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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
APC, antigen-presenting cell; IBD, inflammatory bowel disease; IFN, interferon; TCR, T cell receptor; TGF, transforming growth factor; TLR, toll-like receptor; Th, T helper; Treg, regulatory T; tTreg, thymus-derived Treg

IL-17A-Producing Foxp3+ Regulatory T Cells and Human Diseases

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ABSTRACT

CD4+Foxp3+ regulatory T (Treg) cells play major roles in immune homeostasis. While CD4+Foxp3+ Treg cells act to suppress other immune effector cells, there is growing evidence that they also produce pro-inflammatory cytokines, such as IL-17A, in inflammatory conditions. The pro-inflammatory cytokine milieu, toll-like receptor (TLR) signaling, and specific transcription factors are important for the production of IL-17A by CD4+Foxp3+ Treg cells. In particular, IL-17A-producing CD4+Foxp3+ Treg cells express RORγt, the T helper (Th) 17-specific transcription factor, in addition to Foxp3. IL-17A-producing CD4+Foxp3+ Treg cells are also involved in the pathogenesis of various diseases. Here we review the mechanisms underlying the induction of IL-17A-producing CD4+Foxp3+ Treg cells and the roles of these cells in human disease.

Keywords: Regulatory T-cells; IL-17A; Inflammation; Pro-inflammatory cytokine

INTRODUCTION

CD4+CD25+Foxp3+ regulatory T (Treg) cells are important for the regulation of the immune response. They contribute to maintenance of immune homeostasis by suppressing excessive immune responses, and their dysregulation is involved in various human diseases, including autoimmune diseases, allergy, and cancer (1-5). It is therefore essential to understand exactly how they are modulated in inflammatory environments.

Conventional Treg cells exert their suppressive effect via cell-cell contact-dependent and contact-independent mechanisms. In particular, they produce anti-inflammatory cytokines such as IL-10, transforming growth factor (TGF)-β, and IL-35. However, there is growing evidence that Treg cells secrete pro-inflammatory cytokines in inflammatory conditions (6-13).

Treg cells that produce pro-inflammatory cytokines can be categorized into 2 types according to the stability of their expression of Foxp3. One type is reprogrammed Treg cells, also known as exTreg cells (14,15), that lose Foxp3 expression and acquire the characteristics of T helper (Th) cells, such as the secretion of pro-inflammatory cytokines in immune dysregulated conditions (16). The other type is Th-like Treg cells that stably express Foxp3. These cells express lineage-specific transcription factors and produce pro-inflammatory cytokines (12,17). Th1-like Treg cells express the Th1-specific transcription factor T-bet in addition to Foxp3 and produce a Th1-type cytokine, interferon (IFN)-γ (17). Th17-like

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Treg cells express RORγt in addition to Foxp3 and produce IL-17A (12). In autoimmune diseases, these inflammatory Treg cells are likely to play a role as pathologic effector T cells, thereby contributing to inflammation and to tissue injury (16). During infection by pathogens, inflammatory Treg cells may help elimination of the pathogens by secreting pro-inflammatory cytokines like Th1 or Th17 effector cells. However, it is also possible that they contribute to excessive inflammation and thus exacerbate host injury during infection.

Although inflammatory conversion of Treg cells has been reported in many pathologic conditions, several questions remain: How are inflammatory Treg cells induced? What are their roles in various diseases? And how are their functions modulated? Addressing these questions could allow inflammatory Treg cells to be therapeutic targets for the treatment of human diseases. In this review, we describe the current knowledge of inflammatory Treg cells, particularly IL-17A-producing Treg cells, with an emphasis on their induction and the roles they play in human disease.

CD4$^+$FOXP3$^+$ Treg CELLS

CD4$^+$CD25$^+$Foxp3$^+$ Treg cells negatively control the effector functions of diverse immune cells. Many studies have demonstrated their key roles in preventing autoimmune diseases and inflammatory diseases as well as their potential to control the rejection of transplanted grafts (18). Indeed, their absence in Scurfy mice and in patients with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome, which are caused by defects in the gene that encodes the Foxp3 protein, results in lethal lympho-proliferation and in multiple autoimmune diseases (18).

CD4$^+$CD25$^+$Foxp3$^+$ Treg cells are classified based on their development into naturally occurring thymus-derived Treg (tTreg) cells (also known as natural Treg cells) and into peripherally-derived Treg (pTreg) cells (also known as induced or adaptive Treg cells) (19-21). tTreg cells develop during the process of T cell maturation in the thymus under T cell receptor (TCR) engagement with self-antigens, and these cells play a key role in the maintenance of self-tolerance (22). tTreg cells constitute the majority of the peripheral Treg cell pool in naïve mice and in human cord blood. Conversely, pTreg cells are generated predominantly at peripheral sites of inflammation and at environmental interfaces, such as in the gut.

Conventional Treg cells exert their immune-suppressive functions through various mechanisms, including 1) metabolite inhibition mediated by CD25 or CD39; 2) dendritic cell-dependent inhibition by CTLA-4 or LAG-3; and 3) the production of immunosuppressive, anti-inflammatory cytokines, such as IL-10, TGF-β, and IL-35 (23). However, emerging evidence shows that Treg cells can produce pro-inflammatory cytokines such as IL-17A in inflammatory conditions related to various human diseases.

THE BIOLOGY OF IL-17A-PRODUCING CD4$^+$FOXP3$^+$ Treg CELLS

The origin of IL-17A-producing CD4$^+$Foxp3$^+$ Treg cells
IL-17A-producing Foxp3$^+$ Treg cells can develop from conventional CD4$^+$Foxp3$^+$ Treg cells by plastic changes. When murine CD4$^+$Foxp3$^+$ Treg cells are cultured in Th17 differentiation-

inducing conditions, the Treg cells can produce IL-17A ([24]). In the same conditions, naïve CD4$^+$ T cells also produce IL-17A and transiently express Foxp3 ([24]). That study showed that conventional Treg cells that stably express Foxp3 can produce IL-17A, although the majority of IL-17A-producing CD4$^+$Foxp3$^+$ T cells were CD4$^+$ T cells that transiently expressed Foxp3.

As noted above, CD4$^+$Foxp3$^-$ T cells can transiently express Foxp3 upon TCR stimulation ([25],[26]). Unlike conventional Treg cells, these activation-induced Foxp3$^+$ cells do not stably express Foxp3, and ultimately, they stop expressing it. These cells do not have immune-suppressive functions. Therefore, it has been suggested that non-suppressive CD4$^+$Foxp3$^+$ cells, which easily lose their ability to express Foxp3, are derived from conventional CD4$^+$ T cells in the periphery.

Markers of IL-17A-producing CD4$^+$Foxp3$^+$ Treg cells

The expression of RORγt, the Th17 lineage-specific transcription factor, is the most important characteristic of IL-17A-producing Treg cells ([12]) although all RORγt$^+$Foxp3$^+$ Treg cells are not IL-17A-producing cells. The expression of CCR6, CD49d, IL-1R-β, and CD161, as well as the absence of human leukocyte antigen-DR (HLA-DR), have been described as cell surface markers of IL-17A-producing Foxp3$^+$ Treg cells ([7],[27-30]). Collectively, IL-17A-producing Foxp3$^+$ Treg cells can be defined as CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^-$HLA-DR$^-$CD49d$^+$CD161$^+$Foxp3^{lo}RORγt$^+$CCR6$^+$ T cells. However, further investigation is needed to more precisely define IL-17A-producing Treg cells.

Within the human CD4$^+$Foxp3$^+$ T cell population, the expression patterns of Foxp3 and CD45RA define 3 subpopulations: the Foxp3^{lo}CD45RA$^+$ resting Treg cell subpopulation, the Foxp3^{hi}CD45RA$^-$ activated Treg cell subpopulation, and the Foxp3^{lo}CD45RA$^-$ non-suppressive subpopulation ([31]). The majority of IL-17A-producing Foxp3$^+$ T cells belong to the Foxp3^{lo}CD45RA$^-$ non-suppressive subpopulation. However, the exact nature of the Foxp3^{lo}CD45RA$^-$ non-suppressive subpopulation remains somewhat unclear. A recent study suggested CD15s as a marker that differentiates between the activated suppressive Treg cell subpopulation and the non-suppressive subpopulation. It is not clear yet whether CD15s$^-$Foxp3$^+$ T cells produce IL-17A, although they are known to actively produce IFN-γ and IL-2 ([32]).

The induction of IL-17A-producing CD4$^+$Foxp3$^+$ Treg cells

The pro-inflammatory cytokine milieu is the most important factor for the induction of IL-17A production by CD4$^+$Foxp3$^+$ Treg cells. IL-6, IL-23, and IL-1β are required for Treg cells to differentiate into IL-17A-producing Th17-like Treg cells ([24],[29],[33-36]). In the presence of TGF-β, IL-6 can induce CD4$^+$Foxp3$^+$ Treg cells to produce IL-17A ([37]). As in Th17 cells, IL-6-dependent STAT3 activation is also required, along with RORγt and RORα, for IL-17A expression by Treg cells ([36],[38]). A recent study of human Treg cells suggested that IL-6 promotes the expression of PIM1 kinase, which specifically phosphorylates Foxp3 protein at Ser$_{422}$, and this negatively regulates Foxp3 protein activity *in vitro* ([39]).

Stimulation of toll-like receptors (TLRs) is another important factor that has been implicated in the production of IL-17A by CD4$^+$Foxp3$^+$ Treg cells ([24],[40]). TLRs are expressed not only by innate immune cells and antigen-presenting cells (APCs) ([41],[42]), but also by CD4$^+$ T cells, including Foxp3$^+$ Treg cells ([41],[43-49]). In murine models, when TLR2 that is expressed on APCs is stimulated by its ligands, the APCs produce pro-inflammatory cytokines, and the suppressive function of Treg cells is reversed ([36],[49-54]). When conventional CD4$^+$Foxp3$^+$ Treg cells are stimulated by TLR2 ligands in the presence of APCs and Th17-inducing cytokines like IL-6,

IL-23, and IL-1β, the Treg cells can produce IL-17A without losing Foxp3 expression, although 20% of Treg cells lose Foxp3 (13,24). These data indicate that excessive TLR2 stimulation may contribute to the induction of IL-17A-producing Treg cells in inflammatory environments.

Specific transcription factors have important roles in the induction of IL-17A-producing Treg cells. IL-17A-producing, Th17-like Treg cells express RORγt in addition to Foxp3, suggesting that RORγt is involved in IL-17A expression in Foxp3⁺ Treg cells. Th17 cells and Treg cells are more closely related in terms of their differentiation than are Th1 or Th2 cells, because TGF-β is involved in the differentiation of both Th17 and Treg cells (55-57). While TGF-β signaling is required for the expression of both Foxp3 and RORγt expression, the induction of Foxp3 or RORγt is determined by collaborating cytokines. Specifically, TGF-β works in concert with IL-2-dependent STAT5 activation for Foxp3 induction, whereas it works in concert with IL-6-dependent STAT3 activation for RORγt induction (15,58). In the absence of IL-6, Foxp3 inhibits Th17 differentiation by antagonizing the functions of RORγt and RORα (58). However, excessive IL-6 in the milieu overcomes the inhibitory effect of Foxp3 and induces the development of IL-17A-producing Treg cells in a RORγt- and STAT3-dependent manner (7,38). One study showed that Treg cells from patients with STAT3 loss-of-function mutations were unable to produce IL-17A (33), indicating that IL-6-mediated STAT3 activation is critical for the production of IL-17A by Treg cells. Moreover, STAT3 is not only essential for the induction of IL-17A-producing Treg cells, it is also required for the suppression of Th17-mediated pathogenesis by Treg cells in mice (59,60). Regarding the suppressive functions of IL-17A-producing, Th17-like Treg cells, some studies have shown that their suppressive functions are impaired, while others showed that they retain their suppressive ability (61).

IL-17A-PRODUCING FOXP3⁺ Treg CELLS AND HUMAN DISEASES

Inflammatory diseases

Psoriasis is a chronic inflammatory skin disease that is closely associated with Th17 effector cytokines (62). In psoriasis, Treg cells are dysfunctional and have insufficient suppressive activity (63). The presence of IL-17A-producing, Th17-like Treg cells in human inflammatory conditions was first described in psoriasis patients (12,64-66). In human psoriatic skin, CD4⁺Foxp3⁺ T cells exhibit a memory phenotype in that they express CD45RO and show a high capacity to produce IL-17A, IL-2, and IFN-γ (29,64,65). These IL-17A-producing T cells are regarded as *bona fide* Treg cells because the TCR Vβ sequences of IL-17A-producing T cells overlap very little with those of effector CD4⁺ T cells (65).

Treg cells are critical for commensal tolerance in the intestine, and a lack of intestinal tolerance can lead to inflammatory bowel disease (IBD) (67-69). Indeed, a number of studies have investigated the protective roles of Treg cells in murine models of IBD. It is well known that the number of IL-17A-producing Treg cells is increased in both the intestinal mucosa and the circulation of IBD patients compared to healthy controls (10,70-72). Although one study reported that the suppressive functions of these cells are defective (72), other study has shown that IL-17A-producing Treg cells from colitis samples retain their immuno-suppressive capacity (70). These cells suppress the proliferation of effector T cells *in vitro*, supporting the notion that they are not reprogrammed to lose their suppressive functions (7,70). Taken together, these data suggest that the inflammatory conditions in IBD can induce IL-17A production by conventional Treg cells *in vivo*.

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In rheumatoid arthritis, patients have been reported to have a higher level of IL-17A-producing Treg cells than healthy controls (33). These cells produce IFN-γ and IL-2 along with IL-17A (73,74). The ability of these cells to suppress the immune activity of effector T cells is comparable to that of conventional Treg cells from healthy subjects, indicating that these IL-17A-producing Treg cells do not lose their suppressive capacity.

Cancer

In cancer patients, CD4⁺Foxp3⁺ Treg cells are considered to contribute to the immunosuppressive tumor microenvironment (75). Analyses of human tumor-infiltrating lymphocytes in melanoma, ovarian, breast, and colon cancers indicated that a substantial fraction of CD4⁺Foxp3⁺ Treg cells express pro-inflammatory cytokines such as IL-17A and IFN-γ (76,77). These IL-17A-producing Treg cells, which were isolated from various tumors, had a strong suppressive function *in vitro* (70). These data indicate that although the precise roles of these cells in tumor progression must be investigated further, they may affect the induction of active inflammation and tumor development while still retaining their suppressive function on effector T cells.

A recent study reported interesting results in colorectal cancer regarding the pro-inflammatory cytokine-secreting, non-suppressive Foxp3^lo^CD45RA⁻ subpopulation (31,78). Colorectal cancer patients were divided into 2 groups based on the degree of infiltration of CD4⁺Foxp3^lo^CD45RA⁻ T cells (78). Patients with abundant infiltration of CD4⁺Foxp3^lo^CD45RA⁻ T cells had a significantly better prognosis than those with predominant infiltration of CD4⁺Foxp3^hi^CD45RA⁻ activated Treg cells. The development of such pro-inflammatory cytokine-producing CD4⁺Foxp3^lo^CD45RA⁻ T cells may depend on cytokines such as IL-12 and TGF-β, which are produced by tumor tissues. Interestingly, the presence of CD4⁺Foxp3^lo^CD45RA⁻ T cells was related to bacterial invasion to tumor tissues by intestinal bacteria, particularly *Fusobacterium nucleatum*. This study demonstrated that pro-inflammatory cytokine-producing CD4⁺Foxp3^lo^CD45RA⁻ T cells can be induced by a specific species of bacteria and suggested that they might contribute to tumor control.

Infectious diseases

There are a few reports about the presence of pro-inflammatory cytokine-producing CD4⁺Foxp3⁺ Treg cells in infectious diseases. In oropharyngeal candidiasis models, IL-17A-producing Treg cells arise transiently in infected mice (24,79-81). IL-17-producing Treg cells do not contribute to the pathology during acute candida infection in mice.

In a study of patients with chronic hepatitis B virus (HBV) infections, the relative frequency of CD4⁺Foxp3^lo^CD45RA⁻ T cells, which are pro-inflammatory cytokine-secreting, non-suppressive cells, was increased in patients with acute-on-chronic liver failure and correlated with severe liver injury (82). The results of that study suggested that CD4⁺Foxp3^lo^CD45RA⁻ T cells may contribute to immune-mediated liver injury during chronic HBV infection. In infections by other types of pathogens, researchers need to examine the specific roles and induction mechanisms of CD4⁺Foxp3⁺ Treg cells that produce pro-inflammatory cytokines, particularly IL-17A.

CONCLUSION AND PERSPECTIVE

IL-17A-producing CD4⁺Foxp3⁺ Treg cells have been extensively investigated in various inflammatory diseases (**Fig. 1**). Pro-inflammatory cytokines, TLR signaling, and Th17 lineage-related transcription factors all play roles in the induction of IL-17A-producing Treg cells
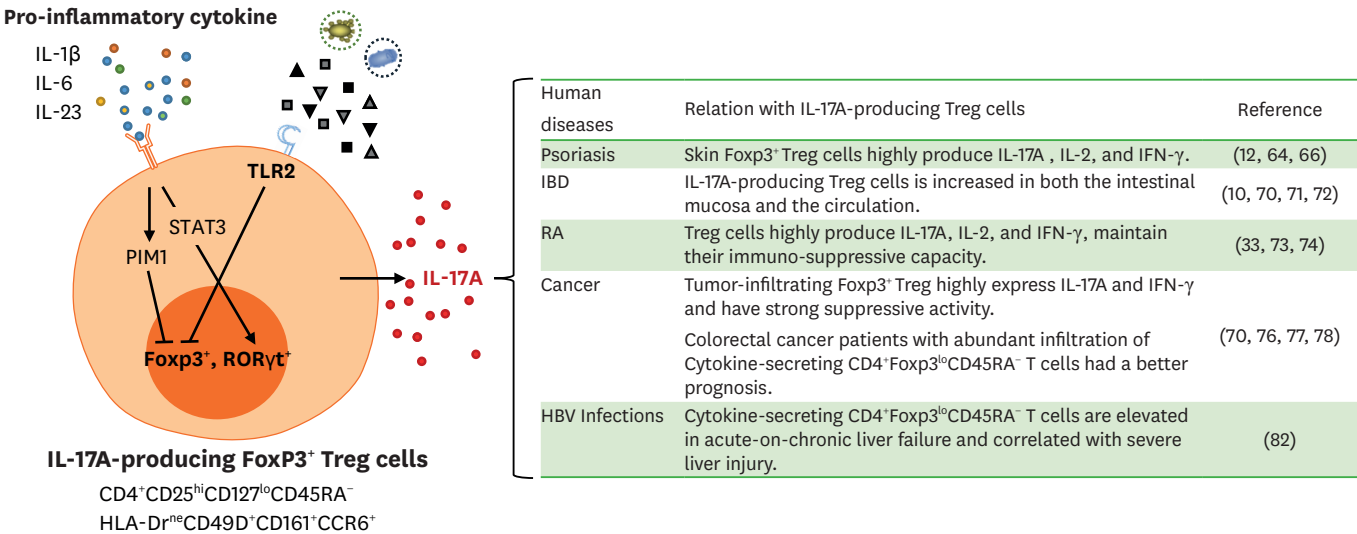
Figure 1. IL-17A-producing CD4$^+$FOXP3$^+$ Treg cells in human disease. IL-17A-producing Treg cells express RORγt in addition to Foxp3. They can be induced by pro-inflammatory cytokines and TLR2 signals. Previous findings regarding IL-17A-producing Foxp3$^+$ Treg cells in human disease are summarized. RA, rheumatoid arthritis; HBV, hepatitis B virus.

(**Fig. 1**). The functions of IL-17-producing CD4$^+$Foxp3$^+$ Treg cells depend on environmental cues, including the local cytokine milieu, metabolites, and possibly the origins of the cells. However, the specific characteristics of IL-17A-producing Treg cells and their mechanisms of action merit further investigation. For example, the differences between the essential functions of conventional Th17 cells vs. IL-17A-producing CD4$^+$Foxp3$^+$ Treg cells are not completely clear. Understanding how IL-17A-producing Treg cells are modulated in healthy subjects and in various pathologic conditions is important as these cells may serve as therapeutic targets for the treatment of human diseases.

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