NORTHWESTERN UNIVERSITY

Steps Toward Engineering the Immune System: Understanding Lymphoid Cell Regulation in the Intestine

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ABSTRACT

Intestinal immunity is a critical contributor to host health. The immune system in the intestine maintains both defense against pathogens and homeostasis of intestinal tissue, which is exposed to environmental influences, including microbes and ingested foods. Proper regulation of the immune response is required to prevent damage to the host. We examined the regulation of the immune system in the intestine by interrogating host and microbial factors. We identified a pair of *Helicobacter* species that had a negative influence on the proliferation of a subset of innate lymphoid cells (ILCs) that are important for the defense against bacteria. This opens the door for future investigations that explore the molecular factors produced by microbes that may influence the maintenance of ILCs in the intestine. In addition to microbial factors that influence regulation, host factors, like the aryl hydrocarbon receptor (Ahr), a transcription factor that regulates gene expression in response to environmental signals, play an important role. Therefore, we investigated the role of Ahr in regulatory T cells (Tregs), a T cell subset that prevents exuberant immune responses. We found that Ahr regulates the expression of key homing and retention molecules on Tregs, which reduced Treg frequencies in the large intestine and increased frequencies in the circulation and liver. This dysregulation of Treg homing resulted in greater harm to the host under models of intestinal inflammation. We also identified the role of Ahr in Group 2 innate lymphoid cells (ILC2s), which protect the host against tissue damaging pathogens (e.g., allergens and helminths) and help in tissue repair. We discovered that Ahr plays a role in negatively regulating the immune response of ILC2s by regulating the expression of key functional genes. Together, our studies identified key regulatory mechanisms that can be used to engineer new therapies that promote balance in the immune system and can potentially treat or prevent disease.

ACKNOWLEDGEMENTS

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CHAPTER 1. INTRODUCTION

The intestine contains one of the highest concentrations of immune cells in the body. The immune cells in the gut protect the intestine from pathogens and help maintain tissue homeostasis. However, dysregulation of immune function can lead to disease. Functional deficits in immunity increase the susceptibility of the intestine to microbial infections. Unregulated immune activation can result in autoimmune diseases such as Celiac disease, an improper immune response to dietary antigens, or inflammatory bowel disease (IBD), a dysregulated response to the endogenous microbes in the gut. Our studies focus on the cellular and molecular mediators of immune regulation in the intestine, with the goal of understanding the mechanism of their effects and contributing to the development of new therapies based on that understanding.

The intestine is populated by a multitude of microbes that exist, for the most part, in a commensal (neutral or positive) relationship with the host. These microbes include members from bacteria, archaea, fungi, viruses and protozoans, and are collectively referred to as the host's microbiota. The microbiota have a direct influence on the development, maintenance and activity of the immune system. The microbiota are now viewed as an essential part of a healthy host, and they participate in cross-regulation between their populations and immune cells. In Chapter 3, we examine a host-microbe relationship that negatively affects the frequency of an innate immune population in the intestine and can serve as a model for future investigations of host-microbe interactions.

From the molecular perspective, we examine the role of transcription factors that are highly expressed in the intestine, such as the aryl hydrocarbon receptor (Ahr). Ahr is an inducible transcriptional regulator, which is activated by endogenously- and environmentally-derived signaling molecules. These molecules derive from the class of poly-cyclic hydrocarbons, such as the toxin, dioxin, and the tryptophan metabolite, kynurenine. Ours and other studies have identified a role for Ahr in multiple cell types in the gut, including both hematopoietic and non-hematopoietic cells. In Chapters 4 and 5, we examine the role of Ahr in lymphoid immune cells. Using genetic deletion of Ahr in mice, we examine the functional role of Ahr in regulatory T cells and innate lymphoid cells in the intestine. Looking at the differential expression of the genes in Ahr-deficient immune cells, we identify the transcriptional target genes of Ahr, and we propose a role for Ahr in both the optimal function and balance of immune cells in the intestine.

In Chapter 6, we examine the findings from the studies described in earlier chapters to propose methods of regulation in the intestine. We examine how these regulatory methods might be utilized to develop new therapies. We will discuss the potential to engineer therapies targeting the immune system, and what challenges may be addressed.

CHAPTER 2. BACKGROUND

Material presented in this background chapter was published as a review article in *Cellular and Molecular Life Sciences* (Bostick and Zhou 2016).

2.1 Innate lymphoid cells in intestinal immunity and inflammation

Innate lymphoid cells (ILCs) are a newly identified subset of immune cells of lymphoid origin, although some members – natural killer (NK) and lymphoid tissue inducer (LTi) cells – have been known for some time (Artis and Spits 2015; Hazenberg and Spits 2014; Kiessling et al. 1975; McKenzie, Spits, and Eberl 2014; Mebius, Rennert, and Weissman 1997). ILCs reside at the mucosal barriers and participate in the control of commensal and pathogenic microbes, as well as wound repair and healing. Unlike adaptive lymphoid cells, such as T and B cells, innate lymphoid cells lack antigen-specific receptors. They act quickly upon cytokine stimulation, releasing additional cytokines that facilitate the control of infections and tissue damage. Interestingly, their cytokine expression profiles mirror the cytokine profiles of the various T cell helper subsets (Zhou 2012). It has been recommended that the subtypes of ILCs be categorized, based on their cytokine production and key transcription regulators, into three groups: Group 1 (ILC1), Group 2 (ILC2), and Group 3 (ILC3) (Figure 2.1) (Spits et al. 2013).

The development and function of ILCs have been the focus of intense research over the last few years. Much work has gone into identifying the critical roles of ILCs in the respiratory, urogenital,

and oral-gastrointestinal tracts in health and disease. There are many excellent reviews that describe innate lymphoid cell biology (Artis and Spits 2015; Bordon 2014; Cortez, Robinette, and Colonna 2015; Diefenbach, Colonna, and Koyasu 2014; Gasteiger and Rudensky 2014; Hazenberg and Spits 2014; Klose and Diefenbach 2014; Philip and Artis 2013; L. Rankin et al. 2013; Sonnenberg and Artis 2012; Spits et al. 2013; Spits and Cupedo 2012; von Moltke and Locksley 2014; Walker, Barlow, and McKenzie 2013). In this background chapter, we will focus on the function of ILCs in the gut and the role they play in health and disease.

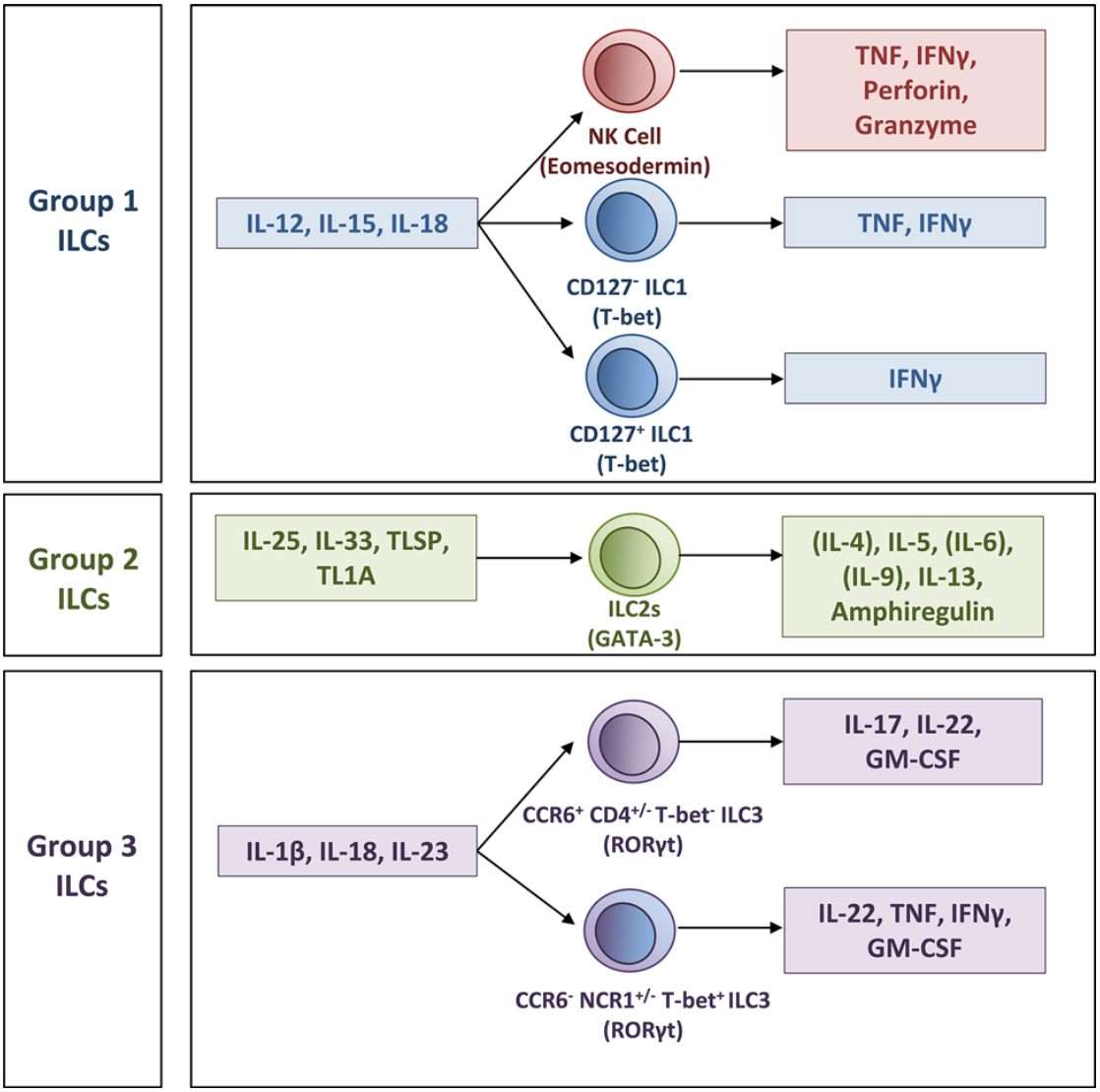


Figure 2.1

Innate lymphoid cells (ILCs) can be categorized, based on their cytokine production and key transcriptional regulators, into three groups: Group 1 - ILC1s (T-bet) and natural killer cells (Eomesodermin), Group 2 - ILC2s (GATA-3) and Group 3 – ILC3s (RORγt). ILCs act quickly upon cytokine stimulation, releasing additional cytokines that facilitate the control of infections and tissue damage. [GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, interferon γ; NCR, natural cytotoxicity receptor; RORγt, RAR-related orphan receptor gamma t; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

2.1.1 Group 1 ILCs

Group 1 ILCs, consisting of natural killer (NK) and ILC1s, are primarily involved in resistance against intracellular pathogens, viruses, and tumors (Bernink et al. 2013; Fuchs et al. 2013; Klose et al. 2014; Robinette et al. 2015; Waldhauer and Steinle 2008). NK cells have cytotoxic functionality, similar to cytotoxic $CD8^+$ T cells (J. C. Sun and Lanier 2011). In contrast, ILC1s have limited cytotoxicity and are similar to T helper 1 (Th1) cells (Fuchs et al. 2013; Klose et al. 2014; Maloy and Uhlig 2013). When stimulated by IL-12, IL-15, or IL-18, NK cells and ILC1s secrete tumor necrosis factor (TNF) and interferon gamma (IFN-γ), and rely on T-box transcription factor (T-bet) as a key transcription factor (Bernink et al. 2013; Fuchs et al. 2013; Klose et al. 2014; Robinette et al. 2015). In mice, ILC1s are identified as a lineage negative, $CD127^+$ $NKp46^+$ $T\text{-}bet^+$ population in the gut lamina propria (Klose et al. 2014). In addition, there is an intraepithelial ILC1 population that expresses CD103, CD160, and NK1.1 (Fuchs et al. 2013). In humans, ILC1s can be identified as either $CD127^{lo}$ intraepithelial ($CD56^+NKp44^+CD103^+$) or $CD127^{hi}$ lamina propria ($CD56^-c\text{-}Kit^-NKp44^-$) cells (Bernink et al. 2013; Klose et al. 2014). Initially classified as ILC1s, natural killer cells are defined by their expression of the transcription factor eomesodermin, which distinguishes them from other Group 1 ILCs. In support, studies indicate that ILC1s, which do not express eomesodermin, derive from a separate and distinct progenitor from NK cells and have different requirements for development (Fuchs et al. 2013; Klose et al. 2014).

2.1.2 Group 2 ILCs

Group 2 ILCs (ILC2s) protect the host against helminth invasion, participate in wound healing, and are implicated in allergic responses (Chang et al. 2011; Eiwegger and Akdis 2011; Halim, MacLaren, et al. 2012; Ho et al. 2015; Miljkovic et al. 2014; J. Mjösberg, Bernink, Golebski, et al. 2012; J. M. Mjösberg et al. 2011; Moro et al. 2010; Neill et al. 2010; Price et al. 2010; Roediger et al. 2013; Roediger and Weninger 2015; Wong et al. 2012; Yang et al. 2011). In mice, ILC2s are typically identified by the expression of CD25, KLRG1, ICOS, or ST2 (a subunit of the IL-33 receptor complex). In humans, ILC2s are identified by the expression of CD161, ST2 and CRTH2 (J. M. Mjösberg et al. 2011). When stimulated with IL-25, IL-33 or thymic stromal lymphopoietin (TSLP), ILC2s primarily produce IL-5 and IL-13 (Yang et al. 2011; Chang et al. 2011; J. M. Mjösberg et al. 2011; Eiwegger and Akdis 2011; Halim, MacLaren, et al. 2012; Price et al. 2010; Fallon et al. 2006). ILC2s, under certain conditions, can also produce IL-4, IL-6, IL-9, and amphiregulin (J. Mjösberg, Bernink, Golebski, et al. 2012; Moro et al. 2010; Neill et al. 2010; Doherty et al. 2013; Turner et al. 2013; Monticelli et al. 2011). Similar to T helper 2 (Th2) cells, the transcription factor, GATA-3, also acts as a key transcription factor for ILC2s (J. Mjösberg, Bernink, Golebski, et al. 2012; Hoyler et al. 2012). ILC2s have been identified in different anatomic locations including: fat tissue, spleen, nasal tissue, lung, intestine, and skin. ILC2s require RAR-related orphan receptor alpha (RORα), T cell factor 1 (TCF1), and Notch signaling for their development (Halim, MacLaren, et al. 2012; Neill et al. 2010; Wong et al. 2012; Fallon et al. 2006; Yang et al. 2013). In the lung and nasal polyps of patients with chronic rhinosinusitis, ILC2s participate in allergic inflammation (J. M. Mjösberg et al. 2011; Halim, MacLaren, et al. 2012; Ho et al. 2015; Miljkovic et al. 2014). More recently, ILC2s have been identified in the skin,

and are involved in skin inflammation (e.g., aptopic dermatitis) (Roediger et al. 2013; B. S. Kim et al. 2013; Salimi et al. 2013).

2.1.3 Group 3 ILCs

Group 3 ILCs (ILC3s) are a heterogeneous population of cells that are involved in resistance to bacterial and fungal infections, control of the commensal bacteria, and development and repair of lymphoid tissues (Satoh-Takayama et al. 2008; Takatori et al. 2009; Cella et al. 2009; Mortha et al. 2014; Sonnenberg et al. 2012, 2011; Munneke et al. 2014; Luci et al. 2009; Scandella et al. 2008). When activated, ILC3s secrete IL-17A, IL-22, TNF and granulocyte macrophage colony-stimulating factor (GM-CSF) (Satoh-Takayama et al. 2008; Takatori et al. 2009; Cella et al. 2009; Mortha et al. 2014; Vacca et al. 2015; Cupedo et al. 2009; Crellin et al. 2010). The RAR-related orphan receptor gamma t (RORγt) and aryl hydrocarbon receptor (Ahr) are key transcription factors that drive the development, maintenance, and function of ILC3s (J. Qiu and Zhou 2013). ILC3s can be divided into $CCR6^+$ cells, which include $CD4^-$ and $CD4^+$ cells, and $CCR6^-$ cells, which express NKp46 in mice or NKp44 in humans ($NKp46/NKp44^+$ ILC3s, NK22, or NCR^+ ILC3s) (Artis and Spits 2015). Fetal $CCR6^+$ ILC3s or lymphoid tissue inducer cells (LTi), participate in the development of gut-associated lymphoid tissue (GALT) (Mebius, Rennert, and Weissman 1997). Human fetal LTi cells lack NKp44 expression, and are precursors to $ROR\gamma t^+$ NK-like cells (Cupedo et al. 2009). Similarly, adult LTi-like cells in mice can upregulate NKp46 expression and become $ROR\gamma t^+$ NK-like cells, or lose expression of RORγt, to become $NKp46^+$ $ROR\gamma t^-$ cells (Vonarbourg et al. 2010). $CCR6^+$ adult LTi-like cells participate in defense against extracellular pathogens (through the production of IL-17A and IL-22) and the repair of lymphoid

tissue (Takatori et al. 2009; Sonnenberg et al. 2011; Scandella et al. 2008). CCR6$^-$ ILC3s produce IL-22 and IFN-γ and rely on the transcription factor, T-bet (Reynders et al. 2011; Powell et al. 2012; Sciumé et al. 2012; Klose et al. 2013; Glatzer et al. 2013). In mice, Notch signaling is important for both adult LTi-like and NKp46$^+$ ILC3 development, although loss of Notch signaling has a more significant effect on NKp46$^+$ ILC3s (Cherrier, Sawa, and Eberl 2012; L. C. Rankin et al. 2013; J. S. Lee et al. 2011; Possot et al. 2011). The importance of Notch signaling in human ILC3s has not been confirmed. Additionally, ILC3s may lose RORγt expression and become exILC3s that adopt an ILC1-like profile (Scandella et al. 2008; Vonarbourg et al. 2010; Klose et al. 2013). More recently, the conversion of CD127$^+$ ILC1s into ILC3s and then back into ILC1s has been reported in humans (Bernink et al. 2015). The conversion of CD127$^+$ ILC1s is mediated by the expression of IL-23 and IL-1β from CD14$^-$ dendritic cells. Additionally, retinoic acid, in cooperation with IL-23 and IL-1β from CD14$^-$ DCs, increases the expression of RORγt in ILC1s. Conversely, ILC3s can become exILC3 and convert into CD127$^+$ ILC1s through the influence of IL-12 and IL-18 produced by CD14$^+$ DCs (Bernink et al. 2015).

2.2 The role of innate lymphoid cells in protective gut immunity

ILCs in the gut protect against many types of pathogens: viruses, intracellular and extracellular bacteria, fungi, and parasites (Klose et al. 2014; Price et al. 2010; Neill et al. 2010; Klose et al. 2013; Campbell and Hasegawa 2013; Hernández et al. 2015). ILCs act quickly, releasing cytokines that stimulate innate and adaptive immune cells and intestinal epithelial cells (Figure 2.2 and 2.3).

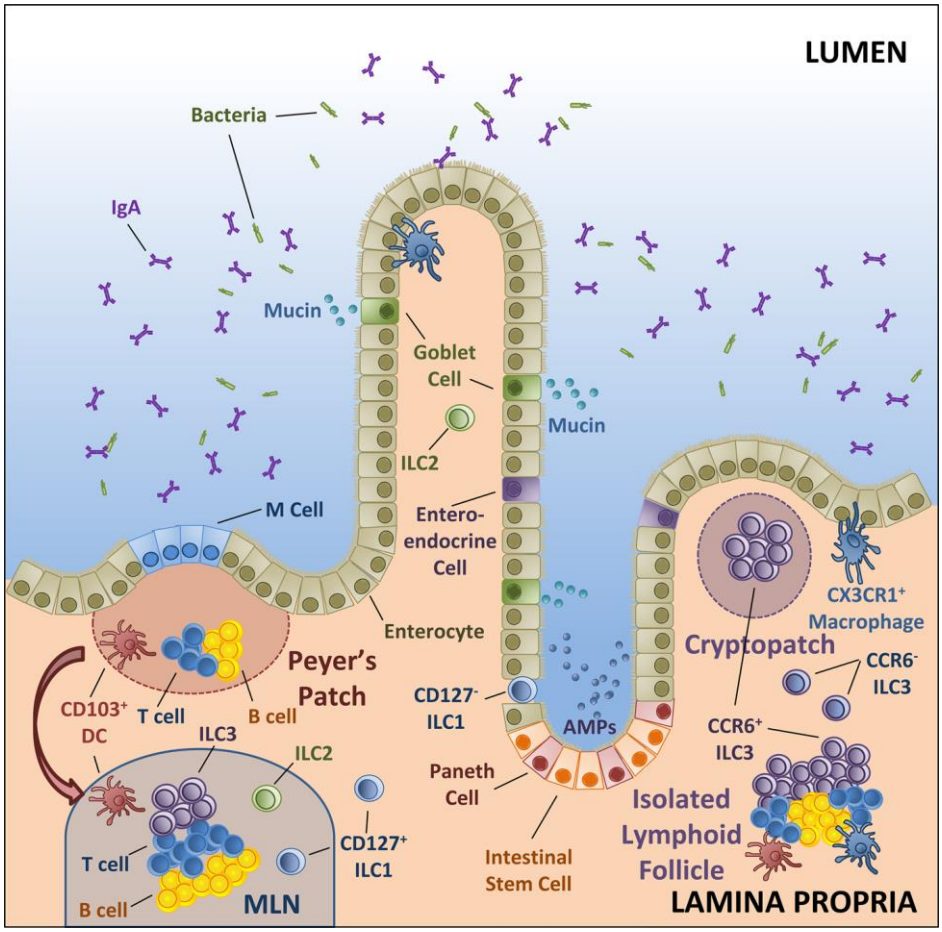


Figure 2.2

By cooperating with epithelial cells and other innate and adaptive immune cells, ILCs participate in the control and tolerance of commensal bacteria. MAMPs (microbe-associated molecular patterns) from commensal bacteria in the intestinal lumen activate epithelial cells and CX3CR1^{+} macrophages, which continuously sample the lumen contents. Additionally, MAMPs and whole bacteria translocate from the lumen into the lamina propria through manifold cells (M cells) located in Peyer's Patches. CD103^{+} dendritic cells take up bacteria and antigen and migrate to the mesenteric lymph nodes, where they interact with T and B cells. In the lamina propria, ILC3s participate in the formation of Peyer's patches, cryptopatches and isolated lymphoid follicles (ILFs), where immune cells aggregate. Cryptopatches mature into ILFs populated with T, B, and antigen presenting cells that facilitate cell-cell interactions. ILC3-induced ILFs are important nodes for the production of IgA, which is transported through the epithelial cells into the lumen. Although ILC3s are the most numerous ILC population in the intestine, ILC1s and ILC2s also play important roles in gut immunity. Intraepithelial ILC1s produce IFN-γ and participate in immunity against intracellular pathogens. ILC2s produce IL-13 which acts on goblet cells, enhancing mucin production during parasite infections. [DC, dendritic cells; MAMPs, microbe-associated molecular patterns]

2.2.1 Cooperation between ILCs and intestinal epithelial cells

ILCs cooperate with intestinal epithelial cells (IECs) to respond to intestinal insults through cytokine and chemokine signaling, or cell-cell interactions. IECs can sense and respond to commensal bacteria and pathogens in the intestinal lumen, as well as injury, and express IL-7, IL-15, IL-25, IL-33, TSLP and TGFβ (Vonarbourg et al. 2010; Pichery et al. 2012; Satoh-Takayama et al. 2010; Zeuthen, Fink, and Frokiaer 2008; Zaph et al. 2008; Atarashi et al. 2011). The cytokines and other soluble factors released by IECs regulate immune responses, including ILCs (Peterson and Artis 2014; J. M. Mjösberg et al. 2011; Neill et al. 2010; Vonarbourg et al. 2010; Satoh-Takayama et al. 2010).

2.2.1.1 Cytokine signaling

IEC expression of IL-7, IL-15, and TGFβ regulates the homeostasis of ILC1s in the mouse and human intestine (Figure 2.3A) (Klose et al. 2014; Fuchs et al. 2013; Vonarbourg et al. 2010; Satoh-Takayama et al. 2010). IL-7 is an important regulator of T cell homeostasis in the thymus, but it also is expressed by epithelial cells in peripheral tissues, such as the intestine and skin, where it participates in the homeostasis of IL-7Rα$^+$ (CD127$^+$) lymphoid cells (e.g., ILCs, T cells, and B cells) (Lodolce et al. 1998). In *Il7*$^{-/-}$ or *Ilra7*$^{-/-}$ mice, which lack expression of IL-7 or the IL-7 receptor, natural killer (NK) cells expand as the numbers of ILCs in the intestine decrease due to their IL-7 dependence (Vonarbourg et al. 2010; Satoh-Takayama et al. 2010). Instead, NK cells depend on IL-15 produced by IECs and DCs for their maintenance in the intestine (Lodolce et al.

1998; Bernink et al. 2013). Intestinal NK cells are absent in mice lacking the IL-15 receptor (IL-15Rα), but there is little effect on intraepithelial ILC1 numbers in the gut (Fuchs et al. 2013). Thus, IL-15-mediated control of homeostasis provides another distinction between NK cells and ILCs, in addition to eomesodermin expression. However, IL-15 does influence the function of ILC1s. In humans and mice, IL-15 from IECs and DCs induces IFN-γ production from ILC1s, and works in cooperation with IL-12, secreted by intestinal DCs, to promote the expression of high levels of IFN-γ (Figure 2.3A) (Fuchs et al. 2013). IFN-γ produced by ILC1s was demonstrated to be essential for protection against infection by the enteric pathogen, *Clostridium difficile*, in a mouse model of the disease (Abt et al. 2015). Additionally, IFN-γ acts on IECs to increase the production of IL-7 (W. Zhang et al. 2015). IFN-γ may contribute to a feedback loop that controls the homeostasis of IL-7-dependent ILCs. In humans, TGF-β maintains the expression of CD103 in intraepithelial ILC1s, helping to maintain them at the intestinal epithelial barrier (Fuchs et al. 2013). The effect of TGF-β on murine ILC1s has not been tested.

IEC-derived cytokines (e.g., IL-25, IL-33, and TSLP) drive type 2 immune responses through the activation of ILC2s (Figure 2.3B). IL-25 plays a critical role in immunity against helminths, such as *Nippostrongylus brasiliensis* or *Trichinella spiralis* (Monticelli et al. 2011; Angkasekwinai et al. 2013). ILC2s respond to IL-25 by producing IL-13 (J. M. Mjösberg et al. 2011; Neill et al. 2010; Fallon et al. 2006; Camelo et al. 2012). IL-13 drives mucus production from goblet cells in the gut, which facilitates helminth expulsion (Figure 2.3B). IL-25 drives an inflammatory subset of ILC2s in the lung, called iILC2s, which express the receptor for IL-25, but express low levels of the receptor for IL-33 (ST2) (Huang et al. 2014). In mice infected with *N. brasiliensis*, iILC2s

proliferate early and produce IL-13, but then transition to $ST2^+$ natural ILC2s (nILC2s) within a few days (Huang et al. 2014). Transfer of sorted iILC2s is enough to control the worm burden in the intestine of a T cell- and ILC-deficient (*$Rag2^{-/-}Il2rg^{-/-}$)* mice (Huang et al. 2014). In the gut, most ILC2s highly express the IL-33 receptor ($ST2^+$) (J. M. Mjösberg et al. 2011; Neill et al. 2010; Roediger and Weninger 2015; Rostan et al. 2015). The IL-33 receptor is composed of two proteins: IL-1 receptor-related protein (IL-1RL1/ST2) and IL-1 receptor accessory protein (IL-1RAcP); IL-33 binds to the ST2 protein, but signaling can only occur through the IL-1RAcP protein (Chackerian et al. 2007). Upon IL-33 binding to ST2 on ILC2s, NF-κB and mitogen-activated protein kinase signaling induce the expression of IL-5 and IL-13 (Hashiguchi et al. 2015). IL-33 has been shown to synergize with TSLP to increase IL-5 and IL-13 production in ILC2s in the lung, nasal tissue, and skin (J. Mjösberg, Bernink, Golebski, et al. 2012; B. S. Kim et al. 2013; Iijima et al. 2014; Halim, Krauss, et al. 2012). Additionally, IL-33 induces the expression of amphiregulin by ILC2s in the gut (Monticelli et al. 2011). Amphiregulin produced by ILC2s signals through the epidermal growth factor receptor (EGFR) on IECs to induce the production of mucus and participate in tissue repair (Brestoff et al. 2015). Therefore, ILC2s can play a protective role in the gut through amphiregulin-mediated tissue protection during injury.

Cytokines produced by IECs can either positively or negatively regulate ILC3s. IECs can positively regulate ILC3s through the secretion of IL-7, which induces signaling that stabilizes RORγt and influences the expression of IL-22 (Vonarbourg et al. 2010; Satoh-Takayama et al. 2010). However, IL-25 produced from IECs negatively regulates IL-22 production by ILC3s through $IL\text{-}17RB^+$ DCs (Figure 2.3C) (Sawa et al. 2011). IL-22 is a member of the IL-10 family

of cytokines, and its production by ILC3s is dependent on the aryl hydrocarbon receptor, Ahr (J. S. Lee et al. 2011; Kiss et al. 2011; J. Qiu et al. 2012). IL-22 binding on epithelial cells induces a STAT3-dependent increase in the production of anti-microbial molecules and stimulates proliferation, required to maintain barrier integrity (J. Qiu et al. 2012; Zheng et al. 2008; Dudakov, Hanash, and van den Brink 2015). Additionally, IL-22 induces the expression of the pro-inflammatory cytokine IL-18 by IECs, contributing to host defense against extracellular pathogens (Muñoz et al. 2015).

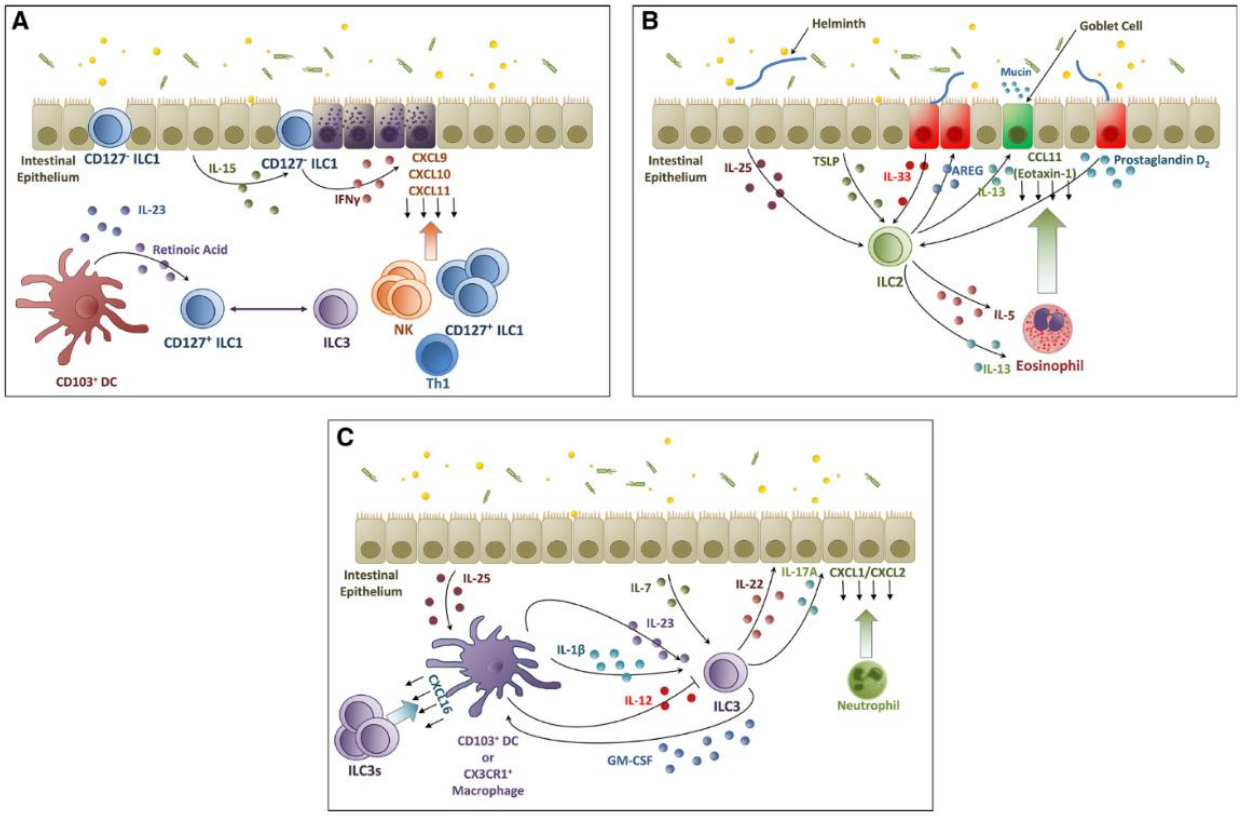


Figure 2.3

(A) ILC1s help protect against intracellular pathogens, viruses, and tumors. IL-15 from intestinal epithelial cells (IECs) induces IFN-γ production from ILC1s. IFN-γ acts on IECs to increase the expression of the chemokines, CXCL9, CXCL10 and CXCL11, which attract CXCR3^{+} leukocytes, including T helper 1 (Th1) cells, ILC1s and NKp46^{+} ILC3s. Retinoic acid, in cooperation with IL-23 from DCs, increases the expression of RORγt in ILC1s and induces their conversion into ILC3s. (B) ILC2s protect against helminth invasion, participate in wound healing, and are implicated in allergic responses. Stimulation by IL-25, IL-33 or thymic stromal lymphopoietin (TSLP) induces ILC2s to produce IL-5 and IL-13. IL-13 drives mucus production from goblet cells in the gut, which facilitates helminth expulsion. IL-33 enhances the expression of amphiregulin by ILC2s in the gut. Amphiregulin plays a tissue-protective role in the gut by participating in injury repair. Prostaglandin D2 (PGD2), which is a pro-inflammatory molecule, also induces chemotaxis of ILC2s and enhances their expression of type 2 cytokines. (C) ILC3s are involved in resistance to bacterial and fungal infections, control of the commensal bacteria, and development and repair of lymphoid tissues. IECs can positively regulate ILC3s through the secretion of IL-7, which induces signaling that stabilizes RORγt. Secretion of pro-inflammatory cytokines (e.g. IL-1β and IL-23) by activated antigen presenting cells induces the production of IL-17A, IL-22 and GM-CSF by ILC3s, while IL-12 suppresses ILC3 function and mediates their conversion into ILC1s. IL-17A participates in the recruitment of neutrophils, an important effector cell for extracellular pathogen immunity, by inducing the expression of CXCL1 and CXCL2 by IECs. IL-22 induces the

production of antimicrobial peptides in intestinal epithelial cells, which is critical for the containment of commensal. GM-CSF is an important cytokine that participates in the homeostasis of mononuclear phagocytes in the intestine. Additionally, CXCL16 produced by DCs recruits ILC3s which express CXCR6. [AREG, amphiregulin; DC, dendritic cells; IECs, intestinal epithelial cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, interferon γ]

2.2.1.2 Chemokine signaling

Chemokines expressed by IECs control ILC trafficking and localization. Although there are limited studies focused on chemokine signaling in ILCs, clues can be inferred by studies of other lymphocytes and shared expression patterns of chemokine receptors on ILCs and T cells. Under inflammatory conditions, IECs express the chemokines, CXCL9, CXCL10 and CXCL11, which attract $CXCR3^+$ leukocytes, including T helper 1 (Th1) cells, ILC1s, and $NKp46^+$ ILC3s (CXCR3 expression on ILCs is based on microarray analysis) (Robinette et al. 2015; Groom and Luster 2011). IEC expression of CCL20 and CCL25 facilitates the recruitment of $CCR6^+$ and $CCR9^+$ ILC3s to the gut, respectively (Griffith, Sokol, and Luster 2014). Gut ILC2s express CCR9 and integrin α4β7, which they acquire during their development in the bone marrow, independent of retinoic acid (Neill et al. 2010; M. H. Kim, Taparowsky, and Kim 2015). On the other hand, retinoic acid from DCs can imprint a gut-homing phenotype on ILC1s and ILC3s (but not ILC2s) by upregulating CCR9 and the integrin α4β7 (M. H. Kim, Taparowsky, and Kim 2015). Additionally, IECs in the colon express CCL28, which recruits CCR10-expressing T lymphocytes to the colon (W. Wang et al. 2000; Xiong et al. 2012). Increased numbers of ILCs (ILC1s, ILC2s, and ILC3s) expressing CCR10 were observed in the circulation of patients who had undergone allogenic hematopoietic stem cell transplantation (HSCT) (Munneke et al. 2014). Thus, similar to its expression on T cells, CCR10 expression on ILCs may recruit them to the colon or skin in HSCT patients, as another CCR10 ligand, CCL27, is produced by skin keratinocytes (Pan et al. 2000).

2.2.1.3 Cell-cell interactions

IECs can interact with ILCs through cell-cell interactions to modulate ILC activation. Intraepithelial ILC1s express the integrin αE(CD103)β7, which can bind E-cadherin on IECs (Fuchs et al. 2013; Van den Bossche et al. 2012). Like other intraepithelial leukocytes, the expression of CD103 could help ILC1s localize to the intracellular spaces of the intestinal epithelium (Van den Bossche et al. 2012). In healthy skin, KLRG1 expressed on ILC2s interacts with E-cadherin expressed on epithelial cells and inhibits ILC2 cytokine production (Salimi et al. 2013). Skin epithelial damage decreases the expression of E-cadherin and removes the suppressive effect of KLRG1 on ILC2s (Salimi et al. 2013). KLRG1 may also play an inhibitory role on ILC2s in the intestine, but this has not been tested. Additionally, KLRG1 is expressed on natural killer cells, and plays an inhibitory role, preventing lysis of E-cadherin-expressing cells (Ito et al. 2006).

The expression of natural toxicity receptor ligands on transformed epithelial cells can activate NKp44 receptor on human ILC3s (Glatzer et al. 2013). In humans, NKp44 receptor stimulation of ILC3s from the tonsils or colon, together with cytokine stimulation (e.g., IL-1β and IL-23), enhances the production of ILC3 cytokines (e.g., IL-22, GM-CSF, and TNF) (Glatzer et al. 2013). However, in mice, the function of NKp46 in ILC3s remains elusive (Satoh-Takayama et al. 2009).

2.2.2 ILC cross-talk with innate immune cells

Two major subsets of $MHCII^{+}CD11c^{+}$ antigen presenting cells are present in the intestine and interact with ILCs: $CD103^{-}CD11b^{+}CX3CR1^{+}$ mononuclear phagocytes ($CX3CR1^{+}$ MNPs) and

$CD103^+CD11b^+CX3CR1^-$ dendritic cells ($CD103^+$ DCs) (Bogunovic et al. 2012). Additionally, there is a minor population of $CD103^+CD11b^-CX3CR1^-$ DCs ($CD11b^-$ DCs) (Bogunovic et al. 2012). $CD11b^-$ DCs reside in lymphoid aggregations such as isolated lymphoid follicles and Peyer's Patches; their function is not completely understood (Bogunovic et al. 2009).

$CX3CR1^+$ MNPs are a highly phagocytic population that participates in the clearance of invading pathogens and damaged epithelial cells. $CX3CR1^+$ mononuclear phagocytes support ILC3 production of IL-22 during colitis by producing large amounts of the cytokines IL-23 and IL-1β (Figure 2.3C) (Longman et al. 2014; Manta et al. 2013). IL-22 production induced by pro-inflammatory cytokines, IL-1β and IL-23, is mediated by IL-1R/MyD88 and STAT3 signaling pathways, respectively (Reynders et al. 2011; Y. Lee et al. 2013; Guo et al. 2014; Hughes et al. 2010). TLR-stimulated $CX3CR1^+$ MNPs induce more IL-22 expression in ILC3s than $CD103^+$ DCs, and ILC3s were observed in close proximity to $CX3CR1^+$ MNPs. Further, depletion of $CX3CR1^+$ MNPs resulted in reduced IL-22 production and more severe pathology in *Citrobacter rodentium* infection (a mouse model of human enteropathogenic *Escherichia coli* infection) (Longman et al. 2014). A more recent study showed the dependence of ILC3-mediated IL-22 production on $CX3CR1^+$ MNPs by CXCL16-CXCR6 axis-mediated recruitment of the $NKp46^+$ ILC3 subset to the gut (Satoh-Takayama et al. 2014).

$CD103^+$ DCs respond to bacterial pathogen-associated molecular patterns (PAMPs), migrate to the lymph nodes, and prime naïve T cells, dependent on CCR7 expression (Bogunovic et al. 2009).

$CD103^+$ DCs secrete retinoic acid and TGFβ, which induce regulatory T cells (Coombes et al. 2007; C.-M. Sun et al. 2007; Kang et al. 2007; Mucida et al. 2009). They also have the ability to imprint a gut homing phenotype to T cells in the lymph nodes by upregulating CCR9 and α4β7 (Johansson-Lindbom et al. 2005; Benson et al. 2007; Menning et al. 2010; Siewert et al. 2007). Retinoic acid produced by $CD103^+$ DCs can also enhance IL-22 production from ILC3s through binding of the retinoic acid receptor (RAR) to the *Il22* locus (Mielke et al. 2013; Spencer et al. 2014). In mice and humans, intraepithelial ILC1s respond to IL-12, secreted by DCs and macrophages, and increase IFN-γ production (Fuchs et al. 2013). In response to macrophage-derived IL-1β, ILC3s participate in a feedback loop by producing GM-CSF that acts on mononuclear phagocytes to control their numbers in the intestine (Mortha et al. 2014).

Molecules from the TNF family can modulate ILC function. Lymphotoxin produced by ILC3s influences IL-22 expression, through a DC-mediated feedback loop; blocking the lymphotoxin receptor, LTβR (LTBR/Tnfrsf3) on DCs, decreases IL-23 expression by DCs and reduces IL-22 production by ILC3s during *C. rodentium* infection (Tumanov et al. 2011). TL1A (Tnfsf15), expressed by myeloid cells and epithelial cells during inflammation, induces an expansion of ILC2s, independent of IL-25 or IL-33, through DR3 (Tnfrsf25) signaling (Yu et al. 2014; Meylan et al. 2014). Activated ILC2s express glucocorticoid-induced TNFR family related gene (GITR/Tnfrsf18) (Moro et al. 2010). However, the role of GITR in ILC2s has not been investigated. ILC3s express CD40L (Tnfsf5), which participates in the induction of antibody secretion by splenic B cells (Magri et al. 2014).

Cytokines produced from ILCs can help to attract and retain other innate effector cells, including other ILCs, eosinophils, and neutrophils. IFN-γ produced by ILC1s can induce the expression of chemokines (CXCL9, CXCL10, and CXCL11) by intestinal epithelial cells that attract $CXCR3^+$ NK cells, ILC1s and T cells (Figure 3A) (Dwinell et al. 2001; Groom and Luster 2011). Eosinophils, activated by IL-5 produced by ILC2s, are essential for the repair of tissue injuries and the clearance of helminth infections (Figure 3B) (Nussbaum et al. 2013). Additionally, IL-5 and IL-13 from ILC2s maintain eosinophils in the small intestine, which promote IgA production from B1 B cells (Nussbaum et al. 2013; Jung et al. 2015). In the lung and mesenteric lymph nodes, ILC2s produce IL-9, which acts in an autocrine manner to promote the survival of ILC2s during *N. brasiliensis* infection (Turner et al. 2013; Gerlach et al. 2014). Finally, IL-17A produced by ILC3s induces the expression of chemokines (e.g., CXCL1 and CXCL2) and growth factors (e.g., granulocyte-colony stimulating factor) in inflamed tissues that recruit neutrophils into the intestine (Figure 3C) (Griffith, Sokol, and Luster 2014; Bulek et al. 2011).

More recently, studies have found that eicosanoids produced by innate effector cells, such as granulocytes, shape ILC immune function (J. M. Mjösberg et al. 2011; Doherty et al. 2013; Xue et al. 2014). ILC2s respond to eicosanoids: lipid-derived modulators of immune function produced by mast cells, eosinophils, and macrophages. ILC2s in the lung and bone marrow express the cysteinyl leukotriene receptor (CysLTR1), and signaling by cysteinyl leukotriene D_4 (LTD4) induces the production of IL-4, IL-5 and IL-13 (Doherty et al. 2013). In the blood, lungs, intestine,

and skin, human ILC2s express CRTH2 (chemoattractant receptor-homologous molecule expressed on TH2 cells). CRTH2 and the prostaglandin D2 receptor (DP1) are two distinct receptors for prostaglandin D_2 (PGD2), which is a pro-inflammatory molecule that also induces chemotaxis and the expression of type 2 cytokines by ILC2s (Figure 3B) (J. M. Mjösberg et al. 2011; Xue et al. 2014). In contrast to LTD4 and PGD2, lipoxin A_4 (LXA4), a pro-resolving eicosanoid, can inhibit the production of IL-13 by ILC2s in the lung (Barnig et al. 2013).

2.2.3 Dialog between ILCs and the adaptive immune system

ILCs can interact with adaptive immune cells through cytokines or cell-cell interactions. Growing evidence indicates that ILCs participate in a dialog with T cells, and this dialog is mediated, in part, through the expression of MHC class II (MHCII) molecules on ILC2s and ILC3s (Hepworth et al. 2013; Oliphant et al. 2014; Mirchandani et al. 2014; Korn et al. 2014). Depending on the expression of co-stimulatory molecules, MHCII:TCR interaction can result in T cell activation, anergy, or deletion (Hepworth et al. 2013; Oliphant et al. 2014; Hepworth et al. 2015). ILC2s in the lung express MHCII molecules with co-stimulatory molecules (CD80 and CD86), and can activate T cells (Oliphant et al. 2014). In turn, activated T-cells express IL-2, which induces the proliferation of ILC2s (Oliphant et al. 2014; Mirchandani et al. 2014). In contrast, ILC3s in the intestine express MHCII molecules but lack expression of CD80 and CD86 co-stimulatory molecules (Hepworth et al. 2013). T cell receptor interaction with MHCII expressed on intestinal ILC3s is thought to promote tolerance due to the lack of co-stimulation. When commensal bacteria-specific T cells encounter antigen presented by MHCII molecules on $CCR6^+$ ILC3s, T cell apoptosis is induced (Hepworth et al. 2015). Although, ILC3s do not express CD80 and CD86,

they do express CD30L and OX40L, which may play a role in the maintenance of $CD4^+$ T cell memory (Withers et al. 2012). Interestingly, $CD4^+$ T cells control ILC3 numbers through a TCR:MHCII interaction-dependent mechanism as well; however, more studies are needed to identify exactly how ILC3 numbers are controlled by T cells (Korn et al. 2014).

ILC3-induced isolated lymphoid follicles (ILFs) are important nodes for T-cell-independent IgA production by B cells (Tsuji et al. 2008; Bouskra et al. 2008). Although ILC3s play a role in promoting optimal IgA production, they are not required (Kruglov et al. 2013) *Id2*$^{-/-}$ mice which lack ILCs and $CD11c^+CD11b^+$ DCs have IgA^+ plasma cells in the lamina propria (Uematsu et al. 2008). A recent study provides mechanistic evidence that human ILC3s may directly interact with B cells, as expression of tumor necrosis factor (TNF), lymphotoxin alpha (LTα), B-cell activating factor (BAFF), and CD40L by ILC3s can stimulate splenic B cells to mature and become plasma cells (Magri et al. 2014).

The interaction of ILCs with adaptive immune cells is facilitated by ILC migration to draining mucosal lymph nodes (Mackley et al. 2015). Equal numbers of ILC1s, ILC2s, and ILC3s enter most peripheral lymph nodes, including inguinal, popliteal, brachial, mediastinal and mesenteric lymph nodes. However, in the mesenteric lymph nodes (MLNs) and mediastinal lymph nodes (mdLNs), $CCR6^+$ ILC3s are the majority (Mackley et al. 2015). In the lymph nodes, $CCR6^+$ ILC3s localize to the interfollicular zones (Mackley et al. 2015). This localization may help facilitate the interaction of ILC3s with T and B cells. The migration of $CCR6^+$ ILC3s to the mesenteric and

inguinal lymph nodes is CCR7-dependent. However, the migration of other ILC subsets is not dependent on CCR7 (Mackley et al. 2015).

2.3 ILCs in intestinal tissue development and homeostasis

ILCs participate in the development of gut-associated lymphoid tissue (GALT). A detailed review of lymphoid tissue development in the gut is not presented here, but can be found elsewhere (Randall and Mebius 2014; van de Pavert and Mebius 2010). Normal GALT development starts with the induction of the formation of Peyer's patches, before birth and independent of the gut microbiota, by fetal lymphoid tissue inducer (LTi) cells (Mebius, Rennert, and Weissman 1997). However, the formation of cryptopatches and isolated lymphoid follicles (ILFs) is a post-natal event mediated by ILC3s, and their maturation in the small intestine is dependent on gut microbiota (Bouskra et al. 2008). Colonic ILFs were reduced upon conventionalization of germ-free mice, suggesting that they may develop differently than small intestine ILFs (Donaldson et al. 2015).

In addition to formation of GALT, ILCs could potentially play a role in intestinal functions that are regulated by neurons (Robinette et al. 2015; Nussbaum et al. 2013). ILCs can sense and secrete neuropeptides, indicating potential crosstalk between ILCs and peripheral neurons (Robinette et al. 2015; Nussbaum et al. 2013). ILC2s in the lungs and intestine express the vasoactive intestinal peptide (VIP) receptor 2, Vipr2 (Nussbaum et al. 2013). ILC2s and adult LTi-like cells secrete Bmp2, which may play a role in regulating gastrointestinal motility, although this has not been tested (Robinette et al. 2015).

2.4 Regulation of commensals by ILCs

ILCs support critical mechanisms for bacterial control and anatomical containment: mucus production, anti-microbial peptide and IgA secretion, and barrier integrity (Sonnenberg and Artis 2012; Moro and Koyasu 2015). ILC3s produce IL-22, which acts on epithelial cells to enhance the secretion of anti-microbial peptides and promotes the anatomical containment of bacteria (J. Qiu et al. 2012; Zheng et al. 2008). IL-22 deficiency leads to bacterial dissemination (Sonnenberg et al. 2012; J. Qiu et al. 2013; Medina-Contreras et al. 2011). IL-22 from ILC3s is critical for the containment of *Alcaligenes* and segmented filamentous bacteria (SFB) (J. Qiu et al. 2013; Sonnenberg et al. 2012; V. F.-S. Shih et al. 2014). In addition to producing IL-22, ILC3s support the production of IgA by B cells, an important mediator in the control of commensal bacteria. ILC3s support IgA production by participating in ILF formation and secreting soluble LTα, which stimulate IgA production to control gut microbiota (Tsuji et al. 2008; Bouskra et al. 2008; Kruglov et al. 2013). Further, ILC3s contribute to the tolerant immune environment of the gut through MHCII interactions with T cells without co-stimulation (Hepworth et al. 2013, 2015). Finally, ILC3s maintain helpful commensals through the IL-22- and LTα- mediated induction of fucosylation of proteins expressed on the surface of epithelial cells (Pickard et al. 2014; Goto et al. 2014). Commensal bacteria, like nonpathogenic *E. coli* and *B. acidifaciens*, which express fucose-metabolizing enzymes and importers, are maintained by fucose metabolism, and protect the host from pathogens (Pickard et al. 2014; Goto et al. 2014).

2.5 Regulation of ILCs by commensals

The role of bacteria in the regulation of ILCs is nevertheless controversial. There are conflicting reports about the effects of the microbiota on the development and maintenance of ILCs. The consensus is that the development and maintenance of LTi-like ILC3s is not affected by the presence or absence of gut microbes (Satoh-Takayama et al. 2008; Munneke et al. 2014; Vonarbourg et al. 2010; Reynders et al. 2011; Sawa et al. 2011). The conflicting reports identify differing roles for the microbiota in the maintenance and function of NKp46^{+} ILC3s. Early reports identified that the numbers of NKp46^{+} ILC3s in the intestine and their IL-22 producing function were dependent on the microbiota (Satoh-Takayama et al. 2008; Munneke et al. 2014; Vonarbourg et al. 2010). However, following studies indicated the opposite, that in germ-free mice IL-22 producing NKp46^{+} ILC3s in the intestine were either not affected or increased (Reynders et al. 2011; Sawa et al. 2011).

There are only a few studies that have examined ILC2 numbers in germ-free mice. In one report, ILC2 numbers in the lung were comparable in germ-free mice and specific pathogen-free (SPF) mice (Monticelli et al. 2011). However, in another study, germ-free mice had more ILC2s (as indicated by lineage negative, GATA-3^{+} cells) in the small intestine than conventionally housed mice (Kernbauer, Ding, and Cadwell 2014). The differences in the studies may be attributed to the specific tissues examined; in the small intestine the microbiota may have more influence on the homeostasis of ILC2s than in the lung.

2.6 The role of ILCs in nutrition and host metabolism

2.6.1 Nutrition

There is growing understanding of the effect of nutrition and metabolism on the development and function of the immune system, and the immune system's influence on metabolism. ILCs are responsive to nutrients like vitamin A and D (Mielke et al. 2013; Spencer et al. 2014; Chen et al. 2015; van de Pavert et al. 2014). Retinoic acid, the bioactive metabolite of vitamin A, enhances IL-22 production from ILC3s by binding of the retinoic acid receptor at the *Il22* locus (Mielke et al. 2013). Retinoic acid regulates the expression of gut-homing receptors (CCR9 and integrin α4β7) in ILC1s and ILC3s, but not ILC2s (M. H. Kim, Taparowsky, and Kim 2015). Vitamin A not only affects the function and homing of ILCs, but also their development. Maternal retinoids control the development of ILC3s in offspring through increased binding of RXR (retinoid X receptor) and RAR (retinoic acid receptor) to the *Rorc* locus to promote its transcription (van de Pavert et al. 2014). Further, vitamin A deficiency increases the number of ILC2s by increasing the expression of IL-7Rα, which increases ILC2 survival (Spencer et al. 2014). In the same study, it was shown that vitamin A deficiency decreases the number of ILC3s in the gut of adult mice, consistent with a previous study (van de Pavert et al. 2014). Finally, knockout of the vitamin D receptor increases the protection of mice from *C. rodentium* infection by increasing the number of ILC3s in the intestine, indicating that vitamin D may have a suppressive effect on ILC3s with a precise mechanism yet to be identified (Chen et al. 2015).

2.6.2 Metabolic homeostasis

Recent work has demonstrated that ILCs are involved in the regulation and development of fat tissue and are an important component of metabolic homeostasis (Brestoff et al. 2015; M.-W. Lee et al. 2015; Y. Qiu et al. 2014; Molofsky et al. 2013; Hams et al. 2013; X. Wang et al. 2014).

A role for ILC1s in metabolic homeostasis has not been identified and remains unclear. Although ILC1s can be identified in the fetal and adult liver, their effect on the liver has not been studied (Klose et al. 2014). The two significant cytokines produced by ILC1s, IFN-γ and TNF, have been shown to play roles in metabolic homeostasis by influencing macrophages in adipose tissue (Chawla, Nguyen, and Goh 2011). However, there are no studies identifying a direct role for cytokines produced by ILC1s in metabolic homeostasis.

Healthy adipose tissue contains a number of immune cells that express a type 2 cytokine profile, including Natural Killer T (NKT) cells, eosinophils, alternately activated macrophages (AAMs) and ILC2s (Hams et al. 2013; Wu et al. 2011; Bosurgi et al. 2013). ILC2s in cooperation with eosinophils and AAMs regulate adiposity, beige conversion of white adipose tissue, and beige fat biogenesis (Hashiguchi et al. 2015; Nussbaum et al. 2013; Y. Qiu et al. 2014; Molofsky et al. 2013; Wu et al. 2011). IL-25 or IL-33 activation of ILC2s results in an ILC2-dependent increase of eosinophils and AAMs in adipose tissue (Molofsky et al. 2013; Hams et al. 2013). ILC2s were identified as the predominant source of IL-5 in adipose tissue, and IL-5 promotes the development, activation and proliferation of eosinophils (Nussbaum et al. 2013; Yamaguchi et al. 1988). AAMs

are induced by eosinophil-derived IL-4 and IL-13, express arginase-1 (Arg-1) and IL-10, and promote glucose homeostasis (Molofsky et al. 2013). Deficiency in IL-5 expression or eosinophils results in greater adiposity and disrupts glucose homeostasis mediated by adipose AAMs (Wu et al. 2011).

In the cold-stress response, adipocyte progenitors may be induced to produce beige fat cells in a mechanism that is dependent on eosinophils, AAMs, and catecholamines from myeloid cells (Y. Qiu et al. 2014). ILC2s may also play a role in the cold-stress response through the promotion of eosinophils by IL-5 production, but this has yet to be confirmed. Alternatively, ILC2s can directly induce the biogenesis of beige fat through the production of methionine-enkephalin peptide, which upregulates UCP1 in white fat cells (Brestoff et al. 2015). Together, these data demonstrate a critical role for ILC2s in the development of adipose tissue and metabolic homeostasis.

ILC3s also play a role in metabolic homeostasis through the production of IL-22 and lymphotoxin β (LTβ). ILC3s are the most significant producers of IL-22 in the gut (Sawa et al. 2011; J. Qiu et al. 2012). IL-22 alleviates metabolic disorders through changes in metabolism in the liver (X. Wang et al. 2014). Upon treatment with exogenous IL-22-Fc (IL-22 conjugated to the antibody Fc domain), mice with obesity induced by diet or *db/db* mice (obesity induced by genetics) had reduced serum levels of triglycerides and cholesterol, reduced fasting glucose, and increased insulin sensitivity (X. Wang et al. 2014). In addition, LTβ receptor-deficient mice gain less weight when fed a high fat diet (HFD) compared to wildtype controls (Scandella et al. 2008). High fat

diet-fed LTβR$^{-/-}$ mice had reduced levels of *Il23a* and *Il22* transcripts. Consistent with reduced IL-22 expression, expression of the anti-microbial peptide, RegIIIγ, was reduced, and there was an expansion of segmented filamentous bacteria (SFB) (J. Qiu et al. 2013). ILC3s were identified as the required source of IL-22 necessary for weight gain after HFD treatment (Scandella et al. 2008). Collectively, these studies demonstrate that IL-22 produced from ILC3s is an important regulator of metabolic homeostasis, in addition to its previously identified roles in the maintenance of barrier integrity and anti-microbial peptide production.

2.7 ILCs in intestinal disease

2.7.1 Chronic inflammation in the intestine

Intestinal ILCs can play pathogenic roles in diseases such as ulcerative colitis, Crohn's disease and celiac disease. The frequency of ILC1s is increased in the intestines of patients with Crohn's disease (Bernink et al. 2013). ILC1s in Crohn's disease patients may play a role in the inflammation by their production of IFN-γ (Strober and Fuss 2011). IL-15 has been identified as a central mediator in the pathogenesis of celiac disease through its stimulation of IFN-γ production from intraepithelial lymphocytes, indicating a possible role for IL-15-responsive ILC1s in the disease (Mei et al. 2012). In an oxazolone-induced model of colitis, ILC2s and NKTs play a pathogenic role by promoting IL-13-driven inflammation, which is ameliorated by blocking IL-25 (Camelo et al. 2012). ILC3s can contribute to chronic inflammation in the intestine through pathology driven by IL-23 signaling (Buonocore et al. 2010; Geremia et al. 2011; Cox et al. 2012). The role of ILC3s in chronic inflammation was first elucidated by the observation that IL-23 drives pathology in mice by activating IL-23 receptor-expressing ILCs (ILC3s), which mediate

inflammation through the production of IL-17A and IFN-γ (Buonocore et al. 2010). This observation was supported by the finding that IL-23-responsive ILCs (ILC3s) are increased in patients with IBD (Bernink et al. 2013; Geremia et al. 2011). Additionally, IL-23 signaling mediates the consequences of chemically-induced mouse model of colitis (Cox et al. 2012). Although ILC3s may contribute to chronic inflammation under certain circumstances, their deficiency can also lead to inflammation and disease. In mice, loss of RORγt^{+} cells (T helper 17 [Th17] cells and ILC3s) in the intestine leads to enhanced pathology under inflammatory conditions induced by the gut microbiota (Lochner et al. 2011). The enhanced pathology was mediated by increased tertiary lymphoid tissue generated by B cells and increased neutrophil infiltration (Lochner et al. 2011). The exaggerated response of the immune system, in the case of ILC3-deficiency, indicates that ILC3s are an important component of a balanced immune response to commensal bacteria. Future therapies that address chronic inflammation mediated by ILC3s must take into account their protective function in controlling the microbiota.

2.7.2 ILCs in food allergy

The incidence of food allergy is increasing in developed countries, and research indicates complex interactions between diet, nutrition, the environment, and the immune system (J. Mjösberg, Bernink, Peters, et al. 2012). The role of ILCs in food allergy has been explored only in a limited way. ILC2s participate in allergic inflammation in the lung and produce cytokines (e.g., IL-5 and IL-13) that are known to contribute to the recruitment of eosinophils (Nussbaum et al. 2013; Jung et al. 2015). However, in the gut, depletion of ILC2s in a peanut allergy model only had an effect on eosinophil response, but not on the Th2-driven initiation of allergic response (Chu et al. 2014).

2.7.3 Graft versus host disease

ILC3s play a protective role in graft versus host disease (GVHD) (Munneke et al. 2014; Hanash et al. 2012). In mice, IL-22 produced by ILC3s after bone marrow transplant protects intestinal stem cells from inflammatory damage (Hanash et al. 2012). Activated ILC3s circulating in the blood of patients before and after transplantation are associated with reduced susceptibility to GVHD (Munneke et al. 2014).

2.7.4 Cancer

Chronic inflammation in the intestine has been associated with a higher risk of the development of colon cancer (Boulard et al. 2012; Kirchberger et al. 2013; Chan et al. 2014). The risk for colon cancer development is associated with a genetic locus expressed in the hematopoietic compartment (Kirchberger et al. 2013). ILC3s were identified as the relevant immune cell, and their expression of IL-22 was found to promote colon cancer (Boulard et al. 2012; Kirchberger et al. 2013). Another study demonstrated that IL-23 activates ILC3s and can induce gut tumorigenesis (Chan et al. 2014).

2.8 New roles for ILCs in host health and disease

Our understanding of innate lymphoid cell biology is growing at a tremendous pace. ILCs are critical regulators of intestinal homeostasis. New roles for ILCs have recently been identified in metabolic homeostasis, development of tolerance to commensals, graft versus host disease, and

cancer (Munneke et al. 2014; Brestoff et al. 2015; Hepworth et al. 2013, 2015; M.-W. Lee et al. 2015; Y. Qiu et al. 2014; Molofsky et al. 2013; Hams et al. 2013; X. Wang et al. 2014; Hanash et al. 2012; Boulard et al. 2012; Kirchberger et al. 2013).

Recent studies are revealing details about the development and function of ILCs (Robinette et al. 2015; Klose et al. 2014; A. W. Y. Lim and McKenzie 2015; Constantinides et al. 2014). Genome-wide expression studies of ILCs using microarrays and RNA-Seq may provide clues to the transcriptional identity and functional potential of ILC subsets. Direct comparison between ILCs (ILC1s, ILC2s, and ILC3s) and their T cell counterparts (Th1, Th2, and Th17/22) may reveal both similarities and differences in their transcriptional regulatory network. A recent study by the Immunological Genome Consortium examined the transcriptional expression profiles of several ILC subsets in various tissues, revealing a core ILC signature and potential new functions (Robinette et al. 2015). ILC subtype-specific genes can be used in the future as markers for the cell populations, both in basic research laboratories and in the clinic. Further studies are required to identify the key regulatory circuitry responsible for the differentiation and function of innate lymphoid cells, and generate a more complete picture of the innate lymphoid cell subsets in the intestine and other tissues.

CHAPTER 3. RESULTS: Negative Regulation of Group 3 Innate Lymphoid Cells by Non-gastric Helicobacter Species from an Immunocompromised Mouse

3.1 Introduction

Innate lymphoid cells (ILCs) are a subset of immune cells that are involved in the protection and homeostasis of mucosal and barrier tissues, but sometimes play pathogenic roles in disease (Ebbo et al. 2017; Bostick and Zhou 2016). ILCs can be divided into "helper-like" (Group 1, 2, and 3 ILCs: ILC1, ILC2, and ILC3) and cytotoxic (Natural Killer: NK) ILCs. Both "helper-like" and cytotoxic ILCs express molecules (e.g., cytokines) that promote immunity, but NK cells have the additional capacity to kill other cells through the expression of cytotoxic molecules. "Helper-like" ILCs are mainly tissue-resident at steady-state (with the exception of a population of circulating ILC1), but enter circulation under chronic inflammatory conditions in both mice and humans (Gasteiger et al. 2015; Teunissen et al. 2014; Villanova et al. 2014; Huang et al. 2014). In contrast, NK cells are readily found in the circulation, but may also establish tissue residency (Gasteiger et al. 2015). P-selectin glycoprotein ligand-1 (PSGL-1; *Selplg*) is expressed on the surface of immune cells, and plays a key functional role in their recruitment from the circulation to areas of inflammation and homing to secondary lymphoid tissues during steady-state (Veerman et al. 2007; Haddad et al. 2003). The critical signals involved in driving the movement of ILCs between tissue residence and circulation remain to be determined. Specifically, whether PSGL-1 plays an important role in innate lymphoid cell (ILC) function in the intestine has not been clearly defined.

In this study, we sought to determine the role of PSGL-1 in ILC residence in and circulation from the intestine by crossing PSGL-1 knockout mice with Rag1 knockout mice, which lack T and B cells, but retain ILCs. Strikingly, we found that *Rag1*$^{-/-}$ *Selplg*$^{-/-}$ double knockout mice had severely reduced ILC3s in the colon. The phenotype was transferable to other *Rag1*$^{-/-}$ mice after co-housing, and was not dependent on PSGL-1 deficiency. Moreover, the loss of ILC3s was ameliorated by antibiotics treatment, implicating the microbiota as the major driving factor for the immune phenotypes. Using selective antibiotics and 16S rRNA gene sequencing, we identified that the acquisition and overgrowth of pathobionts *Helicobacter typhlonius* and *Helicobacter apodemus* contributed to a decrease in the proliferative capacity and subsequent loss of ILC3s in the colon.

In human and murine studies, *Helicobacter* species induce pathogenic responses in their hosts, especially under conditions of compromised immunity (J. G. Fox 2002; J. G. Fox et al. 2011). Gastric *Helicobacter* species, such as *H. pylori*, are clinically significant inducers of stomach ulcers and tumors (James G. Fox and Wang 2007). Non-gastric *Helicobacter* species, which populate the intestine rather than the stomach, can induce strong T cell responses and promote the activation and proliferation of both effector and regulatory T cells (Chai et al. 2017; Maloy et al. 2003). Previous studies demonstrated that ILCs participate in the pathogenic response to *Helicobacter*, by promoting the production of pro-inflammatory cytokines (Chai et al. 2017; Buonocore et al. 2010; Coccia et al. 2012; Pearson et al. 2016; Powell et al. 2012). Here, we demonstrate *Helicobacter*-induced suppression of ILC3s, uncovering a previously unrecognized negative regulation of innate lymphoid cells by non-gastric *Helicobacter* species.

3.2 Materials and Methods

Mice

All of the mice in this study were maintained in Specific Pathogen Free (SPF) facilities at Northwestern University or the University of Florida. Mice were of both sexes, littermates, and 6–10 weeks old unless otherwise indicated. C57BL/6 and *Rag1*$^{-/-}$ were purchased from Jackson Laboratory. *Rag1*$^{-/-}$ mice were crossed with *Selplg*$^{-/-}$ mice to generate *Rag1*$^{-/-}$*Selplg*$^{-/-}$ mice. The Institutional Animal Care and Use Committees of Northwestern University and the University of Florida approved all studies with mice.

Bacterial cultures, fecal collection and administration

Helicobacter strains [*Helicobacter apodemus* (MIT 18-1095S), *Helicobacter typhlonius* (MIT 18-1095F), *H. typhlonius* (MIT 97-6810) and *H. rodentium* (MIT 95-1705)] inoculum were collected and frozen in 20% glycerol/Brucella broth at an OD600 of 1.0. Mice were orally gavaged with 200uL of OD600 1.0 bacterial inoculum every other day, a total of three times. Freshly collected feces from *Rag1*$^{-/-}$, *Rag1*$^{-/-}$*Selplg*$^{-/-}$, or mice previously gavaged with *Rag1*$^{-/-}$*Selplg*$^{-/-}$ feces were homogenized in 20% glycerol in sterile PBS and stored as a slurry at -80C. Fecal slurries were pooled, centrifuged, and washed with sterile PBS before resuspension in sterile PBS for gavage (200 uL per mouse, once).

Isolation of Lymphocytes from Intestinal Lamina Propria and Flow Cytometry

Isolation of intestinal lamina propria cells and flow cytometry were done as previously described (J. Qiu et al. 2012). CD16/32 antibody (eBioscience) was used to block the non-specific binding to Fc receptors before surface staining. Lymphocytes isolated from intestinal lamina propria were stained with antibodies against following makers: GATA3 (APC, PE-Cy7), RORγt (Brilliant Violet 421, PE), T-bet (PE-Cy7), Eomes (PerCP-eFluor 710), Ahr (APC), CD117 (PE), ST2 (PE), KLRG1 (PerCP-eFluor 710), CD4 (Alexa Fluor 488), NKp46 (AmCyan, PerCP-eFluor 710), GM-CSF (Alexa Fluor 488), IFN-γ (APC), IL-13 (Alexa Fluor 488), IL-5 (APC), IL-22 (APC), IL-17A (PerCP-eFluor 710), CD45.2 (APC, PerCP-Cy5.5), CD90.2 Brilliant Violet 421), CD117 (APC), CD11c (PE-Cy7) and Siglec-F (Alexa Fluor 647). For staining of ILC1 and NK cells, Lineage marker mix (Lin) contained APC-Cy7 or APC-eFluor 780-CD3, CD19 and Ly6G. For staining of ILC2 and ILC3 cells, Lin contained APC-Cy7 or APC-eFluor 780-CD3, CD5, CD19, B220, CD11b, CD11c, Ter119 and Ly6G. For nuclear transcription factor staining, cells were fixed and permeabilized with Foxp3 staining buffer Kit (eBioscience). For cytokine staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 hours and Brefeldin A (2 μg/ml) was added 2 hours before cells were harvested. The live and dead cells were discriminated by Live/Dead violet viability kit (Invitrogen) or Zombie Aqua Fixable Viability Kit (Biolegend).

Quantitative Reverse-Transcription RT-PCR

RNA from proximal colon tissue (30mg) from *Rag1*$^{-/-}$ and *Rag1*$^{-/-}$*Selplg*$^{-/-}$ mice was isolated with Trizol reagent (Invitrogen). cDNA was synthesized using GoScript™ Reverse Transcription

kit (Promega). Fecal DNA was extracted by Quick-DNA Fecal/Soil Microbe Kit (Zymogen Research) or E.Z.N.A. Stool DNA Kit (Omega Bio-tek). Real-time RT-PCR was performed using SYBR Green (Biorad) with the primers. Measurements were made in duplicate wells, and results were normalized to those obtained with *Actin* for mouse samples and Eubacteria 16S rRNA gene for bacteria.

RNA-seq and analysis

Whole proximal colon tissue (30mg) from ***Rag1***$^{-/-}$ and ***Rag1***$^{-/-}$***Selplg***$^{-/-}$ mice (two biological replicates for each group) was homogenized in Trizol reagent (Invitrogen). RNA was subsequently extracted and total RNA was treated with poly-A enrichment kit and RNA-seq libraries were generated. Barcoded samples were pooled and sequenced over one lane on an Illumina HiSeq 2500 instrument (University of Chicago Genomics Core) to produce 50 bp single-end reads. De-multiplexed raw data files from the sequencing core were analyzed for quality control using FastQC (Babraham Bioinformatics). Reads were mapped (STAR aligner) to the mm10 assembly of the *Mus musculus* genome (National Center for Biotechnology Information) and filtered for uniquely mapped reads. Genome visualization tracks (bedgraph files) were uploaded to the University of California Santa Cruz (UCSC) Genome Browser for visual comparison of expression levels (Meyer et al. 2013; Patel and Jain 2012). Quantitated relative mRNA expression levels (normalized read counts) were calculated based on exon regions using STAR and the mm10 reference genome annotations. Significantly changed genes were identified by DESeq2. Genes found to be significantly changed (q-value ≤ 0.05; fold change

≥ 1.5) were used for pathway analysis with GSEA software and the Molecular Signature Database (MSigDB).

16S rRNA sequencing and analysis

For microbiome analyses, fecal DNA was isolated and amplified with Illumina Miseq compatible primers targeting the 16S rRNA V4–V5 region. Amplicons were purified by QIAquick Gel extraction kit (Qiagene, Madison, WI) and quantified by Qubit 2.0 Fluorometer (Invitrogen, Grand Island, NY) and Kapa SYBR fast qPCR kit (Kapa Biosystems, Inc., Woburn, MA). Equal amounts of amplicons were pooled with 10% of Phix control to generate the DNA library. Sequencing was performed on the Illumina Miseq (Illumina, Inc., San Diego, CA). Sequence analyses were performed using QIIME v.1.9.0. After checking the quality of the sequenced reads, 8 nucleotides (nt) barcodes were extracted from both forward and reverse reads to generate a barcode library. Forward and reverse reads were then joined, and sequence libraries were split based on their corresponding barcodes. We used an open reference operational taxonomic unit (OTU) picking strategy to select OTUs (with 97% identity threshold). Taxonomy was assigned based on the Greengenes reference database. A taxonomic table for each taxonomic level was generated based on the OTU table and bar charts were generated. Differentially significant features at each level were identified by Mann-Whitney (pairwise comparisons) or Kruskall-Wallis test ($p < 0.05$).

Bone Marrow Transfer

Bone marrow ($4x10^6$ cells in total) from *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$*Selplg*$^{-/-}$ mice were intravenously injected into *Rag1*$^{-/-}$ mice irradiated at 550 rads twice with 5 hr intervals. Recipient mice were treated with antibiotics (sulfamethoxazole and trimethoprim suspension, Hi-Tech Pharmacal) for 2 weeks after injection and were analyzed 11 weeks after transfer.

Citrobacter rodentium Infection and Colony-forming Units (CFUs)

Citrobacter rodentium (DBS100, ATCC51459) was cultured overnight in LB medium and the cell density was determined by OD600 measurement. 10^{10} CFUs of bacteria in 200 μl PBS was gavaged orally into each mouse. Body weight was monitored at indicated time points. Fecal contents were plated on MacConkey plates after serial dilution, and the CFUs of *C. rodentium* were counted and normalized to fecal weight after incubation at 37°C for 24 hours.

Dextran Sodium Sulfate (DSS)-Induced Colitis

DSS colitis was induced in mice by providing 2.5% (w/v) DSS (molecular mass = 36–50 kDa, MP Biomedicals) in drinking water for 6 days. Mice were monitored daily for body weight loss, stool consistency, and blood in the stool.

Statistical Methods

Unless otherwise noted, statistical analysis was performed with the unpaired two-tailed Student's t test on individual biological samples with GraphPad Prism. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.3 Results

3.3.1 *Rag1*$^{-/-}$*Selplg*$^{-/-}$ mice have reduced numbers of ILC3s in the colon

To investigate the role of PSGL-1 in ILC maintenance and/or function in the gut, we bred *Rag1*-deficient mice with PSGL-1-deficient mice (*Rag1*$^{-/-}$*Selplg*$^{-/-}$: referred to as RPS), and examined ILC population frequencies among lamina propria lymphocytes (LPL) in the small and large intestines. We observed a significant decrease of ILC3 (CD3^{-}RORγt^{+}) in the large intestine of RPS mice compared to *Rag1*$^{-/-}$ mice (Figure 3.1A and 3.1B), but no significant difference in the small intestine (Figure 3.2B). ILC3s are a heterogeneous population of cells, composed of T-bet^{-} adult lymphoid tissue inducer-like cells, which may or may not express CCR6 or CD4, and T-bet^{+} cells, which can upregulate NKp46 (Klose et al. 2013; L. C. Rankin et al. 2013; Sciumé et al. 2012). Both T-bet^{+} and T-bet^{-} populations showed decreases in frequency in the large intestine, with a more marked loss in the T-bet^{+} ILC3 population (Figure 3.1C and 3.1D). Additionally, we noted a trend towards increased ILC2 frequencies in the large intestine of RPS mice, presumably due to changes in homeostatic balance due to the loss of ILC3s (S. Li et al. 2018) (Figure 3.2B). RORγt^{-} T-bet^{+} ILC (ILC1 and NK cell) frequencies were significantly decreased in RPS compared to control *Rag1*$^{-/-}$ mice (Figure 3.1A and 3.2C). Together, RPS mice demonstrated significantly reduced ILC3 frequencies in the large intestine with a notable reduction of T-bet^{+} ILC3s.

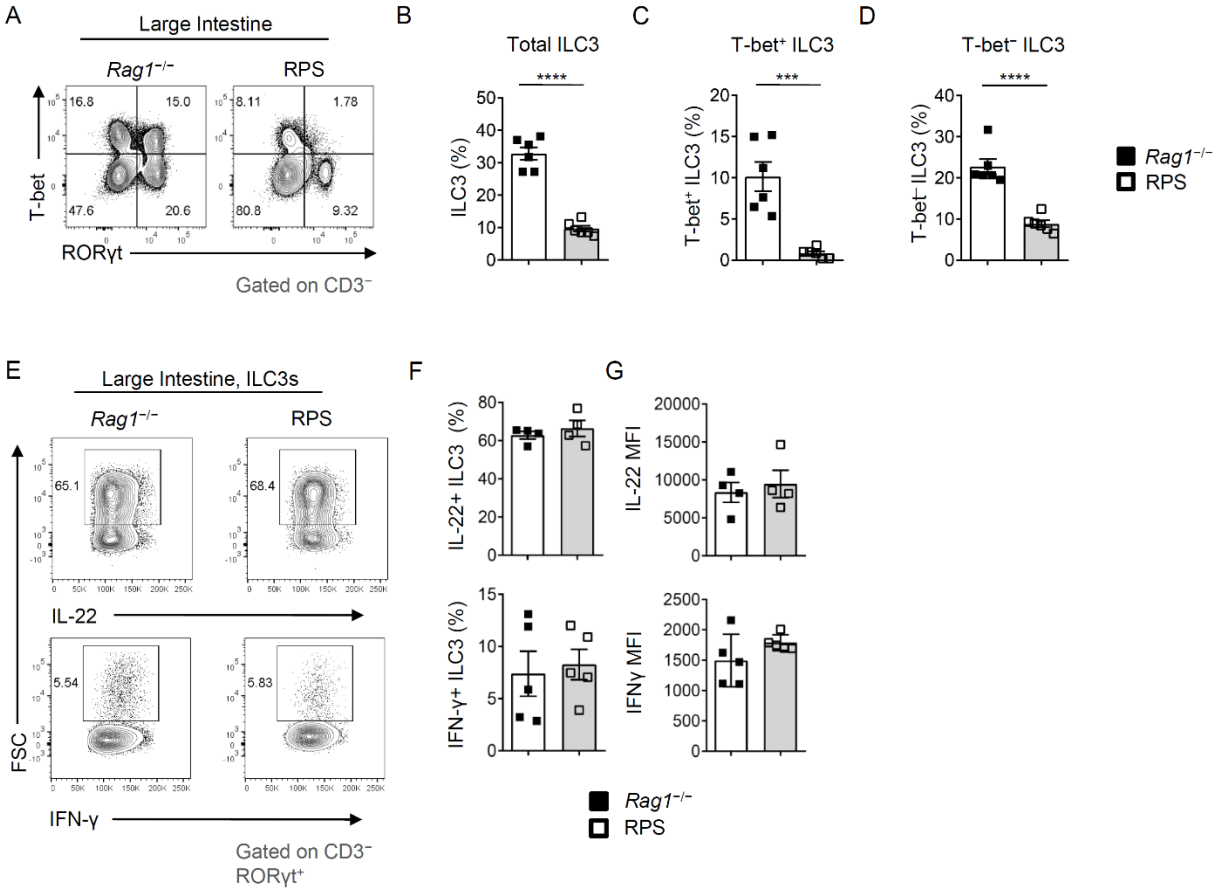


Figure 3.1 ILC3s frequencies are reduced in *Rag1*$^{-/-}$ *Selplg*$^{-/-}$ (RPS) mice.

(A) Representative flow cytometry plots of RORγt and T-bet staining gated on CD3^{-} cells from the lamina propria of the large intestine (LI) from RPS compared to *Rag1*$^{-/-}$ mice. (B) Quantification of ILC3 (CD3^{-} RORγt^{+}), (C) T-bet^{+} ILC3 and (D) T-bet^{-} ILC3 frequencies. (E) Representative flow cytometry histogram plots of IL-22 and IFN-γ staining from ILC3s in the LI lamina propria. (F) Quantification of IL-22^{+} and IFN-γ^{+} ILC3 frequencies. (G) Mean fluorescence intensity (MFI) of IL-22 and IFN-γ signal in large intestinal ILC3s. (A-D) Data are pooled from four independent experiments (n=6 per group). (E-G) Data are pooled from two independent experiments (n=4-5 per group). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

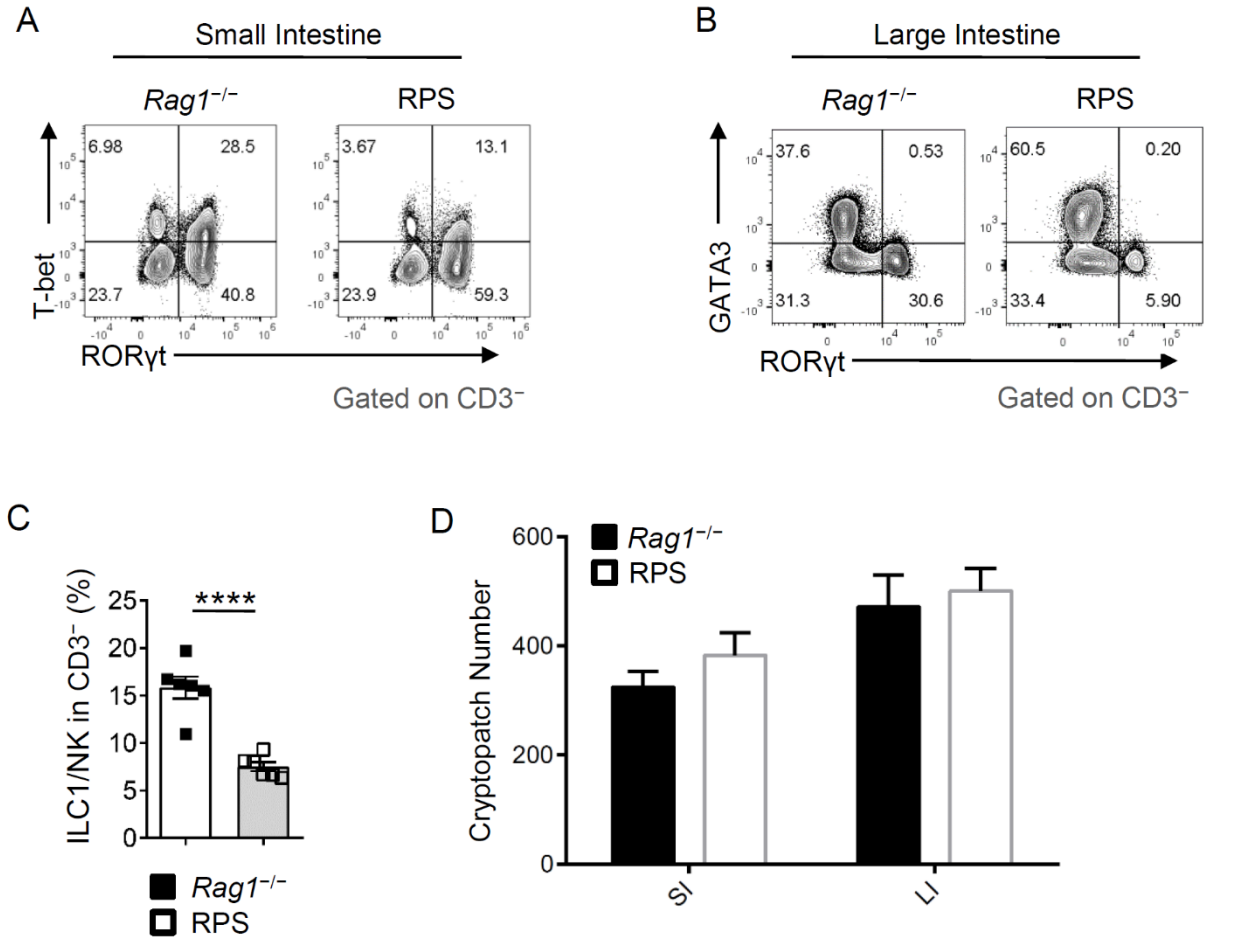


Figure 3.2 RPS mice have normal small intestinal ILC3s and number of intestinal cryptopatches.

(A) Representative flow cytometry plots of RORγt and T-bet staining gated on CD3$^-$ cells from the lamina propria of the small intestine (SI) from RPS compared to *Rag1*$^{-/-}$ mice. (B) Representative flow cytometry plots of RORγt and GATA3 staining gated on CD3$^-$ cells from the lamina propria of the large intestine (LI) from RPS compared to *Rag1*$^{-/-}$ mice. (C) Quantification of ILC1/NK (T-bet$^+$ RORγt$^-$) cell frequencies from the large intestine lamina propria of RPS compared to *Rag1*$^{-/-}$ control mice. (D) Cryptopatch numbers in the small (SI) and large (LI) intestine. (A,B) Representative flow data shown from two independent experiments. (n=2 per group). (C) Data are pooled from two independent experiments (n=5-6 per group). (D) Data from one of two independent experiments with two biological replicates per group. $^{****}P < 0.0001$. Error bars indicate s.e.m.

To evaluate the functional competence of ILC3s in PSGL-1-deficient mice, we first examined cytokine production. In large intestinal ILC3s, there was no change in the frequency of IL-22- or IFN-γ-positive cells (Figure 3.1E and 3.1F). Additionally there was no change in the MFI (mean fluorescence intensity) of these cytokines, indicating that the ability of ILC3s to produce cytokines was not affected on a per cell basis (Figure 3.1G). ILC3s play an important role in the development of tertiary lymphoid structures in the intestines. Examination of cryptopatches by fluorescent microscopy revealed a reduction of ILC3s populating these structures, despite no reduction in their numbers (data not shown and Figure 3.2D). These data suggested that PSGL-1 might play a role in either the homing or maintenance, but not the function, of ILC3s in the large intestine.

3.3.2 A transmissible microbiota population isolated from *Rag1*$^{-/-}$*Selplg*$^{-/-}$ mice suppresses ILC3s in the colon

Initial experiments compared RPS mice to non-littermate *Rag1*$^{-/-}$ control mice. Unexpectedly, when we used co-housed, littermate controls generated through heterozygous breeding **(**Figure 3.3A**)**, we observed no difference in ILC3 frequency between *Rag1*$^{-/-}$*Selplg*$^{-/-}$ and *Rag1*$^{-/-}$*Selplg*$^{+/-}$ controls. Specifically, *Rag1*$^{-/-}$*Selplg*$^{+/-}$ and RPS littermate mice both showed reduced ILC3 frequencies compared to non-littermate, non-co-housed *Rag1*$^{-/-}$ mice (Figure 3.3B and 3.3C). T-bet^{+} ILC3s had the most significant decrease in *Rag1*$^{-/-}$*Selplg*$^{+/-}$ mice co-housed with RPS mice (Figure 3.3C). These data suggested that the ILC3 phenotype observed in RPS littermate mice was transmissible and might be attributed to microbiota that were introduced at the time of generating the double knockout mice, and not to the knockout of *Selplg*. Therefore, to clarify the contribution of the microbiota to the phenotype, we treated the RPS mice with a broad-spectrum cocktail of

antibiotics (Ampicillin, Vancomycin, Metronidazole Neomycin and Gentamicin). After 12 days of treatment, we observed that ILC3 percentages in RPS mice had increased (Figure 3.3D and 3.3E). Notably, the increase in ILC3s was predominantly in the T-bet^{-} ILC3 population, but T-bet^{+} ILC3s either did not recover or only partially recovered (Figure 3.3D and 3.3E). Next, we performed a bone marrow transfer experiment to test the contribution of the genotype (Figure 3.4A). *Rag1*$^{-/-}$ mice were lethally irradiated and bone marrow from either *Rag1*$^{-/-}$ or RPS mice were transferred intravenously. After two months, we examined ILC3 frequencies. ILC3 frequencies were lower in mice that were irradiated and received a *Rag1*$^{-/-}$ bone marrow transfer compared to non-irradiated *Rag1*$^{-/-}$ mice, presumably due to partial recovery of ILC3s after bone marrow reconstitution. Nonetheless, no difference in ILC3 frequencies was observed in mice that received *Rag1*$^{-/-}$ versus RPS bone marrow (Figure 3.4A-C), suggesting that the genotype alone could not account for the ILC3 loss. Moreover, gavage of RPS feces into *Rag1*$^{-/-}$ mice reduced ILC3 frequencies in the colon (Figure 3.3F and 3.3G, Figure 3.4D and 3.4E), but not the small intestine (Figure 3.4F and 3.4G), indicating that the ILC3 phenotype was induced by the microbiota in RPS feces. However, ILC3s were not reduced when wildtype C57BL/6 mice were gavaged with RPS feces, suggesting that adaptive immunity can prevent the loss of ILC3 after RPS feces gavage (Figure 3.3F and 3.3G).

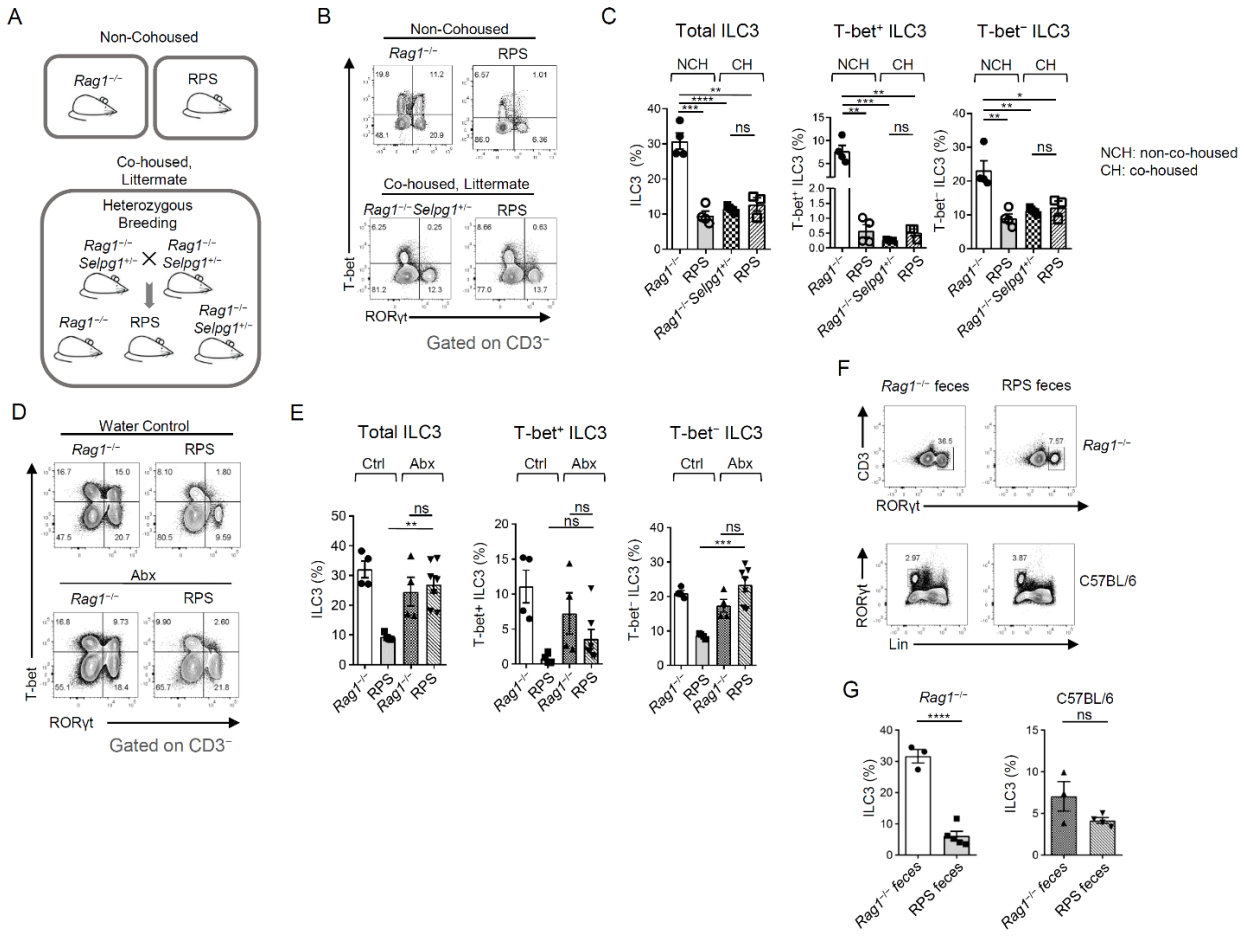


Figure 3.3 The microbiota from RPS mice induce a transmissible dysbiois and loss of ILC3s in littermate and gavaged mice.

(A) Illustration of secondary co-housed/non-co-housed littermate experiment. (B) Representative flow cytometry plots of RORγt and T-bet staining gated on CD3$^-$ cells from the lamina propria of the large intestine (LI) from non-co-housed (top) and co-housed (bottom) RPS, *Rag1*$^{-/-}$ or *Rag1*$^{-/-}$*Selpg1*$^{+/-}$ mice. (C) Quantification of ILC3 (Total, T-bet$^+$ and T-bet$^-$) frequencies from *Rag1*$^{-/-}$*Selplg*$^{+/-}$ compared to RPS littermate/co-housed or non-cohoused mice. (D) Representative flow cytometry plots of RORγt and T-bet staining gated on CD3$^-$ cells from the lamina propria of the large intestine (LI) from water-treated (Ctrl; top) and antibiotics [Ampicillin, Neomycin, Vancomycin, Gentamicin and Metronidazole cocktail] (Abx; bottom) RPS compared to *Rag1*$^{-/-}$ mice. (E) Frequencies of ILC3s (Total, T-bet$^+$ and T-bet$^-$ ILC3s) in antibiotics-treated mice comparing RPS and *Rag1*$^{-/-}$ mice. (F) Representative flow cytometry plots of RORγt and CD3$^-$ staining (top) or Lineage and RORγt staining (bottom) gated on lymphocytes from the lamina propria of the large intestine. (G) Quantification of ILC3 frequencies 12 days after indicated fecal gavage into *Rag1*$^{-/-}$ (left) and C57BL/6 (right) mice. (B,C) Data are pooled from two independent experiments (n=3-4 per group). (D,E) Data are pooled from two independent experiments (n=4-7

per group). (F,G) Data are pooled from two independent experiments (n=3-5 per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

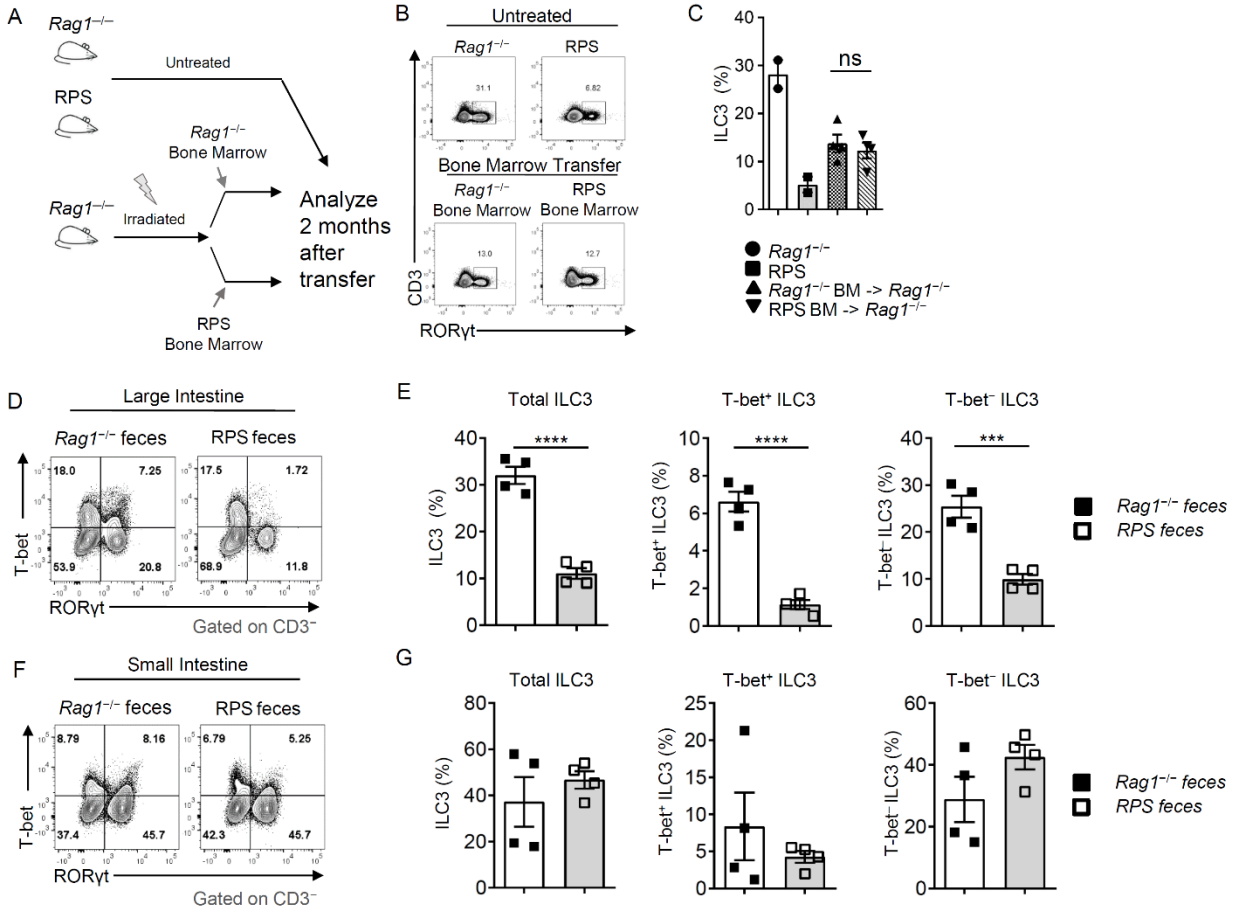


Figure 3.4 The microbiota from RPS mice induce a transmissible dysbiois and loss of ILC3s.

(A) Illustration of bone marrow transfer experiment. (B) Representative flow cytometry plots of RORγt and CD3 staining gated on lymphocytes from the lamina propria of the large intestine. (C) Quantification of ILC3 frequencies in the large intestine of *Rag1*$^{-/-}$ mice receiving RPS or *Rag1*$^{-/-}$ bone marrow compared to untreated RPS or *Rag1*$^{-/-}$ control mice. (D) Representative flow cytometry plots of RORγt and T-bet staining gated on CD3^{-} cells from the lamina propria of the large intestine. (E) Quantification of ILC3 frequencies from the lamina propria of the large intestine of *Rag1*$^{-/-}$ mice gavaged with RPS feces compared to *Rag1*$^{-/-}$ mice mice gavaged with *Rag1*$^{-/-}$ feces. (F) Representative flow cytometry plots of RORγt and T-bet staining gated on CD3^{-} cells from the lamina propria of the small intestine. (G) Quantification of ILC3 frequencies from the lamina propria of the small intestine *Rag1*$^{-/-}$ mice gavaged with RPS feces compared to *Rag1*$^{-/-}$ mice mice gavaged with *Rag1*$^{-/-}$ feces. (B,C) Data from one experiment (n=2-4 per group). (D-G) Data are pooled from two independent experiments (n=4 per group). $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

Feces collected from *Rag1*$^{-/-}$ mice that were previously gavaged with primary RPS feces (2° RPS feces) could also reduce ILC3 percentages in the colon of *Rag1*$^{-/-}$ mice after gavage, indicating that an actively growing or self-sustaining agent is responsible for the ILC3 reduction in the colon (Figure 3.5A-C). This was further supported by the result that heat-killed 2° RPS feces could not significantly decrease ILC3s compared to non-treated feces (Figure 3.5D and 3.5E). Although there was a decrease in ILC1 (Lineage^{-}T-bet^{+}RORγt^{-}EOMES^{-}) frequencies and an increase in ILC2 frequencies, neither ILC1 nor ILC2 cell counts showed significant change after gavage with 2° RPS feces (Figure 3.5F and 3.5G). There was a significant increase of NK cells (Lineage^{-}T-bet^{+}RORγt^{-}EOMES^{+}) after gavage (Figure 3.5F and 3.5G). Furthermore, similar to enhanced gut inflammation observed in primary RPS mice (Figure 3.6A and 3.6B), 2° RPS fecal gavage resulted in increased inflammation and cell infiltration, which coincided with the loss of ILC3s (Figure 3.6C and 3.6D). Increased neutrophils and eosinophils were observed in the large intestine 12 days after gavage (Figure 3.5H and 3.5I). Together, these data implicated the microbiota as the major contributing factor to the loss of ILC3s and inflammation observed in RPS mice.

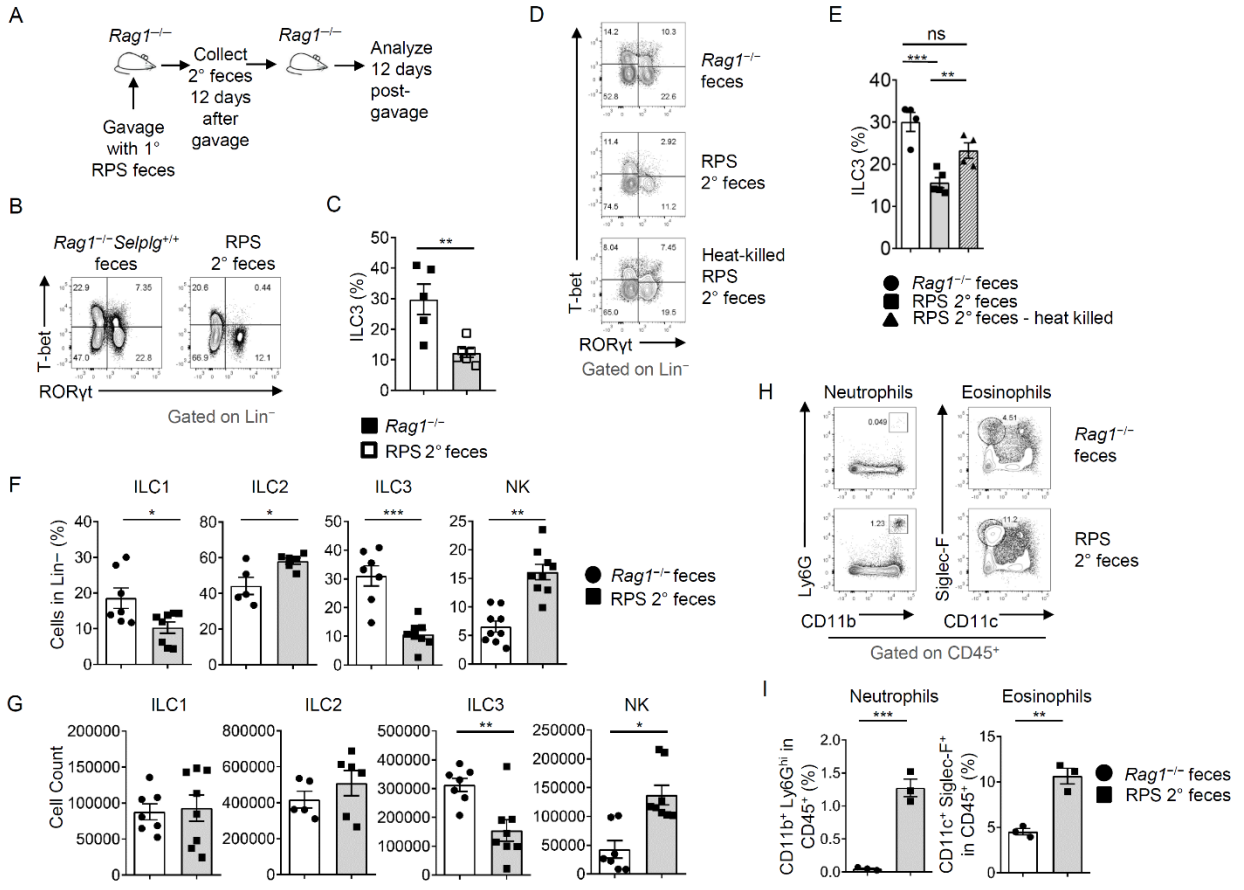


Figure 3.5 Serially-gavaged RPS feces continues to suppress ILC3 frequencies and is associated with inflammation.

(A) Illustration of secondary (2°) RPS feces gavage experiment. (B) Representative flow cytometry plots of RORγt and T-bet gated on Lineage$^-$ cells from the lamina propria of the large intestine. (C) Quantification ILC3 frequencies from the large intestine lamina propria of mice gavaged with secondary (2°) RPS feces. (D) Representative flow cytometry plots of RORγt and T-bet gated on Lineage$^-$ cells from the lamina propria of the large intestine. (E) Quantification of ILC3 frequencies after gavage with heat-killed or non-treated 2° feces. (F) Quantification of ILC frequencies and (G) counts after gavage with 2° RPS feces or *Rag1*$^{-/-}$ control feces. (H) Representative flow cytometry plots of (Neutrophil; CD11b and Ly6g) and (Eosinophil; CD11c and Siglec-F) staining gated on CD45$^+$ cells from the lamina propria of the large intestine. (I) Quantification of neutrophil and eosinophil frequencies in the large intestine lamina propria of *Rag1*$^{-/-}$ mice gavaged with *Rag1*$^{-/-}$ or (2°) RPS feces. (B,C) Data are pooled from two independent experiments (n=5-6 per group). (D,E) Data are from one experiment (n=4-5 per group). (F,G) Data are pooled from three independent experiments (n=5-8 per group). (H,I) Data are representative from one of two independent experiments (n=3 per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Error bars indicate s.e.m.

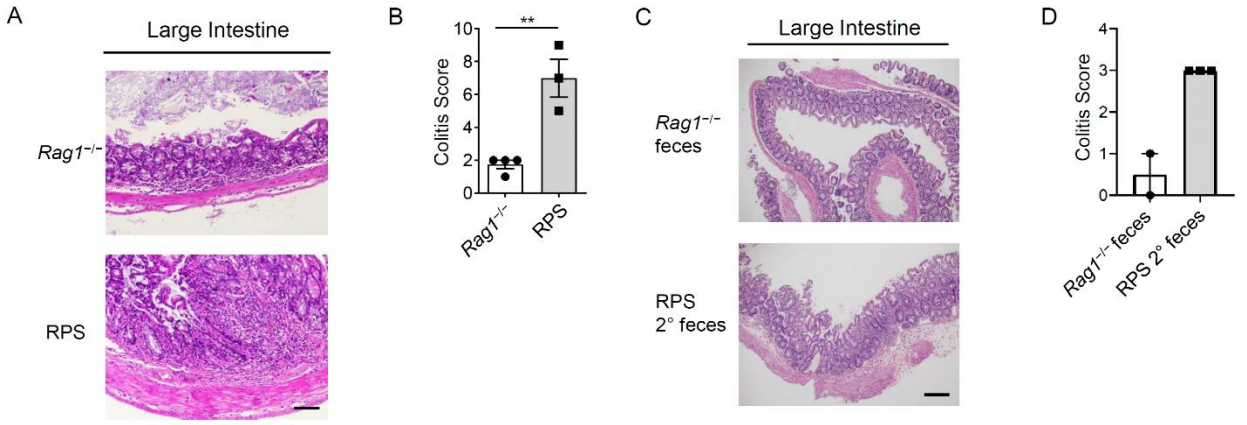


Figure 3.6 Serially-gavaged RPS feces continues to suppress ILC3 frequencies and is associated with inflammation.

(A) Representative histology images of the proximal colon of RPS compared to *Rag1*$^{-/-}$ mice (scale bar: 150 □m). (B) Colitis scores from the large intestine of RPS compared to *Rag1*$^{-/-}$ mice. (C) Representative histology and scoring (D) from the proximal colon of mice gavaged with RPS 2° feces compared to *Rag1*$^{-/-}$ feces (scale bar: 150 □m). (A,B) Data are representative from one experiment (n=3-4 per group). (C,D) Data are representative from one of two independent experiments (n=2-3 per group). $^{*}P < 0.05$; $^{**}P < 0.01$. Error bars indicate s.e.m.

3.3.3 Introduction of RPS microbiota reduces proliferation of ILC3s and enhanced loss of T-bet^{+} ILC3

Changes initiated by the microbiota in the host colon environment of RPS mice could contribute to the loss of ILC3s. To test this, we examined transcriptional changes in the large intestine of *Rag1*$^{-/-}$ mice 12 days after gavage with 2° RPS feces by RNA-seq. Differential gene analysis identified 2815 significantly changed genes (1402 downregulated; 1413 upregulated; [q≤0.05; fold change≥1.5]) in the colon after gavage (Figure 3.7A). Many of the increased genes were related to inflammation and response to infection, including *Tnf, Ifng, Il1b, Il11* and *Il6* (Figure 3.7A). Pathway analysis confirmed that cytokine and chemokine signaling were increased, along with Toll-like and NOD-like receptor pathways (Figure 3.7B, Figure 3.8A and 3.8B). Detailed examination of chemokines and cytokines revealed that genes *Cxcl1, Cxcl2, Cxcl3*, *Cxcl9* and *Cxcl10* related to the chemotaxis of inflammatory cells, such as neutrophils and NK cells, were highly upregulated, consistent with their increased frequencies as demonstrated by flow cytometry (Figure 3.7C, 3.5G and 3.5H). Notably, genes related to ILC3 homeostasis were significantly decreased, including *Il7*, *Il15* and *Kit* (Figure 3.5A and 3.5C). To confirm the RNA-seq results, we examined the transcription of genes in whole colon tissue that are involved in inflammation and the homeostasis of ILCs in the gut, after gavage with 2° RPS feces by qRT-PCR. At day 12 after gavage, we observed no significant difference in pro-inflammatory cytokine transcripts for *Il18 and Il23*, but a significant increase in *Il12a* and *Tnf* in the colon (Figure 3.7D). Previous studies have shown that IL-12 can contribute to ILC3 plasticity, inducing their conversion into ILC1s (Bernink et al. 2015). Additionally, it has been demonstrated that IL-7 influences the maintenance of RORγt expression in ILC3s, and without IL-7, ILC3s become ILC1-like with

increased T-bet and reduced RORγt expression (Vonarbourg et al. 2010). We observed increased expression of *Il12a* (Figure 3.7D), but ILC1s remained unchanged or decreased in percentage after gavage (Figure 3.5F), indicating that ILC3 conversion to ILC1 through plasticity was possible, but not likely a major contributor to the loss of ILC3s.

Changes in homeostatic molecules were also confirmed, with reductions in both *Il7* and *Il15* transcripts (Figure 3.7E**)**, both of which have shown to support ILC3 maintenance in the intestine (Vonarbourg et al. 2010; Robinette et al. 2017). Similar transcriptional changes of cytokines were observed in the cecum (Figure 3.8C). Previous studies have shown that increased IL-25 can reduce ILC3 numbers in the intestine (Sawa et al. 2011), but RPS mice had decreased *Il25* expression (Figure 3.7E). The aryl hydrocarbon receptor (Ahr) is a key transcription factor involved in the maintenance and function of ILC3s (Kiss et al. 2011; J. S. Lee et al. 2011; J. Qiu et al. 2012), and we observed reduced Ahr activity in colonic tissue, indicated by the 5-fold reduction of target gene transcripts (*Cyp1a1* and *Ahrr*) in the colons of mice gavaged with RPS feces (data not shown). However, when we examined transcriptional expression in sort-purified ILC3s, we did not observe any significant difference in the expression of *Cyp1a1* or *Ahrr* (Figure 3.7F), indicating that the overall reduction in Ahr activity in the colon was likely due to the loss of Ahr-expressing cell populations, rather than a change in Ahr activity. This was supported by the observation that the remaining ILC3s in the large intestine after gavage with secondary feces did not have reduced IL-22^+ ILC3 frequencies (data not shown), consistent with the data of primary RPS mice (Figure 3.1E-G). Together, these data indicate that secondary RPS feces gavage can induce a pro-inflammatory environment with reduced homeostatic molecules.

Reduction of homeostatic cytokines, such as IL-7 and IL-15 could contribute to reduced proliferation or survival of ILC3s (Vonarbourg et al. 2010; Robinette et al. 2017; J. Qiu et al. 2012). Thus, we performed a kinetic study of the ILC3 compartment after introduction of RPS 2° feces. Our results showed that at day 3 after gavage with RPS 2° feces there was a significant reduction in $Ki67^+$ ILC3s (Figure 3.7G and 3.7H), with a slight reduction observed as early as day 1 and significant reduction at day 6 (Figure 3.8D-G). We observed no increase in apoptosis in ILC3s, at any time point (Figure 3.7G and 3.7H, Figure 3.8D-G), suggesting that the loss of ILC3s in the large intestine after RPS 2° feces gavage may be mediated by a reduction in proliferation, but not apoptosis. In contrast to day 12 after gavage (Figure 3.7E), when we examined *Il7* and *Il15* expression at day 3, we observed no change or a slight increase in *Il7*, suggesting an alternate mechanism for the early proliferation changes (Figure 3.8H). Therefore, we examined other potential molecules related to the regulation of ILC3 proliferation at early time points after gavage. Ahr and c-Kit (CD117), an Ahr-regulated growth factor receptor, have been shown to control ILC3 proliferation (Klose et al. 2013; Kiss et al. 2011; J. S. Lee et al. 2011; J. Qiu et al. 2012). We examined Ahr and c-Kit expression in ILC3s from the large intestine at day 3 after gavage and found no changes in the expression of either molecule (Figure 3.8I). Together, these results suggest that dysbiosis may lead to the early proliferation changes and reduction of homeostatic promoting factors that are important for sustaining ILC3 compartment.

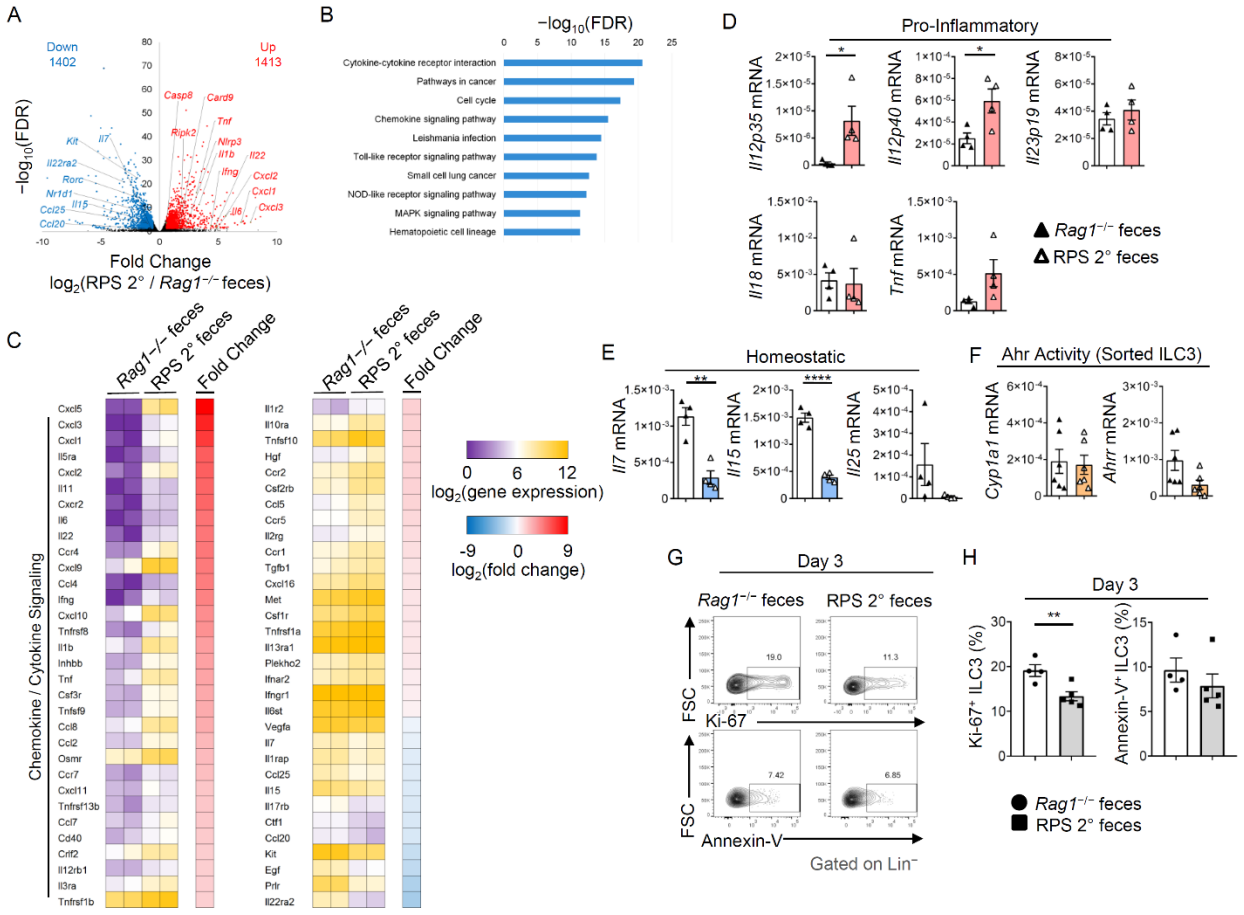


Figure 3.7 The microbiota from RPS mice induces colon changes that result in reduced ILC3 proliferation.

(A) Volcano plot indicating significantly upregulated (Up) and downregulated (Down) genes [q≤0.05; fold change≥1.5] in the large intestines of *Rag1*$^{-/-}$ mice gavaged with (2°) RPS feces compared to mice gavaged with *Rag1*$^{-/-}$ feces. (B) Top 10 pathways identified by pathway analysis of differentially regulated genes (2815 genes) using the KEGG database. (C) Heatmap of gene expression [log2(read counts)] and gene fold change (log2) from genes identified in the cytokine/chemokine pathways. (D) Gene expression relative to *Actin* as measure by RT-qPCR for indicated pro-inflammatory genes and (E) ILC homeostatic genes expressed in the proximal colon of mice gavaged with (2°) RPS feces compared to mice gavaged with *Rag1*$^{-/-}$ feces. (F) Gene expression relative to *Actin* as measure by RT-qPCR for Ahr target genes in FACS-purified ILC3s from the large intestine of mice gavaged with (2°) RPS feces compared to mice gavaged with *Rag1*$^{-/-}$ feces. (G) Representative flow cytometry plots of RORγt and T-bet staining gated on Lineage^{-} cells from the lamina propria of the large intestine. (H) Quantification of Ki67^{+} (top) and AnnexinV^{+} (bottom) ILC3 frequencies from the lamina propria of the large intestine at day 3 after gavage with (2°) RPS or *Rag1*$^{-/-}$feces. (A-C) Data are representative from one experiment with

two biological replicates per group. (D-F) Data are representative from one of two independent experiments (n=4-6 per group). (G,H) Data are pooled from two independent experiments (n=4-5 per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

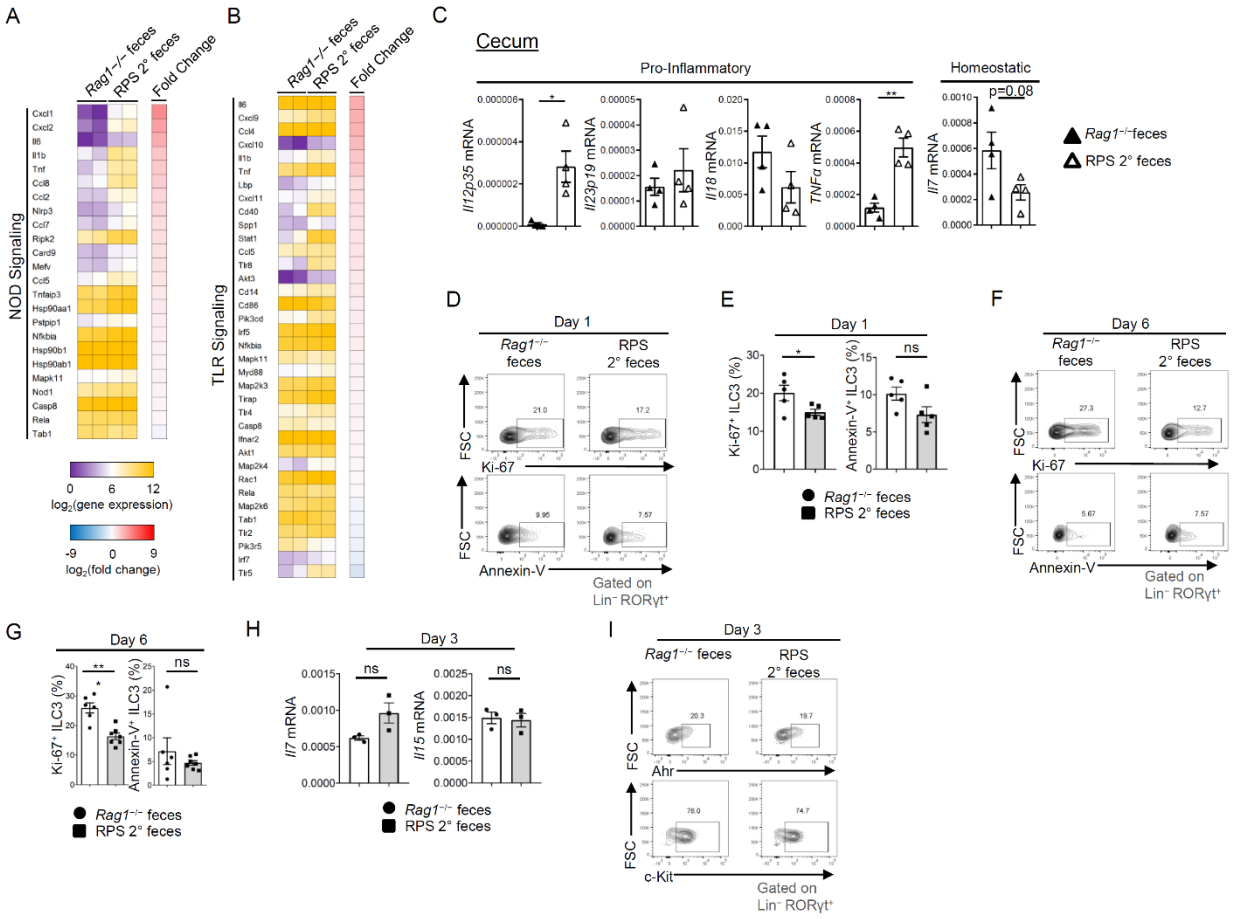


Figure 3.8 The microbiota from RPS mice induces colon changes that result in reduced ILC3 proliferation.

(A) Heatmap of gene expression [log_2(read counts)] and gene fold change (log_2) from genes identified in NOD signaling and (B) TLR signaling pathways. (C) Expression of pro-inflammatory cytokines or homeostatic molecules in the cecum of mice gavaged with RPS feces compared to *Rag1*$^{-/-}$ feces control. (D) Representative flow cytometry plots of Ki67 and AnnexinV staining gated on Lin$^-$ RORgt$^+$ cells from the lamina propria of the large intestine. (E) Quantification of Ki67$^+$ and AnnexinV$^+$ ILC3 frequencies from the lamina propria of the large intestine at day 1 after gavage with *Rag1*$^{-/-}$ or RPS feces, and (F,G) at day 6 after gavage. (H) Expression of homeostatic molecules in the proximal colon of of mice gavaged with RPS feces compared to *Rag1*$^{-/-}$ feces control at day 3. (I) Representative flow cytometry plots of Ahr and Kit staining of ILC3s from the large intestine lamina propria at day 3 after gavage with RPS feces compared to *Rag1*$^{-/-}$ feces. (A,B) Data are representative from one experiment with two biological replicates per group. (C) Data are representative from one of two independent experiments (n=4 per group). (D-G) Data are pooled from two independent experiments (n=5-6 per group). (H,I) Representative data from one

of two independent experiments. (n=3 per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ Error bars indicate s.e.m.

3.3.4 Helicobacter species induce gut dysbiosis and are associated with the loss of ILC3s in immunocompromised mice

To identify the microbiota species that may mediate the change in proliferation and loss of ILC3s, we performed 16S rRNA gene sequencing to compare the microbial composition in RPS, *Rag1*$^{-/-}$, and *Rag1*$^{-/-}$ mice co-housed with RPS mice. Sequencing data revealed that compared to *Rag1*$^{-/-}$ mice, RPS mice have perturbed microbiota, characterized by outgrowth of *Lactobacillus*, *Prevotella*, *Allobaculum* and *Helicobacter* species (Figure 3.9A and 3.9B) and decreased bacterial diversity (Shannon Index) (Figure 3.9C). Further, this perturbed microbiota was transmissible to co-housed *Rag1*$^{-/-}$ mice (Figure 3.9A and 3.9C).

Prevotella and *Helicobacter* species are known pathobionts in immunocompromised mice, and contribute to inflammation (J. G. Fox et al. 2011; Scher et al. 2013). To narrow down possible bacterial agents that contributed to the phenotype, we treated mice with broad-spectrum antibiotics mix (Ampicillin, Neomycin, Vancomycin, Gentamicin and Metronidazole) or individually with antibiotics that targeted specific classes of bacteria (e.g., anaerobes, gram-positive, etc.). We observed that loss of ILC3s was mediated by a population of bacteria that were Vancomycin- and Metronidazole-resistant but susceptible to Gentamicin or a combination of all antibiotics (Figure 3.9D-F, Figure 3.10A). We proceeded to assess the fecal microbial materials from antibiotics-treated mice by 16S rRNA gene sequencing to identify the bacteria that were differentially present among the antibiotics treatment groups. Based on analysis, we identified four species that correlated with reduced ILC3s and susceptibility or resistance to the antibiotics combinations: *Helicobacter typhlonius*, *Mucispirillum schaedleri*, an unclassified species from the

Coprobacillaceae family, and an unclassified *Allobaculum* species. (Figure 3.9H). Neither *Mucispirillum*, *Allobaculum*, nor *Coprobacillaceae* have been shown to induce inflammation in mice. Rather, they have been associated with outgrowth under inflammatory conditions (Robertson et al. 2005; Rooks et al. 2014; Schwab et al. 2014; Zenewicz et al. 2013). *Helicobacter* species have been known to initiate inflammation in immune-deficient mice (Powell et al. 2012; J. G. Fox 2002). We found in our gavage experiments that higher *H. typhlonius* shed in the feces of mice strongly correlated with lower total large intestinal ILC3 (Pearson $r = -0.84$, p-value < 0.0001) and T-bet$^+$ ILC3 (Pearson $r = -0.59$, p-value $= 0.0003$) frequencies (Figure 3.9I); hence, the expansion of *H. typhlonius* in RPS mice warranted further investigation.

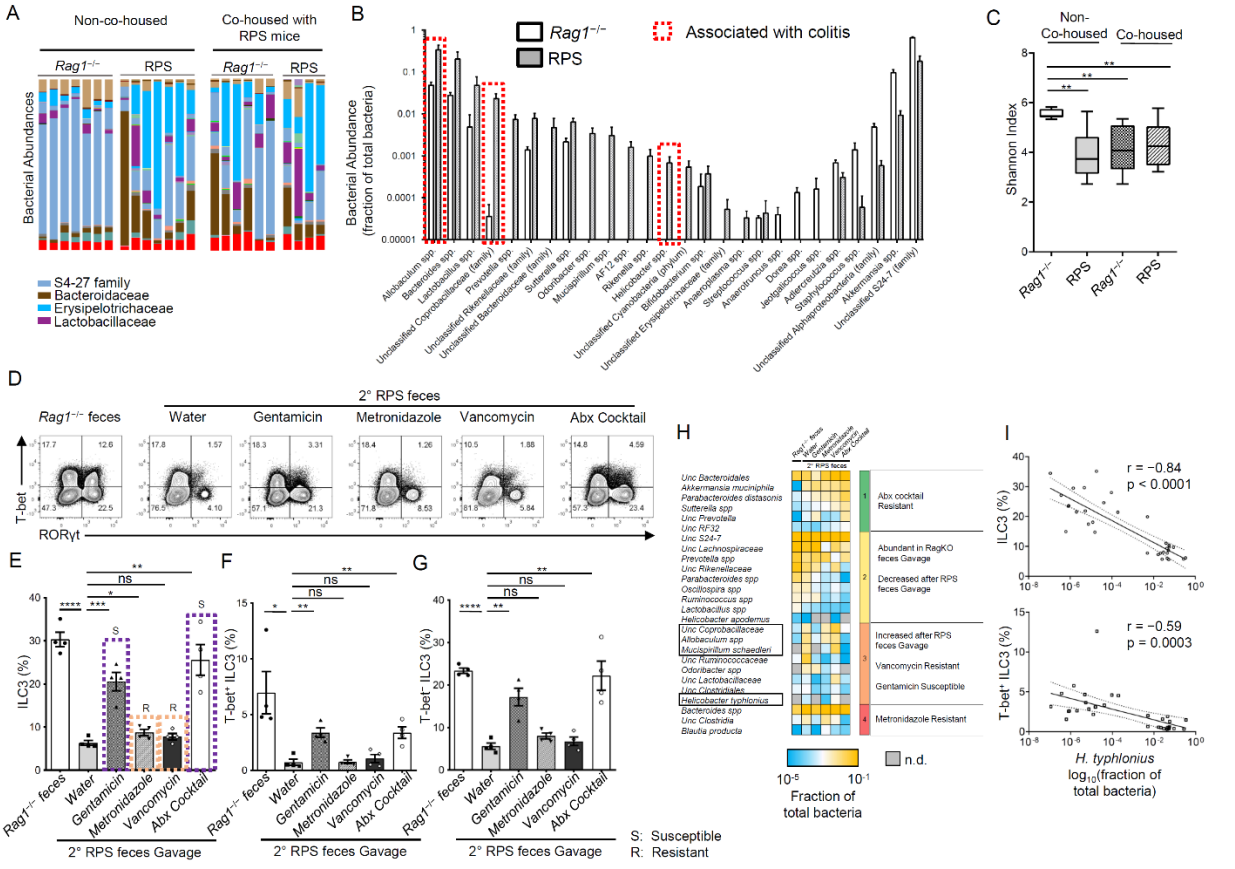


Figure 3.9 *Helicobacter typhlonius* is associated with gut dysbiosis and loss of ILC3s.

(A) Bacterial abundances (family) by 16S rRNA sequencing in non-co-housed *Rag1*$^{-/-}$ and RPS (*Rag1*$^{-/-}$*Selplg*$^{-/-}$) mice compared to co-housed *Rag1*$^{-/-}$ and RPS mice. (B) Quantification of bacterial abundance expressed as a fraction of total bacteria for significantly changed ($p \leq 0.05$, Mann-Whitney test) species. (C) Shannon index measuring bacterial diversity comparing non-co-housed and co-housed *Rag1*$^{-/-}$ and RPS mice. (D) Representative flow cytometry plots of RORγt and T-bet staining gated on lymphocytes from the lamina propria of the large intestine. (E) Quantification of Total, (F) T-bet^{+} and (G) T-bet^{-} ILC3 frequencies in the large intestine of RPS 2° feces-gavaged *Rag1*$^{-/-}$ mice after antibiotics treatment. [S: susceptible; R: resistant]. (H) Heatmap indicating the abundance of bacteria as measured by 16S rRNA sequencing after selective antibiotics treatment or untreated controls (n.d. not detected). (I) Scatterplots indicating *H. typhlonius* abundance [log 10 (fraction of total bacteria)] compared to total ILC3 (top plot) or T-bet+ ILC3 (bottom plot) frequencies. Regression lines (solid line) and 95% confidence interval (dashed lines) plotted. Pearson correlation (r) and p-value indicated. (A-C) Data are representative from one experiment with four to seven biological replicates per group. (D-G) Data are pooled from two independent experiments (n=4 per group). (H) Data are representative from one experiment with two biological replicates per group. (I) Data are pooled from five experiments (n=33). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

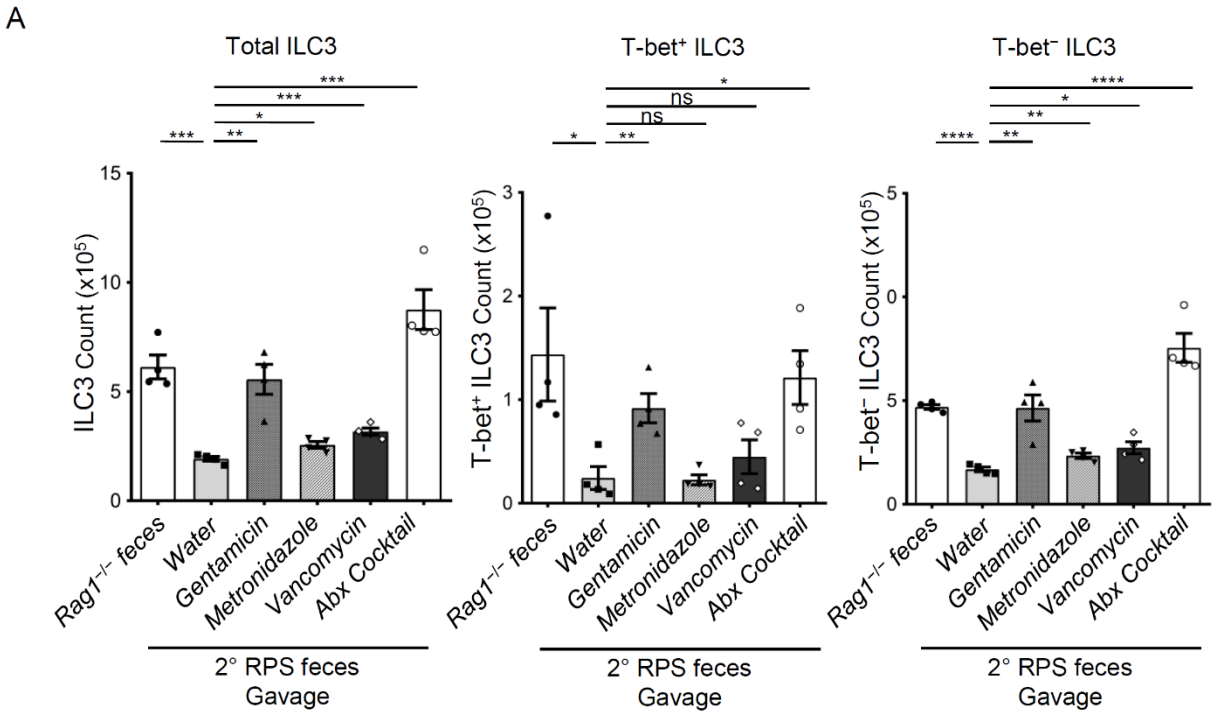


Figure 3.10 A gentamicin-sensitive and vancomycin-/metronidazole-resistant microbiota population is associated with ILC3 loss.

(A) ILC3 counts (Total, T-bet^{+} and T-bet^{-}) in the large intestine of RPS 2° feces-gavaged *Rag1*$^{-/-}$ mice after antibiotics treatment. (a) Data are pooled from two independent experiments (n=4 per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

3.3.5 *Helicobacter typhlonius* and *Helicobacter apodemus* promote the loss of ILC3s in the colon

H. typhlonius induces murine colitis within an immunocompromised background, such as *Il10*$^{-/-}$, *Prkdc*^{scid} and *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ mice (Powell et al. 2012; Franklin et al. 1999; J. G. Fox et al. 1999). Thus, we aimed to isolate the *Helicobacter* strains that may be responsible for the observed phenotypes (i.e., reduction of ILC3s) from RPS 2° feces. While *H. apodemus* (MIT 18-1095S) were present in both *Rag1*$^{-/-}$ and RPS 2° feces, *H. typhlonius* (MIT 18-1095F) was identified only in RPS 2° feces (Figure 3.12A). To elucidate the contribution of these specific strains to the ILC phenotype observed, we gavaged *Rag1*$^{-/-}$ mice with either *H. apodemus*, *H. typhlonius*, or a mixture of the two bacteria and examined ILC populations and *Helicobacter* species in fecal contents 12 days later (Figure 3.12B). Compared to the *Rag1*$^{-/-}$ mice gavaged with *H. rodentium*, *H. apodemus* and/or *H. typhlonius* single or combined transfaunation reduced large intestinal ILC3s (Figure 3.11A and 3.11B), particularly T-bet^{+}RORγt^{+} ILC3 (Figure 3.11C and 3.11D, Figure 3.12C), and increased NK cell frequencies and counts (Figure 3.11E and 3.11F, Figure 3.12D) in the large intestine, consistent with the phenotype observed after gavage with RPS 2° feces. Large intestinal ILC1 frequencies and cell counts were reduced in mice after gavaging with *H. apodemus* and/or *H. typhlonius* (Figure 3.11E and 3.11G, Figure 3.12D). ILC2 frequencies slightly increased, but there was a decrease in cell number (Figure 3.11H and 3.11I, Figure 3.12D). In addition, although we observed increased IL-22^{+} and GM-CSF^{+} ILC3 frequencies (Figure 3.12E and 3.12F), their absolute numbers were decreased in *Helicobacter* or RPS feces-gavaged mice (Figure 3.12H and 3.12I), consistent with the reduction of ILC3s. IL-17A^{+} ILC3s had a trend of

increase, but was only significant in the *H. typhlonius*, *H. apodemus/H. typhlonius* mixed, and *H. rodentium* treatment groups (Figure 3.12G and 3.12J). Of note, endogenous *H. apodemus* (MIT 18-1095S) present in *Rag1*$^{-/-}$ mice did not cause reduction of ILC3s but transfunation of pure *H. apodemus* bacteria with large quantities led to reduction of ILC3s.

3.3.6 *H. typhlonius* and *H. rodentium* induce a strong IFN-γ response

Similar to the RPS 2° feces gavage, gavaging mice with either *H. typhlonius* or *H. apodemus* induced a strong IFN-γ response, notably expressed by ILC1s and infiltrating NK cells but not by ILC3s (Figure 3.13A-C). We also observed an increase of a lineage^{+} (CD3, CD19 and Ly6g), IFN-γ^{+} population of cells in mice gavaged with RPS 2° feces, *H. typhlonius*, *H. apodemus*, or mixed treatments, which was absent in *H. rodentium*-gavaged mice (Figure 3.13D-F). In *Rag1*$^{-/-}$ mice, this lineage^{+} population is likely infiltrating Ly6g^{+} neutrophils. Together, these data indicate that the two specific *Helicobacter* strains present in RPS mice, *H. apodemus* (MIT 18-1095S) and *H. typhlonius* (MIT 18-1095F), both of which may cooperatively contribute to the loss of T-bet^{+} ILCs and the induced inflammation. Indeed, the mixed 50:50 *Helicobacter* inoculation treatment most closely resembled gavage with RPS 2° feces (Figure 3.11B), but this does not exclude the possibility that other endogenous bacterial species to our mouse colonies or enriched in the RPS feces, including *Prevotella*, potentially contribute to the described phenotypes (Figure 3.9B and 3.9H).

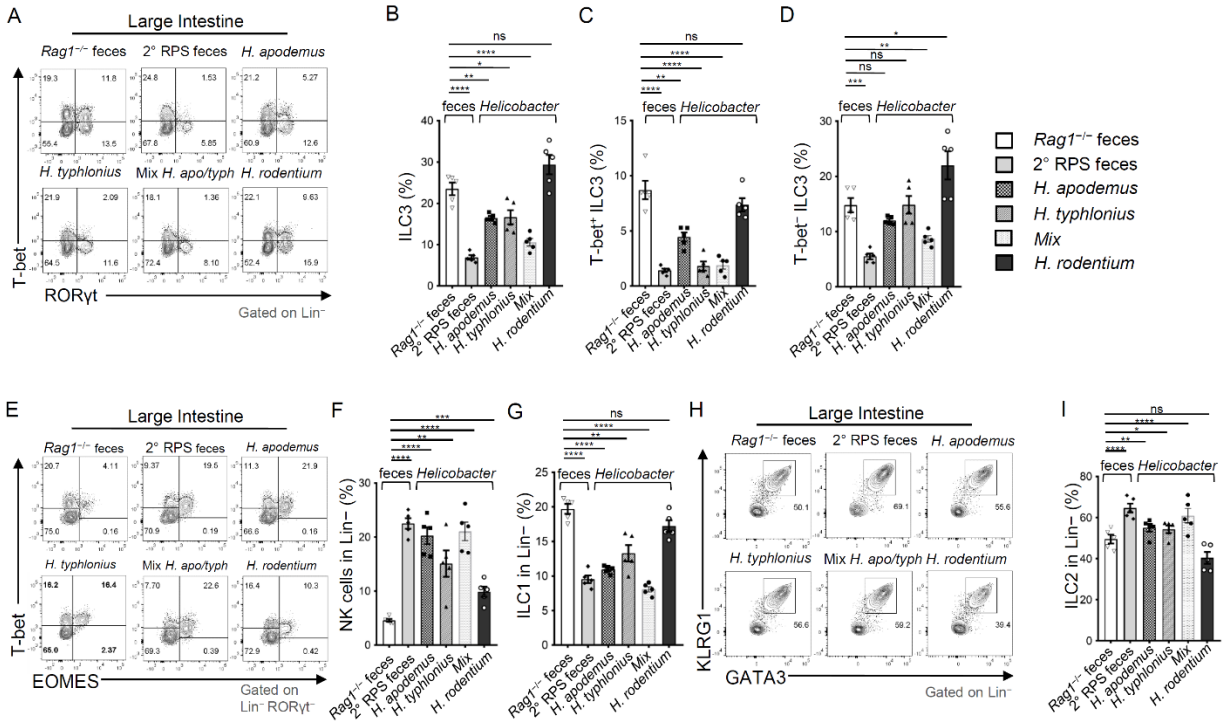


Figure 3.11 *Helicobacter typhlonius* and *Helicobacter apodemus* promote the loss of ILC3s in the colon.

(A) Representative flow cytometry plots of RORγt and T-bet staining gated on Lineage$^-$ cells from the lamina propria of the large intestine. (B) Quantification of ILC3, (C) T-bet$^+$ ILC3, and (D) T-bet$^-$ ILC3 frequencies from the lamina propria of the large intestine after gavage with previously isolated *Helicobacter* strains. (E) Representative flow cytometry plots of EOMES and T-bet staining gated on Lineage$^-$ RORγt$^-$ cells from the lamina propria of the large intestine. (F) Quantification of ILC1 (RORγt$^-$T-bet$^+$EOMES$^-$) and (G) NK cells (RORγt$^-$T-bet$^+$EOMES$^+$) from the lamina propria of the large intestine after gavage with *Helicobacter* strains isolated from RPS mice. (H) Representative flow cytometry plots of GATA3 and KLRG1 staining gated on Lineage$^-$ cells from the lamina propria of the large intestine. (I) Quantification of ILC2 frequencies from the lamina propria of the large intestine after gavage with previously isolated *Helicobacter* strains. (A-I) Data are pooled from three independent experiments (n=5 per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

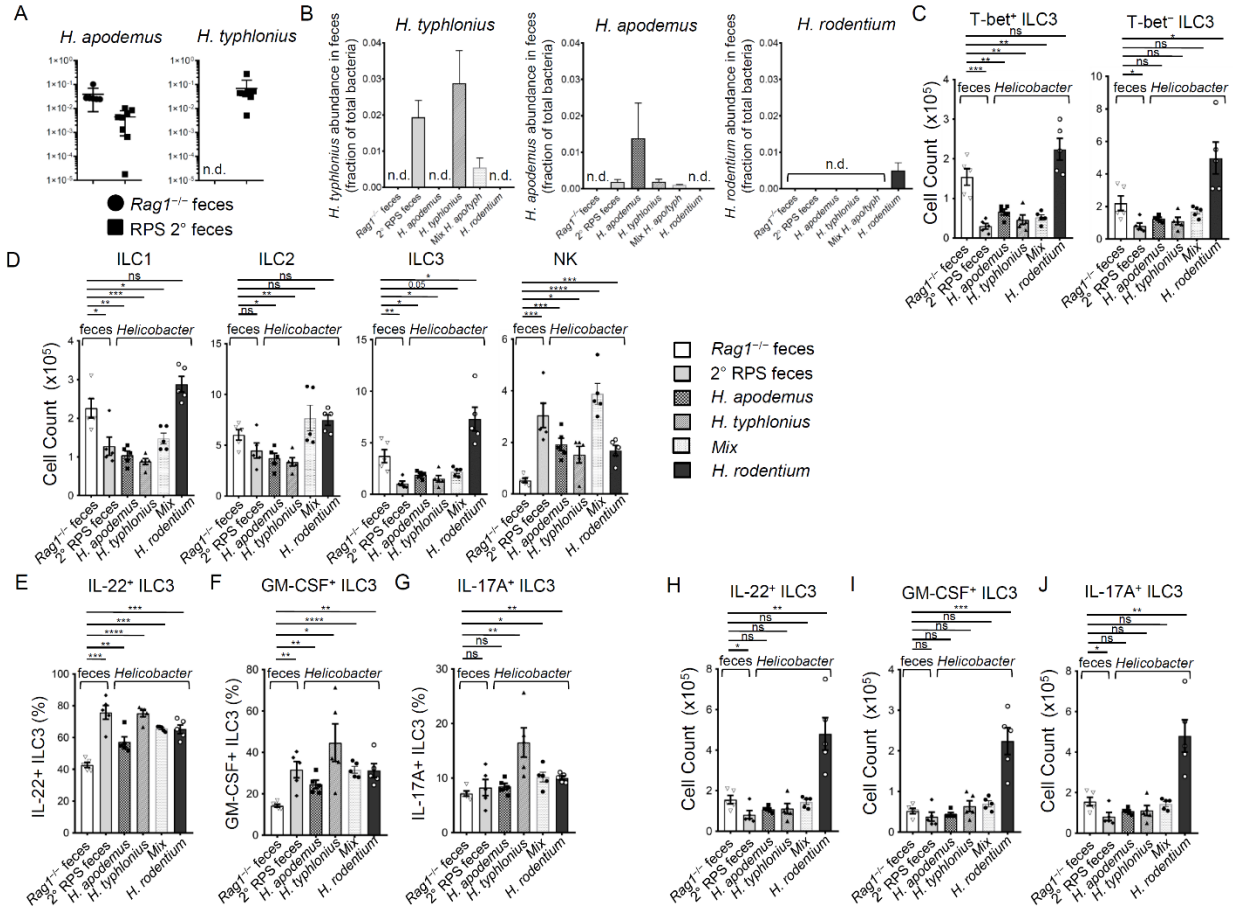


Figure 3.12 Specific Helicobacter strains reduce ILC3s in the colon.

(A) *H. apodemus* and *H. typhlonius* bacterial abundances in *Rag1*$^{-/-}$ or 2° RPS feces, as measured by qPCR of the bacterial 16S rRNA gene. (n.d. indicates not detected) (B) Bacterial abundances relative to total bacteria for *H. typhlonius* (left), *H. apodemus* (center), and *H. rodentium* (right) after gavage. (C) Cell counts of T-bet^{+} (left) and T-bet^{-} (right) ILC3s in the large intestine after gavage with *Helicobacter* species. (D) Cell counts of ILC1, ILC2, ILC3, and NK. (E) Quantification of IL-22^{+}, (F) GM-CSF^{+} and (G) IL-17A^{+} ILC3s frequencies and counts (H-J) in the large intestine after gavage with *Helicobacter* strains. (A) Data are pooled from two independent experiments (n=6-8 per group). (B-J) Data are pooled from three independent experiments (n=5 per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

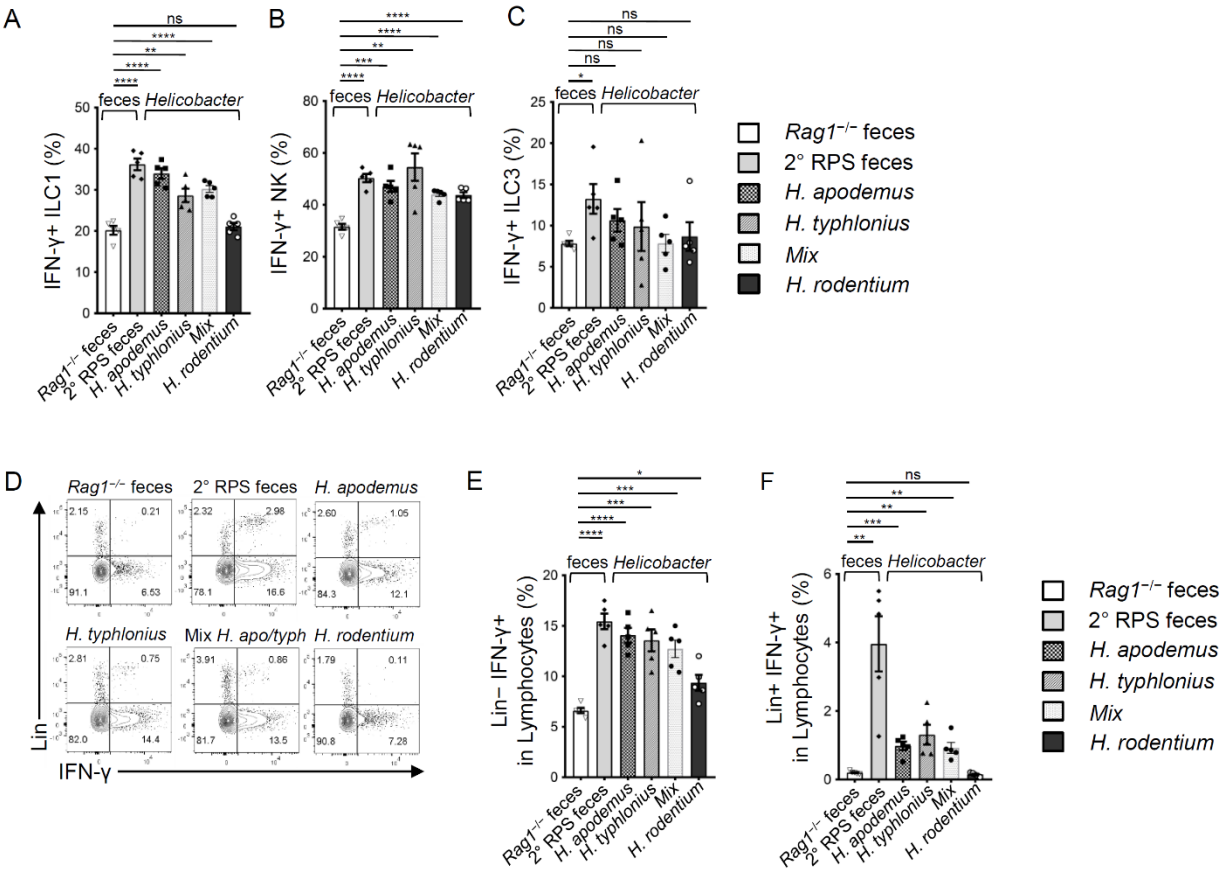


Figure 3.13 *Helicobacter typhlonius* and *Helicobacter apodemus* induce a strong IFN-γ response.

(A) Quantification of IFN-γ^+ ILC1, (B) NK, and (C) ILC3 frequencies after gavage with *Helicobacter* strains. (D) Representative flow cytometry plots of IFN-γ and Lineage staining gated on lymphocytes from the lamina propria of the large intestine. (E) Quantification of Lin^+ and (F) Lin^- IFN-γ^+ lymphocyte frequencies from the lamina propria of the large intestine after gavage with *Helicobacter* strains isolated from RPS mice. (A-F) Data are pooled from three independent experiments (n=5 per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

Thus, we speculated that the loss of ILC3s might result in reduced protective immunity of mice infected with *H. typhlonius* or *H. apodemus*. To this end, we determined the action of *Helicobacter* in two models of colitis, Dextran Sodium Sulfate (DSS)-induced colitis and *Citrobacter rodentium* infection (Sawa et al. 2011; Collins et al. 2014; Sugimoto et al. 2008), both of which depend on ILC3s for protective immunity. We induced DSS colitis after *Helicobacter* gavage and found that *Helicobacter*-gavaged mice had increased weight loss compared to *Rag1*$^{-/-}$ feces-gavaged control mice (Figure 3.14A and 3.14B). Intriguingly, the *H. rodentium*-gavaged control mice, which did not show a reduction of ILC3s after gavage (Figure 3.11A), had increased weight loss, indicating that *Helicobacter* had additional effects, other than the resulting reduction of ILC3s, that can influence the DSS colitis phenotype (e.g., wasting disease) (Figure 3.14B). Since DSS treatment caused ILC3 reduction, the role of *Helicobacter* in regulating ILC3-mediated protection was unclear in the aforementioned model (Figure 3.14C). During *C. rodentium* infection, *Helicobacter* gavage led to a trend of more body weight loss (Figure 3.14D and 3.14E). Moreover, *Helicobacter*-gavaged mice had more dissemination of bacteria to spleen compared to *Rag1*$^{-/-}$ mouse feces-gavaged control mice, potentially due to intestinal barrier disruption caused by *Helicobacter* infection (Figure 3.14F). Together, these data suggest that *Helicobacter spp*. may induce prolonged colonic inflammation and tissue damage that lead to dissemination and wasting disease.

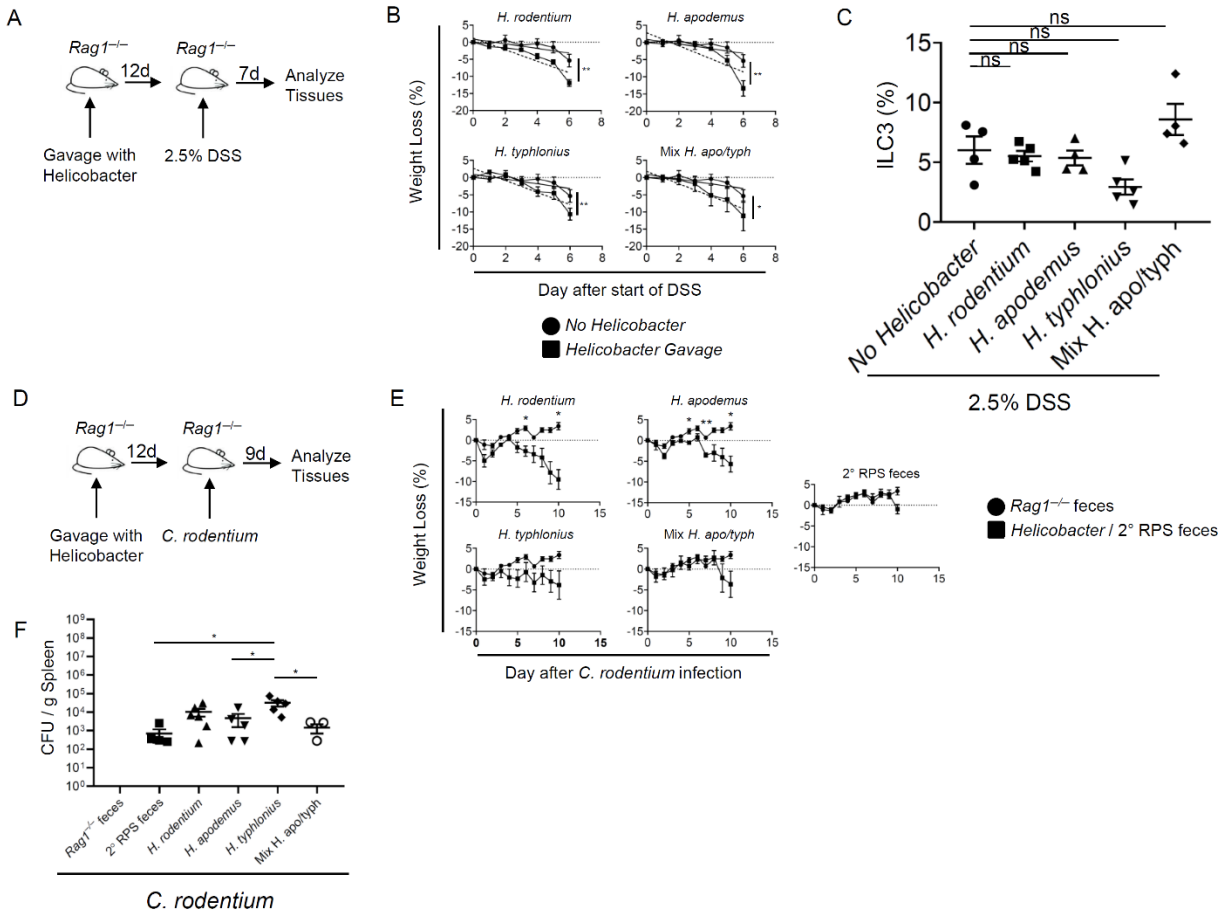


Figure 3.14 Helicobacter induce colonic inflammation that leads to bacterial dissemination.

(A) Illustration of DSS colitis experiment. (B) Weight loss (%) after gavage with indicated *Helicobacter* species followed by 2.5% DSS treatment. Statistics represent significantly different slopes of regression lines. (C) Quantification of ILC3 (Lin^- $ROR\gamma t^+$) frequencies from the lamina propria of the large intestine after indicated treatments. (D) Illustration of *C. rodentium* experiment. (E) Weight loss (%) after gavage with indicated *Helicobacter* species followed by *C. rodentium* infection. Statistics shown are multiple t tests calculated for each day with adjusted p-values (Holm-Sidak method) indicated for significant days. (F) CFU per gram spleen from mice after indicated *Helicobacter* or feces treatment followed by *C. rodentium* infection. Statistics shown are one-way ANOVA with Tukey's multiple comparisons test adjusted p-values. (B,C) Data from one experiment (n=4-5 per group). (E,F) Data from one experiment (n=4-6 per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Error bars indicate s.e.m.

3.4 Conclusions

We started our investigation examining the role of PSGL-1 in ILC biology, but discovered an important interaction between the microbiota and host immune cells. We favored the possibility that RPS mice acquired, by chance, pathobiont *Helicobacter* species in our mouse facility. These *Helicobacter* species function as key contributors to the reduction of ILC3s in the large intestines of immune-compromised mice that lack adaptive immunity. However, we could not rule out the possibility that PSGL-1 deficiency helped initiate the fortuitous outgrowth of the *Helicobacter* species in RPS mice. Further study is needed to determine if loss of PSGL-1 is an initiating factor or dispensable for the observed phenotype. Nevertheless, our data show that in *Helicobacter*-gavaged *Rag1*$^{-/-}$ mice, inflammation and pathology, as noted by increased cell infiltration and cytokine expression, is negatively correlated with the frequency of ILC3s. It remains to be determined if the loss of ILC3s is responsible for gut inflammation or vice versa. Specifically, whether some component of the inflammatory response contributes to the changes in ILC3 proliferation, remains to be determined.

Complicated actions of the microbiota regulate the development and maintenance of ILC3s. Early reports identified that the numbers of NKp46^{+} T-bet^{+} ILC3s in the intestine and their production of IL-22 were dependent on gut microbiota (Vonarbourg et al. 2010; Sanos et al. 2009; Satoh-Takayama et al. 2008). Additionally, T-bet expression in CCR6^{-} ILC3s was found to be positively regulated by the microbiota. Subsequent studies indicated the opposite, that in germ-free mice, intestinal IL-22-producing NKp46^{+} T-bet^{+} ILC3s were either not affected, or increased, indicating that gut microbes may be dispensable for controlling the number of IL-22-producing ILC3s (Sawa

et al. 2011; Reynders et al. 2011). Similarly, the development and maintenance of adult T-bet^{-} ILC3s were not affected by the presence or absence of gut microbes (Sawa et al. 2011; Sanos et al. 2009; Reynders et al. 2011). In contrast, our data showed that the outgrowth of two pathobionts, *H. apodemus* and *H. typhlonius*, from immunocompromised mice correlated with the reduction of ILC3s, and broad-spectrum antibiotics treatment restored T-bet^{+} ILC3s in RPS mice. These data suggest that certain microbes in the gut microbiota can suppress ILC3s at least in a host with an immune-compromised condition.

New non-gastric *Helicobacter* species are increasingly identified in cases of diarrhea and bacteremia in humans and animals (J. G. Fox 2002; Araoka et al. 2018; Fujiya et al. 2016). *Helicobacter hepaticus* infection in immunocompromised mice has been widely used as a model of inflammatory bowel disease, and chronic inflammation induced by *H. hepaticus* infection in *Rag2*$^{-/-}$ mice promotes colon cancer (J. G. Fox et al. 2011; Buonocore et al. 2010; Ge et al. 2017). Similarly, we identified increased pro-inflammatory cytokines after *H. apodemus* or *H. typhlonius* gavage in *Rag1*$^{-/-}$ mice. Powell and colleagues showed that *H. typhlonius* promotes gastrointestinal pathology in *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ ulcerative colitis (TRUC) mice (Powell et al. 2012). Additionally, they found that IL-17A-producing ILCs contributed to the colitis, implicating ILC3s (Powell et al. 2012). Accordingly, treatment with agents that deplete ILCs (e.g., anti-CD90, anti-IL-7Ra antibodies) reduced the pathology of *Helicobacter*-induced colitis (Buonocore et al. 2010; Powell et al. 2012). Consistently, our data also showed that transfaunation of *Helicobacter spp.* can increase production of IL-17, IFN-γ, and GM-CSF by ILCs in *Rag1*$^{-/-}$ mice. Thus, these data indicate that ILC3s may play a pathogenic role by producing pro-inflammatory cytokines in

immune-compromised mice lacking the adaptive immune system (Buonocore et al. 2010; Coccia et al. 2012; Pearson et al. 2016).

However, in the presence of adaptive immunity, ILC3 induction/activation by *Helicobacter* spp. plays a protective role by limiting effector T cell responses towards *Helicobacter*. Indeed, a recent study found that ILC3s negatively regulate the adaptive immune response to *H. typhlonius* (Melo-Gonzalez et al. 2019). Antigen presentation by ILC3s suppresses the effector T cell response to *H. typhlonius*, suggesting that ILC3s, along with regulatory T cells, may be responsible for limiting effector T cell-mediated pathology in the response to *Helicobacter* in the colon. *H. typhlonius* and *H. apodemus* are both potent inducers of antigen-specific effector and regulatory T cell responses (Chai et al. 2017). The addition of IL-10 receptor blocking antibodies in a model of DSS-induced colitis results in the expansion of *H. typhlonius* and *H. apodemus* in the population of mucosa-associated bacteria. This is consistent with previous reports indicating that IL-10-deficient mice are particularly susceptible to colonization by enteric *Helicobacter*, and that regulatory T cells suppress *Helicobacter*-induced pathology, dependent on IL-10 (Maloy et al. 2003; J. G. Fox et al. 1999). These data suggest a balance between the induction of innate immunity and effector T cells by *Helicobacter*, and the induction of regulatory responses (e.g., regulatory T cells). In our study, C57BL/6 wild type mice gavaged with *Helicobacter* do not lose ILC3s, unlike *Rag1*-deficient mice, suggesting that the adaptive immunity could sustain ILC3s in the presence of *Helicobacter*.

Cell number changes in ILC3s in the colon suggested changes in apoptosis or proliferation induced by *Helicobacter* infection, but we did not observe increased apoptosis at any time after gavage. Instead, we observed a decrease in proliferative capacity. The decrease of *Il7* and *Il15* in the colon at day 12 after gavage presented a potential mechanism for compromised of ILC3 maintenance. Examining the host response, we identified a strong induction of IFN-γ in the colon, produced mostly by NK cell, ILC1s and neutrophils. IFN-γ has been shown to negatively regulate ILC2 proliferation and cytokine production in the lung (Molofsky et al. 2015), but little is known about the effect of IFN-γ on ILC1s and ILC3s in the intestine during inflammation, and requires further study. Factors from *H. apodemus or H. typhlonius* may work directly or indirectly on immune cells in the colon. Further study is required to identify and test specific factors from these species. Genomic sequencing of *H. typhlonius* and *H. apodemus* indicate that these bacteria contain a number of virulence factors whose contribution to the ILC3 phenotype would need to be tested through knockout mutations (J. Kim et al. 2018; Frank et al. 2015). Nonetheless, in this study we discovered that *Helicobacter apodemus* (MIT 18-1095S) and *Helicobacter typhlonius* (MIT 18-1095F) introduction into immunocompromised mice are suppressive of ILC3s, and can serve as a model for future investigations of the host-microbe interactions that maintain ILC3s in the gut.

CHAPTER 4. RESULTS: The aryl hydrocarbon receptor preferentially marks and promotes gut regulatory T cells

This work was published in *Cell Reports* (Ye et al. 2017). Results discussed are limited to the direct contributions of the dissertation author.

4.1 Introduction

A fundamental question is how environmental cues instruct regulatory T cell (Treg) development and function to maintain host immune homeostasis. Nutrients and metabolites that are produced by microbiome and diet can act in the gut on Tregs to trigger tolerance. The aryl hydrocarbon receptor (Ahr) is an environmental sensor that detects not only xenobiotic ligands such as environmental pollutants (e.g., dioxin) but also physiological compounds generated by host cells, microbiota, and diet (e.g., amino acid tryptophan metabolites) (Zhou 2016). Thus, deciphering the Ahr-mediated molecular pathways in Tregs offers the potential for developing novel therapies to treat immune dysregulation. The role of Ahr in the immune cells has only been recently appreciated, when Ahr was identified as a molecular link between the environment and the host immune system. It has been reported that Ahr is downregulated in the intestinal tissue of patients with inflammatory bowel disease (IBD), thus highlighting the clinical relevance of the Ahr pathway in human autoimmunity (Monteleone et al. 2011). Ahr has been relatively well studied in T helper (Th)17 cells and group 3 innate lymphoid cells (i.e., ILC3s) for its role in induction of effector cytokines (e.g., interleukin [IL-17] and IL-22) (Esser, Rannug, and Stockinger 2009; J.

Qiu and Zhou 2013). However, its role in regulatory T cells (Tregs), specified by the Forkhead transcription factor Foxp3, remains controversial, with conflicting data showing Ahr expression in Tregs and either positive or negative regulation of Treg differentiation by Ahr. Of note, these data are largely derived from loss-of-function analysis using Ahr complete null mice or gain-of-function analyses by ligand administration (Nguyen et al. 2013; Stockinger et al. 2014; Zhou 2016). These approaches may confound the interpretation of the results because the broad expression of Ahr in other cell types will likely influence Treg development and/or function. For example, Ahr-deficient macrophages produce more IL-6 (Kimura et al. 2009), a cytokine known to suppress Foxp3 expression (Bettelli et al. 2006). In addition, Ahr deficiency in ILC3s leads to aberrant outgrowth of gut commensal segmented filamentous bacteria (SFB), causing elevated intestinal Th17 cells (J. Qiu et al. 2013) that have a reciprocal relationship with Tregs during differentiation (Bettelli et al. 2006; Zhou et al. 2008). Thus, it is essential to elucidate the Treg cell-autonomous role of Ahr in mucosal immunology by genetic approaches.

In spite of shared expression of Foxp3, tissue-resident Tregs may have different gene expression profiles or functions compared to their counterparts in lymphoid organs or transforming growth factor β (TGF-β)-induced Treg cells *in vitro* (iTregs), suggesting a complex mode of tissue-specific regulation of Tregs by Foxp3 and co-factors (Burzyn, Benoist, and Mathis 2013). It remains elusive how the surrounding environment contributes to the differentiation, maintenance, and function of these tissue-resident Tregs. Microbiota and dietary metabolites (e.g., retinoic acid and short-chain fatty acids) have been shown to influence the differentiation and function of gut Tregs,

highlighting the crucial environmental effects on Tregs (Atarashi and Honda 2011; Bollrath and Powrie 2013; Mucida, Park, and Cheroutre 2009).

Ahr belongs to the basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family of proteins. The PAS domains consist of two regions, PAS-A and PAS-B, and are known to function as an interface for dimerization with the Ahr nuclear translocator (ARNT) and in ligand binding (Stevens, Mezrich, and Bradfield 2009). An Ahr deletion mutant lacking the PAS-B domain (ΔPAS-B) has been shown to constitutively dimerize with ARNT, bind to DNA, and activate transcription in a ligand-independent manner (i.e., constitutively active [CA]-Ahr) (McGuire et al. 2001). Ahr expression was thought to be ubiquitous in vertebrate cells; however, recent data suggest that its expression is regulated by certain environmental cues (e.g., cytokines) (Kimura et al. 2009; Quintana et al. 2008; Veldhoen et al. 2008; Zhou 2016). Previous studies expressing the constitutively active Ahr ΔPAS-B (i.e., CA-Ahr) in transgenic mice attempted to examine *in vivo* activation of Ahr (Andersson et al. 2002; Nohara et al. 2005; Stockinger et al. 2014; Tauchi et al. 2005; Zhou 2016). However, in these studies, CA-Ahr was expressed in mice under the control of artificial genes and/or promoters out of the *Ahr* endogenous context (i.e., human CD2 minigene, SV40 and/or keratin-14 promoter), likely confounding the data interpretation in regard to the role of Ahr in Tregs (Stockinger et al. 2014). Here, we developed a conditional knockin mouse model that expressed CA-Ahr and an internal ribosomal entry site (IRES)-GFP under the regulation of the *Ahr* endogenous locus (Ahr^{CAIR} mice) coupled with $Foxp3^{Yfp\text{-}Cre}$ approach to provide insights into *in vivo* expression and activation of Ahr in a Treg-specific manner. In addition, we utilized $Ahr^{f/-}$ $Foxp3^{Yfp\text{-}Cre}$ mice to probe the cell-intrinsic role of Ahr in Tregs. Systemic analysis of Tregs

in different anatomic locations revealed a gut-specific expression pattern of Ahr in Tregs and a role of Ahr in directing Treg gut homing and function.

4.2 Materials and Methods

Mice

All mice used in this study were maintained in SPF or Germ-free facilities at Northwestern University and the University of Florida. The mice were littermate controlled and were 6–10 weeks old unless otherwise indicated in the text. C57BL/6-SJL (CD45.1) mice, $Ahr^{f/f}$ mice, and *Cd4-cre* mice were purchased from Taconic Farms and *EIIa-cre* mice were purchased from Jackson Laboratory. $Foxp3^{Yfp\text{-}Cre}$ mice and $Ahr^{-/-}$mice were described previously (Fernandez-Salguero et al. 1995; Rubtsov et al. 2008). All studies with mice were approved by the Animal Care and Use Committee of Northwestern University and the University of Florida.

Isolation of Intestinal LPLs and Flow Cytometry

The isolation of intestinal lamina proprial cells and flow cytometry were done as previously described (J. Qiu et al. 2012). Splenocytes and peripheral lymph nodes were made into single cell suspension and $CD4^+$ T cells were purified with $CD4^+$ T cell isolation kit (Stemcell). Antibodies were purchased from eBioscince, BD Pharmingen or TONBO. CD16/32 antibody was used to block the nonspecific binding to Fc receptors before all surface staining. For nuclear transcription factor staining, cells were fixed and permeabilized with Foxp3 staining buffer Kit

(eBioscience). For cytokine staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 hours and Brefeldin A (2 μg/ml) was added 2 hours before cells were harvested. The live and dead cells were discriminated by Live and Dead violet viability kit (Invitrogen). Sample acquisition was performed on FACSCantoII or LSRFortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (version 10.2; Tree Star).

RNA-seq and Analysis

$CD4^+YFP^+$ Tregs were sorted by flow cytometry from the large intestinal LPL cells of $Ahr^{f/+}$ $Foxp3^{Yfp\text{-}Cre}$ or littermate $Ahr^{f/-}$ $Foxp3^{Yfp\text{-}Cre}$ mice. About 8×10^5 sorted Tregs pooled from 4-5 mice per group were lysed in Trizol (Invitrogen), and RNA was subsequently extracted with RNAeasy Mini Kit (Qiagen). Total RNA was treated with Ribo-zero kit and RNAseq libraries were generated using kits from Illumina. Barcoded samples were pooled and sequenced over 2 lanes on an Illumina HiSeq 2500 instrument (University of Chicago Genomics Core) to produce 50 bp single-end reads. De-multiplexed raw data files from the sequencing core were concatenated and uploaded to the Galaxy website (Pennsylvania State University) for analysis (Blankenberg et al. 2010; Giardine et al. 2005; Goecks et al. 2010). Quality control data was generated with the NGS Toolbox quality control tools (Patel and Jain, 2012)(Patel and Jain 2012). Based on the quality analysis and recommendation of the sequencing core, low-quality 3' bases were trimmed from each of the reads using the NGS Toolbox FASTQ trimmer tool (Patel and Jain 2012). The trimmed reads were mapped (with TopHat2 fast splice junction mapper for RNA-Seq) to the January 2012 annotation of the *Mus musculus* genome (National Center for Biotechnology

Information, mm10 assembly) and filtered for uniquely mapped reads (D. Kim et al. 2013). Genome visualization tracks (bedgraph files) were uploaded to the University of California Santa Cruz (UCSC) Genome Browser for visual comparison of expression levels (Meyer et al. 2013; Patel and Jain 2012). Quantitated relative mRNA expression levels (RPKM) were calculated based on exon regions using the SeqMonk software (Babraham Bioinformatics) and the mm10 reference genome annotations (Kent et al. 2002; Mortazavi et al. 2008). Significantly changed genes were found by the Intensity Difference method as outlined and recommended by SeqMonk documentation, and by DESeq analysis. Genes found to be significantly changed, after adjusting for multiple test comparisons, in both Seqmonk and DESeq (q-value ≤ 0.05, and filtered on max RPKM ≥ 1) were clustered (k-means) based on log2-transformed mean-centered RPKM values with the Cluster3 software (Anders and Huber 2010; de Hoon et al. 2004; Eisen et al. 1998). The cluster analysis was visualized with gplots software (http://cran.r-project.org/web/packages/gplots).

Realtime RT-PCR

RNA from sorted cell populations was isolated with Trizol reagent (Invitrogen). cDNA was synthesized using GoScript™ Reverse Transcription kit (Promega). Real-time RT-PCR was performed using SYBR Green (Biorad) and different primer sets. Reactions were run using the MyiQ™2 Two-Color Real-Time PCR Detection System (Biorad). The results were displayed as relative expression values normalized to β-actin.

Assay for Transposase-Accessible Chromatin Sequencing (ATAC-Seq) and Analysis

Large intestinal $CD4^+TCR\beta^+YFP^+$ Tregs were sorted and subjected to ATAC-Seq according to a published protocol (Buenrostro et al. 2013) with a modification in the library purification step. Briefly, $5x10^4$ sorted Tregs were washed once with 50 □l PBS, and then lysed in lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% IGEPAL CA-630) on ice. Pelleted nuclei were resuspended in transposition reaction mix with Tn5 transposase (Illumina) and incubated at 37℃ for 30 min with gentle shaking. The DNA was purified with MinElute PCR Purification Kit (Qiagen) and was used as a template of PCR with 10 cycles of amplification. The library was cleaned up with 1.2x SPRIselect beads (Beckman Coulter), to exclude the small fragments, before sequencing with Illumia HiSeq 2500. ATAC-Seq reads were mapped to the mouse genome (mm9) with bowtie2 (Langmead and Salzberg, 2012). The mapped reads were filtered using samtools (H. Li et al. 2009), keeping only the uniquely aligned reads, and bedgraph files (scaled to 10 million reads) were made with bedtools (Quinlan and Hall 2010).

Statistical Methods

Unless otherwise noted, statistical analysis was performed with the unpaired Student's t test on individual biological samples. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$

4.3 Results

4.3.1 Ahr deficiency leads to alteration of the transcriptional program in intestinal Tregs

Tregs may utilize multiple mechanisms to exert the suppressive functions that maintain immune homeostasis and prevent autoimmuninty (Vignali 2012). To investigate the role of Ahr in intestinal Treg transcriptional programming, we purified the large intestinal YFP$^+$ (Foxp3$^+$) Tregs from *Ahr*$^{f/+}$ *Foxp3*$^{Yfp\text{-}Cre}$ or *Ahr*$^{f/-}$ *Foxp3*$^{Yfp\text{-}Cre}$ mice by fluorescence-activated cell sorting (FACS) and performed a genome-wide analysis of mRNA expression by high throughput sequencing (RNA sequencing [RNA-Seq]). Gene expression profiling analysis indicated that the majority of genes in gut Tregs were not significantly affected by genetic ablation of Ahr, indicating no drastic perturbation of the Treg transcriptional program in the absence of Ahr under the steady-state (Figure 4.1A). This was consistent with the observation that *Ahr*$^{f/-}$ *Foxp3*$^{Yfp\text{-}Cre}$ mice did not develop spontaneous autoimmunity (data not shown). However, a small cohort of genes (n = 59) displayed deregulated expression in steady state gut Tregs upon deletion of Ahr ($\geq$ 1.5 fold; $q \leq 0.05$, p-values adjusted for multiple test comparisons) (Figure 4.2). Specifically, the expression of certain genes that are important for Treg homing and/or function in the gut (e.g., *Ccr6, Gpr15, Itgae,* and *Rgs9*) was decreased in Ahr-deficient gut Tregs (Figure 4.1B). The expression of granzyme genes that are associated with Treg cytotoxic function (e.g., *Gzma*) was also downregulated in the absence of Ahr (Figure 4.1B). On the other hand, Th1-associated genes were found to be expressed at increased levels in Ahr-deficient Tregs. For example, the expression of the cytokine *Ifng*, chemokine *Ccl5,* and the Th1-associated key transcription factor *Tbx21* was enhanced in Ahr-deficient Tregs (Figures 4.1B and 4.1C). The expression of other genes that are associated with Treg function (e.g., *Ctla4*, *Il2ra, Il10, Tnfrsf18, Runx1, Runx3, Entpd1,* or *Nt5e*) was not significantly altered in the

absence of Ahr, indicating a selective requirement for Ahr in Foxp3^{+} Treg transcriptional regulation (data not shown). Additionally, gene set enrichment analysis using gene sets derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database identified two pathways that were significantly enriched: Cytokine-cytokine receptor interaction and Chemokine signaling pathway (q-value ≤ 0.01). Differentially regulated genes related to the pathway analysis include: *Ccl4*, *Ccl5*, *Ifng*, *Csf1*, and *Ccl20* (data not shown) (Mootha et al. 2003; Subramanian et al. 2005). Together, these data suggest that Ahr may regulate intestinal Treg development, maintenance, homing/retention, and function by multiple mechanisms.

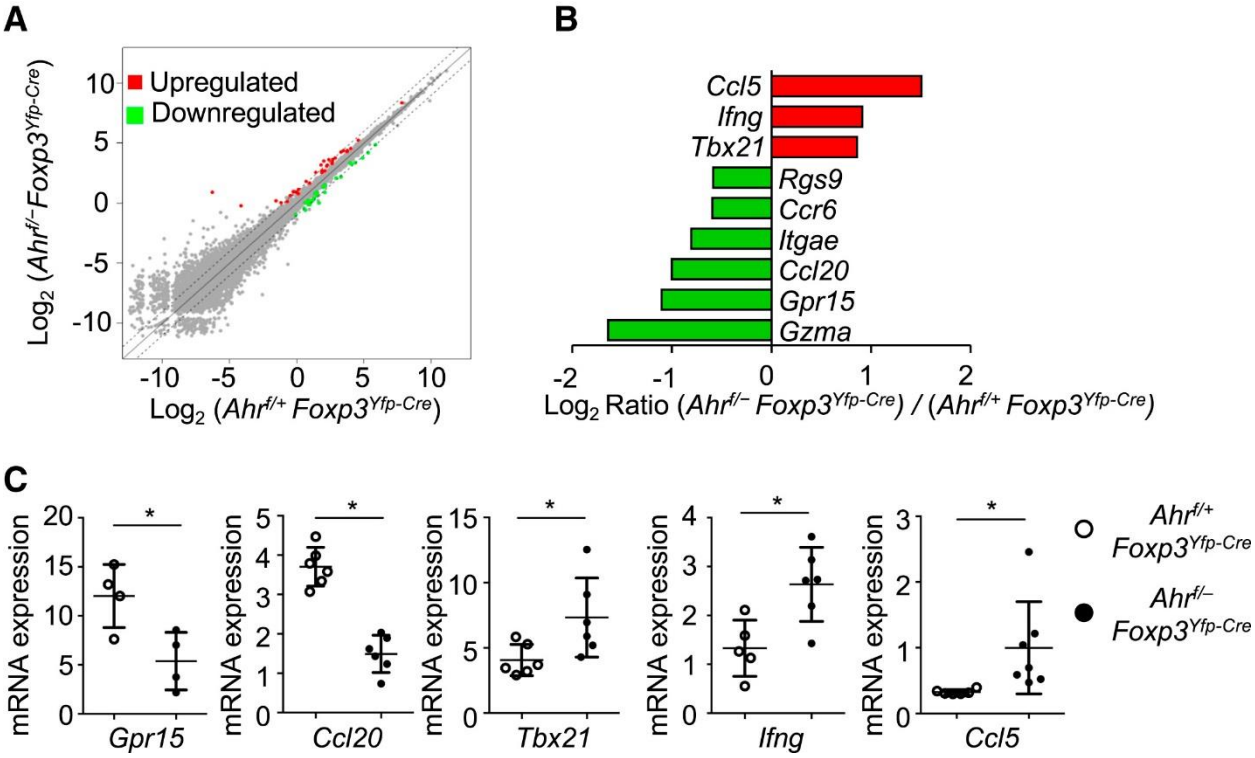


Figure 4.1 Regulation of the Intestinal Treg Transcriptional Program by Ahr

(A) Large intestinal Tregs were sorted from *Ahr*$^{f/+}$*Foxp3*$^{Yfp\text{-}Cre}$ (control) or littermate *Ahr*$^{f/–}$ *Foxp3*$^{Yfp\text{-}Cre}$ mice by flow cytometry and subjected to genome-wide mRNA analysis (RNA-seq). The dot plot shows a comparison of gene expression between the two groups. Genes significantly changed (q value ≤ 0.05) in Tregs are highlighted in red (upregulated) and green (downregulated). Dashed lines delineate 2-fold change. (B) Log_2 ratio of the indicated gene expression derived from RNA-seq of (*Ahr*$^{f/–}$*Foxp3*$^{Yfp\text{-}Cre}$) / (*Ahr*$^{f/+}$*Foxp3*$^{Yfp\text{-}Cre}$) Tregs. Data are derived from two independent experiments. (C) Large intestinal Tregs were sorted from *Ahr*$^{f/+}$*Foxp3*$^{Yfp\text{-}Cre}$ or littermate *Ahr*$^{f/–}$*Foxp3*$^{Yfp\text{-}Cre}$ mice, and the mRNA expression of the indicated genes was analyzed by real-time RT-PCR. Dots represent biological repeats. Data are shown as mean ± SD.

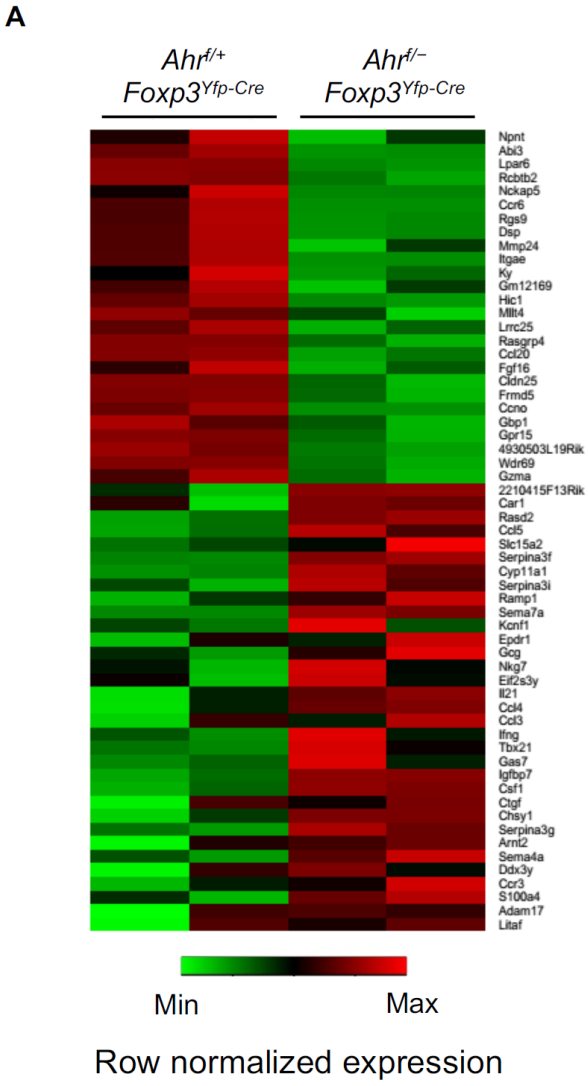


Figure 4.2 Heatmap of Diferrentially-Regulated Treg Genes

(A) Heat map representation of genes with a statistical significant difference (q-value ≤ 0.05) between large intestinal Tregs from mice of the indicated genotypes (presented as k-means clustered and normalized gene expression).

4.4 Conclusions

Our data revealed a distinct expression pattern of Ahr in different tissue-resident Tregs. Under the steady state, tissue-resident Tregs in the gut expressed highest amounts of Ahr. High Ahr expression in gut-associated Tregs, especially in peripherally induced Nrp1^{-} Tregs, may represent a mechanism of tissue adaptation, rendering gut Tregs readily activated by environmental cues (e.g., ligands that are abundantly present in the gut) to exert their suppressive function locally for gut homeostasis. Consistent with the literature (Atarashi and Honda 2011; Geuking et al. 2011), our data showed that lack of gut microbiota caused Treg reduction, especially RORγt^{+}Nrp1^{-} Tregs. We cannot rule out the possibility that Ahr activity is regulated by the microbiota through Ahr ligands. However, the microbiota appeared to play a dispensable role for Ahr expression in gut Tregs, suggesting a model in which microbiota regulate Tregs via mechanism(s) independent of Ahr expression (e.g., by regulating retinoic acid and short-chain fatty acids to affect Foxp3 expression) (Arpaia et al. 2013; Benson et al. 2009; Coombes et al. 2007; Furusawa et al. 2013; Mucida et al. 2007; Nolting et al. 2009; Schambach et al. 2007; Singh et al. 2014; Smith et al. 2013; C.-M. Sun et al. 2007).

Our data suggest a complex role for Ahr in different subsets of Tregs. Of note, there was no apparent defect in iTreg differentiation when Ahr-deficient naïve CD4^{+} T cells were stimulated by TGF-β *in vitro* (data not shown). Likewise, comparable *in vivo* Treg differentiation by ovalbumin (OVA) treatment was observed in recipient mice that adoptively transferred with naïve non-Treg cells from *Ahr*$^{f/-}$ *Foxp3*$^{Yfp\text{-}Cre}$ OT-II mice, compared to those from *Ahr*$^{f/+}$ *Foxp3*$^{Yfp\text{-}Cre}$ OT-II mice

(data not shown). In addition, Tregs in the thymus were not affected by Ahr deficiency, indicating that Ahr is dispensable for the thymic development of Tregs.

Our studies using loss-of-function and gain-of-function approaches demonstrate that Ahr promotes the expression of the gut homing and activation marker CD103, encoded by *Itgae*, in Tregs. Of note, deletion of *Itgae* in Tregs itself is not sufficient to affect Treg cell function (Annacker et al. 2005) in a T cell-transfer model of colitis, suggesting that other mechanism(s) may compensate for the loss of CD103 in Tregs. Consistent with this notion, we also observed a decrease of Gpr15, a recently identified orphan guanine nucleotide-binding protein (G protein)-coupled receptor that plays a key role in homing of Tregs to the large intestine (S. V. Kim et al. 2013). CCL20 can be expressed by Th17 cells that are abundantly present in the gut (Yamazaki et al. 2008), in agreement with the involvement of CCR6-CCL20 axis as an autocrine and/or paracrine recruitment/retention mechanism for Th17 cells and Tregs homing to mucosal tissues especially during inflammation (A. Y. S. Lee et al. 2013). Thus, decreased CCR6/CCL20 expression in Ahr-deficient Tregs may affect their homing to the gut. In agreement, genetic ablation of Ahr in Tregs caused reduction of Tregs, especially $Nrp1^{-}$ Tregs, only in the large intestinal lamina propria but not in other tissues. In addition, a competitive homing assay showed the most marked reduction of Ahr-deficient Tregs in the large intestine, consistent with the important role for Ahr in Treg gut homing.

Our mouse model (i.e., *Ahr*^{CAIR}) allows the expression of C-Ahr under the regulation of the endogenous Ahr locus and simultaneously marks Ahr expression by a GFP reporter to track Ahr expression and function in a cell-type-specific manner in vivo after crossing to Cre transgenic mice. However, several important considerations need to be kept in mind. First, the reporter GFP reflects Ahr transcription. Second, we are using a destabilized GFP protein, and its half-life is likely different from endogenous Ahr protein. Third, the Ahr locus drives not only GFP expression but also CA-Ahr in Ahr reporter mice, and CA-Ahr may feed back and influence the expression of endogenous Ahr. Thus, we have examined and compared Ahr expression both by GFP in Ahr reporter mice and by intracellular staining and real-time RT-PCR of endogenous Ahr protein/mRNA in wild-type mice. Our data suggest that Ahr mRNA and protein expression can be reported by GFP. It is important to point out that the protein expression of the CA-Ahr allele was considerably lower compared with the wildtype allele in Tregs, as shown in immunoblotting, caused by a still unknown mechanism. Nevertheless, in line with the phenotypes that we observed using Ahr ligands, our data favor a model in which CA-Ahr driven by the endogenous locus control regions in *Ahr*$^{CAIR/+}$ *Foxp3*$^{Yfp\text{-}Cre}$ mice was physiologically active.

In the model of T cell transfer-mediated colitis, we were unable to transfer gut Tregs because of the limited cell number and therefore used splenic Tregs. It is important to note that the Ahr-expressing Tregs (GFP^{+}YFP^{+} cells) in *Ahr*$^{CAIR/+}$ *Foxp3*$^{Yfp\text{-}Cre}$ mice co-expressed RORγt. In addition, Ahr-expressing Tregs in the spleens of *Ahr*$^{CAIR/+}$ *Foxp3*$^{Yfp\text{-}Cre}$ mice showed more activated phenotypes, as revealed by increased percentages of CD44^{hi}CD62L^{low} Tregs (data not shown). Despite no apparent Treg phenotype in the spleen under the steady-state conditions, defective

functions of the splenic Ahr-deficient Tregs in suppressing T cell transfer-mediated colitis was observed, consistent with the more suppressive function of RORγt$^+$ Tregs and/or activated Tregs in various animal models of disease (K. S. Kim et al. 2016; Luo et al. 2016; Ohnmacht et al. 2015; Sefik et al. 2015). In addition, upon adoptive transfer to a lymphopenic Rag1-deficient environment, Ahr-deficient splenic Tregs showed enhanced proinflammatory cytokine production (i.e., IFN-γ and IL-17) in the gut, consistent with their impaired suppressive function.

Although Ahr was co-expressed with RORγt in Tregs, overall RORγt$^+$ Tregs did not show a reduction in Ahr-deficient mice. In addition, no alteration of RORγt transcripts in Ahr-deficient Tregs was not detected by RNA-seq analysis. These data suggest that Ahr is unlikely to regulate RORγt transcription in Tregs. Ahr was reported to regulate *in vitro* Foxp3 expression induced by TGF-β (Quintana et al. 2008). However, our data suggest that Ahr did not regulate Foxp3 expression *in vivo*, as evidenced by lack of detectable changes in transcription of *Foxp3* gene by RNA-seq analysis, Treg-specific demethylated region (TSDR) methylation status at the *Foxp3* locus by bisulfite sequencing, or chromatin conformation by ATAC-seq. In addition, we did not observe Foxp3 stability changes upon deletion of Ahr in Tregs. Together, these data suggest a model that Ahr preferentially marks gut Tregs and multiple mechanisms of action of Ahr may work in concert to mediate Treg gut adaptation. Perturbation of the Ahr pathway may lead to impaired Treg cell homing and function and thus diminish its capacity to control inflammation in the gut.

CHAPTER 5. RESULTS: Aryl Hydrocarbon Receptor Signaling Cell Intrinsically Inhibits Intestinal Group 2 Innate Lymphoid Cell Function

This work was published in *Immunity* (S. Li et al. 2018). Results discussed are limited to the direct contributions of the dissertation author.

5.1 Introduction

Innate lymphoid cells (ILCs) represent an emerging family of cell types, including natural killer (NK) cells (which express both transcription factors T-bet and Eomes), group 1 ILCs (ILC1s; which express T-bet but not Eomes), group 2 ILCs (ILC2s; which express transcription factor GATA3), and group 3 ILCs (ILC3s; which express transcription factor RORgt) (Artis and Spits 2015). ILCs play important roles in tissue remodeling and innate immunity, particularly at barrier surfaces (Ebbo et al. 2017; Klose and Artis 2016). Various tissues contain distinct ILC subsets. For instance, the liver is enriched with ILC1s; the lung contains ILC2s in mice under the steady state despite the existence of other ILCs in humans; in contrast, the mouse gut harbors all three major ILC subsets (S. Li et al. 2018; Simoni and Newell 2018). It is unknown how various ILCs are kept in balance for gut immune homeostasis and how the host orchestrates a diverse population of ILCs to control infection and inflammation.

The aryl hydrocarbon receptor (Ahr) is a ligand-dependent environmental sensor, and its transcriptional activity requires ligand-induced nuclear translocation and dimerization with its

partner, the aryl hydrocarbon receptor nuclear translocator (ARNT) (Cella and Colonna 2015). Best known for sensing environmental toxins [e.g., 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin), Ahr can also respond to endogenous ligands generated from host cells, diet, and microbiota (Zhou 2016). Ahr is expressed in barrier tissues (e.g., the gut and skin) by immune cells, such as lymphocytes, and by tissue structural cells, such as epithelial and stromal cells (Stockinger et al. 2014). The presence of Ahr-expressing cells in those locations could represent a mechanism of tissue adaptation by allowing the host to readily respond to external environmental changes. Consistent with this notion, regulatory T (Tregs) cells in the gut express the higher amounts of Ahr than those in other lymphoid or non-lymphoid tissues (Ye et al. 2017).

Recent studies have shown that Ahr plays important roles in regulating ILCs. For example, Ahr is required for liver-resident ILC1 maintenance (L. H. Zhang et al. 2016). Ahr is also essential for ILC3 maintenance and function (Kiss et al. 2011; J. S. Lee et al. 2011; J. Qiu et al. 2012). Ahr-expressing ILC3 regulate intestinal T helper 17 (Th17) cells and microbiota and thus gut inflammation (Hepworth et al. 2013; J. Qiu et al. 2013). Mechanistically, Ahr works cooperatively with transcription factors RORγt and STAT3 to induce interleukin-22 (IL-22) for anti-bacterial immunity (Guo et al. 2014; J. Qiu et al. 2012). Ahr activity in ILC3 can be inhibited by a zinc-finger transcription factor, Ikaros, via a mechanism involving disruption of Ahr-ARNT complex (S. Li et al. 2016). Despite the progress of our understanding of Ahr in ILCs, how Ahr expression is regulated in ILCs and its function in other ILCs (e.g., ILC2s) are unknown. In this study, we show that among various ILCs in the gut, Ahr is expressed at the highest levels by ILC2s. Specific chromatin events mark a tissue adaptation of Ahr expression in gut ILC2s. In contrast to its

function in promoting ILC3 maintenance, Ahr suppresses ILC2 function, thus representing a critical node of regulation of the ILC balance for gut immunity against various pathogenic infections.

5.2 Materials and Methods

Mice

All the mice in this study were maintained in Specific Pathogen Free (SPF) facilities at the University of Florida. Mice were littermates and were 6–8 weeks old unless otherwise indicated. *Ahr*$^{-/-}$ (Fernandez-Salguero et al. 1995), *Ahr*^{CAIR} (Ye et al. 2017), *Rorc*-cre and *Rorc*$^{gfp/gfp}$ (Eberl and Littman 2004; Z. Sun et al. 2000), and *Il33*$^{-/-}$ mice (Pichery et al. 2012) were previously described. *Ahr*$^{f/f}$ mice, and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were purchased from Taconic Farms. *Vav1*-cre and *EIIa*-cre mice were purchased from Jackson Laboratory. All studies with mice were approved by the Animal Care and Use Committee of the University of Florida.

Isolation of Lymphocytes from Intestinal Lamina Propria, Lung, or Adipose Tissue, and Flow Cytometry

Isolation of intestinal lamina propria cells and flow cytometry were done as previously described (J. Qiu et al. 2012). CD16/32 antibody (eBioscience) was used to block the non-specific binding to Fc receptors before surface staining. To isolate lymphocytes from lung or adipose tissue, blood was perfused out of circulatory system. Lung or epididymal fat tissue was collected and digested

in complete RPMI medium containing DNase I (150 □g/ml, Sigma) and collagenase IV (300 U/ml, Sigma) (for the lung) or collagenase II (2 □g/□l, Sigma) (for the fat) at 37°C in 5% CO2 incubator for 1-1.5 hours. The digested lung or fat tissues were smashed and filtered through 100 □m cell strainer. Mononuclear cells were then harvested from the interphase of an 80% and 40% Percoll gradient after a spin at 2500 rpm for 20 min at room temperature. Lymphocytes isolated from intestinal lamina propria were stained with antibodies against following makers: GATA3 (PE-Cy7, eBioscience), RORγt (PE, eBioscience), T-bet (PE-Cy7, eBioscience), Eomos (PerCP-Cy5.5, eBioscience), Ahr (APC, eBioscience), ST2 (PE, MD Bioproducts), KLRG1 (PerCP-Cy5.5, eBioscience), CD127 (APC, eBioscience), IL-13 (FITC, eBioscience), IL-5 (Brilliant Violet 421, Biolegend), Areg (Biotin, R&D), IL-22 (APC, eBioscience), CD45.2 (PerCP-Cy5.5, eBioscience), CD90.2 (APC-Cy7, eBioscience), CD127 (PE, eBioscience), CD25 (PE-Cy7, eBioscience), α4β7 (APC, eBioscience), Flt3 (PerCP-Cy5.5, eBioscience), CD117 (APC, eBioscience), and Sca-1 (PE-Cy7, eBioscience). Lymphocytes isolated from the lung or adipose tissue were stained with antibodies against following makers: GATA3 (PE-Cy7, eBioscience), Ahr (APC, eBioscience), KLRG1 (PerCP-Cy5.5, eBioscience), CD127 (APC, eBioscience). Lineage marker mix (Lin) contained APC-Cy7-CD3, CD5, CD19, B220, Ly6G, CD11b, CD11c, and Ter119 (eBioscience). To sort CLP, CHILP, ILC2P, ILC2 and ILC3, Lin contained FITC-CD3, CD19, B220, Ly6G, FcεR1, CD11c, CD11b, Ter119, NK1.1, and CD16/CD32 (Tonbo). For intracellular staining of Ahr in ILC1 and NK cells, Lin contained APC-Cy7-CD3, CD5, CD19 and B220 (eBioscience). For nuclear transcription factor staining, cells were fixed and permeabilized with Foxp3 staining buffer Kit (eBioscience). For cytokine staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 4 hours and Brefeldin A (2 μg/ml) was added 2 hours before cells were

harvested. The live and dead cells were discriminated by Live and Dead violet viability kit (Invitrogen) or Zombie Aqua Fixable Viability Kit (Biolegend).

Quantitative Real-Time RT-PCR

RNA of sorted cells from the gut was isolated with Trizol reagent (Invitrogen). cDNA was synthesized using GoScript™ Reverse Transcription kit (Promega). Real-time RT-PCR was performed using SYBR Green (Biorad) and different primer sets.

Chromatin Immunoprecipitation (ChIP) assay

Sorted ILC2 ($Lin^{-}KLRG1^{+}CD90^{+}$) were cultured with IL-2 (10 ng/ml), IL-7 (10 ng/ml) and IL-33 (10 ng/ml) in RPMI medium for 5 days. For ChIP of Ahr, cells were treated with FICZ (200 nM) for 4 hours before harvest. Cells were cross-linked with 1% formaldehyde for 15 minutes. Chromatin was sheared by sonication with Bioruptor Pico (30" on and 30" off for 25 cycles) and immunoprecipitated with anti-Ahr (Enzo Life Science), or anti-GATA3 (BD Biosciences), or anti-Gfi1 (Abcam) using iDeal ChIP-Seq Kit for transcription factors (Diagenode). Eluted DNA was used for real-time PCR analyses using specific primers.

RNA-seq and analysis

ILC2 ($Lin^{-}KLRG1^{+}CD90^{+}$) were sorted by flow cytometry from the gut of littermate $Ahr^{+/+}Rag1^{-/-}$ or $Ahr^{-/-}Rag1^{-/-}$. About 1 X 10^6 ILC2 were pooled from 5-6 mice per group, and lysed in Trizol (Invitrogen). RNA was subsequently extracted with RNAeasy Mini Kit (Qiagen). Total RNA was treated with Ribo-zero kit and RNAseq libraries were generated using kit from Illumina. Barcoded samples were pooled and sequenced over 2 lanes on an Illumina HiSeq 2500 instrument (the University of Chicago Genomics Core) to produce 50 bp single-end reads. De-multiplexed raw data files from the sequencing core were analyzed for quality control using FastQC (Babraham Bioinformatics). Reads were mapped (with TopHat2 fast splice junction mapper for RNA-Seq) to the mm10 assembly of the Mus musculus genome (National Center for Biotechnology Information) and filtered for uniquely mapped reads (D. Kim et al. 2013). Genome visualization tracks (bedgraph files) were uploaded to the University of California Santa Cruz (UCSC) Genome Browser for visual comparison of expression levels (Meyer et al. 2013; Patel and Jain 2012). Quantitated relative mRNA expression levels (FPKM) were calculated based on exon regions using Cufflinks and the mm10 reference genome annotations (Trapnell et al. 2010). Significantly changed genes were identified by Cuffdiff (Trapnell et al. 2010). Protein-coding genes found to be significantly changed (q-value ≤ 0.05, and filtered on max FPKM ≥ 1) were used for pathway analysis with GSEA software and the Molecular Signature Database (MSigDB) (Subramanian et al. 2005).

Transposase-Accessible Chromatin Sequencing (ATAC-Seq) and Analysis

Sorted ILC2 ($Lin^{-}KLRG1^{+}CD90^{+}$) from the gut were subjected to ATAC-Seq according to the published protocol Buenrostro et al. 2013) with a modification in the library purification step, which was described previously (Ye et al. 2017). ATAC-Seq reads were mapped to the mouse genome (mm9) with bowtie2 (Langmead and Salzberg 2012). The mapped reads were filtered using samtools (H. Li et al. 2009), keeping only the uniquely aligned reads, and bedgraph files (scaled to 10 million reads) were made with bedtools (Quinlan and Hall 2010). ATAC-seq peak locations were identified using Homer (Heinz et al. 2010). Differentially-expressed ATAC-seq peaks were identified by first quantifying peak signal using HTSeq-count, and then using DESeq2 for differential analysis (Anders, Pyl, and Huber 2015; Love, Huber, and Anders 2014). ATAC-seq signal heatmaps were generated by ngs.plot.r (Shen et al. 2014).

Chromatin Immunopreciptiation Sequencing (ChIP-seq) and Analysis

Sorted ILC2 ($Lin^{-}KLRG1^{+}CD90^{+}$) from the large intestine of *Rag1*-deficient mice (*Rag1*$^{-/-}$*Ahr*$^{+/+}$, *Rag1*$^{-/-}$ *Ahr*$^{-/-}$), were expanded in *in vitro* culture and stimulated with the Ahr ligand, 6-formylindolo[3,2-b]carbazole (FICZ) for the last 4 hours.

Statistical Methods

Unless otherwise noted, statistical analysis was performed with the unpaired two-tailed Student's t test on individual biological samples with GraphPad Prism. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.3 Results

5.3.1 The Ahr Locus in Gut ILC2s Has a Unique Chromatin Structure

To determine the potential mechanism underlying the gut-specific expression of Ahr in ILCs, we analyzed chromatin accessibility at the Ahr locus by using the assay for transposase-accessible chromatin using sequencing (ATAC-seq) data generated from various ILC lineages (H.-Y. Shih et al. 2016; Ye et al. 2017), and several chromatin features at the Ahr locus emerged. Despite various levels of expression of Ahr in ILC subsets and Tregs, the chromatin conformation was generally open at the transcription start site of *Ahr* (Figure 5.1A and 5.1B). At least six regions (boxed in red) identified at the Ahr locus showed unique gut-ILC2-specific ATAC peaks, i.e., peak 1 (_8 kb), peak 2 (+7.6 kb), peak 3 (+14 kb), peak 4 (+24 kb), peak 5 (+41 kb), and peak 6 (+54 kb), indicating open chromatin (Figure 5.1B). These peaks were neither evident in other ILCs (NK, ILC1s, or ILC3s), nor in lung ILC2 (Figure 5.1B). Gut Tregs also expressed higher amounts of Ahr compared to Tregs in the lymphoid organs (e.g., thymus, spleen, and lymph nodes) and other tissue-resident Tregs in the lung, fat, skin, and liver (Ye et al. 2017). However, these gut ILC2-specific open chromatin events at the *Ahr* locus were mostly absent in gut Tregs (Figure 5.1B). Together, these data suggest that these chromatin-accessible regions could function as transcriptional enhancers and are responsible for tissue-specific and/or high expression of Ahr by ILC2s in the gut.

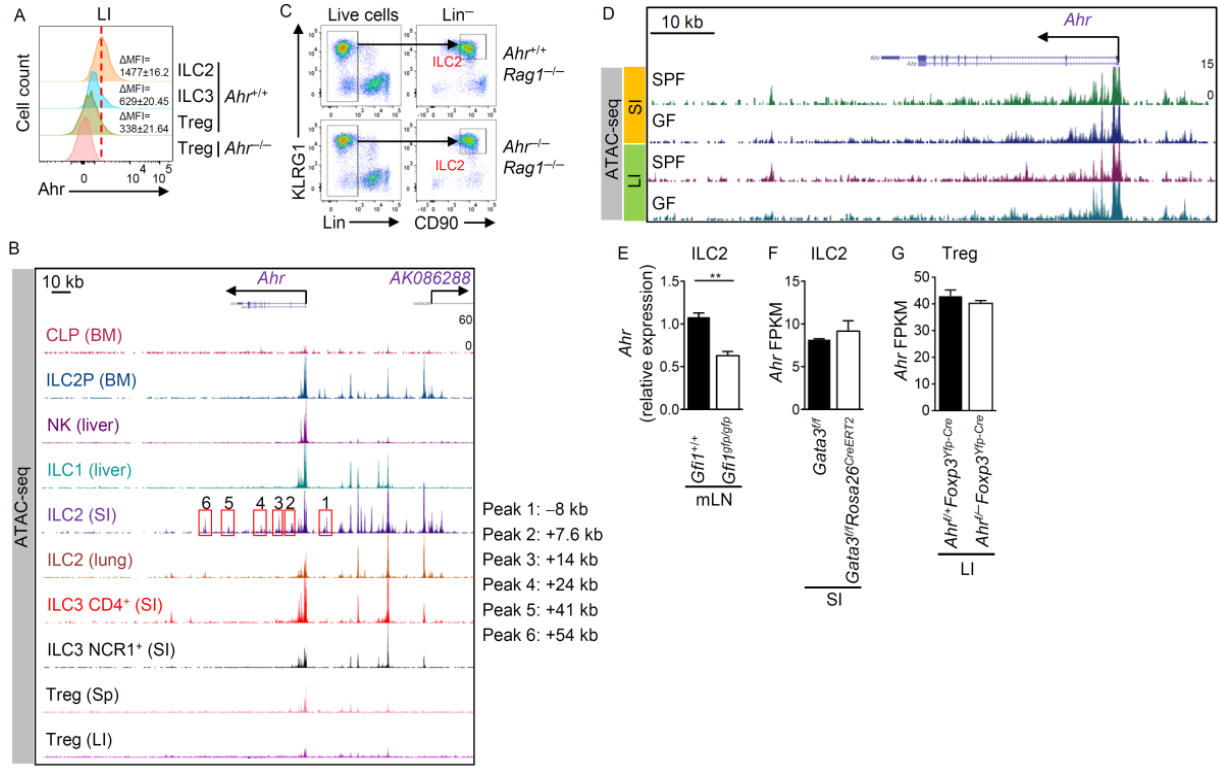


Figure 5.1 Chromatin accessibility at the *Ahr* locus in ILC progenitors, mature ILCs, and Treg cells

(A) Intracellular staining of Ahr in ILC2s (Lin–GATA3+RORγt–), ILC3s (Lin–RORγt+GATA3–), and Treg cells (Lin+Foxp3+) isolated from the lamina propria of large intestine (LI). Data are shown as mean ± SEM (n=5 per group). ΔMFI was calculated as follows: MFI of indicated populations – MFI of corresponding population from *Ahr–/–* mice. (B) Representative ATAC-seq signals at the *Ahr* locus in indicated cell populations. Analysis is based on published ATAC-seq data (Shih et al, 2016; Ye et al. 2017). Red boxes highlight the gut ILC2-specific peaks with indicated distance downstream or upstream from the transcription start site. (C) Sorting strategy of ILC2s from the gut. (D) Representative ATAC-seq signals at the *Ahr* locus in gut ILC2s (Lin–KLRG1+CD90+) of SPF or GF C57BL/6 wildtype mice. (E) Relative Ahr expression in the mesenteric lymph nodes (mLN) of Gfi1-sufficient (Gfi1+/+) or deficient (Gfi1gfp/gfp) ILC2s analyzed from published microarray data (Spooner et al., 2013). (F) Fragments Per Kilobase of transcript per Million mapped reads (FPKM) of Ahr in RNA-seq with sorted ILC2s from SI of *Gata3f/f* or *Gata3f/fRosa26CreERT2* mice injected with tamoxifen for 2 to 3 days (Yagi et al., 2014). (G)FPKM of Ahr in RNA-seq from Treg cells sorted from LI of *Ahrf/+Foxp3Yfp-Cre* or *Ahrf/– Foxp3Yfp-Cre* littermate mice (Ye et al. 2017).

5.3.2 Ahr regulates gut-specific chromatin events at the *Ahr* locus

We next sought to determine whether deletion of Ahr could affect chromatin accessibility at the Ahr locus. To exclude the confounding impact on the ILC2 phenotypes by the adaptive immune system (Halim et al. 2016; Oliphant et al. 2014), we sorted gut ILC2s ($Lin^{-}KLRG1^{+}CD90^{+}$)) (Pelly et al. 2016) from $Ahr^{-/-}Rag1^{-/-}$ or littermate $Rag1^{-/-}$ mice. ATAC-seq conducted with the sorted intestinal ILC2s revealed that without prominent changes of other gut ILC2-specific peaks, certain peaks, including peaks 3 and 6, were markedly decreased in the absence of Ahr, suggesting that Ahr regulates its own locus chromatin accessibility (Figure 5.2). Consistent with unchanged Ahr expression, the gut-ILC2-specific chromatin events at the Ahr locus (e.g., peak 6) were minimally affected (without statistical significance) in GF mice compared with SPF mice, as revealed by ATAC-seq analysis (Figure 5.1D).

Transcription factors can auto-regulate their transcription by binding to their own locus (e.g., the promoter and/or enhancers). To test the potential self-regulation of Ahr, we conducted chromatin immunoprecipitation (ChIP) of Ahr with ILC2s sorted from $Ahr^{-/-}$ or $Ahr^{+/+}$ mice. Among two Ahr-dependent ATACseq peaks, peak 6 (located around 54 kb downstream of the transcription start site) showed enrichment of Ahr (Figure 5.3A), suggesting a differential requirement for Ahr binding in chromatin accessibility. Together, these data show that Ahr regulates chromatin remodeling at its own locus in gut ILC2s.

5.3.3 Cooperative action of Ahr and Gfi1 promotes Ahr transcription in ILC2

Through in silico motif analysis of the peak 6 region, we identified the evolutionarily conserved consensus sequences for Gfi1 and Gata3, transcription factors that play important roles in ILC2 development and function (data not shown). Recruitment of Gfi1 and GATA3 to peak 6 was further confirmed by a previously published ILC2 ChIP-sequencing (ChIP-seq) analysis (Spooner et al. 2013; Zhong et al. 2016) (Figure 5.2). These data prompted us to assess the potential synergy between Ahr and other key ILC2 regulators, which could potentiate the *Ahr* transcription. To this end, we performed Gfi1 and GATA3 ChIP experiments with sorted large intestinal ILC2s from $Ahr^{-/-}$ or $Ahr^{+/+}$ littermate mice. Strikingly, Ahr deficiency in ILC2 significantly reduced the recruitment of Gfi1 and GATA3 to the peak 6 region (Figures 5.3B and 5.3C), suggesting that binding of Gfi1 and GATA3 to the *Ahr* locus is dependent on Ahr, presumably facilitated by Ahr-directed permissive chromatin changes. In accordance to these binding events at the *Ahr* locus, Ahr-deficient ILC2 showed reduced Ahr expression (Figure 5.3D) (Spooner et al. 2013), while deletion of GATA3 has minimal effect on Ahr expression in ILC2 (Yagi et al. 2014). Together, these data suggest that Ahr may cooperate with Gfi1 to promote its own transcription in a cell-type specific manner.

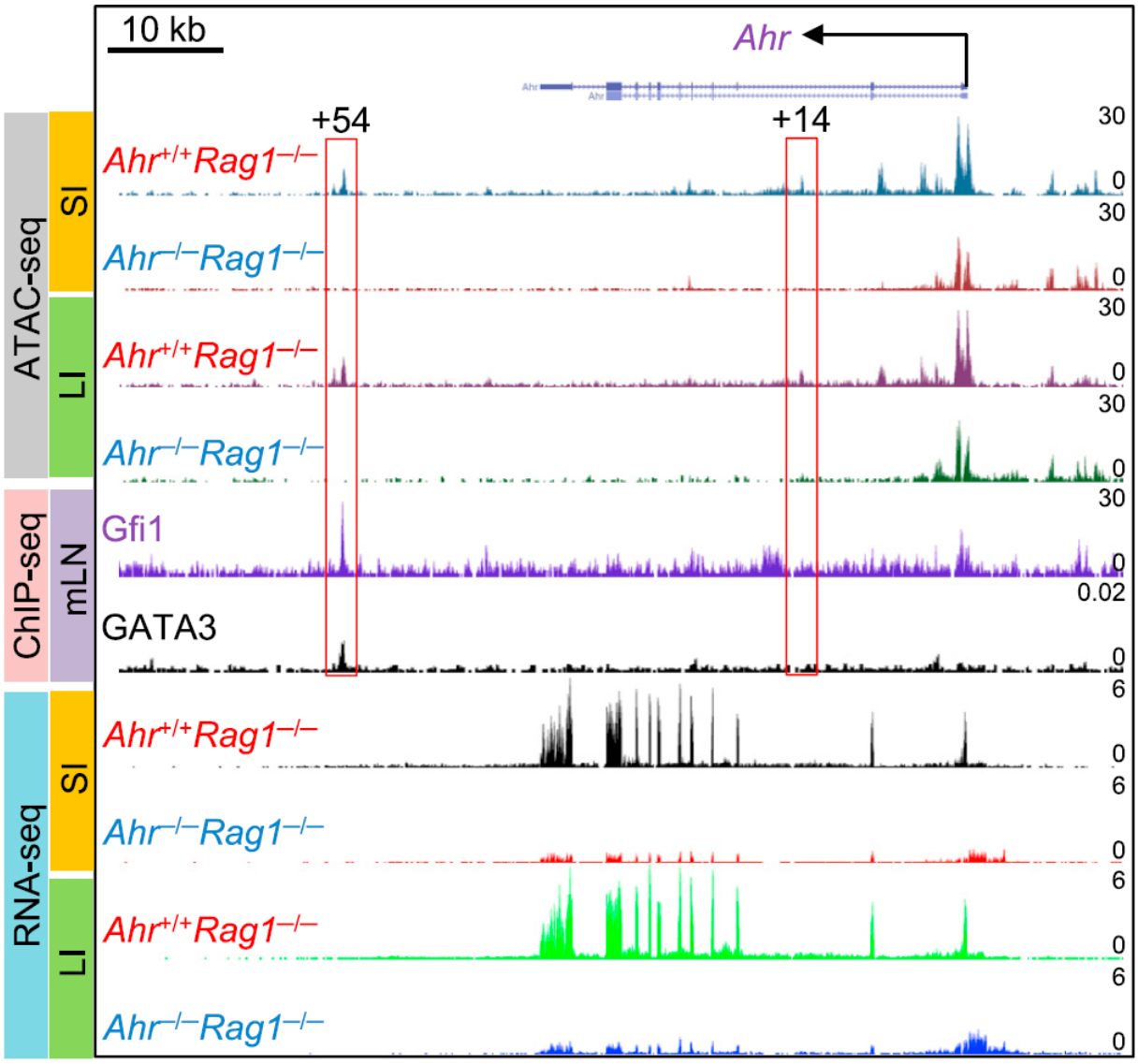


Figure 5.2 Ahr Is Highly Expressed by Gut ILC2s with Unique Chromatin Features at the Ahr Locus

Top: representative ATAC-seq tracks at the Ahr locus in ILC2s sorted from the SI or LI of *Ahr*$^{+/+}$*Rag1*$^{-/-}$ or *Ahr*$^{-/-}$*Rag1*$^{-/-}$ littermate mice. Middle: recruitment of Gfi1 or GATA3 to the Ahr locus in ILC2s as measured by analysis of published ChIP-seq data. Bottom: representative RNA-seq tracks at the Ahr locus in ILC2s sorted from the SI or LI of *Ahr*$^{+/+}$*Rag1*$^{-/-}$ or *Ahr*$^{-/-}$*Rag1*$^{-/-}$ littermate mice.

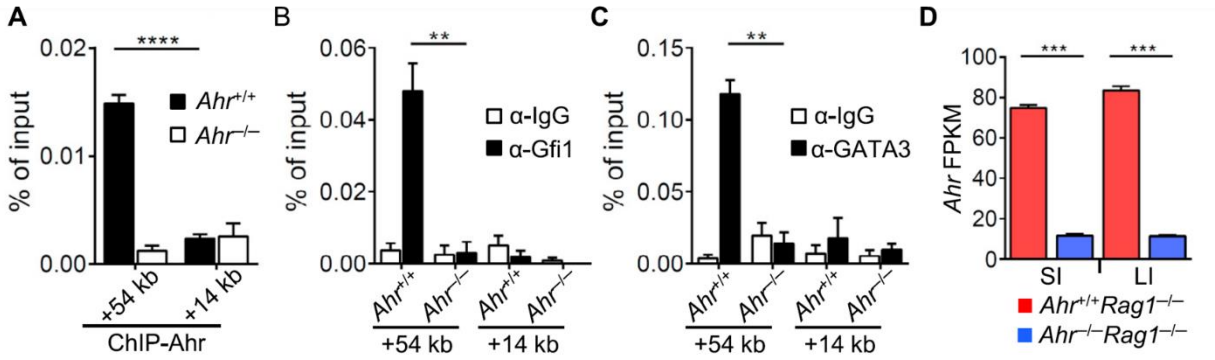


Figure 5.3 Ahr may cooperate with Gfi1 to promote its own transcription

(A–C) ILC2s ($Lin^{-}KLRG1^{+}CD90^{+}$) sorted from LI of $Ahr^{+/+}$ or $Ahr^{-/-}$ littermate mice were expanded in vitro and subjected to a ChIP assay. Enrichment of Ahr (A), Gfi1 (B), or GATA3 (C) at the site 54 kb (+54 kb) or 14 kb (+14 kb) downstream of the transcription start site was determined by real-time PCR. Data represent two independent experiments and are shown as mean ± SEM (n = 3). Rabbit IgG isotype antibody was used as a negative control for ChIP of Gfi1 or GATA3. (J) FPKM of Ahr in RNA-seq of sorted ILC2s from the SI or LI of $Ahr^{+/+}Rag1^{-/-}$ or $Ahr^{-/-}Rag1^{-/-}$ littermate mice. The q value was generated by Cufflinks analysis as described in the STAR Methods. ***q < 0.001.

5.3.4 Ahr deficiency alters ILC2 transcriptional program

To gain more insight into the regulation of ILC2 transcriptional program by Ahr, we performed genome-wide analysis of mRNA expression with RNA-sequencing (RNA-seq) data of gut ILC2s. Gene expression analysis identified 424 genes significantly changed (q-value ≤ 0.05, fold change ≥ 1.5) out of 11,286 expressed genes (fragments per kilobase of transcript per million mapped reads [FPKM] ≥ 1) in Ahr-deficient ILC2s; among these genes, 278 were upregulated and 146 genes were downregulated in the small intestine of *Ahr*$^{-/-}$*Rag1*$^{-/-}$ mice (Figure 5.4A). Large intestinal ILC2s that lacked Ahr showed 168 significantly changed genes out of 11,458 expressed genes, among which 109 were upregulated and 59 were downregulated (Figure 5.4A). Despite an overlapping set of differentially expressed genes, unique genes were found to be regulated by Ahr in small or large intestinal ILC2s, consistent with the distinct features of these two organs (Figure 5.4B). When cross-referencing to the previously published 32 ILC2-characteristic protein-coding genes whose expression is at least 4-fold higher than that in other ILC subsets in the small intestines (Robinette et al. 2015), we found differential regulation of these genes (16 out of 32 for the small intestine and 12 out of 32 for the large intestine) by Ahr (Figures 5.4C and 5.4D). Together, these data suggest that Ahr regulates a broader set of ILC2 gene transcription that is not limited to the ILC2 signature program.

We further determined the impact of Ahr on the expression of genes that have been implicated in the literature to play an important role in transcriptional control of ILC2s (S. Li, Bostick, and Zhou 2017; Sonnenberg and Artis 2015). In the small intestine, *Gfi1*, *Tcf7*, *Nfil3*, *Id2*, *Ets1*, and *Tox*

expression was increased in Ahr-deficient ILC2s (q value ≤ 0.05), whereas *Gata3, Rora, Bcl11b,* and *Ehmt2* (G9a) expression was not significantly changed (Figure 5.4E). In the large intestine, *Gfi1* was upregulated (q value ≤ 0.05) in the absence of Ahr, whereas the other key regulators mostly remained statistically unchanged or downregulated (e.g., *Tox*) (Figure 5.4E). Together, these data suggest that Ahr selectively regulates a subset of ILC2-related genes in the gut.

Genes that were increased in the small intestines of *Ahr*$^{-/-}$*Rag1*$^{-/-}$ mice showed enrichment in pathways involved in cytokine-cytokine-receptor interaction, the chemokine signaling pathway, and the T cell receptor signaling pathway (Figure 5.5A). Downregulated genes showed enrichment in pathways involved in adherens junctions, Jak-STAT signaling, tight junctions, and lysosome and lysine degradation signaling pathways. The cytokine-cytokine-receptor interaction pathway also showed downregulation in Ahr-deficient ILC2s, most likely from the downregulation of IL-2 in Ahr-deficient ILC2s (data not shown). Together, these data suggest that Ahr targets various signaling pathways to regulate ILC2 gene transcription.

5.3.5 Ahr regulates chromatin accessibility at select gene loci in ILCs

Globally, ATAC-seq peaks did not change between Ahr-deficient and Ahr-sufficient ILC2s or ILC3s sorted from *Ahr*$^{-/-}$*Rag1*$^{-/-}$ and *Ahr*$^{+/+}$*Rag1*$^{-/-}$ littermate mice. However, loss of Ahr did affect a number of specific peaks in ILC2s (around 1.5% of total peaks for the small intestine and 1.4% of total peaks for the large intestine) and ILC3s (around 7.4% of total peaks for the small intestine and 6.6% of total peaks for the large intestine) (Figure 5.5B-F), indicating that Ahr does

not function as a general chromatin remodeler in ILCs but instead regulates selective chromatin events in the genome. The differentially expressed genes in ILCs correlated with the changes in ATACseq peak signals (e.g., *Il1rl1* for ILC2s and *Il22* for ILC3s) (Figure 5.4F and 5.5F). Chromatin changes observed at the Ahr and *Il1rl1* loci in Ahr-deficient ILC2s on the *Rag1*$^{-/-}$ background were similar to those on the C57BL/6 background (data not shown). The location of ATAC-seq peaks in the genome were mostly unchanged between *Ahr*$^{-/-}$and *Ahr*$^{+/+}$ ILC2s or ILC3s, and most peaks were located in the promoters (18%–23%), introns (39%–41%), and intergenic regions (29%–37%) (Figure 5.4G). However, the differentially regulated peaks were preferentially located in the introns (48%–51%) or intergenic regions (41%–44%), but not the promoters (3%–6%), of ILCs (Figure 5.4H), suggesting a critical role for Ahr in regulating ILC distal regulatory gene elements (e.g., enhancers).

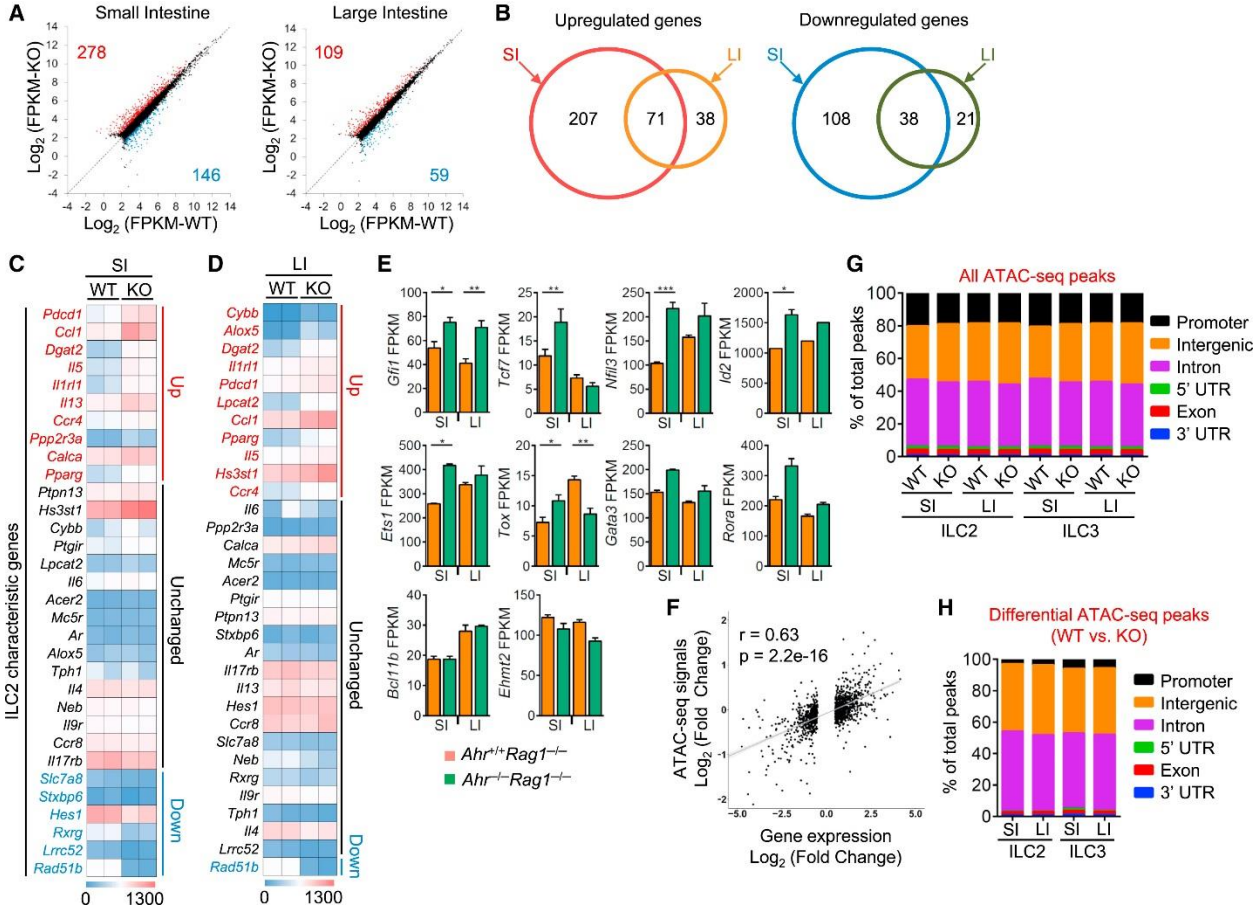


Figure 5.4 Ahr Deficiency Alters the ILC2 Transcriptional Program and Affects the Chromatin **Landscape in ILCs**

(A) Scatterplot of $\log_2$ (FPKM) gene expression compares ILC2s sorted from littermate *Ahr*$^{+/+}$*Rag1*$^{-/-}$ (wild-type [WT]) or *Ahr*$^{-/-}$*Rag1*$^{-/-}$ (knockout [KO]) mice. Differentially expressed genes (q value $\leq$ 0.05, fold change $\geq$ 1.5) are highlighted in blue (downregulated) or red (upregulated). (B) Venn diagrams of upregulated and downregulated genes identified in (A) indicate the overlap of differentially expressed genes regulated by Ahr in the ILC2s between the SI and LI. (C and D) Heatmap of ILC2-characteristic genes in the SI (C) or LI (D) of littermate *Ahr*$^{+/+}$*Rag1*$^{-/-}$ (WT) or *Ahr*$^{-/-}$*Rag1*$^{-/-}$ (KO) mice. (E) FPKM of key transcriptional regulator genes in ILC2s from the SI or LI of littermate *Ahr*$^{+/+}$*Rag1*$^{-/-}$ or *Ahr*$^{-/-}$*Rag1*$^{-/-}$ mice. The q value was generated by Cufflinks analysis as described in the STAR Methods. $*q < 0.05$, $**q < 0.01$, $***q < 0.001$. (F) Scatterplot comparing the log2 fold change (KO/WT) of ATAC-seq signals with the log2 fold change (KO/WT) of RNA-seq signals. The r value represents an r2 goodness-of-fit value for linear regression. (G) Global annotation of ATAC-seq peak locations in ILC2s and ILC3s from the SI and LI of littermate *Ahr*$^{+/+}$*Rag1*$^{-/-}$ (WT) or *Ahr*$^{-/-}$*Rag1*$^{-/-}$ (KO) mice. (H) Annotation of differentially expressed ATAC-seq peak locations in ILC2s and ILC3s from the SI and LI of littermate *Ahr*$^{+/+}$*Rag1*$^{-/-}$ (WT) or *Ahr*$^{-/-}$*Rag1*$^{-/-}$ (KO) mice.

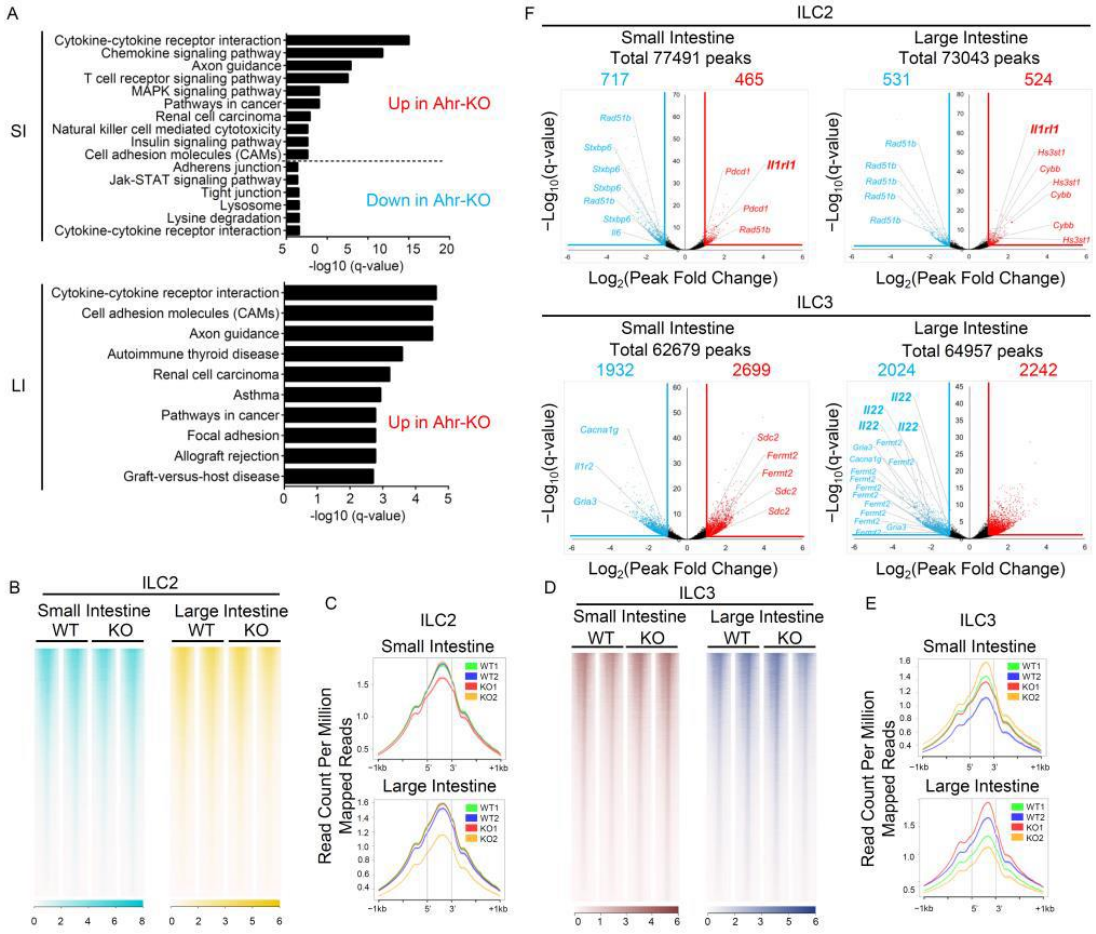


Figure 5.5 Ahr regulates specific gene pathways and chromatin remodeling events in ILCs.

(A) Pathway analysis of differentially-expressed genes identified by RNA-seq in ILC2s (q-value ≤ 0.05) from the small intestine (SI) and large intestine (LI) of *Ahr*$^{+/+}$*Rag1*$^{-/-}$ (WT) or littermate *Ahr*$^{-/-}$*Rag1*$^{-/-}$ (KO) mice. (B, D) ATAC-seq signal across all peak locations comparing ILC2s or ILC3s from SI and LI of *Ahr*$^{+/+}$*Rag1*$^{-/-}$ (WT) or *Ahr*$^{-/-}$ *Rag1*$^{-/-}$ (KO) littermate mice. (C, E) Average ATAC-seq peak signal (Reads Per Million mapped reads; RPM) centered on all peak locations (signal is calculated from 5' to 3' end of the peaks ±1kb) of ILC2s or ILC3s. (F) Volcano plots indicating fold changes between *Ahr*$^{+/+}$*Rag1*$^{-/-}$ (WT) and littermate *Ahr*$^{-/-}$*Rag1*$^{-/-}$ (KO) ILC2s or ILC3s in ATAC-seq signals and q-value. Differentially-expressed peaks (RPM ≥ 1, q-value ≤ 0.05, and fold change ≥ 2) are highlighted in blue (decreased in KO) or red (increased in KO). The differential peaks at the ILC2- or ILC3-characteristic or -associated gene loci were annotated.

5.3.6 Ahr exploits an existing enhancer landscape to modulate target gene expression

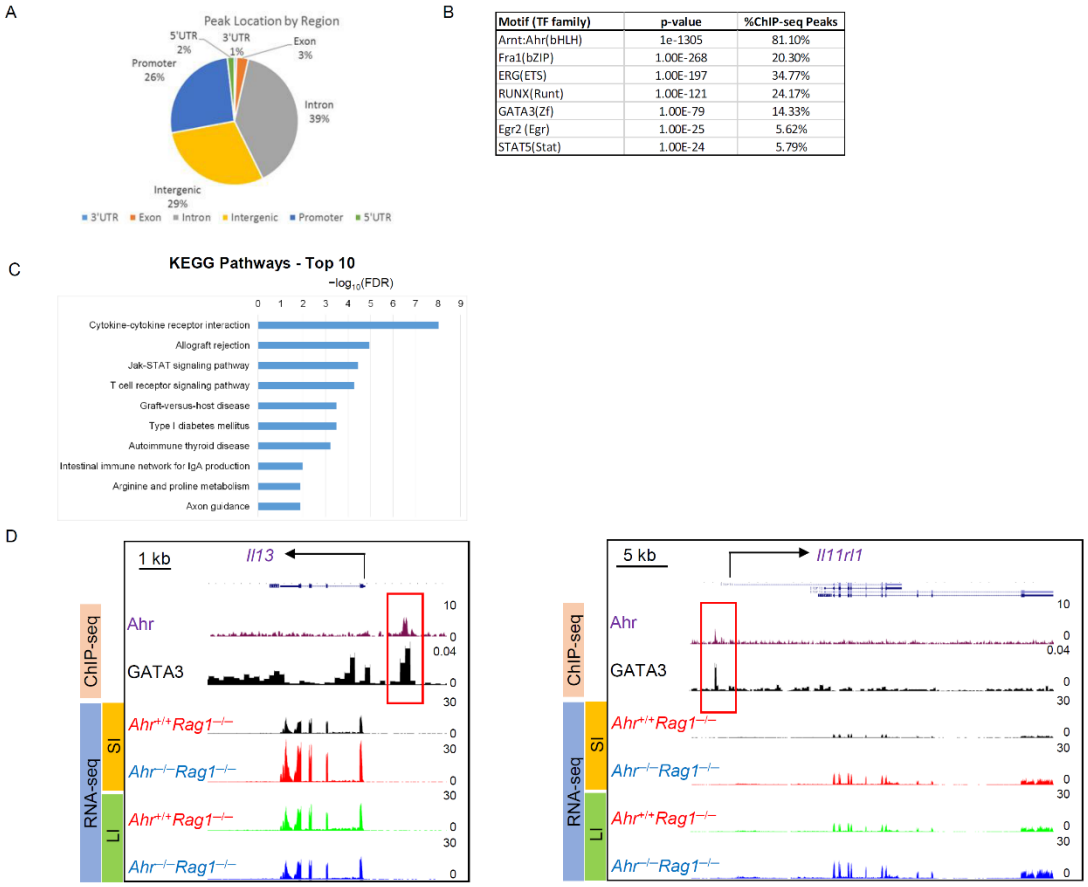
To determine the role of Ahr binding in Group 2 innate lymphoid cells (ILC2s), we performed ChIP-seq experiments with Ahr wildtype and knockout ILC2s from the large intestine. We found that Ahr bound 3434 locations in ILC2s. The majority (71%) of bound sites were found in proximity to gene loci [promoters (26%), introns (39%), exons (3%), 5'UTR (2%) and 3'UTR (1%)], and about a third (29%) of Ahr binding sites were found at putative enhancers in intergenic regions (Figure 5.6A). This distribution of binding sites is consistent with the role of Ahr as a transcriptional regulator.

Motif enrichment analysis indicated the canonical Ahr motif (5'-T(G/C)GCACGCAA-3') as the most prevalent motif found at Ahr binding sites (81% of binding sites), and the motif predominantly located around ChIP-seq peak centers (Figure 5.6B). Additional transcription factor motifs were found at Ahr ChIP-seq peaks, including motifs for the Runx (e.g., Runx2 and Runx3), Ets (e.g., Ets1, Ets2 and Spi1) and AP-1 (e.g., Fos, Fosl2, Jun, and JunB) families of transcription factors (Figure 5.6B). These transcription factor families are widely expressed in lymphocytes and prevalent at enhancers, which is consistent with reports of the importance of Runx and Ets1 in lymphocyte development and AP-1 family members in lymphocyte function. In addition, the motif for the ILC2 lineage-defining transcription factor, GATA3, was also identified at Ahr binding sites (Figure 5.6B). These data indicate that Ahr binding in ILC2s is associated with transcription factors that are important for lymphocyte (e.g., Runx and Ets) and ILC2 (e.g., GATA3) development and function.

Some transcription factors act as pioneer factors, increasing the accessibility of DNA so that other transcription factors can bind. Other transcription factors exploit existing enhancer landscapes and bind where chromatin is accessible. To identify Ahr's mode of operation, we previously performed ATAC-seq experiments to identify accessible chromatin regions in ILC2s, and evaluated the effect of Ahr deficiency on chromatin accessibility (Figure 5.5). Loss of Ahr only caused significant changes (FC $\geq$ 2, FDR$\leq$ 0.05) in chromatin accessibility in about 1.5% of the peaks identified in ILC2s, indicating that rather than participating in *de novo* chromatin remodeling, Ahr exploits the existing enhancer landscape to modulate gene expression (Figure 5.5F).

To elucidate the relationship between Ahr binding and gene expression, Ahr ChIP-seq peak locations were annotated with gene names, and gene pathway analysis was performed with Gene Set Enrichment Analysis. Combining our previous RNA-seq experiments with Ahr ChIP-seq data, we found that the loss of Ahr in ILC2s resulted in transcription changes in 483 genes in ILC2s from both the small and large intestine. Of those 483 genes, Ahr bound 151 genes (31%), identified by proximity to the closest transcription start site. Pathway analysis of the differentially-expressed genes associated with Ahr binding identified cytokine and chemokine signaling, Jak-STAT signaling, and T cell receptor signaling, in the top pathways, indicating that Ahr may modulate these critical signaling pathways in ILC2s (Figure 5.6C).

Previous studies of Ahr indicated that it might have a context-dependent functional role across cell and tissue types. The molecular mechanism behind this context-dependent role is unkown, but the association between GATA3 binding sites and Ahr binding sites in ILC2s might be important. Therefore, we examined the overlap between GATA3 binding sites and Ahr binding sites at genes that were differentially expressed. We found that 83 genes had co-binding of GATA3 and Ahr, and among these genes were important ILC2 functional molecules, including *Il13, Il1rl1 and Ahr* (Figure 5.2 and Figure 5.6D). This suggests that Ahr may work in cooperation with cell or tissue specific transcription factors to mediate gene regulation.



Motif (TF family)	p-value	%ChIP-seq Peaks
Arnt:Ahr(bHLH)	1e-1305	81.10%
Fra1(bZIP)	1.00E-268	20.30%
ERG(ETS)	1.00E-197	34.77%
RUNX(Runt)	1.00E-121	24.17%
GATA3(Zf)	1.00E-79	14.33%
Egr2 (Egr)	1.00E-25	5.62%
STAT5(Stat)	1.00E-24	5.79%

Figure 5.6 Ahr exploits an existing enhancer landscape to modulate target gene expression.

(A) Distribution of Ahr binding site by annotated genome regions [UTR: untranslated region]. (B) Motif frequencies identified within Ahr peaks. (C) Pathway analysis of differentially expressed genes bound by Ahr. (D) Co-binding of Ahr and GATA3 as indicated by ChIP-seq in ILC2s and changes in gene expression (RNA-seq) at the *Il13* locus (left) and *Il1rl1* locus (right).

5.4 Conclusions

The molecular mechanisms underlying the balance of ILC subsets in tissues are poorly understood. Here, we have shown that the expression of Ahr in gut ILCs represents an adaptation to external environment and is essential for intestinal immunity through regulation of the ILC2-ILC3 balance.

Previous studies have shown that enhancer landscapes characterizing T cell lineages are pre-established and strongly influenced by environmental stimuli (Samstein et al. 2012; H.-Y. Shih et al. 2016; Xu and Smale 2012). We hypothesized that unique chromatin events (enhancers) are specified in gut ILC2s by environmental cues and are responsible for promoting tissue-specific Ahr expression in gut ILC2s. We speculated that one candidate of such stimuli would be the microbiota, which are abundantly present in the gut. The fact that, compared with gut ILC2s from SPF mice, gut ILC2s from GF mice had minimal differences in chromatin remodeling at the *Ahr* locus and unaltered expression of *Ahr* supports a model in which the microbiota might play a dispensable role for Ahr transcription in ILC2s, consistent with the microbiota-independent mechanism of Ahr expression in gut Treg cells (Ye et al. 2017). Our data also suggest that deprivation of Ahr dietary ligand has a minimal or modest impact on Ahr expression or activity in ILC2s. Together, these data suggest that a complex interaction among microbiota, diet, and probably other unidentified environmental factors in the gut could specify Ahr expression in intestinal immune cells.

Among the gut-ILC2-specific chromatin events at the Ahr locus, the remodeling of peak 6 was dependent on Ahr, indicating that Ahr facilitates its own gene chromatin accessibility. Despite high expression of Ahr in gut Treg cells (Ye et al. 2017), peak 6 was absent at the Ahr locus, and accordingly the transcription of Ahr was unaffected in Ahr-deficient Treg cells. These data support a cell-type-specific positive-feedback role of Ahr in its own transcription in ILC2s. Our attempt to determine the role of peak 6 as an enhancer in the standard reporter assay by using transient transfection did not yield conclusive data, consistent with the fact that its function in chromatin remodeling is presumably not involved in transient-transfection assays (Zhou et al. 2007). Thus, the function of peak 6 needs to be studied at an organismal level by the generation of mice with a mutation in this element and subsequent evaluation of immune responses (e.g., ILC2 compartment) under steady-state physiological conditions and in disease.

Gfi1 is a positive regulator of ILC2s (e.g., it promotes ST2 expression) (Spooner et al. 2013). However, the observation that Gfi1 binds to peak 6 and promotes the expression of Ahr, a negative regulator of ILC2s, suggests a self-limiting mechanism to prevent exuberant ILC2 responses. Co-occupancy of Ahr and Gfi1 at the *Il1rl1* promoter and increased Gfi1 expression and recruitment to the *Il1rl1* promoter in Ahr-deficient ILC2s favor a model where Ahr keeps the ILC2 effector functions in check by suppressing the Gfi1-ST2 pathway, e.g., through the counteraction of Gfi1 at the *Il1rl1* promoter.

Ablation of the IL-33-ST2 pathway has been shown to relieve experimental colitis, including the 2,4,6-trinitrobenzenesulfonicacid- and dextran-sulfate-sodium-induced colitis models in mice (Sedhom et al. 2013; Zhu et al. 2015). Consistently, increased amounts of IL-33 and soluble ST2 have been shown in the colons of patients with inflammatory bowel disease (Pastorelli et al. 2010), in line with the proinflammatory nature of type 2 immunity (e.g., IL-13) (Neurath 2014). However, a recent report has shown that the ILC2-associated effector molecule Areg participates in tissue repair in the gut (Monticelli et al. 2015). ILC2s can also express IL-9 to promote epithelial cell maintenance in the lung (Mohapatra et al. 2016). These data support a model where ILC2s at different stages of disease and/or some subset of ILC2s (i.e., $Areg^+$ ILC2s or IL-9^+ ILC2s) might have a protective function in tissue damage. $Areg^+$IL-5^- or $Areg^+$IL-13^- cells were unaffected in Ahr-deficient mice. In addition, IL-9 expression was unchanged in Ahr-deficient ILC2s (data not shown). These data indicate that Ahr might play a differential role in regulating ILC2 subsets that express distinct cytokines.

Plasticity exists in certain ILCs in the sense that they can convert into other subsets under different conditions (Colonna 2018). Lack of Bcl11b in ILC2s leads to loss of ILC2 functions but a gain of ILC3 features (Califano et al. 2015). Given the role of Ahr in regulating ILC3 maintenance, it remains to be determined whether Ahr deficiency could convey the plasticity of ILC3s; however, this hypothesis needs to be carefully tested, e.g., through a fate-mapping genetic approach.

Genetic ablation of Ahr enhanced gut ILC2 function, consistent with enhanced immunity to adult *H. polygyrus bakeri* infection. Whether Ahr plays a similar role in other helminth infections remains to be determined. Ahr deficiency has been shown to cause compromised immunity against *C. rodentium* as a result of a loss of IL-22$^+$ ILC3s (Kiss et al. 2011; J. S. Lee et al. 2011; J. Qiu et al. 2012). Strikingly, Ahr expression in RORgt$^+$ cells alone is sufficient to maintain a functional ILC3 compartment and control *C. rodentium* infection. Because Ahr plays a differential role in various immune cells, pharmacological modulation of Ahr expression and/or activity could affect host immunity in a complex manner. Inhibition of Ahr promotes ILC2s that are beneficial for the host to mount an efficient immunity against worm infection but could also contribute to type-2-associated gut inflammation, such as ulcerative colitis and food allergy (Neurath 2014; Stefka et al. 2014; Tordesillas, Berin, and Sampson 2017). On the other hand, Ahr activation could promote anti-bacterial immunity through enhancement of ILC3s and control autoimmunity by promoting Treg cell functions (Gutiérrez-Vázquez and Quintana 2018; Zhou 2016).

Although Ahr is expressed highly in mouse gut ILC2s, it remains to be determined whether this expression pattern is also evident in humans. Specifically, careful comparison of Ahr expression in ILCs among different human tissues is needed. Published high-throughput single-cell sequencing data show the existence of Ahr in human tonsil ILC2s; however, the level of its expression is lower than that in tonsil ILC3s (Björklund et al. 2016). In addition, Ahr mRNA is detectable in ILC2s from human peripheral-blood mononuclear cells, and TNF-like ligand 1A (TL1A) together with IL-25 and IL-33 can upregulate Ahr transcription in these cells (A. I. Lim et al. 2016). Thus, future efforts are needed to elucidate the molecular mechanisms by which Ahr is

expressed in different tissue milieu under the steady state and/or during inflammation. Understanding how Ahr regulates the gut ILC balance could provide new therapeutic opportunities in disease treatment and/or prevention.

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

In the work described in the previous chapters, we show that both host and microbial mechanisms mediate the regulation of lymphoid cells in the intestine. The host transcription factor, aryl hydrocarbon receptor (Ahr), regulates the expression of genes that promote the homing and function of regulatory T cells (Tregs) in the intestine and the balance of innate lymphoid cell subsets. Bacteria, such as non-gastric *Helicobacter* strains, can negatively regulate the proliferation of Group 3 innate lymphoid cells (ILC3s) in the large intestine. Together these findings provide insight into the mechanisms involved in the regulation of intestinal immunity, and offer potential targets for the development of therapeutics.

6.1 Immune regulation prevents host damage

The molecular effectors that recruit lymphoid cells to the intestine and maintain them are central to a well-regulated immune response. This regulation is necessary so that the immune system can support defense against pathogens and tissue homeostasis, but also limit its response to prevent harm to the host. Here we identify chemokines and cytokines as critical molecules in this regulation. Lymphoid cells depend on the expression of chemokines and chemokine receptors for their proper migration and localization within lymphoid and non-lymphoid tissues. Cytokines provide signals that modulate immune cell proliferation, apoptosis, cytotoxic function and the transcription of other effector molecules. Regulation of chemokines and cytokines occurs at many levels: transcription of secreted molecules and receptors, post-transcriptional regulation of

mRNAs, regulation of signaling downstream of receptors and surface expression of receptor proteins.

6.2 Chemokine signaling regulates immunity through proper immune cell localization

Tregs are a diverse population that populate both lymphoid and non-lymphoid tissues. The non-lymphoid Treg populations have tissue-specific gene expression and functionality. Treg populations in the intestine express chemokine receptors Ccr6 and Ccr9, in addition to Gpr15, CD103 (*Itgae*), and integrin α4β7, to help with their localization and/or retention in the tissue. Ccr9 and CD103 expression are induced by retinoic acid, and their expression on Tregs promotes their localization to the small intestine (Coombes et al. 2007; C.-M. Sun et al. 2007). Tregs in the small intestine participate in the maintenance of tolerance to ingested antigens. This is demonstrated by genetic deletion of Ccr9 or integrin α4β7, which results in decreased Treg numbers in the small intestine and disruption of tolerance to orally administered antigens (Cassani B., et al. 2011)(Cassani et al. 2011). Gpr15 has been shown to promote the homing of conventional T cells and Tregs to the large intestine (S. V. Kim et al. 2013). CD103 and Ccr6 promote interactions between Tregs with intestinal epithelial and other immune cells, respectively, which promote the retention of Tregs in the intestine (Chaudhry et al. 2009; Coombes et al. 2007; C.-M. Sun et al. 2007). The importance of Ccr6 expression on Tregs was demonstrated by Stat3-deficient Tregs, which have significantly reduced expression of Ccr6. These Stat3-deficient Tregs had similar suppressive capabilities to wild type Tregs, but failed to home to the large intestine and could not control T cell-mediated inflammation in the intestine (Chaudhry et al. 2009).

Dysregulation of chemokine signaling results in immune deficiencies that prevent the control of inflammation, as similarly observed in the case of Ahr-deficient Tregs. Ahr-deficient Tregs express lower levels of the chemokine receptor, Ccr6, the integrin, CD103, and the gut-homing receptor Gpr15, which collectively results in their reduced retention and homing to the large intestine and higher Treg frequencies in the liver and circulation (Ye et al. 2017). We found that decreased intestinal Treg frequencies made the host more susceptible to exuberant immune responses in the intestine.

6.3 Regulation of the balance of immune cell subsets promote proper immune response

The cellular and molecular mediators of the homeostatic balance of ILCs is an area of active research. In the intestine, all innate lymphoid cell subsets are present in both mice and humans. ILC2s are a subset of innate lymphoid cells that mediate protection against helminth infections, but also are critical effector cells in the repair and homeostasis of the intestine. We show that ILC2s in the intestine express high levels of Ahr, and we identified that Ahr acts as a cell-intrinsic negative regulator of ILC2 function (S. Li et al. 2018). In contrast, Ahr promotes ILC3 maintenance and function in the intestine (J. Qiu et al. 2012). We found that Ahr binds to the promoter of genes that stimulate ILC2 function, such as *Il1rl1* (ST2), a component of the IL-33 receptor, in order to negatively regulate its expression. Ablation of Ahr in ILC2s leads to enhanced immunity against helminth infection in mice, but its ablation in ILC3 leaves mice susceptible to *Citrobacter rodentium* infection. Therefore, in these ILC subsets, Ahr plays a key role regulating their function and maintaining balanced immunity.

In addition to host factors, bacteria can also play a role in regulating the balance of lymphocytes that populate the intestine. Ours and other studies have found that germ-free (GF) mice or mice treated with antibiotics have a marked reduction of RORγt$^+$ Tregs in the large intestine, leading to the conclusion that the development of this population is dependent on bacteria (Ye et al. 2017; M. H. Kim, Taparowsky, and Kim 2015). Studies have shown that RORγt$^+$ Tregs suppress the immune response directed at bacteria, thus acting as an important component of regulation. Different bacterial species have varying ability to promote RORγt$^+$ Tregs (Sefik et al. 2015; Ohnmacht et al. 2015). However, the mechanisms behind the variation in the ability to induce Tregs are still in the process of being elucidated. Bacterial products, such as the short chain fatty acids, butyrate and acetate, can promote the induction of Tregs by disrupting the deacetylation of histones around critical Treg genes, such as *Foxp3* (Arpaia et al. 2013; Furusawa et al. 2013). Additionally, the polysaccharide A (PSA) from *Bacteroides fragilis* can induce Tregs, suggesting that bacterial induction of Tregs may involve multiple and diverse mechanisms (Mazmanian et al. 2005).

6.4 New immunotherapies target the key regulatory hubs of immunity

Together the findings in the studies outlined in the previous chapters provide some insight into how the mechanism of immune regulation may be used for therapeutic benefit. Indeed, cytokines, chemokines and integrins are already targets of therapeutic biologics, such as engineered monoclonal antibodies. Engineered antibodies have specificity for their targets, and are used to treat a number of diseases including cancer, autoimmune diseases and certain infections. A newly developed monoclonal antibody therapy, Vedolizumab, targets the gut-homing integrin, α4β7, and

is currently being used to treat patients with Crohn's disease or ulcerative colitis that do not respond to anti-TNF therapy (Sandborn et al. 2013).

Our findings suggest that promotion of Gpr15 signaling could be beneficial for diseases with uncontrolled T cell responses in the intestine, such as ulcerative colitis. However, the ligand for Gpr15 is still unknown. We demonstrated that Ahr can promote the expression of Gpr15 on Tregs (Ye et al. 2017). Ahr is a ligand-activated transcription factor; therefore, its activity can be manipulated by the increasing or restricting ligand availability. However, pharmalogical manipulation of Ahr activity could have mixed effects on host immunity due to the diverse roles Ahr plays in the hematopoietic and non-hematopoietic compartments. Advances in targeted drug delivery might overcome this potential problem.

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