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Regulation of Intestinal Immune Homeostasis by
Innate and Adaptive Immune Cells

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Regulation of Intestinal Immune Homeostasis by Innate and Adaptive Immune Cells

By

Duke Geem
B.A., Boston University, 2008

Advisor: Timothy L. Denning, Ph.D.

An abstract of
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Abstract

Regulation of Intestinal Immune Homeostasis by
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By Duke Geem

The intestinal immune system interacts with a multitude of foreign antigens in the environment and must elicit appropriate pro-inflammatory or tolerogenic immune responses. Crucial to mediating host defense and immune tolerance are T-helper (Th) 17 and Foxp3+ regulatory T (T_{reg}) cells, respectively, which are specialized CD4+ T cell subsets enriched in the lamina propria (LP). Currently, the cellular and molecular networks that regulate their development remain to be fully defined. To begin investigating the development of intestinal Th17 and Foxp3+ T_{reg} cell responses, the tissue sites important for the differentiation of these cells must first be elucidated. As previous studies have implicated the mesenteric lymph nodes (mLN) and GALT to be the site of intestinal CD4+ T cell responses, the requirement for these structures in supporting intestinal Th17 and Foxp3+ T_{reg} cells was examined. In lymphotoxin-deficient mice that are void of secondary lymphoid organs—including mLN and GALT, normal frequencies and absolute numbers of intestinal Th17 and Foxp3+ T_{reg} cells was observed. These results suggested that Th17 and Foxp3+ T_{reg} cell differentiation may occur within the intestinal LP under the regulation of local antigen presenting cells and the microbiota. Mechanistic studies of intestinal Th17 cell development following colonization by segmented filamentous bacteria (SFB) revealed requirements for MHC class II on CD11c+ cells and antigenic stimulation. This SFB-driven Th17 cell differentiation was dependent on IL-1 signaling and inhibited by eosinophils, which constitutively produced IL-1 receptor antagonist. Moreover, analysis of the Foxp3+ T_{reg} cells in the intestine revealed site-specific differences between the small intestine (SI) and large intestine (LI) in terms of composition and reactivity. Foxp3+Helios+ thymically-derived (t)T_{reg} cells were the predominant population in the intestine with greater abundance in the SI than the LI. Correspondingly, conventionalization of germ-free mice robustly promoted Foxp3+ T_{reg} cell development in the LI, but not in the SI, highlighting distinct reactivity to the gut microbiota between these two sites. Collectively, our findings define important tissue sites required for the intestinal Th17 and Foxp3+ T_{reg} cell development and underscore the contribution of the local milieu in regulating their differentiation and function.

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Chapter 1: Introduction

The intestine is unique organ that facilitates the exchange of waste and nutrients for the host and all the while serving as a home to more than a 100 trillion bacteria that comprise the gut microbiota (1). Amid the plethora of enteric antigens encountered, the intestinal immune system serves to protect the host from colonization and invasion by pathogens while also promoting tolerance to foreign antigens deriving from food, commensal microbes, and other innocuous sources (2, 3). Such immune surveillance and discrimination of enteric antigens are performed in part by antigen-presenting cells (APCs), which dictate the differentiation and migratory pathways of responding lymphocytes (4, 5). CD4+ T cells are a major T lymphocyte population in the lamina propria and play a crucial role in intestinal immune homeostasis. Two subsets of CD4+ T cells that are markedly enriched in the lamina propria are the T-helper (Th) 17 and Foxp3+ regulatory (T_{reg}) cells that contribute to host defense and immune tolerance, respectively (6-8). The dysfunction of Th17 and Foxp3+ T_{reg} cells are associated with autoimmune and inflammatory diseases such as inflammatory bowel disease (IBD) (9-11), and consequently, elucidating the cellular and cytokine networks that regulate the development of these cells in the intestine may be of clinical relevance for the development of novel therapies.

Previous research has focused on how secondary lymphoid organ (SLO) APCs induce T cell differentiation, which has led to the widely accepted model that steady-state intestinal CD4+ T cell differentiation occurs in the mesenteric lymph nodes (mLN)(12). However, little is known regarding T cell differentiation

mediated by intestinal APCs in situ. Over the past several years, in vitro evidence has emerged demonstrating that intestinal APCs are endowed with the capacity to induce T_{reg} or Th17 cell responses, depending upon the type of APC and the context of stimulation (13-15). Furthermore, research from our lab and others have determined that intestinal macrophages and dendritic cells (DCs) are distinct in their localization and ability to induce T_{reg} and Th17 cell differentiation (13, 14). The overall goal of this dissertation is to define the location of intestinal Th17 and Foxp3+ T_{reg} cell differentiation by: 1) evaluating if naïve CD4+ T cells migrate to the intestinal lamina propria, 2) assessing the requirements for the mLN, GALT, and other secondary lymphoid organs, and 3) elucidating the contribution of the microbiota and APCs. In addition to gaining insights into the complex nature of intestinal CD4+ T cell differentiation induced by intestinal APCs, it is hoped that these studies will provide new ideas for the development of agents that alter APC and/or T cell function for use as immunomodulatory agents in the treatment of IBD.

Th17 cells

The characterization of Th17 cells in 2005 expanded our understanding of inflammation, autoimmune diseases, and host defense (6, 16, 17). Following the discovery of the p19 cytokine chain and the identification of IL-23 (18), CD4+ T cells that secrete IL-17A were characterized to be pathogenic mediators of experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA)—experimental models of multiple sclerosis (MS) and rheumatoid

arthritis (RA), respectively (16, 19). This discovery of Th17 cells modified the understanding at the time that IFNγ-producing Th1 cells were the primary mediators of autoimmunity (20). Subsequently, a role for Th17 cells has emerged in orchestrating innate and adaptive immune responses for host defense against extracellular pathogens and the in the pathogenesis of various autoimmune diseases and chronic inflammatory conditions.

A functional profile of Th17 cells

Th17 cells are a subset of CD4+ T cells that are distinguished by their production of IL-17 cytokine family members, particularly IL-17A and IL-17F. These pro-inflammatory cytokines activate immune and non-immune cells to secrete factors, such as granulocyte colony-stimulating factor (G-CSF) and IL-8 that promote neutrophil development and recruitment (21). Additionally, Th17 cells can directly mobilize bone marrow granulocytes and monocytes via granulocyte macrophage colony-stimulating factor (GM-CSF) and enhance the antimicrobial responses and barrier function of epithelial cells through the production of IL-22. The inherent plasticity of Th17 cells further expands their functional profile as Th17 cells can become pathogenic Th1-like cells that secrete IFN-γ (22-24), transdifferentiate into T_{reg} cells that express IL-10 (25), or acquire a follicular helper T cell phenotype in Peyer's patches to regulate high-affinity IgA responses in the intestine (26). Thus, the robust pro-inflammatory nature and the functional plasticity of Th17 cells underscore the importance of these in mediating host defense and their pathogenic potential.

Th17 cells are enriched in mucosal tissue surfaces of the lungs and intestine where they play an important role in protecting the host against colonization and invasion by pathogens. The Th17 cell response and IL-17A signaling mediates the clearance of extracellular bacteria such as *Klebsiella pneumoniae* (27), *Streptococcus pneumoniae* (28), and *Citrobacter rodentium* (29). Moreover, fungal pathogens like *Candida albicans* are capable of inducing polarized Th17 cell responses (30, 31), and accordingly, individuals with hyperimmunoglobulin E syndrome (or Job's syndrome) that are unable to generate effective Th17 cell responses are highly susceptible to recurrent *C. albicans* and *Staphylococcus aureus* infections in the skin and lung (32, 33). Together, these studies support a role for Th17 cells as sentinels in barrier surfaces where they promote neutrophillic and antimicrobial responses for the clearance of extracellular pathogens.

Since the initial characterization of Th17 cells in contributing to the pathogenesis of EAE (16, 19), dysregulated Th17 cell responses have been implicated in various human autoimmune diseases and chronic inflammatory conditions. In accordance with the pathogenic role of Th17 cells in EAE, microarray analysis of MS lesions have yielded increased transcripts of Th17 cell-associated genes such as *Il6*, *Il17*, and *Il1r1* (34). Furthermore, the levels of a micro-RNA that promotes Th17 cell differentiation, miRNA326, in peripheral blood mononuclear cells of MS patients are correlated with disease severity (35). In rheumatoid arthritis, infiltrating T cells of the synovial fluid robustly express IL-17A and other Th17 cell-associated cytokines (36-38), and correspondingly, IL-

17A levels in the synovium are correlated with joint destruction (39). Furthermore, genome-wide association studies (GWAS) of IBD patients have identified polymorphisms in several genes involved in Th17 cell development such as *Il23r* and *stat3* (40). Together, these studies highlight the potential of targeting Th17 cells and the IL-17 axis for the treatment of autoimmune diseases.

Molecular development of Th17 cells

Seminal animal and in vitro studies have elucidated key regulatory cytokines important for the differentiation of Th17 cells. The activation of naïve CD4+ T cells in the presence of IL-6 and TGF-β1 promotes differentiation along the Th17 cell pathway (41-43). IL-6 serves to activate STAT3, which subsequently regulates the transcription of *Rorc*, *Il17*, and *Il23r* (44) while TGF-β1 at optimally low levels function to inhibit T-bet and GATA-3 expression (45). The subsequent exposure of RORγt+ Th17 cells expressing IL-23 receptor (IL-23R) to IL-23 further increases IL-23R expression and promotes the maturation and pathogenicity of Th17 cells via the induction IL-22, GM-CSF, and IFN-γ (46-48). Additionally, T-bet and TGF-β3 are associated with the pathogenic signature of Th17 cells (49-51). In the absence of TGF-β1, IL-1β together with IL-6 and IL-23 are sufficient to produce epigenetic modification of Th17 cell signature genes and yield encephalitic T-bet+ Th17 cells (46). The role of several other cytokines, such as IL-21 (52-54), and the transcription factors aryl hydrocarbon receptor (AhR)(55), BATF, and IRF4 (56, 57) among others highlight the complexity of the transcriptional network regulating Th17 cell differentiation.

Apart from the conventional development of Th17 cells from naïve CD4+ T cells, auto-reactive Th17 cells that develop in the thymus, referred to as natural Th17 (nTh17) cells have recently been described (58). For the development nTh17 cells, TGF-β is required while the role of IL-6 is uncertain based on two contrasting reports (58, 59). Furthermore, these cells express CD44, inducible costimulator (ICOS), and the Th17 cell-associated genes *Rorc*, *Il23*, and *Il22*. Besides RORγt, nTh17 cells may also be identified based on their high expression of the transcription factor promyelocytic leukemia zinc finger (PLZF) and can produce high levels of IL-17A and IL-22 in response to IL-23 and IL-1β without TCR stimulation, similar to other innate lymphocyte populations (59). Nonetheless, there is a paucity of information on nTh17 cells and additional studies elucidating the developmental requirements and in vivo function of nTh17 cells are warranted.

Development of Th17 cells in vivo

Given their importance in host defense, Th17 cells are enriched in mucosal tissue, particularly in the intestinal LP, and their development is driven by the microbiota. This requirement for the microbiota has been demonstrated based on the paucity of Th17 cells in mice treated with antibiotics from birth or housed in GF conditions (60). Recently, the Gram-positive commensal bacteria referred to as segmented filamentous bacteria (SFB) have been shown to potently induce Th17 cells in the intestine (29, 61). In contrast to other commensal bacteria, SFB are capable of breaching the mucus layer and tightly

adhering to the intestinal epithelium (62). Interestingly, colonization by SFB is vendor-specific as B6 mice purchased from Taconic (Tac) and Charles River Laboratories are colonized by SFB while mice from The Jackson Laboratory (JAX) are devoid of SFB. Indeed, the horizontal transfer of SFB from Tac B6 to JAX B6 mice has been shown to potently induce intestinal Th17 cells in JAX B6 and provides an experimental system to investigate how microbial signals may be transduced to modulate the host immune system (29, 61).

Currently, the cells and cytokines governing the development of intestinal Th17 cells in response to SFB are not well defined. Although CD11b+ LP DCs (13, 14, 63) and serum amyloid A (SAA) (29) are thought to contribute to intestinal Th17 cell induction, the fundamental questions of where and how does SFB promote intestinal Th17 cell differentiation have yet to be addressed. To begin exploring the local cytokines, APCs, and other immune cells that regulate SFB-driven intestinal Th17 cell development, the pertinent tissue sites must first be elucidated. Accordingly, SFB may serve as an important model in understanding where and how different components of the gut microbiota and their derivatives may regulate the development of adaptive immune responses in the host.

Following sections are adapted from:

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Foxp3+ T_{reg} cells

Regulatory T (T_{reg}) cells are an important component of the adaptive immune system that suppresses inflammation and helps to maintain homeostasis. The T_{reg} compartment comprises a heterogeneous population in terms of development, phenotype, and suppressive functions. Recently, a system of nomenclature has been proposed to describe these various T_{reg} cells and will be implemented into this review (64). Foxp3+ T_{reg} cells are one T_{reg} subset that constitutively expresses Foxp3 and the high affinity α-chain of IL-2 receptor, CD25 (7, 8). Foxp3+ T_{reg} cells can arise from two developmentally distinct pathways in vivo: Foxp3+ thymically-derived (t)T_{reg} cells or Foxp3+ peripherally-derived (p)T_{reg} cells, which are naïve CD4+ T cells that upregulate Foxp3 in extra-thymic tissues and become functionally suppressive (65). Foxp3+ T_{reg} cells may also be induced in vitro from naïve CD4+ T cells in specific culture conditions containing TGF-β1 and are referred to as in vitro-induced Foxp3+ (i)T_{reg} cells.

The development of Foxp3+ T_{reg} cells

Foxp3+ tT_{reg} cells arise as a distinct lineage of CD4 single positive (SP) thymocytes following high-affinity TCR-MHC interactions and co-stimulatory signals (66, 67). Thymic selection of tT_{reg} cells is distinct from conventional T cells in that high affinity interactions with self-antigens and MHC does not invoke apoptosis through negative selection but instead promotes survival, a process termed “agonist selection” (67). Accordingly, the TCR reactivity of Foxp3+ tT_{reg} cells was originally thought to be directed toward self-antigens. However, Pacholczyk et al. recently reported that high-affinity, auto-reactive TCRs were not required for tT_{reg} development (68), and in fact, the TCR repertoire of tT_{reg} cells can react to bacterial antigens from the colon (69). CD4 SP thymocytes selected along the tT_{reg} cell lineage become CD25+Foxp3- precursor cells that subsequently upregulate Foxp3 in response to cytokines activating the STAT5 signaling pathway (70, 71). The intracellular signaling events during tT_{reg} cell development yields distinct epigenetic patterns that distinguish them from pT_{reg} or iT_{reg} cells. For example, tT_{reg} cells exhibit CpG hypomethylation of T_{reg}-associated genes and a unique methylation pattern of the T_{reg}-specific demethylation region (TSDR) compared to iT_{reg} and conventional T cells (72, 73). Foxp3+ tT_{reg} cells may also be distinguished from extra-thymic T_{reg} cells based on their higher expression of Helios, an Ikaros transcription factor family member, and the cell surface molecule, Neuropilin-1 (74, 75). Following thymic selection and egress, Foxp3+ tT_{reg} cells seed peripheral tissues including the intestine beginning at postnatal day 3 in mice (76) while in humans, Foxp3+ cells have

been observed in the LP of the small and large intestines as early as 23 weeks of gestation (77).

In contrast to tT_{reg} cells, Foxp3+ T_{reg} cells can develop extra-thymically when naïve CD4+ T cells are activated in the appropriate milieu, such as that in the intestinal LP and GALT, resulting in the upregulation of Foxp3 and the gain of immunosuppressive functions. One cytokine important for extra-thymic T_{reg} cell differentiation is TGF-β1, which is secreted by epithelial cells and T cells in the intestine (78-80). Also produced in the intestine is retinoic acid (RA), a vitamin A metabolite, that potentiates extra-thymic T_{reg} differentiation and imprints CD4+ T cells for intestinal homing via the upregulation of CCR9 and α4β7 (81, 82). Intestinal DCs and macrophages express retinaldehyde dehydrogenases, the enzymes important for the biosynthesis of RA, and are adept at promoting Foxp3+ T_{reg} cell differentiation (13-15, 83). Previous animal studies examining intestinal antigen presentation and oral tolerance provide evidence that intestinal Foxp3+ pT_{reg} cells can differentiate in the mesenteric lymph nodes (mLN) upon antigenic stimulation by migratory CD103+ LP DCs (84). Thereafter, a subset of activated CD4+ T cells become gut-tropic Foxp3+ T_{reg} cells that home to the intestinal LP and expand in response to IL-10 produced by CX3CR1+ macrophages (85).

Currently, numerous questions remain regarding the development of intestinal Foxp3+ T_{reg} cells. The tissue sites where intestinal Foxp3+ T_{reg} cells develop, and accordingly the contribution of Foxp3+ tT_{reg} and pT_{reg} cells to the intestinal Foxp3+ T_{reg} cell pool, remains to be investigated. Given the differences

in antigen reactivity and functional niches occupied by Foxp3+ tT_{reg} and pT_{reg} cells, insight into the tissue sites required would aid in directing research to understand the relevant cellular and cytokine networks mediating intestinal Foxp3+ T_{reg} cell development and function.

Mechanisms of immune suppression by Foxp3+ T_{reg} cells

Collectively, T_{reg} cells possess different immunosuppressive functions and play an important role in regulating the intestinal immune system. Through the secretion of anti-inflammatory cytokines and the engagement of immunoregulatory cell surface molecules, T_{reg} cells inhibit pro-inflammatory cytokine production, downregulate costimulatory molecules on antigen presenting cells (APCs), and modulate T cell proliferation and differentiation (86-89). Specific cytokines, including TGF-β1, IL-10, and IL-35, serve not only to dampen the immune response but are also involved in pT_{reg} and iT_{reg} cell differentiation (79, 90-92). In vivo imaging of T_{reg} cells has highlighted the propensity of these cells to engage in stable, long-lasting interactions with DCs in lymph nodes, which results in the inhibition of CD4+CD25- T cell priming (93). Thus, DCs, macrophages and other APCs in the intestine and mLN may be important immunomodulatory targets for T_{reg} cells (94, 95). T_{reg} cells may also express cell surface CD39 and CD73, which can catalyze ATP, ADP, and AMP to produce the immunosuppressive metabolite, adenosine (96-98). The function of CD39 and CD73 is particularly relevant in the intestine where ATP produced by host cells and the microbiota can support the development of colitogenic Th17

cells (60). Accordingly, catabolism of ATP by T_{reg} cells may function to limit pro-inflammatory Th17 responses in the intestine. Additional mechanisms of T_{reg} cell-mediated suppression include cytolysis of effector T cells mediated by the granzyme-perforin pathway (99, 100), and deprivation of proliferating T cells from pro-survival cytokines, like IL-2, that is associated with the induction of colitogenic T cell apoptosis (101); Fig. 1.1). Taken together, T_{reg} cells are capable of employing a variety of immunosuppressive mechanisms to suppress inflammation and promote tolerance in the intestine.

Regulation of intestinal Foxp3+ T_{reg} cells by the gut microbiota

Recent studies have highlighted the importance of the gut microbiota in regulating intestinal Foxp3+ T_{reg} cell homeostasis. In mice, polysaccharide A (PSA) derived from the commensal bacteria *Bacteroides fragilis (B. fragilis)* induces IL-10-producing Foxp3+ T_{reg} cells capable of suppressing pro-inflammatory Th17 cell responses and preventing experimental colitis (102). This immunoregulatory function role of PSA was mediated through plasmacytoid DCs and TLR2 expressed on Foxp3+ T_{reg} cells (103). Besides *B. fragilis*, commensal bacteria deriving from different *Clostridia* strains were discovered to support Foxp3+ T_{reg} cell development in the mouse colon (104). In fact, specific mixture of 17 *Clostridia* strains derived from the human gut microbiota promoted Foxp3+ T_{reg} cell development through the production of short-chain fatty acids (SCFAs) that enhanced TGF-β1 secretion from the intestinal epithelium. Interestingly, several reports published thereafter have demonstrated that microbiota-derived

SCFAs can mediate epigenetic modifications and directly augment Foxp3+ T_{reg} cell differentiation and function (105, 106). Emerging research in probiotics have also suggested that their therapeutic effects may be in part by inducing Foxp3+ T_{reg} cell development and function. Treatment of mice with a probiotic mixture containing *Lactobacilli acidophilus*, *L. casei*, *L. reuteri*, *Bifidobacteria bifidium*, and *Streptococcus thermophiles* increased Foxp3+ Treg cell differentiation and suppression in vitro (107). These findings were further translated to animal models of chronic inflammatory conditions, including IBD, whereby treatment with the probiotic mixture increased the migration of Foxp3+ T_{reg} cells to inflamed tissue and reduced disease activity. Taken together, the gut microbiota serves an important role in supporting the development and function of the Foxp3+ T_{reg} cell compartment in the intestine.

The following is an excerpt adapted from:
Harusato, A., Flannigan, KL., Geem, D., and Denning, TL. Phenotypic and functional profiling of mouse intestinal antigen presenting cells. *J Immunol Methods*. doi: 10.1016/j.jim.2015.03.023.

Intestinal macrophages and DCs

Intestinal APCs play a key role in regulating host-defense and immune tolerance to the multitude of enteric antigens that occupy and translocate from the lumen. In response to invading pathogens, intestinal APCs orchestrate the innate and adaptive arms of immunity to prevent and clear infections while immunosuppressive and anergic mechanisms are elicited to promote tolerance to

antigens derived from food, self, and other innocuous sources. Macrophages and DCs constitute the major APCs in the intestine and contribute to maintaining immune homeostasis in part through the regulation of CD4+ T cells. In particular, recent studies have elucidated the exceptional ability of intestinal macrophages and DCs to induce Th17 cells and Foxp3+ regulatory T (T_{reg}) cells that mediate inflammatory or tolerogenic immune responses under steady-state, respectively.

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Flannigan, KL., Geem, D., Harusato, A., and Denning, TL. Intestinal antigen presenting cells: Regulators of homeostasis and inflammation. *The American Journal of Pathology*. doi: 10.1016/j.ajpath.2015.02.024.

Development and phenotypic characterization of intestinal macrophages and DCs

The tissue microenvironment plays a key role in regulating the differentiation of macrophages and DCs from myeloid progenitor cells. In the intestine, the local milieu is shaped by the microbiota, enteric antigens, and immune cells that collectively contribute to the developmental outcome of macrophage and DC precursors entering the intestine. Intestinal macrophages, for example, are maintained and replenished by Ly6C+ monocytes that continually enter the intestine during the steady-state and inflammation, a process referred to as the “monocyte” waterfall. These Ly6C+ monocytes subsequently differentiate into resident intestinal macrophages through a series

of intermediary stages (108-111). The monocytes that give rise to intestinal macrophages are originally derived from macrophage-DC progenitors (MDPs), which are the same bone marrow progenitors that can give rise to intestinal DCs (112). The ultimate fate of MDPs in the intestine is thus determined by specific cytokines and growth factors in the tissue microenvironment that dictate different developmental programs. The maturation of monocytes that give rise to intestinal macrophages is under the control of the colony-stimulating factor 1 (CSF1) receptor and its stimulation by CSF1. Accordingly, the number of intestinal macrophages is significantly reduced in CSF1 receptor-deficient mice (113) and in mice treated with anti-CSF1 receptor antibody (114). *Csf1*$^{op/op}$ mice, which have a mutation in the gene encoding CSF1, also have markedly reduced numbers of intestinal macrophages (115). MDPs can alternatively differentiate into common DC progenitors (CDPs) that are the precursors of conventional DCs and plasmacytoid DCs (pDCs). CDPs can give rise to pre-DCs that develop into peripheral DCs, including intestinal CD103+ DCs, in a FMS-like tyrosine kinase 3 (Flt3)-dependent manner (113). Thus, intestinal CD103+ DCs expand in vivo in response to Flt3L (116) and are substantially decreased in mice deficient for Flt3 or Flt3L (113). Other growth factors can further influence the homeostasis of different subsets of DCs as highlighted by data demonstrating that CD103+CD11b+ intestinal DCs require CSF2 receptor stimulation via CSF2 (formerly granulocyte macrophage colony stimulating factor) for development in the steady state, but is dispensable for the differentiation of inflammatory DCs

(113). The future identification of additional mediators that control macrophage and DC development may further our understanding of their ontogeny.

Studies investigating intestinal macrophage and DC development have gained support from recent advancements in the phenotypic characterization of these cells. Analyses of cell morphology and surface markers have allowed for the clear distinction of intestinal macrophages and DCs from one another as well as defining different subsets of each population. When examining cellular structure, macrophages can typically be identified by the presence of large phagocytic vacuoles in the cytoplasm while DCs exhibit dendrite-like projections (117). In addition to microscopy, multi-color flow cytometry has been instrumental in distinguishing intestinal macrophage and DC populations from each other, as well as from additional cell types. Clear identification of APCs from collagenase digested intestinal cells can be achieved by inclusion of the two core markers: CD45 to select for leukocytes and major histocompatibility complex (MHC) II to mark cells with exogenous antigen presenting ability. Additional markers can then be used to define populations of macrophages and DCs. Initial work investigating cell surface markers expressed by intestinal APCs relied upon the presence of F4/80 and the alpha X integrin, CD11c. F4/80 has longstanding use as a macrophage-specific marker and when used in combination with the core APC markers, CD45 and MHCII, can discern macrophages from DCs in the healthy intestine (118, 119). On the other hand, the utility of CD11c as a DC-specific marker is limited due to the fact that intestinal macrophages and DCs both express moderate to high levels of this antigen precluding clear delineation

of DCs from macrophages in the intestine (13, 14, 120-123). A similarly complex issue exists with regards to CD11b as it is expressed by nearly all macrophages, but also a subset of intestinal DCs as well as eosinophils and neutrophils (121). In order to ensure exclusion of these cells, the eosinophil-specific marker, Siglec-F, and the neutrophil-specific marker, Ly6G, can be used during analysis of intestinal macrophages and DCs (124). Another marker that has gained particular attention on intestinal APCs is the chemokine receptor, CX3CR1, which is involved in the extension of trans-epithelial dendrites into the intestinal lumen during bacterial infection (125). While CX3CR1 is highly expressed on resident intestinal macrophages (126, 127) that are located in the lamina propria, as well as the smooth muscle layer of the intestine (128), it can also be expressed at intermediate levels by some DCs during inflammation (109, 129). The high affinity IgG receptor, CD64, has also been used to specifically identify intestinal macrophages (110, 130, 131). Beyond F4/80, CD11b, CX3CR1, and CD64, intestinal macrophages can also be further identified by the differential expression of CD14, CD68, TLR2 and IL-10 receptor (121).

Resident intestinal DCs can be distinguished from macrophages primarily by their expression of CD103 and lack of CX3CR1 (132, 133). CD103+CX3CR1- DCs can further be divided into CD11b+ and CD11b- subsets both of which express CCR7 and can migrate to mesenteric lymph nodes (mLN) and imprint gut homing markers on naïve T cells (127). Therefore at the steady-state, intestinal DCs can be defined among APCs (CD45+MHCII+) as CD11b+/- CD11c+F4/80-CD103+CX3CR1-CD64- cells and may be contrasted from

macrophages, which are CD11b+CD11c+/-F4/80+CD103-CX3CR1+CD64+ cells (Fig. 1.2) (14, 109). This panel can also be utilized in complex scenarios such as inflammation, however it must be noted that certain cell surface markers can change expression in the presence of inflammatory stimuli. Inclusion of additional markers can help meet specific experimental needs. Ultimately, continued advancements in the identification and characterization of specific markers for intestinal macrophages and DCs will further aid in elucidating the complex biological functions of these cells.

Intestinal macrophages and DCs in the steady-state

Homeostatic functions of intestinal macrophages

During the steady-state, intestinal macrophages maintain tolerance towards food antigens and the intestinal microbiota without compromising their ability to react to microbes that breach the epithelial barrier. To control bacteria that translocate past the epithelium, intestinal macrophages are highly phagocytic and have robust bactericidal activity (134). Upon uptake of bacteria however, intestinal macrophages do not produce a strong respiratory burst or synthesize nitric oxide, two potentially damaging processes (135, 136). Resident intestinal macrophages also express very low levels of toll-like receptors (TLRs) and associated signaling machinery, and do not produce inflammatory cytokines such as IL-1, -6, -12, -23, or TNF after exposure to bacterial signals (13, 134, 137-140). In mice, this state of “inflammatory anergy” is largely attributable to IL-10 that is constitutively expressed by intestinal macrophages. When IL-10 or IL-

10 receptors (IL-10R) are blocked, intestinal macrophages become highly responsive to TLR ligands (13, 138, 141, 142). These in vitro data provide evidence that the ability of macrophages to produce and/or respond to IL-10 are both involved in regulating their pro-inflammatory responsiveness. Recent in vivo data have clarified this issue by illustrating the requirement for IL-10R signaling in macrophages in restraining inflammation. In these studies specific deletion of IL-10R, but not IL-10 itself, in CX3CR1+ resident macrophages led to the development of spontaneous colitis (143). Functional analyses found that IL-10R deficient macrophages displayed exaggerated pro-inflammatory responses with very little IL-10 production (95). Additionally, the transfer of wild-type intestinal macrophages, but not IL-10R deficient macrophages, prevented colitis in the T cell transfer model of colitis. These changes in IL-10R-deficient macrophages were also observed in humans with IL-10R deficiencies that develop very early onset IBD (95). Collectively, these data strongly support the concept that intestinal macrophage-mediated tolerance of the microbiota is maintained by responsiveness to IL-10 produced by non-macrophage cells. Likely sources of IL-10 in the intestine are CD4+Foxp3+ regulatory T cells (144) and Type 1 regulatory cells (145). Additionally, other factors such as TGF-β (134, 146) and PPAR-γ (147, 148) may help to regulate the hyporesponsiveness of intestinal macrophages towards luminal antigens. This may be particularly relevant for human intestinal macrophages, which exhibit inflammatory anergy yet do not spontaneously secrete IL-10 (134, 139).

Despite their hyporesponsiveness to inflammatory stimuli, intestinal macrophages actively promote tolerogenic immune responses during the steady-state. One way intestinal macrophages do this is by inducing Foxp3+ Treg cells, which are essential in suppressing inflammation and establishing oral tolerance. Production of IL-10 by intestinal macrophages can lead to the induction (13), maintenance (149) and expansion (85) of Foxp3+ Treg cells in vitro and in vivo. Indeed, initial studies found that intestinal macrophages co-cultured with naïve CD4+ T cells could strongly induce the differentiation of Treg cells in an IL-10-dependent manner (13). Intestinal macrophage derived IL-10 is important for Treg cell induction in vivo as illustrated by the fact that CX3CR1-deficient mice display a loss of oral tolerance coinciding with abolished IL-10 production and blunted Treg cell proliferation in the lamina propria (85). Whether human intestinal macrophages can similarly influence Treg cell function is currently unknown. Interestingly, human intestinal macrophages express CCL20 (MIP3α), the ligand for CCR6, which is expressed on IL-10-producing induced Treg cells and this axis may lead to close interactions between macrophages and Treg cells in the intestine (150).

The ability of intestinal macrophages to modulate Treg cell abundance in vitro and in vivo may directly or indirectly inhibit the differentiation of pro-inflammatory CD4+ T cells. For example, loss of intestinal macrophages due to CX3CR1 or CX3CL1 deficiency resulted in enhanced T-helper 17 (Th17)-driven colitis, which was reversed by the adoptive transfer of CX3CR1+ macrophages (126). Deficiency of CD11b results in defective oral tolerance and enhanced

Th17 responses, an effect that may be associated with reduced intestinal macrophages (151). The ability of macrophages to suppress Th17 responses in the intestine may result from inhibiting the Th17-promoting functions of CD103+CD11b+ DCs (13). Notably, resident macrophages do not readily migrate to mesenteric lymph nodes in the presence of intestinal microbiota (152), and therefore must exert these regulatory functions locally in the lamina propria (85). Overall, intestinal macrophages can regulate themselves and neighboring immune cells through a variety of innate and adaptive immune mechanisms that ultimately aid in the prevention of pathological inflammation.

Contributions of DCs to Intestinal Homeostasis

In addition to macrophages, DCs are also found in the intestine where they drive tolerogenic responses through their communication with the adaptive immune system. It is now well appreciated that a large proportion of DCs in the intestine express CD103 and can be further subdivided into CD11b+ and CD11b- subsets. These CD103+ DCs express high levels of CCR7, which allows for constitutive migration to mLN at the steady-state (84). Examination of oral tolerance to soluble food antigens illustrated that this migratory ability of intestinal DCs plays an influential role in establishing immune tolerance. Indeed, removal of lymph nodes or deletion of CCR7 interfered with the proper establishment of oral tolerance (84). A fundamental mechanism by which intestinal DCs appear to promote oral tolerance is through the generation of Foxp3+ Treg cells. Seminal studies revealed that CD103+ DCs isolated from either the small intestinal lamina

propria or mLNs were able to induce the differentiation of Treg cells from naïve CD4+ T cells in the presence of TGF-β and retinoic acid (RA) (15, 81, 83). Interestingly, RA production by DCs is involved in the upregulation of the gut homing markers, α4β7 and CCR9, on T cells (82, 153). A loss of this homing ability abrogates oral tolerance thus demonstrating the importance of DC imprinting on T cells for immune tolerance. In addition to inducing Treg cells, intestinal DCs can also influence Th17 responses. In particular, CD103+CD11b+ DCs are able to drive Th17 differentiation in the lamina propria of mice in a process that is dependent upon the transcription factor interferon regulatory factor 4 (IRF4) and production of IL-6 and IL-23 (63, 154). Depletion of DCs or loss of IRF4 function correlates with a significant decrease in Th17 cell numbers. CD1c+CD11b+ DCs, the human equivalent of mouse CD103+CD11b+ DCs, also expressed IRF4 and are similarly capable of promoting Th17 responses (154). CD103+CD11b+ DCs also appear to be an obligate source of IL-23 that is required for survival after infection with the attaching-and-effacing pathogen *Citrobacter rodentium*(155).

Given the divergent roles of intestinal DCs in promoting Treg and Th17/22 responses, it is important to consider how the same subsets of intestinal DCs can impact opposing T cell responses in vivo. Intestinal DCs exhibit plasticity in influencing adaptive responses based on the specific microenvironment they encounter (156, 157). Consistent with this notion, the density of CD103+CD11b+ DCs throughout the intestine correlates with number of Th17 cells, with both being abundant in the small intestine and rare in the colon. In contrast, DCs and

macrophages that preferentially promote Foxp3+ Treg cells are most abundant in the colon where a higher abundance of Treg cells can be found (14). The ability of intestinal DCs to stimulate Th17 responses is also depedent on the presence of unique microbiota, specifically segmented filamentous bacteria (SFB) (14, 29). Although not completely understood, other less prominent subsets of DCs in the intestine can also influence adaptive immune responses. Intestinal CD103-CD11b+ cells are a heterogeneous population of both macrophages and DCs (158). CCR2+ DCs from this CD103-CD11b+ population constitutively express IL-12/IL-23p40 and harbor the ability to drive IL-17A production by T cells in vitro (158). Additionally, CD103-CD11b- DCs, which are a minor subset of intestinal DCs, may also be involved in the differentiation of Th17 cells in certain situations (159).

The involvement of intestinal macrophages and DCs in promoting distinct adaptive immune responses has led to many new intriguing questions regarding their involvement in antigen acquisition and presentation. With the close proximity of the microbiota to the lamina propria, it has been proposed that macrophages and DCs can directly sample luminal contents. CX3CR1+ lamina propria cells, most likely macrophages, can extend dendrite-like processes into the intestinal lumen and capture bacteria (125). While the physiological importance of this activity remains unclear, it may be involved in defending against invasive pathogens (125). Interestingly, CX3CR1+ cells, which do not migrate to mLN, preferentially take up antigen compared to migratory CD103+ cells, which are inefficient at sampling and acquiring antigen from the intestinal

lumen (160). The conundrum of how intestinal DCs acquire antigen when macrophages are the main phagocytic cells in the steady-state intestine was recently clarified. Mazzini et al. elegantly demonstrated that CX3CR1+ macrophages can efficiently uptake luminal antigen and transfer it to CD103+ DCs via a mechanism mediated through direct cell-to-cell gaps junctions. Deletion of connexin 43, a protein component of gap junctions, specific to CD11c+ cells prevented this antigen transfer and diminished the ability of CD103+ DCs to present antigen and induce Treg cell differentiation in vitro and prevented the establishment of oral tolerance in vivo (161). Macrophages are not the only cells that can take part in antigen transfer to intestinal CD103+ DCs. Small intestine goblet cells have been reported to function as passages delivering low molecular weight soluble antigens from the intestinal lumen to underlying CD103+ DCs cells (162) and intestinal CD103+ DCs can directly sample bacterial antigens upon migration into the epithelium (160). The relative contribution and functional importance of these various antigen acquisition pathways remain to be elucidated.

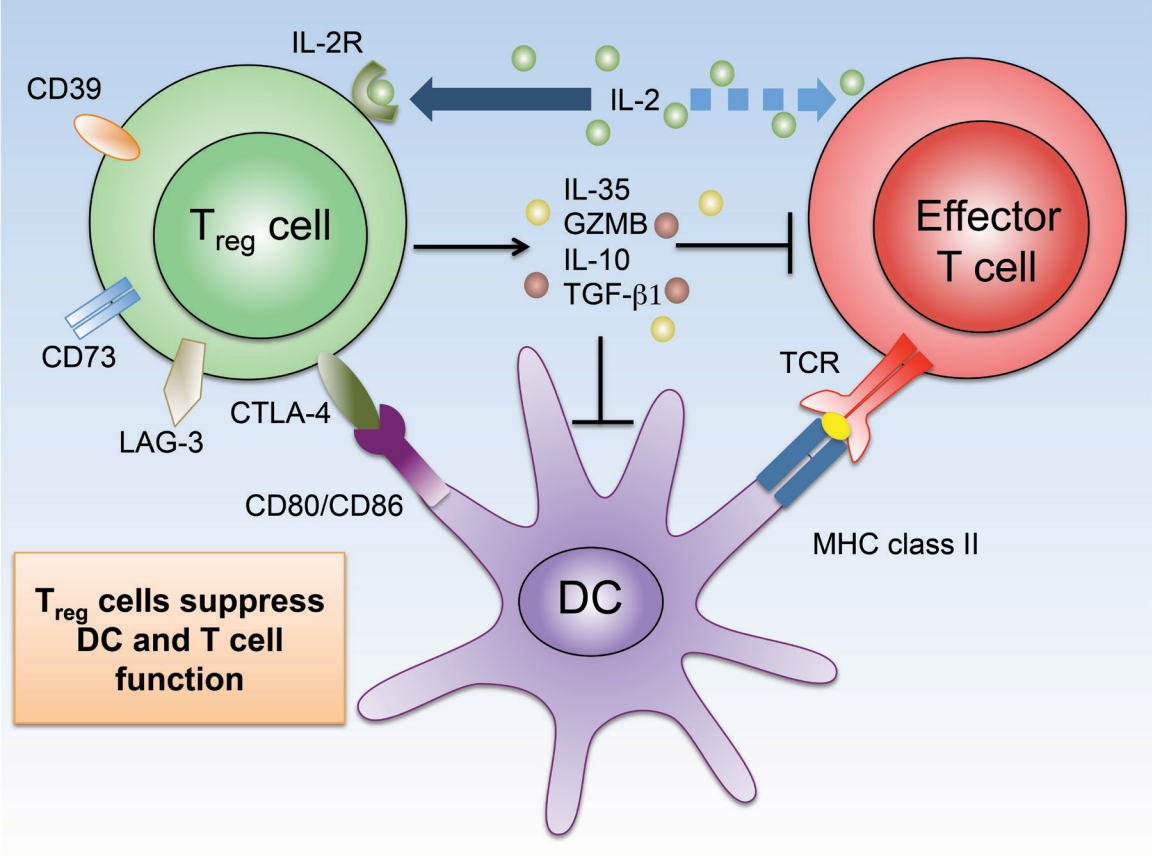


FIGURE 1.1. Mechanisms of T_{reg} cell-mediated suppression. T_{reg} cells inhibit pro-inflammatory cytokine production, impede antigen presentation and/or modulate T cell survival through the secretion of immunosuppressive cytokines (IL-10, TGFβ, and IL-35) and the engagement of immunoregulatory cell surface molecules (CTLA-4 and LAG-3) with their respective ligands on target cells. Additional mechanisms of T_{reg} cell-mediated suppression include: the conversion of ATP to adenosine by CD73 and CD39, IL-2 deprivation of effector T cells, and granzyme (GZMB)- or perforin-dependent killing of responder T cells. This figure is adapted from: Geem, D., Harusato, A., Flannigan, K., and Denning, TL. (2015). Harnessing regulatory T cells for the treatment of inflammatory bowel disease. *Inflammatory Bowel Diseases*. 21(6):1409-18.

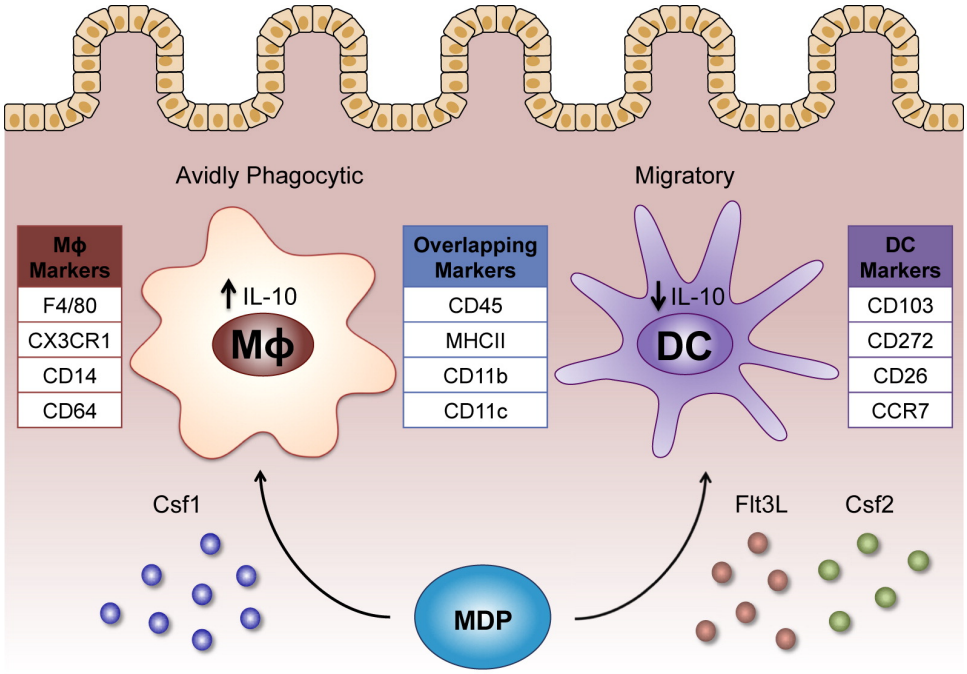


FIGURE 1.2. Distinguishing characteristics of mouse intestinal macrophages (MΦ) and dendritic cells (DCs). Colony-stimulating factor 1 (Csf1) favors the differentiation of intestinal macrophages from macrophages and DC progenitors (MDPs), whereas FMS-like tyrosine kinase 3 ligand (Flt3L) and colony-stimulating factor 2 (Csf2) enhance the differentiation of MDPs into the DC lineage. After populating the intestine, macrophages and DCs can be identified by various cell surface markers. Antigens expressed predominantly by intestinal macrophages include F4/80, CX3CR1, CD14, and CD64, whereas intestinal DCs express CD103, CD272, CD26, and CCR7. Additional markers, including CD45, major histocompatibility complex (MHC) II, CD11b, and CD11c, overlap across both cell types. Macrophages and DCs in the intestine also exhibit a functional dichotomy. Intestinal macrophages are avidly phagocytic and constitutively

produce IL-10, in contrast to intestinal DCs, which efficiently migrate to mesenteric lymph nodes (mLNs) and produce lower levels of IL-10. This figure is adapted from: Flannigan, KL., Geem, D., Harusato, A., and Denning, TL. Intestinal antigen presenting cells: Regulators of homeostasis and inflammation. *The American Journal of Pathology*. doi: 10.1016/j.ajpath.2015.02.024.

Chapter 2: Specific Microbiota-Induced Intestinal Th17 Differentiation Requires MHC II but not GALT and Mesenteric Lymph Nodes

Adapted from:

Geem, D., Medina-Contreras, O., McBride, M., Newberry, RD., Koni, PA., and Denning TL. (2014). Specific Microbiota-Induced Intestinal Th17 Differentiation Requires MHC Class II but Not GALT and Mesenteric Lymph Nodes. *J Immunol.* 193: 431-438. http://www.jimmunol.org/content/193/1/431.long

Abstract

Interleukin (IL)-17 expressing CD4+ T lymphocytes (Th17 cells) naturally reside in the intestine where specific cytokines and microbiota, such as segmented filamentous bacteria (SFB), promote their differentiation. Intestinal Th17 cells are believed to initially differentiate in the GALT and/or mesenteric lymph nodes (mLN) upon antigen encounter and subsequently home to the lamina propria (LP) where they mediate effector functions. However, whether GALT and/or mLN are required for intestinal Th17 differentiation, and how microbiota containing SFB regulate antigen-specific intestinal Th17 cells remain poorly defined. Here we observed that naïve CD4+ T cells were abundant in the intestinal LP prior to weaning and that the accumulation of Th17 cells in response to microbiota containing SFB occurred in the absence of lymphotoxin (LT)-dependent lymphoid structures and the spleen. Furthermore, the differentiation of intestinal Th17 cells in the presence of microbiota containing SFB was dependent on MHC II expression by CD11c+ cells. Lastly, the differentiation of antigen-specific Th17 cells required both the presence of cognate antigen and microbiota containing SFB. These findings suggest that microbiota containing SFB create an intestinal milieu that may induce antigen-specific Th17 differentiation against food and/or bacterial antigens directly in the intestinal LP.

Introduction

CD4+ T lymphocytes constitute a principal component of the adaptive immune system that functions together with innate immune cells to afford host protection against infection and tissue damage (163). The ability of CD4+ T cells to effectively respond to an extensive array of bacteria, viruses, helminthes, and other microbes is the result of a broad T cell receptor (TCR) repertoire and the capacity to differentiate into specific effector subsets. Among the best studied of these effector subsets are Th1 cells, which secrete IFN-g and provide protection against intracellular pathogens, and Th2 cells, which produce IL-4 in response to extracellular bacteria and parasites (164). More recently, Th17 cells were identified as a population of CD4+ T cells distinct from classical Th1 or Th2 cells (6). Th17 cells were shown to be distinct from classical Th1 or Th2 cells and are defined by expression of their hallmark cytokine IL-17A (referred to as IL-17) and can also produce IL-17F, IL-21 and IL-22. Regulated IL-17 production by CD4+ T cells aids in the clearance of extracellular pathogens and fungi in part due its role in recruiting and activating neutrophils. Uncontrolled Th17 responses, however, can lead to pathological tissue damage and have been implicated in numerous infectious, autoimmune, and inflammatory diseases in mice and humans (21).

The in vitro differentiation and expansion of naïve CD4+ T cells along the Th17 lineage is dependent on TCR signaling in the presence of key cytokines including TGF-β1, IL-6, and IL-1β along with the downstream transcription factors of STAT3, IRF4, BATF and RORγt (165). IL-23 does not appear to be required for initial Th17 differentiation, but IL-23 receptor is expressed by developing Th17

cells and IL-23 can stimulate further differentiation, expansion and survival of Th17 cells (41). In vivo, many of the effects of Th17 cells are linked to IL-23 and specific blockade of the p19 subunit of IL-23 ameliorates experimental autoimmune encephalomyelitis, collagen induced arthritis, and colitis (166). Thus, IL-23 and Th17 cells are considered attractive targets for treatment of several autoimmune and inflammatory diseases.

While Th17 cells are induced during infectious and pathological states, they are constitutively present at mucosal surfaces, especially in the intestinal LP (167). The development of intestinal Th17 cells is dependent on the gut microbiota as mice treated with antibiotics from birth and germ-free mice are deficient in these cells (60). Interestingly, SFB are spore-forming, Gram-positive commensal bacteria that adhere tightly to intestinal epithelial cells (IECs) and robustly induce intestinal Th17 cells in mice (61, 168). Since ex vivo culture conditions for SFB have not been defined, many investigations of microbiota-induced Th17 responses have relied upon differences in the SFB status of mice from different vendors. In particular, B6 mice from Jackson Laboratory are void of SFB and consequently harbor a paucity of intestinal Th17 cells, while those from Taconic Laboratory are colonized by SFB and have an appreciable population of intestinal Th17 cells (29). Additionally, horizontal transmission of microbiota containing SFB from Taconic-derived mice to Jackson-derived mice is sufficient to induce Th17 cells in the latter. Although SFB can induce intestinal Th17 cells in vivo, the role for specific signaling pathways regulating this intestinal Th17

development are still being defined and appears to be independent of MyD88 and Trif but may be amplified by ATP (60) and/or serum amyloid A (29).

Currently, numerous aspects of intestinal Th17 differentiation remain poorly defined. Following SFB adherence to epithelium, it is believed that CD11b+ LP dendritic cells (DCs) induce Th17 cells (13, 14, 63, 169, 170), however the requirements for GALT and mLN along with MHC II-mediated antigen presentation requires further investigation. Utilizing mice deficient in LT-dependent lymphoid structures, we demonstrate that colonization by microbiota containing SFB induced intestinal Th17 cell differentiation independent of mLN and GALT, however, MHC II expression by CD11c+ cells and cognate antigen were required —indicating that intestinal Th17 cell differentiation may occur *in situ* in the intestinal LP. These findings suggest that microbiota containing SFB create an intestinal milieu that may induce antigen-specific Th17 differentiation against food and/or bacterial antigens directly in the intestinal LP.

Materials and Methods

Mice

Age- and sex-matched C57BL/6 (B6), B6.129S2-*Lta*^{tm1Dch}/J (*Lta*$^{-/-}$), SPLx *Lta*$^{-/-}$ and B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J (MHC II$^{\Delta/\Delta}$), B6.Cg-Tg (Itgax-cre)1-1Reiz/J (CD11c-cre), B6. SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1) and B6.PL-*Thy1*^{a}/CyJ (Thy1.1), and B6.Cg-Tg(TcraTcrb)425Cbn/J (OTII) mice were purchased from The Jackson Laboratory (JAX). The purchase of *Lta*$^{-/-}$, and B6 (*Lta*$^{+/+}$) controls from Jackson Labs was done to specifically control the SFB status of recipient mice in our

SFB-containing microbiota transfer studies. Immediately upon arrival at Emory University *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice were cohoused. All SFB-containing microbiota transfer studies using *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice were initiated within 2 weeks of arrival from JAX in order to avoid unintentional colonization by SFB in our animal facility. Importantly, immediately prior to the introduction of SFB-containing microbiota, all *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice were verified to be void of SFB (as determined by qPCR detection for SFB DNA in cecal contents and fecal pellets). These measures were also taken for SPLx *Lta*$^{-/-}$, MHC II$^{\Delta/\Delta}$, and associated JAX B6 control mice. *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice are not commercially available and age- and sex-matched littermate controls were provided by R.D. Newberry. These mice were cohoused and the experiments were performed on-site at Washington University-St. Louis. MHC II^{FF} mice provided by P.A. Koni, and CD11c-cre mice were crossed to generate MHC II$^{\Delta DC}$ mice that are hemizygous for CD11c-cre while MHC II^{FF} litter- and cage-mate mice were used as controls. B6 mice purchased from Taconic were utilized as donors of intestinal microbiota for fecal transfer experiments. Mice were maintained under specific pathogen-free conditions and animal protocols were reviewed and approved by the Institute Animal Care and Use Committee of Emory University.

Antibodies and reagents

The following antibodies were purchased from eBioscience: IFNγ (XMG1.2), CD90.1 (H1S51), CD69 (H1.2F3), CD45RB (C363.16A), CD45.1 (A20), Vα2 (B20.1), IL-17A (eBio17B7), CD8α (eBioT4/11.8), CD25 (PC61.5), CD3ε

(eBio500A2), and RORγ(t)-PE (B2D). Antibodies purchased from BD Biosciences were: TCRβ (H57-597), Vβ5 (MR9-4), CCR6 (140706), IL-17A (TC11-18H10), Vα2 (B20.1), and CD4 (RM4-5). Dead cells were identified using the fixable Aqua dead cell staining kit (Invitrogen). The following biotin-conjugated antibodies (eBioscience) were used for negative selection in conjunction with anti-biotin and anti-APC microbeads (Miltenyi Biotec): CD8α (53-6.7), Ly-6G (RB6-8C5), F4/80 (BM8), TER-119 (TER-119), CD11b (M1/70), NK1.1 (PK136), CD11c (N418), CD19 (eBio1D3). Isolation of LP cells and flow cytometry was performed as previously described (126).

Preparation and gavage of cecal contents containing SFB

The cecal contents from Taconic B6 mice were resuspended in 5 ml of sterile PBS, filtered through a 100 μm cell strainer, and 150 ul of the homogenate was gavaged twice into each mouse with 3 hr between each gavage. The homogenate was verified to contain SFB via qPCR (29). Recipient mice were utilized within two weeks of arrival from the JAX and verified to be void of SFB prior to gavage and colonized post-gavage based on qPCR analysis of fresh fecal pellets.

In vivo Th17 differentiation

Naïve CD4+ T cells were enriched via negative selection utilizing magnetic-activated cell sorting to deplete cells expressing: CD25, CD19, CD11b, CD11c, NK1.1, F4/80, Ly-6G, CD8α, and Ter119 on MACS LS columns with anti-biotin

and anti-APC microbeads (Miltenyi Biotec). Frequency of CD4+IL-17A+ T cells (<1%) was verified using flow cytometry on the LSR II (BD). 5 x 10^6 cells were injected i.v. into CD45.2 congenic hosts. Recipients were gavaged with cecal contents from Taconic mice on the following day. After 10 days, recipients were harvested for assessment of intestinal Th17 differentiation.

For experiments involving the co-transfer of naive Thy1.1+ OT-II cells, these cells were mixed at a 1:1 ratio with CD45.1+ cells, and a total of 10^7 cells were injected i.v. On the following day, mice were gavaged with cecal contents from Taconic mice and/or fed albumin from chicken egg white (Sigma-Aldrich) in the drinking water (15 mg/ml) for 10 days.

Statistics

Statistical analyses were performed with Prism software (Graph-Pad Software) using the Student's *t* test. Error bars represent SEM as indicated and *p* values equal to or less than 0.05 were considered statistically significant while *p* values greater than 0.05 were considered not statistically significant (N.S.).

Results

Naïve CD4+ T cells are present in the intestinal LP independent of LT-dependent lymphoid structures. The mLN are specialized secondary lymphoid organs (SLO) that drain the intestine, and as such are a site where LP DCs can migrate to present antigens to naive CD4+ T cells (12). These observations suggest that mLN may be the primary site for naïve CD4+ T cell priming and differentiation into Th17 cells that home to the intestinal LP. Hence, an enrichment of Th17 cells in the mLN would be expected relative to other SLO that do not drain the intestine. To investigate if the mLN are indeed enriched for Th17 cells, a comparative analysis was conducted to assess the proportion of these cells induced by SFB-containing microbiota in the spleen (Spl), peripheral lymph nodes (pLN), mLN, small intestine (SI) LP, and large intestine (LI) LP of JAX B6 mice gavaged SFB-containing cecal contents (SFB^+ CC). The frequency of Th17 cells in the Spl and pLN was similar to that of the mLN, with all being less than 0.5% of total CD4+ T cells (Supplementary Fig. 2.1). Additionally, the frequencies of Th17 cells were significantly higher in the SI LP and LI LP (comprised 14% and 11% of total CD4+ T cells respectively), relative to those observed in the Spl, pLN, and mLN (Supplementary Fig. 2.1). These findings demonstrate that Th17 cells are not significantly enriched in the mLN compared to other SLO that do not drain the intestine and that the enrichment of Th17 cells in the intestinal LP may be due to CD4+ T cell priming and differentiate in situ.

In order to begin investigating whether CD4+ T cells may be primed and differentiate into Th17 cells within the intestine, we first determined whether

naïve CD4+ T cells are present in the intestinal LP during development. Further, we used mice void of SLO—including the lymph nodes, Peyer's patches, and isolated lymphoid follicles—as a result LT signaling deficiency (*Lta*$^{-/-}$) to investigate the differentiation of Th17 cells in the absence of mLN and other SLO. Ontogeny studies were conducted to characterize the proportion of naïve CD4+ T cells in the SI LP and LI LP of B6 (*Lta*$^{+/+}$) and *Lta*$^{-/-}$ mice, and the Spl was used to provide a comparison between the intestine and a peripheral lymphoid organ. In the Spl of *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice, ~70% of the CD4+ T cells were characterized as naïve (CD45RB^{hi} Foxp3-) independent of age (Fig. 2.1A, 1B). These CD45RB^{hi} Foxp3- CD4+ T cells were further verified as naïve due to their lack of expression for the activation and memory markers CD25, CD44, and CD69 (data not shown). Interestingly, ~80% of the SI LP and LI LP CD4+ T cells in both *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice were CD45RB^{hi} Foxp3- at 1 week of age and this frequency decreased to ~60% in the SI LP and ~40% in the LI LP at 3 weeks and remained at ~20% in both the SI LP and LI LP into adulthood (Fig. 2.1C). Additionally, the absolute cell numbers for cell subsets were not altered and were similar at the various points (data not shown). Taken together, our results demonstrate that appreciable numbers of naïve CD4+ T cells are present in the intestine and do not require mLN or other LT-dependent lymphoid structures for their accumulation at this site.

Intestinal Th17 differentiation takes place in the absence of the GALT, mLN, and other LT-dependent lymphoid structures. To examine the requirements

for GALT, mLN and other LT-dependent lymphoid structures in intestinal Th17 differentiation, *Lta*$^{+/+}$ and *Lta*$^{-/-}$ from JAX, which had undetectable levels of SFB DNA (data not shown), were gavaged vehicle (PBS) alone or SFB^{+} CC isolated from Taconic B6 mice, and intestinal Th17 differentiation of recipient mice was assessed 10 days post-gavage. Interestingly, colonization of *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice with SFB^{+} CC induced robust differentiation of intestinal Th17 cells, increasing their frequency to ~15% in the SI LP and ~10% in the LI LP (Fig. 2.2A, 2.2B). Similar trends were observed for absolute cell numbers (Fig. 2.2C). The levels of SFB in *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice at day 10 post-gavage were comparable to TAC B6 based on quantitation of SFB, and this induction of intestinal Th17 cells by SFB-containing microbiota was not observed in the IEL compartment (data not shown). Furthermore, these intestinal Th17 cells were confirmed to be *bona fide* Th17 cells as they expressed the nuclear orphan receptor RORγt, which is both necessary and sufficient for the Th17 program, as well as the chemokine receptor, CCR6 (Fig. 2.2D). Additionally, these intestinal Th17 cells were negative for IL-10 based on flow cytometry (data not shown; positive control included) and therefore do not appear to be regulatory Th17 cells (47). To address the possibility that the Spl may be a site for microbiota-driven intestinal Th17 differentiation in the absence of the GALT, mLN and other LT-dependent lymphoid structures, similar experiments were performed in splenectomized (Splx) *Lta*$^{-/-}$ mice as well as, lymphotoxin β receptor deficient (*Ltbr*$^{-/-}$) mice, an additional model of SLO deficiency. Both Splx *Lta*$^{-/-}$ and *Ltbr*$^{-/-}$ mice yielded similar results as observed in *Lta*$^{-/-}$ mice (Fig. 2.3), and these findings confirm

that intestinal Th17 differentiation induced by SFB-containing microbiota does not require the Spl, mLN or other LT-dependent lymphoid structures and may occur directly in the intestinal LP.

MHC II is required for intestinal Th17 differentiation induced by SFB-containing microbiota. The differentiation of intestinal Th17 cells is promoted by SFB-containing microbiota along with specific cytokines/factors that are secreted by CD11b+CD103+ LP DCs (29). Whether LP DCs or other antigen presenting cells are promoting intestinal Th17 differentiation via presentation of antigen(s) on MHC II, or via other biological functions, remains unclear. Hence, we employed a naïve CD4+ T cell and specific microbiota transfer system to evaluate intestinal Th17 differentiation in B6 (MHC $II^{+/+}$) and MHC II-deficient (MHC $II^{\Delta/\Delta}$) JAX mice. To do so, naïve polyclonal CD4+ T cells were enriched from the Spl and peripheral lymph nodes of CD45.1 mice (purity >99% IL-17A-CD4+ T cells) and adoptively transferred into CD45.2+ MHC $II^{+/+}$ and MHC $II^{\Delta/\Delta}$ mice that were void of SFB. One day later, recipients were gavaged SFB^{+} CC, and Th17 differentiation was assessed amongst both the host and donor CD4+ T cells 10 days post-gavage. This experimental system enabled us to study the role of MHC II in modulating the ability of adoptively transferred naïve CD4+ T cells to differentiate into Th17 cells upon conditioning with SFB-containing microbiota in a defined timeframe. As expected, a paucity of CD4+ T cells were observed in MHC $II^{\Delta/\Delta}$ mice since MHC II is essential for the proper development and survival of CD4+ T cells (Supplementary Fig. 2.2A-C; (171)). Interestingly,

intestinal Th17 differentiation induced by SFB-containing microbiota was significantly attenuated in MHC II$^{\Delta/\Delta}$ mice based on frequency and cell number for host and donor (Fig. 2.4A, 2.4B) lamina propria lymphocytes (LPL), relative to MHC II$^{+/+}$ mice. Approximately 30-fold reduction in the proportion of Th17 cells was observed for host (Fig. 2.4B, *top left panel*) and donor (Fig. 2.4B, *top right panel*) intestinal Th17 cells in MHC II$^{\Delta/\Delta}$ mice relative to MHC II$^{+/+}$ mice, while for absolute cell numbers, >70- and >16-fold reductions were observed in host (Fig. 2.4B, *bottom left panel*) and donor LPL (Fig. 2.4B, *bottom right panel*), respectively. The abrogation of intestinal Th17 induction by SFB-containing microbiota was not due to impaired survival of the donor CD4+ T cells since the number of donor CD4+ LPL were similar on day 10 post-gavage (Fig. 2.4C). However, the deletion of MHC II and thus, antigenic stimulation appeared to be important for the induction of intestinal Th17 cells by SFB-containing microbiota since the differentiation of intestinal Foxp3+ Treg (Fig. 2.4D, left panel) and Th1 cells (Fig. 2.4D, right panel) were also reduced in absence of MHC II globally. Collectively, these results establish that intestinal Th17 differentiation induced by SFB-containing microbiota is dependent upon MHC II.

To investigate the cell lineage for which MHC II expression is required, we examined mice specifically lacking MHC II on CD11c-expressing cells (MHC II$^{\Delta DC}$). Both MHC II$^{\Delta DC}$ and MHC II^{FF} mice were littermate controls and co-housed in the same cage. MHC II was verified to be absent on CD11c+CD103+ LP DCs isolated from MHC II$^{\Delta DC}$ mice (Supplementary Fig. 2.3A) and the loss of MHC II on CD11c+CD103+ LP DCs in MHC II$^{\Delta DC}$ mice did not affect their abundance as

the frequency and cell number were similar (Supplementary Fig. 2.3B; data not shown). Intestinal Th17 development examined 10 days post-gavage of SFB^+ CC demonstrated significantly less Th17 cells in intestinal LP of MHC $II^{\Delta DC}$ mice in comparison to MHC II^{FF} mice (5% versus 15% of CD4+ T cells, respectively), and this was specific to Th17 cells since intestinal Foxp3+ Treg and Th1 cells remained similar (Fig. 2.5A, 2.5B). In addition, deletion of MHC II on CD11c+ DCs did not dramatically affect pro-inflammatory cytokine expression (Supplementary Fig. 2.4) nor impair CD4+ T cell accumulation and abundance in the intestinal LP (Fig. 2.5C; data not shown). Altogether, these data highlight the importance of MHC II on CD11c+ DCs and suggests that DCs may be specialized in providing antigenic stimulation to promote the development of Th17 cells in response to SFB-containing microbiota.

Cognate antigen promotes intestinal Th17 differentiation in the presence of SFB-containing microbiota. Our previous data demonstrates that Th17 differentiation induced by SFB-containing microbiota is MHC II-dependent (Fig. 2.4, Fig. 2.5), however, specific SFB-derived antigens that may induce intestinal Th17 cells have not yet been defined. Thus, we investigated whether a model food antigen is sufficient using an antigen-specific CD4+ T cell transfer system. Naïve CD45.1+ CD4+ T cells and Thy1.1+ OT-II cells were enriched from Spl and pLN, respectively, and were mixed at a 1:1 ratio followed by adoptive transfer into $CD45.2^+$ JAX B6 recipients void of SFB (data not shown). The purity of donor cells was verified to be >99% IL-17A-CD4+ T cells (data not shown).

One day later, recipients were gavaged SFB^+ CC and/or fed the cognate antigen for OT-II cells, chicken ovalbumin (OVA), for 10 days in the drinking water. On day 10, mice were euthanized and intestinal Th17 differentiation was assessed amongst host and donor (both CD45.1+ and Thy1.1+ OT-II) CD4+ T cell populations (Fig. 2.6A). As expected, host CD4+ LPL (CD45.1-Thy1.1-) differentiated into Th17 cells following gavage of SFB^+ CC (Fig. 2.6B, 2.6C). The donor CD45.1+Thy1.1-CD4+ LPL responded similarly to the host LPL (CD45.1-Thy1.1-) in robustly differentiating to Th17 cells following gavage of SFB^+ CC, relative to mice that were not gavaged (Fig. 2.6B, 2.6D). Importantly, OT-II LPL (CD45.1-Thy1.1+Va2+Vb5+) differentiated into Th17 cells comparable to host CD4+ LPL (CD45.1-Thy1.1-) and donor CD45.1+Thy1.1-CD4+ LPL only in mice given SFB^+ CC and OVA (Fig. 2.6B, 2.6E). In the context of OVA without SFB^+ CC, a small proportion of the OT-II cells differentiated into Th17 cells. With the absence of cognate antigen, mice gavaged SFB^+ CC yielded a paucity of donor OT-II cells and the corresponding Th17 cells were negligible (data not shown). Overall, both cognate antigen and specific microbiota are required for robust intestinal Th17 differentiation and the cognate antigen does not have to be of bacterial (SFB) origin.

Discussion

Here, we demonstrate that the accumulation of naïve CD4+ T cells in the intestine and the development of intestinal Th17 cells in response to microbiota containing SFB did not require the Spl, mLN, and other LT-dependent lymphoid

structures. Furthermore, using a CD4+ T cell transfer system, intestinal Th17 differentiation was shown to require MHC II expression by CD11c+ cells and could be induced by a model food antigen. These results suggest that specific components of the microbiota are important in conditioning the local intestinal milieu to facilitate the differentiation of Th17 cells upon antigenic stimulation by LP DCs in situ.

The intestine is unique among organs in that it harbors large numbers of Th17 and Foxp3+ regulatory T cells in the steady-state (21). The presence of these T cell subsets in the intestinal LP is profoundly influenced by specific components of the microbiota (29, 61, 102, 172), and their metabolites (172, 173) since these bacteria interact directly with intestinal epithelial cells (174) and may be sampled by underlying LP DCs and macrophages (160). The site of this steady-state CD4+ T cell differentiation has been assumed to be in the mLN based upon several key observations: 1) naïve CD4+ T cells primarily traffic through secondary lymphoid tissues and not the intestine, 2) Peyer's patch (PP) and LP DCs migrate to the mLN where they present antigens to naïve T cells resulting in their expansion and induction of gut homing molecules (132, 153, 175, 176), and 3) delivery of soluble antigen via the oral route induces Foxp3+ T cell differentiation in the mLN (15, 85, 177). Importantly, none of these observations are inconsistent with CD4+ T cell differentiation taking place directly within the intestinal LP. Additionally, previous reports demonstrated that PP and colonic patches are dispensable for the differentiation of intestinal Th17 cells (60).

While CD4+ T cells primarily traffic through the Spl and lymph nodes, we show that they are abundant in the intestinal LP both before and after weaning. In fact, naïve T cells can enter various non-lymphoid organs as part of a normal migratory pathway (178). While intestinal DCs do migrate via the afferent lymphatics to the mLN (179), this process is dramatically augmented by inflammatory stimuli (180) suggesting that in the steady-state only a fraction of DCs migrate to the mLN, while the majority remains in the LP. Macrophages are also abundant in the intestinal LP and their migration to the mLN is regulated by the microbiota (127, 152). Thus, the intestinal LP contains all of the necessary requirements for CD4+ T cell priming and differentiation: abundant numbers of naïve CD4+ T cells, MHC II bearing DCs and macrophages, and a microbiota-induced local milieu.

While our data demonstrate that GALT and mLN are not required for SFB-induced intestinal Th17 differentiation, they do not imply that these lymphoid structures play no role in this process. In fact, LT-dependent lymphoid structures have been reported to influence steady-state intestinal Th17 cells in mice lacking SFB (181) and our data are consistent with these observations since *Lta*$^{-/-}$ mice from JAX void of SFB demonstrated a reduction in intestinal Th17 cells that was associated with a skewing of intestinal CD4+ T cells toward the Th1 subset such that there were significantly more Th1 cells in both the SI LP and LI LP relative to *Lta*$^{+/+}$ mice (data not shown). Thus, the GALT/mLN and intestinal LP may make unique and perhaps overlapping contributions to intestinal Th17 differentiation

depending on whether they are “naturally-derived” (58, 182) or induced in response to specific components of the microbiota.

Currently, the antigenic specificity of Th17 cells that reside in the intestinal LP at steady-state remains undefined (183). The requirement for specific components of the microbiota indicates that the TCR repertoire of these cells may be reactive to bacterial antigens (29). Cytokines and other factors within the intestinal tissue may also promote Th17 differentiation upon priming of naïve CD4+ T cells by microbial-, self-, and/or food-derived antigens. The reduced intestinal Th17 development we observed in MHC II$^{\Delta/\Delta}$ mice colonized with SFB strongly suggests that antigenic stimulation of naïve CD4+ T cells is essential for intestinal Th17 differentiation, and conditioning by specific components of the microbiota alone is insufficient to drive this process. Furthermore, Th17 differentiation of naïve OT-II cells within the intestinal LP of mice was only observed when both OVA and SFB-containing microbiota were present. These findings are consistent with in vitro studies demonstrating that both TCR stimulation and specific cytokines are required for Th17 differentiation (41, 43). The antigen reactivity of intestinal Th17 cells is clearly not limited to SFB since OVA, a food antigen, is sufficient. While SFB has been shown to promote the development of Th17 cells, the role of other bacteria in the SFB-containing microbiota that may influence intestinal Th17 cell differentiation cannot be excluded in our SFB^{+} CC transfer system. Thus, the antigen specificity of intestinal Th17 cells may encompass reactivity to select bacteria, food- and/or self-antigens. Further, investigations into the TCR specificity of “natural” and

induced Th17 cells are clearly warranted. In summary, our findings highlight several previously unappreciated aspects of specific microbiota-induced Th17 differentiation and suggests that the intestinal LP may be an important site for this process.

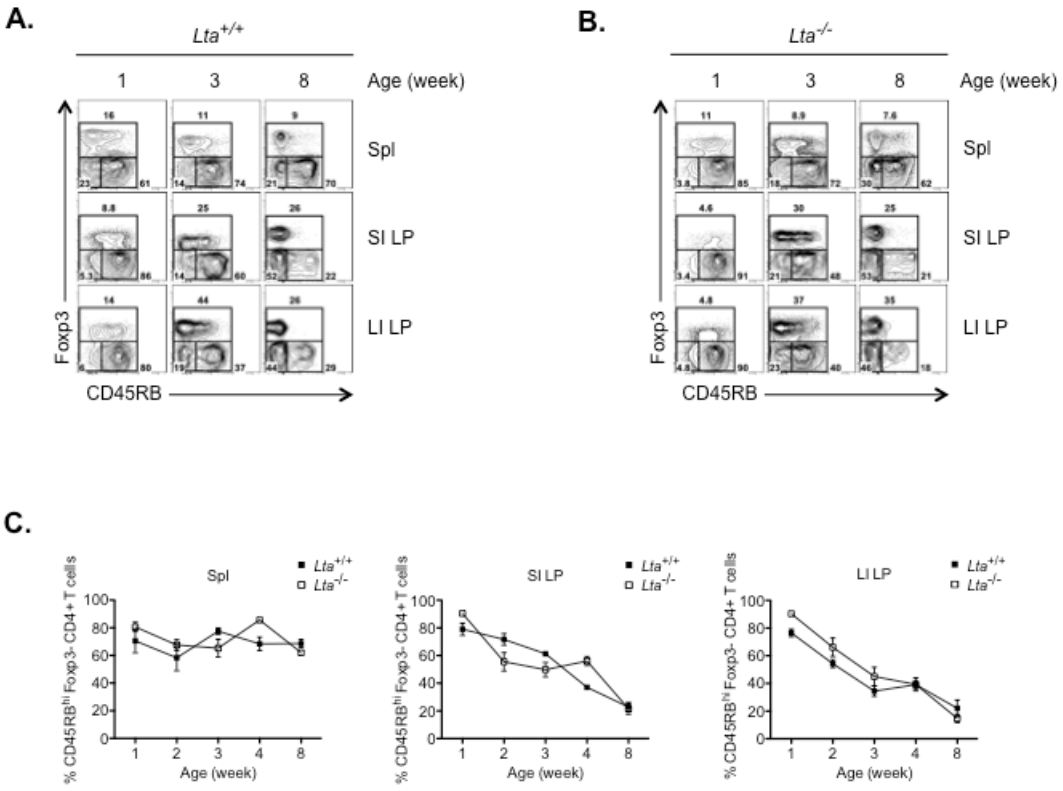


FIGURE 2.1. Naïve CD4+ T cells are present in the intestinal LP independent of LT-dependent lymphoid structures. Ontogeny of naïve CD45RB^{hi} Foxp3- CD4+ T cells in the spleen (Spl) and intestinal lamina propria (LP) was investigated utilizing flow cytometry. Representative FACS plots of cells pre-gated on TCRβ and CD4 and assessed for the expression of CD45RB and Foxp3 in *Lta*$^{+/+}$ (A) and *Lta*$^{-/-}$ mice (B). CD45RB^{hi} Foxp3- CD4+ T cells were further verified to be negative for the memory markers of CD44 and CD25 (data not shown). (C) Frequencies of CD45RB^{hi} Foxp3- CD4+ T cells in the Spl, small intestine (SI) LP, and large intestine (LI) LP of *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice during development into adulthood. Samples for week 1 and 2 were pooled for each age group due to small size of the organs. Data are representative of at least two independent experiments with three to eight mice per age group.

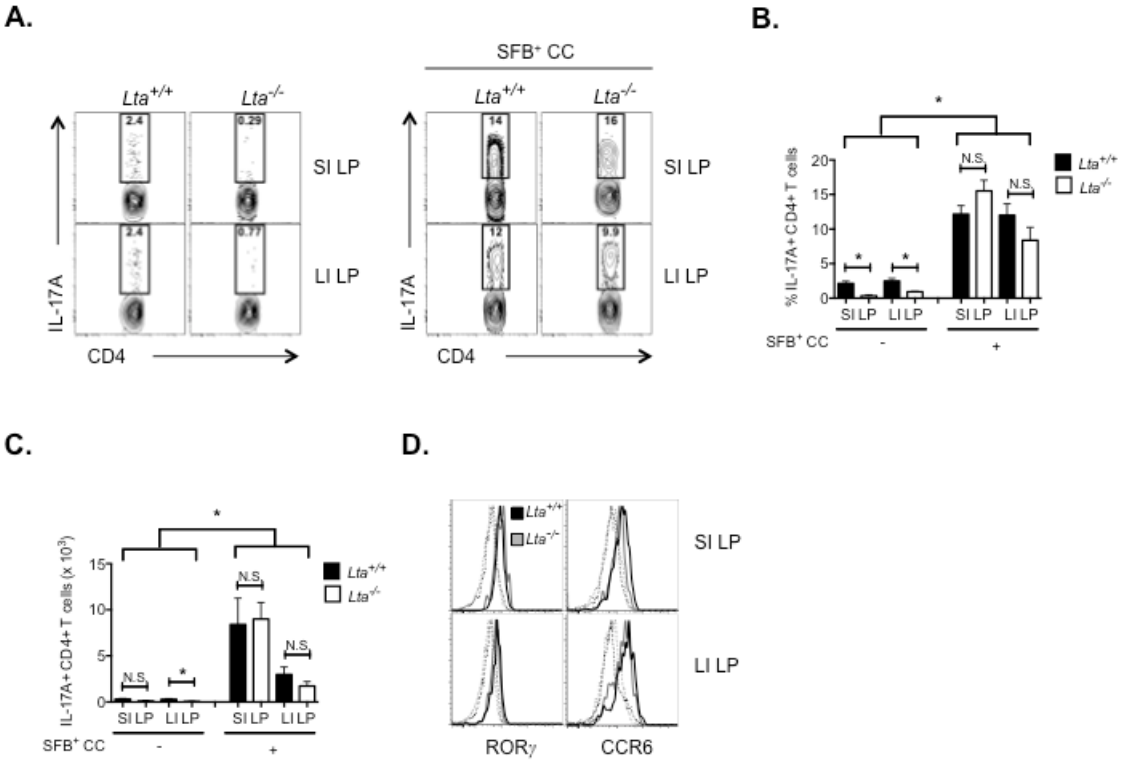


FIGURE 2.2. Intestinal Th17 differentiation driven by SFB-containing microbiota takes place in the absence of the GALT, mLN, and other LT-dependent lymphoid structures. JAX *Lta*$^{+/+}$ and JAX *Lta*$^{-/-}$ mice void of SFB were gavaged PBS or SFB-containing cecal contents (SFB^{+} CC) and intestinal Th17 differentiation was assessed 10 days later. (A) Representative FACS plots of intestinal Th17 cell frequencies in *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice on day 10 post-gavage. (B) Comparison of intestinal Th17 cell frequencies and numbers (C) between *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice. Data are representative of at least two independent experiments with three to four mice per group for A and six to nine mice per group for B. (D) Expression of RORγt and CCR6 by intestinal Th17 cells induced by SFB-containing microbiota. Bolded histograms are pre-gated on IL-17A+ CD4+ T cells while histograms not

bolded are pre-gated on IL-17A- CD4+ T cells. Data in D are representative of two independent experiments with four mice per group. Error bars represent SEM. *, $p \leq 0.05$; not statistically significant (N.S.), $p > 0.05$ using a Student's *t* test.

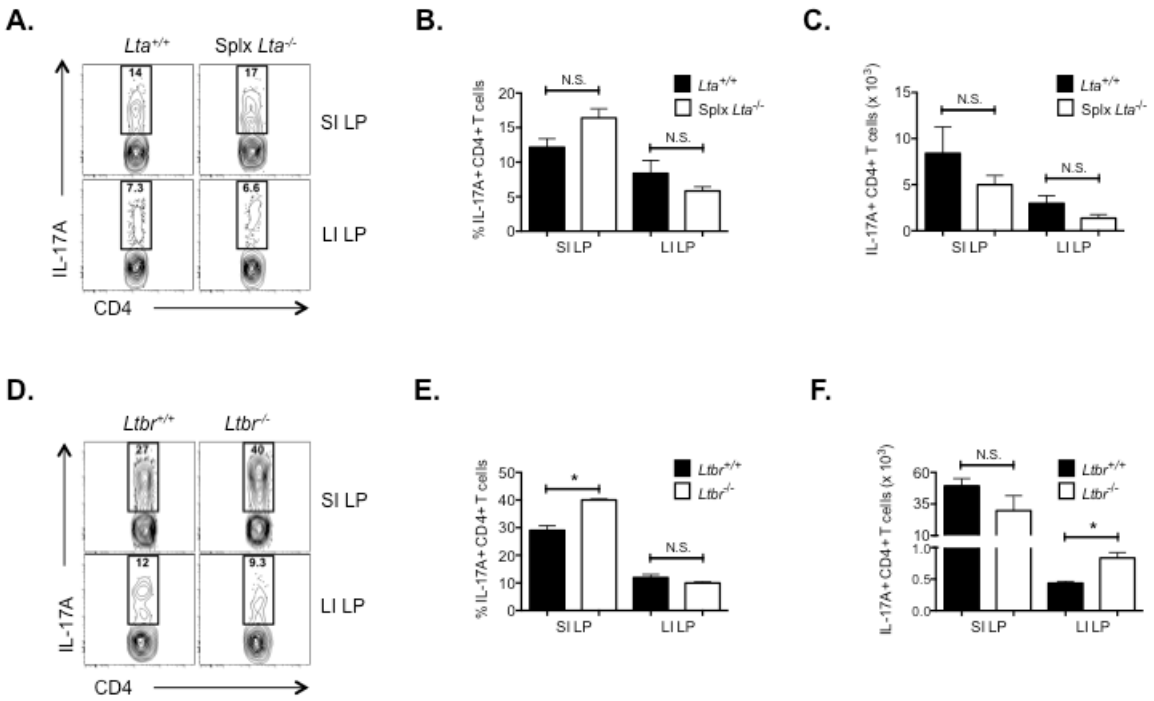


FIGURE 2.3. Intestinal Th17 differentiation in additional models of SLO deficiency. (A) Representative FACS plots of intestinal Th17 cells driven by SFB-containing microbiota for JAX *Lta*$^{+/+}$ and JAX Splx *Lta*$^{-/-}$ mice. Comparison of intestinal Th17 cell frequencies (B) and numbers (C) for *Lta*$^{+/+}$ and Splx *Lta*$^{-/-}$ mice. (D) Representative FACS plots of intestinal Th17 cells in *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice. Comparison of intestinal Th17 cell frequency (E) and number (F) in *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice. Data are representative of two independent experiments with

four mice per group. Error bars represent SEM. *, $p \leq 0.05$; not statistically significant (N.S.), $p > 0.05$ using a Student's *t* test.

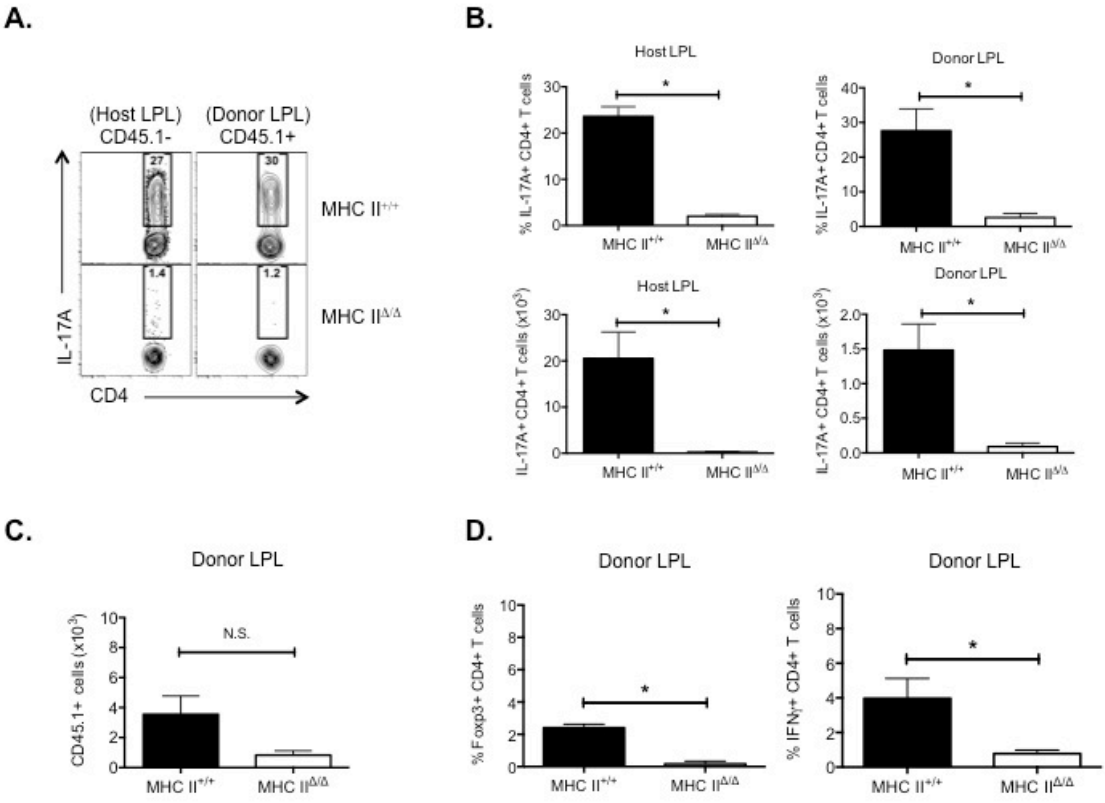


FIGURE 2.4. MHC II is required for intestinal Th17 differentiation induced by SFB-containing microbiota. (A-D) Naïve polyclonal CD4+CD25- T cells enriched from CD45.1 mice were adoptively transferred into JAX CD45.2 MHC $II^{+/+}$ and JAX CD45.2 MHC $II^{\Delta/\Delta}$ mice on day -1. Recipients were gavaged SFB^{+} CC on the next day and intestinal Th17 differentiation was assessed amongst lamina propria lymphocytes (LPL) in the small intestine on day 10. (A) Representative FACS plots of microbiota-induced Th17 cells of host and donor SI LPL in MHC

$II^{+/+}$ and MHC $II^{\Delta/\Delta}$ mice. (B) Comparison of microbiota-induced Th17 differentiation for host LPL and donor LPL frequency (upper panels) and cell number (lower panels). (C) Number of donor CD45.1 CD4+ T cells amongst LPL in MHC $II^{+/+}$ and MHC $II^{\Delta/\Delta}$ mice. (D) Frequency of Foxp3+ Treg (left panel) and Th1 (right panel) cells amongst donor CD4+ LPL in MHC $II^{+/+}$ and MHC $II^{\Delta/\Delta}$ mice. Data are representative of four mice per group from two independent experiments. Error bars represent SEM. *, $p \leq 0.05$; not statistically significant (N.S.), $p > 0.05$ using a Student's *t* test.

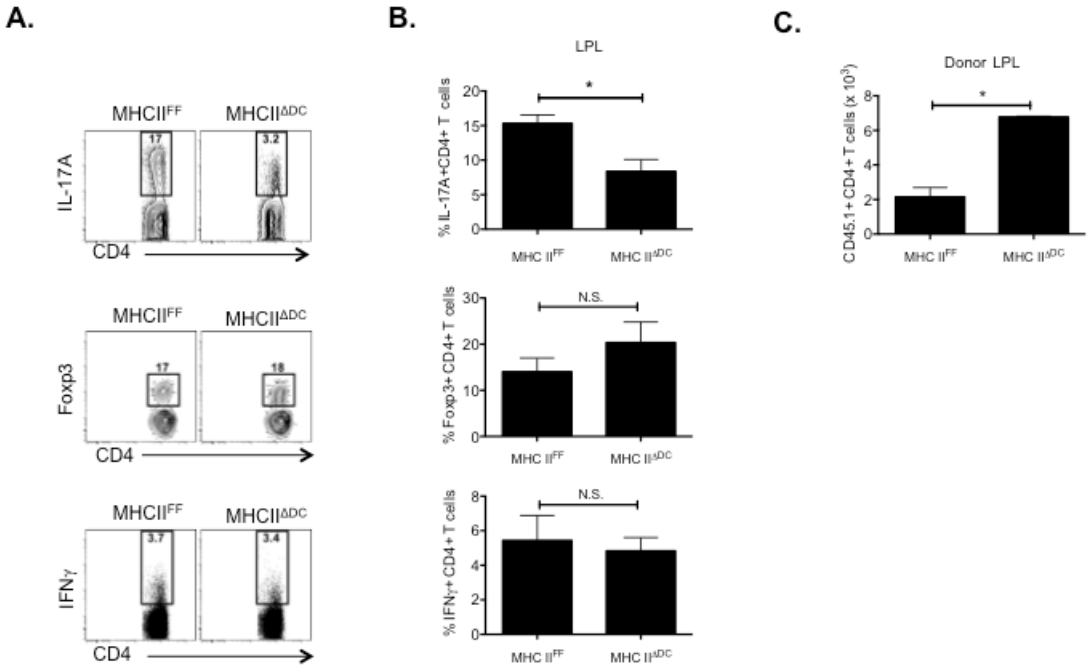


FIGURE 2.5. MHC II expression on CD11c+ cells is important for intestinal Th17 differentiation induced by SFB-containing microbiota. Litter- and cage-mate MHC

II-floxed (MHC II^{FF}) mice and CD11c-cre MHC II^{FF} (MHC II$^{\mathbf{\Delta DC}}$) mice were gavaged with SFB^{+} CC and CD4+ T cell differentiation amongst the LPL of the small intestine was assessed on day 10 post-gavage. Representative FACS plots (A) and corresponding bar graphs (B) of Th17 (top panels), Foxp3+ Treg (middle panels), and Th1 (bottom panels) cells amongst LPL for MHC II$^{\Delta DC}$ and MHC II^{FF} mice. (C) Accumulation of donor CD45.1 CD4+ T cells in the SI LP of MHC II$^{\Delta DC}$ and MHC II^{FF} mice on day 10 post-gavage. Data are representative of two independent experiments with four to five mice per group. *, $p \leq 0.05$; Not statistically significant (N.S.), $p > 0.05$ using a Student's *t* test.

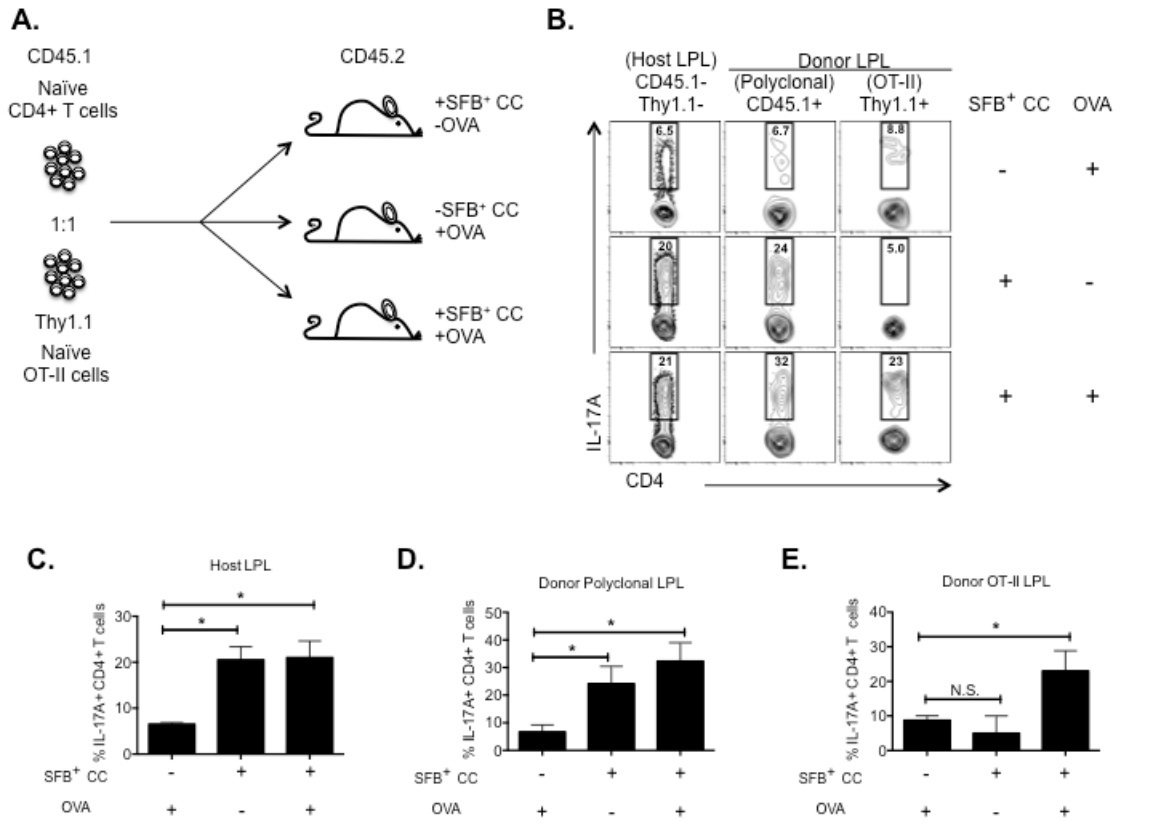
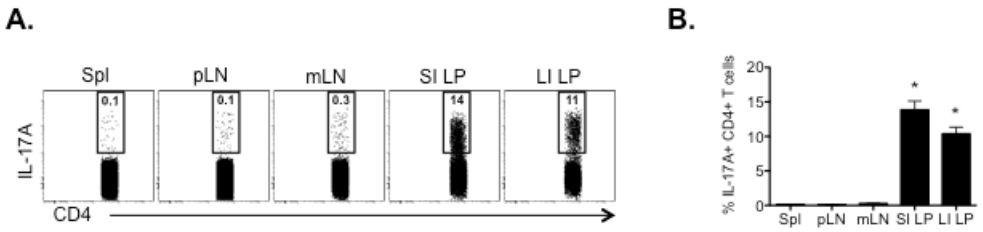
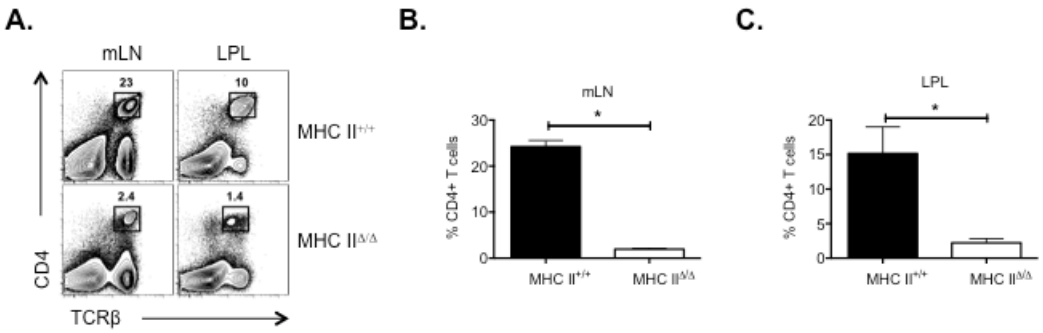


FIGURE 2.6. Intestinal Th17 differentiation induced by SFB-containing microbiota is dependent on antigenic stimulation and the conditioned intestinal microenvironment. (A) Naïve CD4+CD25- cells were enriched from the Spl and LN of CD45.1 and OT-II mice, respectively, and adoptively transferred at a 1:1 ratio into JAX CD45.2 B6 mice void of SFB on day -1. On day 0, mice were gavaged SFB^+ CC and/or fed chicken ovalbumin (OVA) in the drinking water for 10 days. On day 10, mice were euthanized, SI LPL were isolated, and intestinal Th17 differentiation amongst CD4+ T cells of the host (CD45.1-Thy1.1-) and donor CD45.1+Thy1.1- and donor CD45.1-Thy1.1+ OT-II (Va2+Vb5+) cells was assessed. (B) Representative FACS plots for host and donor SI LPL evaluated

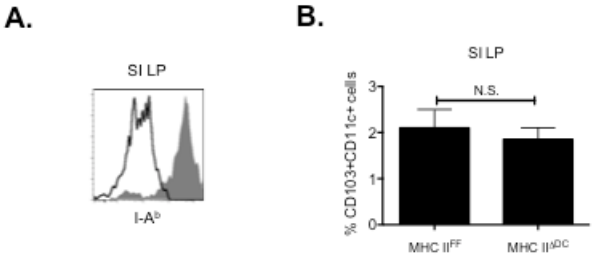
for IL-17A expression in the three different conditions of: OVA only, SFB^+ CC only, or OVA and SFB^+ CC. FACS plots were pre-gated on TCRβ+CD4+ cells. Comparison of intestinal Th17 induction relative to OVA only group for host (C), donor CD45.1+ (D), and donor Thy1.1+ OT-II LPL (E). Data are representative of two independent experiments with three to four mice per group. Error bars represent SEM. *, $p \leq 0.05$; not statistically significant (N.S.), $p > 0.05$ using a Student's *t* test.



SUPPLEMENTARY FIGURE 2.1. Th17 cells are enriched in the intestinal LP and not the SLO. (A) Representative FACS plots pre-gated on TCRβ+ CD4+ cells and showing the proportion of Th17 cells in the Spl, pLN, mLN, SI LP, and LI LP of JAX B6 mice gavaged with cecal contents containing SFB. (B) Comparison of Th17 cell frequencies in the intestinal LP relative to the mLN and other SLO. Data are representative of at least four independent experiments with ten mice. Error bars represent SEM. *, $p \leq 0.05$ using a Student's *t* test.

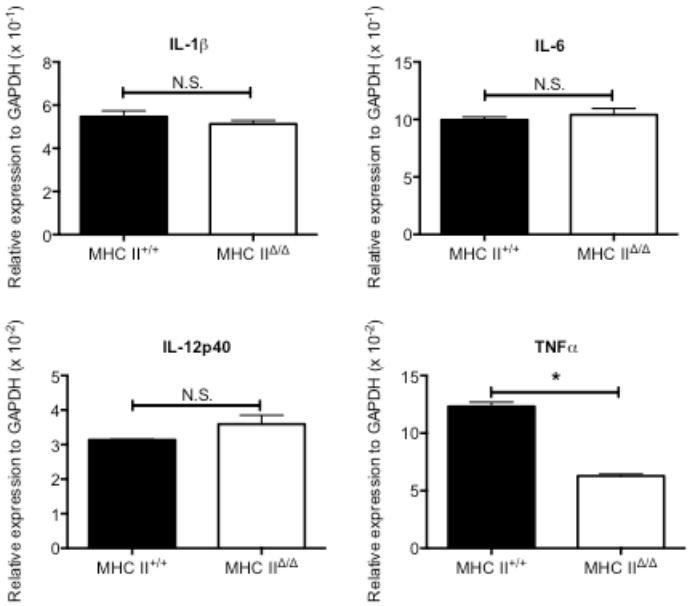


SUPPLEMENTARY FIGURE 2.2. Effects of MHC II deficiency on CD4+ T cells in the mLN and SI LP. (A) Representative FACS plots of TCRβ+CD4+ cells in the mLN and SI LP of MHC $II^{+/+}$ and MHC $II^{\Delta/\Delta}$ mice. (B) Comparison of CD4+ T cell frequencies in mLN (B) and LPL (C) between MHC $II^{+/+}$ and MHC $II^{\Delta/\Delta}$ mice. Data are representative of two independent experiments with four mice per group. Error bars represent SEM. *, $p \leq 0.05$ using a Student's *t* test.



SUPPLEMENTARY FIGURE 2.3. Effects of MHC II deficiency on CD11c+ cells on intestinal DCs. (A) Representative FACS plot for $I\text{-}A^b$ expression on DCs in the small intestinal LP of MHC $II^{\Delta DC}$ (solid black line) and MHC^{FF} (shaded, gray

histogram) mice. (B) Frequency of CD103+CD11c+ cells in the SI LP amongst CD45+ cells are similar between MHC II^{FF} and MHC $II^{\Delta DC}$. Data are representative of two independent experiments with two mice per group for each experiment. Error bars represent SEM. Not statistically significant (N.S.), $p > 0.05$ using a Student's *t* test.



SUPPLEMENTARY FIGURE 2.4. Expression of pro-inflammatory cytokines by intestinal DCs. JAX MHC $II^{+/+}$ and JAX MHC $II^{\Delta/\Delta}$ mice were gavaged SFB-containing cecal contents and DCs were enriched from the small intestine lamina propria on day 10 post-gavage. Following mRNA isolation, cDNA was generated and expression of specific pro-inflammatory cytokines was assessed by qRT-PCR. Data are representative of two independent experiments with four mice per

group. Error bars represent SEM. *, $p \leq 0.05$; not statistically significant (N.S.), $p > 0.05$ using a Student's *t* test.

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Footnotes

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[3]Abbreviations used in this article: JAX, Jackson Laboratory; LI, large intestine; LP, lamina propria; LPL, lamina propria lymphocytes; LT, lymphotoxin; mLN, mesenteric lymph nodes; OVA, ovalbumin; pLN, peripheral lymph nodes; PP, Peyer's patches; SFB, segmented filamentous bacteria; SFB^+ CC, SFB-containing cecal contents; SI, small intestine; Spl, spleen.

Chapter 3: Intestinal Th17 cell differentiation in response to SFB-containing microbiota is regulated by IL-1/IL-1R1 axis and eosinophils

Abstract

A subset of CD4+ T cells that are enriched in mucosal surfaces and mediate host defense to extracellular pathogens are the T helper (Th) 17 cells. Characterized by the expression of the master regulatory transcription factor, RORγt, Th17 cells secrete cytokines of the IL-17 family, namely IL-17A and IL-17F, to mobilize neutrophilic infiltration of tissue and to stimulate anti-microbial responses by epithelial cells. The association of dysregulated Th17 cell responses with various autoimmune diseases and inflammatory conditions indicate that understanding the development and function of these cells may have important clinical implications. Recent studies investigating Th17 cell responses to the gut microbiota have identified segmented filamentous bacteria (SFB) to be a species of commensal bacteria that potently induces intestinal Th17 cells. However, the cytokines and immune cells that govern this process have yet to be fully elucidated. Here, we report that intestinal Th17 cell responses to SFB-containing microbiota were dependent on IL-1/IL-1R1 axis and regulated by lamina propria (LP) eosinophils. Intestinal eosinophils constitutively secreted the IL-1 receptor antagonist (IL-1RA), and depletion of these cells reduced IL-1RA in the small intestine and enhanced Th17 cell responses to SFB-containing microbiota. Together, these findings indicate that colonization by SFB-containing microbiota promotes the development of intestinal Th17 cells through

IL-1 signaling and eosinophils may be important in regulating this process through the production of IL-1RA.

Introduction

Th17 cells represent a unique subset of CD4+ T cells that promotes innate and adaptive immune responses for host defense (6). Enriched in mucosal tissue under steady-state, Th17 cells function as sentinels at barrier surfaces that can promote neutrophilic tissue infiltration through the secretion of the IL-17 cytokine family members IL-17A and IL-17F (21). These pro-inflammatory cytokines can activate immune and non-immune cells to produce factors such as granulocyte colony stimulating factor (G-CSF) and IL-8 to stimulate neutrophil development and recruitment. Additionally, Th17 cells can also express granulocyte macrophage colony stimulating factor (GM-CSF) and IL-22 to augment bone marrow neutrophil and monocyte production and induce anti-microbial responses by the epithelium, respectively. As Th17 cells have a proclivity to drive tissue inflammation, dysregulated Th17 cell responses are implicated in the pathogenesis of various autoimmune diseases and inflammatory conditions (17). Consequently, understanding the factors that regulate Th17 cell development and function may hold important clinical implications.

Seminal research investigating the development of Th17 cells in vitro and in vivo have highlighted the importance of various cytokines and transcription factors enabling the phenotype and function of Th17 cells. Concurrent with T cell receptor (TCR) signaling, key cytokines such as TGF-β1, IL-6 (41-43), TGF-β3 (49), and IL-1β (46) are important for activating the transcriptional networks associated with STAT3 (44), IRF4, BATF, and RORγt that drive Th17 cell differentiation (56, 57). In vitro Th17 cell differentiation studies coupled with

animal studies have begun to uncover the requirements of cytokines important for Th17 cell development and homeostasis in vivo.

At the interface with the gut microbiota, the intestinal lamina propria (LP) is constitutively enriched with Th17 cells in the steady-state. The development of Th17 cells is dependent on the presence of commensal bacteria since mice treated with broad-spectrum antibiotics from birth or those in germ-free conditions are deficient in Th17 cells (60). In particular, segmented filamentous bacteria (SFB) are Gram-positive, spore-forming commensal bacteria that adhere to intestinal epithelial cells (62), and colonization of mice by SFB promotes intestinal Th17 cell development (29, 61). In fact, colonization by SFB-containing microbiota possesses adjuvant-like properties that stimulates the maturation of the adaptive immune system in the intestine and is implicated in establishing a cytokine milieu that drives Th17 cell differentiation in situ upon antigenic stimulation. However, the SFB-induced cytokine signals that promote Th17 cell differentiation have not been well characterized.

Eosinophils are a population of granulocytes that are abundant in the small intestine and contribute to inflammation, tissue remodeling, and wound healing (184). The association of eosinophils and their secreted products with a number of inflammatory gastrointestinal disorders has implicated these cells to be pro-inflammatory and pathogenic. However, the functional characterization of eosinophils in the intestine has begun only recently following advancements in being able to identify these cells phenotypically (124, 185-188). Indeed, eosinophils express an array of pattern recognition receptors and cytokines, such

as TGF-β1, IL-6, and IL-1β, that can modulate the intestinal immune system (189-191). In fact, mice deficient in eosinophils demonstrate impairments in the generation and maintenance of IgA plasma cells and perturbations in CD103+ DCs and T cells—especially the Foxp3+ regulatory $T_{(reg)}$ cell pool—in the intestine (192). As eosinophils are capable of sensing microbial signals and in turn secrete cytokines that may regulate CD4+ T cell responses, these cells may play an important role in regulating specific microbiota-driven Th17 cell development.

In the current study, we investigated the requirement for the IL-1/IL-1R1 axis and eosinophils in mediating intestinal Th17 cell development following colonization by SFB-containing microbiota. Utilizing a previously established specific microbiota transfer system, we demonstrate that neutralization of IL-1β or blockade of IL-1R1 abrogated intestinal Th17 cells responses. Additionally, eosinophils FACS-sorted from the small intestine constitutively produced IL-1 receptor antagonist (IL-1RA), and the loss of eosinophils yielded a reduction of IL-1RA in the small intestine and enhanced Th17 cell responses to SFB-containing microbiota. These findings demonstrate that IL-1/IL-1R1 axis is required for intestinal Th17 cell development in response to SFB-containing microbiota and eosinophils may modulate this IL-1-dependent process via the production of IL-1RA.

Materials and Methods

Mice

Age- and sex-matched C57BL/6 (B6) mice were purchased from The Jackson Laboratory (JAX) to specifically control the SFB status of recipient mice in our SFB-containing microbiota transfer studies. All SFB-containing microbiota transfer studies were initiated within 2 weeks of arrival from JAX in order to avoid unintentional colonization by SFB in our animal facility. Importantly, immediately prior to the introduction of SFB-containing microbiota, mice were verified to be void of SFB (as determined by qPCR detection for SFB DNA in cecal contents and fecal pellets). B6 mice purchased from Taconic were utilized as donors of homogenized cecal contents for SFB-containing microbiota transfer experiments. Mice were maintained under specific pathogen-free conditions and animal protocols were reviewed and approved by the Institute Animal Care and Use Committee of Georgia State University.

Antibodies and reagents

The following antibodies were purchased from eBioscience: IFNγ (XMG1.2), IL-17A (eBio17B7), CD25 (PC61.5), CD3ε (eBio500A2), and RORγ(t)-PE (B2D). Antibodies purchased from BD Biosciences were: TCRβ (H57-597), IL-17A (TC11-18H10), and CD4 (RM4-5). Dead cells were identified using the fixable Aqua dead cell staining kit (Invitrogen). Isolation of LP cells and flow cytometry

was performed as previously described (126). Neutralizing antibodies for anti-mouse IL-1β (B122), anti-mouse IL-1R1 (BE0256) and isotype control (Hamster IgG) were purchased from BioXCell. Anti-mouse Siglec-F (238047) and isotype control (rat IgG) were purchased from R&D SYSTEMS.

Specific microbiota transfer studies

The cecal contents from Taconic B6 mice were resuspended in 5 ml of sterile PBS, filtered through a 100 μm cell strainer, and 150 ul of the homogenate was gavaged twice into each mouse with 3 hr between each gavage. The homogenate was verified to contain SFB via qPCR (29). Recipient mice were utilized within two weeks of arrival from the JAX and verified to be void of SFB prior to gavage and colonized post-gavage based on qPCR analysis of fresh fecal pellets. Mice were euthanized and organs were harvested to assess Th17 cell differentiation at 1 week post-gavage. To examine the role of IL-1/IL-1R1 axis for intestinal Th17 cell differentiation induced by SFB-containing microbiota, 200 μg of neutralizing antibodies against mouse anti-IL-1β or anti-IL-1R1 or isotype control were injected intraperitoneally (IP) on days -2, 0, 2, 4, and 6. Similarly to investigate the role of eosinophils, 20 μg of anti-mouse Siglec-F or isotype control was injected IP on days -2, 0, 2, 4, and 6.

In vitro Th17 cell differentiation studies

Lamina propria eosinophils (defined as SSC^{hi} CD45+I-A^b-CD11b+Siglec-F+ cells), macrophages (defined as CD45+I-A^b+CD11b+F4/80+ cells), and DCs (defined as: CD45+I-A^b+CD11c+CD103+ cells) were FACS-sorted and co-cultured with naive CD4+CD25- T cells pooled from the spleen and lymph nodes in Th17 cell polarizing conditions with TGF-β1 (1 ng/ml) and IL-6 (50 ng/ml) for 90 hours. CD4+ T cell and DCs were co-cultured at 5:1 ratio with or without eosinophils at a 1:1 ratio with CD4+ T cells. CD4+ T cell and macrophages were co-cultured at 10:1 ratio with or without eosinophils at a 1:1 ratio with CD4+ T cells.

Statistics

Statistical analyses were performed with Prism software (Graph-Pad Software) using the Student's *t* test. Error bars represent SEM as indicated and *p* values equal to or less than 0.05 were considered statistically significant.

Results

Intestinal Th17 cell differentiation induced by SFB-containing microbiota is dependent on the IL-1/IL-1R1 axis

The role of IL-1 signaling in the development of intestinal Th17 cells has remained elusive. Thus, we further investigated the role of IL-1/IL-1R1 axis in intestinal Th17 cell development in response to SFB-containing microbiota. JAX B6 mice, initially void of SFB and with a paucity of intestinal Th17 cells, were gavaged SFB-containing cecal contents (SFB^+ CC) on day 0 and injected with neutralizing antibodies to IL-1β, IL-1R1, or isotype control on days -2, 0, 2, 4, and 6. Assessment of intestinal Th17 cell induction on day 7 post-gavage showed significantly reduced frequencies in mice treated with anti-IL-1β (13 ± 0.67%) and anti-IL-1R1 (6.4 ± 2.09%) antibodies relative to isotype control (28 ± 2.49%, respectively; Fig. 3.1A, 3.1B). Furthermore, reductions in absolute Th17 cell numbers were observed following IL-1β and IL-1R1 blockade relative to isotype controls (1345 ± 159 cells, 1747 ± 487 cells, and 2828 ± 62 cells, respectively). These data suggest that the IL-1/IL-1R1 axis is important for intestinal Th17 cell differentiation driven by SFB-containing microbiota.

Eosinophils inhibit Th17 cell differentiation mediated by DCs and macrophages in vitro

In seeking to define immune cell populations that may regulate this IL-1 dependent intestinal Th17 cell development, we focused our attention on

eosinophils, which are enriched in the intestinal lamina propria and secrete various cytokines—including IL-1—capable of regulating CD4+ T cell responses. Accordingly, a recent study indicated that the loss of eosinophils was associated with a decrease in intestinal CD103+ T cells although the specific effector subsets were not characterized (192). As the role of eosinophils in regulating intestinal Th17 cell differentiation has not been explored, we began by assessing the regional distribution of eosinophils along the small intestine. Eosinophils (defined as: SSC^{hi} CD45+I-A^{b}-CD11b+Siglec-F+ cells) were most abundant in the duodenum but decreased in the jejunum and ileum based on cell frequencies (Fig. 3.2A, 3.2B) and numbers (Fig. 3.2C). Intriguingly, the regional distribution of eosinophils resembled that of Th17 cells (Fig. 3.2) suggesting an interaction between these two immune cell populations.

Next, the role of eosinophils in regulating Th17 cell differentiation was assessed in vitro. In utilizing intestinal LP DCs or macrophages as APCs, naïve CD4+ T cells were co-cultured in Th17 cell conditions with or without eosinophils. The frequency of Th17 cells was 40% when naïve CD4+ T cells were cultured with LP DCs, which is expected since LP DCs are potent inducers of Th17 cells (Fig.3.2D). However, Th17 cell differentiation was blunted to 14% in the presence of eosinophils (Fig. 3.2D). Furthermore co-culture with LP macrophages induced IL-17A production in 16% of CD4+ T cells but this frequency decreased to 4.7% with the addition of eosinophils to the culture (Fig. 3.2E). Taken together, these data indicate that eosinophils may serve to abrogate Th17 cell differentiation in the intestine.

Depletion of eosinophils augments intestinal Th17 cell differentiation in response to SFB containing microbiota

Since eosinophils inhibited Th17 cell differentiation in vitro (Fig. 3.2D, 3.2E), the role of eosinophils in regulating Th17 cell responses to SFB-containing microbiota was subsequently investigated in vivo. JAX B6 were colonized by SFB-containing microbiota on day 0 and injected with depleting anti-Siglec-F antibody on days -2, 0, 2, 4, and 6. On day 7 post-gavage, a significant depletion of eosinophils was verified for both frequency (Fig. 3.3A, 3.3B) and cell number (Fig. 3.3C). Furthermore, assessment of intestinal Th17 cell induction in response to SFB-containing microbiota demonstrated a significant increase in frequency (2.87 ± 0.24%, 23.00 ± 6.66%, 36.71 ± 2.88% for control, SFB^+ CC, and SFB^+ CC with anti-Siglec-F, respectively; Fig. 3.3D, 3.3E). Correspondingly, the absolute number of Th17 cells was also increased for mice treated with anti-Siglec-F and SFB^+ CC (15790 ± 1559 cells) relative to SFB^+ CC alone (5823 ± 727.5 cells) and control mice (3194 ± 1664 cells) that did not receive SFB^+ CC nor anti-Siglec-F (Fig. 3.3F). Taken together, depletion of eosinophils robustly augmented intestinal Th17 cell responses to SFB-containing microbiota indicating that eosinophils may be important for regulating microbiota-driven Th17 cell responses.

Intestinal eosinophils secrete IL-1 receptor antagonist

To begin exploring specific cytokine(s) that intestinal eosinophils may secrete to regulate Th17 cell responses, FACS-sorted eosinophils were stimulated with the three TLR ligands: PAM3CSK4, flagellin, and CpG. Thereafter, a PCR cytokine array detected IL-1 receptor antagonist (IL-1RA) to be the most constitutively expressed cytokine by intestinal eosinophils (data not shown). Indeed, FACS-sorted intestinal eosinophils were verified to be a potent source of IL-1RA relative to levels observed in the duodenum of *Il1rn*$^{+/+}$ mice while IL-1RA was undetectable in *Il1rn*$^{-/-}$ mice (Fig. 3.4A). The production of IL-1RA along the small intestine resembled the distribution of eosinophils with IL-1RA protein being highest in the duodenum and decreasing in the jejunum and ileum (Fig. 3.2A, 3.4B). Furthermore, depletion of eosinophils with anti-Siglec-F antibody reduced tissue IL-1RA from the small intestine similar to background levels observed for *Il1rn*$^{-/-}$ mice (Fig. 3.4C). These data demonstrate that intestinal eosinophils are a potent source IL-1RA and may regulate SFB-driven Th17 cell responses by blunting IL-1 signaling.

Dicussion

Here we demonstrate a requirement for the IL-1/IL-1R1 axis in intestinal Th17 cell development driven by SFB-containing microbiota and a regulatory role for eosinophils. Blockade of IL-1β and IL-1R1 with neutralizing antibodies blunted intestinal Th17 cell development in response to SFB-containing microbiota. Furthermore, eosinophils were demonstrated to be a robust source of IL-1RA

and inhibited Th17 cell differentiation in vitro while depletion of eosinophils in vivo augmented intestinal Th17 cell responses following SFB colonization.

Presently, numerous questions remain as to how commensal bacteria, such as SFB, can modulate the development and function of intestinal Th17 cells. In the context of SFB-driven intestinal Th17 cell responses, serum amyloid A and TGF-β1 have been shown to be required while IL-21, IL-23, Ripk, MyD88 and Trif signaling were dispensable (29, 168). Interestingly, a recent report by Shaw et al., demonstrated a requirement for IL-1β in microbiota-driven Th17 cell development although the specific role of SFB was not investigated (193). These seemingly disparate observations may be explained in part by compensatory mechanisms to facilitate intestinal Th17 cell responses. Indeed, several different regulatory cytokines, including IL-1β, IL-6, TGF-β1, and TGF-β3, are capable of promoting Th17 cell differentiation (41-43, 46, 49) and consequently, the absence of a specific cytokine may enable Th17 differentiation through other signaling pathways in vivo. The present study suggests that acute intestinal Th17 cell responses require IL-1β but additional studies are warranted to elucidate the specific cytokine milieu induced by SFB colonization and whether compensatory cytokine signaling can occur.

Recent studies have started to elucidate the immune cells mediating SFB-driven intestinal Th17 cell differentiation. Our lab and others have demonstrated MHC class II expression on LP DCs is required for promoting SFB-driven intestinal Th17 cell responses, indicating that LP DCs provide antigenic stimulation to CD4+ T cells (194, 195). Additionally, macrophages are a potent

source of IL-1β in the intestine (193, 196) and may be providing IL-1 signals required for Th17 cell development in response to SFB. The production of IL-1β by macrophages is dependent on both the microbiota and MyD88 signaling as macrophages from GF or MyD88-deficient mice showed blunted IL-1β expression (193). Thus both LP DCs and macrophages may function together to maintain intestinal Th17 cell homeostasis such that antigenic stimulation from DCs induces CD4+ T cells to upregulate IL-1R1 and colonization by SFB may stimulate TLRs on LP macrophages to produce IL-1β and mediate Th17 cell differentiation.

Currently, the role of eosinophils in regulating intestinal CD4+ T cell responses remain to be defined and is a nascent area of research. A recent study examining the contribution of eosinophils to intestinal homeostasis noted a reduction of intestinal Foxp3+ T_{reg} cells in mice lacking eosinophils (192). The authors proposed that eosinophils may contribute to levels of active TGF-β1 in the LP to support Foxp3+ T_{reg} cells. Though not mutually exclusive, our observation that intestinal eosinophils produce high levels of IL-1RA provides an alternative explanation such that IL-1 signaling may be augmented in the absence of eosinophils and this inhibits Foxp3+ T_{reg} cell differentiation. The reduction of IL-1RA in the intestinal tissue following eosinophil depletion also corroborates our observation of enhanced Th17 cell responses to SFB. Since eosinophils are capable of secreting an array of effector cytokines (189), the possibility of these cells regulating Th17 cell development indirectly through other immune cells cannot be excluded and additional mechanistic studies examining

how eosinophils may regulate intestinal CD4+ T cell responses are needed. Nonetheless, our findings highlight the role for the IL-1/IL-1R1 axis and eosinophils in regulating Th17 cell development driven by SFB-containing microbiota. These results help to further elucidate the complex cytokine and cellular networks governing the interaction between the gut microbiota and the intestinal immune system.

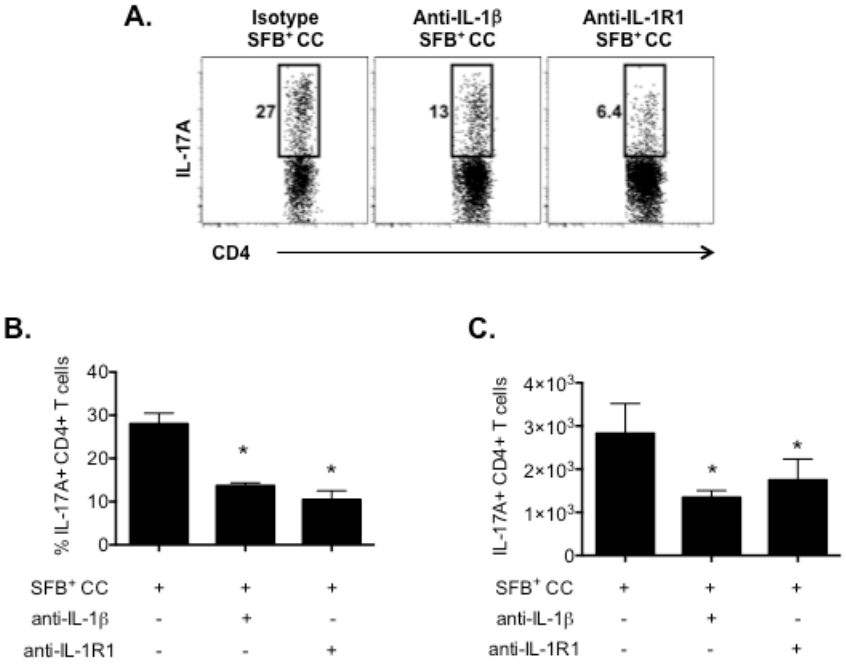

FIGURE 3.1: Intestinal Th17 cell differentiation induced by SFB-containing microbiota is dependent on the IL-1/IL-1R1 axis. JAX B6 mice were colonized by SFB-containing microbiota on day 0 and injected with anti-IL-1β, anti-IL-1R1, or isotype on day -2, 0, 2, 4, and 6. Small intestinal lamina propria Th17 cell induction was assessed on day 7. Cells are pre-gated on TCRβ+CD4+ cells. (A) Representative FACS plots of intestinal Th17 cell frequencies on day 7 post-gavage. Comparison of intestinal Th17 cell frequency (B) and number (C). Error bars represent SEM. *, $p \leq 0.05$.

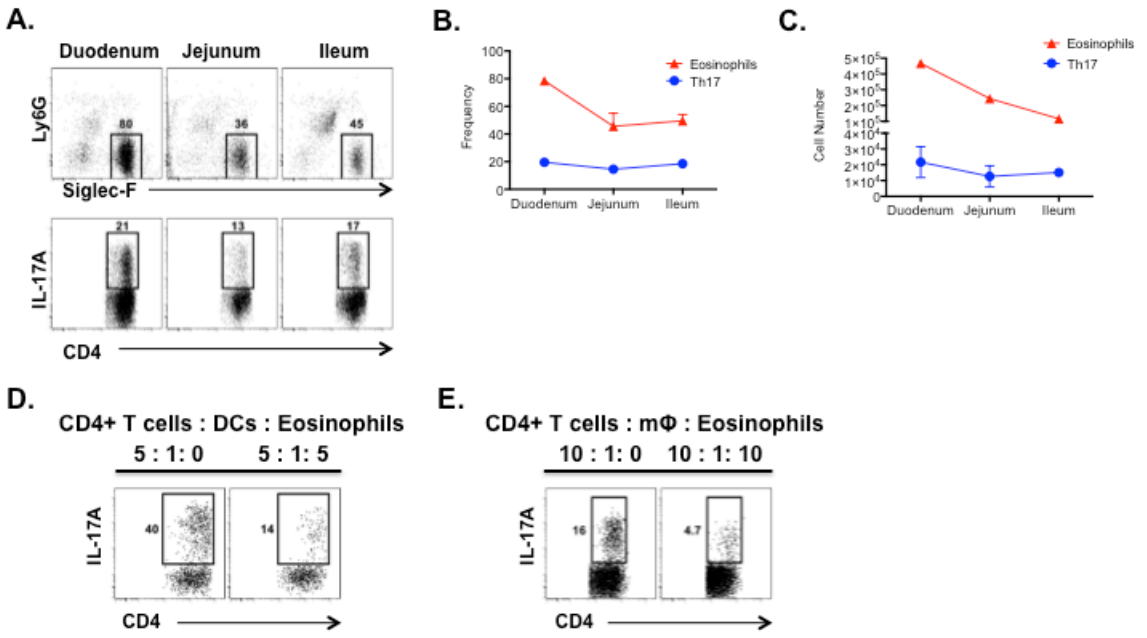

FIGURE 3.2: Intestinal eosinophils inhibit Th17 cell differentiation in vitro. (A) Representative FACS plots of intestinal eosinophils (top panels) Th17 cell (bottom panels) frequencies in the duodenum, jejunum, and ileum. Eosinophils were pre-gated on CD45+I-A^b-CD11b+ cells and Th17 cells were pre-gated on TCRβ+CD4+ cells. Eosinophil and Th17 cell frequency (B) and number (C) along the small intestine. Error bars represent SEM. (D and E) Lamina propria eosinophils, macrophages, and DCs were FACS-sorted and co-cultured with naive CD4+CD25- T cells pooled from the spleen and lymph nodes in Th17 cell polarizing conditions with TGF-β1 and IL-6 for 90 hours. (D) Representative FACS plots of Th17 cell frequency when CD4+ T cell and DCs were co-cultured at 5:1 ratio with or without eosinophils at a 1:1 ratio with CD4+ T cells. E) Representative FACS plots of Th17 cell frequency when CD4+ T cell and

macrophages were co-cultured at 10:1 ratio with or without eosinophils at a 1:1 ratio with CD4+ T cells.

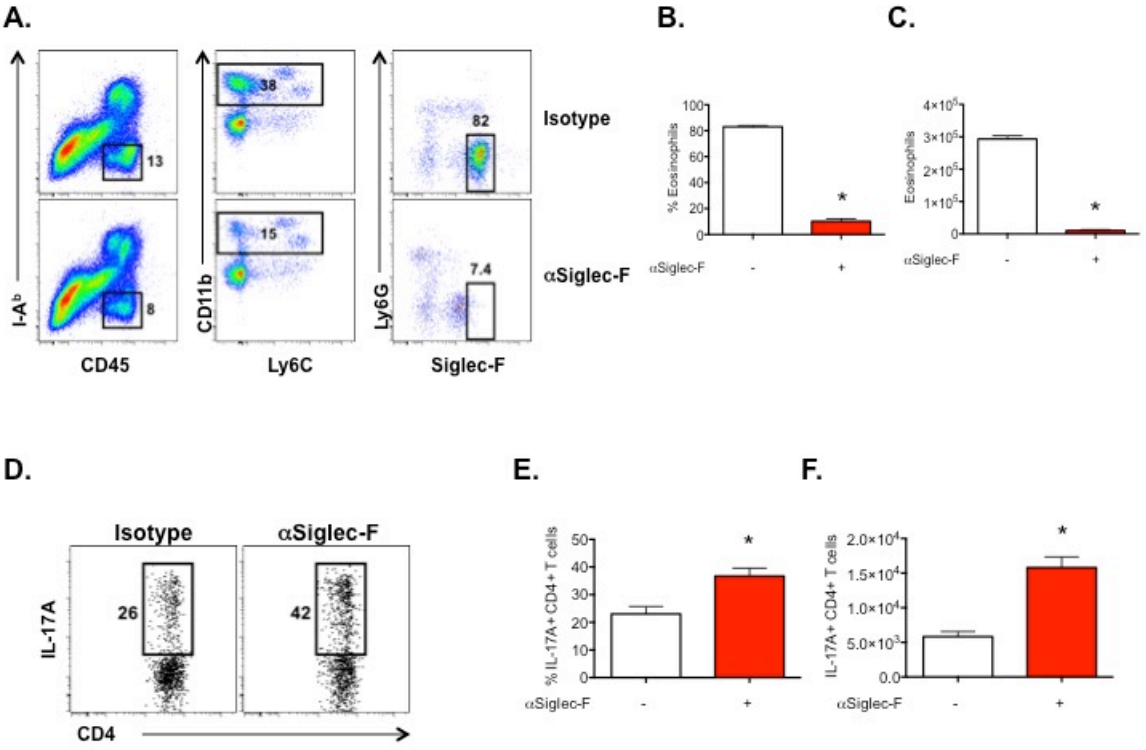

FIGURE 3.3: Depletion of eosinophils augments intestinal Th17 cell differentiation in response to SFB-containing microbiota. JAX B6 mice were colonized by SFB-containing microbiota on day 0 and injected with anti-Siglec-F or isotype antibody on day -2, 0, 2, 4, and 6. Small intestinal lamina propria Th17 cell induction and eosinophil depletion was assessed on day 7. Th17 cells were pre-gated on TCRβ+CD4+ cells and eosinophils were pre-gated on CD45+I-A^b-CD11b+ cells. (A) Representative FACS plots of intestinal eosinophil frequencies on day 7 post-gavage to verify depletion. Frequency (B) and cell number (C) of eosinophils on day 7. (D) Representative FACS plots of Th17 cells on day 7 post-gavage. Frequency (E) and cell number (F) of Th17 cells in the small intestine

lamina propria on day 7 post-gavage. Error bars represent SEM. *, $p \leq 0.05$ using a Student's *t* test.

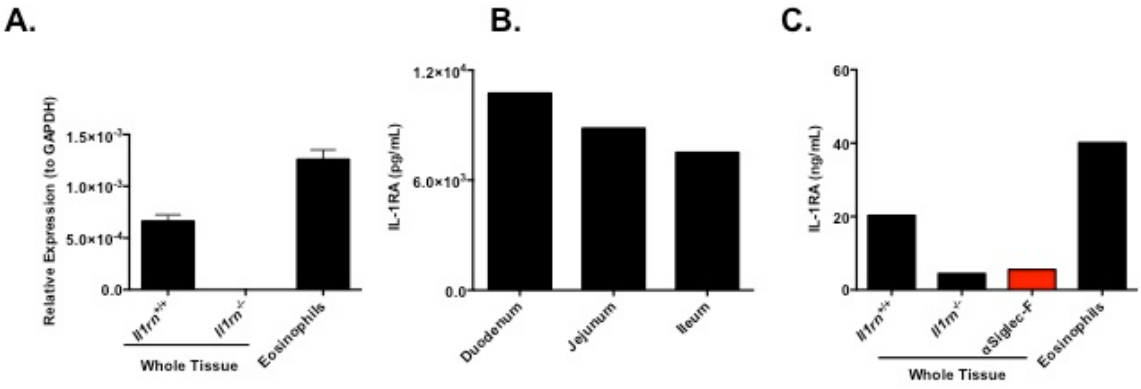

FIGURE 3.4: Intestinal eosinophils secrete IL-1 receptor antagonist (IL-1RA)**.** (A) Tissue expression of IL-1RA by qPCR from the duodenum of *Il1rn*$^{+/+}$ and *Il1rn*$^{-/-}$ and eosinophils FACS-sorted from the small intestine. (B) IL-1RA levels in supernatant of total LP cells cultured for 24 hrs from different regions of small intestine was assessed by ELISA. (C) IL-1RA levels quantitated by ELISA from 24 hr culture supernatant of total LP cells from the duodenum of *Il1rn*$^{+/+}$ and *Il1rn*$^{-/-}$ mice, eosinophils FACS-sorted from the small intestine, and total LP cells from duodenum of mice injected with anti-Siglec-F on days -2, 0, 2, 4, and 6.

Chapter 4: Foxp3+ peripheral T_{reg} cells are more abundant in the colon relative to the small intestine and does not require the GALT and mesenteric lymph nodes.

Abstract

Foxp3+ regulatory T($_{reg}$) cells are an important component of the adaptive immune system that function to suppress inflammation and promote immune tolerance. In the intestine, this population is enriched in the intestinal lamina propria (LP) and helps to establish tolerance to enteric antigens such as those deriving from food and commensal bacteria. As Foxp3+ T_{reg} cells can develop in the thymus (tTreg cells) or in peripheral tissues (pTreg cells) with distinct biological functions, the contribution of these two populations to the intestinal Foxp3+ T_{reg} cell pool is unknown. Previous studies have implicated the mLN and GALT to be the primary site for intestinal Foxp3+ T_{reg} cells development mediated by LP dendritic cells (DCs). In this study we demonstrate in using multi-color flow cytometry the presence of normal Foxp3+ T_{reg} cell frequencies and cell numbers in the intestinal LP of mice lacking all lymphotoxin (LT)-dependent secondary lymphoid organs (SLO) including the mLN and GALT. Despite normal Foxp3+ T_{reg} cell abundance in the intestine, LTα-deficient mice demonstrated defective antigen-specific Foxp3+ pT_{reg} cell induction in the small intestine (SI), but correspondingly had an increase in Foxp3+ T_{reg} cells that expressed Helios, a marker for Foxp3+ tT_{reg} cells. In contrast, Foxp3+ pT_{reg} cell induction in the large intestine (LI) was independent of the mLN and GALT, and naïve CD4+ T cells were enriched in the LI along with the proportion of Helios-

Foxp3+ pT_{reg} cells. Lastly, conventionalization of germ-free (GF) mice induced Foxp3+ T_{reg} cells in the LI but not the SI. Collectively, these results highlight differences in Foxp3+ T_{reg} cell composition and tissue site requirements between the SI and LI LP. The Foxp3+ T_{reg} cell population in the SI is predominantly comprised of Foxp3+ tT_{reg} cells and pT_{reg} cell induction is dependent on the mLN and GALT, while a greater proportion of LI Foxp3+ T_{reg} cells are pT_{reg} cells that are reactive to the gut microbiota and differentiate independent of SLOs.

Introduction

Regulatory T cells represent a heterogeneous population of cells that function to suppress inflammation and promote immune tolerance to self and foreign antigens. A unique subset of T_{reg} cells are CD4+ T cells that express high levels of the master regulatory transcription factor Foxp3 and as a result are endowed with the capacity for immunosuppression (7, 8). Foxp3+ T_{reg} cells regulate the immune system utilizing various mechanisms that include the production of anti-inflammatory cytokines, expression of immunoregulatory cell surface molecules, cytolysis, and cytokine deprivation of effector T cells (86, 87, 99-101). Consequently, Foxp3+ Treg cells functionally inhibit the production of pro-inflammatory cytokines, decrease the expression of co-stimulatory molecules on antigen presenting cells (APCs), and impede T cell proliferation and survival (86-89). Given their vital role in maintaining immune homeostasis, impairments in the development or function of Foxp3+ T_{reg} cells are associated with a breakdown in immune tolerance and the pathogenesis of autoimmune diseases.

The Foxp3+ T_{reg} cell compartment is comprised of thymically-derived Foxp3+ tT_{reg} cells and peripherally-derived Foxp3+ pT_{reg} cells, each distinct in development (65). In the thymus, the input of strong costimulatory signals along with high-affinity interactions between the T cell receptor (TCR) and major histocompatibility complex (MHC) enable CD4+ thymocytes to develop into Foxp3+ tT_{reg} cells through a process called “agonist selection” (66, 67). The resultant signaling events induce unique epigenetic modifications that differentiate Foxp3+ tT_{reg} from pT_{reg} cells and conventional T cells such as CpG

hypomethylation of T_{reg}-associated genes and a distinct methylation pattern of the T_{reg}-specific demethylation region (TDSR) (72, 73). Phenotypically, Foxp3+ tT_{reg} cells may be distinguished from pT_{reg} cells based on their high expression of Helios, the Ikaros transcription factor family member, and neuropilin-1, a cell surface co-receptor (74, 75, 197).

Alternatively, naïve CD4+ T cells that are activated in the appropriate cytokine milieu can express Foxp3 and gain immunoregulatory functions to become Foxp3+ pT_{reg} cells. The microenvironment provided by the intestine is especially adept in generating these cells where cytokines that promote Foxp3+ pT_{reg} cell differentiation like TGF-β1 and retinoic acid (RA) are abundant (78-82). In particular, intestinal DCs and macrophages express retinaldehde dehydrogenases, which are important for the biosynthesis of RA, and relative to APCs from other tissues, intestinal LP DCs and macrophages are potent inducers of Foxp3+ T_{reg} cells in vitro (13-15, 177). Nonetheless, previous studies investigating the site of intestinal antigen presentation and oral tolerance have implicated the mLN to be a site of Foxp3+ pT_{reg} cell differentiation whereupon migratory CD103+ DCs from the intestinal LP provide antigenic stimulation to naïve CD4+ T cells in the mLN (84). Subsequently, a fraction of the activated CD4+ T cells are thought to become gut-tropic Foxp3+ pT_{reg} cells and home to the intestine where they expand in response to IL-10 produced by LP CX3CR1+ macrophages (85).

The intestinal LP is the largest reservoir of Foxp3+ T_{reg} cells where these cells provide a crucial function in promoting tolerance to enteric antigens and yet

the relative contribution of tT_{reg} and pT_{reg} cells that comprise the intestinal Foxp3+ T_{reg} cell pool is unknown. Discerning the proportion of Foxp3+ tT_{reg} and pT_{reg} cells that comprise the intestinal pool may be of biological importance as previous studies have suggested distinct functional niches for Foxp3+ tT_{reg} and pT_{reg} cells in vivo. Accordingly, Foxp3+ tT_{reg} cells are implicated in preventing autoimmunity while Foxp3+ pT_{reg} cells may serve to limit mucosal inflammation (198, 199).

Here, we demonstrate normal Foxp3+ T_{reg} cell frequencies and cell numbers in the intestinal LP of mice void of all lymphotoxin (LT)-dependent secondary lymphoid organs (SLO), including the mLN and GALT. Although the total pool of Foxp3+ T_{reg} cells in the intestine was normal, LTα-deficient demonstrated defective Foxp3+ pT_{reg} cell induction in the OT-II-ovalbumin (OVA) feeding model along with a decreased proportion of Helios-Foxp3+ T_{reg} cells in the small intestine (SI). In contrast, Foxp3+ pT_{reg} cell induction in the large intestine (LI) was independent of the mLN and GALT, and naïve CD4+ T cells were enriched in the LI along with the proportion of Helios-Foxp3+ T_{reg} cells. Furthermore, Foxp3+ T_{reg} cells in the LI demonstrated a microbiota-dependent induction unlike those in the SI upon conventionalization of germ-free (GF) mice. Collectively, these results highlight differences in Foxp3+ T_{reg} cell composition and tissue site requirements between the SI and LI. The Foxp3+ T_{reg} cell population in the SI is predominantly comprised of Foxp3+ tT_{reg} cells and pT_{reg} cell induction is dependent on the mLN and GALT, while a greater proportion of Foxp3+ T_{reg} cells in the LI are pT_{reg} cells that differentiate independent of SLOs in

response to the microbiota.

Materials and Methods

Mice

Age- and sex-matched C57BL/6 (B6), B6.129S2-*Lta*^{tm1Dch}/J (*Lta*$^{-/-}$), SPLx *Lta*$^{-/-}$ and B6.PL-*Thy1*^{a}/CyJ (Thy1.1), and B6.Cg-Tg(TcraTcrb)425Cbn/J (OTII) mice were purchased from The Jackson Laboratory (JAX). *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice are not commercially available and age- and sex-matched littermate controls were provided by R.D. Newberry. These mice were cohoused and the experiments were performed on-site at Washington University-St. Louis. Mice were maintained under specific pathogen-free conditions and animal protocols were reviewed and approved by the Institute Animal Care and Use Committee of Georgia State University.

Antibodies and reagents.

The following antibodies were used from ebioscience unless otherwise specified: Helios-Alexa Fluor 647 (22F6; Biolegend), CD90.1 conjugated to PE-Cy7 or eFluor450 (H1S51), TCRβ conjugated to PE-Cy7 or FITC (H57-597; BD), CD69-FITC (H1.2F3), Vβ5 conjugated to FITC or PE (MR9-4; BD), Foxp3 conjugated to PE or eFluor 450 (FJK16S), CD45RB-PE (C363.16A), CD45.1-PerCP-Cy5.5 (A20), Vα2-APC (B20.1), CD3ε-eFluor 450 (eBio500A2), CD152-APC (UC10-4B9), FR4-PE-Cy7 (eBio12A5), CD73-eFluor 450 (eBioTY/11.8), Vα2-FITC (B20.1; BD), CD25-APC (PC61.5), CD4-PerCP-Cy5.5 (RM4-5; BD). Dead cells

were identified using the fixable Aqua dead cell staining kit (Invitrogen). The following biotin-conjugated antibodies (ebioscience) were used for negative selection in conjunction with anti-biotin and anti-APC microbeads (Miltenyi Biotec): CD8α (53-6.7), Ly-6G (RB6-8C5), F4/80 (BM8), TER-119 (TER-119), CD11b (M1/70), NK1.1 (PK136), CD11c (N418), CD19 (eBio1D3).

Isolation of intestinal LP lymphocytes.

Isolation of LP cells was performed as previously described (126). Briefly, small intestines were removed and carefully cleaned of their mesentery, and Peyer's patches were excised. Small and large intestine were opened longitudinally, washed of fecal contents, cut into pieces 0.5 cm in length, and placed in an orbital shaker at 250 rpm for two sequential 20-minute intervals in HBSS with 5% FBS and 2 mM EDTA at 37°C to remove epithelial cells. After each shake, media containing epithelial cells and debris was discarded. The remaining tissue was minced and incubated in an orbital shaker at 200 rpm and 37°C for 20 minutes in HBSS with 5% FBS, 1.5 mg/ml collagenase IV (Sigma-Aldrich), and 40 U/ml DNase I (Roche). Cell suspensions were collected and passed through a 100-μm strainer and pelleted by centrifugation at 1500 rpm. The pellet was resuspended in 10ml of 45% Percoll (GE Healthcare) and underlaid with 70% Percoll then centrifuged at 2000 rpm for 20 min at 20^{o}C. The interface was harvested and washed.

Flow cytometry.

Isolated LP lymphocytes were re-suspended in PBS containing 5% FBS. Live

cells were identified using an Aqua Dead Cell Staining Kit accordingly to the manufacturer's instructions, and Fc receptors were blocked with the antibody anti-FcγRIII/II (2.4G2) for 15 minutes at 4°C. After incubation, the cells were stained at 4°C for 30 minutes with fluorescence-labeled antibodies. Samples were then washed 2 times in PBS containing 5% FBS and intracellular staining was performed using a Foxp3 fixation/permeabilization kit (eBioscience). Flow cytometric analysis was performed on a LSR II (BD).

In vitro suppression assay

Isolated intestinal LPL were sorted for $CD4^+CD25^+$ cells and co-cultured with splenic DCs and CFSE-labeled $CD4^+CD25^-Thy1.1^+$ responder cells at a suppressor to responder ratio of 2:1. Lymphocytes were stimulated to proliferate with anti-CD3 antibody for 3 days *in vitro*. CFSE dilution was used to measure proliferation, and responders stimulated in the absence of suppressors were used to assess suppression.

In vivo Foxp3+ T_{reg} cell differentiation studies

Naive CD4+CD25- T cells from spleen and peripheral lymph nodes were sorted (>99% purity) from ovalbumin (OVA; 323-339) peptide-specific TCR-transgenic (OTII) Thy1.1 mice. Each WT or LTα KO recipient was adoptively transferred 3 x 10^6 OTII Thy1.1 cells. Twenty-four hours following the transfer of cells, recipients were fed OVA via the drinking water at 20mg/ml for 5 days and switched to

normal drinking water for 6 days. Lymphocytes from the intestinal LP and MLN were isolated and directly stained for Foxp3.

Statistics

Statistical analyses were performed with Prism software (Graph-Pad Software) using the Student's *t* test. Error bars represent SEM as indicated and *p* values equal to or less than 0.05 were considered statistically significant while *p* values greater than 0.05 were considered not statistically significant (N.S.).

Results

Foxp3+ T_{reg} cells are enriched in the intestinal LP and their presence is independent of CCR7

The mLN are lymphoid structures that drain the lymphatics from the intestine and are implicated to be the primary site where LP DCs migrate via a CCR7-dependent manner and present enteric antigens to naïve CD4+ T cells (84, 127). Furthermore, previous studies have reported the mLN to be important for intestinal Foxp3+ T_{reg} cell development and the establishment of oral tolerance to food antigens (85). If the mLN are indeed the primary site of intestinal Foxp3+ T_{reg} development, an enrichment of these cells in the mLN would be expected relative to the intestine and other SLO that lack the specialized APCs and cytokine milieu conducive for Foxp3+ T_{reg} development. Interestingly, the frequencies of Foxp3+ T_{reg} cells were higher in theSI LP and LI LP (30.8 ± 2.00 % and 36.8 ± 1.82%, respectively) relative to the mLN (14.6 ± 0 50%; Fig. 4.1A, 4.1B). Furthermore, the frequency of Foxp3+ T_{reg} cells in the mLN was comparable to the peripheral lymph nodes (10.6 ± 0.46%) and spleen (12.4 ± 0.37%; Fig. 4.1A, 4.1B). These data suggested that the mLN were not the primary site of intestinal Foxp3+ T_{reg} cell development.

As intestinal LP DCs are implicated to carry enteric antigens to the mLN via CCR7 and provide antigenic stimulation to naïve CD4+ T cells for Foxp3+ T_{reg} cell induction, we investigated whether Foxp3+ T_{reg} cell homeostasis was perturbed in *Ccr7*$^{-/-}$ mice. On the contrary, intestinal Foxp3+ T_{reg} cells were more abundant in the absence of CCR7 (Fig. 4.1C, 4.1D), and the absolute cell

numbers in the SI LP and LI LP were elevated in *Ccr7*$^{-/-}$ mice relative to *Ccr7*$^{+/+}$ controls (Fig. 4.1E). Together, these results suggest that CCR7-dependent migration of LP DCs to the mLN was dispensable for intestinal Foxp3+ T_{reg} cell homeostasis.

Intestinal Foxp3+ T_{reg} cell development does not require the mLN, GALT, and other LT-dependent lymphoid structures

Since Foxp3+ T_{reg} cells were enriched in the intestinal LP as opposed to the mLN and the CCR7-dependent migration of LP DCs to the mLN was dispensable, the requirement of the mLN for intestinal Foxp3+ T_{reg} cell homeostasis was examined in lymphotoxin α-deficient (*Lta*$^{-/-}$) mice that are void of mLN, GALT, and other lymphotoxin-dependent lymphoid structures. The frequency of Foxp3+ T_{reg} cells was similar between *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice in the SI LP (30 ± 2.1% versus 27 ± 2.1%, respectively; Fig. 4.2A) and LI LP (38 ± 1.9% versus 40 ± 3.2%, respectively; Fig. 4.2A). Comparable absolute number of Foxp3+ T_{reg} cells was observed in the SI LP and LI LP of *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice as well (Fig. 4.2B). To verify that these were indeed bona fide Foxp3+ T_{reg} cells quantitated in the intestine of *Lta*$^{-/-}$ mice, expression for the following T_{reg} cell-associated markers was assessed: folate receptor 4 (FR4), the ecto-5'-nucleotidase CD73, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and the alpha chain of the IL-2 receptor (CD25). Indeed, intestinal Foxp3+ T_{reg} cells from *Lta*$^{-/-}$ mice expressed these T_{reg} cell-associated markers and at levels indistinguishable from *Lta*$^{+/+}$ mice (Fig. 4.2C). Additionally, LP T_{reg} cells from

Lta$^{+/+}$ and *Lta*$^{-/-}$ mice were similar in terms of functional suppression based on the in vitro suppression assay at a responder-to-suppressor ratio of 2:1 (Fig. 4.2D). To address the possibility that the Spl may be a site for intestinal Foxp3+ T_{reg} cells in the absence of the GALT, mLN and other LT-dependent lymphoid structures, similar experiments were performed in splenectomized (Splx) *Lta*$^{-/-}$ mice as well as, lymphotoxin β receptor deficient (*Ltbr*$^{-/-}$) mice, an additional model of SLO deficiency. Both Splx *Lta*$^{-/-}$ and *Ltbr*$^{-/-}$ mice yielded similar results as observed in *Lta*$^{-/-}$ mice (Fig. 4.3), and these findings confirm that intestinal Foxp3+ T_{reg} cell homeostasis does not require the Spl, mLN or other LT-dependent lymphoid structures and may occur directly in the intestinal LP.

Distinct requirements for mLN, GALT, and other LT-dependent lymphoid structures in mediating Foxp3+ T_{reg} cell differentiation in the SI LP versus the LI LP

The normal intestinal Foxp3+ T_{reg} cell homeostasis observed in the absence of the mLN, GALT, and other LT-dependent lymphoid structures directed us to examine Foxp3+ T_{reg} cell differentiation in vivo utilizing the OTII-OVA feeding model (15, 83, 85). Naïve CD4+CD25- CD4+ T cells were FACS-sorted from OTII Thy1.1 mice (>99% purity) and adoptively transferred into recipient *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice on day 0 and given OVA drinking water (20 mg/ml) for 5 days followed by normal drinking water for 6 days. Intestinal Foxp3+ T_{reg} cell differentiation was assessed amongst transferred Thy1.1+CD4+ T cells expressing the transgenic TCR Vβ5+Vα2+ in the SI LP, LI LP, and mLN on day

11 post-transfer. Foxp3+ T_{reg} cell differentiation was observed to be robust in the SI LP (67.1 ± 5.6%) and LI LP (85.7 ± 4.6%) while poor in the mLN (11.1 ± 2.2%) of *Lta*$^{+/+}$ mice (Fig. 4.4A, 4.4B). Interestingly, Foxp3+ T_{reg} cell differentiation in *Lta*$^{-/-}$ mice was impaired in the SI LP (24.6 ± 5.6%) but similar to *Lta*$^{+/+}$ mice in the LI LP (82.0 ± 3.3%; Fig. 4.4A, 4.4B). The absolute cell number corresponded with the frequencies being highest in the SI LP of *Lta*$^{+/+}$ mice, blunted in the SI LP of *Lta*$^{-/-}$ mice, and similar in the LI LP (Fig. 4.4C). These data suggest distinct requirements for the mLN, GALT, and other LT-dependent lymphoid organs between the SI LP and LI LP for Foxp3+ T_{reg} cell differentiation whereby Foxp3+ T_{reg} cell differentiation in the SI LP was dependent on these structures.

Foxp3+ tT_{reg} cells constitute majority of the Foxp3+ T_{reg} cell reservoir in the intestine with more in the SI LP than the LI LP.

Since Foxp3+ T_{reg} cell differentiation in the SI LP was impaired in *Lta*$^{-/-}$ mice yet the total frequency and cell number was comparable to *Lta*$^{+/+}$ mice, the composition of Foxp3+ tT_{reg} and pT_{reg} cells in the intestine was examined considering the hypothesis that a compensatory increase in Foxp3+ tT_{reg} cells may occur in *Lta*$^{-/-}$ mice. Using the previously established Helios expression as a marker for Foxp3+ tT_{reg} cells, majority of the Foxp3+ T_{reg} cells in the thymus expressed Helios in *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice as expected (~80% of Foxp3+ T_{reg} cells; Fig. 4.5). Intriguingly, the abundance of Foxp3+Helios+ tT_{reg} cells in the SI LP of *Lta*$^{-/-}$ mice was increased relative to *Lta*$^{+/+}$ mice (84.4 ± 2.2% versus 71.0 ± 1.5%, respectively; Fig. 4.5) but similar in the LI LP (53.8 ± 5.5% versus 64.7 ±

2.4%, respectively; Fig. 4.5). The proportion of Foxp3+Helios+ tT_{reg} cells was also significantly greater in the SI LP relative to the LI LP ($P < 0.0001$). Together, these results suggest that Foxp3+ tT_{reg} cells constitute majority of the Foxp3+ T_{reg} pool in the intestine with more being in the SI LP relative to the LI LP, and Foxp3+ tT_{reg} cells may be increased in *Lta*$^{-/-}$ mice to compensate for the poor Foxp3+ pT_{reg} cell induction.

Foxp3+ pT_{reg} cells reactive to the gut microbiota are enriched in the LI LP

The differences in the abundance of Foxp3+Helios+ tT_{reg} cells between the SI LP and LI LP indicated the corollary that Foxp3+Helios- pT_{reg} cells are enriched in LI LP. Subsequently, the TCR reactivity of the Foxp3+ T_{reg} cells was assessed between the SI LP and LI LP since the differences in TCR reactivity may correspond with the differential representation of Foxp3+ pT_{reg} cells between these two tissue sites. Thus, Foxp3+ T_{reg} cell induction was analyzed in GF and GF litter-mates that were conventionalized for two weeks. Interestingly, conventionalized mice showed more Foxp3+ T_{reg} cells in the LI LP relative to GF mice (~49% versus ~24%, respectively) but remained comparable in the SI LP (Fig. 4.6A). As data from *Lta*$^{-/-}$ mice indicated that Foxp3+ pT_{reg} cell induction may occur in situ (Fig. 4.4), the abundance of naïve CD4+ T cells in the SI LP and LI LP was compared between GF and conventionalized mice. Utilizing CD45RB^{hi} expression to characterize naïve CD4+ T cells, majority of the CD4+ T cells were CD45RB^{hi} in the Spl of GF and conventionalized mice (79% and 76%, respectively; Fig. 4.6B) as expected. However, CD45RB^{hi} CD4+ T cells were

higher in the LI LP relative to SI LP of GF mice (61% versus 26%, respectively; Fig. 4.6B), and conventionalization was associated with a decrease in CD45RB^{hi} CD4+ T cells of only the LI LP (29%) but remained similar to GF mice in the SI LP (25% versus 26%, respectively; Fig. 4.6B). These data suggest that naïve CD4+ T cells reactive to the gut microbiota are more abundant in the LI LP than the SI LP and may differentiate into Foxp3+ pT_{reg} cells upon conventionalization.

Discussion

In study, we demonstrate that maintenance of the intestinal Foxp3+ T_{reg} cell pool does not require CCR7 and the mLN, GALT, and other LT-dependent SLO. Additionally, we show distinct requirements for LT-dependent SLO and the composition of Foxp3+ T_{reg} cell pool between the SI LP and the LI LP. Foxp3+ pT_{reg} cell differentiation in response to the food antigen OVA required the mLN, GALT, and other LT-dependent SLO in the SI LP but not in the LI LP as noted in *Lta*$^{-/-}$ mice. Nonetheless, majority of the Foxp3+ T_{reg} cells in the intestine expressed Helios, and thus may be characterized as tT_{reg} cells. A greater abundance of Foxp3+Helios+ tT_{reg} cells were in the SI LP while more Foxp3+Helios- pT_{reg} cells were in the LI LP. Corresponding with a higher proportion of Foxp3+Helios- pT_{reg} cells in the LI LP, Foxp3+ T_{reg} cells could be induced in the LI LP following conventionalization of GF mice while the Foxp3+ T_{reg} cells in the SI LP remained unchanged. In parallel, naïve CD4+ T cells are more abundant in the LI LP relative to the SI LP of GF mice and decreased robustly upon conventionalization.

The intestinal LP maintains the largest reservoir of Foxp3+ T_{reg} cells in the steady-state, however the ontogeny of these cells has not been directly characterized. The maintenance of intestinal Foxp3+ T_{reg} cells is thought to be dependent on the mLN based on previous studies demonstrating that naïve CD4+ T cells traffic primarily through SLO, the mLN drains the lymphatics from the intestinal LP and GALT, DCs from the intestinal LP and Peyer's patch can migrate to the mLN and provide antigenic stimulation and imprinting to naïve CD4+ T cells to upregulate gut-homing molecules CCR9 and $\alpha4\beta7$ (124, 145, 168, 169), and feeding of a soluble antigen induces Foxp3+ T_{reg} cells in the intestine (15, 83, 170). Although we show that the maintenance of intestinal Foxp3+ T_{reg} cells is independent of CCR7, the mLN, GALT, and other LT-dependent SLO, these previous reports are not inconsistent with the data presented in this study. Instead, the possibility is highlighted for complementary developmental pathways that comprise the Foxp3+ T_{reg} cell pool in the intestine.

In addition to the generation of Foxp3+ pT_{reg} cells in the mLN, Foxp3+ tT_{reg} cells may migrate directly to the intestinal LP, which is consistent with the abundance of Foxp3+Helios+ tT_{reg} cells in the SI and LI LP as well as the normal frequency and number of Foxp3+ T_{reg} cells in *Lta*$^{-/-}$ mice. In CNS1 KO mice with that are impaired in Foxp3+ pT_{reg} cell generation, an appreciable population of Foxp3+ T_{reg} cell remained in both the SI and LI LP further supportive of Foxp3+ tT_{reg} cells being capable of trafficking directly to the intestine (199). Although the gut-homing molecules CCR9 and $\alpha4\beta7$ have been shown be induced on CD4+ T cells via retinoic acid and mediate trafficking of Foxp3+ pT_{reg} cells to the SI LP

(79, 80), the role of these gut-homing molecules or if other specific chemokines and chemokine receptors regulate Foxp3+ tT_{reg} cell homing to the SI LP is unknown. Moreover, recent studies demonstrate an important role for G protein-coupled receptor 15 (GPR15) in mediating CD4+ T cell accumulation, including Foxp3+ T_{reg} cells, to the LI LP (200, 201). Though Foxp3+Helios+ and Foxp3+Helios- T_{reg} cells express GPR15 in the colon, whether this molecule differentially modulates Foxp3+ tT_{reg} and pT_{reg} cell trafficking to the LI LP was not investigated. Further studies dissecting the mechanisms regulating the trafficking of Foxp3+ pT_{reg} and tT_{reg} cells to the intestine is warranted.

Another developmental pathway for intestinal Foxp3+ T_{reg} cell maintenance may be in situ differentiation of naïve CD4+ T cells that migrate directly into the LP. Indeed, the trafficking of naïve CD4+ T cells through non-lymphoid tissue has been previously reported (178), and we have demonstrated that naïve CD4+ T cells can populate the intestinal LP independent of the mLN, GALT, and other LT-dependent SLO (194). In situ Foxp3+ pT_{reg} cell differentiation may be more plausible in the LI LP where Foxp3+Helios- pT_{reg} cells constitute a larger portion of the total Foxp3+ T_{reg} cell pool and Foxp3 induction occurred in the LI LP of *Lta*$^{-/-}$ mice in the absence of the mLN and GALT. This was in contrast to the SI LP where ~70% of the Foxp3+ T_{reg} cells expressed Helios and Foxp3+ pT_{reg} cell induction was impaired in *Lta*$^{-/-}$ mice, which corresponded with a higher (~87%) Foxp3+Helios+ tT_{reg} cell pool. Thus, these data suggest that Foxp3+ T_{reg} cells in the SI LP are predominantly Foxp3+ tT_{reg} cells and pT_{reg} cell differentiation requires the mLN and GALT, while Foxp3+

pT_{reg} cells are more abundant in the LI LP and differentiation is independent of the mLN and GALT.

Lastly, the induction of Foxp3+ T_{reg} cells in the LI LP, as opposed to the SI LP, upon conventionalization of GF mice suggests differences in TCR reactivity of between Foxp3+ T_{reg} cells in the SI and LI LP. Foxp3+ T_{reg} cells in the LI LP seem to be reactive to the gut microbiota, and this is in agreement with a previous study demonstrating that antibiotic treatment modulated Foxp3+Helios- T_{reg} cell population in the colon but not the SI (172). In fact the same group also reported that specific *Clostridia* strains are capable of inducing colonic Foxp3+ T_{reg} cells (104). Indeed, TCR analysis of colonic Foxp3+ T_{reg} cells were noted to be reactive to microbial antigens derived from the commensal bacteria relative to the TCRs of T_{reg} cells from other tissues (69, 202). Thus, these data suggest distinct antigenic reactivity between the Foxp3+ T_{reg} cells in the SI and LI LP with Foxp3+ T_{reg} cells in the LI LP comprised of more pT_{reg} cells and correspondingly being reactive to foreign antigens such as those deriving from food and microbes while Foxp3+ T_{reg} cells in the SI LP are predominantly tT_{reg} cells that respond to self antigens.

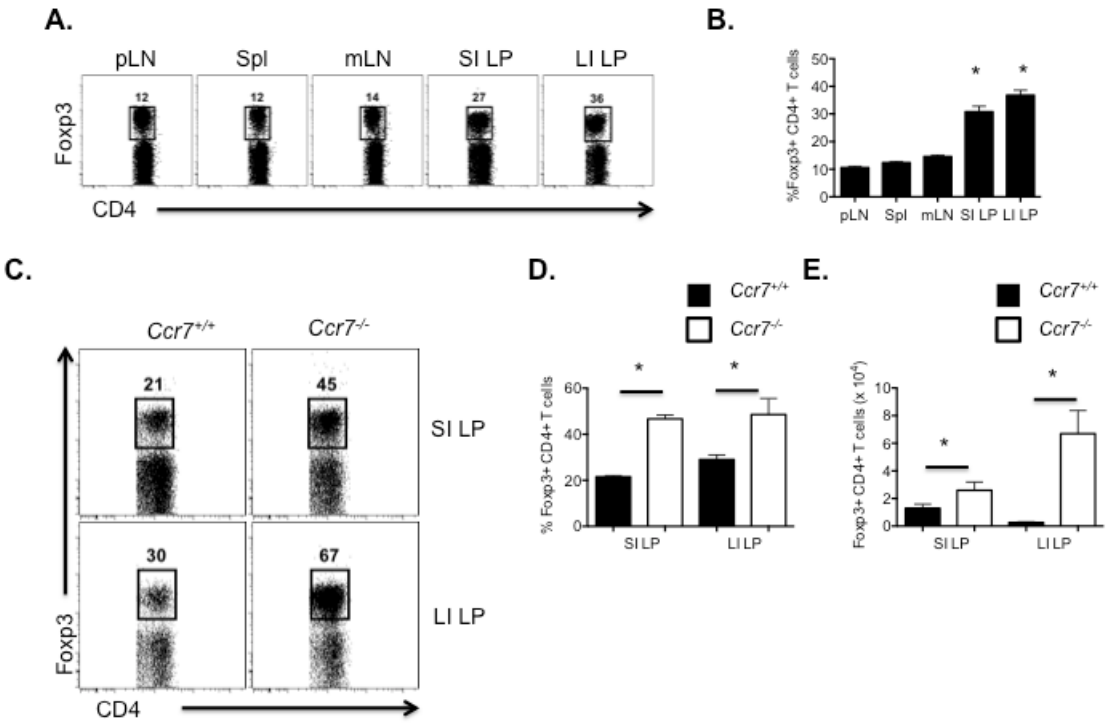

FIGURE 4.1: Foxp3+ T_{reg} cells are enriched in the intestinal LP independent of CCR7. (A) Representative FACS plots pre-gated on TCRβ+ CD4+ cells and showing the proportion of Foxp3+ T_{reg} cells in the Spl, pLN, mLN, SI LP, and LI LP of B6 mice. (B) Comparison of Foxp3+ T_{reg} cell frequencies in the intestinal LP relative to the mLN and other SLO. (C) Representative FACS plots of Foxp3+ T_{reg} cells pre-gated on TCRβ+ CD4+ cells in the SI LP and LI LP of *Ccr7*$^{+/+}$ and *Ccr7*$^{-/-}$ mice. Comparison of Foxp3+ T_{reg} cell frequency (D) and cell number (E) between *Ccr7*$^{+/+}$ and *Ccr7*$^{-/-}$ mice. Error bars represent SEM. *, $p \leq 0.05$ using a Student's *t* test.

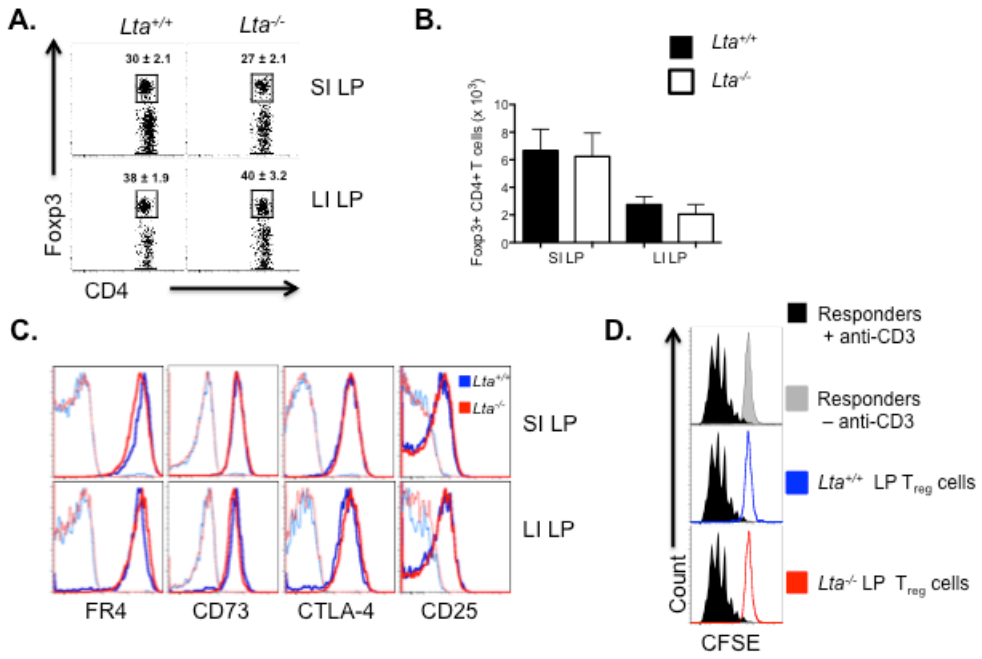

FIGURE 4.2: Foxp3+ T_{reg} cell development is independent of the mLN, GALT, and other LT-dependent lymphoid structures. (A) Representative FACS plots, pre-gated on CD4+ TCRβ+ cells, and frequencies of Foxp3+ T_{reg} cells in the SI and LI LP of *Lta+/+* and *Lta*$^{-/-}$ mice. (B) Absolute cell number of Foxp3+ T_{reg} cells in the SI and LI LP of *Lta+/+* and *Lta*$^{-/-}$ mice. (C) Expression of T_{reg} cell markers Foxp3+ T_{reg} cells in the SI and LI LP of *Lta+/+* and *Lta*$^{-/-}$ mice. Unbolded histograms represent unstained controls. (D) In vitro suppression assay of intestinal LP T_{reg} cells from *Lta+/+* and *Lta*$^{-/-}$ mice. Isolated intestinal LPL were sorted for $CD4^+CD25^+$ cells and co-cultured with splenic DCs and CFSE-labeled $CD4^+CD25^-Thy1.1^+$ responder cells at a suppressor to responder ratio of 2:1. Lymphocytes were stimulated to proliferate with anti-CD3 antibody for 3 days *in vitro*. CFSE dilution was used to measure proliferation, and responders stimulated in the absence of suppressors (black histogram) were used to assess suppression. All panels are pre-gated on $CD4^+Thy1.1^+$cells.

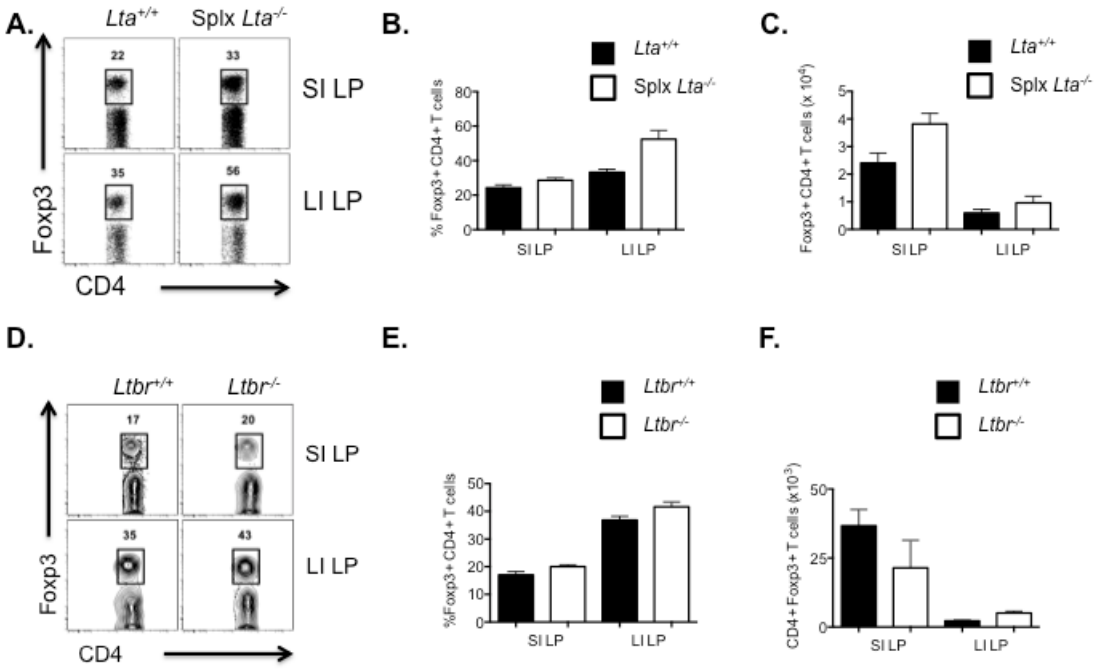

FIGURE 4.3: Intestinal Foxp3+ T_{reg} cell development in additional models of SLO deficiency. (A) Representative FACS plots of intestinal Foxp3+ T_{reg} cell for JAX *Lta*$^{+/+}$ and JAX Splx *Lta*$^{-/-}$ mice. Comparison of intestinal Foxp3+ T_{reg} cell frequencies (B) and numbers (C) for *Lta*$^{+/+}$ and Splx *Lta*$^{-/-}$ mice. (D) Representative FACS plots of intestinal Foxp3+ T_{reg} cells in *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice. Comparison of intestinal Foxp3+ T_{reg} cell frequency (E) and number (F) in *Ltbr*$^{+/+}$ and *Ltbr*$^{-/-}$ mice. Error bars represent SEM.

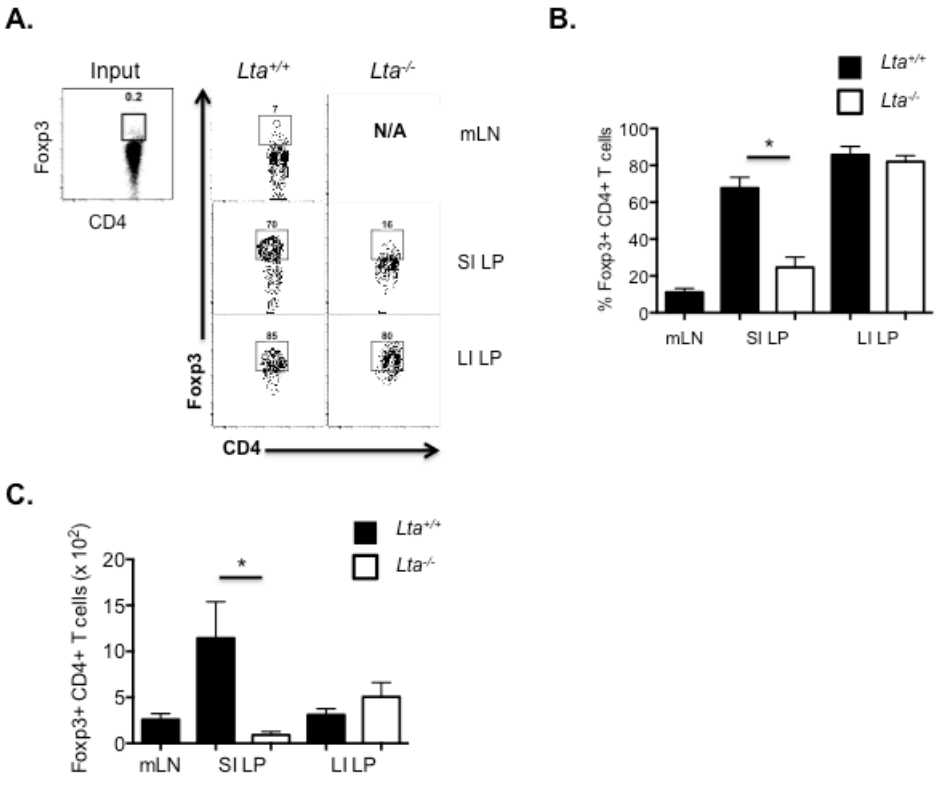

FIGURE 4.4: Distinct requirements between the SI and LI for LT-dependent lymphoid structures in Foxp3+ T_{reg} cell differentiation. Differentiation of naïve CD4+CD25- T cells from OT-II Thy1.1 congenic mice after adoptive transfer into Thy1.2 *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice and fed ovalbumin (OVA) water. (A) Representative FACS plots of Foxp3 induction comparing *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice. Frequency (B) and cell number (C) of Foxp3+ T_{reg} cells induced from OT-II cells. Data are pre-gated on $V_{\alpha 2}$+$V_{\beta 5}$+CD4+Thy1.1+ cells. Error bars represent SEM. *, $p \leq 0.05$ using a Student's *t* test.

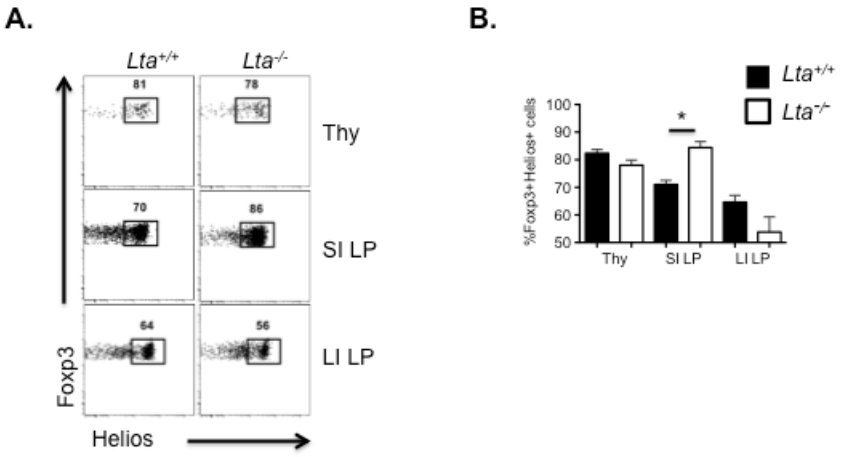

FIGURE 4.5: Foxp3+Helios+ tT_{reg} cells are more abundant in the SI LP in the absence of the mLN, GALT, and other LT-dependent lymphoid structures. (A) Representative FACS plots of Helios expression by TCRβ+CD4+Foxp3+ cells in *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice. (B) Comparison of Foxp3+Helios+ tT_{reg} cell abundance in *Lta*$^{+/+}$ and *Lta*$^{-/-}$ mice. Error bars represent SEM. *, $p \leq 0.05$ using a Student's *t* test.

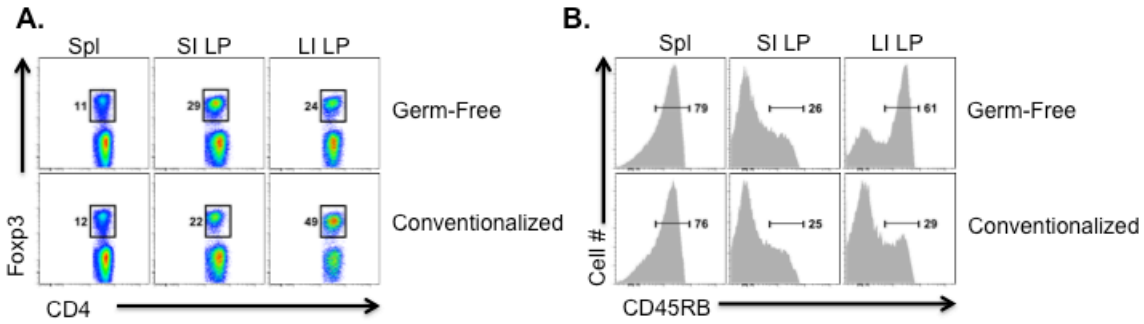

FIGURE 4.6. The microbiota drives Foxp3+ T_{reg} cell differentiation in the LI LP. Germ-free mice were conventionalized at 6 weeks of age. Mice were euthanized at 8 weeks of age and organs were harvested to characterize the CD4+ T cell population. (A) CD4+ T cells in the SPL, SI LP, and LI LP were isolated and stained for Foxp3 to assess Foxp3+ T_{reg} cell induction after conventionalization. (B) Histograms depicting the frequencies of naïve $CD45RB^{hi}$ CD4+ T cells in the SPL, SI LP, and LI LP of germ-free and conventionalized mice. FACS plots are pre-gated on CD4+TCRβ+ cells.

Chapter 5: Summary and Discussion

The intestine is a vital organ that serves as a specialized site where food antigens, the gut microbiota, and the host immune system interact. Enriched in the intestine are Th17 and Foxp3+ T_{reg} cells that function to protect the host against invasive pathogens while promoting tolerance to antigens derived from innocuous sources and suppressing inflammation (203), respectively. In seeking to understand the development of intestinal Th17 and Foxp3+ T_{reg} cell responses, this dissertation sought to 1) evaluate if naïve CD4+ T cells migrate to the intestinal LP, 2) assess the requirements for the mLN, GALT, and other SLO, and 3) elucidate the contribution of the microbiota and APCs. Indeed, we observed that naïve CD4+ T cells represented an appreciable portion of the total CD4+ T cell pool even in adulthood and their presence was independent of the mLN, GALT, and SLO. Furthermore, the mLN, GALT, and SLO were not required for maintenance of the intestinal Th17 and Foxp3+ T_{reg} cell. Lastly, while specific microbiota containing SFB and antigenic stimulation by intestinal DCs were required for promoting intestinal Th17 cell responses, the Foxp3+ T_{reg} cell compartments in the SI and LI LP showed distinct composition and requirements for the microbiota. Foxp3+ T_{reg} cells in the SI LP were predominantly Helios expressing Foxp3+ tT_{reg} cells that were not regulated by the microbiota while more Foxp3+Helios- pT_{reg} cells were present in the LI LP and induced by the microbiota. These findings highlight the distinct developmental pathways that contribute to maintaining intestinal Th17 and Foxp3+ T_{reg} cell homeostasis and

the importance of the local cytokine and cellular milieu governing their development.

The intestinal LP as a site for CD4+ T cell differentiation

Previous studies investigating oral tolerance and intestinal antigen presentation have implicated the mLN to be the primary site of CD4+ T cell differentiation. This paradigm was based on the key observations that: 1) lymphatics from the intestinal LP and PP drained into the mLN and carried with them migratory DCs that primed CD4+ T cells in the mLN (84, 204), 2) naïve CD4+ T cells traffic through SLO, and indeed, the mLN are enriched with naïve CD4+ T cells, 3) oral delivery soluble antigens induced Foxp3+ T_{reg} cell differentiation in the mLN (15, 83, 170), and 4) the mLN were required for the induction of systemic oral tolerance (84). Together, these findings implicated the mLN to be crucial for the development of intestinal Th17 and Foxp3+ T_{reg} cell responses.

In addition to previous studies that highlight the importance of the mLN in regulating intestinal CD4+ T cell responses, accumulating evidence suggests that the intestinal LP may also serve as a site for intestinal CD4+ T cell differentiation. Present in the intestinal LP are tissue resident APCs capable of inducing robust Th17 and Foxp3+ T_{reg} cell differentiation, a plethora of enteric antigens, and a milieu rich in key regulatory cytokines, such as TGF-β1, IL-1β, retinoic acid, IL-6, and ATP, that is conditioned by the gut microbiota (3, 5). Thus, provided a pool of naïve CD4+ T cells, the intestinal LP would be a tissue site conducive for CD4+ T

cell differentiation. Indeed, the presence of naïve CD4+ T cells in non-lymphoid tissues, such as the intestine, has been previously documented (178), but the extension of these findings in the intestine of *Lta*$^{-/-}$ mice devoid of SLO indicates that naïve CD4+ T cells may migrate directly to the intestinal LP without imprinting in the mLN and GALT to upregulate gut-homing markers (194). Thus, the intestinal LP may also serve a site for CD4+ T cell differentiation as it contains a diverse population of APCs, an array of antigens, an appreciable pool of naïve CD4+ T cells, and a cytokine milieu conditioned by the microbiota.

The naïve CD4+ T cells that migrate to the intestine may derive from recent thymic emigrants (RTEs) or from the mature T cell pool that circulates through SLO, however the biological processes that enable a subset of these cells to home to the intestine have yet to be fully characterized. Previous reports of thymocytes and RTEs expressing the gut-homing molecules CCR9 and $\alpha4\beta7$ and capable of migrating to the SI substantiates the plausibility of RTEs as a source of naïve CD4+ T cells in the intestine (205, 206). This indicates that gut tropism may be acquired during thymic development, but whether RTEs utilize additional chemokine receptors or integrins for intestinal homing is unknown. Additionally, characterizing the antigen reactivity of intestinal RTEs may yield distinct TCR repertoires for functional characterization. Since RTEs express recombination-activating genes (RAG) for several weeks after thymic emigration (206), utilizing RAG reporter mice (commercially available) would enable analysis of the RTE composition in the intestinal naïve CD4+ T cell pool and permit comparative functional analysis of RTEs and non-RTEs in the intestinal LP.

Additionally, assessing for differential homing mechanisms of RTEs and non-RTEs to the SI and LI LP would be important given the recent identification of GPR15, which is mediates CD4+ T cell homing to the LI LP in mice (200, 201). In light of the FDA approval for vedolizumab, a humanized monoclonal antibody against $\alpha4\beta7$, in the treatment of UC and CD (207, 208), the discovery for additional chemokine receptors and integrins that regulate naïve CD4+ T cell homing to the intestine may be of clinical importance for the treatment of IBD.

Unraveling the mystery of how specific commensal bacteria can regulate Th17 cells and autoimmunity

The association of SFB colonization with the induction of intestinal Th17 cells was a seminal discovery that began to resolve the parameters regulating Th17 cell development in vivo (29, 61). The mechanism by which SFB could induce intestinal Th17 cell responses was proposed to be through serum amyloid A (SAA) based on elevated intestinal expression in whole tissue from on mono-association studies and in vitro culture conditions (29). However, the pertinent cellular and molecular processes required were unknown. Our results demonstrating that intestinal Th17 cell differentiation occurs in the absence of the mLN and GALT under the regulation by local LP DCs and the microbiota-conditioned milieu underscores the intestine as an additional site for intestinal CD4+ T cell differentiation. Furthermore, the requirement for MHC class II on DCs and stimulation by cognate antigen suggests that colonization by SFB promotes a cytokine milieu conducive for Th17 cell development upon antigenic

stimulation. In the context of two recent studies reporting approximately 50-60% of the Th17 cells to be reactive to SFB antigens (195, 209), our data indicates that 40-50% of Th17 cells develop in SFB-conditioned milieu upon recognizing other enteric antigens that are not derived from SFB. Consequently, the adjuvant-like properties of SFB may be detrimental to the host if auto-reactive CD4+ T cells in the intestine are activated in this milieu to differentiate into pro-inflammatory Th17 cells. Indeed this provides mechanistic insight into how colonization by SFB exacerbates experimental models of autoimmunity (210) whereby auto-reactive Th17 cells that differentiate in the intestine may subsequently re-circulate in the host and promote autoimmune disease upon reactivation by self antigens. Taken together, these findings highlight the importance of the microbiota composition in regulating the intestinal milieu, host immune responses, and autoimmunity.

The regulation of intestinal Th17 cells by SFB-containing microbiota: limitations and future directions

Although we and others have suggested intestinal LP DCs to provide antigenic stimulation for intestinal Th17 cell differentiation based on the use of CD11c for conditional deletion and/or expression of MHC II (195), a role for intestinal macrophages cannot be excluded albeit these cells are less efficacious in promoting Th17 cell differentiation (5, 13, 14). Intestinal macrophages express CD11c and CD11b similar to intestinal DCs and distinguishing the contributions between these two APCs in providing antigenic stimulation requires the use of

more lineage-specific markers such that conditional deletion of MHC II is driven by F4/80 or Lys-M for macrophages or zbtb46 for DCs (5, 211). Furthermore as the intestinal LP DC compartment is comprised of heterogeneous subsets, the differential contribution of CD103+CD11b- and CD103+CD11b+ DCs may be examined utilizing Batf3 (212) or Notch2 (169) for conditional deletion of MHC II, respectively.

The use of the cecal contents from Tac B6 mice for our specific microbiota transfer studies does not preclude a potential role for other commensal bacteria in our results. Nonetheless, horizontal transfer of SFB from Tac B6 to JAX B6 mice via co-housing or oral gavage have yielded complementary results to studies with SFB mono-association or gavage of SFB mono-associated feces (29, 61). Importantly, our published findings are consistent with several other reports that have utilized SFB mono-association studies or gavaged feces from SFB mono-associated mice (195, 209). The culture conditions required to grow SFB in vitro have been discerned only recently (213), and this development may further advance our understanding of the host immune response to SFB.

Currently, the specific microbial components of SFB along with the downstream immune cells and cytokines that mediate intestinal Th17 cell differentiation are unknown. Considering the adherence of SFB to the intestinal epithelium, how SFB regulates IEC and IEL biology remains to be defined. Since genomic analysis of SFB indicate the expression of flagellin (214, 215) and both TLR5 and NLRC4 are expressed by the intestinal epithelium (216, 217), it would be interesting to investigate if the adjuvant-like properties of SFB that promote

intestinal Th17 cell differentiation are mediated via the TLR5/NLRC4 pathway. Additionally, as SAA was the most upregulated gene following SFB mono-association (29), the importance of serum amyloid A (SAA) in regulating SFB-driven intestinal Th17 cell differentiation has not been examined in vivo. Assessing the induction of intestinal Th17 cells in mice deficient of SAA or its known receptor formyl peptide receptor 2 following SFB colonization would explore the importance of this acute phase reactant protein in promoting effector CD4+ T cell responses. Lastly, our data for eosinophils in functioning to limit intestinal Th17 cell responses via IL-1RA requires further validation in *Il1rn*$^{-/-}$ mice to verify if intestinal Th17 cell development upon SFB colonization is enhanced and if reconstitution with IL-1RA-sufficient eosinophils can mitigate excessive induction of these cells.

The composition of the intestinal Foxp3+ T_{reg} cell pool

The results presented here suggest that the Foxp3+ T_{reg} cell pool in the intestine does not require the mLN and GALT, and in fact may be enriched with Foxp3+ tT_{reg} cells. Quantitation of intestinal Foxp3+ T_{reg} cells in *Ccr7*$^{-/-}$ and LT-deficient mice indicates that the antigen presentation by migratory LP DCs in the mLN is dispensable for the maintenance of the intestinal Foxp3+ T_{reg} cell pool. Instead the use of Helios as a marker for Foxp3+ tT_{reg} cells reveals that more than 60% of the Foxp3+ T_{reg} cells in the intestine express Helios and may be thymically-derived. Furthermore, the expression of Helios in these intestinal Foxp3+ T_{reg} cells corresponded with additional tT_{reg} cell markers, FR4 (218) and

neuropilin-1 (219), in our studies to further substantiate their thymic origin (data not shown). Nonetheless, assessing the methylation status of the TDSR (72) would unequivocally distinguish the Foxp3+ tT_{reg} and pT_{reg} cells that comprise the intestinal Foxp3+ T_{reg} cell compartment.

Our comparative analysis of Foxp3+ T_{reg} cells in the SI and LI LP has indicated differences in composition and antigen reactivity. Previous studies examining oral tolerance and Foxp3+ T_{reg} cell induction to a food antigen have primarily focused on the SI (15, 83, 170), and indeed, we found Foxp3+ pT_{reg} cell differentiation in the SI LP to be dependent on the mLN. In the absence of the mLN, the proportion of Foxp3+Helios+ tT_{reg} cells was increased in *Lta*$^{-/-}$ mice similar to that observed in CNS1^{KO} mice (75), which are void of Foxp3+ pT_{reg} cells. This suggests that the proportion of Foxp3+ tT_{reg} cells may increase to compensate for the deficiency in pT_{reg} cells in the SI LP. In contrast, Foxp3+ pT_{reg} differentiation in the LI LP was independent of the mLN and highlights the potential for in situ Foxp3+ T_{reg} cell differentiation similar to Th17 cells. Accordingly, a larger proportion of the Foxp3+ T_{reg} cells in the LI LP do not express Helios relative to the SI LP, and naïve CD4+ T cells were abundant in the LI LP of GF that decreased with conventionalization and corresponded with an increase in Foxp3+ T_{reg} cells. These latter findings also suggest differences in antigen reactivity since conventionalization of GF mice induce Foxp3+ T_{reg} cells only in the LI LP while the SI LP remained unaffected, indicating that Foxp3+ T_{reg} cells in the LI LP are more reactive to the microbiota. Indeed, these results are consistent with previous studies that report specific components of the

commensal microbiota, such as *Clostridium* strains (104, 172) and *B. fragilis* (102, 103), regulating colonic Foxp3+ T_{reg} cells. Consistent with the anatomical functions of the intestine and the distribution of the microbiota whereby the SI is important for nutrient absorption and the LI harbors the largest concentration of bacteria, Foxp3+ pT_{reg} cells in the SI LP may promote immune tolerance to food antigens while Foxp3+ pT_{reg} cells in the LI LP promote tolerance and restrain aberrant immune responses to commensal bacteria (4). Future studies comparing the antigen reactivity of Foxp3+ T_{reg} cells in the SI and LI LP along with the immune cells and cytokines that regulate Foxp3+ pT_{reg} cell differentiation may be important for manipulating the Foxp3+ T_{reg} cell compartment in these tissue two sites for therapeutic purposes.

As intestinal Foxp3+ T_{reg} cells are enriched with Helios+ tT_{reg} cells, it is unclear how Foxp3+ tT_{reg} cells home to the intestine and whether the same mechanisms that govern Foxp3+ pT_{reg} cell homing apply. The gut-homing molecules $\alpha4\beta7$ and CCR9 are important for Foxp3+ T_{reg} cell migration to the SI (79, 80) while GPR15 regulates homing to the LI in mice (192, 193). Sorting intestinal Foxp3+ tT_{reg} and pT_{reg} cells and conducting a comparative analysis for integrins and chemokine receptors may help to elucidate distinct gut-homing mechanisms utilized by each population.

Determining the mechanisms by which Foxp3+ tT_{reg} and pT_{reg} cells can be differentially increased in the intestine may have important clinical implications for Foxp3+ T_{reg} infusion therapy in the treatment of autoimmune diseases and chronic inflammatory disorders like IBD. Previous studies investigating the

efficacy of Foxp3+ tT_{reg} and pT_{reg} cells in the treatment of autoimmunity have demonstrated distinct functional niches with Foxp3+ tT_{reg} cells being important for preventing lethal autoimmunity while Foxp3+ pT_{reg} cells serving to suppress pathologic inflammation (190, 191). Indeed, Foxp3+ tT_{reg} cell immunotherapy alone into Foxp3-deficient mice was insufficient to prevent autoimmune disease but required Foxp3+ iT_{reg} cells as well (190). Similar results were observed in the context of treating chronic T cell-mediated colitis in mice as Foxp3+ tT_{reg} cells were unable to prevent disease without iT_{reg} cells (220). Furthermore, in the absence of Foxp3+ pT_{reg} cells, $CNS1^{KO}$ mice mature into adulthood but develop a severe Th2-like mucosal inflammation (191). Consequently, manipulating the ratio of Foxp3+ tT_{reg} to pT_{reg} cells by modulating differential homing or antigen-specific expansion may be important relevant for the treatment of autoimmunity and chronic inflammation with T_{reg} infusion therapy.

Collectively, our studies provide novel insights into the development of Th17 cells and Foxp3+ T_{reg} cells in the intestine. In conjunction with previous studies that attribute the mLN to be a site of intestinal CD4+ T cell differentiation, we provide compelling evidence utilizing various mouse models that intestinal Th17 and Foxp3+ T_{reg} cell development is independent of the mLN and GALT suggesting the intestinal LP to serve as an additional site for intestinal CD4+ T cell differentiation (Fig. 5.1). Given the current need for identifying new cellular and molecular targets in the treatment of IBD, our findings may serve to direct translational research into focusing on relevant APCs and cytokines in the

intestinal LP that recruit and modulate pro-inflammatory Th17 and tolerogenic Foxp3+ T_{reg} cell responses.

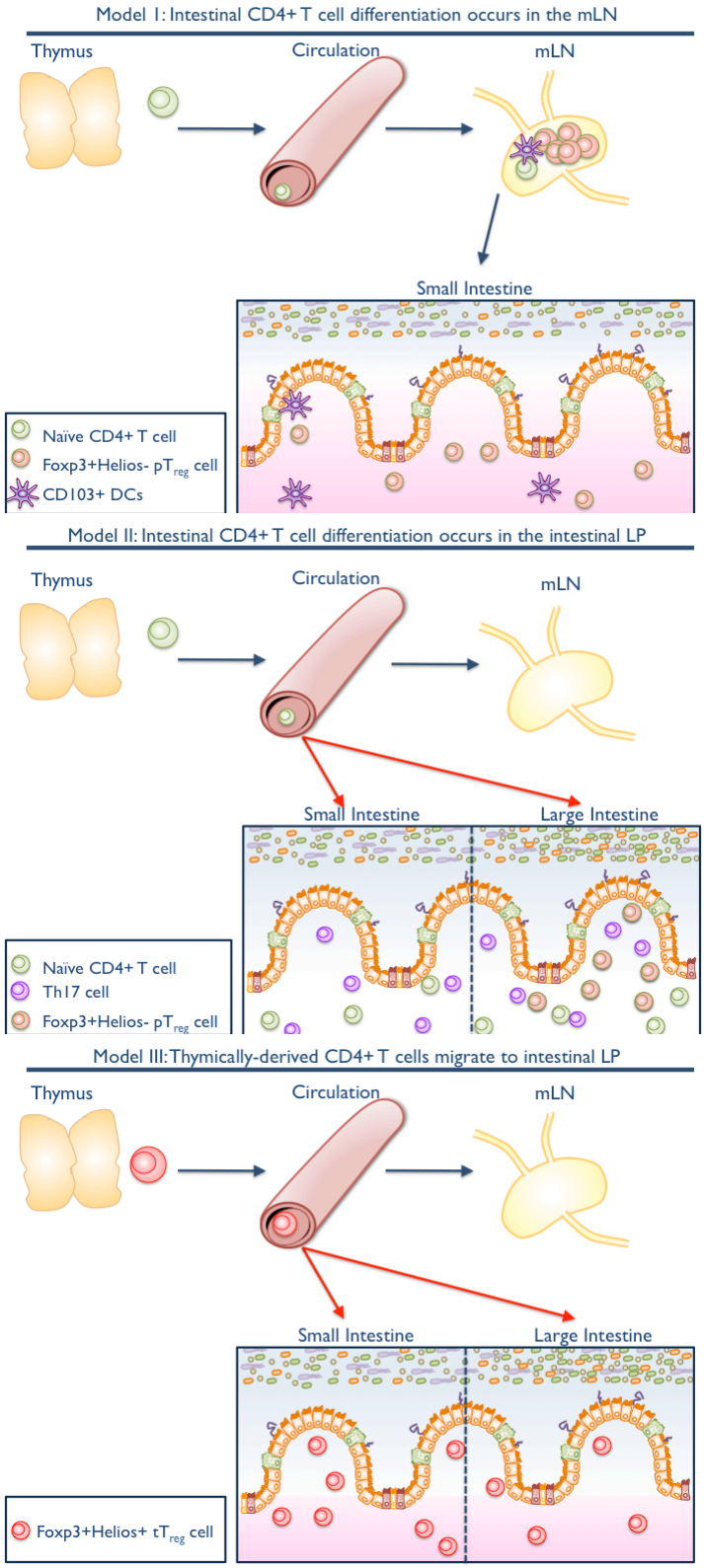
Model I: Intestinal CD4+ T cell differentiation occurs in the mLN
Thymus
Circulation
mLN
Small Intestine
Naïve CD4+ T cell
Foxp3+Helios- pTreg cell
CD103+ DCs
Model II: Intestinal CD4+ T cell differentiation occurs in the intestinal LP
Thymus
Circulation
mLN
Small Intestine
Large Intestine
Naïve CD4+ T cell
Th17 cell
Foxp3+Helios- pTreg cell
Model III: Thymically-derived CD4+ T cells migrate to intestinal LP
Thymus
Circulation
mLN
Small Intestine
Large Intestine
Foxp3+Helios+ tTreg cell

FIGURE 5.1: Complementary pathways for intestinal CD4+ T cell development. (Top panel) Model I, intestinal CD4+ T cell differentiation occurs in the mLN and migrate to the intestinal LP, seems to be applicable for Foxp3+ pT_{reg} cell development in the SI. (Middle panel) Model II whereby naïve CD4+ T cells may migrate directly to the intestinal LP and differentiate under regulation by resident APCs and local cytokine milieu is consistent for intestinal Th17 cell development in both the SI and LI and Foxp3+ pT_{reg} cell development in the LI. (Bottom panel) Model III is pertinent for Foxp3+ tT_{reg} cells that develop in the thymus and home directly to the intestinal LP.

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