Mini Review

Mechanism of Th17 cell differentiation in the intestinal lamina propria

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IL-17-producing Th17 cells are a subset of CD4$^+$ T cells that have been implicated in various chronic inflammatory and autoimmune diseases. Th17 cells selectively and constitutively reside in the intestinal lamina propria. Recent studies using germ-free mice indicate that the development of lamina propria Th17 cells are dependent on the stimulation by intestinal commensal bacteria. In this review, we summarize the recent advances in our understanding of the mechanisms of intestinal Th17 synthesis in mice. We also propose a model in which commensal bacteria-derived factors, including ATP, activate a unique subset of dendritic cells, leading to the differentiation of Th17 cells in the intestinal lamina propria *in situ*.

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Introduction

The intestinal mucosa is continuously exposed to a large number and diverse array of commensal bacteria and food antigens. The intestinal mucosa is also a major site for the invasion of pathogenic bacteria, viruses and fungi. Thus the mucosal immune system must enforce tolerance towards commensal bacteria and food antigens and simultaneously remain reactive to potentially pathogenic microbes. Breakdown of this balance results in inflammatory bowel diseases (IBDs). To maintain this balance, the intestinal mucosal immune system uses an arsenal of unique and diverse immune cell populations, including immunoglobulin A (IgA)-producing plasma cells, $\gamma\delta$T cells and CD4$^+$ T cells dominated by a Th1 or Th2 phenotype. In addition, recent studies have revealed that CD4$^+$ T cells in the intestinal mucosa comprise significant numbers of IL-17-producing cells ("Th17 cells")[1-3].

Th17 cells are characterized by their production of IL-17A, IL-17F, IL-21 and IL-22[4,5]. These Th17 cytokines act on a broad range of immune and non-immune cells and regulate granulopoiesis, neutrophil recruitment and induction of antimicrobial peptides. Indeed, Th17 cells play critical roles in the regulation of host defense against a variety of infections by fungi and bacteria, such as *Klebsiella pneumoniae, Streptococcus pneumoniae and Citrobacter rodentium*[2,6,7]. However, depending on the circumstances, Th17 cells may serve as an auto-reactive inflammatory cell population and lead to severe autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis and IBDs[5,8]. Thus the differentiation of Th17 cells should be strictly controlled.

Importantly, Th17 cells are particularly abundant in the intestinal mucosa and are present even in the steady state. Here, we focused on the cellular and molecular mechanisms of Th17 differentiation in the intestinal mucosa, and discuss the role of dendritic cells (DCs) in sensing factors derived from intestinal commensal bacteria for Th17 generation.

Th17 cell differentiation

The differentiation of Th17 cells from naïve $CD4^+$ T cells requires multiple cytokines, including TGF-β and IL-6[2, 5, 8-11]. TGF-β induces the expression of the retinoic acid-related orphan receptor RORγt, which is known to be the master transcription factor of the Th17 lineage[1]. Despite its induction of RORγt, however, TGF-β alone is unable to initiate Th17 differentiation in vitro. This is because TGF-β also induces Foxp3, which is the essential transcription factor for the differentiation and function of regulatory T cells (Tregs)[12]. Foxp3 inhibits RORγt-directed IL-17 expression through binding to RORγt[13]. In the presence of IL-6 or IL-21, which activate STAT3, TGF-β-induced Foxp3 expression is greatly reduced and the RORγt level is further upregulated, favoring Th17 cell differentiation. Interferon regulatory factor 4 (IRF4) is also essential for Th17 cell differentiation[14]. IRF4 is induced by T cell receptor signaling and is required for the expression of RORγt. Thus a combination of multiple transcription factors, including RORγt, STAT3 and IRF4, regulates the differentiation program for the Th17 lineage.

It should be noted that the Th17 cells are not all the same, and different environments induce Th17 cells with different characteristics[15]. For example, the presence of ligands for aryl hydrocarbon receptor (AHR) affects the expression of IL-22, but not other Th17 cytokines[16]. Furthermore, although TGF-β and IL-6 are essential for the "initiation" of Th17 differentiation, they are not sufficient to induce fully inflammatory Th17 cells. During stimulation with TGF-β and IL6, Th17 cells express IL-21, which acts on Th17 cells in an autocrine manner[17,18]. IL-21 mediates the expansion of Th17 cells and, at the same time, upregulates the expression of IL-23 receptor via activation of STAT3[19]. As a result, the differentiating Th17 cells become responsive to IL-23. In the presence of the pro-inflammatory cytokine IL-23, Th17 cells further expand and mediate a complete Th17 response[15,19]. Indeed, despite the fact that the development of Th17 cells in *Citrobacter rodentium*-infected mice is promoted by TGF-β and IL-6, IL-23 is indispensable for the Th17 response that protects mice against *C. rodentium*-driven colitis[2]. In the presence of excess amounts of IL-23, Th17 cells become pathogenic and induce autoimmune diseases in mice. Indeed, a recent study used a T cell transfer model of experimental allergic encephalomyelitis (EAE) and showed that Th17 cells are expanded in the presence of IL-23, but not in the presence of TGF-β plus IL-6, and induce diseases in mice[15]. IL-23 has also been shown to mediate intestinal inflammation[20,21]. Moreover, in humans, it has been shown that variants of the IL23R gene are linked to susceptibility to IBDs[22].

Commensal bacteria-mediated Th17 differentiation

Th17 cells are present at high frequencies in the small and large intestinal lamina propria but not in the spleen, mesenteric lymph nodes or Peyer's patches of healthy mice housed in a specific-pathogen-free (SPF) environment[1,3,23]. In the small and large intestines, about 10% of $CD4^+$ cells are IL-17^+ cells, whereas only a very small proportion of $CD4^+$ T cells express IL-17 in extra-intestinal sites at steady state. The number of Th17 cells in the intestinal lamina propria increases with age, correlating with the development of intestinal microflora[3]. Furthermore, in germ-free mice or antibiotic-treated mice, the numbers of lamina propria Th17 cells are greatly reduced[3,23]. Interestingly, mice that are obtained from different commercial vendors have marked differences in the number of Th17 cells in their lamina propria: C57BL/6 mice from the SPF facilities of Taconic Farms have higher numbers of Th17 cells than those from the Jackson Laboratory[23]. The difference in the presence of intestinal Th17 cells is correlated with the presence of members of the cytophaga-flavobacter-bacteroides (CFB) phylum[23]. Thus it is likely that Th17 cells are induced in the lamina propria in response to specific components of the commensal microflora. It is of interest that, in contrast to the above notion, a recent report showed that commensal bacteria may inhibit Th17 differentiation via IL-25[24]. Therefore, further studies are needed to clarify these discrepancies.

The gut microbiota comprises approximately 1000 species of commensal bacteria, the component of which is shaped by the dynamic interaction with the host. It is now known that commensal bacteria play an integral role in the development of the optimal mucosal immune system by providing various components of immune stimulators. The microbe-specific molecules (pathogen-associated molecular patterns: PAMPs) are recognized by pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). TLRs are membrane-bound receptors that recognize extracellular or endosomal PAMPs such as lipopolysaccharide (LPS), peptidoglycan, and bacterial DNA. MyD88 and

Trif are essential adaptor proteins that transduce TLR signaling. Two groups have independently shown that *Myd88* and *Trif* doubly-deficient mice had normal numbers of lamina propria Th17 cells in the small and large intestines, and concluded that Th17 development in the lamina propria is independent of TLR signaling, at least at steady state[3,23]. However, a possible caveat to this interpretation is that these mutant mice might have altered intestinal microflora. Indeed, it was shown that Myd88 deficiency changes the composition of the distal gut microbiota: *Myd88*$^{-/-}$ mice have a significantly lower Firmicutes/Bacteroidetes ratio compared with *Myd88*$^{+/-}$ mice[25]. Furthermore, another group showed that *Tlr9*-deficient mice have decreased numbers of lamina propria Th17 cells[26]. In addition, the differentiation of intestinal Th17 cells is also enhanced by a TLR5-dependent pathway, because flagellin activates TLR5^{+} lamina propria DCs to induce Th17 cell differentiation *in vitro*[27]. Thus to confirm the involvement of TLR signaling in Th17 differentiation, gnotobiotic studies using germ-free mice deficient for MyD88 or TLRs colonized with specific Th17-inducing bacteria need to be performed.

In addition to TLR ligands, bacteria have been shown to generate and secrete large amounts of extracellular ATP[28]. ATP is a well-known source of intracellular energy transfer; in addition, it also serves essential roles in extracellular signaling processes. Extracellular ATP binds to and activates the cell-surface ionotropic (P2X) and metabotropic (P2Y) purinergic receptors, which deliver intracellular signals via ion channels or G-proteins, respectively[29,30]. In the context of the immune system, extracellular ATP is known to modulate immune cell functions, such as phagocytosis, chemotaxis and cytokine production[29,30]. In particular, much attention has been focused on ATP signaling via the P2X7 receptor, which cooperates with NLRP3 (previously known as CIAS1 and NALP3) signaling and activates caspase-1 through the assembly of a cytosolic protein complex that is known as the "inflammasome"[31]. The formation of the inflammasome is required for conversion of the immature form of IL-1β to the mature form. Interestingly, it has been reported that a set of SNPs located in a regulatory region of NLRP3 are associated with Crohn's disease[32]. ATP is quickly degraded in the extracellular space by ATPases into ADP, AMP or adenosine forms. However, intestinal commensal bacteria seem to produce high amounts of ATP that are beyond the capacity of host ATPases. Indeed, the ATP concentrations in the intestinal luminal contents of SPF mice are very high, but are at very low levels in germ-free mice or antibiotics-treated mice[3]. Moreover, high ATP concentrations can be detected in the supernatant of in vitro cultured intestinal commensal bacteria. Addition of the culture supernatant of intestinal commensal bacteria markedly enhanced the differentiation of IL-17-expressing cells, and this Th17 differentiation was severely inhibited by the presence of the ATP degrading enzyme, apyrase. Furthermore, treating germ-free mice with ATP markedly increased the numbers of IL-17-producing CD4^{+} cells[3]. Thus extracellular ATP is one of the critical Th17-inducing factors produced by intestinal commensal bacteria.

In the context of the role of extracellular ATP in the immune regulation, it is interesting to note that the immune suppressive activity of Tregs is due, at least in part, to their capacity to hydrolyze extracellular ATP through the enzymatic activity of CD39 and CD73 expressed on their membrane[33,34]. CD39 is an ectoenzyme that hydrolyzes ATP/UTP and ADP/UDP to the respective nucleosides such as AMP. CD73 is a ecto-5'-nucleotidases that degrade extracellular nucleoside monophosphates to nucleosides (e.g., adenosine). Adenoine activates adenylyl cyclases by triggering the cognate Gs-protein coupled receptor A2a, which has a non-redundant role in the attenuation of inflammation and tissue damage *in vivo*. Thus, elucidating the entire picture of the regulation of the development of intestinal Tregs, Th17 cells and other types of cells by ATP and its metabolites ADP, AMP, and adenosine will provide valuable information toward our understanding of the complex system of intestinal mucosal immunity as well as the establishment of innovative ATP-targeted approaches for treating patients with IBDs.

Unique dendritic cells in the intestinal lamina propria for Th17 differentiation

It was previously believed that intestinal bacteria are taken up exclusively by M cells located in the follicle-associated epithelium of Peyer's patches. However, it is now known that commensal bacteria and their components are also directly taken up by CD11c^{+} DCs at mucosal sites. DCs in the intestine can extend their dendrites into the intestinal lumen and directly acquire luminal bacteria, a process that is dependent on the CX3C-chemokine receptor 1 (CX3CR1)[35,36]. Accordingly, *Cx3cr1*$^{-/-}$ mice exhibit defective luminal sampling by DCs and impaired resistance to *Salmonella typhimurium* infection[36], suggesting a crucial role for luminal sampling in the development of protective immune responses in the gut.

There are multiple subpopulations of DCs that differentially regulate the immune response depending on signals from microbes and microenvironments[37,38]. For example, a CD103 (also known as αE-integrin) -expressing DC population in the lamina propria preferentially promotes the generation and homing of

Tregs to the intestinal mucosa[39,40]. CD103$^+$ DCs express retinal dehydrogenases (RALDH) and produce high amounts of the vitamin A metabolite retinoid acid (RA). RA, produced by CD103$^+$ dendritic cells, mediates the expression of the gut-homing receptors on T cells and, at the same time, enhances Treg differentiation in the gut by cooperating with TGF-β. Another subset of lamina propria DCs that is positive for inducible nitric oxide synthase (iNOS) has been shown to express B cell activating factor (BAFF; also known as BLyS) and a proliferation-inducing ligand (APRIL), and promotes IgA class switching in B cells[41].

Because only a very small proportion of CD4$^+$ T cells express IL-17 in mesenteric lymph nodes or Peyer's patches, it is likely that Th17 cells are produced in the lamina propria *in situ* by lamina propria DCs activated by ATP, TLR ligands or other molecules derived from the commensal microflora. In this context, CD70^{high}CD11c$^+$ cells, which are selectively present in the intestinal lamina propria, express high levels of ATP sensors, P2X and P2Y receptors, and several genes including IL-6, IL-23, integrin-αV and integrin-β8 in response to ATP stimulation[3]. Integrin-αV and integrin-β8 are involved in the conversion of the latent form of TGF-β to the active form via triggering degradation of latency-associated protein (LAP) binding to TGF-β[42,43]. In the *in vitro* culture system, CD70^{high}CD11c$^+$ cells preferentially induce the differentiation of naïve CD4$^+$ T cells into Th17 cells[3]. Another report has shown that lamina propria CD11b^{high}CD11c$^+$ cells also preferentially induce Th17 cells *in vitro*[44]. Both CD70^{high}CD11c$^+$ cells and CD11b^{high}CD11c$^+$ cells express CX3CR1, a chemokine receptor that mediates the extension of cellular dendrites of dendritic cells between the tight junctions of epithelial cells to take up luminal bacteria. CD70^{high}CD11c$^+$ and CD11b^{high}CD11c$^+$ populations seem to, at least partially, overlap with each other, and both play a critical role in the Th17 differentiation in the lamina propria.

On the basis of the recent findings described above, we propose a model for Th17 differentiation in the intestinal lamina propria where a specific subset of lamina propria DCs expressing CD70 or CD11b is constitutively activated by intestinal commensal bacteria and produces IL-6, IL-23 and TGF-β, resulting in the continuous production of Th17 cells in the lamina propria (Fig.1). A specific component of the microbiota, such as ATP and TLR ligands, but not simply the presence of bacteria, seems very likely to be required for Th17 cell induction. As described above, because the presence of CFB phylum in the intestine is correlated with the development of lamina propria Th17 cells, specific members of the microbiota might selectively affect Th17 differentiation. It is important to further understand the specific types of Th17-inducing intestinal bacteria as well as Th17-inducing antigen-presenting cell subsets during steady state and during disease states.

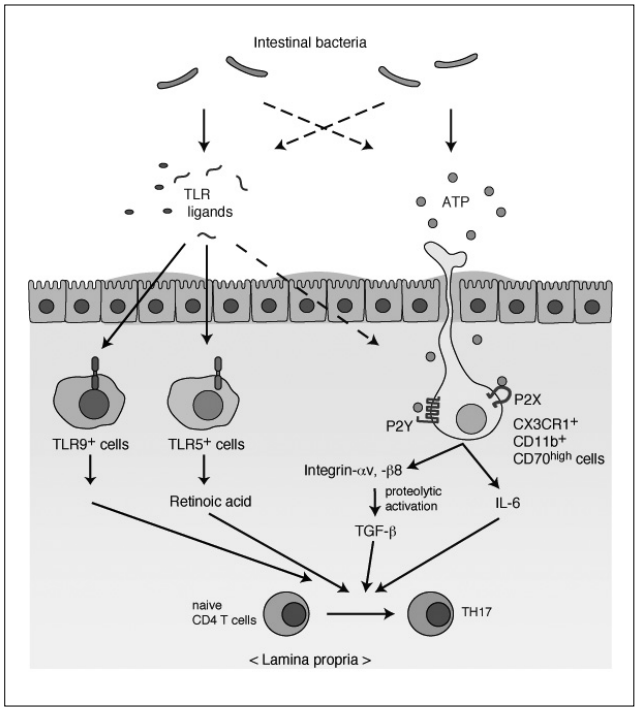

Fig.1 A hypothetical model for Th17 differentiation in the intestinal lamina propria

The intestinal microflora is composed of a variety of bacteria with different characteristics; each of which produce stimulatory factors for the host immune system. A subset of commensal bacteria produces and secretes high amounts of extracellular ATP. CD70^{high}CX3CR1$^+$ DCs, which extend their dendrites into the lumen, sense the extracellular ATP via P2X and P2Y receptors and produce IL-6 and TGF-β-activating enzymes to promote differentiation of Th17 cells in the intestinal lamina propria. In addition to ATP, intestinal bacteria produce TLR5 and TLR9 ligands, which activate lamina propria DCs and contribute to Th17 differentiation.

Concluding remarks

Recent findings have indicated that a specific environment is present in the lamina propria and supports the generation of Th17 cells. In addition to the Th17 cells, it is interesting to note that the lamina propria is also the largest tissue reservoir for Tregs. Foxp3$^+$ Tregs represent 20-40% of the CD4$^+$ T cells in the lamina propria in the small and large intestine[23, 45, 46]. Tregs and Th17

cells share TGF-β as a major cytokine controlling their development. Thus in the TGF-β-rich environment of the gut, T cells may be particularly inclined to differentiate towards the Treg and Th17 programs. It is important to study why the intestinal lamina propria is rich in the active form of TGF-β . Furthermore, such a unique cytokine milieu and a biased T cell differentiation program occur as a result of interactions between commensal bacteria, each with distinct characteristics, and intestinal immune and non-immune cells, both with different functional bias. Thus more studies are required to understand the interactions between intestinal bacteria, DCs, T cells and other non-immune cells in the gut lamina propria. In particular, more comprehensive approaches, such as three-dimensional imaging of the immune system in a living animal, would provide better understanding of the dynamics of the interaction between commensal bacteria and the innate and adaptive immune systems. Such studies may offer therapeutic insight into IBDs.

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