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Mini-review

MicroRNAs in regulatory T cells

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Introduction

Regulatory T cells (Tregs) are a specialized subset of CD4 T cells. As their name suggests, they play a crucial role in controlling and regulating immune responses. Tregs can actively block nearly all aspects of immune responses by suppressing the functions of an array of cell types, including conventional CD4 T helper cells, cytotoxic T lymphocyte (CTL) activity, B cell activation and antibody production, antigen-presenting cell (APC) function and maturation, and natural killer (NK) cell activation. To accomplish their role as global "brakes" on immunity, Tregs can adapt themselves into phenotypically and functionally distinct subsets. Under certain condition, Tregs even lose their master transcriptional factor Foxp3 and reprogram into immune-boosting cells.

MicroRNAs (microRNAs) are small single-stranded noncoding RNAs (20–25 nt) that are the key post-transcriptional regulators of gene expression. Currently, the miRBase database (http://www.mirbase.org/) contains 28,645 entries representing hairpin precursor microRNAs (v21, June 2014). Emerging evidence suggests that microRNAs play a critical role in regulating Treg activation, differentiation, suppressive function, and the stability of Treg cells.

Heterogeneity and plasticity of Treg

Based on their origin, Tregs can be divided into two subpopulations: 1) tTregs that are primarily generated in the thymus, and 2) pTregs that are generated extrathymically at peripheral sites [1]. All of these Treg cells are initially $CD4^+CD25^+$ T cells and were later shown to express a unique Forkhead box protein 3 (Foxp3) [2,3]. Foxp3 acts as a master transcriptional factor that lineage-specializes Treg cells, including expression of CD25 (IL-2Rα) and CTLA-4 [3]. The Treg function and part of the transcriptome rely on high and constitutive expression of Foxp3 in Tregs; mutation of the Foxp3 gene or Foxp3-deficiency in *Scurfy* mice results fatal autoimmune disease [4]. Moreover, deletion of Foxp3 from mature Tregs leads to loss of Treg identity and the conversion into effector T cells [5].

Similar to conventional T CD4 cells, MHC-TCR engagement is also a critical step for Tregs to gain potent suppressive activity. Tregs can be divided into central Tregs (cTregs) and effector Tregs (eTregs) according to their activation status [6]. cTregs account for the vast majority of peripheral and secondary lymphoid Tregs, also referred to as resting or naive Tregs. cTregs are phenotypically similar to conventional naive T cells and express both CD62L and CCR7, which aid in circulation between lymphoid organs. eTregs only account for a small proportion of Tregs in the secondary lymphoid organs and are rather distributed in various tissues and organs. They are considered as a group of antigen-activated Tregs that express high levels of activating molecules (*e.g.*, CD44, ICOS, and KLRG1) and various chemokine receptors responsible for tissue location but display low expression of CD62L and CCR7 [6]. Conditional deletion of TCRα after Treg maturation blocks eTreg generation and causes a severe autoimmune phenotype resembling *Scurfy* mice, indicating that eTregs are likely direct players in immunosuppression [7].

Effector Treg cells can further upregulate different master transcription factors to develop into more specific effector Treg cells. Those transcription factors from other T helper lineages can upregulate the corresponding chemokine receptors and contribute to the better migration of effector Tregs to yield specific inflammation inhibition of effector T cells. For instance, $T\text{-bet}^+$ Treg expression of CXCR3 more effectively inhibits Th1 effector cells [8]. Similarly, the Th2 transcription factor IRF4 and chemokine receptor

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CCR4 are likely required for Treg cells to control the Th2-mediated autoimmune response [9], and Rorγt-CCR6 and Bcl6-CXCR5 are necessary to shut down Th17-mediated experimental autoimmune encephalomyelitis (EAE) and aberrant germinal center responses, respectively [10,11]. Treg cells are also present in a variety of non-lymphoid tissues such as the skin, intestinal mucosa, lung, liver, adipose tissue, tumors, placenta, and damaged muscle. Studies demonstrate that Treg cells resident in visceral adipose tissue (VAT) can further up-regulate the tissue-specific transcription factor PPARγ, thereby helping Treg cells to survive and function better in tissues [12]. Effector Tregs can exert their functions through multiple suppressive mechanisms: 1) inhibitory cytokine release, in which Tregs secrete inhibitory cytokines such IL10, TGF-β, and IL35 to prevent prolonged inflammatory reactions; 2) cytolysis, which is similar to the CTL granzyme-dependent pathway used to kill target cells; 3) metabolic disruption, in which Tregs function as IL-2 sinks or express CD39 to remove extracellular ATP that is considered a "natural adjuvant"; and 4) modulation of APC function [13]. Producing suppressive cytokines is a key molecular mechanism for Tregs to conduct long-range suppressive functions. Interestingly, we found that IL35-producing Tregs are a distinct effector population from the IL10-producing subset, and those two types of effectors play complementary roles in maintaining self-tolerance [14]. Thus, effector Tregs are highly heterogeneous and functionally specialized, which allows them to serve as master regulators, effectively inhibiting highly diversified immune responses.

Although the activation process and functional specialization are essential for Tregs to gain potent inhibitory activity, over-activation in combination with an inflammatory environment can promote conversion of Tregs from immune-suppressing cells to immune-boosting cells. Several studies report that Treg cells can become unstable, lose Foxp3 expression and their suppressive capacity, and acquire features reminiscent of effector T cells in response to the local environmental and inflammatory cues [15,16]. More recently, by using a novel tTreg fate-mapping mouse (Foxp3 ΔCNS1-Cre), we found that only ~1% of mature tTregs lose Foxp3 expression in secondary lymphoid organs, indicating that tTregs are stable under homeostatic conditions. However, TCR engagement and sequential functional specialization of tTregs lead to the generation of Foxp3 instability and reprogramming into the T helper lineage [17].

Stable Foxp3 expression in Tregs is also subject to higher-order regulation by epigenetic modifications of the conserved non-coding sequences (CNS) in the Foxp3 locus [18]. In particular, tTregs display stable Foxp3 expression that is associated with specific demethylation of an evolutionarily conserved element in the CNS (also referred to as the TSDR) of the Foxp3 locus. Demethylation of CNS2 in Treg cells is mediated by Tet-dependent oxidization and favors the recruitment of multiple transcription factors such as Cbfβ, Runx1, STAT5, and Foxp3 itself to the CNS2 to further ensure stable Foxp3 expression. Previous studies demonstrate that genetically deleting the CNS2 enhancer of Foxp3 results in a destabilized Treg lineage, and CNS2-deficient mice develop spontaneous autoimmunity and chronic inflammation [19].

IL-2-induced STAT5 phosphorylation is essential for the maintenance of cTreg homeostasis and their Foxp3 stability. cTregs express high levels of CCR7 and the high-affinity IL-2 receptor CD25, facilitating their T cell zone localization and acquisition of the IL-2 signal [6]. Once Tregs recognize an antigen, Treg activation leads to down-regulation of CD25 but up-regulation of ICOS, which provides different homeostasis signals mainly through the PI3K/AKT pathway. Activation PI3K and downstream mTOR signaling promote Treg activation [20]. However, this signal also induces Foxp3 instability [21], indeed over -activation of either mTOC1 or mTOC2 is detrimental to Tregs suppressive activity and Foxp3 stability [22–24]. Thus, appropriate activation is necessary for tTreg differentiation in specific immune microenvironments, but over-activation raises the risk of Foxp3 instability. IL-2 and ICOS control the two opposite sides of stability and activation, promoting functional specification. In addition, inflammatory cytokines such IL-4 and IL-6 coordinately induce Treg instability [25,26].

Generation of the plasticity of Foxp3 can help Tregs distinguish self and non-self-antigens. Both conventional T cells and tTregs are generated in the thymus, and most thymocytes that bind with high affinity undergo clonal deletion. However, some self-agonist ligands with medium-high affinity preferentially develop Tregs. In the periphery, because high-affinity self-peptide-MHCs are deleted by thymic negative selection, self-peptide-MHCs may provide a 'just right' activation window for tTregs. Similar to conventional T cells, tTregs have the potential to recognize foreign antigens. For conventional T cells, high-affinity TCR-MHC engagement may preferentially generate dominant T cell clonal expansion to clear the infection. However, due to the lack of negative section, foreign TCR-MHC engagement in tTregs may too strongly induce ICOS/PI3K signaling that is detrimental to Treg suppression activity and further terminates Foxp3 expression, reprogramming the Tregs into immune-boosting cells (Fig. 1).

Knockout of Dicer and DGCR8/Drosha in Tregs

microRNA precursors are found in the genome in various contexts, including in the introns of noncoding or coding transcripts, as well as exonic regions. Initially, microRNAs are transcribed by RNA polymerase II to generate long primary microRNA (pri-microRNA) transcripts. Like protein coding mRNAs, pri-microRNAs are capped and polyadenylated. The pri-microRNAs are then folded into secondary configurations containing imperfectly base-paired stem loops that are endonucleolytically cleaved into approximately 70-nt hairpin structures (known as pre-microRNAs) by the nuclear microprocessor complex, of which the core components are the RNaseIII type endonuclease Drosha and the DiGeorge critical region 8 (DGCR8) protein [27,28]. The pre-microRNAs are subsequently exported from the nucleus to the cytoplasm by Exportin-5 via a Ran-GTP-dependent mechanism, and Exportin-5 independent mechanism [29,30]. In the cytoplasm, pre-microRNAs are cleaved near the terminal loop by another RNase III-type enzyme, Dicer, with its cofactor TRBP (TAR RNA-binding protein 2, also known as TARBP2), leading to RNA duplexes of approximately 22 nt [31]. After cleavage by Dicer, one of the two strands of the RNA duplexes (passenger microRNA) is released and degraded, while the other strand, mature microRNA, is retained and loaded into the RNA-induced silencing complex (RISC) comprising an Argonuate (AGO) protein and a glycine-tryptophan repeat-containing protein of 182 kDa (GW182) [32]. Once processed and loaded into the RISC, the mature microRNAs are able to bind to the 3′ untranslated region (UTR) of certain target mRNAs based on sequence complementarity, resulting to direct translational inhibition, mRNA degradation, or a combination of the two [33,34].

Two categories of RNase III enzyme Drosha/DGCR8 and Dicer, which generate pri-microRNAs into pre-microRNA and mature microRNA respectively [35], have been investigated in Tregs. The function of Dicer is not limited to canonical microRNA biogenesis; it is also required for small inhibitory RNAs (siRNAs) and mirtrons [36]. In contrast, Drosha and DGCR8 are more specific for microRNA biogenesis. Dicer ablation in the stage of thymic development (CD4-Cre-Dicer$^{flox/flox}$) results in moderately reduced T cell numbers, particularly the reduced frequency of Treg cells, and causes mild immune pathology affecting the colon, lung, and liver [37,38]. Strikingly, deletion of Dicer specifically in the Treg lineage by using Foxp3-Cre stains leads to the development of