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Approaches to Discriminate Naturally Induced Foxp3$^+$ Treg cells of Intra- and Extrathymic Origin: Helios, Neuropilin-1, and Foxp3$^{RFP/GFP}$

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Abstract

CD4$^+$ regulatory T (Treg) cells expressing the transcription factor fork head box P3 (Foxp3) prevent catastrophic autoimmunity and maintain immune homeostasis throughout life, and are being increasingly implicated in non-immune functions, such as the control of metabolic and regenerative processes in mice and humans. Early studies have attributed a major role of the thymus and intrathymic Foxp3$^+$ Treg lineage commitment (referred to as 'tTreg' cells) in the establishment and maintenance of the mature Foxp3$^+$ Treg cell pool residing in peripheral lymphoid tissue. In addition, numerous experimental modalities have been shown to instruct Foxp3$^+$ Treg cell commitment in peripheral, initially naïve CD4$^+$Foxp3$^-$ T cells, which includes the induction of a Foxp3$^+$ Treg cell phenotype and suppressor function by TGF-β *in vitro* ('iTreg' cells) and by sub-immunogenic T cell receptor stimulation *in vivo* ('pTreg' cells). This led to the hypothesis that, under physiological conditions, the induction of pTreg cells may also contribute to the peripheral Foxp3$^+$ Treg cell compartment in the steady state of nonmanipulated, immunocompetent mice. However, until recently, studies on developmental Foxp3$^+$ Treg cell heterogeneity have been hampered by the lack of suitable markers to discriminate naturally induced tTreg and pTreg cells. Here, we provide an overview of recently proposed approaches to track such developmental sub-lineages, with a particular emphasis on Helios, Neuropilin-1, and Foxp3$^{RFP/GFP}$ mice, in which Foxp3^{RFP+} tTreg and pTreg cells are stably marked by differential GFP expression.

Keywords: T cell development; tTreg; pTreg; iTreg; Foxp3

Introduction

Apart from the artificial induction of a Foxp3$^+$ regulatory T (Treg) cell phenotype in post-thymic, initially naïve CD4$^+$Foxp3$^-$ T cells [1], there is accumulating evidence that, under physiological conditions, Foxp3$^+$ Treg cells are generated both within the thymus (referred to as 'tTreg' cells) and extrathymically at peripheral sites ('pTreg' cells). Reminiscent of Foxp3$^+$ tTreg cell development mediated by developmental progression of CD4$^+$CD8$^-$ single-positive (CD4SP) thymocyte precursors with a CD25$^+$Foxp3$^-$ phenotype [2], mature Foxp3$^+$ pTreg cells continuously differentiate from precommitted CD4$^+$Foxp3$^-$CD25$^+$ pTreg cell precursors in peripheral lymphoid tissues of nonmanipulated mice [1,3] and act in concert with tTreg cells to enforce immune tolerance [4-7]. The identification of immediate CD4$^+$Foxp3$^-$CD25$^+$ pTreg cell precursors provided direct evidence that extrathymic developmental pathways feed into the overall pool of mature Foxp3$^+$ Treg cells in the steady state [3]. However, the lack of unambiguous markers to faithfully discriminate naturally induced Foxp3$^+$ tTreg and pTreg cells remained a considerable limitation of experimental efforts to directly address the processes that generate such developmental sub-lineages and govern their incorporation into the mature Treg cell pool, which exhibits remarkable phenotypic heterogeneity [8]. A universal marker to discriminate murine Foxp3$^+$ tTreg and pTreg cells should preferably fulfill several criteria: i) exclusive expression either by tTreg or pTreg cells; ii) its expression status should be highly stable and preserved under pro-inflammatory conditions; and iii) it should enable the isolation and sub-fractionation of viable cells for downstream applications, such as gene expression surveys, functional studies, and Foxp3$^+$ Treg cell-based therapies. Moreover, a murine marker with evolutionary conservation across species may facilitate translation to human CD4$^+$FOXP3$^+$ Treg cells. While a marker combining all above characteristics remains to be identified, several approaches to discriminate tTreg and pTreg cells have recently been proposed: the Ikaros transcription factor Helios (Ikzf2) [9], the surface marker Neuropilin-1 (Nrp-1) [10,11], and differential GFP expression in Foxp3^{RFP+} Treg cells of Foxp3$^{RFP/GFP}$ mice [7].

Endogenous markers for tTreg/pTreg cell discrimination: Helios and Nrp-1

Helios: Initial studies by Shevach and colleagues [9] suggested that Helios might be selectively expressed in Foxp3$^+$ tTreg cells. This

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conclusion was based on three key findings: i) In the steady state, Helios was found to be co-expressed in the vast majority (≥ 99%) of developing Foxp3⁺ CD4SP thymocytes, whereas its expression was restricted to ~70% of peripheral CD4⁺Foxp3⁺ Treg cells; ii) Foxp3⁺ iTreg cells generated from naïve CD4⁺Foxp3⁻ T cells *in vitro* by TCR stimulation in the presence of added TGF-β maintained a Helios⁻ phenotype; and iii) Foxp3⁺ pTreg cells generated from TCR transgenic CD4⁺Foxp3⁻ T cells *in vivo* after adoptive transfer into immunocompetent recipients and subsequent oral antigen administration also failed to up-regulate Helios expression. Overall, these data are consistent with the interpretation that the Foxp3⁺Helios⁻ phenotype of ~30% of peripheral Treg cells is indicative of their extrathymic developmental origin.

Subsequent studies [12-15] further corroborated the initial observation [9] that TGF-β-driven Foxp3⁺ iTreg cell generation fails to promote Helios co-expression, when TCR stimulation was mediated by plate-bound anti-CD3 monoclonal antibodies (mAbs) ± co-stimulatory anti-CD28 mAbs. However, TCR stimulation through peptide antigen-loaded splenocytes [12,13] or anti-CD3/CD28 mAb-coated beads (i.e. in the absence of antigen-presenting cells; APCs) [15] resulted in robust Helios expression in ~50% of newly generated CD4⁺Foxp3⁺ iTreg cells, irrespective of whether or not IL-2R signaling was abrogated by neutralizing anti-IL-2 mAbs (Figure 1A). *In vivo*, and in contrast to oral antigen feeding [9], Foxp3⁺ pTreg cell induction by intravenous antigen administration [12] or recombinant anti-DEC-205 Ab-mediated delivery of antigen to DEC-205⁺ DCs (Figure 1B) [16-18] resulted in Helios co-expression in the majority of *de novo* induced Foxp3⁺ pTreg cells, although continued TCR stimulation is required to maintain high levels of Helios expression [12].

We also tracked Helios expression in the progeny of physiological CD4⁺(CD8⁻)CD25⁺Foxp3⁻ Treg cell precursors from thymus [2] and peripheral lymphoid tissues [3] of nonmanipulated Foxp3^{GFP} mice. In the absence of deliberate TCR and TGF-βR stimulation, developmental progression of thymic CD25⁺Foxp3⁻ CD4SP tTreg cell precursors in IL-2-supplemented cultures were accompanied by Helios expression in the majority of differentiated Foxp3⁺ tTreg cells (Figure 1C, left panel). In contrast, only ~50% of lymph node-derived CD4⁺CD25⁺Foxp3⁻ pTreg cell precursors acquired Helios expression during *in vitro* differentiation into Foxp3⁺ pTreg cells (Figure 1C, right panel). When we track naturally induced Foxp3⁺ Treg cells of intrathymic (RFP⁺GFP⁺ tTreg) and peripheral (RFP⁺GFP⁻ pTreg) origin in peripheral lymphoid tissues of nonmanipulated Foxp3$^{RFP/GFP}$ mice (see below), it became clear that >90% of mature RFP⁺GFP⁺ tTreg cells expressed Helios, whereas ~50% of their RFP⁺GFP⁻ pTreg cell counterpart were Helios⁻ [7].

In summary, induction of Helios expression during Foxp3⁺ Treg cell development appears more context-dependent than initially anticipated and may not be exclusive to tTreg cells. Nevertheless, lack of Helios expression identifies a significant proportion of Foxp3⁺ pTreg cells in lymphoid tissues of steady-state mice. Clearly, some modalities of Foxp3⁺ iTreg and pTreg cell generation promote Helios expression, which might be transient, unless continuous TCR stimulation is provided [12,19]. The mechanisms underlying differential Helios expression in developmental Foxp3⁺ Treg cell sub-lineages are likely to include differences in co-stimulatory and/or cytokine receptor signaling during the induction phase of Foxp3 expression. In any case, transgenic mice with Helios-dependent fluorochrome reporter expression [20]

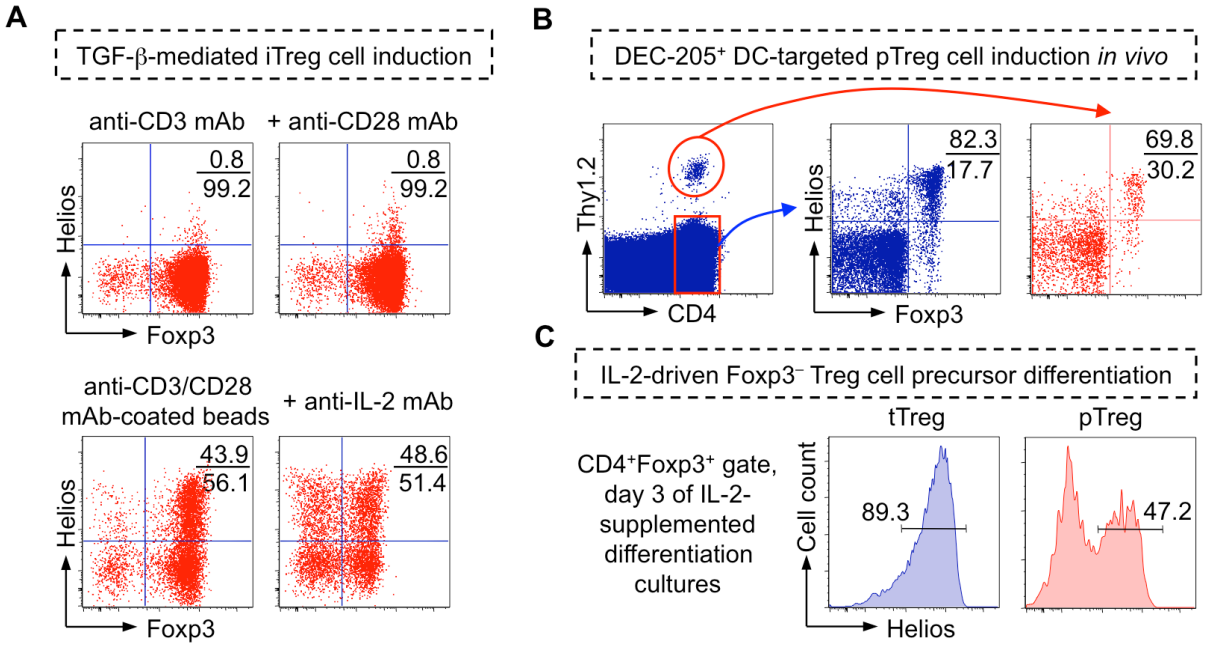

Figure 1: Helios expression in extrathymically induced Foxp3⁺ Treg cells. Flow cytometry of Foxp3 and Helios protein was performed at day (A, C) three and (B) 14 using the fluorochrome-conjugated mAbs FJK-16s and 22F6, respectively. (A) TGF-β-mediated Foxp3⁺ iTreg cell induction. Naïve CD4⁺ T cells (CD62L^{high}CD25⁻ Foxp3^{GFP-}) were FACS purified from peripheral lymphoid organs of Foxp3^{GFP} mice and TCR stimulated in the presence of added IL-2 (100 U/ml) and TGF-β (5 ng/ml). TCR stimulation, top panels: plate-bound anti-CD3 mAbs (5 µg/ml) ± soluble anti-CD28 mAbs (1 µg/ml); bottom panels: anti-CD3/CD28 mAb-coated beads (2 beads/cell) ± neutralizing anti-IL-2 mAbs (20 µg/ml). (B) DEC-205⁺ DC-targeted Foxp3⁺ pTreg cell induction *in vivo*. Immunocompetent Thy1.1⁺ recipients of initially naïve Thy1.2⁺CD4⁺Foxp3⁻Helios⁻ T cells with transgenic TCR-HA$_{107-119}$ expression were injected with anti-DEC-205/HA$_{107-119}$ fusion antibodies (50 ng), and induced Foxp3 and Helios expression was assessed among gated Thy1.2⁺CD4⁺ T cells. Lines with arrowheads illustrate the gating scheme. (C) Precommitted Foxp3^{GFP-} Treg cell precursors were FACS isolated from thymus (CD4⁺CD8⁻CD25⁺Foxp3^{GFP-}) and lymph nodes (CD4⁺CD25⁺Foxp3^{GFP-}) of Foxp3^{GFP} mice and cultured with IL-2 (1000 U/ml) in the absence of deliberate TCR and TGF-βR stimulation. Numbers in histograms show percentages of Helios co-expression among differentiated Foxp3⁺ tTreg (left) and pTreg (right) cells. Adapted from Schallenberg et al. [3,15].

will pave the way to fully establish the relationship between Helios$^+$ and Helios$^-$ Treg cells. Another unresolved question is whether Helios expression can be dynamically regulated during the lifetime of mature Foxp3$^+$ Treg cell populations.

Nrp-1: Two studies [10,11] proposed that differential Nrp-1 expression can be exploited to distinguish Foxp3$^+$ tTreg and pTreg cells under non-inflammatory conditions: the majority of developing Foxp3$^+$ CD4SP thymocytes and ~70% of peripheral CD4$^+$Foxp3$^+$ Treg cells were Nrp-1^{high}, whereas low Nrp-1 expression levels on Foxp3$^+$ Treg cells correlated with their extrathymic developmental origin in certain experimental settings of pTreg cell induction; this included homeostatic proliferation in lymphopenic recipient mice, free antigen administration (oral, intravenous, or *via* subcutaneously implanted osmotic mini-pumps), and spontaneous pTreg cell differentiation in Rag$^{-/-}$ mice with transgenic expression of a myelin basic protein (MBP)-reactive TCR. Consistent with the important role of the microbiota in intestinal pTreg cell homeostasis, Foxp3$^+$Nrp-1^{low} Treg cells were severely reduced in the lamina propria of germ-free mice, whereas this intestinal subset was enriched in mice housed under specific pathogen-free conditions, as compared to other secondary lymphoid tissues [10]. Also, in peripheral lymphoid tissues of mice with partial pTreg cell deficiency due to targeted deletion of an enhancer element (CNS1) within the *Foxp3* gene locus that mediates TGF-β responsiveness during iTreg and pTreg cell development [5,21], the proportion of Nrp-1^{low} cells among CD4$^+$Foxp3$^+$ Treg cells was ~2-fold reduced, as compared to CNS1-proficient mice [10].

Nonetheless, the correlation between extrathymic Treg cell development and their Foxp3$^+$Nrp-1^{low} phenotype may not be absolute, both *in vitro* and *in vivo*: While CD4$^+$Foxp3$^-$ TCR-MBP$^+$ T cells failed to up-regulate Nrp-1 expression upon TGF-β-mediated Foxp3$^+$ iTreg cell generation, irrespective of whether TCR stimulation was mediated by anti-CD3/CD28 mAbs or peptide antigen-loaded APCs [11], polyclonal CD4$^+$Foxp3$^-$ T cells significantly up-regulated Nrp-1 expression during TGF-β-driven Foxp3 induction upon anti-CD3/CD28 mAb-mediated TCR stimulation [10]. Along the same lines, oral antigen feeding of lymphopenic recipients of ovalbumin-reactive CD4$^+$Foxp3$^-$DO11.10$^+$ T cells promoted induction of Foxp$^+$3 pTreg cells with a predominantly Nrp-1^{low} phenotype [10], but oral antigen feeding of Rag1$^{-/-}$ × DO11.10$^+$ mice promoted Nrp-1^{high} expression on up to 70% of *de novo* induced Foxp3$^+$DO11.10$^+$ pTreg cells [7]. Some of the observed discrepancies in Nrp-1 up-regulation during iTreg/pTreg cell generation can potentially be attributed to differences in TCR/co-stimulatory signaling and the kinetics of transient Nrp-1 expression [7,10]. Still, a Nrp-1$^{low/-}$ phenotype is suitable to track Foxp3$^+$ pTreg cells, at least under non-inflammatory/sub-immunogenic conditions and in the intestinal lamina propria.

Correlating Helios and Nrp-1 expression: In adult mice, Helios and Nrp-1 expression by peripheral Foxp3$^+$ Treg cells closely correlate in that the majority of Helios$^+$ cells co-express Nrp-1 [10,11,20], but a significant fraction of Foxp3$^+$Helios$^-$ Treg cells is Nrp-1^{high}, resulting in <40% of Foxp3$^+$Helios$^-$ Treg cells with a Nrp-1^{low} phenotype [20]. One plausible explanation for discordant Helios and Nrp-1 expression is that the Nrp-1^{low} phenotype may not represent an immutable characteristic of Foxp3$^+$ pTreg cells. In fact, while TCR-MBP$^+$ Treg cells remained Nrp-1^{low}, when stimulated *in vitro* with peptide antigen-loaded APCs [11], TCR stimulation using anti-CD3/CD28 mAbs [10] or anti-CD3/CD28 mAb-coated beads [7] up-regulated Nrp-1 expression on polyclonal Foxp3$^+$Nrp-1^{low} Treg cells. Upon adoptive transfer into otherwise nonmanipulated immunocompetent recipient mice, FACS-purified Nrp-1$^+$ and Nrp-1^{low} Treg cell populations largely maintained their respective Nrp-1 expression status [10,11]; in contrast, adoptively transferred Foxp3$^+$Nrp-1^{low} Treg cells increased Nrp-1 expression levels in lymphopenic recipients [11] and at site of acute inflammation, such as the central nervous system of mice with acute experimental autoimmune encephalomyelitis [11], indicating that Nrp-1 expression can indeed be up-regulated under pro-inflammatory conditions.

In steady-state Foxp3$^{RFP/GFP}$ mice (see below) [7], >45% of lymph node-derived RFP$^+$GFP$^-$ pTreg cells expressed Nrp-1 *ex vivo*, at levels comparable to that of RFP$^+$GFP$^+$ tTreg cells. *In vitro*, RFP$^+$GFP$^-$ pTreg cells that initially lacked Nrp-1 expression readily acquired Nrp-1 expression in IL-2-supplemented TCR stimulation cultures. These observations further support the notion that the Nrp-1 expression status of naturally induced tTreg and pTreg cells can be dynamically regulated under T cell stimulatory conditions. Consistently, at day 16 after injection of NOD.Rag1$^{-/-}$ mice with CD4$^+$Foxp3$^-$BDC2.5$^+$ T cells exhibiting a naïve CD62L^{high}CD44^{low} phenotype, ~70% of homeostatically converted Foxp3$^+$ pTreg cells expressed high levels of Nrp-1 [7].

Differential BAC.Foxp3^{GFP} reporter expression in Foxp3^{RFP+} Treg cells as a transgenic approach for tTreg/pTreg cell discrimination

Our studies in the Foxp3$^{RFP/GFP}$ model were initiated by the serendipitous observation that peripheral lymphoid tissues of mice with Foxp3 BAC-driven expression of a Cre recombinase-GFP (Cre-GFP) fusion protein (BAC-Foxp3$^{Cre-GFP}$) [22] harbor a sizable CD4$^+$ Treg cell population that is GFP$^-$ but expresses Foxp3 protein [7,23], as judged by intracellular staining using FACS-purified CD4$^+$CD25$^+$GFP$^-$ T cells and fluorochrome-conjugated anti-Foxp3 mAbs (Figure 2). To assess whether such peripheral Foxp3$^+$GFP$^-$ Treg cells resulted from down-regulated BAC-Foxp3$^{Cre-GFP}$ reporter expression, we performed genetic fate mapping studies employing BAC-Foxp3$^{Cre-GFP}$ × R26Y mice, in which cells are marked by ROSA26-driven expression of yellow fluorescent protein (YFP) after Cre recombinase-mediated, irreversible excision of a loxP-flanked STOP cassette [24]. However, rather than transgene silencing in initially Foxp3$^+$GFP$^+$ Treg cells (e.g. by epigenetic mechanisms), these studies established that the BAC-Foxp3$^{Cre-GFP}$ reporter was transcriptionally inactive throughout the entire development and lifespan of the Foxp3$^+$GFP$^-$ Treg cell population [7]. Consistently, in BAC-Foxp3$^{Cre-GFP}$ mice additionally expressing RFP from an IRES downstream of the Foxp3 coding region (Foxp3$^{IRES-RFP}$) [25], essentially all peripheral Foxp3$^+$GFP$^-$ Treg cells co-expressed Foxp3$^{IRES-RFP}$ (Figure 3A) [7]. Conversely, a considerable fraction of peripheral Foxp3$^{IRES-RFP+}$ Treg cells consistently lacked any BAC-Foxp3$^{Cre-GFP}$ expression, and this RFP$^+$GFP$^-$ population increased from ~20% in adult to up to 45% in aged mice (Figure 3A).

Selective BAC-Foxp3$^{Cre-GFP}$ reporter activity in Foxp3$^+$ tTreg cells but not iTreg/pTreg cells

To determine the origin of peripheral Foxp3$^+$ Treg cells with a RFP$^+$GFP$^-$ phenotype, we next analyzed the activity of the BAC-Foxp3$^{Cre-GFP}$ reporter in thymic and peripheral pathways of Foxp3$^+$ Treg lineage commitment.

Intrathymic BAC-Foxp3$^{Cre-GFP}$ reporter activity: In the thymus of Foxp3$^{RFP/GFP}$ mice, the vast majority of GFP-expressing CD4$^+$CD8$^+$ double-positive (DP) and CD4SP cells co-expressed RFP, and intrathymically injected RFP$^-$GFP$^-$CD25$^+$ CD4SP tTreg cell precursors efficiently up-regulated both RFP and GFP during developmental

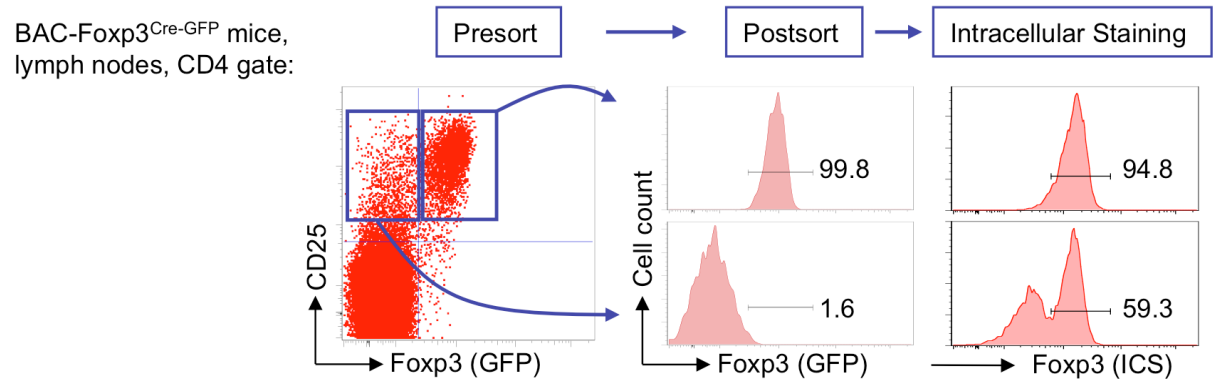

Figure 2: Tracking CD4⁺Foxp3⁺ Treg cells that lack GFP expression in peripheral lymphoid tissues of BAC-Foxp3$^{Cre-GFP}$ mice. The dot plot depicts presort analysis of CD25 and GFP expression among gated CD4⁺ T cells from lymph nodes of BAC-Foxp3$^{Cre-GFP}$ mice. Histograms show postsort analysis of Foxp3-driven GFP expression (left) and Foxp3 expression, as revealed by intracellular staining (ICS) using anti-Foxp3 mAbs (right), among indicated postsort populations. Adapted from Schallenberg et al. [23].

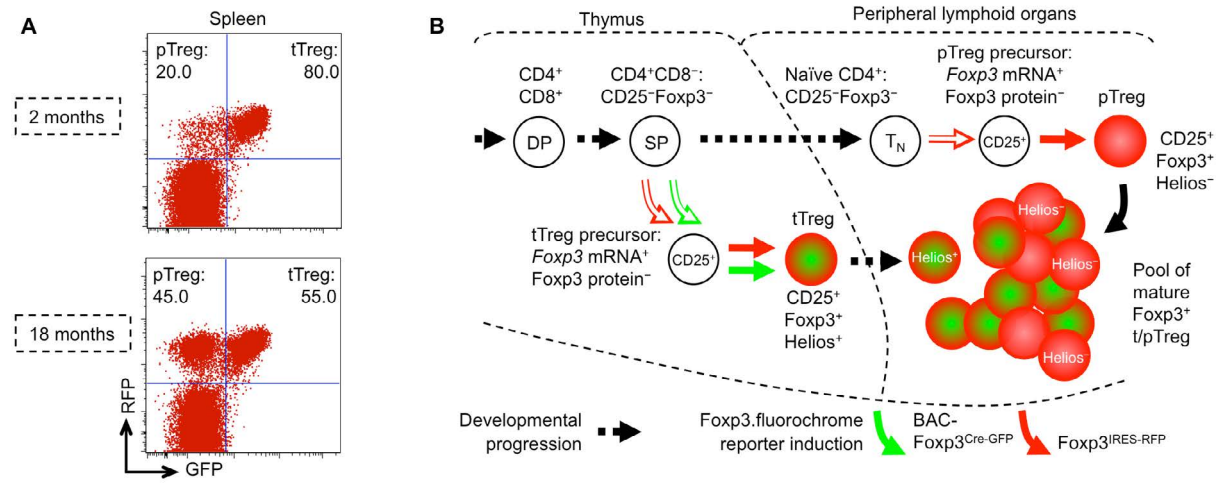

Figure 3: Discrimination of mature Foxp3^{RFP+} tTreg and pTreg cells in peripheral tissues of Foxp3$^{RFP/GFP}$ mice based on differential GFP expression. (A) Flow cytometry of RFP/GFP expression among CD4-gated cells in spleen of 2-month-old (top) and 18-month-old (bottom) Foxp3$^{RFP/GFP}$ mice. Numbers in dot plots indicate percentages of GFP⁻ pTreg cells and GFP⁺ tTreg cells among total RFP⁺ Treg cells in the respective quadrant. (B) In the thymus, tTreg-cell development is mediated by CD4⁺CD8⁻ single-positive (SP) thymocytes that exhibit a CD25⁺Foxp3⁻ phenotype (Foxp3 mRNA⁺, Foxp3 protein⁻) and are precommitted to up-regulate Foxp3 expression upon IL-2 receptor signaling. BAC-Foxp3$^{Cre-GFP}$ expression is induced in such Treg cell lineage committed CD4SP precursors and maintained in their peripheral tTreg cell progeny after thymic export. In contrast, outside the thymus, IL-2 promotes the differentiation of precommitted CD25⁺Foxp3⁻ pTreg precursors (Foxp3 mRNA⁺, Foxp3 protein⁻) into Foxp3⁺ pTreg cells that fail to up-regulate BAC-Foxp3$^{Cre-GFP}$ expression, resulting in mature Foxp3⁺GFP⁻ pTreg cells. Foxp3$^{IRES-RFP}$ expression marks all peripheral Foxp3⁺ Treg cells, irrespective of their GFP expression status. In the steady state, Helios⁻ Treg cells are enriched among RFP⁺GFP⁻ pTreg cells, although this correlation is not absolute. Adapted from Petzold et al. [7].

progression to Foxp3⁺ CD4SP thymocytes; consistently, in peripheral lymphoid tissues of recipient mice, the progeny of intrathymically injected GFP⁻CD25⁺ CD4SP tTreg cell precursors exhibited a RFP⁺GFP⁻ tTreg cell phenotype [7]. Furthermore, in a well-characterized double-transgenic model (TCR-HA × Pgk-HA) of intrathymic Foxp3⁺ Treg cell induction [26], recognition of a thymic epithelium-derived model antigen (influenza hemagglutinin, HA) by developing TCR-HA⁺Foxp3⁻ CD4SP cells promoted intrathymic induction and peripheral accumulation of TCR-HA⁺Foxp3⁺ tTreg cells with a RFP⁺GFP⁺ phenotype [7]. These data indicate that the BAC-Foxp3$^{Cre-GFP}$ reporter is efficiently upregulated during tTreg cell lineage commitment *in vivo*. Consequently, Foxp3-driven RFP and GFP co-expression in peripheral CD4⁺Foxp3⁺ Treg cells closely correlates with their thymic developmental origin (Figures 3A and 3B).

Extrathymic BAC-Foxp3$^{Cre-GFP}$ reporter activity: In striking contrast to intrathymic tTreg lineage commitment, the BAC-Foxp3$^{Cre-GFP}$ reporter was refractory to GFP up-regulation in post-thymic, peripheral CD4⁺Foxp3⁻ T cells in various settings of Foxp3 induction:

i. During Foxp3⁺ iTreg cell generation in TGF-β-supplemented TCR stimulation cultures, initially naïve CD4⁺Foxp3⁻ T cells failed to up-regulate BAC-Foxp3$^{Cre-GFP}$ expression, despite efficient induction of Foxp3 protein and Foxp3$^{IRES-RFP}$ expression; this striking defect was already established at the transcriptional level and maintained under conditions of enhanced Foxp3 induction, e.g. by the addition of excessive amounts of TGF-β or the vitamin A metabolite retinoic acid. The selective BAC-Foxp3$^{Cre-GFP}$ reporter inactivity *in vitro* could also not be over

come by pharmacological inhibition of DNA methyltransferases and CpG methylation by 5-Aza-2-deoxycytidine (5-aza--dC) [18,27], not withstanding efficient 5-aza-dC-mediated Foxp3$^+$ iTreg cell generation in the absence of added TGF-β.

ii. The differentiation of lymph node-derived physiological CD4$^+$CD25$^+$Foxp3$^-$ pTreg cell precursors into Foxp3$^+$ pTreg cells, either *in vitro* in IL-2-supplemented cultures (in the absence of deliberate TCR or TGF-βR stimulation) or upon adoptive transfer into IL-2-proficient recipient mice, coincided with the up-regulation of Foxp3$^{IRES\text{-}RFP}$ expression, whereas expression of the BAC-Foxp3$^{Cre\text{-}GFP}$ reporter remained below the detection limit. Additionally, several well-established experimental modalities of Foxp3$^+$ pTreg cell induction *in vivo* failed to promote BAC-Foxp3$^{Cre\text{-}GFP}$ reporter expression, including lymphopenia-driven proliferation of CD4$^+$CD25$^-$Foxp3$^-$ T cells expressing a transgenic BDC2.5 TCR after adoptive transfer into Rag1$^{-/-}$ recipients, adoptive transfer of CD4$^+$CD25$^-$Foxp3$^-$ BDC2.5$^+$ T cells into fully immunocompetent recipients and subsequent DEC-205$^+$ DC-targeted delivery of minute amounts of a mimotope peptide to steady-state DCs, as well as oral ovalbumin feeding of Rag2-deficient Foxp3$^{RFP/GFP}$ mice with transgenic DO11.10 TCR expression.

Harnessing Foxp3$^{RFP/GFP}$ mice for studies on developmental Treg cell heterogeneity

The above outlined experiments make a strong case for a scenario in which the activity of the BAC-Foxp3$^{Cre\text{-}GFP}$ reporter is exclusively confined to tTreg lineage commitment due to a previously unrealized defect in post-thymic CD4$^+$ T cells. This finding provides a unique opportunity to track viable populations of naturally occurring RFP$^+$GFP$^+$ tTreg and RFP$^+$GFP$^-$ pTreg cells in peripheral lymphoid tissues of otherwise nonmanipulated Foxp3$^{RFP/GFP}$ mice (Figure 3B). Consistently, Foxp3$^+$ Treg cells with a RFP$^+$GFP$^-$ phenotype are enriched in Peyer's patches of the small intestine of Foxp3$^{RFP/GFP}$ mice [7], a site known to support pTreg cell induction [28]. Consistent with their distinct developmental origins, global transcriptional signatures of RFP$^+$GFP$^+$ and RFP$^+$GFP$^-$ Treg cells from the same anatomical location (e.g. spleen or lymph nodes) exhibit considerable differences in the expression of genes encoding T-cell transcription factors, chemokine receptors, and effector molecules implicated in suppressor function [7]. Thus, the sub-phenotypes of tTreg and pTreg cells are largely specified by their developmental origin, rather than by microenvironmental imprinting. Another key requisite for exploiting differential GFP expression among RFP$^+$ Treg cells in Foxp3$^{RFP/GFP}$ mice as a universal marker to discriminate tTreg and pTreg cells is the preservation of their GFP expression status under pro-inflammatory conditions. In fact, the overwhelming majority of FACS-purified populations of RFP$^+$GFP$^+$ and RFP$^+$GFP$^-$ Treg cells stably maintained their fluorochrome expression status under TCR stimulatory conditions, both *in vitro* (APCs + anti-CD3 mAbs or titrating amounts of anti-CD3/CD28 mAb-coated beads ± IL-2, either alone or in the presence of cytokine-secreting CD4$^+$ T effector cells) and under highly inflammatory conditions *in vivo* (lymphopenia-induced proliferation, autoimmune diabetes, *etc.*) [7]. Additionally, although RFP$^+$GFP$^+$ tTreg and RFP$^+$GFP$^-$ pTreg cells were similarly effective in controlling the activation and proliferation of co-cultured conventional CD4$^+$ T effector cells *in vitro*, the assessment of their suppressor function *in vivo* revealed marked qualitative differences between both developmental Treg cell sub-lineages: the *in vivo* accumulation of adoptively transferred RFP$^+$GFP$^+$ tTreg and RFP$^+$GFP$^-$ pTreg cell populations was largely comparable, but RFP$^+$GFP$^+$ tTreg cells more potently suppressed lymphopenic proliferative expansion of co-transferred conventional CD4$^+$ T cells, and RFP$^+$GFP$^-$ pTreg cells were far more efficient in the autoimmune protection of insulin-producing cells in an BDC2.5-based adoptive transfer model of type 1 diabetes [7].

Conclusion and Future Perspectives

While the exact mechanistic cause for the restricted BAC-Foxp3$^{Cre\text{-}GFP}$ reporter activity during tTreg cell differentiation remains to be determined, the differential expression of GFP in Foxp3$^{RFP/GFP}$ mice provides a unique opportunity to track viable populations of RFP$^+$GFP$^+$ tTreg cells and RFP$^+$GFP$^-$ pTreg cells in otherwise nonmanipulated mice. Importantly, the GFP expression status (either GFP$^+$ or GFP$^-$) of Foxp3$^+$RFP$^+$ Treg cells is preserved under pro-inflammatory conditions, which represents a key advantage of the Foxp3$^{RFP/GFP}$ model. Future studies on the synergistic action of tTreg and pTreg cells [4-6] under physiological conditions [7] will be facilitated by the recent generation of Foxp3$^{RFP/GFP}$ mice on genetically well-defined backgrounds that are commonly used for immunological research (e.g. C57BL/6, BALB/c, NOD, *etc.*). This may include studies on the relative contribution of tTreg and pTreg cells in clinical settings of exacerbated (chronic infections by microbes and parasites, cancer) and insufficient (autoimmunity, allergies, transplant rejection) Treg cell suppressor activity. Additionally, genome-wide single-cell transcriptomics of RFP$^+$GFP$^+$ tTreg cells and RFP$^+$GFP$^-$ pTreg cells may help identifying a surface marker (or a combination of surface markers) suitable to discriminate developmental CD4$^+$FOXP3$^+$ Treg cell sub-lineages in humans.

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