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Review Article

Advances in distinguishing natural from induced Foxp3$^+$ regulatory T cells

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Abstract: For more than a decade now, the regulatory T (Treg) cell has widely been considered as a critical subpopulation of T cells which can suppress effector T cell responses as well as suppressing the activity of other immune cells, such as mast cell, dendritic cells, and B cells. Treg cells have been broadly characterized as comprising of two main populations: thymus-derived natural Treg (nTreg) cells, and peripherally generated induced Treg (iTreg) cells. Both subsets have similar phenotypic characteristics and comparable suppressive function against T cell-mediated immune response and diseases. However, both Foxp3 positive Treg subsets exhibit some specific differences such as different mRNA transcripts and protein expression, epigenetic modification, and stability. These subtle differences reinforce the notion that they represent unique and distinct subsets. Accurately distinguishing iTregs from nTregs will help to clarify the biological features and contributions of each Treg subsets in peripheral tolerance, autoimmunity and tumor immunity. One difficult problem is that it has not been possible to distinguish iTregs from nTregs using surface markers until two recent articles were published to address this possibility. This review will focus on very recent advances in using molecular markers to differentiate these Treg subsets.

Keywords: Treg, Foxp3, Helios, neuropilin 1

Introduction

Ever since Sakaguchi's group identified CD4 and CD25 double positive cells as suppressive T cells [1], regulatory T cells (Tregs) have been considered as a promising immunotherapy for clinical diseases, but there have been many difficulties in finding specific markers to identify this unique cell type. A great advance was made in this regard when the nuclear transcription factor Foxp3 was identified as a specific marker for Tregs [2]. Tregs have a prominent suppressive activity under inflammatory conditions to prevent pathogenic damage, but they also have the unfortunate tendency to protect tumors from host immunologic surveillance [3-5]. Tregs can also be used therapeutically in organ transplantation conditions to suppress the reaction of T cells to allo-antigens [3, 6-8]. These kinds of functions represent a double-edged sword under different pathogenic conditions, a trait which necessitates the judicious application of Tregs in clinical diseases.

Tregs are represented by two populations: iTregs and nTregs

Numerous reports have demonstrated that Foxp3 positive Tregs can be found in two varieties: natural Treg (nTreg) cells and adaptive or induced Treg (iTreg) cells. These suppressive cells develop from thymic TCR high affinity T cells selection and peripherally generated Foxp3$^+$ T cells under immunogenic or subimmunogenic antigen stimulation *in vivo*, respectively [9]. In addition, there are two other subsets (CD4 positive interleukin-10 producing Tr1 cells and transforming growth factor -beta-producing Th3 cells) of Foxp3$^-$ iTregs that also possess suppressive function [10]. Here we will focus only on Foxp3$^+$ nTregs and Foxp3$^+$ iTregs. Safe and effective clinical usage of these cell popu-

lations absolutely requires that we have a reliable method for accurately identifying these cell subsets.

The features that differentiate nTregs from iTregs are not universally accepted. It has been reported that the generation of iTregs may result from stimulation with foreign antigen such as intestinal flora and environmental/food allergens, whereas nTreg generation maybe driven by the need of controlling autoantigen reactions so as not to cause autoimmunity diseases [11]. Some researchers believe that the differential TCR repertoire plays an important role in the formation of Tregs *in vivo* [11]. However, that may not be the case. Yadav, *et al* [12] reported that a myelin basic protein (MBP)–TCR-transgenic (Tg) recombination activation gene (RAG)–deficient mouse developed Treg cells in the periphery but not in the thymus. Since MBP ubiquitously expressed in the periphery and thymus, the explanation that mice has no nTregs may be that the generation of nTregs requires both specific TCR signaling along with other (unknown) stimulatory signals in thymus. Fousteri *et al* [13] reported another insulin-specific TCR Tg mouse exhibited an absence of Tregs in thymus but did find them in the periphery, despite the detection of preproinsulin-2 protein expression in thymic medullary epithelial cells. However, these findings were contrasted with another paper in which hemagglutinin (HA) -specific TCR Tg mouse exhibited HA expression in thymus, inducing thymocytes to differentiate into nTregs [14]. In general, the generation of nTreg in thymus requires self-antigen specific TCR and RAG-related factors.

iTregs can also be induced *in vitro* from $CD4^+CD25^-Foxp3^-$ cells in the presence of IL-2 and TGF-β [4, 15, 16]. Recent studies have shown that iTregs can be generated in a TCR-Tg mouse model in which the mouse is completely devoid of thymus derived nTregs [12], implicating that the production of iTregs *in vivo* is independent of nTregs. Adoptive transfer of antigen-specific naive $CD4^+$ T cells into lymphopenic RAG-deficient mice that express an endogenous antigen will result in autoimmune symptoms resembling graft-versus-host disease with a spontaneous late recovery. This kind of recovery is associated with the generation of $Foxp3^+$ regulatory T cells in the periphery. In the absence of IL-2, this scenario results in progressive disease instead [17]. Thus, the generation of iTregs is independent of the thymus but does rely on the presence of IL-2 [11]. In addition, IL-2 is also essential for the generation of iTregs *in vitro* [18, 19]. Functionally, nTregs suppress B cell response through their killing ability that is similar to NK cells [20-23], whereas iTregs suppress B cells independent of killing (Liu Y et al. unpublished data).

Although Treg cells constitute a stable cell lineage to maintain immune homeostasis in a dynamic environment, $Foxp3^+$ T cells still retain some plasticity. Our group and others have reported that nTreg are not stable under inflammatory conditions, and have demonstrated that they may actually transit to a Th17 phenotype in the presence of IL-6 [24-26]. In contrast iTreg do not exhibit this plasticity, highlighting their stability under inflammatory conditions *in vitro* and *in vivo*. In a collagen-induced arthritis mouse model, iTreg cells exhibited a superior suppression of osteoclastogenesis and bone erosion relative to nTregs [27]. However, both iTregs and nTregs are able to upregulate expression of Th1-associated molecules, including T-bet, CXCR3, and IFN-γ in the presence of IL-12 stiumulation, conditions which are found not only in human diseases but also in mouse models [28, 29].

One major hurdle facing the clinical usage of Treg cells is the problem of their stability. In contrast to TGF-β-induced iTregs, nTregs maintain the Foxp3 expression and suppressive activity upon restimulation in the absence of TGF-β *in vitro* [30]. It has been shown that epigenetic modifications in the CpG-rich Treg-specific demethylated region (TSDR) of the Foxp3 locus are related to the stability of Foxp3 [31]. These epigenetic TSDR modifications have been used to distinguish nTregs from iTregs [32]. Demethylation of the TSDR region correlates with the stability of Foxp3 gene. OVA-specific iTreg cells were capable of maintaining high levels of Foxp3 expression for a week *in vivo,* but they rapidly lost Foxp3 expression upon stimulation with OVA. This effect was abrogated through the addition of exogenous IL-2. IL-2 treatment stabilized Foxp3 expression in iTregs and enhanced the demethylation of TSDR [33]. Thus, the unstable phenotype of iTregs may be related to a strong methylation in the TSDR of Foxp3 promoter. Analyzing the demethylation status of the TSDR in the FOXP3

locus may aid in the differentiation of iTregs from nTregs, because reports show that iTregs are predominately methylated at the TSDR, while nTregs are mostly demethylated in this region [28, 30, 34, 35].

Other markers distinguishing nTregs from iTregs

Both Tregs express the canonical Treg markers, CD25, Foxp3, GITR and CTLA4, but nTregs exhibit a higher expression of PD-1 (programmed cell death-1, pdcd1), neuropilin 1 (Nrp1), Helios (Ikzf2), and CD73 compared with iTregs [12]. It should be noted that the with the exception of Helios and Nrp1, none of those molecules can individually provide the specificity necessary to distinguish nTregs from iTregs.

Helios, first identified nTreg marker

Helios protein, encoded by the Ikaros gene, was found primarily in the centromeric regions of T cell nuclei [36]. Using a DNA microarray analysis on freshly isolated mouse $CD4^+CD25^+$ nTreg cells, Sugimoto *et al* first reported that Helios may be a specific marker for natural Treg cells [37]. However a Helios deficiency mouse model has demonstrated that Helios is not essential for T cell differentiation and function [38]. On the other hand, Getnet *et al* [39] have reported that Helios is upregulated in human nTreg cells, and can bind to the Foxp3 promoter. Inhibiting Helios expression with siRNA oligonucleotides results in down-regulation of Foxp3 and significantly attenuates nTreg suppressive functions. Thornton et al [40] reported that although 100% of thymic nTreg cells expressed Helios, Helios expression in peripheral lymphoid tissues was approximately 70% of $Foxp3^+$ T cells in mice and humans. Because $Foxp3^+$ Tregs in the periphery consisted of both thymus derived nTregs and adaptive iTregs induced in second lymphoid tissues, a 70% Treg-associated Helios expression suggests that 70% of the peripheral Tregs were nTregs. Neither mouse nor human TGF-β-induced iTreg cells express Helios *in vitro* (Chen MG et al. unpublished data). Ag-specific iTreg cells induced *in vivo* also fail to express Helios [40]. Kim *et al* [32] further investigated the methylation status of the TSDR in human $CD4^+$ subsets, $Foxp3^-$, $Foxp3^+Helios^+$ and $Foxp3^+Helios^-$ cells. The results demonstrated that $Foxp3^-$ T cells express a fully methylated TSDR. In contrast, $Foxp3^+Helios^+$ cells were fully demethylated, whereas the TSDR region of the $Foxp3^+Helios^-$ subset was 45% methylated, implicating that $Helios^+$ Tregs were thymus derived. When stimulated with PMA and ionomyin *in vitro*, 5%-20% of the $Foxp3^+Helios^-$ subpopulation expanded and produced IL-2, IL-17A, or IFN-γ, whereas the $Foxp3^+Helios^+$ subset secreted lower frequencies of these cytokines [32]. Thus, Helios may potentially serve as a specific marker of thymic-derived nTreg cells.

In a tumor microenvironment, tumor-infiltrating Tregs dominantly consist of $Helios^+Foxp3^+$ cells, with markedly lower expression of CCR4 compared to circulating Tregs [41]. Peripheral blood from renal cell carcinoma (RCC) patients has a T cell subset that expresses both Foxp3 and Helios, which can be expanded by IL-2 treatment [42]. Almost 80 percent of $Foxp3^+$ Tregs in human metastatic lesions express Helios [43], indicating that increased Treg cells in tumor mainly consist of thymic-derived rather than tumor-induced iTregs.

Recently, some researchers have argued that Helios is not suitable as a marker to distinguish nTregs from iTregs cells [44]. Akimova *et al* [44] reported that Helios can be induced during T cell activation and proliferation, but regresses under resting conditions not only in human and murine Tregs but also in $CD4^+$ and $CD8^+$ T cells. In addition, they reported that Helios is selectively upregulated in CD4 T cells during Th2 and follicular helper T (Tfh) cells responses but not Th1 responses in mouse, while Helios deficiency does not participate in the Th2 and Tfh differentiation, implicating an uncertain function for Helios in T helper cells [45]. Gottschalk *et al* [46] reported that Helios is also expressed in $Foxp3^+$ iTreg both *in vitro* and *in vivo*. Helios expression in adoptively transferred TCR transgenic T cells was only transient without a second peptide stimulation. Their interpretation of these data was that Helios may not be suitable as a marker to differentiate nTregs from iTregs. In addition, Zabransky *et al* [47] also found that Helios cannot be induced by immobilized anti-CD3/soluble anti-CD28 TCR signal stimulation *in vitro*, but there were about 30 percent of $Foxp3^+Helio^+$ cells production when TCR signaling was provided with anti-CD3/CD28 microbeads. Furthermore, these $Helios^+$ iTregs exhibited a greater expression of GITR and CD103, with higher suppressive function than conventional Tregs. These findings further support the

contention that Helios may not be suitable as a specific nTreg marker.

Neuropilin 1: a new marker for nTreg cells

Neuropilin 1 (Nrp1) is a receptor for members of the vascular endothelial growth factor (VEGF) family that function in promoting angiogenesis and also as a receptor for the semaphorin family that secret neuronal guidance polypeptides [48]. Dunja *et al* [49] first reported that Neuropilin 1 is a marker of Treg cells. Neuropilin 1 was highly expressed in $CD4^+CD25^+$ Treg cells, in where Foxp3 expression connected with Nrp1 expression. In addition, new data indicate that Nrp1 plays a role in the suppressive function of Tregs. Neutralization of Nrp1 activation abrogates the suppressive ability of Tregs [49]. However, mice lacking functional Nrp1 on T cells display normal thymocyte, peripheral, conventional and $CD4^+CD25^+Foxp3^+$ regulatory T cell populations [50], suggesting that Nrp1 does not participate in the differentiation and maturation of T cells.

Not only T cells express Nrp1, it is also expressed on tumor cells as well. Gray *et al* [51] demonstrated that overexpression of Nrp1 actually reduced tumor cell growth and migration *in vitro*, suggesting that Nrp1 may have different functions for T lymphocytes and tumor cells. However, in the tumor microenvironment, Nrp1 acts as a key regulator of $Foxp3^+$ Treg cells infiltrating to the tumor tissue. Unfortunately, this results in a reduced anti-tumor immune response and promotes tumor progression [52]. T cell specific deletion of Nrp1 results in a significantly decreased tumor infiltrating $Foxp3^+$ Treg cell population and a enhanced activation of tumor killing $CD8^+$ T cells [52].

Battaglia *et al* [53] confirmed the role of Nrp1 in human Treg cells. $Nrp1^+$ Tregs are anergic *in vitro* and exert contact-dependent mechanisms to suppress effector T cell proliferation and cytokine secretion [53]. In addition, the function of $Nrp1^+$ Tregs was superior to that of $Nrp1^-$ Tregs, which can suppress effector T cell proliferation and cytokine production both *in vivo* and *in vitro* via TGF-β but not IL-10 [53, 54]. In an experimental autoimmune encephalitis (EAE) mouse model, conditional knockout of Nrp1 on $CD4^+$ T cells results in increased EAE severity [54]. Thus, $CD4^+Nrp1^+$ T cells play an important role in the maintenance of immune homeostasis.

Interestingly, a minor $CD4^+Foxp3^-Nrp1^+$ T cell subset can be detected in in human secondary lymphoid organs and peripheral blood [53, 55]. Nrp1 expression on Treg ($CD4^+CD25^+Foxp3^+$) cells is higher than $CD4^+CD25^-Foxp3^-$ cells in mouse thymus, blood, lymph nodes and spleen, but no differences of its expression are seen in human thymus, blood, lymph nodes and tonsil [55]. Nrp1 expression can be induced on peripheral blood T lymphocytes ($CD4^+$ or $CD8^+$ T cells) upon *in vitro* stimulating with plate-bound anti-CD3 and anti-CD28 stimulating antibodies [55]. It is unfortunate that this study did not evaluate the function of the $CD25^+Nrp1^+$ cells activated in this manner.

Another interesting finding is that Nrp1 is a high-affinity receptor for free LAP, LAP-TGF-β1, and active TGF-β1, whose combination promotes Treg cell activity [56]. Nrp1 combines with TGF-β to enhance canonical TGF-β downstream of Smad2/3 signaling [57]. Weiss *et al* further verified that $Nrp1^-$ Treg cells increase Nrp1 expression when stimulated with TGF-β *in vitro* [58].

Recently, there were two papers published to identify Nrp1 as a marker which distinguishes nTregs from iTregs [12, 58]. Using a myelin basic protein (MBP)–TCR-transgenic (Tg) recombination activation gene (RAG)–deficient mouse model, Yadav *et al* showed that this mouse lacked $CD4^+Foxp3^+$ Treg cells in the thymus, but Tregs emerged in the periphery after 4-6 weeks age even without the thymus, thus they could be defined as iTregs [12]. These peripheral iTreg cells produced *in vivo* consist of only 6% of Nrp1 expression and 25% of Helios expression, while WT control Tregs were composed of 57% of Nrp1 expression and 60% of Helios expression [12]. These data imply that while both Nrp1 and Helios are mainly expressed on nTreg cells, Nrp1 may serve as a better marker for nTregs. Further evidence came in the form of iTreg cells generated *in vivo* with specific peptide stimulation. These iTregs were predominantly $Nrp1^{low}$ [12]. In the intestinal or colonic lamina propria, a signature location for the peripheral induction of Tregs, the percentage of $Nrp1^-Foxp3^+$ Treg cells was higher than in secondary lymphoid tissues [12, 58]. When compared with $Nrp1^+Foxp3^+$ cells, $Nrp1^-Foxp3^+$ cells exhibit lower expression of *Nrp1*, *Helios* and *Swap70* transcripts and higher expression of *Dapl1* and *Igfbp4* transcripts, while both Tregs

express similar levels of Foxp3 mRNA [58]. Both $Nrp1^{low}$ iTreg and $Nrp1^{hi}$ nTreg cells were able to comparably suppress autoimmunity which might correlate to specific TCR signaling [12]. About 1% $Foxp3^{-}Nrp1^{+}$ cells were detected in the thymus and secondary lymphoid organs, but further investigation using intrathymic injection suggested that these $Foxp3^{-}Nrp1^{+}$ cells did not up-regulate Foxp3 expression after 2 weeks of injection, then this population may not be related to the Treg cell lineage [58].

The vast majority of tumor infiltrating lymphocytes Treg cells are $Nrp1^{-}Foxp3^{+}$ iTreg cells, while splenic $Foxp3^{+}$ Treg cells in the same mice were predominantly $Nrp1^{+}$ [58]. Unexpectedly, in inflammatory environments such as central nervous system (CNS) of mice with chronic spontaneous experimental autoimmune encephalomyelitis (EAE), the majority of Treg cells were $Nrp1^{+}$, while most of splenic Treg cells were also $Nrp1^{+}$ in the same mice [58]. One possible explanation is that some antigens and inflammatory cytokines may drive Nrp1 expression with unknown regulated mechanism(s).

Summary

$Foxp3^{+}$ nTreg and iTreg cells may have different functions, some of which are overlapping. However, it's still hard to define exactly the true diversity of Tregs due to paucity of markers to distinguish these unique cell types. A recently published paper has proposed a new promising candidate, Nrp1, as a $Foxp3^{+}$ nTreg marker. Nrp1 expression on nTreg may provide a superior marker to- or be used in conjunction with Helios to identify these cells. The major difference is that Nrp1 is a cell surface marker and thus can be used in the isolation and characterization of functional (live) nTregs for further study. Although there are still some confusing/conflicting data demonstrating that iTregs produced under inflammatory conditions express some level of Nrp1, this protein may serve as an excellent marker for nTreg cells under normal physiological conditions. While much work remains to be done concerning Nrp1 expression, function and distribution, its immediate utility is of great value to those studying Tregs. Furthermore, the excitement generated surrounding its description as a marker that can distinguish nTregs from iTregs highlights the need to find additional cell surface markers that will serve this important function.

Conflict of interest statement

The authors declare no competing financial interests.

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