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Limiting host-microbiota interactions to control intestinal inflammation

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Limiting host-microbiota interactions to control intestinal inflammation

by

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Dedication

My doctoral dissertation is dedicated to my parents, Toa and Kien Cao, and to my sister Jessica.

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Limiting host-microbiota interactions to control intestinal inflammation

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The host intestinal tract lives in constant balance with the commensal microbiota residing in the intestinal lumen and immune responses must be tightly controlled. A strong intestinal barrier separates the immune system from resident microflora, and multiple mechanisms exist to reinforce the epithelium to limit microbial exposure to the immune system, including a thick mucus layer, secretion of antimicrobial peptides, and production of IgA. However, how Th17 cells contribute to intestinal immunity is still largely unknown.

Our current studies indicate that Th17 cells play a critical role in controlling microbial contact with the epithelium. IgA is produced in large quantities and secreted into the intestinal lumen where it neutralizes pathogens and prevents microbes from penetrating the epithelium and inducing inflammatory responses. IgA must be actively secreted by epithelial cells after binding to the polymeric immunoglobulin receptor (pIgR), and is limited by the

rate of pIgR expression and translocation. Th17 cells can directly induce pIgR expression from intestinal epithelial cells, and increase levels of luminal IgA. During episodes of intestinal injury, the induction of pIgR and increase in IgA as mediated by Th17 cells affords increased protection against microbial breach and infection, thereby preventing inflammatory responses.

Keeping the intestinal lamina propria clear of microbial ligands is critical for maintaining intestinal homeostasis and preventing the onset of colitis. We showed that the presence of LPS can inhibit regulatory T cell generation, thereby disrupting immune regulation and clearing a path for unchecked inflammatory responses. TLR4 signaling through both antigen-presenting cells, and on T cells themselves is capable of inhibiting regulatory T cell generation. Upon the induction of a chronically inflamed state, regulatory T cell function is altered and hinders restoration of tissue homeostasis. We show that chronically-inflamed tissues contain increased numbers of regulatory T cells that have acquired effector T cell capability through production of inflammatory cytokines, while retaining a regulatory T cell phenotype.

Taken together, our studies reveal a dynamic interaction between the host intestinal environment and microbiota. Understanding of these mechanisms before and after the onset of colitis may direct therapeutic approaches to control or prevent chronic inflammation.

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List of Abbreviations

AID	Activation-induced cytidine deaminase
APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B cell-activating factor
BCR	B cell receptor
CSR	Class switch recombination
DC	Dendritic cell
DNA	Deoxyribonucleic acid
FACS	Fluorescence-activated cell sort
FBS	Fetal bovine serum
Foxp3	Forkhead box protein p3
GALT	Gut-associated lymphoid tissue
i.v.	Intravenous
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
iTreg	induced regulatory T cell
LPL	Lamina propria lymphocyte

LPS	Lipopolysaccharide
MACS	Magnetic cell sorting
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
PCR	Polymerase chain reaction
pIgR	Polymeric immunoglobulin receptor
RA	Retinoic acid
Rag	Recombination activating gene
RNA	Ribonucleic acid
RORγt	Retinoic acid-related orphan receptor gamma t
SCFA	Short-chain fatty acid
SFB	Segmented filamentous bacteria
SHM	Somatic hypermutation
STAT	Signal transducer and activator of transcription
T-bet	Tbx-21
TCR	T cell receptor
Tg	Transgenic
TGF	Transforming growth factor
Th-	T helper cell
TLR	Toll-like receptor
TNF	Tissue necrosis factor
Treg	regulatory T cell

tTreg	thymic regulatory T cell
WT	Wild type

INTRODUCTION

Chapter 1: Overview of the Intestinal Immune System

The gastrointestinal tract constitutes the largest mucosal surface in the human body, covering over 300m^2 in surface area and is in constant exposure to a wide variety of food antigens, a diverse array of commensal bacteria, and invasive pathogens. In order to deal with the constant exposure to external antigenic stimuli, the intestinal immune system is required to distinguish between pathogenic and commensal bacteria as well as food antigens. An important feature of the intestinal immune system is to protect the intestinal environment against infection while tolerating the normal microbiota and dietary antigens[1]. The dysregulated initiation and chronic activation of these intestinal immune responses results in chronic inflammatory disorders of the intestinal tract, including inflammatory bowel disease, food allergies, and celiac disease[2]. In order to prevent aberrant activation of the intestinal immune system, multiple strategies have evolved to confine commensal bacteria to the intestinal lumen through a combination of physiological and immunological mechanisms. The coordination of these multiple layers of defense allows the abundant and diverse microbiota to thrive while concurrently limiting intestinal inflammation.

A single layer of tightly-connected epithelial cells separates the vast bacterial load in the intestinal lumen from the underlying mucosa. Remarkably, the intestinal epithelium provides an efficient barrier to prevent the infiltration of the microflora into the intestinal lamina propria. As a result of the constant movement within the lumen, the epithelial layer is renewed every 5 days after the epithelial cells are sloughed off and replaced by epithelial cells arising from stem cells located in the crypts of the villi[3]. The epithelial layer is coated with a layer of mucus that protects the apical epithelium from contact with bacteria, as commensal bacteria are unable to penetrate the outer layer of

mucus[4]. Secreted by specialized epithelial cells called goblet cells, the mucus layer is a key mechanism for preventing bacterial contact with the epithelium and subsequent intestinal immune system[5].

Also present within the mucus layer are diverse antimicrobial peptides that kill bacteria. Produced by epithelial cells, antimicrobial peptides come from diverse protein families, including cathelicidins, defensins, and C-type lectins, and are regulated by independent mechanisms[6]. Most antimicrobial peptides contain bactericidal activity, particularly by disrupting the cell wall or inner membranes of bacteria[5, 7, 8]. In effect, these antimicrobial peptides function similarly to antibiotics. All intestinal epithelial cells secrete some form of antimicrobial peptide and it becomes clear that they function to protect the epithelial layer. After secretion, most peptides are retained in the mucus layer, providing an increasingly concentrated bactericidal barrier next to the intestinal epithelium, all of which is responsible for limiting bacterial exposure to the epithelium and intestinal immune system.

Innate immune cells are present in the intestinal tract in great numbers, and further facilitate the tolerance of the commensal microbiota. The key roles that dendritic cells (DC) and macrophages play as antigen-presenting cells, and for establishing the cytokine environment reveals a delicate balance in innate cell function for directing the CD4 T cell response. Dendritic cells constantly patrol the intestinal tract and play a critical role in the decision of whether to initiate proinflammatory immune responses or promote anti-inflammatory immune regulation[9]. During intestinal homeostasis, intestinal DCs tend to favor immunoregulatory responses to maintain healthy, steady-state conditions. Macrophages are the most abundant phagocytic cells in the intestines, and are responsible for the clearance of any microorganisms that cross through the epithelium. However, like intestinal DCs, intestinal macrophages tend to be more tolerogenic than peripheral macrophages, and typically do not promote inflammation in response to antigens[10].

Chapter 2: Intestinal Lamina propria T cells

T cells are lymphocytes that arise from the bone marrow, and mature in the thymus gland, and each express a unique T cell receptor that allows each T cell to bind to a specific antigen. Based on TCR domain structures, T cells are classified into conventional αβ T cells or γδ T cells[11]. αβ T cells are responsible for antigen-specific cellular immunity, while γδ T cells are non-MHC-restricted and do not require antigen-presentation, and are involved in primary immune responses. In circulation, the large majority of T cells consist of αβ T cells, and only 1-5% are γδ T cells[12]. However, a large number of γδ T cells reside within the small intestine, specifically interspersed throughout the epithelium. These intraepithelial lymphocytes (IEL) are most γδ T cells, and regulate the continuous turnover of epithelial cells[13] and strengthen the epithelial layer through secretion of growth factors[14]. γδ T cells also secrete a number of cytokines, including IFNγ[11], IL-17[11, 15], and IL-22[15], and can prevent infection[16] as well as control intestinal inflammation[15].

αβ T cells express either CD4 or CD8 on their surfaces, resulting in MHC II and MHC I antigen-presentation restrictions, respectively. This divides T cells into two functionally distinct populations for T helper (CD4+) and cytotoxic T cells (CD8+). The prime focus of the present studies will be CD4+ αβ T cells in the intestine and its associated compartments.

Coordination between the innate and adaptive immune systems is required for the swift and complete clearance of pathogens, both systemically and in the intestinal tract. Antigen-presenting cells (APCs) present antigens on MHCII to CD4 T cells, which then differentiate into IFN-γ-producing Th1 cells, IL-4-producing Th2 cells, IL-17-producing Th17 cells, or adaptive Treg cells, based on the cytokine cues present in the environment from APCs and other local factors[17]. IL-12, produced by activated APCs[10], stimulates T

cell Signal Transducer and Activator of Transcription (STAT) 4 activation, and induces IFNγ production, which activates STAT1 in the responding CD4 T cells and up-regulates expression of T-bet, the master transcription factor for Th1 cells[18]. T-bet then induces T cell IFNγ production and responding cells function as Th1 cells. To generate Th2 cells, IL-4 signals through STAT6 and GATA3. GATA3 is the master transcription factor of Th2 lineage, and can work in collaboration with IL-2-activated STAT5 to induce IL-4 production to continue induction of Th2 cells[17]. GATA3 and STAT5 can also impair STAT4 activation, thereby antagonizing Th1 cell induction[19]. Induced peripheral Treg cells are generated via the transcription factor FoxP3, which is induced by TGFβ. Notably, thymic $Foxp3^+$ Treg cells (tTreg) arise out of the thymus. Analyses of the TCR repertoires between peripheral and thymic Treg cells suggest that thymic Treg cells may respond to self-antigens and as such tTreg cells may be a surviving population of Th cells that had been marked for clonal deletion in the thymus[20-22]. While TGFβ alone induces Treg cells through Foxp3, IL-6 can inhibit FoxP3 induction and together, IL-6 and TGFβ induce Th17 cell differentiation. Enhancement of STAT3 signaling by IL-6, with SMAD3 signaling from TGFβ induces RORγt, the master transcription factor for Th17 cells[23]. Th17 cells uniquely produce IL-17A, IL-17F, and IL-22 amongst other cytokines. IL-23 from innate cells is crucial for the function of Th17 cells, as well as maintaining their survival[24]. Follicular helper T cells (Tfh) are a another type of Th cells that are found within lymphoid tissues. Their presence within B cell follicles leaves them in prime position to directly support B cell expansion and differentiation[25], particularly through expression of various surface markers, and the cytokine IL-21[26]. Tfh cells are defined by their surface expression of the chemokine receptor CXCR5, as well as the transcription factor Bcl-6[27]. It is not completely clear which factors are required for Tfh generation, however it appears that STAT3 activation (likely through IL-6, IL-21, or IL-27)[27] is required.

The unique lineages of these T cells reveals that CD4 T cells have evolved to adopt important roles in responding to pathogenic challenges and organizing adaptive immune responses by "sounding the alarm" through cytokines and chemokines to activate and/or recruit target cells. Th1 cells target intracellular bacteria and viruses, Th2 cells respond against large extracellular parasites such as nematodes and helminthes, and Th17 cells respond against extracellular bacteria and fungi. Treg cells promote anti-inflammatory effects, limiting collateral damage and restoring homeostasis once infections are cleared.

The gastrointestinal tract acts as a natural reservoir for Th17 and Treg cells. As the intestines harbor an extreme bacterial load, Th17 and Treg cells exist in a balance to maintain a healthy intestinal environment. In mice, Th17 cells are generated by IL-6 and TGFβ, inducing RORγt and the production of IL-17A, IL-17F, IL-21 and IL-22, in addition to other proinflammatory cytokines including TNF-α and GM-CSF. Through their cytokine panel, Th17 cells are critical for controlling extracellular pathogens, thereby substantiating the high populations of Th17 cells in the intestines and airway, mucosal surfaces that are exposed to the external environment and that come in constant contact with microbes. IL-17A and IL-17F induce potent inflammatory responses by inducing the expression of other proinflammotry cytokines, chemokines, and metalloproteinases. Their effects can target a wide range of cells, as the IL-17R is almost ubiquitously expressed[28]. Notably, IL-17A and IL-17F potentiates strong responses from neutrophils, through recruitment, activation, and subsequent migration into local tissues. Deficiency in IL-17 and the absence of a strong neutrophil response leaves hosts greatly susceptible to a number of bacterial and fungal infections[29-31]. Th17 cells also have a role in protective immunity by regulating antimicrobial peptides as IL-17A induces production of β-defensin 2, and IL-22 induces RegIIIγ from epithelial cells. Presence of these microbicidal peptides is key for maintaining separation between the epithelium and the microflora[5].

Disruptions in the balance between effector T cells and Treg cells results in dysregulated immune responses against the resident commensal microbiota and rampant inflammation often ensues. Treg cells are defined by the expression of the transcription factor Foxp3, and are generated in the thymus, or induced in the periphery by TGFβ. Treg cells exert immune regulation by producing TGFβ and IL-10, two universally-pleiotropic cytokines[32]. Treg cells provide a necessary check upon the immune system, as mice deficient in TGFβ or Foxp3 die within weeks of birth from hyperproliferation of effector T cells and extensive multiorgan inflammation[33, 34]. Deficiency in IL-10 also results in spontaneous colitis[35]. Therefore, Treg cells balance out the function of both innate and adaptive immune cells in the intestine and restrict inflammation against the commensal microflora to maintain homeostasis. When $CD45RB^{hi}$ naïve effector T cells are transferred into immunodeficient $Rag^{-/-}$ mice, high frequencies of Th1 and Th17 cells are rapidly generated in the intestinal tract and cause colitis[36]. $CD45RB^{hi}$ T cells do not expand and cause disease when transferred into germ-free mice, demonstrating that the pool of effector cells are responding to stimulation from commensal antigens[37]. If Treg cells are co-transferred with $CD45RB^{hi}$ T cells, intestinal inflammation is alleviated, indicating that Treg cells are critical for limiting a hyperactive immune response to innocuous commensal antigens.

Chapter 3: B cells and intestinal IgA

B cells are lymphocytes that arise from the bone marrow and each expresses a unique B cell receptor that allows a B cell to bind to a specific antigen. After migrating to secondary lymphoid tissues (e.g. spleen, lymph nodes, Peyer's patches), B cells reach maturation and as such, comprise the humoral wing of the adaptive immune response. After recognition of their cognate antigen through their specific B cell receptor, B cells rapidly proliferate and produce highly-diversified antibodies in order to identify and neutralize foreign antigens. The binding of antibody to foreign antigens and/or infected cells usually results in rapid clearance by the immune system. This, coupled with the longevity of pools of memory B and T cells, leads to the effective elimination of pathogens by the adaptive immune system.

As their name implies, $CD4^+$ helper T cells were originally described based on their ability to help B cells to generate strong specific antibody responses to neutralize and clear infections. B cells form conjugates with antigen-activated T cells, and the profiles of the T cells determine the isotype class switching undertaken by the B cells. It has long been known that Th1 and Th2 cells have provided B cell help, where their signature cytokines IFNγ and IL-4 induce class switching to IgG2a or IgG1 and IgE, respectively.

B cells are present in the intestinal tract in very high frequency where they primarily function to maintaining mutualism with the microbiota. The high frequency of B cells and subsequent antibody production is paramount for controlling bacterial penetration into the intestinal mucosa. By producing enormous quantities of IgA into the intestinal lumen, the microbiota becomes coated in IgA, preventing bacteria from penetrating the mucus layer through agglutination and neutralizing bacterial receptors and

toxins[38, 39]. IgA is the most common immunoglobulin subtype produced in the human body. With 3 to 5 grams of IgA being produced each day in adults, IgA production dwarfs the combined amount of the other antibody subtypes. As such, approximately 80% of all antibody-secreting cells are found in the intestine, and a vast majority of these cells are actively producing IgA[40].

Production of IgA occurs in the intestinal lamina propria, whereas luminal secretion of IgA relies on active transport across the intestinal epithelium. This process is mediated by the polymeric immunoglobulin receptor (pIgR), located on all epithelial cells. Covalent binding of dimerized IgA to pIgR on the basolateral side of epithelial cells results in the transcytosis of the IgA complex, followed by its secretion through to the lumen. Cleavage of pIgR leaves a segment of the pIgR covalently bound to IgA and improves the stability of the complex, now known as secretory IgA (sIgA)[41]. The expression of pIgR on epithelial cells is critical for the secretion of IgA, and for intestinal protection. $pIgR^{-/-}$ mice have drastically decreased luminal IgA, which is attributed to slight mucosal leakiness, and not any mechanism of secretion. As a result, the serum level of IgA is increased as the abundance of intestinal IgA enters circulation[42]. By inducing epithelial damage using oral administration of dextran sulfate sodium, the intestine becomes highly vulnerable to bacterial penetration, and severe inflammation arises from the resulting infections. IgA plays a critical role in preventing DSS-mediated inflammation, and $pIgR^{-/-}$ mice display more severe illness after DSS exposure[43].

The total pool of antigens that are recognized by sIgA is broad, and varies between high-affinity and low-affinity antibodies. High-affinity IgA generally neutralizes microbial proteins and toxins and prevents pathogen invasion. Low-affinity IgA is less-specific against particular microbes, but functions overall to regulate and confine commensal bacteria in the intestinal lumen. Through immune exclusion, low-affinity IgA targets and binds conserved microbial motifs in order to limit bacterial adherence to the epithelium, thereby passively inhibiting microbial infiltration of the intestinal epithelial

layer[44, 45]. In addition to controlling commensal motifs in the intestinal lumen, it has also been reported that IgA can neutralize microbial antigens within intestinal epithelial cells, during active transcytosis[46].

Mature naïve B cells primarily reside in the mesenteric lymph node or Peyer's patches and await activation from antigen exposure through the BCR. Subsequently, B cells undergo antibody-diversification through somatic hypermutation (SHM), and mediate antibody isotype switching through class-switch recombination (CSR). SHM introduces point mutations into immunoglobulin exons and allow for the generation of high-affinity antibodies. CSR remodels the heavy chain locus within the genome, and replaces the constant region exons that encode IgM and IgD with the constant region exons that encode IgG, IgA, or IgE in order to become isotype-switched antibody-secreting cells[47, 48]. Importantly, B cells that undergo CSR to IgA-producing cells have excised the constant region exons that encode all other antibody isotypes, such that differentiation is permanent as genes encoding all other antibody isotypes have been physically excised from the genome. Both SHM and CSR are facilitated by the DNA-modifying enzyme AID[49]. Notably, human B cells produce two IgA subtypes: IgA1 and IgA2, of which IgA2 is most common in mucosal regions and is more resistant than IgA1 to degradation by bacterial enzymes. However, mouse B cells only produce one IgA subtype[44].

Given the overall importance of IgA-mediated immune exclusion, and the overwhelming amount of IgA produced and secreted into the intestinal lumen (~5g/day), it comes as no surprise that many mechanisms are present for the regulation and induction of IgA in the intestinal environment. IgA induction occurs through two pathways: in a CD4 T cell-dependent manner, or a T cell-independent pathway. Most antigens initiate IgA responses through a T cell-dependent pathway that occurs within the lymphoid follicles of the MLN or Peyer's patches. Together with cytokine cues and BCR triggering, T cells provide CD40 ligand through direct cell-to-cell contact to prompt

CD40 signaling within B cells, inducing AID expression and setting off SHM and CSR. However, CD40 ligand is not sufficient to elicit an IgA response, thus requiring other cytokine cues. The conventional T cell-dependent pathway requires 5-7 days to generate the protective high-affinity antibody response[44]. The intestinal tract also utilizes the T cell-independent pathway of IgA induction, which occurs significantly faster, and is generally associated with the production of low-affinity antibodies focused against conserved bacterial motifs typically recognized by Toll-like receptors. As its name denotes, engagement of the BCR in the absence of CD40 ligand and T cell interaction can begin to instigate T cell-independent IgA induction. TLR activation is also paramount for T cell-independent IgA induction. TLR signaling on B cells with BCR engagement induces AID expression and subsequent SHM and CSR[50]. TLR signaling on innate immune cells and epithelial cells elicits expression of the two signaling molecules BAFF and APRIL, both of which are able to initiate IgA CSR in B cells[51].

The intestinal environment provides the ideal environment for facilitating the induction of IgA, as it is rich in the molecular cues that direct IgA CSR and antibody production. The majority of antigen-specific intestinal B cells are located within the mesenteric lymph nodes MLN and Peyer's patches PP, where they await antigen recognition and become antigen-secreting cells. This process is very rapid within the PP, as a result of constant stimulation by commensal bacteria, germinal centers are readily detectible in the PP[52]. B cell antigen-recognition is also not a requisite for germinal center formation in the MLN and PP, as bacterial ligands can activate TLRs on B cells and induce germinal center formation with the help of $CD4^+$ T follicular helper cells (Tfh)[53]. Contrary to peripheral lymph nodes, Peyer's patches are rich in cytokines and growth factors to induce IgA CSR and IgA production, rather than IgG. Dendritic cells are the major innate immune cell population in Peyer's patches and serve to act as immune sentinels and the key cell group for antigen processing and presentation. It has been shown that PP DCs function differently from peripheral or splenic DCs, and promote an

anti-inflammatory program. Analysis of PP DCs has shown that the constant microbial stimuli conditions PP DCs to secrete factors to induce IgA production. Stimulation of follicular DCs with TLR ligands[54] results in the induction of TGFβ, BAFF, and APRIL, critical for both T-dependent[55] and –independent[56] IgA isotype switching. Co-culture of PP FDCs with B cell leads to enhanced generation of IgA^+ B cells[54]. Further studies have revealed that PP DCs produce considerable amounts of IL-6 and retinoic acid after uptake of antigen, or in response to LPS, relative to splenic DCs[57, 58]. IL-6 selectively enhances IgA production from IgA^+ B cells, whereas retinoic acid augments cytokine-mediated IgA production, as well as enhancing TGFβ-mediated CSR[59, 60].

CD4 T cells are integral in establishing an effective IgA response, in terms of antigenic specificity as well as quantity. T cells initiate T-dependent antibody responses by CD40 ligand-CD40 binding and induce CSR and B cell proliferation. Analysis of $TCR\beta\delta^{-/-}$ mice, which lack the β- and δ- chains of the TCR and subsequently lack all functional T cells, display severely reduced fecal IgA levels. We have previously shown that regulatory T cells can promote IgA production from naïve B cells. Transfer of $CD25^+$ Treg cells into T cell-deficient $TCR\beta\delta^{-/-}$ mice partially restores intestinal IgA levels, while the depletion of $CD25^+$ Treg cells from wild-type mice greatly decreases IgA[61]. By utilizing transgenic Treg cells that respond to CBir1, an immunodominant bacterial flagellin, our previous work indicated that Treg cells are able to direct IgA responses against a specific antigen, as well as augmenting overall IgA responses against unrelated commensal antigens. Cultures of Treg cells with intestinal B cells increased both CBir1-specific IgA as well as total IgA. From this, Treg cells induced IgA CSR from naïve B cells through production of TGFβ, as well as augmenting overall IgA production by facilitating IgA^+ B cells proliferation and survival[61].

Chapter 4: Commensal Bacteria and the Adaptive immune response

The intestinal surface interfaces with a tremendous bacterial load. The diversity within the bacterial community is readily apparent, as the microflora is composed of at least 500-1000 species, containing an estimated 3.3 million non-redundant genes[62]. Given the immense population residing within the intestine, the microbial population has a profound influence upon human physiology, nutrition, and disease. The intestinal immune system and the commensal microflora have evolved together throughout time to take advantage of this unique symbiotic relationship. Accordingly, the immune system employs a variety of mechanisms to shape the microbial population to maximize homeostasis and limit colonization by pathogenic organisms. In turn, various members of the commensal microflora are able to shape the development of the immune system.

The effect that microbial colonization has upon the intestinal environment is best illustrated in germ-free mice, which lack any microbial colonization. Histological images of the intestines reveal that germ-free mice possess an underdeveloped immune system, and appear histologically similar to tissues from neonatal mice before commensal microbiota colonization. The development of gut-associated lymphoid tissues, including Peyer's patches, is greatly diminished, and with that, levels of CD4 T cells and B cells are greatly reduced[63]. Additionally, $CD4^+$ T cell and B cell function is compromised in germ-free mice, as germinal centers are mostly absent in the intestine, and the spleen and lymph nodes have abnormal morphology which lack structured B- and T cell- zones[64]. As such, serum IgG and intestinal IgA is greatly reduced[65, 66]. Colonization of these mice with a representative microflora reverses all of these abnormalities within weeks[66]. Gene expression profiles of intestinal epithelial cells also changes greatly upon microbial colonization, particularly genes that strengthen barrier immunity[67].

A number of bacterial components have been found to exhibit some influence upon the function of the intestinal adaptive immune system. Among the most far-reaching are the effects of TLR signaling upon intestinal lymphocytes. The interaction of TLRs with microbial ligands has a clear role in innate immune responses by activating innate cells and shaping their production of cytokines and chemokines. Direct TLR activation on B cells and CD4 T cells can also regulate their function, independent of innate antigen-presenting cells. While the adaptive immune system is conditioned to respond to antigen-specific triggers, the activation of TLRs by broad, but conserved microbial ligands can modulate the adaptive response. Activation of TLRs on B cells in conjunction with BAFF or APRIL can initiate T cell-independent humoral immunity. In the absence of concomitant signals, TLR signaling can induce the upregulation of TACI, the receptor for BAFF and APRIL, ensuring a rapid antibody response. The combination of signals from TLRs and BAFF or APRIL induces AID expression, setting off SHM[51]. Activation of NF-κB is important for the induction of AID, likely explaining why CSR requires signals from both BAFF/APRIL as well as TLRs[68, 69]. As such, TLR ligands can boost specific antibody responses, and have been utilized as vaccine adjuvants[70], whereas TLR-deficiency can result in deficient antibody production[71].

TLR ligands can also affect CD4 T cell responses. MyD88$^{-/-}$ CD4 T cells have decreased proliferation in vitro, in spite of TCR activation through anti-CD3 antibody stimulation and presence of appropriate costimulation via anti-CD28 antibody. Substitution of TLR ligands in lieu of anti-CD28 further reveals that wild-type T cells can respond to TLR signaling, which can also act as costimulation in conjunction with TCR activation[72]. Using transfer models of colitis, CD4 T cell function appears impaired in the absence of MyD88. The CD45RB^{hi} population of CD4 T cells is comprised of naïve effector cells that cause colitis when transferred to immunodeficient Rag2$^{-/-}$ mice after interaction with the microbiota. However, MyD88$^{-/-}$ CD45RB^{hi} T cells are unable to cause colitis. Further, co-transfer to wild-type Treg cells with wild-type CD45RB^{hi} T

cells can prevent the onset of colitis, however, MyD88$^{-/-}$ Treg cells are not capable of preventing colitis[72]. During severe intestinal inflammation, there is extensive tissue damage, leading to disruption of the epithelial barrier. This allows increased breach of the epithelium by microbes, which can subsequently exacerbate colitis by introducing TLR ligands into the environment. Our work has revealed that, during chronic intestinal inflammation, TLR4 signaling decreases Foxp3^{+} Treg frequencies in the intestines. Additionally, LPS and TLR4 signaling can inhibit the generation of Treg cells.

While the entire microbiota has a profound influence on the intestinal environment, and the host as a whole, distinct members of the microbiota can engage specific components of the immune system. As such, the composition of the intestinal microbiota can be one factor for susceptibility to inflammatory bowel diseases. An increasing number of commensal bacteria are being identified that can promote anti-inflammatory responses through the induction of Treg cells. Colonization of germ-free mice with *Bacteroides fragilis* led to lymphoid organogenesis and a restoration of the intestinal immune system. It was then discovered that these effects were due to the production of *B. fragilis* polysaccharide A (PSA)[73]. PSA treatment was also able to induce IL-10 from CD4 T cells and reduce experimental colitis[74]. *B. fragilis* and other commensal species can also breakdown undigestible fibers, and produce short chain fatty acids (SCFA) as a result of fermentation. Notably, these SCFA are also capable of increasing Foxp3^{+} Treg cells, as well as increasing IL-10 production independently of TGFβ, and can ameliorate effector T cell-driven colitis[75].

Specific commensal bacteria have been shown to influence other aspects of the intestinal immune system as well. In particular, colonization of mice with segmented filamentous bacteria (SFB) restores the strong production of IgA in the intestine. Colonization of germ-free mice with uncharacterized gut flora that lacks SFB will partially restore IgA production, but to a lesser extent than in mice colonized solely with SFB. Upon monoassociation with SFB, levels of IgA-secreting cells expand rapidly,

thereby also increasing serum IgA titers, indicating that SFB colonization is a strong proponent for maturation of the intestinal immune system[76]. The dynamic regulation between the microflora and the immune system becomes apparent in this case, as SFB are able to induce the IgA response. In turn, the secretion of IgA into the lumen functions to control and shape the commensal environment, as analysis of the microflora in $AID^{-/-}$ mice (which lack class switching and somatic hypermutation, and cannot direct an antigen-specific IgA response) revealed an extreme overgrowth and expansion of the anaerobic SFB population[39]. Other innate immune mechanisms, including antimicrobial peptides were not affected by the expansion of SFB, indicating that the IgA response is strongly and specifically shaped by the SFB population. Overall, the composition of the commensal microbiota is a product of its environment. Indeed, this led to the observation that mice from two different suppliers had very different frequencies of Th17 cells in the intestines. Further analysis revealed that mice colonized by SFB contained high numbers of Th17 cells, whereas the absence of SFB correlated with decreased levels of Th17 cells[77, 78]. These reports present a case where a single phylogenetic group of bacteria are critical for the induction of two seemingly-independent mechanisms of immune control within the intestines. Here, I demonstrate that Th17 cells play a vital role in regulating luminal IgA levels through the induction of pIgR on epithelial cells, and as such, controlling secretion of IgA and contributing to the maintenance of intestinal homeostasis.

RESULTS

Chapter 5: Th17 cells upregulate polymeric Ig receptor and intestinal IgA and contribute to intestinal homeostasis

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INTRODUCTION

T helper 17 (Th17) cells, a subset of $CD4^+$ T cells that primarily secrete Interleukin-17 (IL-17)A (also referred to as IL-17), IL-17F, IL-21, and IL-22, have been shown to be present in the intestinal lamina propria (LP), where they encounter a large number and diverse array of microbiota, commensal fungi, and food antigens[79]. Although accumulating evidence demonstrates that Th17 cells play a pathogenic role in a variety of inflammatory conditions[80], there is considerable controversy as to whether they also contribute to the maintenance of intestinal immune homeostasis. Both protective and pathogenic functions of the Th17 cytokine IL-17 have been reported in patients with inflammatory bowel diseases (IBD) and in experimental colitis. IBD patients often have increased levels of IL-17 in inflamed tissues[81, 82]. Specific inhibition of IL-17-producing Th17 cells by anti-IL-23p19 monoclonal antibody prevents, as well as treats, colitis in an adoptive T cell transfer model, further confirming a role for the IL-23/Th17 pathway in the pathogenesis of colitis[83]. Furthermore, IL-17 deficiency results in resistance to TNBS-induced colitis[82]. However, IL-17- and/or IL-17F-deficiency does not prevent

colitis mediated by transfer of naive $CD4^+$ T cells. Adoptive transfer of IL-$17^{-/-}$ $CD45RB^{hi}$ T cells, compared to wild type counterparts, induced a more severe wasting disease when transferred into $RAG^{-/-}$ mice, indicating a protective role of IL-17[84]. DSS-induced colitis has also provided conflicting reports of IL-17 involvement in intestinal inflammation[85, 86]. However, whether and how Th17 cells protect against chronic intestinal inflammation is still not understood.

IgA is enriched in mucosal secretions of the intestine[87]. Both T cell-dependent and T cell-independent mechanisms regulate intestinal IgA production[45]. IgA functions in the neutralization and clearance of extracellular pathogens by preventing adherence and access to epithelial surfaces[87]. Notably, germ-free mice that lack microbiota exhibit very low levels of intestinal IgA. Colonization with commensal microbiota restores IgA production. In particular, colonization with segmented filamentous bacteria (SFB) selectively increases IgA production and secretion[76, 88]. It has been separately reported that colonization of germ-free mice with SFB also selectively increases levels of Th17 cells in the intestines[78, 89]. The observations that SFB can induce both Th17 cells and IgA indicate that there could be a link between Th17 cells and IgA production/secretion. Produced by plasma cells in the mucosa, IgA secretion relies on transport across the intestinal epithelium, which is mediated by the polymeric Ig receptor (pIgR) expressed on the basolateral surface of epithelial cells[41]. After translocation, a portion of the pIgR is covalently linked to IgA and secreted in the form of secretory IgA (sIgA), thereby improving stability of the complex[90]. Expression of the pIgR is vital to IgA-mediated innate protection[42]. The rate of IgA secretion is limited by the rate in which IgA binds to the pIgR, and is therefore ultimately dictated by the expression levels of the pIgR[41]. Reductions in pIgR expression lead to decreased IgA-mediated protection against luminal antigens[42]. Previous studies inflicting epithelial injury and colitis revealed that secretory antibodies significantly contribute to protection of the intestinal mucosa and that mice deficient in the pIgR displayed greater disease than did wild-type mice[43]. A recent study

further demonstrated that Th17 cells increase pIgR expression in the bronchial epithelium in response to inhaled antigen[91]. However, whether and how Th17 cells regulate intestinal IgA and pIgR expression, and whether the Th17-IgA axis contributes to intestinal homeostasis are unknown. In this report, we demonstrate that Th17 cells contribute to the maintenance of host immune homeostasis against microbiota at least partially via IL-17 induction of epithelial pIgR expression, thereby increasing IgA secretion into the lumen. In the context of intestinal inflammation, mice that lack IL-17 signaling displayed more severe inflammation than their counterparts, correlating with decreased pIgR expression and subsequent IgA secretion.

MATERIALS AND METHODS

Mice. C57BL/6 and TCRβxδ$^{-/-}$ mice were obtained from Jackson Laboratory. IL-17R$^{-/-}$ mice were kindly provided by Amgen. CBir1 flagellin-specific TCR transgenic (CBir1-Tg) mice were maintained in the Animal Facilities at University of Texas Medical Branch. 8-12 week-old mice were used for all experiments. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the UTMB. All the mice strains were bred in the UTMB animal facility, and housed together from 3 weeks of age. All mice contain SFB as verified via PCR..

Antibody and reagents. Antibodies against IL-17A, CD45.2, and avidin were purchased from Biolegend. Neutralizing antibody to IL-17A was kindly provided by Merck. Mouse recombinant IL-6, IL-12, and human recombinant IL-17A, TNFα, TGFβ1 were purchased from R&D Systems. Antibodies against IgA were purchased from Kirkegaard & Perry Labs. Antibodies against pIgR and Actin were purchased from Santa Cruz Biotechnology. Anti-μ was purchased from Jackson ImmunoResearch Laboratories. Antibodies against phosphorylated NF-κB-p65 and total NF-κB-p65 were purchased from Cell Signaling. NF-κB inhibitor Bay11-7082, PI3K inhibitor LY294002, and all-trans-retinoic acid were purchased from Sigma-Aldrich.

Polarization of Th17 and Th1 cells. $CD4^+$ T cells were isolated from spleens of CBir1 Tg mice using anti-mouse CD4-magnetic beads (BD Biosciences) as previously described[61]. To polarize Th17 cells, CBir1-Tg $CD4^+$ T cells were cultured with 10ng/ml TGFβ1, 20ng/ml IL-6, 10μg/ml anti-IFNγ, and 10μg/ml anti-IL-4[92] with irradiated splenic APC. After 5 days, cells were stimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml), and isolated with a capture complex of avidin, biotinylated-CD45.2, and biotinylated-IL-17A antibodies. Cells were counterstained with fluorescence-labeled antibodies for IL-17A, CD4, and CD45.2, and sorted by flow cytometry with >97% purity. To polarize Th1 cells, CBir1-Tg $CD4^+$ T cells were cultured with 10ng/ml IL-12 and 10μg/ml anti-IL-4.

Fecal pellet preparation. Fecal pellets were homogenized in PBS containing 0.04mg/ml soybean trypsin inhibitor, 20mM EDTA, and 2mM PMSF and centrifuged to remove bacteria and insoluble debris as described previously[93]. Commensal bacterial lysate was prepared by homogenizing cecal contents and centrifuging to remove insoluble debris as described previously[93].

ELISA. 96-well plates (Nunc) were coated with 1μg/ml anti-IgA (Kirkegaard & Perry Labs) or 0.5μg/ml anti-pIgR (R&D Systems) or 1μg/ml of commensal bacterial lysate overnight at 4°. The plates were washed in PBS/Tween and blocked in PBS with 1% BSA. Fecal samples were diluted 1/100 and a 2-fold serial dilution was made. Samples were incubated at room temperature for 2 hours. 0.25μg/ml of biotinylated anti-IgA (KPL) was added for one hour, followed by HRP-conjugated streptavidin (KPL) for one hour. Plates were developed using a two-component TMB substrate (KPL) according to the manufacturer's instructions, and plate was analyzed at 450nm. Results were quantified by normalizing to standard concentrations of IgA (Southern Biotechnology Associates).

Quantitative Real-Time PCR. RNA was extracted with TRIzol (Invitrogen) and followed by cDNA synthesis with Revertaid reverse transcriptase (Fermentas). Quantitative PCR was performed using TaqMan Gene Expression Assays. Predesigned primers and probes for *PIGR* and *GAPDH* were ordered from Applied Biosystems, and data were normalized to *GAPDH* mRNA expression.

Dextran sulfate sodium induction of colitis. As described previously[94], dextran sulfate sodium (DSS) (MP Biomedicals) was dissolved into drinking water and administered to mice *ad libitum*. For acute colitis, 2.5% w/v DSS was administered over seven days, followed by 3 days of fresh water. For chronic colitis, 1.75% DSS was administered for seven days, followed by 3 days of fresh water and repeated over 60 days.

Histopathologic assessment. At necropsy, the small intestine, cecum, and colon were separated and Swiss rolls of each prepared. Tissues were fixed in 10% buffered formalin and paraffin embedded. 5μm sections were sliced, stained with H&E, and blindly scored by an experienced pathologist. Histological scoring was performed using a modification of scoring system reported previously[95]. In brief, longitudinal sections were examined for crypt epithelial hyperplasia, degeneration, loss; goblet cell loss; crypt exudate; LP and submucosal inflammatory cell accumulation; submucosal edema; mucosal ulceration; and transmural inflammation. Each lesion component was scored 1, 2, or 3 for mild, moderate, or severe, respectively (intensity), and 0 for absent, or 1, 2, 3, or 4 for 25, 50, 75, or 100% of the tissue affected, respectively (extent). The total lesion severity score was calculated by summation of the products of extent and intensity scores for each individual lesion component.

TGF-β bioassay. As described previously[96], MFB-F11 cells are embryonic fibroblasts from *Tgfb1*$^{-/-}$ mice that are stably transfected with a reporter plasmid consisting of TGF-β responsive Smad-binding elements coupled to a secreted alkaline phosphatase reporter gene. Secreted alkaline phosphatase activity shown as

chemiluminescence units was measured using Great EscApe SEAP Chemiluminescence kit 2.0 (Clontech) following the manufacturer's instructions and represents biologically active TGF-β activity.

Bacterial enumeration. Mesenteric lymph nodes were isolated and homogenized in 500μL PBS. 10μL was spotted onto blood agar plates (BD Biosciences) in serial dilution and incubated at 37°C under aerobic and anaerobic conditions. Anaerobic cultures were placed in a sealed jar with a lit candle to induce a microaerophilic environment

Statistical analysis. For comparisons between samples, levels of significance were determined by Student's *t* test in Prism 5.0 (Graphpad). Where appropriate, mean ± SEM is represented on graphs. $*p < 0.05$; $**p < 0.01$.

RESULTS

Low levels of intestinal IgA and pIgR in IL-17 receptor deficient mice

Analysis of fecal content in mice deficient in IL-17R (IL-17R$^{-/-}$) revealed that the level of IgA was significantly decreased in the absence of IL-17 signaling as compared to wild-type mice (**Figure 1A**). It has been shown that the pIgR mediates the translocation of IgA into intestinal lumen, and a portion of the pIgR is secreted with IgA to improve stability[90]. Further analysis of fecal content revealed that the level of the pIgR was also significantly reduced to a similar level as IgA in IL-17R$^{-/-}$ mice (**Figure 1B**), indicating that the deficiency in intestinal IgA is partially due to a decrease in secretion. *Pigr* mRNA was also decreased in both the small intestines and large intestines of IL-17R$^{-/-}$ mice (**Figure 1C**), indicating that the reduction in fecal pIgR levels was not from variable levels of protein degradation. While TLR signaling on epithelial cells can regulate pIgR expression[97, 98], the large intestines contain significantly greater numbers of microflora

than the small intestines. These data indicate that IL-17 signaling regulates pIgR expression independent of microbiota.

Transfer of Th17 cells results in increased pIgR and IgA in TCRβxδ$^{-/-}$ mice

Although both T cell-dependent and -independent pathways are involved in regulation of IgA production, CD4^{+} T cells play a significant role in the induction of the pIgR and secretion of IgA into the intestine, as TCRβxδ$^{-/-}$ mice have significantly lower amounts of fecal IgA[61] (**Figure 2A**), as well as pIgR (**Figure 2B**). Because IL-17 is predominantly produced by Th17 cells which are enriched in the intestine, we asked whether the presence of Th17 cells could influence pIgR expression and intestinal IgA secretion. We generated Th17 cells by polarizing CD4^{+} T cells from CBir1 Tg mice, which are specific for an immunodominant microbiota antigen[61, 99], under standard Th17 conditions with TGFβ and IL-6, and transferred them into TCRβxδ$^{-/-}$ mice. Th17 cells were also generated from OTII transgenic mice, which are specific for the model antigen ovabumin (OVA) that is not present in intestinal lumen, and transferred into TCRβxδ$^{-/-}$ mice to serve as a control for antigen-specific stimulation in the intestines. The mice were sacrificed and *Pigr* mRNA expression was measured in intestinal tissue 30 days later. Intestines displayed significant increases in *Pigr* mRNA after transfer of CBir1 Th17 cells, as compared to native TCRβxδ$^{-/-}$ mice receiving only PBS or OVA-specific OTII Th17 cells (**Figure 2C**). Increases in fecal IgA and pIgR were apparent after approximately 1 week and continued to increase for the duration of the experiment (**Figure 2D**). This is consistent with a recent report that revealed that specific antigen-stimulation was required for Th17 cells to induce pIgR from the bronchial epithelium[91]. T regulatory cells (Treg) have been shown to promote intestinal IgA production through production of TGFβ[61]. As Th17 cells are not stable and are able to convert into Treg cells[100, 101], we measured TGFβ production in the intestine of TCRβxδ$^{-/-}$ mice that

received CBir1 Th17 cells or PBS to determine if TGFβ was involved in Th17 cell promotion of intestinal IgA. The intestines from both groups of mice produced TGFβ at a comparable level (**Figure 2E**). Neutralization of IL-17A significantly decreased the amount of IgA present in the fecal content (**Figure 2F**). Adoptive transfer of CBir1 Th1 cells slightly increased total IgA and antigen-specific IgA, but not to the extent seen in the transfer of CBir1 Th17 cells. Furthermore, neutralization of IL-17A decreased fecal IgA levels comparable to the transfer of CBir1 Th1 cells, signifying that the increases in intestinal IgA as a result of Th17 cell transfer is not solely due to the presence of T cell-mediated help, but that IL-17A contributes to IgA secretion as well. Collectively, our data indicates that Th17 cells increases pIgR expression and IgA secretion *in vivo*.

Th17 cells induced B cell IgA production *in vitro*.

To determine whether Th17 cells directly induce B cell IgA production *in vitro*, splenic IgD^+ B cells were cultured with *in vitro* polarized CBir1 Tg Th17, Th1, and unpolarized T cells (Th0). B cells were cultured with anti-μ, CD40L, TGFβ, and retinoic acid (RA) to serve as positive control[59]. B cells were also cultured with *in vitro* polarized OTII Th17, Th1, and Th0 cells, without the presence of OVA. Total IgA in the supernatant was measured at day 5. As shown in **Figure 3**, CBir1 Th17 cell greatly promoted IgA production, whereas CBir1 Th1 and Th0 cells only slightly enhanced IgA production. However, OTII T cells did not promote IgA production in the absence of their cognate antigen, indicating that the T cell activation and production of effector cytokines are required for Th17 cell-mediated induction of IgA. Taken together, Th17 cells were more adept at promoting IgA secretion in an antigen-specific manner, both by directly inducing IgA production as well as pIgR expression.

IL-17 directly induces pIgR expression from epithelial cells through NF-κB and PI3K

To further elucidate the role of IL-17 on the induction of pIgR, we asked if IL-17 signaled directly upon intestinal epithelial cells to produce pIgR, or whether there was another intermediate. Treatment of HT-29 human colon epithelial cells with human IL-17A resulted in an increase of *PIGR* mRNA, in a time and dose-dependent manner, appearing as soon as two hours after IL-17A treatment (**Figure 4A**). This induction of *PIGR* mRNA also mirrors the induction by TNFα (referred to as TNF), which is also produced by Th17 cells[102], and is known to be a potent stimulator of pIgR[103]. Most notably, the combination of human IL-17A and TNF resulted in very strong induction of *PIGR* at all time points (**Figure 4A and B**). This increase in pIgR expression was greater than expected from the two cytokines alone, and suggests a strong synergism between IL-17A and TNF. The effect of IL-17A and synergism of IL-17A and TNF appeared to last beyond 24 hours, as *PIGR* mRNA steadily increased, whereas the effect of TNF began to decline at 24 hours (**Figure 4A**).

Previous reports have detailed that IL-17 can stimulate a number of cytokines and anti-microbial peptides, and that this upregulation occurs through NF-κB[80, 104] as well as PI3 kinase activation[104]. In order to ascertain the mechanisms of IL-17A-mediated *PIGR* mRNA induction, we examined the effect of IL-17A and the synergism of IL-17A and TNF on NF-κB activation. IL-17A was able to rapidly induce phosphorylation of p65, indicative of activated NF-κB signaling. (**Figure 4C and D**).

Next we questioned whether IL-17-induced pIgR was mediated through the NF-κB and PI3K pathways. We included inhibitors specific for NF-κB (Bay11-7082, 10μM) and PI3K (LY294002, 10 μM) pathways to HT-29 cells cultured with IL-17A and TNF and *PIGR* mRNA was measured 4 h later. Blocking NF-κB activity greatly reduced levels of *PIGR* mRNA induced by IL-17A, TNF, or the combination of both IL-17A and TNF

(**Figure 4E**). However, inhibition of either pathway alone does not result in significant abrogation of *PIGR* transcription, which could be due to the short treatment time as it has been demonstrated that *PIGR* mRNA response to TNF stimulation in HT-29 cells peaks at 24 h[105, 106]. Blocking both pathways at once resulted in significant downregulation of *PIGR* mRNA under all treatments, however did not completely shut down *PIGR* transcription, therefore signifying that while NF-κB and PI3K signaling may be identified as the major pathways involved, they do not appear to be the only pathways activated.

More severe colitis in IL-17R$^{-/-}$ mice with chronic DSS

Previous reports have presented conflicting results on the role of IL-17 in IBD. Some reports have suggested a pathogenic role for IL-17 in the development of colitis[82, 86], whereas other work details that IL-17 may alleviate disease[85]. Next, we wanted to assess if there was a functional deficiency in epithelial protection in the absence of IL-17 signaling. We subjected IL-17R$^{-/-}$ mice to intestinal injury through DSS administration to determine if the decrease in intestinal IgA played a significant role in protecting the epithelium. Therefore we settled upon a sub-optimal dose of DSS that would not inflict significant injury in wild-type mice, but still injure the IL-17R$^{-/-}$ mice. Fecal pellets were collected and IgA and pIgR levels were quantified before colitis induction. Administration of 1.75% DSS induced colitis after five days in the IL-17R$^{-/-}$ and control mice, and continued over six cycles of seven days of DSS administration, followed by three days of fresh water. Disease progression was characterized by weight loss and visual examination of loose/bloody stool every 48 hours. As shown in **Figure 5A**, the IL-17R$^{-/-}$ mice displayed more significant disease as witnessed by increased weight loss, loose, mucoidal, and bloody stool. Weight loss and recovery in the control mice were responsive shortly after the switch from DSS to water. IL-17R$^{-/-}$ mice showed a delayed recovery in weight at the end of the first cycle, and continued to display irregular

responses to the treatment cycles. As a whole, IL-17R$^{-/-}$ mice suffered from a more severe colitis than the control mice, detailing that IL-17 provides significant protection in chronic DSS colitis. Whereas the control mice recovered their weight after the initial cycle of DSS, the IL-17R$^{-/-}$ mice repeatedly lost over 10% of their body weight with each cycle. Interestingly, mice that expressed the lowest levels of fecal IgA and pIgR under healthy conditions before DSS administration went on to exhibit a more severe disease and more severe weight loss than mice that expressed higher levels of IgA and the pIgR (**Figure 5B and C**).

Blockade of IL-17 increases severity of acute colitis in response to DSS

To further address the nature of IL-17 in the context of IBD, we injected a neutralizing antibody to IL-17A into C57BL/6 mice, followed by DSS administration. As shown in **Figure 6A-C**, mice that received neutralizing antibody to IL-17A demonstrated more severe colitis than mice receiving a control antibody after ten days, as measured by weight loss and histological examination. The differences were seen in weight loss after six days of DSS administration although it did not reach statistical significance (**Figure 6A**), and the histopathological scores (Figure 6B-C), confirming a protective role of IL-17 in DSS-induced intestinal inflammation.

Increased commensal bacterial stimulation in IL-17 receptor deficient mice

Our data indicates a role of IL-17 in maintenance of intestinal homeostasis. We then questioned if the lack of IL-17 signaling would result in more commensal bacterial translocation with increased systemic response to commensal bacterial activities. There were more bacteria in the MLN of IL-17R$^{-/-}$ mice compared to that in wild-type mice (**Figure 6D**). Consistent with our previous observations[107], there was no serum IgG against commensal bacterial antigens in wild-type mice, but significant serum IgG

against the bacterial antigens was observed in wild-type mice immunized i.v. with commensal A4 bacteria[108]. In contrast, analysis of serum antibody titers revealed detectable levels of IgG specifically directed against commensal bacterial antigens in IL-17R$^{-/-}$ mice (**Figure 6E**). This signifies an important role for IL-17 signaling in the prevention of bacterial translocation across the epithelium, thereby limiting the activation of inflammatory responses against innocuous commensal antigens, both in the intestinal tract as well as systemically.

DISCUSSION

Despite enormous bacterial challenge, the host intestine establishes a mutualistic relationship with the microbiota. Multiple mechanisms have evolved to regulate this relationship. The intestinal tract has been shown as a natural site for Th17 cell development, which is stimulated by specific species of microbiota[89], with SFB being recently identified as one of such stimulators[78]. Although both pro- and anti-inflammatory functions of Th17 have been demonstrated in different experimental systems[82-86], the enrichment of Th17 cells in the intestine suggests a role for these cells in mucosal homeostasis and more specifically in the containment of the vast local microbiota. In consistency with this argument, our data demonstrated that Th17 cells are able to promote intestinal IgA secretion via induction of epithelial cell pIgR expression, thereby contributing to the maintenance of host immune homeostasis to microbiota.

One of the most important strategies to generate immune protection and maintain intestinal homeostasis is the production of IgA[87], which is the primary antibody in the gut. IgA regulates the microbiota, and bacteria in turn adapt to IgA by altering their gene expression patterns[109]. Although IgA also plays a role in host resistance to infection, it

has been argued that the major role of IgA in the intestine is in maintaining the balance between the host and its microbiota[110]. In the absence of pathogen exposure, specific pathogen free mice have abundant levels of IgA, whereas germ-free mice have very low levels of IgA[87]. B cell IgA production can be stimulated by DC-B cell or epithelial cell-B cell interactions via BAFF, APRIL, iNOS, and TLR ligands, or utilizing T cell help and a number of cytokines including TGFβ, IL-4, IL-6, and IL-10[45]. Although the relative contribution of T cell-dependent and –independent regulation to intestinal IgA production is still not completely understood, decreased levels of intestinal IgA in T cell-deficient TCRβxδ$^{-/-}$ mice compared to wild-type mice indicates a predominant role of the T cell-dependent pathway[61, 110]. However, which T cells provide help and the sources of cytokines needed for intestinal IgA production in the mucosa is still largely unclear. Although TGFβ has been shown as a crucial cytokine in promoting IgA class switching[45], and Treg cell production of TGFβ greatly contributes to intestinal IgA production[61], it cannot completely explain why high levels of IgA are present only in the intestine, but not other lymphoid tissues even though TGFβ are also present in those sites. Our data indicated that repletion of Th17 cells promoted intestinal IgA secretion in the TCRβxδ$^{-/-}$ mice. Blockade of Th17 cytokine IL-17 decreased intestinal IgA (**Figure 2**). Additionally, IL-17R deficiency resulted in lower intestinal IgA secretion compared to wild-type mice (**Figure 1**), indicating that Th17 cells and their signature cytokine IL-17 greatly contribute to intestinal IgA secretion. Promotion of IgA secretion is not due to Treg cells that were converted from Th17 cells as the intestinal tissues produced TGFβ at a similar level. Several types of innate cells have been recently identified in the intestines that could also provide sources of IL-17 to promote intestinal IgA production[111-113]. Indeed, a previous report showed that RORγt^{+} LTi cells but not RORγt^{+} CD4^{+} T cells induced T cell-independent LP IgA production in the absence of Peyer's Patches[114]. In RORγt deficient mice, transfer of RORγt^{+} LTi cells induced isolated lymphoid follicle (ILF) formation as well as LP IgA. However, transfer of RORγt^{+} CD4^{+} T cells did not

induce ILF or PP formation, nor intestinal IgA, indicating that in the absence of PP and ILF, Th17 cells would not be activated, thus would not produce cytokines required for induction of intestinal IgA. Several recent elegant studies demonstrated that communal microbiota greatly impact intestinal Treg, Th17 cells and IgA responses. SFB preferably induces intestinal Th17 cells[78] and IgA[78, 88], whereas colonization with Clostridium species and Schaedler flora which contain 8 known commensal bacteria including Clostridium induces Tregs[115, 116]. Interestingly, failure to activate Treg cells results in the induction of Th17 cells, thus commensal bacteria regulate the balance between Tregs and Th17 cells. As Tregs have been shown to promote intestinal IgA response[61], and we now show that Th17 cells are also able to upregulate intestinal IgA, the microbiota greatly influence intestinal IgA responses at least partially through regulation of Treg and Th17 cells.

IgA translocation across the intestinal epithelium is mediated by the polymeric immunoglobulin receptor (pIgR)[87]. IgA function in the intestinal lumen is dependent upon pIgR expression and thus, reduction in pIgR expression has been shown to lead to decreased IgA-mediated protection against luminal antigens[41]. Intestinal pIgR expression was lower in TCRβxδ$^{-/-}$ mice compared to wild-type mice, indicating a role for T cells in the induction of pIgR (**Figure 2**). Consistent with a previous report describing IL-17-mediated pIgR expression in airway epithelial cells[91], repletion of Th17 cells restored intestinal pIgR expression in TCRβxδ$^{-/-}$ mice and IL-17R deficiency resulted in lower expression of intestinal pIgR, demonstrating that Th17 and IL-17 signaling regulate intestinal epithelial pIgR expression. Indeed, treatment with IL-17 greatly increased HT-29 epithelial cell expression of pIgR, alone or synergistically with TNF. IL-17 was able to activate NF-κB p65 signaling in intestinal epithelial cells (**Figure 4**). Blockade of NF-κB signaling and PI3 kinase activity with selective chemical inhibitors inhibited IL-17 induction of pIgR. Interestingly, both pathways work independently in IL-17 signaling as the inhibition of either pathway did not result in strong abrogation of *PIGR* transcription;

only blockade of both pathways resulted in significant downregulation of *PIGR* mRNA. Intestinal Th17 cells require cognate luminal antigen stimulation to produce effector cytokines. Once cytokines are produced by the activated T cells, they regulate intestinal IgA production in an antigen non-specific manner.

Both intestinal pIgR and IgA have been implicated in maintenance of intestinal immune homeostasis, as deficiency of either pIgR or IgA results in greater commensal bacterial translocation across the intestinal epithelium, and more severe intestinal inflammation in response to DSS[42, 43, 107]. Thus, Th17 cell regulation of intestinal pIgR and IgA could play a crucial role in protection against intestinal inflammation induced by mucosal breach by commensal flora. Indeed, there was higher level of systemic anti-commensal bacterial IgG in IL-17R$^{-/-}$ mice but not in wild-type mice **(Figure 6E)**, indicative of the presence of commensal bacteria in the systemic immune system. This revealed that deficiency of IL-17 signaling resulted in more commensal bacterial translocation from lumen, and sequentially, to more severe intestinal inflammation in response to DSS **(Figure 5)**. Consistent with these observations, we also found higher numbers of bacteria in the mesenteric lymph nodes of IL-17R$^{-/-}$ mice **(Figure 6D)**. This is likely due to impaired intestinal pIgR expression and IgA secretion, although the induction of a number of cytokines and anti-microbial peptides from epithelial cells by IL-17 could also contribute to IL-17-mediated protection against intestinal inflammation. However, we cannot exclude the possibility that wild-type and IL-17R$^{-/-}$ mice may have differences in the composition of their respective gut microbiota which could have contributed to our results.

In summary, our data demonstrate that enriched microbiota antigen-specific Th17 cells protect the host from chronic inflammation and contribute to intestinal immune homeostasis by regulating epithelial pIgR expression, thereby promoting intestinal IgA. However, it certainly does not mean that this is the only function of Th17 cells that contributes to intestinal immune homeostasis as Th17 cells and IL-17 have been shown to

stimulate a number of cytokines and anti-microbial peptides which also contribute to the regulation of host immune responses to microbiota[104]. Treg cells have been shown to greatly promote intestinal IgA production via directly promoting B cell IgA class switching through production of TGFβ, now we show that Th17 cells promote IgA translocation across the intestinal epithelium via induction of pIgR by IL-17. Thus Treg and Th17 cells coordinately regulate intestinal IgA production and secretion. A deficiency in either pathway will result in decreased intestinal IgA, and disruption of intestinal immune homeostasis.

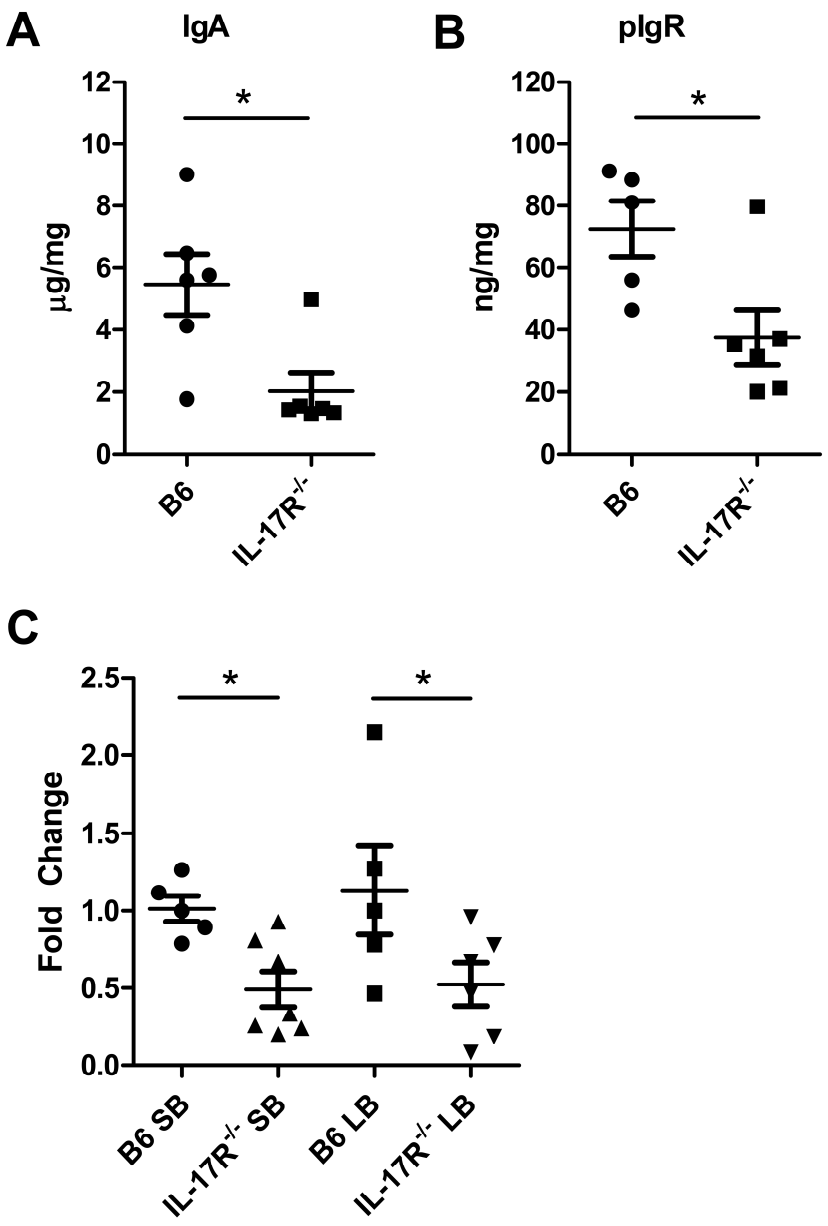

Figure 1. Intestinal IgA secretion and pIgR expression is decreased in IL-17R$^{-/-}$ mice. (**A, B**) Fecal pellets were collected from age-matched 8 week old wild-type or IL-17R$^{-/-}$ mice which were co-housed from 3 weeks old. IgA (**A**) and pIgR (**B**) levels were quantified through ELISA and normalized to total protein. *p<0.05. **(C**) *Pigr* mRNA was analyzed from intestinal tissue from wild-type or IL-17R$^{-/-}$ mice by RT-PCR. *Pigr* expression values were normalized to *Gapdh* expression. Significant differences are compared between respective tissues. *p<0.05 compared to wild-type mice. SB: small bowel: LB: large bowel

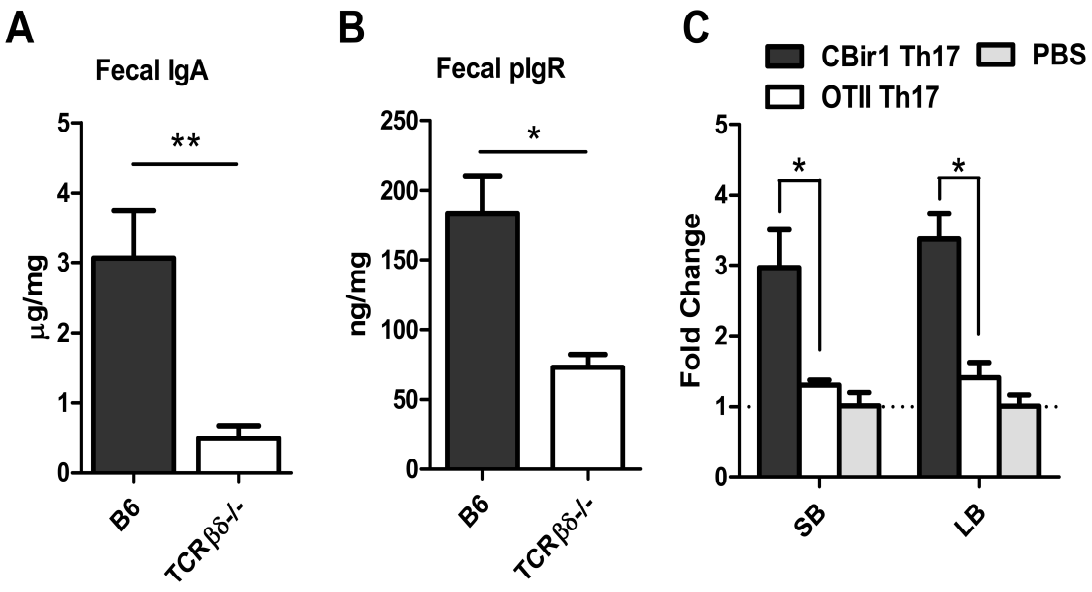

Figure 2. TCRβxδ$^{-/-}$ mice have lower intestinal pIgR and IgA production, and transfer of Th17 cells into TCRβxδ$^{-/-}$ mice increases pIgR expression and IgA secretion. (A, B) Fecal pellets were collected from age-matched 8 week old wild-type and TCRβxδ$^{-/-}$ mice. IgA (**A**) and pIgR (**B**) levels were quantified through ELISA and normalized to total protein. *p<0.05, **p<0.01. N=5 mice per group. (**C**) In vitro-polarized Th17 cells from CBir1 or OTII TCR transgenic mice were transferred IV into TCRβxδ$^{-/-}$ mice. After 30 days, intestinal tissue was obtained from Th17 recipients or control TCRβxδ$^{-/-}$ mice receiving PBS, and *Pigr* mRNA was analyzed from intestinal tissue by RT-PCR. mRNA was normalized to *Gapdh* mRNA. Significant differences are compared to respective tissues. *p<0.05. SB: small bowel: LB: large bowel.

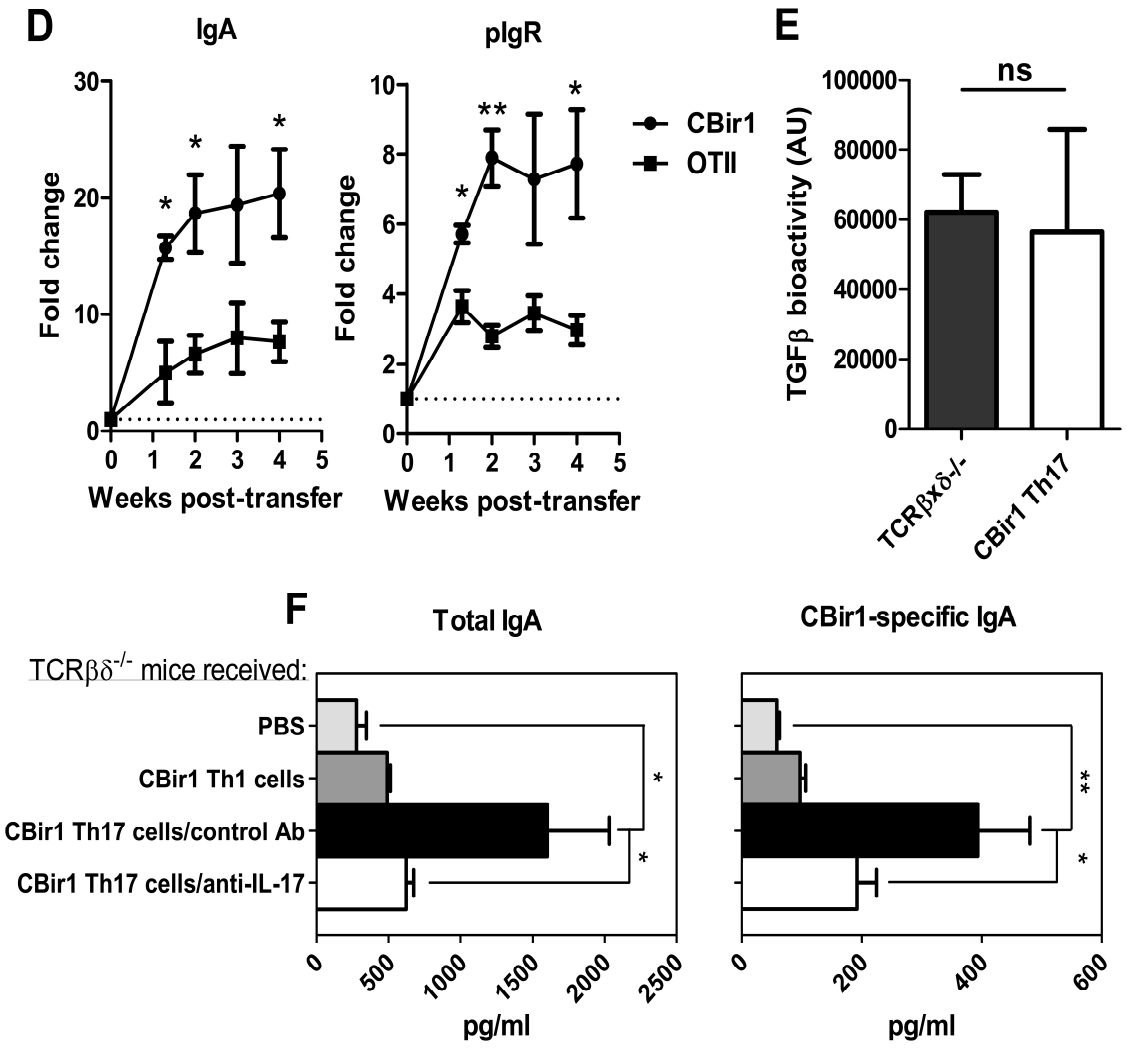

Figure 2. TCRβxδ$^{-/-}$ mice have lower intestinal pIgR and IgA production, and transfer of Th17 cells into TCRβxδ$^{-/-}$ mice increases pIgR expression and IgA secretion. (**D**) Fecal pellets were collected from Th17 cell recipients during the course of the experiment. IgA and pIgR levels were quantified through ELISA and normalized to total protein. Changes in expression over time are expressed as a fold change from individuals pre-transfer. *p<0.05, **p<0.01. N=4 mice. (**E**) Intestinal biopsies from CBir1 Th17 recipients or control TCRβxδ$^{-/-}$ were cultured for 24 hours. Supernatant was collected and cultured with MFB-F11 cells. Secreted alkaline phosphatase was measured as a reflection of TGFβ bioactivity. (**F**) Th17 and Th1 cells from CBir1-Tg mice were transferred IV into TCRβxδ$^{-/-}$ mice. Recipient mice were subsequently injected with a neutralizing antibody to IL-17A, or isotype control. Fecal pellets were collected from recipient mice, and total IgA and CBir1-antigen-specific IgA were quantified through ELISA. *p<0.05, **p<0.01. N=4 mice per group.

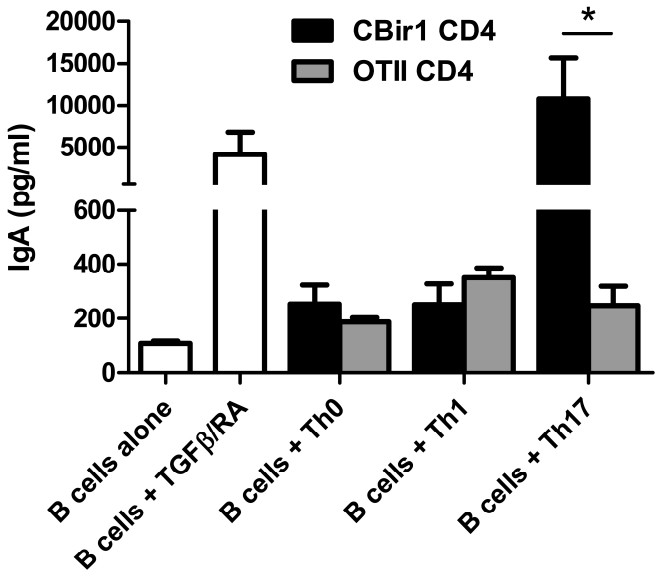

Figure 3. Th17 cells induce IgA production from B cells. In vitro-polarized Th1, Th17 or unpolarized (Th0) cells from CBir1 Tg or OTII mice were co-cultured with splenic IgD^+ B cells in the presence of CBir1 antigen. B cells were also cultured with anti-μ, CD40L, with or without TGFβ and retinoic acid. Five days later, supernatant was collected and total IgA production was quantified by ELISA. *$p<0.05$.

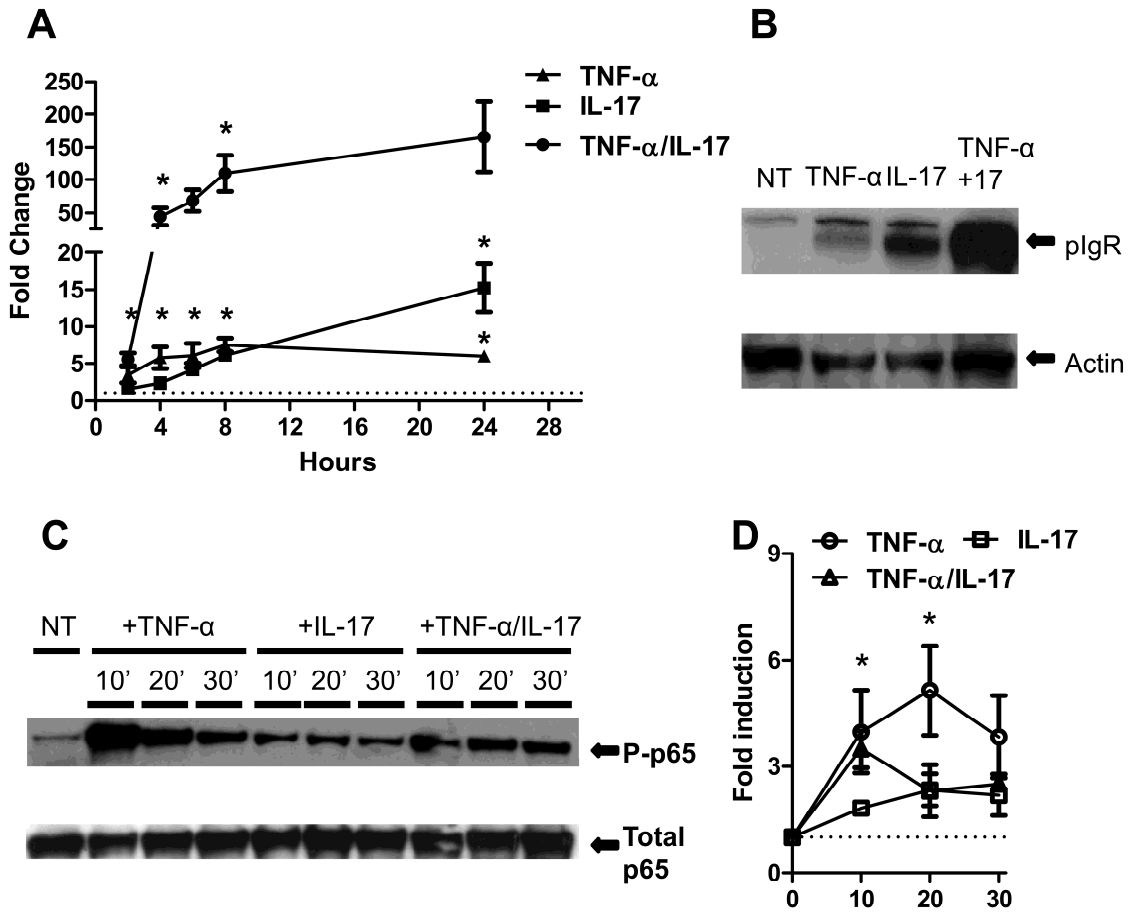

Figure 4. IL-17 upregulates pIgR in epithelial cells through activation of PI3K and NF-κB pathway (A) HT-29 cells were treated with human TNF (10ng/ml), IL-17A (20ng/ml), or TNF and IL-17A for the hours indicated. *PIGR* mRNA expression was analyzed by RT-PCR, and normalized to *GAPDH* mRNA. Significant differences are compared to non-treated controls. *$p<0.05$ compared to non-treated cells. Data reflects three independent experiments. (**B**) HT-29 cells were treated with TNF, IL-17A, or TNF and IL-17A for 48 hours. PIgR expression was detected by Western blot, with Actin as a loading control. One of two experiments with similar results was shown. (**C, D**) HT-29 cells were treated with TNF, IL-17A, or TNF and IL-17A for the time indicated. Phosphorylated NF-κB p65 was detected by western blot (**C**), with total NF-κB p65 as a loading control. (**D**) Relative increase of phosphorylated p65 over non-treated cells, as a percentage of total NF-κB p65. One of two experiments with similar results was shown. *$p<0.05$ of IL-17 treated cells compared to non-treated cells.

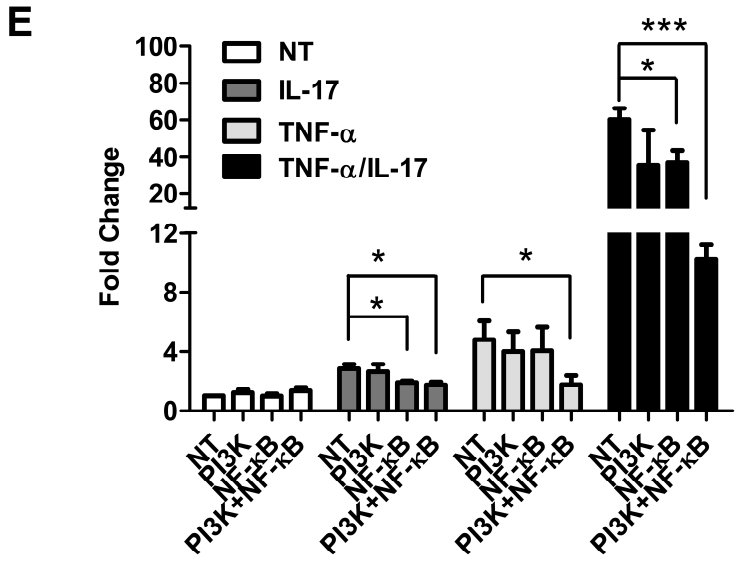

Figure 4 (cont'd). IL-17 upregulates pIgR in epithelial cells through activation of PI3K and NF-κB pathway (**E**) HT-29 cells were treated with PI3K inhibitor LY249002 (10uM) or NF-κB inhibitor Bay11-7082 (10uM) for one hour, then treated with TNF, IL-17A, or TNF and IL-17A for four hours. *PIGR* mRNA expression was analyzed by RT-PCR, and normalized to *GAPDH* mRNA. Significant differences are compared to nontreated controls. *$p<0.05$ compared to non-treated cells. Data reflects five independent experiments.

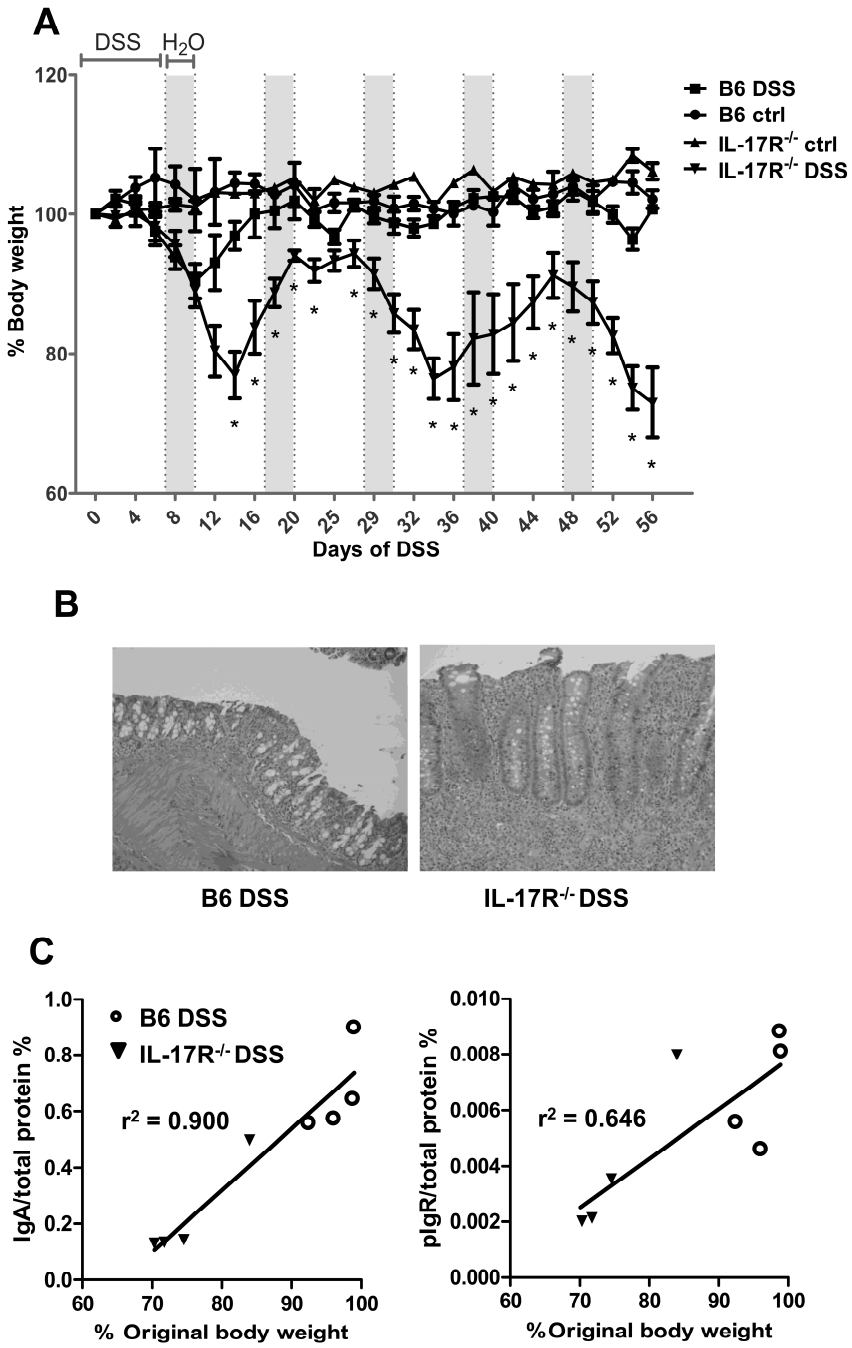

Figure 5. IL-17R$^{-/-}$ mice suffer worsened colitis as a result of decreased pIgR and IgA secretion. (**A**) Age-matched wild-type and IL-17R$^{-/-}$ mice, which had been co-housed from 3 weeks old, were administered 1.75% DSS in drinking water and weight was measured every 2 days. After 7 days of DSS, drinking water was replaced with fresh water for 3 days, and the cycle was repeated over 60 days. Weights are shown as a percentage of individual weight on Day 0. Significant differences are compared between strains on DSS. *$p<0.05$ compared to wild-type mice. N=4 mice per group. (**B**) Colonic histopathology of DSS-treated mice after 60 days of DSS administration. (**C**) IgA and pIgR in fecal pellets were quantified from mice by ELISA before DSS administration, and plotted against their individual body weight after 54 days.

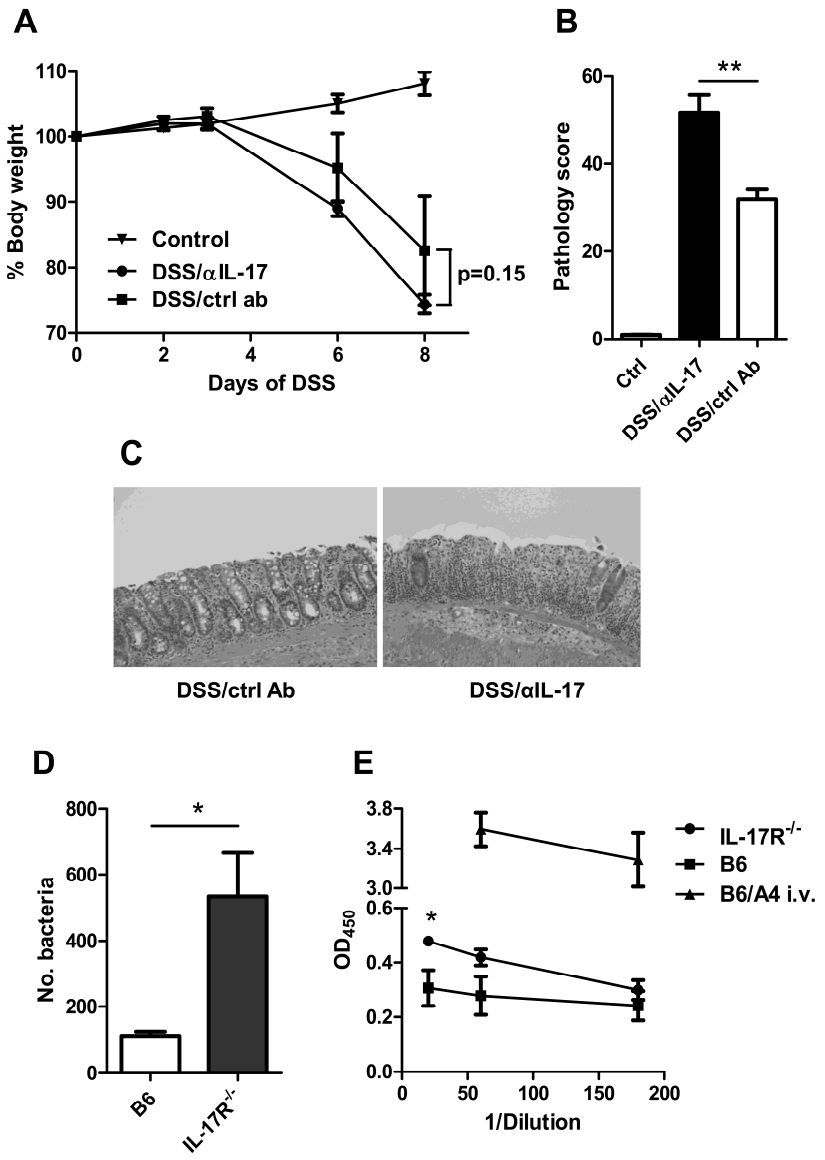

Figure 6. Blockade of IL-17 induces more severe colitis from DSS administration and bacterial translocation is increased in IL-17R$^{-/-}$ mice. (**A**) C57BL/6 mice were IP injected with a neutralizing antibody to IL-17A, or isotype control, and administered DSS for seven days. Weights are shown as a percentage of body weight on Day 0. (**B**) Pathological score of colitis was examined by blind histological observation 10 days after DSS administration. N=4 mice per group. **p<0.01 compared to the mice treated with control mAb. (**C**) Colonic histopathology of the DSS-treated mice after 10 days of DSS administration. (**D**) Mesenteric lymph nodes were harvested from wild-type or IL-17R$^{-/-}$ mice under aseptic conditions. MLN homogenates were cultured onto blood agar plates and incubated in aerobic and anaerobic conditions at 37°C. Aerobic cultures were incubated overnight, anaerobic cultures were incubated for 3 days. *p<0.05 compared to wild-type mice. N=3 mice. (**E**) Serum IgG against commensal bacterial lysate were quantified from wild-type or IL-17R$^{-/-}$ mice by ELISA. N=4 mice per group. *p<0.05 compared to wild-type mice. Wild-type mice were injected i.v. with 200 μg of A4 bacteria to indicate relative amount of serum IgG.

Chapter 6: Th17 cell control of IgA

INTRODUCTION

Th17 cells are present in the intestinal lamina propria, where they encounter a diverse assortment of microbiota, fungi, and food antigens. Through production of inflammatory cytokines, including IL-17A, Il-17F, IL-21, TNFα, and GM-CSF, pathogenic functions have been described for Th17 cells during inflammatory autoimmune disorders, including inflammatory bowel diseases (IBD). Indeed, patients with IBD have increased levels of IL-17 in inflamed tissues and particularly in their inflamed lesions. Blockade of Th17 cells by anti-IL-23p19 neutralizing antibody prevents, as well as treats colitis in an adoptive T cell-transfer model[83]. However, IL-17 deficiency did not prevent colitis initiated by the transfer of naïve CD4 T cells[84]. Adoptive transfer of CD45RBhi T cells induces colitis when transferred into immunodeficient $Rag^{-/-}$ mice after robust Th1 and Th17 responses. However, transfer of IL-$17^{-/-}$ CD45RBhi T cells induced a worsened wasting disease after transfer into $Rag^{-/-}$ mice, reflecting a protective role for IL-17 during colitogenesis[84]. It remains unclear whether and how Th17 cells protect against chronic inflammation.

Within the intestine, B cells produce large amounts of IgA, and IgA is enriched in mucosal secretions. IgA functions to neutralize and aid in clearance of extracellular pathogens by preventing adherence to epithelial surfaces and limiting access to the intestines and the immune system. The high production of IgA is driven by microbial colonization of the intestine, as germ-free mice have very low levels of IgA, and low levels of IgA+ B cells, whereas colonization with commensal bacteria restores IgA production[87]. Notably, monocolonization of germ-free mice with segmented filamentous bacteria (SFB) selectively increases IgA production and secretion[76], and intestinal IgA-deficiency in wild-type mice leads to SFB overgrowth[39]. It has also been independently

reported that SFB colonization selectively increases populations of Th17 cells in the intestines[77, 78]. With the observations that SFB colonization can control both Th17 cells and IgA production, therein suggests a link between Th17 cell function and IgA production.

As with all subtypes of CD4 T cells, Th17 cells exhibit influence over B cell responses. Transfer of Th17 cells into T cell-deficient TCR$\alpha^{-/-}$ mice resulted in increased serum IgG titers across all measured subtypes (IgG1, IgG2a, IgG2b, and IgG3), with strongest increases in IgG1 and IgG2b[117]. Furthermore, transfer of Th17 cells induced the generation of germinal centers in the spleen and draining lymph nodes, structures that are mostly lacking in the absence of T cells. These effects were dependent on both IL-17 and IL-21, as transfer of Th17 cells into IL-17RA$^{-/-}$ or IL-21R$^{-/-}$ mice could not increase the number of germinal centers present. Direct addition of IL-17 to B cells in vitro triggered production of IgG2a and IgG3, whereas IL-21 induced production of IgG1, IgG2a, IgG2b and IgG3[117], indicating that Th17 cells are formidable B cell helpers in generating systemic IgG responses. The effects of IL-17 on IgG induction is further demonstrated in the role of IL-17 during systemic lupus erythematosus (SLE), characterized by autoreactive B cells and pathogenic autoantigen antibody production. Patients with SLE have increased serum levels of IL-17 and BAFF, both of which promote survival and antibody production from autoantigen B cells[118, 119]. Neutralization of IL-17 in SLE patient serum reduces antibody production, demonstrating that IL-17 can work in conjunction with BAFF to augment antibody production[120]. However, the role of Th17 cells and IL-17 to influence mucosal IgA production has not been fully investigated.

MATERIALS AND METHODS

Mice. TCRβx$\delta^{-/-}$ mice were obtained from Jackson Laboratory. CBir1 flagellin-specific TCR transgenic (CBir1-Tg) mice were maintained in the Animal Facilities at

University of Texas Medical Branch. 8-12 week-old mice were used for all experiments. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the UTMB. All the mice strains were bred in the UTMB animal facility, and housed together from 3 weeks of age. All mice contain SFB as verified via PCR..

Antibody and reagents. Antibodies against IL-17A, B220, $\alpha_4\beta_7$, were purchased from Biolegend. Mouse recombinant IL-1β, IL-6, IL-12, IL-23, and human recombinant IL-17A, TGFβ1 were purchased from R&D Systems. Recombinant IL-21 was purchased from eBioscience. Antibodies against IgA and IgD were purchased from Kirkegaard & Perry Labs and Southern Biotechnology. Anti-μ was purchased from Jackson ImmunoResearch Laboratories. All-trans-retinoic acid was purchased from Sigma-Aldrich. Anti-biotin microbeads from Miltenyi were used to sort naïve IgD^+ B cells.

Polarization of Th17 and Th1 cells. $CD4^+$ T cells were isolated from spleens of CBir1 Tg mice using anti-mouse CD4-magnetic beads (BD Biosciences) as previously described[61]. To polarize Th17 cells, CBir1-Tg $CD4^+$ T cells were cultured with 10ng/ml TGFβ1, 20ng/ml IL-6, 10μg/ml anti-IFNγ, and 10μg/ml anti-IL-4[92] with irradiated splenic APC. To polarize Th1 cells, CBir1-Tg $CD4^+$ T cells were cultured with 10ng/ml IL-12 and 10μg/ml anti-IL-4.

Fecal pellet preparation. Fecal pellets were homogenized in PBS containing 0.04mg/ml soybean trypsin inhibitor, 20mM EDTA, and 2mM PMSF and centrifuged to remove bacteria and insoluble debris as described previously[93].

ELISA. 96-well plates (Nunc) were coated with 1μg/ml anti-IgA (Kirkegaard & Perry Labs) overnight at 4°. The plates were washed in PBS/Tween and blocked in PBS with 1% BSA. Fecal samples were diluted 1/100 and a 2-fold serial dilution was made. Samples were incubated at room temperature for 2 hours. 0.25μg/ml of biotinylated anti-IgA (KPL) was added for one hour, followed by HRP-conjugated streptavidin (KPL) for one hour. Plates were developed using a two-component TMB substrate (KPL) according to the manufacturer's instructions, and plate was analyzed at 450nm. Results were

quantified by normalizing to standard concentrations of IgA (Southern Biotechnology Associates).

Quantitative Real-Time PCR. RNA was extracted with TRIzol (Invitrogen) and followed by cDNA synthesis with Revertaid reverse transcriptase (Fermentas). Quantitative PCR was performed using TaqMan Gene Expression Assays. Predesigned primers and probes for *aicda* and *gapdh* were ordered from Applied Biosystems, and data were normalized to *GAPDH* mRNA expression. To detect germ-line α transcripts the primers IαF (5′-CCA GGC ATG GTT GAG ATA GAG ATA G-3′) and CαR (5′-GAG CTG GTG GGA GTG TCA GTG-3′) were used[48]. Circular transcripts were detected by a nested PCR using the outer primers Iμ4 (5′-ACC CTG GAT GAC TTC AGT GT-3′) and Iαup4 (5′-CAT CTG GAC TCC TCT GCT CA-3′) followed by the inner primers IαF (5′-CCA GGC ATG GTT GAG ATA GAG ATA G-3′) and CμR (5′-AAT GGT GCT GGG CAG GAA GT-3′)[48]. Post-switch α transcripts were detected with the primers IμF (5′-GAG CTG GTG GGA GTG TCA GTG-3′)[121] and CαR. Aliquots of PCR products were visualized by electrophoresis on 1.5% agarose gels.

Statistical analysis. For comparisons between samples, levels of significance were determined by Student's *t* test in Prism 5.0 (Graphpad). Where appropriate, mean ± SEM is represented on graphs. $*p < 0.05$; $**p < 0.01$.

RESULTS

Th17 cells induce IgA production *in vitro* and *in vivo*

$CD4^+$ T cells play a considerable role in the induction of IgA in the intestine, as $TCR\beta x\delta^{-/-}$ mice have significantly lower amounts of fecal IgA[61]. Previous reports have shown that Th17 cells can strongly induce IgG responses *in vivo*, and when cultured directly with B cells *in vitro*[117]. In order to determine if Th17 cells could also control IgA responses, we generated CBir1 Th17, Th1, and unpolarized T cells (Th0) and cultured

them directly with splenic IgD^+ B cells. As shown in **Figure 3**, levels of IgA in the supernatant were significantly increased when cultured with CBir1 Th17 cells, whereas, Th1 and Th0 only slightly enhanced IgA production. To determine whether Th17 cells could induce intestinal IgA production *in vivo*, we generated Th17 cells *in vitro* under standard conditions with TGFβ and IL-6 (Th17β), as well as through a recently described alternative differentiation pathway using IL-6, IL-1β, and IL-23 ($Th17_{23}$)[122] and transferred them into TCRβxδ$^{-/-}$ mice. Recipient mice were sacrificed 32 days after transfer, and frequencies of IgA^+ B cells were measured in the spleen and intestines and levels of IgA were measured from feces and serum and compared to naïve TCRβxδ$^{-/-}$ mice. Interestingly, transfer of either group of Th17 cells only slightly enhanced populations of IgA^+ B cells in the spleen (**Figure 7**), however, total IgA titers in the serum increased more than 10-fold in comparison to naïve TCRβxδ$^{-/-}$ mice. Analysis of intestinal B cells revealed greater numbers of IgA^+ B cells in the intestine (**Figure 8**). Notably, both groups of Th17 cells primarily expanded populations of $B220^-IgA^+$ cells, possibly indicating that Th17 cells induced intestinal B cells to transition to antibody-secreting plasma cells. Consistent with this observation, we compared the concentration of fecal IgA from TCRβxδ$^{-/-}$ mice before and after transfer of Th17 cells. Both groups of Th17 cells were capable of inducing strong intestinal IgA responses as measured from 2 weeks after Th17 cell transfer, albeit to slightly different degrees. Given that we used CBir1 Tg Th17 cells that respond to the immunodominant bacterial flagellin CBir1, our transferred cells would become activated and exhibit effector function after migrating to the intestine and encountering the intestinal antigen. That in part explains why we observe that the expansion in B cells primarily occurs in the intestine. However, it is of note that the expanded IgA^+ B cell population does not appear to enter the circulation, as few IgA^+ B cells were found in the spleen. Instead, the increased levels of IgA results in both increased secretion into the intestine, as well as increased circulation in the serum.

Collectively, our data indicates that Th17 cells increase IgA production from B cells *in vivo*.

IL-21 augments TGFβ-mediated IgA CSR

To further elucidate the influence of Th17 cells upon B cells, we investigated whether Th17 cell cytokines could moderate B cell differentiation. From **Figure 8**, we observed that Th17 cells could increase populations of IgA^+ B cells. However, it was not yet clear if IgA production was a result of increased germ-line production of IgA, or if Th17 cells were mediating B cell differentiation into IgA^+ B cells. In order for B cells to fully differentiate, activated B cells undergo class switch recombination (CSR), by which the Ig heavy chain locus becomes rearranged and portions of the locus become looped-out and excised from the genome. The physical recombination of the Ig locus is facilitated by the enzyme activation-induced cytokine deaminase (*aicda*)[47]. B cells undergo IgA CSR after exposure to TGFβ, and the loci for IgG, IgE, and IgM are removed from the genome. As such, these B cells are permanently differentiated and can only produce IgA. CSR is a rapid process, however, the looped-out chromosomes stay within the cell for 24-48 hours[47], and continue to be transcribed until they are degraded. As a result of the continued transcription, detection of these mRNAs via RT-PCR are indicative of cells that are actively undergoing CSR to IgA-producing cells. To determine if Th17 cell cytokines can induce CSR, splenic B cells were cultured with anti-μ and CD40L for B cell activation and to mimic T cell contact. TGFβ and/or IL-21 were added to the cultures, and RNA was collected daily to detect when CSR began. As shown in **Figure 9**, TGFβ immediately induced germ-line production of IgA, with CSR to IgA commencing on day 3. Transcription of IgA after CSR was also detectable on day 3, in accordance with CSR induction. As expected[117, 123], treatment of B cells with IL-21 alone did not induce germ-line IgA, nor CSR to IgA. However, treatment with both TGFβ and

IL-21 continued to induce IgA production and CSR. Furthermore, CSR and post-class switched IgA was detected one day earlier when IL-21 was added with TGFβ. Analysis of *aicda* mRNA, required to initiate CSR to any Ig subtype, revealed that TGFβ and IL-21 each induced aicda, with TGFβ inducing IgA CSR and IL-21 inducing IgG CSR. Interestingly, treatment with both TGFβ and IL-21 greatly enhanced *aicda* transcription from day 2 on, correlating with the appearance of circular transcripts and post-class switched IgA transcripts seen on day 2. Collectively, our data reveals that IL-21 itself does not facilitate IgA CSR, however, potently augments and accelerates TGFβ-mediated IgA CSR. However, IL-17 does not induce *aicda*, nor does it enhance TGFβ-induced *aicda*.

IL-21 and retinoic acid cooperate to induce IgA

Previously, we showed that IL-21 can augment TGFβ-mediated IgA CSR, thereby resulting in increased numbers of differentiated IgA^+ B cells. To determine whether IL-21 influences IgA production, splenic B cells were cultured with anti-μ and CD40L with TGFβ and/or IL-21. IgA from the supernatant was measured by ELISA after 5 days of culture. As seen in **Figure 10**, IL-21 did not induce IgA production *in vitro*, and only slightly increased TGFβ-induced IgA production. However, addition of retinoic acid (RA) greatly increased IgA in conjunction with both TGFβ and IL-21. Surprisingly, RA and IL-21 also increased IgA, in the absence of exogenous TGFβ.

IL-21 induces mucosal homing on B cells

Our data indicated that IL-21 and RA cooperate to induce IgA production in vitro, whereas each molecule alone does not induce IgA. Retinoic acid functions primarily to induce the expression of homing molecules on lymphocytes to migrate to the intestinal tract[96, 124]. We then questioned if IL-21 could synergize with RA to influence mucosal

homing through induction of $\alpha_4\beta_7$. Ligation of $\alpha_4\beta_7$ on lymphocytes with mucosal addressin cell adhesion molecule (MAdCAM-1) on endothelial vessels results in lymphocyte migration into the intestinal tract. splenic B cells were cultured with anti-μ and CD40L with TGFβ, IL-21, or IL-17, with or without RA to determine if Th17 cell cytokines could induce intestinal migration. Five days after culture, cells were analyzed for $\alpha_4\beta_7$ expression via FACS. As expected, retinoic acid was able to induce $\alpha_4\beta_7$ expression, which was enhanced with TGFβ and RA (**Figure 11**). Cooperation was also observed with IL-21 and RA but not quite to the degree as TGFβ and RA. Interestingly, IL-21 alone was able to induce $\alpha_4\beta_7$ expression, which was increased synergistically with RA. Notably, IL-17 had no visible effect on $\alpha_4\beta_7$ induction, both alone and in conjunction with RA.

DISCUSSION

The gastrointestinal tract exists in a mutualistic relationship with the commensal flora within. Multiple mechanisms have evolved to regulate and conserve homeostasis between the immune system and microbiota. The intestines act as a natural reservoir for Th17 cells, as well as for IgA-producing B cells, both of which are stimulated by specific species of microbiota. While Th17 cells have been implicated in many proinflammatory disorders, the enrichment of Th17 cells in the intestine suggests that these cells contribute to maintaining intestinal homeostasis by containing the vast population of commensal microflora. In consistency with this argument, our data demonstrated that Th17 cells are able to promote IgA production by influence B cell migration to the intestine, and regulating differentiation of B cells to produce IgA, thus contributing to the maintenance of intestinal homeostasis.

Although TGFβ has been shown as a crucial cytokine in promoting IgA class switching, it does not completely explain why only the intestine contains high levels of IgA, as TGFβ is also present in high levels in other lymphoid tissues while IgA is decreased relative to the intestine. Our data reveals that Th17 cells are able to promote intestinal IgA production and expansion of IgA-secreting B cells when repleted into TCRβxδ$^{-/-}$ mice. This effect appears independent of increases in TGFβ, as similar experiments have indicated that our transferred Th17 cells do not convert to Treg cells, and do not produce TGFβ (**Figure 2E**). Notably, both Th17 cells differentiated from TGFβ or IL-1β and IL-23 are capable of increasing IgA production and IgA^{+} B cells levels within the intestine. While Th17 cells derived from IL-1β, IL-6, and IL-23 are believed to be more proinflammatory[122] and thus more pathogenic in the intestine, it appears that their function remains capable of inducing IgA and promoting epithelial protection.

Th17 cells mediate inflammatory responses through a number of unique cytokines, namely IL-17A, IL-17F, IL-21, and IL-22. Our results indicate that most of the effects observed *in vivo* may be attributed to IL-21 function. Normally, IL-21 induces B cells to undergo CSR and produce large amounts of IgG[117]. However, TGFβ can override IL-21-mediated CSR to IgG in favor of class switching to IgA in mucosal tissues[123]. Our results indicate that IL-21 is a potent inducer of CSR, as indicated by *aicda* transcription, and when combined with TGFβ, results in highly specific and accelerated induction of CSR to promote IgA-secreting B cells. This may indeed help to explain the mechanism by which the intestinal environment is so favorable for IgA^{+} B cells. As described previously, the intestinal environment is rich in molecular cues to promote IgA production and differentiation. Our data reveals that the combination of TGFβ, IL-21, and retinoic acid (RA) induces very strong IgA production from B cells. It is interesting to note that IL-21 and RA is still capable of inducing IgA production in the absence of exogenous TGFβ (**Figure 10**). As we began with naïve splenic B cells, it remains to be

investigated whether RA and IL-21 can induce IgA CSR, or if they only induce strong germ-line transcription of IgA. Alternatively, RA and IL-21 may regulate one of three TGFβ receptors on B cells, thereby making B cells more receptive to endogenous TGFβ available in our FBS-supplemented *in vitro* culture medium, or the B cells themselves may be induced to produce TGFβ and direct their own differentiation. We have previously observed that RA can induce TGFβ from dendritic cells, but it remains to be seen whether the same effect occurs in B cells[96]. The observation that IL-21 and RA can cooperate in the regulation of B cell homing through $\alpha_4\beta_7$ suggests that Th17 cells can indirectly regulate the development of IgA^+ B cells outside of the intestinal tract. Environments such as the bone marrow are rich in retinoic acid and IL-21[96, 125, 126], and may influence naïve B cells from the bone marrow to migrate en masse to the intestines, where the environment is ripe for the induction of IgA^+ B cells.

In summary, our data demonstrates that enriched microbiota-specific Th17 cells protect the host from chronic inflammation and contribute to maintaining intestinal homeostasis by regulating IgA production from B cells.

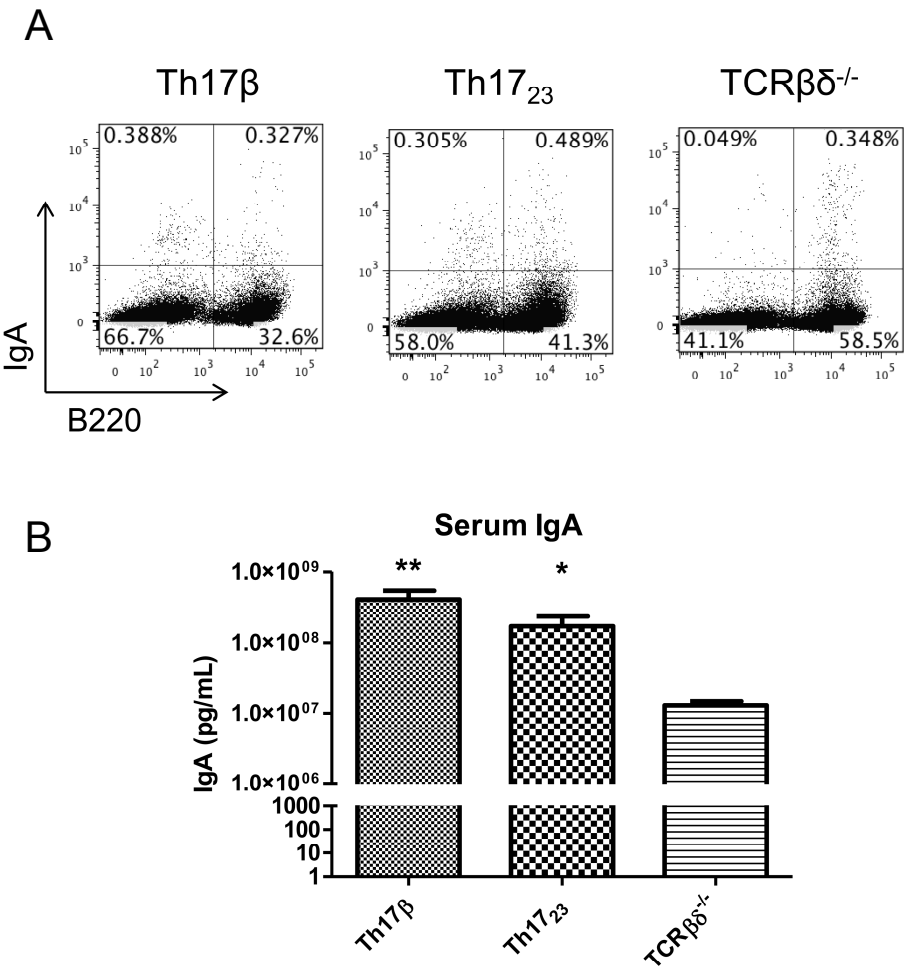

Figure 7. Transfer of CBir1 Th17 cells into TCRβxδ$^{-/-}$ mice increases systemic IgA. (**A**) On day 32 post-Th17 cell transfer, expression of surface IgA on splenic B cells of CBir1 Th17 cell recipients or control TCRβxδ$^{-/-}$ mice was determined by flow cytometry. FACS plots are representative of 2 or 4 mice per group. (**B**) On day 32 post-Th17 cell transfer, IgA in the serum was measured by ELISA. *p<0.05, **p<0.01. N = 4 mice per group.

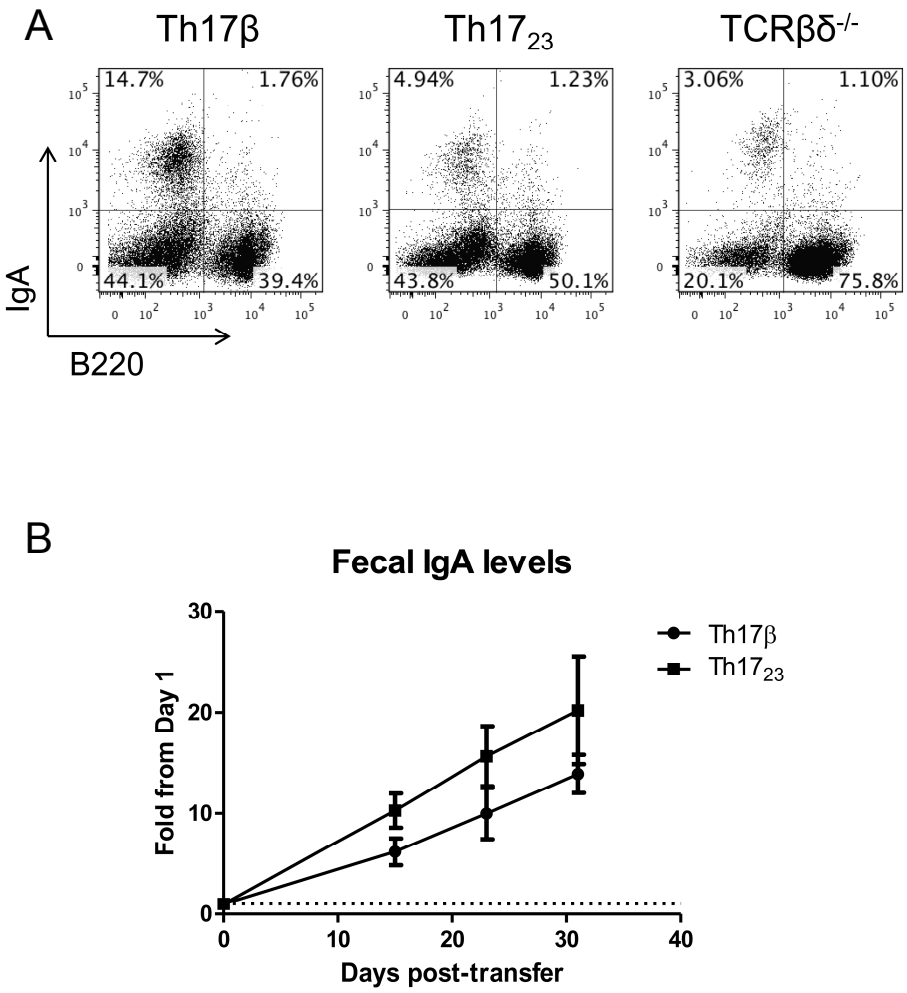

Figure 8. Transfer of CBir1 Th17 cells into TCRβxδ$^{-/-}$ mice increases IgA^{+} B cells and intestinal IgA production. (**A**) On day 32 post-Th17 cell transfer, expression of surface IgA on intestinal lamina propria B cells of CBir1 Th17 cell recipients or control TCRβxδ$^{-/-}$ mice was determined by flow cytometry. FACS plots are representative of 2 or 4 mice per group. (**B**) Fecal pellets were collected from Th17 cell recipients during the course of the experiment. IgA levels were quantified through ELISA and normalized to total protein. Changes in expression over time are expressed as a fold change from individuals pre-transfer. N = 4 mice per group.

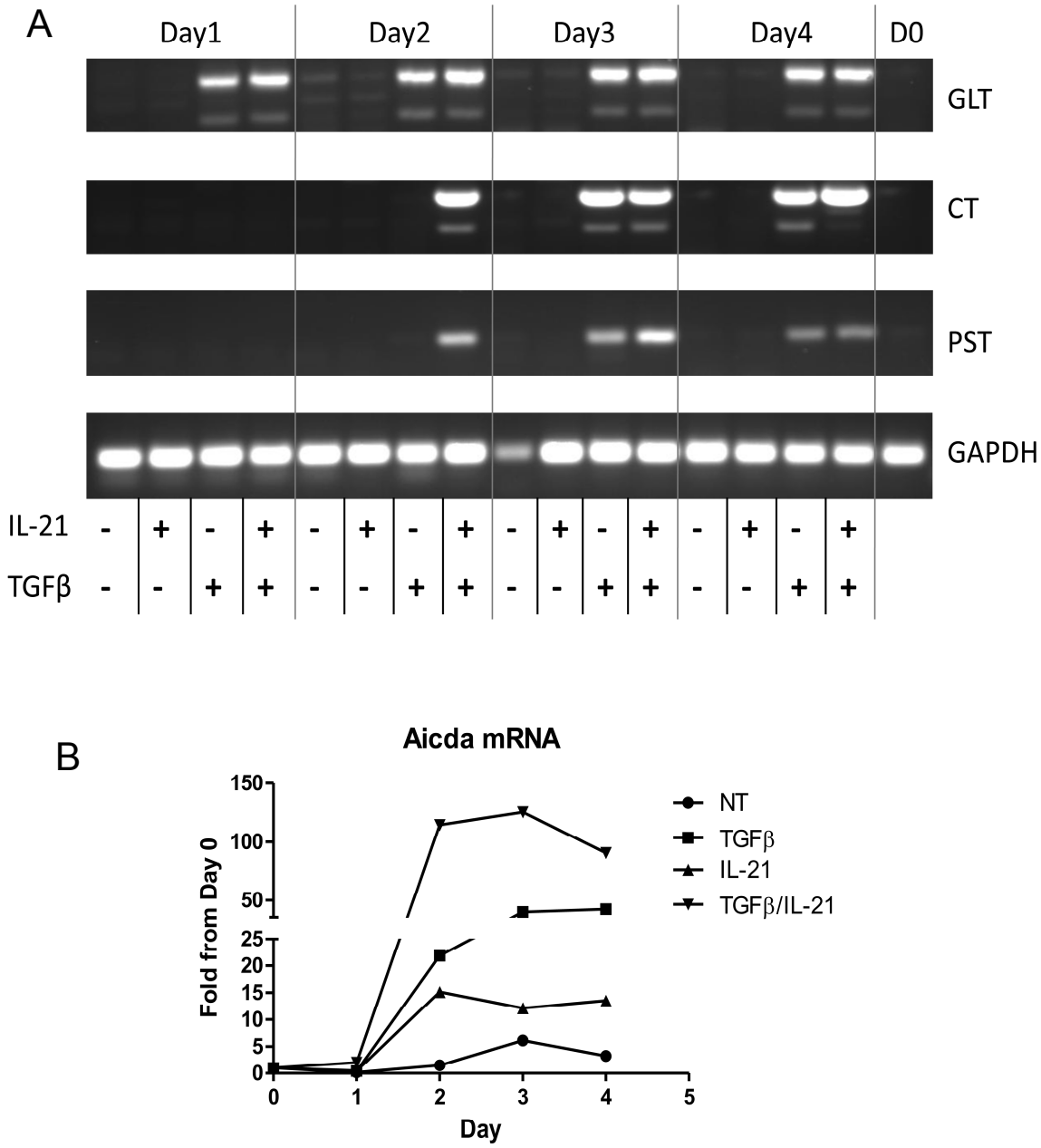

Figure 9. IL-21 enhances TGFβ-mediated class switching towards IgA. Naïve IgD^{+} B cells were activated with anti-μ and anti-CD40 in the presence of TGFβ or IL-21, or a combination of TGFβ and IL-21. (**A**) RNA was collected from cultured B cells on various days during culture and molecular markers for IgA CSR were analyzed by RT-PCR. GLT = α germ-line transcripts. CT = circular transcripts. PST = α post-switch transcripts (**B**) *Aicda* mRNA was analyzed from cells on various days during culture by RT-PCR. *Aicda* expression values were normalized to *Gapdh* expression. Data is reflective of two independent experiments.

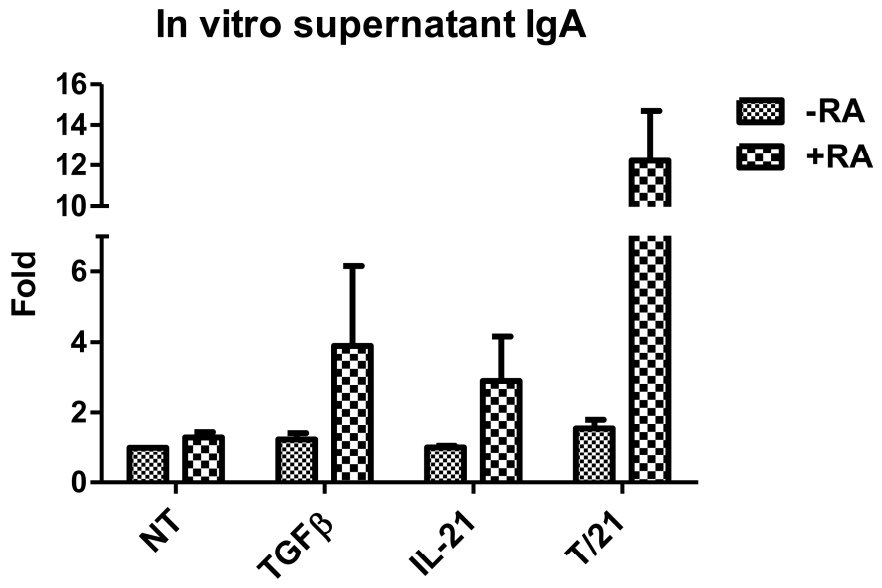

Figure 10. IL-21 and retinoic acid cooperate to enhance TGFβ-mediated IgA production. Naïve IgD^+ B cells were activated with anti-μ and anti-CD40 in the presence of TGFβ or IL-21 or retinoic acid, or a combination of TGFβ, IL-21, and/or retinoic acid. On day 5, IgA was measured from the supernatant by ELISA. Data is reflective of 4 independent experiments.

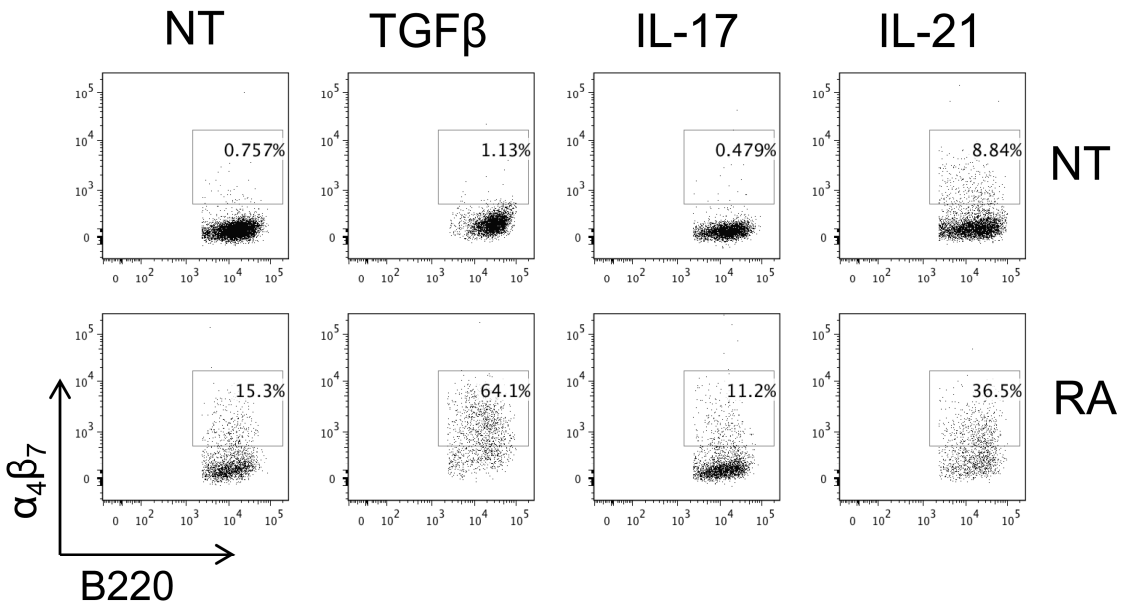

Figure 11. IL-21 induces mucosal homing markers alone and in synergism with retinoic acid. Naïve IgD^+ B cells were activated with anti-μ and anti-CD40, and cultured with TGFβ, IL-17A, or IL-21, with or without retinoic acid. On day 5, $\alpha_4\beta_7$ was measured by flow cytometry. FACS plots are representative of 2 independent experiments. NT, no treatment.

Chapter 7: TLR4 regulates IFN-γ and IL-17 production by both natural and induced Foxp3$^+$ Tregs during intestinal inflammation

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INTRODUCTION

The gastrointestinal tract provides a major entry for dietary and microbial antigens into the body. Among the substantive microbial population, both beneficial commensal microbiota and potential pathogens reside in the gut. A key mechanism of regulation in the intestinal tract lies with regulatory T cells, capable of surveying a wide array of immune responses in order to maintain intestinal homeostasis and protect the intestine from inflammation. The majority of regulatory T cells are defined by expression of the transcription factor Forkhead box P3 (Foxp3), which is critical for Treg cell development and stability[127]. The catastrophic inflammation observed in the absence of Foxp3[127, 128] demonstrates that regulatory T cells are vital for immune regulation and restraint of the inflammatory effects of effector T cells. Foxp3$^+$ Treg cells arise in two separate origins, differing in both location and TCR repertoire[20]. CD4$^+$ T cells with TCR affinities towards self-antigens become Foxp3$^+$ Treg cells in the thymus, and are denoted natural Treg cells (nTreg), whereas Foxp3$^+$ Treg cells can also be induced in the periphery after antigen presentation and are denoted inducible Treg cells (iTreg)[20]. Deficiency in Treg cells results in chronic intestinal inflammation and colitis can be prevented and cured by transfer of Treg cells, showcasing a key role for Tregs in the maintenance of intestine homeostasis[129]. Paradoxically, however, Foxp3$^+$ Tregs are increased in inflamed intestinal tissues in both animal models and patients with IBD[101, 130, 131], and most of them express effector cytokines, such as interferon-γ (IFNγ) and interleukin (IL)-17[130, 132, 133]. Anti-TNFα treatment suppresses inflammation in a majority of patients with IBD with a concomitant decrease in local mucosal Tregs and increase in

peripheral Tregs[134, 135], indicating that inflammation drives Treg expansion and subsequently promotes accumulation in inflamed lesions. However, the factors regulating Treg expansion and expression of effector cytokines in inflamed intestine are largely unknown.

Although a majority of Treg cells continue to express high levels of Foxp3 after transfer into non-inflamed hosts, many reports demonstrate the plasticity of the Treg differentiation and maintenance program[32, 136]. Foxp3$^+$ Treg cells can fully convert into Th1, Th17, and/or T follicular helper cells in the intestine, especially during inflammation[130, 137]. This is manifested by the loss of Foxp3 and acquisition of T-bet (Th1), Rorγt (Th17) or BCL-6 (Tfh) by Treg cells[137, 138]. However, it is unclear whether nTreg or iTreg cells have different propensities for acquiring effector function, and how microbial exposure regulates this process. The interaction of the commensal microbiota with TLRs plays an important role in innate immune responses, e.g., the activation of TLRs on dendritic cells and macrophages results in production of proinflammatory cytokines and chemokines. Conversely, the intestinal microbiota can promote immune regulation through the activation of TLRs on CD4$^+$ T cells, independent of antigen-presenting cells[139, 140]. Deficiencies in TLR4 or TLR9 in vivo, coupled with IL-10 deficiency, result in spontaneous colitis that is significantly worse than that seen in IL-10$^{-/-}$ mice, demonstrating that TLR4 and TLR9 have regulatory roles in CD4$^+$ T cells[35, 141]. Notably, Treg cells in TLR4$^{-/-}$IL-10$^{-/-}$ mice express very high levels of inflammatory cytokines, including GM-CSF, IFN-γ, and IL-17; and have decreased suppressive capability[35]. Activation of MyD88, a downstream effector molecule of most TLRs, has also been shown to be important for Treg cells to control intestinal inflammation in vivo[72]. In this report, we demonstrate that LPS influences the generation and expansion of Tregs through TLR4 signaling on both antigen-presenting cells (APC) and CD4$^+$ T cells in a MyD88-dependent, but TRIF-independent manner. Both Helios$^+$Nrp1$^+$ nTregs and Helios$^-$ Nrp1$^-$ iTregs produce effector cytokines in the intestines. TLR4 signaling

influences Treg cells expressing effector cytokines, however it does not appear to favor either Treg cell subset. Furthermore, Treg expansion under intestinal inflammation occurs in the intestine but not in the periphery.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), B6. TRIF^{LPS2} and B6.RAG1$^{-/-}$ mice were purchased from Jackson Laboratory. B6.CBir1 flagellin-specific TCR transgenic (CBir1 Tg) mice, B6.MyD88$^{-/-}$, B6.TLR4$^{-/-}$, B6.IL-10$^{-/-}$, B6.TLR4$^{-/-}$IL-10$^{-/-}$ mice[35] , and B6.SOCS3 conditional knockout (LysMCre-SOCS3$^{fl/fl}$)[142] were maintained in the Animal Facilities at the University of Texas Medical Branch, the University of Chicago, and the University of Alabama at Birmingham. Age-matched and co-housed mice of 8 to 12 weeks were used in these experiments. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch, the University of Chicago, and the University of Alabama at Birmingham.

Antibodies and reagents. Fluorochrome-conjugated anti-mouse CD4 (RM4-5), Helios (22F6), IL-17A (TC11-18H10), and IFNγ (XMG1.2) antibodies and recombinant TGFβ were purchased from Biolegend. Anti-mouse Nrp1 was purchased from R&D Systems. Anti-mouse Foxp3 (FJK-16s) was purchased from eBioscience. Lipopolysacharride (0111:B4) was purchased from Sigma Aldrich. FTY720 was purchased from Cayman Chemical.

Isolation of lamina propria cells. As described previously[130], large intestines were removed, sliced and digested by Collagense IV. The cells were resuspended in 40% Percoll and carefully overlaid onto 70% Percoll. The interface containing the lamina propria lymphocytes was collected. Lymphocytes were restimulated with phorbol 12-myristate 13-acetate (50ng/ml), ionomycin (750ng/ml), and monensin for 5 hours before labeling with antibodies for flow cytometry.

CD4$^+$ T cell purification, *in vitro* polarization of Treg cells, and bone marrow dendritic cell generation. CD4$^+$ T cells were isolated by using anti-mouse CD4-magnetic beads (BD Biosciences). CD4$^+$ T cells from B6, MyD88$^{-/-}$ or TRIF^{LPS2} mice

were cultured with plate-bound αCD3 (1μg/ml) and αCD28 under Treg-polarizing conditions (5ng/ml TGFβ), or in the absence of polarizing cytokines. Irradiated splenocytes from B6, MyD88$^{-/-}$or TRIF$^{-/-}$ mice were cultured with CD4^{+} T cells from B6 mice with soluble αCD3 (1μg/ml) with or without Treg polarization, or with CD4^{+} T cells from CBir1-Tg mice with CBir1 flagellin peptide under Treg polarization.

Bone marrow dendritic cells were generated from B6 or SOCS3 conditional knockout femurs. Bone marrow cells were cultured with GM-CSF (Biolegend) for 8 days, and cultured with CD4^{+} T cells from CBir1-Tg mice with CBir1 flagellin peptide under Treg polarization.

Statistical analysis. Levels of significance were determined by Student's *t* test in Prism 5.0 (Graphpad). P values of < 0.05 were considered to be statistically significant.

RESULTS

TLR4 regulates expansion and inflammatory cytokine expression by Foxp3^{+} Treg cells in the intestines of colitic mice

Our previous studies demonstrated that during intestinal inflammation, Foxp3^{+} Treg cells accumulated in the inflamed lesions and substantial numbers of such Treg cells expressed the effector cytokines IFN-γ and IL-17 in the inflamed colon[35, 130]. However, the factors responsible for driving Treg expansion and expression of these effector cytokines and whether nTregs or iTregs are capable of producing these cytokines remain unclear as recent reports have offered conflicting results[136, 143]. We have previous shown that deficiencies in both IL-10 and TLR4 resulted in severely aggravated intestinal inflammation, thereby suggesting a critical role of TLR signaling in controlling pro-inflammatory activities[35]. In order to assess how TLR4 regulate nTreg and iTreg cell expression of effector cytokines in the presence and absence of inflammation, we

analyzed Foxp3$^+$ Treg cell expression of effector cytokines from wild-type (WT) B6, TLR4$^{-/-}$, IL-10$^{-/-}$, and TLR4$^{-/-}$IL-10$^{-/-}$ mice. We have reported previously that TLR4$^{-/-}$IL-10$^{-/-}$ mice developed more severe colitis compared to IL-10$^{-/-}$ mice, and there was no inflammation in the intestines of wild-type and TLR4$^{-/-}$ mice[35]. Examination of cytokine production by CD4$^+$ T cells showed that IFNγ-producing Th1 cells and IL-17-producing Th17 cells were increased in spleen of colitic IL-10$^{-/-}$ mice compared to that of wild-type mice, and were further amplified in the intestinal lamina propria (LP) (**Figure 18**). Deficiency of TLR4 alone did not affect CD4 T cell cytokine production in wild-type mice. There was, however, a considerable increase in the number of cells that produce both IFN-γ and IL-17 in colitic IL-10$^{-/-}$ mice, which were further increased in TLR4$^{-/-}$IL-10$^{-/-}$ mice.

Interestingly, Foxp3$^+$ Treg cells were increased in intestinal LP but not in the spleen of colitic IL-10$^{-/-}$ mice compared to wild-type mice (**Figure 12**). However, the presence of Foxp3$^+$ Treg cells was increased in both spleen and LP of TLR4$^{-/-}$ mice compared to that of wild-type mice, which were further increased in TLR4$^{-/-}$IL-10$^{-/-}$ mice (**Figure 12**). These data are consistent with the hypothesis that TLR4 signaling inhibits Treg cell expansion, although differences in microbiota composition between strains may account for some of the observed differences. In addition to the increase of Foxp3$^+$ Treg cells, there was an increase in Foxp3$^+$ Treg cells that produce IL-17, but not IFNγ in the intestines of TLR4$^{-/-}$ mice. Interestingly, there were more Foxp3$^+$ Treg cells expressing both IFNγ and IL-17 in the intestines of IL-10$^{-/-}$ mice, and TLR4 appeared to play a role in controlling Foxp3$^+$ Treg cell cytokine production, because the levels of Foxp3$^+$ Treg cells expressing both IFN-γ and IL-17 is further increased in TLR4$^{-/-}$IL-10$^{-/-}$ mice compared to that in IL-10$^{-/-}$ mice (**Figure 12**).

Both Helios$^+$ Nrp1$^+$ and Helios$^-$ Nrp1$^-$ Foxp3$^+$ Treg cells produce IFN-γ and IL-17 in the intestines of colitic mice

Although it has been shown that Treg cells can convert into effector cytokine producing T cells, it is unclear whether both nTreg and iTreg or only iTregs produce effector cytokines, as nTregs are thought to be relatively stable[143]. In order to investigate the nTreg cells that produce effector cytokines, we chose two markers, Helios and Neuropilin-1 (Nrp1), to distinguish nTreg and iTreg. Currently the co-expression of both markers is the best identifier for nTregs, whereas iTregs do not express either marker[144, 145]. We assessed the difference in cytokine production from Foxp3$^+$ Treg cells between Helios$^+$ Nrp1$^+$ nTregs and Helios$^-$ Nrp1$^-$ nTregs in the context of TLR4 deficiency and inflammation. We found that both Helios$^+$ Nrp1$^+$ and Helios$^-$ Nrp1$^-$ Tregs produced IL-17 and IFN-γ in the spleen and LP under steady state conditions. Analysis of intestinal Tregs in wild-type mice revealed a higher presence of Helios$^-$ Nrp1$^-$ Tregs vs Helios$^+$Nrp1$^+$ Tregs than was found in the spleen. Although TLR4$^{-/-}$IL-10$^{-/-}$ mice had higher frequencies of Treg cells in the intestines, the ratio of Helios$^-$ Nrp1$^-$ Tregs vs Helios$^+$Nrp1$^+$ Tregs was conserved between strains (**Figures 13A and 13B, and Figures 19 and 20**). In wild-type mice, Helios$^+$Nrp1$^+$ Tregs had a higher propensity to produce IFNγ or IL-17 in the intestines, as opposed to the spleen, where Helios$^+$Nrp1$^+$ Tregs only produced IFNγ with little IL-17. Helios$^+$Nrp1$^+$ Tregs produced higher levels of IFNγ compared to that of Helios$^-$ Nrp1$^-$ Tregs in both the spleen and LP. LP Helios$^+$Nrp1$^+$ Tregs produced more IL-17 than did Helios$^-$ Nrp1$^-$ Tregs (**Figure 13B**) whereas both Helios$^-$ Nrp1$^-$ Tregs and Helios$^+$Nrp1$^+$ Tregs cells produced IL-17 at similar levels in the spleen (**Figure 13A**). In IL-10$^{-/-}$ mice, IFNγ and IL-17 production by both Helios$^-$ Nrp1$^-$ Tregs and Helios$^+$Nrp1$^+$ Tregs in the spleens and LP were increased compared to that in wild-type mice, however, the pattern remained the same, i.e. Helios$^+$Nrp1$^+$ Tregs produced higher level of IFNγ compared to Helios$^-$ Nrp1$^-$ Tregs, and both Helios$^-$ Nrp1$^-$ Tregs and Helios$^+$Nrp1$^+$ Tregs

produced IL-17 at similar levels (**Figure 13B**). Although IL-17 and IFNγ production by both Helios$^-$ Nrp1$^-$ Tregs and Helios$^+$Nrp1$^+$ Tregs was not different from wild type in TLR4$^{-/-}$ mice, TLR4 deficiency in IL-10$^{-/-}$ mice significantly increased production of IL-17 and IFNγ by both Helios$^-$ Nrp1$^-$ Tregs and Helios$^+$Nrp1$^+$ Tregs. Notably, the Foxp3$^+$ Treg cells expressing IFNγ and IL-17 were predominantly Helios$^+$Nrp1$^+$ Tregs in the TLR4$^{-/-}$IL-10$^{-/-}$ mice. Collectively, these data demonstrated that while the absence of TLR4 signaling does not affect Treg cell effector cytokine production alone, it augments Treg cell effector cytokine production during intestinal inflammation in the absence of IL-10.

Foxp3$^+$ Treg cells expand in the intestine during intestinal inflammation

We then investigated whether Treg expansion occurred systemically or within the tissue at the interface of microbial exposure and interaction. We used the CBir1 T cell transfer model in order to examine the location of Treg expansion. The transfer of CD4$^+$ T cells from CBir1 TCR transgenic mice, which are specific for an immunodominant commensal bacterial antigen CBir1 flagellin, into immunodeficient RAG$^{-/-}$ mice induces colitis in the recipients[61, 130]. CBir1 CD4 T cells were intravenously transferred into recipient RAG$^{-/-}$ mice, and treated orally with FTY720 on the same day of cell transfer and every other day thereafter for 4 weeks, which inhibits lymphocyte egress from the lymph nodes[146]. In treated mice, CD4$^+$ T cells activated in the intestines are expected to remain within the intestinal compartment.

Consistent with our previous observations[130], both FTY720 treated and control CBir1 T cell-reconstituted RAG$^{-/-}$ mice developed colitis. Analysis of the mice receiving CBir1 CD4$^+$ T cells revealed substantial numbers of Th1 cells, Th17 cells, as well as Foxp3$^+$ Treg cells in the spleen and LP (**Figure 14**). The intestine contained the highest percentage of Foxp3$^+$ Treg cells producing IFNγ, IL-17, or both IFNγ and IL-17. Treatment with FTY720 greatly decreased the Foxp3$^+$ Treg cells in spleen but not in the

MLN and intestine. Notably, the proportion of IFNγ^{+}Foxp3^{+} Treg cells was greatly increased in the intestine after FTY720 treatment. IL-17^{+}Foxp3^{+} T cells also increased to a lesser extent. Collectively, these data demonstrate that the expansion and subsequent production of effector cytokines by Foxp3^{+} Treg cells occur primarily in the inflamed intestine with eventual systemic circulation.

TLR4 signaling downregulates development of Foxp3^{+} Treg cells through both CD4^{+} T cells and antigen-presenting cells in a MyD88-dependent and TRIF-independent manner

We next investigated how TLR4 signaling regulates Foxp3^{+} Treg cells. As TLR4$^{-/-}$ mice had a higher frequency of Treg cells in the intestine, where CD4^{+} T cells would have the most contact with bacteria and TLR ligands, we questioned whether TLR4 signaling inhibited induction of Foxp3^{+} Treg cells, and whether TLR4 signaling on CD4^{+} T cells or antigen-presenting cells was responsible for downregulation of Foxp3^{+} Treg cell induction. We first cultured CBir1 CD4 T cells with splenic APCs under Treg conditions with TGF-β in the presence or absence of a series of doses of LPS. As shown in **Figure 15A**, LPS inhibited development of Foxp3^{+} Tregs in a dose-dependent manner under Treg polarizing conditions; however, it did not affect Foxp3 expression on unpolarized T cells in the absence of TGFβ (**Figure 15B**). Interestingly, while LPS did not affect IFNγ and IL-17 production by Foxp3^{-} T cells under neutral (Th0) conditions, it inhibited IFNγ production by Foxp3^{-} T cells under Treg cell conditions. Because CD4^{+} T cells express TLR4 and respond to its ligand LPS[141], we treated CD4^{+} T cells with plate-bound anti-CD3 and anti-CD28 with TGF-β in the presence or absence of LPS to determine the role of LPS-TLR4 signaling in T cells on Treg cell differentiation. As shown in **Figure 15C**, LPS-TLR4 signaling in T cells inhibited Treg cell development.

TLR4 signals through two separate pathways: the MyD88 pathway and the TRIF pathway[147] . In order to determine the mechanism behind TLR4 inhibition of Foxp3^{+}

Tregs, we used MyD88$^{-/-}$ or TRIF$^{-/-}$ CD4^{+} T cells to identify which pathway was responsible for the effects directly in T cells. After treatment with LPS and TGF-β, MyD88$^{-/-}$ CD4^{+} T cells exhibited no discernible change in induction of Foxp3^{+} Treg cells, whereas LPS downregulated Foxp3^{+} Treg cell induction in TRIF$^{-/-}$ CD4^{+} T cells (**Figure 16A**), revealing that CD4^{+} T cell TLR4 inhibition of Foxp3^{+} Treg cells depends on MyD88 but not TRIF.

As LPS signaling on APC drastically inhibited Foxp3^{+} Treg cells at a much greater level compared to TLR4 signaling solely on CD4^{+} T cells (**Figures 15B and 15C**), we then examined the role of the MyD88 and TRIF pathways in APCs by co-culturing wild-type CD4^{+} T cells with MyD88$^{-/-}$ or TRIF$^{-/-}$ APCs under Treg conditions in the presence or absence of LPS. As addition of LPS in the culture of CD4^{+} T cells with wild-type APCs under Treg conditions almost completely inhibited induction of Foxp3^{+} Treg cells, addition of LPS did not result in a decrease of induction of Foxp3^{+} Treg cells in the absence of APC MyD88 signaling (**Figure 16B**). In contrast, when wild-type CD4^{+} T cells were cultured with TRIF$^{-/-}$ APCs, LPS inhibited induction of Foxp3^{+} Treg cells at a similar level to that with wild-type APC. Collectively, these data demonstrated that LPS modulates Foxp3^{+} Treg cells in a MyD88-dependent, TRIF-independent manner. This effect is mediated both directly on CD4^{+} T cells as well as indirectly through APCs.

SOCS3 mediates LPS-TLR4 inhibition of Foxp3^{+} Treg cell development

LPS-TLR4 signaling stimulates SOCS3 expression and STAT3 activation, and SOCS3 serves as a key negative regulator of the LPS-TLR4 pathway through inhibition of STAT3[148]. Therefore, SOCS3 has a crucial role in modulating STAT3 activation and inflammatory gene expression. We next investigated whether SOCS3 regulates LPS inhibition of Foxp3^{+} Tregs. We first generated bone marrow-derived DC from wild type mice and mice with a conditional SOCS3 deletion in myeloid cells (LysMCre-SOCS3$^{fl/fl}$), and stimulated the BMDCs with LPS. SOCS3-deficient BMDC expressed higher levels

of activation of STAT3 (**Figure 17A**), and produced higher levels of IL-1β, IL-6, IL-12 IL-23 and TNFα compared to levels in wild-type BMDC (**Figure 17B**). To determine whether SOCS3 regulates LPS-TLR4 inhibition of $Foxp3^+$ Tregs, we cultured wild-type $CD4^+$ T cells with wild-type and SOCS3-deficient BMDC with anti-CD3 under Treg conditions with TGF-β. In order to delineate the effect of SOCS3, we selected a low dose of 1 ng/ml of LPS which only partially inhibited induction of $Foxp3^+$ Treg cells. As shown in **Figure 17C**, deficiency of SOCS3 in APC enhanced LPS downregulation of $Foxp3^+$ Treg cells. Collectively, these data indicated that SOCS3 negatively regulates LPS inhibition of $Foxp3^+$ Treg cell development.

DISCUSSION

Inflammatory bowel disease is dependent on the microbiota for providing signals to modulate the immune system and control inflammation. An inability to properly regulate the intestinal environment coupled with constant exposure to microbial ligands induces chronic inflammation. In both patients with IBD and experimental colitis, high levels of $Foxp3^+$ Tregs are found to produce effector cytokines within the inflamed intestine[130-132], potentially intensifying inflammation or stifling regulation. Recently, it has been shown that microbial ligands modulate $CD4^+$ T cell activation. Although $CD4^+$ T cells respond in an antigen-specific manner, the presence of TLR ligands can potentiate $CD4^+$ T cell responses[149, 150]. Deficiency in TLR4 on $CD4^+$ T cells aggravates colitis in $IL\text{-}10^{-/-}$ mice by increasing Treg and effector cell production of IFN-γ and IL-17[35, 141]. Because Treg cells can arise either from the thymus or in the periphery, there is a question of whether there are differences in immunoregulation and cytokine production between Treg subsets. It has been reported that Helios and Nrp1 are predominantly expressed by nTregs, although not absolutely. $Helios^+Nrp1^+Foxp3^+$ is considered to be the best indicator of nTregs whereas $Helios^-$ $Nrp1^-$ $Foxp3^+$ is the best indicator of

iTregs[144, 145]. We report here that both Helios^{+}Nrp1^{+} and Helios^{-} Nrp1^{-} Foxp3^{+} Treg cells can be induced to express IFN-γ and/or IL-17 under both homeostatic conditions and inflammatory conditions, a process which is negatively regulated by TLR4 in a MyD88-dependent, but TRIF-independent manner.

It has been consistently observed that Foxp3^{+} Tregs accumulate in inflamed tissue of animal models of colitis and IBD patients[131, 151]. While it would appear that the influx of Tregs is a response at controlling extensive inflammation, a substantial number of them produce effector cytokines. This highlights the challenge of using expanded Treg cell populations to treat autoimmune diseases, which might result in the uncontrolled production of effector cytokines by Treg cells[152-154]. Although recent reports demonstrated that encounter with microbiota resulted in the peripheral generation of Treg cells, and microbiota antigens play an important role in shaping the colonic Treg population[155, 156], it is still highly debatable whether only iTreg can convert into effector cytokine-producing T cells or nTreg cells can also do so under certain conditions in the intestine. Here, we aimed to distinguish whether nTregs or iTregs had varying propensities for acquiring effector characteristics in the intestines, and how inflammation regulated such process. In contrast to some previous reports[157] indicating that nTregs were relatively stable and resistant to conversion into Th1 or Th17 cells, our findings revealed that Helios^{+}Nrp1^{+}Foxp3^{+} Tregs produced IFNγ and IL-17 at a higher frequency, and even in greater numbers than Helios^{-} Nrp1^{-} Foxp3^{+} Tregs. These data corroborate previous findings that iTregs were more resistant than nTregs to conversion to Th17 cells after treatment with IL-6[158]. Severe inflammation was not a prerequisite for Treg conversion as it can be seen in the intestine of healthy wild-type mice, however, the degree of inflammation did contribute to the acquisition of effector cytokines in Treg cells. Given our previous observations that the acquisition of effector cytokines can occur in tandem with the loss of Foxp3 expression from “ex-Treg” cells; at this point it remains

unclear how the chronic inflammation in TLR4$^{-/-}$IL-10$^{-/-}$ mice affects stability of Foxp3 expression in either nTreg or iTreg cells.

Interestingly, although we observed an increase in intestinal Foxp3^{+} Tregs in TLR4 deficient mice housed in the animal facilities at University of Chicago and at University of Texas Medical Branch, there was no observable difference in Foxp3^{+} Treg numbers between the colons of WT and TLR4$^{-/-}$ mice housed in the animal facilities at Massachusetts General Hospital in our previous studies[35], indicating that different commensal bacteria differentially stimulate gut TLR4 signaling and host immune responses. The acquisition or loss of particular bacterial populations may have altered the propensity for induction of Foxp3 in the context of TLR4 signaling, as it has been reported that identical strains of mice often have considerable phenotypic variation in their intestinal microbiota when relocated to different facilities.

During colitis, antigen-specific Tregs induced by microbial signals produce effector cytokines that accumulate not only in the inflamed intestine but also in the spleen (**Figure 14**). That raises an important question: where does Treg cell expansion and conversion into effector cytokine producing T cell occur? Are they induced systemically or locally in the mucosa where they are activated after encountering their cognate microbial antigens in the intestinal lumen? Treatment with FTY720, which inhibits lymphocyte egress from lymph nodes[146], decreased total Foxp3^{+} Tregs as well as cytokine-expressing Foxp3^{+} Tregs in the spleens of colitic RAG$^{-/-}$ mice which received CBir1 Tg T cells. However, FTY720 treatment did not affect Foxp3^{+} Tregs in the LP, indicating that Foxp3^{+} Treg expansion and production of effector cytokines occurred in the intestine, where they were activated by their cognate microbiota antigens, and migrated into systemic lymph organs.

Given the importance of Foxp3^{+} Tregs in IBD, considerable work has already been done to investigate the roles of TLRs upon Treg function[139, 150]. In the absence of MyD88-signaling, CD4^{+} T cells proliferate poorly in response to anti-CD3 stimulation in

the presence of various TLR agonists. Furthermore, MyD88-deficient Tregs fail to protect against CD45RB^{hi} T cell-induced colitis[72]. However, although a previous report identified no role for TLR4 stimulation directly on CD4$^+$ T cells[157], we demonstrated that LPS treatment at the time of activation did downregulate Foxp3$^+$ Treg cells. Furthermore, it appears that intrinsic MyD88 signaling in CD4 T cells was primarily responsible for regulating Foxp3 induction, as deficiency in TRIF had no effect on Foxp3$^+$ Tregs after LPS treatment. While TRIF signaling appeared to contribute to cytokine production in effector cells[159], intrinsic TRIF signaling did not alter LPS-inhibition of Foxp3$^+$ Treg development.

Activation of CD4$^+$ T cells requires the presentation and co-receptor binding by APCs. Thus, the effects of LPS cannot be isolated solely on CD4$^+$ T cells or on the APCs. When APCs are taken into account, LPS strongly abrogates Foxp3$^+$ Treg cell development under Treg conditions. With the decrease in Foxp3$^+$ Tregs, increases in IFN-γ and IL-17 are also observed, thereby indicating that polarizing cytokines are being produced by APCs upon LPS exposure, which in turn inhibits induction of Foxp3$^+$ Treg cells and skews CD4$^+$ T cell differentiation. The LPS-TLR4 pathway has been shown to stimulate SOCS3 expression and STAT3 activation, with SOCS3 serving as a crucial negative regulator of LPS-TLR4 responses by inhibiting activation of STAT3 and NF-κB, thus limiting proinflammatory cytokine production[148]. By using BMDC from myeloid-specific SOCS3$^{-/-}$ mice, we demonstrated that SOCS3 negatively regulated LPS-TLR4 inhibition of Treg Foxp3 expression, in that deficiency of SOCS3 further enhanced LPS inhibition of Foxp3$^+$ Tregs. This is most likely mediated by higher levels of proinflammatory cytokines production in SOCS3$^{-/-}$ DC in response to LPS, such as IL-1, IL-6, IL-12 and IL-23, which have been shown able to inhibit Foxp3$^+$ Tregs[32, 142, 160] .

In summary, our data demonstrated that both Helios$^+$ Nrp1$^+$ nTregs and Helios$^-$ Nrp1$^-$ iTregs are able to produce IFN-γ and/or IL-17 in the intestine and that intestinal inflammation promotes Treg cell accumulation and effector cytokine production. TLR4

negatively regulates Treg cell generation through a MyD88-dependent, and TRIF-independent manner. TLR4 regulation of $Foxp3^+$ Tregs acts indirectly through APC as well as directly through $CD4^+$ T cells. These data thus provide insights into Treg cell regulation under TLR4-mediated conditions.

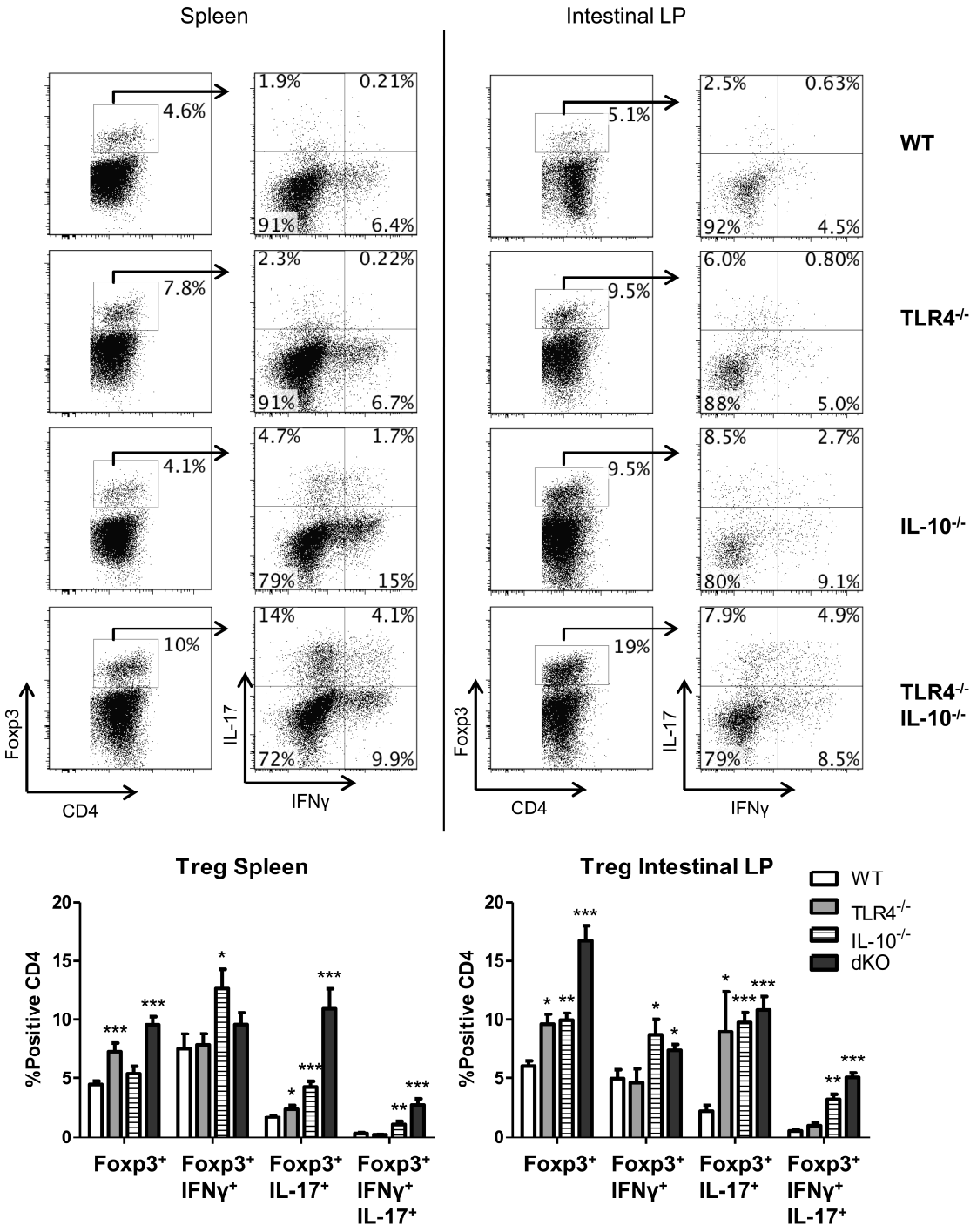

Figure 12. Absence of TLR4 increases intestinal Treg cell cytokine production. CD4 T cells were isolated from the intestinal lamina propria of TLR4$^{-/-}$, IL-10$^{-/-}$, TLR4$^{-/-}$IL-10$^{-/-}$ and WT mice, and analyzed for IFNγ and IL-17 production by flow cytometry gated on Foxp3^{+} cells. Bar charts of Foxp3^{+}IFNγ^{+}, Foxp3^{+}IL-17^{+}, and Foxp3^{+}IFNγ^{+}IL-17^{+} are calculated based on total CD4^{+}Foxp3^{+} T cells. *p>0.05, **p>0.01, ***p>0.001 compared to wild-type B6 mice. Data are representative of 3 or more experiments of 2 or more mice per group with similar results.

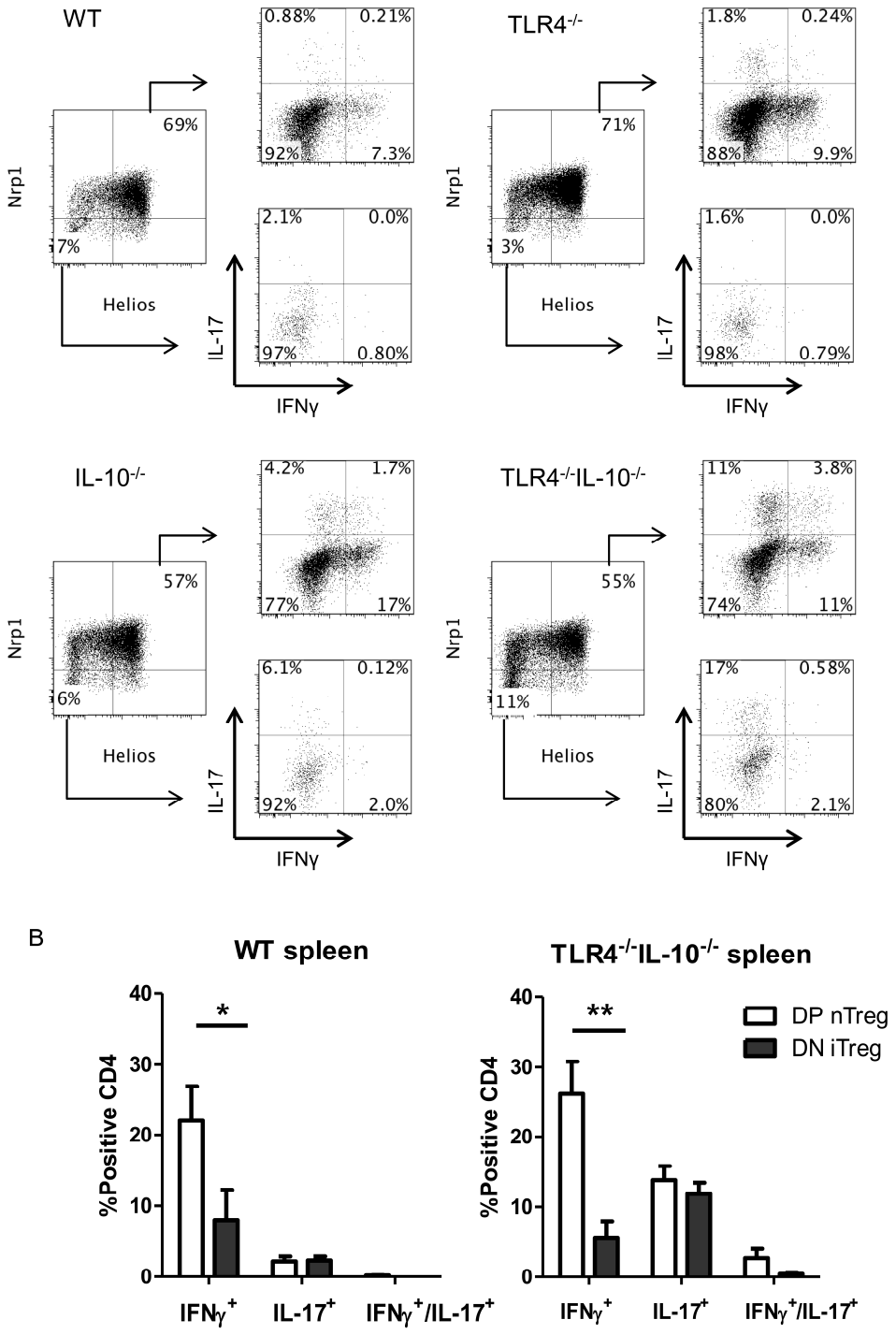

Figure 13. Helios^{+}Nrp1^{+} Treg and Helios^{-} Nrp1^{-} Treg cells differentially express IFNγ and IL-17. A. Treg (CD4^{+}Foxp3^{+}) cells were separated into Helios^{+}Nrp1^{+} and Helios^{-} Nrp1^{-} Treg cells, and analyzed for IFNγ and IL-17 expression from the spleen and intestinal lamina propria of TLR4$^{-/-}$, IL-10$^{-/-}$, TLR4$^{-/-}$IL-10$^{-/-}$ and WT mice. *p>0.05, **p>0.01, ***p>0.001. Data are representative of 3 or more experiments of 2 or more mice per group.

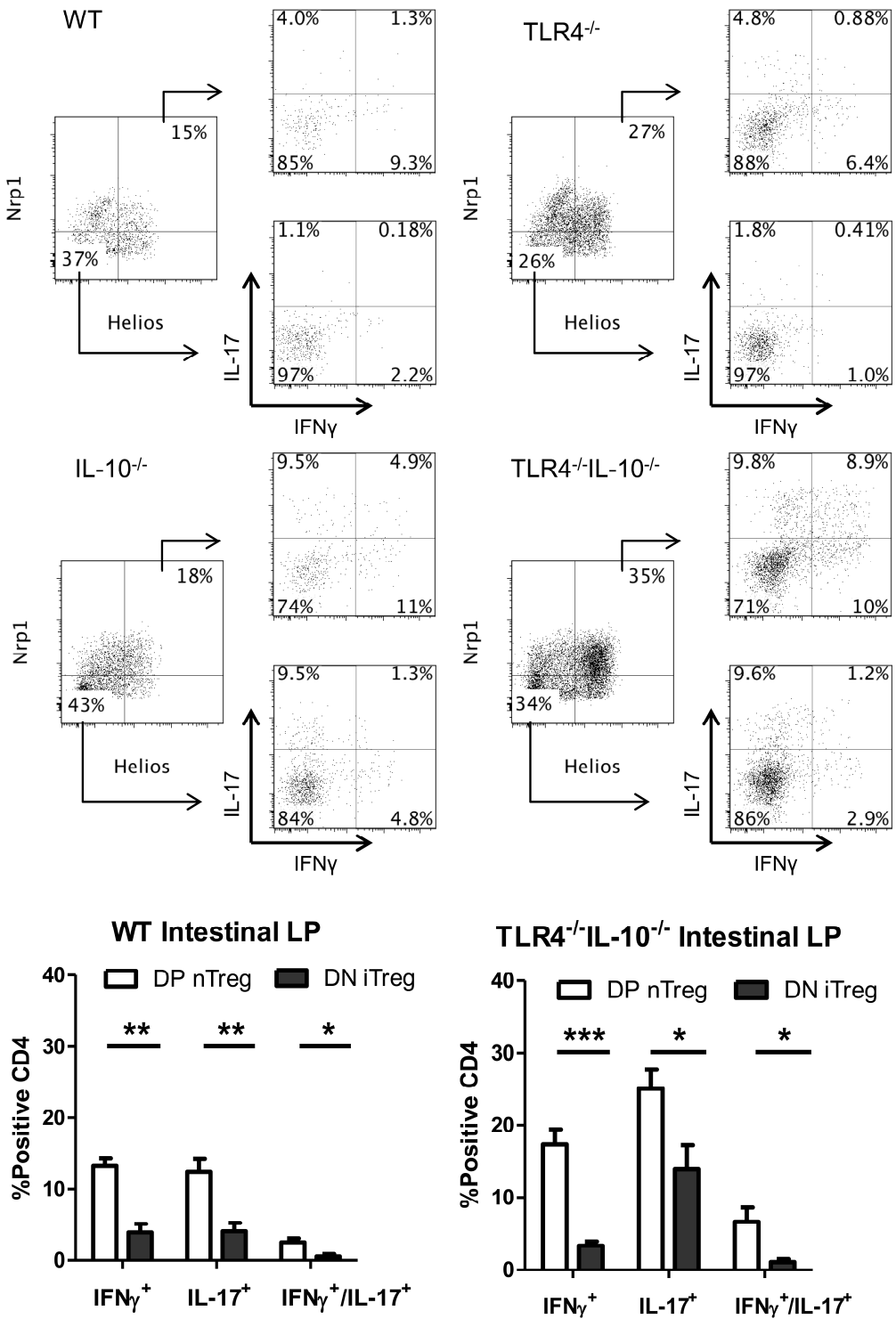

Figure 13 (cont'd). Helios^{+}Nrp1^{+} Treg and Helios^{-} Nrp1^{-} Treg cells differentially express IFNγ and IL-17. B. Helios^{+}Nrp1^{+} Treg cells and Helios^{-} Nrp1^{-} Treg cells from the spleen and intestinal lamina propria of TLR4$^{-/-}$ IL-10$^{-/-}$ and WT mice were analyzed for IFNγ and IL-17 production. *p>0.05, **p>0.01, ***p>0.001. Data are representative of 3 or more experiments of 2 or more mice per group.

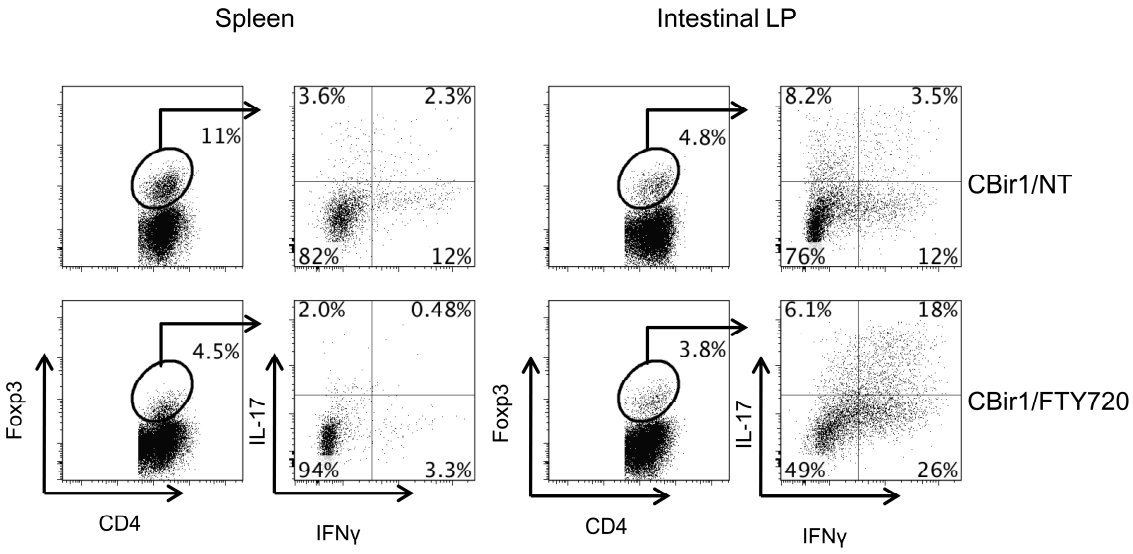

Figure 14. Expansion and acquisition of effector function by Treg cells occurs in the intestine. $0.25x10^6$ CBir1 CD4 cells were intravenously transferred into $Rag2^{-/-}$ mice. Recipient mice were gavaged with PBS or FTY720 every 2 days. Four weeks post-T cell transfer, Treg cells were isolated from the spleens and intestinal lamina propria of CBir1 CD4 T cell recipient mice and analyzed for Foxp3, IFNγ, and IL-17 production by flow cytometry. Data are representative of 2 experiments of 4 mice per group with similar results.

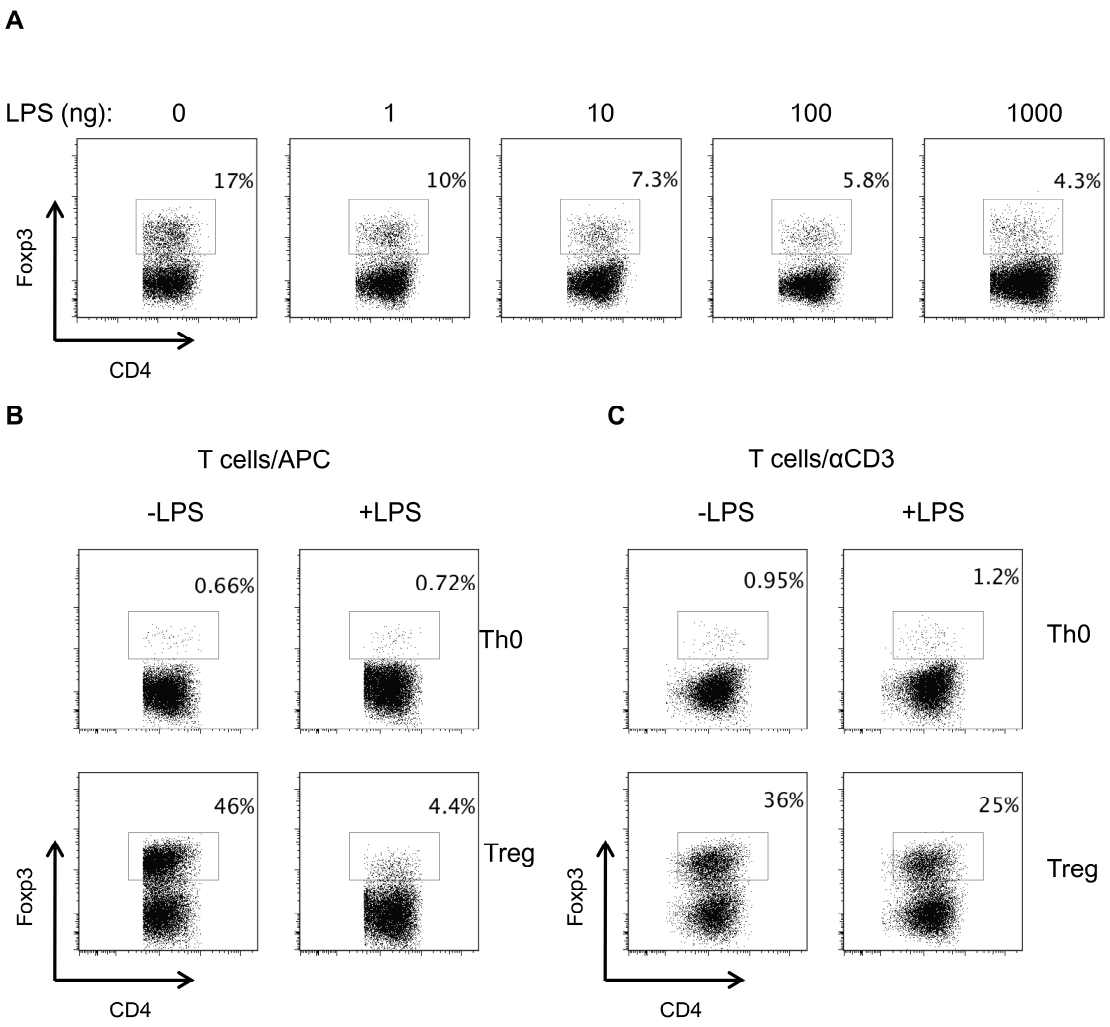

Figure 15. LPS inhibits Foxp3 Tregs. A. CBir1 CD4 T cells were co-cultured with irradiated splenocytes with CBir1 peptide and 5ng/ml TGFβ to induce Foxp3 induction. Cells were also treated with or without varying concentrations of LPS and analyzed by flow cytometry after 5 days. **B**. CBir1 CD4 T cells were co-cultured with irradiated splenocytes with CBir1 peptide in the absence of polarization (Th0), or with 5ng/ml TGFβ (Treg). Cells were also treated with or without LPS (1000ng) and analyzed by flow cytometry after 5 days. **C.** CD4 T cells were cultured with plate-bound αCD3 (1μg) and αCD28 (2μg/ml) in the absence of polarization (Th0), or with 5ng/ml TGF-β (Treg). Cells were also treated with or without LPS (1000ng) and analyzed by flow cytometry after 5 days.

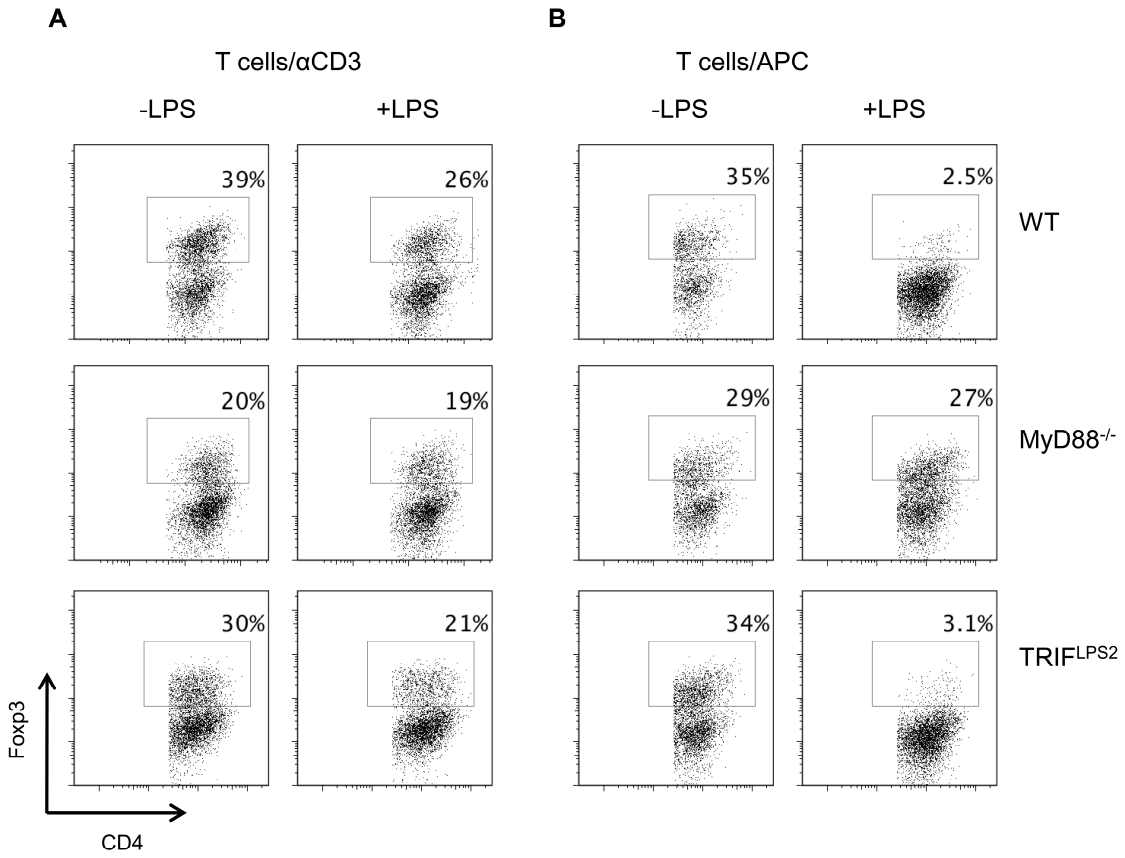

Figure 16. LPS partially inhibits Treg generation through MyD88 signaling on CD4 cells and antigen-presenting cells. **A**. MyD88$^{-/-}$, TRIF$^{-/-}$, or WT CD4 cells were cultured with plate-bound αCD3 (1μg) and αCD28 (2μg/ml) with 5ng/ml TGF-β (Treg), in the presence or absence of LPS (1μg) and analyzed by flow cytometry after 5 days. **B**. MyD88$^{-/-}$, TRIF$^{-/-}$, or WT irradiated splenocytes were cultured with WT CD4 cells with plate-bound αCD3 (1μg) and 5ng/ml TGFβ (Treg), in the presence or absence of LPS (1000ng) and analyzed by flow cytometry after 5 days.

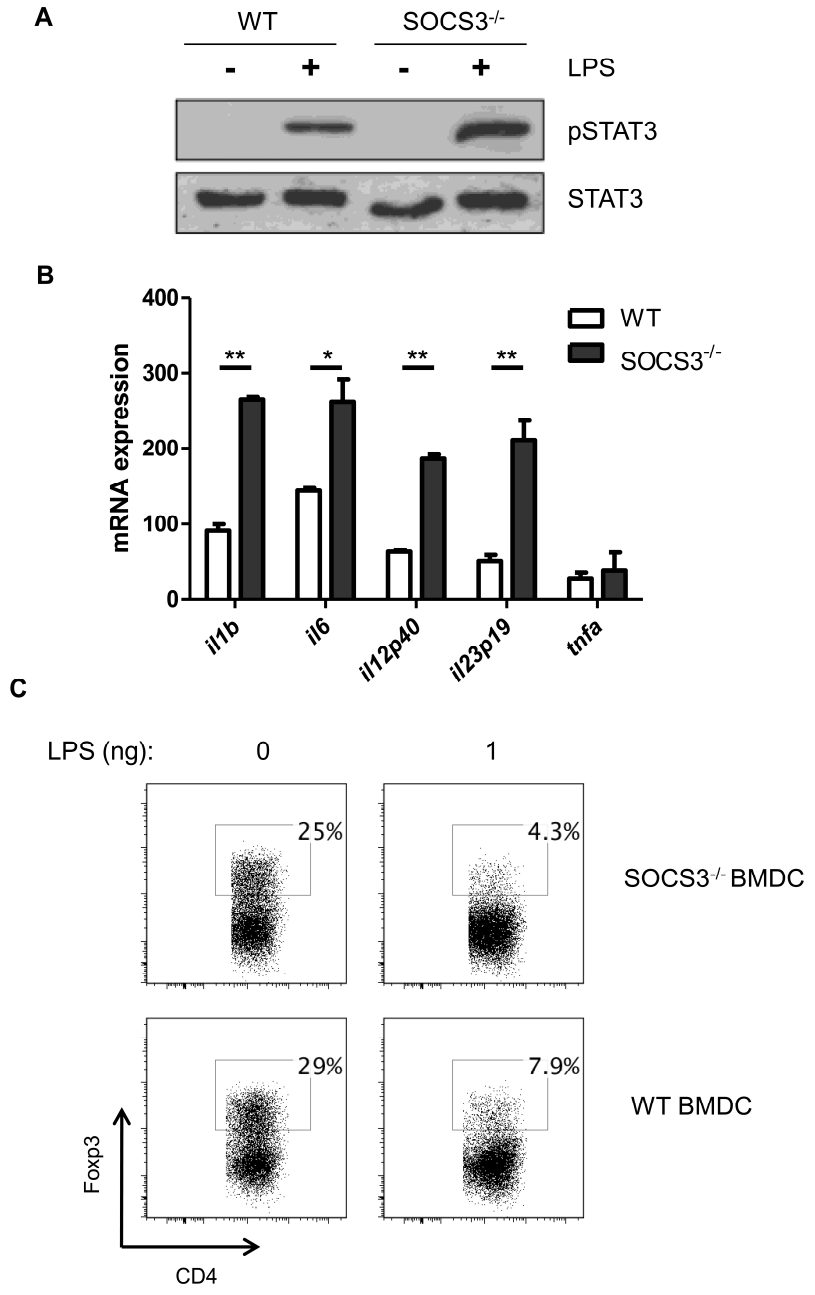

Figure 17. SOCS3 mediates LPS inhibition of Foxp3 Tregs. Bone marrow dendritic cells from SOCS3$^{-/-}$ or WT mice were derived with GM-CSF after 8 days. **A**. BMDCs were treated with medium (UN) or LPS (10ng/ml) for 4 h. Cell lysates were subjected to immunoblotting with anti-Phospho-STAT3, anti-STAT3, and GAPDH antibodies. **B.** Primary BMDCs from SOCS3fl/fl and LysMCre-SOCS3fl/fl mice were treated with medium (UN) or LPS (10ng/ml) for 4 h. mRNA was analyzed for IL-1β, IL-6, IL12p40, IL-23p19 and TNFα by qRT-PCR. Representative of three independent experiments. *p>0.01, **p>0.001. **C.** BMDC were co-cultured with CBir1 CD4 cells with TGFβ (5ng/ml), in the presence or absence of LPS (1ng) and analyzed by flow cytometry after 5 days.

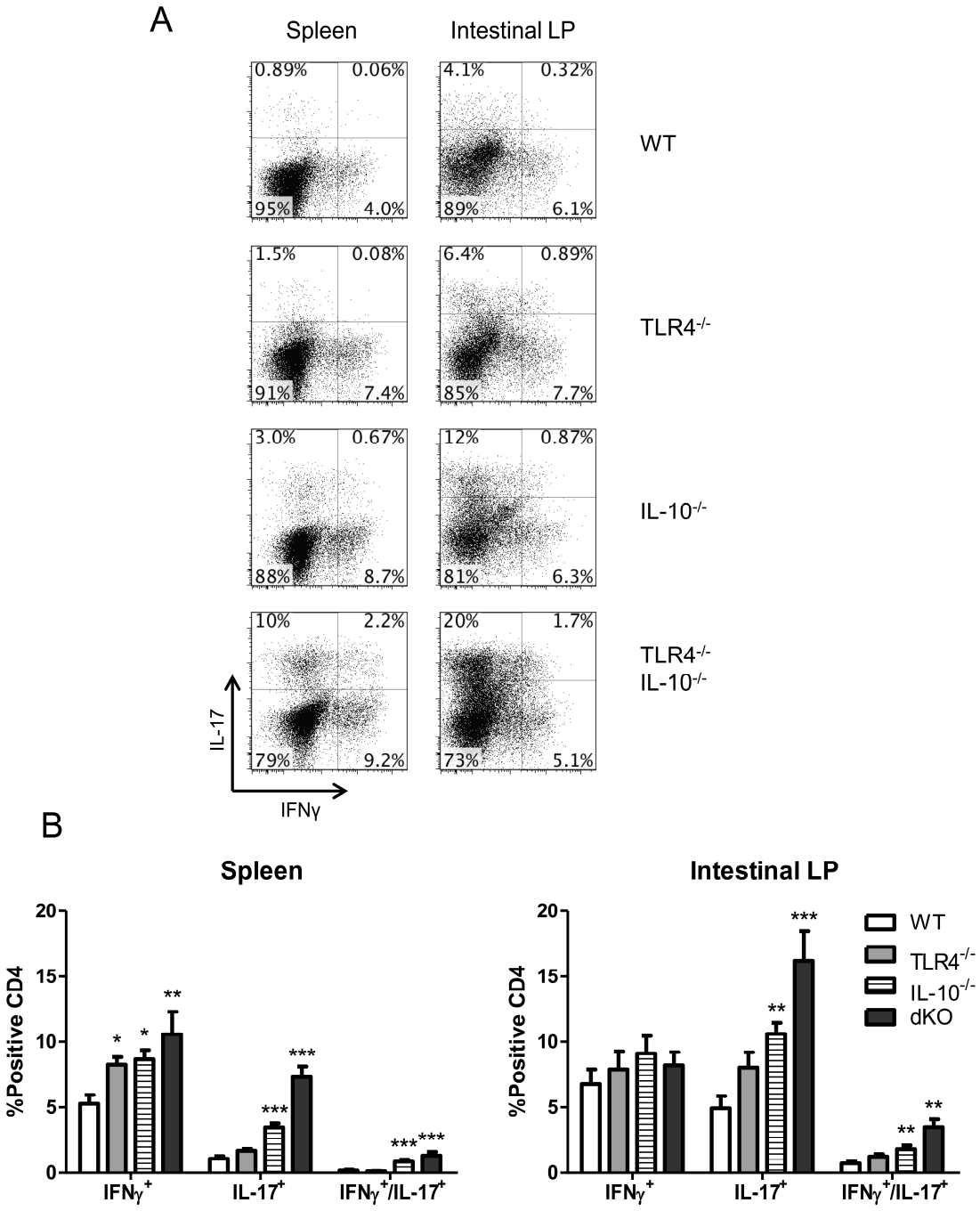

Figure 18. Absence of TLR4 increases Treg cell cytokine production. Spleen CD4 cells from TLR4$^{-/-}$, IL-10$^{-/-}$, TLR4$^{-/-}$IL-10$^{-/-}$, and WT mice were analyzed for IFNγ and IL-17 production. *p>0.05, **p>0.01, ***p>0.001 compared to WT mice. Data are representative of 3 or more experiments of 2 or more mice per group with similar results.

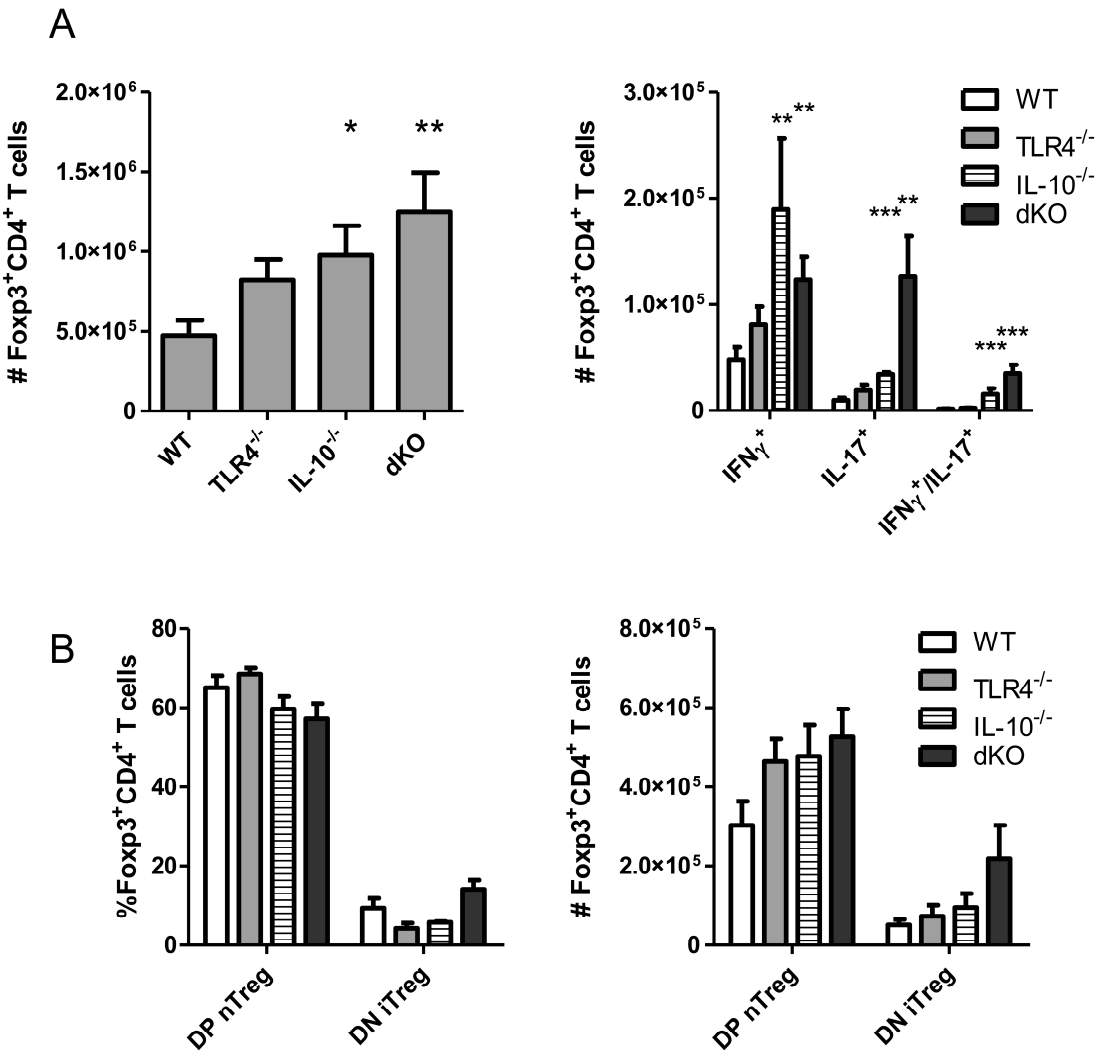

Figure 19. Tregs are increased systemically in the absence of TLR4 and IL-10. (**a**) Spleen CD4 cells from TLR4$^{-/-}$, IL-10$^{-/-}$, TLR4$^{-/-}$IL-10$^{-/-}$ (dKO) and WT mice were counted for Foxp3 expression, and IFNγ and IL-17 in Tregs. (**b**) Tregs were analyzed for Helios and Nrp expression. DP nTreg denotes Helios+Nrp1+ Tregs and DN iTreg denotes Helios-Nrp1- Tregs.

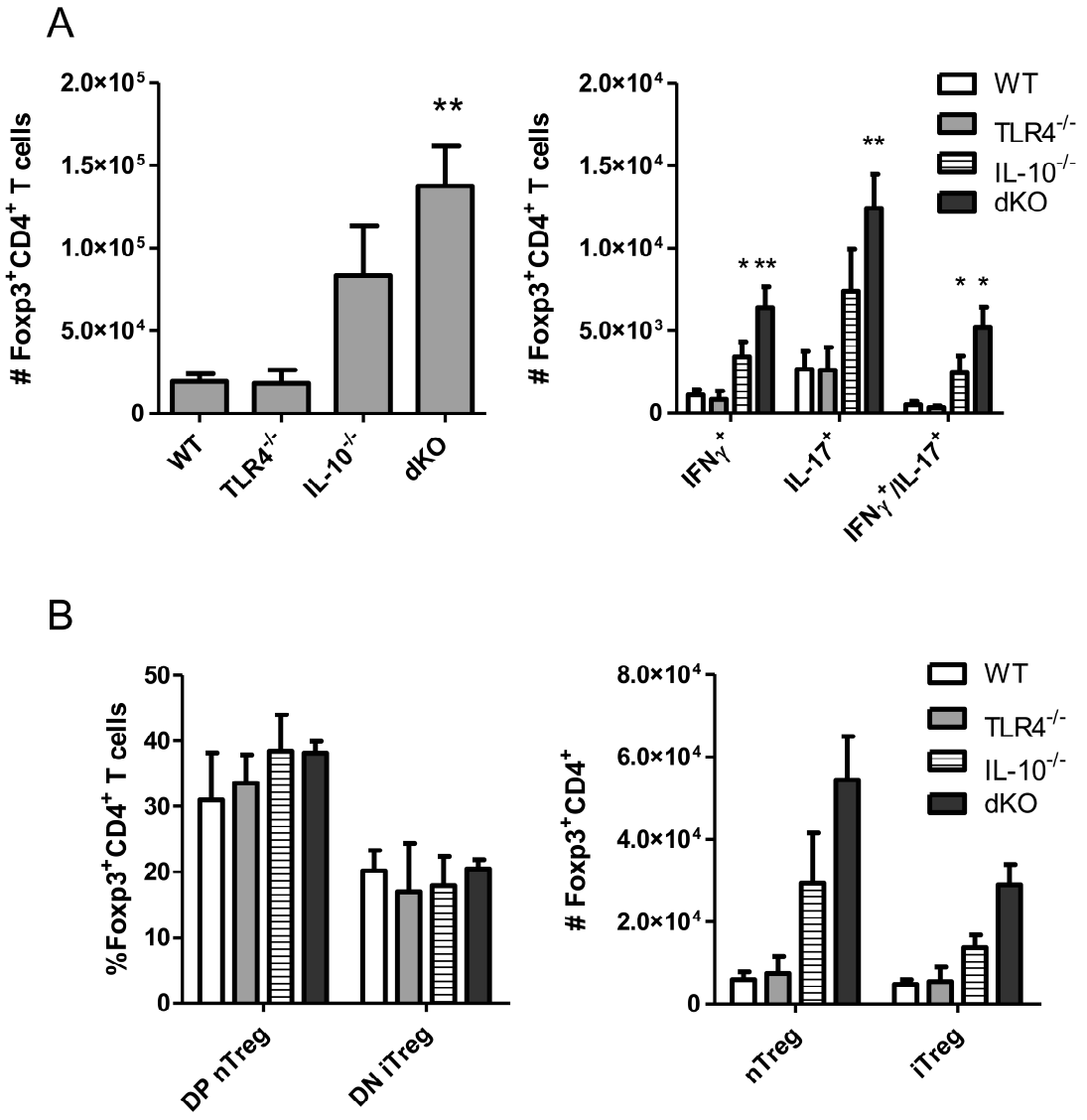

Figure 20. Intestinal Treg cells are increased in the absence of TLR4 and IL-10. (a) Intestinal CD4 cells from TLR4$^{-/-}$, IL-10$^{-/-}$, TLR4$^{-/-}$IL-10$^{-/-}$ (dKO) and WT mice were counted for Foxp3 expression, and IFNγ and IL-17 in Tregs. (**b**) Tregs were analyzed for Helios and Nrp expression. DP nTreg denotes Helios^{+}Nrp1^{+} Tregs and DN iTreg denotes Helios^{-}Nrp1^{-} Tregs.

SUMMARY AND CONCLUSION

The host intestinal tract lives in constant balance with the commensal microflora within. Inflammatory responses by the immune system against the microbiota must be tightly controlled, lest the egregious antigenic load set off chronic immune activation and inflammation. A wide variety of mechanisms exist to control inflammatory responses and maintain and/or restore intestinal homeostasis. In my dissertation studies, I discuss how Th17 cells contribute to IgA-mediated protection, and the deleterious effects that chronic inflammation and inflammatory microbial ligands have upon regulatory T cells and the restoration of homeostasis.

The intestinal immune system is comprised of many immune cells that are capable of initiating strong proinflammatory responses. As the intestinal environment is in constant contact with the microbiota, it is crucial that the intestinal immune system limits its exposure to the gut flora. The host immune cells and the microbiota have evolved various mechanisms to improve immune exclusion, such that the intestines and microflora can live in symbiotic harmony. A key aspect to maintaining immune exclusion is the construction of an ample barrier to separate the microflora from the immune cells patrolling the lamina propria. Though the epithelial barrier is composed of only one monolayer of enterocytes, the layer is coated in a thick layer of mucus that is difficult for bacteria to penetrate. The secretion of antimicrobial peptides by enterocytes, as well as the secretion of IgA reinforces the mucus layer into a bactericidal barrier to further prevent bacterial penetration, thereby limiting the number of antigens that are presented to the intestinal adaptive immune system. As these mechanisms are vital for intestinal homeostasis and the general health of the host, many factors are involved in their regulation. However, it is not fully understood which factors contribute to maintaining intestinal homeostasis.

In the present study, I investigated the relation between intestinal Th17 cells and intestinal IgA production and secretion. Previous observations have hinted at a relationship between Th17 cells and IgA-producing B cells. Independent studies showed that colonization of mice with SFB enhanced levels of IgA-producing B cells[76], and concomitantly increased Th17 cells in the intestines[77, 78]. Our initial studies of mice deficient in IL-17 signaling (IL-17R-/-) revealed a significant decrease in luminal IgA, directly implicating Th17 cells in regulation of intestinal IgA. As intestinal epithelial cells serve as the intermediary between IgA production, and IgA secretion into the lumen, I revealed that Th17 cells and IL-17 induce polymeric immunoglobulin receptor (pIgR) expression on intestinal epithelial cells. As epithelial cells express IL-17R, Th17 cells are able to act directly upon epithelial cells to induce pIgR, by signaling through both PI3K and NF-κB. By itself, IL-17 is able to significantly increase pIgR expression from epithelial cells, however, when combined with TNFα, expression is strongly enhanced, in an apparently synergistic manner. Given that TNFα is also produced by Th17 cells, it appears that production of IL-17 may partially function to augment gene expression induced by TNFα. With low concentrations of TNFα, this characteristic may be important in maintaining homeostasis. This phenomenon has been observed previously with a number of chemokines induced by TNFα. IL-17 is able to prolong the half-life of various TNFα-induced mRNAs that are typically unstable[161-163], although it is thought that IL-17 cannot directly induce many of these genes. Here, I present that IL-17 can directly induce pIgR expression, while also synergizing with TNFα, though it is yet to be seen if IL-17 also stabilizes pIgR mRNA in a similar manner.

To contend with the immense bacterial load, both humans and mice secrete a large amount of IgA. As I initially recorded decreased luminal IgA in mice deficient in IL-17 signaling, I questioned whether the decrease in IgA would hamper intestinal protection, or whether the amount of IgA that remained could sufficiently protect the intestine from infection. By inducing epithelial breakage through oral administration of

dextran sulfate sodium, there is increased bacterial penetration and subsequent inflammation. Mice deficient in pIgR lack all luminal secretory IgA, and only a small amount of IgA leaks out from the lamina propria, however it lacks the stability afforded by pIgR binding. These pIgR-/- mice displayed more profound disease than wild-type mice[43], indicating that secretory IgA as a whole is critical for guarding the epithelium, particularly during intestinal injury. In line with this, I observed that after DSS administration, IL-17R-/- mice suffered from worsened disease compared to wild-type mice as indicated by increased weight loss, as well as severely inflamed tissue pathology. It becomes clear that the high presence of IgA is imperative for controlling intestinal bacteria, and preventing immune system exposure and infection, particularly during intestinal injury. Accordingly, the inability to contain microbial antigens can have detrimental effects on the intestinal environment.

The induction of IgA is mediated by the immunoregulatory molecule TGFβ. While Th17 cells do not produce TGFβ, transfer of Th17 cells into T cell-deficient mice was able to greatly increase intestinal IgA production. We found that the Th17 cell cytokine IL-21 could act in coordination with TGFβ to greatly accelerate B cell differentiation to IgA-producing cells. In an isolated environment, IL-21 serves to induce IgG responses, however the intestinal environment provides many molecular cues to mediate and augment IgA production. As such, the dichotomous nature of Treg and Th17 cells actually work in conjunction to regulate IgA production (**Figure 21**).

I found that chronic intestinal inflammation can profoundly alter regulatory T cell function, and LPS –TLR4 signaling plays a detrimental role in the generation of peripheral regulatory T cells. The use of colitic IL-10$^{-/-}$ mice serves provide a model to investigate the effects of chronic inflammation upon regulatory T cell function, as disease is propagated by dysregulated immune responses from effector T cells. In light of the inflamed environment, the immune system attempts to induce anti-inflammatory measures to restore intestinal homeostasis, as evidenced by the increased frequency of

Treg cells observed in inflamed environments. However, I found that, despite the increase in Treg cells, colitis remains unmitigated in IL-10$^{-/-}$ mice. Furthermore, Treg cell function is hampered as more Treg cells acquire effector cell characteristics and produce the inflammatory cytokines IFNγ and IL-17, while retaining Foxp3 expression. The conversion of regulatory T cells into effector T cells is a phenomenon that has been critically analyzed in both IBD patients and in animal models. Our studies[130] and others[32] have attempted to elucidate the inflammatory factors that influence Treg cell generation and function.

As the intestinal immune system exists in a delicate balance with the microbiota, there also exists a delicate balance between the effector T cell response and regulatory T cells. Often, slight insults can be enough to tip the scales towards dysregulated inflammation, particularly in genetically susceptible individuals. As I have illustrated above, immune exclusion is a key component in preventing the initiation of inflammation, and that IgA greatly contributes to this protection. However, my work has revealed that the presence of LPS can impair Treg cell generation. LPS is the prototypical TLR ligand and can potentiate highly proinflammatory responses from the immune system by triggering TLR4. In my studies, I found that the presence of LPS in culture with naïve T cells and antigen-presenting cells could greatly diminish the number of Foxp3^{+} Treg cells that were generated from the culture. A 10-20 fold decrease in the frequency of Treg cells may very well disrupt the balance of effector and regulatory T cells. As both T cells and APCs express TLR4, I investigated the contributing roles of both T cell-intrinsic TLR4 signaling and APC-intrinsic signaling upon Foxp3 inhibition. Generation of Treg cells with LPS in an APC-free system allowed us to investigate whether TLR4 signaling could directly modulate Foxp3 induction. While I originally observed a 10-20 fold decrease in Foxp3 induction by LPS when APCs were present, TLR4 signaling on T cells still decreased Foxp3 expression by approximately one-third, indicating that LPS can prevent regulatory T cell responses by signaling through both T

cells and innate immune cells.

It has been proposed that one method of restoring the balance between effector and regulatory T cells is to increase the number of Treg cells by autologous transfer[154, 164]. By isolating a pool of Treg cells from the periphery of an IBD patient, expanding the pool in vitro, and transfusing increased numbers of Treg cells, restoration of intestinal homeostasis is attempted via improved immunoregulation through increased Treg cell number and function. However, the observations that Treg cells are present in inflamed environments and that they produce inflammatory cytokines raises concerns over the effectiveness of the proposed Treg therapy. As Treg cells have two different origins, I investigated whether thymic Treg or peripherally-induced Treg cells are more inclined to acquire effector status. In the chronically-inflamed intestines of IL-10-/- mice, both thymic Treg and induced Treg cells produce IFNγ and IL-17, and these cytokine-producing Treg cells can be found in the spleen as well as the intestine, indicating that these cells can enter systemic recirculation. As such, it becomes clear that any proposed therapy utilizing pools of Treg cells will need to overcome the propensity for acquiring effector function when under the influence of proinflammatory cytokines.

Taken together, our studies regarding the maintenance of intestinal homeostasis through the regulation of intestinal barrier immunity and adaptive immune responses reveals a highly dynamic interaction between the immune system and the commensal microflora. Th17 cells strengthen the epithelial barrier by inducing passive immune mechanisms from epithelial cells. The induction of polymeric immunoglobulin receptor, as well as antimicrobial peptides, leads to improved control of the microbiota and provides an improved defense of the intestinal lamina propria during intestinal injury and limiting microbial breach and infection. Breach of the intestinal barrier can have detrimental effects by disrupting the balance between inflammation and regulatory mechanisms, effectively tipping the threshold to initiate pathogenic chronic inflammation. Our studies to appreciate the mechanisms that contribute to protecting the

intestinal barrier and promote immune exclusion improves understanding of the events that may initiate chronic intestinal inflammation, and may help direct approaches to prevent the onset of colitis in genetically-susceptible individuals, as well as improving regulatory T cell-based therapies.

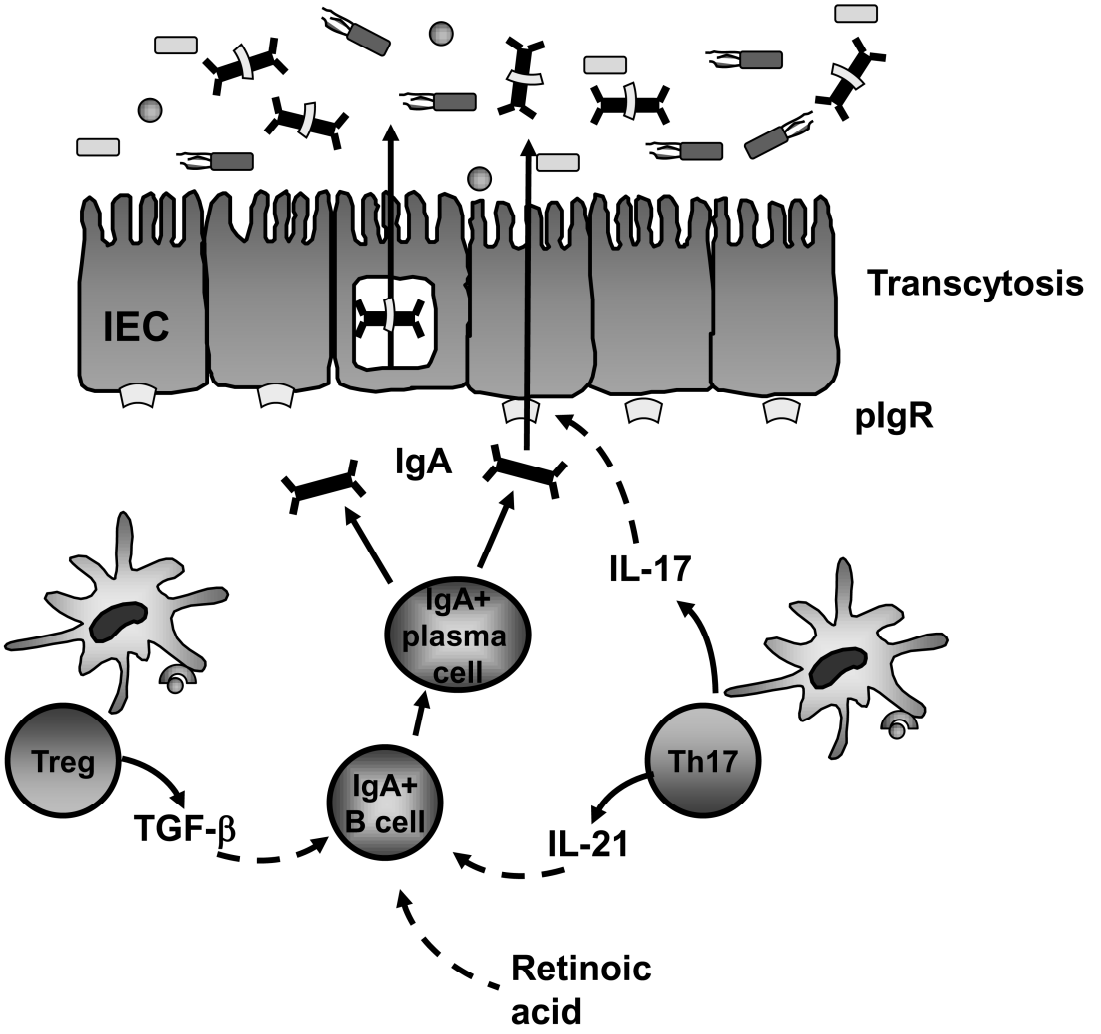

Figure 21. Coordinate regulation of intestinal IgA production and secretion by Treg and Th17 cells. TGFβ produced by Treg cells drives naïve B cells to differentiate into IgA-producing cells. Retinoic acid from stromal cells and IL-21 from Th17 cells accentuates the effect of TGFβ and increases IgA^+ B cell differentiation. Polymeric IgA then binds to pIgR expressed on intestinal epithelial cells, causing transcytosis of pIgR-bound pIgA, and the IgA complex is secreted into the lumen as sIgA. IL-17 from Th17 cells increases pIgR expression from IECs and increases the rate of sIgA secretion into the lumen.

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