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REVIEW

Foxp3$^+$ regulatory T cells: differentiation, specification, subphenotypes

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Regulatory T cells (T_{reg} cells) characterized by expression of the transcription factor Foxp3 play a key role in immune homeostasis. Rather than a monomorphic population strictly determined by Foxp3 as a 'master regulator', the emerging view is one of T_{reg} cells as a population with many levels of complexity. Several regulatory factors partake in the control of their transcriptional 'signature', with Foxp3 being a key regulator but insufficient and unnecessary to specify all aspects of the lineage. Distinct subphenotypes of Foxp3$^+$ T_{reg} cells are found in different anatomical locations. Some subphenotypes specifically control different facets of effector T cell function and, perhaps surprisingly, share transcriptional control elements with the very cells they regulate. This review will focus on these novel aspects of T_{reg} cell diversity.

Any biological system involves negative feedback, and it is now recognized that regulatory T cells (T_{reg} cells) play key roles in the maintenance of lymphoid homeostasis in a number of immune circumstances. These cells maintain tolerance to self and control autoimmune deviation[1,2], prevent runaway responses to pathogens or allergens, help maintain a balance with obligate microbial flora[3], and facilitate tumors' escape from immune monitoring[4]. Although several distinct lineages may participate in these functions, an important population was initially identified in the mouse as CD4$^+$CD25$^+$ or CD4$^+$CD45RB$^-$ and was able to control autoimmunity elicited by thymectomy or lymphopenic complementation[5,6]. A firm molecular definition for these cells came about with the discovery that they express the forkhead–winged helix transcription factor Foxp3 (refs. 7–10) and that deficiencies in Foxp3 underlie the lymphoproliferation and multiorgan autoimmunity of *scurfy* mutant mice and human patients with immunodysregulation polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome[11]. Foxp3$^+$ T_{reg} cells use the αβ T cell antigen receptor (TCR) for antigen recognition and have a broad TCR repertoire similar in size but largely distinct in composition relative to that of CD4$^+$ conventional T cells (T_{conv} cells)[12–14]. The mechanisms of action of T_{reg} cells are clearly pleiomorphic, and several modes and mediators of their activity that are not mutually exclusive have been described, whose relative importance has yet to be sorted out[15]. Because of their fundamental importance for immune function and because of their great potential for therapeutic modulation, Foxp3$^+$ T_{reg} cells have attracted extraordinary interest.

A wide array of mice with conditional knockout of genes and mice expressing transgenes that report T_{reg} cell existence or function have been constructed, and these have been the subject of intense genomic, genetic and epigenetic investigation. More genome-wide transcriptional profiles have been generated on T_{reg} cells than on any other immune cell type, which has resulted in the definition of a canonical 'T_{reg} signature' that distinguishes T_{reg} cells from T_{conv} cells, at least in their resting states in lymphoid organs[16–21] (**Fig. 1**). The T_{reg} signature includes genes overexpressed or repressed in T_{reg} cells (in a proportion of 2 to 1), genes that encode proteins with a wide range of cellular locations and functions: cell surface receptors, signaling kinases and transcription factors. With bioinformatic treatments that can detect fine variations, up to ~1,500 genes are found to be differentially expressed in T_{reg} cells[21], but none or very few of these differences are absolute; instead, these variations correspond to quantitative differences between T_{reg} cells and T_{conv} cells. Nor are they specific, as almost all transcripts overexpressed in T_{reg} cells can also be found in non–T cell lineages. A fraction of these T_{reg} signature genes have also been identified in chromatin immunoprecipitation experiments with antibody to Foxp3 (refs. 22,23). However, the overlap between differentially expressed genes and *Foxp3*-bound genes is not absolute, in part because of the technical limitations of chromatin immunoprecipitation and in part because Foxp3 does not control all aspects of the T_{reg} signature (discussed below).

Differentiation and specification in the thymus

Two origins have been described for Foxp3$^+$ cells, whose numeric and functional importance remain a matter of debate. The first is the thymus, where Foxp3$^+$ cells are generated roughly in sync with positive selection of conventional CD4$^+$ T cells. The second is the periphery, where a number of triggers induce the expression of Foxp3 in T_{conv} cells; we will refer to this event as 'conversion', avoiding the 'natural versus adaptive' terminology, which could lead to the mistaken belief that some T_{reg} cells would be unnatural or innate (which is untrue, as all T_{reg} cells express rearranged antigen receptors that define adaptive lymphocytes). We will first deal with the establishment and transcriptional control of the thymus-derived population before considering the generation and function of converted T_{reg} cells.

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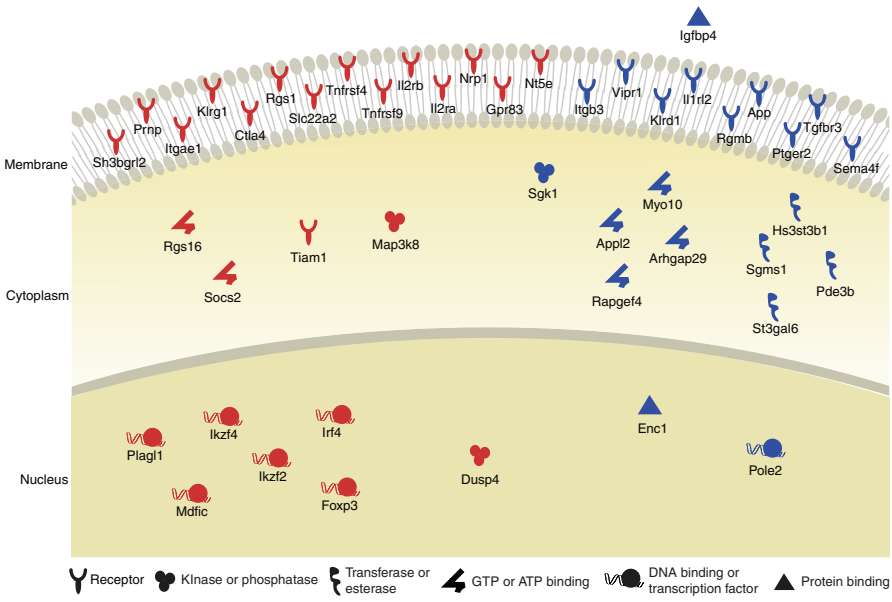

Figure 1 T_{reg} cell signature genes and their cellular localization. The most differentially expressed genes from a consensus T_{reg} signature[21], either overexpressed (red) or underexpressed (blue) in resting T_{reg} cells of spleen or lymph node relative to T_{conv} cells. Gene products are grouped according to cellular localization, and their putative functions are identified by symbols (key).

How immature thymocytes are selected into the T_{reg} cell alternative lineage remains an unresolved question. There is strong genetic variability in their selection and homeostasis, which is perhaps surprising for a population of such importance in immunoregulation[24–26]; $Foxp3^+$ cells are first detected among immature $CD4^+CD8^+$ double-positive cells, but the majority are probably generated from cells that already underwent positive selection[16,27], mainly along the $CD4^+$ single-positive lineage. $CD8^+$ $Foxp3^+$ cells are normally very rare but can be observed in experimental conditions of thwarted selection of the $CD4^+$ lineage[16,28,29] and perhaps in human patients treated with antibody to CD3 (ref. 30). Cells entering the T_{reg} lineage can thus be thought of as cells that have already committed to maturation and differentiation along the main $CD4^+$ or $CD8^+$ lineages. Although the rare $Foxp3^+$ $CD4^+CD8^+$ double-positive cells have yet to be profiled, it is clear that the transcriptional T_{reg} signature is established very early on, with its main characteristics being already present in $CD4^+$ single-positive cells[21]. Positive selection of T_{reg} cells requires TCR—major histocompatibility complex (MHC) molecular interaction, as for T_{conv} cells[16] but with a stronger dependence on costimulatory signals through CD28 (refs. 31,32). The different TCR repertoires of T_{reg} cells and T_{conv} cells indicate that commitment to the T_{reg} lineage must directly or indirectly reflect differential signals received from the TCR. Engagement by agonist ligands favors the selection of T_{reg} cells either by inducing differentiation along the lineage, as observed in transgenic systems[33–36], or because $Foxp3^+$ cells are inherently more resistant to clonal deletion[37–39]. It would be an oversimplification, however, to extrapolate that all T_{reg} cells are necessarily self-reactive. Not only are the data on self-reactivity of T_{reg} cells of normal mice contradictory and not definitive[40–42], but also the significant proportion of TCR sequences that are shared by thymic T_{reg} cell and T_{conv} cell repertoires[12–14] indicates that many T_{reg} cells are no more self-reactive than are T_{conv} cells. Rather than a sharp self-reactive versus non–self-reactive dichotomy distinguishing T_{reg} from T_{conv} cells, it is probably more useful to consider a probabilistic determinism in which each TCR has, in a given MHC environment, a distinct probability to promote commitment along the T_{reg} lineage, with self-reactivity being one but not the only determinant. Further along this line, it has been shown that the proportion of cells that mature into the T_{reg} lineage is strikingly dependent on the precursor frequency of a given clone (with very little selection occurring above a frequency of 1%)[43]. This phenomenon could not be accounted for by a helper effect of additional polyclonal cells but most likely cannot be accounted for by intraclonal competition for MHC-peptide complexes, much as T cells of the same specificity compete during antigen- or lymphopenia-driven population expansion. These observations explained the puzzling mystery that all MHC class II–restricted TCR–transgenic mice made monoclonal by crossing onto the recombination-activating gene–deficient background have essentially no $Foxp3^+$ cells and will mandate a reexamination of past data. Limiting niches have been reported for positive selection of conventional repertoires[44,45], but the niche size for a given T_{reg} TCR specificity seems one or two orders of magnitude smaller[43].

The Hsieh and Farrar groups have described a two-step T_{reg} cell differentiation process in which a $Foxp3^-$ $CD25^{hi}$ population already enriched in TCR sequences 'preferentially' found in mature T_{reg} cells is the first intermediate. Exposure to interleukin 2 (IL-2) can then convert these intermediates into fully differentiated $CD25^+Foxp3^+$ cells[46,47]. This importance of IL-2 in eliciting Foxp3 expression is consistent with the profound T_{reg} cell defects in mice lacking the IL-2 receptor or the IL-2 signal transducers Jak3 or STAT5 (ref. 2). However, most evidence indicates that transforming growth factor-β (TGF-β) is not required for thymic selection of T_{reg} cells as it is for their later homeostasis in the periphery[48–50], except perhaps in the neonatal period, as T cells devoid of TGF-β receptor I show a slight delay in the appearance of thymic T_{reg} cells[51].

How these differential TCR signals are translated into 'preferential' T_{reg} cell commitment is beginning to be better understood. Several milestones have been put down that etch a putative map of how the differential TCR signals are channeled through signaling pathways to induce T_{reg} cell differentiation (**Fig. 2**). Activation of the transcription factor NF-κB pathway seems particularly important for T_{reg} cell differentiation, more so than for normal T cells, as deficits in several elements that link the TCR to NF-κB have been proven highly deleterious for T_{reg} cell development. Mice with conditional knockout of PKC-θ, Bcl-10, CARMA1 or IKK2 have defective T_{reg} cell selection[52–57]. These four molecules draw a fairly clear path from the TCR to NF-κB activation. The MAPK kinase kinase TAK1 (also called Map3k7) is also essential for T_{reg} cell selection[58,59], although this observation is harder to pinpoint on signaling maps, as TAK1 is involved in cytokine signaling (TGF-β, IL-2, IL-15) as well as TCR signaling. However, signaling through the kinase Akt pathway has a negative effect on T_{reg} cell thymic selection, as constitutively active Akt impairs the thymic differentiation of T_{reg} cells, as well as their conversion by TGF-β[60,61], consistent with a positive effect of the kinase mTOR inhibitor rapamycin on T_{reg} cell selection and population expansion[60–65]. These effects are probably related to

the enhanced induction of Foxp3 and corresponding dearth of effector cytokines that occur after TCR stimulation of mature T cells lacking mTOR. This activity seems attributable to the TORC2 complex[66]. Thus, it is possible that the TCR signals that promote selection into the T_{reg} cell lineage are those that elicit a particular balance of transduction along the NF-κB and Akt-mTOR pathways (**Fig. 2**).

How these signals are then translated into specific activation of *Foxp3* and other controllers of the T_{reg} signature remains mysterious. A number of regulatory factors and pathways affect the activity of the *Foxp3* locus[67], but most of them are ubiquitous effectors of cellular activation, alone or in combination (such as AP1, NFAT and CREB).

Foxp3 as a lineage-specification factor?

From the differential inputs described above, how is the T_{reg} cell signature established and which transcriptional regulators forge it? After the discovery of mutations in the gene encoding the transcription regulator Foxp3 as the root of lymphoproliferative and autoimmune disease in *scurfy* mice and patients with IPEX and the results of early transduction experiments[8–10], Foxp3 was seen as the 'master regulator' of the T_{reg} cell lineage, with its presence being necessary and sufficient to specify their phenotype and function. This dogma is still represented in many reviews and in Introductions to primary articles. Yet several arguments have progressively accumulated to erode this view of Foxp3 as the unique specification factor of the lineage[21,68].

First was the description of 'wannabe' T_{reg} cells in the thymus by the Rudensky and Chatila laboratories[20,69]: in *Foxp3*–green fluorescent protein reporter mice in which the green fluorescent protein insert destroys the encoded Foxp3 protein, a substantial number of cells have several of the characteristics of T_{reg} cells, including transcriptional activity at the *Foxp3* locus, high expression of the majority of T_{reg} cell signature genes (including canonical genes such as *Il2ra*, *Nrp1*, *Ctla4* and *Icos*) and low expression of *Il2* (the last being of particular interest because suppression of IL-2 had been thought to be a direct and unique result of Foxp3 action). However, these cells were unstable and exerted no suppressive activity (an interpretation confounded by the fact these 'wannabe' cells themselves adopted effector characteristics). This observation was also consistent with the existence of T_{reg}-like cells in some patients with IPEX[70]. Similarly, it proved possible to elicit a substantial portion of the T_{reg} cell signature in cells devoid of Foxp3 (for example, in TGF-β-treated *scurfy* mutants or by homeostatic conversion *in vivo* of *Foxp3*-null cells; refs. 21,71; **Fig. 3**). Second, careful analysis of cells in which Foxp3 was expressed by direct transduction, or by induced conversion (for example, *in vitro* culture with TGF-β with or without retinoic acid or *in vivo* exposure to agonist or *in vivo* homeostatic expansion) showed that Foxp3 could restore at most about one third of the T_{reg} cell signature transcripts[19,21,71]. In addition, the functional efficacy of Foxp3-transduced or TGF-β-converted cells has variably ranged from highly efficacious to largely inactive, for reasons that remain puzzling although probably related to the stability of Foxp3 expression in TGF-β-converted T_{reg} cells[67] (discussed below). These results indicate that expression of Foxp3 alone does not always suffice for a suppressor phenotype[9,10,21,72–74]. Thus, Foxp3 seems neither absolutely necessary nor uniformly sufficient to specify many aspects of the T_{reg} cell phenotype.

What factors in addition to Foxp3 control the T_{reg} cell signature? A sizeable fraction probably originates from IL-2 through STAT5 (refs. 21,75), consistent with the two-step model for T_{reg} cell selection, in which IL-2 plays a central role. Bioinformatic meta-analyses of T_{reg} cell datasets demonstrating the existence of a group of genes coregulated with *Foxp3* but not induced directly by it suggested the presence of a higher-order regulatory network[21]. In this alternative hypothesis, Foxp3

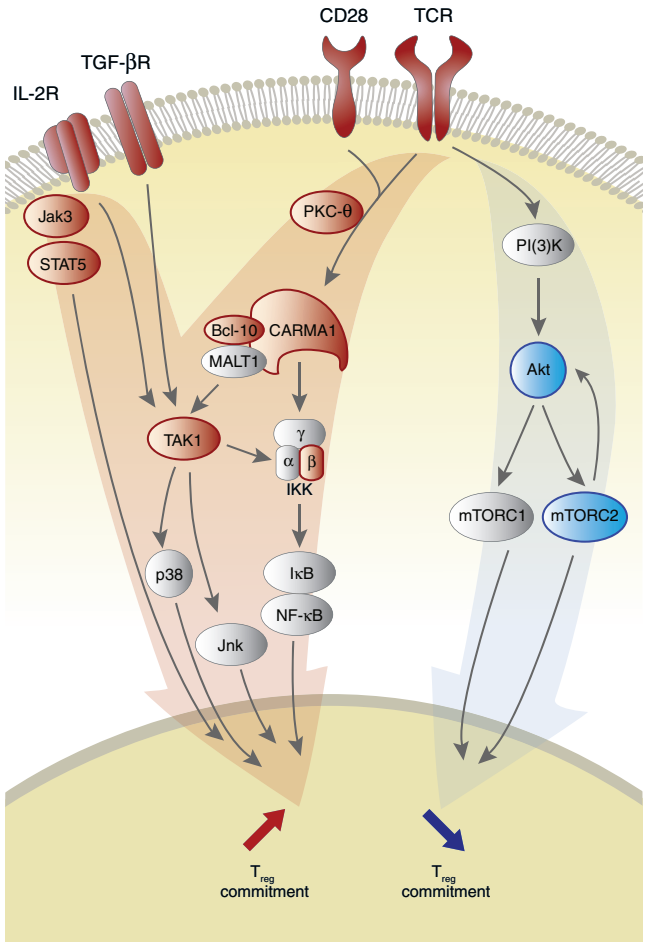

Figure 2 Differential signaling induces or inhibits T_{reg} cell differentiation. Engagement of the NF-κB pathway 'downstream' of the TCR and of IL-2–STAT5 promotes T_{reg} cell differentiation, whereas activation of the Akt-mTOR arm inhibits this, as suggested by the gene knockouts that diminish (red) or increase (blue) commitment to the T_{reg} lineage.

would serve as an important activator or suppressor of a set of genes (some of which are essential for suppressor function) but would be complemented by other transcriptional regulators that control their own set of transcripts in the T_{reg} cell signature. These controls can be complementary and synergistic, as a given T_{reg} cell signature gene can be activated by several pathways (for example, CD103 responds to Foxp3 as well as the combination of IL-2–STAT5 and TGF-β independently of Foxp3). To use a political metaphor, Foxp3 is a *primus inter pares* (a member of an oligarchy) rather than a dictatorial master regulator.

Converted T_{reg} cells

As mentioned above, naive T_{conv} cells can be induced to express Foxp3 by a variety of means (for example, within 2–4 days of activation in the presence of IL-2 and TGF-β *in vitro*[76,77]; within 8–14 days of exposure *in vivo* to subimmunogenic agonist peptide delivered by osmotic minipumps or peptide coupled to antibody to DEC205 in transgenic systems[78,79] or polyclonal systems[80]; after exposure to antigen delivered through mucosal surfaces[81–83]; or as a result of lymphopenia-driven homeostatic proliferation[71,84,85]). In theory, conversion is an attractive mechanism, as it allows lymphocyte pools to adapt to immunogenic conditions, to dampen an overactive acute inflammation or to curtail the response to a chronic unresolved challenge. Of note, this

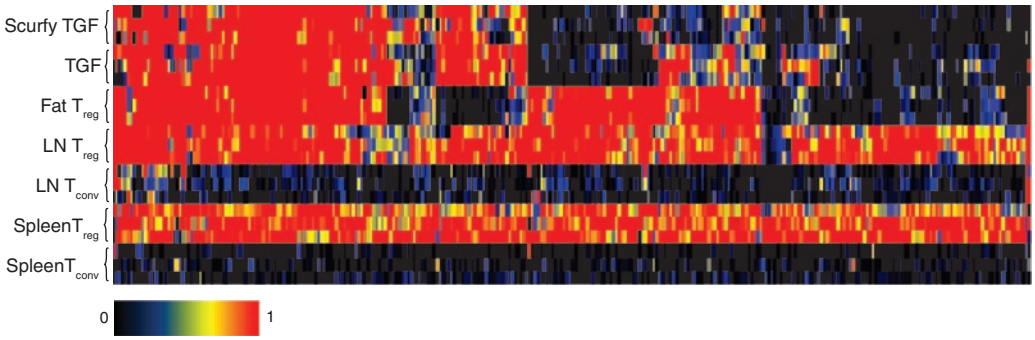

Figure 3 Different segments of the T_{reg} signature appear in different contexts. Heat map of the transcripts of a consensus T_{reg} signature, normalized to the expression in splenic T_{conv} cells and T_{reg} cells (0 and 1, respectively, presented as hierarchical clustering). Lymph node (LN) T_{reg} cells have essentially identical profiles, but only a fraction of the signature is present in T_{reg} cells from adipose tissue or after *in vitro* conversion with TGF–β. Most of the signature transcripts acquired after TGF-β-induced conversion are Foxp3 independent, as they are also present in cultures of Foxp3-deficient *scurfy* mutant cells (top).

concept represents a departure from the paradigm of clonal selection that has served immunology well for several decades; this departure is not truly necessary, as the breadth of the T_{reg} cell TCR repertoire as it emerges from the thymus can certainly provide T_{reg} cells reactive against any given antigen-MHC complex, these antigen-specific T_{reg} cells being amplified *in situ* just as T_{conv} cells (T_{reg} cells actually divide more *in vivo* than T_{conv} cells, contrary to their anergic activity *in vitro*[36,86,87]). Another view, consistent with the instability of Foxp3 expression observed in some of these conversion settings (discussed below) is that transiently eliciting inhibitory functions may be a way of quickly 'fine-tuning' the first steps of a local immune response.

The observation that conversion can occur during experimental manipulation leaves open the question of the true contribution of peripheral conversion to the overall T_{reg} cell pool and whether this is a focused response occurring at specific inflammatory locations. There has been a tendency in the literature to interpret observations of local T_{reg} cell accumulation as reflecting conversion from T_{conv} cells, rather than simple migration, retention and proliferation of antigen-specific T_{reg} cells, but actual evidence for either is often missing. The question is of heuristic importance (are T_{reg} cells a distinct lineage or one of several states into which naive $CD4^+$ T cells can differentiate?) and practical importance (can conversion be a therapeutic target?). In nonimmunized and nonlymphopenic mice, the CDR3 sequences of TCRs expressed by T_{reg} cells isolated from peripheral lymphoid organs largely resemble those of thymic T_{reg} cells, with no peripheral accentuation of the overlap between repertoires that would result from conversion[12–14,88]; this finding suggests that the global contribution of converted T_{reg} cells in lymph nodes and spleen is limited. A more focused analysis using TCR sequences as 'barcodes' to look for evidence of conversion in a setting of autoantigen recognition, *a priori* more favorable to detect conversion events, also failed to bring evidence for any substantial numeric contribution[89]. Similarly, T_{reg} cells are found in brain inflammatory lesions in mice with experimental autoimmune encephalomyelitis as a result of the migration of thymus-derived T_{reg} cells rather than conversion[90]. In infectious settings, the available evidence often points to the recruitment and expansion of antigen-specific T_{reg} cell populations, rather than conversion[91,92]. An interesting handle on the question may be provided by mice lacking CARMA1 (ref. 54). As discussed above, thymic selection of the $Foxp3^+$ lineage is profoundly deficient in these mice, but Foxp3 can be very effectively induced in their mature T_{conv} cells by exposure to IL-2 and TGF-β *in vitro.* Interestingly, the lamina propria of CARMA-1 deficient mice shows a substantial contingent of T_{reg} cells (~37% of a normal pool), far more than in mesenteric or other lymph nodes (8–3%). Although the possibility of localized expansion of rare thymic precursors cannot be ruled out, this distribution could be interpreted to reflect peripheral conversion induced by TGF-β more uniquely in the gut-associated tissue than in spleen or other lymph nodes.

From a functional standpoint and with the exception of the variable results obtained with the T_{reg} cells induced by TGF-β discussed above, cells converted *in vivo* can be functionally quite effective[71,78,83,84]. In the DO11.10 system, T_{reg} cells elicited by exposure to antigen through the gut can protect from airway inflammation[83]. $Foxp3^+$ cells converted from $Foxp3^-$ precursors in conditions of homeostatic expansion are as effective as resting T_{reg} cells in protection against colitis elicited by transfer of naive T_{conv} cells into a lymphopenic host; indeed, these converted T_{reg} cells function even more effectively when combined with resting lymph node T_{reg} cells, which suggests that they brought a complementary phenotype or an enriched antigenic specificity[71]. This observation is compatible with the notion that these 'neo–T_{reg} cells', generated in lymphopenic conditions, are particularly adept at regulating immunopathology occurring in precisely the same triggering conditions of the lymphopenic host.

From a genomic standpoint, converted T_{reg} cells are clearly different from thymus-derived T_{reg} cells, as demonstrated for TGF-β-T_{reg} cells and for T_{reg} cells induced in lymphopenic conditions[21,71]. In both instances, only a fraction of the T_{reg} cell signature was elicited (~35%); although canonical transcripts (*Foxp3* and *Itgae* (encoding CD103)) were expressed, others were not differentially expressed (*Il2ra* and *Ctla4* for TGF-β-T_{reg} cells, *Ikzf4* (Helios) and *Itgb8* for homeostatically induced T_{reg} cells). Nor are these fractions similar, and T_{reg} cells converted in different scenarios each have a subtly different subset of the entire signature (M. Feuerer *et al.*, unpublished data).

It is unclear what relationship exists between the signaling pathways that promote the selection of the T_{reg} cell lineage in the thymus and those that elicit conversion in the periphery. Some appear shared (the importance of TCR engagement, of IL-2 and of Akt). Some appear distinct, in particular the effect of TGF-β (apparently dispensable in the thymus; clearly involved in some but not all instances of peripheral conversion) or of IL-6 and the transcription factors that control the differentiation of IL-17-producing cells. The latter point is of particular interest given observations of shared requirements in conditions that elicit the differentiation of IL-17-producing T helper cells (T_H-17 cells) or conversion to a $Foxp3^+$ phenotype *in vitro*[93,94]. Both processes require TGF-β, but IL-6, by inducing expression of the transcription factor RORγt, effectively shuts down Foxp3 induction[95,96]. Interestingly, RORγt and Foxp3 are both induced during the early phase of a TGF-β-induced response and physically interact, but Foxp3 wins out by shutting down RORγt[97]. There is no evidence that a similar interaction occurs between Foxp3 and RORγt in the thymus (for example, RORγt-deficient mice have normal thymic T_{reg} cells), but might other members

of the large family of nuclear receptors play a corresponding role?

Conversely, there is also emerging evidence suggesting that the T_{reg} cell transcriptional programs are not necessarily permanently etched, but that the Foxp3 transcriptional cassette may be expressed in a reversible manner, transiently or for more extended periods of time (refs. 67,98,99 and J. Bluestone, personal communication). Instability was linked to the differential stability of Foxp3 expression as a function of epigenetic changes at the *Foxp3* locus[67], and it may confer additional flexibility to the application of regulatory functions. In addition, in the realm of the T_{reg} cell versus T_H-17 cell relationships mentioned above, it is interesting to note that strong IL-17-inducing conditions can elicit a shutdown of Foxp3 and induction of IL-17 production from a fraction of outwardly established Foxp3$^+$ cells[96,100].

Functional subphenotypes of Foxp3$^+$ T cells

Soon after the original description of Foxp3 in CD25$^+$CD4$^+$ T cells, subsets of this population were identified by differential expression of cell surface markers. Several of these subsets probably correspond to markers of activation or memory that, as for activated T_{conv} cells, allow them to home to locations other than the secondary lymphoid organs. One of the best characterized examples is the integrin $\alpha_E\beta_7$ (CD103), which binds E-cadherin. It is typically expressed on 20–30% of Foxp3$^+$ cells in secondary lymphoid organs and on a higher percentage of T_{reg} cells in tissues such as the lung, skin and lamina propria of the gut[17,101,102]. The functional relevance of CD103 expression in the T_{reg} cell population is highlighted by the greater potential of T_{reg} cells to access and be retained in peripheral tissues during infection or acute inflammatory insults[17,91]. CD103 is under multifactorial and complex regulation: it is directly responsive to Foxp3 after retroviral transduction *in vitro* but can be induced by TGF-β in a Foxp3-independent manner (for example, in naive CD4$^+$ T cells from *scurfy* mice cultured *in vitro* with TGF-β[21]), and it is strongly expressed in thymic derived and converted Foxp3$^+$ cells after antigen exposure or homeostatic expansion (refs. 17,29,102,103 and M. Feuerer, J. Hill, D. Mathis and C. Benoist, unpublished data). The 'activated-memory' CD103$^+$ T_{reg} cell subset can be further subdivided by the expression of markers typical of natural killer cells, such as KLRG1, CD49b and CD38 (refs. 29,104), whose functional relevance is unclear today.

Other T_{reg} cell subsets, in contrast, correlate with specific tissue localizations. For instance, the chemokine receptor CCR4 is not expressed on thymic T_{reg} cells but is found on an unusually high percentage of extralymphoid Foxp3$^+$ cells in the skin[102]. CCR4$^+$ T_{reg} cells also appear in skin draining lymph nodes after subcutaneous immunization, and their functional relevance is highlighted by the inflammatory manifestations that develop in mice in which *Ccr4* is conditionally deleted specifically in T_{reg} cells. CCR4-deficient T_{reg} cells function normally in *in vitro* inhibition assays, are competitively fit and are able to control many of the peripheral tissue manifestations of autoimmunity in *scurfy* mice but cannot control inflammation in the skin or lungs due in part to their impaired ability to migrate or be retained in these tissues. Similar results have also been obtained with mice that lack the skin-homing receptor α-1,3-fucosyltransferase VII (refs. 105,106). Thus, the ability of T_{reg} cells to protect against autoimmune damage in a particular organ requires the ability of T_{reg} cells to home to that organ; the mere expression of Foxp3 coupled with functional efficacy in particular *in vitro* or *in vivo* assays does not necessarily equate to a *bona fide* T_{reg} cell. A distinct population of Foxp3$^+$ cells residing in the adipose tissue has been described, its presence or absence correlating with pathological manifestations of obesity and insulin resistance (M. Feuerer *et al.*, unpublished data). Here again, these cells express only a subset of the T_{reg} signature (**Fig. 3**) but also express other transcripts that may account for their particular location and effector function. It remains to be resolved whether these T_{reg} cell subpopulations are elicited and acquire their particular characteristics after antigen encounter (for example, perhaps in contact with particular antigen-presenting cells or adventitious stimuli at the time of TCR triggering and/or conversion) or whether a diversity of transcriptional programs are preselected in the thymus, in addition to the bedrock program imparted by Foxp3, with the cells being later selected through differential homing and antigenic specificity. In this respect, it is interesting to note that T_{reg} cells in different lymph nodes have quite different TCR repertoires[88].

Although some of these subphenotypes correspond to differential activation or tissue localization of T_{reg} cells, two reports also indicate that transcriptional submodules in T_{reg} cells are needed for the regulation of different immune functions and that T_{reg} cells do so by involving transcriptional control elements from the very cells they are regulating. This has been shown in the context of the T helper type 1 (T_H1) transcription factor T-bet (encoded by *Tbx21*) and the T_H2- and T_H-17-related transcription factor IRF4 (encoded by *Irf4*)[107,108]. These regulators of lineage development have been studied for their ability to influence cytokine production, but they also help coordinate a much broader transcriptional program in T cells as well as other immune cell types[109,110]. The main outcome of deleting *Irf4* uniquely in Foxp3$^+$ T_{reg} cells is strong overexpression of IL-4 and IL-5 (and to a much more modest extent IL-17) but not of other cytokines in T_{conv} cells, and a massive increase in the production of immunoglobulin G1 and immunoglobulin E by B cells; these features are not typical of Foxp3-deficient mice. Interestingly, the transcriptional program of *Irf4*-deficient T_{reg} cells show a small number of changes, many of which affect characteristic T_H2 transcripts such as *Maf* or *Ccr8* or other chemokine receptors such as those encoded by *Ccr2* or *Ccr6*. Coimmunoprecipitation from primary cell extracts indicates that IRF4 and Foxp3 are physically associated in T_{reg} cells, which suggests that these two transcription factors might act together to control a T_{reg} cell subsignature. Indeed, combined analysis of the T_{reg} signature and of the IRF4 'footprint' shows that many genes controlled by IRF4 belong to the T_{reg} signature but that IRF4 affects only a limited subsegment of the T_{reg} signature.

A role for T-bet in T_{reg} cells has been demonstrated by another route in studies of the expression of CXCR3 on T_{reg} cells[109]. As in conventional T_H1 cells, *Cxcr3* expression in T_{reg} cells was dependent on T-bet (clearly not a factor expected to mediate immunoregulation by T_{reg} cells!), which was induced after TCR stimulation in the context of the activation of dendritic cells by antibody to CD40, classically a T_H1-inducing condition. T-bet-deficient T_{reg} cells survived less well and were less effective than their wild-type counterparts at controlling type 1 inflammatory responses *in vivo*.

Thus, both of the reports discussed above suggest that T_{reg} cells use the same transcriptional regulators as the cells they restrain to generate adapted 'subsignatures' or transcriptional cassettes needed to control a particular facet of the immune response. A thorough transcriptional analysis needs to be performed, but the IRF4 'footprint' in T_{reg} cells might be expected to be a composite of some of the elements it controls in T_H2 cells and of other elements that it uniquely activates in T_{reg} cells (for example, as a result of combinatorial transactivation by IRF4-Foxp3 complexes).

Why the match between regulator and 'regulatee'? One scenario is that shared transcriptional factors would allow the expression of shared surface molecules responsible for homing of the helper T cell and its specific Foxp3$^+$ regulator to the same anatomical location, with shared location explaining the apparent specificity of regulation; such a shared location could be macroscopic (homing to particular tissues such as the gut) or microscopic (particular subsections of T cell areas in secondary

lymphoid organs). The fact that chemokine receptor expression is one of the main consequences of *Irf4* deletion in T_{reg} cells might support this hypothesis, consistent with the requirement for chemokine receptors for T_{reg} cell function[102,105,111]. Alternatively, competition for a specific but common survival factor might be involved.

There may also be a parallel here to the role of T-bet in T cells and B cells[112]. In T cells, T-bet controls interferon-γ and T_H1-like responses, whereas in B cells it facilitates class switching to the immunoglobulin G2a isotype, precisely the isotype whose use is enhanced by T_H1 cells. Although this is perhaps a mere coincidence, this independent instance of a situation where regulator and regulatee cells share the same specification factor may open the following line to speculation: there is advantage in having the same transcriptional cassettes expressed in both sides of a regulator–regulatee cell (or function) pair because it ensure coevolution of the same partners.

Conclusion

The studies discussed here underscore the complexity of transcriptional and phenotypic regulation in T_{reg} cells, in which multiple factors control the bedrock signature as well as the different subfunctions and subphenotypes. The notion of a unimodal program of T cell differentiation may hold little relevance to the complexity that is inherent to T_{reg} cell populations *in vivo*. Clearly, the extent of this diversity and how stable or interrelated these T_{reg} cells subphenotypes may be is not known. But this complexity will need to be considered when devising therapeutic strategies based on T_{reg} cells.

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