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Adaptive immune responses in intestinal homeostasis and experimental inflammatory bowel disease

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Adaptive immune responses in intestinal homeostasis and experimental inflammatory bowel disease

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Dedication

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Adaptive immune responses in intestinal homeostasis and experimental inflammatory bowel disease

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The intestine, a dwelling place for trillions of commensal bacteria, is constantly exposed to numerous ligands and antigens from foods and microbiota. Intestinal homeostasis involves a balance between anti-inflammatory and pro-inflammatory signals. Intestinal epithelial barrier, commensal bacteria, and immune cells, along with the interactions between the three players maintain a toleranic environment to food and microbiota. Conversely, when a dysregulated interaction occurs in genetically susceptible individuals, the homeostasis breaks down and chronic intestinal inflammation develops. $CD4^+$ T cells are important mediators for both intestinal homeostasis and colitis development. The intestine is a preferential site for induction of Treg, Th1 and Th17 cells. The differentiation, plasticity and functions of $CD4^+$ T cells in intestines are intensely studied, but still incompletely defined. The differentiation of peripherally induced Tregs and Th17 cell population is reciprocally regulated in the intestine. In addition to the regulation by master

transcriptional factors and STATS signaling, the ERK pathway is also involved in regulating or fine-tuning T cell lineage commitment. ERK differentially regulates Tregs and Th17 cell development by positively regulating Th17 and negatively regulating Treg cell differentiation. My studies indicated that the inhibition of ERK decreased IL-6 induction of RORγt while it enhanced TGF-β induction of Foxp3. Moreover, ERK inhibitor-treated T cells under Th17 conditions possessed a suppressive function in vitro because they produced more IL-10 and TGF-β. Furthermore, ERK inhibitor-treated T cells under Th17 polarization conditions had a decreased potency to induce colitis in vivo. Although accumulating evidence demonstrates that differentiated $CD4^+$ T cells preserve plasticity to alter phenotypes under various conditions, it is still unclear how stable Th1 cells are and whether Th1 cells can convert into Th17 cells. I demonstrated that Th1 cells converted into Th17 cells under inflammatory conditions in the mouse intestines. TGF-β, IL-6 and IL-2, but not hypoxia factors, differentially regulated Th1 to Th17 conversion. TGF-β induction of Runx1 and RORγt, was crucial for the conversion. Taken together, my studies revealed the interference with the ERK pathway could represent a therapeutic treatment for inflammatory bowel diseases and demonstrated that Th1 cells convert into Th17 cells under inflammatory conditions in intestines, which was mediated by TGF-β induction of Runx1.

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AMP	Antimicrobial peptide
APC	Antigen-presenting cell
ChIP	Chromatin Immunoprecipitation
CMRA	Chloromethyl rhodol acetate
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Foxp3	Forkhead box protein p3
HIF	Hypoxia inducible factor
i.v.	Intravenous
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
LPL	Lamina propria lymphocyte
LPS	Lipopolysaccharide

MACS	Magnetic cell sorting
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
NK	Natural killer
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PP	Peyer's patches
PRR	Pattern recognition receptors
RA	Retinoic acid
Rag	Recombination activating gene
RNA	Ribonucleic acid
RORγt	Retinoic acid-related orphan receptor gamma t
RORα	Retinoic acid-related orphan receptor A
RT-PCR	Real time PCR
Runx	Runt-related transcription factor
SFB	Segmented filamentous bacteria
siRNA	Small interfering RNA
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
Treg	Regulatory T cell
WT	Wild type

INTRODUCTION

Chapter 1: Intestinal mucosa homeostasis

1.1 THE BALANCE OF TOLERANCE AND IMMUNITY IN INTESTINES

The intestinal mucosal site is the entrance for numerous stimulation of and antigens from foods and microorganisms. This entrance is susceptible to invasion by viruses, bacteria, fungi and parasites. To combat potential pathogens, mammals have evolved mucosa associated lymphoid tissues with specialized innate and adaptive immune cells. The mucosal immune system itself contains most of the lymphocytes and immunoglobulin in the body. On the other hand, the intestinal immune system has evolved multiple mechanisms to maintain tolerance responses to digested foods and gut commensal flora.

Intestinal homeostasis involves a balance between anti-inflammatory and pro-inflammatory signals. Under steady state conditions, regulatory mechanisms of intestinal immune system dominantly take control to promote intestinal tolerance. The contributors to tolerance include an intact intestinal epithelial barrier, regulatory types of antigen presenting cells, Treg cells and IgA production. During infection or inflammatory conditions, with microbiota shifts and disrupted intestinal epithelial barrier, innate and adaptive immune cells are activated by pathogen-associated molecular patterns (PAMPs) derived from pathogens or endogenous damage-associated molecular patterns (DAMPs) from injured or dying host cells[1]. In the inflamed sites, proinflammatory immune cells are recruited, and expanded and trigger strong immune responses. Under normal circumstances, inflammation is acute. Once pathogens are cleared or certain stress is relieved, the gut environment will promptly return to homeostatic conditions. However, in genetically susceptible individuals, a failure in pathogen clearance or dysfunction of

immunoregulation may results in chronic inflammation, for example, inflammatory bowel diseases (IBD).

In Chapter One, I will discuss the major players which contribute to intestinal homeostasis as well as the interactions among those players.

1.2 INTESTINAL EPITHELIAL BARRIER

The intestinal surface covers a huge area of approximately 100 m^2 that is composed of a single layer of intestinal epithelial cells (IECs). This single layer of cells forms a barrier between the intestinal lumen and intestinal lamina propria. IECs comprise several specialized cell types, including enterocytes, goblet cells, and Paneth cells. Each IEC subtype contributes to barrier integrity through distinct functions. Enterocytes, the most abundant cell type, absorb dietary nutrients, form the surface epithelium and ensure barrier function through an apical brush border and intercellular tight junctions. Intercellular tight junctions, which seal the apical epithelium, prevent the traffic of fluid and small molecules through the intercellular gaps. Paneth cells reinforce the defense of the enterocyte by secreting a broad range of antimicrobial peptides (AMP) including α-defensins (cryptidins), β-defensins, and cathelicidins[2, 3]. These AMPs confer broad spectrum antimicrobial properties. Goblet cells secrete mucin glycoproteins that assemble to form a thick mucus layer overlying the epithelium[4, 5]. The mucus layer forms a matrix that has a crucial role in concentrating secreted antimicrobial peptides and secretory IgA, close to the epithelial surface. Studies have reported that the disruption of tight junctions and deficiency in the secretion of mucin and defensin results in a colitis phenotype in mice, which reveals an important homeostatic role of each type of intestinal epithelial cells[6].

1.3 INTESTINAL COMMENSAL BACTERIA

Upon birth, mammals begin to acquire commensal microorganisms from their surroundings[7]. The intestinal environment forms a diverse microbial ecosystem. Among all populated microorganisms, commensal bacteria are the dominant and best-studied types. The numbers of commensal bacteria are 10 times greater than the cell numbers in the human body[8]. The combined gene numbers of commensal communities are at least 100 times greater than the gene numbers of the human genome[8]. The constant interaction between commensal bacteria and the human intestine has a great influence in maintaining a homeostatic balance between tolerance and immune responses. Commensal bacteria profoundly influence the development of the human mucosal immune system, e.g., nutrient absorption and digestion...etc. Complementarily, the pathogenic state of human diseases can cause a shift in the species and in the diversity of commensal communities.

The intestine is home to large communities of indigenous bacteria. Microbial communities in the intestine have a high level of diversity at the species level but low phylum-level diversity. In all vertebrates, intestinal microbial communities are dominated by two phyla: the Firmicutes and the Bacteroidetes[9-11]. The Firmicutes are Gram-positive bacteria that include numerous species belonging to the Clostridia class, in addition to the Enterococcaceae and Lactobacillaceae families and Lactococcus species. The Bacteroidetes are Gram-negative bacteria comprised of several *Bacteroides* species. Prominent intestinal *Bacteroides* species include *Bacteroides thetaiotaomicron*, *Bacteroides fragilis* and *Bacteroides ovatus*[10]. The remaining intestinal bacteria, comprising less than 10% of the total population, belong predominantly to the Proteobacteria and Actinobacteria phyla [10].

1.4 PATTERN RECOGNITION RECEPTORS ON INTESTINAL EPITHELIAL CELLS AND COMMENSAL BACTERIA- REGULATION OF INTESTINAL EPITHELIAL CELLS

Pattern recognition receptors (PRRs) expressed by IECs include Toll-like receptors (TLRs) and NOD-like receptors (NLRs). In general, ligation of TLRs and NLRs results in the activation of innate immune responses. It is thought that the initial contact between IECs and intestinal microorganisms is through the interaction between PRRs of IECs and microbial PAMPs. There have been very few reports that identify structural differences between the PAMPs of pathogenic and commensal bacteria which allow host cells to easily discriminate between the two classes of microorganisms[12]. In contrast, some evidence has shown that the decreased expression and selective distribution of PRRs on IECs are part of the mechanisms for IEC to discriminate between commensals and pathogenic bacteria. For example, the slight expression of TLR4 and CD14 on IECs ensure the minimal responses to LPS from Gram-negative bacteria[13-15]. Similarly, the limited level of expression of TLR2 results in hyporesponsiveness in IECs when the cells encounter Gram-positive bacteria[16]. In another example, TLR5, which recognizes bacterial flagellin, is reported to have a restricted expression on the basolateral surface of IECs so that it is mainly activated by invading bacteria[17]. Similar to the distribution in other cell types, TLR3, TLR7, TLR8 and TLR9 are found in intracellular endosomal organelles while NLRs are expressed in the cytoplasm[18, 19]. These intracellular PRRs only recognize invading bacteria, but not commensal bacteria present in the intestinal lumen. In addition, various host and microbial factors may modulate and fine-tune the interaction between PRRs of IECs and microbial PAMPs. Some pathogenic flagella are reported to not only interact with TLR5 but also with TLR2[20]. The interactions of PAMPs with multiple PRRs may determine the level of host responses.

Complementarily, some evidence demonstrates that commensal bacteria actively modulate functions of IECs. The basal level of interaction between PRRs and commensal bacteria maintains the intestinal epithelial barrier without initiating a cascade of innate

immune responses. For example, TLR signals stimulate AMP expression from IECs[21-23], modulate intracellular tight junctions[24, 25] and induce the release of protective cytokines such as IL-18, which is essential for epithelial barrier integrity and repair[26, 27]. Commensal bacteria also trigger PRR signaling which cooperates with other cellular stress responses, which contribute to intestinal homeostasis, such as autophagy and ER stress. The autophagy pathway, induced by PRR signaling or ER stress response, promotes the secretion of AMPs as well as mucins and enhances the clearance of pathogens[28]. Notably, NOD2, a member of NLRs, is highly expressed in Paneth cells and regulates their secretion of AMPs[29] and has been reported to stimulate autophagy by interacting directly with an autophagy gene ATG16L1[30]. The regulatory role of NOD2 is essential in both IECs and intestinal immune cells, especially in antigen-presenting cells. Polymorphisms in *NOD2*, *ATG16L1* and ER stress response genes, including *XBP1*, *AGR*2 and *ORMDL3*, are risk factors for inflammatory bowel disease (IBDs)[31, 32].

1.5 INTESTINAL DENDRITIC CELLS AND MACROPHAGES

Intestinal DCs and macrophages have a distinctive phenotype compared with their counterparts in lymphoid or other tissues. These distinct phenotypes of intestinal DCs and macrophages are thought to contribute to maintenance of gut homeostasis. Under homeostatic conditions, mucosal DCs and macrophages are anti-inflammatory. Among heterogeneous mucosal DC subsets, $CD103^+$ $CD11b^-$ DCs are short-lived[33] and have a preferential induction of Treg cells, gut homing receptors on IgA-secreting B cells and T cells[34-37]. More information about $CD103^+$ DCs will be addressed in 1.7. It is also reported that one subset of DCs, which expresses αvβ8-integrin, mediates the activation of latent TGF-β and serves as a source of intestinal TGF-β[38]. Human intestinal macrophages have strong phagocytic and bactericidal activity; however, they lack CD14 expression, which is required for TLR4 activation[39]. These cells also showed an impaired

ability to produce proinflammatory cytokines when being activated with a TLR4 ligand and other stimuli[39]. It is reported that intestinal $CX_3CR1^+F4/80^+$ monocyte-derived macrophages constantly produce high level of IL-10 in response to microbiota through MyD88 pathway[40] and inhibit $CD4^+$ T cell proliferation through a cell-cell contact[41]. These $CX_3CR1^+F4/80^+$ macrophages can project dendrites through the single-cell intestinal epithelial layer and sample antigens directly from the intestinal lumen in a CX_3CR1 dependent manner[42]. Alternatively, antigens flow through goblet cells and are collected by the $CX_3CR1^+F4/80^+$ macrophages in the lamina propria[43]. $CD103^+$ DCs present in the lamina propria then receive antigens from the macrophages. This transfer of antigen from $CX_3CR1^+F4/80^+$ macrophages to $CD103^+$ DCs is dependent on the gap junction molecule connexin 43(Cx43)[43]. The $CX_3CR1^+F4/80^+$ macrophages reside in lamina propria and have poor antigen presenting ability to prime $CD4^+$ T cells. In contrast, $CD103^+$ DCs can migrate to draining lymph nodes and prime $CD4^+$ T cell effeciently[44]. In addition, specialized M cells located in the Peyer's patches sample luminal antigens by selective endocytosis and the antigens are then taken up by nearby DCs. Through this process, intestinal DCs can sample antigens from the commensal microflora[45]. It has been reported that the microbial sensing through PRRs drives complementary functions in IECs and hematopoietic cells. While the basal level of PRR signaling in IECs play a protective role through maintaining the intestinal epithelial barrier, aberrant PRR signaling in hematopoietic cells tends to drive chronic inflammation[46].

1.6 INTESTINAL REGULATORY T CELLS

Among various T-cell populations with anti-inflammatory functions, regulatory T cells (Treg), including $Foxp3^+CD25^+CD4^+$ Tregs and $Foxp3^-CD4^+$ IL-10-secreting Tr1 cells, play a particularly important role in regulating intestinal homeostasis[47]. The

signature transcription factor Foxp3 expressed in $Foxp3^+$ Tregs is required for cell development as well as regulatory activity. $Foxp3^+$ Tregs can be generated in the thymus or be induced from naive $CD4^+$ T cells in the periphery in the presence of TGF-β. Deficiency in the gene encoding Foxp3 results in immune dysregulation and a fatal inflammatory disease in mice and X-linked IPEX syndrome in humans[48]. $Foxp3^+$ Treg cells are particularly abundant in the intestinal mucosa. It has been reported that the intestine is a preferential site for Treg induction which occurs by interaction with microbiota[49-51]. Peripherally induced Tregs and the Th17 cell populations are likely to be reciprocally regulated in the intestine[52, 53]. While TGF-β is required for the differentiation of both Tregs and Th17 cells, cytokines which activate STAT3 pathways, such as IL-6 and IL-21, along with the presence of TGF-β, will drive naïve CD4 cells to a Th17 cell fate[54]. The later may mean that extraneous stimulation resulting in production of proinflammatory IL-6 and IL-21 allows the inflammatory response to override Treg-cell induction and promotes Th17 effector cell responses for host defense.

1.7 EPITHELIAL CELL- REGULATION OF IMMUNE CELL FUNCTION

How can the intestine tolerate numerous stimulations and antigens from foods and trillions of intestinal bacteria? Some mechanisms have been proposed to promote the induction of tolerogenic DCs which sequentially drive the differentiation of regulatory T cells under steady state conditions. IECs regulate DC function through their secretion of immunoregulatory mediators, including thymic stromal lymphopoietin (TSLP), TGF-β and retinoic acid (RA)[55, 56]. In addition, IECs express a range of metabolic enzymes, including indoleamine 2,3-dioxygenase (IDO), which is involved in the induction of Treg cells[57, 58]. It has been demonstrated that TSLP limits DC-derived IL-12 production and DC capacity to promote Th1-cell differentiation[56]. TGF-β is a regulatory cytokine which affects gut DCs and is important for the generation of $Foxp3^+$ Treg cells[52, 55]. In the

presence of RA and TGF-β, DC precursors give rise to $CD103^+$ tolerogenic DCs[33, 59]. Again, through RA and TGF-β, these $CD103^+$ tolerogenic DCs promote the peripheral induction of $Foxp3^+$ Treg[34, 35]. Conversely, RA inhibits the generation of Th17 and thus regulates the balance between Th17 effector cell and T regulatory cell populations in gut[52, 53]. Another effect of RA is to induce gut homing receptors, α4β7 integrin and CCR9, on T cells and IgA-secreting B cells[36, 37]. Interestingly, gut-associated $CD103^+$ DCs express retinal dehydrogenase, which is an enzyme required for generation of RA from vitamin A[60]. It has been demonstrated that activation of TLR2 signaling upregulates the expression of retinal dehydrogenase in DCs, indicating that the interaction between gut-associated DCs and commensal bacteria may contribute to oral tolerance[61].

1.8 COMMENSAL BACTERIA- REGULATION OF IMMUNE CELL FUNCTION

Commensal bacteria, along with their secretion and metabolites can regulate host immune responses. Some commensal bacteria preferentially induce a specific type of T helper cell response. For instance, colonization with *Clostridium* strains from IV and XIVa clusters promotes $FoxP3^+$ Treg generation in intestinal lamina propria[49, 50]. Segmented Filamentous Bacteria (SFB) preferably promote intestinal Th17 generation[62]. It has been demonstrated the colonization of commensal bacteria in general inhibits Th2 responses in intestinal lamina propria[63, 64]. Microbial secretions and metabolites can also play a regulatory role. For example, short-chain fatty acids (SCFA), including acetate, propionate and butyrate, are products of the fermentation of fiber by gut microbes and reported to be immunomodulatory. SCFA are ligands of G protein–coupled receptor (GPR43), which is mainly expressed on innate immune cells. GPR43-deficient mice are susceptible to inflammation in multiple experimental disease models including colitis models[65]. Polysaccharide A, produced by commensal bacteria *Bacteroides fragilis*, promotes induction of regulatory T cells and local IL-10 induction through TLR2

signaling[49]. Gut flora DNA as a TLR9 ligand suppresses Treg conversion and thus possesses potential as a vaccine adjuvant [66]. ATP released from commensal bacteria to intestinal lumen promotes the generation of Th17 cells[67], which promote IgA production[68]. In summary, commensal bacteria and their products can cause pro-inflammatory or anti-inflammatory effects and shape the development and function of the mucosal immune system. The regulation generates a homeostatic response which is hyporesponsive to microbiota and actively defensive against pathogens. The dysregulated interactions between the host immune system and microbiota could result in a loss of tolerance and perpetuate intestinal inflammation.

Chapter 2: Pathogenesis of Inflammatory bowel disease

2.1 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD), a chronic disease, which is influenced by environmental risk factors, arises as a result of dysregulation of the gastrointestinal immune system and the host flora. The major types of IBD are Crohn's disease and ulcerative colitis. The two main diseases have both overlapping and distinct clinical and pathological features. The common clinical manifestations include persistent diarrhea, intestinal bleeding, and abdominal cramps. In ulcerative colitis, the key features include continuous inflammation and ulcers throughout inflamed area and diffusion inflammation in colonic mucosa[69]. In Crohn's disease, inflammation is typically transmural but can be granulomatous, patchy and segmental. Involvement of the terminal ileum is most common, although any site of the gastrointestinal tract may be affected [69].

IBD constitutes a significant health burden in developed countries and impacts the quality of life of some 1.4 million individuals in North America and 2.2 million individuals in Europe[70]. Although developing countries have historically reported a lower prevalence of IBD, the incidence of IBD has dramatically increased in those nations, e.g. China and India, as they have become industrialized[71, 72]. This increase may be indicative of the influence of environmental factors, including lifestyle, hygiene, diet, and use of antibiotics, all of which play a role in triggering IBDs. In addition to environmental factors, the genetic factors play a stronger role in Crohn's disease than in ulcerative colitis. The importance of genetic susceptibility has been established through human genome-wide association studies and over 100 IBD susceptibility loci have been identified. The risk loci include deficiency or polymorphisms in PRRs, autophagy, responses to ER stress, epithelial barrier, immune tolerance, T cell regulation and others[73-75].

As mentioned earlier, the microbiota itself actively participates in intestinal homeostasis. The shifts in microbiota can both be a cause or a result of intestinal inflammation. Dysbiosis, a condition with an imbalance in microbiota composition, induces or perpetuates inflammation. Numerous studies have demonstrated that the microbiota shifts during inflammatory bowel disease, usually with a decrease in Firmicutes and an expansion in some proteobacteria, mostly enterobacteriaceae[76, 77]. In addition, a loss in bacteria with potential anti-inflammatory features, such as *Faecalibacterium prausnitzii*, during inflammation is reported[78]. Although an expansion of proteobacteria or enterobacteriaceae is regarded as a mark of dysbiosis, current studies do not indicate a single organism responsible for IBD. Rather, increasing evidence show that a dysregulated host immune response to microbiota is essential to inflammatory bowel disease. The environmental and genetic factors have a great influence on shaping the composition of the gut microbiota and on determining which members and components of the microbiota are targeted by pathogenic effector T cells.

2.2 RESPECTIVE ROLES OF TH1 AND TH17

Although human-genome wide association studies and numerous mutant mouse models revealed over 100 risk loci of IBD, among which many mutant mice develop IBD do not do so when being crossed onto a $Rag^{-/-}$ background[74, 79]. The latter finding indicates that an adaptive immune response to the microbiota is required in the development of intestinal inflammation, particularly effector $CD4^+$ T cell responses. Many experimental colitis models have supported the crucial role of $CD4^+$ T cells in the pathogenesis of colitis. $Rag^{-/-}$ mice, which are deficient in mature T and B cells, do not spontaneously develop IBD, while the transfer of Treg-depleted $CD4^+$ effector T cells into $Rag^{-/-}$ mice induces IBD in the recipient mice[80]. In addition, development of colitis is dependent on commensal bacteria. Germ-free $Rag^{-/-}$ mice receiving $CD4^+$ effector T cells

do not develop colitis. It confirms that $CD4^+$ effector T cells responsive to antigens from gut microbiota play a key role in the pathogenesis of colitis. Among various antigens and ligands from microbiota, bacterial flagellins not only serve as TLR5 ligands but are also identified as immunodominant antigens in half of all Crohn's disease patients and in numerous experimental colitis models[81]. A T cell adoptive transfer colitis model with $CD4^+$ cells specific for CBir1 flagellin from A4 bacteria has been established[82]. Consistent with other adoptive transfer models, the transfer of CBir1 TCR-specific $IFN\gamma^+$ Th1 or $IL\text{-}17^+$ Th17 cells potently induces colitis, revealing that Th1 and Th17 cells are important mediators of intestinal inflammation[83-85]. The production of IL-12 and IL-23, which is essential for Th1 and Th17 responses, is elevated in the inflamed lesions of Crohn's disease[86-89]. The neutralizing antibody against IL-12/IL-23 p40 is effective to attenuate colitis in patients with Crohn disease[90, 91]. A novel therapy using neutralizing antibody against IL-23 p19 has been proven capable of treating colitis in several mouse IBD models[92, 93]. The findings from genome-wide association studies confirmed that the *IL23R* and *IL12B* gene loci are strongly associated with Crohn's disease and ulcerative colitis[74].

Required for immunity against intracellular bacteria and viruses and implicated in the pathogenesis of Chron's disease, Th1 cells are characterized by IFNγ production and expression of transcriptional factor T-bet. Th1 differentiation is initiated by the activation of STAT1 which is induced by IFNγ or IL-27 secreted from NK cells or APCs and sequentially upregulates T-bet. T-bet collaborates with transcription factors activated following TCR signaling to promote IFNγ production. IFNγ, along with IL-2, induces the expression of IL-12 receptor and increases cell sensitivity to IL12 through activation of STAT4 and STAT5. T-bet induces the activation of Runx3, which further promotes IFNγ production. These transcriptional factors collaborate to mediate Th1 cell differentiation and lineage commitment[94-96].

The differentiation of Th2 cells involves the induction of transcriptional factor GATA3. GATA3 activation is mediated by IL-4 and the sequential STAT6 activation. IL-2-induced STAT5 activation is also involved in the initial stage of Th2 differentiation. Collaborating with other signaling, GATA3 further promotes IL-4 transcription. This establishes a positive-feedback loop to drive the differentiation of Th2 cells, which are characterized by the expression of IL-4, IL-5 and IL-13[94, 97]. Increasing evidence demonstrated that very few IL-4$^+$ Th2 cells are present in healthy intestines without colonies of parasitic infection[98], which is at least partially attributed to the suppression of Th2 responses by microbiota[63, 64]. Although the number of IL-4$^+$ cells is not strongly associated with ulcerative colitis, the production of IL-5 and IL-13 is reported to be elevated in the inflamed intestine[99, 100]. Some studies clarified the observations by the finding that a group of non-classical CD1d-restricted NKT cells are responsible for the elevated production of IL-5 and IL-13 because this cell population expands during an active ulcerative colitis[100].

The differentiation of Foxp3$^+$ Tregs cells and Th17 cells are closely associated. With the stimulation from TGF-β, both Foxp3 and RORγt, the lineage specific transcriptional factors of Tregs cells and Th17 cells respectively, are induced in the common precursor of all T helper subsets, CD4+ naïve cells[52]. The strength of TCR stimulation, STAT3 activating cytokine signaling, oxygen supply, the presence of RA, and other factors further determine the cell fate[101-105]. When naïve CD4$^+$ cells receive weak TCR stimulation under TGF-β/IL-6 Th17-skewing conditions, Treg differentiation is favored[101]. Naïve CD4$^+$ cells that receive strong TCR signaling and TGFB/IL-6 prefer Th17 fate[101]. In the absence of the STAT3-activating cytokine IL-6, Foxp3 inhibits the function of RORγt and blocks Th17 cell differentiation. Conversely, in the presence of IL-6, the activation of STAT3 and the expression of RORγt become dominant and therefore promote the development of Th17 cells[106, 107]. IL-2-induced STAT5 activation attenuates STAT3 activation and blocks Th17 development[108]. Hypoxia inducible factor

1α (HIF1α) forms a transcriptional complex with RORγt and p300 to promote IL-17 transcription while HIF1α binds Foxp3 and targets Foxp3 for degradation.[105] RA promotes Treg while suppresses Th17 cell development; however, when in conjunction with IL-15, RA can activate DCs to release proinflammatory cytokines IL-12 and IL-23 and thus triggers favoring Th1 and Th17 responses[109]. Th17 cells produce several cytokines, including IL-17, IL-21 and IL-22[93]. Although IL-23 is dispensable for Th17 differentiation, it mediates Th17 effector function and pathogenicity[110, 111].

2.3 PLASTICITY OF TREGS AND $CD4^+$ EFFECTOR T CELLS IN INFLAMMATORY INTESTINES

Some types of intestinal immune cells possess different phenotypes and functions under steady-state conditions and during inflammation. Different subsets of intestinal DCs dominate in different settings. $CD103^+$ DCs contributing to tolerance under steady-state conditions are replaced by CX_3CR1^+ DCs during inflammation[33]. Unlike intestinal DCs in which, in different settings, one subset is replaced by another of a distinct origin under, $CD4^+$ T cells are reported to preserve plasticity to change in functions. The conversion between $CD4^+$ T cell subsets is heavily influenced by the cytokine milieu and other environmental factors[112]. Under steady-state conditions, Th17 cells are largely IL-17 single-positive, while in colitis settings, significant proportions of the cells are IL-17 and IFNγ double-positive[113]. This transition of Th17 cells into IFNγ-producing T cells during inflammation is driven by IL-12, IL-23 and correlates with increased expression of T-bet[84, 113-115]. The presence of $IL\text{-}17^+IFN\gamma^+$ T cells in the inflamed mucosa, but not in the steady state, suggests that these cells may contribute to the pathogenesis of disease. In the CBir1 TCR Tg T cell transfer colitis model, transfer of Th17 cells induces more severe colitis than the transfer of Th1 cells in Rag-/- recipient mice. The severity may be attributed to $IL\text{-}17^+IFN\gamma^+$ T cells derived from Th17 cells[84]. Also, in the CBir1 T cell

model, Th17 cell derived IL-17 promotes the production of IL-12 and IL-23 from mucosal DCs and thus induce IFNγ production and Th1 responses[84].

The plasticity of Th1 cells under different inflammatory settings is relatively less studied. Th1 cells are considered one of the most stable T helper subsets through numerous T cell transfer studies and epigenetic studies[94, 97, 116, 117]. However, in recent years, some reports demonstrate the plasticity of Th1 cells. In a mouse model infected with the gastrointestinal helminth *Nippostrongylus brasiliensis*, IFNγ$^+$ Th1 cells converting into IL-4$^+$ Th2 cells and IFNγ$^+$IL-4$^+$ double-positive cells was observed when Th1 cells were adoptively transferred[118]. The results are consistent with early studies on the reversibility between established Th1 and Th2 cells[119]. In another study, it was demonstrated that human Th1 cells can convert into Foxp3$^+$ Treg cells in human-into-mouse xenogeneic GVHD. Under a tumor microenvironment, the Th1 to Treg conversion was mediated by overexpression of the programmed death ligand-1 (PDL1) through inactivation of STAT1[120]. These studies illustrate the abundant in vivo differentiation plasticity of Th1 cells in distinct disease settings. More studies are required to clarify and confirm the functions of Th1 cells in homeostatic and inflammatory settings.

RESULTS

Chapter 3: ERK mitogen-activated protein kinase differentially regulates Th17 and Treg cell development and contributes to the pathogenesis of colitis

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3.1 INTRODUCTION

Ulcerative colitis and Crohn's disease are collectively termed inflammatory bowel disease, a chronic inflammation of the intestines[121]. The intestines encounter a daily heavy antigen load from food, commensal bacteria, and occasionally evading pathogens[122]. Among multiple regulatory mechanisms that maintain the balance between mucosal tolerance and immune responses, $CD4^+$ T cells play a crucial role in maintaining intestinal homeostasis. While Tregs suppress intestinal inflammation[123], commensal bacterial, antigen-specific Th1 and Th17 cells have been implicated in the pathogenesis of IBD[87]. The development of different T cell subsets is regulated by a combination of the expression of a master transcriptional regulator and the phosphorylation of a particular STAT protein stimulated by distinct cytokines[124]. IL-12 and IFN-γ promote Th1 cells through activation of T-bet/STAT4. TGF-β and IL-2 stimulate Treg cell development through activation of Foxp3/STAT5. Additionally, it drives Th17 cell

differentiation in the presence of IL-6 through activation of RORγt/STAT3. However, accumulating evidence indicates that other signaling pathways may also be involved in regulating or fine-tuning T cell lineage commitment. The ERK MAPK signal transduction pathway is comprised of Ras, Raf, MEK1/2, and ERK1/2[125]. It has been shown that blockade of ERK attenuates Th17 cell-mediated autoimmune diseases in mouse models, such as EAE (experimental autoimmune encephalomyelitis)[126]. Such attenuation is partially due to ERK dependent IL-1β/IL-23 production in dendritic cells, which stabilize Th17 lineage commitment[126], and also possibly due to the involvement of the ERK MAPK pathway in the signaling of TGF-β and IL-6.

TGF-β alone induces both Foxp3 and RORγt expression in $CD4^+$ T cells, and Foxp3 inhibits RORγt activity and promotes Treg cell differentiation[52, 106, 107] . In the presence of IL-6, TGF-β-induced Foxp3 expression is suppressed, and the combined signaling of TGF-β and IL-6 synergistically induces RORγt expression and promotes Th17 cell differentiation[107]. Once binding its receptors, TGF-β activates the Smad pathway[127], including Smad2/3 and Smad4, which have been reported to be immediate mediators downstream of TGF-β receptors and key in mediating TGF-β-induced development of Treg, but dispensable in Th17 cells[128, 129]. The ERK MAPK pathway is involved in the signaling of both TGF-β and IL-6; however, it is not completely understood how it regulates the differentiation of Th17 cells and Tregs. Although it has been shown that the blockade of ERK inhibits Treg differentiation but does not influence Th17 differentiation[130], other reports demonstrate that inactivation of ERK with small molecule inhibitor U0126 promotes Treg generation[131] as well as Th17 cell differentiation[132]. In this study, I carefully examined the ERK MAPK pathway downstream of TGF-β and IL-6 signaling and assessed their roles under different conditions for T cell differentiation. I found that the blockade of ERK under Th17 polarization conditions inhibited Th17 cell differentiation by reducing RORγt expression and promoted Treg by enhancing Foxp3 expression, whereas the blockade of ERK under

Treg polarization conditions enhanced Treg development. Furthermore, microbiota antigen-specific T cells generated under Th17 conditions in the presence of ERK inhibitors exhibited less capability to induce inflammation, as they induced much less colitis when transferred into $Rag^{-/-}$ mice. My data thus demonstrate that the ERK MAPK pathway promotes $CD4^{+}$ T cell differentiation into Th17 cells, while inhibiting Treg cell development, and contributes to the pathogenesis of IBD.

3.2 MATERIALS AND METHODS

Mice. C57BL/6 (B6), CD45.1 congenic C57BL/6, and B6.$Rag^{-/-}$ mice were obtained from The Jackson Laboratory and maintained in the animal facilities of the University of Texas Medical Branch. CBir1 TCR Tg mice[82] were bred in the animal facilities of the University of Texas Medical Branch. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch.

Reagents and Antibodies. RPMI 1640, HEPES, penicillin-streptomycin, FBS, 2-mecaptoethanol and sodium pyruvate were purchased from Life Technologies (Carlsbad, CA); CellTracker CMRA, TRIzol reagents, from Invitrogen (Carlsbad, CA); and qScript reverse transcriptase from Quanta Biosciences. Reagents for TaqMan gene expression assays were from Applied Biosystems (Carlsbad, CA). Small molecule inhibitors PD098059 and U0126 ethanolate, EDTA, Collagenase type IV was obtained from Sigma-Aldrich (St. Louis, MO). SMARTpool siRNAs specific for murine MEK1, MEK2 and non-targeting siRNA were purchased from Dharmacon (Lafayette, CO). Anti-mouse CD3 (145-2C11), CD28 (37.51) antibodies, recombinant IL-6, TGF-β1, and IL-1β, IL-23 were from Cell signaling, BioLegend and R&D Systems. Anti-mouse IFN-γ (XMG1.2) and anti-IL-4 (11B11) neutralizing monoclonal antibodies were purchased from BD Biosciences (San Diego, CA). Fluorochrome-conjugated anti-mouse CD4 (RM4-5 and

GK1.5), IL-17 (TC11-18H10), and IFN-γ (XMG1.2) antibodies were purchased from BioLegend (San Diego, CA) and anti-mouse CTLA4 (UC10-4F10-11) from BD Biosciences. Anti-mouse IL-17F (eBio18F10), Foxp3 (FJK-16s), and Foxp3 staining buffer sets were purchased from eBioscience (San Diego, CA). Live/Dead dye indicating cell viability was obtained from Invitrogen (Carlsbad, CA).

Isolation of $CD4^+CD62L^+$ and $CD4^+$ T cells and *in vitro* T cell cultures. $CD4^+CD62L^+$ T cells were isolated using $CD4^+CD62L^+$ T cell isolation kit II from MACS Miltenyi Biotec, following manufacturer's instructions. $CD4^+$ T cells were isolated by using anti-mouse CD4 magnetic beads (BD Biosciences) as previously described[133]. $CD4^+$ T cells were cultured with irradiated, wild-type splenic APCs or stimulated with plate-bound anti-CD3 (5 ng/ml) and soluble anti-CD28 (2 ng/ml) at 37°C in humid air with 5% CO_2 under classical Th17-polarizing conditions with TGF-β (2 ng/ml), IL-6 (30 ng/ml), and anti-IFNγ (10 μg/ml)/anti-IL-4 (10 μg/ml), alternative Th17 polarization conditions with IL-1β (10 ng/ml), IL-6 (30 ng/ml), IL-23(10 ng/ml), and anti-IFNγ (10 μg/ml)/anti-IL-4 (10 μg/ml), or Treg-polarizing conditions with TGF-β (5 ng/ml) .

siRNA transfection. siRNA transfection in $CD4^+CD62L^+$ T cells was performed according to the manufacturer's instructions (Lonza, Amaxa mouse T cell nucleofector kit). Briefly, $1x10^6$ $CD4^+CD62L^+$ T cells were transfected with 50-100 nM siRNA, followed by stimulation with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml). At 6-8 hours after transfection, different cytokines were added into cell cultures. Knockdown efficiency was measured 48 hr post transfection. The expression of IFNγ, IL-17 and Foxp3 were determined 4-5 days later by flow cytometry.

Flow cytometry. As described previously[82], cells were stimulated for 5 hr with PMA (50 ng/ml) and ionomycin (750 ng/ml), with monensin added for the last 3 hr. The cells were fixed and permeabilized by using a Foxp3 staining buffer set. Staining was performed for Live/Dead dye, CD4, CTLA4, Foxp3, IL-17(A), IL-17F, and IFNγ by

using fluorescence-conjugated Abs, and the cells were sampled on a LSRII Fortessa flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). The gating strategy was shown in **Figure 9**.

RT-PCR. Total RNA was extracted with TRIzol reagent and followed by cDNA synthesis. Quantitative PCR reactions were performed by using TaqMan Gene Expression Assays for IL-21, Rorc, Foxp3 on a Bio-Rad iCycler (Bio-Rad, Hercules, CA), and all data were normalized to GAPDH mRNA expression.

ELISA. Mouse IL-17(A), IL-22 and IL-10 ELISA kits were purchased from Biolegend. The detection for each cytokine was performed following manufacturer's instructions.

SEAP Chemiluminescence Assay. MFB-F11 cells are embryonic fibroblasts from TGF-β-1 KO mice transfected with a reporter plasmid consisting of TGF-β responsive Smad-binding elements coupled to a secreted form of human alkaline phosphatase (SEAP) reporter gene. Samples were processed using Great EscAPe SEAP Chemiluminescence kit 2.0 (Clontech). Measured by GloMax™ 96 Microplate Luminometer (Promega), the SEAP activity, representing biologically active TGF-β activity, was shown as chemiluminescence units.

Immunoblotting. Protein (20 μg) in cell lysates were separated by electrophoresis on 12% SDS-polyacrylamide gels and probed with phospho-ERK antibody (Cell signaling) as described previously[134]. Membranes were stripped and reprobed for total ERK.

T cell suppression assay. CD45.1 B6 $CD4^+$ T cells were labeled with CMRA (5 μM) and cultured in 24-well plates at 2 X 10^5 cells per well with 2 X 10^5 irradiated splenic APCs in the presence of $2X10^5$ cells per well of different CD45.2 effector T cells. The cells were harvested 3 days late. Cytokine production in $CD45.1^+$ T cells and CMRA intensity were analyzed by flow cytometry by gating on the $CD45.1^+$ cells.

Preparation of lamina propria lymphocytes. Lamina propria lymphocytes were isolated as previously described[123]. Briefly, for removal of epithelial cells and intraepithelial lymphocytes, the entire intestines were rinsed, sliced into small pieces, and incubated at 37°C for 40 min. The tissues were then digested for 50 min at 37°C with stirring. The liberated cells were collected by passage through a stainless steel sieve and a 100 μM cell strainer (BD Falcon). The isolated cells were pooled together and separated on a 40/75% discontinuous Percoll gradient (Amersham Pharmacia Biotech). The cell yield was typically 2-3 million lymphocytes per mouse with 90% cell viability.

Histopathologic Assessment. At necropsy, cecum, and colon were separated and Swiss rolls of each were prepared. Tissues were fixed in 10% buffered formalin and paraffin embedded. Following paraffin embedding, 5-μm sections were prepared, stained with H&E and visualized with an Eclipse 80i microscope (Nikon; Melville, NY). The severity of tissue damage was quantified by cellular infiltration, hyperplasia, and edema. A score of 0-3, denoting increasingly severe abnormality, was assigned for each of these parameters and added together for a combined histological score. Quantitative measurements were obtained using NIS-Elements BR3.2 software (Nikon).

Statistical analysis. For comparisons between groups, levels of significance were determined by unpaired, two tailed Student's t test in Prism 4.0 (GraphPad Software). A p value <0.05 was considered statistically significant and shown as *.

3.3 RESULTS

3.3.1 INHIBITION OF ERK SIGNALING BLOCKS THE IN VITRO DIFFERENTIATION OF TH17 CELLS

To examine the role of ERK MAPK in Th17 development, I used a selective inhibitor of MEK, an ERK-activating enzyme, PD098059, which is a small molecule readily crossing cell membrane, to block ERK activation during $CD4^+$ T cell

differentiation. Because a high dose of ERK-activating enzyme inhibitor (ERK inhibitor) inhibits cell proliferation[135], for the purpose of studying $CD4^+$ T cell differentiation, I utilized a concentration range of 5-10 μM of PD098059 to allow proliferation of $CD4^+$ T cells and blockade of ERK MAPK to occur simultaneously[136]. I first cultured naïve $CD4^+$ $CD62L^+$ T cells from CBir1 Tg mice, which are specific for the immunodominant commensal bacterial CBir1 antigen[82], under classical Th17- polarizing conditions with TGF-β, IL-6, and anti-IFNγ/anti-IL-4[107] in the presence or absence of PD098059. About 2.5% of freshly isolated splenic $CD4^+$ $CD62L^+$ T cells of CBir1 Tg mice were $Foxp3^+$, and less than 0.05% produced IL-17A (also termed IL-17). Th17 conditions greatly promoted CBir1 $CD4^+$ T cell production of IL-17 (**Fig 1A**). I observed that in the presence of PD098059, $CD4^+$ T cells cultured under classical Th17-polarizing conditions had decreased IL-17 and IL-17F expression and an up-regulation in Foxp3 expression (**Figs 1A**). IL-17 expression was inhibited in a dose-dependent manner (**data not shown**). The total amounts of IL-17 in culture supernatants were also decreased whereas IL-10 and TGF-β were increased when ERK activation was blocked. The amounts of IL-22 were low and showed no obvious difference in both conditions (**Fig 1B**). I observed similar results when another ERK inhibitor, U0216, was used in experiments described above (**Fig 7**). As Th17 cells can also be induced under alternative Th17- polarizing conditions with IL-1β, IL-23, IL-6 and anti-IFNγ/anti-IL-4[137], I added PD098059 into such cultures. I observed a reduced IL-17 production, but Foxp3 expression was not up-regulated (**Fig 1C)**. IL-17 and IL-22 production were decreased when ERK activation was blocked (**Fig 1D**). Under both classical and alternative Th17 polarizing conditions, addition of PD098059 did not affect the expression of CTLA4 and IL-21 (**Figs 1A-1D**). To determine whether the effect of ERK inhibitors on Th17 cell development was due to TCR affinities, Irepeated the ERK inhibitor treatments by using two other $CD4^+$ T cell systems: OTII $CD4^+$ T cells, which specifically recognize ovalbumin (OVA), and B6 $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 antibodies, and obtained similar

results, in that addition of ERK activation inhibitors inhibited Th17 cells but promoted Treg cell development (**Fig 7**). Collectively, these results indicated that the ERK MAPK pathway promoted Th17 differentiation and suppressed Treg differentiation.

3.3.2 KNOCKING DOWN ERK-ACTIVATING ENZYMES BLOCKS TH17 CELL DIFFERENTIATION.

As both ERK inhibitors PD098059 and U0216 are not absolutely specific for the ERK MAPK pathway, I used MEK siRNA, which specifically inhibits the expression of ERK- activating enzyme MEK. Transfection of B6 $CD4^+$ T cells with MEK1 and MEK2 siRNA, which are specific for MEK1 and MEK2, respectively, were characterized with a decreased expression of ERK-activating enzymes and thus decreased ERK phosphorylation/activation levels. Transfection with MEK1 siRNA and MEK2 siRNA suppressed about 50% of MEK1 and MEK2 expression at 48 hr post transfection, when compared with T cells transfected with non-targeting control siRNA (**Fig 2A**), and inhibited $CD4^+$ T cells' development into Th17 cells when T cells were cultured under classical (TGF-β/IL-6/anti-IFNγ/anti-IL-4) (**Figs 2B and 2C)** or alternative Th17-polarizing conditions (IL-6/IL-1/IL-23) (**Figs 2D and 2E**). However, transfection with these siRNAs promoted Treg cell development when cultured under classical Th17-polarizing conditions (**Figs 2B).**

3.3.3 BLOCKADE OF IL-6-INDUCED ERK ACTIVATION DOWN-REGULATES RORΓT EXPRESSION AND REDUCES IL-6-MEDIATED FOXP3 SUPPRESSION DURING DIFFERENTIATION OF TH17 CELLS.

With the observation that the blockade of ERK activation under Th17-polarizing conditions differentially regulated the expression of Foxp3 and IL-17, I next investigated the role of ERK activation mediated by IL-6 or mediated by TGF-β in regulation of *foxp3*

and *rorc*, the gene that encodes RORγt. IL-6 stimulated $CD4^+$ T cell ERK phosphorylation started from 10 min post IL-6 administration (**Figs 3A and 3B**). However, ERK phosphorylation was inhibited by treatment with PD098059 (**Fig 3C**). To examine whether the ERK MAPK pathway was involved in IL-6 induction of RORγt expression, I cultured OT II $CD4^+$ T cells with IL-6 in the presence or absence of ERK inhibitor and measured RORγt expression by RT-PCR. As shown in **Fig 3D**, IL-6-induced RORγt expression was decreased with the addition of ERK inhibitor. It has been shown that TGF-β induces the expression of both Foxp3 and RORγt in $CD4^+$ T cells, and Foxp3 physically binds to RORγt and inhibits RORγt function[106, 138]. In addition to directly inducing RORγt, IL-6 inhibits TGF-β-induced Foxp3, thus further enhancing RORγt functions[138]. To determine whether inhibition of ERK activation also blocked IL-6 suppression of TGF-β-induced Foxp3 expression, I cultured OT II T cells with TGF-β along or together with IL-6 in the presence or absence of ERK inhibitor. As shown in **Figure 3E**, IL-6 inhibited TGF-β-induced Foxp3, which was consistent with the previous studies. Interestingly, addition of ERK inhibitor released the suppression of IL-6 on Foxp3 expression and resulted in the recovery of Foxp3 expression. Collectively, these data demonstrated that the ERK MAPK pathway mediates IL-6 suppression of Foxp3 and up-regulation of RORγt.

3.3.4 INHIBITION OF TGF-B-MEDIATED ERK SIGNALING ENHANCES FOXP3 EXPRESSION.

I then determined whether ERK activation regulated TGF-β-induced Foxp3 expression. I cultured CBir1 $CD4^+CD62L^+$ T cells with TGF-β in the presence or absence of PD098059. Addition of ERK inhibitor PD098059 during Treg differentiation up-regulated Foxp3 expression (**Fig 4A**). The elevated Foxp3 expression did not cause significant changes in production of IL-10 and TGF-β (**Figs 4B and 4C**). Collectively,

these data showed that ERK inhibitor PD098059 was able to enhance Foxp3 expression induced by TGF-β during Treg development. As my data did not reconcile with a previous report showing that addition of ERK inhibitor PD98059 markedly attenuated Foxp3 expression in TGF-β–primed $CD4^+$ T cells[130] and addition of ERK inhibitor U0126 didn't change the level of *foxp3* transcript in TGF-β/IL-6–primed $CD4^+$ T cells[132], I also included U0126, in addition to PD98059, in two culture systems: naïve $CD4^+$ T cells cultured in the presence or absence of TGF-β. Interestingly, U0126 and PD98059 differentially regulated Foxp3 expression under different $CD4^+$ T cell culture conditions. Consistent with a previous report[131], I found that addition of U0126 promoted Foxp3 expression when $CD4^+$ T cells were stimulated with anti-CD3/CD28 in the absence of TGF-β but did not affect Foxp3 expression in the presence of TGF-β (**Fig. 4D)**. In contrast, PD98059 did not promote T cell Foxp3 expression when stimulated with anti-CD3/CD28 in the absence of TGF-β but promoted Foxp3 expression induced by TGF-β. Collectively, these data demonstrated that although both ERK inhibitors U0126 and PD98059 promoted Foxp3 expression, they functioned differentially depending on the status of Treg cell differentiation.

3.3.5 ERK INHIBITOR-TREATED T CELLS UNDER TH17 CONDITIONS EXHIBITED SUPPRESSIVE FUNCTIONS IN VITRO.

I have shown previously that Treg cells co-expressing Foxp3 and IFNγ maintain their suppressive functions[83]. As treatment with ERK inhibitor increased Foxp3 expression during Th17 cell differentiation, I investigated whether such ERK inhibitor-treated T cells under Th17 conditions had a regulatory function. I cultured CD45.2 B6 $CD4^+$ T cells under Th17 polarization conditions with TGF-β and IL-6 in the presence or absence of ERK inhibitor. I also cultured CD45.2 B6 $CD4^+$ T cells with TGF-β to generate Treg cells to serve as positive controls. Five days later, their IL-17 and Foxp3

expression was determined by flow cytometry (**Fig. 5A**). I then measured their inhibitory function by co-culture with CMRA-labeled CD45.1 B6 $CD4^+$ T cells. CMRA-CD45.1 $CD4^+$ T cell proliferation was determined by CMRA dilution and IFNγ production by intracellular staining. As shown in **Fig. 5**, the ERK inhibitor-treated T cells under Th17 conditions inhibited $CD4^+$ T cell activation by inhibiting both proliferation (from 89.3% to 64.9%) and IFNγ production (from 33.7% to 5.83%) at a comparable level to that of Treg cells. Th17 cells also inhibited $CD4^+$ T cell proliferation and IFNγ production to a lesser degree, possibly due to competing culture nutrients with $CD4^+$ T cells (**Fig. 5B-E**). Taken all together, those data indicated that ERK inhibitor-treated T cells under Th17 conditions possessed a regulatory function which could be very likely due to the increased Foxp3 expression by T cells.

3.3.6 ERK INHIBITOR-TREATED T CELLS UNDER TH17 CONDITIONS HAVE DECREASED POTENCY TO INDUCE COLITIS.

Since the blockade of ERK activation during Th17 differentiation inhibited Th17 cell development and enhanced Foxp3 expression, I then investigated whether the blockade of ERK activation limited the inflammatory capacity of such T cells *in vivo*. As I have shown previously that the adoptive transfer of CBir1 Th17 cells induced colitis in $Rag^{-/-}$ recipient mice[139], I cultured CBir1 $CD4^+$ T cells under Th17 polarization conditions with TGF-β and IL-6 in the presence or absence of ERK inhibitor, and transferred them into $Rag^{-/-}$ mice, respectively. Freshly isolated splenic CBir1 $CD4^+$ T cells were also transferred into $Rag^{-/-}$ mice to serve as controls. The mice were sacrificed at 5 weeks post-adoptive T cell transfer, the severity of intestinal inflammation was assessed, and $CD4^+$ T cells from the spleen, MLN and intestinal lamina propria were characterized in the recipient mice. Th17 cells induced severe colitis in $Rag^{-/-}$ recipients, whereas ERK inhibitor-treated T cells under Th17 conditions only induced mild intestinal

inflammation (**Figs 6A-B**). An equivalent number of IFNγ$^+$ CD4$^+$ T cells were found in the intestinal lamina propria in both groups of Rag$^{-/-}$ recipients and slight higher in spleen and MLN of Rag$^{-/-}$ mice receiving ERK inhibitor-treated T cells under Th17 conditions. However, the percentage of IL-17$^+$ and IL-17$^+$IFNγ$^+$ effector CD4$^+$ T cells were consistently lower in Rag$^{-/-}$ recipients of ERK inhibitor-treated T cells under Th17 conditions in intestinal lamina propria, MLN (**Figs 6C-D**). In contrast, there were more Foxp3$^+$ T cells in Rag$^{-/-}$ recipients of ERK inhibitor-treated T cells under Th17 conditions compared to that in Rag$^{-/-}$ recipients of Th17 cells (**Fig 6D**). Freshly isolated CD4$^+$ T cells induced severe colitis at the levels comparable to that of Th17 cells. Collectively, these data demonstrated that ERK inhibitor-treated T cells under Th17 conditions exhibited a decreased capability to induce colitis. To determine whether ERK inhibitor treatment also reduced the pathogenicity of naïve T cells, I treated CD4$^+$CD62L$^+$ T cells from CBir1 Tg mice with or without PD098059, and then transferred them into Rag$^{-/-}$ mice to define their abilities to induce the colitis. ERK inhibitor treatment did not alter the pathogenicity of naïve T cells in the induction of colitis (**Fig 8**).

3.4 DISCUSSION

Multiple molecular mechanisms regulate T cell differentiation. While TGF-β drives T cell expression of Foxp3 to differentiate into Treg cells, it also stimulates Th17 cell development through induction of RORγt in the presence of IL-6[107, 140]. Although the IL-6-STAT3 axis has a central role in the development of Th17 cells and TGF-β-Smad axis in Treg cell differentiation, accumulating data indicate the involvement of other signaling molecules of IL-6 and TGF-β pathways in regulating or fine-tuning Th17 and Treg cell commitment[126, 130, 133, 141, 142]. In this report, I investigated how the ERK MAPK pathway, which is downstream of both TGF-β and IL-6, regulated the development of Th17 and Treg cells, and its role in regulating the pathogenesis of inflammatory bowel

diseases. To this end, I found that the ERK MAPK pathway differentially regulated Th17 and Treg development, in that the blockade of ERK activation enhanced TGF-β-induced Foxp3 expression and Treg cell development. The blockade of IL-6-induced ERK activation inhibited induction of Th17 cells, whereas it promoted Treg cell development. Importantly, ERK inhibitor-treated T cells under Th17 conditions exhibited a reduced capability to induce intestinal inflammation *in vivo*.

TGF-β is a prerequisite for induction of $CD4^+$ T cell Foxp3 expression and differentiation into Treg cells. It has been shown that the Smad pathway is required for TGF-β induction of $CD4^+$ T cell Foxp3 expression; however, accumulating data also demonstrated roles for other downstream signaling pathways. Among Smad-independent, TGF-β-signaling pathways, MAPKs, including ERK, JNK, and p38, play important roles in mediating the intracellular responses to TGF-β[127, 143]. Although previous studies demonstrated that inhibition of ERKs markedly attenuated[130] or had no effects[142] on Foxp3 expression in TGF-β–primed $CD4^+$ T cells, my data in the current report indicated that inhibition of ERK activation by both chemical inhibitors PD98059 and U0126, or siRNAs promoted $CD4^+$ T cell expression of Foxp3 not only under Treg polarization conditions with TGF-β alone, but also under Th17 conditions with TGF-β and IL-6, which was consistent with recent reports that ERK pathways negatively regulated Foxp3 expression at least partially in a TGF-β-dependent manner[102]. In the presence of TGF-β, the ERK2 pathway suppressed a large program of gene expression, effectively limiting the differentiation of $Foxp3^+$ Treg cells[144]. Thus, my studies support the notion that the ERK pathway inhibited Treg cell development. Foxp3 expression is negatively regulated by CpG island methylation[145]. It has been shown that in the absence of DNA methyltransferase I (Dnmt1), TCR stimulation was able to induce Foxp3 expression[146] and ERK pathways inversely regulated the expression of DNA methytransferases[131]. As both ERK inhibitors U0126 and PD98059 suppressed ERK activation-mediated DNA methylation[131, 147], they also promoted transcription of *foxp3* gene. It is still not clear,

however, why the two inhibitors functioned differentially under different T cell culture conditions. But this could provide an explanation at least partially on the disparity of ERK inhibitors on Treg cell Foxp3 expression among different reports.

Controversial data have also been reported on the role of ERK pathways in Th17 cell development. Lu et al. reported that the inhibition of ERK did not attenuate Th17 cell production[130]; however, some other studies demonstrated that the ERK pathway negatively regulated Rorc expression and Th17 cell development, in that ERK inhibited STAT3 and Th17 cell differentiation[148]. Further, inhibition of ERK signaling enhanced Th17 differentiation and effector cytokine expression, and ERK-inhibited Th17 cells exhibited an increased pathogenic potency to induce autoimmunity *in vivo*[132]. There are also reports that activation of ERK MAPK signaling promoted Th17 cell differentiation through TLR-induced DC IL-23 and IL-1b production[126]. Inhibition of ERK MAPK suppressed IL-23 and IL-1β production by dendritic cells stimulated with TLR or dectin-1 agonists, and thus suppressed the ability of TLR-activated dendritic cells to induce Th17 cells. Treatment with an ERK inhibitor attenuated autoimmune diseases, such as EAE, through inhibition of Th17 responses[126, 149]. In the current study, addition of ERK inhibitors suppressed IL-6-induced RORγt expression and promoted TGF-β-induced Foxp3 expression (**Figs 3 and 4**). As it has been shown that Foxp3 functionally inhibits RORγt[138], my data indicated that activation of the ERK pathway promoted Th17 development, probably by mediating IL-6 induction of RORγt as well as inhibiting TGF-β-induced Foxp3 and thus releasing Foxp3 inhibition of RORγt. Although I used both chemical ERK inhibitors, including PD98059 and U0126, as well as siRNA specific for ERK activation, and three different CD4 T cell systems, these data were different from an early report by Tan and Lam[132], which showed that inhibition of ERK promoted Th17 cell development under Th17 condition by using one ERK inhibitor U0126. It is still not clear the exact reasons behind such disparity. Among many possibilities, although I observed addition of both ERK inhibitor PD98059 and U0126 inhibited Th17 cells under

Th17 conditions (**Figs 1, 2 and 7**), I also observed a slightly elevated IL-17 expression when $CD4^+$ T cells were treated with U0126 but not with PD98059 under TCR stimulation with or without TGF-β in the absence of IL-6 (**Fig 4D)**, indicating that U0126 and PD98059 may function differently in regulation of Th17 cell development under different conditions. This may explain at least partially the difference between my data and studies done by Tan and Lam [132]. Compared to PD98059, U0126 may serve as a stronger DNA demethylation regent and activate transcription of Th17 related genes under certain conditions, for example the gene encoding Th17 transcription factor, *rorα* gene, which expression is inversely regulated by CpG methylation.

It is now established that different T cell subsets are not stable and can convert into other subsets of T cells[97]. Under certain circumstances, T cells co-express Foxp3 and effector cytokines, i.e. there are $Foxp3^+IFN\gamma^+$, $Foxp3^+IL\text{-}4^+$, and $Foxp3^+IL\text{-}17^+$ T cells. I also identified, in a previous study, the T cells which co-expressed Foxp3/IFNγ/IL-17 during intestinal inflammation, although their functional roles are still unclear. Moreover, while I did not directly investigate T cell plasticity in the current study, my data indicated that ERK inhibitor-treated T cells under Th17 conditions exhibited a decreased pathogenic potency to induce intestinal inflammation *in vivo*. Thus it could provide a potential therapeutic treatment for IBD and possible other autoimmune diseases.

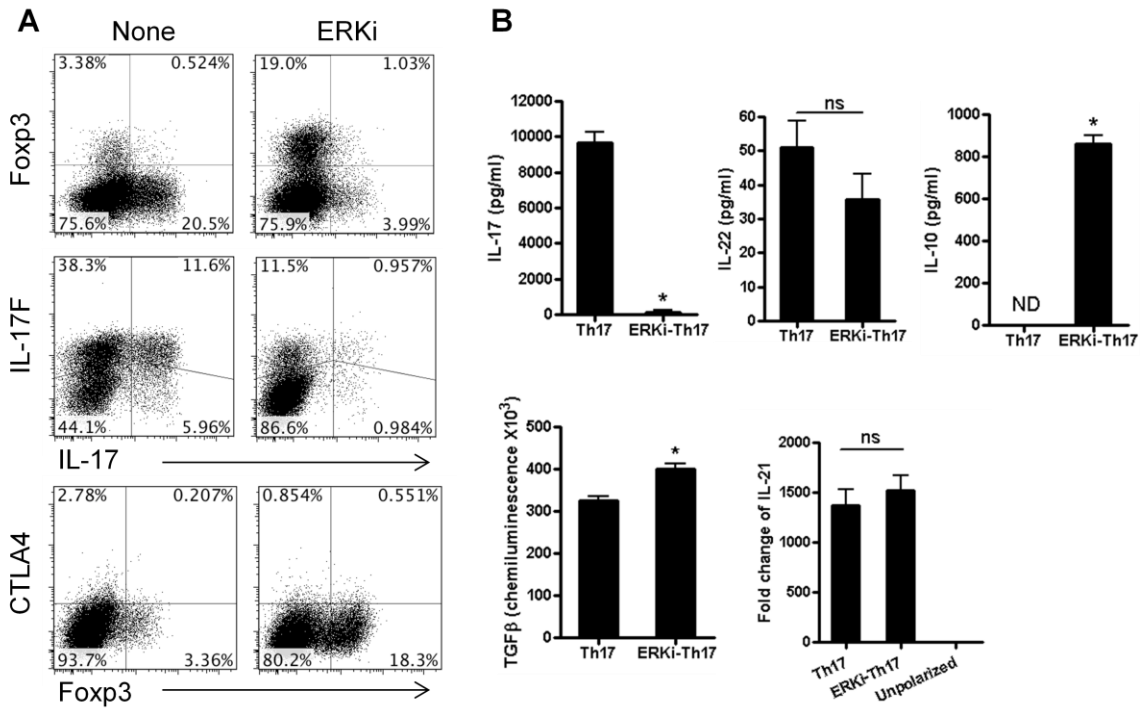

Figure 1: Treatment with ERK inhibitor suppresses Th17 cell but promotes Treg cell development.

$CD4^+CD62L^+$ T cells from CBir1 Tg mice were cultured with APCs and CBir1 antigen in the presence of PD098059 under TGF-β+IL-6 (**A-B**) Th17-polarizing conditions for 4-5 days. The expression of IL-17, IL-17F, CTLA4 and Foxp3 were examined by FACS analysis. The total IL-17 and IL-22 production in Day 4 or 5 culture supernatants were detected by ELISA. Equal numbers of Day4 cells were washed and cultured in fresh medium for 24 hours and the amount of IL-10 production in overnight soup was detected by ELISA. The bioactivity of TGF-β was determined by SEAP Chemiluminescence Assay. The mRNA level of IL-21 was determined by RT-PCR. RT-PCR, ELISA and SEAP assay results are shown as mean +/- SD of 3 samples. Data are representatives of two experiments. * *P< 0.05.*

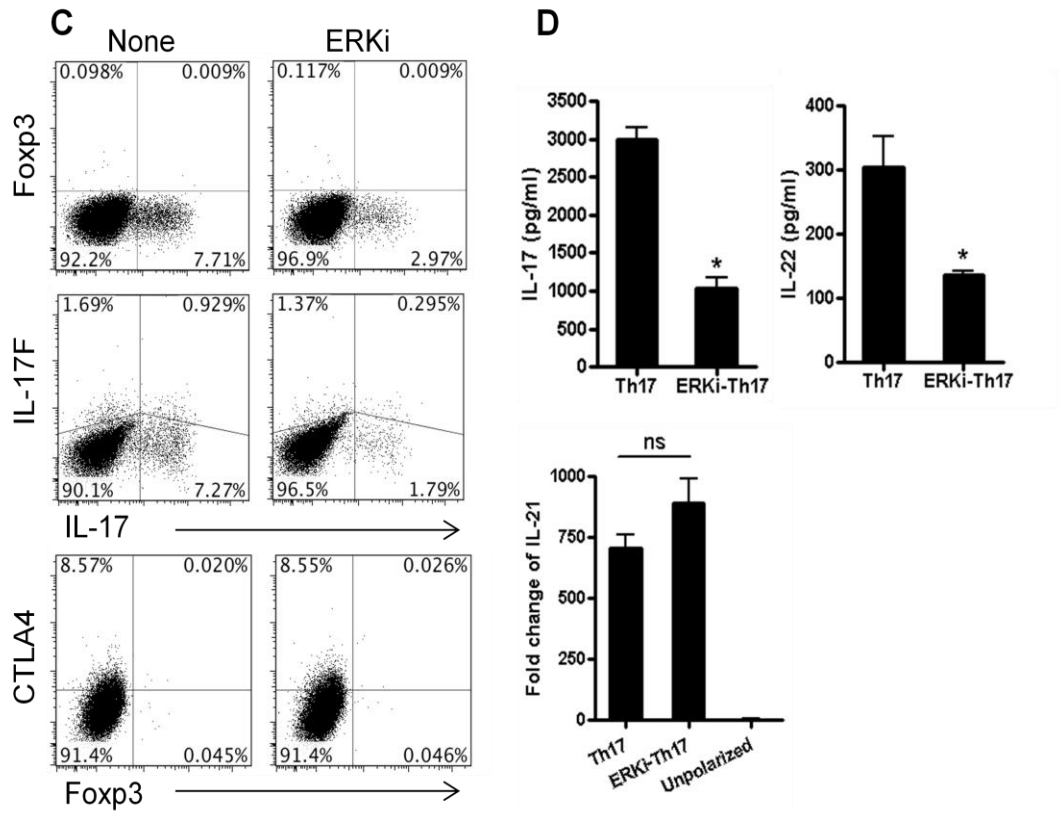

Figure 1: Treatment with ERK inhibitor suppresses Th17 cell but promotes Treg cell development. (Cont'd)

$CD4^+CD62L^+$ T cells from CBir1 Tg mice were cultured with APCs and CBir1 antigen in the presence of PD098059 under IL-1β+IL-6+IL-23 **(C-D)** Th17-polarizing conditions for 4-5 days. The expression of IL-17, IL-17F, CTLA4 and Foxp3 were examined by FACS analysis. The total IL-17 and IL-22 production in Day 4 or 5 culture supernatants were detected by ELISA. Equal numbers of Day4 cells were washed and cultured in fresh medium for 24 hours and the amount of IL-10 production in overnight soup was detected by ELISA. The bioactivity of TGF-β was determined by SEAP Chemiluminescence Assay. The mRNA level of IL-21 was determined by RT-PCR. RT-PCR, ELISA and SEAP assay results are shown as mean +/- SD of 3 samples. Data are representatives of two experiments. * *P< 0.05.*

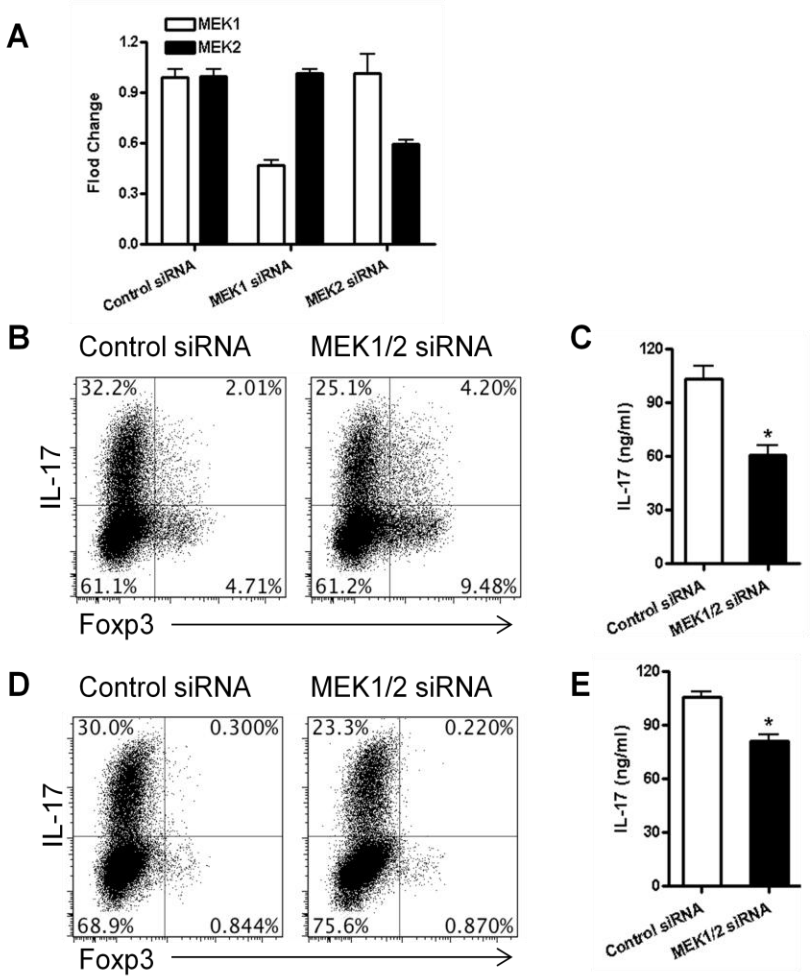

Figure 2: Transfection with MEK siRNA decreases Th17 cells and enhances Treg cells.

Splenic $CD4^+CD62L^+$ T cells from C57BL/6 mice were transfected with MEK1 siRNA, MEK2 siRNA or control siRNA, and stimulated with anti-CD3/anti-CD28. (**A**) siRNA knockdown efficiency was confirmed by RT-PCR at 48 hrs post transfection. The cells were allowed to rest at 37°C in an incubator for 6 hours after transfection with siRNA and cultured under Th17-polarizing conditions with TGF-β/IL-6/anti-IFNγ/anti-IL-4 (**B-C**) or IL-1β/IL-23/IL-6/anti-IFNγ/anti-IL-4 (**D-E**). The expression of IL-17 and Foxp3 was examined by flow cytometry (**B, D**). IL-17 production in culture supernatants was measured by ELISA (**C, E**). RT-PCR and ELISA results are shown as mean +/- SD of 3 samples. Data are representatives of two experiments. * *P< 0.05.*

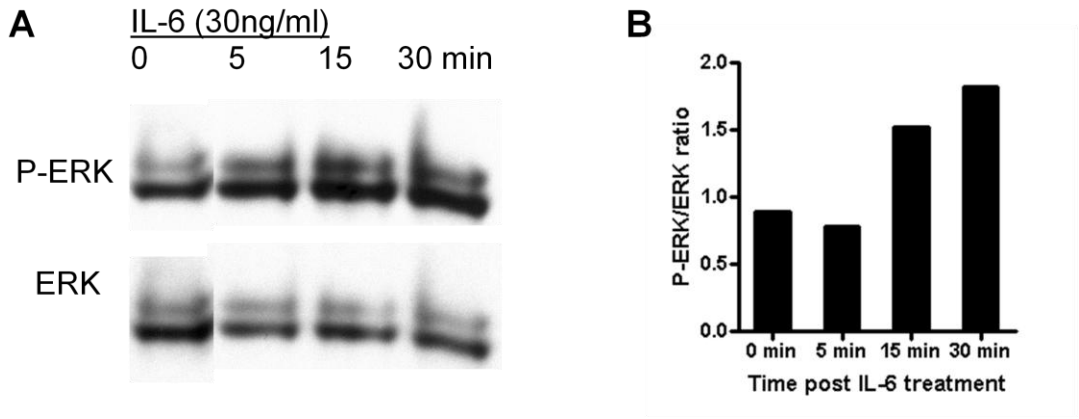

Figure 3: Blockade of IL-6-induced ERK activation downregulates RORγt and upregulates Foxp3 expression.

(A) Splenic $CD4^+$ T cells from CBir1 Tg mice were stimulated with IL-6 (30ng/ml) for 0, 5, 15, 30 min respectively and examined using western blot for ERK and phosphorylated-ERK (P-ERK) expression. (B) The bands from western blot were quantified using Image J analysis and shown as P-ERK/ERK ratio. One representative of two experiments was shown.

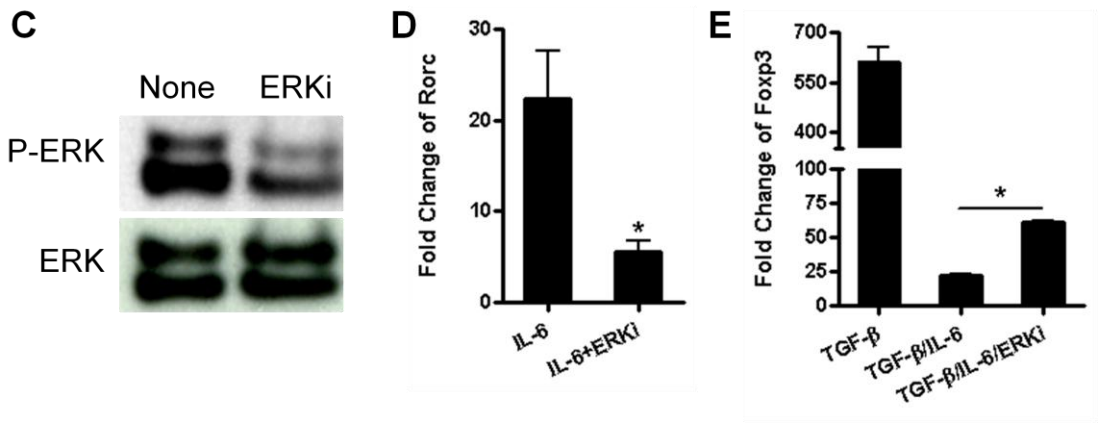

Figure 3: Blockade of IL-6-induced ERK activation downregulates RORγt and upregulates Foxp3 expression. (Cont'd)

(C) $CD4^+CD62L^+$ T cells from C57BL/6 mice were activated with α-CD3/CD28 stimulation in the presence or absence of ERK inhibitor PD098059 5uM for 2hr, 37°C incubation, followed by IL-6 (30ng/ml) treatment for 10 min. The expression of phosphorylated ERK and total ERK were examined by western blot. **(D)** OTII $CD4^+$ T cells were cultured with APCs and OVA antigen in the presence of IL-6 with or without 5μM ERK inhibitor PD098059 for 4 days. The expression of Rorc was detected by RT-PCR. **(E)** OTII $CD4^+$ T cells were cultured with APCs and OVA antigen with TGF-β alone or together with IL-6 in the presence or absence of 5uM ERK inhibitor PD098059 for 4 days. The expression of Foxp3 was detected by RT-PCR. RT-PCR results are shown as mean +/- SD of 3 samples. Data are representatives of at least two experiments. * *P< 0.05.*

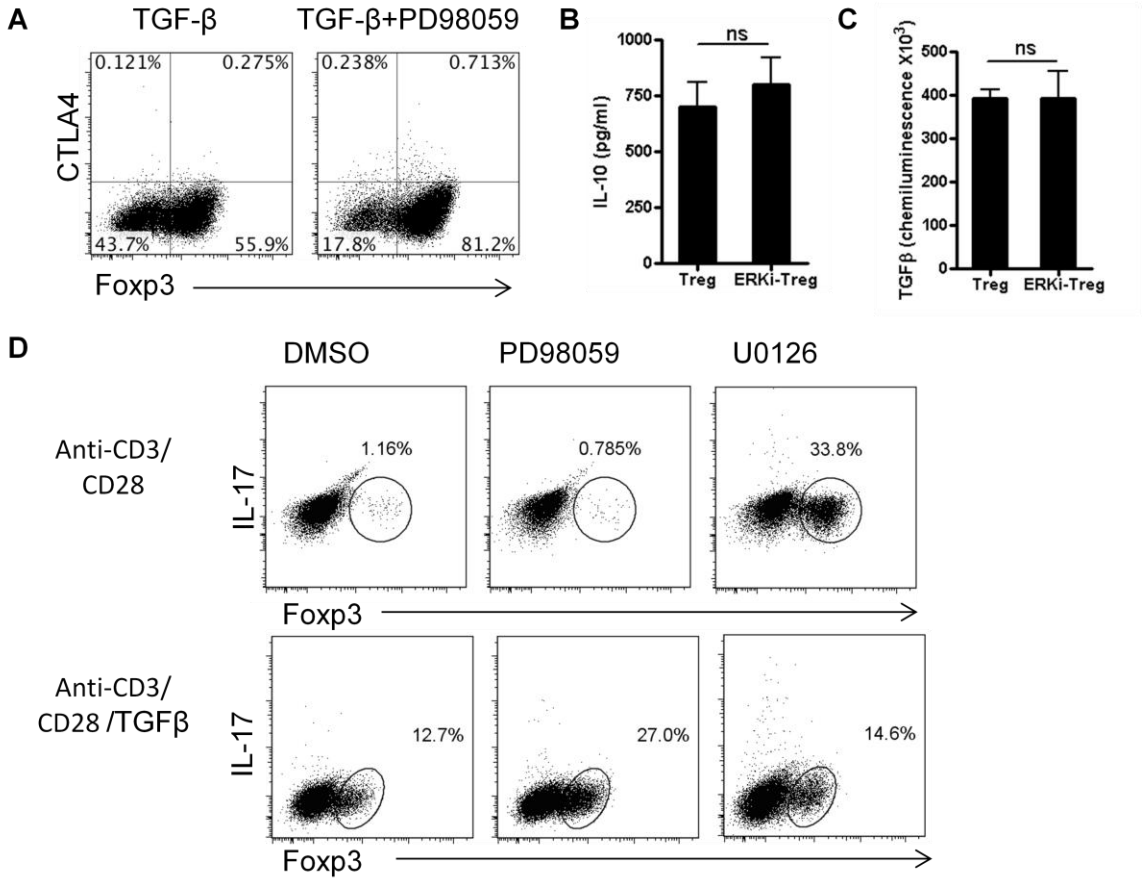

Figure 4: Inhibition of ERK activation enhances $CD4^+$ T cell Foxp3 expression.

Splenic $CD4^+CD62L^+$ T cells from CBir1 Tg mice were cultured with APCs and CBir1 antigen with TGF-β in the presence or absence of 5 μM PD098059 for 5 days. CTLA4 and Foxp3 expression was determined by flow cytometry (**A**), IL-10 production in the supernatants was detected by ELISA (**B**), and the bioactivity of TGF-β was determined by SEAP Chemiluminescence Assay (**C**). (**D**) Splenic $CD4^+CD62L^+$ T cells from C57/B6 mice were culture with anti-CD3/CD28 in the presence or absence of PD098059 5uM or U0126 5uM for 4 days in the presence or absence of TGF-B (5ng/ml). The expression of IL-17 and Foxp3 was examined by flow cytometry. RT-PCR results are shown as mean +/- SD of 3 samples. Data are representatives of at least two experiments. No significance (ns) *P> 0.05.*

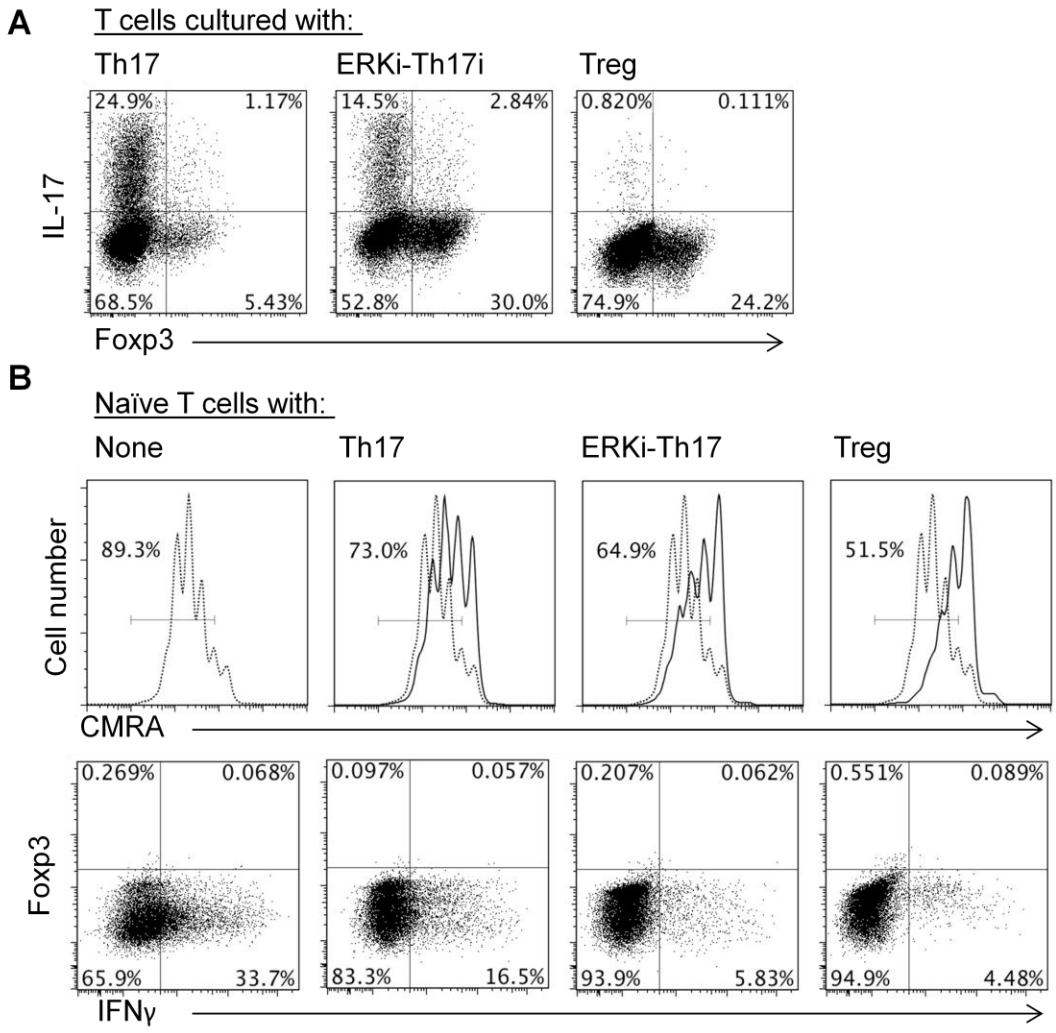

Figure 5: ERK inhibitor-treated Th17 cells exert suppressive function.

CD45.2 B6 $CD4^+$ T cells were cultured under Treg conditions with TGF-β or under Th17-polarizing conditions with TGF-β/IL-6/anti-IFN-γ/anti-IL4 in the presence or absence of 5 μM of ERK inhibitor PD098059 for 5 days. (**A**) The expression of IL-17 and Foxp3 was examined by FACS. These T cells were then co-cultured with CMRA-labeled CD45.1 B6 $CD4^+$ T cells for 3 days. The CMRA dilution (**B**) and expression of IFN-γ (**C**) of CD45.1 $CD4^+$ T cells were measured by flow cytometry. (**D-E**) Bar chart represents aggregate data with mean +/- SD of two experiments. Results are representatives of two experiments. * *P< 0.05.*

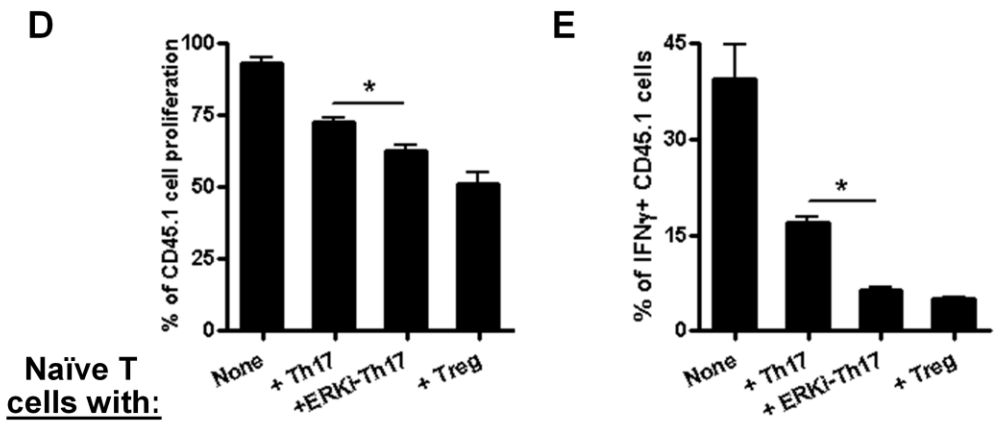

Figure 5: ERK inhibitor-treated Th17 cells exert suppressive function. (Cont'd)

CD45.2 B6 $CD4^+$ T cells were cultured under Treg conditions with TGF-β or under Th17-polarizing conditions with TGF-β/IL-6/anti-IFN-γ/anti-IL4 in the presence or absence of 5 μM of ERK inhibitor PD098059 for 5 days. (**D-E**) Bar chart represents aggregate data with mean +/- SD of two experiments. Results are representatives of two experiments. * *P< 0.05.*

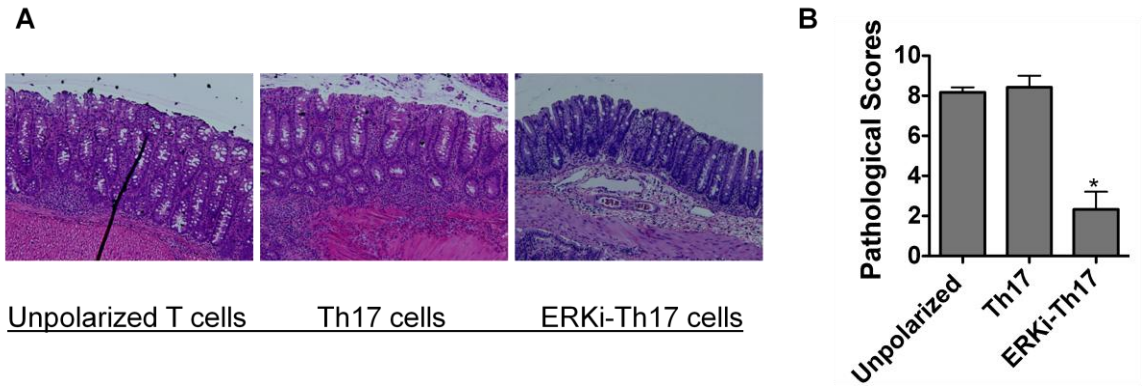

Figure 6: ERK inhibitor-treated Th17 cells have decreased pathological potency to induce colitis.

Splenic $CD4^+$ T cells from CBir1 Tg mice were cultured under classical Th17 differentiation conditions in the presence or absence of PD098059 for 5 days. Then, Day5 10^6 effector cells as well as unpolarized CBir1 $CD4^+$ cells were i.v. injected into $Rag^{-/-}$ mice respectively. After 5 weeks post-T cell transfer, the severity of intestinal inflammation was assessed by histological analysis. (**A)** Pathological scores. **(B**) Histopathology.

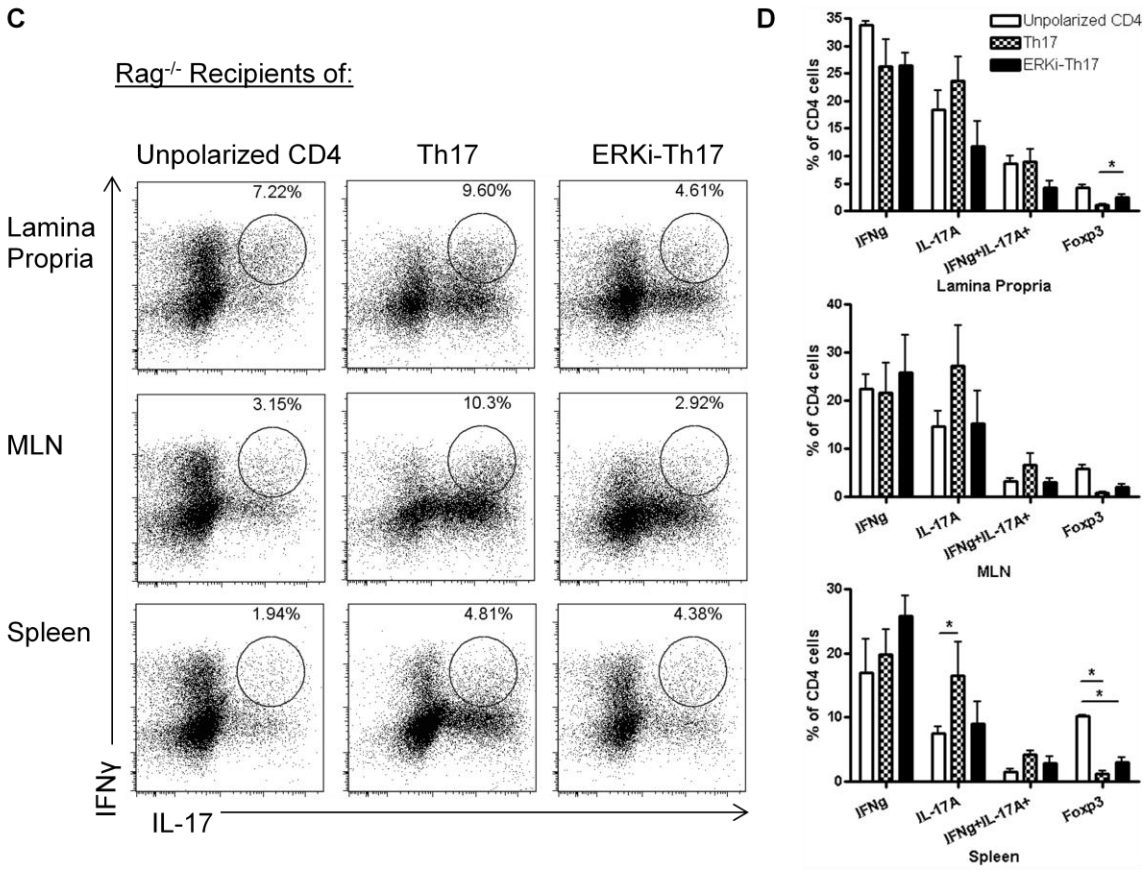

Figure 6: ERK inhibitor-treated Th17 cells have decreased pathological potency to induce colitis. (Cont'd)

Splenic $CD4^+$ T cells from CBir1 Tg mice were cultured under classical Th17 differentiation conditions in the presence or absence of PD098059 for 5 days. Then, Day5 10^6 effector cells as well as unpolarized CBir1 $CD4^+$ cells were i.v. injected into $Rag^{-/-}$ mice respectively. After 5 weeks post-T cell transfer, the severity of intestinal inflammation was assessed by histological analysis. (**C**) $CD4^+$ T cells were isolated from spleen, MLN, intestinal lamina propria of recipient mice and re-stimulated with PMA/Ionomycin and measured for expression of IFN-γ, IL-17 and Foxp3 by flow cytometry. The IFNγ+IL-17+ double positive $CD4^+$ cell population was gated. (D) The percentage of cytokine expressing T cells over live $CD4^+$ cells in each organ was shown. Data are representatives of at least two experiments. Bar chart represents aggregate data with mean +/- SD of two experiments.

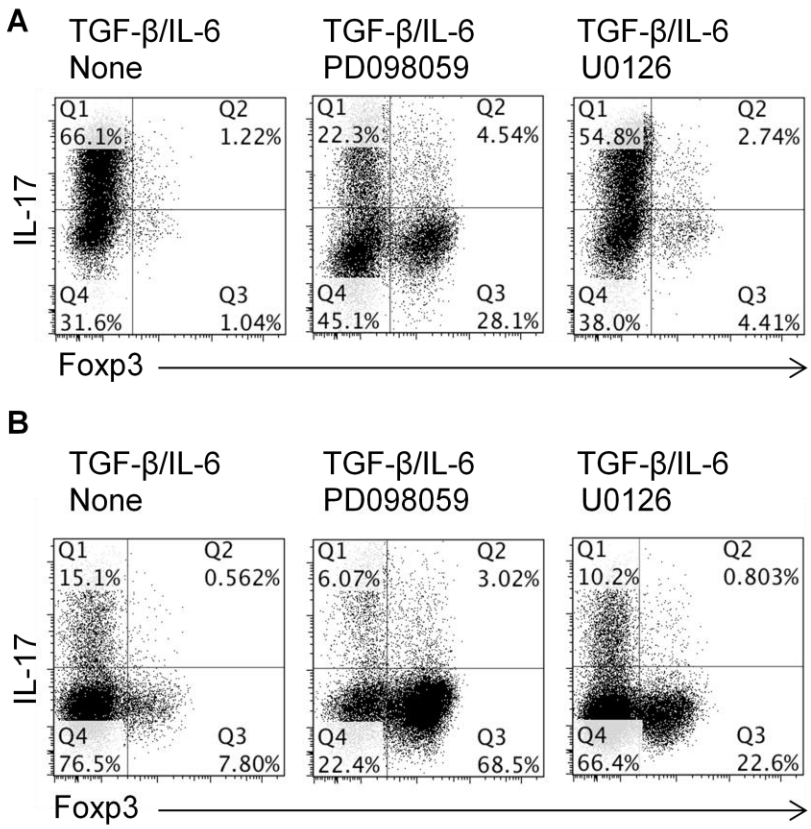

Figure 7: Inhibition of ERK signaling blocks in vitro Th17 differentiation.

$CD4^+$ T cells from OTII (**A**) and B6 mice (**B**) were cultured with irradiated APCs and OVA antigen or stimulated with anti-CD3/anti-CD28 antibodies respectively in the presence or absence of ERK inhibitor PD098059 and U0126 under classic Th17 polarizing conditions for 5 days. The expression of IL-17 and Foxp3 were examined by FACS analysis. One representative of two experiments is shown.

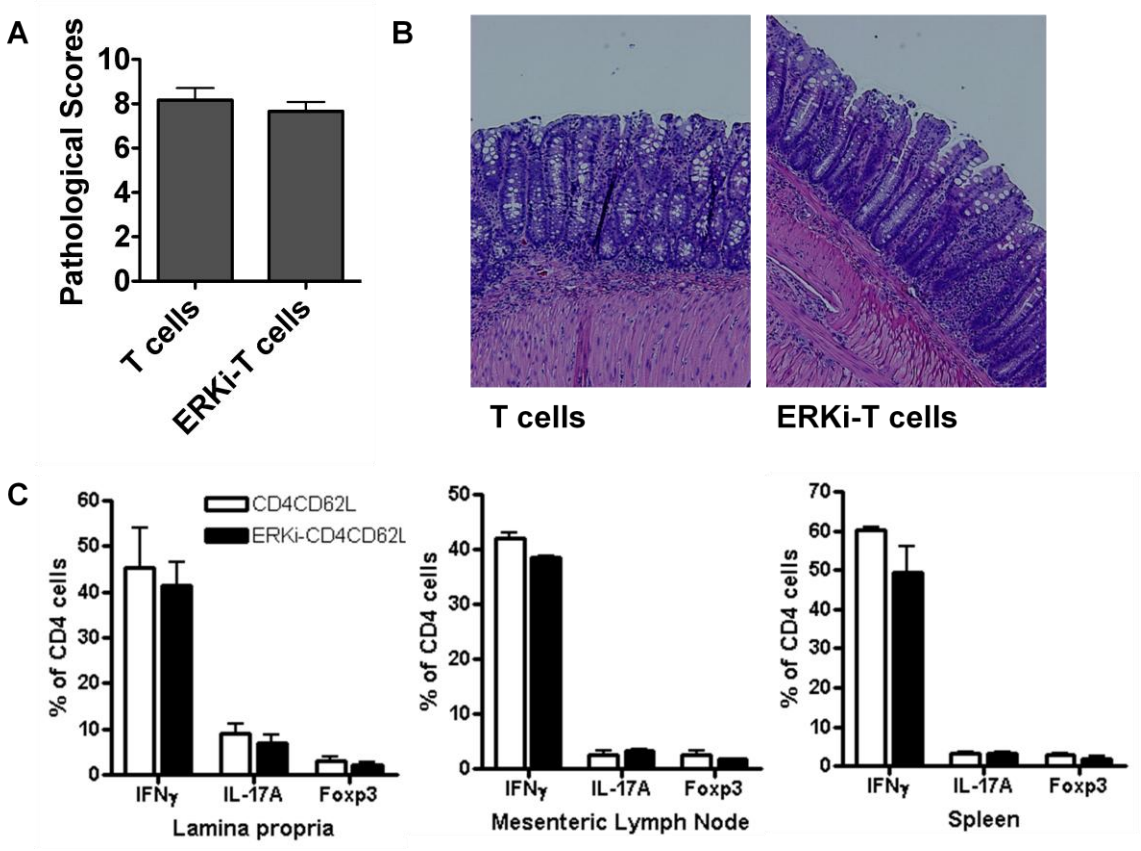

Figure 8: ERK treatment has little effect on pathological potency of naïve T cells to induce colitis.

Splenic CD4+CD62L+ T cells from CBir1 transgenic mice were incubated in the presence or absence of ERK inhibitor PD098059 for 2hr, 37°C. Then, 10^6 CD4+CD62L+ T cells were i.v. injected into Rag-/- mice. After 7 weeks post-T cell transfer, the severity of intestinal inflammation was assessed by histological analysis. (A) Pathological scores. (B) Histopathology of colons. (C) CD4+ T cells were isolated from spleen, MLN, intestinal lamina propria of recipient mice and re-stimulated with PMA/Inomycin and measured for expression of IFN-γ, IL-17 and Foxp3 by flow cytometry. The percentage of cytokine expressing T cells over totally live CD4 T cells in each organis shown.

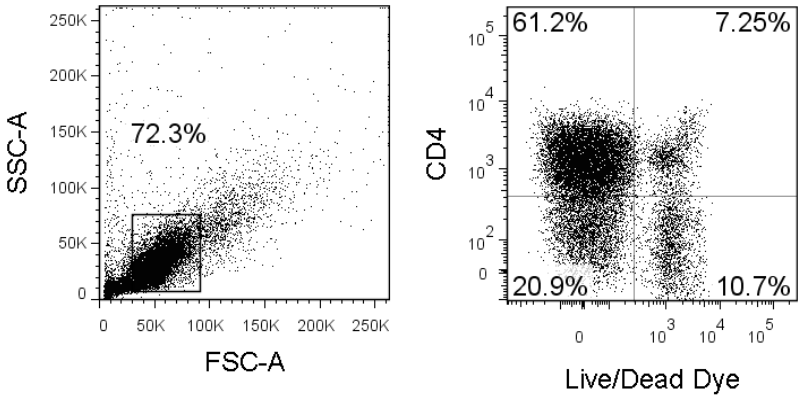

Figure 9: Gating strategy for FACS analysis.

(A) Cells were first gated in a FSC and SSC scale to remove cell debris and **(B)** followed by gated in a CD4 and live/dead cell dye scale to remove $CD4^-$ APCs and dead cells. Only live $CD4^+$ cells population was analyzed.

Chapter 4: TGF-β converts Th1 cells into Th17 cells through stimulation of Runx1 expression under inflammatory conditions in intestines

4.1 INTRODUCTION

As a major interface with the external environment and a site of huge immune challenge, the intestine harbors trillions of commensal bacteria in addition to viral and fungal species. Multiple levels of regulations control intestinal homeostasis, including innate and adaptive mechanisms[150, 151]. Among multiple specialized lymphocyte populations within the intestinal tract, $CD4^+$ T cells constitute a major part of the adaptive immune system and orchestrate diverse host protective and homeostatic responses. However, accumulating evidence indicates that $CD4^+$ T cells, once dysregulated, also play crucial roles in the pathogenesis of chronic intestinal inflammation, resulting in inflammatory bowel disease (IBD), which consists of Crohn's disease and ulcerative colitis[121]. $CD4^+$ T cells are divided into functionally distinct subsets by their production of distinct cytokines. Th1 cells produce IFNγ and regulate cellular immunity against intracellular pathogens, whereas Th17 cells produce IL-17 and IL-22 and provide a host response against extracellular pathogens by recruiting neutrophils[152, 153]. Both Th1 and Th17 cells have been implicated in the pathogenesis of IBD in humans and mice, and accumulating evidence indicates that Th1 and Th17 cells present in the intestines during inflammation may differ from steady-state populations in terms of their effector functions[84, 154]. Whereas steady-state Th1 and Th17 cells are largely $IFN\gamma^+$- and $IL\text{-}17^+$-single positive, respectively, significant portions of the $CD4^+$

T cells accumulating in inflamed intestines are IFNγ$^+$ IL-17$^+$ double positive[84, 85]. Those populations have been proposed to play a crucial role in the pathogenesis of IBD as well as other autoimmune diseases[111, 155]. However, the mechanisms involved in the development of IFNγ$^+$ IL-17$^+$ double positive CD4$^+$ T cells are still largely unknown.

Environmental factors, cytokine milieu, and O_2 supply are essential determinants for CD4$^+$ T cell differentiation. In addition to TCR stimulation and co-stimulatory molecules, cytokines serve as a third signal during T cell differentiation. IL-12 promotes Th1 cell development through induction of transcription factor T-bet, whereas TGF-β and IL-6 promote Th17 cell development through induction of transcription factor RORγt. Hypoxia and sequential induction of hypoxia-inducible factor, HIF1α, have been shown to facilitate Th17 generation[105]. However, there is growing evidence that differentiated CD4$^+$ T cells preserve the flexibility to alter phenotypes upon external stimulation, revealing the plasticity of CD4$^+$ T cells, especially in the intestines under inflammatory conditions. It has been shown that high levels of local IL-12 and IL-23 convert Th17 cells into Th1 cells with abrogation of IL-17 production and a parallel gain of IFNγ production in inflamed intestines[83, 156]. Moreover, conversion of Th17 cells into Th1 cells has been shown to be essential for the development of various autoimmune diseases, including EAE and type 1 diabetes[111, 155]. However, while the plasticity of Treg and Th17 are well-documented[97, 116], the stability of Th1 remains controversial. Some epigenetic studies revealed high stability for Th1 cells[94, 117], a finding which is supported by numerous reports of Treg and Th17 cells conversion into Th1 cells but not vice versa [83, 97, 116]. However, several recent reports of Th1 conversion into Th2, Treg and Tfh cells under various conditions argue against the absolute stability of Th1 lineage[118, 120, 157].

In this study, I investigated the role of microbiota antigen-specific Th1 cells in the induction of colitis and the stability of Th1 cells under inflammatory conditions in intestines by using IFNγ$^{Thy1.1}$ CBir1 TCR transgenic reporter mice, which are specific for an immunodominant microbiota antigen, CBir1. To this end, I demonstrated that transfer of purified CBir1-specific IFNγ^{+}Th1 cells induced colitis in Rag$^{-/-}$ mice, and, in the inflamed intestines, these Th1 cells can convert into IL-17^{+} Th17, but not Foxp3^{+} Treg cells. I also found that TGF-β and IL-6, but not IL-1β, IL-23, and hypoxia factors, regulated Th1 conversion into Th17 cells. Further, TGF-β induction of transcriptional factor Runx1 was essential for the conversion, in that silencing Runx1 by siRNA inhibited Th1 conversion into Th17 cells.

4.2 MATERIAL AND METHODS

Mice. C57Bl/6 mice were purchased from Jackson Laboratory. CBir1 TCR transgenic (CBir1 Tg) mice[82] and IFNγ$^{Thy1.1}$ CBir1 Tg reporter mice[83] were maintained in the animal facilities of the University of Texas Medical Branch. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Medical Branch.

Antibodies and Reagents. RPMI 1640, HEPES, penicillin-streptomycin, FBS, 2-mecaptoethanol and sodium pyruvate were purchased from Life Technologies (Carlsbad, CA); TRIzol reagents were from Invitrogen (Carlsbad, CA) and qScript reverse transcriptase from Quanta Biosciences. Reagents for TaqMan gene expression assays were from Applied Biosystems (Carlsbad, CA). EDTA, Collagenase type IV was obtained from Sigma-Aldrich (St. Louis, MO). SMARTpool siRNAs specific for murine HIF1α, Runx1 and non-targeting siRNA were purchased from Dharmacon (Lafayette, CO). Anti-mouse CD3 (145-2C11), CD28 (37.51), IL-2 (JES6-1A12) antibodies,

recombinant IL-6, TGF-β1, and IL-1β, IL-23 were from Cell signaling, BioLegend and R&D Systems. Anti-mouse IFN-γ (XMG1.2) neutralizing monoclonal antibodies were purchased from BD Biosciences (San Diego, CA). Fluorochrome-conjugated anti-mouse CD4 (RM4-5 and GK1.5), IL-17 (TC11-18H10), and IFN-γ (XMG1.2) antibodies were purchased from BioLegend (San Diego, CA) from BD Biosciences. Foxp3 staining buffer sets were purchased from eBioscience (San Diego, CA). Live/Dead dye indicating cell viability was obtained from Invitrogen (Carlsbad, CA).

Isolation of $CD4^+$ T cells and in vitro T cell cultures. $CD4^+$ T cells were isolated by using anti-mouse CD4 magnetic beads (BD Biosciences) as previously described[133]. $CD4^+$ T cells were cultured with irradiated, wild-type splenic $CD4^-$ APCs 37°C in humid air with 5% CO_2 under classical Th1-polarizing conditions with mIL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml). Th1 cells were sorted with FACSAria sorter and cultured under classical Th17-polarizing conditions with TGF-β (5 ng/ml), IL-6 (30 ng/ml), and anti-IFNγ (10 μg/ml)/anti-IL-2 (2.5 μg/ml), alternative Th17 polarization conditions with IL-1β (10 ng/ml), IL-6 (30 ng/ml), IL-23(10 ng/ml), and anti-IFNγ (10 μg/ml)/anti-IL-2 (2.5 μg/ml). ChIP-IT Express Enzymatic kit, antibodies against histone H3K9ac and histone H3K9me3 were purchased from Active Motif (Carlsbad, CA). Rabbit IgG, the negative control for immune precipitation, was obtained from Cell Signaling. Primers for real time PCR were ordered from Integrated DNA Technologies (Table. 1).

siRNA transfection. Sorted Th1 cells were sub-cultured for an extra 2 days to allow cells to reach the logarithmic growth phase optimal for transfection. siRNA transfection in sub-cultured Th1 cells was performed according to the manufacturer's instructions (Lonza, Amaxa mouse T cell nucleofector kit). Briefly, $1x10^6$ sub-cultured Th1 cells were transfected with 100 nM siRNA. The cells were allowed to rest at 37°C in an incubator for 6-24 hours after transfection with siRNA and cultured with anti-CD3 (1μg/ml) and anti-CD28 (2 μg/ml) and different conversion conditions. Knockdown

efficiency was measured at 24 hr post transfection. The mRNA expression of IL-17, Rorc, Rorα, Runx1 and BATF were determined by RT-PCR.

Flow cytometry. As described previously[82], cells were stimulated for 5 hr with PMA (50 ng/ml) and ionomycin (750 ng/ml), with monensin added for the last 3 hr. The cells were fixed and permeabilized by using a Foxp3 staining buffer set. Staining was performed for Thy1.1, CD4, Live/Dead dye, IL-17, and IFNγ by using fluorescence-conjugated Abs, and the cells were sampled on a LSRII Fortessa flow cytometer (BD Biosciences). Data were analyzed by using FlowJo software (Tree Star).

Real time PCR (RT-PCR). Total RNA was extracted with TRIzol reagent, and this was followed by cDNA synthesis. Quantitative PCR reactions were performed by using TaqMan Gene Expression Assays for Rorc, Rorα, Runx1, and BATF on a Bio-Rad iCycler (Bio-Rad, Hercules, CA), and all data were normalized to GAPDH mRNA expression.

ELISA. Mouse IL-17(A) ELISA kits were purchased from Biolegend. The detection for each cytokine was performed following the manufacturer's instructions.

Preparation of lamina propria lymphocytes. Lamina propria lymphocytes were isolated as previously described[123]. Briefly, for removal of epithelial cells and intraepithelial lymphocytes, the intestines were rinsed, sliced into small pieces, and incubated at 37°C for 40 min. The tissues were then digested with collagenase for 50 min at 37°C with stirring. The liberated cells were collected by passage through a stainless steel sieve and 100-μM cell strainer (BD Falcon). The isolated cells were pooled together and separated on a 40/75% discontinuous Percoll gradient (Amersham Pharmacia Biotech). The cell yield was typically 2-3 million lymphocytes per mouse with 90% cell viability.

Histopathologic assessment. As previously described[83], at necropsy, the cecum, and colon were separated, and Swiss rolls of each prepared. Tissues were fixed in 10% buffered formalin and paraffin embedded. Following paraffin embedding, 5-μm sections

were prepared, stained with H&E and visualized on an Eclipse 80i microscope (Nikon; Melville, NY).

Hypoxia chamber. FACS sorted IFNγ$^{Thy1.1}$ Th1 cells were cultured under hypoxic conditions (5% CO_2, 0.1% O_2 and 94.9% N_2) for 4-5 days. The hypoxic environment was created by purging 95% N_2 and 5% CO_2 (AIRGAS USA, Texas City, TX) into an environmental chamber by using a ProOx Oxygen controller (Biospherix, Lacona, NY) to achieve the desired oxygen concentration.

Chromatin immunoprecipitation (ChIP) assay. As previously described[158], a ChIP assay was performed according to the manufacturer's instructions. Briefly, histones were cross-linked to DNA with 1% formaldehyde and incubated for 5 minutes at room temperature. The DNA was sheared with an enzymatic shearing cocktail for 10 minutes at 37°C. Magnetic beads and 2 μg of each antibody were used to capture chromatin. Immunoprecipitates were eluted and reverse cross-linked. For RT-PCR, 1.5 μl of the eluted DNA and 40 cycles of amplification were used and all data normalized to input DNA. RT-PCR products were subjected to electrophoresis on 1% agarose gels, stained with ethidium bromide.

Statistical analysis. For comparisons between groups, levels of significance were determined by one-way ANOVA with Tukey's t test and unpaired, two-tailed Student's t test in Prism 4.0 (GraphPad Software). A p value of <0.05 was considered statistically significant and shown as *.

4.3 RESULTS

4.3.1 IL-17$^+$IFNγ$^-$, IL-17$^-$IFNγ$^+$, AND IL-17$^+$IFNγ$^+$ CD4$^+$ T CELL POPULATIONS WERE PRESENT IN INFLAMED INTESTINES OF THE MICE WITH COLITIS

Our research group has previously established a microbiota antigen-specific T cell-mediated animal model of colitis by adoptive transfer of CD4$^+$ T cells from CBir1

TCR-transgenic (CBir1 Tg) mice, which are specific for an immunodominant bacterial commensal flagellin, into $Rag^{-/-}$ mice[82]. To examine the phenotypes of $CD4^+$ T cells presenting in the inflamed intestines, I isolated CBir1 Tg $CD4^+$ T cells and transferred them intravenously (i.v.) into $Rag^{-/-}$ mice for colitis development. I also injected PBS into $Rag^{-/-}$ mice to serve as negative controls. At 4 weeks post-adoptive transfer, the $Rag^{-/-}$ recipient mice were scarified, and the histopathology of the small intestine, large intestine, and cecum determined. As shown previously, no $Rag^{-/-}$ mice receiving PBS developed colitis; however, the $Rag^{-/-}$ recipient mice that received CBir1 T cells developed severe colitis (**Fig. 10A and B**). The small intestines of these recipient mice did not show any inflammation (data not shown). $IFN\gamma^+$ $IL\text{-}17^-$ Th1 and $IL\text{-}17^+$ $IFN\gamma^-$ Th17 cells, as well as significant proportions of $IL\text{-}17^+$ $IFN\gamma^+$ double-positive $CD4^+$ T cells, were present in the lamina propria of inflamed intestines (**Fig. 10C**). Of note, the levels of $IL\text{-}4^+$ Th2 cells were undetected in the inflamed intestinal lamina propria (**data not shown**). Collectively, these data indicated that microbiota antigen-specific Th1 and Th17 cells, and especially $IL\text{-}17^+$ $IFN\gamma^+$ double-positive $CD4^+$ T cells, are able to induce colitis.

4.3.2 IFNΓ$^+$ TH1 CELLS CONVERTED INTO IL-17-PRODUCING TH17 CELLS BUT NOT FOXP3$^+$ TREG CELLS IN THE INFLAMED INTESTINES

Our research group previously demonstrated that $IL\text{-}17^+$ Th17 cells could convert to $IFN\gamma^+$ Th1 cells through IL-17 induction of mucosal innate IL-12 and IL-23 production in inflamed intestines[84], which could at least partially explain the presence of $IL\text{-}17^+IFN\gamma^+$ T cells. However, as recent reports demonstrated the conversion of Th1 cells into Th2, Tregs, and Tfh under various conditions[118, 120, 157], I investigated whether $IFN\gamma^+$ Th1 cells could also convert into $IL\text{-}17^+$ Th17 cells, and thus contribute to the generation of $IL\text{-}17^+IFN\gamma^+$ T cells under inflammatory conditions. Taking advantage of $IFN\gamma^{Thy1.1}$ CBir1 Tg reporter mice[83], I generated Th1 cells under standard Th1 polarizing conditions

and then sorted them by using a FACS sorter. I was able to sort IFNγ$^{Thy1.1+}$ CD4^{+} Th1 cells with a purity of 98.9% **(Fig. 11A and 11B)**, and I then transferred the sorted CBir1 Tg IFNγ$^{Thy1.1+}$ Th1 cells to Rag$^{-/-}$ mice. At 8 weeks post Th1 cell transfer, the recipient mice developed mild colitis. When I determined the phenotypes of CD4^{+} T cells isolated from the intestinal lamina propria, most Th1 cells were found to have lost expression of IFNγ (over 65%). Although there was no Foxp3 expression by those adoptively transferred Th1 cells, which is consistent with our previous reports[83], significant portions of CD4^{+} T cells expressed IL-17 **(Fig. 11C)**, including both IL-17 single-positive and IL-17^{+}IFNγ^{+} double-positive T cells. Collectively, these data indicated that conversion of Th1 cells into Th17, but not Treg, cells occurred under inflammatory conditions in the intestines.

4.3.3 TGF-B, IL-6 AND IL-2 DIFFERENTIALLY REGULATE THE CONVERSION OF TH1 CELLS INTO TH17 CELLS.

Among cytokines present at an elevated level in inflamed intestines of colitic mice, Th17-skewing cytokines, TGF-β, IL-6, IL-1β and IL-23[159], could potentially induce Th1 conversion into Th17 cells, whereas IL-2 would antagonize Th17 responses. IL-2 not only affects T cell proliferation, but also regulates T cell differentiation by differentially regulating key cytokine receptors, IL-12Rβ2 and IL-6R, and by activating STAT5, which competes with STAT3 for the IL-17 promoter binding site, resulting in the enhancement of Th1 but the inhibition of Th17 cell differentiation [96, 108, 160]. Some evidence also showed that the consummation of IL-2 in the microenvironment can enhance Th17 cell induction [161]. Hence, I then investigated which cytokines promoted Th1 conversion into Th17 cells. In conjunction with the neutralization of IL-2 and IFNγ, I treated differentiated and sorted CBir1 Tg IFNγ$^{Thy1.1+}$ Th1 cells with various cytokines, including TGF-β/IL-6/IL-23 and IL-1β/IL-6/IL-23. I examined the expression of IFN-γ and IL-17

in Day 5 cultures with FACS analysis. Neutralization of IL-2 and IFNγ alone did not induce Th1 cell expression of IL-17. While IL-1β/IL-6/IL-23 only induced Th1 conversion into Th17 cells at a minimal level, TGF-β/IL-6/IL-23 greatly induced Th1 cells to express IL-17 but not Foxp3 **(Fig. 12A and Fig. 17)**. I then examined the impact of individual cytokines on Th1 conversion into Th17 cells. I found TGF-β or IL-6 alone exerted a minimal effect, while the combination of TGF-β/IL-6 induced a substantial Th1 to Th17 cell conversion **(Fig. 12B-D)**. Treatment with IL-1β or IL-23 alone did not stimulate IL-17 production (**data not shown**).

4.3.4 HYPOXIA AND SEQUENTIAL INDUCTION OF HYPOXIA-INDUCIBLE FACTOR, HIF1A, HAVE DISPENSABLE ROLES IN TH1 CONVERSION INTO TH17 CELL CONVERSION.

Hypoxic conditions are associated with intestinal inflammation[162]. Hypoxia and the sequential induction of hypoxia-inducible factor, HIF1α, have been shown to facilitate Th17 cell differentiation[105, 163], which is impaired in HIF1α-deficient $CD4^+$ T cells. HIF1α directly induces RORγt expression and works with RORγt and p300 to trigger IL-17 expression[105]. To determine whether hypoxia and the expression of HIF1α promote Th1 conversion into Th17 cells, I examined the roles of hypoxia and HIF1α by two approaches. I firstly cultured CBir1 Tg $IFN\gamma^{Thy1.1+}$ Th1 cells under conversion conditions with TGF-β/IL-6 and anti-IL-2/anti-IFNγ under hypoxic or normoxic conditions for 5 days and examined T cell IL-17 expression by using FACS analysis. I found that Th1 cells cultured under hypoxic conditions did not promote a higher expression of IL-17 than did the cells cultured under normoxic conditions **(Fig. 13A and 13B)**, indicating that hypoxia is not as significant a factor for Th1 conversion into Th17 cells. To further confirm these results, I utilized HIF1α siRNA to knock down HIF1α expression in CBir1 Tg $IFN\gamma^{Thy1.1+}$ Th1 cells before culturing the cells under conversion conditions with TGF-β/IL-6 and anti-IL-2/anti-IFNγ. Transfection with HIF1α siRNA

suppressed about 60% of HIF1α expression at 24 h post-transfection, when compared with the results when CBir1 Tg IFNγ$^{Thy1.1+}$ Th1 cells were transfected with non-targeting control siRNA **(Fig. 13D)**. I found that HIF1α knockdown in Th1 cells did not enhance IL-17 production **(Fig. 13E and 13F)**. Collectively, these data suggested to us that hypoxia and HIF1α are dispensable in Th1 conversion into Th17 cells. On the other hand, TGF-β and IL-6 consistently promoted Th1 conversion under both hypoxic and normoxic conditions.

4.3.5 TGF-β AND IL-6 REGULATED EXPRESSION OF RORα, RORC AND RUNX1 IN TH1 CELLS

I then investigated the mechanisms involved in the TGF-β/IL-6 promotion of IL-17 production in Th1 cells. It has been shown that RORγt, RORα, Runx1, and Batf regulate IL-17 expression and Th17 cell differentiation[107, 152]. To determine whether TGF-β and IL-6 regulate expression of those transcription factors in Th1 cells, I treated CBir1 Tg IFNγ$^{Thy1.1+}$ Th1 cells with TGF-β, IL-6, or both in conjunction with neutralizing antibodies against IL-2 and IFNγ for 4 days, and determined gene expression by real-time PCR. I found that TGF-β promoted Th1 cell expression of Rorc, Rorα, and Runx1. IL-6 promoted expression of Rorc and Rorα at a lower level than that with TGF-β. However, IL-6 did not affect Runx1 expression. In addition, IL-6 further enhanced TGF-β-induced expression of Rorc but not of Rorα and Runx1 (**Figure 14A-C**). TGF-β and IL-6, either alone or in combination, did not affect Batf expression in Th1 cells (**Figure 14D**).

4.3.6 TGF-β-INDUCED RUNX1 MEDIATED TH1 CONVERSION INTO TH17 CELLS

It has been shown that Runx1 induces transactivation of the *rorc* promoter and promotes IL-17 production[164]. I found that treatment with TGF-β induced the transcription of Runx1 in IFNγ$^{Thy1.1+}$ Th1 cells as early as 24 hr post treatment (**data not shown**). To determine the role of TGF-β-induced Runx1 in Th1 conversion into Th17 cells, I used Runx1 siRNA to knock down Runx1 expression in IFNγ$^{Thy1.1+}$ Th1 cells, and then treated the cells with TGF-β and anti-IL-2/anti-IFNγ antibodies. I examined via real-time PCR whether Runx1 knockdown would influence the expression of genes associated with Th17 cells. I was able to knock down 50% of Runx1 expression in Th1 cells after transfection with Runx1 siRNA (**Fig. 15A**). Knockdown of Runx1 greatly decreased the expression of IL-17, RORγt and RORα (**Fig. 15B-D**), indicating that TGF-β-induced Runx1 plays an essential role in Th1 conversion into Th17 cells.

4.3.7 TGF-β AND IL-6 INCREASED THE ACCESSIBILITY OF RUNX1 BINDING SITES IN PROMOTERS OF RORC AND IL-17A

The consensus Runx1- and RORγt-binding sites in human and mouse *il-17* loci was reported previously[138]. The Runx1 binding site, TGTGGT, is located at a promoter region, ranging from -2 kb to the start codon. RORγt-binding sites, AGGTCA and TGACCT, are located in both the promoter region and a distal upstream CNS-5 enhancer region. On the other hand, potential Runx1-binding regions in the mouse *rorc* gene have been described previously[165]. From there, I further narrowed down and identified three Runx1-binding sites in the *rorc* locus by aligning human and mouse *rorc* loci. Three Runx1-binding sites containing a perfect consensus binding sequence for Runx1, TGTGGT, were identified in human and mouse *rorc* loci **(Fig. 17)**. Notably, the mouse *rorc* gene has 4 transcripts or splice variants. Three out of four transcripts start from the third exon. The Runx1-binding sites are located upstream of the third exon. Similarly, the

human *rorc* gene also has 4 transcripts. Three out of four transcripts start from the fourth exon. Three Runx1-binding sites are located upstream from the fourth exon. The similarity indicates that, in both mouse and human *rorc* gene, the binding of Runx1 protein to regulatory regions of the *rorc* gene possibly influences the transcription of the majority of Rorc transcripts.

Histone modification markers are widely used to investigate the plasticity of $CD4^+$ T cell differentiation[124, 166]. To examine the effect of TGF-β on accessibility of Runx1- and RORγt-binding sites located at mouse rorc and *il-17* genes in Th1 cells, I performed a ChIP assay by using antibodies against H3K9ac and H3K9me3. The acetylation on the 9th lysine of H3 histone indicates an accessible state of the transcriptional factor binding sites, while the trimethylation on the same amino acid indicates a silent state of the binding sites. I found that in $IFN\gamma^{Thy1.1+}$ Th1 cells treated with TGF-β/IL-6, the levels of histone H3K9 acetylation in Runx1-binding sites of the *rorc* promoter were increased, whereas the levels of histone H3K9 trimethylation in these binding sites were decreased compared to those in control Th1 cells **(Fig. 16A and B)**. Consistently, treatment with TGF-β/IL-6 increased the levels of histone H3K9 acetylation at the Runx1- and RORγt-binding sites in the *il-17* promoter, whereas the levels of histone H3K9 trimethylation in these binding sites were decreased compared to the levels in control Th1 cells **(Fig. 16C and D)**. The level of histone H3K9 acetylation in RORγt-binding site in the CNS-5 enhancer of *il-17* locus was also increased while the level of histone H3K9 trimethylation was decreased in TGF-β/IL-6 treated Th1 cells. Taken together, these data indicated to us that TGF-β/IL-6 treatments enhance the accessibility of Runx1- binding sites in mouse promoters of *il-17* and *rorc* and also of the RORγt-binding site in the *il-17* promoter, thereby sequentially increasing IL-17 production in Th1 cells.

4.4 DISCUSSION

It is increasingly clear that both Th1 and Th17 cells reactive to commensal bacterial antigens are effector T cells driving colitis development both in experimental colitis and human IBD[92, 167]. With the progression of colitis, not only IFNγ$^+$single-positive and IL-17$^+$single- positive CD4$^+$ T cells, but also IFNγ$^+$ IL-17$^+$ double-positive CD4$^+$ T cells, accumulate in the inflamed intestines. Our research group previously demonstrated that the severity of disease correlated with the proposition of IFNγ$^+$ IL-17$^+$ double-positive CD4$^+$ T cells in the lamina propria[84]. High levels of IFNγ$^+$ IL-17$^+$ CD4$^+$ T cells are also present in the inflamed lesions from patients with IBD in contrast to their absence in the steady state, possibly meaning that such cells may contribute to the pathogenesis of colitis. However, it is still not clear how IFNγ$^+$ IL-17$^+$ CD4$^+$ T cells are generated in the inflamed tissues. Our research group and others have shown previously that commensal bacterial antigen-specific, IL-17-producing Th17 cells can convert into IFNγ-producing Th1 cells in the intestines under inflammatory conditions, mediated by local innate cell production of IL-12 and IL-23[84, 156], which thus contributes to the generation of the pathogenic IFNγ$^+$ IL-17$^+$ CD4$^+$ T cells. My current study further demonstrated that commensal bacterial antigen-specific, IFNγ-producing Th1 cells can also convert into IL-17-producing Th17 cells during intestinal inflammation, thereby providing a new mechanism driving the generation of the pathogenic IFNγ$^+$ IL-17$^+$ CD4$^+$ T cells during colitis development.

The plasticity of CD4$^+$ T cells has been investigated intensively both in mice and humans. It has been shown that under certain conditions, especially an inflammatory state, Treg cells can convert into Th1, Th17, and Tfh cells; moreover, Th17 cells can convert into Treg, Th1, and Tfh cells. Among all CD4$^+$ T cell lineages, Th1 cells have been considered as one of the most stable subsets[116]. However, some recent reports

demonstrated that Th1 cells could convert into Th2, Tfh and Treg cells in vivo under various conditions[118, 120, 157]. A conversion of IFNγ$^+$ Th1 cells into IL-4$^+$ Th2 cells and IFNγ$^+$IL-4$^+$ double-positive cells was observed when Th1 cells were adoptively transferred to a mouse model infected with the gastrointestinal helminth *Nippostrongylus brasiliensis*[118]. In an elegant recent study, it was demonstrated that human Th1 cells can convert into Foxp3$^+$ Treg cells in human-into-mouse xenogeneic GVHD, which was mediated by overexpression of the programmed death ligand-1 (PDL1) through inactivation of STAT1[120]. However, it is still unclear whether Th1 cells can convert into Th17 cells. By using IFNγ$^{Thy1.1}$ CBir1 TCR-transgenic reporter mice, whose TCR is specific for an immunodominant microbiota antigen, I investigated the stability of Th1 cells under intestinal inflammatory conditions. In inflamed intestines, transfer of purified, CBir1-specific IFNγ$^+$ Th1 cells induced colitis in Rag$^{-/-}$ mice, and IFNγ$^+$ Th1 cells lost IFNγ expression and converted into IL-17$^+$-single-positive Th17 cells, as well as IFNγ$^+$ IL-17$^+$ CD4$^+$ T cells, but not Foxp3$^+$ Treg cells (**Figure 1**). Interesting, TGF-β, which is present at high levels in inflamed intestinal tissues, promoted Th1 conversion into Th17 cells, and this was enhanced by IL-6. In contrast, IL-1β and IL-23, which have been shown to induce Th17 cells co-expressing T-bet and RORγt[111], and hypoxic conditions, which are associated with intestinal inflammation[162], did not affect Th1 conversion into Th17 cells, although hypoxia and the sequential induction of hypoxia-inducible factor, HIF1α, have been shown to facilitate Th17 cell differentiation from naïve CD4$^+$ T cells[105, 163].

RORγt has been implicated as a key transcription factor driving Th17 cell differentiation and production of IL-17. Among multiple genes which regulate the expression and function of RORγt, Runx1 has been found to induce RORγt expression, influence Th17 cell differentiation by forming a complex with RORγt, and upregulating IL-17 expression by binding to its enhancer and promoter[138]. T-bet was reported to interact with Runx1, and this interaction blocked the Runx1-mediated transactivation of

Rorc, which encoded RORγt, and also blocked the Runx1- and RORγt-mediated synergetic activation of IL-17 transcription[165]. Data from my studies demonstrate that TGF-β promoted Th1 cell expression of Runx1, as well as that of Rorc, Rorα, and IL-17. Knockdown of Runx1 expression in Th1 cells significantly down-regulated the TGF-β induced expression of Rorc, Rorα, and IL-17, thereby suggesting that the TGF-β induction of Runx1 expression in Th1 cells plays a crucial role in Th1 conversion into Th17 cells. By using a ChIP assay, I further demonstrated that TGF-β increased the levels of histone H3K9 acetylation in Runx1-binding sites of promoters of *rorc* and *il-17* genes, as well as in the RORγt binding site in *il-17* promoter. Whereas TGF-β decreased the levels of histone H3K9 trimethylation in these binding sites, indicating that TGF-β treatment may enhance the accessibility of Runx1-binding sites in mouse *rorc* promoter, sequentially increasing IL-17 production in Th1 cells.

In summary, my current study demonstrates for the first time that commensal bacterial, antigen-specific Th1 cells can convert into Th17 cells in intestines under inflammatory conditions. Interestingly, TGF-β plays a key role in driving Th1 conversion into Th17 cells through induction of Runx1 expression, as well as epigenetic regulation of Runx1. Thus, Th1 conversion into Th17 cells during intestinal inflammation could also contribute to the generation of pathogenic IFNγ$^+$ IL-17$^+$ CD4$^+$ T cells. However, it is still unclear whether Th1 cells in a steady state can also convert into Th17 cells. Thus, it will be important to determine the relationship between mucosal IFNγ$^+$-single-positive Th1 cells and IL-17$^+$-single-positive Th17 cells in terms of in vivo plasticity, as well as the developmental pathways of Th1 cells present in the steady state or under inflammatory conditions in intestines.

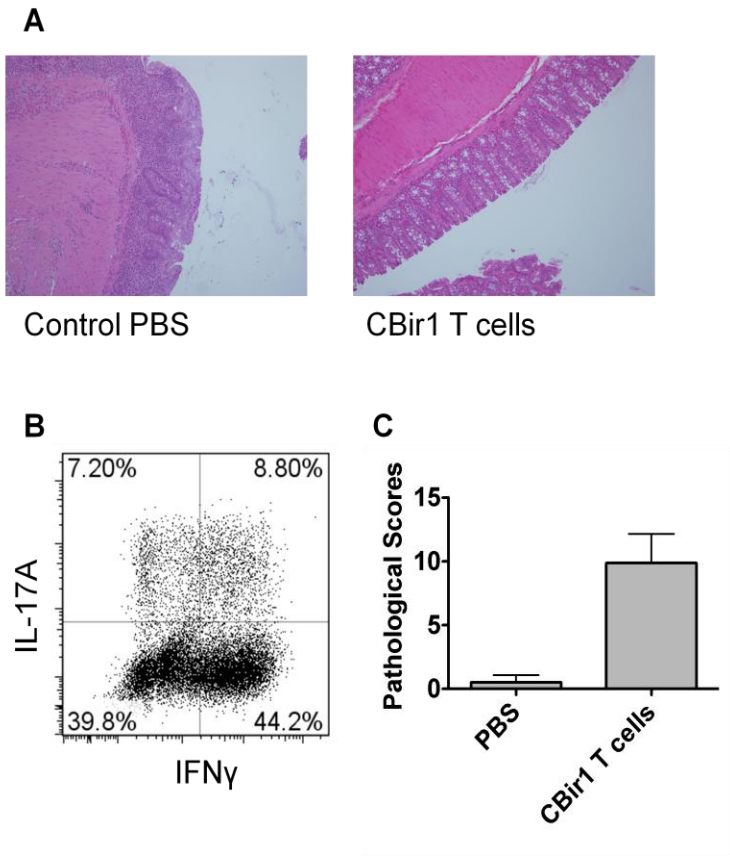

Figure 10: IL-17^{+}, IFNγ^{+}, and IL-17^{+}IFNγ^{+} CD4^{+} cells were found in the lamina propria of colitic mice.

Control PBS or naïve splenic CD4^{+} T cells from CBir1 Tg mice were i.v. injected into Rag$^{-/-}$ mice. After 4 weeks post T cell transfer, CD4^{+} cells were isolated from colon lamina propria of Rag$^{-/-}$ recipient and measured for expression of IFN-γ and IL-17 by flow cytometry. The severity of colonic inflammation was assessed by histological analysis. (A) Histopathology. (B) CD4^{+} T cells. (C) Pathological scores.

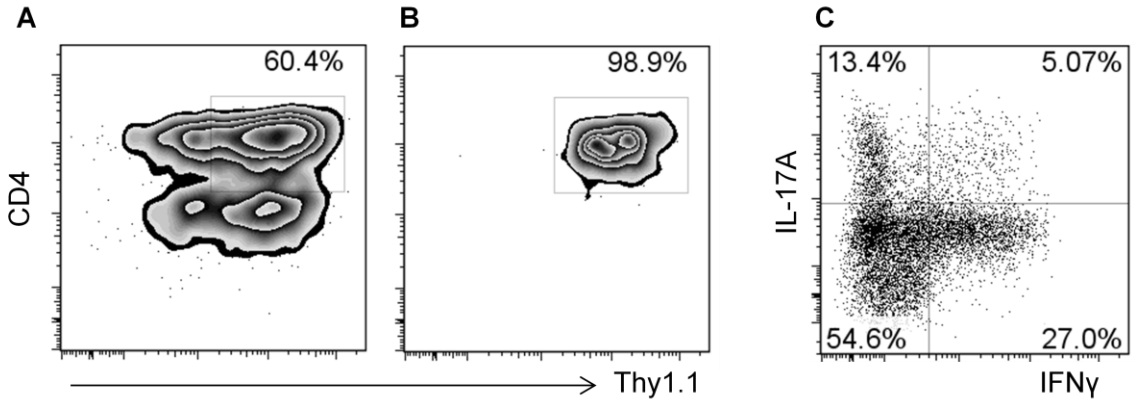

Figure 11: IFNγ-producing Th1 cells converted into IL-17-producing Th17 cells in inflamed intestines.

Splenic $CD4^+$ T cells from $IFN\gamma^{Thy1.1}$ CBir1 TCR Tg reporter mice were cultured under classical Th1 differentiation conditions with mIL-12/anti-IL-4 for 4 days and stimulated with anti-CD3 (1μg/ml) and anti-CD28 (2μg/ml) for 3 hours. $IFN\gamma^{Thy1.1+}CD4^+$ double-positive cells were sorted with a purity of 98.9%. (A) Before sorting. (B) After sorting. (C) 8 wk post-adoptive transfer of sorted CBir1 Tg $IFN\gamma^{Thy1.1+}$ $CD4^+$ cells to $Rag^{-/-}$ mice. $CD4^+$ cells from the lamina propria of $Rag^{-/-}$ recipient mice were isolated and measured for expression of IFN-γ and IL-17 by flow cytometry.

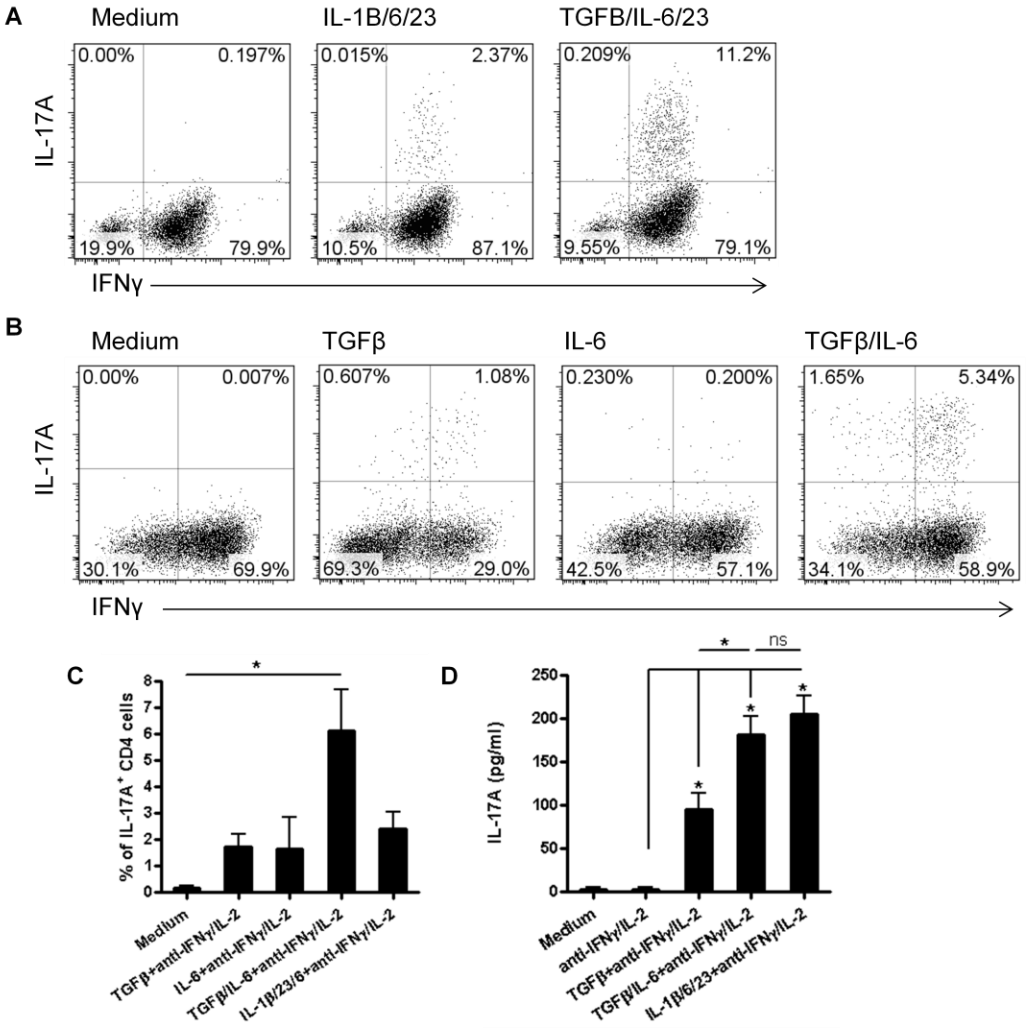

Figure 12: TGF-β and IL-6 stimulated Th1 cells to convert into IL-17-producing Th17 cells.

FACS-sorted CBir1 Tg IFNγ$^{Thy1.1+}$ CD4^{+} cells were cultured with irradiated splenic CD4^{-} APCs and different cytokines, (A) IL-1β/IL-6/IL-23 or TGF-β/IL-6/IL-23 and (B) TGF-β, IL-6 or TGF-β/IL-6, in conjunction with anti-IL-2 and anti-IFNγ antibodies for 5 days. The expression of IL-17 and IFNγ was examined by flow cytometry. (C) Bar chart represents aggregate flow cytometry data with a mean of +/- SD of three experiments. (D) The total IL-17 production in Day 5 culture supernatants was detected by an ELISA. Bar chart represents aggregate ELISA data with mean +/- SD of three experiments. *$p<0.05$, One-way ANOVA with Tukey's t test.

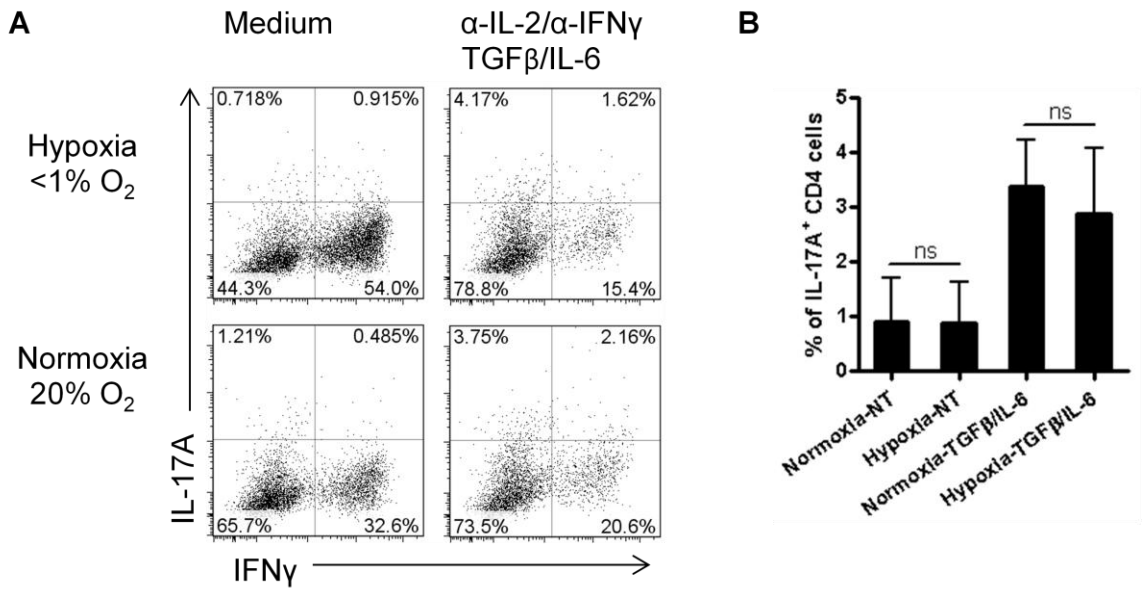

Figure 13: Hypoxia and hypoxia-induced factor 1α had a dispensable role in Th1 conversion into Th17 cells.

(A) FACS-sorted CBir1 Tg IFNγ$^{Thy1.1+}$ CD4^{+} cells were cultured with irradiated splenic CD4^{-} APCs and TGF-β/IL-6 in the presence of anti-IL-2 and anti-IFNγ antibodies under hypoxic or normoxic conditions for 5 days. The expression of IL-17 and IFNγ of CD4^{+} cells was examined by FACS analysis. (B) Bar chart represents aggregate flow cytometry data with mean +/- SD of two experiments.

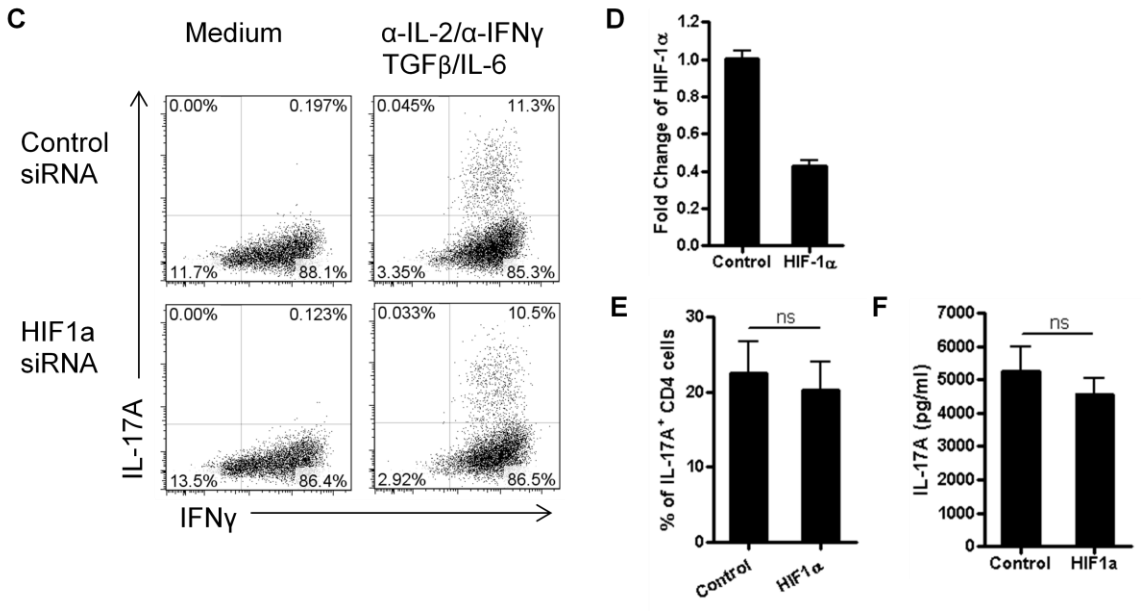

Figure 13: Hypoxia and hypoxia-induced factor 1α had a dispensable role in Th1 conversion into Th17 cells. (Cont'd)

(C) FACS-sorted CBir1 Tg IFNγ$^{Thy1.1+}$ CD4^{+} cells were cultured with irradiated splenic CD4^{-} APCs for 2 days, transfected with HIF1α siRNA or control siRNA, and stimulated with anti-CD3/anti-CD28 TGF-β and IL-6 in the presence of anti-IL-2 and anti-IFNγ antibodies for 5 days. The expression of IL-17 and IFNγ was examined by flow cytometry. (D) siRNA knockdown efficiency was confirmed by RT-PCR at 24 hrs post transfection. (E) Bar chart represents aggregate flow cytometry data with mean +/- SD of two experiments. (F) IL-17 production in Day 5 culture supernatants was detected by an ELISA. Bar chart represents aggregate ELISA data with mean +/- SD of two experiments.

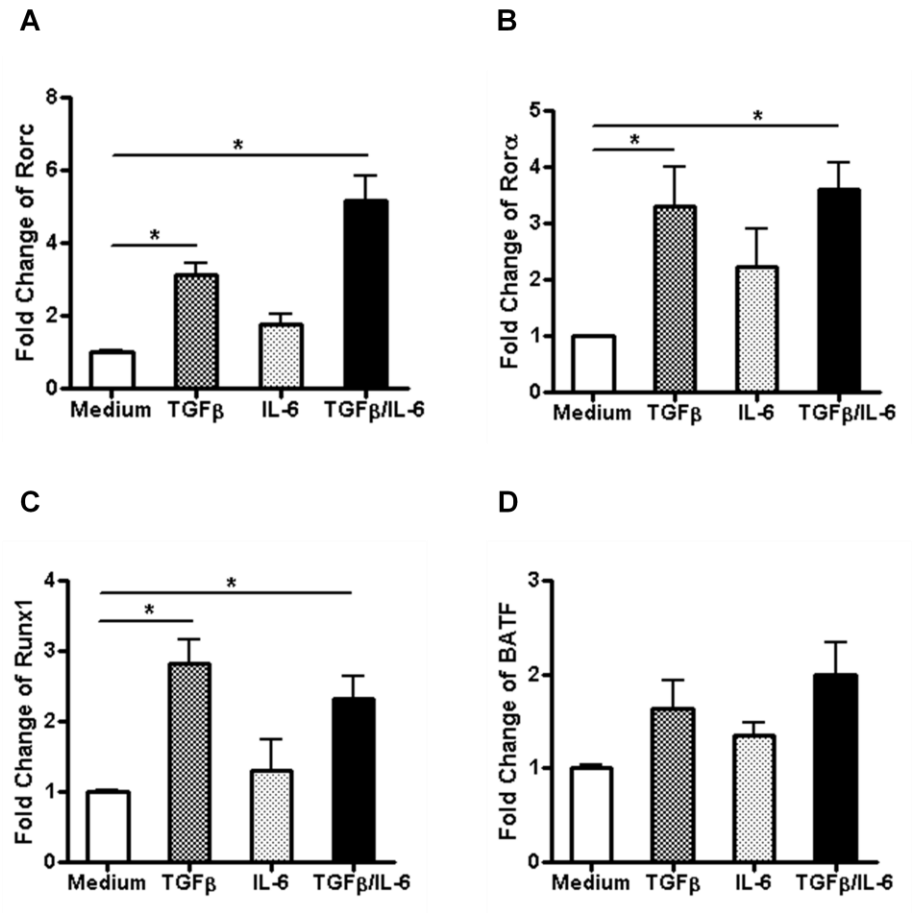

Figure 14: TGF-β dominated the induction of Th17-associated genes in Th1 cells.

CBir1 Tg IFNγ$^{Thy1.1+}$ CD4^{+} cells were cultured with irradiated splenic CD4^{-} APCs with TGF-β, IL-6 or TGF-β/IL-6, in conjunction with anti-IL-2 and anti-IFNγ antibodies for 4 days. The expression of different transcription factors was detected by RT-PCR. (A) Rorc. (B) Rorα. (C) Runx1. (D) Batf. *$p<0.05$, One-way ANOVA with Tukey's t test.

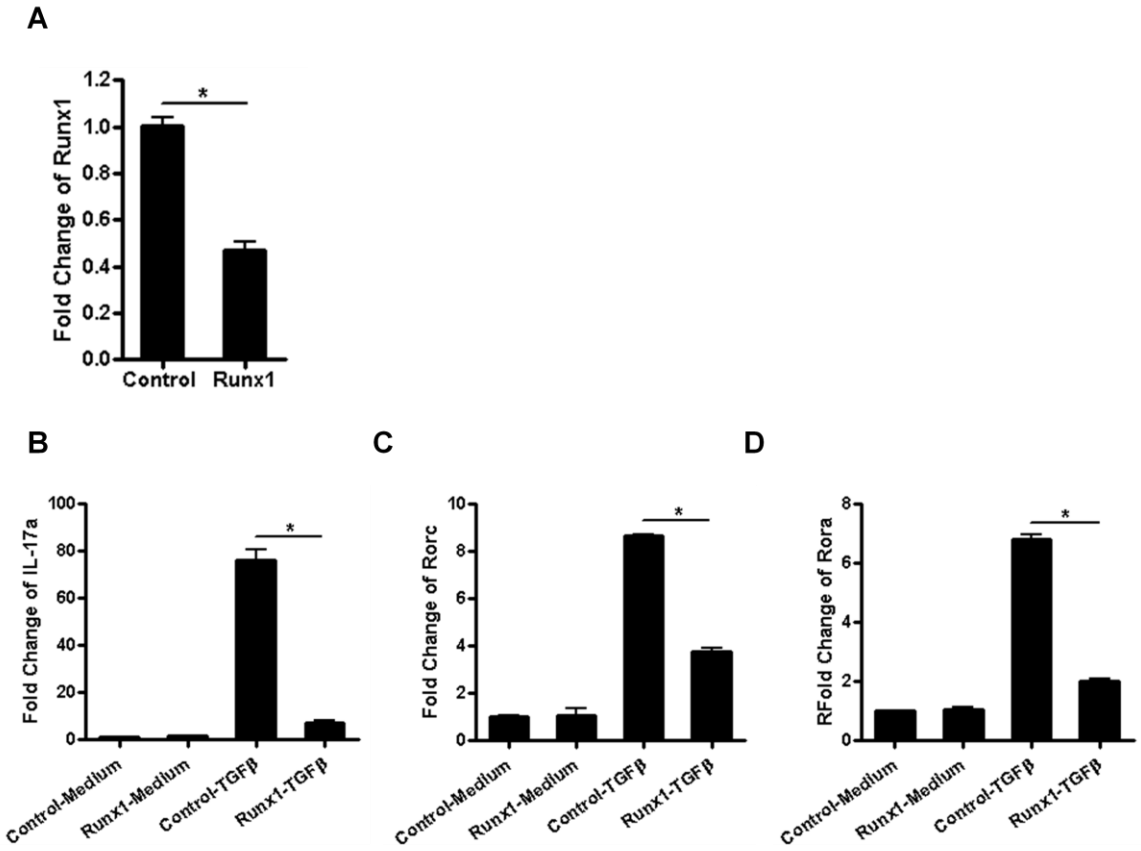

Figure 15: TGF-β-induced Runx1 played a crucial role in Th1 conversion into Th17 cells.

CBir1 Tg IFNγ$^{Thy1.1+}$ CD4^{+} cells were cultured with irradiated splenic CD4^{-} APCs for 2 days, transfected with Runx1 siRNA or control siRNA, allowed to rest for 24 hours and stimulated with anti-CD3/anti-CD28 with TGF-β and anti-IL-2/anti-IFNγ for 2 days. (A) siRNA knockdown efficiency was confirmed by RT-PCR at 24 hrs post transfection. The expression of (B) IL-17, (C) Rorc and (D) Rorα was detected by RT-PCR. Results are representative of two experiments. *$p<0.05$, Student's *t*-test.

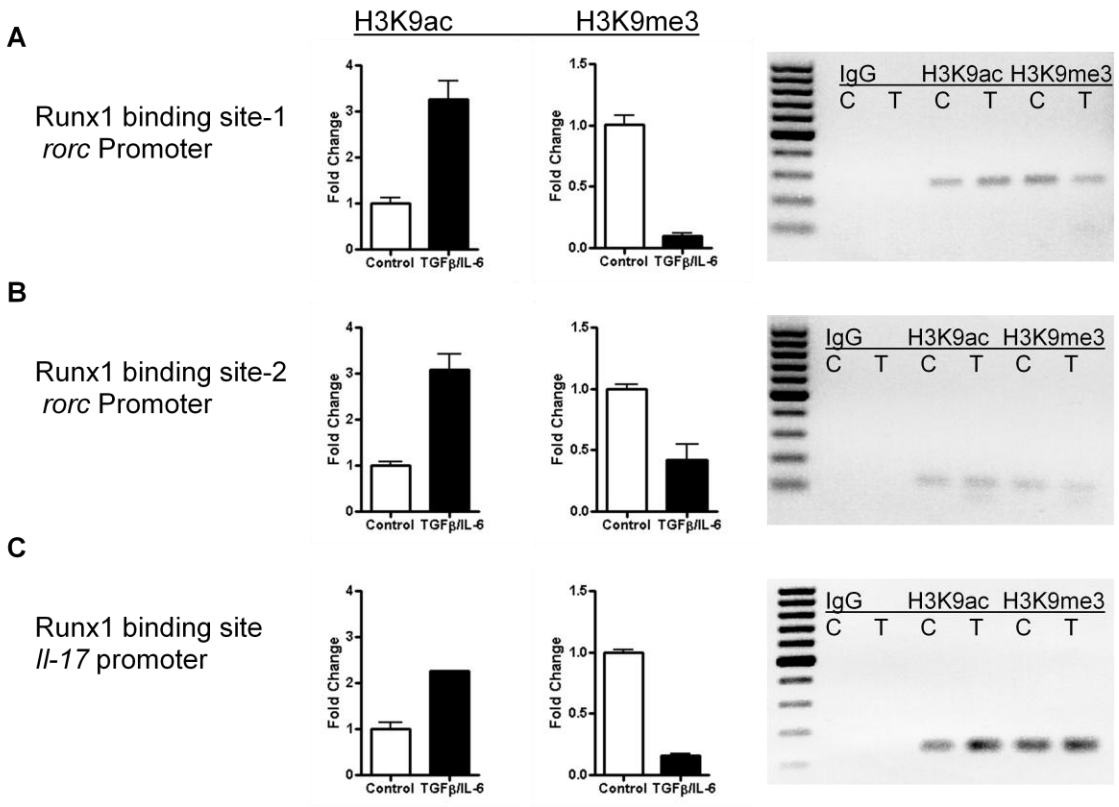

Figure 16: Treatment with TGF-β and IL-6 increased the accessibility of RORγt and Runx1 binding sites in Th1 cells.

CBir1 Tg IFNγ$^{Thy1.1+}$ CD4^{+} cells were cultured with irradiated splenic CD4^{-} APCs with TGF-β/IL-6 and anti-IL-2/anti-IFNγ for 24 hrs. Cellular DNA and proteins were fixed, sheared and immunoprecipitated with antibodies against H3K9ac, H3K9me3, or control IgG. The accessibility of multiple Runx1- and RORγt-binding sites on *il-17*, and *rorc* genes were analyzed by RT-PCR. Equal volumes of RT-PCR products were subjected to electrophoresis. Results are representative of two experiments. (A) Runx1-binding site-1 on *rorc* promoter. (B) Runx1-binding site-2 on *rorc* promoter. (C) Runx1-binding site on *il-17* promoter.

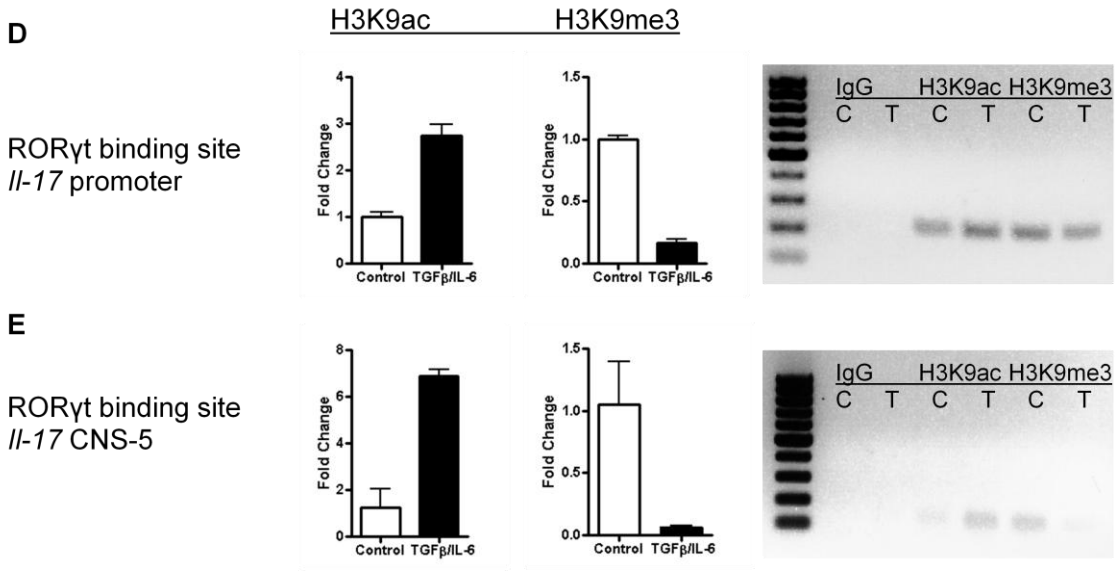

Figure 16: Treatment with TGF-β and IL-6 increased the accessibility of RORγt and Runx1 binding sites in Th1 cells. (Cont'd)

CBir1 Tg IFNγ$^{Thy1.1+}$ CD4^{+} cells were cultured with irradiated splenic CD4^{-} APCs with TGF-β/IL-6 and anti-IL-2/anti-IFNγ for 24 hrs. Cellular DNA and proteins were fixed, sheared and immunoprecipitated with antibodies against H3K9ac, H3K9me3, or control IgG. The accessibility of multiple Runx1- and RORγt-binding sites on *il-17*, and *rorc* genes were analyzed by RT-PCR. Equal volumes of RT-PCR products were subjected to electrophoresis. Results are representative of two experiments. (D) RORγt-binding site on *il-17* promoter. (E) RORγt-binding site on *il-17* CNS-5 enhancer.

Figure 17: Identification of consensus Runx1 binding sites in human and mouse rorc locus.

(A) Alignment of intron regions of mouse and human rorc genes. The location of Runx1 consensus binding sites are underlined in the aligned sequences. Alignment was made by Blast basic local alignment search tool, program BLASTN 2.2.28+.

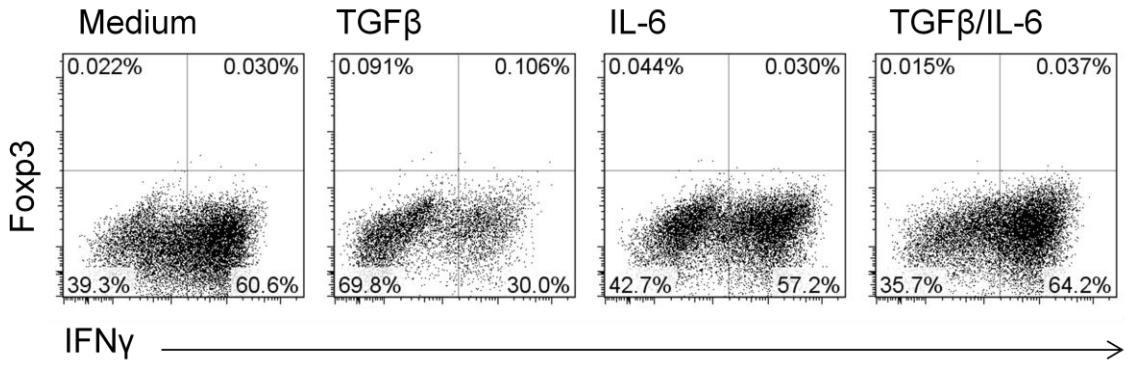

Figure 18: TGF-β and IL-6 did not stimulate Th1 cells to convert into Foxp3^{+} Treg cells.

FACS sorted CBir1 Tg IFNγ$^{Thy1.1+}$ CD4^{+} cells were cultured with irradiated splenic CD4^{-} APCs and different cytokines in the presence of anti-IL-2 and anti-IFNγ antibodies for 5 days. The expression of Foxp3 was examined by flow cytometry.

A

Pair	Gene	Binding Site and sequence	Sequence	Location
1	*rorc*	Runx1, TGTGGT	F: 5' CCCAAGGGGTGCCAAGTTAC 3' R: 5' CAGAGCCTCTCTCCAGGCTT 3'	0.1 kb upstream of the 3rd extron.
2	*rorc*	Runx1, TGTGGT	F: 5' CGGCTGTAGCTGTGGTTTTG 3' R: 5' GTATGATGACAGGCACCCCA 3'	2 kb upstream of the 3rd extron.
3	*il-17a*	Runx1, TGTGGT	F: 5' GCCCATAAAGAAGCCAATGTTGT 3' R: 5' CAGCCTAGGCAATGTCCTCTT 3'	Promoter, -1.5 Kb
4	*il-17a*	Rorγt, AGGTCA	F: 5' TGGGACCTAATGACCCCCATA 3' R: 5' AGGCTGAAAGACAGTGGAGAC 3'	Promoter, -0.9Kb
5	*il-17a*	Rorγt, TGACCT	F: 5' CCGTTTAGACTTGAAACCCAGTC 3' R: 5' GTACCTATGTGTTAGGAGGCGC 3'	Enhancer CNS-5, -5 Kb

Table 1: Sequences of primers used for quantification of DNA from ChIP assays, their genomic locations and Runx1/RORγt binding sites flanked by primers.

(A) Primer pairs were designed for real time PCR in ChIP assay. The sequences of the 5th pair are obtained from a previous report.

CHAPTER 5: SUMMARY AND CONCLUSION

Multiple studies in patients with IBD and experimental colitis models reveal the dual role of $CD4^+$ T cells as important mediators in intestinal homeostasis and colitis development. In addition to their roles in IBD, $CD4^+$ T cells broadly mediate the pathogenesis of chronic inflammatory diseases, as well as the immunity against infectious diseases, and tumors. Multiple specialized T effector lineages are implicated in the development of chronic inflammation and are required for hosts to eliminate threats from various microorganisms. Conversely, regulatory T helper cells suppress the autoimmunity mediated by effector T cells and maintain the systemic homeostasis. Therefore, a complete understanding of the factors regulating T helper cell differentiation and conversion are fundamental for investigating human diseases.

In the present study, I found that a specific ERK MAPK pathway differentially regulated the differentiation of Tregs and Th17 cells **(Illustration 1)**. Although the development of T-cell subsets is mainly regulated by a master transcriptional regulator and phosphorylation of the STAT protein in response to distinct cytokine stimulation, accumulating data indicate that other signaling pathways are also involved in regulating or fine-tuning T-cell lineage commitment. I investigated the role of ERK, mitogen-activated protein kinase (MAPK), in Th17 and Treg cell development. I demonstrated that blockade of ERK activation inhibited Th17-cell development while upregulating Treg cells under Th17 polarization conditions. Inhibition of ERK decreased IL-6 induction of RORγt, but enhanced TGF-β induction of Foxp3, and ERK inhibitor-treated T cells under Th17 conditions possessed suppressive function in vitro because they produced more IL-10 and TGF-β and inhibited naïve T-cell proliferation and IFNγ

production at levels comparable with that of Treg cells. Furthermore, ERK inhibitor-treated T cells under Th17 polarization conditions had a decreased potency to induce colitis in vivo. Collectively, my data demonstrated that the ERK pathway differentially regulates Th17- and Treg-cell differentiation, and thus interferences with the ERK pathway could represent a therapeutic treatment for inflammatory bowel diseases and other Th17-related autoimmune diseases

Differentiated $CD4^+$ T cells preserve plasticity to alter phenotypes under various conditions. The direction of conversion from one lineage to another is dependent on the intrinsic stability of a certain lineage and extraneous stimulation from the environment. I confirmed the speculation that, similar to other relatively unstable T helper cell subsets, Th1 cells can transition to another specific subset according to disease settings. By using $IFN\gamma^{Thy1.1}$ CBir1 TCR-transgenic reporter mice, whose TCR is specific for an immunodominant microbiota antigen, I investigated the stability of Th1 cells under intestinal inflammatory conditions. Transfer of purified, CBir-specific $IFN\gamma^+$ Th1 cells induced colitis in $Rag^{-/-}$ mice and converted into IL-17^+ Th17, but not $Foxp3^+$ Treg, cells in the inflamed intestines. TGF-β and IL-6, but not IL-1, IL-23, and hypoxia factors, regulated Th1 conversion into Th17 cells **(Illustration 2)**. TGF-β induction of transcriptional factor Runx1 is crucial for the conversion, in that silencing Runx1 by siRNA inhibited Th1 conversion into Th17 cells. Furthermore, by using ChIP assays, I demonstrated that TGF-β enhanced histone acetylation but inhibited histone trimethylation of Runx1- and RORγt- binding sites on *il-17* or *rorc* genes in Th1 cells. In conclusion, my data demonstrate that Th1 cells convert into Th17 cells under inflammatory conditions in intestines, and this conversion is mediated by TGF-β induction of Runx1.

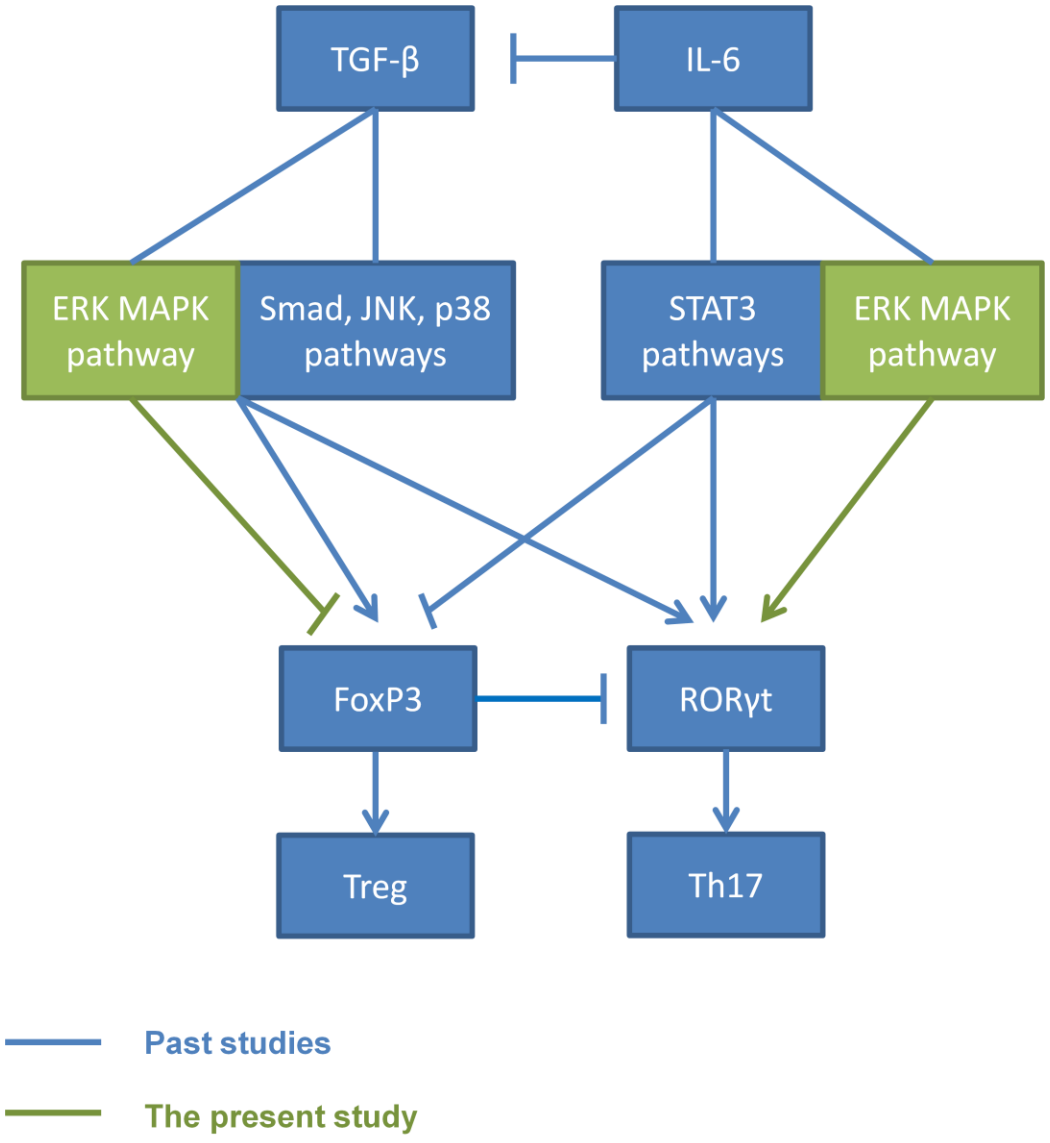

Illustration 1: The ERK MAPK pathway differentially regulates the differentiation of Treg and Th17 cells.

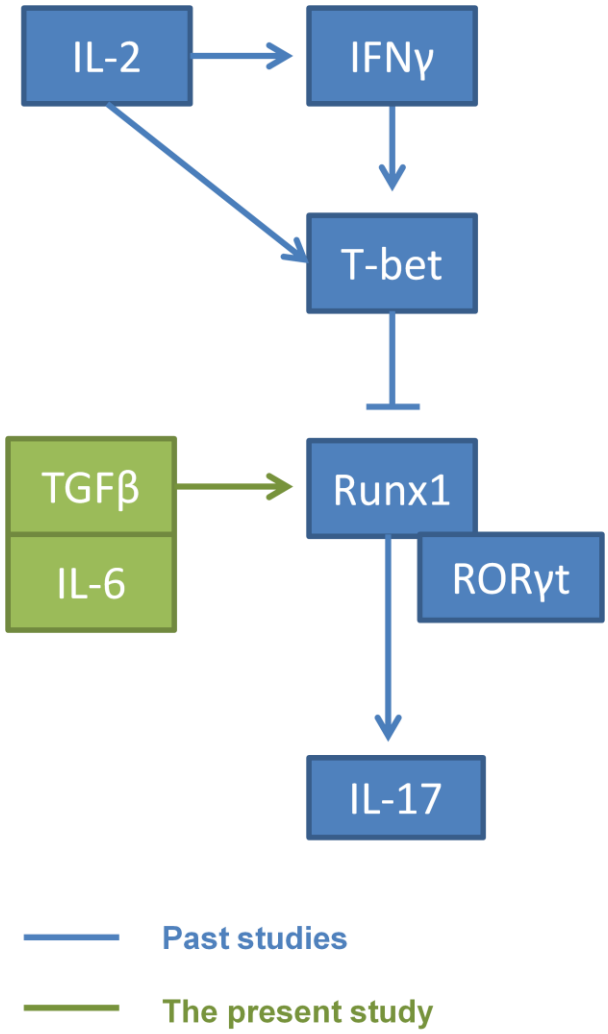

Illustration 2: IL-2, TGF-β and IL-6 differentially regulate Th1 conversion into Th17 cells.

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Publications

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