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Mechanisms of human FoxP3$^+$ T_{reg} cell development and function in health and disease

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Summary

Regulatory T (T_{reg}) cells represent an essential component of peripheral tolerance. Given their potently immunosuppressive functions that is orchestrated by the lineage-defining transcription factor forkhead box protein 3 (FoxP3), clinical modulation of these cells in autoimmunity and cancer is a promising therapeutic target. However, recent evidence in mice and humans indicates that T_{reg} cells represent a phenotypically and functionally heterogeneic population. Indeed, both suppressive and non-suppressive T_{reg} cells exist in human blood that are otherwise indistinguishable from one another using classical T_{reg} cell markers such as CD25 and FoxP3. Moreover, murine T_{reg} cells display a degree of plasticity through which they acquire the trafficking pathways needed to home to tissues containing target effector T (T_{eff}) cells. However, this plasticity can also result in T_{reg} cell lineage instability and acquisition of proinflammatory T_{eff} cell functions. Consequently, these dysfunctional CD4$^+$FoxP3$^+$ T cells in human and mouse may fail to maintain peripheral tolerance and instead support immunopathology. The mechanisms driving human T_{reg} cell dysfunction are largely undefined, and obscured by the scarcity of reliable immunophenotypical markers and the disregard paid to T_{reg} cell antigen-specificity in functional assays. Here, we review the mechanisms controlling the stability of the FoxP3$^+$ T_{reg} cell lineage phenotype. Particular attention will be paid to the developmental and functional heterogeneity of human T_{reg} cells, and how abrogating these mechanisms can lead to lineage instability and T_{reg} cell dysfunction in diseases like immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, type 1 diabetes, rheumatoid arthritis and cancer.

Keywords: antigen specificity, cell therapy, human immunology, regulatory T cells, regulatory T cell dysfunction

Introduction

Regulatory T (T_{reg}) cells are essential mediators of peripheral tolerance to self and non-self-antigens [1]. T_{reg} cells achieve this immunoregulatory control through multiple suppressive mechanisms that inhibit cells of innate immunity, antigen-presenting cell (APC) functions, as well as adaptive B, CD4$^+$ or CD8$^+$ effector T (T_{eff}) cell responses [2]. Pioneering experiments identified these potently immunosuppressive cells as CD4$^+$CD25$^+$ T cells as the transfer of CD25-depleted splenocytes into lymphopenic mice conferred multi-organ autoimmunity [3]. Homologous CD4$^+$CD25^{high} human counterparts were identified shortly thereafter [4–8]. In 2003, forkhead box P3 (FoxP3) was identified as the lineage-defining transcription factor of T_{reg} cells. Indeed, gene deletion or abrogation of its functions caused severe inflammatory autoimmune disorders in mice and humans by abolishing T_{reg} cell development [9–11].

FoxP3$^+$ T_{reg} cells can be categorized into two ontogenic categories: thymic-derived/natural T_{reg} (tT_{reg}) cells and

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peripheral/induced T_{reg} (pT_{reg}) cells. The former develop within the thymus from single-positive $CD4^+$ thymocytes following a moderate- to high-avidity T cell receptor (TCR) engagement with self-antigens on major histocompatibility complex (MHC)-II molecules by medullary thymic epithelial cells [12]. The latter arise in the periphery from naïve, $CD4^+$ conventional T (T_{conv}) cells that are antigen-activated in the presence of FoxP3-inducing cytokines [transforming growth factor (TGF)-β, interleukin (IL)-2], dietary constituents (retinoic acid) or drugs (glucocorticoids, rapamycin) [13]. Both T_{reg} cell types play central roles in the global immunoregulatory potential in hosts. Alterations in their development, homeostasis or function may predispose to a variety of disease conditions including allergy, autoimmunity, graft rejection, cancer and response to immunotherapies.

Current research is focused on developing novel therapies to enhance endogenous T_{reg} cell functions *in vivo* with cytokines and small drugs, use *ex-vivo* manipulated T_{reg} cells in autologous adoptive transfers to promote immunoregulation in settings of autoimmunity, and induce antigen-specific T_{reg} cells to strengthen tolerance in allergic inflammation [14]. However, T_{reg} cells represent a phenotypically and functionally diverse array of cell subsets with differing effector functions and fates in circulation and tissues [15,16]. Here, we provide an overview of the factors and mechanisms influencing the development and heterogeneity of T_{reg} cells in human health and disease.

FoxP3, the master regulator of T_{reg} cell lineage commitment

FoxP3, a 431 amino acid forkhead winged helix family transcriptional regulator, is the master transcription factor driving the genetic programming of T_{reg} cells [17]. Natural or experimental mutations of the *foxp3* gene result in congenital deficiencies in T_{reg} cell development and function. These lead to spontaneous, multi-organ, immune pathology in *scurfy* mice and humans with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome [9–11].

FoxP3 acts primarily as a transcriptional repressor of key genes involved in T cell activation and effector functions, including proliferation and synthesis of proinflammatory cytokines [e.g. IL-2, IL-4, IL-17A and interferon (IFN)-γ], all the while endowing the cell with potent suppressive functions [18,19]. Sustained expression of FoxP3 in T_{reg} cells is required for lineage commitment and stability, and several key mechanisms including cytokine signaling, epigenetic control of the *foxp3* locus and interactions of FoxP3 with other proteins, contribute to the regulation of FoxP3 expression and, consequently, maintenance of peripheral tolerance (Fig. 1).

Cytokine control of FoxP3+ T_{reg} cell homeostasis

IL-2 is necessary for global T_{reg} cell homeostasis by promoting their development, survival and function in the thymus and periphery [20–22]. IL-2 activates the signal transducer and activator of transcription (STAT)-5, which binds to several sites on the *foxp3* promoter to enhance FoxP3 expression and thus establish the T_{reg} cell genetic program. A defining feature of T_{reg} cells, unlike other T cell subsets, is their constitutive expression of CD25, the α chain of the heterotrimeric high-affinity IL-2R. Indeed, T_{reg} cells have a higher sensitivity to IL-2 signaling than T_{eff} cells due to preferential binding of IL-2 through high expression of CD25 and higher activity of PP1 and PP2A phosphatases which modulate IL-2 signaling [23]. Defects in IL-2 signaling (e.g. mutations in CD25) can give rise to IPEX-like autoimmunity as a consequence of T_{reg} cell dysfunction [24].

TGF-β is another essential cytokine promoting the development of T_{reg} cells. In conjunction with TCR stimulation, TGF-β mediates the conversion of $CD4^+$ $FoxP3^-$ naïve T_{conv} cells into iT_{reg}/pT_{reg} cells (*in-vitro*/*in-vivo*) [25]. TGF-β signaling activates Smad2/3 transcription factors, which bind directly to enhancer regions of the *foxp3* locus to promote FoxP3 expression in these pT_{reg} cells [25,26]. TGF-β signaling is also critical for tT_{reg} cell development and function. Although a $CD4^+$ T cell-specific deletion of the TGF-β receptor II (TGF-βRII) in mice had no defect in T_{reg} cell frequencies, mice nevertheless developed multi-organ T cell-mediated autoimmunity, suggesting a defect in immunoregulation [27,28]. Moreover, a specific knock-out of the TGF-β receptor I (TGF-βRI) in murine T cells curtailed tT_{reg} cell generation within the first week of life of neonatal mice, supporting a requirement for TGF-β in tT_{reg} cell development [29]. Additionally, intrathymic injection of precursor $CD4^-CD8^-$ thymocytes from $cd4^{Cre}tgfbr1^{fl/fl}$ mice into syngeneic wild-type hosts failed to yield tT_{reg} cells [30]. Thus, global T_{reg} cell development is dependent on TGF-β signalling.

Epigenetic regulation of FoxP3 expression

Enzymatic demethylation of specific cytosine–phosphate–guanine (CpG) motifs within the intronic enhancer evolutionary conserved non-coding sequence (CNS) 2, also called the T_{reg}-specific demethylated region (TSDR), is another critical mechanism maintaining FoxP3 expression in T_{reg} cells and is a feature that is absent in T_{conv} cells [31–33]. Demethylation of these critical non-coding sequences opens up the *foxp3* enhancer region to large multi-molecular complexes containing FoxP3, c-Rel, nuclear factor of activated T cells (NFAT), STAT-5, runt-related transcription factor 1-core-binding factor (Runx1-CBF)β, cAMP responsive element-binding/activating transcription factor (Creb/ATF) and ETS proto-oncogene 1 (Ets1). These multi-molecular complexes bring the CNS2

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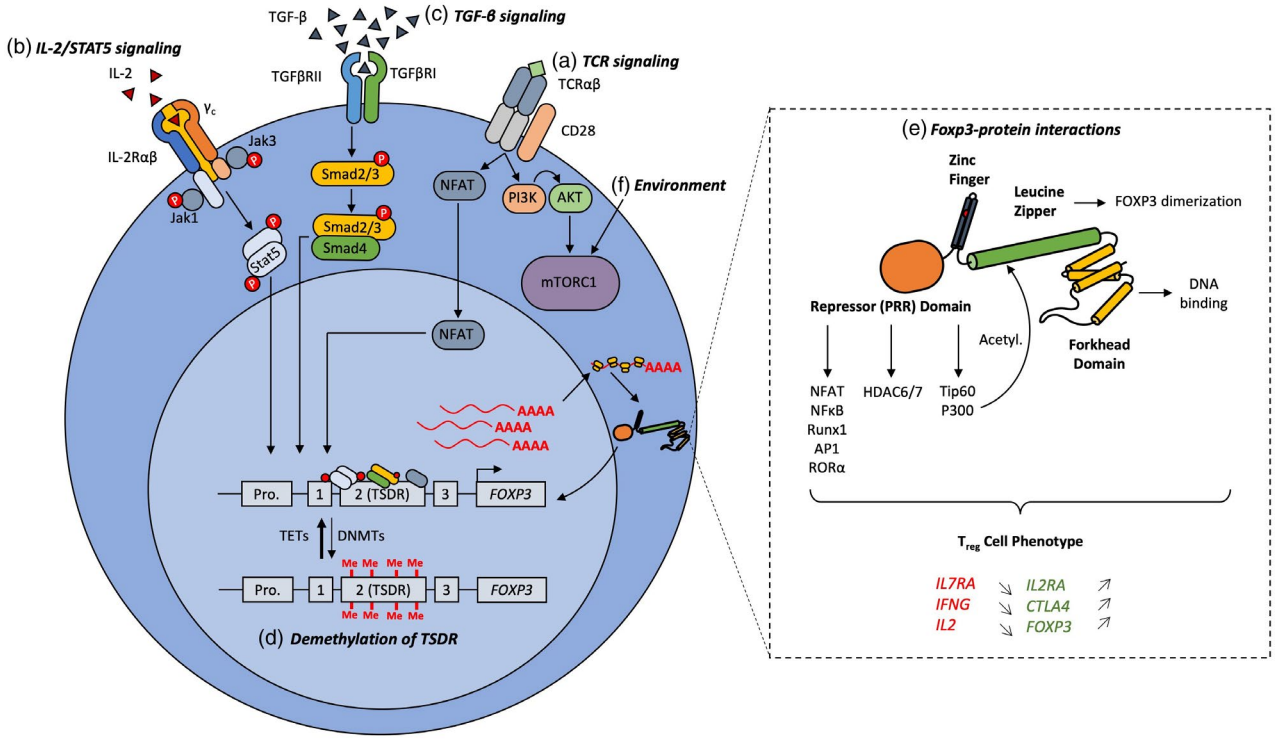

Fig. 1. Mechanisms preserving the stability of the regulatory T cell (T_{reg}) phenotype. T_{reg} cell lineage stability is reliant on the strength of forkhead box protein 3 (FoxP3) expression. There are several mechanisms in place to ensure robust FoxP3 expression in T_{reg} cells. **A,** T cell receptor (TCR) signaling leads to nuclear factor of activated T cells (NFAT) binding to the CNS2 region of the *foxp3* locus for transactivation of gene expression. **B,** Constitutive High level of CD25 expression, the interleukin (IL)-2 receptor α, on the T_{reg} cell surface confers a high sensitivity to IL-2 in the environment. IL-2 signaling through Janus kinase (Jak)1 and Jak3 result in signal transducer and activator of transcription (STAT-5) phosphorylation and dimerization and subsequent translocation into the nucleus. Phosphorylated (p)STAT-5 binding to the conserved non-coding DNA sequence (CNS)2 drives FoxP3 expression. **C,** Transforming growth factor (TGF)-β signaling through TGF-βRI and TGF-βRII result in Smad2/3 phosphorylation, association with the transcription Smad4 and the translocation of the complex into the nucleus. Smad2/3/4 bind to the *foxp3* promoter and drive FoxP3 expression. In the presence of TCR signaling, TGF-β-driven FoxP3 expression in naïve $CD4^+$ conventional T (T_{conv}) results in induced (i)T_{reg}/peripheral (p)T_{reg} induction. **D,** To enable transcription factor binding to the *foxp3* locus enhancer region, certain sites are specifically demethylated in T_{reg} cells. In the CNS2 enhancer region, this is referred to as the T_{reg}-specific demethylated region (TSDR). Demethylation of the TSDR is mediated by the 10–11 translocation (Tet) family demethylases Tet1 and Tet2. DNA methyl transferases (Dnmt) such as Dnmt1 methylate the TSDR and destabilize Foxp3 expression. **E,** Once FoxP3 is expressed, it heterodimerizes and can associate with many different binding partners (~700), including transcription factors, histone deacetylases and histone acetyl transferases. Binding to these proteins are necessary for transcriptional repression of various genes (*il7ra*, *ifng*, *il2*) and activation of others (*il2ra*, *ctla4*, *foxp3*). **F,** Significant focus has been devoted to studying the environmental signals controlling FoxP3 expression. Phosphoinositide-3-kinase–protein kinase B (PI3K-Akt) signaling downstream TCR and CD28 signaling is needed for transient mammalian target of rapamycin complex 1 (mTORC1) activation and consolidation of the T_{reg} cell phenotype. However, chronic activation of mTORC1 (e.g. through environmental signals such as glutamine) result in sustained mTORC1 activation and therefore deregulation of T_{reg} cells. Thus, mTORC1 inhibitors (e.g. rapamycin) are used in the *in-vitro* expansion of T_{reg} cells.

into contact with the *foxp3* promoter, thereby driving FoxP3 expression. Indeed, it was recently demonstrated that demethylation of the CNS2 resulted in enhanced T_{reg} cell lineage stability by increasing T_{reg} cell sensitivity to IL-2 through greater STAT-5 occupancy of the *foxp3* enhancer [34]. Accordingly, recruitment of the DNA methylase Dnmt1 to the foxp3 promoter and its subsequent methylation severely impaired the maintenance of FoxP3. Demethylation of CNS2 in T_{reg} cells is mediated by the 10–11 translocation (Tet) family of demethylases, which are themselves recruited to the *foxp3* locus by STAT-5 [35]. Deletion of Tet1 and Tet2 in murine T_{reg} cells yields severe autoimmunity as a consequence of poor T_{reg} cell lineage commitment.

CNS1 is also important for T_{reg} cell lineage commitment. Deletion of CNS1, which contains a binding site for Smad2/3 upstream of CNS2, abrogates pT_{reg} cell development in mice and leads to inflammation at mucosal sites [36]. Moreover, the newly identified CNS0 was also shown to be necessary for T_{reg} cell stability [37]. CNS0 is bound by the genomic organizer Satb1 in $FoxP3^-$ tT_{reg} cell thymic precursors and alters chromatin accessibility in order to orchestrate the

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changes needed for future FoxP3 expression and T_{reg} cell development in mice.

FoxP3 binding-partners and T_{reg} cell stability

FoxP3 imparts its functions by directly or indirectly binding to ~ 700 transcriptional promoters, either repressing or activating the target genes [38,39]. However, the transcriptional control of most genes inherent to the T_{reg} cell transcriptional program is achieved by the indirect binding of FoxP3 through other molecular factors. For example, complexes between FoxP3 and NFAT, nuclear factor (NF)-κB, acute myeloid leukemia 1 (AML1)/Runx1, activator protein 1 (AP1) and retinoic acid orphan receptor (ROR)α transcription factors are known to impair the NFAT/AP1 transcriptional program that leads to *il2* expression in T_{conv} cells, thereby maintaining anergy in T_{reg} cells [1]. To identify other FoxP3 binding partners, Rudra *et al*. expressed the prokaryotic biotin ligase, BirA, and FoxP3 with an N-terminal BirA ligase biotinylation site in a murine T cell hybridoma line to purify protein complexes containing FoxP3 and identified 361 binding partners by mass spectrometry [40]. Along with FoxP3, these binding partners formed a complex transcriptional program with multiple positive and negative feedback loops consolidating the T_{reg} cell transcriptional program. Hence, FoxP3 is necessary but not sufficient for maintaining the T_{reg} cell phenotype [40,41].

Key binding partners of FoxP3 include several histone deacetylases (HDAC) such as HDAC1, 7 and 9, and histone acetyltransferases (HAT), such as the Tat-interacting protein 60 (TIP60) and p300 [42–44]. Interactions with these proteins are necessary for transcriptional silencing of target genes as well as for protein modifications that enhance FoxP3 function. For example, acetylation of FoxP3 upon complexing with p300 allows it to evade proteasomal degradation, which constitutes a post-translational mechanism of FoxP3 stability [43]. Furthermore, we have recently shown that abrogating the FoxP3–Tat-interacting protein 60 (TIP60) interaction, a defect caused by the germline *foxp3*^{A384T} IPEX mutation, relieves human T_{reg} cell suppressive capacity while retaining major aspects of the T_{reg} cell phenotype including the repression of proliferation and inflammatory cytokines [45]. Restoring this interaction using a TIP60 allosteric modifier rescued FoxP3 functions and T_{reg} cell suppressive capacity *in vitro* and *in vivo*. Thus, several mechanisms maintain FoxP3 stability and consequently establish the requisite transcriptional program to ensure FoxP3$^+$ T_{reg} cell functional development.

Functional heterogeneity among human FoxP3$^+$ T_{reg} cells

Despite FoxP3 ensuring a robust immunosuppressive phenotype, significant functional heterogeneity exists among human FoxP3$^+$ T_{reg} cells. We previously developed a single-cell strategy to examine the phenotypical and functional heterogeneity of human CD4$^+$CD25^{high}CD127^{low} T_{reg} cells relative to FoxP3 expression from blood of healthy individuals [16]. CD4$^+$CD25$^{high/bright}$ T_{reg} cells, albeit highly enriched in suppressive FoxP3$^+$ T cells, harbour a pool of bona fide FoxP3$^+$ T_{reg} cells with compromised suppressive function, despite the maintenance of hallmark phenotypic, epigenetic and transcriptional features of T_{reg} cells. These FoxP3$^+$ T_{reg} cells with compromised suppressive function also produce proinflammatory cytokines such as IL-2, IL-17 and IFN-γ following polyclonal activation [16]. Whether this heterogeneity relates to FoxP3$^+$ T_{reg} cell subsets that have acquired unique effector functions or T_{reg} cells losing their phenotype remains to be determined. These non-suppressive CD25^{high} FoxP3$^+$ cells in healthy peripheral blood may be involved in the onset of autoimmune or inflammatory states, and further characterization is needed to understand more clearly their roles in health and disease.

We recently identified Helios, an Ikaros family transcription factor, to be preferentially expressed on suppressive T_{reg} cells as opposed to non-suppressive T_{reg} cells in human blood during immune quiescence and in disease [46]. Although, Helios was initially proposed as a marker for differentiating between tT_{reg} and iT_{reg} cells, there are no data supporting this definition in humans. We further demonstrated that co-expression of the surface receptors T cell immunoreceptor with immunoglobulin (Ig) and ITIM domains (TIGIT) and Fc receptor-like 3 (FcRL3) identified most Helios$^+$ T_{reg} cells in the periphery and were absent from T_{eff} cells at the steady state and following TCR stimulation [46].

Antigen-specific T_{reg} cell functions

Heterogeneity in T_{reg} cell function also lies in TCR specificities that drive their developmental ontogeny, peripheral homeostasis and effector functions. As stated earlier, tT_{reg} and pT_{reg} cells differ in their ontogenies. Consequently, tT_{reg} cells possess a diverse, self-restricted TCR repertoire distinct from T_{conv} cells, whereas pT_{reg} cells retain the antigen specificities of their naïve T_{conv} precursors. *In-vitro* evidence shows that tT_{reg} cell suppressive function is TCR activation-dependent, and although antigen non-specific suppression has been described, antigen-specific signals are largely viewed as necessary for optimal tT_{reg} cell suppressive functions within the periphery [47–49]. For example, pancreatic autoantigen-specific T_{reg} cells and myelin basic protein (MBP)-specific T_{reg} cells preferentially prevent disease in mouse models of type-1 diabetes (T1D) and multiple sclerosis, respectively [50–52]. Nevertheless, naïve (CD25^{low}CD45RA$^+$) non-antigen-experienced tT_{reg} cell populations are critical for homeostasis of the global

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tT_{reg} cell pool [53]. Expressing high levels of the pro-survival molecule Bcl-2 and relying on IL-7 signaling in the periphery, naïve tT_{reg} cells are rapidly proliferative and readily differentiate into potently suppressive memory tT_{reg} cells upon TCR engagement by their cognate self-antigen [53,54].

Allergy represents the best-studied area regarding the functional consequences of pT_{reg} cell antigen specificity. Barrier tissues are constantly exposed to a myriad of environmental, dietary and commensal microbial antigens requiring homeostatic immune control mediated by the local T_{reg} or T_{eff} cell antigen-specific repertoires. Consequently, they are particularly specialized sites for the *de-novo* generation of pT_{reg} cells in order to ensure immune homeostasis and maintain tolerance [55,56]. A break of T_{reg} cell-mediated tolerance often results in allergic inflammation at these sites. Specialized APCs, such as alveolar macrophages in the lungs and $CD103^+$ dendritic cells (DCs) in the intestinal mucosa, favour pT_{reg} cell differentiation via a TGF-β- or retinoic acid-dependent process [57–59]. In the lungs, the TCR specificities from pT_{reg} cells are often restricted to common aeroantigens (e.g. house dust mite, plant pollen, birch and *Aspergillus fumigatus*), and dampen allergic inflammation [60]. Bacher *et al.* recently developed an antigen-reactive T cell enrichment technology to study aeroantigen-specific T_{reg} cell function in adults [60]. The technique relied on the detection and isolation from peripheral blood mononuclear cells (PBMC) of rare antigen-specific T_{reg} and T_{eff} cells by the use of 4-1BB (CD137) or CD40L (CD154) expression, respectively [60]. The authors showed that T_{reg} cells specific to common aeroantigens were enriched in allergic adults and existed at greater numbers than aeroantigen-specific T_{eff} cells, thus leading to the hypothesis that aeroantigen-specific T_{reg} cells suppressed T_{eff} cell expansion. In other barrier tissues such as the gut, pT_{reg} cells maintain tolerance towards the diversity of commensal microorganisms and food antigens to which the immune system is continuously being exposed [55,56].

Antigen-specific T_{reg} cells have also been implicated to play a role in autoimmunity and cancer. Ooi *et al.* recently demonstrated that T_{reg} cells with a TCR self-restriction towards the type IV collagen α chain (peptides 135–145) presented specifically on human leukocyte antigen D-related (HLA-DR1) provided dominant resistance to Goodpasture's syndrome, a rapidly progressive and fatal autoimmune disease of the kidneys and lungs, in humanized mice [48]. In rheumatoid arthritis (RA), TCR-sequencing of synovial $CD14^-CD4^+CD25^{high}CD127^-$ T_{reg} cells yielded a few expanded clonotypes [61]. These T_{reg} cells were enriched in suppressive and activated ($HLA\text{-}DR^+$) T_{reg} cell subsets, thereby demonstrating a functional relevance for T_{reg} cell antigen-specificity in RA. In cancer, T_{reg} cells play a key role in promoting immune-evasion by cancer cells. For example, increased frequencies of T_{reg} cells specific to NY-ESO-1, an immunogenic tumor antigen, are observed in peripheral blood of melanoma patients [62], and their frequency correlates with metastatic potential and poor prognosis.

Functional plasticity, or instability, of T_{reg} cells

We and others have shown that adoptive transfer of $FoxP3^+$ T_{reg} cells into lymphopenic mice resulted in a loss of FoxP3 expression, loss of suppressive activity and consequential acquisition of inflammatory characteristics [15]. Experiments conducted in *foxp3*^{GFPCre}*Rosa26-loxP-stop-loxP-YFP* fate mapping mice showed that a significant proportion of T_{reg} cells that expressed FoxP3 at one point in time (YFP^+) lost FoxP3 expression (GFP^-) in homeostatic settings [15]. These YFP^+GFP^- cells, termed *ex*FoxP3, were phenotypically similar to memory T_{eff} cells and produced proinflammatory cytokines. It is unclear whether *ex*T_{reg} cells originate from *bona fide* committed tT_{reg} cells, from an uncommitted $CD25^{low}$ subset found therein, or if they represent T_{eff} cells that have not completely converted into $FoxP3^+$ pT_{reg} cells [63]. Whether T_{reg} cell plasticity occurs in the normal pathophysiology of human disease is currently unknown.

Extrinsic factors promoting the instability of T_{reg} cell function

Cytokine signaling, repeated antigen exposure and methylation patterns within the *foxp3* promoter are key factors enabling the stability of T_{reg} cell function. IL-6 is a proinflammatory cytokine known to counteract IL-2 signaling through STAT-3 dimers which occupy STAT-5-binding sites of the *foxp3* locus, thereby attenuating FoxP3 expression [22]. Moreover, IL-6 in the presence of TGF-β can favour the development of Th17 cells over pT_{reg} from naïve $CD4^+$ T cells in mice and humans [64]. Whether T_{reg} cells can themselves convert into Th17 cells, thereby demonstrating true plasticity, remains unknown in both humans and mice. The nature and strength of antigenic stimulation also influences T_{reg} stability. Development of tT_{reg} cells in the mouse thymus depends on strong TCR stimulation leading to demethylation of the TSDR and subsequent binding of STAT-5 to the *foxp3* locus [65]. In contrast, induction of murine pT_{reg} cells is improved when TCR stimulation is weaker and co-stimulation is reduced [66]. In humans, repeated TCR stimulation of $CD4^+CD25^{high}CD127^{low}$ T_{reg} cells attenuated, and even completely abrogated, FoxP3 expression while increasing production of proinflammatory cytokines [67], although the *ex*FoxP3 cells identified here may have originated from contaminating T_{eff} cells (see Conclusion).

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Functional adaptation of T_{reg} cells

Local inflammatory signals can drive $CD4^+$ T cells to undergo functional plasticity to acquire specialized effector functions in inflammatory sites. Growing evidence indicates that $FoxP3^+$ T_{reg} cells can also acquire tissue-specific adaptations that promote their homing to inflammatory sites for the control of immune responses driven by various T_{eff} cell lineages. T_{reg} cells achieve this by up-regulating the transcription factors of other T cell lineages in the presence of specific polarizing conditions, namely T-bet (Th1), interferon regulator factor 4 (IRF4) and GATA binding protein 3 (GATA3) (Th2) and STAT-3 and RORγt (Th17) [68–71]. For example, expression of CCR6 (CD196), a chemokine receptor characteristic of Th17 cells, driven by STAT-3 expression in T_{reg} cells, is thought to dampen Th17 cell-mediated inflammation and tissue pathology in crescentic glomerulonephritis, a potent inflammatory kidney disease [72]. However, the co-expression of secondary transcription factors may also represent a transition where T_{reg} cells convert into T_{eff} cells as the acquired transcription factors may endow T_{reg} cells with the ability to produce proinflammatory cytokines. Indeed, human and mouse $ROR\gamma t^+$ T_{reg} cells obtained through *in-vitro* Th17 polarizing conditions produce IL-17A and demonstrate attenuated suppressive capacities despite intact FoxP3 expression [71,73]. Whether these cells can completely convert into $FoxP3^-$ T_{eff} cells or whether this plasticity is reversible remains to be determined. Moreover, whether this occurs *in-vivo* in humans is completely unknown.

T_{reg} cell dysfunction in human disease

How T_{reg} cell dysfunction occurs and impacts the outcome of human disease is an important question. Here, we will highlight a few key mechanisms leading to T_{reg} cell dysfunction in human disease: (i) genetic defects, (ii) abrogation of T_{reg} cell-promoting signals, (iii) presence of T_{reg} cell destabilizing factors and (iv) co-opting T_{reg} cell suppressive function.

Congenital T_{reg} cell defects: IPEX syndrome, the human 'FoxP3 knock-out'

The most extreme example of T_{reg} cell dysregulation occurs in IPEX syndrome, a sex-linked congenital disease that is frequently fatal within infancy. It is largely caused by single loss-of-function germline point mutations in the *Foxp3* locus that abrogate T_{reg} cell function to different degrees [9]. More than 60 such mutations have been identified with a clinical spectrum whose severity is dependent on the nature of the mutation and the protein domain affected [74]. Mutations in the N-terminal proline-rich repressor (PRR) domain often result in improper T_{reg} cell suppressive function and production of proinflammatory cytokines (e.g. E70H and T108M) [75]. Moreover, IPEX mutations on the leucine zipper domain (e.g. ΔE251) prevent FoxP3 from exerting transcriptional control of target genes and are often associated with severe clinical manifestations of autoimmunity. However, most IPEX cases are caused by mutations in the forkhead (FKH) domain of FoxP3, which either prevent DNA-binding (e.g. R397W and I363V) or interactions with other proteins that aid in orchestrating the T_{reg} transcriptional program (e.g. A384T) [45,75]. Importantly, the presence of a mutation does not necessarily imply diminished FoxP3 protein expression levels (e.g. F373A [76]), T_{reg} numbers (e.g. A384T [45]) or even loss of T_{reg} suppressive functions (e.g. R347H). Clearly, the IPEX case demonstrates that, simple enumeration of T_{reg} cells is often not sufficient to make claims about T_{reg} cell function *in vivo*.

Developmental and homeostatic T_{reg} cell dysfunction in autoimmunity

The IL-2/IL-2R pathway is necessary for the thymic development and peripheral homeostasis of T_{reg} cells, and congenital or homeostatic disruption of key components of this pathway can provoke T_{reg} dysregulation and give rise to a spectrum of diseases ranging from IPEX-like autoimmunity [24] to type 1 diabetes (T1D).

Implications for T1D pathogenesis. Mouse studies show that a hallmark of diabetes onset is the apoptosis of pancreatic islet T_{reg} cells, alongside decreased CD25 and Bcl-2 expression, suggesting local IL-2 deprivation [77]. In some T1D cohorts, patients have decreased IL-2 production [78], and diminished CD25 expression within $FoxP3^+$ T_{reg} cells [79]. Long *et al*. found that IL-2 sensitivity of $CD4^+CD25^+$ T_{reg} cells was decreased in diabetic patients compared to healthy controls [80]. However, this observation was not seen by other studies [23], or only reproduced when looking at subjects bearing T1D-predisposing mutations on genes of the IL-2 pathway (*IL2RA* and *PTPN2*) [81,82]. Indeed, the *IL2RA* susceptibility haplotype was associated with decreased sensitivity to low doses of IL-2 *in vitro*, diminished suppressive function, lower CD25 expression and lower levels of FoxP3 expression by $Helios^+$ T_{reg} cells under limiting conditions of IL-2 (Fig. 2A) [81]. There is still no consensus on whether T_{reg} cell frequencies correlate with T1D onset [81,83]. Furthermore, some have argued that T_{eff}cells from diabetic subjects are resistant to suppression by T_{reg} cells [84], possibly through a STAT-3-dependent mechanism [85].

In-vitro, as stated earlier, diminished FoxP3 expression is correlated with loss of a suppressive phenotype and secretion of proinflammatory cytokines [67]. Indeed,

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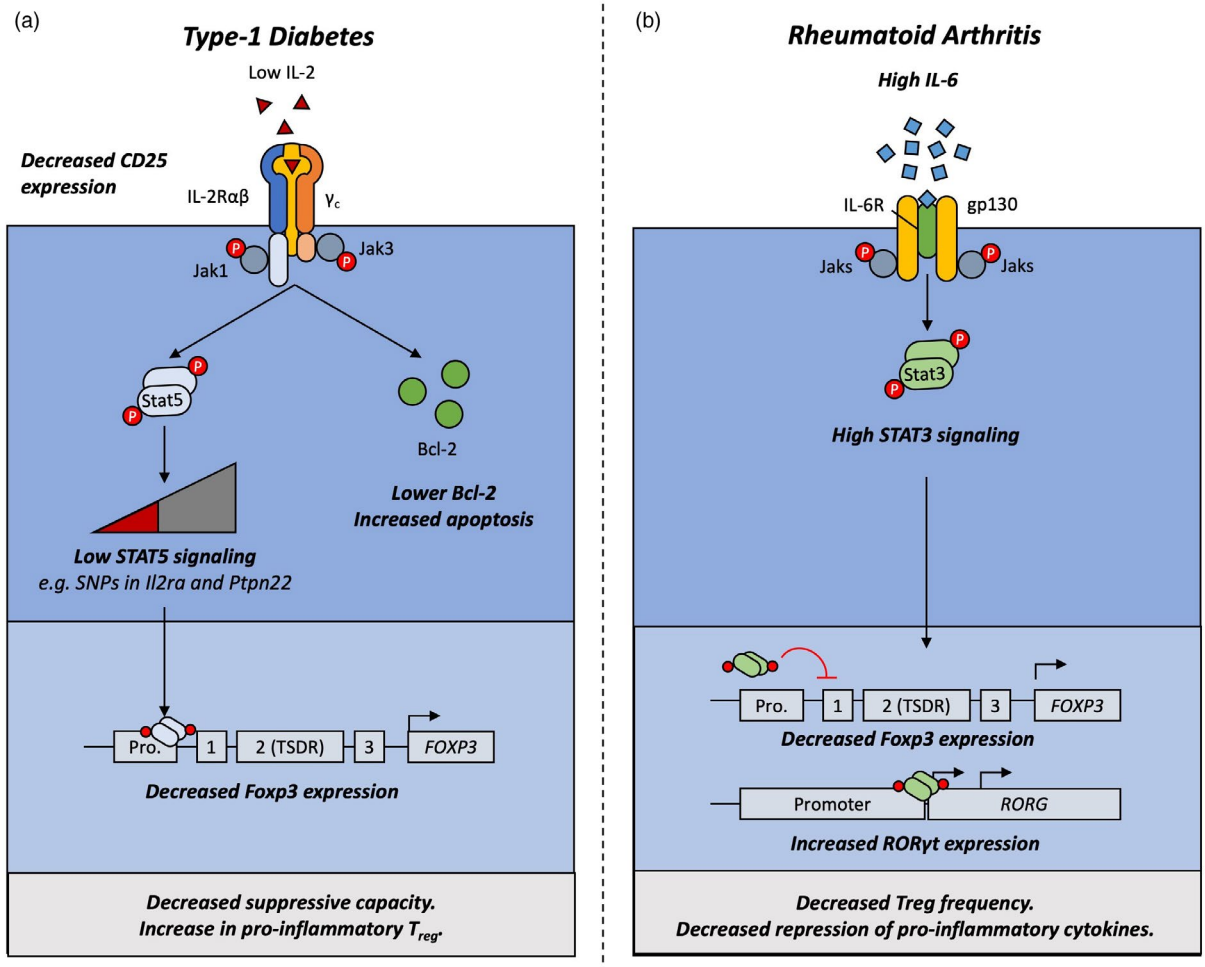

Fig. 2. Mechanisms driving T_{reg} cell dysfunction in type-1 diabetes and rheumatoid arthritis. Forkhead box protein 3 (FoxP3) expression is destabilized by extrinsic factors in type-1 diabetes and rheumatoid arthritis. **A,** Local deprivation in interleukin (IL)-2 and diminished sensitivity to IL-2 increases susceptibility to apoptosis through diminished B-cell lymphoma 2 (Bcl-2) production. Furthermore, lack of this positive signal reduces phosphorylated signal transducer and activator of transcription (pSTAT)-5 activation and occupancy of the *foxp3* promoter, leading to diminished FoxP3 expression. As a result, regulatory T cells (T_{reg}) have a lower suppressive capacity *in vitro* and can start secreting proinflammatory cytokines. **B,** High levels of IL-6 in the inflammatory pannus of rheumatoid arthritis patients trigger STAT-3 signaling through the IL-6 receptor (IL-6R). STAT-3 occupies the STAT-5-binding sites on the *foxp3* locus, which attenuates FoxP3 expression. Furthermore, STAT-3 binding to the *rorc* promoter enhances retinoic acid orphan receptor (ROR)γt expression, the T helper type 17 (Th17) master transcription factor. As a result, Th17 cells develop preferentially over T_{reg} cells during disease flares.

patients with either new-onset [86] or established diabetes [87] have an increased frequency of proinflammatory, cytokine-secreting, less suppressive T_{reg} cells in their peripheral blood. However, it is as yet unknown if this dysfunction is linked causally to poor IL-2 signaling or if it is a consequence of islet inflammation. Altogether, these results indicate that susceptibility mutations in genes of the IL-2 pathway impair T_{reg} cell sensitivity to IL-2 signals, leading to diminished pSTAT-5 levels and reduced expression levels of FoxP3. This dysregulation could ultimately lead to defective suppression by T_{reg} cells.

Translational relevance for immunotherapy. Defects in IL-2 signaling preferentially tamper with T_{reg} cell homeostasis. Therefore, Low-dose IL-2 therapy could specifically stimulate the T_{reg} compartment and re-establish immune homeostasis in diseases such as T1D, graft-*versus*-host disease (GVHD) [88] and hepatitis C virus (HCV)-induced vasculitis [89]. The DF-IL-2 trial showed low-dose aldesleukin [recombinant human (rh)IL-2] increased T_{reg} cell frequency in a dose-dependent manner [90]. In the DILT1D trial, a single dose of aldesleukin improved T_{reg} suppressive function *in vitro* [91]. In *Alopecia areata*, an

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autoimmune disease targeting hair follicles, hair regrowth upon treatment with rhIL-2 was associated with the recruitment of $CD4^+CD25^+FoxP3^+$ cells at the site of lesion and persisted for 2 months after the end of treatment, suggesting that T_{reg} cells require a threshold of IL-2R activation to acquire migratory capacity [92].

Finding a therapeutic window that allows for specific activation of T_{reg} cells over other immune cell subsets will be key to the successful development of low-dose IL-2 therapy. As harmful cell types such as NK cells, $CD4^+$ and $CD8^+$ T_{eff} cells constitutively express the intermediate-affinity IL-2R, the potential for accelerating the course of the disease exists. Indeed, Todd *et al* reported that for all doses tested at day 1 post-administration, the plasma concentrations of aldesleukin was higher than the activation threshold of NK cells and activated memory T_{eff} cells [91]. To circumvent these issues, one approach is the engineering of IL-2 superagonists to improve durability and selectivity through increased affinity, prolonged half-life and lower doses. One example is IL-2/CD25 fusion proteins, where IL-2 is bound to CD25 by a non-cleavable linker to increase the persistence of IL-2 and reduce binding to the intermediate-affinity IL-2R [93]. Another investigated compound is IL-2-anti-IL-2 monoclonal antibody immune complexes (IL-2IC) [94–96], where IL-2 is bound to the IL-2IC antibody such that the CD25-binding epitope is exposed and the CD122 (IL-2Rβ)-binding epitope is blocked (e.g. clone JES6-1), preferentially inducing the expansion of T_{reg} cells over T_{conv} cells. Moreover, a human IL-2-anti-IL-2 monoclonal antibody (F5111.2) immune complex was generated to preferentially enhance human T_{reg} cell proliferation in humanized mice, and successfully used to ameliorate autoimmunity and GVHD in non-obese diabetic (NOD) mice [97]. Finally, Sockolosky *et al.* devised a strategy to selectively stimulate engineered T cells in the context of T cell therapy. The infused T cells express a mutant ortho-IL-2Rβ receptor that signals through the native STAT-5 pathway but does not bind to wild-type IL-2. Instead, these receptors bind ortho-IL-2, an engineered cytokine–receptor complex that acts as an agonist of ortho-IL-2Rβ but not of any form of the wild-type IL-2R [98]. They applied this strategy for the expansion of tumor-reactive $CD8^+$ cytotoxic T lymphocytes (CTL) in a mouse model of melanoma. However, a similar approach could be conceived to expand engineered ortho-IL-2Rβ T_{reg} cells and enhance the efficacy of T_{reg} cell therapy.

Inflammation-mediated destabilization of T_{reg} cell function

Proinflammatory cytokines such as IL-6 and TNF-α can interfere with the stability of FoxP3 expression in T_{reg} cells, alter the T_{reg}/T_{eff} balance locally or systemically and ultimately provoke a loss of peripheral tolerance. Indeed, IL-6 and TNF-α are readily over-expressed in a number of autoimmune and chronic inflammatory conditions and prompts us to consider its relevance as a target of immunotherapy in autoimmunity.

The case for rheumatoid arthritis (RA). The inflammatory environment of the synovial pannus in RA represents an obvious setting where cytokine-mediated T_{reg} cell dysfunction may occur. High levels of IL-6 may inhibit T_{reg} cell homeostasis and function and enhance the development of proinflammatory T cell subsets. One evidence in support of IL-6-mediated dysfunction of T_{reg} cells is the preferential development of Th17 cells over T_{reg} cells in the periphery of RA patients [99,100]. This reciprocal regulation can be explained as, in the presence of TGF-β, IL-6 enhances the expression of RORγt through STAT-3 while repressing FoxP3 expression (Fig. 2B).

Another proinflammatory cytokine affecting T_{reg} cell function is TNF-α, which signals via the TNF-RII receptor, and can subsequently down-regulate FoxP3 expression and alter T_{reg} cell suppressive function [101]. TNF-α may also impair T_{reg} cell function by altering the formation of the immunological synapse between APCs and T_{reg} cells. Here, PKCθ plays a role in the integration of TCR and CD28 signals in T_{eff} cells upstream of NF-κB. Contrary to T_{conv} cells, PKCθ is sequestered from the immunological synapse in T_{reg} cells. TNF-α promotes the recruitment of PKCθ to the TCR in T_{reg} cells, and through downstream Akt signalling inhibits their suppressive capacity (Fig. 2B). Consistently, *in-vitro* administration of a PKCθ inhibitor on T_{reg} cells from RA patients enhanced their suppressive function [102].

However, studies evaluating the function of T_{reg} cells in RA reveal inconsistent findings. While in new-onset patients or during disease flares the frequency of T_{reg} cells in circulation is diminished [103], T_{reg} cell frequencies are normal in patients with clinically managed disease. However, a defect in repression, and not in suppressive function, of proinflammatory cytokines was reported in T_{reg} cells in RA [104], linked to defective CTLA-4 expression [105]. Furthermore, Van Amelsfort *et al.* reported that synovial T_{reg} cells from RA patients have a very activated phenotype and that synovial T_{eff} cells are resistant to suppression [106]. Indeed, in addition to dysregulating T_{reg} cells, IL-6 and TNF-α signaling render T_{eff} cells resistant to suppression via a protein kinase B (PKB)-dependent mechanism [107].

Translational relevance for immunotherapy. IL-6 and TNF also contribute to systemic symptoms such as fever and asthenia, and fuel tissue damage through recruitment of neutrophils to the inflamed joints and the differentiation

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of osteoclasts through the NF-κB pathway. Consequently, antibodies directed against these cytokines were developed to block their signaling. Anti-TNF pharmacological agents such as etanercept, adalimumab and infliximab are a major part of the therapeutic arsenal available to treat RA. Anti-TNF treatment induces an increase in circulating T_{reg} cells in responding patients, which correlates with a decrease in the titres of C-reactive protein, a biomarker of inflammation [108]. Tocilizumab, an IL-6R inhibitor, has been authorized as a second line of treatment for RA patients, after failure of methotrexate or anti-TNFs. As observed with anti-TNF drugs, the clinical benefits of tocilizumab treatment are accompanied by an increase in the frequency of peripheral T_{reg} cells after 6 months of treatment [101].

Co-opting T_{reg} cell suppressive function in cancer

Cancer immunity provides a setting where increased T_{reg} cell suppression contributes to cancer onset, progression and metastasis. To sustain their growth and gain the potential to metastasize, tumors develop a variety of tumor-induced immunosuppressive mechanisms to escape anti-tumor immunity, such as the expression or secretion of anti-inflammatory mediators [indoleamine 2,3-dioxygenase (IDO), IL-10, TGF-β], and the recruitment of a wide variety of suppressive leukocytes such as T_{reg} cells, myeloid-derived suppressor cells (MDSCs), tolerogenic DCs and tumor-associated macrophages (TAMs).

Establishment of a T_{reg} cell niche in the tumor microenvironment. Tumor cells contribute to the establishment of a T_{reg} cell niche by expressing IDO, an enzyme involved in tryptophan degradation. Its expression diminishes tryptophan availability and produces metabolites that induce T cell apoptosis [109] while promoting increased T_{reg} cell frequencies in the tumor infiltrate (Fig. 3). Reprogramming of T_{reg} cells into Th17 cells has been shown to promote early anti-tumor CTL responses [110]. However, IDO inhibits this process by suppressing IL-6 secretion in DCs and through the GCN2 kinase pathway in $FoxP3^+$ cells. Finally, IDO expression also silences the mTORC2/Akt pathway, thus stabilizing the T_{reg} cell lineage inside the tumor microenvironment [111].

T_{reg} cells play an essential role in establishing immunosuppression in the tumor microenvironment. Indeed, depletion of T_{reg} cells with an anti-CD25 monoclonal antibody (mAb) leads to tumor clearance in murine syngeneic tumor models [112]. T_{reg} cells infiltrate several types of human cancers, including melanoma, breast, pancreas and liver [113]. Higher frequencies of tumor-infiltrating T_{reg} cells have been correlated with worse prognosis and metastatic potential. In the example of ovarian cancer, a high frequency of infiltrating T_{reg} cells and a decreased CTL to T_{reg} cell ratio have both been associated with reduced survival of patients [114,115], consistent with T_{reg} cells suppressing anti-tumoral immune response. Tumor-infiltrating T_{reg} cells also display a very activated phenotype and have high levels of expression of immune checkpoint molecules such as CTLA-4 (CD152), programmed cell death 1 (PD-1) (CD279) [116] and TIGIT [117], and activation markers such as GITR [118]. These data point towards enhanced activity of T_{reg} cells in the tumor microenvironment contributing to immune evasion.

Translational relevance for immunotherapy. Immune check point inhibitors were developed following the rationale that these biologics compete with the binding of natural ligands to the target co-inhibitory receptor, thus alleviating T_{conv} and CTL inhibitory signaling. Numerous molecules targeting these pathways have been developed successfully for the treatment of many cancers, such as anti-CTLA-4 mAbs (ipilimumab, tremelimumab) and anti-PD-1 mAbs (nivolumab, pembrolizumab).

Studies now show that the protective effect of some of these biologics was through impairment of T_{reg} cell function. Since it is abolished in $Fc\gamma R^{-/-}$ mice, the protective effect of anti-CTLA-4 is very likely mediated by antibody-dependent cell-mediated cytotoxicity (ADCC) [119]. Indeed, depletion of T_{reg} cells in a mouse model of melanoma was dependent on the presence of FcγR-expressing macrophages infiltrating the tumor [120]. In melanoma patients, ADCC assays showed depletion of T_{reg} cells through the interaction of ipilimumab and $Fc\gamma RIIIA^+$ monocytes. Furthermore, responders to ipilimumab treatment have increased proportions of macrophages in their tumors [121], and treatment efficacy is correlated with decreasing frequencies of T_{reg} cells in tumors [122]. In contrast, tremelimumab, another anti-CTLA-4 mAb, likely functions without ADCC of T_{reg} cells as it suppresses T_{reg} cell function without affecting cell numbers [123]. Therefore, research is needed to assess the impact of check point inhibitors on the functional dynamics of T_{reg} cell subsets in blood and in particular within the tumor microenvironment.

For example, the role of CTLA-4 in T_{reg} cell suppressive function is well established. CTLA-4 acts in a cell-intrinsic manner by competing with CD28 for its shared ligands. It also acts on APCs by inducing IDO expression [124] and reducing surface expression of CD80 and CD86 through endocytosis and down-regulating transcription of their mRNA [125]. This leads to the emergence of tolerogenic DCs and limits the availability of co-stimulatory ligands to T_{eff} cells.

Conversely, PD-1 is also highly expressed on T_{reg} cells, but its role is not well identified. In melanoma patients,

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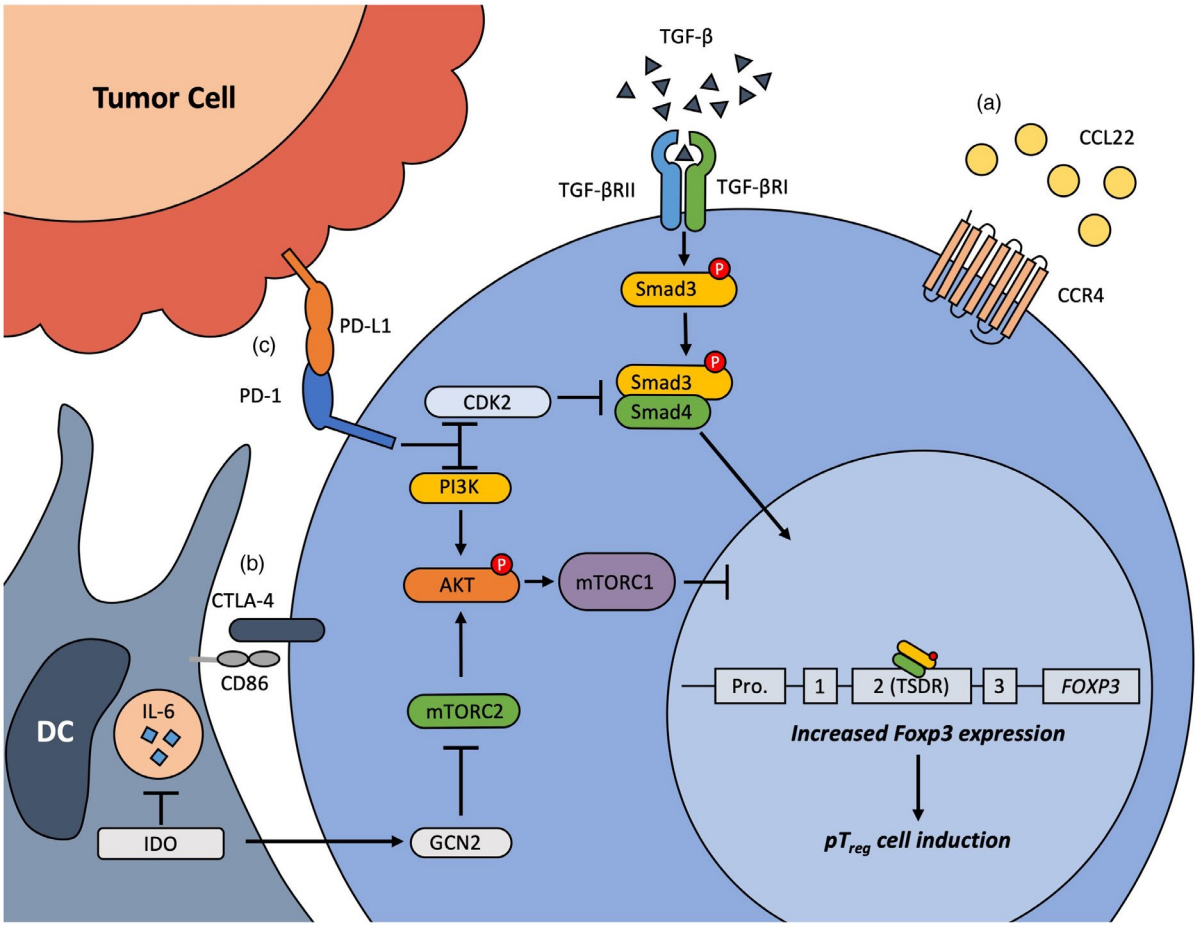

Fig. 3. Mechanisms promoting regulatory T cell (T_{reg}) development and immunosuppression in the tumor microenvironment. **A,** T_{reg} cells are recruited to the tumor through chemokine attraction. **B,** Interaction of T_{reg} cells with antigen-presenting cells (APCs) through cytotoxic T lymphocyte antigen (CTLA)-4 deprive T effector (T_{eff}) cells of co-stimulatory signals, polarizes dendritic cells (DCs) towards a tolerogenic phenotype and induces the expression of indoleamine 2,3-dioxygenase (IDO), which catabolizes metabolites, thereby inducing T_{eff} cell apoptosis. Furthermore, it inhibits the reprogramming of T_{reg} cells into Th17 cells by suppressing interleukin (IL)-6 secretion and promotes T_{reg} cell lineage stability by inhibiting the transient mammalian target of rapamycin complex 1/protein kinase B (mTORC2/Akt) pathway. **C,** Tumor cells express programmed cell death ligand 1 (PD-L1), which binds programmed cell death 1 (PD-1) at the surface of T_{reg} cells. The PD-1 pathway stabilizes forkhead box protein 3 (FoxP3) expression by inhibiting the phosphoinositide-3-kinase (PI3K)/Akt pathway and synergizes with transforming growth factor (TGF)-β by diminishing the level of Smad3 necessary to promote the conversion of naïve $CD4^+$ T cells into peripheral (p)T_{reg} cells, while inducing T_{eff} cell exhaustion.

in-vitro treatment with nivolumab down-regulated FoxP3 expression in sorted T_{reg} cells [126] and inhibited T_{reg} cell suppressive function [127]. However, PD-$1^{-/-}$ mice have a similar frequency of circulating T_{reg} cells to their wild-type counterparts, and do not display a diminished suppressive capacity [128]. Nevertheless, PD-$1^{-/-}$ $CD4^+$ T cells have a diminished capacity to differentiate into pT_{reg} cells when transferred into lymphopenic hosts [128]. Indeed, the PD-L1/PD-1 pathway plays a role in the development of pT_{reg} cells by synergizing with TGF-β signaling through Smad3 to promote the conversion of naïve T cells into iT_{reg} cells. Furthermore, PD-1 signaling inhibits the PI3K/Akt pathway, which is known to destabilize FoxP3 expression [129]. Taken together, these results suggest that PD-1 does not necessarily play a role in T_{reg} cell suppressive function but regulates their homeostasis and stability, thus contributing to the regulation of the T_{reg}/T_{eff} cell balance.

Conclusion and future perspectives

Despite the enormous efforts by the immunological community to characterize the molecular and cellular basis of T_{reg} cell development and function in health and disease, several knowledge gaps remain in this area

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which future research must address. Various mechanisms ensure a robust and sustained immunosuppressive phenotype in T_{reg} cells. However, there is significant functional and phenotypical heterogeneity that remains to be captured in human disease. One of the future priorities relate to the urgent need for novel strategies to monitor T_{reg} cell function in human blood more effectively, and in particular in tissues, in states of health and disease. Part of the problem lies with the phenotypical markers used to distinguish human T_{reg} cells and subsets found therein from a variety of T_{conv} cells. Both CD25 and FoxP3, the quintessential markers of T_{reg} cells, are up-regulated on T_{conv} cells upon TCR stimulation, and can only be used in situations of immune quiescence, not states of immune activation or inflammation. Other markers have been proposed to overcome this issue. For example, T_{reg} cells are often defined as $CD127^{low}$ given the inverse correlation between CD127 and FoxP3 expression [130,131]. Nevertheless, this gating strategy fails to eliminate activated T_{eff} cells and excludes a large proportion of *bona fide* T_{reg} cells. More recently described T_{reg} cell markers include HLA-DR, CTLA-4, CCR6, GARP, CD15s, CD39, CD49d, CD147, TNFRII, GITR and LAP (Table 1) [132]. Nonetheless, these markers are readily modulated on the surface of T_{reg} and T_{conv} cells consequently impeding proper discrimination and their use for downstream functional and phenotypical studies. We recently demonstrated that TIGIT and FcRL3 are reliable and specific markers for identifying and sorting $Helios^+$ T_{reg} cells, and given the stable suppressive phenotype of these cells, we envision that sorted $TIGIT^+FcRL3^+CD25^{high}CD127^{low}$ cells can be isolated and clinically manipulated for therapeutic use [46].

Another issue that has thus far prevented a proper definition of T_{reg} cell function in human disease is the lack of discrimination of antigen-specific responses, particularly in conventional, *in-vitro* polyclonal suppression assays. These methods mask the important effects of antigen-specific T_{reg} cells that may serve different physiological roles than other T_{reg} cell subsets (e.g. aeroantigen-specific T_{reg} cells maintaining tolerance to airborne

Table 1. List of human T_{reg} cell markers

Location	Marker	T_{reg}	T_{eff}
Surface	CD25 (IL-2Rα)	High expression on most cells	Up-regulated with activation
	CD127 (IL-7Rα)	Low/negative expression	Down-regulated with activation
	TIGIT[1]	High expression correlating with Helios	Up-regulated with activation
	CD307c (FcRL3)[1]	High expression on $Helios^+$ cells	Low/negative expression
	HLA-DR	Expression on terminally differentiated cells	Up-regulated with activation
	CD15s (Sialyl Lewis x)	Up-regulated with activation	Weakly up-regulated with activation
	GARP	High expression on activated cells	Not expressed
	CD39	Up-regulated with activation	Not expressed
	CD49d	Down-regulated with activation	Highly expressed
	CD120b (TNF-RII)	High expression	Up-regulated with activation
	CD357 (GITR, TNF-RSF18)	High expression	Up-regulated with activation
	LAP	High expression on activated cells	Not expressed
	CD147 (Basigin/Emmprin)	Constitutive expression	Up-regulated with activation
	CCR6	Expression on memory T_{reg} cells only	Expressed on Th17 cells and with activation
Intracellular	FoxP3	High expression	Transient, high up-regulation with activation
	Helios	High expression	Not expressed
	CD152 (CTLA-4)	High intracellular expression	Not expressed intracellularly

[1]When TIGIT and FcRL3 are used in conjunction, they capture most $Helios^+$ memory T_{reg} cells, which have a stably immunosuppressive phenotype.

IL = interleukin; TIGIT = T cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibition motif (ITIM) domains; FcRL3 = Fc receptor-like protein 3; GARP = glycoprotein-A repetitions predominant protein; HLA-DR = human leukocyte antigen D-related; TNF = tumor necrosis factor; GITR = glucocorticoid-induced TNF-R-related protein; CTLA-4 = cytotoxic T lymphocyte antigen; Th17 = T helper type 17; T_{reg} = regulatory T cell; T_{eff} = effector T.

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allergens, but not necessarily counteracting inflammation following pulmonary viral infections). Novel methodologies are emerging now to understand more clearly the antigen-specific effects of T_{reg} cells in human disease. MHC-II tetramer technology, which has largely been ineffective at reliably isolating rare $CD4^+$ TCR specificities, is being vastly improved through adjustments such as barcoding to improve the number of epitopes studied, or dual staining of identically loaded tetramers with different fluorochromes, in conjunction with surface markers such as CD137 to improve specificity and isolate antigen-stimulated T_{reg} cells by flow cytometry. Ultimately, these important advancements will enable us to monitor T_{reg} cell functions in unprecedented depth, thereby enhancing our understanding of T_{reg} cells in human health and disease.

Finally, several hurdles affect the therapeutic potential of T_{reg} cells in disease. Novel strategies will need to be developed to overcome the limitations related to survival and cell persistence *in vivo*, stability of the T_{reg} cell functional phenotype and selective engagement or repression of antigen-specific responses in defined disease settings. In this regard, a better understanding of the genetic factors [single nucleotide polymorphisms (SNPs) or epigenetic], mechanisms of transcriptional regulation (splice isoforms, miRNAs and transcription factor activity) and post-translational modifications (phosphorylation, acetylation or ubiquitination) influencing FoxP3 gene and protein expression or activity will be required to further modulate the function of endogenous, or adoptively transferred expanded T_{reg} cells in therapy.

Disclosures

There are no competing interests.

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