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REVIEW

The role of all-*trans* retinoic acid in the biology of Foxp3$^+$ regulatory T cells

Zhong-Min Liu[1,5], Kun-Peng Wang[2,3,5], Jilin Ma[2,4] and Song Guo Zheng[1,2]

Regulatory T (Treg) cells are necessary for immune system homeostasis and the prevention of autoimmune diseases. Foxp3 is specifically expressed in Treg cells and plays a key role in their differentiation and function. Foxp3$^+$ Treg cells are consisted of naturally occurring, thymus-derived Treg (nTreg) and peripheral-induced Treg (iTreg) cells that may have different functional characteristics or synergistic roles. All-*trans* retinoic acid (atRA), a vitamin A metabolite, regulates a wide range of biological processes, including cell differentiation and proliferation. Recent studies demonstrated that atRA also regulates the differentiation of T helper (Th) cells and Treg cells. Moreover, atRA also sustains nTreg stability under inflammatory conditions. In this review, we summarize the significant progress of our understanding of the role(s) and mechanisms of atRA in Treg biology.
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INTRODUCTION

Autoimmunity is a heterogeneous disorder, which includes at least 80 diseases and is controlled by complex genetic and environmental factors. The pathogenesis of autoimmunity is hypothesized to result from a breakdown of immune tolerance, including central and/or peripheral mechanisms, and this loss of control ultimately culminates in autoimmune diseases.[1] Whereas the immune system plays an important role in the prevention of autoimmune diseases through self-tolerance mechanisms, it must be efficient in protecting the host from insult by exogenous pathogens. In this regard, the CD4$^+$ T-cell represents the chief protagonist, and plays a critical role in controlling the adaptive immune system.[2] A current paradigm in immunology is that autoimmunity is elicited by an imbalance between pathogenic T cells and Foxp3$^+$ regulatory T (Treg) cells.[3] These Treg cells prevent autoimmune and inflammatory diseases by suppressing the activities of deleterious effector T helper (Th) cells.[4]

CD4$^+$CD25$^+$Foxp3$^+$ Treg cells are a specialized CD4$^+$ T-cell lineage that plays a central role in maintaining self-tolerance, and the dysfunction of these cells is implicated in the development of various autoimmune diseases.[5–7] CD4$^+$CD25$^+$Foxp3$^+$ Treg cells comprise at least two distinct subsets in the periphery, natural Treg cells (nTreg cells) produced by the thymus after recognition of high-affinity self-antigen and then move to the periphery, and induced Treg cells (iTreg cells) that are converted from conventional non-Treg cells as a consequence of peripheral exposure to antigens in the presence of transforming growth factor-beta (TGF-β) signaling.[8] The comparison of the similarities and differences between nTreg and iTreg cells has been previously reviewed.[3,9,10]

Foxp3$^+$ iTreg cells can be induced *ex vivo* by TGF-β or IL-10.[11,12] Although many factors may promote the differentiation and development of iTreg cells, TGF-β and its receptor signaling pathway is critical because Foxp3$^+$ iTreg cells cannot be induced without a TGF-β signal.[13,14] IL-2 is also important for the development and maintenance of iTreg cells.[15] All-*trans* retinoic acid (atRA), a vitamin A metabolite, regulates a wide range of biological processes, including cell differentiation and proliferation. Recent studies revealed that atRA regulates the differentiation of Th cells and Foxp3$^+$ Treg cells.[16,17] Additionally, atRA promotes the development and function of CD4$^+$ iTreg cells,

[1]Research Center for Translational Medicine, East Hospital, Tongji University School of Medicine, Shanghai, China; [2]Division of Rheumatology, Penn State Hershey College of Medicine, Hershey, PA, USA; [3]Division of Liver Surgery, First Affiliated Hospital of Nanjing Medical University, Nanjing, China and [4]Division of Rheumatology, Immunology and Nephrology, Zhejiang Provincial Traditional Chinese Medicine and Western Medicine Hospital, Hangzhou, China
[5] These authors contributed equally.
Correspondence: Dr Song Guo Zheng, Division of Rheumatology, Department of Medicine, Penn State University Hershey College of Medicine, 500 University Drive, PA 17033, USA.
E-mail: szheng1@hmc.psu.edu
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although its effect on CD8$^+$ iTreg cells is minimal.[18–21] Moreover, atRA also helps preserve nTreg cell stability under inflammatory conditions.[22,23] In this review, we summarize our understanding of the role of atRA in Treg cell biology, its related molecular mechanisms and potential clinical application for patients with autoimmune diseases and who need organ transplantation.

FOXP3 AND TREG CELL SUBSETS

Foxp3, an X chromosome linked factor that controls Treg cell development and function, is the major transcription factor for determining the fate and identity of Treg cells and is specifically expressed in Treg cells.[24,25] Foxp3 is generally postulated to positively control Treg cell function in a binary fashion, because its expression in conventional T cells is sufficient to specify immune-suppressive activities.[7] Foxp3 is critically involved in the development and function of Treg cells, its expression appears to play a necessary role in governing Treg cell action. Treg cells also prevent autoimmune and inflammatory diseases by suppressing the potentially deleterious activities of Th cells.[4] In contrast, the downregulation of Foxp3 or Foxp3 deficiency results in multiorgan autoimmune diseases. For example, downregulation of Foxp3 in antigen-experienced Treg cells coincides with the onset of pro-inflammatory and immunoregulatory cytokine secretion, such as IL-2, IFN-γ and IL-10, in these cells.[26] Recent data indicate that mature Foxp3$^+$ Treg cells express the highest levels of neuropilin-1 (Nrp-1), which is usually expressed on thymus-derived natural regulatory T cells. This suggests that the overwhelming majority of thymus-derived, natural Treg cells express Nrp-1.[27] Similarly, Helios provides an additional marker for the discrimination of nTreg cells from iTreg cells, although its specificity remains a concern.[28,29] Nrp-1 also identifies Foxp3$^+$ cell stability because Nrp-1$^+$ nTreg cells are more stable compared with Nrp-1$^-$ nTreg cells. Nrp-1$^+$ nTreg cells have lower methylation levels in the Treg cell-specific demethylated region.[30] The Treg cell-specific demethylated region colocalizes with conserved non-coding sequence-2 of Foxp3, a region involved in the maintenance of Foxp3 expression.[31]

One paradigm of immunology is that autoimmunity is elicited by an imbalance between pathogenic T and Foxp3$^+$ Treg cells. The pathophysiology driven by autoimmune diseases can alter the phenotypic and functional activity of Treg cells. Foxp3 expression in Treg cells is closely associated with their functional activities. The plasticity of Foxp3 expression by nTreg cells under inflammatory conditions may also play an important role in infectious diseases, in which early inflammatory cytokines induced by the innate immune response may not only downregulate Treg cell function, but may also change Treg cells into T effector cells locally in the infected tissues, thereby enhancing immunity.[1] The adoptive transfer of nTreg cells prevents the initiation and development of autoimmune diseases in many animal models; however, the therapeutic effect of nTreg cells on autoimmune diseases remains unsatisfactory. The key reason is that inflammatory cytokines, such as IL-6, TNF-α and IL-1, may decrease Foxp3 expression and subsequently reduce the functional activity of nTreg cells.[22,23,32–36]

THE STABILITY OF TREG CELL SUBSETS

Recent studies demonstrated that nTreg cells from both mouse and human are instable and dysfunctional under inflammatory conditions.[7,32,34,35,37,38] These cells not only lose their suppressive ability after encountering inflammatory environments, but they can convert into pathogenic cells that may actually accelerate the inflammatory process.[1] In addition, the repeat expansion of nTreg cells, even in the absence of pro-inflammatory cytokines, can also result in the loss of Foxp3 expression. This finding has very important implications for clinical utility because nTreg cells initially exist as a very small cell population.[26] It is therefore critical to identify approaches that maintain Foxp3 expression and Treg cell function during expansion, particularly under inflammatory conditions.

Rapamycin (RAPA) may be an ideal candidate for promoting nTreg cell stability. RAPA, an mTOR kinase inhibitor, is an immunosuppressive drug that inhibits effector T-cell proliferation, migration and cytokine production,[39] and can selectively promote the expansion of suppressive human CD4$^+$CD25^{hi} Foxp3$^+$ T cells isolated from healthy donors and patients with diabetes.[40,41] It remains unclear whether RAPA selectively suppresses the expansion of non-Treg cells, thereby indirectly promoting the expansion of Foxp3$^+$ Treg cells.[16] Although a comparison study has shown that both RAPA and atRA had similar effects on promoting and stabilizing Treg cells during their expansion,[42] a more recent study demonstrated that atRA exhibits superior efficacy relative to RAPA for stabilizing nTreg cells under inflammatory conditions.[23] The mechanism by which atRA stabilizes nTreg cells is discussed below.

iTreg cells exhibit several characteristic differences in stability and functionality relative to nTreg cells. Whereas IL-6 can convert nTreg cells into Th17 and Th1 cells, it does not have this effect on iTreg cells.[32] Conversely, iTreg cells are stable and function effectively in an inflammatory environment.[32,43] It is likely that TGF-β treatment reduces IL-6 receptor expression and thereby suppresses its signaling pathway.[32] Therefore, iTreg cells may play a complementary role to nTreg cells, particularly in response to self-antigens, which are not expressed in the thymus. It is possible that under inflammatory conditions, the induction of iTreg cells is suppressed and self-reactive cells develop directly into effector-memory T cells and promote autoimmune disease. Whereas TGF-β is crucial for promoting the development of iTreg cells, the presence of IL-6 interferes with the ability of TGF-β to promote this differentiation.[44] However, because iTreg cells are stable and functional under inflammatory conditions, after they have been induced, they can expand *ex vivo* following adoptive transfer for cell-based therapeutic treatment of patients with autoimmune inflammatory diseases.[3,45]

ATRA AND TREG CELL FUNCTION

atRA, the primary biologically active metabolite of vitamin A, plays vital roles in embryonic development, vision, skin

homeostasis and reproduction, and it also crucial for maintenance of the immune system.[46] atRA produced by dendritic cells facilitates the *de novo* generation of Foxp3$^+$ Treg cells from naive CD4$^+$CD25$^-$ T cell populations in mice,[47,48] but also suppress the *de novo* differentiation of naive CD4$^+$ cells into Th17 cells.[22] The effect of atRA on Treg and Th17 cells is dependent upon the RA receptor/retinoid X receptor heterodimer.[49,50] Because the pathogenesis and development of many autoimmune diseases is affected by the imbalance between Treg and Th17 cells, the role of atRA in regulating this balance may greatly affect the progress of autoimmune diseases.

atRA appears to promote gut homing of CD4$^+$ T cells by inducing CCR9 and α4β7 expression, and the expression of these molecules also indicates that a given population of T cells respond to atRA.[51] An initial study showed that atRA suppresses Th1 but promotes Th2 cells.[52] Vitamin A deficiency results in immune dysfunction *via* excessive IFN-γ production and impaired antibody responses. A recent study reported that atRA inhibits Th17 cell differentiation but promotes Foxp3$^+$ Treg cells,[17,18,53] although the role of atRA in CD4$^+$ and CD8$^+$ iTreg cell differentiation may be different.[21] The orphan nuclear receptor, RORγt, has been implicated in the gene transcription of Th17 cells. TGF-β induces high levels of RORγt and further promotes Th17 cell development in the presence of IL-6. However, the addition of atRA to cultures containing TGF-β and IL-6 greatly reduces RORγt expression and Th17 cell differentiation.[54]

The key role played by atRA in immune tolerance is *via* the induction of iTreg cells. atRA plays a crucial role in maintaining gut mucosa tolerance to commensal bacteria and food antigens through the induction of both Foxp3$^+$ Treg cells and IL-10-producing Treg cells.[55,56] atRA is primarily produced by CD103$^+$ dendritic cells in the intestine. These CD103$^+$ dendritic cells originate in the lamina propria, but migrate to the mesenteric lymph nodes where they drive the differentiation of gut-homing Foxp3$^+$ Treg cells through the production of retinoic acid from dietary vitamin A.[57] Whereas TGF-β alone is not sufficient to drive the development of human iTreg cells, the addition of atRA provides the necessary stimulus for human iTreg cell induction, demonstrating its value in clinical translation.[19] The study of molecular mechanisms demonstrated that although atRA does not significantly affect the phosphorylation levels of Smad2/3, it promotes iTreg cell induction in CD4$^+$ cells isolated from Smad3 knockout and Smad2 conditional knockout mice. By contrast, atRA markedly increases the activation of the ERK1/2 signaling pathway, and the resultant signaling promotes Foxp3 expression.[20] Although DNA methylation at the Foxp3 gene locus affects Foxp3 expression and maintenance by Treg cells,[58] atRA enhances the differentiation and stability of iTreg cells in the absence of any alteration of DNA methylation. Instead, atRA acts *via* increased histone methylation and acetylation within the promoter and conserved non-coding DNA sequence elements at the Foxp3 gene locus;[20] however, atRA can inhibit the methylation of the Foxp3 gene of nTreg in the presence of inflammatory cytokines.[23]

Interestingly, atRA also helps maintain Foxp3 expression during the nTreg cell expansion process.[22] This has recently been extended to human nTreg cells.[23] Compared with RAPA, the effect of atRA on stabilizing Foxp3 expression and Treg cell function is superior under inflammatory conditions.[23] atRA promotes the expression of cytotoxic T lymphocyte antigen 4(CTLA-4), a cell-surface receptor typically expressed by Treg cells on the majority of TGF-β-generated Foxp3$^+$ cells. The B7/CTLA-4 signal is crucial for the development and function of iTreg cells.[59] atRA also enhances the expression of surface TGF-β on nTreg cells,[23] which is another possible mechanism for nTreg cell stabilization. In addition, atRA also downregulates IL-6R expression and IL-6R signaling on nTreg cells, rendering nTreg cells resistant to the pro-inflammatory cytokine, IL-6, which is usually elevated in autoimmune diseases.[60] The key role atRA plays in promoting iTreg cells development and nTreg cells stabilization is summarized in Figure 1. These characteristics highly suggest that atRA-treated nTreg cells from patients with rheumatoid arthritis and other autoimmune diseases could potentially be used to control disease development and even cure patients. The combination of atRA and TGF-β also provides another approach to develop Treg cell therapy for patients with autoimmune diseases and the prevention of allograft reject in patients with organ transplantation.

CONCLUSIONS

Treg cells are a distinct lineage of CD4$^+$ T cells that are essential for maintaining immune system homeostasis by promoting self-tolerance and restraining excessive immune responses. Many mechanisms are involved in Treg cell development and function. Foxp3 is the most specific hallmark of Treg cell subsets. Foxp3

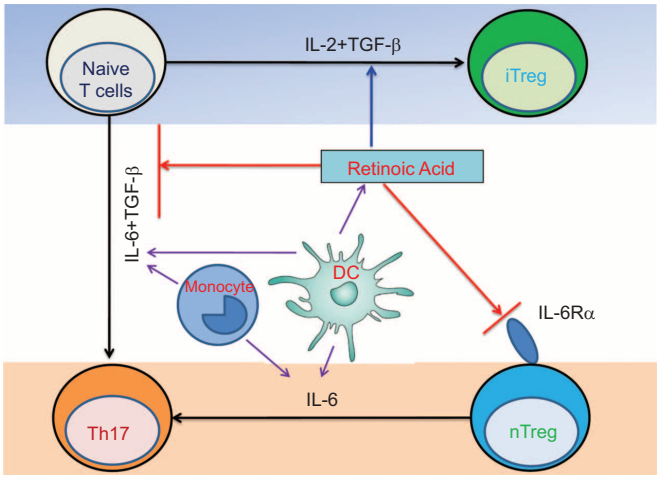

Figure 1 Immunomodulatory effects of atRA on CD4$^+$ T-cell subsets. atRA maintains immune homeostasis by working with TGF-β to promote Treg cell induction from naive T cells, while inhibiting Th17 cell induction in the presence of inflammatory cytokines such as IL-6. In addition, atRA inhibits Th17 differentiation from nTreg cells by reducing their expression of IL-6Rα. atRA, all-*trans* retinoic acid; TGF, transforming growth factor; Treg, regulatory T.

expression and stability are closely related to the functionality of Treg cells. Foxp3 expression on nTreg cells is unstable in the presence of IL-6 and other pro-inflammatory cytokines. RAPA and/or atRA can stabilize nTreg cells, but atRA has superior effects on nTreg cell stabilization under inflammatory conditions. atRA also promotes the differentiation of TGF-β-induced iTreg cells and inhibits Th1 and Th17 cell differentiation. These results highlight the role of atRA in promoting the development of iTreg cells and stabilizing the phenotype and function of nTreg cells, indicating that approaches with atRA-primed Treg cells have potential therapeutic value for patients with autoimmune diseases and those undergoing organ transplantation.

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