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Gut microbiota promotes hematopoiesis tocontrol bacterial infection

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Summary

The commensal microbiota impacts specific immune cell populations and their functions at peripheral sites, such as gut mucosal tissues. However, it remains unknown whether gut microbiota control immunity through regulation of hematopoiesis at primary immune sites. We reveal that germ-free mice display reduced proportions and differentiation potential ofspecific myeloid cellprogenitors of both yolk sac and bone marrow origin. Homeostaticinnate immune defects may lead to impaired early responses to pathogens. Indeed, following systemic infection with *Listeria monocytogenes*, germ-free and oral antibiotic treated mice display increased pathogen burden and acute death. Re-colonization of germ-free mice with a complex microbiota restores defects inmyelopoiesis and resistance to *Listeria*. Thesefindingsreveal that gut bacteriadirect innate immune cell development via promoting hematopoiesis, contributing to our appreciation of the deep evolutionary connection between mammals and their microbiota.

Introduction

The vast majority of our interactions with bacteriaare symbiotic in nature, consisting of colonization by a complex and diverse microbiota that inhabit humans for life.Rather than causing inflammation, commensal microbes largely direct beneficial immune functions and

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often engender health. In particular, the microbiota shapesglobalimmune cell repertoires, thereby altering host susceptibility to inflammation and infection at sites of colonization(Hill et al., 2012; Kamada et al., 2012; Mazmanian et al., 2008; Naik et al., 2012). Specific gut bacteria or bacterial products have been shown to suppressintestinal inflammation(e.g., colitis) in micethrough a variety of immune mechanisms (Atarashi et al., 2013; Round and Mazmanian, 2010; Smith et al., 2013).Furthermore, the impact of commensal microbes on host immune responses is not limited to mucosal interfaces, but extends tosystemic compartments; gut microbes regulate immune responses that influence organ-specific autoimmunity in animal models of multiple sclerosis, rheumatoid arthritis and type 1 diabetes(Lee et al., 2011; Markle et al., 2013; Wu et al., 2010). While numerous examples illustrate how the microbiota contributes to immune function at mucosal and systemic sites, little is known about the influencesofgut bacteria on cellular development within primary immune tissues.

The immune system begins to develop *in utero*, but full maturation requires both genetic and environmental signals that further shape immunity after birth. Lymphoid and myeloid cells develop largely from hematopoietic stem cells (HSCs) within primary tissues, where molecular cues orchestrate immune cell differentiation from uncommitted HSCs and progenitor cells via regulation of transcription factors and epigenetic modifications (Weissman, 1994). Additionally, certain phagocyte populations (including Langerhans cells and microglia), derived from embryonic precursors, are maintained independently of HSCs(Sieweke and Allen, 2013). Genetic contributions (i.e., molecular cues encoded by the host genome) to lineage commitment pathways that control the myeloid repertoire are well studied (Georgopoulos, 2002). However, environmental factors that influence hematopoiesis have not been extensively defined. Based on emerging data that the microbiota represents an integral environmental factor in shaping numerous features of the immune system, we reasoned that gut bacteria may be controlling central immunity. We report herein that commensal microbes promote the maintenance of both HSC and embryonic-derived myeloid cells during steady-state conditions. The absence of commensal microbes leads to defects in severalinnate immune cell populations (including neutrophils, monocytes and macrophages) within systemic sites. By controlling the differentiation of innate immunity, the gut microbiota prepares the host to rapidly mount immune responses upon pathogen encounter, as germ-free and antibiotic treated mice are impaired in clearance of systemic bacterial infection. Our study reveals that gut microbesevolved to actively shape immunity at its core—via regulation of hematopoiesis.

Results

Germ-Free Animals Display Global Defects in Innate Immune Cells

The commensal gut microbiota profoundly influencescellular proportions, migration and functions of various immune cell subsets. Recent studies have provided numerous examples illustrating how gut bacteria modulate innate and adaptive immune responses at mucosal surfaces during infection, inflammation and autoimmunity(Kamada et al., 2013; Round and Mazmanian, 2009). With such pervasive effects, we reasoned that the microbiota may regulate hematopoiesis—the developmental programming of the immune system. Initially,

to determine if the microbiota has global effects on systemic immune cell populations, we profiled myeloid cells in the spleen of colonized (SPF; specific pathogen free) and germ-free (GF) mice. Indeed, GF animals display reduced proportions and total numbers of F4/80^{hi} and F4/80^{lo}cells compared to SPF mice (Figures 1A-C).F4/80^{hi} cells are mainly macrophages, while F4/80^{lo} splenocytes are a heterogeneous population of macrophages, monocytes and neutrophils (Schulz et al., 2012). Intriguingly, all three cell subsets are reduced in GF mice(Figure S1A). Furthermore, treatment of SPF mice with antibiotics also results in diminished myeloid cell populations in the spleen (Figure S1B). Thus, gut bacteria dynamically influence innate immune cell proportions at secondary immune sites in the periphery.

Myeloid cell precursors differentiate into various phagocyte lineages that are stored in the bone marrow, which are a major source of cells that populate peripheral tissues(Geissmann et al., 2010). The reduction of splenic macrophages, monocytes and neutrophils in GF mice suggests that defects in host immunity may include compromiseddevelopment in primary immune sites. Accordingly, we observed a reduction of myeloid cells within the bone marrow of GF mice (Figures 1D-F). A similar decrease was observed in the liver, a site of alternative immune cell development (Figure S1C). A global defect in myeloid cell populations in primary immune sites of GF mice demonstrates that gut bacteria shape the architecture of the immune system early in cellular development.

Commensal Microbes Enhance Myelopoiesis

We reasoned that reductions in several phagocytic cell subsets in GF mice may reflect a primary defect in the maintenance of myeloid cell populations. To test if commensal microbes promote myelopoiesis, we pulsed SPF and GF mice with 5-Ethynyl-2′-deoxyuridine (EdU), a thymidine analog, to compare the percentage of dividing leukocytes. Both F4/80^{hi} and F4/80^{lo} phagocytes from GF mice showed reduced EdU incorporation compared to SPF animals (Figure 2A,B). F4/80^{hi} macrophages are largely derived from embryonic yolk sac progenitors and are maintained independently of HSCs (Schulz et al., 2012; Sieweke and Allen, 2013). F4/80^{lo} leukocytes, however, are of hematopoietic origin and reduced EdU incorporation by these cells in GF mice indicates defects in the expansion and/or differentiation of bone marrow progenitor cells (Schulz et al., 2012). These studies uncover a role for commensal microbes in promoting the maintenance of both splenic yolk sac-derived and HSC-derived myeloid cells.

The reduction of F4/80^{lo} cells in GF mice led us to further investigate the contribution of commensal microbes on HSCs and myeloid progenitor cells in the bone marrow. No differences were detected in the proportion or differentiation potential of LKS^{+} cells (HSCs and multipotent progenitors; MPPs), LKS^{-} cells (total lineage-restricted progenitors), or common myeloid progenitor cells (CMPs) between SPF and GF mice (Figure S2A-F). Remarkably, GF miceare significant reduced in the proportion of bone marrow granulocyte and/or monocyte progenitors (GMPs), identified as LKS^{-} CD34^{+} FcγR^{hi} cells (Figure 2C). GMPs consist of progenitor cells, downstream of HSCs and CMPs during hematopoiesis, with restricted myeloid differentiation potential (Akashi et al., 2000). To further examine the effects of gut microbiota on innate immune cells, we tested if commensal microbes affect

the differentiation potential and self-maintenance capacity of GMPs. Methylcellulose culture of LKS$^-$ CD34$^+$ FcγR^{hi} cells from GF mice displayed reduced granulocyte (G-CFU) and monocyte (M-CFU) colony formation compared to cells from SPF mice (Figure 2D). Furthermore, passage of LKS$^-$ CD34$^+$ FcγR^{hi} cells isolated from GF mice in primary methylcellulose culture yielded reduced recovery of c-Kit$^+$ CD11b$^-$ progenitor cells compared to SPF GMPs (Figure 2E). This suggeststhe ability of GMPs to maintain cells with progenitor potential isdefective in the absence of commensal microbes(Rodrigues et al., 2008). Consistent with this notion, secondary cultures of unfractionated cells derived from GF GMPs generated fewer colonies compared to cells isolated from SPF mice (Figure 2F). The commensal microbiota therefore promotes steady-state myelopoiesis by specifically maintaining GMP proportions and enhancing their differentiation to mature myeloid cells in the bone marrow.

Extramedullary hematopoiesis (outside the bone marrow) further contributes to the maintenance and inflammatory responses of tissue-resident phagocytic cells (Jenkins et al., 2011; Massberg et al., 2007; Robbins et al., 2012; Swirski et al., 2009). We therefore investigated whether commensal microbes influence the hematopoietic potential of progenitors located in the spleen. Similar to GMPs from the bone marrow, splenocytes isolated from GF mice displayed reduced colony formation in methylcellulose, compared to SPF mice, with significant reductions in both neutrophil and monocyte production (Figures 2G, H). Overall, we conclude that the microbiota shapes innate immune profiles by promoting myeloid progenitor development and differentiation in the bone marrow and extramedullary sites, revealing that gut bacteria control immunity at its core—during hematopoiesis.

Tissue-Resident Phagocytes Mediate Protection by Commensal Microbes

Innate immune cells are the first responders to infection, mediating early pathogen control and coordinating downstream immune reactions(Kastenmuller et al., 2012; Shi and Pamer, 2011). We sought to test the impact of commensal microbes on myeloid cell differentiation by employing infection models where innate immunity is vital for an effective immune response. SPF and GF mice were infected intravenously (*i.v.*) with the model pathogen, *Listeria monocytogenes*. SPF mice challenged systemically with *L. monocytogenes* effectively control infection, as previously described (Figure 3A) (Serbina et al., 2012; Shi et al., 2011). However, GF mice rapidly succumb at the same inoculum (Figure 3A). Heightened susceptibility to infection among GF mice was associated with a significant increase in splenic and liver bacterial burden 24 and 72 hours post-infection (hpi), demonstrating a defect in early resistance to *Listeria* infection (Figures 3B, C and Figure S3A). Susceptibility to infection is not restricted to *L. monocytogenes*, as GF mice also displayed increased disease burden following systemic challenge with *Staphylococcus aureus* (Figure S3B). Interestingly, SPF mice treated orally with broad-spectrum antibiotics were also impaired in controlling *Listeria*, indicating protection by commensal microbes is an active process and is subject to loss following depletion of gut microbiota (Figure 3D). Collectively, these data reveal that commensal microbes are critical for rapid and potent systemic immune responses to acute bacterial infection.

To confirm that defects in myelopoiesis contribute to increased disease burden in GF mice, phagocytic cells were depleted with clodronate-loaded liposomes (CL) prior to infection with *L. monocytogenes*(van Rooijen et al., 1996). CL pre-treatment increased susceptibility to *Listeria* infection (Figure 3E), confirming the importance of resident cells in pathogen resistance(Aichele et al., 2003; Kastenmuller et al., 2012). Importantly, depletion of resident phagocytes rendered both SPF and GF mice equally susceptible to infection, resulting in similar splenic disease burden 24 hpi (Figure 3E), and rapid death within 48 hpi (Figure 3F). While functional defects inmyeloid cellsmay potentially contribute to increased disease in GF mice, we did not detect differences during *in vitroListeria* killing by macrophages from SPF or GF mice (Figure S3C). Furthermore, CD11b+ myeloid cells isolated from either SPF or GF donors were equally sufficient in providing protection when transferred into GF mice prior to infection (Figure 3G),suggesting reduced cell proportions are likely the primary defect in GF mice. These studies confirm the importance of microbiota-driven myelopoiesis in promoting host resistance during systemic infection.

Effective responses to *L. monocytogenes* requires coordination between innate and adaptive immune cells, resulting in pathogen clearance and protective immunity (Pamer, 2004). Thus, we investigated whether additional immune cells beyond tissue-resident phagocytes may mediate commensal-derived protection to *Listeria* infection. We show that adaptive immunity is not required for protection by the microbiota during acute infection (Figure S3D), nor are GF mice deficient in developing long-term protective immunity against subsequent infection (Figure S3E). Furthermore, the selective expansion of myeloid cells during acute infection, called emergency hematopoiesis, which is necessary for mediating delayed resistance to *L. monocytogenes* (following 48 hpi), was maintained in GF mice (Figure S3F). Finally, while there are fewer inflammatory neutrophils and monocytes recruited to the spleen following infection (Figure S3G), a possible consequence of increased apoptosis (Figure S3H), these cells were not required for commensal-mediated protection against *L. monocytogenes* (Figure S3I, J). Together, these findings demonstrate that hematopoietic defects in tissue-resident myeloid cells prior to infection of GF mice (i.e., during cell development) is the primary cause of impaired control of *Listeria*.

CommensalBacterialSignals Mediate Maintenance of Myelopoiesis

The molecular mechanism(s) by which commensal microbes promote steady-state expansion of bone marrow- and yolk sac-derived myeloid cells remains unknown. Microbial associated molecular patterns (MAMPs) and microbial metabolites, such as short chain fatty acids (SCFAs) have been shown to modulate various aspects of the host immune response (Chu and Mazmanian, 2013; Clarke et al., 2010; Smith et al., 2013). Furthermore, MyD88 (an adaptor for recognition of many MAMPs) was recently shown to promote GMP expansion and differentiation (Fiedler et al., 2013). Accordingly, we sought to address whether commensal-derived factors are involved in the maintenance of myeloid cells under naïve conditions. Re-colonization of GF mice with a complex microbiota andoral treatment with MAMPs, but not SCFAs, was sufficient to promote recovery of GMP-derived myeloid cells (neutrophils and monocytes) within the bone marrow (Figure 4A, B). Importantly, only re-colonization of GF mice with an SPF microbiota was sufficient to restore splenic populations of F4/80^{hi} macrophagesand F4/80^{lo} splenocytes (i.e., neutrophils, monocytes

and macrophages) (Figure 4C and Figures S4A-D). Therefore, while MAMP treatmentis necessaryfor the maintenance of bone marrow-derived myeloid cells, colonization with a live and complex microbiota is required to promote complete myelopoiesis (including yolk sac-derived macrophages). Finally, only re-colonization of GF animals, and not oral MAMP treatment, was sufficient to restore the defect in GF mice to systemic challenge with *L. monocytogenes* (Figure 4D and data not shown).Collectively, these studiesreveal that the microbiota provides complex molecular signals that actively promote the hematopoietic differentiation of myeloid cells, resulting in peripheral phagocyte populationsthat function as sentinels for the early detection and control of systemic bacterial infection.

Discussion

Advances in understanding host-microbial symbiosis have revealed that, remarkably, the gut microbiota controls the phenotype, migration and activity of multiple innate and adaptive cells(Belkaid and Naik, 2013; Chu and Mazmanian, 2013). Disruption or alteration of commensal communities impacts host susceptibility to various disorders, particularly at sites of microbial colonization such as the intestines, respiratory mucosa and skin epithelium(Kamada et al., 2013). In addition to modulating functional immune outcomes, the microbiota is necessary for maintaining systemic populations of neutrophils in the circulation and $CD4^+$ T cells in the spleen(Bugl et al., 2013; Mazmanian et al., 2005), suggesting a possible contribution by gut microbiota to the development of the immune system. Herein, we revealthat gut bacteria regulatehematopoiesiswithin primary immune sites, providing a unifying explanation for previous observations of the widespread effectsby the microbiota on the immune system. Specifically, our study uncovers that the microbiota promotes steady-state myeloid cell development by driving the expansion of yolk sac-derived macrophages, as well as enhancing the numbers and differentiation potential of GMPs in the bone marrow. We propose a model whereby a primary defect in hematopoiesis in GF or antibiotic-treated mice compromisesmultiple tissue-resident innate immune cell populations prior to infection, leading to blunted early responses upon subsequent pathogen encounter (see diagram in Figure S4E). While our studies focus on innate immunity due to its role in rapid control of early *Listeria* infection, impaired microbiota-mediated hematopoiesis may alsoextend to the adaptive immune system, providing an explanation for observations that peripheral T, B and iNKT cell populations are altered in GF mice (Ivanov et al., 2008; Macpherson and Uhr, 2004; Mazmanian et al., 2005; Olszak et al., 2012).

How commensal microbes (presumably in the gut) are able to control immune responses in distant sites such as the bone marrow remains incompletely understood. It has recently been shown that mice deficient in MyD88 signaling display reductions in systemic myeloid cell populations and GMP numbers(Fiedler et al., 2013; Yanez et al., 2013), similar to our findings in GF mice. Further, as microbial ligands have been detected in systemic sites, including the bone marrow (Clarke et al., 2010), commensal-derived MAMPs that originate in the gut may mediate steady-state myelopoiesis in primary immune sites. Accordingly, we show that oral treatment with MAMPs is sufficient to rescue GMP-mediated expansion of neutrophils and monocytes. However, MAMP treatment aloneis inadequate to expand splenic $F4/80^{hi}$and $F4/80^{lo}$ cells, indicating additional commensal-derived signals are necessary to influence site-specific HSC and yolk sac-derived myeloid cells. Interestingly,

re-colonization of adult GF mice with SPF microbiota is insufficient to restore splenic F4/80^{hi} macrophages to the levels found in SPF mice. This may suggest that complete rescue requires either colonization from birth or colonization with specific microbes that were not transferred into GF mice. In addition to MAMPs translocating from the gut into the circulation, other explanations for how the microbiota affects hematopoiesis may include a role for myeloid cell growth factors. In support of this notion, preliminary data suggest that GF mice are reduced in M-CSF transcript levels in the gut (data not shown), though further work is need to uncoverthe complex molecular mechanism(s) by which commensal bacteria signal from the gut to distant primary immune organs.

Finally, we speculate that these findings may be relevant to human infections. The spread of antibiotic-resistance among pathogens, paired with a dwindling supply of effective antibiotics,has necessitated alternative strategies to combatinfections (Khosravi and Mazmanian, 2013). Evidence that depletion of the microbiota leads to transient immune suppression suggests factors that disrupt commensal microbes, including clinical antibiotic use may, paradoxically, be a risk factor for susceptibility to opportunistic pathogens. The concepts proposed herein, if validated in humans, may herald future medical approaches that combine antibiotics with immunomodulatorymicrobial molecules as revolutionary combination treatments to address the reemerging crisis of infectious diseases.

Experimental Procedures

Animal Studies

Specific pathogen-free (SPF) C57BL/6 mice were purchased from Taconic Farms. Germ-free (GF) C57BL/6 and C57BL/6 Rag$^{-/-}$ mice were bred and raised in sterile gnotobiotic flexible film isolators at the California Institute of Technology. Mice at 8-12 weeks of age were infected via retro-orbital injection with 3×10^4 colony forming units (CFU) of *Listeriamonocytogenes* 10403S. Splenic and liver bacterial CFU were assessed 24-72 hpi by microbiological plating. For microbiota depletion studies, SPF mice were treated with 1 mg/ml of ampicillin (Auromedics), neomycin sulfate (Fisher), streptomycin (Sigma) and 0.5 mg/ml of vancomycin (Sagent) in the drinking water for 4-5 weeks. Mice were taken off antibiotics 4 days prior to infection. Antibiotic-treated and untreated SPF mice were infected with 3×10^4 CFU of *L. monocytogenes* and splenic bacterial burden was assessed 72 hpi. GF mice were recolonized by gavage with cecal contents of SPF mice. Alternatively, GF mice were treated with MAMPs through the addition of heat killed *Escherichia coli* strain Nissle(Lodinova-Zadnikova and Sonnenborn, 1997)or autoclaved cecal contents from SPF mice in water ($\sim1\times10^9$ CFU/ml indrinking water). For treatment with short chain fatty acids,sodium proprionate (Sigma), sodium butyrate (Sigma), and sodium acetate (Sigma) was added to drinking water at previously described concentrations (25mM, 40mM and 67.5mM, respectively)(Smith et al., 2013). Mice were re-colonized or treated with microbial ligands or metabolites for 4 weeks prior to cellular analysis and infectious studies. Animals were cared for under established protocols and IACUC guidelines from the California Institute of Technology.

Cellular Analysis

Spleens were either mechanically disrupted viapassage through 100 μm mesh filters (BD Biosciences) or digested in 0.5 mg/ml of Collagenase D (Roche) and 0.5 mg/ml of DNase I (Worthington). Bone marrow was collected by flushing femurs with PBS containing 0.5% BSA and 5mM EDTA. Single cell suspensions were removed of red blood cells (RBC lysis buffer, Sigma). Mature myeloid cells were evaluated by staining with antibodies to GR1 (RB6-8C5), Ly6C (HK 1.4), CD11b (M1/70), CD115 (AFS98) and F4/80 (BM8). Mouse hematopoietic stem and progenitor cells (HSPCs) were isolated from bone marrow by a combination of MACS magnetic bead purification (Miltenyi) and fluorescence activated cell sorting (FACS). Lineage marker-negative cells (Lin^-) were first separated using a MACS lineage cell depletion kit (containing antibodies against CD5 (53-7.3), CD45R (B220; RA3-6B2), CD11b, Gr-1, 7-4 (15BS) and Ter-119 (Ter-119)) and an autoMACS Separator (Miltentyi). Lin^- cells were then further stained with c-Kit (CD117; 3C1), Sca-1 (D7), CD16/CD32 (93), CD34 (RAM34). Populations of LKS^+ cells (Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^+$; HSCs and MPPs), Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^-$ (LKS^-) $CD34^+$ $Fc\gamma R^{lo}$ cells (CMPs) and LKS^- $CD34^+$ $Fc\gamma R^{hi}$ cells (GMPs) were analyzed by flow cytometry. LKS^- $CD34^+$ $Fc\gamma R^{hi}$ cells were FACS sorted using an Aria cell sorter (BD Biosciences). Steady-state cell proliferation was measured by intraperitoneal (*i.p.*) injection of 500 μg EdU (Life Technologies) and EdU incorporation among splenic myeloid cells was measured 24 hours later via Click-it EdU assay kit (Life Technologies). Antibodies were purchased from eBioscience, BD Bioscience, Miltenyi or Biolegend. Data were collected on a FACSCalibur or LSR Fortessa (BD Bioscience) and analyzed with FlowJo software (TreeStar).

Cell Depletion and Adoptive Transfer

Resident phagocytes were depleted by intravenous(*i.v.*) treatment with 100 μl of clodronate-loaded liposomes (CL; FormuMax) 48 hours prior to infection. $CD11b^+$ splenocytes were isolated from naïve SPF and GF mice using CD11b microbeads (Miltenyi). 2×10^6 $CD11b^+$ cells (>90% purity) were transferred into GF recipients, 24 hours prior to infection with *L. monocytogenes*. CFU burden was assessed 24 hpi.

CFU Assays

To evaluate hematopoietic potential, 1×10^3 Lin^- or 1×10^3 LKS^- $CD34^+$ $Fc\gamma R^{hi}$ cells or 2×10^5 splenocytes were plated in triplicate in MethoCult GF M3434 (StemCell Technologies) methylcellulose-based medium and incubated for 7 days in 37°C with 5% CO2, after which the colonies were counted on the basis of their morphological characteristics in accordance with the manufacturer's instructions. On the same day, cells were harvested, counted and stained for c-Kit and CD11b expression for progenitor quantification by flow cytometry. For re-plating assays, 5×10^4 cells from the first culture were plated in triplicate in a secondary culture of fresh MethoCult GF M3434, and colonies were counted after 7 days of incubation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Germ-free mice are deficient in spleen and bone marrow resident myeloid cell populations
- Gut microbes impact both yolk sac- and stem cell-derived myeloid cell development
- Microbiota promotes early resistance to systemic *Listeria monocytogenes* infection
- Re-colonizationrestores immune integrity against systemic Listeriosis in germ-free mice

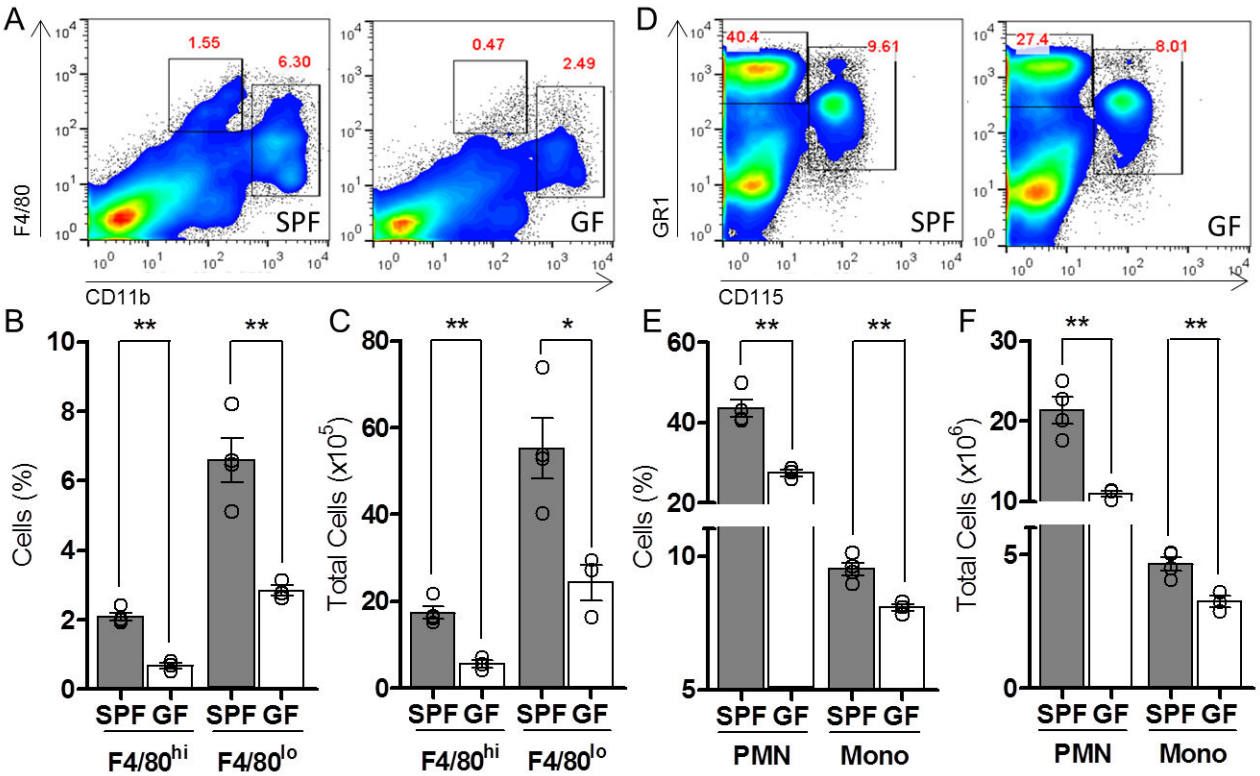

Figure 1. GF Mice Are Deficient in Resident Myeloid Cell Populations in the Spleen and Bone Marrow

(A-C) Splenic phagocyte profile among SPF and GF mice. Representative flow cytometry plots (A), cell proportions (B), and total cell number (C) of $CD11b^{lo}$ $F4/80^{hi}$ and $CD11b^{hi}$ $F4/80^{lo}$ splenic cells in SPF and GF mice. (D-F) Bone marrow populations of neutrophils ($Gr1^{hi}$ $CD115^{neg}$) and monocytes ($Gr1^{hi}$ $CD115^{hi}$) among SPF and GF mice. Representative flow cytometry plots (D), cell proportions (E) and total cell number (F) within the bone marrow of SPF and GF mice. For all panels, data are representative of at least 3 independent trials with n≥ 4 mice / group. Each symbol represents data from a single animal. Error bars represent standard error of mean (SEM). *p<0.05, **p<0.01. PMN: polymorphonuclear cells; Mono: monocytes. See also Figure S1.

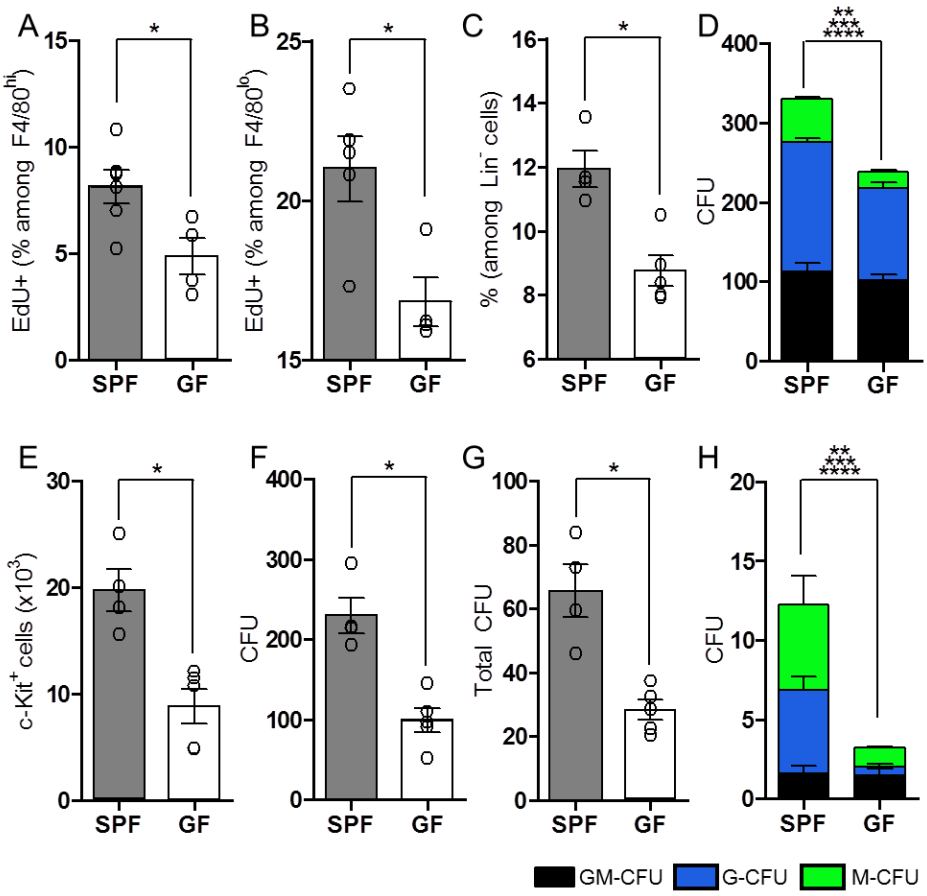

Figure 2. The Microbiota Directs Myelopoiesis

The percentage of F4/80^{hi} CD11b^{+} (A) and F4/80^{lo} CD11b^{+} (B) splenocytes with incorporated EdU, following single dose administration. (C) The frequency of LKS^{-} CD34^{+} Fc γ R^{hi} granulocyte and/or monocyte progenitors (GMPs) among lineage negative (Lin^{-}) progenitors from bone marrow of SPF and GF mice, as assessed by flow cytometry. (D) Distribution of cell types following purified LKS^{-} CD34^{+} Fc γ R^{hi} cell culture in methylcellulose medium. Colonies were identified and counted to assess the proportion of granulocyte-monocytes (GM-CFU; black), granulocytes (G-CFU; blue) and monocytes (M-CFU; green). (E) Total numbers of c-Kit^{+} CD11b^{-} progenitors from methylcellulose cultures of LKS^{-} CD34^{+} Fc γ R^{hi} progenitors, as assessed by flow cytometry. (F) Cells harvested from methylcellulose cultures of LKS^{-} CD34^{+} Fc γ R^{hi} progenitors were re-plated at equal numbers in fresh methylcellulose, and cultured to assess their colony forming capacity. (G and H) Splenic cells isolated from SPF and GF mice were cultured in methylcellulose to assess the colony forming capacity of progenitors from SPF and GF mice. Total CFUs (G), and GM-CFUs, G-CFUs and M-CFUs (H) are shown. For each panel, data are representative of at least 2-3 independent trials with n≥ 4/ group. Each symbol represents data from a single animal. Error bars represent SEM. *p<0.05 for all panels. **p<0.05 (comparing total CFU between SPF and GF for (D) and (H)), ***p<0.05 (comparing G-CFU between SPF and GF for (D) and (H)), ****p<0.05 (comparing M-CFU between SPF and GF for (D) and (H)). CFU: colony forming units. See also Figure S2.

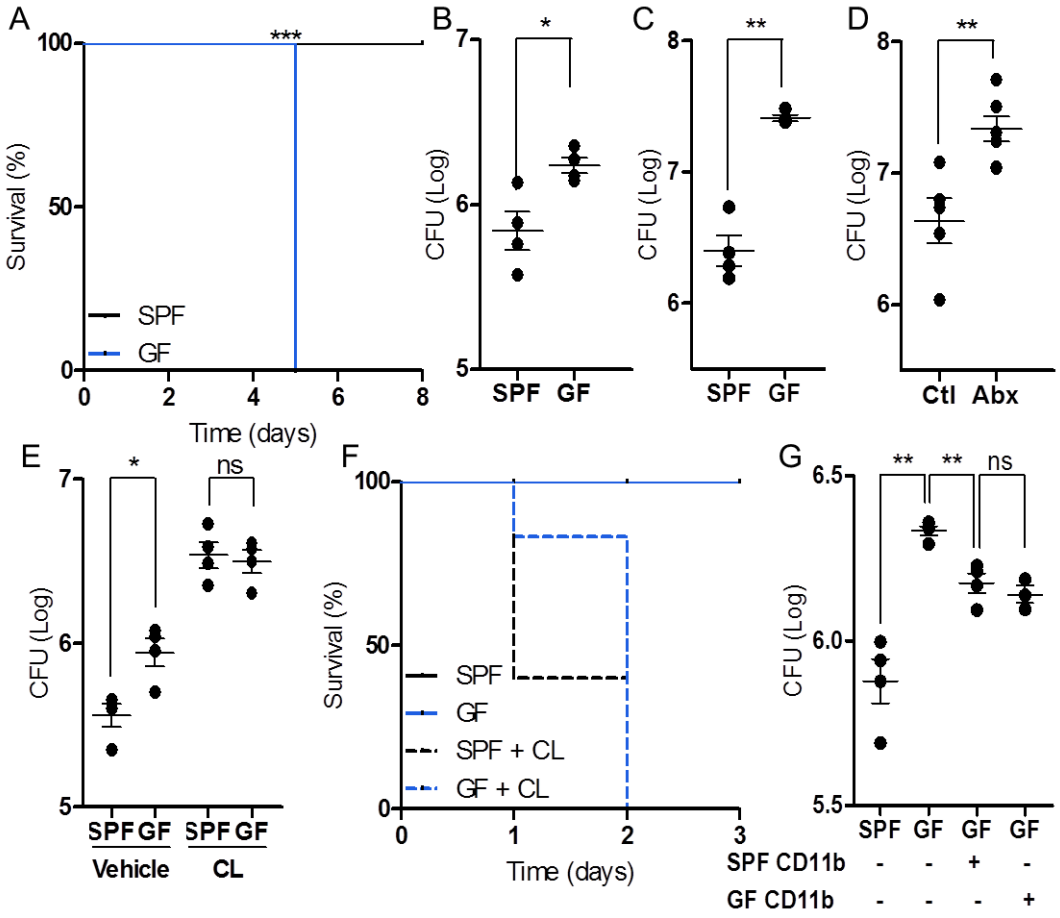

Figure 3. The Microbiota Promotes Early Resistance to Systemic Infection by *L. monocytogenes* via Tissue-Resident Cells

(A-C) SPF and GF mice infected with *L. monocytogenes* and assessed for survival (A) and splenic bacterial burden at 24 (B) and 72 (C) hours post- infection (hpi). SPF mice treated with antibiotics (Abx) and untreated controls (Ctl) were infected with *L. monocytogenes* and splenic bacterial burden was measured 72 hpi (D). SPF and GF mice depleted of tissue-resident cells prior to infection with *L. monocytogenes* and assessed for splenic bacterial burden 24 hpi (E) and survival (F). Splenic bacterial burden, 24 hpi, following transfer of splenic $CD11b^+$ cells from SPF or GF donors (G). For all panels, data are representative of at least 2-3 independent trials with n≥ 4/ group. Each symbol represents data from a single animal. Error bars represent SEM. *p<0.05, **p<0.01, *** p<0.05 log-rank test used for survival curves in (A). CL: clodronate-loaded liposomes. See also Figure S3.

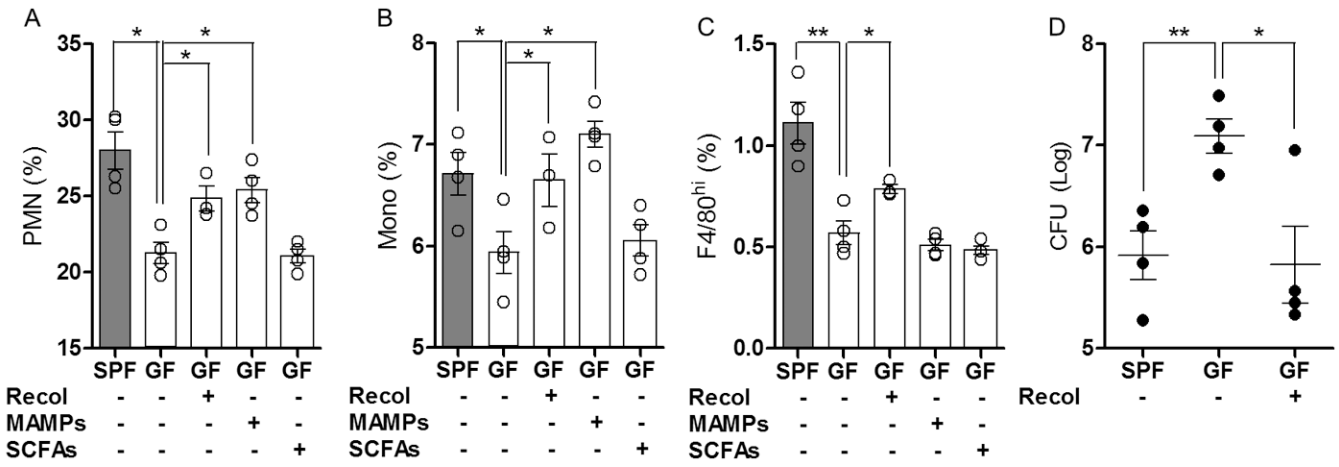

Figure 4. Re-colonization of GF Mice Restores Immune Integrity Against Systemic Listeriosis

(A) Neutrophil ($GR1^{hi}$ $CD115^{-}$) and (B) monocyte ($GR1^{hi}$ $CD115^{+}$) bone marrow profiles from SPF, GF, re-colonized GF mice and MAMP or SCFA-treated GF mice. (C) $F4/80^{hi}$ splenic macrophage profile among SPF, GF, re-colonized GF mice and GF mice treated with MAMPs or SCFAs. (D) Splenic bacterial burden 72 hpi among SPF, GF and re-colonized GF mice infected with *L. monocytogenes*. For all panels, data are representative of at least 2 independent trials with n≥4/ group. Each symbol represents data from a single animal. Error bars represent standard error of mean (SEM). *p<0.05, **p<0.01. Recol: re-colonized; MAMPs: molecular associated molecular patterns; SCFAs: short chain fatty acids. See also Figure S4.