REVIEW

Retinoid-Related Orphan Receptor ROR γ t in CD4⁺ T-Cell—Mediated Intestinal Homeostasis and Inflammation

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Retinoic acid—related orphan receptor (ROR)- γ t, the master transcription factor of the Th17 subset of CD4⁺ Th cells, is a promising target for treating a host of autoimmune diseases. ROR γ t plays a vital role in the pathogenesis of inflammatory bowel diseases—Crohn disease and ulcerative colitis—caused by untoward reactivity of the immune system to the components of the intestinal microbiome. The mammalian intestinal tract is a highly complex and compartmentalized organ with specialized functions, and is a privileged site for the generation of both peripherally induced regulatory CD4⁺ T cells (Tregs) and effector Th17 cells. As Th17 cells can be proinflammatory in nature, the equilibrium between effector Th17 and Treg cells is crucial for balancing intestinal homeostasis and inflammation. Recent findings suggest that ROR γ t, in addition to Th17 cells, is also expressed in peripherally induced, colonic regulatory CD4⁺ T cells. Therefore, ROR γ t is expressed in both effector and regulatory subsets of CD4⁺ T cells in the intestine. The present review discusses the role of ROR γ t in cellular and molecular differentiation of Th17 and Treg, and examines how targeting ROR γ t in inflammatory bowel disease therapy could influence the development of these two diverse subsets of immune cells with opposing functions. (*Am J Pathol* 2020, 190: 1984–1999; <https://doi.org/10.1016/j.ajpath.2020.07.010>)

Retinoic acid (RA)—related orphan receptor (ROR)- γ t is a ligand-dependent transcription factor (TF) that plays a vital role in regulating the adaptive immune response. Starting from the development of CD4⁺ T cells in the thymus until their maturation in peripheral lymphoid tissues, including the intestine, ROR γ t plays an indispensable role in coordinating the development and function of CD4⁺ T cells.^{1,2} In addition to its crucial involvement in thymocyte maturation and lymphoid follicle development, ROR γ t has drawn extensive interest as the master TF of Th17 cells, which are abundantly present in the intestine.³ Although ROR γ t-expressing CD4⁺ T cells are implicated in the pathogenesis of inflammatory bowel disease (IBD)—a disease that affects over 1.6 million Americans—our understanding of ROR γ t-driven mechanisms contributing to disease pathogenesis remains incomplete. Due to the development of various synthesis modulators that can alter ROR γ function, ROR γ t has become a prime candidate for pharmacologic intervention to repress the function of proinflammatory Th17 cells that

contribute to the pathology of several autoimmune diseases.⁴ Major pharmaceutical companies are involved in developing ROR γ modulators, some of which are in early clinical trials.⁵ While it is acknowledged that proinflammatory Th17 cells with a Th1-like signature are the principal pathogenic cells in IBD, how ROR γ t contributes to this mechanism of pathogenicity is poorly understood.⁶ Surprisingly, targeting of Th17 cells in Crohn disease (CD) patients by blocking IL-17 worsens colitis, indicating that only a subset of Th17 cells are pathogenic and that targeting the entire Th17 cell population is counterproductive.^{7,8}

Recently, ROR γ t was found to be expressed in a subset of intestinal Tregs with enhanced immunosuppressive function (ROR γ t⁺ Tregs) compared to the ROR γ t-nonexpressing

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Tregs.^{9,10} Contrary to an earlier notion that forkhead box (FOX)-P3 and ROR γ t have mutually antagonistic functions,¹¹ ROR γ t is stably co-expressed with FOXP3 and plays a collaborative role in this specific subset of colonic Tregs. The discovery of ROR γ t⁺ Tregs demonstrated that in addition to regulating the differentiation of effector Th17 subset, ROR γ t could control the function of a regulatory subset of Th cells. Therefore, targeting ROR γ t would affect both the pro- and anti-inflammatory pathways and give rise to undesirable consequences. Hence, a therapeutic strategy is needed for selectively targeting the pathogenic ROR γ t-expressing Th17 cells during IBD without affecting the regulatory function of ROR γ t⁺ Tregs. The divergent mechanistic roles of ROR γ t in the development of two opposing Th subsets necessitates further characterization of the effector and regulatory functions of ROR γ t. In this review, the signaling pathways that induce ROR γ t in various CD4⁺ T-cell lineages present in the intestine and the role of ROR γ t in intestinal homeostasis and inflammation are discussed.

Types of Th Subsets in the Intestine

As the intestine encounters millions of microbes, it forms a protective barrier between the bloodstream and the external environment. The intestinal immune system maintains a delicate balance between promoting immune tolerance and mounting an inflammatory immune response. CD4⁺ T cells, present within the epithelial layer and lamina propria of the intestine, play a crucial role in the decision-making process of mounting an appropriate immune response during their persistent encounter with a plethora of microbes. Several types of CD4⁺ Th cells, each with unique functionalities, are present in the intestine and are briefly described in the context of IBD. Many subsets of intestinal CD4⁺ T cells show developmental flexibility (plasticity) and can frequently transition from one phenotype to another based on their surrounding inflammatory milieu.¹²

Th1

The Th1 subset of CD4⁺ Th cells expresses their master TF, T-box TF TBX21 (T-bet), and predominantly produces interferon (IFN)- γ and tumor necrosis factor (TNF)- α . Th1 differentiation is initiated by IL-12 secreted by antigen-presenting cells and offers protection against intracellular pathogens. Th1 cells are also potent drivers of autoimmunity and play a key role in the pathogenesis of IBD. High-IFN γ -producing Th1 cells are present in the intestinal tract of CD patients.¹³

Th2

IL-4 signaling drives Th2 differentiation through the induction of STAT6, which in turn activates lineage-specific TF GATA binding protein 3 (GATA3). Th2 cells target helminths and contribute to chronic inflammatory diseases, such as asthma and allergy. Th2 cells secrete IL-4, IL-5, IL-

13, and IL-33 as their signature cytokines. Although the role of Th2 in IBD is controversial, expressions of IL5, IL13, and IL33 mRNAs are found to be significantly elevated in UC patients.¹⁴

Th17

Th17 differentiation is driven by the cytokines IL-6 and transforming growth factor (TGF)- β , which induce the expression of the lineage-specific TF ROR γ t and the signature cytokines IL 17A and 17F.¹⁵ Th17 cells fight against extracellular bacteria and fungi and are also powerful drivers of autoimmunity. Both active CD and UC patients show an increased number of IL-17-expressing cells in the intestine.¹⁶

Th22

Differentiation of Th22 cells is initiated by IL-6 and IL-23 and suppressed by TGF- β .¹⁷ Although the developmental axis of Th22 overlaps with that of the Th17 subset, this newly discovered subset of Th cells produces IL-22 without IL-17 co-production and expresses the TFs aryl hydrocarbon receptor (AhR) and T-bet.¹⁷ Th22 cells mediate their protective effect against inflammation, tissue damage, and bacterial infection by producing antimicrobial peptides.^{15,18} A significant reduction in the frequency of Th22 cells has been observed in the inflamed tissues of UC patients, but its association with IBD pathogenesis remains unknown.¹⁹

T-Regulatory Cells

Tregs are induced by TGF- β and express the master TF FOXP3. Although the thymus is the primary organ for the generation of Tregs (tTregs) that develop during the lymphocyte-maturation process in order to acquire the knowledge of the self and nonself antigens, Tregs are also generated in the peripheral lymphoid tissues (pTregs).²⁰ Both tTregs and pTregs are anti-inflammatory subsets of CD4⁺ T cells that maintain immune homeostasis and restrict autoimmune response by suppressing the deleterious activities of proinflammatory effector CD4⁺ Th cells. A large number of Tregs found in the gut actively produce IL-10.²¹ Like other CD4⁺ Th subsets, pTregs are also a heterogeneous population, and a specific subpopulation of pTregs co-express ROR γ t (ROR γ t⁺ Tregs) in the intestine.¹⁰ Despite evidence that Tregs are crucial for the maintenance of intestinal homeostasis, Tregs isolated from the intestinal mucosa of IBD patients show no obvious defects in their frequency, indicating that their suppressor function might be compromised in IBD patients.^{22,23}

Role of ROR γ t in T-Cell–Mediated Immunity

The nuclear receptor superfamily is a group of structurally related, ligand-dependent TFs that function as key

regulators of genes that control various physiologic processes during embryonic development as well as in adulthood.² RORs α , β , and γ are orphan nuclear receptors, as the identification of natural endogenous ligands for these receptors has been controversial. Several cholesterol-biosynthesis intermediates are involved in regulating ROR γ t function in the development of lymph nodes and Th17 cells.²⁴ Ligand-bound RORs bind as monomers to specific ROR response elements (RORE) in the DNA, consisting of the consensus core motif AGGTCA, preceded by an A/T-rich sequence. By binding with the DNA, RORs can either activate or repress the gene expression in a cell type-specific manner, depending on the types of interacting ligand (agonist, inverse agonist, or antagonist), which additionally facilitates the binding of co-activators or co-repressors.²⁴

The ROR subfamily contains three genes, ROR α (*RORA*; alias NR1F1), ROR β (*RORB*; alias NR1F2), and ROR γ (*RORC*; alias NR1F3), with each ROR form encoded by a separate gene. The *RORA* and *RORC* genes produce several isoforms that are generated through a combination of alternative promoter usage and gene splicing. In humans, four different isoforms of ROR α (α 1 to α 4) are found. The *RORB* gene encodes the protein ROR β , and the *RORC* gene translates into two distinct isoforms, namely ROR γ 1 and ROR γ 2(t).²⁵ *RORA* mRNA has been detected in multiple tissues, including heart, brain, skin, muscle, lung, spleen, testis, ovary, thymus, and peripheral blood leukocytes. *RORB* mRNA expression is less diverse and is most abundant in the brain, pineal gland, and eye. While ROR γ 1 transcripts are ubiquitously expressed, ROR γ t transcripts are restricted to the thymocytes, a specific subset of peripheral CD3⁺CD4⁺ T cells and a population of non-T cells (CD3⁻CD4⁺ cells) that are involved in the development of lymph nodes of the small intestine known as Peyer patches.²⁶ ROR γ t controls important immunologic functions during T-cell maturation, both in the thymus (thymopoiesis) as well as in the peripheral lymphoid tissue. It regulates apoptosis of immature lymphocytes during thymopoiesis and promotes lymphoid organogenesis in the periphery.²⁷ By regulating the cellular level of the antiapoptotic factor B-cell lymphoma—extra large (Bcl-XL), it promotes the survival of immature thymocytes, as evidenced in mice lacking Ror γ , which lose thymic expression of Bcl-XL.²⁸ Thus ROR γ t enhances the survival of CD4⁺CD8⁺ thymocytes and controls the duration of survival of the thymocytes by enabling them to undergo positive selection in the thymus. ROR γ t is also required for the development of lymph nodes and Peyer's patches.²⁶ In its absence, there is a loss of a population of cells, the lymphoid tissue-inducer cells, which are early progenitors of lymphoid organs. Subsequently, it was found that ROR γ t is necessary for the development of Th17 cells, a subset of Th cells that is abundant at mucosal interfaces, including intestinal mucosa. The primary function of Th17 cells is to contain the infection from extracellular bacteria and fungi, but an exaggerated Th17 response can also cause tissue

inflammation.^{3,29,30} In the intestine, Th17 cells are abundantly present in the lamina propria, where they protect the host against infectious pathogens, but their hyperactivation causes inflammation leading to IBD.^{3,6} There is an increased prevalence of Th17 cells along with an increased IL-17A level in the serum and mucosa of UC and CD patients compared to healthy individuals.^{16,31,32} It is the characterization of ROR γ t as the master regulator of Th17 cells that catapulted ROR γ t to prominence as a potential pharmacologic target in the therapy of IBD. Paradoxically, in the intestine, ROR γ t is co-expressed in a population of peripherally induced regulatory T cells (pTregs) together with FOXP3, the master TF for Tregs.^{9,10} Quite unexpectedly, colonic ROR γ t⁺ Tregs demonstrate an enhanced immunosuppressive function and prevent intestinal inflammation more effectively than do ROR γ t-nonexpressing pTregs. Although many studies have elucidated the function of ROR γ t in Th17 development, how ROR γ t regulates pTreg function is poorly understood. As ROR γ t is expressed in two cellular subsets with opposite functions, therapeutic targeting of ROR γ t will have both a pro- and anti-inflammatory effect. Therefore, a clear understanding of diverse ROR γ t-driven pathways, in both Th17 as well as Tregs, is necessary for selective targeting of the pathogenic signaling pathways.

Types of ROR γ t-Expressing CD4⁺ T Cells in the Intestine: Th17, Tr17-Like, and ROR γ t⁺ Tregs

In addition to its expression in CD4⁺CD8⁺ thymocytes during thymic development, ROR γ t participates in the development of four subsets of CD4⁺ Th cells in the intestine: nonpathogenic Th17 cells, pathogenic Th17 cells, ROR γ t⁺ Tregs, and T-regulatory 17-like (Tr17-like) cells (Figure 1). While pathogenic and nonpathogenic Th17 cells are effector CD4⁺ Th cells, ROR γ t⁺ Tregs and Tr17-like cells are regulatory in function. The role of ROR γ t in the development of Th17 cells is well characterized. ROR γ t is necessary for the development of Th17 cells and induces the cytokines IL-17A and IL-17F, which control infection with extracellular pathogens and promote tissue inflammation. In chronic intestinal inflammation or other autoimmune disorders, ROR γ t is often co-expressed with T-bet in a pathogenic class of Th17 cells that co-produce IL-17 and IFN γ .^{33,34} Whether pathogenic and nonpathogenic effector Th17 cells develop independently or emerge along a common developmental axis is controversial. However, in the intestine, ROR γ t is also co-expressed in a population of pTregs along with FOXP3.^{9,10,35} ROR γ t⁺ Tregs are not found in the thymus and do not express markers of tTregs. In the colon, ROR γ t⁺ Tregs show an enhanced suppressive function compared to ROR γ t-deficient Tregs. Another striking difference between ROR γ t-expressing Th17 and Treg cells is that ROR γ t⁺ Tregs do not express signature Th17 cytokines such as IL-17A and IL-17F. The fourth subset of ROR γ t-expressing CD4⁺ T cells is the intestinal Tr17-like cells.^{36,37} They are differentiated in inflammatory conditions

Figure 1 Subsets of retinoic acid–related orphan receptor gamma t (ROR γ t)-expressing CD4⁺ T cells in the intestine. Intestinal ROR γ t⁺ CD4⁺ T cells can be broadly categorized into two subtypes: effector Th17 cells, which are either nonpathogenic or pathogenic based on co-expression of T-box transcription factor 21 (T-bet) and interferon gamma (IFN γ) (**top panel**), and regulatory CD4⁺ T cells, which co-express forkhead box P3 (FOXP3) (ROR γ t⁺ Tregs) with or without IL-17 expression (**bottom panel**). However, in patients with Crohn disease, a subset of ROR γ t-expressing CD4⁺ T cells, similar to thymically derived ROR γ t-expressing Tr17 cells, is found to co-express ROR γ t and FOXP3 and to secrete IL-17 (Tr17-like), unlike the ROR γ t⁺ Tregs.

and display combined characteristics of Th17 and Treg cells. Tr17-like cells, unlike colonic ROR γ t⁺ Tregs, readily induce IL-17. As opposed to thymically derived Tr17 cells, Tr17-like cells are found in the colon of CD patients and bear a strong phenotypic resemblance to Tr17 cells.^{37,38} These IL-17⁺FOXP3⁺ Tr17-like cells, present in the inflamed intestinal mucosa of CD patients, express high levels of ROR γ t, suggesting that they closely resemble Tr17 cells. Strikingly, the development of all of the ROR γ t-expressing subsets of CD4⁺ Th cells is contingent on IL-6/STAT3 signaling.^{9,38,39} How IL-6/STAT3 signaling, a potent repressor of FOXP3, contributes to the development of suppressor populations of FOXP3–co-expressing ROR γ t⁺ Tregs remains a puzzle. It is important to understand that in inflammatory conditions, intestinal CD4⁺ T cells, including ROR γ t⁺ Tregs and ROR γ t⁺ Th17 cells, are an assortment of phenotypically intermediate subpopulations that can transdifferentiate in response to

environmental cues by altering their effector or suppressor function. Therefore, whether the developmental dependence of the ROR γ t-expressing regulatory T cells on IL-6 signaling predisposes them to transdifferentiating into Th17-like or Tr17-like cells is a pertinent question. Colonic pTregs have been shown to be neither phenotypically nor functionally stable and can transdifferentiate into Th17-like cells.^{40,41} In infectious colitis caused by an enteropathogenic bacterium, *Citrobacter rodentium*, FOXP3-expressing CD4⁺ T cells were readily induced and transdifferentiated to IL-17–producing cells with concomitant down-regulation of FOXP3.⁴⁰ Whether these FOXP3⁺ Tregs also express ROR γ t has not been investigated. It is conceivable that intestinal Th17, ROR γ t⁺ Tregs, and Tr17-like cells emerge from common FOXP3-expressing CD4⁺ T-progenitor cells, which are the dominant phenotype in intestinal homeostasis. During inflammation, pTregs might change their phenotype, acquire ROR γ t expression, and function in accordance with the immediate spatiotemporal milieu. A detailed understanding of how ROR γ t modulates the developmental plasticity of these diverse cell types will lead to a better understanding of the biology of these cells.

Cytokines and Transcription Factors Regulating ROR γ t Expression

The two cytokines that control ROR γ t induction in Th17 cells are IL-6 and TGF β 1, pro- and anti-inflammatory cytokines, respectively.^{30,42} In presence of IL-6 and TGF β 1, T-cell receptor (TCR)-activated CD4⁺ T cells optimally induce ROR γ t and differentiate into Th17 cells.^{3,30,43} In a study in mice, colonic T cells from *Il6*^{-/-} mice did not express ROR γ t and showed compromised Th17 differentiation.³ IL-6, on engaging with IL-6R on activated CD4⁺ T cells, was reported to induce expression of cytokine IL-21 and cytokine receptor IL-23R.³⁹ Although the functions of IL-21 and IL-23 are not strictly restricted to Th17 development, it was initially thought that autocrine production of IL-21 in T cells, along with antigen-presenting cell–derived IL-23, was necessary for the induction of ROR γ t. In addition to IL-6, TGF β 1 is needed for optimal differentiation of Th17. It has been demonstrated that Th17 cells are a crucial source of TGF β 1, which operates in an autocrine way to promote the stability of Th17 cells.⁴⁴ As TGF β 1 is a recognized repressor of IL-23R and an inducer of FOXP3 expression, how it participates with IL-6 to induce ROR γ t remained unclear initially.¹¹ TGF β 1 can induce both FOXP3 and ROR γ t.¹¹ IL-6 stabilizes the expression of ROR γ t during Th17 development by opposing FOXP3-mediated inhibition of ROR γ t. TGF β 1-induced binding of SMAD3 protein to *FOXP3* enhancer assists in the generation of FOXP3⁺ Treg cells in the gut.^{45,46} However, the mechanism by which TGF β 1-induced SMAD signaling affects ROR γ t transcription remained unknown for a long time. Recently, it was shown that during Th17

differentiation, TGFβ1 signaling, partly via the activation of SMAD proteins 2 and 3, prevents a repressive association of the SMAD4–SKI protein complex with *RORC* promoter.⁴⁷ In addition to the repression of FOXP3, IL-6–driven phosphorylated STAT3 binds to *RORC* promoter to induce its transcription.⁴⁸ Accordingly, STAT3-deficient CD4⁺ T cells fail to induce RORγt.⁴⁸ In addition to RORγt induction, phosphorylated STAT3 directly binds to *IL17A* and *IL17F* gene promoters and induces their transcriptions.³⁹ The activation of STAT3 by IL-6 alone is not sufficient for the induction of robust RORγt expression and requires TGFβ1 signaling.⁴⁹ Furthermore, TGFβ1 signaling limits the expression of genes encoding T-bet, GATA3, and other Th1- and Th2-associated TFs to stabilize RORγt expression during Th17 differentiation. Thus, RORγt is optimally induced by the synergistic interaction of IL-6 and TGFβ1, which are abundant in the intestine during enteropathogenic infection or autoimmune inflammation.

Besides these central activators of RORγt, several other Th17 lineage–associated pioneering TFs up-regulate RORγt during Th17 differentiation. These include IFN-regulatory factor (IRF)-4 and the basic leucine zipper ATF–like TF (BATF), which are induced on TCR stimulation.^{50,51} *BATF*^{−/−} and *IRF4*^{−/−} T cells show significantly reduced induction of RORγt, along with a compromised Th17 differentiation.^{50,51} However, the overexpression of Rorγt in Cd4⁺ T cells from either *Batf*^{−/−} or *Irf4*^{−/−} mice failed to restore IL-17 production. This finding suggests that the two TCR-activated TFs cooperate with IL-6–driven STAT3 signaling to optimize *RORC* and *IL17* transcriptions. It should be noted that BATF and IRF4 are involved in the development of multiple Th cell lineages. BATF plays a crucial role in the generation of T-follicular Th cells, a specialized subset of CD4⁺ Th cells that help B cells in the germinal center to make high-affinity antibodies.⁵² IRF4 and BATF are also essential for the development of Th2 cell–mediated immune response.^{53,54} IRF4 and BATF are induced after TCR engagement and are further enhanced by cytokine signaling. These TFs are named pioneer TFs, as they alter chromatin accessibility across multiple Th lineages. How these pioneer TFs aid in the induction of RORγt during Th17 development is not clear. Likely, they bind to the chromatin to alter the accessibility of the target genes. During early stages of Th17 differentiation, cooperative binding of STAT3, BATF, and IRF4 enable chromatin accessibility of many genes, including *RORC*, and allow for consequent recruitment of RORγt to regulate specific Th17-associated genes.⁵⁵ Interestingly, BATF represses the *FOXP3* gene by inducing a closed chromatin configuration in *FOXP3* promoter during Treg development.⁵⁶ Similarly, a high level of FOXP3 is induced in *IRF4*^{−/−} Th17 cells, indicating that IRF4 can directly inhibit *FOXP3* transcription or indirectly prevent IL-6–mediated suppression of FOXP3.^{51,57} Therefore, it is tempting to speculate that these pioneer TFs not only promote RORγt-mediated gene programming but also

simultaneously antagonize FOXP3-mediated Treg programming during Th17 development (Figure 2).

In addition to IL-6– and TGFβ1-induced Th17 differentiation, another subset of Th17 cells has been described that develops in the absence of TGFβ1 signaling. These Th17 cells are proinflammatory in nature and are characterized as pathogenic Th17 cells. IL-1 and IL-23 act in concert with IL-6 to induce the development of these pathogenic Th17 cells that express a high level of T-bet and a low level of RORγt.³⁴ IL-23 has a potent STAT4-activating function, which leads to the induction of T-bet.⁵⁸ It is possible that TGFβ1 induces a robust expression of RORγt that restricts the pathogenicity of Th17 cells by limiting T-bet induction. Alternatively, TGFβ1 also restricts the development of pathogenic Th17 cells by suppressing the expression of IL-23R.³⁴ Naïve CD4⁺ T cells do not express IL-23R, denoting its limited role in initiating Th17 development. Although initial reports suggested that IL-23 was a driver of RORγt induction, it is becoming increasingly clear that IL-23 acts to induce the pathogenicity in the developing Th17 cells.^{17,59,60} Th17 cells comprise several heterogeneous populations, with some capable of promoting inflammation. This variation can also rest on their exposure to TGFβ1, a cytokine whose concentration differs among various organs.⁶¹ As TGFβ1 signaling shows high basal activity in the intestine, TGFβ1-independent Th17 development in the intestine is an unlikely phenomenon.⁶¹ Little is known about the network of interactions controlling RORγt expression in pathogenic Th17 cells that develop in the absence of TGFβ1 signaling. Paradoxically, despite the capacity of TGFβ1 to induce optimal RORγt expression during Th17 development, Th17 cells grown in presence of TGFβ1 can also be pathogenic, as they retained the capacity to cause inflammation in a model of chronic colitis.⁶ It is likely that IL-23 induces T-bet in Th17 cells by reducing RORγt expression in inflammatory conditions.⁶² Alternatively, nonpathogenic Th17 cells might lose RORγt expression during colitis, converting them into the pathogenic Th1-like Th17 phenotype with a dominant Th1 program.

Role of RORγt-Expressing Th17 Cells in Intestinal Inflammation

The inflammatory microenvironment in the intestine of IBD patients induces the generation of specific CD4⁺ Th-cell subsets that have the capacity to transdifferentiate to related phenotypes. In inflammation of the intestine, proinflammatory cytokines such as IL-1β, IL-6 and IL-23 break down the immune tolerance and promote the generation of pathogenic Th17 and Th1 cells.¹⁸ Two independent studies have demonstrated an abundant presence of IL-17 in the gut of IBD patients. Enhanced levels of *IL17A* and *IL17F* transcripts have been found in the intestinal mucosa of CD patients compared to healthy controls.^{32,63} Moreover, a correlation between disease progression and level of IL-17 produced by peripheral blood mononuclear cells has been

Figure 2 Network-based interaction among various transcription factors that reciprocally modulate retinoic acid–related orphan receptor gamma t (ROR γ t) and forkhead box P3 (FOXP3) expression. *RORC* and *FOXP3* are reciprocally controlled by three layers of interactions—cytokines, cytokine-receptors, and cytokine-induced STAT transcription factors—along with the pioneer transcription factors such as interferon-regulatory factor (IRF)-4 and basic leucine zipper ATF-like transcription factor (BATF). Members of each of these three levels can potentially interact with each other, as well as with the members of the other levels, to determine the extent of ROR γ t and FOXP3 inductions. Similarly, ROR γ t and FOXP3 can interact with each other to determine the differentiation of CD4⁺ T cells toward the effector or regulatory phenotype. The analysis depicted in this figure was made in String software version 11 (ELIXIR, Cambridge, UK; <https://string-db.org>, last accessed April 25, 2020) using default settings. Results represent automated, unsupervised text mining of PubMed results. Two types of results were integrated into the network diagram: (1) proteins that share the same biological context (green) and (2) experimentally validated interprotein relationships (violet). Nodes represent genes, while edges show putative direct, indirect, or correlational relationships. HIF, hypoxia-inducible factor; TGF, transforming growth factor.

reported in UC patients.⁶⁴ MicroRNAs 22 and 155, which promote Th17 differentiation, are elevated during IBD.⁶⁵ A genome-wide association study in IBD patients similarly indicated a crucial role of Th17 cells in IBD pathogenesis.⁶³ In accordance with an expansion of Th17 cells during colitis, the *RORC* transcript level is elevated in the colons of both UC and CD patients.⁶⁶ Data mining using the single-nucleotide polymorphism–based approach showed that *RORC* single-nucleotide polymorphism (rs4845604) was associated with CD and UC.⁶⁷ Additionally, a genome-wide association study in IBD patients identified several polymorphisms in genes associated with the induction of ROR γ t, such as *STAT3* and *IL23R*.⁶⁸ Taken together, these observations support the involvement of the ROR γ t in IBD pathogenesis. Experimental studies have indicated that the co-expression of T-bet along with ROR γ t, rather than an the expression of ROR γ t alone, enables Th17 cells to become pathogenic.^{6,33,34} Many autoimmune disorders, including IBD, have been associated with IFN γ and IL-17 co-producing Th17 cells at the site of inflammation. However, the role of ROR γ t in the development of pathogenic potential in Th17 cells is unclear. Of note, Th17 cells overexpressing ROR γ t can repress T-bet expression.⁶⁹ Therefore, the gradual loss of ROR γ t expression allows the Th17 cells to become more permissive to T-bet expression. The expression of receptors for specific cytokines such as IL-23 likely diverts the Th17 cells toward a Th1 pathway, with concomitant acquisition of T-bet during inflammation. Indeed, IL-23 appears to play a fundamental role in the terminal differentiation and expansion

of pathogenic Th17 cells. During the time of its discovery, IL-23 was regarded as a sibling of IL-12 with shared IL-12p40 subunit.⁵⁸ IL-23 produced by innate cells of the intestine promotes transdifferentiation of Th17 to Th1-like Th17 cells by up-regulating T-bet, through a STAT4-dependent pathway.³³ IL-23R polymorphism has been genetically linked to human IBD.⁷⁰ TGF β 3 is one of the downstream mediators of IL-23 signaling that mediates the pathogenic function of Th17 cells.⁷¹ IL-23 enhances the expression of, and maintains the endogenous level of, TGF β 3 in Th17 cells. Whereas TGF β 1 is necessary for optimal ROR γ t expression in nonpathogenic Th17 cells, TGF β 3, despite a comparable ROR γ t-inducing capacity, encourages pathogenic Th17 development by promoting high T-bet. As TGFs β 3 and β 1 both signal through the same receptor TGF β RII, how TGF β 3 selectively mediates the pathogenic functions in Th17 cells is not clear.

Further characterization of pathogenic versus nonpathogenic Th17 subsets is required for specifically targeting the pathogenic Th17 cells. Similar to Th17 cells, the role of ROR γ t in IBD is not fully understood despite the fact that ROR γ t-null T cells failed to induce colitis in an adoptive transfer model of experimental colitis.⁷² Pharmacologic inhibition of Ror γ t provided therapeutic benefit in a mouse model of intestinal inflammation by reducing the frequency of Th17 cells.⁷³ However, clinical trials of the front-runner ROR γ t inhibitors were either discontinued or suspended recently due to a lack of efficacy along with safety concerns.⁷⁴ A spectrum of theories surrounds this intriguing subject. It has been shown that ROR γ t

and Th17 cells play a beneficial role in IBD, as targeting Th17 cells worsened disease outcome in CD patients. It has also been indicated that the pathogenic functions of Th 1 and 17 subsets are disease specific, as the frequency of IFN γ -secreting T cells in the peripheral blood was correlated with disease severity in CD but not UC.^{64,66} In the colon of UC patients, there was a significant increase in IL17 mRNA in CD4⁺ T cells, while IFNG was increased in CD. The reason for the selective development of pathogenic populations of Th 1 and 17 cells in the colons of CD and UC patients, respectively, is ambiguous. One of the plausible explanations is the heterogeneity of the Th17 population that consists of two subpopulations: stable Th17 cells and Th1-like Th17 cells that could transition to Th1 in chronic inflammation. Alternatively, the difference in Th17 phenotypic stability might be contingent on the expression levels of ROR γ t and IL-23R. While Th17 cells with low IL-23R and high ROR γ t expressions play a tissue-protective role by resisting T-bet acquisition, those with high IL-23R and low ROR γ t expressions might readily gain T-bet and promote tissue inflammation. This specific hypothesis needs to be fully addressed to ascertain the role of ROR γ t in intestinal homeostasis and inflammation. However, a simple interpretation of the functions of ROR γ t became more complicated after the discovery of colonic ROR γ t-expressing Tregs.

Role of ROR γ t-Expressing Treg Cells in Intestinal Inflammation

Tregs are suppressors of immune response and the primary inducers of peripheral tolerance. Mutation in the human *FOXP3* gene leads to spontaneous multiorgan inflammation, including severe intestinal inflammation.⁷⁵ According to their developmental origin, Tregs are divided into two subpopulations: tTregs, which emerge from the thymus, and peripheral Tregs (pTregs), which are induced in the secondary lymphoid organs in the periphery.

tTregs constitutively express CD25, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), glucocorticoid-induced TNFR-related protein (GITR), and FOXP3 and play a crucial role in maintaining homeostasis within the body by identifying self-antigens and preventing inflammatory responses to chronic inflammatory stimuli.⁷⁶ The differentiation of tTregs is dependent on IL-2-, STAT5-, and TGF- β -driven signaling pathways. On the other hand, pTregs can be generated by the activation of FOXP3⁻ naïve CD4⁺ T cells by the resident tolerogenic DCs of the intestine.⁷⁷ In homeostasis, in addition to secreting TGF β 1 and IL-10, tolerogenic intestinal DCs produce RA in response to commensal antigens and promote the differentiation of naïve CD4⁺ T cells into FOXP3⁺ Tregs. TGF- β , RA, and short-chain fatty acids produced in the immunosuppressive environment promote pTreg development. Similar to Th17 cells, the role of TGF- β is essential for pTreg development.¹⁵ However, for tTreg development, the necessity of TGF β signaling is disputed.⁷⁸ pTregs and tTregs differ in their developmental aspects, including epigenetic regulation and

stability. They have distinct regulatory functions due to the difference in their developmental origin and TCR repertoires.⁷⁹ Despite similarity in their function, their roles are often nonredundant. tTregs alone are not sufficient for the maintenance of intestinal immune homeostasis. The differentiation of naïve T cells into pTregs is required for the prevention of chronic colitis, indicating its nonredundant role that complements tTregs.⁸⁰ While it has been difficult to classify pTregs, at least three groups of tTregs have been identified: Tr17, T-bet⁺ Tregs, and GATA3⁺ Tregs. T-regulatory 17 (Tr17) cells express IL-10 and IL-17 and are derived from FOXP3⁺ tTregs exposed to IL-6/STAT3 signaling in an antigen-specific manner.³⁸ Tr17 cells express ROR γ t and show immunosuppressive activity toward antigen-specific effector Th17 cells. Similar to Tr17, T-bet⁺ Tregs are phenotypically stable and regulate Th1-induced inflammation.⁸¹ Treg-specific GATA3 deletion also results in intestinal pathology with excess Th2 cytokine production from effector T cells.⁸² In mice with *Gata3*^{-/-} Tregs, defects in peripheral homeostasis were caused by an impaired suppressive response.⁸³ The transcriptomic difference among these tTreg subsets beyond their markers has not been elucidated. How the expression of ROR γ t, T-bet, or GATA3 in tTregs endows them with the capacity to suppress specific effector populations is not clear. Proinflammatory cytokines that promote differentiation of a specific effector CD4⁺ Th subset might also act on tTregs to induce the corresponding master regulators along with FOXP3. For example, an inflammatory milieu marked with a high IL-12 level will promote Th1 differentiation and induce T-bet expression in tTregs. Co-expression of effector cell-associated TF might also influence the homing properties of tTregs and enable them to co-localize with the effector cell population. This phenomenon poses the vital question of how tTregs exert a suppressor response without transdifferentiating to an effector phenotype. In cancer, Tregs expressing IFN γ have compromised suppressor function that promotes antitumor immunity. But in infectious-disease models, T-bet⁺ Tregs are a potent suppressor of the immune response.⁸¹ How the diverse Treg populations function in a context-dependent manner is still unclear and remains an area of active investigation.

In addition to Tr17 tTregs, ROR γ t is co-expressed in intestinal pTregs, together with FOXP3.^{9,10} ROR γ t⁺ Tregs are not found in the thymus and do not express markers that characterize tTregs. ROR γ t⁺ Tregs also differ from Th17 cells in that they do not express Th17 signature cytokines (IL-17A and IL-17F). In mice with Ror γ t deficiency in Foxp3-expressing Tregs, severe oxazolone-induced colitis and trinitrobenzene-sulfonic acid-induced colitis developed.^{9,10} The adoptive transfer of ROR γ t⁺ Tregs in the T-cell-transfer model of chronic colitis revealed a higher suppressive capacity compared to that in FOXP3⁺ROR γ t⁻ Tregs.³⁵ This observation was further confirmed by their capacity to suppress the proliferation of activated Th17 cells *in vitro*. An epigenetic study has revealed significant demethylation at Treg-specific signature genes such as *FOXP3*, *CTLA4*, *TNFRSF18*, *IKZF4*, hinting that these cells have a stable regulatory profile.³⁵ This finding may be

surprising on several levels. ROR γ t typically promotes the differentiation of Th17 cells that are associated with tissue inflammation in chronic intestinal inflammation. How the same TF regulates the function of two disparate subsets with opposing functions is puzzling. As with many colonic CD4⁺ T cells, ROR γ t⁺ Tregs are predominantly induced by the intestinal microbiota. In germ-free mice, the frequency of ROR γ t⁺ Tregs was significantly reduced. These findings demonstrate that microbiota-induced ROR γ t⁺ Tregs are involved in controlling intestinal inflammation. Moreover, RA favors the development of ROR γ t⁺ Tregs.⁹ Notably, signaling mediated by IL-6 and IL-23 is required for their optimal differentiation.⁹ How these proinflammatory cytokine-mediated signaling pathways promote the regulatory function of this subset remains unknown. The incapacity of ROR γ t⁺ Tregs to produce IL-17 has been attributed to the B lymphocyte-induced maturation protein (BLIMP)-1 TF, its expression in intestinal pTregs has been described as crucial for suppressing Th17-associated cytokines and restricting the proinflammatory potential of ROR γ t⁺ Tregs.⁸⁴ In the absence of BLIMP-1, ROR γ t⁺ Tregs lost suppressor function and promoted intestinal inflammation, as BLIMP-1 prevented IRF-4 binding to the IL17A/IL17F locus. Taken together, these observations suggest that ROR γ t expression in Tregs enhances the suppressor function of colonic Tregs. That ROR γ t enhances the Treg suppressor function raises several questions about the function and stability of ROR γ t⁺ Tregs: What is the central role of ROR γ t in promoting the suppressor function of pTregs?; How do antagonistic TFs such as ROR γ t and FOXP3 cooperate to modulate the regulatory function of pTregs?; How does ROR γ t differentially regulate pTreg versus Th17 cell development?; and Why is IL-6/STAT3 signaling, which opposes Treg development, necessary for ROR γ t⁺ Treg development and function? The discovery of ROR γ t⁺ Tregs contradicts the concept that FOXP3 and ROR γ t are antagonistic TFs, a notion stemming primarily from the findings from *in vitro* experiments.¹¹ The existence of ROR γ t⁺ Tregs attests to the fact that the two TFs can act collaboratively, consistent with overlap between their chromatin-binding sites.⁸⁵ Contrary to the inference drawn from experimental models, patient-based data contradict the stability and suppressive function of ROR γ t⁺ Tregs. ROR γ t⁺ Tregs in IBD patients showed low suppressive capacity and elevated the expression of proinflammatory cytokines.⁸⁶ Patients with IBD have been reported to have a significantly higher prevalence of IL-17-producing ROR γ t⁺ Tregs compared with that in healthy control subjects. Importantly, the capacity of IL-17⁺ ROR γ t⁺ Tregs to suppress autologous T-cell proliferation was reduced by approximately 60% in patients with IBD. Although it is difficult to reconcile the experimental and patient-derived data, it is plausible that ROR γ t⁺ Tregs are not a stable population and can readily transition to IL-17-producing Tregs with reduced suppressor function. The dynamic developmental relationship between suppressor Tregs and

effector Th17 cells could play a vital role in harmonizing intestinal homeostasis with inflammation.

Role of ROR γ t in Treg-Th17 Developmental Axis in Intestinal Homeostasis and Inflammation

What makes the Treg-Th17 axis intriguing is that the two lineages have interlinked development pathways due to the common requirement of TGF- β , but with reciprocal inhibition of the key TFs ROR γ t and FOXP3 (Figure 3). The most substantive evidence of their developmental overlap came from a lineage-tracing study showing that a significant proportion of ROR γ t⁺ Th17 cells expressed FOXP3 early in the development, with the extinction of FOXP3 during late developmental stages.¹¹ The study showed that CD4⁺ T cells receiving a TGF- β signal gained the potential to develop into either Treg or Th17 lineage. That RA could reciprocally modulate Treg and Th17 differentiation is also evidence of their linked development.⁸⁷ During pTreg development, FOXP3 induction by TGF β restrains Th17 differentiation by inhibiting ROR γ t. IL-6 inhibits FOXP3, which allows ROR γ t to be dominantly expressed, leading to Th17 development.¹¹ However, in the complex intestinal environment, where RA is abundant, the IL-6-driven STAT3 level falls short of inducing Th17 development from pTregs.⁴⁰ For a long period after the discovery of Th17 cells, it was not clear how Th17 cells are copiously found in the intestine despite the abundance of RA that potently inhibits Th17 development and induces FOXP3⁺ Tregs. It was later revealed that colonic Tregs possess dormant Th17 programming during intestinal homeostasis.⁴⁰ By repressing suppressor of cytokine signaling protein (SOCS)-3, a potent inhibitor of STAT3, IL-1 signaling assists in the amplification of the IL-6-driven STAT3 level to redirect the developmental programming of Treg toward Th17 differentiation during intestinal inflammation.⁴⁰ As RA predominantly boosts IL-2-driven STAT5 signaling to stabilize FOXP3 expression, the action of IL-1 signaling results in rapid shifting of the dominant RA-driven STAT5-modulated genetic programming in Tregs toward a STAT3-driven Th17 developmental program. In mice with bacteria-induced colitis, this alteration in the genetic programming of Treg by IL-1 resulted in the emergence of host-protective Th17 cells in the inflamed colonic mucosa.^{40,41} Therefore, transdifferentiation of colonic Tregs to effector Th17 cells requires IL-1 signaling for opposing RA-mediated repression of Th17 differentiation. The conversion of colonic Tregs to Th17 cells provides an example of a symbiotic developmental relationship between the Tregs and Th17 cells that modulate the balance between intestinal homeostasis and inflammation. Other studies have highlighted additional mechanisms for the cooperative relationship between Th17 and Treg cells. IL-2 is a cytokine that can reciprocally modulate Treg and Th17 development.⁸⁸ Studies have shown that Tregs promote Th17 development by consuming the Th17 cell-inhibitory cytokine IL-2 and simultaneously enhancing their own development.^{89,90} There is other

Figure 3 Cytokines and transcription factors that regulate the induction of retinoic acid–related orphan receptor gamma t (RORγt). T-cell receptor–activated CD4⁺ T cells stimulated with IL-6 and transforming growth factor-beta (TGF-β) lead to optimal induction of RORγt. Engagement of IL-6 with IL-6R induces STAT3 phosphorylation that binds to the *RORC* promoter to induce its transcription. TGFβ can induce both forkhead box P3 (FOXP3) and RORγt expressions via SMAD-driven pathways, but also limits the expression of genes encoding T-box transcription factor 21 (T-bet), GATA binding protein 3 (GATA3), and other Th1- and Th2-associated transcription factors. Although retinoic acid (RA) does not have a direct role in RORγt induction, it enhances IL-2/STAT5 signaling to stabilize FOXP3-mediated regulatory T-cell (Treg) programming and oppose RORγt induction. IL-1/IL-1R1 signaling plays a unique role by repressing *SOCS3* transcription, thereby amplifying STAT3 level, which competes with RA-driven STAT5 to reciprocally regulate Treg and Th17 differentiation. Several transcription factors reciprocally modulate *RORC* and *FOXP3* gene expressions. Pioneer transcription factors such as interferon-regulatory factor (IRF)-4 and basic leucine zipper ATF-like transcription factor (BATF) act on the chromatin to change the accessibility of specific Th17-associated genes, including *RORC*, while simultaneously antagonizing FOXP3-mediated Treg programming. Similarly, runt-related transcription factor (RUNX)-1 influences Th17 differentiation by inducing RORγt expression, but interacts with FOXP3 to down-regulate its expression. Hypoxia-inducible factor (HIF)-1α, a key metabolic sensor, transcriptionally activates RORγt, but binds to FOXP3 to target it for proteasomal degradation. RA, retinoic acid; RAR, RA receptor.

mechanistic evidence suggesting that Th17 cells and Tregs could emerge through a linked pathway. The cytokine-induced TFs STAT3 and STAT5 have been reported to reciprocally promote and inhibit Th17 and Treg development.^{40,88} The runt-related TF 1 protein (RUNX1) has been reported to influence Th17 differentiation by promoting RORγt induction and down-regulating FOXP3 expression.⁹¹ In another study, hypoxia-inducible factor (HIF)-1α, a key metabolic sensor, formed a tertiary complex with RORγt to enhance Th17 development through direct transcriptional activation of RORγt. At the same time, HIF-1α opposed Treg development by binding to FOXP3 and targeting it for proteasomal degradation.⁹² Taken together, these findings suggest that the developmental linkage between these two lineages is such that the suppression of one encourages the development of the other. The basis of this correlation is dependent not only on the developmental overlap but also on common cytokine requirements and the plasticity of Tregs.

As the evidence supporting the transdifferentiation from Tregs to Th17 accumulates, examples of the conversion of Th17 cells to Tregs, that is, from an effector to a regulatory phenotype, are uncommon. However, it has been demonstrated that in homeostatic conditions, intestinal Th17 cells cease IL-17 production, and a percentage of ex-Th17 cells gain

regulatory phenotype similar to the type 1 Tregs (Tr1s) (CD4⁺FOXP3[−]IL-10⁺ cells).⁹³ The reprogramming of Th17 cells into Tr1s is irreversible, and the converted Tr1 cells demonstrate anti-inflammatory properties by averting Th17-mediated colitis. A similar mode of transdifferentiation of Th17 cells to FOXP3-expressing Tregs has not been shown. If discovered, it will be a seminal discovery, as it would substantiate that effector T cells can be reverse-programmed to Tregs. Such a finding will have an enormous clinical application in the treatments of autoimmune diseases and cancer.

Role of Gut Microbiota in the Induction of RORγt-Expressing CD4⁺ T cells

The microbiota consist of thousands of bacterial species, eukaryotic microorganisms, archaea, viruses, and bacteriophages.⁹⁴ The intestinal microbiota continually interact with the host mucosa and regulate the gut immune response. The epithelial cells, as well as the innate immune cells of the intestine, are equipped with the pattern-recognition receptors, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)–like receptors (NLRs), and retinoic acid-inducible gene I–like

Figure 4 Microbiota control the induction of the intestinal regulatory T cell (Treg)–Th17 axis. Microbiota can direct the differentiation of Th17 as well as Treg cells. Segmented filamentous bacteria (SFB) induce Th17 through serum amyloid A (SAA). SFB adhere to intestinal epithelium to induce SAA production, which promotes the production of IL-23 and IL-6 from antigen-presenting cells to favor intestinal Th17 development. Extracellular ATP derived from the microbiota also acts to induce Th17 cells. Interaction of β -glucans produced by fungi with dectin-1 induces dendritic cells (DCs) to produce IL-23, which assists in Th17 development. Many pathogen-associated molecular patterns of the microbes can stimulate DCs and macrophages to induce production of proinflammatory cytokines. Microbiota can likewise direct the differentiation of Tregs. Several commensal microbes such as *Clostridia* are unique inducers of colonic Tregs in that they generate short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, which are metabolites of the bacterial fermentation process. SCFAs can promote the accumulation of Tregs by affecting T cells, DCs, and intestinal epithelial cells via diverse mechanisms, including inducing the secretion of transforming growth factor-beta (TGF- β). Polysaccharide A derived from the human commensal bacterium *Bacteroides fragilis* also induces Tregs. Additionally, *Bifidobacterium adolescentis*, one of the human symbiont bacterial species, has been recognized as a potent inducer of Th17 cells as well as retinoic acid–related orphan receptor gamma t (ROR γ t⁺) Tregs. FOXP3, forkhead box P3; PAMP, pathogen-associated molecular pattern.

receptors (RLRs), which continuously sample the conserved molecules of the microbes, known as pathogen-associated molecular patterns (PAMP). During homeostasis, how commensal bacteria fail to provoke an effector CD4⁺ T-cell response is not clearly understood. The observation that commensal bacteria species, such as *Clostridium*, increase the frequency of pTreg cells raises the possibility that bacterial products may favor the preferential expansion of pTregs.^{95,96} These commensals might induce the production of the immunosuppressive molecules from intestinal epithelial cells and innate immune cells.⁹⁷ During infection or inflammation, pathogenic microbial molecules bind to pattern-recognition receptors, leading to the induction of cytokines from antigen-presenting cells that result in the differentiation of effector CD4⁺ T-cell subsets, leading in turn to the loss of Treg-mediated tolerance.

It has been challenging to identify which of the species or genera of the gut microbiome are the dominant inducers of Th17 or Treg cells and contribute to intestinal inflammation or homeostasis. A number of studies have suggested a role of the microbiota in influencing ROR γ t level in the Th17 and Treg cells of the gut⁹⁸ (Figure 4). A study in germ-free mice showed diminished populations of ROR γ t⁺ Th17 and ROR γ t⁺ Treg cells.⁹ Mice treated with broad-spectrum antibiotics, which eliminate the intestinal bacteria, had reduced Th17 and

ROR γ t⁺ Treg frequencies in the lamina propria.^{3,10} Recolonization of germ-free mice with fecal homogenates containing a diverse microbiome induced the generation of Th17 and ROR γ t⁺ Treg cells in the intestine.^{99,100}

The induction of specific effector or regulatory Th subsets by the microbiota depends primarily on two factors: location and the species of bacteria. Different compartments of the intestine are colonized with different species of commensal bacteria.¹⁰¹ In conventionally raised mice, the elevated frequency of Th17 cells is found in the lamina propria of the ileum and colon (higher bacterial load), as opposed to the duodenum, and jejunum (lower bacterial load).¹⁰² Various bacterial species play a role in the induction of Th17 and Treg cells in homeostasis. SAA proteins secreted by segmented filamentous bacteria–activated epithelial cells induce IL-6, IL-23, and IL-1 β , from DCs, which are crucial for Th17-cell differentiation.^{99,103} Other bacterial products that drive intestinal Th17 cells have also been elucidated. Flagellin, a bacterial polymeric protein of flagellum, plays a role in regulating Th17-cell differentiation by activating DCs through TLR5 to secrete IL-23.¹⁰⁴ TLR recognition of intestinal bacteria by macrophages drives IL-1R signaling in resident T cells that assist in the homeostatic differentiation of Th17 cells.¹⁰⁵ Extracellular ATP derived from the microbiota also activates Th17 differentiation.¹⁰⁶ Other than bacteria, fungal β -glucans, through dectin-

1, selectively induce DCs to produce IL-23, which participates in Th17 differentiation/maturation.¹⁰⁷

On the other hand, Tregs are abundant in intestinal lamina propria, and their accumulation and induction depend on the commensal microorganism *Clostridium*, an exceptional inducer of colonic Tregs.^{96,108} The colonization of *Clostridium*-induced intestinal epithelial cells to produce TGF- β robustly increases the frequency of Tregs in the colon.⁹⁶ Likewise, bacterial polysaccharide A derived from the commensal bacterium *Bacteroides fragilis* could protect mice from T-cell-mediated colitis in a TLR2-dependent manner through the induction of Tregs.⁷⁹ Recent studies have shown that short-chain fatty acids and other gut commensal-derived bacterial-fermentation metabolites induce colonic Treg differentiation through multiple pathways. Short-chain fatty acids, particularly butyrate, directly induce extrathymic Treg accumulation by inhibiting histone deacetylases, which constitutively suppress histone H3 acetylation at the *FOXP3* enhancer.^{109,110} In addition to activating T cells, butyrate can modulate the function of DCs by inhibiting the induction of inflammatory cytokines to favor Treg differentiation.¹⁰⁹ Short-chain fatty acids could induce TGF- β 1 production in intestinal epithelial cells and directly contribute to the differentiation of colonic Tregs.⁹⁵

The role of microbiota in the induction of ROR γ t in CD4⁺ T cells is an emerging area of research. Strains of *Bifidobacterium* or probiotics containing *Bifidobacterium* (naxabiotic) are potent inducers of both ROR γ t⁺ Th17 cells and Helios[−] ROR γ t⁺ Treg cells, without causing any intestinal inflammation.¹¹¹ Moreover, the relative proportion of microbiota-induced Th17 cells compared to ROR γ t⁺ Tregs could be predictive of human IBD. Compared to those in healthy donors, the transfer of microbiota from IBD patients into germ-free mice showed increased numbers of intestinal ROR γ t⁺ Th17 cells, and decreased numbers of ROR γ t⁺ Tregs with exacerbated disease.¹⁰⁰ These findings suggest that the difference in the composition of the microbiome could increase susceptibility to intestinal inflammation due to a varying capacity to induce Th17 cells versus ROR γ t⁺ Tregs.

Taken together, these findings indicate that various microbial species could differentially induce Th17 and Treg generation by modulating diverse molecular pathways. The identification of the specific metabolites used by the microbiota to alter the molecular mechanisms guiding effector versus regulatory T-cell differentiation will provide vital information on how the gut microbiota regulate the immune response in homeostasis and inflammation, and may offer potential therapeutic options for treating human IBD.

Targeting Th17 Cells and ROR γ t in the Therapy of IBD

The pathogenesis of IBD depends on several factors, such as environmental factors, type of immune response, genetic

background, and the composition of gut microflora.²³ The strategy for IBD therapy is aimed at suppressing the uncontrolled inflammatory response. Currently, a new group of drugs, biologics and biosimilars, which are partly or completely derived from animal or human sources, is used in the treatment of IBD patients.¹¹² Monoclonal TNF α antibodies (infliximab, adalimumab, certolizumab, and golimumab), integrins (vedolizumab and natalizumab), and an antagonist of IL-12 and IL-23 (ustekinumab) have been approved for use in IBD therapy. Although TNF α antibodies are considered as first-line biologics in the treatment of moderate to severe CD, both vedolizumab and ustekinumab have been reported to be more effective in the therapy of naïve IBD patients than in TNF α treatment-unresponsive patients.¹¹³ However, antibody against the shared subunit (p40) of IL-12/IL-23 showed limited effect in the treatment of CD patients resistant to anti-TNF α therapy. Antibodies to the p19 subunit of IL-23 (risankizumab, brazikumab) have shown promising effect, with reduced inflammation score, in the treatment of anti-TNF α therapy-resistant CD patients, possibly by allowing IL-12-mediated Th1 immune response against intracellular pathogens. Multiple reports have highlighted that Th17 cells are associated with IBD.^{32–34,63,64,66} However, their role in IBD pathogenesis remains unresolved. Although targeting of Th17 cells has been efficacious in psoriasis, it has been reported to be harmful in CD patients.¹¹⁴ Blocking of IL-17A (secukinumab and ixekizumab) or IL-17RA (brodalumab) has been reported to worsen colitis outcome.^{7,8} Neutralization of IL-17A, either by antibody treatment or by genetic ablation, has been associated with aggravated intestinal inflammation in the dextrose sodium sulfate-mediated colitis model.¹¹⁵ There could be multiple reasons for the failure to treat IBD patients by blocking IL-17. First, IL-17 can play a protective role in colitis. Second, IL-17 is also produced from innate immune cells, such as invariant natural killer T cells (iNKT) and lymphoid tissue inducer-like cells (LTi), which can be crucial for protection by ensuring rapid host defense and maintenance of barrier function.¹¹⁶ Additionally, in an acute model of colitis, a protective role of IL-17A produced by ROR γ t⁺ $\gamma\delta$ T cells has been indicated in terms of maintenance, protection, and the repair of epithelial barrier in intestinal mucosa.¹¹⁷

As the master TF of Th17 cells, ROR γ t has been the focus of new drug discovery in many autoimmune disorders, including psoriasis, IBD, rheumatoid arthritis, and multiple sclerosis.⁷⁴ In 2016, several companies disclosed their interest in developing small-molecule ROR γ /ROR γ t inhibitors. There are eight ROR γ t inhibitors in active clinical trials (Phase I/II), mainly in psoriasis. Unfortunately, most of the ROR γ t inhibitors have failed to fulfill expectations.⁷⁴ Similar to the failure in targeting IL-17 to treat IBD patients due to nonspecific targeting of multiple cellular sources of IL-17, targeting ROR γ t is also likely to target multiple cell types, as it is expressed in both innate and adaptive cells, including invariant natural killer T cells, lymphoid tissue-inducer cells, natural killer p46 cells, Th17 cells, and Tregs. Now it is clear that ROR γ t-expressing

FOXP3⁺ Tregs constitute a separate regulatory T-cell subset that maintains immune tolerance in the intestine. Therefore, the scenario has become much more complex, as targeting ROR γ t will also affect the suppressor population of intestinal Tregs that will contribute to the aggravation of IBD.

Conclusions and Perspectives

Historically, a specific CD4⁺ Th subset has been associated with a unique lineage-specifying master TF that guides Th-cell differentiation to a terminally differentiated, or fixed, phenotype. Now it appears that the master TFs often act in a combinatorial manner in various subsets of CD4⁺ T cells, resulting in a broad assortment of effector and regulatory phenotypes that can often interconvert. One of the serious disagreements with the fixed nature of T-helper cell differentiation comes from the relationship between pTregs and Th17 cells. Despite the contrasting functions of regulatory pTreg and effector Th17 cells, their origin of development is largely overlapping. The optimal development of both subsets requires TGF- β signaling. TGF- β induces both FOXP3 and ROR γ t, the master regulators of Treg and Th17, respectively. However, the induction of ROR γ t by TGF- β does not drive the differentiation program to bona fide Th17 cells that produce the cytokine IL-17. For a long time, FOXP3 was considered an inhibitor of ROR γ t that prevented its transcriptional competence by physically interacting with ROR γ t—an observation primarily made in *in vitro* cultured T cells. It was proposed that in inflammatory conditions, proinflammatory cytokines, such as IL-6 and IL-23, potently suppress FOXP3, which liberates ROR γ t from the grip of FOXP3. After the recent discovery of a novel regulatory cell type that co-expresses FOXP3 and ROR γ t and possesses an enhanced regulatory function in the intestine, a dent is made in the previous perception. Now, it is acknowledged that ROR γ t and FOXP3 could have a collaborative interaction in which the expression of both TFs enables colonic Tregs to become more suppressive. Nearly half of the colonic Tregs stably express both FOXP3 and ROR γ t. The discovery of colonic ROR γ t⁺ Tregs came with several caveats. First, because of its expression in proinflammatory Th17 cells, ROR γ t has become an attractive clinical target of treatment in a wide variety of autoimmune diseases, including IBD. It is now known that targeting ROR γ t in the treatment of IBD might have both pro- and anti-inflammatory consequences, as it will also target the colonic Tregs and compromise the immune tolerance of the intestine. Second, the discovery suggested that the long-regarded antagonistic master TFs can collaborate to bestow a novel functionality on Tregs, as deleting ROR γ t from FOXP3-expressing Tregs makes the host more susceptible to intestinal inflammation, including colitis. Third, the existence of ROR γ t⁺ Tregs changes the notion of ROR γ t as a potent transcriptional regulator of IL-17, as the induction of IL-17 is not readily observed in colonic ROR γ t⁺ Tregs, even in inflammation. These observations raise several

critical questions: How does ROR γ t contrastingly regulate the effector function of Th17 cells and the suppressor function of Tregs? Is there a component of microbiota that can specifically induce ROR γ t⁺ Tregs without inducing Th17 cells? How can the enhanced immunosuppressive function of ROR γ t⁺ Tregs be incorporated into IBD therapy? The growing association of IBD in the etiology of multiple diseases, including cardiovascular disease and colon cancer, makes addressing these questions a priority. The answers will provide insight into the selective manipulation of ROR γ t to target ROR γ t⁺ Th17 cells as opposed to ROR γ t⁺ Tregs.

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