Gut Microbiota Promote Hematopoiesis to Control Bacterial Infection

Thesis by

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In Partial Fulfillment of the Requirements for the degree

of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2014

(Defended 3/24/14)

ACKNOWLEDGEMENTS

The work presented here could not be accomplished without the support and guidance of Sarkis Mazmanian, the members of the Mazmanian lab, and my committee members. Further, I am indebted to my collaborators for their contributions. I would like to thank the USC-Caltech MD/PhD program for providing me the opportunity to pursue training as a physician scientist. Finally, none of this could have been accomplished without the love and support of my better half. Marburg, you are my light. Thank you for being such a rad cat.

ABSTRACT

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 of specific myeloid cell progenitors of both yolk sac and bone marrow origin. Homeostatic innate 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. Recolonization of germ-free mice with a complex microbiota restores defects in myelopoiesis and resistance to *Listeria*. These findings reveal that gut bacteria direct innate immune cell development via promoting hematopoiesis, contributing to our appreciation of the deep evolutionary connection between mammals and their microbiota.

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Chapter 1

INTRODUCTION

The discovery of antibiotics in the last century is one of the most significant achievements of modern medicine. Pathogens that once devastated entire civilizations, such as *Mycobacterium tuberculosis*, could finally be controlled, suggesting a triumph over infectious disease. However, the rampant rise of antibiotic resistance among pathogens, compounded by a drying pipeline of novel antibiotic development by pharmaceutical companies, has rendered current therapeutic strategies ineffective. As such, it is speculated that we are entering a post-antibiotic era where pathogens once again reign with limited opposition, and a minor scrape may pose the risk of a fatal infection (Alanis, 2005; Kahrstrom, 2013). To combat the renewed threat of pathogenic microorganisms, clinical approaches toward eradicating infectious disease must evolve.

The recent increase in the severity and incidence of *Clostridium difficile*-associated diarrhea (CDAD) is emblematic of medicine's current failings as well as its possible future. The disruption of intestinal microbiota, most commonly by antibiotics, prompts infection by *C. difficile*, resulting in disease that ranges from mild diarrhea to fulminant colitis (Bartlett, 2002). Once fatal, the advent of antibiotics consigned infection to a manageable disease. However, the spread of antibiotic-resistant, hypervirulent strains in recent years has created an epidemic that is exceedingly difficult to manage (Loo et al., 2005). Currently, 20–25% of patients experience relapsing disease, further reflecting the reduced efficacy of antibiotic therapy (Bartlett, 2002). Besieged by an unrelenting pathogen, clinicians began to supplement patients with the fecal contents of healthy donors in an

attempt to reestablish the natural resistance afforded by the microbiota against *C. difficile*. Fecal transplantation embraces the hygiene hypothesis, which argues that microbial exposure, particularly commensal microbes, is beneficial to host health. This approach of administering microbes to combat disease is in shocking contrast to standard medical practices of the last century that, abiding by the principles of germ theory, indiscriminately target microbes as a means of promoting individual health. Yet, as fecal transplantation achieves a 91% primary cure rate (Brandt et al., 2012), it insists upon a reassessment of our clinical strategy toward preventing and treating infectious disease and suggests a possible role of commensal microbes in mediating protection against pathogenic microorganisms.

The commensal microbiota is primarily comprised of indigenous bacteria that colonize the external interfaces of its host. Co-evolution has resulted in microbes with extensive and diverse impacts on multiple aspects of host biology, including nutrient acquisition and immune development (Kau et al., 2011; Round and Mazmanian, 2009). Appropriately, conditions that disrupt the symbiotic host-microbial coexistence significantly alter predisposition to a wide spectrum of metabolic and inflammatory disorders, which include diabetes, metabolic syndrome, inflammatory bowel disease, asthma and multiple sclerosis (Hill et al., 2012; Lee et al., 2011; Mazmanian et al., 2008; Vijay-Kumar et al., 2010). We further advance these studies by revealing the microbiota is essential in maintaining immune integrity against pathogenic microbes by driving immune development within primary and secondary lymphoid tissues. To provide background for these studies, Chapter 2 will review current literature regarding the influence of commensal microbiota on host immune responses, particularly as it relates to promoting resistance against infectious disease. Chapter 3 will present new data revealing the contribution of the microbiota in maintaining systemic populations

of myeloid cells by driving steady-state hematopoiesis. Chapter 4 will show this influence is essential for promoting resistance against systemic bacterial infection. Finally, Chapter 5 will summarize and contextualize these new finding, as well as discuss future directions regarding how this work may contribute to the prevention and treatment of infectious disease.

Chapter 2

THE MICROBIOTA PROTECTS AGAINST INFECTION

The development of enteric infection, following antibiotic use, has long been observed in both clinical practice and in animal models of disease (Bartlett, 2002). This observation suggests that the commensal microbiota is essential in protecting against infection by pathogenic microorganisms. The utilization of animal models to study the microbiota, including germ-free (GF) mice that lack microbial exposure, has revealed significant insight into the diverse and intricate contribution of the commensal microbes towards mediating resistance against infectious disease.

Commensal Microbes Directly Resist Enteric Pathogens

The commensal microbiota achieves resistance against opportunistic infection, in part by competing for resources required by pathogens to establish infection. GF mice are highly susceptible to enteric infection with *Salmonella enterica* serovar Typhimurium (STm), a human-specific enteric pathogen, and *Citrobacter rodentium*, a murine pathogen used to model infection with enterohemorragic and enteropathogenic *Escherichia coli* (Kamada et al., 2012; Ng et al., 2013; Sekirov et al., 2008). However, colonization of GF mice with isolated commensal microbes protects against infection by STm or *C. rodentium*, in part due to the enhanced glycan acquisition capabilities of the transferred bacteria which outcompete and eventually displace pathogenic microbes (Kamada et al., 2012). Alternatively, antibiotic-depletion of the microbiota in colonized mice (Specific pathogen free; SPF) results in a spike in free glycans, which promotes pathogen

expansion and increases the risk for enteric infection (Ng et al., 2013). These findings reveal direct competition by commensal microbes for nutrients as a means of limiting infection by pathogens at sites of colonization.

Recent studies show that certain enteric pathogens actively trigger host inflammation which favors pathogen invasion and dissemination (Lupp et al., 2007; Sekirov et al., 2008; Stecher et al., 2007). Further, these reports surprisingly demonstrate that pathogen-induced inflammation adversely affects the microbiota, depleting populations of beneficial bacteria. By reducing the numbers of commensal microbes via triggering inflammatory responses, pathogens have unimpeded nutrient access. Collectively, there is growing evidence for the notion that pathogens and symbiotic bacteria are engaged in an 'evolutionary combat', with the host serving as the battlefield. However, instances in which commensal microbes are unable to directly prevent invasion by pathogenic microbes, protection is indirectly achieved by commensal microbes through the modulation of host immune responses.

Gut Microbes Promote Barrier Immunity

In addition to competing with invading pathogens, the commensal microbiota directly promote host immune responses which are protective against infection. Immune modulation by commensal microbes is essential for establishing host-microbial coexistence (Round and Mazmanian, 2009). We now appreciate that this influence extends into supporting protection against infectious disease

both by promoting barrier immunity as well as priming immune defenses against pathogen insult (Figure 1-1).

Immune modulation by the microbiota occurs through commensal-derived signals, such as microbial associated molecular patterns (MAMPs). Host recognition of MAMPs is achieved by pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs). At mucosal surfaces, these commensal-derived signals drive epithelial production of mucin, secretion of immunoglobulin A (IgA), and the expression of antimicrobial peptides (AMPs), which collectively limit microbial (beneficial and pathogenic) contact with mucosal tissue (Hooper and Macpherson, 2010; Moreau et al., 1978; Petersson et al., 2011). One such example is commensal-driven expression of RegIIIγ by intestinal epithelial cells (Figure 1-2). RegIIIγ is a C-type lectin that possesses antimicrobial activity against Gram-positive microbes (Cash et al., 2006). Expression of RegIIIγ requires TLR recognition of commensal MAMPs (Vaishnava et al., 2011). As such, disruption of the microbiota, as through antibiotic treatment, reduces production of RegIIIγ, resulting in a breakdown of barrier immunity. As a consequence, antibiotic-treated mice are highly susceptible to opportunistic infection with enteric pathogens such as vancomycin-resistant enterococcus (VRE) (Brandl et al., 2008). VRE is a common cause of antibiotic-associated diarrhea and, similar to *C. difficile*, exceedingly difficult to treat. However, supplementation of antibiotic-treated mice with purified MAMPs is sufficient to prime RegIIIγ expression and achieve resistance against infection. Herein is another example of how current treatment strategies predispose the host to secondary infections, and how efforts to maintain the integrity of the microbiota or supplement it during antibiotic treatment may be effective in limiting susceptibly to opportunistic pathogens.

The Microbiota Primes Mucosal Immune Resistance to Pathogen Invasion

Under conditions in which barrier resistance fails, commensal microbes continue to limit pathogen infection and dissemination by enhancing immune clearance responses. One such means by which the microbiota promotes host resistance is through priming expression of interleukin-1 β (IL-1β). IL-1β is a proinflammatory cytokine that is expressed in an inactive form (pro-IL-1β), which is subsequently cleaved by caspases following inflammasome activation (Rathinam et al., 2012). Intestinal mononuclear phagocytes isolated from GF mice express reduced levels of pro-IL-1β, compared to cells isolated from mice with an intact microbiota (specific pathogen-free; SPF) (Franchi et al., 2012). Cleavage of pro-IL-1β into its active form occurs after challenge with pathogenic microorganisms, such as STm, but not following exposure to commensal microbes. This would suggest that commensal microbes promote pro-IL-1β expression among intestinal mononuclear cells, which is specifically activated following pathogen insult. This selective activation of proinflammatory cytokines is one possible means by which the host is able to distinguish between beneficial and harmful microbes. Appropriately, commensal-driven pro-IL-1β expression enhances resistance to enteric infection with STm.

Additional mucosal immune responses are influenced by the microbiota include driving differentiation of T-helper 17 (Th17) cells and expression of IL-22 by intestinal NKp46^{+} cells (Ivanov et al., 2009; Satoh-Takayama et al., 2008). While the role of these cells in mitigating host-commensal co-existence remains unknown, both cell types are critical in combating mucosal infection with *C. rodentium*. It appears that the microbiota may drive certain immune responses,

including the production of pro-IL-1β, with the primary purpose of promoting resistance to pathogenic infection.

Commensal Microbes Prevent Infection at Colonization Sites Beyond the Gut

While many studies investigating the contribution of the microbiota in resisting infections have focused within the gut, colonization by commensal microbes at other epithelial surfaces also affords protection against pathogenic microorganisms. Skin microbes prime local development of Th1, Th17 and IL-17^+ gamma-delta T cells (Naik et al., 2012). Cutaneous T cell differentiation by commensal microbes is achieved through MAMP-driven IL-1β signaling. This response is independent of the intestinal microbiota as oral antibiotic treatment, which reduces intestinal Th1 and Th17 cells, has no effect on the immune profile within the skin. Furthermore, colonization of GF mice with the prominent skin commensal *Staphylococcus epidermidis* is sufficient to rescue the defective immune response in GF mice. Priming of these immune responses by skin microbes is instrumental in promoting resistance against cutaneous infection with *Leishmania major*. Here we see compartmentalized immune modulation by commensal microbes leads to site-specific protection against infectious disease.

Immune protection is also achieved by commensal microbes residing within the respiratory mucosa. Antibiotic-treated mice display reduced resistance to influenza infection (Ichinohe et al., 2011). Disease susceptibility is characterized by defective IL-1β production as well as reduced dendritic cell recruitment and T cell priming. As a consequence, antibiotic-treated animals display attenuated

T cell and B cell responses following viral infection. Interestingly, depletion of the microbiota did not enhance susceptibility to infection with herpes simplex virus type 2 or *Legionella pneumophila*, indicating specificity for pathogens to which the microbiota promotes resistance. Intranasal inoculation with purified MAMPs, such as LPS, is sufficient to restore protective immunity to infection, as is, surprisingly, intrarectal MAMP administration. These findings suggest that the imunoprotective properties of commensal microbes are not limited to the sites of colonization, but rather may extend to distal compartments and may even support host resistance against systemic infection.

Commensal Microbes Promote Host Resistance to Systemic Infection

While commensal microbes are physically restricted to external sites of colonization, their influence on host immune responses extends into systemic compartments. This concept was revealed with the finding that GF mice display a diminished splenic $CD4^+$ T cell profile (Mazmanian et al., 2005). Monocolonization of GF mice with *Bacteroides fragilis*, a prominent intestinal microbe, is sufficient to promote $CD4^+$ T cell development within the spleen. The role of commensal microbes in driving systemic immune maturation suggests that disruption of the microbiota may compromise host resistance to systemic infection.

Deliberate depletion of the microbiota reduces resistance to systemic infection with Lymphocytic Choriomeningitis Virus (LCMV) (Abt et al., 2012). Antibiotic-treated mice display increased viral burden as a consequence of attenuated anti-viral immune responses following infection.

Macrophages isolated from antibiotic-treated mice are deficient in type I and II interferon (IFN) signaling, as well as in controlling viral replication *ex vivo*. This defect in innate immune resistance contributes to an impaired adaptive immune response, which includes deficient expansion and cytolytic activity of LCMV-specific $CD8^+$ T cells, as well as reduced serum titers of anti-LCMV IgG. Furthermore, anti-viral immunity among microbiota-depleted mice may be further compromised by altered transcriptional regulation proinflammatory genes following infection. Splenic mononuclear cells, isolated from GF mice, demonstrate reduced production of proinflammatory cytokines following stimulated with purified MAMPs (Ganal et al., 2012). This defective response is associated with reduced transcription of various inflammatory response genes due to chromatin modification of the promoter region. These studies reveal a remarkable role for commensal microbes in programing host systemic defense responses during steady-state conditions. Furthermore, as this influence is reversible, temporary depletion of the microbiota is sufficient to compromise systemic immune resistance to pathogen invasion.

In addition to priming anti-viral immune responses during steady-state conditions, commensal microbes may also protect against systemic bacteremia. Neutrophils isolated from the bone marrow of antibiotic-treated or GF mice are attenuated in killing extracellular pathogens *Staphylococcus aureus* and *Streptococcus pneumonia, ex vivo* (Clarke et al., 2010). This defect was reproduced in mice deficient in Nod1, a PRR which recognizes peptidoglycan derived meso-diaminopimelic acid (mesoDAP), but not in mice deficient in other PRRs. As mesoDAP is expressed by commensal microbes, it was speculated that the microbiota may directly prime neutrophil killing activity. Appropriately, peptidoglycan from intestinal microbes is detected within the bone marrow, indicating that commensal microbes are able to directly stimulate neutrophils within systemic

tissues. Furthermore, neutrophil antimicrobial activity among antibiotic-treated mice is rescued following stimulation with Nod1 ligand. While it remains to be shown that the absence or disruption of the microbiota actually reduces resistance to bacterial infection, these collective findings suggest that immune priming by commensal microbes is critical in promoting host resistance against systemic infections.

Defects in Host-Microbial Symbiosis May Predicate Susceptibility to Infection

Factors that determine an individual's susceptibility to infectious disease remain largely unknown. Here we suggest that environmental and genetic influences that disrupt the microbiota or impede host sensing of commensal-derived signals may confer vulnerability to pathogen infection (Figure 1-3). As discussed earlier, depletion of the microbiota through antibiotics is sufficient to compromise host immune function and increase the risk of opportunistic infection. Other environmental factors that disrupt the composition of the microbiota, including gastrointestinal infection or diet, additionally may serve as a risk factor for disease (Bäckhed et al., 2007; Lupp et al., 2007). Susceptibility to infection may even persist long after exposure to the microbiota-disrupting agent. Tracking the intestinal commensal profile among patients taking oral antibiotics show a recovery in the composition of the microbiota following cessation of therapy (Dethlefsen and Relman, 2011). However, there is a delay of several weeks to months between the final antibiotic administration and recovery of the microbiota to the pre-treatment composition. In animal models this delay was associated with increased susceptibility to infection, reflecting the persistent consequences of antibiotic therapy (Ubeda et al., 2010). Additionally, certain individuals display alterations for up to four years after antibiotic treatment, indicating a defect in microbiota resilience

(Jakobsson et al., 2010). We speculate that such a defect, while asymptomatic, may compromise the protective contribution of the commensal microbiota to host immunity and weaken resistance against pathogenic insult.

Defects in host sensing of the beneficial influence of commensal microbes may also serve as a risk factor for disease. Nod2 is an intracellular PRR that recognizes muramyl dipeptide, a conserved structural moiety of bacterial peptidoglycan (Maeda et al., 2005). Nod2 signaling promotes expression of Paneth cell α-defensin, a class of antimicrobial peptides that, similar to RegIIIγ, limits microbial contact with host tissue (Kobayashi et al., 2005). As a consequence of the diminished α-defensin production, Nod2-deficient mice display heightened susceptibility to gastroenteritis by *Listeria monocytogenes*. Furthermore, as homozygous mutations in this receptor are associated with increased incidence of Crohn's disease, defects in host sensing of commensal signals may be a risk factor for inflammatory bowel disease (IBD) by reducing clearance of pathogenic bacteria (Maeda et al., 2005). Indeed, the finding that adhesive and invasive *E. coli* (AIEC) are tightly associated with the intestinal epithelium among patients with Crohn's disease may support this notion (Eaves-Pyles et al., 2008).

Finally, the genetic selection of one's microbiota composition may reflect individual susceptibility to infection. NIH Swiss (NIH) mice are naturally resistant to gastrointestinal infection with *C. rodentium* compared to C3H/HeJ (HeJ) mice, which develop lethal disease (Willing et al., 2011). Resistance among NIH mice is associated with increased expression of IL-22 and RegIIIβ, relative to HeJ mice. As the microbiota drives the expression of both antimicrobial mediators, susceptibility

to infection may be a function of gut bacterial community composition. To test this hypothesis, HeJ mice were depleted of microbiota through antibiotic treatment, and colonized with intestinal microbes from NIH mice. The bacterial community profile of transplanted mice was shown to resemble that of the NIH donor. Remarkably, transfer of commensal microbes from NIH to HeJ mice is sufficient to promote resistance to infection. Protection is associated with increased expression of IL-22 and RegIIIβ, and protection is lost following neutralization of IL-22. Reciprocally, transplantation of HeJ microbiota to NIH mice increased disease burden to *C. rodentium*. Finally, pups in the subsequent generation inherit the microbiomes transferred to their parents. Offspring display resistance patterns to *C. rodentium* infection relative to their microbiota composition, rather than their genetics. These data suggest that familial history of infectious disease may not only reflect the inheritance of susceptibility genes, but possibly the vertical transmission of a microbiota that is less protective against pathogen challenge.

Conclusion

The evidence summarized in this review suggests that disruption of the microbiota through environmental influences may compromise immune function, leading to increased susceptibility to infectious disease. These studies emphasize the importance of commensal microbes in mediating host immune integrity during infection with pathogenic microorganisms. While these studies have focused on the contribution of the microbiota in modulating functional responses by mucosal and systemic immune cell populations (including cytokine processing, production of antimicrobial peptides and phagocytic activity), little is known regarding the role of commensal microbes in mediating immune cell development. In the next three chapters, we present new data that

demonstrates that commensal microbes promote hematopoiesis to expand myeloid cells that populate systemic sites, and serve as a first line defense against infectious disease.

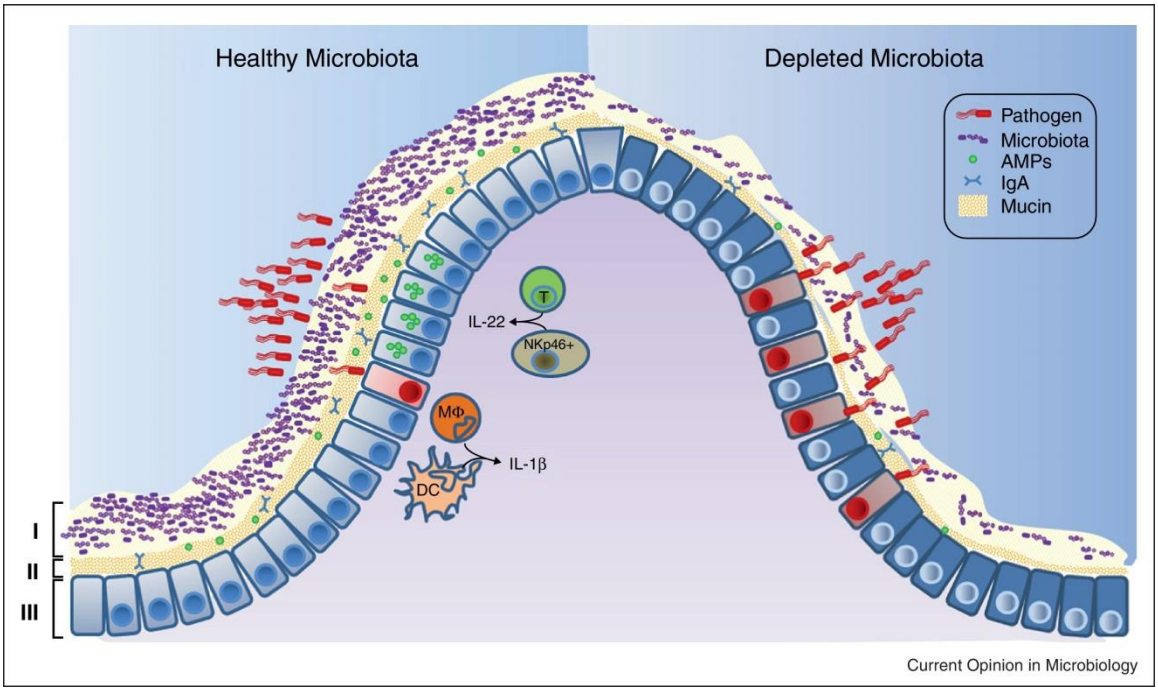

Figure 1-1. The Intestinal Microbiota Promotes Three Levels of Protection Against Enteric Infection (I) Saturation of colonization sites and competition for nutrients by the microbiota limit pathogen association with host tissue. (II) Commensal microbes prime barrier immunity by driving expression of mucin, immunoglobulin A (IgA) and antimicrobial peptides (AMPs) that further prevent pathogen contact with host mucosa. (III) Finally, the microbiota enhances immune responses to invading pathogens. This is achieved by promoting IL-22 expression by T cells and NKp46^{+} cells, which increases epithelial resistance against infection, as well as priming secretion of IL-1β by intestinal monocytes (MΦ) and dendritic cells (DCs), which promotes recruitment of inflammatory cells into the site of infection. Under conditions in which the microbiota is absent, such as following antibiotic treatment, there is reduced competition, barrier resistance and immune defense against pathogen invasion.

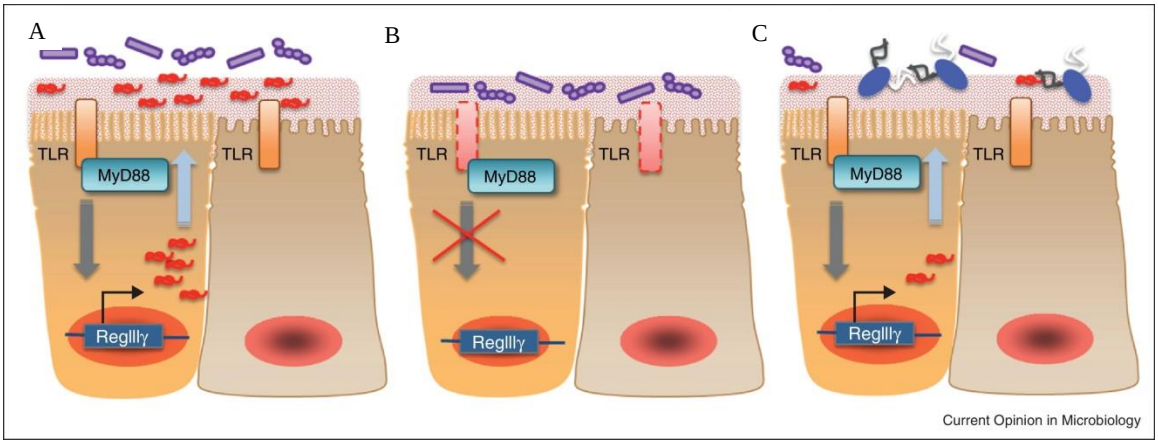

Figure 1-2. The Commensal Microbiota Primes Barrier Immunity Direct stimulation of epithelial Toll-like receptors (TLRs) by commensal MAMPs primes expression of RegIIIγ (A). Production of RegIIIγ is essential to limit microbial contact with host mucosa. As such, defects in TLR function result in deficient RegIIIγ expression, which leads to an increased association of commensal microbes with host tissue, as well as a heightened risk of infection with enteric pathogens (B). Additionally, reduced TLR stimulation as a consequence of the depletion of the microbiota is sufficient to reduce RegIIIγ expression and render the host susceptible to infection (C).

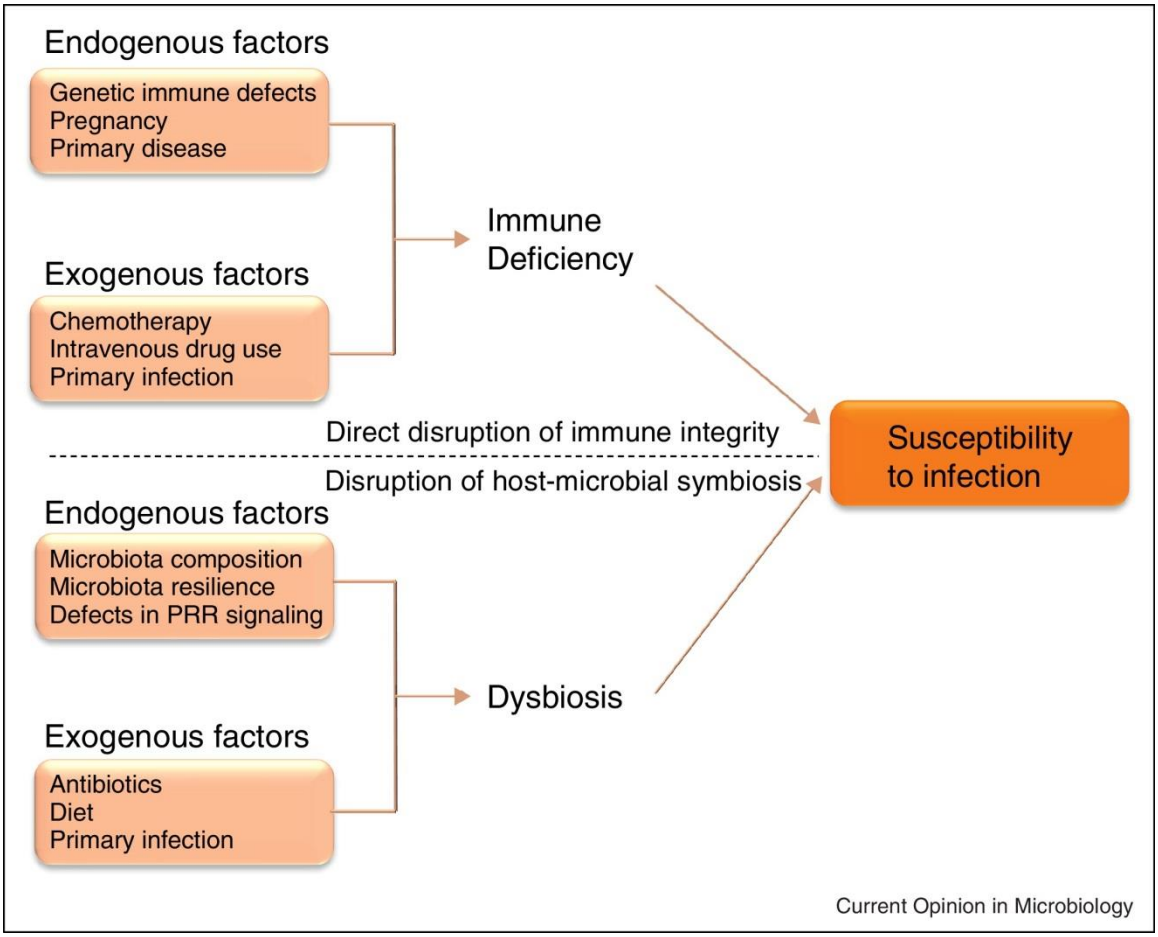

Figure 1-3. Disruption of Host–Microbial Symbiosis as a Risk Factor for Infectious Disease

Exposure to pathogenic microorganisms is often insufficient to cause disease. Rather, susceptibility to infectious disease reflects deficient immune resistance to pathogen challenge. As such, exogenous and endogenous factors that directly compromise individual immune function (including genetic immune defects and chemotherapy) are significant risk factors for infection. We extend this model by proposing that the factors that disrupt the protective benefits of the commensal microbiota similarly compromise individual immune integrity and may predispose to infectious disease.

Chapter 3

GUT MICROBES DRIVE STEADY-STATE HEMATOPOIESIS

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 several innate immune cell populations (including neutrophils, monocytes and macrophages) within systemic sites. By controlling the differentiation of innate immune cells, 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 microbes evolved to actively shape immunity at its core, via regulation of hematopoiesis.

Germ-free Animals Display Global Defects in Innate Immune Cells

The commensal gut microbiota profoundly influences cellular 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 3-1A-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 3-2A). Furthermore, treatment of SPF mice with antibiotics also results in diminished myeloid cell populations in the spleen (Figure 3-2B). 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, and 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 compromised development in primary immune sites. Accordingly, we observed a reduction of myeloid cells within the bone marrow of GF mice (Figures 3-3A-C). A

similar decrease was observed in the liver, a site of alternative immune cell development (Figure 3-2C). 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 3-4A, 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 3-5A-F). Remarkably, GF mice are significantly reduced in the proportion of bone marrow granulocyte and/or monocyte progenitors (GMPs), identified as LKS^{-} $CD34^{+}$ $Fc\gamma R^{hi}$ cells (Figure 3-6A). 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^{-} $CD34^{+}$ $Fc\gamma R^{hi}$ cells from GF mice displayed reduced granulocyte (G-CFU) and monocyte (M-CFU) colony formation compared to cells from SPF mice (Figure 3-6B). Furthermore, LKS^{-} $CD34^{+}$ $Fc\gamma R^{hi}$ cells isolated from GF mice in primary methylcellulose culture yielded fewer of c-Kit^{+} $CD11b^{-}$ progenitor cells compared to SPF GMPs (Figure 3-6C). This suggests that the ability of GMPs to maintain cells with progenitor potential is defective 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 3-6D). The commensal microbiota therefore promotes steady-state myelopoiesis by specifically maintaining GMP proportions and enhancing their differentiation into 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 3-7A-B). 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.

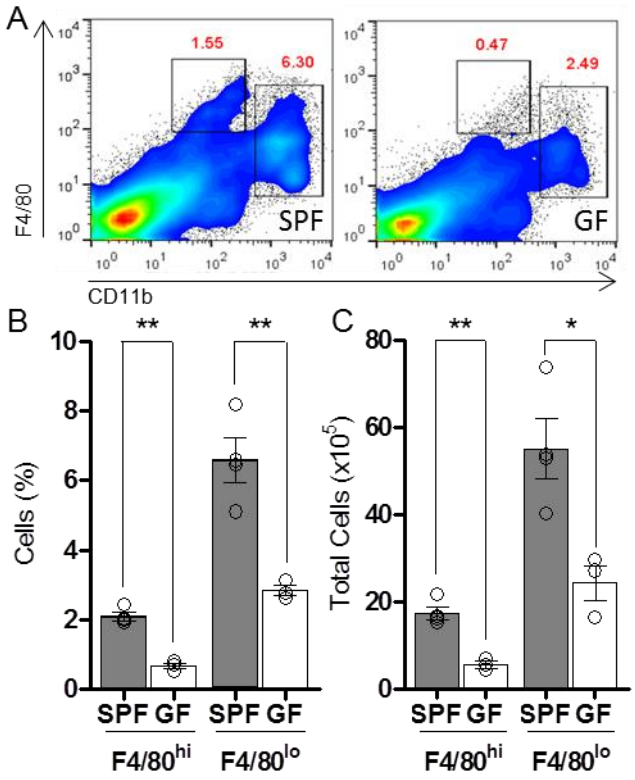

Figure 3-1. GF Mice Are Deficient in Resident Myeloid Cell Populations in the Spleen (A-C) Splenic phagocyte profile among SPF and GF mice. Representative flow cytometry plots (A), cell proportions (B), and total cell number (C) of $CD11b^{lo}$ $F4/80^{hi}$ and $CD11b^{hi}$ $F4/80^{lo}$ splenic cells in SPF and GF mice. For all panels, data are representative of at least 3 independent trials, with $n \geq 4$ mice / group. Each symbol represents data from a single animal. Error bars represent standard error of mean (SEM). *p<0.05, **p<0.01.

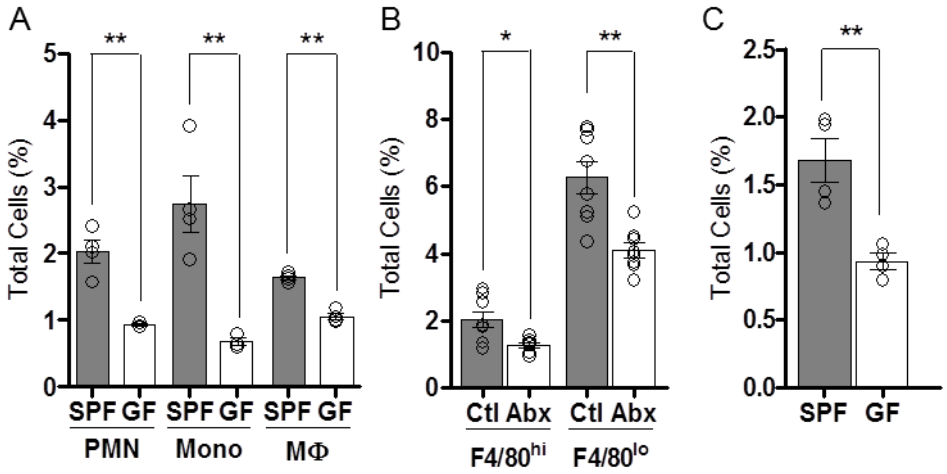

Figure 3-2. GF and Antibiotic-Treated Mice Have Reduced Populations of Myeloid Cells in Systemic Sites (A) Frequency of splenic neutrophils ($CD11b^+$ $GR1^{hi}$ $Ly6c^{lo}$), monocytes ($CD11b^+$ $Ly6c^{hi}$ $GR1^{hi}$) and macrophages ($CD11b^+$ $GR1^-$ $F4/80^{lo}$) among SPF and GF mice. (B) Frequency of splenic $CD11b^+$ $F4/80^{hi}$ and $CD11b^+$ $F4/80^{lo}$ phagocytes among untreated mice (Ctl) and SPF mice treated with oral antibiotics (Abx). (C) Frequency of liver $CD11b^+$ $F4/80^{hi}$ macrophages recovered from SPF or GF mice. Error bars represent SEM. Data are representative of 2-3 independent trials, with $n \geq 4$. *$p<0.05$, **$p<0.01$. PMN: polymorphonuclear cells; Mono: monocytes; MΦ : macrophages.

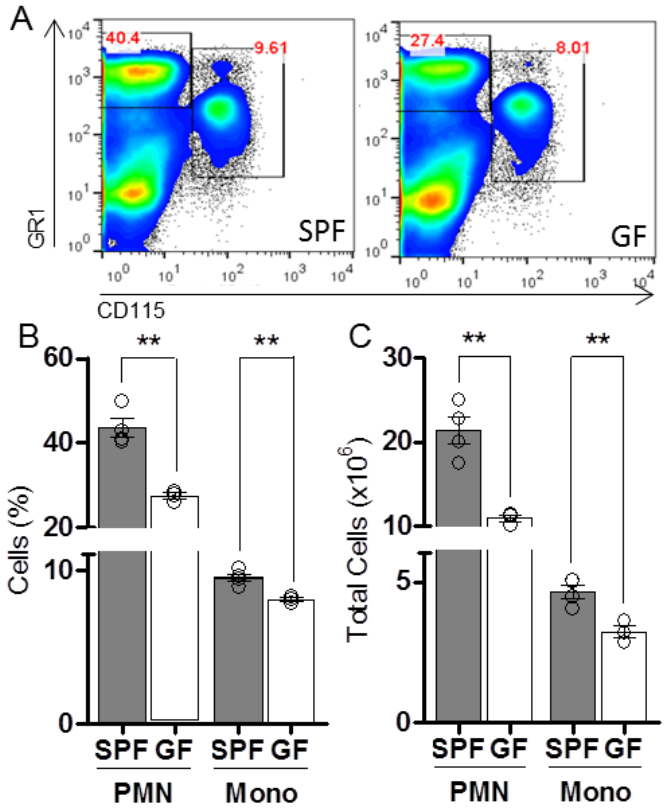

Figure 3-3. GF Mice Are Deficient in Bone Marrow Myeloid Cell Populations (A-C) Bone marrow populations of neutrophils ($Gr1^{hi}$ $CD115^{neg}$) and monocytes ($Gr1^{hi}$ $CD115^{hi}$) among SPF and GF mice. Representative flow cytometry plots (A), cell proportions (B) and total cell number (C) within 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.

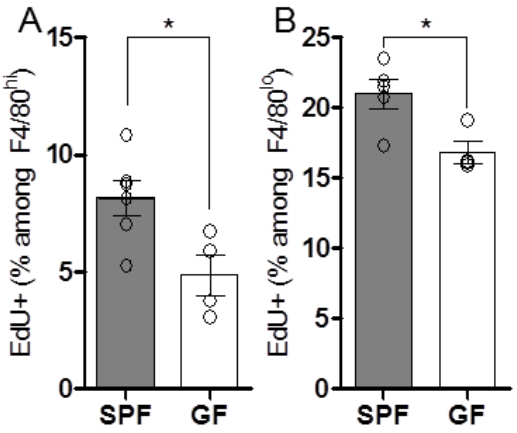

Figure 3-4. The Microbiota Promotes Expansion of Splenic Myeloid Cells The percentage of F4/80^{hi} CD11b^{+} (A) and F4/80^{lo} CD11b^{+} (B) splenocytes with incorporated EdU, following single dose administration. For all panels, data are representative of 2 independent trials, with n≥ 4 mice / group. Each symbol represents data from a single animal. Error bars represent standard error of mean (SEM). *p<0.05, **p<0.01.

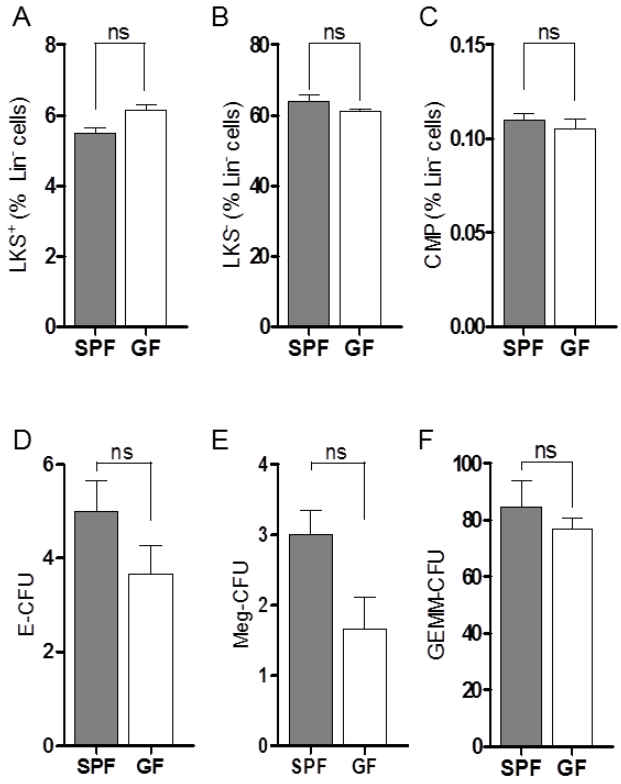

Figure 3-5. GF Mice Have Normal Proportions and Differentiation Potential of HSCs and Early Myeloid Progenitors in the Bone Marrow (A) Proportion of LKS^+ cells (Lin^- c-Kit^+ $Sca\text{-}1^+$; HSCs and MPPs), (B) LKS^- cells (Lin^- c-Kit^+ $Sca\text{-}1^-$; lineage-restricted progenitors) and (C) CMPs (LKS-$CD34^+$ $Fc\gamma R^{lo}$) among total progenitors (Lin^- cells) of SPF and GF mouse bone marrow. (D-F) Unfractionated bone marrow progenitor cells (Lin^- cells) from SPF and GF mice cultured in methylcellulose to assess the colony forming potential of progenitors. (D) E-CFU; erythrocyte colony forming units, (E) Meg-CFU; megakaryocyte CFU, (F) GEMM-CFU; Granulocyte/erythrocyte/monocyte/megakaryocyte CFU. Error bars represent SEM. Data are representative of 3 independent trials, with $n \geq 4$. Error bars represent SEM. ns: non-significant.

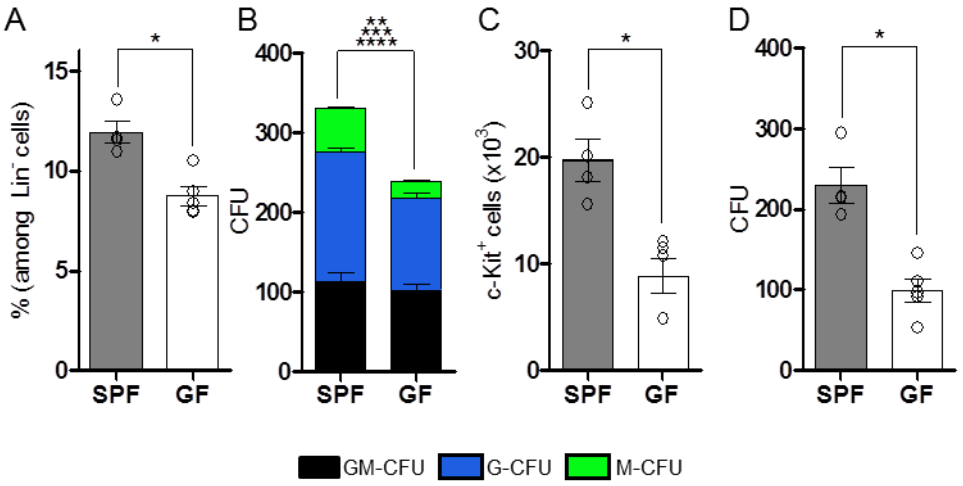

Figure 3-6. The Microbiota Directs Myelopoiesis (A) The frequency of LKS$^-$ CD34$^+$ FcγR^{hi} granulocyte and/or monocyte progenitors (GMPs) among lineage negative (Lin$^-$) progenitors from bone marrow of SPF and GF mice, as assessed by flow cytometry. (B) Distribution of cell types following purified LKS$^-$ CD34$^+$ FcγR^{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). (C) Total numbers of c-Kit$^+$ CD11b$^-$ progenitors from methylcellulose cultures of LKS$^-$ CD34$^+$ FcγR^{hi} progenitors, as assessed by flow cytometry. (D) Cells harvested from methylcellulose cultures of LKS$^-$ CD34$^+$ FcγR^{hi} progenitors were re-plated at equal numbers in fresh methylcellulose, and cultured to assess their colony forming capacity. 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 (B)), ***p<0.05 (comparing G-CFU between SPF and GF for (B)), ****p<0.05 (comparing M-CFU between SPF and GF for (B)). CFU: colony forming units.

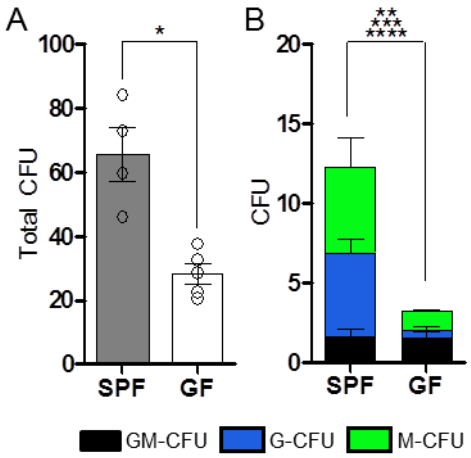

Figure 3-7. Commensal Microbes Promote Extramedullary Hematopoiesis (A and B) Splenic cells isolated from SPF and GF mice were cultured in methylcellulose to assess the colony forming capacity of progenitors from SPF and GF mice. Total CFUs (A), and GM-CFUs, G-CFUs and M-CFUs (B) are shown. For each 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 (B)), ***p<0.05 (comparing G-CFU between SPF and GF for (B)), ****p<0.05 (comparing M-CFU between SPF and GF for (B)). CFU: colony forming units.

Chapter 4

MICROBIOTA-DRIVEN HEMATOPOIESIS PROTECTS AGAINST SYSTEMIC INFECTION

Commensal microbes have previously been shown to influence functional responses by various phagocytic cells during bacterial and viral infection (Clarke et al., 2010; Franchi et al., 2012; Ganal et al., 2012; Ichinohe et al., 2011). However, the role of the microbiota in promoting hematopoiesis, and its contribution towards host health, has not been previously studied. As revealed in Chapter 3, naïve GF animals display reductions in both proportions and total cell numbers of tissue-resident F4/80^{hi} and F4/80^{lo} phagocytes compared to SPF mice (Figures 3-1A-C). Furthermore, treatment of SPF mice with antibiotics also results in diminished resident phagocytic cells (Figure 3-2B). Tissue-resident cells are essential in mediating acute resistance against pathogenic microorganisms by restricting bacterial dissemination, as well as coordinating the recruitment of additional immune cells to the site of infection (Pamer, 2004; Sieweke and Allen, 2013). Therefore, we investigated whether the reduced populations of these phagocytic cells, as a consequence of absent or diminished colonization by commensal microbes, increases susceptibility to infectious disease.

Tissue-Resident Phagocytes Mediate Protection by Commensal Microbes

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 4-1A) (Serbina et al., 2012; Shi et al., 2011). However, GF mice rapidly succumb at the same inoculum (Figure 4-1A). 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 4-1B-D). Susceptibility to infection is not restricted to *L. monocytogenes*, as GF mice also displayed increased disease burden following systemic challenge with *Staphylococcus aureus* (Figure 4-1E). Interestingly, SPF mice treated orally with broad-spectrum antibiotics are also impaired in controlling *Listeria*, indicating that protection by commensal microbes is an active process and is subject to loss following depletion of gut microbiota (Figure 4-1F). 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 4-2A,B), 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 4-2A), and rapid death within 48 hpi (Figure 4-2B). While functional defects in myeloid cells may potentially contribute to increased disease in GF mice, we did not detect differences during *in vitro Listeria* killing by macrophages from SPF or GF mice (Figure 4-2C).

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 4-2D), suggesting that 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 4-3A), nor are GF mice deficient in developing long-term protective immunity against subsequent infection (Figure 4-3B). 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 4-3C) (Serbina et al., 2009; Serbina et al., 2003). Finally, while there are fewer inflammatory neutrophils and monocytes recruited to the spleen following infection (Figure 4-3D), a possible consequence of increased apoptosis (Figure 4-3E), these cells were not required for commensal-mediated protection against *L. monocytogenes* (Figure 4-3F, G). Together, these findings demonstrate that hematopoietic defects in tissue-resident myeloid cells prior to infection of GF mice (i.e., during steady-state hematopoiesis) is the primary cause of impaired control of *Listeria.*

Commensal Bacterial Signals 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. Recolonization of GF mice with a complex microbiota and oral treatment with MAMPs, but not SCFAs, was sufficient to promote recovery of GMP-derived myeloid cells (neutrophils and monocytes) within the bone marrow (Figure 4-4A, B). Importantly, only recolonization of GF mice with an SPF microbiota was sufficient to restore splenic populations of F4/80^{hi} macrophages and F4/80^{lo} splenocytes (i.e., neutrophils, monocytes and macrophages) (Figure 4-4C and data not shown). Therefore, while MAMP treatment is necessary for 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 recolonization of GF animals, and not oral MAMP treatment, was sufficient to restore the defect in GF mice to systemic challenge with *L. monocytogenes* (Figure 4-4D and data not shown). Collectively, these studies reveal that the microbiota provides complex molecular signals that actively promote the hematopoietic differentiation of myeloid cells, resulting in peripheral phagocyte populations that function as sentinels for the early detection and control of systemic bacterial infection.

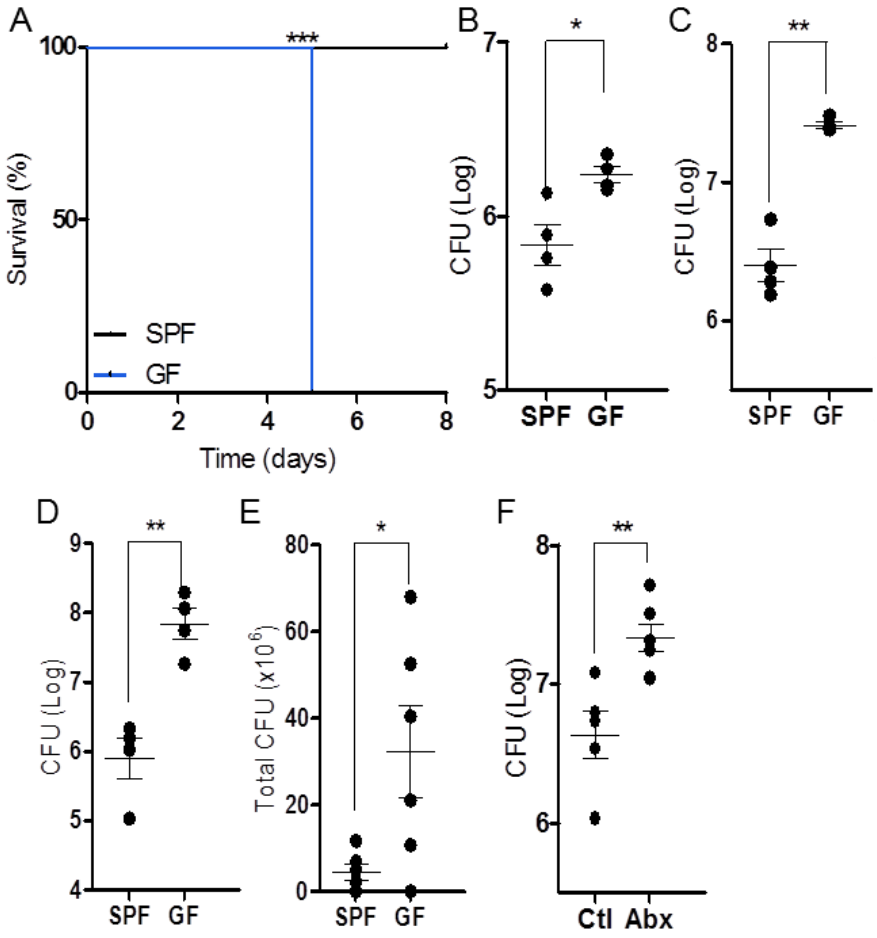

Figure 4-1. The Microbiota Promotes Early Resistance to Systemic Infection (A-C) SPF and GF mice were infected with *L. monocytogenes* and assessed for survival (A) and splenic bacterial burden at 24 (B) and 72 (C) hours post- infection (hpi). (D) Liver bacterial burden among SPF and GF mice, 72 hpi. (E) SPF and GF mice infected with *S. aureus.* Kidney bacterial burden assessed 5 days post-infection . (F) SPF mice treated with antibiotics (Abx) and untreated controls (Ctl) were infected with *L. monocytogenes* and splenic bacterial burden was measured 72 hpi. 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).

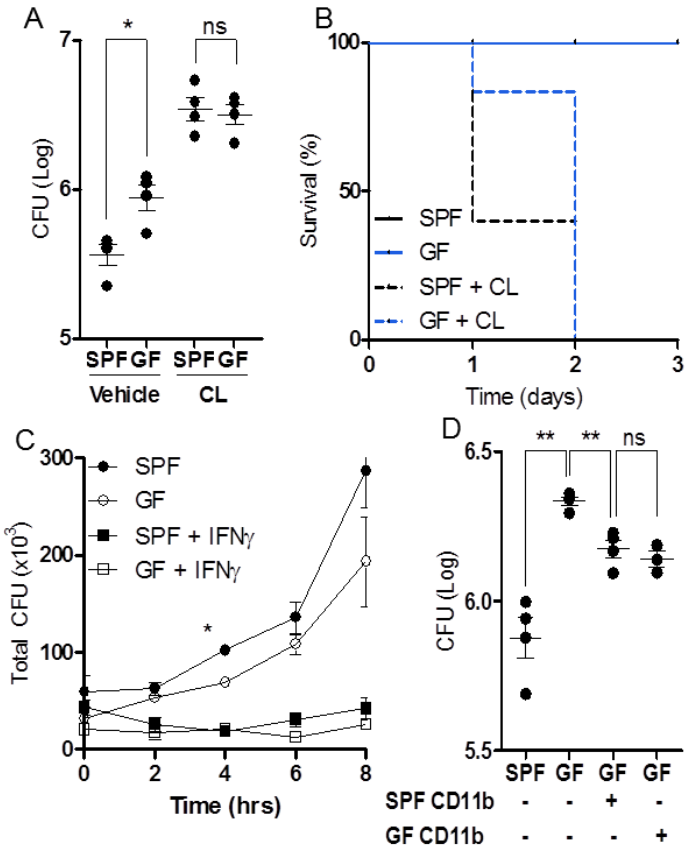

Figure 4-2. The Microbiota Promotes Resistance to Infection via Tissue-Resident Cells (A-C) SPF and GF mice depleted of tissue-resident cells prior to infection with *L. monocytogenes* and assessed for splenic bacterial burden 24 hpi (A) and survival (B). (C) Peritoneal macrophages isolated from SPF or GF mice, untreated or stimulated with interferon-γ (IFNγ), infected with *L. monocytogenes*. Recovery of intracellular bacteria measured over time. Data is non-significant for all time points measured, except where indicated (untreated SPF vs. GF, 4 hpi). (D) Splenic bacterial burden, 24 hpi, following transfer of splenic $CD11b^+$ cells from SPF or GF donors . 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. CL: clodronate-loaded liposomes.

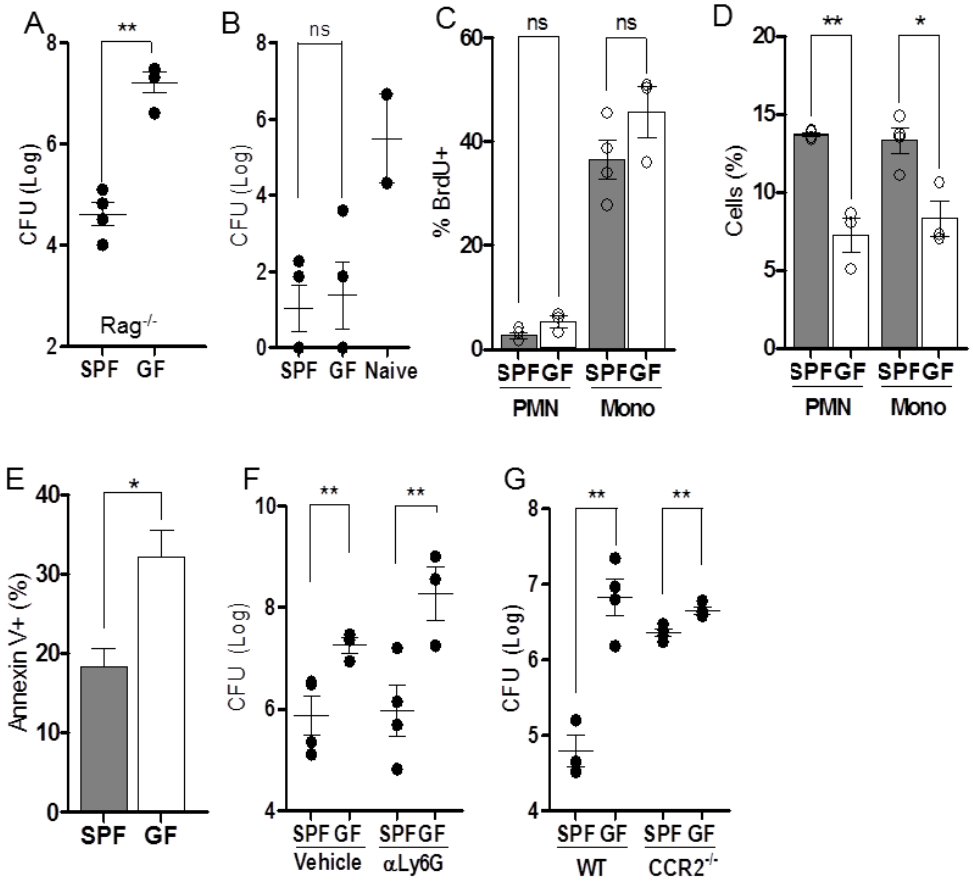

Figure 4-3. Resident Phagocytes Mediated Commensal-Enhanced Protection Against Infectious Disease (A) SPF and GF Rag$^{-/-}$ mice infected with *L. monocytogenes*, splenic bacterial burden assessed 72 hpi. (B) SPF and GF mice were immunized with *L. monocytogenes* Δ*actA*. 45 days after immunization, SPF and GF mice, as well as naïve, non-immunized SPF controls, were infected with wild-type (WT) *L. monocytogenes*. Splenic bacteria burden of the WT strain was measured at 72 hpi. Note: two of the four naïve, non-immunized SPF mice died following infection, prior to the 72 hour time point (data not shown). (C) BrdU incorporation among bone marrow neutrophils (CD11b^{+} GR1^{hi}) and monocytes (CD11b^{+} CD115^{+}), 72 hpi. (D) Percentage of splenic neutrophils (Gr1^{hi} Ly6C^{lo}) and monocytes (Gr1^{hi} Ly6C^{hi}) among SPF and GF mice, 72 hpi. (E) Annexin V^{+} bone marrow monocytes, 72 hpi. (F) SPF and GF mice infected with *L.*

monocytogenes, following neutrophil depletion. Splenic bacterial burden assessed at 72 hpi. (G) Splenic bacterial burden of SPF and GF mice, reconstituted with bone marrow from WT or $CCR2^{-/-}$ mice, 72 hpi. SPF mice reconstituted with $CCR2^{-/-}$ bone marrow display a two-fold reduction in splenic CFUs compared to GF $CCR2^{-/-}$ mice. For all panels, data are representative of 2-3 independent trials, with $n \geq 4$/ group. Each symbol represents data from a single animal. Error bars represent SEM. *$p<0.05$, **$p<0.01$. PMN: polymorphonuclear cells; Mono: monocytes.

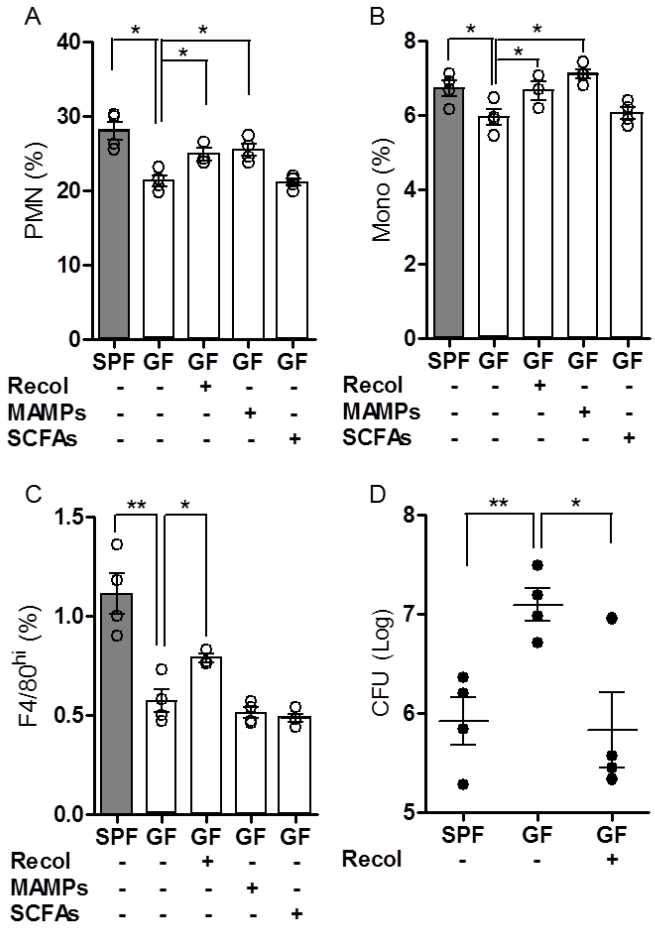

Figure 4-4. Recolonization of GF Mice Restores Immune Integrity Against Systemic Listeriosis (A) Neutrophil ($GR1^{hi}$ $CD115^{-}$) and (B) monocyte ($GR1^{hi}$ $CD115^{+}$) bone marrow profiles from SPF, GF, recolonized GF mice and MAMP or SCFA-treated GF mice. (C) $F4/80^{hi}$ splenic macrophage profile among SPF, GF, recolonized GF mice and GF mice treated with MAMPs or SCFAs. (D) Splenic bacterial burden 72 hpi among SPF, GF and recolonized 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: recolonized; MAMPs: molecular associated molecular patterns; SCFAs: short chain fatty acids.

Chapter 5

FINDINGS AND DISCUSSION

Advances in understanding host-microbial symbiosis have revealed that the gut microbiota control the phenotype, migration and activity of multiple innate and adaptive immune 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 circulating populations of neutrophils 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 reveal that gut bacteria regulate hematopoiesis within primary immune sites, providing a unifying explanation for previous observations of the widespread effects by the microbiota on the immune system.

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. Furthermore, as a consequence of the reduced populations of resident phagocytes, which serve as a first line defense against invading pathogens, GF mice are more susceptible to systemic infection with *L. monocytogenes*. Interesting, despite multiple immune abnormalities having been previously described in GF mice, the increased susceptibility to systemic infection with *L. monocytogenes* appears to be specific to the reduced

proportions of resident myeloid cells. Previous studies have shown that commensal microbes prime neutrophil killing of S*treptococcus pneumoniae* and *Staphylococcus aureus* (Clarke et al., 2010). Further, the microbiota enhances host resistance to viral infection by promoting expression of type-1 interferon by splenic phagocytes (Abt et al., 2012). However, we were not able to detect defects in the functional activity of phagocytes isolated from GF mice, as related to protection against *L. monocytogenes*. Peritoneal macrophages isolated from naïve SPF and GF mice displayed equivalent killing of *Listeria*, *ex vivo*. Additionally, SPF and GF phagocytes expressed similar levels of TNFα and NO following infection, which is essential for limiting *Listeria* dissemination (data not shown). Finally, splenic phagocytes isolated from SPF and GF donors were equally sufficient to provide protection against infection when transferred into GF recipients. These data suggests that a primary defect in the maintenance of resident phagocytes in GF animals is responsible for the increased susceptibility to systemic infection. However, it remains possible that the microbiota primes additional immune responses, not described here, that further contributes to mediating host protection against infectious disease.

While our studies reveal steady-state hematopoiesis is compromised in GF mice, emergency hematopoiesis, or the selective expansion of myeloid cell following infection, is maintained independent of commensal microbes. One possible explanation for this contrast is that the expression of cytokines and growth factors following infection, as well as direct stimulation by microbial ligands, may rescue hematopoietic defects in GF mice otherwise present under steady-state/non-inflammatory conditions. We propose a model whereby a primary defect in hematopoiesis in GF or antibiotic-treated mice compromises multiple tissue-resident innate immune cell populations prior to infection, leading to blunted early responses upon subsequent pathogen encounter (see diagram in Figure 5-1). While tissue-resident phagocytes directly mediate early

resistance to infection, these cells are also essential for recruiting additional phagocytes (monocytes) which is essential for maintaining resistance (Coombes et al., 2012; Kastenmuller et al., 2012). GF mice therefor display exacerbated disease severity as a consequence of diminished phagocyte recruitment into infected tissues. While our studies focus on innate immunity due to its role in rapid control of early *Listeria* infection, impaired microbiota-mediated hematopoiesis may also extend 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 are 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 in GF mice. However, MAMP treatment alone is inadequate to expand splenic F4/80^{hi} and F4/80^{lo} cells, indicating that additional commensal-derived signals are necessary to influence site-specific HSC- and yolk sac-derived myeloid cells. Interestingly, recolonization 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 microbial ligands or metabolites translocating

from the gut into the circulation to directly stimulate progenitor cells, 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 uncover the 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. Evidence that depletion of the microbiota leads to transient immune suppression suggests factors that disrupt commensal microbes, including that clinical antibiotic use may, paradoxically, be a risk factor for susceptibility to opportunistic pathogens. Furthermore, the spread of antibiotic-resistance among pathogens, paired with a dwindling supply of effective antibiotics, has necessitated alternative strategies to combat infections (Khosravi and Mazmanian, 2013). As certain commensal microbes have been previously shown to express molecules with unique immunomodulatory properties, it is possible such microbial products may be developed into therapeutics to treat infectious diseases. Whereas traditional antibiotics work by through direct microbicidal activity, indiscriminately killing both pathogenic and commensal microbes, immunomodulatory therapeutics would enhance host immune responses to promote pathogen clearance. Such a strategy may specifically target pathogenic microbes and thereby reduce the risk of secondary inflammatory disease caused by depleting commensal microbial communities, as currently occurs with antibiotic use. The concepts proposed herein, if validated in humans, may herald future medical approaches that combine antibiotics with immunomodulatory microbial molecules as revolutionary combination treatments to address the reemerging crisis of infectious diseases.

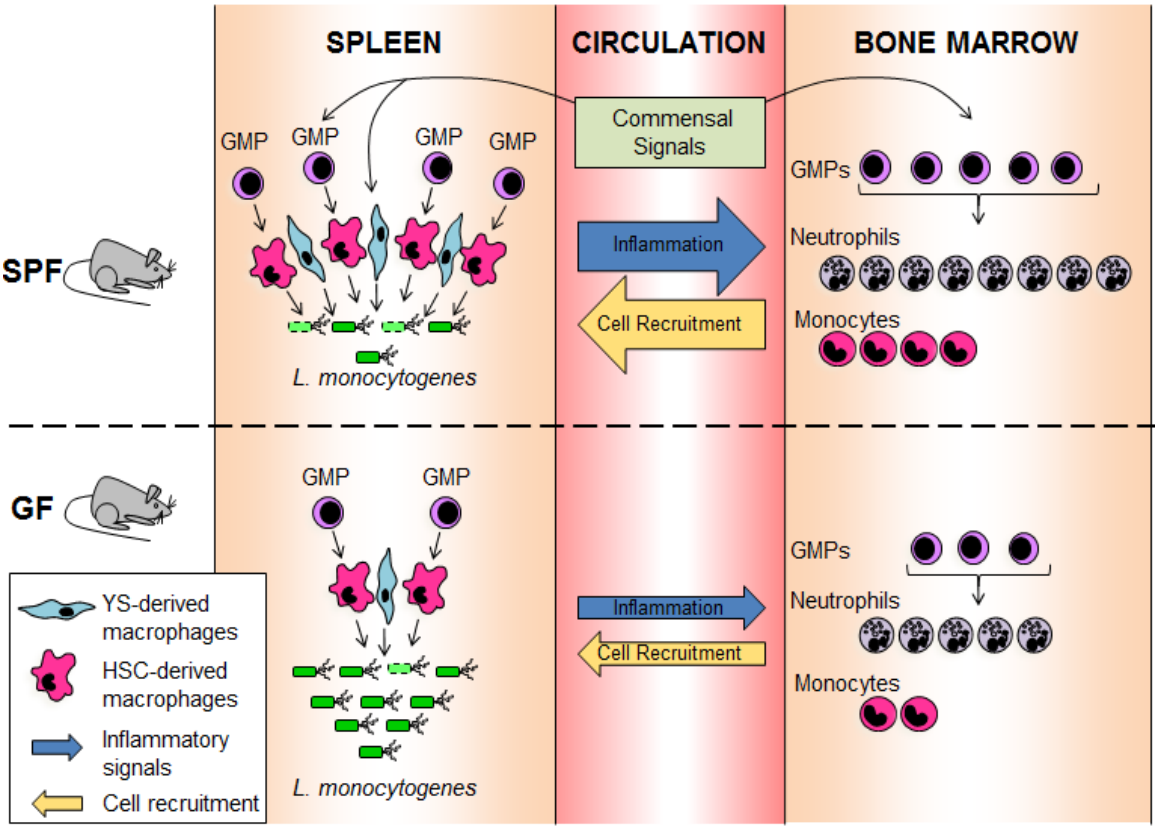

Figure 5-1. A Proposed Model For How the Microbiota Mediates Host Resistance to Systemic Infection Commensal microbes stimulate bone marrow and splenic myelopoiesis during naïve conditions (in the absence of infection), expanding systemic pools of mature myeloid cells in SPF mice that are essential for restricting pathogen dissemination upon acute infection. GF mice have reduced proportions and differentiation potential by GMPs during the steady-state, as well as diminished expansion of yolk sac-derived macrophages, impairing the immune response to infection with *L. monocytogenes*. This model suggests that conditions under which the microbiota is disrupted may result in deficient expansion of myeloid cells, compromising host resistance to infectious disease.

Material and Methods

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 $3x10^4$ colony forming units (CFU) of *Listeria monocytogenes* 10403S. Splenic and liver bacterial CFU were assessed 24-72 hpi by microbiological plating. In some experiments, SPF and GF mice were immunized with 3x104 CFU *L. monocytogenes* Δ*actA* (Lara-Tejero and Pamer, 2004), and immunized mice and non-immunized controls were infected with 2x105 CFU of wild-type (WT) *L. monocytogenes* 45-day post immunization, with splenic bacterial burden measured 72 hpi. Alternatively, SPF and GF mice were infected with $1x10^7$ CFU of *S. aureus* (strain Newman) via tail vein injection and kidney bacterial burden assessed 5 days post-infection. 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 $3x10^4$ 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 (~$1x10^9$ CFU/ml in drinking 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 recolonized 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 via passage 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-Kit^+ $Sca\text{-}1^+$; HSCs and MPPs), Lin^- c-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). To measure cell proliferation during *Listeria* infection, mice were injected *i.p.* with 100 μg BrdU (Sigma), and BrdU incorporation among progenitor and mature myeloid cells was determined 3 hours later via a BrdU detection kit (eBioscience). Apoptosis and cell viability was assessed by staining with Annexin V (eBioscience) and 7-Aminoactinomycin-D (Invitrogen). *Listeria*-killing assays were conducted as previously described (Portnoy et al., 1989). Briefly, peritoneal macrophages were collected from naïve SPF and GF mice. Adherent cells were stimulated with 100 U/ml of interferon gamma (IFNγ) (PeproTech) or left untreated for 24 hours. Macrophages were washed and infected with *L. monocytogenes* at a multiplicity of infection (MOI) of 10. Cells were washed 30 minutes later and fresh media with 5 μg/ml of Gentamycin (Phoenix) was added. Cells were washed and lysed at various time points to quantitate intracellular *Listeria* via microbiological plating. 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). $2x10^6$ $CD11b^+$ cells (>90% purity) were transferred into GF recipients 24 hours prior to infection with *L. monocytogenes*. CFU burden were assessed 24 hpi. $CCR2^{-/-}$ chimeras were generated by transferring bone marrow from WT or $CCR2^{-/-}$ donors into SPF or GF recipients that had been lethally irradiated (1000 rads) 48 hours prior. Mice were infected with 3x104 CFU of *L. monocytogenes* 8 weeks post reconstitution, and

splenic bacterial burden was assessed 72 hpi. For neutrophil depletion, SPF and GF mice were injected *i.p.* with 0.5 mg of anti-Ly6G antibody (Bioxpress), or saline control, 24 hours prior to infection with *L. monocytogenes*.

CFU Assays

To evaluate hematopoietic potential, 1×10^3 Lin^- or $1x10^3$ LKS^- $CD34^+$ $Fc\gamma R^{hi}$ cells or $2x10^5$ splenocytes were plated in triplicate in MethoCult GF M3434 (StemCell Technologies) methylcellulose-based medium and incubated for 7 days in 37^{o}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, $5x10^4$ 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.

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The work presented in Chapters 1-5 is collected from published articles (Khosravi and Mazmanian, 2013) and (Khosravi et al., 2014).

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