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Defining Interactions Between Lymphoid Tissue-Resident Commensal Bacteria and the Host Immune System

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Defining Interactions Between Lymphoid Tissue-Resident Commensal Bacteria and the Host Immune System

Abstract
The mammalian gastro-intestinal tract is colonized by trillions of beneficial commensal bacteria that are essential for promoting normal host physiology. While the majority of commensal bacteria are found in the intestinal lumen, many species have also adapted to colonize different anatomical locations in the intestine including the surface of intestinal epithelial cells (IECs) and the interior of gut-associated lymphoid tissues. Distinct tissue localization of commensal bacteria permits unique interactions between these microbes and the mammalian immune system and collectively influences intestinal immune cell homeostasis. Conversely, dysregulated localization of commensal bacteria can lead to inappropriate activation of the immune system and is associated with numerous chronic infectious, inflammatory and metabolic diseases. Therefore, regulatory mechanisms that control proper anatomical containment of commensal bacteria are essential to maintain tissue homeostasis and limit pathology. Although the roles of luminal and epithelial-associated commensal bacteria on immune cell homeostasis have been extensively studied, how lymphoid tissue-resident commensals (LRC) modulate host immune responses is not well understood. Data in Chapter 2 will describe interactions between a genetically related family of LRCs that can colonize dendritic cells and induces lymphoid tissue-specific immune responses. Further, Chapter 3 will provide evidence that LRC-induced immune responses are mutually beneficial for itself and the host. Specifically, LRC-induced interleukin (IL)-22 enhances its own microbial colonization and LRC-induced IL-10 limits tissue inflammation during intestinal injury.

Dysregulated anatomical localization of commensal bacteria during chronic inflammatory conditions is typically associated with extra-intestinal complications including anemia or dysregulated iron homeostasis. Results in Chapter 4 support a role for the iron-regulatory hormone hepcidin in modulating LRC colonization of DCs and identify hepcidin as a critical regulator of immune cell homeostasis and tissue repair following intestinal injury. Collectively, studies presented in this thesis define mutually beneficial interactions between mammalian hosts and commensal bacteria in intestinal-associated lymphoid tissues that regulate immune cell homeostasis and host responses to tissue inflammation or injury.

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DEFINING INTERACTIONS BETWEEN LYMPHOID TISSUE-RESIDENT COMMENSAL BACTERIA AND THE HOST IMMUNE SYSTEM

Thomas Chia Ting Fung

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DEFINING INTERACTIONS BETWEEN LYMPHOID TISSUE-RESIDENT COMMENSAL BACTERIA AND THE HOST IMMUNE SYSTEM

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ABSTRACT

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Thomas Chia Ting Fung

Gregory F. Sonnenberg

David Artis

The mammalian gastro-intestinal tract is colonized by trillions of beneficial commensal bacteria that are essential for promoting normal host physiology. While the majority of commensal bacteria are found in the intestinal lumen, many species have also adapted to colonize different anatomical locations in the intestine including the surface of intestinal epithelial cells (IECs) and the interior of gut-associated lymphoid tissues. Distinct tissue localization of commensal bacteria permits unique interactions between these microbes and the mammalian immune system and collectively influences intestinal immune cell homeostasis. Conversely, dysregulated localization of commensal bacteria can lead to inappropriate activation of the immune system and is associated with numerous chronic infectious, inflammatory and metabolic diseases. Therefore, regulatory mechanisms that control proper anatomical containment of commensal bacteria
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Attributions

The contents of this thesis include portions of modified text and figures from the following published manuscripts:


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Chapter 1
Introduction

Trillions of commensal microorganisms collectively termed the microbiota colonize the mammalian gastrointestinal tract (GI). Most of these microbes belong to three bacteria phyla: Bacteroidetes, Firmicutes and Proteobacteria. Recent work has demonstrated a critical role for commensal bacteria in host nutrient metabolism, colonization resistance against invading pathogens and development and maturation of the immune system. Maintenance of proper host-commensal interactions is essential for host health as dysregulated interactions are often associated with and contribute to chronic inflammatory diseases. Despite recent findings describing the molecular and cellular mechanisms that promote healthy host-commensal bacteria interactions, our understanding of 1) the anatomical localization of commensal bacteria in health and disease, 2) the role of distinct commensal bacteria communities on immune cell homeostasis and 3) the functional consequences of commensal bacteria colonization for the host, is incomplete and requires further investigation.

Most commensal bacteria in mammalian hosts are found in the lumen of the GI tract and are physically separated from the intestinal epithelium. However, some bacteria are able to bypass this physical barrier to colonize the surface of intestinal epithelial cells (IECs). More recently, commensal bacteria signatures were detected in the interior of intestinal-associated lymphoid tissues such as
Peyer’s patches and the mesenteric lymph node, suggesting that commensals colonize these immune cell-rich sites previously thought to be devoid of bacteria. Based on these findings, commensal bacteria can be classified by their anatomical localization as i) luminal, ii) epithelial-associated or iii) lymphoid tissue-resident. Most studies in the past decade have focused on how the host pathways recognize and interact with luminal and epithelial-associated commensal bacteria. However, much less is known about how lymphoid tissue-resident commensal bacteria (LRC) colonize and interact with the host. The aim of this thesis is to comprehensively investigate and define interactions between LRCs and the host immune system. This Chapter will introduce our current understanding of intestinal commensal bacteria anatomical localization in the context of a healthy mammalian GI tract and during chronic inflammation. The end of this Chapter will also introduce the role of iron homeostasis in host-microbe interactions and immune cell function. **Chapter 2** will highlight the cellular mechanisms by which LRCs gain access to and colonize intestinal-associated lymphoid tissues and characterize the immunological consequences of LRC colonization. Further, **Chapter 3** will investigate the role of LRC-induced immune pathways on commensal bacteria colonization and tissue protection during intestinal injury. Finally, **Chapter 4** will explore the role of iron homeostasis and the iron-regulatory hormone hepcidin in LRC colonization and immune cell homeostasis.
1.1 Anatomical localization of intestinal commensal bacteria in the healthy mammalian GI tract

The mammalian GI tract is colonized by trillions of beneficial microbes including bacteria, fungi and viruses, which collectively influence host intestinal physiology. Levels of commensal bacteria in the GI tract have been reported to be as high as $10^{14}$ organisms, with over 1000 different bacterial species represented (Backhed et al., 2005; Ley et al., 2008; Ley et al., 2006; Turnbaugh et al., 2007; Xu et al., 2007). Some of the most well characterized roles of commensal bacteria include promoting efficient host nutrient absorption and protection from pathogen colonization (Brestoff and Artis, 2013; Buffie and Pamer, 2013; Cani, 2014; Flint et al., 2012; Kamada et al., 2013a; Musso et al., 2011). In addition, an expanding body of literature has identified critical roles for commensal bacteria in the development of the host immune system and maintenance of immune cell homeostasis (Hooper et al., 2012; Ivanov and Honda, 2012).

In contrast to the ability of commensal bacteria to confer host-beneficial properties, dysregulated interactions between commensal bacteria and the host are associated with many chronic infectious, inflammatory and metabolic diseases such as inflammatory bowel disease (IBD), chronic viral infection, obesity, cancer and cardiovascular disease (Hooper et al., 2012; Kamada et al., 2013b). Progression of disease in these settings has been proposed to occur either by changes in the composition of the microbiota known as dysbiosis and/or bacterial translocation (Blumberg and Powrie, 2012; Brenchley and Douek, 2012;
Brenchley et al., 2006; Henao-Mejia et al., 2012; Hooper et al., 2012; Kamada et al., 2013b; Loh and Blaut, 2012; Manichanh et al., 2012; Wang et al., 2011). Although many studies have identified and characterized dysbiosis during disease, much less is known about the role of commensal bacteria localization in disease development and pathogenesis.

The majority of our current knowledge on the role of the microbiota in health and disease is derived from studies describing luminal commensal bacteria. However, emerging studies suggest that, in the steady state, specific commensal populations colonize distinct compartments of the intestine and selectively alter immune cell homeostasis to provide host tissue protection. For example, the Firmicutes phylum member *Clostridium* spp. was demonstrated to colonize the lumen of the colon while the Bacteroidetes phylum member *Bacteroides fragilis* was shown to colonize both the lumen and crypts of the colon (Atarashi et al., 2013; Atarashi et al., 2011; Lee et al., 2013; Round et al., 2011; Round and Mazmanian, 2010). In contrast, the gram-positive segmented filamentous bacteria (SFB) colonize the intestine by adhering tightly to epithelial cells of the terminal ileum in mice (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). These studies highlight that the anatomical localization of commensal bacteria in the intestine can be categorized into at least two groups: (i) luminal and (ii) epithelial-associated. More recently, additional studies have described gut-associated lymphoid tissues as a novel and unexpected site for commensal bacterial colonization in healthy mammals. These commensal species, herein
referred to as lymphoid tissue-resident commensal bacteria (LRC), were shown to colonize the interior of Peyer’s patches (PPs) of healthy mice, primates and humans (Kunisawa and Kiyono, 2012; Obata et al., 2010; Sato et al., 2013; Sonnenberg et al., 2012). Despite our growing understanding of host-commensal bacteria relationships, how these interactions are influenced by commensal bacteria colonization in different compartments of the intestine is not well understood. This Chapter will discuss how anatomically distinct commensal populations, including (i) luminal, (ii) epithelial-associated or (iii) lymphoid tissue-resident (Figure 1), are recognized by the immune system, influence immune cell function and are anatomically restricted via host and bacterial intrinsic mechanisms. Furthermore, this Chapter will review current literature on how dysregulated commensal bacteria localization in mice and humans may contribute to a variety of chronic inflammatory diseases.

1.1.1 Luminal commensal bacteria
Analyses of commensal bacteria composition in the mammalian GI tract have mainly focused on 16S rDNA sequencing of luminal contents from the intestine. In healthy mice, luminal commensal bacteria are largely represented by three phyla: Bacteroidetes, Firmicutes and Proteobacteria. Within these groups, approximately 70% are Bacteroidetes, 20% are Firmicutes and less than 2% are Proteobacteria (Eckburg et al., 2005; Hill et al., 2010; Ley et al., 2005; Ley et al., 2006). Consistent with the intestine being a hypoxic environment, most luminal
commensal bacteria, especially Bacteroidetes and Firmicutes members, are obligate anaerobes (Colgan and Taylor, 2010; Glover and Colgan, 2011). Furthermore, many of these bacteria are not culturable using current techniques and have only been identified through 16S rDNA sequencing (2012; Salzman et al., 2002). Using this technique to interrogate interactions between the host and luminal commensal bacteria, an increasing body of data has revealed that members of both Bacteroidetes and Firmicutes have profound roles in shaping the host immune system.

The Bacteroidetes phylum is comprised predominantly of gram-negative, rod-shaped, anaerobic bacteria. Within this phylum, the genus Bacteroides is highly represented along the mammalian GI tract and has been reported to reach as high as $10^{10} - 10^{11}$ organisms per gram of human feces (Wexler, 2007). Many members of the Bacteroides genus including \( B. \text{ thetaiotaomicron} \) and \( B. \text{ fragilis} \) are well adapted to thrive in the GI tract (Wexler, 2007) and have been described to aid in host metabolism by processing complex polysaccharides into smaller compounds for host absorption (Comstock, 2009). Studies interrogating the interactions between Bacteroides and the host immune system have focused primarily on one species, \( B. \text{ fragilis} \) (Figure 2). An identifying feature of this bacterium is the presence of a capsular polysaccharide complex composed of eight capsular polysaccharides, of which polysaccharide A (PSA) is the most highly expressed (Surana and Kasper, 2012). While PSA can elicit pro-inflammatory responses such as production of interleukin (IL)-12, tumor necrosis...
factor alpha (TNFα) and interferon gamma (IFNγ) through recognition by dendritic cells (DCs) (Surana and Kasper, 2012), it also has potent anti-inflammatory properties and can stimulate interleukin (IL)-10-producing CD4⁺ Foxp3⁺ Tregs in the colon (Round and Mazmanian, 2010). Surprisingly, this response was independent of antigen presenting cells (APCs) but dependent on T cell recognition of PSA by Toll-like receptor (TLR) 2 (Round et al., 2011). In mice, this immunomodulatory response was required for intestinal colonization by *B. fragilis* and was associated with protection from experimental colitis (Round and Mazmanian, 2010). *B. fragilis* could also produce glycosphingolipids that inhibit invariant natural killer T (iNKT) cell proliferation, likely by interfering with lipid loading on CD1d molecules (An et al., 2014). Functionally, modulation of iNKT cell homeostasis in the colon by *B. fragilis* glycosphingolipids protected mice from iNKT cell-mediated intestinal inflammation. In a separate report, it was demonstrated that *B. fragilis* could produce immunostimulatory glycolipids that bind CD1d and activate iNKT cells in mice and humans (Wieland Brown et al., 2013), suggesting a dual role for Bacteroides glycolipids in regulating immune cell homeostasis.

Patients with autism spectrum disorder (ASD) often present with GI symptoms (Adams et al., 2011; Buie et al., 2010; Coury et al., 2012; Kohane et al., 2012). In recent findings, *B. fragilis* was demonstrated to correct behavioral abnormalities and restore increased intestinal permeability and altered luminal commensal bacteria composition in a mouse model of ASD (Hsiao et al., 2013).
Notably, oral treatment with PSA-deficient *B. fragilis* or wild-type *B. thetaiotaomicron* but not *Enterococcus faecalis* also improved anxiety-like, repetitive and communicative behavior of mice with ASD, suggesting that host protection by Bacteroides is not specific to *B. fragilis*. Furthermore, it was demonstrated that oral treatment of ASD mice with *B. fragilis* could limit elevated levels of the neurotoxic, commensal bacteria-derived serum metabolite 4-ethylphenylsulfate. These findings suggest that Bacteroides play important roles in host physiology beyond the intestine by modulating the metabolic activity of other commensal bacteria.

Within the Firmicutes phylum, which is comprised of gram-positive, spore forming, obligate anaerobes, the most studied group of commensal bacteria in regard to modulation of immune cell function is the class Clostridia (Figure 2). Similar to *B. fragilis*, *Clostridium* spp. isolated from mouse and human feces can promote the differentiation and function of CD4⁺ Foxp3⁺ Tregs in the mouse colon and are sufficient to protect mice from experimental colitis (Atarashi et al., 2013; Atarashi et al., 2011). Colonization of germ-free mice, which have enlarged ceca, by chloroform-resistant *Clostridium* spp. resulted in significantly reduced cecum size due to *Clostridium*-dependent fermentation of dietary fiber and production of short-chain fatty acids (SCFAs) as metabolic by-products (Furusawa et al., 2013). Consistent with the hypothesis that gut metabolism and immune cell homeostasis are two related processes, optimal induction of colonic Treg responses by *Clostridium* spp. was dependent on metabolism of dietary fiber and
subsequent production of SCFAs (Arpaia et al., 2013; Atarashi et al., 2013; Furusawa et al., 2013; Smith et al., 2013b).

The cellular and molecular mechanisms by which SCFAs influence immune cell function have begun to be elucidated. SCFAs could directly promote Treg immune-suppressive function through Treg-intrinsic expression of the SCFA receptor, GPR43 (Smith et al., 2013b). Butyrate also promoted acetylation at the Foxp3 locus and the Foxp3 protein (Arpaia et al., 2013; Furusawa et al., 2013), which have been previously shown to enhance Foxp3 stability and function (van Loosdregt et al., 2010; Zhang et al., 2012). Alternatively, butyrate and propionate could function independently of Tregs by suppressing DC production of pro-inflammatory cytokines and augmenting their ability to induce peripheral Treg differentiation (Arpaia et al., 2013). Furthermore, the SCFA butyrate was shown to promote Tregs at extra-intestinal tissues such as the spleen and peripheral lymph nodes (Arpaia et al., 2013). More recently, it was demonstrated that sensing of SCFAs by the metabolite receptors GPR43 on non-hematopoietic cells promote activation of the NLRP3 inflammasome and is critical for SCFA-induced host protection from intestinal inflammation and injury (Macia et al., 2015). Collectively, bacteria from the genus Clostridium are prominent members of the luminal microbiota that induce tissue protective, immunoregulatory responses through production of microbial-derived metabolites.

Anatomical restriction of B. fragilis and Clostridium spp. to the lumen of the intestine is dependent on the presence of physical and biochemical barriers
that limit interactions of commensal bacteria with IECs and potential translocation to the underlying connective tissues of the intestinal lamina propria (Figure 2). Mucus, which is secreted by specialized epithelial cells known as goblet cells, forms a dense gel-like layer on the apical surface of the intestinal epithelium and is composed of mucin glycoproteins including the highly glycosylated mucin MUC2 (Johansson et al., 2011). In the colon, mucus is organized into an inner mucus layer, which is approximately 50 µm thick in mice and is firmly attached to the surface of epithelial cells, and an outer non-epithelial attached layer, which is approximately 100 µm thick in mice (Johansson et al., 2011). Luminal commensal bacteria in the colon are restricted to either the lumen or the outer mucus layer whereas the inner mucus is mostly devoid of commensal bacteria with the exception of some epithelial-associated species (Johansson et al., 2011), which will be discussed in the following section. Although *B. fragilis* colonizes the lumen, it could bind intestinal mucins and was found within the colon mucus layer of gnotobiotic mice dual-colonized with *E. coli* and *B. fragilis* (Huang et al., 2011). These data are supported by the finding that in the distal colon, members of Bacteroidales are found near the inner mucus layer while Firmicutes are found more evenly distributed throughout the lumen (Earle et al., 2015). In studies interrogating how the microbiota regulates mucus production and integrity, it was demonstrated that the colon inner mucus layer of germ-free mice was penetrable to bacteria-sized beads and that colonization of these mice with feces from conventional animals restored mucus integrity and anatomical
segregation of commensal bacteria from the epithelium at 6 weeks post-colonization (Johansson et al., 2015).

In the small intestine, the mucus layer, although much less defined than in the colon, is about 50 µm thick in mice and also promotes physical separation between most luminal commensal bacteria and the epithelium (Johansson et al., 2011). In mice deficient in the mucin glycoprotein MUC2, physical separation between commensal bacteria and the intestinal epithelium was lost resulting in the development of spontaneous colitis (Shan et al., 2013; Van der Sluis et al., 2006). It was described that MUC2 in the mucus layer of the small intestine also actively participated in promoting immune tolerance to luminal antigens by conditioning intestinal DCs (Shan et al., 2013). Recognition and uptake of MUC2 by DCs enhanced their expression of the immunoregulatory cytokines Il10, Tgfβ1 and limited their ability to induce T cell proliferation and production of the proinflammatory cytokine IFNγ (Shan et al., 2013). These data indicate that mucus secretion is an important mechanism that restricts dissemination of luminal commensal bacteria and supports immunological tolerance to luminal commensal antigens.

In addition to mucus, antimicrobial peptides (AMPs) are small molecular weight proteins secreted into the lumen of the intestine that limit colonization of commensal bacteria and promote physical separation between commensal bacteria and the intestinal epithelium. AMPs are produced by virtually all IECs but the expression of a subset of AMPs such as the C-type lectin regenerating
islet-derived protein 3γ (REGIIIγ) is enriched in specialized IECs such as Paneth cells in the small intestine (Hooper et al., 2012). AMPs are concentrated to the epithelial surface to limit apical attachment of commensal bacteria normally restricted to the lumen (Vaishnava et al., 2011). It was also demonstrated that production of REGIIIγ could be induced by TLR signaling, as IEC-intrinsic deletion of Myd88 resulted in loss of commensal-epithelium segregation (Vaishnava et al., 2011). Collectively, these data suggest that recognition of commensal bacteria by TLRs initiates regulatory pathways that promote anatomical restriction. Recently, it was shown that the REGIII family proteins recognize and target commensal bacteria by binding to surface carbohydrates and forming an oligomeric membrane-permeabilizing pore (Mukherjee et al., 2014). The gram-negative bacteria-derived component lipopolysaccharide (LPS) inhibited REGIIIα pore-forming activity, providing a mechanism by which REGIII family proteins are bactericidal to gram-positive but not gram-negative bacteria. Other AMPs such as lipocalin 2 and calprotectin (S100A8/9), which limit microbial access to micronutrients such as zinc, manganese and iron, are likely candidates that control the gram-negative commensal populations in the intestinal lumen (Behnsen et al., 2014; Corbin et al., 2008; Diaz-Ochoa et al., 2014; Kehl-Fie et al., 2011; Liu et al., 2012).

A third mechanism by which the host limits commensal attachment to the epithelium is through secretory IgA. IgA is the most abundant immunoglobulin produced in mammals and play a critical role in immune defense at the intestinal
barrier surface. Induction of IgA in the intestine occurs predominantly in the PPs and isolated lymphoid follicles (ILFs) of the small intestine and, similar to mucins and AMPs, is highly dependent on the microbiota (Cerutti and Rescigno, 2008; Macpherson et al., 2012; Macpherson et al., 2008). IgA secreted by plasma cells in these tissues is covalently bound to the polymeric immunoglobulin receptor (pIgR) and transported through IECs via intracellular vesicles. Once released into the lumen, IgA restricts commensal bacteria from associating with the epithelium by binding to the surface of bacteria and neutralizing their ability to trigger inflammation (Cerutti and Rescigno, 2008; Macpherson et al., 2012). Mice-deficient in pIgR displayed increased penetration of commensal bacteria to the mesenteric lymph node (mLN) and systemic antibody responses to commensal bacteria. IgA can be induced by both T-dependent and T-independent mechanisms. While high affinity IgA from T-dependent pathways is important for protection from mucosal pathogen colonization, low-affinity, polyreactive IgA from T-independent pathways is generally necessary for confining commensal bacteria to the intestinal lumen (Cerutti and Rescigno, 2008; Macpherson et al., 2008). In support of a role for IgA in anatomical containment of commensal bacteria to the intestinal lumen, it was shown that IgA selectively labels colitogenic bacteria in the intestine such as members of Prevotellaceae, Helicobacter and SFB and likely limits their ability to stimulate the host immune system (Bunker et al., 2015; Palm et al., 2014). Interactions between the luminal microbiota and IgA are likely reciprocal as varying IgA levels in the gut is in part a
function of microbiota composition (Moon et al., 2015). Taken together, mucus, AMPs and secretory IgA are three of the main host mechanisms that cooperatively serve as important regulators that restrict commensal bacteria to the lumen of the GI tract.

1.1.2 Epithelial-associated commensal bacteria

Although most commensal bacteria are restricted to the intestinal lumen, a small fraction can penetrate the mucus layer and colonize the surface of IECs. Sequencing of 16S rDNA on the surface of the intestinal epithelium has revealed the presence of epithelial-associated commensal bacteria that include members of the Bacteroidetes and Firmicutes phyla at levels of about 2-3 logs less than those observed for luminal bacteria (Hill et al., 2010; Vaishnava et al., 2011). The most notable example in mice is SFB, a member of the Firmicutes phylum, which colonizes and attaches to IECs of the terminal ileum (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Colonization of mice with SFB results in elevated Th17 cell responses in the small intestine lamina propria, which are associated with both intestinal tissue protection from mucosal pathogen infection and susceptibility to mouse models of autoimmune arthritis and multiple sclerosis (Lee et al., 2011; Wu et al., 2010). Recent studies addressing the mechanisms by which SFB induces Th17 cells demonstrated that a diverse array of cellular and molecular pathways are involved. Host-specific attachment to IECs by SFB was necessary for the generation of Th17 cell responses as they were induced in
mice only following colonization with mouse but not rat SFB (Atarashi et al., 2015). In additional studies, it was demonstrated that local induction of Th17 responses in the lamina propria was dependent on epithelial cell production of serum amyloid proteins 1 and 2 (SAA1/2) (Ivanov et al., 2009; Sano et al., 2015). SFB stimulated production of IL-22 by innate lymphoid cells (ILCs), which activates epithelial STAT3 to promote SAA1/2 (Sano et al., 2015). Therefore, SFB is an epithelial-associated commensal bacterium that elicits selective immune responses beneath the intestinal epithelium.

Direct recognition of SFB is likely mediated by lamina propria mononuclear phagocytes (MNPs) that actively sample bacteria in the intestinal lumen (Figure 3). Subsets of lamina propria MNPs that express the fractalkine receptor CX₃CR1 have been shown to sample antigens by extending transepithelial dendrites across the intestinal epithelium (Niess et al., 2005; Rescigno et al., 2001). Although these phagocytes are believed to be non-migratory and only stimulate local mucosal immune responses, one report suggests that they could carry commensal bacteria antigens to the mLN to stimulate both T cell and IgA responses (Diehl et al., 2013). Migration of CX₃CR1-expressing cells to the mLN and subsequent mucosal immune responses were regulated by MYD88-dependent recognition of commensal bacteria (Diehl et al., 2013). In contrast, it was recently demonstrated that CX₃CR1⁺ cells in the small intestine can acquire and transfer soluble antigens via the gap junction protein Connexin 43 to CD103⁺ DCs, which then migrate to
draining lymph nodes to induce antigen-specific T cell responses (Mazzini et al., 2014). In recent studies, however, it was recently shown that intestinal CX3CR1⁺ but not CD103⁺ mononuclear phagocytes are responsible for antigen-specific Th17 cell induction by SFB (Panea et al., 2015). Although further investigation is necessary to delineate the precise contribution of MNPs to recognition of epithelial-associated commensal bacteria, it can be concluded that generation of immune cell responses by these bacteria collectively requires lamina propria phagocytes that actively sample bacteria attached to the intestinal epithelium.

Similar to their functions in regulating luminal commensal bacteria, host mechanisms such as AMPs and IgA are critical regulators of epithelial-associated commensal bacteria. Consistent with the specificity of REGIIIγ for gram-positive bacteria, REGIIIγ downstream of MYD88 signaling was demonstrated to restrict expansion of SFB on the epithelial surface as Reg3g-deficient mice had increased epithelial colonization of SFB and another epithelial-associated bacterium Eubacterium rectale in the small intestine (Vaishnava et al., 2011). Increased commensal colonization at intestinal epithelial surfaces due to loss of REGIIIγ resulted in increased frequencies of lamina propria T helper 1 (Th1) but not Th17 cells. Since total levels of SFB were not altered in Reg3g-deficient mice, these data suggest that dysregulated epithelial colonization by SFB may skew CD4 T cells towards a Th1 cell response. A role for AMPs in restricting colonization of epithelial-associated commensal bacteria was also demonstrated in mice ectopically expressing the human α-defensin gene Defa5.
Analyses of the microbiota composition in the distal small intestine of these mice demonstrated that while the amount of total bacteria was similar to littermate controls, Defa5-transgenic (Defa5-Tg) mice had significantly reduced colonization of SFB. In support of a role for AMPs in regulating epithelial-associated bacterial species, cytokine responses that promote AMP expression, such as IL-22, were recently demonstrated to suppress SFB colonization (Qiu et al., 2013; Upadhyay et al., 2012). In addition, pathways and molecules associated with IL-22 production in the intestine, such as the aryl hydrocarbon receptor (Ahr) and RORγt+ ILC responses also direct commensal colonization of the intestinal epithelial surface (Qiu et al., 2013). Ahr-deficient mice, which have reduced RORγt+ ILCs, demonstrated expansion of SFB and increased small intestine Th17 cells (Qiu et al., 2013). Finally, IgA is also involved in restricting colonization by SFB as demonstrated by expansion of this commensal bacterium in the feces of Iga-deficient mice (Suzuki et al., 2004).

Collectively, these data highlight a critical role for these biochemical and cellular mechanisms in regulating colonization of SFB on the intestinal epithelial surface.

Commensal bacteria that are found in the lumen of the intestine can also be epithelial-associated. Electron microscopy (EM) and immunofluorescence revealed that B. fragilis could penetrate the inner mucus layer and colonize the crypts of the large intestine. This was demonstrated to be dependent on both host-derived and bacterial-intrinsic factors. Colonic crypt colonization by B. fragilis was dependent on induction of Tregs by PSA (Round et al., 2011).
follow-up study by the same group, it was shown that expression of commensal (ccf) genes by B. fragilis could promote colonization of the bacteria at colonic crypts (Lee et al., 2013). It was hypothesized that host-derived glycan structures could induce the expression of ccf genes, consistent with the dependence of Bacteroides spp. on host-derived carbohydrates for intestinal colonization and survival (Wexler, 2007). It has also been reported that B. fragilis, although an anaerobe, can tolerate and benefit from nanomolar concentrations of oxygen, which is higher at the epithelial surface relative to the lumen of the intestine (Baughn and Malamy, 2004). Additionally, although Clostridium spp. typically reside in the intestinal lumen, EM analysis of the colon of Clostridium spp. colonized mice also demonstrated their presence on the epithelial surface (Atarashi et al., 2013). In support of a role for Clostridium spp. in modulating epithelial cell responses, cecal extracts from Clostridium-colonized mice could induce primary IECs to produce the Treg-inducing cytokine TGF-β (Atarashi et al., 2013). Taken together, these data suggest that commensal bacteria-elicited immune responses and commensal bacteria-intrinsic factors cooperatively permit stable intestinal colonization by epithelial-associated commensal bacteria. Further analyses are required to determine whether the immune cell responses promoted by Bacteroides and Clostridia members are associated with their localization in either the lumen or the epithelial surface. Chapter 2 of this thesis will compare tissue-specific immune responses induced by epithelial-associated commensal bacteria versus LRCs.
1.1.3 Lymphoid tissue-resident commensal bacteria

Tissues beyond the intestinal epithelium are not typically thought of as sites for commensal bacteria colonization. Although pathogens can penetrate the intestinal epithelium, evade macrophage killing and in some cases thrive in intestinal tissues, commensal bacteria that breach the intestinal barrier are typically phagocytosed and cleared by lamina propria macrophages (Macpherson and Harris, 2004; Smith et al., 2011; Varol et al., 2010). However, recent data provided evidence that some commensal species could stably colonize the interior of gut-associated lymphoid tissues such as PPs and mLN of healthy mice and humans (Obata et al., 2010; Sonnenberg et al., 2012) (Figure 4). In a seminal study by Kiyono and colleagues, sequencing of 16S rDNA in the interior of intestinal lymphoid tissues revealed the presence of specific groups of commensal bacteria including *Alcaligenes* spp., *Ochrobactrum* spp., *Serratia* spp. and *Burkholderia* spp. *Alcaligenes* spp. represented the most abundant lymphoid tissue-resident commensal in the PPs. It was observed that 16S rDNA of some of these bacteria were detected in the PP DCs of healthy mice, suggesting the possibility of intracellular survival in DCs. These data support a hypothesis in which lymphoid tissue-resident commensal bacteria can preferentially access host lymphoid tissues and evade clearance by host immune cells. Indeed, *Alcaligenes* spp. can be transcytosed by specialized IECs known as M cells from the lumen of the small intestine to PPs (Sato et al., 2013).
It is interesting to note that all of the lymphoid tissue-resident species identified so far are also aerobic, environmental microbes, resistant to a broad spectrum of antibiotics and associated with opportunistic nosocomial infections, particularly in the respiratory tract (Amoureux et al., 2013; Chain et al., 2011; Davies and Rubin, 2007; Mahenthiralingam et al., 2008). Some of these species, such as *Burkholderia* spp. and *Serratia* spp. are well adapted to survive on inanimate surfaces and also in host cells (Fedrigo et al., 2011; Valvano et al., 2005). Whether these features are related to their unique colonization pattern in the intestine is not well understood and requires further investigation. However, analyses in *Alcaligenes*-colonized mice demonstrated that *Alcaligenes* spp. is sufficient to promote robust *Alcaligenes*-specific and non-*Alcaligenes*-specific IgA (Obata et al., 2010). Furthermore, B cell-deficient CBA/N *xid* and Iga-deficient mice had reduced numbers of *Alcaligenes* spp. in the PP. These data suggest that in contrast to luminal and epithelial-associated commensal bacteria, which are restricted by IgA, optimal colonization by *Alcaligenes* spp. is dependent on IgA-producing B cells (Obata et al., 2010). Consistent with the notion that mucosal DCs are restricted from migrating beyond the mLN to systemic lymphoid tissues (Macpherson and Uhr, 2004), *Alcaligenes*-specific IgG responses were not detected in the periphery of *Alcaligenes*-colonized mice (Obata et al., 2010).

Recently, anatomical containment of the lymphoid tissue-resident-commensal bacteria *Alcaligenes xylosoxidans* (also referred to as *Achromobacter xylosoxidans*) was demonstrated to be dependent on IL-22
produced by RORγt+ ILCs (Sonnenberg et al., 2012) (Figure 4). *Rag1*-deficient mice depleted of ILCs experienced low-grade systemic inflammation that was associated with dissemination of *Alcaligenes* spp. to the liver, spleen and blood (Sonnenberg et al., 2012). Administration of IL-22 or the AMP calprotectin to ILC-depleted *Rag1*-deficient mice partially restored anatomical containment of *Alcaligenes xylooxidans*, suggesting a role for IL-22-induced factors such as AMPs in restricting *Alcaligenes* to intestinal lymphoid tissues (Sonnenberg et al., 2012). More recently, it was revealed that secretory IgA from breast milk could restrict translocation of *Ochrobactrum anthropi* from the neonatal gut to the draining lymph node (Rogier et al., 2014), suggesting a role for IgA in anatomical containment of other lymphoid tissue-resident commensal bacteria. These data provide potential mechanisms for anatomical containment of LRCs and also highlight that dysregulated localization of normally beneficial commensal bacteria can promote pathological inflammation, a condition often associated with human chronic inflammatory diseases. This idea will be discussed further in the following section. However, despite these advances, lymphoid tissue-resident commensal bacteria are still the least understood class of bacteria that colonize the intestine. Studies in *Chapter 2 and 3* will interrogate the functional significance of LRC colonization in the context of immune cell homeostasis and intestinal injury.
1.2 Anatomical localization of intestinal commensal bacteria during chronic inflammatory diseases

Analyses of the composition and localization of the human and mouse microbiota have established that in some contexts, commensal bacteria can promote chronic inflammatory disease through dysbiosis and/or bacterial translocation (Hooper et al., 2012; Kamada et al., 2013b). Most studies thus far have focused on how changes in the composition of the luminal microbiota can influence the development of disease. For example, IBD and obesity were associated with a dysbiotic microbiota that was sufficient to transfer disease to healthy hosts following cohousing or fecal transfer (Garrett et al., 2007; Vijay-Kumar et al., 2010). The role of dysbiosis in other chronic diseases such as AIDS and cancer has been extensively reviewed elsewhere (Cho and Blaser, 2012; Elinav et al., 2013; Ivanov and Honda, 2012; Kaur et al., 2011; Manichanh et al., 2012; Pflughoeft and Versalovic, 2012; Schwabe and Jobin, 2013). Alternatively, prebiotic and probiotic administration of beneficial microbes have been reported to promote intestinal health and limit pathologic inflammation (Bron et al., 2012; Lebeer et al., 2010). However, an emerging theme of the studies performed in the last decade is that dysregulated localization, not just dysbiosis, of normally beneficial commensal bacteria can be detrimental to the host and is often a major contributing factor to the development and pathogenesis of chronic inflammatory diseases. Chronic viral infections, IBD, metabolic syndromes and cancer have all been associated with dysregulated localization of commensal bacteria. The
following sections will highlight disease settings where anatomical restriction of luminal, epithelial-associated and lymphoid tissue-resident commensal bacteria is disrupted and may contribute to chronic inflammatory disease.

1.2.1 Chronic viral infections

Chronic viral infections are strongly associated with the loss of intestinal barrier function, commensal bacteria translocation to peripheral tissues and induction of systemic inflammation in humans (Ancuta et al., 2008; Balagopal et al., 2008; Brenchley et al., 2006; Jiang et al., 2009; Redd et al., 2009). A seminal finding by Douek and colleagues demonstrated that patients chronically infected with HIV, and those that have progressed to AIDS, exhibit elevated plasma LPS levels, suggesting that translocation of gram-negative bacteria components or whole gram-negative bacteria occurs in these diseases (Ancuta et al., 2008; Brenchley et al., 2006). Consistent with elevated plasma LPS, the peripheral blood of AIDS patients exhibited signs of systemic immune activation such as the presence of elevated serum IFNα, activated monocytes and activated circulating CD8\(^+\) T cells, hallmarks that contribute to viral replication, dissemination and disease progression (Ancuta et al., 2008; Brenchley et al., 2006; Marchetti et al., 2008). Interestingly, SIV-infected sooty mangabeys, which are natural hosts of SIV that do not develop AIDS, did not exhibit loss of intestinal barrier function, bacterial translocation or systemic inflammation (Estes et al., 2010). Bacterial translocation has been observed in other chronic viral infections such as HCV.
and HBV. Patients infected with HCV and HBV had elevated plasma LPS, which was associated with parameters of systemic immune activation such as elevated serum levels of the TLR4-co-receptor CD14 and IL-6 (Sandler et al., 2011). Taken together, these data suggest that loss of anatomical restriction of gram-negative commensal bacteria-derived products contribute to the pathogenesis of chronic viral infections.

Although it remains unclear whether whole commensal bacteria are found in the periphery of these patients or which commensal species promote disease progression, recent studies suggest that HIV and HCV-infected patients have elevated serum IgG responses to the lymphoid tissue-resident commensal bacteria Alcaligenes (Sonnenberg et al., 2012; Tatro et al., 2014). In support of a role for these bacteria in disease pathogenesis and systemic inflammation, levels of Alcaligenes/Achromobacter-specific IgG were associated with hallmarks of AIDS such as reduced CD4+ T cell counts and increased proportion of activated CD4+ T cells, and parameters of HCV progression including increased serum bilirubin and decreased serum albumin and platelets. Potential translocation of Achromobacter/Alcaligenes members in HIV-infected patients agrees with previous reports that Burkholderiales 16S rDNA, which includes the genus Achromobacter and Alcaligenes, was present in the peripheral blood of some patients (Merlini et al., 2011). Other groups of bacteria such as members of the orders Enterobacteriales, Lactobacillales and Pseudomonadales were also identified in the serum of these patients. Altogether, these data suggest that HIV
infection is strongly associated with dysregulated anatomical localization of various commensal bacterial communities.

Loss of cellular pathways that promote intestinal epithelial barrier function may also contribute to bacterial translocation observed in chronic HIV and HCV infection. RORγt+ ILCs, which are potent sources of IL-17 and IL-22, and the CD103+ immunoregulatory DCs were found to be dramatically reduced in SIV infection (Klatt et al., 2012; Li and Reeves, 2012; Xu et al., 2012). More recently, it was demonstrated that ILC3s are irreversibly depleted during acute HIV infection (Kloverpris et al., 2016; Zhang et al., 2015). These data are consistent with the role of RORγt+ ILCs in regulating the anatomical containment of SFB and LRCs (Qiu et al., 2013; Sonnenberg et al., 2012). Given that antiretroviral therapy is inadequate to completely control bacterial translocation (Merlini et al., 2011), further work is required to define the pathways that regulate intestinal barrier integrity and systemic inflammation in chronic viral infections. It is also important to highlight that patients with chronic viral infections often exhibit intestinal, metabolic and cardiovascular symptoms, which have all been associated with dysregulated localization of commensal bacteria (Gandhi et al., 2012; Keating et al., 1995; Samaras et al., 2007; Sheikh et al., 2008). Therefore, it is possible that bacterial translocation observed in HIV and HCV patients may be related to the onset of these diseases.
1.2.2 Inflammatory bowel disease

IBD, which includes Crohn’s disease (CD) and ulcerative colitis (UC), is strongly associated with dysregulated localization of commensal bacteria in humans. It is well documented that dysregulated host-commensal bacteria interactions, as indicated by parameters such as elevated serum LPS and bacterial DNA, play a major role in the pathogenesis of IBD by promoting inappropriate immune activation and chronic inflammation (Brenchley and Douek, 2012; Gardiner et al., 1995; Gutierrez et al., 2009; Lakatos et al., 2011; Obermeier et al., 2005). Changes in localization of commensal bacteria in IBD have been observed in the form of both bacterial translocation and increased bacterial colonization on the surface of the intestinal epithelium. Studies have implicated a role for increased colonization of the ileum by an adherent-invasive strain of *Escherichia coli* (AIEC). The predominance of AIEC epithelial colonization was demonstrated to contribute to chronic inflammation and immunopathology in human IBD (Darfeuille-Michaud et al., 2004; Lee et al., 2011; Small et al., 2013). To analyze the composition of disseminated commensal bacteria in gut-associated lymphoid tissues, one recent report identified that *Escherichia* and *Shigella* were enriched in the mLN of CD patients (O’Brien et al., 2014; Small et al., 2013). Similar findings were reported by a study that sequenced 16S rDNA from micro-dissected intestinal biopsies. Facultative anaerobes such as Enterobacteriaceae were enriched in micro-dissected ulcer biopsies of CD patients (De Hertogh et al., 2006). Collectively, these data suggest that increased epithelial and lymphoid
tissue colonization by commensal bacteria are hallmarks of and may contribute to the pathogenesis of IBD. To further investigate bacterial translocation to extra-intestinal sites in IBD, one study identified the presence of *Enterococcus faecalis*, a common member of the luminal flora, in the visceral fat of UC and CD patients (Zulian et al., 2013). In addition, it was recently demonstrated that pediatric CD patients exhibit increased serum IgG specific for *Alcaligenes* spp. (Sonnenberg et al., 2012), suggesting that impaired localization and systemic dissemination of LRCs is also associated with disease pathogenesis. Studies in Chapter 3 of this thesis will directly interrogate the role of LRC colonization in a mouse model of acute intestinal injury.

While it is unclear whether translocation of commensal bacteria is the cause or effect of IBD, several genetic polymorphisms in genes involved in host-microbial interactions are strongly associated with IBD (Jostins et al., 2012). Among these, the most prevalent are polymorphisms in *Atg16l1, Il23r, Nod2* and *Il10r* (Cho, 2008; Gutierrez et al., 2014). ATG16L1, a component of the autophagy machinery, is involved in intracellular degradation of bacteria (Cadwell et al., 2008). IL-23R is expressed on innate and adaptive immune cells that promote immunity to intestinal pathogens and maintain intestinal barrier function (Duerr et al., 2006). NOD2 is a pattern recognition receptor (PRR) that recognizes peptidoglycan from bacteria and triggers anti-bacterial immune responses (Cho and Abraham, 2007). IL-10 is a potent anti-inflammatory cytokine produced by innate and adaptive immune cells that maintain tissue
homeostasis by suppressing pro-inflammatory responses (Saraiva and O'Garra, 2010; Shah et al., 2012). Therefore, loss of function mutations in these genes may contribute to enhanced bacterial translocation and intestinal inflammation.

1.2.3 Metabolic diseases

Diabetes and obesity were two of the first extra-intestinal diseases to be associated with commensal bacteria-derived chronic inflammation (Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2006). In a mouse model of diet-induced obesity, mice fed a high-fat diet (HFD) for four weeks had increased concentrations of bacterial DNA and LPS in the blood, mesenteric adipose tissue and mLN as well as increased adherence of commensal bacteria to the intestinal epithelium (Amar et al., 2011; Cani et al., 2007). Analysis of translocated bacteria species in the mesenteric adipose tissue identified members of the family Enterobacteriaceae and Enterococcus spp. and Lactobacillus spp. (Amar et al., 2011), which are commensal bacteria commonly found in the lumen of the intestine. Translocation of commensal bacteria was associated with a loss of intestinal barrier integrity (Amar et al., 2011), increased levels of IL-1, IL-6 and TNFα in adipose depots and the onset of diabetes. Many of these parameters were markedly reduced in HFD-fed mice lacking the intracellular PRR Nod1 (Amar et al., 2011; Shi et al., 2006), TLR4 (Shi et al., 2006) or the TLR4-coreceptor CD14 (Amar et al., 2011; Cani et al., 2007), suggesting that recognition of translocated commensal bacteria can drive tissue inflammation and disease
pathology. In support of these findings, repeated subcutaneous injections of LPS to mice fed a normal chow (NC) was sufficient to recapitulate many of the diabetic symptoms observed in mice fed a HFD (Cani et al., 2007). Altogether, these findings indicate that impaired anatomical localization is a hallmark and significant contributor of diet-induced obesity.

Obesity is associated with significant changes in the composition of the luminal microbiota. For example, mice deficient in TLR5 are highly susceptible to diet-induced obesity, which was associated with a dysbiotic microbiota that could confer disease to wild-type germ-free mice following fecal transfer (Vijay-Kumar et al., 2010). In contrast, mice deficient in lymphotoxin β signaling are resistant to diet-induced obesity, and cecal contents from HFD-fed lymphotoxin β receptor (Ltbr)-deficient mice were less efficient in promoting weight gain in wild-type germ-free recipients compared to cecal contents from HFD-fed wild-type mice (Upadhyay et al., 2012). Interestingly, normal chow diet (NCD)-fed Ltbr-deficient mice, which lacked IL-23 and IL-22, demonstrated increased colonization by SFB. Whereas SFB levels were reduced in HFD-fed wild-type, SFB levels in HFD-fed Ltbr-deficient mice remained high relative to wild-type controls. Furthermore, administration of IL-22 or IL-23-Ig restored HFD-induced changes in SFB and obesity in Ltbr-deficient mice. Although bacterial translocation was not analyzed in these studies, it is interesting to speculate that specific groups of commensal bacteria that promote or limit disease are more or less likely, respectively, to disseminate to adipose depots and subsequently trigger chronic
inflammation. Nevertheless, these data support the hypothesis that specific immune pathways and/or commensal bacteria communities can promote (e.g. LTβ signaling, Enterobacteriaceae) or limit (e.g. TLR5, SFB) the development of obesity.

Other metabolic diseases are also associated with dysbiosis and loss of normal localization of commensal bacteria in the intestine. For example, plasma LPS levels are significantly elevated in patients with chronic hepatitis, alcoholic and non-alcoholic liver disease and have been associated with severe liver dysfunction (Garcia-Alvarez et al., 2012; Lin et al., 1995; Wiest et al., 2014). In a mouse model of non-alcoholic fatty liver disease (NAFLD), it was demonstrated that a dysbiotic microbiota in inflammasome-deficient mice could promote hepatic steatosis and Tnf expression due to translocation of TLR4 and TLR9 agonists through the portal circulation to the liver (Henao-Mejia et al., 2012). Although translocation of intact bacteria was not detected in the portal vein or liver, these data suggest that translocation of commensal-derived products alone to extra-intestinal sites can promote immune activation and tissue pathology in this mouse model of liver disease. More recently, it was shown that emulsifiers commonly found in processed foods disrupt the mucus barrier on the surface of the intestinal epithelium and result in altered microbiota composition on the surface of the intestinal epithelium (Chassaing et al., 2015). These changes were associated with the development of metabolic disease, which could be recapitulated by transfer of the altered microbiota to germ-free mice. Collectively,
a disturbance of metabolic homeostasis is often associated with changes in anatomical localization of commensal bacteria.

1.2.4 Cardiovascular diseases

An increasing body of literature has revealed that the inflammatory and metabolic effects of the gut microbiota contribute to the development and pathogenesis of cardiovascular diseases (Libby, 2002, 2006). Several studies have identified changes in the composition of luminal commensal bacteria and investigated the mechanisms by which commensal-dependent metabolic pathways can impact disease. In one study, fecal microbiota analyses of healthy controls and atherosclerosis patients demonstrated a selective enrichment of the genus Collinsella and reduction in the genus Eubacterium and Roseburia in patients (Karlsson et al., 2012). By measuring the metabolic activity of the luminal microbiota, it was determined that the microbiota of healthy controls was enriched for genes involved in the synthesis of anti-inflammatory and anti-oxidant compounds whereas the microbiota of atherosclerosis patients was enriched for genes in the peptidoglycan biosynthesis pathway. These data suggest that changes in composition of luminal commensal bacteria can alter the inflammatory and metabolic status of the host to promote atherosclerosis. In addition, a separate study demonstrated that increased levels of the commensal-dependent dietary metabolite, trimethylamine-N-oxide (TMAO), were associated with an increased risk of adverse cardiovascular events (Tang et al., 2013). This
is supported by studies in which metabolism of the dietary metabolite L-carnitine, which is abundant in red meat, produces TMAO and accelerates onset of atherosclerosis (Koeth et al., 2013). Taken together, these findings establish a link between luminal commensal bacteria, metabolism and atherosclerosis. Despite these findings, much less is known about the role of disrupted anatomical localization of commensal bacteria in cardiovascular disease. However, one study sequenced 16S rDNA from atherosclerotic plaques of human patients and demonstrated the presence of bacteria that are normal inhabitants of the intestinal lumen including members of the Bacteroidaceae, Enterobacteriaceae and Lachnospiraceae families (Koren et al., 2011). These data suggest that dissemination of commensal bacteria or commensal bacteria-derived products to the circulation may be associated with the development and pathogenesis of atherosclerosis. However, additional studies are necessary to interrogate the origin, cause and contribution of commensal bacteria in atherosclerotic plaques.

1.2.5 Cancer

As previously discussed, translocation of commensal bacteria is commonly observed in chronic liver diseases and can contribute to liver fibrosis and cirrhosis (Garcia-Alvarez et al., 2012; Lin et al., 1995; Wiest et al., 2014). Hepatocellular carcinoma (HCC) is a leading cause of death in patients who develop fibrotic and cirrhotic livers due to chronic inflammation. In support of the
hypothesis that bacterial translocation promotes liver tumor development, \textit{Tlr4}-
deficient mice, germ-free mice and mice treated with oral antibiotics were protected from hepatocarcinogenesis in a mouse model of chronic liver injury (Dapito et al., 2012). This is consistent with a previous report demonstrating that LPS-induced liver fibrosis was reduced in \textit{Myd88}-deficient mice and that hepatic satellite cells is the dominant target by which TLR4 ligands promote liver fibrosis (Seki et al., 2007). Another cancer associated with dysregulated anatomical localization of commensal bacteria is colorectal cancer (CRC). Bacterial translocation has been observed in the mLN and serum of CRC patients (Chin et al., 2007; Klein et al., 2013; Lescut et al., 1990). These patients also exhibited cachexia and elevated serum pro-inflammatory cytokine levels and had a poor prognosis. However, studies on specific groups of commensal bacteria that disseminate in human colon cancer are limited. \textit{Fusobacterium nucleatum}, a rare member of the human intestinal microbiota, was demonstrated to be strongly associated with human colorectal carcinoma and was found to be enriched in human colonic adenomas (Kostic et al., 2013). In contrast to commensal bacteria such as the enterotoxigenic \textit{B. fragilis} and AIEC which promote colitis-associated cancer (Arthur et al., 2012; Wu et al., 2009), \textit{F. nucleatum} did not promote colitis in mice, but rather increased tumor multiplicity, recruited tumor-promoting myeloid cells and generated a pro-inflammatory environment that favored tumor development. Since \textit{F. nucleatum} was also enriched in the fecal microbiota of CRC patients, it is likely that increased luminal burden of \textit{F. nucleatum}
contributes to the accumulation of this bacterium in colonic adenomas. Response to chemotherapy and immunotherapy was recently demonstrated to be dependent on anatomical localization of the microbiota. In contrast to the role for *F. nucleatum* in promoting colon adenomas, cyclophosphamide chemotherapy commonly used for solid malignancies injures the small intestine, which results in bacterial translocation and activation of anti-tumor CD4 T cell responses (Viaud et al., 2013). In a separate study, treatment with the chemotherapeutic agent oxaliplatin activated the immune system that was dependent on microbiota priming of myeloid cells and reactive oxygen species production (Iida et al., 2013). More recently, two studies demonstrated that the efficacy of cancer immunotherapy via checkpoint inhibitors required the presence of a complete microbiota (Sivan et al., 2015; Vetizou et al., 2015). However, whether the chemotherapeutic agent oxaliplatin and cancer immunotherapies promote anti-tumor immunity by altering localization of commensal microbes is not known. Future investigations should determine how dysregulated commensal bacteria localization can promote or limit cancer development.

1.3 Iron homeostasis in health and disease

Iron is an essential nutrient for the survival of all living organisms. In humans, iron is absorbed from the diet through enterocytes in the duodenum, which is then redistributed to the rest of the body in the form of intracellular and extracellular iron stores. Because iron is a critical element for many human
biological processes, its uptake, storage and transport are tightly regulated by multiple molecular mechanisms. Central to this iron-regulatory network is the hormone hepcidin (Figure 5). Hepcidin is a 25-amino acid peptide that is expressed and secreted primarily by liver hepatocytes. It binds to and results in the degradation of the iron exporter ferroportin, a membrane-bound protein that is expressed on duodenal enterocytes and reticuloendothelial cells such as macrophages (Nemeth et al., 2004b; Zhang et al., 2011). As a result, hepcidin-ferroportin interactions inhibit iron uptake through the intestine and promote iron sequestration in macrophages (Figure 6). To maintain homeostatic levels of intracellular and extracellular iron, hepcidin expression is regulated by numerous factors including plasma iron levels, inflammatory signals and erythropoiesis (Kautz et al., 2014; Nemeth et al., 2004a). Consistent with a role for iron in the survival and proliferation of microorganisms including pathogens, multiple studies support the hypothesis that hepcidin-mediated iron regulation is a critical host pathway to limit pathogen infection (Cassat and Skaar, 2013; Drakesmith and Prentice, 2012; Ganz and Nemeth, 2015). However, whether hepcidin-mediated iron regulation controls host-commensal bacteria interactions and the immunity system is not well understood. The following sections will explore the role of hepcidin and iron homeostasis in the context of host-microbe interactions and immune cell function.
1.3.1 Role of iron homeostasis and hepcidin in host-pathogen interactions

During inflammation, hepcidin is upregulated and subsequent hepcidin-ferroportin interactions result in a reduction in plasma iron levels via inhibition of iron uptake and sequestration of iron within reticuloendothelial cells (Nemeth et al., 2004b). Since most microbial pathogens trigger inflammatory immune responses and require iron for survival, hepcidin-mediated hypoferraemia has been proposed to be a host-protective response to limit pathogen proliferation (Cassat and Skaar, 2013; Drakesmith and Prentice, 2012; Ganz and Nemeth, 2015). Indeed, hepcidin-deficient mice are susceptible to infections caused by Gram-negative bacteria such as *Vibrio vulnificus* and *Yersina enterocolitica* (Arezes et al., 2015; Quenee et al., 2012). Hepcidin-deficient mice treated with hepcidin agonists that induce hypoferraemia were rescued from infection-induced mortality, suggesting that hepcidin alone is sufficient to provide host protection (Arezes et al., 2015). Furthermore, sera from hepcidin-deficient mice supported *in vitro* growth of *V. vulnificus* to a greater extent than sera from wild-type mice, suggesting that the susceptibility of hepcidin-deficient mice to infection is due to elevated systemic iron. Collectively, these data support the hypothesis that hepcidin-mediated plasma iron reduction acts to limit the bioavailability of iron to invading extracellular pathogens.

Since some pathogens infect the host by colonizing the intracellular compartment of host cells such as macrophages, hepcidin-mediated sequestration of intracellular iron may actually promote infection by these
intracellular pathogens (Figure 6). Indeed, studies in mouse models revealed that macrophages from hepcidin-deficient mice are unable to support the growth of intracellular microbes such as *Mycobacteria tuberculosis* and *Salmonella enterica* serovar *enterica* Typhimurium (Nairz et al., 2009; Olakanmi et al., 2007). Therefore, although hepcidin may serve to restrict infection by extracellular pathogens, re-distribution of intracellular versus extracellular iron by hepcidin may promote the survival of pathogens that thrive within host cells. Collectively, these data suggest that dysregulated iron homeostasis is associated with altered susceptibility to infectious disease. However, more studies are necessary to define the role of hepcidin-mediated iron regulation in host protection from extracellular versus intracellular microbial pathogens. Chapter 4 of this thesis will investigate the role of hepcidin in the generation of anti-microbial immune responses by innate and adaptive immune cells.

### 1.3.2 Role of iron homeostasis in host-commensal bacteria interactions

The host GI tract is colonized with trillions of microbes that provide multiple beneficial functions. Similar to humans and microbial pathogens, many of these microbes depend on iron for their survival and persistence in the host. Therefore, the iron-regulatory hormone hepcidin could be a critical regulator of host-commensal bacteria relationships. In support of this hypothesis, a recent study reported that regulation of liver hepcidin expression during acute intestinal inflammation is dependent on the immune environment and composition of the
intestinal microbiota (Shanmugam et al., 2014). During DSS-induced intestinal injury, wild-type mice downregulated expression of liver hepcidin whereas Il10−/− mice, which are susceptible to intestinal inflammation, upregulated expression of liver hepcidin. Differential regulation of hepcidin in wild-type and Il10−/− mice was dependent on the intestinal microbiota as co-housing Il10−/− mice with wild-type mice abolished this effect. More recently, it was shown that commensal bacteria-induced IL-1β secreted by macrophages promotes hepcidin expression in hepatocytes (Lee et al., 2005; Shanmugam et al., 2015). Collectively, these data support the hypothesis that commensal bacteria communities modulate hepcidin expression.

_Lactobacillus_ is a common human commensal bacterium frequently used as probiotics. Unlike most organisms many _Lactobacillus_ species have little to no requirement for iron and are rapidly outcompeted by other commensal bacteria that thrive in the presence of elevated iron such as during dietary supplementation and intestinal bleeding (Bailey et al., 2011). To determine the effect of iron supplementation on microbiota composition, one study demonstrated that anemic African children who received iron-fortified biscuits had a high ratio of fecal enterobacteria to probiotic lactobacilli and bifidobacteria (Zimmermann et al., 2010). These changes were associated with an increase in calprotectin levels, a marker of intestinal inflammation. These data suggest that that treatment of anemia using iron replacement therapy may have adverse effects and result in a pathogenic and inflammatory gut microbiota. This is indeed
supported by the poor efficacy of iron supplementation in the management of anemia caused by inflammatory bowel diseases (Rogler and Vavricka, 2014; Stein and Dignass, 2013). More studies are necessary to determine 1) the role of iron homeostasis in regulating host-commensal relationships during a state of health and disorders associated with iron deficiency and 2) whether the iron regulatory hormone hepcidin influences iron-mediated changes to host-commensal interactions. **Chapter 4** of this thesis will explore the role of hepcidin on host-LRC interactions.

### 1.3.3 Role of iron homeostasis and hepcidin in immune cell function

Dysregulated iron homeostasis in animals has been associated with changes in innate immune function. In a mouse model of acute wounding, iron supplementation skewed macrophage activation to a pro-inflammatory M1 phenotype and impaired wound healing (Sindrilaru et al., 2011). Treatment of iron-supplemented animals with the iron chelator deferoxamine restored wound healing, suggesting that iron is necessary and sufficient to promote M1 macrophage polarization. During infection, *Hfe*<sup>−/−</sup> mice, which have impaired hepcidin expression and thus decreased intracellular iron stores, are highly susceptible to *Salmonella* and *Mycobacterium avium* infection (Gomes-Pereira et al., 2008; Wang et al., 2008). These data are in contrast to the previous reports that hepcidin-deficient macrophages are unable to support intracellular bacteria growth due to reduced intracellular iron levels (Nairz et al., 2009; Olakanmi et al.,
The authors hypothesized that susceptibility of $Hfe^{-/-}$ mice to intracellular bacterial infection may be due to an inability of knockout animals to mount an innate immune response to the pathogen, as $Hfe^{-/-}$ peritoneal macrophages expressed lower levels of the pro-inflammatory cytokines IL-6 and TNFα following LPS or Salmonella exposure (Wang et al., 2008). This defect was restored in wild-type macrophages treated with a cell-permeable iron chelator or overexpressing the iron exporter ferroportin, suggesting that reduced pro-inflammatory cytokine production is a result of low intracellular iron. Interestingly, iron is selectively involved in the microbial sensing through the TLR4 but not the TLR2 and TLR3 pathways (Wang et al., 2009). Altogether these data suggest that high intracellular iron levels enhance pro-inflammatory cytokine production by macrophages. Chapter 4 of this thesis will interrogate the role of hepcidin-mediated iron homeostasis on cytokine production by innate and adaptive immune cells.

Several studies in humans have demonstrated that impaired iron homeostasis results in dysregulated immune responses. In children with iron-deficient anemia, one study showed that serum IL-6 levels and the oxidative burst capacity of neutrophils were significantly reduced compared to healthy controls (Ekiz et al., 2005). In contrast, a separated study demonstrated that monocytes in patients supplemented with iron produced less IL-6 in response to IFNγ (Sonnweber et al., 2011). Although these results suggest that iron is a critical regulator of innate immune cell function, it is likely that iron may have both
direct effects on immune cell function and indirect effects potentially through modulation of the endogenous microbiota. More studies are necessary to dissect the interplay between iron homeostasis, the immune system and the gut microbiota.

1.3.4 Regulation of hepcidin expression

Hepcidin expression is regulated at the level of transcription by three main cell-extrinsic factors: inflammatory stimuli, erythropoiesis and plasma iron levels (Figure 5). Cytokines that activate the STAT3 pathway including IL-6 and IL-22 are strong inducers of hepcidin production (Armitage et al., 2011; Nemeth et al., 2004a; Smith et al., 2013a). Regulation of hepcidin expression by inflammation is consistent with the fact that hepcidin is strongly upregulated in the context of infection and tissue inflammation. While inflammatory signals upregulate hepcidin, erythropoietic signals such as erythroferrone downregulate hepcidin to increase iron availability for erythropoiesis (Kautz et al., 2014). In order to maintain homeostatic levels of systemic iron, hepcidin expression is tightly controlled by plasma iron levels. High plasma iron promotes hepcidin expression to halt iron absorption through the intestine and inhibit its release from intracellular stores (Ganz and Nemeth, 2015).
1.4 Outline of thesis

Commensal bacteria colonize distinct sites along the mammalian GI tract. While most commensal bacteria are found in the lumen or on the surface of the intestinal epithelium, recent studies report the presence of commensal bacterial signatures in the interior of intestinal-associated lymphoid tissues. The focus of this thesis is to examine interactions between LRCs and the host immune system. **Chapter 2** will explore the cellular mechanisms by which LRCs colonize intestinal-associated lymphoid tissues and the immunological consequences of LRC colonization. **Chapter 3** will investigate the role of LRC-induced immune responses in the context of LRC colonization and intestinal injury. Finally, **Chapter 4** will define the role of iron homeostasis and the iron regulatory hormone hepcidin on LRC colonization and immune cell homeostasis.
**Figure 1.** Anatomical localization of commensal bacteria in the mammalian gastrointestinal tract.

Commensal bacteria in the mammalian GI tract can be classified by their anatomical localization as (i) luminal, (ii) epithelial-associated or (iii) lymphoid tissue-resident. Commensal bacteria are important for promoting normal host physiology. In the healthy mammals, most commensal bacteria occupy the lumen of the intestine while some are found associated with the intestinal epithelium. Recent studies have identified that commensal bacteria can also inhabit gut-associated lymphoid tissues in the steady state.
Figure 2. Interactions between the host immune system and luminal commensal bacteria.

Luminal commensal bacteria comprise of the majority of commensal bacteria in the mammalian intestine and are physically separated from immune cells. Colonization of the GI tract by luminal commensal bacteria promotes tissue protective and inhibits pathological immune responses. Anatomical containment of luminal commensal populations is mediated by physical and biochemical barriers such as IECs, tight junctions, mucus, antimicrobial peptides (AMPs) and secretory immunoglobulin A.

Figure 2. Luminal commensal bacteria comprise of the majority of commensal bacteria in the mammalian intestine and are physically separated from immune cells. Colonization of the GI tract by luminal commensal bacteria promotes tissue protective and inhibits pathological immune responses. Anatomical containment of luminal commensal populations is mediated by physical and biochemical barriers such as IECs, tight junctions, mucus, antimicrobial peptides (AMPs) and secretory immunoglobulin A.
Figure 3. Interactions between the host immune system and intestinal epithelial-associated commensal bacteria.

Figure 3. Colonization of the mammalian GI tract by epithelial-associated commensal bacteria promotes pro-inflammatory immune responses and is regulated by distinct host mechanisms. SFB is a model epithelial-associated commensal bacterium that colonizes the small intestine by adhering to IECs. SFB can promote pro-inflammatory immune responses that are associated with both pathogen immunity and autoimmunity. Some members of the luminal commensal flora such as *B. fragilis* and Clostridia can also colonize the epithelial surface of the colon and may interact with IECs.
Figure 4. Interactions between the host immune system and lymphoid tissue-resident commensal bacteria.

Figure 4. Lymphoid-tissue resident commensal bacteria can colonize gut-associated lymphoid tissues such as Peyer's patches, isolated lymphoid follicles and mesenteric lymph nodes. Lymphoid tissue-resident commensal bacteria share many properties: they are typically aerobic, environmental microbes that are broadly resistant to antibiotics. One member of this group, *Alcaligenes* spp., is a potent inducer of intestinal IgA responses. Anatomical containment of *Alcaligenes* spp. is mediated by IL-22-producing RORγt+ innate lymphoid cells. Loss of this pathway results in dissemination of *Alcaligenes* spp., systemic inflammation and tissue pathology.
Figure 5. Regulation of iron homeostasis by the hormone hepcidin.

Hepcidin is a iron-regulatory hormone that is expressed primarily by hepatocytes. Hepcidin interacts with the membrane-bound iron exporter, ferroportin (FPN) expressed on duodenal enterocytes, splenic macrophages and hepatocytes and results in its degradation. Hepcidin-ferroportin interactions therefore regulate plasma iron levels by inhibiting dietary iron absorption and iron release by splenic macrophages and hepatocytes. Expression of hepcidin is upregulated by plasma iron and inflammatory cytokines and downregulated by erythropoiesis. Figure modified from Ganz and Nemeth 2012 Biochimica et Biophysica Acta.
Figure 6. Role of iron homeostasis in host-pathogen interactions.

Iron is an essential nutrient for both mammals and microbial pathogens. Regulation of systemic iron levels by hepcidin differentially influences the survival and growth of extracellular and intracellular pathogens. In the absence of hepcidin expression where iron export by ferroportin (FPN) is uninhibited, increase in extracellular iron levels favors the growth of extracellular pathogens but may inhibit infection by intracellular pathogens. In the presence of hepcidin expression where iron export by ferroportin is inhibited, an increase in intracellular iron levels now favors growth of intracellular pathogens but inhibits infection by extracellular pathogens.
Chapter 2
Lymphoid tissue-resident commensal bacteria colonize DCs and promote tissue-specific immune cell responses

2.1 Abstract
Dysregulated immune responses against commensal bacteria are associated with the pathogenesis of chronic inflammatory diseases, and can be prevented by maintaining physical separation between commensal bacteria and intestinal immune cells. However, recent reports demonstrate that selective species of commensal bacteria can reside within intestinal-associated lymphoid tissues of healthy mammals. How this unique colonization occurs and functionally influences the immune system is unclear. This Chapter will demonstrate that lymphoid tissue-resident commensal bacteria (LRC) can colonize and persist in murine dendritic cells and modulate their cytokine production. In germ-free and antibiotic-treated (ABX) mice, LRCs colonize intestinal-associated lymphoid tissues and induce multiple members of the IL-10 cytokine family, including dendritic cell-derived IL-10 and group 3 innate lymphoid cell (ILC3)-derived IL-22. Unlike SFB, which colonize the epithelial surface of the small intestine and promote lamina propria Th17 cell responses, LRCs selectively promote Th17 cell responses in the Peyer’s patch and mesenteric lymph nodes but not in the small intestine lamina propria. Collectively, the data in this Chapter reveal a novel host-commensal bacteria dialogue whereby LRCs colonize intestinal-associated...
lymphoid tissues and interact with dendritic cells to promote tissue-specific immune responses.

2.2 Introduction

Trillions of microorganisms constitutively colonize the mammalian gastrointestinal (GI) tract and are essential to establish and maintain a state of health (Belkaid and Hand, 2014; Turnbaugh et al., 2007). These microorganisms include commensal bacteria that aid in nutrient metabolism and resistance to pathogen infection (Brestoff and Artis, 2013; Buffie and Pamer, 2013; Flint et al., 2012), as well as the development and maturation of the immune system (Belkaid and Hand, 2014; Honda and Littman, 2012; Hooper et al., 2012; Ivanov and Honda, 2012). In the healthy intestine, most commensal bacteria are restricted to the lumen of the GI tract or found associated with the surface of the intestinal epithelium and remain physically separated from the immune cells that populate the lamina propria and intestinal-associated lymphoid tissues (Chow et al., 2010; Chu and Mazmanian, 2013; Hooper et al., 2012; Hooper and Macpherson, 2010). Physical separation of commensal bacteria from immune cells is achieved by multiple physical and biochemical mechanisms that include intestinal epithelial cells, tight junction proteins, and secretion of anti-microbial peptides, mucus and immunoglobulin A (Chow et al., 2010; Chu and Mazmanian, 2013; Hooper et al., 2012; Hooper and Macpherson, 2010). Anatomical segregation of commensal bacteria from the immune system, often referred to as the “demilitarized zone”, is
essential to prevent pathologic immune responses against commensal bacteria (Hooper et al., 2012; Hooper and Macpherson, 2010). Consistent with this, translocation and dissemination of commensal bacteria across the intestinal epithelium lead to the generation of pro-inflammatory immune cell responses and are often associated with the pathogenesis of chronic infectious, inflammatory and metabolic diseases (Brenchley and Douek, 2012; Brenchley et al., 2006; Kamada et al., 2013; Loh and Blaut, 2012; Manichanh et al., 2012; Musso et al., 2011; Zhao, 2013).

Several recent studies suggest that a subset of commensal bacteria can colonize the interior of intestinal-associated lymphoid tissues of healthy mammals (Fung et al., 2014; Kunisawa and Kiyono, 2012; Obata et al., 2010; Sato et al., 2013; Sonnenberg et al., 2012). One report demonstrated by 16S rDNA sequencing and fluorescence in situ hybridization (FISH) that the interior of isolated lymphoid follicles (ILFs), Peyer’s patches (PPs) and mesenteric lymph nodes (mLN s) from healthy mice, non-human primates and humans contain multiple groups of bacteria (Obata et al., 2010). These bacteria include Alcaligenes spp., Achromobacter spp., Bordetella spp. and Ochrobactrum spp. 16S rDNA for many of these lymphoid tissue-resident commensal bacteria (LRCs) were found associated with CD11c⁺ dendritic cells (DCs), suggesting a role for DCs in lymphoid tissue colonization. In a subsequent study, it was demonstrated that IL-22 and group 3 innate lymphoid cells (ILC3) are important in preventing systemic dissemination of one LRC, Alcaligenes xylosoxidans, and
subsequent induction of systemic inflammation (Sonnenberg et al., 2012). These data highlight that intestinal-associated lymphoid tissues are potential sites for colonization by commensal bacteria and innate immune pathways maintain anatomical segregation between LRCs and the systemic immune system. Despite these findings, the mechanisms by which commensal bacteria colonize immune cell-rich lymphoid compartments associated with the intestinal tract of healthy mammals, and the functional significance of this colonization to the host, remain undefined. This Chapter will demonstrate that lymphoid tissue-resident commensal bacteria can colonize the intracellular compartment of murine DCs, modulate DC cytokine production in a viability-dependent manner and promote local Th17 cell and ILC3 responses in intestinal-associated lymphoid tissues in vivo.

2.3 Methods

2.3.1 Mice
C57BL/6 and Rag1−/− mice were purchased from Jackson Laboratories and used at 6-12 weeks of age. Conventional mice used as controls in gnotobiotic mice experiments were purchased from Jackson Laboratories and co-housed with mice bred in our conventional animal facility for at least 7 days before use. C57BL/6 and Rag1−/− mice used as controls for knockout mice were either bred in the same animal facility as the knockout mice, purchased from Jackson
Laboratories and co-housed with mice bred in our SPF animal facility for at least 7 days or littermates as indicated to control for microbiota differences. All conventional mice were maintained in specific pathogen-free facilities at the University of Pennsylvania or Weill Cornell Medical College. Germ-free and gnotobiotic C57BL/6 and Rag1−/− mice were maintained within sterile vinyl isolators at the University of Pennsylvania or Weill Cornell Medical College Gnotobiotic Mouse Facility and monitored for germ-free or gnotobiotic status by aerobic and anaerobic culturing. Additional microbiology testing was performed on feces from mice under experimentation and at the endpoint of the experiment to confirm germ-free or monoclonization status. Germ-free C57BL/6 mice were monocolonized with the mouse LRC isolate *Bordetella* spp., or SFB by oral gavage or cohousing with soiled bedding from previously monocolonized mice for at least 10 days. To establish LRC colonization in ABX-treated mice, conventional mice were treated in the drinking water with a limited ABX cocktail of ampicillin (1 mg/ml, Sigma) and gentamicin (1 mg/ml, Gemini Bio-Products) for C57BL/6 mice or ampicillin (1 mg/ml), gentamicin (1 mg/ml) and neomycin (0.25 mg/ml, Sigma) for *Rag1*−/− for 3 days and then orally gavaged with the clinical LRC isolate (LRCR), *Achromobacter*. Antibiotic cocktails were supplemented with 1 packet of artificial sweetener (Sweet’N Low) per 250 ml. All mice were used at least 10 days post LRCR colonization. All experiments were performed according to the guidelines of the University of Pennsylvania or Weill Cornell Medical College Institutional Animal Care and Use Committee.
2.3.2 Microbiology

The LRC strain, *Bordetella* spp., used to colonize BMDCs or monocolonize germ-free mice was originally isolated from the spleen of an anti-CD90.2 mAb treated conventional *Rag1*−/− mice using a previously defined protocol (Sonnenberg et al., 2012). *E. coli* (human isolate) and *Ochrobactrum* spp. used to colonize BMDCs and the ABX-resistant (ampicillin, gentamicin, neomycin) LRC (LRCR), *Achromobacter* used to colonize antibiotic-treated mice were human clinical isolates kindly provided by Kaede V. Sullivan (Children’s Hospital of Philadelphia). Bacterial identities were determined by genomic sequencing (Wellcome Trust Sanger Institute) and confirmed by genus-specific 16S rDNA primers. *E. coli* R9 (ATCC 35328), *Enterobacter cloacae* (ATCC 13047) and gentamicin-sensitive *Achromobacter* spp. (ATCC 31444) used to colonize BMDCs were purchased from American Type Culture Collection. Feces from SFB-monocolonized mice were obtained from Dr. Yoshinori Umesaki and Dr. Tatsuichiro Shima (Yakult). All bacterial strains used to colonize germ-free mice and for *in vitro* DC assays were grown in LB broth and incubated at 250 RPM, 37°C for 16-20 hours. For enumeration of tissue and fecal CFUs, the spleen, liver, feces, mesenteric lymph nodes (mLN) and Peyer’s patches (PP) were isolated, homogenized in sterile PBS and plated on Brain Heart Infusion (BHI) agar supplemented with 5% defibrinated horse blood. When culturing LRCR from tissues and feces, BHI blood agar plates were supplemented with 25 µg/ml ampicillin.
2.3.3 Bone marrow-derived and primary dendritic cell assays

Bone marrow-derived DCs were generated by culturing bone marrow cells from C57BL/6, Myd88\(^{−/−}\), Tlr2\(^{−/−}\) and Tlr4\(^{−/−}\) mice in the presence of 20 ng/mL GM-CSF for 8-10 days. Culture media was replaced with fresh media every 3 days. Frequencies of CD11c\(^{+}\) cells were ≥ 95%. To prepare primary splenic and mLN DCs, C57BL/6 mice were first injected subcutaneously in the right flank with 5 x 10\(^6\) cells GM-CSF-expressing B16 melanoma (kindly provided by Jedd D. Wolchok, MSKCC). 10 days later, CD11c\(^{+}\) cells were purified from Liberase TL (Roche)-digested spleen and mLN using CD11c positive selection beads (Miltenyi Biotec). Cell purities in the spleen were approximately 90% CD11c\(^{+}\) CD11b\(^{+}\) of non-T and B cells. For intracellular bacterial survival assays, BMDCs were seeded on 6-well plates at 5 x 10\(^6\) cells/well in antibiotic-free media and co-cultured with bacteria at an MOI of 50 for 2 hours, harvested and washed 3 times with sterile PBS. BMDCs were then plated in media containing gentamicin (100 µg/mL) at 200,000 cells/well on 48-well plates and lysed with sterile water every 48 hours to enumerate CFUs. For ELISA and qPCR, BMDCs were co-cultured with bacteria at an MOI of 50 for 2 hours and then gentamicin-treated to kill extracellular bacteria. Culture supernatants and cell lysates were harvested for ELISA and qPCR, respectively, 24 hours later. Heat killing was performed by incubating bacterial suspensions at 70°C for 60 min. For transmission electron microscopy, BMDCs colonized with *Bordetella* spp. for 5 days were harvested, processed for imaging by the University of Pennsylvania Electron Microscopy
Resource Laboratory and imaged using the Jeol-1010 transmission electron microscope. For microarray analysis, BMDCs were co-cultured with bacteria for 2 hours, washed extensively with PBS, incubated in media containing gentamicin (100 µg/mL) and lysed in TRIzol on day 4. All cell incubations were performed at 37°C and 5% CO₂.

2.3.4 Immunofluorescent detection of intracellular bacteria
BMDC-bacteria co-cultures were harvested on days 0, 2 and 5 and transferred to glass slides via cytospin. BMDCs were fixed with 4% PFA for 30 minutes at room temperature and stained with the Live/Dead BacLight Bacterial Viability Kit according to the manufacturer’s instructions (Invitrogen). Cells were mounted with VectaShield mounting medium containing DAPI (Vector Laboratories) and imaged on the Nikon Eclipse Ti Fluorescence Inverted Microscope. PFA fixation did not affect the ability of the bacterial viability kit to distinguish between live and dead bacteria.

2.3.5 Gene expression profiling and quantitative real-time PCR
RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. cDNA was generated using Superscript reverse transcriptase II (Invitrogen). Quantitative real-time PCR was performed using SYBR green chemistry (Invitrogen) and QuantiTect Primer Assays (Qiagen) on the ABI 7500 real-time PCR system (Applied Biosystems). Samples were
normalized to β-actin and displayed as fold change over PBS-treated BMDCs or antibiotic-treated mice. For microarray analysis, BMDCs colonized with the LRC, *Bordetella* spp., for 4 days were lysed directly in TRizol. RNA was isolated, amplified, reverse-transcribed to cDNA and hybridized to an Affymetrix GeneChip (Mouse Gene 1.0ST) by the University of Pennsylvania Molecular Profiling Facility. Relative expression data was normalized by Z score transformation.

2.3.6 Flow cytometry

mLN and PP were harvested, and single-cell suspensions were prepared at necropsy. For small intestine lamina propria preparations, intestines were isolated, attached fat and PPs were removed and tissues were cut open longitudinally. Luminal contents were removed by shaking in cold PBS. Epithelial cells and intra-epithelial lymphocytes were removed by shaking tissue in stripping buffer (1 mM EDTA, 1 mM DTT and 5% FCS) at 37°C for 30 minutes. The lamina propria layer was isolated by digesting the remaining tissue in 1 mg/mL collagenase/dispase (Roche) and 20 µg/mL DNase I (Sigma-Aldrich) for 30 minutes at 37°C. For flow cytometric analyses, cells were stained with antibodies against the following markers: anti-NK1.1 (clone PK136, eBioscience), anti-CD3 (clone 145-2C11, eBioscience), anti-CD5 (clone 53-7.3, eBioscience), anti-CD11c (clone N418, eBioscience), anti-CD4 (clone GK1.5, Abcam), anti-CD8α (clone 53-6.7, eBioscience), anti-B220 (clone RA3-6B2, eBioscience), anti-CD45 (clone 30-F11, eBioscience) and anti-CD11b (clone M1/70, eBioscience). For
intracellular staining of RORγt, cells were fixed and permeabilized with the Foxp3 intracellular staining buffer set (eBioscience) and stained with anti-RORγt (clone B2D, eBioscience). For intracellular cytokine staining, cells were stimulated ex vivo by incubation for 4 h with 50 ng/mL PMA, 750 ng/mL ionomycin, 10 µg/mL Brefeldin A (all obtained from Sigma-Aldrich) and 50 ng/mL rmIL-23 (eBioscience) and permeabilized as indicated above and stained with anti-IL-17A (clone eBioTC11-18H10.1, eBioscience), anti-IFN-γ (clone XMG1.2, eBioscience) and anti-IL22-02 (mouse cytokine detection antibody developed by Pfizer). Dead cells were excluded from analysis using the Live/Dead Aqua Viability Kit (Invitrogen). Flow cytometry data collection was performed on the LSR II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.).

2.3.7 Histology

Tissues from the small and large intestines were fixed with 4% PFA, embedded in paraffin, and 5 µm sections were cut and stained with H&E.

2.3.8 Bacterial genome sequencing, assembly, and annotation.

DNA was prepared and sequenced using the Illumina Hi-Seq platform with library fragment sizes of 200-300 bp and a read length of 100 bp at the Wellcome Trust Sanger Institute, as previously described (Harris et al., 2010). De novo assembly of 32 genomes was performed with Velvet v. 1.2.10 (Zerbino and Birney, 2008),
SSPACE v. 2.0 (Boetzer et al., 2011) and GapFiller v 1.1 (Boetzer and Pirovano, 2012) using an in-house pipeline developed at the Wellcome Trust Sanger Institute. *De novo* assemblies were annotated with Prokka v. 1.5-1 (Seemann, 2014) using an in-house pipeline developed at the Wellcome Trust Sanger Institute, which is available on GitHub (https://github.com/sanger-pathogens/Bio-AutomatedAnnotation).

### 2.3.9 Phylogenetic analysis

The fetchMG v. 1.0 (http://www.bork.embl.de/software/mOTU/fetchMG.html) was used to extract the protein information based on 40 universal single copy marker genes (Ciccarelli et al., 2006; Sorek et al., 2007) from each of the studied genome including 32 draft genomes and 15 reference genomes (Table S1). The protein sequences were then concatenated and aligned with MAFFT v. 7.205 (Katoh and Standley, 2013), and a maximum-likelihood tree was constructed using RAxML v. 7.8.6 (Stamatakis, 2006) with 100 bootstrap replicates under the Gamma + WAG model. The tree was visualized using SplitsTree v. 4.13.1 (Huson and Bryant, 2006).

### 2.3.10 Statistical analyses

Results represent mean ± SEM and statistical analyses were performed by unpaired student’s t-test, one- or two-way ANOVA with or without multiple comparisons tests as indicated in figure legends.
2.3.11 Data accessibility

Sequenced microbial genome data have been deposited in the European Nucleotide Archive under study number ERP012121. Array data have been deposited in GEO under accession number GSE76731.

2.4 Results

2.4.1 Lymphoid tissue-resident commensal bacteria are phylogenetically distinct from luminal commensal bacteria

16S rDNA for several groups of bacteria have been detected in the interior of PPs and the mLN of healthy mice (Obata et al., 2010; Sonnenberg et al., 2012). These LRCs, including *Achromobacter* spp., *Alcaligenes* spp., *Bordetella* spp. and *Ochrobactrum* spp., belong exclusively to the α- and β-proteobacteria groups, which are not highly represented in sequence analyses of the luminal contents of the intestine (Eckburg et al., 2005; Hill et al., 2010; Ley et al., 2005; Ley et al., 2006). We constructed a phylogenetic tree using in-house sequenced and publically available reference genomes of α-, β-, γ-proteobacteria and Bacteroidia members (Table 1). Phylogenetic analyses indicated that members of the α-proteobacteria and β-proteobacteria LRCs form two distinct clades from γ-proteobacteria and Bacteroidia members, which are typically found in the lumen of the intestine and not associated with intestinal lymphoid tissues in healthy mammals (Figure 7A).
2.4.2 Lymphoid tissue-resident commensal bacteria colonize the intracellular compartment of murine dendritic cells *in vitro*

16S rDNA for LRCs have been detected in CD11c+ cells from PPs and the mLN of healthy mice (Obata et al., 2010; Sonnenberg et al., 2012), suggesting LRCs may colonize DCs. To test this, bone marrow-derived DCs (BMDCs) were cocultured with intestinal bacteria predicted to be LRCs or representative intestinal microbes not predicted to be LRCs (non-LRCs), and the ability of each bacterium to survive in DCs was determined using a gentamicin protection assay. LRCs and non-LRCs were both internalized by DCs, but the numbers of viable non-LRCs declined rapidly within the first three days of culture, while viable LRCs persisted at high levels on days 2 and 4 (Figure 7B). LRCs but not non-LRCs were also able to colonize and persist in primary DCs isolated from the mLN and spleen (Figure 7C and 7D). Transmission electron microscopy (TEM) demonstrated intracellular colonization of DCs by LRCs as noted by the presence of dense bacterial clusters within intracellular vesicles (Figure 8A). To investigate the kinetics of intracellular survival of LRCs on a single-cell level, LRC and non-LRC-colonized BMDCs were analyzed using an immunofluorescence assay that distinguishes between live and dead bacteria. Both LRCs and non-LRCs were able to initially colonize BMDCs, however, the number of BMDCs containing viable bacteria was significantly greater for LRC-colonized BMDCs on days 2 and 5 (Figure 8B and 8C). Similar to the results using TEM, LRCs appear to form dense clusters in BMDCs beginning at day 2 post-colonization. However,
these clusters were not observed in non-LRC-colonized DCs (Figure 8B). Together, these data suggest that, in contrast to luminal-resident commensal bacteria, LRCs have the ability to efficiently colonize and persist in murine DCs.

2.4.3 Lymphoid tissue resident-commensal bacteria modulate dendritic cell cytokine production in a viability- and TLR-dependent manner

Innate immune cells such as DCs and macrophages respond to microbial stimuli by producing pro- and anti-inflammatory cytokines that shape subsequent adaptive immune cell responses. In the intestine, these responses are critical in regulating tissue homeostasis, immunity and inflammation (Bekiaris et al., 2014; Coombes and Powrie, 2008; Rescigno, 2010). Furthermore, DCs and macrophages can sense viability-associated molecular patterns in bacteria to induce distinct cytokine responses (Sander et al., 2011). Based on the ability of LRCs to colonize and persist in murine DCs, we sought to characterize DC cytokine induction following exposure to live versus heat-killed LRCs. To begin to test this, genome-wide transcriptional profiling was performed on DCs treated with PBS, live or heat-killed Bordetella spp., a model LRC isolated from our mouse colony, at 4 days post-exposure. Analysis of canonical DC-derived cytokines revealed that DCs colonized with live LRCs expressed greater levels of the pro- and anti-inflammatory cytokines Il6, Il23a, Il18, Il12a, Il12b, Il10 and Tgfb2 compared to PBS-treated DCs (Figure 9A). Notably, the induction of mRNA and protein for several of these cytokines, including IL-1β, IL-6, IL-10, IL-
12p40 and IL-23, by live LRCs was detected as early as 24 hours post co-culture (Figure 9B and 9C), and the induction of IL-10 and IL-23 mRNA (Figure 9A and 9B) and secretion of IL-1β, IL-10 and IL-23 protein (Figure 9C) did not occur in DCs exposed to only heat-killed LRCs, suggesting that viability-dependent factors expressed by LRCs induce selective DC cytokine responses. Primary splenic DCs exposed to live LRCs also produced IL-1β, IL-6, IL-10 and IL-12p40 (Figure 9D). Efficient induction of both viability- (IL-1β, IL-10, IL-23) and non-viability-dependent cytokines (IL-6, IL-12p40) required MYD88, suggesting a role for Toll-like receptors (TLRs) in recognition of LRCs (Figure 10A). Consistent with this, production of IL-1β and IL-23 was significantly reduced in Tlr4−/− but not Tlr2−/− BMDCs (Figure 10B). Collectively, these data indicate that LRCs can effectively colonize murine DCs in vitro and promote distinct pro- and anti-inflammatory cytokine responses by DCs in a viability- and TLR-dependent manner.

2.4.4 Lymphoid tissue-resident commensal bacteria colonize intestinal-associated lymphoid tissues in gnotobiotic mice

Most commensal bacteria found in the intestine are restricted to the lumen or the surface of the intestinal epithelium (Chow et al., 2010; Chu and Mazmanian, 2013; Hooper et al., 2012; Hooper and Macpherson, 2010). However, 16S rDNA for multiple groups of bacteria are enriched in the ILFs, PPs and mLN of healthy mice (Obata et al., 2010; Sonnenberg et al., 2012). To test whether these LRCs can stably colonize and survive in intestinal-associated lymphoid tissues, germ-
free mice were monocolonized with *Bordetella* spp., a model LRC isolated from our mouse colony. At day 10 post-inoculation, viable LRCs were consistently detected in the mLN (Figure 11A), PPs (Figure 11B) and fecal contents of the small intestinal lumen (Figure 11C), but not the spleen and liver. However, colonization by LRCs was not associated with macroscopic or microscopic inflammation in the intestine at 2 weeks post colonization (Figure 11D). These data support the hypothesis that LRCs can colonize and persist in intestinal-associated lymphoid tissues of healthy mammals.

2.4.5 Lymphoid tissue-resident commensal bacteria promote tissue-specific Th17 cell responses in gnotobiotic mice

Commensal bacteria modulate the innate and adaptive immune system in the GI tract (Belkaid and Hand, 2014; Honda and Littman, 2012; Hooper et al., 2012; Ivanov and Honda, 2012). Based on the ability of live LRCs to elicit IL-1β, IL-6, IL-23 and TGFβ, which are potent enhancers of Th17 cell responses (Korn et al., 2009), we hypothesized that LRCs may promote Th17 cell responses in colonized lymphoid tissues. To test this, we analyzed CD4+ T cells in the PP, mLN and small intestine lamina propria (SI LP) of conventionally-housed (CNV), germ-free, LRC-monocolonized mice and mice monocolonized with segmented filamentous bacteria (SFB), an epithelial-associated commensal bacterium which has previously been shown to induce robust Th17 cell responses in the SI LP (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Consistent with published
reports, germ-free mice lacked Th17 cells in the mLN (Figure 12A), PP (Figure 12B) and SI LP (Figure 12C) compared to CNV mice, and SFB was sufficient to promote robust Th17 cell responses in all three of these tissues. In contrast, LRCs promoted Th17 cell responses in the mLN and PP (Figure 12A, 12B and 12D) but failed to promote Th17 cell responses in the SI LP (Figure 10C and 10D), suggesting tissue-specific modulation of the immune system. Analysis of LRC-elicited Th17 cells in the PP revealed that a subset of these cells co-expressed the IL-10 family cytokine, IL-22 (Figure 13A and 13B). Accumulation of Th17 cells was selective as compared to Th1 cells, since LRCs did not significantly promote IFNγ-producing CD4+ T cell responses in the mLN, PP or SI LP (Figure 13C). Collectively, these data indicate that LRCs selectively promote Th17 cell responses at intestinal-associated lymphoid tissues.

2.4.6 Lymphoid tissue-resident commensal bacteria promote tissue-specific ILC3 responses in gnotobiotic mice

ILC3 also respond to the cytokines IL-1β, IL-6 and IL-23, express the transcription factor RORγt, and have important functions in lymphoid tissue organogenesis, immunity to pathogens and intestinal tissue homeostasis through production of effectors cytokines IL-17A and IL-22 (Artis and Spits, 2015; Diefenbach et al., 2014; McKenzie et al., 2014; Qiu and Zhou, 2013; Sonnenberg and Artis, 2012, 2015). Based on the ability of live LRCs to elicit DC-derived IL-1β, IL-6 and IL-23, we hypothesized that LRCs may promote ILC3 responses in
intestinal-associated lymphoid tissues. To test this, we measured ILC3 frequencies in the PP, mLN and SI LP of CNV, germ-free, SFB-monocolonized and LRC-monocolonized mice. Consistent with published reports, germ-free mice have reduced frequencies of ILC3 in the SI LP as compared to CNV mice (Klose et al., 2013; Satoh-Takayama et al., 2008). Monocolonization by SFB did not significantly alter ILC3 frequencies in the PP, mLN and SI LP (Figure 14A-D). In contrast, LRC-monocolonized mice revealed a significant increase in ILC3 frequencies in the PP and mLN (Figure 14A, 14B and 14D) but not in the SI LP as compared to germ-free mice (Figure 14C and 14D). Notably, ILC3s in the PP of LRC-monocolonized mice produced IL-22 (Figure 15A). However, frequencies and total numbers of IL-22+ ILC3 were comparable between germ-free and LRC-colonized mice (Figure 15B). Collectively, these data suggest that LRCs modulate tissue-specific ILC3 responses in the intestine.

2.4.7 Lymphoid tissue-resident commensal bacteria colonize intestinal-associated lymphoid tissues in antibiotic-treated mice

ILC3 and IL-22 responses induce anti-microbial proteins in the intestine (Artis and Spits, 2015; Diefenbach et al., 2014; McKenzie et al., 2014; Rutz et al., 2013; Sonnenberg and Artis, 2012, 2015; Sonnenberg et al., 2011), suggesting that LRCs may induce immune responses that limit its own colonization or the colonization of other bacteria. To investigate the role of LRC-elicited immune responses in LRC-host interactions, we established a mouse model of LRC...
colonization by treating conventional C57BL/6 mice with a limited cocktail of antibiotics (ABX) followed by inoculation with an antibiotic-resistant LRC, *Achromobacter* (LRC\textsuperscript{R}). We confirmed that, similar to the results using LRC-monocolonized mice, LRC\textsuperscript{R} could be cultured from the mLN and PPs of LRC\textsuperscript{R}-colonized C57BL/6 mice in which LRC\textsuperscript{R} was the dominant bacterial species in the intestinal lumen (Figure 16A and 16B). Furthermore, analyses of ILC3 responses in LRC\textsuperscript{R}-colonized C57BL/6 mice revealed a significant increase in IL-22\textsuperscript{+} ILC3 frequencies as compared to ABX-treated controls (Figure 16D). Collectively, these data suggest that LRCs can colonize intestinal-associated lymphoid tissues not only in gnotobiotic but also in antibiotic mice.

### 2.4.8 Lymphoid tissue-resident commensal bacteria promote innate immune cell responses in a tissue-specific manner in antibiotic-treated mice

Live LRC\textsuperscript{R} were also detected in the mLN of LRC\textsuperscript{R}-colonized *Rag1\textsuperscript{-/-}* mice (Figure 16C), suggesting that lymphoid tissue colonization does not require the adaptive immune system and allowing us to interrogate interactions between LRCs and the innate immune system. To investigate whether DC colonization and cytokine modulation by LRCs occur in the absence an adaptive immune system *in vivo*, we measured LRC\textsuperscript{R} 16S rDNA and cytokine gene expression in CD11c\textsuperscript{+} cells isolated from the mLN of LRC\textsuperscript{R}-colonized *Rag1\textsuperscript{-/-}* mice. LRC\textsuperscript{R} 16S rDNA was detected in CD11c\textsuperscript{+} cells from LRC\textsuperscript{R}-colonized but not in control ABX-treated
mice (Figure 16E), suggesting that in this model LRCs or LRC-derived nucleic acids are associated with murine DCs isolated from lymphoid tissues of healthy mice. Furthermore, CD11c+ cells from LRC\(^R\)-colonized mice expressed increased levels of \(I/1b\), \(I/l0\) and \(I/23a\) as compared to CD11c+ cells from control ABX-treated mice (Figure 17A). Induction of \(I/1b\) and \(I/23a\) was associated with an increase in \(I/17a\) and \(I/22\) expression in the whole mLN but not the small intestine of LRC\(^R\)-colonized \(Rag1^{−/−}\) mice (Figure 17B and 17C). Altogether, these data demonstrate that LRCs modulate tissue-specific DC and ILC3 responses in antibiotic-treated mice.

**2.5 Discussion**

The findings in this Chapter define a unique host-commensal bacteria dialogue whereby direct interactions between LRCs and the mammalian immune system orchestrate tissue-specific immune responses. While most studies interrogating host-microbiota interactions to date have focused on populations of commensal bacteria residing in the intestinal lumen or attached to the intestinal epithelium, here we examine an under-appreciated class of commensal bacteria that has the potential to reside closely associated with immune cells in the interior of intestine-associated lymphoid tissues. This colonization pattern is paradoxical to our current understanding of interactions between the immune system and commensal bacteria, which suggests that anatomical segregation must occur to limit the development of pathologic immune responses (Brenchley and Douek,
The data from this Chapter identifying the immunological role of commensal bacteria colonization in lymphoid tissues represent a key advance in our understanding of host-commensal bacteria interactions. Furthermore, these studies suggest that ongoing host-microbiota studies should consider not only the composition of luminal and epithelial commensals but also commensal bacteria communities that colonize organized lymphoid tissues associated with the intestine such as the mLN, PPs and ILFs.

Interrogating interactions between the host immune system and several species of LRCs revealed that LRCs but not luminal members of the microbiota could colonize and persist within murine DCs and modulate selective DC cytokine responses in a viability-dependent manner. Induction of IL-1β, IL-6, IL-10 and IL-23 by non-LRCs such as *E. coli* has been demonstrated by other groups (Hayashi et al., 2013; Manuzak et al., 2012; Sander et al., 2011), suggesting that activation of DCs does not account for differences in intracellular bacterial survival. Constitutive colonization of intestinal DCs by LRCs likely maintains expression of viability-dependent cytokines, including IL-1β, IL-10 and IL-23. This is in direct contrast to luminal-resident or epithelial-attached commensal bacteria in which viable bacteria belonging to these groups are not typically found beyond the intestinal epithelium (Hooper et al., 2012; Hooper and Macpherson, 2010; Obata et al., 2010). Therefore, these commensal bacteria predominantly influence immune cells through indirect interactions, for example
via production of metabolites or modulation of intestinal epithelial cell responses (Arpaia et al., 2013; Furusawa et al., 2013; Goto et al., 2014; Ivanov et al., 2009; Pham et al., 2014; Pickard et al., 2014; Smith et al., 2013). It is likely that LRCs may provide more direct and continuous stimulation to the immune system. Furthermore, the data in this Chapter suggest that induction of tissue-specific immune responses by commensal bacteria is linked to their anatomical localization in the GI tract and associated lymphoid tissues. Consistent with this, the epithelial-associated commensal SFB, induces potent SI LP Th17 cell responses that are dependent primarily on physical attachment to intestinal epithelial cells, production of epithelial cell-derived SAA1/2 and activation of the IL-23R-IL-22 axis. (Atarashi et al., 2015; Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Sano et al., 2015). Similarly, Bacteroides fragilis predominantly colonizes the colonic crypts in mice and promotes robust regulatory T cell responses in the underlying colon lamina propria (Lee et al., 2013; Round and Mazmanian, 2010). This Chapter demonstrates that LRCs colonize and activate DCs to selectively promote Th17 cell and ILC3 responses within intestinal-associated lymphoid tissues.

Collectively, the findings in Chapter 2 demonstrate that colonization of lymphoid tissues by commensal bacteria modulates the host immune system in a tissue-specific manner. Further interrogation of LRC colonization in health and disease will be critical to understand the complex microbial contexts where LRCs influence tissue immunity and inflammation. Understanding the mechanisms by
which LRCs modulate tissue-specific immune cell function may inform the development the therapeutics for the treatment of chronic inflammatory diseases.
Figure 7. Lymphoid tissue-resident commensal bacteria are phylogenetically distinct from luminal commensal bacteria and selectively colonize murine dendritic cells.

Figure 7. (A) Maximum-likelihood tree of 47 bacterial genomes based on 40 universal single copy marker genes. Red symbols indicate published reference genomes. Blue symbols and text indicate in-house sequenced genomes. Scale bar indicates number of substitutions per site. (B) Bone marrow-derived dendritic cells (BMDCs) were co-cultured with representative LRCs or luminal-resident commensals and bacterial survival was measured at 0, 48 and 96 hours. Data are representative of at least 2 independent experiments. (C) Primary DCs isolated from the mesenteric lymph node (mLN) and (D) spleen of C57BL/6 mice were co-cultured with the mouse-derived LRC, \textit{Bordetella} spp. or luminal commensal, \textit{E. coli} ATCC 35328, and bacterial survival was measured at 0, 48 and 96 hours. l.o.d., limit of detection. Data are representative of 2 independent experiments.
Figure 8. Lymphoid tissue-resident commensal bacteria colonize the intracellular compartment of bone marrow-derived dendritic cells.

(A) *Bordetella*-colonized BMDCs were analyzed at day 5 post co-culture by transmission electron microscopy. Images are representative of 10 individual bacteria-containing DCs. (B) *Bordetella*- and *E. coli*-colonized BMDCs were analyzed at day 0, 2 and 5 post-co-culture by immunofluorescence. Scale bar – 5 µm. Data are representative of 2 independent experiments. (C) Percentage of BMDCs containing live (green) bacteria was quantified across 7-16 distinct fields of view. Infection efficiency (BMDCs containing live or dead bacteria/total BMDCs) on day 0 was approximately 15% for *Bordetella* spp. and 30% for *E. coli*. Data are represented as mean ± SEM. Statistics were performed using unpaired, two-tailed, student’s t test. ***, p < 0.0001. ND, not detectable.
Figure 9. Lymphoid tissue-resident commensal bacteria modulate dendritic cell cytokine responses in a viability-dependent manner.

Figure 9. (A) Genome-wide transcriptional profiling was performed on BMDCs co-cultured with live or heat-killed *Bordetella* spp. for 4 days. Numbers in colored legend represent Z scores. (B) BMDCs co-cultured with live or heat-killed *Bordetella* spp. for 24 hours were analyzed for cytokine gene expression by qPCR. (C) BMDCs co-cultured with live or heat-killed *Bordetella* spp. for 24 hours were analyzed for cytokine protein secretion by ELISA. (D) Production of IL-1β, IL-6, IL-10 and IL-12p40 was measured in supernatants from *Bordetella*- or *E. coli*-spleen DC co-cultures at 96 h post-co-culture. qPCR and ELISA data are representative of at least 2 independent experiments. Data are represented as mean ± SEM. Statistics in B and C were performed using unpaired, two-tailed, student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.
Figure 10. Lymphoid tissue-resident commensal bacteria modulate dendritic cell cytokine production in a MYD88- and TLR-dependent manner.

Figure 10. (A) C57BL/6 and Myd88−/− BMDCs were treated with PBS or co-cultured with Bordetella spp. for 24 hours and production of IL-1β, IL-6, IL-10, IL-12p40 and IL-23 was measured in the culture supernatant by ELISA. (B) C57BL/6, Tlr2−/− and Tlr4−/− BMDCs were treated with PBS or co-cultured with Bordetella spp. for 24 hours and production of IL-1β and IL-23 was measured in the culture supernatant by ELISA. Data are representative of 2 independent experiments. Statistics were performed using two-way ANOVA with Sidak’s multiple comparisons test. ****, p < 0.0001.
Figure 11. Lymphoid tissue-resident commensal bacteria colonize intestinal-associated lymphoid tissues but do not cause pathological inflammation in gnotobiotic mice.

Figure 11. (A) Mesenteric lymph node (mLN), (B) Peyer’s patch (PP) homogenates and (C) small intestinal lumen contents from GF and *Bordetella* spp. (LRC)-monocolonized mice were cultured to determine bacterial CFUs. Data in (A) and (B) are representative of at least 2 independent experiments. (D) H&E staining of the small intestine and colon of conventional (CNV), germ-free (GF) and mice LRC-monocolonized mice.
Figure 12. Lymphoid tissue-resident commensal bacteria promote tissue-specific Th17 cell responses in gnotobiotic mice.

Figure 12. (A) mLN, (B) PP and (C) small intestine lamina propria (SI LP) of conventionally-housed (CNV), GF, SFB-monocolonized and LRC-monocolonized mice were analyzed for frequencies of IL-17A$^+$ and IFN$\gamma$^+ CD4$^+$ T cells by flow cytometry. (D) Frequencies of Th17 cells in CNV, GF, SFB-monocolonized and LRC-monocolonized mice. Values represent frequencies of IL-17A$^+$ cells among CD4$^+$ T cells. One-way ANOVA, mLN and SI LP - ****p < 0.0001; PP - **p < 0.01. Cells in all flow cytometry plots are gated as live, Lineage$^+$ and CD4$^+$. Lineage cocktail consists of CD3, CD5, CD8$\alpha$, CD11b and NK1.1. Data are represented as mean ± SEM. Data are pooled from 2 independent experiments for a total of 6-8 mice per group. Statistics in D were performed using one-way ANOVA with unpaired, two-tailed, student’s t test with no correction for multiple comparisons. *, p < 0.05; **, p < 0.01; ****, p < 0.0001.
Figure 13. Lymphoid tissue-resident commensal bacteria promote tissue-specific IL-22-producing Th17 cell responses in gnotobiotic mice.

Figure 13. (A and B) PPs of CNV, GF, SFB-monocolonized and LRC-monocolonized mice were analyzed for frequencies of IL-22-producing Th17 cells by flow cytometry. Values represent frequencies of IL-22^{+}IL-17A^{+}CD4^{+} T cells. One way ANOVA, mLN - ****p < 0.0001; PP - **p < 0.01; SI LP - *p < 0.05. Data are pooled from 2 independent experiments for a total of 6-8 mice per group. (C) Frequencies of Th1 (IFNγ^{+}of Lineage^{+}CD4^{+}) cells in the mLN, PP and SI LP of CNV, GF and LRC-monocolonized mice. Lineage cocktail consists of CD3, CD5, CD8α, CD11b and NK1.1. One-way ANOVA, mLN - **p < 0.01; PP - ****p < 0.0001; SI LP - ****p < 0.001. Data representative of 3 independent experiments. Statistics in panel B were performed using one-way ANOVA with unpaired, two-tailed, student’s t test with no correction for multiple comparisons. Statistics in panel C were performed using one-way ANOVA with uncorrected Fisher’s LSD test. *, p < 0.05; **, p < 0.01; p < 0.001; ****, p < 0.0001.
Figure 14. Lymphoid tissue-resident commensal bacteria promote tissue specific ILC3 responses in gnotobiotic mice

Figure 14. (A) mLN, (B) PP and (C) SI LP of CNV, GF, SFB-monocolonized and *Bordetella* (LRC)-monocolonized mice were analyzed for frequencies of ILC3 cells by flow cytometry. Cells in (A) were gated as live and lineage¹ (CD3⁻, CD5⁻, CD8α, CD11b⁻, B220⁻, NK1.1⁻). Cells in (B) and (C) were gated as live, CD45⁺ and lineage⁻. (D) Quantification of ILC3 frequencies in the mLN, PP and SI LP of CNV, GF, SFB-monocolonized and LRC-monocolonized mice. mLN ILC3 values represent frequencies of CD45⁺RORγt⁺ of lineage⁻ cells. PP and SI LP ILC3 values represent frequencies of CD90⁺RORγt⁺ of lineage⁻ cells. One-way ANOVA, mLN - ****p < 0.0001; PP - *p < 0.05; SI LP - **p < 0.01. Data are pooled from 2 independent experiments for a total of 6-8 mice per group. Data are represented as mean ± SEM. Statistics in panel D were performed using one-way ANOVA with uncorrected Fisher’s LSD test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 15. Lymphoid tissue-resident commensal bacteria do not elicit IL-22-producing ILC3 in gnotobiotic mice.

Figure 15. (A) PPs of CNV, GF, SFB-monocolonized and LRC-monocolonized mice were analyzed for frequencies of IL-22⁺ ILC3 cells by flow cytometry. Cells are gated as live, lineage⁻, CD90⁺ and RORγt⁺. Data are pooled from 2 independent experiments for a total of 6-8 mice per group. (B) Numbers of total or IL-22⁺ ILC3 in the mLN of CNV, GF, SFB-monocolonized and LRC-monocolonized mice. Data are representative of 2 independent experiments with 4-5 mice per group. Data are represented as mean ± SEM.
Figure 16. Lymphoid tissue-resident commensal bacteria colonize antibiotic-treated mice.

Figure 16. Bacterial CFUs were determined in antibiotic (ABX)-treated control and ABX-resistant *Achromobacter* (LRC<sup>R</sup>)-colonized C57BL/6 (A) PP and (B) mLN homogenates or (C) *Rag1<sup>−/−</sup>* mLN homogenates. Data are representative of at least 2 independent experiments. (D) PP of CNV, ABX-treated and ABX-resistant *Achromobacter* (LRC<sup>R</sup>)-colonized C57BL/6 mice were analyzed for frequencies of IL-22<sup>+</sup> ILC3. One-way ANOVA, *p* < 0.01. (E) LRC<sup>R</sup> 16S rDNA was measured by PCR in sorted CD11c<sup>+</sup> cells from ABX-treated control or LRC<sup>R</sup>-colonized *Rag1<sup>−/−</sup>* mLN. Data are representative of 2 independent experiments with 3 mice per group. Statistics in panel D were performed using one-way ANOVA with uncorrected Fisher’s LSD test. **, *p* < 0.01.
Figure 17. Lymphoid tissue resident-commensal bacteria promote tissue-specific innate immune cell responses in antibiotic-treated mice.

(A) Expression of Il1b, Il10 and Il23a was measured in sorted CD11c+ cells from ABX-treated control or LRCR-colonized Rag1−/− mLN. Data pooled from 2 independent experiments for a total of 6 mice per group. (B) mLNs and (C) small intestines of CNV, ABX-treated and LRCR-colonized were analyzed for expression of Il17a and Il22. Data pooled from 2 independent experiments for a total of 6-8 mice per group. One-way ANOVA, mLN Il17a – ****p < 0.0001; mLN Il22 – **p < 0.01; SI Il17a – ***p < 0.001; SI Il22 – **p < 0.01. Statistics in panel A were performed using unpaired, two-tailed, student's t-test. Statistics in panels B and C were performed using one-way ANOVA and uncorrected Fisher's LSD test. **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.
Table 1. List of publically available reference genomes used in this thesis.

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<td>NZ_ACQA01000001.1</td>
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<td><em>Rikenella microfusus</em> DSM 15922</td>
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Chapter 3

Lymphoid tissue-resident commensal bacteria-induced immune pathways are required for bacterial colonization and host tissue protection

3.1 Abstract

Trillions of commensal bacteria colonize the gastrointestinal tract of mammals. Although most commensal bacteria are found restricted to the luminal or epithelial surface of the intestine, some commensals, referred to as lymphoid tissue-resident commensal bacteria (LRC), can colonize the interior of intestinal-associated lymphoid tissues. Chapter 2 demonstrated that LRCs elicit members of the IL-10 cytokine family, including DC-derived IL-10 and ILC3-derived IL-22, within intestinal-associated lymphoid tissues but not the small intestine lamina propria. This Chapter will explore the functional consequences of these LRC-induced immune pathways. IL-22 production enhances LRC colonization by restricting the colonization of other commensal microbes. Furthermore, LRC-induced IL-10 and provides host protection from lethal intestinal damage by limiting intestinal tissue injury and systemic inflammation. Collectively, these data reveal a novel host-commensal bacteria dialogue whereby selective subsets of commensal bacteria interact with DCs to induce tissue-specific responses that are mutually beneficial for both the host and microbe.
3.2 Introduction

Commensal microorganisms, such as bacteria, fungi and viruses that colonize our gastrointestinal tract are essential for the maintenance of host health. Animals that lack commensal microbes or are depleted of commensals through the use of oral antibiotics are often highly susceptible to mouse models of infectious, inflammatory and metabolic diseases. Germ-free animals are unable to restrict colonization and infection by the bacterial pathogens *Citrobacter rodentium*, *Listeria monocytogenes*, *Salmonella* serovar *Typhimurium* (Inagaki et al., 1996; Kamada et al., 2012; Stecher et al., 2005). The mechanisms for enhanced susceptibility to bacterial colonization is largely dependent on lack of competition from the endogenous commensal microbiota, In the presence of a complex microbiota, which often utilize the same nutrients as pathogens, pathogenic bacteria are rapidly outcompeted. Collectively, the endogenous microbiota protects the host from invasive microbes by providing colonization resistance.

The endogenous microbiota stimulates the development of intestinal and systemic immune responses against invading pathogens. For example, germ-free mice colonized with the epithelial-associated commensal SFB generate Th17 cell responses that are protective against colonization by *Citrobacter rodentium* (Ivanov et al., 2009). On the other hand, the microbiota has also been shown to prime regulatory T cell responses, which are responsible for limiting pro-inflammatory and pathologic immune cell responses such as Th1 and Th17.
cells. For example, the luminal commensals *Bacteroides fragilis* and *Clostridium* spp. promote Treg responses that are protective in multiple mouse models of intestinal inflammation (Atarashi et al., 2011; Round and Mazmanian, 2010). On the other hand, generation of pro-inflammatory immune responses by commensal bacteria may be detrimental to the host. Indeed, SFB-colonized animals, which have elevated intestinal and systemic Th17 responses, are more susceptible to experimental autoimmune encephalitis and autoimmune arthritis (Lee et al., 2011; Wu et al., 2010).

In contrast to the hypothesis that commensal bacteria drive tissue inflammation in colitis, germ-free mice, antibiotic-treated mice and mice lacking the bacterial recognition adaptor protein MYD88 are all highly susceptible to DSS-induced colitis (Ayres et al., 2012; Kitajima et al., 2001; Rakoff-Nahoum et al., 2004). These data support the hypothesis that recognition of the endogenous microbiota and microbiota-dependent host physiological changes are protective in the context of intestinal inflammation. These data also suggest that a defined consortium of commensal bacteria offers intestinal tissue protection. Indeed, colonization of germ-free mice with *Bacteroides fragilis* or *Clostridium* spp. as previously discussed is host protective during colitis (Atarashi et al., 2011; Round and Mazmanian, 2010). However, other commensals, such as *Prevotella* and *Helicobacter*, do not provide host protection and instead drive disease development (Palm et al., 2014). Despite these findings, the functional significance of bacteria colonization by LRCs has not been explored.
Host biological pathways are critical for the maintenance of healthy interactions with commensal bacteria through modulation of intestinal barrier functions including production of anti-microbial peptides, mucus and IgA. One pathway that is critical for maintenance of intestinal barrier function is the production of IL-22. IL-22 is an IL-10 cytokine family member that stimulates the production of antimicrobial peptides and delivers pro-proliferative signals to cells necessary for host tissue homeostasis (Rutz et al., 2013; Sonnenberg et al., 2011). In support of a role for IL-22 in regulating the microbiota, \( \textit{Il22}^{\textit{-/-}} \) mice have a dysbiotic microbiota that is transmissible and colitogenic (Zenewicz et al., 2013). Mice that lack molecular factors upstream of the IL-22 pathway such as \( \textit{Rorc}^{\textit{-/-}} \) and \( \textit{Ahr}^{\textit{-/-}} \) mice also demonstrate expansion of the epithelial-associated commensal SFB (Qiu et al., 2013; Upadhyay et al., 2012). Furthermore, IL-22 has been shown to suppress the luminal commensal \textit{E. coli} in the context of \textit{Salmonella} infection. Collectively, it can be concluded that IL-22 is a critical cytokine that restricts luminal and epithelial-associated commensal bacteria colonization. Despite this, the role of IL-22 induced by LRCs is not known and will be explored in this Chapter.

\textbf{Chapter 2} demonstrated that LRCs can colonize the interior of DCs \textit{in vitro} and intestinal-associated lymphoid tissues \textit{in vivo} to elicit tissue-specific Th17 and IL-22 responses. This Chapter will demonstrate that, in contrast to previously identified roles for IL-22 in limiting intestinal colonization by commensal bacteria (Qiu et al., 2013; Rutz et al., 2013; Shih et al., 2014;
Upadhyay et al., 2012), IL-22 enhances LRC colonization of intestinal-associated lymphoid tissues. On the other hand, LRC-induced IL-10 limits pro-inflammatory responses in the steady state and could protect mice in a model of intestinal damage. These data collectively suggest that LRCs engage in a mutualistic dialogue with mammalian hosts by eliciting members of the IL-10 cytokine family.

3.3 Methods

3.3.1 Mice
C57BL/6 and Rag1−/− mice were purchased from Jackson Laboratories and used at 6-12 weeks of age. Conventional mice used as controls in gnotobiotic mice experiments were purchased from Jackson Laboratories and co-housed with mice bred in our conventional animal facility for at least 7 days before use. C57BL/6 and Rag1−/− mice used as controls for knockout mice were either bred in the same animal facility as the knockout mice, purchased from Jackson Laboratories and co-housed with mice bred in our SPF animal facility for at least 7 days or littermates as indicated to normalize for microbiota differences. All conventional mice were maintained in specific pathogen-free facilities at the University of Pennsylvania or Weill Cornell Medical College. Germ-free and gnotobiotic C57BL/6 and Rag1−/− mice were maintained within sterile vinyl isolators at the University of Pennsylvania or Weill Cornell Medical College Gnotobiotic Mouse Facility and monitored for germ-free or gnotobiotic status by
weekly aerobic and anaerobic culturing. Germ-free and gnotobiotic $\text{Il10}^{+/+}$ and $\text{Il10}^{-/-}$ mice were maintained at the National Gnotobiotic Rodent Resource Center (University of North Carolina, Chapel Hill). Additional microbiology testing was performed on feces from mice under experimentation and at the endpoint of the experiment to confirm germ free or monocolonization status. DSS experiments using gnotobiotic mice were performed in a biosafety cabinet, and the gnotobiotic status of mice was confirmed by microbiology testing. Germ-free C57BL/6 mice were monocolonized with the mouse LRC isolate *Bordetella* spp., or SFB by oral gavage or cohousing with soiled bedding from previously monocolonized mice for at least 10 days. To establish LRC colonization in ABX-treated mice, conventional mice were treated in the drinking water with a limited ABX cocktail of ampicillin (1 mg/ml, Sigma) and gentamicin (1 mg/ml, Gemini Bio-Products) for C57BL/6, $\text{Il22}^{-/-}$ and $\text{Il17a}^{-/-}$ mice or ampicillin (1 mg/ml), gentamicin (1 mg/ml) and neomycin (0.25 mg/ml, Sigma) for $\text{Rag1}^{-/-}$ and $\text{Rag1}^{-/-} \text{Il22}^{-/-}$ for 3 days and then orally gavaged with the clinical LRC isolate ($\text{LRC}^R$), *Achromobacter*. Neomycin (0.25 mg/ml), metronidazole (0.5 mg/ml, Sigma) and vancomycin (0.25 mg/ml, Chem-Impex International) were added to the limited ABX cocktail as indicated. Antibiotic cocktails were supplemented with 1 packet of artificial sweetener (Sweet’N Low) per 250 ml. For cytokine treatments *in vivo*, rmIL-22 or PBS control (kindly provided by Pfizer) was injected i.p. at 25 µg/mouse every 2 days for 1 week and control- or IL-22-Fc (kindly provided by Pfizer) was injected i.p. at 50 µg/mouse every 3 days for 1 week. First injections were administered 1 day
prior to LRC\textsuperscript{R} colonization. For quantification of Th17 cells in the absence of IL-10, either LRC-monocolonized \textit{Il10}\textsuperscript{-/-} mice, or LRC-monocolonized GF C57BL/6 mice treated with 500 µg/mouse anti-IL-10R (clone 1B1.3A, Bio X Cell) i.p. every 3 days for 1 week, was used. All mice were used at least 10 days post LRC\textsuperscript{R} colonization. All animals used are on a C57BL/6 background with the exception of \textit{Il10}\textsuperscript{-/-} and \textit{Il10}\textsuperscript{+/+} germ free mice, which are on a 129S6/SvEv background. All experiments were performed according to the guidelines of the University of Pennsylvania or Weill Cornell Medical College Institutional Animal Care and Use Committee.

3.3.2 Microbiology
The LRC strain, \textit{Bordetella} spp., used to colonize BMDCs or monocolonize germ-free mice was originally isolated from the spleen of an anti-CD90.2 mAb treated conventional \textit{Rag1}\textsuperscript{-/-} mice using a previously defined protocol (Sonnenberg et al., 2012). The ABX-resistant (ampicillin, gentamicin, neomycin) LRC (LRC\textsuperscript{R}), \textit{Achromobacter} used to colonize antibiotic-treated mice were human clinical isolates kindly provided by Kaede V. Sullivan (Children’s Hospital of Philadelphia). Bacterial identities were determined by genomic sequencing (Wellcome Trust Sanger Institute) and confirmed by genus-specific 16S rDNA primers. All bacterial strains used to colonize GF mice and for \textit{in vitro} DC assays were grown in LB broth and incubated at 250 RPM, 37°C for 16-20 hours. For enumeration of tissue and fecal CFUs, the spleen, liver, feces, mesenteric lymph
nodes (mLN) and Peyer’s patches (PP) were isolated, homogenized in sterile PBS and plated on Brain Heart Infusion agar supplemented with 5% defibrinated horse blood. When culturing LRC\textsuperscript{R} from tissues and feces, BHI blood agar plates were supplemented with 25 µg/ml ampicillin.

### 3.3.3 Quantitative real-time PCR

RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. cDNA was generated using Superscript reverse transcriptase II (Invitrogen). Quantitative real-time PCR was performed using SYBR green chemistry (Invitrogen) and QuantiTect Primer Assays (Qiagen) on the ABI 7500 real-time PCR system (Applied Biosystems). Samples were normalized to β-actin and displayed as fold change over antibiotic-treated mice.

### 3.3.4 Flow cytometry

Peyer’s patches were harvested and single-cell suspensions were prepared at necropsy. For flow cytometric analyses, cells were stained with antibodies against the following markers: anti-NK1.1 (clone PK136, eBioscience), anti-CD3 (clone 145-2C11, eBioscience), anti-CD5 (clone 53-7.3, eBioscience), anti-CD11c (clone N418, eBioscience), anti-CD4 (clone GK1.5, Abcam), anti-CD8α (clone 53-6.7, eBioscience), anti-B220 (clone RA3-6B2, eBioscience), anti-CD45 (clone 30-F11, eBioscience) and anti-CD11b (clone M1/70, eBioscience). For intracellular staining of ROR\textsuperscript{yt}, cells were fixed and permeabilized with the Foxp3
intracellular staining buffer set (eBioscience) and stained with anti-RORγt (clone B2D, eBioscience). For intracellular cytokine staining, cells were stimulated ex vivo by incubation for 4 h with 50 ng/mL PMA, 750 ng/mL ionomycin, 10 µg/mL Brefeldin A (all obtained from Sigma-Aldrich) and 50 ng/mL rmIL-23 (eBioscience) and permeabilized as indicated above and stained anti-IL22-02 (mouse cytokine detection antibody developed by Pfizer). Dead cells were excluded from analysis using the Live/Dead Aqua Viability Kit (Invitrogen). Flow cytometry data collection was performed on the LSR II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.).

### 3.3.5 DSS-induced intestinal damage

Mice were administered 2% DSS (MW 36,000-50,000, MP Biochemicals) in their drinking water ad libitum for the number of days indicated and then placed on regular drinking water. Disease severity was cumulatively scored based on rectal bleeding (out of 2), fecal consistency (out of 2), general appearance (out of 4), weight loss (out of 4) and rectal temperature (out of 4). For antibody treatments during DSS, mice were administered i.p. 500 µg/mouse of rat IgG, anti-IL22-01 (mouse neutralizing antibody developed by Pfizer) or anti-IL-10R (clone 1B1.3A, Bio X Cell) 1 day prior to DSS treatment, then on days 2, 5 and 7. Mice were sacrificed on day 8.
3.3.6 Histology

Tissues from the small and large intestines were fixed with 4% PFA, embedded in paraffin, and 5 µm sections were cut and stained with H&E.

3.3.7 Statistical analyses

Results represent mean ± SEM and statistical analyses were performed by unpaired student’s t-test, Mann-Whitney test, or one- or two-way ANOVA with or without multiple comparisons tests as indicated in figure legends.

3.4 Results

3.4.1 Loss of IL-22 is associated with impaired colonization by lymphoid tissue-resident commensal bacteria

Multiple reports suggest that IL-17A and IL-22 limit intestinal colonization of the commensal bacterium SFB (Qiu et al., 2013; Rutz et al., 2013; Shih et al., 2014; Upadhyay et al., 2012). Therefore, we hypothesized that induction of IL-17A and IL-22 by LRCs would be important in limiting its own colonization in intestinal-associated lymphoid tissues. To examine the role for IL-17A and IL-22 in LRC colonization, ABX-treated C57BL/6, Il17a<sup>−/−</sup> and Il22<sup>−/−</sup> mice were inoculated with LRC<sup>R</sup> and lymphoid tissue colonization was assessed 10 days post-inoculation. Based on the requirement for IL-22 in restricting Alcaligenes dissemination (Sonnenberg et al., 2012), we also predicted that colonization of Il22<sup>−/−</sup> mice with
LRC<sup>R</sup> would result in systemic dissemination of LRC<sup>R</sup>. However, systemic dissemination of LRC<sup>R</sup> was not observed in the absence of either IL-17A or IL-22 (Figure 18A). Furthermore, analysis of intestinal-associated lymphoid tissues revealed an impaired ability of LRC<sup>R</sup> to colonize the mLN and PPs of Il22<sup>−/−</sup> but not C57BL/6 or Il17a<sup>−/−</sup> mice (Figure 19A and 19B). These data suggest that IL-22 may also facilitate colonization of lymphoid tissues by commensal bacteria. ILC3s are major producers of IL-22 in the intestine of healthy mammals (Artis and Spits, 2015; Diefenbach et al., 2014; McKenzie et al., 2014; Sonnenberg and Artis, 2012, 2015; Sonnenberg et al., 2012). To test whether innate cell-derived IL-22 could impact lymphoid tissue colonization by commensal bacteria, we inoculated ABX-treated Rag<sup>1</sup>−/− and Rag<sup>1</sup>−/−Il22<sup>−/−</sup> mice with LRC<sup>R</sup> and measured lymphoid tissue colonization. Similar to the findings using lymphocyte-sufficient Il22<sup>−/−</sup> mice, inoculation of Rag<sup>1</sup>−/−Il22<sup>−/−</sup> mice with LRC<sup>R</sup> did not result in systemic dissemination of LRC<sup>R</sup> (Figure 18B), but resulted in impaired LRC<sup>R</sup> colonization in the mLN (Figure 19C). Collectively, these results suggest that induction of ILC-derived IL-22 by LRCs is important to facilitate its colonization of lymphoid tissues.

3.4.2 Impaired lymphoid tissue-resident commensal bacteria colonization in Il22<sup>−/−</sup> mice is dependent on a dominant and transmissible microbiota

Expression of the IL-22 receptor is restricted to non-hematopoietic cells and IL-22-IL-22R signaling regulates antimicrobial responses in the intestinal lumen and
the composition of the microbiota (Qiu et al., 2013; Shih et al., 2014; Sonnenberg et al., 2011; Zenewicz et al., 2013). Therefore, rather than a direct effect on DCs, we hypothesized that in the absence of IL-22 there may be an expansion of commensal bacteria that outcompete and limit LRC entry and colonization. To investigate this hypothesis, we measured luminal bacterial colonization in C57BL/6, Il22−/−, Il17a−/−, Rag1−/− and Rag1−/−Il22−/− mice that were treated with a limited ABX cocktail followed by LRCR inoculation. At 10 days post-inoculation, we detected an absence of fecal LRCR in Il22−/− and Rag1−/−Il22−/− but not in C57BL/6, Rag1−/− and Il17a−/− mice (Figure 20A and 20B). Il22+/− littermates raised under the same conditions as Il22−/− mice also have impaired ABX-mediated fecal LRC colonization, and administration of additional antibiotics including neomycin, metronidazole and vancomycin, restored fecal LRC colonization in Il22−/− mice and Il22+/+ littermates (Figure 20C). Furthermore, cohousing Il22−/− mice with C57BL/6 mice impaired fecal and lymphoid tissue LRC colonization in C57BL/6 mice (Figure 20D). Altogether, these data indicate that IL-22 promotes LRC colonization by limiting competition with other commensal microbes.

3.4.3 Exogenous administration of IL-22 enhances lymphoid tissue-resident commensal bacteria colonization

To further interrogate the role of IL-22 in regulating LRC colonization, we pursued a gain-of-function approach. Consistent with a role for IL-22 in promoting lymphoid tissue colonization by LRCs, exogenous administration of recombinant
mouse IL-22 to LRC\textsuperscript{R}-colonized \textit{Rag1}\textsuperscript{−/−} mice significantly enhanced LRC\textsuperscript{R} levels in the mLN (Figure 19D). These data suggest that IL-22 is sufficient to promote lymphoid tissue colonization by LRCs.

### 3.4.4 IL-22 regulates lymphoid tissue-resident commensal bacteria colonization in the presence of a complex microbiota

The LRC\textsuperscript{R} colonization model requires the use of antibiotics that eliminate most commensal bacteria in the intestine. To test the requirement for IL-22 in LRC colonization in the presence of a complex microbiota, we measured levels of the endogenous LRC, \textit{Alcaligenes} by fluorescence in situ hybridization (FISH) in conventional \textit{Il22}\textsuperscript{−/−} mice and littermate controls. Compared to \textit{Il22}\textsuperscript{+/+} littermates, conventional \textit{Il22}\textsuperscript{−/−} mice have reduced \textit{Alcaligenes} puncta in the PP and mLN (Figure 21A and 21B). These data demonstrate that IL-22 plays a critical role in enhancing the colonization of intestinal-associated lymphoid tissues by LRCs in the presence of a complex microbiota.

### 3.4.5 Loss of IL-10 results in elevated lymphoid tissue-resident commensal bacteria-induced Th17 cell responses in gnotobiotic mice

Although induction of Th17 cell responses were observed in the PP and mLN of LRC-monocolonized mice, the magnitude of these responses was lower than that observed in SFB-monocolonized mice (Figure 12A-D), raising the possibility that LRCs are less efficient at inducing Th17 cell responses or are actively limiting
Th17 cell responses. Since LRCs could promote IL-10 by DCs (Figures 9A-D and 17A) and IL-10 has been shown to suppress IL-17 responses (Liu et al., 2011a; McGeachy et al., 2007; Ouyang et al., 2011), we hypothesized that IL-10 may restrain LRC-induced Th17 cell responses. Consistent with this hypothesis, Th17 cells were significantly increased in the mLN (Figure 22A and 22B) and PP (Figure 22C and 22D) of LRC-monocolonized Il10−/− mice compared to LRC-monocolonized wild type mice. Collectively, these data demonstrate that LRCs selectively promote local Th17 cell responses in intestinal-associated lymphoid tissues, and that the magnitude of these responses is limited by co-induction of IL-10.

3.4.6 Lymphoid tissue-resident commensal bacteria colonization provides host protection from DSS-induced intestinal inflammation and injury

IL-22 is important for regulating immune homeostasis and maintenance of the intestinal barrier following infection or tissue damage (Ouyang et al., 2011; Rutz et al., 2013; Sonnenberg et al., 2011). The finding that LRCs induce IL-22 raises the possibility that LRCs may provide intestinal protection following tissue damage. To test this, we employed a model of intestinal damage where administration of the chemical, dextran sodium sulfate (DSS), induces significant damage to the colonic epithelium. Compared to CNV mice, mice lacking commensal bacteria or treated orally with a broad-spectrum cocktail of antibiotics are highly sensitive to intestinal damage induced by DSS and succumb to
disease (Ayres et al., 2012; Kitajima et al., 2001; Maslowski et al., 2009). To test the functional significance of LRC colonization following DSS treatment, we administered 2% DSS to CNV, ABX-treated alone and LRC<sup>R</sup>-colonized Rag1<sup>−/−</sup> mice for 6-7 days, then removed DSS, re-administered normal drinking water and monitored their survival, weight loss and parameters of inflammation. Compared to CNV mice, which survived throughout the duration of the experiment (21 days), ABX-treated mice exhibited significant systemic and intestinal morbidity and succumbed to disease by day 10 post DSS administration (Figure 23A, 23C and 23D). In contrast, LRC<sup>R</sup>-colonized mice were largely protected from DSS-induced mortality, and demonstrated significantly reduced weight loss and elevated red blood cell counts as compared to ABX-treated controls (Figure 23A-D). Further, analysis of intestinal tissue damage on day 5 post DSS administration revealed that although CNV mice did not exhibit significant intestinal damage or inflammation in the colon at this low dose of DSS, the colons of ABX-treated mice displayed severe loss of crypt architecture and extensive inflammation (Figure 24). In contrast, LRC<sup>R</sup>-colonized mice had visible but reduced intestinal tissue damage and inflammation (Figure 24). LRC<sup>R</sup>-conferred protection from DSS-induced mortality was also observed in gnotobiotic C57BL/6 (Figure 25A) and Rag1<sup>−/−</sup> (Figure 25B) mice monocolonized with the mouse-derived LRC, *Bordetella* spp., suggesting that host protection can occur in the absence of other commensal bacteria. Collectively, these data
demonstrate that colonization of lymphoid tissues by selective commensal bacteria is host beneficial in the context of intestinal damage and inflammation.

3.4.7 Lymphoid tissue-resident commensal bacteria-induced IL-10 provides host protection from DSS-induced intestinal injury and systemic inflammation

To investigate whether LRC-induced IL-22 is providing host protection in LRC\textsuperscript{R}-colonized mice, we administered anti-IL-22 neutralizing antibody to LRC\textsuperscript{R}-colonized \textit{Rag1}\textsuperscript{−/−} mice during exposure to DSS. Anti-IL-22-treated mice exhibited comparable weight loss to IgG-treated mice (Figure 26C), suggesting that IL-22 is not involved in LRC-mediated host protection from DSS. As the anti-inflammatory cytokine IL-10 was also induced upon LRC colonization both in the steady state and during DSS exposure (Figure 9, 17A and Figure 26A), we interrogated whether innate cell-derived IL-10 is providing host protection in LRC\textsuperscript{R}-colonized mice. To test this, we administered anti-IL-10R neutralizing antibody to LRC\textsuperscript{R}-colonized \textit{Rag1}\textsuperscript{−/−} mice during exposure to DSS. Blockade of IL-10-IL-10R interactions resulted in increased weight loss and colonic inflammation as compared to IgG-treated controls (Figure 26D, 27). ABX-treated \textit{Rag1}\textsuperscript{−/−} mice on DSS water display elevated levels of serum IFN\textsubscript{γ}, which was significantly diminished in LRC\textsuperscript{R}-colonized \textit{Rag1}\textsuperscript{−/−} mice (Figure 26B). Conversely, IL-10R neutralization in LRC\textsuperscript{R}-colonized mice partially restored serum IFN\textsubscript{γ} levels observed in ABX-treated mice (Figure 26B). Treatment of ABX-only control \textit{Rag1}\textsuperscript{−}
animals with anti-IL-10R during DSS exposure did not result in further weight loss or colon pathology compared to isotype-treated animals (Figure 28A-B). Collectively, these data suggest that LRC colonization protects mice from acute intestinal injury and systemic inflammatory responses through induction of innate cell-derived IL-10.

3.5 Discussion

This Chapter explores the role of LRC-induced immune pathways in the steady state and in the context of acute intestinal injury. The data suggest that LRCs induce IL-10 and IL-22, members of the IL-10 cytokine family, to establish beneficial host-commensal interactions (Figure 29).

Multiple reports suggest that induction of the ILC3-IL-22 pathway limits host colonization by pathogenic attaching and effacing bacterial pathogens such as *Citrobacter rodentium* or the commensal bacterium SFB (Qiu et al., 2013; Rutz et al., 2013; Shih et al., 2014; Sonnenberg et al., 2011; Upadhya et al., 2012). In contrast, the data in this Chapter demonstrate that IL-22 facilitates colonization of lymphoid tissues by LRCs. *Il22*−/− mice harbor intestinal microbes that limit LRC colonization in the lumen likely through competition for space or nutrients. This is consistent with IL-22-induced antimicrobial functions on gram-positive commensal bacteria (Hooper and Macpherson, 2010; Rutz et al., 2013; Sonnenberg et al., 2011). This also agrees with a report demonstrating a role for IL-22-induced lipocalin and calprotectin in enhancing *Salmonella* infection by
suppressing commensal Enterobactericeae (Behnsen et al., 2014). Critically, recombinant IL-22 enhanced LRC colonization in Rag1−/− mice, and reduced endogenous LRC colonization was observed in CNV Il22−/− compared to littermate controls. Reduction of endogenous LRCs in CNV Il22+/− mice may be due to dysbiosis as Il22−/− mice have an altered and a transmissible microbiota (Zenewicz et al., 2013). These data collectively suggest that IL-22 indirectly promotes LRC colonization in multiple contexts by restricting colonization of competing intestinal microbes.

It was previously reported that ILC3-derived IL-22 is a critical pathway that maintains anatomical containment of LRCs in intestinal-associated lymphoid tissues in healthy mice and that transient loss of this pathway results in dissemination of Alcaligenes to systemic lymphoid tissues (Sonnenberg et al., 2012). In the current study, systemic dissemination of a distinct but related LRC, Achromobacter was not observed in the absence of IL-22. Rather, Il22+/− and Rag1+/− Il22−/− mice had impaired lymphoid tissue entry and colonization. Therefore, the findings in this Chapter highlight a multifaceted role for IL-22 in maintaining anatomical localization of LRCs, by both promoting lymphoid tissue colonization and limiting systemic dissemination.

LRC colonization is sufficient to protect wild type and lymphocyte-deficient mice from lethal intestinal damage, supporting the hypothesis that host recognition of commensal bacteria provides beneficial microbial stimulation to limit intestinal injury. It was previously demonstrated that mice lacking the pattern
recognition receptors TLR2, TLR4 or the downstream adaptor molecule MYD88 are also susceptible to lethal DSS-induced intestinal damage (Rakoff-Nahoum et al., 2004), suggesting that direct recognition of microbial signals is critical for maintenance of the intestinal barrier. Despite our understanding of microbial recognition pathways in host protection from intestinal injury, the downstream signals induced are not fully understood. The data in this Chapter suggest that MYD88-dependent IL-10 induction by LRCs is one pathway that provides tissue protective functions in the context of intestinal injury and systemic inflammation. These data are consistent with the anti-inflammatory and tissue protective roles of IL-10 in the intestine (Asseman et al., 1999; Kuhn et al., 1993; Ouyang et al., 2011) and that innate and adaptive IL-10 responses in the intestine are dramatically driven by the microbiota (Atarashi et al., 2011; Chiu and Ching, 2014; Geuking et al., 2011; Liu et al., 2011b; Ueda et al., 2010). The data in this Chapter support a model whereby, following LRC colonization, IL-10 produced by DCs in intestinal-associated lymphoid tissues has local effects in limiting Th17 cell responses in lymphoid tissues in the steady state, as well as distal effects on the intestinal epithelium and systemic circulation in the context of intestinal damage.

Collectively, the findings in this Chapter demonstrate a previously unrecognized and mutually beneficial dialogue between the host and lymphoid tissue-resident commensal bacteria (Figure 29). Colonization of lymphoid tissues by commensal bacteria modulates the host immune system in a tissue-specific
manner and confers protective effects in the context of intestinal damage. From an evolutionary perspective, there may be multiple beneficial functions for permitting colonization of mammalian lymphoid tissues by commensal bacteria. However, it is likely that a tightly regulated balance between beneficial and pathologic commensal colonization must be maintained in order to prevent pathologic inflammation.
Figure 18. Colonization of IL-22-deficient animals by lymphoid tissue-resident commensal bacteria is not associated with bacterial dissemination.

![Graph](image)

**Figure 18.** (A) Bacterial CFUs were determined in spleen homogenates of LRC\(^R\)-gavaged C57BL/6, \(Il17a^{-/-}\) and \(Il22^{-/-}\) mice. (B) Bacterial CFUs were determined in spleen homogenates of LRC\(^R\)-gavaged \(Rag1^{+/+}\) and \(Rag1^{-/-}Il22^{+/+}\) mice. ND, not detectable.
Figure 19. Loss of IL-22 is associated with impaired colonization by lymphoid tissue-resident commensal bacteria.

Figure 19. (A and B) mLN and PP homogenates from LRC\textsuperscript{R}-colonized C57BL6, \textit{Il17a}\textsuperscript{-/-} and \textit{Il22}\textsuperscript{-/-} mice were cultured to determine CFUs. Data are representative of at least 2 independent experiments. (C) mLN homogenates from LRC\textsuperscript{R}-colonized \textit{Rag1}\textsuperscript{-/-} and \textit{Rag1}\textsuperscript{-/-} \textit{Il22}\textsuperscript{-/-} mice were cultured to determine CFUs. Data pooled from 2 independent experiments for a total of 5-6 mice per group. (D) mLN homogenates from control or recombinant mouse IL-22 (rIL-22)-treated \textit{Rag1}\textsuperscript{-/-} mice were cultured to determine CFUs. Data representative of 2 independent experiments using recombinant mouse IL-22 or IL-22-Fc. Data are represented as mean ± SEM. Statistics in panels D were performed using unpaired, two-tailed, student’s t test. *, \( p < 0.05 \); ND, not detectable.
Figure 20. Impaired lymphoid tissue-resident commensal bacteria colonization in IL-22-deficient mice is dependent on the presence of competing microbiota.

Figure 20. (A and B) Fecal CFUs were determined in LRC<sup>R</sup>-gavaged C57BL/6, II17a<sup>−/−</sup>, II22<sup>−/−</sup>, Rag1<sup>−/−</sup> and Rag1<sup>−/−</sup>II22<sup>−/−</sup> mice. Data representative of at least 2 independent experiments. (C) Fecal CFUs were determined in ampicillin and gentamicin (AG)-treated, LRC<sup>R</sup>-gavaged C57BL/6, II22<sup>+</sup> and II22<sup>−/−</sup> mice. (D) Fecal CFUs were determined in AG-treated, LRC<sup>R</sup>-gavaged C57BL/6, II22<sup>+</sup> and II22<sup>−/−</sup> mice following treatment with neomycin, metronidazole and vancomycin (NMV) for 1 week. One-way ANOVA, ****, p < 0.0001. (D) Fecal and mLN CFUs were determined in AG-treated, LRC<sup>R</sup>-gavaged C57BL/6, C57BL/6 mice co-housed with II22<sup>−/−</sup> (C57BL/6 CH) and II22<sup>−/−</sup> mice. Data in B, C and D are representative of 2 independent experiments. Statistics in panel C were performed using one-way ANOVA and the Tukey’s multiple comparisons test. **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.
Figure 21. IL-22 regulates lymphoid tissue-resident commensal bacteria colonization in the presence of a complex microbiota.

Figure 21. (A) Whole PP and (B) mLN were stained with *Alcaligenes*-specific 16S FISH probes ALBO (red) and BPA (green), and wheat germ agglutinin (blue). Data are representative of 2 independent experiments with 2-5 mice per group. Number of puncta per mm$^3$ of tissue was quantified. Data are represented as mean ± SEM. Statistics were performed using unpaired, two-tailed, student's t test *, $p < 0.05$. 
Figure 22. Loss of IL-10 results in elevated lymphoid tissue-resident commensal bacteria-induced Th17 cell response in gnotobiotic mice.

**Figure 22.** Frequencies of Th17 cells (gated as RORγt<sup>+</sup>FOXP3<sup>-</sup> of Lineage<sup>+</sup>CD4<sup>+</sup>) in the (A and B) mLN or (C and D) Peyer’s patch of LRC-monocolonized Il10<sup>+/+</sup> or Il10<sup>−/−</sup> mice. Data representative of 2 independent experiments with 2-5 mice per group using Il10<sup>−/−</sup> monocolonized mice or C57BL/6 monocolonized mice with anti-IL-10R treatment. One-way ANOVA, mLN, ****p < 0.0001; PP, **p < 0.01. Cells in all flow cytometry plots are gated as live, Lineage<sup>+</sup> and CD4<sup>+</sup>. Lineage cocktail consists of CD3, CD5, CD8α and NK1.1. Data are represented as mean ± SEM. Statistics shown in panels B and D were performed using one-way ANOVA with unpaired, two-tailed, student’s t test and no correction for multiple comparisons. *, p < 0.05; **, p < 0.01.
Figure 23. Lymphoid tissue-resident commensal bacteria colonization provides host protection from DSS-induced mortality and weight loss.

Figure 23. CNV, ABX-treated and ABX-resistant Achromobacter (LRC<sup>R</sup>)-colonized- colonized <i>Rag1</i>−/− mice were administered 2% DSS in their drinking water for 6 to 7 days and then placed on regular drinking water. Mice were monitored for survival (A) and weight loss (B) for up to 21 days. Data in panel A are pooled from 2 independent experiments for a total of 8-9 mice per group. (C) Disease score for CNV, ABX and LRC<sup>R</sup>-colonized <i>Rag1</i>−/− mice treated with 2% DSS in drinking water for 6 days. (D) Red blood cell (RBC) counts for ABX and LRC<sup>R</sup>-colonized <i>Rag1</i>−/− mice treated with 2% DSS in drinking water for 8 days. Data pooled from 2 independent experiments for a total of 4-7 mice per group. Data are represented as mean ± SEM. Statistics in panels B, C and D were performed using unpaired, two-tailed, student’s t test with no correction for multiple comparisons. * <i>p</i> < 0.05; ***, <i>p</i> < 0.0001.
Figure 24. Lymphoid tissue-resident commensal bacteria colonization provides host protection from DSS-induced intestinal injury.

Figure 24. CNV, ABX-treated and ABX-resistant Achromobacter (LRC<sup>R</sup>)-colonized mice were administered 2% DSS in their drinking water for 6 to 7 days and then placed on regular drinking water. Mice were monitored for survival. Mice were sacrificed on day 6 and analyzed for colon tissue pathology and inflammatory infiltrate by H&E. Data are representative of 2 independent experiments. Scale bar – 100 µm.
Figure 25. Lymphoid tissue-resident commensal bacteria provide host protection from DSS-induced mortality in gnotobiotic mice.

Figure 25. (A) CNV, GF and *Bordetella*-monocolonized C57BL/6 mice were administered 2% DSS in the drinking water for 5 days and then placed on regular drinking water. CNV, n = 3; GF, n = 7; *Bordetella*-monocolonized, n = 9. (B) GF and *Bordetella*-monocolonized *Rag1*−/− mice were administered 2% DSS in the drinking water for 7-9 days and then placed on regular drinking water for the duration of the experiment. GF, n = 6; *Bordetella*-monocolonized, n = 6. Data in panels A and B are pooled from 2 independent experiments.
Figure 26. Lymphoid tissue-resident commensal bacteria-induced IL-10 provides host protection from DSS-induced weight loss and systemic inflammation.

Figure 26. (A) Analysis of Il10 expression in the colon of 2% DSS-treated CNV, ABX-treated or LRC<sup>R</sup>-colonized Rag1<sup>−/−</sup> mice at day 6 post-DSS treatment. Data in A are pooled from 2 independent experiments with a total of 5-7 mice per group. (B) Serum IFNy on day 8 post-DSS treatment. Data in panel B are pooled from 3 independent experiments for a total of 8-12 mice per group. One-way ANOVA - **p < 0.01. (C and D) ABX and LRC<sup>R</sup>-colonized Rag1<sup>−/−</sup> mice given rat IgG, anti-IL-22 or anti-IL-10R were treated with 2% DSS in the drinking water for 8 days and monitored for weight loss. Data in C are representative of 2 independent experiments. Data in D represent pooled data from 3 independent experiments. Data are represented as mean ± SEM. Statistics were performed using one-way ANOVA with uncorrected Fisher’s LSD test (B, D) or Mann-Whitney test with no correction for multiple comparisons (A). *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 27. Lymphoid tissue-resident commensal bacteria-induced IL-10 provides host protection from DSS-induced intestinal injury.

Figure 27. ABX, LRC\textsuperscript{R}-colonized \textit{Rag1}\textsuperscript{-/-} mice given rat IgG or anti-IL-10R were treated with 2\% DSS in the drinking water for 8 days and colon pathology was assessed on day 8. Scale bar – 100 \(\mu\)m. Data are representative of 2 independent experiments.
Figure 28. Blockade of IL-10-IL-10R interactions in antibiotic-treated mice is not associated with enhanced intestinal tissue damage induced by DSS.

Figure 28. (A) Weight loss and (B) colon pathology of ABX-treated, rat IgG or anti-IL-10R-injected Rag1−/− mice given 2% DSS for 9 days. Scale bar – 100 µm. Data are representative of 2 independent experiments.
Figure 29. Lymphoid tissue-resident commensal bacteria promote members of IL-10 cytokine family to establish mutualism.

Figure 29. Lymphoid tissue-resident commensal bacteria (LRC) colonize DCs in host intestinal-associated lymphoid tissues to elicit pro- and anti-inflammatory cytokines. Colonization of lymphoid tissues by LRCs is also associated with the induction of tissue-specific Th17 cell and ILC3 responses. IL-22 enhances LRC colonization in lymphoid tissues by limiting colonization of other commensal bacteria. LRC-induced IL-10 limits intestinal tissue damage and suppresses systemic inflammation.
Chapter 4
The iron-regulatory hormone hepcidin regulates host-microbe interactions and immune cell homeostasis

4.1 Abstract
Chronic inflammatory diseases are often associated with altered iron homeostasis. For example, patients with inflammatory bowel disease frequently exhibit anemia that is dependent on increased expression of the iron-regulatory hormone hepcidin. Hepcidin is expressed and secreted by hepatocytes and acts to limit iron absorption in the intestine and iron release from intracellular stores. Despite our understanding of hepcidin-mediated iron regulation, its role in immune cell function and host-commensal bacteria relationships is not well understood. This Chapter demonstrates that, in addition to hepatocytes, bone marrow-derived dendritic cells (BMDC) can express hepcidin and upregulate its expression following exposure to LRCs. Furthermore, dendritic cell (DC)-intrinsic hepcidin is required for intracellular colonization by LRCs. Examination of hepcidin-deficient mice also revealed elevated levels of the pro-inflammatory cytokines *Il1b* and *Il6* and increased frequencies of IL-17A- and IFNγ-producing CD4 T cells in intestinal-associated lymphoid tissues. Consistent with a role for Th17 and Th1 cells in the pathogenesis of intestinal inflammation, hepcidin-deficient mice exhibit delayed recovery from acute intestinal injury. Altogether, these results highlight a previously unrecognized role for hepcidin in immune...
homeostasis and host-commensal bacteria interactions at the steady state and during intestinal inflammation.

4.2 Introduction

Iron is an essential nutrient for all living organisms. The uptake, transport and storage of iron are tightly regulated biological processes that are coordinated by multiple molecular components. In mammals, one critical pathway involves the iron-regulatory hormone hepcidin. Hepcidin, a 25-amino acid peptide that is secreted primarily by liver hepatocytes, binds to and results in the degradation of the membrane-bound iron exporter ferroportin (Cassat and Skaar, 2013; Ganz and Nemeth, 2015; Zhao et al., 2013). As a result, hepcidin-ferroportin interactions control iron levels in the plasma and intracellular stores. Hepcidin expression is regulated cell-extrinsically by three major factors: plasma iron levels, inflammatory cytokines and erythropoiesis. Studies have demonstrated that upregulation of hepcidin during inflammation functions as a host protective pathway to prevent extracellular pathogen infection by limiting their access to iron. For example, hepcidin-deficient mice are highly susceptible to infection by the pathogens *Vibrio vulnificus* and *Yersinia enterocolitica* (Arezes et al., 2015; Quenee et al., 2012). Conversely, hepcidin deficiency may be protective during infection by obligate intracellular pathogens due to low intracellular iron, an essential nutrient for these microbes. Mice with a mutation in HFE, an adaptor for iron sensing, have reduced intracellular iron due to impaired expression of...
hepcidin (Wang et al., 2008). Hfe$^{-/-}$ mice displayed prolonged survival and enhanced control of bacterial replication during septicemia from the intracellular pathogen *Salmonella Typhimurium* (Nairz et al., 2009). Furthermore, Hfe$^{-/-}$ macrophages had reduced bacterial load compared to wild-type macrophages when infected with *Salmonella Typhimurium*. Altogether, these results demonstrate that hepcidin-regulated iron homeostasis is a critical pathway for the maintenance of antibacterial host responses.

Although the interaction between hepcidin and ferroportin has been well characterized, the role of hepcidin in regulating host-commensal relationships and immune cell function is not well understood. This Chapter will demonstrate that in addition to hepatocytes, DCs are a cellular source of hepcidin *in vitro*, and in the absence of hepcidin, LRC colonization in DCs is impaired. In the absence of hepcidin *in vivo*, mice exhibit elevated pro-inflammatory cytokine production and IL-17A$^{+}$ and IFNγ$^{+}$ CD4 T cell responses in the gut draining lymph node. Consistent with a role for Th1 and Th17 cells in the pathogenesis of intestinal inflammation, loss of hepcidin is associated with exacerbated tissue damage and delayed recovery from acute intestinal injury. These results define iron homeostasis as a critical pathway regulating immune cell homeostasis and host-commensal bacteria relationships in the intestine and associated lymphoid tissues.
4.3 Methods

4.3.1 Mice

C57BL/6 mice were purchased from Jackson Laboratories. Hamp1⁻/⁻ mice on a C57BL/6 background were generated by Sophie Vaulont (Lesbordes-Brion et al., 2006) and kindly provided by Stefano Rivella (Children’s Hospital of Philadelphia). Genome scanning for 153 SNPs confirmed that Hamp1⁻/⁻ mice are 98.67% C57BL/6 (Jackson Laboratories). Age and gender matched C57BL/6 mice used as controls were purchased from Jackson Laboratories and cohoused with Hamp1⁻/⁻ mice for at least 3 weeks before use. Analysis of fecal 16S rDNA qPCR for groups of common mouse commensal bacteria showed that microbiota of Hamp1⁻/⁻ mice did not differ significantly from wild-type mice at the phylum and genus levels following cohousing. All experiments were performed according to the guidelines of the Weill Cornell Medical College Institutional Animal Care and Use Committee.

4.3.2 Bone marrow-derived dendritic cells

Bone marrow-derived DCs were generated by culturing bone marrow cells from C57BL/6 and Hamp1⁻/⁻ mice in the presence of 20 ng/mL GM-CSF for 8-10 days. Culture media was replaced with fresh media every 3 days. Frequencies of CD11c⁺ cells were ≥ 95%. For intracellular bacterial survival assays, BMDCs were seeded on 6-well plates at 5 x 10⁶ cells/well in antibiotic-free media and co-
cultured with bacteria at an MOI of 50 for 2 hours, harvested and washed 3 times with sterile PBS. BMDCs were then plated in media containing gentamicin (100 µg/mL) at 200,000 cells/well on 48-well plates and lysed with sterile water every 48 hours to enumerate CFUs. For gene expression analyses, BMDCs were co-cultured with bacteria at an MOI of 50 for 2 hours and then gentamicin-treated to kill extracellular bacteria. Cell lysates were prepared 24 hours later. All cell incubations were performed at 37°C and 5% CO₂.

4.3.3 Gene expression profiling and quantitative real-time PCR

RNA was isolated from BMDCs using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was generated using Superscript reverse transcriptase II (Invitrogen). Quantitative real-time PCR was performed using SYBR green chemistry (Invitrogen) and QuantiTect Primer Assays (Qiagen) on the ABI 7500 real-time PCR system (Applied Biosystems). Samples were normalized to β-actin and displayed as fold change over PBS-treated BMDCs. For microarray analysis, BMDCs colonized with the LRC, *Bordetella* spp., for 4 days were lysed directly in TRIzol. RNA was isolated, amplified, reverse-transcribed to cDNA and hybridized to an Affymetrix GeneChip (Mouse Gene 1.0ST) by the University of Pennsylvania Molecular Profiling Facility. Relative expression data was normalized by Z score transformation.
4.3.4 DSS-induced intestinal damage

Mice were administered 2.5% DSS (MW 36,000-50,000, MP Biochemicals) in their drinking water ad libitum for 7 days and then placed on regular drinking water. Mice were monitored for weight loss throughout the course of the experiment.

4.3.5 Flow cytometry

Mesenteric lymph nodes were excised and single-cell suspensions were prepared at necropsy. For large intestine lamina propria preparations, intestines were excised, attached fat and PPs were removed and tissues were cut open longitudinally. Luminal contents were removed by shaking in cold PBS. Epithelial cells and intra-epithelial lymphocytes were removed by shaking tissue in stripping buffer (100 I.U./ml penicillin, 100 µg/ml streptomycin, 1 mM EDTA, 1 mM DTT and 5% FCS) at 37°C for 30 minutes. The lamina propria layer was isolated by digesting the remaining tissue in 1 mg/mL collagenase/dispase (Roche) and 20 µg/mL DNase I (Sigma-Aldrich) for 30 minutes at 37°C. For flow cytometric analyses, cells were stained with antibodies against the following markers: anti-CD3 (clone 145-2C11, eBioscience), anti-CD5 (clone 53-7.3, eBioscience), anti-CD4 (clone GK1.5, Abcam), and anti-CD45 (clone 30-F11, eBioscience). For intracellular staining of RORγt, cells were fixed and permeabilized with the Foxp3 intracellular staining buffer set (eBioscience) and stained with anti-RORγt (clone B2D, eBioscience). For intracellular cytokine staining, cells were stimulated ex
vivo by incubation for 4 h with 50 ng/mL PMA, 750 ng/mL ionomycin, 10 µg/mL Brefeldin A (all obtained from Sigma-Aldrich) and permeabilized as indicated above and stained with anti-IL-17A (clone eBioTC11-18H10.1, eBioscience), anti-IFN-γ (clone XMG1.2, eBioscience) and anti-TNFα (clone MP6-XT22, eBioscience). Dead cells were excluded from analysis using the Live/Dead Aqua Viability Kit (Invitrogen). Flow cytometry data collection was performed on the LSR Fortessa (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.).

4.3.6 Histology
Tissues from the large intestines were fixed with 4% PFA, embedded in paraffin, and 5 µm sections were cut and stained with H&E.

4.3.7 Statistical analyses
Results represent mean ± SEM and statistical analyses were performed by unpaired student’s t-test, one- or two-way ANOVA with or without multiple comparisons tests as indicated in figure legends.

4.3.8 Data accessibility
Array data have been deposited in GEO under accession number GSE76731.
4.4 Results

4.4.1 Bone marrow-derived dendritic cells upregulate hepcidin expression following exposure to lymphoid tissue-resident commensal bacteria

In Chapter 2, microarray-based gene expression profiling was performed on DCs colonized with the mouse-derived LRC, *Bordetella* spp. compared to DCs treated with PBS. Analysis of these results indicated that colonization of *Bordetella* significantly altered the expression of genes associated with iron storage, transport and uptake, which include those that encode for transferrin, transferrin receptor, ferroportin, lipocalin 2 and hemoglobin beta-chain (Figure 30). Hepcidin, encoded by *Hamp1*, was also highly upregulated in *Bordetella*-colonized DCs and is considered to be a master regulator of systemic iron homeostasis. Upregulation of *Hamp1* in bacterial-colonized DCs was confirmed by real time PCR (Figure 31). To investigate whether bacterial-derived products are sufficient to induce hepcidin, BMDCs were treated with various TLR ligands for 24 hours and hepcidin expression was measured. Consistent with exposure to whole bacteria, BMDCs exposed to LPS, Pam₃CSK₄ and CpG DNA upregulated *Hamp1* (Figure 31). Collectively, these data suggest that in addition to hepatocytes, BMDCs are a cellular source of hepcidin and BMDCs can upregulate hepcidin in response to bacteria *in vitro.*
4.4.2 Loss of hepcidin in bone marrow-derived dendritic cells results in impaired intracellular bacterial survival by lymphoid tissue-resident commensal bacteria

Data from Chapter 2 demonstrated that LRCs could persist and survive in bone marrow-derived and primary DCs (Figure 7B-D). Given the role of hepcidin in modulating intracellular iron, an essential nutrient for most microbes, and that hepcidin is upregulated in BMDCs following LRC colonization, one hypothesis is that Hamp1−/− BMDCs will not support intracellular colonization by LRCs. To test this, wild-type or Hamp1−/− BMDCs were exposed to the mouse LRC, Bordetella spp. or the non-LRC, E. coli, and intracellular survival was assessed over time. Although Bordetella spp. initially colonized Hamp1−/− BMDCs, the amount of live Bordetella spp. that survived in Hamp1−/− BMDCs was significantly reduced at 48 and 96 hours post-colonization (Figure 32). In contrast, E. coli survival was not affected by hepcidin deficiency. Collectively, these results suggest that DC-intrinsic hepcidin is important for efficient intracellular colonization by LRCs.

4.4.3 Loss of hepcidin is associated with altered immune cell homeostasis in vivo

Hepcidin is induced in vivo by inflammatory cytokines, such as IL-6, generated during host exposure to microorganisms including fungi, bacteria and viruses (Nemeth et al., 2004). In Hamp1−/− mice, levels of serum and liver iron are significantly increased whereas splenic iron levels are significantly decreased
(Lesbordes-Brion et al., 2006). Despite these data, the role of hepcidin in immune cell homeostasis *in vivo* has not been well characterized. To test this, *Hamp1*−/− mice were analyzed for expression of innate immune cytokines. Gene expression analyses of innate cell-derived cytokines in the mLN of *Hamp1*−/− mice indicated elevated expression of the pro-inflammatory cytokines *Il1b* and *Il6* but not *Il10* and *Il12b* (Figure 33). Collectively, these data suggest that hepcidin or hepcidin-regulated iron homeostasis controls innate immune function *in vivo*.

IL-1β and IL-6 are innate immune cytokines that promote CD4 T cell responses, namely Th17 cells. We hypothesize enhanced *Il1b* and *Il6* expression in *Hamp1*−/− mice may lead to elevated Th17 cell responses. To test this, we analyzed the frequency of IL-17A+ CD4 T cells in wild-type and *Hamp1*−/− mice. CD4 T cells from the spleen, mLN and colon of *Hamp1*−/− mice had elevated expression of IL-17A (Figure 34A). Notably, hepcidin-deficiency does not result in selective induction of Th17 cells as IFNγ-producing Th1 cells were also induced (Figure 34A). Consistent with elevated Th17 cells, frequencies of Foxp3+ or Foxp3− RORγt+ CD4 T cells were increased in *Hamp1*−/− mice (Figure 34B). Altogether, these results demonstrate that loss of hepcidin *in vivo* is associated with elevated proinflammatory CD4 T cell responses.
4.4.4 Altered immune cell homeostasis in hepcidin-deficient mice is not due to intestinal dysbiosis

Since commensal bacteria regulate intestinal and systemic Th17 responses, we tested whether elevated Th17 responses in $Hamp1^{-/-}$ are due to changes in the composition of the microbiota. We compared $Hamp1^{-/-}$ mice to wild-type mice cohoused with their knockout counterparts for at least 3 weeks. Analysis of fecal 16S rDNA from representative groups of the normal mouse microbiota indicated that levels of Bacteroides, Firmicutes, SFB, Lactobacillus and Enterobacteriaceae in the intestine of $Hamp1^{-/-}$ mice did not significantly differ from co-housed wild-type mice (Figure 35). Altogether, these results suggest that dysregulated innate and adaptive immune cell homeostasis in $Hamp1^{-/-}$ is not due to dysbiosis but more likely due to interactions between hepcidin itself or hepcidin-mediated iron homeostasis and the host immune system.

4.4.5 Loss of hepcidin is associated with delayed intestinal repair from DSS-induced intestinal injury

Th1 and Th17 cells can promote tissue pathology and inflammation in mouse models of intestinal injury (Neurath, 2014; Weaver et al., 2013). We hypothesize that elevated proinflammatory CD4 T cells in $Hamp1^{-/-}$ mice may predispose them disease during intestinal inflammation. To test this, we administered DSS to wild-type and $Hamp1^{-/-}$ mice. Although weight loss between wild-type and $Hamp1^{-/-}$ mice were not significantly different during DSS treatment, $Hamp1^{-/-}$
mice demonstrated delayed recovery from DSS-induced weight loss (Figure 36A). Consistent with this, analysis of tissue pathology revealed that there were increased inflammatory infiltrates and loss of crypt architecture in the colons of \textit{Hamp1\textsuperscript{-/-}} mice compared to wild-type colons during recovery from DSS (Figure 36B). Analysis of immune cell function during recovery from DSS indicated that frequencies of IL-17\textsuperscript{+}, IFN\textsubscript{\gamma}\textsuperscript{+} and TNF\textalpha\textsuperscript{+} CD4 T cells were significantly increased in the colons and mLN of \textit{Hamp1\textsuperscript{-/-}} mice (Figure 37 and 38A). Furthermore, \textit{Hamp1\textsuperscript{-/-}} mice had increased frequencies of neutrophils in the intestine (Figure 38B). Collectively, these results suggest that elevated pro-inflammatory immune responses in \textit{Hamp1\textsuperscript{-/-}} are associated with exacerbated tissue damage and delayed recovery from acute intestinal injury.

\textbf{4.5 Discussion}

This Chapter demonstrates that in addition to hepatocytes, BMDCs are a source of hepcidin \textit{in vitro}. This finding parallels a previous report, which provided evidence that macrophages and neutrophils upregulate the expression of hepcidin mRNA and protein in response to \textit{Mycobacteria tuberculosis}, \textit{Pseudomonas aeruginosa} and \textit{Streptococcus} (Peyssonnaux et al., 2006; Sow et al., 2007). Despite the fact that hepcidin gene expression is upregulated however, further studies are necessary to determine whether hepcidin protein production and secretion is also increased in bacteria-stimulated BMDCs. \textit{In vivo}, hepatocytes appear to be the primary source of hepcidin at the steady state. To
address whether hepcidin is expressed by immune cells in vivo, hepcidin expression should be measured in lymphoid organs and compared to the liver during steady state and inflammation. Since DCs, macrophages and neutrophils are some of the first cellular responders to sites of inflammation (Peyssonnaux et al., 2006; Sow et al., 2007), it is interesting to speculate that these cells may provide an early, localized source of hepcidin, which acts to limit iron available to invading pathogens during infection or colonization with commensal bacteria. In contrast, hepatocytes respond rapidly to systemic inflammation by upregulating hepcidin and may act to control systemic sequestration of iron from invasive pathogens. One hypothesis may be that there is a division of labor between immune cells and hepatocytes for promoting localized versus systemic iron sequestration. One approach to determine whether there are differential functions for hepcidin derived from immune cells relative to hepatocytes is to generate bone marrow chimeric animals that lack hepcidin either in the hematopoietic or non-hematopoietic compartments. Models of local or systemic bacterial infection can then be applied to these mice.

Hepcidin-deficient mice have elevated systemic iron levels (Lesbordes-Brion et al., 2006). The data in this Chapter examining immune cell function in the gut-draining lymph nodes of Hamp1−/− mice reveal a previously unrecognized role for hepcidin in immune cell homeostasis (Figure 39). Loss of hepcidin in vivo was associated with elevated pro-inflammatory cytokines IL-1β, IL-6 and enhanced Th1 and Th17 cell responses. Multiple factors could contribute to
these effects. Expression of hepcidin by the liver at steady state results in inhibition of iron release from macrophages and hepatocytes. Therefore, changes in intracellular iron levels by hepcidin may lead to altered immune cell homeostasis. Consistent with this idea, altered iron homeostasis is often associated with dysregulated systemic immune responses in vivo (Balouchi et al., 2014; Cunningham-Rundles et al., 2000; Ekiz et al., 2005). Although there is no known role for hepcidin itself in regulating immune responses, it is possible that it may directly control cytokine production by innate and adaptive immune cells through a novel pathway. Further investigation using gain and loss of function approaches such as iron supplementation and chelation is necessary to determine whether hepcidin-mediated iron homeostasis plays a role in modulating immune cell function at the steady state.

Given the result that loss of hepcidin is associated with elevated pro-inflammatory cytokine responses including IL-1β, IL-6 and IL-17A, which are critical members of innate and adaptive anti-bacterial immunity, one would predict that hepcidin-deficiency may actually confer protection against bacterial pathogens. However, there are multiple reports suggesting that hepcidin deficiency is associated with susceptibility to extracellular bacterial infections primarily due to an increased bioavailability of iron to invading pathogens (Arezes et al., 2015; Quenee et al., 2012). The relative contribution of immune versus iron defects in the context of antibacterial immunity is not well understood and should be the focus of investigation.
Hepcidin-deficient mice exhibit delayed recovery from DSS-induced intestinal inflammation. These data are consistent with a pathogenic role for the pro-inflammatory cytokines IL-1β and IL-6 in this model. Furthermore, intestinal inflammation is often associated with elevated Th17 cells (Weaver et al., 2013), which is a feature of Hamp1−/− mice at the steady state. It appears that loss of hepcidin does not influence disease outcome during DSS treatment but rather results in delayed recovery and tissue repair. As a result, mechanisms of repair in the host such as the production of IL-22, M2 macrophages and various growth factors should be measured in Hamp1−/− mice.

This Chapter demonstrates that in addition to hepatocytes, DCs are a potent source of hepcidin in vitro. In vivo, loss of hepcidin was associated with elevated pro-inflammatory cytokine expression, elevated Th17 cell responses and delayed repair from DSS-induced intestinal injury (Figure 39). These results represent a key advance in our understanding of the interplay between iron homeostasis and immune cell function and may inform future therapeutic strategies for the treatment of anemia associated with chronic inflammation.
Figure 30. Lymphoid tissue-resident commensal bacteria alter the expression of genes associated with iron uptake, transport and storage in bone marrow-derived DCs.

Figure 30. BMDCs were treated with PBS or colonized with *Bordetella* spp. and analyzed for gene expression by microarray on day 4. Numbers in colored legend represent Z scores.
Figure 31. Lymphoid tissue-resident commensal bacteria upregulate hepcidin in bone marrow-derived dendritic cells.

Figure 31. (A) BMDCs were stimulated with *Bordetella* spp. (MOI 50), LPS (100 ng/ml), Pam3CSK4 (100 ng/ml) or CpG DNA (2 µg/ml) for 24 hours and *Hamp1* expression was analyzed by qPCR. Statistics were performed using one-way ANOVA and Sidak’s multiple comparisons test. **** $p < 0.0001$. 

![Graph showing fold change over PBS for different stimuli](image-url)
Figure 32. Hepcidin regulates intracellular survival of lymphoid tissue-resident commensal bacteria in bone marrow-derived dendritic cells.

Figure 32. Wild-type BMDCs were co-cultured with *E. coli* or *Bordetella* spp. for 2 hours and levels of live intracellular bacteria was assessed by culturing. Data are representative of two independent experiments. Statistics were performed using student’s t-test. l.o.d., limit of detection. ***, p < 0.001; **** p < 0.0001.
Figure 33. Loss of hepcidin *in vivo* is associated with altered expression of pro-inflammatory cytokines in the mesenteric lymph node.

**Figure 33.** Expression of *Il1b, Il6, Il10* and *Il12b* were analyzed in the mesenteric lymph node of wild-type and *Hamp1*−/− mice by qPCR. Data are pooled from three independent experiments. Statistics were performed using student’s t-test. * p < 0.05; **, p < 0.01.
Figure 34. Loss of hepcidin *in vivo* is associated with elevated pro-inflammatory CD4 T cell responses in the mesenteric lymph node.

**Figure 34.** CD4 T cells in the mLN of wild-type and *Hamp1<sup>−/−</sup>* mice were analyzed for production of (A) IL-17A and IFNγ or (B) Foxp3 and RORγt following PMA and ionomycin stimulation. Cells in flow cytometry plots are gated as live, CD45<sup>+</sup>, CD3<sup>+</sup>, CD5<sup>+</sup>, CD4<sup>+</sup>. Data are representative of three independent experiments. Statistics were performed using student’s t-test. **, *p* < 0.01; ***, *p* < 0.001; ****, *p* < 0.0001.
Figure 35. Altered immune cell homeostasis in *Hamp1*−/− mice is not due to intestinal dysbiosis.

Figure 35. 16S rDNA for different groups of bacteria were measured in the feces of wild-type and *Hamp1*−/− mice. Units on the y-axis represent bacterial-specific 16S rDNA relative to universal 16S rDNA. SFB, segmented filamentous bacteria. Data are pooled from three independent experiments.
Figure 36. Loss of hepcidin in vivo is associated with delayed recovery from DSS-induced intestinal injury.

Figure 36. Wild-type and Hamp1−/− were treated with 2.5% DSS for 7 days and then placed on regular drinking water. (A) Weight loss was measured throughout the course of the experiment and (B) colon pathology was assessed on day 12. Scale bar – 200 µm. Data are representative of two independent experiments. Statistics were performed using student’s t-test. **, p < 0.01.
Figure 37. Loss of hepcidin in vivo is associated with elevated frequencies of pro-inflammatory CD4 T cell responses during recovery from DSS-induced intestinal injury.

**Figure 37.** CD4 T cells in the (A) mLN and (B) colon of DSS-treated (day 12) wild-type and Hamp1−/− mice were analyzed for production of IL-17A and IFNγ following PMA and ionomycin stimulation. Cells in flow cytometry plots are gated as live, CD45+, Lineage+, CD4+. Lineage cocktail consists of CD3, CD5, CD8α, CD19, NK1.1. Data are representative of two independent experiments. Statistics were performed using student’s t-test. * p < 0.05; ***, p < 0.001.
Figure 38. Loss of hepcidin *in vivo* is associated with elevated frequencies of intestinal TNFα⁺ CD4 T cells and neutrophils during recovery from DSS-induced intestinal injury.

**Figure 38.** Frequencies of (A) TNFα⁺-producing CD4 T cells (CD3⁺CD5⁺CD4⁺) and (B) neutrophils (CD3⁻CD5⁻CD11b⁺Ly6G⁺) were analyzed in the colon of DSS-treated mice (day 12) following PMA and ionomycin stimulation. Data are representative of two independent experiments. Statistics were performed using student's t-test. ***, p < 0.001.**
Figure 39. Hepcidin and iron homeostasis regulate immune cell function.

In wild-type mice, hepcidin controls systemic iron levels by inhibiting dietary iron intake and iron release from macrophages. Data from Chapter 4 suggest that hepcidin expression and the associated reduction in systemic iron levels act to suppress the production of the pro-inflammatory cytokines IL-1β, IL-6 by innate immune cells and IL-17A, IFNγ and TNFα by CD4 T helper cells. Hepcidin may regulate the immune cell function independent of iron. In the mice that lack hepcidin, systemic iron levels increase due to uninhibited iron absorption and iron release from macrophages. Elevated systemic iron may lead to enhanced production of pro-inflammatory cytokines due to the release of reactive oxygen species (ROS). Elevated pro-inflammatory cytokine production in Hamp1<sup>−/−</sup> mice may be responsible for impaired tissue homeostasis following acute intestinal injury.
Chapter 5
Summary, discussion and future directions

5.1 Lymphoid tissue-resident commensal bacteria belong exclusively to the Proteobacteria phyla

The mammalian intestine is colonized trillions of bacteria that belong predominantly to three phyla: Bacteroidetes, Firmicutes and Proteobacteria (Ley et al., 2005; Turnbaugh et al., 2006). Chapter 2 of this thesis identified that LRCs belong exclusively to the alpha- and beta-Proteobacteria classes. Collectively, Proteobacteria are classified as gram-negative bacteria that differ from their Bacteroidetes and Firmicutes counterparts in their requirement for oxygen. It is therefore possible that the metabolic requirements for Proteobacteria may be linked to their intestinal colonization pattern (Figure 40). In support of this, several reports identified that there is a unique spatial segregation of Proteobacteria versus Bacteroides and Firmicutes in the lumen of the intestine. For example, Proteobacteria are typically found in close proximity to the intestinal epithelial wall, where oxygen concentration is the highest compared to the center of the intestinal lumen (Albenberg et al., 2014; Flint et al., 2012; Ringel et al., 2015; Yasuda et al., 2015). Therefore, sampling of luminal contents by phagocytic dendritic cells and macrophages that populate the intestinal lamina propria may be biased towards Proteobacteria members. Further investigation is
necessary to link the metabolic requirements of commensal bacteria communities with their distinct colonization pattern in the host intestine.

Proteobacteria are found not only in the mammalian GI tract but also in the environment. In fact, many of the strains predicted to be LRCs such as *Bordetella*, *Achromobacter* and *Ochrobactrum* are the causative agents for opportunistic infections in immunocompromised individuals (Hagiya et al., 2013; Melvin et al., 2014; Spilker et al., 2013). Specifically, strains of *Bordetella* and *Achromobacter* have been associated with respiratory tract infections in patients with cystic fibrosis (Melvin et al., 2014; Spilker et al., 2013). These data support the hypothesis that while LRCs inhabit mammals and maintain immune cell homeostasis in the presence of an intact and otherwise healthy immune system, their proximity to lymphocytes in immune cell-rich tissues can quickly alter their interactions with the host to become pathogenic. Therefore, future studies assessing the beneficial versus pathological functions of LRCs should do so in the context of healthy and diseased animals or patients to clearly identify host and microbial-specific factors that regulate a healthy host-LRC interaction.

Proteobacteria levels frequently bloom during intestinal inflammation and have been associated with the pathogenesis of IBD (Melvin et al., 2014; Spilker et al., 2013). In light of the data in Chapter 3 that LRCs, which are Proteobacteria members, promote tissue protective properties in the context of acute intestinal injury, one may hypothesize that Proteobacteria that colonize a healthy GI tract are largely beneficial, especially those that colonize lymphoid
tissues. However, during loss of intestinal barrier function, the pathogenic members of Proteobacteria such as adherent invasive *E. coli* strains and *Helicobacter pylori* expand, become dominant members of the microbiota and contribute to disease. Therefore, understanding the role of LRCs in the context of other pathogenic Proteobacteria members is essential and should be a focus for future investigations. The biological implications of studying host-LRC interactions in the context of a replete microbiota will be described in the following section.

5.2 Understanding LRC colonization in the context of a complex microbiota

The results from Chapter 2 demonstrated that LRCs promote tissue-specific immune responses in gnotobiotic and antibiotic-treated mice where LRCs are the dominant bacterial population in the intestine. However, in nature, the composition of the gut microbiota reflects a complex community of microorganisms that have a diverse array of biological properties and functions. Further experimentation is necessary to investigate host-LRC interactions in the presence of a complex microbiota. However, this approach may be met with multiple challenges. Despite the fact that 16S rDNA for bacteria belonging to LRCs can be detected in the interior of intestinal-associated lymphoid tissues, live bacteria cannot be cultured from the gut-draining mesenteric lymph node of conventional mice that LRCs are predicted to colonize. This may either reflect the absence of live LRCs in conventional mice or that LRCs colonize lymphoid
tissues but maintain a dormant state and thus cannot be cultured using laboratory methods (Figure 41). The former hypothesis is supported by the finding that commensal bacteria, through a MYD88-dependent mechanism, inhibit trafficking of a specialized subset of CX3CR1-expressing phagocytic cells that sample the intestinal lumen (Diehl et al., 2013). Depletion of the gut microbiota using broad-spectrum antibiotics, similar to the experimental approach used to colonize antibiotic-treated mice with LRCs resulted in translocation of a non-virulent strain of *Salmonella* to the mLN and spleen. Developing a mouse model whereby live LRCs selectively colonize lymphoid tissues in the presence of other luminal and epithelial-associated commensal bacteria should take into account these factors.

The infant GI tract is typically colonized with more Proteobacteria compared to the adult microbiota (Arrieta et al., 2014; Shin et al., 2015). One intriguing theory is that colonization with LRCs early in life may provide heightened immune cell maturation in lymphoid tissues. Over time, colonization by Bacteroidetes and Firmicutes may act to limit LRC colonization in lymphoid tissues to prevent pathologic inflammation. This hypothesis is supported by data demonstrating that early exposure to antibiotics may predispose humans to chronic inflammatory diseases such as allergic airway inflammation and obesity (Melvin et al., 2014; Spilker et al., 2013). Whether this reflects altered LRC colonization early in life is unknown and should be a subject of future investigation.
5.3 Commensal bacteria communities modulate tissue-specific immune cell function

The gut microbiota that colonize the mammalian GI tract collectively influences the development and maturation of the host immune system (Hooper et al., 2012; Ivanov and Honda, 2012). Over the last decade, many reports have shown that commensal bacteria found in different sites of the intestine influence the local tissue immune environment. Data from Chapter 2 demonstrated that a novel class of commensal bacteria, lymphoid tissue-resident commensal bacteria (LRC), colonizes the interior of intestinal-associated lymphoid tissues such as the Peyer’s patch and mesenteric lymph node. Within these tissues, LRCs promote local ILC3 and Th17 cell responses but do not promote small intestine lamina propria responses despite detectable LRCs in the lumen. This is in contrast to the epithelial-associated commensal segmented filamentous bacteria (SFB), which promotes potent Th17 responses in both intestinal-associated lymphoid tissues and the small intestine lamina propria (Atarashi et al., 2015; Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Sano et al., 2015). Induction of tissue-specific immune responses is therefore consistent with the colonization pattern of the commensal in the intestine, supporting the notion that commensal bacteria influence local immune responses.

The data in Chapter 2 also revealed that LRCs promote IL-10 production by DCs and that IL-10 acts to limit Th17 cell responses in LRC-monocolonized mice. This may be an evolutionary conserved feature adopted by commensal
bacteria, especially LRCs, in order to limit the development of pathologic inflammation but simultaneously promote maturation of the host immune system. The finding that LRCs induce DC-derived IL-10 parallels the discovery that commensal populations promote regulatory T cell responses that limit pathologic inflammation (Atarashi et al., 2013; Atarashi et al., 2011; Round et al., 2011; Round and Mazmanian, 2010). The human commensal *Bacteroides fragilis* was demonstrated to colonize colonic crypts and is associated with elevated colonic Treg responses (Lee et al., 2013). Similarly, Gram-positive commensal bacteria from the class Clostridia colonize the lumen of the large intestine and also promote potent Treg responses in the underlying lamina propria (Atarashi et al., 2013; Atarashi et al., 2011). Whether LRCs also promote Tregs has not been investigated and warrants future investigation. However, given the ability of LRCs to promote IL-6 and IL-23 *in vitro*, it is likely that LRCs can promote the subset of Tregs that express RORγt as this subset is induced by these cytokines (Ohnmacht et al., 2015; Sefik et al., 2015).

LRCs may have the capacity to bypass intestinal barrier functions to colonize lymphoid tissues. Therefore, it can be hypothesized that LRCs have the capacity to modulate immune responses beyond the intestine. Indeed, neutralization of IL-22-producing ILC3 was demonstrated to result in systemic dissemination of LRCs, which resulted in elevated IL-6 and TNFα (Sonnenberg et al., 2012). Whether LRCs can modulate extra-intestinal immune responses, such as in secondary lymphoid tissues beyond the intestine, at the steady state
requires further experimentation. However, the cellular mechanisms by which LRCs promote tissue-specific immune effects appear to be direct communication between the bacteria and immune cells in the intestine (Figure 42). This is in contrast to the mechanism by which the epithelial-associated commensal SFB elicits Th17 cell responses. In the case of SFB, bacterial attachment is required for and leads to production of SAA1/2 by the epithelium that then induces a Th17 cell response (Atarashi et al., 2015; Sano et al., 2015). Since LRCs do not elicit small intestine lamina propria Th17 cell responses, it is possible that LRCs do not have the ability to attach to intestinal cells and/or regulate their biology. LRCs may be able to access intestinal-associated lymphoid tissues exclusively by being recognized and captured by mononuclear phagocytes that sample intestinal lumen contents. Further research is necessary to decipher the precise molecular and cellular pathways by which LRCs promote tissue-specific immune responses.

5.4 Beneficial properties of lymphoid tissue-resident commensal bacteria colonization in the host intestine

Data from Chapter 3 demonstrated that IL-22 is required for the colonization of LRCs in the mesenteric lymph node. The mechanism of this pathway may involve the production of IL-22-dependent antimicrobial peptides such as the REG3 proteins, which limit the growth of both competing Gram-positive and Gram-negative commensals (Cash et al., 2006; Lehotzky et al., 2010; Mukherjee
et al., 2014; Sonnenberg et al., 2011; Vaishnava et al., 2011). In addition, Chapter 3 provided evidence that exogenous administration of IL-22 to LRC-colonized mice enhanced LRC colonization. The mechanism of this pathway may also be due to upregulation of IL-22-dependent antimicrobial peptides and suppression of competing commensal bacteria. However, given the role of the iron-regulatory hormone hepcidin in intracellular survival of LRCs in vitro, administration of IL-22 may lead to hepcidin-mediated changes in iron homeostasis that favors LRC colonization in lymphoid tissues (Figure 43).

IL-10 but not IL-22 produced by LRC-colonized DCs provide host tissue protection during acute intestinal injury. However, as IL-22 is known to have pro-proliferative functions on inflamed and damaged tissues (Sonnenberg et al., 2011), whether LRC-induced IL-22 plays a role for LRC-induced recovery from acute intestinal injury should be investigated. Therefore, the mechanism by which LRCs protect mice from DSS may involve the coordination of both IL-10 and IL-22, where IL-10 provides protection from acute tissue damage whereas IL-22 may provide signals that promote tissue homeostasis following acute injury. Given the role for IL-22 in enhancing LRC colonization in lymphoid tissues and the fact that IL-22 has tissue protective properties, it would also be interesting to test the hypothesis that IL-22-induced effects may be dependent on LRC colonization (Figure 43).

IL-10 is induced by LRCs both in the gut-draining lymph node and colon lamina propria. In addition, IL-10 appears to function systemically as blockade of
IL-10-IL-10R interactions result in elevated serum IFNγ. The key question of whether lymphoid tissue colonization contributes to and is required for LRC-mediated tissue protection is not well understood but requires further experimentation using approaches that selectively deplete lymphoid colonization by LRCs or mice that lack secondary lymphoid organs. It is likely that in the presence of a complex microbiota, LRCs adopt a more strict lymphoid colonization pattern whereas in antibiotic-treated mice, LRCs can exert its tissue protective effects through interactions with immune cells in both the lamina propria and lymphoid tissues. An alternative explanation for intestinal tissue-protective effects by LRCs may lie in the ability of LRC-primed immune cells to traffic to the site of inflammation. Direct stimulation of immune cells in lymphoid tissues by LRCs may provide a more rapid response by directing tissue-protective lymphocytes (such as those that produce IL-10 and IL-22) to the site of injury. Mice that lack gut homing molecules or trafficking machinery will be able to determine whether LRCs employ this pathway for host-protection.

5.5 Analysis of commensal bacteria in lymphoid tissues during chronic inflammatory diseases

Although the data from Chapter 3 suggest that LRC have beneficial tissue-protective properties for the host, its unique colonization pattern in intestinal-associated lymphoid tissues may result in pathologic inflammation when mechanisms of intestinal barrier function such as the production of mucus and
antimicrobial peptides are impaired. This is supported by translocation of LRCs and development of systemic inflammation in animals that are depleted of IL-22+ ILCs (Sonnenberg et al., 2012). In humans, this possibility was demonstrated in HIV patients that have enhanced systemic immune responses to commensal bacteria. One study showed that patients with HIV have elevated systemic IgG specific for the LRC, *Alcaligenes* (Tatro et al., 2014). Whether systemic immune responses to *Alcaligenes* is due to bacterial translocation from lymphoid tissues is unclear and warrants further investigation. However, these data highlight the dangers of commensal bacteria colonization in lymphoid tissues and thus more research is necessary to understand the mechanisms by which the host harnesses the beneficial but limits the pathological functions of LRCs.

**5.6 Interplay between commensal bacteria, the host immune system and iron homeostasis during health and disease**

Iron is an essential nutrient for all living organisms including mammals, pathogenic and commensal bacteria. Iron scavenging from the environment is therefore of critical importance to all organisms. In the mammals, mechanisms are present to sequester iron for host biological processes and limit their access from pathogenic microbes. Microbes on the other hand have also evolved molecular strategies to scavenge iron. Therefore, there is a constant “tug-of-war” between pathogenic microbes, commensal microbes and mammals for iron. In mammals, one pathway that acts to limit iron available to invading pathogens
involves the iron-regulatory hormone hepcidin (Arezes et al., 2015; Quenee et al., 2012). The receptor for hepcidin, ferroportin, is expressed on macrophages and hepatocytes and functions to export iron from these intracellular stores. Binding of hepcidin to ferroportin results in its degradation and therefore inhibition of iron release into the extracellular space (Cassat and Skaar, 2013; Ganz and Nemeth, 2015). Although there is substantial evidence for the role of hepcidin in limiting iron availability to pathogenic microbes, its role in regulating host-commensal bacteria interactions is much less understood. Chapter 4 demonstrated that loss of hepcidin in vivo was associated with elevated expression of proinflammatory cytokines. Upregulation of the cytokines such as IL-1β and IL-6 in hepcidin-deficient mice was associated with elevated Th17 cell responses. The mechanism by which loss of hepcidin in vivo leads to dysregulated cytokine production is unknown. However, one possibility is that generation of a pro-inflammatory immune environment is due to expansion of members of the microbiota in the presence of iron overload. As SFB is a potent inducer of intestinal and systemic Th17 cell responses (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009), fecal 16S rDNA for SFB was measured and the results determined that SFB levels were comparable between hepcidin-deficient and wild-type mice. One experiment to interrogate whether other members of the microbiota may be responsible for elevated Th17 cells will involve the use of broad-spectrum antibiotics or derivation of hepcidin-deficient animals under germ-free conditions. Given the DC-intrinsic role for hepcidin in LRC colonization
in vitro, one may also speculate whether altered Th17 cell responses are due to dysregulated host-LRC interactions. One hypothesis is that in the absence of hepcidin, levels of intracellular LRCs are reduced leading to less production of IL-10 and unrestrained Th17 cell responses. Experiments measuring the level of LRCs in lymphoid tissues of Hamp1−/− mice should address this possibility (Figure 44).

An alternative explanation for elevated pro-inflammatory immune responses in hepcidin-deficient mice is due to elevated production of reactive oxygen intermediates (Figure 39). Under the setting of iron overload, the redox potential of iron generates reactive oxygen species and hydroxyl radicals that can damage the lipids, DNA and protein of host tissues (Cassat and Skaar, 2013; Ganz and Nemeth, 2015). Production of ROS and cellular toxicity has been associated with increased expression of pro-inflammatory cytokines and increased Th17 cell responses (Naik and Dixit, 2011; Zhi et al., 2012). Future experiments using gain and loss of function approaches will be necessary to determine whether ROS levels are elevated in Hamp1−/− mice and are involved in hepcidin-mediated immune regulation.

The animals used in Chapter 4 as controls for hepcidin-deficient mice were age and gender-matched mice that were purchased from Jackson Laboratories. These mice were cohoused with hepcidin-deficient mice for at least 3 weeks prior to use to normalize their microbiota composition. Although the data suggest that loss of hepcidin leads to significant immune abnormalities, future
experiments should compare $Hamp1^{+/}$ mice to littermate controls to rule out the possibility that the phenotypes observed are due to animals that have been reared under different environments. Experiments comparing littermates and knockout mice generated from breeding $Hamp1^{+/}$ mice are currently on going to address this concern.

5.7 Concluding remarks

Commensal bacteria communities are essential inhabitants of the mammalian GI tract. These bacteria aid in nutrient metabolism, prevent pathogen colonization and promote maturation of the immune system. Our current understanding of commensal bacteria colonization suggest that they colonize at least three distinct anatomical sites: i) the intestinal lumen, ii) the intestinal epithelial surface and iii) intestinal-associated lymphoid tissues. Commensal bacteria from each of these three sites appear to interact with the host immune system in distinct manners. Luminal commensal bacteria are physically separated from the mucosal surface by antimicrobial peptides and mucus and influence host immune cell functions primarily through production of bacterial metabolites. Epithelial-associated commensal bacteria can colonize the surface of intestinal epithelial cells and interact with the host by altering the function of intestinal epithelial cells. Data in this thesis demonstrated that LRCs interact with the host via colonization of lymphoid tissues and activate innate and adaptive immune cells such as DCs, ILCs and CD4$^+$ T cells. A comprehensive understanding of how these three
commensal populations interact and coordinate immune cell homeostasis is an area for future investigation and would involve the development of novel gnotobiotic mouse models. Understanding the contexts by which anatomically distinct commensal communities interact with the host may inform the development of immunologic- and bacterial-based therapeutic strategies for the treatment of chronic inflammatory diseases in people.
Figure 40. Spatial segregation of commensal bacteria in the intestine based on oxygen requirements.

Figure 40. Lymphoid tissue-resident commensal bacteria are predominantly Proteobacteria, while luminal commensals are predominantly Firmicutes or Bacteroidetes. Proteobacteria are typically gram-negative obligate aerobes or facultative anaerobes and thus differ from luminal microbes by their requirement for oxygen. Oxygen concentration is higher near the surface of the intestinal epithelium compared to the center of the lumen. Therefore, Proteobacteria may be preferentially localize to the intestinal epithelium and thus are more readily sampled by innate immune cells in the mucosa and poised to colonize intestinal-associated lymphoid tissues.
Figure 41. Chapters 2 and 3 demonstrate that LRCs regulate immune cell and tissue homeostasis in gnotobiotic or antibiotic mice where LRCs are the dominant bacterial species in the intestine. In order to fully define LRC colonization, future studies should investigate host-LRC interactions in the presence of a complex microbiota in conventional mice. Specifically, whether LRCs impact immune cell function in the same manner in gnotobiotic/antibiotic mice should be assessed. Previous work demonstrated that LRCs could not be cultured from intestinal-associated lymphoid tissues of conventional mice. Future work should address whether this is due to lack of bacterial viability or LRCs adopting a dormant metabolic state.
Figure 42. Commensal bacterial communities regulate tissue-specific immune cell homeostasis through distinct pathways.

Epithelial-associated commensals

Lymphoid resident commensals

Luminal commensals

Figure 42. Epithelial-associated, lymphoid tissue-resident and luminal commensals regulate immune cell homeostasis through distinct pathways. Epithelial-associated commensals such as SFB physically attach to epithelial cells and promote the production of serum amyloid A (SAA). SAA conditions dendritic cells (DCs) to drive Th17 cell responses. On the other hand, lymphoid tissue-resident commensals colonize the intracellular compartment of DCs and promote DC-derived IL-1β, IL-6 and IL-23. These cytokines are likely responsible for LRC-induced Th17 and ILC3 responses. Luminal commensal bacteria regulate immune cell function likely through the production of bacterial-derived metabolites such as short chain fatty acids (SCFAs). SCFAs condition DCs and epithelial cells to promote regulatory T cell responses.
**Figure 43.** IL-22-induced hepcidin expression may promote LRC colonization and host-beneficial properties.

**Figure 43.** Chapter 3 of this thesis demonstrated that IL-22 enhances host colonization by lymphoid tissue-resident commensal bacteria. Given the role of IL-22 in hepcidin expression and the role of hepcidin in LRC colonization, it is possible that IL-22-induced LRC colonization is dependent on hepcidin upregulation. IL-22 has potent tissue-protective effects by modulating antimicrobial peptide production and tissue regeneration. Given the role of LRCs in host tissue protection during acute intestinal injury, future experiments should test whether IL-22 regulates tissue homeostasis in part through modulation of LRC colonization.
Figure 44. Regulation of immune cell homeostasis by hepcidin through modulation of lymphoid tissue-resident commensal bacteria colonization.

Figure 44. Chapter 5 demonstrated that hepcidin promotes lymphoid tissue-resident commensal bacteria colonization in vitro and limits proinflammatory cytokine production by CD4 T helper cells. Given that LRCs promote IL-10, which restrains Th17 cell responses, hepcidin may regulate CD4 T cell responses through modulation of LRC colonization. In the presence of hepcidin, intracellular colonization by LRCs is favored and thus results in IL-10 production and suppression of CD4 T cell responses. In the absence of hepcidin, intracellular LRC colonization and IL-10 production are impaired. As a result, proinflammatory cytokine production by CD4 T cells is uninhibited.
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