locally, directly and/or indirectly, to promote differentiation of HSPCs. During ehrlichial infection MyD88-mediated signals act directly in CD4 T cells to promote their activation and ability to secrete IFN-γ. Regulation of hematopoiesis by Th cells during infection raises important questions about how hematopoietic function is maintained in the face of chronic infections where CD4 T cells are increased (or decreased, as in HIV infection) or exhibit a more activated phenotype. Thus, our findings highlight a previously unappreciated role for MyD88 in modulating hematopoietic function.

Disclosures

The authors have no financial conflicts of interest.

References